Ca^{2+}-activated K^+ Efflux Limits Complement-mediated Lysis of Human Erythrocytes

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Abstract

The lytic effect of complement on human erythrocytes has been reported by others to increase when Na^+ is substituted for K^+ in the external medium. In this paper we have investigated the hypothesis that net loss of K^+ through a K^+ transport pathway protects erythrocytes from complement-induced colloidosmotic swelling and lysis. Antibody-sensitized human erythrocytes containing different intracellular cation concentrations (nystatin treatment) were exposed to low concentrations of guinea pig serum in media of different cation composition; complement lysis was assessed by the release of hemoglobin and the volume of the surviving cells estimated by their density distribution profiles. Complement-dependent swelling and lysis of erythrocytes (*a*) were limited by the presence of an outwardly directed K^+ electrochemical gradient and (*b*) were enhanced by carbocyanine, a specific inhibitor of the Ca^{2+}-activated K^+ transport pathway, and by absence of Ca^{2+} in the external medium. We propose that during complement activation a rising cytosolic calcium triggers the Ca^{2+}-activated K^+ permeability pathway, the Gardos effect, produces a net K^+, Cl^- and water loss, and thus limits the colloidosmotic swelling and lysis of erythrocytes.

Introduction

Lysis of erythrocytes by complement results from the insertion of a hydrophobic macromolecular complex of the terminal complement proteins, C5b, 6, 7, 8, and 9 (membrane attack complex, MAC),[1] into the lipid bilayer of a target's cell membrane. The inserted MAC disrupts the functional impermeability of the cell membrane, allowing a net influx of salt and water, which leads to colloidosmotic swelling and lysis of the erythrocyte (reviewed in references 1 and 2). It has been widely accepted that insertion of one MAC complex in the erythrocyte membrane would suffice to eventually lyse the cell (3). However, we have recently reported that under conditions of limited complement activation the MAC may cause a large but transient increase in cell membrane permeability, and the affected cells do not lyse (4). In addition, we also observed that the cells surviving sublytic complement attack do not swell even though they experience a substantial increase in the permeability of their membranes, documented by a marked reduction in intracellular K^+ (K_i^+) and an increase in intracellular Na^+ (Na_i^+). This observation suggested that when the MAC-induced increase in membrane permeability is transient, K^+ loss from the cells might prevent the net gain of cations and thereby avoid colloidosmotic swelling. Many types of animal cells that contain high K_i^+ restore their cell volume after hypotonic swelling by a net loss of K^+ plus Cl^- with concomitant loss of cell water (reviewed in references 5 and 6). In this paper we provide evidence that the Ca-activated K^+ transport pathway, known as the Gardos effect (7), limits the extent of submaximal complement lysis in normal human erythrocytes.

Methods

Complement reagents and assays. An isotonic Veronal buffer solution containing either 140 mM NaCl or 140 mM KCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM dextrose, 0.1% gelatin, and 5 mM Veronal sodium, pH 7.3 (GVB.D^{2+}) was used in the hemolytic assays. Human erythrocytes were obtained from heparinized blood and washed three times with isotonic choline chloride solution (140 mM choline chloride, 1 mM $MgCl_2$, 10 mM Tris-MOPS, pH 7.4, at 4°C) and three times with Veronal-buffered solution containing 140 mM NaCl, 0.1% gelatin, 0.04 M EDTA, and 5 mM Veronal sodium, pH 7.3 (GVB-EDTA). Then the cells were sensitized at 4°C for 30 min and at 37°C for an additional 30 min with a subagglutinating concentration of rabbit anti-human red cell antibody (BCA/Cappel Laboratories, Cochranville, PA) diluted in GVB-EDTA (5×10^8 cells/ml). Erythrocytes with complexed antibody (EA) were washed three times with GVB-EDTA, two times with isotonic choline chloride solution, and finally, with GVB.D^{2+} before use.

Pools of sera were derived from blood obtained by cardiac puncture of carbon dioxide narcotized outbred Hartley guinea pigs. Human serum genetically deficient in C8 (8, 9) was provided by Dr. Peter Rice, Boston University Medical School. The sera used as complement sources were aliquotted and stored at −70°C; new aliquots were used for each experiment. For hemolytic assays, varying quantities of serum were diluted in 0.5 ml GVB.D^{2+} containing 3×10^8 cells/ml, incubated for varying times at 37°C, and the reaction terminated by the addition of 2.0 ml of isotonic NaCl solution and centrifugation. The hemoglobin in the supernatant was measured by the absorbancy at either 541 or 414 nm.

