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Immobilization of C3b with retention of functional activity

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We compared eight commercially available, pre-activated affinity chromatography supports for ability to immobilize C3b that would retain functional activity. Pre-activated supports that we studied were: cyanogen bromide activated agarose, *N*-hydroxysuccinimide activated agarose, Reacti-Gel HW-65, Actigel A aldehyde activated agarose, thiopropyl activated agarose, 1,4-bis(2,3-epoxypropoxy) butane activated agarose, Reacti-Gel GF-2000 and tresyl activated agarose. The amount of C3b immobilized by each support varied from 81% for Actigel A aldehyde activated agarose to only 19% for Reacti-Gel GF-2000. We examined the functional capacity of the C3b immobilized on these various supports to participate in the alternative pathway. Immobilized C3b was mixed with factors D and B of the alternative pathway and examined over time for ability to consume factor B hemolytic activity. C3b immobilized on thiopropyl activated agarose consumed factor B at a rate comparable to unbound fluid phase C3b. C3b immobilized on other supports was less active in participating in factor B consumption. Thus, we have demonstrated the ability to immobilize C3b onto a solid matrix with the immobilized C3b retaining the ability to participate in the alternative pathway. This immobilized C3b can be used to fractionate substances with high C3b binding affinity.

Key words: Affinity chromatography; Complement; C3b; Agarose

Introduction

In 1968 Cuatrecasas described the technique by which protein could be coupled to a solid support. Since that time, affinity chromatography has been widely used to purify proteins as well as to study the ability of substances to bind to immobilized protein. Indeed, there are currently dozens of different types of commercially available pre-activated supports to which proteins can be coupled. However, when proteins are coupled to an insoluble matrix two conditions should be met: (1) the support should efficiently immobilize the protein, and (2) the immobilized protein should retain functional capacity. The present study was undertaken to examine the capacity of eight different commercially available pre-activated supports to bind C3b, the major cleavage fragment of the third component of complement (C3), without destroying the ability of C3b to participate in the alternative pathway of complement.

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Abbreviations: C3, third component of complement; C3b, cleavage fragment of complement; CDI, 1,1-carbonyldiimidazole; CNBr, cyanogen bromide; $DGVB^{2+}$, dextrose, gelatin, veronal-buffered saline with calcium and magnesium; EAC4b,3b, sheep erythrocytes, antibody and complement components C4b and C3b; EDTA, ethylenediamine tetraacetate; EDTA-GVB, EDTA solution containing GVB; GVB, gelatin, veronal buffered saline; HSA, human serum albumin; PBS, phosphate-buffered saline; Z, number of lytic events per cell.

Materials and methods

Activated supports

All of the agarose based supports used in this study were 4% cross-linked. Cyanogen bromide (CNBr) activated agarose, 1,4-bis(2,3-epoxypropoxy) butane activated agarose, thiopropyl activated agarose, tresyl activated agarose and *N*-hydroxysuccinimide agarose were purchased from Sigma Chemical Company, St. Louis, MO. Reacti-Gel HW-65, a CDI activated Fractogel and Reacti-Gel GF-2000, a CDI activated Trisacryl were purchased from Pierce Chemical Company, Rockford, IL. Actigel A, an aldehyde activated agarose, was purchased from ICN ImmunoBiologicals, Cleveland, OH.

Complement components and buffers

Buffers used in complement assays were: half isotonic veronal-buffered saline containing 0.1% gelatin, pH 7.5 (GVB), GVB containing 0.5 mM magnesium, 0.15 mM calcium, and 2.5% dextrose ($DGVB^{2+}$), and GVB containing 40 mM ethylenediamine tetraacetate (EDTA) (EDTA-GVB). C3, B, D, and P were purified to homogeneity and quantified as previously described (Hunsicker et al., 1973; Fearon et al., 1975, 1977; Tack et al., 1976). Rat serum, used as the source of terminal complement components, was purchased from Rockland (Gilbertsville, PA) and was diluted 1/20 in 40 mM EDTA-GVB.

Protein concentrations were determined by using Folin and Ciocalteu's phenol reagent (Sigma Chemical Company) by the method of Lowry et al. (1951). Sodium cyanoborohydride was purchased from Sigma Chemical Company.

