Specificity of the thioester-containing reactive site of human C3 and its significance to complement activation

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The specificity of the thioester-containing site in three plasma proteins is regulated by elements of their protein structures other than the thioester bond itself. Human C4A and α 2-macroglobulin preferentially form amide linkages while human C3 primarily forms ester linkages with hydroxyl groups. We have examined the thioester in C3 and found evidence of strong preferences for certain carbohydrates, indications of selectivity for specific positions on those carbohydrates and a preference for terminal sugars in polysaccharides. A testable set of rules are derived from these findings which predict preferred attachment sites on polysaccharides. A computer model of the effect of different reactivities on activation of the alternative pathway of comp-

lement suggested that organisms might greatly alter their susceptibility to complement with small changes in carbohydrate structure. While a random selection of 20 biological particles showed no correlation between activation and C3b attachment efficiency, subsets of related organisms differing primarily in their surface polysaccharide exhibited stronger correlations. The strongest correlation occurred in a series of the yeasts (*Cryptococcus neoformans*) possessing capsular polysaccharides with one, two, three or four branching xylose sugars per repeating unit. These organisms exhibited capture efficiencies for metastable C3b from 12% (one-xylose strain) to 41% (four-xylose strain).

INTRODUCTION

A central question remaining largely unanswered in the biology of complement is why some micro-organisms activate the alternative pathway of complement poorly and are virulent, while others activate the pathway well [1–3]. Although there are clearly many different defences against complement, numerous studies have shown that surface polysaccharides play an important role in resistance to alternative pathway activation [1,4–18]. We have examined in this study the specificity of the thioester bond in activated C3 and asked whether micro-organisms might alter complement activation by changing their surface polysaccharides.

An intramolecular thioester bond is present in three plasma proteins: C3, C4 and α 2-macroglobulin [19–21]. While other studies have examined the reactivity of thioesters in the native proteins [19,22,23], the present study is only concerned with the quite different specificity of the activated thioester, which has been examined in greatest detail by Law, Dodds and others [24–31]. Proteolytic activation of native C3 results in the release of the C3a fragment (M_r 9000) and the formation of a metastable form of C3b (M_r 176000) which possesses a short-lived (100 μ s), highly reactive, thioester site [32,33]. The activated thioester of metastable C3b shows a strong preference for ester formation with hydroxyl groups over amide formation [23,34,35]. The C4B isotype of C4 exhibits a preference for amide bond formation and the structural basis for this specificity has been localized to a single amino acid [31,36]. Reaction of metastable C3b with hydroxyl groups on the surface of micro-organisms attaches C3b to the surface and leads to enhanced phagocytosis, increased antibody production and cell damage or lysis [1–3].

Activation of the alternative pathway of complement is particularly sensitive to C3b attachment, due to its unique

amplification process [1–3,37]. After the first C3b molecule is attached [38] all subsequent deposition proceeds by amplification of the first C3b via a bimolecular C3 convertase (C3b,Bb) formed by C3b and Factor B. Each new C3b may also form a C3 convertase and deposit additional C3b molecules on the surface. This amplification process is capable of depositing 1×10^6 to 1×10^8 C3b molecules on the surface of an activating particle 5–20 min after contact with human blood [39,40].

Although many studies have examined the attachment of C3b to pathogenic and non-pathogenic organisms [1,3,4,40–43], the specificity of this reaction has not been characterized. Other studies have examined the attachment efficiency of small molecules with activated C3b [25–27,33], but none of these studies examined the biological role of this specificity. In the present communication we report a quantitative examination of the selectivity expressed at this site for monosaccharides and small oligosaccharides and an examination of a variety of biological organisms which reveals correlations between C3b attachment efficiency and alternative pathway activation.

MATERIALS AND METHODS

Reagents

Sugars and alcohols were purchased from Sigma (St. Louis, MO, U.S.A.); α 1–4 polyglucose oligosaccharides (maltose, maltotriose, etc.) were from Boehringer–Mannheim (Indianapolis, IN, U.S.A.); α 1–6 polyglucose oligosaccharides were prepared as described previously [10]. Carbohydrate stock solutions were prepared by weight and were verified by refractive index. Veronal-buffered saline (VBS) contained 5 mM barbital and 145 mM NaCl, pH 7.4. Gelatin veronal-buffered saline (GVB) was VBS with 0.1% gelatin and GVBE was GVB containing 10 mM EDTA.

