STUDIES ON IMMUNE CELLULAR INJURY

II. Functional Role of C'1 Esterase in Immune Cytotoxicity*

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It was demonstrated in the preceding paper (1) that incubation of primary isolates of normal human amnion cells with specific rabbit antibody and normal human serum resulted in immune cellular injury as measured by two independent criteria of cytotoxicity. It was further shown that the factors in normal human serum which were required in addition to specific antibody for production of cytotoxicity were indistinguishable from components of hemolytic complement and calcium and magnesium ions. Thus, no fundamental differences were apparent between requirements for immune injury of human amnion cells (immune cytotoxicity) and immune injury of sheep or human erythrocytes (immune hemolysis) (2, 3).

The sequence of action of the components of complement in immune hemolysis was deduced by Pillemer, Seifter, and Ecker (4). In further dissection of this complex system, Mayer, Levine, and their associates showed that early events in immune hemolysis involved reactions between the sensitized erythrocyte and the first and fourth components of complement (C'1 and C'4) in the presence of Ca^{++}. The resulting complex could then react with the second component of complement (C'2) in the presence of Mg^{++}, forming a complex which could react with the third component (C'3) in the absence of divalent cations. The latter reaction effected injury of the erythrocyte leading to hemolysis (2). The biochemical as opposed to the descriptive events in immune hemolysis were unknown.

A large body of evidence has accumulated from this laboratory and independently from Becker's laboratory that C'1 is a proenzyme which may be activated by antigen-antibody reactions (5). The active enzyme is an esterase (C'1 esterase) capable of hydrolyzing certain synthetic amino acid esters, such as *N*-acetyl-L-tyrosine ethyl ester and *p*-toluenesulfonyl-L-arginine methyl

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ester, and of interacting with C'4 and possibly C'2. Human and various other serums contain a naturally occurring inhibitor, a heat-labile α_2-globulin, which can block, under appropriate conditions, both the esterolytic and complement-interacting properties of C'1 esterase (6–9). The inhibitor, partially purified from human serum by ammonium sulfate fractionation and column chromatography, contains only small amounts of serum trypsin and plasmin inhibitors (8). The relative specificity of such purified preparations of inhibitor provided an important reagent for studies on the functional role of C'1 esterase in various immune phenomena requiring complement.

Using partially purified preparations of human C'1 esterase (10) and human serum inhibitor of C'1 esterase (8) in appropriately separated reactions, it has been possible to demonstrate that a reaction between antigen-antibody and C'1 esterase is the first detectable event in complement-fixation (9) and immune human hemolysis (Donath-Landsteiner reaction described in reference 3). The present paper is concerned with the initiating role of human C'1 esterase in immune cytotoxicity with normal human amnion cells and specific rabbit antibody, using uptake of trypan blue as the index of cell death. The results of these experiments represent a partial biochemical definition of one step of immune cellular injury.

Materials and Methods

Human C'1 Esterase.—C'1 was prepared from pooled human serum and activated to C'1 esterase by published procedures (10). The preparation, at pH 7.4, ionic strength 0.15, was adsorbed at 1° with an equal volume of human erythrocytes (blood group AB, Rh-positive), centrifuged free of all insoluble material, diluted in barbital buffer of pH 7.4, ionic strength 0.15 to a concentration of 10 units per ml., and stored in small aliquots at −20°. Activity and solubility were unimpaired by storage for at least 1 month. One unit of C'1 esterase is defined as that amount of enzyme which will liberate 0.5 micromol of titratable acid during incubation for 15 minutes at 37° with *N*-acetyl-L-tyrosine ethyl ester under standardized conditions of assay (6, 7). The preparation of C'1 esterase used contained 30 μg. of nitrogen per unit of enzyme.

Human Serum Inhibitor of C'1 Esterase.—Inhibitor was prepared by chromatography on a Dowex-2 chloride column of the supernatant from precipitation of human serum with 40 per cent ammonium sulfate (8). The final preparation, at pH 7.4, ionic strength 0.15, was stored in small aliquots at −20° at a concentration of about 30 units per ml. Activity and solubility were unimpaired by storage for at least several months. One unit of inhibitor stoichiometrically inhibits the esterolytic activity of 10 units of C'1 esterase under standardized conditions of assay (7). The preparation of inhibitor used contained 8 μg. of nitrogen per unit.

