575-587.
Tsong, T. Y. (1975), *Biochemistry 14*, 1542-1547.
Vallee, P. B., and Williams, R. C., Jr. (1975), *Biochemistry 14*, 2574-2580.
Wassarman, P. M., and Burgner, J. W. (1972), *J. Mol. Biol. 67*, 537-542.
Wetlaufer, D. B., and Ristow, S. (1973), *Annu. Rev. Biochem. 42*, 135-158.

# Limited Degradation of the Third Component (C3) of Human Complement by Human Leukocyte Elastase (HLE): Partial Characterization of C3 Fragments†

J. C. Taylor,‡ I. P. Crawford, and T. E. Hugli*,§

ABSTRACT: The third component of human complement (C3) was digested with human leukocyte elastase (HLE). Digestion proceeds in two stages under controlled conditions of proteolysis. The initial event of HLE attack produces cleavage of the C3 $\alpha$ chain near the amino terminus forming an $\alpha'$ chain and releasing an 8500 molecular weight fragment ($\alpha$4) which is immunologically and chemically similar to the C3a anaphylatoxin. The purified $\alpha$4 fragment is lacking in both anaphylatoxic or chemotactic activity. More extensive digestion of C3 with HLE results in a selective degradation of the $\alpha'$ chain to form three principle fragments of 36 000 ($\alpha$1), 28 000 ($\alpha$2), and 24 000 ($\alpha$3) molecular weight. Digestion of C3 with HLE either for extended periods of time (2 h) or at high ratios of enzyme to C3 (1:20) does not affect the covalent integrity of the $\beta$ chain. Specificity of HLE cleavage sites on the protein substrate is consistent with that previously observed with model synthetic substrates. However, the ability of HLE to cleave the peptide linkage between aliphatic residues and a cationic residue was not predicted from model substrate studies. Fragments $\alpha$1 to $\alpha$4 account for approximately 90% of the total $\alpha$-chain structure. Fragments $\alpha$2 and $\alpha$4 are not covalently bonded to the disulfide-linked complex of $\alpha$1, $\alpha$3, and $\beta$ chain. In addition, the $\alpha$2 fragment cross-reacts with anti-C3d; C3d is a natural catabolite of C3 formed by the action of a plasma enzyme C3b inactivator on C3b. The C3d fragment contains the labile C3 binding site. Distribution of the carbohydrate on C3 is divided between both $\alpha$ and $\beta$ chains. After extensive HLE digestion, all of the stainable carbohydrate on the $\alpha$ chain is detected on the $\alpha$3 fragment. The carbohydrate moiety represents a significant marker for future structural analysis. Characterization of the HLE fragments from the $\alpha$ chain includes identification of $NH_2$- and COOH-terminal residues and the respective amino acid compositions. A corresponding characterization of the isolated $\alpha$ chain of C3 permits a tentative alignment of the major HLE fragments from the $\alpha$ chain. A molecular model derived from an alignment of the HLE fragments of C3 compares favorably with the tentative C3 model reconstructed from fragments produced by plasma enzymes. C3 is selectively cleaved into C3a and C3b during complement activation and further degradation of C3b by plasma enzymes produces $\alpha$-chain fragments closely resembling the degradation products obtained after HLE digestion of C3.

Crude lysates of lysosomal granules from human peripheral leukocytes have been found to induce alterations in a number of serum proteins involved in inflammation and tissue injury. Lysosomal extracts incubated with high- and low-molecular-weight kininogens cause the release of a kinin (Movat et al., 1973). This kinin-generating activity has been purified recently and is thought to be due to leukocyte elastase (Movat et al., 1976), a lysosomal protease comprising approximately 16% of the total granular extract (Janoff, 1973; Taylor and Crawford, 1975). Crude lysosomal lysates have also been shown by Taubman et al. (1970) and Ward and Zvaifler (1971) to produce a factor from serum and from the fifth component of human complement which is chemotactic for rabbit polymorphonuclear (PMN) leukocytes. Goldstein and Weissman (1974) noted that a C5a[1]-like fragment produced by the lysosomal proteases acts to release additional lysosomal enzymes from isologous, cytochalasin-B-treated human PMN leukocytes. Proteolytic degradation of other components of the complement system such as $C\overline{1s}$ (Taubman and Lepow, 1971) and C2 (Taubman et al., 1970) has previously been described. However, none of the proteolytic alterations mentioned above have been well characterized biochemically. Such a study would help clarify the molecular events occurring during the inflammatory process as well as elucidate the substrate specificity of the proteases contained in leukocyte lysosomes.

Human leukocyte elastase (HLE) is a serine protease consisting of a single polypeptide chain with a molecular weight between 22 000 (Taylor and Crawford, 1975) and 32 000 (Ohlsson and Olsson, 1974a). A highly basic glycoprotein, HLE, appears as a characteristic set of four isozymes after

† From the Departments of Molecular Immunology and Microbiology, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received January 17, 1977.* This is publication number 1254 from the Scripps Clinic. This work was supported in part by Grants HL 16411, HL 20220, and HL 15309 from the United States Public Health Service and by a Grant-in-Aid (74-864) from the American Heart Association.

‡ Postdoctoral Fellow (PF-940) of the American Cancer Society. Present address: Division of Thoracic Diseases, Mayo Clinic, Rochester, Minn. 55901.

§ Recipient of an Established Investigatorship (72-175) from the American Heart Association.

[1] Symbols used for the complement components conform to the recommendations of the World Health Organization Committee on Complement Nomenclature (1968). A bar above the complement component (e.g., C1s) signifies an enzymatically active form.

acrylamide gel electrophoresis at pH 4.5 (Janoff, 1973; Taylor and Crawford, 1975). Current evidence suggests that this electrophoretic heterogeneity is due primarily to size and charge differences resulting from quantitative differences in the carbohydrates attached to the molecule (Taylor and Crawford, 1975). The major serum inhibitor of HLE is $\alpha$1-antitrypsin (Ohlsson, 1971; Ohlsson and Olsson, 1974b). The enzyme is also inhibited by $\alpha$2-macroglobulin (Ohlsson and Olsson, 1974b), and synthetic inhibitors such as serine protease active-site titrants and *N*-acetyl-Ala-Ala-Pro-Ala-chloromethyl ketone (Tuhy and Powers, 1975).

