Observations on Cytotoxic Effect of Antihistiocyte Serum.* (26518)

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Guinea pig anti-rabbit histiocyte serum exerts a cytotoxic effect on living histiocytes in the presence of high concentrations of complement. Although cytotoxic action of antibody, especially on tumor cells, has been reported(1-7), the role of whole complement or complement components in the cytotoxic reaction has been neglected until recently(8). This paper describes preliminary observations on some of the characteristics of guinea pig antirabbit histiocyte antibodies and their cytotoxicity for live histiocytes.

The cell designated as a histiocyte in this paper is a large mononuclear cell elicited by oil in the peritoneal cavity of the rabbit. It has morphologic and tinctorial characteristics very similar to the blood monocyte and is synonomous with the wandering macrophage.

Materials and methods. Antibody production. Whole histiocytes harvested in the manner given below were mixed into a modified Freund's adjuvant (no BCG added). Each guinea pig received 3 doses of one ml antigen subcutaneously at weekly intervals. One week later, 0.25 ml saline-suspended histiocytes were injected into each foot pad. Sensitization was followed by measuring precipitating antibody against soluble antigens extracted in Grabar's solution conservatrice(12) from histiocytes which were ground in liquid nitrogen. From 10 to 30 mg of antigen (as whole cell nitrogen) was required per animal for production of precipitating antibody against these soluble antigens.

Histiocyte production. Histiocytes were harvested from the peritoneal cavity of New Zealand white rabbits (3 to 5 lb) 5 days after injection of 50 ml of Klearol. Cells were washed from the cavity with Grabar's solution conservatrice (SC)(12). Oil was separated from cells by separatory funnel. Cells were sedimented at 250 g for 10 minutes and washed 3 times in SC. Packed cells were used for the immunizing antigen. About 10 to 20% of the cells were non-viable at time of harvest.

Complement (C′). Commercial,‡ dehydrated guinea pig complement was reconstituted according to package directions.

Dye. 1% trypan blue in 0.85% saline, filtered and diluted 1:5 in saline, was used in cytotoxic tests.

Cytotoxic test. To a 4-inch screw cap test tube was added: 1.0 ml antiserum diluted 1:10 or more, 1.0 ml cell suspension (1×10^6/ml in 0.85% NaCl and 5% bovine serum albumin), 0.5 ml 0.0012 M Ca^{++} solution in 0.85% NaCl and 0.5 ml 0.004 M Mg^{++} solution in 0.85% NaCl. The mixture was brought to 37°C in a water bath. One ml of complement, diluted 1:5 and brought to 37°C, was added. The test mixture was incubated 60 minutes at 37°C; tubes were frequently hand-mixed. The mixture was centrifuged for 2 minutes, supernatant discarded, and cells resuspended in 0.5 ml diluted trypan blue. The cell-dye suspension was held at room temperature (25°C) for 3 to 5 minutes. A drop of the mixture was placed on 3×5 microscope slide covered with 22×40 mm coverslip, and examined by high power objective. The number of cells with blue-stained nuclei in a total of 200 cells was recorded. The percent dead cells was corrected on the basis of 100% live cells in the control suspension.

Kinetic tests. For the kinetic studies multiples of the 4.0 ml test dose were placed in a flask, samples withdrawn periodically and treated as described above. Time was measured from the moment of addition of complement. Unless otherwise noted, all reagents were brought to 37°C before adding complement.

Experimental results. The cytotoxic activity of the antihistiocyte serum was heat stable

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TABLE I. Cytotoxic Effect of Antihistiocyte Serum in Presence of Varying Amounts of Complement. (Activity recorded as % cells killed.)

	ml complement			
ml serum	.05	.1	.2	.4
.1	72	80	82	92
.05	32	71	57	78
.025	28	40	43	69
.0125	16	26	20	41

(56°C, 1 hour) and could be absorbed from the serum by whole live histiocytes without qualitatively affecting the serum's gel diffusion pattern with soluble antigens. A reciprocal relationship was observed between amount of antibody and amount of complement which gave maximal killing effect. (*See* Table I).

No extra calcium or magnesium ions were added to the system in this experiment, and SC solution was employed instead of normal saline.

In excess complement, maximal rate and extent of killing was dependent upon amount of antibody present in the system. Maximal cytotoxicity of 0.05 ml of antiserum pool used in these experiments was reached 50 minutes after addition of complement.

Combined effect of calcium and magnesium ions on cytotoxic reaction. Two flasks were set up, one with 10 test doses for the control, (I) the other with 30 test doses (II). No additional Ca^{++} or Mg^{++} was added to flask II. Disodium ethylenediamine tetraacetate (EDTA-50μM) was added, which was enough to bind the estimated Ca^{++} and Mg^{++} in antiserum and in complement solutions. Four ml portions were removed from each flask over a period of 50 minutes after complement was added. At 60 minutes the remaining contents of flask II were divided (flask IIa and IIb), and Ca^{++} and Mg^{++} sufficient to tie up all the EDTA, with excess for maximal free ion concentration, was added to flask IIa. Additional samples were removed and counted from both flask IIa and flask IIb over a period of 50 minutes.