All other pertinent materials and methods are described in the preceding paper (1). R1 and R2, the human serum reagents deficient respectively in C'1 and C'2, were prepared only by the method of dialysis.

Design of Experiments.—Immune cytotoxicity was separated into the following reaction stages. All centrifugations were performed at 1000 r.p.m. (205 *G*).

Reaction I.—1.4×10^5 human amnion cells were sensitized with rabbit anti-human amnion γ_2-globulin at 22°, centrifuged, and washed with mixture 199 containing 10 per cent

complement-depleted human serum, as described in the preceding paper (1). The amount of γ_2-globulin used corresponded to 60 μg. of nitrogen.

Reaction II.—The sensitized amnion cells were reacted for 30 minutes at 15° with 0.5 ml. of a dilution of human C'1 esterase in mixture 199 containing 10 per cent complement-depleted human serum and then centrifuged and washed with 0.5 ml. of the same diluent at 15°. The time and temperature of reaction were selected on the basis of parallel experiments on the interaction of pneumococcal specific polysaccharide, Type III-rabbit anti-Type III aggregates with human C'1 esterase. These experiments showed that interaction occurred within a few minutes at low temperatures and was progressively inhibited at temperatures greater than 22° (9). The choice of 15° for this reaction with amnion cells was an arbitrary compromise between conditions favoring interaction and avoiding cold injury of the cells.

Barbital buffer of pH 7.4, ionic strength 0.15, with or without added Ca^{++} and Mg^{++}, was used as diluent in experiments involving the use of salts of EDTA. The sensitized amnion cells were reacted for 30 minutes at 15° with 0.5 ml. of a solution in barbital buffer of C'1 esterase and EDTA of indicated concentrations and then centrifuged and washed with 0.5 ml. of barbital buffer at 15°. This substitution of diluents was made because of previous findings that mixture 199 interfered with the chelation of Ca^{++} by EDTA (1).

For experiments on the effect of premixing C'1 esterase with serum inhibitor of C'1 esterase, the enzyme and inhibitor were incubated together in barbital buffer without added Ca^{++} and Mg^{++} for 10 minutes at 37° at concentrations 5-fold greater than desired. The mixtures were then diluted 1/5 in mixture 199 containing 10 per cent complement-depleted human serum, cooled to 15°, reacted with sensitized amnion cells, and centrifuged and washed as described above.

The washed product of reaction II, is designated, for convenience, as an amnion cell-antibody-(C'1 esterase) complex.

Reaction IIA.—This reaction was performed only in experiments designed to test the effect of serum inhibitor of C'1 esterase on amnion cell-antibody-(C'1 esterase) complexes; that is, on the product of reaction II. Such complexes were incubated under various conditions of time and temperature with 0.5 ml. of dilutions of inhibitor in mixture 199 containing 10 per cent complement-depleted human serum and then centrifuged and washed at room temperature with 0.5 ml. of the same diluent.

Reaction III.—Products of reactions II or IIA were incubated for 30 minutes at 37° with 0.5 ml. of indicated amounts of various human serum reagents diluted in mixture 199. Barbital buffer with added Ca^{++} and Mg^{++} was substituted for mixture 199 in experiments involving the use of salts of EDTA. Although reaction III was treated operationally as a single step, it was recognized that it was a composite of several reactions requiring, as will be shown below, the participation of C'2, C'3, C'4, and Mg^{++}.

Uptake of Trypan Blue.—At the completion of reaction III, 0.25 ml. of 1 per cent trypan blue was added at once to the total reaction mixture. The tube was gently agitated for 2 minutes and the per cent of stained cells estimated as described in the preceding paper (1).

Controls.—Appropriate diluents were substituted for the various reagents used at each reaction stage. These controls were otherwise manipulated in exactly the same manner as experimental mixtures.

