Further Characterization of the in Vitro Tumoricidal Activity of Staphylococcal Protein A¹

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ABSTRACT

Treatment of plasma or serum from leukemic patients with solid phase staphylococcal Protein A induced leukemic blast cell lysis in vitro, but this effect was relatively independent of the amount of immunoglobulin G (IgG) removed. Samples with approximately equal cytotoxic activity contained markedly different IgG levels, while samples with similar IgG levels had a wide range of tumoricidal activity. Assays of plasma samples collected during a perfusion of one plasma volume through a Protein A-Sepharose column indicated that the duration of the procedure had a greater effect on cytotoxic activity than did the amount of IgG removed. Neither added leukemic nor normal IgG significantly improved blast cell viability in treated serum. Cytotoxic activity was not dialyzable and concentrated in the M₄ <100,000 fraction of samples separated by filtration. Treated cytotoxic serum samples did not have important Clq binding activity. These results suggest that the in vitro tumoricidal activity of solid phase Protein A is probably due to a toxic substance added to serum during immunoadsorption rather than to its immunoadsorptive capacity.

INTRODUCTION

Infusion of plasma treated with solid phase staphylococcal Protein A has been associated with tumor regressions in both animals and human patients (1-7). The original observations were made using heat and formalin killed whole SAC, but these treatments were often accompanied by severe side effects (8). Consequently, other preparations containing purified Protein A bound to particles such as collodion charcoal, silica, or Sepharose beads were developed (6, 9, 10). While the empirical observation that plasma treatment with these preparations often produces tumor necrosis has been confirmed by several laboratories, the mechanism of action is unclear at present. At least four explanations have been proposed: (a) Protein A immunoadsorption removed IgG-containing antibodies and immune complexes which interfere with tumor immunity, thereby enhancing host tumoricidal activity (1); (b) bacterial products (either Protein A or other cell components) which are directly toxic to tumor cells are eluted from the particles (11); (c) Protein A stimulates the immune system through its activity as a mitogen or by increasing interferon production (12-15); and (d) the matrix particles activate complement and generate anaphylatoxins (16). These mechanisms are not mutually exclusive and more than one may be operative in vivo. However, the optimal use of Protein A preparations depends upon a better understanding of the most important mechanism(s), since treatment strategies would be quite different. For example, if removal of “blocking” antibodies is critical, then immunoadsorption might be adequately induced by brief exposure of plasma to Protein A or by direct injection of soluble Protein A. Likewise, if activated complement or tumoricidal bacterial products are the active agents, these can be administered more efficiently by other methods to obtain a tumoricidal response.

The current studies were performed to investigate the role of immunoglobulin in Protein A-induced cytotoxicity. We have previously reported that in vitro solid phase Protein A treatment of serum or plasma from leukemic patients, but not normal individuals, induces blast lysis which is not complement dependent nor cell mediated (17, 18). While this model system does not reflect the complex interactions of the in vivo treatment systems, it seems well suited for investigating the specific role of immunoglobulin as a putative blocking factor. For the most part, these studies were performed with leukemic blast cells and plasma from four patients with refractory end-stage acute leukemia who were being treated with a Protein A-Sepharose immunoadsorption system (19).

MATERIALS AND METHODS

Assays of Protein A-associated tumoricidal activity were performed as previously described (17, 18). Leukemic blast cells were obtained from adult patients with acute myelogenous leukemia. Heparinized peripheral blood samples were mixed with CMRL 1066 culture medium containing glutamine and penicillin-streptomycin and were placed on a Ficoll-Hypaque gradient (25 min at 400 x g). Interface cells were washed three times with medium, tested for viability by trypsin blue exclusion, and resuspended to 2 x 10⁶ cells/ml. Blood samples were obtained from patients in flagrant relapse and essentially all cultured cells were myeloblasts. In some experiments autologous serum was treated with SAC as a 10% (w/v) suspension (Pansorbin; Calbiochem, La Jolla, CA) as previously described (17). In other experiments autologous citrated plasma was obtained during a perfusion treatment with Protein A-Sepharose columns. The perfusion technique and the clinical characteristics of the treated patients are reported separately (19). The columns (Immuno-Sorba SpA CL 4B) were provided by the Gambro Corp. (Lund, Sweden) and contained approximately 400 mg of Protein A. A total of 2000 ml of plasma was usually perfused through a single column at a rate of 10 ml/min. Treated serum or plasma or control (untreated) serum or plasma was diluted with culture medium and used to suspend leukemic blast cells. One ml of suspension (15% leukemic serum, 5 x 10⁶ leukemic cells) was incubated for 24 to 36 h at 37°C with 5% CO₂. Cell number was then determined by Coulter counter and viability by trypsin blue exclusion. Results were expressed as viable cells in treated serum or plasma divided by viable cells in untreated serum or plasma times 100. Determinations of statistical significance were performed with the paired t test (20).

