A Study of Multinucleated Tumor Cells Demonstrating the Effect of Transplant Duration on Serum Changes in Cancer-bearing Hamsters

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SUMMARY

A cancer-induced change occurs in the serum of golden hamsters bearing transplantable cheek pouch tumors. The appearance of this change is a function of time after transplantation and the number of tumor cells injected into the animals. The serum change is caused by $1.5 \times 10^6$ tumor cells within 24 hr after transplantation. Removal of the cheek pouch by surgical excision caused the serum change to disappear within 6 to 8 days. The criterion used to detect these changes was the ability of the serum to reduce the number of hamster ascites multinucleated cells in vitro. The serum change appears to be associated with the seromucoid fraction of cancer serum.

INTRODUCTION

In 1959, Stevens and Schwenk (8) reported a new, multinucleated, hamster ascites tumor that had been developed in female golden hamsters, *Mesocricetus auratus*, from a methylcholanthrene-induced spindle cell sarcoma, nongonadal in origin (3). The ascites form of the methylcholanthrene-induced sarcoma was produced according to the method described by Klein (2).

Inoculation of this ascites tumor into male and ovariectomized hamsters significantly increased the percentage of multinucleated cells in the ascites tumor, compared with intact normal female hosts (4). It was then found that estrogens but not other steroids reduced the number of multinucleated cells both *in vivo* and *in vitro* (4). Subsequent *in vitro* studies were performed with washed cells suspended in an artificial culture medium in place of the previously undefined ascites fluid. Addition of estrone to the cells in this medium did not reduce the percentage of multinucleation unless the *in vitro* incubate was fortified with ascites fluid or with serum or plasma from cancer-carrying hamsters (5). Heterologous cancer serum (human and rat) also reduced the percentage of multinucleated tumor cells, whereas serum or plasma from normal individuals usually did not (7). On the basis of these results, it appeared that an active substance was present in hamster ascites fluid and cancer serum of several species that, together with estrogen, could cause a reduction in the number of multinucleated tumor cells.

Subsequent studies suggested that the serum change occurred in the seromucoid fraction of hamster serum or ascites fluid and was associated with the serum orosomucoid fraction (6).

Since cancer serum, together with estrogen, can cause a decrease in the number of multinucleated tumor cells *in vitro*, it was suggested that this decrease was a result of a cancer-induced serum change in seromucoids. Within this context, the present study was designed to determine (a) the length of time required for the appearance of the active substance after the injection of a known number of cancer cells into the hamster cheek pouch and (b) the length of time required for the disappearance of the factor after the excision of the tumor.

MATERIALS AND METHODS

Female golden hamsters, *Mesocricetus auratus*, weighing between 120 and 150 g, were used in all of the following experiments. Experimental and control animals were kept on a 12-hr light and dark cycle, and all animals were given a constant supply of Purina laboratory chow and tap water.

Two animals carrying ascites tumor 9 to 10 days were sacrificed with chloroform. The tumors from both animals were withdrawn with a sterile, 16-gauge needle and a 20-ml syringe, pooled in 45-ml plastic centrifuge tubes, and centrifuged at 6000 rpm for 5 min. The supernatant ascites fluid was then decanted. The cells were washed in an equal volume of sterile Hanks' balanced salt solution and recentrifuged for 5 min at 6000 rpm. The supernatant was then discarded and replaced with an equal volume of sterile 0.9% NaCl solution. Total tumor cell counts per ml were made with a Coulter Model B counter and then diluted with sterile 0.9% NaCl solution to obtain either $1.0 \times 10^6$ or $1.0 \times 10^5$ tumor cells/0.1 ml.

Female golden hamsters were anesthetized with pentobarbital sodium, 0.15 ml/100 g body weight. The left cheek pouch was everted with forceps and held flat against a corkboard with dissecting pins. A 21-gauge needle was used to...
inoculate the aboral end of the cheek pouch with 0.1 ml of diluted cancer cell preparation. The pouch was then replaced to a normal position.

All cheek pouch tumors were allowed to grow a total of 3 days, after which time the animals were anesthetized with pentobarbital sodium, and their pouches were grossly examined for the presence of solid tumor masses, tied off at the proximal end with No. 3 surgical silk, and excised.

