Tumoricidal Response following Perfusion over Immobilized Protein A: Identification of Immunoglobulin Oligomers in Serum after Perfusion and Their Partial Characterization

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ABSTRACT

Previously, we showed that perfusion of plasma from hosts bearing breast adenocarcinoma over immobilized staphylococcal protein A resulted in objective tumor regressions. In the present study, sera perfused in vitro over immobilized staphylococcal protein A were analyzed by physicochemical and immunochemical methods to characterize newly formed products. Sera from normal and breast adenocarcinoma-bearing dogs showed increased levels of C1q-binding IgG after perfusion over a strain of staphylococcus that is protein A rich (Cowan I), but not protein A deficient (Woods 46). C1q binding levels were also increased in normal and tumor-bearing canine or human sera which were perfused over purified protein A immobilized in collodion charcoal (PACC), and this increase was localized in sucrose density gradient fractions ranging from 7S to 19S. Polycrylamide gel electrophoresis analysis of the high-molecular-weight fraction in postperfusion canine sera, isolated by G-200 fractionation and immunoaffinity chromatography, showed predominantly heavy and light immunoglobulin chains of canine IgG. Furthermore, protein A was released from PACC after perfusion with serum or solutions containing IgG or albumin from humans, dogs, and chickens. After serum perfusion over PACC, protein A was identified in the effluent by additional studies as follows: (a) polycrylamide gel electrophoresis analysis showed that eluted 125I-protein A comigrated with the protein A marker; (b) postperfusion C1q-binding complexes, isolated by gel filtration under dissociating conditions and affinity chromatography on IgG-Sepharose showed a single precipitin band with normal human (protein A reactive) but not chicken (protein A unreactive) serum. Protein A released from PACC which appeared in postperfusion sera was associated with immunoglobulins in macromolecular complexes since (a) eluted 125I-protein A was largely (NH4)2SO4 and polyethylene glycol precipitable, whereas free protein A was not, and it sedimented in sucrose density gradient fractions distributed beyond the 7S marker, compared to free protein A which localized below 7S; (b) radiolabeled protein A eluting from PACC after serum perfusion showed 8-fold greater binding to C1q-coated tubes compared to free protein A; and (c) increased C1q-binding IgG in postperfusion sucrose density gradient fractions corresponded to the appearance of protein A in parallel.

INTRODUCTION

Protein A is a constituent of the cell wall of many strains of Staphylococcus aureus, which binds selectively to immunoglobulins and immune complexes from many mammalian species at sites located in the IgG-Fc region. These complexes display diverse biological activity, including complement binding and activation (6, 9, 11–13, 23). Previously, in studies of dogs with spontaneous mammary adenocarcinoma, an excellent model of human breast cancer, plasma was circulated over SAC, which were immobilized in a microporous membrane filtration system and placed on line with plasma emerging from a continuous-flow plasma-cell separator (27). Shortly after perfusion of one plasma volume over Protein A-bearing staphylococci, tumor necrosis followed by objective tumor regressions were observed. These effects were not seen when plasma was passed over Protein A-deficient staphylococci (27). Findings of tumor regressions in our experimental canine system were confirmed in an independent study (8). We refined the perfusion system, and similar necrotic responses were observed after plasma perfusion over purified PACC (28). This system was recently used to treat 5 consecutive patients with advanced breast adenocarcinoma, and after repeated plasma perfusion, objective tumor regressions were noted in 4 patients (29).

In prior studies of patients with breast adenocarcinoma, we...
noted the rapid onset of acute pain, as well as gross and histopathological changes in chest wall tumors after extracorporeal perfusion of small volumes of plasma over PACC (29). These results suggested that these effects might be produced by factors generated in plasma after passage over PACC. To test this hypothesis, the procedure was technically simplified by eliminating the on-line extracorporeal circulation system and, instead, small aliquots of autologous or homologous plasma, previously collected by phlebotomy, were perfused over PACC off-line, followed by direct infusion into patients (29). Patients treated entirely with the off-line plasma perfusion showed acute tumor-inflammatory responses, and tumor-regressive effects similar to those observed with the on-line extracorporeal system (29). Moreover, the tumoricidal responses in both dogs and man were accompanied by serological and immunohistochemical changes, including increased solid-phase C1q-binding IgG, and a decline in C3 associated with deposits of IgG and C3 within tumor tissue (27, 29).

From the foregoing observations, it appeared that the interaction of plasma with staphylococcal protein A resulted in the formation of products which were capable of activating host responses and producing tumoricidal activity, as well as some of the side effects of the procedure (28-30). Therefore, we attempted to identify and characterize these products. For this purpose, we developed an in vitro perfusion system similar to the off-line system used in our patients, in which sera from tumor-bearing or normal dogs and patients were passaged over SAC or PACC. Preperfusion and postperfusion serum samples were analyzed by physicochemical and immunoochemical methods and compared. The results of the present study show that perfusion of normal or tumor-bearing serum over SAC or PACC results in the generation of C1q-binding complexes, composed predominantly of immunoglobulins and protein A. We postulate that these complexes may be a major constituent responsible for the previously described tumoricidal activity and toxicity of this therapeutic system (29, 30).

MATERIALS AND METHODS

Animals and Sera

Sera obtained from dogs with histologically confirmed mammary adenocarcinoma were used in this study. Normal dog sera were obtained from animals raised in a controlled environment and examined at regular intervals by a veterinarian. Sera from all animals were collected and stored at −70° until use. Normal human sera were obtained from volunteers with no evidence of active or chronic disease. Tumor-bearing sera were obtained from patients with breast adenocarcinoma who had a clinically evident tumor burden at the time serum was obtained.

