SURFACE-ASSOCIATED HEPARIN INHIBITS
ZYMOSAN-INDUCED ACTIVATION OF THE HUMAN
ALTERNATIVE COMPLEMENT PATHWAY BY AUGMENTING
THE REGULATORY ACTION OF THE CONTROL PROTEINS
ON PARTICLE-BOUND C3b*

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The alternative pathway of human complement serves as a humoral recognition
mechanism in a nonimmune host for cell surfaces presented by a variety of bacteria
(1-4), fungi (5, 6), parasites (7), and heterologous (8-10), virus-infected (11, 12), or
neoplastic (13, 14) mammalian cells. The continuous low-grade interaction of the
alternative pathway components C3, B, D, and properdin (P)1 (15, 16) generates the
major cleavage fragment of C3, C3b, which may attach covalently to bystander
surfaces (17). Particle-bound C3b binds B in a Mg++-dependent reaction (18) and
subsequent cleavage of B by D generates the amplification convertase, C3b,Bb (19,
20), which is stabilized by the uptake of P (21). Whether a particle is activating or
nonactivating is determined by the relative capacity of its surface to assemble and
maintain the function of the amplification convertase in the presence of the control
proteins, β1H and C3b inactivator (C3bINA) (22, 23). One biochemical characteristic
that determines the capacity of a particle surface to activate the human alternative
complement pathway is a relative deficiency of sialic acid (24, 25). Sialic acid
promotes high-affinity association between particle-bound C3b and β1H without
influencing the affinity of C3b for B, and thus impairs the formation of the amplifi-

* Supported in part by grants AI-07722, AM-08816, and RR-05669 from the National Institutes of
Health, and in part by the Medical Research Service of the Veterans Administration.
‡ Postdoctoral fellow of the Delegation Generale la Recherche Scientifique et Technique, France.
§ Recipient of a Research Career Development Award K04 AI 00245 from the National Institutes of
Health.
1 Abbreviations used in this paper: C3bINA, C3b inactivator; CNBr, cyanogen bromide; DGVB**, half-
isotonic GVB** with 2.5% dextrose; GVB**, GVB containing 0.15 mM calcium and 0.5 mM magnesium; GVB-EDTA, GVB containing 0.04 M EDTA; GVB-Mg-EGTA, GVB containing 2 mM magnesium and 8 mM EGTA; NHS-EDTA, normal human
serum diluted in EDTA; NHS-Mg-EGTA, normal human serum diluted 1:4 in GVB-Mg-EGTA; P,
properdin; PBS**, phosphate-buffered saline containing 0.6 mM Ca++ and 0.4 mM Mg**; Sepharose-
CNBr, CNBr-activated Sepharose lacking heparin; Sepharose-heparin, CNBr-activated Sepharose with
coupled heparin; Zy, zymosan; Zy,C3b; C3b-bearing Zy particles; Zy-CNBr, CNBr-activated Zy particles
lacking heparin; Zy-heparin, CNBr-activated Zy particles with coupled heparin.
cation convertase at the C3b site (26). In addition, β1H displaces Bb, the catalytic unit, from any amplification convertase sites that may have formed (27, 28) and augments cleavage inactivation of particle-bound C3b by C3bINA (27, 29). The inverse relationship between the membrane content of sialic acid and the capacity of the cell to activate the alternative pathway by deposition and maintenance of the amplification convertase on the surface was established by demonstrating the effects of chemical modification (24) or enzymatic removal (24, 25) of this moiety on sheep erythrocytes, and was confirmed by showing the same relationship with natural, genetically determined variations of sialic acid on mouse erythrocytes (9).

