The Cytotoxicity of Serum for Mouse Mammary Cancer Cells

II. The Effects upon Cells in Culture*

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Samples of antiserum representative of tissues known to contain the mammary tumor virus have been found to be cytotoxic for mouse mammary cancer cells upon in vitro exposure before homoiotransplantation (10, 17, 19, 20). In the first paper of the present series (20), it was demonstrated that cytotoxic activity can be produced by antiserum representative of cells known to contain the virus whether from normal tissues or from a mammary carcinoma. Moreover, evidence was presented to show that the samples of antiserum representative of tissues without the virus had limited inhibitory effect.

It is the purpose of the present paper to describe the cytotoxic effects upon cellular cultures that resulted from contact in vitro with assorted samples of antiserum.

MATERIALS AND METHODS

Preparation of cell cultures.—The coverslip method (8) was employed for cultures. The tissues were cut by a sharp-edged cataract knife into pieces from 1 to 2 mm. in size, quickly washed with five changes of Hank’s balanced salt solution (13), and placed in a drop of chicken plasma on a coverslip. When the explants had been placed in position, the plasma was coagulated by adding a small drop of chick embryo extract, the coverslip was inserted in the bottom of a Pyrex test tube (180 X 15 mm.), 1.5 ml. of fluid medium was added, and the tube was sealed with a solid rubber stopper. The tubes were placed at an angle of approximately 5 degrees to permit the nutrient fluid to cover the explants, and cultures were kept at 37.5°C. until the explants showed sufficient growth, usually for 40–72 hours.

Preparation of medium.—The nutrient solution for growth of the tissues consisted of normal guinea pig serum, 84 per cent (GPS-84); chicken embryonic extract, 5 per cent (CEE-5); and Hank’s balanced salt solution, 81 per cent (H-61). The total amount of fluid in each culture tube was 1.5 ml.

Source of tissues.—The mouse tissues for culture consisted of transplantable mammary carcinomas and fetal intestines removed immediately after the mice had been killed. The mammary tumor explants were derived from mammary cancers that had originated spontaneously in mice of strain A and, thereafter, had been maintained by serial transfer in susceptible hosts of strain AZF, lacking the milk agent. The normal embryonic intestines were obtained from embryos of an AZF, mother lacking the milk agent. A stable strain of human malignant epithelial cells, strain HeLa (8), which had been maintained for months in this laboratory (36) was employed for control purposes.

Samples of antiserum.—Guinea pigs were used for the production of samples of test antiserum. The antigens for immunization were prepared from (a) transplantable mammary carcinomas, (b) normal lactating mammary glands with virus, and (c) normal lactating mammary glands without virus. The procedure for the preparation of antigens for immunization was described previously (20).

Test for cytotoxic activity in vitro.—The test for cytotoxicity in tissue culture was carried out in the following manner. When explants showed sufficient cellular outgrowth, commonly in from 40 to 72 hours, the culture medium overlying the cellular cultures was removed completely by a sterile glass pipette, undiluted test serum was added to permit it to react during incubation, and, after fixed periods of time, the serum was removed and the cultures rinsed with Hank’s solution. The effects, if any, of the test serum were observed by direct microscopic examination before and after the cells had been fixed and stained with Harris’ hematoxylin.

RESULTS

In the first series of experiments, 70-hour cultures of transplantable mouse mammary carcinomas were utilized (Figs. 1–19).

It can be seen from Figures 1–3 that cultivation for 70 hours resulted in epithelial sheets that extended from the explants. The cultures were exposed for from 3 to 5 hours to each of the various test samples of antiserum or to the control normal guinea pig serum. Exposure to normal guinea pig serum was without effect (Figures 1–8). On the other hand, the cultures which had been exposed to mammary tumor antiserum or to the antiserum associated with the normal lactating mammary glands without virus showed marked cytotoxic effects (Figures 9–11). The cultures which had been exposed to antiserum associated with the mammary tumor showed that the cultures had been killed by this preparation (Figures 12–19).
samples representative of mammary gland tissues with the virus, showed extensive degenerative changes (Figures 4–6 and 10–12), as evidenced by shrinkage of the cytoplasm and by pyknosis and irregularity in the shape of the nuclei. These changes were exaggerated with time. The nucleoli were inconspicuous and apparent in few cells. The cell outlines were indistinct, ragged, and irregular.

