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Interaction between the third complement protein and cell surface macromolecules

(binding of third complement component)

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ABSTRACT **The activated form of the third complement protein, C3b, forms a stable complex with components of plasma membranes and particulate entities such as zymosan. The complex resists the action of detergents and protein denaturants as well as extremes of temperature, salt concentration, and pH. It can, however, be broken by exposure to hydroxylamine or by ammonolysis followed by incubation with sodium dodecyl sulfate. Thus, the complex appears to result from a hydrophobic interaction as well as a bond susceptible to nucleophilic attack.**

In the immune system, the recognition and elimination of foreign cells and particles depends in part on whether they bear on their surfaces C3b, the activated form of the third complement protein, C3 (1–3). C3 is comprised of two polypeptide chains, α (110,000 daltons) and β (70,000 daltons). C3b is derived from C3 by an enzymatic cleavage that removes a 9000-dalton polypeptide (C3a) from the NH_2 terminus of the α chain (1–3). C3b binds to cell membranes (1–3) and to particulate material such as zymosan (4), oil droplets coated with *Escherichia coli* lipopolysaccharide and human serum albumin (5), or Sepharose (6), and it cannot be readily dissociated from the surfaces to which it binds (5–7). In view of these properties of C3b, it is of some interest to understand the way in which it binds to receptive surfaces. In this paper we present results of experiments which suggest that hydrophobic interactions and covalent bonding are involved in the formation of the complex between C3b and erythrocytes and zymosan particles.

MATERIALS AND METHODS

Cells, Antisera, and Complement Proteins. Sheep blood, drawn into Alsever's solution, was obtained from a single animal. Rabbit anti-sheep hemolysin was purchased from Microbiological Associates. Guinea pig serum was obtained from Pelfreez Biologicals. Complement proteins C5, C6, C7, C8, and C9 used in assays were obtained from Cordis Laboratories. Guinea pig C1 and C2 were isolated and purified as described (8). Human C3 was isolated and purified from fresh frozen human plasma (9, 10). Different preparations of purified C3 gave hemolytic activities (11) of 10,000–200,000 eu/ml, where eu is defined as the amounts of C3 required to lyse 63% of 1×10^8 sensitized sheep erythrocytes at excess amounts of other complement components. Contaminating proteins in the C3 preparations were evaluated by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis. Relatively small amounts of contaminating material occur in all of our preparations (Figs. 1*c left* and 2*d left*).

Labeling by Iodination. Lactoperoxidase was purchased from Sigma Chemical Co. Immobilized lactoperoxidase on Sepharose beads was purchased from Worthington Biochemical Corp. ^{125}I or ^{131}I was obtained from New England Nuclear Corp. Immobilized lactoperoxidase was used to label C3 with either ^{125}I or ^{131}I (12). There is no loss of hemolytic activity of the C3 after its iodination. Erythrocyte membrane proteins were labeled with ^{125}I using soluble lactoperoxidase (13).

Erythrocyte–Complement Intermediates. Sensitized erythrocytes (EA) were prepared according to Nelson *et al.* (8), and cell–complement intermediates EAC1 and EAC14 according to Borsos and Rapp (14). EAC142 was prepared by incubating EAC14 at 1×10^8 cells per ml with guinea pig C2 (100 eu/ml) at 30° for 15 min in 2.5 mM Veronal buffer, pH 7.4/75 mM NaCl/2.5% dextrose/0.1% gelatin/0.15 mM Ca^{2+}/0.5 mM Mg^{2+} ($DGVB^{++}$). The cells were immediately washed twice in the same buffer at 0° in order to remove contaminating C3b-inactivator. EAC1423 was prepared by incubating freshly prepared EAC142 at 1×10^9 cells per ml with C3 or labeled C3 at 0.1–0.5 mg/ml for 30 min at 30°. The cells were washed twice in ice-cold $DGVB^{++}$. Cells with either ^{125}I- or ^{131}I-labeled C3b will be referred to as EAC1423*. When the cell membrane proteins are labeled, the notation is E*AC1423. A double label is given as E*AC1423*. EAC-C3* are EAC prepared in the presence of ^{125}I-labeled C3.