Intracellular cation composition, Stractan density separation, and density distribution profiles. Erythrocyte Na^+ and K^+ contents were measured by atomic absorption spectrometry as described (10). In some experiments, the intracellular cation composition of the cell was modified using the ionophore nystatin as previously reported (10), and in others, cells were separated by density using discontinuous gradients of Stractan (11). Density distribution profiles were determined by the phthalate method (12).

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Received for publication 20 June 1988 and in revised form 5 January 1989.

1. *Abbreviations used in this paper:* EA, erythrocyte antibody; GPS, guinea pig serum; GVB, isotonic Veronal-buffered saline, pH 7.4, containing 0.1% gelatin; GVB-EDTA, GVB containing 0.04 M EDTA; GVB.D^{2+}, GVB containing 0.15 M $CaCl_2$ and 0.5 M $MgCl_2$; K_i^+, intracellular K^+; K_o^+, extracellular K^+; MAC, membrane attack complex; Na_i^+, intracellular Na^+; Na_o^+, extracellular Na^+.

J. Clin. Invest.

0021-9738/89/05/1466/06 $2.00
Volume 83, May 1989, 1466–1471

Results

The following experiments were performed to test the hypothesis that activation of a K^+ transport pathway, driven by the K^+ electrochemical gradient, might limit the extent of colloid-osmotic swelling and lysis induced by complement activation in human erythrocytes. Sensitized human erythrocytes in which the electrochemical K^+ gradient was modified by varying either the extracellular K^+ (K_o^+) or the K_i^+ concentration were exposed to guinea pig serum (GPS) as a source of complement. In the experiment depicted in Fig. 1, normal EA containing high K_i^+ (95 mmol/liter cell) were resuspended in either 140 mM KCl or 140 mM NaCl medium and then exposed to a dose of complement sufficient to lyse ~ 30% of the cells. After 1 h incubation at 37°C the unlysed EA were recovered by centrifugation and their volume was estimated by the density distribution profile of the cells. When the EA were exposed to complement in 140 mM NaCl medium, the unlysed EA did not swell (Fig. 1). In contrast, the unlysed EA did swell when they were exposed to complement in high K^+ medium. The observed changes in cell volume were consistent with the measured changes in the internal cation composition; namely, in Na^+ buffer, the loss of internal K^+ balanced the uptake of Na^+, whereas in K^+ buffer, the total cation content increased due to net K^+ influx (Fig. 1, *inset*).

The differential swelling observed in KCl as compared with NaCl media was also associated with different amounts of lysis (Fig. 2 *a*). When a given concentration of GPS lysed ~ 30% of the EA in NaCl medium, the same concentration of GPS lysed two times more EA in KCl medium (lysis in KCl/lysis in NaCl = 2±0.2 (mean±SD; n = 6). Similar effects of external cations on complement lysis of sheep and human erythrocytes had been reported previously (13, 14). To establish whether the observed difference between lysis in Na^+- and K^+-containing media was due to different kinetics of lysis, as determined by the time required to lyse 50% of the total number of cells lysed ($t_{1/2}$), or to lysing different numbers of EA, the time course of lysis in both media was followed. In the experiment depicted in Fig. 2 *b*, the $t_{1/2}$ of lysis in Na^+ and K^+ media was the same: 8 min. In contrast, the end point lysis in KCl medium (69%) was twofold higher than the value observed in NaCl medium (35%). Thus, the cation composition of the media influenced the total number of EA lysed, but not the kinetics of lysis. Since replacing external Na^+ for K^+ abolishes the electrochemical K^+ gradient, these results suggested a possible influence of the K^+ electrochemical gradient on immune lysis of erythrocytes.

