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Comparison of the activity of polyanions and polycations on the classical and alternative pathways of complement

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Abstract: Polyanions and polycations inhibit activity of the alternative and classical pathways of complement. We compared polyanions (commercial porcine heparin, chondroitin sulfate A, chondroitin sulfate B (dermatan sulfate), chondroitin sulfate C and heparatin sulfate) with polycations (salmon sperm protamine sulfate, poly-L-lysine, poly-L-arginine, polybrene and a synthetically prepared portion of platelet factor 4) for ability to inhibit alternative and classical pathway activity. The polyanions had considerably more activity on the alternative than on the classical pathway, whereas the polycations more profoundly inhibited classical than alternative pathway activity. For example, heparin, a polyanion, at 1.0 μg (7.7×10^{-7} M based upon an M_r average of 13000)/10^7 cellular intermediates, inhibited alternative pathway activity and classical pathway activity by 77 and 14%, respectively, whereas protamine sulfate, a polycation, at 0.25 μg/10^7 cellular intermediates, inhibited these two pathways by 34 and 98%, respectively. These studies suggest that the capacity to inhibit complement activity is a common feature of highly charged substances and the polyanions preferentially inhibit the alternative pathway while polycations preferentially inhibit the classical pathway. In vivo these highly charged substances could play an important role in the tissues in regulating the activity of both pathways of complement.

Key words: Polyion; Polyanion; Polycation; Classical pathway; Alternative pathway

Introduction

Polyionic substances have the ability to regulate complement activation. Heparin, a polyanion, and its fragments, inhibit generation of fluid-phase alternative pathway C3 convertase and cell-bound classical and alternative pathway C3 convertases (Ecker and Gross, 1929; Raepple et al., 1976; Loos et al., 1976a; Weiler et al., 1978; Kazatchkine et al., 1981; Sharath et al., 1985; Linhardt et al., 1988). Protamine sulfate, a polycation, inhibits fluid-phase and cell-bound C3 convertases as well as the activity of a preformed C3 convertase (Weiler, 1983). A variety of other polyions have also been shown to have the capacity to inhibit complement activity (Baker et al., 1975; Raepple et al., 1976; Kazatchkine et al., 1981; Weiler and Gleich, 1988). Polyions have been shown to be capable of potentiating the action of C1 esterase inhibitor (Caughman et al., 1982), interfering with Clq binding to immune complexes (Raepple et al., 1976), inhibiting Cls interac-

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Abbreviations: EDTA, ethylenediamine tetraacetate; GVB, half-isotonic veronal-buffered saline, pH 7.5, containing 0.1% gelatin; DGVB^{++}, GVB containing 2.5% dextrose, 0.5 mM magnesium and 0.15 mM calcium; EDTA-GVB, GVB containing 40 mM EDTA; RC-EDTA, rat serum diluted 1:20 in EDTA-GVB; gpC-EDTA, guinea pig serum, diluted 1:15 in EDTA-GVB; *Z*, the average number of hemolytic sites per cell; C2, C3, C4, C5, C6, C7, C8, C9, classical pathway complement components; B, P, D, alternative pathway complement components; EAC1,4b and EAC4b,3b, classical and alternative pathway cellular intermediates.

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tion with C4 and C2 (Loos et al., 1976b), enhancing or suppressing reactive lysis (Baker et al., 1975, 1976), inhibiting generation of cell-bound or fluid-phase alternative pathway convertase, inhibiting preformed alternative pathway convertase, and impairing factor H-mediated decay of the alternative pathway convertase (Weiler, 1983).

The present studies were designed (a) to compare the inhibitory effects of a variety of polyanions and polycations on both pathways of complement and (b) to determine whether polyions share a common ability to regulate convertase activity. These studies suggest that the capacity to inhibit complement activity is shared by most charged substances. Furthermore, polyanions preferentially inhibited the alternative pathway and polycations preferentially inhibited the classical pathway of complement. Activity of the polyions increased with an increase in net charge of the molecule. Thus, highly charged molecules in vivo could serve to regulate both pathways of complement, with polyanions inhibiting the alternative pathway and polycations suppressing the classical pathway.

