# THE RESPONSE OF HUMAN TUMOUR CELLS TO RADIATION AND CYTOTOXIC DRUGS

IAN EDWARD SMITH

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Institute of Cancer Research,
Sutton,
Surrey.

# ABSTRACT

Important differences between human cancers and experimental animal tumours limit the potential of the latter as models for cancer therapy research. This thesis describes the development of a clonogenic assay for human tumour cells, allowing for the first time the radiosensitivity and chemosensitivity of these to be measured in a way previously restricted to cells from experimental animal tumours and established tumour cell lines. The technique involves the use of agar in diffusion chambers implanted into murine peritoneal cavities, the mice acting both as "incubators" and as a source of nutrients for cell growth. Human tumour xenografts have been used for most experiments, although the use of the assay to clone cells from human tumours taken direct from the patient without xenograft passage has also been investigated.

With this assay, the radiosensitivities of cells from several human tumours were measured, both in vitro and in vivo, and the dose survival curves so obtained provided information on the mechanisms underlying the clinical response of different human tumours to radiotherapy. The system was also used to demonstrate the enhancing effect of the radiosensitising drug Ro-07-0582 on the radiosensitivity of hypoxic tumour cells in vivo.

Studies on the sensitivity of human tumour cells to cytotoxic drugs showed important differences compared with cells from experimental animal tumours, and some correlation was shown

between cell survival in the assay and clinical response to chemotherapy.

The advantages of this assay over those based on animal tumours as a tool to complement clinical cancer therapy research are discussed, and suggestions are proposed for its further use.

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# CHAPTER ONE

## GENERAL CONSIDERATIONS

# CHAPTER 1:

# GENERAL CONSIDERATIONS

This thesis is about the development of a laboratory technique for studying the response to therapy of human tumour cells. It is based on a simple premise: experimental animal tumours have a therapeutic response which frequently differs from that of their human counterparts and are therefore of limited value as research tools in this field; laboratory systems derived directly from human tumour material might prove to be more useful and valid models for study, if such systems could be developed.

## 1.1: THE USES AND LIMITATIONS OF EXPERIMENTAL ANIMAL TUMOURS

Experimental animal tumours propagated in inbred host animals [Gross, 1943] have long been a cornerstone of cancer research. Their development has allowed meaningful and reproducible quantitative measurements of tumour behaviour to be made, the influence of genetic and extraneous factors on tumour development to be studied, and epidemiological influences to be assessed. More recently these tumour systems have increasingly come to be employed in therapeutic aspects of cancer research. Where this research has devoted itself to general therapeutic principles, further important contributions have been made. For example, it has recently been demonstrated in the murine Lewis lung carcinoma that cells within very small tumours of less than 0.5mm diameter are more radiosensitive and more chemosensitive than cells within

larger tumours of the same type [Steel and Adams, 1975; Shipley et al, 1975]. This reinforces the clinical concept of early adjuvant radiotherapy or chemotherapy for clinically undetectable micro-metastases at the time of treatment of the primary tumour.

But experimental animal tumours have also been used in a much more specific way to predict clinical response to individual cytotoxic agents, and to plan the scheduling of combination therapy. For example, a series of animal tumours are used in screening programmes for new anti-cancer drugs throughout the world. And it has been advocated that cell cycle kinetic data obtained from murine leukaemias should be extrapolated to develop elaborate drug scheduling regimes in clinical cancer chemotherapy [Bergsagel, 1969; Vietti et al, 1971; Price et al, 1975].

It is in these latter areas of research that the value of animal tumour models becomes questionable. Important differences exist between these and human tumours, both in their cell proliferation kinetics [reviewed by de Vita, 1971; Steel, 1973; van Putten, 1974; Tubiania and Malaise, 1976], the therapeutic significance of which will be discussed in Chapter 10, and in their inate sensitivity to specific cytotoxic agents.

Some examples of the latter can be quoted here. The nitrosoureas are a group of compounds first detected in the N.C.I. (National Cancer Institute, Bethesda) drug screening programme and developed for clinical use over the last 15 years. Some of these, including BCNU (1, 3, bis 2-chloroethyl-1-nitrosourea), CCNU

(1,2-chloroethyl-3 cyclohexyl-1-nitrosourea) and Methyl-CCNU are amongst the most active agents ever studied against experimental animal tumours, both quantitatively and in the range of their anti-tumour activity. Yet their clinical activity in man has proved in general modest and rather disappointing. In particular BCNU has been shown to cure murine leukaemias and the C3H mammary cancer [Schabel, 1976], whereas this drug has little activity in human leukaemias and is relatively ineffective in human breast cancer [Slavik, 1976]. DTIC (Diamino-triazeno-imidazole-carboxamide), another N.C.I.-developed drug, has a wide range of experimental tumour activity, with cures reported for L1210 leukaemia; it is inactive, however, in the B-16 mouse melanoma [Venditti, 1976]. Conversely, in man this drug is of little value in leukaemia but is one of the few agents to which human melanoma shows any significant response rate [Comis, 1976]. Vincristine is a highly active agent in the induction therapy of acute lymphoblastic leukaemia in children and yet shows no activity against any of three commonly studied murine lymphocytic leukaemias (L1210, AKR, L5178Y) [Dykes and Nelson, 1977]. And finally, L-asparaginase, an enzyme with specific clinical activity in acute lymphoblastic leukaemia, is inactive against all five tumours used until recently in the N.C.I. programme, and would therefore have been completely missed by their screen [Connors and Jones, 1970].

## 1.2: HUMAN TUMOUR XENOGRAFTS

These important differences which limit the use of experimental animal tumours as models for clinical cancer therapy research have not, of course, gone unrecognised, and their use has been defended largely on the grounds that no other more appropriate model has been available for this type of work. In recent years, however, techniques have been developed for the transplantation of human tumours into immune-suppressed animals. These xenograft tumours can be maintained and propagated by serial passage in the laboratory and therefore present an opportunity to carry out on human tumour material the type of therapeutic research previously restricted to animal systems.

Details of the development of human tumour xenografts and the grounds for believing that they may represent a more valid model for clinical therapy will be described in the next Chapter. Here it can be stated that while their long-term value as a research tool has yet to be clearly established, there is nevertheless promising circumstantial evidence to suggest that they may respond to therapy in a similar way to the parent human tumour; indeed two human tumour xenografts have recently been added to the standard animal tumour series used in the N.C.I. drug screening programme.

At present there are at least two important aims in human tumour xenograft research. The first is to establish more clearly their validity as models for cancer therapy in the clinic; the second is to investigate what techniques for measuring tumour response might be most appropriately applied to this system.

This thesis concerns research directed mainly at the second of these problems. So far the response to treatment of xenografts has been measured mainly by tumour growth delay techniques in which tumour growth in the treated animal is compared with that in untreated controls (Chapter 3). In experimental animal tumours, important additional information can also sometimes be obtained on the specific effect of such treatment on individual tumour cells, using clonogenic assay techniques (Chapter 3). At the start of this research project no such reproducible assay system for studying individual human tumour cells had been described (although my colleague Mrs. Doreen Courtenay working at the Institute of Cancer Research was developing an in vitro assay technique for this purpose). However, a clonogenic cell assay system using agar in diffusion chambers (Chapter 4) had recently been developed for human bone marrow [Gordon, 1974]. My project therefore was first to study whether this technique could be adapted for human tumour cells, and second to use the assay to study the effects on these cells of irradiation and of cytotoxic drugs.

Before the results of these studies are presented, the development and use of both human tumour xenografts and clonogenic assay techniques, which are fundamental to this work, will be reviewed.

# CHAPTER TWO

## HUMAN TUMOUR XENOGRAFTS

# CHAPTER 2:

# HUMAN TUMOUR XENOGRAFTS

The ethical and practical problems which restrict research on human tumours in patients, and the limitations to experimental animal tumours already discussed, have stimulated attempts for many years to grow human tumour material in animals. The fundamental problem thwarting such attempts is of course the rapid and complete rejection of foreign tissue by the immune defence mechanisms of the host animal.

## 2.1: THE DEVELOPMENT OF XENOGRAFT TECHNIQUES

### 2.1.a: Sites of "Immune Protection"

The earliest efforts to overcome this immune response were aimed at trying to grow tumour tissue at certain sites in the animal which appeared to be relatively protected from host immune mechanisms. The anterior chamber of the eye with its absence of direct blood supply was an obvious site for study, and human tumour biopsy material has occasionally been grown in this site [Greene, 1942]. However, the techniques involved are difficult, the percentage of failures to take high, the growth rate of successfully implanted tumours slow and transplantability poor.

The hamster cheek pouch has also been found to confer some degree of immune protection on implanted human tumours [Lemon et al, 1952; Handler et al, 1956; Patterson et al, 1957], particularly when the hamster is further immune-suppressed with

cortisone or irradiation [Handler et al, 1956; Patterson et al, 1957]. With this technique human tumours have been grown and transplanted through repeated animal passage, the tumours in the cheek pouch maintaining with serial passage the histological characteristics of the parent human tumour. An attractive feature of this site is that growth of the implanted tumour can be visualised using a perspex observation chamber inserted into the hamster, and important observations on the development of a blood supply necessary for successful growth have been made in this way [Smith, 1969a].

Another approach to overcome host immune response has been based on the relative immune "tolerance" of new-born animals. This has been exploited to grow pulmonary and subcutaneous tumours in new-born rats less than 24 hours old from human cancer cell lines [Kutner and Southam, 1960] and subsequently directly from human cancers [Southam, 1966]. However, the take-rate for tumours was low both in rat lungs and subcutaneously, and tumours rarely grew to more than a few millimetres in diameter. This raised doubts about whether viable tumours were in fact being grown or whether in this system the host animals were merely serving as a nutrient medium for short term tissue culture.

2.1.b: Immune Suppressed Hosts

A more rewarding approach to the problem of immune rejection of implanted tumour material has been the development of techniques for suppressing the immune response of the host animal. This was first successfully achieved by Toolan [1951] who

managed to grow subcutaneous tumours from human cancer tissue implanted into albino rats and hamsters previously treated with whole body irradiation. Some of these tumour xenografts could be transplanted through two or more passages, but in the initial studies tumour masses proliferated usually for only an 8 to 10 day period, and raised doubts about tumour viability. Later Toolan [1953] achieved a greater tumour-take rate in irradiated animals subsequently treated with cortisone; and these grew for a longer, but still self-limiting period. Despite its shortcomings, this technique was important in demonstrating that the propagation of human tumour material in immune suppressed animals was a practical possibility, and the method was in fact used in what appears to have been the first study of an anti-cancer agent on a human tumour grown in vivo in the laboratory: the oncolytic effect of Egypt virus on a human epidermoid carcinoma [Toolan and Moore, 1952].

A considerable advance in techniques for host animal suppression came with the appreciation that thymus-derived lymphocytes (T-lymphocytes) play an important role in rejecting xenografts [Davies et al, 1966]. It was found that human tumour xenografts could be grown in mice depleted of T-lymphocytes and several techniques were devised to achieve this. These included thymectomy at birth [Osoba and Auersperg, 1966], treatment of mice with antilymphocyte or antithymocyte serum [Phillips and Gazet, 1967, 1970; Arnstein et al, 1972; Stanbridge et al, 1975],

a combination of thymectomy and antilymphocyte serum [Davis and Lewis, 1968; Cobb, 1972, 1973; Cobb and Mitchley, 1974; Stanbridge et al, 1975], thymectomy and whole body irradiation, usually followed by marrow reconstitution [Castro, 1972; Cobb and Mitchley, 1974; Mitchell et al, 1974; Franks et al, 1975; Pickard et al, 1975; Stanbridge et al, 1975], and thymectomy, antilymphocyte serum and whole body irradiation [Berenbaum et al, 1974]. These techniques represented a major breakthrough in xenograft research and permitted the successful growth in the T-cell depleted mouse of viable, vascularised human tumours which could be readily propagated through serial animal passage.

Genetically athymic* 'nude' mice with severe or complete T-lymphocyte depression have also been used to grow and propagate a variety of human tumour xenografts [Rygaard and Povlsen, 1969; Giovanella et al, 1974; Helson et al, 1975; Povlsen and Jacobsen, 1975; Stanbridge et al, 1975]. These animals are expensive and require elaborate laboratory facilities for their breeding and maintenance. On the other hand they have the advantage of not requiring time-consuming immune-suppressive procedures before use. Their xenograft 'take' rate is also probably slightly

*It has been recently suggested that these mice may have a residual thymus and an associated low level of circulating T-lymphocytes [Raff, 1973].

better: Giovanella and Stehlin [1974] report a 'take' rate of over 80% for primary human tumours, whereas only 50-70% of tumours take in artificially immune suppressed mice [Castro, 1972; Berenbaum, 1974]. 'Take' rates are themselves dependent on tumour type: in our experience at this laboratory, oat cell carcinomas, melanomas and to a lesser extent colonic carcinomas have high 'take' rates, whereas only 10% of breast carcinomas can be established as xenografts.

Basically, however, there is no doubt that both types of mouse have the ability to propagate xenografts, and the decision on which to select is principally dependent at present on the type of laboratory facilities locally available.

## 2.2: THE VALIDITY OF THE HUMAN TUMOUR XENOGRAFT AS A MODEL FOR CLINICAL THERAPY

Compared with experimental animal tumours, human tumour xenografts are expensive and time-consuming to produce, capricious to maintain and often adapt with difficulty to standard experimental procedures (e.g. the production of single cell suspensions - see later). Their use can only be justified on the assumption that they offer a more valid model than animal tumours for studies relating to clinical cancer therapy. It is therefore of fundamental importance in xenograft research to verify whether this is indeed the case in practice, or whether they are merely eccentric and irrelevant laboratory artefacts.

2.2.a: Xenograft Histology and Karyotype

There are several approaches to this problem, of which the first is the study of xenograft tumour morphology and histology. Many human tumour xenografts developed in recent years have been derived from carcinomas of the large bowel. The histology of these xenografts, even after multiple passages, has usually shown a close resemblance to that of the parent tumour: they maintain cellular appearance and function (e.g. mucin-producing), cellular arrangement (e.g. acinar), and the degree of differentiation of the original [Castro, 1972; Cobb, 1973; Helson et al, 1975; Pickard et al, 1975]. A similar close histological resemblance to the original has also been described for other tumour xenografts including an oat cell carcinoma of lung [Kopper and Steel, 1975], a malignant melanoma [Povlsen and Jacobsen, 1975] and a neuroblastoma grown from a cell line derived from the original tumour [Helson et al, 1975]. Chromosomal analyses are described in most published studies on human tumour xenografts and these invariably demonstrate tumour cells to be of human origin.

There are two interesting histological differences frequently observed between xenografts and originals, however. Pickard et al [1975] point out that the stroma in their xenografts, inevitably of murine origin, had a fine reticular appearance with few inflammatory cells, in contrast to the dense stromal and inflammatory reaction exhibited by the original tumours. This seems scarcely unexpected in view of the differing environments

of the growing xenograft and the original tumour, and it is surprising that it has not been more frequently commented on in other reports. The other, and less predictable difference, is that xenograft tumours only rarely appear to metastasise in the host animals, even when their histology shows a very poor degree of differentiation and a high mitotic index. Occasional exceptions have been reported, the most significant being 18 metastases from 63 tumour implants, most of which were however from HeLa cells rather than fresh tumour biopsies [Franks et al, 1975]. A single metastasis of a human colonic tumour in a hamster brain has also been reported [Goldenberg, 1970], as have metastases from 3 melanoma xenografts to lymph nodes and lung [Giovanella et al, 1973]. But these reports are exceptional, and in general macroscopic metastases from human tumour xenografts are not usually seen. Indeed even the intravenous injection of up to $10^6$ xenograft tumour cells into immune-suppressed mice failed to grow pulmonary tumours [Mr. R. George, personal communication].

This curious phenomenon may be associated with the observation that xenografts invade with the advancing tumour edge continuous with the main tumour mass and without outlying separate islands of tumour cells seen in original specimens [Pickard et al, 1975]. All this may indicate some residual host immune response to the xenograft despite immune-suppression and inevitably must raise doubts about the interpretation of studies on tumour response to therapy. Further evidence suggesting a residual immune response is discussed below.

2.2.b: Xenograft Response to Therapy

The most direct test of the xenograft as a valid model for clinical therapy studies is to see whether its response to therapy resembles that of the parent tumour. This is less easy to investigate than it might at first appear. The problem is primarily a practical one: not all tumour biopsies can at present be successfully grown as xenografts and therefore clinical tumours whose response to therapy can be measured may fail to provide derived xenografts; conversely not all patients from whom successful tumour xenografts have been established are subsequently treated in a way that allows comparison with xenograft response to therapy. Much of what data has so far been assembled on the therapeutic response of xenografts is therefore of a general nature, and lacks a specific correlation between the human tumour and its own xenograft.

Smith [1969a] studied the effects of nitrogen mustard and methotrexate on a series of human tumour xenografts implanted into the hamster cheek pouch and found a considerable variation in the response of different tumours, which he felt might be analagous to the variation in clinical response anticipated for these agents. He subsequently demonstrated that the response to cytotoxic drugs of the rat Walker 256 tumour was the same when grown as a xenograft in the hamster cheek pouch as *in situ* in the rat, and extrapolated that the same might reasonably be anticipated for human tumours [Smith, 1969b].

Sheard et al [1971] also showed that the rank order of effectiveness of 4 drugs for a rat tumour was the same in the species of origin as when grown as a xenograft in immune-suppressed mice.

Cobb and Mitchley [1974] studied the effects of several chemotherapeutic agents on 2 human colonic carcinoma xenografts and found that of these drugs only 5-fluorouracil (5-FU) achieved complete tumour control, supporting the clinical observation that 5-FU is relatively more effective than most other drugs in what is usually a chemoresistant tumour [BMJ Editorial, 1976]. Povlsen and Rygaard [1974] reported a good response of a Burkitt's lymphoma xenograft to cyclophosphamide, which parallels the response seen in patients. Similarly Povlsen and Jacobsen [1975] treated a melanoma xenograft with various drugs of which DTIC (dimethyl-triazeno-imidazole-4-carboxamide) was the most effective and of which 5-FU had no effect whatsoever. This again correlates with clinical experience in the chemotherapy of melanomas, DTIC being one of the few agents to achieve any response in this notoriously chemoresistant tumour [Carter and Friedman, 1972; Comis, 1976]. Unfortunately in neither of these studies were the patients' original tumours treated with chemotherapy.

This was also the limitation to a detailed study on the effects of various agents on growth delay of 3 xenografts derived from 2 colonic carcinomas and one oat cell carcinoma of lung [Kopper and Steel, 1975]. Despite the lack of direct clinical correlation to back

up this study, it was of interest that the colonic tumours responded best to 5-FU whereas the oat cell xenograft showed most response to cyclophosphamide, findings which again agree well with clinical observations on the chemosensitivity of these tumours [Holland and Frei, 1973]. An unexpected finding in this study was that some of the oat cell xenografts showed permanent regressions after cyclophosphamide at a calculated level of cell kill which would not have been expected to achieve long term tumour control; and the authors considered the possibility that a residual host immune response was still operating in the thymectomised irradiated mice. Obviously this would require careful consideration in interpreting the results of tumour growth delay and tumour case studies with cytotoxic drugs in the xenograft system.

Very few studies have so far attempted to compare directly the response of a xenograft with the response of the original tumour in the patient. Burt et al [1966] described a bladder carcinoma in a patient which responded to radiotherapy and 5-FU but not to vinblastine; this subsequently retained these sensitivities when treated as a xenograft in an immune-suppressed hamster. At present there is only one other study of which I am aware where the attempt has been made to correlate xenograft response to chemotherapy with clinical response in the patient. Novak, working in the same laboratory as myself, has studied xenograft tumour growth delay after cytotoxic drugs from patients being treated with chemotherapy for advanced colo-rectal carcinoma, and has so far

found that the xenograft showing maximal response to therapy is from one of the few patients in the study who obtained an objective tumour response to chemotherapy [personal communication].

This important and painstaking work provides the most direct indication so far that xenografts may indeed be the valid models sought for laboratory studies on clinical chemotherapy. With the important proviso that xenograft tumour response may be influenced by some degree of residual host immune activity, the evidence suggested that further studies into developing this new technique, including an attempt to produce a clonogenic tumour cell assay, would seem worthwhile.

# CHAPTER THREE

## CLONOGENIC CELL SURVIVAL ASSAYS

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## 3.1: GROSS MEASUREMENTS OF TUMOUR RESPONSE TO TREATMENT

The response of experimental tumours to cytotoxic agents can be measured in several ways. First, the percentage increase in life span [% ILS] of treated animals compared to untreated controls can be calculated. This approach is particularly suitable for widely disseminated tumours including leukaemias and is commonly used in experiments where new agents or combinations of agents are screened for cytotoxic activity. For localised tumours, measurement of growth delay after treatment is often more appropriate, and most studies with human tumour xenografts have used this method. In tumour growth delay experiments some parameter of gross tumour growth, for example volume doubling time, is compared in the treated tumour to that of untreated controls. This technique is appealingly similar to the clinician's measurement of the extent and duration of tumour regression in his patients after treatment, and is therefore an attractive tool for the laboratory study of anti-cancer agents.

However these gross studies of tumour response are clearly limited in the type of information they can provide. What they measure and what the clinician measures in his patient is merely an effect; they can provide little insight into the elaborate interplay of mechanisms whereby this effect is mediated.

Changes in the rate of tumour growth after treatment may depend on a variety of factors. These include first the fraction of tumour cells killed by the cytotoxic agent under study. Second, the effect of cell kill on tumour size in turn depends on the balance between the rate of dead cell removal on the one hand and the rate of cell repopulation on the other. Third, a variety of host tissue response factors may also affect the size of the tumour: these can include a non-specific acute inflammatory response with cellular infiltrate, vascular changes and tissue oedema; in addition, the possibility of a specific host immune response, at least against some tumours, has also been argued, and immunotherapeutic approaches to exploit this are at present under intensive study [Southam and Friedman, 1976]. Evidence suggesting the possibility of a residual host immune against human tumour xenografts has already been mentioned (Chapter 2.2.b).

If the actual mechanisms underlying the effect of cytotoxic agents on tumour growth are to be understood, and hence if a more rational basis for designing new techniques of therapy is to be achieved, then it is first necessary to be able to study not merely the end effect of therapy as in tumour growth delay experiments but also these individual mechanisms which underlie that effect.

In particular it is important to be able to measure the specific effects of cytotoxic agents directly on tumour cells, and not merely on the gross tumour, in a quantitative way. For this reason assays for cell survival after exposure to these agents have been developed in recent years.

## 3.2: CELL SURVIVAL ASSAYS

Tissue growth, maintenance and repair are based on the existence in both tumours and normal tissue of what are termed stem cells. These are cells which have the capacity to proliferate and give rise to generations of daughter cells which may themselves in turn either further proliferate, or else differentiate, mature and eventually die. The factors controlling these fundamental processes and their inter-relationship are intricate and only partly understood; they have recently been reviewed [Cairnie et al, 1976].

Cell survival assays are experimental techniques designed to study and measure in an appropriate artificial environment cells from tumours or normal tissues which have this capacity for proliferation. The artificial environment employed in the assay is either an in vitro culture system, or an in vivo organ of a living animal e.g. spleen or lung. Details of these systems will be described below.

If a single cell suspension is made from the tissue under study and introduced into such a system, then the cells with proliferative capacity in that suspension can undergo a series of divisions to form colonies or clones of cells. Colonies thus formed can eventually be counted after an appropriate time interval, and the number of colonies grown represents the number of parent cells with proliferative capacity in the original cell suspension. By dividing the number of colonies grown from a cell suspension of treated tissue with that from an untreated control, a quantitative measurement of

the fraction of cells with colony-forming ability surviving after the treatment can be obtained. The technique thus devised is called a colony-forming or clonogenic cell survival assay.

## 3.3: THE DEVELOPMENT OF MAMMALIAN CLONOGENIC CELL SURVIVAL ASSAYS

For many years it proved technically impossible to grow cellular colonies from single mammalian cells in vitro in a manner analogous to that employed for bacteria and viruses. In 1955, however, Puck and Marcus in a now classic paper described an in vitro method for growing colonies from single cell suspensions of the so-called HeLa cell line originally derived from a human cervical carcinoma. In their original experimental design, single cells were plated out on glass slides supported in a Petri dish above a layer of heavily irradiated and therefore non-dividing "feeder" cells. These latter were believed to provide some unknown conditioning factor to the suspension medium which enabled the single cells to reproduce to the point where colonies of cells could become self-sustaining. The supporting medium itself was supplemented with horse and human serum and cultures were incubated in 5% $CO_2$. Later, it was found that gentle trypsinisation of the initial cell suspension eliminated the need for a "feeder" layer. Agar was added to the medium in some experiments both to decrease loss by diffusion of any necessary metabolites escaping from the cells and to limit the tendency of individual colonies to migrate [Puck et al, 1956].

Using these techniques, single cells grew visible colonies with a diameter of about 1mm (800 - 2000 cells) in about 10 days after plating, with a plating efficiency of 100% (i. e. all plated cells grew colonies). Thus it became possible to assay the effect of external agents on the colony-forming ability of mammalian cells, and the first study on cell survival after in vitro irradiation was published within the year [Puck and Marcus, 1956]; (Chapter 7. 1).

Colonies from many other cell lines were subsequently grown using this technique and initially the assays were mainly used to study the effects of in vitro irradiation on different types of mammalian cell (Chapter 7. 1). However, the artificiality of these laboratory propagated cell lines greatly limited their use in studying the effects of therapy on normal tissues growing in vivo under physiological conditions.

## 3.4: CLONOGENIC CELL ASSAYS FOR ANIMAL MARROW

The development of an elegant spleen colony assay for cells from normal mouse marrow by Till and McCulloch [1961] was an important advance. Their assay was based on the fact that the intravenous injection of marrow cells into syngeneic mice previously exposed to supralethal whole body irradiation leads to the formation of colonies of proliferating erythroid and myeloid precursor cells at various stages of differentiation in the spleens of these animals; these colonies could be counted as discrete nodules 10 or 11 days after injection. In this technique the plating efficiency was only one hundredth that of Puck and Marcus, but the marrow cell count

included all nucleated cells, a large proportion of which were already differentiated and had presumably lost the capacity to give rise to a new generation of proliferating cells. Apart from this the two methods had striking analogies: in both a small number of individual cells could be inoculated from which colonies of cells grew and could be counted, and in both the viability of the inoculated cells and hence the effects of external agents could be assayed. The spleen colony assay moreover has one practical advantage: maintenance of a constant biochemical medium, pH, humidity and temperature control, of such critical importance in the in vitro culture system, is provided for "de gratis" by the mouse host. Although the histology of the spleen colonies could be readily studied, the exact nature of the progenitor cell giving rise to colonies was uncertain and was therefore designated "colony forming unit-spleen" (CFU-S).

Other techniques for assaying marrow colony-forming cells were later described. Pluznick and Sachs [1965] and Bradley and Metcalf [1966] developed an in vitro plating technique similar to that originally described by Puck and Marcus; here however, the addition of agar to the culture medium overcame the tendency of plated cells to "wander" in the medium and allowed colonies to be grown at a higher density than in monolayer without the problems of overlap or of "feeding" of small colonies from larger ones. For these reasons the addition of agar to culture medium has become a widespread practice in most in vitro colony assay techniques. This in vitro assay had the great advantage over the earlier spleen colony assay that it did not require the use of syngeneic rodent hosts and

therefore raised the possibility of similar studies in higher animals and in humans.

The principal disadvantage of all in vitro assays for marrow colony forming cells is that the addition to the culture medium of an external factor is necessary to promote colony growth. This so-called colony stimulating factor (CSF) is obtained from various sources: these include feeder layers of white blood cells [Pike and Robinson, 1970], feeder layers of kidney embryo cells [Brown and Carbone, 1971], "conditioned" medium in which feeder cells have been previously grown [Iscove et al, 1971; Bull et al, 1973] or human urine [Harris and Freireich, 1970]. The exact nature of CSF is the subject of intensive biochemical research at present, but from the practical point of view, both the standardisation of colony stimulating factor and variations in the terminology associated with different sources of factor complicate analysis and comparison of data from the in vitro assay.

More fundamentally, the colony progenitor cell in the in vitro assay does not appear to be the same as that in the spleen colony assay. Stained histological preparations of marrow colonies indicate that the in vitro soft agar assay selects out a less primitive progenitor cell already committed to forming granulocytes and macrophages but not capable of giving rise to the erythroid elements also found in spleen colonies [Metcalf and Moore, 1971]. By convention, therefore, the two types of marrow colony forming cell are described as CFU-C (for in vitro soft agar culture) and CFU-S (for spleen

colony growth).

## 3.5: CLONOGENIC CELL SURVIVAL ASSAYS FOR OTHER NORMAL TISSUES

Cells from normal tissues other than bone marrow do not readily form colonies in agar culture, but the development of elegant alternative in vivo techniques has led in recent years to the study of clonogenic cells from cartilage, skin and intestinal epithelium. The recovery of growth cartilage in rats after irradiation is marked by the appearance of clones of dividing cells, and this physiological repair process was exploited as a colony cell survival assay for growth cartilage in rats, colonies of about 250 cells being counted microscopically 25 days after in vivo irradiation [Kember, 1967]. A similar technique for assaying surviving epithelial cells of mouse skin after irradiation was described by Withers [1967]. Here small numbers of epithelial cells surviving in areas of irradiated skin proliferated to form visible nodules after 10-20 days; by counting regenerating nodules per unit area after measured doses of irradiation the relationship of cell survival with dose could be calculated on the assumption that nodules are formed by the proliferation of single surviving cells. Cell survival in murine intestinal epithelium has been measured by irradiating in vivo a strip of jejunum, temporarily exteriorised; microscopic clones of regenerating mucosa are counted 13 days later. Again in this technique it is assumed that each clone arises from a single surviving colony-forming cell [Withers and Elkind, 1968]. An obvious attraction of these techniques

is the physiological basis of their experimental design, and recently one has been used for sophisticated studies on the ratiosensitivity of skin clonogenic cells in both a well-oxygenated and a hypoxic environment [Denekamp et al, 1974].

Finally, in considering cell survival studies on normal animal tissues, mention must be made of a curious technique for assaying the viability of mouse oocytes after irradiation [Lin and Glass, 1962]; here the end-point was not colony formation but, logically enough, embryo-development after post-irradiation implantation into mated female mice. The radiosensitivity measured by this technique lay well outside the range for all other mammalian tissues however (Chapter 9.1), and this inevitably raised doubts about the validity of the assay method.

## 3.6: TUMOUR CELL SURVIVAL ASSAYS

Although the original in vitro technique of Puck and Marcus was satisfactory for growing colonies from cell lines, some of which had been originally derived from tumours, the culture environment provided by this relatively simple system initially proved inadequate to support the more rigorous demand of cells from tumours growing in the animal. The first cell survival assays therefore had to depend on in vivo techniques.

The earliest tumour studied in this way was a spontaneously arising lymphocytic leukaemia in CBA mice and the number of viable leukaemic cells in suspension was assayed by limiting dilution technique [Hewitt and Wilson, 1959]. In this method, several dilutions

of leukaemic cells were injected into syngeneic mice and the number of cells required to induce leukaemia in 50% of the injected mice after a 90-day observation period calculated ($TD_{50}$).

Limiting dilution assays have subsequently been used to study cell survival in many experimental animal tumours; these include a round cell sarcoma [Hewitt and Wilson, 1961], the Ehrlich ascites tumour [Silini and Hornsey, 1962], the AKR mouse lymphoma [Bush and Bruce, 1964], the MTG-B adenocarcinoma [Clifton et al, 1966], a rat rhabdosarcoma [Reinhold, 1966], the C3H mouse fibrosarcoma [Frindel et al, 1967], a well differentiated murine squamous carcinoma [Hewitt, 1967], an osteosarcoma [van Putten, 1968], the KHT mouse sarcoma [Kallman et al, 1967] and the Lewis lung carcinoma in mice [Steel and Adams, 1975].

Limiting dilution assays have the advantage of great sensitivity and are capable of measuring cell survival fractions down to $10^{-6}$. This however, must be balanced against the large animal numbers demanded by the experimental design of the technique. A more serious disadvantage is the possibility of tumour cell antigenicity to the recipient animals, particularly when non-syngeneic recipients must be used. Occasionally host immune reactions can be circumvented by using new born animals [Silini and Hornsey, 1962], but clearly the assay could not be used for cross-species tumours and in particular for tumours from human patients (unless of course effective immune-suppressive therapy were first given to recipient mice); even where syngeneic animals are available however the

possibility of tumour cell-specific antigenicity must be investigated and the experimental design appropriately modified and standardised [Kallman et al, 1967; Steel and Adams, 1975].

Two other in vivo assay systems have also been employed to measure tumour cell survival. The spleen colony assay used to study haemopoetic colony forming cells has been adapted for malignancies of the haemopoetic system including cells from the AKR mouse lymphoma [Bush and Bruce, 1964; Bruce et al, 1966], the L1210 leukaemia [Wodinsky et al, 1967], a mouse plasma cell tumour [Bergsagel and Valeriote, 1968] and mouse myelomas [Park et al, 1971]. A lung colony assay similar in principle to the spleen colony assay but applicable to non-haematological malignant cells was proposed by Williams and Till [1966] who found that polyoma virus-transformed cells from rat embryo tissue injected intravenously into rats grew large macroscopic lung nodules within 17 days. Subsequently this system has been used to grow lung colonies from the KHT sarcoma [Hill and Bush, 1969] and the Lewis lung carcinoma [Shipley et al, 1975].

Both these assays have the attraction over limiting dilution techniques of rapidity, the colony growth end-point being achieved in 2-3 weeks compared with up to 12 weeks for the latter; furthermore the experimental design requires far fewer animals. However the lung colony assay is often less sensitive than limiting dilution methods; Shipley et al [1975] found for example a cloning efficiency for untreated tumour cells of only 0.2 to 0.3%, which for cell

survival after high doses of irradiation required more cells than could be recovered from the treated tumour. A more important disadvantage which both these assays inevitably share with the limiting dilution technique and indeed with any design of *in vivo* assay is host immune response unless syngeneic animals are used as recipients; they are therefore useless for assaying cell survival of tumours from larger animals and humans.

This problem therefore emphasised the need for an *in vitro* cloning assay for tumour cells and stimulated research despite the recognised difficulties in artificially fulfilling the rigorous nutritional and metabolic demand of such cells. One approach was the selecting out of tumour cell lines which could be adapted to grow both in culture and in the animal. Frindel *et al* [1967] developed an *in vitro* assay for a C3H mouse fibrosarcoma grown originally from an established cell line. Conversely Barendsen and Broerse [1969] devised a similar assay for a tumour which initially arose *in vivo* and was subsequently adapted for tissue culture. This was achieved by culturing *in vitro* a suspension of tumour cells from a radiation-induced rat rhabdosarcoma; once a cell line had been established a clone of cells was isolated and reinoculated into a rat where it developed a tumour; repeated alternate *in vitro* and *in vivo* passages eventually produced a tumour adapted for growth both in animals and in culture. In the same way the EMT6 tumour was developed from a mouse mammary tumour and its ability to grow in culture used to develop an *in vitro* colony forming assay [Rockwell and Kallman, 1973].

Although colonies from single cell suspensions of these specially adapted tumours could be grown in culture conditions similar to those employed originally by Puck and Marcus for their HeLa cell lines, in vitro cloning of tumours not previously adapted to culture conditions was eventually achieved only with a much more rigorous experimental design. The first such tumour to be assayed in this way was a mouse plasma cell tumour for which a spleen colony assay had previously been developed; the growth of colonies from single tumour cell suspensions was achieved only when several critical requirements were met: these included the use of a kidney tubule feeder layer, the daily addition of fresh culture medium and the addition of human serum, unnecessary for the growth of haemopoetic cell colonies in the same system [Park et al, 1971]. Thomson and Rauth [1974] grew colonies of cells in vitro from the widely studied KHT mouse fibrosarcoma; here they found the addition of 10% foetal calf serum and Bacto agar were important for colony growth; no growth was seen with Noble agar or methyl cellulose. In vitro colony growth of cell suspensions from the Lewis lung tumour and the B16 melanoma in mice was achieved by Courtenay [1976]; here an incubation atmosphere of only 5% oxygen and the addition of rat red blood cells to the culture medium were important factors. Recently an in vitro colony assay for a rat brain tumour has been described, requiring 10% foetal calf serum and a layer of heavily irradiated "feeder" cells [Rosenblum et al, 1975].

Complex though these criteria for culture have proven to be, the development of in vitro colony forming cell assays for animal tumour was an important advance. They are in general less elaborate and easier to carry out than parallel in vivo assays, they are often more rapid in that colonies can be counted microscopically at an early stage, they are certainly more economical eliminating the need for recipient animals, and they offer some opportunity for studying specific requirements of individual growth factors. But most important, they eliminate the problem of host immune factors which limit the scope of in vivo assays, and raise the possibility of developing a similar assay for human tumour cells.

## 3.7: CLONOGENIC ASSAYS FOR HUMAN MARROW CELLS

In the 1960s successful attempts to grow in vitro colonies from animal haemopoietic tissues inevitably stimulated contemporary efforts to grow similar colonies from human bone marrow. In 1967, Senn et al described the successful growth of colonies in agar from human marrow cell suspensions, using a technique very similar to that previously described for the in vitro growth of mouse marrow colonies [Pluznick and Sachs, 1965; Bradley and Metcalf, 1966]. Colony stimulating factor was provided by the addition of a "feeder" layer of mouse-kidney tubules to the agar-culture medium. Subsequently Pike and Robinson [1970] described a similar method for colony growth of human marrow cells; here however a "feeder" layer of human peripheral white blood cells was used. Both

techniques grew colonies that contained principally granulocytic precursor cells, with a low plating efficiency of approximately 16 to 50 colonies per $10^5$ cells. These observations were rapidly confirmed by other studies which demonstrated, as in animal marrow assays, the necessity for some source of colony-stimulating factor. Inconsistencies in the levels of CSF generated by these sources have therefore proved a disadvantage in this in vitro system and repeated careful standardisation of controls are necessary. For this reason the development of a diffusion chamber technique for growing human marrow colonies without requiring the addition of a source of colony stimulating factor was of some importance [Gordon et al, 1975]. This technique will be described in more detail below (Chapter 4).

## 3.8: COLONY FORMATION IN HUMAN ACUTE LEUKAEMIA

Analysis of colony-formation by the marrow of patients with acute leukaemia is complex. Some experimenters have found that the marrow of patients with acute myeloid leukaemia in relapse grows no colonies [Senn et al, 1967; Iscove et al, 1971; Brown and Carbone, 1971]; others have reported occasional small abortive cluster-colonies of less than fifty cells [Harris and Freireich, 1970; Greenberg et al, 1971; Bull et al, 1973], and very rarely apparently normal colonies [Bull et al, 1973]. Moore et al [1974] found that the marrow of 17% of a series of patients with acute myeloid leukaemia grew colonies in agar, and that most of the rest grew large or small clusters. Moore claimed that the ability of leukaemia marrow to grow

colonies or clusters was of prognostic significance, patients with small cluster formation being more likely to achieve remission. This observation has yet to be confirmed by others, and in general it would appear that acute myeloid leukaemia blasts do not often form colonies though they may form small clusters. Recently it has been reported that acute myeloid leukaemia blasts can be stimulated to form colonies of more than 500 cells in soft agar by the addition of phytohaemagglutinin, electron-micrographs demonstrating nuclear "pockets" in colony cells consistent with chromosome abnormalities in the leukaemic cell opoulation [Dicke et al, 1976]. The significance of these artificially-stimulated colonies is hard to assess at present.

Colony formation by marrow cells from patients with acute lymphoblastic leukaemia has been less intensively studied, although one report suggests that normal granulocytic colony formation in methyl cellulose was depressed by leukaemic lymphoblasts in patients in relapse, but recovered once remission was achieved [Duttera et al, 1973]. Leukaemic lymphoblasts do not themselves grow colonies, nor usually do normal lymphocytes. Again however it has recently been reported that normal human peripheral blood lymphocytes can be stimulated to grow large lymphoblast colonies in agar by phytohaemagglutinin or pokeweed mitagen (PWM) [Fibach et al, 1976]. Histological staining demonstrated that the colonies were composed of lymphoblasts. Rosetting techniques and immunofluorescent staining showed the PWM-induced colonies contained both T and B lymphoblasts whereas PHA-induced colonies contained only T lymphoblasts.

While this technique may prove of value as an immunological tool, the artificiality of the system at present makes its significance hard to assess.

## 3.9: COLONY FORMATION FROM HUMAN TUMOURS

Chronic granulocytic leukaemia (CGL) is the only human tumour which at present consistently grows colonies from single tumour cells. Brown and Carbone [1971] showed that large granulocytic colonies could be grown in methyl cellulose from both bone marrow and peripheral blood samples of patients with CGL. The malignant nature of these colonies was implied by their growth from peripheral blood cells, an occurrence not seen with normal peripheral blood, and later verified by the demonstration of the so-called Philadelphia marker chromosome, characteristic of chronic granulocytic leukaemia, in some colonies [Chervenick et al, 1971]. Colonies were not however grown from a patient in the blast-crisis end stage of this disease (Ibid). Theoretically CGL would seem to provide an excellent opportunity for developing a colony-forming cell assay system to study therapy in this disease, particularly when the cell sampling technique, namely venipuncture, is so simple. Indeed my initial plans had been to include chronic granulocytic leukaemia in my research project. In practice however, the rarity of the disease, and the medical indications for treatment to reduce the peripheral leukaemia cell blood count once diagnosis has been made, greatly limits the scope and potential for this type of research work except over an extended period of time.

Colony growth in agar of cells from human solid tumours has occasionally been described. McAllister and Reed [1968] grew agar colonies from trypsin-dispersed cell suspensions of 9 out of 17 paediatric tumours studied. The original specimens included 3 Wilm's tumours, 2 ependymomas, 2 sarcomas, 1 glioma and 1 embryonal carcinoma. Eagle's medium with 10% foetal calf serum and agar was used for colony culture without the addition of "feeder" cells or conditioned culture medium. Plating efficiency was very low, ranging from one to 133 colonies per $10^6$ cells plated out, after a 20 day incubation period. The authors claimed that the histological characteristics in the colonies reflected the features of cells from the original tumours. No tumour induction was achieved by colony implantation into adolescent hamsters, although this seems scarcely surprising in view of the immunological affront of heterologous tissue to the recipient animal. Cell suspensions plated out in this study from 5 normal foetal tissues all failed to grow colonies. Subsequently, the same group [McAllister et al, 1969] grew agar colonies from 2 cell lines originally derived from an embryonal rhabdosarcoma; the pitfalls in assuming that a tumour derived cell line maintains the characteristics of the original tumour cells are notorious but the authors described cytological, histochemical, electron micrographic and chromosomal evidence compatable with both the cell line and the derived colonies resembling the original tumour cells.

More recently, cells from a malignant pleural effusion in a child with rhabdosarcoma grew irregularly shaped colonies of 50 to 200 cells after 7 days incubation in both agar and methyl cellulose [Altman et al, 1975]. A problem inherent in the type of experiment is that inflammatory and immune reactive cells might reasonably be anticipated in such an effusion and might themselves give rise to colonies. In this case however, the authors described electron-micrograph features of the colony cells which they claimed were characteristic of rhabdosarcoma cells in the original tumour. Considering the general interest in developing colony-forming cell assays in general over the last 20 years, there appears to be surprising few other reports in the literature on attempts to grow colonies from human tumours. To some extent this reflects the technical difficulties involved, but there is also a major practical problem which has presumably suppressed interest in what would otherwise be a most useful investigative tool. The development of an assay for human tumour cells would require serial tumour samples over an extended period of time, first to standardise the method and then to run the assay for the agents under study. However, ethical considerations would nearly always rule out the possibility of repeated biopsies of an untreated tumour in a patient, over a prolonged period.

