Effect of Serial Passage in Nude Athymic Mice on the Growth Characteristics and Chemotherapy Responsiveness of 13762 and R3230AC Mammary Tumor Xenografts


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ABSTRACT

Serial passage of the R3230AC and 13762 rat mammary adenocarcinomas for 20 generations in nude athymic mice revealed changes in biological and chemotherapy response characteristics indicating a tumor-host relationship that questions long-term stability of the tumor xenograft-nude mouse test system. Unresponsiveness to L-phenylalanine mustard (NSC 8806), characteristic of the R3230AC tumor, remained stable, but attempts to reestablish progressive growth in syngeneic hosts were unsuccessful. Responsiveness to L-phenylalanine mustard, a characteristic of the 13762 tumor marked by oncology and many complete remissions in syngeneic hosts, was significantly reduced with no complete remissions after the tenth passage. When tumors were reestablished in syngeneic hosts, tumor growth pattern and responsiveness to L-phenylalanine mustard returned to normal by the second passage. Clearly definable acinar structures typical of the R3230AC and 13762 adenocarcinomas were markedly reduced in the R3230AC and disappeared in the 13762 after serial passage in nude mice. Acini were still absent from the 13762 tumor after being reestablished for three passages in syngeneic females. The chromosomal modes of both tumors were unchanged. These results stress the need for careful monitoring of growth and of biological and chemotherapeutic response characteristics and for periodic replacement of tumor lines to assure long-term stability of the tumor xenograft-nude mouse test system.

INTRODUCTION

Increasing clinical acceptance of early chemotherapy as an adjuvant to surgery and emphasis on the use of combinations of chemotherapeutic agents as well as therapeutic modalities have accentuated the need for tumor test systems that could predict the clinical success of new as well as of combinations of existing chemotherapeutic agents and treatment modalities. The advent of the nude mouse with its thymic aplasia (8) has provided the experimental chemotherapist with a seemingly simple means of addressing the question, "What could be more predictive of a human tumor response than a human tumor," even as a xenograft in a nude athymic host?

The feasibility of establishing human tumors as successful xenografts has been amply demonstrated (4, 5, 12, 18, 19). The idea of establishing human tumors derived from various organs in serial transplantation to serve as preclinical test systems for drug and therapy evaluation is a logical consequence and certainly worthy of an intensive evaluation. However, one should be aware that, although the nude mouse reportedly lacks the classical cell-mediated rejection mechanisms, this apparently clear-cut distinction has recently been qualified. Using a xenogeneic anti-T cell antiserum, Lamelin et al. (7) found that 1% of cells in spleen from nude mice were labeled in an indirect immunofluorescence assay. More recently, Raff (15) reported that 0.2 to 0.8% of spleen cells and 0.4 to 1.6% of lymph node cells from nude mice from various sources fluoresced when exposed to specific fluorescein-conjugated anti-Thyl (8) antibody. These reports and those of Pritchard and Micklem (14) and Andersson et al. (1) leave little doubt that nude mice harbor T-cells. On the other hand, numerous reports strongly suggest that in nude mice thymus-dependent functions are absent (3, 6, 9, 17, 20). It appears likely, therefore, that nude mice may have immunologically immature T-cells capable of differentiation into immunocompetent T-cells. The studies of Ramseier (16) show what appear to be T-cell precursors in spleens of nude mice that can be activated to recognize alloantigens specifically.

Regarding humoral factors, the IgM response of nude athymic mice to antigens may be augmented in the absence of suppressor T-cells and, while IgG and IgA responses are markedly diminished in the absence of helper T-cells, the capability for responsiveness, however impaired, is nevertheless there (2, 10, 11, 20, 21). Therefore, the immunogenetic relationship between the tumor xenograft and nude athymic host still is not quite the same as in the autochthonous or even in the syngeneic systems. The possibility of changes in the cellular or structural composition of serially transplanted tumor xenografts, as a consequence of the selective pressures resulting from the immunological elimination or suppression of highly antigenic clones, must be of real concern.

In view of the possibility that immunoselective pressures may alter the cellular composition of xenografts during repeated passaging, there is also the question of the stability of the chemotherapy responsiveness of these test systems. The study that forms the basis for this report addressed itself to these 2 questions by monitoring the histology and the responsiveness to L-PAM (2) of 2 rat mammary...
adenocarcinomas passed in nude athymic mice for 20 transplant generations.

MATERIALS AND METHODS

The 2 rat mammary tumors used in this study were selected because (a) they were both histologically definable as adenocarcinomas; (b) they both originated in and are 100% transplantable in syngeneic Fischer 344 females; and (c) they respond differently to treatment with L-PAM, i.e., the R3230AC tumor is unresponsive, whereas the 13762 tumor is very responsive, a responsiveness characterized by a high percentage of complete remissions followed by regrowth of drug-resistant tumors.

