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SECTION MEETINGS

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University of Illinois	November 10, 1959
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A Serum Cytolytic Factor Active against HeLa and Other Established Cell Strains.* (25393)

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Immune lysis has invariably been associated with serum complement since its discovery more than 6 decades ago. Four components together with divalent cations are required for the activity of complement. More recently, evidence has been obtained that additional factors are involved(1). (In the present communication complement is understood to refer to the aforementioned normally occurring serum factors, which together with specific antibody bring about cytolysis and bactericidal and virucidal effects as well.) During investigations concerned with immunological properties of human tumor cells (2) the presence of a lytic system in normal serum differentiated from complement was revealed. This lytic system is present in serum as an inactive complex consisting of a cytolytic factor (CF) coupled with an inhibitor (CFI). Both of these factors have been prepared in a relatively pure state from human and from horse serum. It has been established that the serum cytolytic factor, under appropriate conditions, causes rapid and complete destruction of human tumor cells *in vitro*. This communication reports in summary form the characteristics of the cytolytic system and the methods by which its components are prepared and studied. The present work had its origin in studies of antigenicity of pooled

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malignant human tissue by means of cytotoxic reactions in cell cultures(2). Those studies clearly showed that independently of their origin(3), human cancer cells possessed in common an antigen linked to a lipoprotein(4) which was not demonstrable in normal tissues. The same antigenicity was also demonstrated on cells of the established atypical cell lines HeLa (Gey) and Detroit-6. Antiserum prepared in horses(2) immunized with antigen from pooled human carcinomas(2) displayed characteristic cytolytic effects on the aforementioned cell lines(2). The cytolytic activity remained undiminished following absorption with normal human tissues(2) but the activity was completely removed upon absorption with antigen derived from any of 35 individual human carcinomas(3). Indeed, these findings have since been extended to show that the specifically absorbing antigenic structures were present without exception in a total of 60 carcinomas.

Methods. Purified horse antibody to pertinent antigen of pooled human carcinomas was prepared by binding of the virtually insoluble antigen with excess antibody in the cold and eluting the antibody from the antigen under conditions of increased ionic strength and temperature according to the method used by Heidelberger and Kendall for purification of pneumococcus antibody(5). Other attempts to obtain antibody preparations were: precipitation according to Felton(6); Cohn fractionation (Methods 6 and 10)(7,8); zone electrophoresis(9); continuous curtain electrophoresis(10,11,12); chromatography on CMC and DEAE cellulose(13,14) as well as on calcium phosphate gel(15); precipitation with Na_2SO_4; by $(NH_4)_2SO_4$ and isoelectric precipitation. In these ways preparations were obtained with varying amounts of specific antibody, the activity of which was titrated in triplicate series of cell cultures. For details see(2,4). A common feature of the preparations was their ability to cause retraction and agglutination of monolayers of HeLa and Detroit-6 cells. Unfractionated immune serum not only had the same effect but also caused lysis of the cells(2). As the immune serum was fractionated to concentrate the antibody by removal of other serum constituents, the resultant preparations displayed less and less of the lytic effect. In fact, the most potent and purest antibody preparations, prepared by means of elution and continuous curtain electrophoresis,† were devoid of lytic activity. Cytolysis is known to require participation of serum complement. This seems to be true *e.g.*, for the actions of rabbit antisera to HeLa cells(16,17). Accordingly, it was anticipated that the addition of fresh serum to purified antibody would reconstitute the lytic system. This was found to be the case; addition of fresh normal horse or human serum to purified specific antibody consistently provided a system which was lytic for HeLa cells. The determining factors in the cytolytic reaction such as those of time, temperature, pH and concentration had been elucidated previously(2). This information was therefore applied and the test was standardized so that with antibody held constant the reaction was read as a function of the activity of the accessory lytic factors contributed by normal serum. Once the conditions for this reaction had been established the identity with complement of the lytic factors, contributed by fresh serum, was investigated.

Results. Serum which had been frozen at −20°C for 6 months and serum inactivated by heating for 45 min. at 56°C were equal to fresh serum in providing cytotoxic activity when combined with specific antibody. This undiminished potency of heat-inactivated serum has been observed in hundreds of tests. Actually the activity of serum withstood heating to 71°C for 30 minutes but 30 minutes at 73°C resulted in total loss of activity. Subsequently serum was separated into euglobulin and pseudoglobulin fractions; the C′1 and C′3 containing euglobulin was inactive while the pseudoglobulin, containing C′2 and C′4, carried all the activity. Treatment of serum with ammonia under conditions known to inactivate C′4 and with zymosan in a manner which effects removal of C′3(18), did not adversely affect the cytolytic activity.

While seeking to determine whether complement was involved in the cytolytic effect, the requirement for calcium and magnesium ions was examined. Normal serum was sub-

† Apparatus: Spinco Model CP.

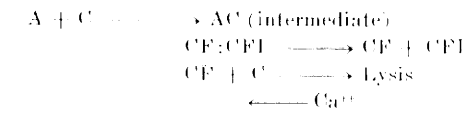
jected to protracted dialysis against distilled water. Except for calcium and magnesium the salt concentration of the dialyzed serum was restored. Different amounts of calcium and magnesium were then added; these were without effect on the cytolytic events. However, when the concentration of calcium was increased to an amount well above that present in serum, cytolysis was totally suppressed. This effect was obtained also with barium and strontium but not with a considerable number of other divalent cations.

