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Hematoporphyrin Phototherapy of Neoplastic Tumor Models

by

© Douglas Ross Hamilton

A THESIS
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To Kathy

I couldn't have done it without you.

ABSTRACT

The inhibiting effect of the activation of hematoporphyrin derivative (HpD), by an Argon ion dye laser (wavelength 630 nanometers), was investigated in three different laboratory rats bearing three different types of tumor. In all cases laser phototherapy with HpD significantly interrupted the growth of all neoplasms and prolonged the survival all rats bearing these tumors.

To further understand the behaviour of malignant neoplasms to this type of phototherapy the 9L-glioma in tissue culture was exposed to HpD in various concentrations and different intensities, wavelength and dose of light. The results confirm the the wavelength dependent nature of HpD phototherapy and the importance of incident light intensity when considering dosimetry.

The findings of this study showed that 630 nanometers is the optimal wavelength for fiber optical delivery of light to the site of deep seated tumors where optical attenuation is significantly greater for shorter wavelenghts.

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CHAPTER 1

INTRODUCTION

1.1 HISTORY OF HEMATOPORPHYRIN

The photosensitizing activity of porphyrins is now well known and has been the subject of many studies.

In 1898, Oscar Raab observed that low concentration dyes promoted rapid killing of paramecium upon illumination. The photodynamic properties of these dyes could not be exploited in these experiments without the presence of oxygen. The use of hematoporphyrin in the study of photodynamics came soon after the original studies by Raab. The first study was by Hausmann in 1908, he reported the destruction of paramecium and red blood cells. He also described in some detail, the symptoms of hematoporphyrin sensitized mice upon exposure to light.

The first human experiment using hematoporphyrin was in 1913. This was the famous self-experiment of

Meyer-Betz, in which 200 milligrams of hematoporphyrin was injected intravenously; this resulted in marked erythema and edema of exposed parts of the body.

The majority of these early studies were performed to further the understanding of the group of diseases called "porphyrias". These were diseases which affected the porphyrin metabolism of certain mammals and in some cases, caused photosensitivity or excretion of red urine ("wine urine"). It was in the early twentieth century that researchers realized that some porphyrins had "photodynamic action" on certain exposed parts of the body. The affinity of porphyrins for malignant tissue was reported by Auler and Bayer, and by Figge and his co-workers in independent reports in 1942.

In 1948, Figge and Weiland injected mice with porphyrins and noted red fluorscence in a sarcoma and mammary carcinoma upon exposure to ultraviolet light. One year later they reported observations of fluorescence in areas of trauma, in the greater omentum, and in the lymph nodes of mice injected with hematoporphyrins. In 1955 Rassmussen-Taxdal and his co-workers used intravenous doses of 500 and 1000 milligrams and studied ten cases of malignant neoplasm.

Eight of the ten malignant lesions demonstrated red fluoresence under the activating light. The two lesions that did not fluoresce under appropriate conditions were an ependymoma of the cervical spinal cord and an adenocarcinoma of the prostate gland. They noted maximum differentiation of the neo-plastic tissue from surrounding normal tissue occurring between 24 to 48 hours after injection of hematoporphyrin derivative. It was after these findings that researchers began to investigate the tendency of fluorescent compounds to accumulate in neoplastic tissue and thus provide a method for the detection of malignancy.

Lipsom , Baldes and Olsen (1961) , in a series of reports expanded on the usefulness of hematoporphyrin with respect to their affinity for malignant neoplasm. One of their main findings was that smaller doses could be used. This was significant because hematoporphyrin derivative did have some side effects. These series of reports are the first which examined the absorption and fluorescence spectra of HpD (hematorporphyrin derivative) clearly illustrating its superior fluorescent properties over crude hematoporphyrin.

The next report Lipsom , et al. wrote was the first of several clinical reports dealing with the use of HpD

in humans. Lipsom used hematoporphyrin primarily in endoscopic work, attempting to find fluorescence in neoplasms of the esophagus and tracheo-bronchial tree. This report contains several photographs demonstrating red fluorescence , which is typical of hematoporphyrin fluorescence seen in malignant tissue. The most significant contribution in this report was that apparently, all epithelial neoplasms will fluoresce after intravenous administration of HpD, if the ultraviolet light is delivered in sufficient quantity. A later report by Lipsom , gave further clinical experience with the same HpD, and again the results were identical in that all epithelial neoplasms appeared to have an affinity for HpD and the typical fluorescence occurs if the ultraviolet light source is strong enough.

There are certain clinical drawbacks to the diagnostic use of HpD. Since HpD is injected intravenously, there are certain side effects which result. Some of these symptoms are common with certain types of abnormal porphyrin metabolism. As stated before, experimental porphyrin photosensitization in man was first established in 1913 by Meyer-Betz, who injected himself with 200 milligrams.

of HpD. Most symptoms disappear after a period of several days with erythema lasting longer with continuous exposure to sunlight.

1.2 THE USE OF FLUORESCENT DYES IN THE DETECTION OF MALIGNANT NEOPLASMS

Ever since Hausmann discovered the "photodynamic effects" of hematoporphyrin, the search for photosensitive dyes with similar properties was also being considered. Dougherty (1974), demonstrated that fluorescein behaved similar to HpD and two years later, Thompson, et al. (1974) reported the use of acridine orange and an argon laser to treat mouse epithelial tumors. In every case, the investigator demonstrated a certain degree of localization of the dye in tumor tissues. Several dyes such as fluoresceins (Moor 1953), tetracyclines (Rall 1957, Phillips 1960, Vassar 1960, Sandlow 1963, Sherman 1963), and berberine sulfate (Mellors 1952) have been similarly studied and shown to be preferentially retained in neoplastic tissue as compared to normal tissue. Hematoporphyrin has been widely studied and has been known to accumulate in a variety of animal and human tumors.

Rassmussen-Taxdal, et al. studied the localization and distribution of hematoporphyrin in rats using a Walker 256 carcinosarcoma. Their results showed the tumors to retain 10 milligrams dye per gram of tissue but with no retention in control muscle tissue, 24 hours after the operation. These results were obtained using an intraperitoneal injection of 80 milligrams dye per gram of body weight. Detection in other tissues was checked for by fluorescent techniques and they were found to be negative. In a study by Gregorie, et al., it was found that out of 226 patients with various types of tumors, 76% of those tumors which were malignant (classified histologically) had positive hematoporphyrin fluorescence .

1.3 PROPERTIES OF HPD

After the discovery of the [illegible]ity of certain neoplasms for HpD, the mecha[illegible]of selectivity remained to be elucidated. Usin[illegible] fluorescent microscopy technique, Sanderson (19[illegible]oted that HpD localizes in or on malignant cells, dep[illegible]on their viability. Living tumor cells showed [illegible]uoresc[illegible]e confined to the cytoplasm, whereas non-[illegible]ab[illegible] cells exhibited diffuse fluorescence resulting from [illegible]rence

of HpD to the cell membrane . Accumulation of HpD in viable cells was time and dose dependent, with optimal fluorescence demonstrable 3 to 5 hours after addition of HpD to tumor cell cultures. This showed that the respose of a malignant cell to HpD had two phases. The initial phase is characterized by the granular cytoplasmic fluorescence . This initial phase is then followed by a diffuse cytoplasmic fluorescence. The fluorescence of both phases depended directly on the concentration of HpD and the time of exposure. Normal tissue had little affinity for HpD and any fluorescence present was found to be similar to the fluorescence found by Hughes (1960). The real reasons as to why HpD is selective for neoplasms in situ is not completely understood. There have been many suggestions that it might be in some way selective to the membrane. This was thought after Fleischer (1966) and Swchothorst (1970) found essentially all cellular potassium was released from erythrocyte cells exposed to hematoporphyrin before hemolysis occurred. This suggests early damage to the membrane. Mammalian erythrocytes have been a common experimential cell to study cellular levels of photodynamic action because the photodynamic action appears to be a pure membrane

effect.

The amount of research that has been conducted in the field of phototherapy since 1970 is quite extensive. This thesis will deal with the therapeutic aspects of hematoporphyrin derivative in three animal tumor models and how those results will further understanding of this treatment modality.

CHAPTER 2

HEMATOPORPHYRIN DERIVATIVE

2.1 GENERAL INFORMATION

Commercial hematoporphyrin is mixture of dicarboxylic porphyrins from obtained treated whole blood or from the degradation of hemoglobin. Early studies (prior 1975) yielded inconsistent results when assessing the tumor localizing potential of hematoporphyrin. It is now recognized that the components of hematoporphyrin which localize in tumors are "impurity components" of the crude preparation (Lipsom 1961). Hematoporphyrin derivative (HpD), prepared by acid recrystallization of crude hematoporphyrin (Lipsom 1961) , has been shown to be the porphyrin derivative with the greatest affinity for malignant cells (Dougherty 1978).

The exact molecular composition of this substance is as yet unknown. It is prepared by treating

hematoporphyrin hydrochloride with a glacial-acidic--acid-sulphuric acid mixture and neutralizing to a pH of 6 with 3% sodium acetate. The precipitate is washed and dried and can be stored in the dark at -20 C until required. An injectable solution is made by mixing 1 part HpD with 50 parts by volume of 0.1N sodium hydroxide (Forbes 1980). The pH is brought to 7.2 to 7.4 by addition of 0.1N hydrochloric acid and isotonic by addition of sodium chloride. A final solution containing 5 milligrams/ml is made and sterilized by millipore filtration (Lipsom 1961 ,Gregorie 1968, Dougherty 1975).

Thin layer chromatography (TLC) of HpD has shown that it consists of at least 4 porphyrin components, 50 to 70% of the total fraction consisting of pure hematoporphyrin (Moan 1982). Reverse phase liquid chromatographic analysis also showed HpD consists of at least 4 separate components (Culbreth 1979 ,Kessel 1983).

Pure hematoporphyrin differs from protoporphyrin only in the hydration of two vinyl groups to form hydroxylethyl groups. Other synthetic, fluorescent porphyrins have been shown to localize in neoplastic tissue; among these are tetraphenylporphine-sulfonate

(TPPS4) (Carrano 1978/79), and tetracarboxylphenyl-porphyrin (TCPP) (Musser 1978). The phototherapeutic activity of these derivatives remains unknown. Further, Kohn and Kessel reported the photo destruction of mamalian cells in culture by two porphyrins: NSC 19665 (a dicarboxylic porphyrin) and NSC 407318 (a monocarboxylic porphyrin). The tumor localizing properties of these porphyrins is still unknown.

HpD can be toxic in high doses. The lethal dose in mice is over 180 mg/kg in the dark (Lipsom 1961, Wise 1967). In man the main problem is caused by skin photosensitization (Forbes 1980, Lipsom 1967, Dougherty 1975). No gastrointestinal symptoms have been noted and serum chemistry and blood clotting are unchanged (Perria 1980). The photosensitivity is dose-related and can be avoided by keeping the subject in subdued lighting and by wearing a cream to protect the skin to exposed light. The skin reactions are similar to that of a second degree burn and can occur up to 30 days after administration (Forbes 1980, Dougherty 1976). Hematoporphyrin derivative accumulates preferentially in tumors when administered to tumor bearing animals (Lipsom 1961, Diamond 1972,

Kelly 1975, Dougherty 1975, Carpenter 1977, Gomer 1979, Winkleman 1970, Kessel 1982, Christensen 1981) or human subjects (Kelly 1976, Zawirsaka 1979, Dougherty 1978/1979, Kinsey 1978, Rassmussen-Taxdal 1955, Forbes 1980 , Moan 1982, Kessel 1983).

The excitation spectrum of pure hematoporphyrin in aqueous solution varies with the solvent, the pH and temperature. In saline the excitation peak is at 396 nanometers but in serum it is shifted to between 400 and 407 nanometers. (Lipsom 1960). The fluorescence also depends on the solvent, pH and temperature. fluorescence can also be quenched by oxygen, some ions and by coloured materials.

When illuminated with light in the violet region of the spectrum (maximum excitation at 400-407 nanometers), HpD fluoreseces emitting an orange red color (600-700 nanometers). Selective uptake of HpD in tumors, monitored by illumination with violet or UV light, becomes evident 4 to 48 hours after administration (Diamond 1972) in doses of 2 to 5 mg/kg. In general, the fluorescence was greater at the tumor periphery and where malignant cells invade normal tissue (Lipsom 1961, Dougherty 1975, Carpenter 1977) and necrotic areas of the tumor fluoresced the least

(Carpenter 1977).

The concentration of HpD was 9 to 10 times greater in animal tumors than in contiguous muscle or epithelium 24 to 48 hours after administration (Dougherty 1975, Winkleman 1970,). In some instances, lymphatic tissue (Kelly 1975, Rassmussen-Taxdal 1955), vascular tissues (Kelly 1975), and connective tissues (Kelly 1975) within hours fluoresced more brilliantly than the rest of the tumor. HpD is also localized in human lymphatic tissue, as well as traumatized tissues (Rassmussen-Taxdal 1955). Ulceration and blood can mask the fluorescence of HpD (Carpenter 1977, Gomer 1979), thus it is sometimes difficult to observe fluorescence in spleen , kidney and liver because of their rich blood supply. HpD has been shown to fluoresce in pre-malignant bladder epithelium in human subjects (Kelly 1975), whilst normal bladder epithilium in dogs will not when HpD is applied topically or systemically (Hamilton 1981).

Studies performed using Carbon-14 labeled HpD and tritiated HpD injected intraperitoneallly in mice bearing subcutaneous transplantable mammary carcinoma indicated that the porphyrin is present in liver , kidney and spleen in greater concentrations than in the

tumor at all times between 24 and 72 hours post-injection (Gomer 1979). However, concentrations of HpD in muscle and skin were less than in tumor at all times (between 24 and 72 hours). Concentrations of HpD in the lung were less than in tumor at 24 hours but equal to those in tumor at 72 hours. The chemical identity of the labelled compounds present in tumor and tissues was not determined. Interestingly, 24 hours after injection of HpD, fluorescence (under blue light) was observed in and around the tumor mass only and no fluorescence was observed in any other tissue, including the liver and spleen (Gomer 1979).

Two synthetic porphyrins, TPPS4 and TCPP showed a high affinity for murine tumors (sarcoma and solid 1210) relative to non-malignant tissues (Carrano 1978), being present at concentrations in tumor 10 times greater than in muscle 96 hours after injection. At that time, however, concentrations of TPPS4 in the kidney, the organ through which this chemical is cleared, were greater than those in tumor or other organs (Carrano 1977).

2.2 BASIS OF TUMOR LOCALIZING ABILITY OF HPD

Although the selective accumulation of HpD in tumors is well documented, few studies have investigated the basis of this important phenomenon. Sanderson (1979) and his colleagues have reported that the fluorescence of HpD associated with neoplastic cervical cells in vitro was bright red-orange and lasted several minutes after exposure to light. Normal cervical cells showed less affinity for HpD and the fluorescence of HpD associated with these cells was pale and lasted only a few seconds upon exposure to light. Currano and his colleagues studied the uptake of another selective porphyrin, TPPS4, by a normal and an oncogenic (Hep-2) cell line. The normal cell line was basicallly impermeable to the porphyrin but a slow transport process was observed which was shown to be mediated and dependent upon intact production of cellular energy. The oncogenic cell line, Hep-2, took up TPPS4 rapidly and the process was non-linear reaching a plateau after two hours. A dose-response study indicated low affinity (passive diffusion) and high affinity (mediated) uptake processes. It was sugested by the authors that the oncogenic cell type is "leaky" to the porphyrin, a factor which could

contribute to the localizing ability of TPPS4 in tumors. This latter study, limited to only one type of normal and oncogenic cell, provides little information on the role, if any, of uptake processes, and diffusion in particular, in the localization of porphyrins. It is interesting that HpD does not influence the ability of tumors to accumulate met allic radionuclides, such as Gallium-67 , Iron-59 and Zinc-65 which localize preferentially in tumors (Phillips 1978).

Experiments performed using radio-labelled HpD indicated that, although concentrations of the prophyrin were greater in mouse spleen , liver and kidney than in mammary carcinoma (transplanted subcutaneously) 24 hours post-injection, intense fluorescence was detectable in the tumors at that time (Gomer 1979). It was suggested that fluorescence is quenched in the blood-rich organs.

Moan and Christensen (1982) found evidence that the main components in HpD which played a major role in photo-sensitization were those which are least polar. They suggested that another factor of importance to be considered in HpD cellular uptake was the tendency of porphyrins to dimerize and aggregate in aqueous solutions. Kessel and Chou (1983) used

non-aqueous gel exclusion and reverse phase liquid chromatography to conclude that porphyrin localization is mediated by hematoporphyrin components which are the most hydrophobic in the preparation and that this apparent hydrophobicity may be derived from a hydrogen bonding phenomenon, rather than from the absence of hydrophilic functional groups. Brief incubations of the more hydrophilic agents of HpD to cells showed poor porphyrin accumulation and therefore poor photo-sensitization (Kessel et al. 1982, Kessel and Kohn 1982, Kohn et al. 1979). Kessel (1982) suggested hematoporphyrin crosses the cell membrane with difficulty and that tumor localization can result from intracellular conversion of a permeable HpD component to a poorly diffusible product such as hematoporphyrin. Reverse phase analysis showed that HpD is complex mixture containing the original starting material HP, dehydration products (hydroxyethal-vinyl-deuteroporphyrin HVD, protoporphyrin PP) and other unidentified components (Kessel 1983). The hypothesis that the more hydrophobic components of HpD are accumulated by neoplastic cells was supported when no detectable uptake of the highly polar sulfur (S-35) labeled sulfonate porphryin product was observed

(Kessel 1983). Studies in the mouse , using radioactive HpD (Gomer and Dougherty 1980) suggest the retention of at least 50% of the total injected material for 48 hr.. Kessel (1982) found that and improved tumor:skin ratio could be obtained by using the more hydrophobic components from HpD. Dougherty (1982) separated porphyrin aggregates present in aqueous solution and suggested that these might be more suited for phototherapy since the "localizing" components of HpD are highly aggregated in aqueous solution. These recent advances in the determination of the main photosensitizing components of HpD (Bonnett et al. 1980, Moan et al. 1982, Dougherty et al. 1983) have led to the refinement of HpD which are the more purified porphyrin aggregates which composed approximately 40 percent of the original HpD.

Mew et al. (1983) was able to conjugate HpD to tumor specific monoclonal antibodies. Female mice (DBA/2J or B6D2F Jackson Laboratory) were innoculated with M1 rhadomyosarcoma and treated with Anti-M-hematoporphyrin conjugates intravenously. The tumor growth was suppressed only in those groups treated with the conjugated HpD. This procedure has opened up a new area of phototherapy research where a

more tumor specific destruction can be achieved and the resultant systemic toxicity is reduced.

2.3 HEMATOPORPHYRIN DERIVATIVE AND PHOTOTHERAPY

2.3.1 Description Of The Photodynamic Phenomenon

HpD is a photosensitizer of the photodynamic type; that is, it causes the photo-oxidative damage of biomolecules, macromolecules and small molecules exposed to light in the presence of oxygen (Dougherty 1976). The photo-dynamic process involves the following sequence:

Illumination of porphyrins yield sequentially the excited singlet states and triplet states of the dye. The sensitizer in the triplet state then reacts by two major pathways -

1. Interaction with another molecule by a hydrogen or electron transfer process, producing reactive radicals and/or

2. Transferring its energy primarily to molecular oxygen to yield ground state dye and excited singlet state oxygen, a reactive species which in turn oxidizes various substrates.

HpD in cells shows a broad absorption in the red region with a peak in the red at 630 nm (Dougherty 1975). Murine tumor cells exposed to 5x10-4 molar HpD in vitro and illuminated with light at 630 nm (1 milliwatts/square centimeters) were killed (99.9%) after 10 minutes (Dougherty 1975/1976); 1 to 3 billion quanta per cell were thus required to induce cell death . In another study, the minimum requirement for 100% kill of neuroblastoma cells exposed in vitro to 2x10-5 molar HpD was 6 minutes of exposure to white light intesities of 0.06 milliwatts per square centimeter. The presence of serum greatly reduces the extent of cell kill obtained by light exposure at a certain HpD concentration (Sery 1979, Moan 1979). HpD is known to bind to serum proteins (Sery 1979, Moan 1979), possibly to hemopexin (a glycoprotein involved in the transport of circulating prophyrins to the liver for elimination). Cellular uptake of HpD is reduced up to 75% due to serum protein binding in the presence of 10% serum (Moan 1979). Experiments performed in vitro have provided evidence that cellular mechanisms operate which repair sublethal damage induced by phototherapy (Dougherty 1975), as has been shown with the use of high energy irradiation. This contention is supported

by the demonstration that:

1. A shoulder exists on curves of percent survival versus time of illumination of HpD-treated cells (Dougherty 1975, Moan 1979, Christensen 1979, Granelli 1975) and

2. Cell kill is more efficient for all populations irradiated at 4 degrees centigrade (a temperature at which repair mechanisms do not operate) than at 37 degrees (Moan 1979).

The repair has been shown to take place either during or immediately after irradiation (Moan 1979). Further, if illumination of HpD-treated cells takes place in fractionated doses, cell kill is not equal to that obtained with a single course of light exposure (Moan 1979). Increased membrane fluidity , as observed in illuminated liposomes (Delmelle 1978) , may favour the uptake of more HpD and greater sensitivity to light in subsequent illuminations. Finally, retinoblastoma and glioma cells washed free of dye required longer periods of light exposure in vitro to be destroyed (Sery 1979, Granelli 1975). Human bladder carcinomas growing subcutaneously in immunosuppressed mice were severely damaged by treatment with HpD and light (Kelly

1975); normal bladder tissue was relatively resistant to identical treatment. Further, combination treatment with HpD and light produced a 48% total cure rate for mice bearing a spontaneous mammary carcinoma and the transplanted SMT-F tumor and for rats with the Walker 256 carcinosarcoma and a carcinogen-induced mammary carcinoma (Dougherty 1975). These encouraging results in laboratory animals have been substantiated in the clinical environments (Kelly 1976, Dougherty 1975/1978, Forbes 1980, Hauro 1983). Dougherty and his colleagues (1979), using HpD (2.5 mg/kg administered intravenously) activated by red light effectively controlled local and regional chest wall recurrences of breast carcinoma. In another study (Dougherty 1978), the same group obtained complete or partial remissions in 111 or 113 cutaneous or sub-cutaneous malignant tumors (carcinoma of breast , colon , prostate , squamous cell , basal cell and endometrium ; malignant melanoma , chondrosarcoma and angiosarcoma) by treatment with HpD (2.5 mg/kg administered intravenously) followed by irradiation three days later. No type of tumor was found to be completely unresponsive to phototherapy. Also high density illumination of carcinoma of the bladder , following

the administration of HpD and exposure by surgery led to effective tumor destruction (Kelly 1976, Haruo et al. 1983). Haruo et al. (1983) treated 46 superficial bladder tumors in 9 patients using an Argon-ion-dye-laser and systemically administered HpD and found total remission in 5 of 6 patients with tumors less than 1 cm. in size.

2.3.2 Mechanism Of Cell Kill By Photosensitization With Hematoporphyrin Derivative.

Available evidence suggests that, in biological systems, HpD in the excited triplet state reacts according to Pathway II (see Section 3.3.1); that is, it transfers its energy to ground state molecular oxygen, a reactive species which then oxidizes cellular molecular structures (Dougherty 1976, Moan 1979, Weishaupt 1970). These conclusions were obtained by demonstrating that:

1. Deoxygenation prevents cell kill by HpD and light (Dougherty 1976,Moan 1979)

2. Cell kill by HpD activation is more effective in heavy water (it is known that the lifetime of singlet oxygen is about 10 times longer in heavy water (Moan 1979).

3. The photodynamic inactivation of TA-3 mouse mammary carcinoma cells was completely inhibited by 1,3-diphenyl-isobenzofuran, an effective singlet oxygen trapping agent (Weishaupt 1976).

In general molecular oxygen is in its triplet state (O-3), but most chemical compounds are in the singlet state (O-1), and the reaction between triplet state oxygen and singlet state compounds seldom happens. Therefore, non-specific oxygen peroxidation of subcellular organelles is due mostly to

1. Super Oxide anion radical

2. Hydrogen Peroxide

3. Singlet Oxygen

4. Hydroxyl radical generated by Hydrogen Peroxide or Super Oxide

When saturated fatty acids are peroxidized singlet oxygen or a hydroxyl radical, hydroperoxide is

generated. The hydroperoxide is also regarded as active oxygen because this induces further lipid peroxidation by radical chain reactions (Asada 1978).

At the cellular level, the photodynamic process results in the oxidation of aminoacids, primarily histidine and possibly tyrosine and trytophan (Dubbelman 1978). A reaction between free amino groups and photoxidation products of the above mentioned amino acids results in the cross-linking of membrane proteins (Dubbelman 1978), as has been shown by electrophoretic analysis (Kohn 1979, Dubbelman 1978, Girotti 1978/1979). Further evidence indicates that the formation of disulfide bonds is not involved in the cross-linking of membrane proteins (Dubbelman 1978, Girotti 1978/1979). Conflicting evidence suggests that lipid peroxidation (a mechanism sometimes involving Pathway I, see Section 3.3.1), may not (Girotti 1979) mediate the observed protein damage. Girotti has observed that BHT and N,N1-diphenyl-p- phhenylenediamine, agents which inhibit lipid peroxidation, did not influence membrane protein cross-linking.

