STUDIES ON IMMUNE CELLULAR INJURY

I. Cytotoxic Effects of Antibody and Complement*

By ANN ROSS,‡ M.D., and IRWIN H. LEPOW,§ M.D.

(From the Institute of Pathology, Western Reserve University, Cleveland)

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The requirement for a group of normal serum factors, collectively designated as complement, for injury or destruction of cells sensitized with antibody has been recognized for more than 70 years (1). The participation of complement in immune destruction of erythrocytes (immune hemolysis) has been most intensively studied, primarily because of technical feasibility. Considerable attention has also been given to the destruction of Gram-negative bacteria by antibody and complement (immune bacteriolysis). Although some evidence is available that normal serum factors resembling complement also participate with antibody in the killing of mammalian cells other than erythrocytes (immune cytotoxicity) (2–13), this activity of complement has been least clearly defined.

Investigation of the phenomenon of immune cytotoxicity is of fundamental biological interest from the standpoints of general mechanisms of cellular injury and of specific mechanisms of tissue damage in hypersensitivity states. Although an ultimate aim of such investigation is elucidation of mechanisms of immune injury of human cells *in vivo*, it appeared desirable initially to establish *in vitro* a model system in which factor requirements and reaction sequences could be studied with greater facility.

The model system consisted of freshly isolated normal human amnion cells, rabbit anti-human amnion antibody and normal human serum. The amnion cell was selected because of the desire to establish the model with normal fixed-tissue cells of human origin, the ready availability of sterile human placentas and the relative ease with which suspensions of amnion cells could

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‡ Research Fellow, The Helen Hay Whitney Foundation.

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be prepared from them. The humoral constituents of the system were also of human origin when this was possible. For this reason, normal human serum was used almost exclusively as the source of normal serum factors required for immune cytotoxicity and exclusively as a source of growth-promoting factors in tissue culture media. Recourse to a heterologous reagent was necessary in the case of anti-human amnion antibody; for convenience, this was prepared in the rabbit.

Two criteria of cytotoxicity were employed in this investigation: (*a*) metabolism-inhibition, measuring the diminished production of metabolic acids by injured cells (7, 14); and (*b*) uptake of trypan blue, measuring the loss of ability of injured cells to exclude this acid dye (2, 15). The results of the experiments to be described demonstrated that antibody, the components of hemolytic complement and calcium and magnesium ions were all required for immune cytotoxic effects on human amnion cells. An initial separation of reaction stages in this system is presented in the following paper (16).

Materials and Methods

Human Serum.—Human blood collected without anticoagulant was allowed to clot at room temperature for 1 to 3 hours and was stored at 1° overnight for maximal clot retraction. The serum was separated by centrifugation at 1°. Pools of serum from 10 to 25 donors were employed in most experiments.

Guinea Pig Serum.—Guinea pig blood was drawn either by cardiac puncture or exsanguination from the carotid artery and the serum separated as described for human serum. Lyophilized and freshly reconstituted guinea pig serum (Carworth Farms, New City, New York) was used equally satisfactorily in some experiments.

Human Complement Reagents.—Complement or fresh normal serum is designated C′. The components of complement are indicated by the symbols C′1, C′2, C′3, and C′4. Serum fractions or reagents which are deficient in C′1, C′2, C′3, or C′4 are designated R1, R2, R3, and R4, respectively. These reagents were employed for titration of components of complement and for determination of the requirement for the various components of complement for immune cytotoxicity. It is recognized that C′3 has been separated into two components (17) and that still another previously unrecognized serum constituent may be required for complement function (18). However, the operating definition of complement as a system with four components has been utilized in this investigation.

R1 and *R2* were prepared as routine by the method of dialysis (19, 20). In a few experiments, R1 was also prepared either by dilution (19) with subsequent lyophilization and reconstitution to 1.5 times the original serum volume with barbital-Ca^{++}-Mg^{++} buffer, or by incubation of serum with trisodium ethylenediaminetetraacetate (21). R2, the most labile of the serum reagents, was stabilized by suspension in either 0.3 M sodium chloride or pH 7.4 barbital-Ca^{++}-Mg^{++} buffer of ionic strength 0.30 and clarified by centrifugation at 46,000 *G* for 15 minutes. After sterile filtration (see below) and immediately prior to use, the reagent was adjusted to pH 7.4, if necessary, and diluted with sterile water to ionic strength 0.15 and a final dilution of 1/1.5 with respect to original serum. *R3* was prepared by incubation of serum with zymosan (19, 22) and *R4* by incubation of serum with either hydrazine or ammonia (19, 23). Because of the cytotoxic effect of hydrazine, R4 was prepared with ammonia for all experiments involving amnion cells. R4 prepared with either ammonia or hydrazine was equally satisfactory for the titration of C′4 in the hemolytic system; the hydrazine reagent was used as routine. R1, R2, R3, and R4 were non-hemolytic in amounts used in the various

tests, were not anticomplementary, and recombined at test levels to give 100 per cent hemolysis.

Complement-fixed (CF) serum (24), *streptokinase-treated (SK) serum* (25), and *RP* (serum deficient in properdin) (22) were prepared according to published procedures. *Heated serum* was prepared by heating pooled normal human serum for 30 minutes at 56°. Serum heated for 30 minutes at 48° or 52° was used in some experiments but, unless otherwise indicated, the term heated serum refers to 56°.

Complement-depleted serum, deficient in all four components of complement, was made by first incubating serum with zymosan to inactivate C'3, then incubating with ammonia to inactivate C'4, and finally heating for 30 minutes at 56° to inactivate C'1 and C'2. The resulting reagent, free of detectable components of complement, was slightly anticomplementary. It was possible however to use this reagent with appropriate controls.

Serum and serum reagents used in experiments on immune cytotoxicity were sterilized by filtration through Selas 01 and 015 or single 02 candle filters (Selas Corporation of America, Philadelphia) or Millipore HA filters (Millipore Filter Corp., Bedford, Massachusetts). They were either maintained at 1° and used on the day of preparation or stored at −60° in a mechanical freezer. Serum and serum reagents were always tested after filtration and storage and were used only if they met the required criteria in the hemolytic system.

Barbital Buffer (19, 22).—Barbital buffer at pH 7.4, ionic strength 0.15, containing 1.5×10^{-4} M Ca^{++} and 5×10^{-4} M Mg^{++}, was employed as diluent in hemolytic assays, unless otherwise indicated.