*S. aureus*, Immobilized Protein A

Cowan I (SAC) (ATCC 2530) and Woods 46 (SAW) (ATCC 1830) strains were cultured, fixed with 0.5% formaldehyde, and stored at 70°C until use. For *in vitro* perfusion studies, the heat-killed and formalin-stabilized staphylococci were washed in 0.15 M NaCl, and 10 g were resuspended to a final concentration of 1% in 0.15 M NaCl. The staphylococcal suspension was then pumped at a rate of 20 ml/min into a modified 0.2-μm pleated filter (Pelican Instrument Co., Ann Arbor, MI) until the bacteria were embedded in the membrane and all 0.15 M NaCl had passed through the filter. The filter was then emptied of residual fluid by air expulsion. In perfusion studies, the filter was loaded with 100 ml of serum which were then perfused through the system by forced-air pressure at a rate of 5 ml/min. Effluent fractions (1 to 5 ml) were collected and stored at −70° until use. In selected studies, normal or tumor-bearing sera were simultaneously perfused by dual channel tubing through filters containing SAC and SAW, which were arranged in parallel. Effluent samples from each system were collected and stored at −70°. Purified protein A (Pharmacia, Piscataway, NJ; 5 mg), was immobilized in 30 g of collodion charcoal and washed with PBS by a modification of previously described methods (25, 26).

Perfusion Studies

For perfusion studies, 6 g of charcoal containing approximately 1 mg of immobilized protein A were spread in a thin layer between two 20 mesh stainless steel screens in a Lexan-stainless steel cartridge. The PACC was washed with 500 to 1000 ml of 0.01 M PBS, pH 7.5. After removal of residual PBS, 5 to 50 ml of sera were introduced into the cartridge and incubated with PACC for 5 min at 25°. The serum was evacuated from the chamber by gravity, and 1- to 5-ml effluent fractions were collected. In selected studies, 1 mg of canine albumin was immobilized in collodion charcoal in a fashion similar to that for protein A and similarly incubated with human or canine serum. In other studies, 10 ml of normal human, canine, or chicken serum, solutions of human, canine, or chicken IgG or albumin, were perfused into a 0.15 M NaCl solution-filled Lexan chamber containing PACC with 125I-protein A immobilized together with unlabeled protein A. Similar experiments were carried out with collodion charcoal-containing immobilized canine albumin, together with 125I-labeled canine albumin. Effluent fractions (1 ml) were collected and radioactivity was measured.

Antisera and F(ab’2) Fragments

Purified F(ab’2) fragments of goat IgG specific for human IgG, IgM, IgA, and C3 (Cappel Laboratories, Cochranville, PA) were used. F(ab’2) fragments of goat or rabbit IgG fractions (Cappel Laboratories) specific for canine IgG, IgM, IgA, and C3 were prepared as previously described (24). Briefly, IgG fractions of these antisera were digested with pepsin in 0.1 M acetate buffer, pH 4.1, using an enzyme/substrate ratio of 1/100 (w/w) for 20 hr at 37°. The pH was adjusted to 8.0 by the addition of solid Tris salt, and F(ab’2) was separated from the other fragments by gel filtration on Sephadex G-200 with PBS, pH 7.4, as the eluting buffer. The preparations were free of intact IgG and Fc fragments, as shown by double immunodiffusion.

Radioiodination of Protein

Purified protein A, canine or human IgG, IgM, and F(ab’2) fragments were labeled with 125I by the method of McConahey and Dixon (20). The mean specific activities were as follows: protein A, 0.35 to 0.40 μCi/μg; canine and human IgG, 0.15 to 0.25 μCi/μg; canine albumin, 0.40 μCi/μg; F(ab’2) fragments, 0.24 to 061 μCi/μg. In selected studies, protein A was labeled with 125I with a mean specific activity of 0.9 to 1.0 μCi/μg by the method of Bolton and Hunter (4).

Measurement of C1q-binding Complexes, Immunoglobulins, and Protein

C1q binding complexes were measured by a standard solid-phase C1q-binding radioimmunoassay, as previously described (27). In some experiments, radioiodinated F(ab’2) fragments specific for human or canine IgG, IgM, IgA, or C3 were used to detect components of C1q-binding complexes. In preliminary experiments using 125I-C1q, we determined that approximately 1.2 μg of C1q were bound per tube. In some studies, results were referred to a standard curve using AHG or ACG, and were expressed as μg equivalents AHG or ACG/ml. Mean normal values for normal canine and human sera were 20.1 ± 5.7 (S.D.) and 18.6 ± 2.6 μg equivalents ACG or AHG/ml, respectively. In other studies, results were given as ng of anti-canine or anti-human IgG, IgM, IgA, or C3.
bound. For the detection of C1q-binding IgG in sucrose density gradient fractions, or concentrates of postperfusion effluents, 0.2- to 0.5-ml aliquots were assayed. Preliminary experiments showed that increasing amounts of protein A, tested up to a concentration of 100 μg/ml and added to normal canine serum, gave a linear increase in the quantity of complexes that bound to C1q-coated tubes.

Canine or human immunoglobulins were measured in serum by a nephelometric assay, as previously described (10). In selected studies, quantitative measurements of canine IgG were performed by the method of Mancini et al. (19), using radial immunodiffusion in agarose gels (Miles Laboratories, Elkhart, IN). Protein concentrations of serum were measured by the method of Lowry et al. (18). The protein content of solubilized PEG-precipitated protein was determined by the method of Bradford (5) (Bio-Rad Laboratories, Richmond, CA).