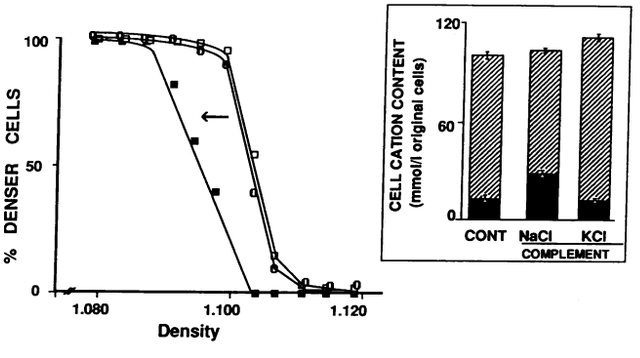

Figure 1. Phthalate density distribution profile of sensitized human erythrocytes (EA) before and 1 h after exposure to GPS in Na^+ and K^+ media. Density profile of EA before incubation with complement (○); density profiles of unlysed EA after exposure to GPS in Na^+ (□) and K^+ buffer (■). EA (2×10^8/ml) were exposed to 1:30 dilution of GPS and then incubated for 60 min at 37°C. At the end of the incubation the unlysed cells were separated by centrifugation and resuspended at ~ 50% hematocrit, and the density phthalate distribution profile was determined as described in Methods. *Inset,* Intracellular cation content of EA before and after exposure to serum in Na^+ and K^+ media. Before and after exposure to serum an aliquot of EA was washed five times with cold isotonic choline chloride and then suspended in washing solution at ~ 50% hematocrit for determination of hematocrit and cell Na^+ (1:50 dilution; *solid bars*) and K^+ (1:500 dilution; *hatched bars*) by atomic absorption spectrometry.

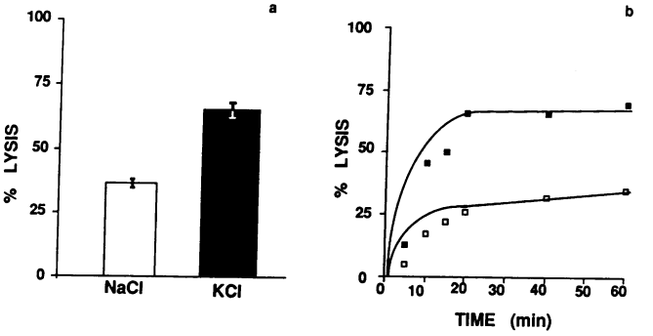

Figure 2. Effect of replacing external K^+ for Na^+ upon lysis of EA by complement. (*a*) EA (2×10^8/ml) were suspended in either Na-GVB.D^{2+} (*open bar*) or K-GVB.D^{2+} (*solid bar*) and then exposed to a dilution of GPS at 37°C; after 60 min the samples were diluted with 2 ml saline solution and centrifuged to determine the hemoglobin content of the supernatant (OD at 541 nm). 100% lysis was determined by diluting a sample of the cell suspension in distilled water. Values are the mean±SEM of six experiments. (*b*) Time course of the lysis in Na^+ and K^+ media. 5 ml of cells suspended in either Na-GVB.D^{2+} (□) or K-GBVD^{2+} (■) (2×10^8 EA/ml) were exposed to a 1:25 dilution of GPS at 37°C. At 5, 10, 15, 20, 40, and 60 min aliquots of 0.6 ml were removed and diluted in 2 ml of saline solution and centrifuged to determined the amount of lysis as in *a*.

To investigate further the role of the K^+ electrochemical gradient on complement-induced lysis of erythrocytes, the K^+ gradient across the EA membranes was modified by changing the internal, instead of the external, cation composition of the cells. Cells containing either Na^+ as the predominant internal cation (high Na^+ cells: Na_i^+ = 95 mmol/liter cells; K_i^+ = 2 mmol/liter cell) or half Na^+ and half K^+ (50/50 cells: Na_i^+ = 52 mmol/liter cells; K_i^+ = 50 mmol/liter cells) were prepared using the ionophore nystatin. As a control for nystatin effects, EA were also treated with nystatin but prepared to contain only K^+ instead of only Na^+ (high K^+ cells: K_i^+ = 99; Na_i^+ = 0.2 mmol/liter cell). Then complement lysis was assayed in 140 mM NaCl, 70 mM NaCl-70 mM KCl, and 140 mM KCl media. Replacing internal Na^+ for K^+ resulted in a significant increase of complement-mediated lysis (Fig. 3 and Table I). Furthermore, any maneuver that abolished the outwardly directed K^+ electrochemical gradient either by increasing the external K^+ (high K^+ cells in K^+ medium), or by decreasing the internal K^+ (high Na^+ cells in Na^+ medium), or by both changes (50/50 cells in 70 mM NaCl-70 mM KCl medium) resulted in increased complement-mediated lysis of EA (Table I). In contrast, replacing Na^+ in the external medium by isotonic choline chloride substitution did not affect complement

