AN EXAMINATION OF CROSS-RESISTANCE TO PHOTODYNAMIC THERAPY AND ULTRAVIOLET LIGHT IN RODENT CELLS USING A VIRAL CAPACITY ASSAY

BY

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ABSTRACT

Photodynamic therapy (PDT) for cancer utilizes the localised delivery of light to activate a photosensitizing drug (such as Photofrin) which is selectively retained by tumour tissues. Although this treatment modality is undergoing clinical trials, the mechanism of PDT cytotoxicity is not fully understood. One approach to understanding the mechanism of action of PDT is to study cell mutants showing alterations in their response to PDT.

The capacity of mammalian cells to support virus replication has been used as an assay to compare cellular response to ultraviolet (UV) light and other cytotoxic agents. Cellular capacity has been previously employed to measure cellular recovery following UV irradiation and to predict the sensitivity of cells to several anticancer drugs.

In this work, I have examined the use of the capacity assay to examine the sensitivity of cells to PDT treatment. RIF-1 mouse fibrosarcoma cells and a PDT resistant derivative, RIF-8A; as well as Chinese hamster ovary (CHO) cells and CHO-MDR (multi-drug resistant) mutant cells were studied. Consistent with the clonogenic survival of these cells after PDT, the viral capacities of RIF-8A and CHO-MDR cells were greater than those of RIF-1 and CHO cells respectively following PDT treatment.

The capacity assay was also used to examine the relative ability of RIF cells to

recover from PDT damage. These capacity experiments show that recovery from PDT damage is greater in RIF-8A cells compared to RIF-1 cells, suggesting that RIF-8A cells have an enhanced repair capacity for PDT damage compared to RIF-1 cells.

The response of CHO cells and the RIF cells to ultraviolet (UV) light irradiation was also examined. No difference in the UV sensitivity of CHO-N and CHO-MDR cells was found. However, the RIF-8A cell line showed a cross-resistance to UV. The survival of viral DNA synthesis for UV-irradiated virus was also greater in RIF-8A cells compared to RIF-1 cells, suggesting that RIF-8A cells have an increased capacity for the repair of UV-induced DNA damage. It is possible that the increased resistance to PDT of RIF-8A may also result from an increased repair of PDT-induced DNA damage in RIF-8A cells.

The identification and characterization of a PDT-sensitive cell mutant was also investigated. The CHO-AUXB1 cell mutant was found to show an increased sensitivity to PDT and UV compared to the CHO-PRO- parent line. This sensitivity of the CHO-AUXB1 mutant may also result from a deficiency in the repair mechanism of PDT-induced and UV-induced damage.

PREFACE

This thesis is presented in 5 chapters. The first chapter is an introduction to explain and review general concepts presented in the thesis. The second chapter presents data from initial experiments used to determine the appropriate parameters for the capacity experiments described in chapters three and four. The third chapter is the first section of the study written in preparation to be submitted for publication. In this chapter, I examine the use of the capacity assay to examine the sensitivity of rodent cells to PDT and UV. The capacity assay is also used to examine the ability of rodent cells to recover from PDT damage. The fourth chapter is the second section of the study also written in preparation to be submitted for publication. In this chapter I examine the cross-sensitivity of a CHO mutant to PDT and UV. These chapters are followed by a summary chapter of all work, including possible future directions for this work.

All work presented in this thesis has been carried out by the author with the exception of the clonogenic survival experiments. In chapter three, the split-dose clonogenic survival experiments were carried out by Paula Deschamps. In chapter four, the clonogenic survival experiments and the drug uptake experiments were carried out by Merna Espiritu.

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DEDICATIONS

This work is dedicated to my family and Bruno, for their continued confidence in what I could accomplish.

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I would like to thank my supervisor, Dr. A. J. Rainbow for his time and patience, without whose help and guidance I would not have completed this study.

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I would finally like to thank the other inhabitants of lab 406, Murray, Kelly, Bruce, Todd, Lianne, Chun-Yip and Cathy, who made 'life in the lab' a great one.

CHAPTER ONE

INTRODUCTION

INTRODUCTION

A. Cancer and cancer therapies.

Cancer is a small name that encompasses a broad category of diseases that involve the presence of malignant cells. Generally, the malignant cell does not grow faster than normal cells and goes through all four phases of the cell cycle. The cancer cell can be easily defined by three characteristics which render it virtually lethal to the human body. i) Tumour cells are defective in regulatory mechanisms that normally prevent inappropriate proliferation (Weinberg, 1985; Goustin et al., 1986). ii) Tumour cells continue to proliferate under circumstances in which normal cells do not seem to grow. Normal cells stop dividing when cells contact one another and a confluent monolayer is formed. This is termed contact inhibition. Transformed cells will undergo several changes which lead to the loss of contact inhibition (Abercrombie, 1970). iii) Tumour cells are also resistant to conditions which would produce serious damage to other cells, such as exposure to chemotherapeutic agents (for a review see Moosa et al., 1991 and Rubin, 1993).

The fight against cancer is progressing forward with continual advances in the mechanics of the disease and innovative new approaches in treatment. Conventional means of cancer treatment include chemotherapy, radiation and surgery and combinations of these three modalities. Although all these therapies are being used

with great success, there are a number of limitations to their effectiveness. First with regards to chemotherapy, the use of extremely toxic anticancer agents such as alkylating agents, antibiotics, antimetabolites, specific mitotic inhibitors, steroidal hormones and miscellaneous drugs such as cis-platinum complexes (as reviewed in Moosa, 1991) poses a threat to all cells of the body, both healthy and cancerous. As a result, a number of side effects are common, which include diminished platelet and white cell production, toxic effects on the kidneys and auditory nerves, hair loss, nausea and vomiting. Chemotherapy is also often marked by limited efficacy, such as an induced drug resistance. Secondly, with regards to radiation therapy and surgery, both are limited by the localization of the tumour site and inaccessibility.

B. Photodynamic Therapy (PDT).

B.1. History.

Photodynamic therapy is a novel cancer therapy that utilizes the localized delivery of light to activate a photosensitizer which is selectively retained by tumour tissue, resulting in tumour tissue necrosis. Photodynamic therapy requires three simultaneously present components for cytotoxicity: a sensitizer, light and oxygen.

In the early 1900's the use of light-activated materials to treat human cancer was first introduced to treat skin tumours using topical eosin, activated by white light (Jesionek and Tappenier, 1903). No subsequent clinical work was carried out until the mid-1970's following the development of the hematoporphyrin derivative (Hpd). Early

work on the feasibility of PDT as a potential treatment for cancer was initiated by Lipson et al., (1961), who were able to purify a hematoporphyrin derivative that was better localized in tumour tissue than previously used photosensitizers. The purified hematoporphyrin had a greater tendency to accumulate in tumour tissue rather than the normal surrounding tissue.

Work by Diamond et al., (1972) utilized the administration of hematoporphyrin followed by light therapy to kill glioma cells in culture and to destroy porphyrin-containing gliomas transplanted subcutaneously into rats. In similar types of experiments, it was shown that animal tumours can be largely destroyed by the combination of fluorescein and visible light and Hpd and light (Dougherty, 1974; Dougherty et al., 1975).

B.2. Types of Photosensitizers used and Light Delivery.

The ideal properties of an effective photosensitizer can be easily summarized. i) A sensitizer should be red or near infrared light absorbing and the photosensitizer must be non-toxic to the patient, with low skin photosensitizing potency. In the visible and ultraviolet (UV) range of wavelengths, only red and near infrared light is not absorbed appreciably in tissue. The photosensitizer should therefore absorb strongly in the red but not absorb elsewhere in the solar spectrum. Since the photosensitizer is delivered intravenously to the patient, there are initially high concentrations throughout the body. This property will reduce the patient's susceptibility to photodamage by sunlight. In the 600-1000 nm spectral region, light is scattered to

a small extent by most mammalian tissues. Light of this wavelength is poorly absorbed by endogenous chromophores such as melanin, cytochromes and haemoglobin. As a result, red light possesses a high penetration power into human tissues and can be selectively absorbed by photosensitizing agents (Jori, 1992). ii) Some degree of selective retention of the dye in tumour tissue is needed to ensure for the selective destruction of tumour tissue, leaving surrounding tissue undamaged. iii) The sensitizer should be well photoactivated by efficiently generating cytotoxic species, usually singlet oxygen, that will destroy tissue. iv) A fluorescent photosensitizer provides a monitor of the location of the sensitizer in the tissue. v) The sensitizer should have a defined chemical composition and preferably be water soluble (as reviewed in Dougherty, 1987; as reviewed in MacRobert and Phillips, 1992).

B.2.i. Photofrin II.

Photofrin II (PH II) (Quadra Logic Technologies Inc., Vancouver, British Columbia), a purified form of the hematoporphyrin derivative, is currently being utilized in Phase III clinical trials. PH II is a mixture of monomeric and oligomeric hexar derivatives of hematoporphyrin units linked via ether or ester bonds (figure 1, as modified from Dougherty and Marcus, 1992) (as reviewed in Dougherty, 1987; Byrne et al., 1990; Jori, 1992).

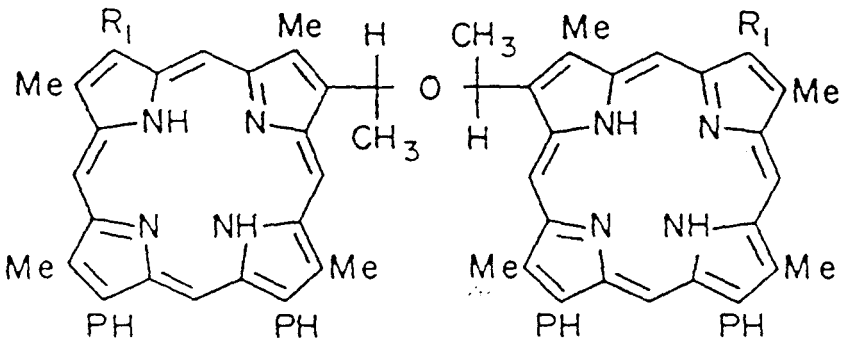
B.2.ii. Other types of Photosensitizers.

Chlorins. The chlorins are reduced, hydrophillic porphyrins. The chlorins absorb

Figure 1: Structure of Photofrin.

The porphyrin trimer is a major component of Photofrin. The R groups represent $CH(OH)CH_3$ or $CH = CH_2$. The PH groups represent $(CH_2)2COOH$.

(as modified from Dougherty and Marcus, 1992)


R1
Me
H
CH3
Me
R1
Me
O
NH
N
CH3
H
NH
N
Me
N
NH
N
NH
Me
Me
Me
Me
PH
PH
PH
PH

strongly in the 640-700 nm range (red light). Their absorption properties can be altered as a result of their chelation with metals (Spikes, 1990; Pandey et al., 1991). The chlorins include chlorin e6 (Gomer and Ferrraro, 1980; Nelson et al., 1987); purpurins (Spikes, 1990) and benzoporphyrin derivatives (Kessel, 1989; Richter et al., 1990; Richter et al., 1991).

Pthalocyanines. Pthalocyanines are synthetic porphyrins that absorb strongly in the 675-700 nm range. They can be chelated with a variety of metals (aluminum and zinc) and these metals enhance their phototoxicity (Kol, 1986; as reviewed in Spikes, 1986; Stern et al., 1990).

Cationic sensitizers. Lipophilic cationic dyes such as rhodamine 123 have been investigated. Unlike porphyrin derivatives, cationic dyes cause direct photodamage to the tumour tissue while sparing the epidermis and dermis. Higher light doses are required and some of these dyes exhibit toxicity in the absence of light (Modica-Napolitano and Aprille, 1987).

Porphines. The synthetic porphyrins known as porphines are meso-tetra-hydroxyphenylated porphyrins. The sulfonated derivative TPPS4 is H_2O soluble and exerts its phototoxicity by inhibition of microtubular function (Kessel et al., 1987; Kreimer-Birnbaum, 1989).

A variety of new photosensitizing systems will soon be evaluated in clinical trials. The main attributes of these second generation photosensitizers are that they have: i) a lower potential for prolonged photosensitization; ii) more rapid uptake and clearance from tumours and iii) activation by longer, more penetrating wavelengths

of light. Two second generation photosensitizers in Phase I trials are benzoporphyrin derivative monoacid ring A (BPD-MA) and mono-1-aspartyl chlorin e6 (NPe6) (Allan et al., 1992; Lui et al., 1992).

B.2.iii. Light Source and Delivery.

Light exposure of the tumours is carried out between 48 and 72 hours post-injection of the photosensitizer using a laser light at 630 nm wavelength. Conventional incandescent or arc lamps with wavelength filtering are used as illumination sources in the laboratory and for treating surface lesions. Most PDT applications use lasers as the light source largely because high light power can be coupled into single strand optical fibres for endoscopic or interstitial use. Presently, PDT dose determination is in terms of incident light fluence and the administered quantity of the photosensitizer. The differences in light penetration in individual tumours is not measured by this protocol. It partially accounts for the differences due to irradiation geometry and ignores the variation in photosensitizer uptake by the tumour. The depth distribution of the light, the concentration of the photosensitizer in the tumour and the tumour oxygenation must also be included to estimate an accurate PDT dose (as reviewed in Wilson, 1989).

B.3. Selective Tumour Retention.

A number of studies have shown a difference in the preferential uptake of photosensitizer when comparing normal and tumour tissue, depending on the

photosensitizer being used and the specific cancer tissue being treated (Bellnier et al., 1989; Meyer et al., 1991; Biolo et al., 1994). Tumour to tissue ratios with most sensitizers currently being used in laboratory and clinical studies, range from two to one (2:1) to five to one (5:1) (as reviewed in Pass, 1993), which is sufficient for effective photodestruction of the tumour tissue with limited damage to normal tissue.

One of most important features of photosensitizers is their selective retention in tumour tissues. Bugelski et al., (1981) utilized autoradiographic techniques to compare Hpd distribution in normal and tumour tissue of the mouse. They concluded that tissue factors such as vascular permeability, lack of adequate lymphatic drainage and nonspecific binding of serum proteins to stromal elements may be responsible for, or contribute to, the preferential uptake and retention of Hpd in both human and animal tumours. Kessel and Chou (1983), by measuring absorbance and through chemical structure analysis, suggested that tumour localization of Hpd in mice can result from intracellular conversion of a permeable Hpd component to a poorly diffusible product that cannot escape tumour tissue. The acidity of tumour tissue favours hematoporphyrin and hematoporphyrin derivative incorporation (Brault, 1990). The number of low-density lipoproteins (LDL) receptors is also increased in tumour cells and as a result, it is thought that porphyrin accumulation by tumours results from its delivery to cells via the LDL pathway because of the hydrophobicity of the photosensitizer (Kessel, 1981; Jori et al., 1984; Reyftman, 1984; Kessel, 1986a; as reviewed in Maziere et al., 1990). Tumour uptake and retention of porphyrins seems to be related to a number of properties specific to tumour tissue, such as a leaky

vasculature, poor lymphatic drainage, the presence of macrophages that take up aggregated material, low pH, an elevated concentration of lipoprotein receptors and the deposition of newly synthesized collagen in tumours (as reviewed in Moan, 1986b).

Novel methods of enhancing the tumour to normal skin drug ratio are being developed. They include liposomal drug formulations to enhance low density lipoprotein receptor mediated drug uptake by tumours (Biolo et al., 1994) and antibody-conjugated photosensitizers to more specifically target tumours (Mew et al., 1985).

B.4. Mechanisms of Damage.

Three pathways of photodynamic destruction of tumour tissue have been proposed. The primary mechanism of tumour destruction in PDT is mediated by cytotoxic singlet oxygen as well as free radicals.

The sensitizer is transformed from its ground state (singlet state) into an electronically excited state (triplet state) via a short lived excited singlet state. The excited triplet state can undergo two kinds of reactions: i) it can react directly with either substrate or solvent to form radicals and radical ions which then react with oxygen to produce oxygen radicals or ii) it can directly react with oxygen to form singlet oxygen. The activated oxygen species then interacts with tissue and results in tumour necrosis (as reviewed in Dougherty and Marcus, 1992; as reviewed in Henderson and Dougherty, 1992). The chemiluminescence caused by

photosensitization of porphyrins in saline has provided evidence that the second type of reaction, which directly produces singlet oxygen occurs significantly in the photosensitizing reaction (Takemura et al., 1992).

Photodynamic therapy may also destroy the tumour indirectly by damaging its vascular system, which results in hypoxia and tissue death (Nelson et al., 1987; van Geel et al., 1994).

Recent studies of immunosuppression, modulation of cyclooxygenase and cytokine release have supported the immunological involvement in cytotoxicity by photodynamic therapy (Evans et al., 1990; Fingar and Weiman, 1990; Henderson and Donovan, 1989; Lynch et al., 1989; Nseyo et al., 1990; Liu et al., 1994).

Apoptosis also is believed to play a role in cellular death resulting from PDT. Apoptosis is defined as programmed cell death (Williams et al., 1992). Evidence of apoptosis has been seen in a number of studies of cells treated with PDT using chloroaluminum pthalocyanine (AlPc) and PH II (Agarwal et al., 1991; Oleinick et al., 1991; Agarwal et al., 1993). Activation of programmed cell death (apoptosis) has also been identified as the cytotoxic mechanism in the treatment with a number of chemotherapeutic agents (Barry et al., 1990; Walker et al., 1991; Cotter et al., 1992). However, a recent study of mammalian carcinoma cell lines found that not all cell lines showed an apoptotic response following PH II-PDT (He et al., 1994). Thus, although apoptosis can occur during PDT-induced death, apoptosis is not required for cell death following PDT.

B.5. The Damaging Species in PDT.

There is a great deal of indirect evidence to suggest that singlet oxygen is the major damaging species in PDT. When oxygen is not present in the system or is present at extremely low levels, cells are resistant to PDT. Under anaerobic conditions the cytotoxicity of Hpd and red light was completely inhibited in Raji cells (Lee See et al., 1984). Mitchell et al., (1985) found that hypoxic Chinese hamster V79 cells were extremely resistant to the lethal effects of Hpd and light. **In vivo** experiments also supported the finding that at low oxygen levels there was reduced effectiveness of PH II-mediated photodynamic cell kill (Henderson and Fingar, 1987; Chapman et al., 1991b). Further, the use of quenchers of singlet molecular oxygen produced efficient inhibition of the photodestructive process in photochemical systems (Weishaupt et al., 1976; Krasnovsky et al., 1992).

There are a number of acute cellular and vascular effects of photodynamic therapy on cells and tissues.

B.6. The Cellular Effects.

As reviewed in Moan (1990), the diffusion length of singlet oxygen is limited to approximately 1 μm, which suggests that damage from singlet oxygen, as in PDT, must occur very near the site of generation of the singlet oxygen species. Three primary targets of porphyrin-sensitized photodynamic inactivation of cells have been identified: cell membranes, mitochondria and DNA (as reviewed in Moan, 1986b).

It has been shown that the site of photosensitization varies as a function of

time (Kessel, 1986b; Chapman et al., 1991a), thus the site of damage is related to the localization of the photosensitizer at the particular time of irradiation.

B.6.i. Membrane damage.

The first type of damage to result from the PDT (Hpd) cytotoxic process is membrane damage (Kohn and Kessel, 1979; Svenstrom et al., 1980; Bellnier and Dougherty, 1982; as reviewed in Valenzano, 1987). This damage involves oxidation of membrane lipids and cholesterol, a process termed lipid peroxidation (Thomas and Girotti, 1989; as reviewed in Girotti, 1990). Crosslinking of membrane proteins may occur as well as the oxidation of amino acids. Girotti (1976) presented evidence, using an early photosensitizer (protoporphyrin IX) and blue light, of significant cross-linking in the membrane prior to the onset of immediate haemolysis. Membrane damage (mediated by protoporphyrin, Hpd and PH II) also includes disruption of cell surface enzymes and transport systems (Kessel, 1977; Dubbelman et al., 1980; Biade et al., 1992).

B.6.ii. Mitochondrial Damage.

Mitochondrial damage is also believed to be a primary cytotoxic target of PDT. Fluorescence analysis has shown intracellular binding of Hpd to mitochondria (Berns et al., 1982). Mitochondria proved to be the most lethal photosensitive target in comparative studies relating phototoxicity and subcellular localization (Woodburn et al., 1992). There are significant photoradiation-induced (Hpd) inhibitions of important

mitochondrial enzymes, such as succinate dehydrogenase and cytochrome c oxidase (Hilf et al., 1984). Porphyrin-mediated PDT has also shown to cause inhibition of ATPase (PH II) (Atlante et al., 1990) and uncoupling of oxidative phosphorylation (Hpd) (Boeghiem et al., 1988). Hpd-photosensitization has also been shown to result in reduced cellular ATP levels (Hilf et al., 1986). In a recent study by Sharkey et al., (1993) it was demonstrated that there is a possible association between mitochondrial alterations and resistance to PH II-mediated PDT. The characterization of a PDT-resistant murine cell line, RIF-8A revealed striking differences in mitochondrial appearance and kinetics in comparison to the parental RIF-1 cells. The RIF-8A cells contained smaller mitochondria with more electron-dense matrices. For an equivalent rate of oxygen consumption, RIF-8A cells contained a greater intracellular ATP pool, suggesting an altered energy metabolism when compared to RIF-1 cells. A human ovarian carcinoma cis-diamminedichloroplatinum (II) (cisplatin) resistant line was found to be cross-resistant to PDT. The mitochondria in these cells demonstrate striking similarities to the mitochondria of the RIF-8A cells (Andrews and Albright, 1992). The RIF-8A cells were also found to exhibit a cross-resistance to cisplatin (Sharkey et al., 1993).

B.6.iii. DNA Damage.

DNA is also damaged by photodynamic treatment. Although very little amounts of Hpd accumulate in the nucleus compared to other cellular components, DNA damage is possible if singlet oxygen reacts with DNA that is sufficiently near the

nuclear membrane. DNA-protein cross-links, single strand breaks, sister chromatid exchanges, alkali labile lesions and DNA-DNA cross-links have been detected following PDT mediated by both Hpd and pthalocyanine (Gomer, 1980; Fiel et al., 1981; Ramakrishnan et al., 1988 and 1989; Oleinick et al., 1991; Gantchey, 1994).

There is evidence for a mutagenic action following PDT. The treatment of L5178Y (LY) cells with pthalocyanine plus light and Photofrin II plus light is mutagenic at the thymidine kinase (tk) locus (Evans et al., 1989; Rerko et al., 1992). Deahl et al., (1993) showed that Photofrin II and AlPc photosensitization-induced mutation at the heterozygous tk locus in strains of LY cells were caused by large DNA lesions. V79-379A cells were shown to be significantly mutagenic at the hypoxanthineguanine phosphoribosyl transferase (hprt) locus following treatment with tetra(3-hydroxyphenyl) porphyrin (3THPP) plus light (Noodt et al., 1993).

There is an inhibition of DNA repair enzymes in PDT (Hpd) treated cells (Musser et al., 1977; Munson, 1979; Crute et al., 1986; Boeigheim et al., 1987; Dubbelman, 1992). Kvam and Stokke (1994) used a method to label the sites of DNA repair after (meso-tetra (4-sulfonatophenyl) porphyrin-mediated) PDT. Using the proliferating cell nuclear antigen (PCNA) protein, which binds selectively to damaged DNA and participates in excision repair, they showed that the periphery of the nuclei was selectively labelled. This is direct evidence of the induction of DNA damage and the localization of repair sites.

DNA replication has also been found to be affected by Hpd-mediated PDT treatment. Lin et al. (1986) have shown that there is an inhibition of DNA and protein

synthesis and cell division by photoactivated hematoporphyrin derivative in hamster ovary cells.

B.7. The Vascular Effects.

It is believed that for the **in vivo** situation the vasculature appears to be the major initial target (PH II) (Henderson et al., 1985). This is the development of microscopic and macroscopic PDT tissue damage. The sequence of events begins with clumps of aggregated platelets, followed by transient vasoconstriction, vasodilation and eventual complete blood stasis and haemorrhage (PH II) (as reviewed in Henderson and Dougherty, 1992). A number of groups have shown that tumour cell death following Hpd-mediated PDT is the indirect result of destruction of tumour blood circulation (Selman et al.,1984; Star et al., 1986; Chauduri et al., 1987). Fingar et al., (1990 and 1992) have shown that following PH II-mediated PDT, there is localized release of thromboxane (an eicosanoid - a biologically active molecule which is synthesized from lipid precursors in response to cellular damage), from cells or platelets causing vasoconstriction and platelet aggregation which lead to vascular stasis. PDT elicits systemic toxicity in the form of a traumatic shock syndrome, also characterized by the release of prostaglandins (an eicosanoid). Traumatic shock can be produced by tissue injury which leads to inadequate peripheral perfusion. The final result is circulatory collapse (Reed et al., 1989; Ferrario and Gomer, 1990; Tromberg et al., 1990). van Geel et al., (1994) examined the influence of PH-mediated PDT on vascular perfusion in two mouse tumours. The most severe reductions in tumour

blood flow were associated with the longest regrowth delays indicating a major role for vascular damage in tumour response to PDT.

The specific PDT cytotoxic pathway could possibly involve a number of simultaneous sites of damage, or possibly a sequence of damages that lead to cell death. It appears that for the **in vivo** situation, most of the tumour cells are initially sublethally damaged and die subsequently from vasculature collapse (Henderson and Fingar, 1994).

B.8. The Late Effects.

The late or side effect of PDT is prolonged cutaneous photosensitivity. All patients undergoing PH II-PDT will remain photosensitive to light for up to 6 weeks after drug injection (Razum et al., 1987; Roberts et al., 1989; Dougherty et al., 1990).

B.9. The Treatment.

The patient usually receives Photofrin II intravenously at a dose of 2 mg/kg of body weight. Two or three days after injection, the patient undergoes light irradiation to the tumour site (as reviewed in MacRobert and Phillips, 1992; as reviewed in Nseyo, 1992) (figure 2, as modified from MacRobert and Phillips, 1992).

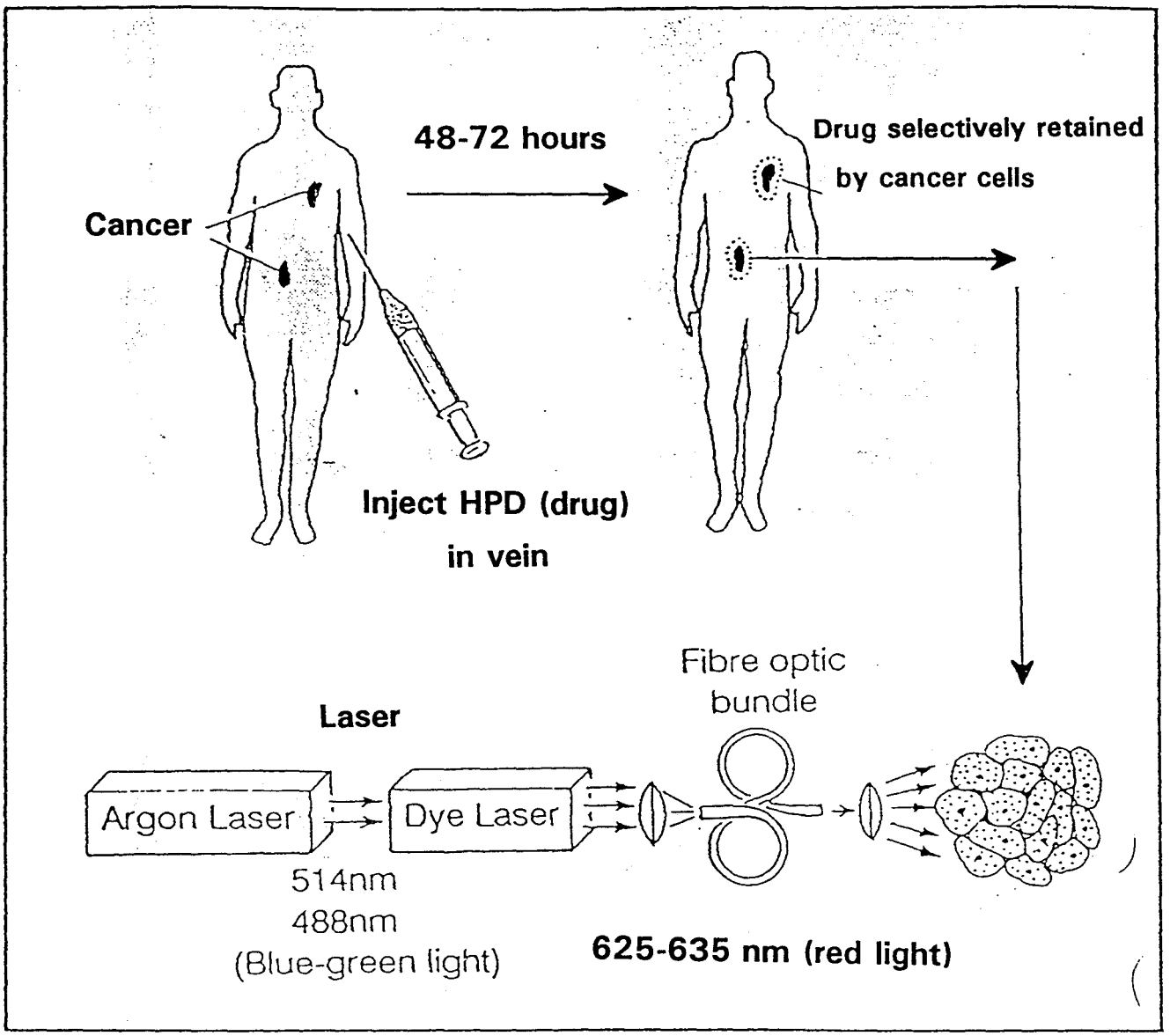
B.10. Tumour Response in Clinical Trials.

PDT is currently in Phase III clinical trials, although the exact mechanism of

Figure 2: Photodynamic Therapy Treatment.

The patient usually receives Photofrin II intravenously at a dose of 2 mg/kg of body weight. Two or three days after injection, the patient undergoes light exposure to the tumour site.

(as modified from MacRobert and Phillips, 1992)


48-72 hours
Cancer
Drug selectively retained
by cancer cells
Inject HPD (drug)
in vein
Fibre optic
bundle
Laser
Argon Laser
Dye Laser
514nm
488nm
(Blue-green light)
625-635 nm (red light)

cytotoxicity is not clearly defined. The Phase III study is the most rigorous and extensive type of scientific clinical investigation of a new drug or therapy. Cancers being currently investigated as potentially benefitting from PDT are superficial bladder cancer, gastrointestinal cancer, endobronchial lung cancer, gyneologic malignancies, head and neck cancer, intracranial tumours, ocular cancer and of cutaneous and subcutaneous tumours (Li et al, 1990; as reviewed in Pass, 1992; as reviewed in Spinelli and Dal Fante, 1992; as reviewed in Marcus, 1992).

Alternative uses of PDT, in conjunction with the treatment of cancer sites, include the use of PDT for the palliation of obstructing tumours. Also, the selective concentration of the photosensitizer in tumour tissue has allowed its use as a diagnostic tool by means of fluorescence detection (Dougherty, 1987; Mang et al., 1993). Other than oncological use, some laboratories are utilizing PDT as a possible means of purifying virus-infected blood (Allen et al., 1994).