Sucrose Density Gradient Fractionation

Serum samples (1.5 ml) obtained before and after perfusion over SAC or PACC were layered on 15 ml of 10 to 40% (w/v) sucrose density gradients in PBS. The samples were centrifuged at 40,000 rpm for 6 hr at 4° with a reorienting TV865B vertical rotor in a Sorvall OTD-65 ultracentrifuge, and 1.2-ml fractions were collected from the bottom of the gradient. The positions of 7S and 19S were determined with purified canine IgG and IgM (Pel-Freeze Biologicals, Rogers, AK) markers run in parallel gradients.

Chromatography

Gel filtration chromatography was carried out on Sephrose 6B or Sephadex G-200 (Pharmacia). Buffers used were PBS, and 3-ml fractions were collected.

SDS-PAGE

The high-molecular-weight IgG isolated by affinity chromatography was analyzed by PAGE in 7.5 or 10% gels, using the buffer systems of Laemmli (14). The samples to be analyzed were mixed in a final solution containing 5% urea, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.01% bromophenol blue and heated for 1 min at 100° before application to gels. In other studies, normal canine serum was passed over PACC which contained 125I-protein A (1 to 2 μCi), together with unlabelled protein A (total immobilized protein, 1 mg). The postperfusion serum was incubated with 5% PEG for 60 min at 25° and then centrifuged at 5000 x g for 15 min. The precipitate was resuspended in PBS and analyzed by SDS disc PAGE. After electrophoresis, the gel was sliced into 2-mm segments and radioactivity measured.

Treatment of Sera with PEG and Ammonium Sulfate

Normal or tumor-bearing sera (50 ml) were passed into a 0.15 M NaCl-filled Lexan chamber containing PACC with 125I-protein A immobilized together with unlabeled protein A (total immobilized protein A, approximately 1 mg). An additional 200 ml of 0.15 M NaCl was perfused through the chamber to ensure removal of serum. Effluent fractions containing the peak 125I (50 ml) were pooled and treated as follows: (a) to 15 ml of effluent an equal volume of saturated (NH₄)₂SO₄ was added, incubated for 30 min at 4°, and centrifuged at 1500 x g for 30 min at 4°; and (b) to 15 ml of effluent, 5 ml of 20% PEG were added, incubated for 60 min at 25°, and centrifuged at 5000 x g for 15 min at 4°. In all samples, radioactivity in the precipitates was measured. In all instances, free 125I protein A used in the immobilization process was similarly treated.

Evaluation of Stability of Immobilized Protein A

To evaluate the possible release of protein A from PACC during perfusion of serum over it, the following studies were performed. Radiolabeled protein A (1 to 2 μCi) was mixed with 1 mg of unlabeled protein A and immobilized in colloidion charcoal, as described above. Greater than 95% of the radiolabeled protein A was incorporated into the colloidion charcoal. The PACC was placed in a Lexan chamber and washed with 3000 ml of 0.15 M NaCl. Normal human or chicken sera (10 ml) were then perfused through the chamber at a flow rate of 2 ml/min. Radioactivity which eluted from the PACC after perfusion with serum or solutions containing IgG or albumin was measured in the effluent samples. The quantity of protein A released was calculated as a percentage of the total radioactivity added to the PACC after correction for the amount lost in the immobilization and washing procedures.

Isolation of Complexes and Identification of Components

Method 1. High-molecular-weight canine IgG in postperfusion sera was isolated by a modification of a previously described procedure (1). Postperfusion sera (3 ml) were passed through Sephadex G-200, and fractions in the void volume peak were collected. The void volume peak was passed through Sepharose 4B to which goat IgG specific for γ chain of canine IgG was bound. The complexed protein was eluted with 0.1 M glycine-HCl buffer, pH 2.5. The eluate was adjusted to pH 7.5 with 5 N NaOH and concentrated to a final volume of 0.2 to 0.3 ml by negative-pressure dialysis. This sample was then examined by SDS-PAGE and immunodiffusion.

Method 2. Postperfusion sera were incubated with 5% PEG and centrifuged, and the precipitate was resuspended and passed over Sephadex G-100 at pH 3.0, as in Method 1, above. The effluent was neutralized and concentrated as above. To isolate protein A, the effluent was treated further as follows: to remove small quantities of contaminating IgG, the included fractions were incubated with an equal volume of saturated (NH₄)₂SO₄, at pH 3.0 for 30 min at 4°, then centrifuged at 5000 x g for 15 min at 4°C. The supernatant was dialyzed extensively against H₂O at 4° and lyophilized. The protein was resuspended in PBS, passed over IgG-Sepharose 4B, and bound protein was eluted with 0.1 M glycine-HCl buffer, pH 2.5. The pH was then adjusted to 7.5 with 5 N NaOH, concentrated to a volume of 0.1 ml by negative-pressure dialysis, and analyzed for the presence of protein A by immunodiffusion in 1% agarose, using 20 μl of normal human or chicken sera per well, with continuous addition of 20-μl aliquots of the test sample containing putative protein A.

