ABSTRACT

The plasma of dogs afflicted with mammary carcinoma was perfused through chambers bearing Staphylococcus aureus Cowan strain I in an attempt to remove tumor-promoting, immunosuppressive immune complexes from the peripheral blood of these animals. In this canine model of spontaneous mammary carcinoma, reduction of breast and/or soft-tissue tumor (posttreatment size equal to 0 to 50% of pretreatment tumor size) was observed in five of the ten animals so treated. Immune complexes capable of blocking lymphocytotoxicity were measured pre- and postimmunosorption; removal was more efficient in the five responders (four of six complexes) than in nonresponders (one of ten complexes), although statistical significance was not attained. The reduction of tumor size seen in soft-tissue sites was not always accompanied by a similar reduction of tumor size in visceral sites, and surgical resection of residual soft-tissue tumor nodules remaining after immunosorption treatment was required to achieve a complete response in two responding animals. No significant decrease in tumor size was observed in the control group, perfused without immunoadsorbent, nor in five additional tumor-bearing animals infused with normal dog plasma which had been passed through S. aureus Cowan strain I-containing chambers. These data indicate that immunoadsorption of tumor-bearing host plasma can result in reduction in size of canine mammary adenocarcinoma but that the response is dependent on the site of the tumor (s.c. versus visceral) and may require utilization of other modalities to achieve a complete disappearance of the tumor.

INTRODUCTION

The existence of an immune response to tumors has been documented by many investigators (1–5, 7–10, 12, 13, 15–18, 20–26, 29). This response may, however, be abrogated by circulating factors (free tumor antigen, antitumor antibodies, or immune complexes) which appear to possess the ability to block tumor-directed cytotoxicity. Such factors have been described in both humans and animals with growing neoplasms (1, 2, 4, 5, 7–9, 12, 13, 16, 18, 20, 29). It has been determined that protein A, found in the cell walls of SAC, can avidly bind both immune complexes and free IgG (human subclasses 1, 2, and 4) via the Fc portion of the immunoglobulin molecule (7, 16, 19). Passage of autologous plasma through chambers containing protein A bearing Staphylococcus aureus in both tumor-bearing dogs and in a human cancer patient was associated with evidence of tumor necrosis and clinical improvement (2, 3, 29, 30). It has not, however, been established that quantitative reductions in tumor mass occur in such hosts when compared to a separate, concurrent control group, nor have the potential antitumor effects of materials which might be leached from the bacterial paste by plasma perfusion been evaluated. The present study was undertaken to answer such questions and to determine if alterations of immune complexes in tumor-bearing animals would be associated with such reductions in neoplastic mass, as might be observed.

MATERIALS AND METHODS

The test animals used were dogs with biopsy-proven mammary adenocarcinoma. All of the animals had measurable tumor in the breast and/or soft tissues, with no disease detectable elsewhere as determined by physical examination, chest and skeletal survey X-ray, and routine clinical chemistry screens. Fifteen dogs were randomly assigned to the treatment group (10 animals) or to the control group (5 animals); these dogs were treated on a continuous flow cell separator as described below. Five additional dogs were nonrandomly assigned to the infusion group, which did not use the cell separator; these animals were smaller in size than the treatment or control groups, and our preliminary investigations indicated that the extracorporeal volume demanded by the cell separator would result in excessive depletion of the intravascular volume.