It is obvious that PDT is an exciting and innovative investigational therapy for human cancer. Advances in light delivery systems and development of new photosensitizers will make PDT a very valuable therapeutic tool. Inevitably this will result in the improved ability of physicians to treat certain types of human cancers. With stringent localization of drug and focal delivery of light to the tumour site, there will be increased reduction of toxicity to the cancer patient, unlike conventional chemotherapy.

C. Predicative Assays of Tumour Response.

C.1. Chemotherapy and Radiotherapy.

The use of an **in vitro** system for quantifying inherent cellular sensitivity to chemotherapy and radiotherapy has become an important tool to predict human tumour response on an individual patient basis. A number of studies have attempted to assess the correlation of **in vitro** sensitivity of cell lines derived from human tumours with the clinical responsiveness of tumours.

C.1.i. Clonogenic Survival Assay.

Clonogenic cell survival, to date, is the most widely used assay in determining the cellular response to anticancer agents, including radiation. The clonogenic survival assay, simply measures the ability of cells to reproduce and form a colony in culture. Clinical studies have shown that the clonogenic assay can accurately predict drug sensitivity (Shrivastav et al., 1980; Tanigawa et al., 1982; Tveit et al., 1982; Kirkwood and Marsh, 1983; Gazdar et al., 1990; vanHoff et al., 1990; Parchment et al., 1992) and radiosensitivity (Michalowski, 1984; Twentyman et al., 1984; as reviewed in Peters, 1990) of human tumour biopsy cells.

The use of the clonogenic survival assay is limited by the poor plating efficiency of human tumour cells, clumping artifacts and the duration of the assay (Bertoncello et al., 1982; Selby et al., 1983; Alley and Lieber, 1984), as a result, a number of short-term assays have been developed.

C.1.ii. The Colorimetric Assay.

The colorimetric assay, utilizes the exclusion of vital dyes as a method to assay growth rate. Cells from an established cell line or from a tumour biopsy specimen are plated into multi-well plates. The plates are then treated with a cytotoxic drug or radiation and allowed to grow for several days. The cells are stained and cell growth quantitated based on the assumption that only viable tumour cells are stained. The density of the stain in each well is measured by a spectrophometer and correlates with the number of surviving cells. The most widely used colorimetric assay is the tetrazolium (MTT) assay (Carmichael et al., 1987; Alley et al., 1988).

More recently, the technique has been modified to utilize dyes that can measure the total protein content or total DNA content of a well. One of the latter is the Hoescht DNA fluorochrome assay. The assay requires exposure of nuclear DNA to a fluorochrome which, when bound to DNA, is excited to 360 nm to emit at 460 nm. DNA fluorescence is measured by a spectrophometer and correlates with the number of cells present. These clonogenic assays do not seem suitable as predicative assays for tumour response, but seem more beneficial as wide use in an initial screening of anti-cancer drugs.

C.1.iii. The Cell Adhesive Matrix Assay.

The basis of the cell adhesive assay is to provide tumour cells from a biopsy specimen with an optimal substrate for cell attachment and growth. The surface of plastic dishes is coated with a mixture of fibronectin and fibrinopeptides (CAM) and

cells are grown in a special medium supplemented with hormones and growth factors. Cells are seeded into 24-well plates and treated. Following treatment, cells are grown for about 2 weeks, after which they are fixed and stained with crystal violet. Growth is quantitated by computerized image analysis of the stained cell monolayer of each well. This assay is relatively new and has not been proven as a predictive assay for clinical response (Baker et al., 1986).

C.1.iv. Isotope Uptake Assay.

DNA biosynthesis is another measure used to quantitate cellular sensitivity to anticancer agents. The amount of incorporated tritium labelled thymidine (^3H) into nucleic acid is used as a measure of the sensitivity of the cells to the treatment (Friedman and Glaubiger, 1982; Twentyman et al., 1984).

C.1.v. Cellular Capacity Assay.

The cellular capacity assay measures the ability of a cell to support viral growth (Coohill et al., 1977). Physical or chemical treatment of cell monolayers can decrease the cellular capacity to support viral infection. A decrease in cellular capacity for supporting viral infection has been noted following treatment by a number of agents such as proflavin and light, angelicin or 8-methoxypsoralen and UV light (Lytle et al., 1974; Bockstahler et al., 1976; Coppey and Nocentini, 1976; Lytle and Hestler, 1976; Coppey et al., 1979a).

The cellular capacity assay does not require replication of cellular DNA and has

several advantages over the other methods (mentioned above) which are dependent on host cell replication.

The cellular capacity assay has been used to screen anticancer drugs for their effectiveness on tumour biopsy cells and was able to distinguish sensitive from resistant cell lines as clearly as the clonogenic survival assay (Parsons et al., 1986).

C.2. Photodynamic Therapy.

A predicative **in vitro** assay has not been utilized to assay the clinical response of human tumours to photodynamic therapy.

D. Cells with Differing PDT Response.

Studies have identified a number of cell lines with a defined biochemical defect to show varied PDT response. The isolation and examination of these cell lines is considered of interest in order to help identify the specific mechanism of PDT cytotoxicity.

D.1. Resistant Cell Lines.

A number of multi-drug resistant (MDR) cells have been tested for their cross-resistance to PDT. Murine melanoma MDR cells have shown to be cross-resistant to anthrapyrazole, PD 110095 and to rhodamine plus light (Kessel, 1989; Edwards et al., 1990). Chinese hamster ovary MDR cells have exhibited a cross-resistance to Photofrin or hematoporphyrin derivative (Singh et al., 1991; Mitchell et al., 1988).

The MDR phenotype is associated with specific alterations in tumour cell membranes and tumour cell pharmacology, specifically, increases in high molecular weight cell surface glycoprotein and decreases in the ability of cells to accumulate and retain drug respectively (as reviewed in Beck, 1987).

A murine radiation-induced fibrosarcoma (RIF-1) cell was used to isolate (PH II-mediated) PDT-resistant variants (Luna and Gomer, 1991; Singh et al., 1991). The RIF-8A cell line was found to have mitochondrial alterations, both morphologically and metabolically, compared to its normal counterpart, RIF-1 (Sharkey et al., 1993). Response to PDT was determined in a human ovarian cell line (2008) and its cisplatin resistant counterpart, C13. The C13 cell line was shown to be cross-resistant to PDT (Sharkey et al., 1993). Mitochondrial alterations were identified in the C13 cell line in comparison to the 2008 cell line (Andrews and Albright, 1992).

D.2. Sensitive Cell Lines.

Murine leukaemic cells (LY-R) deficient in the repair of UV-induced damage exhibit a sensitivity to the cytotoxic effects of pthalocyanine and PH II-mediated PDT, but not in the LY-S cells which have an enhanced sensitivity to ionizing radiation (Evans et al., 1989; Ramakrishnan et al., 1989; Rerko et al., 1992; Deahl et al., 1993).

E. Cross Resistance and/or Sensitivity of PDT with other Agents.

Mutant cell lines exhibiting a resistance or sensitivity to a DNA damaging agent

often show a cross-resistance or cross-sensitivity to other DNA damaging agents (as reviewed in Collins, 1993). The various profiles of cross sensitivity or cross resistance can provide evidence for different biochemical defects.

E.1. UV Light.

It was found that the murine leukaemic cell line, LY-R, which is sensitive to UVC-radiation because of its deficiency in the excision repair of UV-induced dimers, shows an increased sensitivity to PDT using AlPc and PH II (Evans et al., 1989; Ramakrishnan et al., 1989; Rerko et al., 1992; Deahl et al., 1993). Gomer et al., (1983) found that cells sensitive to Hpd and light, exhibited a similar sensitivity to UV light.

E.2. Cisplatin.

The RIF-8A PDT-resistant cell line also showed a cross resistance to cisplatin. Similarly the cisplatin-resistant cell line, C13 showed a cross-resistance to PDT (Sharkey et al., 1993).

E.3. X-rays.

LY-R cells, which are sensitive to PDT, exhibited the opposite cytotoxic response to X-ray irradiation (Evans et al., 1989; Rerko et al., 1992; Deahl et al., 1993).

F. DNA Repair.

The sensitivity and or resistance of DNA damaging agents is often related to a reduced or increased DNA repair capability.

F.1. Mechanisms of DNA Repair.

The most general DNA repair pathway that has been observed is where damaged or inappropriate bases are excised from the genome and replaced by the normal nucleotide sequence.

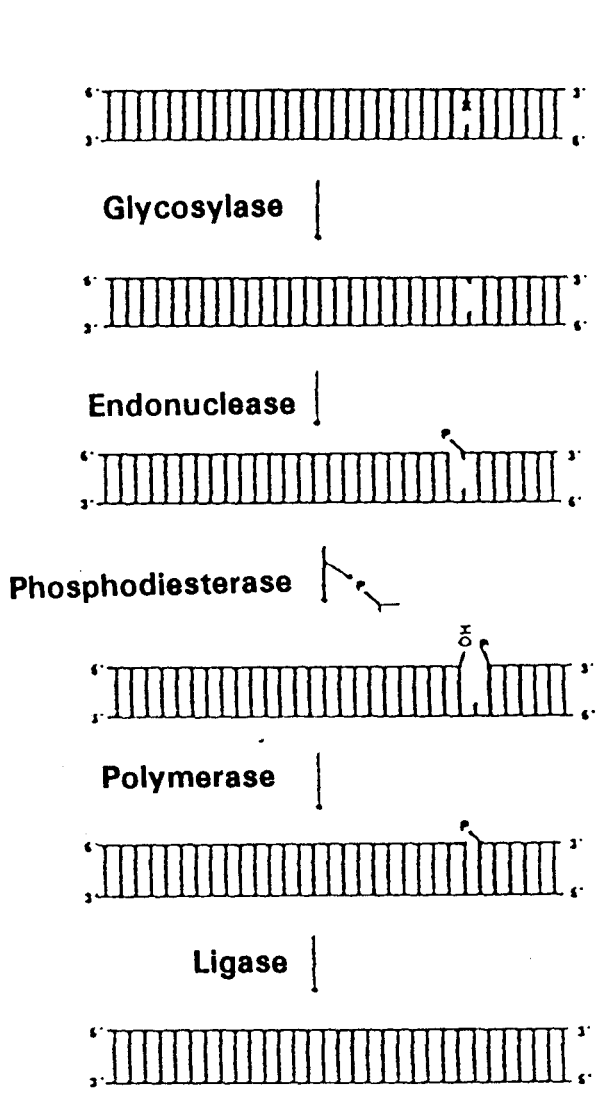
DNA damage caused by deleterious agents, if not repaired by an error-free process, may lead to biological consequences. Some possible consequences in cells are reproductive inactivation, mutation, carcinogenic transformation, and senescence. In higher organisms, the resulting consequences may include debilitating disease, cancer, genetic diseases, and aging. Excision of damaged DNA can be accomplished by one of two ways: base excision repair removes inappropriate bases from the genetic code allowing their appropriate counterparts to be re-introduced, while nucleotide excision repair removes bulky nucleotide adducts from DNA and replaces the excised moiety with the correct sequence of nucleotides (as reviewed in Freidberg, 1985; as reviewed in Barnes et al., 1993).

Base excision repair (figure 3a, as modified from Barnes et al., 1993) is mediated by DNA glycosylase and apurinic/apyrimidinic (AP) endonucleases. The first step involves a DNA glycosylase generating an AP site by cleaving the glycosidic bond between the damaged base and the deoxyribose-phosphate backbone. Secondly, one

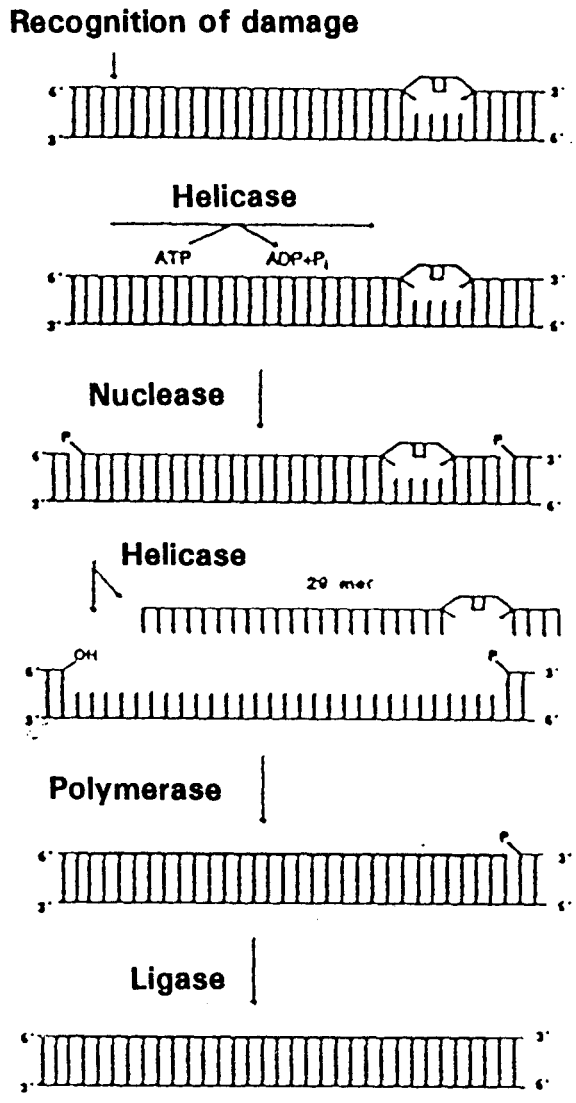
Figure 3: DNA Excision Repair Pathways.

Figure 3a depicts the base excision repair of a cyclobutane pyrimidine dimer, the x represents an altered base. Figure 3b depicts the nucleotide excision repair of a cyclobutane pyrimidine dimer in human cells.

(as modified from Barnes et al., 1993)


Glycosylase
Endonuclease
Phosphodiesterase
Polymerase
Ligase

3a


Recognition of damage
Helicase
ATP
ADP+Pi
Nuclease
Helicase
20 mer
OH
Polymerase
Ligase

3b

of the phosphodiester bonds of the AP site is cut by an AP endonuclease. This is followed by elimination of the AP section of the backbone by the activity of an AP endonuclease or exonuclease. The final step is the filling in and sealing of the gap by polymerase and ligase respectively (as reviewed in Hickson and Harris, 1988; as reviewed in Barnes et al., 1993; as reviewed in Hoeijmakers, 1993).

Mammalian nucleotide excision repair is a complex pathway. Enzymes involved have not been clearly defined but a general idea of the steps involved has been proposed (figure 3b, as modified from Barnes et al., 1993). The accumulated evidence from the study of a number of inherited syndromes that result from defects in DNA repair pathways, suggest that it depends upon the concerted action of multiple proteins. Simply, nucleotide excision repair involves the recognition of the aberrant nucleotides and consequent incision of the defective strand at a significant distance from either sides of the damage. The segment containing the defect is removed and DNA polymerase activity creates a complementary strand from the undamaged template. This is followed by ligation of the newly synthesized segment to the pre-existing strand (as reviewed in Barnes et al., 1993; as reviewed in Hoeijmakers, 1993; as reviewed in Cleaver, 1994).

F.2. DNA Repair Mutants.

UV light sensitive rodent cell lines that have been derived from Chinese hamster ovary (CHO) cell lines have been extremely useful in DNA repair studies (Busch et al., 1980; Thompson et al., 1980; as reviewed in Busch et al., 1989). Mutants sensitive

to UV light and other agents which induce bulky DNA adducts have been comparatively used in studies with repair proficient cell lines to look at repair capabilities following a particular treatment (as reviewed in Collins, 1993). More importantly they have proven useful in the isolation of human DNA repair genes. Because of the problems associated with transfecting DNA into untransformed human fibroblasts (Hoeijmakers et al., 1987), human DNA sequences have been transfected into CHO repair deficient mutants in an effort to identify human genes which can correct the hamster repair deficiency. This process has resulted in the identification of at least 6 ERCC (Excision Repair Cross Complementing) genes, of which four of the genes have been identified as deficient in specific human DNA repair deficient diseases (as reviewed in Hoeijmakers, 1993).

F.3. Effects of Deoxyribonucleotide Pool Imbalance.

An important feature of DNA metabolism is the polymerization of deoxyribonucleotides (dNTP's). Intracellular levels of deoxyribonucleotides (dNTPs), precursors of DNA, play an important role in the processing of DNA damage. Investigators have found that alterations in dNTP pool levels have been found to be mutagenic and to modify cellular sensitivity to the lethal and mutagenic effects of DNA-damaging agents (as reviewed in Kunz, 1982; as reviewed in Haynes and Kunz, 1988; as reviewed in Kunz, 1988).

It was shown that thymidine kinase (tk) deficient cell lines have an increased sensitivity to the DNA damaging agents such as UV light, ethyl methane sulfonate

(EMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Meuth, 1983; McKenna et al., 1985; Rainbow, 1989c). Thymidine kinase is a salvage enzyme which permits a rapid increase in the concentration of deoxythymidine triphosphate (dTTP) to a level which may ensure the correction of any imbalance in the deoxyribonucleoside phosphate pools. Ayusawa et al., (1983) showed that a cell mutant with altered ribonucleotide reductase and thymidylate synthase show a sensitivity to the DNA damaging agent bleomycin. These enzymes are involved in the formation of functional dNTP's. Mutants were found to have abnormal levels of deoxyribonucleoside triphosphate pools, especially those of deoxyadenosine triphosphate. Amara et al., (1991) found that adenine phosphoribosyl transferase (aprt) deficient mutants showed a sensitivity to killing and mutagenesis by EMS and MMS. They were found to have significantly decreased levels of dATP and dTTP nucleotides and decreased levels of all four ribonucleoside triphosphates (ATP, GTP, CTP and UTP) relative to wild-type cells.

Imbalances in dNTP pools facilitate replication errors. The error frequency of various prokaryotic DNA polymerases was found to be dependent, in part, on the relative concentrations of deoxyribonucleotides in the reaction mixture. Replication errors increased with the concentration of the incorrect nucleotide or with a decrease in the concentration of the correct nucleotide. Thus, it was suggested that the fidelity of the **in vitro** replication of synthetic templates can be reduced by biasing the relative dNTP pools. Studies on natural templates also showed that dNTP pool imbalances decrease the fidelity of replication (as reviewed in Kunz, 1982).

Decreased levels of dTTP has shown to produce the greatest amount of effects on cells. In addition to cell killing, starvation for thymine nucleotides can induce a variety of genetic and cytological effects in both prokaryotes and eukaryotes (as reviewed in Kunz, 1982).

F.4. Assays to Detect DNA Repair.

F.4.i. Molecular Assays.

A number of molecular assays have been utilized to detect repair, however the unscheduled DNA synthesis (UDS) assay is the most widely used. It is an autoradiographic technique to detect repair synthesis. Eukaryotic cells carry out semi-conservative DNA synthesis only during the DNA synthesis (S) phase of the cell cycle. If mammalian cells in culture are allowed to grow for a short period of time in the presence of tritium labelled thymidine and are then examined autoradiographically, cells in the (S) phase of the cell cycle show intense labelling of their nuclei with silver grains, whereas cells not in the S phase show essentially no detectable grains. However, if the cells in culture undergo repair synthesis of DNA, the presence of silver grains is also manifested in the non S phase cells (as reviewed in Friedberg, 1985).

F.4.ii. Split Dose Experiments.

DNA repair can also be examined by using experiments designed to investigate differing responses to DNA damage utilizing split-dose protocols. The protocol involves

dose fractionation with a suitable time interval between doses. Dose fractionation is known to alter the cytotoxic effects of hyperthermia and ionizing radiation and hematoporphyrin or pthalocyanine-mediated photodynamic therapy (Elkind et al., 1967; Winans et al., 1972; Ben-Hur et al., 1974; Henle and Leper, 1979; Moan and Christiansen, 1979; Wolters et al., 1985; Koval, 1988; Baumann, 1992). An increased survival from the dose fractionation suggests the presence of cellular repair mechanisms for the repair of sublethal damage.

F.4.iii. Host Cell Reactivation Assay (HCR).

HCR measures the ability of the host cell to reactivate DNA-damaged virus. The extent of reactivation is a reflection of constitutive levels of repair in the infected host cell line. HCR of irradiated virus has been investigated in several different cell types. A comparison of the HCR in repair-deficient cell lines with their normal counterpart effectively reveals differential repair capabilities. (Day, 1974; Rainbow, 1980; Rainbow and Howes, 1982; Rainbow, 1989a; Rainbow, 1989b; Rainbow, 1991).

F.4.iv. Cellular Capacity Assay.

Previous studies have shown that the capacity assay can be used to compare the recovery and/or repair of mammalian cells following exposure to DNA damaging agents. It was shown that the capacity of normal human fibroblasts in comparison to xeroderma pigmentosum (XP) fibroblasts (repair deficient fibroblasts) increased when viral infection was delayed following UV damage (Lytle et al., 1976; Coppey et al.,

1979b). This restored capacity was also seen in monkey kidney cells and human skin cells when treated with angelicin plus light (Coppey et al., 1979a). This recovery is thought to result from the ability of repair mechanisms to remove cellular DNA damage during this time period.

G. The Proposed Study.

Previous work from the Hamilton Regional Cancer Centre (HRCC) derived a PH II-mediated PDT resistant mouse fibrosarcoma cell line (RIF-8A) to use in identification of the cytotoxic mechanism of PDT. The RIF-8A cells were found to have both morphological and biochemical mitochondrial alterations compared to the RIF-1 cell line (Singh et al., 1991; Sharkey et al., 1993). A CHO-MDR cell line was also found to be cross-resistant to PDT. Further examination showed that the resistance to PDT in the CHO-MDR cell line was a result of altered drug uptake (Singh et al., 1991).

In this work, I have examined the use of a viral cellular capacity assay to predict the cellular PDT sensitivity of several rodent cell lines. Results using the cellular capacity assay showed that RIF-1 cells show an increased sensitivity to PDT in comparison to RIF-8A cells and CHO-N cells show an increased sensitivity to PDT in comparison to CHO-MDR cells. Results using the capacity assay are consistent with results using the colony forming assay previously published (Singh et al., 1991). These results suggest the cellular capacity assay can be utilized to test human tumour biopsies for sensitivity to PDT.

In this work, I have further characterized the RIF-8A PDT-resistant line. The

infection of the mouse fibrosarcoma cells (RIF-8A and RIF-1) 6 hours after PDT treatment compared to infection immediately after infection showed that RIF-8A cells have an increased capacity compared to RIF-1 cells, to support viral infection 6 hours post PDT damage. This suggests that RIF-8A cells has an enhanced repair capacity for PDT damage in comparison to RIF-1 cells.

A cross-resistance to UV light of the RIF-8A cell line compared to the RIF-1 cell line was also found using the cellular capacity assay. A similar cross-sensitivity to PDT and UV light in rodent cells has been reported previously (Gomer et al., 1983; Evans et al., 1989; Ramakrishnan et al., 1989; Rerko et al., 1992; Deahl et al., 1993).

Using the cellular capacity assay, I also detected an increased PDT sensitivity for the CHO-AUXB1 cell mutant compared to its parental cell line. This increased sensitivity to PDT in the CHO-AUXB1 cell line was confirmed using the colony survival assay. This PDT sensitive cell line also showed a cross-sensitivity to UV light.

CHAPTER TWO

PARAMETERS OF ADENOVIRUS DNA SYNTHESIS AND THE HOST CELL REACTIVATION (HCR) OF PDT-TREATED ADENOVIRUS IN RODENT CELLS

INTRODUCTION

This study utilizes the endpoint of adenoviral DNA synthesis to assess the capacity of several PDT and UV treated rodent cell types for viral infection. In order to undertake such a study it was first important to determine a number of parameters associated with adenoviral DNA synthesis in the Chinese hamster ovary (CHO) cells and the radiation-induced mouse fibrosarcoma (RIF) cells to be examined.

Adenoviruses (Ad) are non-enveloped double stranded DNA viruses. The adenovirus genome is transcribed and replicated in the nucleus of the host cell and therefore can utilize transcriptional enzymes of the host for the generation of viral mRNA. The adenovirus encodes its own DNA polymerases but depends on the host cell for many of the other functions involved in the synthesis of viral DNA.

The replicative cycle of adenoviruses is divided into early and late phases, with the latter commencing by definition, with the onset of viral DNA replication.

The early phase includes the transcription of the viral genome into 4 differentially spliced mRNAs. The E1 region, which is subdivided into E1A and E1B codes for more than 10 proteins which are involved in the transcriptional activation of other viral proteins. The E2 region is subdivided into E2A and E2B. The E2 region codes for proteins involved in DNA replication (Linne et al., 1977; Lichy et al., 1982; Friefeld et al., 1983). The E3 region is nonessential for viral replication in tissue culture, it functions in modulating the host response to Ad infection (Bachenheimer

and Darnell, 1975; Persson et al., 1979; Paabo et al., 1986; Gooding et al., 1988; Carlin et al., 1989). The E4 region codes for a number of other proteins.

The onset of viral replication signals the late phase of adenoviral infection. Most of the late viral-encoded proteins synthesized are the virion structural proteins or their precursors.

Productive infection of virus occurs in permissive cells and is characterized by the production of infectious progeny. This is best achieved in human cells. Infection of non-permissive cells is characterized by variable early gene expression and the absence of viral DNA replication and late viral gene expression, with no viral progeny evident. Cells semi-permissive to infection also exist, whereby infection results in some degree of expression of viral genes with a lesser amount of viral progeny or no progeny being produced (for a review see Fields, 1990).

Rodent cells are semi-permissive for infection with adenovirus type 2 and adenovirus type 5. Studies have shown that Chinese hamster ovary (CHO) cells do replicate Ad DNA and although viral DNA synthesis is delayed, viral DNA does accumulate to normal levels by 40 to 48 hours post-infection (Longiaru and Horwitz, 1981; Eggerding and Pierce, 1986). Viral DNA synthesis in rodent cells is an endpoint of viral expression that is influenced by the time after infection at which viral DNA is measured. It was therefore important to examine the time course of viral DNA synthesis following the infection of CHO and RIF cells, in order to determine an appropriate time after infection to measure viral DNA synthesis for the capacity experiments.

Recombinant viruses have been used for the delivery and the expression of foreign genes in a number of cell lines (Berkner, 1988). A number of viruses have been examined as vectors for the delivery and the expression of foreign genes in both tissue culture and immunization experiments, including adenovirus (Graham and Prevec, 1991). A number of Ad recombinant vectors have been utilized in this study. The Ad5(denV) contains the bacteriophage T4 pyrimidine dimer DNA glycosylase (denV) gene inserted into the deleted E3 region (Colicos et al., 1991). The denV gene encodes an endonuclease activity specific for thymine dimers (Friedberg and King, 1971). Thymine dimers are produced in DNA following irradiation with ultraviolet (UV) light (Friedberg, 1985). The Ad5(ERCC1) contains the human DNA excision repair cross complementing (ERCC) gene number 1 (Rainbow and Castillo, 1992). The ERCC1 gene corrects the UV sensitivity of an excision repair deficient CHO mutant cell line, 43-3B from complementation group 1 (Westervald et al., 1984). Previous work has shown that infection with both these recombinant vectors increased the amount of viral DNA synthesized in CHO cells and murine cells compared to infection with wild type Ad5 or Ad5(lacZ) (Arnold, 1992). The Ad5(lacZ) contains the ß-galactosidase (lacZ) gene in the deleted E3 region.

The two assays utilized in this study are the cellular capacity assay and the host cell reactivation (HCR) assay. The cellular capacity assay measures the ability of physically or chemically treated cells to support viral replication. The HCR assay measures the ability of host cells to repair damaged viral DNA (for further details refer to chapter 1).

The HCR of PDT damaged viral DNA was used to detect any differences in viral DNA synthesis in repair deficient and repair proficient CHO cell lines. These CHO repair deficient cell mutants are sensitive to UV light and other agents which induce bulky DNA adducts (as reviewed in Busch et al., 1989; as reviewed in Collins, 1993) and have been used in comparative studies with repair proficient cell lines to look at repair capabilities following a particular treatment. The UV20 cell line exhibits an increased sensitivity to UV compared to its parental cell line, AA8. The UV20 cell line also exhibits an increased sensitivity to the cross-linking agent, mitomycin C (MMC) and the alkylating agent, ethyl methanesulphonate (EMS) (Thompson, 1980). The UV5 exhibits an increased sensitivity to UV, MMC and EMS compared to its parental line, AA8. However, its sensitivity to the two cross-linking agents is decreased in comparison to the UV20 cell line (Thompson, 1980). The UV41 cell line exhibits an increased sensitivity to UV and MMC when compared to AA8, and as with UV20 it shows a marked sensitivity to MMC (Hoy et al., 1985).

All the parameters of viral DNA synthesis identified in this preliminary work were used in subsequent viral capacity experiments.

MATERIALS AND METHOD

A. Cells and Viral Strains.

The **CHO normal** (CHO-N) cell is a CHO cell line that is defective in folypolyglutamate synthetase (Taylor and Hanna, 1977 and 1979). It is derived from the CHO-PRO- cell line (McBurney and Whitmore, 1974). It was provided by Dr. V. Ling, Ontario Cancer Institute, Toronto, Ontario.

The **CHO multi-drug resistant** (CHO-MDR) cell line, containing the p-glycoprotein gene (CH^RC5), is derived from the CHO-N cell line (Ling and Thompson, 1974). It was provided by Dr. V. Ling, Ontario Cancer Institute, Toronto, Ontario.

AA8 is a normal CHO cell line that appears to be functionally heterozygous at the aprt locus (Thompson et al., 1979). It was provided by Dr. L. Thompson, Lawrence Livermore, California, with the help of Dr. G. Whitmore, Physics Division, Ontario Cancer Institute, Toronto, Ontario.

UV20, **UV5** and **UV41** cells are members of the rodent excision repair complementation group 1, 2, and 4 respectively (Thompson et al., 1980; Busch et al., 1980; as reviewed in Busch et al., 1989; as reviewed in Collins, 1993). They were derived from the AA8 cell line and are deficient in the incision step of DNA nucleotide excision repair. These mutant cell lines were obtained from Dr. L. Thompson with the help of Dr. G. Whitmore.

RIF-1 is a radiation-induced mouse fibrosarcoma tumour cell line (Luna and Gomer, 1991). It was originally provided by Dr. B. Henderson, Roswell Park Memorial Institute, Buffalo, New York and obtained from Dr. G. Singh, Hamilton Regional Cancer Centre and McMaster University, Department of Pathology, Hamilton, Ontario.

RIF-8A is a radiation-induced mouse fibrosarcoma tumour cell line resistant to photodynamic therapy. It was derived from the RIF-1 cell line (Singh et al., 1991). It was obtained from Dr. G. Singh, Hamilton Regional Cancer Centre and McMaster University, Department of Pathology, Hamilton, Ontario.

All cells types were grown as monolayers in alpha-minimal essential media (α-MEM) fully supplemented with deoxyribonucleosides and ribonucleosides (Gibco BRL, cat#11900-024), 10% fetal bovine serum (Gibco BRL, cat#16000-028), 4% of an antibiotic-antimycotic solution (penicillin 10,000 units per ml, fungizone 25 μg per ml and streptomyocin 10,000 μg per ml (Gibco BRL, cat#15240-013)) and 4% of L-glutamine (Gibco BRL, cat#16000-028).

