BREAKDOWN OF C3 AFTER COMPLEMENT ACTIVATION
Identification of a New Fragment, C3g, Using Monoclonal Antibodies*

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The reaction mechanisms of C3 are central to both the classical and alternative pathways of complement activation and have in consequence been extensively studied (see 1 and 2 for reviews). The activation of C3 by cleavage to C3b is brought about by the C3-converting enzymes of the classical and alternative pathways, as well as by a number of noncomplement enzymes. C3b is subsequently inactivated and prevented from further participation in the C3b feedback cycle and in the hemolytic reaction by cleavage to C3bi by factor I, which splits C3b when the latter is combined with factor H. C3bi is the C3 conversion product that appears in serum in vitro after complement activation by either pathway, and it is fairly clear that the C3 product, which was known as "beta 1A" in much of the earlier complement literature, is C3bi and not C3c, the fragment with which beta 1A has usually been identified (3). C3bi does, however, undergo further breakdown both in serum and when cell bound. It was originally shown by Lachmann and Müller-Eberhard (4) that the conglutinable form of C3 bound on cells (cell-bound C3bi) could be cleaved by 1 µg/ml of trypsin to lose its conglutinability. This was accompanied by the elution of C3c from the cell, C3d remaining bound. In serum, too, C3 gave rise, on aging, to two fragments, one of which was described as beta 1A and the other, a fast-migrating fragment, as alpha 2D (5). Alpha 2D was generally equated with C3d (3). As will be pointed out in this paper, this identification is not wholly correct.

Antigenic analysis with polyclonal antisera to C3 had shown the existence of distinct antigenic specificities in C3a, C3c, and C3d, as well as antigenic determinants found only in native C3 (5, 6). Studies with monoclonal anti-C3 antibodies (7) showed one that reacted with C3c, one with C3d, and one that showed an unusual pattern of reactivity that has now been found to react with the fragment here called C3g. By the use of the monoclonal antibodies, the breakdown of C3bi both on cells and in plasma has been reinvestigated, and the results of this investigation are presented herein.

Materials and Methods

Monoclonal Anti-C3 Antibodies. The three monoclonal antibodies are described as clone 3, clone 4, and clone 9 and have been fully described (7). Unless stated to the contrary, ascites from tumor-bearing rats was used as the source of monoclonal antibodies without further purification.

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Where the monoclonal antibodies were labeled with $^{125}$I, immunoglobulin G (IgG) fractions of the ascites were prepared by fractionation on DEAE-Cellulose. Iodination of monoclonal antibodies was performed using the iodogen procedure (8), and offering 1 mCi of $^{125}$I for every mg of protein.

Complement Components. C3 was purified at the Scripps Clinic and Research Foundation by the method of Tack and Prahl (9), and at the Medical Research Council Centre by the method of Harrison and Lachmann (10). C3 was radiolabeled both by the iodogen procedure and by the Bolton and Hunter (11) procedure. Factor H was purified as described in (10). Factor I was purified substantially as described in (4). Bovine conglutinin was purified from bovine serum as described (12). Cobra venom factor was purified from Naja Naja venom as described (13).

Enzymes. The enzymes thermolysin, cathepsin G, leukocyte elastase, and kallikrein were kindly donated by Dr. Alan Barrett of the Strangeways Laboratory, Cambridge, England. Trypsin was purchased from Sigma Chemical Co., Ltd., London, England. Plasmin was purified by chromatography of whole plasma on Sepharose-lysine, which is used as an early step in the fractionation of C3 (10). Sepharose-lysine columns, after extensive washing, were eluted with 0.2 M epsilon-aminocaproic acid, and the plasminogen dialyzed and concentrated by ultrafiltration. Plasminogen was activated with streptokinase obtained from Sigma Chemical Co., Ltd. The amount of streptokinase used was titrated to obtain optimal lysis of fibrin on a fibrin plate. The concentration of plasminogen was then compared with that in undiluted human serum, again assessed by lysis of a fibrin plate. The fibrin plates were prepared as described (14), and plasminogen concentrations were measured by the diameter of the zone of fibrinolysis produced.

