Serotherapy of Primary Rat Mammary Carcinoma: Inhibition by Ethylenedinitrilotetraacetic Acid but not by [Ethylenedinitrilotetraacetic Acid]

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ABSTRACT

We reported inhibition of growth of primary rat mammary carcinomas after infusions of tumor-bearer plasma absorbed with Protein A-Sepharose or inactivated CNBr Sepharose. Absorbed plasmas were depleted of the third component of complement (C3) (other complement components defined similarly) and C5 but not C1, C4, or C2. These results suggested that activation of the alternative pathway of complement might be involved in the observed antitumor effects. To test this concept sera were treated with ethylenedinitrilotetraacetic acid or ethylenedinitrilotetraacetic acid before absorption with Protein A-Sepharose. Ethylenedinitrilotetraacetic acid, by chelating calcium and magnesium, prevents activation of both the alternative and classical complement pathways. Ethylenedinitrilotetraacetic acid, by chelating calcium but not magnesium, permits activation of the alternative pathway but inhibits activation of the classical complement pathway. Sera in the presence or absence of chelating agent were absorbed with Protein A-Sepharose twice at room temperature. After absorption calcium was added to the sera. Rats were treated by i.v. injection of sera twice a week for 2 weeks. Measurements of tumor size were made weekly for 5–7 weeks and then tumor weight was determined. Groups were compared for size of index and total tumors. The results can be summarized as follows: (a) tumor-bearer sera after absorption did not inhibit the growth of rat primary mammary carcinomas; (b) tumor-bearer sera after absorption with Protein A-Sepharose showed significant consumption of C3 and did inhibit tumor growth; (c) C3 depletion and did inhibit the growth of primary rat mammary tumors. The results indicate that EDTA-treated sera absorbed with Protein A-Sepharose twice at room temperature, after absorption calcium was added to the sera, were treated by i.v. injection of sera twice a week for 2 weeks. Measurements of tumor size were made weekly for 5–7 weeks and then tumor weight was determined.

INTRODUCTION

We (1) and others (2) have described inhibition of growth of primary rat mammary carcinomas after infusion of plasma obtained from tumor-bearing animals. We found that the growth of primary rat mammary tumors was retarded when Buffalo/N rats were treated with plasma from tumor-bearing animals absorbed with inactivated CNBr Sepharose or Protein A-Sepharose. These observations suggested that Protein A was not an absolute requirement for this type of antitumor effect and that a factor(s) that can acquire antitumor activity after suitable treatment in vitro might exist in the plasma of rats with primary cancer.

Immunochemical studies (3) indicated that the alternative complement pathway is activated during absorption of rat plasma with Sepharose derivatives. The titer of functionally active C3 decreased after absorption or rat plasma with either Sepharose 4B, inactivated CNBr Sepharose, or Protein A-Sepharose. Although the titer of C3 decreased, the titer of functionally active C1 and C4 was unchanged or increased after absorption of rat plasma. Consumption of C3 during absorption with Sepharose derivatives required Mg²⁺, not Ca²⁺. EDTA, a chelating agent which binds both Ca²⁺ and Mg²⁺ prevented the consumption of C3 during absorption of plasma with CNBr Sepharose or Protein A-Sepharose. EGTA, a chelating agent which binds Ca²⁺ to a greater degree than Mg²⁺, did not prevent C3 consumption. The consumption of C3 without concomitant decrease of C1 and C4 and the requirement for Mg²⁺ but not Ca²⁺ are hallmarks of activation of the alternative pathway of complement.

To correlate the results of immunochemical studies with the activity of plasmas in vivo, sera were treated to prevent or permit activation of the alternative pathway, absorbed with Protein A-Sepharose, and then administered to tumor-bearing rats. The results indicate that EDTA-treated sera absorbed with Protein A-Sepharose had no decrease in C3 functional activity and did not inhibit growth of primary rat mammary tumors. EGTA-treated sera absorbed with Protein A-Sepharose showed C3 depletion and did inhibit the growth of primary rat mammary tumors. The results are consistent with a role of the alternative pathway of complement in the observed antitumor effects.