Erythrocyte Membrane Preparations. Erythrocyte membranes were prepared from ghosts by the method of Findlay (15). Prior to $NaDodSO_4$/polyacrylamide gel electrophoresis, the final pellet of ghosts was solubilized by heating for 3 min at 100° in a solubilizing buffer (0.05 M Tris (pH 6.8)/2% $NaDodSO_4$/10% glycerol/0.001% bromphenol blue). Protein content of the samples was determined according to Lowry *et al.* (16). Before electrophoresis mercaptoethanol was added to the samples to a concentration of 2%.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gradient slab gels were prepared according to Laemmli (17) and run in an apparatus modified from Reid and Bieleski (18). Gels were stained and destained according to Fairbanks *et al.* (19) and dried (20). The dried gels were mounted on posterboard and autoradiograms were obtained using Kodak No-screen x-ray film.

Cylindrical $NaDodSO_4$/polyacrylamide gels were prepared with the Laemmli buffer system. A 1-cm stacking gel was placed on top of a 9-cm running gel in a glass tube with 5 mm

Abbreviations: Terminology for complement components is that recommended by the World Health Organization Committee on Complement Nomenclature (1968): C1, C2, C3, C4, C5, C6, C7, C8, and C9, the nine complement components in the classical pathway. E, sheep erythrocytes; EA, erythrocytes sensitized with the appropriate antibody; EAC1, EAC14, EAC142, and EAC1423, cell–complement intermediates with the designed complement proteins attached to the cell; EAC, EA that have been lysed with guinea pig serum; $NaDodSO_4$, sodium dodecyl sulfate; $DGVB^{++}$, 2.5 mM Veronal buffer, pH 7.4/75 mM NaCl/2.5% dextrose/0.1% gelatin/0.15 mM Ca^{2+}/0.5 mM Mg^{2+}.

inner diameter. After electrophoresis the gels were stained, and destained, and sliced into 1-mm slices. Radioactivity in the slices was measured in a Packard γ-counter.

For the two-dimensional electrophoresis, solubilized membrane polypeptides were separated by electrophoresis on a $NaDodSO_4$/polyacrylamide (5%) slab gel. A strip containing the polypeptides of interest was cut out and dialyzed against the sample buffer (0.05 M Tris, pH 6.8/2% $NaDodSO_4$/2% mercaptoethanol). For electrophoresis in the second dimension, the strip was placed horizontally on a $NaDodSO_4$/polyacrylamide slab gel containing a 6–12% polyacrylamide gradient. After electrophoresis, the gel was stained and destained and an autoradiogram was prepared. In the experiments described here, the gel strip from electrophoresis in the first dimension was treated with hydroxylamine (1 M, pH 9) for 1 hr at room temperature before it was dialyzed against the sample buffer.

Hydroxylamine and Ammonia Treatments. A membrane pellet of EAC1423* was dissolved in a minimal volume of 0.2% $NaDodSO_4$/15 mM Tris (pH 7.4). Added to this was an equal volume of 2 M hydroxylamine hydrochloride titrated to pH 10.5 with 50% NaOH. The pH of the mixture was above pH 10, as indicated by pH paper. The sample was incubated at 37°, and 0.1-ml aliquots were withdrawn after 5, 10, 30, and 90 min and immediately cooled. Excess NH_2OH was removed by dialysis, and the aliquots were analyzed by electrophoresis. EAC1423* membrane polypeptides were also treated with an ammonolysis reagent (21) containing 30% ammonium hydroxide/glacial acetic acid/20% $NaDodSO_4$/glycerol/mercaptoethanol (82:7:5:5:1) and analyzed by electrophoresis.