Complement Intermediates. The preparation of sheep erythrocytes (E), their sensitization with antibody (preparation of EA), and the preparation of yeast-treated human and guinea pig complement (R3 reagents) were performed as described (15). The intermediate EA treated with complement (EAC) was made by treating 1 ml of 10% EA with 1 ml of human R3 reagent for 2 min at 37°C followed by washing. This intermediate contains, as far as C3 fragments are concerned, a mixture of C3b and C3bi. More defined intermediates were made as follows: 1 ml of 10% EA was treated with 1 ml of guinea pig R3 reagent for 5 min at 37°C to make the intermediate EAC142. The mixture, after incubation, was chilled to 4°C and washed rapidly. It was then treated with C3 using 1 mg of C3 for 1 ml of 10% EAC142. Incubation was continued for 15 min at 37°C with stirring. The EAC3b was washed and used as such. To convert EAC3b to EAC3bi, the cells were treated with purified preparations of factor H and factor I for 15 min at 37°C. The EAC3bi were washed and used as such. To convert EAC3bi to EAC3d, the cells were treated with a final concentration of 2 µg/ml of trypsin for 5 min at 37°C. The cells were then spun down and resuspended in a concentration of 5 µg/ml of soy bean trypsin inhibitor and washed twice more. The treatment of EAC3bi with enzymes other than trypsin is described in Results.

Polyvalent Antisera. Polyvalent antibody to C3 reacting with both C3c and C3d was kindly given by Dr. J. Bradwell, Immunodiagnostic Research Laboratories, University of Birmingham, Birmingham, England. An antiserum reacting predominantly with C3d was kindly given by Ortho Diagnostics, Raritan, NJ. An antiserum reacting exclusively with C3c was kindly given by Dr. H. Müller-Eberhard, Scripps Clinic and Research Foundation. Anti-rat IgG antiserum was raised by immunization of a rabbit with rat IgG. This antiserum was absorbed with EAC for use in the agglutination and agglutination inhibition reactions.

Coprecipitation Assays. In these assays, immunoelectrophoresis was performed on microscope slides. 1% agarose in 0.05 M veronal buffer, pH 8.6, was used as support, and electrophoresis was carried out at 7 mA/slide at 4°C until the albumin Bromophenol blue marker had reached the end of the slide. The antibody slots were filled with a mixture of polyclonal anti-C3 and $^{125}$I-labeled monoclonal antibodies. Development was allowed to take place for 24-48 h. The slides were then exhaustively washed, dried, stained with Coomassie Brilliant Blue, and dried again. Finally, they were submitted to autoradiography, usually allowing an exposure of 1-3 d.

Agglutination Assay for Monoclonal Antibodies. Monoclonal antibodies were titrated in three steps in microtiter plates using 50-µl vol. 50 µl of 0.3% of complement intermediate cells were

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1 Abbreviations used in this paper: E, sheep erythrocytes; EA, erythrocyte antibody; EAC, EA treated with complement; PAGE, polyacrylamide gel electrophoresis.
added and mixed well. The cells were allowed to settle completely at 4°C and then read for agglutination. At this stage, agglutination titers were very low and were not recorded. The plates were then centrifuged for 1 min at 1,000 rpm, and the supernatant was gently shaken off. The cells were resuspended in 50 µl of rabbit anti-rat IgG, diluted 1:400. Cells were then allowed to settle once more, and the agglutination patterns read visually.

**Assay of C3 Fragments by Inhibition of Agglutination.** Sources of C3 fragments were diluted in microtiter trays in 50-µl vol, and 50 µl of the monoclonal antibody was added (ascites diluted 1:250,000) and the mixture incubated for 1 h at 4°C. The plates were then centrifuged at 1,000 rpm for 1 min and the supernate gently shaken off. The cells were resuspended in 50 µl 1:400 sheep anti-rat IgG and allowed to settle. Agglutination was read by pattern.

