Immunoregulatory Markers in Rats Carrying Dunning R3327 H, G, or MAT-LyLu Prostatic Adenocarcinoma Variants

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ABSTRACT

The Dunning R3327 tumor represents a system for studying prostate cancer in Copenhagen × Fischer rats. Animals bearing variant sublines (H, G, and MAT-LyLu) differing in growth rate, differentiation, hormone responsiveness, and metastatic ability were assessed for three immunological markers.

Spleens were passed through a tissue sieve, and mononuclear cells were obtained by Ficoll-Hypaque centrifugation. These were assayed for leukocytic subsets using monoclonal antibodies. An adherent population was isolated and evaluated using thin-layer chromatography for conversion of radioiodinated arachidonic acid to E series prostaglandins. Finally, sera from these animals were assayed for levels of circulating immune complexes using polyethylene glycol precipitation. Data from 52 rats bearing the various tumors were obtained, correlated with subline aggressiveness, and compared to 15 controls.

Each tumor group demonstrated significantly lower helper/suppressor T-cell ratios than controls, probably due to general tumor presence. In addition, the most aggressive R3327 MAT-LyLu variant had significantly increased prostaglandin E synthesis by adherent spleen cells compared to the H or G sublines and significantly increased levels of circulating immune complexes relative to the H1 subline. G1 subline values for both prostaglandin E and circulating immune complexes were intermediate, suggesting that these markers correlate better with tumor aggressiveness than helper/suppressor T-cell ratios.

INTRODUCTION

In a continuation of a preliminary study, the Dunning R3327 rat prostatic adenocarcinoma was used to evaluate immunoregulation in tumor sublines differing in rate of growth, hormone responsiveness, degree of differentiation, and ability to metastasize. Additional tumor markers have now been evaluated in efforts to correlate tumor aggressiveness with alterations in immunoregulatory markers.

Urological tumors including those of the prostate are immunogenic and frequently provoke lymphocytic infiltration or granulomatous reactions. Such responses suggest an immunological responsiveness by the host towards the tumor. The role of prostaglandins in prostate cancer is also unclear. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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MATERIALS AND METHODS

The Dunning Tumor Model. The Dunning R3327 prostatic adenocarcinoma is carried in male Copenhagen × Fischer F1 rats. The H subline is a well-differentiated prostatic adenocarcinoma, possessing androgen-sensitive columnar epithelial cells and numerous well-differentiated acini containing both prostate specific acid phosphatase and secretory material. The tumor is slow growing, hormonally sensitive, and will involute upon castration or estrogen administration. An...
an androgen-sensitive variant; however, unlike the H tumor, it expresses neither involution nor cessation of growth following castration (25). An additional tumor has been derived termed the R3327 AT subline. This is a rapidly growing, anaplastic, hormone-insensitive variant which grows equally well in females and intact or castrated males. This R3327-AT tumor gave rise to an additional subline which exhibits a high and predictable pattern of metastatic dissemination. This R3327 MAT-LyLu tumor (Metastatic AT LYmph nodes, LUng) metastasizes to the lymph nodes and to the lungs (26). Comparisons between the R3327 Dunning sublines are listed in Table 1.

**Implantation of Tumors.** Tumors were carried in Copenhagen x Fischer F1 rats by s.c. injection of 10^6 viable tumor cells obtained by collagenase digestion into the left flank. Viability was determined throughout these experiments by exclusion of 0.4% trypan blue dye.

A total of 67 rats were utilized and distributed into groups as follows: control, non-tumor bearing, 15; those bearing the H subline, 11; those bearing the G subline, 15; and those bearing the MAT-LyLu subline, 26. At the time of implantation animals were sexually mature and weighed approximately 200–300 g. Tumor progression was determined at least once a week using micropipettes.

Animals which bore tumors of the various sublines were sacrificed when tumor volumes were equivalent. This occurred approximately 3 wk following implantation of the MAT-LyLu subline, 3 mo following implantation of the G subline, and 9 mo following passage of the H subline. Animals comprising each of the groups were removed randomly from our animal facilities when tumor sizes were comparable. Differences in immunological exposure occurring from the time of tumor implantation until animal sacrifice were unavoidable due to the differences in subline growth rates.

