

Oxygen-carrying Perfluorochemical Emulsion as an Adjuvant to Radiation Therapy in Mice¹

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ABSTRACT

The potential of an oxygen-carrying perfluorochemical emulsion (PFCE) to enhance radiation damage in Lewis lung tumor growing in C57BL/6J × DBA/2J F₁ mice was examined. PFCE and 95% O₂:5% CO₂ (carbogen) breathing caused a significant enhancement of single-fraction radiation damage measured by the growth delay assay. The dose-response effect of PFCE was very broad; doses as small as 0.5 ml/mouse were effective, and doses of 0.3 to 0.4 ml/mouse gave maximal enhancement. The peak dose-modifying factor was 2.8 ± 0.6 (S.E.). The addition of 0.3 ml of perfluorochemical-free annex solution with carbogen breathing produced a smaller enhancement in tumor growth delay; the dose-modifying factor was 1.5 ± 0.2. When the perfluorochemical treatment was added to a fractionated course of radiation therapy, a dose-modifying effect of 1.8 ± 0.3 was obtained. Oxygen-carrying PFCE may provide a nontoxic, clinically useful method of increasing the effectiveness of radiation therapy and of certain chemotherapeutic agents.

INTRODUCTION

Oxygen tension in tissues depends upon vascular supply and metabolic removal. Tumors have areas of intermittent and irregular blood flow. External pressure on capillaries resulting from unbalanced cell proliferation causes blood vessels to collapse (2, 8, 9, 14, 19). Tumor growth observations *in situ* reveal rapidly opening and closing blood vessels (33, 34, 40). Both effects lead to zones of necrosis and areas of hypoxia within tumors.

Hypoxia may be a limiting factor in radiotherapeutic efficacy (3). A variety of methods have been used to overcome this problem. The effectiveness of hyperbaric oxygen is limited by constriction of the vasculature and the toxicity of oxygen (16). Organic radiosensitizers such as misonidazole were developed out of a search for compounds which would mimic the action of oxygen. However, neurological complications have limited the total administered dosage of misonidazole; thus far, clinical trials have been unsuccessful (2, 6, 7, 21, 22). High linear energy transfer radiations such as neutrons have a minimal oxygen effect, but difficulties in calculating the radiobiological effect in hydrogen-rich tissues have resulted in unacceptable normal tissue damage (24). Hypoxic cell-selective cytotoxic agents offer a promising approach; however, few of these agents are available at present for clinical evaluation (35). The use of oxygen-carrying PFCE² represents a new approach to the problem of hypoxia.

Perfluorochemicals have excellent oxygen- and carbon dioxide-carrying capacity (12). Oxygen solubility expressed as a function of partial pressure (pO₂) will approach a saturation level

in chelates, such as hemoglobin in erythrocytes. In PFCE, solubility is a linear function of pO₂ (11-13, 27). To fully utilize the oxygen-carrying capacity of these materials, high partial pressures of oxygen are used. Unlike hemoglobin, where oxygen is bound to the molecule, the solvent action of the perfluorochemicals for oxygen does not involve any kind of chemical or chelating process. The gas molecules situate themselves in the spaces between the molecules (11, 13). The uptake and release of oxygen from PFCE are completely reversible, and the rate is twice as fast as chelation to hemoglobin. At least 90% of the emulsion particles in the preparation which we used are less than 0.2 μm in diameter, much smaller than RBC (average diameter, 5 to 10 μm).

In this paper, we describe the enhancement in tumor growth delay of the Lewis lung tumor observed with several *i.v.*-administered doses of PFCE and 95% O₂:5% CO₂ (carbogen) breathing in single-dose and fractionated radiation treatment protocols.

MATERIALS AND METHODS

Fluosol-DA 20% (Green Cross Corp., Osaka, Japan) was obtained from Alpha Therapeutics Corp. (Los Angeles, CA). The stem emulsion consists of 40% (w/v) perfluorochemicals: 7 parts perfluorodecalin; 3 parts perfluorotripropylamine; Pluronic F-68 (2.7%, w/v); and yolk phospholipids (0.4%, w/v) as emulsifiers; and glycerol (0.8%, w/v) as a cryoprotecting agent. The annex solution (Krebs-Ringer's bicarbonate solution) furnishes the preparation with physiological osmolarity, and hydroxyethyl starch (3.0%, w/v) provides osmotic pressure. The stem emulsion and annex solution are mixed (1:1) to make Fluosol-DA 20%. The combined surface area of the emulsion particles is 1.82 × 10⁶ sq cm/liter available for oxygen diffusion (about 100 times the surface area of the RBC in whole blood). The half-life of Fluosol-DA *in vivo* is about 12 hr (12).