Although the capacity to recognize a relative deficiency of cell-surface sialic acid represents a mechanism by which the alternative pathway could lead to phagocytosis and cytolyis of a foreign cell in a nonimmune host, the failure of mammalian sera to lyse normal homologous cells indicates the existence of additional cell-surface constituents that modulate the capacity of a particle to activate the alternative pathway. The proteoglycan, heparan sulfate, has been identified as a cell-surface constituent of cultured human fibroblasts (30) and endothelial cells (31) and of certain animal (32-35) and tumor cells. Heparin resembles heparan sulfate in having glucuronic or iduronic acid in a disaccharide pair with glucosamine. Heparin and heparan sulfate differ from chondroitin sulfates, dermatan sulfate, and keratan sulfate in the nature of their glycosidic linkages and in being the only glycosaminoglycans with N-sulfation in addition to O-sulfation, although more glucosamine in heparin than in heparan sulfate is N-sulfated rather than N-acetylated (36, 37). Because of the availability of heparin, the coupling of this glycosaminoglycan to an activating particle was assessed for its effect on the activating capacity of that particle. Coupled heparin glycosaminoglycan suppressed the capacity of zymosan to activate the human alternative pathway by a dose-dependent facilitation of the regulatory action of the endogenous control proteins, β1H and C3bINA, on particle-bound C3b.

Materials and Methods

Preparation of Heparin and of Zymosan-Heparin Particles. 400 mg of commercial heparin glycosaminoglycan from hog intestine (H-3125, Sigma Chemical Co., St. Louis, Mo.) were dissolved in 4 ml of 0.01 M Tris HCl, pH 8.0, containing 1.0 M NaCl and applied to a 1- × 30-cm column of Dowex 1X2 (100-200 mesh, chloride form; Bio-Rad Laboratories, Richmond, Calif.) equilibrated with the same buffer. The column was eluted in a stepwise fashion with 0.01 M Tris, pH 8.0, containing 1.0 M and 3.0 M NaCl (38, 39). The heparin was recovered in the 3.0-M NaCl eluate, dialyzed against 10 volumes of distilled H2O for 18 h at room temperature, and concentrated to ~50 mg/ml by dialysis against polyethylene glycol, mol wt 20,000 (Fisher Scientific Co., Pittsburgh, Pa.) in a Spectrapor 1 membrane (Spectrum Medical Industries Inc., Los Angeles, Calif.). Heparin was assayed by metachromasia with azure A (Fisher Scientific Co.) (40, 41) with use of a reference curve established with commercial heparin that was linear between 10 and 100 μg/ml, and also by uronic acid content relative to a heparin standard with use of the carbazole reaction as modified by Bitter and Muir (42).

Zymosan (Zy) (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) was boiled for 45 min at 0.15 M NaCl, washed, and stored as a 25-mg/ml suspension in 0.15 M NaCl at 2°C. 125 mg of Zy in 5 ml of 0.1 M NaHCO3, pH 8.5, were incubated with an equal volume of 0.1 M NaHCO3 containing 90 mg of cyanogen bromide (CNBr) for 10 min at 4°C, during which a pH of 10.5 was maintained by addition of 2.5 M NaOH (43). The CNBr-activated Zy-particles were washed once in ice-cold distilled H2O and three times in ice-cold 0.1 M NaHCO3, resuspended in 5 ml of 0.1 M NaHCO3, and incubated with various amounts of heparin overnight at 4°C with gentle stirring. Unsubstituted groups on the Zy-heparin
particles were blocked by a further incubation for 1 h in 0.2 M Tris, pH 8.0, at room temperature. The Zy-heparin particles were then washed four times in 0.2 M Tris, pH 8.0, and once in Veronal-buffered saline, pH 7.5 (VBS), containing 0.1% gelatin (GVB), adjusted to a concentration of 1 × 10⁹/ml in GVB with a model 2F Coulter counter (Coulter Electronics Inc., Hialeah, Fla.) and stored at 2°C. CNBr-treated Zy particles lacking heparin (Zy-CNBr) were prepared in parallel. The amount of heparin on CNBr-activated Zy particles with coupled heparin (Zy-heparin) was determined by hydrolytic release of the glycosaminoglycan side chains with 0.5 M NaOH for 18 h at room temperature (41, 44) and quantitation of the heparin in the supernate after sedimentation of the particles. No metachromatic material was released from Zy-CNBr particles and the material registering in the uronic-acid assay (which approximated 3.2 μg of uronic acid/10⁹ particles) was subtracted for the calculation of heparin release from Zy-heparin by the uronic-acid assay. Approximately 1% of the input of heparin was coupled to CNBr-activated Zy, whereas no heparin was bound to Zy particles processed in the same manner.