MATERIALS AND METHODS

Animals. Four-week-old female Buffalo/N rats were obtained from Charles River Laboratories, Kingston, NY, through the Animal Genetics and Production Branch, Division of Cancer Treatment, and housed at the National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD. The animals were caged in groups of two and maintained on a diet of Purina laboratory chow checkers and tap water, which was available to animals at all times. Sera from rats lacked antibodies to Sendai virus.

Induction of Mammary Tumors. N-Nitroso-n-methylurea (ICN Pharmaceuticals, Plainview, NY) (2.25 mg/100 g body weight for initial experiments; 3 mg/100 g body weight for subsequent experiments) was injected i.v. into 50-day-old female rats. (The carcinogen contained 25% stabilizing material.)

Allocation of Rats into Experimental Groups. Starting 3 months after injection of carcinogens, rats were examined for the presence of mammary tumors. Rats with single mammary tumors were entered into therapy experiments 1–2 weeks after detection of tumors. (Rats entered into experiments had received carcinogen 3–4 months before therapy.) From rats ranked in order of increasing tumor size, animals were organized in sets of three. From each set of three animals, rats were randomly distributed among three experimental groups. Each experimental group contained eight animals. The mean tumor volume at the start of treatment in a representative experiment (Fig. 4) was 420 mm³ ± 20 (SE). All rats were entered into treatment at the same time.

The abbreviations used are: C3, third component of complement (other complement components defined similarly); ACD, anticoagulant citrate dextrose solution; EGTA, ethylenedinitrilotetraacetic acid; HBSS, Hank's buffered salt solution without calcium or magnesium.
Absorption of Plasma and Sera. Protein A-Sepharose was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Protein A-Sepharose (1.5 g) was swollen with HBSS without calcium or magnesium (15 ml) for 30 min at room temperature. The gel suspension was washed twice with 15 ml of HBSS (900 g, 5 min, room temperature) resuspended in HBSS, and then used for absorption of plasma or sera. Blood was collected from anesthetized rats by cardiac puncture. For experiments with plasma, blood was anticoagulated with ACD (1, 3). For experiments with serum, blood was allowed to clot overnight at 4°C; then serum was separated by centrifugation at 1650 × g for 15 min. Sera were kept at -70°C until the day of absorption. On the day of treatment, sera or plasma (5 ml) was absorbed in 15-ml conical centrifuge tubes with Protein A-Sepharose (55 mg). Absorptions were performed at room temperature on a rocking platform for 1 h. Fluids were removed after centrifugation at 900 × g for 5 min and admixed with fresh absorbent for 1 additional h. Absorbed sera or plasma were filtered through a 0.45 micron filter (Milllex-HA, Millipore Corp., Bedford, MA 01730) before injection. Tumor-bearer sera or plasma was obtained from rats with mammary tumors at least 30 mm in diameter.

Chelating Agents. EDTA (S-311; Fisher Scientific Company, Fair Lawn, NJ 07410) and EGTA (E-4378; Sigma Chemical Company, St. Louis, MO 63178) were prepared as 100 mM stock solutions (pH 7.6). One volume of stock chelating agent (0.5 ml) was added to 9 volumes of sera or plasma (4.5 ml). CaCl₂ was prepared as a 100 mM stock solution. Just before injection absorbed sera containing chelating agents were recalcified by the addition of 1 volume of stock CaCl₂ solution to 9 volumes of sera or plasma.

Titration of Functionally Active Rat C3. The conditions for titrations of rat C3 are given in Ref. 3.

Treatment. Rats were treated by infusion into the tail vein of sera or plasma twice a week for 2 weeks. The dose of sera or plasma is given in the legends to Figs. 1–5. To facilitate i.v. injection rats were anesthetized by inhalation of methoxyflurane (Metofane; Pitman Moore, Washington Crossing, NJ 08560) and oxygen delivered by a veterinary anesthesia machine (Heidbrink; Ohio Medical Products, Madison, WI 53707). Control rats were treated by injection of HBSS (2.5 ml).