Abbreviations used: C3, native, haemolytically functional C3; NHS, normal human serum; VBS, veronal-buffered saline; GVB, VBS containing 0.1% gelatin; GVBE, GVB containing 10 mM EDTA.

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Purified proteins

Complement proteins C3 [44,45], Factors B [37], D [46] and H [47] were purified from normal human plasma as described. Radioiodination of C3 to between 0.5 and 1.0 $\mu\text{Ci}/\mu\text{g}$ was accomplished using ^{125}I in glass tubes coated with Iodogen (Pierce Chemical Co., Rockford, IL, U.S.A.). Before each experiment ^{125}I -C3 was repurified on a Mono S column (Pharmacia, Piscataway, NJ, U.S.A.) as described previously [45].

Microbial strains and growth conditions

The bacterial strains used in this study were group B *Streptococci* COH 31-15 and COH 1, *Escherichia coli* XL1 Blue (Invitrogen, San Diego, CA, U.S.A.), NM 522, PRC 116, PRC 104, ATCC 35218, ATCC 25922, ATCC 12792 and ATCC 33780, *Salmonella* strains *S. typhimurium* SH5771, *S. enteritidis* SH4340, *S. montevideo* SH5770 and *S. typhimurium* TV119 (*Salmonella* strains were obtained from Dr. J. Schweinle at Yale University). All bacterial strains were grown in Luria-Bertani broth at 37 °C, harvested during logarithmic growth and fixed with 0.3 % glutaraldehyde. *Cryptococci neoformans* isolates of serotypes A (strain 271), B (strain 182), C (strain 191) and D (strain 52) were obtained from K. J. Kwon Chung and J. E. Bennett (NIH, Bethesda, MD, U.S.A.). Growth characteristics on CGB agar [48] were used to confirm the isolates as *C. neoformans* var. *neoformans* (serotypes A and D) or *C. neoformans* var. *gattii* (serotypes B and C). The yeast cells were grown on liquid synthetic medium [49] on a gyratory shaker for 72 h at 30 °C. The yeast cells were fixed with 0.3 % glutaraldehyde, washed and resuspended in GVB.

Cation-exchange chromatography of C3 and C3b

C3 and C3b were separated on a Mono S HR 5/5 column (Pharmacia) [45]. Samples were diluted in buffer A (50 mM sodium phosphate, pH 6.0), microfuged, injected and the column washed with 5 ml of buffer A. Elution was with a linear gradient of 22.0 ml from 0 % to 35 % buffer B (50 mM sodium phosphate, 500 mM NaCl, pH 6.0).

C3b attachment inhibition assay

Inhibition of C3b attachment by sugars or alcohols was studied by measuring the attachment of ^{125}I -C3b to zymosan coated with C3b,Bb. C3b was first deposited on 1×10^{10} zymosan particles by resuspension in 0.2 ml of 10 mg/ml C3, addition of 5 μg of trypsin and incubation for 10 min at 22 °C. This was repeated, the cells were washed and the deposited C3b was amplified by addition of 35 μg of Factor B and 0.5 μg of Factor D in 150 μl of GVB. After addition of 50 μl of 10 mM NiCl_2 and incubation for 5 min at 22 °C, 5 μl of 0.2 M EDTA was added, mixed and 50 μl of C3 (500 μg) was added. After 1 h at 22 °C the cells were washed and the amplification procedure was repeated (using double the amount of B, D, and C3) until the desired numbers of C3b/zymosan were achieved [14,39,50]. ZymC3b,Bb was made by mixing 1×10^7 ZymC3b (1×10^5 C3b/zymosan) with 5.0 μg of Factor B and 0.2 μg of Factor D in 200 μl of GVB containing 1 mM NiCl_2 . The reaction mixture was incubated at 22 °C for 5 min and stopped by adding GVBE. Immediately thereafter, 20 μl of ZymC3b,Bb was mixed with 10 μl of ^{125}I -labelled C3 (0.08 μCi) and various concentrations of inhibitors in a total volume of 40 μl . After 15 min at 37 °C with mixing, the percentage

of C3b attached was determined by centrifuging C3b-coated particles through 20 % (w/v) sucrose in GVBE to separate bound from free ^{125}I -C3b. Controls utilized ZymC3b without enzyme.

IC₅₀ determination

The concentration of sugar causing inhibition of 50 % of the attachment to zymosan was taken as the IC₅₀. This value represents the concentration required to react with 50 % of the metastable C3b molecules before they react with the target. Other similar studies have used different particles [27,33] or small radioactive molecules such as [^3H]glycerol [25,26,35] as the target.