CNBr-activated Sepharose with coupled heparin (Sepharose-heparin) was prepared by incubating 20 mg of heparin with 3 ml of packed Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) activated by 90 mg of CNBr (45), and the particles were then processed as for the preparation of Zy-heparin. To prepare Zy particles bearing human transferrin or human serum albumin, 240 μg of transferrin or 150 μg of albumin were incubated with ~10⁹ CNBr-activated Zy particles under the same conditions as for coupling heparin. The quantity of protein coupled was calculated from the decrease in protein content of the supernate after the coupling reaction.

Enzymatic Removal of Heparin from the Zymosan-Heparin Particles. Crude heparinase was prepared from Flavobacterium heparinum by the method of Linker and Hovingh (46); the preparation had 3 U of heparinase/100 μl and contained other mucopolysaccharidases, but had no detectable proteolytic activity. 1 U of enzyme is defined as the quantity that catalyzes the formation of 1 μmol of unsaturated uronides per h at 37°C from a heparin substrate. Zy-heparin particles, 2 × 10⁹, in phosphate-buffered saline containing 0.6 mM Ca²⁺, and 0.4 mM Mg²⁺ (PBS⁺⁺) were incubated with 0.055–1.5 U of heparinase at 37°C for designated intervals. The particles were washed twice in PBS⁺⁺, twice in GVB, and examined for residual heparin. Zy-heparin particles, 2 × 10⁹, were also incubated with 6 U of chondroitin ABC lyase (Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.) (1 U of enzyme catalyzes the formation of 1 μmol of product per h) in 0.25 M NaCl, 0.17 M Na acetate, and 0.2 M Tris HCl, pH 8.0, containing 50 mg/ml bovine serum albumin for 1 h at 37°C. Under these conditions, 6 U of chondroitin ABC lyase degraded 25 μg each of a mixture containing equal amounts of chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate, as assessed by gel filtration of the mucopolysaccharides before and after degradation (47, 48).

Assays and Preparation of Alternative Pathway Proteins. B (49), C3 (50), P (23), D (21), B1H (28), and C3bINA (51) were purified to homogeneity as assessed by polyacrylamide gel electrophoresis of their reduced forms in the presence of sodium dodecyl sulfate (52). C3 and B hemolytic activities were assayed as described (53, 54). C3 was trace labeled with ¹²⁵I (New England Nuclear, Boston, Mass.) by use of insoluble lactoperoxidase (Worthington Biochemical Corp., Freehold, N. J.) (55) to a sp act of 85,000 cpm/μg without loss of functional activity. GVB containing 0.15 mM calcium and 0.5 mM magnesium (GVB⁺⁺), half-isotonic GVB⁺⁺ with 2.5% dextrose (DGVB⁺⁺), GVB containing 0.04 M EDTA (GVB-EDTA) (56), and GVB containing 2 mM magnesium and 8 mM EGTA (GVB-Mg-EGTA) (57) were prepared as described.

Activation of the alternative pathway by Zy particles was examined by measuring inactivation of B and C3 in normal serum which had been diluted in GVB-Mg-EGTA to prevent classical pathway activation (57, 58). 2.5 × 10⁹ particles were suspended in 0.025 ml of 0.15 M NaCl and added to 0.2 ml of normal human serum diluted 1:4 in GVB-Mg-EGTA (NHS-Mg-EGTA). The reaction mixtures were incubated with agitation at 37°C, during which duplicate 0.01-ml samples were removed at timed intervals and added to either 1 ml of ice-cold DGVB⁺⁺ or GVB containing 5 mM EDTA for subsequent assay of residual C3 or B hemolytic activity.