IgG levels were assayed with a Beckman Immunochemistry Analyzer and Beckman Nephelometry Reagents (Beckman Instruments, Inc., Brea, CA). Immune complexes were determined by the 125I-C1q binding assay, as previously described (21). Normal and leukemic IgG were obtained by perfusing citrated plasma through a Protein A-Sepharose column, eluting the protein with acidic buffers, dialyzing against 0.015 M phosphate, concentrating by lyophilization, and resuspending in 0.9% NaCl solution. The eluate consisted of 90% IgG, 4.6% IgA, and 5.3% IgM by immunoelectrophoresis. Normal or leukemic IgG was added to some cultures to a final concentration of 1500 mg/dl.

Whole or SAC treated sera were dialyzed with Spectra/Por 1 tubular...
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RESULTS

To test the relationship between Protein A removal of interfering substances (such as blocking antibodies) and induction of cytotoxic activity, we collected plasma samples at 30-min intervals from above and below a 200-ml Protein A-Sepharose column during perfusion treatments. Extraction of IgG is initially extensive, but over a period of time the absorptive capacity of the column saturates, and little additional extraction occurs after about 150 min (1500 ml of plasma perfused) (19, 22). Consequently, IgG levels in plasma samples collected from below the column vary widely during a perfusion treatment. The data in Fig. 1 represent observations from three treatments of two patients. IgG levels in these samples ranged from 96 to 5890 mg/dl. As expected, plasma obtained from below the column contained less IgG than did samples from above the column, except in one case. Overall, the correlation between cytotoxic activity and IgG levels was poor (r = 0.18), and the slope of the line representing the best fit by the method of least squares was nearly flat. Samples with approximately equal cytotoxic activity (for example, 50–60%) contained markedly different IgG levels (195 to 4760 mg/dl), while samples with roughly equal IgG levels (i.e., 3000 mg/dl) manifested cytotoxic activity over the range of 0–70%. Therefore, simple removal of leukemic IgG does not predictably lead to enhanced cytotoxic activity.

The data from a single perfusion treatment are shown in Fig. 2. Extraction of IgG was nearly complete during the first pass through the column (time zero), and incubation of autologous leukemic blasts in the treated plasma resulted in a 46% reduction in cell viability compared to the sample collected above the column. At subsequent time points, the samples from above the column contained approximately the same amount of IgG, but this plasma had progressively more cytotoxic activity. Perhaps more striking was the observation that despite increasing IgG levels in samples collected below the column as the perfusion treatment continued, the cytotoxic activity increased as well. Only 17% of the leukemic blasts remained viable after incubation in plasma collected at 120 min from below the column. Therefore, it appears that the duration of the procedure is more important for the cytotoxic effect than is the amount of IgG removed.

We next evaluated the importance of IgG to the cytotoxic activity by adding leukemic and normal IgG to Protein A-treated sera. As shown in Table 1, SAC treatment of leukemic serum reduced blast cell viability to approximately one-half that of cells in untreated serum. While cultures with leukemic or normal IgG added to SAC-treated serum showed slightly improved viability, these differences were not statistically significant when analyzed by the paired t test. Therefore, added IgG does not appear to significantly block cytotoxicity in this system. These experiments also do not support the notion that immunoadsorption removes a growth-supporting IgG from leukemic serum. Blast cell viability was decreased by culture in serum-free medium, but normal and leukemic IgG were equally effective in partially supporting viability, and neither enhanced cellular proliferation when added to untreated serum (Table 1).