Materials and Methods

Polyions

Polyanions. Preservative-free heparin was obtained from Fellows Medical Divisions, Chromalloy Pharmaceuticals, Oak Park, MI, and from Hepar Industries, Franklin, OH, and quantitated as previously described (Weiler et al., 1978; Sharath et al., 1985), Heparatin sulfate (heparan monosulfate, bovine kidney) and chondroitin sulfate B (porcine skin) were obtained from ICN ImmunoBiologicals, Lisle, IL. Chondroitin sulfate A (whale cartilage) and chondroitin sulfate C (shark cartilage) were obtained from Sigma Chemical Company, St. Louis, MO. All these polyanions are polydisperse and range in M_r from 10000 to 30000 (Linhardt et al., 1986).

Polycations. Protamine sulfate, poly-L-arginine HBr 40000, poly-L-lysine HBr 15000, poly-L-lysine HBr 3700 and polybrene (M_r average 5000 to 10000) were obtained from Sigma.

PF4-13, a polycation, which contains the carboxy-terminal 13 amino acids of platelet factor 4 (Osterman et al., 1982), was synthesized using solid-phase peptide (Merrifield, 1963) chemistry on a BioSearch model 9500 automated peptide synthesizer (BioSearch, San Rafael, CA) on 1 g of 4-methylbenzhydrylamine resin using a *t*-boc protection scheme (Merrifield, 1963; Barany and Merrifield, 1984). We monitored the growing peptide using a ninhydrin assay and cleaved the peptide from resin using a standard hydrogen fluoride protocol (Stewart and Young, 1984; Houghten et al., 1986). Once the anisole scavenger had been removed by ether extraction, the peptide was eluted from the resin using 15% acetic acid and freeze-dried. The crude peptide was then purified using reverse-phase high-performance liquid chromatograhy on a C_{18} column (19 mm × 15 cm μBondaPak C_{18} column (Waters Corp., Milford, MA); 4 ml/min; gradient, 100% H_2O containing 0.1% trifluoroacetic acid to 90% acetonitrile containing 0.04% trifluoroacetic acid over 40 min). The major peak was then pooled, freeze-dried and shown to have a single peak on reverse-phase high-performance liquid chromatography and the desired composition on amino acid analysis.

Complement buffers and components

Buffers used in the hemolytic assays were: half-isotonic veronal-buffered saline, pH 7.5, containing 0.1% gelatin (GVB), GVB containing 2.5% dextrose, 0.5 mM magnesium and 0.15 mM calcium ($DGVB^{++}$) and GVB containing 40 mM ethylenediamine tetraacetate (EDTA) (EDTA-GVB). C3, B, D and P were purified to homogeneity and quantitated as previously described (Tack and Prahl, 1976; Hunsicker, 1973; Fearon and Austen, 1975, 1977). Rat serum, as a source of terminal components for alternative pathway assays, was purchased from Rockland, Inc. (Gilbertville, PA) and diluted 1:20 in EDTA-GVB (RC-EDTA). Guinea pig serum, as a source of terminal components for classical pathway assays, was purchased from

Rockland and diluted 1:15 in EDTA-GVB (gpC-EDTA).

Complement assays

EAC1,4b and EAC4b,3b cellular intermediates were prepared as described (Fearon et al., 1973; Harrison and Lachmann, 1986). Complement assays employed these cellular intermediates to examine the ability of the polyions to inhibit cell-bound classical and alternative pathway convertases as previously described (Weiler et al., 1978; Weiler, 1983).