In contrast to these results, no evidence of alternative effect was observed in the cultures which had been exposed to antiserum samples representative of mammary gland tissues without virus (Figures 7–9). These findings were confirmed by observations upon eight cultures, or more, under four experimental conditions.

In the next series of experiments, 48-hour cultures of transplantable mouse mammary cancer were exposed to various samples of serum for 16 hours (Figures 13–18). Cellular degeneration was observed when the explants had been exposed to tumor antiserum (Figures 16–18). Contrariwise, the control cultures with normal guinea pig serum showed no detectable damage (Figures 13–15).

Since the tumor antiserum had been prepared from antigen derived from mouse tissues, experiments were carried out for control purposes to assess the role of antibodies to mouse tissues per se.

The effects of tumor antiserum sample upon epithelial sheets extending from explants of embryonic mouse intestines were tested. The intestines were obtained from embryos of an AZF1 mother lacking the mammary tumor virus. It can be seen from Figures 19–24 that the mouse intestinal cells were not damaged upon exposure to normal guinea pig serum or to tumor antiserum produced in guinea pigs. These results made it apparent that the cytotoxicity of antisera samples representative of tissues with the virus may be specific for mammary tumor cells; it is probably not related to antibody representative of normal mouse tissues. Unequivocal evidence for specificity will require cross-absorption studies and the employment of antigenic materials from mice of identical genetic constitution.

The demonstration of the cytotoxicity of antisera representative of mouse mammary cancer cells left open the possibility that the cytotoxic effect may be due to malignancy per se. In accord with this possibility, the effect of the mouse mammary cancer antiserum was tested upon a strain of human malignant epithelial cells, strain HeLa (8, 36). The results of this experiment are represented by Figures 25–27.

It can be seen from Figures 25–27 that mouse mammary cancer antiserum produced in guinea pigs had no apparent effect upon a strain of human malignant epithelial cells. These results indicated that the cytotoxic property of the antiserum is not necessarily due to malignancy per se.

Finally, attempts were made to develop cytotoxic antibodies in the homologous species. Mice of C strain origin were given repeated injections of strain A tumor which had been maintained in AXZbF1 mice. Thereafter, the mice were sacrificed, and the serum and lymph nodes were collected for test samples. Forty-eight-hour cultures of transplantable mouse mammary cancer of A strain were exposed to the undiluted pooled serum for a period of 3 hours. As it can be seen from Figures 28–30, no apparent effect was noticed.

Since there was a possibility that immune substances may be present in the lymph nodes of the immunized mice (21), a homogenized suspension of the pooled lymph nodes was prepared. This preparation was added to the 48-hour cultures of mouse mammary cancer cells, allowed to react for 3 hours and observed microscopically (Figures 31–33).

The absence of damaging effect was accepted as evidence that cytotoxic antibodies had not been elicited in the homologous species under the experimental conditions reported here.

DISCUSSION

The studies here described provided additional evidence of the cytotoxicity for mouse mammary cancer cells of samples of antiserum representative of mouse tissues known to contain the mammary tumor virus. Evidence was presented in previous studies (10, 17, 19, 20) that the antiserum was specific for mammary tumor cells. These results indicated that the cytotoxic property of the antiserum is not necessarily due to malignancy per se.