Sensitized Sheep Erythrocytes.—Sterile sheep erythrocytes in Alsever's solution (Cappell Laboratories, West Chester, Pennsylvania) were washed and sensitized with rabbit hemolysin (Certified Blood Donor Service, Inc., Jamaica, New York, or generously supplied by Dr. H. D. Piersma, Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York), as described previously (22).

Hemolytic Complement Assays.—Titrations of hemolytic complement and components of complement were performed according to previously published methods (19, 23, 24). The 50 per cent endpoint was employed for all component titrations and for determining the titer of complement-fixing antibody in antiserums using serial double-dilution techniques. Either the 50 per cent ($C'H_{50}$) or 100 per cent ($C'H_{100}$) endpoint was used for determining the hemolytic activity of serum or recombinations of serum reagents. The complement-equivalent of non-hemolytic serum reagents was based on the hemolytic activity ($C'H_{100}$ units) of the normal human serum from which these reagents were prepared.

Ethylenediaminetetraacetic Acid (EDTA).—The sodium, magnesium, and calcium salts of EDTA (generously provided by Geigy Chemical Co., Ardsley, New York) were dissolved in distilled water and adjusted to pH 7.4 at a final stock concentration of 0.15 M.

Hanks' Balanced Salt Solution (BSS).—Stock solutions A and B (26), prepared with analytic reagent grade chemicals and water-soluble phenol red (Hartman-Leddon Co., Philadelphia), were filtered separately through 01 and 015 or single 02 candle filters and stored at 1°. Prior to use, the stock solutions were mixed, diluted 10-fold with distilled water, and brought to pH 7.6–7.8 with 1.4 per cent sodium bicarbonate previously autoclaved at 10 pounds per square inch for 10 minutes. A second sterile filtration was performed as above and antibiotics were then added: 100 units per ml. of crystalline penicillin potassium (Squibb), 100 μg. per ml. of streptomycin base equivalent (Lilly), and 100 units per ml. of mycostatin (Squibb nystatin).

Mixture 199.—The 10-fold concentrate without sodium bicarbonate (Microbiological Associates, Inc., Bethesda, Maryland) was diluted 10-fold with distilled water and fortified if necessary with heated human serum or complement-depleted serum. The pH was adjusted to 7.6–7.8 with autoclaved 1.4 per cent sodium bicarbonate and the solution sterilized by filtration through a Millipore HA filter or through candle filters in the manner described for

BSS. Antibiotics were added exactly as for BSS and the mixture stored at 1° for use within 3 to 4 weeks.

Trypsin.—A 1 per cent aqueous solution of twice recrystallized trypsin containing approximately 50 per cent $MgSO_4$ (Worthington Biochemical Co., Freehold, New Jersey) was sterilized by filtration through a Millipore HA filter, stored at 1° and used within 2 weeks.

Human Amnion Cells.—The procedure for obtaining amnion cells from human placentas was a modification of that described by Dunnebacke and Zitcer (27). Placentas were obtained under sterile conditions from the Obstetrical Services of University Hospitals and of St. Luke's Hospital, Cleveland, primarily from deliveries by elective caesarian section. Vaginal deliveries yielded satisfactory placentas with lesser frequency. Immediately following delivery, the placenta was placed in a sterile Pyrex pan covered with sterile aluminum foil. Within 4 hours of delivery, and usually within 1 hour, the umbilical cord was cut off, the placenta rinsed with sterile BSS, and the amnion stripped from the chorion and cut into several large pieces which were placed in successive washes of BSS until free of all visible blood. After washing, the amnion was either trypsinized immediately or stored at room temperature overnight in mixture 199 containing 20 per cent heated human serum and trypsinized the following day. If the amnion was to be trypsinized immediately, the pieces were placed in a 32 ounce prescription bottle containing 350 ml. of BSS, 40 ml. of 1 per cent trypsin was added gradually with constant agitation, and autoclaved 1.4 per cent sodium bicarbonate was then added to bring the pH to 7.7–7.8. The suspension was incubated in a water bath at 37° for 2.5 to 4 hours with manual shaking every 20 minutes. Sodium bicarbonate was added as needed to maintain the pH at 7.7–7.8. After incubation, the fluid containing the suspended amnion cells was passed through four layers of cotton gauze. The pieces of amnion withheld by the gauze were washed with 100 ml. of mixture 199 containing 20 per cent heated human serum and the washings added to the original suspension. The pooled suspension was centrifuged at room temperature at 1000 r.p.m., (225 *G*) for 10 minutes, the supernatant discarded, and the residue resuspended to a volume of 25 ml. in mixture 199 containing 20 per cent heated human serum. This suspension was centrifuged at 500 r.p.m. (45 *G*) for 5 minutes at room temperature and the supernatant discarded. The average yield from a single placenta was 4.0 ml. (1.5 to 5.3 ml.) of packed cells. Following resuspension to approximately 10 per cent by volume in mixture 199 containing either 10 per cent heated human serum or 10 per cent complement-depleted serum, microscopic examination revealed individual rounded cells of uniform appearance. The cell suspension was used immediately or stored at room temperature in stoppered tubes. The cells usually maintained viability, with several changes of medium, for at least 1 week.

If the amnion was not trypsinized on the day it was obtained, the washed pieces were stored overnight at room temperature in 100 ml. of mixture 199 containing 20 per cent heated human serum, transferred to BSS the next morning, and trypsinized as above. Both procedures provided equally satisfactory cells by the criteria of metabolic activity and exclusion of trypan blue.

All procedures were performed in an isolated room equipped with air filters. All media were tested for sterility in thioglycollate broth or brain-heart infusion. Aseptic techniques were used in all manipulations involving the placenta or materials to be used in association with the amnion cells. In short term experiments, in which trypan blue uptake was used as the indicator of cytotoxicity, only stock solutions were handled aseptically.

Amnion Antigen.—Amnion cells, for use as antigen in the preparation of rabbit anti-human amnion serum, were obtained by the method outlined above except that after the initial centrifugation the cell pack was resuspended and washed at least 4 times in 0.15 M NaCl. After each centrifugation, erythrocytes at the surface of the cell pack were removed with a capillary pipette. The washed cells, as free as possible of erythrocytes, were resuspended in 14 volumes of 0.15 M NaCl and partially disrupted by successively freezing the suspension

at −60° and thawing at 37° a total of 5 times. The resulting suspension was divided into small aliquots and stored at −20° until used. The antigen was stable for 2 to 4 months, as measured by its ability to participate in a complement-fixation test with rabbit anti-human amnion serum.

