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The effect of fluosol-DA and oxygenation status on the activity of cyclophosphamide in vivo*

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Summary. The addition of Fluosol-DA followed by carbogen breathing increased the antitumor effect of cyclophosphamide as measured by both tumor growth delay and tumor cell survival assays. Under air breathing conditions, cyclophosphamide (100 mg/kg) administered i.p. five times on alternate days produced a tumor growth delay in the FSaIIC fibrosarcoma of 8.0±0.8 days. Adding Fluosol-DA (0.3 ml) to treatment with cyclophosphamide followed by carbogen breathing increased tumor growth delay to 11.4±3.6 days, which was not statistically significantly different from that obtained with the drug plus carbogen breathing without Fluosol-DA. As the dose of Fluosol-DA was increased and administered with drug treatment followed by carbogen breathing for 6 h, increasing tumor growth delays of 15.0±1.5 days, 18.1±1.7 days and 29.4±2.2 days were observed with 0.1 ml, 0.2 ml and 0.3 ml Fluosol-DA, respectively. When 0.1 ml Fluosol-DA was administered in combination with cyclophosphamide and immediately followed by 1 h of hyperbaric oxygen (3 atm), a tumor growth delay of 13.7±1.2 days was observed. With 0.2 ml Fluosol-DA under these conditions, the tumor growth delay increased to 23.2±1.6 days, and with 0.3 ml Fluosol-DA the tumor growth delay was 35.6±3.2 days. Single doses of cyclophosphamide with and without Fluosol-DA (0.3 ml) and various conditions of oxygenation were used in an FSaIIC fibrosarcoma tumor cell survival assay. The addition of Fluosol-DA to this single-dose protocol produced a five- to tenfold increase in tumor cell kill compared to air-breathing drug-treated animals. There was no significant difference in the toxic effect of any of the treatment conditions on bone marrow.

Introduction

A broad range of human tumors respond to cyclophosphamide. These include malignant lymphoma, leukemia, multiple myeloma, neuroblastoma, retinoblastoma, sarcoma, and carcinomas of the ovary, testis, breast and lung [1, 42]. Cyclophosphamide may be regarded as a prodrug which undergoes a complex metabolism in vivo. 4-Hydroperoxycyclophosphamide is the initial metabolite formed after administration of cyclophosphamide [4, 21], and phosphoramide mustard is probably the major biologically active alkylating compound derived from cyclophosphamide [13].

As with other anticancer agents, the development of tumor resistance to cyclophosphamide is a clinical problem. Evidence for a major role of aldehyde dehydrogenase in cyclophosphamide resistance has been seen in studies with both L1210 leukemia and P388 leukemia drug-resistant cell lines [9, 19, 30, 31]. Boon and Parsons [3] studied a human melanoma cell line made resistant to cyclophosphamide in vitro, and several other cyclophosphamide-resistant sublines of murine tumors have been developed in vivo [10, 14, 22].

Enhanced tumor growth delay and tumoricidal effects resulting from combination treatment with radiosensitizers and chemotherapeutic agents were first reported in 1980 by Clement et al. [7] and Rose et al. [27]. To date, the alkylating agents melphalan, cyclophosphamide and CCNU have shown the greatest increases in antitumor effect without a concomitant increase in normal tissue toxicity in combination with misonidazole of all the drugs tested [23, 24, 27, 28]. Tannock [32] found that misonidazole enhanced the effect of cyclophosphamide in both large and small KHT tumors. The mechanisms of potentiation of these drugs by misonidazole are currently unknown; however, altered pharmacokinetics, inhibition of DNA damage repair and enhancement in direct cell kill have been implicated [6, 29].