EXPERIMENTAL

I. Effect of Concentration of C'1 Esterase on Immune Cytotoxicity.—Amnion cells sensitized with antibody were reacted with various dilutions of human C'1 esterase, centrifuged, and washed, as described above. The resulting complexes were then incubated with 3 units of R1 as a source of C'2, C'3, and

C'4. Cytotoxicity, as measured by uptake of trypan blue, was observed only when cells were exposed to antibody, C'1 esterase, and R1 (Table I). If any one of these constituents was omitted, detectable cellular injury did not occur. The minimal concentration of C'1 esterase effecting optimal cytotoxicity under the conditions of this experiment was 0.02 unit, corresponding to about 0.6 μg. of nitrogen in the preparation employed. It is emphasized that C'1 esterase did not contain hemolytic C'1 activity but was nevertheless capable of preparing sensitized amnion cells for injury by R1.

TABLE I

Effect of Concentration of C'1 Esterase on Immune Cytotoxicity

Reaction I (22°, 10 min.)		Reaction II (15°, 30 min.)	Reaction III (37°, 30 min.)	Cytotoxicity
Amnion cells	Antibody*	C'1 esterase	R1	Stained cells
No./0.25 ml.	*dilution/0.25 ml.*	*unit/0.5 ml.*	*unit/0.5 ml.*	*per cent*
1.4×10^5	1/20	1.0	3	89
1.4×10^5	1/20	0.25	3	91
1.4×10^5	1/20	0.06	3	90
1.4×10^5	1/20	0.04	3	96
1.4×10^5	1/20	0.02	3	90
1.4×10^5	1/20	0.01	3	65
1.4×10^5	1/20	0.005	3	58
1.4×10^5	1/20	0.0025	3	29
1.4×10^5	1/20	0.0013	3	19
1.4×10^5	1/20	0	0	6
1.4×10^5	1/20	0	3	12
1.4×10^5	1/20	1.0	0	11
1.4×10^5	0	1.0	3	12

* CF titer, 1/384; see previous paper (1).

II. Requirement for C'2, C'3, and C'4 for Immune Cytotoxicity Following Interaction of Cell-Antibody Complex with C'1 Esterase. Amnion cells sensitized with antibody were reacted with 0.05 unit of C'1 esterase in the usual manner and then incubated with 3 units of R1, R2, R3, or R4. Significant cytotoxicity, compared with controls, occurred only when reaction III was performed in the presence of C'2, C'3, and C'4 (R1) (Table II). It had been demonstrated previously that C'1, C'2, C'3, and C'4 were required for immune cytotoxicity (1). This experiment therefore indicated that C'1 esterase could be substituted for C'1 and that R1 was a satisfactory source of C'2, C'3, and C'4.

III. Effect of Various Concentrations of C'1 Esterase, Heat-Inactivated C'1 Esterase, and R1 on Immune Cytotoxicity.—Amnion cells sensitized with antibody were reacted with 0.05 or 0.5 unit of C'1 esterase or heat-inactivated C'1

esterase (56°, 30 minutes) in the usual manner and then incubated with various concentrations of R1. The results (Table III) demonstrated that heat-inactivated esterase, which is without esterolytic and complement-interacting properties, was inactive in preparing sensitized amnion cells for immune injury. The data also showed that the concentration of R1 rather than of C'1 esterase was the limiting factor in producing cytotoxicity under conditions of the experiment. The extent of uptake of trypan blue increased with increasing concentration of R1 but was not affected by increasing the concentration of C'1 esterase in reaction II. Optimal cytotoxicity was achieved with either 0.05 or 0.5 unit of

TABLE II

Requirement for C'2, C'3, and C'4 for Immune Cytotoxicity Following Interaction of Cell-Antibody Complex with C'1 Esterase

Reaction I (22°, 10 min.)		Reaction II (15°, 30 min.)	Reaction III (37°, 30 min.)	Cytotoxicity
Amnion cells	Antibody	C'1 esterase	Complement reagent	Stained cells
No./0.25 ml.	*dilution/0.25 ml.*	*unit/0.5 ml.*	*units/0.5 ml.*	*per cent*
1.4×10^5	1/20	0.05	3 units R1	97
1.4×10^5	1/20	0.05	3 units R2	13
1.4×10^5	1/20	0.05	3 units R3	6
1.4×10^5	1/20	0.05	3 units R4	5
1.4×10^5	1/20	0	3 units R1	18
1.4×10^5	1/20	0	3 units R2	15
1.4×10^5	1/20	0	3 units R3	7
1.4×10^5	1/20	0	3 units R4	5
1.4×10^5	1/20	0	0	8
1.4×10^5	1/20	0.05	0	7

esterase in reaction II and 3 units of R1 in reaction III. This amount of R1 frequently caused a small amount of cytotoxicity in controls unexposed to C'1 esterase, as shown in Table III.

