THE RADIOSENSITIVITY OF HUMAN NEUROBLASTOMA CELLS GROWN AS MULTICELLULAR TUMOUR SPHEROIDS.

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Abstract

Multicellular Tumour Spheroids (MTS) are a useful *in vitro* models of human cancer. Two cell lines - NB1-G and 1MR-32 - derived from two human neuroblastomas, were grown as MTS and were subjected to single, split and fractionated irradiation.

The NB1-G MTS line is radiosensitive, with low capacity for repair of sublethal damage, which indicated that NB1-G may be a suitable cell line to test the theoretical advantage of hyperfractionation.

The single dose response of 1MR-32 MTS, suggested that, intypically for neuroblastoma, 1MR-32 cells possessed a significant 'shoulder' on the cell survival curve.

Fractionated radiation regimes were designed to be theoretically isoeffective for damage to late responding normaltissues (calculated using the linear-quadratic mathematical model with $\frac{\alpha}{\beta}$ = 3GY). The radiation responses of MTS were evaluated using the end-points of regrowth delay and 'proportion cured'

Regimens using smaller doses per fraction were found to be markedly more effective in causing damage to the NB1-G MTS, as assessed by either end-point.

The isoeffective regimens caused approximately equal damage to 1MR-32 spheroids also.

The findings were consistent with a substantial repair capacity for 1MR-32 MTS and implied that the well-known clinical heterogeneity of neuroblastoma might extend to its cellular radiobiology.

These experimental findings support the proposal that

hyperfractionation should be a therapeutically advantageous strategy in the treatment of tumours whose radiobiological properties are similar to those of the MTS neuroblastoma line NB1-G but not in the case of 1MR-32 MTS.

On the basis of these results, it seems plausible that hyperfractionation would not be a universally advantageous strategy, but one whose efficacy is likely to depend on being able to select apropriate tumours for this form of treatment.

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Chapter 1

NEUROBLASTOMA: THE CLINICAL PROBLEM

Nature of Neuroblastoma

Neuroblastoma - Neuroblastoma Sympathicum - originates from neural crest cells which normally give rise to sympathetic ganglia and the adrenal medulla. (1). It is the most primitive of the sympathetic nervous system tumours and is also the most common extracranial solid malignant tumour in childhood, which accounts for ∧10% of paediatric cancers. (2,3,4,5). The tumours may occur anywhere from the superior cervical ganglia down the sympathetic chain to the bladder. The median age of diagnosis is 2½ years old and 90% of patients will be diagnosed within the first five years of life. (5).

Neuroblastoma is characterized by early dissemination and 70% of patients have tumour spread beyond the primary site at the time of diagnosis. The sites of dissemination are the lymph nodes, bone marrow, bone, liver and skin, with the liver metastases mostly occuring in young infants (< 6 months old) and the bone metastases more prevalent in older children. (2,5).

Neuroblastomas often contain areas of differing degrees of maturation with the presence sometimes of fully mature ganglion cells.

Etiology of Neuroblastoma

The Etiology of Neuroblastoma is unknown, however, environmental factors are suggested by the fact that the tumour is uncommon in children in certain

geographical areas. (6).

It has been observed that there is a significantly reduced incidence of neuroblastoma in black children in tropical Africa in comparison with American black children, which suggests the presence of exogenous influences on the etiology. (2). In the United States of America it is less common in black children than in white children. (6).

In children with neuroblastoma, there seemed to be no association with immune deficiency syndrome or congenital defects. There is, however, an established link between neuroblastoma and foetal hydantoin syndrome i.e. women who took hydantoin anticonvulsants when pregnant. (3).

Pathology of Neuroblastoma

Macroscopically, neuroblastoma appears encapsulated with poorly defined margins which infiltrate the surrounding tissue.

Microscopically, neuroblastoma is composed of small, round or slightly elongated cells with scant cytoplasm.

The first sign of differentiation is seen by the formation of rosette-like structures - a grouping of cells around a tangle of young nerve fibres, although these are not always present. (3,6).

Neuroblastoma cells have cytoplasmic structures consisting of neurofilaments, neurotubules and neurosecretory granules. The granules represent cytoplasmic accumulations of catecholamines. (3,6).

It is thought that coarse ultrastructural evaluation of undifferentiated neuroblastoma may have prognostic value.

Clinical Manifestations.

The clinical manifestations of neuroblastoma often depend upon the location of the primary tumour. Neuroblastoma tumour tissue can occur anywhere that sympathetic neural tissue normally occurs. In more than 50% of cases, a primary tumour is found in the Retroperitoneal region which arises in the adrenal medulla or a sympathetic ganglion. (5).

In some tumours, there is extension through to the spine and children may present with signs of spinal cord compression.

Late recognition of the intraspinal component has resulted in permanent paraplegia, which is unfortunate because patients with dumb-bell shaped neuroblastoma have a very good chance of survival. (3,5)

Horner's Syndrome is the result of an involvement of the cervical sympathetic ganglion and is associated with enopthalmus, ptosis of the upper eyelid, slight elevation of the lower lid and constriction of the pupil. (3).

When neuroblastoma arises in the opthalmic sympathetic nerve, Heterochromia iridis - a difference in the colour between the two iridis - may result. (3,5). A particular pattern of spread peculiar to neuroblastoma is the association of orbital and liver metastases.

Meningeal or Intercerebral metastases may occur but are rare. (6).

Biochemical Features

Increased levels of Catecholamines and their metabolites are found in the urine of $>90\%$ of

neuroblastoma patients. This is thought to be due to their increased production or to their defective storage within the tumour cells. (6).

The most frequently assayed urinary catecholamines are Vanillymandelic Acid (VMA) and Homovanillic Acid (HVA).

VMA is elevated in the urine of 75 - 96% of neuroblastoma patients and an HVA elevation is found in 68 - 95%. (3,6). The VMA/HVA ratio has been shown to have prognostic significance with the most favourable outcome associated with VMA/HVA ratios greater than or equal to 1.5 (3). A patient occasionally shows no sign of increased catecholamines although a periodic assay of urinary VMA/HVA levels have proved to be a good prognostic test with good reliability (80 - 90%) (3).

The amount of HVA does not matter to prognosis, although the higher the level of VMA, the better the prognosis. (3).

Increased levels of HVA and VMA have also been found in plasma. Plasma Carcinoembryonic Antigen(CEA) levels are increased in a number of malignant and non-malignant diseases. (3,6).

Complement levels increase and fluctuate during the course of neuroblastoma, they often rise with recurrence but decrease if the disease becomes terminal. (3).

Increased levels of Neuron-Specific-Enolase (NSE) have also been found in neuroblastoma tissue, although it is not neuroblastoma specific.

Gene Amplification It has been demonstrated by

Kohl et al that there are two metaphase chromosome anomalies in many human neuroblastoma cell lines. (7). These are long, non-banding homogeneously staining regions (HSR's) and small, paired chromatin bodies known as Double Minutes (DM's). It is thought that the HSR's and DM's of human tumours are likely to be manifestations of amplified genes.

The amplification process is associated with the transposition of the sequences involved from the short arm of chromosome two to the long arm of chromosome one. The frequent occurrence of DM's and HSR's in neuroblastoma tumours and cell lines indicated that amplification was a general property. (7). Biedler et al speculated that the HSR's were in some way functionally involved in the excessive production of one or more proteins specific to the malignant neuronal cells. (8). Kohl et al suggested that the commonly amplified sequence was representative of a gene - N-MYC - whose production was necessary for the proliferation of neuroblastoma tumour cells. (7).

Brodeur et al have shown that N-MYC is amplified in >90% of human neuroblastoma cell lines irrespective of the cytogenetic form of the amplified DNA. The amplification, however, is only found in 38% of primary tumours and the reasons for this are unknown. (9).

It has been demonstrated by Gilbert et al that one or more genes on chromosome 1p were involved in neuroblast transformation and that amplification of certain onc genes contribute to the ability of a tumour to metastasize. (10).

Staging and Diagnosis

The most widely used staging system for neuroblastoma was devised by Evans et al in 1971. (2,3,5,6).

Stage 1 — tumour is confined to organ or structure of origin.

Stage 11 — tumour extending in continuity beyond the organ or structure of origin but not crossing the midline. Regional lymph nodes may be involved.

Stage 111 — tumour extending in continuity beyond midline. Regional lymph nodes may be involved bilaterally.

Stage 1V — remote disease involving skeleton, parenchymatous soft tissue or distant lymph node groups.

Stage 1V-S — otherwise classified as having Stage 1 or 11 but with remote disease confined to one or more of the following sites - liver, skin or bone marrow.
Most patients with this stage are less than one year of age.
Spontaneous regression occurs. (2).

There are biological differences between Stage 1V and 1V-S e.g. there are increased serum ferritin levels in Stage 1V but not Stage 1V-S. (2,3,6). Increased serum ferritin levels are associated with an unfavourable prognosis. (3).

Prognostic Factors.

The prognosis of neuroblastoma is affected by the following factors:-

(a) Age of the patient at the time of diagnosis.

Infants who remain free of neuroblastoma for one year are usually cured but older children have been known to experience recurrences many years after initial diagnosis. (3).

Children less than one year and older than six years do better than children between the ages of one and six.

Children older than six have a less aggressive disease and may live 5 years or longer before succumbing to their illness. (6).

(b) Clinical stage at the time of diagnosis.

This is the single most important prognostic factor and together with the patient's age are the two independent variables which are of the greatest prognostic significance. (3,5,6).

Patients with Stages 1 and II generally have good prognosis. Few children with Stages III and IV neuroblastoma - regional or widespread metastatic disease - survive disease free for more than two years. For all stage of disease beyond localized, completely excised tumours, the infant of less than one year has a significantly better remission rate and survival rate than the older child of equivalent stage. (5)

(c) Localization of Primary Tumour.

Children with abdominal primary tumours have the worst prognosis, and in this group, those with nonadrenal primaries have a better prognosis than

those whose tumours originate from the adrenal glands. (1,6).

(d) Lymphocyte Count

Spontaneous remission and maturation have been attributed to, in part, host immune defense mechanisms. (3,6). Lymphocytes of most patients cured of neuroblastoma and those which have active disease, have been known to inhibit the growth of neuroblastoma cells in tissue culture - a phenomenon not seen in lymphocytes from control subjects. This cytotoxicity appears to be mediated by T-cells. (3,6).

Other factors involved in the prognosis of neuroblastoma include site of metastases, lymph node involvement and the histological make-up of the tumour i.e. degree of differentiation. (1,5,6).

The presence of tumour in regional lymph nodes markedly alters the prognosis in these patients and they have a clinical course much the same as the child with more widely disseminated tumour. (5).

Vaage et al summed up the most favourable prognostic circumstances:-

(1) tumour diagnosed within the first three months of life.

(2) tumour localized in cervicotheracic region.

(3) clinical Stage 1

(4) tumour histologically well differentiated

and(5) normalization of levels of post-operatively excreted catecholamine derivatives (VMA and HVA) (1).

Present Treatment Methods.

Therapy of neuroblastoma is often difficult to evaluate because of the tumours unpredictable course and the lack of uniform criteria for defining treatment response.

Before surgery, however, complete evaluation for metastatic disease should be carried out with computerized axial tomography (CAT) of the abdomen, liver scan and bone scan. Urinary catecholamine levels should be obtained.

Surgery

Where at all possible, total surgical removal of Stage I and II should be undertaken. The two years disease free survival of this group varies from 84% when complete resection is performed, to 63% when only partial resection is possible. There is no evidence to suggest that further therapy improves survival even when macroscopic disease is left behind. (5,6).

The exact rôle of surgery in Stage III and IV tumours has yet to be defined, although it has been shown that there is no improvement in overall survival using debulking surgery for Stage IV patients.

When surgery is performed following other treatment, however, the survival period is longer than if no surgery was used. (6).

Radiation Treatment

Neuroblastoma is a radiosensitive tumour, but the exact rôle of radiation therapy in it's treatment still remains to be defined.

Radiation therapy does not seem to benefit patients

with Stage 1 and 11 disease, even when macroscopic tumour remains following surgery. (3,6).

It is normal to irradiate residual Stage III tumour after surgery or even before to reduce tumour size. It is not known whether radiation improves survival in these cases. (3,6)

Radiation therapy clearly plays a role in the palliative treatment of patients with Stage IV disease. (6).

Chemotherapy

Chemotherapy is used for the majority of patients with disseminated neuroblastoma. The variety of drugs and drug combinations that have become available over the past 20 years have significantly increased the tumour response rate (3,6).

Combination chemotherapy with cyclophosphamide, vincristine and adriamycin in two and three drug combinations has not resulted in a definite increase in the complete remission rate.

Since cisplatin and VM-26 were added to cyclophosphamide and vincristine, however, there seems to have been a difference observed in recent years. It has been concluded, therefore, that at the present there has been no confirmed increase in the median survival of children with metastatic neuroblastoma (Evans Stage III and IV) over the past 30 years. (11).

Carli et al looked at 26 different chemotherapeutic drugs used as single agents and found that only the 6 named below achieved a clinical response in at least 20% of the patients.

Cyclophosphamide.	Cis-platinum.
Vincristine.	VM-26.
Adriamycin.	Peptichemo. (5,6).

There is some evidence that aggressive combination chemotherapy may improve the survival rate in older children and adults with metastatic disease.

Nitschke et al treated 33 children with Stage IV neuroblastoma with intensive chemotherapy. The drugs used were Cyclophosphamide, Vincristine, Papaverine and Trifluoro-methyl-2-deoxyridin. The therapeutic value of the four drug combination was limited due to side-effects related to myelosuppression which resulted in severe complications. (12).

Voûte et al used a four drug schedule of Vincristine, Prednisolone, Cyclophosphamide and Methotrexate. Complete remissions were achieved but could not be maintained. (13).

Finklestein et al looked at 104 Stage IV neuroblastoma patients, using a triple drug regimen of Cyclophosphamide, Imidazole, Carboxamide and Vincristine, and a similar regimen plus Aariamycin. They found no significant difference in the median survival observed in children with Stage IV metastatic neuroblastoma, although overall survival significantly improved. It was found that the children under one and older than six at the time of diagnosis, had > 40% greater chance of enjoying prolonged tumour-free survival. (14).

If systemic treatment is needed in certain stage IV - S patients, gentle chemotherapy is often very successful. (2).

Late Effects of Treatment

Radiation Therapy

(a) Skeletal Effects.

Irradiation of growing bone reduces growth rate and causes early fusion of the epiphyses.

(b) Soft Tissue Effects.

Failure of muscle development.

(c) Endocrine Organ Effects.

Children treated in the early years were irradiated throughout the whole abdomen and so the girls' ovaries and the boys' testes received the total dose of radiation. Most of the children had complete ovarian/testicular failure and required hormonal replacement for pubertal development to take place.

Children with neuroblastoma in the neck had their Thyroid Gland irradiated and the thyroid dysfunction that resulted was compensated with increased levels of thyroid stimulating hormone.

Chemotherapy

Since treatment with cytotoxic drugs is fairly recent, not much is known about the late effects of

chemotherapy.

Limitations of Chemotherapy

The effectiveness of chemotherapy is compromised when cells are not proliferating or when tumour growth kinetics are almost zero, as seen in the necrotic centre. Cells in less vascularised areas will only be exposed to lower concentrations of the chemotherapeutic agent. (4).

The response rates to drugs in children under two years of age at diagnosis are usually as high as 60% but the cure rates may be 10% to 25% with disseminated disease. (4).

Combination Therapy

Due to the immediate enhancement of radiation by certain of the cytotoxic agents, one might expect that there will be greater late effects although there is only limited evidence of this. (2) Neuroblastoma patients don't usually have both treatments now.

Radiochemotherapeutic treatment of minimal residual disease in neuroblastoma was studied by Zucker et al. (15)

In the low risk patients, the early and late hazards of therapy were found to be higher than the benefits and Zucker concluded that even radiotherapy after partial removal and chemotherapy in Stage II patients was questionable.

Proposals for New Treatment.

Due to the limited success in the treatment of patients with disseminated neuroblastoma, several new experimental approaches are being investigated. This section will attempt to summarize what has been

done.

Antibody Targeted Radiotherapy

A quick and accurate diagnosis of malignant disease is essential, in order that the appropriate treatment may commence without delay.

Most malignancies can be diagnosed from the clinical picture and the results from histopathological and cytological analysis, although neuroblastoma can be difficult to differentiate from other small blue round cell tumours. (16).

Monoclonal antibodies were proposed as good diagnostic and therapeutic tools in 1982 and have a high degree of tumour specificity - not absolute tumour specificity but operational specificity - and availability. (16,17).

Antibodies which do exist are used for in vitro diagnosis of tumour type and in vivo detection of metastatic spread of tumour. (17). If such antibodies can be radiolabelled without any loss of immunologic specificity, they may be able to deliver cytotoxic amounts of radiation, cytotoxic drugs or toxins to the tumour. (18,19).

In order to minimize normal tissue irradiation, target cell specificity and a high extraction co-efficient are necessary with any radionuclide.(18). Other factors influencing it's applicability are tumour cell retention time and the rate of catabolized radionuclide. (18).

The choice of radionuclide for antibody mediated radiotherapy is a difficult problem. Long range β^- particle emitters like ^{131}I have been used so far but short range

- particle emitters like At are under consideration. For short - range emitters, it is important that every cell be targeted which may not be possible with single antibodies due to reported antigenic heterogeneity in some tumours.

In order to overcome heterogeneity in antigen expression seen in neuroblastoma, it is necessary to use a panel of monoclonal antibodies and currently there are 11 different anti-neural antibodies in use to assist in the diagnosis of neuroblastoma. (16,17). Such heterogeneity could be due to the fact that cells blocked at different stages of maturation express different profiles of introcellular or cell membrane antigens. There is an occasional cross-reactivity, the mechanism for which is unknown. (16).

Kemshead et al used a panel of monoclonal antibodies to differentiate between leukaemia, lymphoblastic lymphoma and neuroblastoma. The panel of monoclonal antibodies offered a rapid and accurate adjunct to conventional techniques in the diagnosis of neuroblastoma. (16).

Cheung et al used 3 different monoclonal antibodies which were cytotoxic to neuroblastoma in the presence of complement. All identified neuroblastoma in patient specimens and, using indirect immunofluorescence, they could reproducibly detect $<0.1\%$ tumour cells seeded in bone marrow cells. (19).

Studies using monoclonal antibodies conjugated with low doses of either ^{123}I or ^{131}I have demonstrated the possibility of targeting antibodies to primary and

metastatic tumour sites in patients with neuroblastoma.

UJ13A is a monoclonal antibody which is known to bind to human neuroblastoma cells. Preclinical studies have demonstrated that radiolabelled UJ13A is taken up into human neuroblastoma xenografts established in nude mice, although no antibody uptake was recorded when experiments were repeated with an antibody known not to bind to human neuroblastoma. The levels of the radio-labelled antibody conjugate fell more rapidly in mouse blood and organs, than in tumour tissue. (17,20).

Before antibody-mediated radiotherapy can be used to it's full capacity, certain objectives need to be achieved e.g. preservation of immunologic specificity after labelling, elimination of cross-reactivity with normal tissue antigens and the use of monoclonal antibodies of human rather than murine origin. (18).

MIBG - Targeted Radiotherapy

The compound Meta-Iodobenzylguanidine (MIBG) is an analogue of a precursor of epinephrine and norepinephrine and so had a strong affinity for the adrenal medulla and adrenergic storage vesicles.(17,21).

MBG follows the metabolic pathway of norepinephrine and tissues which have reservoirs of hormone retain norepinephrine and norepinephrine-like compounds e.g. MIBG.

Neuroblastoma tumour cells can synthesize and store catecholamines. (21,22).

Radio iodinated MIBG allows safe, non invasive, sensitive and specific scintigraphic location of

neuroblastoma tumour cells (22) and is useful in locating primary tumour as well as residual, recurrent or metastatic disease. (21).

In patients with bone marrow metastases, systemic uptake may lead to a high dose being delivered to bone marrow stem cells. (21). Harvested bone marrow may be cleaned using specific antibodies and reinfused after therapy to circumvent the bone marrow toxicity.

The therapeutic possibilities afforded by ^{131}I-MIBG may give a new role for radiotherapy in the treatment of children with neuroblastoma.

Total Body Irradiation and Bone Marrow Rescue

Some of the newer experimental approaches in the treatment of patients with disseminated neuroblastoma include Total Body Irradiation (TBI), which is used as a systemic therapeutic agent.

Total Body Irradiation (TBI) with bone marrow rescue is being used increasingly in the management of children with first or second remission. (23).

TBI as a preparation for Bone Marrow Transplant (BMT) has three functions.

(1) Immune Suppression

This is essential for the graft to 'take'

(2) Elimination of Malignant Cells from the body

This seems to be more successful if the number of tumour cells has been reduced by previous intensive treatments.

(3) Bone Marrow Ablation

There is some evidence that grafted bone marrow can only be successfully established if 'space' in the marrow cavity is made by a huge deletion of

haemopoietic elements by TBI. (23).

Wheldon et al looked at log cell kill after low and high dose TBI on human neuroblastoma cells in vitro. (2). They predicted that low dose TBI achieved only modest cell kill but high dose TBI with marrow rescue could reduce the number of clonogenic cells by a factor of $10^4 - 10^5$, without severe side-effects.

Bone marrow stem cell toxicity is frequently the dose-limiting factor with conventional cytotoxic drug and/or radiation therapy. (17,25,26). This can be overcome by using autologous or allogenic bone marrow rescue. Bone marrow contamination by metastases is often present at diagnosis which may limit the availability of uncontaminated marrow for haemopoietic reconstitution. (17,26,27).

Several techniques have been developed to ablate tumour cells from harvested bone marrow to be used for autologous bone marrow e.g. treatment of bone marrow with drugs or physical procedures relying on differential centrifugation.

Immunological procedures include coating malignant cells with antibody for opsonisation, treating bone marrow with antibody and complement or with antibodies to which either drugs or toxins have been attached. (17).