Lewis lung tumor (30-32) was carried in male C57BL/6J mice (The Jackson Laboratory). For tumor growth delay and surviving fraction experiments, 2 × 10⁶ tumor cells prepared from a brei of several stock tumors were implanted *i.m.* into the gastrocnemius muscles of mice. The Lewis lung tumor was grown in 8- to 10-week-old male C57BL/6J × DBA/2J F₁ (hereafter called B6D2F₁) mice (The Jackson Laboratory). When the tumors were approximately 50 cu mm in volume (about 1 week after tumor cell implantation), PFCE, in volumes ranging from 0.05 to 0.6 ml/mouse, was administered by tail vein injection. The animals were then allowed to breath air or were placed in circulating carbogen chambers. One hr later, the animals were treated with a single dose of ¹³⁷Cs γ-rays of 10, 20, or 30 Gy or with multiple 3-Gy fractions to the tumor-bearing limb (Gamma Cell 40, Atomic Energy of Canada, Ltd.; dose rate, 0.88 Gy/min) continuously flushed with air or carbogen. In the fractionated radiation experiments, the tumors received two to five 3-Gy treatments. The fractions were delivered twice a day with at least 6-hr interval. The animals receiving 3 and 4 fractions were given a dose of 0.3 ml Fluosol-DA on each of 2 days; the animals receiving 5 and 6 fractions were given a dose of 0.3 ml Fluosol-DA per day for 3 days. The animals were placed in a circulating carbogen atmosphere for 1 hr prior to and during both the morning and afternoon radiation treatments. The shielded portion of the animal received less than 2% of the delivered

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² The abbreviation used is: PFCE, perfluorochemical emulsion(s).
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dose. The animals were anesthetized (Nembutal) during the radiation treatment. Tumor size was followed by thrice weekly measurements. The experimental end point was the number of days post-tumor cell implantation for the tumors to reach a volume of 500 cu mm (~10 times the initial treatment volume) (28). Untreated tumors reach 500 cu mm in approximately 14 days. Statistical comparisons were made using the Student *t* test. Data from the tumor growth delay experiments were analyzed using a computer program written in Basic for the Apple II+ microcomputer. The program first derives the best-fit curve for each individual set of tumor volume data and then calculates the median, mean, and S.E. for each experimental group. The day on which each tumor reached 500 cu mm and the median, mean, and S.E. are then derived. A second program provides statistical comparisons between any number of groups using the *t* test and deriving degrees of freedom and *p* values. Each experimental group had 7 mice, and each experiment was repeated at least twice; therefore, the minimum number of tumors examined at each point was 14.

RESULTS

PFCE and carbogen breathing significantly enhanced X-ray-induced tumor growth delay. The PFCE itself had no effect on the growth or on the growth delay produced by X-rays in air. Chart 1 shows the dose-response effect obtained over a Fluosol-DA dosage range from 0 to 0.6 ml/mouse. The dose-response curve is very broad, peaking at 0.2 to 0.4 ml Fluosol-DA (or 8 to 16 mg Fluosol-DA per kg). The tumor growth delay observed with low doses of Fluosol-DA (0.05 or 0.1 ml) was significantly different from that obtained without Fluosol-DA addition at the *p* < 0.002 level.

In all of the experiments described in this paper, treatment with PFCE was performed by addition of a volume of fluid to the intravascular volume. Plasma is a significant oxygen carrier in circumstances of low hematocrit (26, 27). We attempted to distinguish between the contributions of the complete PFCE and the annex solution to tumor growth delay. Table 1 shows the growth delay of Lewis lung tumor in animals treated with 0.3 ml of annex solution or 0.3 ml of Fluosol-DA. In all cases, the animals were maintained in a circulating 95% oxygen atmosphere