Rabbit Anti-Human Amnion Serum.—Male New Zealand white rabbits weighing 3 to 4 kg. were bled from a marginal ear vein and injected in the contralateral marginal ear vein with amnion antigen. The dose and schedule of inoculation varied but good results were obtained with 3 daily injections of 0.5 to 1.0 ml. followed by 4 days' rest for 3 successive weeks. After an additional 3 weeks of rest, a series of 3 daily injections was repeated. Six days after the last injection the rabbits were again bled from the ear and the serum separated. Additional antiserum was obtained by further booster injections and bleedings according to the same schedule. Serums of sufficient potency on the basis of titer of complement-fixing antibody (see below) were pooled and frozen at −60° after no treatment or at −20° after either (*a*) heating for 30 minutes at 56°, (*b*) complement-depletion as described above, or (*c*) isolation of γ_2-globulin by the method of Nichol and Deutsch (28).

Complement-Fixing (CF) Titers of Preparations of Rabbit Anti-Human Amnion Antibody.—CF titers of heated or complement-depleted rabbit anti-human amnion serum and of rabbit anti-human amnion γ_2-globulin were performed in the following manner: 0.2 ml. of serial dilutions of the antibody preparation, 0.1 ml. of a dilution of guinea pig serum containing 2 $C'H_{100}$ units, and 0.2 ml. of an optimal dilution of amnion antigen were incubated for 60 minutes at 37°; 1.0 ml. of sensitized sheep erythrocytes was then added and the mixtures incubated further for 30 minutes at 37°. Appropriate antigen, antibody, complement, and sensitized erythrocyte controls were always included. The CF titer was expressed as the greatest dilution of antibody which would permit 50 per cent hemolysis under test conditions. Treated antiserums were diluted, if necessary, to a CF titer of 1/192. The preparation of anti-human amnion γ_2-globulin had a CF titer of 1/384 and contained 4.8 mg. of nitrogen per ml. New batches of antibody were adjusted to the same CF titer as previous batches by performing CF titers simultaneously on old and new preparations and by further reference to a standardized egg albumin–rabbit-anti-egg albumin system.

Antigen for Adsorption Experiments.—Guinea pig kidney antigen was prepared by published methods (29). *Human AB erythrocytes* were obtained from defibrinated blood and suspended in an equal volume of modified Alsever's solution (29). The cells were washed 3 times with 0.15 M NaCl prior to use. *Sheep erythrocytes* in Alsever's solution were treated in the same manner as human AB erythrocytes. *Human amnion cells* were prepared as described for immunization of rabbits and the insoluble residue used for adsorptions.

Mineral Oil.—Heavy mineral oil (drakeol No. 35, Pennsylvania Refining Co., Butler, Pennsylvania), for use in the metabolism-inhibition test, was autoclaved at 15 pounds per square inch for 15 minutes.

Trypan Blue.—A 1 per cent suspension of trypan blue (National Aniline Chemical Co., New York) in 0.15 M NaCl was clarified by low speed centrifugation for 5 minutes and stored in a brown bottle or shielded from direct light at all times.

General.—Glassware used in cytotoxicity experiments was washed with calgon–sodium metasilicate (30); other glassware was cleaned with sodium dichromate–sulfuric acid. All glassware was rinsed thoroughly with double distilled water.

EXPERIMENTAL

I. Metabolism Inhibition as a Criterion of Immune Cytotoxicity

A. General Considerations.—A modification of the metabolism-inhibition technique of Salk and Ward (14) was used as one criterion of immune cytotoxicity. The test is based on acid production by cells metabolizing in a suitable

medium containing phenol red as indicator. The amount of acid produced, resulting in a decrease in pH determined colorimetrically with a pH comparator block containing phenol red standards, is a function of the number of cells and their metabolic activity. Acid production by a given number of cells may therefore be taken as an index of cellular function and absence of acid production by the same number of cells as an index of cytotoxicity.

A major purpose of this investigation was to determine the relationship of hemolytic complement and its components to normal human serum factors required for immune cytotoxicity. Accordingly, it was necessary to exclude complement from the supporting medium and from the source of antibody and then to add known amounts in the form of human serum or serum reagents. For many experiments, this could be accomplished by using mixture 199 containing 10 per cent heated human serum as supporting medium and heated rabbit anti-human amnion antiserum as antibody. However, since heated serum contains small amounts of C'3 and relatively large amounts of C'4, it was mandatory that complement-depleted human serum and rabbit antiserum be substituted for the corresponding heated serums in all experiments designed to test the participation of C'3 and C'4 in immune cytotoxicity. On the other hand, either heated or complement-depleted serums could be used with comparable results in experiments testing the participation of C'1 and C'2 in immune cytotoxicity.

B. Design of Experiments.—The following constituents were added aseptically to sterile 12 x 75 or 13 x 100 mm. test tubes:

1. 0.05 ml. of heated or complement-depleted rabbit anti-human amnion antiserum having a CF titer of 1/192.
2. Various volumes of human or guinea pig serum or human serum reagents, diluted as required in mixture 199.
3. Mixture 199 to make a total volume at this step of 0.5 ml.
4. 0.25 ml. of serial 2-fold dilutions of human amnion cells suspended and diluted in mixture 199 containing either 10 per cent heated human serum or 10 per cent complement-depleted human serum. The original 10 per cent cell suspension was initially diluted to a concentration of $3.1 \pm 0.7 \times 10^7$ cells per ml., as determined by hemocytometer count. Dilutions of this standardized suspension were then made serially to 1/512.
5. 0.5 ml. of mineral oil to exclude air-borne contamination.

Controls lacking antibody, serum or serum reagents, or amnion cells were included and the same volume of appropriate diluent was substituted.

The tubes were incubated at $37° \pm 0.5°$ for 5 days. The pH was determined daily to the nearest 0.2 pH unit by visual comparison with a pH comparator block containing phenol red standards. The least dilution of cells at which the pH after 5 days of incubation was equal to or greater than the initial pH was taken as the endpoint. Experimental and control series were performed at least in duplicate. Two standard deviations from the mean (95 per cent confidence limits) was $\pm$2-fold or one dilution of amnion cells for any single experiment.

