Modification of the Immunologic Response to Human Choriocarcinoma in the Hamster Cheek Pouch by Heterologous Antilymphocyte Serum

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SUMMARY

Because the immune response to human choriocarcinoma growing in the hamster cheek pouch makes it difficult to evaluate the responsiveness of the tumor to drugs, we have studied this immune response in untreated hamsters and hamsters immunologically suppressed with heterologous antilymphocyte serum (ALS).

Untreated animals were immunized by the tumor beginning as early as 6 days after implantation. Once an animal rejected the tumor, it would not accept a second implant of that or any other strain. This immune state was due to species-specific antigens, for it could be induced by injection of whole human blood.

Treatment of hamsters with ALS resulted in a higher incidence of growth of tumors from a given donor tumor, increased tumor volume, and longer tumor survival. These effects were the result of the potent immunosuppressive action of ALS and may facilitate the establishment of heterotransplant tumors for each patient to be evaluated for therapy. Methotrexate had less oncolytic effect on choriocarcinoma in ALS-treated hamsters than in untreated hamsters. This suggests that the evaluation of drugs may be more reliable in the absence of the immune response to these xenogeneic tumors, for the strain of choriocarcinoma used was not responding to Methotrexate when obtained from the patient.

INTRODUCTION

The response of human choriocarcinoma, growing in the cheek pouch of golden Syrian hamsters, to oncolytic agents has been used as a means of selecting drugs for clinical trial (11). In an effort to extend these studies, we encountered difficulty in differentiating the immunologic rejection of the tumor by the hamster from the oncolytic effect of the drug. Because of this initial difficulty, we have evaluated the immune response of untreated hamsters to human choriocarcinoma and have studied a method of suppression of this response. Since antilymphocyte serum (ALS) is the most potent immunosuppressive agent available (14), and is not oncolytic, its effect on the hamster's immune response to human choriocarcinoma has been evaluated. The response to Methotrexate of tumor growing in the pouch of ALS-treated hamsters has been compared with the response in hamsters not undergoing immunosuppression.

MATERIALS AND METHODS

Hamsters

Female golden Syrian hamsters (Mesocricetus auratus) of 6 weeks of age or older were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Maryland.

Tumor

The tumors used in these studies were the WO, RE, and CA strains of the Erwin-Turner human choriocarcinoma, which were originally isolated by Hertz from patients with gestational choriocarcinoma (10). All 3 strains can be maintained by serial transplantation in untreated hamsters.

Tumor-implanting Technic

The technic used for implanting tumors into the cheek pouch of anesthetized hamsters has been described previously (10). Implants measuring 1.0–1.5 mm in each dimension were used. Matched study groups were obtained by random selection from animals which had been implanted with fragments from a single donor tumor. Tumor growth was evaluated by observation, palpation, measurement, and histologic evaluation. A tumor was considered viable when it had a reddish-purple color, firm consistency, and enlargement to at least 20 times its initial volume. These criteria were used successfully in our laboratory for selecting donor tumors for 50 successive transplant generations. The viability of each tumor used as a donor for a study or removed from a hamster at the end of a study was confirmed by histologic evaluation and by the growth of fragments of these tumors when reimplanted into the cheek pouches of untreated hamsters. Tumor volume was determined from 3 perpendicular diameters using the formula
for determining the volume of an oblate spheroid (8). Mean tumor volume was determined by dividing the sum of the volumes of the viable tumors in the study by the total number of animals bearing these tumors. Relative tumor frequency at any period in the study was determined by dividing the number of animals with viable tumors at that time by the total number of animals in the study.

The percent of donor implants which developed into viable tumors was variable depending upon the quality of the donor tumor employed. Our cumulative average in untreated hamsters was 75%. Particular attention was employed to select uniform tumor fragments for implantation into study and control animals. For each donor tumor employed in an experiment, tumor fragments were also implanted into the cheek pouches of untreated hamsters in order to determine the quality of the donor tumor. When it was necessary to use more than one donor tumor for a study, animals bearing implants from each donor tumor were evenly divided between the experimental and control groups.

