INFECTION AND IMMUNITY, Nov. 1983, p. 594–598
0019-9567/83/110594-05$02.00/0

Vol. 42, No. 2

Classical Complement Pathway Activation by Antipneumococcal Antibodies Leads to Covalent Binding of C3b to Antibody Molecules

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Received 29 April 1983/Accepted 12 August 1983

We have examined whether or not a physical relationship exists between antipneumococcal antibodies (Ab) and C3b when Ab activate the classical complement pathway on the surface of pneumococci (Pn). After sensitization with ^{125}I-labeled Ab, Pn were sequentially incubated with purified C1, C4, C2, and biotinylated C3. Ab molecules were then eluted from Pn, and C3b-associated molecules were purified on avidin-Sepharose. Both ^{125}I-labeled immunoglobulin G (IgG) and [^{125}I]IgM bound to C3b; the association was stable to incubation in 1% sodium dodecyl sulfate at 37°C. The association was only partially reversed by incubation in 1 M hydroxylamine–0.5% sodium dodecyl sulfate (pH 10.5), implying that Ab and C3b were linked by amide as well as ester bonds. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of dithiothreitol and NH_2OH eluates from the avidin-Sepharose showed that C3b bound to both heavy and light chains of the Ab. Moreover, the ability to bind to erythrocyte C3b receptors could be transferred to unsensitized Pn by eluates from Pn on which Ab had activated the classical pathway. These covalent complexes of Ab and C3b may be especially important in the opsonization of Pn by Ab and complement.

The need for antipneumococcal antibodies (Ab) in efficient host defense against infections with pneumococci (Pn) has long been appreciated (13). The molecular basis for this phenomenon has been examined in a guinea pig model of experimental infection (4, 15). In this model, we recently have shown that type-specific Ab are effective opsonins in Pn bacteremia because they activate complement. Although the alternative complement pathway mediates bacterial clearance in the absence of Ab, the in vivo protective effect of both immunoglobulin G (IgG) and IgM Ab requires classical complement pathway activation. Since C3b is known to be the most important opsonin of the complement system (11), our findings led us to investigate the association between Ab and C3b during Ab-induced classical pathway activation on the surface of Pn.

MATERIALS AND METHODS

Pn. Pn R36a (no. 27336; American Type Culture Collection, Rockville, Md.) were obtained in lyophilized form, reconstituted, and maintained as previously described (4). For each experiment, R36a were grown to log phase in Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy broth, washed three times in phosphate-buffered saline, and then heat killed by incubation for 30 min at 56°C, a temperature sufficient to inactivate the Pn autolysin (24).

Anti-Pn Ab. IgG and IgM Ab to R36a were prepared and purified as described elsewhere (4). Ab were radiolabeled with ^{125}I either with iodobeads (Pierce Chemical Co., Rockford, Ill.), according to the manufacturer's instructions or with the Bolton-Hunter reagent (New England Nuclear Corp., Boston, Mass.) as described elsewhere (3). For some experiments, nonradiolabeled Ab were biotinylated as described elsewhere (5).

Sensitization of Pn with Ab and complement. Heat-killed Pn were treated with [^{125}I]Ab and washed in phosphate-buffered saline, and then the uptake of radiolabel was quantitated. These Pn-Ab complexes were used to assemble the classical pathway C3 convertase, C4b2a, on the surface of the Pn by sequential incubation with purified C1, C4, and C2 exactly as previously described (4), except that human C4 and C2 were used in place of guinea pig components. Biochemically pure C3 (12) that had been biotinylated without the loss of hemolytic activity (1) was then reacted with the organisms for 60 min at 30°C. The Pn were then washed extensively in phosphate-buffered saline.

Elution of Ab and C3b from the Pn surface. After being washed, the Pn were incubated for 30 min at 37°C in 1% sodium dodecyl sulfate (SDS)–0.0375 M Tris (buffer A), pH 8.0. Preliminary experiments showed that this incubation removed the vast majority of Ab from the Pn. However, buffer A released less

TABLE 1. Binding of ^{125}I-labeled anti-Pn Ab to A/S via biotinyl-C36[a]

Treatment of Pn	% Elution from Pn of ^{125}I-labeled anti-Pn Ab		% Binding to A/S by eluted ^{125}I-labeled anti-Pn Ab	
	IgM[b]	IgG[c]	IgM	IgG
Incubation with biotinyl-C3 before elution with buffer A	90	84.2	14.6[d]	9.2[d]
Incubation with unbiotinylated C3 before elution with buffer A	90.5	84	0.3	1.5
Incubation with biotinyl-C3 before elution with buffer A (A/S presaturated with biotin)	90	84.2	0.9	0.8

[a] As described in the text, 2×10^9 Pn were sensitized with radiolabeled Ab and classical pathway components through C3. The organisms were incubated in buffer A for 30 min at 37°C. The eluate was next incubated with 0.5 ml of A/S for 12 h at room temperature in buffer A. After five 2-ml washes with buffer A, no more radioactivity washed off the A/S.