Fig. 1 indicates that the cytotoxic reaction was dependent on one or more of the divalent cations. Increased killing in the EDTA control after 80 minutes was possibly due to pH changes, as the system was not buffered.

When no Ca^{++} or Mg^{++} was added, minimal killing effect was observed over a period of 70 minutes. If Mg^{++} alone was added, the reaction was maximal without addition of Ca^{++}. There was an optimal concentration of Ca^{++} in the minimal system due to the calcium concentration in guinea pig serum present, whereas concentration of Mg^{++} was sub-optimal. The results indicate a requirement for Mg^{++}, but do not indicate whether or not Ca^{++} is required.

The reaction of complement in the cytotoxic system was compared to the reaction in the sheep red cell-rabbit hemolysin system. Sensitized histiocytes were subjected to complement, Ca^{++} and Mg^{++} at 0°C for one hour in an attempt to form HAC'_{142} cells analogous to EAC'_{142} cells of Levine *et al.*(9,10). These cells are designated as C'_{0_0} cells and the supernatant removed from these cells as C'_{0_0} super. The antiserum did not kill the cells in the presence of complement at 0°C. Fig. 2 presents data comparing the rate of cytotoxic reaction when the following supernatants at 37° were added to the C'_{0_0} cells: C'_{0_0} super (Curve 1); fresh complement with no additional Ca^{++} or Mg^{++} (Curve 2); heated complement (15 minutes 56°C) (Curve 3) and fresh complement in the presence of 0.02M EDTA (Curve 4). There was no difference between curve 4 and its control with EDTA but without complement. Each mixture was incubated at 37°C and sampled as described above. The results indicate that HAC'_{142} cells were not formed at 0°C because neither the heated complement nor the EDTA-treated complement, as sources of C'3, mediated the cytotoxic reaction. There is evidence that pretreatment of the cells shortened the time necessary for the reaction to reach a maximum. C'_{0_0} cells formed in the presence of 0.001 M Ca^{++} and 0.003 M Mg^{++} according to Becker's method(11), gave the same rate and extent of reaction as C'_{0_0} cells formed in presence of the amounts used routinely.

To determine if any complement activity was removed from the supernatant by sensitized cells at 0°C, C'_{0_0} super (37°C) was added to fresh sensitized cells, and to C'_{0_0}

