Replication of an Avian Myxovirus in Tumor Cell Cultures Obtained from Effusions of Mammary Carcinoma Patients

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SUMMARY

In view of the possible use of viruses for the immunotherapy of breast cancer, the replication of a strain of fowl plague virus was studied in the tumor cells of 11 mammary carcinoma patients with malignant effusions. The tumor cells were obtained by centrifugation on iodamide solutions, then cultured in vitro, and infected by a fowl plague virus previously adapted to grow in a mammary carcinoma cell line. Virus multiplication was observed in all cases, a prerequisite for the use of autologous viral oncolytics for immunotherapy in mammary carcinoma patients.

INTRODUCTION

There is strong evidence for the presence of TAA's in human mammary carcinomas (17). The TAA's in general, are weak immunogens (16). Augmentation of the immunogenicity of TAA by infection with viruses has been shown in several systems (1, 2, 4, 14, 15). Enveloped viruses, mostly influenza viruses, were used for this purpose, since the association of viral and host antigens at membrane sites is apparently important for the augmentation of tumor cell immunogenicity (14).

For the use of viruses as immunological potentiators in the immunotherapy of human neoplasms, the virus has to replicate in a tumor cell with the TAA in question. FPV, an influenza A virus, has been adapted to several types of human tumor cells, including a human mammary carcinoma cell line (6, 7, 19), and has been shown to replicate regularly in primary cultures of human leukemic cells (20). Currently, the leukemia-adapted FPV is used for immunotherapy in patients suffering from acute myelogenous leukemia in a prospective clinical trial of the Swiss group for clinical cancer research (22).

This study was designed to test the replication ability of FPV in primary cultures of breast cancer cells from individual patients. Successful virus replication in such cells provides the basis for a clinical trial with mammary carcinoma patients similar to the 1 mentioned above with patients suffering from acute myelogenous leukemia.

MATERIALS AND METHODS

From 14 patients suffering from metastatic mammary carcinoma with pleural effusion or ascites, tumor cells were isolated, put into culture, and infected with an avian myxovirus. Eleven patients had never received cytostatic or hormonal treatment, 2 were under cytostatic drugs for more than 1 year, and, in 1 patient, oophorectomy had been done 3 weeks prior to the pleurocentesis.

Exudate, 400 to 800 ml, was collected in 250-ml plastic flasks (Falcon Plastics, Oxnard, Calif.) containing 1500 units of heparin. Separation of tumor cells from other cells (mainly leukocytes and erythrocytes) of the effusions was done by the following method (10). After centrifugation of the exudate at 200 × g for 10 min at 4°, the cells were washed in MEM and resuspended in 30 ml MEM. This cell suspension was layered on an iodamide [mixture of sodium and methylglucamine salts of 3,5-diacetoamido-2,4,6-triiodo-benzoic acid (Urografin; Schering, Berlin, West Germany)] solution (density of 1.078, pH 7.2) and centrifuged at 200 × g for 30 min at 4°. The cells with a density of less than 1.078 were put on another iodamide solution (density of 1.065, pH 7.2) and centrifuged again at 200 × g for 30 min at 4°. The 3 cell populations, separated according to their densities (>1.078; 1.078 to 1.065; <1.065), were washed in MEM, resuspended in 15 ml of Roswell Park Memorial Institute Medium 1640 with 10% fetal calf serum, and incubated at 36° in three 25-sq cm Falcon plastic bottles. Smears for cytological examination were prepared from each fraction.

Those bottles containing a monolayer of tumor cells (usually after 3 weeks) were washed with MEM and either used for cytological examination or for FPV replication experiments. Then 0.33 ml of a FPV suspension (input titers, see Table 2) was added. The infected cultures were incubated for 45 min at 36° with agitation every 15 min for virus adsorption. MEM, 4.67 ml, with 2% fetal calf serum was then added and incubation was continued. The supernatant was removed (and replaced by 5 ml of fresh medium) at 4, 22, 46, and 94 hr of incubation. The removed samples

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4 The abbreviations used are: TAA, tumor-associated antigen; FPV, fowl plague virus; MEM, Eagle's minimum essential medium.
were checked for hemagglutinin, put into sterile glass ampuls, and kept at −80° until infectivity titrations could be performed.