Tannock [33], in the 16/C murine mammary carcinoma, and Hill and Stanley [18], in the B16 melanoma, found no selective cytotoxicity of cyclophosphamide for aerobic or hypoxic cells. Dixon et al. [11] found that cyclophosphamide was less cytotoxic toward hypoxic cells of a transplanted rat tumor. Both in vitro [34] and in vivo [43, 44] studies have reported an additive relationship of cyclophosphamide with radiation. No direct effect of oxygen has been implicated in the cytotoxicity of simple alkylating agents such as melphalan, cyclophosphamide or the nitrosoureas. However, combined treatment with the perfluorochemical emulsion, Fluosol-DA, and oxygen breathing has been shown to enhance both the tumor growth delay and tumor cell killing by melphalan [39, 40] and four nitrosoureas [41] in the FSaIIC fibrosarcoma. We report here the results of tumor growth delay, tumor cell survival and bone marrow toxicity studies with cyclophosphamide combined with Fluosol-DA and various conditions of oxygen breathing.

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Materials and methods

Drugs. Fluosol-DA 20% (Green Cross Corp., Osaka, Japan) was obtained from Alpha Therapeutics Corp. (Los Angeles, Calif.). The stem emulsion consists of 25% (w/v) perfluorochemicals: 7 parts perfluorodecalin; 3 parts perfluorotripropylamine; Pluronic F-68 (2.7%, w/v); yolk phospholipids (0.4%, w/v) as emulsifiers; and glycerol (0.8%, w/v) as a cryoprotective agent. The annex solution (electrolyte/bicarbonate solution) furnishes the preparation with physiological osmolarity. The stem emulsion particles provide a surface area of 1.82×10^8 cm^2/liter available for oxygen diffusion (about 100 times the surface area of erythrocytes in whole blood). The half-life of Fluosol-DA in vivo is about 12 h [16]. Cyclophosphamide (Cytoxan; Mead Johnson, Syracuse, NY) was purchased from the Dana-Farber Cancer Institute pharmacy.

Tumor. The FSaII fibrosarcoma [26] adapted for growth in culture (FSaIIC) [39] was carried in C3H/FeJ male mice (The Jackson Laboratory, Bar Harbor, Me.). For the experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted i.m. into the legs of C3H/FeJ male mice 8–10 weeks of age.

Tumor growth delay experiments. When the tumors were approximately 50 mm^3 in volume, the perfluorochemical emulsion Fluosol-DA (0.1, 0.2 or 0.3 ml) was injected via the tail vein. Immediately afterward, cyclophosphamide (100 mg/kg) was administered by i.p. injection. Treatment was administered every other day for five injections. The animals were then allowed to breathe air or were placed in a circulating atmosphere of 95% O_2/5% CO_2 (carbogen) for 1, 2 or 6 h or were placed in a hyperbaric chamber (Riemers Engineering, Falls Church, VA) filled with 100% oxygen at 2 or 3 atm for 1 h and then removed to air. The progress of each tumor was measured 3 times weekly until it reached a volume of 500 mm^3. Tumor growth delay was calculated as the days taken by each individual tumor to reach 500 mm^3 compared to the untreated controls. Tumor volume was calculated as a hemiellipsoid. Untreated FSaIIC tumors reach 500 mm^3 in 12.2 ± 0.4 days. Each treatment group had seven animals and the experiment was repeated three times. Days of tumor growth delay are the mean ± SEM for the treatment group compared to the controls.

Data analysis. Data on the delay of tumor growth were analyzed with a BASIC program for the Apple II microcomputer. The program derives the best-fit curve for each set of data, then calculates the median, mean, and SEM for individual tumor volumes and the day on which each tumor reached 500 mm^3. Statistical comparisons were made with the Dunn multiple comparisons test.

