Mechanism of Regression of Mammary Adenocarcinomas in Rats following Plasma Adsorption over Protein A-containing *Staphylococcus aureus*  

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**ABSTRACT**

The plasma from 7,12-dimethylbenz(a)anthracene-induced mammary tumor-bearing rats was adsorbed *ex vivo* with non-viable protein A-containing *Staphylococcus aureus* Cowan I and then injected into the rats, along with its original blood cells. Tumors in the treated rats showed significant (*p < 0.005*) growth inhibition. There were fewer metastatic nodules; cellular cytotoxicity in the presence of plasma was augmented, and there was increased antitumor cytotoxic antibody activity in treated rats. Plasma from sham-treated rats, however, showed blocking activity. It appears that plasma perfusion over *S. aureus* decreases blocking activity and augments antitumor immunoreactivity of plasma. The exact mechanism by which growth of 7,12-dimethylbenz(a)anthracene-induced mammary tumors was inhibited in these treated Sprague-Dawley rats is not known. However, it is hypothesized that the observed tumor regression is at least partly attributable to the augmentation of antitumor immunoreactivity in the treated animals.

**INTRODUCTION**

Recent scientific developments make it increasingly apparent that tumors develop and grow in an otherwise immunocompetent host. The tumors create an immunosuppressive environment in the host in order to protect themselves against an otherwise hostile immune environment. Host lymphocytes which may be competent to kill tumor cells *in vitro* usually remain inactive *in vivo* (2, 35, 36). Tumors can induce both humoral (2, 34-36) and cellular (8, 16, 34) immunosuppressive mechanisms which perhaps interact (11, 18, 34) to impose restrictions on the functions of antitumor immune components. Under induced immunosuppressive conditions, IC appear to play a central role. They counteract the antitumor reactivity of the sensitized lymphocytes (3, 15, 18) and also potentiate the activity of suppressor cells (9, 11, 34). Thus, removal of the IC from the plasma of a tumor-bearing host should unblock the immune activity and allow the host’s immune components to become active against the tumor.

SPA binds with the Fc structure of IgG. The Fc structure of an IC molecule is also open for binding with SPA. SPA has been observed to have a greater affinity for IC than for monomeric IgG (1, 8). Since both blocking antibody and IC have been suspected of playing a role in causing immunosuppression in cancer (2, 34-36), we wished to learn whether: (a) adsorption with SPA of plasma from a tumor-bearing host and reinfusion of the adsorbed plasma would augment the host’s immunoreactivity; and (b) whether this procedure would lead to regression of an established tumor. We reported earlier that perfusion of plasma of tumor-bearing patients (24, 30), dogs (27-30), and rats (22, 27, 30, 31, 33) over SPA led to tumor regression in treated hosts. Subsequent to those reports, other laboratories also described tumor necrosis in dogs (38, 39) and reversal of feline leukemia (14) when adsorption of plasma with SPA (38) was followed by chemotherapy (39) or radiation treatment (14). Although these latter studies were done with different tumor models using different protocols of treatment, they all supported the finding that plasma adsorption over SPA produced tumor regression. In this study, we wanted to determine the mechanism of regression of primary mammary adenocarcinomas in Sprague-Dawley rats following plasma adsorption over SPA and the subsequent reinfusion of the adsorbed plasma.

**MATERIALS AND METHODS**

**Animals.** Female Sprague-Dawley rats were obtained from Charles River Laboratories. When 50 days old, they were fed (20 mg/animal) DMBA in sesame oil which caused most of them (80%) to develop tumor nodules, usually multiple nodules, in various parts of their bodies. This suggests the metastatic nature of this tumor.

*S. aureus* Suspension. *S. aureus* Cowan I (NCTC 8530) and *S. aureus* Wood 46 were grown in CCY broth medium. The bacteria were formalinized (0.5% in phosphate-buffered saline) and heat treated. Bacteria treated in this manner did not show any growth when tested in nutrient broth media. Further details of the method have been described elsewhere (25, 30).

**In Vitro Adsorption of Plasma with SPA.** *S. aureus* Wood 46, or Protein A-Sepharose 4B. Plasma (2 ml) was collected from animals to be treated and transferred to tubes containing a packed SPA pellet (0.5 ml). The plasma was mixed well with SPA and incubated for 0.5 hr at 37°. After incubation, the mixture was centrifuged (3000 rpm, 10 min); the adsorbed plasma was reunited with its original blood cells and injected back into the animal. Plasma samples were incubated with *S. aureus* Wood 46 or Protein A-Sepharose 4B (Pharmacia) in the same manner as with SPA and were injected back into the animal. The samples were stored at −20° for future use.

**Preparation of Lymphocytes from Peripheral Blood and Spleen.** Lymphocytes from spleen and peripheral blood were separated using Lymphoprep (32). Peripheral blood and spleens were collected from both untreated and treated rats.

