C3d Fragment of Complement Interacts with Laminin and Binds to Basement Membranes of Glomerulus and Trophoblast

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Abstract. Two mouse monoclonal antibodies generated against human placental homogenate were found to react specifically with human complement component C3. In immunofluorescence of human tissues, these antibodies gave a bright linear staining outlining the glomerular basement membrane of the adult kidney and the trophoblast basement membrane of placenta. An identical staining pattern was observed with a rabbit C3d antiserum which also prevented binding of the monoclonal antibodies to tissue sections. Only negligible basement membrane staining was observed in the same tissues with antisera to human C3c, C5, IgG, IgA, or IgM. When interactions of C3 with basement membrane proteins were tested in enzyme immunoassays and column chromatography, C3(H2O) was found to bind efficiently to solid-phase laminin. Native C3 from fresh plasma did not bind to laminin but C3 from plasma treated with methylamine bound efficiently. When C3 was cleaved with trypsin, C3b and C3d but not C3c bound to laminin-Sepharose. The interaction of C3 and laminin was inhibited by soluble laminin and by high ionic strength. The results indicate that C3d, a biologically active breakdown product of C3, can be found in glomerular and placental basement membranes in the absence of signs for ongoing local complement activation or immune complex deposition. It is possible that binding affinities between C3 and basement membrane molecules, especially laminin, are involved in the retention of C3d at these sites. Such interactions between C3 and components of the glomerular basement membrane could play important roles in complement-related pathological processes of the glomerulus.

Complement plays a major role in the defense against infections by microorganisms (2, 30, 33). The third component of complement, C3, participates in both the antibody-dependent classical (32) and the antibody-independent alternative pathways of complement activation (27).

Proteolytic cleavage of C3 (185 kD) gives rise to fragments that display important biological activities not associated with intact C3. Initial cleavage releases C3a (9 kD)—a vasoactive peptide and a mediator of inflammation (7, 19). The liberated C3b (176 kD), when bound to cell surfaces or foreign particles (22, 37), may initiate formation of the membrane attack complex (27). Eventually, the bound C3b is cleaved and C3c (140 kD) is lost into the fluid phase, while C3dg (39 kD) remains bound (8, 20). Tryptic cleavage of C3 yields a slightly smaller fragment, the C3d (35 kD).

A low level of C3dg is present in normal human plasma, and higher levels are seen in disorders associated with increased complement consumption (28). Recently, it has been reported that C3d has immunomodulatory functions in addition to its role in complement activation. C3d-K, a kallikrein cleavage fragment of C3, inhibits proliferation of lymphocytes and other cells (38), induces leukocytosis (17), and enhances enzyme secretion by macrophages, whereas C3d may also act as a lymphocyte growth factor (24). In the present study, we show that C3d is present in many trophoblast basement membranes and often in the glomerular basement membrane. When the interaction of C3 with basement membrane proteins was tested in vitro, C3 showed particular affinity to laminin. From trypsin digests of C3, only the C3b and C3d fragments bound to laminin.

Materials and Methods

Immunoprecipitation of Cell Culture Media

Human tumor cell lines and normal fibroblastic cells obtained from American Type Culture Collection (Rockville, MD) and from other sources were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum. For immunoprecipitation, subconfluent cultures were incubated for 24 h in methionine-free minimum essential medium supplemented with glutamine, ITS (insulin/transferrin/selenium; Collaborative Research, Inc., Waltham, MA), and 100 μCi/ml of [35S]methionine (1,100 Ci/mmol; New England Nuclear, Boston, MA). Culture medium was collected, centrifuged, and made 0.5 M with NaCl, 0.1 mM with phenylmethylsulfone fluoride, 0.1% with Tween 20, and 0.1 M with Tris-HCl, pH 7.0. Protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), which had been preincubated with an excess of rabbit anti-mouse IgG antiserum, was saturated with hybridoma ascitic fluid and washed. 1 ml of culture medium was then incubated with the Sepharose beads for 3 h at room temperature. The Sepharose beads were washed five times with 0.5 M NaCl and 0.1% Tween in 0.05 M Tris-HCl, pH 7.2. The samples were then boiled in SDS PAGE sample buffer and 2-mercaptoethanol for 5 min and analyzed by SDS PAGE.
**Immunohistochemistry**