For this reason the development of human tumours which could be propagated as xenografts in immune-suppressed mice was a most interesting step forward and provided the opportunity for the

repeated tumour sampling necessary to develop a colony cell survival assay.

Work on the development of an in vitro agar assay for human tumour xenografts similar to that described above for animal tumours and for human haemopoetic cells is being carried out by my colleague Mrs. V. D. Courtenay in the same laboratory as myself, and preliminary results on this technique have been published [Courtenay and Mills, 1976]. This assay has proved effective, but requires extremely stringent and rigorous culture requirements. Furthermore it is difficult to study the effects of drugs on colony-forming cells in vitro; problems arise in trying to reproduce the biochemical milieu for in vivo metabolism in the artificial culture medium of the assay system.

For these reasons the possibility was raised of developing a second, parallel assay system based on a recently devised technique for assaying haemopoetic precursor cells growing in vivo in diffusion chambers implanted into murine peritoneal cavities.

# CHAPTER FOUR

# THE AGAR IN DIFFUSION CHAMBER ASSAY

# CHAPTER 4:

## THE AGAR IN DIFFUSION CHAMBER ASSAY

The modern diffusion chamber consists of a small disc-shaped perspex container one or both sides of which have been replaced by a millipore filter of a pore size designed to allow the transport of large molecules but not cells (Plate 4.1). Implantation of a cell-filled chamber into the peritoneal cavity of a mouse thus allows the continuous diffusion of vital nutrients and metabolites, but not host reactive cells, across the filter in a physiological milieu maintained relatively constant by the mouse; thus the need for external maintenance of temperature, humidity, pH and fresh culture medium inherent in in vitro techniques is obviated. Details of the design of the chambers used in my research are described in the next chapter.

The diffusion chamber technique is not a new one however, and appears to have been invented by Metschnikoff [1887] who needed a method to separate the effects of phagocytes from those of humoral factors. For this purpose he developed thin-walled tubes from the marrow of the reed Phragmites communis as diffusion chambers. Later Nocaul and Roux [1898] used the technique to grow microbes, including mycoplasma, which failed to grow well in vitro; these researchers introduced microbe-containing nutrient media into bags made from porous colloiden which were then implanted into experimental animals. Using the same type of colloiden bag, Bisceglie [1934] was the first to study tumour growth by the diffusion chamber

PLATE 4.1

0.22$\mu$m Millipore Filter Diffusion Chambers. Details of manufacture described in Chapter 5.4.

technique and the method duly became recognised as being of value in the culture of cells and tissues which otherwise grew poorly *in vitro*.

The modern perspex and millipore-filter diffusion chamber described above was developed by Algire *et al* [1954] to prove the importance of cellular immunity in transplantation rejection phenomena. More recently intraperitoneal diffusion chambers have been used to study haemopoietic stem cell growth and differentiation [Boyum and Borgstrum, 1970; Brievik *et al*, 1971; Rothstein *et al*, 1971]. However, these measurements could initially only be made by comparing total and differential cell counts before and after chanber implantation [Boyum *et al*, 1972; Tyler *et al*, 1972]; colony assays akin to those carried out with *in vitro* techniques were not attempted. The proliferation kinetics of experimental animal tumour cells have also been studied in diffusion chambers [Schumann *et al*, 1975]. Here changes in kinetic parameters were measured by pulse-cytophotometry, and again no attempt was made to develop a colony assay system.

The development of the diffusion chamber technique for studies on colony growth was achieved by Gordon [1974] who conceived the elegantly simple idea of introducing agar into the cell suspension culture medium before loading into chambers. Thus a semi-soft agar colony-forming cell culture, previously incubated *in vitro* in Petri dishes, could now be maintained under more physiological conditions in a mouse peritoneal cavity. Murine

marrow cells were found to grow colonies in this system, and these were morphologically identical with colonies produced in the in vitro method but without the need for colony stimulating factor and the standardisation problems so involved. Later the technique was also shown to grow colonies from human marrow cells [Gordon et al, 1975]; here however, pre-treatment of chamber-bearing mice with whole-body irradiation was required for colony growth. This rather curious prerequisite subsequently proved necessary in my research, and is discussed in detail in Chapter 5.

Using the agar in diffusion chamber technique, cell survival assays for the effect of irradiation and cytotoxic drugs on murine and human haemopoietic precursor cells have been carried out [Gordon, 1975; Gordon and Douglas, 1976], and give the same results as those obtained from in vitro assay techniques [Gordon, 1975]. Apart from eliminating the need for colony stimulating factor when studying marrow cells, the agar in diffusion chamber assay has one other important technical advantage over in vitro assays already mentioned: it is possible, by injecting the host mouse after chamber implantation, to study the effect of cytotoxic drugs on individual cells under physiological conditions which allow normal drug metabolism to proceed. Similar in vitro studies are less satisfactory in that artificial manipulation of the biochemical milieu, for example by the addition of liver microsomal extracts, is necessary to mimic physiological condition for drug metabolism.

These encouraging results with colony-forming cells from human bone marrow, and the inherent advantages of the system over in vitro techniques, held out the hope that it might be possible to develop the agar in diffusion chamber technique for growing colony-forming cells from human tumour xenografts. This would allow for the first time the effects of radiation and cytotoxic drugs on clonogenic human tumour cells to be assayed directly and would do away with the necessity to extrapolate from experimental animal tumours or tumour cell lines. Subsequent chapters in this thesis describe the development of such a technique and of the results so obtained.

# CHAPTER FIVE

## THE DEVELOPMENT OF A CLONOGENIC CELL ASSAY FOR HUMAN TUMOUR XENOGRAFTS USING AGAR IN DIFFUSION CHAMBERS

# CHAPTER 5:

# THE DEVELOPMENT OF A CLONOGENIC CELL ASSAY FOR HUMAN TUMOUR XENOGRAFTS USING AGAR IN DIFFUSION CHAMBERS

## 5.1: THE ORIGIN OF THE HUMAN TUMOUR XENOGRAFTS USED IN THESE STUDIES

Eleven human tumour xenografts were used in these studies, and their origins are listed in Table 5.1. They include an anaplastic pancreatic carcinoma (HX32); four colonic carcinomas, three of which were poorly differentiated (HX18, HXUR5, HXK9) and one moderately differentiated (HXK1); two rectal carcinomas, one of which was poorly differentiated (HXK4) and one well differentiated (HX12); two oat cell carcinomas of lung (HX29 and HX33); one melanoma (HX34) and one anaplastic carcinoma of the uterus (HX35).

Four of the biopsies from which the xenografts were established came from the primary tumour, and the other seven from metastatic deposits, as described in the Table.

The original tumour types were clearly established on clinical and histological grounds, with one important exception. This was the anaplastic pancreatic carcinoma (HX32) widely used in experiments to be described in this Thesis. This tumour was obtained at laparatomy from a 34-year old man presenting with widespread peritoneal metastases which in the opinion of the surgeon originated from the pancreas. Histological specimens showed a poorly differentiated tumour, probably an adeno-carcinoma,

| Code No. | Tumour | Cell Suspension Technique |
|---|---|---|
| HX32 | Pancreatic carcinoma, anaplastic* | Collagenase + Trypsin |
| HX18 | Colonic adenocarcinoma, poorly differentiated | Trypsin |
| HXUR5 | Colonic carcinoma, anaplastic | Trypsin |
| HXK1 | Colonic adenocarcinoma, mod. differentiated* | Trypsin |
| HXK9 | Colonic adenocarcinoma, poorly differentiated* | Trypsin |
| HX12 | Rectal adenocarcinoma, well differentiated | Trypsin |
| HXK4 | Rectal adenocarcinoma, poorly differentiated | Trypsin |
| HX29 | Oat cell carcinoma of lung* | PBS** |
| HX33 | Oat cell carcinoma of lung* | PBS |
| HX34 | Melanoma*** * | PBS |
| HX35 | Uterine carcinoma, anaplastic* | PBS |

*Metastasis Biopsy **PBS - Phosphate-Buffered Saline ***After Short Term Monolayer Culture (Chapter 5.2)

TABLE 5.1

The Origins, Histology and Techniques for Preparing Single Cell Suspensions of 11 Human Tumour Xenografts

compatible with a pancreatic origin but not diagnostic of this. The patient died soon after the operation and unfortunately no post mortem was performed to add to this information. Throughout this thesis, therefore, the HX32 xenograft will be referred to as a pancreatic carcinoma, but implicit in this is the reservation that this was merely the most likely diagnosis.

## 5.2: XENOGRAFT IMPLANTATION

These xenograft tumours were established during the last 3 years by Mr. R. Pickard, F.R.C.S., Mr. K. Novak, F.R.C.S. and Mr. J. Gibb working in the same laboratory as myself, and to them I express my gratitude for this painstaking work which allowed my own studies to proceed. Details of their technique have been described elsewhere [Pickard et al, 1975], but the method used, including some subsequent modifications, can be summarised as follows: tumour biopsy specimens obtained at operation were finely chopped and washed in medium TC199. In early experiments small pieces of about 8-10mm$^3$ were implanted subcutaneously into the flanks of immune-suppressed syngeneic CBA/lac mice (see below) (Plate 5.1). Later, it was found that the injection of a "mush" of tumour cells injected by 19 guage needle intramuscularly into the thighs of the mice was an easier and more efficient way of producing tumours.

Both male and female CBA/lac mice were used for growth and propagation of the xenografts. They were immune suppressed by thymectomy at 3 to 4 weeks of age and 2 to 4 weeks later treated

PLATE 5.1

Bilateral Human Pancreatic Carcinoma (HX32) Xenografts Growing Subcutaneously in the Flanks of Immune-Suppressed CBA/lac Mouse

with 900 rad whole body irradiation with "rescue" by intravenous injection of syngeneic bone marrow cells within 6 hours of irradiation. Tumour implantation was carried out approximately 3 weeks later.

One xenograft, melanoma HX34, was prepared in a slightly different way. The original biopsy specimen was considered too small for xenografting, and a cell suspension was therefore initially grown in monolayer culture. Cell numbers were built up by subculturing, and after a six week period cells were harvested and implanted by injection into the thighs of host mice as described for other xenografts. It would ideally have been better not to have had to resort to culture techniques at all, as with the other 10 xenografts. There was good evidence however to suggest that melanoma cells had been maintained in this short term culture and had not been overgrown by non-malignant or contaminant cells: tumours grew and could be passaged in the mice after implantation; these were characterised as melanomas by their dark pigmentation and by the demonstration of melanosomes in electron micrograph sections of tumour cells [Dr. K. Dawson, personal communication]. This and other evidence confirming the human origin of the xenografts will be described in more detail in Chapter 6.

## 5.3: CELL SUSPENSIONS OF TUMOUR XENOGRAFTS

Tumours excised from mice freshly killed by neck dislocation were washed and finely chopped in phosphate-buffered saline (PBS)

under sterile conditions using a crossed-scalpel technique. Further treatment to produce a viable single cell suspension was then determined empirically for each tumour and details are shown in Table 5.1. The preparation of single cell suspensions of 4 tumours used in experiments to be discussed in subsequent chapters will be described here. These illustrate the different types of treatment required.

5.3.a: Pancreatic Carcinoma (HX32)

The finely chopped pieces of this tumour were incubated in collagenase (Worthington) 2mg/ml in Ham's F12 medium with 15% special Bobby calf serum (Gibco Biocult) at 37°C for 30 minutes in Falcon tubes. The material was then rinsed twice in PBS and trypsinised for 5 minutes at 37°C with 0.05% trypsin (Bacto trypsin Difco). The trypsin-in-PBS supernatant was then decanted off, the cellular material incubated for a further 5 minutes at 37°C in Ham's F12 medium without trypsin and a cell suspension produced in the supernatant layer by three or four vigorous shakes of the tube. This supernatant was then added to 15% special Bobby calf serum to inactivate any residual trypsin activity, the cells were re-suspended in fresh medium and serum after gentle centrifugation for 2 minutes, and the suspension was finally filtered through a 30$\mu$m polyester mesh (Henry Simon, Stockport) to remove clumps and debris. The single cell suspension so obtained was kept at 4°C.

5.3.b: Colonic Carcinoma (HX18)

For this tumour, incubation with collagenase was found to be unnecessary, and the finely chopped tumour material was incubated

with trypsin 0.05% in PBS for 5 minutes. The supernatant was then decanted off, the cellular material incubated for a further 5 minutes at 37°C in Ham's F12 medium without trypsin, and again a cell suspension produced in the supernatant layer by three or four vigorous shakes of the tube. The final single cell suspension was then achieved by the same steps as described for pancreatic carcinoma HX32.

5.3.c: Oat Cell Carcinoma (HX33)

A single cell suspension of this tumour was obtained without the need for either trypsin or collagenase. Finely chopped pieces of tumour were incubated in PBS for 10 minutes at 37°C, and the tube then vigorously shaken 3 or 4 times to produce a cell suspension in the supernatant layer. Cells were then re-suspended in Ham's medium and serum and filtered as described for the previous two xenografts.

5.3.d: Melanoma (HX34)

The same technique was used to produce a single cell suspension of this tumour as for oat cell carcinoma HX33.

Viability for suspension of all tumours was assessed by dye exclusion using lissamine green, and a viable cell count was made using a haemocytometer. Viability of single cells in suspension maintained at 4°C remained fairly constant for a period of at least 4 hours, assessed both by dye exclusion and by the fraction of the original cell count growing colonies in agar. Exceptions to this general observation were the 2 oat cell carcinomas of lung, the small tumour cells of which began to lose the ability to exclude dye after 45 minutes in suspension. These tumours were therefore correspondingly more

difficult to study, and cells from these were always loaded into chambers within less than an hour of preparing the initial suspension.

## 5.4: MANUFACTURE, FILLING AND IMPLANTATION OF DIFFUSION CHAMBERS

Diffusion chambers were made by attaching a Millipore filter (pore size 0.22$\mu$m) to one side of an acrylic ring (inside diameter 12.5mm; wall thickness 2.5mm; depth 3.5mm) by the solvent action of acetone. A circular glass coverslip was glued to the other side of the ring with MF cement (Millipore Corporation, Bedford, Massachusetts; Catalogue No. XX.70.000.00), completing a chamber whose volume was approximately 0.4ml (Plate 4.1). Completed chambers were sterilised before use in dry heat at 80$^{o}$C for 18 hours.

Molten agar, held at 44$^{o}$C in a water bath, was added to the required number of cells for assay suspended in Ham's medium and serum at 4$^{o}$C to produce a final agar concentration of 0.3%. (The ratio of cell suspension at 4$^{o}$C to agar at 44$^{o}$C was 9:1 and therefore there was no possibility of overheating of cells in the final mixture.) 0.35ml of this mixture was injected into each chamber through a small hole in the acrylic ring, and allowed to set at room temperature. The holes were then sealed with paraffin wax and the chambers implanted into the peritoneal cavity of 11-12 week old male C57Bl mice, under ether anaesthesis.

A diagram of the complete experimental technique is shown in Figure 5.1.

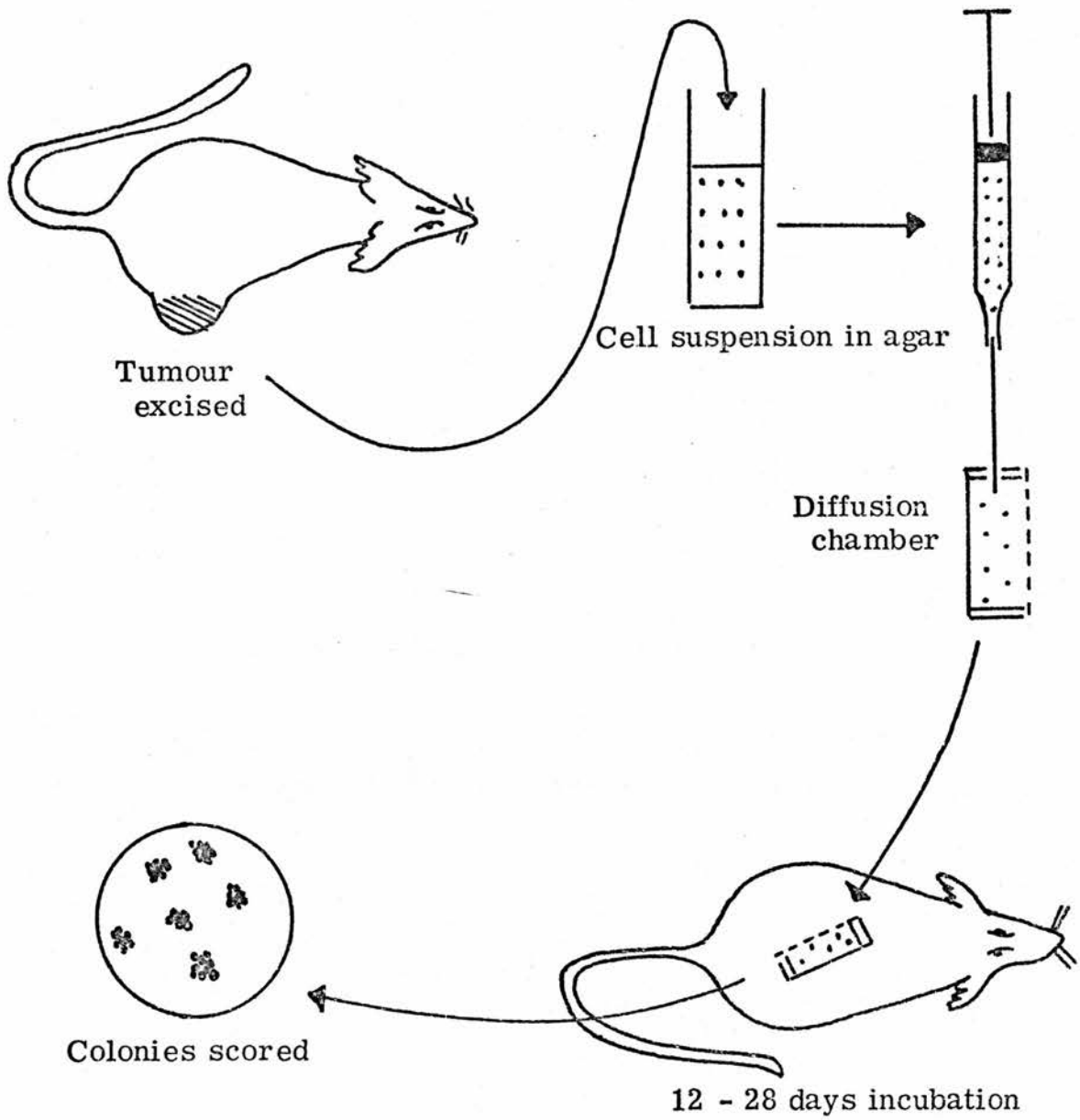



FIGURE 5.1

Agar in Diffusion Chamber Assay Technique for Clonogenic Human Tumour Cells

## 5.5: COLONY COUNTING AND DEFINITIONS OF "COLONIES" AND "CLUSTERS"

Chamber-bearing mice were killed by cervical dislocation, and the Millipore filter of each chamber removed with a scalpel blade; colonies were then counted in the chamber under a binocular microscope at a magnification of x50. Colonies were defined as aggregates of at least 50 cells; clusters were defined as aggregates of between 20 and 50 cells. The optimum incubation period for colony formation was determined by colony counting at various times after implantation until there was no further increase in colony numbers (Chapter 5.10).

## 5.6: COLONY GROWTH FROM CELL SUSPENSIONS OF HUMAN TUMOUR XENOGRAFTS

Using this technique, cell suspensions of all 11 tumours studied reproducibly grew agar colonies in diffusion chambers. However, an essential pre-requisite for successful colony growth was pre-treatment of the chamber-bearing C57Bl mice; little or no colony growth from any of the xenografts was achieved in untreated mice. This point is of great importance and will be discussed in detail in a later section of this chapter (Chapter 5.12).

## 5.7: COLONY MORPHOLOGY

Interestingly, there was considerable variation in colony morphology between different tumours. Most produced colonies composed of rather loosely clumped cells (Plate 5.2), while a few,

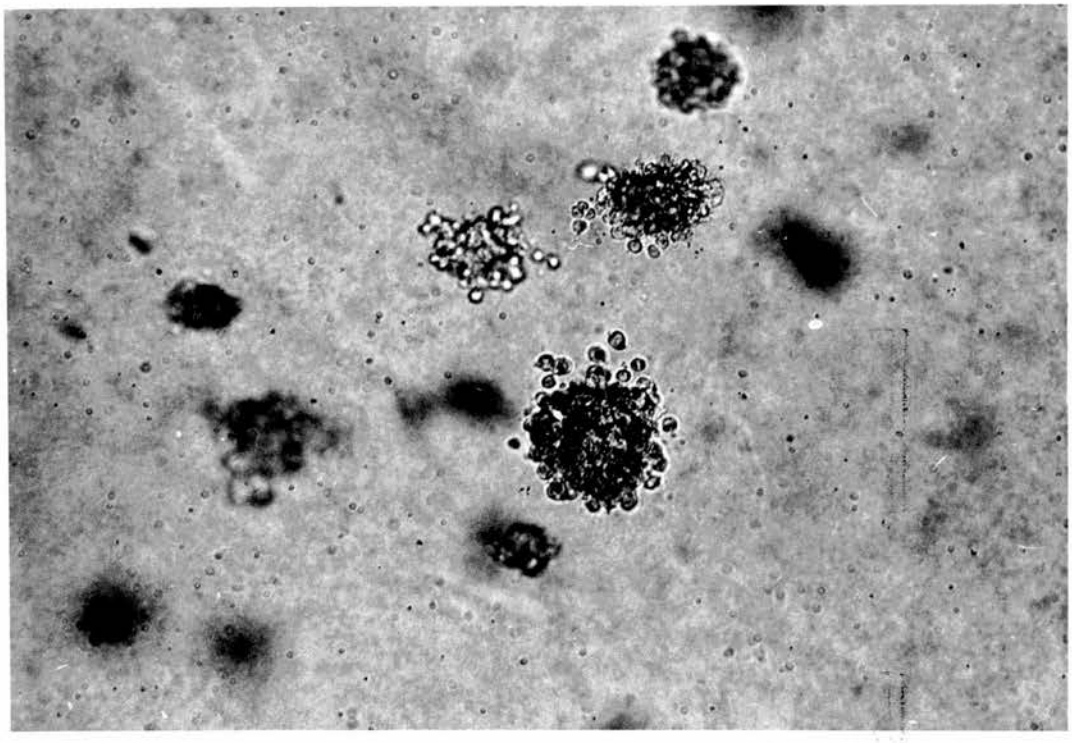


PLATE 5.2

Agar Colonies from Cells of Pancreatic Carcinoma Xenograft HX32 (x 100)

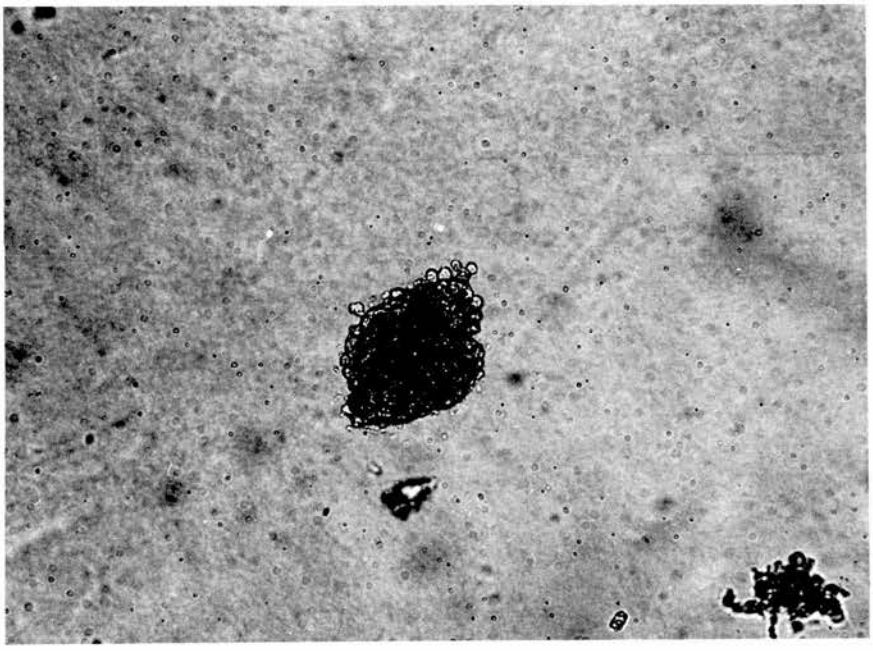


PLATE 5.3

Agar Colony from Cells of Melanoma Xenograft HX34 (x 100)

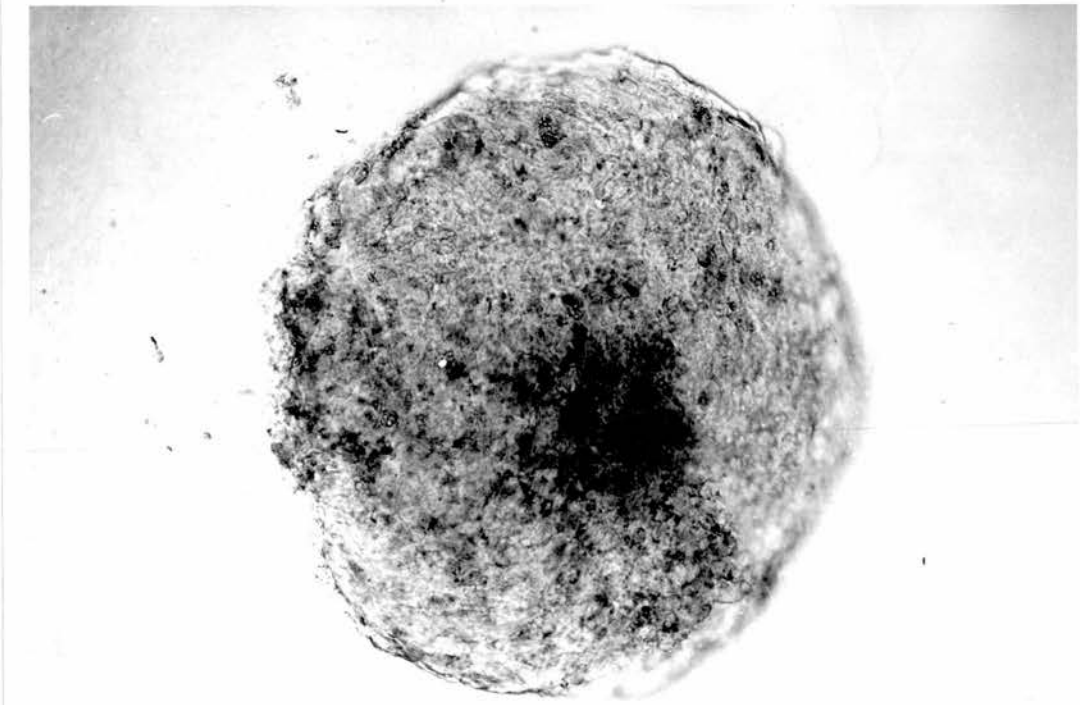


PLATE 5.4

Agar Colony from Cells of Colonic Carcinoma Xenograft HX18 (x 100)

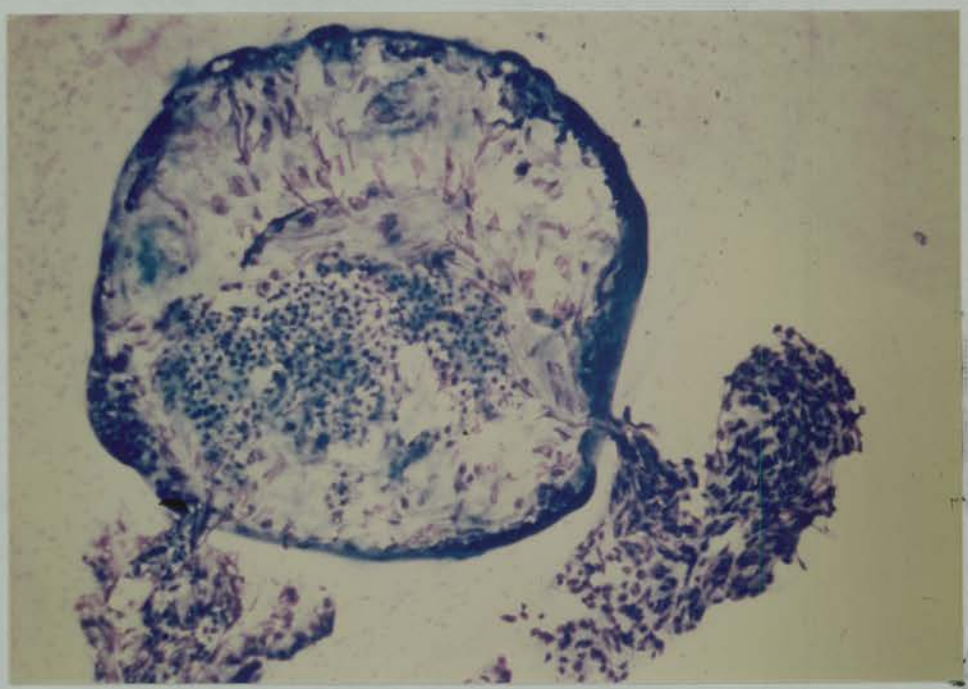


PLATE 5.5

Giemsa-Stained Preparation of Colonic Carcinoma Xenograft HX18 Colony Removed from Agar, Showing Mucinous Capsule "Leaking" Cells at 2 Sites

including melanoma HX34 and uterine carcinoma HX35 produced more densely packed colonies (Plate 5.3). One tumour, colonic carcinoma HX18, produced colonies in the form of tightly packed encapsulated spheres (Plate 5.4). Giemsa staining of one such colony removed from agar showed an extraordinary mucinous capsule surrounding the tumour cells (Plate 5.5). (The technique used here is described in Chapter 6.) The morphology of colonies from each individual tumour remained absolutely constant in different experiments and with different passages.

## 5.8: LINEARITY

A few of these xenografts, and in particular pancreatic carcinoma HX32 and colonic carcinoma HX18, were used in experiments to be described in later chapters where the effects of irradiation and cytotoxic drugs on colony cell survival were studied. To establish the validity of the technique as an assay for this purpose, the relationship between the number of tumour cells introduced into the diffusion chambers and the subsequent yield of colonies was investigated. A linear relationship was demonstrated for pancreatic carcinoma HX32 over a range of $0.1 \times 10^3$ to $1.75 \times 10^3$ cells per chamber (Fig. 5.2) and for colonic tumour HX18 over a range of $0.5 \times 10^3$ to $4.5 \times 10^3$ cells per chamber (Fig. 5.3). The diffusion chamber technique could therefore be used as a valid assay for the effects of irradiation and drugs on the survival of clonogenic human tumour cells, at least over these ranges.

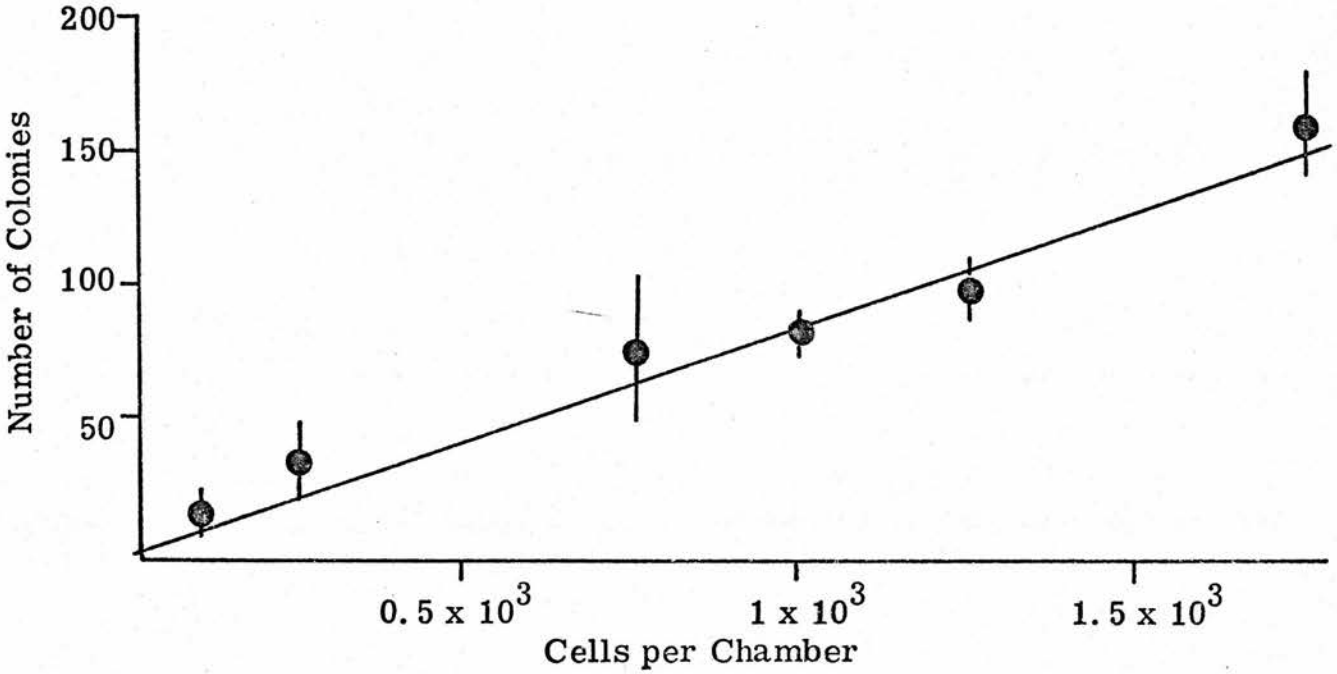



FIGURE 5.2

The Relationship Between the Number of Cells Cultured in Agar in Diffusion Chambers and the Number of Colonies Scored, for Human Pancreatic Carcinoma Xenograft HX32 (Vertical Bars Represent ± Standard Error of the Mean)

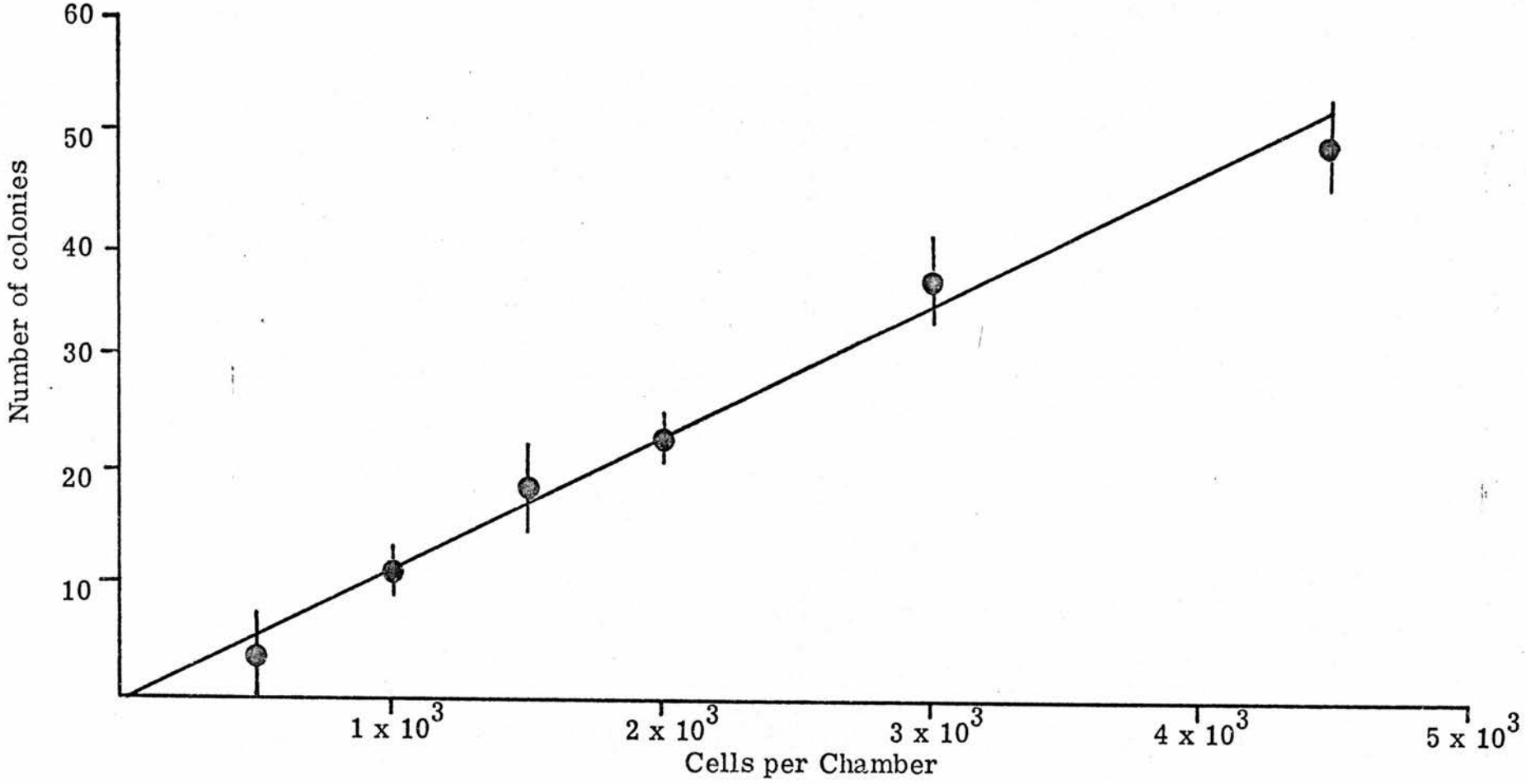



FIGURE 5.3 Human Colonic Carcinoma Xenograft HX18

The Relationship Between the Number of Cells Cultured in Agar in Diffusion Chambers and the Number of Colonies Scored (Vertical Bars Represent ± Standard Error of the Mean)

## 5.9: INCUBATION PERIOD

Details of the incubation period for colony growth for each xenograft are given in Table 5.2. This ranged from only 12 days for the melanoma HX34, and the anaplastic uterine carcinoma HX35 to 28 days for the well differentiated rectal carcinoma HX12 and a moderately well differentiated colonic carcinoma HXK1. Most tumours had an incubation period of about 21 days, with a tendency for colonies from the less differentiated tumours to grow more rapidly than those from well differentiated ones. Despite the range of incubation times between different tumours, this remained generally constant for each tumour type in different passages and experiments.

## 5.10: THE GROWTH OF CLUSTERS AND COLONIES

Clusters (20-50 cells) and colonies (greater than 50 cells) from the pancreatic tumour xenograft HX32 were counted at various times after chamber implantation (Fig. 5.4). Initially, many more clusters than colonies were seen, but the cluster count decreased as colony count increased showing that most clusters continued growing to form colonies. A few colonies began to appear around the ninth day, after which the number of colonies rapidly increased until about the 18th day and then plateaued at about 130 colonies per 1000 cells plated. About 70 clusters per 1000 cells plated persisted once the colony count had levelled out, giving a final colony to cluster ratio of approximately 2:1. This illustrates that not all clusters

| Code No. | Tumour | Incubation Period (Days) |
|---|---|---|
| HX32 | Pancreatic carcinoma, anaplastic | 18 |
| HX18 | Colonic adenocarcinoma, poorly differentiated | 21 |
| HXUR5 | Colonic carcinoma, anaplastic | 21 |
| HXK1 | Colonic adenocarcinoma, mod. differentiated | 28 |
| HXK9 | Colonic adenocarcinoma, poorly differentiated | 21 |
| HX12 | Rectacl adenocarcinoma, well differentiated | 28 |
| HXK4 | Rectal adenocarcinoma, poorly differentiated | 21 |
| HX29 | Oat cell carcinoma of lung | 21 |
| HX33 | Oat cell carcinoma of lung | 18 |
| HX34 | Melanoma | 12 |
| HX35 | Uterine carcinoma, anaplastic | 12 |

TABLE 5.2

Incubation Periods for Colony Growth of Human Tumour Xenografts

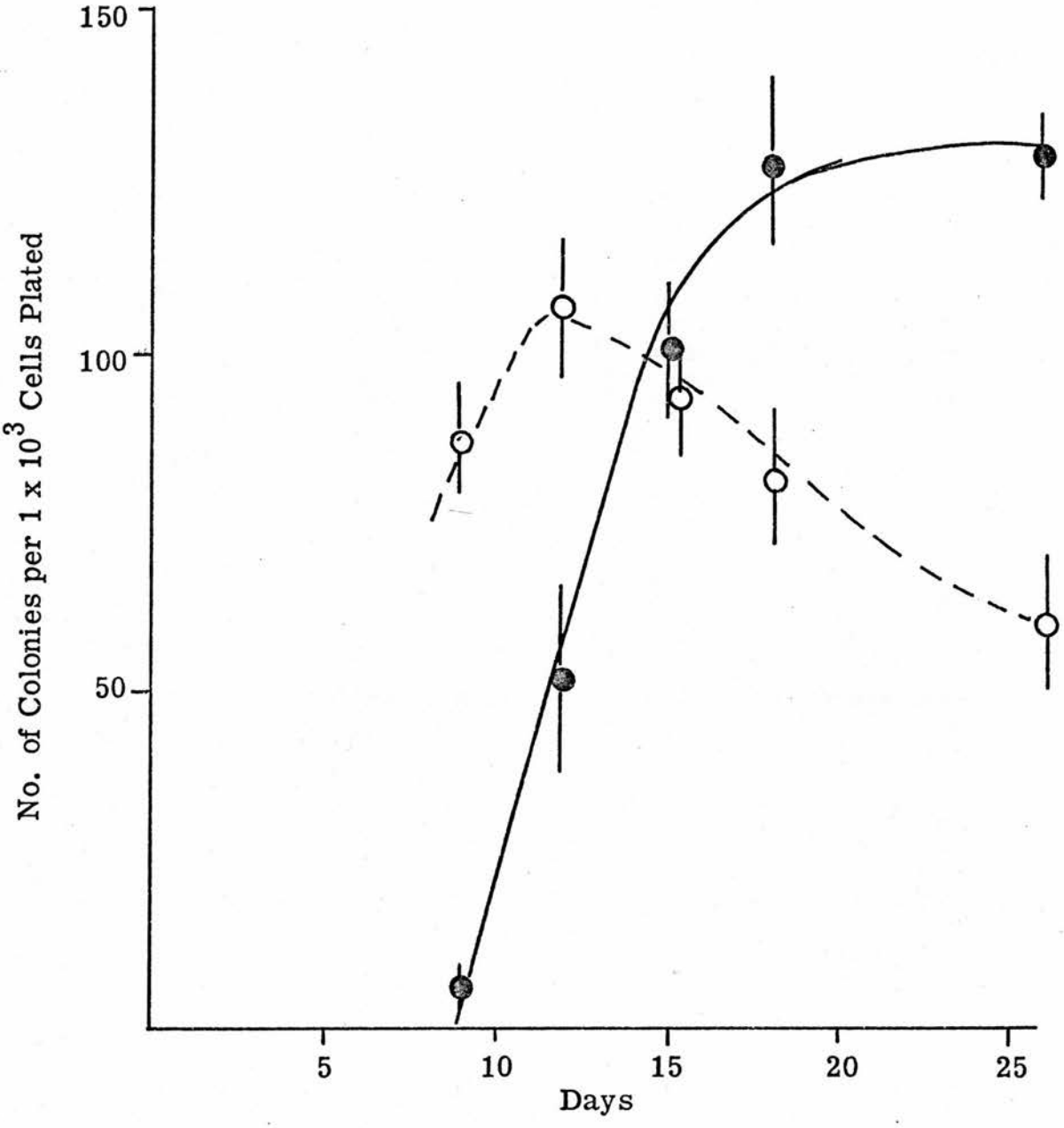



FIGURE 5.4

The Development of Clusters (○) and Colonies (●) with Time for Cells From Pancreatic Carcinoma Xenograft HX32 (Vertical Bars Represent ± Standard Error of the Mean)

become colonies; some plated cells appear to have potential for a limited number of divisions but are incapable of forming colonies of 50 cells or more. This observation becomes important in later chapters in which the effects of radiation and cytotoxic agents on colony growth were studied.