Preparation of C3b and assay for functional capacity

C3b was cleaved from C3 by using cobra venom factor convertase which had been immobilized on cyanogen bromide activated Sepharose 6B as described previously (Weiler et al., 1978). EAC4b,3b were prepared as described by Fearon et al. (1973).

Functional activity of the C3b was demonstrated as follows: 1 μg of C3b was added to 0.02 ng factor B and 10 ng factor D in a final volume of 0.2 ml of $DGVB^{2+}$ and incubated for 60 min at 37°C. 10 μl aliquots of this mixture were removed at time intervals and diluted in 1.0 ml $DGVB^{2+}$. Factor B consumption was measured as follows: the aliquots from the previous step containing factor B were further diluted in $DGVB^{2+}$ and added to 0.2 ml $DGVB^{2+}$ which contained 1×10^7 EAC4b,3b, 200 μg P and excess D and incubated for 30 min at 30°C. Then 0.3 ml of rat complement diluted 1/20 in 0.04 mM EDTA-GVB was added to each tube and the incubation was continued at 37°C for 60 min. Next, 1.5 ml saline was added to each tube except the 100% lysis tube to which 1.5 ml water was added. Reagent blank tubes contained the same cellular intermediates except that factor B was not present. The tubes were mixed and centrifuged. The extent of lysis was determined by measuring optical density of the supernatant at 414 nm. This value was used to calculate the average number of lytic events per cell (Z) as described (Kabat et al., 1960).

Coupling C3b to activated supports

For each matrix, the amount of support used was roughly 25 times what would be necessary for a 1:1 binding ratio based on manufacturers' specifications. All supports were swollen or re-swollen and washed in accordance with the manufacturers' suggestions as follows: CNBr activated agarose (27.5 mg) was re-swollen in 1.0 ml of 0.1 M HCl for 15 min, washed three times with 0.1 M HCl and three times with 0.1 M bicarbonate buffer with 0.5 M NaCl, pH 8.7. Thiopropyl activated agarose stock suspension (200 μl) was washed six times with deaerated water. Wet Reacti-Gel HW-65 gel (100 μl) was washed six times with 0.1 M bicarbonate buffer, pH 8.7. Wet Reacti-Gel GF-2000 (100 μl) was washed six times with 0.1 M bicarbonate buffer, pH 8.7. 51.6 mg of 1,4-bis(2,3-epoxypropoxy) butane activated agarose was re-swollen in 0.5 ml water for 15 min and then washed three times in water and three times in 0.1 M bicarbonate buffer, pH 8.7. Actigel A aldehyde activated agarose stock suspension (200 μl) was washed six times in PBS, pH 7.2. Tresyl activated agarose (30.5 mg) was re-swollen in 0.5 ml of water and washed five times with PBS. *N*-hydroxysuccinimide activated

agarose (333 μl) stock suspension was washed three times in chilled acetate buffer, pH 4.5 and 3 times in 0.1 M bicarbonate buffer, pH 8.7.

All of the washed supports were adjusted to a final volume of 200 μl. Actigel A aldehyde activated agarose required acceleration of the activation as follows: 100 μl of 0.25 M sodium cyanoborohydride was added to 100 μl of the support, mixed thoroughly, and 50 μl of 1.0 mg/ml C3b was immediately added to the activated support. 50 μl of 1.0 mg/ml C3b was also added to each of the other supports resulting in 50 μg of C3b being present in 250 μl final volume for each support. 50 μg of HSA was added to 200 μl of Actigel A aldehyde activated agarose to serve as a control in later experiments. The proteins and supports were coupled at 4°C for 12 h.

Following the coupling, the support solutions were centrifuged and 100 μl of supernatant was assayed for protein content using Folin and Ciocalteu's phenol reagent as described by Lowry. All supports were treated with 1.0 M ethanolamine for 2.5 h at 4°C to block remaining uncoupled sites. The supports were again washed six times, each with the same buffer used to couple to that support.

Detection of functional C3b activity on solid matrix

We modified the C3b assay described above to detect functional C3b on the surface of the supports: appropriate volumes of each support to which C3b had been coupled were removed so that 1 μg of C3b matrix was used in the assay. An equivalent amount of HSA-Actigel A aldehyde activated agarose which did not contain C3b was used as a negative control; 1 μg of standard C3b was used as the positive control in this assay. In each case, 1 μg of C3b or 1 μg of C3b immobilized on the support was added to 0.02 ng B and 10 ng D in a final volume of 0.2 ml and was incubated for 60 min at 30°C; 10 μl aliquots of the mixture were taken at various time intervals, added to 1 ml $DGVB^{2+}$ and the tubes were centrifuged to remove the insoluble matrix. The remaining supernatant was assayed for functional factor B hemolytic activity as described above.

