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Perspectives and Commentaries

Antitumor Effects of Immobilized Protein A and Staphylococcal Products: Linkage between Toxicity and Efficacy, and Identification of Potential Tumoricidal Reagents

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INTRODUCTION

IMMOBILIZED protein A (SPA) has been used as experimental cancer therapy in animal models and in man. Striking antitumor responses were frequently observed and prompted an intensive search for the active bioreactants and the mechanism of action of this most unusual approach to cancer therapy. In recent years substantial clinical, biochemical and immunological research has been carried out in this area and several biomolecules which appear to contribute to the tumoricidal effects of plasma perfused over immobilized SPA have been identified. These and other major developments will be reviewed and based upon these findings we will advance an hypothesis to explain the tumoricidal activity of SPA-perfused plasma. This unifying concept may also reconcile some seemingly disparate observations obtained with various SPA perfusion systems and, perhaps, serve as a blueprint for future investigations in this field.

EARLY EXPERIENCE WITH IMMOBILIZED *STAPHYLOCOCCUS AUREUS* ORGANISMS OF THE COWAN I STRAIN

In 1976 Bansal *et al.* demonstrated tumoridical effects in a patient with colon carcinoma after perfusion of plasma over heat-inactivated and formalin-stabilized *Staphylococcus aureus* Cowan I (SAC), which was immobilized in a microporous filtration system [1]. The work was largely inspired by that of Hellstrom *et al.*, who showed that circulating specific blocking factors in the plasma of animals and patients with cancer suppressed the ability of their own lymphocytes to destroy tumor cells *in vitro* [2]. The objective of Bansal's work was to subtract these 'blocking factors' from the circulation of the tumor-bearing patient. This objective seemed ideally satisfied with the use of an extracorporeal perfusion system since quantitative removal of the blocking factors required exposure of the total plasma volume to the immunoadsorptive *Staphylococcus aureus* organism. The tumoricidal effect in this patient was, indeed, associated with reduction in circulating blocking factors and transient decline in Clq binding. Clinically, chills, fever, a transient decline in blood pressure and pain in the tumor site accompanied the antitumor response.

The system of Bansal *et al.* was then extended by Terman *et al.* to dogs with spontaneous mammary adenocarcinoma [3]. Tumor necrosis was observed in these cutaneous canine tumors within 24 hr after perfusion and healing of several large ulcerated tumorous areas occurred in the ensuing days. Serologically, Clq binding and tumor-associated antibodies were increased following treatments while serum C3 levels declined. Since Clq binding levels actually increased, tumoricidal effects could not be ascribed to the subtraction of circulating immune complexes. Nor did the tumoricidal effects appear to be due to removal of circulating blocking factors since the column capacity for binding of immune complexes and immunoglobulin was small and phlebotomy of up to 50% of the total plasma volume of several of these animals failed to induce a tumoricidal effect. Two independent groups

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working at The National Institute of Health have confirmed the antitumor effects of plasma perfused on over *Staphylococcus aureus* in experimental animal models [4, 5]. Holohan *et al.* in particular confirmed Terman's observations in an identical dog model [4] and Sukumar *et al.* observed reduction of established rat mammary carcinomas with plasma perfused over SPA immobilized on Sepharose [5]. Today the tumoricidal effect of plasma perfused over *Staphylococcus aureus* perfusion systems has been demonstrated in various additional experimental animal systems by Cooper and Masinello [6], Gordon *et al.* [7], Jones *et al.* [8] and Liu *et al.* [9].

USE OF PURIFIED SPA IN THE COLLODION-CHARCOAL (PACC) SYSTEM

Because some of the early studies suggested that SPA was one of the substances involved in generating the tumoricidal process, Terman *et al.* subsequently immobilized commercially purified SPA in a collodion-charcoal matrix (PACC). This new system was tested for toxicity and efficacy in the dog model [10] and then extended to patients with advanced breast carcinoma [11]. A major observation emerged from these studies. Tumoricidal effects were characterized by rapid onset of acute pain as well as hyperemia, edema and vesicle formation in the chest wall tumor in most of the treated patients after direct infusion of only a small volume (200 ml) of plasma which was perfused over PACC. Therefore it appeared that antitumor activity was mediated by tumoricidal materials within the plasma perfused over PACC rather than by removal of blocking factors. Terman's finding that PACC perfused plasma from a patient with breast carcinoma was capable of inducing tumor-killing effects when given to another breast cancer patient might also support this point of view. Of additional interest was that PACC perfused plasma induced toxicities [12], antitumor responses and even histologic changes [13] in post-treatment tumor tissues that were almost identical to those noted by Bansal *et al.* in humans [1] and Terman *et al.* in dogs [3] with plasma perfused over SAC. This implied that the tumoricidal materials present in plasma perfused over SAC and PACC were similar or identical in nature.