The FPV (A/Turkey/England/63, Hav1 Nav3, Langham strain) that we used in these experiments had been adapted to the human mammary carcinoma cell line BT 20 as previously described (19).

Virus infectivity assays were performed in 16- x 100-mm glass tubes containing chicken embryo fibroblast monolayer cultures. The monolayers were infected with serial 10-fold dilutions of virus in MEM without serum. Six tubes/dilution were used. After 72 hr of incubation at 37°, the tubes were screened for cytopathogenic effect. Tubes with incomplete destruction of the monolayers were checked for hemagglutinin. The 50% tissue culture infectious doses were calculated according to the method of Reed and Muench (18). The hemagglutinin titrations were done by WHO standard procedures.

RESULTS

No tumor cells could be cultivated in 5 experiments where we tried to cultivate tumor cells from malignant effusions from mammary carcinoma patients without the iodamide separation. However, of a total of 14 patients, 11 effusions yielded, after the iodamide separation, monolayer cultures that, by cytological examination, proved to consist of tumor cells.

Table 1 shows the results of the cytological examination of the unseparated effusions and the results of the cell cultures after the iodamide separation. Of the 14 original effusions, 11 were examined cytologically. In 8 of the 11 effusions, tumor cells were detected.

After centrifugation on the iodamide solutions the composition of the different cell populations was as follows: (a) density < 1.065 (Fig. 1B), mainly tumor cells and lymphocytes, a few monocytes; (b) density 1.065 to 1.078, mainly lymphocytes and fewer tumor cells than in the lighter fraction, a few monocytes; (c) density > 1.078 (Fig. 1C), mainly erythrocytes and neutrophilic granulocytes, occasionally a few tumor cells.

From the 1st 6 effusions, only the cells with a density of < 1.065 and those with a density of > 1.078 were cultured. As seen in Table 1, tumor cell monolayers were always obtained with the cells of a density of < 1.065, provided tumor cells were found in the smears of the original effusion. In 1 of the 3 cases where no tumor cells could be detected in the smears of the original effusion, tumor cells could be observed after the iodamide solution centrifugation, and in this case a tumor cell monolayer grew in culture. The culturing of cells of a density of 1.065 to 1.078 was also successful. In 5 of 8 effusions, tumor cell monolayers developed. In the fraction with the cells of a density > 1.078, only in 2 instances could a tumor cell monolayer be obtained.

Only tumor cell monolayers from the fraction with a density of < 1.065 were used for experiments with FPV. A strong virus replication was found in all of the 11 tumor cell cultures. The virological data are shown in Table 2. The virus input was always between 50 and 150 infectious particles/culture. Since the whole culture supernatant was removed at 4, 22, 46, 70, and 94 hr of incubation (and

<table>
<thead>
<tr>
<th>Tumor cell monolayers obtained with cells of different densities from malignant effusions from patients with mammary carcinomas</th>
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<tr>
<td>Tumor cells present in the smears of the original effusions</td>
<td>Tumor cell monolayer obtained with the cells of a density of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1.065</td>
<td>1.065-1.078</td>
<td>&gt;1.078</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8/8*</td>
<td>4/5</td>
<td>2/8</td>
</tr>
<tr>
<td>No</td>
<td>1/3</td>
<td>0/2</td>
<td>0/3</td>
</tr>
<tr>
<td>Not done</td>
<td>2/3</td>
<td>1/1</td>
<td>0/3</td>
</tr>
<tr>
<td>11/14</td>
<td>5/8</td>
<td>2/14</td>
<td></td>
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</tbody>
</table>

* Number of complete tumor cell monolayers in culture/number of effusions tested.

<table>
<thead>
<tr>
<th>FPV replication in primary cultures of human mammary carcinomas</th>
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<tbody>
<tr>
<td>Patient</td>
<td>Virus input*</td>
<td>Virus production from 0 to 4 hr of incubation* Maximal virus production</td>
</tr>
<tr>
<td>1</td>
<td>2.0</td>
<td>2.2</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
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<tr>
<td>3</td>
<td>2.1</td>
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<td>1.3</td>
</tr>
<tr>
<td>11</td>
<td>1.9</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* Log 50% tissue culture infectious dose.
* Total amount of virus produced during the incubation periods mentioned (log 50% tissue culture infectious dose).
* HA, hemagglutination.
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... replaced by fresh medium as described in “Materials and Methods”), the total amount of virus replication could be measured in each interval between the hours mentioned.