Torinuki (1980) has implicated singlet oxygen as the mechanism by which HpD destroys lysosomal membranes and

the formation of liperoxide. Lysosomal enzymes, acid-phophotase and beta-glucuronidase were released from rat liver lysosomes when exposed to 400 nanometer radiation in the presence of HpD. Torinuki prevented this release by the introduction of vitamin-E, diazabicyclo-octane, bovine serumalbumin, superoxide dismutase or d-Mannitol. Reduction of nitroblue-tetrazolium was not observed when hematoporpyrin was excited by 400 nanometer radiation. It was therefore deduced that superoxide anion radical was not primarily generated.

It has been suggested by Patterson (1981) that HpD phototherapy produces intracellular superoxide and that the destruction of neoplastics cells is mediated by the lack of mitocondrial superoxide disutase. Patterson's in vitro findings, coupled with the known deficiency of mitrocondrial superoxide dismutase in tumor cells, supports the hypothesis that the tumorcidal effect of HpD phototherapy may be secondary to superoxide production. Patterson suggested that the HpD is unstable in the presence of light and that upon exposure its porphyrin ring breaks down. They also suggest that 02 is reduced to superoxide during this oxidation since nitrotetrazolium blue, which is reduced

by superoxide , is reduced under these conditions. The amount of nitrotetrazolium blue reduced was decreased in the presence of superoxide dismutase thus implicating superoxide as the entity responsible for tumor destruction in HpD phototherapy. The enzyme superoxide dismutase is believed to be in all oxygen metabolizing cells but lacking in most anaerobes. This is thought to be because its physiological function is to provide a defence against the potential damaging activivies of superoxide radical generated by aerobic metabolic reactions (Oberly and Buttner 1979). It has also been thought that HpD production of hydrogen peroxide can inhibited by superoxide dismutase since superoxide is a precursor in some hydrogen peroxide production pathways.

Harihan et al. (1980) established a method to monitor the production of hydroxyl radicals in cell system exposed to HpD and red light. It has suggested that the production of hydroxyl radical may yet be another mode of HpD photosensitized induced cell death.

Numerous membrane-associated functions are altered as a consequence of the photodynamic treatment of cells, including inactivation of membrane-bound enzymes (Sery 1979, Kessel 1977) [e.g. 5' 1-nucleostidase],

and interference with the membrane transport of nucleosides (Kessel 1977) and amino acids (Kohn 1979,Kessel 1977). Perturbation of the permeability barriers to actinomycin C uptake was also a consequence of illumination of porphyrin-treated (5x10-6M) leukemia L1210 cells in vitro (Kessel 1977), as was an enhanced cellular binding of the fluorogenic dye 8-anilino-1-naphthalene sulfonate. The latter observation could be a consequence of the observed reduction of all surface electronegativity (i.e. increased cell surface hydrophobicity) of cells treated with HpD and light in vitro (Kohn 1979, Kessel 1977). At higher porphyrin concentrations (10 times greater), inactivation of cytosolic nucleoside kinase and inhibition of sugar transport was evident (Kessel 1977). Photodynamic damage to plasma membranes may cause an increase in membrane fluidity (Delmelle 1978), a phenomenon which could explain some of the membrane alterations discussed above.

Incorporation of nucleotide precursors into DNA was unaffected by the illumination of cells incubated in the presence of HpD concentrations which reduced cell viability (Allison 1964).

Evidence provided by the morphological examination

of HpD-treated cells following exposure to light further supports the contention that damage to the plasma membrane mediates, at least in part, cell death. Following illumination of HpD-treated cells in vitro, blebs appeared within seconds on the membrane of human NHIK 3025 cells, and the cells began swelling within minutes (Moan 1979). Further, the nucleoli became more distinct and nuclear and plasma membranes soon showed evidence of degradation (Moan 1979).

Subcellular structures, other than the plasma membrane, appear to be involved in the cell killing effect of phototherapy. HpD is known to be concentrated in cells (Dougherty 1976) and granular distribution of HpD-associated fluorescence has been shown in the cytosol (Dougherty 1975, Carpenter 1977). Further, the lysosomes of cells cultured in the presence of a porphyrin (uroporphyrin-I) fluoresced bright red due to the uptake of porphyrin (Allison 1964). Following exposure to light the lysosomal membranes became leaky, as observed by diffuse acid phosphatase staining of the cytosol of unfixed cells, and cell death ensued (Allison 1964/1966).

Allison et al (1966) suggested that photosensitivity

was partially due to damage of lysosomes by free radicals which were generated from porphyrin and light.

It has been suggested that vitamin-E scavanges active oxygen (Asada 1976, Watabiki 1975). Since Vitamin-E and hydrocortisone protected skin cell lysosomes against photodynamic damage in the presence of phylloerythrin , a free radical mechanism has been suggested for this effect (Slater 1966). These experiments suggest to us that photodynamic killing by HpD involves, at least in part, the rupture of cell lysosmes resulting the release of hydrolytic enzymes in the cytosol (Torinuki 1980). The release of hydrolytic enzyme was also prevented with diazabicyclo-octane (Torinuki 1980). It was assumed that the superoxide scavanging property of this substance was responsible. The observation that protoporphyrin caused mitochrondrial dysfunction (Stumpf 1979) might suggest an influence of HpD on mitochrondrial function. Porphyrins are known to bind to the mitochrondrial outer membrane and to be transported into mitochondria by a mechanism dependent upon transmembrane K+ gradient (Koller 1978).

When cells were exposed to HpD, no fluorescence was

observed in the nucleus (Dougherty 1975, Carpenter 1977, Moan 1979). Further, there was no evidence of single strand breaks in DNA as a result of the photodynamic inactivation of in vitro human NHIK 3025 cells, and binding of HpD to pure DNA could not be demonstrated (Moan 1977). However, others have shown that a synthetic porphyrin which localizes in tumors selectively, meso-tetra(4-N-methyl-pyridyl)-porphine tetra-perchlorate, binds to calf thymus DNA by intercalation and by external electrostatic association (Fiel 1979). A variation in cytological responses was demonstrated by Frisch (1976) who divided the photodynamic effect of HP on cells into "TYPE1"and "TYPE2" effects, the former being a membrane effect causing blebs to form on the membrane and rapid cell lysis whilst the later inactivated cells by mechanisms not involving the cell membrane. The TYPE1 effect could be created by incubation of cells in HP after 5 minutes whilst considereably longer times were required to induce the TYPE2 effect. Christensen (1983) incubated NHIK 3025 cells for long periods of time in HpD and found that one fraction is easily removed when washed with a serum rich medium and that another fraction remains bound to cells for a long time. The

easily removed component was found not to contribute to the photosensitivity of the cells whilst the tightly bound component produced a cellular photosensitivity which was proportional to the cellular contents of porphryin. It was also concluded that the photoactivation of the tightly bound fraction of HpD induces less damage to the outer membrane and probably more intracellular damage than irradiation of cells after a short period of exposure with the derivative. By photoactivation of the strongly bound porphyrins the cells did not show any immediate memebrane damage. Little information is avialable on the type of cell damaged inccurred in vivo during HpD phototherapy. Christensen (1983) suggested that since the association between HpD and the tumor cells is probably strong, one might expect that cytological responses typical for the tightly bound fraction will be dominating. However, Bugelski et al. (1981) observed bleb formation near vascular structures 15 minutes after treatment of mouse tumors in vivo.

2.3.3 Cell Cycle Dependency Of Photodynamic Cell Destruction By Hematoporphyrin Derivative.

Experiments have shown that the sensitivity of cells in vitro to photodynamic damage involving HpD varies according to the cell cycle stage: sensitivity is least in early G1 phase, increases through G1 and early S phases to reach a maximum in mid S phase and decreases during late S and G2 phases (Christensen 1979). A greater than 100 times variation was observed between the sensitivity of cells occupying early G1 phase and mid S phase (Chistensen 1979). Results suggest this variation with the cell cycle sensitivity is due to more active cellular mechanisms for the repair of photo-induced damage in G1, G2 and M phases (Christensen 1979).

2.3.4 Pharmacology

Hematoporphyrin was introduced clinically early in this century as an antidepressant (Roxas 1978); it has been replaced in this respect by more modern drugs. Recent experiments suggest that the antidepressant activity of HpD is mediated by 5-hydroxytryptamine (5-HT) or a 5-HT-like action of hematoporphyrin itself

on the hypothalamic-hypophyseal-adrenal axis (Roxas 1978). Hematoporphyrin antagonizes the action of 5-HT at the serotoninergic receptor (Franzone 1978). It is not known which component of hematoporphyrin exerts these actions or whether this component is present in HpD.

Experiments in which HpD is employed to detect or treat cancerous lesions in animals and humans employed doses of 1.5 to 5 mg/kg administered intravenously. Following the i.v. administration of 7 mg/kg hematoporphyrin to human subjects, plasma hematoporphyrin concentrations were 520 ug/dl after one hour and declined over a period of 42 days biphasically indicating existence of at least two pools of hematoporphyrin, with half-life decay times of 16 hours and 12 days (Zalar 1977). Hematoporphyrin binds to plasma proteins, possibly hemopexin (Sery 1979, Moan 1979), and is metabolized in the liver and excreted (via the bile) by the gastrointestinal tract (Kinsey 1978).

Photosensitivity to sunlight is the main adverse reaction to HpD. If the skin of patients who have received HpD is exposed to sunlight, a reaction ranging from redness and mild swelling to blister formation and

extensive swelling results (Dougherty 1978/1978, Zalar 1977), depending on the duration of exposure. This reaction may be seen for 10 to 50 days after administration of HpD. Experiments (Zalar 1977) have shown that administration of B-carotene affords a small degree of protection against this reaction, but is not sufficient to protect the patient. In contrast, results reported by others suggest orally administered B-carotene decreases markedly the photosensitivity of most patients (Matthews-Roth 1972).

Early experiments have suggested that intra-arterial calcium disodium edetate protects mice against the photosensitizing action of HpD (Langhof 1961), as do intra-peritoneal injections of dithioerythritol (Harber 1972). In this respect it is interesting that reduced glutathione impaired protoporphyrin IX sensitized hemolysis (Strom 1977). Under hypoxic conditions, photodynamic damage to tissue is markedly reduced (Langhof 1961). Some evidence, based upon early studies, exists that mice injected subcutaneously with hematoporphyrin and illuminated for an extended period subsequently develop skin tumors. It is not known whether photodynamic treatment can induce skin cancers in humans. Santamaria has reviewed the literature

which suggest that, under certain circumstances, hematoporphyrin may be carcinogenic in laboratory animals. However, there is no evidence to date that HpD is carcinogenic in man under any circumstances.

CHAPTER 3

THE BRAIN TUMOR MODEL

3.1 INTRODUCTION

The study of primary tumors of the nervous system have been complicated by the existence of multiple cell types within a single tumor. While fairly uniform composition may be found in benign intracranial human tumors (meningiomas and schawannomas) or well differentiated gliomas (oligodendrogliomas), many of the most common and biologically active neoplasms are not uniform. In many tumors normal cellular constituents may be intermingled with neoplastic cells. A normal biological characteristic of a glial cell is its ability to insinuate its cellular extention among other neural fibers and processes. This condition seems to be exaggerated in the glioma. An advancing glioma is usually poorly deliniated and often impossible to grossly identify. Within a glioma

multiple cell types may exist. Even a tumor derived from a single glial cell type such as a glioblastoma multiform may have zones of varying morphology.

The amount of DNA present in these cells have been used to correlate certain properties of neoplasms (Lapham 1959). Large or giant cell types in glioblastomas characteristically contain large amounts of DNA per cell and have a low rate of division whilst the small spindle types containing smaller amounts of DNA divide more rapidly. These characteristics which have been known to exist in human tumors can be reproduced in animal models by the use of oncogenic agents (Sehgnan 1939., Zimmerman et al 1943, Diamond 1970, Kirsch 1972).

3.2 TUMOR GROWTH AND MODEL DEVELOPMENT

The growth of a tumor and how its morphology changes depends on its environment. The simple spherical model can be used to explain many of the growth parameters of basic tumor growth. These spherical models can be easily replicated in many experimental animal models using many different cell lines. The predictability of the behaviour of these models make them ideal for

evaluating the effectiveness of a potential treatment modalilty in a controlled enviroment.

A basic spherical tumor can be broken down into a collection of shells. The outermost shell of rapid cell division and high metabolism encloses an intermediate shell of viable cells but with reduced rates of metabolism. The central or inner core of the tumor will contain cells which are hypoxic yet viable to cells which are in various stages of degeneratio: The center if the tumor may just be a necrotic cyst of dead tissue from which the original tumor was derived.

Kinetic studies of the various mitotic phases of these tumors have shown rapid cell division in the periphery of these tumors with a greater proportion of cells in the resting phase in the central region (Wilson et al. 1972). The recognition of such zones may be of significance when considering the eff iveness of HpD in phototherapy. In the glioma there exists evidence that the centrally placed zones behave as if they were in a chronic state of hypoxia (Lehrer 1972). Singlet oxygen trapping in tumor models have been found to negate the effects of phototherapy (Weishaupt et al 1976). It is therefore questionable as to whether phototherapy will be successful in

destroying neoplastic tissues under these conditions.

The glioma found occuring normally in the brain usually has finger-like extensions which infiltrate surrounding brain. Normal and malignant glial cells may line up at the borders of the zone of necrosis and normal tissue, forming a pallisading effect to enclose the tumor. Studies have shown the most viable and rapidly growing outer zone may show fewer differences in biochemical characteristics from normal brain than the more centrally located zones. This property may be significant when evaluating the penetration of a chemoterapeutic agent in this zone and its oncolytic results. The presence of the blood brain barrier may restrict the ability of the agent to communicate with the neoplasm.

3.3 THE BLOOD BRAIN BARRIER

The first evidence that a structural barrier was interposed between blood and brain was based on the exclusion of protein bound dyes after their infusion into the vascular system. The term Blood Brain Barrier was first used by Stein (1921) to describe the inability of certain dyes to stain the nervous system (Lee 1976). This ability to restrict the flow of large

molecules from the lumen of a capillary to the surrounding tissue has raised questions as to the effectivness of HpD phototherapy on primary tumors of the brain.

The growth of primary and secondary tumors of the brain require the stimulation of capillary growth when the tumor size is greater than a few millimeters (Bren 1976). The role of angio-genesis provided by the host enables capillaries to penetrate the tumor and provide it with the necessary metabolites. The formation of these new vessels is mediated by tumor angio-genesis factor (TAF) (Folkman 1971). The most abundant source of TAF is found to be from human glioblastomas (Kelly et al. 1976). These capillary growths vary in accordance to their relationship to the tumor epicenter and the grade of the tumor malignancy. At the periphery of gliomas there is extensive new blood vessel formation with some vessels very similar to those found in normal tissue and some showing various signs of immaturity. The type of alterations seen in these blood vessels include fenestration, wide intra-cellular junction, increase in pinocytotic vessels, and folding luminal surfaces. This alteration in vessel growth causes the break down in the

blood brain barrier. This endothelial proliferation occurs in 95% of the gliomas and in some metastatic tumors (Long 1979).

The ability of HpD to accumulate in an area of neoplastic tissue where the blood brain barrier does not exist has not been disputed. The main concern of most neuro-oncologists researching HpD phototherapy is how well does HpD penetrate those areas of the brain where blood brain barrier exists. In order for the selective retention or concentration of HpD to occur, the porphyrin must communicate with the neoplasm. The glioma's tendency to insinuate itself among normal tissue and processes where the blood brain barrier is intact make it a difficult entity to irradicate using large molecular oncolytic agents. The presence of the blood brain barrier will help restrict the exposure of normal tissue to HpD and therefore increase the concentration ratio of HpD in maliganant tissue to normal tissue. It now seems that the only way to mediate the communication of HpD to glioma at the outer periphery of the tumor is to incur the temporary osmotic alteration of the of the blood brain barrier. Osmotic disruption would enable HpD to penetrate areas of normal tissue where neoplastic growths have not yet

affected the blood brain barrier through the secretion of (TAF). Generally osmotic insult leads to the diffusive movement of large macro-molecules across the barrier and the loss of blood brain barrier integrity is reversed within a few hours (Paulson et al. 1978). In primates such barrier disruptions can be done without apparent neurological damage to the subject (Raport 1972).

3.4 BRAIN ADJACENT TO TUMOR

Although attention of most aspects of phototherapy has been focused on the exchange of HpD between blood and tumor, the effective treatment of intracranial tumors will depend on the properties of brain adjacent to tumor. Relativly little is known about the exchange of chemotheraputic drugs between blood and brain adjacent to tumor (BAT) (Levin 1972). These regions are significantly important since invasive malignant cells in the BAT are in areas of intact blood brain barrier. Since viable rapidly dividing neoplastic tissue can be expected found in areas of the brain where normal capillary permability exists as opposed to areas of increased permeability such as that found in main tumor masses. Findings by Levin

(1975) have suggested that the increased permeability of tumor capillaries creates a concentration gradient which mediates the flow of substances such as sodium, urea and water from the tumor mass into BAT. If this phenomenon exits it may negate the need to incur osmotic insult to the blood brain barrier in order to facilitate the communication of HpD with neoplastic tissue in an area of intact blood brain barrier.

3.5 CURRENT TREATMENT METHODS

Brain cancer is a pernicious and intractable disease that does not respond well to modern treatment modalities. The present average survival time following diagnosis of most malignant brain tumors is limited to months.

One strange characteristic of intrinsic tumors of the nervous system, the majority of which are gliomas, is the rarity with which they spread beyond the nervous system (McDonald et al. 1975). All gliomas are malignant in that they are locally invasive and complete resection is not usually possible. In some cases the tumors may metastasize by means of communication to the ventricle or other areas were cerebrospinal fluid is present. It has been thought

that since these tumors can be confined to the cerebro-spinal axis that radiotherapy might successfully control these tumors. However, the radiation doses needed to irradicate these tumors often exceed the tolerence levels for that of normal tissue. A common approach to treating many of these malignant tumors is combined protocol of chemotherapy and radiotherapy . Most malignant central nervous system tumors are associated with a high degree of morbidity and mortality despite the many advances in chemotherapy and radiotherapy.

Surgery only rarely cures malignant brain tumors. Generally, by the time a proper diagnosis of the tumor is made, invasion of deep seated areas of the brain has already occured. As a consequence, sub-total resection of these tumors is usually the only surgical option available. This in itself internally decompresses intracranial tissue which in some cases and restores function. It can be generally stated surgery performed on a patient with a malignant tumor provides a pallative effect with the possibility of enhancing the response of the neoplasm to other treatment modalities such as radiation therapy and/or chemotherapy .

Tumors of the central nervous system comprise 2 to 5

percent of all tumors (of course these rates are geographically dependent). Eight percent involve the brain and 20 percent involve the spinal cord. Twenty to forty percent of brain tumors are metastatic lesions (lung , breast , kidney , melanoma and the gastro-intestinal tract). In one study 70 percent of cases had multiple deposits (Kahn 1969).

Gliomas make up 50 percent of all primary brain tumors. Glioblastomas comprise 50 percent of all gliomas. They seed most frequently in the cerebrum and occur most frequently between the ages of 40 to 60 years. Gliomas comprise 23 percent of all spinal cord tumors.

CHAPTER 4

TREATING THE 9L GLIOMA IN VITRO

4.1 ABSTRACT

To further understand the behaviour of malignant neoplasms to this type of phototherapy the 9L glioma in tissue culture was exposed to HpD in various concentrations and varying intensities, wavelength and dose of laser light . The results confirm the wavelength dependent nature of HpD phototherapy and the importance of incident intensity when considering dosimetry. The findings of this study showed that 630 nanometers is the optimal wavelength for fiber optical delivery of light to the site of deep seated tumors where optical attenuation is significantly greater for shorter wavelengths.

4.2 IN VITRO TUMOR MODEL

The 9L glioma is a continuous cell culture which grows as a monolayer. Two days previous to light exposure 40,000 cells in 2 milliliters were placed in every second well in a 4 X 6 well culture tray. The primary cell culture was trypsinized, spun down and then resuspended to the appropriate concentration for delivery to the wells. Every second well was populated so there could be no exposures of light from one tray affecting the next populated well. A total of 12 wells were populated per tray. One day before the exposure of the cells to light the medium was exchanged with HpD containig media in all wells containing cells. The HpD (Photophyrin 1 Oncology Research Inc.) was added to the cell medium in concentrations from 10-4 to 10-7 molar. Light treatment was applied to each populated well in various doses intensity and wavelength. The light source was a Krypton-ion-laser or Coherent Inova-20 Argon-ion laser pumping a Rhodamine 6G dye laser . The ouptut of the laser was passed through a 50% beam splitter where 1/2 of the beam went to the target and the remaing 1/2 went to a coherent 210 thermopile laser power meter. The target beam was expanded using a triplet lens and an adjustable

telescope and the resulting beam was collumated using three adjustable shutters (fig. 4.1). The trays were mounted on a table fitted with a tube through which light could pass through the table and the wells could sit in the tube thus isolating the exposed cell from the rest of the cells. The cells not to be exposed were protected with an optical shield which fitted over the top of tray. The shield had one hole, the exact diameter of a well, so the light could pass through the top shield, through the well, and out the bottom of the table. This experimental setup enabled the easy measurement of incident light intensity using a second power meter of the same type before and after exposure. The power meter used after the beam splitter was used to measure output stabilty of the laser system during the exposure of a cell. The second meter was used to calibrate incident intensity before and after each well was exposed. The duration of the exposure was timed using a digitally controlled optical shutter manufactured on site. The wavelength was measured using a (SPEX Inc.) spectrometer and compared against the settings of a calibrated triple plate birefringent intra-cavity filter mounted in the Coherent 599 Dye Laser. The output mode of the laser

was intermittently checked by inspecting the image of the output beam using a concave mirror. The laser mode was kept at TMoo at all times. The expanded beam was truncated so only the inside 30% of the beam was actually used. This assured us of a constant distribution of output power over the whole cross section of the beam. The image of the beam incident to the wells was checked for interference patterns which may arise from misalignment of the optics. This initially presented problems which caused the interference rings of viable and non viable cells in the wells when stained. Since the output power of the dye laser is greater than 1 watt at any wavelength used in this experiment we could afford to lose power in many places so as to deliver a reliable monochromatic source of incident radiation. Since the output bandwidth of the laser is less than 5 nanometers this experimental configuration is a true source of monchromatic light which does not present the wavelength dosimetry complications of filtered white light sources. The absorption spectrum (fig 4.2) of HpD in human serum has five major peaks in the visible region. The selection of the treatment wavelengths were based on this curve. The Argon-ion dye and

Kypton-ion-laser configuration enabled us to examine the dose , intensity and wavelength dependence of HpD phototherapy on the 9L Glioma . The wavelengths chosen were 647, 630, 590, 573 and 514 nanometers. The 647 nanometer output was delivered from a Kypton-ion-laser with red mirror optics and an intra-cavity prism . The 630,590 and 573 outputs were supplied by the Argon-ion-dye-laser configuration using Rhodamine 6G as the dye to be optically pumped. The 514 nm line was supplied by the Argon Ion laser alone using the green optics and an intra-cavity prism . The incident treatment intensities used were 10,30,40 and 100 milliwatts. with doses ranging from 1/2 to 10 joules. The treatment of 6 wells were used for each intensity ,wavelength and dose. Since wavelength sensitivity of the 9L Glioma to HpD phototherapy varied, the applied dose depended on the treatment wavelength (fig. 4.3). The temperature was monitored in wells containing cells which were given maximum intensity and dose using a copper-constantin thermocouple. The temperature in these cells did not rise more than 1 degree centigrade during the light exposure. Upon the wells being exposed they were returned to the incubator for 6 hours. Cell death was determined using the Trypan blue

vital staining technique. In order for a data point to considered relevant data, all six wells must have shown 100% cell death. It was not considered accurate to estimate the fractional survival of these wells using this staining method. The vital stain method enabled us to quickly adjust our dose,intensity and wavelength parameters to establish quickly what was required for 100% kill.