IV. Sequence of Reactants Required for Immune Cytotoxicity.—These experiments were designed to test the effect of varying the order in which antibody, C'1 esterase and R1 were reacted with human amnion cells. Individual reactants were added to cells in individual reaction stages in sequences indicated in Table IV. Following each reaction, the cells were centrifuged and the sediments washed with 0.5 ml. of mixture 199 containing 10 per cent complement-depleted human serum. Reactions involving antibody (0.25 ml. of 1/20 dilution of rabbit anti-human amnion γ_2-globulin) were performed at 22° for 10 minutes; R1 (3 units), 37° for 30 minutes; C'1 esterase (0.1 unit), 15° for 30 minutes,

TABLE III

Effect of Various Concentrations of C'1 Esterase, Heat-Inactivated C'1 Esterase, and R1 on Immune Cytotoxicity

Reaction I (22°, 10 min.)		Reaction II (15°, 30 min.)	Reaction III (37°, 30 min.)	Cytotoxicity
Amnion cells	Antibody	C'1 esterase	R1	Stained Cells
No./0.25 ml.	*dilution/0.25 ml.*	*unit/0.5 ml.*	*units/0.5 ml.*	*per cent*
1.4×10^5	1/20	0.05	1	31
1.4×10^5	1/20	0.05	2	75
1.4×10^5	1/20	0.05	3	89
1.4×10^5	1/20	0.05	4	95
1.4×10^5	1/20	0.5	1	26
1.4×10^5	1/20	0.5	2	79
1.4×10^5	1/20	0.5	3	92
1.4×10^5	1/20	0.5	4	96
1.4×10^5	1/20	0.05—heated*	3	18
1.4×10^5	1/20	0.5—heated	3	16
1.4×10^5	1/20	0	0	7
1.4×10^5	1/20	0.05	0	10
1.4×10^5	1/20	0.5	0	6
1.4×10^5	1/20	0	2	11
1.4×10^5	1/20	0	3	19
1.4×10^5	1/20	0	4	25

* C'1 esterase previously heated for 30 minutes at 56°.

TABLE IV

Sequence of Reactions Required for Immune Cytotoxicity

Order of reactants*	Cytotoxicity
	Stained cells
	per cent
Cell + antibody + C'1 esterase + R1	87
Cell + diluent + C'1 esterase + R1	12
Cell + antibody + C'1 esterase + diluent	9
Cell + antibody + diluent + R1	10
Cell + C'1 esterase + antibody + R1	6
Cell + antibody + R1 + C'1 esterase	14

* For explanation, see text.

except when C'1 esterase was used as the final reactant and the reaction was performed at 37° for 30 minutes.

Immune cytotoxicity was observed only when the amnion cells were first reacted with antibody, then with C'1 esterase, and finally with R1 (Table IV).

Omission of any one of these reactants or variation of the sequence of reactants resulted in absence of cytotoxicity. It was concluded that C'1 esterase could react with amnion cells only after the cell had reacted with antibody and that only the product of the reaction between sensitized amnion cells and C'1 esterase was susceptible to injury by C'2, C'3, and C'4.

