Antigenic Differences between Normal and Polyoma Virus-transformed Hamster Cells

I. A Quantitative Study of the Cytotoxic Effect of Antisera

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SUMMARY

Rabbit antisera against hamster embryo cells and hamster cells which had been transformed by polyoma virus were assayed by single cell technics. Antiserum killed cells in the presence or absence of complement, and the two mechanisms of inactivation appear to be different. Kinetics of cell-killing by antibody and complement are described. The transformed cells studied appear to have less surface antigen than embryo cells. Specific antibody to polyoma virus-transformed hamster embryo cells could not be found in an antiserum prepared against one such polyoma-transformed hamster cell line. The methodology is reported since it has proved capable of resolving antigenic differences between mammalian cells in the sense that an unabsorbed antiserum having equally strong sensitizing effects on two different cell types could be shown to specifically sensitize only one cell type after exhaustive absorption with the other.

Polyoma virus infection of mouse and hamster cells appears to bring about specific antigenic changes in the resulting neoplastic cells. Habel (4), Sjögren (11), Sachs (10), Atanasiu (1), and others have provided evidence that polyoma tumor transplants are more easily rejected by susceptible hosts if these animals have been previously immunized with polyoma virus. Presumably, there is induction of the new antigen in an immunized animal's cells, followed by an immune response which endows it with a specific (though weak) resistance to subsequent challenge by polyoma tumor cells. The resistance appears unrelated to antibody against the virus (4). Spleen and lymph node cells, rather than humoral antibodies, mediate the response. Fogel and Sachs could not find heterologous antibodies to the new antigen by in vitro tests (2).

In vitro evidence for the new antigen is needed to facilitate study of its location, chemical and physical properties, and factors governing its appearance. Attempts were made in vitro to detect a specific antigen in transformed hamster cells with heterologous antiserum. Although our initial attempt was also negative, the general methodology is reported in this article, since it has proved capable of resolving certain antigenic differences between mammalian cells and is the basis of a continuing study of this problem.

MATERIALS AND METHODS

Cells.—Hamster cell line “261” was isolated from in vitro-infected hamster embryo cells by Dr. K. Bayreuther. It rapidly produced tumors in hamsters. In vitro these cells have a generation time of 10 hours in modified Eagle's medium with 20 per cent calf serum. Single “261” cells plated on glass or plastic without feeder layer produce colonies several millimeters in diameter in 4–5 days. In this case the proportion of single cells which gave rise to colonies (plating efficiency) was 50–70 per cent.

Normal hamster embryo cells in secondary culture were maintained in 20 per cent fetal bovine serum in modified Eagle's medium. Although it is difficult to establish permanent cultures of such cells, 1–3 per cent were capable of producing macroscopic colonies in 5 days when plated in the above medium. This permitted them to be used in
assays of antibody and complement in the work described.

BHK is a tissue culture line of baby hamster kidney cells established by Dr. M. Stoker. This line gave slowly progressive tumors only many weeks after inoculation of 5 million cells into adult hamsters, presumably owing to a low frequency of "spontaneously" malignant variants. Its usual plating efficiency was about 30 per cent.

"651" is a variant of BHK which produced tumors rapidly from low density inocula. Plating efficiency was about 30 per cent.

PY-576-18 and PY-578-29 are typical in vitro transformed tumor cell lines isolated by Dr. M. Vogt. Plating efficiency was about 20 per cent. PY-576-18 showed evidence for the new antigen as detected in animal transplantation tests (by Dr. Vogt).

Media.—Eagle's medium with fourfold concentration of amino acids and vitamins was supplemented with 20 per cent calf or fetal bovine serum in single cell platings or with 10 per cent serum in maintaining continuous lines. Four-ounce glass prescription bottles or plastic Petri dishes were used in plating experiments. All cells were maintained at 37° C. in a CO2 incubator at 90—100 per cent humidity; 0.025 per cent trypsin was used to disperse cells in single cell platings and in transferring stock cultures.

