The Influence of Electrochemical Gradients of Na\(^+\) and K\(^+\) upon the Membrane Binding and Pore Forming Activity of the Terminal Complement Proteins

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Summary. The hemolytic activity of the terminal complement proteins (C5b-9) towards erythrocytes containing high potassium concentration has been reported to be dramatically increased when extracellular Na\(^+\) is substituted isotonically by K\(^+\) (Dalmasso, A.P., et al., 1975, J. Immunol. 115:63-68). This phenomenon was now further investigated using resealed human erythrocyte ghosts (ghosts), which can be maintained at a nonlytic osmotic steady state subsequent to C5b-9 binding: (1) The functional state of C5b-9-treated ghosts was studied from their ability to retain trapped [\(^{14}\)C]-sucrose or [\(^{3}\)H]-inulin when suspended either in the presence of Na\(^+\) or K\(^+\). A dramatic increase in the permeability of the ghost membrane to both nonelectrolytes - in the absence of significant hemoglobin release - was observed for C5b-9 assembly in the presence of external K\(^+\). (2) The physical binding of the individual \(^{125}\)I-labeled terminal complement proteins to ghost membranes was directly measured as a function of intra- and extracellular K\(^+\) and Na\(^+\). The uptake of \(^{125}\)I-C7, \(^{125}\)I-C8, and \(^{125}\)I-C9 into membrane C5b-9 was unaltered by substitution of Na\(^+\) by K\(^+\). (3) The binding of the terminal complement proteins to ghosts subjected to a transient membrane potential generated by the K\(^+\)-ionophore valinomycin (in the presence of K\(^+\) concentration gradients) was measured. No significant change in membrane binding of any of the C5b-9 proteins was detected under the influence of both depolarizing and hyperpolarizing membrane potentials. It can be concluded that the differential effect of Na\(^+\) versus K\(^+\) upon the erythrocyte membrane is not due to an effect upon the binding of the complement proteins to the membrane per se, but upon the functional properties of the assembled C5b-9 pore site.

Key Words membrane pore · complement · membrane permeability · erythrocyte ghost · membrane potential

Introduction

The cytolytic activity of the serum complement system resides in the capacity of the five terminal proteins - C5b, C6, C7, C8, and C9 - to bind to bilayer lipid and increase membrane permeability to aqueous solute, thereby collapsing transmembrane electrochemical gradients [9, 20]. The equilibration of Na\(^+\) and K\(^+\) across the C5b-9 damaged plasma membrane is accompanied by the colloid-osmotic expansion of cell water, leading ultimately to its hydrostatic rupture and cell death [13, 19]. Evidence for a direct membranolytic activity of these proteins (independent of osmotic water movement) has also been considered [10, 32]. Because the ability of the C5b-9 proteins to collapse transmembrane electrochemical gradients is integral to their cytolytic function, it is of particular interest to explore how the interaction of these molecules with the plasma membrane is influenced by the ionic milieu of the cell, including physiological electrochemical gradients. In early studies undertaken with antibody-sensitized red blood cells (RBC), it was noted that the hemolytic activity of the C5b-9 proteins towards erythrocytes containing high potassium concentrations (HK RBC) was dramatically augmented by isotonic substitution of Na\(^+\) by K\(^+\) in the suspending medium [2, 4, 6]. By comparison, the lytic activity of these proteins towards erythrocytes with high Na\(^+\) content (LK RBC) was always increased with respect to HK RBC and was only slightly influenced by the extracellular cation [2, 4, 6]. The effect of hypotonic and hypertonic media on the complement-mediated hemolysis of sheep HK and LK RBC has been extensively studied by Lauf [18, 19].

In a recent study undertaken by Dalmasso and colleagues [7], it was demonstrated that the effect of extracellular cations upon the hemolysis of antibody-sensitized HK RBC exposed to the complement proteins occurred subsequent to C5b67 assembly, either in the binding of C8 and C9 to the membrane site, or, in their consequent interaction with the membrane leading to cytolysis. These results raise the intriguing possibilities that Na\(^+\) and K\(^+\) exert differential effects upon the physical and...
chemical properties of these pore-forming proteins or, alternatively, upon the physiological changes experienced by the immune-damaged membrane subsequent to C5b-9 pore formation.

In order to gain additional insight into how ion gradients across biological membranes might influence the functional activity of these key serum proteins, we have directly measured both the quantitative physical binding and the membrane pore-forming function of the isolated C5b-9 proteins, when added to a model membrane system (resealed human erythrocyte ghost) which can be maintained under nonlytic conditions in both Na+ and K+ containing media [28].

Materials and Methods

Solutions

All solutions were freshly prepared using H2O obtained by reverse osmosis and ultrafiltration (Millipore, Boston, MA). Except where indicated otherwise, all buffers contained 0.02% NaN3. KCl-Tris: 165 mM KCl, 2 mM Tris, pH 7.2. NaCl-Tris: 165 mM NaCl, 2 mM Tris, pH 7.2. KCl-Tris (NaCl-Tris)-albumin: KCl-Tris (or NaCl-Tris) made 0.5% (wt/vol) in fatty acid-free albumin (Sigma, St. Louis, MO). K-sucrose-cushion: 45% (wt/vol) sucrose in 50 mM KCl, 25 mM Tris, pH 7.2. Na-sucrose-cushion: 45% (wt/vol) sucrose in 50 mM NaCl, 25 mM Tris, pH 7.2. When indicated (see below), solutions were made 10-5 M in valinomycin (Sigma) by addition from 0.33 mg/ml ethanol stock.