[b] Molecules per Pn, 6,040.

[c] Molecules per Pn, 5,550.

[d] The percent binding is 6- to 50-fold greater than that with the other treatments, implying that [^{125}I]Ab is sticking specifically to the A/S via biotinyl-C3.

than 1% of [^{3}H]choline (which is incorporated into Pn cell walls) and of [^{75}Se]methionine (as a marker of the internal compartment) from heat- or glutaraldehyde-killed radiolabeled Pn under identical conditions. Buffer A supernatants were incubated with avidin-Sepharose (A/S) (2) for 12 h at room temperature to allow the binding of biotinyl-C3b in solution. After five washes with buffer A, the binding of [^{125}I]Ab to A/S was quantitated to discover whether radiolabeled Ab had become associated with biotinyl-C3. In some experiments, biotinylated IgG and IgM were used to sensitize the Pn and then to deposit [^{125}I]C3 via the classical pathway convertase.

Elution of C3b from A/S. To examine the nature of the bond formed between Ab and C3b on Pn, we incubated A/S bearing [^{125}I]Ab bound to biotinyl C3b with 1 M NH_2OH–0.5% SDS–0.02 M Tris (buffer B; pH 10.5) at 37°C for 1 h. The release of specifically bound Ab was calculated by the formula (^{125}I released from A/S − ^{125}I released from A/S presaturated with biotin)/(total ^{125}I bound to A/S − total ^{125}I bound to A/S presaturated with biotin).

To determine the immunoglobulin chains to which C3b attached, we incubated [^{125}I]IgG bound to A/S via biotinyl-C3b for 30 min at 37°C in buffer A containing 50 mM dithiothreitol (DTT) to reduce intramolecular disulfide bonds. The reduction of interchain disulfide bonds in the presence of SDS releases, from A/S, immunoglobulin chains not covalently linked to C3b. The A/S was then washed four times in buffer A-DTT. The IgG-associated ^{125}I remaining of the resin did not change between washes 3 and 4, implying that all DTT-releasable counts had been washed from the resin. The resin was then washed twice with distilled water to remove the DTT and finally incubated with buffer B for a further 30 min at 37°C to release radiolabeled immunoglobulin chains bound via ester bonds to biotinyl-C3b. The DTT and hydroxylamine eluates from A/S were analyzed by SDS-polyacrylamide gel electrophoresis (18) followed by autoradiography.

Interaction of Ab-C3b complexes with the C3b receptor. To test whether C3b that was bound to Ab could interact with the C3b receptor, we used an immune adherence assay (21). To test the ability of Ab-C3b complexes to transfer immune adherence, we sensitized 2×10^9 Pn with IgM Ab and classical pathway components through [^{125}I]C3 with Ab alone or by incubation in C2-deficient serum containing [^{125}I]C3 that had been absorbed with Pn at 0°C to remove Ab. Sensitized Pn were washed and eluted by a 2-h room temperature incubation in 1 M glycine, pH 3.3. After neutralization by extensive dialysis in phosphate-buffered saline, the eluates were reincubated with 5×10^8 unsensitized Pn for 2 h. After being washed, these Pn were tested for immune adherence, which was quantitated as previously described (4).

RESULTS AND DISCUSSION

Table 1 shows that 90% of IgM and 84.2% of IgG were eluted from Pn by incubation in buffer A. Both IgG and IgM bound to A/S after complement activation through biotinyl-C3, implying that an SDS-stable association between Ab and C3b was formed during classical pathway activation. Only 0.3% of the [^{125}I]Ab bound to A/S when unbiotinylated C3 was used rather than biotinyl-C3, and only 0.9% of the [^{125}I]Ab bound when A/S was presaturated with biotin, confirming that the radiolabeled Ab bound to A/S via biotinyl-C3. To confirm the SDS-stable binding of C3 to Ab, we used biotinylated IgG and IgM anti-Pn Ab to sensitize Pn and then to deposit [^{125}I]C3. Table 2 shows that slightly more than half of the [^{125}I]C3b was eluted by incubation in buffer A. SDS-polyacrylamide gel electrophoresis showed that all the C3 α' chain in this eluate migrated with a M_r greater than 200,000, whereas the size of the unbound α' chain is only 110,000. This suggests that the buffer A-eluted C3 was not nonspecifically bound to the bacterial surface. Moreover, the stability of the Ab-C3b bond after being boiled in SDS suggests that this bond is covalent. This is consistent with the known mechanism of C3b bond formation (19, 23). Some buffer A-eluted [^{125}I]C3b was bound specifically to A/S by both biotinyl-IgG and biotinyl-IgM and accounted for approximately 10% of the total C3b originally bound to the Pn.