**Preparation of Tumor Cells.** A single-cell suspension of tumor cells was prepared by stirring fine pieces of tumor with 0.078% trypsin solution (Grand Island Biological Co.). The cells were washed 3 times with 10% fetal bovine serum (Flow Laboratories) containing CTM. The cells were finally suspended in CTM and counted for viability by the
trypan blue dye exclusion test. Only suspensions with >90% viability were used.

**Tumor Measurement.** The diameter of each tumor was measured from 3 different directions each week using a slide caliper, and the volume of each tumor was calculated using the formula described by Epstein and Bennett (6).

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

where \(D_1, D_2,\) and \(D_3\) are measurements of diameter from 3 different directions.

If animals had more than one tumor, volumes of all of the tumors were summed to arrive at the total tumor volume in that animal.

**Assay of Effector Cell Cytotoxicity in Either the Absence or Presence of Plasma.** Cytotoxic activity of PBL and splenic lymphocytes in either the presence or the absence of plasma was determined as described previously (4). Effector lymphocytes (100 µl) and \(^{51}\)Cr-labeled tumor cells (100 µl) were mixed in effector:target cell ratios of 25:1, 50:1, and 100:1 in Falcon microtissue culture plates. The culture plates were incubated for 4 and 18 hr at 37° in a humidified incubator containing 5% CO₂ in air. Two sets of controls were maintained with each experiment. In one set, labeled target cells were mixed with 0.1% detergent solution (sodium dodecyl sulfate) to obtain the total radioactivity. In another set, the target cells were incubated with CTM only, to account for spontaneous release of radioactivity into the supernatant. The percentage of cytotoxicity was determined using the formula

\[
\% \text{ of cytotoxicity} = \left(\frac{\text{cpm in experimental tubes} - \text{cpm for spontaneous release}}{\text{Total radioactivity} - \text{spontaneous release}}\right) \times 100
\]

**Antibody- and Complement-mediated Cytotoxicity Assay.** Cytotoxic antibody activity of the plasma was determined using rabbit serum complement (1:12) and \(^{51}\)Cr-labeled tumor cells. Controls were maintained for determining total radioactivity and spontaneous release of radioactivity, for monitoring background cytotoxicity due to the plasma from both treated and untreated rats, and to analyze the degree of rabbit serum complement lysis. The details of the method have been described elsewhere (23).

**Statistical Analysis.** The significance of increases or decreases in tumor volume was computed by the median test. Student’s one-tailed \(t\) test was used to determine significance of PBL-mediated cytotoxicity in the absence or presence of plasma. The significance of cytotoxic antibody activity was calculated by the median test also.

**RESULTS**

**Effect of Plasma Adsorption on the Growth of DMBA-induced Tumor.** Two months following DMBA feeding, the
animals with tumors were divided into 2 groups. One group received plasma adsorptions every week, while the other received sham treatment; i.e., plasma from these animals was handled identically during incubation, centrifugation, etc., except for addition of the immunoadsorbent. Tumor measurements were taken periodically, and total tumor volume in each animal was calculated, comparing it with the original tumor volume of the animal. The data are presented in Table 1. It is apparent that tumor size in most of the treated rats (8 of 11) remained unchanged for some time and then became smaller (5 of 8) in diameter than those in the sham-treated controls. Tumors of a few animals (2 of 11) regressed completely. Some animals (3 of 11) did not show any response. During the time when tumor nodules in the plasma-adsorbed animals usually remained constant in number, in 8 of 11 animals, most of the sham-treated animals (5 of 6) showed an increase in the number of tumor nodules, indicating that metastatic spread of the tumors. Changes in tumor volume of both the sham-treated and the treated groups were found to be significant ($p < 0.05$).

In a few treated animals (Animals 8 to 11), the plasma adsorption was continued for 11 weeks. Two animals (Animals 5 and 6) in the control group received sham treatment during the same period. After 11 weeks of treatment, the animals were kept for observation for another 2 months. Three experimental animals (Animals 8 through 10) showed continued tumor regression. One experimental animal (Animal 11) and 2 control animals (Animals 5 and 6) showed progressive tumor growth (Table 1, Footnote c). At the end of the 2 months, all of the animals were killed to obtain their plasma and PBL for mechanism studies.

It should be mentioned that animals in which plasma was adsorbed with non-protein A-containing S. aureus Wood 46, rather than with protein A-containing adsorbent, did not show similar tumor regression. Further, tumor-bearing animals in which plasma was either filtered after SPA adsorption or adsorbed with Protein A-Sepharose 4B did not show tumor regression to the extent observed with SPA adsorption only. This suggests involvement of some bacterial component in the observed tumor regression. Investigations are under way to learn more about this phenomenon.