Binding C3b to Zymosan. Zymosan particles, obtained from ICN Pharmaceutical, Inc., were prepared for use according to ref. 4. Zymosan-C3* particles were prepared by incubating zymosan at 1 mg/ml, guinea pig serum at 1:10 dilution, and ^{125}I-labeled C3 at about 10 μg/ml, in $DGVB^{++}$ at 37° for 30 min. They were then washed twice in $DGVB^{++}$. The radioactivity in the original mixture recovered on the particles was 10–15%. Particles incubated with ^{125}I-labeled C3 in the absence of serum or with heat-inactivated serum retain at most 3% of the radioactivity.

Hydroxylamine Treatment of C3. C3 was treated with hydroxylamine exactly as described (22). The reaction was carried out at room temperature for 20 min, and samples were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. C3 in Tris buffer in hydroxylamine solution at pH 9 was also prepared and analyzed.

RESULTS

C3b Bound to Sheep Erythrocyte Cell Membranes. EAC1423* cells were prepared as described in *Materials and Methods*. C3 is in excess in the preparation of these cells, and the conversion of C3 to C3b is about 50% of the C3 input. Between 10 an 15% of the converted C3 is found on the surface of EAC1423* cells. The bound C3b is equivalent to about 50,000 molecules per erythrocyte, and the binding is specific, for little conversion of C3 to C3b or uptake of C3b was detected when EAC14 cells were incubated with either ^{125}I-labeled C3 or a mixture of labeled C3 and C3b. The binding of C3b to ghost membranes appears to be very tight since no significant amounts of C3b were removed from the membranes either by washing them in high salt or by freezing and thawing them repeatedly.

C3b Bound to Cell Membrane Proteins. The $NaDodSO_4$-solubilized polypeptides of EAC1423* were analyzed by gel electrophoresis. The profile of Coomassie blue-stained polypeptides (Fig. 1*b left*) is essentialy identical to that obtained from osmolytically lysed ghosts of untreated sheep erythrocytes (Fig. 1*a left*) except for bands that represent C3bα and C3β. In the autoradiogram shown in Fig. 1 *right*, bands other than those corresponding to C3bα and C3β can be seen, having molecular weights in excess of C3bα. When EAC1423* cells are prepared, the supernatant shows only excess unbound C3α, C3bα, and C3β polypeptides. Electrophoresis of a $NaDodSO_4$-solubilized mixture of EAC14 and ^{125}I-labeled C3b also shows only C3α, C3bα, and C3β, suggesting that the occurrence on EAC1423 cells of new polypeptides of unusually high molecular weight is not induced by the detergent.

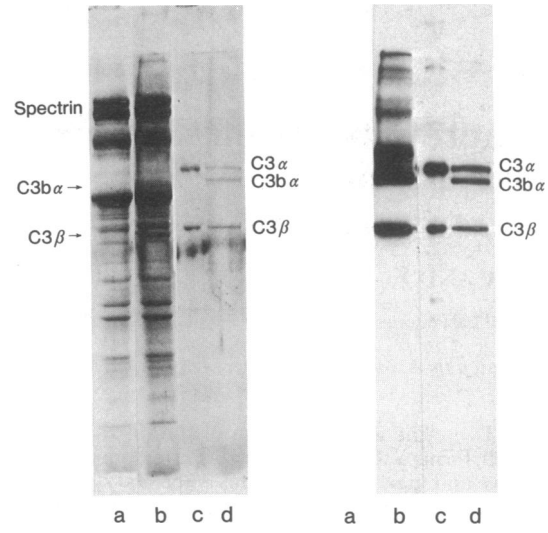

FIG. 1. (*Left*) $NaDodSO_4$-solubilized membrane samples of E (*a*) and EAC1423* (*b*), analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis on a slab of 7–15% polyacrylamide. Ghosts were solubilized by heating in a boiling water bath for 3 min in 2% $NaDodSO_4$/2% mercaptoethanol. Except when noted otherwise, this and subsequent gels were loaded with 10–20 μg of protein. A sample of ^{125}I-labeled C3 (*c*) and the supernatant of the reaction mixture from the preparation of EAC1423* cells (*d*) were run as reference bands of C3 and C3b polypeptides. (*Right*) Autoradiogram of the gel shown at *left*. The gel was exposed to Kodak No-Screen x-ray film for 2 days. The film was developed in KLX developer for 5 min and was fixed in Kodak rapid fixer for 9 min at 68°. All autoradiograms were prepared in a similar way.