The minimal virus production was always found between 0 and 4 hr of incubation. Maximal virus production occurred between 24 and 46 or between 46 and 70 hr of incubation in all cases, except 2 where maximal FPV production was detected between 70 and 94 hr after infection. The maximal hemagglutinin titer was observed at the time of maximal production of infectious FPV or 24 hr thereafter. The hemagglutinin reached its peak earlier in 1 patient only (Patient 2).

Chart 1 shows the complete data of a representative FPV replication experiment in a primary culture of mammary carcinoma cells (Patient 11).

**DISCUSSION**

The adaptation of an avian myxovirus (FPV) to a human mammary carcinoma cell line (BT 20) was shown in previous work from this laboratory (19). In the present report we have demonstrated that this virus replicates also in primary cultures of human mammary carcinoma cells. The 2 main aspects of these experiments, to be discussed here, are the production of tumor cell monolayer cultures from mammary carcinomas and the FPV replication in these cultures.

Breast cancer patients present often with malignant pleural effusion or ascites. This source of tumor cells was chosen because the cells are in small aggregates or in single cell suspension and should therefore be brought easily in monolayer cultures. On the other hand the tumor cell suspension is always contaminated by a variety of cells (lymphocytes, neutrophilic granulocytes, monocytes) that might inhibit tumor cell growth in vitro. (a) Lymphocytes are known to be cytotoxic in vitro for autologous tumor cells, even from progressor patients, provided the patient's serum is eliminated (9). (b) Neutrophilic granulocytes were shown to contain lysosomes (3) from which interferon might be released (21), exerting then its antitumor activity (8). (c) The effect of monocytes on cancer cells in vitro has recently been demonstrated (11). It is therefore not surprising that all attempts at obtaining tumor cell monolayers from these tumor cell-leukocyte mixtures failed.

The success in obtaining tumor cell monolayer cultures from every single effusion (see Table 1) after the iodamide separation is probably due to the separation of certain leukocytes from the cancer cells. Lymphocytes alone [this is certainly surprising in view of the results of Hellström et al. (9)] did not inhibit the formation of a tumor cell monolayer. The tumor cells and the lymphocytes were found in the same fraction. In the neutrophilic granulocyte-rich fraction, tumor cells were found only occasionally, but tumor cell monolayers developed also in 2 of 8 cases. Neutrophilic granulocytes alone thus do not seem to be the cause of the failures to obtain tumor cell cultures with the unseparated cells. These observations suggest therefore that the simultaneous presence of lymphocytes and other cells (neutrophilic granulocytes or monocytes) is deleterious to the formation of tumor cell monolayers in vitro.

The FPV replication in these primary cultures of human mammary carcinomas was excellent. The adaptation of the virus to human mammary carcinoma cells seems to be optimal. The production of infectious virus is almost as good as with the original virus in the original host cell [chicken embryo fibroblasts (6)]. The dynamics of virus production corresponds well with that of other influenza A viruses, i.e., almost no virus production during the first hours, a time when influenza A viruses are in the latent period (5), and then maximal virus replication between 24 and 72 hr of incubation.

In conclusion we can say that the most important prerequisite for the clinical application of myxovirus oncolysate in the immunotherapy of mammary carcinoma is fulfilled, i.e., the successful replication of a myxovirus in tumor cells from individual mammary carcinoma patients. This replication appears to be necessary for the creation of the intimate association of viral and cellular antigens at various stages of the infectious process. These hapten-carrier relationships are thought to form the basis of the immunological potentiation by viruses (13).
ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Smears of a malignant pleural effusion from a mammary carcinoma patient (Table 2, Patient 2). Giemsa stained, × 1400. A, unseparated effusion; B, cells with a density of < 1.065 (mainly tumor cells); C, cells with a density of > 1.078 (mainly erythrocytes and granulocytes).
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