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Attempts to Increase Intratumoral Blood Flow in the Rat Solid Walker 256 Tumor by the Use of the Perfluorocarbon Emulsion Fluosol-DA

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Abstract—*We have examined the effect of the perfluorocarbon emulsion, Fluosol-DA 20% (FDA), on blood flow in rats bearing an advanced solid Walker 256 tumor implanted s.c. Blood–FDA exchange in unanesthetized rats maintained under 100% oxygen was accomplished by simultaneous arterial withdrawal and i.v. infusion until the hematocrit was less than 4%. Control rats were maintained under 100% oxygen but did not undergo any exchange. Regional blood flow studies in tumors of control and FDA-exchanged rats were performed using [^{14}C]iodoantipyrine and quantitative autoradiography. FDA–blood exchange did not increase flow to the whole tumor. Similarly, the pattern of regional flow within the tumor, which was determined in histologically distinct areas—including dense and normocellular, necrotic and peripheral zones invading into muscle and connective tissue—was not substantially altered. Flow to cerebral tissue was increased two-fold, although flow to normal tissues including temporalis muscle, skin and diaphragm was not altered. These results show that FDA–blood exchange does not enhance vascular flow in solid Walker 256 tumor implanted s.c. in the rat.*

INTRODUCTION

ONE OF THE reasons that solid tumors may be refractory to chemotherapy is the presence of poorly perfused regions in the tumor which prevent the achievement of effective concentrations of an anti-cancer drug throughout the tumor [1–3]. A perfluorochemical emulsion (PFCE) such as Fluosol-DA 20% (FDA) has sufficient oxygen carrying capacity at high partial pressures of oxygen to enable it to serve as an artificial blood substitute in animals and in humans [4–9]. In experimental rodent tumors, i.v. administration of a PFCE including FDA or Fluosol-43 in an oxygen environment enhances anti-cancer drug treatment [10–14]. In addition, the radiation sensitivity of the mouse Lewis lung carcinoma and FSA-IIc fibrosarcoma tumors are increased by i.v. treatment with FDA in an oxygen environment [15, 16]. A PFCE such as FDA possesses a smaller particle size compared with red blood cells [17] and a lower viscosity as compared with blood [5]. These characteristics of PFCE may allow for increased perfusion, and thereby possibly enhanced drug distribution throughout a tumor. We have examined the effect of FDA on local blood flow in tumor and normal tissues of control and FDA-exchanged rats using quantitative autoradiographic methods. Our results indicated that FDA–blood exchange did not increase regional blood flow to the tumor. Although blood flow to cerebral tissue was increased approx. two-fold, flow to several other normal tissues was not altered. A preliminary report of our investigation has already appeared [18].

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MATERIALS AND METHODS

Chemicals

FDA 20% (Green Cross Corp., Osaka, Japan) was obtained as a generous gift from the Alpha Therapeutic Corporation, Los Angeles, CA. Since repetitive freezing and thawing of the emulsion should be avoided [19], the emulsion was thawed

once, divided into 40 ml aliquots, and then stored at −20°C. In this way only the amount of FDA emulsion needed for a given experiment was subject to rethawing. 4-[*N*-Methyl-^{14}C]iodoantipyrine ([^{14}C]IAP), 40–60 mCi/mmol, was obtained from New England Nuclear Corp. (Boston, MA); isotopic purity was > 98% as determined by chromatography in two solvent systems [20].

Animal model and tumors

Male Wistar rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). The Walker 256 carcinosarcoma, maintained by intraperitoneal (ascites) passage, was obtained from an intramural NIH source. The tumor was maintained in rats weighing 150–200 g by weekly i.p. inoculations of 1×10^6 cells. Animals were caged in an air-conditioned room lighted from 6 a.m. to 6 p.m. and had free access to a standard Purina laboratory chow diet and tap water. Rats, weighing 350–400 g, were inoculated s.c. with 1×10^6 Walker 256 ascites cells in the dorsal scruff of the neck. Regional blood flow experiments were carried out on days 6–12 after tumor inoculation at which time the tumors from control or FDA-exchanged animals were 0.98 ± 0.21 and 1.29 ± 0.18 g, respectively. Experiments to determine the tissue–blood partition coefficient were carried out on days 3–5 after tumor inoculation at which time the tumors from control or FDA-exchanged animals were 0.24 ± 0.04 and 0.22 ± 0.04 g, respectively.