4.3 RESULTS OF THE 9L GLIOMA IN VITRO PHOTOTHERAPY

The wavelength dependence of HpD phototherapy in vitro seems to closely follow the HpD absorption characteristics (fig. 4.2). The decrease in absorbance of HpD in the region of 590 nanometers corresponds directly with an apparent decrease in HpD activation when compared to 630 nm and 573 nm. The lower error bar in all figures represents the highest dose delivered where there was not total cell death as determine by the trypan blue staining method. The HpD concentration which provided the most useful results for comparing the effects of varying wavelength was 10-5 molar (fig 4.3). Concentrations of 10-6 molar HpD required large doses of radiation to obtain 100% kill (8 Joules at 100 milliwatts/square centimeter and 50

Joules at 22 milliwatts/square centimeter). We could not cause 100% cell death at doses less than 100 Joules with intensities of 100 milliwatts/square centimeter (fig. 4.4) using HpD concentration of 10-7 molar. The results of this study are in agreement with the findings of Kinsey (1981) which show that light of wavelengths in the green region of the spectrum are very efficient in killing malignant neoplasms in tissue culture exposed to HpD (fig. 4.5). The intensity dependence of the 9L Glioma is quite pronounced and should be a major consideration when attempting to calculate to total effective dose on a deep seated neoplasm. Since the light intensity from an optical fiber will decrease with distance the total dose needed to irradicate neoplasic tissue will increase with distance away from the fiber. Total integrated dose , in some cases, will not be a reliable method of determining the dose required to destroy neoplastic tissue in vivo.

figure 4.1
In vitro Laser Experimental Setup

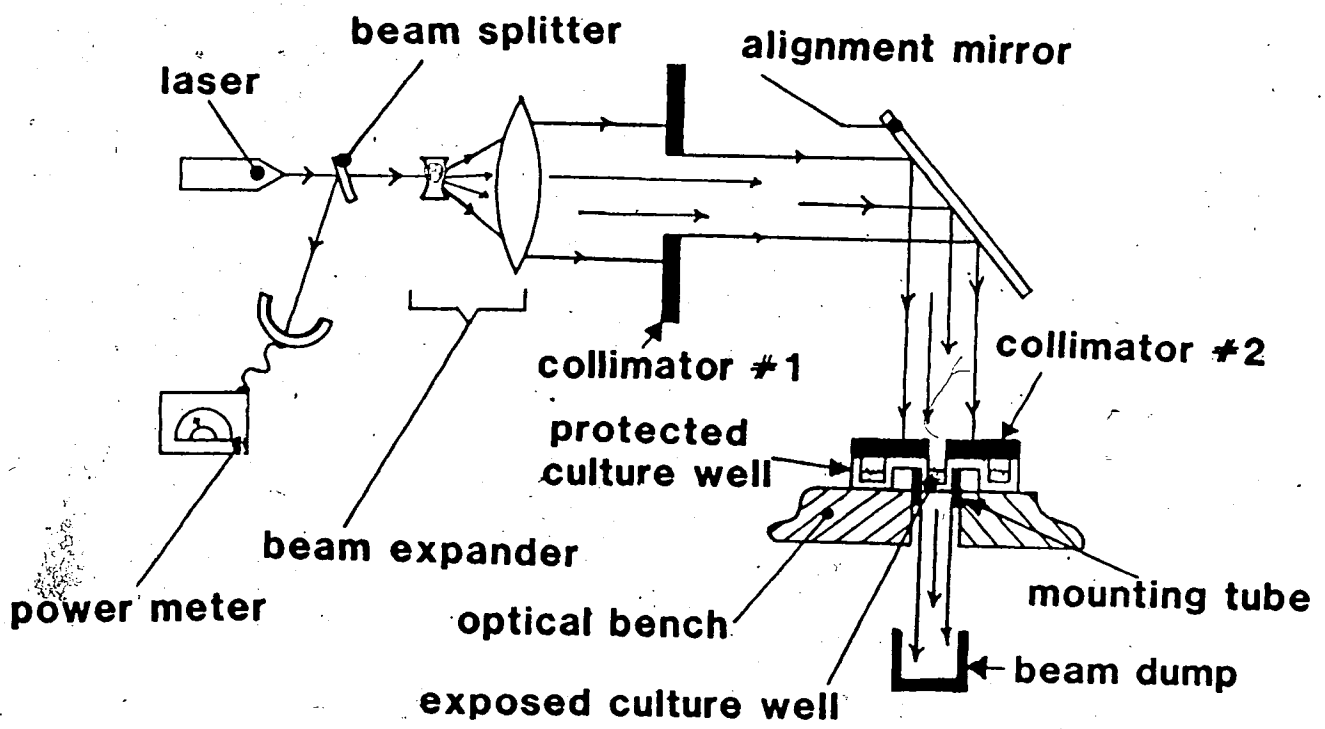
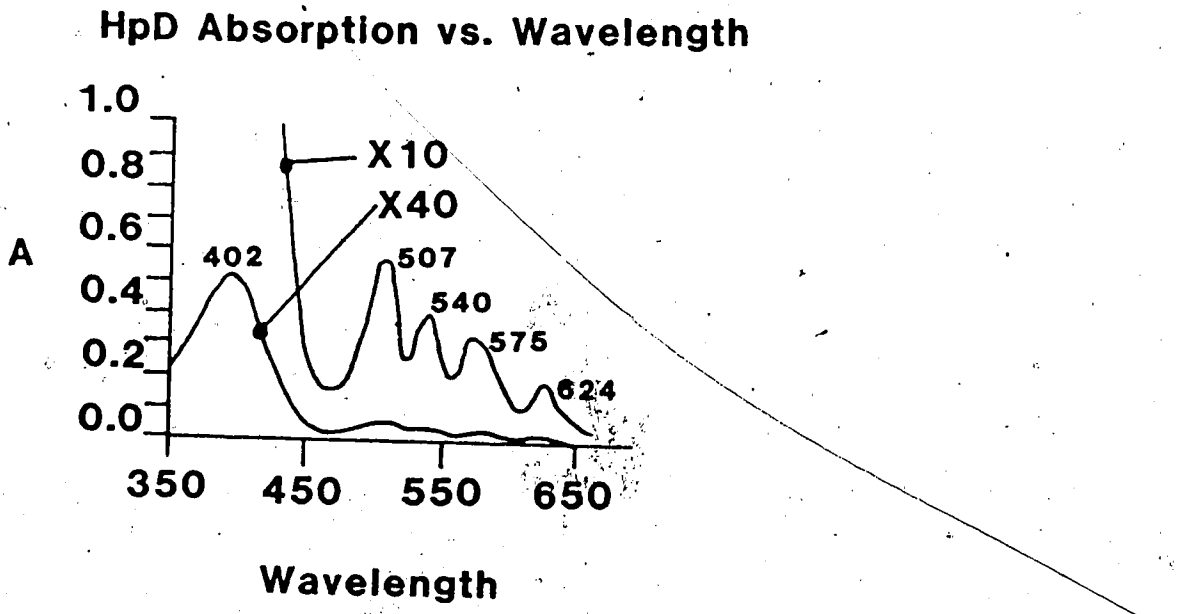


FIGURE 4.2



Absorption spectrum of HpD in human serum showing 5 major absorption peaks, all in the visible. Greatest tissue penetration and least absorption by HpD occur at 624 nm; least tissue penetration and highest HpD absorption is at 402 nm.

figure 4.3

Dose required to produce 100% Kill vs. Wavelength

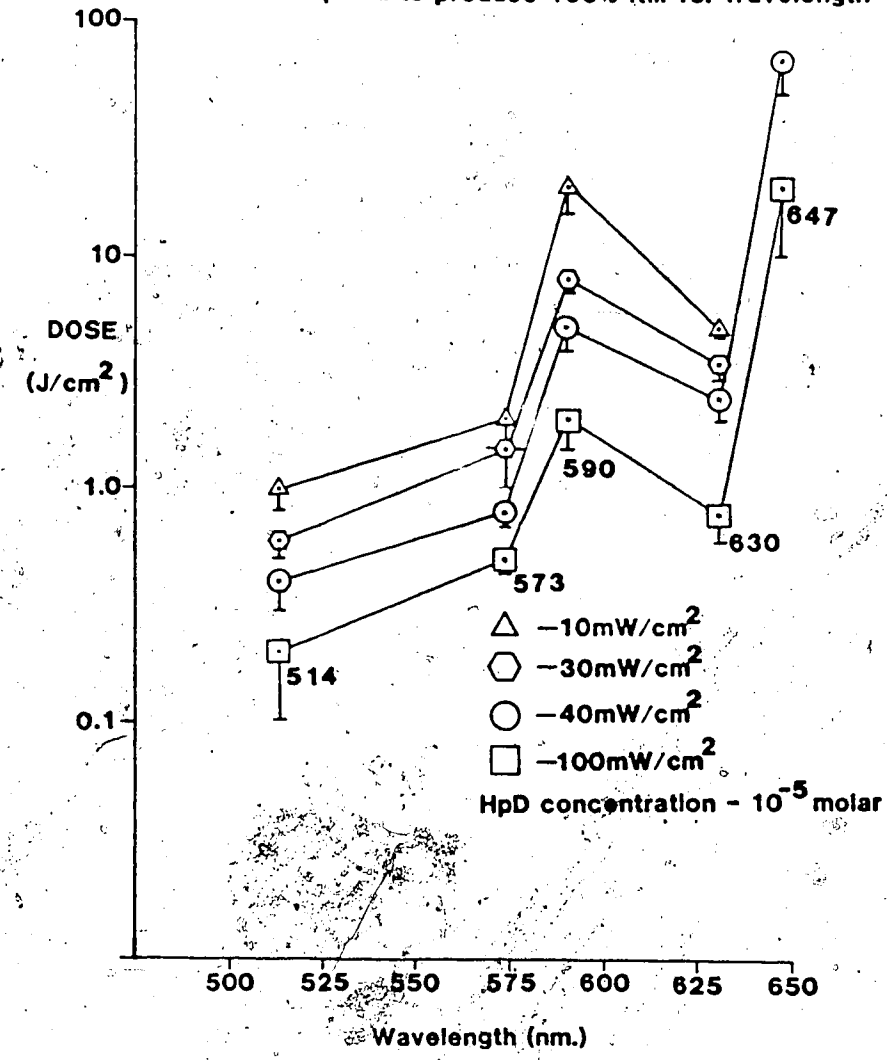


figure 4.4

Dose required to produce 100% Kill

vs.

HpD Concentration

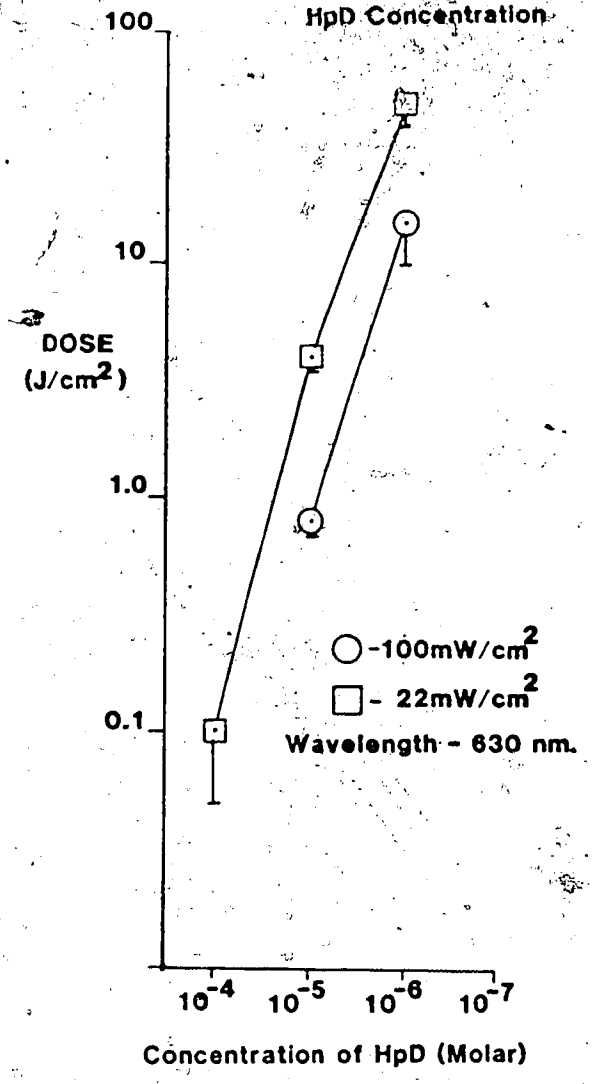
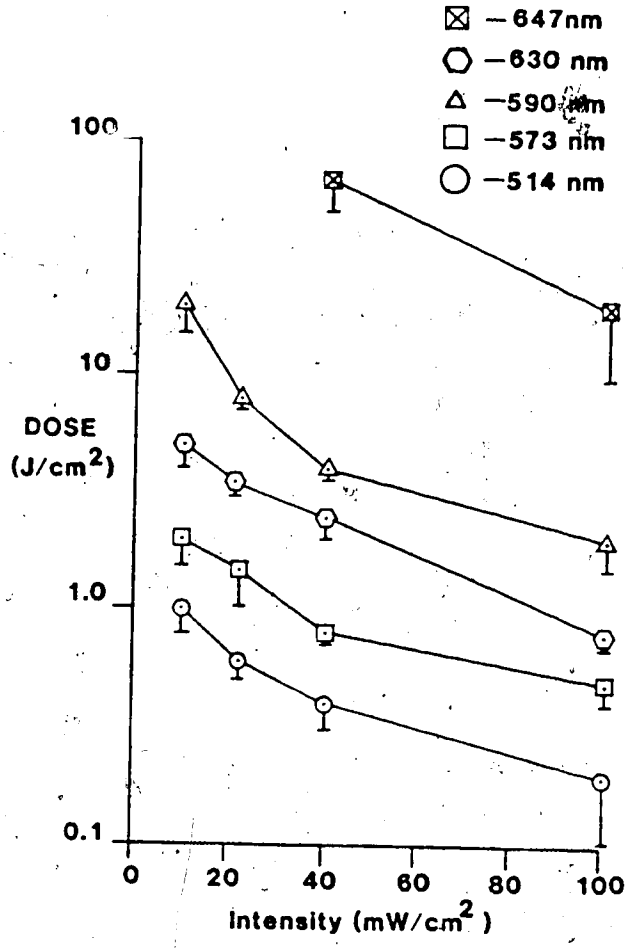


figure 4.5

Dose required to produce 100% Kill
vs.
Incident Intensity



CHAPTER 5

TREATMENT OF THE SUBCUTANEOUS 9L GLIOMA

5.1 ABSTRACT

The inhibiting effect of the activation of hematoporphyrin derivative (HpD), by an Argon-ion-dye-laser (Wavelength 630 nm), was investigated in the Fischer 344 rat bearing subcutaneous implants of the 9L glioma . Tumor growth was monitored by measuring the tumor volume with constant force calipers . In control animals and those that were pretreated with HpD alone (10 mg/kg intraperitoneal injection, i.p.) or laser light alone (2000 Joules at 100 milliwatts), a predictable exponential growth pattern of the cancer was observed. Animals were pretreated with HpD (10 mg/kg by i.p.) 48 hours prior to the fiberoptic , intratumor delivery of laser radiation (2000 Joules at 100 milliwatts), when the tumor had reached a volume of approximately 1.5

cubic centimeters. Seventy-two hours after the combined laser and HpD treatment the tumor underwent coagulation necrosis and the tumor volume rapidly increased from a value of 1.77 +/- 0.5 cubic centimeters (Mean with Standard Deviation, referto fig 5.10), to a value of 5.6 +/- 1.2 cubic centimeters compared with a mean of 3.9 +/- 0.8 cubic centimeters. in animals who had not received laser phototherapy . Rats treated with HpD and laser light survived longer (mean survival 52.7 days +/- 14.6 standard deviation) than the other animals treated with laser alone or HpD alone (mean survival 34.5 days +/- 6.8 standard deviation). Laser phototherapy with HpD significantly interrupts the growth of the highly malignant 9L glioma and prolongs the survival of Fischer 344 rats bearing this neoplasm.

5.2 INTRODUCTION

Dougherty (1981) performed phototherapy on 14 tumors in pet cats and dogs. This was the first paper to address the problems of clinical application of fiber optically delivered laser light to the tumor site. Kinsey et al. (1983) addressed the problems of thermal effects generated from phototherapy and showed

that cures could be effected with light alone when applied in powers high enough to cause thermal damage. Tomio, et al. (1983) treated subcutaneous solid Yoshida Hepatoma using a filtered xenon light source (590 to 690 nanometers) with no recurrance. Granelli (1975) performed subcutaneous tumor treatmnents using a white light source and a lucite rod as the method of delivery.

The first attempt to show that nervous system tumors produced fluorescence after administration of porphyrins was by Rassmussen-Taxdal et al.. In a study looking for fluorescence in various types of human tumors following the intravenous administration they showed that 500 milligrams. of hematoporphyrin produced fluorescence in an olfactory groove menigioma but not in an ependymoma of the cervical cord for reasons which they could not explain. In each case the hematoporphyrin was given by intravenous injection infusion over approximately 6 hours at least 24 hours before surgery. The tumors were then examined for fluorescence under an ultraviolet light. No patient experienced any undesirable effects from this study even though some recieved as much as 1000 milligrams of hematoporphyrin.

Wise and Taxdal have shown that hematoporphyrin will not cross the blood brain barrier but will cross a damaged blood brain barrier. They produced injury to the brains of guinea pigs and dogs either by electro-cautery or needling and administered hematoporphyrin. The brains were examined for fluorescence under ultraviolet light 24 to 72 hours later. In all cases fluorescence was seen in the area of the lesions. This was effect was found to be maximal at 48 hours. fluorescence was also seen in the area postrema and the pituitary stalk, both regions lacking the blood brain barrier. They have also given 5 patients 500 milligrams of hematoporphyrin by intravenous infusion and have been able to delineate the locations and limits of gliomas at surgery.

In 1972 Diamond et al. first reported on the photodynamic destruction of a nervous system tumor. In vitro experiments were conducted using the 9L glioma grown in tissue culture and then incubated in either 10-5 or 10-6 molar hematoporphyrin for 150 minutes in the dark. Some cells were then kept in the dark as a control while others were exposed to light the source being eight 20 watt fluorescent cool lamps. The cells were stained with trypan blue to test for survival. It

was found that 100% cell death could be produced after 50 minutes of exposure at 10-5 molar HpD and 93% death after 120 minutes at 10-6 molar HpD. Those cells kept in the dark showed no destruction.

Diamond's in-vivo experiments invloved the subcutaneous innoculation of 9L glioma cells into the flanks of Fischer 344 rats. After 19 to 21 days the rats were given 10 mg/kg hematoporphyrin derivative intraperitoneally. over the next 3 to 5 days the tumors were exposed to light for a total of 3 to 5 hours. The light source was a high intensity light bulb directed through a lucite rod to intact skin overlying the tumor. Histological examination of the tumors a week later showed massive coagulation necrosis with only a rim of viable cells remaining along the periphery of the tumor most remote from the light source.

In 1975 the same group reported further results with same tumor model. The subcutaneous tumors were irradiated with a more intense light source and the lucite rod was passed through a skin incision so as to apply the tip closer to the tumor. At the time of treatment the tumors measured 1.5 to 2.0 centimeters in diameter. Again any viable cells were seen at the

periphery of the tumor most remote from the light source. Further in vitro studies showed that treated cells still succumbed to phototherapy even though they were washed before exposure.

The first report on the photodynamic action of light and hematoporphyrin derivative on human gliomas was in 1978 (Signorelli et al.). Cells from a glioblastoma multiform were grown in tissue culture . They were incubated with 10-5 molar hematoporphyrin derivative prior to being exposed to light from eleven 15 watt neon light bulbs. Microscopic examinantion after treatment showed degenerative changes when compared to controls. In particular they tended to lose their cytoplasmic processes, showed large nuclear and cytoplasmic vacuoles and the loss of their glifibrillary structure.

Sery reported on the killing of retinoblastoma cells with hematoporphyrin derivative and light. The light source was a 15 watt cool white fluorescent bulb giving an irradiance of 6.0 microwatts/square millimeter. He found that the minimum concentration of hematoporphyrin to produce 100% kill was 1.67 X 10-5 molar. The minimum time to produce cell death at this intensity and hematoporphyrin concentration was 6 minutes. He

was also able to show that by increasing the plasma concentration, the cytotoxic effect was markedly reduced and even heat inactivated serum inhibited the photodynamic effect. This result was thought to be due to binding of the porphyrin to hemopexin .

The first report on the treatment of human patients using phototherapy was by Perria et al. from the University of Genoa in late 1980. Hematoporphyrin derivative was used with a Helium-Neon laser as the light source, delivering 25 milliwatts of power. Nine patients (8 gliomas and 1 sarcoma) were given hematoporphyrin ranging in dose from 2.5 to 10 mg/kg by intravenous infusion between 24 to 96 hours before surgery. During the operation the brain was examined under ultraviolet light and in all cases fluorescence was observed. Standard tumor excisions were performed after which the tumor bed was irradiated with 630 nanometer radiation. Attempts were made to administer 9 Joules/square centimeter to the residual tumor as this was calculated to produce cell death up 1.5 centimeters deep. Examination of some of the brains at post mortem showed coagulation necrosis in the walls of the resected area. No cures were reported and this was thought to be due to residual tumor existing beyond the

range of light.

Forbes reported similar results in Southern Australia (1980). They treated 3 gliomas using hematoporphyrin derivative 5 mg/kg, and light from an Argon-ion-dye-laser using Rhodamine B dye. In one patient they were able to confirm necrosis when they reoperated for recurrence. In each case the light was given during surgery.

Laws et al. (1981) treated 5 patients using 5 mg/kg Hematoporphyrin derivative and fiberoptically delivered laser light from an Argon-Ion-Dye-Laser running at 630 nanometers with a distal fiber output of 250 to 400 milliwatts. The result at that time were inconclusive but seem to show promise for the technique. Laws reported that it took approximately 300 milliwatts of red light from a 400 micron fiber to activate 5 to 10 cubic centimeters. of a subcutaneous murine soft tissue tumor. The Phototherapy Research Group at the University of Alberta has been investigating hematoporphyrin phototherapy in number of different animal tumor models. It has been the policy of the group to examine in more depth the effects of this therapy on predictable models and to investigate methods which might help improve the clinical

application of this therapy. To further investigate the use of phototherapy for the treatment of cancer, HpD was administered to Fischer 344 rats bearing subcutaneous implants of the 9L glioma and the HpD in these tumors was activated by light from an Argon-Ion--Dye-Laser.

5.3 THE DEVELOPMENT OF THE SUBCUTANEOUS TUMOR MODEL

The first subcutaneous tumor model to be used for the evaluation of phototherapy at the University of Alberta was the 9l Glioma . The method used was based on Diamonds (1972, 1975) two papers using the same tumor line. The protocol labeled R3(DH) was devised in May 1982 and was finished by the end of December 1982. In may 1982 it was decided that the effects of fiber-optically delivered laser light in conjunction with HpD on subcutaneous 9L tumors in Fischer 344 rats should be examined. As a consequence of this a protocol was written and the experiment was commenced on May 28, 1982. The concept of R3(DH) was based on the ability to innoculate male Fischer 344 rats with a bolus of 9L tumor cells subcutaneously. It was observed that these tumors grew 95% of the time. The R3(DH) experiments performed to May 28, 1982 were

conducted only to check the reliability of the tumor take and not to see how well the tumor growth pattern fit any particular growth model. It became obvious by the end of July 1982 that the tumor growth patterns observed in R3(DH) could not be modelled to show the objectives set out in May.

Those objectives were:

1. To determine the growth response of the 9L subcutaneous tumor to Hematoprophyrin phototherapy
2. To address the problem of fiber-optic delivery of phototherapeutic radiation.

In order to establish that an alteration in the tumor growth pattern has occurred due to the effects of phototherapy; the normal growth pattern of the tumor had to be established. Upon examining the data collected from May to July 1982 absolutely no predictable parameters in the tumor growth pattern could be isolated except for the 95% tumor take. Even though the tumor take was 95% the tumors did not take at the same time. The experimental procedure required the innoculation of one tumor on each flank of the rat. The injection method used did not control the depth of injection into the skin of the number of cells per

injection. This may have been the cause of the independent seeding times of the tumors. The seeding time is defined by the author to be the time after innoculation required for the tumor to reach a volume 1000 cubic millimeters (The seeding volume) measured using constant force calipers. The difference in seeding times was totally unpredictable and at times presented problems in measurement. On some of the animals one tumor would be so large it could be said that it affected the growth of the other by virtue of its nutritional load on the host. One noticable fact was that if the tumors seeded at approximately the same time their growth was always exponential upon reaching the seeding volume.

The tumors did not always grow with a simple geometry. This presented problems because the shape of the tumor was treated mathematically as if it were an ellipsoid .

In August 1982 an experiment was conducted to test the procedure of 2nd generation transplants and to see if it could produce a more consistent seeding time . The first eight tumors (four rats) had seeding times within 2 days of each other. The model derived to predict the behaviour of the subsequent tumors was a

simple one:

1. Upon reaching a volume of 1000 cubic millliliters the tumor will grow exponentially with time at least until it reaches a volume of 6000 mm3.

2. The difference in the seeding time for the tumors on any rat will not exceed 3 days and the average seeding time will be 10 days +/- 3 days.

3. The tumor geometry will closely resemble an ellipsoid with no diameter differing from the mean of the other two by more than 30 percent.

4. The doubling time for the tumor over the exponential growth period will not be less than 2.5 days or greater than 4.5 days.