Cells were grown in 75 cm^2 plastic screw-cap bottles (Corning Inc., Corning, New York, cat#25115-75) in a humidified incubator at 37°C with 5% CO_2. The medium from confluent monolayers was aspirated and the monolayers were rinsed in phosphate buffered saline (PBS), (1x, diluted in double distilled water from a 10x stock (0.8g NaCl, 2g KCl, 1.15g Na_2HPO_4 and 0.2g KH_2PO_4)), 2 ml of trypsin (2x, diluted in PBS from a 10x stock) was added. Within five minutes, the bottle was rapped sharply to dislodge the cells. 8 ml of media was added to the dish to dilute the cell suspension for subculturing. Confluent cell cultures were usually diluted at a ratio

of 1 to 10 (1:10) for subculturing.

The human adenovirus Ad5 and the recombinant viruses Ad5(denV), Ad5(lacZ) and Ad5(ERCC1) were used in the experiments.

Ad5(denV) contains the bacteriophage T4 pyrimidine dimer DNA glycosylase (denV) gene inserted into the deleted E_3 region (Colicos et al, 1991).

Ad5(lacZ) contains the ß-galactosidase gene (lacZ) inserted into the deleted E_3 region and was provided by Dr. Frank Graham, Departments of Biology and Pathology, McMaster University, Hamilton, Ontario.

Ad5(ERCC1) contains the 1.1 kb cDNA of the human DNA repair gene ERCC1 inserted into the deleted E_3 region of the virus (Rainbow et al., 1992).

Preparation of Viral Stocks. 293 human embryonic kidney cells (Graham et al., 1977) were grown to confluency in 150 cm^2 dishes. The cells were infected at a multiplicity of infection (MOI) of 1-2 pfu/cell in 4 ml of α-MEM with 1% antibiotic-antimycotic. Following a 90 minute period, the dishes were overlayed with 16 ml of complete growth media. Once the cytopathic effect (CPE) was evident, usually 48 to 72 hours post-infection, the cell suspension was collected and transferred to 50 ml conical tubes (Corning Inc., Corning, New York, Cat#25331-50). The cell suspension was centrifuged at 1000 rpm for 10 minutes to pellet the cells. The supernatant was then discarded and the cells from each tube were separately resuspended in 1-2 ml of a filter sterilized solution of 10% glycerol in α-MEM.

Titering of Viral Stocks. Virus was released from the 293 cells into the 10%

glycerol/α-MEM by freeze/thawing three times. The viral stock was then serially diluted in α-mem and 1% antibiotic-antimycotic. Confluent monolayers of 293 cells were infected with the various dilutions. Following a 90 minute time period, the cells were overlayed with a mixture of 10% Newborn Calf Serum (Gibco BRL, cat#26010-025), 1% antibiotic-antimycotic, 0.2% yeast extract and 0.5% agarose in 2X F11 medium (Gibco BRL, cat#611-00-087). When the plaques were large enough to count, approximately 6-8 days post-infection, the monolayers were fixed and stained with crystal violet (1% crystal violet, 10% methanol, and 18% formaldehyde solution in 1X PBS). The titre of the viral stock was then calculated using the number of plaques formed by each dilution.

B. Determination of Adenoviral DNA Synthesis.

Preparation of Cells. Preconfluent cells were rinsed with 1x PBS, trypsinized and resuspended in a small volume of complete growth medium. A haemocytometer (American Optical Company, Buffalo, USA) was used to determine the number of cells present in the suspension. The cells were seeded in 24-well Linbro plates (Flow Laboratories Inc., Hamden, CT) at 1 x 10^5 to 2 x 10^5 cells per well, depending on the specific cell line being utilized. The cells were then returned to the 37°C incubator for 24 hours to allow for cellular attachment.

Infection of Cells. Viral stocks were diluted in α-MEM supplemented with 1% antibiotic-antimycotic and used to infect cells at a multiplicity of infection (MOI) of 10

or 40 pfu/cell. The growth media was aspirated from the seeded wells and 0.2 ml of virus suspension was added to each well. After incubating at 37°C for a period of 90 minutes, during which the plates were gently rocked at 15 minute intervals, each well was overlayed with 1 ml of pre-warmed fully supplemented α-MEM. At two to four hours after the overlay, the media from each well was aspirated (to remove unabsorbed or unattached virus) and replaced with 0.5 ml of pre-warmed fully supplemented α-MEM.

Harvesting of Samples. Samples were collected at 4 hours, 24, 48, 72, 84, and 96 hours, by the addition of 0.2 ml of lysis solution (4 mg/ml pronase, 40 mM tris pH 8.0, 40 mM EDTA pH 8.0, 2.4% SDS) added directly to the 0.5 ml of growth medium in each well. Following the addition of the lysis solution, the plates were returned to 37°C for a period of 1 to 3 hours. After this time, the contents of the wells were collected and transferred to microfuge tubes which were stored at -20°C until DNA extraction.

DNA Extraction. One volume (0.7 ml) of phenol:chloroform (1:1) was added to each sample. The mixture was vortexed, centrifuged and the aqueous phase transferred to a second microfuge tube, one volume (0.7 ml) of chloroform:isoamyl alcohol (24:1) was then added. The mixture was vortexed and centrifuged and the aqueous phase transferred to a third microfuge tube. To this mixture, 28 μl of 5 M NaCl was added, followed by two volumes (1.4 ml) of cold absolute ethanol. The samples were then stored at -20 °C overnight and subsequently centrifuged at 14,000 rpm at 4°C for 30 minutes to pellet the DNA. The supernatant was poured off and the

DNA pellets dried. The dried DNA samples were resuspended in 30 μl of TE buffer (10mM Tris, 1mM EDTA pH 8.0) at -20°C.

Slot Blotting of DNA Samples. The DNA was slotblotted to GeneScreen Plus (Dupont cat#NEF-976) using a Minifold II Slot Blot System (Schleicher & Schuell, Prod.#SRC972/0), according to manufacturer specifications. Briefly, the DNA samples were thawed at room temperature. The DNA sample was then denatured on 0.25 N NaOH for 15 minutes, chilled on ice, and then diluted with SSC (20x stock: 175.3 g NaCl, 88.2 g of sodium citrate in 800 ml of H_2O) to a final concentration of 0.125 M NaOH and 0.1x SSC and then slot-blotted onto the membrane. The membrane was removed and allowed to dry.

Preparation of Radioactive DNA Probe - Random Primer Extension. Radioactively labelled DNA probes were made using a Klenow labelling procedure (Sambrook et al., 1989), using Ad 2 DNA (Gibco BRL, cat#15270-010) as the template.

In a screwtop microfuge, 200 ng of Ad2 DNA was added to 3.5 μl of double distilled water and boiled for 5 minutes. It was then quenched on ice for 3 minutes, and centrifuged to collect the condensate (approximately 10 seconds). To the DNA was added, 2 μl of the cocktail nucleotide, 5 μl of the random primer, 50 μCi of the radioactive nucleotide (10 mCi/ml) and 2 μl of klenow (in glycerol). This was incubated at 37°C for 45 minutes. The radioactive DNA probe was diluted with TE buffer pH 8.0 to a final volume of 250 μl. The efficiency of the probe was determined by placing 1 μl of the radioactive DNA probe on each of two discs of Whatman DES1 filter paper (Whatman International Ltd., Maidstone, England; cat#3658-324). One of the discs

was then washed with 0.5 M Na_2HPO_4, sterile water followed by cold 100% ethanol. Each of the discs (both washed and unwashed) were placed in two labelled scintillation vials (with 5 ml of scintillation fluid) and the radioactivity calculated.

Hybridization of Probes to DNA samples. Membranes were briefly soaked in 2x SSC, then rolled between nylon meshes and inserted into a hybridization tube (Hybaid, HB-OV-BL). A pre-hybridization buffer (0.15 g Bovine Serum Albumin, 7.5 ml 1M NaH_2PO_4, 2.25 ml deionized formamide, 5.25 ml 20% SDS) was added to the tube and was incubated at 60°C for a minimum of two hours in a Hybaid Mini Hybridization Oven (Bio/Can Scientific; serial# 0165). After this period, the probe was denatured by heating at 100°C for 10 minutes and added to the tube without incidence to the membranes. This was hybridized for a minimum of 15 hours at 60°C.

At the end of the hybridization period, the probe solution was discarded, and the membrane washed three times with a 150 mM NaH_2PO_4/1% SDS solution (15 minutes at 60°C then two more times for 30 minutes at 65°C, respectively), and finally once with a 30 mM NaH_2PO_4/.1% SDS solution (for 30 minutes at 50°C). Membranes were dried, then taped to Whatman paper (Whatman International Ltd., Maidstone, England; cat#3030-917) and wrapped in Saran wrap. The membranes were placed face-up in a Phosphorimager screen and allowed to develop (for varying time periods). The DNA was then quantitated using a Phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, California).

C. Determination of Viral DNA Synthesis in PDT Experiments.

Photosensitizing Drug. Photofrin II was obtained by Quadralogic Techonologies Inc. (Vancouver, B.C., Canada). It was diluted to a 2.5 mg/ml suspension using α-MEM, from an original stock of 10 mg/ml.

Light Source. Plates were exposed to red light using a 21" x 32.75" light box, illuminated by a parallel series of 12 fluorescent tubes (Phillips type TL/83), enclosed on top with a sheet of clear Plexiglass and filtered with red acetate filters (no. 19, Roscolux, Rosco, California). The emission spectrum of the light source was measured using a Jobin-Yvon model CP-200 spectrometer and a Princeton Instruments CCD. The energy fluence rate was 0.34 $W/m^2/sec$ in the wavelength band of 640 nm. Exposure for 5 minutes resulted in incident energy fluence of 102 J/m^2.

Preparation of cells. The cells were seeded in 24-well Linbro plates (Flow Laboratories Inc., Hamden, CT) as previously described. The cells were then returned to the 37°C incubator for 4 hours to allow for cellular attachment.

Addition of Drug. After this 4 hour period, the media was removed and replaced with 0.5 ml of fresh, fully supplemented α-MEM; 0.1 ml of a Photofrin suspension at appropriate concentrations was added to each well. The cells were then incubated at 37°C for 18 hours. The addition of the drug and all steps following were carried out in minimal light conditions until addition of the pronase solution.

Irradiation of Cells. Following this 18 hour incubation, the drug containing media was removed and replaced with 0.5 ml of fully supplemented α-MEM. The cells were then exposed to red light (at room temperature) for five minutes.

Harvesting and Quantitating of DNA. Samples were collected at: i) between 2

and 4 hours (the initial amount of virus absorbed; used as a background sample) and ii) at 48 or 72 hours (the maximum amount of virus replicated). DNA was harvested and quantitated as previously described.

D. The Host Cell Reactivation of PDT-Treated Adenovirus.

Preparation of Cells. The cells were prepared as previously described.

The Photosensitizing Drug and the Light Source. The photosensitizing drug utilized and the light source are as previously described.

Treatment of Virus. Virus stocks were diluted to appropriate concentrations for infection in α-MEM supplemented with 1% antibiotic-antimycotic.

Before the addition of the drug, 0.1 ml of virus suspension was removed, to be used as a control. 250 μg/ml of drug was added to 0.5 ml of the remaining virus suspension. The virus-drug suspension was then incubated at 37°C for 6 or 24 hours.

Irradiation of Virus. Prior to infection of the cells, 5 aliquots of 0.1 ml of drug-treated virus were placed into 17 x 100 mm^2 plastic transparent tubes (Becton Dickinson Labware, New Jersey; cat#4-2058-0) and exposed with red light at an energy fluence rate of 0.34 W/m^2/sec for time periods of 0 to 6 hours.

Infection of Cells. Cells were infected with PDT-treated or non-treated Ad5(lacZ) as previously described.

Harvesting and Quantitating of DNA. Viral DNA was harvested and quantitated as previously described.

RESULTS

Adenoviral DNA Synthesis in CHO and RIF cells.

The time course of adenovirus DNA replication in CHO cells is shown in figure 1A. The relative amount of viral DNA synthesized is plotted on a logarithmic scale against time post-infection on a linear scale. It can be seen that the maximum amount of viral DNA replicated was detected at about 72 hours post-infection for each of the three viruses tested. A greater amount of viral DNA replication occurred when the infection of the CHO cells was carried out at 40 MOI compared to 10 MOI. At 40 MOI, the amount of viral DNA synthesized by 72 hours post-infection in Ad5(denV) infected CHO cells was approximately 10 fold the level synthesized in both Ad5(lacZ) and Ad5(ERCC1) infected CHO cells.

The time course of adenovirus DNA replication in RIF cells is shown in figure 1B. It can be seen that the maximum amount of viral DNA replicated was detected at about 48 hours post-infection for each of the three viruses tested. A similar amount of viral DNA replication was detected when the infection of the RIF cells was carried out at an MOI of 10 or 40 pfu/cell. The amount of viral DNA detected at 48 hours post-infection in Ad5(denV) infected RIF cells was approximately 5 fold greater than the amount of viral DNA synthesized in Ad5(lacZ) infected cells and approximately 2 fold the amount of viral DNA synthesized in Ad5(ERCC1) infected RIF cells. Sample collection at times greater than 48 hours after infection of RIF cells resulted in reduced

amounts of viral DNA detected under all the conditions tested. This reduction in viral DNA detected could result from degradation of viral DNA at later times after infection.

The amount of viral DNA synthesized in CHO cells was approximately 5 fold the amount of viral DNA synthesized in RIF cells at the time of maximum detectable amount of viral DNA.

Determination of Dark Toxicity and Light Toxicity in PDT Experiments.

The effect of the photosensitizer alone on viral DNA synthesis in RIF cells is shown in figure 2A. The amount of viral DNA synthesis following drug only treatment was determined relative to the amount of viral DNA replicated in untreated cells for each dose point. Points were plotted on a logarithmic scale against Photofrin dose on a linear scale. Similar for RIF-1 and RIF-8A cells there is a reduction in the amount of viral DNA synthesized to about 80% (range 105% to 50%), at the highest Photofrin concentrations used (20 μg/ml).

The effect of the light source alone on viral DNA synthesis in RIF cells is shown in figure 2B. It can be seen that the amount of viral DNA synthesis in RIF-1 cells and RIF-8A cells is not significantly different, following exposure to red light for 5 minutes.

The amount of viral DNA synthesized in the untreated cells from the drug only experiment was not significantly different from the amount of viral DNA synthesized following light treatment only (data not shown).

The Host Cell Reactivation (HCR) of PDT-treated Adenovirus.

A number of experiments were attempted in order to determine the HCR of PDT-treated adenovirus in mammalian cells.

In order to observe a reduction in survival of viral DNA synthesis following infection with PDT-treated adenovirus, it was found necessary to give extremely high concentrations of Photofrin (250 μg/ml) together with long exposure times with red light (2 to 6 hours). Furthermore, large reductions in viral DNA synthesis were found following treatment of the virus with drug alone compared to light alone (5 to 10 fold) at the high concentrations of drug (250 μg/ml) employed for the infection with the Ad5(denV) and large reductions were found following treatment with light alone compared to drug alone (8 to 10 fold) at the long exposure time (6 hours) employed for the infection with the Ad5(lacZ).

HCR experiments were attempted using several CHO cell lines including CHO mutants known to be deficient in the excision repair of UV-damaged DNA. The adenovirus suspension was incubated with Photofrin for 6 or 24 hours and then exposed to red light for 0 to 6 hours. The control utilized in all experiments was not exposed to red light.

As shown in figure 3A and 3B, after 4 to 6 hours of red light exposure there is a reduction of about 50 percent survival of viral DNA synthesis following infection of CHO cells with PDT-treated Ad5(denV). This is seen both for the 24 and the 6 hour drug treatment of virus. No significant difference in HCR of PDT treated Ad5(denV) was detected in the excision repair proficient AA8 cells and the excision repair

deficient UV5 and UV41 cells. In a single experiment using 6 hour PDT-treated Ad5(lacZ) for infection, a substantially greater reduction in survival of viral DNA synthesis occurred (98 percent) after 2 hours of red light exposure, as shown in figure 3C. The HCR of PDT treated Ad5(lacZ) in the UV20 cells was less than that for UV5, UV41 and AA8 cells for the one light dose for which reliable data was obtained. However, due to the large reduction in viral DNA synthesis following treatment of virus with light alone (compared to drug alone), the decreased survival of viral DNA synthesis following infection of UV20 cells with PDT-treated Ad5(lacZ) is questionable.

Figure 1A: Time Course of Adenovirus Replication in CHO cells following Infection with Ad5(denV), Ad5(lacZ) and Ad5(ERCC1).

The time course of viral DNA replication following infection of Ad5(denV), Ad5(lacZ) and Ad5(ERCC1) was investigated in CHO cells. Cells were infected at an MOI of 10 or 40 pfu/cell and the viral DNA extracted from the infected cells at the indicated time points.

Open squares - Ad5(ERCC1), 40 pfu/cell

Open circles - Ad5(denV), 40 pfu/cell

Open triangles - Ad5(lacZ), 40 pfu/cell

Closed squares - Ad5(ERCC1), 10 pfu/cell

Closed circles - Ad5(denV), 10 pfu/cell

Closed triangles - Ad5(lacZ), 10 pfu/cell

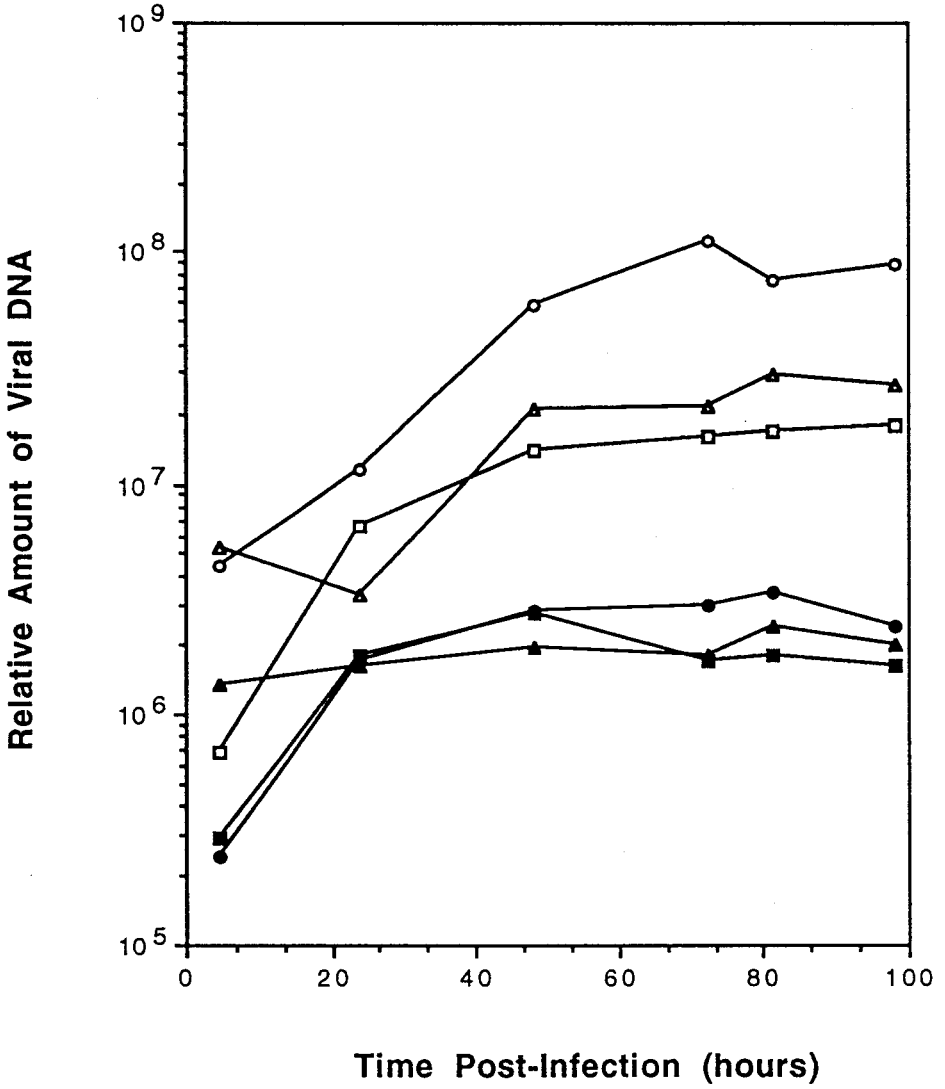

Relative Amount of Viral DNA
10^9
10^8
10^7
10^6
10^5
0
20
40
60
80
100
Time Post-Infection (hours)

Figure 1B: Time Course of Adenovirus Replication in RIF cells following Infection with Ad5(denV), Ad5(lacZ) and Ad5(ERCC1).

The time course of viral DNA replication following infection of Ad5(denV), Ad5(lacZ) and Ad5(ERCC1) was investigated in CHO cells. Cells were infected at an MOI of 10 or 40 pfu/cell and the viral DNA synthesized was extracted from the infected cells at the indicated time points.

Open squares - Ad5(ERCC1), 40 pfu/cell

Open circles - Ad5(denV), 40 pfu/cell

Open triangles - Ad5(lacZ), 40 pfu/cell

Closed squares - Ad5(ERCC1), 10 pfu/cell

Closed circles - Ad5(denV), 10 pfu/cell

Closed triangles - Ad5(lacZ), 10 pfu/cell

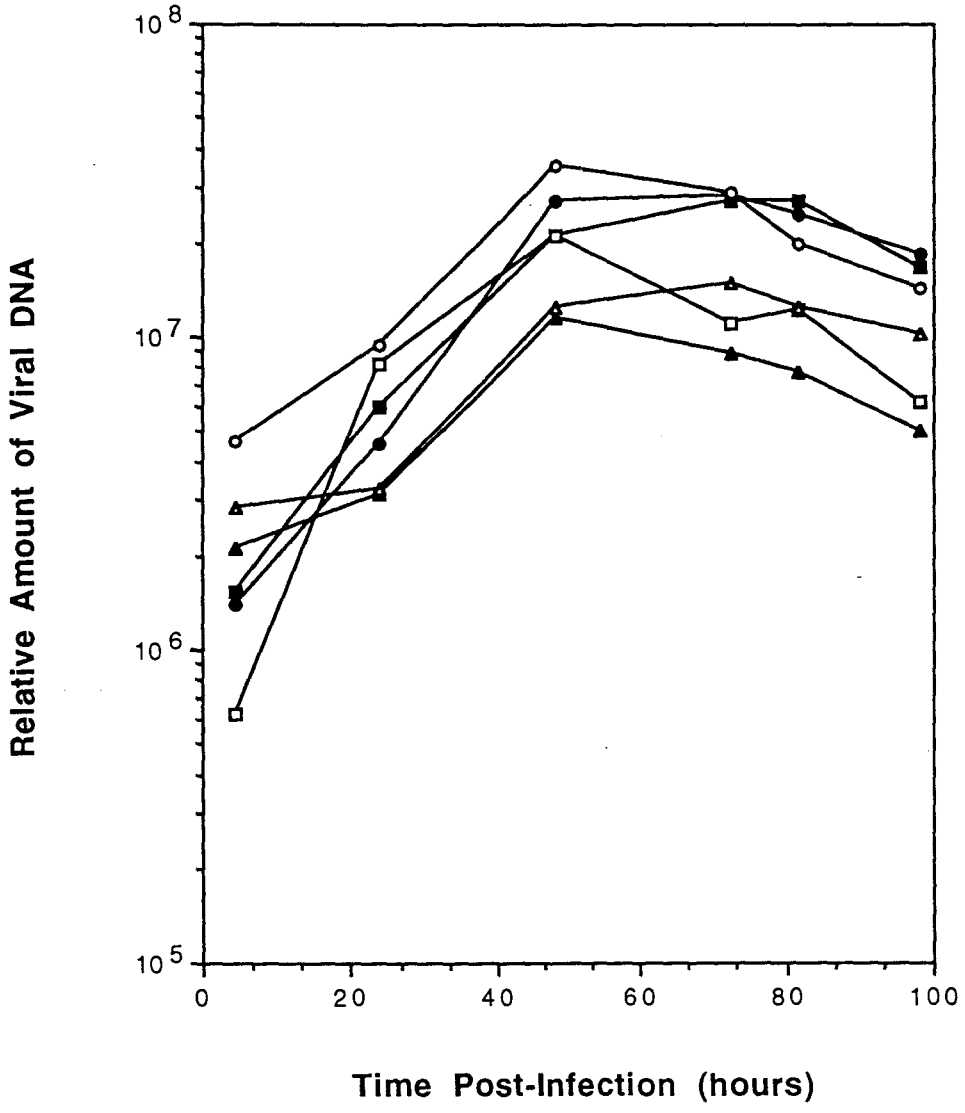

Relative Amount of Viral DNA
10^8
10^7
10^6
10^5
0
20
40
60
80
100
Time Post-Infection (hours)

Figure 2A: The Amount of Viral DNA Synthesis in RIF cells, Treated with Photofrin only, following Infection with Ad5(denV).

The relative amount of viral DNA synthesis following infection with Ad5(denV) at an MOI of 40 pfu/cell was investigated in RIF cells treated with Photofrin only. The survival of viral DNA synthesis was scored at 48 hours post-infection. Results are for a single experiment carried out in duplicate trials. Curves were fitted by linear regression.

Open Circles - RIF-1 cells

Closed Circles - RIF-8A cells

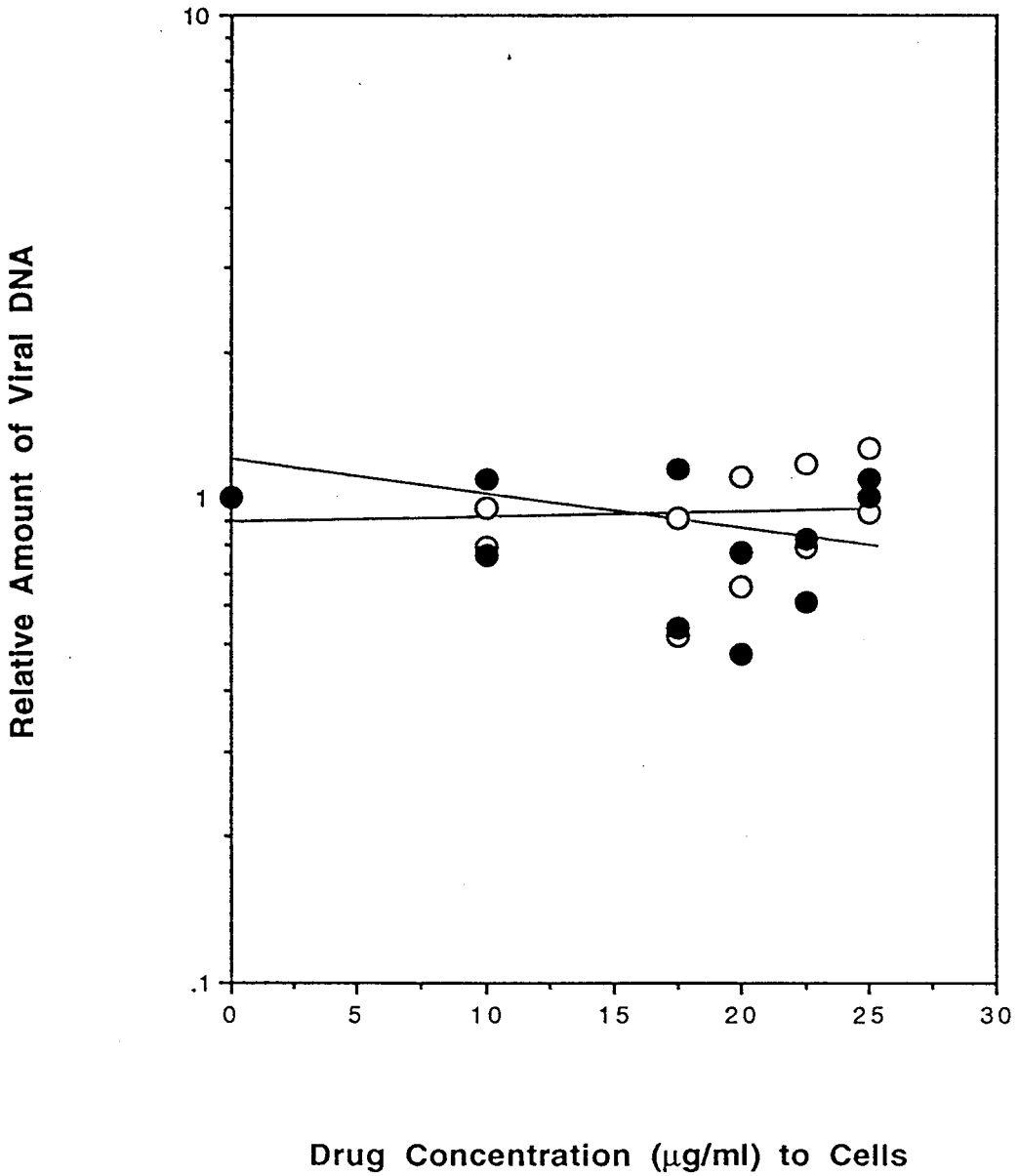

Relative Amount of Viral DNA
10
1
.1
0
5
10
15
20
25
30
Drug Concentration (μg/ml) to Cells

Figure 2B: Histogram of the Relative Amount of Viral DNA Synthesis of RIF Cells Treated with Light only.

The relative amount of viral DNA synthesis following infection with Ad5(denV) at an MOI of 40 pfu/cell was investigated in RIF cells exposed to red light for 5 minutes at an energy fluence rate of 0.34 $W/m^2/sec$. The amount of viral DNA synthesized was scored at 48 hours post-infection. Results are from a single experiment carried out in duplicate trials. The mean amount of viral DNA synthesis for a number of samples and the standard error of the mean was calculated.