To assess the function of particle-bound C3b, particles were prepared (23) by incubation of 0.15 × 10⁹ Zy, Zy-CNBr, and Zy-heparin (13.1 × 10⁹ molecules of heparin/particle),
respectively, with 105 μg of unlabeled or 125I-labeled C3, 50 μg of B, and 0.05 μg of D in GVB containing 1.5 mM Mg²⁺ for 1 h at 30°C. The particles were washed twice in EDTA and once in GVB++. The number of 125I-C3b molecules bound per particle was 520 for Zy, 1,320 for Zy-CNBr, and 1,520 for Zy-heparin. Zy,C3b, Zy-CNBr,C3b, and Zy-heparin,C3b particles were incubated with 0.15 μg of B and 0.05 μg of D in 0.2 ml of GVB++ containing 1.5 mM Mg²⁺ at 37°C. At timed intervals, 0.01-ml samples were removed, added to 0.2 ml of GVB++, and centrifuged for 2 min at 1,300 g; the supernates were assayed for residual B hemolytic activity. Nonspecific adsorption of B to the particles was assessed in parallel reaction mixtures in which B was incubated with the particles in the absence of D or in GVB-EDTA in the presence of D and this figure was subtracted from the B input to permit calculation of specific B inactivation. The percentage of C3b-dependent inactivation of B for each type of particle was directly related to the input of particle-bound C3b.

Results

Inhibition by Surface-bound Heparin of Zymosan-induced Activation of the Alternative Pathway in Normal Human Serum. The relative capacity of Zy, of Zy-CNBr, and Zy-heparin particles, prepared in a ratio of 29 mg of heparin to ~10⁸ CNBr-activated Zy particles, to activate the alternative pathway was compared in NHS-Mg-EGTA. B and C3 inactivation by Zy and Zy-CNBr increased progressively throughout the period of observation and exceeded 50% after 30 min. In contrast, Zy-heparin had no capacity to activate the alternative pathway as B and C3 consumption was limited to that observed in the absence of particles (Fig. 1).

Zy-heparin particles bearing different amounts of heparin were compared for their capacity to inactivate B in NHS-Mg-EGTA during a 45-min incubation. The Zy-CNBr particles that had not been incubated with heparin inactivated 90% of the B activity. The amount of heparin present in the initial coupling reaction or bound to the particle was inversely related to the capacity of Zy-heparin to inactivate B (Fig. 2). The correlation between B inactivation and the amount of heparin bound (r = 0.97, P <0.01) is such that complete inhibition of activating capacity, as calculated by linear regression, would be achieved by 11.5 × 10⁶ molecules/particle.

The coupling of 1.1 × 10⁶ molecules of human transferrin or 0.9 × 10⁶ molecules of human serum albumin/particle to Zy-CNBr did not alter the capacity of particles to inactivate B and C3 in NHS-Mg-EGTA. When the particles bore 8.3 × 10⁶ molecules of heparin, which represents a weight equivalent to that of the coupled proteins, the inactivation of B was reduced from 82 to 13%, and of C3 from 78 to 15%. Treatment of a replicate portion of Zy-heparin with 1.5 U of heparinase/0.2

![Fig. 1. Time course of inactivation of B (A) and C3 (B) in NHS-Mg-EGTA induced by zymosan (●), Zy-CNBr (□), and Zy-heparin (▲) as compared to spontaneous inactivation (◇).](image)
HEPARIN ON ZYMOSAN INHIBITS ALTERNATIVE PATHWAY ACTIVATION

1206

HEPARIN ON ZYMOSAN INHIBITS ALTERNATIVE PATHWAY ACTIVATION

Fig. 2. Dose-response relationship between the amount of heparin in the coupling reaction (△) or the amount of heparin bound to zymosan and the capacity of the particles to induce B inactivation in NHS-Mg-EGTA. Particle-bound heparin was assessed either by metachromatic (●) or uronic acid (○) assay.

× 10⁸ particles for 2 h restored their B and C3 inactivating capacity to that of the original Zy-CNBr, whereas treatment with 6 U of chondroitin ABC lyase/0.2 × 10⁸ particles had no effect.