SPA therapy was further advanced in a human study by Bertram *et al.* employing a modified PACC system [14, 15]. Significant antitumor responses were induced by infusion of small aliquots (100 ml) of PACC-perfused plasma and also akin to Terman's earlier human trial antitumor responses, and toxicities occurred rapidly after infusion of the PACC-treated plasma. In the studies of both Bertram *et al.* and Terman *et al.* toxicities consisted mostly of rigors, chills and fevers, while hypotension and signs of early pulmonary edema were observed in addition by Terman *et al.* [3]. The pathophysiological basis of the cardiopulmonary toxicities were defined by Young *et al.* in a prospective study [12] in which hypotension was caused by a decline in peripheral vascular resistance while the pulmonary edema apparently was due to a pulmonary capillary leak syndrome.

During the trial of Bertram *et al.* the interesting observation emerged that toxicities may not merely represent side-effects of therapy but may be a critical requisite for optimal tumoricidal effects of SPA-perfused plasma. All patients who showed antitumor effects also had systemic side-effects of fevers, chills and rigors. Conversely, all patients in this study who lacked systemic side-effects after plasma therapy not only failed to obtain antitumor responses but were the only individuals of this trial with tumor progression during therapy [15]. This linkage between toxicity and antitumor activity might, in retrospect, also have been present in the human study of Terman *et al.*, where the patient with the most extensive antitumor response experienced the most severe systemic toxicity [11, 12]. Moreover, in Håkansson *et al.*'s study only the patient (patient 3) who experienced substantial toxicities obtained a significant antitumor response [16]. Toxicities again were those of fever, chills and rigor. Similarly, Kinet *et al.* noted objective tumor regressions with a protein A silica column in four of eight patients which were associated with side-effects of hypotension, chills, fever and tumor pain, whereas none of the patients in a group treated with a heat-denatured protein A column showed any antitumor effect or toxicity [17]. Identical toxicities were also present in the patient treated by Bansal *et al.* with the SAC perfusion system [1], who also showed an antitumor effect [1].

It should be noted that although the toxicity was occasionally severe, it was, indeed, manageable. Hypotension responded well to volume expansion and the use of vasoactive drugs such as metarminol and/or dopamine [11, 12]. Antipyretics when given at the onset of fever successfully attenuated the febrile response [14] and the frequently observed rigors could easily be controlled with the judicious use of intravenous demerol [14]. Bronchospasm apparently occurred mostly in patients with pulmonary metastases and could be reversed with the use of bronchodilators and short-term corticosteroids [12].

OBSERVATIONS FROM CLINICAL STUDIES USING ADDITIONAL SPA SYSTEMS

After the description of the tumor-killing effects using the SAC organism or the PACC perfusion system [1, 3, 11, 14], additional perfusion systems appeared, and most of these studies are compiled in a recent volume of the *Journal of Biological Response Modifiers* [18]. Matrices such as methacrylate, silica and Sepharose containing SPA covalently bound were employed andused either in on-line or off-line mode. While tumor killing effects frequently occurred, in general they tended to be less striking, less consistent and less rapid in onset than the reactions noted with the SAC or the collodion-charcoal systems. Toxicities induced by these systems also appeared to be less severe, consistent with the concept that toxicity may be essential for maximal antitumor effects. This notion seemed also to be supported by a trial with the PACC system which was carried out at the National Cancer Institute [19]. Despite the use of identical quantities of immobilized SPA that were employed in a prior study [11, 12], toxicity typical for PACC therapy [11, 12] was not observed and no clinical antitumor responses were noted [19]. The different outcomes in these various trials may reflect qualitative or quantitative differences in the products formed or generated in plasma after perfusion over these columns, as will be discussed below. These differences may also reflect major variations in commercial protein A and the quantity of contaminating elements which, according to one manufacturer, may be due to multiple changes which were introduced into the fermentation step of the production process over the past several years in an effort to increase the yield [Inganass, Pharmacia, personal communication]. The latter possibility is supported by the recent detection of substantial quantities of enterotoxins contaminating commercial protein A preparations [20], Terman and Langone, personal communication]. Therefore it appears that careful analysis of the character of observed toxicities may provide valuable clues to the nature and identity of the tumoricidal substances present in SPA-perfused plasma.