**Immunization Studies**

The immunologic status of hamsters which had rejected a strain of choriocarcinoma was evaluated by observing the growth of a second implant of the same tumor strain, or a different tumor strain, in the opposite cheek pouch. The effect of immunizing animals with whole human blood was also evaluated. Four injections of 0.2 ml of type O, Rh positive human blood were given intraperitoneally at weekly intervals. Tumor implantation was done one week following the last injection. The time required for immunization of hamsters by tumor growing in the cheek pouch was evaluated by amputating the tumor-bearing pouch at various intervals following implantation. The pouch of an anesthetized hamster was fully everted and amputated at least 2 cm proximal to the visible tumor margin. All of the amputated tumors were evaluated histologically.

**Antilymphocyte Serum**

ALS was prepared in New Zealand rabbits by a modification of the method of Gray et al. (9). Single cell suspensions containing 150 X 10^6 cells from thymus glands of weanling hamsters were mixed with equal volumes of complete Freund’s adjuvant and injected into the foot pads. Five weeks later 150 X 10^6 thymus cells were injected intravenously daily for 5 days. Rabbits were bled by cardiac puncture at varying intervals beginning one week after the last booster injection. Serum was separated from clotted blood by centrifugation, heated to 56°C for 30 minutes, and stored at -20°C. The serum was not absorbed with red cells. Lymphoagglutinin titers were determined by a modification of the methods of Payne (18) and Dausset (5). Lymphoagglutinin titers of the antisera used in these studies varied from 1:32 to 1:512. The same batch of antiserum was used for all animals in an experiment. ALS was given subcutaneously at varying doses and intervals as described for each experiment.

The effects of ALS on human choriocarcinoma growing in the hamster cheek pouch was evaluated in a variety of experiments.

**Dose-Response Effect.** Groups of 12 hamsters received 0.025 ml, 0.05 ml, and 0.1 ml ALS subcutaneously 3 times a week beginning at the time of tumor implantation. The same tumor used in these animals was simultaneously implanted into 18 control hamsters. Relative tumor frequencies and mean tumor volumes were determined weekly. Each animal was weighed weekly.

**Prolonged Administration.** Ninety-eight hamsters were given 0.1 ml ALS subcutaneously 3 times a week for 10 weeks beginning at the time of tumor implantation. The same tumor was implanted simultaneously into 38 controls. ALS was discontinued at that time because the tumor size was interfering with the ingestion of food. Tumors persisting at the end of the study were implanted into untreated hamsters as a test of viability and were also evaluated histologically. All animals were examined for metastases.

**Effects on Immunization and Established Immunity.** Amputation and reimplantation studies were carried out in animals receiving ALS to determine if ALS affected initiation of immunization of the hamsters by choriocarcinoma growing in the cheek pouch. Tumors were amputated 7 days after implantation into 6 hamsters which received ALS. ALS (0.1 ml) was given every other day for 3 doses beginning on the day of tumor implantation. Seven untreated controls, which had received the same donor tumor, underwent pouch amputation at the same time. Viability of each of these tumors was verified histologically. Four weeks after amputation, tumors were reimplanted into these 2 groups, as well as a third group of untreated hamsters, to determine if ALS had affected the time of immunization.

The effect of ALS on established immunity was evaluated by reimplanting tumors into hamsters that had previously rejected a viable tumor. Tumors were implanted into 21 animals that had previously rejected the WO strain choriocarcinoma and also into 21 controls. The previous rejectors received 0.1 ml ALS subcutaneously 3 times a week.

**Subcutaneous Tumor Implantation.** Implants from the same donor tumor were placed subcutaneously into 20 hamsters and into the cheek pouches of 10 untreated hamsters. Ten of the hamsters with subcutaneous implants received 0.1 ml ALS 3 times a week. All tumors were excised 2 weeks after implantation, measured, and evaluated histologically.

**Antilymphocyte Serum and Chemotherapy Studies**

WO strain choriocarcinoma from one donor animal was implanted into 3 groups each consisting of 20 hamsters. Each group of 20 received 8, 12, or 16 mg/kg body weight Methotrexate i.p. 3 times a week beginning the day of implantation. Ten of the animals in each group also received 0.1 ml ALS s.c. 3 times a week. The animals were anesthetized, the pouches were everted, and the tumors were measured twice a week beginning one week after implantation. Relative tumor frequency and mean tumor volumes were determined at the time of each inspection.
RESULTS

Untreated Hamsters

Growth and Regression of Tumor. The 3 strains of human choriocarcinoma used in these studies have a characteristic growth pattern and appearance which have previously been described qualitatively by Hertz (10). The histology of the WO strain growing in the cheek pouch is shown in Fig. 1.