The use of fluorescence activated cell sorting for cleansing tumour cells from bone marrow experimentally has produced good results but it is too slow. (17).

Kemshead et al developed an alternative approach

to fluorescent sorting - the use of a Magnetic Separation procedure. (17,26,28).

This method removes neuroblastoma cells from bone marrow by means of monoclonal antibodies conjugated to magnetic polystyrene microspheres. The separation of the cells is on the basis of their iron content and it is possible now to target microspheres (using Magnetite) to any type of cell using monoclonal antibodies. (17,26,28).

Using the magnetic separation device, it is possible to purge approximately 5×10^9 bone marrow cells of at least 99.9% of tumour cells in 3 hours. (17).

This method is advantageous because it allows rapid estimation of tumour cell removal from the 'magnetic negative' fraction and testing and absorption of complement is not required. (26).

The indirect method of attaching beads to tumour cells using monoclonal antibodies and anti-mouse Immunoglobulin has several advantages over a direct system where microspheres are coated with monoclonal antibodies. It is more efficient and economical; the separation technique depletes tumour cells from bone marrow to a level of at least 1:1000 and 1:10000 nucleated cells; the use of the technique for T-cell depletions from bone marrow to be used for allogenic transplantation is being investigated and the new beads require less anti-mouse Immunoglobulin to coat their surface. (26).

Kemshead et al looked at new M450 magnetic beads and discovered them to be more effective than the older M330 beads. (28). The new beads have hydroxyl groups

on the surface, allowing monoclonal antibodies, anti-mouse immunoglobulin or other antibody binding molecules to be covalently linked to the surface of the microspheres.

Tumour cell contamination of harvested bone marrow from neuroblastoma patients for use in autologous marrow transplantation may adversely affect patient survival. It is still not known whether tumour cells which are re-infused are clonogenic and what numbers of viable cells would be necessary for re-establishment of the disease.

Reynolds et al looked at two methods for sensitive detection of neuroblastoma cells in bone marrow. (29). The first method was for use in model systems. The viable neuroblastoma cells were premarked with the DNA stain Hoechst 33342 (H342) and seeded into normal marrow for purging experiments.

The fluorescence of H342 stained cells, allowed detection of one H342 stained cell in one million marrow cells.

Counterstaining the mixture with trypan blue, reduced the H342 fluorescence in non-viable cells, therefore, limiting the detection to viable cells only.

The second method could be used in model systems or clinical bone marrow specimens. It relied on the specific staining of neuroblastoma cells with an antiserum to Neuron Specific Enolase (NSE) and/or with anti-neuroblastoma monoclonal antibodies. If the haemopoietic cells were removed from the marrow before staining i.e. enriching the neuroblastoma cells in the sample, it allowed detection of neuroblastoma cells at

1:100,000 using NSE alone.

In spite of all the recent advances that have been made in chemotherapy and monoclonal antibodies, the therapy of neuroblastoma still remains unsatisfactory and in order to determine how treatment would best be improved by the addition of radiotherapy which is believed to be an effective agent, further studies of the radiobiology of neuroblastoma are very important.

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RADIOBIOLOGICAL CONSIDERATIONS

It has been known for a long time that reproduction is the most radiation sensitive activity of mammalion cells. (1,2,4).

Irradiation, at dose levels which have a high probability of reproductive sterilization, has no effect upon cellular metabolism or on the ability of specialised cells to synthesize tissue-specific proteins. Radiation sterilized cells with a few exceptions (e.g. some small lymphocytes, oocytes) stay physically and biochemically competent until cell division is attempted and cell death results. This is an indication that radiation-induced functional impairment of tissue is mediated through the reproductive sterilization of proliferative cells. (2).

For most tissues, it is thought that physiological functional impairment is a direct result of the loss of reproductive integrity by the proliferative cells, especially the clonogenic cells of the tissue. concerned i.e. a failure of radiation-sterilized clonogenic cells to keep pace with the need to replace functional cells which are being lost from the tissue at a characteristic rate. The loss of this ability as a function of the radiation dose is described by the Dose Survival Curve.(3).

The deposition of radiant energy and the infliction of radiochemical injury are random events. The dose-response relationship for cell killing is essentially

exponential i.e. for a given dose increment, the same proportion, not the same number of cells is killed. (17).

The relationship between the radiation dose absorbed and the proportion of cells which 'survive' can be seen in a cell survival curve, which is a special form of dose-effect curve, and is based on viable cell counting i.e. the counting of colonies originating from single cells. The ability of a single cell to grow into a large colony is proof that it has retained it's reproductive integrity.

Mammalian cell survival curves are usually presented with dose plotted on the linear scale and surviving fractions on a logarithmic scale.

At low doses of radiation, there is an initial shoulder which becomes straight or almost straight at high doses on the semi-logarithmic plot. The slope is expressed in terms of the dose required to reduce the number of clonogenic cells by the factor of e^{-1}, i.e. to 37% of their previous value - called the 37% dose slope and is designated Do.

The extrapolation number - n - is found by extrapolating the straight portion of the survival curve until it cuts the 'surviving fraction axis'. The extrapolation number is important because it is a measure of the initial shoulder. (3).

The intercept of the extrapolated curve with the 100% survival level is called Dq - the quasithreshold dose.

Dq and n are interrelated because both are a measure of shoulder size (Dq is not a threshold but

a mathematical term) (17).

As the dose rate of irradiation is decreased, the efficiency of cell killing per Gray has been shown experimentally to decrease too, i.e. the 'effective D_o' increases (17).

Models for estimating equal effects when different fractionation schedules are used, have been evolved to the stage of using reasonable dose-response curves to represent the biological effect of each fraction, plus a separate time factor.

There are three dose-response formulae which are commonly used:

(1) The Linear Quadratic Model.

(2) The Multi-target Model (provided it has an initial slope superimposed i.e. 2 component slope)

(3) The Repair - Misrepair Model (16)

The Linear Quadratic Model

The L-Q Model is very useful for describing the in vitro survival curves of human tumour cell lines. It offers valid approximations for all clinically used doses, has only 2 parameters to be determined and is very easy to use in practical applications (16).

The L-Q Model assumes that the sterilization of clonogenic 'target cells' in the tissue during radiation is the cause of the observed effects. The target cells have a dose-response curve for clonogenic survival which is linear quadratic in dose (29).

From the evidence obtained from single hit killing by high LET radiation, it was suggested that the

inactivation of a cellular target, should only require the passing of one densely ionizing particle through the DNA double strand. This would mean that the 'high LET' components of neutron irradiation would cause lethal lesions in direct proportion to the dose. The term attributed to this was α- thus making the αD portion of the equation.

With low LET radiation then, lesions would only accumulate as the square of the dose i.e. one lesion requires energy deposition by 2 independent electrons within the target volume. The sublesions caused must interact to cause lethal lesions. This was termed βD^2 in the equation.

The entire equation is:-

$$\ln f = - (\alpha D + \beta D^2) \qquad (13)$$

The Multitarget Model

The Multitarget, two component (TC) model requires 2 independent parameters - D_o and n. It originates from the theory that a cell contains n targets - all alike - and if all are inactivated, the cell cannot proliferate. Hits are considered to be distributed randomly in uniformly irradiated homogenous cell populations. (17,18,30).

If, however, one of the targets is not inactivated, the cell is still capable of proliferation. (13).

The Repair/Misrepair Model

This hypothesis suggests that the surviving fraction of cells in a mutagen-treated population is proportional to the number of potentially lethal lesions that are not repaired (30).

It is thought that, over a certain range of dose rate, physical or chemical lesions from statistically independent charged particle tracks do not interact. So, only lesions from singly charged particle tracks need to be considered in the physical and chemical time domains. These lesions produce longer lived biological lesions, some of which are enzymatically repaired and some interact with other lesions.

The Model, therefore, has 2 parts:-

(1) deals with repair and/or interaction with other biological lesions and (2) deals with the production of biological lesions through evolution of the physical and then the chemical lesions. (31).

The cell survival models discussed so far, were designed on the assumption that a relatively insensitive region to a dose effect curve signifies the requirement for an accumulation of damaging events, if the end - point is to be achieved. It has been suggested that, if cells survive by the accumulation of damage, they must surely contain a repair mechanism which becomes less effective as the dose or the number of incipiently lethal lesions increases, until it ceases to operate. (13).

The initial slope of the dose-response curve is of major importance because, at lower doses (2 Grays or less) repair processes are at their most efficient, so the 'initial' slope represents the fullest expression of repair.

Two distinct mechanisms of cell killing are thought to produce a shouldered cell survival curve - one being single hit killing (a single lethal injury) and

the other being an accumulation of sufficient sublethal lesions whose interaction cause death. (17,18)

As the dose increases, presence of a shoulder on the survival curve of most mammation cells is an indication that the efficiency of cell killing per Gray increases (up to a certain limit depending upon cell type).

The manifestation of a shoulder on a survival curve was assumed to be evidence that radiation - induced damage must accumulate within the cell before a final event could prove lethal. The damage which was assumed - leaving the cell still viable - became known as 'sublethal damage' and the recovery which restored the cells' capacity to accumulate sublethal damage was known as the recovery (or repair of) sublethal damage.

Repair of Sublethal Damage/Elkind Recovery.

Sublethal damage can be repaired in a matter of hours unless additional sublethal damage is added e.g. from a second dose which will interact to produce lethal damage.

It was discovered, after X-rays started to get used clinically, that their biological effectiveness was usually lowered when low dose rates were used or when the total dose was delivered in fractions. It was the investigation into dose fractionation which helped the discovery of the sparing effect observed when a reduction in dose rate was employed. It was demonstrated that it was linked with the manifestation of shoulders in survival curves.(18). Elkind and Sutton completed a series of experiments on mammalion cells in which two sufficiently large

radiation doses were given, several hours apart. The shoulder on the first survival curve was reproduced on the second survival curve. The first dose reduced survival to the terminal exponential region of the survival curve where all surviving cells could be considered to have accumulated one less than the lethal number of hits. The full recovery of the shoulder on the second dose survival curves was an indication that all the sublethal damage had been repaired in the interval between the two doses (13,17,24)

The method employed for investigating Elkind Recovery involves the use of split dose radiations and measures the survival as a function of time between two doses.

Elkind and Sutton discovered that, as the time between doses was increased, survival increased until a maximum was reached after two hours recovery. When the length of recovery interval was increased further, the survival fell to a minimum and then rose to a plateau level. This was explained by taking into account the partial synchrony imposed on a heterogeneous cell population after the first radiation dose. The cells which survived the first dose were those in the more resistant phases and the surviving population would be partially synchronized. (10,13)

Bryant et al discovered that the speed of recovery in algal cells was dependent upon the partial pressure of oxygen i.e. Elkind recovery was an energy dependent process (13)

The actual nature of sublethal damage and it's

repair are not yet fully understood. (19).

Repair can be suppressed by exposure of cells to metabolic inhibitors e.g. cyanide, severe hypoxia or cold, before and after irradiation. So, the increased radio-resistance of hypoxic cells *in vivo* could be partly offset, in fractionated treatment, by their reduced capacity for repair of sublethal damage. (17,20).

Elkind demonstrated that there was a close connection between radiation damage/repair and the normal synthetic processes of cells. (23).

It is generally accepted that one cannot associate *all* DNA damage *e.g.* all strand breaks, with cell killing and one cannot connect all DNA repair with cell repair.

Efficient strand rejoining occurs in cells which will stop dividing and eventually lyse, indicating that the enzymatic machinery of cells consists of small, multiple replicated molecules which are therefore not high in the hierarchy of radiation - sensitive targets. (22).

In conclusion then, it is thought that DNA damage is the primary cause of cell killing and repair of DNA damage is causally related to cell repair.

The molecular and cellular damage/repair processes take place, in and around the replisome *i.e.* where DNA undergoes the sequential transition from a super helix of a single duplex to two superhelixes of replicated DNA.(22).

The importance of sublethal damage repair for radiotherapy is that, within certain limits, the total

dose required to achieve a certain level of cell killing increases as the number of dose fractions increases.

Lethal Damage

This type of damage is irrepairable, irreversible and leads to death.

Potentially Lethal Damage

This is radiation damage which could cause cell death under some post irradiation culture conditions but not in others e.g. nutritional deprivation, low temperatures and inhibition of protein synthesis by cycloheximide, all of which prevent the progress of cells through the division cycle (3,17)

Clinical Radioresistance

The extent of radiation damage observed in tissue is dependant upon several factors, including tissue responsiveness to radiation, and it is known that there are multiple biological factors involved in clinical radioresponsiveness.

The operational definition of 'Tumour Radioresistance' is: 'A tumour is clinically radioresistant if it regrows within the irradiated region, regardless of it's rate of regression'. (6)

Causes of Clinical Radioresistance

Tumour Related Factors

(a) Number of clonogenic cells

A clonogenic cell is one which is capable of regenerating the tumour and must belong to the stem cell compartment or be capable of being recruited back into the pool. Since the killing of tumour cells is random, tumours with a large number of clonogens are

more difficult to destroy than tumours with a few clonogens of similar radio-sensitivity. It is impossible to quantitate the number of clonogens in human tumours and so one cannot predict radioresistance based on a large number of clonogens.(5)

When a homogenous group of tumours are exposed to graded irradiation doses, a Tumour Control Probability curve (TCP) is produced, which implies that tumour populations are heterogeneous and sub populations of studied tumours vary in their 'radioresistance'.(5).

(b) Hypoxia

As solid tumours grow, the tumour vasculature often fails to keep up with the increase in the number of tumour cells. As cells become more than $\prec$180 microns distant from the nearest capillary, they become hypoxic (oxygen deficient) and die producing a necrotic centre in the tumour. Tumours do not have to be large and bulky or necrotic to contain hypoxic cells.

For ionizing radiation, the oxygenation status of the cells is considered to be an important factor in determining response. Hypoxic cells are thought to be three times less sensitive than oxygenated cells to X-ray radiation.(3,17)

The shape of mammalion cell survival curves after exposure to X-rays in the presence and absence of oxygen is seen to be the same. The difference is the magnitude of the dose required to cause a given degree of biological damage. The ratio of hypoxic to aerated doses required to produce the same

biological effect is the same at all survival levels and the ratio is known as the 'Oxygen Enhancement Ratio'. (3,17).

(c) Tumour Kinetics

There have been many theories put forward to explain the phenomenon of tumour recurrence, including rapidly proliferating tumours which can significantly repopulate during a conventional treatment regimen and slowly growing tumours which may be radioresistant due to poor redistribution throughout the division cycle during dose fractions, to quote only two.

(d) Intrinsic Radioresistance

This is defined as 'cellular radioresistance' manifested by asynchronous, well oxygenated populations of tumour cells in vitro. (6).

There are two kinds demonstrated:
(1) is associated with a large shoulder on a radiation cell survival curve indicating a capacity for 'Elkind type repair', and (2) is associated with plateau phase cultures with clinical resistance which have an increased ability to repair Potentially Lethal Damage.

It is now agreed that the initial slope of a cell survival curve at 2 Gray and lower is the region of interest. (26,27,32). The 'final slopes' represented by D_o refer to a minority of cells and are largely irrelevant in fractionated radiotherapy.

Since n and D_o are the currently used parameters and are calculated from the high dose portion of the graph, they cannot be used to define the low dose

radiosensitivity *in vitro* of a cell line whose survival curve has an initial shoulder.

A single model free parameter, the mean activation dose - $\bar{D}$ - is the area under the survival curve and has been suggested for the characterization of human tumour cell line radiosensitivity at clinically relevant doses.

$\bar{D}$ depends upon the initial portion of the *in vitro* survival curve and it has been shown that the parameters S_2, $\bar{D}$ and $(\alpha D + BD^2)$ are the most suitable for the characterization of human cell radiosensitivity. (27,28).

Fertil and Malaise looked at *in vitro* survival curves (low dose portion only) at 2 Gray (S_2) and established a correlation between S_2 and the clinical radioresponsiveness of the group from which the tumours were derived. (5,26). This was confirmed by Deacon et al who demonstrated that 2 Gray was the optimum dose level to compare the biological data with clinical radioresponsiveness. (26).

The observed differences in *in vitro* radiosensitivity as a function of the histology of the tumour, argue strongly for the role of certain intracellular factors in clinical responsiveness (5,26).

Clinical responsiveness is known to be linked to the histology of the tumour and it is known that intrinsic radioresponsiveness varies from one histological cell type to another. (5,26,27,28).

Weichselbaum completed studies on radiosensitivity and radiation damage repair in human cell lines. He discovered that some radio-incurable tumour produced

cell lines which possessed the ability to repair potentially lethal damage in plateau phase cultures.

The cell lines found to be most efficient at potentially lethal damage repair had been derived from patients who had failed clinical radiation therapy. The surviving fraction following radiation could be a function of high $\underline{D_o}$/inherent radioresistance and/or the capacity to accumulate sublethal damage or recovery over a period of hours. The maximum survival potential of a tumour cell, therefore, could be an important factor in clinical radiocurability. (9).

The more resistant types of human tumour e.g. gliomas do have significantly more intrinsic radioresistance to low doses than other types of tumour.(26,27,28).

Parshad et al completed experiments on the chromosomal radiosensitivity of 13 human tumours from diverse tissue origins and discovered that all the tumour cells displayed enhanced G_2 chromosomal radiosensitivity, compared to normal cells.(25).

The effect, therefore, which tumour radioresistance has on patient survival is dependant upon the type of cancer present and the level of disease control.(6).

(2) Host Related Factors

(a) The Volume Effect

The complications in 'deep-seated' tissues are dependant upon the volume irradiated, therefore, when large treatment volumes are needed to encompass known

or suspected disease, the clinical radioresistance of a tumour increases.

(b) Dose Limiting Normal Tissues.

The total biological dose of radiation given to a tumour is dependant upon the dose-limiting organ/ tissue within the treatment site and so, depending upon the relevant normal tissue, a tumour might be said to be clinically more difficult to treat in one site than another.

(c) Patho-physiological Factors.

These affect the tumour directly e.g. by increasing the number of hypoxic cells in an anaemic state.

(d) Host Defenses

This is unlikely to affect the clinical problem of radioresistance.

(3) Technical Factor.

These include error in dose delivery and geographical miss i.e. the need to encompass all the tumour.

Multiple Dose Fractions and Dose Survival Curves.

On a multifraction irradiation dose survival curve, after allowing for complete sublethal damage repair between doses, the slope will have an increasingly downward trend as the dose per fraction decreases.

The dose per fraction will eventually reach the zone where the majority of cell killing is due to single hit events and not the accumulation of sublethal damage. When the dose per fraction is small enough, the slope of the fractionated survival curve is determined by the single hit component of cell killing and this represents the limiting slope

irrespective of further fractionation (17).

Repair of subcellular injury, regeneration, cell cycle redistribution and reoxygenation all contribute to differentials in fractionated dose response between various normal tissues and tumours. Studies by Withers examined fractionated dose responses of the three generic tissues types: Acute/ Early responding normal tissue, Slowly/Late responding normal tissue and Tumour tissue.(7).

Regeneration

When radiation treatment is continued over several weeks, surviving stem cell regeneration is the most important contributer to the differential sparing of early responding tissues relative to both slowly responding normal tissues and, most of all, tumour tissue.

The onset of regeneration depends upon the development rate and severity of injury which, in turn, is dependant upon tissue kinetics and, to a lesser extent, dose given. The latency in the onset of regeneration of the surviving cells is also important and may last from days to weeks depending upon the tissue e.g. G.I. Tract - within a few days and the skin - within 3-4 weeks (7,17).

It is believed that the target cells for late sequelae do not display significant regeneration throughout the course of a conventionally fractionated regimen.

Tumours can accelerate their growth rate due to cytocidal effects of radiotherapy. Delay between initial radiation and the onset of regrowth is

probably due to the proliferation kinetics of the tumour clonogens and to a certain extent, the size and delivery pattern of the dose. The onset of regrowth is slower after a set of multiple fractions than after a large single dose of radiation.

Repair of Cellular Injury

Early responding normal tissue do not display as large a capacity for cellular repair than late responding normal tissue (7). Late responding normal tissues determine the tolerance doses which are dependant upon the late responding tissue in question and the volume irradiated. This could be attributable to the fact that target cells for late effects have the ability to repair more sublethal damage than the target cells in acutely responding tissues (7,8).

The α/β ratio of the Linear-Quadratic model describes the susceptibility of tissues to single and multihit killing with late responding normal tissues responding as though they were more susceptible to multihit mechanisms. The β/α ratio is a measure of tissue sensitivity to changes in size of dose per fraction. Late responding normal tissues are characterized by larger values of β/α than acute responding normal tissues (8,17).

There is no experimental evidence to suggest that tumour cells are capable of more repair of sublethal demange than normal cells, although some studies have shown that, due to hypoxia or low levels of cyclic AMP, tumour cells may have a reduced repair capacity (10).

Repair of sublethal damage due to dose fractionation should spare tumour cells to about the same extent as acutely responding normal tissues, but to a lesser extent than it spares slowly responding normal tissues.

Reoxygenation

Reoxygenation is not a factor in the response of acute or late responding normal tissues, although reoxygenation during a radiation treatment course, sensitizes the hypoxic fraction of tumour cells (10).

Although the mechanism of reoxygenation is not fully understood, several factors may play a part:
(1) Death of some of the tumour cell population will result in a reduction in oxygen consumption and the dead cells' removal could reduce the average intercapillary distance.
(2) Loss of tumour substance may help to improve blood flow due to a reduction in tissue tension and
(3) an increase in vascularization may occur (3,11).

Reoxygenation can account for the success of fractionated radiotherapy relative only to single-dose treatment. It cannot account for a favourable differential between normal and neoplastic tissue responses since normal tissues are usually well oxygenated (10).

Redistribution

The effect of redistribution is to reduce the probability that surviving cells will be in resistant phases of the cell cycle at times of subsequent doses of radiation (17).