The coverslip method (3) was employed in culturing tissues from (a) transplantable mammary carcinomas derived from A strain mice and (b) for control purposes, fetal mouse intestines and a strain of human malignant epithelial cells, strain HeLa (8, 36). These cultures were exposed for from 8 to 6 hours to various samples of antiserum which had been prepared in guinea pigs. It was learned that mouse mammary cancer cells in culture were not damaged from exposure to normal guinea pig serum or to antiserum samples representative of tissues

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lacking the mammary tumor virus. Contrariwise, the cultures were severely damaged by samples of antiserum which had been prepared by utilizing tissues containing the mammary tumor virus. Finally, the control cultures of embryonic mouse intestine and human malignant epithelial cells were not damaged by various samples of antiserum.

Lambert and Hanes (24) and Lambert (23) were the first to employ the technic of tissue culture for study of cytotoxic substances. They utilized this approach for investigations of immunity to rat and mouse sarcomas. These tumors grew vigorously in plasma of normal animals; they showed little or no growth when cultured in plasma from animals which had been immunized by the injection of sarcomatous tissue. Lumsden (27–29) and Lumsden and Kohn-Speyer (30) reported the successful demonstration by tissue culture methods of cytoxins for cells of carcinoma M63 and of Jensen's rat sarcoma. Phelps (32) and Ludford (26) were unable to confirm this work. Niven (31) employed the tissue culture technic to demonstrate that rabbit antiserum representative of tissues of mouse embryos had cytotoxic effects upon normal epithelial cells (kidney, liver, intestine) as well as upon tumor cells of carcinoma M63, and a spontaneous adenocarcinoma of the mouse. More recently, Favorite and Cheever (5) were unable to show any significant differences in the growth rate of Brown-Pearce carcinoma cells which had immune or normal serum in the nutritive fluid. Pomerat (33) showed that cells of a Walker rat sarcoma 319 were inhibited in vitro by homologous (antirat) reticulo-endothelial immune serum (REIS), but not by heterologous (antichick) REIS in a similar concentration.

Other observations on the utilization of the tissue culture technic for the study of cytoxins have been concerned with the broader problems of their action on body cells in general. Antisera to various cells have been tested in tissue culture. For example, the list includes bone marrow and spleen (6, 34, 35), embryonic tissues (22, 31, 37), heart tissues (4, 14, 23), leukocytes (25), kidney cells (38), and brain cells (12). These studies indicate that cells of diverse sources can be employed as antigens for production of antisera, which are capable of destroying the corresponding cells in vitro.

Evidence for the antigenicity of the mouse mammary tumor virus has been established by a variety of serologic technics (1, 2, 7, 11, 15, 16, 18). However, the relationship of the antibody, or antibodies, concerned to the pathogenesis of the mouse mammary tumor is not known. Support for the anticipated finding that protective antibodies can result came (a) from the demonstration that samples of antiserum, representative of tissues known to contain the mammary tumor virus, were capable upon admixture before injection of rendering mammary cancer cells innocuous (20) and (b) from the direct observation made herein of the cytocidal effect of antibody upon mouse mammary tumor cells grown in vitro. This example of "protective" antibody is extraordinary. Thus, these findings suggest that the protective capacity results from the ability of the antibody to destroy cells which otherwise would prove lethal upon homoiotransplantation and not from a direct inactivation or inhibition of the pathogenicity of a nociocuous agent, such as a virus.

Lumsden (26) reported that antirat sarcoma serum was found to kill human cancer cells in vitro. Moreover, the antiserum which was prepared by employing as antigen human mammary cancer tissue was shown to be lethal for rat sarcoma cells. These results suggest that the cytotoxic effect may be related to malignancy per se. However, the results of the present study support the thesis that the cytotoxicity of mouse mammary cancer antiserum may be specific for mammary tumor cells. The evidence was the cytocidal effect of antiserum for mammary tumor cells and the failure of antiserum to injure mouse intestinal cells or a strain of human malignant epithelial cell, strain HeLa (8, 36).