At requisite times throughout the experiments, a maximum of 2 blood samples were drawn by heart puncture from each animal while it was under light ether anesthesia. Each blood sample was placed in a 10-ml plastic centrifuge tube, heparinized, and centrifuged. A 0.5-ml aliquot of each plasma sample was placed in a 25-ml siliconized Erlenmeyer flask, coded, and frozen until used.

Briefly, the tumor in vitro incubation procedure was as follows. Female golden hamsters that had been ovariectomized 14 days previously were inoculated i.p. with 0.25 ml of ascites tumor. Ten days later, the ascites fluid was removed from 4 or more animals, pooled, and centrifuged. The precipitated cells were resuspended in cold Hanks' solution, recentrifuged, and then suspended in Medium 199. This procedure resulted in washed tumor cells suspended in a known medium.

"Test aliquots" were prepared from 4 ml of the above preparation, 0.5 ml of an estrone solution, and 0.5 ml of the serum to be tested. The final volume was 5 ml and contained 1.5 to 2.0 X 10^7 tumor cells/ml and 0.1 µg estrone per ml. Control experiments were prepared in the same manner, except that test serum was not added to the final incubate. Standard experiments contained 4 ml of tumor preparation, 0.5 ml of estrone solution, and 0.5 ml of ascites fluid. Duplicate 2.5-ml aliquots were incubated in 25-ml Erlenmeyer flasks for 6 hr at 37°C in a Dubnoff metabolic bath in an atmosphere of 95% O2:5% CO2. The shaker was operated at 60 rpm.

After incubation, the cells were smeared, fixed, and stained. Results were determined by counting 1000 tumor cells for each sample and calculating the percentage of multinucleated cells.

Preparation of the hamster ascites tumor cells for incubation, in vitro incubation procedures, assay techniques, and statistical analysis have been previously described in detail (5).

RESULTS

In the initial series of experiments, 1.5 X 10^6 ± 10% tumor cells were injected into the left cheek pouches of normal female golden hamsters. Twenty-four hr after transplantation, blood was drawn from the animals and the plasma was prepared and frozen. Three days after transplantation, additional blood samples were drawn and the cheek pouches were examined for the presence of solid tumor masses and then excised. The results from these experiments are presented in Table 1. Plasma from 91% of all hamsters given injections of 1.5 X 10^6 ± 10% tumor cells developed the ability to decrease the number of multinucleated cells by 24 hr after transplantation. By 3 days, 100% of the hamsters exhibited plasma activity, and at this time their tumors were excised.

Since a significantly large number of animals developed activity by 24 hr, another experiment was conducted to determine whether or not the plasma became positive prior to this time. Seven animals were given injections of 1.5 X 10^6 ± 10% tumor cells, and blood samples were taken at requisite times throughout the experiments, a maximum of 2 blood samples were drawn by heart puncture from each animal while it was under light ether anesthesia. Each blood sample was placed in a 10-ml plastic centrifuge tube, heparinized, and centrifuged. A 0.5-ml aliquot of each plasma sample was placed in a 25-ml siliconized Erlenmeyer flask, coded, and frozen until used.

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<table>
<thead>
<tr>
<th>Test group</th>
<th>No. of animals tested</th>
<th>Time after transplantation (days)</th>
<th>% multinucleated cells</th>
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<tr>
<td>Control</td>
<td>15.50 ± 0.15a</td>
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<tr>
<td>Standard</td>
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<tr>
<td>Cheek pouch-plantation, 1.5</td>
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<tr>
<td>× 10^6 ± 10%</td>
<td>11.50 ± 0.21b</td>
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<td>tumor cells</td>
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<thead>
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<th>Test group</th>
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<tbody>
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<td>Standard</td>
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<tr>
<td>Cheek pouch-plantation, 1.5</td>
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<tr>
<td>× 10^6 ± 10%</td>
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<tr>
<td>tumor cells</td>
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<td>× 10^6 ± 10%</td>
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<td>regenerating liver cells</td>
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[Table 1: The in vitro effect of plasma from hamsters bearing cheek pouch tumors on hamster multinucleated cells: influence of time and cell number]

[Table 2: The in vitro effect of plasma from hamsters bearing cheek pouch tumors on hamster multinucleated cells: influence of tumor excision and time]

[Table 3: The in vitro effect of plasma from hamsters inoculated in the cheek pouch with regenerating liver cells]
Temporal Changes in Cancer-bearing Hamster Serum

12 hr afterwards. Subsequent in vitro incubation of this plasma indicated that no animals were active (see Table 1).