C. Definition of Cytotoxicity Index (C.I.).—The *cytotoxicity index* (C.I.) was defined as the reciprocal of the least dilution of cells producing no acid with serum or serum reagent in the absence of antibody divided by the reciprocal of the least dilution of cells producing no acid with serum or serum reagent in the presence of antibody. For example, if with serum reagent

without antibody the least dilution of cells producing no acid was 1/256 while with serum reagent plus antibody the least dilution of cells producing no acid was 1/8, then the C.I. was 256/8 or 32. Thus, in the presence of serum reagent and antibody in this example 32 times as many cells were required to produce the same metabolic effect as could be produced with serum reagent in the absence of antibody. Since the experimental error was ±2-fold, a C.I. of 0.5 to 2 was interpreted as absence of cytotoxicity and a C.I. of 4 or greater as an indicator of the presence of cytotoxicity.

TABLE I

Requirement for Normal, Heat-Labile Serum Factors and Antibody for Cytotoxicity (Metabolism-Inhibition)

Antiserum*	Normal serum‡	C.I.§
1. Fresh	None	16
2. Heated	None	1
3. "	Fresh guinea pig	16
4. "	" human	16
5. "	Heated human	1
6. None	Fresh rabbit	1–2
7. "	" guinea pig	1–2
8. "	" human	1–2
9. None	Heated rabbit + fresh guinea pig	1–2
10. "	" " + fresh human	1–2

* Rabbit anti-human amnion antiserum; fresh = unheated; heated = 56° for 30 minutes; CF titer, 1/192.

‡ Guinea pig and human serums used at volumes equivalent to 10 $C'H_{100}$ units of complement; normal rabbit serum used at 0.05 ml. of undiluted serum to correspond to amount of antiserum used; fresh = unheated; heated = 56° for 30 minutes.

§ Calculated as defined in text for lines 3 to 5; for lines 1 and 2 and 6 to 8 calculated on basis of metabolism of cells in indicated serums *vs.* metabolism in diluent alone; for lines 9 and 10, calculated on basis of metabolism of cells in heated normal rabbit serum and fresh guinea pig or human serum *vs.* metabolism in heated normal rabbit serum.

D. Requirement for Normal, Heat-Labile Serum Factors and Antibody.— Initial experiments were designed to determine qualitatively whether normal heat-labile serum factors and antibody were necessary for cytotoxicity. The sources of these factors were normal human serum, normal guinea pig serum, normal rabbit serum, and unheated and heated rabbit anti-human amnion antiserum. The results, summarized in Table I, demonstrated that metabolism-inhibition occurred only in the presence of both antibody and normal, heat-labile serum factors. Both antibody and normal serum factors were supplied by unheated rabbit anti-human amnion antiserum. If the antiserum was heated, cytotoxicity was abolished but could be restored by fresh normal guinea pig or human serum. The cytotoxicity of human serum could not be demonstrated

following heating for 30 minutes at 56° or in the absence of antibody. These qualitative experiments therefore confirmed and extended previous observations (2–7, 9–13) and provided the basis for subsequent experiments on the nature of the normal, heat-labile, human serum factors required for immune cytotoxicity.

E. Optimal Concentrations of Normal Human Serum and Antibody.—Attention was next directed toward determination of the optimal concentration of normal serum required for immune cytotoxicity. Human serum was used exclusively for this purpose throughout the remainder of this investigation. When antibody was maintained constant at 0.05 ml. of heated rabbit anti-human amnion antiserum (CF titer = 1/192), optimal cytotoxicity was achieved at a volume of normal human serum (about 0.1 ml.) equivalent to 5 $C'H_{100}$ units of complement.

When normal human serum was maintained constant at 5 $C'H_{100}$ units, optimal cytotoxicity was achieved at a volume of 0.05 ml. of heated rabbit anti-human amnion antiserum (CF titer = 1/192). Since the concentration of amnion cells was always varied as an integral part of each test and in view of the preceding experiments, these conditions were used for subsequent studies.

In separate experiments, it was found that adjustment of various batches of rabbit anti-human amnion antiserum to the same CF titer (1/192) resulted in uniform cytotoxic indices in the presence of a constant amount of normal human serum and with the same preparation of amnion cells. Significant differences could not be detected among the cytotoxic activities of various preparations of antibody (heated or complement-depleted rabbit anti-human amnion antiserums or the γ_2-globulin derived from the antiserum) when they were compared in a single experiment at the same CF titer in the presence of a constant amount of normal human serum.

F. Specificity of the Antibody.—A limited investigation was made of the specificity of rabbit anti-human amnion antiserum with respect to reaction with human amnion cells, major human blood group antigens, and Forssman antigen. Heated antiserum was adsorbed at 0° for 60 minutes with equal volumes of amnion antigen, washed sheep erythrocytes, or washed human erythrocytes (Type AB, Rh-positive) or with 5 volumes of 20 per cent suspension of guinea pig kidney. The mixtures were stirred frequently and then centrifuged at 0°. The CF titers and immune cytotoxic activities of the adsorbed and unadsorbed antiserums were compared, using amnion antigen and amnion cell suspensions respectively (Table II). Adsorption with amnion antigen resulted in complete or nearly complete loss of both CF titer and immune cytotoxic activity. Adsorption with sheep or human erythrocytes or guinea pig kidney did not affect significantly either CF titer or immune cytotoxic activity. It was concluded that rabbit anti-human amnion antiserum reacted, in both the CF test and metabolism-inhibition test, with an antigen of the amnion cell which was distinct from major human blood group antigens or Forssman antigen. Unadsorbed antiserum was therefore used as routine.

G. Relation of Normal Human Serum Factors to Hemolytic Complement.— The preliminary experiments demonstrated that both antibody and normal serum factors were required for cytotoxicity by the criterion of metabolism-inhibition and that the activity of normal serum was abolished by heating for 30 minutes at 56°. A series of experiments was then undertaken to compare the factors in normal human serum with hemolytic complement.

1. Heat-lability: Aliquots of human serum were heated for 30 minutes at 48°, 52°, and 56° and then tested for cytotoxic and hemolytic activities (Table III). At 48°, residual cytotoxic activity was proportional to residual complement activity; at 52° and 56°, both cytotoxic and hemolytic activities of human serum were abolished.

TABLE II

Effect of Various Adsorptions on CF Titer and Immune Cytotoxic Activity (Metabolism-Inhibition) of Heated Rabbit Anti-Human Amnion Antiserum

Adsorbing agent	CF titer	C.I.
None	1:192	64
Amnion antigen	1:8	2
Human erythrocytes (AB, Rh-positive)	1:192	64
Sheep erythrocytes	1:192	64
Guinea pig kidney*	1:144	128

* Data corrected for dilution of antiserum with guinea pig kidney antigen.

