THE MECHANISM OF ACTION OF THE C3b INACTIVATOR (CONGLUTINOGEN-ACTIVATING FACTOR) ON ITS NATURALLY OCCURRING SUBSTRATE, THE MAJOR FRAGMENT OF THE THIRD COMPONENT OF COMPLEMENT (C3b)*

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The fixation of the third component of complement (C3) results in many important biological phenomena, among which are (*a*) immune adherence (1), (*b*) enhancement of phagocytosis (2, 3), (*c*) the release of an anaphylatoxin which is a potent releaser of histamine (4), and (*d*) the feedback activation of the alternative pathway (5, 6). The physiological mechanisms involving C3 fixation require the generation of a C3 convertase which may occur by two separate pathways. C3 convertase can be generated, in the form of $C\overline{42}$, by the so-called classical pathway of activation or in the form $\overline{C3b,B}$ by the alternative or properdin pathway (7). In both cases, C3 is converted to C3b by cleavage of a small peptide, C3a. Normal human serum contains an inactivator of activated C3b. This C3b inactivator or conglutinogen-activating factor (KAF) has been shown to inhibit both immune hemolysis and the immune adherence properties of C3b and to cause cleavage of C3b in the fixed and fluid-phase stages (8–11).

Although it is known that the C3b inactivator is not depleted during its reaction with C3b and that C3b treated with the C3b inactivator becomes extremely sensitive to proteolytic digestion by trypsin and "trypsin-like" enzymes (9), the exact molecular nature of the action of the C3b inactivator on C3b has not been studied. In an effort to delineate the products of this interaction, purified C3b and C3b inactivator were allowed to react for various specific lengths of time and the products of these reactions were then analyzed.

Materials and Methods

Purified Serum Proteins. C3 was purified by a modification of the method of Nilsson and Müller-Eberhard (12) and subsequently labeled with ^{125}I by the iodine monochloride technique (13). C3b inactivator was also purified from normal human serum (14). 20 mg of cobra venom factor (CVF) was isolated from *Naja naja* venom (Sigma Chemical Co., St. Louis, Mo.) by DEAE-cellulose chromatography and gel filtration on Sephadex G-200 (15) and subsequently conjugated to 2 ml of packed cyanogen bromide-treated 4B Sepharose beads (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). 0.1 ml of CVF-Sepharose was reacted with 0.4 ml of fresh normal human serum at 37°C for 15 min in order to generate C3 convertase. The CVF-Sepharose beads were then centrifuged and washed with chilled phosphate-buffered saline. C3b was generated by incubation of purified C3 with serum-exposed, washed CVF-Sepharose beads for 45 min at 37°C which results in the loss of hemolytic C3 activity (L. Halbachs and P. J. Lachmann, unpublished observations).

Fresh human serum was saturated to 20% with sodium sulphate. The precipitate was discarded, and the supernate was chromatographed on O-(carboxymethyl)cellulose as previously described to

* Supported in part by U. S. Public Health Service grant No. AI-05877.

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isolate a fraction containing partially purified factor B and factor D (16).

Enzymes and Inhibitors. Trypsin (Sigma Chemical Co.) was stored in HCl under liquid N_2 before use. Diisopropylfluorophosphate (DFP) was purchased from Sigma Chemical Co. and used at a final concentration of 10^{-3} M. Antrypol was obtained as Suramin-BP from FBA Pharmaceuticals, Inc., New York, and was used to render C3b resistant to KAF at a concentration of 1 mg/ml (14).

Reactions. All reactions were carried out at 37°C in 10-ml plastic tubes placed in a rotary-stir water bath. Time-course reactions were stopped at the designated time by addition of antrypol to the reaction vessel and removal of the tube from the 37°C bath. All necessary dilutions were made with 0.01 M sodium phosphate buffer at pH 7.

Polyacrylamide Gel Electrophoresis. Reaction products were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) according to the methods of Weber and Osborn (17). The reaction mixture was at first dialyzed for 6 h against 0.1 M sodium phosphate buffer pH 7 and then prepared in 0.01 M sodium phosphate pH 7 containing 1% SDS and 1% 2-mercaptoethanol and applied to the polyacrylamide gels. After destaining, gels were cut into 2-mm slices, placed in 1mM proprionic acid, and analyzed for radioactivity.