V. Requirement for Ca^{++} for Formation of Amnion Cell-Antibody-(C'1 Esterase) Complex.—Amnion cells sensitized with antibody were reacted in the usual manner with 0.05 unit of C'1 esterase in the presence and absence of 5×10^{-3} M sodium, magnesium, or calcium salts of EDTA. The salts of EDTA, at pH 7.4

TABLE V

Requirement for Ca^{++} for Formation of Amnion Cell-Antibody-(C'1 Esterase) Complex

Reaction I (22°, 10 min.)		Reaction II (15°, 30 min.)*		Reaction III (37°, 30 min.)	Cytotoxicity
Amnion cells	Antibody	C'1 esterase + EDTA		R1	Stained cells
No./0.25 ml.	*dilution/ 0.25 ml.*	*unit/0.5 ml.*	*5×10^{-3} M*	*units/0.5 ml.*	*per cent*
1.4×10^5	1/20	0.05	—	3	75
1.4×10^5	1/20	0.05	Na_3 EDTA	3	16
1.4×10^5	1/20	0.05	Na_2Mg EDTA	3	19
1.4×10^5	1/20	0.05	Na_2Ca EDTA	3	88
1.4×10^5	1/20	0	—	0	16
1.4×10^5	1/20	0	—	3	9
1.4×10^5	1/20	0.05	—	0	4
1.4×10^5	1/20	0	Na_3 EDTA	3	11
1.4×10^5	1/20	0	Na_2Mg EDTA	3	9
1.4×10^5	1/20	0	Na_2Ca EDTA	3	3

* Barbital buffer without added Ca^{++} and Mg^{++} used as diluent and for washing of complexes; see text.

and ionic strength 0.15, and the C'1 esterase were diluted in barbital buffer. The product of the reaction was washed with 0.5 ml. of barbital buffer and then incubated with 3 units of R1 diluted in mixture 199. Cytotoxicity was inhibited by both the sodium and magnesium salts of EDTA but not by the calcium salt of EDTA (Table V). None of the salts of EDTA was cytotoxic in the absence of C'1 esterase. These results were consistent with a requirement for Ca^{++} for the formation of amnion cell-antibody-(C'1 esterase) complex.

VI. Requirement for Mg^{++} for Cytotoxic Activity of R1 on Amnion Cell-Antibody-(C'1 Esterase) Complex.—Sensitized amnion cells were reacted in the usual manner with 0.1 unit of C'1 esterase diluted in mixture 199 containing 10 per cent complement-depleted human serum. The washed complexes were then incubated with 3 units of R1 in the presence and absence of 5×10^{-3} M sodium, magnesium, or calcium salts of EDTA, using barbital buffer as diluent. Cyto-

toxicity was inhibited only by the sodium salt of EDTA (Table VI). These results were consistent with a requirement for Mg^{++} for the cytotoxic activity of R1 on amnion cell-antibody-(C'1 esterase) complexes. The reaction with R1, operationally treated as a single reaction, was a resultant of a series of reactions involving several components of complement. No attempt was made to determine the precise locus of action of Mg^{++} in this reaction sequence.

VII. Inhibition of Immune Cytotoxicity by Human Serum Inhibitor of C'1 Esterase.—A. Effect of Premixing C'1 Esterase and Inhibitor.—At this point in the investigation, it had been shown that a partially purified preparation of

TABLE VI

Requirement for Mg^{++} for Cytotoxic Activity of R1 on Amnion Cell-Antibody-(C 1 Esterase) Complex

Reaction I (22°, 10 min.)		Reaction II (15°, 30 min.)	Reaction III (37°, 30 min.)*		Cytotoxicity
Amnion cells	Antibody	C'1 esterase	R1 + EDTA		Stained cells
No./0.25 ml.	*dilution/0.25 ml.*	*unit/0.5 ml.*	*units/0.5 ml.*	*$5 \times 10^{-2} M$*	*per cent*
1.4×10^5	1/20	0.1	3	—	96
1.4×10^5	1/20	0.1	3	Na_3 EDTA	5
1.4×10^5	1/20	0.1	3	Na_2Mg EDTA	90
1.4×10^5	1/20	0.1	3	Na_2Ca EDTA	94
1.4×10^5	1/20	0	0	—	9
1.4×10^5	1/20	0	3	—	12
1.4×10^5	1/20	0.1	0	—	8
1.4×10^5	1/20	0.1	0	Na_3 EDTA	7
1.4×10^5	1/20	0.1	0	Na_2Mg EDTA	8
1.4×10^5	1/20	0.1	0	Na_2Ca EDTA	5