**Polyacrylamide Gel Electrophoresis (PAGE).** PAGE analysis was carried out by standard techniques (16).

**Results**

**The Antigenic Determinants Recognized by Three Monoclonal Antibodies Against C3**

*Reaction with cell-bound C3 studied by antiglobulin agglutination reactions.* Table I shows the agglutination titers obtained. Clone 3 and clone 4 show the reactivity to be expected of anti-C3d and anti-C3c, respectively. Weak reactivity of clone 4 is, however, seen with EAC3d cells. This can be ascribed to the fixation of some C3 to EAC142 in a form that is insusceptible to cleavage by I and H. Clone 9 reacts exclusively with a neoantigen exposed in C3bi. To this extent it resembles bovine conglutinin, which reacts with a carbohydrate determinant exposed only in C3bi (4). Clone 9 however, does not react with the same determinant as conglutinin. Thus, the reaction of clone 9, unlike that of conglutinin, is not dependent on calcium or inhibited by EDTA. Clone 9 is not inhibited by 0.1 M N-acetylglucosamine, which inhibits conglutination (17), nor does it react with zymosan in the absence of complement as conglutinin does (18) (data not shown).

**Studies by coprecipitation using radiolabeled monoclonal antibodies and the breakdown products of purified C1 and of C3 in serum.** In these experiments the source of C3 is subjected to electrophoresis and then precipitated with a polyvalent anti-C3 antibody, to which is added a radiolabeled monoclonal antibody. Two precautions have been found necessary. One is to use only one monoclonal antibody on each slide. Because monoclonal antibodies have been found to diffuse through

| Table I |
| --- |
| **Reaction of Monoclonal Anti-C3 Antibodies with Complement Intermediates by Antiglobulin Agglutination** |
| **Cell intermediate** | EAC | EAC3b | EAC3bi | EAC3d |
| Clone 3 | 729 | 2187 | 2187 | 2187 |
| Clone 4 | 243 | 729 | 2187 | 81 (3) |
| Clone 9 | 243 | 0 | 243 | 0 |
| Bovine K | +++ | 0 | +++ | 0 |

Cell intermediates were made as described in Materials and Methods. Cells were incubated with dilutions of the monoclonal antibodies, washed, and finally agglutinated by anti-rat Ig. Figures shown are the highest dilutions giving agglutination. Figure in parentheses is a prozone. Reactions with bovine conglutinin (bovine K) (10 minimal agglutinating doses) were performed by direct agglutination.
precipitin lines (7), they can produce staining of lines in unexpected parts of the slide, which can cause confusion if more than one monoclonal antibody is present on a slide. A further problem is that changes in the antigenic structure of C3 may occur after electrophoresis, so that a line in the C3 position might be C3b or even C3bi or a line in the C3b/C3bi position might be a mixture of C3c and C3d. To minimize the risk of post-electrophoretic breakdown, only purified IgG fractions have been used as antisera in the trough. It can be seen (Fig. 1) that C3b and C3bi cannot be distinguished by their electrophoretic mobility in these conditions and both give lines in the "beta 1A" position.

C3bi is split to C3c and C3d by trypsin. C3c is slightly faster in mobility than C3bi and C3d is slightly slower. This is in contrast to the situation using the aged, cobra venom-treated serum, which contains C3c and a faster fragment precipitated by anti-C3d. This fragment is alpha 2D and is the C3d-containing fragment generated by enzymes occurring in human serum.

On autoradiography clone 3 (anti-C3d) reacts as expected with C3, C3b, C3bi, C3d, and the C3 in normal human serum but not with C3c. Clone 4 (anti-C3c) reacts with C3, C3b, C3bi, and C3c, but not with C3d or alpha 2D. Clone 9 reacts with C3 and with C3b (which was unexpected, as it failed to react with cell-bound C3b). It reacts strongly with C3bi but with neither of the two trypsin digest products shown by precipitation—C3c and C3d. On the other hand, it does react strongly with the alpha 2D line. There are, therefore, at least two differences between alpha 2D and