Tumor excision assay. When the tumors were approximately 50 mm^3 in volume (about 1 week after tumor cell implantation) the perfluorochemical emulsion Fluosol-DA (0.3 ml) was injected via the tail vein. Immediately afterward cyclophosphamide (100, 200 or 300 mg/kg) was administered by i.p. injection. The animals were then allowed to breathe air or were placed in a circulating atmosphere of carbogen for 2 or 6 h or were placed in a hyperbaric chamber filled with 100% oxygen at 2 or 3 atm for 1 h and then removed to air. Mice were killed 24 h after treatment to allow for full expression of drug cytotoxicity and repair of potentially lethal damage and then soaked in 95% ethanol. The tumors were excised under sterile conditions in a laminar flow hood and minced to a fine brei with two scalpels. Four tumors were pooled to make each treatment group. Approximately 0.2–0.3 g tumor brei was used to make each single-cell suspension. All reagents were sterilized with 0.22-μm Millipore membranes and were added aseptically to the tumor cells. Each sample was washed in 20 ml of alpha-minimal essential medium (α-MEM) in a 50-ml centrifuge tube, after which the liquid was gently decanted and discarded. The samples were resuspended in 450 units/ml collagenase (Sigma, St. Louis, Mo.) and 0.1 mg/ml deoxyribonuclease (DNase; Sigma) and incubated for 10 min at 37° C in a shaking water bath. The samples were centrifuged at 200 *g* and the supernatant was discarded. The samples were resuspended as above and incubated for another 15 min at 37° C. One milliliter of 0.1 mg/ml DNase was added and incubation was continued for 5 min at 37° C. The samples were then filtered through two layers of sterile gauze. The samples were washed twice, then resuspended in α-MEM supplemented with 10% FBS. These single-cell suspensions were counted and plated at three different cell concentrations in duplicate for the colony-forming assay. One week later the plates were stained with crystal violet and colonies of more than 50 cells were counted. The untreated tumor cell suspensions had a plating efficiency of 8%–12%. The results are expressed as the surviving fraction ± SEM of cells from treated groups compared to untreated controls.

Bone marrow toxicity. Bone marrow was taken from the same animals used for the tumor excision assay. A pool of marrow from the femurs of two animals was obtained by gently flushing the marrow through a 23-gauge needle using ice-cold McCoy's 5A medium (Grand Island Biological Co.), supplemented with 5% FBS and nonessential amino acids (100×; 2 ml/liter) (Grand Island Biological Co.), sodium pyruvate (50 μg/ml), L-glutamine (146 μg/ml), L-asparagine (8 μg/ml), L-serine (4.2 μg/ml), vitamins (100×; 2 ml/liter), penicillin (50 units/ml) and streptomycin (50 μg/ml) (Grand Island Biological Co.) [25]. The cells were washed and resuspended in supplemented medium. Granulocyte-macrophage colony forming units (CFU-GM) were measured as follows: Bone marrow cells were suspended in supplemented McCoy's 5A medium containing 15% FBS, 0.3% agar (Difco, Detroit, Mich) and 10% conditioned medium as a source of colony-stimulating activity. The colony-stimulating activity supplement was prepared by incubating L-929 mouse fibroblasts (2500 cells/ml; Microbiological Associates, Bethesda, Md.) with 30% FBS in McCoy's 5A medium for 7 days in a humidified 5% CO_2 atmosphere at 37° C. The supernatant containing colony-stimulating activity was obtained by centrifugation of the medium at 10000 *g* for 10 min at 4° C followed by filtration under sterile conditions [5]. The bone marrow cell cultures were incubated for 7 days in a humidified 5% CO_2 atmosphere at 37° C and then fixed with 10% glutaraldehyde. Colonies of at least 50 cells were scored on an Acculite Colony counter (Fisher, Springfield, NJ). The results from three experiments, in which each group was measured at these cell concentrations in duplicate, were averaged. The results are expressed as the surviving fraction of treated groups compared to untreated controls.

Results

The addition of Fluosol-DA followed by carbogen breathing increased the antitumor effect of cyclophosphamide as measured by both tumor growth delay and tumor cell survival assays. Under air-breathing conditions, cyclophosphamide (100 mg/kg) administered i.p. five times on alternate days produced a tumor growth delay in the FSaIIC fibrosarcoma of 8.0±0.8 days. Adding an i.v. injection of 0.1, 0.2 or 0.3 ml Fluosol-DA to this treatment regimen led to no significant change from the tumor growth delay produced by cyclophosphamide alone (Fig. 1). When the cyclophosphamide-treated animals were allowed to breathe carbogen for 2 h immediately following drug administration, a tumor growth delay of 9.0±1.5 days resulted. Adding Fluosol-DA (0.3 ml) to treatment with cyclophosphamide followed by carbogen breathing increased tumor growth delay to 11.4±3.6 days, which was not statistically significantly different from the delay obtained with the drug plus carbogen breathing without Fluosol-DA.