Blood flow

Regional blood flow in tumor and flow to normal tissues was measured using [^{14}C]IAP as previously described [21–24]. The animals were anesthetized with halothane:nitrous oxide:oxygen (1.5:70:30, v/v/v). Bilateral artery and vein catheters (PE-50, polyethylene tubing) were inserted, the animals were immoblized below the midthorax by using plaster bandage that did not impinge on the s.c. tumors, and the animals were allowed to recover from the anesthesia for at least 1 h while body temperature was maintained at 37°C using heat lamps. The animals were then placed under a plexiglass container into which 100% oxygen gas was administered at a rate of 10 l/min. Using a dual Harvard 940 infusion pump the animals were exchange-transfused with FDA by simultaneous i.v. infusion and arterial withdrawal [25] at a rate of 1 ml/min, until their hematocrit was less than 4%. Control tumor-bearing animals did not undergo blood exchange and were maintained under the same enhanced oxygen conditions. Arterial blood pressure, pO_2, pCO_2, pH, and body temperature were monitored during the recovery period from surgery, the blood–FDA exchange period, and just prior to each experiment; arterial blood pressure was monitored throughout the experimental period. Because we were concerned about the possibility of acidosis in animals maintained in a 95% oxygen–5% carbon dioxide atmosphere we carried out initial studies in both normal and FDA-exchanged animals in which we compared the effects of pure oxygen or 95% oxygen–5% carbon dioxide on blood pressure, arterial blood gases and pH. We found that a 95% oxygen–5% carbon dioxide atmosphere caused a consistent decrease in arterial blood pH of approx. 0.1 pH unit and an increase in pCO_2 of 9–12 mmHg. Therefore we carried out all our experiments in an oxygen atmosphere.

To measure blood flow an infusion of 40 μCi of [^{14}C]IAP was administered into the right femoral vein over 35 s, according to an increasing infusion schedule which resulted in continuously increasing blood levels of [^{14}C]IAP. Serial 5-s arterial blood samples were obtained during the infusion. The animal was decapitated proximal to the cervical tumor at 30 s, the tumor and adjacent tissue was immediately removed and a small sample (10–200 mg) was obtained for liquid scintillation counting. The remaining tissue was frozen in liquid Freon, dipped in embedding matrix, and stored at −80°C prior to sectioning. Samples of brain, skin adjacent to the tumor, temporal muscle and diaphragm were also promptly removed, weighed and dissolved in NCS tissue solubilizer (Amersham, Arlington, Heights, IL). Plasma samples, for radioactivity measurements, from control or FDA-exchanged animals were obtained by centrifugation of blood samples. In a companion paper we showed that the blood/plasma and blood/aqueous layer partition ratios of [^{14}C]IAP was essentially unity. This permitted plasma (or supernatant) rather than whole blood (or blood-FDA mixture) measurements of radioactivity [26]. Radioactivity was measured in a Beckman LS 350 liquid scintillation spectrometer using external standard quench correction.

Partition coefficient of IAP

To measure the tissue-to-blood partition coefficient of IAP, animals were anesthetized with i.p. pentobarbital (40 mg/kg), the femoral arteries and veins were catheterized and a complete evisceration (esophagus to rectum) and ligation of both renal pedicles was performed to eliminate iodoantipyrine metabolism during the experiment [21]. One group of animals underwent blood–FDA exchange and the control group was maintained under 100% oxygen as described above. [^{14}C]IAP (50 μCi) was injected i.v. and timed arterial blood samples were obtained. Animals were decapitated at either 45 or 60 min; blood and tissue was processed as described above.