An interaction between C3b and other proteins is implied by the results shown in Fig. 1. That these new polypeptides arise from an interaction between C3b and membrane proteins is demonstrated by the results of double-labeling experiments in which membrane proteins carried ^{125}I and C3 carried ^{131}I. Samples of membranes from E*AC1423, EAC1423*, and E*AC1423* cells were analyzed by electrophoresis. The pattern of labeling of polypeptides derived from E*AC1423* cells is very similar to that of EAC1423* cells (Fig. 2 *left*). The pattern of labeling seen after the decay of ^{131}I (Fig. 2 *right*) shows only the ^{125}I label from membrane proteins; this pattern is identical to that of E*AC1423.

Dissociation of C3b from Erythrocyte Membrane Proteins. The binding of C3b to sheep erythrocyte membrane proteins is not affected by either extraction of ghosts with high salt or by repeated freezing and thawing. It is also unaffected by raising the pH from 7 to 11. Furthermore, the complex is resistant to extraction with Triton X-100, for both the pellet and supernatant fractions from C3b-labeled cells retain the high molecular weight polypeptides that are characteristic of the C3b–membrane protein association. The addition of 10 M urea to the protein solubilizing buffer of 2% $NaDodSO_4$/2% mer-

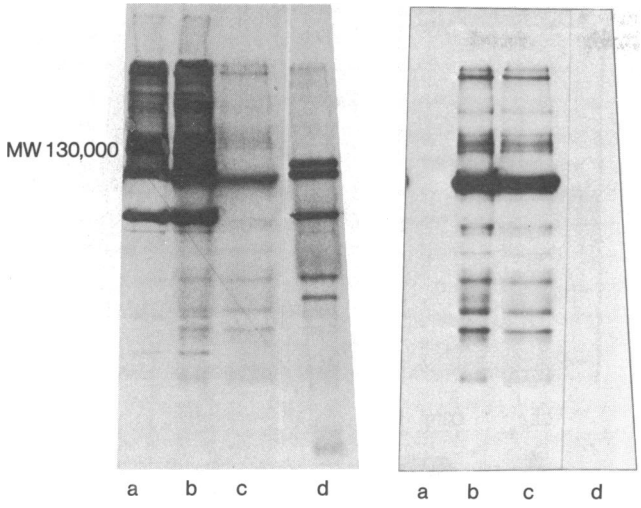

FIG. 2. The polypeptides obtained from sheep erythrocyte membranes labeled with ^{125}I and C3 labeled with ^{131}I. Membrane samples of EAC1423* (*a*), E*AC1423* (*b*), and E*AC1423 (*c*) were prepared and run on a 7–15% $NaDodSO_4$/polyacrylamide gradient slab gel. Supernatant of the reaction mixtures in the preparation of EAC1423* (*d*) was also analyzed by electrophoresis. (*Left*) Autoradiogram prepared from the gel immediately after it was stained and dried. (*Right*) Autoradiogram prepared from the same gel 3 months later when the isotope ^{131}I had decayed and radioactivity from ^{125}I could be detected.

captoethanol is also without effect on the C3b–membrane protein complex. The complex can be broken, however, when EAC1423* membrane polypeptides are treated with hydroxylamine. This is demonstrated by the results of an experiment in which solubilized EAC1423* membrane polypeptides were incubated with hydroxylamine for various lengths of time and then analyzed by gel electrophoresis. Slices were obtained from the gels, radioactivity was measured, and the percent radioactivity recovered from the region of the gel above the C3bα band was determined. The amount of radioactivity in the high molecular weight region decreased as the length of incubation in NH_2OH increased (Fig. 3). Most of the radioactivity is recovered at the C3bα band. However, it should be noted that on prolonged incubation with hydroxylamine, a peak of molecular weight about 25,000 progressively shows up in the low molecular region (Fig. 3). A similar result is obtained if the EAC1423* membrane polypeptides are treated with the ammonolysis reagent.