Human placentas were obtained from cesarean sections or normal-term deliveries. Other human tissues were from autopsy cases with no history of a pathologic process in the organ studied. Newborn monkey tissues were obtained from the Primate Research Center (Alamogordo, NM). After being transported under cooled conditions, the tissues were snap-frozen in liquid nitrogen and 6-μm frozen sections made, air dried, and fixed in cold acetone. Mouse tissues were obtained from adult and newborn BALB/c and C57Bl mice (Bantam and Kingman Inc., Fremont, CA). Adult mice received a tail vein injection of 17 μg phenylmethylsulfonyl fluoride and 1.7 mg EDTA in 0.5 ml of phosphate-buffered saline (Pi/NaCl) (for 0.2 mM phenylmethylsulfonyl fluoride and 10 mM EDTA in an estimated circulating volume of 5 ml) 10 min before sacrifice. Control mice received no injection. The mice were then anesthetized with methoxyflurane inhalation and the kidneys were snap-frozen in situ by using liquid nitrogen. Similarly, close-to-term placentas were obtained from BALB/c mice. Also, newborn BALB/c mouse kidneys were snap-frozen. Frozen mouse tissues were processed as human and monkey tissues.

For immunofluorescence, sections were treated for 2 h with hybridoma culture medium diluted 1:2 or ascitic fluid diluted 1:100-500, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) at 1:40 dilution. Staining of sections with rabbit antisera was performed as described (25). The specimens were observed under a Zeiss microscope equipped for phase contrast and epillumination. For double staining, sections were treated as above with monoclonal antibodies followed by FITC-conjugated goat anti-mouse IgG, and then with rabbit anti-human C3d antiserum at 1:100 dilution followed by tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Cappel Laboratories) at 1:40 dilution. Inhibition of the binding of monoclonal antibodies to tissue sections was carried out by pretreating the sections with rabbit anti-human C3d antiserum at 1:30 dilution overnight and subsequently staining with the monoclonal antibodies as above. For elution experiments, unfixed frozen sections were treated with 1 M NaCl, 1 M acetic acid, 6 M guanidine, or 8 M urea for 30-80 min, washed, air dried, fixed with acetone, and stained for C3c and laminin.

For the studies on the binding of human C3 to cell cultures, 1 mg/ml of C3 (Behring Diagnostics, San Diego, CA) was incubated for 1 h at 37°C with unfixed cultures and tissue sections or cultures fixed with 3.5% acetic acid, paraformaldehyde, and subsequently the cultures and sections were stained with C3d antiserum.

**Monoclonal Antibodies and Antisera**

Monoclonal antibodies to basement membrane-associated antigens were produced by hybrids of mouse myeloma cells and spleen cells of mice immunized with a homogenate of human placental membranes (16). Two IgG1 (κ) antibodies, 3F3 and 4G3, which displayed identical staining patterns of all tissues tested, were used in this study. Rabbit anti-C3c, anti-C3d, and anti-C3 antisera were from Behring Diagnostics, and rabbit anti-C3c and anti-C3d antisera were also purchased from Dako Corp. (Santa Barbara, CA). The goat anti-C3 antibody was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Rabbit anti-human IgM was from Antibodies Inc. (Davis, CA), and rabbit anti-human IgG and rabbit anti-human IgA were from Behring Diagnostics.

**Enzyme Immunoassays**

The assays were carried out essentially as described (8). Plastic microtiter wells (Flow Laboratories, McLean, VA) were coated with various concentrations of laminin (Bethesda Research Laboratories, Gaithersburg, MD), type I collagen (Bethesda Research Laboratories), or fibronectin (BD) at room temperature overnight. The washings terminating the coating and antibody incubations were carried out in Pi/NaCl containing 0.05% Tween 20. Human C3 (Behring Diagnostics) was added at different concentrations in Pi/NaCl and 0.05% Tween 20. For other binding assays, fresh human plasma was prepared in the presence of 10 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and an aliquot was reacted with 0.2 M methionine (Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C, pH 8, as described (29). Both untreated and treated aliquots were added to microtiter wells at different dilutions in Pi/NaCl and 0.05% Tween 20. Antiserum to human C3, C3c, and C3d were diluted in Pi/NaCl and 0.05% Tween 20 to 1:1,000-10,000 and incubated in wells. Binding was detected using anti-IgG conjugated with alkaline phosphatase (Sigma Chemical Co.). All incubations were done at 37°C for 2 h. Alkaline phosphatase reaction product was measured with a multi-channel photometer (Titertek Multiscan, Flow Laboratories, McLean, VA). All assays were done in duplicates.