When carbogen breathing was extended to 6 h following administration of cyclophosphamide, a tumor growth delay of 11.7±1.3 days was observed (Fig. 1). With increasing doses of Fluosol-DA administered with drug treatment followed by carbogen breathing for 6 h, increasing tumor growth delays were observed: 15.0±1.5 days, 18.1±1.7 days and 29.4±2.2 days with 0.1 ml, 0.2 ml and 0.3 ml Fluosol-DA respectively. These increases in tumor growth delay are significantly different from those achieved with the drug followed by carbogen breathing for 6 h ($P < 0.01$, $P < 0.001$ and $P < 0.0005$, respectively).

The amount of oxygen carried by Fluosol-DA increases linearly with the partial pressure of oxygen [15–17]; therefore, the effect of hyperbaric oxygen was examined in combination with cyclophosphamide (100 mg/kg) and Fluosol-DA (0.3 ml). As the atmospheric pressure was increased from 1 to 3 atm, there was a marked increase in tumor growth delay (Fig. 2). At normal atmospheric pressure (1 atm; carbogen breathing for 1 h), the tumor growth delay was 11.2±2.5 days; at 2 atm. (100% oxygen, 1 h), the tumor growth delay was 14.8±1.8 days, and at 3 atm. (100% oxygen, 1 h), the tumor growth delay was 35.6±3.2 days. These results were statistically significantly different from those in the air-breathing controls ($P < 0.01$, $P < 0.001$ and $P < 0.0005$, respectively).

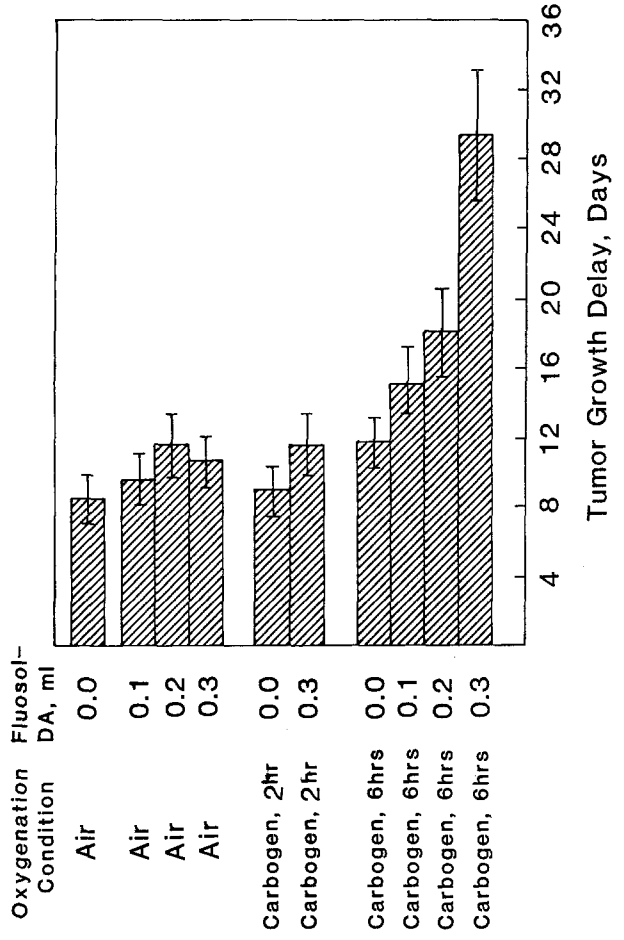

Fig. 1. Growth delay of the FSaIIC fibrosarcoma produced by five doses of cyclophosphamide (100 mg/kg) on alternate days with and without Fluosol-DA (0.1, 0.2 or 0.3 ml) under various conditions of oxygenation. Each point represents the mean ± SEM of three independent experiments with seven animals per group (21 animals per point)