## 5.11: PLATING EFFICIENCY

The mean plating efficiency of each tumour was fairly constant, although the range between different tumours varied from 0.3% for one of the oat cell lung tumours (and 1.5% for the other) to 16% for the melanoma. Details of the plating efficiency for each xenograft are given in Table 5.3. It was not possible to assess the extent to which this wide range reflected differences in the fraction of cells with proliferative capacity in different tumours in situ, or instead represented a laboratory artefact based on varying susceptibilities of different tumours to the trauma involved in producing cell suspensions. It was of interest that most, but not all, of the poorly differentiated and clinically aggressive tumours had a relatively high fraction of colony forming cells. On the other hand, the two oat cell carcinomas of lung, also in general clinically aggressive, had low plating efficiencies. These may have been associated with the unusually rapid loss of viability in single cell suspensions of these tumours (Chapter 5.3), giving an artificially high count of viable cells introduced into the chambers.

| Code No. | Tumour | Mean Plating Efficiency | Range for Individual Experiments |
|---|---|---|---|
| HX32 | Pancreatic carcinoma, anaplastic | 11.0% | 9.0 - 14.0% |
| HX18 | Colonic adenocarcinoma, poorly differentiated | 1.9% | 0.9 - 2.4% |
| HXUR5 | Colonic carcinoma, anaplastic | 1.1% | 0.9 - 1.2% |
| HXK1 | Colonic adenocarcinoma, mod. differentiated | 0.5% | 0.4 - 0.5% |
| HXK9 | Colonic adenocarcinoma, poorly differentiated | 3.5% | -* |
| HX12 | Rectal adenocarcinoma, well differentiated | 1.1% | -* |
| HXK4 | Rectal adenocarcinoma, poorly differentiated | 11.5% | 10.5 - 13.0% |
| HX29 | Oat cell carcinoma of lung | 1.5% | 1.0 - 1.6% |
| HX33 | Oat cell carcinoma of lung | 0.3% | 0.25 - 0.4% |
| HX34 | Melanoma | 16.0% | 15.0 - 18.0% |
| HX35 | Uterine carcinoma, anaplastic | 14.0% | 12.0 - 16.0% |

*One Experiment Only

TABLE 5.3

Mean Plating Efficiencies for Cell Suspensions from Human Tumour Xenografts

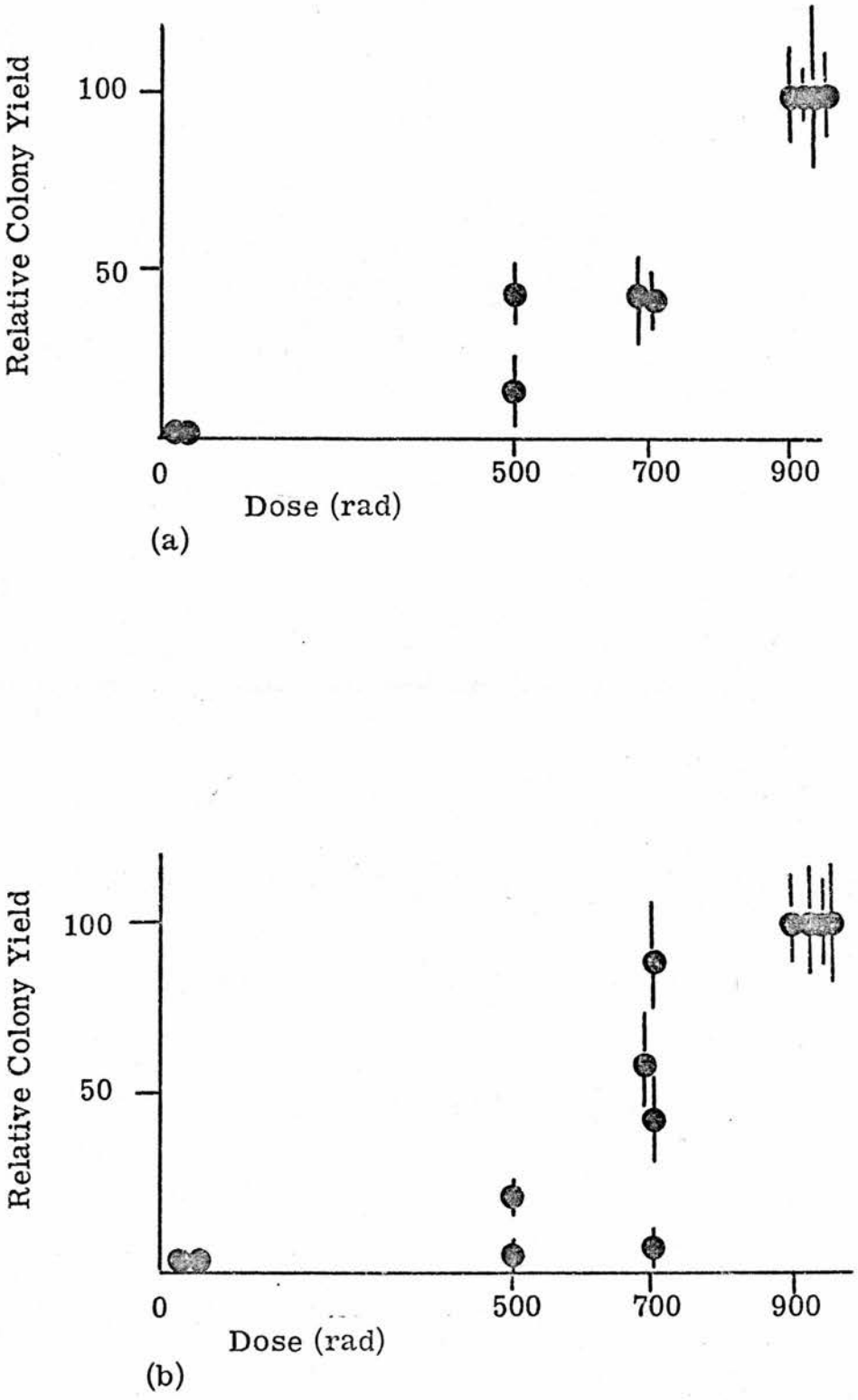



FIGURE 5.5

The Influence of Pretreatment Whole-Body $^{60}$Co $\gamma$-Irradiation on Plating Efficiency for Colonic Carcinoma HX18 (a) and Pancreatic Carcinoma HX32 (b). The Results for Each Pretreatment are Expressed as a Percentage of the Yield Achieved Using 900 rad-Irradiated Mice. (Vertical Bars Represent $\pm$ Standard Error of the Mean)

## 5.12: PRE-TREATMENT OF CHAMBER-BEARING MICE AS A PRE-REQUISITE FOR COLONY GROWTH

The ability of this system to grow colonies of tumour cells, and the plating efficiency for each tumour, was critically dependent on pre-treatment of the chamber-bearing C57Bl mice, and little or no colony growth was seen in untreated animals from any tumours. This observation had previously been made for human bone marrow cell colonies grown in agar in diffusion chambers [Gordon et al, 1975], and it is of interest that Schumann et al [1975] were unsuccessful in growing human tumour cells for proliferation kinetic studies in diffusion chambers implanted into untreated animals. The effect of different types of pre-treatment was studied in detail for 2 xenografts, the pancreatic carcinoma HX32 and colonic carcinoma HX18 and results are shown in Figures 5.5 to 5.8.

## 5.13: WHOLE BODY IRRADIATION

The first type of pre-treatment investigated was whole body irradiation. This was given to mice 3 hours before chamber implantation from a $^{60}$Cobalt source at a rate of around 50 rad per minute. For this purpose, the mice were restrained in position in perspex chambers.

It will be seen from Figures 5.5a and 5.5b that the plating efficiency increased for both tumours as the dose of irradiation was increased up to a lethal dose of 900 rads. Gordon et al [1975] had previously observed the same effect using this technique for human

marrow. 900 rad whole body irradiation produces fatal bone marrow toxicity in mice around the ninth day after its administration. This is unimportant in human bone marrow experiments where colonies grow in eight days (ibid), but it presented serious problems in human tumour studies which required much longer incubation periods for successful colony formation. Initially the only solution was to transplant chambers to fresh batches of newly irradiated mice every ninth day, despite the drain on both mice and time this involved.

## 5.14: CYTOSINE ARABINOSIDE AND WHOLE BODY IRRADIATION

However, it had been found by Dr. John Millar, working in the same laboratory but on a different project, that death after 900 rad and even 1000 rad whole body irradiation to C57Bl mice could be prevented by prior treatment with cytosine arabinoside in a dose of 200mg/kg injected intra-peritoneally 1 to 3 days before incubation [Millar et al, 1976]. This in itself was an extraordinary finding with possible long term therapeutic implications, and the underlying mechanism is being investigated at present. Clearly this is outside the scope of this thesis, but the observation itself suggested a technique whereby chambers could be implanted into mice pre-treated with cytosine arabinoside 24 to 48 hours before 1000 rad whole body irradiation and thus be maintained for the whole incubation period without the need for further transplantation. The question posed was whether colonies would grow with the same plating efficiency in this system as in chambers transplanted every

ninth day into a new batch of 900 rad irradiated mice. The results of these experiments are shown in Figure 5.6 and demonstrate that for both tumours the plating efficiency was the same within the limits of experimental error whether the chambers remained in the same mice for the entire duration of the incubation period or were transplanted every ninth day into newly irradiated mice. The saving in mice and labour thus achieved resulted in the cytosine arabinoside pre-treatment technique being adopted as standard procedure for most of the experiments subsequently described in this thesis.

## 5.15: CYCLOPHOSPHAMIDE

Smith and van Dierendonk demonstrated that 200mg/kg cyclophosphamide given intraperitoneally was many times more effective than other pre-treatment schedules for promoting lung colony formation from C22LR mouse osteosarcoma [personal communication]. The simplicity of this regime suggested that it might be worth studying the effect of cyclophosphamide pre-treatment in the agar diffusion chamber system. However, cyclophosphamide 200mg/kg given intraperitoneally to C57Bl mice 24 hours before chamber implantation was ineffective, achieving a mean plating efficiency of only 5.5% (HX32) and 14% (HX18) of that reached with high dose whole body irradiation (Fig. 5.7). Cyclophosphamide pre-treatment was therefore not studied further, although possibly more successful results might

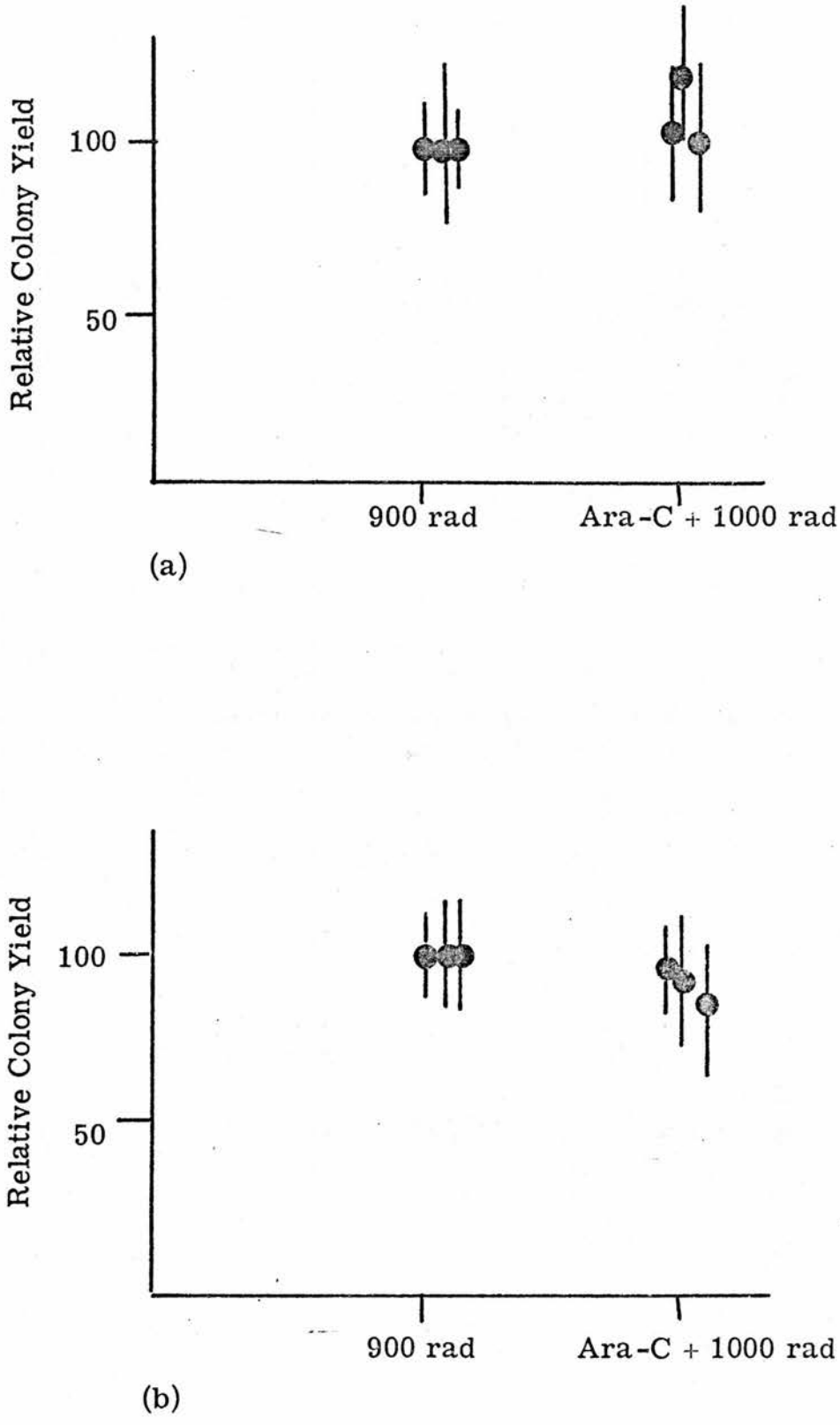



FIGURE 5.6

The Influence on Plating Efficiency of 900 rad Whole-Body Irradiation (Transplanting Chambers Every 9th Day) Compared with Cytosine Arabinoside (Ara-C) 200mg/kg i.p. 24 hours Before 1000 rad Whole-Body Irradiation (No Chamber Transplantation) for Colonic Carcinoma HX18 (a) and Pancreatic Carcinoma HX32 (b). Results Expressed as for Figure 5.5.

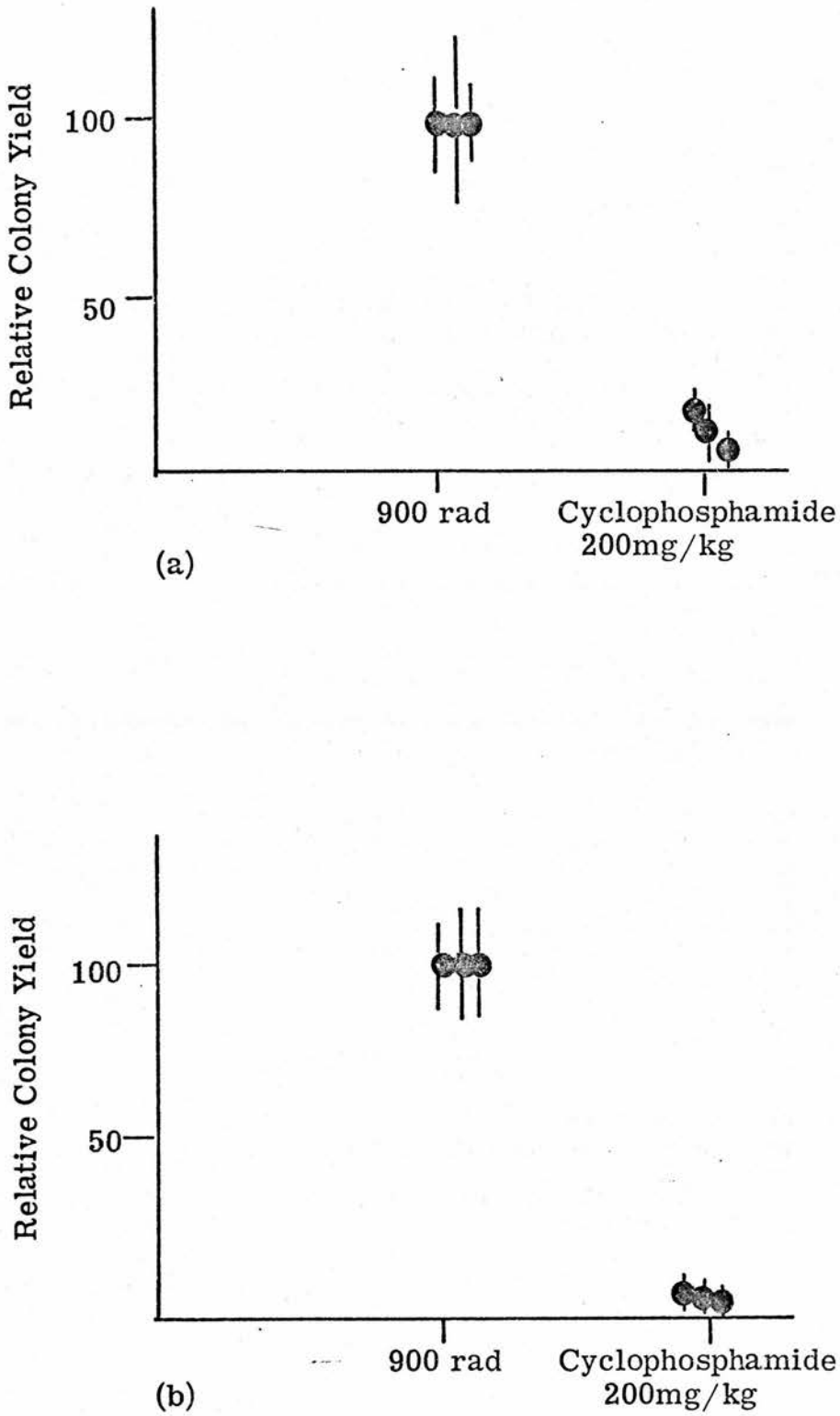



FIGURE 5.7

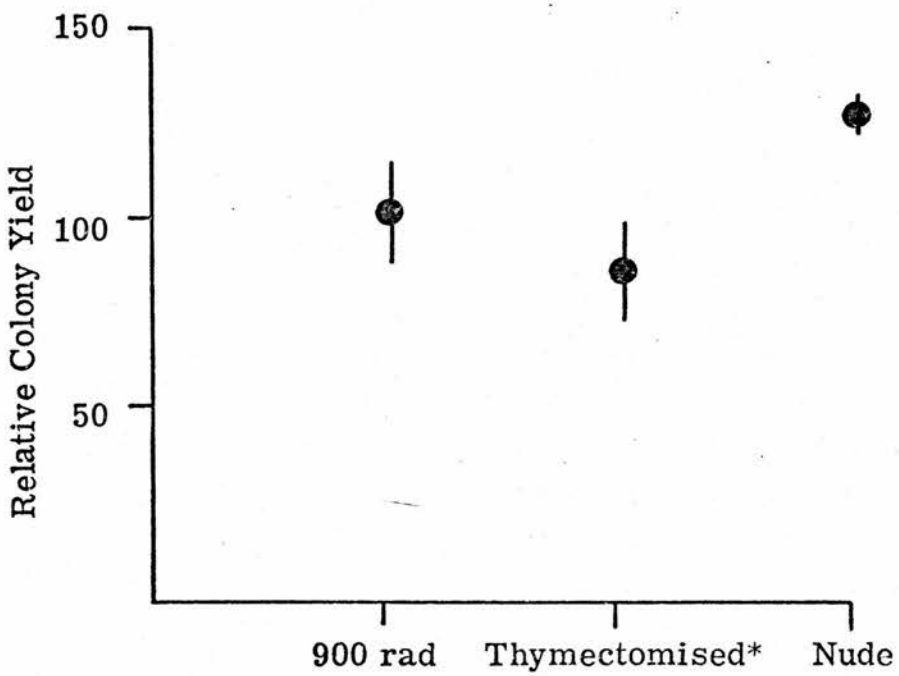
The Influence on Plating Efficiency of Pretreatment with 900 rad Whole-Body Irradiation Compared with Cyclophosphamide 200mg/kg i.p. for Colonic Carcinoma HX18 (a) and Pancreatic Carcinoma HX32 (b). Results Expressed as in Figure 5.5.

have been achieved by varying the time interval of administration before chamber implantation.

## 5.16: IMMUNE-SUPPRESSED AND "NUDE" MICE

Although the time, labour and expense in producing CBA/lac mice immune-suppressed by thymectomy, whole body irradiation and marrow re-constitution as previously described (Chapter 5.2) ruled these out as routine chamber-bearing hosts, chambers containing cells from pancreatic carcinoma HX32 were implanted into these animals without further pre-treatment in one study in an attempt to elucidate the mechanism of action of the pre-treatment phenomenon. In this environment, colonies grew almost as well as in whole body irradiated animals, with a plating efficiency of 83 $\pm$ 12% that of 900 rad-irradiated mice (Fig. 5.8).

An opportunity also became available during the early stages of my research to carry out a single experiment on the ability of genetically athymic "nude" mice to sustain colony growth of cells from pancreatic carcinoma xenograft HX32. Chambers were implanted into the peritoneal cavities of 5 untreated "nude" mice and incubated for 18 days as usual. Here, the plating efficiency was 125 $\pm$ 4% that achieved by the 900 rad pre-treatment technique (Fig. 5.8) and the range of colony counts from individual chambers was less than that for irradiated animals. This perhaps suggested a greater uniformity of effect between each animal than




*Chapter 5.2

FIGURE 5.8

A Comparison of the Influence on Plating Efficiency of Chambers Implanted into 900 rad Whole-Body Irradiated Mice, Thymectomised Whole-Body Irradiated Mice and Nude Mice for Pancreatic Carcinoma HX32. Results Expressed as in Figure 5.5.

between those artificially pre-treated in the laboratory. Clearly no statistical significance can be placed on a single experiment, however, and lack of further availability of "nude" mice at present has prevented the verificiation of these initial observations. In practice, cost would probably rule out the standard use of these animals as chamber-bearing hosts in any case, but the experiment was of interest in trying to elucidate the mechanisms of action which underlie the pre-treatment phenomenon.

## 5.17: WHY IS PRE-TREATMENT NECESSARY?

The reason for pre-treatment of chamber-bearing animals as an essential pre-requisite for colony growth is not at all clear. At first sight, suppression of a host immune response against the human tumour cells would seem to be operating, and there is some circumstantial evidence to support this. First pre-treatment is not required for syngeneic tissues: C57Bl Lewis lung tumour and marrow cells will grow colonies in this system without pre-treatment of chamber-bearing animals [Gordon, 1974; Personal observations]. Second, the observation that colony growth is promoted in animals experimentally or genetically depleted of T-lymphocytes favours an innumological basis for the phenomenon.

However, it is hard to reconcile this with the experimental design of the system: both the "affector" and "effector" arms of the T-lymphocyte specific immune response to foreign antigens are believed to involve direct interaction of host cells with antigen

[Basten and Mitchell, 1976a],], and yet the 0.22$\mu$m Millipore filter forms a complete barrier to cellular movement in and out of the diffusion chambers. Large molecules can of course diffuse across the filter and it could be postulated that "shed" tumour cell antigens diffuse out of the chamber and initiate a host response, but this seems unlikely since such antigens are currently believed to provide only a very weak antigenic stimulus [Alexander, 1974]. Likewise, the affector arm of the immune response might be solely medicated by immunoglobulins; again this would be unexpected in a system based primarily on T-cell deprivation provided by thymectomised or "nude" mice. If non-cellular immune mechanisms are operating here they would therefore appear to lie outwith current concepts of immunology. Indeed this diffusion chamber system might prove a useful technique for their further investigation.

Alternatively, a completely non-immunological process may be operating. It is theoretically possible that T-cell depletion, achieved by whatever mechanism, is associated with the production of some factor which is essential for colony growth of human tumour cells. This possibility is not so implausible as it might at first seem: it has already been established by Courtenay for example that human tumour colony formation in vitro demands very exacting growth requirements (Chapter 3.9) and the same may be true of the diffusion chamber system.

Much work remains to be done in elucidating the mechanism underlying the pre-treatment effect and this might provide important

new information on immune or other factors associated with tumour cell growth. For the purposes of this thesis, however, my approach has been merely to make the observation, without pursuing underlying mechanisms, and instead to use the system to study radiation and cytotoxic drug effects on the human tumour cell colonies so produced.

# CHAPTER SIX

## EVIDENCE THAT DIFFUSION CHAMBER COLONIES ORIGINATE FROM HUMAN TUMOUR CELLS

# CHAPTER 6:

# EVIDENCE THAT DIFFUSION CHAMBER COLONIES ORIGINATE FROM HUMAN TUMOUR CELLS

The most critical point underlying the entire basis of the diffusion chamber technique as a valid assay system is that the colonies grown by this method are indeed derived from human tumour cells and not from some other cell type. Several techniques were used to demonstrate this.

## 6.1: GENERAL CONSIDERATIONS

Circumstantial evidence is very much against non-malignant stromal cells from these tumours giving rise to colonies. Some normal tissues, and in particular fibroblasts, can of course be grown in monolayer cultures but such cells do not produce colonies in agar either in vitro [McAllister and Reed, 1968] or in diffusion chambers [personal observation with human foetal fibroblasts]. Furthermore one might expect the same stromal elements to be present for different xenograft tumours, albeit perhaps in different proportions, and it would therefore be surprising that these same stromal cells would produce colonies whose morphology differs so strikingly depending on the tumour under study (Plates 5.2, 5.3, 5.4). On purely circumstantial grounds therefore it seems unlikely that colonies in this system are derived from stromal cells.

A more plausible alternative candidate for colony formation from xenograft cell suspensions might be the macrophage. This cell has been demonstrated in both animal and human tumours [Evans, 1972; Currie, 1976] and may well play an important role in tumour rejection [Alexander, 1976; Lancet Editorial, 1976]. There appears to be no published data on the existence of mouse macrophages in human tumour xenografts, and it therefore seemed relevant to investigate this possibility and to exclude the macrophage as the basic colony-forming cell in the diffusion chamber system.

## 6.2: MACROPHAGES IN THE HX32 PANCREATIC CARCINOMA XENOGRAFT

Macrophages cannot always be confidently identified either by histological staining techniques or by direct microscopic observation in suspension. This therefore raised technical problems in studying their existence and possible colony-forming potential in xenograft cell suspensions. Fortuitously, however, cell suspensions of one xenograft, pancreatic carcinoma HX32, consistently demonstrated two distinct cell populations of different size, in approximately equal proportions. The possibility that one of these populations might consist of macrophages was investigated by two separate techniques.

In the first, the xenograft cell suspension was incubated at 37$^{o}$C for 10 minutes in a haemocytometer and then examined for macrophages which adhere to, and spread over, the haemocytometer

cover slip [Evans, 1972]. In the second, the cell suspension was centrifuged at 800 r.p.m. for 10 minutes with sheep red blood cells (RBCs) coated with the IgG fraction of hyper-immune rabbit anti-sheep RBC serum. These sheep RBCs rosette with Fc-receptor sites on macrophages, and rosettes so formed can be recognised and counted in a haemocytometer [Basten and Mitchell, 1976b]. Each of these techniques demonstrated that approximately 50% of cells in the HX32 tumour were macrophages, and that furthermore these corresponded with the smaller of the two cell types seen in suspension under direct light microscopy. The larger of the cell types did not take part in either of these reactions and were therefore not macrophages. (I am grateful to Dr. Graham Currie for technical advice and assistance with these macrophage experiments.)

The next problem was to establish which of the two cell populations was giving rise to colonies in agar. Crude cell separation was achieved by allowing the original suspension sediment under gravity in Ham's medium and serum for one hour, after which a suspension containing approximately 80% of the smaller cells could be recovered from the uppermost layers and a similarly enriched suspension of larger cells could be obtained from the bottom layers. The colony forming ability of each of these suspensions was then separately measured in diffusion chambers using the standard technique previously described.

The plating efficiency of these differential cell suspensions always correlated with the original percentage of large cells, demonstrating that these, and not the macrophages were forming colonies.

## 6.3: COLONY MORPHOLOGY

One of the problems involved in studying colony morphology is that colonies are difficult to remove intact from agar; nevertheless this is necessary to allow satisfactory uptake of dye by colony cells in histological preparations. Eventually the following technique was employed: colonies were removed from agar by Pasteur pipette under a binocular microscope, placed on a clean slide, squashed under a coverslip and immersed in a freezing mixture of liquid nitrogen. The coverslip was then flicked off the frozen slide and the cells adhering to the slide were stained with Giemsa.

This technique proved particularly successful for studying the closely packed, encapsulated colonies grown from colonic carcinoma HX18. Giemsa-stained preparations of colonies grown from this tumour showed undifferentiated neoplastic cells with high-nuclear: cytoplasmic ratio, all basically of the same type (Plate 5.5). Comparison with histological sections of the original tumour xenograft showed that the colony cells were entirely compatible with an origin from that tumour and indeed from the original biopsy removed from the patient at surgery (Dr. A. Mackay,

Consultant Pathologist, Royal Marsden Hospital).

Similar studies on the other xenografts were less successful at producing intact colonies, but the cells obtained from colony fragments again had the cytological features of tumour cells resembling those found both in the xenograft tumours and the original human cancers.

## 6.4: CHROMOSOMAL ANALYSIS

A direct chromosomal analysis was carried out on cell suspensions of all 11 xenografts by my colleague Miss J. Mills. The technique used was as follows: the tumour cell suspension was incubated with colcemid 0.4$\mu$g/ml in Ham's medium for 2-3 hours at 37$^{o}$C, and then resuspended three times in Clark's fixative (3 parts methanol : 1 part glacial acetic acid) at 4$^{o}$C for 30 minutes each time. Drops of the final cell suspension were heat dried on a clean glass slide and stained with Giemsa. Analysis was repeated from time to time for different passages of the same tumour.

All xenograft cell suspensions analysed in this way were of human karyotype with typical acrocentric and metacentric chromosomes which are lacking in mouse karyotypes. For example, cells of pancreatic carcinoma HX32 had a range of 42 to 68 chromosomes and a mode of 62, with extra chromosomes coming from groups C, D and E. Only the larger of the 2 cell populations of this tumour were of human karyotype; occasionally a mouse karyotype could be demonstrated in the smaller, macrophage population

known not to form colonies (Chapter 6.2). One tumour, colonic carcinoma HX18, had an added large acrocentric marker chromosome, persisting throughout repeated passage.

## 6.5: ELECTRON MICROGRAPH STUDIES: MELANOSOMES

Melanocytes and malignant melanoma cells can be further characterised by distinctive sub-cellular cytoplasmic organelles called melanosomes, demonstrated by electron microscopy. These are elongated structures 0.7 x 0.3$\mu$ in size, and are associated with the biosynthesis of melanin [Seigi et al, 1963]. Melanosomes were demonstrated in colony-forming cells from xenograft HX34 by electron microscopy, further confirming their melanoma origin; the colonies grown from this tumour were correspondingly darkly pigmented. (I am grateful to Dr. Kent Dawson for carrying out these electron micrcscopy studies.)

In conclusion, evidence based on morphology, chromosomal analysis, the exclusion of non-malignant cells as candidates for colony formation, and in one case the demonstration of characteristic sub-cellular organelles, all strongly support the assumption that the colonies grown in this assay system are indeed derived from human tumour cells and not from some other cell type.

# CHAPTER SEVEN

## THE IN VITRO IRRADIATION RESPONSE OF FOUR HUMAN TUMOUR XENOGRAFTS

# CHAPTER 7:

# THE IN VITRO IRRADIATION RESPONSE OF FOUR HUMAN TUMOUR XENOGRAFTS

## 7.1: INTRODUCTION

Although the effect of ionising radiation on the reproductive capacity of bacteria and viruses had been studied for many years by plating and plaque-forming techniques [Lea, 1946], similar measurements on mammalian cells were not possible until Puck and Marcus [1955] developed the technique described in Chapter 3.3 for growing colonies of plated HeLa cells in culture.

Using this technique they found that exposure of HeLa cells to ionising irradiation before plating out in Petri dishes limited colony growth: some cells disappeared completely, some remained single, some formed bizarre "giant forms" suggesting continued growth but with immediate loss of reproductive capacity, some formed "abortive" colonies of less than 50 cells suggesting loss of reproductive capacity after 3 or 4 divisions and some grew colonies as before [Puck and Marcus, 1956].

As the dose of irradiation was increased the ability of single cells to grow colonies progressively decreased, and the colony-forming cell survival curve so produced was of "sigmoid" or "C" type. This type of cell survival curve has subsequently been found for almost all mammalian cells so far studied after irradiation, and is pertinent to data arising from my own research; it will be described in some detail and is shown schematically in Figure 7.1.

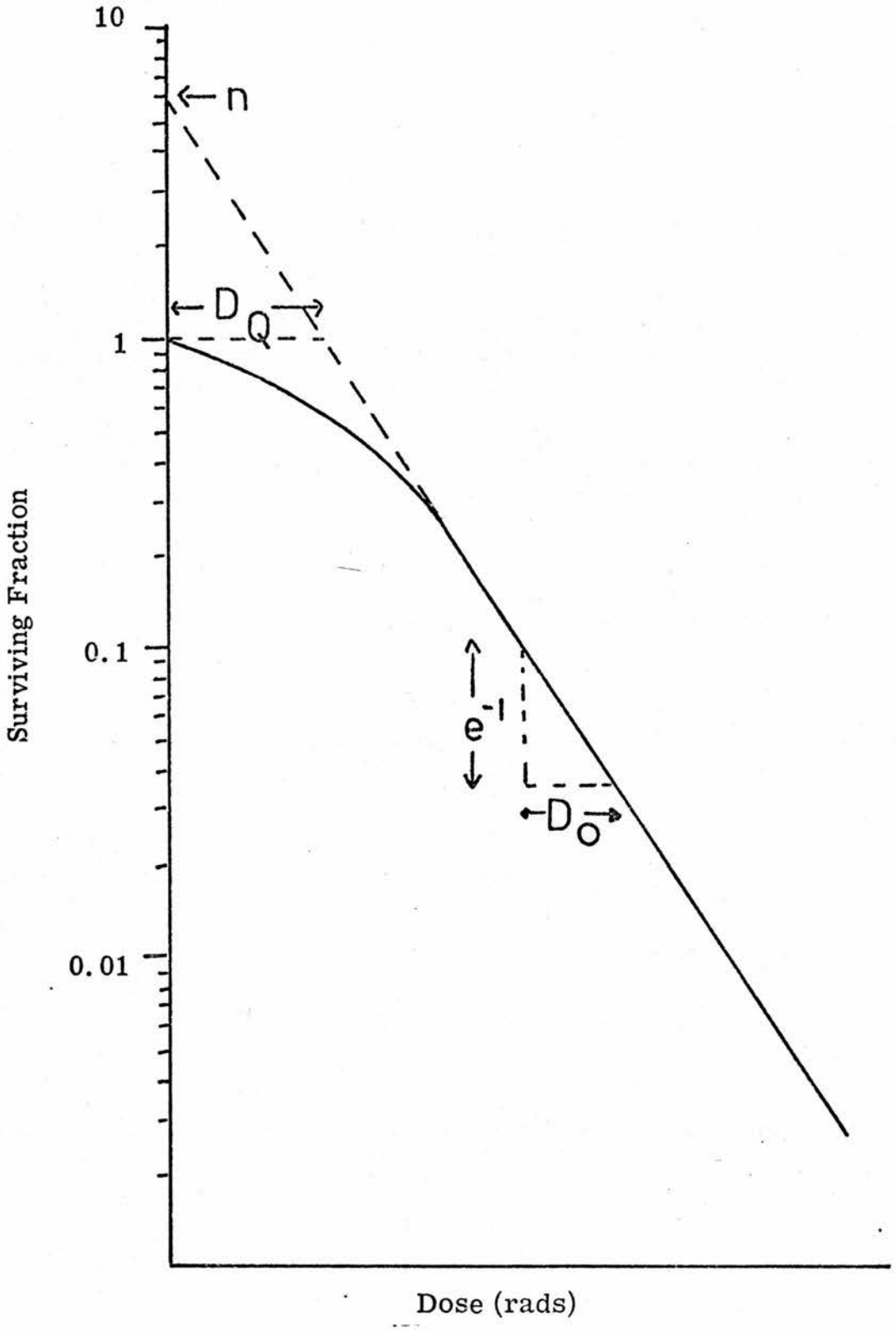



FIGURE 7.1

Schematic Representation of Sigmoid-Type Radiation Cell Survival Curve for Mammalian Cells

This type of curve has an initial "shoulder" or pseudo-threshold region followed by a region of exponential decline [Puck and Marcus, 1956; Alper et al, 1962], and can be characterised by two parameters: the first measures the slope of the exponential region on a semi-logarithmic plot and is conventionally expressed as $D_O$, that being the dose required to reduce survival to $e^{-1}$ (37%) in the exponential portion; the second measures the width of the shoulder region and is conventionally expressed either as n, the value obtained by extrapolating the exponential portion of the survival curve to zero on a semi-logarithmic plot, or as $D_Q$, the dose obtained by extrapolating the exponential portion of the survival curve to the point where it crosses the 100% survival level (all shown in Figure 7.1).

The radiobiological significance of this "C form" type of curve is that it consistutes evidence for a single-hit multi-target mechanism* for mammalian cell kill† by irradiation. The shoulder

*The single-hit multi-target theory of cell kill by irradiation means that several points in the cell must be hit each by a single quantum of irradiation to cause loss of proliferative capacity; if some but not all these points are hit then damage is sub-lethal and the cell can recover.

†Cell "kill" in clonogenic cell assays is by convention taken to mean loss of unlimited proliferative capacity.

portion of the curve represents the ability of cells to accumulate a certain amount of sub-lethal damage, presumably through "non-hitting" of some targets, before loss of proliferative capacity occurs [Elkind and Sutton, 1959]. This differs from the simple exponential irradiation survival curve without an initial shoulder region found for many bacteria, which suggests a single-target single-hit mechanism of killing [Lea, 1946].

The slope of the exponential tail of the curve, which is inversely proportional to $D_O$, gives a measure of the inate radiosensitivity of cells in vitro under standard, fully oxygenated conditions, and provides the basis for comparison of the inate radiosensitivity of cells from different tissues and species. Puck and Marcus [1956] found the $D_O$ for HeLa cells to be 96 rad, which suggested that the proliferative capacity of mammalian cells was many times more sensitive to ionising irradiation than those of micro organisms; equivalent values for E. coli bacteria are 4,000 rad [Lea, 1946] and for viruses around 40,000 rad [Watson, 1950].

Both the method of Puck and Marcus and other types of cell assay described in Chapters 3 and 4 have subsequently been used to measure the in vitro radiosensitivities of a whole range of mammalian tissues. These include laboratory cell lines [reviewed by Whitmore and Till, 1964; and Okada, 1970], experimental animal tumours [Hewitt and Wilson, 1961; Silini and Hornsey, 1962; Bush and Bruce, 1964; Reinhold, 1966; Hewitt, 1967; Brown and Berry, 1968; Reinhold and Debree, 1968; Hill and Bush, 1969; Rockwell and

Kallman, 1973; Thomson and Rauth, 1974; Shipley et al, 1975; Courtenay, 1976], haemopoietic precursor cells from animal bone marrow [Robinson et al, 1967; Chen and Schooley, 1970; Senn and McCulloch, 1970; Gordon, 1975], human bone marrow precursors [Senn and McCulloch, 1970; Gordon, 1975], cells from normal tissues [Whithers, 1967a; Withers and Elkind, 1968] and recently human tumour cell lines [Barranco et al, 1971; Thomson et al, 1975; Weichsenbaum et al, 1975].

From these data it has emerged that the in vitro radiosensitivities of mammalian cells under well oxygenated conditions appear to lie within a fairly narrow range: nearly all mammalian cells have a $D_O$ between 70 and 200 rad, and the large majority a considerably narrower range than this, with $D_O$ between 90 and 150 rad. Exceptions to this general observation have been occasionally reported: radiosensitive mutant strains of radiation-induced L5178Y leukemic cells in DBA/2 mice were shown to have a $D_O$ as low as 35 rad, depending on the medium used [Beer et al, 1963]; at the other extreme, Lin and Glass [1962] studied the effects of in vitro irradiation on the survival of mouse oocytes and found a $D_O$ of 300 rad; in this assay the end-point was not colony formation but embryo development after post-irradiation implantation into mated female mice (Chapter 3.5). Such exceptions have so far been described very rarely and do not invalidate the general observation that the inate radiosensitivities of nearly all mammalian cells so far studied in vitro lie within a fairly narrow range.

In striking contrast to these in vitro studies is the wide range of clinical radiotherapy response of human tumours, long recognised by radiotherapists [Moss et al, 1973]. It has been assumed that these observed clinical differences must be based on factors other than inate differences in the cellular radiosensitivity of human tumours; various explanations, including for example differences in tissue hypoxia within tumours and differences in the kinetics of tumour cell repopulation after irradiation, have been postulated to explain this [Berry, 1974; Lamerton, 1974; Nias, 1974]. While mechanisms such as these may undoubtedly be relevant in influencing the clinical response of a tumour to radiotherapy, it is important to note that the argument dismissing the possibility of inate differences in radiosensitivity at the cellular level as an explanation of observed clinical differences is based purely on extrapolation of in vitro studies from other mammalian tissues; impressive though the volume of these data may be, no direct studies on the in vitro radiosensitivity of clonogenic human tumour cells appear to have been published, through lack of a suitable assay technique.

It was therefore decided to study the response to in vitro irradiation of four human tumour xenografts in the diffusion chamber assay system. From the xenografts available, tumour types were selected to some extent on the basis of recognised differences in their clinical radiosensitivities.

## 7.2: METHODS EMPLOYED FOR IN VITRO IRRADIATION OF XENOGRAFT TUMOUR CELLS

### 7.2.a: Tumour Xenografts and Cell Suspensions

The 4 human tumour xenografts selected for these experiments were a pancreatic carcinoma (HX32), a colonic carcinoma (HX18), an oat cell carcinoma of lung (HX33) and a melanoma (HX34). Details of these have been described in Chapter 5 and Tables 5.1 - 5.3, as have the techniques used to produce single cell suspensions (Chapter 5.3.a, 5.3.b, 5.3.c, 5.3.d).

### 7.2.b: Irradiation

Tumour cell suspensions in Ham's F12 medium and 15% special Bobby calf serum were irradiated in vitro in air at room temperature from a $^{60}Co$ $\gamma$ source at a dose rate of 400 rad per minute. During irradiation, the cell suspensions were held in Falcon tubes, themselves suspended in water in larger tubes to ensure adequate build-up of irradiation dose.

Measured aliquots of cells were then mixed with agar to a final agar concentration of 0.3% and loaded into diffusion chambers for intraperitoneal implantation as described in Chapter 5.

### 7.2.c: Colony Counting

The technique for colony counting, the criteria used for defining colonies, and the incubation periods for each tumour have already been described in Chapter 5.