Attachment efficiency relative to water

The reactivity of compounds relative to the reactivity of water was calculated based on the following rationale. The IC₅₀ represents a concentration of inhibitor R-OH where half of the metastable C3b molecules have reacted with R-OH and fail to reach the surface in active form. C3 activated in the presence of R-OH at its IC₅₀ reacts approximately equally well with R-OH and with water (C3b attachment without competitors was between 10 and 12 % in these assays). Dividing the molar concentration of water (corrected for salts and R-OH) by the concentration of R-OH at IC₅₀ yields the reactivity of R-OH relative to water.

Measurement of C3b attachment efficiency with purified components

Two factors were found to be critical to accurate measurement of the attachment efficiency. First, after large numbers of C3b molecules had bound to particles the attachment efficiencies of subsequent C3b molecules were similar no matter what particle was used. Presumably this observation was due to C3b attachment to C3b on the surface [39,51–53]. Therefore, the measurements made here report the attachment efficiency of the first few C3b molecules attached. Secondly, because not all of the C3 in reaction mixtures was activated the percentage bound was corrected for the actual percentage of C3 cleaved in each assay to determine the attachment efficiency.

In order to form the C3-cleaving enzyme, C3b,Bb, on a surface a limited number of C3b molecules were first attached randomly from the fluid phase [14,39,50]. Packed cells (200 μl) were mixed with 380 μg of C3, 13 μg of Factor B, and 0.2 μg of Factor D in 140 μl of GVB containing 3.5 mM NiCl_2 . After 10 min at 37 °C, the cells were washed and resuspended at 50 % cell suspension in GVB. C3b,Bb was generated as described in the C3 attachment inhibition assay above. The particles were immediately incubated with 0.16 μCi of ^{125}I -C3 in 160 μl of GVB at 37 °C for 15 min. Half of the reaction mixture was layered on 20 % (w/v) sucrose and centrifuged to separate bound from free ^{125}I -C3b for calculation of the % C3 bound (the percentage of total C3 that bound). The other half was mixed with Mono S buffer A, centrifuged and the supernatant injected on a Mono S column to determine the ratio of c.p.m. in the C3 and C3b peaks. Attachment efficiency was calculated after determining the % C3 cleaved. For this an expression was derived equating the ratio c.p.m. C3/c.p.m. C3b (from the Mono S column) to an expression correcting the % C3 cleaved for the proportion of C3b bound to the particles and not loaded on the Mono S column:

$$\frac{(\text{c.p.m. C3})}{(\text{c.p.m. C3b})} = \frac{100\% - (\% \text{ C3 cleaved})}{(\% \text{ C3 cleaved}) - (\% \text{ C3 bound})} \quad (1)$$

Solving for % C3 cleaved, the only unknown, gives:

% C3 cleaved (pure) =

$$\frac{100 + (\% \text{ C3 bound})(\text{c.p.m. C3})/(\text{c.p.m. C3b})}{(\text{c.p.m. C3})/(\text{c.p.m. C3b}) + 1} \quad (2)$$

The true attachment efficiency for purified C3, corrected for the proportion of C3 not activated, was then calculated according to eqn. (3).

$$\text{Attachment efficiency (pure C3)} = 100 \times \frac{(\% \text{ C3 bound})}{\% \text{ C3 cleaved (pure)}} \quad (3)$$

Eqn. (3) gives the percentage of the C3 activated at the surface that attached to the surface.

Measurement of C3b attachment efficiency with normal human serum (NHS)

C3b attachment in NHS was studied by incubating 250 μl of 50 % particle suspension in GVB with 500 μl of NHS containing 1.4 μCi of ^{125}I -C3 and 250 μl of GVB containing 10 mM MgEGTA, at 37 °C. The ^{125}I -C3 was purified on Mono S just before use. Samples (40 μl) were taken out at various time intervals, layered on 20 % (w/v) sucrose in GVB and centrifuged to separate bound and free C3b. Attachment efficiencies were determined during the amplification phase [39] of the activation time course. Controls contained 10 mM EDTA.