The mammary adenocarcinoma designated R3230AC originated spontaneously in the laboratory of Wilhelmina F. Dunning, Papanicolaou Research Institute, Miami, Fla. It metastasizes only occasionally. The 13762 mammary adenocarcinoma, induced with 7,12-dimethylbenzanthracene in the laboratory of Albert Segaloff, Ochsner Medical Foundation, New Orleans, La., has a high incidence of metastases. Both tumors have been carried in serial transplantation in syngeneic females for over 8 years in the authors' laboratory. For this study both tumors were passaged in nude athymic females received from the Frederick Cancer Research Center, Frederick, Md. The animals were housed in restricted, pathogen-free facilities at the Mason Research Institute, Worcester, Mass.

Tumor grafts were implanted s.c. on Day 0 as 2-mm cubed pieces, by means of a 13-gauge trocar, on the right side midway between the axilla and inguinal areas. Treatment with L-PAM was initiated on Days 12 or 14 when tumors were well established in the early log phase of measurable growth. L-PAM was administered p.o. at 3.2 mg/kg/injection 3 times weekly in Klucel (0.3 g hydroxypropyl cellulose:0.9 g NaCl in 100 ml distilled water; Carter-Glogau Laboratories, Melrose Park, Ill.) Tumor sizes were measured with calipers 3 times weekly and were plotted as the average of the largest and smallest diameters.

Tumor sections were taken for histology at time of transplantation, i.e., in early log phase of measurable growth, and were fixed in Stieve's solution, paraffin embedded, and stained with hematoxylin and eosin. Chromosome analyses were made on tumors in midlog phase of measurable growth. Tumor-bearing animals were treated with colchicine, and chromosome spreads were prepared and stained with lactic-acetic orcein.

RESULTS

Response to Chemotherapy

The responsiveness of established R3230AC mammary adenocarcinoma xenografts to L-PAM in the 2nd, 10th, and 20th transplant generations in nude athymic mice is compared as tumor growth curves in Chart 1. The lack of response of the treated tumors illustrated in the 2nd transplant generation persisted through the 20th passage.

Chart 2 illustrates tumor growth and regression curves of control and L-PAM-treated xenografts of the 13762 mammary adenocarcinoma in the 2nd, 6th, 10th, and 20th transplant generations in nude athymic mice. The oncolytic effect of L-PAM as well as the emergence of drug-resistant tumors despite continuing therapy, demonstrated in the second and sixth passages, mimic the response of this tumor system to L-PAM in the syngeneic host. However, responsiveness to L-PAM treatment progressively decreased during subsequent serial transplantation as indicated by Passages 10 and 20, although emergence of drug-resistant tumors, after maximum remission, persisted.

Histological Characteristics

The effect of serial transplantation in nude athymic mice on the histology of the R3230AC tumor is illustrated by the following observations.

First Passage Diagnosis. Well-differentiated carcinoma...
of the breast. Eighty % of this tumor mass is composed of an epithelial carcinoma containing many acini. There are many mitoses, and there is about 20% necrosis and fibrous tissue (Fig. 1). The appearance is typical of this tumor passed in syngeneic hosts.

**Tenth Passage Diagnosis.** Moderately-well-differentiated adenocarcinoma of the breast. The tissue submitted consists of a very cellular noncircumscribed mass composed of cells arranged in sheets and nests. Slight acinar development was present. The cells comprising the mass were moderately pleomorphic with oval, round, and irregular nuclei and a moderate amount of amphophilic cytoplasm. Nuclear:cytoplasmic ratios were high, and mitotic figures were moderate in number. A fine connective tissue stroma and vascular supply support the mass. The epithelial:stromal ratio was about 99:1 (Fig. 2).

**Twentieth Passage Diagnosis.** Poorly differentiated adenocarcinoma of the breast. Tumor mass is less well differentiated than in first and tenth passages where one can observe some acinar development (Fig. 3).

The effect of serial transplantation in nude athymic mice on the histology of the 13762 tumor is illustrated by the following histopathology reports.

**First Passage Diagnosis.** Well-differentiated adenocarcinoma of the breast. There is about 80% epithelium with many acini. Necrosis and fibrous tissue amount to about 20%. The tumor is growing rapidly and is very invasive as is seen by remnants of muscular tissue (Fig. 4). The appearance is typical of this tumor passed in syngeneic hosts.