Measurements of Tumor Size. Three dimensions of tumors were measured with a vernier caliper. The volume of the tumor was calculated according to the equation

\[
\text{Tumor volume} = \frac{\pi}{6} (D_1 + D_2 + D_3 - 0.9)^3
\]

Fig. 1. Inhibition of growth of primary rat mammary carcinomas after infusion of tumor-bearer plasma absorbed with Protein A-Sepharose. A, median index tumor volume; B, median cumulative tumor volume; O, Group A, HBSS; •, Group B, tumor-bearer plasma absorbed with Protein A-Sepharose. Dose per treatment: Group A, 2.0 ml HBSS; Group B, 1.2 ml absorbed plasma plus 0.8 ml HBSS (from diluent trapped in absorbent). In this and all subsequent figs., median tumor volume of each group is plotted versus time after the first i.v. treatment. +, median tumor volume at that time was significantly different (\(P < 0.05\)) from the control group; * next to the number for median tumor weight, median tumor weight was significantly different (\(P < 0.05\)) from the control. The results of the Koziol analysis were as follows: A, Group B versus Group A, \(P = 0.026\); B, Group B versus Group A, \(P = 0.019\).

In immunochemical studies (3) of plasma absorbed with Sepharose derivatives we found that the substance used to anticoagulate the blood had a profound effect on C3 consumption and might in turn influence the efficacy of plasma transfer therapy. Of particular importance was the variability of C3 consumption in blood anticoagulated with ACD. When Mg²⁺ was added to blood anticoagulated with ACD, complete C3 consumption occurred. These results suggested that if serum could substitute for plasma, results of treatment might be more consistent and subsequent experiments with chelating agents would be simpler to interpret. Accordingly, rats with primary rat mammary carcinoma were treated with HBSS, with tumor-bearing sera absorbed with Protein A-Sepharose, or with tumor-bearing sera not absorbed with Protein A-Sepharose. The results (Fig. 2) indicate that absorbed tumor-bearer sera inhibited tumor growth. The tumor growth curve of rats treated with absorbed tumor-bearing sera was significantly different (\(P = 0.006\)) from that of control animals and animals treated with nonabsorbed sera (modified Koziol analysis) (Fig. 2A). The growth curve of rats treated with nonabsorbed sera was not significantly different (\(P = 0.27\)) from that of control animals (Fig. 2A).

Compounds that chelate Mg²⁺ inhibit activation of the alternative pathway of complement. To determine whether activation of the alternative pathway of complement during absorption with Protein A-Sepharose correlated with antitumor activity, sera were pretreated with EDTA before absorption and administration to rats. In agreement with previous observations (3), functionally active C3 was depleted in sera absorbed with Protein A-Sepharose; C3 depletion did not occur in sera pretreated with EDTA (10 mM) (Table 1). Because sera containing EDTA (10 mM) killed one of three treated rats, sera (containing chelating agents) were recalcified after absorption and before
administration to rats. No deaths were observed in rats given injections of recalified sera containing chelating agents. Addition of Ca²⁺ to absorbed sera did not change the titer of functionally active C3 (data not shown). The results (Fig. 3) indicate that EDTA inhibited generation of sera with ability to inhibit the growth of primary rat mammary tumors. The tumor growth curve of rats treated with EDTA-treated sera absorbed with Protein A-Sepharose was not significantly different (P = 0.08) from that of control animals (Fig. 3A). The tumor growth curve of tumor-bearing sera absorbed with Protein A-Sepharose was significantly different (P = 0.007) from that of control animals (Fig. 4A). In this experiment sera not treated with EDTA but absorbed with Protein A-Sepharose inhibited tumor growth as assessed by measurement of index or cumulative tumor volume (P = 0.05) (Fig. 4B) but did not show statistically significant inhibition of growth of index tumor volume (P = 0.08) (Fig. 4A).