Once it had been established that 100% of the hamsters exhibited a positive plasma change by 3 days, experiments were conducted to determine when the serum change disappeared. At varying times after the tumors had been excised, blood samples were drawn from each group of animals exhibiting serum activity at 3 days to determine when the serum change disappeared. The results are presented in Table 2. Of all animals tested 5 days after tumor excision, 100% exhibited positive plasma activity. This percentage decreased to 45.5 on the 6th day and to 38 on the 7th day. By the 8th day, only 9% of the animals were still active. Zero % activity, 100% inactivity, occurred on the 9th and 10th days.

Several cheek pouches, each of which had received an inoculation of 1.5 X 10⁶ tumor cells 3 days previously, were fixed in 10% neutral formalin, sectioned in paraffin, and stained with Harris’ hematoxylin and eosin. Microscopic examination of each slide revealed the presence of a small, well-defined tumor with a central necrotic area and an actively growing cortical region.

The appearance of a specific, cancer-induced serum change is most likely related to the number of cancer cells present in the host. This hypothesis was examined by transplanting 1.0 X 10⁵ ± 10% instead of 1.5 X 10⁶ ± 10% tumor cells into the cheek pouch. Their plasma was tested for activity (see Table 1).

It can be seen in Table 1 that no activity was present in the plasma up to 4 days after the smaller tumor cell inoculum. However, examination at that time revealed small tumor nodules that, if allowed to continue growing, eventually caused the plasma to become positive. No attempt was made in this study to correlate the size of the tumor cell inoculum with the time required for plasma activity to appear.

Control experiments were run with regenerating liver cells as a source of noncancerous tissue. The regenerating liver cells were obtained from female hamsters 70% hepatectomized 3 days previously. Two groups of female golden hamsters received cheek pouch transplantsations of 1.5 X 10⁶ ± 10% regenerating liver cells. Four days later, blood samples were obtained from the animals and the plasma was tested for activity (see Table 3). A decrease in multinucleation did not occur in any of the samples.

DISCUSSION

The experiments described here show that a cancer-induced change occurs in the serum of golden hamsters bearing transplantable cheek pouch tumors and that the appearance of this change is a function of the time after transplantation and the number of cells injected into the animals. The criterion used to detect this change was the ability of the serum to decrease the number of multinucleated cells in vitro. Once the tumors were excised and the host was free of cancerous tissue, the serum change disappeared within 6 to 8 days.

An evaluation of these data suggests that the presence or absence of activity may be attributed to an increase or decrease in serum factor pool size. Since the factor appears to be a constituent of the seromucoid fraction, it is probably subject to normal metabolic pathways that would degrade it after a period of time. If the presence of cancer directly stimulates the production of factor, and breakdown of factor normally takes place at some time after its formation, it follows that a net drop in factor pool size will occur once the host is free of cancerous tissue. By the 6th and 7th days postexcision, a significant net drop had occurred, so that the plasma from many of the animals could no longer decrease multinucleation.

Of particular interest is the identification of the serum factor responsible for inducing cleavage in multinucleated ascites tumor cells. Recently reported results indicate the serum factor shows an isoelectric point and electrophoretic mobility consistent with its being a simple or complex polypeptide or protein (1). Further studies suggest that it occurs in the seromucoid fraction of serum or ascites fluid of hamsters carrying the HA ascites tumor and may be orosomucoid (6). Many reports have shown increases in the glycoprotein fraction of serum not only in clinical and experimental cancer but also in rheumatic fever, pneumonia, and rheumatoid arthritis, as well as in other pathological conditions (9). However, the hamster multinucleated cell system appears to be specific for seromucoid changes associated with cancer.

In view of the provisionally demonstrated specificity of the hamster multinucleated cell system for seromucoids in cancer serum but not in other pathological conditions, further investigations with this system might be useful not only in monitoring serum for a protein associated with neoplasia but also in isolating a natural product specific for cancer serum.

REFERENCES

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