The effect is difficult to assess in early

responding normal tissues because it is overshadowed by the protective effect of regeneration.

The target cells for late sequelae are slow to proliferate and so undergo little cell cycle redistribution during a multifractionation regimen. Late responding normal tissues are less sensitized by redistribution which, therefore, results in less severe late reactions for a given level of tumour control.

It is likely, but unproven, that both normal and malignant proliferative tissues may self sensitize themselves by redistribution during clinical multifraction regimens in a manner similar to the sensitization of tumours by reoxygenation. This would cause an increase in the therapeutic ratio obtained with dose fractionation by allowing the tumour clonogen population to 'self-sensitize' itself in the absence of a similar effect in slowly responding cells whose depletion leads to late injury.

There are two detectable phases through which a cell passes during a division cycle - mitosis, which is visible microscopically, and DNA synthesis which is detected by the uptake of radioisotope labeled DNA precursors. Between mitosis (M) and DNA synthesis (S) there is a gap (G_1) and after DNA synthesis, before the next mitosis, a second gap (G_2). (10,12,25).

Sinclair and Morton irradiated Chinese hamster cells at different cell cycle phases. They found that the most sensitive cells were in mitosis (M) or at the G_1:S phase with the most resistant cells at the

late S phase and in the 'middle' of the long G_1 phases. (10,12).

Slowly proliferating cells may stay in a radioresistant phase post irradiation but rapidly proliferating cells will redistribute themselves into more sensitive phases in time for subsequent dose fractions.

Hyperfractionation

Withers defined Hyperfractionation as the division of radiation treatment into smaller than conventional doses per fraction without a change in the overall treatment duration. (7).

The aim of Hyperfractionation is to increase the therapeutic differential between late responding normal tissues and tumour tissues. Acute responses would be expected to parallel tumour responses and so, if slowly responding tissues are treated to their tolerance by increasing the dose sufficiently, acute normal tissue responses should be increased in parallel with an improvement in tumour control probability.

The rationale behind Hyperfractionation is that due to a larger repair capacity of sublethal injury, slowly responding tissues are spared more by reducing the dose per fraction, than are tumours. (7,14,15).

The responses of acute responding normal tissues are increased, although they can be tolerated with good nursing care, and, if this is not possible, then an extension of the overall time would have to be introduced.

If cell cycle redistribution restored the surviving proliferative cells to the starting - more

radiosensitive asynchronous state, then it would be more effective, in it's sensitizing influence, if the doses per fraction were smaller and the fractionation intervals were more frequent. (7,14).

Since reduction occurs in all cycling cell, no therapeutic gain will occur relative to normal epithelial cells which determine acute reactions.(14).

When the radiation dose is fractionated, sublethal injury is repaired over a period of hours. In low dose regions of cell survival curves, the majority of cell killing is the result of single hits. The accumulation of sublethal injury has a low probability of causing cell death because the number of lethal lesions per cell is usually less than the lethal number (14,17).

Dose per Fraction

The exact dose-response relationships for late responding normal tissues have not been measured over a dose range of 1-2 Gray. The dose range of the initial exponential region of cell survival curves is also uncertain.(21).

The biologic equivalence sought in the conventional and hyperfractionated regimens is in late - not acute - effects. Increased acute effects may make it difficult to give the appropriate dose for equivalent late effects in exactly the same overall treatment time.

With higher fractional doses, which reduce survival beyond the initial exponential region of a survival curve, accumulation of sublethal injury is the major cause of cell death. Repair of

sublethal damage after every larger fraction means that extra dose has to be delivered in the next fraction to bring the average number of sublethal lesions per surviving cell back to the lethal threshold. Therefore, the total dose required to produce the same effect is increased when the number of fractions in this size range is increased. (12,14,17).

Experimental studies have suggested that a basic difference exists between the survival curves of the target cells determining acute and late normal tissue effects. A difference which causes late responses to be more critically affected by changes in dose fractionation.(14).

A well known clinical manifestation of this difference when changing to fractionation regimens with fewer, larger doses per fraction is the observation of large increases in late effect incidences with little or no change in acute effects. (12,14).

It is suggested that Hyperfractionation is advantageous, in that, when late damage is the limiting factor, it allows higher total doses to be given in a larger number of smaller fractions than usual.

Fractionation Interval

It is thought that sublethal damage repair in acutely responding normal tissues is achieved in 3 hours.(7).

This may not be true for late effects tissues or for all acute effect ones. An ideal fractionation interval should be at least 6 hours to ensure that

sublethal repair is complete in the dose limiting late responding normal tissues. If repair is complete in acutely responding tissues (including the tumour) but not in the late responding normal tissue, the influence of the most important rationale for Hyperfractionation - Differential Repair - is lost, or at least reduced.

The overall treatment time should be the same in Hyperfractionated and conventional treatment, although there are advantages and disadvantages to prolongation of treatment time.

The disadvantages are:

(1) the tumour cells have more time to proliferate therefore making ultimate cure less likely and

(2) it is inconvenient for the patient and it is expensive.

The advantages are:

(1) it spares the acute reactions since compensatory proliferation in skin or mucosa accelerates at 2-3 weeks after starting an irradiation course,

(2) it permits adequate reoxygenation to occur in tumours before the end of the treatment.

(14,15).

Potential Therapeutic Gain.

The increase in tolerance dose for slowly responding late effects tissues has not been experimentally determined for doses of <2.6 Gray for lung and this has produced inconsistant data.

If tumours show the same fractionation response as acutely responding normal tissues, the escalation

is the dose required for an isoeffect (equal Tumour Control Probability) determined by using an α/β ratio of 10 Gray would be 7-8%,

that is $2n \times 1.08\ Gy = n \times 2\ Gy$.

So, if late effect tissues can tolerate a 15-25% increase in dose, when n fractions of 2 Gy are replaced by 2n fractions, then the therapeutic gain would lie between

$$\frac{1.15}{1.08} = 1.065 \text{ and } \frac{1.25}{1.08} = 1.16$$

equivalent to an increase in tumour effective dose of 6.5 - 16% for the same tolerance dose in late responding tissues. (7).

The gain would be further increased if, the greater number of fractionation intervals and smaller doses per fraction, resulted in a greater response due to cell cycle redistribution, with no change in slowly developing normal tissue responses.

The impact of Therapeutic Gain Factors of 1.065 - 1.16 on Tumour Control Probability would depend upon the steepness of Tumour Control Probability curves.

In conclusion then, it is generally agreed that malignant tumours have a lesser repair capacity for sublethal damage and have faster proliferation kinetics than normal tissues, under steady state conditions.

Deacon et al, Fertil and Malaise and Weichselbaum have suggested that, due to the vast diversity in the radiobiology of human tumour cells - with respect to 'intrinsic radiosensitivity' and 'post irradiation damage repair', Hyperfractionated treatment regimens may not be suitable for every malignant tumour type. (9,5,26,28).

It is therefore desirable to complete experimental studies on individual tumours wherever possible.

Hyperfractionation and Neuroblastoma

Wheldon et al suggested that neuroblastoma may be a suitable candidate for Hyperfractionation, although this has not, so far, been attempted clinically (33). The radiobiological rationale for Hyperfractionation in Neuroblastoma is based mainly on single dose cell capacity for the repair of sublethal damage as discovered by Wheldon et al (32). The extension, therefore, of the investigation to include split-dose and fractionated irradiation would appear to be the next step.

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THE TUMOUR SPHEROID MODEL

Cellular aggregates of animal cells have been used in developmental biology and experimental cancer research for several decades. As long ago as 1944, Holtfreter described a method he used to generate stable, spherical aggregates of embryonic cells. (1).

In 1959, Dabrowska - Piaskowska examined the 'histo-formative capacities' of cell aggregates of C3H mammary tumour and vaginal epithelioma cells, and discovered that there was a remarkable structural similarity between tumour cell aggregates and the original tumour tissue. (2).

In 1967, McAllister et al described a pronounced similarity between the cytological characteristics of the primary tumours, the respective colonies and the tumours induced by in vivo implantation of the cultured cells. It was also reported that the almost spherical colonies consisted of a central, necrotic area, surrounded by a run of viable cells with interspersed, degenerated forms. (3).

Sutherland et al were the first to study the responses of cell aggregates to radiation therapy using Chinese Hamster V79 lung cells. It was because of their almost perfect spherical shape that the aggregates were called 'multi-cell spheroids' or 'multicellular spheroids'. (4,5).

It was noted that the cellular aggregates bore a close resemblance to nodular structures observed in C3H mouse mammary carcinomas. These experiments

showed that a multi-component survival curve was generated when mammalian cells in the form of Multicellular Tumour Spheroids (MTS) were irradiated. The curve was seen to be similar to that reported for solid animal tumours and several types of human tumours and so multicellular tumour spheroids were henceforth considered as in vitro models for examining the basic biological properties of cancer cells in vivo (4,5).

Characteristics of Multicellular Tumour Spheroids.

Growth of spheroids

The volume growth kinetics of MTS are known to be similar to that of solid tumours. There are 3 phases of spheroidal growth which are distinguishable.

The first phase is the initiation phase and is characterized by the aggregation of single cells to form small clumps and then a period of geometric, exponential growth until the spheroid diameter is between 50 - 200 um. (6,7,8).

At this point, the cell cycle distribution changes within the spheroid, resulting in an accumulation of resting cells at the centre of the spheroid and a sequestering of the proliferating cells at the periphery. (8).

As the MTS increases in size, more diffusion gradients of nutrients and catabolites set in and it becomes a 2 layered solid, with an outer, well-nourished and proliferative 'crust' and an inner, deprived and non-proliferative 'mantle'. (6).

This leads to a progressive diminution of the growth fraction and to a second phase of growth,

characterized by a linear expansion of the spheroid diameter with time. (8).

During the second phase, the cell cycle distribution starts to alter, towards an increasing accumulation of non-proliferating cells in central regions of the spheroids. (9).

At a later stage, which is line characteristic, the MTS develops a central 'necrotic' core which continues to increase proportionally as the overall size increased. Central necrotic cells are observed occasionally at ~200 μm diameter and are clearly evident at 300 μm. (4).

Eventually the MTS, like solid tumours in vivo, are heterogeneous cellular masses with three layers - the outer, proliferative crust, the middle, viable but non-proliferative mantle and a central, necrotic core. (6).

Sutherland et al suggested that the fraction of cells in the intermediate zone increased as the spheroid grew, which accounted for the reduction in the growth fraction. (5).

At later times of plateau and senescence, cell shedding of the MTS and cell packing in the interior may be appreciable.

These do not significantly affect the growth rate during the initial and linear growth phases because if this were the case, then the growth rate would be less than linear. (6).

In clinical oncology, it seems likely that the pattern of linear increase in diameter with time might also apply to 'micrometastases' in vivo, in the period

before the development of intratumoural blood supply. When this occurs, the diffusion of metabolites and catabolites is no longer solely through the external surface of the solid aggregate and different conditions apply. (6).

As the spheroids enlarge further, their growth progressively slows down and the MTS reach a maximum diameter. (8).

Spheroids whose maximum diameter has been achieved, can be maintained in culture for several weeks with practically no increase in their volume, irrespective of however often the medium is replenished.

Folkman et al demonstrated that the same saturation in volume growth was obtained when spheroids were implanted into animals in such a way that vascularization of the implant, from the host, was prevented. (10).

Landry et al developed a growth model for spheroids which took into account parameters such as cell size, cell doubling time and cell shedding rate. (8).

Yuhas et al demonstrated a correlation (for 3 murine cell lines) between tumour growth rate and spheroid growth rate, while no such correlation could be demonstrated between tumours and standard monolayers. (11)

Structure of Spheroids

Yuhas and Li looked at 7 different tumour lines and discovered that the outer, proliferative crust had a constant thickness which was characteristic for each cell line.

They discovered that, if the cell cycle times were the same, the tumour lines with the thick, proliferative crust grew faster than those with the thin, proliferative layers. (12).

Conger and Ziskin demonstrated that the mantle maintained a constant thickness and so the entire 'viable' rim had a constant thickness as the diameter increased over the full range of linear growth. (6). Their findings that crust thickness was line specific and different for different cell lines supported the findings of Yuhas and Li.

Since the nutrient and oxygen concentration of the medium at the MTS surface is constant and if the metabolic rate is constant with time, it follows that a gradient of nutrients in, and catabolites out, will be established within the MTS.

If there is a nutrition or catabolite threshold level at which proliferation ceases, then it is easy to see why the proliferative crust thickness is constant.

Freyer and Sutherland completed experiments in dissociating EMT6/RO MTS to produce 8 subpopulations of cells originating from a varying depth within the spheroid. (9).

The cells located further into the spheroid (50 - 100 μm) usually remained viable and were capable of forming colonies. They were smaller in size than outer cells and no longer incorporated ^{3}H - thymidine into their DNA. The cells were not incapable of such incorporation but were dormant.

The cells located further than 100 µm from the spheroid surface were also arrested in their cell cycle progression, but, in addition, many of their inner region cells had a reduced viability and a markedly reduced ability to form colonies. The cells located nearest the necrotic centre were apparently not far from cell death and lysis. (9).

It has been outlined previously that spheroids possess a histological structure similar to that of solid tumours as regards the distribution of vital and necrotic areas. This is also, at least partly true for the extracellular matrix.

Nederman et al identified similar matrix components such as glycosaminoglycans or collagen in tumour spheroids and their respective tumours. (13).

When the spheroid cells were grown as monolayers, the amount of synthesizing matrix materials appeared to be reduced or even absent.

This is an illustration that the degree of structural and functional differentiation in the primary tumour may be retained in spheroids rather than in monolayer cultures. (14).

Many recent publications on multicellular spheroids are concerned with the simulation of various tumour therapies, since the in vivo response of cancer cells to treatment may be reproduced in spheroids more closely than in conventional monolayer cultures.

Contact Resistance

The reaction of multicellular spheroids to ionizing radiation has been investigated most intensively and

one of the most interesting observations is the finding that some cells grown as spheroids are more resistant to ionizing radiation than the same cells grown as monolayer under otherwise similar conditions. (15). Sutherland and Durand suggested that this provided one possible mechanism for tumour radioresistance in vivo (16).

The 'contact effect' or 'contact resistance' is poorly understood. However, it has been suggested that it may be correlated with the occurrence of gap junctions and, with some restrictions, of electrical coupling between cells. (17).

The activity of adenylate cyclase in coupled cells cultured in vitro as multicellular tumour spheroids falls to a level much lower than under any other culture conditions. (18).

Variations in CAMP, which are capable of modulating the permeability of gap junctions, can modify contact resistance.

Changes in Ca^{2+} and H^{+} ion concentrations, can also alter the permeability of gap junctions. (17).

Although the molecular mechanism of the contact effect is still unknown, it has been hypothesized that the radiocurability of tumour cells may be enhanced by manipulation of CAMP, H^{+} or Ca^{2+} ions, using appropriate drugs. (17).

The local eradication of a tumour in vivo by irradiation is influenced directly by the radiosensitivity of the cell population, the number of viable cells in the tumour at the time of treatment, cellular hypoxia, radiation quality, dose and dose

rate.

Other factors which may influence observed tumour responses are host-tumour interactions (immunological or other) effects of the radiation on the vascular supply and the number of 'surviving' cells required to regrow and thus re-establish the tumour.

Use of End-points

A variety of techniques have been developed to quantify the effects of ionizing radiation on tumour cells. Durand examined the use of a variety of end-points which could be intercompared when using MTS as an *in vitro* tumour model system. (19). The end-points examined included growth delay and 'cure'.

Growth delay is conventionally defined as the time taken for the median volume of each individual MTS to reach some multiple of its original volume (e.g. X10).

Multicellular tumour spheroids are considered to have been 'cured' i.e. *all* the clonogenic sterilized, if they fail to regrow - with regrowth defined as reaching some multiple of their volume - by some specified time. (e.g. one month).

Regrowth curves may theoretically allow estimation of cell curvival *in situ* as long as the surviving cells' growth continues as in the un-irradiated case. If this is the case, then regrowth curves may be interpreted as simple displacements of the control growth curve, with the displacement providing a measure of the magnitude of cell kill assumed to have taken place. Extrapolation of the regrowth curve to zero time

yields an estimate (V_E) of the 'effective volume' from which the spheroid appears to have regrown. This is to say, it represents the volume of viable cells following treatment. The ratio V_E to the measured volume V_O (which represents the volume of all the cells initially present) provides a measure of the cellular surviving fraction (S) immediately following treatment. (20). Some pitfalls in this type of analysis have recently been pointed out by Moore et al. (21).

Experimental Radiotherapy of Tumour Spheroids

Rofstad et al studied the radiation response of MTS initiated from a human melanoma xenograft using cell survival growth delay and spheroid cure as end-points. (22)

The relationship between these end-points was analysed and the radiation response of the spheroids was compared with the parent xenograft.

The D_o values calculated from spheroid cure curves were similar to those of the cell curvival curves measured in soft agar.

The specific growth delays, as well as the D_o values, calculated from cure curves were similar for spheroids and tumours, when the data for the latter was corrected for the presence of hypoxic cells.

Rofstad et al used 5 melanoma cell lines and discovered that the cell survival curves for cells irradiated after disaggregation of the spheroid were equal to those previously reported for cells from disaggregated tumours of the same melanomas. That

is to say, that the cellular radiosensitivity was the same whether the cells were grown as spheroids in liquid-overlay culture or as solid tumours *in vivo* *i.e.* Contact resistance was not seen for those cell lines. (23).

Evans et al set up experiments to determine whether a parallelism existed between the clinical behaviours of specific human tumour types - neuroblastoma and melanoma - and the biological behaviour of the same tumour types grown as MTS. (24). The growth delay data correlated with the clinical response of the tumours.

Evans et al concluded that MTS provided an *in vitro* tumour model system which was amenable to the study of common radio-biological end-points including cure, regression and cell-survival which was likely to be of clinical relevance.

Although neuroblastoma is a clinically radioresponsive neoplasm, it is fatal in three out of every four cases, due to it's pattern of early dissemination. (25).

Micrometastases are often present at the time of diagnosis and because tumour spheroids *in vitro* are considered to be an appropriate model for avascular micrometastases *in vivo*, it would seem appropriate to use tumour spheroids as an *in vitro* model for the treatment of neuroblastoma with T.B.I.

Materials and Methods

Origin of NB1-G

NB1-G cell line was obtained by growth in monolayer culture of cells released by enzymatic disaggregation of a human tumour xenograft originated from tumour fragments obtained by surgical excision of a stage 1V abdominal neuroblastoma in a 2 year old boy.

In culture, NB1-G cells synthesize catecholamines, have neurosecretory granules visible by electron microscopy and an aberrant but identifiable human karyotype. In situ DNA hybridization studies have revealed the presence of multiple copies of the human oncogene N-MYC (1).

Origin of 1MR-32

1MR-32 was obtained from a Stage 1V abdominal mass in a 13 month old boy and was mechanically disaggregated and set down in flasks i.e. a primary culture.

1MR-32 is an established cell line (1967), it is hyperdiploid and consists of a mixed culture of at least 2 morphologically distinct cell types.

The catecholamine synthesis of IMR - 32 is not known. Neurosecretary granules have not been reported.

The second cell type are found in minute numbers and are relatively large, well-spread fibroblast like cells. (2) The cell line shows several features characteristic of human neuroblastoma including the existence of chromosomal Homogeneously Staining Regions

(HSRs) and an amplified copy number of the human proto-oncogene N-MYC (3).

Monolayer Culture.

NB1-G and 1MR32 cells were plated into 75 cm^2 flasks containing 20 mls of medium (MEM) with foetal calf serum (10%) at a concentration of 5 x 10^5 cells. The flasks were then incubated at 37°C in an atmosphere of 5% CO_2 at 100% humidity.

Spheroid Culture

Monolayer cultures of NB1-G and 1MR-32 were trypsinized, cell suspensions were obtained and, using the 'agar-underlay' method, were seeded to initiate MTS production. (4) In each 25 cm^2 flask, base-coated with 1% Noble-agar, 10^6 cells were seeded into 5 mls of medium (MEM) containing 10% foetal calf serum. Incubation took place in a humid atmosphere (100%) at 37% with 5% CO_2 content. After 2 - 3 days, the Spheroids were large enough to be individually 'harvested' with a Pasteur pipette. The spheroids selected those with a diameter of $\sim$ 250 μM - were transferred individually to agar coated wells of 24 well test plates (Linbro). The plates were incubated as described and 0.5 mls of medium was added weekly to each well.

Determination of Spheroid Growth Curves.

Using an automated image analysis scanning system (see Twentyman 1982), thrice weekly measurements of the cross-sectional area of the individual MTS were used to determine the growth of MTS. (5).

It was assumed that the MTS were spherical and their cross-sectional measurements were converted to

estimates of volume. By plotting the median volume value for each MTS against each day of measurement for each experimental group, spheroid growth curves were obtained.

Irradiation Procedures

The MTS were placed in test-plates for the irradiations which were carried out using a Co^{60} treatment unit. The dose rate was approximately 1 Gy/min and to ensure the maximum deposition of energy per well, a perspex 'build-up' was used.(1cm)

Single Dose Studies

The single dose studies were carried out to evaluate the simple dose radiosensitivity of NB1-G and 1MR-32 spheroids as assessed by the growth delay end-point. The doses ranged from 50 cGy to 300 cGy increasing in 50 cGy instalments.

Split Dose Studies

The Split-dose Studies were carried out to determine the split-dose radiosensitivity of NB1-G and 1MR-32 spheroids using the growth delay end point.

The dose range was between 2 x 25 cGy and 2 x 100 cGy with the time interval between the two doses at 6 hours.

The split-dose method was employed to investigate whether NB1-G and 1MR-32 spheroids were capable of repairing sublethal damage.

Fractionated Treatment Regimens

Using the linear-quadratic model (with $\frac{\alpha}{\beta}$ = 3 Gy) a series of treatment regimens were designed which were calculated to be isoeffective for late responding normal tissues (see Appendix).