Briefly, the classical pathway assay using purified components was performed by incubating tubes containing a reaction mixture consisting of 1×10^7 EAC1,4b, guinea pig C2 pre-titered to provide an average of one hemolytic event per cell (one Z of lysis) when all other components were in excess (Nelson et al., 1966) and 10 units each of C3, C5, C6 and C7 (Diamedix Corporation, Miami, FL), with and without polyion in 0.4 ml $DGVB^{++}$. After 30 min at 30°C in a shaking water bath, 10 units each of C8 and C9 (Diamedix) in 0.1 ml $DGVB^{++}$ were added to each tube and the incubation was continued for an additional 60 min at 37°C. Then 1.5 ml saline was added to each tube except the 100% lysis tube, which received water. The tubes were shaken, centrifuged and lysis was determined by measuring the optical density of the supernatant at 414 nm to determine the average number of hemolytic sites (Z) per cell. Reagent blank tubes contained cellular intermediates and complement except that C2 was omitted. Non-inhibited tubes contained cellular intermediates and complement but no polyion and were constituted so that there would be one Z of lysis.

The classical pathway assay was also run using serum (gpC-EDTA) as a source of terminal components. Tubes containing 1×10^7 EAC1,4b and guinea pig C2 (pre-titered to provide one Z of lysis) were incubated for the t_{max} at 30°C with and without polyion. Then 0.3 ml gpC-EDTA was added to each tube and the incubation was continued for an additional 60 min at 37°C. Finally, 1.5 ml saline was added and lysis was determined as described above.

Briefly, activity on the alternative pathway was assessed using EAC4b,3b produced by incubating 1×10^9 EAC1,4b with an excess of guinea pig C2 and 100 μg C3. We incubated 1×10^7 EAC4b,3b with approximately 0.02 ng factor B (limited factor B), 250 ng factor P, and an excess of factor D and various concentrations of polyion in 0.2 ml $DGVB^{++}$ for 30 min at 30°C in a shaking water bath. Then 0.3 ml of RC-EDTA was added to each tube and the incubation was continued for an additional 60 min at 37°C. Next, 1.5 ml saline was added to each tube except the 100% lysis tube, which received water. The tubes were then shaken, centrifuged and lysis was determined by measuring the optical density of the supernatant at 414 nm to determine Z. Reagent blank tubes contained cellular

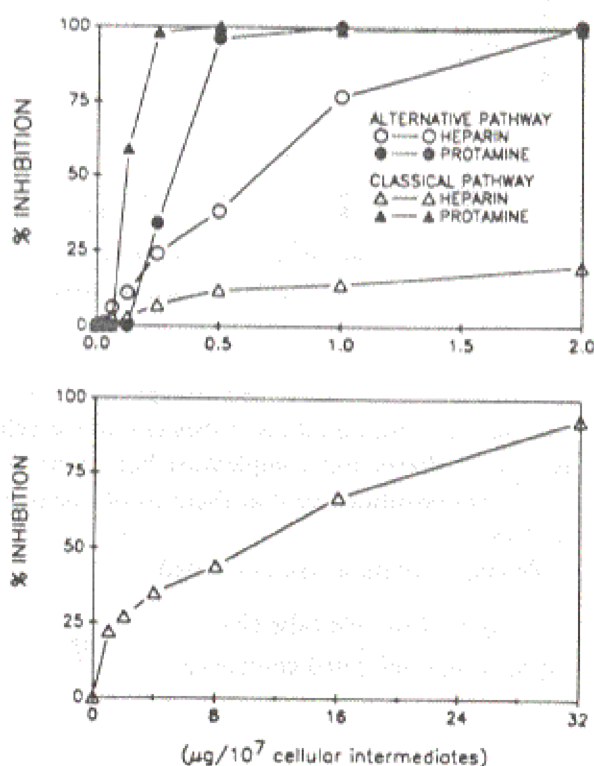

Fig. 1. Inhibition of alternative and classical pathways by heparin and protamine. The top graph demonstrates that heparin more profoundly inhibited the alternative pathway than the classical pathway, while protamine more profoundly inhibited the classical pathway than the alternative pathway. The bottom graph demonstrates that heparin is capable of profoundly inhibiting classical pathway convertase if sufficient heparin is present.