The percentage of native C3 was determined from the ratio of radioactivity in the C3 peak to the total radioactivity eluting from Mono S (% C3 Exp). NHS treated identically, but without activating particles, was run as the control (% C3 NHS). The percentage of C3 remaining unactivated was the ratio of these values corrected for the C3b removed by attachment to the particle:

$$\% \text{ C3 remaining (NHS)} = \frac{(\% \text{ C3 Exp})(100 - \% \text{ C3 bound})}{(\% \text{ C3 NHS})} \quad (4)$$

and

$$\% \text{ C3 cleaved (NHS)} = 100 - [\% \text{ C3 remaining (NHS)}] \quad (5)$$

Finally, the attachment efficiency in NHS was calculated from the measured percentage bound corrected for the % C3 cleaved (NHS) to yield the true proportion of the activated C3 which bound:

$$\text{Attachment efficiency (NHS)} = 100 \times \frac{(\% \text{ C3 bound})}{[\% \text{ C3 cleaved (NHS)}]} \quad (6)$$

Data analysis by non-linear regression

Binding data were plotted and fit by non-linear regression analysis using the method of Marquart (GraFit v2.0 from Erithacus Software, London, U.K.) and the equation for simple single-site inhibition:

$$\% \text{ of Max. C3b bound} = 100/[1 + (\text{Inhibitor concn.})/IC_{50}] \quad (7)$$

Statistical analysis of these fits yielded S.E.M.s of less than $\pm 5\%$ of IC_{50} for the determinations reported here.

RESULTS

Comparison of assay methods

Several versions of the attachment inhibition assay have been employed for the measurement of C3 attachment efficiencies [25–27,29,33]. One assay measures inhibition of C3b attachment to radioactive compounds with both the radioactive compound and the inhibitor in the fluid phase [25,26,29]. A second type of assay measures competition of a fluid-phase inhibitor with attachment of ^{125}I -C3b to a particle [27,33]. A preliminary comparison of the fluid-phase assay (using [^3H]glycerol as the target) with the attachment assay used in the present study (using zymosan) showed that the IC_{50} values for seven different inhibitory compounds differed less than 2.0-fold between the two procedures (results not shown) and the statistical accuracy of the zymosan assays was much higher. The agreement in IC_{50} values between the two assays and the near perfect fit to theoretical binding curves (Figure 1) suggested that the all-fluid-phase assay and the particle assay are mechanistically similar [25,26,29,30,54].

Specificity of the activated thioester for sugars

IC_{50} values were determined for a large number of mono-saccharides using the zymosan-binding assay. The observed IC_{50} values varied 12-fold (Figure 1 and Table 1). Ribose was found to be the most effective sugar, while inositol was least effective in inhibiting the attachment of C3b to zymosan. Glucose required more than 2-fold higher concentrations than xylose to inhibit binding by 50 %. The S.E.M.s were less than $\pm 5\%$ for all IC_{50} values reported here. As will be shown below even apparently small differences in attachment efficiency may have a dramatic effect on the progress of alternative pathway activation, due to their influence on every cycle in the amplification process.

Figure 1 Inhibition of ^{125}I -C3b attachment to zymosan by various concentrations of xylose (●) or inositol (▲)

Zymosan particles bearing surface-bound C3 convertase (C3b,Bb) were incubated with ^{125}I -C3 and increasing concentrations of monosaccharide for 15 min at 37 °C. The percentage of C3b attached to zymosan was determined by centrifuging particles through 20 % (w/v) sucrose. The data were normalized by setting 100 % bound equal to the average binding of C3b in the absence of competitor. Solid lines were generated by non-linear regression analysis using the software GraFit and eqn. (7) for single-site competitive inhibition.

Table 1 Reactivity of the activated thioester with sugars and alcohols

Reactivity was measured by determining the concentration of sugar required to inhibit 50% of the attachment of ^{125}I -C3b to particles as shown in Figure 1. Relative reactivity was the ratio between the reactivity with sugar and the reactivity with water (hydrolysis). Non-linear regression analysis showed S.E.M.s to be less than $\pm 5\%$ for all IC_{50} measurements.