The human complement system contains more than 20 humoral proteins which, through specific interactions, contribute to the immune defense mechanism (Müller-Eberhard, 1975). The most abundant of all the complement components is C3, occupying a central position in the complement-activation process. Previous reports have claimed that C3 is activated by lysosomal enzymes, but no anaphylatoxin activity is generated according to Taubman et al. (1970). Others have reported that lysosomal proteases release a chemotactic factor from purified C5 but not from C3 (Ward and Hill, 1970). Although these digestions were not performed with purified leukocyte elastase, this major lysosomal protease is believed responsible for much of the proteolysis induced by crude leukocyte granular lysates. We have therefore attempted to characterize the molecular events resulting from digestion of the third component (C3) of human complement by lysosomal elastase purified from human leukocytes. In the present study, we report the highly specific cleavage of human C3 by HLE under nearly physiological conditions. Products of C3 degradation by HLE are compared with C3 products formed by plasma enzymes described by Bokisch et al. (1975). Alignment of the fragments of C3 based on chemical characterization of the HLE digestion products provides a tentative model of the C3 molecule which should prove useful in future studies of the primary structure of this major serum protein.

## Materials and Methods

*Purification of Human Leukocyte Elastase (HLE).* The purification of HLE from lysosomal extracts of human leukocytes has been described in detail elsewhere (Taylor and Crawford, 1975). $\beta$-Alanine was omitted from all buffers during the purification procedure; otherwise, the conditions were identical to those previously described by Taylor and Crawford (1975).

*Digestion of the Third Component (C3) of Human Complement by HLE.* Purified C3 was dialyzed at 4 °C for 24 h against 0.02 M Tris[2]-HCl, pH 7.4, containing 10% sucrose and 0.15 M NaCl. The protein concentration after dialysis was 5 mg/mL. Magnesium chloride was then added to a final concentration of 5 mM, followed by addition of HLE. Molar ratios during digestion of C3 by HLE were either 20:1 (150:0.85 $\mu$g) at 21 °C or 52:1 (150:0.34 $\mu$g) at 37 °C. Phenylmethanesulfonyl fluoride was added at various intervals in a 200-fold molar excess over HLE in order to terminate proteolysis. In some experiments, *N*-acetyl-Ala-Ala-Pro-Ala-chloromethyl ketone, a specific inhibitor of HLE (Tuhy and Powers, 1975), was used instead of $PhCH_2SO_2F$ to terminate the digestion. This inhibitor was kindly provided by Dr. James Powers of the Georgia Institute of Technology.

*Polyacrylamide Gel Electrophoresis.* Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to separate the polypeptide fragments of C3 produced during digestion by HLE. Acrylamide gels (7%) and buffers were as described by Laemmli (1970). The samples were prepared for electrophoresis by incubation either at 21 or 37 °C for 1–2 h in 12.5 mM sodium phosphate (pH 7.6) with 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 3.4 mM $PhCH_2SO_2F$ (Weber and Osborn, 1969). Following this treatment, an equal volume of Tris-HCl (25 mM) containing glycine (0.19 M), sodium dodecyl sulfate (0.1%), glycerol (20%), bromophenol blue (0.005%), and 10% 2-mercaptoethanol was added, and the mixture was layered onto the gel. Electrophoresis, staining, and destaining of gels were done as previously reported (Taylor and Crawford, 1975). The molecular weights of the digestion products were estimated by the procedure of Weber and Osborn (1969) using $\beta$-galactosidase, ovalbumin, chymotrypsinogen A, and pancreatic ribonuclease (Worthington) as standards. Carbohydrate was detected by Schiff reagent (Harleco, Gibbstown, N.J.) using the procedure of Kapitany and Zebrowski (1973). The gels used for carbohydrate staining were fixed in a mixture of acetic acid, ethanol, and water (10:45:45, v/v). The sodium dodecyl sulfate was removed by electrophoresis at 150 mA for 6 h in 5% acetic acid prior to staining with the Shiff reagent.

*Isolation of C3 and the C3 Fragments Generated by HLE.* Human C3 was purified from freshly drawn blood supplied by the San Diego Blood Bank. The C3 was isolated according to the procedure described by Tack and Prahl (1976). A 10-mL sample containing 44 mg of human C3 was digested with 140 $\mu$g of HLE. Radiolabeled C3 (12 $\mu$Ci) was added before digestion to facilitate isolation of the fragments. The $^{125}$I-labeled C3 (1.2 $\mu$Ci/$\mu$g) was prepared by a lactoperoxidase-catalyzed iodination according to the method of David and Reisfeld (1974). C3 was digested for 3.5 h at pH 7.2 and 37 °C and then applied to a Sephadex G-100 column (4.5 × 50 cm). The C3 digest was eluted from the column at 15 mL/h with 0.15 M sodium acetate at pH 3.7 and room temperature. Material from the first peak eluted from the Sephadex G-100 column was pooled, reduced with 5 mM dithiothreitol in 1% sodium dodecyl sulfate and then separated by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 4.5 × 10 cm gel according to the method of Jovin et al. (1964). Protein bands were visualized after electrophoresis by staining a strip of the gel with amido black stain. Protein was recovered from slices of the preparative polyacrylamide gel by diffusion into 5% acetic acid. The sodium dodecyl sulfate was removed from the protein samples by exhaustive dialysis against 5% acetic acid.