Results

C3b was immobilized onto seven different supports with the efficiency of these immobilizations ranging from 19–81%. Actigel A aldehyde activated agarose immobilized 81%. Thiopropyl agarose, CNBr activated agarose and *N*-hydroxysuccinimide agarose immobilized about 50%. The remaining supports immobilized 40% or less. Tresyl activated agarose was dropped from further consideration when it disintegrated upon centrifugation.

We next examined the functional activity of the immobilized C3b. We compared the ability of fluid phase (uncoupled) C3b with immobilized C3b to consume factor B in the presence of factor D. Fig. 1 shows that C3b immobilized on thiopropyl agarose supported consumption of factor B activity similar to fluid phase C3b. C3b immobilized on Actigel A aldehyde activated agarose had less activity in supporting consumption of factor B. C3b immobilized on CNBr activated agarose gave variable results, having good activity in one experiment but no activity in another. C3b on Reacti-Gel HW-65, *N*-hydroxysuccinimide activated agarose, Reacti-Gel GF 2000 and 1,4-bis(2,3-epoxypropoxy) butane activated agarose, as well as the control matrix (HSA-Actigel A aldehyde activated agarose) did not support the consumption of factor B.

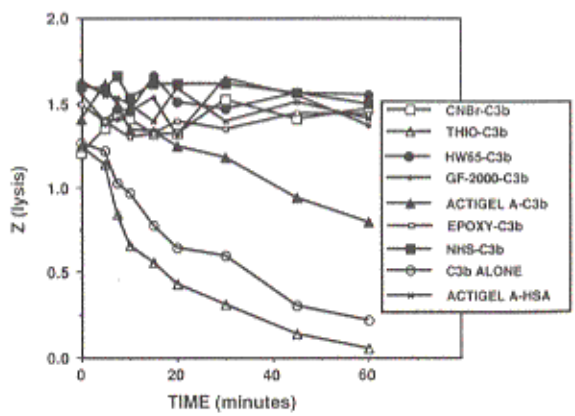

Fig. 1. Comparison of C3b functional activity while bound to each support vs. standard C3b.

Discussion

We examined the ability to immobilize C3b onto a solid matrix with retention of functional activity. We assayed C3b functional activity using a modification of the fluid phase assay for C3b activity (Weiler, 1978). In this model for the alternative pathway, highly purified factor B binds to C3b (to form C3b,B) which is acted upon by D (to form C3b,Bb). Bb then decays allowing C3b to bind additional B causing B hemolytic activity to decrease over time. Using this assay we have established that C3b can be immobilized and still retain the capacity to participate in the alternative pathway of complement.

Actigel A activated aldehyde agarose immobilized the most C3b but the immobilized C3b had slightly less alternative pathway activity than did fluid phase C3b. Conversely, thiopropyl agarose immobilized only about 50% of the C3b added, but the C3b immobilized on thiopropyl agarose demonstrated activity comparable to fluid phase C3b in supporting B consumption. These studies demonstrate for the first time the capacity for C3b to be immobilized onto a solid matrix and yet to retain ability to participate in the alternative pathway of complement (Fig. 1).

This will allow us to use this immobilized C3b for a variety of studies which depend upon the immobilization of *functional* C3b. For example, heparin is one substance that is known to interact with C3b. Heparin binding to C3b inhibits the formation and activity of the alternative pathway convertase, C3b,Bb (Weiler et al., 1978; Linhardt et al., 1988; Weiler et al., 1989). Nevertheless the discrete binding site for heparin on C3b and the minimal oligosaccharide sequence necessary to inhibit complement activation have yet to be determined. This determination will be important if specific oligosaccharides are to be selected with enhanced complement binding activity which could lead to the development of new drugs to control complement activation.

The present study provides the initial techniques by which C3b can be immobilized with retention of functional activity. Consequently, we can now produce large quantities C3b immobilized on thiopropyl activated agarose, knowing that the immobilized C3b has retained functional capacity. This will enable us to separate heparin and other substances with high affinity for C3b to study interactions with C3b.

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