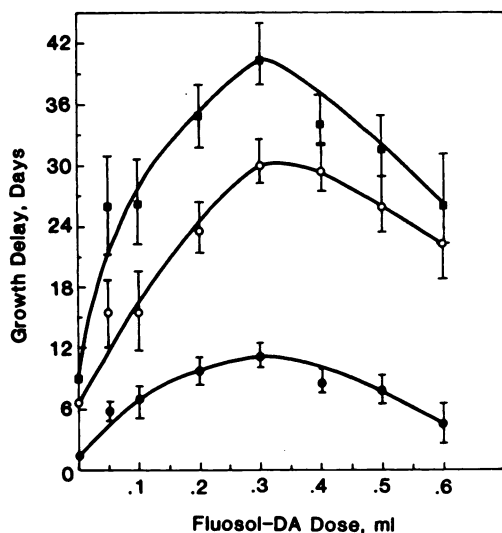


Chart 1. Dose-response effect for Fluosol-DA with carbogen breathing for 1 hr prior to and during X-ray treatment in the Lewis lung tumor. Radiation was delivered in single doses of 10 Gy (●), 20 Gy (○), or 30 Gy (■). Points, mean growth delay; bars, S.E.

Table 1
Growth delay of Lewis lung tumors treated with annex solution or PFCE and carbogen breathing for 1 hr prior to and during irradiation

Treatment condition	Growth delay (days)		
	10 Gy	20 Gy	30 Gy
Carbogen breathing	1.6 ± 0.5 ^a	6.2 ± 1.0	8.8 ± 1.0
Carbogen breathing and annex solution	3.6 ± 1.0	12.6 ± 1.5	19.7 ± 2.0
Carbogen breathing and Fluosol-DA	8.5 ± 1.5	28.5 ± 2.5	36.3 ± 2.0

^a Mean ± S.E.

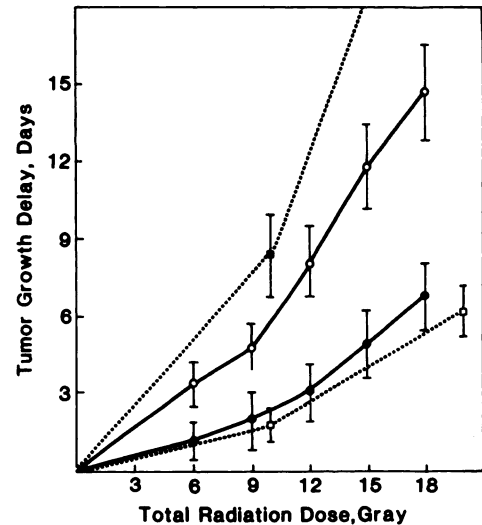


Chart 2. Growth delay of the Lewis lung tumor produced by fractionated X-ray treatments with carbogen breathing for 1 hr prior to and during each treatment in the presence (○) and absence (●) of Fluosol-DA. Radiation was delivered in 3-Gy fractions twice per day, and 0.3 ml of Fluosol-DA was administered once per day. ---, growth delay of the Lewis lung tumor produced by single-dose X-ray treatments with carbogen breathing for 1 hr prior to and during treatment in the presence (■) and absence (□) of Fluosol-DA (37). Points, mean growth delay; bars, S.E.

for 1 hr prior to and during irradiation. Based on the data obtained at a point giving 6 days of tumor growth delay, the dose-modifying factor observed with annex solution was 1.45 ± 0.21 (S.E.); with PFCE, the dose-modifying factor was 2.76 ± 0.56. In the PFCE plus carbogen plus 30 Gy group, 70% of the animals died on or about Day 60 of metastatic disease with no palpable tumor at the primary site. Similarly in the 20-Gy group, 46% of the animals died on or about Day 60 with no palpable tumors at the primary site. Therefore, 60 days was used as a ceiling point for tumor growth delay, potentially causing an artificial leveling of effect of the 20- and 30-Gy points. In all other groups, all tumors eventually regrew.

The results adding PFCE to fractionated radiation are shown in Chart 2. The radiation was delivered in 3-Gy fractions in the morning and afternoon, and 0.3 ml of Fluosol-DA was administered each morning. Since the half-life of Fluosol-DA is about 12 hr, PFCE was present for each radiation treatment. In all cases, the animals inspired 95% oxygen for 1 hr prior to and during each X-ray fraction. Without PFCE treatment, fractionated radiation is slightly more effective than single-dose radiation. Addition of PFCE to the fractionated radiation treatment protocol produces a dose-modifying effect of 1.6 ± 0.3 compared to fractionated radiation alone.