Compound	No. of hydroxyl groups	IC_{50} (mM)	Relative reactivity per mol
Water	1	—	(1)
Monosaccharides			
Inositol	6	870	56
Fucose	4	297	179
Glucose	5	161	336
Arabinose	4	159	340
Rhamnose	4	156	347
Galactose	5	149	360
Glucosamine	4	137	396
<i>N</i> -Acetyl- β -galactosamine	4	134	403
Galactosamine	4	127	427
<i>N</i> -Acetyl- β -glucosamine	4	117	462
Fructose	5	117	467
Mannose	5	111	491
Sialic acid	6	96	561
Xylose	4	81	674
Ribose	4	69	793
Disaccharides			
Melibiose	8	201	261
Gentiobiose	8	109	494
Maltose	8	107	507
Sucrose	8	91	592
Trisaccharides			
Maltotriose	11	79	678
Raffinose	11	65	831
Melezitose	11	63	850
Tetrasaccharides			
Stachyose	14	162	313
Maltotetraose	14	82	644
Dextran tetramer	14	80	661
Deoxy-sugars			
2-Deoxyglucose	4	88	618
6-Deoxyglucose	4	227	236
6-Deoxygalactose (Fucose)	4	297	179
6-Deoxy-L-mannose (Rhamnose)	4	156	347
Alcohols			
Methanol	1	137	404
Ethanol	1	175	314
<i>n</i> -Propanol	1	112	492
<i>n</i> -Butanol	1	138	419
Isopropanol	1	386	141
<i>t</i> -Butanol	1	944	55
Ethylene glycol	2	146	377
Glycerol	3	95	579

The relative reactivity of sugars compared with the reactivity of water is also given in Table 1. Ribose was 793 times more reactive than water on a molar basis and the average reactivity per -OH on ribose was 198 times that of water. This comparison highlights the selectivity of the thioester site in metastable C3b for carbohydrates as opposed to hydrolysis.

Positional preferences in sugars

The relative reactivity of individual -OH groups at specific positions on sugars was examined by testing the reactivity of

Figure 2 Effect of polysaccharide size on reactivity with C3b

Zymosan bearing surface-bound C3 convertase (C3b,Bb) was incubated with ^{125}I -C3 and increasing concentrations of α 1-4 polyglucose (amylose) oligosaccharides for 15 min at 37 °C. Bound C3b was separated from free protein by centrifugation through 20% (w/v) sucrose. IC_{50} and relative reactivity per -OH group were calculated for each oligosaccharide as described in the Materials and methods section.

deoxy-sugars (Table 1). A comparison of the reactivity of glucose and galactose with that of their 6-deoxy forms suggests that this position is considerably more reactive than the average hydroxyl group. In glucose and galactose the reactivity lost by removal of the 6-OH was 100 and 181 (times that of water, respectively) versus reactivities of 59 and 45 for the average -OH in the rest of the molecules (Table 1). Similarly, examination of the di-, tri- and tetrasaccharides in Table 1 shows that most of the differences may be accounted for by the number of primary -OH groups (6-OH or 1-OH groups).

Removal of the 2-OH of glucose, resulted in a 2.3-fold increase in reactivity of the average -OH group, which may be related to release of steric hindrance as will be described below.

Positional preferences in polysaccharides

Metastable C3b formed during complement activation does not generally encounter isolated monosaccharides. In order to analyse the reactivity with higher polymers and keep the variables to a minimum, a series of oligosaccharides composed solely of glucose units was examined. The results from the α 1-4-linked amylose-derived oligosaccharides (maltose, maltotriose, etc.) are shown in Figure 2. The IC_{50} decreased rapidly between monomer and trimer then showed little change up to the 12-mer. However, to interpret these results correctly it is necessary to compare the relative reactivities/hydroxyl group because neither the IC_{50} nor the relative reactivity/mol take into account the changes in the number of -OH groups per molecule. Viewed in this way (Figure 2b) it is apparent that the reactivity of the monomer, dimer and trimer are not significantly different except for the greater number of reactive groups on each molecule. The decrease in reactivity of -OH groups in the higher polymers suggests that sugars occupying central positions have low reactivity relative to the ends. Calculating the relative reactivity per end in the dimer maltose ($507/2 = 254$), subtracting this from the reactivity of the heptamer [$686 - (2 \times 254) = 178$] and dividing by the number of interior sugar residues ($178/5 = 36$) yields a 7:1 ratio ($254/36$) between the reactivities of end residues and interior residues respectively. Similar data were found for the α 1-6 polyglucose series of oligosaccharides (results not shown).

Table 2 Theoretical dependence of the amplification rate on C3b attachment efficiency

Computer model of the effect of attachment efficiency on rates of amplification of C3b on a particulate surface assuming that the first C3b molecule was bound after 1 s and that each C3 convertase formed immediately and then operated at 56% of the $V_{\max}$ [55], as expected in undiluted human plasma in the absence of control factors.