Chart 1 shows the results in 326 consecutive untreated hamsters. Most viable tumors reached their maximum size at 2 weeks and were rejected by 3 weeks. A few tumor implants exhibited delayed onset of growth, but they then went through a characteristic growth and rejection pattern. As noted in Chart 1, the average volume of tumors exhibiting this delayed growth was somewhat greater than the average size of those showing earlier growth. There were no metastases in these untreated hamsters. The maximum mean tumor volume in untreated hamsters was 0.8 ml. At some time during the 5 weeks, 76.4% of these animals developed viable tumors. The ovaries and uteri of these animals showed marked evidence of tumor gonadotropin production, as evaluated by appearance, weight, and histology. Measurable amounts of human chorionic gonadotropin were present in the serum of tumor-bearing animals (21).

Immunization Studies

Reimplants of all 3 tumor strains failed to grow in 58 hamsters which had previously rejected WO strain tumors. As noted in Table 1, the viability of the donor tumors used in these reimplantations was confirmed by the development of 46 viable tumors in 60 untreated control animals. One strain of choriocarcinoma, therefore, immunized these hamsters to all 3 strains of this tumor.

Table 1

<table>
<thead>
<tr>
<th>Chorio strain</th>
<th>WO strain rejections</th>
<th>Controls</th>
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<tr>
<td>WO</td>
<td>0/20</td>
<td>16/21</td>
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<tr>
<td>CA</td>
<td>0/18</td>
<td>16/18</td>
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<tr>
<td>RE</td>
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Growth of 3 strains of human choriocarcinoma implanted into other pouch of previous rejectors of WO strain and controls.

Animals immunized to human antigens by human blood injections developed only one viable tumor in 20 implanted animals. The same tumor grew in 17 of 20 nonimmunized control animals. Immunity to this tumor, therefore, results from the response to xenogeneic antigens common to human blood and to all 3 strains of choriocarcinoma.

The time of immunization was studied by amputating tumors at 2-day intervals from Day 4 to Day 14 after implantation. Twenty-three animals with histologically viable tumors at the time of amputation were reimplanted with tumor fragments 4 weeks later. When tumor was reimplanted in the 7 animals with amputations on Day 4, 7 viable tumors developed. When tumor was implanted in the 16 animals undergoing amputation on Days 6 to 14, only 3 viable tumors developed. The 3 animals which subsequently grew tumors had undergone pouch amputation at Day 6 (one animal) and Day 12 (2 animals). Tumors used in these reimplantation studies grew in 26 of 32 control animals. This indicates that the animals were not immunized by tumor growing in the pouch only 4 days, whereas the majority of animals were immunized by tumors growing longer.

Effects of Antilymphocyte Serum

Dose-Response Effects. Chart 2 shows that increasing doses of ALS result in a greater percentage of viable tumors, an increase in size, and longer survival. These effects are consistent with a dose-related suppression of the immune response by the hamster to this tumor xenograft and also indicate that ALS did not have an oncolytic effect.

Prolonged Administration. Chart 3 presents the effects of administration of 0.1 ml of ALS 3 times a week for 10 weeks. These effects are contrasted with the results in a series of untreated controls which received the same tumor. Mean tumor volumes are several times greater than any tumor ever seen in an untreated hamster. Viable tumors persisted in the cheek pouch in gradually diminishing numbers until the end of the study. Two of these animals had pulmonary metastases.
Fig. 2 shows the metastasis in the right lung of one of these animals. This could not be explained by direct invasion by the tumor, for it was implanted in the left cheek pouch. Metastases of choriocarcinoma in hamsters have never been observed in our laboratory, except in animals receiving ALS.