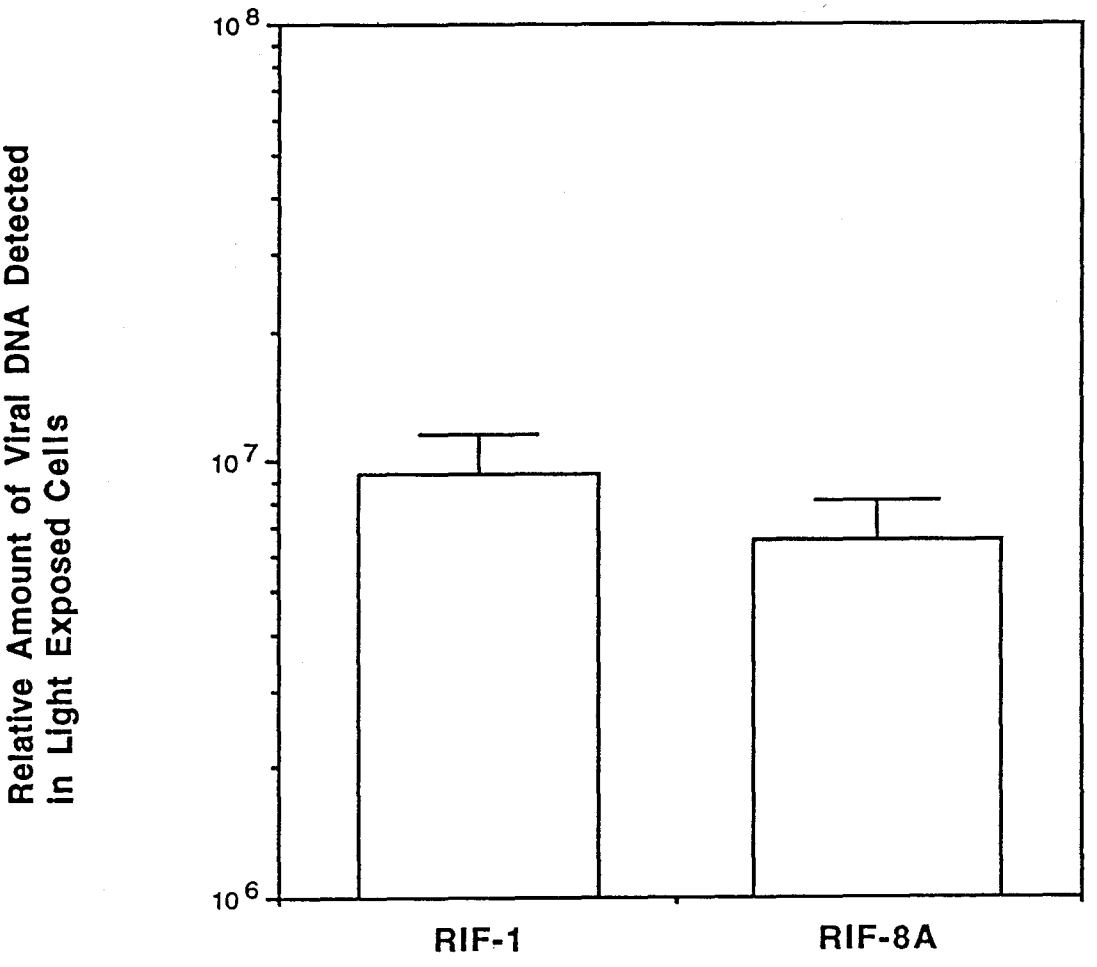

Relative Amount of Viral DNA Detected
In Light Exposed Cells
10 8
10 7
10 6
RIF-1
RIF-8A

Figure 3: Survival of Viral DNA Synthesis in CHO Cells following Infection with PDT-treated Ad5(denV) or PDT-treated Ad5(lacZ).

In each experiment, cells were infected with PDT-treated Ad5(denV) or PDT-treated Ad5(lacZ) at an MOI of 40 pfu/cell. The surviving fractions of viral DNA detected were scored at 72 hours post-infection. Each point represents the logarithmic mean of duplicate or triplicate trials of a single experiment and the standard error of the mean is shown by the bar or contained within the symbol.

Right Panel (3A):

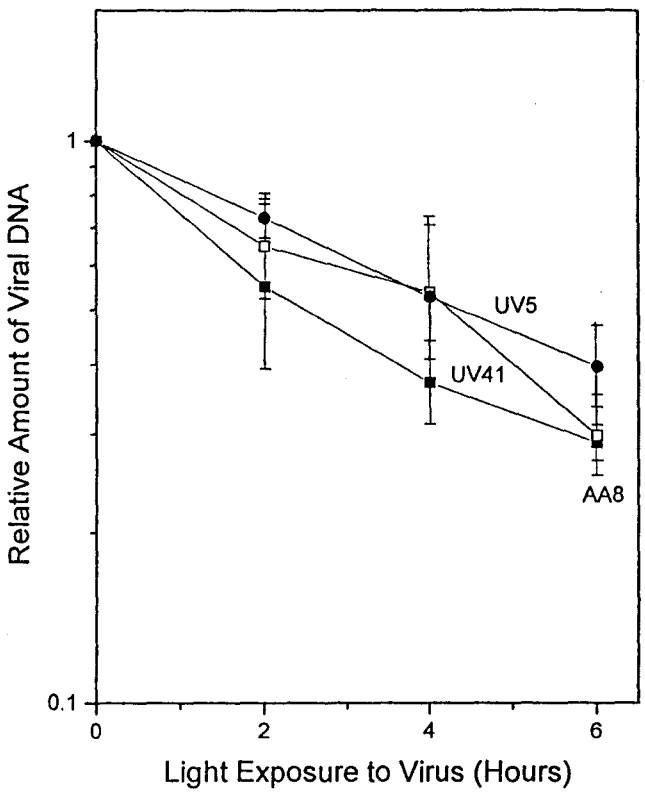
Closed squares - AA8, Ad5(denV)
Closed circles - UV5, Ad5(denV)
Open squares - UV41, Ad5(denV)

Middle Panel (3B):

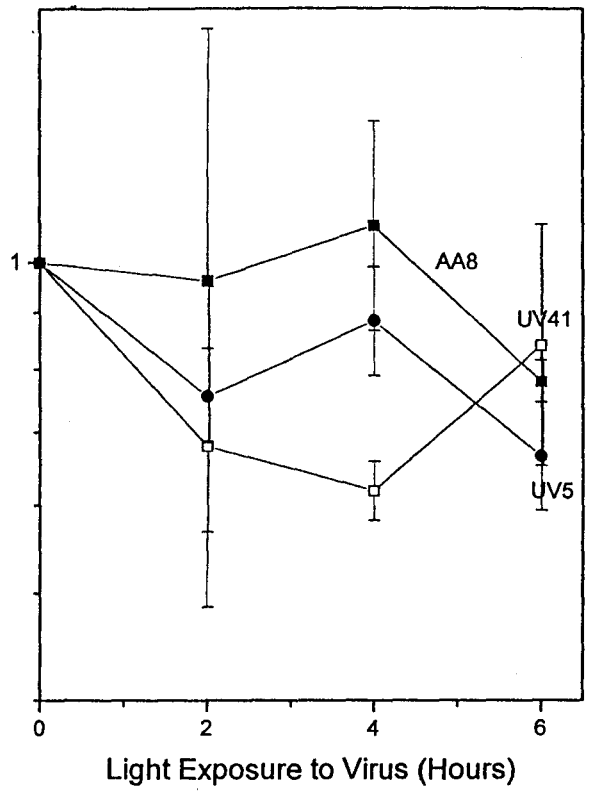
Closed squares - AA8, Ad5(denV)
Closed circles - UV5, Ad5(denV)
Open squares - UV41, Ad5(denV)

Left Panel (3C):

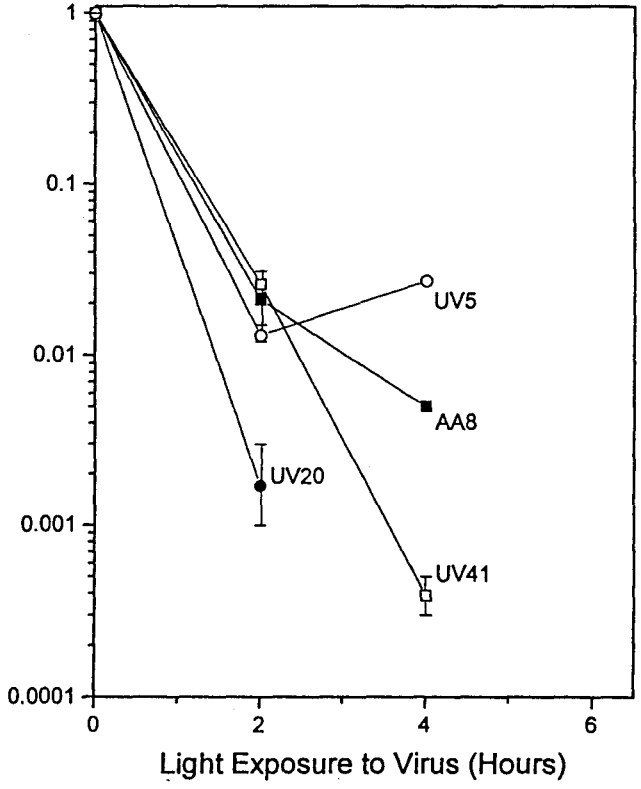
Closed squares - AA8, Ad5(lacZ)
Closed circles - UV20, Ad5(lacZ)
Open squares - UV41, Ad5(lacZ)
Open circles - UV5, Ad5(lacZ)


Relative Amount of Viral DNA
1
0.1
0
2
4
6
Light Exposure to Virus (Hours)
UV5
UV41
AA8

3a


1
0
2
4
6
Light Exposure to Virus (Hours)
AA8
UV41
UV5

3b


1
0.1
0.01
0.001
0.0001
0
2
4
6
Light Exposure to Virus (Hours)
UV5
AA8
UV20
UV41

3c

DISCUSSION

Adenoviral DNA Synthesis in CHO and RIF cells.

The time course of adenovirus DNA replication was determined for CHO and RIF cells in order to ascertain the time after infection for detection of maximum viral DNA replication. The collection of each sample at maximum viral DNA detection allows the greatest reduction in viral replication to be examined following PDT.

As shown in figure 1A, maximal viral DNA replication was detected at approximately 72 hours post-infection in CHO cells for all 3 recombinant viruses. Infection in RIF cells produced maximum viral DNA detection at approximately 48 hours post-infection for all 3 recombinant viruses (figure 1B). The collection endpoints used for all subsequent experiments were 72 and 48 hours post-infection for CHO cells and RIF cells respectively.

The amount of viral DNA detected in RIF cells decreased at time points greater than 48 hours post-infection. This decrease in the amount of viral DNA detected could be due to the degradation of viral DNA in the infected cell at these later times after infection. The degradation of the viral DNA into small fragments may inhibit detection by the extraction and detection methods being utilized to quantitate viral DNA, especially adherence of DNA to the nitrocellulose used in slot blotting.

The effect of MOI was also examined in these experiments. Rodent cells are semi-permissive for infection with class C adenoviruses which include Ad2 and Ad5

(as reviewed in Fields, 1990). It was thought that varying the MOI of the virus used for infection might affect the time course of viral DNA synthesis and alter the amount of viral DNA synthesized in the infected cell. For the CHO cells, a greater amount of viral DNA replication was achieved with infection at an MOI of 40 compared to 10 pfu/cell. However for the infection of RIF cells, the amount of viral DNA synthesis did not differ between the two MOI's utilized.

In preliminary experiments (data not shown), the replication of wildtype adenovirus was similar to that following infection of cells with the Ad5(lacZ) virus. The amount of viral DNA synthesis was greater following infection with the Ad5(denV) and Ad5(ERCC1) compared to infection with wildtype Ad5. Similar results have been reported in earlier work by Arnold, (1992) who showed that viral DNA synthesis in CHO cells and murine cells following infection with either Ad5(denV) or Ad5(ERCC1) was increased. The increase in viral DNA replication for infection with Ad5(denV) and Ad5(ERCC1) suggests that the proteins coded for by these genes (denV and ERCC1) enhance viral DNA replication in CHO cells and the RIF cells. It is possible that the denV protein could enhance repair of PDT induced thymine dimers and the ERCC1 protein could be involved in the excision repair of PDT induced DNA damage.

Infection of CHO and RIF cells with Ad5(denV) resulted in relatively high amounts of viral DNA synthesis, so that, a significant reduction in viral DNA synthesis due to PDT treatment of cells could be examined for a wide range of Photofrin concentrations. For this reason, the Ad5(denV) virus was used for all subsequent

capacity experiments using PDT treated CHO and RIF cells.

The denV gene codes for a protein that has been shown to incise UV-damaged DNA, specifically at sites of pyrimidine dimers (Gordon and Haseltine, 1980; Nakabeppu et al., 1982). For this reason, some of the HCR and capacity experiments carried out using UV irradiation, the Ad5(lacZ) virus was used as well as the Ad5(denV) virus, in order to insure that results were not influenced by the repair of UV damaged DNA due to the presence of the denV gene.

Determination of Dark Toxicity and Light Toxicity.

Figure 2A shows little decrease in the amount of viral DNA synthesis in the RIF cells, produced by Photofrin II at the highest drug concentrations being utilized in this study. In PDT experiments using the colony forming assay, there was a reduction in survival to about 70% (range 30% to 80%) at the highest drug concentrations used (20 μg/ml) (private communication with Merna Espiritu). The measure of dark toxicity gives an indication of the toxicity of the photosensitizer alone on the cells. The toxicity of the hematoporphyrin derivative (Hpd) has been reviewed previously by Dougherty, (1986). The LD_{50} dose in mice is about 275 mg/kg for Hpd. The cause of death is due to liver toxicity. Photosensitizers for clinical use have been developed to be non-toxic at the required doses used for the treatment of human cancers. Doses used in clinical treatment are approximately 2 mg/kg, this dose is a fraction of the dose utilized to produce liver toxicity in the reported study.

Figure 2B shows that the light alone is not affecting viral DNA replication

following infection of the RIF cell lines. A comparison of the amount of viral DNA synthesis in the control value of the drug only treatment and in the light only experiments indicates that the amount of viral DNA synthesis is not sufficiently different. In PDT experiments, using the colony forming assay, the number of colonies counted in the untreated plates of the drug colony experiment was similar to the number of colonies counted following light only treatment (private communication with Merna Espiritu).

The Host Cell Reactivation of PDT-damaged Adenovirus.

Experiments on the HCR of PDT damaged adenovirus were carried out in order to examine whether this approach would yield differences in cellular capacity to repair PDT damaged viral DNA. Practical difficulties were encountered when performing these experiments using the currently available light and drug delivery system.

There was a large reduction in viral DNA synthesis following treatment of the virus with the drug alone. Furthermore, very high concentrations of Photofrin together with long exposure times to red light were required in order to detect significant virus inactivation. It is not known whether the effects of viral DNA synthesis observed are due to damage to viral DNA, viral proteins or both. The contribution of oxygen levels and temperature on the viral suspension during light exposure may also contribute to the observed effects. It will be important to answer these questions and develop a more practical experimental protocol if experiments using PDT-treated virus are to be continued.

Not withstanding the above concerns, some conclusions and speculations can be made regarding the results of experiments performed so far.

Results using PDT-treated Ad5(denV) and Ad5(lacZ) suggest a similar HCR for the excision repair proficient AA8 cells and the excision repair deficient mutants, UV5 and UV41. The reduced HCR value in UV20 cells in one experiment is not considered sufficient evidence to suggest the UV20 mutant has any differences in its PDT response at this time. Furthermore, virus treated with light alone resulted in a large reduction (8 to 10 fold) in viral DNA synthesis compared to virus treated with drug alone. Therefore, it is possible that the UV20 cells show a reduced HCR because of their reduced ability to repair light-damaged and/or heat-damaged viral DNA (produced as a result of the long exposure times with red light), rather than from a reduced ability to repair PDT damaged viral DNA. Previous work from our laboratory and elsewhere has shown a similar sensitivity of AA8 and UV20 cells to PDT treatment using both cellular capacity for viral DNA synthesis and colony formation (data not shown). Taken together, these results suggest that the excision repair deficiencies of the UV5, UV41 and UV20 CHO cell mutants do not confer an increased PDT sensitivity to these cells. A normal PDT sensitivity for excision repair deficient XP groups A, B, and C has also been reported previously (Gomer et al., 1988; Nocentini, 1992). The CHO cell mutants UV5, UV41 and UV20 and excision deficient XP cells are known to be deficient in the initial incision step of excision repair, indicating that a deficiency in this first step of incision repair does not affect the cellular response to PDT.

Also, considered of interest is the reduction in viral DNA synthesis of PDT-treated Ad5(lacZ) compared to PDT-treated Ad5(denV), following infection of the CHO cell lines. The increased survival of viral DNA synthesis in CHO cells following infection with the PDT-treated Ad5(denV) could result from the presence of the denV gene. Previous studies have shown an increased survival of viral DNA synthesis in human fibroblasts following infection with UV irradiated Ad5(denV) compared to the infection with UV irradiated Ad5(lacZ) (Arnold, 1992). These results could reflect the effects of the denV gene on viral DNA replication and/or repair of viral DNA lesions following infection of CHO cells. However, the large reduction in viral DNA synthesis in virus treated with light alone compared to drug alone suggests that treatment conditions could have been altered in the single experiment using PDT-treated Ad5(lacZ) for infection, resulting in the increased survival of viral DNA synthesis following infection of CHO cells with Ad5(denV) compared to Ad5(lacZ). It is also possible the increased survival of viral DNA synthesis in CHO cells following infection with the PDT-treated Ad5(denV) compared to PDT-treated Ad5(lacZ) could result from a difference in the heat stability of the two recombinant viruses.

Results presented in chapters 3 and 4 indicate an increased PDT sensitivity for the CHO-AUXB1 mutant and an increased PDT resistance for the RIF-8A cell line. If the necessary improvements could be made to the protocol for HCR of PDT-treated virus, then it would be of interest to examine the HCR of PDT-treated adenovirus following infection of these cells and their counterparts showing normal PDT sensitivity.

CHAPTER THREE

CROSS-RESISTANCE OF THE RIF-8A MOUSE FIBROSARCOMA CELL LINE TO PDT AND UV LIGHT MEASURED USING A VIRAL CAPACITY ASSAY

CROSS-RESISTANCE OF THE RIF-8A MOUSE FIBROSARCOMA CELL LINE TO PDT AND UV LIGHT MEASURED USING A VIRAL CAPACITY ASSAY

ABSTRACT

Photodynamic therapy (PDT) utilizes the localized delivery of light to activate a photosensitizing drug (such as Photofrin) which is selectively retained by the tumour tissues. Previous reports have shown that viral capacity can be used as an **in vitro** probe for drug sensitivity in human tumours (Parsons et al., Eur. J. Cancer Clin. Oncol., 2, 401, 1986). In this work we show the feasibility of using a viral capacity assay as an indicator of cellular sensitivity to PDT. RIF-1 mouse fibrosarcoma cells and a PDT resistant derivative, RIF-8A; as well as chinese hamster ovary (CHO) cells and CHO-MDR (multi-drug resistant) mutant cells were studied. Consistent with the clonogenic survival of these cells, the viral capacities of RIF-8A and CHO-MDR cells were greater than those of RIF-1 and CHO cells respectively following PDT treatment.

The RIF cell lines were also further characterized with respect to their repair capability and their response to UV light. Data suggests that RIF-8A cells have an increased capacity to repair PDT damage, as well as an increased capacity to repair

UV-induced DNA damage compared to the RIF-1 cell line. Thus, it is possible that altered repair capability of the RIF-8A cell line for PDT damage, possibly DNA damage, may be responsible for its PDT resistance.

INTRODUCTION

Photodynamic therapy (PDT) is a novel treatment in cancer therapy, currently in Phase III clinical trials for a number of human cancers (as reviewed in Marcus, 1992; as reviewed in Pass, 1993). Although this treatment modality is undergoing clinical trials, the mechanism of PDT cytotoxicity is not fully understood.

Photodynamic therapy utilizes the localized delivery of light to activate a photosensitizing drug (such as Photofrin), which is selectively retained by tumour tissue (Bugelski et al., 1981; Kessel and Chou, 1983; as reviewed in Moan, 1986b; Brault, 1990; Maziere et al., 1990).

The photodynamic process requires three simultaneously present components for cytotoxicity: a sensitizer, light and oxygen. The mechanism of cytotoxicity involves the generation of singlet oxygen when the photosensitizer becomes excited, this singlet oxygen is believed to be the major damaging species in PDT (Lee See et al., 1984; Mitchell et al., 1985). PDT induces both cellular and vascular damage of the cells and tissue, which result in tumour necrosis. The cellular effects include membrane damage (Kohn and Kessel, 1979; Bellnier and Dougherty, 1982; Valenzano, 1987; Biade et al., 1992); mitochondrial damage (Woodburn et al., 1992; Hilf et al., 1984; Hilf et al., 1986; Sharkey et al., 1993) and DNA damage (Gomer, 1980; Fiel et al., 1981; Ramakrishnan et al., 1988; Evans et al., 1989; Ramakrishnan et al., 1989; Oleinick et al., 1991; Rerko et al., 1992; Deahl et al., 1993; Noodt et

al., 1993; Gantchey et al., 1994).

Studies have identified a number of cell lines which exhibit an abnormal PDT response. PDT and drug resistant cell lines have been used by several investigators in order to examine the characteristics of PDT cytotoxicity. This has been done by looking at either the PDT susceptibility of cells resistant to other treatments or at the nature of PDT-induced resistance.

A number of multi-drug resistant (MDR) cell lines have been found to be cross-resistant to PDT (Mitchell, 1988; Kessel, 1989; Edwards et al., 1990; Singh et al., 1991). The MDR phenotype is associated with specific alterations in tumour cell membranes and tumour cell pharmacology, specifically, increases in high molecular weight cell surface glycoproteins and decreases in the ability of cells to accumulate and retain drug respectively (as reviewed in Beck, 1987). However, there is no such cross-resistance to PDT, utilizing mesoporphyrin and light, found in a murine leukaemia MDR cell line, P388/ADR (Kessel and Erickson, 1992).

Comparative studies using PDT sensitive and PDT resistant lines will possibly identify targets for PDT killing **in vitro**. Singh et al., (1991) derived a PH II-mediated PDT resistant murine cell line, RIF-8A. Examination of this PDT-resistant cell line and its parental cell line, RIF-1, revealed mitochondrial alterations between the two cell lines (Sharkey et al., 1993). The RIF-8A cells contained smaller mitochondria with more electron dense matrices. The RIF-8A cells were double in size compared to the RIF-1 cells. Owing to the large size difference between RIF-8A cells and RIF-1 cells, there was an increase in ATP content and succinate dehydrogenase activity per cell

in RIF-8A cells. There was an absence of an accompanying significant increase in oxygen consumption in these cells. It has been suggested that RIF-8A cells have an altered energy metabolism when compared to RIF-1 cells since for an equivalent rate of oxygen consumption, RIF-8A contain a greater intracellular pool of ATP.

Comparative studies between the RIF-8A and the RIF-1 cell lines and the CHO-MDR and CHO-N cell lines found that there were no alterations in drug uptake for the RIF cell lines. This suggested that the mechanism responsible for PDT resistance in the RIF-8A cell line is different from that in the CHO-MDR cell line. A cisplatin resistant human ovarian cell line, C13, was shown to be cross-resistant to PDT and the RIF-8A cell line shown to be cross-resistant to cisplatin (Sharkey et al., 1993). The similar mitochondrial alterations noted in RIF-8A cells were also noted in C13 cells (Andrews and Albright, 1992).

PDT sensitive cell lines have also been identified. Murine leukaemic cells deficient in the excision repair of UV-induced damage exhibit a sensitivity to pthalocyanine and PH II-mediated PDT (Evans et al., 1989; Ramakrishnan et al., 1989; Rerko et al., 1992; Deahl et al., 1993).

The intrinsic sensitivity of cells may important in assessing tumour response to PDT. The clonogenic assay, to date, is the most widely used **in vitro** assay in determining tumour response to other cancer therapies. Clinical studies have shown that the clonogenic assay can accurately predict drug sensitivity (Shrivastav et al., 1980; Tanigawa et al., 1982; Tveit et al., 1982; Kirkwood and Marsh, 1983; Gazdar et al., 1990; vanHoff et al., 1990; Parchment et al., 1992) and radiosensitivity

(Michalowski, 1984; Twentyman et al., 1984; as reviewed in Peters, 1990) of human tumours. The use of the clonogenic assay is primarily limited by the poor plating efficiency of human tumour cells and as a result a number of non-clonogenic and short term assays have been developed to quantify the sensitivity of human tumour cells to various cancer therapies.

In this study the capacity of PDT-treated cells for viral DNA synthesis assays is used as a measure of PDT response for different cell types. The cellular capacity assay measures the ability of a cell to support viral growth (Coohill et al., 1977). Physical or chemical treatment of cell monolayers can decrease the cellular capacity to support viral infection. A decrease in capacity for supporting viral infection has been found in cells treated with a number of agents such as proflavin and light, angelicin or 8 methoxypsoralen and UV light (Lytle et al., 1974; Bockstahler et al., 1976; Coppey and Nocentini, 1976; Lytle and Hestler, 1976; Coppey et al., 1979a). Parsons et al., (1986) showed that the cellular capacity assay correctly predicts the sensitivity of antitumour agents for their effectiveness on tumour cells. The host cell reactivation (HCR) assay compares the ability of the host cell to repair and/or replicate damaged viral DNA. HCR of irradiated or chemically treated virus has been investigated in several different cell types and found to be a sensitive and a quantitative measure of the DNA repair capacity of the infected cell (Day, 1974; Rainbow and Howes, 1979; Day, 1980; Hayward and Parsons, 1984; Scudiero et al., 1984).

In this study we show the feasibility of using a cellular capacity assay for

adenovirus DNA synthesis as a measure of the sensitivity of cells to PDT. Using the capacity assay and an HCR assay, we show a cross resistance of RIF-8A cells to UV light. The results of a delayed capacity assay and the HCR assay suggest the increased resistance of RIF-8A to UV and PDT result from elevated levels of repair of UV and PDT damage in RIF-8A cells compared to RIF-1 cells.

MATERIALS AND METHOD

Cells and Viruses.

RIF-1 cells were originally provided by Dr. B. Henderson, Roswell Park Memorial Institute, Buffalo, New York. **RIF-8A** cells, having the PDT-resistant phenotype, were derived from RIF-1 cells (Luna and Gomer, 1991) as described in Singh et al., (1991). The **CHO-N** parental line and **CHO-MDR** cells were obtained from Dr. V. Ling, Ontario Cancer Institute, Toronto, Ontario. The CHO-MDR cell line, containing the p-glycoprotein (CH^RC5), was derived from its parent line as described by Ling and Thompson, (1974). Both cell types were grown as monolayers in alpha minimal essential media (α-MEM) fully supplemented with deoxyribonucleosides and ribonucleosides (Gibco BRL, cat#11900-024), 10% fetal bovine serum (Gibco BRL, cat#16000-028), 4% of an antibiotic-antimycotic solution (penicillin 10,000 units per ml, fungizone 25 μg per ml and streptomycin 10,000 μg per ml) (Gibco BRL, cat#15240-013) and 4% of L-glutamine (Gibco BRL, cat#25030-016) in 75 cm^2 plastic flasks. Cells were grown in a humidified incubator at 37°C with 5% CO_2.

The human adenovirus Ad5 and the recombinant viruses Ad5(denV) and Ad5(lacZ) were used in the experiments. Ad5(denV) contains the bacteriophage T4 pyrimidine dimer DNA glycosylase (den V) gene inserted into the deleted E3 region (Colicos et al., 1991). Ad5(lacZ) contains the ß-galactosidase (lacZ) gene inserted into the deleted E3 region and was provided by Dr. Frank Graham, Departments of Biology

and Pathology, McMaster University, Hamilton, Ontario.

Photosensitizer and Light Source

Photofrin II was obtained by Quadralogic Technologies Inc. (Vancouver, B.C. Canada). It was diluted to a 2.5 mg/ml solution using α-MEM, from an original stock of 10 mg/ml.

Plates were exposed to red light using a 21" x 32.75" light box illuminated by a parallel series of 12 fluorescent tubes (Philips type TL/83), enclosed on top with a sheet of clear Plexiglass and filtered with red acetate filters (No. 19, roscolux, Rosco, CA). The emission spectrum of the light source was measured using a Jobin-Yvon model CP-200 spectrometer and a Princeton Instruments CCD. The energy fluence rate was 0.34 $W/m^2/sec$ in the wavelength band of 640 nm. Exposure for 5 minutes resulted in incident energy fluence of 102 J/m^2.

Cellular Capacity Assay for PDT-treated cells.

The capacity of RIF cells and CHO cells to support adenovirus DNA replication following PDT treatment was determined as follows.

Preconfluent cells were rinsed with 1x PBS (diluted from a 10x stock in double distilled water (0.8g NaCl, 2g KCl, 1.15g Na_2HPO_4 and 0.2g K_2PO_4)), 2 ml of trypsin (2x, diluted in PBS from a 10x stock) was added and cells were resuspended in 8 ml of complete growth medium. A haemocytometer was used to determine the number of cells present in the suspension. Cells were seeded in 24-well Linbro plates at 2 x

10^5 cells/well and allowed 4 hours for cellular attachment. After this 4 hour period, the medium was removed and replaced with 0.5 ml of fresh medium and 0.1 ml of a Photofrin II suspension of an appropriate concentration was added to each well. At this point all subsequent steps were carried out in minimal light conditions until the addition of pronase solution used in the first step of viral DNA extraction. After an 18 hour incubation, the drug containing media was removed and replaced with 0.5 ml of fresh media. The cells were then exposed to red light at a fixed light fluence rate of 0.34 $W/m^2/sec$ for 5 minutes.

Cells were then infected with virus either immediately or 6 hours after PDT treatment. The cells were infected with 0.2 ml of the adenovirus suspension at a multiplicity of infection (MOI) of 40 pfu/cell for 90 minutes, during which time the plates were gently rocked at 15 minute intervals. Each well was then overlayed with 1 ml of growth medium. The medium was replaced at two to four hours after the overlay with 0.5 ml of growth medium to remove unabsorbed virus and samples taken to quantitate the amount of virus absorbed. After 48 hours (for RIF cells) and 72 hours (for CHO cells) the amount of viral DNA replication was quantitated.

Samples were collected by the addition of 0.2 ml of lysis solution (4 mg/ml pronase, 40 mM tris pH 8.0, 40 mM EDTA pH 8.0, 2.4 % SDS) directly to the 0.5 ml of growth medium in each well. Following the addition of the lysis solution, the plates were returned to 37°C for 1 to 3 hours. After this time, the contents of the well was collected and the DNA extracted using the phenol:chloroform method. One volume (0.7 ml) phenol:chloroform was added to each well. The mixture was

vortexed, centrifuged and the aqueous phase transferred to a second microfuge tube, one volume of isoamyl alcohol:chloroform was then added. The mixture was vortexed, centrifuged and the aqueous phase transferred to a third microfuge tube. DNA was precipitated with 5 M NaCl. The DNA was slot blotted to nitrocellulose and probed with Ad2 DNA (Gibco BRL, cat#15270-010) as a template as described in Sambrook et al., (1986). DNA was then quantitated using a Phosphorimager, and ImageQuant software (Molecular Dynamics, Sunnyvale, California).

Split dose experiments for PDT treated RIF cells.

The clonogenic survival of RIF cells following fractionated doses of PDT was determined as described in Singh et al., (1991) with the following modifications.

Preconfluent cells were seeded in 100 mm tissue culture plates at 2 x 10^3 cells/plate and allowed to adhere for 4 hours. The medium was then removed and replaced with drug-containing medium at an appropriate drug concentration. All subsequent steps were carried out in minimal light conditions until the additon of the stain. Following a 24 hour incubation, the drug-containing medium was removed and replaced with fresh medium. Following various time periods of efflux, cells were exposed to either single or split light doses. Cells were then allowed to grow for 5 days and subsequently fixed and stained with methylene blue (0.5 g methylene blue, 70 ml ethanol, 30 ml double distilled water) and colonies of 20 cells or more counted and scored.

UV Treatment to cells and virus.

The cellular capacity of UV-irradiated cells and the host cell reactivation (HCR) of UV irradiated virus were executed in a similar way to PDT experiments.

For the cellular capacity experiments cells were seeded in 24-well Linbro plates as for the PDT experiments. Following a 24 hour incubation period, the medium from the wells was aspirated and replaced with 0.2 ml of pre-warmed 1x PBS. The cells were then irradiated at a number of different UV fluences. After irradiation, the 1X PBS was removed from each well and UV-irradiated cells were immediately infected with adenovirus and viral DNA replication quantitated as previously described.

