Adenocarcinoma R-3327 of the Copenhagen Rat as a Suitable Model for Immunological Studies of Prostate Cancer

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SUMMARY

An experimental animal model for the study of host-prostatic tumor cell interactions has been described. R-3327, a line of prostatic adenocarcinoma of the Copenhagen rat, has been proven to be immunogenic to its syngeneic host as evidenced by two different in vitro cell-mediated immune assays. Specificity of the responses has been ascertained on the basis of absence of response: (a) of nonimmune lymphocytes to the R-3327 tumor antigen(s); (b) of R-3327 immune lymphocytes to several normal tissues including normal prostate; (c) of immune lymphocytes to unrelated squamous cell prostatic carcinoma of the Copenhagen rat. Furthermore, the presence of tumor has an effect in several nonspecific aspects of host response, inducing splenomegaly, heightened responses to nonspecific mitogens in lymphocyte transformation assay, and increased levels of killer cell action. Since there are many histological, biochemical, and functional analogies between this tumor line and human prostate carcinomas, this system appears to be suitable for immunological and possible immunotherapeutic studies of this type of neoplasia.

INTRODUCTION

It is generally believed that the immune status of a host is an important factor in the development of malignant disease. Cell-mediated immune reactions have been implicated in the control or modification of tumor growth in animal systems and human subjects in a wide variety of carcinomas of diverse origins (1, 3, 5). In the case of prostatic neoplasia, few detailed immunological studies are available, mainly due to the lack of suitable experimental models in which tumor-specific responses could be clearly demonstrated.

The R-3327 prostatic adenocarcinoma of the Copenhagen rat has been hormonally and morphologically characterized in our laboratories (13, 14). We have found that this tumor line is androgen dependent and that it possesses specific receptors for dihydrotestosterone. With this model system we have been able to demonstrate specific responses to tumor antigens. The following is a report of our findings.

MATERIALS AND METHODS

Animals. Male Copenhagen rats obtained from the National Cancer Institute stocks at Tulane University, New Orleans, La., were used. These animals were approximately 6 months old at the initiation of the experiments. At least 6 animals were used in each experimental group.

Tumors. Prostatic adenocarcinoma R-3327 was originally described and established as a transplantable tumor by Dunning (2). The prostatic squamous cell carcinoma R-3327-A was derived from a 5th transfer generation of R-3327. This tumor differs from the parent line in histological characteristics, growth rate, lack of androgen receptors, and insensitivity to androgen presence (14). Implants of minced tumor (approximately 1 cu mm) were made s.c. with trocar needles on the flanks of the animals. The tumors became palpable after 50 days. Animals were used when tumors weighed approximately 1 g.

Preparation of Antigen Extracts. Antigen extracts were prepared by mincing the prostatic tumors or normal tissues (prostate, muscle, or kidney) and homogenizing the fragments with glass tissue grinders. Tissue extracts were centrifuged at low speed (100 × g) in HBSS,3 and the protein concentration of the supernatants was determined by A280 nm readings in a Beckman DU spectrophotometer.

Preparation of Responding Cells. Spleen cells were forced through stainless steel mesh by squeezing tissue fragments with siliconized rubber stoppers. The screens were rinsed with 10 ml HBSS. To obtain a suspension of single cells, clumps were removed by pushing the suspension through a syringe containing loosely packed nylon fibers. The suspension was then centrifuged and washed once in HBSS. RBC were eliminated by hypotonic shock as previously described (9). The cell suspension was pushed a 2nd time through another nylon mesh sieve. The cells were washed once in HBSS and incubated for 1 hr at room temperature on glass surfaces to remove adherent cells.

Lymphocyte Transformation Assay. The splenocytes from normal and tumor-bearing rats were cultured in the growth medium described by Heber-Katz et al. (6) according to the previously described method (9). In brief, cells were adjusted to a concentration of 1 × 10⁶ cells/ml. Triplicate cultures (Falcon plastic tubes; Falcon Plastics, Oxnard Calif.) of 0.5-ml volumes were incubated for 4 days in a 5% CO₂ atmosphere at 37°C in the presence of tissue extracts.

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PHA (Difco Laboratories, Inc., Detroit, Mich.), Con A (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), or growth medium alone, which served as base-line control. Eighteen hr prior to termination, the cultures were pulsed with 2 μCi of [methyl-3H]thymidine (specific activity, 3 Ci/m mole; Schwarz/Mann, Orangeburg, N. Y.). Samples were twice precipitated with trichloroacetic acid, extensively washed with 0.85% NaCl solution, and dried in ethanol. The pellets were dissolved in hydroxide of Hyamine (Packard Instrument Co., Downers Grove, Ill.), and the extent of radioisotope incorporation was determined in a Packard Tri-Carb Model 3330 scintillation spectrometer after the samples were placed in scintillation vials with a mixture of Spectraflo (Amersham/Searle Corp., Arlington Heights, Ill.) and toluene. Data were expressed in cpm per 0.5 x 10⁶ cells culture and/or stimulation indices (SI) defined by the formula:

\[
\text{SI} = \frac{\text{cpm of stimulated cultures}}{\text{cpm of base-line growth medium cultures}}
\]

Data were statistically evaluated by the Wilcoxon test of ranks.