Viability.—The criterion of cell killing was loss of capacity of single cells to produce macroscopic colonies in 5—6 days.

Staining colonies.—Two milliliters of 0.5 per cent methylene blue were added directly to the nutrient medium and allowed to stand for 30 minutes. The bottles were gently rinsed in tap water, and the stained colonies were counted without magnification.

Complement.—Normal guinea pig or rabbit serum provided complement. It was stored at —20° C.

Antiserum.—Adult rabbits were given four injections of cells or cell membrane fractions each week for 4 weeks; sera were collected 5—7 days after the last injection. The first injection each week was given subcutaneously, the last three intravenously. Each injection contained 5—10 million cells or cell membrane fractions from many cells.

Cell membrane fraction.—The procedure of Herzenberg and Herzenberg was followed (8). A cell particulate fraction was collected by centrifuging cell homogenates at 60,000 × g in a potassium bromide solution of specific gravity 1.23. The cell membrane fraction floated to the top and was easily scooped off. Direct observation with phase microscopy confirmed that the pellicle was mainly packed cell membranes, but since it may have contained other cell fractions the designation "cell membrane fraction" is not used strictly but only for convenience in the text.

Three types of experiments were performed.

1. Antiserum dilution vs. colony formation.—Replicate single cell platings (500 "261" cells or 5,000—10,000 hamster embryo cells) were made into dishes or bottles containing progressive dilutions of antiserum in the presence or absence of complement. Antiserum concentration was related to the proportion of colonies that developed, with reference to a sample exposed to pre-immune serum, and handled in an identical way. The suppression of colony formation by antibody will be referred to as cell killing.

2. Kinetic study of cell killing by antiserum and complement.—Single cell suspensions of between 2 × 10^4 and 4 × 10^6 cells/ml in medium with 10 per cent calf serum were exposed to antiserum and complement at 37° C. Samples were removed at intervals, diluted sufficiently to abolish further killing by antiserum, and then plated for survivors. Survival is referred to the proportion of colonies formed, as in case (1). Attachment of the cells to glass or to one another during the reaction was prevented by gentle pipetting and agitation; evidence for this was by direct microscopic observation as well as cell platings.

3. Kinetics of complement killing of cells previously sensitized with antiserum.—Cells in suspension were first exposed to antibody in the absence of complement. After 1 hour they were centrifuged down and resuspended in medium containing complement but no antibody. Samples were then plated for survivors, as in the kinetic study. Control cells were exposed to pre-immune serum and then were subjected to an identical complement treatment. This two-step treatment avoided interference of anticomplementary materials present in absorbed sera.

In all these experiments a hemacytometer was used to determine cell concentrations and to verify that the cells were indeed single. All platings (except in the few cases indicated) were done in duplicate. In the replicate plates the number of colonies formed generally varied by not more than 15 per cent of their mean.

Cells undergoing damage by complement reveal this by swelling and extruding their contents. For a review of this subject see Green and Goldberg (9). The damage was easily observed in the hemacytometer and was a guide to the taking of samples in kinetic studies. Suppression of colony-forming capacity of cells is, however, a more sensitive and objective criterion of effect; furthermore, it is less
subject to doubt and is readily applicable to following a wide range of dose effect. For these reasons it was used as the general experimental approach.

RESULTS
Cell killing by antiserum: extended exposure with or without complement.—Chart 1 shows a dose-effect relationship between antiserum concentration and suppressive effects on the colony formation of single "261" cells. Colonies arising from cells which survived antiserum and complement were normal in size (measurements not shown). Without complement, the same degree of cell killing was obtained only at antiserum concentrations 50–100

Chart 1 clearly shows that hamster cells were sensitive to antiserum against transformed cells and indeed were more sensitive to it than the transformed cells which were used as antigen. This will be discussed below.

Kinetic study of cell killing by antibody and complement.—Chart 2 shows an experiment in which "261" cells were treated with an antiserum and complement in suspension.