### 7.2.d: Calculation of Surviving Fraction of Clonogenic Cells

The surviving fraction of colony-forming cells after each dose of irradiation was calculated as the plating efficiency (number of colonies produced per 100 cells plated) of the irradiated cells divided by the plating efficiency of unirradiated controls.

## 7.3: RESULTS OF IN VITRO IRRADIATION

(For a description of the statistical methods used to analyse the results of data presented in this and subsequent chapters, see Appendix One.)

The in vitro irradiation of tumour cell suspensions from all 4 xenografts decreased the fraction of colony-forming cells in the suspension compared with that of controls. Many more single cells and small clusters (20-50 cells) were seen in the irradiated cell chambers than in the controls, suggesting that irradiation of clonogenic human tumour cells inhibits further cell division immediately for some cells but allows a limited number of further divisions before eventual loss of proliferative capacity in others. Giant cells of the type reported for HeLa cells after irradiation by Puck and Marcus [1956], and mentioned earlier (Chapter 7.1), were not seen here.

The relationship between in vitro irradiation dose and clonogenic cell survival for the 4 human tumour xenografts studied is shown in the dose-survival curves of Figures 7.2, 7.3, 7.4 and 7.5. The $D_O$ and $D_Q$ values which define these curves are summarised in Table 5.1.

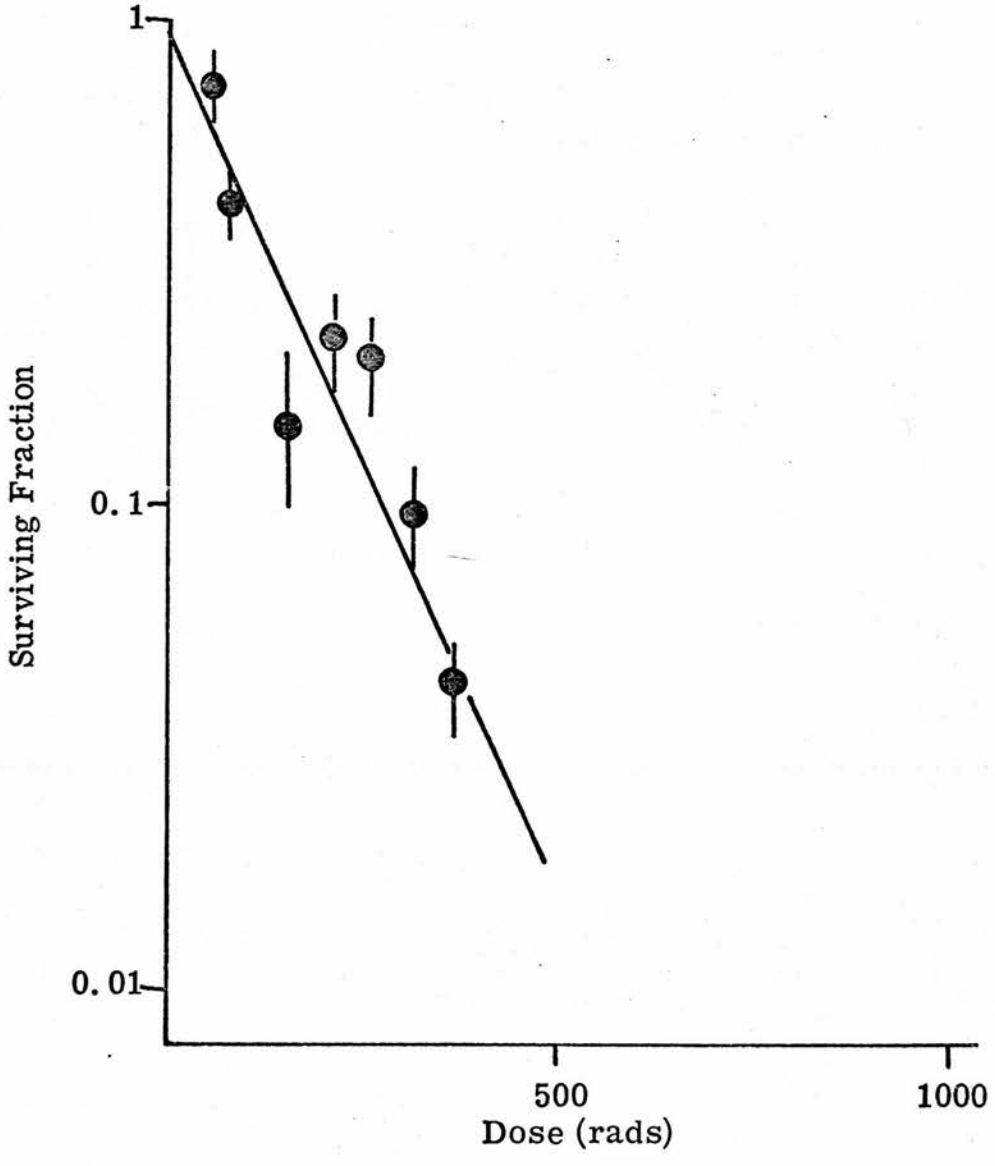



FIGURE 7.2

In Vitro $^{60}$Co $\gamma$-Irradiation Response of Cells from Human Colonic Carcinoma HX18 (Vertical Bars Represent $\pm$ Standard Error of the Mean)

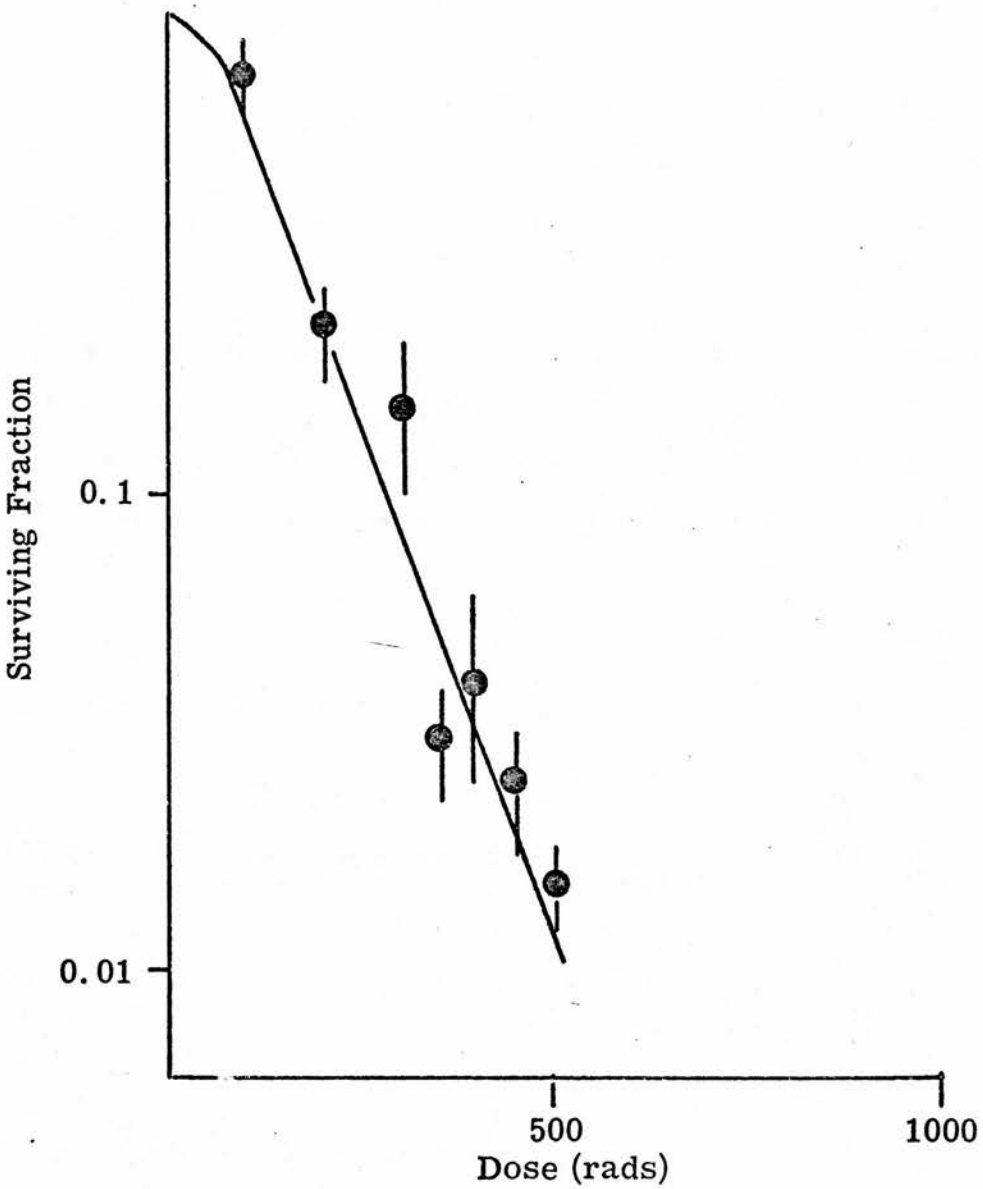



FIGURE 7.3

In Vitro $^{60}$Co $\gamma$-Irradiation Response of Cells from Human Pancreatic Carcinoma Xenograft HX32 (Vertical Bars Represent $\pm$ Standard Error of the Mean)

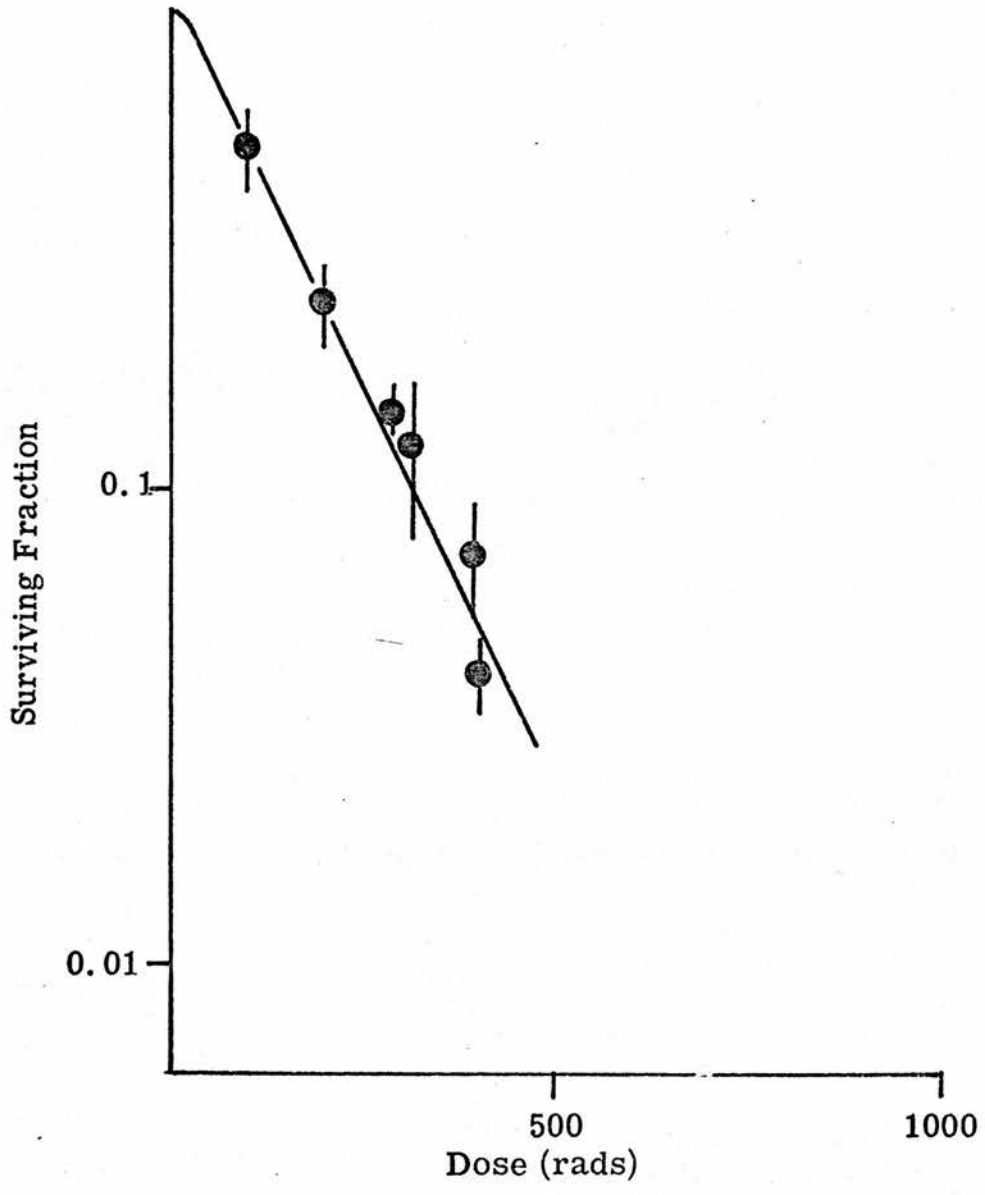



FIGURE 7.4

In Vitro $^{60}$Co $\gamma$-Irradiation Response of Cells from Human Oat Cell Carcinoma Xenograft HX33 (Vertical Bars Represent $\pm$ Standard Error of the Mean)

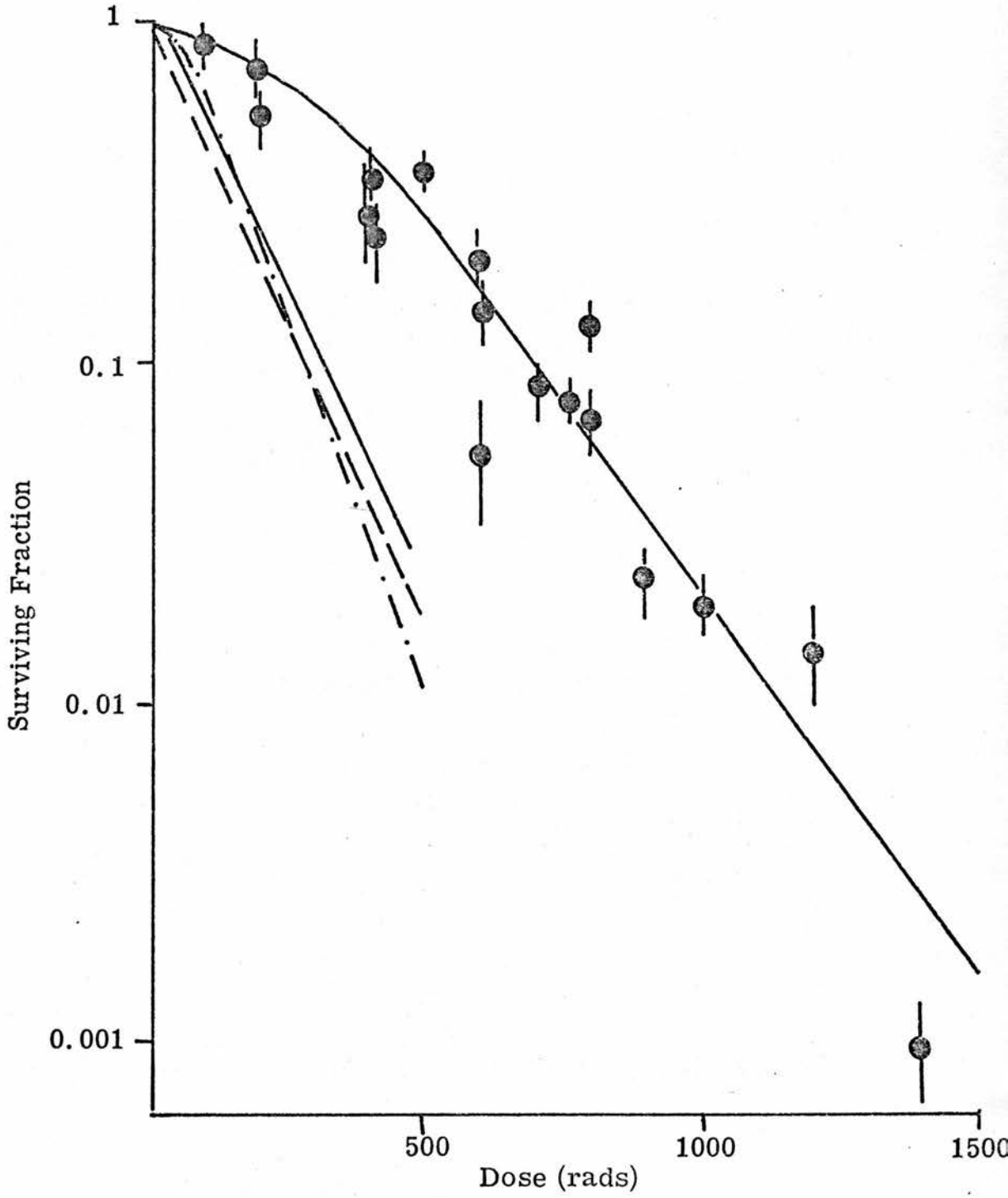



FIGURE 7.5

In Vitro $^{60}$Co $\gamma$-Irradiation Response of Cells from Human Melanoma Xenograft HX34 (Vertical Bars Represent $\pm$ Standard Error of the Mean)

Survival Curves of HX32 (-·-·-), HX18 (— — —) and HX33 (———) are Superimposed (Figs. 7.2, 7.3 and 7.4)

| Tumour | $D_O$ (rad) | Range* | $D_Q$ | Range* |
|---|---|---|---|---|
| HX18 | 123 | (105 - 147) | -9 | (-60 - (+)27) |
| HX32 | 100 | ( 81 - 126) | 59 | ( 17 - 93) |
| HX33 | 135 | (121 - 152) | 12 | (-29 - (+)45) |
| HX34 | 192 | (175 - 212) | 230 | ( 83 - 331) |

*$\pm$ Standard Error of the Mean

TABLE 7.1

$D_O$ and $D_Q$ Values for In Vitro Radiation Cell Survival Curves for Human Tumour Xenografts HX18, HX32, HX33 and HX34

The cell survival curves for three of these tumours, pancreatic carcinoma HX32, colonic carcinoma HX18, and oat cell carcinoma HX33, were very similar, with small initial "shoulder" regions followed by a region of exponential decline. The $D_O$ value of the exponential region for each tumour, derived by linear regression analysis (see Appendix One for details), was as follows:- HX32 100 (+26-19) rad, HX18 123 (+24-18) rad and HX33 135 (+17-14) rad. The $D_Q$ value was 59 (+34-42) rad for HX32, -9(+36-69) rad for HX18 and 12 (+33-41) rad for HX33 (figures in brackets represent $\pm$ one standard error of the mean).

The in vitro irradiation cell-survival curve of melanoma xenograft HX34 differed from that of the other three tumours in two respects, as can be seen from Figure 7.5 and Table 7.1. First, the $D_O$ value of 192 (+20-17) rad was higher than the $D_O$ for the other three tumours. This difference reached statistical significance compared with pancreatic xenograft HX32 ($p < 0.05$). Second, the width of the initial "shoulder" for melanoma cells, with a $D_Q$ value of 230 (+101-127) rad, was greater than that of the other three tumours. This difference reached statistical significance compared with colonic carcinoma HX18 ($p < 0.05$) and oat cell carcinoma HX33 ($p = 0.05$).

Because of the unexpected nature of these findings, more in vitro irradiation experiments were carried out on the melanoma tumour than on the other tumours, with more resultant points on the dose-survival curve to verify the data. It will nevertheless be seen that only one point has been obtained at the very high dose of 1400 rad, and this point has a critical effect on the slope of the exponential part of the curve. (If this point is excluded from the calculations the estimate of $D_O$ rises to 233 rad.) Unfortunately it has not been possible to obtain further points at this dose with accuracy and confidence, and this is due to technical reasons: to obtain surviving fractions as low as 0.001 it is necessary to load each diffusion chamber with $1 \times 10^6$ cells; these produce sufficient abortive "clusters" of cells after incubation to form a dense, semi-confluent background of cells against which accurate colony-counting is extremely difficult. Nevertheless, my colleague Mrs. Courtenay has also obtained a few points in this region for melanoma cells using an in vitro agar assay (Chapter 3) and these corresponded well with my own data and in particular with the one point I have been confidently able to obtain at 1400 rad. For this reason I have felt it justifiable to include this point in calculating $D_O$ for melanoma cells.

## 7.4: DISCUSSION

The first and fundamental observation from these in vitro studies is that the effect of irradiation on the proliferative capacity of clonogenic human tumour cells can be quantified using this assay

system in a way similar to that employed for cells of other mammalian tissues and that therefore human tumour cells are now for the first time amenable to a wide range of radiobiological investigations previously limited to cells from experimental animal tumours and established tumour cell lines.

It is of interest that the in vitro radiosensitivities of cells from pancreatic carcinoma HX32, colonic carcinoma HX18, and oat cell carcinoma HX33 were similar to one another. Furthermore the $D_O$ values established here for these tumours lay within the same fairly narrow range previously established for nearly all other mammalian cells (Chapter 7.1). This does not correlate well with the clinical response to radiotherapy usually observed for these tumour types: oat cell carcinomas of lung have a radiotherapy response rate of over 80% [Carr et al, 1972] and a low incidence of tumour recurrence within the locally irradiated field [Rissanen et al, 1968]; in contrast, colonic and pancreatic carcinomas are in general clinically more radioresistant and failure to achieve local control with conventional radiotherapy is common [Moertel, 1973].

If these xenograft tumours can be considered typical of tumours encountered in the clinic, then this discrepancy between experimental and clinical observations implies that the intrinsic radiosensitivity of tumour cells as established in vitro is not a major factor in determining the therapeutic response in the patient, and suggests that other extrinsic factors, including perhaps tumour cell hypoxin in vivo, must be involved. To this extent, these findings

therefore support the views of many radiobiologists, based on previous experimental animal tumour data (Chapter 7.1).

Studies on the in vitro radiosensitivity of the fourth xenograft, melanoma HX34, would however appear to contradict this general hypothesis. Here a clear difference was found between the cell survival curve for this tumour compared with the other three. First, the $D_o$ for melanoma tumour cells was higher than that for the other tumours, reflecting a greater intrinsic radioresistance, and indeed was very much at the upper limit of the range of $D_o$ values so far obtained for mammalian cells of whatever type (Chapter 7.1).

But the other important feature differentiating the melanoma cell survival curve from that of the other tumours was its much wider initial "shoulder". The implication of these data is therefore that melanoma cells from this xenograft irradiated in vitro have a greater capacity for repair of sub-lethal radiation damage (Chapter 7.1) than cells of the other tumours.

Conventional radiotherapy is usually given in fractionated doses of around 300 rad. From Figure 7.5 it can be seen that this dose achieves about one decade kill of clonogenic pancreatic, colonic and oat cell carcinoma cells; however, because of their larger "shoulder" only 40% of melanoma cells would be killed by this dose, with a surviving fraction of 0.6. To reduce the surviving fraction of melanoma cells to the same as that of the other cells treated with 300 rad, a much

larger dose of about 700 rad would be required (Fig. 7.5).

What makes this radiobiological observation of clinical interest is that melanomas are notoriously resistant in the clinic to conventional radiotherapy given in small fractionated doses [Rubin et al, 1974], and it is tempting to speculate from the data found here that larger fractionated doses might be more successful in exceeding the capacity of melanoma cells for repair sub-lethal damage and thus achieving greater cell kill with better tumour control.

There are of course serious limitations to extrapolating this type of data to the clinic. The first problem is that only one melanoma has been studied in this assay system and this may not be representative of the tumour type in general. Further studies with a series of melanomas are indicated to clarify this point. However it is of interest in this context that similar wide initial "shoulders" have been reported for established human melanoma cell lines irradiated in vitro [Barranco et al, 1971; Thomson et al, 1975]. Obviously further studies with a series of melanoma xenografts are now indicated to clarify this point.

The second and more fundamental problem with the extrapolation of in vitro data to clinical therapy is that many factors may operate in vivo to modify the intrinsic radiosensitivity of tumour cells. This point will be developed in the next chapter, but it has already been implied by these experiments with the demonstration that a pancreatic and a colonic tumour have an in vitro radiosensitivity

no greater than that of an oat cell lung carcinoma. In vivo irradiation studies are therefore of importance in adding to and modifying the initial information provided by these in vitro experiments, and the next step in this project was therefore to investigate the radiosensitivity of the human tumour xenograft model in vivo.

# CHAPTER EIGHT

## THE IN VIVO IRRADIATION RESPONSE OF A HUMAN PANCREATIC CARCINOMA XENOGRAFT, UNDER AIR-BREATHING AND HYPOXIC CONDITIONS

# CHAPTER 8:

# THE IN VIVO IRRADIATION RESPONSE OF A HUMAN PANCREATIC CARCINOMA XENOGRAFT, UNDER AIR-BREATHING AND HYPOXIC CONDITIONS

## 8.1: INTRODUCTION

Although the response of human tumour cells to *in vitro* irradiation can be measured using the techniques described in the last chapter, important factors may modify this response when cells are irradiated *in vivo* within the tumour. It is known, for example, that many animal tumours contain a significant fraction of hypoxic cells whose resistance to irradiation is increased [Gray *et al*, 1953; Suit and Maeda, 1967; Van Putten and Kallman, 1968; Barendsen and Broerse, 1969; Thomlinson and Craddock, 1969; Hewitt and Sakamoto, 1971; Rockwell and Kallman, 1973; Shipley *et al*, 1975], and it has been reasonably postulated that the same may be true of human cancers and may perhaps be a factor in preventing local tumour control with radiotherapy [Thomlinson and Gray, 1955; Duncan, 1973].

Techniques for studying tumour cell survival after *in vivo* irradiation are therefore required to complement basic *in vitro* data if clonogenic assays are to be of any value in studying the role of hypoxia and other factors which underlie tumour response to radiotherapy in the clinic. The principle behind such techniques is of course a simple one: whereas a tumour is first excised and a cell suspension prepared for irradiation to produce an *in vitro* survival

curve, an in vivo curve is produced by first irradiating the tumour in situ within the animal and subsequently excising it to produce a cell suspension for assay. The surviving fraction of cells irradiated in vivo is obtained by dividing the plating efficiency of the irradiated tumour by that of an unirradiated control.

While an entire in vitro dose survival curve can be obtained using aliquots of cell suspension from a single tumour, the in vivo assay requires a tumour-bearing animal for each irradiation dose. The experimental technique is therefore more elaborate, time-consuming and expensive, and it was therefore decided to concentrate resources on studying only one xenograft, for the experiments to be described.

## 8.2: METHODS USED FOR IN VIVO IRRADIATION STUDIES

### 8.2.a: Tumours

The pancreatic xenograft HX32 was selected for the in vivo irradiation experiments to be described; this particular tumour was chosen for two reasons. Firstly it has a relatively high plating efficiency compared with most of the other xenografts (Table 5.3) and therefore lends itself to detailed study in this assay system. Secondly, pancreatic carcinoma is of particular radiobiological interest in that it is an example of a human tumour which is relatively radioresistant and which frequently poses the radiotherapist with the problem of failure of local control [Moertel, 1973].

Tumours growing intramuscularly in the thigh muscles of immune-suppressed C57Bl mice (Chapter 5.2) were used in these experiments, and in vivo irradiation was carried out 3 to 4 weeks after implantation, by which time tumours had grown to a diameter of 0.5 to 1cm.

8.2.b: Irradiation Technique

Tumour-bearing mice were immobilised with nembutal anaesthesia in a dose of 60mg/kg injected intra-peritoneally 5 to 10 minutes before irradiation was started. Irradiation was administered locally to the tumour-bearing limb at a dose rate of 400 rad/minute, from a $^{60}Co$ $\gamma$ source at a distance of 25cm and a 3mm perspex sheet was placed in front of the tumours to allow adequate build-up of the absorbed dose. The rest of the animal was protected during irradiation by 13cm thick lead shielding (Figure 8.1).

8.2.c: Air-Breathing and Anoxic Assays

Tumours in air-breathing animals were assayed by killing the animal, dissecting out the irradiated tumour under sterile conditions and preparing a cell suspension as previously described (Chapter 5). This was carried out either immediately after irradiation (0 hour air-breathing assay) or 18 hours later (18 hour air-breathing assay). This latter assay was carried out to see whether cell survival after irradiation in vivo is influenced by the length of time the irradiated cells remain in the tumour before assay. This point will be discussed in detail below (Chapter 8.4).

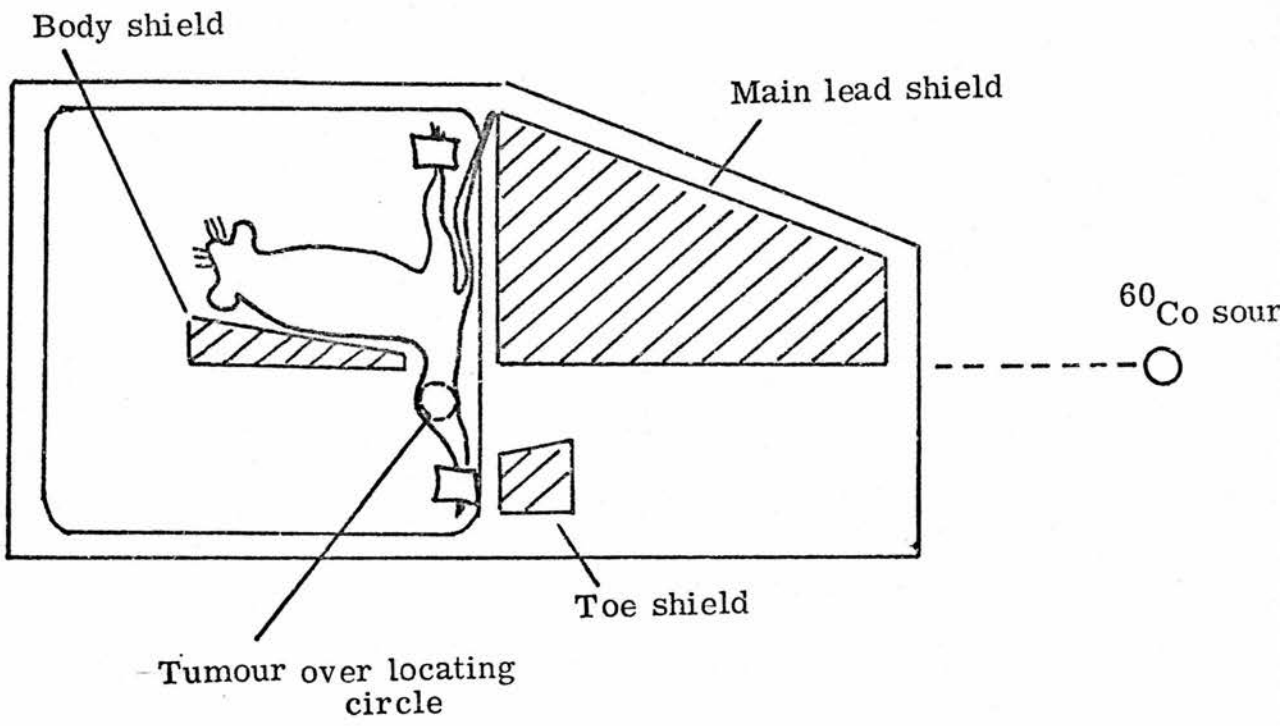



FIGURE 8.1

Experimental Set-Up for In Vivo $^{60}$Co $\gamma$-Irradiation of Hind Limb Xenograft Tumours in Nembutal-Anaesthetised Mice

In vivo irradiation of hypoxic tumours was achieved by killing the animal and keeping it at 37°C for 15 minutes before irradiating the tumour-bearing leg; this delay allowed tissue oxygen to be metabolised and rendered the tumour hypoxic. The tumour was immediately dissected out after irradiation and a cell suspension prepared and plated out in the usual way.

## 8.3: RESULTS

The cell survival curves for pancreatic carcinoma HX32 tumour cells irradiated in vivo: (1) in air-breathing mice assaying at 0 hours after irradiation; (2) in air-breathing mice assaying at 18 hours after irradiation; and (3) in hypoxic mice are shown in Figure 8.2, to which the in vitro irradiation cell survival curve obtained for this tumour in experiments described in Chapter 7 has also been added. The parameters with standard errors for each of these curves, calculated by linear regression analysis, are given in Table 8.1.

The hypoxic in vivo dose survival curve had an initial "shoulder" portion followed by a region of exponential decline. The value of $D_O$ for this curve was calculated as 351 (+26-23) rad, and $D_Q$ was 222 (+50-58) rad.

The "air-breathing 18 hour" assay curve had a scarcely discernible "shoulder" portion followed by a portion of exponential decline parallel to that of the hypoxic curve within the limits of experimental error but displaced vertically downwards because of the smaller "shoulder". For this curve $D_O$ was calculated as 313 (+20-17) rad.

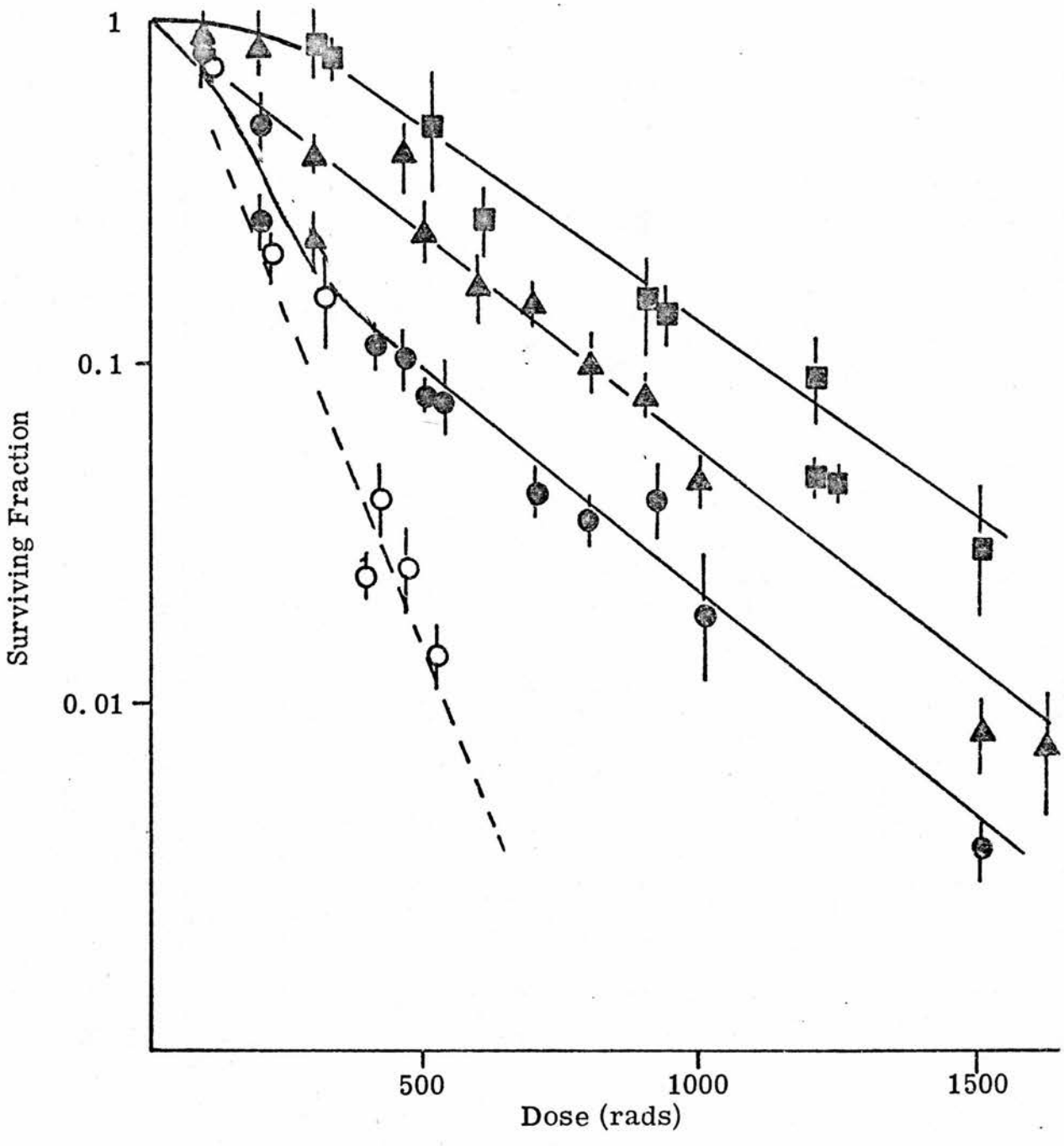



FIGURE 8.2

The In Vivo and In Vitro Response of Pancreatic Carcinoma Xenograft HX32 to $^{60}$Co $\gamma$-Irradiation (Vertical Bars Represent $\pm$ Standard Error of the Mean)

○ In Vitro ● In Vivo Airbreathing 0hrs

▲ In Vivo Airbreathing 18hrs ■ In Vivo anoxic

| Assay | $D_O$ (rad) | Range* | $D_Q$ (rad) | Range* |
|---|---|---|---|---|
| Anoxic - 0 hours | 351 | 328 - 377 | 222 | 164 - 272 |
| Air-Breathing - 0 hours | 325 | 303 - 351 | ** | |
| Air-Breathing - 18 hours | 313 | 296 - 333 | 59 | 11 - 103 |

*$\pm$ Standard Error of the Mean

**Intercepted y axis at $0.4 \pm 0.1$

TABLE 8.1

$D_O$ and $D_Q$ Values for In Vivo Radiation Cell Survival Curves for Pancreatic Carcinoma HX32 (i) Under Anoxic Conditions (ii) In Air-Breathing Animals, Assayed Immediately After Radiation (iii) In Air-Breathing Animals Assayed 18 Hours Later

The "air-breathing 0 hour" assay curve was biphasic: it had a small initial "shoulder" followed by a short portion of steep exponential decline; at a dose of between 200 and 400 rad, the curve then developed a second portion of exponential decline this time less steep than the first part, with a $D_O$ calculated as 325 (+26-22) rad. This distal exponential portion extrapolated back to intercept the y axis at $0.4 \pm 0.1$.

## 8.4: INTERPRETATION OF RESULTS AND DISCUSSION

These results are most easily discussed by considering initially the relationship between the *in vitro* dose-survival curve, the "air-breathing 0 hour" *in vivo* curve, and the anoxic *in vivo* curve. The significance of the 18 hour air-breathing curve will be discussed later (Chapter 8.4).

### 8.4.a: Oxygen Enhancement Ratio

The shapes of these first 3 dose-survival curves for the human pancreatic carcinoma xenograft HX32 resemble earlier findings for experimental animal tumour systems similarly irradiated [Powers and Tolmach, 1963; van Putten and Kallman, 1968; Barendsen and Broerse, 1969; Rockwell and Kallman, 1973; Shipley *et al*, 1975], and the salient features have been interpreted as follows: the $D_O$ of the anoxic irradiation curve (351 rad) was much greater than that of the *in vitro* irradiation curve under fully oxygenated conditions ($D_O$ = 100 rad) with a significance $p < 0.001$.

This demonstrates the increased radioresistance of human pancreatic carcinoma cells from HX32 xenograft under hypoxic conditions. Similar findings have been made for all other mammalian cells previously studied. This enhancing effect of oxygen can be quantified by what is referred to as the Oxygen Enhancement Ratio (O.E.R.). This is obtained from the ratio of the slopes of the exponential regions of the 2 curves ($D_O$ anoxic : $D_O$ oxygenated in vitro) providing these originate from a common point; if this condition does not hold then clearly the ratio of the surviving fraction of cells irradiated in the presence or absence of oxygen will change over a range of doses [van Putten and Kallman, 1968].

In these studies, this criterion is met for the anoxic and oxygenated in vitro cell survival curves within the limits of experimental error, and the O.E.R. for human pancreatic carcinoma xenograft HX32 is calculated as 3.5.

8.4.b: Hypoxic Cells in Tumours of Air-Breathing Animals

It can be seen from Figure 8.2 that the hypoxic cells have not only a higher inate radioresistance than oxygenated cells but also a greater capacity to repair sub-lethal damage, as evidenced by the wider shoulder of their cell survival curve. Thus not only is a greater dose of radiation required to kill a given fraction of hypoxic cells compared with oxygenated cells in the exponential part of the curve, but so also is a greater initial dose of radiation required to overcome sublethal damage repair and allow cell kill to proceed exponentially.

The biphasic nature of the survival curve for tumour cells irradiated in the air-breathing animal and then immediately assayed (air-breathing 0 hour assay) is interpreted as demonstrating a mixed population of oxygenated and hypoxic cells in this tumour [van Putten and Kallman, 1968; Rockwell and Kallman, 1973]. Initially, at low doses of irradiation the more radiosensitive oxygenated cells with less capacity for repair of sublethal damage will be preferentially killed, producing a cell survival curve which approximates towards that of oxygenated cells irradiated in vitro. As these oxygenated cells are killed so the proportion of surviving cells that are hypoxic increases until, beyond doses of 400 rad, this comes to represent nearly all the remaining tumour cells in the air-breathing animals. Thus beyond doses of 400 rad, the slope of the curve decreases, with a higher $D_O$ which reflects the greater radioresistance of residual hypoxic cells.

There is an important assumption in this argument, expounded by Elkind [1970], and that is that the response to $\gamma$-irradiation of chronically hypoxic cells (in the tumour of the air-breathing animals) is the same as that of acutely hypoxic cells (in the tumour of the animal killed immediately prior to assaying). There is some evidence that this may not always be the case with respect to repair of sub-lethal damage, which may be less or slower in chronically hypoxic cells [Suit and Urano, 1969; Shipley et al, 1975]. However, my findings here that the $D_O$ values for the exponential portions of the two curves are the same within the limits of experimental error

suggest that acute and chronically hypoxic cells probably do respond in the same way to irradiation at least beyond the initial shoulder portion.

### 8.4.c: Calculation of the Hypoxic Fraction

On the basis of the foregoing argument the fraction of hypoxic cells in the air-breathing tumour can be calculated from the ratio of hypoxic : oxygenated + hypoxic cells, which is equivalent to the air-breathing : hypoxic ratio in the terminal portions of the two curves. Thus, from the data of Figure 8.2, the hypoxic fraction for human pancreatic carcinoma xenograft HX32 is 0.19.

### 8.4.d: Clinical Implications of a Hypoxic Fraction in Pancreatic Carcinoma Xenograft HX32

Any attempt to extrapolate the results of these studies to human cancers in the clinic is limited by an important difference between xenografts and their parent tumours: the basis of tumour hypoxia is believed to be impaired vascularity within the tumour, and whereas the actual tumour cells in our xenografts have been shown to be of human origin, the supporting connective tissue stroma and vascular supply must derive from the host mouse. Histological differences in the connective tissue stroma of some xenografts compared with that of their parent human tumour have been described [Pickard et al, 1975] and it may not therefore be that the vascular distribution of oxygen within the pancreatic tumour xenograft studied here is an accurate replica of that in the patient's original tumour.

This is clearly a theoretical qualification to the use of any xenografts for investigating the clinical problem of hypoxia, but there exists comparative experimental evidence from animal tumour studies that offers the basis for some encouragement. Using similar experimental techniques to our own, the hypoxic fraction of cells in several animal tumours have been measured: a hypoxic fraction of 0.15 was found for a C3H mouse mammary carcinoma in a tumour-bearing animal under normal aerobic conditions [Suit and Maeda, 1967], 0.14 for a KHT mouse sarcoma [van Putten and Kallman, 1968], 0.15 for a rat rhabdosarcoma [Barendsen and Broerse, 1969], 0.33 for the EMT 6 tumour [Rockwell and Kallman, 1973] and 0.36 for the Lewis lung tumour [Shipley et al, 1975].

Thus the hypoxic fraction of 0.19 for the pancreatic carcinoma xenograft lies well within the range found for these experimental animal tumours with their own natural blood supply, and perhaps therefore it may not be completely invalid to extrapolate data on hypoxic cells in xenograft models despite the theoretical objections implicit in their murine-derived stroma.