Cytotoxicity Assays. The method of Perlmann and Perlmann (11) was used for the assessment of mitogen-induced cytotoxic activity of lymphocytes against chicken RBC or of ADCMC with rabbit antibody bound to chicken RBC. A method devised in our laboratories (8) for the specific activation of lymphocytes by tumor extracts, in which lymphocytes become killer cells of nonrelated target cells was used as a measure of specific cell-mediated immunity to tumor antigens. In brief, this method consists of incubation of glass-nonadherent splenocytes for 20 hr at 37°C in a 5% CO₂ atmosphere in the presence of different concentrations of tissue extracts and ⁵¹Cr-labeled chicken erythrocytes. The effector:target cell ratio used was 25:1. Roswell Park Memorial Institute Medium 1640 supplemented with 5% heat-inactivated fetal bovine serum was used. At the end of the incubation period, the cultures were centrifuged at 400 x g for 10 min at 4°C. Supernatants and pellets were counted in a Scientific Products, Model AW 1450 γ-counter.

Controls for mitogen- or antigen-induced cytotoxicity included target cells with inducer but without lymphocytes and target cells with growth medium alone. A control for ADCMC contained normal rabbit serum. Percentage release of ⁵¹Cr was determined according to the formula:

\[
\text{% release of } ^{51}\text{Cr} = \frac{A - B}{C - B} \times 100
\]

where A is the percentage of chromium released in stimulated cultures; B is the release in cultures containing nonlabeled target cells in place of lymphocytes, i.e., nonspecific release in the presence of inducer; and C is the maximum release resulting from physical disruption.

RESULTS

Transplantation of the R-3327 prostatic tumor induced splenomegaly in Copenhagen rats. Splenomegaly increased in comparison with those of normal rats (0.72 ± 0.03 versus 0.36 ± 0.02 g) although the tumors were in the early stages of growth (approximately 1 g weight).

A study of the effect of T-cell mitogens on the blastogenic transformation of splenocytes from normal and tumor-bearing rats revealed that the presence of the tumor greatly increased the lymphocyte reactivity. In Table 1 it is evident that lymphocytes from rats bearing R-3327 tumors had higher thymidine uptakes in response to PHA and Con A than did those of normal rats. The nonstimulated growth medium control cultures did not differ significantly in the 2 groups.

For detection of immune reactions to tumor antigens in animals bearing tumors, several concentrations of tumor extracts were tested in the lymphocyte transformation assay (Chart 1). Splenocytes from normal rats failed to respond to any concentration of tumor extract tested. In sharp contrast the splenocytes from R-3327-bearing rats were reactive to several concentrations of the antigenic extracts. The best responses were obtained with concentrations of 0.2 and 0.1 ⁰/₀ of tumor extracts. High concentrations of antigen (1 ⁰/₀) were not effective in eliciting positive responses, a prozone effect that we have observed previously in other tumor systems (8).

To ascertain whether the responses obtained were a true reflection of specific immunity to tumor antigens, we included several controls. Lymphocytes from normal and R-3327-bearing rats were tested against a battery of normal tissues (prostate, muscle, and kidney) and against extracts of squamous cell carcinoma tumor line R-3327-A (Table 2). Lymphocytes from normal rats did not respond in the blastogenic transformation assay when confronted with any of the normal or tumor tissue extracts. In contrast, lymphocytes from R-3327-immune rats were responsive to extracts of the R-3327 tumor, although they were not stimulated to increased thymidine uptake by normal muscle, kidney, or prostate tissues or by extracts of the

Table 1

<table>
<thead>
<tr>
<th>Incorporation of [³H]thymidine after mitogen stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpmp/0.5 x 10⁶ cells culture</td>
</tr>
<tr>
<td>Normal rats</td>
</tr>
<tr>
<td>Growth medium</td>
</tr>
<tr>
<td>1.989 ± 0.201a</td>
</tr>
<tr>
<td>PHA, 1.800μg</td>
</tr>
<tr>
<td>93.935 ± 3.475g (47.12)</td>
</tr>
<tr>
<td>Con A, 1 μg</td>
</tr>
<tr>
<td>147.112 ± 9.337g (73.96)</td>
</tr>
<tr>
<td>Tumor-bearing rats</td>
</tr>
<tr>
<td>1.767 ± 0.183g</td>
</tr>
<tr>
<td>124.185 ± 6.007g (70.28)</td>
</tr>
<tr>
<td>277.952 ± 8.984g (157.30)</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
* Final concentration of mitogen per culture.
* Significantly different by the Wilcoxon test of ranks (2α = 0.01).
* Number in parentheses, stimulation index.
unrelated tumor line, the prostatic squamous cell carcinoma.