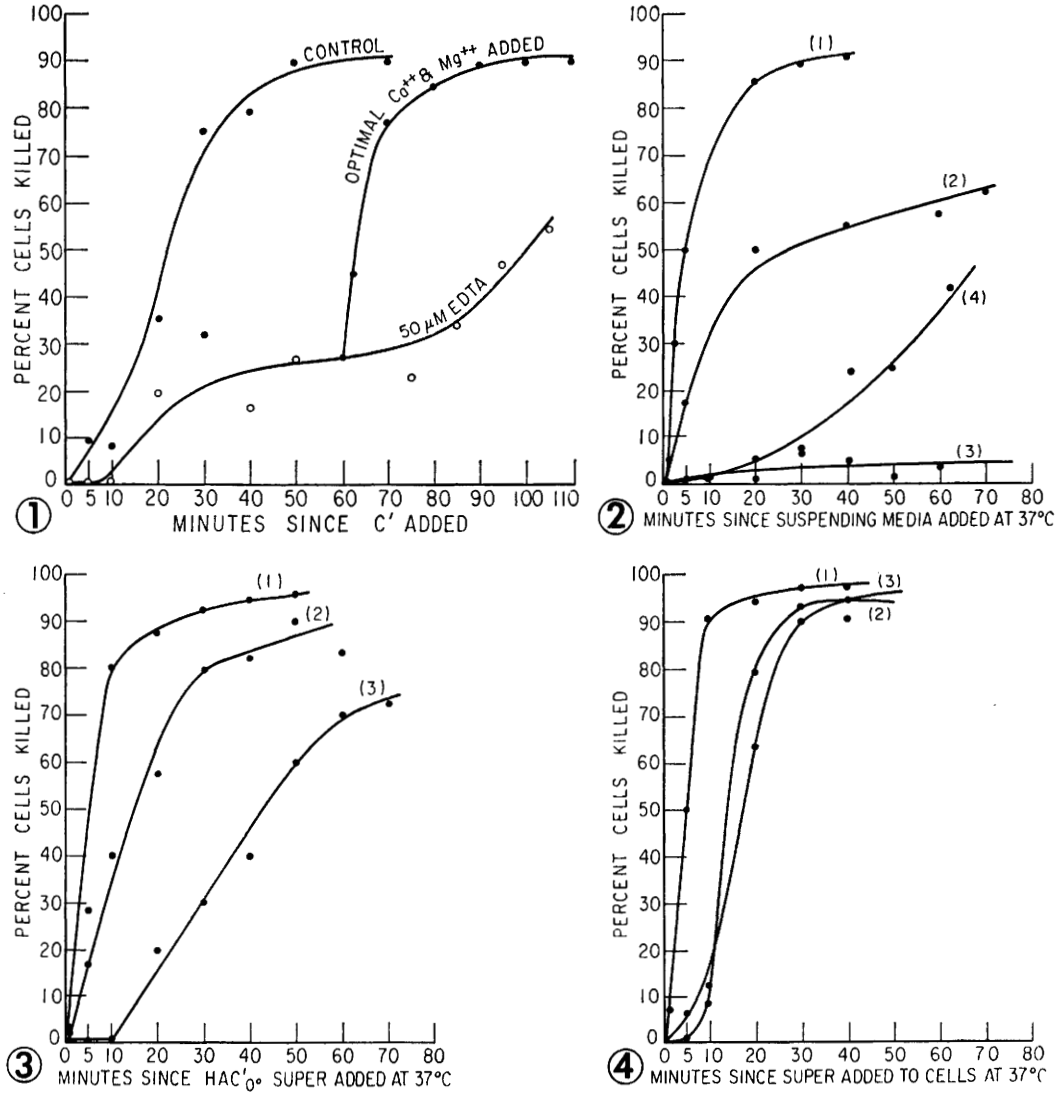

FIG. 1. Effect of divalent ions (Ca^{++} and Mg^{++}) on rate of cytotoxic reaction of antihistiocyte serum.

FIG. 2. Effect of addition of various suspending media to HAC'_{Oo} cells at 37°C on rate of cytotoxic reaction. (Curve 1) C'_{Oo} super; (Curve 2) fresh complement with no additional Ca^{++} or Mg^{++}; (Curve 3) heated complement; (Curve 4) fresh complement in presence of 0.02 M EDTA.

FIG. 3. Rate of cytotoxic reaction of HAC'_{Oo} super on: (Curve 1) HAC'_{Oo} cells, (Curve 2) fresh, sensitized cells, (Curve 3) unsensitized cells at 37°C.

FIG. 4. Effect of complement previously treated with unsensitized histiocytes on: (Curve 1) previously sensitized cells, (Curve 2) unsensitized cells after antiserum is added to the altered super. Curve 3 shows cytotoxic reaction of supernatant fluid taken from sensitized cells on fresh cells (test for unfixed antibody).

cells (37°C) as control. Addition of C'_{Oo} super to sensitized cells which had not had previous contact with complement resulted in cytotoxicity at a slower rate, and to a lower maximum (Fig. 3, curve 2) than occurred in the control reaction (Fig. 3, curve 1). The reaction of C'_{Oo} super with unsensitized cells (Fig. 3, curve 3) suggested residual antibody

activity in the C'_{0o} super. Evidence for residual antibody is seen in Fig. 4, curve 2, which shows the cytotoxic reaction of supernate removed from sensitized cells (to which complement had not been added) and in turn added to fresh unsensitized cells in presence of complement.

It was possible that histiocytes alone affected complement and resulted in production of a toxic material. Histiocytes were treated with complement at 0°C in the absence of antibody. The supernatant of these cells, designated "altered" C'_{0o} super, was tested for cytotoxic activity by adding it to fresh unsensitized cells at 37°C. The results shown in Fig. 4, curve 1, show that preincubation of complement with cells alone had no effect on the rate of the reaction. Fig. 4, curve 3, shows the effect of "altered" C'_{0o} super (mixed with antiserum at 37°C) added to fresh cells as compared to the reaction of "altered" C'_{0o} super added to previously sensitized cells (Fig. 4, curve 1). The lag evident in curve 3 suggests that sensitization of the cells is a rate-limiting reaction under the conditions of the experiment.

Discussion. The results of these preliminary experiments suggest that the reaction of guinea pig complement in the histiocyte immune system differs in certain characteristics from its reaction in the red cell immune system. The inability to form C'142 cells at 0°C recalls the human complement red cell system. The large amount of complement necessary in the histiocyte system is not explained by anticomplementary effects of the reaction constituents. Tests using reagents of complement(13) show that R1, R2, R3 (the latter prepared both by formalin(14) and Zymosan), E, M & H do not replace fresh guinea pig serum, supporting the view that complement is the constituent which is active in the cytotoxic system.

Summary. Preliminary observations of the cytotoxic action of guinea pig antirabbit histiocyte serum mediated by large amounts of guinea pig complement have been presented. The data suggest that guinea pig complement does not act in the same manner in this system as it does in the sheep-red cell system with respect to the amount required and to its action at 0°C.

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