Autoradiography and histology

Sections of tumor and adjacent tissue were prepared for histology and autoradiography as previously described [22, 27]. Sequential measurements of absorbance within 5 × 50 μm elements of the tissue autoradiographic image were made using a computerized high-speed scanning microdensitometer and disc storage system similar to that previously described [28]. To convert the X-ray film images to tissue radioactivity (nCi/g), the mean absorbance of each image produced by the ^{14}C standards was measured and a standard curve which related absorbance to tissue radioactivity was generated for each film.

Calculation and measurements

Regional measurements of blood flow were obtained using a computer-controlled cursor-outlining routine on the video monitor that could outline selected tissue areas for measurement. The autoradiographic and histological images from adjacent tissue sections (6–15 sections depending on tumor size) were registered (aligned) in two different channels of the image array processor (with a maximum x,y axis error of about 100 μm) and the region of interest was outlined on the channel containing the histological image. The blood flow data are expressed as the mean and standard deviation of all individual measurements (50 × 50 μm pixels) within the defined region of interest. Whole-tumor measurements are given by the average of all measurements within the histologically defined cross sectional area integrated along the Z-axis (length) of the tumor. Statistical comparison of unpaired data was by Student's t-test.

RESULTS

Tumor description and histology

A small subcutaneous mass in the posterior neck is usually palpable by 3–4 days after tumor inoculation. Histological examination of hematoxylin and eosin stained sections indicated that the cells were densely packed except for small foci of necrosis; larger tumors had larger necrotic areas but cyst formation was rare. An invasion zone into muscle and connective tissue could usually be identified.

Determination of tissue-to-blood and tissue-to-FDA partition coefficient for IAP

The physiological parameters of the animals at the time of the blood flow and partition coefficient studies are shown in Table 1. The measurement of local blood flow in these studies is based on an operational equation derived by Kety [29, 30] and applied to tumor (and brain tissue) using [^{14}C]IAP as previously described [21]. One of the constants in this equation is the tissue-to-blood partition coefficient. Based on the work of Sakurada *et al.* [21] as well as previous studies from this laboratory, the tissue-to-blood partition coefficient for IAP was found to be 0.8. Because it was possible that the tissue-to-blood partition coefficient of IAP might be different than its tissue-to-FDA partition coefficient, we determined the value of the partition coefficient in tumor and several normal tissues in control and FDA-exchanged rats. Blood levels of [^{14}C]IAP declined between 5 and 20 min and then remained essentially constant (Fig. 1). The partition coefficients calculated from the 45 and 60 min experiments were the same; the results from these experiments were combined (Table 2). The partition coefficients of IAP for tumors from control animals ranged between 0.83 and 0.94 for either the whole tumor or histologically distinct regions including packed cell areas, and invasive areas into either muscle or connective tissue (Table 2). In contrast, in animals which had undergone FDA–blood exchange the partition coefficients ranged between 1.36 and 1.65 for the whole tumor and discrete histological areas (Table 2). The increase in the partition coefficient of IAP due to FDA was not unique to tumor since similar increases were observed in various normal tissues from the rat including brain, temporal muscle, skin and diaphragm.

Regional blood flow in tumour

Blood flow in tumors of control and FDA-exchanged rats indicated that FDA did not alter flow in either the whole tumor or in selected histologically distinct areas of the tumors (Table 3 and Fig. 2). Similarly, no differences in flow were observed in those areas of either muscle or connective tissues which were invaded by the tumor. Flow to central-necrotic regions of the tumor was low compared to either the whole tumor or in other regions of the tumors in both control and FDA-exchanged rats. Similarly, flow in various organs was unaffected by blood–FDA exchange except for brain where a two-fold increase in flow was measured in several brain regions (Table 3).