NB1-G

Two sets of regimes were used on two separate occasions. One set of regimens (A) was calculated to be late - responsive - isoeffective to a single dose treatment of 2.5 Gy. The other set (B) was late-responsive - isoeffective to a single dose treatment of 4 Gy.

Each set of regimes consisted of five isoeffective schedules ranging from one to eight treatment fractions. The two sets of regimes may be regarded as corresponding to two different 'effect levels' (A $\equiv$ 1 x 2.5 Gy, B $\equiv$ 1 x 4 Gy) for damage to late responding normal tissues. Table 1 contains all the details of the schedules.

1MR-32

The treatment regime was designed to be 'late-responsive - isoeffective' to a single dose of 2.5 Gy. Five different isoeffective regimes were used, ranging from one to eight treatment fractions. These are summarized in Table 2.

It was considered necessary to have an inter-fraction interval of at least 6 hours and so the split and fractionated doses were given twice daily.

Estimation of Growth Delay

Using the MTS median growth curves, the growth

delay value for each experimental group was determined.

Estimation of Cell Survival from Regrowth Curves

If it is assumed that regrowth delay is mostly due to cell sterilization, and that irradiation has little effect on the growth kinetics of the surviving cells, then by back extrapolating the regrowth curves to zero time, an estimation can be made of median cell survival. (see p 161).

'Proportion cured'

MTS.were considered to have been 'cured' (i.e. all the clonogenic cells sterilized) if they failed to regrow (defined as reaching at least 3 X original volume) by 1 month following treatment. In practice there was a clear demarcation between regrowing and non regrowing MTS at the time. The 'proportion cured' was then evaluated as the fraction of non-growing MTS relative to the total number of MTS originally present in the treatment group.

TABLE 1

Normal tissue effect level	Fraction Number	Dose/ Fractionation (Gy)	Total time (days)	Total dose (Gy)
A	1	2.50	0	2.50
	2	1.52	0.25	3.04
	4	0.89	1.25	3.56
	6	0.63	2.25	3.78
	8	0.49	3.25	3.92
B	1	4.00	0	4.00
	2	2.53	0.25	5.06
	4	1.54	1.25	6.16
	6	1.13	2.25	6.78
	8	0.90	3.25	7.20

TABLE 2

Treatment schedule	Fraction Number	Dose/ Fractionation (Gy)	Total time (days)	Total dose (Gy)
A	1	4.00	0	4.00
B	2	2.53	0.25	5.06
C	4	1.54	1.25	6.16
D	6	1.13	2.25	6.78
E	8	0.90	3.25	7.20

DISCUSSION OF RESULTS.

NB1-G Single Dose Irradiation

Spheroid Growth Curves

The NB1-G MTS in test plates were irradiated with a single dose of 50, 100, 150, 200, 250, 300 or 350 cGy.

In fig.1 it can be seen that the unirradiated spheroids grew exponentially with doubling times of ~2.5 days until a diameter of 800 - 1,000 μM was reached after which growth slowed with a progressive lengthening of the doubling time.

Irradiated spheroids exhibited either a static phase, or a regression phase (dose dependant) which was followed by a recovery of the growth curve to a pattern similar to that for unirradiated spheroids.

The exponential portions of the regrowth curves were, to a reasonable approximation, parallel to each other and to the corresponding portion of the control growth curve.

Very few spheroids failed to regrow following irradiation in the dose range used (50 - 350 cGy) so that no 'cure correction' was necessary to the growth date.

Growth Delay

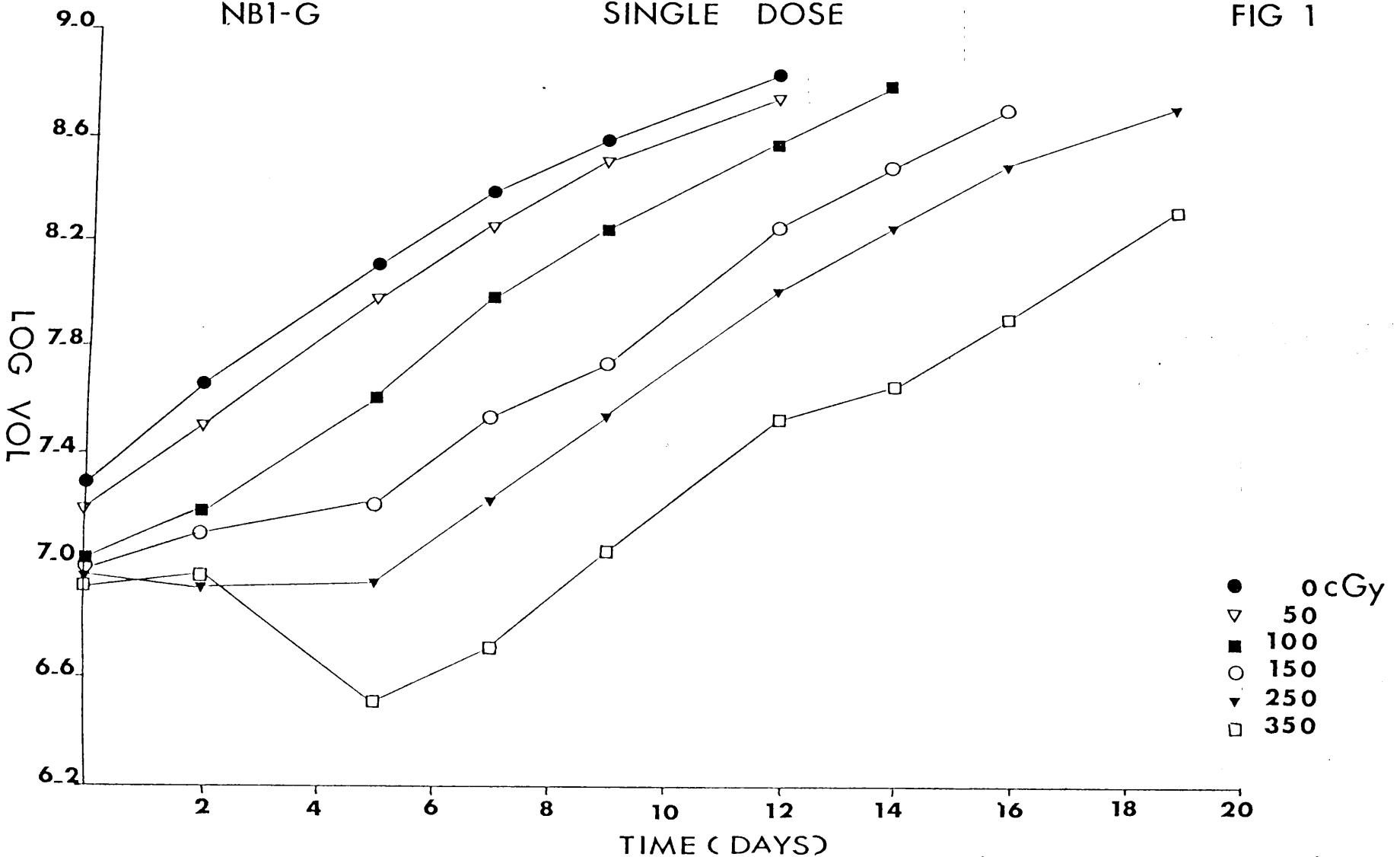
It can be seen from the growth delay curve in fig.2 that, after an initial region of shallow slope, the curve steepens as the dose is increased.

NB1-G Split Dose Irradiation

Spheroid growth curves

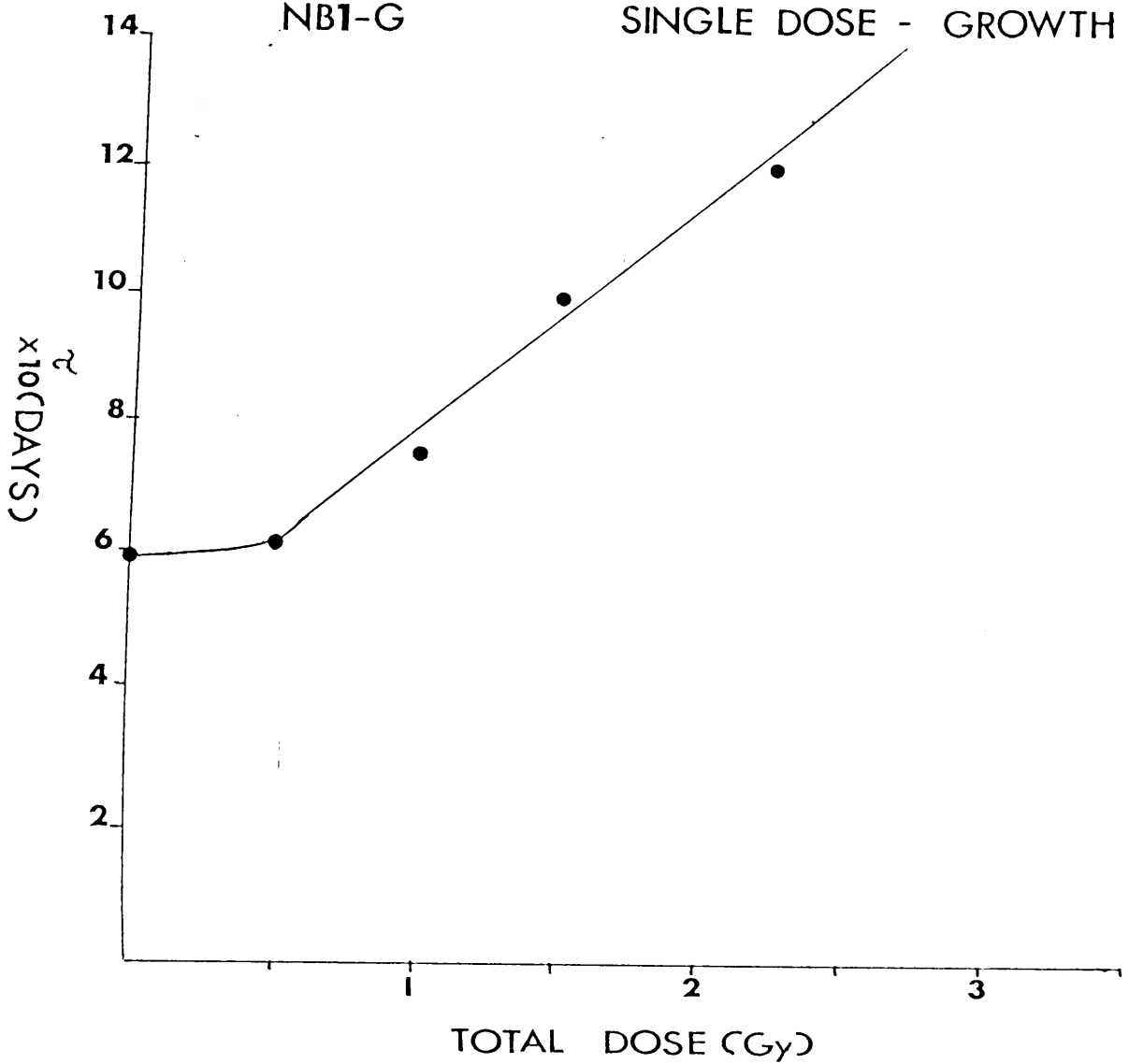
The NB1-G MTS in test plates were irradiated with

NB1-G SINGLE DOSE FIG 1

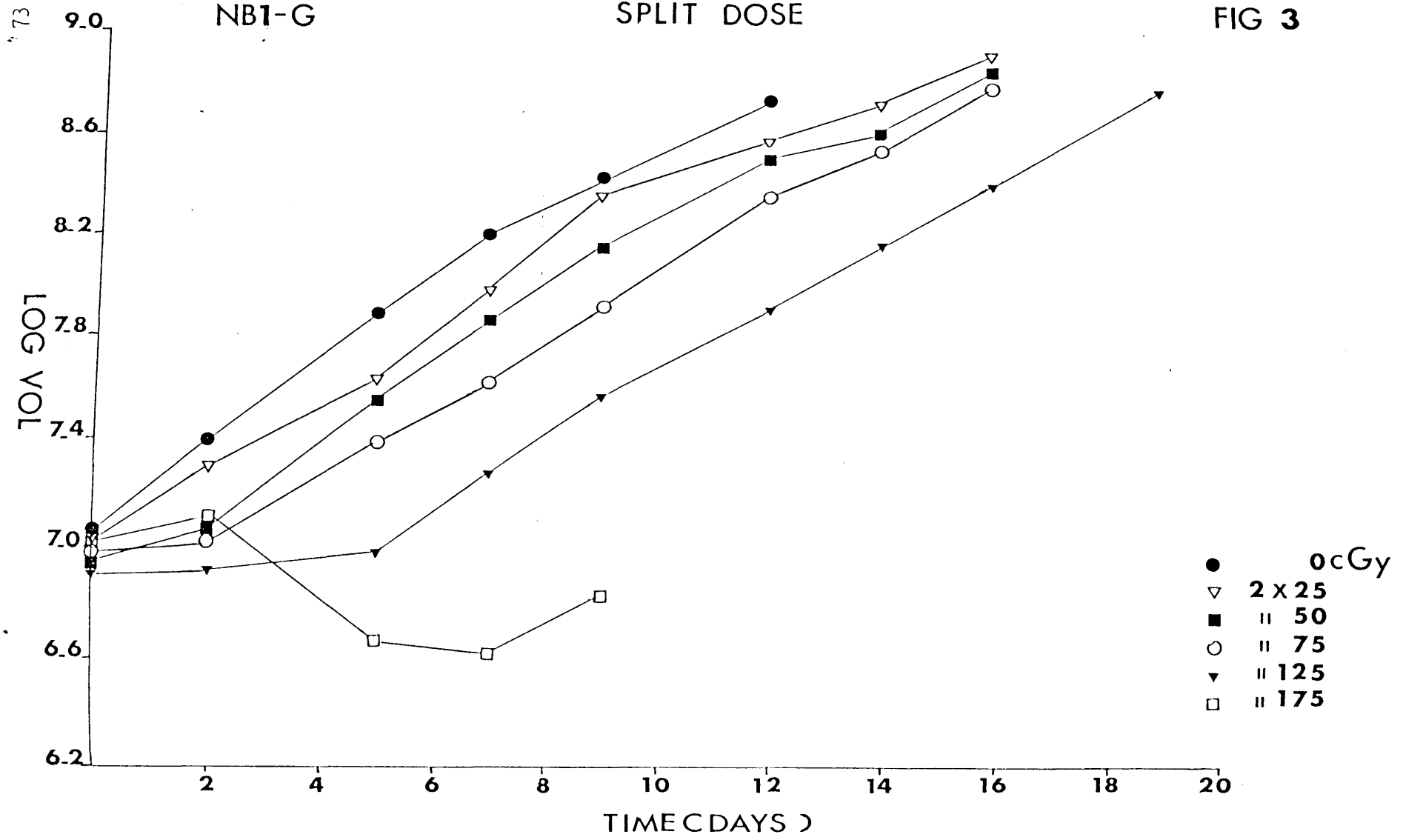


NB1-G SINGLE DOSE - GROWTH DELAY

FIG 2



NB1-G SPLIT DOSE FIG 3



NB1-G SPLIT DOSE - GROWTH DELAY FIG 4

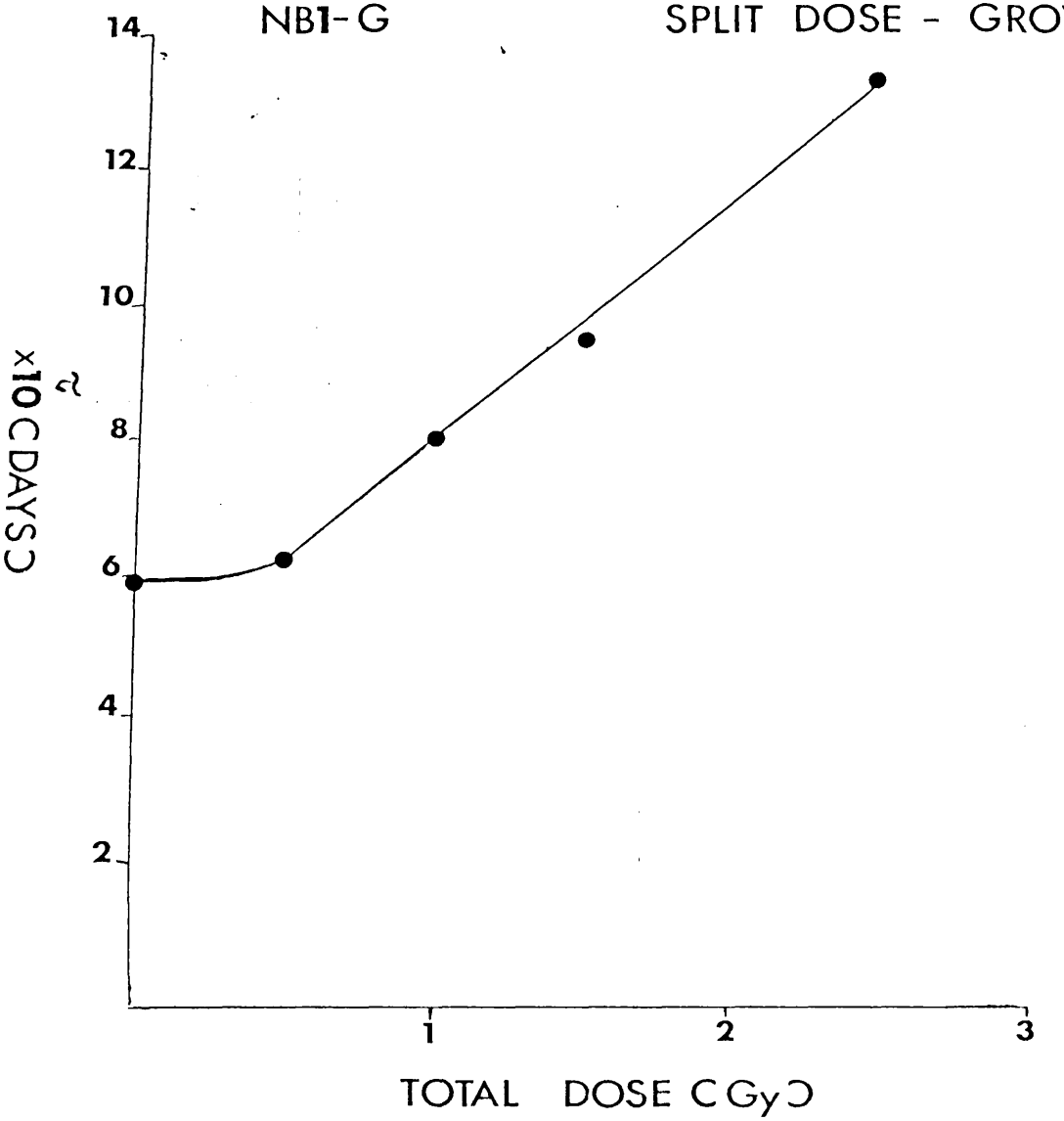
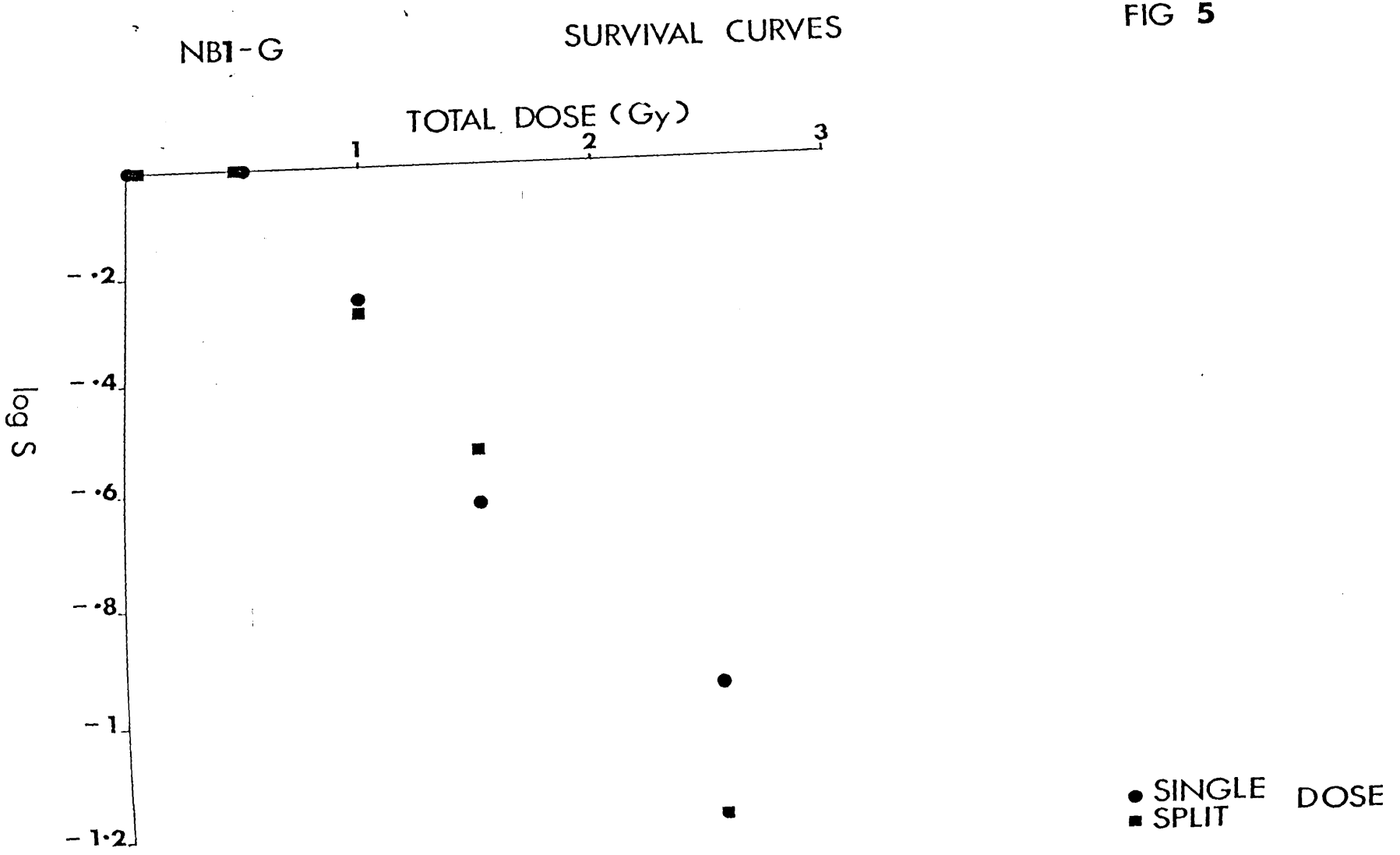


FIG 5


NB1-G
SURVIVAL CURVES
TOTAL DOSE (Gy)
1
2
3
log S
-·2
-·4
-·6
-·8
-1
-1·2
SINGLE DOSE
SPLIT

a split dose of 2 x 25, 2 x 50, 2 x 75 or 2 x 100 cGy.