These findings clearly showed that the lysis of HeLa cells was caused by accessory factors in serum other than complement. Consequently a system or mechanism different from any hitherto described was operating to bring about cytolysis. The magnitude of the lytic effect was of such proportions as to render it inconceivable that the factor or factors responsible for final fragmentation of the cell could exist in serum in an active state. This is self-evident, from the fact that these cells normally are propagated in the presence of serum. It was therefore postulated that activation of the accessory mechanism involved release from the influence of an inhibitor. Such release was assumed to occur as a consequence of the antigen-antibody reaction known to take place on the cell surface. Antigen-antibody reactions are influenced by van der Waals' forces; a mutual partial discharge of the reacting molecules occurs, leading to a decrease of solubility. Inasmuch as many types of antigen-antibody reactions on the surface of cells lead to these same consequences it was visualized that changes in the physical, rather than the chemical state, were involved. The forces which release lytic activity from initially inert serums are therefore likely to be relatively weak, possibly involving shifts in colloidal-chemical equilibria. Application of physical forces such as an electrical field and/or changes of ionic environment might therefore effect the same changes. If this were the case, and if the lytic factor could be prepared in an active state freed from its inhibitor, one would expect that the lytic factor itself would produce lysis even in the absence of antibody. Whatever the ultimate explanation, it was considered worthwhile to attempt the separation of the active lytic factor and its inhibitor. Toward this end serum was subjected to continuous curtain electrophoresis. With M/150 phosphate buffer at a pH of 7.9 a fraction was obtained which migrated slightly faster than albumen. Of the 25-30 fractions collected from each run only this fraction was found to exert lysis of HeLa, Detroit-6, heart, and conjunctiva cells all of which are known to possess atypical (malignant) properties. However, the same fraction had no discernible effect on freshly established cultures of dog kidney and testes; monkey kidney (second generation); rabbit spleen suspension; and human and sheep erythrocytes before or after sensitization with isoantibody and amboceptor, respectively.

The isolated cytolytic factor (CF) could be further purified by electrophoresis. It was readily adsorbed on calcium phosphate gel, $BaSO_4$, kaolin, Bentonite, polyvinyl chloride, and on CMC and DEAE cellulose. However, attempts to elute CF from these adsorbents were unsuccessful. The activity of CF was suppressed by calcium ions at levels of 0.5-1.0 mg/ml. Heating to 73°C for 30 minutes destroyed CF, but 71°C for 30 minutes was tolerated. Addition of whole serum to CF stoichiometrically inhibited its cytolytic effect, presumably as a consequence of excess of reactive inhibitor.

CF could be inhibited completely by a single serum fraction (CFI) precipitated from whole serum by decrease in ionic strength to 0.01 at a pH of 7.9. This fraction was relatively homogeneous by electrophoretic criteria. An inactive complex consisting of CF and inhibitor (CFI) could be prepared by mixing the 2 fractions and allowing them to stand at 0°C for 20-40 hours. The inactive complex thus obtained exerted no cytolytic effect. However, addition of specific antibody to this inactive complex resulted in full cytolysis. The cytolytic system, as it was first observed with whole serum and specific antibody, could thus be reconstituted. This sequence of events in the interaction of antibody, cell, and the CF : CFI complex may be depicted as follows:

Schema for immune activation of CF



Key: A = antibody; C = cell surface; CF = cytolytic factor; CFI = cytolytic factor inhibitor.

Specific antibody reacts with receptors on the cell surface resulting in a complex which, provided the cell exhibits active pinocytosis, is rapidly brought inside the cell. Thus the antigen-antibody complex is exposed only momentarily to factors present in the environment of the cell. The specific antigen-antibody reaction effects the release of CF from its native state of combination with inhibitor. The CF thus liberated at the cell surface causes a reaction, the results of which are immediate cessation of the movements of cytoplasmic granules and subsequent fragmentation of the cell. In the presence of Ca-ions, at levels well above the physiologic, CF no longer exerts these effects.

Many investigators have observed cytotoxic effects of serum on tumor cells; some of these observations extend back many years. In most instances, if not all, the heat-labile factors and the mechanism of the effect were not known. For the following reasons it is clear that the test system employed in the present work was not influenced by any of the previously reported serum activities: a) CF is not inactivated at 56°C; b) the cell strains used were propagated in a medium containing whole serum; c) whole serum by itself caused no lytic effect, indeed, serum suppressed the lytic effect of CF.

The interaction of CF and CFI in conjunction with the reactions of antigen-antibody has led to the recognition of a lytic system which had not been described heretofore. The information on CF as part of a biologic system is presently insufficient for the full interpretation of its significance. It is nonetheless apparent that a factor with these distinctive properties could have important implications for the reaction of the host to neoplasia.

Summary. Human serum was shown to contain a cytolytic factor coupled to an inhibitor. The lytic factor could be released by antibody specific to structures on the surface of human tumor cells. This factor could also be dissociated from its inhibitor by dialysis and obtained in a relatively pure state following electrophoresis. It then displayed a lytic effect on 4 established cell strains of human origin.

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