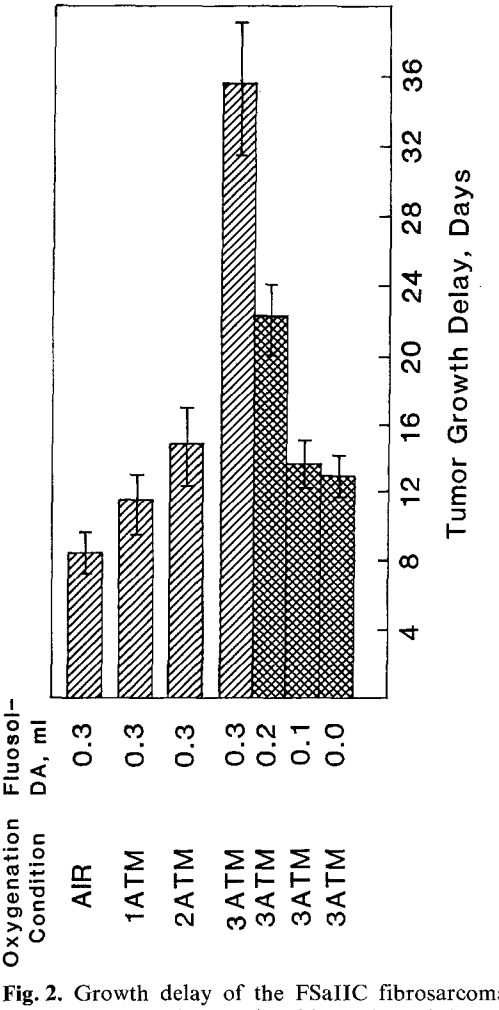

Fig. 2. Growth delay of the FSaIIC fibrosarcoma produced by five doses of cyclophosphamide (100 mg/kg) on alternate days and Fluosol-DA (0.3 ml) under conditions of air breathing, carbogen breathing (1 atm, 2 h) or hyperbaric 100% oxygen (2 or 3 atm, 1 h). Each point represents the mean ±SEM of three independent experiments with seven animals per group (21 animals per point)

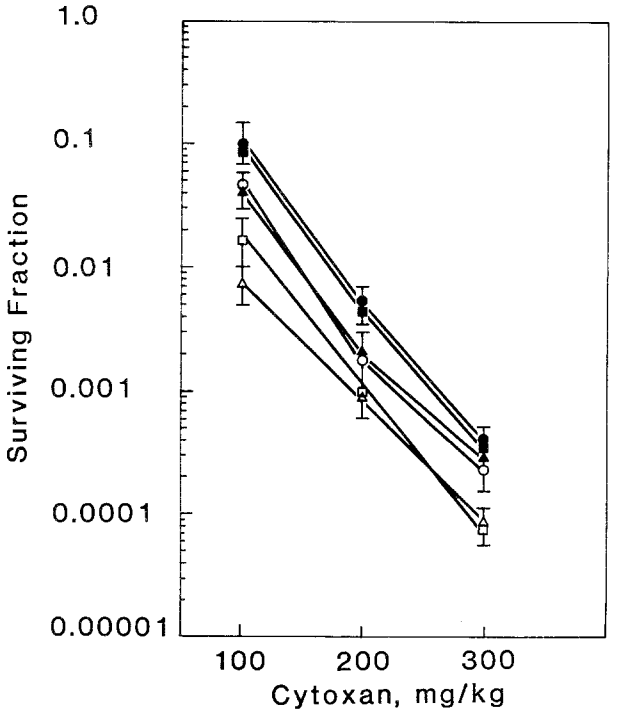

Fig. 3. Survival of cells from FSaIIC tumors treated with various doses of cyclophosphamide (Cytoxan) and Fluosol-DA. The treatment groups are: ● cyclophosphamide and air breathing; ○ cyclophosphamide followed by carbogen for 2 h; ■ cyclophosphamide and Fluosol-DA with air breathing; □ cyclophosphamide and Fluosol-DA followed by carbogen breathing for 6 h; ▲ cyclophosphamide followed by hyperbaric oxygen (3 atm) for 2 h; △ cyclophosphamide and Fluosol-DA followed by hyperbaric oxygen (3 atm) for 1 h. Each point represents the mean of four independent experiments