TABLE III

Effect of Heat on Immune Cytotoxicity (Metabolism-Inhibition) and Hemolytic Complement Activity of Human Serum

Temperature	C.I.	Human serum
°C.		$C'H_{100}$ *units/ml*
Unheated	64	42
48	32	20
52	2	0
56	0.5	0

2. Complement-fixation and treatment with streptokinase: Further similarities between cytotoxic and hemolytic complement activities of human serum were observed when normal human serum was incubated with either a heterologous antigen-antibody aggregate formed *in situ* or a solution of streptokinase (varidase, Lederle). Each of these procedures destroyed all or nearly all of both the cytotoxic and hemolytic activities of human serum (Table IV). Neither the C.I. nor complement activity was significantly affected when serum was incubated with either antigen or antibody alone.

3. Serum reagents deficient in individual components of complement: R1, R2, R3, and R4, human serum reagents deficient respectively in C'1, C'2, C'3, and C'4 and conforming to previously indicated criteria in the hemolytic system, were each tested at two levels for cytotoxic activity in the presence of the standard amount of rabbit anti-human amnion antiserum. The anti-

TABLE IV

Effect of Complement-Fixation and Streptokinase Treatment on Immune Cytotoxicity (Metabolism-Inhibition) and Hemolytic Complement Activity of Human Serum

Treatment of human serum	C.I.	Human serum
		$C'H_{100}$ *units/ml.*
None	32	42
Complement-fixation	2	0
Streptokinase-treatment	4	0

Aliquots of human serum incubated for 60 minutes at 37° with either 8 μg. of purified pneumococcal capsular polysaccharide, Type III and 200 μg. of rabbit anti-S-III antibody nitrogen per ml. of serum or with 1000 units of streptokinase (varidase, Lederle) per ml. of serum.

TABLE V

Immune Cytotoxicity (Metabolism-Inhibition) of Human Serum and Serum Reagents at Two Concentrations of Hemolytic Complement

Reagent	C.I. in presence of antibody and			
	5 $C'H_{100}$ units of reagent		1 $C'H_{100}$ unit of reagent	
	Average	Range	Average	Range
Normal human serum	48	16–128	8	2–32
R1	4	2–8	2	0.5–8
R2	2	0.5–2	1	0.5–1
R3	2	—	1	—
R4	2	—	1	1–2

serum was either heated or complement-depleted for experiments with R1 and R2; complement-depleted antiserum was used exclusively for experiments with R3 and R4. The data presented in Table V represent a summary of 11 experiments in which each reagent was tested a total of 3 to 11 times. Normal human serum from which the reagents were prepared was tested in each experiment. It will be noted that the cytotoxic activity of human serum was greater at a higher concentration of serum but that cytotoxic activity at either level of reagent was diminished or entirely abolished when any one of the 4 components of complement was not present. R2, R3, and R4 were consistently inactive

but slight residual cytotoxicity was usually encountered with R1. This was observed not only when R1 was prepared by the dialysis procedure, as shown in Table V, but also when this reagent was prepared by the method of dilution or of incubation with Na_3EDTA. The effect was also observed with R1 prepared by dialysis from 5 individual pools of human serum which were compared simultaneously. Definitive interpretation of these findings with R1 was not possible, although it may be inferred from data in the following paper that amounts of C'1 which were not detectable by hemolytic assay were sufficient to cause significant cytotoxicity in the presence of C'2, C'3, and C'4. The experiments summarized in Table V clearly suggested a requirement for the 4 components of complement for immune cytotoxicity.

TABLE VI

Immune Cytotoxicity (Metabolism-Inhibition) of Recombinations of Human Serum Reagents Compared with Normal Human Serum at Equivalent Levels of Hemolytic Complement

Reagent combination	C.I. of combination	C.I. of normal human serum at equivalent levels of C'
R1 + R2*	24	8
R1 + R3	64	16
R1 + R4	8	16
R2 + R3*	8	8
R2 + R4	16	16
R3 + R4	8	4

* Each reagent recombined at the 5 unit level; C.I. of starred combinations and their corresponding values for C.I. of normal human serum at equivalent concentrations of complement represent geometric average of 2 separate experiments; all other values represent single experiments for each combination and an equivalent concentration of complement in the form of normal human serum.

4. Recombination of serum reagents deficient in individual components of complement: Hemolytic complement activity of any one of the serum reagents R1, R2, R3, and R4 may be restored by addition of any of the 3 remaining reagents. It was therefore of direct importance to compare the cytotoxic and hemolytic activities of the 6 possible recombinations of the 4 serum reagents in order to test further the relationship to hemolytic complement of the normal human serum factors required for immune cytotoxicity.

The hemolytic activity of each recombination of serum reagents was determined and found to vary between 20 and 67 per cent of the equivalent volume of normal human serum. The cytotoxicity of each of these recombinations with known hemolytic complement activity was then compared with the cytotoxicity of a volume of normal human serum equivalent to the same hemolytic complement activity. The results (Table VI) showed excellent agreement between C.I. of the combinations and C.I. of human serum at equivalent levels of

hemolytic complement for all recombinations except R1 + R2 and R1 + R3. Even with these recombinations, the discrepancies appeared to be within limits of experimental errors.

5. Serum reagents deficient in properdin: Human serum rendered deficient in properdin by a single adsorption with zymosan at 17° (RP) and highly deficient by a second adsorption with zymosan at 37° (RPb) (31) were compared in the metabolism-inhibition test with normal human serum at equivalent levels of hemolytic complement. The cytotoxic activities of RP and RPb were directly proportional to their hemolytic complement activities. It was concluded, therefore, that properdin was not involved in immune cytotoxicity in the system under investigation.

II. Trypan Blue Uptake as a Criterion of Immune Cytotoxicity

A. General Considerations.—The investigations summarized above demonstrated that both antibody and normal serum factors were required for cytotoxicity by the criterion of metabolism-inhibition and indicated positive correlations by a variety of approaches between the properties of the normal serum factors and the components of hemolytic complement. These observations were tested by a second criterion of cytotoxicity. The principle of exclusion of trypan blue by viable cells was adopted as a rapid, technically simple indicator of cytotoxicity for this purpose.

It was also desirable to devise a test system for immune cytotoxicity which would be more readily susceptible to separation of reaction stages than the metabolism-inhibition procedure. The study described in the following paper was therefore anticipated in the design of these experiments.