To assess whether limited treatment with heparinase of a nonactivating Zy-heparin particle would yield particles with incremental activating capacities, 0.2 × 10⁹ Zy-heparin particles (13.1 × 10⁶ molecules of heparin/particle) were treated with 0.06 or 0.3 U of heparinase for 5, 10, 20, or 30 min at 37°C, and the residual heparin on each batch was assessed. There was a significant inverse correlation (r = 0.98, P < 0.01) between the residual bound heparin and the relative capacity of the particles to induce B inactivation in NHS-Mg-EGTA after a 45-min interaction (Fig. 3). Complete inhibition of the activating capacity of Zy requires 12.9 × 10⁶ molecules of heparin/particle, as calculated by linear regression; this number is similar to that observed when bound heparin was varied based upon its concentration in the coupling step (Fig. 2).

The inhibitory effect of surface-bound heparin on the capacity of another alternative pathway-activating particle to induce B and C3 consumption in NHS-Mg-EGTA was examined with CNBr-activated Sepharose lacking heparin (Sepharose-CNBr) and Sepharose-heparin before and after treatment with 1.5 U of heparinase/0.2 ml of packed beads for 2 h at 37°C. 70% (vol/vol) Sepharose-CNBr, Sepharose-heparin, heparinase-treated Sepharose-CNBr, and heparinase-treated Sepharose-heparin in 0.2 ml of GVB were incubated with 0.25 ml of NHS-Mg-EGTA, and samples were assessed for residual C3 and B function after 0-, 5-, 15-, 30-, and 45-min incubations at 37°C. Sepharose-CNBr, whether or not treated with heparinase, induced progressive B and C3 consumption that reached 70 and 52%, respectively, at 45 min. Sepharose-heparin had no capacity to activate the alternative pathway, as B and C3 consumptions were limited to that observed in the absence of particles, namely, 2 and 3%. Heparinase treatment of Sepharose-heparin beads restored the B and C3 inacti-
vating capacity to that observed with Sepharose-CNBr, thereby confirming the inhibitory effect of the coupled glycosaminoglycan on a second activating particle.

**Augmentation by Surface-bound Heparin of Inactivation of Bound C3b by C3bINA and β1H.** The inhibitory effect of coupled heparin on the capacity of a particle to activate the human alternative pathway could be a result of the failure of such particles to accept C3b from the fluid phase, of the impaired function of particle-bound C3b, or of augmented inactivation of particle-bound C3b by the regulatory proteins. The formation of C3b-bearing particles by a fluid phase reaction mixture containing 125I-labeled C3, as described in Materials and Methods, did not reveal impaired uptake of C3b in the presence of bound heparin. Numbers of particles bearing equivalent amounts of C3b, namely, $2 \times 10^7$ Zy-heparin,C3b, $2.3 \times 10^7$ Zy-CNBr,C3b, and $5.6 \times 10^7$ Zy,C3b, inactivated B in the presence of D as a first order reaction (Fig. 4). Furthermore, B consumption by Zy-heparin,C3b equaled that by Zy-CNBr,C3b and exceeded that by Zy,C3b, thereby indicating that heparin did not impair the function of particle-bound C3b.

