Third Component of Human Complement: Resolution into Two Factors and Demonstration of a New Reaction Intermediate.* (25026)

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In presence of Ca^{++} and Mg^{++}, sensitized sheep erythrocytes (EA) react with C'3 deficient human serum (R3) to form a complex ($EAhuC'_A$) which exhibits activities of C'1, C'2 and C'4.† If this complex is incubated with serum containing an agent which binds Ca^{++} and Mg^{++}, thus preventing further action of C'1, C'2 and C'4, the cells are hemolyzed by C'3, and the extent of hemolysis can be used as a measure of C'3 concentration(1,2). Evidence has been accumulating, both for human(3) and guinea pig(4,5,6) systems, that more than one factor is involved in this cation independent stage of immune hemolysis. This paper describes the resolution of human C'3 into 2 factors which act sequentially in the lysis of $EAhuC'_A$, and the demonstration of a new reaction intermediate.

Materials and methods. Preparation of EA, R3, $EAhuC'_A$ and ethylenediamine tetraacetate (EDTA) buffer are described in(7). The anion exchanger used, diethylaminoethyl (DEAE) cellulose, is a commercial product containing 0.40 meq/g of ionizing groups on a Solka-Floc lattice. Optical densities were measured with Beckman Model DU spectrophotometer. The fractionation procedure, briefly outlined here, will be published in greater detail elsewhere. A preliminary, low temperature, ethanol precipitation was performed on 20 ml of pooled human serum (pH 5.6, ionic strength 0.1, alcohol 9%, serum 3%). The precipitate was dissolved in 4 ml of 0.08 M phosphate buffer, pH 7.5. Spectrophotometric assay(7) showed that approximately 10-fold enrichment of total C'3 could be achieved in this way with negligible losses. The dialysed fraction was placed on 20 x 1.1 cm DEAE cellulose column of the type developed by Peterson and Sober(8), and subjected to continuous salt concentration gradient elution at constant pH of 7.5(9). The starting buffer was 0.02 M in phosphate and 0.02 M in NaCl. Concentration of NaCl in the eluate was allowed to reach a calculated value(10) of 0.16 M, during which time (3½ hours) twenty 6 ml fractions were collected. The whole operation was carried out in a cold room at 2°C. The eluate fractions were diluted 1 → 20 in EDTA buffer and tested for C'3 by incubation with $EAhuC'_A$. None of them caused lysis. It was suspected that the C'3 had been separated into 2 distinct components, both of which were essential for lysis of $EAhuC'_A$. To test this hypothesis, all eluate fractions were recombined and assayed for C'3 in pairs. The hypothesis was confirmed when one such recombination gave rise to a high degree of lysis. The 2 components have been designated C'_a and C'_b, in accordance with recent publication by Mayer and his group(11) on hemolysis by guinea pig C'.

Results. The first component to emerge from the column, immediately after "break through," has been arbitrarily termed C'_a, and the second, emerging towards the end of elution, C'_b. Optical densities at 541 mμ of the $EAhuC'_A$ hemolysates resulting from incubation with separate and combined fractions from a typical fractionation are shown in Table I.

After separation of the two C'3 factors, formation of a complex between $EAhuC'_A$ and C'_a, and its subsequent lysis by C'_b, were demonstrated as follows: At 0°C, 50 ml aliquots of a suspension of $EAhuC'_A$ were placed in 3 tubes, A, B and C. After centrifuging at 2°C and pouring off the supernatants, the sedi-

TABLE I. Lytic Activity of Fractions C'_a and C'_b.

Test solution	Optical density of $EAhuC'_A$ hemolysate
2 ml 1 → 10 C'_a + 2 ml EDTA buffer	.047
2 ml 1 → 10 C'_b + 2 ml EDTA buffer	.049
4 ml EDTA buffer	.053
2 ml 1 → 10 C'_a + 2 ml 1 → 10 C'_b	1.19

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† C'1, C'2, C'3 and C'4 represent the 4 recognized components of complement.