B. Design of Experiments.—In all of the experiments to be described, amnion cells were reacted with rabbit anti-human amnion γ_2-globulin. The cells were then centrifuged and washed in order to minimize the anti-complementary activity of the γ_2-globulin. On the basis of a series of preliminary experiments, the following procedure was adopted. A suspension of trypsinized human amnion cells in mixture 199 containing 10 per cent complement-depleted human serum was adjusted by dilution in this diluent to a concentration of 5.5×10^5 cells/ml. by hemocytometer count. In a series of 12 x 75 mm. test tubes, 0.25 ml. of cell suspension and 0.25 ml. of a 1/20 dilution of rabbit anti-human amnion γ_2-globulin (CF titer undiluted = 1/384) in mixture 199 were mixed and incubated for 10 minutes at room temperature (19–23°). The mixtures were centrifuged for 5 minutes at 1000 r.p.m. (205 *G*) at room temperature, the supernatant fluids were decanted and discarded, and the residues resuspended in 0.5 ml. of mixture 199 containing 10 per cent complement-depleted human serum. Centrifugation and decantation were repeated and the tubes containing the washed residues were stoppered, kept at room temperature, and used within a 7 hour period. As shown in subsequent tables, these antibody-coated cells were viable by the criterion of exclusion of trypan blue. For controls, unsensitized amnion cells were prepared by omission of antibody in the same experimental procedure.

The amount of antibody used (60 μg. of γ_2-globulin nitrogen) represented a 2.5 to 5-fold excess over the minimum amount required to give optimal uptake of trypan blue by this number of amnion cells after incubation for 30 minutes at 37° in the presence of 2 $C'H_{100}$ units of human serum. The sensitization of amnion cells by rabbit anti-human amnion γ_2-

globulin was therefore performed according to the same principles employed for the sensitization of sheep erythrocytes by rabbit hemolysin for titrations of hemolytic complement.

The immune cytotoxic activity of normal human serum and serum reagents was tested by appropriate dilution in mixture 199 so that the desired amount of reagent was contained in 0.5 ml. of solution. This volume was then added to the previously prepared washed residues of sensitized amnion cells. The cells were suspended by mixing and the mixtures were incubated for 30 minutes at 37°. Controls consisted of 0.5 ml. of the same dilutions of serum reagents in mixture 199 added to unsensitized amnion cells or of 0.5 ml. of mixture 199 con-

TABLE VII

Immune Cytotoxicity (Trypan Blue Uptake) of Human Serum and Serum Reagents at Several Concentrations of Hemolytic Complement

Reagent	Per cent stained cells in presence of indicated $C'H_{100}$ units of human serum or serum reagents*				
	None	0.5 unit	1 unit	2 units	4 units
Normal human serum	5	21	75	100	—
R1	7	—	—	7	22
R2	7	—	—	10	23
R3	7	—	—	8	6
R4	8	—	—	5	6
RP	5	48	90	—	—
Complement-fixed serum	5	—	—	3	6
Streptokinase-treated serum	5	—	—	5	7
Serum heated for 30 min. at: 48°	6	24	70	—	—
" " " " " " 52°	6	—	—	7	4
" " " " " " 56°	5	—	—	9	5

* For normal serum, for serum heated for 30 minutes at 48°, and for RP, the indicated levels of complement were obtained by direct hemolytic titration; for all other reagents, the indicated levels of complement were calculated on the basis of the hemolytic activity of the serum from which the reagent was prepared.

taining 10 per cent complement-depleted human serum added to sensitized or unsensitized amnion cells. After incubation, 0.25 ml. of 1 per cent trypan blue was added, the tubes were shaken gently at room temperature for 2 minutes (32), an aliquot was placed in hemocytometer chambers, and the per cent of stained cells was determined at once by microscopic examination of at least 100 cells under low power magnification (× 100). The start of incubation of individual tubes was adjusted so that after incubation for 30 minutes sufficient time was available for staining and counting before the next sample was ready for staining.

C. Relation of Normal Human Serum Factors to Hemolytic Complement.—A series of human serum reagents, similar to those employed in the metabolism-inhibition test, was tested for cytotoxic activity on sensitized amnion cells by the criterion of trypan blue uptake. The results (Table VII) clearly demon-

strated that normal human serum was required for immune cytotoxicity, that inactivation of one or more of the components of complement by a variety of

TABLE VIII

Immune Cytotoxicity (Trypan Blue Uptake) of Recombinations of Human Serum Reagents Compared with Normal Human Serum at Equivalent Levels of Hemolytic Complement

Reagent combination*	Actual hemolytic complement	Per cent stained cells	
		Observed	Calculated
	$C'H_{100}$ *units*		
R1 + R2	1.5	92	97
R1 + R3	0.56	72	75
R1 + R4	1.1	93	96
R2 + R3	1.1	92	96
R2 + R4	1.5	96	97
R3 + R4	0.78	90	89

* Each reagent recombined at the 2 unit level.

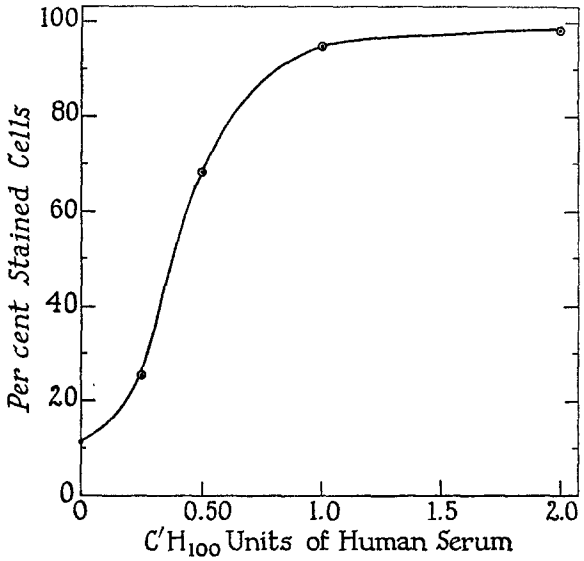

FIG. 1. Standard curve of hemolytic *vs.* cytotoxic activity of human serum (trypan blue uptake); see Table VIII.

procedures resulted in loss of cytotoxicity, and that properdin was not involved in the phenomenon.

The 6 possible recombinations of R1, R2, R3, and R4 were tested for both hemolytic and cytotoxic activities (Table VIII). A standard curve of hemolytic activity of human serum *vs.* immune cytotoxicity was established (Fig. 1).