![Fig. 1. Coprecipitation assay using radiolabeled monoclonal antibodies. Immunelectrophoreses were set up as shown in top row and electrophoresed until the albumin marker reached the right hand end of the slide. After development, washing, and staining for protein (2nd row) autoradiography was performed. C3 was used at 1 mg/ml, and the breakdown products were generated from this with little dilution, as follows: C3b was generated from C3 using EAC142 (0.1 ml packed cells/mg C3 for 30 min at 37°C); C3bi was generated from C3b using purified factors I and H (1% by weight of C3b treated) for 1 h at 37°C; C3c + C3d was generated from C3bi by cleavage with trypsin 2 μg/ml for 10 min at 37°C. The reagents made from whole serum (NHS) containing 0.01 M azide were: NHS/CVF, serum treated with 5 μg purified CVF/ml for 45 min at 37°C; NHS/CVF/aged, NHS/CVF incubated at 37°C for 20 h; NHS/CVF/aged (60°C 1 h), NHS/CVF/aged heated at 60°C for 1 h. This treatment destroys the antigenicity of C3d.](image-url)
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C3d. They differ in electrophoretic mobility, and alpha 2D carries the antigenic determinant reacting with clone 9, whereas C3d does not.

**Does Trypsin Digestion of C3bi Produce a Nonprecipitable Fragment Reacting with Clone 9?**

To answer this question, C3 fragments were tested for their ability to inhibit the binding of clone 9 to EAC3bi. Table II shows the results for the C3 fragments tested by the inhibition assay described in Materials and Methods. Purified C3 gives much better inhibition of clone 3 and clone 4 than it does of clone 9. A variety of C3 preparations have been tested, and the more native preparations give the lower inhibition of clone 9. C3b does not inhibit clone 9 at the concentrations tested, and one may therefore conclude that the clone 9-reactive antigen is not exposed. C3bi inhibits all three monoclonal antibodies well. Of particular interest is the result with the tryptic digest of C3bi, which on precipitation analysis shows only C3c + C3d. This digest inhibits clone 9 well and therefore must contain a fragment carrying the determinant for clone 9 that is not capable of being precipitated. It is proposed to call this fragment C3g.

**The Size of Alpha 2D and C3d.**

Alpha 2D, by virtue of containing C3g, should be larger than C3d. To see whether this was the case, immuno-electrophoresis was carried out on serum that had been supplemented with 125I-C3 and then treated with cobra venom factor (CVF) at 37°C for 20 h; using anti-C3d antiserum to precipitate the alpha 2D formed. The anodal part of the alpha 2D line, which can readily be separated from the C3c line, was cut from the immunoelectrophoretic plate the agarose gel broken up by freezing and thawing, and the protein was eluted by boiling with sodium dodecyl sulfate. This alpha 2D preparation was then subjected to PAGE and autoradiography and compared with C3 and a tryptic digest of C3bi. The results are shown in Fig. 2. It can be seen that the alpha 2D is ~8K larger at 41K than is the C3d fragment at 33K. A low molecular weight band can be seen on the track containing trypsin-digested C3bi but cannot be given a size on this gel.

**Can Cell-bound C3bi be Converted to Cell-bound Alpha 2D?**

The summary of the reaction of the three monoclonal antibodies with soluble C3 fragments is shown in the upper part of Table III. It can be seen that C3bi, alpha 2D, C3c, and C3d can be distinguished by the pattern of reaction with the monoclonal antibodies. Similarly, it is therefore possible to detect cell-bound alpha 2D showing reaction with clone 3 and clone 9 but not with clone 4. Such reactivity was found initially on cells of patients with cold hemagglutinin disease in vivo, but in the present experiments this question

**Table II**

| Fragments tested (undiluted = 250 μg/ml) | Titer of inhibition of agglutination of EAC3bi using: |
|-----------------------------------------|-----------------------------------------------------|
|                                         | Clone 3  | Clone 4  | Clone 9  |
| C3                                      | 27       | 27       | 5        |
| C3b                                     | 81       | 81       | 0        |
| C3bi                                    | 81       | 243      | 243      |
| Trypsin-treated C3bi (C3c + C3d + C3g)  | 81       | 243      | 243      |
Fig. 2. Autoradiograph of PAGE on 9% polyacrylamide of alpha 2D globulin cut from an immunoelectrophoresis plate (track 1) compared with a tryptic digest of C3bi (track 2) and with native C3 (track 3). Numbers on the right of the figures are molecular weights in kiloDaltons.