NATURE OF REACTANTS RESPONSIBLE FOR TOXICITY AND POSSIBLY ANTITUMOR EFFECT

Since we postulated that plasma perfused over PACC contained products that caused tumor necrolysis and toxicities as well, an attempt will be made to identify some of the products. The nature of these factors may devolve into two categories: those products generated after interaction of plasma components with immobilized SPA and those leached from the column in the course of plasma perfusion. Leached materials might consist of enterotoxins which are a natural product of the *Staphylococcus aureus* organism. Indeed, in a recent study approximately 5% of a commercially purified SPA preparation was identified as enterotoxins A [20] and, using sensitive radioimmunoassays, several enterotoxins were detected in commercial protein A preparations, with enterotoxin B being the most abundant, representing up to 0.05% contamination [Terman and Langone, personal communications]. SPA itself, the major component of the perfusion systems, may detach from the column and appear in small quantities in the effluent plasma. Finally, an example of leakage-independent interaction of plasma components with the SPA matrix would be activation of complement and generation of biologically active complement by-products.

Leached enterotoxins in the effluent from PACC

Enterotoxins are known to be contaminating protein A preparations and if released from the matrix into the effluent plasma could exert potent biological activity almost identical to that observed in the clinical setting. Enterotoxins induced hemodynamic changes in monkeys [21] that were strikingly similar to those observed in humans by Young *et al.* [12] with the PACC system and by Messerschmidt *et al.* with the SAC system [22], characterized by a decline in peripheral vascular resistance with an increase in cardiac output. Additional side-effects noted in the course of SPA therapy, such as fever, nausea, vomiting and respiratory distress, have also been described in experimental animals after feeding or infusion of small quantities of enterotoxins [20]. Indeed, the clinical features of the toxic shock syndrome in man known to be induced by several enterotoxins bear a striking similarity to those described after PACC perfusion therapy [1, 12, 22, 23, 24]. Similarly, the ultrastructural damage to tumor endothelial cells observed by Daskal *et al.* [13] shortly after PACC perfusion therapy was strikingly similar to the pulmonary endothelial lesions induced by enterotoxin B infusion in monkeys [25]. Tumoricidal properties of enterotoxins are less well studied but it seems of interest that most enterotoxins are highly mitogenic to human lymphocytes [26]. In fact, concentrations as low as 0.1–1 ng/ml may induce potent lymphoproliferation [27] and such concentrations might easily be achieved by perfusion of plasma over SPA preparations containing approximately 0.001% enterotoxins as con-

taminants. The recent *in vitro* demonstrations of interleukin [28] and interferon [20] produced by staphylococcal enterotoxins further supports their potential role in fever production during perfusions as well as immunostimulation, mitogenesis and clonal expansion.