DISCUSSION

The inability of anticancer drugs to distribute effectively throughout all portions of a solid tumor, particularly in poorly perfused areas containing both necrotic and viable tissue, remains one of the major dilemmas of cancer chemotherapy [27]. Regional tumor blood flow is therefore an important determinant of the efficacy of cancer chemotherapy. Vasoactive drugs and other techniques have been used in attempts to increase tumor blood flow [2, 31, 32]. Several characteristics of the synthetic blood substitute FDA, including low viscosity and small particle size as compared with blood [5, 6],

Table 1. Physiological parameters of control and FDA-exchanged animals

Physiological parameter	Partition coefficient experiments Control (6)	Partition coefficient experiments FDA-exchanged (6)	Blood flow experiment Control (4)	Blood flow experiment FDA-exchanged (6)
pH	7.35 ± 0.01*	7.41 ± 0.01	7.39 ± 0.02	7.42 ± 0.04
$paCO_2$	37.2 ± 2.2	33.0 ± 2.2	33.9 ± 1.6	33.6 ± 2.1
paO_2	369 ± 13	483 ± 24	401 ± 34	497 ± 35
Hematocrit	38.8 ± 1.2	2.8 ± 0.5	39.3 ± 1.1	3.7 ± 0.9
Blood pressure	118 ± 7	106 ± 2	132 ± 4	112 ± 3

Rats bearing a s.c. Walker 256 tumor were prepared surgically and the physiological parameters determined as described in Materials and Methods.
*Values are the mean ± S.E. with the number of individual animals indicated in parentheses.

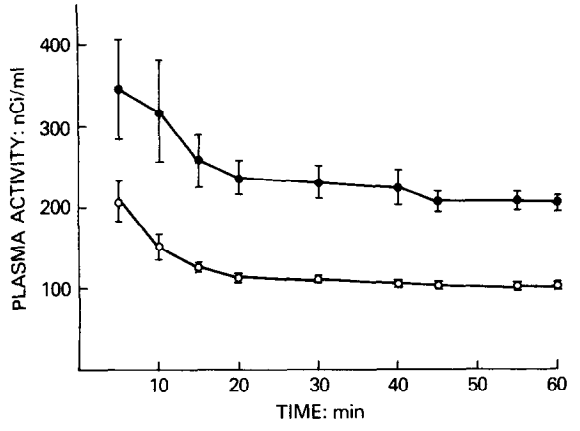

Fig. 1. The plasma activity–time curve of [^{14}C]IAP in eviscerated animals maintained under 100% *oxygen is shown:* (○) *represent the FDA–blood exchange animals;* (●) *represent control animals. All animals were given a single i.v. injection of* [^{14}C]*IAP* (50 μCi). *Each point represents the mean ± S.E. of three animals per group.*

prompted us to determine whether FDA–blood exchange could provide a means of increasing flow in an experimental solid tumor. Our experiments in rats bearing a s.c. implanted Walker 256 carcinosarcoma measured local blood flow using quantitative autoradiographic techniques and correlated these measurements to morphological features of the tumors. The validity of the IAP blood flow measurement technique in conscious rats has been established [21–24] and passive restraint in an enhanced oxygen environment does not result in undue stress. Frequently the animal is dozing during the study and arterial blood gases, except for pO_2, are within the normal range.

None of the tumor regions identified showed an increase in flow following FDA–blood exchange. Similarly, flow to several normal tissues was not affected by FDA–blood exchange; however, flow to cerebral tissue was increased approx. two-fold. Our blood flow measurements in tumors of approx.

Table 2. Tissue-to-blood and tissue-to-FDA partition coefficient for IAP

	Control Tissue-to-blood ratio	FDA-exchanged Tissue-to-FDA ratio
Description of tumor region		
Whole tumor	0.87 ± 0.03(5)*	1.40 ± 0.07(6)
Packed cell	0.83 ± 0.04(5)	1.37 ± 0.05(6)
Invasive into muscle	0.94 ± 0.05(5)	1.65 ± 0.05(3)
Invasive into connective tissue	0.94 ± 0.05(5)	1.48 ± 0.05(6)
Normal tissue		
Temporal muscle	0.77 ± 0.08(6)	1.37 ± 0.03(6)
Skin adjacent to tumor	0.85 ± 0.03(5)	1.39 ± 0.11(6)
Diaphragm	0.86 ± 0.04(6)	1.46 ± 0.05(6)
Brain, frontal pole	0.78 ± 0.03(6)	1.46 ± 0.09(6)
Brain, medulla	0.77 ± 0.03(6)	1.39 ± 0.07(6)

Rats bearing a s.c. Walker 256 tumor were prepared surgically and the partition coefficient for IAP was determined as described in Materials and Methods.
*Values are mean ± S.E. with the number of individual tumors, as well as regional areas which are analyzed, indicated in parentheses.
Significantly different from control, at $P < 0.001$.