*Identification of $NH_2$- and COOH-Terminal Residues.* The $NH_2$-terminal amino acids of C3 digestion products were identified as follows: after cleavage of C3 by HLE and addition of $PhMe_2SO_2F$, the mixture was dialyzed for 4 h (4 °C) against 0.1 M Tris-HCl, pH 9, containing 10% sucrose and 0.15 M NaCl. Dansyl chloride (Pierce, 10%, w/v in acetone) was diluted 1:2 with acetone. One-twentieth volume of the 1:2 dansyl chloride was added to the protein solution, and the mixture was covered with Parafilm and incubated at 37 °C for 1 h. The dansylated proteins were then dialyzed 24–48 h (4 °C) against 0.02 M Tris-HCl, pH 7.4, with 10% sucrose and 0.15 M NaCl added and prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described above. Approximately 600 $\mu$g of protein was layered over each gel and electrophoresis was carried out as previously reported (Taylor and Crawford, 1975). The electrophoretic pattern of the dansylated proteins was identical to that of nondansylated digests of C3 visualized by protein staining. The C3 products

[2] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DTT, dithiothreitol.

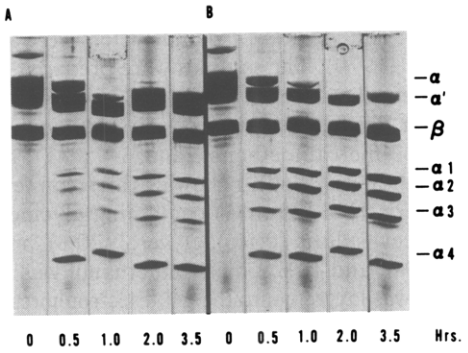


FIGURE 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels containing 2-mercaptoethanol-reduced human C3 and C3 digestion mixtures. (A) The C3 was digested by HLE at a molar ratio of 20:1 (C3:HLE) in 0.02 M Tris-HCl containing 10% sucrose, 5 mM $MgCl_2$, and 0.15 M NaCl at pH 7.4 and 21 °C for the period of time indicated in the figure. (B) Digestion of C3 by HLE at a molar ratio of 52:1 (C3:HLE) and at 37 °C under conditions otherwise similar to those used for the digests shown in panel A.

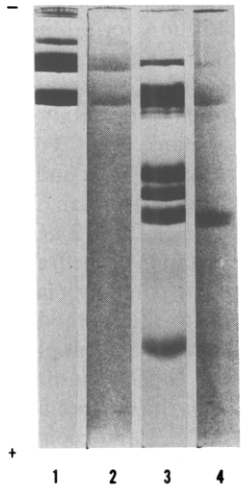


FIGURE 2: Analysis for carbohydrate in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels containing reduced C3 and a reduced HLE digest of human C3. Duplicate sets of gels were prepared for individual protein and carbohydrate staining. Gel 1: Human C3 stained for protein by amido black stain. Gel 2: Human C3 stained for carbohydrate by Schiff stain. Both the α and β chains of C3 contain stainable carbohydrate. Gel 3: HLE digest of C3 (52:1 (C3:HLE), pH 7.2 and 37 °C for 5 h) stained for protein. Gel 4: HLE digest of C3 stained for carbohydrate, demonstrating that the carbohydrate on the α chain is associated with the α3 fragment.

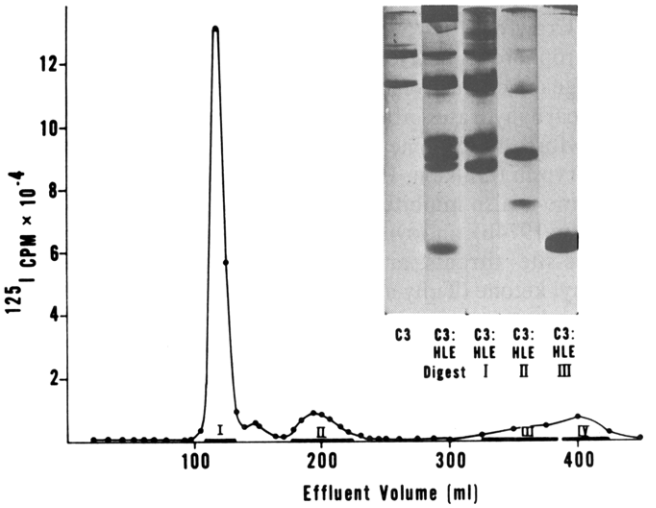


FIGURE 3: Gel filtration of an HLE digest of C3. A sample of 44 mg of C3 was digested by 142 μg of HLE at pH 7.2 and 37 °C for 3.5 h. Radiolabeled C3 ([$^{125}$I]C3, 12 αCi) was introduced initially to permit monitoring of the proteolytic fragments by radioactivity. The digestion mixture was applied to a column (4.5 × 50 cm) of Sephadex G-100 without prior chemical reduction. Elution was performed at 15 mL/h with 0.15 M sodium acetate at pH 3.7 and room temperature. The fragments recovered in each pool were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as shown in the inset. Pool I contains a complex of intact β chain and the fragments α1 and α3 as shown in the corresponding gel (C3:HLE I). Pool II contains only the α2 fragment (C3:HLE II) and pool III contains the α4 fragment (C3:HLE III).

of elastase cleavage were located under an ultraviolet light, cut out, and hydrolyzed 16 h at 110 °C in 6 N HCl. Protein bands from four gels were combined for each $NH_2$-terminal determination. The hydrolysate was dried under vacuum, dissolved in pyridine-$H_2O$ (1:1), dried again, and then extracted three times with ethyl acetate. Thin-layer chromatography of the dansyl amino acid derivative was performed as reported by Weiner et al. (1972).

COOH-terminal residues of purified fragments were identified by either carboxypeptidase A or Y digestion. The carboxypeptidase A digests were performed in 0.05 M sodium bicarbonate at pH 8.5 and 37 °C.

*Amino Acid Analysis.* Samples were placed in 6 N HCl in Pyrex tubes, and the tubes were evacuated and sealed. Samples were hydrolyzed at 110 °C for 24 h unless otherwise stated. Amino acid analyses were performed with a Beckman Autoanalyzer (Model 121) modified for two-column operation according to the procedures of Spackman et al. (1958). Analyses were also performed on protein fragments contained in polyacrylamide gel slices according to the method of Houston (1971). Each gel was freed of background amino acids, sodium dodecyl sulfate and buffer salts by electrophoresis at 150 mA in 5% acetic acid for 6 h. The protein-containing bands were then sliced from the gels and lyophilized before being hydrolyzed as described by Houston (1971).