Attachment efficiency (%)	Time (s)...	Number of C3b molecules attached		
		30	60	90
2.5		2	4	9
5		4	18	77
6		5	31	179
7		7	54	412
8		9	94	942
9		12	161	2143
10		16	189	4830
11		21	472	10808
12		27	801	24011
15		58	3812	252411

Figure 3 Effect of glucose on C3b attachment to zymosan during alternative pathway activation in NHS

Deposition of C3b on zymosan was measured by incubating 500 μ l of NHS containing 2 μ g of 125 I-C3 (1.4 μ Ci), with 225 μ l of GVB or GVB containing 711 mM glucose, 25 μ l of 0.1 M MgEGTA and 250 μ l of zymosan (50% in GVB) at 37 °C. Samples (50 μ l) were removed at different times and layered on 250 μ l of 20% (w/v) sucrose in a microfuge tube. After centrifugation the radioactivity present in pellet and supernatant was determined and used to calculate the percentage of total C3 bound.

Reactivity of the thioester with simple alcohols

Primary alcohols (Table 1) from methanol to butanol exhibited essentially the same reactivity, but the reactivity of secondary

and tertiary alcohols dropped 3-fold and 7-fold respectively, in agreement with previous results of Law et al. [25]. The 3-fold difference found with alcohols is similar to the difference described above between 6-position -OH groups (primary) versus other -OH positions (secondary) in monosaccharides.

Computer modelling of the effect of C3b attachment efficiency on alternative pathway activation

The biological significance of differences in the attachment efficiency of C3b to different polysaccharides was modelled using numerical integration to examine the dependence of amplification on C3b attachment efficiency. Table 2 shows the results of the simulation. The highly simplified model assumed that all cells received one initiating C3b after 1 s in serum; that all attached C3b could form a C3 convertase; that each C3 convertase cleaved one C3 per second (56% of $V_{\max}$ [55], which is the rate expected in undiluted human plasma); and that no control systems were present (neither properdin nor Factors H and I). The result, while not physiological, is useful in illustrating the powerful influence attachment efficiency may have on the amplification of C3b on a surface. The difference between 5% and 15% in the attachment efficiency of activated C3b results in a difference of 77 versus 252411 C3b/cell after 90 s. A difference as small as 1% (8% versus 9%, Table 2) results in attachment of 942 versus 2143 C3b/cell in the model. While not a mechanistically correct model, this analysis demonstrates the large differences in activating potential which may result from seemingly minor differences in attachment efficiency due to the influence on each successive step in the alternative pathway amplification process.

Effect of reduced attachment efficiency on complement activation

Experimental verification of the principle illustrated by the model was obtained by altering C3b attachment efficiency by adding glucose to complement assays (Figure 3). The presence of 160 mM glucose during activation of the alternative pathway inhibited both the rate and extent of C3b uptake, delaying attainment of the 50% level from 7 to 13 min and reducing the maximum C3b uptake to 5.4%. The predisposition of hyperglycaemic diabetics to infection may be exacerbated by just such a phenomenon [56].

Correlation of complement activation on biological particles with C3b attachment efficiency

Model structures of the four major capsular polysaccharides of the yeast *C. neoformans* have been described [48,49,57] in which the surface polysaccharide is decorated with one (serotype D), two (serotype A), three (serotype B) or four (serotype C) xylose residues branching out from each trimannose repeating unit of the polysaccharide chain. Figure 4 shows that both the maximum amount of C3b bound and the rate of complement activation in NHS increased with the expected number of xylose units on the polysaccharides of *C. neoformans*. The attachment efficiency of C3b examined in the purified C3 system showed a strong dependence on xylose content with the single-xylose strain (serotype D) binding only 12% of the C3 activated at its surface, while the four-xylose strain (serotype C) bound 41% (Figure 5a). The attachment efficiency of C3b in NHS also increased with increasing xylose content, although not as dramatically (Figure 5b). A significant correlation ($r = 0.90$) between attachment efficiency measured with purified C3 and that measured in NHS was also found. When a similar comparison was extended to 20 largely randomly selected activating particles (Figure 6), their

Figure 4 Time course of C3b deposition on four strains of *C. neoformans* in NHS

The number of xylose residues per unit in model structures of the four *C. neoformans* serotypes D (strain 52), A (strain 271), B (strain 182) and C (strain 191) is one, two, three and four respectively [57]. The percentage of C3 deposited was determined by incubating 250 μ l of different serotypes of *C. neoformans* (5×10^7 cells) with 500 μ l of NHS containing 2 μ g of 125 I-C3 (1.4 μ Ci), 225 μ l of GVB and 25 μ l of 0.1 M MgEGTA at 37 °C. The percentage of C3 bound after the indicated time intervals was determined by centrifuging 40 μ l of reaction mixture through 250 μ l of 20% (w/v) sucrose.