SPA containing immunoglobulin oligomers in the effluent from PACC

Sizeable amounts of SPA which was detached from the collodion-charcoal were detected by Balint *et al.* in plasma after it was passed over a modified PACC perfusion system [29]. In subsequent studies the quantity of SPA released from the charcoal was found to depend upon the amount of immobilized SPA, and ranged from 0.1 to 1.2 mg of SPA/100 ml of perfused plasma [Terman, personal communication]. Other determinations revealed increased Clq binding activity in post-perfusion sera and this activity could be identified as polyethylene glycol-precipitable oligomers which contained SPA, immunoglobulins and complement components in various ratios [29]. SPA was thus released into the effluent and formed macromolecular complexes, with molecular weights ranging from 600,000 to more than 2 million daltons. The major immunoglobulins in the complexes seemed to be IgG and IgM; IgA and C3 were occasionally present [29]. Biologically, SPA-Ig complexes are known to induce histamine release from mast cells and also cause release of serotonin by activation of platelets. SPA-Ig complexes may also be capable of depositing in the tumor neovasculature and as such might exert potent local antitumor activity [Terman, unpublished observations]. Tumor destruction may also be mediated by SPA-Ig complex-induced expansion of immunocyte populations and SPA itself is a well-recognized lymphocyte mitogen [30]. Indeed, SPA-induced IgG production by B cells *in vitro* appears to be altered remarkably by the presence of interleukin-2 [31]. Bertram showed that the concentration of leached SPA in effluent plasma was insufficient for induction of lymphocyte mitogenesis; however, if SPA were complexed to IgG in the plasma, it might be a far more powerful mitogen than free SPA [32]. While the exact *in vivo* potential of these complexes as well as their interaction with interleukins, enterotoxins, anaphylatoxins and C3b is presently unknown, their widespread *in vivo* distribution and multitude of biological activities would suggest, theoretically, that they are a major component of SPA perfusion therapy.

Complement activation and complement by-product generation

Another well-recognized biological activity of SPA is activation of complement and generation and complement by-products [30]. Langone *et al.* recently demonstrated formation of C3a, C4a and C5a in serum after incubation with the SAC organism and C3a after incubation with PACC [34]. C3a seems to possess direct *in vitro* tumoricidal activity [35] and Kassel *et al.* showed that the administration of normal serum to C5-deficient lymphoma-bearing mice resulted in marked tumor regression [36]. Free Clq was recently demonstrated in the effluent from SPA-Sepharose [37] and a crude preparation with characteristics of Clq caused tumor growth retardation in a mouse B16 melanoma model [6]. Zymosan-activated plasma and the partially purified complement components C3b and C3u had a similar effect in the same murine system [Cooper, personal communication]. Immunologically, C5a was shown to be immunopotentiating, and to induce release of interleukin-1 by its interaction with monocytes [38], while factor C3b apparently is capable of inducing strong lymphoproliferation [39]. These effects might lead to expansion of tumor-specific lymphocyte clones, while the recently demonstrated interaction of anaphylatoxins with polymorphonuclear and endothelial cells [40-42] might cause *in vivo* margination and aggregation of neutrophils in neovasculature with release of oxidants and proteases, lysis of endothelium [43] and resultant inflammatory edema formation. Anaphylatoxins may also be implicated in some of the toxic responses to perfusion treatment such as hypotension and bronchospasm in view of their known experimental properties of vasodilatation [44] and leukotriene release *in vitro* [45].

Mitogenicity of perfused plasma

In the course of a phase I/II study Bertram *et al.* described a potent immunostimulatory component in PACC-perfused plasma of most of his patients [14]. Such perfused plasma was found to be highly mitogenic to normal lymphocytes and mitogenic potency was similar to Concanavalin A, a powerful lymphocyte mitogen. This immunostimulatory component required the presence of SPA on the collodion-charcoal; however, it did not appear to be derived from SPA leached from the PACC column. Of considerable significance was that the symptoms of fever, chills and rigors were produced only by mitogenic plasma whereas plasma devoid of mitogenicity caused no systemic reactions. This linkage applied also to antitumor effects, which

occurred only in patients with mitogenic plasma, while tumor progression was noted in those whose PACC-perfused plasma failed to become mitogenic [14]. This study thus substantiated earlier assumptions that immunostimulation apparently is an important mechanism by which SPA therapy achieves antitumor effects. The correlative aspect of *in vitro* mitogenesis with *in vivo* symptomatology might also be of great importance for selecting patients who may be amenable to SPA therapy. The nature of the stimulating factor remains to be elucidated but enterotoxins, SPA-Ig complexes, complement factor C3b, anaphylatoxins or combinations thereof working synchronously or synergistically may account for this activity.

PROPOSED MECHANISM OF THE ANTITUMOR EFFECTS AND TOXICITY

We may now advance a hypothesis to explain the tumor killing effects of plasma perfused over the PACC column.