*Protein Determinations.* Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

## Results

*C3 Fragments Generated by HLE.* HLE induces a highly selective cleavage of human C3 under nearly physiological conditions. Limited proteolysis occurs at pH 7.4, 21 °C, and C3:HLE molar ratios of 20:1. At these conditions a low-molecular-weight fragment (α4)[3] is released from the α chain of C3 (Figure 1A; 0.5–1 h), leaving a modified α chain (α′, mol wt = 100 000). Extensive digestion at 21 °C (2.0 and 3.5 h, Figure 1A) gave evidence of three additional fragments which are designated as α1, α2, and α3. Digests at 37 °C of C3 by HLE at molar ratios of 52:1 are shown in Figure 1B. The time course of digestion clearly shows that the four fragments generated (α1–α4) arise at the expense of the α chain. The β chain of human C3 appears to be resistant to proteolysis by HLE at pH 7.4, even after extended periods of incubation (e.g., 16 h at 37 °C). However, when C3 is digested by HLE at pH 8.3, nearer the pH optimum of the enzyme, the β chain is no longer resistant and both the α and β chains are degraded to numerous small fragments.

[3] The nomenclature α1, α2, α3, and α4 was adapted as a systematic means of identifying the major C3 α-chain fragments produced by HLE digestion according to their relative mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels.

TABLE I: Amino Acid Composition of the $\alpha 4$ Fragment Recovered From an HLE Digest of C3.[a]

| Amino Acid | Residues/mole[b] $\alpha 4$ | Human C3a | Δ C3a-$\alpha 4$ |
|---|---|---|---|
| Lys | 7.0 (7) | 7 | |
| His | 1.0 (1) | 2 | 1 |
| Arg | 9.3 (9-10) | 11 | 1-2 |
| Asp | 5.1 (5) | 5 | |
| Thr | 3.0 (3) | 3 | |
| Ser | 3.5 (3-4) | 4 | 0-1 |
| Glu | 9.4 (9) | 9 | |
| Pro | 2.2 (2) | 2 | |
| Gly | 3.3 (3) | 4 | 1 |
| Ala | 2.6 (2-3) | 4 | 1-2 |
| ½-Cys[c] | 5.5 (6) | 6 | |
| Val | 3.1 (3) | 3 | |
| Met | 2.7 (3) | 3 | |
| Ile | 1.9 (2) | 2 | |
| Leu | 5.1 (5) | 7 | 2 |
| Tyr | 1.8 (2) | 2 | |
| Phe | 2.9 (3) | 3 | |
| Total res | 68-71 | 77 | |

[a] The $\alpha 4$ fragment was recovered from a 3.5-h and 37 °C HLE digest of C3 after gel filtration on Sephadex G-100. The material analyzed above came from pool III of the separation shown in Figure 3. [b] Residues per mole values were based on the assumed molecular weight of 8500. The data represents an average of three separate analyses. [c] Half-cystine was determined as cysteic acid after performic acid oxidation (Moore, 1963).

TABLE II: $NH_2$- and COOH-Terminal Analyses of the $\alpha 4$ Fragment From C3 Digested with HLE.

| | Method | Res Identified | 2 h (mol/mol of $\alpha 4$) | 16 h (mol/mol of $\alpha 4$) |
|---|---|---|---|---|
| $NH_2$ terminal | Dansylation[a] | Ser | | |
| COOH terminal | Carboxypeptidase A digestion[b] | Ala | 0.8 | 1.0 |
| | | Ser | 0.4 | 0.6 |
| | | His | 0.4 | 0.5 |
| | | Glu | 0.3 | 0.4 |

Interpretation: Two molecular species of $\alpha 4$ are generated by HLE[c]

| | | |
|---|---|---|
| (1) 0.5-0.6 mol/mol | 1-68 69 70 71<br>-Ala-Arg-Ala-Ser-COOH | 1-71 $\alpha 4$ |
| (2) 0.4-0.5 mol/mol | 1-65 66 67 68<br>-Arg-Gln-His-Ala-COOH | 1-68 $\alpha 4$ |

[a] Dansylated amino acid was identified according to the method of Weiner et al. (1972). [b] Carboxypeptidase A digestion was performed at a 1% (w/w) enzyme to peptide ratio as described in the text. [c] Cleavage sites were assigned based on the known primary structure of human C3a anaphylatoxin (Hugli, 1975).

TABLE III: End-Group Analysis of C3 Fragments Generated by Human Leukocyte Elastase.

| C3 fragment | Amino terminus[a] | Carboxyl terminus[b] | Estimated mol wt |
|---|---|---|---|
| $\alpha 1$ | Lys- | -Gly-Ser | 36 000 |
| $\alpha 2$ | Ala- | (Ala,Ser)-Leu | 28 000 |
| $\alpha 3$ | Ala- | -Thr | 25 000 |
| $\alpha 4$ | Ser- | -Gln-His-Ala<br>-Ala-Ser | 8 500 |
| $\alpha'$ chain | Ala- | -Gly-Ser | 100 000 |
| $\alpha$ chain | Ser- | -Gly-Ser | 108 000 |
| $\beta$ chain | ND | -Ala | 74 000 |

[a] Residues were assigned as the dansyl derivative by thin-layer chromatographic analysis (Weiner et al., 1972). [b] Residues were assigned based on enzymatic degradation by carboxypeptidase A.

If the HLE digest of C3 at 37 °C is separated on sodium dodecyl sulfate gels in the absence of DTT or 2-mercaptoethanol only three bands are seen (not shown). A complex of the $\alpha 1$, $\alpha 3$, and $\beta$ chains is contained in a slow-moving band, with the $\alpha 2$ and $\alpha 4$ polypeptides migrating to the same positions they occupy after reduction. Molecular weight estimates were obtained for the C3 polypeptide fragments based on their relative migration in sodium dodecyl sulfate gels. The fragments were estimated to be 36 000, 28 000, and 25 000 for $\alpha 1$, $\alpha 2$, and $\alpha 3$, respectively. The $\alpha 4$ fragment was determined to be 8500 ± 500. Only the $\alpha 3$ fragment is visualized by carbohydrate staining, as illustrated in Figure 2.