Table III

Summary of Specificity of Anti-C3 Monoclonal Antibodies and Bovine Conglutinin

| Reaction with soluble C3 fragments | Clone 4 anti-c | Clone 3 anti-d | Clone 9 anti-g | Bovine conglutinin |
|-----------------------------------|---------------|---------------|---------------|-------------------|
| C3                                | ++*           | ++‡           | ++            | ++                |
| C3b                               | ++            | ++            | ++            | +                 |
| C3bi                              | ++            | ++            | ++            | ++                |
| C3c                               | ++            | ++            | ++            | ++                |
| Alpha 2D                          | 0             | 0             | ++            | ++                |
| C3d                               | 0             | 0             | 0             | ++                |

| Reaction with cell-bound C3 fragments | C3b | C3bi | Alpha 2D | C3d |
|---------------------------------------|-----|------|----------|-----|
|                                      | ++  | ++   | ++       | ++  |
|                                      | ++  | ++   | ++       | ++  |
|                                      | 0   | ++   | ++       | 0   |

* Coprecipitation assay: left of each pair.
‡ Inhibition of agglutination: right of each pair.
§ Not applicable because fragment does not precipitate.
¶ Binding assay or agglutination.

was investigated by the proteolysis of cells bearing C3bi using a variety of different proteolytic enzymes.

For these experiments EAC3bi were treated with dilutions of various enzymes for 30 min at 37°C, washed, and tested with monoclonal anti-C3 and bovine conglutinin. A number of enzymes were tested, and the results are summarized in Table IV. Some

Lachmann, P. J., C. Voak, R. G. Oldroyd, M. D. Downie, and P. C. Bevan. Analysis of C3 fragments found on red blood cells coated with complement in vivo and in vitro. Manuscript submitted for publication.
TABLE IV

Proteolysis of Cell-bound C3

| Concentration of enzyme (ng/ml) to destroy reactivity with: | Clone 3 | Clone 4 | Clone 9 | Bovine conglutinin |
|------------------------------------------------------------|--------|--------|--------|-----------------|
| Trypsin                                                    | >3000  | 120    | 370    | 120             |
| Thermolysin                                                | >3000  | 120    | 1100   | 120             |
| Cathepsin G                                                | >3000  | 370    | 370    | 370             |
| Leukocyte elastase                                         | >3000  | 370    | 370    | 370             |
| Kallikrein                                                 | >3000  | >3000  | >3000  | >3000           |
| Plasmin*                                                   | >1:1   | 1:4    | >1:1   | 1:8             |

Enzymes that can generate alpha 2D globulin have differential destructive activity on clone 4 and clone 9. These are boxed. At concentrations between those given in the two sides of the box, alpha 2D globulin is generated from C3bi.

* Concentrations compared with those generated in normal human serum with streptokinase.

TABLE V

Loss of Conglutinin Reactivity of EAC3bi on Incubation in Serum with and without Plasmin

| EAC3bi incubated in: | Streptokinase | Lysis on fibrin plate | Doses of conglutinin giving conglutination after incubation at 37°C for: |
|----------------------|---------------|-----------------------|--------------------------------------------------|
|                      |               |                       | 18 h | 41 h |
| NHS/56°C*            | -             | 0                     | 8    |      |
| NHS/56°C filtered through | -       | 0                     | 8    |      |
| NHS/56°C filtered through | +       | ++                    | 8    | No conglutination |
| Sepharose-lysine     | +             | 0                     | 8    |      |

EAC3bi at zero times need one dose of conglutinin to give positive conglutination.

* Normal human serum heated at 56°C for 30 min.

Enzymes (thermolysin is the best example and trypsin is another) split C3bi to alpha 2D at a lower concentration than that needed to bring about the further split of alpha 2D to C3d. On the other hand, leukocyte elastase and cathepsin G cannot be used to make an alpha 2D intermediate, and kallikrein will not destroy C3bi at the concentrations tested. Plasmin in the physiological concentration range gave rise only to alpha 2D.

These experiments show that the split from C3bi leading to alpha 2D is not a split at an alternative site to that producing C3d, but that the two splits are sequential; a fragment carrying the clone 9 determinant is released when alpha 2D is converted to C3d. On the basis of all the findings recorded above it seems appropriate to designate the material hitherto called alpha 2D as C3d,g.