For the HCR experiments, cells were seeded in 24-well Linbro plates as for the PDT experiments. Following a 24 hour incubation period, the cells were infected with UV-irradiated adenovirus and the amount of adenovirus DNA quantitated as previously described. Virus was irradiated prior to infection as described previously in Rainbow (1977). Virus stocks were diluted to appropriate concentrations for infection in α-MEM supplemented with 1% antibiotic-antimycotic. 1 ml of the adenovirus suspension was placed in 35 mm plates and irradiated. During irradiation of virus, the 35 mm plates were kept on ice and the virus suspension continually swirled using a magnetic stir bar.

The cells and the virus were irradiated with a UV lamp (General Electric Germicidal Lamp G8T5) emitting a wavelength of predominantly 254 nm. The incident dose rate was 2 $J/m^2/sec$ as determined with a J-255 shortwave UV meter (Ultraviolet Products, San Gabriel, California).

RESULTS

Capacity of PDT-treated CHO and RIF cells.

The cellular capacity of CHO cells and RIF cells to support adenovirus DNA replication following PDT treatment was assayed. The response was compared to previously published results for the colony forming ability of CHO cells and RIF cells following PH II-mediated PDT treatment (Singh et al., 1991; Sharkey et al., 1993).

A representative cellular capacity curve of PDT-treated CHO cell lines to support the replication of Ad DNA is shown in figure 1A as a function of varying Photofrin concentration and fixed light fluence. The amount of viral DNA synthesis following PDT treatment was determined relative to the amount of viral DNA replicated in untreated cells for each dose point. The log of the relative capacity values for viral DNA synthesis at each dose point were averaged in each experiment to produce a single logarithmic mean and a standard error of the mean as shown in figure 1A. It can be seen that CHO-MDR cells have a greater cellular capacity for viral infection compared to CHO-N cells following PDT treatment. In order to pool results from a number of experiments, data was normalized as a function of the capacity of CHO-N cells versus the ratio of the capacity of CHO-MDR cells to CHO-N cells at each dose point as shown in figure 1B. It can be seen that as the capacity of CHO-N cells decreases, there was an increased capacity of CHO-MDR cells compared to CHO-N cells. Previously published results for the clonogenic survival of CHO-N cells and CHO-

MDR cells plotted in the similar fashion are shown for comparison (Singh et al., 1991).

A representative cellular capacity curve of PDT-treated RIF cell lines to support the replication of Ad DNA is shown in figure 2A. It can be seen that RIF-8A cells have a greater cellular capacity for viral infection than RIF-1 cells following PDT treatment. The data from a number of experiments was pooled in figure 2B. It can be seen that as the capacity of RIF-1 cells decreases, there is an increased capacity of the RIF-8A cells compared to RIF-1 cells. Clonogenic survival of RIF-8A cells and RIF-1 cells are shown on the same plot for comparison purposes (Singh et al., 1991).

Comparison of Clonogenic Survival of RIF-1 and RIF-8A cells for Split Dose PDT Experiments.

Split dose PDT experiments were carried out to examine a difference in recovery and/or repair capabilities of RIF-8A cells and RIF-1 cells following PDT treatment. Previous studies have shown that fractionation of the light dose has resulted in a recovery in clonogenic survival of cells from Hpd-sensitized damage and merocyanine 540-sensitized damage (Bellnier and Lin, 1985; Qiu and Sieber, 1992).

Figure 3 shows results from a typical split-dose survival experiment. It can be seen that splitting the light dose into two fractions given at 18 and 24 hours PDT treatment resulted in an increased colony survival for both RIF-1 and RIF-8A cells. However, the survival of cells given a single light dose was also increased and to a similar extent to the split dose survival when the light exposure was given at 24 hours

as compared to 18 hours after PDT treatment. This suggests that efflux of the drug between 18 and 24 hours after PDT treatment also affects cell survival such that the true extent of repair of sublethal PDT damage could not be determined.

Comparison of Capacity of RIF cells for Immediate and Delayed Infection following PDT Treatment.

The capacity assay has also been used to compare the recovery and/or repair of mammalian cells following exposure to DNA damaging agents (Lytle et al., 1976; Coppey et al., 1979a; Coppey et al., 1979b). In this work, it was of interest to examine differences in the recovery and/or repair capabilities of RIF-8A cells in comparison to RIF-1 cells following PDT damage.

Figure 4A shows a typical cellular capacity curve depicting the capacity of PDT-treated RIF cells to support the replication of Ad DNA immediately following PDT treatment and 6 hours post PDT treatment. It can be seen that there is an increased capacity for viral infection in both RIF-1 and RIF-8A cells 6 hours post PDT treatment. Furthermore, RIF-8A cells have a greater cellular capacity compared to RIF-1 cells when the time of infection is delayed by 6 hours with respect to the time of PDT treatment.

Pooled data from a number of experiments is shown in figure 4B. Data points in figure 4B were fitted to a straight line using linear regression to obtain slopes ($\pm$ standard errors) of -0.73 $\pm$ 0.05 and -0.91 $\pm$ 0.065 for immediate and delayed capacity respectively. It can be seen that as the capacity of RIF-1 cells decreases,

there is a significant increase in the capacity of RIF-8A cells in comparison to RIF-1 cells when a 6 hour period is allowed between PDT treatment and infection.

In order to ensure that the increased amount of viral DNA synthesis was not due to the proliferation of the RIF-8A cells during this 6 hour time delay, both the RIF-1 cells and the RIF-8A cells were counted prior to each infection. The number of cells in both cell lines and at the two infection times was not significantly different (data not shown).

Capacity of CHO and RIF cells following UV treatment and HCR of UV-treated Ad DNA in RIF cells.

Cell lines that exhibit a resistance or a sensitivity to a DNA damaging agent often show a cross-resistance or cross-sensitivity to other DNA damaging agents (as reviewed in Collins, 1993). The murine leukaemic cell line, LY-R, which shows a sensitivity to UV light irradiation also shows a cross-sensitivity to pthalocyanine and PH II-mediated PDT (Evans et al., 1989; Ramakrishnan et al., 1989; Rerko et al., 1992; Deahl et al., 1993). The examination of the cross-resistance profile of cells resistant to PDT can provide insight into the cytotoxic mechanism of PDT. It was therefore considered of interest to examine the capacity of CHO cells and RIF cells following UV light treatment.

A representative cellular capacity curve of UV-treated CHO cell lines to support the replication of Ad DNA is shown in figure 5A. It can be seen that CHO-MDR cells and CHO-N cells are similar in their capacity to support adenovirus DNA replication

following UV light treatment.

A representative cellular capacity curve of UV-treated RIF cell lines to support the replication of Ad DNA is shown in figure 5B. It can be seen that RIF-8A cells have an increased capacity to support Ad DNA replication in comparison to RIF-1 cells following UV treatment. Data from a number of experiments was pooled in figure 5C. It can be seen that as the capacity of RIF-1 cells decreases, the capacity of the RIF-8A cells relative to RIF-1 cells increases indicating an increased resistance of RIF-8A cells to UV light.

The HCR of UV-damaged adenovirus has been used previously as a measure of the cell line's capacity for the repair of DNA damage (Rainbow, 1981). It was therefore of interest to examine the repair capability of the RIF-8A cell line in comparison to the RIF-1 cell line for UV-damaged adenovirus.

Results shown in figure 5D suggest a small, but significant increase in the repair capacity of RIF-8A compared to RIF-1 cells for UV-damaged virus. The relative HCR of RIF-8A cells compared to RIF-1 cells for the three experiments carried out was 2.99 ± 0.010, 1.46 ± 0.001 and 0.948 ± 0.001. The mean relative HCR and the standard error of the mean is 1.799 ± 0.614. The relative HCR is the slope for UV survival of Ad5(lacZ) DNA synthesis in RIF-8A cells expressed as a ratio of that in RIF-1 cells.

Figure 1A: The Capacity of PDT-treated CHO Cells to Support Viral DNA Replication following Infection with Ad5(denV).

The relative amount of viral DNA synthesized in PDT treated CHO- MDR cells (closed symbols) and CHO-N cells (open symbols) following infection with Ad5(denV) at an MOI of 40 pfu/cell was scored at 72 hours post-infection. Cells were incubated with 2.5, 5, 10, 15 or 20 μg/ml of Photofrin II for 18 hours and exposed to 120 J/m^2 of red light. Results shown are for a typical experiment. Each point represents the logarithmic mean of duplicate trials or is a single trial and the standard error of the mean is shown by the bar or is contained within the symbol. Points with arrows indicate that the amount of viral DNA synthesized was lower than .001. Although there was considerable experiment-to-experiment variation, the resistance of the CHO-MDR cells was observed in each of the 6 experiments performed.

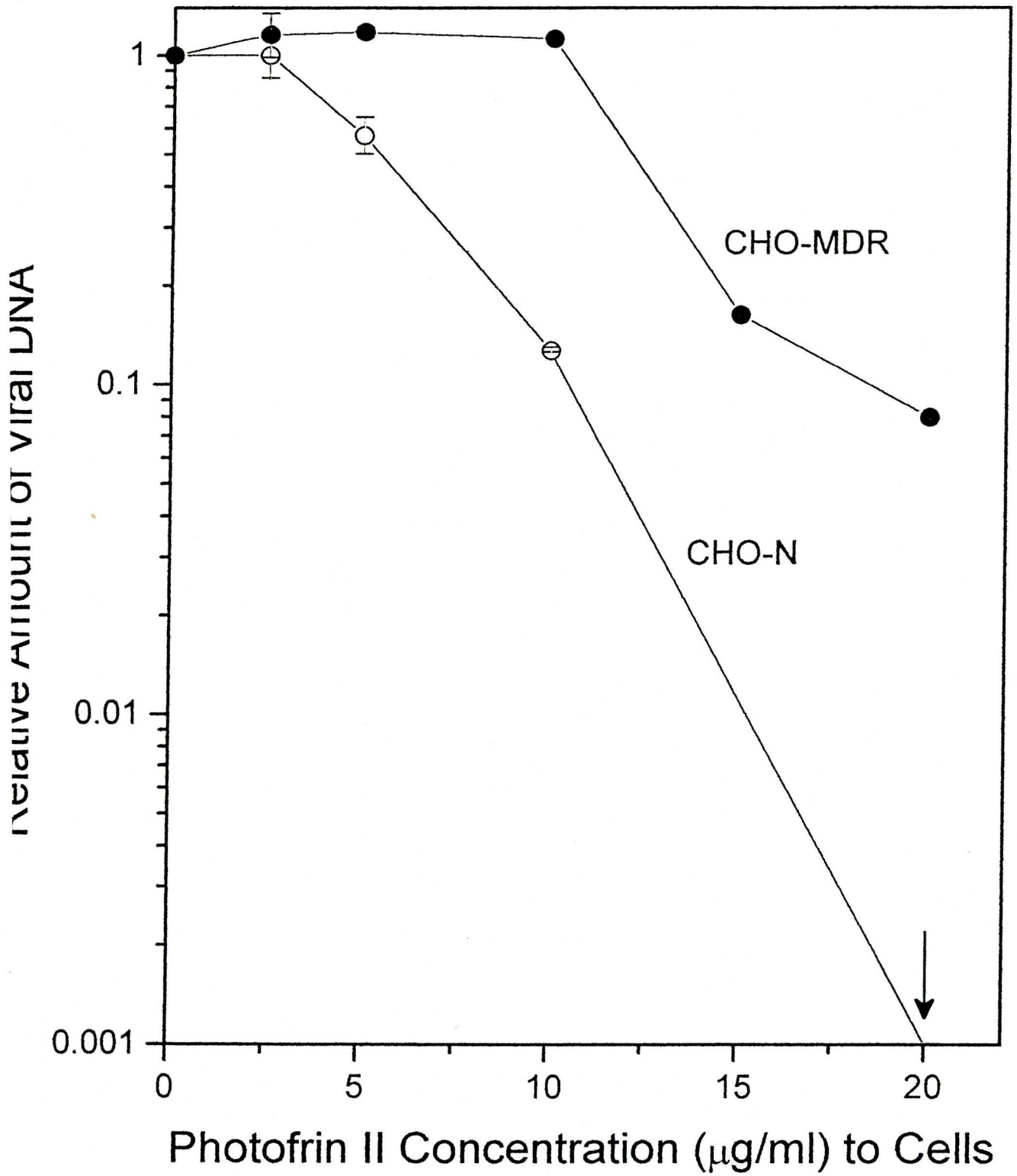

Relative Amount of Viral DNA
1
0.1
0.01
0.001
0
5
10
15
20
CHO-MDR
CHO-N
Photofrin II Concentration (μg/ml) to Cells

Figure 1B: The Capacity of CHO-MDR Cells to Support Viral DNA Replication of Ad5(denV) as PDT Damage to CHO-N Cells Increases.

Points (open symbols) represent the capacity of CHO-N cells as a function of the capacity of CHO-MDR cells relative to CHO-N cells at each drug concentration using pooled data from 6 experiments. The line was fitted using linear regression for the cellular capacity data. Previously published results for the clonogenic survival of CHO-N and CHO-MDR cells following PDT treatment (Singh et al., 1991) are plotted in a similar way (closed symbols) and are shown for comparison purposes.

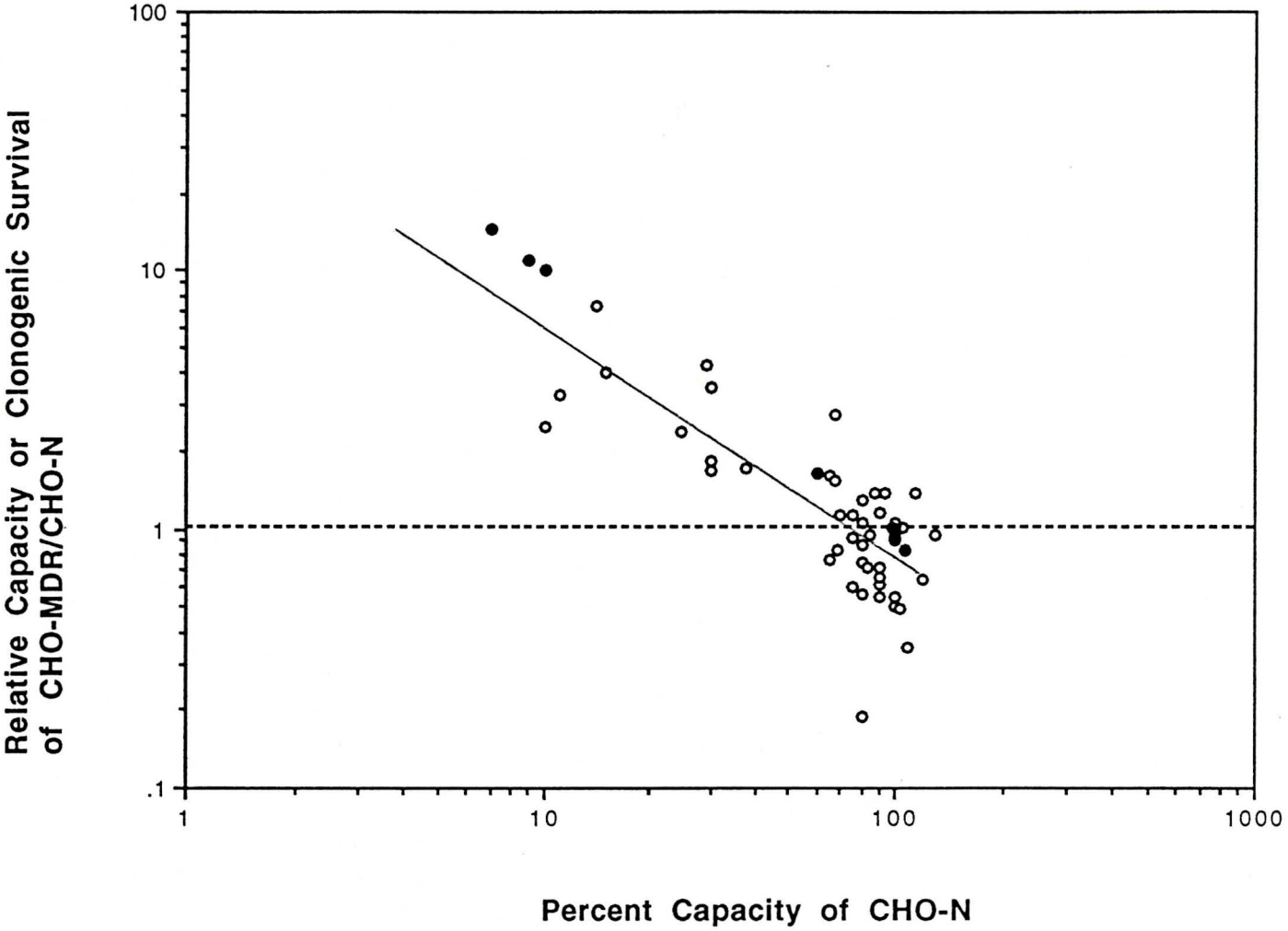

Relative Capacity or Clonogenic Survival of CHO-MDR/CHO-N
100
10
1
.1
1
10
100
1000
Percent Capacity of CHO-N

Figure 2A: The Capacity of PDT-treated RIF Cells to Support Viral DNA Replication following Infection with Ad5(denV).

The relative amount of viral DNA synthesized in PDT treated RIF-8A cells (closed symbols) and RIF-1 cells (open symbols) following infection with Ad5(denV) at an MOI of 40 pfu/cell was scored at 48 hours post-infection. Cells were incubated with 2.5, 5, 10, 15 or 20 μg/ml of Photofrin II for 18 hours and exposed to 120 J/m^2. Results are shown for a typical experiment. Each point represents the logarithmic mean of duplicate or triplicate trials and the standard error of the mean is shown by the bar or is contained within the symbol. Although there was considerable experiment-to-experiment variation, the resistance of the RIF-8A cells was consistently observed in each of the 6 experiments performed.

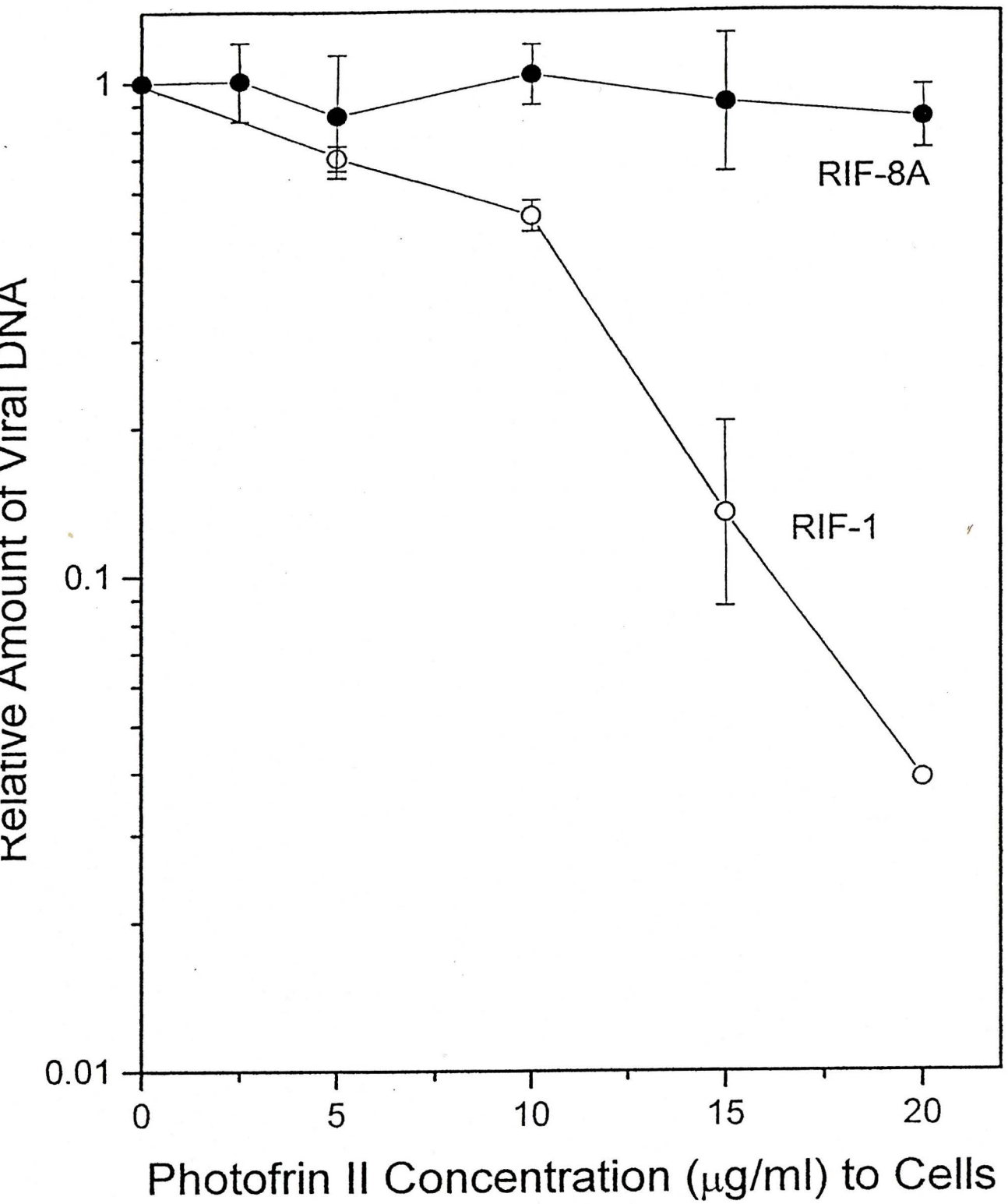

Relative Amount of Viral DNA
1
0.1
0.01
0
5
10
15
20
RIF-8A
RIF-1
Photofrin II Concentration (μg/ml) to Cells

Figure 2B: The Capacity of RIF-8A Cells to Support Viral DNA Replication of Ad5(denV) as PDT Damage to RIF-1 Cells Increases.

Points (open symbols) represent the capacity of RIF-1 cells as a function of the capacity of RIF-8A cells relative to RIF-1 cells at each dose point for pooled data from 6 experiments. The line was fitted using linear regression analysis for the cellular capacity data. Previously published results for the clonogenic survival of RIF-1 and RIF-8A cells following PDT treatment (Singh et al., 1991) are plotted in a similar way (closed symbols) and are shown for comparison purposes.

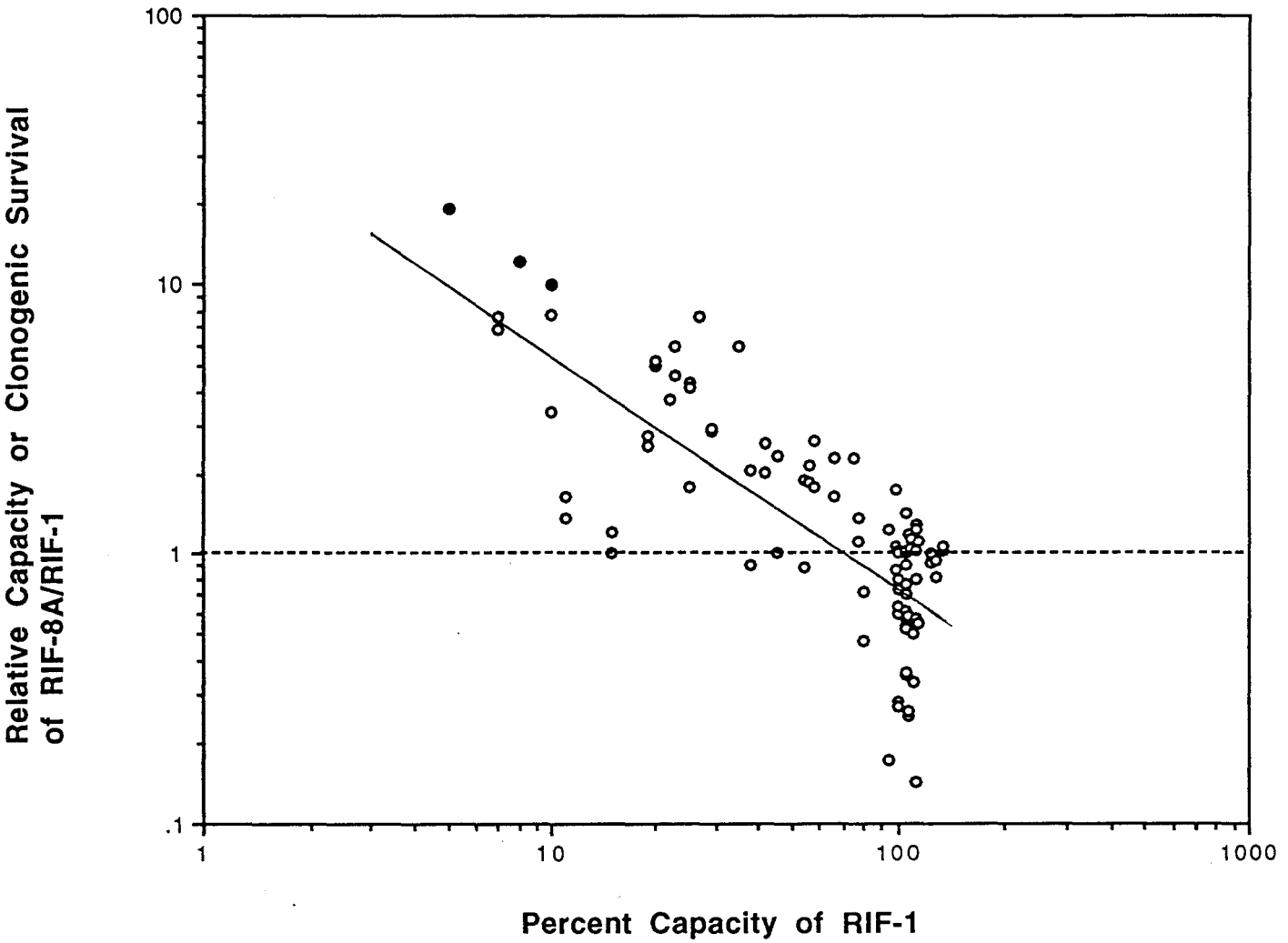

100
10
1
.1
1
10
100
1000
Relative Capacity or Clonogenic Survival of RIF-8A/RIF-1
Percent Capacity of RIF-1

Figure 3: Split-dose Survival of RIF-1 and RIF-8A Cells.

Cells were incubated with 20 μg/ml of Photofrin for 24 hours. The drug containing medium was then removed and replaced with fresh growth medium. Following a period of efflux, cells were exposed to either single or split light doses. For RIF-1 cells single light exposures of 10 minutes were given after either 18 or 24 hours of efflux and split dose was given by 5 minutes at both 18 and 24 hours of efflux. For the RIF-8A cells, the single light exposures were for 15 minutes at either 18 or 24 hours of efflux and the split dose given by 7.5 minutes at both 18 and 24 hours of efflux. Colonies were counted 5 days after exposure. Survival values compared to drug plus no light treatment were obtained from the mean colony counts of the 3 to 6 dishes used for each treatment, the bars indicate the standard error of the mean. Drug alone reduced the colony counts to 39% and 56% for the RIF-1 and RIF-8A cells respectively.

1 - RIF-1, 10 minute exposure at 18 hours
2 - RIF-1, 5 minute exposure at 18 hours plus a second 5 minute exposure at 24 hours
3 - RIF-1, 10 minute exposure at 24 hours

4 - RIF-8A, 15 minute exposure at 18 hours
5 - RIF-8A, 7.5 minute exposure at 18 hours plus a second 7.5 minute exposure at 24 hours
6 - RIF-8A, 15 minute exposure at 24 hours

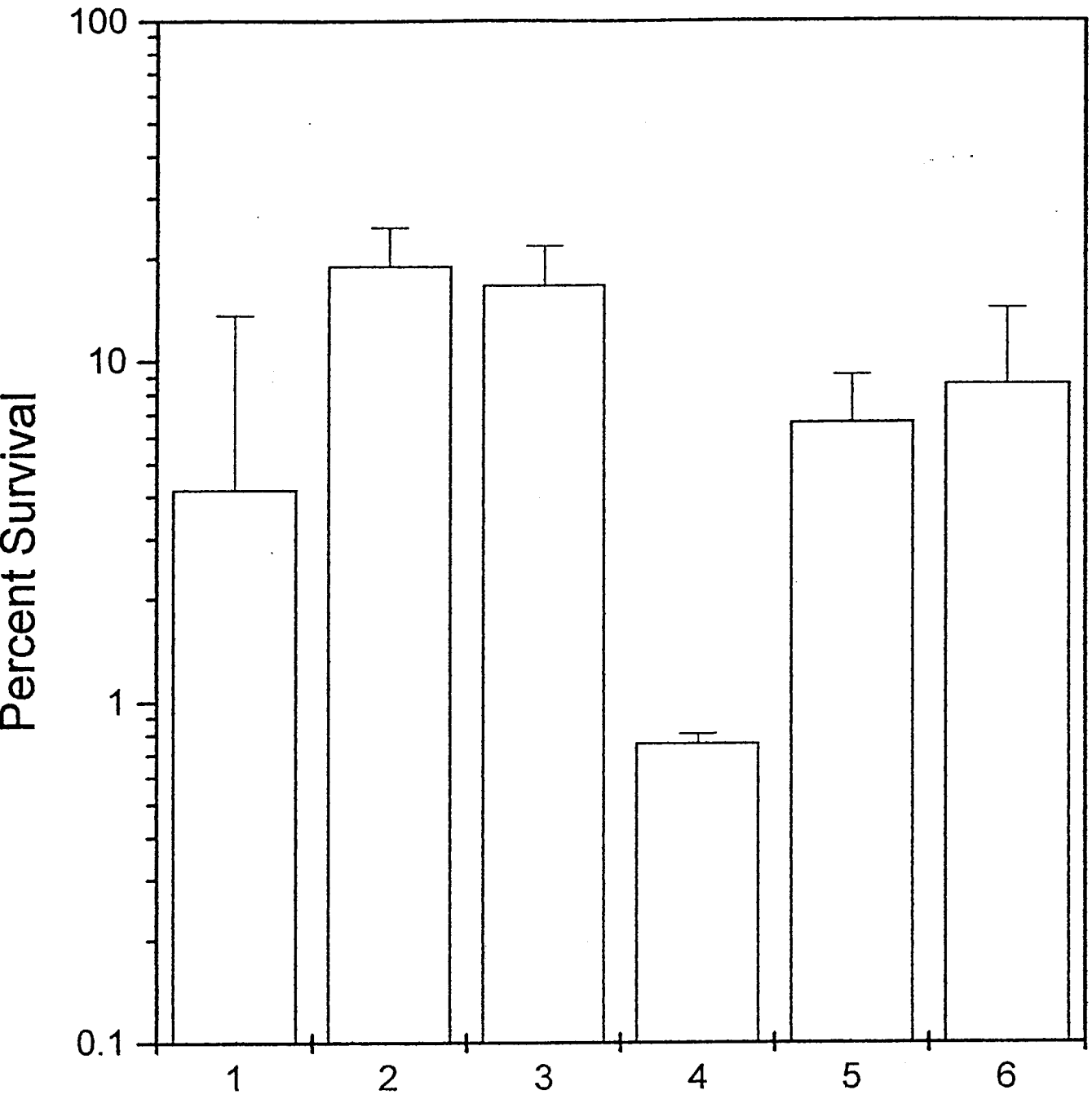

100
10
1
0.1
Percent Survival
1
2
3
4
5
6

Figure 4A: The Capacity of PDT-treated RIF Cells to Support Viral DNA Replication following Infection with Ad5(denV) Immediately following PDT Damage and 6 Hours Post-PDT Damage.