The linkage between antitumor activity and toxicity firmly establishes SPA therapy as an immunomodulatory modality where activation of immunostimulatory circuits leads to simultaneous induction of systemic reactions and antitumor responses. The observation that administration of small aliquots of perfused plasma was able to induce antitumor activity suggests the presence of immunostimulating molecules in such plasma and makes subtraction of blocking reagents by the column a less likely explanation. Antitumor activity of SPA therapy thus appears to be mediated by immunostimulatory molecules which are either released or formed after contact with plasma with the SAC or PACC column. These molecules may be directly tumoricidal, interact with polymorphonuclear cells or stimulate lymphocyte populations inducing proliferation of immunocompetent cells. Activated inflammatory cells may marginate in the tumor microvasculature and release proteases and oxidases causing tumor cell destruction. Systemic enhancement of antitumor activity may be mediated by activated T cell populations leading to production of interleukins and thus expansion of pre-immunized tumor-specific clones. Proliferation of such clones would explain the observed selectivity of SPA-induced antitumor responses, including the presence of tumor-specific cytotoxic antibodies observed in the sera of experimental animals and humans shortly after treatment [1, 3], as well as the profound lymphoproliferation noted in lymph nodes of dogs after SAC perfusion treatments [46].

Several immunostimulatory molecules or activities have been identified over the last few years. Effluent sera from PACC contained SPA-IgG conjugates with Clq-binding activity [26] and PACC-perfused plasma was also found to be strongly mitogenic to human lymphocytes [14]. Complement by-products were shown to be generated during the perfusion process [34] and various enterotoxins were detected in commercial protein A preparations [20]. Each of these four systems may interact with multiple cell populations and thus could account for some or all of the biological effects of SPA therapy. Their relative importance for induction of antitumor activity is presently unknown but they may work together, potentiate each other at the cellular level and ultimately lead to a coordinated activation of a variety of immunologic and inflammatory systems. Maximum tumoricidal responses may occur with conjoint activation while suboptimal responses may reflect a lesser degree or uncoordinated activation of these cellular systems.

The magnitude and character of antitumor responses may thus ultimately depend upon the host's state of reactivity at the time of therapy. In every study, be it of animals or humans, a certain percentage of treated individuals were unresponsive to therapy. Whether this failure of reactivity is due to the presence of potent serum inhibitors of complement by-products [47], neutralizing antibodies to enterotoxins in serum [48], failure of SPA-IgG complexes to deposit in tumor neovasculature (possibly due to aggressive uptake in reticuloendotherial tissue) [49] or insufficient activation of host immunologic or inflammatory cells will be the subject of further investigations.

FUTURE PERSPECTIVES

For each new protein A system conditions of safety and efficacy will have to be established. The nature of the solid support, the ligand used to attach SPA, may influence the optimal quantity of SPA required for functional activity. The interaction of immobilized protein A with plasma and immunoglobulins may differ among the various systems and will require careful determination of the reactants involved, i.e. complement by-products, SPA-Ig complexes, enterotoxins and other, as yet unclassified, materials.

It will be very important that manufacturers begin to approach SPA as a pharmaceutical and that SPA preparations be structurally and functionally standardized, prepared by a homogeneous and reproducible technique, and be carefully tested for Fc-binding activity and additional components such as enterotoxins. Only with such standardized SPA preparations can reproducible conditions of dose, plasma volumes and flow rates be established. The

mitogenicity assay as developed and described by Bertram *et al.* [14] might be a reasonable initial approach for such standardization and might have an important place in predicting which systems are in fact active and which may be used for clinical studies.

The observation that small doses of cytosine arabinoside, an antineoplastic agent, seemed to potentiate the tumoricidal effects of SPA therapy [10, 11] might be of great significance and will need to be further examined. The concept that a combination of small 'non-toxic' doses of chemotherapeutic agents with SPA therapy might be sufficient for induction of maximal antitumor effects could be of great importance for the treatment of human cancer, and the cellular basis of this effect should be studied.

Besides the interaction of SPA therapy with anticancer drugs, future research will need to address a multitude of questions. Most importantly, the relative role in the antitumor effect of each of the major bioreactants which have now been identified will need to be elucidated and the potential synergistic interaction of these factors with inflammatory cells as well as immunocytes will need to be determined. Indeed, the book on SPA therapy has just been opened. However, available data indicate this treatment as a constellation of bioreactive substances formed and released after contact of plasma with PACC or SAC which may coordinately stimulate host immune and inflammatory mechanisms and provide potentially new instruments to activate host antitumor defenses.

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