The relative susceptibility of bound C3b to inactivation either by purified control proteins or by normal human serum diluted in GVB-EDTA (NHS-EDTA) was studied in kinetic experiments. Amounts of Zy,C3b, Zy-CNBr,C3b, and Zy-heparin,C3b ($17.6 \times 10^6$ molecules of heparin/particle) were selected to give a range of 50-65% B inactivation in the presence of D in 60 min at 37°C when the particles were not pretreated with either source of control proteins. Four samples each of $4 \times 10^7$ Zy,C3b, $1.5 \times 10^7$ Zy-CNBr,C3b, $1 \times 10^7$ Zy-heparin,C3b, and $4 \times 10^7$ EAC4b,3b in 0.1 ml GVB** were incubated at 37°C for 60 min, during which replicates of each particle type received 0.1 ml of a 1:25 dilution of NHS-EDTA at 0, 15, 30, and 45 min, respectively. Additional samples of each particle type were incubated with buffer alone for 60 min at 37°C. All reactions were stopped by the addition of 1 ml of ice-cold GVB** and washing of the particles at 4°C. Particles that
had been incubated in buffer alone were defined as having 100% of bound C3b activity as assessed by inactivation of B in the presence of D. C3b on Zy or on Zy-CNBr was relatively resistant to inactivation by control proteins in NHS-EDTA with only 50% loss of function after 60 min. The activity of C3b on Zy-heparin was reduced by >90%, with the rate of inactivation resembling that of C3b on sheep erythrocytes, a nonactivating particle of the human alternative pathway (Fig. 5 A). The experiment was repeated by treating the same four sets of particles with 2 µg of C3bINA and 18 µg of β1H in 0.1 ml EDTA. C3b was rapidly inactivated on sheep erythrocytes and Zy-heparin, whereas C3b on Zy or Zy-CNBr was again relatively resistant to inactivation by control proteins (Fig. 5 B).

The effect of particle-bound heparin on the inactivation of bound C3b by C3bINA was examined for the importance of β1H as a cofactor. 3 × 10⁷ Zy-CNBr,C3b and Zy-heparin,C3b (13.1 × 10⁶ molecules of heparin/particle) were each incubated with 2 µg of C3bINA in the presence and absence of 18 µg of β1H, or with buffer alone. Particles incubated in buffer alone were defined as having 100% of bound C3b activity. C3bINA alone inactivated 10 and 15% of the C3b on Zy-CNBr and Zy-heparin, respectively, by 60 min. In the presence of β1H, the inactivation of C3b was increased to 55% on Zy-CNBr and to 99% on Zy-heparin, emphasizing that bound heparin augments the cofactor role of β1H (Fig. 6).

When the same particles were treated with heparinase at a ratio of 1.5 U per 0.2 × 10⁷ Zy-CNBr,C3b or Zy-heparin,C3b, there was no loss of C3b function. Before and after heparinase treatment, Zy-heparin,C3b inactivated 67% and 63% of B, respectively, in the presence of D. Similarly, Zy-CNBr,C3b inactivated 59% and 66% of B before and after heparinase treatment of the particles. 2 × 10⁷ Zy-CNBr,C3b
and Zy-heparin,C3b with or without prior heparinase treatment were incubated with 2 μg of C3bINA and 18 μg of β1H for timed intervals and then assessed for residual particle-bound C3b activity. C3b on Zy-heparin particles without heparinase pretreat-
HEPARIN ON ZYMOSAN INHIBITS ALTERNATIVE PATHWAY ACTIVATION

Fig. 7. Correlation between the number of molecules of heparin/zymosan particle and the percentage of residual C3b activity on the particles after treatment with purified C3bINA and \( \beta \)1H.

...ment was 100% inactivated by the purified control proteins in 30 min; C3b on the heparinase-treated Zy-heparin particles had regained resistance comparable to that of C3b on Zy-CNBr particles pretreated with buffer or heparinase, in that only 60% of the C3b was inactivated at 60 min.

Some of the particles described in Fig. 3 in terms of their heparin content and alternative pathway-activating capacity were converted to C3b-bearing intermediates in order to examine the dose-response effects of particle-bound heparin on the regulatory function of C3bINA and \( \beta \)1H. \( 2 \times 10^7 \) Zy-heparin, C3b bearing 2.64, 4.48, 7.28, or 13.1 \( \times 10^6 \) molecules of heparin/particle and \( 2 \times 10^7 \) Zy-CNBr, C3b were treated with 2 \( \mu \)g of C3bINA and 18 \( \mu \)g of \( \beta \)1H for 45 min and each particle type was assayed for residual C3b activity. The capacities of untreated particles to induce B inactivation in the presence of \( \tilde{D} \) were comparable and defined as 100% C3b activity for each particle type. Residual C3b activity was inversely correlated to the number of molecules of heparin/particle (r = 0.99, P < 0.01) (Fig. 7), indicating that the susceptibility of particle-bound C3b to the inactivation by the purified control proteins increased with the amount of heparin bound/particle.