**Preparation of Mononuclear Spleen Cells.** Spleens were removed from control and tumor-bearing rats and placed into a 5-ml volume of medium (consisting of RPMI 1640 supplemented with 100 units/ml of penicillin and 100 μg/ml of streptomycin) prior to passage through an EC tissue sieve. The screen was washed twice with 5 ml of additional medium, and the dispersed cells were collected and pooled. Mononuclear cells were removed by Ficoll-hypaque centrifugation (27). The cells were harvested, washed, and resuspended in a small volume, and residual RBC were lysed by suspension in a small volume of 0.85% (NH4)2Cl. Cells were then washed again, counted by hemocytometer, and diluted to 10^7 cells/ml in medium. An aliquot of 0.1 ml was immediately removed for nonspecific esterase activity while the remaining cells were depleted of adherent cells by incubation at 37°C for 45 min in plastic culture dishes. Following incubation nonadherent cells were collected, counted, and again suspended to 10^7/ml prior to measurement of monocytic reactivity. Viability consistently exceeded 95%.

Individual spleen weights were not determined nor were total spleen cell numbers for each leukocytic subset. Portions of representative spleens were removed and fixed for future evaluations, and occasional differences in viscosity following screen passage affected total cell recovery from some of the larger spleens. Monoclonal antibody evaluation of leukocytic subset distribution was deemed best under these circumstances since it is unaffected by efficiency of cell recovery.

**Monoclonal Antibodies.** Monoclonal antibodies (Accurate Chemicals, Westbury, NY) with the specificities listed in Table 2 were utilized.

**Analysis of Spleen Cell Populations.** In duplicate tubes, 100 μl of cell suspensions (10^6 cells) were treated with 10 μl of the appropriately diluted monoclonal antibody. Incubation, on ice for 3–4 hours, preceded washing with phosphate-buffered saline (136 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 0.9 mM NaH2PO4, 0.5 mM MgCl2, pH 7.4) at 4°C. One hundred μl of goat anti-mouse IgG conjugated to horseradish peroxidase (Tago, Burlingame, CO) were added, and incubation was continued on ice. Cells were washed and reacted with diaminobenzidine substrate. Smears were made onto slides, air dried, washed, and counterstained using 1% methyl green. Cells which were scored positive contained either particulate brown deposits or distinct dark brown circles along the periphery of the cells. Monocytes were identified by morphology (using Wright stain) and nonspecific esterase activity (30).

Each preparation was counted at x400 magnification, and the percentage of distribution of each leukocytic subset was determined. The relationship between helper and suppressor T-lymphocyte subpopulations was expressed by the helper-suppressor ratio.

**Conversion of Arachidonic Acid to Prostaglandin E2.** Spleen cells were dispensed into glass scintillation vials to yield 10^7 mononuclear cells per vial. Cells were incubated in a 1-ml volume of medium (RPMI 1640, containing 100 units/ml of penicillin and 100 units/ml of streptomycin), at 37°C for 45 min to permit adherence. Nonadherent cells were removed by aspiration of the supernatant followed by two 1-ml medium washes. Adherent cells, presumably containing both immunoregulatory monocytic and T-cell populations, were then incubated in 1 ml of medium containing 5 μCi of [3H]HETE (New England Nuclear, Boston, MA) for 3 h. Following incubation the supernatant was removed, and the adherent cells were gently washed 3 times before the addition of 1 final ml of medium. This was then acidified as previously described with N HCl and extracted with 3 volumes of diethyl ether. Samples were evaporated to dryness, PGE, and PGE2 markers were then added, and the samples were spotted on silica gel plates (Kodak, Rochester, NY) and subjected to thin-layer chromatography using the organic phase of a solvent consisting of ethyl acetate, isooctane, acetic acid, and H2O in a 9:5:2:10 ratio (11). Upon completion of migration, spots were visualized with iodine vapor, and the area of PGE marker migration was defined. Spots containing [3H]PGE were cut from the silica gel plates and then counted using a scintivue cocktail. The extent to which glass-adherent cells converted [3H]HETE to [3H]PGE was expressed as the percentage of PGE conversion equal to cpm PGE area/total cpm recovered. Calculation of percentage of conversion of radiolabeled arachidonic acid to PGE, rather than quantitation of PGE produced, minimizes any effects of subtle differences in spleen cell adherence. The population of adherent spleen cells obtained appeared consistent throughout all groups.