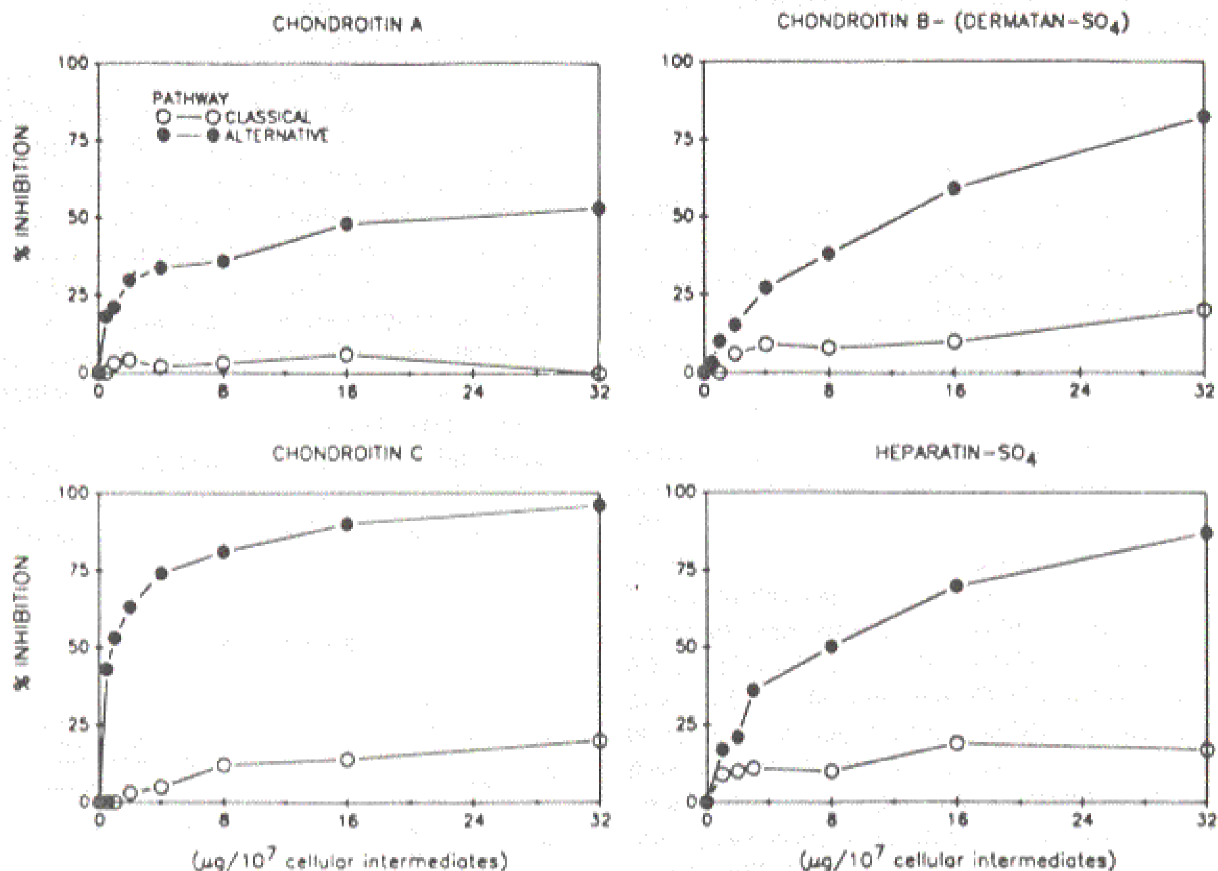

Fig. 2. Inhibition of alternative and classical pathways by various polyanions.

intermediates and complement except that factor B was omitted. Non-inhibited tubes contained cellular intermediates and complement but no polyion and were constituted so that there would be one Z of lysis.

Percent inhibition was calculated:

$$\frac{\text{lysis of sample tube }(Z)}{\text{lysis of non-inhibited control }(Z)} \times 100$$

Results

The polyanions, heparin, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C and heparatin sulfate, all acted in a dose-dependent manner to inhibit effective alternative pathway convertase activity (Figs. 1 and 2). None of these polyanions had more than minimal activity to inhibit classical pathway lysis unless the dose of polyion was increased substantially over the dose necessary to inhibit the alternative pathway. Heparin, for example, at 1 μg (7.7×10^{-7} M)/10^7 EAC4b,3b inhibited the alternative pathway by 77%, whereas it required 16 μg (1.2×10^{-5} M) heparin/10^7 EAC1,4b to inhibit the classical pathway by 67%.