Dissociation of C3b from Zymosan Particles. We have also attempted to dissociate ^{125}I-labeled C3b from zymosan-C3* particles. The particles were first incubated overnight in 2 M NaCl at 37°, followed by two washes in distilled water. About 70% of the radioactivity remains bound. [Goldstein *et al.* (6) found that 70–80% of C3b bound to Sepharose was not removed by incubation in 2 M NaCl at 100° for 15 min.] The washed zymosan particles were then subjected to various treatments designed to release bound C3b. The results (Table 1) show that whereas about 25% of the bound radioactivity is released by $NaDodSO_4$, 50% of it is released after hydroxylamine treatment. If the NH_2OH is followed by either detergent ($NaDodSO_4$ or Triton X-100) or boiling, 75–80% of the bound C3b is released. Only about 5% of the counts remain bound if the particles are treated with $NaDodSO_4$, NH_2OH, and then $NaDodSO_4$ again.

Demonstration of Binding of C3b via Its α-Polypeptide. The binding of C3b via its α-polypeptide can be demonstrated by two-dimensional gel electrophoresis. An EAC1423* membrane sample, solubilized in 2% $NaDodSO_4$/2% mercaptoethanol, was run in the first dimension. An appropriate strip of the gel was cut out, treated with hydroxylamine, and run on the gel for the second dimension. In the autoradiogram shown in Fig. 4, the bulk of high-molecular-weight C3b-membrane protein complexes are no longer present and the radioactivity is recovered in the C3bα polypeptide. The result demonstrates that the bond is between C3bα and the membrane proteins.

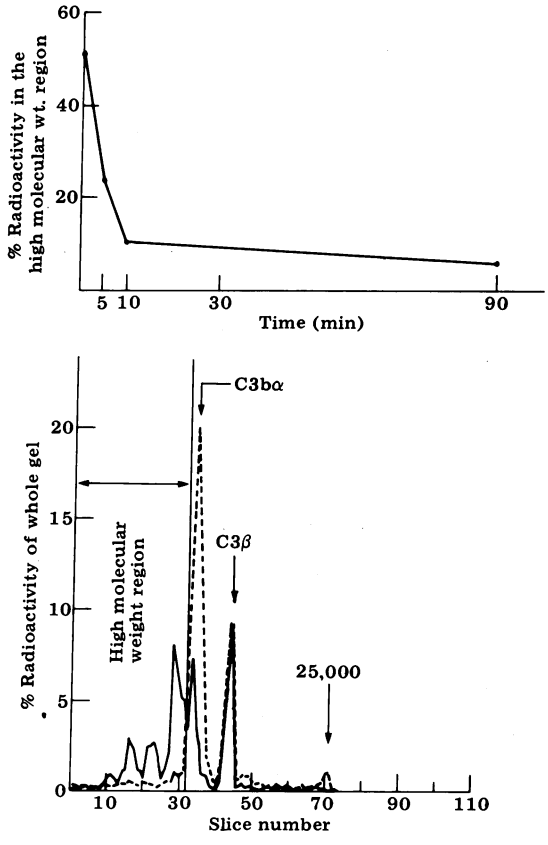

FIG. 3. Effects of hydroxylamine on the association between C3b and erythrocyte membrane proteins. (*Upper*) To one volume of EAC1423* membrane solubilized in 0.2% $NaDodSO_4$ one volume of 2 M hydroxylamine solution was added. The pH of the mixture is about 10. Aliquots of the reaction mixture were taken after 5, 10, 30, and 90 min and excess NH_2OH was removed by dialysis against 0.1% $NaDodSO_4$ in distilled water. The samples were made to 2% $NaDodSO_4$/2% mercaptoethanol and were run on cylindrical gels. The gels were sliced and radioactivity was measured. For each gel, the radioactivity in the high-molecular-weight region is summed and expressed as a percentage of the total radioactivity of the gel. This percentage is compared with that of the untreated sample. (*Lower*) Radioactive gel patterns of untreated EAC1423* membrane polypeptides (solid line) and EAC1423* membrane polypeptides treated with NH_2OH for 10 min (broken line).