In further studies, lymphocytes from animals implanted with the squamous cell prostatic tumor were tested against extracts of the adenocarcinoma line in the lymphocyte transformation assay. Splenocytes from rats bearing the squamous cell carcinoma (Table 3) did not recognize the private antigens of the prostatic adenocarcinoma tumor, which further attests to the specificity of the reactions shown in Chart 1 and Table 2. The animals were fully immunocompetent as evidenced by their responses to the mitogens PHA and Con A.

Cytotoxicity studies were performed to assess the levels of killer cell activity of splenocytes of normal and tumor-bearing rats. Table 4 shows the cytotoxic activity against chicken RBC evoked by stimulation with the mitogens PHA and Con A. PHA was capable of eliciting positive responses in both normal and R-3327-bearing rats. However, the amounts of $^{51}$Cr release were significantly higher in the tumor-bearing animals, which indicates again the increased reactivity of their lymphocytes. Con A did not induce killer cell activity.

Experiments with ADCMC revealed a similar pattern (Chart 2). Positive responses could be obtained with several dilutions of anti-chicken RBC. However, in all cases the responses of the tumor-bearing rats were higher than those of the normal rats.

Chart 3 depicts the results of the new assay of cytotoxicity comprised of specific activation of immune lymphocytes by tumor antigen(s) followed by nonspecific expression of the

Table 2
Specificity of blastogenic responses of R-3327 tumor-bearing rats to homologous tumor extracts

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Antigen concentration ($A_{280}$)</th>
<th>cpm/0.5 x 10⁶ cells culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal rats</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>1.0</td>
<td>1,714 ± 103 (0.98)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1,434 ± 99 (0.82)</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.0</td>
<td>1,364 ± 163 (0.76)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1,556 ± 201 (0.89)</td>
</tr>
<tr>
<td>Prostate</td>
<td>1.0</td>
<td>2,133 ± 247 (1.22)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1,976 ± 193 (1.13)</td>
</tr>
<tr>
<td>R-3327 prostate</td>
<td>1.0</td>
<td>1,836 ± 163 (1.05)</td>
</tr>
<tr>
<td>adenocarcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1,679 ± 109 (0.96)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2,098 ± 218 (1.20)</td>
</tr>
<tr>
<td>R-3327-A squamous cell carcinoma</td>
<td>0.2</td>
<td>1,766 ± 189 (1.01)</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

*a Numbers in parentheses, stimulation index.

Table 3
Lack of a blastogenic response to R-3327 adenocarcinoma antigens of lymphocytes from Copenhagen rats immunized with prostatic squamous cell carcinoma

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>cpm/0.5 x 10⁶ cells culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Copenhagen rats</td>
</tr>
<tr>
<td>None</td>
<td>1,349 ± 99*</td>
</tr>
<tr>
<td>PHA, 1:500</td>
<td>58,951 ± 6,091 (43.7)*</td>
</tr>
<tr>
<td>Con A, 1 μg</td>
<td>115,474 ± 9,889 (85.6)</td>
</tr>
<tr>
<td>Normal prostate*a</td>
<td>1,848 ± 243 (1.37)</td>
</tr>
<tr>
<td>R-3327 prostate adenocarcinoma*a</td>
<td>1,268 ± 180 (0.94)</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

*b Number in parentheses, stimulation index.

Final concentration, 0.2 $A_{280}$.
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Effect of tumor extract on lymphocytes. The injection of tumor extract from the R-3327 adenocarcinoma of the Copenhagen rat into normal rats evoked no cytotoxic reaction. In contrast, tumor extract from the adenocarcinoma but not from normal tissue evoked significant lytic activity on lymphocytes of tumor-bearing rats. A concentration of 1 A28, which was nonstimulatory in the lymphocyte transformation test, was effective in eliciting cell-mediated responses in this assay. Additional controls (not shown in Chart 3) included testing of the splenocytes with tumor extracts from the unrelated prostatic squamous cell carcinoma. No cytotoxicity was elicited with such extracts in lymphocytes from normal and adenocarcinoma-bearing rats.

**DISCUSSION**

Insight into many of the basic biological parameters involved in the etiology and treatment of prostate cancer has been obscured by the lack of a true animal model of prostatic carcinoma. Although much useful data have been obtained on the function of normal prostate with currently available models, these results cannot always be properly extrapolated to the case of a neoplastic prostate.

Adenocarcinoma R-3327 of the Copenhagen rat has already proved useful in biochemical and endocrinological investigations relevant to the cancer of the prostate (13, 14). In the present context it is proving to be a valuable model for immunological investigations. Lubaroff (10) has recently reported that, following intraprostatic injection of R-3327 cells in rats, tumors can be produced that possess the gross and microscopic morphology of typical human adenocarcinoma. Furthermore, he has found that, if the tumors become extremely large, the rats develop metastatic lesions. Our studies thus far have been performed in animals with small tumors. In studies by other investigators as well as ourselves (4, 7, 12, 15), the presence of small tumors is often associated with specific immunological responses to tumor antigens and with heightened immunological reactivities in general. In some of these other systems, progression of tumor to large size is often associated with a loss of immunological reactivity. This aspect has not been tested as yet in the present system.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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Model for Immunological Studies of Prostate Cancer

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