The polycations, protamine sulfate, polybrene, poly-L-lysine HBr 3700, poly-L-lysine HBr 15000, poly-L-arginine HBr 40000 and PF4-13, all acted in a dose-dependent manner to inhibit the classical pathway, with less or no effect on the alternative pathway of complement (Figs. 1 and 3). For example, poly-L-lysine HBr 3700, at 16 μg (4.3×10^{-5} M)/10^7 cellular intermediates, inhibited classical pathway lysis by 91% but had no activity on the alternative pathway. Larger poly-L-lysine and poly-

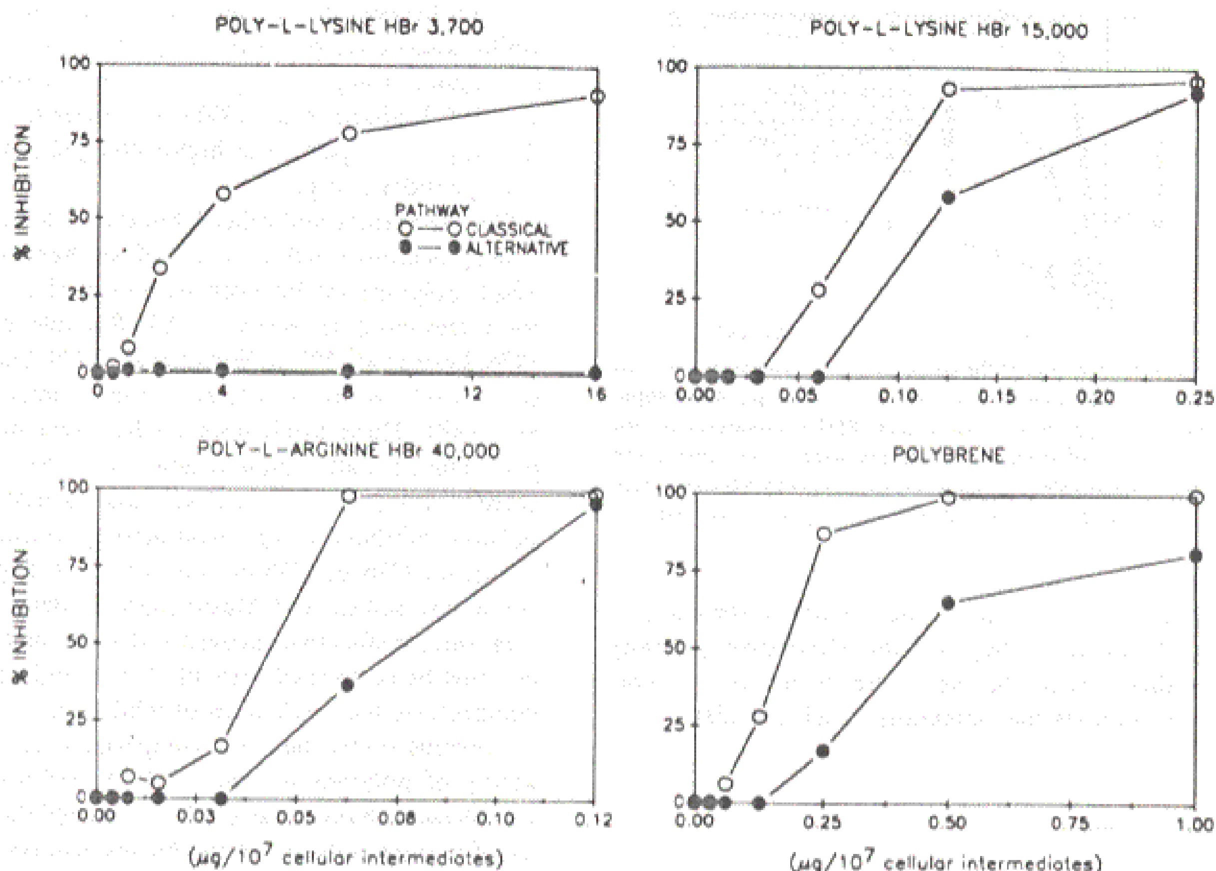

Fig. 3. Inhibition of alternative and classical pathways by various polycations. Sixteen micrograms of poly-L-lysine 3700 is 4.3×10^{-5} M; 0.25 µg poly-L-lysine 15000 is 1.7×10^{-7} M; 0.12 µg poly-L-arginine is 3×10^{-8} M; and 1 µg polybrene is $1\text{-}2 \times 10^{-6}$ M (based upon M_r average for polybrene of 5000 to 10000).

L-arginine molecules inhibited lysis of both pathways but the classical pathway was always more profoundly inhibited at a given dose (Fig. 3). PF4-13 inhibited lysis of the classical pathway in a dose-dependent manner but had no activity on the alternative pathway at any dose tested (Fig. 4).

The classical pathway assay used purified proteins as a source of terminal components (C3-C9), whereas the alternative pathway assay used EDTA-treated serum. Therefore, we investigated whether the source of the terminal components had any effect on the ability of these polyions to inhibit lysis (Fig. 5). Both heparin and protamine sulfate had comparable amounts of