**Isolation and Characterization of C3 and Its Fragments**

The IgG fraction of 3F3 ascitic fluid was precipitated with 18% Na2SO4, collected by centrifugation, redissolved in 0.1 M NaHCO3, and coupled to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals). Human plasma was pre-run through plain Sepharose and gelatin-Sepharose columns and then applied to a 3F3 antibody column. After washing with Pi/NaCl, the adsorbed material was eluted with 1 M acetic acid. C3 thus isolated was used in experiments confirming the identity of the antigen and for some binding experiments.

Human C3 and C5 were purchased from Behring Diagnostics. This preparation of C3 and its fragments (see below) were used for the documented binding experiments of this study. After storage, freezing, and thawing, aliquots of the commercial C3 were tested for incorporation of [14C]methylyamine (New England Nuclear) as described by Howard (18) and were found to incorporate only minimal amounts of the label (1-3 mol %). A similar low level of [14C]methylyamine incorporation was exhibited by human C3 isolated by 3F3 antibody affinity chromatography. Thus, C3 from both these sources was considered to be C3(C3H2O) (35). A high level of [14C]methylyamine incorporation into C3 (>70 mol %) was obtained when fresh plasma was reacted with [14C]methylyamine in 1,000-fold molar excess of plasma C3 and the C3(C3H2O) was isolated by affinity chromatography on laminin-Sepharose.

Human C3 (Behring Diagnostics) was converted to C3b by light trypsinization (5 min at 37°C) as described (29). The result of the digestion was verified by SDS PAGE of samples under reducing conditions. To obtain fragments bearing C3c and C3d determinants, respectively, the C3 was subjected to more extensive trypsin digestion as described (4, 26). The course of the digestion was followed by SDS PAGE of samples at different time points. The material chosen for affinity chromatography from the prolonged trypsin digest did not contain any intact C3 or C3b detectable by protein staining or autoradiography of 35S-labeled material (4). Purified rat laminin-Sepharose and affinity chromatography on rabbit anti-C3 antisera with [14C]methylamine incorporation into C3 (>70 mol %) was obtained when fresh plasma was reacted with [14C]methylyamine in 1,000-fold molar excess of plasma C3 and the C3(C3H2O) was isolated by affinity chromatography on laminin-Sepharose.

**Figure 1.** SDS PAGE of protein recognized by the 3F3 and 4G3 antibodies. Polypeptides were detected by autoradiography (lanes 1-5) and by Coomassie Blue staining (lanes 6-9). Polypeptides immune precipitated by the 3F3 antibody from culture media of [35S]methionine-labeled subconfluent cultures of A-431 cells: (lane 1) untreated and (lane 2) after reduction. Polypeptides immune precipitated from the extracted cell layer of [35S]methionine-labeled SKLMS-1 cells: (lane 3) untreated, and (lane 4) after reduction. (Lane 5) Polypeptides precipitated from the SKLMS-1 cell layer with an unrelated monoclonal antibody. Polypeptides isolated from human plasma by 3F3 antibody affinity chromatography: (lane 6) untreated and (lane 7) after reduction. Purified human C3 (Behring Diagnostics): (lane 8) untreated, and (lane 9) after reduction.
Figure 2. Immunological identification of C3 as the antigen recognized by 3F3 antibodies. Polypeptides bound from human plasma by 3F3 antibodies were analyzed upon SDS PAGE, transferred to nitrocellulose filter, and reacted with rabbit anti-C3c; (lane 1) unreduced and (lane 2) after reduction. (Lane 3) The same material as in lane 1 immunoblotted with rabbit anti-C5, and (lane 4) purified human C5 immunoblotted with C5 antiserum. Molecular mass markers used include myosin (200 kD), β-galactosidase (116 kD), phosphorylase B (92 kD), BSA (66 kD), and ovalbumin (45 kD).

