Tumoricidal Response Induced by Cytosine Arabinoside After Plasma Perfusion over Protein A

Abstract. *In dogs with spontaneous mammary adenocarcinomas, a single nontoxic infusion of cytosine arabinoside after extracorporeal perfusion of plasma over immobilized protein A resulted in a necrotizing response rapid in onset and specific for tumorous tissue. Gross tumoricidal reactions 12 hours after this combined treatment exceeded the algebraic sum of responses to cytosine arabinoside and protein A perfusion treatments alone in the same dogs, implying a synergistic effect between the two. The magnitude, rapidity, and specificity of the tumoricidal response after the combined treatment suggests that it may be an effective chemoimmunotherapeutic approach to breast adenocarcinoma.*

Spontaneous mammary adenocarcinoma occurs commonly in adult female dogs and is considered an excellent model of human breast adenocarcinoma (*1*, *2*). We previously demonstrated that a necrotizing tumoricidal response could be induced by extracorporeal perfusion of plasma over protein A-bearing *Staphylococcus aureus* Cowan 1 (SAC) but not over *S. aureus* Wood 46 (SAW) not bearing protein A (*3*). From analysis in vitro and in vivo, we now recognize that tumor-associated antibodies (TAA) are activated in plasma after perfusion over SAC but not SAW (*3*, *4*) and may contribute to the acute necrotizing tumoricidal responses observed. Indeed, canine immunoglobulin G (IgG) and C'3 bound to tumor cell membranes in situ within 4 hours after extracorporeal perfusion over SAC (*3*). In separate studies in vitro, TAA were shown to work synergistically with cytosine arabinoside (CA) to inhibit tumor cell replication (*5*, *6*). Here we present evidence that a single nontoxic infusion of CA administered to dogs with spontaneous mammary adenocarcinomas after extracorporeal perfusion of plasma over protein A immobilized in collodion charcoal (PACC) results in a necrotizing tumoricidal response that exceeds the reactions observed after extracorporeal perfusion of plasma over PACC or CA infusion alone and the algebraic sum of the reactions to the latter two treatments in the same dogs.

We obtained dogs with histologically proven spontaneous mammary adenocarcinomas ulcerating through the surface of the skin. During a 14-day control period preceding the treatments, all the visible neoplasms grew noticeably. Protein A (Pharmacia) (1.0 mg per kilogram of body weight) was immobilized in collodion charcoal by a slight modification of previously described methods (*7*–*12*). With ^{125}I-labeled protein A used as a marker (unlabeled protein A was used in preparation of the immunoadsorbent), 95.6 to 97.8 percent of the added protein A was bound in the charcoal. The capacity of PACC to bind canine IgG in vitro was demonstrated by perfusing ^{125}I-labeled canine IgG over it and a control column containing no protein A; uptake of IgG was 55 times greater in the columns containing protein A. For studies in vivo, arterial blood from anesthetized dogs was pumped through a filtration plasma-cell separator (Travenol), and then the calculated total volume of plasma for one dog was selectively perfused over the PACC at 10 to 20 ml/min. Formed elements were pumped to a site where they joined the plasma, and reconstituted whole blood was returned to the host. The extracorporeal volume of the PACC and filters was 100 to 150 ml.

Two methods were used to assess the tumoricidal reactions after the individual and combined regimens. In the first method, color photographs were taken of the tumors before each treatment and 12 hours afterward. The optical density of the color transparencies was recorded with a digital densitometer (Graphic Arts) calibrated with reference neutral-gray step wedges; this instrument has a tungsten light source, a red filter, and a photomultiplier tube as detector (*13*–*16*). For standardization and comparison of the readings, the average of ten normal skin readings was determined for each photograph, and this value was subtracted from the average of 30 to 40 readings taken directly from the tumors. The results were scored as the percentage of change in optical density compared to the values obtained before perfusion. In the second method, the percentage of tumor surface area necrotizing 12 hours after each treatment was recorded by three observers who had no prior knowledge of the treatments. The percentages were then averaged (Table 1).