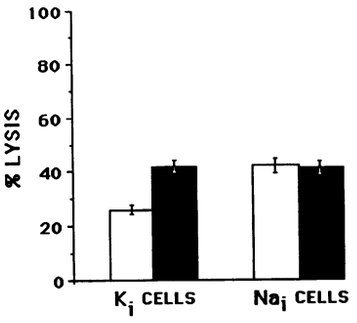

Figure 3. Effect of Na^+ and K^+ buffers on complement lysis of erythrocytes of different internal cation composition. EA were prepared with the ionophore nystatin to contain either K^+ or Na^+ as only intracellular cation (all Na^+ cells: Na_i^+ = 95 mmol/liter cells; all K^+ cells: K_i^+ = 99 mmol/liter cells). The cells (2×10^8/ml) were suspended in either Na-GVBD^{2+} (*open bars*) or K-GVBD^{2+} (*solid bars*) and then exposed to a dilution of GPS for 60 min at 37°C. At the end of the incubation period the samples were diluted in 2 ml of saline solution and centrifuged, and the amount of lysis was calculated as in 2 *a*. The figure represents one of three similar experiments.

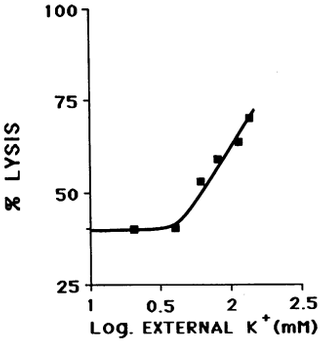

Figure 4. Effect of the electrochemical K^+ gradient on complement lysis of erythrocytes. EA (2×10^8/ml) were suspended in isotonic GVB.D^{2+} buffer of different Na^+ and K^+ concentrations prepared by reciprocal substitution of Na^+ and K^+ GVBD^{2+} buffers. End point lysis (60 min) by GPS was then assayed as in Fig. 2.

lysis of EA (lysis in Na^+-GVB.D^{2+} medium = 30%; lysis in choline chloride-GVB.D^{2+} = 30%). These results further support the conclusion that complement-mediated lysis is affected by the presence and direction of the electrochemical K^+ gradient, and not by the cation composition of the medium.

To determine the dependence of complement-mediated lysis on the magnitude of the electrochemical K^+ gradient, EA were suspended in different isotonic media containing increasing concentrations of external K^+, prepared by the reciprocal substitution of KCl by NaCl, and then exposed to sublytic complement activation. The lysis of erythrocytes was proportional to the K^+ concentration of the external medium and inversely related to the K_i^+/K_o^+ ratio (Fig. 4). This finding is consistent with the hypothesis that in response to the complement lesion there is a net efflux of K^+ mediated by a K^+ transport pathway passively dependent on the normal electrochemical K^+ gradient. Activation of this putative K^+ transport pathway limits the amount of net cation gain through the complement pore and thereby limits the extent of colloidosmotic lysis of erythrocytes.

Several K^+ transport pathways have been described in the human erythrocyte membrane, including (*a*) the ouabain-inhibitable Na^+/K^+ pump (10), (*b*) the bumetanide-inhibitable $Na^+/K^+/Cl^-$ cotransport system, (15), (*c*) the volume-activated and chloride-dependent K^+/Cl^- cotransport system (16, 17), and finally, (*d*) the Ca^{2+}-activated K^+ transport system (7). The role of the first three K^+ transport pathways in protecting against immune lysis of erythrocytes was ruled out by using the specific inhibitors of the transport systems, ouabain (0.1 mM), bumetanide (10 μM; Table II), and by measuring lysis in Na^+ and K^+ buffers in the least dense and in the denser fractions of EA separated by density using the Stractan procedure (11). Since the K/Cl cotransport is present in the least dense (and not in the denser) fraction of human erythrocytes (12), it was expected that if this K^+ transport pathway were involved in the Na^+ vs. K^+ effect, this phenomenon would be present only in the least dense fraction, but this was not observed. It was not possible to test by nitrate substitution for chloride dependence of this K movement, because nitrate inhibited complement activation (data not shown), an effect of chaotropic anions previously reported by others (18).

Exposure of EA to complement does cause a rapid increase in cytosolic Ca^{2+}, which enters through the complement pore (19). To test whether the Ca^{2+}-activated K^+ transport pathway was involved in protecting human EA from complement lysis, EA suspended in either Na^+-GVB.D^{2+} or in K^+-GVB.D^{2+} were exposed to a sublytic complement dose in the presence and absence of the carbocyanine dye diS-C_2-(5), a potent inhibitor of the Ca^{2+}-induced K^+ permeability (20). In the absence of complement, carbocyanine did not produce any lysis of EA (Fig. 5 *a*). When the hemolytic assay was performed in Na^+ medium, carbocyanine (50 μM) mimicked the effect of external K^+ in that it increased the amount of complement-mediated lysis to the same extent observed in K^+ medium (Fig.