In fig.3 the control growth curve follows a characteristically exponential form (volume doubling time ~ 2 - 3 days) until a diameter of ~800 - 1,000 µM after which growth slows progressively.

Growth curves for irradiated MTS displayed a static or regression phase before resumption of growth with growth curves becoming parallel to those of controls. The lateral displacement of irradiated from control curves is seen to increase progressively with dose.

Growth Delay

Growth delay as a function of total dose is graphed in fig.4 and it can be seen that there is an initial region of shallow slope which steepens as the dose is increased. When the growth delay curves for single and split dose (fig 2 & 4) are compared, it can be seen that there is no significant difference between the 2 curves, indicating no sparing effect of dose fractionation as evaluated in terms of growth delay.

Cell Survival Curves

Fig.5 shows derived cell survival curves for both the single and split dose experiments whose growth delay curves are graphed in fig.2 and 4.

As may be seen, both survival curves are nearly exponential in form with little evidence for quadratic curvature, and with only small shoulders apparent. There is also little indication for a biphasic break in the curves as would occur if a significant proportion of the clonogenic cells of the spheroid were hypoxic.

The split dose data is consistent with negligible

capacity for the accumulation of sublethal damage. No significant repair capacity has been found for NB1-G human neuroblastoma MTS subjected to 2 doses of radiation in the total dose range 50 - 350 cGy.

DISCUSSION OF RESULTS

1MR-32 Single Dose Irradiation

Spheroid Growth Curves

The MTS in test plates were irradiated with 9 single dose of 50, 100, 150, 200, 250, 300 cGy.

Fig.6 is representative of the growth curves obtained for control and irradiated MTS.

The control growth curve displays a characteristic exponential growth pattern, until a volume of 800 µM - 1,000 µM per spheroid is reached. Thereafter, growth slows with increasing size. The growth curves for the irradiated MTS show a progressively perturbed pattern as the dose is increased. At higher doses, a static or regressive phase is observed, before growth is resumed, usually becoming parallel to the control curve.

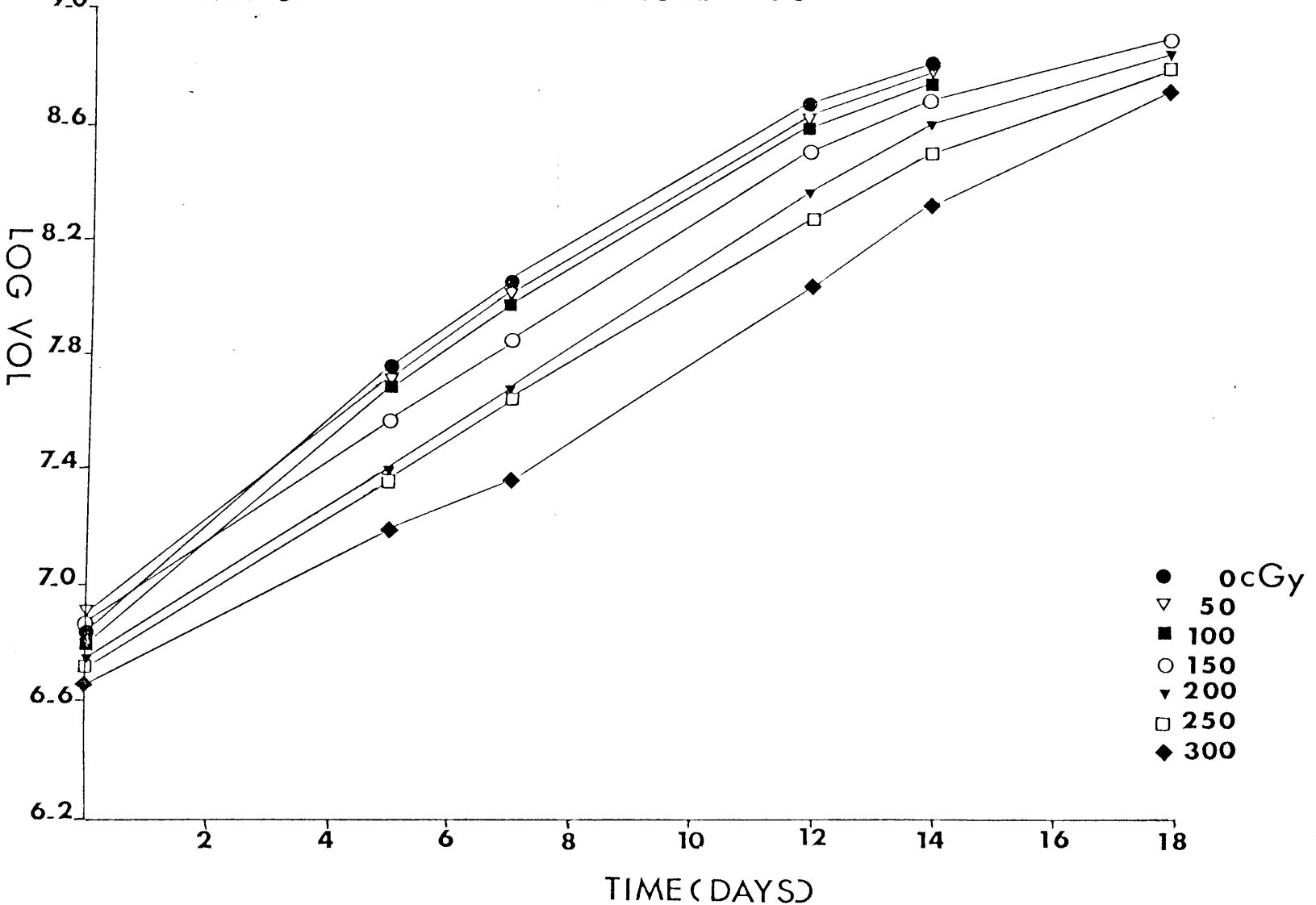
Growth Delay

As can be seen from fig.7 the growth delay curves have an initial region of shallow slope which then steepens with increasing dose. Each separate symbol is representative of a separate experiment to ensure the reproducibility of the results.

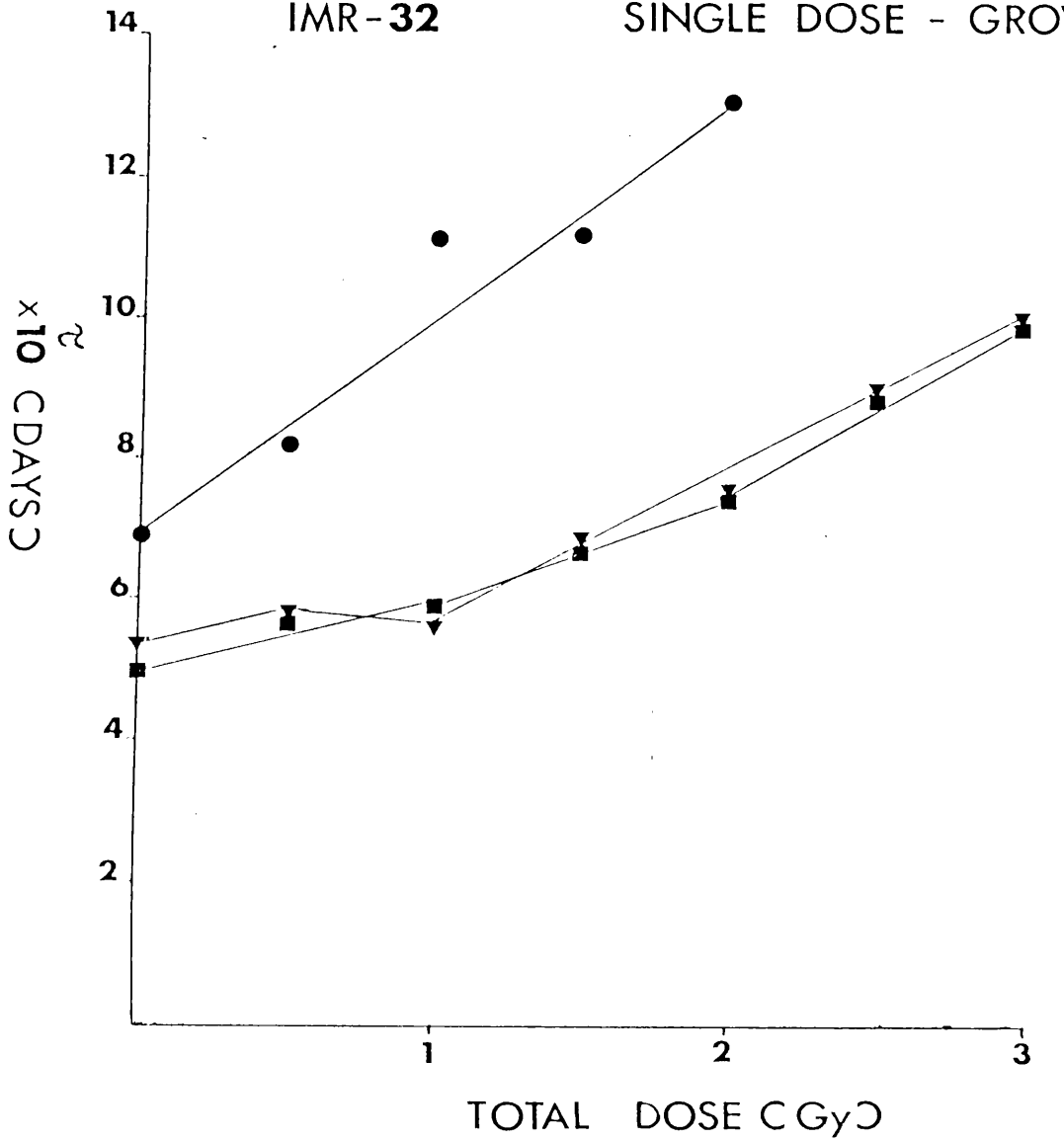
Survival Curves

The survival curves calculated from fig.7 are graphed on fig.8. Each symbol on the graph corresponds to the same symbol on the Growth Delay Curves. As can be seen, the calculated survival curve appears to have an initial shoulder - and then became approximately linear as the dose is increased.

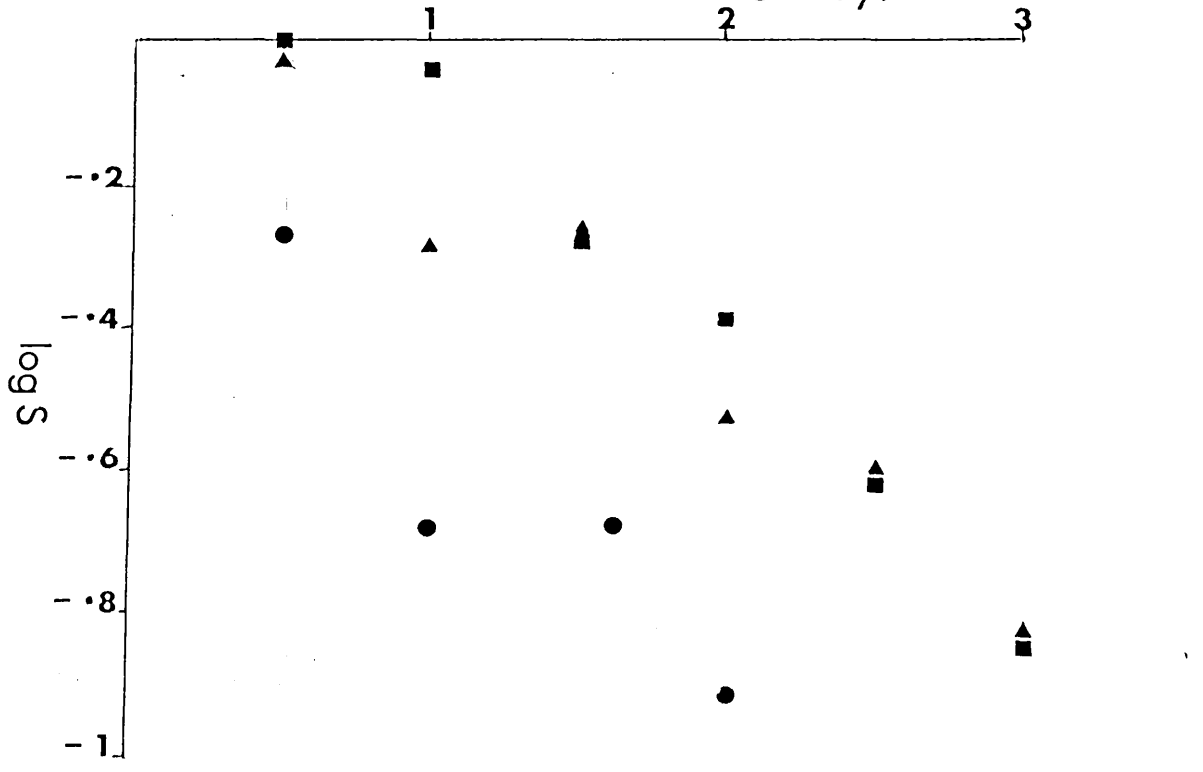
IMR-32 SINGLE DOSE FIG 6



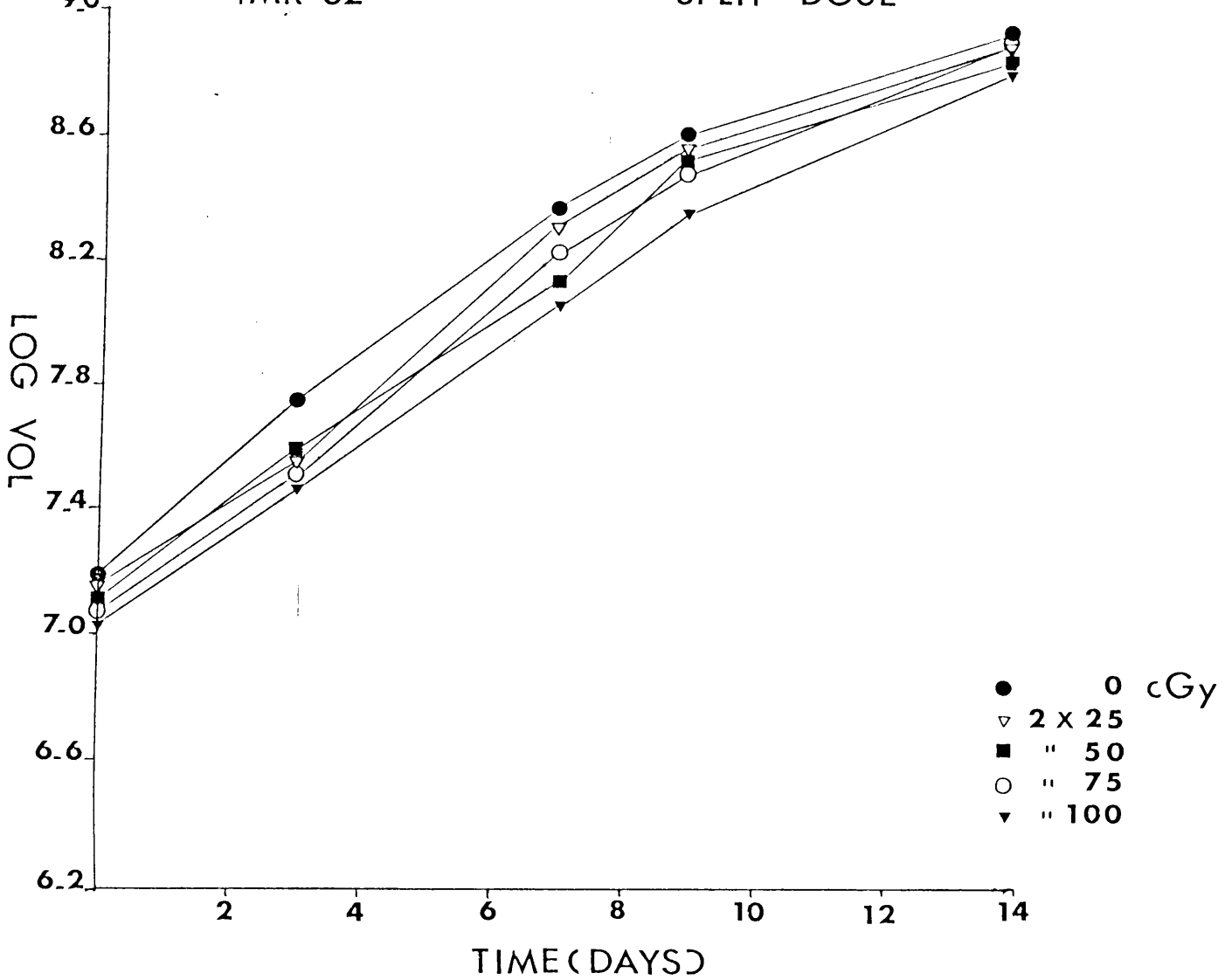
IMR-32 SINGLE DOSE - GROWTH DELAY FIG 7



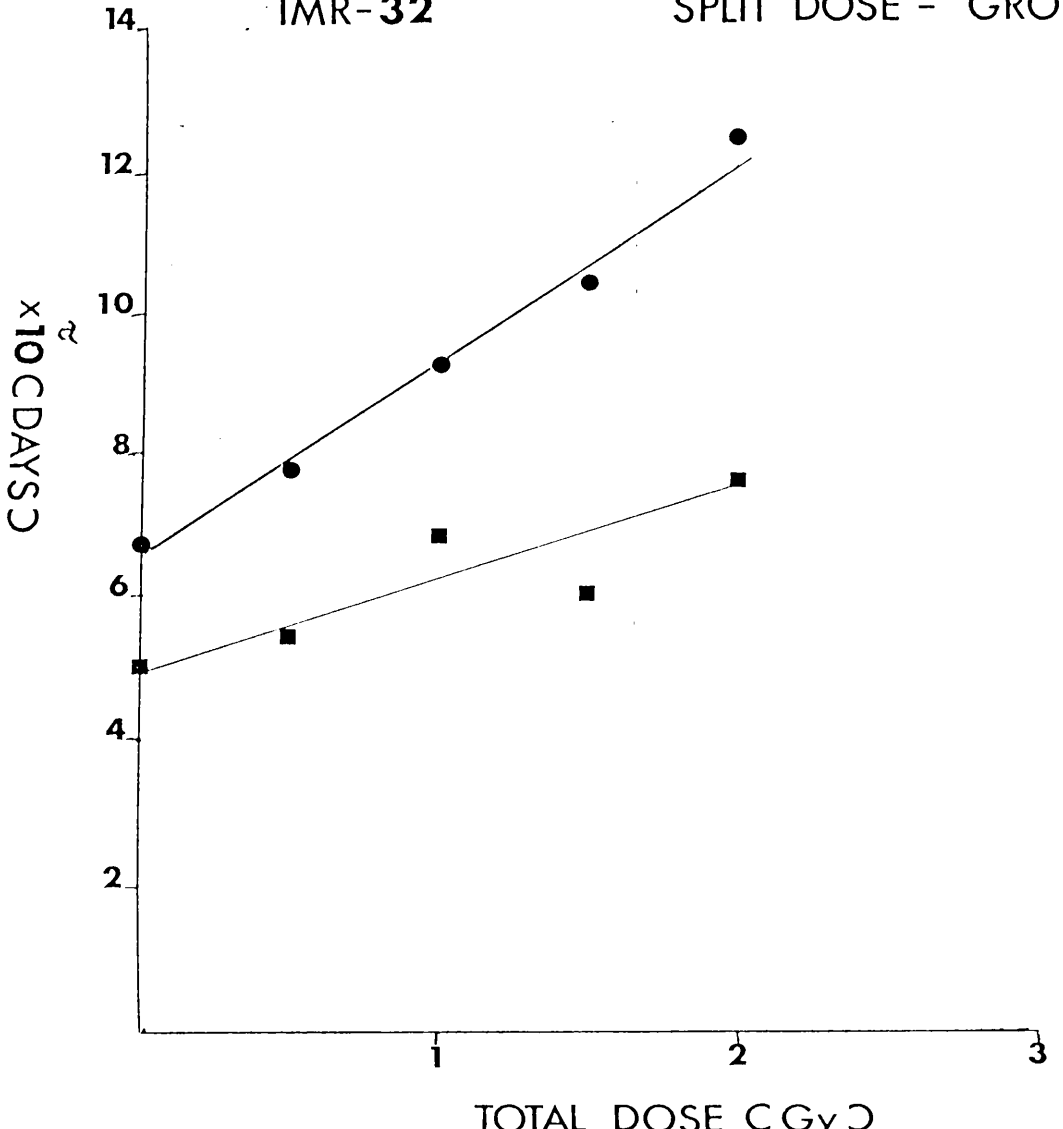
IMR-32 SINGLE DOSE - SURVIVAL CURVES FIG 8



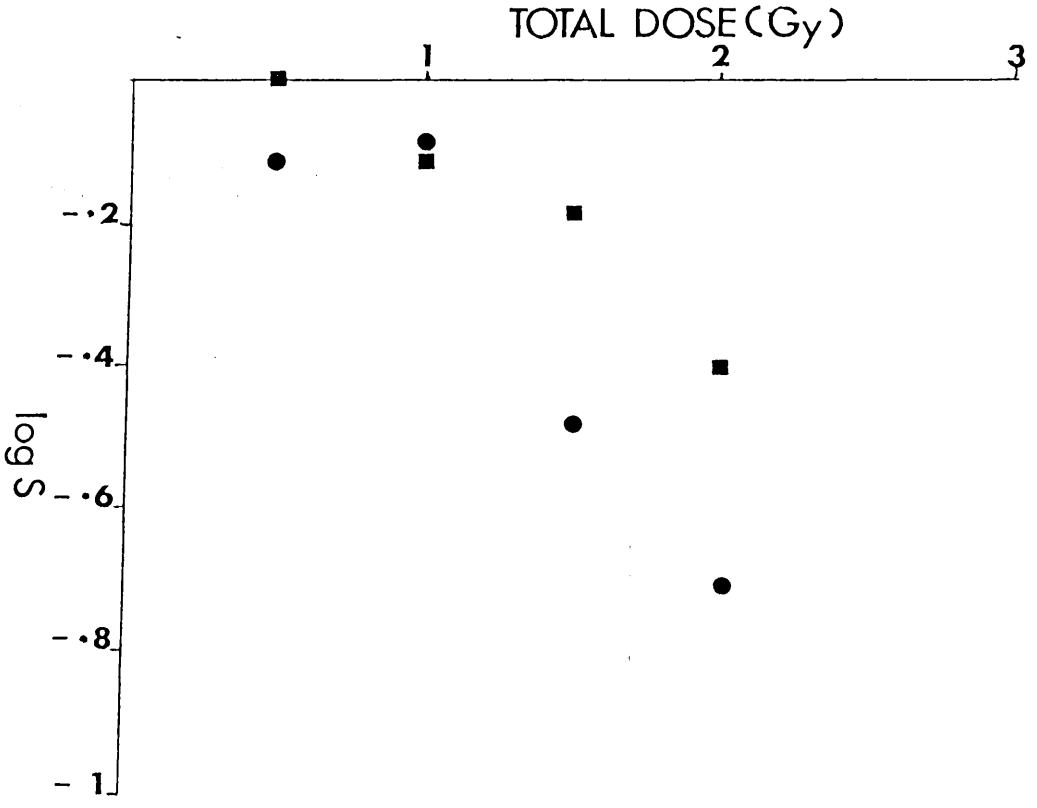
IMR-32 SPLIT DOSE FIG 9



IMR-32 SPLIT DOSE - GROWTH DELAY FIG 10



IMR-32 SPLIT DOSE - SURVIVAL CURVES FIG 11



1MR-32 Split Dose Irradiation

Spheroid Growth Curves

The MTS in test plates were irradiated with split doses of 25, 50, 75 and 100 cGy with a 6 hour interval between the two irradiations.