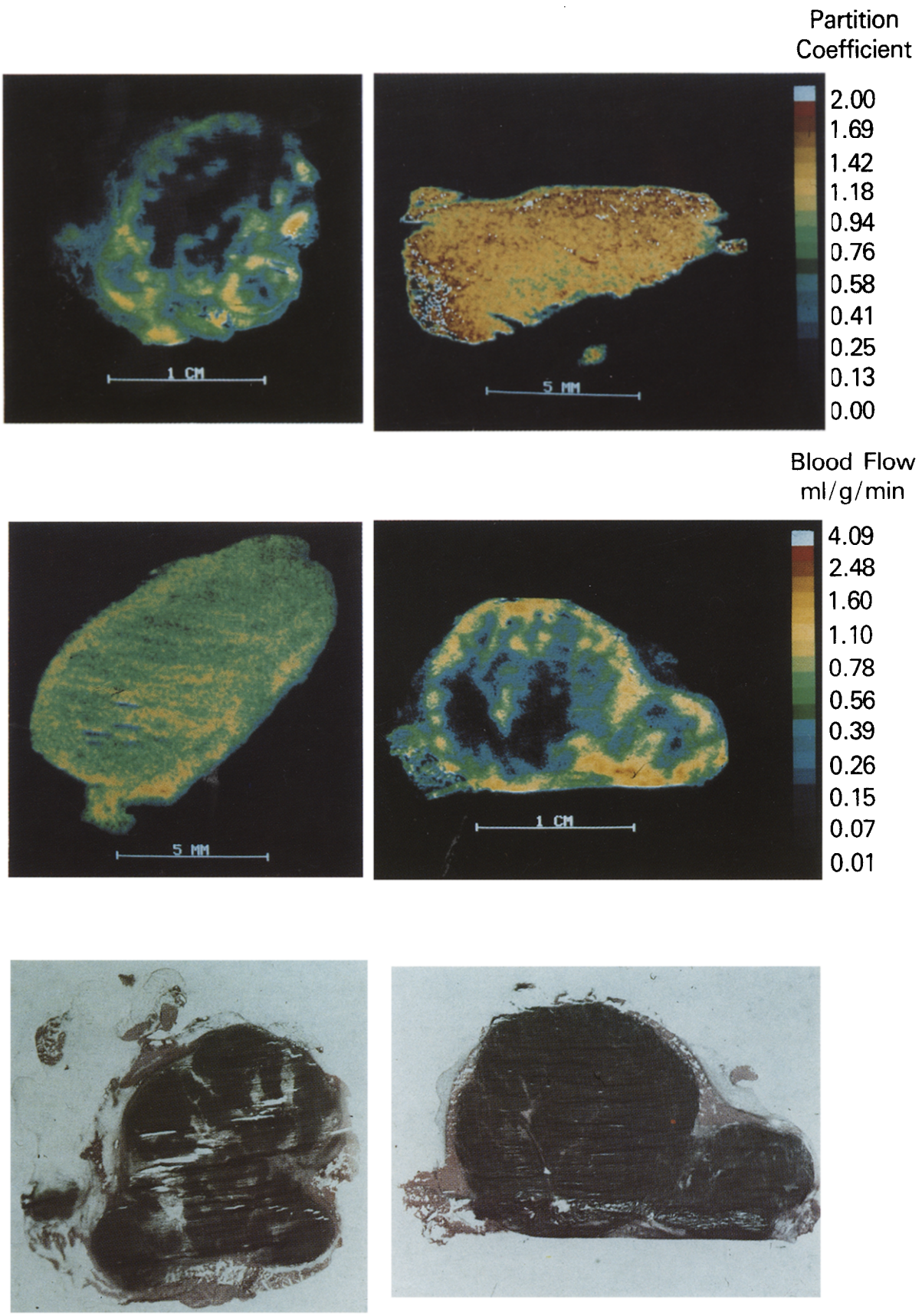

Fig. 2. Autoradiographic images of the [^{14}C]IAP tissue-to-plasma partition coefficient (upper panel) and blood flow (middle panel) in subcutaneous Walker 256 tumors are shown; the corresponding histology for the blood flow images are also shown (lower panel). The autoradiographic images are color-coded to a range of values. The left of each image pair represents the control animals; the right of each image pair represents the FDA–blood exchange animals. Note the uniformity of the partition coefficient within each subcutaneous tumor; the value is significantly higher in the FDA–blood exchange experiment. Local blood flow varies considerably in the subcutaneous tumors and was unaffected by FDA–blood exchange.

Table 3. Effect of FDA on blood flow in tumor and normal tissues

	Control	FDA-exchanged
	Flow (ml/100g/min)	
Description of tumor region		
Whole tumor	26.2 ± 5.8(5)*	24.5 ± 4.4(5)
Packed cell	23.7 ± 3.9(4)	17.4 ± 2.5(4)
Non-necrotic	29.4 ± 4.6(3)	22.4 ± 4.5(4)
Central necrotic	5.5 ± 1.8(3)	3.1 ± 1.0(4)
Invasive into muscle	61.1 ± 18.9(3)	69.9 ± 16.4(4)
Invasive into connective tissue	28.1 ± 6.8(4)	34.5 ± 8.8(5)
Normal tissue		
Temporal muscle	20.1 ± 3.7(7)	33.9 ± 7.9(6)
Skin adjacent to tumor	17.2 ± 2.2(5)	18.6 ± 2.8(5)
Diaphragm	153 ± 25(7)	185 ± 16(6)
Brain, frontal pole	171 ± 30(7)	323 ± 28(6)
Brain, medulla	136 ± 10(7)	178 ± 18(6)

Rats bearing a s.c. Walker 256 tumor were prepared surgically and the blood flow was determined as described in Materials and Methods.

*Values are mean ± S.E. with the number of individual tumors, as well as regional areas which are analyzed, indicated in parentheses.

Significantly different from control, at $P < 0.005$.

1 g from control and FDA-exchanged animals were 26.2 and 24.5 ml/100 g/min, respectively. These values are comparable to that of Song *et al.* [33] who reported a blood flow of 30 ml/100 g/min in 1 g Walker 256 tumors implanted s.c. in the leg of Sprague–Dawley rats. Blood flow in skin of 7.82 ml/100 g/min which they reported is less than our values in control and FDA-exchanged animals which were 17.2 and 18.6 ml/100 g/min, respectively. It should be noted, however, that these investigators measured blood flow using the microsphere technique in anesthetized animals.