inhibition when either purified components (C3-C9) or serum (gpC-EDTA) was used as a source of terminal components to lyse the cells by the classical pathway. Thus, the source of the terminal components did not have any effect on the ability of these polyions to inhibit lysis. Furthermore, this experiment clearly indicates that

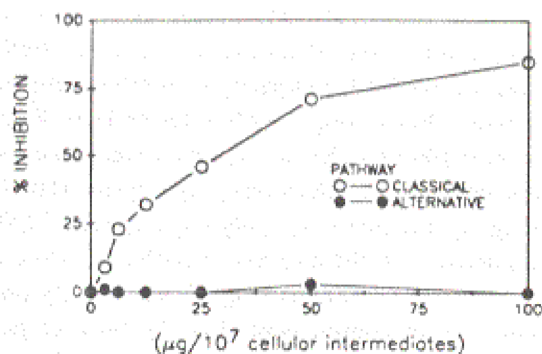

Fig. 4. Inhibition of alternative and classical pathways by PF4-13, the carboxy-terminal 13 amino acids of platelet factor 4. One hundred micrograms of PF4-13 is 6.7×10^{-4} M.

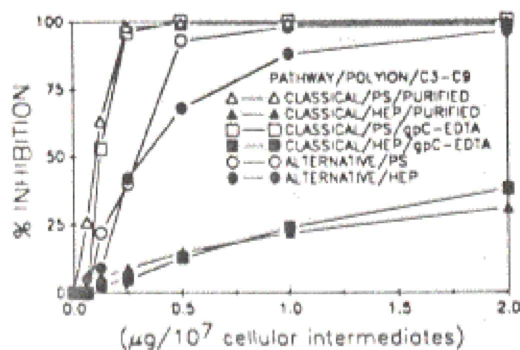

Fig. 5. Comparison of the ability of heparin and protamine sulfate to inhibit classical pathway activity when purified components (triangles) or when serum (gpC-EDTA) (squares) was used as a source of terminal components; capacity of heparin and protamine sulfate to inhibit alternative pathway when rat serum was used as a source of terminal components (circles).

polycations more profoundly inhibited the classical pathway than they did the alternative pathway without regard to the source of the components used to form the membrane attack complex.

Discussion

These studies demonstrate that the capacity to regulate complement activity is shared by a wide variety of highly charged molecules (Figs. 1–4). The present studies also show that polycations more profoundly inhibited classical than alternative pathway activity (Figs. 1 and 3); conversely polyanions more profoundly inhibited alternative than classical pathway activity (Figs. 1 and 2).