**Effects on Immunization and Established Immunity.** The immunization of hamsters by choriocarcinoma growing in the pouch 7 days prior to amputation was apparently not suppressed by the administration of ALS. When tumor was reimplanted in the 6 animals receiving ALS prior to pouch amputation on Day 7, tumor grew in only one of the 6. Reimplantation into the 7 controls who had pouch amputations on the same day resulted in no growth. The tumor used in these regraft studies grew in 23 of 30 untreated controls. Apparently the ALS had little effect on the immunization of these animals.

ALS caused a definite suppression of established immunity. In the 42 rejectors receiving reimplants in the other pouch, tumor grew in 4 of the 21 who received ALS at the time of reimplantation but in none of the 21 untreated rejectors. Tumor grew in only 9 of 21 untreated controls receiving the same tumor at this time. These results demonstrate that ALS interferes with established immunity in this system.

**Subcutaneous Tumor Implantation.** The effects of ALS on subcutaneous tumor growth was evaluated on tumor excised 2 weeks after implantation. Excision for measurement and histologic evaluation was necessary for subcutaneous tumors because the usual method of sequential inspection of cheek pouch tumors could not be used. In the 20 animals with subcutaneous implants, histologically viable tumors were found in 6 of 10 ALS-treated animals and 3 of 10 controls. Viable tumor was present in the cheek pouch of 3 of 10 untreated animals receiving the same tumor. The mean volume of subcutaneous tumor in ALS-treated animals was 0.19 ml, while the volume of subcutaneous tumor in the untreated animals was 0.03 ml. The average volume of tumors in the cheek pouch of untreated animals was 0.17 ml.

**Antilymphocyte Serum and Chemotherapy Studies**

The effect of immunosuppression with ALS on the apparent responsiveness of choriocarcinoma to Methotrexate is shown in Chart 4. The tumors of immunosuppressed animals were less responsive to Methotrexate. This was true for each dose of Methotrexate but was most striking at 16 mg/kg where only one animal without ALS ever had a tumor that met the criteria of viability (color, consistency, and size), whereas 7 of 10 animals with ALS and the same dose of Methotrexate had viable tumors. Tumor volumes were determined and gave similar results. The effect of ALS on the cumulative tumor mass in this study is shown in Chart 5. Cumulative tumor mass was calculated as the product of relative tumor frequency times average tumor volume observed at 8, 10, 15, and 17 days after implantation. At each dose of Methotrexate the ALS-treated animals had a greater cumulative tumor mass. The cumulative tumor mass lines began to converge at higher doses, which suggests that at higher doses of Methotrexate complete suppression of tumor growth would occur even in ALS-treated animals.
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Chart 4. The effects of immunosuppression with heterologous antilymphocyte serum (ALS) on responsiveness of human choriocarcinoma cheek pouch implants to different doses of Methotrexate (MTX). All 6 groups of hamsters received implants from the same donor of WO strain human choriocarcinoma. Relative tumor frequency was calculated as defined in Chart 1.

Chart 5. The cumulative tumor mass in normal and antilymphocyte serum (ALS)-suppressed hamsters receiving different doses of Methotrexate (MTX) (same animals as in Chart 4). Cumulative tumor mass (ml) is calculated as the product of relative tumor frequency (R. T. F.) times the average volume of viable tumors at 4 periods of measurement: 8, 10, 15, and 17 days after implantation.

DISCUSSION

The observation that human gestational choriocarcinoma can be cured by several different drugs is now well established (1, 3, 12, 15, 22). An *in vitro* model to study the responsiveness of this neoplasm to different drugs has several potential uses. The first is the search for new drugs for clinical trials. Another use is of potential importance in the treatment of patients with drugs already proven effective. Because these drugs do respond to one drug but not to another. An *in vitro* method to evaluate the effects of several separate drugs on a patient’s tumor early in treatment would be of help in deciding whether to change the drug being used. At the present time this decision requires evaluation of human chorionic gonadotrophin (HCG) response after several courses of treatment. An *in vitro* chemotherapy model is clinically pertinent only if a tumor responds to more than one drug, and the drugs do not have the same mode of action.