With this proviso, the demonstration in these experiments that about 20% of clonogenic cells in this human tumour xenograft are hypoxic is of considerable interest, in that it reinforces the hypothesis that one of the factors which may clinically prevent local tumour control with radiotherapy is the presence of radioresistant hypoxic foci in human cancers. Furthermore, these studies suggest that hypoxia may not merely be a problem in large tumours with

multiple necrotic areas commonly found in clinical practice, but also in very much smaller nodules. The xenografts irradiated in these studies were only of 0.5 to 1cm diameter; this is equivalent to a human tumour that in some circumstances might be hard to detect clinically, and which, for example, would scarcely be visible on a standard chest x-ray film [Rigler, 1966].

A further experimental approach to the problem of xenograft hypoxia would therefore be to investigate whether extremely small xenograft tumours also have a hypoxic fraction. Shipley et al [1975] have recently shown that although 500mm$^3$ Lewis lung carcinoma tumour had a hypoxic fraction of 0.36, minute 0.5mm$^3$ pulmonary metastases nevertheless had a hypoxic fraction of less than 0.005 with tumour cells correspondingly more sensitive to irradiation. Similar findings have also been described for 0.6mm$^3$ C3H mouse mammary tumours grown subdermally in the ear [Suit and Maeda, 1967].

Were the HX32 xenograft also shown to exhibit this effect when extremely small nodules were irradiated, this finding would be important in supporting the use of "adjuvant" radiotherapy to regions of the body, and in particular to the lungs, without clinical evidence of tumour but where the probability of cryptic micrometastases seemed high.

However, from a practical point of view, research along these lines is thwarted by the lack of a suitable technique for growing the necessary very small xenograft nodules at present.

A second line of research stemming from the demonstration of hypoxia within xenograft HX32 concerns the use of therapeutic techniques designed to overcome hypoxic cell radioresistance. Recently for example, there has been considerable interest in chemical radiosensitisers of hypoxic cells as a means of enhancing tumour response to radiotherapy (Chapter 9). Therefore following on from the experimental findings described in this chapter, it was decided to investigate one such radiosensitiser in the diffusion chamber assay system, and to see whether this might modify the hypoxic exponential tail of the air-breathing dose survival curve of Figure 8.2. The results of this study will be described in the next chapter. First, however, the implications of the 18 hour air-breathing survival curve in Figure 8.2 must be considered.

8.4.e: The "Air-Breathing 18 Hours" Dose Survival Curve

The "air-breathing 18 hours" dose survival curve differs from the "air-breathing 0 hours" curve in that, although the slopes of the exponential tails of both curves are approximately parallel, the curves are nevertheless vertically displaced, with cell survival increased in the 18 hour assay by a factor of 2.5. This is an important observation: if cell survival in the assay system varies depending on the time interval between irradiation and assay, then it is obviously essential to know which of the two time intervals gives a result that more accurately reflects the surviving fraction of cells left undisturbed within the tumour after in vivo irradiation.

There are at least two hypotheses which might explain this discrepancy. The first is that during the 18 hour period between irradiation and tumour excision, "doomed" cells destined not to form colonies as a result of irradiation are lysed or otherwise removed from the tumour such that the fraction of surviving clonogenic cells left within the tumour after 18 hours is raised, although their absolute number remains unchanged. On the basis of this explanation the true surviving fraction would be given by the "0 hour" assay, while that of the "18 hour" assay would be artificially raised.

This explanation is unlikely however. First, no histological changes compatable with cell lysis and removal are seen at so short a time after assay in this xenograft. Second, cell suspensions made from a tumour 18 hours after irradiation show no evidence of irradiation damage, and it is not until 48 hours have elapsed that features of irradiation death begin to appear in these cells observed in monolayer culture [Mrs. V. D. Courtenay, personal communication]. Finally, in the agar colony assay system a large proportion of irradiated cells form clusters of between 4 and 50 cells, which, although too small to be scored as colonies, indicate that irradiation has not immediately destroyed such cells but rather limited their proliferative capacity to a few further divisions. This observation also tends to argue against a significant number of cells being lysed and removed within 18 hours of irradiation.

8.4.f: Repair of Potentially Lethal Damage

An alternative explanation to explain the differences in cell survival obtained from the two assays is that removal of some cells from their tumour "milieu" immediately after irradiation interferes in some way with their ability to repair irradiation damage and survive, if left in situ for 18 hours. The existence of such a phenomenon has been postulated recently by several authors and has been referred to as "repair of potentially lethal damage" [Phillips and Tolmach, 1966; Little et al, 1973; Hahn et al, 1974; Shipley et al, 1975].

There is increasing evidence to suggest that the ability of cells to repair damage caused by irradiation can be influenced by their post-irradiation environment. For example, in vitro survival is enhanced by a reduction in temperature after irradiation [Whitmore and Gulyas, 1967], by a change in the nutritional state of the medium [Belli and Shelton, 1969; Djordevic and Perez, 1970], by inhibition of protein synthesis [Phillips and Tolmach, 1966] and by maintenance of cells in crowded plateau-phase cultures [Little et al, 1973]. Although these in vitro manipulations are of course artificial, some at least might reasonably resemble environmental differences between cells suspended in vitro and those growing in situ within a tumour. Thus it is postulated that cell survival might be increased by this so-called repair of potentially lethal damage if cells were left in situ for some hours after tumour irradiation rather than removed immediately for assay.

Some experimental data exists to support this. Shipley et al [1975] have recently observed a 3 to 6-fold increase in survival of Lewis lung carcinoma cells after in vivo $\gamma$-irradiation (but not fast neutron therapy) if cells were left in situ for up to 24 hours post-treatment. Similar observations have been made for a mouse lymphoma grown as an ascites tumour [Belli et al, 1971], a mouse fibrosarcoma [Little et al, 1973] and the EMT6 mammary sarcoma [Hahn et al, 1974]. This repair effect appears to be time-dependent, and for the EMT6 tumour the surviving fraction increases with time up to 8 hours after irradiation and remains increased for at least 24 hours.

This phenomenon of repair of potentially lethal damage could therefore explain the discrepancies found between the "0 hour" and "18 hour" survival for pancreatic tumour cells found in these experiments. Further studies are now indicated to establish the relationship between increase in survival and time after irradiation; if the same phenomenon is demonstrated after a much shorter time interval than 18 hours, then this would be completely incompatible with the first hypothesis, lysis and disappearance of irradiation-killed cells prior to assay, and would provide compelling further evidence in favour of potentially lethal damage repair for pancreatic xenograft HX32.

From the practical point of view, this is important, not merely for further human tumour assay studies but for all clonogenic assays, in that the removal of cells from a tumour immediately

after irradiation would not allow time for repair of potentially lethal damage and would therefore markedly underestimate the fraction of cells surviving a dose of irradiation. Most of the data obtained in recent years on the effect of in vivo irradiation on clonogenic cells has been based on assays performed immediately after irradiation and are therefore subject to this potentially serious criticism.

# CHAPTER NINE

## THE EFFECT OF RADIOSENSITISER Ro-07-0582 ON THE IRRADIATION RESPONSE OF CELLS FROM A HUMAN PANCREATIC TUMOUR XENOGRAFT

Very recently, the drug referred to in this Chapter as Ro-07-0582 has been given the official generic name Misonidazole.

# CHAPTER 9:

# THE EFFECT OF RADIOSENSITISER Ro-07-0582 ON THE IRRADIATION RESPONSE OF CELLS FROM A HUMAN PANCREATIC TUMOUR XENOGRAFT

## 9.1: HYPOXIC CELLS AND RADIORESISTANCE

In the previous chapter evidence was presented demonstrating that a human pancreatic carcinoma xenograft has a significant proportion of radioresistant hypoxic cells. This is in keeping with previous similar observations for experimental animal tumours. Normal tissues do not usually contain hypoxic cells [Withers, 1965 and 1967b], and it has therefore been argued that this oxygen-dependent difference in radiosensitivity may be an important factor in limiting the chance of local tumour cure with radiotherapy [Thomlinson and Gray, 1955; Duncan, 1973].

Various techniques for overcoming the radio-resistance of hypoxic tumour cells have been studied: these include the use of hyperbaric oxygen, densely ionising radiation including neutrons and $\pi$-mesons, decreased oxygenation of normal tissues, and fractionated radiotherapy dose schedules designed to take advantage of tissue reoxygenation; the possibilities and limitations of these approaches have been reviewed elsewhere [Duncan, 1973].

Recently, interest has also developed in the possible use of chemical radiosensitisers to enhance the effect of radiotherapy on hypoxic tumour cells.

## 9.2: CHEMICAL RADIOSENSITISERS OF HYPOXIC CELLS

In the 1960's, various chemicals were shown to sensitise bacteria to irradiation [Dewey, 1963; Adams and Cooke, 1969; Barnes et al, 1969]. Their mechanisms of action varied [Adams, 1973], but the large majority were specifically active on hypoxic cells alone. These had in common a high electron affinity with an oxygen-mimicking action through which their radiosensitising effect was understood to be mediated, and on which a rationale for further research and development could be based [Adams and Dewey, 1963; Adams and Cooke, 1969; Raleigh et al, 1972]. Many of the early compounds studied failed to sensitise mammalian cells or were cytotoxic, but radiosensitisation of hypoxic Chinese hamster cells grown in monolayer culture was demonstrated with a substituted acetophenone, p-nitroacetophenone (PNAP) [Chapman et al, 1971; Adams et al, 1971]. Subsequently a variety of other compounds proved to have a similar specific effect on hypoxic but not well-oxygenated mammalian cells in vitro: these included nitrofurans [Chapman et al, 1972], nitroimidazoles [Foster and Willson, 1973; Asquith et al, 1974], nitropyrazoles [Asquith et al, 1974] and nitrobenzenes [Adams et al, 1971; Raleigh et al, 1972].

Not all in vitro sensitisers proved effective in vivo, and some were ruled out on the basis of poor water solubility, failure to penetrate hypoxic tissue effectively because of rapid metabolism or a high affinity for serum proteins, or unacceptable host toxicity [Iwamoto et al, 1972; Denekamp et al, 1974; Rauth and Kaufman, 1975].

But some of these chemical radiosensitisers clearly had potential clinical advantages over most other hypoxic cell sensitisation techniques: they were relatively inexpensive, easy to administer, and one, metronidazole (Flagyl), was already in clinical use for the treatment of trichomonas infections.

## 9.3: IN VIVO USE OF METRONIDAZOLE (FLAGYL)

Using a skin cloning assay, Denekamp et al [1974] demonstrated a specific radiosensitising effect on hypoxic epidermal cells in vivo with metronidazole (Flagyl). Subsequently Rauth and Kaufman [1975] showed a similar effect on the hypoxic cell population of solid tumours in C3H mice, with an enhancement ratio of 1.5 at a dose of 1.5G/kg, and tumour cure studies on mammary carcinomas in C3H mice showed that large doses of metronidazole (3G/kg) increased the cure rate of a dose of 3400 rad from 20% in control mice to 70% in drug treated mice [Begg et al, 1974].

In these experiments, maximum radiosensitisation was only achieved with high doses of metronidazole, peak murine serum levels rising to more than 500$\mu$g/ml after intra-peritoneal injection. Such serum levels cannot be achieved in humans without producing unacceptable nausea, and therefore smaller doses must be used clinically. Urtasun et al [1976] recently reported that patients with glioblastoma treated with radiotherapy and metronidazole survived longer than those treated with radiotherapy alone; a criticism of this study however was that the radiotherapy dosage was lower than that normally used in treating this tumour, and metronidazole-treated

patients fared no better than patients reported in other studies treated with more conventional large dose radiotherapy alone.

Studies with metronidazole have been important in demonstrating the feasibility of chemical radiosensitisation, and have stimulated the search for better compounds that might be more effective clinically.

## 9.4: Ro-07-0582*

Metronidazole is a 5-nitro-imidazole, and it therefore seemed logical in the search for more effective clinical radiosensitisers to look at structurally related compounds. One such compound already available was Ro-07-0582 (1-(2-nitro-I-imidazolyl) 3 methoxy-2-propanol), a 2-nitro-imidazole whose structure is shown in Fig. 9.1. This drug had also originally been developed as a possible agent against trichomonas infection by Roche Products Ltd., and was found to be readily absorbed after oral ingestion. Its highly electron affinic structure suggested a possible role as a chemical radiosensitiser, and Asquith et al [1974] demonstrated a very marked radiosensitivity effect in vitro on hypoxic Chinese hamster cells grown in monolayer culture. In this system the drug achieved an enhancement ratio of up to 2.5 which was near the oxygen enhancement ratio of 2.8 for the system. Its effect was maintained in the presence of high concentrations of serum proteins, and its known low toxicity and slow rate of metabolism encouraged in vivo studies.

*Recently renamed Misonidazole.

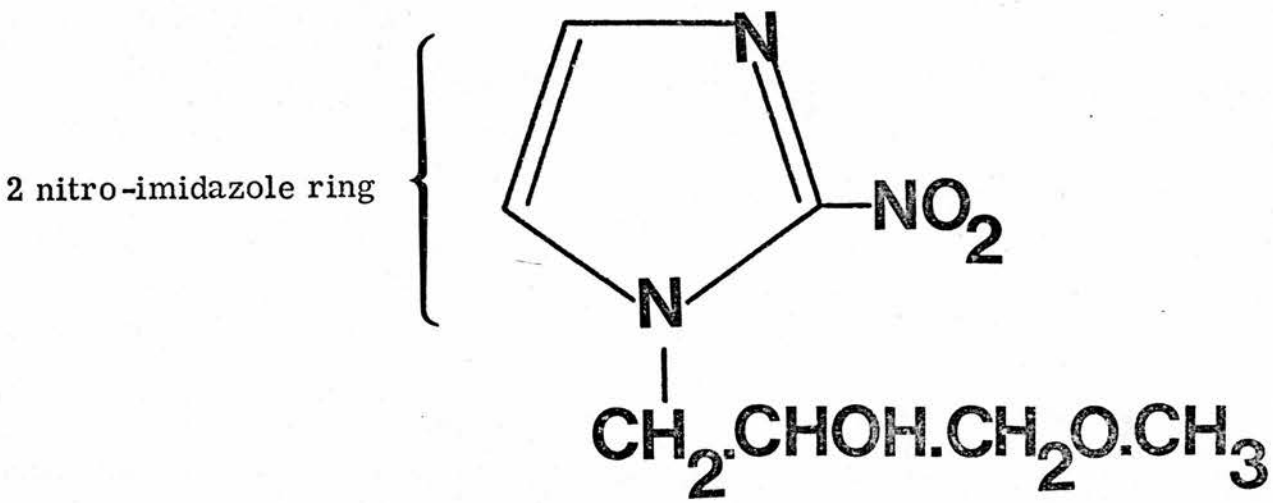



FIGURE 9.1

Structure of Ro-07-0582

Several such studies confirmed the effect of the drug in vivo and demonstrated its superiority over metronidazole as a radiosensitiser. Denekamp et al [1974] found a specific effect of Ro-07-0582 on hypoxic but not oxygenated murine epidermal cells using the skin cloning assay mentioned above (Chapter 9.3); it produced a maximum enhancement ratio of 2.2 compared with 1.7 for metronidazole. In tumour growth delay studies using mammary tumours in CBA mice Ro-07-0582 gave an enhancement ratio of 2.1 compared with 1.6 for metronidazole [Denekamp and Harris, 1975]. In a tumour cure study the drug was found to reduce the dose of irradiation required to control 50% of C3H mouse mammary tumours from 4380 to 2410, giving an enhancement ratio of 1.8 [Sheldon et al, 1974]. compared with 1.4 for metronidazole in a similar study [Begg et al, 1974].

The maximum dosages used in these experiments were high, as for metronidazole, but the therapeutic ratio in animals was greater for Ro-07-0582, and a considerable radiosensitising effect was still seen at much smaller dosages [Denekamp et al, 1974]. These observations encouraged studies into the use of Ro-07-0582 as a radiosensitiser in humans.

In a clinical toxicology study, single oral doses of up to 140mg/kg of Ro-07-0582 in suspension were tolerated before gastrointestinal symptoms, including nausea, vomiting and occasional mild diarrhoea, became unacceptable [Gray et al, 1976]. No neurological or other clinical abnormalities were observed directly attributable

to the drug, despite a report that high doses led to a cerebellar degeneration in dogs [Lennox-Smith, 1975]. At this dosage, peak serum levels of around 200$\mu$g/ml, within the range of radiosensitisation in animal tumours, were achieved between one and three hours after oral ingestion, with a long half-life of 10 to 17 hours [Gray et al, 1976].

Based on these data, clinical testing of Ro-07-0582 has recently been started in patients with malignant disease. In one study its ability to radiosensitise normal tissue rendered artificially hypoxic was tested by observing skin reaction under hypoxic and fully oxygenated conditions with and without Ro-07-0582 [Dische et al, 1976]. Skin reactions to radiotherapy were enhanced under hypoxic but not oxygenated conditions with an enhancement ratio of up to 1.68 at maximum tolerated dosage, calculated on the basis of a skin pigmentation reaction scoring system [Dische and Zanelli, 1976]. The drug was approximately three times as effective at radiosensitisation as metronidazole tested in the same system at dosages comparable in terms of tolerance to side effects.

In some of these patients an attempt was made to measure the enhancing effect of Ro-07-0582 on radiotherapy of metastatic tumour deposits [Thomlinson et al, 1976]. Practical difficulties limited the scope of this study, but an enhancement ratio of 1.2 was estimated in one patient with subcutaneous metastases from carcinoma of the uterine cervix. In a second patient with pulmonary metastases from breast carcinoma no enhancement was found.

Some patients died before measurements could be completed but a qualitative assessment of three other patients suggested that two of these also showed some evidence of enhancement.

Clinical studies of this type constitute the ultimate test of the clinical value of radiosensitisers, but problems arise in their design and interpretation. Estimates of hypoxic tumour cell kill can only be made indirectly from the delay in tumour regrowth after radiotherapy with and without drugs. Recognised differences in the growth rates of similar histological tumours between patients suggest that these studies should therefore best be carried out on multiple metastases in the same patient. Even here it is quite possible that important differences in growth rate, vascularity and response to therapy of such metastases may occur and invalidate interpretation of tumour growth delay measurements. But quite apart from these theoretical differences, practical difficulties exist in finding suitable patients with measurable multiple metastases of fairly uniform size. Such patients, even when found, are often very ill from advanced disease, and ethical considerations may limit optimal design of the clinical experiment.

For these reasons adjuvant laboratory studies with an appropriate experimental system might be of value in assessing the effect of the drug on hypoxic human tumour cells. The agar in diffusion chamber assay seemed to offer this opportunity in that the survival of hypoxic and oxygenated clonogenic tumour cells after irradiation in the presence and absence of the drug could be measured directly in this system and an enhancement ratio calculated.

This latter point will be developed below (Chapter 9).

Human pancreatic xenograft HX32 which had already been shown to have a hypoxic fraction of 0.19 (Chapter 8) was therefore chosen to investigate the effects of Ro-07-0582 in vivo, using the diffusion chamber assay technique.

## 9.5: METHODS: DRUG ADMINISTRATION AND IRRADIATION

In these experiments on pancreatic carcinoma xenograft HX32, some tumour-bearing mice were treated with single dose irradiation alone and some with irradiation preceded by intraperitoneal injection of Ro-07-0582 in a dose of 1mg/G (sic). The drug was dissolved in warm* isotonic saline and then injected intraperitoneally one hour before irradiation. Shorter time intervals have been previously employed in animal tumour studies [Sheldon et al, 1974; Denekamp and Harris, 1975] but the serum half-life of the drug in mice of about $1\frac{1}{2}$ hours encouraged us to allow a longer gap between administration and irradiation to increase the chance of adequate build up of drug concentration in poorly vascularised areas of tumour.

*Ro-07-0582 is poorly soluble in saline, and to achieve the high concentrations required to give the dose per mouse in a total volume of about 0.5ml, the drug was agitated in saline held at $37^{o}$C for about 20 minutes.

The techniques for anaesthetising the mice, irradiating the tumours and preparing cell suspensions for assay have been described previously in Chapters 5, 7 and 8. Assays were carried out in some experiments immediately after irradiation (0 hours) and in others 18 hours later. Unirradiated tumours were again used as controls to calculate surviving fractions.

Some unirradiated animals were treated with Ro-07-0582 alone in a dose of 1mg/G i.p., and tumours were assayed 18 hours later to study whether the drug had any specific cytotoxic effect of its own in this system.

In one series of experiments, tumour cell suspensions were incubated for 1 hour at 37$^{o}$C with Ro-07-0582 in a dose of 0.3mg/ml and then irradiated in vitro under oxygenated conditions to measure whether the drug had any radiosensitising effect on oxygenated tumour cells.

## 9.6: RESULTS

In all cases, the administration of Ro-07-0582 in a dose of 1mg/Gi.p. was well tolerated with no untoward effects noticed in the mice.

The effects of pretreatment on clonogenic cell survival after in vivo irradiation are shown in Figure 9.2 for the 0 hour assay, and in Figure 9.3 for the 18 hour assay. Parameters and standard errors for the cell survival curves are given in Table 9.1.

In both assays an increase in cell kill after single dose irradiation was found in the Ro-07-0582 pretreated animals.

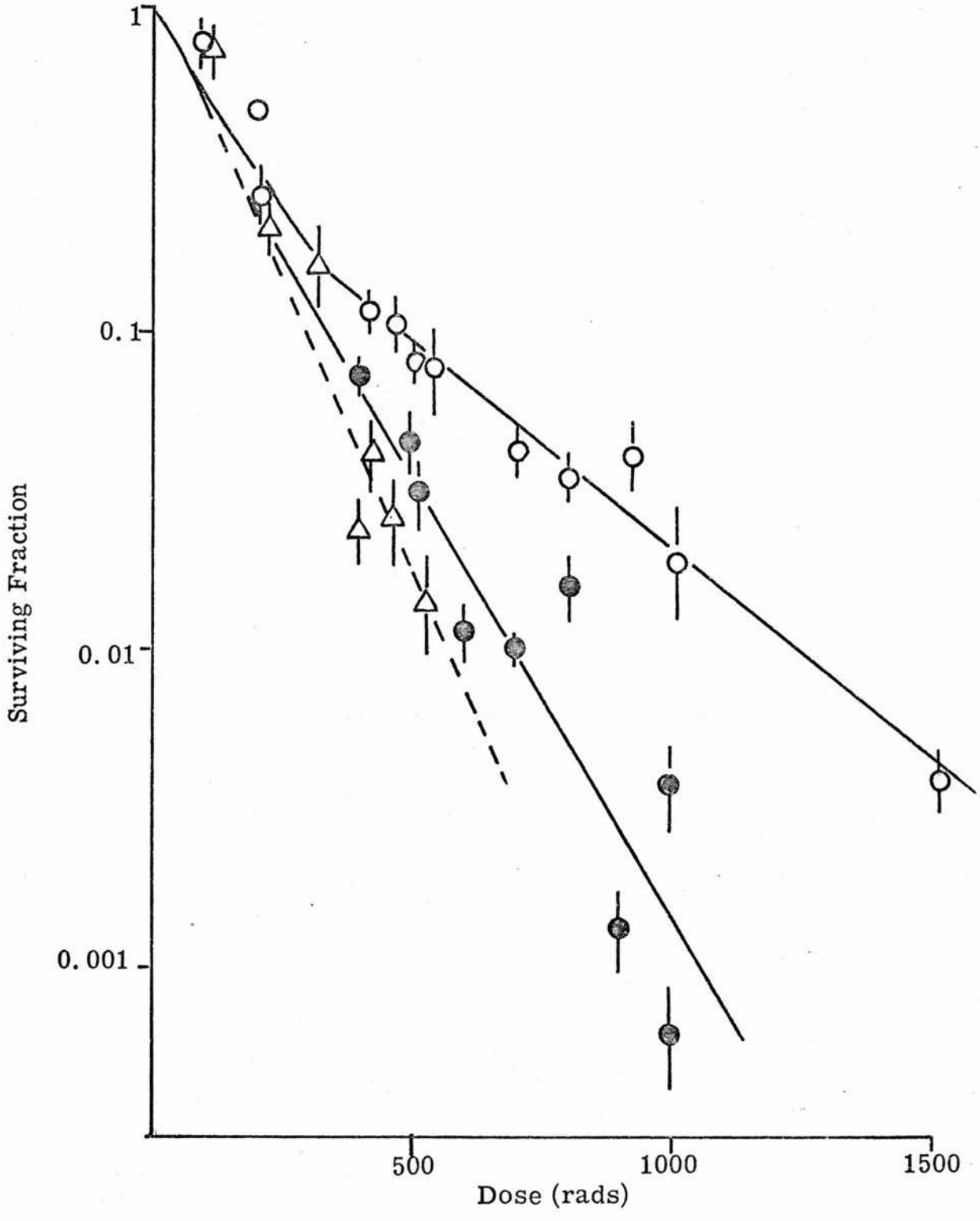



FIGURE 9.2

The Effect of Radiosensitiser Ro-07-0582 on the Response to $^{60}Co$ $\gamma$-Irradiation of Pancreatic Carcinoma Xenograft HX32 in the Air-Breathing Mouse. Assay at 0 Hours (Vertical Bars Represent $\pm$ Standard Error of the Mean)

○ In Vivo Irradiation Alone

● In Vivo Irradiation + Ro-07-0582

△ In Vitro

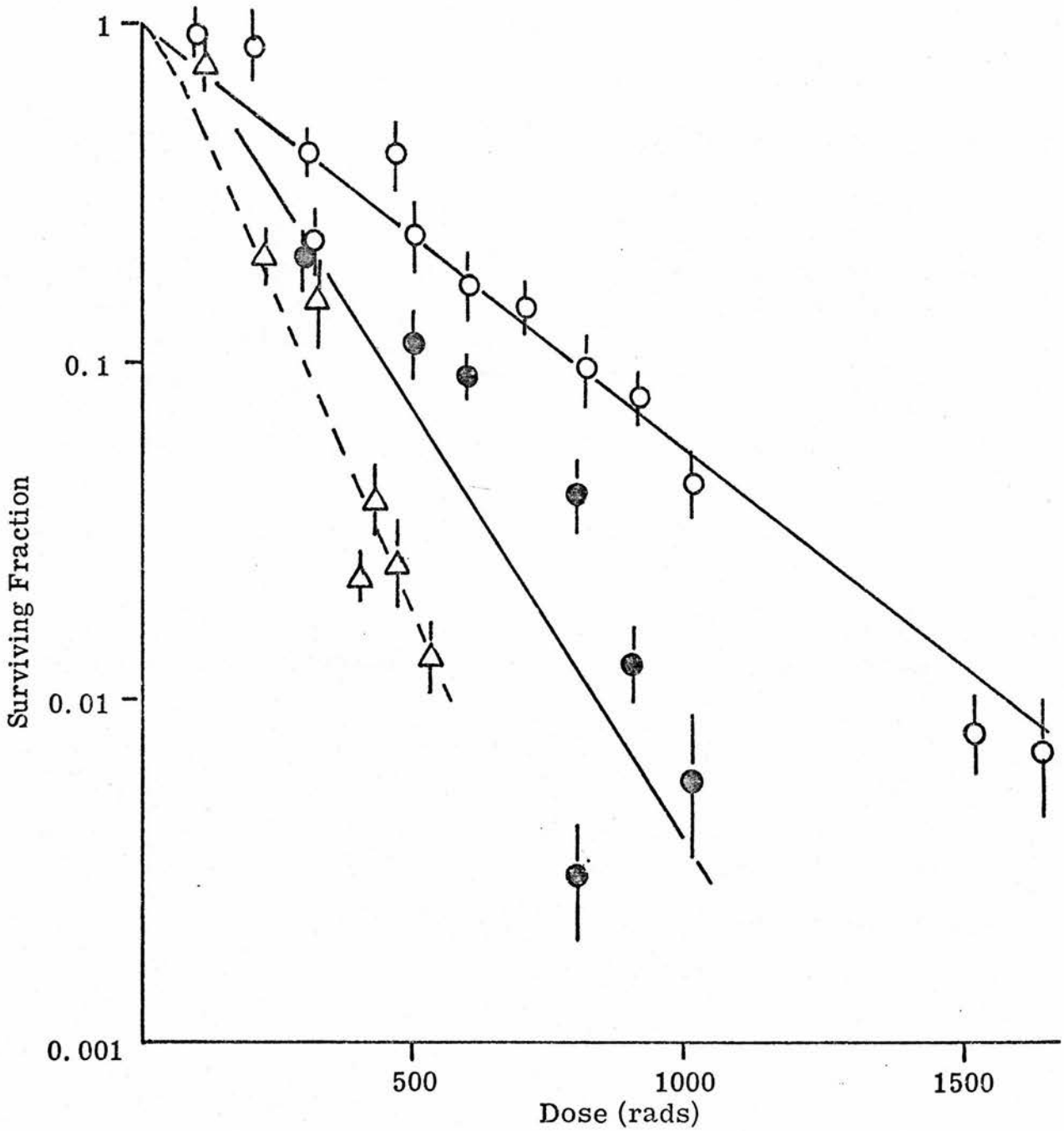



FIGURE 9.3

The Effect of Radiosensitiser Ro-07-0582 on the Response to $^{60}Co$ $\gamma$-Irradiation of Pancreatic Carcinoma Xenograft HX32 in the Air-Breathing Mouse. Assay at 18 Hours (Vertical Bars Represent $\pm$ Standard Error of the Mean)

○ In Vivo Irradiation Alone

● In Vivo Irradiation + Ro-07-0582

△ In Vitro

| | | $D_O$ (rad) | Range* |
|---|---|---|---|
| 0 hour Assay | Radiation alone | 325 | (303 - 351) |
| | Radiation + Ro-07-0582 | 159 | (134 - 195) |
| 18 hour Assay | Radiation alone | 315 | (298 - 335) |
| | Radiation + Ro-07-0582 | 182 | (142 - 253) |

*$\pm$ Standard Error of the Mean

TABLE 9.1

$D_O$ Values for In Vivo Radiation Cell Survival Curves for Human Pancreatic Carcinoma Xenograft HX32 with and without Ro-07-0582 Pretreatment. Assays at 0 hours and 18 hours After Irradiation

| | Colonies per 100 Cells Plated | |
|---|---|---|
| | 1st Experiment | 2nd Experiment |
| Control | 11.4 ± 0.8* | 13.0 ± 1.5 |
| Control + Ro-07-0582 | 12.6 ± 2.1 | 12.3 ± 1.6 |

*± Standard Error of the Mean

TABLE 9.2

Plating Efficiency of Cells from a Control Human Pancreatic Carcinoma Xenograft HX32 Compared with Cells from a Xenograft Treated In Vivo with Ro-07-0582 1mg/G i.p. 18 Hours Previously

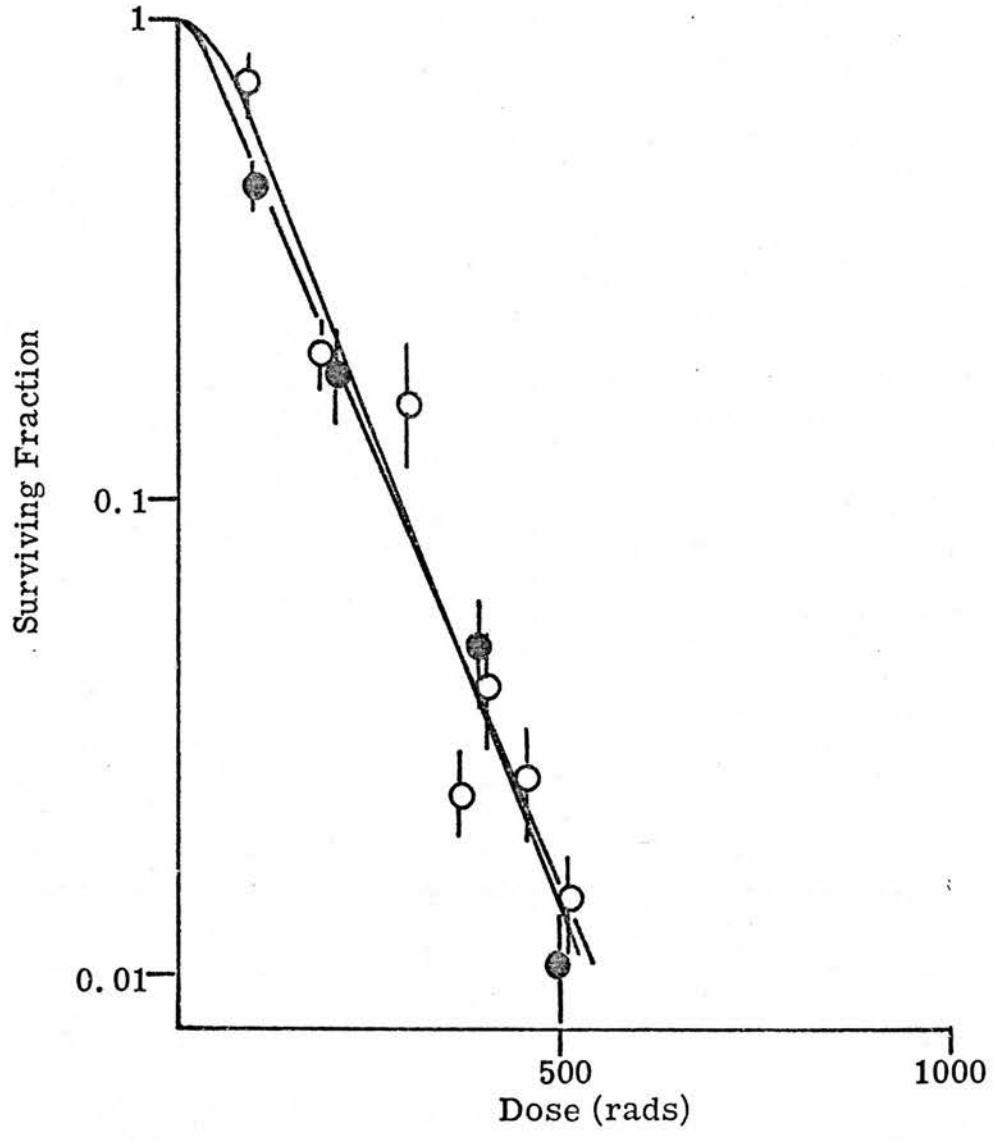



FIGURE 9.4

In Vitro $^{60}$Co $\gamma$-Irradiation Response of Cells from Pancreatic Carcinoma Xenograft HX32, with (●) and without (○) Incubation In Vitro with Radiosensitiser Ro-07-0582 (Vertical Bars Represent ± Standard Error of the Mean)

| | $D_O$ (rad) | Range* | $D_Q$ (rad) | Range |
|---|---|---|---|---|
| In vitro alone | 100 | (81 - 126) | 59 | ( 17 - 93) |
| In vitro + Ro-07-0582 | 112 | (99 - 129) | 17 | (-47 - (+)64) |

*± Standard Error of the Mean

TABLE 9.3

$D_O$ and $D_Q$ Values for In Vitro Radiation Survival Curves for Cells from Pancreatic Xenograft HX32 with and without In Vitro Incubation with Ro-07-0582

The $D_O$ for the 0 hour drug-treated curve was 159 (+36-25) rad compared with 325 (+26-22) rad for the radiation alone control ($p < 0.02$), and the $D_O$ for the 18 hour drug-treated curve was 182 (+71-40) rad compared with 315 (+20-17) rad for the radiation alone control ($p < 0.05$). There was no significant difference between the $D_O$s of the 2 drug-treated curves. These data gave an enhancement ratio for Ro-07-0582 in this system of 2.1 for the 0 hour assay and 1.7 for the 18 hour assay, again calculating the enhancement ratio from the ratio of $D_O$ (irradiation alone) : $D_O$ (irradiation + radiosensitiser) (Chapter 8.4.a).

The plating efficiency of tumours from animals treated with Ro-07-0582 alone, without irradiation is compared with that of untreated controls in Table 9.2. These show no statistically significant difference, and indicate that Ro-07-0582 at a dose of 1mg/G had no specific cytotoxic effect of its own for xenograft HX32 in this system.

Figure 9.4 shows the in vitro irradiation survival curve for cells previously incubated for 1 hour with Ro-07-0582, compared with irradiated controls. Parameters and standard errors for these curves are given in Table 9.3. No significant difference was found between these two curves, and this demonstrates that the drug has no radiosensitising effect on well oxygenated cells.

## 9.7: DISCUSSION

### 9.7.a: Experimental Considerations

These experiments demonstrate that Ro-07-0582 enhances the effect of single dose irradiation on clonogenic tumour cells from

pancreatic xenograft HX32. This enhancement is manifested in an increase in sensitivity of the exponential tail of the air-breathing curve, indicating that the enhancement effect is on hypoxic cells; no enhancement is seen on oxygenated cells irradiated in vitro; and no direct cell-killing effect is seen after treatment with the drug alone. These experiments therefore demonstrate that Ro-07-0582 is indeed a specific hypoxic cell radiosensitiser of human tumour cells, at least from a pancreatic carcinoma.

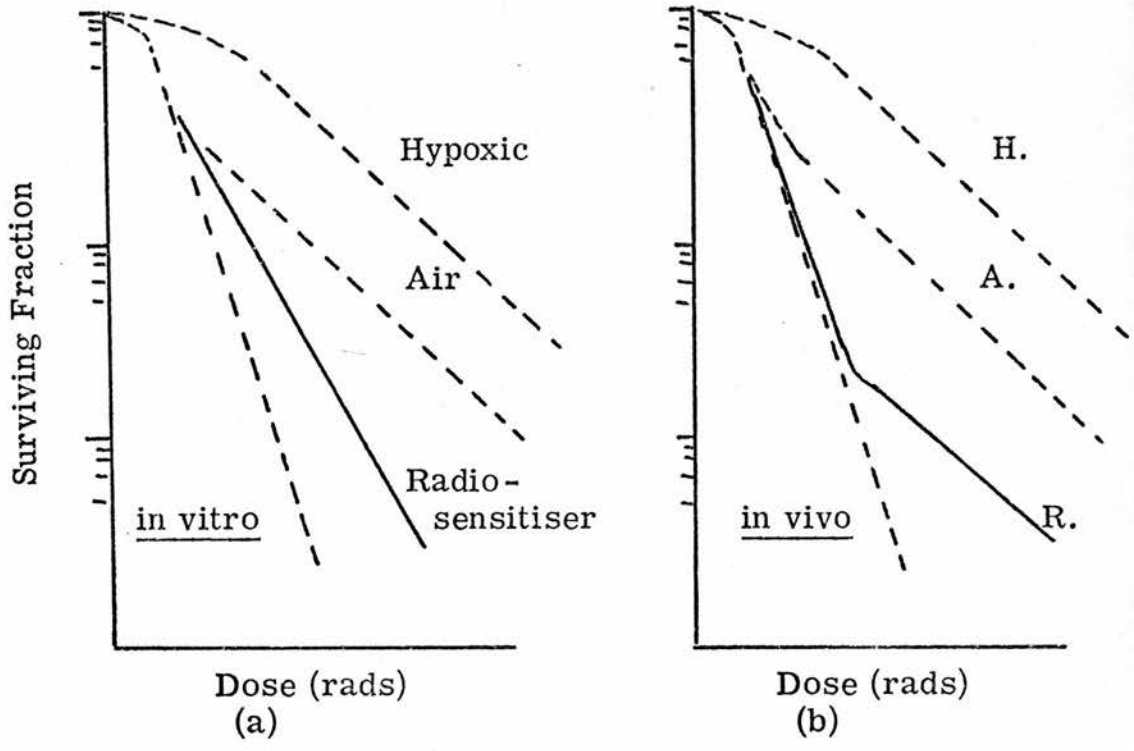
The degree of enhancement measured in the system is similar whether the assay is carried out immediately after irradiation or 18 hours later. This finding is entirely in keeping with the drug's action as a radiosensitiser, and argues against an alternative explanation that it alters cell kill by some post-irradiation effect; in other words this is not merely another manifestation or repair of potentially lethal damage (Chapter 8).

Although a statistically significant difference in the $D_O$ of the Ro-07-0582 curve compared with the oxygenated in vitro curve could not be demonstrated for either the 0 hour or 18 hour assay, the slope of the drug-treated curve was less steep in both cases (Figure 9.2 and 9.3). This suggested that the degree of irradiation enhancement achieved by the drug was not as great as that achieved by oxygen. There are two likely explanations for this: either all hypoxic cells are being sensitised by the drug but its action at a molecular level, whatever that may be, is not as great as

that of oxygen; alternatively it may be in theory just as affective as oxygen, but in practice the drug is not achieving complete penetration and not all hypoxic cells are being sensitised. This difference is important, because it determines whether the timing and size of the dose is adequate, or whether these need modification to achieve greater tumour tissue penetration.

Rauth and Kaufman [1975] have suggested that these two possible mechanisms can be differentiated from the shape of the radiosensitiser curve and this is best illustrated with reference to the two theoretical situations depicted in Figure 9.5 (a) and (b). If all cells are fully sensitised but the activity of the drug is not as great as that of oxygen, then a straight line curve sloping less steeply than the in vitro oxygenated curve would be anticipated, indicating exponential kill of a single population of cells (Figure 9.5 a). If not all hypoxic cells are being sensitised on the other hand, then a biphasic curve would be expected with an initial steep decline indicating kill of sensitised cells, followed by a tail with the same slope as the in vivo air-breathing curve tail, indicating kill of non-sensitised hypoxic cells (Figure 9.5 b). The argument in this latter case is the same as that proposed to explain the biphasic air-breathing curve in Chapter 8.4.b.

The simplest fit by eye for cells irradiated after exposure to Ro-07-0582 in the 0 hour assay, though not necessarily in the 18 hour assay, is a straight line, which would favour the former explanation that all cells are sensitised and that adequate tumour penetration has




From: Rauth and Kaufman [1975]

FIGURE 9.5

Theoretical Diagram Showing Two Possible Effects of a Radiosensitiser (solid lines) on Hypoxic Cell Survival from a Tumour Irradiated in vivo in Air-Breathing Animals

In (a) All Hypoxic Cells are Sensitised

In (b) Only a Fraction of Hypoxic Cells are Sensitised

been achieved. This is in agreement with similar recent findings for animal tumours using metronidazole (Rauth and Kaufman, 1975] and Ro-07-0582 [Rauth et al, 1975]. However, the data are clearly insufficient to exclude with complete confidence a biphasic curve which would favour the latter hypothesis, and the problem could satisfactorily be resolved only by obtaining many more data points.

9.7.b: Clinical Considerations

The results of these experiments obviously justify current clinical trials with Ro-07-0582 to try to enhance local tumour control with radiotherapy. However, it is doubtful whether they are of value in predicting the outcome of such trials.

First, significant enhancement in this system was only seen with doses of irradiation higher than those currently used in most fractionated radiotherapy regimes. It might perhaps be argued from these data that fractions should be delivered in larger doses to exploit more fully any radiosensitising effect.

Second, and more important, is the argument that even if hypoxia is initially present within a tumour, fractionated treatment regimes may allow sufficient re-oxygenation between each treatment dose to render radiosensitisers superfluous. Indirect evidence for this has been obtained from tumour control studies in experimental animal tumours [Suit and Maeda, 1967; Field et al, 1968; Howes, 1969]. And Elkind [1970] has proposed that the hypothesis might be investigated in a clonogenic assay system as follows: tumours are first irradiated with a dose known to reduce the surviving fraction of

cells to a value on the exponential tail of the biphasic air-breathing curve which would imply survival only of hypoxic cells; at some later time graded second doses of irradiation would be given to these tumours to produce a second air-breathing survival curve. If all cells remained hypoxic, this second curve should resemble the hypoxic in vivo curve for HX32 cells shown in Figure 8.2. If on the other hand this curve showed any further biphasic displacement, as in the 0 hour air-breathing curve of Figure 8.2, then this would indicate that re-oxygenation of a fraction of previously hypoxic cells had occurred between the two radiation treatments. This experiment would be quite feasible in the xenograft system, but it would be large and time-consuming, and has not so far been attempted.