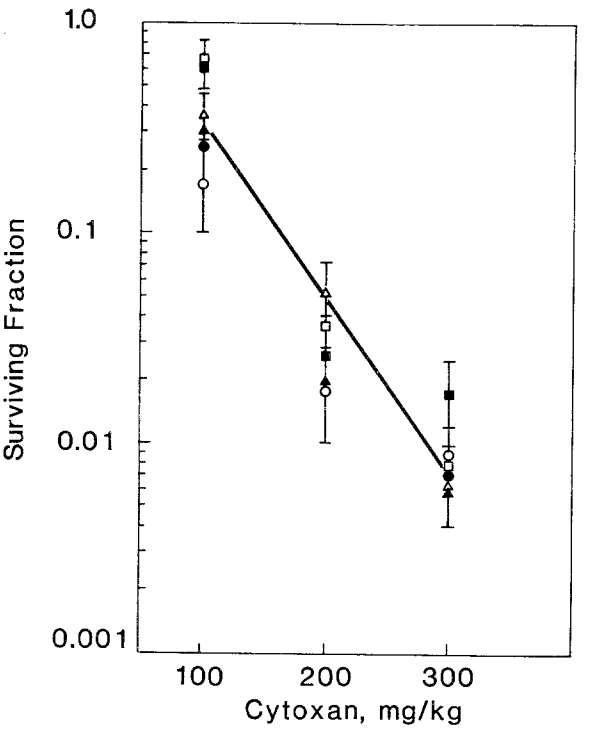

Fig. 4. Survival, measured by the CFU-GM assay, of bone marrow cells from mice treated with various doses of cyclophosphamide (Cytoxan) and Fluosol-DA. The treatment groups are: ● cyclophosphamide and air breathing; ○ cyclophosphamide followed by carbogen for 2 h; ■ cyclophosphamide and Fluosol-DA with air breathing; □ cyclophosphamide and Fluosol-DA followed by carbogen breathing for 6 h; ▲ cyclophosphamide followed by hyperbaric oxygen (3 atm) for 2 h; △ cyclophosphamide and Fluosol-DA followed by hyperbaric oxygen (3 atm) for 1 h. Each point represents the mean of four independent experiments

In the absence of Fluosol-DA, cyclophosphamide treatment followed by 1 h at 3 atm (100% oxygen) resulted in a tumor growth delay of 13.0 ± 1.0 days (Fig. 2). When 0.1 ml Fluosol-DA was administered in combination with cyclophosphamide and immediately followed by 1 h hyperbaric oxygen (3 atm), a tumor growth delay of 13.7 ± 1.2 days was observed. With 0.2 ml Fluosol-DA under these conditions, the tumor growth delay increased to 23.2 ± 1.6 days, and with 0.3 ml Fluosol-DA the tumor growth delay was 35.6 ± 3.2 days. The addition of 0.1 ml Fluosol-DA to drug treatment and hyperbaric oxygen made no difference; however, the addition of 0.2 or 0.3 ml Fluosol-DA produced tumor growth delays statistically significantly different from those with 0.1 ml Fluosol-DA and those without Fluosol-DA ($P < 0.0005$).

Single doses of cyclophosphamide with and without Fluosol-DA (0.3 ml) and various conditions of oxygenation were used in a FSaIIC fibrosarcoma tumor cell survival assay (Fig. 3). Increasing doses of cyclophosphamide continued to produce increasing levels of tumor cell kill following a log-linear curve. Fluosol-DA and air breathing did not change the degree of tumor cell kill by cyclophosphamide. Breathing carbogen for 2 or 6 h or breathing hyperbaric oxygen (3 atm) for 1 h after drug administration produced approximately a twofold increase in tumor cell kill at the two lower doses of cyclophosphamide which disappeared at the highest drug level. The addition of Fluosol-DA to this single-dose protocol produced a five- to tenfold increase in tumor cell kill compared to air-breathing drug-treated animals. This increase in tumor cell killing was maintained over the entire drug dose range.

The toxicity of these cyclophosphamide and Fluosol-DA treatments toward bone marrow under the various oxygenation conditions was assessed by the CFU-GM assay (Fig. 4). There was no significant difference in the toxic effect of any of the treatment conditions on bone marrow.