Discussion

Discrimination by the alternative pathway between activating and nonactivating particles or cells occurs after C3b deposition and is related to the modulation by surface constituents of the interaction of C3b with the control proteins, \( \beta \)1H, and C3bINA (22, 23). The relative amount of membrane sialic acid is inversely related to the relative capacity of sheep or mouse erythrocytes to activate the alternative complement pathway in whole human serum (9, 24). Membrane-associated sialic acid promotes high-affinity binding of \( \beta \)1H to cell-bound C3b without altering the binding of B to C3b (26), thereby impairing formation of C3b,Bb, facilitating extrinsic decay of any C3b,Bb already generated, and enhancing inactivation of C3b by C3bINA. In an analogous manner, the amount of heparin coupled to the surface of Zy particles is inversely related to the capacity of the particles to activate the alternative complement pathway because coupled heparin augments the regulatory action of \( \beta \)1H and C3bINA on particle-bound C3b.

Heparin glycosaminoglycan coupled to Zy impaired in a dose-dependent manner the ability of the particle to activate the alternative pathway as assessed by inactivation of B and C3 in NHS-Mg-EGTA. The suppressive effect of coupled heparin on the
activating function of Zy-CNBr was not duplicated by coupling transferrin or serum albumin, and was not reversed by treating the Zy-heparin particles with chondroitin ABC lyase. The ability of bound heparin to impair the alternative pathway-activating capacity of the carrier particle was not peculiar to Zy but was also observed in comparable experiments with Sepharose. The loss of alternative pathway-activating capacity was significantly correlated (P < 0.01) to the number of molecules of heparin bound/Zy particle when the ratio was adjusted either by varying the amount of heparin in the initial coupling reaction (Fig. 2) or by enzymatically removing graded amounts of heparin from a fully inhibited Zy-heparin particle (Fig. 3). Analysis by linear regression of the effect of each method of adjusting the amount of heparin/particle showed that complete inhibition of activating capacity would be achieved by the presence of at least 11.5 × 10^6 (Fig. 2) or 12.9 × 10^6 (Fig. 3) molecules of heparin/particle.

The inhibition by coupled heparin of the capacity of a particle to activate the human alternative pathway could have been secondary to the inability of such particles to bind C3b from the fluid phase, to the impaired interaction of particle-bound C3b with B and D to form the amplification convertase, or to augmented inactivation of particle-bound C3b by the regulatory proteins. There was equivalent uptake of radiolabeled C3 by Zy-CNBr, a particle retaining activating capacity, and by Zy-heparin during the fluid phase interaction of C3, B, and D. Further, C3b bound to Zy-heparin has the same capacity as C3b bound to Zy-CNBr to interact with B to permit its cleavage by D (Fig. 4), thereby indicating unimpaired function of C3b on Zy-heparin in the absence of control proteins. In contrast to surface-bound heparin, fluid-phase heparin directly interferes with the ability of C3b bound to a nonactivating particle to form C3b,Bb (59). The presence of heparin on Zy did not alter binding or function of C3b, excluding these reactions as the sites of inhibition by surface-bound heparin of alternative pathway activation.

The effect of heparin coupled to Zy was to augment inactivation of bound C3b by the regulatory proteins to an extent comparable to the inactivation of C3b on a nonactivating cell, the sheep erythrocyte. Although human serum chelated with EDTA or the combination of purified C3bINA and β1H were relatively ineffective in removing C3b function from Zy or Zy-CNBr, particles which activate the alternative pathway, the control proteins rapidly inactivated C3b on nonactivating particles such as sheep erythrocytes or Zy-heparin (Figs. 5A and B). The absence of β1H from the purified control protein mixture resulted in almost no inactivation of C3b presented as either Zy-CNBr,C3b or Zy-heparin,C3b, thereby indicating that surface-bound heparin facilitates the cofactor role of β1H for C3bINA by promoting the interaction between β1H and bound C3b (Fig. 6).