Figure 3. (a and c) Phase-contrast photomicrograph of frozen sections of normal human autopsy kidney. (b and d) Staining of the sections with 3F3 antibodies shows a predominantly linear pattern of fluorescence outlining contours of the glomerular basement membrane. Mesangial areas are not stained. Intimal aspects of arteriolar walls show bright fluorescence (arrows). Bars, (a and b) 20 μm; (c and d) 50 μm.

Results

Identification of the Antigen

To identify the antigen recognized by the 3F3 and 4G3 antibodies, several human tumor and normal cell lines were metabolically labeled and the culture media immunoprecipitated for study of secreted radiolabeled proteins. The same major polypeptide chains were precipitated with the 3F3 and 4G3 antibodies from most of the cell lines studied. The unreduced protein migrated as a single band at 185 kD and was, upon reduction, resolved into two major bands at 120 kD and 75 kD (Fig. 1). The protein isolated by detergent treatment of the cell layer displayed one major band at 185 kD which was unaltered after reduction (Fig. 1). Results with the different cell lines are summarized in Table I.

The finding of a hepatoma line among the major producers of the antigen prompted a closer look at known plasma proteins. A major human plasma protein which bound specifically to a 3F3 antibody column, displayed a migration pattern in SDS gels identical to that of the cell culture-derived polypeptides, as well as with human complement component C3 (Fig. 1). After transfer to nitrocellulose filters, the protein isolated from human plasma by antibody affinity chromatography reacted in immunoblotting with rabbit anti-C3c but not with rabbit anti-C5 (Fig. 2). The above findings established the identity of the antigen as C3.
**Immunohistochemical Studies**

Reaction of the 3F3 and 4G3 antibodies with tissue sections was tested by indirect immunofluorescence using adult human and neonatal monkey tissues. Three adult human kidneys from fresh autopsy cases with no record or findings of kidney disease were examined. In two cases, a largely linear pattern of bright fluorescence outlining the glomerular basement membrane was observed (Fig. 3 b). Occasional spotty outlines but no general staining of tubular basement membranes were seen. In the third case, only weak glomerular fluorescence was present. In all three autopsy cases, walls of medium-sized arterioli in the kidney were brightly stained (Fig. 3 d) while diffuse staining of some vessel walls was observed in the liver and in other organs (not shown).

Both human and monkey placentas showed bright fluorescence with both antibodies in the trophoblast basement membranes of the chorionic villi (Fig. 4). Typically, the staining displayed a linear but frequently interrupted pattern giving it a stitched appearance (Fig. 4). The proportion of villar basement membranes stained varied greatly from one placenta to another, ranging from almost 100% to <20%, but none of the eight placentas examined was negative. The other placental basement membranes (namely, the villar capillary basement membrane and the amniotic basement membrane) were negative (Fig. 4). No staining of the villar interstitium or trophoblast cells was observed. Granular or amorphous fluorescence was, however, seen in decidua at the fetomaternal interface in term human placentas (Fig. 4 d). Decidual staining was also present in a 10-wk pregnant uterine mucosa. A basement membrane-type pattern of staining, however, was only found in the glomerulus and the placenta.

After identification of the antigen recognized by the 3F3 and 4G3 antibodies as complement component C3, the tissue localization of C3, C3c, C3d, C5, IgG, and IgM was studied with polyclonal antisera. The C3 antiserum gave a weak linear staining of the glomerular basement membrane in the cases which were strongly positive with 3F3 and 4G3 antibodies (Fig. 5 b) and a weak stitched staining of trophoblast basement membranes. The two C3c antisera used in this study, however, gave no or only faint staining of either base-
ment membrane in any of the cases studied (Fig. 5 d). Stain-
ings with the C5, IgG, IgA, and IgM antisera varied from negative to faintly positive in both the glomerular and trophoblast basement membranes (not shown). In contrast to these, the two C3d antisera gave bright fluorescence in the kidney and the placenta in a pattern similar to that of the 3F3 or 4G3 antibodies (Fig. 5 f).