Method 3. Pre- and postperfusion sera were subjected to sucrose density gradient ultracentrifugation, as described above. Each fraction was incubated with C1q-coated tubes for 18 hr at 4°. The tubes were washed twice with PBS containing 1% bovine serum albumin. To determine the presence of canine IgG, IgM, IgA, or C2, 125I-labeled goat F(ab')₂ fragments specific for canine IgG, IgM, IgA, or C2 were added, the tubes were washed, and radioactivity was measured, as described above. In other studies, 10 ml of effluent serum from PACC or canine albumin collodion charcoal columns were collected. The eluted 125I-protein A or 125I-labeled canine albumin was concentrated by ultrafiltration, using an Amicon XM10 filter (Amicon, Lexington, MA), to a volume of 1 ml, and 0.5-ml samples were incubated with C1q-coated tubes. The tubes were washed and radioactivity was measured. As a control, free 125I-protein A or 125I-labeled canine albumin solution was similarly incubated with C1q-coated tubes and radioactivity was measured.

RESULTS

Changes in C1q Binding in Normal and Tumor-bearing Sera after Perfusion over SAC, SAW, or PACC. Sera from 5 normal dogs or 6 dogs with mammary adenocarcinoma were perfused over SAC, and pre- and postperfusion samples were assayed for C1q-binding activity by radioimmunossay. As shown in Chart 1, C1q-binding levels in postperfusion samples declined at first, then increased above pretreatment values. The initial decrease in C1q-binding levels may represent a dilutional effect from small amounts of residual 0.15 M NaCl in the filter. In contrast to the
observed increases in C1q binding, canine IgG concentrations in postperfusion samples were uniformly reduced below preperfusion levels, as were postperfusion levels of IgM in sera perfused over SAC (see legend to Chart 1).

To determine if protein A on the SAC might be important in the generation of increased C1q binding, sera from normal dogs and dogs with mammary adenocarcinoma were perfused over SAC or protein A-deficient SAW, as described in "Materials and Methods." Samples emerging from the SAC columns showed an increase above preperfusion C1q-binding levels, whereas effluent samples from the SAW columns showed minimal changes from pretreatment levels (Chart 2). As with the above studies, the initial reductions in C1q binding may partially reflect dilutional effects from a small amount of residual fluid in the filters containing SAC or SAW.

Sera from normal dogs and dogs with mammary adenocarcinoma were passed over PACC, as described in "Materials and Methods." In 8 of 8 tumor-bearing sera, and 6 of 8 normal sera, there was an increase in C1q binding in effluent sera (Table 1).

In contrast to perfusion over SAC, there were no significant changes in IgG, IgM, and IgA levels in sera perfused over PACC (data not shown).

Partial Characterization of Complexes Generated in Sera after Perfusion over PACC. Since protein A has been shown to bind canine IgM and IgA (2, 3), we tested for these components, as well as for IgG and C3, in the complexes in sera perfused over PACC. For this purpose, pre- and postperfusion sera were incubated with C1q-coated tubes in order to bind the complexes. As a control, sera were perfused over canine albumin rather than over PACC, and were similarly incubated with C1q-coated tubes. The tubes were washed and excess 125I-F(ab')2 fragments specific for canine IgG, IgM, IgA, or C3 were added, and bound radioactivity was measured. Postperfusion binding levels of anti-IgG and anti-IgM in sera perfused over PACC increased approximately 20% above preperfusion values, but there were no significant changes in anti-IgA or anti-C3 binding. Results were similar for tumor-bearing and normal sera, and similar increases in anti-IgG-binding activity were detected in postperfusion sera from an additional tumor-bearing and a normal dog. In contrast, anti-IgG-binding activity was reduced by 36 to 44% in serum perfused over immobilized canine albumin, while binding of other components was essentially unchanged.

Normal or tumor-bearing human sera were similarly passed over PACC, incubated with C1q-coated tubes, and tested for binding of 125I-F(ab')2 fragments specific for human IgG, IgM,

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**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>Normal sera</th>
<th>Tumor-bearing sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preperfusion</td>
<td>50.9±50.0±51.8</td>
<td>65.3±62.5±68.8</td>
</tr>
<tr>
<td>Postperfusion</td>
<td>63.2±59.0±67.4</td>
<td>43.6±47.3±47.4</td>
</tr>
<tr>
<td>Δ</td>
<td>14.4±14.4±14.4</td>
<td>19.6±19.6±19.6</td>
</tr>
<tr>
<td>Preperfusion</td>
<td>56.9±57.3±60.6</td>
<td>50.2±43.5±53.0</td>
</tr>
<tr>
<td>Postperfusion</td>
<td>63.3±60.0±66.7</td>
<td>52.1±47.5±55.2</td>
</tr>
<tr>
<td>Δ</td>
<td>7.0±7.0±7.0</td>
<td>0.4±0.4±0.4</td>
</tr>
<tr>
<td>Preperfusion</td>
<td>50.0±49.5±51.0</td>
<td>41.7±39.7±43.7</td>
</tr>
<tr>
<td>Postperfusion</td>
<td>57.0±54.8±59.2</td>
<td>55.8±55.0±56.7</td>
</tr>
<tr>
<td>Δ</td>
<td>7.0±7.0±7.0</td>
<td>14.1±14.1±14.1</td>
</tr>
<tr>
<td>Preperfusion</td>
<td>26.9±25.4±28.5</td>
<td>29.6±27.9±31.3</td>
</tr>
<tr>
<td>Postperfusion</td>
<td>30.8±30.1±31.6</td>
<td>30.8±30.1±31.6</td>
</tr>
<tr>
<td>Δ</td>
<td>13.2±13.2±13.2</td>
<td>1.2±1.2±1.2</td>
</tr>
<tr>
<td>Preperfusion</td>
<td>26.0±25.6±28.4</td>
<td>36.0±33.1±39.4</td>
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<tr>
<td>Postperfusion</td>
<td>75.9±70.9±80.0</td>
<td>75.9±70.9±80.0</td>
</tr>
<tr>
<td>Δ</td>
<td>39.9±39.9±39.9</td>
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* Values represent the average of duplicate or triplicate determinations.
* Numbers in parentheses, range of values from duplicate or triplicate determinations.
* Mean ± S.D.
* The increases in post- compared to respective preperfusion levels were statistically significant.
In contrast, there were no significant anti-IgG binding in effluents of 2 of 3 normal sera, and of 4 of 4 increases in anti-IgG, anti-lgA, anti-IgM, or anti-C3 binding in all normal sera, and in 2 of 4 tumor-bearing sera tested; and (d) increased anti-lgM binding in 2 of 4 tumor-bearing sera. In contrast, there were no significant increases in anti-lgG, anti-lgA, anti-lgM, or anti-C3 binding in normal or in tumor-bearing sera perfused over immobilized human albumin (Table 2).