Functional Assay of C3b. The capacity of C3b before and after treatment with the C3 inactivator to activate the alternative pathway was assayed by the inactivation of the hemolytic activity of factor B by C3b in the presence of factor D. The hemolytic activity of factor B was measured by the single radial diffusion assay of Martin and Lachmann (18), which is based on the bystander lysis of guinea pig erthrocytes when the alternative pathway of human C is activated. Agarose plates incorporating 0.5% guinea pig erythrocytes, 5% human serum heated for 20 min at 50°C, 10 mM EDTA, and 7 mM Mg^{++} are used for the assay.

Results

Purified human C3 consists of a single heavy or alpha chain and a single light or beta chain joined by interchain disulphide bonds which can be resolved by polyacrylamide gel electrophoresis in SDS and separated by reduction and alkylation followed by gel filtration chromatography. Purified human C3b, when generated from C3 by CVF-Sepharose convertase, yields three protein bands when resolved by electrophoresis in SDS-polyacrylamide gels as indicated in Fig. 1. The band with a mol wt of 180,500 represents intact C3b, the 110,000 mol wt band represents the alpha chain, and the beta chain moiety of C3b is resolved as a band of mol wt 70,000. Fig. 1 illustrates that after incubation of C3b with purified C3 inactivator for 3 min, the 110,000 band representing the alpha or heavy chain disappears and a new protein fragment of 85,000 mol wt can be discerned. Furthermore, it is apparent from Fig. 1 that the protein band representing the light or beta chain of C3b is unchanged after 3 min incubation with C3 inactivator.

Further incubation of C3b with C3 inactivator results in gradual degredation of the 87,000 dalton fragment. As Fig. 1 indicates, after 3 h of incubation, almost complete disappearance of both the 110,000 fragment of C3b and the 85,000 fragment generated after 3 minutes of C3b inactivator action on the C3b substrate occurs. In fact, almost all of the protein appears in the 70,000 mol wt band after 3 h incubation. The reactions illustrated in Fig. 1 cannot be inhibited by DFP up to a concentration of 10^{-3} M.

Since it is known that the action of C3b inactivator on its cell-bound substrate, C3b renders C3b exquisitely sensitive to proteolytic digestion by trypsin (9), further incubations were carried out in the presence of various concentrations of trypsin. Fig. 2 illustrates that incubation of C3b with trypsin had no effect upon the size of the alpha or beta chains of the C3b as resolved in SDS-polyacrylamide gels.

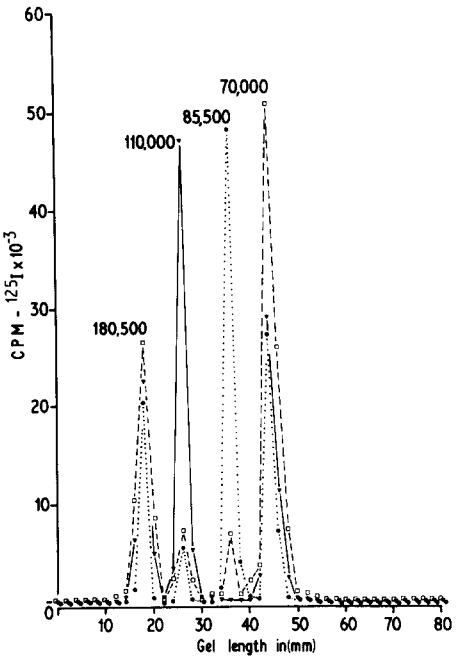

FIG. 1. Polyacrylamide gel electrophoresis in SDS of C3b (▼—▼), C3b reacted with C3 inactivator for 3 min (●· · · ·●), and C3b reacted with C3 inactivator for 3 h (□---□).