The relative amount of viral DNA synthesized in PDT treated RIF-8A cells (closed symbols) and RIF-1 cells (open symbols) following immediate infection (circles) with Ad5(denV) at an MOI of 40 pfu/cell or infection 6 hours post PDT damage (squares) was scored at 48 hours post-infection. Cells were incubated with 10, 17.5, 20, 22.5 and 25 μg/ml of Photofrin II for 18 hours and exposed to 120 J/m^2. Each point represents the logarithmic mean of pooled data from two experiments (each with 4 trials) and the standard error of the mean is shown by the bar or is contained within the symbol. Although there was considerable experiment-to-experiment variation, the increased capacity of the RIF-8A following delayed infection was consistently observed in every experiment (4).

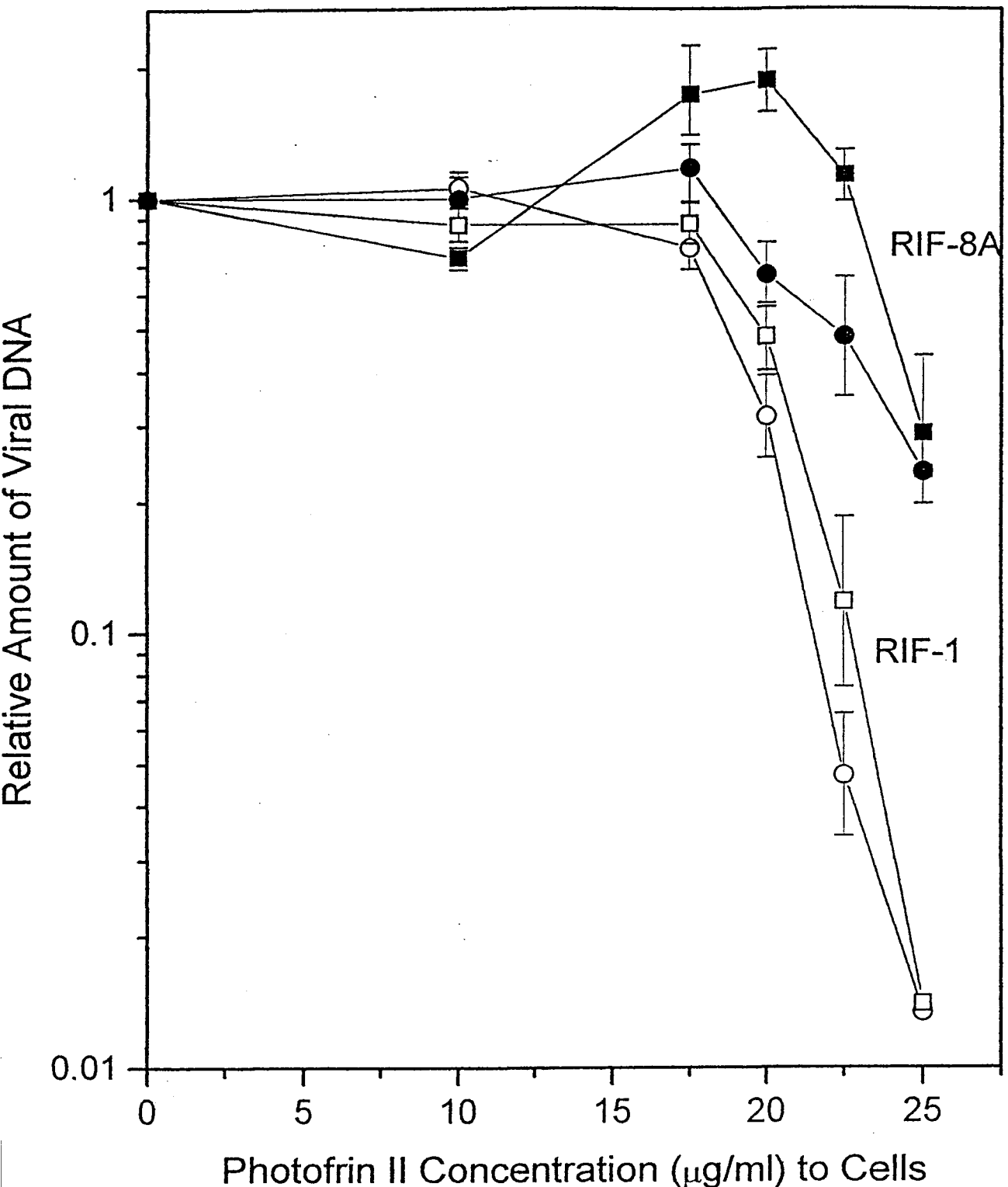

Relative Amount of Viral DNA
1
0.1
0.01
0
5
10
15
20
25
Photofrin II Concentration (μg/ml) to Cells
RIF-8A
RIF-1

Figure 4B: Comparison of the Capacity of RIF-8A Cells to Support Viral DNA Replication of Ad5(denV) as PDT Damage to RIF-1 Cells Increases Immediately following PDT Damage and 6 Hours Post-PDT Damage.

The capacity of RIF-1 cells is plotted as a function of the capacity of RIF-8A cells relative to RIF-1 cells for immediate infection (closed symbols) and for infection 6 hours after PDT treatment of cells (open symbols). The figure shows pooled data from 4 experiments. Lines were fitted using linear regression analysis for the immediate infection data (solid line) and the delayed infection data (broken line).

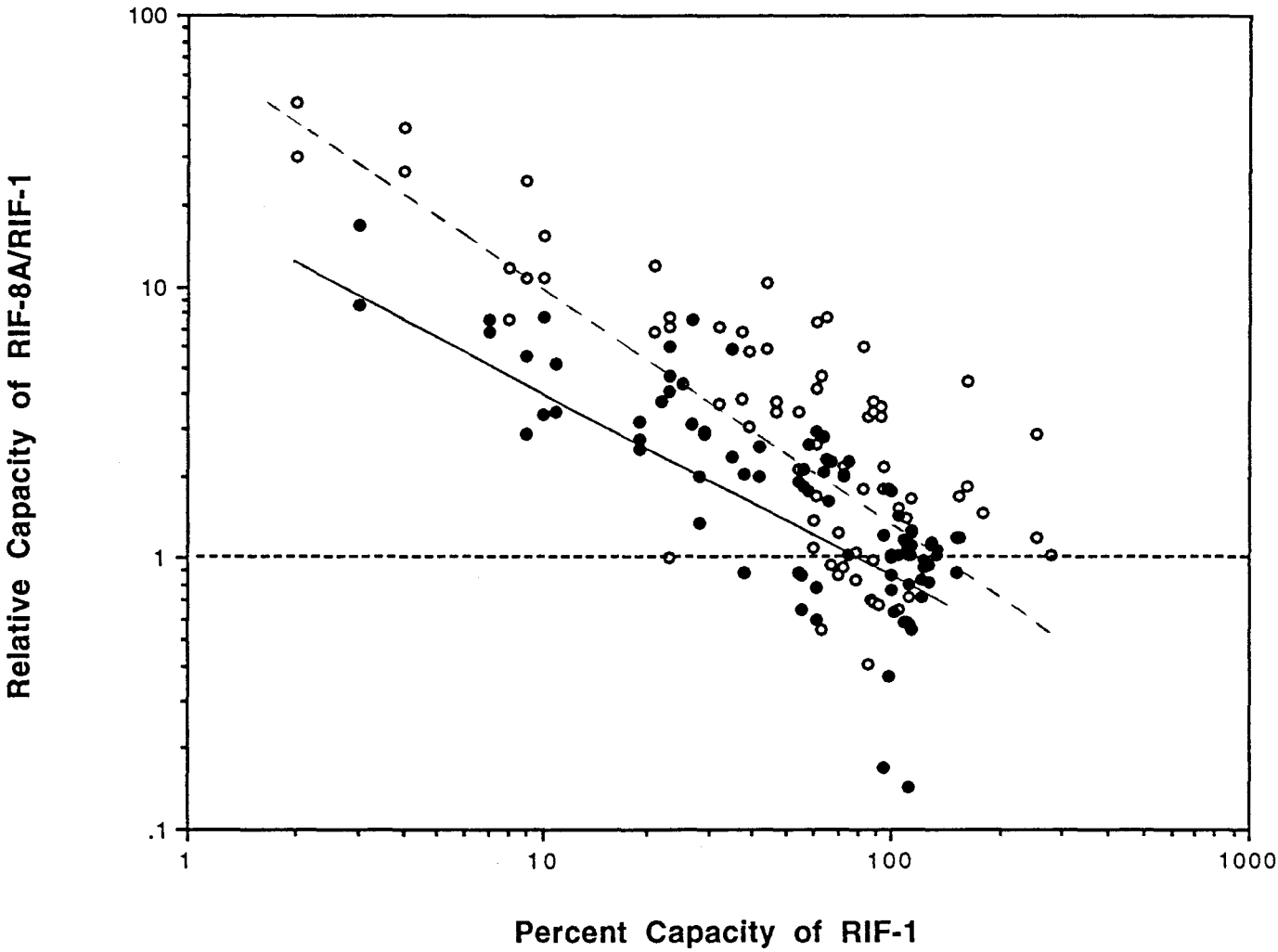

100
10
1
.1
1
10
100
1000
Relative Capacity of RIF-8A/RIF-1
Percent Capacity of RIF-1

Figure 5A: The Capacity of UV-irradiated CHO Cells to Support Viral DNA Replication following Infection with Ad5(lacZ).

The relative amount of viral DNA synthesized in UV-treated CHO-N cells (open symbols) and CHO-MDR cells (closed symbols) following infection with Ad5(lacZ) at an MOI of 40 pfu/cell was scored 72 hours post-infection. Cells were irradiated at a fluence rate of 2 $J/m^2/sec$. Each fluence point represents the logarithmic mean of pooled data from two experiments (each with triplicate trials) and the standard error of the mean is shown by the bar or is contained within the symbol. Similar results were obtained in two other experiments.

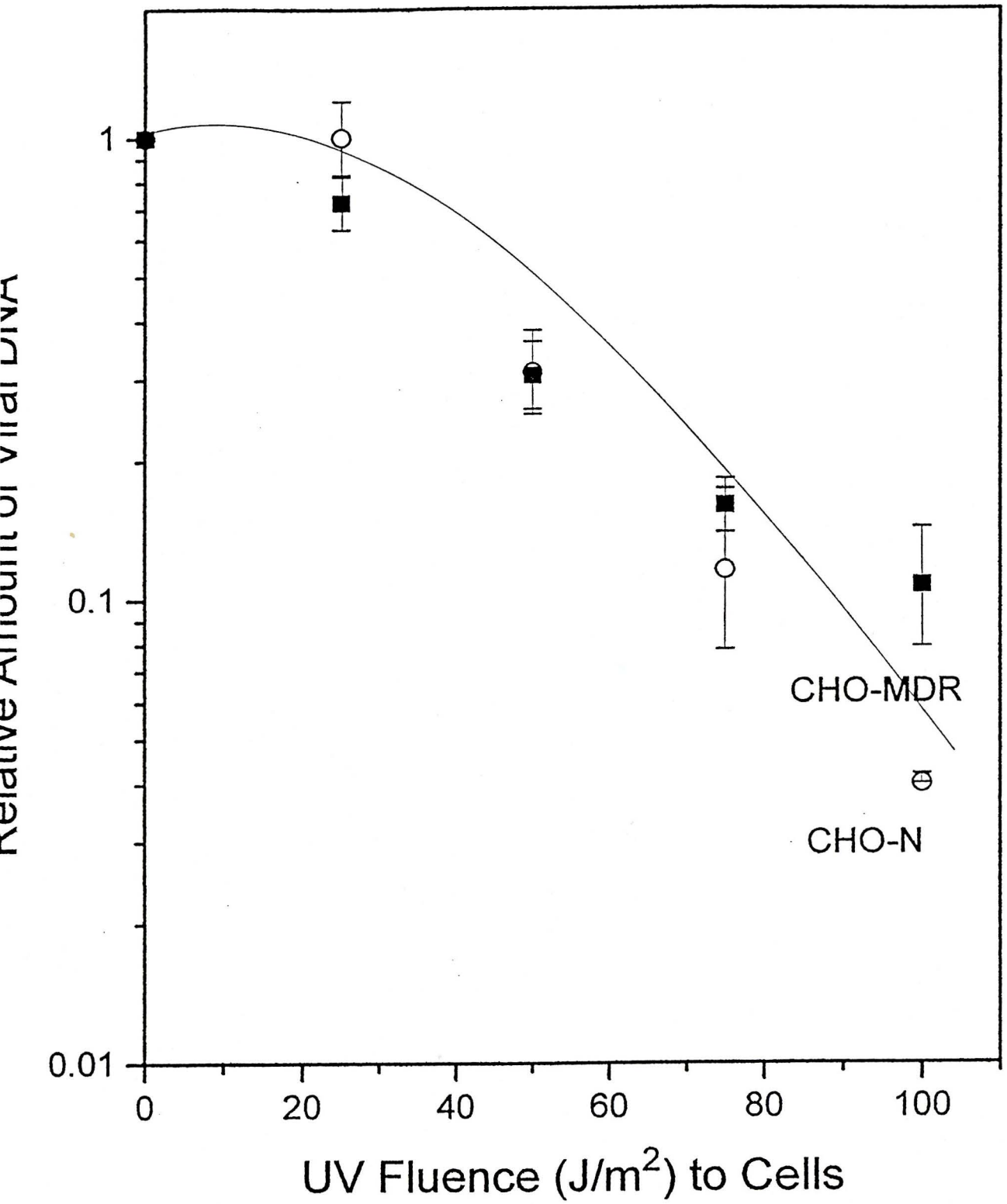

Relative Amount of Viral DNA
1
0.1
0.01
0
20
40
60
80
100
UV Fluence (J/m2) to Cells
CHO-MDR
CHO-N

Figure 5B: The Capacity of UV-irradiated RIF Cells to Support Viral DNA Replication following Infection with Ad5(lacZ).

The relative amount of viral DNA synthesized in UV-treated RIF-1 cells (open symbols) and RIF-8A cells (closed symbols) following infection with Ad5(lacZ) at an MOI of 40 pfu/cell was scored 48 hours post-infection. Cells were irradiated at the indicated fluence points at a fluence rate of 2 $J/m^2/sec$. Each fluence point represents the logarithmic mean of data from 1 experiment with duplicate trials and the standard error is shown by the bar or is contained within the symbol. Although there was considerable experiment-to-experiment variation, the resistance of the RIF-8A cells was consistently observed in each of the 6 experiments performed.

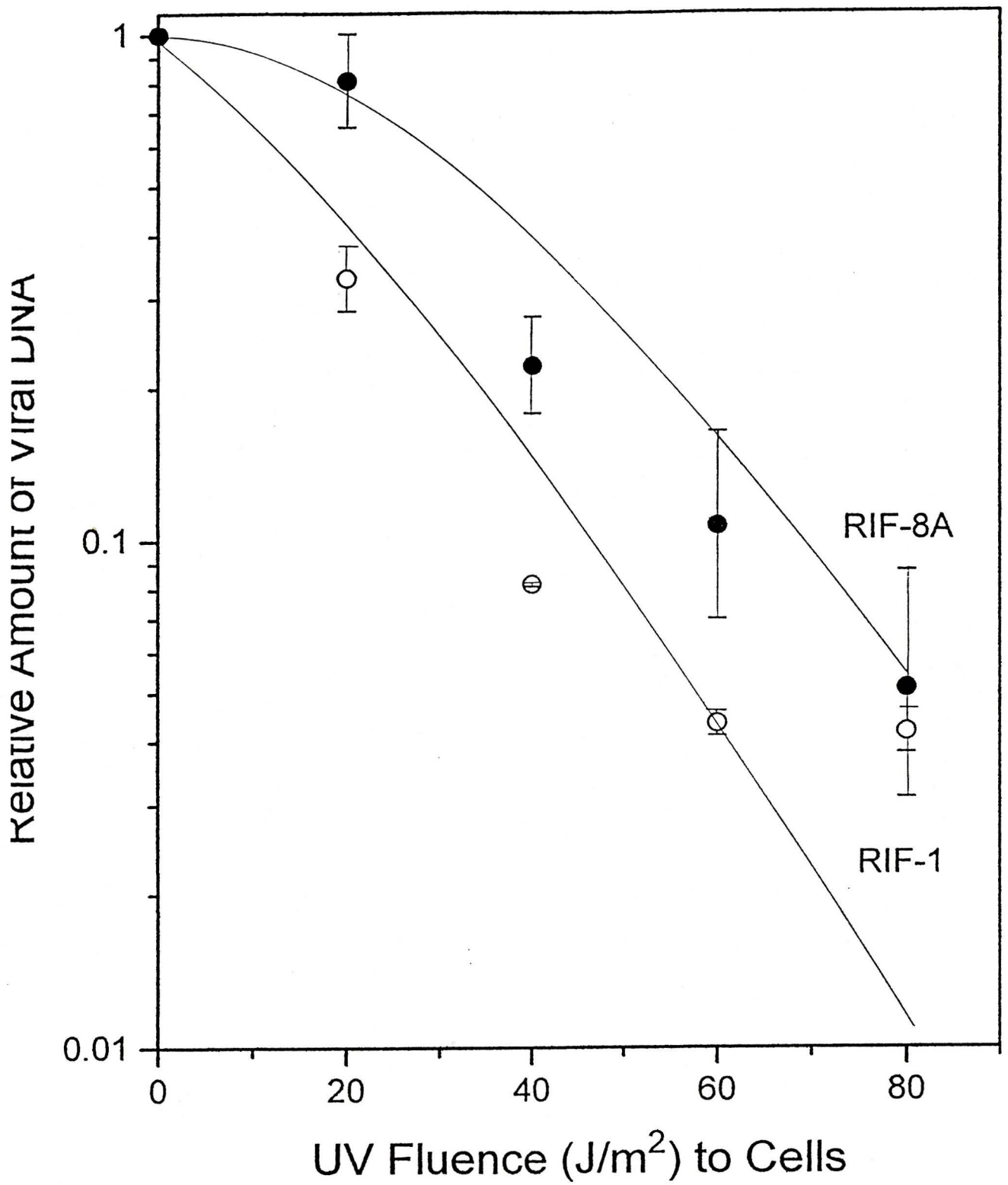

Relative Amount of Viral DNA
1
0.1
0.01
0
20
40
60
80
UV Fluence (J/m2) to Cells
RIF-8A
RIF-1

Figure 5C: The Capacity of RIF-8A Cells to Support Viral DNA Replication of Ad5(lacZ) as UV Damage to RIF-1 Cells Increases.

Points represent the capacity of RIF-1 cells as a function of the capacity of RIF-8A cells to RIF-1 cells at each fluence point using pooled data from 6 experiments. The line was fitted using linear regression analysis.

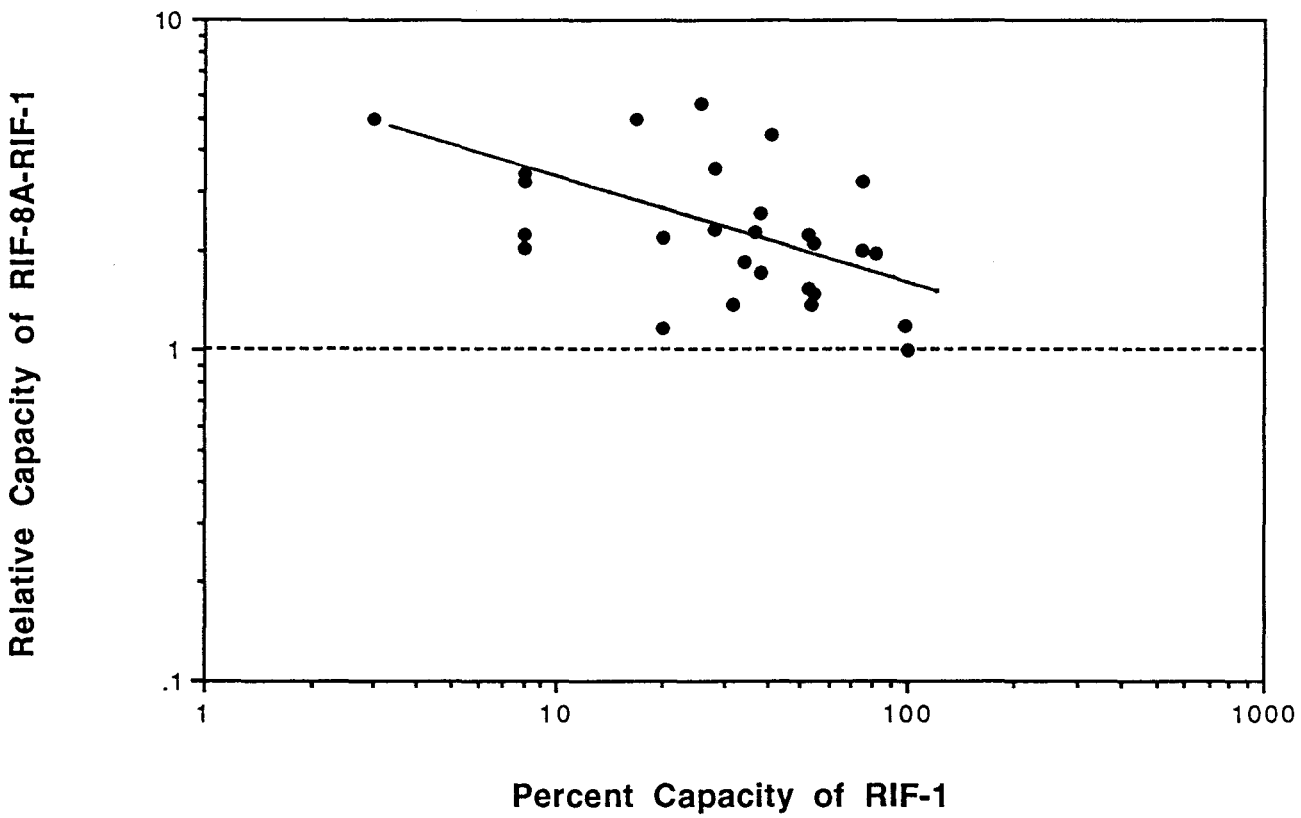
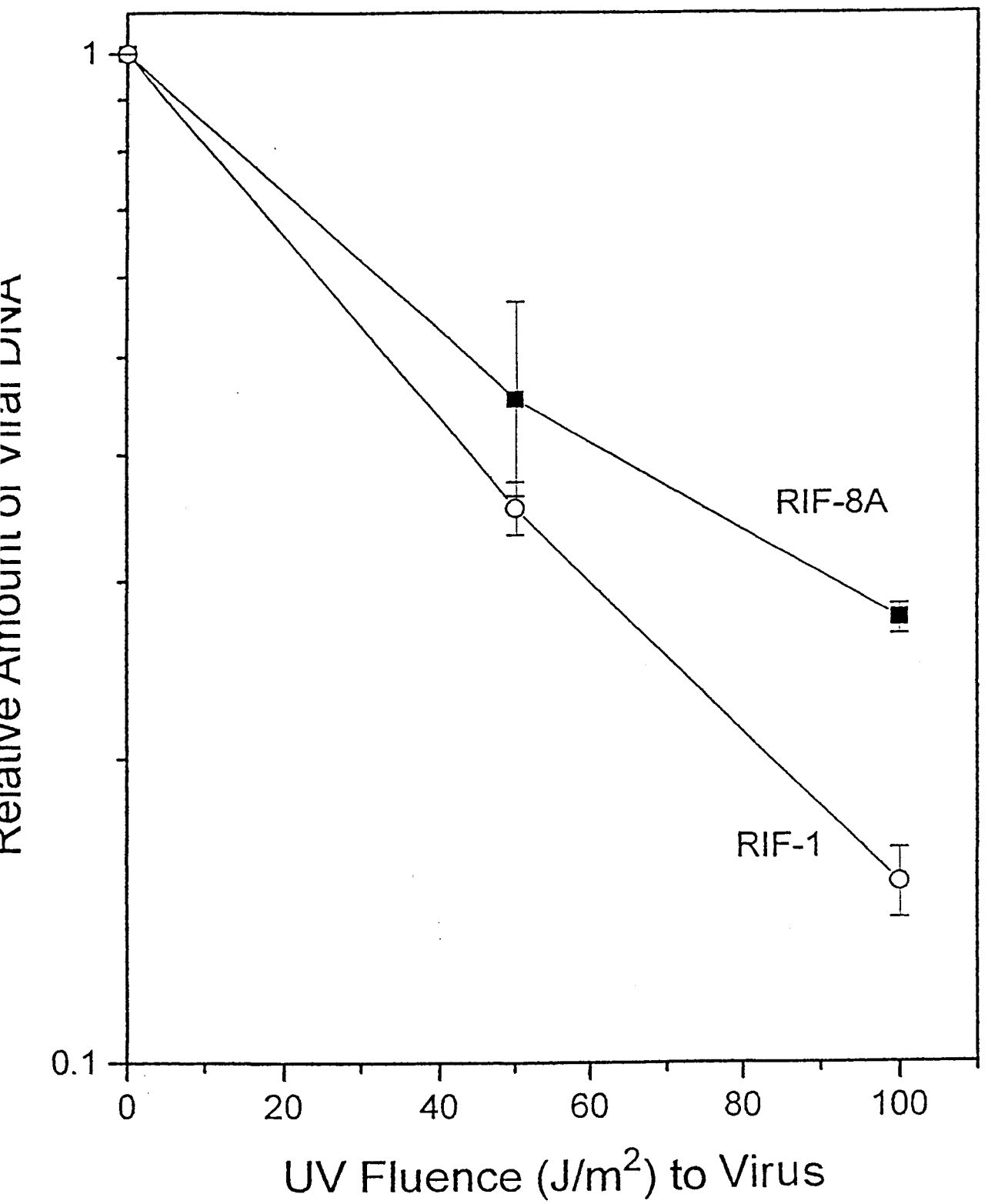

10
1
.1
1
10
100
1000
Relative Capacity of RIF-8A-RIF-1
Percent Capacity of RIF-1

Figure 5D: The Survival of Viral DNA Synthesis of UV-irradiated Ad5(lacZ) in RIF Cells.

The relative amount of viral DNA synthesized in RIF-1 cells (open symbols) and RIF-8A cells (closed symbols) following infection with UV-irradiated Ad5(lacZ) at an MOI of 40 pfu/cell was scored 48 hours post-infection. Viral suspensions were irradiated at a fluence rate of 2 $J/m^2/sec$. Each fluence point represents the logarithmic mean of data from 1 experiment with 4 trials and the standard error of the mean is shown by the bar or is contained within the symbol. Similar results were obtained in two other experiments.


Relative Amount of Viral DNA
1
0.1
0
20
40
60
80
100
RIF-8A
RIF-1
UV Fluence (J/m2) to Virus

DISCUSSION

Cellular capacity has been defined as the ability of a cell to support the growth of a particular virus (Coohill et al., 1977). The cellular capacity has been used to measure the response of mammalian cells to UV light irradiation and has been shown to parallel the response measured by the colony forming assay (Coohill, 1981). Parsons et al., (1986) used the cellular capacity assay as an **in vitro** test utilizing human tumour cells as a screening tool of anticancer drugs and for the prediction of an individual's clinical response to chemotherapeutic treatment. Data correctly predicted sensitivity to a number of anticancer drugs when results were compared to clonogenic survival. In this work, we have shown the feasibility of using the cellular capacity assay as an indicator of cellular sensitivity to PDT.

In this work, the cellular capacity assay revealed differences in PDT response between CHO-MDR cells and CHO-N cells, the multi-drug resistant cell line and its normal counterpart respectively as shown in figure 1. As shown in figure 2, the cellular capacity assay revealed differences in PDT response between RIF-8A cells, the PDT-resistant, and RIF-1 cells, its normal counterpart. It is evident that the cellular capacity for adenovirus DNA synthesis predicts the sensitivity of these four cell lines to PDT and is consistent with the clonogenic survival of these cell lines following PDT.

It is therefore possible that this cellular capacity assay can be extended to test the sensitivity of tumour biopsy cells to photodynamic therapy and help in predicting

tumour response to PDT.

Photodynamic therapy is currently being tested in Phase III clinical trials for a number of human cancers. It's mechanism of cytotoxicity has still not been well defined. It is thought that through the characterization of PDT-resistant cell lines and their normal parental counterpart that a mechanism of cytotoxicity can be targeted to a specific difference between the two cell lines.

Most commonly, MDR cell lines have been tested for cross-resistance to PDT (Mitchell, 1988; Kessel, 1989; Edwards et al., 1990), however, results have been conflicting. Singh et al., (1991) have shown that CHO MDR cells are cross-resistant to PDT using Photofrin and light. They reported that this cross-resistance is achieved by a reduced Photofrin intracellular concentration compared with the wild-type CHO cell line. However, no cross-resistance to PDT was detected utilizing mesoporphyrin and light in P388/ADR cells, a murine leukaemia cell line expressing the multi-drug resistance phenotype (Kessel and Erickson, 1992).

Sensitivity to PDT has also been examined for a human ovarian carcinoma cell line (2008) and its cisplatin resistant counterpart (C13). The C13 cell line was shown to be cross resistant to PH II-mediated PDT and the RIF-8A cell line was shown to be cross resistant to cisplatin (Sharkey et al., 1993). Sharkey et al., (1993) have found mitochondrial alterations in RIF-8A, the PDT resistant cell line, compared to its parental cell line, RIF-1. The RIF-8A mitochondria are morphologically different than the RIF-1 mitochondria and the RIF-8A cells have an altered energy metabolism compared to the RIF-1 cells. Similar mitochondrial alterations to those seen in RIF-8A

have also been noted in the PDT and cisplatin resistant C13 human ovarian carcinoma cells (Andrews and Albright, 1992).

The repair or recovery capabilities of RIF-8A cells compared to RIF-1 cells were investigated in this study in order to further characterize the PDT-resistant cell line and its normal counterpart. Split dose experiments which involve a time lapse between two consecutive doses have been utilized by others to examine the recovery of cell lines to various damaging agents. Dose fractionation has been reported to alter the cytotoxic effects of hyperthermia and ionizing radiation and hematoporphyrin or pthalocyanine-mediated photodynamic therapy (Elkind et al., 1967; Winans et al., 1972; Ben-Hur et al., 1974; Henle and Leper, 1979; Moan and Christiansen, 1979; Wolters et al., 1985; Koval, 1988; Baumann et al., 1992). It has been suggested that the recovery from fractionated doses is a result of an increased capacity of cells to repair sublethal damage. With respect to PDT split dose experiments, conclusive evidence for repair may not be possible from results which show increased survival for split doses because the time lapse between doses could result in the efflux of the drug or the redistribution of the drug to allow for the cells to become less photosensitive. Notwithstanding, Bellnier and Lin, (1985) have shown a recovery from PH II photosensitivity damage following fractionated doses of light and Qie and Sieber (1992) found that exposure of L1210 and K562 leukaemia cells to merocyanine 540 photosensitizer and fractionated doses of light inactivated fewer **in vitro** clonogenic cells than exposure to an equivalent dose of continuous light at 37°C but not at 4°C. In both studies, data suggested that there was a recovery due to repair of PDT

damage and not due to drug efflux. However in the latter study it is possible that there is a different redistribution of the drug between doses at 4°C compared to 37°C which could account for the greater split dose survival at 37°C. At 4°C the biochemical systems of the cell may be slowed down such that drug efflux is altered. In the present work we found evidence for a contribution from drug efflux to the increased colony survival detected in our split PDT light exposure experiments. Consequently, the interpretation of results from our PDT split dose experiments in terms of repair and recovery are inconclusive due to the effects of drug efflux between light exposures on cell sensitivity.