Although experiments with plasma indicated that plasma from non-tumor-bearing rats did not inhibit primary tumor growth (1) we reinvestigated this question by absorbing sera from normal adult female Buffalo/N rats and administering these sera to rats with primary rat mammary tumors. The results (Fig. 5) indicate that sera from normal adult female Buffalo/N rats inhibited tumor growth as evaluated by measurement of index or cumulative tumor volumes. Ray et al. (7) found that the plasma from normal rats absorbed with nonviable Staphylococcus aureus Cowan I cells inhibited growth of mammary tumors.

To compare the results of serotherapy with a treatment known to have antitumor effects in rats with primary mammary cancer, female Buffalo/N rats with primary mammary cancers were treated by oophorectomy. The results (Fig. 6) indicate that oophorectomy led to reduction in size of established rat mammary tumors.

Table 1 C3 content of tumor-bearing sera before and after absorption with Protein A-Sepharose

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Serum pretreatment</th>
<th>Before absorption</th>
<th>After absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>None</td>
<td>9.694 ± 334*</td>
<td>&lt;600 (0)</td>
</tr>
<tr>
<td>3</td>
<td>EDTA</td>
<td>8.828 ± 678 (100)</td>
<td>10.531 ± 1.029 (119 ± 7)</td>
</tr>
<tr>
<td>4</td>
<td>EGTA</td>
<td>8.885 ± 369 (100)</td>
<td></td>
</tr>
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* Mean ± SE for four rats.

Numbers in parentheses, percentage of unasorbed plasma.

Fig. 2. Inhibition of growth of primary rat mammary carcinomas after infusion of tumor-bearing sera absorbed with Protein A-Sepharose. A, median index tumor volume; B, median cumulative tumor volume; O, Group A, HBSS; ◇, Group B, tumor-bearing sera absorbed with Protein A-Sepharose; □, Group C, tumor-bearing sera treated with EDTA before absorption with Protein A-Sepharose. Dose per treatment: Group A, 2.0 ml HBSS; Group B, 2.0 ml nonabsorbed sera; Group C, 1.2 ml absorbed serum plus 0.8 ml HBSS. *, at that time tumor size in Group B was significantly different from tumor size in Group C. Results of Kozioł analysis for A were: Group A versus Group B, P = 0.0057; Group B versus Group C, P = 0.029; for B: Group A versus Group B, P = 0.0026; Group B versus Group C, P = 0.0097; Group A versus Group C, P = 0.076. For explanation of single * and numbers within figs., see legend to Fig. 1.

Fig. 3. Abrogation by EDTA of the generation of antitumor activity in sera during absorption with Protein A-Sepharose. A, median index tumor volume; B, median cumulative tumor volume; O, Group A, HBSS; ◇, Group B, tumor-bearing sera absorbed with Protein A-Sepharose; □, Group C, tumor-bearing sera treated with EDTA before absorption with Protein A-Sepharose. Dose per treatment: Group A, 2.0 ml HBSS; Group B, 1.2 ml absorbed sera plus 0.8 ml HBSS; Group C, 1.2 ml absorbed sera plus 0.8 ml HBSS plus 0.13 ml EDTA (100 mM) plus 0.25 ml CaCl₂ (100 mM). The results of Kozioł analysis for A were: Group A versus Group B, P = 0.0015; Group B versus Group C, P = 0.0027; Group C versus Group A, P = 0.08; for B: Group A versus Group B, P = 0.0097; Group B versus Group C, P = 0.012; Group A versus Group C, P = 0.054. For explanation of single and double * and numbers within figs., see legends to Figs. 1 and 2.