* Barbital buffer with added Ca^{++} and Mg^{++} used as diluent; see text.

human C'1 esterase could participate in a reaction leading to immune cytotoxicity. The identity of an active constituent in this preparation with C'1 esterase rested on two major observations: (*a*) the preparation could be substituted for C'1 and prepared the sensitized amnion cell for injury by C'2, C'3, and C'4; and (*b*) the activity of the preparation in immune cytotoxicity, as well as in esterolysis and ability to interact with complement, was destroyed during heating for 30 minutes at 56°. Additional evidence for the functional role of C'1 esterase in immune cytotoxicity was obtained in experiments which utilized partially purified human serum inhibitor of C'1 esterase as an agent for blocking the effect of C'1 esterase.

Enzyme and inhibitor or heat-inactivated inhibitor (60°, 30 minutes) were diluted in barbital buffer, mixed at concentrations 5-fold greater than desired,

and incubated for 10 minutes at 37°. The mixtures were then diluted 1/5 in mixture 199 containing 10 per cent complement-depleted human serum, cooled to 15°, reacted with sensitized amnion cells in the usual manner, and then incubated with 3 units of R1. Partially purified inhibitor in sufficient concentration completely inhibited the ability of 0.05 unit of C'1 esterase to prepare sensitized amnion cells for immune cytotoxicity (Table VII). If stoichiometric interaction of enzyme and inhibitor were valid at the low concentrations employed in this

TABLE VII

Inhibition of Immune Cytotoxicity by Human Serum Inhibitor of C'1 Esterase: Effect of Premixing C'1 Esterase and Inhibitor

Reaction I (22°, 10 min.)		Reaction II (15°, 30 min.)	Reaction III (37°, 30 min.)	Cytotoxicity
Amnion cells	Antibody	C'1 Esterase(E) + Inhibitor (EI)	R1	Stained cells
No./0.25 ml.	*dilution/0.25 ml*	*unit/0.5 ml. unit/0.5ml.*	*units/0.5 ml.*	*per cent*
1.4×10^5	1/20	0.05 E	3	93
1.4×10^5	1/20	0.05 E + 0.0025 EI	3	71
1.4×10^5	1/20	0.05 E + 0.0075 EI	3	44
1.4×10^5	1/20	0.05 E + 0.01 EI	3	24
1.4×10^5	1/20	0.05 E + 0.02 EI	3	22
1.4×10^5	1/20	0.05 E + 0.04 EI	3	9
1.4×10^5	1/20	0.05 E + 0.08 EI	3	10
1.4×10^5	1/20	0.05 E + 0.04 EI-heated*	3	86
1.4×10^5	1/20	0.05 E + 0.08 EI-heated	3	82
1.4×10^5	1/20	—	0	7
1.4×10^5	1/20	—	3	9
1.4×10^5	1/20	0.05 E	0	6

* Inhibitor previously heated for 30 minutes at 60°.

experiment, the amount of inhibitor for complete inhibition should have been 0.005 unit (0.04 μg. of nitrogen). However, at this level of inhibitor, only partial inhibition was observed. Complete inhibition was achieved at a concentration of inhibitor of 0.04 unit, corresponding to 0.32 μg. of nitrogen in the preparation of partially purified inhibitor employed. Heat-inactivated inhibitor at concentrations of 0.04 and 0.08 unit was inactive or only slightly active in blocking the functional activity of C'1 esterase.

B. Effect of Incubating Amnion Cell-Antibody-(C'1 Esterase) Complexes with Inhibitor.—The preceding experiment demonstrated that it was possible to block the functional activity of C'1 esterase in immune cytotoxicity by incubation of the enzyme with serum inhibitor of C'1 esterase prior to interaction with

sensitized amnion cells. Incubation of preformed amnion cell-antibody-(C'1 esterase) complexes with inhibitor could also diminish or prevent the subsequent cytotoxic activity of R1.