Effect of Plasma Adsorption Therapy on Cytotoxic Activity of PBL in Either the Presence or Absence of Plasma. Both sham-treated and treated animals were killed, and their plasma was prepared. Lymphocytes were obtained from peripheral blood. Tumor cells and lymphocytes were mixed at ratios of 1:25, 1:50, and 1:100, either in the presence or in the absence of plasma (50 μl) from sham-treated and treated animals. The mixture was incubated for either 4 or 18 hr. Consistently reproducible data were obtained using the 1:100 ratio, when the cells were incubated for 18 hr. Cytotoxicity obtained at 18 hr was greater than it was at 4 hr. Therefore, data pertaining to 18 hr of incubation, target:effector cell 1:100, are presented in Table 2 which shows significant ($p < 0.05$) stimulation of PBL-mediated cytotoxicity in the presence of plasma from treated animals but not in the presence of plasma from sham-treated animals. Plasma from treated animals (Animals 1 and 3) which had no tumor regression showed some (2%) potentiation of PBL cytotoxicity. This suggests that mechanisms other than those being discussed might also contribute to the observed tumor regression in the animals. Plasma from sham-treated controls show significant ($p < 0.05$) blocking of PBL-mediated cytotoxicity when compared with plasma from treated animals. In subsequent studies, we have observed that plasma of sham-treated controls also show blocking activity against cells from treated animals. Also, the effect of treated serum was blocked in the presence of serum from sham-treated controls. In a canine venereal tumor model, we found that plasma from treated animals augmented "naive" lymphocytes to show ADCC.

Effect of Adsorbed Plasma on Antibody- and Complement-mediated Cytotoxicity. Cytotoxicity due to plasma antibody was measured using rabbit serum complement (1:12 dilution). The results are shown in Table 3. Plasma from treated animals showed a larger percentage of cytotoxicity than did plasma from sham-treated controls (statistically significant at the level of $p < 0.05$). Plasma from both treated and sham-treated control animals showed no appreciable cytotoxicity in the absence of rabbit serum complement, indicating the complement dependence of this reaction.

DISCUSSION

Our results indicate that adsorption of plasma of Sprague-Dawley rats bearing DMBA-induced primary mammary tumors with SPA can arrest the growth of DMBA tumors or cause them to regress. However, some tumors do not respond to the treatment. Animals in which plasma was adsorbed with Protein A-Sepharose 4B did not show similar regressive effect as that seen with SPA. Our results conform with previous observations of tumor regressions in rats (22, 27, 30, 31, 33) even though a different protocol of immunoadsorption (twice a week) was used in those studies. We are now able to report that plasma from treated animals showed: (a) decreased blocking activity; (b) augmentation of PBL-mediated cytotoxicity of DMBA-induced...
induced tumor cells; and (c) an increase in cytotoxic antibody activity, as measured in vitro against $^{51}$Cr-labeled DMBA-induced tumor cells.

It is known (3, 10, 13, 17) that tumor-bearing hosts have inherent difficulties in mounting an ADCC against autochthonous tumors. It has also been reported that sera from cancer patients can inhibit lymphocytic activity of normal individuals (12, 13). We described earlier that tumor-specific antibodies present in the serum of tumor-bearing hosts cannot function in the presence of serum-blocking factors (22). Mikulski et al. (17) reported that inhibition of effector cell function in the ADCC reaction using cancer patients' sera is different from the inhibition of effector cell function in cancer patients treated by us, following extracorporeal immunoadsorption of their plasma over SPA (24-30). An increase in body temperature observed in cancer patients subsequent to the infusion of SPA-adsorbed plasma (24-30) has also been observed in some treated rats. Thus, hyperthermic reactions induced in plasma-adsorbed hosts might also have a part in the regression of tumors since hyperthermia is known to induce tumoricidal reactions (7). It is also possible that leaching of some bacterial component(s) during immunoadsorption with SPA might have induced antitumor immune reactions (19-22). We have observed that nonviable SPA can cause regression of both chemically induced fibrosarcomas in C3H/HeJ mice and mammary adenocarcinomas in rats (22). We have recently reported (20, 21) that cancer patients' plasma elutes certain bacterial moieties, including Protein A, during in vitro adsorption with SPA. This study was done using radiolabeled SPA (labeled for proteins, carbohydrates, lipids, and nucleic acid). The adsorbed plasma was passed through a bacterial filter, and the filtered plasma showed an appreciable amount of radioactivity when lipid- and carbohydrate-labeled SPA was used for adsorption. Bacterial proteins, although they leached in much smaller amounts than did lipids and carbohydrates, showed the highest antigenic reactions of the 4 with anti-SPA antisera (20, 21). We have also observed that i.v. infusion of purified Protein A itself causes regression of mammary adenocarcinomas in rats (19). All of these observations taken together imply that both removal of plasma-blocking components and inclusion of leached bacterial components (Protein A and others) are contributory to the observed tumor regression in DMBA-treated rats. Further studies are necessary to delineate the exact role of each of these components in producing tumor regression.

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REFERENCES


Augmented Immune Reactivity after Plasma Adsorption

Table 3

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<tr>
<th>Group</th>
<th>Animal</th>
<th>% of cytolyis $^b$</th>
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<tr>
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$^a$ Rabbit serum complement (1:12 dilution).

$^b$ Statistical significance was determined by the median test. The median cytotoxicity in the experimental group was significant when compared with that of the control group ($p < 0.05$).
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