Fig.9 demonstrates the growth curves obtained from the control and irradiated MTS. The control growth curve displays a characteristic growth pattern until a volume of 800 μM - 1,000 μM per spheroid is reached. The growth rate then slows, with increasing size. The lateral displacement of irradiated from control curves is seen to increase progressively with dose.

Growth Delay

The growth curves obtained from two experiments are graphed on fig.10 The two symbols represent two separate experiments.

Survival Curves

The survival curves are graphed on fig.11. The two survival curves appear to indicate the presence of a shoulder which would imply that 1MR-32 spheroids has an ability to accumulate and repair sublethal damage.

NBI-G Hyperfractionation.

Spheroid Growth Curves

Median regrowth curves following treatment are only available for all treatment groups in the case of regimes. late-effect equivalent at effect level A ($\equiv$1 x 2.5 Gy) At effect level B ($\equiv$1 x 4 Gy) the high values of 'proportion cured' mode definition of median regrowth curves impossible in most cases. Results for effect level A are presented in fig.12 This shows a progressive lateral displacement of regrowth curves with increasing total dose, the doses delivered by the fractionation regimes indicated (See Table 1) In most cases, the regrowth curves returned to become parallel to the control curve

(6).

Growth Delay

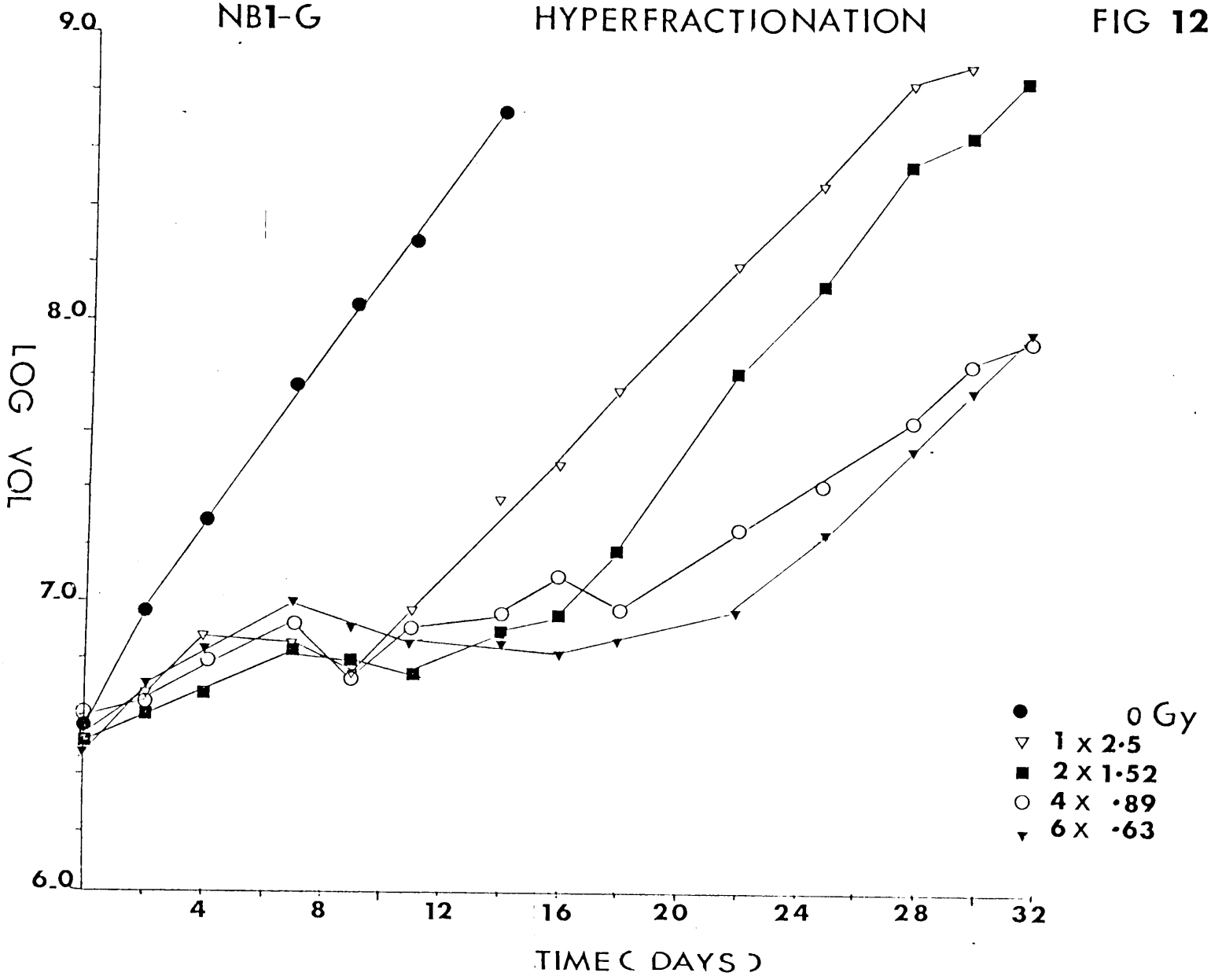
Fig.13 shows (for regimes at effect level A) the increase in regrowth delay as a function of total dose delivered by the various regimes. (See Table 1) Regrowth delay is seen to have a distinct dose-response relationship, with upward curvature.

Fig.14 displays the regrowth delay curve for irradiated MTS as a function of fraction size. (Data available for level A regimes only). This delay obtained decreases with increasing fraction size.

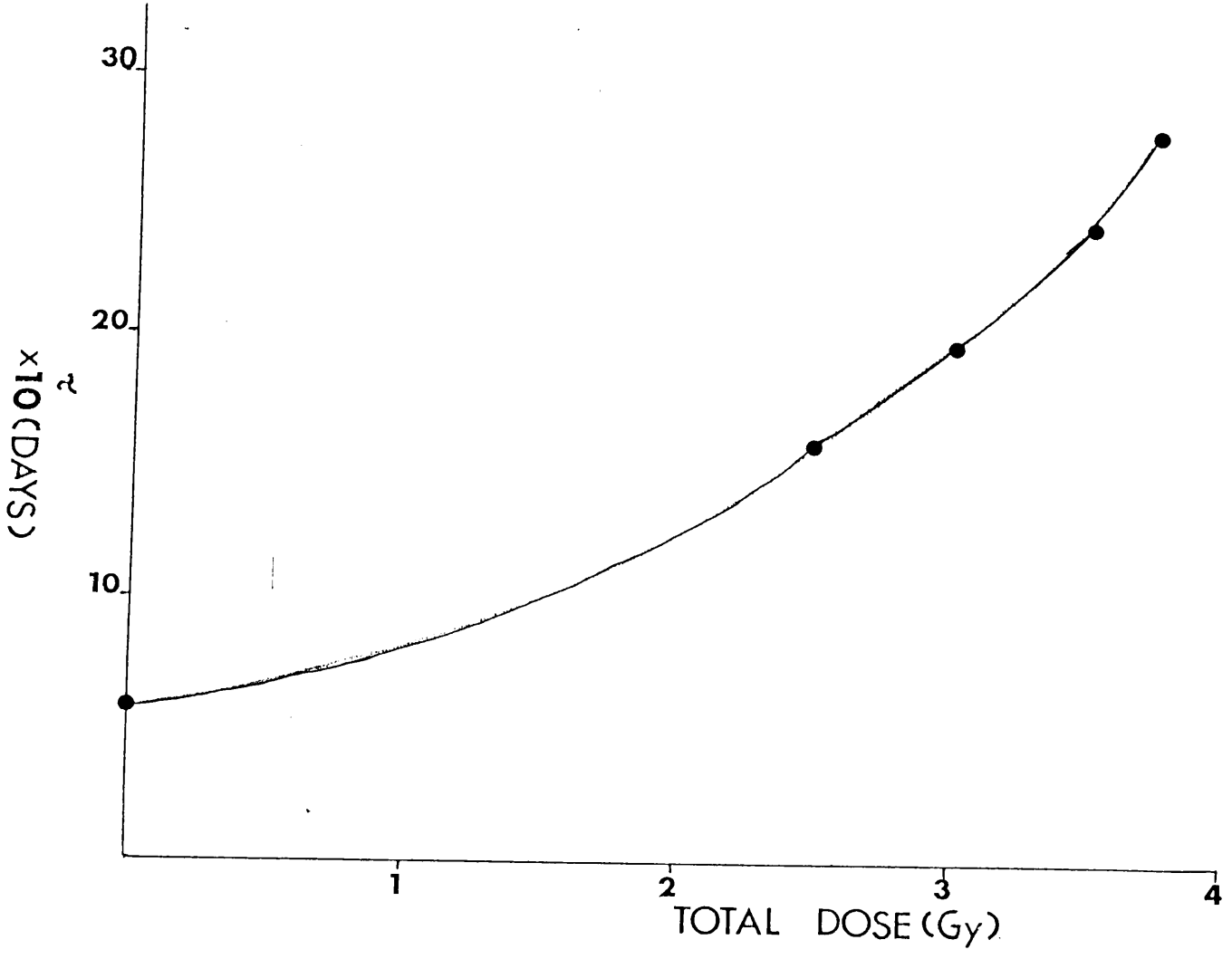
Survival Curves

The regrowth curves (at effect level A) generally showed a return to parallelism with control growth curves and therefore satisfied the condition for

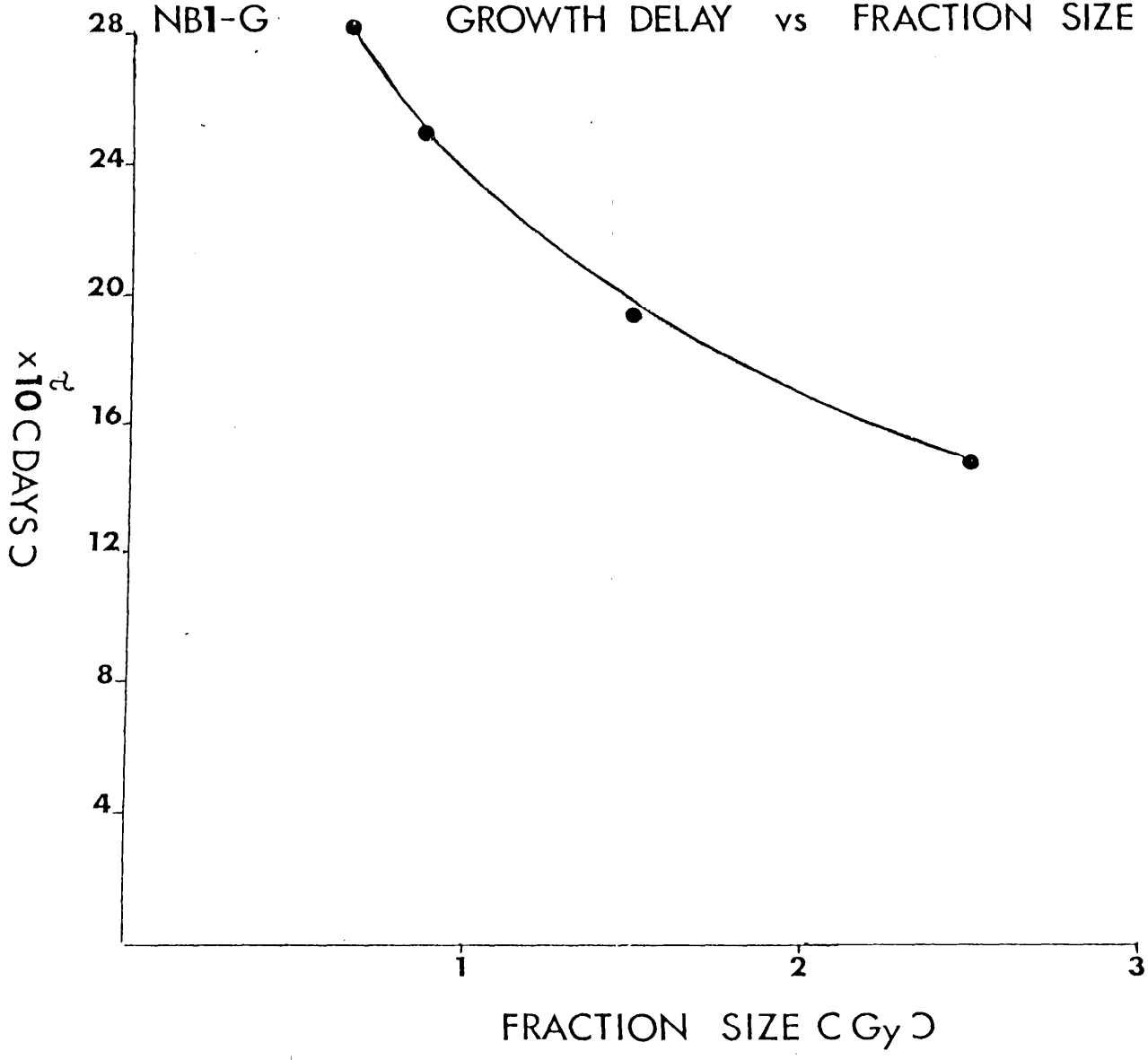
NB1-G HYPERFRACTIONATION FIG 12



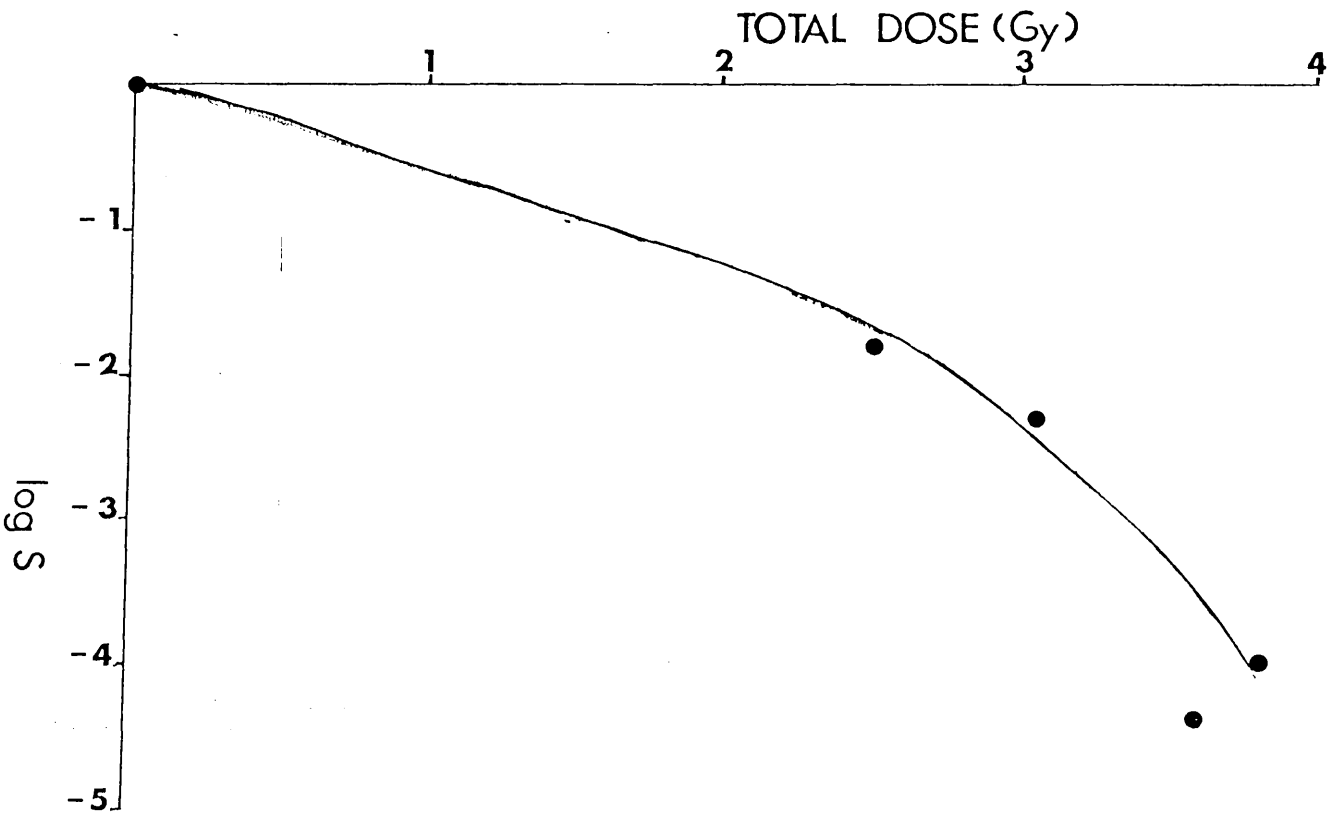
NB1-G HYPERFRACTIONATION FIG 13



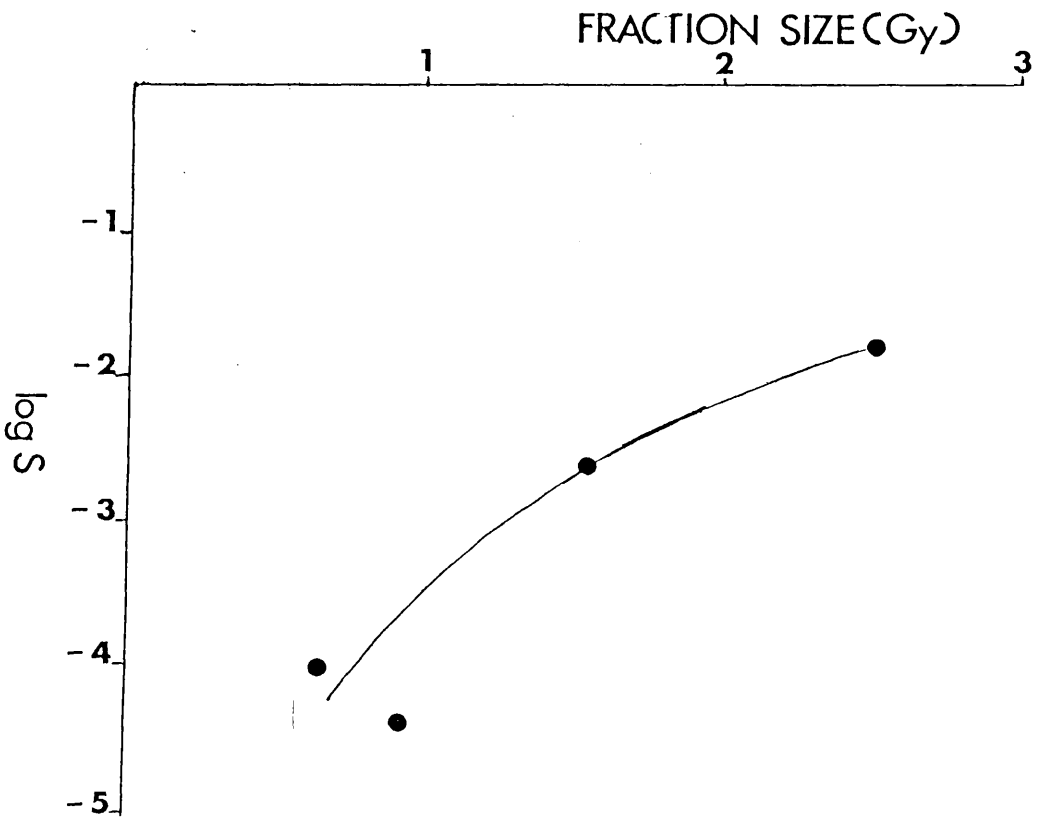
NB1-G GROWTH DELAY vs FRACTION SIZE FIG 14