Discussion

An enhancement in antitumor activity in combination with Fluosol-DA and the breathing of a 95% oxygen atmosphere has been observed with several antineoplastic drugs as well as with X-rays. Of the alkylating agents, significant increases in tumor growth delay have been seen with melphalan [39, 40] busulfan [35], thiotepa (unpublished results) and several nitrosoureas [41] in combination with Fluosol-DA and carbogen breathing for 1 h following drug administration. Under those conditions very little effect was seen with *cis*-diamminedichloroplatinum (II) or cyclophosphamide [35]. Unlike the other alkylating agents, cyclophosphamide is activated to an alkylating species by the liver. Peak alkylating activity levels from this drug in man were found to occur 2–3 h post drug administration, and Juma et al. found the $t_{1/2\beta}$ of the plasma

alkylating activity in man to be 7.7 h [20]. In general, a plateau-like level of plasma alkylating activity is maintained for at lease 6 h after cyclophosphamide administration in man [8]. In the FSaIIC fibrosarcoma, although 1 or 2 h of carbogen breathing post cyclophosphamide and Fluosol-DA administration did not produce a significant effect, extending the carbogenbreathing period to 6 h resulted in a threefold increase in tumor growth delay (Fig. 1).

Perfluorochemicals have excellent oxygen- and carbon dioxide-carrying capacity [15–17]. To fully utilize the oxygen-carrying capacity of these materials, high partial pressures of oxygen are used. Unlike hemoglobin, where oxygen is chelated to the molecule, the solvent action of the perfluorochemicals for oxygen does not involve any kind of chemical or chelating process. The amount of oxygen carried by the perfluorochemicals increases linearly with the partial pressure of oxygen; therefore, the perfluorochemical emulsion will carry more oxygen under hyperbaric conditions [12]. Although hyperbaric conditions were maintained for only 1 h after administration of cyclophosphamide and Fluosol-DA, there was a marked increase in tumor growth delay with increasing atmospheric pressure. At 3 atm, doses of Fluosol-DA corresponding to 8–12 ml/kg were found to be significantly better than a dose corresponding to 4 ml/kg. This is very similar to results obtained with a combination of Fluosol-DA, carbogen breathing and radiation therapy [37, 38].

A difference was seen in the results obtained in the tumor growth delay studies and the tumor cell survival assay. Whereas tumor growth delay increased in both extended carbogen breathing and hyperbaric oxygen breathing conditions, these effects were not seen in the tumor excision assay. Whether carbogen breathing was maintained for 2 or 6 h or oxygen was maintained at 3 atm for 1 h, the tumor cell killing assayed at 24 h post administration of drug and Fluosol-DA was the same (Fig. 3). This dichotomy between the results of these two standard assay methods has occurred before [36] and suggests that Fluosol-DA must have other effects on the tumor and/or host which operate at greater than 24 h post treatment and that these other effects are influenced by the level of oxygenation immediately after drug administration. The tumor cell survival results indicate that the direct cytotoxic effect of cyclophosphamide/Fluosol-DA may be maximal with carbogen breathing for 2 h at five- to tenfold greater cell kill than with air breathing at the therapeutic dose of the drug, since neither extended carbogen breathing (6 h) nor hyperbaric oxygen (1 h, 3 atm) produced significantly greater tumor cell killing than 2 h carbogen. On the other hand, toxicity to bone marrow, which is a well-oxygenated tissue, was not influenced by the presence of Fluosol-DA or any of the various oxygenation conditions.

The effects of hypoxia [2, 34], pH and culture growth stage [2] on cell killing in culture by activated cyclophosphamide have been explored. No major effect of hypoxia was seen, and both normally oxygenated and hypoxic cells were more sensitive to the drug at acidic pH. In some experimental tumors, hypoxic cells have been found to preferentially survive cyclophosphamide therapy [18]. One possible reason for this observed resistance of hypoxic cells may be limited penetration of these cells by the activated species of the drug. Although Fluosol-DA may in some way alter the pharmacodistribution of the activated drug species, this does not explain the involvement of oxygenation status in tumor response to treatment, and implies the existence of other tumor and/or host effects in drug/Fluosol-DA/oxygen therapies. Experiments are in progress to address these issues.

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