**Twelfth Passage Diagnosis.** Poorly differentiated adenocarcinoma of the breast. The tissue submitted consists of a noncircumscribed mass of epithelial cells and connective tissue arranged in sheets and supported by fine vascular and connective tissue stroma. The mass is very cellular and the cells are moderately pleomorphic with oval, round, and irregularly shaped vesicular nuclei. There is a small to moderate amount of amphophilic cytoplasm, and there are numerous mitotic figures. The epithelial:stromal ratio is about 99:1. The tumor has lost its acinar pattern (Fig. 6).

After 20 transplant generations in the nude athymic mouse, the 13762 tumor was transplanted back into syngeneic female Fischer 344 rats. The histology in the first 3 passages is illustrated by the following report.

The tissue submitted consists of a noncircumscribed mass of epithelial cells arranged in sheets and supported by a poorly differentiated connective tissue stroma. There are numerous mitotic figures and extensive necrosis throughout the mass. The epithelial cells are very crowded and pleomorphic with oval, round, and irregularly shaped nuclei and small amounts of amphophilic cytoplasm. The epithelial:stromal ratio is about 95:5. The acinar character of the original tumor has been lost. Diagnosis, mammary carcinoma.

After 20 transplant generations in the nude athymic mouse, the R3230AC tumor was transplanted back into syngeneic female Fischer 344 rats. Tumor grafts grew to an average diameter of 8.4 mm and then regressed. The histology of a regressing tumor having an average diameter of 6 mm is as follows.

Tissue submitted in no way resembles an adenocarcinoma. Elements present include connective tissue stroma, infiltrated by numerous mononuclear inflammatory cells, mainly plasma cells and lymphocytes. A rare neoplastic epithelial cell occurs singly or in a small nest in the stroma. It appears to be a rejection reaction.

**Chromosome Studies**

The distributions of chromosome numbers in rat mammary adenocarcinomas R3230AC and 13762 in syngeneic hosts, and after 20 transplant generations in nude athymic mice, are compared in Table 1. There was no change in either the modal number of 75 or in the distribution of chromosome numbers to the right and left of the mode in the R3230AC tumor. Although the modal number of 45, characteristic for the 13762 tumor, also did not change.

Table 1

*Distribution of chromosome numbers in rat mammary adenocarcinomas R3230AC and 13762 in rats, and after 20 transplant generations in nude athymic mice*

| Tumor    | Host          | 48-57 | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | >80 | Total cells counted |
|----------|---------------|-------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|                       |
| R3230AC  | Rat           |       | 2  | 1  | 1  | 2  | 4  | 4  | 7  | 4  | 18 | 40 | 13 | 3  | 1  | 100                      |
|          | nu/nu mouse   |       | 2  | 1  | 2  | 2  | 1  | 2  | 2  | 1  | 3  | 7  | 6  | 2  | 48 | 10 | 7  | 1  | 1  | 100                      |

| Tumor    | Host          | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48-57 | Total cells counted |
|----------|---------------|----|----|----|----|----|----|----|----|----|----|----|--------|                       |
| 13762    | Rat           | 1  | 1  | 1  | 3  | 7  | 5  | 14 | 17 | 44 | 3  | 1  | 1  | 100                |
|          | nu/nu mouse   | 1  | 2  | 3  | 1  | 8  | 9  | 6  | 61 | 2  | 6  | 2  | 100                |

**Instability of Rat Mammary Tumor Xenografts in Nude Mice**

Animals bearing tumors in midlog phase of measurable growth were treated with colchicine. Chromosome spreads of tumor tissue were prepared and stained with lactic-acetic orcein.
after prolonged growth in the nude athymic host, there was a slight shift to the left in frequency of chromosome numbers, i.e., to 44 and 43.

Growth in Syngeneic Hosts

After 20 transplant generations in nude athymic hosts, both mammary tumors were reimplanted into syngeneic rat females; the resulting growth curves are shown in Chart 3. Two attempts were made to reestablish the R3230AC tumor in originally 100% susceptible (lethal growth) hosts. In the first there was 1 “no take”, and growth was followed by regression in 9 animals. In the second attempt 10 out of 10 animals showed growth followed by regression.

In contrast, grafts of the 13762 tumor from nude athymic mice all grew progressively when reimplanted into Fischer 344 females. Chart 3 illustrates the first and second passages in syngeneic rat hosts. Since graft sizes were the same, it is important that lag time (period from day of implantation to day of first measurable growth) was significantly shorter in the second passage. Tumor growth rate appears to have returned to normal. Responsiveness to L-PAM was tested in the second passage. The overall response is not as dramatic as is typical of this tumor system. However, 6 of 10 tumors were in complete remission by Day 60. Except for the second and sixth passages in nude athymic mice (Chart 2), no complete remissions had been induced in this tumor system with L-PAM while in the nude athymic host.