Table I. Effect of Changing the Intracellular and Extracellular Cation Composition on Complement-induced Lysis of Human Erythrocytes

	% Lysis		
	Cation content of the medium (millimolar)		
Cell cations	140 NaCl	70 NaCl/70 KCl	140 KCl
mmol/liter cell H_2O			
140 NaCl 70	16±1	16±1.5	14±2
NaCl/70 KCl	9±0.5	17±1	18±1
140 KCl	5±0.5	12±0.5	18±1.6

The internal cation content of human EA was changed using the ionophore nystatin (cf. reference 10). The cells were then exposed for 1 h at 37°C to a 1:25 dilution of guinea pig complement in GBV.D^{2+} buffers of different cation compositions: 140 mM NaCl; 70 mM NaCl and 70 mM KCl; and 140 mM KCl. After 1 h the percent of cells lysed was calculated from the hemoglobin released, measured by OD at 414 nm.

Table II. Effect of Different Inhibitors of K^+ Transport on Complement-mediated Lysis of Human Erythrocytes

Inhibitor	% Lysis
Control	15±1
Ouabain (100 μM)	15±2
Bumetanide (10 μM)	15±2
Carbocyanine (10 μM)	15±1
Carbocyanine (50 μM)	28±3

EA were exposed for 1 h at 37°C to a 1:20 dilution of GPS in Na-GBV.D^{2+} containing maximal inhibitory concentrations of ouabain (100 μM), bumetanide (10 μM), and two different concentrations of carbocyanine (10 and 50 μM). After 1 h the percent of cells lysed was calculated from the hemoglobin released, measured by OD at 414 nm.

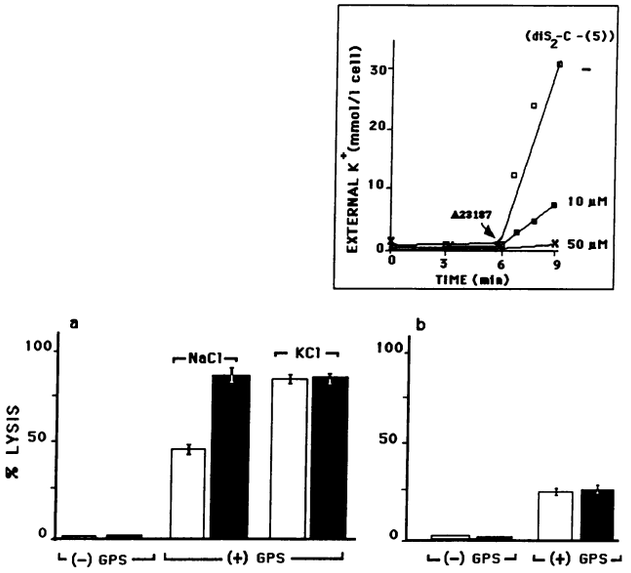

Figure 5. Effect of carbocyanine dye (diS-C_2-[5]) on complement-mediated lysis of human and high K^+ sheep erythrocytes. A cell suspension (10^7 EA/ml) of human EA in either Na^+-$GVBD^{2+}$ or K^+-$GVBD^{2+}$ (*a*) and high K^+ sheep EA (*b*) were incubated for 60 min at 37°C with (+) and without (−) a dilution of GPS in the presence (*solid bars*) or absence (*open bars*) of carbocyanine dye diS-C_2-(5) (50 μM). At the end of the incubation period lysis was estimated as in Fig. 2 but hemoglobin was determined by the OD at 414 nm (to avoid interference by carbocyanine). Values are mean±SEM of three experiments, each performed in triplicate samples. (*Inset*) Inhibition by diS-C_2-(5) of the Ca-induced K^+ transport pathway triggered by the calcium ionophore A23187. EA were suspended at an hematocrit ~ 1% in Na-GVB.D^{2+} (nominally K^+ free, 0.150 mM $CaCl_2$). Aliquots of 1 ml were removed at 0, 3, and 5 min, layered on top of 0.3 ml of phthalate in an Eppendorf microtube, and then centrifuged in an Eppendorf microcentrifuge for 20 s. At 5.5 min ionophore A23187 (1 μM final concentration) was added and samples were removed and spun down through phthalate at 6, 7, 8, and 9 min. K^+ in the supernatants was determined by atomic absorption spectrometry as described in Methods. The efflux of K^+ (mmol/liter cells per h) in the absence (□) and presence of 10 μM (■) and 50 μM (×) diS-C_2-(5) was calculated from the slope of the curves relating the K^+ concentration in the supernatants (millimoles/liter cells) vs. time (minutes).