Previous studies have shown that the capacity assay can be used to compare the recovery and/or repair of mammalian cells following exposure to DNA damaging agents. The restoration of capacity of normal human fibroblasts was greater compared to that in xeroderma pigmentosum (XP) fibroblasts (repair deficient fibroblasts) when viral infection was delayed following UV damage (Lytle et al., 1976; Coppey et al., 1979a). A restored capacity following a delay between cell treatment and viral infection was also seen in monkey kidney cells and human skin cells when treated with angelicin plus light (Coppey et al., 1979b).

As shown in figure 4, there was an increased cellular capacity of RIF-8A cells in comparison to RIF-1 cells when the time between PDT treatment and infection is increased. Performing linear regression on the two data sets, it was confirmed that there is an increased capacity of the RIF-8A cell line compared to RIF-1 when 6 hours is allowed between damage and infection. This suggests that RIF-8A cells have an

enhanced repair capacity in comparison to RIF-1 cells.

In eukaryotic cells, it has also been shown that there is an induction of a number of genes following treatment by DNA damaging agents (Fornace et al., 1988). These genes have been termed DNA damage inducible (DDI) genes and are induced by different types of DNA damaging agents and also by other types of stresses, such as heat shock. These genes are associated with many different cellular processes including signal transduction, intercellular signalling, growth control, responses to tissue injury, inflammation, DNA repair, responses to oxidative stress and other protective responses (as reviewed in Fornace, 1992). Protic et al., (1988) have shown that DNA excision repair is enhanced in mammalian cells in response to DNA damage by mitomycin C, a cross-linking agent. The enhanced levels of viral DNA synthesis detected in the PDT treated RIF-8A cells when infection was carried out at 6 hours after cell treatment (figure 4A) may reflect an induced expression of genes by PDT which are involved in the replication of viral DNA.

Studies have shown that **in vitro** photosensitization (by benzoporphyrin and PH II) enhances the transcription and translation of stress proteins (heat shock proteins (hsp) and glucose-regulated proteins (grp)) (Curry and Levy, 1993; Gomer et al., 1991; Fisher et al., 1993). The activation of stress proteins can modulate the sensitivity of cells to physical, chemical and oxidative injury. Luna et al., (1994) have shown that early response genes such as c-fos, c-jun, c-myc and erg1 are induced following PH II-mediated PDT. These genes code for protein products that act as transcription factors and therefore may play a role in the regulation of cell responses

following endogenous or exogenous stresses. It is possible that a greater response is being induced in the RIF-8A cell line compared to the RIF-1 cell line that enhances the repair of PDT damage and/or enhances DNA replication of adenovirus.

Mutant cell lines exhibiting a resistance or sensitivity to a DNA damaging agent often show a cross resistance or cross-sensitivity to other DNA damaging agents (Singh et al., 1991; Sharkey et al., 1993; as reviewed in Hickson and Harris, 1988; Chao et al., 1991). The various profiles of cross sensitivity or cross resistance can provide evidence for different biochemical defects. It was found that the mouse lymphoma cell line, LY-R, which is sensitive to UV light irradiation because of its deficiency in the excision repair of UV-induced dimers, shows an increased sensitivity to PDT using AlPc and PH II plus light compared to LY-S, a mouse lymphoma cell line which does not show this sensitivity to UV light irradiation (Evans et al., 1989; Ramakrishnan et al., 1989; Rerko et al., 1992; Deahl et al., 1993). It was thus considered of interest to test the response of the CHO cell lines and the RIF cell lines to UV light irradiation.

The CHO-MDR cell line did not show any significant difference in cellular capacity to support virus replication in comparison to CHO-N cells following UV light irradiation (figure 5A). This suggests that the PDT resistance of CHO-MDR, which is due to its differential drug uptake ability does not overlap with the pathway for resistance to UV light. RIF-8A cells exhibit an increased cellular capacity to support viral replication following UV light irradiation compared to RIF-1 cells (figure 5B and 5C). This suggests that the mechanism of PDT resistance of the RIF-8A cells, may be

related to their resistance to damage following UV light irradiation.

To further assess the difference in DNA repair between RIF-1 and RIF-8A cells, host cell reactivation (HCR) studies were carried out with UV light as the damaging agent. The HCR assay measures the ability of the host cell to repair and hence replicate UV damaged viral DNA. The extent of reactivation is a reflection of constitutive levels of repair in the host cell line. As illustrated in figure 5D, the RIF-8A cell line has an increased ability to repair UV damaged viral DNA. This suggests that RIF-8A has an increased repair capability for UV-damaged DNA compared to RIF-1 cells. Previous work in our laboratory has shown that a variety of rodent cells exhibit differences in their repair capability for UV-damaged DNA (Arnold, 1992).

A number of investigators have shown that DNA damage is induced following PDT and that cellular repair mechanisms are altered following PDT (Musser et al., 1977; Munson, 1979; Gomer, 1980; Fiel et al., 1981; Crute et al., 1986; Lin et al., 1986; Boeigheim et al., 1987; Ramakrishnan et al., 1988; Evans, 1989; Ramakrishnan et al., 1989; Oleinick et al., 1991; Dubbelman, 1992; Rerko et al., 1992; Deahl et al., 1993; Noodt et al., 1993; Gantchey, 1994; Kvam and Stokke, 1994). Thus it is reasonable to suggest that RIF-8A exhibits an increased resistance to PDT compared to RIF-1 cells because of an altered capability to repair PDT damage, possibly DNA damage.

Studies have shown the presence of large amounts of DNA-protein cross-links, smaller amounts of single strand breaks, sister chromatid exchanges, alkali labile lesions and DNA-DNA cross-links following Hpd and pthalocyanine-mediated PDT

(Gomer, 1980; Fiel et al., 1981; Ramakrishnan et al., 1988 and 1989; Oleinick et al., 1991). There is evidence for a mutagenic action following PDT (Hpd and pthalocyanine) at the thymidine kinase (tk) locus (Evans et al., 1989; Rerko et al., 1992; Oleinick et al., 1991) and at the hypoxanthineguanine phosphoribosyl transferase (hprt) locus (tetra (3-hydroxyphenyl) porphyrin) (Noodt et al., 1993). Deahl et al., (1993) showed that PH II and pthalocyanine-mediated PDT induced mutations at the tk locus were caused by large DNA lesions.

It has been suggested that differences in cellular sensitivities to pthalocyanine-mediated PDT could be caused by differences in their capacity to process DNA lesions (Ramakrishnan et al., 1988; Evans et al., 1989; Ramakrishnan et al., 1989). Ramakrishan et al., (1988) showed an increased amount of DNA-protein cross links in V79 CHO cells following PDT. These DNA lesions were not repaired in contrast to lesions produced by gamma-radiation (single strand breaks), thus suggesting varying repair kinetics for different types of DNA lesions. There was an increased sensitivity to the cytotoxic effects of PDT in leukemic cells deficient in the repair of UV-induced damage (LY-R) but not LY-S cells which have enhanced sensitivity to ionizing radiation (double strand breaks). (Evans et al., 1989; Ramakrishnan et al., 1989). Kvam and Stokke (1994) used a method to label the sites of DNA repair after PDT (meso-tetra (4-sulfanatophenyl) porphyrin). Using the proliferating cell nuclear damage (PCNA) protein, which binds selectively to damaged DNA and participates in excision repair, they showed that the periphery of the nuclei was selectively labelled. This is direct evidence of the induction of DNA damage and the localization of repair sites following

PDT.

Studies have shown the inactivation of enzymes involved in replication and repair following Hpd-mediated PDT. Both RNA polymerase (Munson, 1979; Musser et al., 1977) and DNA polymerases (Crute et al., 1986) were reported to be inactivated by PDT. Other reports show that ligase activity is indirectly lowered by PDT inactivation of the protein ADP-ribosyl transferase (ADPRT) (Dubbelman et al., 1992), which is important for efficient repair of DNA.

However, data provided by Gomer et al., (1988) has shown that there is no significant difference of PH II-mediated PDT sensitivity between human repair-deficient fibroblasts (XP and AT) and normal fibroblasts. Results by Nocentini (1992) have shown no significant differences in the clonogenic survival of XP and FA (Fanconi anaemia) fibroblasts in comparison to normal fibroblasts following hematoporphyrin photosensitization. The sensitivity to PDT of certain cell lines with varied repair capabilities has resulted in varied findings. This simply can be due to the specific nature of the damage and repair mechanisms involved. For example, DNA repair defects in both AT and XP cells possibly do not overlap with the repair processes required for repair of PDT damage. XP is deficient in the initial incision step of the excision repair pathway (as reviewed in Friedberg, 1985). In comparing the repair kinetics of DNA damage induced by X-rays, UV light and the methylating agent, MMS of L929 fibroblasts, Boeigheim et al., (1987) reported data suggesting that Hpd-mediated PDT inhibits one or more of the enzymes involved in excision repair beyond the incision step. Therefore, it is possible that there is an overlap between nucleotide

excision repair and the mechanism for repair of PDT damage, yet the exact repair pathway might involve other enzymes and other steps.

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CHAPTER FOUR

CROSS-SENSITIVITY TO PHOTODYNAMIC THERAPY AND UV LIGHT IN THE CHO-AUXB1 CELL MUTANT DEFICIENT IN FOLYPOLYGLUTAMATE SYNTHETASE

CROSS-SENSITIVITY TO PHOTODYNAMIC THERAPY AND UV LIGHT IN THE CHO-AUXB1 CELL MUTANT DEFICIENT IN FOLYPOLYGLUTAMATE SYNTHETASE

ABSTRACT

Photodynamic therapy (PDT) utilizes the localized delivery of light to activate a photosensitizing drug (such as Photofrin) which is selectively retained by the tumour tissues. In this work, we have used both clonogenic survival and viral capacity as indicators of cellular response to PDT for a CHO cell mutant line, CHO-AUXB1 and its parental line, CHO-PRO-. The CHO-AUXB1 cell mutant is a triple auxotroph, deficient in the folypolyglutamate synthetase enzyme. The CHO-AUXB1 cell line was found to have an increased sensitivity to PDT compared to the CHO-PRO- cell line.

The CHO-AUXB1 cell line also showed a cross-sensitivity to ultraviolet (UV) light irradiation compared to CHO-PRO- cells. We suggest that CHO-AUXB1 cells have an altered repair capability both for PDT-induced damage and UV-induced damage.

INTRODUCTION

Photodynamic therapy is a novel treatment in cancer therapy that utilizes the localized delivery of light to activate a photosensitizing drug (such as Photofrin), which is selectively retained by tumour tissue (Bugelski et al., 1981; Kessel and Chou, 1983; as reviewed in Moan, 1986b; Brault, 1990; Maziere et al., 1990). Photodynamic therapy is currently in Phase III clinical trials for a number of human cancers (as reviewed in Marcus, 1992; as reviewed in Pass, 1993).

Three simultaneously present components are required for the photodynamic process: a sensitizer, light and oxygen. The mechanism of cytotoxicity involves the generation of singlet oxygen when the photosensitizer becomes excited, this singlet oxygen is believed to be the major damaging species in PDT (Lee et al., 1984; Mitchell et al., 1985).

Studies have detailed numerous types of damage that result from the photodynamic process. However, the mechanism of PDT cytotoxicity has not been defined.

PDT induces both cellular and vascular damage of the cells and tissue, which result in tumour necrosis. The cellular effects include membrane damage (Kohn and Kessel, 1979; Bellnier and Dougherty, 1982; Valenzano, 1987; Biade et al., 1992); mitochondrial damage (Hilf et al., 1984; Hilf et al., 1986; Woodburn et al., 1992; Sharkey et al., 1993) and DNA damage (Gomer, 1980; Fiel et al., 1981;

Ramakrishnan et al., 1988; Evans et al., 1989; Ramakrishnan et al., 1989; Oleinick et al., 1991; Rerko et al., 1992; Deahl et al., 1993; Noodt et al., 1993; Gantchey et al., 1994). Although the critical targets for PDT killing **in vitro** are not yet established, it is possible that those factors which can influence the magnitude of PDT damage to membranes, mitochondria and DNA will influence the sensitivity of cells to PDT.

A number of cell lines have been identified that show altered response to PDT. Cells which carry the multi-drug phenotype show a cross resistance to PDT because of altered drug uptake (Mitchell, 1988; Kessel, 1989; Edwards et al., 1990; Singh et al., 1991). Mitochondrial alterations have been identified in cell lines resistant to PDT (Sharkey et al., 1993). However, there has been very limited examination of the PDT response of defined cell mutants. Investigators have examined the differential cell photosensitivity for a murine leukeamic cell line, LY-R, deficient in the excision of UV-induced DNA damage. The LY-R cells exhibited a cross-sensitivity to pthalocyanine-mediated PDT (Evans et al., 1989; Ramakrishnan et al., 1989). Gomer et al., (1988) have examined the effect of PH II-mediated PDT on human DNA repair deficient fibroblasts (ataxia telangiectasia (AT) and xeroderma pigmentosum (XP)) compared to normal human fibroblasts and found no significant differences in photosensitization for these cell lines. Nocentini (1992) showed no significant difference among three human cell types, XP fibroblasts, normal fibroblasts and Fanconi anaemia fibroblasts following Hpd photosensitization. Both these studies examined the response of XP cells from complementation groups A, B, and C.

It was considered of interest to examine the PDT sensitivity of a defined

Chinese hamster ovary (CHO) mutant cell line. The CHO-AUXB1 cell mutant is deficient in folypolyglutamate synthetase, an enzyme responsible for the formation of polyglutamates (Taylor and Hanna, 1977 and 1979). Polyglutamates are more effective as substrates in the thymidylate cycle and it is believed that this cycle is readily affected by this deficiency (Shane, 1990). Previous studies have shown that mutants with imbalanced dNTP pools, such as thymidine kinase deficient cells, have altered cytotoxicity to a number of DNA damaging agents such as ultraviolet light (UV), ethyl methanesulphonate (EMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Meuth, 1983; McKenna et al., 1985; Rainbow, 1989c). Cells with altered ribonucleotide reductase also show altered sensitivity to general DNA damaging agents (Ayusawa et al., 1983).

Mutant rodent cell lines sensitive to UV light, ionizing radiation and cross-linking agents have proved useful in our knowledge of DNA repair and pathways involved in cellular response to cytotoxic agents (as reviewed in Collins, 1993). In this work we show that the CHO-AUXB1 cell mutant, shows an increased PDT sensitivity compared to its parental cell line CHO-PRO-, utilizing both the cellular capacity assay and the colony forming assay. The cellular capacity assay tests the ability of the cells to support viral infection following PDT treatment, whereas the colony forming assay tests the ability of the cells to form colonies following PDT treatment. We also demonstrate, using the cellular capacity assay, that the CHO-AUXB1 cell mutant is cross-sensitive to UV light irradiation and suggest that altered rate of repair of DNA damage may influence its sensitivity to both PDT and UV.

MATERIALS AND METHOD

Cells and Viruses.

CHO-AUXB1 cells and **CHO-MDR** cells were obtained from Dr. V. Ling, Ontario Cancer Institute, Toronto, Ontario. The CHO-MDR cell line, containing the p-glycoprotein (CH^RC5), was derived from the CHO-AUXB1 cell line (Ling and Thompson, 1974). **CHO-PRO-** cells were obtained from Dr. R. S. Gupta, Department of Biochemistry, McMaster University, Hamilton, Ontario. They are the parental strain of CHO-AUXB1 (McBurney and Whitmore, 1974). All cells types were grown as monolayers in alpha minimal essential media (α-MEM) fully supplemented with deoxyribonucleosides and ribonucleosides, 10% fetal bovine serum (Gibco BRL, cat#16000-028), 4% of an antibiotic-antimycotic solution (penicillin 10,000 units per ml, fungizone 25 μg per ml and streptomycin 10,000 μg per ml (Gibco BRL, cat#15240-013) and 4% of L-glutamine (Gibco BRL,cat# 25030-016) in 75 cm^2 plastic flasks. Cells were grown in a humidified incubator at 37°C with 5% CO_2.

The human adenovirus Ad5 as well as recombinant viruses Ad5(denV) and Ad5(lacZ) were used in the experiments. Ad5(denV) contains the bacteriophage T4 pyrimidine dimer DNA glycosylase (denV) gene inserted into the deleted E3 region (Colicos et al., 1991) and Ad5(lacZ) contains the ß-galactosidase (lacZ) gene inserted into the deleted E3 region and was provided by Dr. Frank Graham, Departments of Biology and Pathology, McMaster University, Hamilton, Ontario.

Photosensitizer and Light Source.

Photofrin II was obtained by Quadralogic Technologies Inc. (Vancouver, B.C. Canada). It was diluted to a 2.5 mg/ml solution using α-MEM, from an original stock of 10 mg/ml.

Plates were exposed to red light using a 21" x 32.75" light box illuminated by a parallel series of 12 fluorescent tubes (Philips type TL/83), enclosed on top with a sheet of clear Plexiglass and filtered with red acetate filters (No. 19, roscolux, Rosco, CA). The emission spectrum was measured using a Jobin-Yvon model CP-200 spectrometer and a Princeton Instruments CCD. The energy fluence rate was 0.34 $W/m^2/sec$ in the wavelength band of 640 nm. Exposure for 5 minutes resulted in incident energy fluence of 102 J/m^2.

Cellular Capacity Assay for PDT-treated cells.

The capacity of CHO cells to support adenovirus replication following PDT treatment was determined as follows.

Preconfluent cells were rinsed with 1x PBS (diluted from a 10x stock in double distilled water (0.8g NaCl, 2g KCl, 1.15g Na_2HPO_4 and 0.2g K_2PO_4)), 2 ml of trypsin (2x, diluted in PBS from a 10x stock) was added and they were resuspended in 8 ml of medium. A haemocytometer was used to count the number of cells in the suspension. Cells were seeded in 24-well Linbro plates at 2 x 10^5 cells/well and allowed 4 hours for cellular attachment. After this 4 hour period, the medium was removed and replaced with 0.5 ml of fresh medium. 0.1 ml of a Photofrin II

suspension of an appropriate concentration was added to each well. At this point all subsequent steps were carried out in minimal light conditions until the addition of pronase solution used in the first step of viral DNA extraction. After an 18 hour incubation, the drug containing media was removed and replaced with 0.5 ml of fresh media. The cells were then exposed to red light at a fixed light fluence rate of 0.34 $J/m^2/sec$ for 5 minutes.

Treated cells were immediately infected with 0.2 ml of the adenovirus suspension at a multiplicity of infection (MOI) of 40 pfu/cell for 90 minutes, during which time the plates were gently rocked at 15 minute intervals. Each well was overlayed with 1 ml of growth medium. The medium was then replaced at two to four hours after the overlay to remove unabsorbed virus and samples taken to quantitate the amount of virus absorbed. After 72 hours, 0.2 ml of lysis solution was added (4 mg/ml pronase, 40mM tris pH 8.0, 40 mM EDTA pH 8.0, 2.4% SDS) directly to the 0.5 ml of growth medium in each well. Following the addition of the lysis solution, the plates were returned to 37°C for 1 to 3 hours. After this time, the contents of the well was collected and the DNA extracted using the phenol:chloroform method. One volume (0.7 ml) phenol:chloroform was added to each well. The mixture was vortexed, centrifuged and the aqueous phase transferred to a second microfuge tube, one volume (0.7 ml) of isoamyl alcohol:chloroform was then added. The mixture was vortexed, centrifuged and the aqueous phase transferred to a third microfuge tube. DNA was precipitated with 5M NaCl. The DNA was slot blotted to nitrocellulose and probed with Ad2 DNA (Gibco BRL, cat#15270-010) as a template as described in

Sambrook et al., (1986). DNA was then quantitated using a Phosphorimager, and ImageQuant software (Molecular Dynamics, Sunnyvale, California).

Qualitative Assay for Cellular Response to PDT.

An estimate for cell survival of CHO-PRO- cells and CHO-AUXB1 cells following PDT treatment was determined as described in Collins and Johnson, (1987) with the following modifications.

Preconfluent cells were seeded in 24-well Linbro plates at 1 x 10^5 cells/well and allowed to adhere for 4 hours. The medium was then removed and replaced with drug-containing medium containing an appropriate Photofrin concentration. All subsequent steps were carried out in minimal light conditions until the addition of the stain. The cells were incubated for 18 hours and then the drug-containing media was removed and replaced with fresh media. The plates were then exposed to red light at a fixed light fluence rate of 0.34 W/m^2/sec for 5 minutes. Cells were allowed to grow for 72 hours and then stained with methylene blue (0.5 g methylene blue, 70 ml methanol, 30 ml millipore water). The cells were then scored by visual inspection.

Clonogenic Survival Assay for PDT treated cells.

The clonogenic survival of CHO cells following PDT treatment was determined as described in Singh et al., (1991).

Preconfluent cells were seeded in 100 mm tissue culture plates at 2 x 10^3 cells/plate and allowed to adhere for 4 hours. The medium was then removed and

replaced with drug-containing medium containing an appropriate Photofrin concentration. All subsequent steps were carried out in minimal light conditions until the addition of stain. The cells were incubated for 18 hours and then the drug-containing medium removed and replaced with fresh medium. The plates were then exposed to red light at a fixed light fluence rate of 9.2 $W/m^2/sec$ for five minutes. Cells were allowed to grow for 5 days and then stained with methylene blue and colonies of 20 cells or more counted and scored.

Cellular Capacity Assay for Ultraviolet (UV) treated cells.

The capacity of UV-treated cells to support adenovirus infection was executed in a similar way to the PDT experiments.

Cells were seeded in 24-well Linbro plates as for the PDT experiments. Following a 24 hour incubation period, plates were irradiated at a number of UV fluence points. Cells were then immediately infected with Ad5(lacZ) and viral replication quantitated as previously described.

The cells were irradiated with a UV lamp (General Electric Germicidal Lamp G8T5) emitting wavelength of predominantly 254 nm. The incident dose rate was 2 $J/m^2/sec$ as determined with a J-255 shortwave UV meter (Ultraviolet Products, San Gabriel, California).

RESULTS

Capacity of PDT-treated CHO Cells.

A representative cellular capacity curve of PDT-treated CHO-AUXB1 cells and its parental cells, CHO-PRO- to support the replication of Ad DNA is shown in figure 1A as a function of varying Photofrin concentration and fixed light fluence. The amount of viral DNA synthesis following PDT treatment was determined relative to the amount of viral DNA replicated in untreated cells. The log of the relative capacity values for viral DNA synthesis at each drug concentration were averaged in each experiment to produce a single logarithmic mean and a standard error of the mean as shown in figure 1A. It can be seen that CHO-AUXB1 cells have a reduced cellular capacity for viral infection compared to CHO-PRO- cells following PDT treatment. The data from a number of experiments was pooled and is shown in figure 1B. In order to pool all experiments, data was normalized as a function of the capacity of CHO-AUXB1 cells versus the ratio of the capacity of CHO-PRO- cells to CHO-AUXB1 cells at each drug concentration for each trial. As the capacity of CHO-AUXB1 cells decreases, there is a increased capacity of CHO-PRO- cells compared to CHO-AUXB1 cells.

Clonogenic Survival of PDT-treated CHO Cells.

The sensitivity of these CHO cells following PDT was also examined using a

modified plating assay, also referred to as the cytotoxicity assay (Collins and Johnson, 1987) and then followed by the conventional plating assay.

CHO-AUXB1 cells show a distinct increased sensitivity to PDT compared to CHO-PRO- cells using the cytotoxicity assay (figure 2A).

Figure 2B shows a typical clonogenic survival curve for CHO-AUXB1 cells and the parental cells, CHO-PRO-, for varying concentrations of Photofrin and a fixed light fluence. CHO-AUXB1 cells show an increased sensitivity following PDT treatment compared to CHO-PRO- cells.

Data from both survival assays reveal that CHO-AUXB1 cells shows an increased sensitivity following PDT treatment compared to CHO-PRO- cells. For comparison purposes, the data from the colony forming assay is presented in conjunction with the cellular capacity data in figure 1B. The CHO-AUXB1 cell line shows a similar increased sensitivity to PDT compared to the CHO-PRO- cell line for both assays.

Capacity of CHO cells following UV Treatment.

Previous studies have shown that cell mutants with imbalanced dNTP pools have shown an increased sensitivity to DNA damaging agents (Ayusawa, 1983; Meuth, 1983; McKenna et al., 1985; Rainbow, 1989). Since the CHO-AUXB1 cell line is believed to have a deficiency in the thymidylate cycle, it was considered of interest to test its sensitivity to UV light treatment.

A typical cellular capacity curve is shown in figure 3A for the capacity of the

UV treated CHO cell lines, CHO-PRO- and CHO-AUXB1, to support the replication of Ad DNA. It can be seen that there is an decreased cellular capacity of CHO-AUXB1 cells following UV damage compared to CHO-PRO- cells. Data from a number of experiments was pooled in figure 3B, which allows a direct comparison of PDT and UV sensitivity of the CHO-AUXB1 cell line. It can be seen that as the capacity of CHO-AUXB1 decreases, the capacity of the CHO-PRO- cells increases compared to CHO-AUXB1 cells. The CHO-AUXB1 cells show a similar sensitivity to both UV and PDT. CHO-AUXB1 cells and CHO-MDR cells showed no significant difference in their capacity following damage by UV.

Results show that CHO-AUXB1 cells have an increased sensitivity to PDT and to UV light treatment compared to the parental, CHO-PRO- cells.

Figure 1A: The Capacity of PDT-treated CHO Cells to Support Viral DNA Replication following Infection with Ad5(denV).

The relative amount of viral DNA synthesized in PDT-treated CHO-PRO- (closed symbols) and CHO-AUXB1 (open symbols) following infection with Ad5(denV) at an MOI of 40 pfu/cell was scored 72 hours post-infection. Cells were incubated with 2.5, 5, 10, 15 and 20 μg/ml of Photofrin II for 18 hours and exposed to 120 J/m^2. Each point represents pooled data from two experiments (each with 3 or 4 trials) and the standard error of the mean is shown by the bar or is contained within the symbol. Although there was considerable experiment-to-experiment variation in CHO-AUXB1 cells and CHO-PRO- cells, the sensitivity of the CHO-AUXB1 cells was consistently observed in each of the 6 experiments performed.

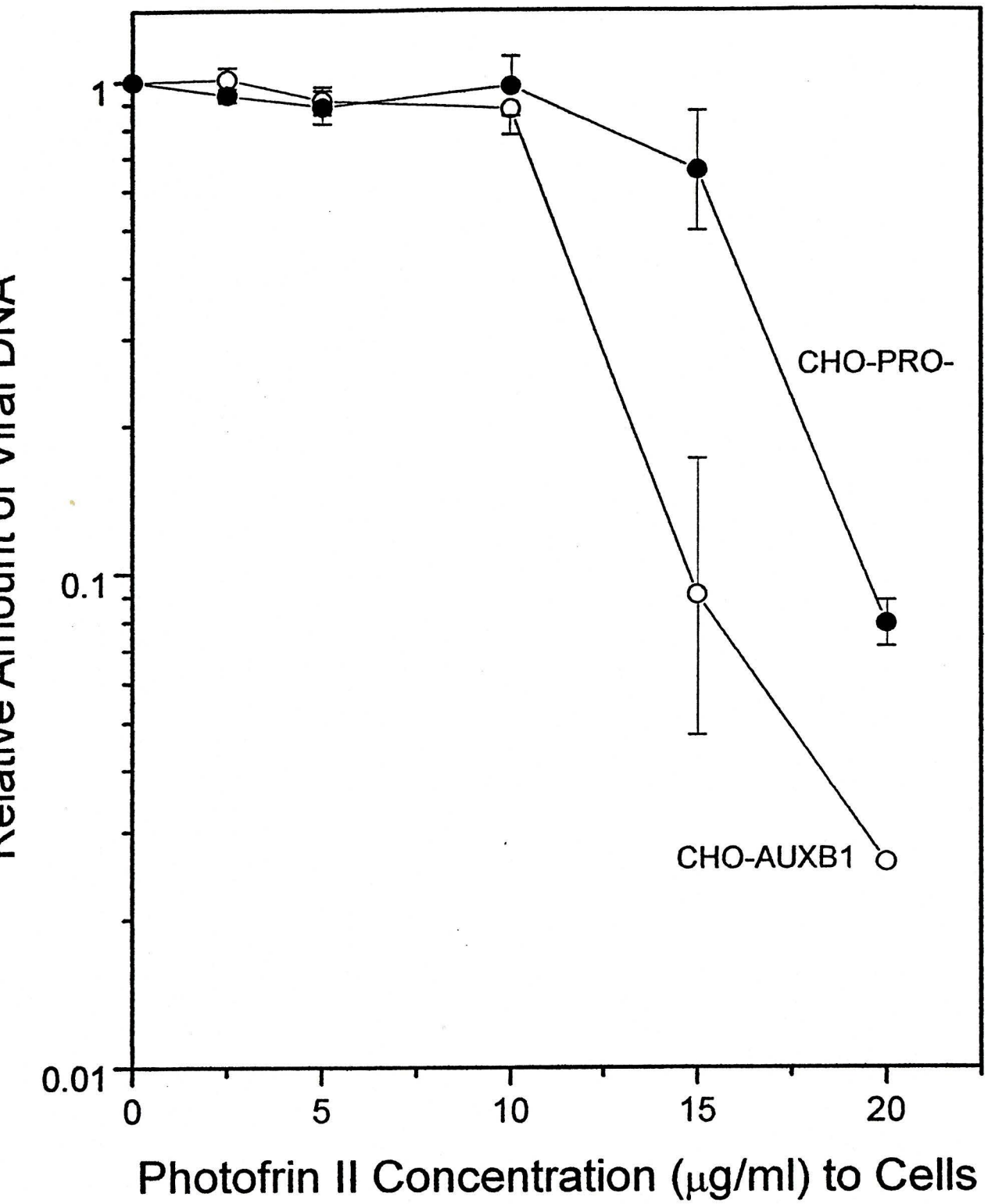

Relative Amount of Viral DNA
1
0.1
0.01
0
5
10
15
20
CHO-PRO-
CHO-AUXB1
Photofrin II Concentration (μg/ml) to Cells

Figure 1B: The Capacity of CHO-PRO- Cells to Support Viral DNA Replication of Ad5(denV) as PDT Damage to CHO-AUXB1 Cells Increases.

Points (closed symbols) represent the capacity of CHO-AUXB1 cells as a function of the capacity of CHO-PRO- cells relative to CHO-AUXB1 cells at each drug concentration for pooled data from a number of experiments (6). For comparison purposes, the data from the colony forming assay is shown by the open symbols. A line was fitted using linear regression analysis for the cellular capacity data.