DISCUSSION

Repeated transplantation and chemotherapy of 2 rat mammary adenocarcinomas in nude athymic mice revealed changes in biological and chemotherapy response characteristics that indicate a tumor-host relationship that questions long-term stability of the tumor xenograft-nude athymic mouse as a test system.

Lack of response to L-PAM, a characteristic of the R3230AC tumor, remained stable. However, responsiveness to L-PAM, a characteristic of the 13762 tumor marked by oncolytic effects and many complete remissions in the syngeneic host, was significantly reduced when passaged in nude athymic animals. Tests of tumors in the 10th and 20th transplant generations revealed a decrease in oncolytic effects by L-PAM with no complete remissions. When this tumor was reimplanted into syngeneic rat hosts, growth pattern and responsiveness to L-PAM appeared to be almost normal by the second passage.

More surprising was the inability of the R3230AC to grow progressively when reimplanted into previously syngeneic rats. Two attempts proved negative. Distribution of chromosome numbers in both tumor systems before and after 20 transplant generations in the nude athymic host revealed no changes that might hint of possible interspecies hybridization, especially in the R3230AC tumor. However, no intensive analysis of the karyotype, before and after 20 transplant generations in the nude athymic mouse, has been made of either tumor.

Both the 13762 and R3230AC mammary tumors are histologically clearly definable as adenocarcinomas. Serial transplantation in nude athymic mice markedly reduced the number of acinar structures observable in the R3230AC tumor, and acinar structures disappeared completely from the 13762 tumor. Acini were still absent from the 13762 tumor after 3 passages in syngeneic female hosts.

The results obtained in this study are somewhat in contradiction to those reported by Povlsen et al. (13). In their studies 2 colon adenocarcinomas, 2 malignant melanomas, and 1 Burkitt’s lymphoma were passaged for 27 to 56 transplant generations over a period of 3.5 to 5.5 years. No changes in the cytological and histological appearance or in chromosome number and growth rates of the individual tumors were observed.

Significantly, the changes in biological and chemotherapeutic response characteristics that were observed in the present study did not involve both mammary tumors in the same manner or to the same degree. One might postulate that changes occurring in tumor xenografts during serial transplantation in nude athymic mice may reflect factors inherent in and peculiar to each tumor. The possibility of changes in the cellular or structural composition of xenografts, associated with biosynthetic functions operative in chemotherapy responsiveness for example, as a consequence of the selective pressures resulting from the immunological elimination or suppression of highly antigenic clones, is certainly a reality of transplantable systems.

The inability of the R3230AC tumor to grow progressively when reimplanted into its rat strain of origin would indicate an acquisition of new antigens. If this were the result of mouse substance replacing rat, one wonders what other tumor characteristics may have been altered that have not been tested for.

The presence of mouse hepatitis virus or Sendai virus, scourges of athymic nude mouse colonies, in the animals used in our studies was not indicated during the time.
frame in which animals were tumor bearing. Except for the terminal cachexia resulting from very large tumors, many of which ulcerated to the surface, xenograft recipients appeared to be in good health. However, since neither tumors nor animals were screened for the common murine viruses during the course of this study, the possibility exists that virus-associated antigens affecting transplantability may have been acquired. Other and very probable possibilities are that serial transplantation in the immunodeficient athymic nude mouse either fails to select against new tumor cell variants, e.g., those generated by somatic mutation, or permits the outgrowth of minor cell populations normally inhibited during serial transplantation in immunocompetent hosts. The significant decrease in chemotherapy responsiveness of the 13762 tumor by the tenth transplant generation in the athymic nude mouse may well be a reflection of the latter possibilities. That such possibilities must be considered in rationalizing the results observed in this study cautions that the tumor xenograft-nude athymic test system is neither autochthonous nor syngeneic. However, the fact that chemotherapy responsiveness was stable for at least 6 transplant generations augurs well for the usefulness of the tumor xenograft-nude athymic mouse test system for preclinical drug evaluation. Freeze-preservation of tissues in early transplant generations for periodic replacement of tumor lines, and careful monitoring of growth and biological and chemotherapy response characteristics will be necessary, however, to assure the stability of these test systems.

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REFERENCES

Figs. 1 to 3. First, 10th, and 20th serial passages of R3230AC in nude mice. H & E, x 400.
Figs. 4 to 6. First, 12th, and 19th serial passages of 13762 in nude mice. H & E, x 400.
Note, in each case, the decrease of differentiation and decrease in the number of acini with increasing serial transfers of these rat mammary adenocarcinomas in nude mice.
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