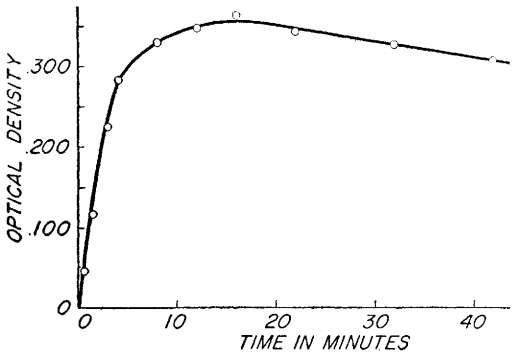

FIG. 1. Formation and decay of intermediate complex EAhuC′$_B$ by action of C′$_a$ on EAhuC′$_A$.

mented cells were resuspended by tapping tubes with the fingers. Tubes were then placed in 32°C water bath and immediately 15 ml of a 1 → 30 dilution of C′$_a$ in EDTA buffer added to A and B, and 15 ml of buffer to C. Mechanical agitation of reaction vessels was maintained during experiment. The C′$_a$ and buffer had been equilibrated at 32°C. Samples (1.5 ml) were removed at intervals and delivered into 9 ml of ice cold EDTA buffer to stop further reaction. The diluted suspensions were centrifuged in the cold within 2 minutes of sampling. After sampling and centrifugation were completed, each lot of sedimented complex from A and C was treated with 3 ml of 1 → 20 dilution of C′$_b$ in EDTA buffer and incubated 1 hour at 32°C. Those from B were incubated at 32°C for one hour with EDTA buffer alone. No samples from control vessels B and C gave any lysis above that of a "cell blank" (EAhuC′$_A$ + EDTA buffer, 1 hour at 32°C – optical density of hemolysate, 0.042). The samples from vessel A, on the other hand, gave lysis which increased rapidly with time of sampling until a maximum was reached, then slowly decreased. The lysate optical densities, corrected for "cell blank," are plotted against sampling time in Fig. 1.

Discussion. The evidence presented indicates that terminal stages of immune hemolysis in the human system may now be formulated:

$$\begin{array}{ccccccc} \text{EAhuC}'_A & \xrightarrow{C'_a} & \text{EAhuC}'_B & \xrightarrow{C'_b} & E^* & \longrightarrow & \text{ghosts} + \text{hemoglobin}, \\ \downarrow & & \downarrow & & & & \\ \text{inactive material} & & \text{inactive material} & & & & \end{array}$$

where EAhuC′$_B$ represents a new reaction intermediate, and E* a cell in a damaged state (12). These final stages, unlike conversion of EA to EAhuC′$_A$ by C′1, C′2 and C′4, require no Ca^{++} and Mg^{++}, and can thus take place in presence of EDTA. Decay of EAhuC′$_A$ to inactive material is known to occur rapidly at 32°C(1) and, unless massive concentrations of C′$_a$ are present, only a relatively small proportion is likely to be converted to EAhuC′$_B$. Decay of EAhuC′$_B$, on the other hand, is comparatively slow (Fig. 1), and it seems probable that most of the complex is converted to E* by C′$_b$ under normal conditions of hemolysis.

Solutions of C′$_b$ have been found to retain their activity over periods of several weeks if kept at –70°C. Solutions of C′$_a$ are less stable, but decomposition may be delayed by addition of gelatin to final concentration of 0.05%. Separation of C′3 into 2 factors and demonstration of the intermediate EAhuC′$_B$ promises to simplify greatly the task of investigating the mechanism of terminal stages of hemolysis.

Summary. Human C′3 has been resolved into 2 factors, C′$_a$ and C′$_b$. These factors acted in the order C′$_a$, C′$_b$, in the conversion of EAhuC′$_A$ to E*. Formation and decay of a new intermediate complex between sensitized erythrocytes and human complement, EAhuC′$_B$ has been demonstrated.

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