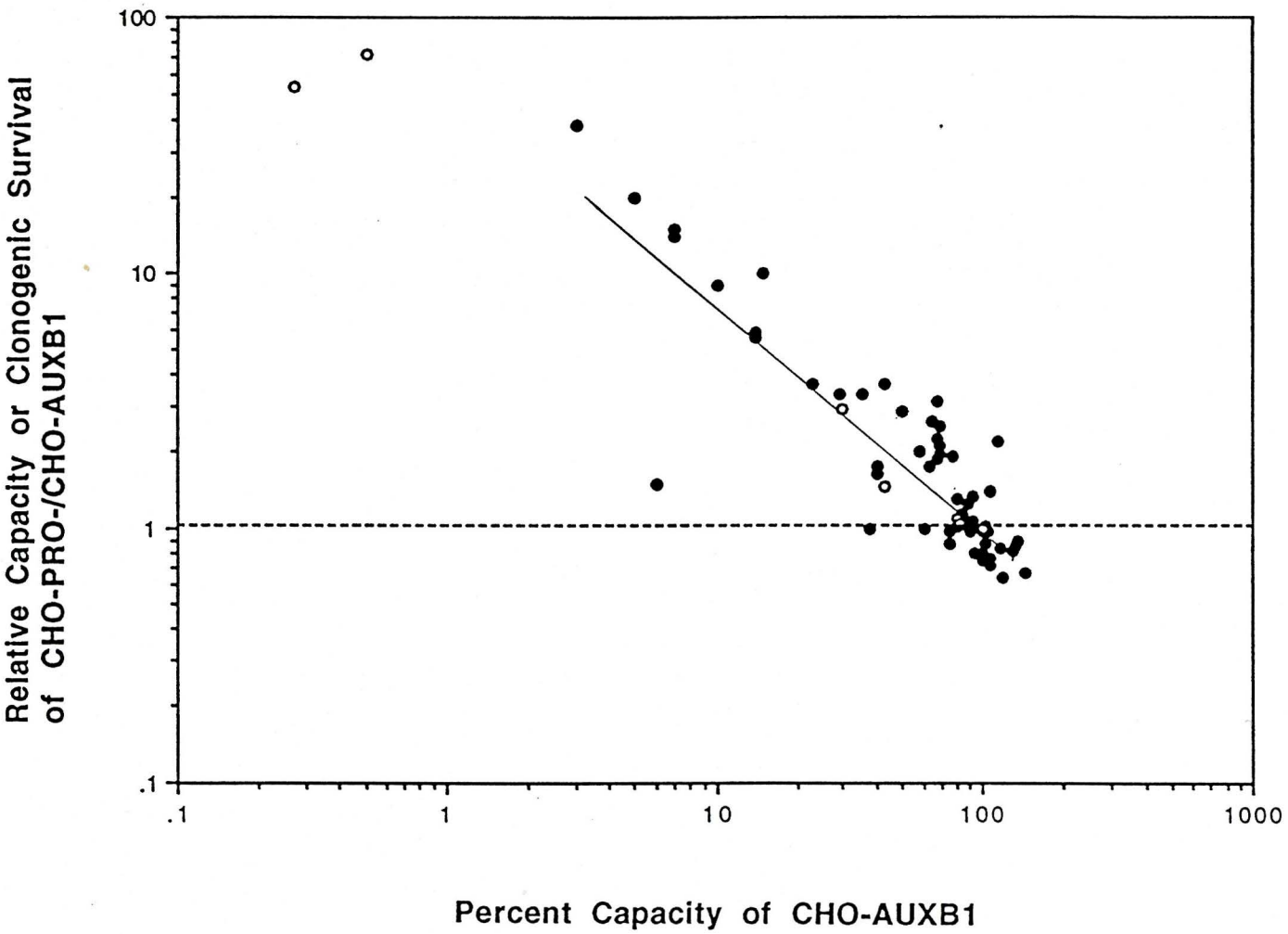

Relative Capacity or Clonogenic Survival of CHO-PRO-/CHO-AUXB1
100
10
1
.1
.1
1
10
100
1000
Percent Capacity of CHO-AUXB1

Figure 2A: PDT Cell Sensitivity Assay for CHO-PRO- Cells and CHO-AUXB1 Cells.

Cells were seeded at 1 x 10^5 cells/well in 24-well dishes and allowed to adhere to the dish for 4 hours. Cells were incubated with 5, 10, 15, 20 and 25 μg/ml of Photofrin II for 18 hours and exposed to 120 J/m^2. 72 hours later, the wells were stained with methylene blue.

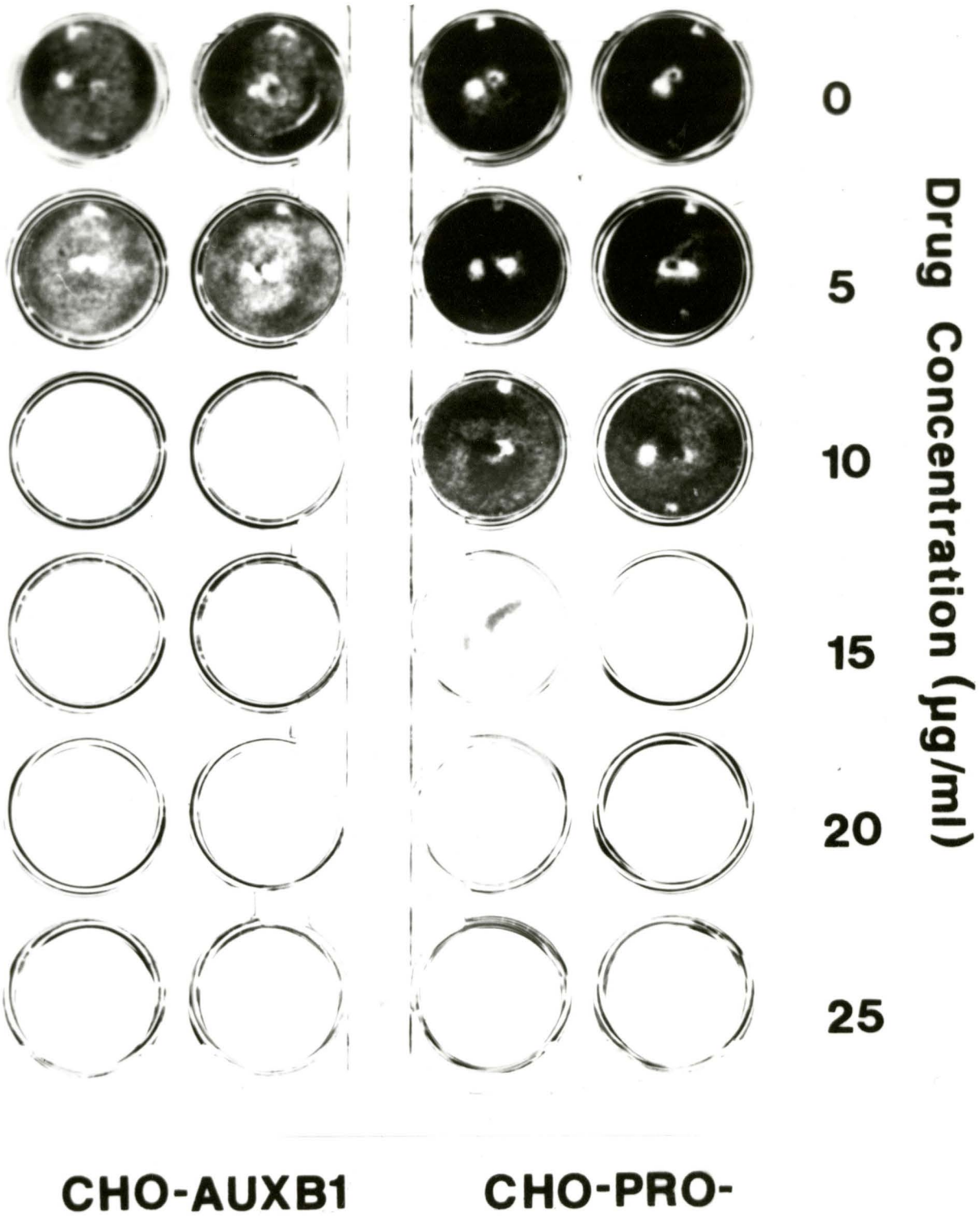

Drug Concentration (µg/ml)
0
5
10
15
20
25
CHO-AUXB1
CHO-PRO-

Figure 2B: The Clonogenic Survival Curve of CHO PRO- Cells and CHO-AUXB1 Cells following PDT Treatment.

The clonogenic survival of CHO-PRO- cells (closed symbols) and CHO-AUXB1 cells (open symbols) was scored 5 days after PDT treatment. Cells were incubated with 5, 7.5 and 10 μg/ml Photofrin II for 18 hours and exposed to 2.7 x 10^3 J/m^2. Figure 2B shows the results of a typical experiment. Survival values compared to no drug and no light treatment were obtained from the mean colony counts of 3 dishes used for each treatment. Drug alone reduced the colony counts 19% and 4% for the CHO-AUXB1 and CHO-PRO- cells respectively. Each point is an average of triplicate trials and the standard error is indicated by the bars or contained within the symbol. Similar results were obtained in 2 other experiments.

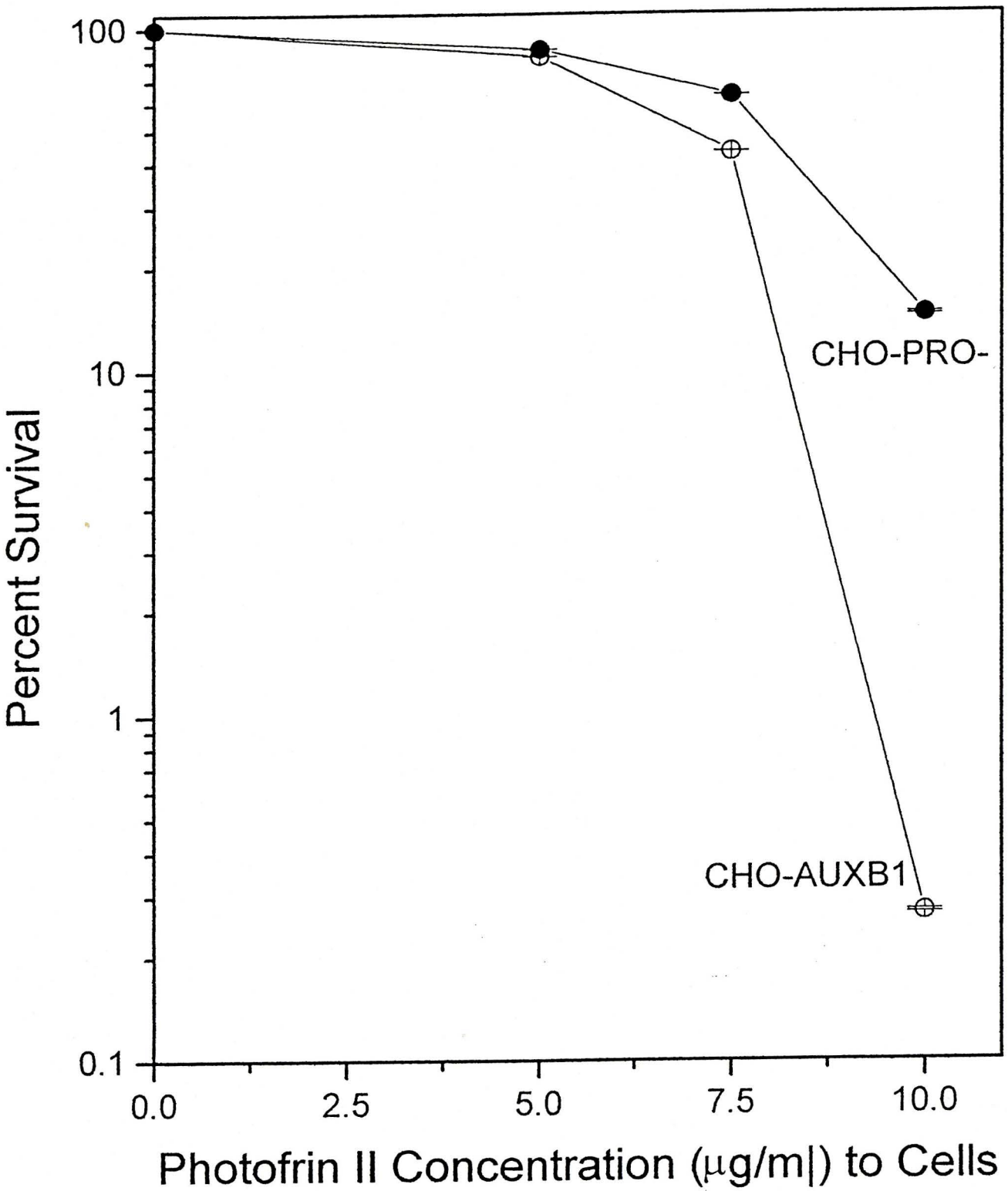

100
10
1
0.1
Percent Survival
CHO-PRO-
CHO-AUXB1
0.0
2.5
5.0
7.5
10.0
Photofrin II Concentration (μg/m|) to Cells

Figure 3A: The Capacity of UV-irradiated CHO Cells to Support Adenovirus DNA Replication following Infection with Ad5(lacZ).

The relative amount of viral DNA synthesized in UV-treated CHO-PRO- cells (closed symbols) and CHO-AUXB1 (open symbols) following infection with Ad5(lacZ) at an MOI of 40 pfu/cell was scored 72 hours post-infection. Cells were irradiated at a fluence rate of 2 $J/m^2/sec$. Each fluence point represents the logarithmic mean of pooled data from two experiments (each with 4 trials) and the standard error of the mean is shown by the bar or is contained within the symbol. Although there was considerable experiment-to-experiment variation in these survival curves, sensitivity of CHO-AUXB1 cells was consistently observed in each of the 4 experiments performed. The cellular capacity of CHO-MDR cells is also shown for comparison purposes.

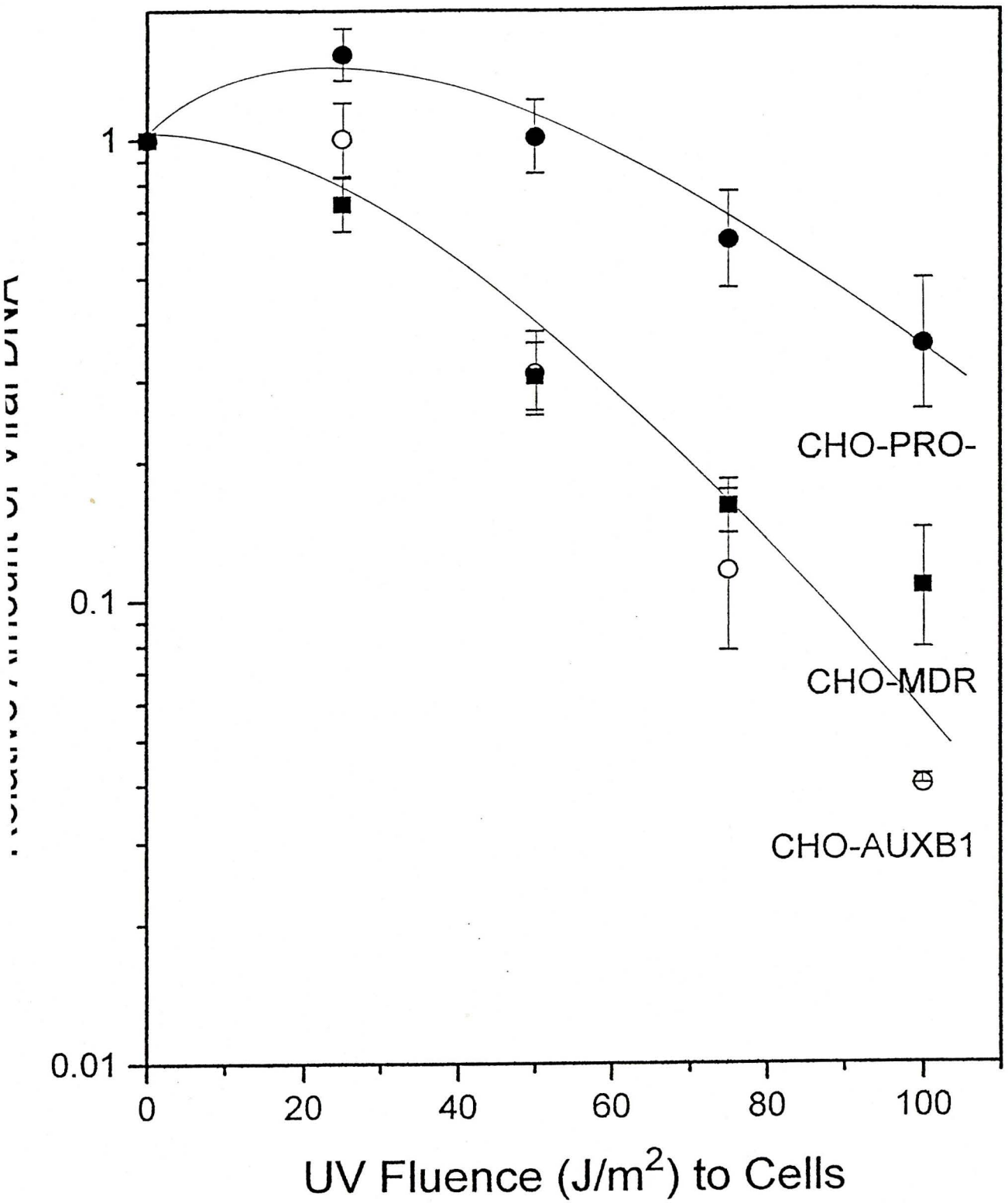
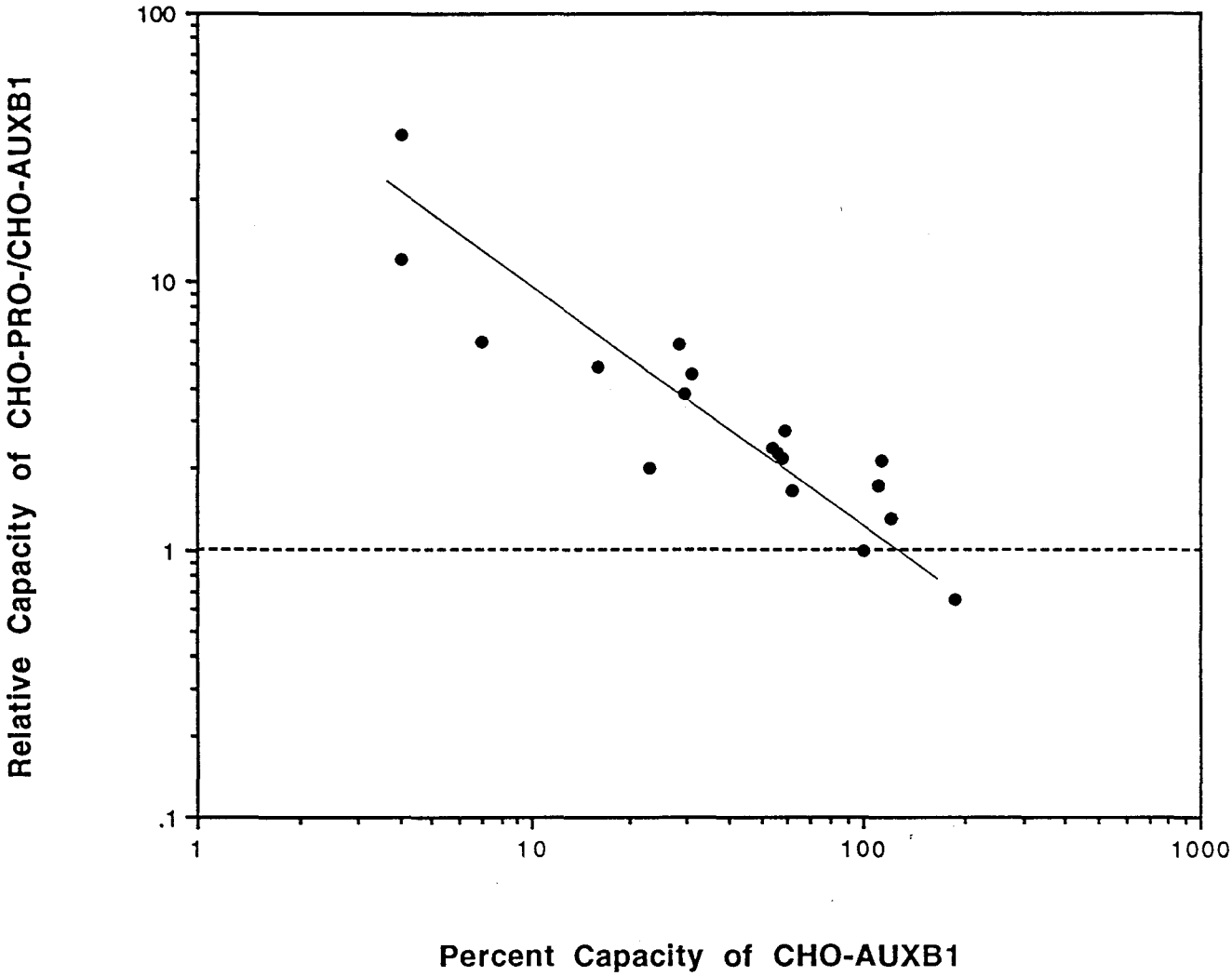

1
0.1
0.01
0
20
40
60
80
100
CHO-PRO-
CHO-MDR
CHO-AUXB1
UV Fluence (J/m2) to Cells

Figure 3B: The Relative Capacity of UV-irradiated CHO-PRO- Cells to Support Viral DNA Replication of Ad5(lacZ) as the Damage to CHO-AUXB1 Increases.

Points represent the capacity of CHO-AUXB1 as a function of the capacity of CHO-PRO- cells compared to CHO-AUXB1 cells at each fluence point using pooled data from 2 experiments, each with 4 trials. The line was fitted by linear regression analysis.


Relative Capacity of CHO-PRO-/CHO-AUXB1
100
10
1
.1
1
10
100
1000
Percent Capacity of CHO-AUXB1

DISCUSSION

Cell lines resistant to PDT treatment have been used by several investigators to examine the characteristics of PDT cytotoxicity. A mouse cell line has been induced to show PH II-mediated PDT-resistance compared to its parental line (Singh et al., 1991; Luna and Gomer, 1991) and a number of cell lines resistant to other modalities have shown a cross-resistance to PDT (Mitchell, 1988; Kessel, 1989; Edwards et al., 1990; Singh et al., 1991; Kessel and Erickson, 1992; Sharkey et al., 1993). However, few studies have examined the response of defined cell mutants to PDT. Results of this study clearly show an increased PDT sensitivity of the CHO-AUXB1 mutant cell line compared to its parental cell line, CHO-PRO-.

The CHO-AUXB1 mutant cell line is the parental cell line of CHO-MDR, (used in previous studies showing cross-resistance to PH II-mediated PDT (Singh et al., 1991 and in chapter 3). The CHO-AUXB1 mutant cell line was derived from a CHO-PRO- cell line (McBurney and Whitmore, 1974) and was found to be a triple auxotroph which requires glycine, adenosine and thymidine for its growth. Later studies by Taylor and Hanna (1977 and 1979) identified the deficiency of the folypolyglutamate synthetase (FPGS) enzyme in the CHO-AUXB1 cell line. FPGS catalyses the synthesis of poly-gamma-glutamate forms of tetrahydrofolate and its coenzyme adducts (Scrimgeour, 1986).

The cellular capacity assay and the colony forming assay both show that CHO-

AUXB1 cells exhibit an increased sensitivity to PDT in comparison to its parental cell line, CHO-PRO- (figure 1). These results also indicate that the cellular capacity assay can be utilized to test the response of cells to PDT, in accordance with previous studies that have shown that the cellular capacity assay can correctly predict the sensitivity of cells to a number of other chemotherapeutic agents (Parsons et al., 1986).

Altered sensitivity of cells to PDT may be due to a number of factors including altered photosensitizer uptake and transport, altered sub-cellular localization of the photosensitizer, photochemical modification or repair of the photodamage. The CHO-AUXB1 cell mutant was found not to have altered drug uptake (data not shown) compared to CHO-PRO- cells indicating that the sensitivity to PDT did not result from an increased retention of Photofrin II.

The CHO-AUXB1 cell line is deficient in the FPGS enzyme. The thymidylate cycle involves a number of enzymes where polyglutamates are more effective as substrates, and it is believed this cycle is readily affected by this deficiency (as reviewed in Shane, 1990). Since the thymidylate cycle is of primary importance in the production of deoxythymidylate (dTTP), this suggests that CHO-AUXB1 could have an altered dTTP pool. A number of cultured mammalian mutants with altered deoxyribonucleoside (dNTP) pools have shown an increased sensitivity to DNA damaging agents. It was shown that thymidine kinase (tk) deficient cell lines have an increased sensitivity to ultraviolet (UV) light, ethyl methane sulfonate (EMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Meuth,1983; McKenna et al., 1985;

Rainbow, 1989). Similarly, it was found that a cell mutant with altered ribonucleotide reductase and thymidylate synthase show a sensitivity to a number of DNA damaging agents (Ayusawa et al., 1983). The increased sensitivity is thought to result from an alteration in excision repair of DNA.

Hpd and pthalocyanine-mediated PDT damages DNA and has been found to produce DNA-protein cross-links, single strand breaks, sister chromatid exchanges, alkali labile lesions and DNA-DNA cross-links (Gomer, 1980; Fiel et al., 1981; Ramakrishnan et al., 1988 and 1989; Oleinick et al., 1991; Gantchey, 1994). Mutations at the tk (thymidine kinase) and the hypoxanthineguanine phosphoribosyl transferase (hprt) locus have also been detected following PDT (PH II, pthalocyanine and tetra (3-hydroxyphenyl) porphyrin) treatment of cells (Evans et al., 1989; Rerko et al., 1992; Deahl et al., 1993; Noodt et al., 1993).

The AUXB1 cell mutant is suggested to have a deficiency in the thymidylate cycle and since deoxythymidylate is a precursor of one of the four building blocks of DNA, it is possible that the AUXB1 cell mutant has a reduced ability to carry out the repair process of PDT damaged DNA due to a reduced supply of DNA precursors.

The sensitivity of CHO-AUXB1 cells and CHO-MDR cells to UV light were relatively similar but greater than that of CHO-PRO- (figure 3). PDT cross-resistance in the CHO-MDR cells is due to altered drug uptake (Singh et al., 1991) and since CHO-MDR cell line is derived from the CHO-AUXB1 cell line, it still retains the FPGS deficiency and thus would exhibit the same sensitivity to UV light as its parental line. The CHO-AUXB1 cell mutant exhibits a cross-sensitivity to UV compared to the

parental CHO-PRO- cells.

Increased UV sensitivity of cell mutants with reduced dNTP pools is thought to result from a reduced rate of nucleotide excision repair of UV-induced DNA lesions (Schor et al., 1975; Burg et al., 1977; Johnson et al., 1987). Thus it is possible that increased sensitivity of the CHO-AUXB1 cell mutant results from a reduced rate of repair of PDT-induced DNA damage. Murine leukaemic cells deficient in the repair of UV-induced damage also exhibit a cross-sensitivity to pthalocyanine-mediated PDT (Evans et al., 1989; Ramakrishnan et al., 1989). The LY-R cells were shown to be have a limited ability to carry out both the incision step and the patch repair step of nucleotide excision repair of UV-induced DNA damage (Szumiel et al., 1988). However, excision repair deficient xeroderma pigmentosum (XP) cells, which are deficient in the incision step of the nucleotide excision repair pathway (as reviewed in Friedberg, 1985), have normal sensitivity to PDT which suggests that the incision step of nucleotide excision repair is not involved in the survival of PDT (Hpd) treated cells. It is possible that the nucleotide excision repair is involved in the repair of PDT-induced DNA damage but not at the incision step, as suggested by Boeigheim et al., (1987). The synthesis of the new DNA segment may be altered. Since the CHO-AUXB1 cell mutant is believed to have decreased dNTP pools, the rate of the synthesis of the new DNA segment would be affected. Lin et al., (1986) have shown that DNA biosynthesis is altered in Hpd treated cells. It is also possible that another incision pathway, that is present in XP cells is responsible for cell survival after PDT or that LY-R cells are deficient in a step common to excision repair of UV damage and

repair mechanism for PDT damage, that is beyond the incision step. It would be considered of interest to examine PDT response of XP variants (Boyer et al., 1990; Kaufman, 1989) that are deficient in post-replication repair and also other UV sensitive cell mutants (Collins, 1993).

In conclusion, it is evident that the CHO-AUXB1 cell mutant line is a PDT sensitive mutant and its sensitivity can be linked to a defect in the thymidylate cycle. Results suggest that this mutation (the absence of FPGS) affects the sensitivity of CHO-AUXB1 cells to both PDT and UV light. Thus, another pathway, other than altered drug uptake, is responsible for PDT sensitivity in the CHO-AUXB1 mutant, possibly the altered repair of PDT induced DNA damage. Thus, it would be beneficial to examine the response of other defined mutants to PDT treatment to help in characterization of the cytotoxicity of PDT.

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CHAPTER FIVE

SUMMARY

SUMMARY

The capacity of PDT treated cells for DNA synthesis of Ad is reduced in CHO-N cells compared to CHO-MDR cells and in RIF-8A cells compared to RIF-1 cells. The difference in capacity of PDT treated cells is similar to their difference in clonogenic survival after PDT. This suggests that the cellular capacity assay can be used to predict cell sensitivity to PDT and can possibly be used to examine sensitivity of human tumour cells and tumour biopsy samples to PDT.

Results of the split-dose experiments did not yield conclusive evidence of repair in RIF cells due to drug efflux problems and other possible interpretations of data. Capacity experiments suggest a recovery from PDT damage in RIF cells which is greater in RIF-8A cells compared to RIF-1 cells. The results of the delayed capacity assay suggest that the increased resistance of RIF-8A to PDT results from elevated levels of repair of PDT damage in RIF-8A cells compared to RIF-1 cells.

Using the capacity assay the RIF-8A cell line shows greater capacity than RIF-1 cells to UV indicating a cross-resistance of the RIF-8A cell line to UV. No difference in sensitivity to UV was seen between the CHO-N cells and the CHO-MDR cells using the cellular capacity assay. Survival of viral DNA synthesis for UV-irradiated Ad5 was also greater in the RIF-8A cells compared to the RIF-1 cells, suggesting a greater repair capacity for UV-induced DNA damage in RIF-8A cells. This suggests that the increased UV resistance of RIF-8A compared to RIF-1 results in part at least, from an

increased repair of UV-induced DNA damage in these cells. It is possible that the increased resistance of RIF-8A to PDT may also result from an enhanced repair from PDT -induced DNA damage in RIF-8A cells. It is evident from the data presented that the repair mechanisms involved in response to PDT damage should be further investigated in order to further characterize the mechanism of PDT cytotoxicity.

This study shows that the CHO-AUXB1 cell mutant is cross-sensitive to PDT and UV. Through the characterization of the mutant cell line, it is believed that this increased sensitivity is due to a decreased capacity for repair of PDT and UV-induced DNA damage. Defined cell mutants have been utilized in a number of comparative studies to examine sensitivity of cells to cytotoxic agents and it appears that the use of defined cell mutants in PDT studies could possibly further characterize PDT cytotoxicity.

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