Proteolytic degradation of human C3 is completely inhibited when the purified HLE is preincubated with specific elastase inhibitor, *N*-acetyl-Ala-Ala-Pro-Ala-chloromethyl ketone. These results confirm that the cleavages of C3 observed in Figure 1 are elastase specific, indicating that there are no other lysosomal proteases in the purified HLE preparation.

*Characterization of the HLE Fragments of the α Chain of C3.* A 3.5-h HLE digest of C3 at pH 7.2 and 37 °C was resolved on a Sephadex G-100 column, yielding three peaks (Figure 3). The sodium dodecyl sulfate gel patterns for DTT-reduced material corresponding to these individual peaks are illustrated in the inset. Pool I contains a complex of three components corresponding to an intact $\beta$ chain and the $\alpha 1$ and $\alpha 2$ fragments. Pool II is composed primarily of the $\alpha 2$ fragment, and pool III contains only the small $\alpha 4$ fragment. Several polypeptides were detected in pool IV by amino acid analysis and end-group analyses, but these were too small to be observed by sodium dodecyl sulfate gel analysis. Since $\alpha 1$ to $\alpha 4$ represent approximately 90% of the estimated molecular weight of the intact $\alpha$ chain, the small peptides in pool IV can comprise, at most, the remaining 10 000 molecular weight of this chain.

Chemical characterization of the $\alpha 4$ fragment from pool III clearly established that this portion of the $\alpha$ chain is structurally similar to the C3a anaphylatoxin molecule. The amino acid composition of $\alpha 4$ is given in Table I along with that of the C3a molecule for comparison. Although the compositions show a remarkable similarity, differences are seen in certain residues. Notably fewer histidine, arginine, alanine, and leucine residues were obtained for $\alpha 4$ as compared to C3a. The reason for these differences immediately became clear when the termini of $\alpha 4$ were examined. The $NH_2$-terminal residue of $\alpha 4$ was identified as a serine (Table II), the same as the $NH_2$-terminal residue in C3a. Determination of the carboxy-terminal residues of $\alpha 4$ was more complicated because several residues were released simultaneously by short-term CpA treatment (e.g., several minutes of digestion). However, longer term digests clarified these results, since fractional molar quantities of three amino acids were released even after 16 h of CpA digestion (see Table II). These data can be readily explained if the HLE is assumed to have cleaved the $\alpha$ chain at two nearby sites releasing two closely related, but distinct,

TABLE IV: Composition of Human C3 Fragments Produced by Human Leukocyte Elastase.

| Amino acid | Residues per mole[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $\alpha 1$[b] | $\alpha 2$ | $\alpha 3$[b] | $\alpha 4$ | $\alpha 1$-$\alpha 4$ | $\alpha$ chain | $\beta$ chain | $\alpha + \beta$ C3 |
| Lys | 24.7 (25) | 15.9 (16) | 15.2 (15) | 7.0 (7) | 63 | 67.9 (68) | 38.3 (38) | 106 |
| His | 4.6 (5) | 3.2 (3) | 4.1 (4) | 1.0 (1) | 13 | 15.6 (16) | 10.3 (10) | 26 |
| Arg | 11.8 (12) | 8.8 (9) | 11.0 (11) | 9.3 (9-10) | 42 | 46.8 (47) | 28.2 (28) | 75 |
| Asp | 39.0 (39) | 22.6 (23) | 20.8 (21) | 5.1 (5) | 88 | 95.1 (95) | 58.1 (58) | 153 |
| Thr[c] | 16.6 (17) | 12.6 (13) | 11.7 (12) | 3.1 (3) | 45 | 52.8 (53) | 46.9 (47) | 100 |
| Ser[c] | 17.8 (18) | 11.8 (12) | 16.0 (16) | 3.5 (3-4) | 50 | 59.8 (60) | 47.3 (47) | 107 |
| Glu | 46.5 (47) | 34.6 (35) | 26.6 (27) | 9.4 (9) | 118 | 138.0 (138) | 84.0 (84) | 222 |
| Pro | 13.1 (13) | 9.7 (10) | 12.3 (12) | 2.2 (2) | 37 | 39.8 (40) | 38.9 (39) | 79 |
| Gly | 15.3 (15) | 16.8 (17) | 6.0 (6) | 3.4 (3) | 41 | 53.7 (54) | 51.4 (51) | 105 |
| Ala | 15.7 (16) | 21.8 (22) | 9.7 (10) | 2.6 (2-3) | 51 | 59.4 (59) | 35.7 (36) | 95 |
| ½-Cystine[d] | 10.9 (11) | 3.3 (3) | 5.0 (5) | 5.8 (6) | 25 | 27.8 (28) | 8.6 (9) | 37 |
| Val | 17.8 (18) | 16.0 (16) | 22.6 (27) | 3.1 (3) | 60 | 66.0 (66) | 65.9 (66) | 132 |
| Met | 7.6 (8) | 6.0 (6) | 5.9 (6) | 2.7 (3) | 23 | 25.8 (26) | 10.5 (11) | 37 |
| Ile | 14.9 (15) | 11.1 (11) | 12.6 (13) | 1.9 (2) | 41 | 46.7 (47) | 31.9 (32) | 79 |
| Leu | 25.0 (25) | 25.6 (26) | 18.4 (18) | 5.1 (5) | 74 | 89.3 (89) | 57.4 (57) | 146 |
| Tyr | 12.1 (12) | 9.3 (9) | 5.9 (6) | 1.8 (2) | 29 | 31.1 (31) | 22.2 (22) | 53 |
| Phe | 11.7 (12) | 10.0 (10) | 8.1 (8) | 2.9 (3) | 33 | 36.0 (36) | 23.3 (23) | 59 |
| Trp[e] | 4.2 (4) | 4.0 (4) | 4.3 (4) | 0.0 (0) | 12 | 11.3 (11) | 6.4 (6) | 17 |
| Total Res | 312 | 245 | 217 | 68-71 | 845 | 964 | 664 | 1 628 |
| Protein mol wt | 36 120 | 27 565 | 24 804 | 8 400 | 96 889 | 109 048 | 73 244 | 182 292 |
| Estimated mol wt | 36 000 | 28 000 | 25 000 | 9 000 | 98 000 | 110 000 | 74 000 | 184 000 |

[a] Reported as moles of amino acid/mole of protein based on the molecular weights of components estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Each composition represents an average of no fewer than duplicate analyses. [b] Determined from polyacrylamide gel slices after sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Houston, 1971. [c] Corrected for destruction during hydrolysis. [d] Estimated as cysteic acid. [e] Tryptophan determined by the procedure of Hugli and Moore, 1972.