was significantly (P = 0.002) different from that of control animals (Fig. 3A). EDTA, by chelating Ca²⁺ to a greater degree than Mg²⁺, inhibits the classical but not the alternative pathway of complement. Pretreatment of sera with EDTA before absorption would test whether chelation of Ca²⁺ or Mg²⁺ interfered with generation of antitumor activity in sera. Both untreated sera and sera treated with EDTA showed pronounced depletion of functionally active C3 (Table 1). EDTA-treated sera absorbed with Protein A-Sepharose inhibited tumor growth as assessed by measurement of index or cumulative tumor volume (Fig. 4). The tumor growth curve of rats treated with EDTA-treated sera absorbed with Protein A-Sepharose was significantly different (P = 0.007) from that of control animals (Fig. 4A). In this experiment sera not treated with EDTA but absorbed with Protein A-Sepharose inhibited tumor growth as assessed by measurement of cumulative tumor volume (P = 0.05) (Fig. 4B) but did not show statistically significant inhibition of growth of index tumor volume (P = 0.08) (Fig. 4A).
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Time (days)

Fig. 4. Generation of antitumor activity using EGTA in sera during absorption with Protein A-Sepharose. A, median index tumor volume; B, median cumulative tumor volume; O, Group A, HBSS; •, Group B, tumor-bearer sera absorbed with Protein A-Sepharose; □, Group C, tumor-bearer sera treated with EGTA before absorption with Protein A-Sepharose. Dose per treatment: Group A, 2.0 ml HBSS; Group B, 1.2 ml absorbed sera plus 0.8 ml HBSS; Group C, 1.2 ml absorbed sera plus 0.9 ml HBSS plus 0.13 ml EGTA (100 mM) plus 0.25 ml CaCl2 (100 mM). The results of Koziol analysis for A were: Group A versus Group B, P = 0.084; Group B versus Group C, P = 0.44; Group C versus Group A, P = 0.0074; for B: Group A versus Group B, P = 0.047; Group B versus C, P = 0.92; Group A versus C, P = 0.0094. For explanation of * and numbers within fig., see legend to Fig. 1.

Fig. 5. Comparison of the ability of sera from tumor-bearer and tumor-free Buffalo/N rats to inhibit the growth of primary mammary adenocarcinomas. A, median index tumor volume; B, median cumulative tumor volume; O, Group A, HBSS; •, Group B, tumor-bearer sera absorbed with Protein A-Sepharose; □, Group C, normal sera absorbed with Protein A-Sepharose. Dose per treatment: Group A, 2.5 ml HBSS; Group B, 1.5 ml absorbed sera plus 1.0 ml HBSS; Group C, 1.5 ml absorbed tumor-bearer sera plus 1.0 ml HBSS. The results of Koziol analysis for A were: Group A versus Group B, P = 0.001; Group A versus C, P = 0.002; Group B versus C, P = 0.25; for B: Group A versus Group B, P = 0.001; Group A versus C, P = 0.001; Group B versus Group C, P = 0.78. For explanation of * and numbers within fig., see legend to Fig. 1.

Fig. 6. Regression of established mammary tumors in female Buffalo/N rats after ovariectomy. A, median index tumor volume; B, median cumulative tumor volume; O, Group A, sham ovariectomy; •, Group B, bilateral ovariectomy. The results of Koziol analysis for A were: Group A versus Group B, P = 0.006; for B: Group A versus Group B, P = 0.005. For explanation of * and numbers within fig., see legend to Fig. 1. Tumor weight is shown on day 70.

DISCUSSION

There are four major observations in this report: (a) inhibition of growth of primary rat mammary tumors by serotherapy was a reproducible observation; (b) the antitumor effect was generated from absorbed plasma or sera; (c) absorbed sera from tumor-bearing or tumor-free adult female Buffalo/N rats were effective in treatment; (d) generation of antitumor activity in sera during absorption with Protein A-Sepharose was inhibited by EDTA but not by EGTA.

The observations presented in this report may be viewed in the context of previous observations with this animal model. The initial observation (1) was that administration of tumor-bearer plasma absorbed with Protein A-Sepharose or inactivated CNBr Sepharose inhibited the growth of primary mammary tumors. Administration of unabsorbed plasma did not inhibit mammary tumor growth. There was no inhibition of growth by administration of quantities of endotoxin calculated to be present in the absorbent. In vitro analyses (3) showed that C3 and C5 were consumed during absorption of tumor-bearer or normal plasma with inactivated CNBr Sepharose or Protein A-Sepharose. The anticoagulant ACD had a major effect on C3 consumption. Increasing quantities of ACD were associated with decreasing C3 consumption. Addition of Mg2+ (10 mM) to plasma anticoagulated with ACD augmented C3 consumption. These observations indicated that Mg2+ concentration limited C3 consumption in plasmas anticoagulated with ACD. To more rigorously link Mg2+ to the activation of blood factors, sera were absorbed in the presence or absence of chelating agents known to bind Ca2+ and Mg2+ or to preferentially bind Ca2+.