C3b has been shown to bind covalently to ligands via ester bonds formed by nucleophile attack on an internal thioester in the C3 mole-

cule (19, 23). This ester bond is cleavable by NH_2OH at pH 10. However, theoretical considerations have led to the postulate that C3b may also form covalent amide bonds with receptive surfaces (17). In support of this hypothesis, Gadd and Reid showed that 74 to 78% of C3b which is bound to IgG in soluble immune complexes was not released by hydroxylamine treatment (10). In our experiments, 39% of the IgM and 53% of the IgG which bound to A/S via biotinyl-C3b remained bound after treatment with buffer B (Fig. 1). When [^{125}I]C3b was bound to A/S via biotinyl Ab, the treatment of A/S with buffer B released 53.2% of the C3b counts from A/S containing biotinyl-IgM, and 66.2% of the [^{125}I]C3b was released from A/S containing biotinyl-IgG. Under identical conditions, [^{3}H]raffinose that was bound to A/S via biotinyl-C3b was completely released. Moreover, the released raffinose chromatographed as a small molecule (i.e., eluted at 1 column volume) on Sephadex G-25. Since raffinose can bind to C3b only by ester bonds, this experiment shows that the conditions employed are sufficient to release, from A/S, the ligands which are bound to C3b by ester bonds. This experiment provides further evidence of a covalent association between C3b and Ab when the classical pathway is activated at the Pn surface and shows that not all C3b is bound to Ab by an ester linkage. This implies that C3b is bound to IgM and IgG on Pn via amide as well as ester bonds. As discussed above, the incubation of sensitized Pn with buffer A released 80 to 90% of bound Ab and about half of the C3b. The incubation of the buffer A-treated Pn with buffer B released 63.0% (IgM-sensitized Pn) and 73.8% (IgG-sensitized Pn) of the remaining C3b, implying that some of the C3b molecules remaining on the Pn are also bound to the bacterial surface by linkages other than ester bonds.

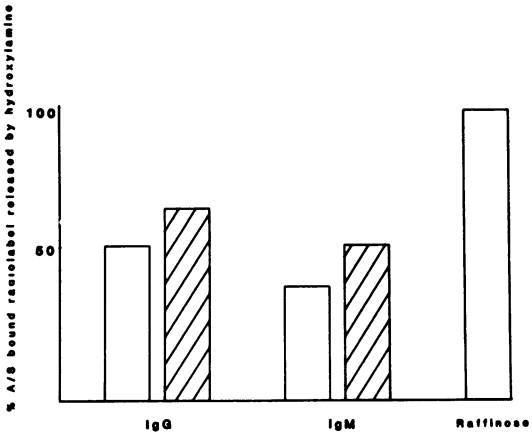

FIG. 1. Release of radiolabel from A/S by hydroxylamine. The percentage of ^{125}I that was bound to A/S and released by a 30-min incubation at 37°C with buffer B is calculated for radiolabeled IgG and IgM, which had been bound to A/S via biotinyl-C3b (open bars). The NH_2OH-mediated release of [^{125}I]C3b is shown when the C3b is bound to A/S via biotinyl IgG or biotinyl IgM (hatched bars). The conditions used were sufficient to cleave C3b-ligand ester bonds, as demonstrated by the 100% release of [^{3}H]raffinose.

TABLE 2. Binding of [^{125}I]C3 to A/S via biotinylated anti-Pn Ab

Sensitization of Pn[a]	% Elution from Pn of [^{125}I]C3b		% Binding to A/S by eluted [^{125}I]C3b	
	IgM	IgG	IgM	IgG
Biotinylated anti-Pn Ab	65.1	47.3	14[b]	29[b]
Unbiotinylated anti-Pn Ab	64.8	46.8	0.4	0.2

[a] After 1.5×10^9 Pn were sensitized with 5,000 molecules of IgG or IgM per Pn, they were incubated with classical complement pathway components through [^{125}I]C3. When C2 was omitted from the reaction, [^{125}I]C3 binding was reduced by more than 90%. A total of 5,000 C3 molecules per organism were specifically bound to IgG- and IgM-sensitized cells. The organisms were incubated with buffer A, and the supernatants were incubated with 0.5 ml of A/S. After five washes with buffer A, the A/S was assayed for bound radioactivity.

[b] The 34- to 58-fold greater binding with biotinylated anti-Pn Ab implies a specific SDS-stable association between Ab and C3b, as did the data from the inverse experiment in Table 1.