**Isolation of Circulating Immune Complexes.** PEG will precipitate proteins including immunoglobulins according to molecular weight. The method described by Bhatti (31) was utilized. Serum was diluted 1:3 in 0.1 M borate EDTA buffer, pH 8.4, and mixed in a 1:9 ratio with 3.75% polyethylene glycol (M, 6000). Controls for each serum sample were similarly mixed with borate EDTA buffer alone to determine nonspecific precipitation. All tubes were run in duplicate and kept at room temperature for 2 h. Levels of immune complexes were determined by absorbance at 450 nm. Results are expressed thus: circulating immune complexes = (Amax × 10^4 with buffer) – (Amax × 10^4 with buffer alone).

These units relate to the absorbance of material specifically precipitated by the PEG after the nonspecific precipitation by buffer alone is subtracted.

<table>
<thead>
<tr>
<th>Tumor subline</th>
<th>Histological characterization</th>
<th>Generation time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3327-H</td>
<td>Well differentiated</td>
<td>15–20</td>
</tr>
<tr>
<td>R3327-G</td>
<td>Poorly differentiated</td>
<td>2</td>
</tr>
<tr>
<td>R3327-MAT-LyLu</td>
<td>Anaplastic</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Table 2 Monoclonal antibody specificity**

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>T-cell subset recognized</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3/13</td>
<td>Total T-cell</td>
<td>28</td>
</tr>
<tr>
<td>W3/25</td>
<td>Helper T-cell</td>
<td>28</td>
</tr>
<tr>
<td>Ox8</td>
<td>Suppressor T-cell</td>
<td>29</td>
</tr>
</tbody>
</table>
RESULTS

The quantitation of W3/13, W3/25, and Ox8 positive cells in both control and tumor-bearing rats is shown in Table 3. A total of 15 non-tumor-bearing control rats was evaluated. Seventy-seven percent of their nonadherent mononuclear spleen cells were T-cells, and these were distributed between helper and suppressor T-cell subsets in a 1.20 ratio. Monocytic representation determined morphologically and by nonspecific esterase activity showed a 23% representation.

When similar data were accumulated from the tumor-bearing groups (11 animals bearing the H subline tumor, 15 animals bearing the G subline, and 26 animals with the MAT-LyLu tumor subline), significant alterations from control values were observed. Animals bearing the slow-growing H subline had a similar representation by total T-cells (77%); however, these were distributed between helper and suppressor T-cell subsets in a 1:0.99 ratio (P < 0.01). Animals bearing the more rapidly growing sublines. As shown in Table 4 these values were not statistically significant. This implies that the altered leukocytic distribution reported in Table 3 are largely effects associated with the Dunning R3327 tumor in general and are not associated with any particular subline characteristic. We are not able to imply any correlation between histological characterization and the immunological values noted here.

Occasional animals from either control or tumor-bearing groups had unexplained enlarged spleens. These yielded proportionally greater total cell recoveries but did not exhibit differences in any of the immune markers.

The conversion of labeled arachidonic acid to E series prostaglandins by adherent spleen cells is illustrated in Fig. 1. Control cells, derived from non-tumor-bearing animals, demonstrate a mean conversion of 1.33 ± 1.1%.

Values obtained from animals bearing the various sublines demonstrate increased conversion by the more aggressive, rapidly growing sublines. Values ranged from 0.22 ± 0.13% by adherent spleen cells obtained from H subline-bearing animals, to 0.86 ± 0.9% from G subline-bearing animals and 2.02 ± 0.85% from MAT-LyLu-bearing animals. This suggests that increasing tumor aggressiveness might be accompanied with enhanced prostaglandin production by adherent cells. Differences between the metastatic growing MAT-LyLu-bearing animals (2.02%) and the slow-growing H subline (0.22%) were significant (P < 0.001) as was the intermediate value obtained from the rapid-growing G subline (0.86%, P < 0.01). Control
animals demonstrate a wide range of individual activities, having individual values within the same range as those obtained from the tumor-bearing animals. This suggests that multiple mechanisms for prostaglandin regulation are probably present in normal animals which become altered by tumor development.