The data in this report demonstrate that activation of the antitumor factor(s) during absorption is inhibited by EDTA but not by EGTA. The differential effect of these two chelating agents on generation of the factor(s) indicates that activation of the antitumor factor is dependent on Mg2+. The specific requirement for Mg2+ for generation of antitumor activity during absorption strongly suggests that activation of the alternative pathway of complement is involved in the observed antitumor effects. This conclusion is supported by the following observations: (a) the alternative pathway of complement is

index and median cumulative tumor volume in treated rats. There was, however, no reduction in median tumor volume of the "subsequently arising" tumors (data not shown). This result indicates that the primary effect of treatment is on the index tumor.
known to be activated during absorption of rat plasmas or sera with Sepharose derivatives (3); (b) inhibition of activation of the alternative pathway of complement by EDTA inhibits generation of sera with antitumor activity. These data suggest that one or more of the complement proteins altered during activation of the alternative pathway may have antitumor activity in vivo.

Whether the antitumor effect of absorbed sera is mediated directly by assembly of complement components on the mammary tumor cell membrane, through effects on tumor microvasculature, through leukocytes mobilized at the tumor site, or some other mechanism is the subject for future investigation. There is precedent for cell death mediated by assembly of the alternative pathway of complement on the surfaces of Raji tumor cells (8). Potent vasoconstrictive materials are generated during activation of the alternative pathway of complement (9).

Our interpretation of these results bear similarities to those of Cooper and Masinello (10) and Cooper and Sim (11). These workers treated mice having B16 melanoma cells in the peritoneal cavity by i.p. injection of sera absorbed with fixed S. aureus cells or Protein A-Sepharose. Sera from mice, guinea pigs, rabbits, and humans contained a precursor that could be activated by absorption to express antitumor activity. Both the precursor and product were labile at 0°C. This factor was present in some but not all partially purified preparations of human C1. In subsequent work (11) i.p. injection of purified preparations of human C3b, human C3 (H2O), cobra venom factor, or guinea pig C3 (H2O) led to inhibition of growth of the B16 melanoma. These authors concluded that substances that activate the alternative pathway of complement in mice have antitumor activity.

The syndrome of respiratory distress that occurs in some patients subjected to hemodialysis of fresh cuprophane dialysis membranes may be relevant (12, 13). The syndrome of respiratory distress occurring during hemodialysis has been linked to activation of the alternative pathway of complement in anticoagulated blood perfused over the new cuprophane membranes. Infusion of blood containing activated complement components leads to sequestration of leukocytes in the lungs of treated patients. Common pathophysiological mechanisms might underlie pulmonary toxicity associated with activation of the alternative pathway of complement and the inhibition of tumor growth observed in patients treated by infusion of absorbed plasma.

Inhibition of growth of primary rat mammary carcinomas by treatment with absorbed plasma was relatively weak. In treated rats median index or median cumulative tumor weight 35 or 50 days after beginning treatment was reduced 2- to 4-fold compared to controls treated with HBSS. In contrast, bilateral oophorectomy, a treatment known to inhibit the growth of primary rat mammary tumors (14), caused tumor regression and reduced median tumor weight 40- to 50-fold. Further attempts to optimize serotherapy in this animal model are needed. Consideration will be given to use of cobra venom factor and purified rat complement components. Absorption of sera at 0°C (a procedure suggested by Cooper (10) to preserve antitumor activity) may be important. Little is known about the optimal dose and schedule of serotherapy in this model.

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REFERENCES

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