What is the Physiological Enzyme that Brings About the Split of C3bi to C3c and Alpha 2D (C3d,g) in the Serum? The reaction breaking down C3bi in the serum is slow. Even in cobra venom-treated serum, where all the C3 is in the form of C3bi, it requires ~18 h to get complete conversion of C3bi to C3c and alpha 2D. The relevant enzyme is
therefore probably present at low concentration. From its specificity plasmin would seem the most likely candidate. However, attempts to show that the breakdown of C3bi (as shown by loss of reactivity with conglutinin) can be retarded by depleting plasminogen or accelerated by activation with streptokinase have both so far been unsuccessful (Table V), and this raises doubts of whether plasmin is indeed enzyme concerned. However, where plasmin inhibitors are absent plasmin will cleave C3bi efficiently.

**C3g Can Be Eluted from Complement-coated Cells.** These experiments were done using 125I-C3. The results shown in Fig. 3 were done with iodogen-labeled C3, but those obtained by Bolton and Hunter (11) reagent were not appreciably different. The sequence of conversions are outlined in Fig. 3. EAC3b was generated from EAC142 and radiolabeled human C3. About 7% of the offered C3 was bound to the cells. The EAC3b was treated with I and H to convert it to C3bi. This process was accompanied by the elution from the cell of 30-40% of the C3 counts (4). This material presumably derives from C3b that was not covalently bound to the cell surface and is eluted in the form of C3bi. It has been assumed that the specific activities of C3bi and C3bi remain the same. On this basis, 32,000 molecules of C3bi/erythrocyte remain in this particular experiment. The cells were then eluted with plasmin to release the C3c and to leave the alpha 2D (C3d,g) on the cells. The specific activity of C3c (818 cps/µg) is higher than that of C3bi (658 cps/µg), whereas that of C3d,g is lower (253 cps/µg). C3d,g cells were then treated with trypsin to release the C3g. This fragment also is of relatively low specific activity (156 cps/µg) compared with the starting C3, and as there is also very little of it, it is not surprising that radioactive C3g bands have not been detected. The C3g preparation was tested by inhibition of agglutination and gave a titer of 27 (corresponding to ~25 µg/ml C3bi) on inhibition of clone 9 but no inhibition at all with clones 3 and 4.

**Discussion**

Fig. 3 shows in schematic form what we now believe to be the breakdown pattern of C3bi. The scheme is similar to that given by Harrison and Lachmann (19) except
for the transposition in the alpha chain of the alpha d,g and the alpha c fragments (and for redesignation of the fragment there called C3e as C3g). It also shows some resemblance to that of Nagasawa and Stroud (20). The sequential nature of the reactions breaking down C3bi has now been established both on cells and in free solution. It is clear that the initial cleavage of C3bi in serum splits the molecule into C3c, which in the case of cell-bound C3bi is eluted from the cell, and the intermediate identified as alpha 2D (C3d,g) on immunoelectrophoretic analysis. This first split of C3bi into C3c and C3d,g without the second split of C3d,g to C3d and C3g can be brought about by low concentrations of a number of proteolytic enzymes, notably trypsin, thermolysin, and plasmin. Some other enzymes—leucocyte elastase and cathepsin G—do not produce the first split without the second. It is not possible from this distribution of enzymic activity to make any predictions as to the chemical nature of the bond split. It is presumably in the tertiary structure of the fragment that the fine specificity rests, which determines which of these cleavages happens first. The enzymes that bring about C3bi breakdown in vivo are still not clearly known. The enzyme that brings about this split extravascularly may well be plasmin, in as much as it has the right specificity, but we were unable to demonstrate any acceleration of the split with streptokinase, nor was it absent in sera passed through sepharose-lysine to remove plasminogen. Thus it seems that there may be other enzymes that are capable of this split. It has recently been suggested (21) that factor I itself may split C3c from cell-bound C3bi. In the fluid phase, however, it has been shown using purified components (19, 22) that factor I does not split C3bi. It is also not clear what enzymes subsequently split C3d,g to C3d and C3g. In spite of the conventional view to the contrary it must be doubted whether this reaction normally goes on in plasma either in vitro or in vivo. In vivo data show that the final fragment of complement activation found on erythrocytes in cold haemolytic antibody disease and in mesangiocapillary glomerulonephritis (23)—two of the diseases in which the most intense complement activation occurs—is alpha 2D (C3d,g) and not C3d or C3g. We have never seen C3d in aged serum, except in one instance when no sodium azide was added to the serum. The split of C3d,g to C3d and C3g may require enzymes not present in normal plasma but derived from either bacteria or from the breakdown of cells at inflammatory sites.