5 *a*). In contrast, the dye did not influence lysis in K^+ medium (Fig. 5 *a*). These results suggest that the Na^+ vs. K^+ effect on complement lysis could be mediated by the Ca^{2+}-activated, carbocyanine-inhibitable, K^+ transport pathway. A concentration of 50 μM dye was required to completely abolish the Ca^{2+}-induced K^+ efflux triggered by the Ca^{2+} ionophore A21387 (Fig. 5, *inset*). In contrast, other inhibitors of the Ca^{2+}-activated K^+ transport, such as quinine and quinidine, failed to influence complement-mediated lysis, but they also failed to completely abolish the K^+ efflux activated by the Ca^{2+} ionophore. Further evidence that carbocyanine does influence complement lysis through inhibition of the Ca^{2+}-activated K^+ efflux was provided by experiments using sheep erythrocytes of the high K^+ type, a cell lacking the Ca^{2+}-activated K^+ channel. In these cells, 50 μM carbocyanine did not influence the immune lysis by complement (Fig. 5 *b*), indicating that the dye does not affect either complement activation or binding of complement components, and that it does not enhance complement-mediated lysis by affecting the target membrane in a detergentlike manner. These results suggest that complete inhibition of the Ca^{2+}-activated K^+ transport pathway enhances complement-mediated lysis of human EA.

To determine whether inhibition of the Ca^{2+}-induced K^+ permeability could also result in swelling of the EA that survive complement lysis, the density distribution profile of the unlysed erythrocytes was determined in NaCl medium in the presence and absence of 50 μM carbocyanine. In the NaCl medium the presence of the dye leads to swelling of the unlysed erythrocytes as determined by the displacement to the left of the density distribution profile (Fig. 6). This result further supports the notion that a net K^+ loss driven by the K^+ electrochemical gradient through the Ca^{2+}-activated K^+ channel limits the extent of the complement-induced colloidosmotic swelling and the subsequent lysis of erythrocytes.

Experiments to test directly for dependence on external Ca^{2+} of complement-induced lysis were also performed. Since Ca^{2+} ions are required for the antibody-triggered activation of the complement cascade, the protocol used in the previous experiments could not be adapted to perform experiments in Ca^{2+}-free medium. Therefore, we used human serum genetically deficient in C8 to first form EAC1-7 cells in the presence of Ca^{2+}. Then external Ca^{2+} was removed and lysis was elicited by addition of purified C8 and C9. Human EA (2×10^{-7}) were first incubated for 30 min at 37°C in $GVBD^{2+}$ and a ¼ dilution of C8-deficient serum. Then the cell suspension was diluted in GVB-EGTA (2 mM EGTA), spun down, and reconstituted in an equal volume of either $GVBD^{2+}$ or GVBD − EGTA (containing 0.5 mM $MgCl_2$ and 2 mM EGTA, but no Ca^{2+}; final free Ca^{2+} concentration < 2 μM). Lysis was elicited by addition of purified terminal complement components C8 (100 CH50 units; Cordis Laboratories Inc., Miami, FL) and C9 (100 ng/ml; Cytotech, San Diego, CA). Fig. 7 shows that removal of Ca^{2+} from the lysis medium results in a significant increase in the lytic effect of the terminal complement components, an effect comparable to the enhancement of lysis observed when external Na is replaced for K (Fig. 2). These experiments support the concept that a Ca^{2+}-activated K^+ transport pathway protects EA from complement-mediated lysis.

Discussion

The present study was conducted to test the hypothesis that volume regulatory mechanisms are involved in the erythrocyte's response to the sublytic damage by complement. We

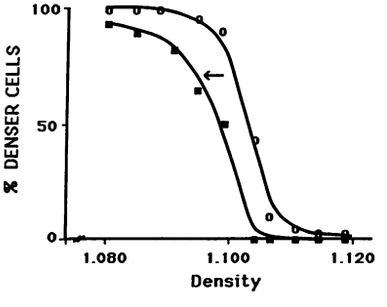

Figure 6. Effect of diS-C_2-(5) on the phthalate density distribution profile of unlysed EA exposed to complement in Na^+ medium. EA (2×10^8/ml) suspended in Na-$GVBD^{2+}$ were exposed to a 1:30 dilution of GPS in the absence (○) and presence (■) of 50 μM diS-C_2-(5), and then incubated for 60 min at 37°C. At the end of the incubation period the unlysed cells were separated by centrifugation and their density distribution profile was determined by the phthalate method as described in Fig. 1.