Treatment Protocol. In the treatment and control groups, access to the circulation was provided by placement of an arteriovenous shunt in the neck. Whole blood was pumped through a cell separator (American Instrument Corp., Silver, Spring, Md.) at an average rate of 40 to 60 ml/min; following separation, cellular elements were returned directly to the animal (Chart 1). Plasma was perfused through a filter chamber of 0.2-μm pore size (Gelman Co., Ann Arbor, Mich.). In the treatment group of 10 animals, this chamber was loaded with culture-negative SAC (30), which had been heat inactivated and formalin fixed ("Pansorbin"; Calbiochem-Behring Corp., LaJolla, Calif.) in a dose of 0.2 g/kg body weight. The immunoadsorbent capacity of the SAC was 1 mg IgG per 50 mg of bacteria. The control group of 5 tumor-bearing dogs was treated in an identical fashion, except that no bacteria were placed in the chamber. For each treatment, perfusion was continued until 100% of the calculated plasma volume had been processed, or until filter plugging resulting in excessive perfusion pressure forced a termination of the procedure. Perfusion was performed weekly for 2 weeks, and the animals were observed for another 2 weeks. Followable soft-tissue tumors were measured every other day in laboratory-owned animals and weekly in privately owned dogs; if a 50% reduction in tumor size (measured as the product of diameters) had not been attained at that point, 2 more weekly perfusions were performed. The 5 infusion group animals were not treated on a cell separator but were infused i.v. with pooled normal dog plasma, which had been passed through a chamber loaded with SAC (0.75 g/kg); this increased ratio was deliberately chosen so as to maximize the likelihood of detecting a real response, which might be secondary to elution of material from SAC, and thus independent of interaction with autologous plasma. Immunoadsorbed plasma volumes infused in these dogs were calculated to be as close as practicable to the median plasma volume (per kg body weight) adsorbed in the treatment group (62 ml/kg). Treatment schedules were similar to those animals treated on the cell separator.

In all 3 groups of animals, posttreatment biopsies were obtained at 55 to 60 days following the initiation of immunoadsorption treatment.
complete autopsies were obtained on all animals which expired, with
the exception of one case wherein permission was denied.

Immunological Studies. Pre- and posttreatment plasma of all ex-
perimental animals was sampled, and a series of immunological eval-
uations was performed. Circulating immune complexes were measured
by Raji cell assay (31) (Raji cells; American Tissue Culture Laborato-
ries, Bethesda, Md.), which had been modified by using 125I-labeled,
dog, heat-aggregated IgG (normal dog IgG obtained from Miles Re-
search Biochemicals, Elkhart, Ind., and labeled with 125I from New
England Nuclear, Boston, Mass.) and calibrated against a binding
curve constructed from incubating the Raji cells with increasing
amounts of unlabeled, heat-aggregated, normal dog IgG. Total circu-
ulating immune complexes were isolated by precipitation with a 3%
solution of polyethylene glycol (Sigma Chemical Co., St. Louis, Mo.)
and separated into individual complexes by ultracentrifugation on a 10
to 40% polyethylene glycol gradient (26). The individual complexes
were recovered, acid was dissociated at pH 2.5 in 0.3 M citric acid
(Sigma), and the dissociated components were separated by agarose
(Sigma) block electrophoresis (14). The separated antibody and anti-
gen components were recovered from the gel and concentrated by
lyophilization, and the antibody components reacted with autologous
tumor biopsy sections by indirect immunofluorescence (goat anti-dog
IgG fluorescein isothiocyanate-labeled antisera; Miles Research Bio-
chemicals) (26).

Following the immunofluorescence studies, the isolated complexes
were classified into 2 groups: (a) those containing tumor-associated
antibody capable of binding to autologous tumor; and (b) those which
did not contain tumor-associated antibody. In both cases, isolated,
intact complexes were incubated for 30 min at 37 °C with dog lympho-
cytes, cytotoxic to heterologous dog tumor cells (dog sarcoma cell
line; American Tissue Culture Laboratories), prior to performing a
microlymphocytotoxicity assay against the target tumor cells. The
degree of primed lymphocyte kill was recorded by measuring the
amount of 51Cr (New England Nuclear) released from the dead target
cells. The degree of cytotoxicity caused by the lymphocytes which had
been preincubated with the isolated complexes was compared with that
causd by primed lymphocytes which had not been preincubated with
the complexes. A decrease of 40% or greater 51Cr release follow-
ing incubation with the immune complexes was considered as evidence
of immune complex-associated blocking of lymphocytotoxicity.