Experiments to determine the effect of FDA on the tissue-to-blood partition coefficient of IAP in tumor indicated that the partition coefficient increased from 0.9 to 1.4 following FDA–blood exchange. This change probably represents a shift of IAP from the intravascular (FDA) compartment to the intracellular tissue compartment (Table 2). The increase in the partition coefficient of IAP following FDA–blood exchange was not unique to tumor since we also found essentially identical increases in several other normal tissues including brain, muscle, skin and diaphragm. We have shown in a related paper that the increase in the tissue-to-blood partition coefficient is due to rapidly reversible binding of IAP to plasma proteins and to the removal of plasma proteins during FDA–blood exchange [26].

For an anticancer drug with physical/chemical characteristics (e.g. molecular size, lipid solubility) and plasma protein binding properties similar to IAP, and whose entry into cells like that of IAP is not transport-limited, it would be expected that the partitioning of such a compound between tissue and the vascular space would be comparable to that of IAP. Since FDA–blood exchange also increased the partition coefficient of IAP in normal tissues from 0.8 to 1.4, it is unlikely that any selective advantage would be obtained with respect to anticancer drug distribution in tumor vs. normal tissues. Our observation that an essentially complete FDA–blood exchange increased the partition coefficient of IAP is not altogether surprising since PFCE have been shown to alter the physiological disposition of selected drugs. For example, in rats which had undergone approx. 60% replacement of blood with FDA the plasma half-life and volume of distribution of sulfamethazine was increased. In contrast, the pharmacokinetics of antipyrine, diazepam and penicillin have been reported as unchanged [34]. In rats which had undergone approx. 90% replacement of their blood with the PFCE Fluosol-43, and were then maintained in an oxygen environment, the plasma half-life of phenytoin was increased and its clearance was decreased, although its total volume of distribution in the animal was unchanged [35].