Figure 5 Correlation between C3b attachment efficiency and xylose content of *C. neoformans* capsule

Correlation of the number of xylose residues per repeating polysaccharide unit in model structures of the *C. neoformans* capsular polysaccharides with the attachment efficiency of the organisms. The percentage of C3b attached in the purified system and in NHS were determined using 125 I-C3 as described in the Materials and methods section. Each point represents the mean of at least three determinations.

attachment efficiencies in serum did not show a significant correlation with C3b attachment efficiencies in the purified system ($r = 0.20$). This was probably due to the many factors other than C3b attachment efficiency which affect activation of the alternative pathway [1–3]. Better correlations were found in sub-groups of organisms, for example, the four *Cryptococci* and four strains of *Salmonella*, with fewer differences other than in their polysaccharides. The *Salmonella* strains exhibited a weak correlation

Figure 6 Comparison of attachment efficiencies of 20 different biological particles

Scatterplot of the C3b attachment efficiency of each organism assayed in the purified system plotted against the attachment efficiency of that organism assayed during complement activation in NHS. Biological particles are identified as: 1, rabbit erythrocytes; 2, zymosan; 3, group B *Streptococci* COH31-15; 4, group B *Streptococci* COH-1; 5, *E. coli* XL1 Blue; 6, *E. coli* NM 522; 7, *E. coli* PRC 116; 8, *E. coli* PRC 104; 9, *E. coli* ATCC 35218; 10, *E. coli* ATCC 25922; 11, *E. coli* ATCC 12792; 12, *E. coli* ATCC 33780; 13, *Salmonella typhimurium* SH 5771; 14, *Salmonella montevideo* 5770; 15, *Salmonella enteritidis* SH 4340; 16, *Salmonella typhimurium* TV 119; 17, *C. neoformans* serotype D (strain 52); 18, *C. neoformans* serotype A (strain 271); 19, *C. neoformans* serotype B (strain 182); 20, *C. neoformans* serotype C (strain 191).

($r = 0.63$) between complement activation in NHS and C3b attachment efficiency.

DISCUSSION

One illustration of the effect of C3b attachment efficiency on complement activation is shown in Figure 4 where four strains of *C. neoformans*, differing in the numbers of xylose branches, were allowed to activate human complement. Deposition of C3b on serotype D with a single xylose branch per repeating trimannose unit [48,49,57] was slow and the maximum C3b attached was only one-quarter of that on serotype C possessing four xylose sugars per unit. Because complement, not the number of yeast particles, was limiting, these observations reflect the attachment efficiency of the yeast not the capsule size as has been reported in another study [40]. *In vitro* measurements demonstrated that these strains bound 12% and 41% respectively of the C3 activated at their surfaces. These findings suggest that the specificity expressed at the thioester site of activated C3b may have a biological relevance not previously demonstrated.

Earlier studies, principally by Law and co-workers [25–27,58] have shown that activated C3b exhibits a strong preference for hydroxyl groups, a preference for certain sugars and poor or non-existent reactivity with amino groups. Law et al. derived an expression defining the binding efficiency (BE) to compare the efficiency of attachment to radioactive compounds [25,26]. Be-

cause the value of BE varies with the concentration of the compound used in the test we have reported IC_{50} values. The IC_{50} value is related to BE such that at a concentration equal to the IC_{50} , the BE of a compound is equal to 0.5. We have also reported the reactivity relative to that of a universal standard, water. These values allow direct comparisons of reactivities per molecule independent of the concentration of the compound, its molecular mass or, when presented as reactivity per -OH, the number of -OH groups per molecule.

Reactivity with sugars and polysaccharides

Analysis of the reactivity of sugars and alcohols reveals a number of features of metastable C3b specificity (Table 1, Figures 1 and 2). A first attempt at formulating generalized rules for this specificity is given below; although the data contain apparent exceptions and modifications may be necessary.

First, the primary -OH in the 6 position appears to be more reactive than the average -OH group in sugars. One-third to one-half of the reactivity of glucose, galactose or mannose (Table 1) was lost in the 6-deoxy forms of these sugars. Comparison of the relative reactivities shows that the 6-OH group was 2.5-fold more reactive than the average secondary -OH groups of these sugars. This figure compares well with the value (2.9-fold) obtained with primary and secondary alcohols (Table 1 and [25]). Not clear, however, is why the primary -OH group of simple alcohols was twice as reactive as the primary -OH groups in sugars and in compounds such as ethylene glycol.