Sensitized amnion cells were reacted with 0.1 unit of C'1 esterase in the usual manner and then incubated for 60 minutes at 22° with various dilutions of partially purified human serum inhibitor of C'1 esterase. After centrifugation and washing, the complexes were further reacted with 3 units of R1. The susceptibility to cytotoxicity of the amnion cell-antibody-(C'1 esterase) complexes de-

TABLE VIII

Inhibition of Immune Cytotoxicity by Human Serum Inhibitor of C'1 Esterase: Effect of Incubating Amnion Cell-Antibody-(C'1 Esterase) Complexes with Inhibitor

Reaction I (22°, 10 min.)		Reaction II (15°, 30 min.)	Reaction IIA (22°, 60 min.)	Reaction III (37°, 30 min.)	Cytotoxicity
Amnion cells	Antibody	C'1 esterase	Inhibitor	R1	Stained cells
No./0.25 ml.	*dilution/ 0.25 ml.*	*unit/0.5 ml.*	*unit/0.5 ml.*	*units/0.5 ml.*	*per cent*
1.4×10^5	1/20	0.1	0	3	83
1.4×10^5	1/20	0.1	0.06	3	66
1.4×10^5	1/20	0.1	0.12	3	59
1.4×10^5	1/20	0.1	0.25	3	47
1.4×10^5	1/20	0.1	0.5	3	33
1.4×10^5	1/20	0.1	1.0	3	23
1.4×10^5	1/20	0.1	1.0—heated*	3	77
1.4×10^5	1/20	0	0	0	4
1.4×10^5	1/20	0	0	3	8
1.4×10^5	1/20	0.1	0	0	7
1.4×10^5	1/20	0	1.0	0	6
1.4×10^5	1/20	0	1.0	3	8

* Inhibitor previously heated for 30 minutes at 60°.

creased during incubation with increasing concentration of inhibitor (Table VIII). More than 70 per cent inhibition was effected by 1.0 unit of inhibitor under the conditions of this experiment. The same concentration of heat-inactivated inhibitor had only slight or absent inhibitory activity.

DISCUSSION

It had been shown previously that specific antibody and the components of hemolytic complement were required for immune cytotoxicity with primary isolates of normal human amnion cells (1). These observations have been extended to an initial separation of reaction stages which are summarized in the following schematic sequence:

I. Human amnion cell + rabbit γ_2-globulin antibody $\rightarrow$ Amnion cell-antibody

II. Amnion cell–antibody + human C'1 esterase $\xrightarrow{Ca^{++}}$ Amnion cell–antibody-(C'1 esterase)

III. Amnion cell–antibody-(C'1 esterase) + C'2 + C'3 + C'4 $\xrightarrow{Mg^{++}}$ Cell death

Attention has been directed primarily to reaction II and the initiation of complement action in immune cytotoxicity by C'1 esterase. Evidence for this functional role of C'1 esterase has been obtained from several observations:

1. The requirement for C'1 in immune cytotoxicity (1) was fulfilled by partially purified preparations of human C'1 esterase which were devoid of hemolytic C'1 activity by the usual methods of assay (5). The amount of enzyme required was less than 1 μg. of nitrogen in an impure fraction.

2. Inactivation of the enzyme by heating for 30 minutes at 56° abolished the activity in reaction II.

3. Incubation of C'1 esterase with a partially purified preparation of human serum inhibitor of C'1 esterase prevented the formation of an active amnion cell-antibody-(C'1 esterase) complex. The amount of inhibitor required was greater than the predicted stoichiometric quantity for the enzyme concentration employed. However, partial inhibition of C'1 esterase was observed with 0.04 μg. and complete inhibition with 0.32 μg. of inhibitor nitrogen in the presence of excess enzyme.

4. Incubation of an active amnion cell-antibody-(C'1 esterase) complex with partially purified inhibitor greatly diminished the extent of reaction III. Relatively high concentrations of inhibitor were required for this purpose.

5. Inactivation of the inhibitor by heating for 30 minutes at 60° also abolished nearly completely its inhibitory activity in preventing the formation of an active amnion cell-antibody-(C'1 esterase) complex or in blocking the activity of a pre-formed active complex.

6. The formation of an active amnion cell-antibody-(C'1 esterase) complex required the presence of Ca^{++}, an ion known to be required for immune cytotoxicity (1) and for the interaction of other antigen-antibody complexes with C'1 (2, 3, 5).