DISCUSSION

Anemic patients with cervical carcinoma or animals bearing transplantable tumors treated with radiation had increased recurrence rates and lower cure rates than did comparable patients with normal hemoglobin levels (1, 18). Recently, Hirst *et al.*³ examined the dependence of radiosensitivity on hematocrit in mice bearing the KHT tumor. By increasing the hematocrit to 50 to 55%, an increase in radiosensitivity was achieved. No further benefit was obtained by further hypertransfusion.³ Drugs which reduce tumor blood flow increase radioresistance (20). The experiments described in this paper demonstrate that i.v. administration of a PFCE combined with breathing 95% O₂ enhances the radiosensitivity of Lewis lung carcinoma *in vivo*. These moderate doses of PFCE produce fluorocrits of 1.4 to 3.4%. Similar observations have been made in 2 other rodent tumors (36, 37). The small size of the emulsion particles may allow the delivery of oxygen to sites which RBC cannot reach. The decrease in the enhancement of tumor growth delay observed at higher doses of the PFCE may occur because RBC do not effectively off-load oxygen in the presence of high concentrations of oxygenated PFCE (26, 27). Alternatively, higher fluorocrits are associated with transiently lower systemic blood pressure and hemodilution (10, 11, 38). Both conditions may paradoxically decrease oxygen chelated to hemoglobin at the capillary level.

In situations of low hematocrit, the oxygen-carrying capacity of plasma can be demonstrated clearly (26, 27). Plasma, like PFCE, carries oxygen in a manner that varies linearly with the partial pressure of oxygen in the atmosphere (27). Plasma is a much less effective oxygen transport agent than perfluorochemicals. When annex solution was added to the blood volume of mice bearing Lewis lung tumor, additional oxygen-carrying capacity was added to the system, reflected by the enhancement of tumor growth delay observed under hypervolemic conditions. With the further addition of the PFCE particles, an additional 2- to 3-fold enhancement of tumor growth delay was observed.

Fractionation is an effective means by which to deliver radiation therapy, because it allows for tumor reoxygenation between doses, thus reducing the hypoxic cell population (5, 17, 23, 28, 39). The fractionated regimen combined with Fluosol-DA and 95% oxygen breathing was more effective than fractionated therapy alone, but it was less effective than single-dose radiation therapy with PFCE and 95% oxygen breathing (Chart 2). The increased efficacy of the single radiation dose-perfluorochemical-oxygen therapy over its fractionated counterpart can be explained by simple survival curve considerations. The fractionated treatment allowed for sublethal damage repair between radiation doses with each treatment; therefore, even if one postulates equivalent radiosensitization with both treatment schemes, the fractionated program would result in increased tumor cell survival and thus decreased tumor growth delay. Since there was equivalent tumor growth delay observed with the fractionated and single-dose schemes without PFCE, the fractionated program without Fluosol-DA must have resulted in some reduction in the hypoxic fraction with a concomitant increase in radiosensitivity. If not, one would expect that the fractionated program, repairing radiation damage after each dose, would have a decreased tumor growth delay. The growth delays were equal; therefore,

³D. G. Hirst, J. L. Hazlehurst, and J. M. Brown. The effect of alterations in hematocrit on tumor sensitivity to X-rays, submitted for publication.

the increase in survival caused by repair must have been balanced by a decrease caused by reoxygenation. This is a pivotal point. If no significant reoxygenation occurred during the 3-day fractionation period, the finding of increased efficacy with PFCE would be irrelevant to the clinical experience (5, 10, 29). On the other hand, if the tumor completely reoxygenated during the time of fractionation, the addition of an oxygen-carrying radiosensitizer would be unnecessary.

Fluosol-DA is being tested as an oxygen transport agent for use during surgery, after hemorrhage, or to minimize ischemic damage after stroke or myocardial infarction (11-13). Since the overall oxygen concentration of the body is changed very little by the addition of the PFCE, there should not be enhancement of X-ray toxicity to most normal tissues (4, 15). The findings described in this paper are of general importance in cancer therapy, because they demonstrate a nontoxic method of delivering molecular oxygen to tissues. Because the efficacy of several drugs as well as radiation is enhanced by oxygen and oxymimetic substances (25), oxygen-carrying PFCE may provide a means of increasing the effectiveness of radiation therapy and of certain chemotherapeutic agents.

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