Similar results (Fig. 2) were obtained when the levels of circulating immune complexes were measured in serum from control and tumor-bearing animals. The values obtained from the control group were widely distributed about a mean value of 12.1 ± 8.1, while values obtained from animals bearing the individual tumor sublines demonstrate increasing mean values, ranging from 10.0 ± 7.4 for the H subline to 24.1 ± 17.2 in rats with the MAT-LyLu tumor. This later value is significantly greater than those obtained from the control (P < 0.05) and H subline (P < 0.05) animals. The values from the G subline animals (17.2 ± 12.3) were intermediate and statistically not different from either group.

**DISCUSSION**

The relationship between urological malignancies and immunological responsiveness is well established (20, 32–36) and by using monoclonal antibodies, quantitation of the leukocytic subsets, presumably involved in such responses, is possible. This technology has detected altered distributions of leukocytic subsets in a variety of urological cancers including those of the prostate.

Previously we have reported that primary tumors derived from patients bearing Stage D prostate cancer have decreased helper-inducer/suppressor-cytotoxic T-cell ratios and increased monocytes and null cell representations when compared to distributions of similar cells obtained from peripheral blood of the same individual (37). This suggests a localized immunological suppression mediated by T-cells within the tumor. In addition, peripheral blood derived from Stage D prostate cancer patients demonstrated an altered leukocytic subset distribution when compared to normal age-matched controls. These patients have significantly decreased total T-cell and helper-inducer T-cell contents, along with increased representation by suppressor-cytotoxic T-cells, monocytes, granulocytes, and null cell types (38). Such alterations resulted in significantly diminished helper-inducer/suppressor-cytotoxic T-cell ratios, which were in part reversible following hormonal therapy.

Leukocytic subset changes have also been reported in renal carcinoma (39) and are also affected by therapy (40). Subset distributions are used by some for monitoring recipients of renal allografts (40). In other areas, these differences have been correlated with in vitro deficiencies in phytohemagglutinin and alloantigen responsiveness (41).

The Dunning tumor model was chosen in these experiments because it represents a reproducible system to study the effects of tumor variability upon immunological markers. Each of the variant sublines chosen differs in degree of differentiation, rate of growth, hormone responsiveness, or predisposition for metastatic dissemination. Such variables are commonly found when one examines human tumor heterogeneity and progression.

These experiments suggest that alterations in the spleen cell distribution of leukocytic subsets are similar to those seen in the peripheral blood of patients having Stage D prostatic carcinoma (40). Such patients also have elevated levels of circulating immune complexes (19, 31). Patients with disseminated solid tumors have increased E series prostaglandin synthesis by peripheral blood adherent cell types, presumably mononuclear (9). This suggests that at least some of the elevated levels of PGE2 found in peritumor effusions of the Dunning R3327 MAT-LyLu tumor may be contributed in part by mononuclear cells similar to adherent cells isolated and cultured from the spleen.

Alterations in leukocytic subset representation, prostaglandin E synthesis, and levels of circulating immune complexes have been implicated as having roles in immunoregulation. Both prostaglandin E2 and immune complexes act upon natural killer cells to inhibit tumor-directed cytotoxicity (42). Such immunosuppressive effects are antagonized by interleukin-2 (43)

The macrophage also bears Fc receptors for immunoglobulins and is central to regulation of both humoral and cellular immune responsiveness. Distribution of these receptors is affected by immune complex presence (44, 45) as is the release of various regulatory molecules such as γ-interferon and prostaglandins (46). These data indicate that more aggressive tumor types, including those with a propensity for metastasis, have increased synthesis of PGE2 and levels of circulating immune complexes, which may coordinate tumor enhancement allowing both markers to play roles in tumor progression.

These results also suggest that helper/suppressor T-cell ratios, although markers for tumor presence, are not as significant as elevated production of PGE2 or levels of circulating immune complexes for correlating tumor aggressiveness. Whether such differences relate to functional alterations of immunologically responsive cells which prevent the animal from rejecting its tumor is unknown. Reversal of such alterations is only the first step to reestablishing or potentiating an antitumor response. What is needed is a model system which may be manipulated in manners not applicable to human experimentation. The Dunning system appears to represent just such a system.

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IMMUNOREGULATORY MARKERS IN DUNNING TUMOR-BEARING RATS

REFERENCES


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