Chart 1.—Survival of "261" cells and hamster embryo cells in antiserum against "261" cells. ■ = Survival of "261" cells in antiserum and complement. Results of three experiments. Under these conditions embryo cells were tenfold more sensitive than "261" cells (data not shown). □ = Survival of hamster embryo cells, complement absent. Results of two experiments. ○ = Survival of "261" cells in antiserum without complement. One experiment. ◊ = Survival of both cell types which was unimpaired in heated pre-immune rabbit serum diluted 1/100 with or without complement.

Note: Complement in these experiments was due to that in normal calf serum plus 1 per cent fresh guinea pig serum. Cells which survived antiserum and complement produced normal-sized colonies. Those which survived high concentrations of antiserum in the absence of complement produced colonies which were tiny.

Anti-embryo cell antiserum or 4 per cent guinea pig serum alone had no detectable effect on survival. When both were present from the start the survival curve has a lag period and then a straight line portion. If cells were exposed to this antiserum alone during the early phase and then 4 per cent guinea pig serum was added at the time indicated by an arrow, rapid killing ensued at a rate equal to that attained by cells which were exposed to complement and antibody from the start.

Chart 3 shows similar experiments in which cell line "261" was exposed to varying concentrations of anti-"261" cell serum in the presence of excess complement. The steep curves on the left show that complement was adequate to permit the very rapid reaction at high antiserum concentrations.

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Doubling the antibody concentration shortened the length of the shoulder by half and made the slope about twice as steep. The relationship of proportionality of the antiserum concentration with the final slope and of inverse proportionality with the length of the shoulder held reasonably well through an eightfold concentration of antiserum. (Pre-immune serum and complement produced essentially no killing in 1 hour in this experiment. However, complement alone did have a strong cytotoxic effect on certain cell types. This factor was controlled in every experiment.) Thus, with a given cell type and with complement in excess, the lengths of the shoulders of such curves and their final slopes provide reliable comparisons of cytotoxic antibody concentrations.

On the other hand, as shown in Charts 3 and 4, anti-"261" cell antiserum at a given concentration produced more rapid killing of embryo cells than "261" cells (smaller shoulder, steeper slope). To further support the notion that embryo cells were more sensitive to this antiserum because they absorbed more antibody per cell, the following experiments were performed.

Antiserum against "261" cell membrane fraction was subjected to absorption on healthy monolayers of equal numbers of embryo cells or "261" cells. Complement was added in known amounts and the sera assayed against the same cell type—"261."

Chart 5 shows that absorption on either cell type reduced the killing power of the sera but that embryo cells were more effective in this. Chart 6 confirmed the above result in a kinetic study of the absorbed sera. Serum absorbed on "261" cells gave steeper killing curves than those absorbed on embryo cells, indicating that less antibody was lost from the serum to the absorbing cells.

The apparent deficit of antigen found on "261" cells does not appear to be accounted for by differences in cell size. This was shown by measuring with an optical micrometer the diameters of "261" and hamster embryo cells suspended by trypsinization.

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The average diameters were found to be 18 μ for "261" cells and 16 μ for hamster embryo cells, with a standard error of the mean of 0.45 μ and 0.40 μ, respectively.

Kinetic studies of cell killing by complement after sensitization with absorbed anti-"261" cell antiserum...
**Chart 5.** Assay of sera against "261" cell membrane fraction after absorption on monolayers of "261" cells or hamster embryo cells. ○ = activity left after 2 hr. on 1.6 × 10⁶ "261" cells. ● = activity left after 2 hr. on 1.6 × 10⁶ embryo cells. □ = activity left after 2 hr. more on a second monolayer of 1.6 × 10⁹ "261" cells. ■ = activity left after 5 hr. more on a second monolayer of embryo cells. ▼ = unabsorbed serum 1/14 for reference. C = pre-immune serum 1/14, heat-inactivated.

Titration of residual antibody was made by assay on "261" cells. Complement: normal amount in calf serum plus 1% guinea pig serum.