TABLE V: Activities of C3 Fragments Generated by Human Leukocyte Elastase.

| HLE Fragments of C3 | Analogous Serum Fragments of C3 | Act. Tested for HLE Fragment |
|---|---|---|
| $\alpha 4$ | C3a | (+) Immunological cross-reactivity with anti-human C3a<br>(−) Smooth-muscle contraction[a]<br>(−) Chemotaxis of polymorphonuclear leukocytes[b] |
| $\alpha'$, $\beta$ | C3b | 90% consumption of C3PA[c] |
| $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta$ | C3c, C3d | 14% consumption of C3PA |
| $\alpha 2$ | C3d | (+) Immunological cross-reactivity with anti-human C3d |

[a] Guinea pig ileum assay for anaphylatoxin activity (Cochrane and Müller-Eberhard, 1968). [b] In vivo analysis of directed leukocyte migration in rabbits (Otani and Hugli, 1977). [c] Assay for functional C3b includes $10^{-3}$ M $Mg^{2+}$, C3PA convertase (C3PAse) and C3 proactivator (C3PA). Following incubation with $\alpha'\beta$ the consumption of C3PA was determined.

$\alpha 4$ fragments. The proposed cleavage sites are given at the bottom of Table II and involve the linkages between Ala-Arg and Ser-His at positions 68, 69 and 71, 72 in the known primary structure of the human C3a molecule (Hugli, 1975). This conclusion is based on the CpA results, is supported by the composition data in Table I, and is generally consistent with the specificity displayed by other elastases (Powers and Tuhy, 1973).

End-group analyses of the larger HLE fragments from C3 were performed and the data are summarized in Table III. Identification of a single residue at the amino-terminal position of fragments $\alpha 1$, $\alpha 2$, and $\alpha 3$ suggests that these fragments result from unique cleavages, unlike the $\alpha 4$ COOH-terminal situation. Amino acid compositions were obtained for the individual fragments and for the intact chains of C3 (Table IV). The sum of the amino acid compositions for polypeptides $\alpha 1$ to $\alpha 4$, while not identical, compared favorably with that of the intact $\alpha$ chain. Assuming that molecular weight estimates for $\alpha$ chain and $\alpha$ fragments are valid, the difference in composition should be small.

*Biological and Immunological Activity of C3 Fragments.* Functional activities and antigenic properties of the C3 fragments isolated from HLE digests were tested. The results are summarized in Table V. The $\alpha 4$ fragment develops a strong precipitin reaction with rabbit antisera to human C3a. The precipitin line to $\alpha 4$ merges with the line formed with human C3a when examined by the double-diffusion technique (Ouchterlony, 1948), indicating near identity of the two antigens. The immunological similarity between $\alpha 4$ and C3a does not extend to the biological activity, since the $\alpha 4$ fragment is unable to induce contraction of the guinea pig ileum. The $\alpha 4$ fragment is also incapable of producing desensitization of the smooth muscle to excitation by C3a, a characteristic of anaphylatoxins known as tachyphylaxis (Jensen, 1972). Neither purified $\alpha 4$ nor the total HLE digest of C3 could stimulate directed migration of polymorphonuclear leukocytes. When introduced to plastic chambers on rabbit skin (Otani and Hugli, 1977), between concentrations of 1.0 and 10 $\mu$M for $\alpha 4$ and 5.0 and 50 $\mu$M for the 20 °C HLE digest (2 h), no chemotaxis was detected. These results provide evidence that neither the $\alpha 4$ fragment nor the HLE digest (20 °C) of C3 possesses chemotactic activity.

The remainder of the C3 molecule, after loss of C3a, is termed C3b. This portion, which serves as an accelerator of the "feedback" enzyme system for complement activation (Mül-

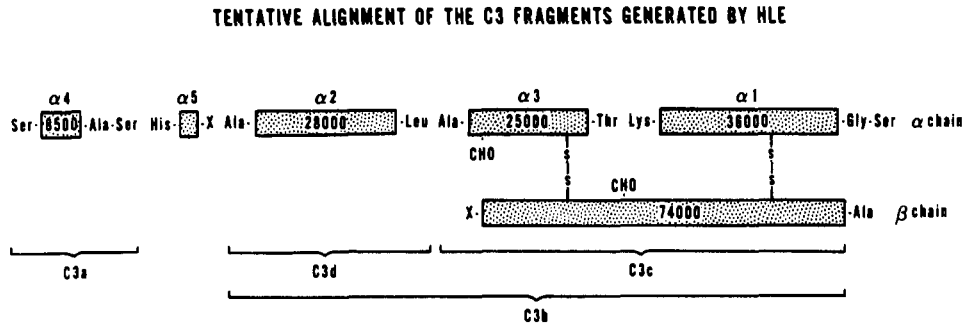


FIGURE 4: A tentative model is given for the human C3 molecule as reconstructed by aligning the major HLE cleavage products of C3. The composite fragments are described in the text. Fragment $\alpha 5$ was not characterized but is known to exist as an interconnecting polypeptide between $\alpha 4$ and $\alpha 2$ based on the primary structural analysis of human C3a. Fragments C3a, C3b, C3c, and C3d are natural catabolites of the parent C3 molecule generated by plasma enzymes.