These studies also show that as the size of the polycations, and therefore the charge itself, increased, so did the capacity to inhibit complement activity. For example, poly-L-lysine HBr 3700 was at least 100-times less active on a weight basis on the classical pathway than was poly-L-lysine HBr 15000 (Fig. 3). Furthermore, poly-L-lysine HBr 3700 had no activity on the alternative pathway, while poly-L-lysine HBr 15000 had significant activity on that pathway of complement (Fig. 3). Smaller poly-L-lysine molecules (7-mers and smaller) had no activity on either pathway of complement (data not shown). We have previously demonstrated a relationship between polyanion size and the ability to inhibit alternative pathway complement activity in our studies of heparin oligosaccharides (Sharath et al., 1985; Linhardt et al., 1988). This same relationship appears to pertain to highly charged polycationic peptides and proteins.

Our laboratory and other laboratories have previously demonstrated a variety of mechanisms by which polyions inhibit both pathways of complement (reviewed in Weiler, 1983; Raepple et al., 1976; Loos et al., 1976a; Weiler et al., 1978; Sharath et al., 1985; Weiler and Gleich, 1988; Linhardt et al., 1988). These mechanism studies were not repeated here for all of these additional polyions presented in this paper. It seems likely that each of these polyions inhibited complement activity through an action on C3b or C4b as was seen with heparin, protamine sulfate and Major Basic Protein; however, this remains to be proved and additional studies must be performed with all of these polyions to demonstrate the mechanism of inhibition.

Previous studies have not compared in detail the ability of polyions to regulate both pathways of complement. Baker et al. (1975) compared the ability of various polyions to inhibit reactive lysis and found that heparin at 2 μg/ml inhibits reactive lysis by 50%. In contrast, protamine sulfate or poly-L-lysine 178000, as well as a number of other polycations, did not in their study inhibit reactive lysis and may have augmented lysis. Kazatchkine et al. (1981) examined the ability of an assortment of polyanions such as heparin to inhibit the alternative pathway of complement and showed that all of the chondroitin sulfates are less active on the alternative pathway than is heparin. These investigators in this paper, however, did not look at the activity of these polyanions on the classical pathway. Raepple et al. (1976) showed that eight polyanions inhibit fluid-phase C1 activity but not the activity of C4 or C2. In their studies, some polyanions were able to inhibit fluid-phase C1 activity much more efficiently than cell-bound C1, whereas other polyanions inhibited fluid-phase C1 and cell-bound C1 with similar efficiency. Loos et al. (1976a,b) extended these studies to examine the mechanism by which polyanions inhibit the classical pathway of complement.

They reported that polyanions prevent the uptake of C2 by EAC4b and the consumption of C2 and C4 by C1s̄ and cause EAC4b,2a to dissociate into EAC4b and C2.

Takada et al. (1987) showed that simple cations (e.g. arginine and lysine) inhibit the conversion of C3 and B in a mixture which also contains factors D and magnesium, whereas the acidic amino acids, aspartic acid and glutamic acid, do not have this effect (Shirahama et al., 1986). They suggested that lysine and arginine prevent the effective interaction of B with C3. These authors only looked at chemical conversion and did not look at hemolytic activity or at the ability of these simple cations to inhibit cell-bound convertase activity. We did not see any effect of simple or small polycations (polylysine or polyarginine polymers as long as 7 amino acids) on the ability to form either classical or alternative pathway cell-bound convertase. Taken together, these previous studies are consistent with our current observations regarding the activity of the polyions on complement activity.

Although not all of the molecules which we have tested exist at the tissue level, many charged molecules are present in the tissues (including heparin, protamine sulfate, Major Basic Protein and the chondroitin sulfates). These highly charged molecules are found in areas in which complement activation occurs and in which complement activation may play an important role in the immune and inflammatory response in vivo. Therefore, highly charged molecules may be important in regulating complement activity. The polycationic proteins (e.g. protamine sulfate and Major Basic Protein) might be expected to inhibit classical pathway complement activity, whereas the polyanions (e.g. heparin or the chondroitin sulfates) might be expected to inhibit alternative pathway activity. Thus, the present studies suggest that highly charged molecules may as a class act to regulate complement activity in a very complex manner.

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