PFCE, given as a single bolus i.v. injection, increases the action of several anticancer drugs in experimental rodent tumors maintained in an oxygen environment. Rats bearing a transplanted intracerebral RG-C_6 tumor were given Fluosol-43 (20 ml/kg, i.v.) and treated with 1,3-bis(2-chloroethyl)-1-nitrosourea in an oxygen environment; survival time following this regimen was increased compared with treatment with the nitrosourea

alone. The authors suggest that Fluosol-43 plus oxygen may have some effects on the oxygenation of hypoxic tumor cells as well as improved blood flow and drug delivery to the poorly vascularized areas in the tumor [10]. Fluosol-43 (20 ml/kg, i.v.) given to rats bearing a transplanted intracerebral 9L tumor and treated with 1,3-bis(2-chloroethyl)-1-nitrosourea in an oxygen environment increased mean survival time compared with rats receiving Fluosol-43 plus the nitrosourea and breathing air. The authors speculate that Fluosol-43 plus oxygen may increase the tumor microcirculation and thereby increased drug accessibility to areas of low blood flow in the tumor [11]. In rats bearing the solid AH130 carcinoma, treatment with FDA (20 ml/kg, i.v.) plus an oxygen environment increased the inhibition of tumor growth by vincristine as compared to treatment with vincristine plus oxygen. Treatment with FDA did not, however, enhance the action of spadicomycin against the rat AH130 carcinoma. Negative results were also observed with 3-[(4-amino-2-methyl-5-pyrimidinyl)ethyl]-1-(2-chloroethyl)-3-nitrosourea against the rat solid Sato lung carcinoma. Measurement of tumor blood flow and pO_2 indicated that both were increased by administration of FDA plus oxygen [12].

FDA can enhance the cytotoxicity of anticancer drugs against a tumor without a comparable increase in cytotoxicity against a sensitive, normal tissue. In mice bearing an established subcutaneous FSA-IIc fibrosarcoma, FDA administered as a single i.v. bolus at a dose of 0.3 ml/mouse (12 ml/kg based on an estimated body weight of 25 g) combined with breathing 95% oxygen–5% carbon dioxide for 2 h increased the therapeutic efficacy of etoposide. This increase was the result of a markedly enhanced tumor growth delay without a concomitant increase in toxicity to bone marrow cells, the latter of which were obtained from treated animals and quantitated by colony forming assay *in vitro* [13]. Again using the mouse FSA-IIc fibrosarcoma system the same investigators showed that FDA followed by 2 h of breathing 95% oxygen–5% carbon dioxide also increased the therapeutic index of a series of nitrosoureas, i.e. there was increased cytotoxicity against tumors without a corresponding increase in toxicity to bone marrow. In these experiments the results were quantified in terms of tumor growth delay as well as tumor and bone marrow cell survival by colony forming assay *in vitro* [14].

PFCE can increase tumor radiosensitivity. FDA administered as a single i.v. bolus at doses of 0.3–0.5 ml/mouse combined with breathing 95% oxygen–5% carbon dioxide, gave a maximal enhancement of radiation therapy against either the mouse Lewis lung carcinoma or the FSA-IIc fibrosarcoma. The authors suggest that the ability of FDA to enhance radiosensitivity of these tumors may be due to FDA-mediated changes in oxygen delivery to the tumor [15, 16].

Conclusions and implications for chemotherapy

Review of the literature in which PFCEs have been administered results in a somewhat confusing picture; the effects on blood flow as well as specific therapeutic regimens have been inconsistent. The spectrum of results could well reflect differences in the experimental tumor systems that have been studied. For the Walker 256 s.c. tumor in the rat, FDA–blood exchange had no effect on blood flow in tumor and systemic organs except for brain.

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