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

Diana M. Lopez and Walter Voigt

Department of Microbiology, Laboratory of Virology [D. M. L.], and Department of Pathology and Dermatology [W. V.], University of Miami School of Medicine, Miami, Florida 33152

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, a 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 cm3) 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 x g) in HBSS, 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 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 x 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° in the presence of tissue extracts.

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2 To whom requests for reprints should be addressed, at Laboratory of Virology, Department of Microbiology, University of Miami School of Medicine, P. O. Box 520875, Biscayne Annex, Miami, Fla. 33152.
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The abbreviations used are: HBSS, Hanks’ balanced salt solution; PHA, phytohemagglutinin; Con A, concanavalin A; ADCMC, antibody-dependent cell-mediated cytotoxicity.
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PHA (Difco Laboratories, Inc., Detroit, Mich.), Con A (Phar- 
macia 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 
\([\text{methyl-}^3\text{H}]\text{thymidine} \) (specific activity, 3 Ci/mmole; 
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 Instru-
ment Co., Downers Grove, Ill.), and the extent of radioiso-
tope incorporation was determined in a Packard Tri-Carb 
Model 3330 scintillation spectrometer after the samples 
were placed in scintillation vials with a mixture of Spectra-
fluor (Amersham/Searle Corp., Arlington Heights, Ill.) and 
toluene. Data were expressed in cpm per 0.5 × 10^6 cells 
culture and/or stimulation indices (SI) defined by the for-
mula:

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

Data were statistically evaluated by the Wilcoxon test of 
ranks.

**Cytotoxicity Assays.** The method of Perlmann and Perl-
mann (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 acti-
vation of lymphocytes by tumor extracts, in which lypho-
cytes 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° in a 5% CO,

ermosphere in the presence of different concentrations of 
tumor extracts and 51Cr-labeled chicken erythrocytes. The 
offector:target cell ratio used was 25:1. Roswell Park Mem-
orial 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 × g 
for 10 min at 4°. Supernatants and pellets were counted in a 
Scientific Products, Model AW 1450 γ-counter.

Controls for mitogen- or antigen-induced cytotoxicity in-
cluded 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 stimu-
lated cultures; B is the release in cultures containing nonla-
beled 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. Spleens of the tumor-
bearing rats were greatly 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-bear-
ing 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 
(Char 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 
A2Ko. High concentrations of antigen (1 A2Ko) were not effec-
tive 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 anti-
gen(s), we included several controls. Lymphocytes from 
normal and R-3327-bearing rats were tested against a bat-
tery 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 con-
fronted with any of the normal or tumor tissue extracts. In 
contrast, lymphocytes from R-3327-immune rats were res-
sponsive to extracts of the R-3327 tumor, although they 
were not stimulated to increased thymidine uptake by nor-
mal muscle, kidney, or prostate tissues or by extracts of the

| Table 1 |

| Incorporation of [3H]thymidine after mitogen stimulation |
|-----------------|-----------------|-----------------|
|                  | cpmp/0.5 × 10^6 cells culture |
| Growth medium    | Normal rats      | Tumor-bearing rats |
| Growth medium    | 1,998 ± 201    | 1,767 ± 183    |
| PHA, 1.800μg     | 93,935 ± 3,473  | 124,185 ± 6,007 |
| Con A, 1 μg      | 147,112 ± 9,337 | 277,952 ± 8,984 |

* 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 51Cr 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

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**Table 2**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Antigen concentration (A_s)</th>
<th>cpm/0.5 × 10⁶ cells culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>1,749 ± 187*</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.0</td>
<td>1,741 ± 103 (0.98)*</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1,410 ± 184 (0.90)*</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.0</td>
<td>1,364 ± 163 (0.78)*</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>R-3327 prostate adenocarcinoma</td>
<td>1.0</td>
<td>1,836 ± 163 (1.05)</td>
</tr>
<tr>
<td>R-3327-A squamous cell carcinoma</td>
<td>0.2</td>
<td>1,679 ± 109 (0.96)*</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2,096 ± 218 (1.20)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,105 ± 240 (1.31)</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

* Numbers in parentheses, stimulation index.

* Significantly different by the Wilcoxon test of ranks (2α = 0.01).

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**Table 3**

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>cpm/0.5 × 10⁶ cells culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Copenhagen rats</td>
<td>Rats bearing squamous cell carcinoma</td>
</tr>
<tr>
<td>None</td>
<td>1,349 ± 99*</td>
</tr>
<tr>
<td>PHA, 1.0 µg</td>
<td>56,951 ± 6,091 (43.7)*</td>
</tr>
<tr>
<td>Con A, 1 µg</td>
<td>115,474 ± 9,888 (85.6)</td>
</tr>
<tr>
<td>Normal prostate*</td>
<td>1,848 ± 243 (1.37)</td>
</tr>
<tr>
<td>R-3327 prostate adenocarcinoma*</td>
<td>1,268 ± 180 (0.94)</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

* Number in parentheses, stimulation index.

* Final concentration, 0.2 A_s.
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effector function against unrelated target cells. No cytotoxic reaction was evoked by tumor or normal tissue extract in the splenocytes of normal rats. In contrast, tumor extract but not normal tissue evoked significant lytic activity on lymphocytes of tumor-bearing rats. A concentration of 1 A280(i, 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

We gratefully acknowledge the technical assistance of Monica Modes and Jan B. Stern.

REFERENCES

1. Bhatnagar, R. M., Zabriskie, J. B., and Rausen, A. R. Cellular Immune Responses to Methylcholanthrene-Induced Fibrosarcoma in Balb/c

Table 4

<table>
<thead>
<tr>
<th></th>
<th>Normal rats</th>
<th>R-3327-bearing rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth medium</td>
<td>6.12</td>
<td>5.48</td>
</tr>
<tr>
<td>PHA, 1:800</td>
<td>36.79*</td>
<td>57.72*</td>
</tr>
<tr>
<td>Con A, 1 μg</td>
<td>7.29</td>
<td>7.19</td>
</tr>
</tbody>
</table>

* Significantly different by the Wilcoxon test of ranks (2α = 0.01).
Model for Immunological Studies of Prostate Cancer


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