To study the degree of similarity in the staining patterns, sections of both placenta and kidney were first stained with 3F3 antibodies and FITC-conjugated anti-mouse IgG and then stained with C3d antiserum followed by TRITC-conjugated anti-rabbit IgG. In such double stainings, the basement membranes in question showed an identical localization of both TRITC and FITC fluorescence. Moreover, if a tissue section was pretreated with C3d antiserum in low dilution (1:10) overnight, and subsequently stained with the 3F3 antibodies followed by a mouse IgG-specific FITC conjugate, no FITC fluorescence could be seen. These experiments indicated that the epitope recognized by the 3F3 antibody lies in immediate proximity to those recognized by the C3d antiserum.

A study of mouse kidneys was undertaken to eliminate the possibility that basement membrane-associated C3d was the result of (artifactual) complement activation and C3d deposition due to blood clotting in human and monkey tissue samples. In all four adult mouse kidneys snap-frozen in situ, the glomeruli displayed C3d fluorescence with a prominent linear component (Fig. 5, g and h). In addition, prominent staining of tubular basement membranes was seen in this species in arch-like or stitched patterns (Fig. 5 g). In contrast, in both newborn monkey and newborn mouse kidney no or only faint glomerular C3d fluorescence was observed while the tubular basement membranes in the newborn mouse showed a pattern of C3d fluorescence similar to that in the adult mouse kidney (not shown). No or only minimal fluorescence for C3c and C5 was found in mouse kidneys.

In an attempt to examine the nature of the binding of C3d in tissues, sections were incubated with 1 M NaCl, 1 M acetic acid, 4 M KSCN, 6 M guanidine hydrochloride, or 8 M urea to remove C3d. No apparent effect on the intensity of C3d fluorescence was seen with 1 M NaCl whereas treatment with 1 M acetic acid removed all C3d immunoreactivity from mouse tubular basement membranes and weakened the C3d fluorescence in mouse glomeruli and human placenta. Laminin immunoreactivity was unaltered by these treatments. No effect of acid on C3d fluorescence in the human kidney was observed. Harsher treatments resulted in loss of tissue integrity.

Binding Studies

Because of the basement membrane-associated tissue localization of C3d, the interaction of soluble C3 with solid-phase basement membrane molecules was studied in enzyme immunoassays and in affinity chromatography on laminin-Sepharose. Intact C3 with the properties of C3(H2O) and its tryptic fragments were used in these studies.

When C3 was added into wells coated with laminin, type IV collagen, or fibronectin, binding to all three proteins occurred to some extent, but binding to laminin was clearly more efficient than binding to type IV collagen or fibronectin (Fig. 6). Binding of C3 was first detectable at a laminin coating concentration of 30 ng/ml, while binding of C3 to type IV collagen or fibronectin only became detectable at 10-fold higher coating concentrations. Binding of C3 to bovine serum albumin (BSA), fetuin, and transferrin was negligible.

To study the specificity of the interaction of soluble C3 and solid-phase laminin, microtiter wells were coated with low amounts (0.2 μg/ml) of laminin, and C3 was added together with soluble laminin at different concentrations. With in-
creasing concentrations of soluble laminin, the binding of C3 to the wells decreased progressively (Fig. 7). On the other hand, soluble BSA, IgM, or transferrin did not decrease the binding of C3 to solid-phase laminin. Increasing concentrations of soluble fibronectin, however, produced some inhibition of the binding of C3 to laminin (Fig. 7) but fibronectin was a less efficient inhibitor than laminin.

The effect of ionic strength on the interaction of C3 and laminin was also tested. Increasing concentrations of NaCl inhibited a progressively larger proportion of the binding of C3 to solid-phase laminin until a maximum of over 90% inhibition was achieved at ~1.0 M NaCl (Fig. 8).