In conclusion, therefore, it would seem unwarranted on theoretical grounds to extrapolate at present these data from single dose irradiation experiments to clinical treatment with fractionated dose radiotherapy. The long term potential of this experimental approach is more likely to emerge if current clinical trials first demonstrate a correlation on purely empirical grounds, i.e. if Ro-07-0582 proves to be as effective in improving tumour response to radiotherapy in the patient as it is in the xenograft model. If a predictive value for the diffusion chamber assay can be established in this way, then the system may prove to be a very useful screening assay for a wide range of potential new radiosensitising compounds prior to their use in more elaborate and time-consuming clinical pilot studies.

# CHAPTER TEN

# THE RESPONSE TO CYTOTOXIC DRUGS OF CLONOGENIC CELLS FROM HUMAN TUMOUR XENOGRAFTS

# CHAPTER 10:

# THE RESPONSE TO CYTOTOXIC DRUGS OF CLONOGENIC CELLS FROM HUMAN TUMOUR XENOGRAFTS

## 10.1: INTRODUCTION

### 10.1.a: Chemosensitivity - Animal Tumours

So far in this thesis, clonogenic cell survival assays have been discussed only in terms of the radiobiological data they can provide. In recent years a large number of chemotherapeutic agents have also been studied in this way using experimental murine tumours. The effect of cytotoxic drugs on cell survival appears to differ from that of radiation in two main ways: first, there is a much greater variation in the steepness of the cell survival curve for a given agent between different tumours; and second, the shape of the curve may differ markedly for different drugs.

Some examples can be quoted. Cyclophosphamide produces an exponential survival curve for most tumours tested, but the dose required to achieve one log kill varies from about 2mg/kg for the exquisitely sensitive PC-5 myeloma [Ogawa et al, 1973] to between 105 and 125mg/kg for the EMT6 tumour [Hahn et al, 1973] and the B16 melanoma [Hill and Stanley, 1975], with the sensitivities of other tumours including L1210 leukaemia, AKR lymphoma, C22RL osteosarcoma and Lewis lung tumour lying between these extremes [Bruce et al, 1966; van Putten and Lelieveld, 1970; Razek et al, 1974; van Putten, 1974; Steel and Adams, 1975]. Other drugs for

which exponential survival curves with a wide range of sensitivities for different tumours have been obtained include BCNU, CCNU and vincristine [Bruce et al, 1969; Lin and Bruce, 1972; Razek et al, 1974; van Putten, 1974; Hill and Stanley, 1975; Steel and Stanley, 1976].

In contrast, some drugs give cell survival curves that are not exponential but fall to a plateau; these include methotrexate and vinblastine for the AKR lymphoma [Bruce et al, 1966] and cytosine arabinoside for AKR lymphoma, L1210 leukaemia and B16 melanoma [Bruce et al, 1969; Edelstein et al, 1974; Hill and Stanley, 1976]. 5-fluorouracil gives a plateau for KHT and EMT6 tumours, though with differing sensitivities of 40% and 5% respectively [Lin and Bruce, 1972; Hahn et al, 1973]. On the other hand this drug gives an exponential survival curve for AKR lymphoma whether given as a single dose or in divided doses [Bruce et al, 1966; Vietti et al, 1971].

Tumour size, the time interval between treatment and sampling, and for certain drugs the duration of exposure have all been shown to influence chemosensitivity measured by cell survival [Bruce et al, 1969; Steel and Stanley, 1976; Hahn et al, 1973] and these must be standardised before comparisons of drug sensitivity can be made. However, these alone cannot fully account for the wide variation in both steepness of slope and shape of cell survival curves after exposure to chemotherapeutic agents.

Bruce, Meeker and Valeriote [1966] in a classic study demonstrated three classes of response to anti-cancer agents for the AKR lymphoma and for mouse marrow using a spleen colony assay, and they interpreted these differences in response on the basis of cell cycle kinetic parameters. In the first class (Type 1 response), obtained with nitrogen mustard and $\gamma$-irradiation, survival curves were approximately exponential and the sensitivities of both marrow and lymphoma cells were similar. This is represented schematically in Figure 10.1a, and is explained on the basis that all cells, both proliferating and non-proliferating, are equally affected by these agents.

In the second class (Type 2 response) which included tritiated thymidine, vinblastine and methotrexate, cell survival decreased as a function of dose to constant values, i.e. the dose survival curves above a certain dose plateaued out. These plateau levels were similar for all drugs in this group but differed markedly between normal and lymphoma cells. This is represented schematically in Figure 10.1b. This type of response is explained on the basis that drugs in this group act only on proliferating cells during a specific phase of the cell cycle and that cells not passing through this vulnerable phase during the period of drug exposure will not be killed however much dosage is increased. The differential plateau effect with these agents is dependent on the fact that the proliferating fraction of lymphoma cells during treatment is much greater than that of bone marrow.

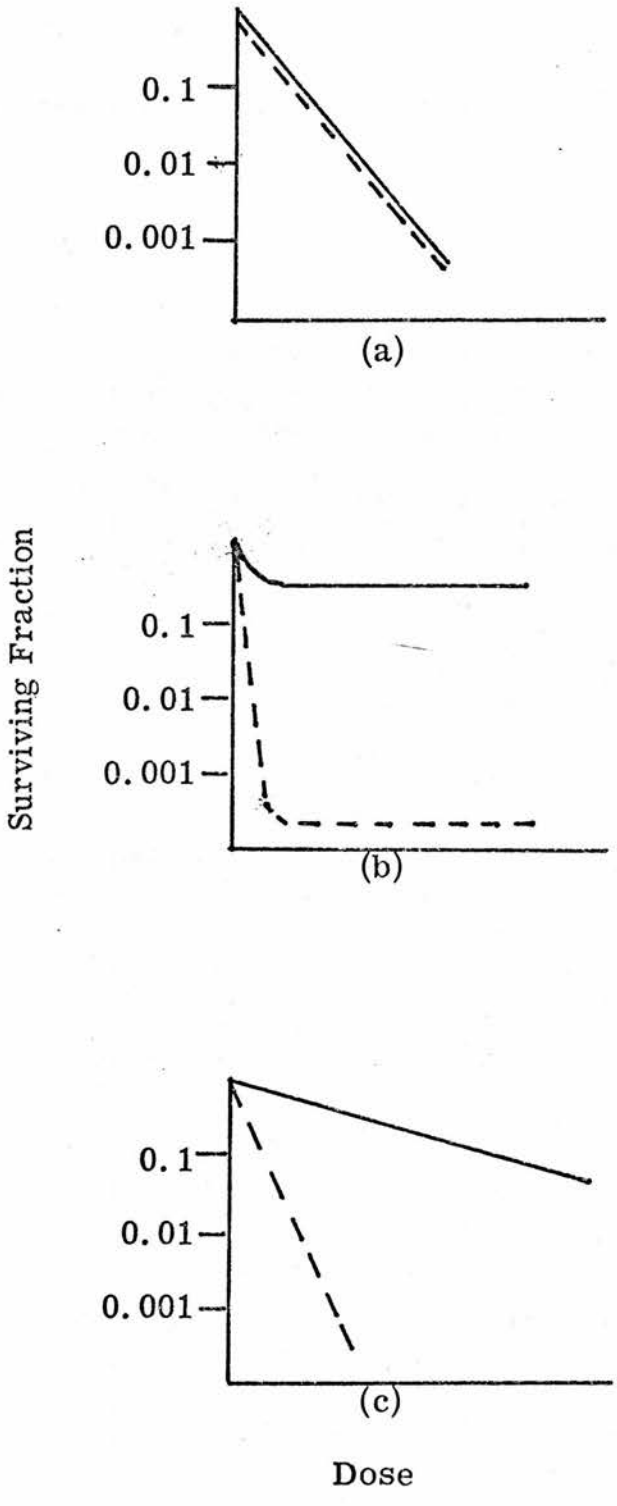
In the third class of response (Type 3 response), obtained with cyclophosphamide, actinomycin D and 5-fluorouracil, survival curves were exponential as in the Type 1 response but with the important difference that here the lymphoma cells were six to tenfold more sensitive than marrow cells. This is represented schematically in Figure 10.1c. This response was interpreted on the basis that Type 3 agents act only on proliferating cells, but throughout all phases of the cell cycle. Since all proliferating cells are at risk cell kill is exponential, but since the fraction of proliferating cells varies between the two types a differential effect is seen.

These observations of Bruce and his colleagues were of importance in their demonstration that the activity of many cytotoxic drugs was closely dependent on the proliferative state of the cell and often on the specific phase of the cell cycle during drug exposure. This has been of considerable clinical value, and has led for example to the concept that high doses of phase-specific (Type 2 response) drugs can be administered in cancer chemotherapy without intolerable toxicity provided the duration of exposure to the drug is short [Goldie et al, 1972].

However, in some instances Bruce's data have been extrapolated in a literal way to the design of clinical chemotherapy regimes on the assumption that careful scheduling of drug administration could exploit similar kinetic differences between human tumour and normal tissues and thus increase tumour response [Price et al 1975; Lampkin et al, 1976].

This assumption is questionable on two grounds. First, others have suggested that differential chemosensitivity between tumour and marrow cells is not always dependent on kinetic differences. Van Putten and Lelieveld [1970] have demonstrated a similar increased sensitivity to cyclophosphamide of C22LR osteosarcoma cells compared with marrow, but have argued that differences in the kinetics of these two tissues are insufficient to explain this differential response and that instead differences in the inate chemosensitivity of the two cell types must be involved, independent of their proliferative activity. And it seems increasingly likely that differential sensitivity must play an important part in explaining many of the observed differences in drug effect for different tumours, as more is learned of the characteristics of their cell cycle kinetics.

The second objection is simply this: even if the interpretation of his data on a kinetic basis is correct for the system Bruce studied, important differences have been demonstrated between the kinetics of human tissues and those of the AKR lymphoma and other experimental leukaemias. These include in particular the smaller fraction of cells proliferating, the longer cell cycle time and the greater heterogeneity of cell cycle times for human tumours so far studied [Clarkson et al, 1965; de Vita, 1971; van Putten, 1974; Tubiana and Malaise, 1976]. It is, sadly, not surprising therefore that no convincing evidence for an increased therapeutic response based on this type of kinetic scheduling appears so far to have been demonstrated in clinical cancer chemotherapy.




From:

Bruce et al, 1966

FIGURE 10.1

Schematic Representation of Cell Survival for Mouse Marrow (——) and AKR Lymphoma (— — —) After Treatment with (a) Type 1 agents (b) Type 2 agents (c) Type 3 agents. See Text for Details (Chapter 10.1)

Unfortunately, similar data on the chemosensitivity of human tumour cells which might more validly be extrapolated to the clinic have not so far been available.

10.1.b: Chemosensitivity - Human Tumours

The reason it has not been possible to repeat similar sophisticated cell survival studies for human tumours exposed to cytotoxic chemotherapy has been the absence of an equivalent clonogenic assay system. Other techniques have been employed in an attempt to quantify the response of human tumour cells to cytotoxic drugs *in vitro* and to correlate this with subsequent clinical response to chemotherapy.

The earliest attempts were made almost 20 years ago [Wright *et al*, 1957; 1962]; in these studies changes in the morphology of cultured cells from human tumours were used as a measure of drug sensitivity. And later, similar studies were attempted in which the uptake of radioactive precursors of nucleic acid and protein synthesis was used to measure chemosensitivity of human tumour slices incubated *in vitro* [Yarnell *et al*, 1964; Bickis *et al*, 1966]. More recently Dendy and his co-workers have carried out painstaking work in this field, and have described a technique for growing cell suspensions of human tumours, and in particular ovarian carcinomas, in short term monolayer cultures which are incubated with a series of cytotoxic drugs to determine chemosensitivity [Dendy, 1968; Dendy *et al*, 1970]. In early experiments, morphological damage to cells in culture was used as a measure of drug sensitivity. Later, the uptake of labelled precursors of DNA

synthesis was used as an end point to measure drug effect. Holmes and Little [1974] used a tissue culture micro-test for predicting the response of human tumours to chemotherapy. In this study single cell suspensions of human tumours were incubated for 72 hours with cytotoxic drugs, the end point for measurement of response being the number of cells remaining as detected by Coulter counting. A similar approach was used by Berry et al [1975] who described a method for studying the chemosensitivity of fresh explants of human tumours cultured in vitro for 7 days with different drugs added to the culture medium; here increase in total cell number after 7 days growth in culture was used as a biological end point.

Some, but not all of the studies, claimed a correlation between the predicted in vitro chemosensitivity, and subsequent clinical response to chemotherapy in the patient, but such data is so far small and inconclusive. Furthermore detailed quantitative data on cell survival is clearly not possible with the various end points for measurement of drug response used. These in vitro systems have several other important limitations. Certain drugs which first require in vivo activation by the liver, including in particular cyclophosphamide, cannot be studied directly in this way. Toxic derivatives of cyclophosphamide can be obtained in vitro by incubating the drug with liver microsomes [Connors, 1970] but attempts to standardise this procedure for in vitro assays have proved unsuccessful in some hands [Denby et al, 1976] and usually this important drug has been excluded from these studies. Secondly,

it is becoming apparent that a good correlation between decreased uptake of labelled precursors and cell kill does not always exist: for example, $^{3}$H-thymidine incorporation initially increases after methotrexate exposure in mammalian cells [Tattersall and Harrap, 1973]. Finally, it is difficult to demonstrate in these short term culture systems that the cells growing in monolayer are indeed all tumour cells and not cells derived from stromal elements. Chromosomal analysis was used in some of these studies to try to identify normal from tumour cells, but this approach can only be applied to dividing cells, and gives no information on non-dividing cells which may also be affected by drugs in these systems.

More recently Berry's group has reported on the reproductive survival of cells from their explant system after exposure to vinblastine [Wells et al, 1976] or nitrogen mustard [Wells et al, 1977], using a cloning assay in which treated cells grow colonies after plating on to irradiated foetal cell layers in Petri dishes.

This approach is of considerable potential interest in that it permits more detailed quantitative data on the effects of different cytotoxic agents on cell survival and allows cell survival curves to be constructed. However, certain features of the assay suggest that some artificial selecting out of unrepresentative cells may occur during the initial monolayer culture and sub culture required for this assay. First, the plating efficiencies of up to 75% for this system are rather higher than that found for most animal tumour systems in which cells are plated out directly from the tumour.

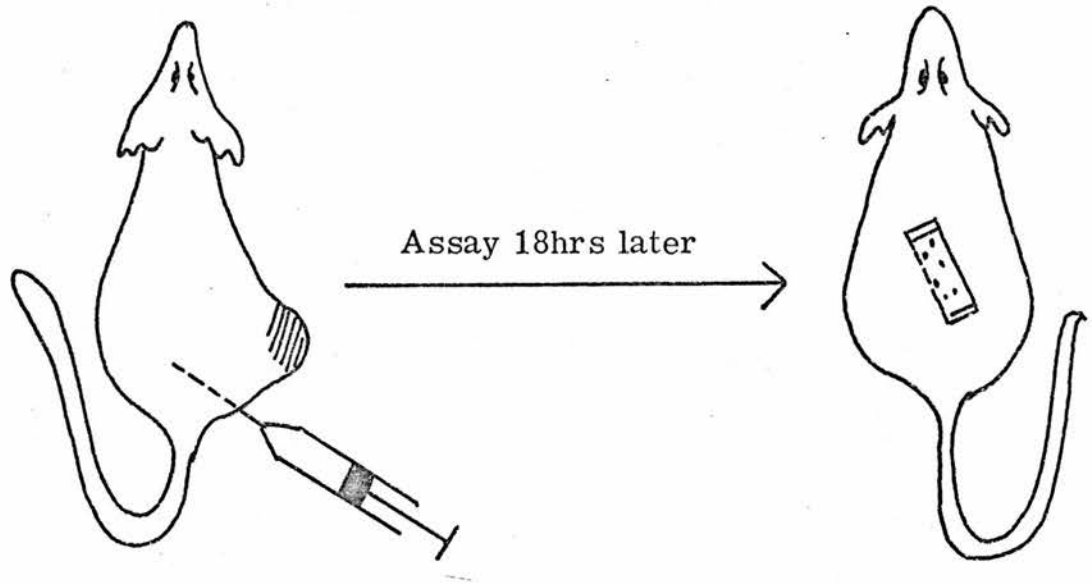
And second the data obtained does not always reflect clinical experience: for example cells from a seminoma were less sensitive to both vinblastine and nitrogen mustard than cells from most other tumours studied including a melanoma; this certainly very much contradicts the clinical response to chemotherapy usually observed for these. Nevertheless this work represented an important attempt to break away from the limitations of cell culture techniques in the measurement of human tumour cell chemosensitivity, and encouraged the hope that other techniques might be adapted to study the chemosensitivity of clonogenic human tumour cells.

## 10.2: METHODS

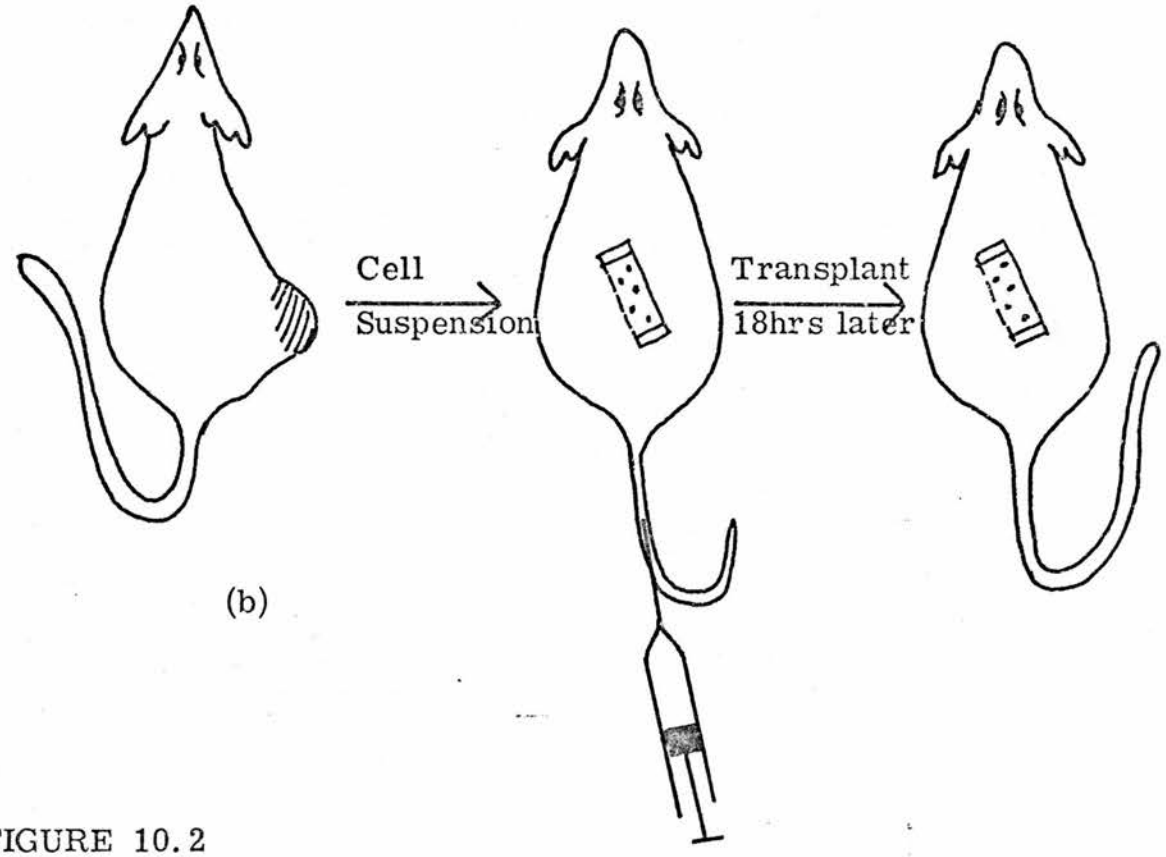
### 10.2.a: Theoretical Approaches: In Vivo and In Mure

The diffusion chamber system can in theory be used to measure the chemosensitivity of cells from human tumour xenografts in two different ways. In the first method, the tumour-bearing mouse is treated with the drug under study, and the tumour subsequently dissected out after an appropriate time interval for assay (Fig. 10.2a). In the second method, a cell suspension of an untreated tumour can be loaded into diffusion chambers for implantation; once this is done the chamber-bearing mouse is treated with the drug and the chamber then transplanted after an appropriate time interval (Fig 10.2b).

The first is obviously an in vivo method whose main advantage is that tumour cells in situ are exposed to drugs under physiological conditions which hopefully might resemble those pertaining in the clinic (with the inevitable proviso that drug metabolism is murine and




(a)




(b)

FIGURE 10.2

Human Tumour Xenograft Cytotoxic Drug Assay Techniques

(a) In vivo by intra-peritoneal drug administration and assay 18hrs later

(b) In the mouse by tail vein injection of chamber-bearing mouse and transplant 18 hours later

not human). The second technique is highly unphysiological: single tumour cells in medium and agar are exposed to drugs under conditions very different to those pertaining in vivo, and data obtained with this technique must be interpreted with this important limitation in mind. This point will be discussed in more detail below (Chapter 10.4.b). But this second assay technique also has two important advantages over the in vivo assay. First, since clonogenic cells of both human tumours and human bone marrow can be assayed in the diffusion chamber system, the relative sensitivities of these to the same cytotoxic agent can be compared using this latter technique. The low therapeutic index and high risk of marrow toxicity associated with most current cancer chemotherapy regimes suggest that such comparative data might be of value in understanding and predicting differential therapeutic response between marrow and tumour tissue. Second, this technique would provide the only way of measuring the chemosensitivity of clonogenic human tumour cells taken directly from the patient, rather than from a xenograft, if the diffusion chamber assay could be adapted to grow colonies directly in this way (Chapter 11).

The feasibility of both methods for assaying the chemosensitivity of human tumour cells was therefore investigated in the experiments to be described. The technique whereby cellular chemosensitivity is assayed by treating chamber-bearing mice raises an interesting question in semantics. The method is not strictly an in vivo one, but neither can it be described as in vitro. A new term seems indicated and since cells are treated after implantation into the mouse, I shall use the term in mure to describe this system.

10.2.b: Experimental Design

Several groups of experiments were carried out. In the first series the in vivo dose-survival characteristics for 2 tumours, pancreatic xenograft HX32 and colonic carcinoma HX18, treated with cyclophosphamide were studied. In the second series of experiments similar studies were undertaken for cells from these tumours treated with the same drug in mure. Once the feasibility of the in mure technique had been established, this was used to compare the sensitivity to cyclophosphamide of human tumour cells with cells from the murine Lewis lung tumour.

In a final series of experiments the comparative chemosensitivity of pancreatic carcinoma HX32 to a series of cytotoxic drugs was measured, treatment being administered in vivo. Similar comparative chemosensitivity studies were made on colonic carcinoma xenograft HXK1 and a rectal carcinoma xenograft HXK4, treated in mure. These latter experiments were designed to test whether similar comparative chemosensitivity studies on human tumour cells direct from the patient might be theoretically feasible.

10.2.c: Xenograft Tumours

4 tumour xenografts were used in these experiments: pancreatic carcinoma HX32, colonic carcinoma HX18, colonic carcinoma HXK1 and colonic carcinoma HXK4. Relevant details of these tumours have already been described (Chapter 5.1 and Tables 5.1 - 5.3). The latter two xenografts were selected for study because information was already available on the clinical behaviour of the

original tumours from which they were derived. Furthermore gross tumour growth delay studies (Chapter 3.1) had also been carried out on these xenografts by my colleague Mr. Novak. These data will be described below (Chapter 10.4.e).

10.2.d: Lewis Lung Tumour

The Lewis lung carcinoma is a transplantable tumour that arose spontaneously in the lung of a C57Bl mouse [Sugiura and Stock, 1955] and has subsequently been widely used in radiobiological and chemotherapeutic aspects of experimental cancer research. It is currently used as one of the standard animal tumours in the NCI drug screening programme. For this reason it seemed an appropriate choice of experimental tumour to compare with the human tumour xenografts in chemosensitivity studies using the diffusion chamber assay.

Single cell suspensions of this tumour were obtained for these experiments by finely chopping the tumour with crossed scalpels, washing the pieces in phosphate-buffered saline and then trypsinising at 37$^{o}$C for 15 minutes with 1:200 trypsin. Subsequent steps to obtain the final single cell suspension were identical to those described for xenografts HX32 and HX 18 (Chapter 5.3).

10.2.e: Cyclophosphamide Administration - In Vivo Assays

Tumours growing intra-muscularly in the thighs of immune-suppressed mice (Chapter 5.2) were used in these experiments, at a time when the tumours had grown to a diameter of 0.5 to 1cm. Cyclophosphamide B.P. (W.B. Pharmaceuticals Ltd.) was dissolved

in saline and administered by intra-peritoneal injection 18 hours prior to assay (Fig. 10.2a).

10.2.f: Cyclophosphamide Administration - In Mure Assays

Single tumour cell suspensions were loaded into diffusion chambers and implanted into the peritoneal cavities of pre-treated C57Bl mice under ether anaesthesia as previously described (Chapter 5.4). At this time, cyclophosphamide was also administered intra-venously by tail vein injection. This route was preferred to that of intraperitoneal injection to prevent artificially high local concentrations of drug developing initially around the implanted diffusion chambers. Chambers were transplanted 18 hours later to a new batch of pre-treated mice, because the combination of cyclophosphamide and total-body irradiation in the same mouse was lethal after a few days (Fig. 10.2b).

10.2.g: Comparative Chemosensitivity Experiments

In these experiments, the comparative chemosensitivity of clonogenic cells from pancreatic carcinoma HX32 to adriamycin, 5-fluorouracil, vinblastine, methotrexate and methyl-CCNU was studied in vivo, and the comparative chemosensitivities of clonogenic cells from colonic carcinoma HXK1 and rectal carcinoma HXK4 to 5-fluorouracil, actinomycin D and methyl-CCNU were studied in mure. (For a note on all the cytotoxic agents used in these studies see Appendix 2.)

It was decided to administer equitoxic doses of each drug to allow comparison of effects on tumour cell kill, and therefore the $LD_{10}$ dose of each drug for C57Bl mice (that dose which is lethal to

10% of this strain of mouse) was given in each of these comparative chemosensitivity experiments. The $LD_{10}$ doses for each drug, and hence the dose used in these experiments, are given in Table 10.1.

All drugs were prepared by dissolving in saline immediately prior to use, except for methyl-CCNU which posed special problems because of its low solubility in aqueous solutions. Methyl-CCNU was therefore obtained in solution by dissolving in dimethyl-sulphoxide (DMSO) subsequently diluted 1:9 with a 5% solution of Tween 80 (BDH Chemicals) in normal saline.

As before, drugs were administered intraperitoneally 18 hours before assay for the *in vivo* studies and intravenously by tail vein injection at the time of chamber implantation for the *in mure* studies, chambers being transplanted 18 hours later.

## 10.3: RESULTS

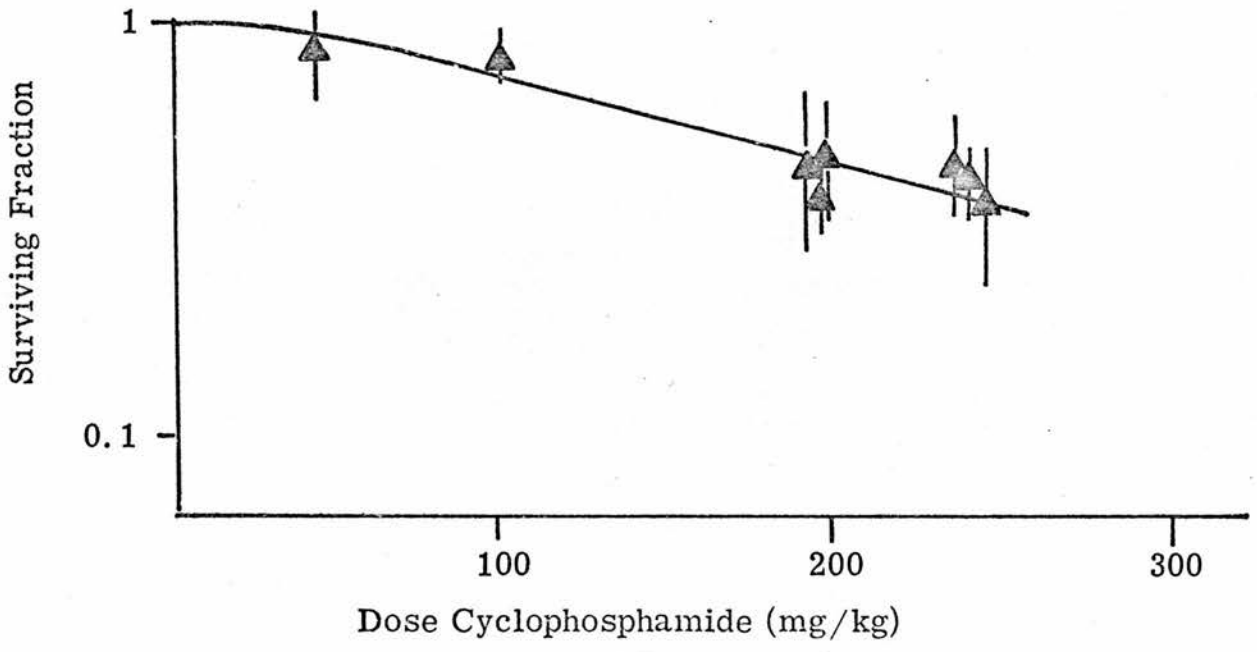
### 10.3.a: In Vivo Cyclophosphamide Dose Survival

The dose survival curves for clonogenic cells from pancreatic carcinoma HX32 and colonic carcinoma HX18 treated with single dose cyclophosphamide *in vivo* are shown in Figure 10.3 and their parameters calculated by linear regression analysis given in Table 10.2. These curves appeared to be exponential but in both cases the range of cell kill was small, with less than one log kill even at the maximum tolerated dose to the mouse. The $D_O$ convention has not been so universally accepted for cell survival curves for cytotoxic drugs as for radiation, and instead the $D_{10}$ (the dose of drug required to

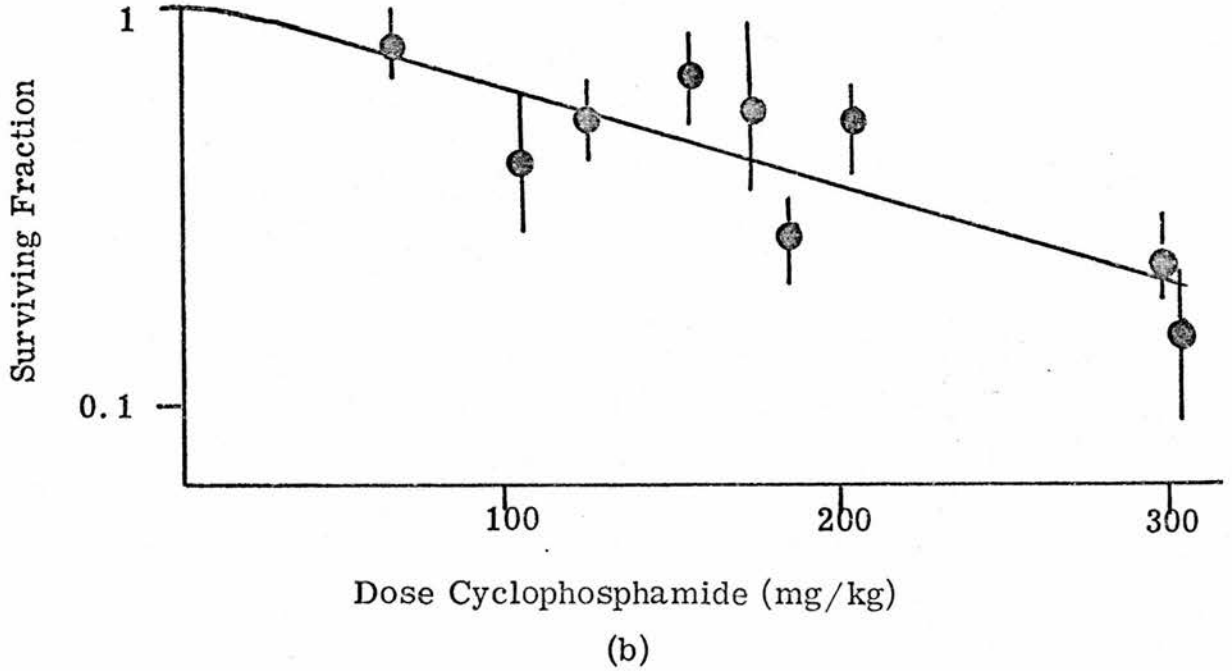
| Drug | $LD_{10}$ Dose for C57Bl Mice |
|---|---|
| Actinomycin D | 0.5mg/kg |
| Adriamycin | 15mg/kg |
| 5-Fluorouracil | 250mg/kg |
| Methotrexate | 200mg/kg |
| Methyl-CCNU | 30mg/kg |
| Vinblastine | 3mg/kg |

TABLE 10.1

Approximate $LD_{10}$ Doses for C57Bl Mice of Actinomycin D, Adriamycin, 5-Fluorouracil, Methotrexate, Methyl CCNU and Vinblastine




(a)




(b)

FIGURE 10.3

Response to Cyclophosphamide of Cells from Pancreatic Carcinoma HX32 (a) and Colonic Carcinoma HX18 (b) Treated In Vivo (Vertical Bars Represent ± Standard Error of Mean)

| Tumour | $D_{10}$(mg/kg) | Range* | $D_Q$ (mg/kg) | Range |
|---|---|---|---|---|
| Pancreatic carcinoma HX32 | 456 | (415 - 508) | 53 | ( 33 - 68) |
| Colonic carcinoma HX18 | 404 | (331 - 520) | 18 | (-37 - (+)53) |

*$\pm$ Standard Error of the Mean

TABLE 10.2

$D_{10}$ and $D_Q$ Values for the Cell Survival Curves for Pancreatic Carcinoma HX32 and Colonic Carcinoma HX18 Xenografts Treated with Cyclophosphamide In Vivo

achieve 1 log cell kill) is often used. This latter notation will be used here. For HX32 the predicted $D_{10}$ was 456 (+52-41)mg/kg and for HX18 404 (+116-73)mg/kg (Table 10.2) although in neither case were doses as large as these actually tested. Pancreatic carcinoma HX32 curve had a small initial shoulder with a $D_Q$ value of 53 (+15-20) rad; colonic carcinoma HX18 curve had no definite shoulder within the range of one standard error, the $D_Q$ value being 18 (+35-55) rad. There was no significant difference between these cyclophosphamide cell survival curve parameters for the two tumours.

10.3.b: In Mure Cyclophosphamide Dose Survival

The dose survival curves for clonogenic cells from pancreatic carcinoma HX32 and colonic carcinoma HX18 treated with single dose cyclophosphamide in mure are shown in Figure 10.4 and their parameters given in Table 10.3.

Cell kill was exponential for both tumours over the range of doses studied, and in both instances cells were significantly more sensitive to cyclophosphamide treated this way than when treated in vivo. The $D_{10}$ value for HX32 cells was 169 (+7-7)mg/kg compared with 456mg/kg treated in vivo ($p < 0.01$), and the $D_{10}$ value for HX18 cells was 105 (+17-12)mg/kg compared with 404mg/kg treated in vivo ($p < 0.005$). Although no difference in sensitivity to cyclophosphamide between two tumours treated in vivo had been demonstrated, a significant difference was found between the two tumours treated with cyclophosphamide in mure ($p < 0.05$). No significant shoulders were demonstrated for either of these curves (Table 10.3).

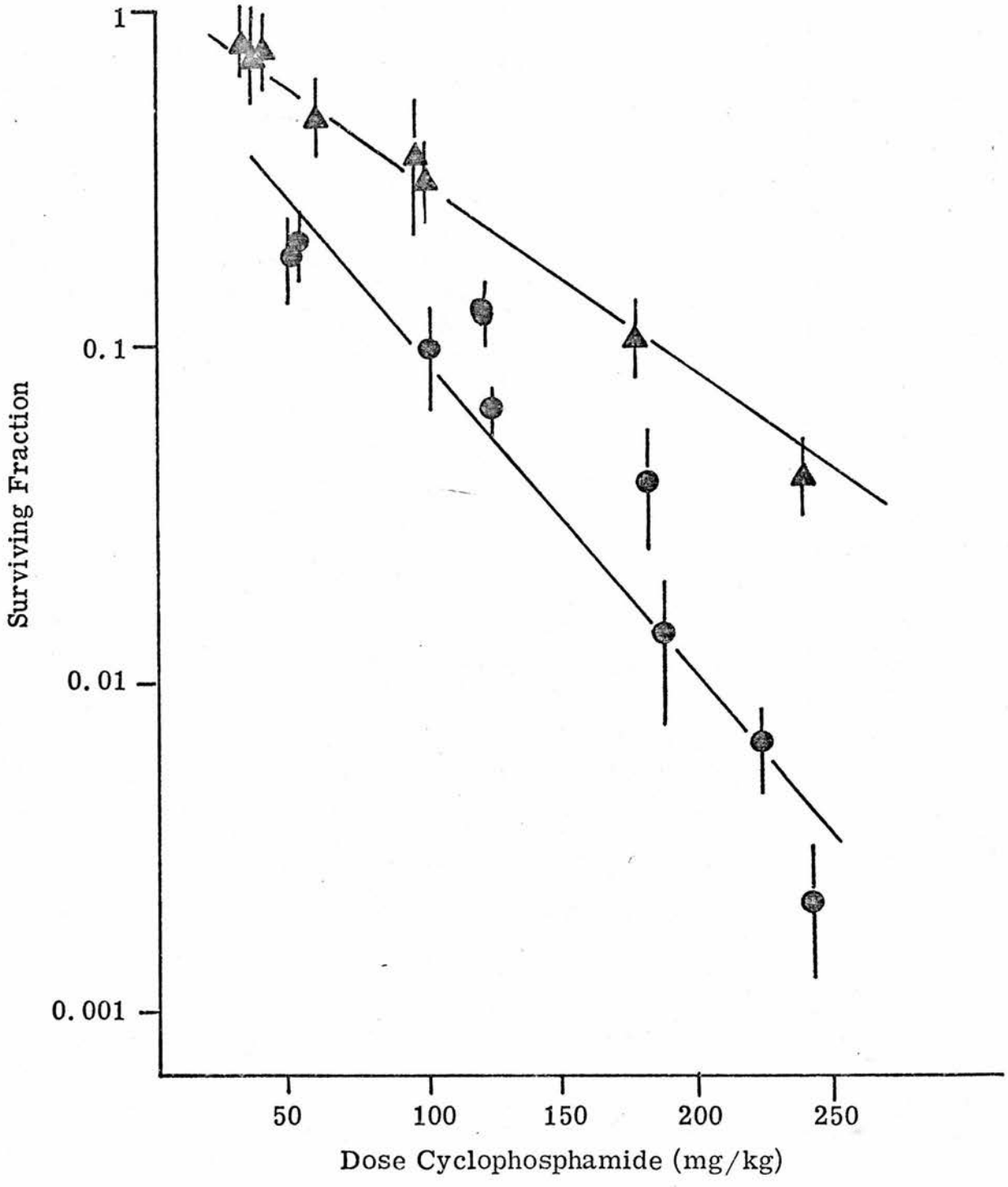



FIGURE 10.4

Response to Cyclophosphamide of Cells From Pancreatic Carcinoma HX32 (▲) and Colonic Carcinoma HX18 (●) Treated In the Mouse (Vertical Bars Represent ± Standard Error of the Mean)

| | $D_{10}$ (mg/kg) | Range* | $D_Q$ (mg/kg) | Range |
|---|---|---|---|---|
| Pancreatic carcinoma HX32 | 169 | (162 - 176) | 9 | ( 4 - 14) |
| Colonic carcinoma HX18 | 105 | ( 93 - 122) | -8 | (-35 - (+)12) |
| Lewis lung tumour | 48 | ( 46 - 51) | -17 | (-26 - (-)10) |
| Human bone marrow** | 37 | ( 34 - 40) | 8 | ( 2 - 13) |

*$\pm$ Standard Error of the Mean

**Data from M.Y. Gordon [personal communication]

TABLE 10.3

10.3.c: Lewis Lung In Mure Cyclophosphamide Dose Survival

The dose survival curve for clonogenic cells from Lewis lung tumour treated with cyclophosphamide in mure is shown in Figure 10.5. Cell kill was again exponential, but Lewis lung tumour cells with a $D_{10}$ value of 48 (+3-2)mg/kg were significantly more sensitive to cyclophosphamide than cells from either the colonic carcinoma HX18 ($p < 0.001$) or from pancreatic carcinoma HX32 ($p < 0.001$). No significant shoulder was found for this curve (Table 10.3).

10.3.d: Human Bone Marrow In Mure Cyclophosphamide Dose Survival

My colleague, Dr. M.Y. Gordon, working in the same department, had previously established a cyclophosphamide cell survival curve for clonogenic cells from human bone marrow also using an agar in diffusion chamber technique, and details of her experimental methods were identical to those used by me (Chapter 10.2.f). She has kindly allowed me to quote her results here, and the comparative cyclophosphamide cell survival data for human marrow and human tumour xenografts (HX18 and HX32) treated in mure are shown in Figure 10.6. Cyclophosphamide cell kill was again exponential for human marrow, but the sensitivity of bone marrow cells was much greater than that of either tumour with a $D_{10}$ value of 37 (+3-3)mg/kg. This difference was statistically significant compared with both colonic carcinoma HX18 ($p < 0.001$) and pancreatic carcinoma HX32 ($p < 0.001$).