**Chart 6.** Kinetic study of killing of "261" cells at 2 × 10⁶/ml in the various absorbed sera of Chart 5. Symbols are the same as in Chart 5. Complement = 4% guinea pig serum.

X = immune serum 1/50 diluted through control embryo supernatant fluids. Each symbol represents the mean of two replicate assays. ⊗ = Pre-immune serum 1/14, heat-inactivated.

**Chart 7.** Killing of "261" cells by complement after exposure to antiserum against "261" cells, 1/960, for various lengths of time. X = control curve for complement only (4% rabbit serum). △ = control curve for serum without complement. ◇ = 5 minutes exposure to antiserum.

The 20-minute curve represents two separate experiments. The other curves represent one experiment. Each symbol is the mean value obtained from two replicate assays.

Cell concentration 3 × 10⁶/ml; colonies produced by cells which survived any of these treatments were grossly equal in size to control colonies.

**Chart 8.** Killing of hamster embryo cells by complement following incubation for 1 hour with antiserum against "261" cells. ○ = 1/960. ◇ = absorbed, 1/24. ■ = absorbed, 1/48. △ = pre-immune serum, 1/24, heat-inactivated.

Complement was supplied by 4% normal rabbit serum. Absorption was on 1.5 × 10⁶ hamster embryo cells. Each symbol represents a single assay expressed as a per cent of the number of colonies found in the initial sample taken less than 1 minute after exposure of sensitized cells to complement.
rum.—Extensive antigenic cross-reaction between embryo cells and “261” cells noted above made it necessary to absorb anti-“261” antiserum with hamster embryo cells and then to seek a residual serum capable of sensitizing “261” cells but not embryo cells. Multiple absorptions (30 minutes at 37°C, 30 minutes at 4°C) of 4 ml. of anti-“261” cell serum on a total 1.5 X 10^8 cells were carried out, and various cell types were tested in the two-step procedure described in “Methods.” An indication of the general form of such experiments is shown in Chart 7.

**Chart 8**—Killing of BHK cells by complement following incubation with antiserum against “261” cells. The serum had been exhaustively absorbed on embryo cells; it is the same as shown in Chart 8. ○ = 1/24 after absorption on embryo cells, cell concentration = 1.7 X 10^9/ml. □ = 1/8 after absorption on embryo cells, cell concentration = 2.5 X 10^9/ml. △ = pre-immune serum 1/24. □ = pre-immune serum 1/8, heat-inactivated. ● = immune serum, unabsorbed 1/960.

Each symbol represents a single assay.

Chart 8 shows that there was little or no residual antibody in the absorbed serum diluted 1/24 when tested on hamster embryo cells. Absorbed serum, at dilution 1/24 or 1/48, and pre-immune serum, at dilution 1/24, all gave the same degree of killing in the presence of 4 per cent normal rabbit serum. Although it was rather severe, this is a regular finding with embryo cells. This shows that there was no anticomplementary activity and that there was no specific antibody activity detectable in the absorbed serum.

Chart 9 shows a similar experiment with BHK cells in which the absorbed serum at 1/24 had very definite sensitizing effects. However, a further absorption of 1.8 ml. of this serum on 4 X 10^8 “651” cells left it devoid of activity against “651” or two typical polyoma-transformed cell lines (PY-576-18, PY-572-29). Thus, absorption with cells not transformed by polyoma virus removed all detectable activity in the serum (tested at 1/12 dilution) against cells transformed by polyoma virus. The result is especially significant, since the absorbed serum was tested at a concentration 80 times as high as one which gave very strong effects unabsorbed on “261,” the cell line which was used as antigen.

Chart 10 shows that an antiserum against cell line PY-576-18, absorbed on 1.5 X 10^9 embryo cells and 5 X 10^8 BHK cells could not sensitize BHK cells. It had very strong sensitizing effects on PY-576-18, the cell line used as antigen. Sensitivity to the dilute unabsorbed antiserum plus complement, as well as to complement alone, was roughly equivalent with these two cell lines. This serum, therefore, clearly discriminated between two hamster cell lines.