To determine the size characteristics of the C1q-binding IgG generated in sera after perfusion over PACC, pre- and post-perfusion effluent samples from normal or tumor-bearing sera were analyzed by sucrose density gradient centrifugation, and fractions were assayed for C1q-binding IgG. Both sera showed increased levels of C1q-binding IgG in fractions greater than 7S, compared to corresponding fractions of preperfusion sera (Chart 3). Similar increases in C1q binding in these gradient fractions were noted when F(ab')2 fragments specific for γ chains of canine IgG were used to detect IgG bound to C1q.

The high-molecular-weight IgG complexes in postperfusion canine sera were then isolated by fractionation on G-200 column chromatography. The void volume peak fractions were rechromatographed over an immunoadsorbent containing γ chain-specific rabbit anti-canine IgG, as described in "Materials and Methods." The retained protein was eluted, neutralized, concentrated, and reduced before analysis by SDS-PAGE. PAGE profiles stained with Coomassie blue revealed distinct bands with molecular weights of 50,000 and 25,000 comigrating with γ and light chains of canine IgG (Fig 1). A lightly stained M, 75,000 band comigrating with the μ immunoglobulin chain of canine IgM was also observed, and probably represents canine IgM present in the complex isolate. Ten μg of the isolated high-molecular-weight IgG complexes examined in double diffusion studies showed a single precipitin band with goat anti-whole canine serum which formed a line of identity with γ chain-specific rabbit anti-canine IgG (data not shown). These studies indicated that high-molecular-weight complexes containing canine IgG and IgM were formed during perfusion of sera over PACC.

Release of Protein A from PACC by Serum and Serum Components. Although high-molecular-weight C1q-binding IgG was formed during perfusion of serum over PACC, PAGE analysis of eluates failed to reveal a band that could be unequivocally identified as protein A, since authentic protein A migrates to a position nearly coincident with canine γ chains. Therefore, release of protein A into serum during perfusion was studied, using trace-labeled 125I-protein A in the PACC as a marker. The results in Chart 4 show that 125I-protein A was released from PACC by perfusion of sera as well as from solutions of albumin or IgG from 3 different species. In addition, the elution was not specific for protein A, since the same solutions also released 125I-labeled albumin and 125I-IgG. The results are expressed as the mean change in C1q-binding IgG in postperfusion gradient fractions compared to corresponding gradient fractions of preperfusion serum; bars, S.D. Comparative increases in C1q-binding IgG were noted in Fractions 1 through 5 (>19S) for normal and tumor-bearing sera. However, unlike normal sera, tumor-bearing sera showed less C1q-binding activity in Fractions 6 and after perfusion over PACC were layered on 10 to 40% sucrose density gradients and ultracentrifuged; each gradient fraction was assayed for Clq-binding radioactivity bound to C1q were as follows: normal sera ranged from 8,317 to 22,943 cpm in Fractions 1 to 3, and from 13,707 to 22,943 cpm in Fractions 4 to 10; tumor-bearing sera ranged from 13,707 to 22,943 cpm in Fractions 1 to 3, and from 23,441 to 40,079 cpm in Fractions 4 to 10.

Table 2

Components of C1q-binding complexes in human serum after passage over PACC or human albumin

<table>
<thead>
<tr>
<th>Immobilized protein</th>
<th>Sample no.</th>
<th>Preperfusion</th>
<th>Postperfusion</th>
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</thead>
<tbody>
<tr>
<td>Normal serum</td>
<td></td>
<td>Preperfusion</td>
<td>Postperfusion</td>
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<tr>
<td>Protein A</td>
<td>1</td>
<td>93.7 ± 1.2</td>
<td>100.4 ± 3.0</td>
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<tr>
<td>Protein A</td>
<td>2</td>
<td>96.4 ± 1.6</td>
<td>118.5 ± 2.3</td>
</tr>
<tr>
<td>Protein A</td>
<td>3</td>
<td>111.6 ± 9.5</td>
<td>114.7 ± 7.2</td>
</tr>
<tr>
<td>Human albumin</td>
<td>4</td>
<td>13.9 ± 1.5</td>
<td>10.8 ± 1.1</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Tumor serum</th>
<th></th>
<th>Preperfusion</th>
<th>Postperfusion</th>
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<tbody>
<tr>
<td>Protein A</td>
<td>1</td>
<td>41.8 ± 2.4</td>
<td>99.2 ± 4.1</td>
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<tr>
<td>Protein A</td>
<td>2</td>
<td>78.2 ± 3.1</td>
<td>95.6 ± 7.4</td>
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<tr>
<td>Protein A</td>
<td>3</td>
<td>59.9 ± 2.9</td>
<td>78.1 ± 2.3</td>
</tr>
<tr>
<td>Protein A</td>
<td>4</td>
<td>19.4 ± 0.7</td>
<td>28.1 ± 0.8</td>
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<tr>
<td>Human albumin</td>
<td>5</td>
<td>44.8 ± 0.7</td>
<td>45.1 ± 0.7</td>
</tr>
</tbody>
</table>