The role of the integrity of C3 in binding to laminin was studied by comparing the binding of C3 from fresh human citrate plasma and from methylamine-treated plasma. No binding of C3 to laminin took place from fresh plasma whereas efficient binding was observed from methylamine-treated plasma (Fig. 9). No binding of C5 or C4 was observed in either case (Fig. 9). Also, isolated [3H]methylamine-labeled C3 bound efficiently to laminin in enzyme immunoassays (not shown).

The affinity of soluble C3 for solid-phase laminin was also tested by passing radioiodinated C3 over laminin coupled to Sepharose. Bound material was eluted with 1 M NaCl and subjected to SDS PAGE. The eluate contained a large proportion of the applied radioactivity, and under nonreducing conditions, the eluted material migrated as a single band at 185 kD (Fig. 10, lane 1) and at 120 kD and 75 kD after reduction. A similar result was obtained when labeled C3b was fractionated on laminin-Sepharose. Only a minimal amount of radioactivity from either preparation bound to a similar bed volume of BSA-Sepharose.

To study which domain(s) of C3 are responsible for the interaction with laminin, a prolonged trypsin digest of radioiodinated C3 containing no intact C3 or C3b was fractionated on laminin-Sepharose, and the bound material was eluted with 1 M NaCl and analyzed by SDS PAGE. The nonbound material contained radioactivity at the position of C3c (140 kD) and at lower molecular weights (Fig. 10, lane 3). Relative to the starting material, radioactivity at the position of C3d (35 kD) was decreased or absent in the nonbound material (Fig. 10, compare lanes 2 and 3). The eluate contained one radioactive band which migrated at 35 kD (Fig. 10, lane 4). In immunoprecipitations of the eluate, a single band at 35 kD was precipitated with C3d antiserum, while only negligible radioactivity was precipitated with C3e antiserum (Fig. 10, lanes 4–6). Thus, in C3 digests, the C3d domain appears to be the main component binding to laminin. In both affinity chromatography and enzyme immunoassay experiments, however, the C3d fragment bound less efficiently to laminin than intact C3 or C3b.

The interaction of C3 and laminin was also studied using cell cultures. Cultures of human PA-1 teratocarcinoma cells, which deposit abundant laminin matrices (our unpublished observations) but secrete no detectable C3 (Table I), were stained in indirect immunofluorescence with C3d antiserum to detect if C3 from calf serum had bound to the matrices. In other experiments, C3(H2O) was added to PA-1 cultures or human tissue sections which were then stained with 3F3 and 4G3 antibodies or C3d antiserum. In these experiments, no newly acquired C3d fluorescence was seen in the extracellular matrices. Also, human cell lines which secrete C3 (Table I) and deposit some extracellular laminin (JAR, BeWo, RD) (1, 31) were stained with 3F3 and 4G3 antibodies, but no staining of extracellular matrices was observed. Obviously, interaction of C3 with assembled basement membrane matrices does not take place under these conditions.

![Figure 8](https://example.com/figure8.png)

**Figure 8.** Inhibition of binding of C3 to solid-phase laminin by increased ionic strength. Different concentrations of NaCl were added to C3 (10 μg/ml) and incubated in wells coated with mouse laminin (○) or BSA (○) (3 μg/ml). Bound C3 was assayed using rabbit C3d antiserum.

![Figure 9](https://example.com/figure9.png)

**Figure 9.** Comparison of the binding of C3 to solid-phase laminin from fresh human plasma (△) and from methylamine-treated fresh human plasma (●). Different dilutions of fresh citrate plasma (with phenylmethylsulfonyl fluoride and EDTA) were added to wells coated with mouse laminin (3 μg/ml) or to wells coated with BSA (△) (3 μg/ml). Bound C3 was assayed using rabbit C3d antiserum.
The present light microscopic results show C3d, a breakdown product of complement, outlining basement membranes of the apparently normal autopsy glomerulus and the placenta. This information agrees with earlier clinical and autopsy materials which indicate that a minor degree of C3 immunoreactivity can be demonstrated in glomeruli of up to 1/3 of clinically normal donor and autopsy kidneys (21, 39).

When evaluating the biological significance of these results, it may be useful to remember that human tissue material, when used to study the interaction of circulating proteins and filter basement membranes, may be subject to artefactual entrapment of proteins due to alteration of hemodynamic conditions before fixation (13). Nevertheless, even under such circumstances, a specific deposition of C3d is interesting and may disclose molecular interactions operative in pathological conditions.