On October 1982 10 rats were innoculated with a second generation tumor previously established from a subcutaneous growth in a separate animal. The primary tumor was derived by the subcutaneous injection of a 100 microliter bolus of 100 million cells per milliliter suspension of 9L glioma cells grown in culture. Second generation tumors were never used as a source of transplants for further experiments. A viable piece of tumor was removed from the primary

source (200 to 300 milligra[illegible] surgically inserted subcutaneously via a small skin incision made on the flank of the second host rat. The 10 rats were broken up into two groups of five. The first group was labelled the control group C9 (graphs E3-C9-R1 through R5) and recieved no HpD or light. The second group was labelled C10 (E3-C10-R1 through R5) and was administered 10 milligrams per kilogram of HpD intraperitoneally 48 hours after their tumors had reached a volume of 1000 cubic millimeters (treatment volume). The rats in C10 did not receive any therapeutic light. Group C11 was given transplants in November 1982 (E3-C11-R1 through R5) and upon their tumor volumes reaching a volume of approximatel 1500 mm3. they were given intra-tumoral doses of 2000 Joules of fiber optically delivered 630 nanometer radiation. Group C12 was given tranplants in November 1982 (E3-C12-R1 through R8) and 48 hours previous to the tumor reaching a treatment volume of 2000 mm3. ,each rat received an intraperitoneal injection of 10 mg/kg . The tumors were then treated with 2000 joule doses of 630 nanometer radiation upon reaching the treatment volume.

It was this subcutaneous tumor model t[illegible] was used

the protocols in the treatment of the 9L Glioma, Morris 7777 Hepatoma and the Dunning RH3327 prostate tumors.

5.4 METHOD

The rapidly growing 9L glioma was selected from tissue culture for subcutaneous implantation in male Fischer 344 albino rats (Charles River Laboratories CDF(F344)/CrlBR, mean weight 250 grams). Throughout all studies each animal was allowed free access to Purina Laboratory Chow and water. The 9L glioma is a transplantable tumor (transplantable in Male Fischer 344 rats only) that arose initially in the Fischer 344 rat after treatment of the animal with N-nitrosomethylurea. Upon the tumor being palpable, the untreated subcutaneous glioma grows rapidly, and it reaches a size of about 10% of body weight of the animal within 7 weeks. Initial studies using subcutaneous inoculations derived from tissue culture (100 microliters of 10 million cells per milliliter) of the tumor in both flanks of 6 rats revealed consistent but eccentric tumor growth and death of each animal within 5 weeks. To produce a tumor model with predictable growth characteristics a skin incision was made on each flank of 5 Fischer 344 rats and a piece of

tumor (approximately 2mm in diameter) derived from an already established subcutaneous growth in a separate animal was transplanted. The incision was then closed with fine silk sutures. Tumor growth was monitored daily using constant force calipers (10 grams force). The tumor diameters were measured in three orthogonal planes. The double fold skin thickness was subtracted from each diameter measurement and the resulting corrected diameters were used to calculate an ellipsoidal tumor volume (Fig. 5.0). If any tumor, upon reaching 1 cubic centimeter had one diameter more than 50 percent larger or smaller than the mean the three measured diameters, the rat hosting that tumor was excluded from the study. Each rat was given one tumor on each flank. If the tumors did not reach a volume of 1 cubic centimeter within 3 days of each other, then the rats hosting those tumors were also excluded from the study. The growth of the tumors was found to be exponential between volumes of 1 cubic centimeter and 6.5 cubic centimeters, with a mean doubling time of 3.9 days. The mean doubling time (fig. 5.9) was derived from the linear regression of the logarithm of the tumor volumes against the number of days after reaching 1 cubic centimeter. Since all

tumors behaved in a predictable expontential fashion upon reaching 1cubic centimeter, all growth data was aligned around tumor volumes of 1 cubic centimeter. Thoughout this and subsequent studies, direct implantation of tumors was undertaken and the animals were anesthetized with Halothane during experimental handling .

5.5 - PHOTOTHERAPY

Four groups of male Fischer 344 rats (refer to Table 5.1) received surgical implantation of the 9L Glioma in the manner as previously described.. Each animal had a tumor in the left and right flank. Group C9 (Fig. 5.2) received no treatment and formed a control group. Groups C10-C12 formed the treatment groups (those groups to receive laser light had only one tumor on each rat treated). These tumors were treated when they had reached a volume of 1.5 cubic centimeters. Treatments consisted of the intra-tumor delivery of laser light alone (2000 Joules at 100 milliwatts Group C11, fig 5.4.2 and the C11 tumors not given laser fig. 5.4.1)), HpD given by i.p. injection in doses of 10 mg/kg (Group C10 fig 5.3) alone, and intratumor delivery of laser light (2000 Joules at 100 milliwatts

- group C12, fig 5.5 and the control tumors that did not receive laser light fig 5.6) 48 hr after the i.p. administration of HpD (10 mg/kg). Tumor growth was monitored by measuring the tumor volume with calipers and the length of survival of each animal was noted. Autopsy was performed on at least 2 animals from each group following their death and adjacent muscle and tumor tissue was removed for light microscopy from animals before and after laser phototherapy (Group C12).

The delivery of light to the tumors was performed using a 600 micron, step index fiber (quartz silica) that was implanted along the longest axis of the tumor. Although this technique provided uniform transillumination of all the treated neoplasms, a rapid decrease of transillumination intensity occurred at the tumor surface following the initiation of the laser treatment. This reduction of transillumination was a result of the baking of tissue and blood onto the fiber tip with the consequent light absorption and heating. To overcome this problem the freshly cleaved end of the fiber was rounded off using a oxy-acetylene micro-torch and about 1 centimeter of the distal end of the fiber was treated with acrylic cement (fig 5.8). The cement

dried onto the quartz core of the fiber to produce a mat surface. The refractive index of the acrylic cement closely matched that of the quartz core so that light readily passed from the core into the coating where it was scattered by the rough outer surface. Hence, the light leaked out of the fiber along the length of the treated tip resulting in a cylindrical radiator, not unlike a miniature fluorescent lamp. Before laser light was administered the skin overlying the tumor was shaved and the animal was immoblilzed on a small metal frame. A 21 gauge needle was inserted into the longest axis of the tumor and 500 ul of normal saline was injected into the centre of the tumor. The acrylic coated fiber, through which light was transmitted from the Argon-ion-dye-laser, was inserted into the needle and the needle was then withdrawn to expose the fiber tip. The fiber tip was then located in the approximate middle of the neoplasm and laser light was administered at a rate of 1 Joule every 10 seconds. During light delivery the intratumor and intraperitoneal temperature of each animal was monitored simultaneously with a 30 gauge copper-constantin thermocouple.

5.6 RESULTS

The results of treatment in all five groups of rats are shown in figures 5.2 through 5.6. Neither HpD alone (10 mg/kg i.p. fig.5.3), or laser light alone (2000J at 100 milliwatts fig 5.4.2) caused any measurable effect on normal tumor growth. After the combined administration of HpD (10 mg/kg) by i.p. injection and laser light, there was a rapid increase in tumor size which was generally noticeable upon inspection of the animal within 6 hours of light delivery (Fig. 5.5). The hard, nodular nature of the subcutaneous glioma changed to a soft mass on palpation and the tumor became a cystic swelling. One tumor on each rat in group C12 did not recieve laser therapy. These tumors (figure 5.6) served as laser controls for the treatment group and did not show any deviation from the normal exponential growth observed with group C9. In one animal that had received laser phototherapy tumor tissue leaked when the skin over the treated tumor was punctured with a needle. In 4 of the 8 rats treated with laser phototherapy some ulceration of skin over the tumor was noted. The rats behaved sluggishly for approximately 2 days after treatment but then returned to apparently normal activity. In 4 out of 8

of the animals that received laser phototherapy with HpD the tumor ulcerated and sloughed away. This striking tumoricidal effect with liquefaction of tissue made subsequent measurements of tumor volume difficult but within 2-5 days of treatment, tumor growth appeared to recover. The mean survival time (fig 5.10) after the implantation of the glioma, in the rats treated with light and HpD was significantly longer (48 days +/- 12 standard deviation) than the mean survival in all other groups of animals (31 days +/- 16.5 standard deviation).

Measurement of temperatures in the tumor, 1 to 10 millimeters from the fiber-optic surface, were uniformly less than 3 C above the simultaneously measured, intraperitoneal temperature of the animal. (fig. 5.6) This implied that the resultant light pattern did not give rise to any "hot spots" at the doses of light used in these experiments and thermal injury was not responsible for the observed tumoricidal effects of laser phototherapy with HpD.

Autopsies, performed after death of the animals, showed malignancy which was manifest most often by direct intraperitoneal invasion of the glioma through the muscles of the flank of the rat. Macroscopic

examination of the abdominal and thoracic organs after death did not reveal major differences between each group of animals and the pattern of tumor invasion appeared similar in all groups.

Light microscopy of sections of the biopsies of tumor tissue that had been removed before and after laser phototherapy revealed the development of extensive coagulation necrosis in the treated neoplasm with total cellular disruption that could be attributed to therapy. However, there was some evidence of survival of islands of glioma cells within the tissue sections removed from neoplasms that had been treated with laser phototherapy and HpD.

The finding of surviving glioma tissue matched the observation of tumor recurrence in the animals after treatment. Most necrosis was noted in the middle of the tumors at areas of the tumor adjacent to entry of the fiberoptic light cable. Sections of tumor tissue that were examined after treatment with HpD alone or laser light alone did not reveal large areas of necrosis. Such changes observed with light microscopy were not seen in the other groups of animals that received HpD alone (10 mg/kg) or laser light (2000 J) alone.

5.7 DISCUSSION

Laser phototherapy with HpD significantly interrupts the growth of the highly malignant 91 Glioma and improves survival time in the Fischer 344 rat bearing this tumor. The data confirms the findings of other investigators (Diamond et al., 1972; Dougherty , 1975; Thomson et al., 1974; Dougherty et al., 1975; Forbes et al., 1980) that light activation of phototoxic dyes such as HpD, may have an important role in the treatment of certain malignancies. The toxic effect of HpD phototherapy on neoplasms is not specific since normal tissue containing HpD can be damaged during illumination. However, in the present study light microscopy of muscle tissue adjacent to tumors treated with HpD did not reveal evidence of necrosis. Despite an apparent non-specific localization of HpD, good therapeutic ratios between tumor response and skin response (phototoxic eruptions) have been reported in clinical trials Dougherty et al., 1977; Dougherty et al., 1978). However, severe and persistent phototoxic reactions to hematoporphyrin administration can be anticipated in a significant proportion of patients, thereby limiting the clinical use of phototherapy (Zalar et al., 1977). The rats used in

this experiment did not show any adverse reactions to the injection of HpD even though they were exposed to normal lighting. First attempts to treat malignant brain tumors using phototherapy (Perria 1980 , Laws et al 1981, Forbes et al. 1981) were inconclusive but proved that the application of activating light to a tumor site in the brain is possible. It would seem after these studies that more investigation into HpD-Light dosimetry will be required. Normal tissue adjacent to a neoplasm could be severly damaged if the interval between HpD administration and light exposure is not optimal. In addition, the therapeutic ratio obtained during phototherapy depends to a large extent on the location of the tumor. This may be significant for tumors of the nervous system where the blood brain barrier exists. The glioma's ability to insinuate itself amongst normal tissue and processes make it a difficult entity to irradicate using large molecular oncolytic agents. The blood brain barrier can be intact in areas of brain adjacent to tumor and therefore may present problems when attempting to expose tumor to certain chemotherapeutic drugs. The permeability characteristics of brain adjacent to tumor for HpD may have to be further investigated to

determine the feasibilty of phototherapy in treating malignant tumors of the nervous system. Temporary osmotic insult to the blood brain barrier might be neccessary to facillitate the communication of HpD to tumor. It would appear that more localized delivery of HpD to tumors by irrigation or by conjugation with a tumor seeking compound such a monoclonal antibody or a lectin has potential to reduce toxicity to normal tissue (Dougherty [illegible], Mew 1983). The theoretical advantages of photo-immunotherapy include a possible reduction in dose levels of HpD and reduction of concentration of the dye in adjacent tissues and skin, thereby reducing local and systemic toxicity. Since blo[illegible] most likely a major determinant of HpD loca[illegible] necrosis of normal tissue adjacent to or within the glioma may be a limiting factor for the concentrations of the dye. In addition, the uptake of tumor seeking conjugates of HpD with monoclonal antibodies directed against neoplasms would be limited by such necrosis. However, small areas of spontaneous necrosis that occurred in areas of the 9L Glioma in the Fischer 344 rat did not appear to interfere with laser phototherapy in this experimental model.

Major factors to be considered in

clinical application of phototherapy include efficient and selective light activation of HpD containing malignant tissue. The modification of the tip of the quartz fiber that was used in the laser system in this study appears to exhibit some advantages for efficient light delivery. The modification of the fiber to behave like a cylindrical radiator offered clear advantages over a simple cleaved fiber. The power flux of 100 milliwatts produced an intensity of approximately 30 watts/square centimeters at the tip of a 600 micron cleaved fiber, whereas the same power flux in a cylindrical radiator would be 0.5 watts/square centimeter at the fiber surface. As a consequence, local heating at the fiber was reduced considerably. A further and less obvious advantage of a cylindrically radiating source over a point source is the relative geometric fall in light intensity as the light propagates away from the source. A cleaved fiber radiating light in a highly scattering meduim does so with a spherical geometry. A properly cleaved fiber projects light with a cone-like geometry, but when inserted into a highly scattering medium like living tissue, the distribution of light behaves as if it were emitted from a point source . Thus for cleaved fibers

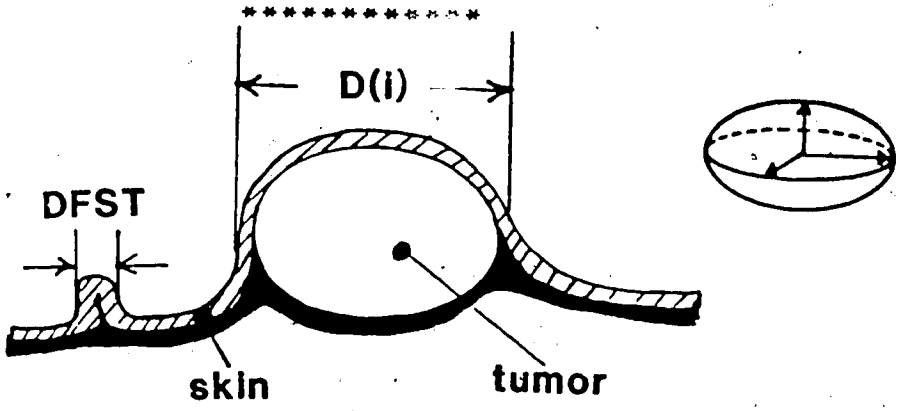
the geometrical reduction of intensity follows the inverse cubed power of the distance from the source. For a cylindrical radiator this reduction in intensity follows the inverse of distance so that uniformity of transillumination is consequently improved in this case. The mode of light delivery that was used in the present study would be suitable for light delivery to endoscopically accessible tumors or malignant lesions that are within reach of a percutaneous or steriotaxic needle insertion.

The demonstration of the lack of preferential uptake of HpD by neoplastic tissue in the present tumor model in comparision to some other organs has important implications for the applications of phototherapy. The use of a controlled point source of light delivery by a laser could lead to less damage to adjacent tissue but the presence of significant amounts of HpD at other areas, such as adjacent tissue and the skin, are important determinants of toxicity. The rapid and striking tumorcidal effects of photoactivated HpD show great promise for the treatment of gliomas and other tumors

This treatment modality for deep seated tumors may become acceptable for clinical use only when a

combination of selective tumor delivery of HpD together with efficient light delivery can be achieved. In the present study only one period of activation of HpD by a laser was used. It may be possible to treat malignancy more effectively with repeated sessions of laser phototherapy . Recently, we have modified our light delivery system for multiple fiber probes which will permit powerful illumination of a larger area of a neoplasm.

FIGURE 5.0



TUMOR VOLUME = (D1-DFST) * (D2-DFST) * (D3-DFST) * 3.14/6

D(i) = EXTERNALLY MEASURED TUMOR DIAMETER

DFST = DOUBLE FOLD SKIN THICKNESS

THE TUMOR VOLUME IS TREATED AS IF IT WERE AN ELLIPSOID. THE EXTERNAL DIAMETERS OF THE TUMOR ARE MEASURED USING CONSTANT FORCE CALIPERS SO AS TO SQUEEZE EVERY TUMOR WITH THE SAME FORCE AND THEREFORE REDUCE THE AMOUNT OF ERROR IN TUMOR COMPRESSION.

TABLE 5.1

Group	Description	Treatment
C9	CONTROL GROUP	NO TREATMENT GIVEN
C10	HpD CONTROL GROUP	10 mg/Kg i.p.
C11	LASER CONTROL GROUP	2000 Joules @ 100mW
C12	PHOTOTHERAPY GROUP	2000 Joules @ 100mW 10 mg/Kg i.p.

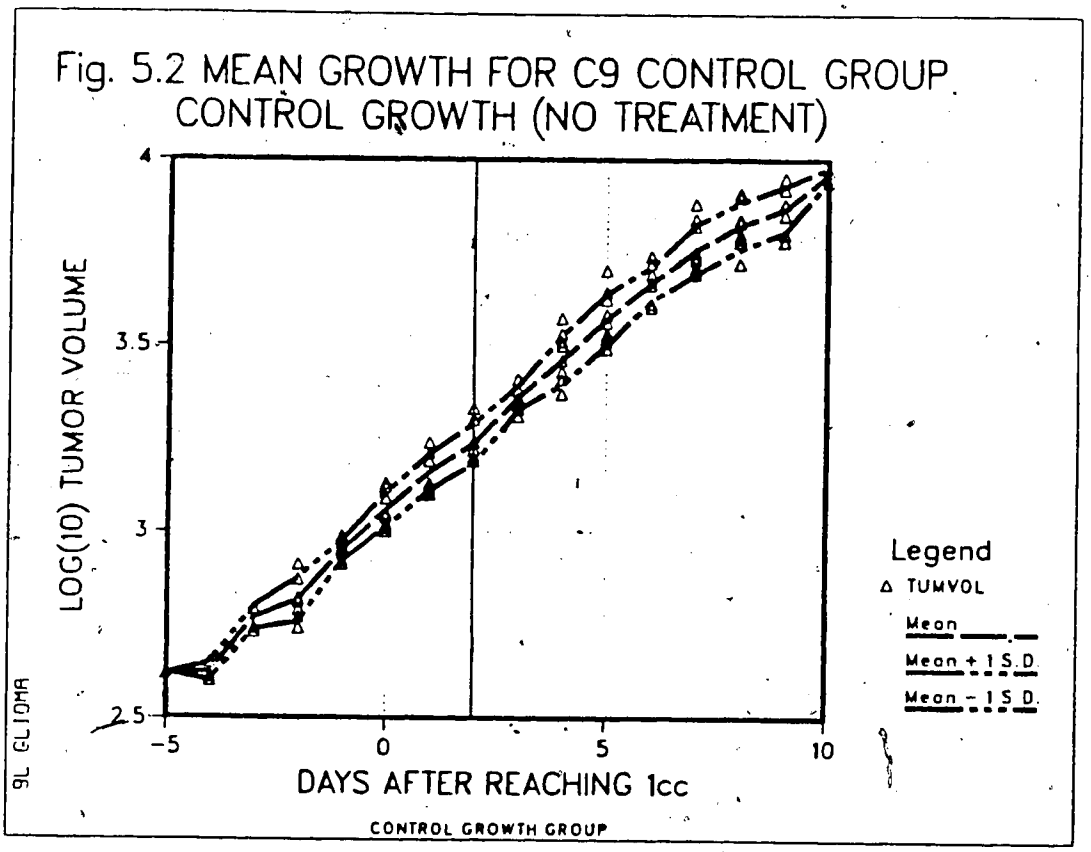


Fig. 5.2 MEAN GROWTH FOR C9 CONTROL GROUP
CONTROL GROWTH (NO TREATMENT)
LOG(10) TUMOR VOLUME
4
3.5
3
2.5
-5
0
5
10
DAYS AFTER REACHING 1cc
Legend
TUMVOL
Mean
Mean + 1 S.D.
Mean - 1 S.D.
9L GLIOMA
CONTROL GROWTH GROUP

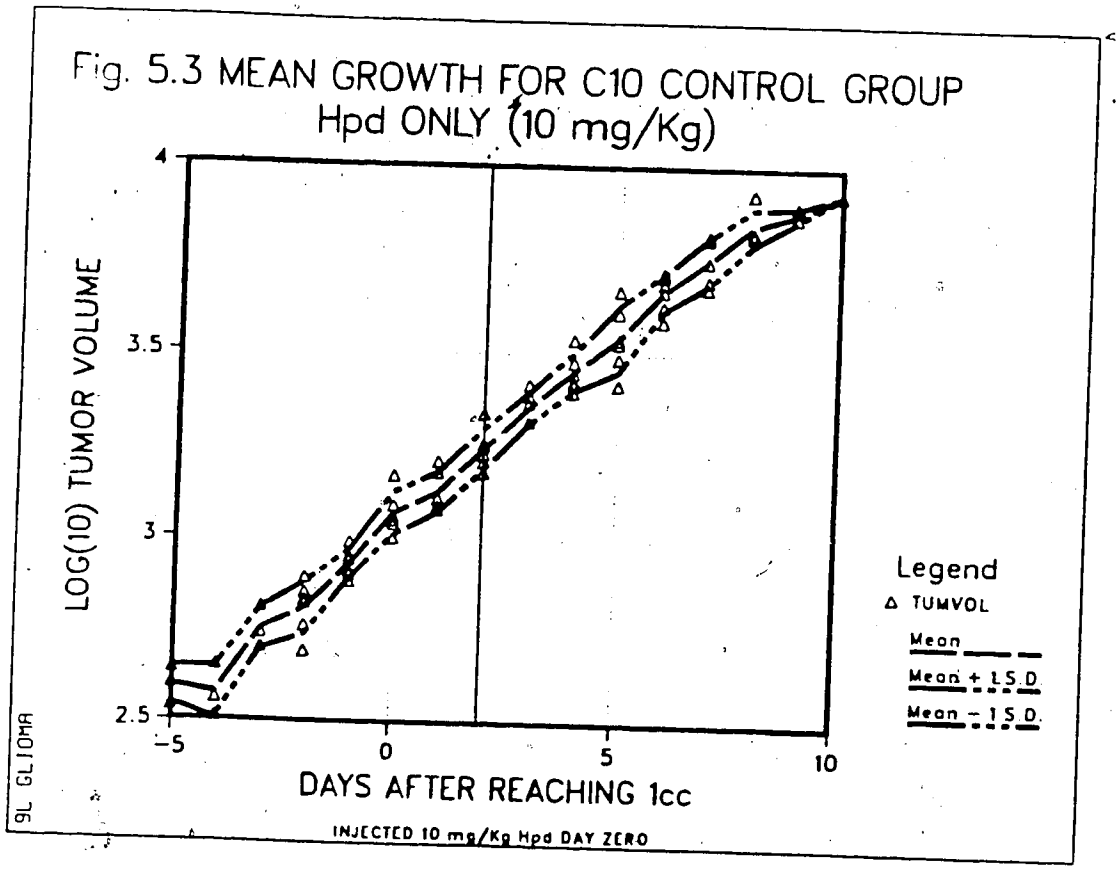


Fig. 5.3 MEAN GROWTH FOR C10 CONTROL GROUP
Hpd ONLY (10 mg/Kg)
LOG(10) TUMOR VOLUME
4
3.5
3
2.5
-5
0
5
10
DAYS AFTER REACHING 1cc
INJECTED 10 mg/Kg Hpd DAY ZERO
9L GLIOMA
Legend
TUMVOL
Mean
Mean + 1 S.D.
Mean - 1 S.D.