ler-Eberhard and Götze, 1972) can be assayed indirectly by measuring its ability to stimulate C3 proactivator convertase (C3Pase) to consume the serum complement component C3 proactivator (C3PA) (Medicus et al., 1976). A 5-$\mu$g sample of C3 digested with HLE for 2 h at 20 °C, as shown in Figure 1A, stimulated a 90% consumption of C3PA after 30 min at 37 °C. The major C3 product after HLE cleavage and release of the $\alpha 4$ fragment should be structurally similar to the C3b molecule generated by complement-activating enzymes in plasma. These results confirm that the major portion of C3, consisting of an $\alpha'$ and a $\beta$ chain after limited HLE digestion, indeed functions efficiently as a C3b analogue. However, prolonged digestion of the C3 by HLE (5 h, 37 °C at 52:1 (C3:HLE)) gave only a 14% consumption of the C3PA when 5 $\mu$g of the digestion mixture was assayed. This loss in C3b-like activity agrees with a similar activity loss associated with treatment of C3b with serum C3b inactivator (Lachmann and Müller-Eberhard, 1968; Ruddy and Austen, 1971). Degradation of C3b by C3b inactivator to form C3c and C3d is analogous to the scission of C3 by HLE to form a complex composed of the $\beta$ chain and $\alpha 1$ and $\alpha 3$ fragments (C3c analogue) and the $\alpha 2$ fragment (C3d analogue). Further support for the similarity of C3d and $\alpha 2$ is provided by the fact that the isolated $\alpha 2$ polypeptide produces a precipitin reaction with rabbit anti-human C3d when tested by immunodiffusion (See Table V).

## Discussion

Proteolytic attack on human C3 by leukocyte enzymes derived from lysosomal lysates was previously described by Taubman et al. (1970). However, their study did not define the proteases responsible for C3 degradation, nor did it characterize the fragments of C3 which were formed. We have shown that a specific and restricted proteolysis of C3 is produced by purified HLE under nearly physiological conditions. A resistance of the $\alpha 1$ to $\alpha 4$ fragments and the entire $\beta$ chain of C3 to further degradation by HLE at pH 7.2–7.4 is apparently due to an inaccessibility of potentially susceptible peptide bonds. This conclusion is borne out by the extensive degradation of C3 observed when the HLE digestion is performed at pH 8.3 or when C3 has been heat denatured before digestion at pH 7.4. The HLE degradation of C3 is orderly, and an important, biologically active intermediate is formed in the course of the early lytic events. The $\alpha'\beta$ intermediate which is formed by removal of the $\alpha 4$ fragment exhibits the same biological behavior as C3b, an activated form of C3 generated by natural serum enzymes (Lachmann and Müller-Eberhard, 1968). The significance of this finding rests in the fact that C3b can participate in a "feedback" enzyme pathway (Müller-Eberhard and Götze, 1972) capable of accelerating additional turnover of C3 in serum. The initial event of C3 activation, namely, formation of a functional C3b moiety, can also be accomplished by HLE under physiological conditions. Therefore, any circulating HLE is fully capable of activating C3 if unchecked by indigenous humoral inhibitors of this leukocytic protease.

Combining chemical, biological, and immunological properties affords characterization of the major C3 fragments recovered from the HLE digests and has permitted construction of a tentative model for the C3 molecule (see Figure 4). Alignment of fragments $\alpha 1$ to $\alpha 4$ is shown and their arrangement was based primarily on end-group analysis. The primary structure of human C3a has recently been reported (Hugli, 1975) and comparison of this structure with the partial $NH_2$-terminal sequence of intact $\alpha$ chain (Tack et al., 1977) established that the anaphylatoxin portion of C3 is derived from the $NH_2$ terminus of the $\alpha$ chain. Evidence that the $\alpha 4$ fragment is indeed an analogue of C3 was provided by both the chemical analysis and the immunological behavior of this fragment. Partial chemical characterization of $\alpha 4$ also explains the absence of any biological activity associated with this C3a-like fragment. The HLE cleavage sites for generation of $\alpha 4$ from C3 occur at either position 68 or 71, several residues $NH_2$-terminal to the arginine at position 77 in the C3a molecule. This particular arginyl residue at the COOH-terminal position in C3a was shown to be essential for anaphylatoxin activity by Bokisch and Müller-Eberhard (1970). Therefore, a deletion of this arginyl residue from the covalent structure of $\alpha 4$ readily explains its biological inactivity.

The $\alpha 1$ fragment contains the same COOH-terminal residues (-Gly-Ser) as the intact $\alpha$ chain and is consequently assigned to the COOH-terminal position in the $\alpha$ chain. Fragments $\alpha 2$ and $\alpha 3$ are arbitrarily assigned, but the apparent antigenic identity between $\alpha 2$ and the C3d portion of the molecule indicates that the $\alpha 2$ fragment should be positioned adjacent to the $NH_2$-terminal anaphylatoxin analogue ($\alpha 4$). This conclusion is, in part, based on previous analyses by Bokisch et al. (1975) of the serum-derived C3a, C3c, and C3d fragments of the parent molecule and the recent chemical data of Tack et al. (1977).

The $\alpha 3$ portion of the $\alpha$ chain contains all of the stainable carbohydrate associated with this chain. Therefore, $\alpha 3$ becomes a particularly significant fragment for the investigation of functional involvement of carbohydrate on the C3 molecule. In addition, the carbohydrate on $\alpha 3$ will serve as an important marker in the future linear structural analysis of C3.

The combined molecular weight of the four fragments accounts for about 90% of the total estimated molecular weight

of the $\alpha$ chain. Several additional small fragments are therefore required to complete the $\alpha$-chain structure. One of these small fragments is predicted to have an $NH_2$-terminal histidine or arginine resulting from HLE cleavage of the linkages at positions 68–69 and 71–72 and has been identified as $\alpha 5$ in Figure 4. At least one small polypeptide must be positioned between the $\alpha 4$ and $\alpha 2$ fragments, since the $NH_2$ terminus of the $\alpha 2$ fragment does not correspond to either residues 69 or 72 of the $\alpha$ chain. Several small fragments formed by the long-term HLE digestion of C3 were detected in the low-molecular-weight fractions after gel filtration (pool IV, Figure 2). Together these account for no more than 10 000 molecular weight of polypeptide.