The scarce immunoreactivity for C3c and the strong reactivity for C3d in the present kidney and placenta material suggest that only the C3d portions of the molecules are retained. The weak staining obtained with the C3 antiserum agrees with this interpretation, since C3c determinants are likely to form the major part of the specificities recognized by a C3 antiserum. Furthermore, the absence or negligible reactions for IgG, IgA, IgM, and C5 at the basement membranes of the glomerulus and the placenta suggest that the presence of C3d is not due to ongoing local complement activation or immune complex deposition. It is possible that the basement membrane-associated C3d deposits reflect earlier in vivo complement activation or proteolytic C3 cleavage with deposition of C3b and subsequent removal of C3c. The basement membrane specificity of C3d deposits, however, suggests that the binding is not solely due to the random reactivity of the thioester linkage of C3d but may result from a specific binding affinity. In fact, the present elution studies suggest that some of the deposited C3d may be noncovalently bound. The precise binding mechanisms of basement membrane-associated C3d remain to be determined but present in vitro results suggest that C3(H2O) as well as C3b and C3d could also be deposited due to interactions with tissue components.

Blood coagulation in postpartal and postmortem human tissues, due to associated cleavage of complement factors (36), might provide an artificial source of reactive C3b. The present experiments with in situ frozen mouse tissues indicate, however, that the phenomenon of basement membrane-associated C3d deposition is not an artifact of blood clotting in tissue samples.

In early immunohistological studies, C3 (determinants) were demonstrated in the trophoblast basement membrane at various stages of normal human pregnancy (25, 41). As the placental C3d of our study shows a similar interrupted pattern of deposition, it seems likely that the present and the previous observations deal with the same phenomenon. Our results, however, indicate that only the C3d part of the molecule is present. The origin and significance of C3d at this location are not clear at present. Conceivably, the deposited C3d could be derived from maternal circulation, or be secreted locally by macrophage-like Hofbauer cells, fibroblasts, or trophoblastic cells, as such cells in culture secrete C3 (35, 40, our present observations). The fetal circulation is a less likely alternative since C3d is not found in the fetal capillary basement membrane which constitutes the first filtration barrier for fetal plasma proteins.

**Discussion**

**Table I. C3 Immunoprecipitated from Culture Media of Human Cell Lines**

| Cell line       | Origin                  | Secretion of C3 |
|-----------------|-------------------------|-----------------|
| IMR-32          | Adrenal neuroblastoma   | −               |
| FL              | Amniotic epithelium     | +               |
| MCF-7           | Breast adenocarcinoma   | ++              |
| MB9812          | Bronchogenic carcinoma  | −               |
| A-431           | Cervical carcinoma      | ++              |
| Caski           | Cervical carcinoma      | ++              |
| SKGIIIa         | Cervical carcinoma      | ++              |
| BeWo            | Choriocarcinoma         | ++              |
| JAR             | Choriocarcinoma         | ++              |
| JEG-3           | Choriocarcinoma         | +               |
| HUVEC           | Cord endothelium        | −               |
| RD              | Embryonal rhabdomyosarcoma | +         |
| IMR-90          | Embryonic fibroblast    | +               |
| MRC-5           | Embryonic fibroblast    | +               |
| WI-38           | Embryonic fibroblast    | +               |
| CRL-1573        | Embryonic kidney        | −               |
| 5838            | Ewing sarcoma           | −               |
| HT-1080         | Femoral fibrosarcoma    | +               |
| MB 8387         | Fibrosarcoma or osteosarcoma | + |
| U-373MG         | Glioblastoma            | +               |
| HepG-2          | Hepatocellular carcinoma| ++              |
| M-21            | Melanoma                | +               |
| PA-1            | Ovarian teratocarcinoma| −               |
| A204            | Rhabdomyosarcoma        | −               |
| SKLMS-1         | Vulvar leiomyosarcoma   | ++              |