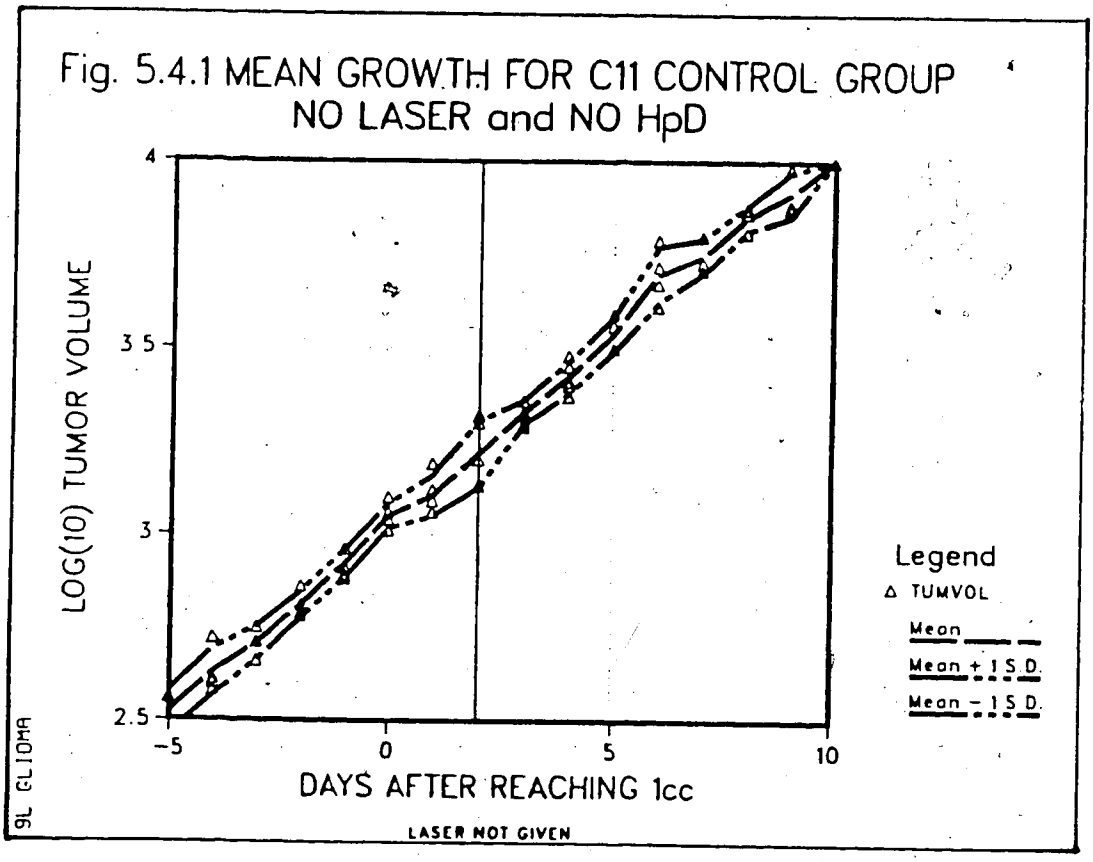
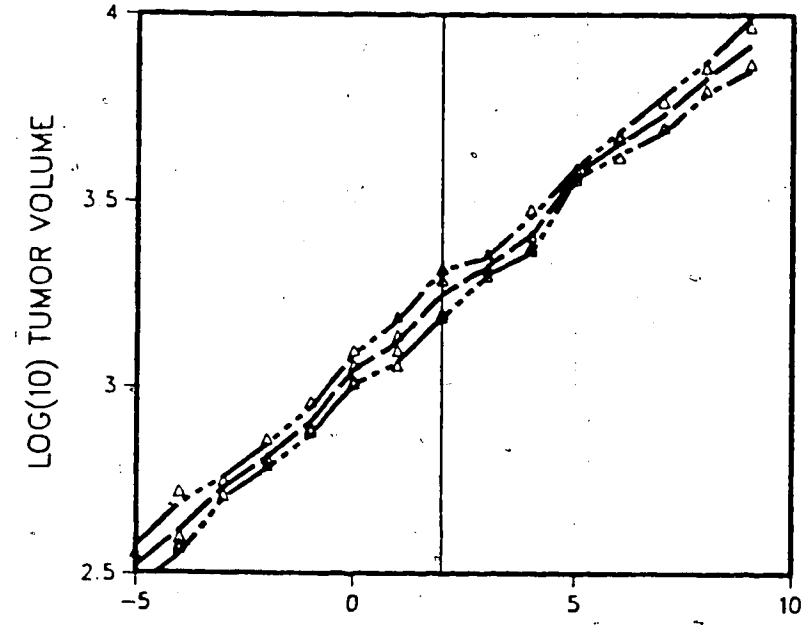


Fig. 5.4.1 MEAN GROWTH FOR C11 CONTROL GROUP
NO LASER and NO HpD
LOG(10) TUMOR VOLUME
4
3.5
3
2.5
-5
0
5
10
DAYS AFTER REACHING 1cc
Legend
Δ TUMVOL
Mean
Mean + 1 S.D.
Mean - 1 S.D.
9L GLIOMA
LASER NOT GIVEN

Fig 5.4.2 MEAN GROWTH FOR C11 TREATMENT GROUP
LASER ONLY (2000 JOULES @ 100mW)



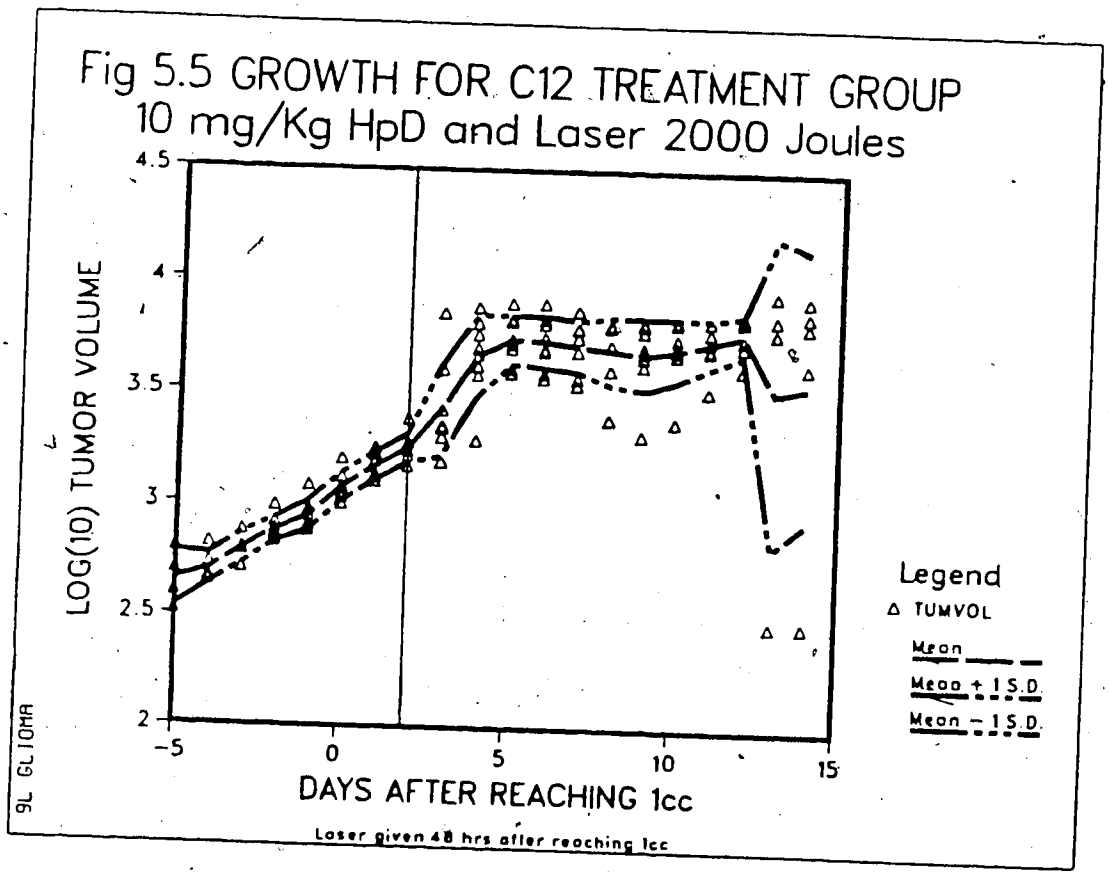


Fig 5.5 GROWTH FOR C12 TREATMENT GROUP
10 mg/Kg HpD and Laser 2000 Joules
LOG(10) TUMOR VOLUME
4.5
4
3.5
3
2.5
2
-5
0
5
10
15
DAYS AFTER REACHING 1cc
Laser given 48 hrs after reaching 1cc
9L GLIOMA
Legend
TUMVOL
Mean
Mean + 1 S.D.
Mean - 1 S.D.

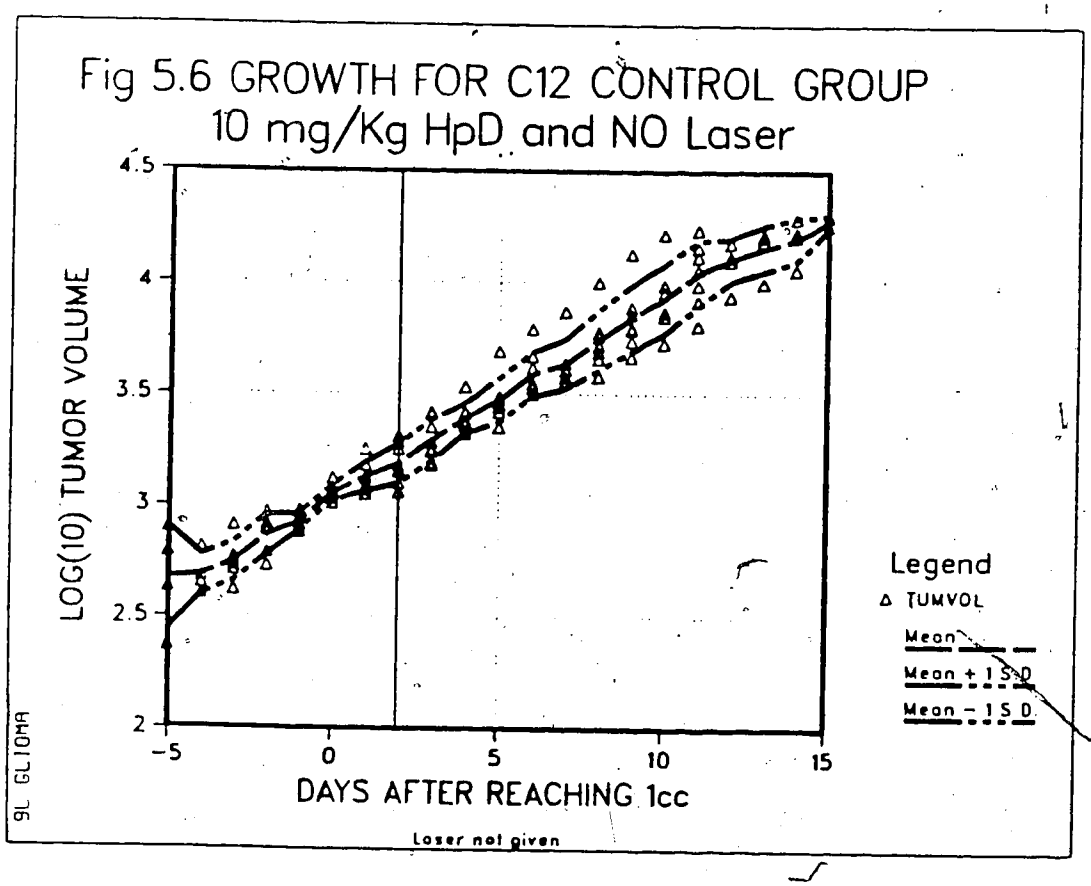


Fig 5.6 GROWTH FOR C12 CONTROL GROUP
10 mg/Kg HpD and NO Laser
LOG(10) TUMOR VOLUME
4.5
4
3.5
3
2.5
2
-5
0
5
10
15
DAYS AFTER REACHING 1cc
Laser not given
9L GLIOMA
Legend
TUMVOL
Mean
Mean + 1 S.D.
Mean - 1 S.D.

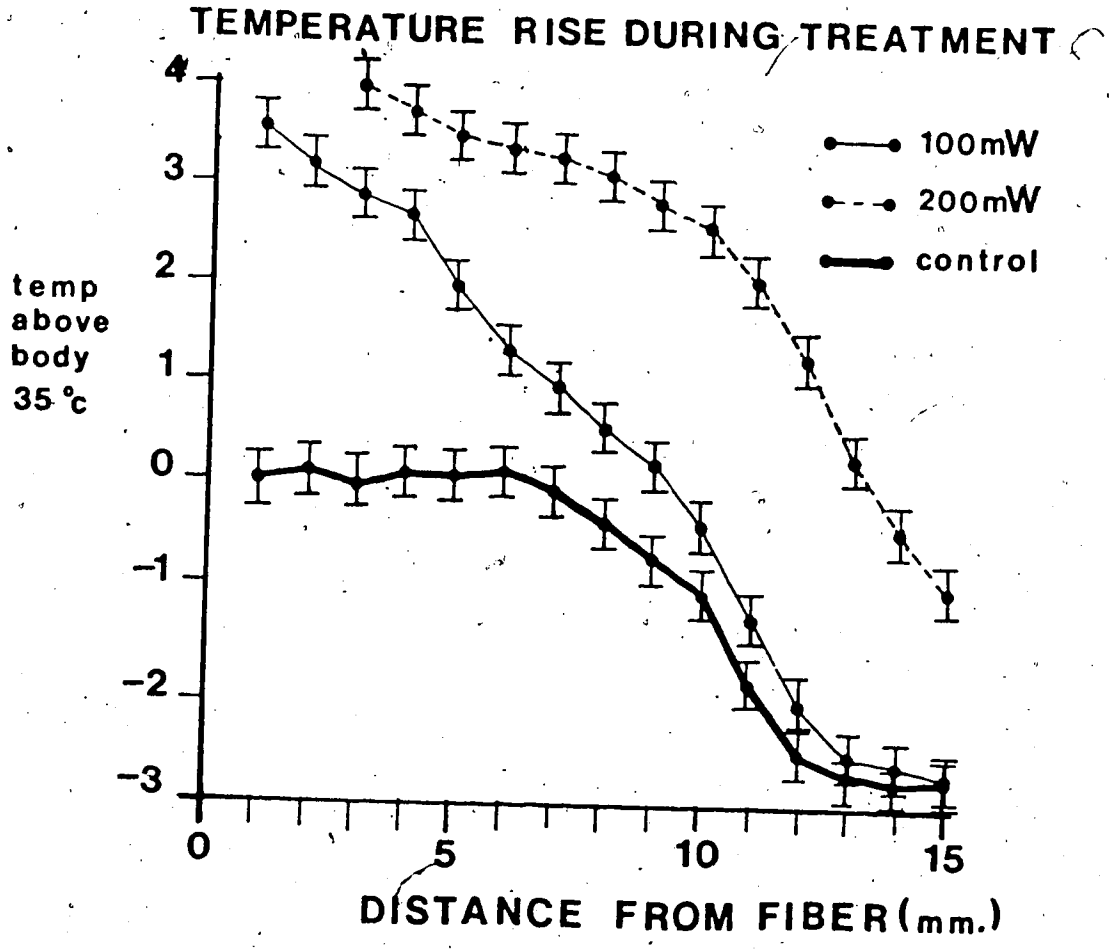

TEMPERATURE RISE DURING TREATMENT
100mW
200mW
control
temp above body 35 °c
4
3
2
1
0
-1
-2
-3
0
5
10
15
DISTANCE FROM FIBER (mm.)

figure 5.7

Quartz Fibre Cylindrical Irradiator

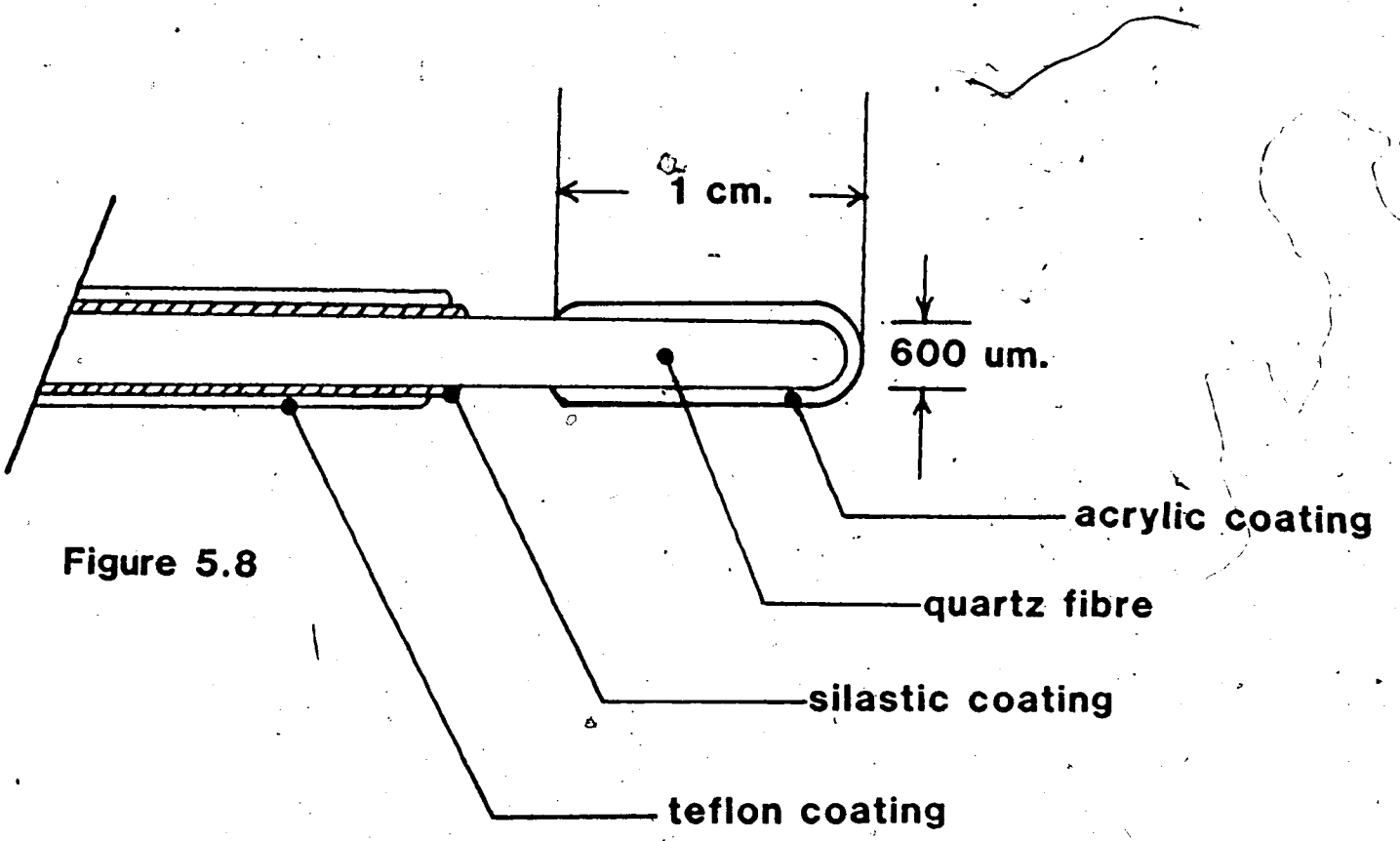


Figure 5.8

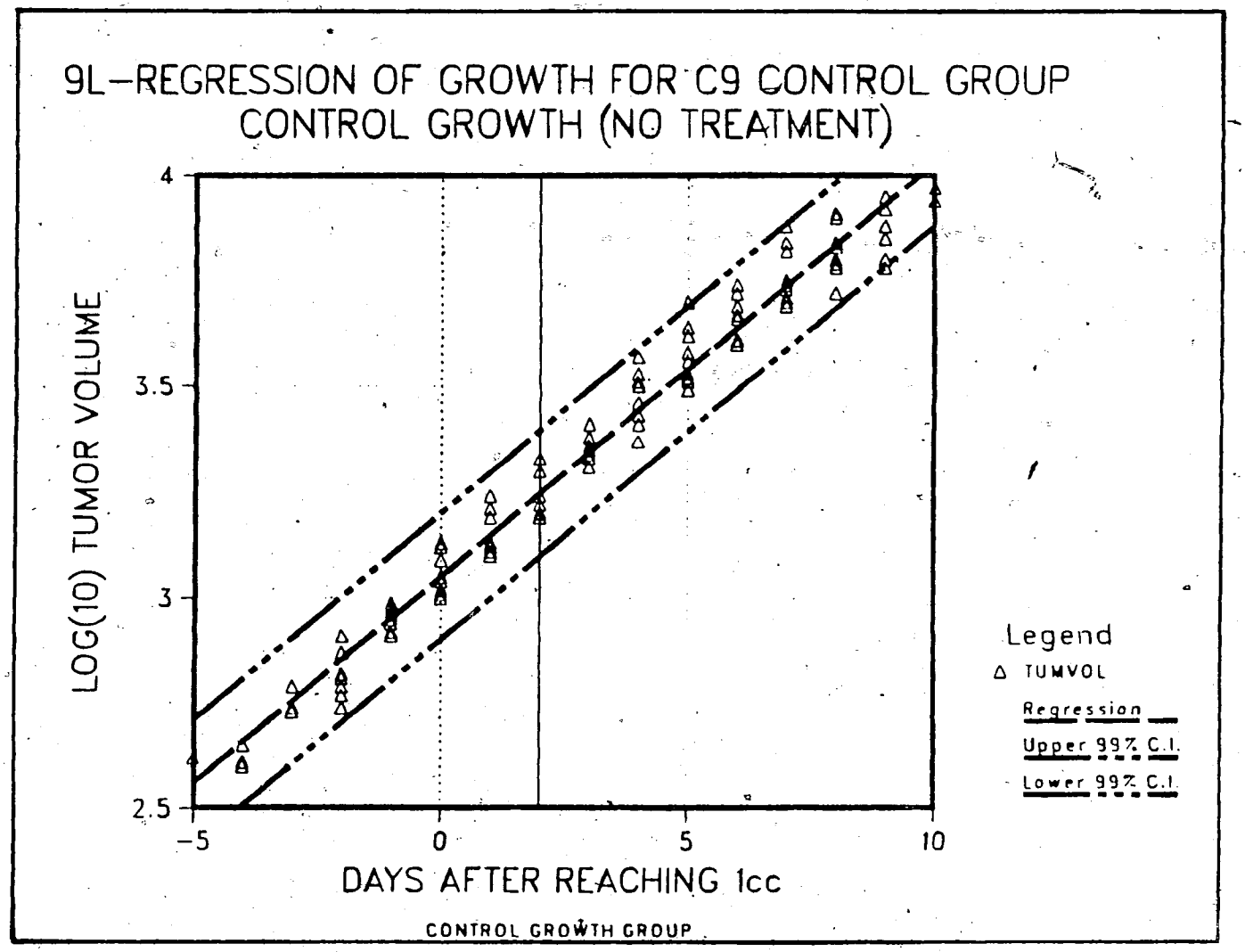

9L—REGRESSION OF GROWTH FOR C9 CONTROL GROUP
CONTROL GROWTH (NO TREATMENT)
LOG(10) TUMOR VOLUME
4
3.5
3
2.5
-5
0
5
10
DAYS AFTER REACHING 1cc
Legend
TUMVOL
Regression
Upper 99% C.I.
Lower 99% C.I.
CONTROL GROWTH GROUP

figure 5.9

TABLE 5.10

TREATMENT GROUP	SURVIVAL TIME	STANDARD DEVIATION
CONTROL	34.5 DAYS	6.8
PHOTOTHERAPY	52.7 DAYS	14.6

TREATMENT GROUP	TUMOR SIZE BEFORE TREATMENT	TUMOR SIZE 72 HOURS AFTER TREATMENT
CONTROL	1.81	3.9
PHOTOTHERAPY	1.77	5.6

CHAPTER 6

TREATING HEPATOMA TUMOR MODEL

6.1 THE HEPATOMA

Carcinoma of the liver is relatively rare in North America. It makes up about three percent of all cancers. There is a very high incidence in Orientals and the Bantu. It occurrs six to ten times more often in males than in females and the average onset time is between sixty and seventy years of age. The overall five year survival rate is less than one percent.

The only definitive treatment for hepatoma is surgical excision. This can be done only in cases where the cancer is localized and there has been no spreading into lymph nodes. Partial hepatectomies have been useful in cases where early diagnosis has occurred (Stanzl 1976).

Radiotherapy is considered of little value since lessions are not radiosensitive. Chemotherapy drugs

administered systemically or by way of the cannulated ,hepatic artery have been reported to effect temporary regression of tumors (Mosely 1967). Methotrexate and 5-FU have had the greatest success in treating hepatoma.

There has been only one attempt to investigate the effects of phototherapy on an animal hepatoma tumor model and the results were quite promising considering the problems this particular neoplasm presents onocologists. Tomio, et al. (1983) treated subcutaneous solid Yoshida Hepatoma using a filtered xenon light source (590 to 690 nm) with no recurrance. The results of Tomio are promising but do not address the problems of clinical feasiblilty. It is hoped that this study will open the door to further research in the photothearpy of the hepatoma.