RESULTS

Of the 10 treatment group animals subjected to plasma
immunoabsorption with SAC, 5 exhibited a 50% or more re-
duction in the size of the original soft-tissue tumor(s) (Table 1).
In 3 of the responding animals (HT-42, 34, and 43), tumor size
reduction occurred in 8 to 11 days following the initial extracorpo-
real immunoabsorption treatment, while 2 other respond-
ers (HT-24 and 36) required 25 and 38 days, respectively, for
50% regression to be accomplished. Biopsies obtained be-
tween 55 and 60 days following initial treatment and at autopsy
in one responder at Day 18 (HT-42) revealed infiltration with
large numbers of mononuclear and inflammatory cells and
tumor necrosis (Fig. 1). However, examination of multiple sec-
tions revealed scattered pockets of apparently viable tumor
cells in all animals. Two responding animals had simultaneous
normal breast biopsies performed (HT-24 and 34), and these
revealed no evidence of inflammatory infiltrates nor cellular
necrosis. An autopsy, performed 18 days after a single immu-
noadsorption treatment in one of the animals (HT-42) in which
dramatic reduction of soft-tissue carcinomatous deposits was
observed, revealed residual tumor in the lung, uterus, and
gastrointestinal tract. A second responding animal (HT-36)
expired at Day 58, and postmortem examination revealed the
presence of carcinoma in abdominal lymph nodes. Reduction
of soft-tissue carcinoma to 11 and 16%- of pretreatment size
was achieved in 2 animals (HT-24 and HT-34, respectively),
but surgical extirpation was required to completely eliminate
gross tumor. The first of these animals remains clinically free
of disease at present, 14 months after completion of immu-
noadsorption and surgical treatment, while the second is simi-
larly in complete remission at 16 months.

All of the treatment group animals exhibited transient in-
creases in WBC counts following extracorporeal immuno-
absorption. Approximately 15% of treatments were associated
with culture-negative transient fever. No consistent alterations
in serum chemistry panels were observed; IgG levels were
unaffected by the immunoabsorption with SAC.

None of the 5 control group animals perfused through blank
chambers exhibited tumor regression; all primary lesions re-
amained at 90% or greater of original size (Table 1). Biopsies
following perfusion demonstrated absent or quite minimal in-
flammatory cell infiltrate and few scattered areas of tumor
necrosis. Likewise, no reduction in tumor size occurred in the
animals infused with immunoabsorbed pooled normal plasma.

There were 7 deaths in the 20 dogs studied: 4 in the
treatment group (2 responders and 2 nonresponders); one
among the 5 control animals; and 2 of the 5 infusion group
dogs. Postmortem was performed in 6 of the 7, and residual
gross tumor was evident in all animals.

A series of tests to detect and characterize circulating
immune complexes was performed on serum samples of the
treatment group dogs as described previously. Such com-
plexes were found in both pre- and posttreatment samples from
all animals (see Table 2).

Complexes were isolated and dissociated, and the antibody
components thus obtained were reacted against paraffin-
embedded sections of autologous tumor by indirect immuno-
fluorescence localization. Tumor-associated antibodies were
found in all animals prior to immunoabsorption; after treatment,
such antibodies were found in 4 of 5 responders and 3 of 5
nonresponders.

The capacity to block cytotoxicity was possessed by both
tumor-associated complexes and by “nonspecific” complexes
which did not contain antibody capable of binding to tumor
cells. Specific blockers were present pretreatment in 4 of 5
animals each in the responding and nonresponding groups;
following immunoabsorption, specific blockers were found in 2
of 5 responders and 3 of 5 nonresponders. Nonspecific block-
ning complexes were present in pretreatment samples of 2 of 5
responders and 5 of 5 nonresponders. Analysis of the post-