Similarly, while the 70,000-dalton β-chain of C3b molecules bound to zymosan particles can be removed with $NaDodSO_4$ and mercaptoethanol, the α-chains remain bound until either the ammonolysis reagent or hydroxylamine is used (Fig. 5).

Attempt to Cleave C3 with Hydroxylamine. It has been reported (23) that hydroxylamine treatment of C3 cleaves the molecule into two fragments, one of which resembles C3a and one which corresponds to C3b. However, we were unable to reproduce this result under conditions identical to those reported in the literature (22, 23). Treatment of C3 with 1 M hydroxylamine at pH 9 also has no effect on the integrity of the molecule.

Table 1. Dissociation of ^{125}I-labeled C3b from zymosan-C3* particles

Treatment sequence	Bound radioactivity[a] Before treatment	After treatment	% remaining bound ± SD
$NaDodSO_4$	10,241(4)	7185	76.1 ± 1.4
NH_2OH	8,507(4)	4205	49.5 ± 2.3
$NaDodSO_4$, NH_2OH	10,706(2)	6016	56.2 ± 4.9
$NaDodSO_4$, NH_2OH, $NaDodSO_4$	9,777(2)	548	5.6 ± 1.0
NH_2OH, $NaDodSO_4$	8,851(2)	2067	23.4 ± 0.5
NH_2OH, Triton	8,584(2)	2162	25.1 ± 1.5
NH_2OH, boiling	10,125(2)	2073	20.4 ± 1.6

Zymosan-C3* particles were prepared as described in the *text*. They were then incubated overnight in 2 M NaCl at 37° followed by two washes in distilled water. $NaDodSO_4$ treatment, incubation in 2% $NaDodSO_4$ for 30 min at 37°; NH_2OH treatment, incubation in 1 M NH_2OH (pH 10) for 90 min at 37°; Triton treatment, incubation in 2% Triton X-100 for 30 min at 37°; boiling, incubation of a suspension of zymosan particles in water in a boiling water bath for 10 min. Each individual treatment was followed by two washes with distilled water.

[a] The radioactivity bound to zymosan particles was determined before and after each treatment sequence and is shown as total counts per 0.1 min. The number of counts represents an average of several determinations; the number of determinations is indicated in parentheses. About 2 mg of zymosan particles were used in each determination.

DISCUSSION

The results of the experiments described here show that C3b bound to erythrocyte membranes or to zymosan particles resists dissociation by action of detergents and protein denaturants as well as by extremes of temperature, salt concentration, and pH. Conceivably the association could be due to hydrophobic interactions or to ionic, hydrogen, or covalent bonds. The complex formed between C3b and erythrocytes and zymosan is not wholly hydrophobic, however, for it cannot be disrupted by detergents such as $NaDodSO_4$ and Triton X-100, and it is apparently not entirely the result of ionic or hydrogen bonds because it remains intact regardless of changes in salt concentration and pH. Since it resists treatment with 2% $NaDodSO_4$ and 10 M urea at 100°, the quaternary structure of C3b is apparently not essential for the maintenance of the complex.

Our observation that the complex can be disrupted by exposure to hydroxylamine or by ammonolysis suggests that the complex is held together by a bond that is susceptible to nucleophilic attack, conceivably either an ester or imidoester bond (21). However, we also observe that the effectiveness of hydroxylamine in releasing C3b from zymosan can be enhanced by the subsequent addition of detergents (see Table 1). It is therefore reasonable to suggest that hydrophobic interactions are in part responsible for the stability of the complex.