Radiochemicals

Na125I was obtained in dilute NaOH solution from Amersham (Chicago, IL). [14C]-sucrose (sucrose [14C], mol wt 342.3, sp act 342.3 mCi/mmol) and [3H]-inulin (inulin, [methoxy-3H], mol wt 5000–5500, sp act 140.5 mCi/g) were obtained from New England Nuclear (Boston, MA).

Complement Proteins

Human complement proteins C5b, C6, C7, C8, and C9 were purified and assayed for functional (hemolytic) and binding activity according to the methods described in Ref. [27].

Radiolabeling of C7, C8, and C9 with Na125I was performed in azide-free phosphate buffered solution at 2 °C using the solid phase glucose oxidase-lactoperoxidase system (Enzymobeads®, BioRad). Unreacted radiolabel was removed by chromatography on Sephadex G25 (PD10 column, Pharmacia). In the case of 125I-C9, the material was subsequently rechromatographed on S200 (0.9 × 60 cm, Pharmacia) to remove trace high molecular weight aggregates found to be generated during labeling. Fatty acid-free bovine albumin carrier was added to 2% (wt/vol) before storage in glycerol (40% wt/vol) at -20 °C. Specific activities of (0.1 to 7) × 106 cpm/μg protein were achieved. The functional activity of each labeled protein was evaluated by methods described in Ref. [27]. In no case did reduction of binding or functional activity of the labeled protein exceed 10%.

Resealed Erythrocyte Ghosts

Resealed ghost membranes of human erythrocytes (ghosts) were prepared by reversible hypotonic hemolysis at 0 °C in the presence of 4 mM MgSO4 according to published methods [11, 25, 28]. Botoncity was restored to 165 mm KCl, 2 mm Tris, pH 7.0–7.2 (HK ghosts) or to 82.5 mm KCl, 82.5 mm NaCl, pH 7.0–7.2 (LK ghosts). Following rescaling (60 min, 37 °C) the sucrose-impermeant ghosts were isolated by density flotation (90 min, 45,000 × g) on either a K-sucrose-(HK ghosts) or Na-sucrose-(LK ghosts) cushion. After washing into either KCl-Tris (HK ghosts) or NaCl-Tris (LK ghosts), the ghosts were suspended to 5 × 109/ml in the same solution. The ghosts were rescaled to and retained 10–14 g hemoglobin per liter cell H2O. For certain experiments, trace quantities (0.51 mCi per liter cell water) of [14C]-sucrose and [3H]-inulin were incorporated into the ghosts prior to rescaling, according to methods previously described [29]. After rescaling, the ghosts were maintained at 0–4 °C and used within 18 hr.

C5b67 Ghosts

1.0 mg C7 was added to each ml of ghost suspension (5 × 108 cells) and the ghosts were rapidly mixed with an equal volume of either KCl-Tris or NaCl-Tris containing 0–1.35 mg/ml C5b6. Following incubation (15 min, 37 °C), the suspension was placed on ice, diluted 20-fold with ice-cold KCl-Tris-albumin (or NaCl-Tris-albumin), and centrifuged (5 min, 45,000 × g) at 2 °C. After two additional washes in the same buffer, the C5b67 ghosts were suspended to 2.5 × 109/ml in either KCl-Tris-albumin or NaCl-Tris-albumin. In certain experiments membrane binding of C5b67 was quantitated from the measured uptake of 125I-C7 during incubation with C5b6, after correction for nonspecific binding of radioactivity to control ghosts exposed only to 125I-C7 (in absence of C5b6). Under these conditions, binding of 125I-C7 to control ghosts always measured less than 0.5% of input and less than 4% of the total uptake measured for ghosts suspended in the presence of C5b6.

C5b9 Ghosts

C5b-9 ghosts were prepared by incubating (60 min, 37 °C) 200 μl volumes of C5b67 ghost suspension (5 × 108 cells) with various quantities of C8 and C9 (see text) in a total volume of 0.5 ml KCl-Tris-albumin or NaCl-Tris-albumin. In certain experiments, 125I-C8 was substituted for C8, or, 125I-C9 for C9. To minimize possible C5b-8 inactivation before C9 binding, the C5b67 ghosts were routinely suspended with C9 (or 125I-C9) before C8 was added [22]. After incubation with C8 and C9, a 50-μl aliquot of each cell suspension was saved and the remaining cells were packed by centrifugation (Beckman Microfuge 12, 15 min, 4 °C). The supernatant was aspirated and saved and each cell pellet washed at 4 °C with 4 × 1 ml vols of either KCl-Tris-albumin or NaCl-Tris-albumin. The washed pellets were lysed in detergent (1% Nonidet P40, Sigma) and saved for assay by gamma counting.

Determination of Specific 125I-C8 and 125I-C9 Uptake

The specific binding of 125I-C8 (in the presence of unlabeled C9) and 125I-C9 (in the presence of unlabeled C8) to C5b67 ghosts was determined on the basis of measured cell-bound radioactivity and cell number (after correction for nonspecific