A key line of evidence for the functional activity of C'1 esterase was derived from experiments on inhibition of immune cytotoxicity by partially purified preparations of a human serum inhibitor of C'1 esterase. The inhibitor, a naturally occurring heat-labile, α_2-globulin, stoichiometrically inhibits the esterolytic activity of relatively high concentrations of C'1 esterase (7, 8). Because of limitations of sensitivity of the esterolytic assay for C'1 esterase, no information is available as yet on the stoichiometry of interaction of enzyme and inhibitor at very low concentration. The failure to achieve complete inhibition of immune cytotoxicity with concentrations of inhibitor which were *calculated* to be stoichiometrically equivalent to the small amount of enzyme employed in the experiments reported here (Table VII) cannot therefore be interpreted rigorously

at this time. However, partial inhibition could be achieved under these conditions with only 40 millimicrograms of inhibitor nitrogen in a fraction with only small amounts of trypsin and plasmin inhibitors. This suggested strongly that inhibition of immune cytotoxicity was indeed a result of inhibition of C'1 esterase. Assuming a molecular weight of 100,000 and a nitrogen to protein factor of 6.25, partial inhibition was observed at a protein concentration of 5×10^{-9} M in a non-homogeneous preparation.

It is emphasized that this profound effect of the serum inhibitor could only be demonstrated by appropriate separation of reactions. When complement action was permitted to occur in a single reaction stage, as in the previous study (1), immune cytotoxicity proceeded in the presence of normal human serum which contains an average of 6.4 units of inhibitor per ml. (7). An explanation for this apparent paradox is emerging from kinetic studies of the relative rates of reaction of low concentrations of C'1 esterase with inhibitor and with C'4 (9). These studies, still in progress, suggest that further action of complement (interaction of C'1 esterase with C'4) proceeds at a much faster rate than inhibition of C'1 esterase by serum inhibitor at enzyme concentrations used in the present investigation. They also indicate that the rate of interaction of enzyme and inhibitor decreases with decreasing concentration of either reactant. Since the actual concentration of C'1 esterase present on amnion cell-antibody-(C'1 esterase) complexes is not known but is probably less than the concentration of enzyme used for reaction, these data perhaps account for the relatively high concentration of inhibitor required to block the activity of amnion cell-antibody-(C'1 esterase) complexes (Table VIII).

No attempt has been made to determine the sequence of action of the components C'2, C'3 and C'4 in reaction III. It was demonstrated, however, that all of these components and Mg^{++} were required for completion of reaction III and that the sequence of reactions I, II and III could not be varied without loss of cytotoxicity. It would be inferred from previous studies of immune hemolysis that C'4, C'2, and C'3 acted in that order and that Mg^{++} was involved in the action of C'2 (2).

It is noteworthy that in several systems which have been studied, an early event in the action of complement has been found to be the formation of antigen-antibody-(C'1 esterase) complexes. Initiation of complement activity in immune cytotoxicity, immune human hemolysis (3), immune hemolysis by guinea pig complement of sheep erythrocytes sensitized with rabbit antibody (11–14), and complement-fixation (9) appears to be dependent on activation of a proenzyme (C'1) and subsequent action of the active enzyme (C'1 esterase). Diverse functions of complement would seem therefore to follow mechanisms of action which at least initially are identical and for which a partial biochemical explanation is available.

SUMMARY

An initial separation of reaction stages in immune cytoxicity is described. Primary isolates of normal human amnion cells were reacted first with specific rabbit γ_2-globulin antibody, then with human C'1 esterase in the presence of Ca^{++}, and finally with human C'2, C'3, and C'4 in the presence of Mg^{++}. Cytotoxicity, as measured by uptake of trypan blue, occurred only when these reactions were performed in the order given and did not occur when any of the constituents was omitted. Inhibition of immune cytotoxicity was achieved with partially purified preparations of human serum inhibitor of C'1 esterase under certain experimental conditions.

The data are discussed in relation to the role of C'1 esterase in initiating complement action and the partial biochemical definition of an early event in immune cytotoxicity.

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