Potent antiserum to hamster embryo cells, absorbed similarly, revealed no such activity (data not shown).

**Chart 9**—Killing of BHK cells by complement following incubation with antiserum against “651” cells. The serum had been exhaustively absorbed on embryo cells; it is the same as shown in Chart 8. ○ = 1/24 after absorption on embryo cells, cell concentration = 1.7 X 10^9/ml. □ = 1/8 after absorption on embryo cells, cell concentration = 2.5 X 10^9/ml. △ = pre-immune serum 1/24. □ = pre-immune serum 1/8, heat-inactivated. ● = immune serum, unabsorbed 1/960.

Each symbol represents a single assay.

**Chart 10**—Cell sensitization by absorbed serum. Response of BHK cells (Chart 10a) and PY576-18 cells (Chart 10b) to complement after sensitization by antiserum against transformed cell line PY576-18 after absorption on 1.5 X 10^9 hamster embryo cells and 5 X 10^8 BHK cells. ● = unabsorbed serum 1/1800. ○ = absorbed serum 1/26. □ = pre-immune serum 1/26, heat-inactivated.

**DISCUSSION**

The goal of this work was an antiserum specific for the new cellular antigen induced in hamster cells transformed by polyoma virus.

A sensitive, quantitative method of detecting an antiserum is by its suppressive effect on the colony-forming capacity of single cells. Although antiserum without complement is effective and can discriminate between different cell types (Chart 1), complement increased 50- to 100-fold the sensitivity of the method and was used throughout this study.
Evidence presented by others (6) suggests that the cytotoxic action of complement on sensitized cells is "single-hit." In other words, a cell may be lysed even if only one antigenic site is activated by complement.

We do not wish to present a detailed analysis of the kinetic curves of cell killing by antiserum and complement, but it should be noted that their apparent "multiple-hit" nature does not conflict with the "one-hit" theory of complement action described by Mayer (6). Presumably, the shoulders observed in our kinetic curves (Charts 7–10) are due to the requirements that all components of complement act sequentially on a site sensitized by antibody before lysis can begin. The shoulders of Chart 3 probably arose owing to the increase by antibody before lysis can begin. The shoulders of Chart 3 probably arose owing to the increase with time of sites sensitized by antibody as well as to the multiple component nature of complement.

Normal-sized colonies were produced by cells surviving an antibody-complement treatment which killed 99 percent of the original cell population (Charts 1, 7). This all-or-none response is entirely consistent with the "single-hit" action of complement. Suppressive effects due to antiserum alone appear to be different in mechanism, since small colonies were produced by cells surviving extended exposure to high antiserum concentration in the absence of complement.

Evidence has been presented showing that there is a positive correlation between the capacity of cells to absorb antibody and their sensitivity to the killing action of antibody and complement. This is in agreement with findings of Möller and Möller (7).

The failure to detect a polyoma-specific antigen in cells of cell line "261" may be attributed to the fact that it had been in continuous culture 6 months by the time this study began and perhaps had lost the antigen. This is a definite possibility, since Weiler (12), Roizman (9), and others have demonstrated the loss of cellular antigens coincident with cell culture in vitro. This report has provided evidence that cells of line "261" possessed less (presumably surface) antigen than hamster embryo cells. Charts 1, 3, and 4 indicate that embryo cells were more sensitive to antibody against transformed cell line "261" than were "261" cells themselves. Moreover, Charts 5 and 6 indicate that embryo cells absorbed this antibody better than "261" cells.

Nonspecific losses of gamma globulin incurred during absorption on large numbers of cells may have eliminated the small fraction of the antibody for which we were searching. Nonetheless, small antigenic differences were clearly revealed (as in Charts 9 and 10) by the present methods which are now being used in a continuing study of the problem of antigenic variation induced by polyoma virus. The absorbed serum of Chart 10 has been tested on a variety of polyoma virus-transformed cell lines and other hamster cell lines. This work will be reported separately.

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