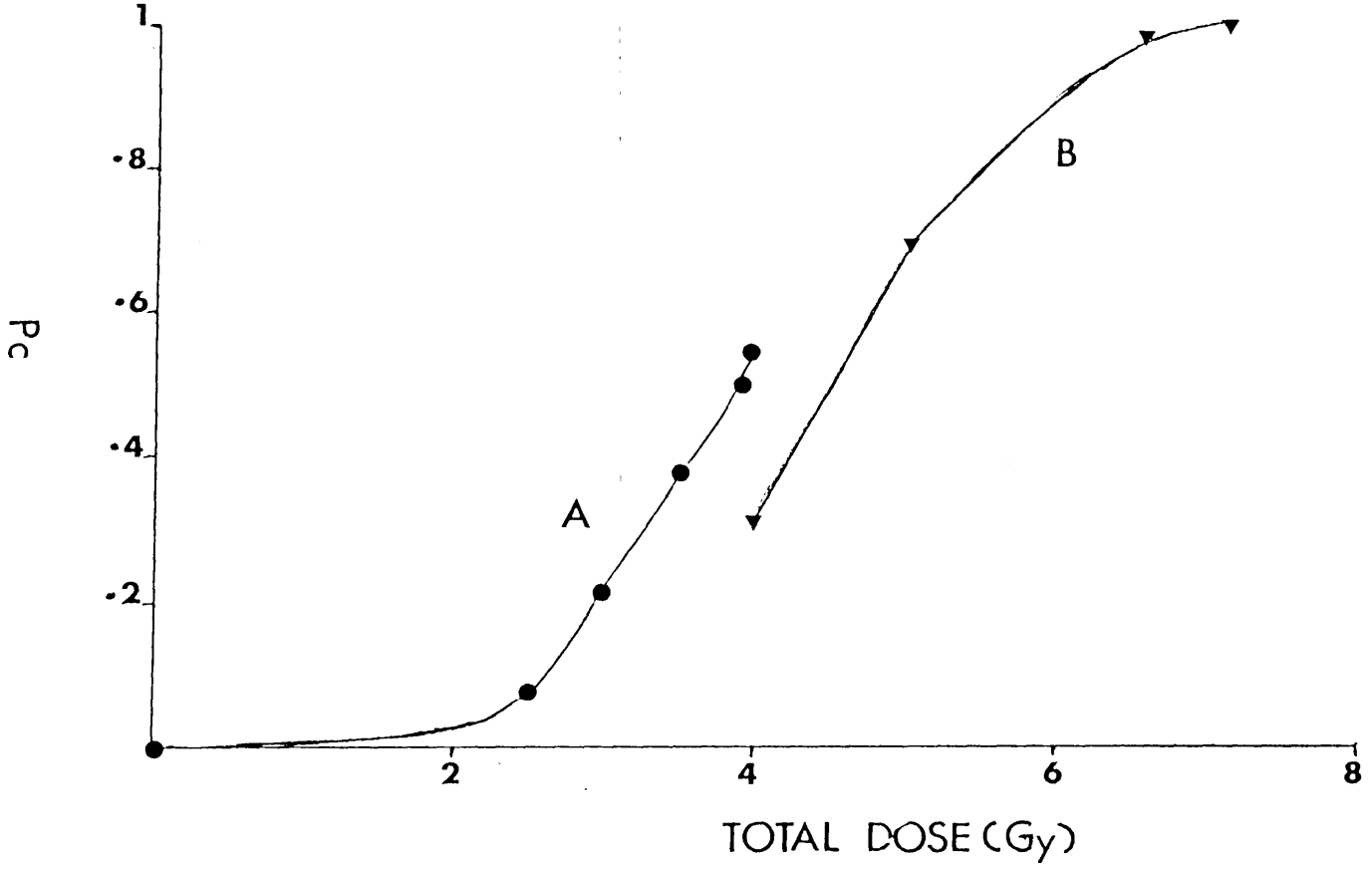
NB1-G HYPERFRACTIONATION - SURVIVAL CURVE FIG 15



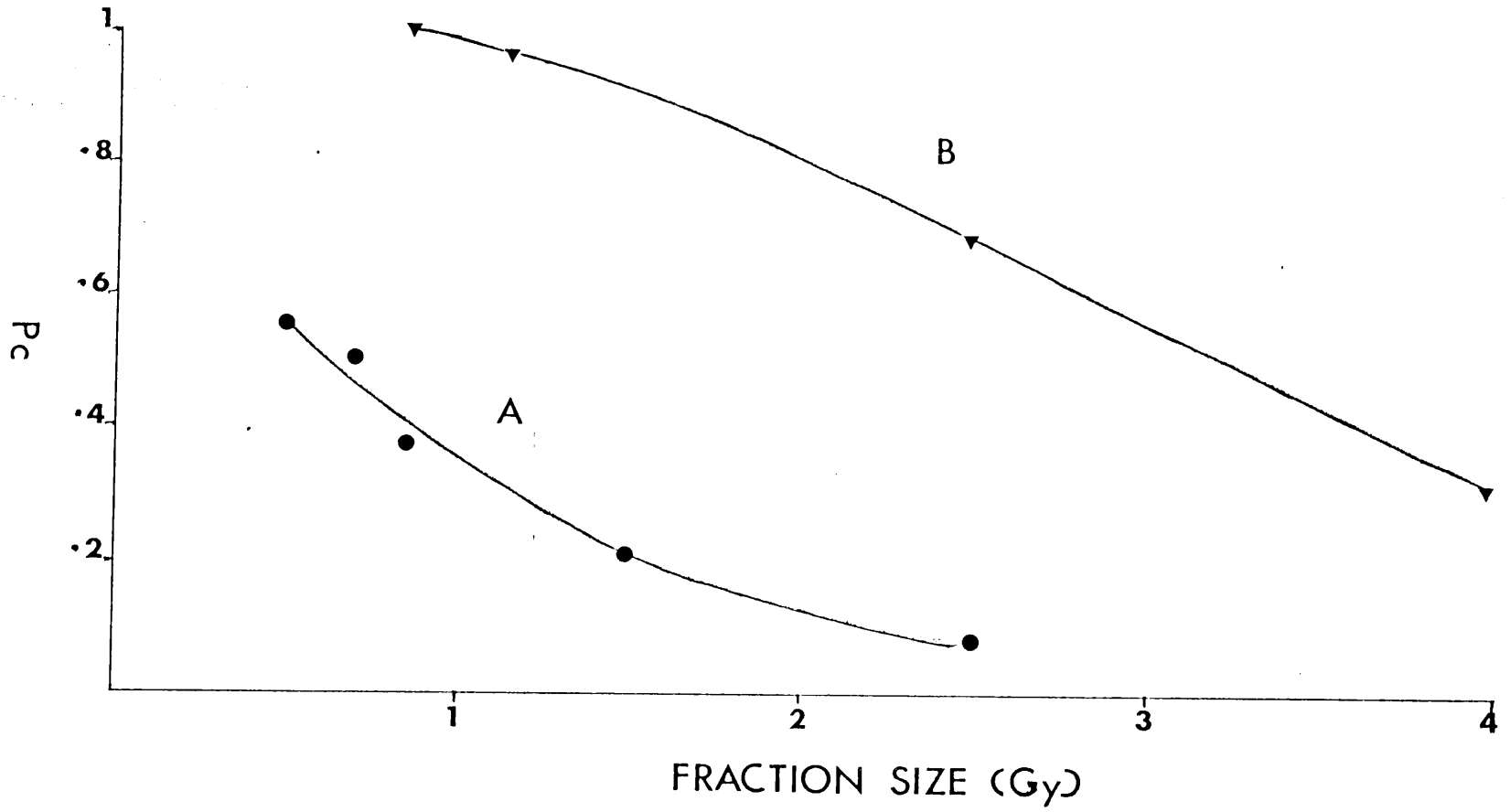
NB1-G SURVIVAL CURVE vs FRACTION SIZE FIG 16



NB1-G HYPERFRACTIONATION FIG 17



NB1-G HYPERFRACTIONATION FIG 18



for estimation of cell survival in situ by back-extrapolation of regrowth curves. Fig.15 shows estimated cell survival as a function of total dose delivered by various regimes. (See Table 1). As can be seen, the responses of NB1-G MTS were dependant upon the total dose given.

Fig.16, shows the estimated cell survival as a function of fraction size and it can be seen that as the fraction size increased, the log cell kill decreased.

Proportion Cured.

In this case, results are available for both effect levels A and B. In fig.17, 'proportion cured' has been plotted as a function of total dose delivered on the various regimes. (See Table 1)

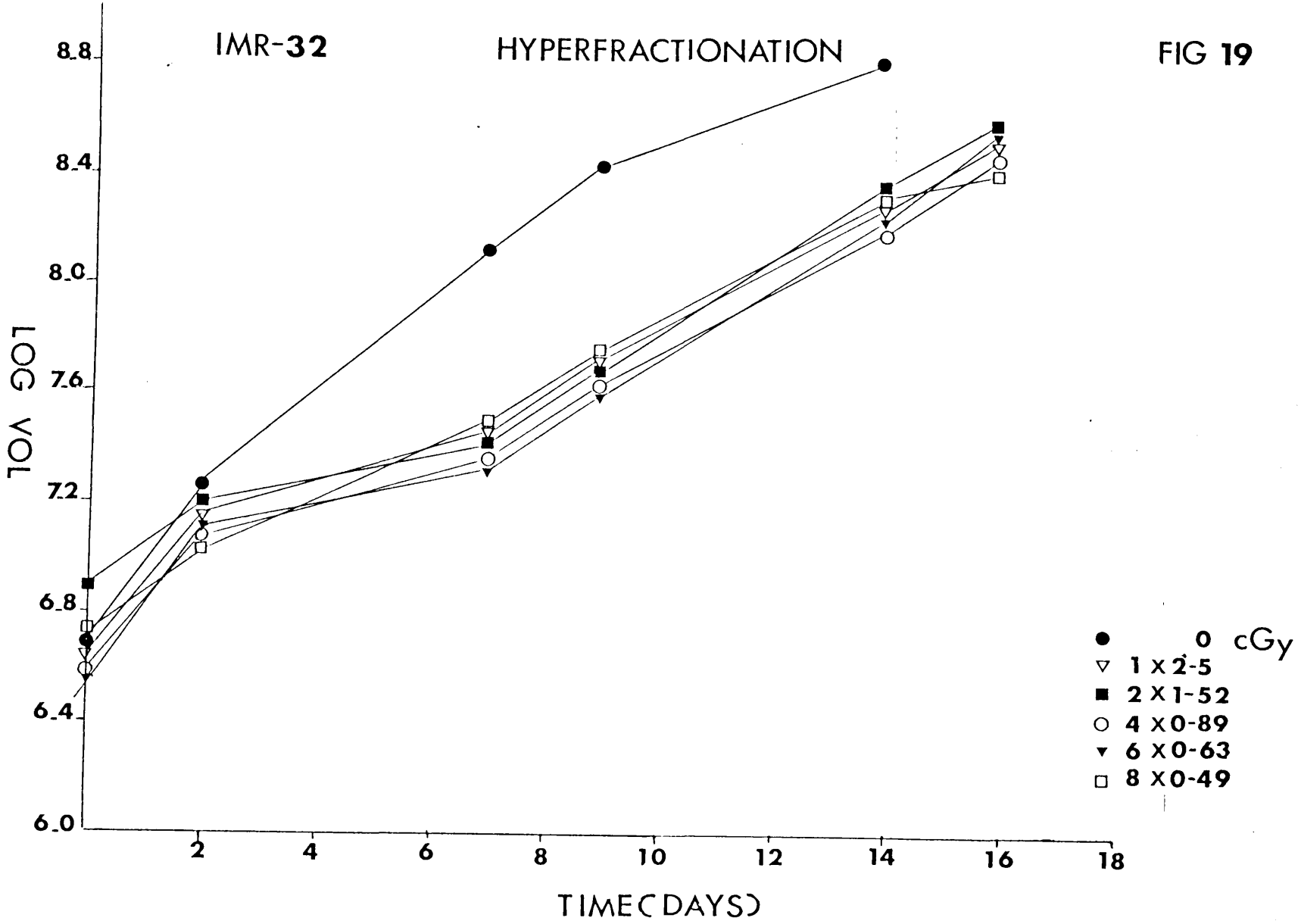
Fig.18 displays 'proportion cured' for irradiated MTS as a function of fraction size, for regimes administered at effect levels A and B.

1MR-32 Hyperfractionation

Spheroid Growth Curves

Fig.19 shows growth curves for control and irradiated MTS. The control growth curves, exhibit a characteristic exponential growth pattern, until a volume of 800 μM - 1,000 μM per spheroid is reached. Thereafter growth slows with increasing size. The growth curves for the irradiated MTS shows an increasingly disturbed pattern as the dose is increased. At higher doses, a static or regressive phase is observed, before growth is resumed, usually becoming parallel to the control curve.

IMR-32 HYPERFRACTIONATION FIG 19



IMR-32 HYPERFRACTIONATION FIG 20

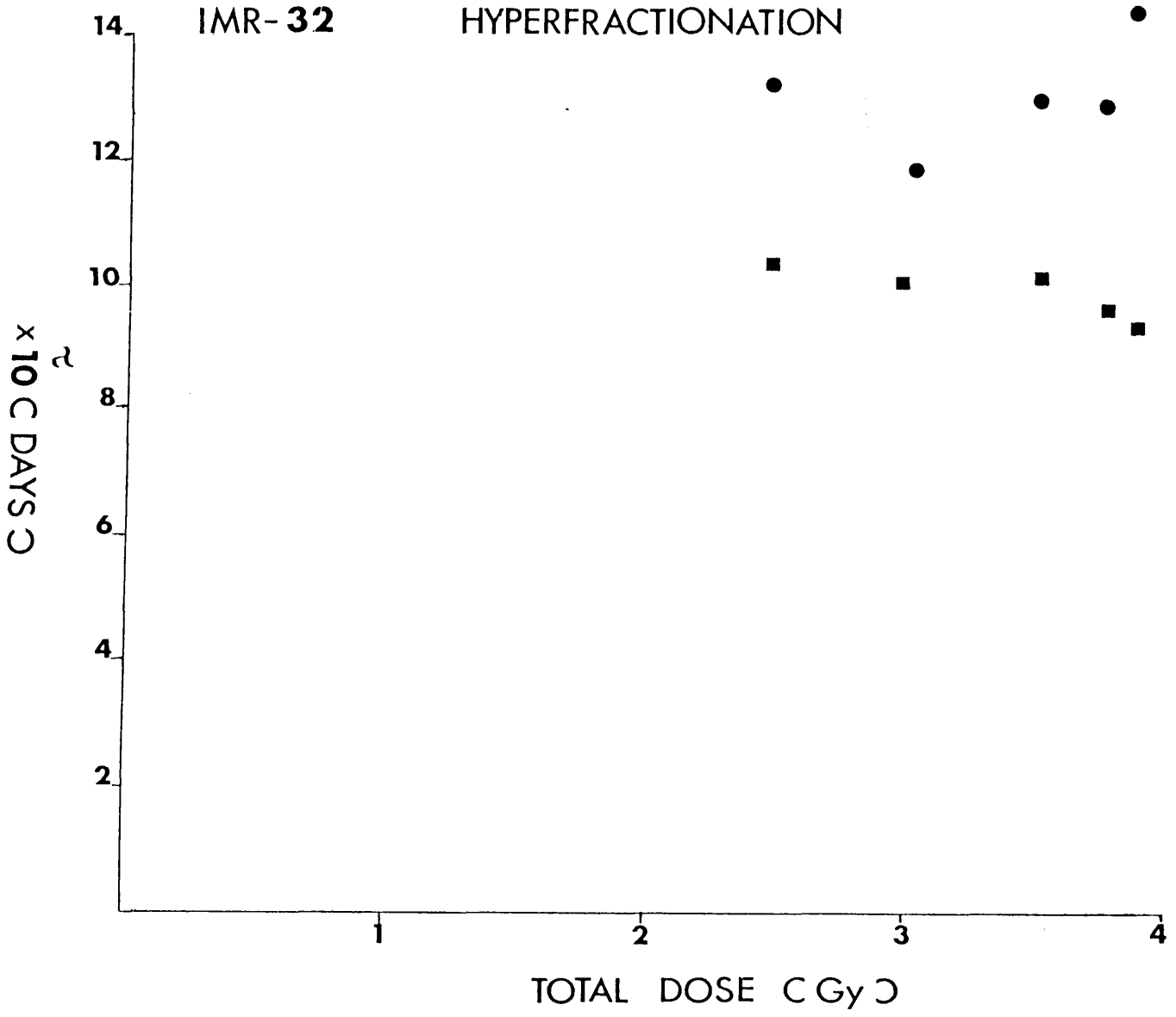
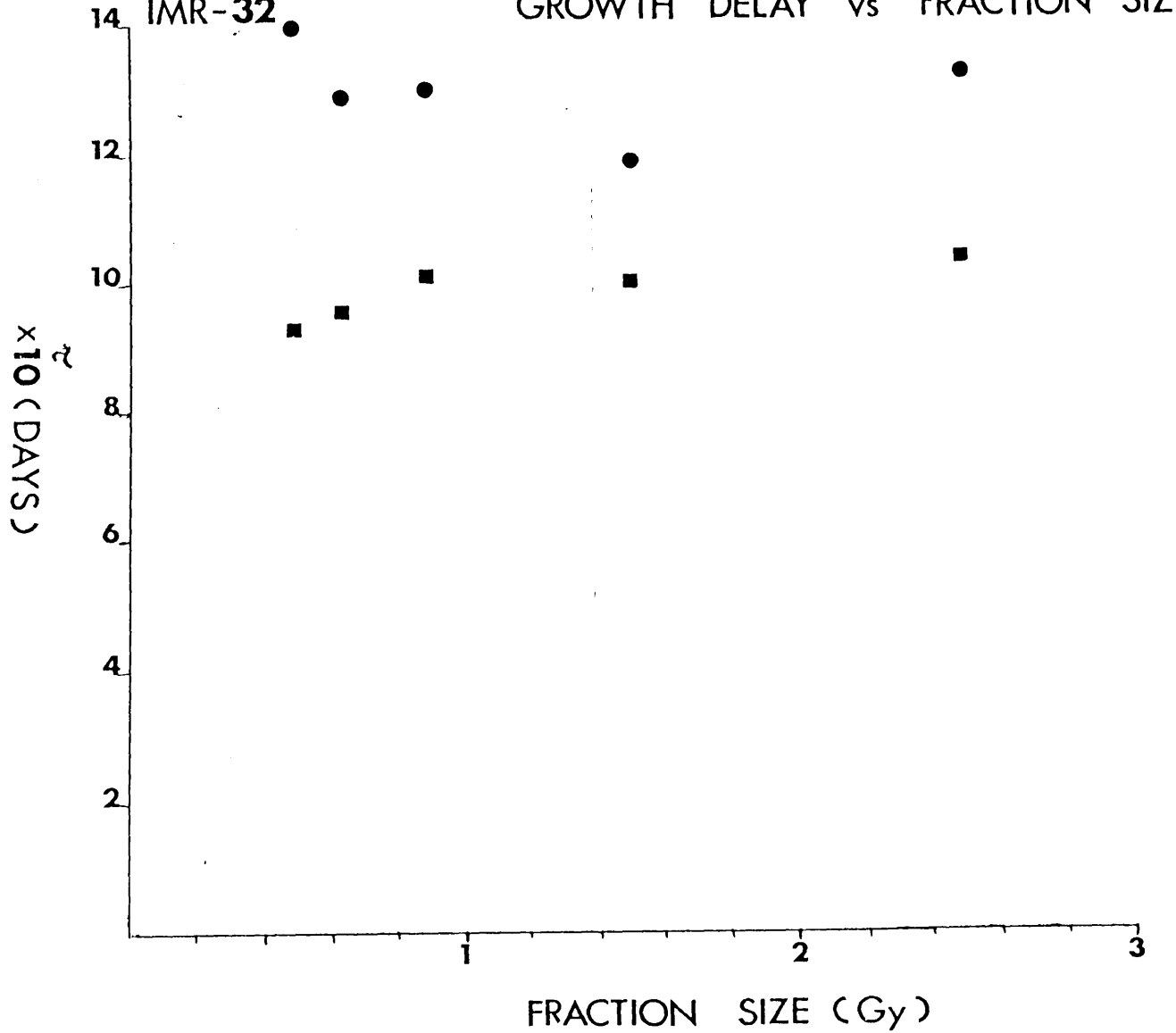
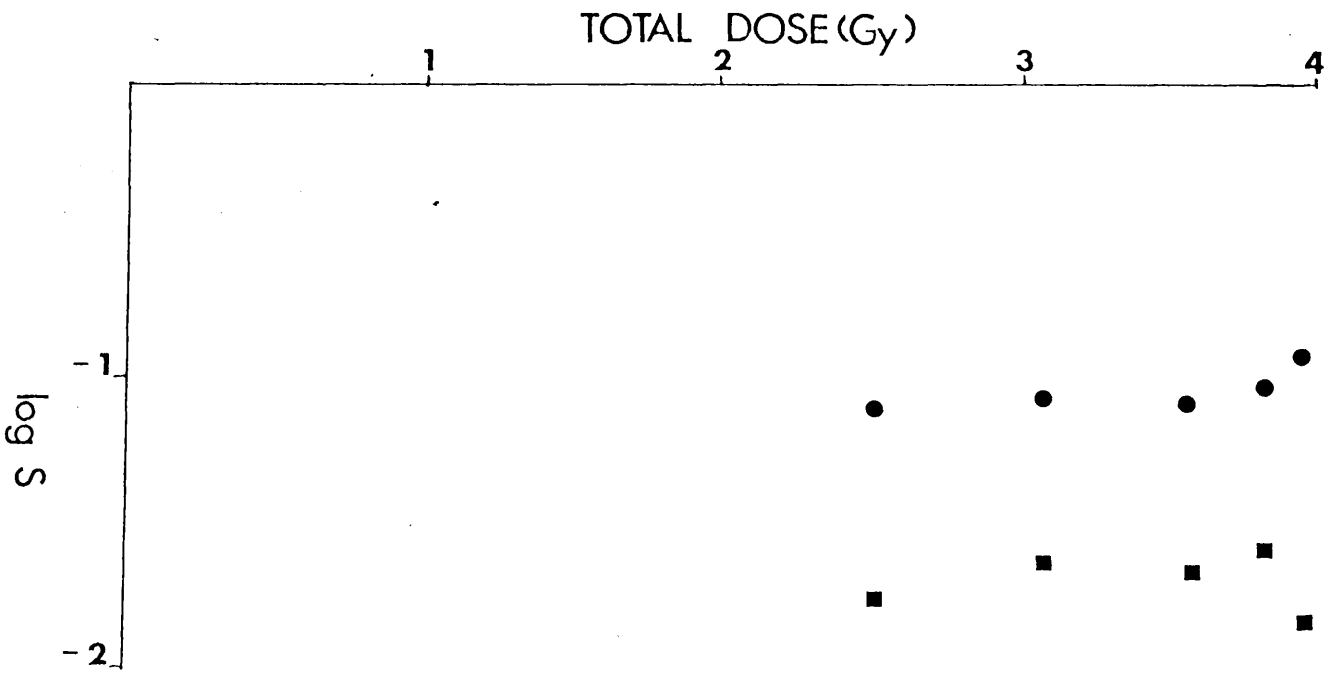