* The relative amount of C3-bound radioactivity precipitated is scored as + or ++. Absence of such radioactivity under conditions used is scored −.
C3d, due to its small size and almost neutral pI (7.5), is among those plasma proteins which could easily enter and traverse the glomerular basement membrane under normal hemodynamic conditions. Some of these small molecular weight proteins, such as lysozyme and β1-glycoprotein III, can also bind to normal glomeruli (5, 6). In the case of highly basic proteins, such as lysozyme (pI 11), binding is thought to be due to attraction of the protein to the anionic charges of basement membrane heparan sulfate proteoglycan (13). More specific mechanisms of binding, however, must be involved when neutral (e.g., C3d) or acidic (e.g., β2-glycoprotein III) small molecular weight proteins bind to the glomerulus. A comparison of the binding behavior of C3d (M, 35,000; pI 7.5; plasma concentration 8 μg/ml) made with three other plasma proteins of comparable size, isoelectric point, and plasma concentration showed that none of these three, β2-glycoprotein 1 (M, 48,000; pI 6.25; plasma concentration 200 μg/ml), C3 activator (β2II) (M, 60,000; pI 6.0; plasma concentration 120–300 μg/ml), or β2-microglobulin (M, 12,000; pI 5.8; plasma concentration 1.5 μg/ml) was found by immunofluorescence in the glomeruli of normal postmortem kidneys (6; our observations). Thus, a study of the binding specificities of C3d appeared warranted.

As C3d antibodies specifically outlined basement membrane contours in tissues, enzyme immunoassays were undertaken to study the interaction of C3 and its fragments with various basement membrane proteins. C3 bound particularly efficiently to laminin, while weaker interactions were observed with type IV collagen and fibronectin. The binding of C3 and the fragments C3c and C3d to fibronectin has been reported previously (5). Binding of C3 to solid-phase laminin could be inhibited by excess soluble laminin, indicating that the interaction also takes place in fluid phase and that it is not dependent on conformational changes of laminin induced by adsorption onto plastic. Partial cross-inhibition by fibronectin suggests that the sites in C3d interacting with laminin and fibronectin may be related.

Information about the domain of C3 responsible for the interaction with laminin was obtained by studying the binding of iodinated C3 fragments to laminin-Sepharose. Prolonged trypsin digestion of C3 yields a large (120 kD) and a small (35 kD) fragment containing C3c and C3d determinants, respectively. Such fragments, used in the present study, display an approximate correspondence to the physiological counterparts released during C3 activation. Our affinity chromatography results indicated that, of the different fragments present in digestion mixtures, only C3b and C3d bound to laminin–Sepharose, while C3c did not. This localizes the binding site to the C3d fragment which is contained in C3b.

Interestingly, no binding of native C3 to laminin was observed from fresh human plasma whereas strong binding took place from methylamine-treated plasma. Methylamine, when reacting with the internal thioester linkage of C3, causes a conformational change of the molecule such that C3(CH2NH2) functionally resembles C3(H2O) and C3b (29, 30). In the present study, the binding of C3(CH2NH2), C3(H2O), C3b(H2O), and C3d to laminin indicates that the interaction is not dependent on the cross-linking capabilities of the internal thioester linkage in C3. Rather, it seems that the conformational state of C3 is essential for the interaction.

Conformational factors could also explain the apparent differences in the binding affinities of C3(H2O) and C3d.

Inhibition of the binding of C3 to laminin by high salt concentrations reveals the importance of ionic forces in the interaction. The charge properties of C3d were investigated using a hydrophilicity plot based on the amino acid sequence of C3d (9). This analysis, however, did not disclose any major hydrophilic domains which could provide an obvious explanation for the interaction. The binding of C3 to laminin, then, may depend on more specific ionic compatibility.

Lack of the binding of exogenous C3 to cell culture matrices and tissue sections could imply that the binding sites of C3 are not readily available in assembled matrices. An interesting direction for future research is to determine whether C3 present in immune complexes can also bind to laminin, as has been suggested for the interaction of C1q and laminin (3). If indeed operative in human disease, the interaction of C3 and laminin could also have significance in diseases like IgA nephropathy and membranoproliferative glomerulonephritis where the alternative complement pathway is activated at the glomerular basement membrane (34).

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