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**Table 1**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Original tumor size (sq cm)</th>
<th>Final tumor size (% reduction of original)</th>
<th>Total plasma processed (mL/kg)</th>
<th>Duration of observations (days)</th>
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<tr>
<td></td>
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<tr>
<td><strong>Treatment group (responders)</strong></td>
<td></td>
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<tr>
<td>HT-42</td>
<td>39</td>
<td>100</td>
<td>75</td>
<td>18^a, b</td>
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<td>HT-24</td>
<td>6</td>
<td>89</td>
<td>82</td>
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<td>HT-34</td>
<td>13</td>
<td>84</td>
<td>53</td>
<td>480</td>
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<td>HT-36</td>
<td>10</td>
<td>71</td>
<td>238</td>
<td>56^a, c</td>
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<td>HT-43</td>
<td>29</td>
<td>50</td>
<td>120</td>
<td>210</td>
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<tr>
<td>Mean</td>
<td>25</td>
<td>71</td>
<td>114</td>
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<tr>
<td>Median</td>
<td>21</td>
<td>77</td>
<td>82</td>
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<td><strong>Treatment group (nonresponders)</strong></td>
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<td>HT-40</td>
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<td>57</td>
<td>57</td>
<td>114^a, i</td>
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<tr>
<td>Mean</td>
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<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>5</td>
<td>65</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Signifies death on study.
^b Pneumonia; local nodal metastases, few (3 to 5 mm) pulmonary metastases, plus metastases in the uterus and gastrointestinal tract; metastatic lesions with patchy areas of necrosis; and inflammatory cell infiltrates.
^c Nonmalignant hemorrhagic cerebral intact, local metastases with areas of cellular necrosis, and inflammatory cell infiltrates.
^d Progressive soft-tissue infection, visceral metastases with no apparent tumor cell necrosis, and minimal inflammatory cell infiltration.
^e Rapid tumor progression, pneumonia, multiple nodal and visceral metastases, and no cellular necrosis or inflammatory cell infiltration.
^f Aspiration pneumonia, multiple nodal and visceral metastases, no apparent cellular necrosis, and minimal inflammatory cell infiltrates.
^g Lost to further observation.
^h Multiple visceral metastases, no cellular necrosis, nor inflammatory infiltrate.
^i Suspected visceral metastases; owners denied permission for necropsy.

Removal of blocking complexes was more effectively accomplished in the responder than the nonresponder animals, in that 6 such complexes were present pretreatment in responders, and 4 were removed via immunoadsorption, whereas 10 pretreatment complexes were present in nonresponders, and only one was removed by immunoadsorption. χ^2 tests were performed comparing pre- and posttreatment samples of responders and nonresponders for total blocking complexes, specific blockers, and nonspecific blockers (6). The observed differences did not achieve statistical significance, with p values greater than 0.10.

**DISCUSSION**

These data indicate a beneficial effect of extracorporeal immunoadsorption utilizing protein A in the form of SAC, in that half of the animals so treated exhibited partial response of primary and/or soft-tissue tumor deposits associated with histological evidence of inflammatory cell infiltrate and tumor cell necrosis. However, it was also clear that apparently viable tumor remained after treatment in every case.

The predicted benefit of perfusion of plasma over the adsorbent was the removal of immunosuppressive material, namely circulating immune complexes, although this hypothesis has not been explicitly stated in previous studies of this therapeutic modality (3, 29, 30). Such complexes have been demonstrated in the sera of dogs with malignant breast disease (9, 29, 30), and their persistence after surgical removal of the tumor was associated with a high likelihood of recurrence (9). Indeed, such complexes were found in the pretreatment sera of all treatment group dogs, and approximately 40% possessed the capacity to block cytotoxicity. Immunoadsorption therapy reduced the number of complexes present, and within the limits of the amount of adsorbent used and plasma treated, this...
lymphocytotoxicity of primed lymphocytes against dog sarcoma target cells. Total complexes, total blocking complexes, and specific and nonspecific blocking immune complexes which did not contain detectable antibody which was directed against the tumor cells in the autologous biopsy but were able to block lymphocytotoxicity of primed lymphocytes against dog sarcoma target cells. The first column indicates the number of antibody-containing complexes over the total number of complexes detected. The second column indicates the number of complexes possessed cytotoxic blocking properties, and amounted to a reduction of approximately 30% of total complexes. In nonresponding animals, a slightly greater proportion of complexes possessed cytotoxic blocking properties, and removal was less effective (one of 10 as opposed to 4 of 6 in responders). However, pre- and posttreatment differences in total complexes, total blocking complexes, and specific and nonspecific blocking complexes were not significant by \( \chi^2 \) tests.