The dogs were given CA (10 mg/kg) intravenously for 4 hours, and their tumors showed no significant morphological changes. Seven to 12 days later, the same dogs underwent extracorporeal perfusion over PACC alone, which resulted in hyperemic responses 4 to 8 hours after perfusion, progressing to focal necroses after 12 hours. Ten to 21 days later, when the gross inflammatory reactions had subsided, each dog underwent a similar extracorporeal perfusion over PACC, followed immediately by intravenous infusion of CA (10 mg/kg) for 4 hours. This resulted in more rapid and diffuse necrosis than after PACC perfusion alone. With this regimen, hyperemic reactions were evident 30 minutes to 2 hours after the CA infusion, progressing to diffuse necrosis during the ensuing 10 hours. In three of five dogs, up to 50 percent of the surface area of the visible lesions healed in 12 to 18 days after this treatment, whereas no significant healing was observed after treatment with PACC or CA alone in the same dogs. Normal mammary glands in these dogs showed no morphological changes during the course of the tumoricidal reactions. When the tumors had regrown to near-original size, 25 to 37 days after the last treatment, all the dogs under-

Table 1. Morphological responses in dog mammary adenocarcinomas after four types of treatment. Morphological changes after PACC + CA significantly exceeded those after individual treatment with CC, CA, or PACC in all dogs tested ($P < .03$, rank-sums test) (*18*). Morphological changes after PACC + CA significantly exceeded the algebraic sum of individual PACC and CA treatments in each dog tested ($P < .03$), implying pronounced synergism between the two treatments. The median response for treatment with CA alone was 0, yet the effect of CA after PACC perfusion was far greater that that of PACC treatment alone. Results are given as the percentage of change in optical density after perfusion. The tumors were evaluated densitometrically 12 hours before each treatment and again 12 hours afterward. Figures in parentheses indicate the percentage of total surface area involved in new necroses (0, none; 1, 1 to 20 percent; 2, 20 to 40 percent; 3, 40 to 60 percent; 5, 80 to 100 percent).

Dog	Lesions		Treatment			
	Number	Size* (cm^2)	CC	CA	PACC	PACC + CA
1	1	16.8 to 20.0	0 (0)	0 (0)	34.8 (2+)	381.7 (5+)
2	1	18.6 to 22.8	0 (0)	0 (0)	77.0 (2+)	264.8 (4+)
3	3	10.4 to 12.6	2.1 (0)	0 (0)	17.0 (2+)	223.1 (4+)
4	1	10.0 to 10.8	0 (0)	0.7 (0)	28.1 (1+)	131.6 (3+)
5	1	8.2 to 8.6	0 (0)	0.6 (0)	5.2 (1+)	28.5 (3+)

*Range of sums of products of perpendicular diameters of tumors at the beginning of each treatment.

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went extracorporeal perfusion over a collodion charcoal column in which no protein A was immobilized; no tumoricidal responses were visible 12 hours later. Three dogs underwent PACC + CA treatment only; their tumors showed pronounced necrosis 12 hours later. Representative results of these regimens are shown in Fig. 1. Table 1 gives graded responses for five dogs.

Toxic reactions to the PACC + CA regimen were minimal except for temperature elevations of 2.5°F in four dogs. All the animals were alert and ambulatory 6 hours after treatment, and there were no significant effects on erythrocyte, leukocyte, or platelet counts for up to 7 days after perfusion. Immunofluorescence studies (*3*) of tumor tissue from dogs 1 and 2 showed that the deposition of canine IgG and C'3 on mammary carcinoma cell membranes 12 hours after PACC and PACC + CA perfusion was more diffuse than that revealed in biopsies before each treatment. Microscopic examination of tumor tissue obtained before and 12 hours after treatment showed more extensive necrosis after the PACC + CA regimen than after PACC alone (*17*). Radioactivity in the protein A columns measured before and after the extracorporeal procedure showed no significant changes; hence there was no discernible release of protein A from the columns into the hosts.