It has been reported that polypeptide fragments of C3 can be desorbed from EAC by incubation in buffer containing 0.15 M NaCl (24), and from lipopolysaccharide-coated oil droplets with $NaDodSO_4$ (5). We found that the radioactivity eluted from EAC1423* membranes with high salt (data not shown) or from zymosan-C3* particles with $NaDodSO_4$ can account for only a portion of the total radioactivity (see Table 1). Stossel *et al.* (5) report that a 70,000-dalton polypeptide of C3b can be eluted from lipopolysaccharide-coated oil droplets with $NaDodSO_4$ and mercaptoethanol (5). This polypeptide is most likely the β-chain of C3b. We obtained similar results when we

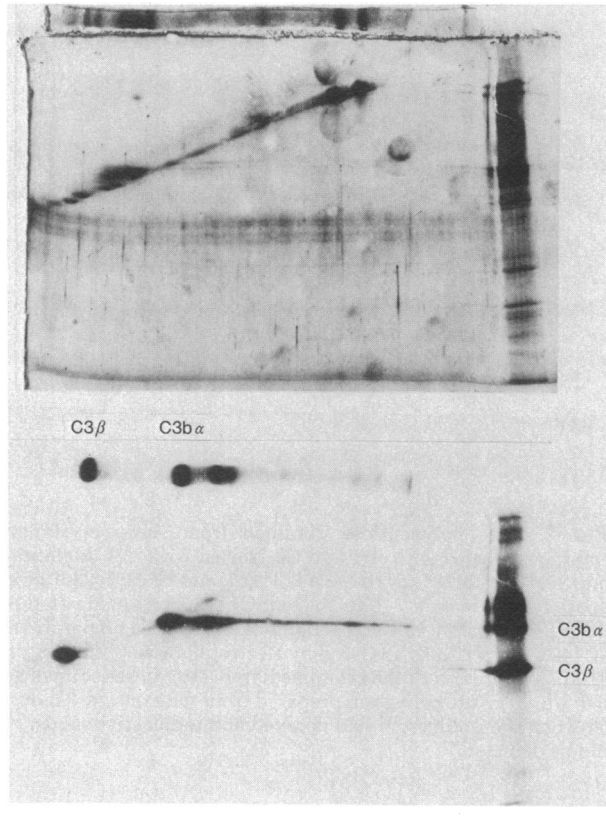

FIG. 4. (*Upper*) Two-dimensional gel electrophoresis of EAC1423* membrane polypeptides. An EAC1423* membrane sample solubilized in $NaDodSO_4$ and mercaptoethanol was run on a $NaDodSO_4$/5% polyacrylamide gel. A strip was cut out and treated with hydroxylamine, 1 M, pH 9, for 1 hr at room temperature. It was loaded onto a 6–12% polyacrylamide gel containing $NaDodSO_4$ for the second dimension electrophoresis. A solubilized EAC1423* membrane sample was run simultaneously with the hydroxylamine-treated strip in the second dimension electrophoresis. An identical, but untreated, strip from the first dimension electrophoresis is shown horizontally on the top of the gel. (*Lower*) Autoradiogram of the gel (*Upper*).

washed zymosan-C3* particles with the same reagents (Fig. 5).

The apparent formation of a covalently linked complex between C3b and receptive surfaces as described here implies that specific receptors for C3b do not exist. Rather, a reactive group or groups in the α-chain of the C3b molecule, hidden until C3 is activated, appear capable of binding to chemical groups that are of common occurrence on cell surfaces and on inert materials such as zymosan. For example, activation of C3 might reveal either —COOH or —$CONH_2$ groups that could conceivably form a bond with a hydroxyl group on a receptive surface. Binding could be to a free hydroxyl group on an amino acid residue of a protein or on a carbohydrate of a glycoprotein, glycolipid, or cell wall polysaccharide. We emphasize, however, that our evidence for such a bond derives only from our experiments which show that nucleophilic agents disrupt the association between C3b and its receptive surface. The binding of C3b to Sepharose (6) and to small polymers of glucose containing *N*-acetyl-glucosamine (25) are of interest in this regard. In view of the common nature of the chemical group or groups on receptive surfaces, it is also important to note that the activation of C3b is a transient phenomenon, for the binding capacity of the molecule is lost if it fails to collide with a suitable receptive surface within a short time (26, 27).