The hemolytic and cytotoxic activities of each reagent recombination were then experimentally determined. In addition, the expected cytotoxic activity of each recombination was calculated by reference to the standard curve. The observed and calculated values for cytotoxicity were in excellent agreement, demonstrating a close correlation between the hemolytic and immune cytotoxic properties of human serum. It will be noted that the values shown in Fig. 1 were different from those given in Table VII, possibly reflecting variations in the sources of cells or in the age of the cell preparation. For this reason, a

TABLE IX

Comparison of the effect of diluents on the cytotoxic (trypan blue uptake) and hemolytic activities of human serum in the presence of various salts of EDTA

Reagent	Salt of EDTA	Cytotoxic and hemolytic activities in indicated diluent			
		Mixture 199		Barbital buffer*	
		Stained cells	$C'H_{50}$ units	Stained cells	$C'H_{50}$ units
	$1.5 \times 10^{-3} M$	*per cent*		*per cent*	
Normal human serum	—	100	118	95	118
" " "	Na	9	0	6	0
" " "	Mg	96	83	3	0
" " "	Ca	92	154	97	125
None	—	4	—	3	—
"	Na	3	—	6	—
"	Mg	3	—	7	—
"	Ca	5	—	8	—

* Without added Ca^{++} and Mg^{++}.

standard curve was obtained simultaneously with the performance of each experiment in order to minimize the effects of such variables.

D. Requirement for Ca^{++} and Mg^{++} for Immune Cytotoxicity.—The procedure of measuring cell viability by trypan blue uptake after incubation for 30 minutes permitted investigation of the requirement for Ca^{++} and Mg^{++} in immune cytotoxicity (10). Both of these cations are required for immune hemolysis of sheep erythrocytes sensitized with rabbit antibody (33) and of human erythrocytes sensitized with human antibody from patients with paroxysmal cold hemoglobinuria (34). However, modification of the test system was required for this purpose.

Human serum was diluted in mixture 199 in the presence of the sodium, magnesium, or calcium salts of EDTA and tested for cytotoxic activity on sensitized amnion cells. The data (Table IX) indicated a requirement for Mg^{++} but not Ca^{++} since

Na_3 EDTA was inhibitory but Na_2 Mg EDTA and Na_2 Ca EDTA were not. The possibility was then considered that mixture 199 contained one or more substances which interfered with the chelation of Ca^{++} by Na_2 Mg EDTA. Such an interference effect has been noted under other circumstances (35). Indeed, when barbital buffer of pH 7.4, ionic strength 0.15 was substituted for mixture 199 as the diluent, a requirement for Ca^{++} as well as Mg^{++} could be shown clearly. In this case, both Na_3 EDTA and Na_2 Mg EDTA inhibited cytotoxicity, but Na_2 Ca EDTA did not (Table IX). None of these salts of EDTA was cytotoxic in control mixtures. The interfering effect of mixture 199 in demonstration of a requirement for Ca^{++} could be also be demonstrated in immune hemolysis (Table IX).

E. Irreversibility of Cytotoxicity.—Experiments were performed to determine if the cytotoxic effect of antibody, complement and Ca^{++} and Mg^{++} was reversible.

Amnion cells sensitized with rabbit anti-human amnion γ_2-globulin in the usual way were incubated at 37° for either 5 or 30 minutes in the presence of 2 $C'H_{100}$ units of fresh human serum diluted in mixture 199. The cell complexes were removed by centrifugation for 5 minutes at 500 R.P.M. (45 G), washed once with mixture 199 containing 10 per cent heated human serum, and suspended in the same diluent to the original cell concentration. Incubation at 37° was then continued and aliquots were withdrawn for staining with trypan blue at intervals up to 120 minutes. No reversibility of cytotoxicity was observed. The extent staining with trypan blue was 96 to 100 per cent regardless of the time of the initial incubation in the complete cytotoxic system or of subsequent incubation of the injured cells in supporting medium.

III. Morphologic Alteration as a Criterion of Immune Cytotoxicity

Attempts to establish morphologic criteria for immune cytotoxicity in the experimental model employed were unsuccessful. Trypsinized amnion cells were grown in monolayers in T flasks and after a confluent layer had formed, the desired serum reagents were added, the flasks were incubated at 37° and the cells were observed microscopically for at least 60 minutes. In parallel with the studies on metabolism-inhibition and on trypan blue uptake, aliquots of cells incubating in the presence of specific antibody and serum reagent were sampled in duplicate at various times (0 to 68 hours) and stained with either Papanicolaou or hematoxylin-eosin. In all of these procedures, there was insufficient difference between the appearance of control and experimental cells to permit conclusions about the effect of antibody and normal serum factors on the morphology of human amnion cells. The cellular lesion which was reflected by the metabolism-inhibition test and by increased uptake of trypan blue was therefore not associated with cytolysis or readily demonstrable morphologic alterations under these conditions.

DISCUSSION

The phenomenon of cytotoxicity and its production by humoral factors has long been of interest to investigators in the fields of immunology and experimental pathology. A variety of experimental models has been established using

cell types ranging from the chick embryo to human cells of malignant origin. In some of these systems, specific antibody has been a sole requirement for cytotoxicity (36, 37); in others, both specific antibody and normal serum constituents have been required (2–13, 38–40); and in still others, normal serum in the absence of demonstrable specific antibody has sufficed (13, 37, 39–41). The present investigation has been limited entirely to *in vitro* experiments, utilizing primary isolates of a normal, fixed-tissue cell of human origin, the amnion cell. Using this experimental model, it was found that cytotoxicity depended on the presence of specific rabbit antibody, normal human serum factors which by a variety of criteria were indistinguishable from the components of hemolytic complement, and calcium and magnesium ions. The requirements for immune cytotoxicity were therefore identical with those for immune hemolysis (33, 34).

Evidence for the identity of the normal human serum factors required for immune cytotoxicity with the components of hemolytic complement rests on the following observations:

1. Requirement for specific antibody for cytotoxic and hemolytic activities of normal serum.

2. Partial loss of cytotoxicity to the same extent as loss of hemolytic activity when normal serum was heated for 30 minutes at 48°; complete loss of both cytotoxic and hemolytic activities when normal serum was heated for 30 minutes at 52° or 56°.

3. Inactivation of cytotoxic and hemolytic activities of normal serum by complement-fixation with antigen-antibody aggregates or by treatment with streptokinase.