Analysis by sucrose density gradient centrifugation, and fractionation failed to reveal a band that could be unequivocally identified as protein A, since authentic protein A migrates to a position nearly coincident with canine γ chains. Therefore, release of protein A into serum during perfusion was studied, using trace-labeled 125I-protein A in the PACC as a marker. The results in Chart 4 show that 125I-protein A was released from PACC by perfusion of sera as well as from solutions of albumin or IgG from 3 different species. In addition, the elution was not specific for Protein A, since the same solutions also released 125I-labeled canine albumin or IgG.

In further studies, canine serum was perfused over PACC containing 125I-labeled protein A as a marker. The postperfusion serum was treated with 5% PEG to precipitate any 125I-protein A-IgG complexes and then analyzed by PAGE. A radioactive

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Table 2

Components of C1q-binding complexes in human serum after passage over PACC or human albumin

<table>
<thead>
<tr>
<th>Immobilized protein</th>
<th>Sample no.</th>
<th>Preperfusion</th>
<th>Postperfusion</th>
</tr>
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<tbody>
<tr>
<td>Normal serum</td>
<td></td>
<td>Preperfusion</td>
<td>Postperfusion</td>
</tr>
<tr>
<td>Protein A</td>
<td>1</td>
<td>93.7 ± 1.2</td>
<td>100.4 ± 3.0</td>
</tr>
<tr>
<td>Protein A</td>
<td>2</td>
<td>96.4 ± 1.6</td>
<td>118.5 ± 2.3</td>
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<tr>
<td>Protein A</td>
<td>3</td>
<td>111.6 ± 9.5</td>
<td>114.7 ± 7.2</td>
</tr>
<tr>
<td>Human albumin</td>
<td>4</td>
<td>13.9 ± 1.5</td>
<td>10.8 ± 1.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tumor serum</th>
<th></th>
<th>Preperfusion</th>
<th>Postperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein A</td>
<td>1</td>
<td>41.8 ± 2.4</td>
<td>99.2 ± 4.1</td>
</tr>
<tr>
<td>Protein A</td>
<td>2</td>
<td>78.2 ± 3.1</td>
<td>95.6 ± 7.4</td>
</tr>
<tr>
<td>Protein A</td>
<td>3</td>
<td>59.9 ± 2.9</td>
<td>78.1 ± 2.3</td>
</tr>
<tr>
<td>Protein A</td>
<td>4</td>
<td>19.4 ± 0.7</td>
<td>28.1 ± 0.8</td>
</tr>
<tr>
<td>Human albumin</td>
<td>5</td>
<td>44.8 ± 0.7</td>
<td>45.1 ± 0.7</td>
</tr>
</tbody>
</table>

4 Mean ± S.D. of triplicate determinations.
5 Postperfusion values were significantly increased above pretreatment levels (p < 0.05).

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As shown in Chart 6, radiolabeled protein A in the postperfusion samples were distributed in high-molecular-weight fractions of the gradient compared to free protein A. Parallel sucrose density gradient fractions of postperfusion sera showed increased C1q-binding IgG compared to pretreatment sera. This suggested that protein A which eluted from PACC into postperfusion sera was bound in complexes with immunoglobulins, and may have contributed to the increased C1q binding observed in postperfusion sucrose density gradient fractions.

To determine if $^{131}$I-protein A eluted from PACC after serum perfusion could bind to C1q-coated tubes, the following experiment was carried out. Ten ml of normal or tumor-bearing canine serum were perfused over PACC containing 1 mg of unlabeled protein A, together with $^{125}$I-protein A, followed by 200 ml of PBS. The effluents (200 ml) containing eluted $^{131}$I-protein A were concentrated to a volume of 10 ml, and 0.5-ml aliquots were incubated with C1q-coated tubes. Free $^{131}$I-protein A was used as a control. Effluent serum samples containing $^{131}$I-protein A demonstrated approximately 8-fold greater binding to C1q-coated tubes compared to free $^{131}$I-protein A. Binding of eluted $^{125}$I-protein A to C1q-coated tubes represented 34 and 37% (mean of triplicate samples) of total added radioactivity from normal and tumor-bearing effluents, respectively, compared to 4.3% for free protein A. Hence, some of the protein A eluted from PACC after serum perfusion appeared to acquire C1q-binding properties and was in a form probably complexed to immunoglobulins.

In further studies, 50 ml of normal or tumor-bearing canine sera were perfused through columns containing $^{125}$I-protein A or $^{128}$I-labeled canine albumin, together with unlabeled protein A or canine albumin, respectively, immobilized in collodion charcoal. The effluent fractions containing the peaks of the radioactivity released from the charcoal were pooled. To 10-ml aliquots of these peaks, an equal volume of saturated (NH₄)₂SO₄ or 5% PEG was added. Results demonstrate that 70 to 80% of the released $^{128}$I-protein A was precipitable with (NH₄)₂SO₄ and PEG, compared to only 5 to 10% of free protein A. In contrast, less than 10% of released albumin was (NH₄)₂SO₄ or PEG precipitable. These findings suggest that protein A but not albumin emerges in postperfusion effluent complexed with immunoglobulins.