Increasing amounts of heparin on the Zy surface were significantly correlated (P < 0.01) with increased numbers of C3b sites that were susceptible to inactivation by C3bINA in the presence of β1H (Fig. 7). By linear regression analysis of this correlation, the number of heparin molecules/particle required to promote total inactivation of bound C3b by purified control proteins was estimated to be 13.8 × 10^6, which corresponds to the amount of heparin required to abolish the activating capacity of Zy in NHS-Mg-EGTA (Figs. 2 and 3). This finding suggests that the augmentation of endogenous regulation by bound-heparin is responsible for the loss of activating capacity by Zy-heparin particles in serum.
Initial studies of specific cell-surface biochemical characteristics that modulated recognition by the alternative pathway involved the conversion of a nonactivating cell, the sheep erythrocyte, into an activating cell by removal of sialic acid residues (24, 25). In the studies presented here, an activating particle, Zy, is transformed into a nonactivating particle by the coupling to its surface of heparin glycosaminoglycan. Although structurally dissimilar, both sialic acid and heparin glycosaminoglycan, when on a surface of a particle, promote the interaction of bound C3b with the regulatory proteins, β1H and C3bINA, to prevent formation and function of the bound amplification convertase, C3b,Bb. Because heparin and heparan sulfate molecules can be specifically bound to nucleated cells (30-35), heparin-related sulfated mucopolysaccharides may modulate the activation of the alternative pathway by some mammalian cells.

Summary

Discrimination by the human alternative pathway between activating and nonactivating particles occurs after deposition of C3b by the continuous low-grade interaction of the alternative pathway components in biologic fluids and is dependent on the modulation by surface constituents of the interaction of bound C3b with the control proteins, β1H, and C3b inactivator (C3bINA). When heparin glycosaminoglycan was coupled to activating particles, such as zymosan or Sepharose, by cyanogen bromide activation, their capacity to activate the human alternative pathway was inhibited. The loss of alternative pathway-activating capacity was directly correlated to the number of heparin molecules bound/zymosan particle, whether the ratio was varied by increasing the amounts of heparin in the initial coupling reactions or by treating a fully inhibited particle with incremental concentrations of heparinase. Analysis by linear regression of the inhibitory effect of each procedure (r = 0.97, r = 0.98, respectively) for adjusting the number of heparin molecules/particle revealed that the dose-response relationships were identical and that complete inhibition occurred with >12 × 10^6 molecules of heparin/zymosan particle. The coupling of heparin to zymosan did not impair the uptake of C3b from the fluid-phase interaction of C3, B, and D, and did not alter the capacity of bound C3b to associate with B so as to permit its inactivation by D. Although the regulatory proteins present in normal serum chelated with EDTA or presented as a combination of purified C3bINA and β1H were relatively inefficient in inactivating C3b function on an activating particle of the alternative pathway such as zymosan or zymosan-cyanogen bromide, the control proteins rapidly inactivated C3b on a nonactivating particle such as a sheep erythrocyte or zymosan with coupled heparin. The increased numbers of C3b sites susceptible to inactivation by C3bINA in the presence of β1H were significantly correlated to the number of molecules of heparin/particle. By linear regression analysis of the correlation (r = 0.99) the number of heparin molecules/particle required to promote total inactivation of bound C3b by purified control proteins was 13.8 × 10^6. This molecular analysis suggests that the action of heparin coupled to an activating particle of the alternative pathway is to promote the interaction between particle-bound C3b and the regulatory proteins, thereby preventing particle-associated amplified C3 cleavage. It is noteworthy that both surface constituents known to maintain
a particle as a nonactivator of the alternative pathway, sialic acid and N-sulfated mucopolysaccharide, act by facilitating the inactivation by regulatory proteins of the function of particle-bound C3b.

Received for publication 9 July 1979.

References

HEPARIN ON ZYMOSAN INHIBITS ALTERNATIVE PATHWAY ACTIVATION

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