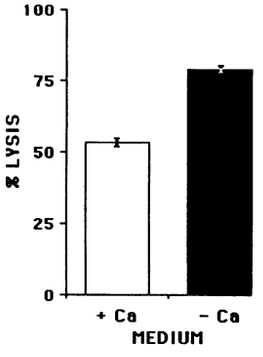

Figure 7. Effect of removal of external Ca^{2+} on complement-mediated lysis of EA. Antibody-sensitized human EA were exposed for 30 min at 37°C to a 1:4 dilution of human C8-deficient serum in Na-GVBD^{2+}. Then the cell suspension was diluted 10-fold in GVB-EGTA (2 mM EGTA), and the EAC1-7 cells were separated by centrifugation and resuspended in either Na-GVBD^{2+} or GVB^{+-}-EGTA (containing 0.5 mM $MgCl_2$ and 2 mM EGTA, but no Ca^{2+}) in the presence and absence of purified C8 (100 CH_{50}) and C9 (100 ng/ml). After 15 min at 37°C lysis was estimated as in Fig. 2 (reading hemoglobin at 414 nm). The values are mean±SE of triplicate determinations. The figure is representative of one of three experiments with similar results.

have previously reported that during complement activation the cells that survive the complement attack experience a large but transient increase of their membrane permeability, which results in an increase of the Na_i^+/K_i^+ ratio (4). An increased leak of the magnitude observed should result in cell swelling, which, surprisingly, was not observed (cf. reference 4 and Fig. 1). We reasoned that, similar to other volume regulatory mechanisms that restore volume after hypotonic swelling, activation of a selective K^+ permeability pathway could balance the net cation gain through the complement pore and therefore limit the extent of swelling and lysis. Direct evidence for the activation of a K^+ transport system operating during sublytic complement attack was not possible to obtain because (*a*) the movement of K^+ through the selective K^+ transport pathway would occur in parallel with exchange of ions through the complement pore, and (*b*) the simultaneous lysis of a subpopulation of EA releases K_i^+ to the extracellular medium.

When external Na^+ was replaced by K^+, and then complement was activated, the unlysed EA were swollen compared with control cells exposed to complement in Na^+ buffer (Fig. 1). This observation cannot be explained by different cation movement through the complement lesion because (*a*) the lesion is not selective for Na^+ or K^+ (21), and (*b*) the steady-state gradient of permeant cations ($Na_i^+ + K_i^+ < Na_0^+ + K_0^+$) maintained by the Na^+/K^+ pump would promote net cation entry independently of the ion species involved. In contrast, the observation is consistent with the possibility that net efflux of K^+ driven by the K^+ electrochemical gradient through a K^+ permeability pathway balances the net cation gain through the complement pore. The absence of the outwardly directed K^+ electrochemical gradient, the driving force of the putative K^+ transport pathway, imbalances the fluxes of cations and allows for net cation gain through the complement lesion, enhancing cell swelling and lysis (Figs. 1 and 2).

Previous work by Dalmasso et al. (13, 14) showed that complement-induced lysis of erythrocytes is significantly increased when K^+ instead of Na^+ is present in the reaction medium. To explain this observation, they proposed that (*a*) K^+ ions might enhance the binding of the terminal complement components to the target membrane, or (*b*) the complement pore could be selectively more permeable for K^+ as compared with Na^+. Further work by Sims and Wiedmer (21) showed that the binding of terminal complement components is similar in Na^+ and K^+ media and that the complement pore does not discriminate between Na^+ and K^+. Moreover, since it is known that one permanent MAC pore is sufficient to lyse an erythrocyte, changing either the number, the size, or the efficiency of the complement lesion for a given target cell could only change the kinetics of lysis, but not the total number of lysed cells, such as occurs when K^+ is replaced for Na^+ in the external medium (cf. references 13 and 14, and Fig. 2). Since we have demonstrated that sublytic complement activation induces a transient change in the permeability of unlysed erythrocytes, an alternative explanation for the K^+ effect on complement-induced lysis could be that K^+ inhibits the recovery/repair process that terminates the transient permeability change. Against such a role for external K^+ is the fact that in EA prepared to contain only internal Na^+ (high Na^+ cells), another maneuver to eliminate the outwardly directed K^+ gradient, the presence of K^+ in the external medium did not affect the magnitude of lysis, as compared with lysis of the same cells in Na^+ medium (Fig. 3). Moreover, lysis of high Na^+ cells in Na^+ medium was increased as compared with lysis of high K^+ cells in the Na^+ buffer, indicating that what indeed influences lysis is not the cation in the external medium but the presence or absence of an outwardly directed K^+ gradient. Furthermore, lysis by complement is linearly proportional to the magnitude of the K^+ gradient (Fig. 4). Several types of cation transport pathways have been described in the human erythrocyte membrane, including the Na^+/K^+ pump, the $Na^+/K^+/Cl^-$ and the K^+/Cl^- cotransport systems, and the Ca^{2+}-activated K^+ transport pathway (7, 10, 12, 15–17). Ouabain (0.1 mM) and bumetanide (10 μM), specific inhibitors of the Na^+/K^+ pump and the $Na^+/K^+/Cl^-$ cotransport, respectively, failed to influence the level of complement lysis, indicating that these transport systems are not involved in protecting erythrocytes from complement lysis. Similar results with ouabain were also reported by de Bracco and Dalmasso (22). The K^+/Cl^- cotransport system is present in the least dense but not in the denser fraction of human erythrocytes (12). Experiments with cells separated by density using Stractan density gradients showed that the increased lysis in K^+ as compared with Na^+ media was present in both the least dense and the denser fractions of cells (results not shown), indicating that the KCl cotransport was unlikely to participate in the volume regulatory response triggered by complement activation.