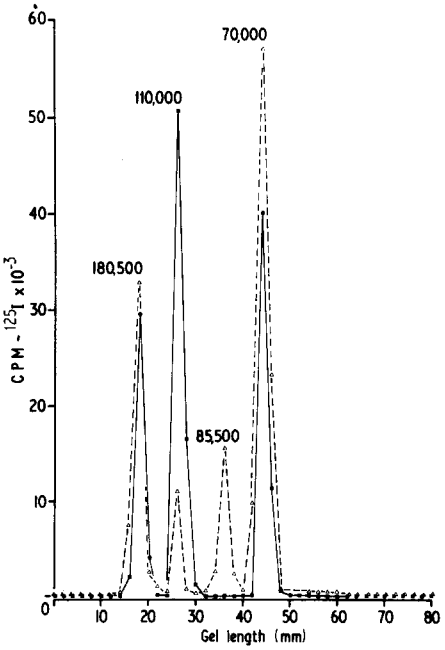

FIG. 2. Polyacrylamide gel electrophoresis in SDS of C3b treated with trypsin for 75 min at 37°C (■—■)or C3b treated with trypsin after prior reaction with C3 inactivator for 3 min (Δ---Δ).

No effect was seen with trypsin concentrations from 0.1 to 10 μg/ml, a concentration of trypsin 100-fold in exesss to that needed to proteolyse C3 inactivator-treated, cell-bound C3b. The results of treatment of C3b preincubated with the C3b inactivator for 3 min with concentrations of trypsin down to 0.1 μg/ml are also shown in Fig. 2. From this it is clear that only the protein bands at the 70,000 mol wt range appear after such trypsin treatment of C3b which has been preincubated for 3 min with the C3b inactivator. Indeed, the results of such treatment are remarkably similar to those seen after incubation of C3b with its inactivator for 3 h.

When 8.6 μg of C3b were interacted with 560 μg of factor B at 37°C for 30 min, the factor B lost its hemolytic capacity as measured by bystander lysis of guinea pig erythrocytes. On the other hand, it required 86 μg of C3 treated for 3 min with C3 inactivator at 37°C to bring about a comparable loss of hemolytic activity of 560 μg of factor B. Thus, C3b treated with C3 inactivator for 3 min lost over 90% of its capacity to inactivate factor B in the presence of factor D.

Discussion

From the results presented here, it is apparent that the C3b inactivator proteolytically cleaves C3b. Furthermore, the reaction of the C3b inactivator is twofold in nature; the first cleavage, which is initiated rapidly, is essentially completed in 3 min. The second reaction, which is much slower, requires 3 h to reach completion. Since electrophoresis of the reaction products in SDS-polyacrylamide gel allows resolution of the heavy and light chains, it is clear that both enzymatic cleavages by the C3b inactivator occur at sites on the heavy chains of the intact molecule. The first reaction product, composed of alpha 85,000, beta 70,000, should properly be designated $C3b^i$, as this reaction product is inactive with regard to the capacity of C3b to activate the alternative pathway. The product of the second reaction, composed of alpha 70,000, beta 70,000, resembles C3c purified from aged serum. The conditions under which the gels were run resulted in failure to detect the smaller cleavage fragments.

The data also clearly indicate that the second slow reaction of the C3b inactivator can also be accomplished by very small concentrations of trypsin upon completion of the first, fast reaction. However, no effect on C3b is seen by trypsin in concentrations 100-fold in excess of this when the first, fast reaction of the C3b inactivator has not occurred. Thus, C3b is relatively trypsin resistant compared with the exquisite sensitivity of $C3b^i$ to tryptic cleavage. It is important to note that since such minute concentrations of trypsin are required to mimic the second, slow reaction of the C3b inactivator, the possibility of its contamination by another trypsin-like protease is very real. Leukocytes and platelets are known to contain significant amounts of trypsin-like proteases, and the possibility of contamination on the scale needed cannot be excluded by the methods employed.

A patient with a genetically determined deficiency of the C3b inactivator has been studied in great detail (19, 20). His serum contains small amounts of C3b so that the alternative pathway in his blood is constantly subject to feedback activation. The intravenous administration of purified C3b inactivator causes a prompt conversion of his C3b to C3c and a cessation of in vivo activation of the alternative pathway in the patient's plasma (21). It appears likely that in the conversion of C3b to $C3b^i$ the capacity of C3b to activate the alternative pathway has been destroyed while the conglutinable and opsonically active fragment of C3 has been formed, facilitating phagocytic uptake of particles coated with this moiety of C3 (22).

Received for publication 6 November 1975.

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