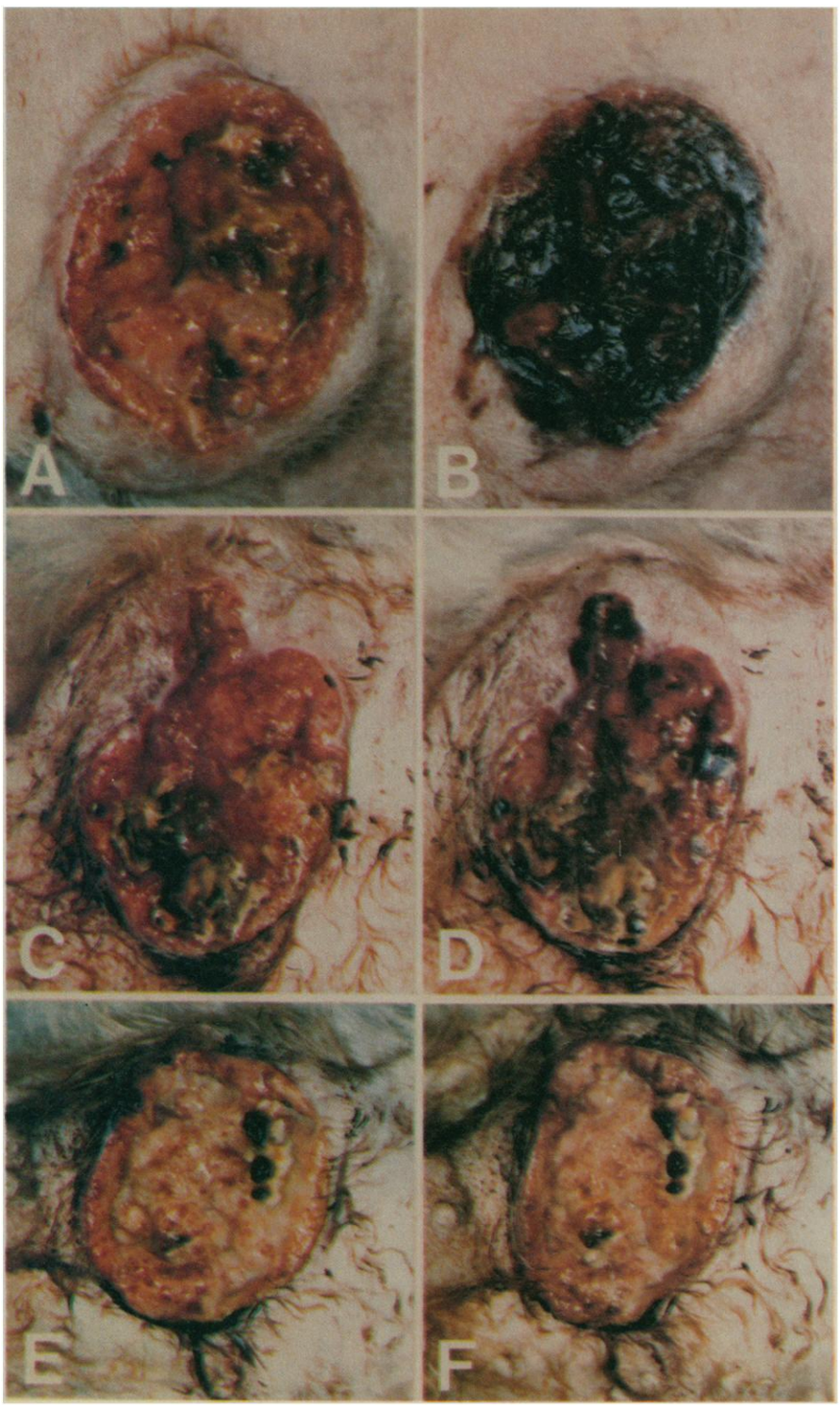

Fig. 1. Gross morphology of large ulcerating mammary adenocarcinoma in dog No. 2 before (A, C, and E) and 12 hours after various treatments. (B) Tumoricidal response after the PACC + CA regimen. (D and F) Reactions after treatment with PACC and CA, respectively.

Tumor necrosis resulting from extracorporeal perfusion over PACC was augmented by administration of a single nontoxic dose of CA after the perfusion. The gross tumoricidal reactions noted after the PACC + CA regimen were substantially greater than the algebraic sum of responses to CA and PACC treatments alone in the same dogs (Table 1). The CA infusion treatment alone induced no significant tumoricidal responses (median = 0); however, when the drug was given after PACC perfusion, the tumoricidal reactions exceeded those noted after PACC alone (Table 1). Hence, it appears that the effect of the drug was potentiated when it was given immediately after PACC perfusion.

The increased magnitude of the tumoricidal responses after the PACC + CA treatment is further supported by evidence of partial healing of visible lesions in three dogs in which no healing was seen after PACC treatment alone. Indeed, more extensive necrosis was observed microscopically in dogs treated with PACC + CA than in the same dogs after PACC treatment alone. Not only was the magnitude of the tumoricidal reactions greater after the PACC + CA regimen, but the necrosis began sooner after the extracorporeal perfusions than after the PACC treatments alone in the same dogs. The acute necrosis observed after the PACC + CA regimen was specific for mammary carcinoma tissue since normal mammary glands were not involved in the inflammatory reactions, and toxicity was minimal. Differences in the tumoricidal effects cannot be attributed to variations in tumor size, since the tumors were of comparable dimensions before each treatment (Table 1). It is unlikely that the tumoricidal responses observed after the PACC + CA regimen

were due to conditioning by prior treatments, since the tumors of three previously untreated dogs showed extensive necrosis 12 hours after the PACC + CA regimen. Nor can the tumoricidal response be ascribed to release of protein A from the immunoadsorbent column, since there were no significant changes in radioactivity in the column before and after extracorporeal perfusion.