We next examined the immunoglobulin chains to which the C3b covalently bound. As described above, [^{125}I]IgG which was bound to A/S via biotinyl-C3b was eluted sequentially with buffer A-DTT and buffer B. When examined by SDS-polyacrylamide gel electrophoresis, both of these eluates contained heavy and light chains (Fig. 2). The elution of both heavy and light chains by DTT implies that, in the IgG-C3b complex, these chains are not covalently bonded to C3b. The elution of both heavy and light chains by NH_2OH implies that, in some cases at least, both heavy and light chains are linked via ester bonds to biotinyl-C3b. This suggests that C3b can bind to either chain of the immunoglobulin molecule during classical complement pathway activation on Pn.

To examine whether the Ab-C3b complexes could interact with C3b receptors, we tested acid eluates of Ab- and complement-sensitized Pn for their ability to transfer immune adherence. Eluates from Pn incubated with Ab and C1 through C3 did transfer immune adherence to unsensitized Pn, but eluates from Pn sensitized with Ab alone or with complement by the alternative pathway did not (Table 3). Thus, C3b

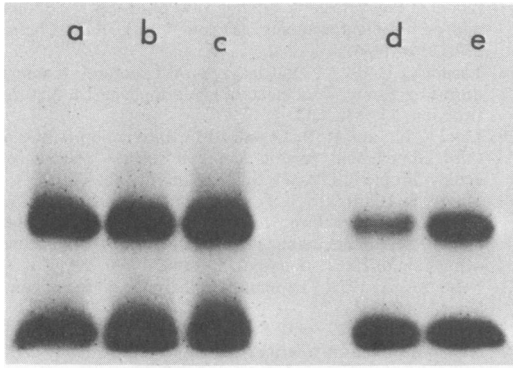

FIG. 2. SDS-polyacrylamide gel electrophoresis (5 to 10%) of anti-Pn IgG. Lane a, IgG anti-Pn Ab; lane b, buffer A eluate from Pn sensitized with [^{125}I]IgG and C1 through C3, including biotinyl-C3; lane c, supernatant of A/S incubation with the material in lane b; lane d, DTT eluate of the material which bound to A/S; lane e, NH_2OH eluate of the A/S which had already been incubated and washed with DTT. The presence of both heavy and light chains in lanes d and e implies that C3b can bind to both heavy and light chains of IgG during classical pathway activation on Pn. Samples a to c were incubated with 50 mM DTT before electrophoresis, but lanes d and e were electrophoresed without further reduction.

which was eluted from Ab-sensitized Pn was able to rebind to unsensitized Pn and mediate biological activity, i.e., bind to C3b receptors. Presumably, the binding of C3b to unsensitized Pn in this way was mediated by Ab molecules which had been eluted from Pn by acid and which rebound to the unsensitized Pn under conditions of neutral pH. These C3b molecules were most likely bound to Ab; C3 that was bound to Pn by an Ab-independent mechanism could not transfer biological activity in this way because acid-eluted C3b could not bind to unsensitized Pn when the pH was readjusted.

In summary, the results of these experiments show that both IgG and IgM Ab may be important binding ligands for C3b during complement activation on Pn. Although reports of IgG binding C3b or C4b in other systems have been published (6, 10, 16), C4b, at least, does not bind to IgM when complement is activated on erythrocytes (7), and no systematic examinations of whether C3 will bind to IgM have been undertaken. On Pn, the IgG or IgM Ab molecule may provide a site where C3b molecules may cluster. These clusters may greatly facilitate phagocytosis by presenting multiple complement receptor ligands to the phagocytic cell. It is interesting to speculate that IgG-C3 complexes may be especially opsonic since the Fc fragment of IgG and C3b would be presented to the phagocytic cell membrane in very close proximity. Indeed, IgG-C3b complexes may be extremely important for the synergy between complement and Ab which has been observed for bacterial phagocytosis in many systems (8, 14). C3b is generally regarded as the signal that mediates binding, and Fc is regarded as the signal mediating the phagocytosis of the particle to which they are bound (9); a covalent complex of IgG and C3b could present both signals simultaneously to the phagocytic cell.

TABLE 3. Ability of Pn eluates to transfer immune adherence[a]

Initial sensitization	[^{125}I]C3 in eluate (cpm)	Immune adherence in second step[b]
Ab, C1–C3	32,000 (9%)	2+
Alternative pathway in serum	21,000 (12%)	—
Ab alone	—	—

[a] As described in the text, Pn sensitized with IgM and [^{125}I]C3 via alternative pathway activiation or IgM and [^{125}I]C3 via classical pathway activation were incubated in acid glycine. The [^{125}I]C3 eluted by this treatment and the percent of initially bound C3 that this represents (in parentheses) are shown in the second column.

[b] Eluates from Pn incubated with Ab alone or with complement in the absence of Ab could not transfer immune adherence (—). However, eluates from Pn incubated with Ab and C1 through C3 did transfer immune adherence.

ACKNOWLEDGMENT

We thank Karen Leighty for excellent editorial assistance.

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