6.2 ABSTRACT

The tumoricidal effect of the activation of hematoporphyrin derivative (HpD), by an Argon-ion-dye-laser (Wavelength 630 nm), was investigated in the Buffalo rat bearing subcutaneous implants of the Morris 7777 hepatoma. Tumor growth was monitored by measuring the

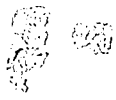
tumor volume with constant force calipers. In (refer to Table 6.1) control animals and those that were pretreated with HpD alone (10 mg/kg intraperitoneal injection) or laser light alone (2000 Joules at 100 milliwatts), a predictable exponential growth pattern of the cancer was observed. Animals were pretreated with HpD (10 mg/kg by i.p.) 48 hours prior to the fiberoptic , intratumor delivery of laser radiation (2000 Joules at 100 milliwatts), when the tumor had reached a volume of approximately 1.5 cubic centimeters. Seventy-two hours after the combined laser and HpD treatment the hepatoma underwent coagulation necrosis and the tumor volume rapidly increased (refer to Table 6.7) from a value of 1.80 +/- 0.7 cubic centimeters (mean +/- one standard deviation). to a value of 5.8 +/- 1.5 cubic centimeters compared with a 3.7 +/- 0.7 cubic centimeters. in animals who had not received laser phototherapy. Rats treated with HpD and laser light survived longer (mean survival 48 days +/- 12 standard deviation) than the other animals treated with laser alone or HpD alone (mean survival 31 days +/- 16.5 standard deviation). Laser phototherapy with HpD significantly interrupts the growth of the highly

malignant Morris 7777 hepatoma and prolongs the survival of Buffalo rats bearing this neoplasm.

6.3 INTRODUCTION

The photodynamic effect of hematoporphyrin derivative (HpD) on malignant cells has been applied to the diagnosis (Lipsom et al., 1967) and treatment of cancer (Diamond et al., 1972; Dougherty et al., 1978). The exposure of mouse or rat tumors that contain HpD to light of appropriate wavelength (630nm) results in death of malignant cells. The photoradiation therapy has been curative in a number of tumor systems including cancer of the breast, prostate, colon, skin, endometrium, bone and angiosarcoma (Dougherty et al., 1975; Dougherty et al., 1978). More recently, light has been delivered by a laser (Dougherty , 1981). A laser, combined with a fiberoptic delivery system, is a more efficient and convenient light source because the laser wavelength may be selected to fall in the optimal range for HpD activation and the fiber may be used to deliver light percutaneously. To further investigate the use of phototherapy for the treatment of cancer, HpD was administered to Buffalo rats bearing subcutaneous implants of the Morris 7777 hepatoma and

the HpD in these tumors was activated by light from an Argon-ion-dye-laser .



6.4 METHODS

6.4.1 Tumor Model

The poorly differentiated and rapidly growing Morris 7777 hepatoma was selected from tissue culture for subcutaneous implantation in Buffalo rats (Simonson Lab, Gilroy, CA, U.S.A., mean weight 251 +/- 20 S.D gm.). Throughout all studies each animal was allowed free access to Purina Laboratory Chow and water. The Morris 7777 hepatoma is a nonmetastasizing transplantable tumor that arose initially in the Buffalo rat after treatment of the animal with 2-N-fluorenyl phthalamic acid , an alkylating agent. The untreated hepatoma grows rapidly, and it reaches a size of about 10% of body weight of the animal within 4-5 weeks (Morris and Criss, 1978). Initial studies using subcutaneous trocar implants of the cancer in both flanks of 6 rats revealed consistent but eccentric tumor growth and death of each animal within 4-5 weeks. To produce a tumor model with predictable growth characteristics a skin incision was made on each flank of 5 Buffalo rats and a piece of tumor (approximately 2mm in diameter) derived from an already established subcutaneous growth in a separate animal was

transplanted. The incision was then closed with fine silk sutures. Tumor growth was monitored daily using constant force calipers (10 grams force). The tumor diameters were measured in three orthogonal planes. The double fold skin thickness was subtracted from each diameter measurement and the resulting corrected diameters were used to calculate and elliipsoidal tumor volume (refer to Fig. 5.0). If any tumor, upon reaching 1 cubic centimeter, had one diameter more than 50 percent larger or smaller than the mean the three measured diameters, the rat hosting that tumor was excluded from the study. Each rat was given one tumor on each flank. If the tumors did not reach a volume of 1 cubic centimeter within 3 days of each other, then the rats hosting those tumors were also excluded from the study. The growth of the tumors was found to be exponential between volumes of 1 cubic centimeter and 6.5 cubic centimeters, with a mean doubling time of 4.07 days. The mean doubling time was derived from the linear regression of the logarithm of the tumor volumes against the number of days after reaching a volume of 1 cubic centimeter. Since all tumors behaved in a predictable exponential fashion upon reaching 1 cubic centimeters all growth data was

aligned around tumor volumes of 1 cubic centimeter. Thoughout this and subsequent studies, direct implantation of tumors was undertaken and the animals were anesthetized with halothane during experimental handling.

6.4.2 Phototherapy

Four groups of Buffalo rats received surgical implantation of the Morris 7777 hepatoma in the manner as previously described. Each animal had a tumor in the left and right flank. Group C9 (Fig. 6.2) received no treatment and formed a control group. Groups C10-C12 formed the treatment groups. These tumors were treated when they had reached a volume of 1.5 cubic centimeters. Treatments consisted of the intratumoral delivery of laser light alone (2000 Joules at 100 milliwatts, Group C11 fig. 6.4.2 and the C11 control group fig. 6.4.1), HpD given by i.p. injection in doses of 10 mg/kg (Group C10 fig. 6.3) alone, and intratumor delivery of laser light (2000 Joules at 100 milliwatts) 48 hr after the i.p. administration of HpD (10 mg/kg) (Group C12 fig 6.5 and the treatment control group fig. 6.6). Tumor growth was monitored by measuring the tumor volume with

calipers and the length of survival of each animal was noted. Autopsy was performed on at least 2 animals from each group following their death and adjacent muscle and tumor tissue was removed from animals before and after laser phototherapy for light microscopy (Group C12).

The delivery of light to the tumors was performed using a 600 micron, step index fiber (quartz silica) that was implanted along the longest axis of the tumor. Although this technique provided uniform transillumination of all the treated neoplasms, a rapid decrease of transillumination intensity occurred at the tumor surface following the initiation of the laser treatment. This reduction of transillumination was a result of the baking of tissue and blood onto the fiber tip with consequent light absorption and heating. The thermal problems experienced in this protocol were similar to those encountered in the 9L Glioma protocol. The same measures were taken to reduce fiber optic light intensities around the fiber. The freshly cleaved end of the fiber was rounded off using a micro-torch and about 1 centimeter of the distal end of the fiber was treated with acrylic cement. The cement dried onto the quartz core of the fiber to produce a

mat surface. The refractive index of the acrylic cement closely matched that of the quartz core so that light readily passed from the core into the coating where it was scattered by the rough outer surface. Before laser light was administered, the skin overlying the tumor was shaved and the animal was immoblilzed on a small met al frame. A 21 gauge needle was inserted into the longest axis of the tumor and 500 ul of normal saline was injected into the centre of the tumor. The acrylic coated fiber, through which light was transmitted from the Argon-ion-dye laser, was inserted into the needle and the needle was then withdrawn to expose the fiber tip. The fiber tip was then located in the approximate middle of the neoplasm and laser light was administered at a rate of approximately 1 Joule/10 sec. During light delivery the intratumor and intraperitoneal temperature of each animal was monitored simultaneously with a 30 gauge copper-constantin thermocouple .

6.5 RESULTS

The results of treatment in all five groups of rats are shown in figures 6.2 through 6.6. Neither HpD alone (10 mg/kg i.p.), or laser light alone (2000J at

100 milliwatts) caused any measurable effect on noraml tumor growth. After the combined administration of HpD (10 mg/kg) by i.p. injection and laser light, there was a rapid increase in tumor size which was generally noticeable, on inspection of the animal, within 6 hours of light delivery (Fig. 6.5). The hard, nodular nature of the subcutaneous hepatoma changed to a soft mass on palpation and the tumor became a cystic swelling. In one animal, that had received laser phototherapy, tumor tissue leaked when the skin over the treated tumor was punctured with a needle. In 2 of the 8 rats treated with laser phototherapy some ulceration of skin over the tumor was noted. The rats behaved sluggishly for approximately 2 days after treatment but then returned to apparently normal activity. In 6 out of 8 of the animals that received laser phototherapy with HpD the tumor ulcerated and sloughed away. This striking tumoricidal effect with liquefaction of tissue made subsequent measurements of tumor volume difficult but within 2-5 days of treatment tumor growth appeared to recover. The survival time (refer table 6.7), after the implantation of the hepatoma , in the rats treated with light and HpD was significantly longer (48 days +/- 12 standard

deviation) than for all other groups of animals (31 days +/- 16.5 standard deviation).

Measurements of temperatures in the tumo , 1 to 10 millimeters from the fiber-optic surface, were uniformly less than 3 C above the simultaneously measured, intraperitoneal temperature of the animal. This implied that the resultant light pattern did not give rise to any "hot spots" at the doses of light used in these experiments and thermal injury was not responsible for the observed tumoricidal effects of laser phototherapy with HpD.

Autopsies, performed after death of the animals, showed malignancy which was manifested most often by direct intraperitoneal invasion of the hepatoma through the muscles of the flank of the rat. Macroscopic examination of the abdominal and thoratic organs after death did not reveal major differences between each group of animals and the pattern of tumor invasion appeared similar in all groups.

Light microscopy of sections of the biopsies of tumor tissue that had been removed before and after laser phototherapy revealed the development of extensive coagulation necrosis in the treated neoplasm with total cellular disruption that could be attributed

to therapy. However, there was some evidence of survival islands of hepatoma cells within the tissue sections removed from neoplasms that had been treated with laser phototherapy and HpD.

The finding of surviving hepatoma tissue matched the observation of tumor recurrence in the animals after treatment . Most necrosis was noted in the middle of the tumors at areas of the tumor adjacent to entry of the fiberoptic light cable. Sections of tumor tissue that were examined after treatment with HpD alone or laser light alone did not reveal large areas of necrosis.

6.6 DISCUSSION

Laser phototherapy with HpD significantly interrupts the growth of the highly malignant Morris 7777 hepatoma and improves survival time in the Buffalo rat bearing this tumor. The data confirms the findings of other investigators (Diamond et al., 1972; Dougherty , 1975; Thomson et al., 1974; Dougherty et al., 1975; Forbes et al., 1980) that light activation of phototoxic dyes such as HpD, may have an important role in the treatment of certain malignancies. Normal tissue adjacent to a neoplasm could be severly damaged if the

interval between HpD administration and light exposure is not optimal. In addition, the therapeutic ratio obtained during phototherapy depends to a large extent on the location of the tumor. The liver is an organ which may pose problems for photoradiation treatment because of its ability to concentrate HpD. The treatment of intrahepatic malignancy by HpD and light delivered percutaneously through a needle inserted into primary or secondary malignancies in liver or the use of phototherapy as an adjunct to the treatment of hepatic neoplasia are intriguing possibilities. However, they may not be feasible without a selective delivery system for HpD or other photoactive dyes. Selective delivery of HpD to a neoplasm by a tumor seeking compound such as a monoclonal antibody (Mew et al., 1983) has obvious advantages since it may be possible to reduce the systemic toxicity of photoactivated dyes. This concept of photoimmunotheray has been proposed by Mew et al. (1983) where HpD conjugated with a tumor-seeking monoclonal antibody appeared to have cancer treatment properties in a mouse tumor model. The theoretical advantages of photoimmunotherapy include a possible reduction in dose levels of HpD and reduction of concentration of the dye

in adjacent tissues and skin, thereby reducing local and systemic toxicity. In addition, the uptake tumor seeking conjugates of HpD with monoclonal antibodies directed against neoplasms would be limited by tumor necrosis. However, small areas of spontaneous necrosis that occurred in areas of the Morris 7777 hepatoma in the Buffalo rat did not appear to interfere with laser phototherapy in this experimental model. Hematoporphyrin derivative seems to accumulate in a wide variety of neoplasms but the effect of factors such as cell type and degree of differentiation of the tumor on HpD uptake have not been studied in detail. In the present study only one period of activation of HpD by a laser was used. It may be possible to treat malignancy more effectively with repeated sessions of laser phototherapy. The use of a controlled point source of light delivery by a laser could lead to less damage to adjacent tissue but the presence of significant amounts of HpD at other areas, such as adjacent tissue and the skin, are important determinants of toxicity. The rapid and striking tumoricidal effects of photoactivated HpD show great promise for the treatment of hepatic and other tumors. However, this treatment modality for deep

seated tumors may become acceptable for clinical use only when a combination of selective tumor delivery of HpD together with efficient light delivery can be achieved.

TABLE 6.1

Group	Description	Treatment
C9	CONTROL GROUP	NO TREATMENT GIVEN
C10	HpD CONTROL GROUP	10 mg/Kg i.p.
C11	LASER CONTROL GROUP	2000 Joules @ 100mW
C12	PHOTOTHERAPY GROUP	2000 Joules @ 100mW 10 mg/Kg i.p.

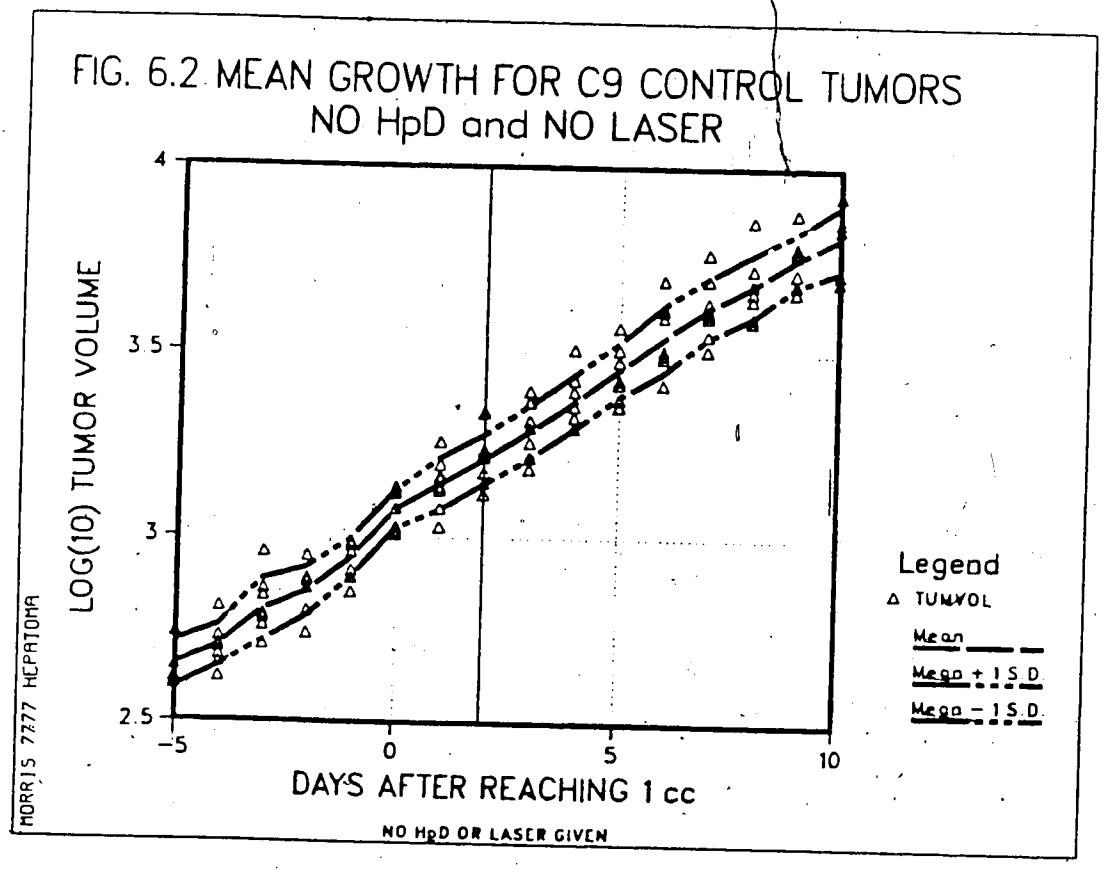


FIG. 6.2 MEAN GROWTH FOR C9 CONTROL TUMORS
NO HpD and NO LASER
LOG(10) TUMOR VOLUME
4
3.5
3
2.5
−5
0
5
10
DAYS AFTER REACHING 1 cc
NO HpD OR LASER GIVEN
MORRIS 7777 HEPATOMA
Legend
TUMVOL
Mean
Mean + 1 S.D.
Mean − 1 S.D.

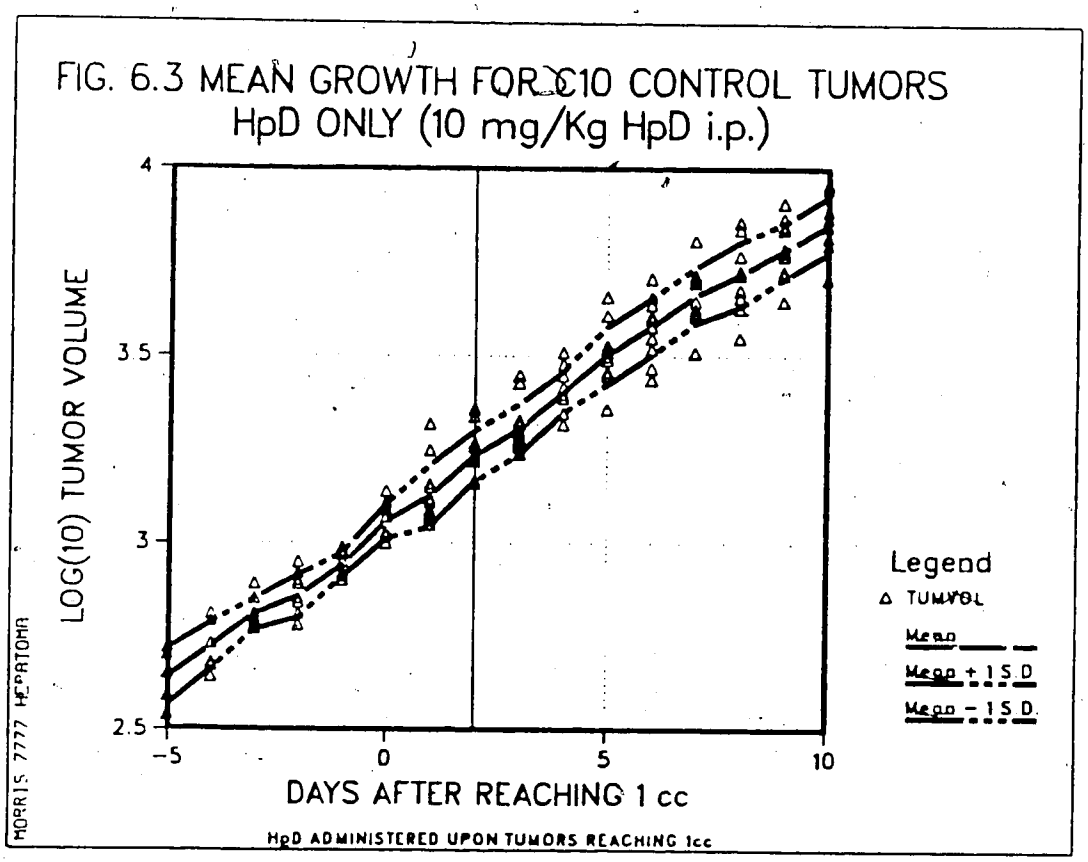
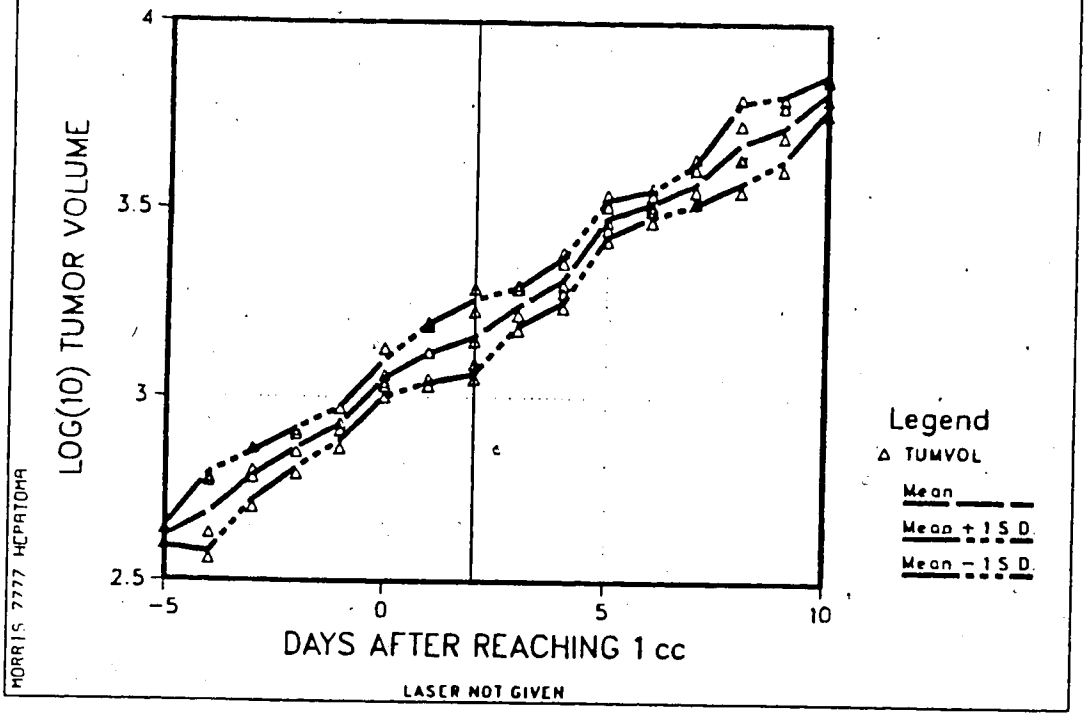


FIG. 6.3 MEAN GROWTH FOR C10 CONTROL TUMORS
HpD ONLY (10 mg/Kg HpD i.p.)
LOG(10) TUMOR VOLUME
4
3.5
3
2.5
-5
0
5
10
DAYS AFTER REACHING 1 cc
HpD ADMINISTERED UPON TUMORS REACHING 1cc
MORRIS 7777 HEPATOMA
Legend
Δ TUMVOL
Mean
Mean + 1 S.D.
Mean - 1 S.D.