4. Complete or nearly complete loss of cytotoxic and hemolytic activities of normal serum in which a single component of complement had been inactivated (R1, R2, R3, and R4); restoration of cytotoxic and hemolytic activities of normal serum when any 2 of the 6 possible recombinations of R1, R2, R3, and R4 were tested.

5. Requirement for calcium and magnesium ions for cytotoxic and hemolytic activities of normal serum.

In certain previous studies in which both antibody and normal serum were required for cytotoxicity, identification of the normal serum constituents with complement was made primarily on the basis of heat-lability (56°, 30 minutes) (2–7, 9–13). One report showed further that complement-fixed normal guinea pig serum, as well as heated normal guinea pig serum, was without cytotoxic activity on chick embryo hearts sensitized with specific guinea pig antibody (42). In addition, Goldberg and Green (10) found that cytotoxic effects observed with specific rabbit γ-globulin and normal rabbit serum could be abolished in the presence of disodium ethylenediaminetetraacetate and restored by the addition of calcium and magnesium ions. These observations have been con-

firmed and extended in the present investigation with human amnion cells, rabbit antibody, and normal human serum. The results clearly support definition of the normal human serum factors required for immune cytotoxicity as the components of hemolytic complement and calcium and magnesium ions.

The model system described in this report had distinctly different normal serum requirements for immune cytotoxicity from that described by Björklund (38), using established lines of HeLa (originally derived from carcinoma of the human cervix), Detroit-6 (human marrow), heart, and conjunctival cells and specific partially purified antibody produced in horses. Björklund's normal serum factor, obtained from either fresh human or horse serum, was inactivated by heat only at temperatures of 73° and greater. Its activity was not abolished by treating serum with either ammonia or zymosan or by isolation from the serum of a pseudo-globulin fraction with only C′2 and C′4 activity. Furthermore, cytotoxicity was not dependent on normal serum levels of calcium or magnesium ions.

Two differences between Björklund's experiments and our own are to be emphasized: the nature of the cell and the source of the antibody. (*a*) In Björklund's work, the antigen used for antibody production was prepared from human tumor tissues. The resulting antiserum in conjunction with normal serum produced cytotoxicity with established human tissue culture lines of malignant origin or with malignant characteristics but was without effect on freshly established lines of human cells or on human or sheep erythrocytes. On the other hand, in the present investigation primary isolates of normal human amnion cells were used both as the source of antigen for antiserum production and as the test cell in immune cytotoxicity. (*b*) Björklund performed his experiments with horse antibody while those reported here employed rabbit antibody. Antibodies produced in the horse are known to have very poor complement-fixing activity while rabbit antibodies are usually highly active in this property (19). It is possible, therefore, that a system not requiring complement was preferentially selected in Björklund's experiments by the use of horse antibody. Parallel experiments with rabbit antibody would be of great interest.

These differences have been elaborated primarily as a means of emphasizing the limitations of generalizations about immune cytotoxicity. Apparently several systems exist for the killing of host cells by immunologic or serologic mechanisms and the particular system selected experimentally may well depend on the nature of the cell, the antibody, and the normal serum employed. In our own experiments, cytotoxicity with primary isolates of normal human amnion cells depended upon the presence of rabbit antibody, the components of human complement, and calcium and magnesium ions. It would be predicted that these requirements would apply to many other normal cells and complement-fixing antibodies but not necessarily to systems with different characteristics.

The concept of humoral cytotoxins apparently unrelated to the presence of specific antibody has existed for many years (2). In this connection, it is of interest that Lumsden (39), Bolande and Todd (41), and others (13, 40) have found that normal serums, in the absence of demonstrable antibody, have cytotoxic activity for certain established cell lines in tissue culture. On the other hand, numerous investigators have demonstrated the necessity for specific cytotoxic antibodies (2–14, 36–40). Ross has delineated five criteria for establishing the participation of specific anticellular antibodies in cytotoxicity (43). All of these criteria have been fulfilled in the present investigation. (*a*) The preimmunization and postimmunization sera from 31 individual rabbits were tested for the presence of anti-amnion antibodies. In none of the preimmunization sera were they found whereas all the postimmunization sera contained them in varying amounts. (*b*) Preliminary control experiments with heated antiserum and heated human serum failed to demonstrate cytotoxicity. (*c*) In no instance was either heated antiserum or complement alone able to elicit cytotoxicity. (*d*) The cytotoxic factor could be adsorbed from postimmunization serum by human amnion cells but not by human AB erythrocytes or sources of Forssman antigen. (*e*) Progressive increase of the titer of cytotoxic antibodies with continued immunization of individual rabbits was demonstrable, although these data have not been presented. If a rabbit was able to produce potent antiserum (CF titer of 1/192 or greater in these experiments), it acquired this level gradually and with repeated stimulation. Some rabbits were never able to produce a potent antiserum and at least 18 of them exhibited a decrease in titer following antigenic stimulation which was continued for over a year.

The conclusions drawn from the data presented have been based in most instances on two independent criteria of cytotoxicity: metabolism-inhibition and uptake of trypan blue. Both of these techniques have been widely used as indicators of cell viability under well defined experimental conditions (2, 5, 6, 8, 14–16, 44–46). Uptake of trypan blue proved to be a rapid, convenient, and reproducible procedure for measuring the cytotoxic activity of normal human serum on human amnion cells sensitized with rabbit antibody. A small percentage of cells used as controls was always stained (1 to 19 per cent, average 8 per cent) and could be attributed to the trypsinization procedure, storage, and manipulations involved in preparation of the sensitized cells. The effect of whole human serum, at the level of either 1 or 2 $C'H_{100}$ units, resulted in at least 75 per cent staining and usually 90 to 100 per cent of sensitized amnion cells. There was therefore a clear difference between uptake of trypan blue by amnion cells in control and experimental series in each protocol. This technique permitted the study of separate reaction stages and the functional role of C'1-esterase in immune cytotoxicity. The results of this investigation are presented in the following paper (16).

SUMMARY

A model system for the investigation of immune cellular injury in primary isolates of a fixed-tissue cell of human origin has been described, using metabolism-inhibition and uptake of trypan blue as independent criteria of cytoxicity. Cytotoxic effects on human amnion cells were produced by the combination of specific rabbit antibody and factors in normal human serum which were indistinguishable from the components of hemolytic complement and calcium and magnesium ions. The data have been discussed in relation to the apparent multiplicity of serologic systems which can effect cellular injury. The nature of the cell and the source of antibody or normal serum constituents have been emphasized as factors which may influence the experimental selection of a given humoral mechanism.

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