Finally, an increase in the K^+ permeability can be triggered by raising the intracellular Ca^+ concentration. Calcium is known to be freely diffusible through the fixed complement pore. In fact, a significant increase in the intracellular Ca^{2+} concentration can be detected even before any measurable release of other ions and macromolecules and can be detected at the C5b-8 stage (23). Presumably, Ca^{2+} also diffuses through the transient complement lesion, which is known to allow for the entry of both Na^+ and glucose into the cell (4). Indirect evidence for a role of the Ca^{2+}-induced K^+ transport pathway was provided using carbocyanine, a specific inhibitor of this K^+ transport pathway. The dye not only increased the amount of complement lysis (Fig. 5), but also promoted swelling of the unlysed erythrocytes (Fig. 6). The use of sheep erythrocytes of the high K^+ type, a cell devoid of the Ca^{2+}-induced K^+ transport system, provided an important control indicating that the dye is not acting on the complement system, nor is it enhancing complement-mediated lysis by its lipophilic effect on the EA membranes, independently of its inhibitory action on the Ca^{2+}-activated K^+ transport system. Such a detergentlike effect of the dye would also have increased the complement-me-

diated lysis of EA in high K medium, which was not observed (cf. Fig. 5). The failure of quinine and quinidine, other known inhibitors of the Ca^{2+}-activated K^+ transport pathway, to influence complement-mediated lysis is not clearly understood but could be related to their failure to completely abolish, at the concentrations used, the Ca^{2+}- plus A23187-induced K^+ efflux from EA. The use of higher concentrations to clarify this point resulted in cell damage expressed as spontaneous lysis of the cells.

The use of C8-deficient serum and purified terminal complement components allowed us to induce complement-mediated lysis in the presence and absence of external Ca^{2+}. The enhancement of lysis observed when Ca was removed from the lysis medium (Fig. 7) provides strong support to the concept that Ca^{2+} entry through the complement pore activates a K^+ transport pathway that balances the cation gain through the complement pore, thereby preventing colloidosmotic swelling and lysis.

Previously described mechanisms by which erythrocytes circumvent complement damage involve the inhibition of complement activation/binding at the cell membrane (reviewed in reference 24). The findings presented here represent the first description of a different type of protective mechanism of erythrocytes against complement lysis, namely, the operation of a Ca^{2+}-activated K^+ transport pathway, stimulated by a rise in cytosolic Ca^{2+}, that balances the change in the internal cation composition induced by the complement lesion. A similar role for the Ca^{2+}-activated K^+ transport pathway in modulating cell cation content and volume has been proposed for human lymphocytes (25) and for cells of the choroidal plexus epithelium (26). Protection against complement damage represents the first physiological role proposed for this transport system in the human erythrocyte, the cell in which this transport pathway was originally described by Gardos 31 years ago.

Acknowledgments

The authors are grateful to Dr. Daniel C. Tosteson and Dr. Magdalena T. Tosteson for their significant contribution to this work. We thank Alane Taratuska and Rosemarie Sime for their excellent technical assistance.

This work was supported by National Institutes of Health grants NS-23367, HL-33768, HL-36076, and 2-P60-HL-15157. Dr. Nicholson-Weller is a Scholar of the Leukemia Society of America.

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