The fragment called C3g in this paper is defined by its reactivity with clone 9. It has been shown that this fragment is part of alpha 2D and can be cleaved from it by trypsin. The C3g antigenic determinant is poorly if at all present in native C3 and is absent from C3b both on cells or in solution. The observation that the anti-C3g antibody reacts on coprecipitation assays with C3 and C3b must be taken to represent changes either induced subsequent to electrophoresis, possibly by contaminating enzymes in the antibody, or brought about by the reaction with the polyclonal anti-C3 itself. This latter mechanism would be analogous to the situation described by Coombs et al. (24) for C4, who showed that, whereas C4 on its own does not react with the C4 receptor on guinea pig erythrocytes, complexes of C4 and anti-C4 will indeed do so. C3g is, however, well exposed in both C3bi and in alpha 2D, which we now propose should be called C3d,g. To this extent C3g antigen is a neoantigen appearing on complement activation, and anti-C3g may therefore find a use in the demonstration of complement activation products. C3e, as a separate acidic fragment of complement, was described by Ghebrihewet and Müller-Eberhard (25), and they
identified their fragment with the leukocytosis-inducing fragment earlier described by Rother (26). C3g as defined by reactivity with clone 9 resembles the fragment described by Ghebrihewet and Müller-Eberhard (25) approximately with respect to molecular weight and to a highly anodal mobility (because alpha 2D is so much more anodal than C3d). However, C3e was derived from C3c, whereas the present fragment is undoubtedly derived from alpha 2D and is not present in C3c. Furthermore, we have been unable to precipitate C3g with any of the polyclonal anti-C3 sera available to us, including that used to precipitate C3e in the earlier study of Ghebrihewet and Müller-Eberhard (25). We were unable to produce precipitation even with a mixture of clone 9 and polyvalent antisera, suggesting that it is difficult to make antibodies to more than one (or two) C3g determinants.

The biological properties of the C3g will be reported together with details of purification in a separate publication, but preliminary testing failed to show consistent production of leucocytosis in rabbits. The identification of C3g with the C3e of Ghebrihewet and Müller-Eberhard is therefore unlikely and it is probably a hitherto unrecognized C3 fragment.

Summary

The physiological breakdown of C3 has been studied using monoclonal anti-C3 antibodies, and it has been found that the later stages of this process—the breakdown of C3bi—is more complex than had previously been recognized. C3bi is the reaction product produced from C3b by the action of factor I which, in the presence of factor H, produces a double cleavage in the alpha chain of C3b.

It is here reported that, both on cells and in the fluid phase, the breakdown of C3bi in serum gives rise to two products: C3c and the product previously described as alpha 2D, which we now propose to designate C3d,g. Alpha 2D differs from C3d in that it contains an additional fragment of ~8,000 mol wt that carries the antigenic determinant for the clone 9 monoclonal anti-C3 antibody. C3g cannot be precipitated by anti-C3 antisera and therefore behaves as a uni- or bideterminant antigen. The cleavage of C3d,g to C3d and C3g does not occur in sterile serum. It is also still uncertain what enzyme cleaves C3bi to C3c and C3d,g in plasma. Plasmin can do so in vitro, but plasminogen-depleted serum can still produce the cleavage. The antigenic determinant recognized by clone 9 in C3 is not exposed in C3 or C3b, but appears as a neoantigen in C3bi (and in C3d,g). Anti-C3g therefore is a potentially useful ligand for detecting complement-activation products. C3g represents a new, highly anionic C3 fragment and seems not to be identical with the C3e fragment described by others.

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