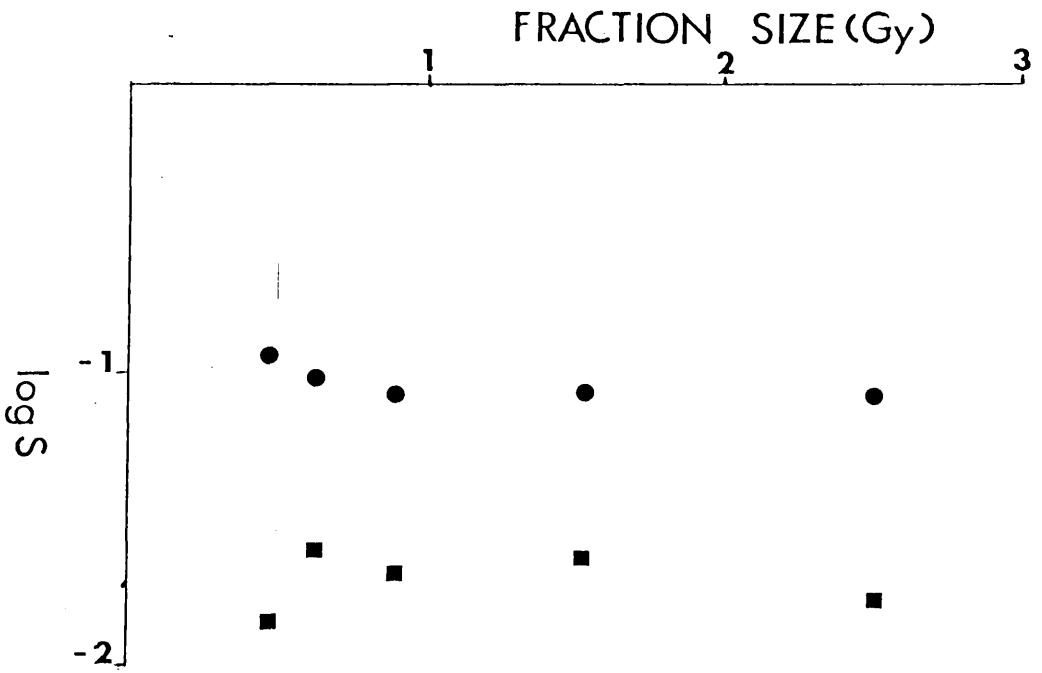
FIG 21


IMR-32
GROWTH DELAY vs FRACTION SIZE
14
12
10
8
6
4
2
τ x10 (DAYS)
1
2
3
FRACTION SIZE (Gy)

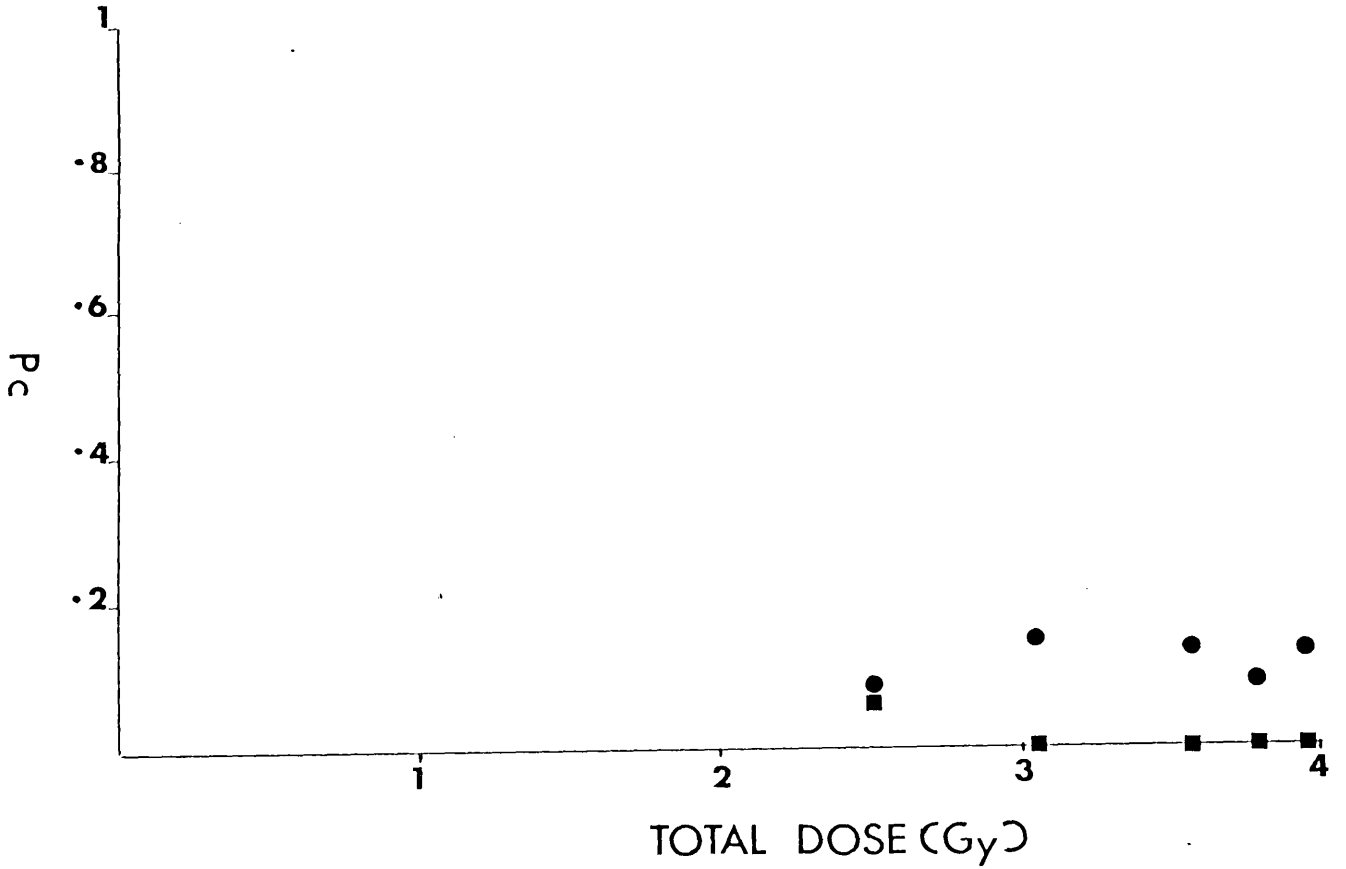
IMR-32 HYPERFRACTIONATION - SURVIVAL CURVES FIG 22



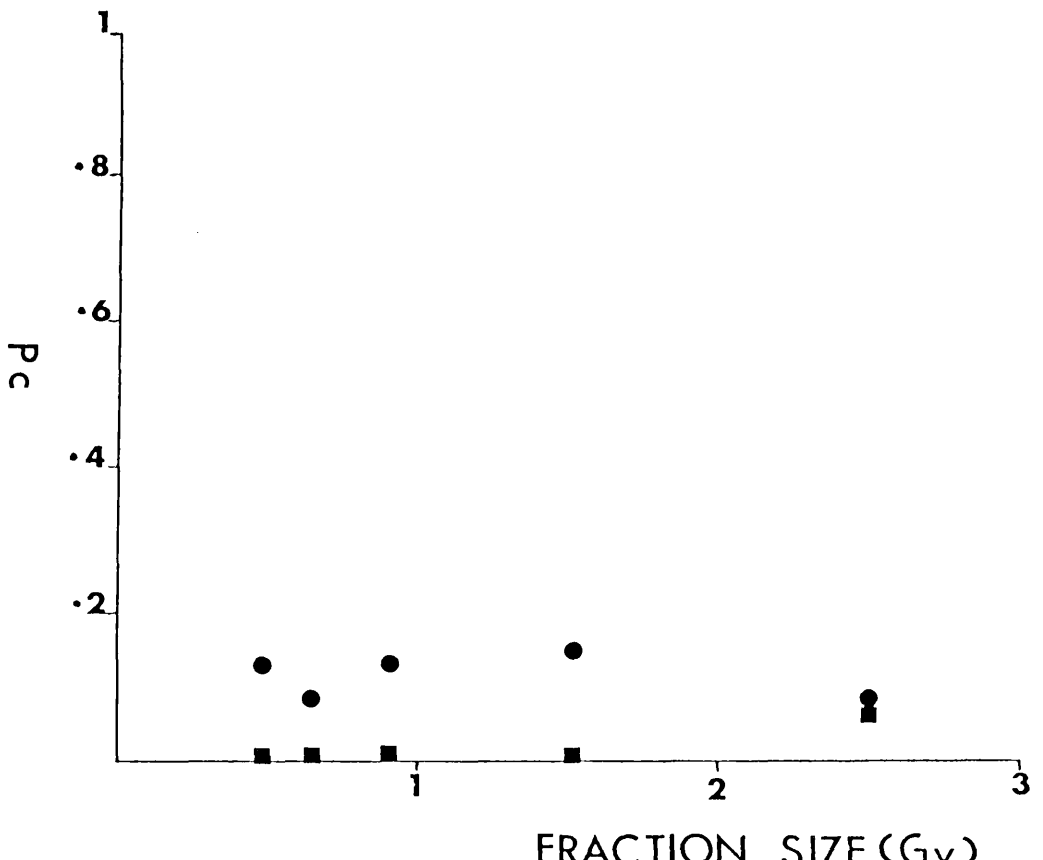
IMR-32 HYPERFRACTIONATION FIG 23



IMR-32 HYPERFRACTIONATION FIG 24



IMR - **32** HYPERFRACTIONATION . FIG **25**



Growth Delay

The growth delay curves for two separate experiments are graphed in fig.20 as a function of the total dose given on the various regimes. Fig.21 shows the two growth delay curves graphed as a function of fraction size. Since the treatment regimes were calculated to be isoeffective for late-responding normal tissue, the lack of any obvious dose-dependance implies that the regimes appear to be isoeffective for 1MR-32 spheroids also.

Survival Curves

The surviving fraction for each experimental group, as a function of total dose, as calculated from back - extrapolation of regrowth curves is graphed on fig.22. The surviving fraction as a function of fraction size is graphed on fig.23 and they similarly show little dependance on total dose and fraction size respectively.

Proportion Cured.

Fig.24, shows proportion cured (Pc) graphed as a function of total dose and fig.25 shows proportion cured graphed as a function of fraction size. It would appear from both graphs that the 1MR-32 spheroids were about equally affected by the alternative schedules. This implies that 1MR-32 spheroids responded similarly to each of the different regimens chosen to be isoeffective for late responding normal tissues.

DISCUSSION AND CONCLUSIONS

In principle, clonogenic survival curves may be deduced from tumour regrowth data by extrapolation of regrowth curves to zero time. Previously, these deductions have been attempted to obtain 'in situ' survival curves for experimental tumours irradiated in vivo, but, the analysis in that situation is complicated by the existance of the tumour bed effect, possible immunological responses, tumour infiltration by host cells, the limited proliferation potential of 'doomed cells' and the difficulty of obtaining accurate measurements of the regrowing tumour over an adequate range of sizes. (1).

The problem in spheroids is simpler, in that, the tumour bed effect, and host cell and immunological effects, are necessarily absent in vitro. It is feasible to make observations over a wide range of sizes and - at least in the present spheroid lines - doomed cell proliferation evidently does not substantially influence growth.

When looking at the survival curve for single dose irradiation of NB1-G spheroids, it can be seen that it is nearly exponential in form, with little evidence for quadratic curvature, and with only a small shoulder apparent. There is also little indication for a biphasic break in the curves, which would occur if a significant proportion of the clonogenic cells of the spheroid were hypoxic.

It can be interpreted that the derived survival curve

is representing the radiosensitivity of a well-oxygenated neuroblastoma line, which has little capacity for accumulation of sublethal damage. Due to the fact that the cells were irradiated as spheroids the response includes any contribution made by 'contact resistance' and may be representative of the radio-sensitivities of similarly sized micrometastases *in vivo*.

The results indicate that NB1-G cells grown as spheroids are quite radiosensitive, being less resistant than the majority of mammalian cell lines grown in monolayer culture. (2,3).

The NB1-G spheroids have a D_q value of 17 cGy which is about average for human neuroblastoma cell lines whose radiosensitivity in cultre has been reported. (4).

These results are broadly consistent with other reports which indicate that neuroblastoma cells *in vitro*, are relatively radiosensitive and may have little or no capacity for cellular repair. (4,5).

Several studies, however, have appeared on the single dose radiosensitivity of human neuroblastoma cells in monolayer (4,5,6) or as MTS (5,7,8).

They reveal a significant heterogeneity in the *in vitro* radiosensitivity of human neuroblastoma cells with D_o values ranging from 66 - 155 cGy.

Single dose irradiation studies on 1MR-32 spheroids imply a significant shoulder on the underlying cell survival curve. The D_q value obtained is close to 1 Gray, which is larger than is seen for the majority of human neuroblastoma cell lines whose

radiosensitivity in culture has been reported. (9). The results reported here are consistent with the interpretation that 1MR-32 cells grown as spheroids possess a significant capacity for repair of sub-lethal damage.

Division of a radiation dose in the range 150 - 350 cGy into two equal fractions spaced 6 hours apart seemed to render that NB1-G spheroids more sensitive to radiation than those exposed to single doses. This result could be explained in terms of cellular redistribution throughout the mitotic cycle but is within experimental uncertanties in the data. There has been no evidence found for any substantial interfraction repair capacity of these MTS. (10)

It has been proposed that a systematic difference may exist in the repair capacities of tumour cells and target cells in normal tissues and that a therapeutic advantage might result from the use of small doses per fraction (Hyperfractionation) by which normal tissues should be preferentially spared. (11,12)

No significant repair capacity was found for NB1-G spheroids subjected to one or two doses of radiation in the total dose rang 50 - 350 cGy. which could be taken as providing a radiobiological rationale for the use of hyperfractionated treatment schedules in the adjuvant radiotherapy of neuroblastoma for which the target micrometastases may have similar radiobiological properties to MTS <u>in vitro</u>.

The split dose irradiation response of the 1MR-32 spheroids appears to indicate the presence of a shoulder, which implies that 1MR-32 spheroids had an

ability to accumulate and repair sublethal damage. The fact that 1MR-32 cells grown as spheroids possess a significant capacity for repair of sub-lethal damage which leads to relatively inefficient cell killing when radiation is given as multiple small fractions. The role of other factors (e.g. redistribution, repopulation, reoxygenation) cannot be excluded, however.

The use of hyperfractionated radiotherapy on NB1-G spheroids was designed to test the hypothesis of an exploitable difference in the radiobiological properties of neuroblastoma MTS relative to late responding normal tissues. The treatment regimes, at each effect level were calculated to be equivalent in their effects on late-responding normal tissues. If NB1-G MTS hadthe same properties as these normal tissues, the regimes should also have been equivalent on their effects on the MTS.

By each end-point, radiation damage to NB1-G MTS increased with total dose, regardless of how it was delivered. Since higher doses could be delivered by using smaller fractions (i.e. utilizing the high tolerance to small fractions of late-responding normal tissues) regimes using smaller fractions were more effective. This can be seen in fig.13 for effect level A and fig.17 for effect levels A and B, where regrowth delay and 'proportion cured' are plotted as functions of fraction size. The growth delay data was not available for effect level B because at the higher effect level, there were too many cures for definition of growth delay.

In general, these results support the superior effectiveness of hyperfractionation used to treat tumours whose cells have low capacity for repair of sublethal damage.

As can be seen from fig.16, there is a lateral displacement of the 'proportion cured' versus dose curves for effect levels A and B. Without this, the upper curve would have been a smooth continuation of the lower. The effect is small and is readily explained by the (accidental) difference in spheroid sizes in the two separate experiments.

However, each experiment considered independently yields the same conclusion, viz increasing proportion cured with increasing total dose.

When taken together, the NB1-G results provide encouragement that hyperfractionation should be a useful strategy in the radiation therapy of at least some human tumours.

The results obtained from the use of hyperfractionated radiation on 1MR-32 spheroids differed to those obtained from the NB1-G spheroids. When the 1MR-32 cells were grown as spheroids, they seemed to possess a significant capacity for the accumulation and repair of sub-lethal damage. This leads to relatively inefficient cell killing when radiation is given as multiple small fractions although the role of other factors such as redistribution, repopulation and regeneration cannot be excluded.

The experiments on 1MR-32 neuroblastoma cells grown as MTS, suggest that the MTS respond to changes in fraction size in the same way as would be anticipated

for late-responding normal tissues.

By each end-point, radiation damage to 1MR-32 MTS did not appear to increase or decrease with increasing dose.

Although higher doses can be delivered by using smaller fractions, regimes using smaller fractions did not appear to be any more effective than regimes using larger fractions. This can be seen in fig.20

When the 'proportion cured' data was plotted as a function of fraction size, it was apparent that fraction size did not have any effect upon the proportion of 1MR-32 spheroids which were cured.

Each experiment, when looked at individually provides the same conclusion - the radiation regimes calculated to be isoeffective for late-responding normal tissues were also isoeffective in the treatment of 1MR-32 spheroids.

The clinical implication of these results is that, should some micrometastases behave similarly to 1MR-32 spheroids, the use of multiple small fractions (hyperfractionation) would not necessarily result in a therapeutic advantage.

It should be noted that the results do not imply any <u>disadvantage</u> of hyperfractionation, only that all regimens with the same effect on late-responding normal tissues would have similar effects on the tumour. 1MR-32 may, however, be untypical of neuroblastoma cell lines in this respect.

OVERALL CONCLUSION

It seems plausible, on the basis of these results, that hyperfractionation would not be a universally advantageous strategy, but one whose efficacy is likely to depend on being able to select appropriate tumours for this form of treatment.

Further work is necessary to establish the generality of the expected advantage of hyperfractionation, even in laboratory models.

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APPENDIX

In calculating total doses to make alternative fractionation regimes isoeffective for damage to late-responding normal tissues, the linear-quadratic model was utilized. This model assumes that gross effects of radiation are attributable to the sterilization of clonogenic 'target cells' in the tissue, whose sterilization is responsible for the observed effects. The target cells have a dose-response curve for clonogenic survival which is linear-quadratic in dose. The level of effect, denoted E, will also then be quadratic in dose, and proportional to number of treatments.

Viz $$E = N(\alpha d + \beta d^2) \qquad (1)$$

Where d is dose per fraction, N the number of treatments given and α and β are parameters characteristic of the tissue concerned. Two treatments utilizing different fractionation regimes (N_1, d_1) and (N_2, d_2) will be isoeffective for damage to that tissue if their effects (E_1 and E_2) are the same.

Viz for equivalence,

$$N_1(\alpha d_1 + \beta d_1^2) = N_2(\alpha d_2 + \beta d_2^2) \qquad (2)$$

dividing through by β gives

$$N_1\left(\frac{\alpha}{\beta} d_1 + d_2^2\right) = N_2\left(\frac{\alpha}{\beta} d_2 + d_2^2\right) \qquad (3)$$

The fraction size for regime 2 such that N_2 fractions will have the same effect as regime 1 is given by the solution of the quadratic equation in d_2 i.e.

$$d_2 = \frac{-\frac{\alpha}{\beta} + \sqrt{\left(\frac{\alpha}{\beta}\right)^2 + \frac{4N_1}{N_2}\left(\frac{\alpha}{\beta} d_1 + d_2^2\right)}}{2} \qquad (4)$$

Hence doses on different regimes can be calculated to give equivalent effects on a tissue if its $\frac{\alpha}{\beta}$ ratio is known. Values of the estimated $\frac{\alpha}{\beta}$ ratio for various normal tissues have recently been collated by Withers et al (7) and by Fowler (8) (See Materials references). Most late-responding tissues are found to have an $\frac{\alpha}{\beta}$ ratio dose to 3 GY. Equation (4) with $\frac{\alpha}{\beta}$ = 3 GY, was used to calculate doses isoeffective for damage to late-responding normal tissues. These regimes are specified in Tables 1 and 2.