The specificity of porcine pancreatic elastase has been defined by Shotten (1970) as peptide bonds involving carboxyl groups of amino acids bearing uncharged, nonaromatic side chains (e.g., valine, alanine, leucine, isoleucine, glycine and serine). The substrate specificity of HLE, however, has not been determined using natural substrates. Tuhy and Powers (1973, 1975) have reported that, while HLE is inhibited by *N*-acetyl-Ala-Pro-Ala-Ala-chloromethyl ketone, porcine pancreatic elastase is unaffected. Janoff and Scherer (1968) also noted a differential inhibition of these elastases in studies using soybean trypsin inhibitor and salivary kallikrein inhibitor. This suggests that the specificity of the human leukocyte and porcine pancreatic proteases may be different. Attack by leukocyte elastase on human C3 also centers on peptide bonds involving carboxyl groups of amino acids with uncharged, nonaromatic side chains (leucine, alanine, serine, and threonine); the adjacent residue whose amino group is liberated can be either basic or neutral (e.g., arginine, lysine, histidine, or alanine). The number of cleavages studied is still too small for us to be able to make general statements about the similarity or differences between the specificity of HLE and pancreatic elastase on native proteins.

One additional conclusion is evident from the selectivity exhibited by the purified preparations of HLE employed. Since no tryptic or chymotryptic-like cleavages were observed, the absence of other proteases is implied. This result, with the highly specific cleavages which did occur, gives strong evidence of purity for the HLE preparations used.

## Acknowledgments

We thank Dr. Otto Götze for kindly performing the functional assays for C3b. The excellent technical assistance of Mrs. Stamatia Gockel is gratefully acknowledged. We thank Mrs. Carol Davis for her assistance in the preparation of this manuscript.

## References

Bokisch, V. A., Dierich, M. P., and Müller-Eberhard, H. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1989.

Bokisch, V. A., and Müller-Eberhard, H. J. (1970), *J. Clin. Invest. 49,* 2427.

Cochrane, C. G., and Müller-Eberhard, H. J. (1968), *J. Exp. Med. 127,* 371.

David, G., and Reisfeld, R. A. (1974), *Biochemistry 13,* 1014.

Goldstein, I. M., and Weissmann, G. (1974), *J. Immunol. 113,* 1583.

Houston, L. L. (1971), *Anal. Biochem. 44,* 81.

Hugli, T. E. (1975), *J. Biol. Chem. 250,* 8293.

Hugli, T. E., and Moore, S. (1972), *J. Biol. Chem. 247,* 2828.

Janoff, A. (1973), *Lab. Invest. 29,* 458.

Janoff, A., and Scherer, J. (1968), *J. Exp. Med. 128,* 1137.

Jensen, J. A. (1972), in Biological Activities of Complement, Ingram, D. G., Ed., Basel, S. Karger, p 136.

Jovin, T., Chrambach, A., and Naughton, M. A. (1964), *Anal. Biochem. 9,* 351.

Kapitany, R. A., and Zebrowsky, E. J. (1973), *Anal. Biochem. 56,* 361.

Lachmann, P. J., and Müller-Eberhard, H. J. (1968), *J. Immunol. 100,* 691.

Laemmli, U. K. (1970), *Nature (London) 227,* 680.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. L. (1951), *J. Biol. Chem. 193,* 265.

Medicus, R. G., Götze, O., and Müller-Eberhard, H. J. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 654 (abstract 2471).

Moore, S. (1963), *J. Biol. Chem. 238,* 235.

Movat, H. Z., Habal, F. M., and Macmorine, D. R. L. (1976), *Agents Actions 6,* 183.

Movat, H. Z., Steinberg, S. G., Habal, F. M., and Ranadive, N. S. (1973), *Lab. Invest. 29,* 669.

Müller-Eberhard, H. J. (1975), *Annu. Rev. Biochem. 44,* 697.

Müller-Eberhard, H. J., and Götze, O. (1972), *J. Exp. Med. 135,* 1003.

Ohlsson, K. (1971), *Scand. J. Clin. Lab. Invest. 28,* 251.

Ohlsson, K., and Olsson, I. (1974a), *Eur. J. Biochem. 42,* 519.

Ohlsson, K., and Olsson, I. (1974b), *Scand. J. Clin. Lab. Invest.* 34, 349.

Otani, A., and Hugli, T. E. (1977), *Inflammation (N.Y.) 2* (in press).

Ouchterlony, O. (1948), *Acta Pathol. Microbiol. Scand. 25,* 186.

Powers, J. C., and Tuhy, P. M. (1973), *Biochemistry 12,* 4767.

Ruddy, S., and Austen, K. F. (1971), *J. Immunol. 107,* 742.

Schmidt, W., and Havemann, K. (1974), *Hoppe-Seyler's Z. Physiol. Chem. 355,* 1077.

Shotten, D. M. (1970), *Methods Enzymol. 19,* 113.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.

Tack, B. F., Morris, S., and Prahl, J. W. (1977), *Biochemistry 16* (in press).

Tack, B. F., and Prahl, J. W. (1976), *Biochemistry 15,* 4513.

Taubman, S. B., Goldschmidt, P. R., and Lepow, I. H. (1970), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 29,* 434 Abstract.

Taubman, S. B., and Lepow, I. H. (1971), *Immunochemistry 8,* 951.

Taylor, J. C., and Crawford, I. P. (1975), *Arch. Biochem. Biophys. 169,* 91.

Tuhy, P. M., and Powers, J. C. (1975), *FEBS Lett. 50,* 359.

Ward, P. A., and Hill, I. H. (1970), *J. Immunol. 104,* 535.

Ward, P. A., and Zvaifler, N. J. (1971), *J. Clin. Invest. 50,* 606.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

Weiner, A. M., Platt, T., and Weber, K. (1972), *J. Biol. Chem. 247,* 3242.