The attempt to induce by the injection of mouse mammary cancer cells the production of cytotoxic antibodies in recipients of homologous species with different antigenic constitution, i.e., resistant mice, was unsuccessful. This finding is in agreement with that of Gorer and Law (9), who were unable to produce in mice neutralizing antibodies to the mammary tumor virus. In the present study, extracts of lymph nodes from the immunized mice also proved innocuous to the cultures of mammary tumor cells. By contrast, Kidd and Toolan (21) reported successful inhibition of mouse lymphosarcoma cells upon admixture with minced suspension of lymph nodes from immune mice. Moreover, serum from the same immune hosts had no effect on the lymphosarcoma cells.

**SUMMARY**

A cytotoxic study on mouse mammary cancer cells was made by utilizing the tissue culture technic. Mouse mammary cancer cells in culture were severely damaged by exposure for from 3 to 6 hours to samples of guinea pig antiserum representative of tissues known to contain the mammary tumor virus. The exposure under similar con-
The cytotoxic effect of antiserum upon cells of a transplantable mouse mammary cancer after 70 hours in cellular culture. Harris' hematoxylin stain. Photographed by Mr. Henry Morris. The magnifications are 65, 160, and 250.

Figs. 1-3.—Normal-appearing cells after exposure for 3 hours to normal guinea pig serum.

Figs. 4-6.—Mouse mammary cancer cells photographed after exposure for 3 hours to tumor cell antiserum produced in guinea pigs. The cells are undergoing degeneration as evidenced by the shrunken cytoplasm, pyknotic nuclei, and indistinct cellular membranes.

Figs. 7-9.—Transplantable mouse mammary cancer cells photographed 5 hours after exposure to guinea pig antiserum representative of the microsome fraction of normal lactating mammary glands without virus. The cells appear normal.

Figs. 10-12.—Degenerated mouse mammary cancer cells; photographed after exposure for 3 hours to guinea pig antiserum representative of the microsome fraction of normal lactating mammary glands with virus.

The effects of antiserum representative of mouse mammary cancer cells and normal cells in culture. Harris' hematoxylin stain. Photographed by Mr. Henry Morris. The magnifications are 65, 160, and 150.

Figs. 13-15.—Normal-appearing mouse mammary cancer cells; photographed after 64 hours in culture and 16 hours after exposure to normal guinea pig serum.

Figs. 16-18.—Degenerated transplantable mouse mammary cancer cells representative of a 64-hour cellular growth; photographed 16 hours after exposure to tumor cell antiserum produced in guinea pigs.

Figs. 19-21.—Forty-six-hour cellular growth of embryonic intestine from mice without virus; photographed 4 hours after exposure to normal guinea pig serum. The cells appear normal.

Figs. 22-24.—Normal-appearing cellular growth of embryonic intestine from mice without virus; photographed after 46 hours, in culture and 4 hours exposure to tumor cell antiserum produced in guinea pigs.
Control observations relating to the effect of mouse mammary cancer cell antiserum upon cancer cells in culture. Harris' hematoxylin stain. Photographed by Mr. Henry Morris. The magnifications are 65, 160, and 250.

Figs. 25-27.—Normal-appearing cells from strain HeLa; photographed after 51 hours in culture and 3 hours after exposure to mouse mammary cancer cell antiserum.

Figs. 28-30.—Transplantable mouse mammary cancer cells essentially normal in appearance; photographed after 51 hours in culture and 3 hours' exposure to serum from C mice which had been immunized with strain A tumor.

Figs. 31-33.—Normal-appearing mouse mammary cancer cells; photographed after 51 hours in culture and 3 hours' exposure to suspension of minced lymph nodes from C mice immunized with strain A tumor.
ditions of cultures to normal guinea pig serum or to antiserum representative of tissues lacking the tumor virus did not damage the cells. Control cultures of embryonic mouse intestines and of strain HeLa, of human malignant epithelial cells, were not damaged by various samples of antiserum. An attempt to produce cytotoxic antibodies in a resistant strain of the same species was unsuccessful.

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