**Molecular Weight of Protein A-containing Complexes.** To determine if IgG from canine serum and staphylococcal protein A were bound together in C1q-binding complexes present in postperfusion serum, the following experiment was carried out: 10 ml of normal canine serum containing $^{131}$I-labeled canine IgG were perfused over PACC containing $^{125}$I-protein A. The effluent was concentrated and then incubated with C1q-coated tubes. Based on the specific activities of the labeled protein A (PA) and IgG, the empirical formula of C1q-binding complexes was calculated to be close to IgG2PA (Table 3). The approximate molecular weight of the protein A-containing complexes ranged from 600,000 to 2,000,000 by chromatography over Sepharose 6B (Chart 7). Hence, it appears that in addition to IgG and protein A, other molecules such as IgA and C3 may be present in the complexes to give a molecular weight greater than 700,000. In the case of human serum, in addition to the high-molecular-weight protein A peak corresponding to the complexes found in canine serum, a second protein A peak appeared close to the molecular weight of IgG. When this smaller peak was rechro-
Chart 4. PACC was loaded into a Lexan chamber and washed with 3000 ml of 0.15 w NaCl solution. Serum or solutions of purified albumin or IgG from various species were then perfused through the columns at 2 ml/min, and radioactivity in the effluent was measured. Perfusion with (A) normal serum, (B) immunoglobulin solutions, or (C) albumin solutions obtained from humans, dogs, or chickens resulted in release of radioactivity from PACC. The release effect by normal sera was not selective for immobilized protein A, since similar leaching of immobilized canine albumin was noted after perfusion with sera from the same 3 species (D). NHS, normal human serum; NCS, normal chicken serum; NOS, normal dog serum.

matographed on Sephadex G-200, it still corresponded to a molecular weight of 160,000.

DISCUSSION

In previous studies, we showed that extracorporeal perfusion of plasma from hosts bearing breast adenocarcinoma over SAC or PACC, resulted in acute tumoricidal responses and objective tumor regressions in both dogs and humans (27–29). In our initial studies in humans, we noted that these tumoricidal effects and tumor regressions were also induced by plasma which was passaged over PACC off-line and administered i.v. Hence, we developed an in vitro system, similar to the off-line perfusion system used in our patients, in which sera from normal or tumor-bearing hosts were passaged over SAC or PACC. The effluent sera from these systems were collected and analyzed by physicochemical and immunochemical methods. The original perfusion system which we used in human studies (29, 30) contained a large internal fluid volume and additional attachments, making it cumbersome for study of products emerging in sera passaged over PACC. In order to conveniently test undiluted samples in large numbers and evaluate compositional changes in the effluent, we designed a model perfusion system using quantities of perfusate and immobilized protein A similar to those utilized in clinical studies (29, 30).
Immunoglobulin Oligomers in Serum after Perfusion over Protein A

Chart 6. Postperfusion normal canine sera containing eluted 125I-protein A was fractionated on 10 to 40% sucrose density gradients. Radiolabeled protein A was distributed in high-molecular-weight fractions. Increased C1q-binding IgG in postperfusion was also noted in parallel gradient fractions, suggesting that protein A which eluted from PACC in postperfusion sera may have contributed to the increased C1q binding.

Table 3
Ratio of IgG to protein A molecules in postperfusion C1q-binding complexes
Refer to text for experimental details.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein A bound (ng)</th>
<th>IgG bound (ng)</th>
<th>Molar ratio IgG/protein A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.25 (12.38)</td>
<td>26.83 (16.77)</td>
<td>2.1 ± 0.73</td>
</tr>
<tr>
<td></td>
<td>7.41 (17.64)</td>
<td>52.24 (32.65)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.62 (20.52)</td>
<td>101.70 (63.56)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.4 (8.1)</td>
<td>32.38 (20.24)</td>
<td>2.74 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>3.6 (8.5)</td>
<td>41.43 (25.59)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, nmol x 10^-9.
* Mean ± S.D. of 3 determinations.
* Average of 2 determinations.

The present investigation focuses on the identification and analysis of products which are formed in sera of normal and tumor-bearing dogs after perfusion over SAC or PACC. Perfusion of canine sera over SAC resulted in an increase of C1q-binding IgG in effluent samples, whereas perfusion of canine sera over protein A-deficient SAW did not. Hence, the presence of protein A on heat-killed and formalin-stabilized SAC appeared to be required to produce the increased C1q-binding activity. This was supported by the observation that similar increases in C1q-binding IgG were noted in effluent canine serum after perfusion over purified protein A. The increased C1q-binding IgG emerging from SAC columns could not be ascribed to nonspecific increases in IgG in effluent samples, since the concentration of this major immunoglobulin class was decreased in the same postperfusion samples which showed increased levels of C1q-binding IgG. However, a decrease in IgG levels did not appear necessary for the formation of C1q-binding IgG, since effluent sera from PACC column showed no significant reductions in canine immunoglobulins.