FIG 6.4.1 MEAN GROWTH FOR C11 CONTROL TUMORS
NO HPD and NO LASER



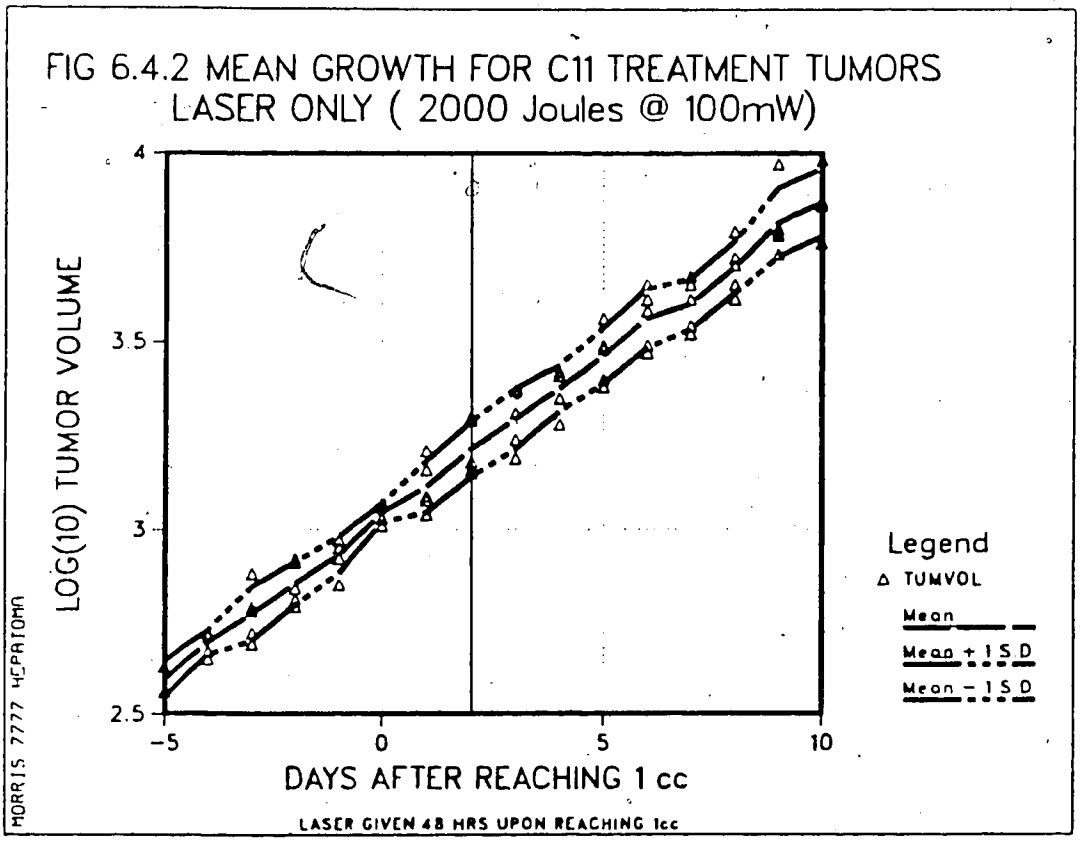


FIG 6.4.2 MEAN GROWTH FOR C11 TREATMENT TUMORS
LASER ONLY (2000 Joules @ 100mW)
LOG(10) TUMOR VOLUME
4
3.5
3
2.5
-5
0
5
10
DAYS AFTER REACHING 1 cc
LASER GIVEN 48 HRS UPON REACHING 1cc
MORRIS 7777 HEPATOMA
Legend
TUMVOL
Mean
Mean + 1 S.D
Mean - 1 S.D

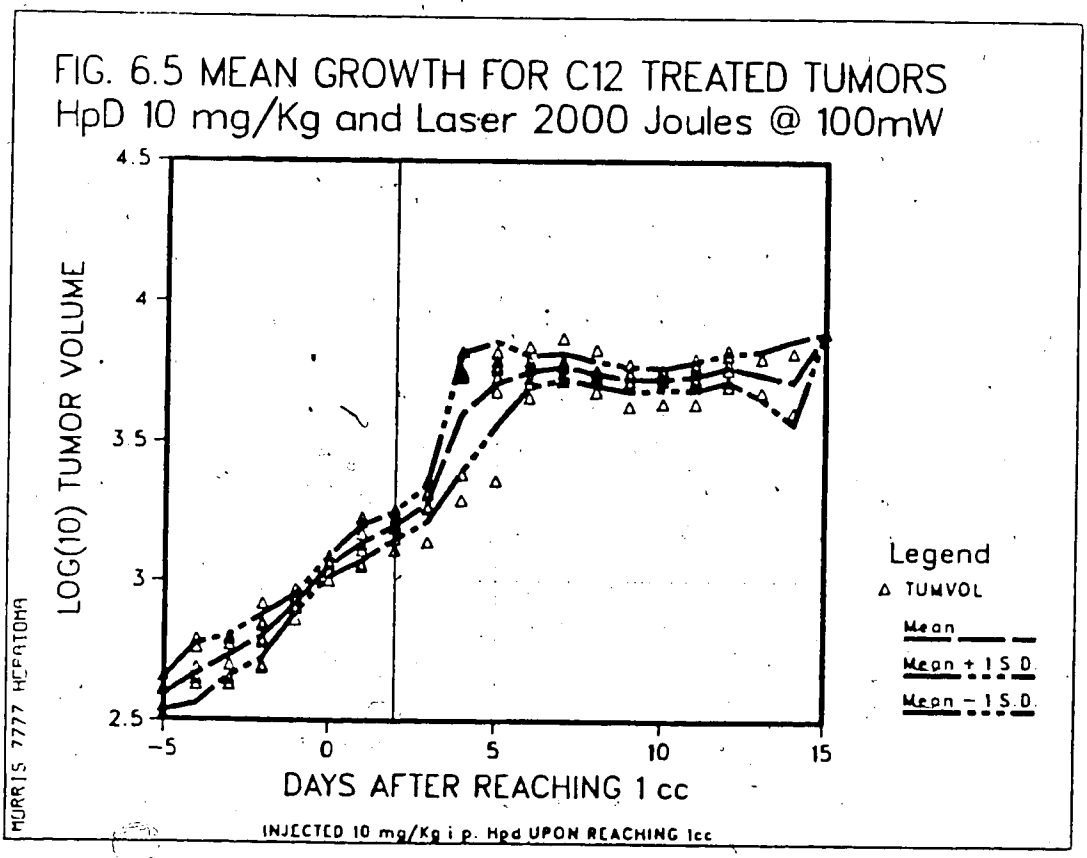


FIG. 6.5 MEAN GROWTH FOR C12 TREATED TUMORS
HpD 10 mg/Kg and Laser 2000 Joules @ 100mW
LOG(10) TUMOR VOLUME
4.5
4
3.5
3
2.5
-5
0
5
10
15
DAYS AFTER REACHING 1 cc
INJECTED 10 mg/Kg i.p. Hpd UPON REACHING 1cc
MURRIS 7777 HEPATOMA
Legend
Δ TUMVOL
Mean
Mean + 1 S.D.
Mean - 1 S.D.

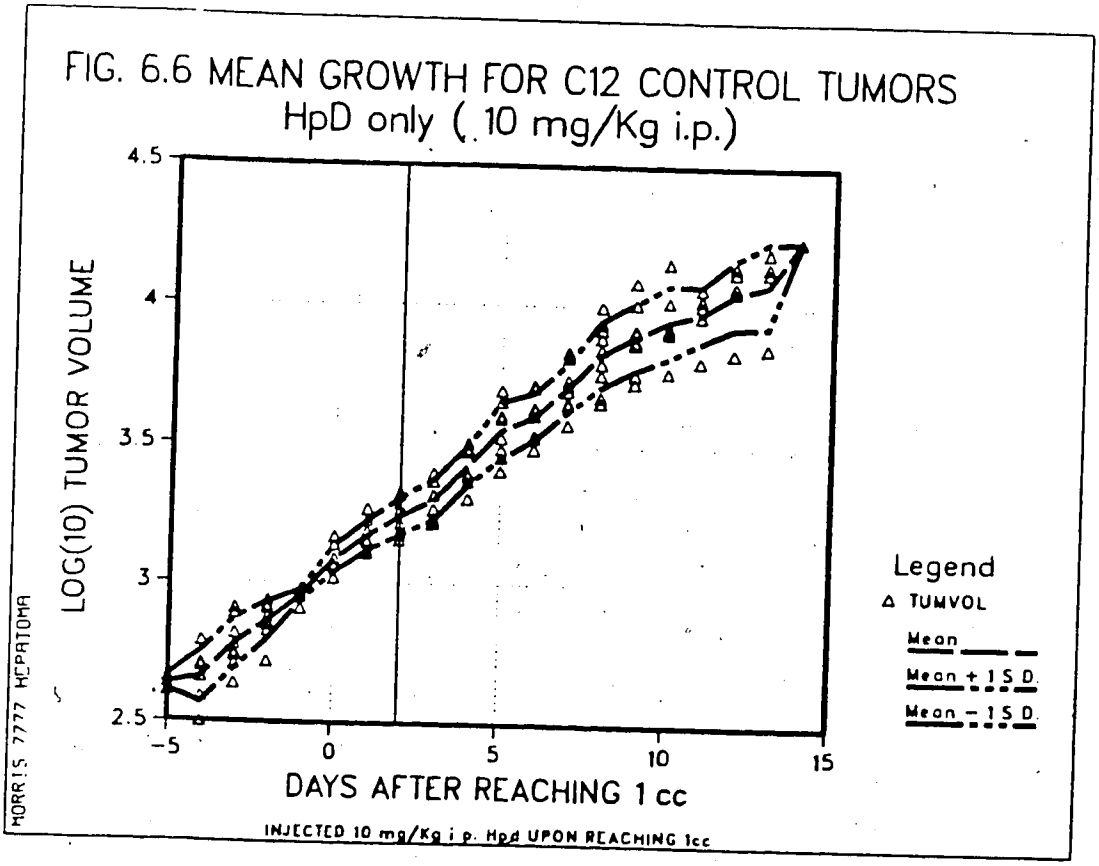


FIG. 6.6 MEAN GROWTH FOR C12 CONTROL TUMORS
HpD only (.10 mg/Kg i.p.)
LOG(10) TUMOR VOLUME
4.5
4
3.5
3
2.5
-5
0
5
10
15
DAYS AFTER REACHING 1 cc
Legend
TUMVOL
Mean
Mean + 1 S.D.
Mean - 1 S.D.
MORRIS 7777 HEPATOMA
INJECTED 10 mg/Kg i.p. HpD UPON REACHING 1cc

TABLE 6.7

TREATMENT GROUP	SURVIVAL TIME	STANDARD DEVIATION
CONTROL	41.3 DAYS	16.5
PHOTOTHERAPY	48.4 DAYS	12

TREATMENT GROUP	TUMOR SIZE BEFORE TREATMENT	TUMOR SIZE 72 HOURS AFTER TREATMENT
CONTROL	1.76	3.7
PHOTOTHERAPY	1.80	5.8

CHAPTER 7

TREATING THE DUNNING R3327 PROSTATE ADENOCARCINOMA

7.1 ABSTRACT

Cancer of the prostate is the most common cancer in men over 50 years of age and is rarely seen in younger age groups. Adenocarcinoma is the usual tumor found with the sarcoma rarely seen.

The tumoricidal effect of the activation of hematoporphyrin derivative (HpD), by an Argon-ion-dye-laser (Wavelength 630 nm), was investigated in the Fischer Copenhagen rat bearing subcutaneous implants of the Dunning R3327 prostate adenocarcinoma. The Dunning R3327H prostatic tumor was bilateraaly transplanted in the flanks of Copenhagen Fischer rats and tumor growth was monitored by measuring the tumor volume with constant force calipers. In control animals and those that were pretreated with HpD alone (20 mg/kg

intraperitoneal injection) or laser light alone (500 Joules at 100 milliwatts), a predictable exponential growth pattern of the cancer was observed. Animals were pretreated with HpD (10 mg/kg by i.p.) 48 hours prior to the fiberoptic, intratumor delivery of laser radiation (500 Joules at 100 milliwatts), when the tumor had reached a volume of approximately 1.5 cubic centimeters. Tumor growth was completely stopped when 500 Joules of fiber optically delivered laser light was administered two days after intraperitoneal injection of 20 mg/kg of Hematoporphyrin derivative. Only 1 Of 13 tumors showed any regrowth 5 weeks after treatment. Laser phototherapy with HpD significantly interrupts the growth of the hormonally sensitive Dunning R3327 prostate adenocarcinoma and prolongs the survival of Fischer Copenhagen rats bearing this neoplasm.

7.2 INTRODUCTION

A laser, combined with a fiberoptic delivery system, is a more efficient and convenient light source because the laser wavelength may be selected to fall in the optimal range for HpD activation and the fiber may be used to deliver light percutaneously. Fiber optic

delivery of laser light to HpD sensitized bladder carcinoma in situ was achieved by Haruo et al.(1981) with total remission in 5 of 6 patients treated. It can be seen that HpD phototherapy may be useful in treating the early states of prostate cancer. To further investigate the use of phototherapy for the treatment of cancer, HpD was administered to Fischer Copenhagen rats bearing subcutaneous implants of the Dunning R3327 prostate adenocarcinoma and the HpD in these tumors was activated by light from an Argon-ion-dye-laser.

7.3 METHODS

7.3.1 Tumor Model

The homonally sensitive and slow growing Dunning R3327 prostate adenocarcinoma was selected for subcutaneous implantation in Fischer Copenhagen rats Throughout all studies each animal was allowed free access to Purina Laboratory Chow and water. The Dunning R3327 prostate adenocarcinoma arose initially in a Copenhagen male rat in 1961. Dunning (1963) cross bred the Copenhagen with Fischer rat and innoculated the flanks of the offspring with 10 milligram grafts of the tumor. This method of

bilateral transplantation was used in the following investigation. To produce a tumor model with predictable growth characteristics a skin incision was made on each flank of 5 Fischer Copenhagen rats and a piece of tumor (approximately 2mm in diameter) derived from an already established subcutaneous growth in a separate animal was transplanted. The incision was then closed with fine silk sutures. Tumor growth was monitored daily using constant force calipers (10 grams force). The tumor diameters were measured in three orthogonal planes. The double fold skin thickness was subtracted from each diameter measurement and the resulting corrected diameters were used to calculate and ellipsoidal tumor volume (refer to fig. 5.0). The growth of the tumors was found to be exponential between volumes of 0.3 cubic centimeters. and 10.0 cubic centimeters, with a mean doubling time of 11.4 days. The mean doubling time was derived from the linear regression of the logarithm of the tumor volumes against the number of days after reaching 1cubic centimeters.. Since all tumors behaved in a predictable expontential fashion upon reaching 0.3 cubic centimeters. all growth data was aligned around tumor volumes of 0.3 cubic centimeters.. Thoughout

this and subsequent studies, direct implantation of tumors was undertaken and the animals were anesthetized with halothane during experimental handling.

7.3.2 Phototherapy

Four groups of Fischer Copenhagen rats received surgical implantation of the Dunning R3327 prostate adenocarcinoma in the manner as previously described. Each animal had a tumor in the left and right flank. Group 1 received no treatment and formed a control group (refer to fig 7.1). Groups 2-4 formed the treatment groups. These tumors were treated when they had reached a volume of 0.3 cubic centimeters. Treatments consisted of the intratumor delivery of laser light alone (500 Joules at 100 milliwatts) (Group 1), HpD given by i.p. injection in doses of 20 mg/kg (Group 3) alone, and intratumor delivery of laser light (500 Joules at 100 milliwatts) 48 hr after the i.p. administration of HpD (20 mg/kg) (Group 4). Tumor growth was monitored by measuring the tumor volume with calipers and the length of survival of each animal was noted.

The delivery of light to the tumors was performed using a 600 micron, step index fiber (quartz silica)

that was implanted along the longest axis of the tumor. Before laser light was administered, the skin overlying the tumor was shaved and the animal was immoblilzed on a small metal frame. A 21 gauge needle was inserted into the longest axis of the tumor and 500 ul of normal saline was injected into the centre of the tumor. The acrylic coated fiber, through which light was transmitted from the Argon-ion-dye laser, was inserted into the needle and the needle was then withdrawn to expose the fiber tip. The fiber tip was then located in the approximate middle of the neoplasm and laser light was administered at a rate of approximately 1 Joule/10 sec. During light delivery the intratumor and intraperitoneal temperature of each animal was monitored simultaneously with a 30 gauge copper-constantin thermocouple.

7.3.3 Laser Phototherapy

The results of treatment in all five groups of rats are shown in Fig. 3. Neither HpD alone (20 mg/kg i.p.), or laser light alone (500J at 100 milliwatts) caused any measurable effect on noraml tumor growth. After the combined administration of HpD (20 mg/kg) by i.p. injection and laser light, there was a rapid

increase in tumor size which was generally noticeable, on inspection of the animal, within 6 hours of light delivery (Fig. 7.2). The rats behaved sluggishly for approximately 2 days after treatment but then returned to apparently normal activity.

Measurements of temperatures in the tumor, 1 to 10 millimeters from the fiber-optic surface, were uniformly less than 3 C above the simultaneously measured, intraperitoneal temperature of the animal. This implied that the resultant light pattern did not give rise to any "hot spots" at the doses of light used in these experiments and thermal injury was not responsible for the observed tumoricidal effects of laser phototherapy with HpD.

7.4 DISCUSSION

Laser phototherapy with HpD significantly interrupts the growth of the highly malignant Dunning R3327 prostate adenocarcinoma and improves survival time in the Fischer Copenhagen rat bearing this tumor. The therapeutic ratio obtained during phototherapy depends to a large extent on the location of the tumor. The prostate gland is an organ which lends itself well to the fiber optic delivery of laser light via the rectum

or the urinary tract. The treatment of prostatic malignancy by HpD and light delivered percutaneously through a needle inserted into primary or secondary malignancies in the capsule or bladder neck is an intriguing possibility. However, they may not be feasible without a selective delivery system for HpD or other photoactive dyes. In the present study only one period of activation of HpD by a laser was used. It may be possible to treat malignancy more effectively with repeated sessions of laser phototherapy. The delivery of laser light to a tumor in the urinary tract and bladder have been investigated in the Medical Laser Lab of the Univeristy of Alberta with very promising results. It would take very little modification of the present urological laser delivery system to enable the illumination of prostate gland for phototherapy. The use of a controlled point source of light delivery by a laser could lead to less damage to adjacent tissue but the presence of significant amounts of HpD at other areas, such as adjacent tissue and the skin, are important determinants of toxicity. The rapid and striking tumorcidal effects of photoactivated HpD show great promise for the treatment of prostatic and other tumors.

TABLE 7.1

Group	Description	Treatment
1	CONTROL GROUP	NO TREATMENT GIVEN
2	HpD CONTROL GROUP	20 mg/Kg i.p.
3	LASER CONTROL GROUP	500 Joules @ 100mW
4	PHOTOTHERAPY GROUP	500 Joules @ 100mW 20 mg/Kg i.p.

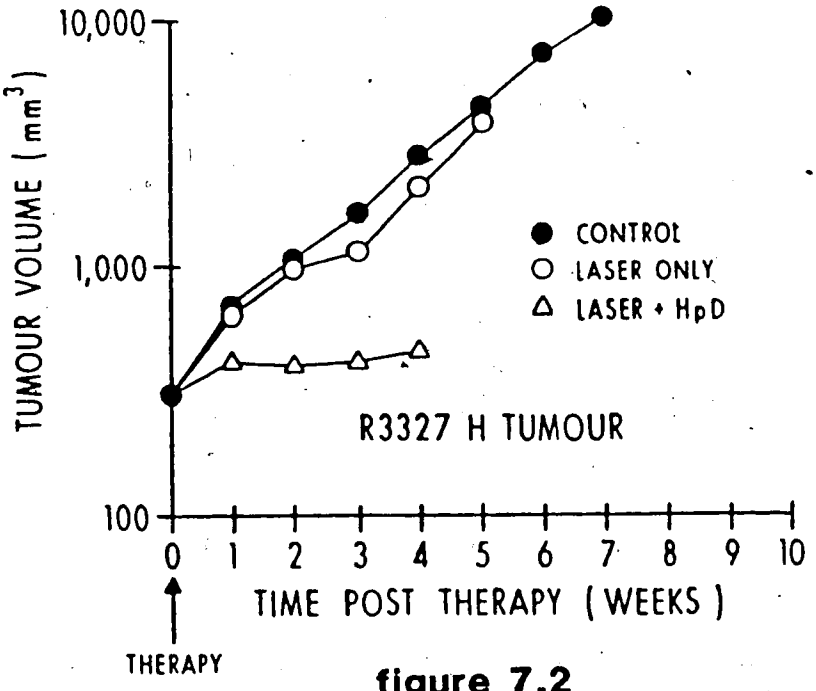

10,000
1,000
100
TUMOUR VOLUME (mm^3)
CONTROL
LASER ONLY
LASER + HpD
R3327 H TUMOUR
0 1 2 3 4 5 6 7 8 9 10
TIME POST THERAPY (WEEKS)
THERAPY

figure 7.2

CHAPTER 8

DISCUSSION OF THE PROJECT

8.1 THE PROJECT HISTORY

This phototherapy project at the University of Alberta was originally started by Dr. John Tulip and myself in the fall of 1979 with the initial investigation into the rejection properties of normal bladder epithelium to HpD. It was orginally thought by the author that the use of an irrigation fluid containing HpD might help assist in the deliniation of malignant and premalignant sites in the bladder. With the use of a Helium-Cadmium laser we looked for fluoresence in normal and traumatized bladder tissue in 25 dogs. We could only observe fluoresence in the bladder upon sacrificing the animal. This seemed to indicate that living bladder mucosa specifically rejected HpD absorption. It was suggested by the author that patients diagnosed with carcinoma in situ

be checked cystoscopically for fluorescence in the malignant sites using irrigation fluid containing HpD. If malignant tissue concentrated or even just retained HpD that was present in irrigation fluid then the selective destruction of the tumor may be possible. Dr. Brian Ritchie was most helpful in these investigations and proved to be very helpful in assisting me in modifying the present laser endoscope system to enable the delivery of blue laser light to the bladder mucosa of the dog. The application of this technique in humans, to test the fluoresence of a true malignant lesion in the bladder, never became a reality. Similar studies were eventually conducted and published by Haruo in 1983 and 1984.

The project involving HpD phototherapy of the 9L-Glioma was first proposed by Dr. John McKean in the winter of 1981 and the experimental protocol was commenced in the summer of 1982 after obtaining the proper funding for the project from M.R.C., M.S.I. and P.C.H.B.. The phototherapy project was expanded at that time to include Dr. Don Boviert who investigated the bio-distibution of HpD in the 9L-Glioma and the Morris 7777 Hepatoma using HPLC. In addition to the 9L-Glioma tumor protocol, two other subcutaneous tumor

model protocols were being by conducted the author (Morris 7777 and Dunning RH3327). The Hepatoma tumor model phototherapy project was conducted by myself under the supervision of Dr. Steven Holt and the Dunning RH3327 tumor protocol was conducted by myself under the supervision of Dr. Charles Thorndyke. These tumor models received similar treatment methods and therefore proved to be very useful in determining the general effectiveness of our methods against malignant tumor models in the rat. Dr. David Hume (Bsc. Fag. Mic. Key. Mou. Se.) was most helpful in assisting with the intricate neurosurgery that was necessary for the blood brain barrier study on dogs (Dr. Hume is probably the best neuro-surgeon the faculty of geology has ever produced.). The In vitro and subcutaneous tumor data was finished in January 1983. Two papers based on this research have been submitted for publication and one additonal paper is planned for a September 1984 submission.

I believe this research can only be conducted further when a more tumor specific version of this drug is found. Mew et al. have had promising results with monoclonal antibody congugates. The tumor models examined in the project would be ideal for the animal

model evaluation of Mew's findings.

8.2 DATA HANDLING METHODS

All tumor data was entered into the University of Alberta computer (Ahmdal 470/V6 using the MTS operating system) and inital tumor volume calculations were performed using the resident fortran compiler. This data was later transfered to a Tektronix model 4052 computer and the tumor growth curves were plotted on a Tektronix 4662 plotter. The results from these calculations were used to produce a paper on the Morris 7777 protocol. It was seen at this time that a different method of data manipulation was required to obtain a better contol group regression coefficient for the determination of the doubling time. Figure 8.2 shows the all control group volume measurements for the Morris 7777 protocol. These volumes are plotted using the number of days after implantation as the time variable. This method is more than adequate when considering each tumor separately but does not produce accurate results when the tumors are treated as a group.

Using a VAX 11/750 at the University of Alberta I was able align the tumor growths around volumes of 1000

cubic millimeters and define that time to be day zero. This is shown in figure 8.1 and it can be easily seen that the standard deviation for tumor growth of the same group of tumors is much lower. All tumors in this project were mathematically treated similar to the above example. All regressions were performed of the VAX using SPSS-X and there were no cases where the p-value of the linear regression of any one tumor was greater than 0.05.

The ellipsiodal volume approximations were checked on a group of five rats where upon their tumors having reached a volume of 3000 cubic centimeters were sacrificed. Their tumor diameters and volumes were checked against the constant force caliper method and all volumes were within ten percent of the actual measured volume (exact tumor volume was based on the water displacement method).

9.3 ENGINEERING PROBLEMS

The engineering problems in this project mostly invloved overcomming the problems of dosimetry and thermal generation. It was decided early on in the project that attempting to measure the amount of light being absorbed by living tissue by a radiating optical

fiber would not be useful due to the optically non homogenous nature of a tumor. Instead, a method was developed to insure that the same amount of light was leaving the fiber at all times by observing the coupling efficiency of the fiber to the laser. Our coupling efficiencies were in the range of 70 to 80 percent. The output power of the fibre was measured using a freshly cleaved 600 micron fiber and a Coherent 210 power meter. It was found that the highest coupling efficiency was produced by ensuring that the alignment of the focused laser on the proximal end of the fiber was such that the least amount of light was reflected back and that the outer sheath on the proximal end of the fiber d not emit light. Since the diameter of the fibre was large it was very easy to couple a focused laser to it. The cheapest and most successful optics to do this turned out to be a 10X objective lens from and ordinary microscope. By mounting the lens on an optical bench with a translating fibre mount we could couple as much as 20 watts of power through the cable without serious thermal problems. This proved very useful when using this laser system for developing a method of fingerprint detection for the RCMP and city police (one

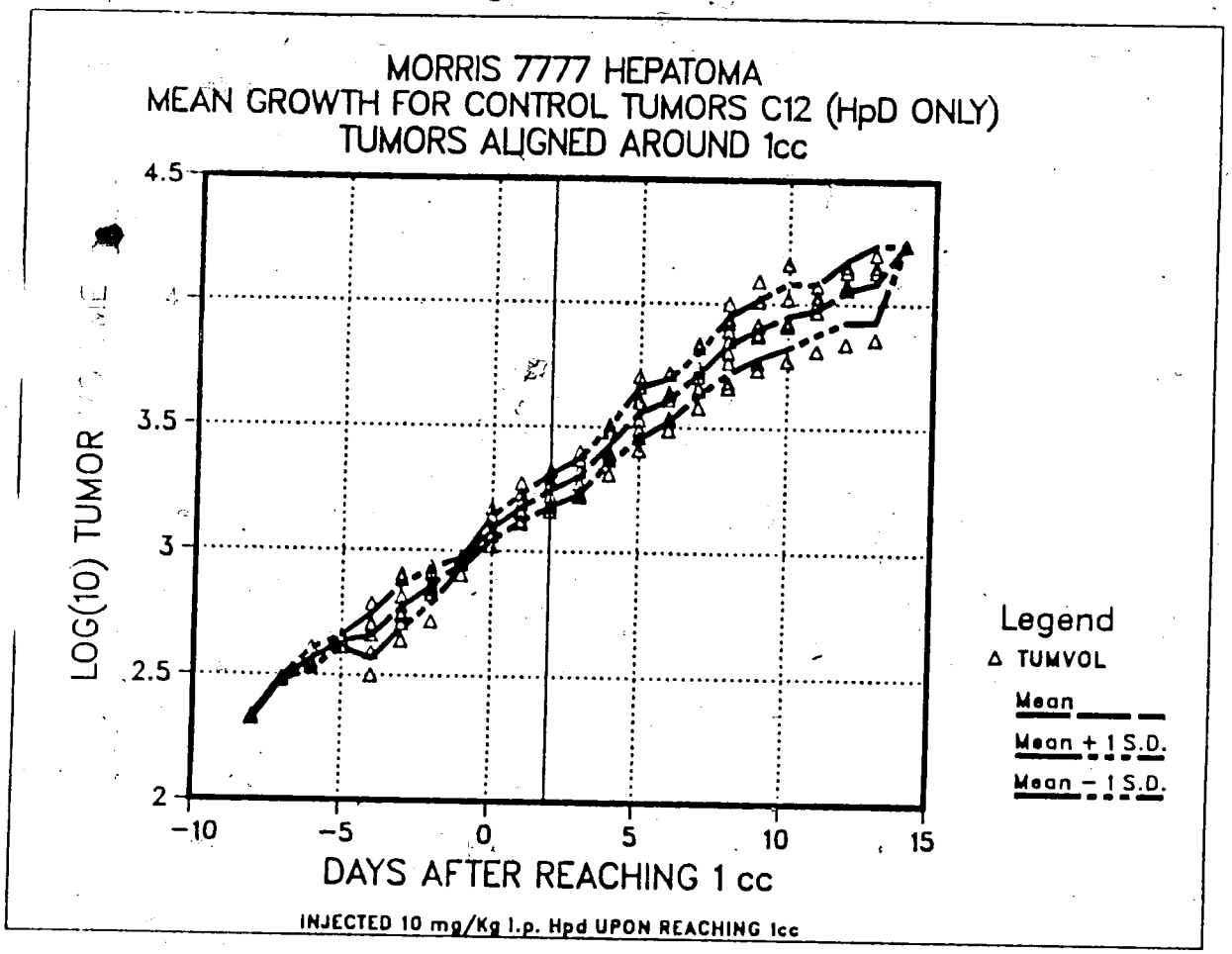
of many smaller projects the author undertook during this project).