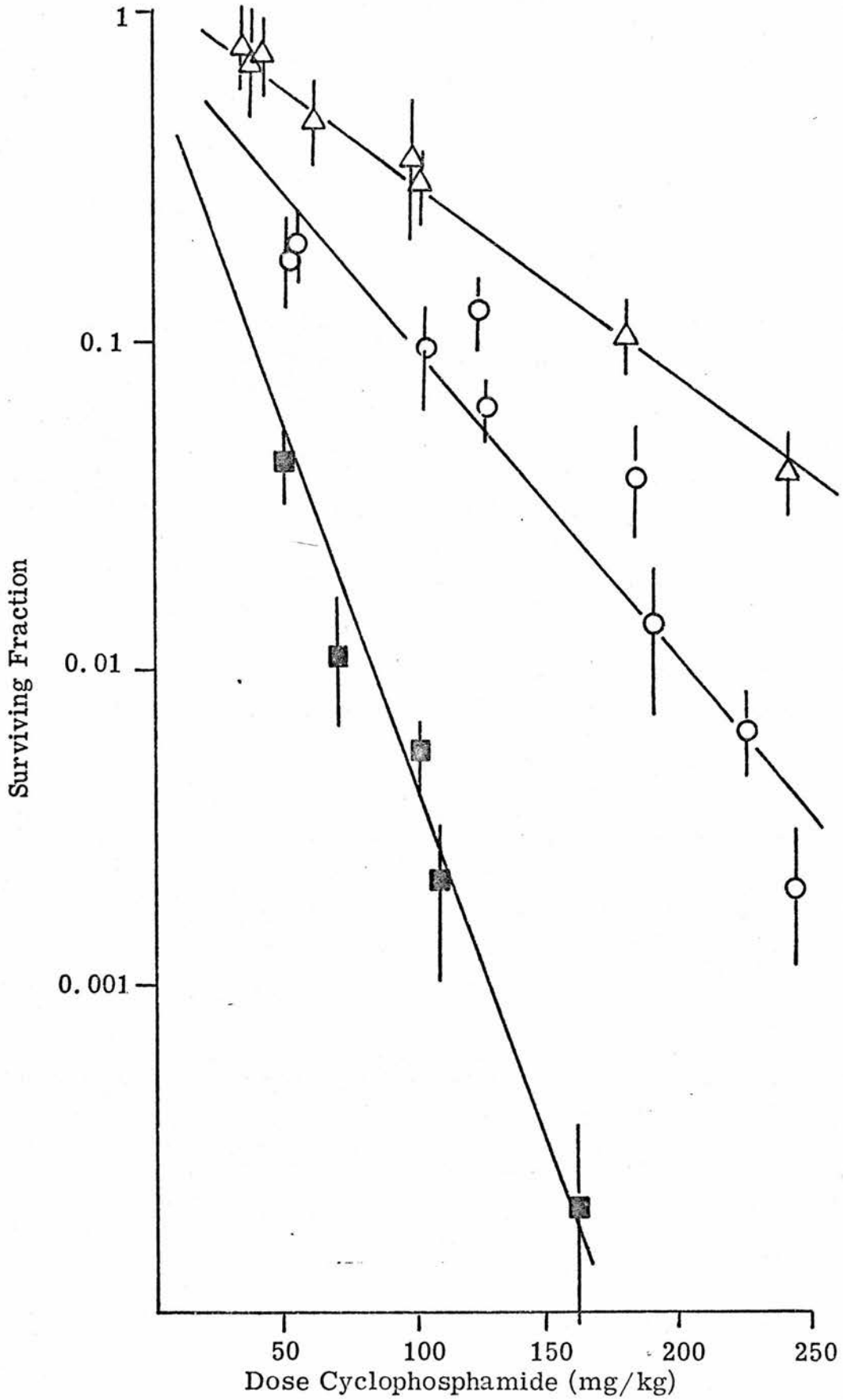



FIGURE 10.5

Response to Cyclophosphamide of Cells from Lewis Lung Tumour (□) Compared with Cells from HX32 (△) and HX 18 (○) Treated In the Mouse (Vertical Bars Represent ± Standard Error of the Mean)

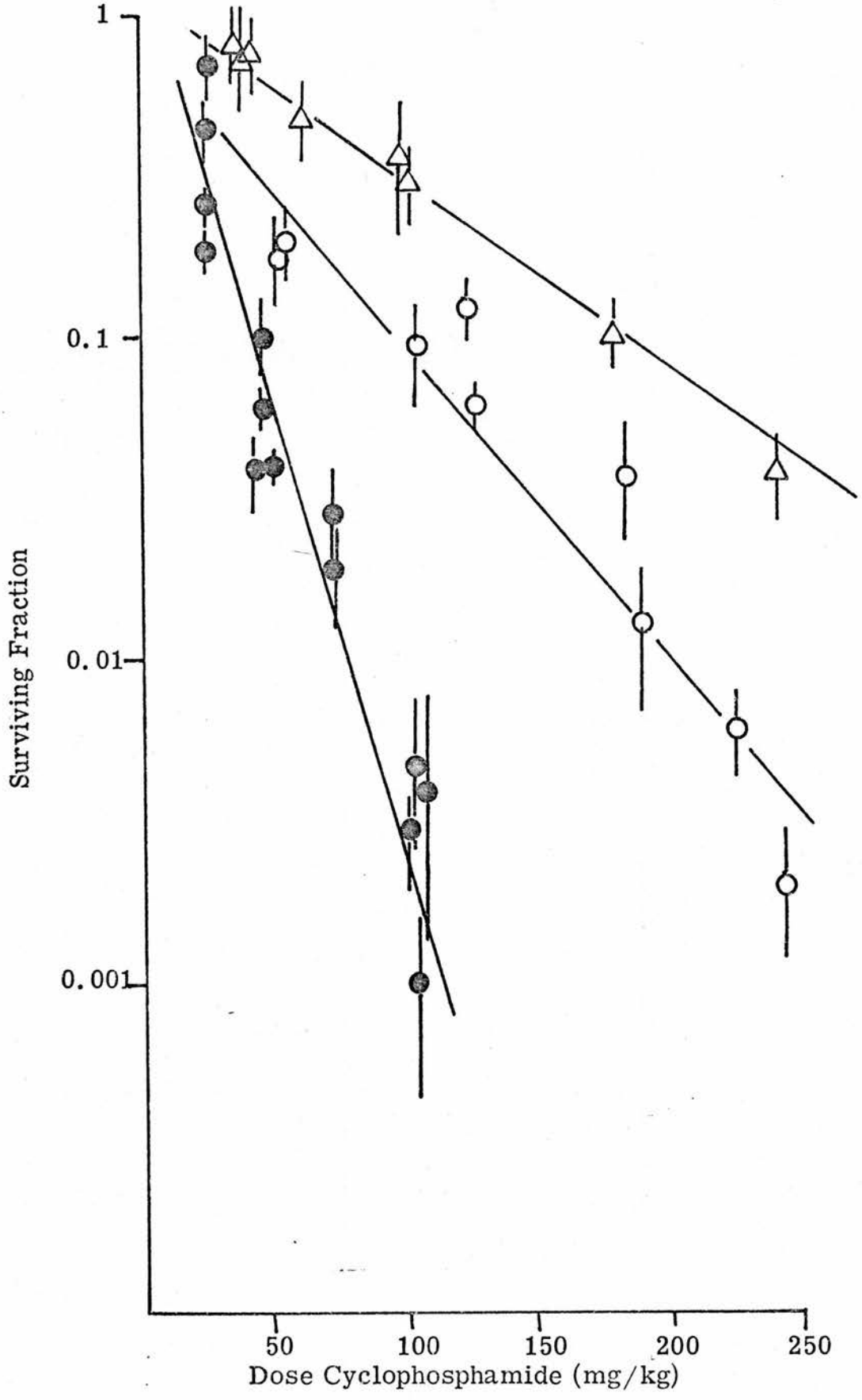



FIGURE 10.6

Response to Cyclophosphamide of Human Bone Marrow Cells (●) [M.Y. Gordon - personal communication] Compared with Cells from HX32 (△) and HX18 (○) Treated In the Mouse (Vertical Bars Represent ± Standard Error of the Mean)

10.3.e: Comparative Chemosensitivity In Vivo of Pancreatic Carcinoma HX32

The surviving fractions (S.F) of cells from pancreatic carcinoma HX32 after treatment in vivo with $LD_{10}$ doses of 5 cytotoxic drugs are shown in Figure 10.7 and the results are as follows:-

after adriamycin 15mg/kg i.p. the S.F was $0.51 \pm 0.06$

after 5-fluorouracil 250mg/kg i.p. the S.F was $0.63 \pm 0.07$

after methotrexate 200mg/kg i.p. the S.F was $0.40 \pm 0.04$

after methyl-CCNU 30mg/kg i.p. the S.F was $0.08 \pm 0.02$

and after vinblastine 3mg/kg i.p. the S.F was $0.49 \pm 0.04$

The increase in cell kill achieved with Methyl-CCNU compared with the other 4 drugs was significant ($p < 0.001$). This experiment however was only carried out once.

10.3.f: Comparative Chemosensitivity In Mure of Colonic Carcinoma HXK1 and Rectal Carcinoma HXK4

The surviving fractions (S.F) of cells from colonic carcinoma HXK1 and rectal carcinoma HXK4 after treatment in mure with 5-fluorouracil, methyl-CCNU and actinomycin D are shown in Figure 10.8 and the results are as follows:- after 5-fluorouracil 250mg/kg i.v. the S.F was $0.42 \pm 0.08$ for HXK1 and $0.73 \pm 0.10$ for HXK4 (this difference was significant at $p < 0.05$); after actinomycin D 0.5mg/kg i.v. the S.F was $0.20 \pm 0.03$ for HXK1 and $0.46 \pm 0.04$ for HXK4 (this difference was significant at $p < 0.002$); after methyl-CCNU 30mg/kg i.v. the S.F was $0.03 \pm 0.01$ for HXK1 and $0.05 \pm 0.01$ for HXK4 (this difference was not statistically significant).

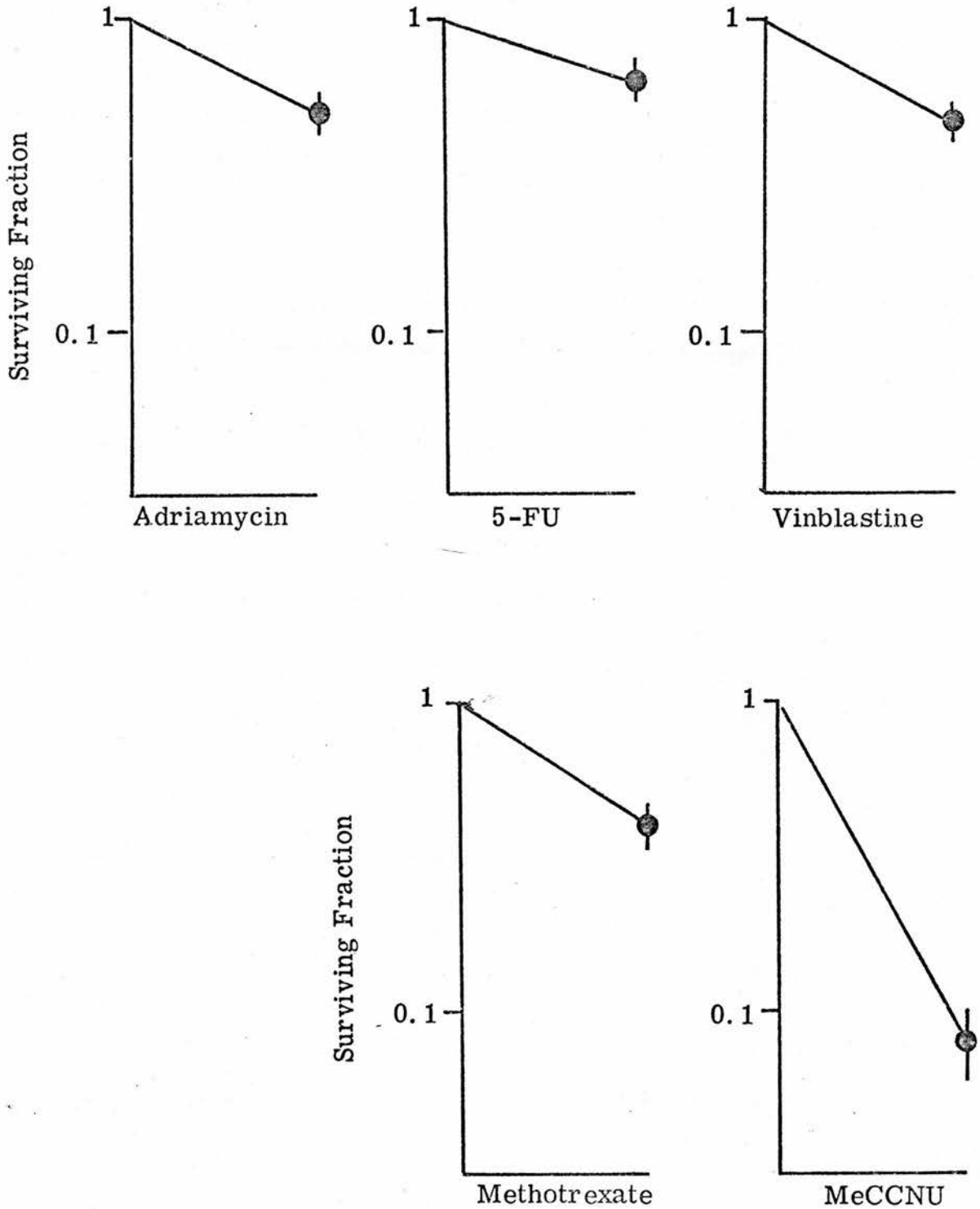



FIGURE 10.7

Response of Cells from Pancreatic Carcinoma Xenograft HX32 to $LD_{10}$ Doses of Adriamycin, 5-FU, Vinblastine, Methotrexate and Methyl CCNU. Treatment In Vivo (Vertical Bars Represent ± Standard Error of the Mean)

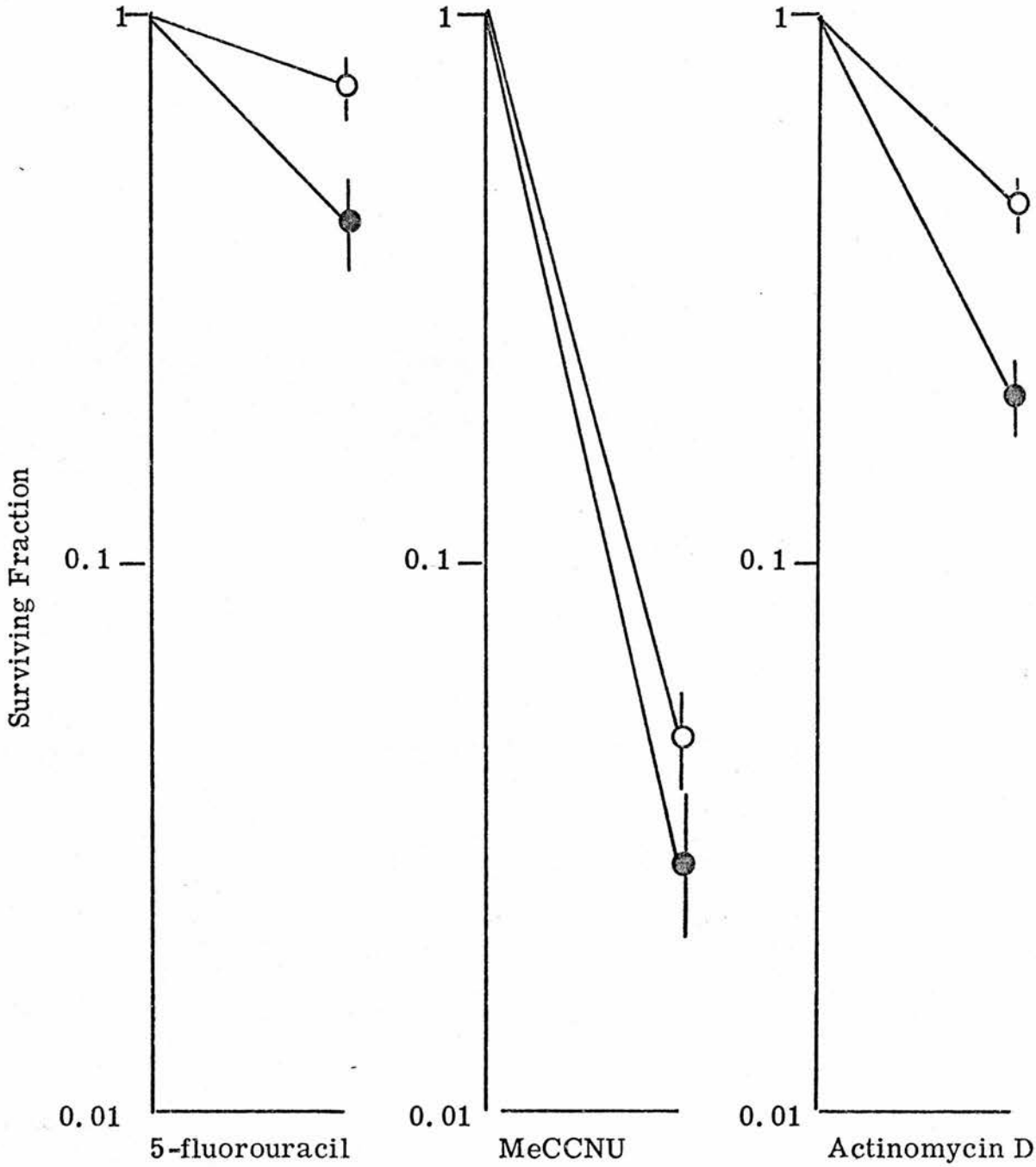



FIGURE 10.8

Response of Cells from Human Colonic Carcinoma Xenografts HXK1 (●) and HXK4 (○) to $LD_{10}$ Doses of 5-Fluorouracil, Methyl-CCNU and Actinomycin D. Treatment In the Mouse (Vertical Bars Represent ± Standard Error of the Mean)

## 10.4: DISCUSSION

The first point to be made about these studies is that they demonstrate the feasibility of measuring the chemosensitivity of clonogenic human tumour cells with the diffusion chamber assay, and they show that it is now possible to provide quantitative information on the effects of cytotoxic drugs previously limited to work with experimental animal tumour models. Both the *in vivo* and the *in mure* techniques can be used to assay chemosensitivity. The latter therefore provides a means for comparing the sensitivity of marrow and tumour cells to cytotoxic drugs, and perhaps for measuring the chemosensitivity of tumour cells removed directly from the patient. An important finding of these experiments, however, is that the sensitivity at least to cyclophosphamide differs depending on which assay is used; this will be discussed below.

### 10.4.a: In Vivo Cyclophosphamide

The *in vivo* cyclophosphamide experiments demonstrate that cells from both the pancreatic and the colonic carcinoma xenografts are very insensitive to this drug when treated *in situ*: the respective doses of 456mg/kg and 404mg/kg to achieve 1 log cell kill are about four times as great as that required for the EMT6 tumour, one of the most cyclophosphamide-resistant experimental animal tumours [Hahn *et al*, 1973], and more than 200 times as great as that required for the PC-5 myeloma [Ogawa *et al*, 1973]. These observations correlate well with clinical experience in the chemotherapy of pancreatic and colonic carcinomas, which have a low

response rate of about 15% to cyclophosphamide [Livingstone and Carter, 1970].

Such a correlation is encouraging and provides circumstantial evidence that the assay may be a valid experimental model for studying the cellular basis underlying clinical response to chemotherapy. It has been calculated on the basis of comparative toxicity data for cytotoxic drugs between mouse and man that the maximum tolerated dose in man is about 1/12 the $LD_{10}$ in mice on a mg/kg basis, and this appears to hold true for most drugs [Freireich et al, 1966]. For cyclophosphamide 300mg/kg, the approximate $LD_{10}$ dose in C57Bl mice, would therefore be eqivalent to 25mg/kg in man, and this is certainly around the maximum dose conventionally used in clinical practice. Yet such a dose in this system produces less than one log cell kill for both tumour xenografts, and if these can be considered in any way representative of human pancreatic and colonic carcinoma in general then the poor clinical response of these tumours to cyclophosphamide can be readily understood. It would obviously be of interest to carry out similar in vivo experiments in xenografts of human tumours known to be clinically sensitive to cyclophosphamide, including for example lymphomas, and attempts to establish such xenografts are under way.

10.4.b: In Mure Cyclophosphamide

It has already been noted that the sensitivity to cyclophosphamide of these two tumours after treatment in mure was significantly greater than when treated in vivo; at the maximum dose of drugs studied in mure, cell kill was increased by one decade for

pancreatic carcinoma HX32 and by two decades for colonic carcinoma HX18. Various factors might be proposed to explain this.

The first is that the trauma of cell suspension and chamber-loading might stimulate some previously "resting" tumour cells in situ into proliferative activity; in other words, "recruitment" of cells from $G_0$ into the cell-cycle might be occurring during the experimental process. Since cyclophosphamide is a cycle-dependent drug (Figure 10.1c), greater cell kill might therefore be achieved by drug exposure in the diffusion chamber system than in situ. Many factors have been shown to trigger resting cells into proliferative activity in experimental systems [van Putten, 1974], and it is possible that this phenomenon may be operating here. However, the magnitude of the difference in cell kill between the two systems argues against this being the only factor involved.

A more likely explanation is that not all cells in the tumours treated in vivo are adequately perfused. Tumour blood supply is maintained mainly by capillary proliferation with larger vessels displaced peripherally; as tumours enlarge the ability of this capillary network to maintain adequate tissue perfusion diminishes, particularly in more central areas of the tumour [Thomlinson, 1973]. This is associated with tissue hypoxia, already discussed in Chapters 8 and 9, and areas of poor perfusion can be demonstrated by intra-arterial dye injection studies [Goldacre and Sylven, 1962]. Rowe-Jones [1968] has demonstrated that the penetration of cytotoxic drugs to such areas is also impaired, and it is very possible that

effective concentrations of active cyclophosphamide metabolites are not uniformly achieved throughout xenograft tumours treated in vivo, and that therefore cell kill in this situation is less than when treatment is given in mure.

A third factor to be considered is the phenomenon of repair of potentially lethal damage. This has already been discussed for cell survival after radiation in the xenograft system (Chapter 8.4.f). Hahn et al [1973] demonstrated that in the EMT6 tumour treated with cyclophosphamide, 5-fluorouracil and bleomycin, cell survival was increased in tumours assayed 24 hours after drug exposure compared with those assayed after 2 hours, and it was postulated that some potentially lethal cytotoxic drug damage might be repaired by factors operating when cells were allowed to remain in situ for some time after drug exposure. From their data a greater than 5-fold increase in survival can be calculated for the tumours treated with cyclophosphamide at maximum dosage. Were the interpretation of Hahn's data correct, and were similar repair factors operating in the xenograft tumours, then it is certainly possible that this might explain differences in survival between cells left in situ after treatment and in cells treated "in isolated" in diffusion chambers. At present however little further is known of the phenomenon of potentially lethal damage repair of tumour cells after cytotoxic drugs and this explanation must therefore remain speculative. It is possible that an in vivo cyclophosphamide experiment assayed 2 hours later might be of value here: if cell survival after in vivo

cyclophosphamide were less assayed at 2 hours compared with 18 hours, this would argue in favour of repair of potentially lethal damage rather than inadequate perfusion as a basis for the differences in cyclophosphamide sensitivity observed between the in mure and in vivo techniques.

Whatever the underlying explanation, it seems reasonable to argue by analogy with in vitro radiation studies that the in mure technique may measure the intrinsic chemosensitivity of clonogenic tumour cells, whereas the in vivo technique may reflect chemosensitivity under physiological conditions, where this intrinsic chemosensitivity is modified by extrinsic factors; these latter may include many processes, of which inadequate tumour perfusion, the ability to repair potentially lethal drug damage in situ, and a significant fraction of "resting" $G_0$ cells have been discussed here. The role and influence of these extrinsic factors are at present perhaps even less well understood for drugs than for radiation (Chapter 8), and their significance may vary for different tumours, thus masking differences in intrinsic chemosensitivity for different tumours. This would explain the finding in these experiments that the in mure sensitivity to cyclophosphamide for pancreatic carcinoma cells and colonic carcinoma cells differed significantly, whereas no such difference was detected with the drug administered in vivo.

### 10.4.c: Lewis Lung Tumour Studies

Previous studies on the murine Lewis lung carcinoma treated with cyclophosphamide in vivo [Steel and Adams, 1975] have shown

that this, like other animal tumours, is very much more sensitive to cyclophosphamide than the pancreatic and colonic carcinoma xenografts treated in vivo here. This marked difference in sensitivity is maintained in the in mure studies and confirms that differences in the intrinsic sensitivity to cyclophosphamide between cells from the Lewis lung tumour and from the human tumour xenografts must exist, independent of other extrinsic factors operating in vivo. It is also of interest that the $D_{10}$ value of 48mg/kg obtained here for the Lewis lung tumour is less than the $D_{10}$ value of around 75mg/kg which I have extrapolated from data on this tumour treated in vivo [Steel and Adams, 1975]. Although a statistical comparison is not possible here, this finding is consistent with the difference in xenograft cyclophosphamide sensitivity obtained in vivo and in mure.

### 10.4.d: Comparative Human Bone Marrow and Human Tumour Cell Studies

The relative sensitivity to cytotoxic drugs of tumour and bone marrow cells is an important factor in determining the success or failure of a cancer chemotherapy regime. Comparative data from experimental animal systems has already been described and the theoretical limitations to their clinical application discussed (Chapter 10.1.a). These limitations are further emphasised in the comparative cyclophosphamide chemosensitivity data for cells from human marrow and human tumours obtained here. Whereas both Bruce and his colleagues [1966] and van Putten and Lelieveld [1970] found their experimental animal tumour cells to be many times more sensitive

to cyclophosphamide than murine marrow, these present studies on human material provide fundamentally different results in that human pancreatic and colonic carcinoma cells are respectively four-fold and two-fold less sensitive than human marrow to this drug.

Obviously this interpretation of these data requires important qualifications. First, both these human tumour types are known to be clinically chemoresistant and it would be of interest to carry out similar comparative studies with chemosensitive tumours, including lymphomas were these available as xenografts. Second, both the data of Bruce and van Putten were obtained from studies in which treatment was given in vivo. But it has already been postulated that the in mure technique measures intrinsic chemosensitivity which may be modified under in vivo conditions by outside factors. Direct comparison of the data is therefore invalid. However, it has been shown here that the sensitivity to cyclophosphamide of these tumour xenografts is even less in vivo than in mure, whereas for marrow sensitivity may not change. (This has been demonstrated at least for murine marrow - Dr. M.Y. Gordon, personal communication.) The conclusion is therefore that the difference in cyclophosphamide sensitivity between human marrow and the two human tumours studied here is probably even greater in vivo than has been measured in mure, and the contrast between this and animal tumour data is therefore emphasised rather than diminished.

Factors other than cell kill are also involved in predicting differential clinical toxicity between marrow and tumours: in particular

the rate of cell repopulation and hence the rate of tissue recovery is probably of importance and information on this would also be necessary in predicting with greater accuracy overall differential response. Nevertheless the findings described here support the clinical observation already referred to above that the response rate of pancreatic and colonic carcinomas to chemotherapy is low, even in doses which produce considerable marrow toxicity [B.M.J. Editorial, 1976]. And it seems reasonable to hope that this kind of assay may be of more value in understanding and predicting clinical chemotherapy response than experimental animal systems in the future.

10.4.e: Comparative Chemosensitivity Studies

These last group of studies were carried out to examine the feasibility of a cytotoxic drug sensitivity test for tumours, analogous to antibiotic sensitivities provided by bacteriology laboratories. They were of a preliminary nature and can be interpreted only in a very limited way; but several observations can be made here.

First, in both the in vivo study and in the in vitro studies differences in drug sensitivity were detected, the $LD_{10}$ dose of methyl-CCNU achieving up to one log greater cell kill than the other drugs studied. This is of some interest in that recently it has been claimed that this drug is more effective than others previously used in the clinical chemotherapy of colonic and pancreatic cancers [Moertel et al, 1976]. Although this might argue in favour of the validity of the assay, it must be pointed out that the clinical

data on methyl-CCNU is controversial and is at present being reassessed.

The second observation was that with the exception of methyl-CCNU none of the agents achieved even one log kill despite being administered in $LD_{10}$ doses equivalent to maximum tolerated doses in man. This, though depressing clinically, again is compatible with the poor clinical response of these tumours generally observed with all current clinical chemotherapy. It is perhaps surprising however that in none of the 3 tumours studied was 5-fluorouracil more effective than any of the other agents tested, since this drug, like methyl-CCNU, has been reported as having greater single agent activity in intestinal cancers than most other drugs studied. Three points must be made in the interpretation of these data: first only three tumours were tested and these may therefore be unrepresentative of their general types; second the assay measures only single dose effects whereas frequency of drug administration may be important clinically, particularly with 5-FU; third metabolism of some of these agents may vary significantly between mouse and man.

The final observation from the studies in which cells from colonic carcinoma HXK1 and rectal carcinoma HXK4 were treated in mure is the correlation with clinical data and with data obtained from tumour growth delay studies.

The patient from whom HXK1 xenograft was obtained was treated for multiple liver metastases with a chemotherapy regime

which included methyl-CCNU and 5-fluorouracil, and was one of the few patients with metastatic colonic carcinoma treated in this way at the Royal Marsden Hospital to make a good objective response. This was assessed by general improvement in clinical condition, improvement in isotopic liver scan and a full to normal level of previously raised serum carcino-embryonic -antigen (CEA) level. On the other hand, the patient from whom HXK4 xenograft was obtained developed clinical evidence of liver metastases soon after surgical removal of the primary tumour and deteriorated very rapidly before chemotherapy could be initiated.

Both these patients' xenografts were treated with 5-fluorouracil and methyl-CCNU in tumour growth delay studies by my colleague Mr. K. Novak who has kindly allowed me to describe his results here. Tumour growth delay was calculated from the ratio (TD treated - TD control) : (TD control), where TD was the median time for the tumour to double in volume. This ratio may be regarded as the number of tumour-doubling times saved by the treatment [Kopper and Steel, 1975].

HXK1 was more sensitive than HXK4 to methyl-CCNU with a growth delay ratio of 5.2 compared with 0.4. HXK1 was also slightly more sensitive than HXK4 to 5-fluorouracil with a growth delay of 1.5 compared with 1.0.

Although increased cell kill measured by the clonogenic assay for HXK1 therefore correlated with a better xenograft response to the same drugs and with a clinical response to chemotherapy seen

infrequently for this tumour type, no detailed conclusions can be drawn from this preliminary study. Indeed it might have been anticipated that the difference in tumour growth delay between the two xenografts would have been associated with a difference in cell kill greater than the two-fold actually measured. The main value of this study is that it at least demonstrates that such experiments are now technically feasible and that further comparative data can therefore be obtained to establish whether a real correlation between clonogenic cell kill and tumour response in the xenograft or the patient actually exists.

# CHAPTER ELEVEN

## COLONY GROWTH WITH CELLS TAKEN DIRECT FROM HUMAN TUMOUR BIOPSIES, USING THE AGAR IN DIFFUSION CHAMBER TECHNIQUE

# CHAPTER ELEVEN

# COLONY GROWTH WITH CELLS TAKEN DIRECT FROM HUMAN TUMOUR BIOPSIES, USING THE AGAR IN DIFFUSION CHAMBER TECHNIQUE

## 11.1: INTRODUCTION

The reasons for assaying clonogenic cells from xenografts, rather than directly from biopsy specimens of human tumours, were described in Chapter 3. Ethical and practical problems in obtaining repeated samples of the same untreated tumour severely restrict the potential of the latter source. Nevertheless, the growth in agar of colonies of human tumour cells have occasionally been reported [McAllister and Reed, 1968; Altman et al, 1975] (details in Chapter 3.9), and opportunities arose from time to time during the course of my research to see whether colony growth direct from tumour biopsy material could be achieved with the diffusion chamber technique.

## 11.2: METHODS

### 11.2.a: Tumours

Seven tumour biopsy specimens in Ham's F12 medium, 15% foetal calf serum, penicillin 200units/ml and streptomycin 100$\mu$g/ml were obtained for study and details of these are given in Table 11.1. Five of the seven were biopsies of the primary tumour and included a rectal adenocarcinoma, a pancreatic adenocarcinoma, two colonic adenocarcinomas and a lymphoma; the other two were

from metastases of an oat cell carcinoma of lung and an anaplastic teratoma of testis.

Because of the difficulty in obtaining single cell suspensions from these biopsies (see below), short term monolayer cultures of cells from 6 melanomas and 3 hypernephromas were also studied in the assay system (Table 11.1); these cultures had been grown in primary tumour cell suspensions in medium 199 in Falcon flasks for a period of between 7 and 14 days. Single cell cell suspensions could be obtained with ease from these cultures by gently trypsinisation with a 0.1% solution in medium 199 for 3 or 4 minutes.

11.2.b: Single Cell Suspensions

The optimum technique for obtaining single cell suspensions from tumour biopsies was established empirically for each tumour in the same way as previously described for xenografts (Chapter 5.3); this is summarised in Table 11.1. In general, it was more difficult to obtain single cell suspensions from these tumour specimens taken direct from the patient than from xenografts: more prolonged periods of trypsinisation were usually required, and frequently only a small yield of single cells was obtained. In most instances the fraction of viable cells in suspension assessed by lissamine green dye exclusion was much smaller than with xenografts.

The technique for obtaining single cell suspensions from monolayer cell cultures is described above.

11.2.c: Assay Technique

Other aspects of the assay technique, including chamber loading, implantation, mouse pre-treatment, colony counting, and the determination of plating efficiency and optimum incubation period were identical to that described for xenografts in Chapter 5. Mouse pre-treatment in these experiments was always with 900 rad whole body irradiation, chambers being transplanted every ninth day.

11.3: RESULTS

Colonies in agar were grown from only 3 out of 7 of the tumour biopsy specimens (from the oat cell carcinoma of lung, the pancreatic carcinoma and one of the colonic carcinomas) as shown in Table 11.1. The plating efficiencies were low: these were 0.1% for the oat cell carcinoma, 1% for the pancreatic carcinoma and 0.25% for the colonic tumour. The corresponding incubation periods were 4 weeks, 5 weeks and 3 weeks. Colonies were as large as from xenograft clonogenic cells for the pancreatic carcinoma with some containing several hundred cells, but were small for the other two tumours and were defined in these as having 30 cells or more with most colonies containing between 30 and 50 cells. No colonies or clusters were grown from the other 4 tumours although in some instances single cells remained structurally intact in the agar for up to six weeks.

In contrast, 7 of the 9 cell suspensions obtained from monolayer culture grew colonies in agar; these included 5 of the 6 melanomas and 2 of the 3 hypernephromas, as shown in Table 11.1. Plating efficiencies ranged from 0.1% to 10% and incubation periods

| Tumour | Origin | Cell Suspension Technique | Incubation Period | Plating Efficiency |
|---|---|---|---|---|
| 1. Rectal adenocarcinoma | Primary biopsy | Trypsin | No growth | - |
| 2. Oat cell carcinoma (lung) | Metastasis biopsy | Phosphate-buffered saline | 4 weeks | 0.1% |
| 3. Teratoma (anaplastic) | Metastasis biopsy | Collagenase + trypsin | No growth | - |
| 4. Pancreatic adenocarcinoma | Primary biopsy | Collagenase + trypsin | 5 weeks | 1.0% |
| 5. Colonic adenocarcinoma | Primary biopsy | Trypsin | No growth | - |
| 6. Lymphoma | Primary biopsy | Trypsin | No growth | - |
| 7. Colonic adenocarcinoma | Primary biopsy | Trypsin | 3 weeks | 0.25% |
| 8. Hypernephroma | Monolayer culture | Trypsin | 3 weeks | 0.5% |
| 9. Melanoma | Monolayer culture | Trypsin | 3 weeks | 0.1% |
| 10. Melanoma | Monolayer culture | Trypsin | 3 weeks | 0.1% |
| 11. Hypernephroma | Monolayer culture | Trypsin | 12 days | 0.2% |
| 12. Melanoma (x2)* | Monolayer culture | Trypsin | 4 weeks | 10.0% |
| 13. Melanoma | Monolayer culture | Trypsin | 5 weeks | 2.0% |
| 14. Melanoma | Monolayer culture | Trypsin | No growth | - |
| 15. Melanoma | Monolayer culture | Trypsin | 3 weeks | 0.2% |
| 16. Hypernephroma | Monolayer culture | Trypsin | No growth | - |

*On second occasion, cells reconstituted after storage for 2 months in liquid $N_2$

TABLE 11.1 Colony Growth in Agar: Cells Taken Direct from Human Tumours

from 12 days to 4 weeks. Colonies were defined as containing 30 cells or more, and were usually small, rarely containing more than 100 cells. A few clusters of less than 30 cells were seen in both the monolayer samples which failed to grow colonies.

Some cells from one melanoma culture (No. 12, Table 11.1) were cryopreserved in liquid nitrogen for 2 months and then reconstituted for assay. These cells also grew colonies with the same plating efficiency as the original.

## 11.4: DISCUSSION

These studies show that colony growth can sometimes be achieved from cells taken directly from human tumours with the diffusion chamber technique, but the system is less consistent than with xenografts, success occurring in only 3 of the 7 tumours so far studied.

It is possible that technical factors may to some extent explain this. In particular, the trauma involved in obtaining good single cell suspensions was in general considerably greater than for xenografts. Dye exclusion studies demonstrated a low fraction of viable cells and even these may have lost clonogenic potential in the suspension process. To support this hypothesis was the fact that the oat cell carcinoma of lung, which alone went into single cell suspension easily, grew colonies; and more strikingly, all but two of the monolayer culture samples from which viable single cell suspensions were easily prepared achieved colony growth.

At present there appears to be no simple solution to this technical problem, although various methods for improving single cell suspensions from human tumours are being investigated. It might for example be possible to extend the technique of preparing initial short term monolayer cultures prior to making a single cell suspension to other tumour types but this process carries with it the risk of the rapid selecting out of unrepresentative tumour clones or even of clones of non-tumour cells adapted for in vitro growth [Sheard et al, 1971; Editorial, Lancet, 1972]. An alternative approach is to study the clonogenic potential of malignant effusions where virtual single cell suspensions already exist. The source of human tumour cells previously reported to grow colonies in agar was malignant pleural fluid in one instance [Altman et al, 1975] and the possibility of growing colonies in diffusion chambers from malignant pleural effusions and malignant ascites is now being studied.

Finally, the single successful attempt so far to grow colonies from reconstituted melanoma cells after cryopreservation in liquid nitrogen is of considerable long term importance. This technique might allow the storage of sufficient cells from a single biopsy sample (or aspiration of a malignant effusion) to enable repeated experiments on the same tumour to be carried out. In this way, detailed radiosensitivity and chemosensitivity studies at present restricted to xenograft tumour cells might become feasible with tumour cells taken direct from the patient.

# CHAPTER TWELVE

## CONCLUSIONS AND PROPOSALS FOR FUTURE STUDIES

# CHAPTER 12:

# CONCLUSIONS AND PROPOSALS FOR FUTURE STUDIES

## 12.1: GENERAL CONSIDERATIONS: CLONOGENIC CELL KILL - TUMOUR RESPONSE

The main contribution of the research described in this thesis has been to demonstrate that the radiosensitivity and chemosensitivity of clonogenic human tumour cells can now be measured in a way previously restricted to experimental animal tumours and to established cell lines (Chapter 1.1 and Chapter 10.1). In this final chapter the areas for further research of possible clinical relevance will be discussed.

A fundamental problem in this and indeed any other clonogenic cell assay is to establish the quantitative relationship, if any, between clonogenic tumour cell survival and gross tumour response after treatment. Only a few studies have so far attempted to examine this. Tumour cure and cell survival for a rat rhabdosarcoma were studied by Reinhold and de Bree [1968] who demonstrated a good correlation and found that the tumour cure rate after a single dose of X-irradiation could be correctly predicted by extrapolation of cell survival data. Steel and Adams [1975] found that the curability of small implants of Lewis lung carcinoma with cyclophosphamide correlated with cell survival data, if allowance were made for the increased clonogenic cell sensitivity to cyclophosphamide of these small implants compared with larger tumours of the same type.

Others, however, have found less simple relationships. Tumour growth delay and cell survival have been compared for the same rat rhabdosarcoma treated with X-irradiation [Hermens and Barendsen, 1969] and a Lewis lung murine carcinoma treated with cyclophosphamide [Steel and Adams, 1975]. In each of these studies the actual clonogenic cell kill measured by assay was much greater than that predicted by backward extrapolation of regrowth data. Likewise, it has been shown for the RIB5 tumour that the induced growth delay for a given level of cell survival varies depending on whether radiation is given under air-breathing or hypoxic conditions [McNally, 1973], and whether radiation is given as X-rays or as fast neutrons [McNally, 1975].

Various explanations might be postulated to explain these discrepancies. Nearly all clonogenic cell assays involve removal of cells from their normal "milieu"* and the artificial environment provided by the assay might alter cell growth characteristics in general and response to cytotoxic agents in particular. Furthermore, the trauma involved in the preparation of single cell suspensions

*Techniques for studying clonogenic cells in situ have occasionally been possible for some normal tissues including growth cartilage [Kember, 1967], skin [Withers, 1967] and intestinal epithelium [Withers and Elkind, 1968] as described in Chapter 3.5

might result in the selecting out of an unrepresentative sub-population of cells for assay.

Occasionally tumour antigenicity might induce a residual host immune response such that tumour cure is obtained with doses of radiation or drug too low to kill all clonogenic tumour cells. This has been established for the EMT6 tumour treated with single dose irradiation [Rockwell and Kallman, 1973], and probably also explains occasional cures observed in a human oat cell carcinoma xenograft with doses of cyclophosphamide predicted by the authors as being too small to kill all clonogenic cells [Kopper and Steel, 1975].

In summary, these studies suggest that some kind of relationship between clonogenic cell survival and tumour response does appear to exist despite the artificial nature of the assays used, but that this is often complex and may vary for different tumours, depending on antigenicity or other factors.

For this reason correlative studies of the type described above are indicated to elucidate further the quantitative relationship between xenograft tumour response and cell survival; such studies might investigate not merely the fraction of clonogenic cells killed but their rate of recovery, as Hermens and Barendsen [1969] have done for the rat rhabdosarcoma. This experimental approach, though important, is elaborate and painstaking, and will inevitably generate data fairly slowly.

A second line of research is therefore also indicated in which relationships between cell survival and tumour response are sought on an empirical basis in the hope that such information may be of qualitative, if not of quantitative, clinical significance. This approach is best illustrated by considering some of the findings described in this thesis, and by using these to indicate further areas of research of clinical relevance.

## 12.2: RADIATION RESPONSE: FUTURE STUDIES

The general conclusion to be drawn from the experiments reported in Chapters 7, 8 and 9 is that the radiation response of cells from human tumour xenografts so far studied is often similar to that previously obtained for cell lines and for experimental animal tumours. For example, the *in vitro* radiosensitivities of all but the melanoma xenograft HX34 lie within the same relatively narrow range found for nearly all other mammalian cells (Chapter 7), and the pancreatic carcinoma HX32 studied *in vivo* has a fraction of radioresistant hypoxic cells of the same order as that previously established for most experimental animal tumours (Chapter 8). It may therefore prove in the long term that human tumour xenografts have no great advantage over animal tumours for many areas of radiobiological investigation. But further human tumour cell data are first required to confirm or refute this hypothesis.

The first unresolved question is the underlying radiobiological basis for variations in the clinical responsiveness to radiotherapy of different tumour types. It has already been discussed in Chapter 7 that, as for experimental animal tumours, these variations are independent of in vitro radiosensitivity differences and must be based on other extrinsic factors; Chapter 8 indicates that hypoxia may be one such factor. But the data from melanoma HX34 suggests that this generalisation may not always hold true: here it is possible that the wide initial shoulder and decreased slope of the in vitro survival curve may in part explain the poor clinical response to radiotherapy of this tumour type.

Further studies are therefore indicated on xenografts of tumours in which the chance of clinical control with radiotherapy is either very poor, e.g. melanomas, gliomas, some types of sarcoma, or very good, e.g. lymphomas, seminomas. These would investigate the extent to which clinical response correlates with intrinsic radiosensitivity characteristics similar to those of melanoma HX34, or with extrinsic factors, including tumour cell hypoxia. It is probable that for some tumour types, both intrinsic and extrinsic factors are involved, and the hypoxic fraction of melanoma HX 34 in vivo in air-breathing animals is currently being studied with this in mind.

Such studies would not necessarily merely be of theoretical interest but could be of clinical relevance. For example, the identification of tumours with a large fraction of hypoxic cells would predict where hypoxic cell radiosensitiser drugs might prove most

effective clinically. And the possible use of the assay as a simple screen for new radiosensitisers prior to clinical trial has already been discussed (Chapter 9).

Likewise, tumours whose in vitro survival curves showed wide initial shoulders might be amenable to different types of radiation. It has been shown for animal tumours and tumour cell lines that the ability to accumulate sub-lethal damage is less with high linear-energy-transfer radiation including fast neutrons, than with X- or $\gamma$-irradiation [Hornsey and Silini, 1961; Shipley et al, 1975; Thomson et al, 1975]. The assay system should be used to study whether a similar reduction in shoulder width with high LET radiation can be obtained for melanomas and other tumours proving to have similar radiobiological characteristics. Such a finding might have important therapeutic implications, and in this context it is of interest that preliminary clinical observations have suggested that melanomas may be more responsive to neutron therapy than to conventional $\gamma$-irradiation [Dr. M. Catterall, personal communication].