Secondly, several comparisons suggest that the reactivity of metastable C3b is increased if the -OH group is next to less bulky groups such as the ring -O- or a -CH₂- (deoxy) group. In 2-deoxyglucose the steric hindrance was removed from between two secondary -OH groups and the reactivity was twice that of glucose even though one -OH group was lost. Similarly, xylose possesses -OH groups at positions 1 and 4, each with a neighbouring -O- or -CH₂-group. Xylose was the most reactive of all the 6-membered-ring sugars examined, yet it contains only four -OH groups. Inositol with six -OH groups lacks -O- and -CH₂- as well as a primary 6-OH and it had the lowest reactivity of any compound tested. The reactivity of ethylene glycol is 50 % higher than that of glycerol due to the fact that the secondary -OH group has low steric hindrance on both sides. This occurs because the end -OH groups are free to rotate back into positions normally occupied by ring atoms in sugars. These and other examples in the data suggest that the thioester in metastable C3b resides in a pocket with restricted access and that bulky groups on either side of a secondary -OH group appear to reduce its reactivity.

Thirdly, the axial/equatorial arrangement of -OH groups may affect reactivity, but the data are not consistent on this point. Xylose (reactivity 674) and arabinose (340) differ only in that arabinose possesses an axial -OH group at position 4. Xylose possesses all equatorial -OH groups. Less dramatic is the effect seen in the 6-deoxyglucose (236)/fucose (179) pair. And the reverse was found in the glucose (336)/galactose (360) and the glucose (336)/mannose (491) pairs. Although the equatorial position appears to be the most reactive, the effect does not appear to override the effects of steric hindrance at neighbouring sites as evidenced by inositol.

Fourthly, no experimental data are available about the preferred sites of attachment of C3b on polysaccharides at the surface of biological particles. To begin to approach this most interesting of questions the reactivity of metastable C3b with a series of pure oligosaccharides of known structures was measured (Figure 2b). The results suggest that there should be a strong

preference for terminal residues or branches of polysaccharides on the surfaces of biological particles, as indicated by the xylose dependence of C3b attachment to *Cryptococcus*.

Biological significance of thioester specificity

Activation of complement on potential pathogens is affected by many different factors. The data presented here suggest that in some cases a significant factor may be the efficiency of C3b attachment. Although C3b attachment is necessary for activation of both pathways of complement, the alternative pathway is especially sensitive to this step due to its reliance on repetitive cycles of C3b deposition during amplification. This is illustrated in principle in Table 2, although in reality the presence of control factors would slow down this process.

Attachment efficiencies on biological particles measured both in NHS and with purified components were very high in this study compared with other studies [33,34,39]. There are four major reasons for this. First, the ¹²⁵I-C3 used here was repurified before each use. Secondly, because half of the C3b may be released in the first 10 min after deposition [39,59], rapid (60 s) centrifugation through sucrose was used instead of washing. Thirdly, the % C3 bound was corrected for the proportion of C3 activated so that it accurately reflects the percentage of C3 which had a chance to bind. Fourthly, in the purified assay only the first few C3b deposited around the enzyme were measured, so the result reflects a property of the innate surface rather than the C3b-coated surface. This effect can be seen in Figure 6 where the large differences observed with purified C3 (12–48 % binding) were reduced in NHS (8–26 %).

The poor correlation of attachment efficiency with purified C3 and with NHS illustrated in Figure 6 is probably due to a variety of differences between the organisms and how they activate complement. These include the presence or absence of complement defence systems on the cells (e.g. complement regulatory proteins, polyanions or sialic acid), differences in the density or positions of bound C3b, the presence of different amounts of natural antibodies in the NHS used and different rates of C3b release. For example, although rabbit erythrocytes exhibited 32 % attachment with purified C3 and 26 % in NHS, sheep erythrocytes exhibited 38 % attachment with purified C3, but no attachment in NHS because the sialic acid on sheep cells prevents activation of the alternative pathway.

The data in Figure 6 make it clear that the ability of metastable C3b to attach to biological particles varies greatly among different organisms. Attachment efficiencies for the initial layer of C3b on a surface varied from 12–48 %. In serum during amplification these efficiencies drop for reasons cited in the previous paragraphs, but significant differences remain. While the range of attachment efficiencies of different *Cryptococci* with purified C3 was 12–41 %, the range in serum was only 8–12 %. Nevertheless such small differences, as predicted by the model in Table 2, may have a profound effect on the progress of complement activation in serum as shown in Figure 4.

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