Hertz (10, 11) and Pierce *et al.* (20) have reported on this responsiveness of human choriocarcinoma growing in the hamster cheek pouch as a chemotherapy model. However, their observations point out some of the limitations of the system. Hertz showed that 5 strains of tumor which were not responsive to Methotrexate in the patient were responsive when in the hamster cheek pouch. Pierce *et al.* (20) reported that human choriocarcinomas of gestational and testicular origin were both responsive to Methotrexate when growing in the hamster cheek pouch. This does not parallel clinical results. Both of these observations suggested that the cheek pouch system in untreated or cortisone-treated hamsters was only a fair method for predicting clinical response. The rapid period of tumor growth followed by a brisk immunologic rejection by the hamster also limited the use of the system because of the difficulty of separating drug effect from immunologic rejection. In an effort to improve the usefulness of the model, we have studied the nature of the immune response to the tumor and the effect of immunosuppression with ALS on this response.

Even though the hamster cheek pouch is an immunologically privileged site (2), our data show that as early as one week after implantation the animal is already immunized. This early immunization may occur because of the invasiveness of this tumor leading to penetration of the layer of areolar tissue in the pouch, which is thought to account for the delay in sensitization of the hamster by tissue growing in the pouch. The period of rapid tumor growth in an untreated hamster occurs after the animal is already immunized but before the immunologic rejection overcomes the inherent growth potential of the tumor. The studies of tumor implants in previous rejectors and in animals immunized with only whole human blood indicate that the response is to species-specific xenogeneic antigens. This finding is in accord with that reported by Hertz (10).

ALS was found to be effective in suppressing the hamster’s immune response to the tumor. ALS was employed because of its potent immunosuppressive activity for normal tissue grafts (17, 24, 25), as well as allogeneic or xenogeneic tumor grafts (7, 13, 19). Our results indicate that ALS was not itself oncolytic and that it allowed increased tumor growth by delaying the immune rejection of the tumor. The finding that ALS did not delay the time of immunization suggests that ALS functions at a later stage of immune-mediated events. This agrees with the hypothesis that ALS depletes or inactivates the immunoreactive lymphocytes which have an essential role in the immune rejection of foreign grafts (14, 17).
It was previously demonstrated that adult thymectomy increased the immunosuppressive effectiveness of ALS in our tumor xenograft system (6). Adult thymectomy delays immunologic maturation of lymphocytes and has a significant immunosuppressive effect only in animals with a deficiency in immunoreactive cells (4, 16). The effectiveness of adult thymectomy in ALS-treated hamsters further indicates that the effectiveness of ALS in our system results from the action of ALS on the immunoreactive cells of the hamster.

Metastases of human choriocarcinoma from the pouch were found only in ALS-treated animals. Hertz (10) has not seen this in 10 years of work with this tumor in untreated or cortisone-treated hamsters. Early in these studies ALS was shown to be more effective than cortisone in suppressing the immune response to this tumor. The addition of cortisone to ALS had little effect on immunosuppression and resulted in increased toxicity. The effectiveness of ALS was not the result of general toxicity since ALS-treated animals bearing tumors for 10 weeks continued to gain weight. The effects of ALS were not limited to cheek pouch tumors since ALS also increased the growth of subcutaneous tumors.

Our studies suggest that immunosuppression with ALS results in a better model for evaluating the response of the tumor to a drug. Sommers et al. recently reported that ALS decreased the chemotherapeutic responsiveness of human melanoma in the cheek pouch of cortisone-treated hamsters (23). This suggests that in the absence of ALS the immune rejection of the tumor interferes with the evaluation of the drug. Our finding that Methotrexate was less oncolytic in ALS-treated hamsters suggests the same conclusion and is encouraging since the tumor strain used came from a patient whose tumor was not responding to Methotrexate.

ACKNOWLEDGMENTS

We would like to thank Dr. Roy Hertz for supplying these 3 tumor strains.

REFERENCES

Fig. 1. WO strain human choriocarcinoma growing in the hamster cheek pouch 10 days after implantation. The tumor is composed of both syncytiotrophoblastic and cytotrophoblastic cells, as well as hemorrhage and necrosis. The outer layer is composed of hamster cheek pouch and cellular reaction to the tumor. H & E, x 100.

Fig. 2. Human choriocarcinoma metastatic to right lung of hamster after implantation in the left cheek pouch of an antilymphocyte serum-treated hamster. The tumor is in an area of consolidated lung tissue and is separated from the liver by the diaphragm. H & E, x 40.
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