Due to the thermal drift of large frame lasers it was neccessary to build a microprocessor controled geared down stepper motor which controlled the alignment optics. Two stepper motors were mechanically coupled to the rear laser alignment mirrors and were controled by a Motorola 6809 microprocessor using an Analog Devices 12 bit A/D converter to measure the optical power output of the laser via an internally mounted intracavity beam spitter coupled to a thermopile. The alignment system kept the laser output power was stable to a 2% drift over 6 hours (this was checked using a Grass chart recorder). The Dye laser did not require a feedback system since its power instabilties were mostly due to the larger pump laser drift. Most of the engineering problems encountered were straight forward and easily solved in a matter of weeks.

In conclusion, the optical and engineering problems that this type of cancer therapy presents are not inherently difficult. The problems which prevent this therapy from becoming a clinical reality will only be solved by the immuno-oncologist, for it is only they

who can deliver this drug specically to the neoplasm. Perhaps when the immuno-oncolgist has solved the problems of the Magic Bullet then a drug will come forth which may be activated by a means other than light. We can only hope.

figure 8.1


MORRIS 7777 HEPATOMA
MEAN GROWTH FOR CONTROL TUMORS C12 (HpD ONLY)
TUMORS ALIGNED AROUND 1cc
LOG(10) TUMOR
4.5
4
3.5
3
2.5
2
−10
−5
0
5
10
15
DAYS AFTER REACHING 1 cc
INJECTED 10 mg/Kg I.p. Hpd UPON REACHING 1cc
Legend
TUMVOL
Mean
Mean + 1 S.D.
Mean − 1 S.D.

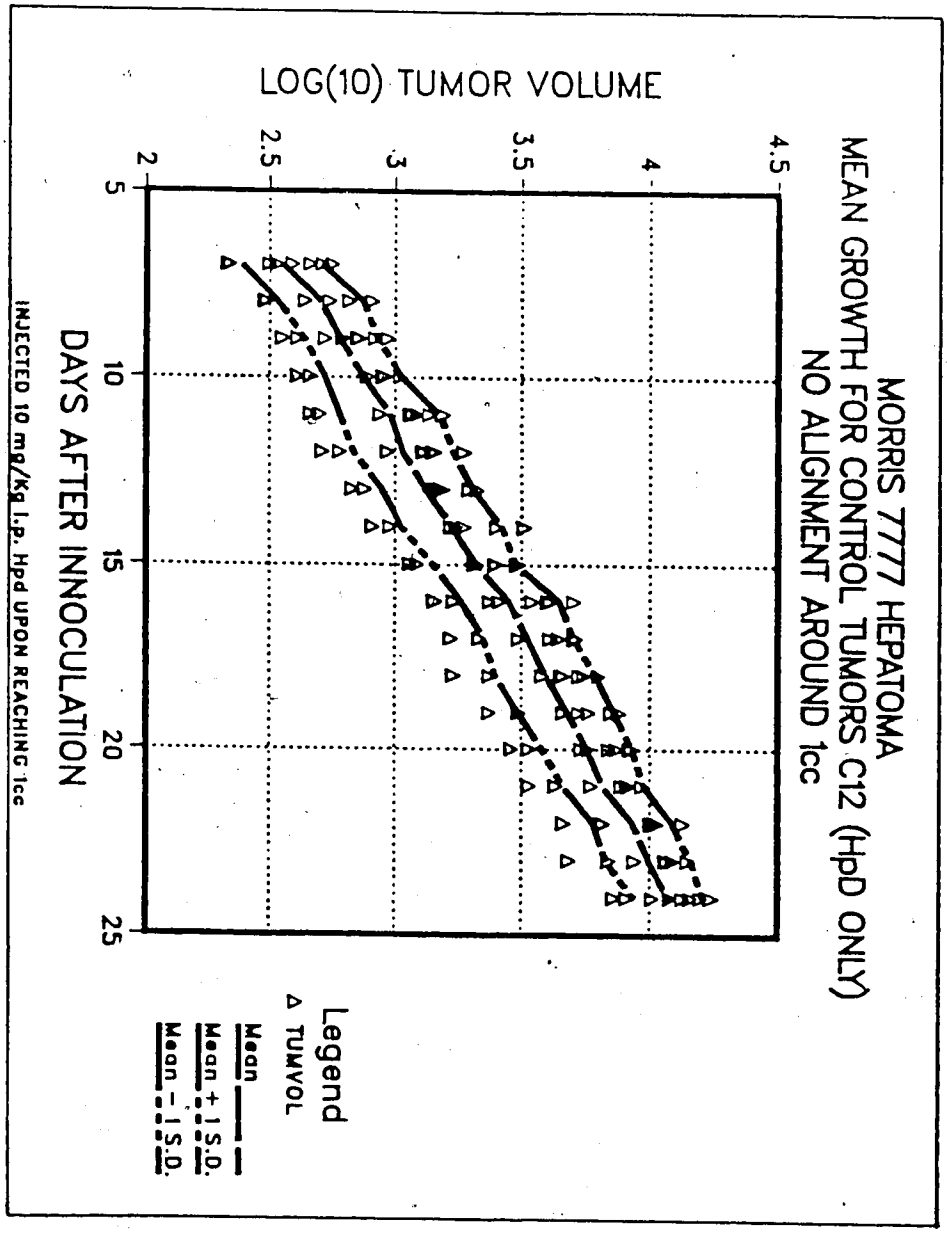

MORRIS 7777 HEPATOMA
MEAN GROWTH FOR CONTROL TUMORS C12 (HpD ONLY)
NO ALIGNMENT AROUND 1cc
LOG(10) TUMOR VOLUME
4.5
4
3.5
3
2.5
2
5
10
15
20
25
DAYS AFTER INNOCULATION
INJECTED 10 mg/Kg I.P. Hpd UPON REACHING 1cc
Legend
Δ TUMVOL
Mean
Mean + 1 S.D.
Mean - 1 S.D.

figure 8.2

CHAPTER 9

BIBLIOGRAPHY

1. Allison, A.C., Young, M.R.,
   - Life Science (Pergamon Press)
   (1964) 3:1407

2. Allison A.C., Magnus, I.A., Young, M.R.,
   - Nature,
   (1966) 209:874

3. Altman, K.I, Salomon, K.,
   - Nature,
   (1960) 187:1124 (London)

4. Andreoni, A., Cubeddu, R., De Silvestri, S., Laporta, P.,
   - Cancer Research
   (1983) 43:2076-2080

5. Asada, K.,
   'Active Oxygen'
   -Protein, Nucleic Acid, and Enzyme
   (1978) 23:200

6. Band, P., Feldstein, M., King, E.G., et al.,
   - In 'International Cancer Congress',
   Buenos Aires,
   (1978) pp. 168

7. Bonnet, R., Ridge, R. J., and Scourides, P.,
   - Chem. Soc. D Chem. Commun.
   (1980) 1980:1189-1199

8. Bonnet, R., Ridge, R. J., and Scourides, P.,
- J. Chem. Soc. Perkin. Trans
(1981) 1:3135-3140

9. Barker, D.S., Henderson, R.W., Storey, E.,
- Br. J. Exp. Pathol.,
(1970) 51:628

10. Benda,P., Lightbody, J., Sato, G.,
Levine, L,. and Sweet, W.
- Science
(1968) 161:370-371

11. Berenbaum, M.C., Bonnett, R., Scourides, P.A.,
- Br. J. Cancer
(1982) 45:571

12. Blum, H.F.,
In 'Carcinogenesis by Ultraviolet Light',
Princeton University Press,
(1959) pp. 160

13. Brem. S.,
- Clin Neurosurg
(1976) 23:440-453

14. Carpenter, R.J., Nell, H.B., Ryan,
R.J., Sanderson, D.R.,
- Ann. Otol. Rinhol. Laryngol.,
(1977) 86:661

15. Christensen, T., Moan, J.,
- Cancer Res.,
(1975) 39:3735

16. Christensen, T., Moan, J., Wibe E.,
Oftebro, R.,
- Br. J. Cancer,
(1979) 39:64

17. Christensen, T., Sandquist, T., Feren, K.,
Waksvik, H., and Moan, J.,
- Br. J. Cancer
(1983) 48:35-43

18. Clezy, P., Hai, T.T., Henderson, R. W.,
and Thuc, L. V.,
- Aust. J. Chem.

(1980) 33:585-597

19. Courtese, D. A., Kinsey, J. H., Woolner, L. B., Payne, W. S., Sanderson D. R., and Fontana, R. S., - Mayo. Clin. Proc. (1979) 54:635-642

20. Culbreth, P., et al., - Clin. Chem., (1975) 25:605

21. Curraco, C.J., Tsutsui, M., and McConnel, S., - Cancer Treat. Rep., (1977) 61:1297

22. Curraco, C.J., Tsutsui, M., and McConnel, S., - Chem. Bio,. Interact., (1978) 21:233

23. Delmelle, M., - Photochem. Photobiol., (1978) 28:357

24. Denekamp, J., Hirst, D.G., Stewart, F.A., Terry, H.H.A., - Br. J. Cancer (1980) 41:1

25. Diamond, I., McDonagh, A.F., Wilson, C.B., Granelli, S.G., Nielsen, S., Jaenicke, R., - Lancet, (1972) 2:1175-1177

26. Dougherty, T.J., - J. Nat. Cancer Inst., (1974) 52:1333-1336

27. Dougherty, T.J., Grindey, G.B., Fiel, R., Weishaupt, K.R., Boyle, P.G., - J. Nat. Cancer Inst., (1975) 55:115

28. Dougherty, T.J., Gomer, C.J., Wieshaupt, K.R., - Cancer Research, (1976) 36:2330

29. Dougherty, T.J., Kaufman, J.E., Goldfarb, A., Weishaupt, K.R., Boyle, D., Mittleman, A. - Cancer Research, (1978) 38:2628-2635

30. Dougherty, T.J., Lawrence, G., Kauffman, J.H., Boyle, D., Weishaupt, K.R., Goldfarb, A., - J. Nat. Cancer Inst., (1979) 62:231-237

31. Dougherty, T.J., - The J. of Investigative Dermatology, (1981) 77:122-124

32. Dougherty, T. J., - Cancer Res. (1982) 42:1188

33. Dougherty T. J., Porphyrin Photosensitization New York Plenum Publishing Corp. (1982) pp. 3-13

34. Dorion, D.R., et al., - Chest, (1979) 76:27

35. Dorion, D.R., Profio, A.E., - Proc. 9th Annual meeting of the American Society for Photobiology (1981) pp. 111

36. Dubbelman, T.M., et al., - Biochem. Biophys. Acta., (1978) 511:141

37. Endrich, B., Intaglietta, M., Reinhold, H.S., Gross, J.F., - Cancer Res., (1979) 39:17

38. Evans, N., Games, D.E., Jackson, A.H., Mattin, S.A., - J. Chromatogr., (1975) 115:325

39. Evans, N., Jackson, A.H., Matlin, S.A.,
Towil, R.S.,
- J. Chromatogr.,
(1976) 125:345

40. Felton, J.S.,
- Workshop on the role Sputum Cytology
- The Asbestos Worker LAC
- USC Compehensive Cancer Center, Los Angles,
(1978)

41. Fiel, R.J., Howard, J.C., Mark, E.H. Gupta, N.D.,
- Nucleic Acid Res.,
(1979) 6:3093

42. Figge, E.H.J., Weiland, G.S., Manganiello, L.O.,
- Proc. Soc. Exp. Bio. and Med.,
(1978) 64:640

43. Figge, E.H.J., Weiland, G.S.,
- Cancer Res.,
(1949) 9:549

44. Folkman, J.,
- N. Engl J. Med.
(1971) 285:1182-1186

45. Fontana, R.S., Sanderson, D.R., Woolner, L.B.,
- Chest,
(1974) 67:511

46. Forbes, I.J., Cowled, P.A., Leong, A.S.Y.,
Ward, A.D., Black, R.B., Blake, A.J.,
- Med. J. Aust.,
(1980) 2:489-493

47. Franzone, J.S., et al.,
- Bull. Chem. Farm.,
(1978) 117:235

48. Girotti, A.W.,
- Photochem. Photobiol.,
(1976) 24:525

49. Girotti, A.W.,
- Biol. Chem.,
(1978) 253: 7186

50. Girotti, A.W.,
- Biochem.,
(1979) 18:4403

51. Gomer, C.J., Dougherty, T.J.,
- Cancer Research
(1979) 39:146-151

52. Granelli, S.G., Diamond, I,. McDonagh, F.F.,
Nilson, C.B., Nielson, S.C.,
- Cancer Res.,
(1975) 35:2567

53. Gray, C.H., Lim, C.K., Nicholson, D.R.,
- Clin. Chem. Acta.,
(1977) 77:167

54. Gregorie, H.B., Horger, E.O., Ward, J.L.,
Green, J.F., Richards, T.,
Robertson, H.C., Stevenson, T.B.,
- Annals of Surgery,
(1968) 167:820

55. Harber, L.C., Hsu, J., Hsu, H., Goldstein, B.B.,
- J. Invest. Dermatol.,
(1972) 58:373

56. Haruo, H., Toshimitsu, M., Norio M.,
Journal of Urology
- (1983) 130:685-687

57. Hayata, Y., Kato, H., Ming-Chih, C., et al.,
- J. Jap. Dis. Chest,
(1977) 15:759

58. Horenhner, P., Rietund, T.,
- J. Chromatogr.,
(1976) 123:414

59. Horst, M.N., Basha, S.M.M., Baunbach, G.A.,
Mansfield, E.H., Roberts, R.M.,
- Anal. Biochem.,
(1980) 102:399

60. Jori, G., Tomio, L., Reddi, E., Rossi, E.,
Corti, L., Zorat, P.L., Calzavara, F.,
- (1983)

61. Kahn, E.A., Taren, J.A., Schneider, R.C. and Crosby, E.C.
'Correlative Neurosurgery'
Springfield: CC Thomas Publ.,
- (1969)

62. Kelly, J.F., Snell, M.E., Berenbaum, M.C.,
- J. Cancer,
(1975) 31:237

63. Kelly, J.F., Snell, M.E.,
- J. Urology,
(1976) 115:150

64. Kelly, P.J., Suddith, R.L., Hutchison, H.T., Werbach, K., and Harber, B.,
J. Neurosurg.
(1976) 44:342-346

65. Kessl, D.,
- Biochem Soc. Trans.,
(1977) 5:139-140

66. Kessel, D.,
- Biochem.,
(1977) 16:3443

67. Kessel, D.,
- Cancer Res.
(1981) 41:1381-1323

68. Kessel, D.,
- Cancer Res.
(1982) 42:1703-1706

69. Kessel, D.,
- Photochem. Photobio.
(1982) 36:99-101

70. Kessel, D., and Rossi, E.,
- Photochem. Photobio.
(1982) 35:37-42

71. Kessel, D., Chou, Ta-Hsu
- Cancer Research
(1983) 43:1994-1999

72. King, E.G., Saccamanno, G., LeRiche, J., et al.,
- In 'XIIIth World Congress on
Disease of the Chest',
Kyoto, Japan,
(1978) pp. 38

73. Kinsey, J.H., Cortese, D.A., Sande
- Mayo Clin. Proc.,
(1978) 53:594-600

74. Kinsey, J.H., Cortese, D.A.,
- Rev. Sci. Instrum.
(1980) 51:1403-1406

75. Kinsey, J.H., Cortese, D.A., Moses, R.L.,
Ryan, R.J., Branum, E.L.,
- Cancer Research
(1981) 41:5020-5026

76. Kinsey, J.H., Cortese, D.A., Nell, H.B.,
- Cancer Research
(1983) 43:1562-1567

77. Kohn, K.W.,
- In 'In Effects od Drugs on the Cell Nucleus'
Eds., Busch, H., Croooke, S.T., Daskal, Y.,
Academic Press, New York,
(1978) pp. 207-238

78. Kohn, K., Kessel, D.,
- Biochem. Pharmacol.,
(1979) 28:2465-2470

79. Koller, M.E., Romslo, I.,
- Biophys. Acta.,
(1978) 503:238

80. Langhof, H.,
Elsevier Publishing Co. Amsterdam,
(1961) pp. 429-430

81. Laws, E.R., Cortese, D.A., Kinsey, J.H.,
Eagan, R.T., Anderson, R.E.,
- Neurosurgery
(1981) 9:672-678

82. Leonard, J.R., Beck, W.L.,
- Laryngoscope,
(1971) 81:365

83. Levin, V.A., Clancy, T.P., Ausman, J. L.,
- Nat. J. Cancer Inst.,
(1972) 42:875-883

84. Lipsom, R.L., Baldes, E.J.,
- Arch. Dermatology,
(1960) 82:508

85. Lipsom, R.L., Baldes, E.J., Olsen, A.M.,
- J. Nat. Cancer Inst.,
(1961) 26:1-12

86. Lipsom, R.L., Baldes, E.J., Gray, M.J.,
- Cancer Research,
(1967) 20:2255-2257

87. Long, D.M.,
- J. Neurosurg.
(1978) 51:53-58

88. Mador, B., Ritchie, B., Meeker, R., Moore, R.,
Elliott, F.G., McPhee, M.S., Chapman, J.D.,
Lakey, W.K.,
- Cancer Treatment Reports
(1982) 66:1837-1843

89. Mathews-Roth, M.M., Pathak, M.A., Parrish, J.,
Fitzpatrick, T.B., Koos, E.H., Toda, K.,
Clemens, W.,
- J. Invest. Dermatol.,
(1972) 59:349

90. Mew, D., Wat, C., Towers, N., Levy, J.G.,
- J. Immunology
(1983) 130:1473-1477

91. Millar, V., Malina, L.,
- J. Chomatogr.,
(1978) 145:290

92. Moan F.,
- In 'Free Radicals in Biology',
Ed. W.A. Pryor.,
Academic Press,

(1976) pp. 51

93. Moan, J., Pettersen, E.O., Christensen, T.,
- Br. J. Cancer,
(1979) 39:398

94. Moan, J., Sommer, S.,
- Photobiochem. Photobiophys.
(1981) 3:93-103

95. Moan, J., Christensen, T., Sommer, S.,
- Cancer Letters
(1982) 15:161-166

96. Moan, J., et al.,
- Cancer Lett.,
(1979) 6:331

97. Moan, J., Christensen, T.,
- Photobiochem. Photobiophys.
(1981) 2:291

98. Mosely, R.V.,
- Surg.
(1967) 61:674-686

99. Musser, D.A., Wagner, J.M., and Danna-Gupta, N.,
- J. Nat. Cancer Inst.,
(1978) 61:1397

100. Patterson, A.R.P., Babb, L.R., Paran, J.H.,
Cass, C.E.,
- Mol. Pharmacol.,
(1977) 13:1147

101. Patrice T., Le Bodic, M., Le Bodic L.,
Spreux, T., Dabouis, G., Hervouet, L.,
- Cancer Research
(1983) 2876-2879

102. Paulson, O. B., and Hertz, M. M.,
- Eur. J. Clin. Invest.
(1978) 8:391-396

103. Peterson, D.A., McKelvey, S., Edmondson, .P.R.,
- Medical Hypotheses
(1981) 7:201-205

104. Perria, C., Capuzzo, T., Cavagnaro, G.,
Datti, R., Francaviglia, N.,
Rivano, C., Tercero, V.E.,
- J. Neurosurg Sci.
(1980) 34:119-129

105. Perria, C.,
- IRCS Medical Science
(1980) 9:57-58

106. Petryka, Z.J., Watson, C.J.A.,
- Anal. Biochem.,
(1978) 84:173

107. Phillips, C.A., Taylor, D.M.,
- Nuklearmedizin,
(1978) 17:262

108. Profio, E.A., Dorion, D.R.,
- Phys. Med. Bio.,
(1972) 22:949

109. Raport, S.I., Bachman, D. S., and Thompson, H.K.,
- Science
(1972) 176:1243-1244

110. Rassmussen-Taxdal, D.S., Ward, G.E.,
Figge, F.H.J., Jr.,
- Cancer,
(1955) 8:78

111. Reinhold, H.S.,
- Eur. J. Cancer,
(1971) 7:273

112. Roxas, M., Calandra, C., Matera, M., Lomagro, M.,
Prata, A., Clementi, G.,
- Acta. Neurol.,
(1978) 33:408

113. Sandberg, S., Romslo, I.
- Chim Acta
(1981) 109:193

114. Sanderson, D.R., Fontana, R.S., Lipsom, R.L.,
Baldes, E.J.,
- Cancer,
(1972) 30:1368

115. Santamaria, L.,
- In'Research Progression in Organic, Biological and Medicinal Chemistry' Eds. Gallo, U., and Santamaria, L., North Holland Publishing Co., Amsterdam, (1972) Vol. III, Part2, pp. 671-684

116. Schwartz, S., Berg, M.B., Bossenmaier, I., Dinsmore, H.,
- Methods Biochem. Anal., (1960) 8:221

117. Sery, T.W.,
- Cancer Res., (1979) 39:96-1000

118. Sigornelli, C.D., Ammirati, M., Tajana, G.,
- Clinica Neurochirurgica (1978) 33:105-112

119. Slater, T.F., Riley, P.A.,
- Nature, (1966) 209:151

120. Smolev, J.K., Warren, D.W., William, W.S., Coffey, D.S.,
- Cancer Treatment Reports (1977) 61:273-287

121. Stanyl, T.E., and Putnam, C.N.,
Int. J. Radiat. Onc. Bio. Phys. (1976) 1:959-964

122. Strom, R., et al.,
- Physiol. Chem. Phys., (1977) 9:53

123. Stumpf, D.A., McCabe, E.R.B., Parks, J.K., Bullen, W.W., Schiffy, S.,
- Biochem. Med., (1979) 21:182

124. Sullivan, R. D., and Semel, C. J.,
- In "Cancer Chemotherapy" Brodsky I., and Kahn, B., eds. New York Grune and Stanton (1972) pp. 453-458

125. Tappeiner, H.N., Jesionek, A.,
- Muench. Med. Wochensohr
(1903) 1:2042-2044

126. Thomson, S.H., Emmett, E.A., Fox, S. H.,
- Cancer Research,
(1974) 34:3124-3127

127. Tomio, L., Zorat, P.L., Corti, L., Calzavara, F.,
Reddi, E., Jori, G.,
- Acta Radiologica Oncology.
(1983) 22:49-53

128. Wieshaupt, K.R., Gomer, C.J., Dougherty, T.J.,
- Cancer Research,
(1976) 36:2326-2329

129. Winkelman, J., Rassmussen-Taxdal, D.S.,
- Bull., John Hopkins Hosp.,
(1970) 107:228-233

130. Wise, B.L., Taxdal, D.R.,
- Brain Research,
(1967) 4:387-389

131. Warrington, R.C.,
J. Natl. Cancer Inst.,
(1978) 61:69

132. Zalar, G.L., Poh-Fitzpatrick, M., Krohn, D.L.,
Jacobs, R., Harber, L.C.,
- Arch. Dermatol.,
(1977) 113:1393

133. Zawirsaka, B.
Neoplasma,
(1979) 26:223