We considered the possibility that immune complex formation or aggregation of IgG during SAC treatment might contribute to the development of cytotoxic activity, since other investigators have shown an increase in Clq binding during ex vivo Protein A adsorption (2, 23, 24). However, when Clq binding was measured in pre- and postcolumn plasma samples, values were in the normal range, and no important differences could be discerned either at the beginning or at the end of a perfusion treatment (data not shown).

Finally, we have begun to study the possibility that SAC treatment adds a cytotoxic substance to serum. Earlier studies from our laboratory provided evidence that cytotoxic activity in treated serum is trypsin sensitive (18), suggesting that the active agent is a protein. To preliminarily characterize the size of this protein, we used dialysis and Amicon filtration. Dialysis did not significantly diminish the cytotoxic activity of SAC-treated serum. In four experiments, leukemic cells cultured with autologous SAC-treated leukemic serum showed markedly re-

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Table 1 Effect of addition of leukemic or normal IgG on leukemic blast cell viability in culture

<table>
<thead>
<tr>
<th>Serum</th>
<th>Addition</th>
<th>Viable cells as % of control</th>
<th>n*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAC-treated</td>
<td>None</td>
<td>47.6 ± 22.9</td>
<td>13</td>
</tr>
<tr>
<td>SAC-treated</td>
<td>AML IgG</td>
<td>62.5 ± 28.1</td>
<td>13</td>
</tr>
<tr>
<td>SAC-treated</td>
<td>Normal IgG</td>
<td>52.5 ± 17.2</td>
<td>13</td>
</tr>
<tr>
<td>Untreated</td>
<td>AML IgG</td>
<td>115.0 ± 55.1</td>
<td>10</td>
</tr>
<tr>
<td>Untreated</td>
<td>Normal IgG</td>
<td>99.2 ± 28.2</td>
<td>10</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>52.1 ± 15.6</td>
<td>10</td>
</tr>
<tr>
<td>None</td>
<td>AML IgG</td>
<td>69.8 ± 23.1</td>
<td>10</td>
</tr>
<tr>
<td>None</td>
<td>Normal IgG</td>
<td>72.9 ± 25.6</td>
<td>10</td>
</tr>
</tbody>
</table>

* n, number of observations using cells and autologous serum from the indicated number of patients. Added IgG was allogeneic.

AML, acute myelogenous leukemia.

Mean ± SD.
duced viability (14 ± 8%; SD) compared with controls, while cells cultured with the same serum SAC-treated and dialyzed showed a similar loss of viability (23 ± 5%) compared to controls. Utilizing Amicon filters of various pore sizes, we found that the cytotoxic activity appeared to be concentrated in the fraction of serum containing molecules of less than \( M_0 \), 100,000. In 10 experiments, incubation of leukemia cells in this allogeneic SAC-treated serum fraction reduced viability to 38.5 ± 25.2% of cells in whole, untreated serum, and this reduction was comparable to that seen in unfraccionated SAC-treated serum (43.5 ± 27.6%). In contrast, blast cell viability was actually greater in the less than \( M_0 \), 50,000 SAC-treated fraction (59.4 ± 38.9% of control) than in either unfraccionated SAC-treated serum or in similarly fractionated untreated serum. Taken together, these preliminary analyses suggest that the toxic substance is a protein, with \( M_0 >8,000 \), and probably in the range of 50,000 and 100,000.

DISCUSSION

The use of Protein A-containing immunoabsorption systems in cancer patients developed from the idea that this bacterial cell wall component might remove circulating blocking factors more specifically than plasma exchange (1). When the predicted tumor necrosis was observed, it was logical to assume that reduction of IgG and/or IgG containing immune complexes was at least partly responsible for the clinical effect. However, subsequent observations have cast doubt upon this interpretation. For example, clinical responses were often noted after treatment of small volumes of plasma with relatively little Protein A, limiting the possible impact on total body levels of immunoglobulin (6). Similarly, infusion of small volumes of SAC-treated plasma resulted in frequent clinical responses, while intensive removal of IgG with Protein A-Sepharose columns was less effective (5). When measured, immune complex levels were usually unaffected or, in fact, increased by Protein A perfusion (2, 5, 23, 24). Finally, some, but not all, laboratories have reported tumor regressions following exposure of plasma to staphylococcal strains, such as Wood 46, Protein A (25), may be involved in the Protein A-induced tumoricidal activity.

REFERENCES

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