Since IgG levels were unaffected by immunoadsorption and since no tumor reduction occurred in the group of dogs treated by infusion of pooled normal plasma passed through chambers prepared with SAC (implying that leaching of antitumor material was unlikely to be the cause of tumor reduction), it would appear that there is a relationship between the ability to remove blocking immune complexes and reduction of tumor mass. Such tumor regressions were occasioned by the use of material having an immunoadsorbent capacity of only 4 mg of IgG equivalent per kg of body weight and absorption of only 70 to 100 ml of plasma per kg over a 60-day period. Whether more intensive immunoadsorption would have been associated with increased tumor regression cannot be inferred from this study. Complete removal may not be possible with SAC immunoadsorption, since at least in humans, IgG3 is not bound. However, it should be noted that 2 of the 5 responding animals each had one blocking complex remaining posttreatment, and one nonresponder had no blocking complexes detected after immunoadsorption; it would then appear that removal of all blockers is neither a necessary nor sufficient condition for initiation of tumor regression in this animal model.

The precise mechanism by which removal of blocking antibodies may induce tumor regression was not addressed in this study. However, the impressive cellular infiltrate seen in posttreatment biopsies suggests a role for cell-mediated immunity. Prior studies (30) have described deposition of immunoglobulin (IgG) on tumor cell surface following SAC adsorption in 4 dogs (of 12 treated) and suggest that tumoricidal response was secondary to tumor-specific antibody action. However, such a relationship may be more gratuitous than causal, since in human studies, IgG deposition has been observed in 35% of primary, untreated breast cancers and 49% of metastatic lesions (23) as well as in untreated primary head and neck tumors (18).

Removal of blocking immune complexes as the initiating event in immune-mediated tumor destruction may be an explanation of the observations made in this investigation. Tumor hosts whose plasma was passed through empty filter chambers showed no reduction in tumor size nor evidence of inflammatory cell infiltrate or necrosis on biopsy. Although enhancement of T-cell activity has been ascribed to the use of the cell separator alone (5, 11), it did not appear that this manipulation was capable of inducing tumor regression in this study. Of equal significance, however, was the observation that passage of pooled normal plasma through SAC with subsequent i.v. infusion into dogs with mammary cancer was without observable gross or histological effect on the tumor. This indicates that it was not some eluate of the bacterial suspension or material generated by interaction of plasma and bacteria which was responsible for tumor cell kill, but it implies that some interaction of bacterial suspension and autologous plasma is required for this effect.

We conclude that plasma immunoadsorption with material able to remove circulating immune complexes capable of blocking cytotoxicity can induce partial regression of local and/or regional soft-tissue tumor deposits, even when moderate amounts of adsorptive material are used, and comparatively modest volumes of plasma are so treated. However, in no case was all tumor eliminated, and in particular, postmortem studies revealed persistence of visceral metastases in one responder (HT-42) virtually exhibiting total necrosis of soft-tissue tumor. Further, 2 long-term survivors (14 and 16 months) required
local surgery following immunoadsorption to render them fully disease free. It would seem that this technique has therapeutic potential in an adjuvant setting and that further investigation must address the role of cell-mediated tumor cell destruction apparently engendered by immunoadsorption.

REFERENCES


Fig. 1. Photomicrographs of tumor sections from animal HT-24 pre- and postimmunoperfusion treatment. H & E. × 132 (A) and × 80 (B).
Regression of Canine Mammary Carcinoma after Immunoadsorption Therapy


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