It is possible that specific interactions between C3b and its receptive surfaces occur in more than one way. For example,

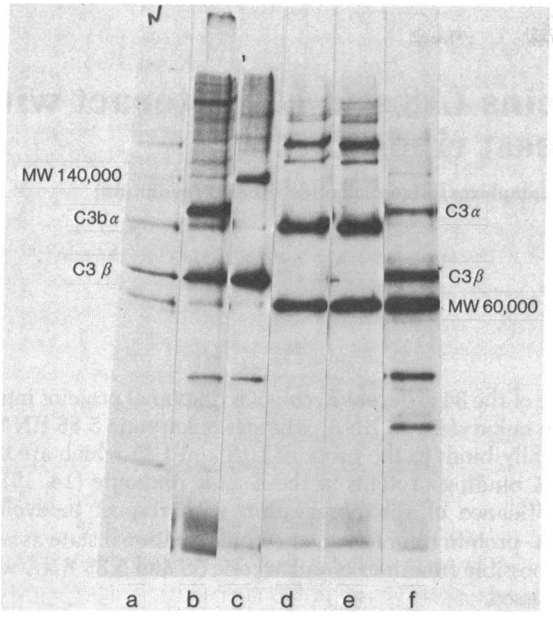

FIG. 5. Autoradiogram of $NaDodSO_4$/polyacrylamide gel electrophoresis of fractions obtained from the elution of C3b from zymosan. C3b-coated zymosan particles were eluted with the following reagents in the order listed: (*i*) $DGVB^{++}$, (*ii*) 2% $NaDodSO_4$, (*iii*) 2% $NaDodSO_4$/2% mercaptoethanol, (*iv*) ammonolysis reagent or 1 M hydroxylamine at pH 9, and (*v*) 2% $NaDodSO_4$/2% mercaptoethanol. Shown in the figure are eluates from (*a*) $DGVB^{++}$, (*b*) 2% $NaDodSO_4$, (*c*) 2% $NaDodSO_4$/2% mercaptoethanol, (*d*) 2% $NaDodSO_4$/2% mercaptoethanol after ammonolysis reagent treatment, and (*e*) 2% $NaDodSO_4$/2% mercaptoethanol after hydroxylamine treatment. All samples were adjusted to a final concentration of 2% $NaDodSO_4$/2% mercaptoethanol, and electrophoresis was carried out on a 6–12% polyacrylamide gradient $NaDodSO_4$ gel. The supernatant of the initial reaction mixture of zymosan, ^{125}I-labeled C3, and serum is also included (*f*) to show the positions of C3α, C3bα, C3β, and the breakdown product of C3bα at molecular weight 60,000.

there may be a population of C3b molecules that bind to the receptive surface in a noncovalent manner. This can clearly explain the two observations that (*i*) in the gels on which EAC1423* membrane polypeptides are separated, there is a band corresponding to C3bα not linked to any membrane polypeptide (see Fig. 1), and (*ii*) there is a significant amount of radioactivity on zymosan-C3* particles that is removable by $NaDodSO_4$. However, we find it is more reasonable to hypothesize that the C3bα band in EAC1423* membranes represents those C3bα polypeptides covalently linked to membrane lipids and/or glycolipids, and that the eluate from zymosan-C3* particles with $NaDodSO_4$ contains C3 rather than C3b molecules (see Fig. 5*b*), indicating that the $NaDodSO_4$-releasable portion radioactivity is from nonspecifically bound C3.

Clearly our understanding of how C3b binds will be incomplete until we know the chemical nature of the reactive group or groups on C3b as well as the nature of the bonds and the mechanism by which they are formed.

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