## 12.3: RESPONSE TO CYTOTOXIC DRUGS: FUTURE STUDIES

In Chapter 1, important differences in the response to cytotoxic drugs of human and experimental animal tumours were described, and the studies reported in Chapter 10 show that such differences are based in large part on differences in intrinsic cellular chemosensitivity. It is therefore my belief that the major long term advantage of this assay over equivalent animal tumour

assays will be less in radiobiology than in chemosensitivity studies.

The first step is therefore to confirm the validity of the assay as a reflection of clinical chemotherapy response. The cytotoxic drug data presented in Chapter 10 provides some circumstantial evidence towards this, first in the marked resistance to various cytotoxic drugs of pancreatic and colonic carcinoma cells, and second in the tentative correlation of cell kill with clinical tumour behaviour in two patients. However, further data are required to substantiate these preliminary observations, and in particular correlative studies in which chemosensitivity in the assay can be compared with the patient's response to chemotherapy need to be carried out. These could be done either via the xenograft system or perhaps directly from the patient treating the cells in mure, if the technique described in Chapter 11 for cloning human tumour cells directly can first be improved.

Second, it is possible that in the long term the in mure assay system might be adapted in this way as a measure of tumour sensitivity to different cytotoxic drugs for individual patients, in a manner analogous to current bacteriological sensitivity testing in the treatment of infection. Such information might be only of qualitative value in view of the differences in sensitivity between the in vivo and in mure assay described in Chapter 10; even this however would be a considerable improvement on current combination chemotherapy regimes designed on a "probability of response" basis. At best, this would allow the selection of drugs to which the tumour

was maximally sensitive, with the possibility of increased clinical response; at least, the system would allow the exclusion from treatment of drugs to which the tumour cells were resistant, thus minimising toxicity to the patient.

Third, a more immediate, and more important, role for the assay is in determining the shape and steepness of dose response curves for cytotoxic drugs against human tumours, in a manner similar to that achieved by Bruce and his colleagues [1966] for the AKR lymphoma (Chapter 10.1). This would provide information on how best to administer drugs clinically, both in terms of dosage and scheduling, and thereby might establish a more rational basis for cancer chemotherapy.

Some examples can illustrate this further. There is a current tendency to administer increasingly large doses of cytotoxic drugs in cancer chemotherapy, based on the argument that the inevitable increase in host toxicity will be outweighed by increased tumour cell kill and hence a better clinical tumour response. At present this strategy is mainly empirical, and depends on the assumption that a steep log linear dose response curve exists for drugs against tumour cell (Figure 10.1a). However, a plateau-type dose response curve (Figure 10.1b) or a shallow curve, as for cyclophosphamide against pancreatic and colonic carcinoma cells (Figure 10.3) would suggest that such a treatment strategy were illogical; I have already suggested that these latter curves may explain the clinical observation that such tumours usually respond

poorly to cyclophosphamide even in doses large enough to produce significant toxicity to normal tissues (Chapter 10.4.a).

A steep log linear dose response curve has recently been demonstrated with the diffusion chamber assay for melphalan against melanoma HX34 cells, and preliminary clinical studies at the Royal Marsden Hospital with high dose melphalan (up to 200mg/$m^2$) undertaken on the basis of this dose survival curve in patients with melanoma have shown several good clinical responses not previously achieved with this drug in conventional dosage [Dr. P. Selby and Dr. T.J. McElwain, personal communication]. This simple study is a good example of how the validity of the chamber assay technique as a model for studying clinical response may be established on an empirical "try-it-and-see" basis. Further dose response curve data of this type may help identify those clinical situations where very high dose therapy is rational.

Clinical combination chemotherapy is commonly used in the treatment of cancer, but again largely on an empirical basis. Here the assay can be used to provide dose-response data for drugs used alone and in combination. Synergistic, additive and antagonistic effects can thus be determined, and a rational basis established for clinical drug combinations of increased therapeutic effectiveness.

An added refinement to experiments of the type described above would be the inclusion of similar data for human bone marrow cells using the diffusion chamber assay, as shown in Figure 10.6. Predictions not merely of tumour response but of associated host

toxicity and hence of therapeutic effectiveness of specific chemotherapy regimes could then be made.

Fourth, the diffusion chamber system could be used to study new agents for cytotoxic activity; however the assay is too elaborate to be used as a general screening system, and its role here would need to be carefully defined. Its best use would probably be in verifying cytotoxic activity against human tumour cells of agents already suspected of having clinical potential, and in defining dose survival curve characteristics following the principles established by Bruce (above and Chapter 10.1.a). Again a specific example can be quoted: vindesine (Eli Lilly) is a new vinca alkaloid similar to vinblastine but with anecdotal reports of a broader therapeutic range including activity against melanomas. The dose survival curve for this agent against malenoma HX34 is about to be studied with the diffusion chamber assay, first to verify its activity compared with other cytotoxic agents and second to establish whether response remains log-linear or develops a plateua, as Bruce found for vinblastine in the AKR lymphoma (Figure 10.2). Such information may be of value in planning the optimal dose and scheduling of this drug in clinical trials.

Finally, and in the long term perhaps most important of all, the assay provides a means of isolating clones of human tumour cells for further biological study. The areas for such study include the fields of biochemistry, pharmacology, and perhaps immunology and genetics. At present the major problem in clinical

cancer chemotherapy is the inadequacy of currently available cytotoxic drugs to treat many tumours effectively. The ability to isolate drug-sensitive and drug-resistant clones of human tumour cells and to investigate in these the biological parameters underlying drug action and the development of resistance might eventually provide a basis for the design of a generation of more effective anti-cancer drugs.

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# APPENDIX ONE

## A NOTE ON STATISTICAL METHODS USED IN CHAPTERS 7 - 10

The original formula defining the radiation cell survival curve for mammalian cells described in Chapter 7.1 was derived by Lea [1946] and is given by: -

$$S.F. = 1 - (1 - e^{-D/D_O})^n \quad \text{if } D > D_O$$

where S.F. is surviving fraction, D is dose to give this survival, $D_O$ is negative reciprocal of slope on the exponential part of the curve on a semi-log plot, and n is the extrapolation number.

To simplify calculations, this approximates to: -

$$\log_e S.F. = \log_e n - D/D_O$$

and this latter formula was used here to calculate the values of slope, intercept and the errors on these by linear regression analysis as formulated in Geigy [1970].

## COMPARISON OF SLOPES FOR STATISTICAL SIGNIFICANCE

An F test (variance - ratio test) was done to see if the variances about both regression lines were similar. If so, a "t" value was calculated and the probability "p" obtained from tables [Bailey, 1959]. If the variances were significantly different a "d" value was calculated as suggested by Bailey [1959] and again "p" obtained from tables.

CALCULATION OF $D_Q$

This was obtained from $D_O$ and n according to the formula: -

$$D_Q = \log_e n \times D_O$$

$$= \frac{-\text{Intercept}}{\text{Slope}} \quad (\text{since } D_O = {}^{-1}/\text{slope}, \text{ and } \log_e n = \text{intercept})$$

Fieller's theorem as laid out in Finney [1952] allowed the Standard Error of this ratio to be estimated and the probability "p" of significant differences to be calculated.

A CDC 6400 computer was used for all calculations and the computer programs were kindly supplied by Dr. J. Millar of this department.

# APPENDIX TWO

## A NOTE ON THE DRUGS USED IN CHAPTER 10 OF THIS THESIS

### ACTINOMYCIN D

An anti-tumour antibiotic derived from soil Streptomyces and developed clinically in the 1950s for the treatment of Wilm's tumour. It acts by binding to helical DNA and also by intercalation between base pairs adjacent to guanine. DNA and protein synthesis are inhibited at higher concentrations. Its main clinical uses are in the treatment of paediatric malignancies including Wilm's tumour, and malignant teratoma and chorion-carcinoma in adults.

### ADRIAMYCIN

A recently developed anthracycline antibiotic derived from Streptomyces peucetius, and very similar in structure to daunorubicin. It acts by both binding and intercalation to form complexes with DNA. It has a broad spectrum of clinical activity, particularly in the treatment of breast carcinoma, lymphomas, acute leukaemias, sarcomas and paediatric malignancies, but cardiotoxicity is a cumulative dose-limiting factor.

### CYCLOPHOSPHAMIDE

An oxazaphosphorine alkylating agent, which is relatively inactive in vitro and requires metabolic activation by the cytochrome P450 system in the liver microsomes. It is the most extensively

used cytotoxic drug in cancer chemotherapy and has activity against lymphomas, acute lymphoblastic leukaemia, carcinomas of the breast, lung, ovary and testis, and in paediatric malignancies. Its powerful immunosuppressive activity has also led to its occasional use in auto-immune disorders.

5-FLUOROURACIL

A pyrimidine analogue anti-metabolite which acts mainly by the inhibition of thymidylate synthetase and hence of DNA synthesis. Its main clinical use has been in the treatment of gastro-intestinal carcinomas where its response rate, though low, is probably better than that of most other agents. Also effective in combination against breast carcinoma.

METHYL-CCNU

One of the nitroso-ureas, a group of compounds whose anti-tumour activity was first detected in the National Cancer Institute drug-screening programme. These compounds, which include BCNU and CCNU, act as alkylating agents. They are highly active against many animal tumours but have proved disappointing in clinical use. They are lipophilic and are therefore used in the treatment of tumours of the CNS with some success, and also as second-line therapy against lymphomas and myeloma. Methyl-CCNU has been reported to be active against gastro-intestinal carcinomas, but response rates vary widely in different studies, and toxicity is considerable.

METHOTREXATE

An anti-folate anti-metabolite whose effects are mediated by the inhibition of dihydrofolate reductase. It has wide clinical activity, and is used in particular against breast and lung carcinoma, and in some paediatric malignancies including osteogenic sarcoma where it is given in high dosage with folinic acid "rescue".

VINBLASTINE

A vinca alkaloid derived, like vincristine, from the periwinkle plant. Its action is to arrest proliferating cells in metaphase, probably by interfering with microtubular function. Both compounds have broad clinical activity, although myelosuppression is the major toxicity with vinblastine compared with neurotoxicity for vincristine, and the latter is therefore more commonly used in combination chemotherapy. Vinblastine is principally used in the treatment of lymphomas, malignant teratoma, chorion-carcinoma and in some paediatric malignancies including neuroblastoma.

## APPENDIX THREE

## PUBLISHED PAPERS ARISING FROM RESEARCH DESCRIBED IN THIS THESIS

Studies on the Radiosensitivity of Human Bone Marrow and Human Tumour Xenografts Using an Agar Diffusion Chamber Technique. Smith I.E. and Gordon M.J. Br. J. Radiol. 49 : 558 (1976) (Abstract).

In Vitro and In Vivo Radiosensitivity of Human Tumour Cells Obtained from a Pancreatic Carcinoma Xenograft. Courtenay V.D., Smith I.E., Peckham M.J. and Steel G.G. Nature 263 : 771 (1976) (Enclosed).

A Colony Forming Assay for Human Tumour Xenografts Using Agar In Diffusion Chambers. Smith I.E., Courtenay V.D. and Gordon M.Y. Br. J. Cancer 34 : 476 (1976) (Enclosed).

The Comparative Sensitivity to Cyclophosphamide of Cells from Human Tumour Xenografts and Human Bone Marrow. Smith I.E. and Gordon M.Y. Antibiotics and Chemotherapy (In Press).

(Reprinted from Nature, Vol. 263, No. 5580, pp. 771–772, October 28, 1976)

# *In vitro* and *in vivo* radiosensitivity of human tumour cells obtained from a pancreatic carcinoma xenograft

HUMAN tumours show a wide range of clinical response to radiotherapy, but the extent to which this reflects differences in intrinsic cellular radiosensitivity, extent of tumour cell hypoxia, or host reaction against the tumour is unknown. For tumours in laboratory animals, various assays for clonogenic cells are available. These have shown that the range of radiosensitivity of aerobic cells is quite narrow[1,2] and that the hypoxic fraction of clonogenic cells within tumours of palpable dimensions is often in the range 10–35%. Until now the lack of satisfactory assay for clonogenic cells has prevented similar studies on human solid tumours treated *in vivo*. We have developed two assays for clonogenic cells from a human metastatic pancreatic carcinoma propagated as a xenograft in immune-suppressed CBA/lac mice. This xenograft was one of a series established by Pickard *et al.*[3].

In the first assay, tumour-cell suspensions were incubated in Ham's medium with the addition of 15% calf serum, 0.3% agar and rat red blood cells in 17-mm diameter test tubes using a double layer technique. Colonies of 50 cells or more were counted at 28 d and the plating efficiency was 20–40%. In the second assay, tumour-cell suspensions in medium, serum and agar were grown in diffusion chambers implanted into the peritoneal cavity of C57BL mice, previously treated with whole-body radiation. Colonies, growing slightly more quickly than in the first assay, were counted at about 18 d and the plating efficiency was 10–20%. Detailed descriptions of the experimental methods are being prepared for publication. Chromosomal analysis of the cells after 16 d growth in culture verified a human karyotype, with aneuploidy ranging from 42 to 68 chromosomes and a mode of 62.

Tumours measuring 5–8 mm in diameter were grown in the leg muscles of immune-suppressed mice and were irradiated *in vivo* using $^{60}$Co $\gamma$ rays. The animals were killed and the tumours removed either immediately after irradiation or 18 h later, and cell suspensions were prepared for assay. Acute hypoxia was induced by killing mice by nitrogen asphyxiation 15 min before the start of irradiation. *In vitro* aerobic irradiation was carried out on freshly prepared tumour-cell suspensions.

The results of *in vitro* irradiation and *in vivo* irradiation in air-breathing and hypoxic conditions are shown in Fig. 1. The two assays agree well and we are unable to detect any systematic differences between them. The *in vitro* cell survival curve has a $D_0$ of 96±9 rad. ($D_0$ is the dose that reduces survival by 63% on the exponential part of the curve.) The *in vivo* air-breathing survival curve for animals killed immediately after irradiation shows an initial sensitive component and a radioresistant component, the $D_0$ of which is 305±16 rad. The $D_0$ values for the terminal parts of the other two curves are not significantly different from this and we have therefore drawn the three curves parallel. From the vertical displacement of the hypoxic curve from the lowest air-breathing curve we calculate an hypoxic fraction of 0.25. The oxygen enhancement ratio is 3.1±0.4. The data obtained on tumours that were taken 18 h after irradiation, are displaced vertically from the other air-breathing curve by a factor of 2.2. This displacement shows that the fractional survival of tumour cells obtained at 18 h after irradiation was greater than when the cells were removed immediately. Two mechanisms may be involved: the repair of potentially lethal damage or the selective death or disappearance during the 18-h period of lethally damaged cells. Repair of potentially lethal damage has been reported in cell populations irradiated *in vitro* and *in vivo*[4,5].

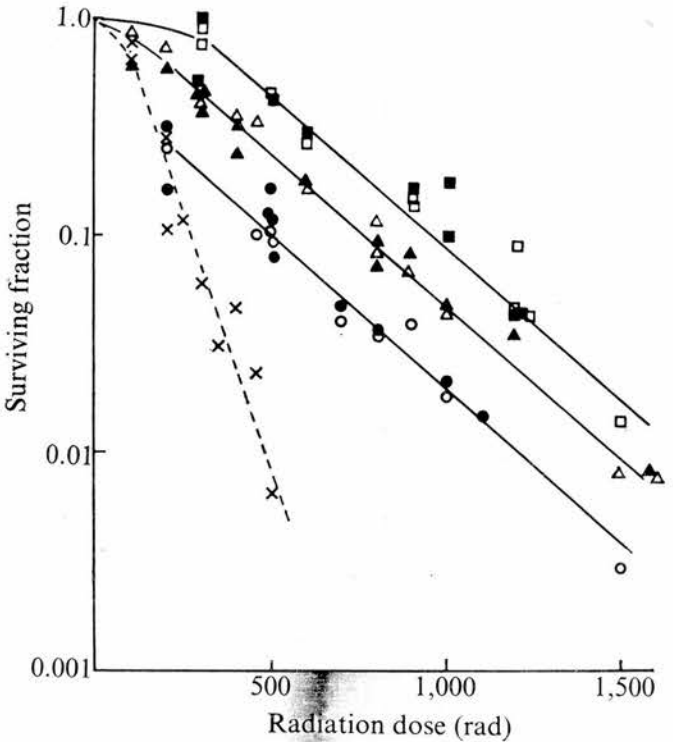


**Fig. 1** Survival curves for the xenografted tumour cells treated with single doses of $\gamma$ radiation. ×, Cells irradiated as a single-cell suspension in aerobic conditions *in vitro* and assayed using the *in vitro* colony assay. The other data are for tumours irradiated *in situ*: ○ ●, assay immediately after irradiation in air-breathing mice; △ ▲, assay 18 h after irradiation in air-breathing mice; □ ■, assay immediately after irradiation in nitrogen-asphyxiated mice. Open symbols indicate the results of the diffusion chamber assay; closed symbols the results of the *in vitro* assay.

The present data suggest that the 18-h curve is parallel to the other two curves down to radiation doses as low as 200 rad, but more data are required to confirm this.

Limited clinical experience with irradiation in pancreatic carcinoma suggests that this is not usually a radioresponsive tumour[6]. It is therefore of interest that the $D_0$ values which we have obtained are within the range of values determined using tumours of laboratory animals. The hypoxic fraction is also no less than has been found in experimental tumours, but the significance of this must be interpreted with caution because of the possible influence on this parameter of vascular stroma derived from the mouse. One interpretation of these data, however, is that the response of pancreatic carcinoma to radiotherapy is limited by the high proportion of hypoxic cells. This provides support for current efforts to improve clinical therapeutic response by the use of chemical radiosensitisers or high LET radiation.

V. D. COURTENAY
I. E. SMITH
M. J. PECKHAM
G. G. STEEL

*Radiotherapy Research Department,*
*Divisions of Radiotherapy and Biophysics,*
*Institute of Cancer Research,*
*Sutton, Surrey, UK*

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buffered saline (PBS) using a crossed-scalpel technique. Further treatment to produce a viable single-cell suspension was then determined empirically for each tumour and details are shown in the Table. Some xenografts required only incubation in PBS at 37°C for 15 min; some required incubation with trypsin 1 : 100 in PBS for between 5 and 10 min; one (pancreatic carcinoma xenograft HX32) required incubation in collagenase 2 mg/ml in Ham's medium with 15% foetal calf serum for 30 min, followed by trypsin 1 : 100 in PBS for a further 5 min, after washing free of serum. All cell suspensions were finally filtered (Simon Polyester Mesh, aperture 30 $\mu$m) and a single-cell suspension obtained in ice-cold Ham's medium and 15% foetal calf serum. Viability was assessed by dye exclusion using lissamine green. Diffusion chambers were then filled with the required number of cells for assay, suspended in Ham's medium, serum and 0·3% agar.

*Filling and implantation of agar diffusion chambers.*—The preparation, filling and implantation of diffusion chambers for this assay were identical to those previously described for bone marrow cells (Gordon, 1974).

*Pretreatment of mice before chamber implantation.*—Pretreatment of C57BL mice used in these experiments was required before implantation for successful growth of colonies in the diffusion chambers (see Results section). Standard pretreatment was with whole-body irradiation from a $^{60}$Co source, 3 h before chamber implantation. When 900 rad whole-body irradiation was used, following the technique employed with bone marrow (Gordon *et al.*, 1975), transplantation of the chambers to newly irradiated mice was required every ninth day, since this dose of irradiation was usually fatal to the mice around this time. Smaller doses of whole-body irradiation were not usually fatal and therefore did not require transplantation of chambers.

It has been found by Dr John Millar, working in this laboratory, that death after 900 rad or even 1000 rad whole-body irradiation to C57BL mice can be prevented by previous treatment with cytosine arabinoside 200 mg/kg i.p. between 1 and 3 days before irradiation. Some chambers were therefore implanted into C57BL mice treated with cytosine arabinoside 200 mg/kg i.p. 48 h before 1000 rad whole-body irradiation, and in these animals transplantation was unnecessary. In other experiments, the mice were pretreated with cyclophosphamide 200 mg/kg i.p. 24 h before implantation instead of whole-body irradiation, and this was well tolerated.

In 2 experiments, chambers were implanted into thymectomized, whole-body irradiated, marrow-reconstituted CBA/lac mice (Pickard *et al.*, 1975) or genetically athymic nude mice, without irradiation or other forms of pretreatment.

*Colony counting.*—Chamber-bearing mice were killed by cervical dislocation, and the Millipore filter of each chamber removed with a scalpel blade. Colonies were then counted in the chamber under a binocular microscope at a magnification of × 50. Colonies were defined as aggregates of at least 50 cells, and clusters as aggregates of between 20 and 50 cells. The optimum incubation period for colony formation was determined by colony counting at various times after implantation until there was no further increase in colony numbers.

## RESULTS

Cell suspensions of all 11 human tumour xenografts so far studied reproducibly grew agar colonies in diffusion chambers, and details of the incubation period, the mean plating efficiency (PE), and the number of experiments performed for each xenograft are shown in the Table. The incubation periods ranged from 12 to 28 days, but were fairly constant for each tumour, and in general colonies from the less differentiated tumours grew more rapidly than those from more differentiated ones. The mean PE of each tumour was fairly constant, but the range between tumours was from 0·3% to 16%.

There was variation in colony morphology between different tumours: some tumours produced colonies composed of fairly loosely clumped cells (Fig. 1a) while others produced densely packed spherical colonies (Fig. 1b), which in one tumour (colonic carcinoma HX18) appeared to develop a mucinous capsule, demonstrated by Giemsa staining of a colony removed from agar (Fig. 1c). The morphology of the colonies from each individual tumour was the same on different occasions.

TABLE.—*Histology and Criteria for Colony Growth of Human Tumour Xenografts*

| Code no. | Tumour | Cell suspension technique | No. of experiments performed | Incubation period (days) | Mean PE (%) | Range (%) |
|---|---|---|---|---|---|---|
| HX 32 | Pancreatic carcinoma, anaplastic (Met)* | Collagenase +trypsin | 26 | 18 | 11 | 9–14 |
| HX 18 | Colonic adenocarcinoma, poorly differentiated | Trypsin | 8 | 21 | 1·9 | 0·9–2·4 |
| XUR 5 | Colonic carcimona, anaplastic | Trypsin | 2 | 21 | 1·1 | 0·9–1·2 |
| HXK 1 | Colonic adenocarcinoma, mod. differentiated (Met) | Trypsin | 2 | 28 | 0·5 | 0·4–0·5 |
| HXK 9 | Colonic adenocarcinoma, poorly differentiated (Met) | Trypsin | 1 | 21 | 3·5 | — |
| HX 12 | Rectal adenocarcinoma, well differentiated | Trypsin | 1 | 28 | 1·1 | — |
| HXK 4 | Rectal adenocarcinoma, poorly differentiated | Trypsin | 3 | 21 | 11·5 | 10·5–13 |
| HX 29 | Oat cell carcinoma of lung (Met) | PBS† | 3 | 21 | 1·5 | 1·0–1·6 |
| HX 33 | Oat cell carcinoma of lung (Met) | PBS | 3 | 18 | 0·3 | 0·25–0·4 |
| HX 34 | Melanoma (Met) | PBS | 6 | 12 | 16 | 15–18 |
| HX 35 | Uterine carcinoma, anaplastic (Met) | PBS | 2 | 12 | 14 | 12–16 |

* Met—Metastasis biopsy.
† PBS—Phosphate buffered saline.

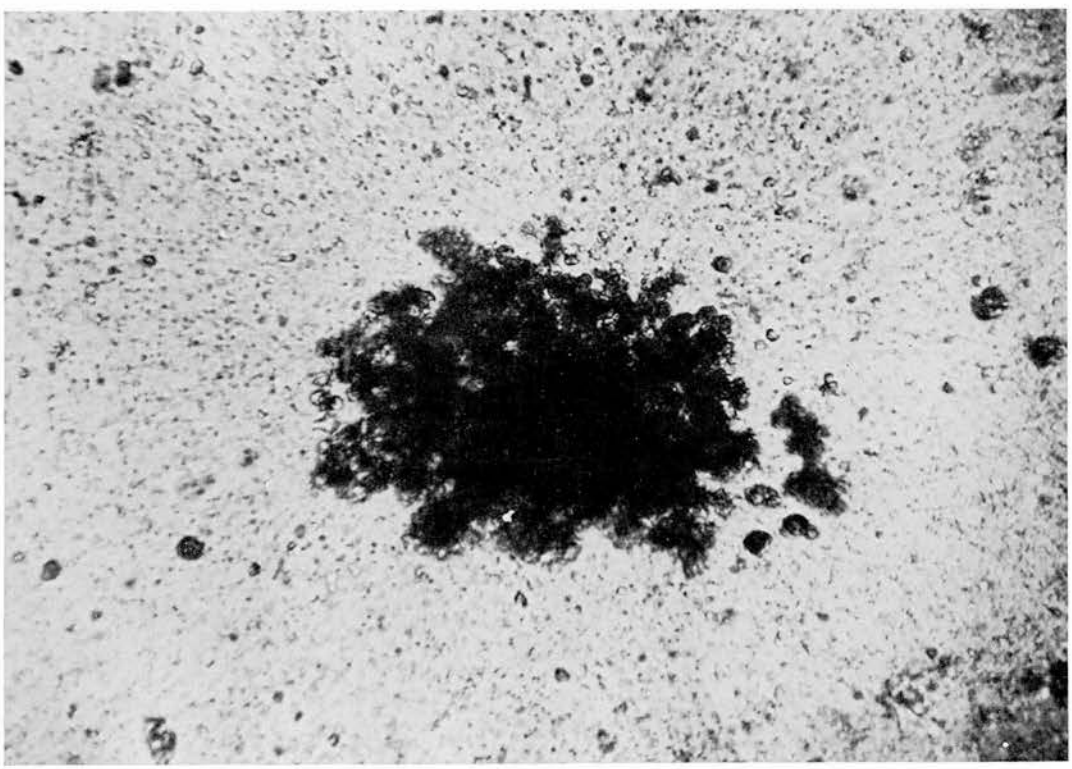

FIG. 1(a)

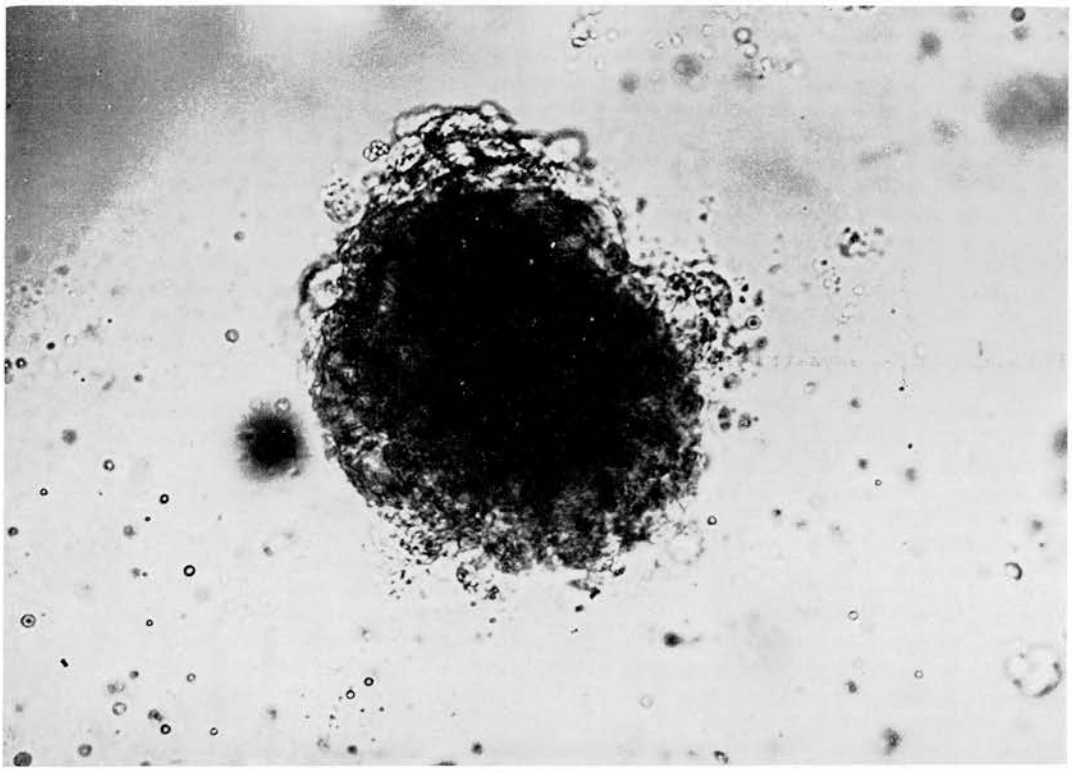

FIG. 1(b)

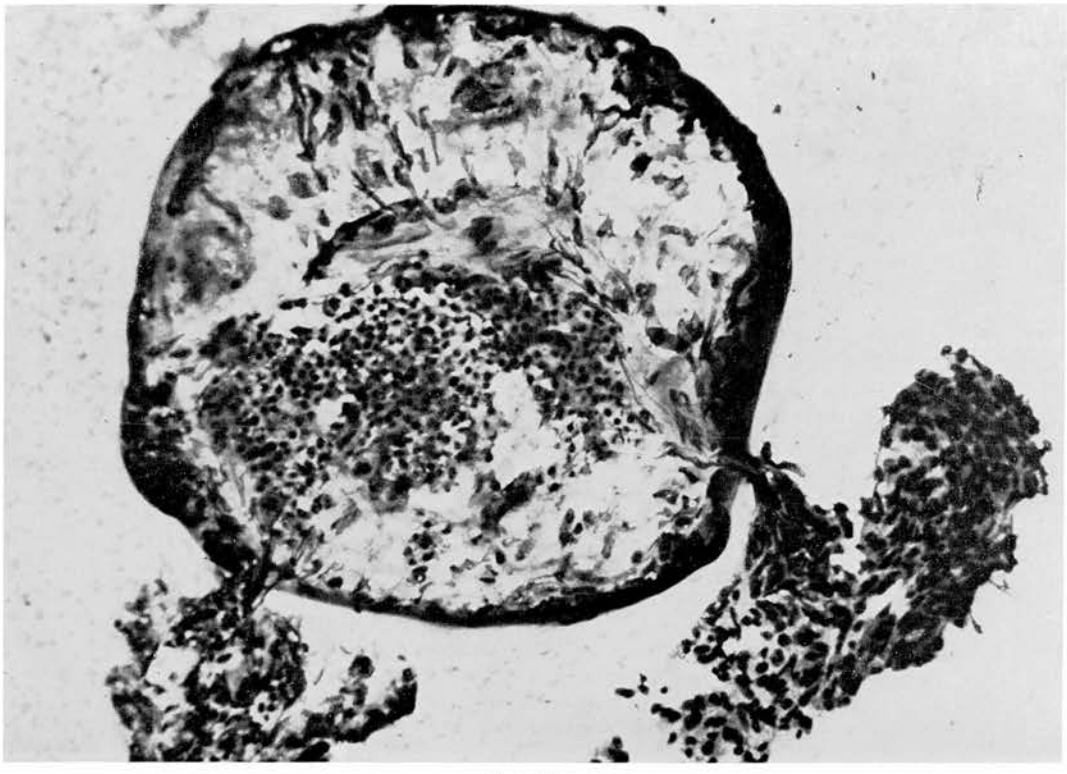

FIG. 1(c)

FIG. 1—(a) Typical agar colony from cells of colonic adenocarcinoma xenograft HXK1. ×350. (b) Typical agar colony from cells of colonic adenocarcinoma xenograft HX18. ×350. (c) Giemsa-stained preparation of HX18 colony removed from agar, showing mucinous capsule "leaking" cells at 2 sites. ×150.

The PE of colonies from each tumour cell suspension depended greatly on the pretreatment of the diffusion chamber host mice. Details of the effects of different pretreatment regimes on PE for 2 human tumour xenografts are shown in Fig. 2. Diffusion chamber colonies did not grow in untreated mice. Whole-body $^{60}Co$ $\gamma$-irradiation to the mice promoted colony growth, and the PE increased with increasing doses of irradiation. Colonies grew as well in chambers maintained for 3 weeks in mice pretreated with cytosine arabinoside 200 mg/kg and 1000 rad (preventing death of the mice) as in chambers transplanted every ninth day into mice pretreated with 900 rad alone. Since the former technique saves mice and labour, this has now been adopted as standard pretreatment. Cyclophosphamide 200 mg/kg i.p., which has been shown to be many times more effective than other pretreatment schedules in allowing lung colony formation from C22LR mouse osteosarcoma (Smink and van Dierendonck, personal communication), was an ineffective pretreatment in this system. PE was almost as high in thymectomized, whole-body irradiated, marrow-reconstituted CBA/lac mice as in the 900-rad pretreated C57BL mice, while chambers implanted into genetically athymic " nude " mice produced the highest PE of all. Practical factors prevented us from using nude mice routinely for this assay.

Chromosomal analyses of cell suspensions from 6 of the xenografts have so far been carried out, and these were all of human karyotype. Four tumours had a normal diploid chromosomal complement, one (colonic carcinoma HX18) had an added large acrocentric marker chromosome, and one (pancreatic carcinoma HX32) showed aneuploidy, with a range of 42 to 68 chromosomes and a mode of 62, with extra chromosomes coming from groups C, D and E.

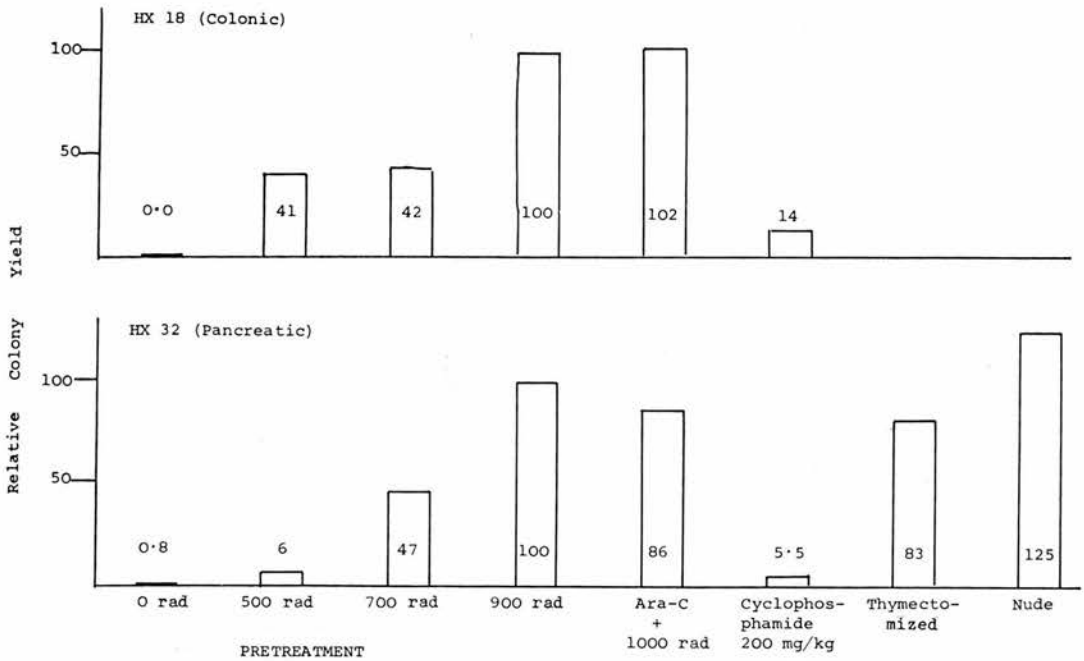


FIG. 2.—The influence of different types of pretreatment on PE for 2 xenografts. The results for each pretreatment are expressed as a percentage of the yield achieved using host mice which had received 900 rad whole-body irradiation (thymectomized and " nude " mice were not used for HX18 xenograft). Each pretreatment result is the mean of at least 3 experiments, except for " nude " mice, when only one experiment was performed.

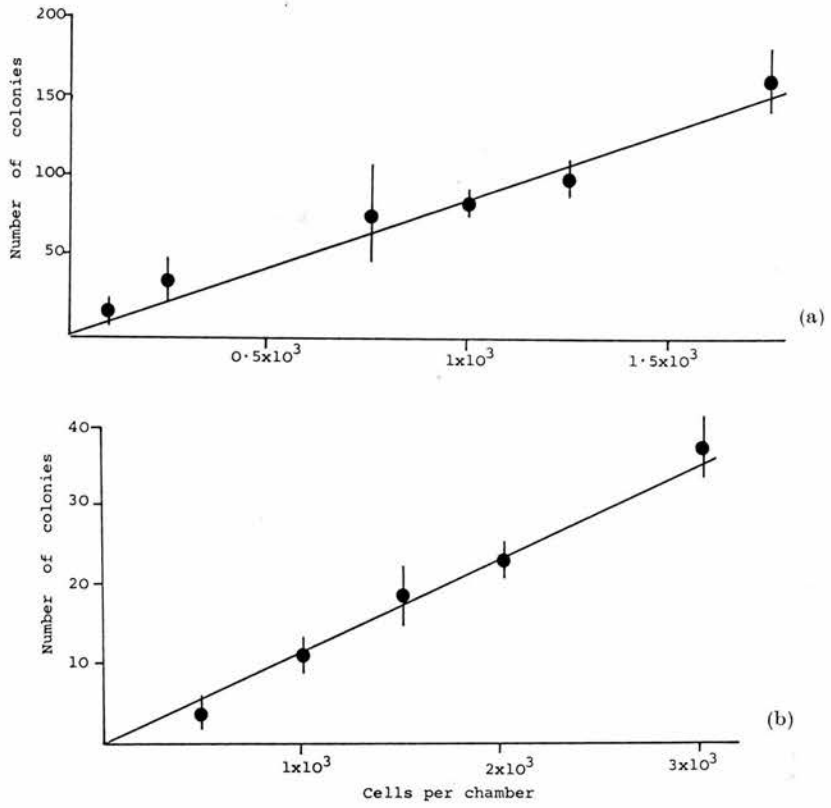


FIG. 3.—The relationship between the number of colonies scored and the number of cells cultured in agar diffusion chambers for 2 xenografts. (a) Pancreatic xenograft HX 32; (b) Colonic xenograft HX 18. Vertical bars represent ± s.e.

Giemsa-stained preparations of colonies grown from colonic carcinoma HX18 showed undifferentiated neoplastic cells with high nuclear cytoplasmic ratio, all basically of the same type; comparison with histological sections of the original tumour xenograft showed that the colony cells were entirely compatible with an origin from that tumour (Dr A. Mackay, Consultant Pathologist, Royal Marsden Hospital).

A linear relationship between the yield of colonies and the number of cells introduced into the chambers was demonstrated for the pancreatic tumour xenograft HX32 over a range from $1 \times 10^2$ to $1{\cdot}75 \times 10^3$ cells per chamber, and for the colonic tumour HX18 over a range from $5 \times 10^2$ to $3 \times 10^3$ cells per chamber (Fig. 3a and b).

The ability of this system to measure the effect of cytotoxic drugs on PE of human colonic carcinoma xenograft HX18 is shown in Fig. 4. In this example, *in vivo* cyclophosphamide i.p. produced a dose survival curve with a small range of cell kill; the surviving fraction at the maximum tolerated dose to the mouse (300 mg/kg) was about 0·2.

## DISCUSSION

Agar colony assays of human bone marrow progenitor cells (Pike and Robinson, 1970; Gordon *et al.*, 1975) and human chronic granulocytic leukaemic cells (Brown and Carbone, 1971; Chervenick

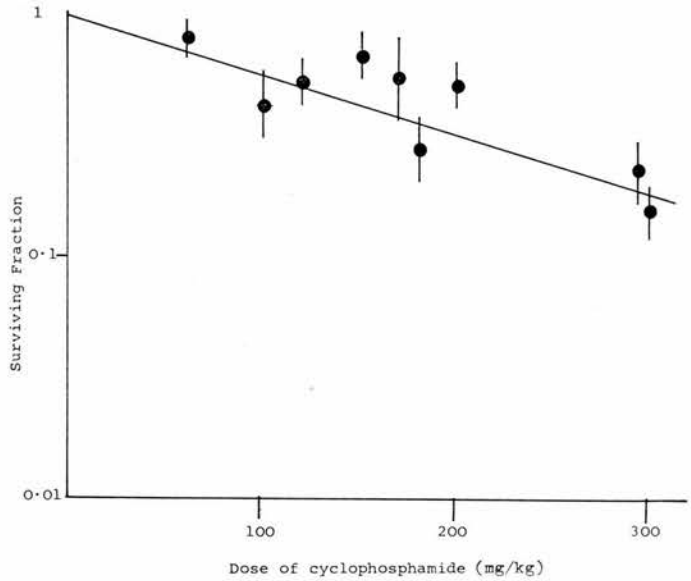


FIG. 4.—The effect of *in vivo* cyclophosphamide on colony survival (colonic tumour HX18). Tumour cell suspensions were made 18 h after i.p. injection. The surviving fraction was calculated as the ratio of the PE of the treated cells to that of the controls. Each point represents the mean of at least 5 chambers. Vertical bars represent ± s.e.

*et al.*, 1971) are well established, and some childhood solid tumours including neuroblastoma, hepatoblastoma, Wilms' tumour and rhabdosarcoma have occasionally been shown to form colonies in agar (McAllister and Reed, 1968; Sandor, 1973; Altman *et al.*, 1975). However, a reproducible quantitative colony assay for human solid tumours does not appear to have been described. Practical difficulties present a major obstacle: preliminary experiments suggest that cell suspensions direct from human tumour biopsies sometimes grow colonies in this system, but it is usually impossible to obtain repeated biopsies of the same human tumour over the prolonged period necessary to develop a reproducible assay. This problem can to some extent be overcome by the use of human tumour xenografts, which can provide a continuous supply of tumour cells from which the necessary criteria for colony growth can be established. An important assumption here is that biological characteristics influencing xenograft response to therapy do not alter with repeated passage: in this laboratory no consistent major changes in growth rate or histology of xenografts have so far been demonstrated after the initial passage from human to mouse (Pickard *et al.*, 1975) and reproducible dose survival curves have been obtained using the same treatment on different passages of xenografts over a period of about 1 year.

All 11 xenografts so far studied grew colonies using agar in diffusion chambers and chromosomal analysis and colony cell morphology demonstrated that the colonies were derived from human rather than murine cell lines. Colonies from each tumour had their own individual incubation period and PE in this system, however, and these parameters must therefore be established empirically on an individual basis for each tumour under study.

Whether the effect of cytotoxic agents on human tumour colony-forming cells in agar correlates with clinical tumour response is a question that has yet to be answered. But the dose response to

cyclophosphamide of the colonic carcinoma HX18 (Fig. 4) offers some encouragement: the sensitivity of this tumour is much less than that established for experimental animal tumours to cyclophosphamide (Bruce, Meeker and Valeriote, 1966; Park *et al.*, 1971; Lin and Bruce, 1972; Ogawa, Bergsagel and McCulloch 1973; Hill and Stanley, 1975; Steel and Adams, 1975) and this is consistent with the clinical observation that human colonic carcinomas do not usually show a marked response to cyclophosphamide. An *in vitro* agar assay using the same xenograft material has also recently been developed in this laboratory, producing dose survival curves which correlate closely with those obtained by the diffusion chamber method (V. D. Courtenay, in preparation). These human tumour colony-forming assays may therefore prove to be more realistic than assays based on experimental animal tumours for extrapolating laboratory tumour response data to clinical cancer therapy.

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