Augmentation of the tumoricidal response after the PACC + CA regimen may be related to observations in which CA, in the presence of TAA, exerted a synergistic inhibitory effect on tumor cell replication in tissue culture (*5*, *6*), perhaps due to antibody-enhanced phosphorylation and subsequent increased incorporation of the drug into the DNA of cell nuclei. The TAA, measured by radioimmunoassay, are now known to be activated in plasma after perfusion over PACC in vivo and in vitro (*3*, *4*). Indeed, canine IgG and C'3 were found deposited in tumor cell membranes 12 hours after treatment with PACC and PACC + CA. The distribution and concentration of these deposits were comparable after the two treatments, but light microscopy showed that necrosis was far greater after the PACC + CA treatment.

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Stress-Induced Eating Is Mediated Through Endogenous Opiates

Abstract. *The interaction of endogenous opiates and stress-induced eating in rats was evaluated by pharmacological manipulation. Eating induced by the tail-pinch method was inhibited by the opiate antagonist naloxone; after being repeatedly stressed over a 10-day period and then given naloxone, the rats behaved in a manner indistinguishable from the "wet-dog" shakes of opiate withdrawal. Thus endogenous opiates may have a role in the control of stress-related eating, a finding that may have therapeutic implications for humans.*

Mildly pinching the tails of rats reliably induces a syndrome of eating, gnawing, and licking in the presence of food (*1*). This response has interesting parallels with stress-induced eating in humans (*2*). The endorphins may be involved in the modulation of feeding drives in food-deprived rats (*3*) and in the development of obesity in genetically obese mice (*4*). In addition, there are striking pharmacological parallels between the behavioral response to tail pinching and schizophrenia (*5*), and a number of studies have suggested a role of endogenous opiates in the genesis of some types of schizophrenia (*6*). We report here that the opiate antagonist naloxone suppresses ingestive behavior without affecting gnawing or licking behavior in rats stressed by the tail-pinch method.

We modified the tail-pinch method by using a plastic hemostat (MacBick Co.), which gives better control of the range of pressures that can be exerted than a surgical hemostat. Testing was carried out in a 22 by 17 cm plastic box containing two pellets of Purina rat chow (6 to 9 g).

There are several fine points to the tail-pinch method. For example, gentle side-to-side oscillations of the hemostat may facilitate the onset of the oral behaviors. (In the majority of animals, the behaviors are induced before the onset of pain, as indicated by squeaking.) In addition, certain methodological precautions are necessary: (i) the hemostat and the hand of the investigator must be kept out of sight of the rats, (ii) the rat's tail must not become twisted, and (iii) the rat must be adapted to the experimental environment and should not be engaged in exploratory behavior at the time tail pinching begins. Provided these caveats are observed, oral behaviors can be reliably induced in all rats tested. However, only 72 percent of all the rats we have tested ate; 20 percent gnawed without ingestion and 8 percent merely licked. In general, once a rat has ingested food in one trial, it will eat during subsequent trials spaced 15 minutes apart.

In the experiment described here we used adult male Sprague-Dawley rats (175 to 225 g) that had not been deprived of food; all had been shown to ingest food during a 2-minute period of mild tail pinching. Naloxone (4 or 2 mg/kg, parenterally) decreased food intake, and this decrease was more marked 30 minutes than 15 minutes after the injection (Fig. 1). Naloxone did not suppress gnawing, which was present in all the naloxone-treated animals, including those that failed to ingest any food. A number of these rats demolished one or both of the pellets without ingesting any of them. Naloxone at 1 mg/kg did not significantly suppress eating, although two of the eight rats given this dose failed to ingest any food. At the highest dose of naloxone (4 mg/kg), a number of the rats squeaked at tail-pinch pressures below those necessary to induce eating, gnawing, or licking during the control trial. For this reason, we pinched these rats again (35 minutes after naloxone was given), using a pressure slightly less than that which elicited squeaking. This pressure was insufficient to induce the oral behaviors in these animals.

In contrast to the effects of naloxone, parenteral injections of saline tended to increase the amount of food ingested during the second and third trials (Table 1). To exclude the possibility that naloxone exerted its effect by decreasing the general arousal of the animal, we used diazepam (0.5 mg/kg) as a sedative control. Not only did diazepam fail to sup-

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