Pre- and postperfusion sera from normal or tumor-bearing dogs were further analyzed by sucrose density gradient ultracentrifugation. Increased levels of C1q-binding IgG sedimenting in fractions greater than 7S were noted in postperfusion sera from both groups. This increase was not due to Fc binding of the anti-canine IgG antibody indicator to any protein A in the complexes, since F(ab')2 fragments of anti-canine IgG also detected elevated C1q binding in similar postperfusion gradient.
fractions. Moreover, PAGE analysis of the high-molecular IgG isolated by G-200 fractionation and affinity chromatography revealed predominantly gamma and light immunoglobulin chains of canine IgG. Immunodiffusion studies confirmed the presence of canine IgG in the isolate, since a precipitin line formed between the isolate and anti-canine IgG gamma chain antisera. Further analysis of the C1q-binding complexes using heterologous and F(ab')2 fragments to human or canine IgG, IgM, IgA, or C3 showed increased binding of anti-IgG and anti-IgM in canine complexes and anti-IgG, IgA, IgM, and C3 in human complexes. Hence, the complexes formed in serum after perfusion over PACC appeared to be of high molecular weight and consisted predominantly of IgG, together with other serum components.

Since we detected an increase in C1q-binding activity in sera after perfusion, we considered the possibility that protein A released from PACC had combined with canine immunoglobulins in sera to produce high-molecular-weight C1q-binding complexes. Using radiolabeled protein A immobilized on PACC with unlabeled protein A, we found that 2 to 16 micrograms of protein A were desorbed from PACC during serum perfusion. Moreover, protein A was identified in serum effluent emerging from the PACC by the following additional studies: (a) PAGE analysis of postperfusion effluent after Sepharose G-100 separation showed a radioactive peak migrating at the protein A marker; and (b) postperfusion complexes isolated by gel filtration and affinity chromatography and then dissociated under acid conditions showed a precipitin line with normal human but not with chicken serum.

The latter experiments showed that protein A was released from PACC and present in serum perfused over PACC. Further studies demonstrated that the released protein A was present in the high-molecular-weight complexes since, (a) sucrose density gradient analysis of postperfusion sera showed that 125I-protein A released from PACC was distributed in high-molecular-weight fractions beyond the 75 marker and was distinct from free protein A; (b) radiolabeled protein A emerging in effluent samples bound to C1q-coated tubes to a greater extent than did free protein A; (c) the increase of released 125I-protein A from PACC in high-molecular-weight sucrose density gradient fractions corresponded to augmentation in C1q-binding IgG in parallel gradient fractions; and (d) a larger percentage of 125I-protein A eluted from PACC and emerging in postperfusion effluent was precipitable with NH4H2SO4 and PEG, compared to free 125I-protein A.

In prior studies, using gross techniques such as pyrogen and Limulus testing of effluent samples emerging from SAC or PACC, leaching of protein A was not detected (27–29). Moreover, we did not detect release of immobilized protein A, probably due to the low mean specific activity of labeled protein A on the colloidion charcoal or SAC. The present model system, using a high mean specific activity of radiolabeled protein A immobilized in PACC enabled us to detect leaching. Indeed, release of protein A from heat-killed and formalin-fixed SAC and of proteins immobilized on colloidion membranes has been previously observed by others (7, 21, 22). The present studies show that release of protein A from PACC occurred not only with serum, but also with solutions of purified serum proteins (Chart 5). Furthermore, this phenomenon was not specific for protein A, since 125I-labeled canine albumin immobilized in colloidion charcoal was also eluted by sera from 3 species. These results suggest that solubilization of immobilized proteins by serum or its components may play an important role in the release of proteins from colloidion charcoal.

Additional studies are under way to further dissect the mechanism of this effect.

Using 125I protein A in the PACC and 131I-lgG added to serum perfused over it, we showed that the empirical formula of the C1q-binding complexes corresponded to [lgG2PA]. With the use of molecular weight standards, the protein A-containing complexes appeared between Mr, 680,000 and 2,000,000. Previous studies have shown that rabbit IgG and protein A form complexes with same empirical formula, but with a molecular formula corresponding to the dimeric structure [lgG2PA]2 with a molecular weight of approximately 700,000. These complexes behaved like IgM in being relatively efficient in binding and activating C1 (16, 17). The complexes we detected may, in fact, be predominantly dimeric, and therefore correspond to the previously described rabbit IgM-like complexes. The higher molecular weight of the former may be due to the presence of components other than IgG and protein A in these complexes, such as IgG, IgM, and C3, as we demonstrated with the use of monospecific F(ab')2 fragments (Table 2). We are currently studying this possibility.

Having isolated and partially characterized complexes that contain protein A, IgG, and other serum components in effluent from PACC, we are currently testing the activity of these complexes in vitro and in vivo, prepared by adding protein A to serum or to monomorphic IgG. We have shown that these model complexes may activate Fc-bearing leukocytes and complement system to generate oxidant and anaphylatoxin activity, respectively, and may have tumoricidal activity in experimental animals. We are currently attempting to prepare these complexes on a preparative scale for testing in vivo. The basis for the selectivity of the tumoricidal effects after infusion may involve their deposition in tumor microcirculation, recruitment of inflammatory cells, and local activation of mediators that may be directly or indirectly tumoricidal.

These studies show that perfusion of normal or tumor-bearing sera over SAC or PACC results in the appearance of increased C1q-binding activity. The high-molecular-weight material consists predominantly of IgG and protein A. After serum or serum component perfusion, protein A desorbs from PACC, appears in the postperfusion (NH4H2SO4 and PEG-precipitable fractions, binds to solid-phase C1q, and coincides with increased C1q-binding IgG in heavy-sedimenting sucrose density gradient fractions. Finally, results to be reported elsewhere suggest that these protein A-lgG complexes play a significant role in the genesis of the antitumor effects and toxicity previously observed in dogs and humans after receiving plasma perfused over PACC or SAC (27–30).

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Tumoricidal Response Following Perfusion over Immobilized Protein A: Identification of Immunoglobulin Oligomers in Serum after Perfusion and Their Partial Characterization


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