TREATMENT OF HUMAN LUNG CANCER WITH INTERFERON AND CYTOXIC AGENTS

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Declaration

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work of which it is a record is my own unless otherwise stated. All sources of information have been acknowledged by means of reference.

R J Fergusson

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Publications arising from the work described in this thesis

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Abbreviations

ACTH	Adrenocorticotrophic hormone
ADR	Adriamycin (doxorubicin)
AFP	Alpha feto protein
ALS	anti-lymphocyte serum
ArA-c	cytosine arabinoside
ATS	anti-thymocyte serum
BRM	Biological Response Modifier
CBDCA	carboplatin
CDDP	cisplatin
CY	Cyclophosphamide
DFMO	α-difluoromethylornithine
HCG	Human chorionic gonadotrophin
IFN	Interferon
i.p.	intraperitoneal
JM40	ethylenediamine-melonato platinum
MSH	melanocyte stimulating hormone
MTD	maximum tolerated dose
MTX	Methotrexate
NSCLC	Non small cell lung cancer
s.c.	subcutaneous
SCLC	Small cell lung cancer
SGD	Specific growth delay
TD	doubling time
Tt	trebling time
TNM	Tumour, nodes, metastases (staging system)

ABSTRACT

The prognosis for patients with lung cancer remains extremely poor. Newer, more effective treatment regimens are required. In this thesis, the effectiveness of combining human recombinant interferon alpha with various established cytotoxic agents was assessed in laboratory models of human lung cancer and a clinical study. The majority of the experimental work was performed on a series of human bronchial carcinoma xenografts established in immune deprived CBA mice. Interferon alone had no cytotoxic effect but appeared to potentiate the activity of various anti-cancer agents in non-small cell tumours. No such effect was seen in two small cell xenografts. Further studies suggested that the dosing schedules of each agent in the combination was important in producing positive effects. The exact mechanisms by which interferon may interact with cytotoxic drugs remains unexplained. It was shown that the results obtained in the xenograft model were not mediated through increased toxicity to the host or by modulation of cell cycle distribution within the tumour cells. Assessment of interferon/drug combinations was also performed using in vitro models of lung cancer. Significant synergistic interactions were not demonstrated. A pilot clinical study investigating the potential of combined treatment with interferon and Cisplatinum was performed in a group of patients with non-small cell lung cancer. The toxicity of the treatment was predictable and an encouraging response rate was seen.

SECTION I - BACKGROUND INFORMATION

Chapter 1

CURRENT MANAGEMENT OF LUNG CANCER

1.1 Introduction

In the Western World lung cancer remains the commonest cause of death from malignant disease in men (Mould 1983). It is only second to breast cancer in this respect in women and the incidence of the disease in the female population has increased at an alarming rate in England and Wales in the last two decades (OPCS 1982). The prognosis of patients with lung cancer remains extremely poor. Until recently it was felt that surgical resection offered the only curative treatment. However it is now recognised that the vast majority of patients with lung cancer have generalised disease at presentation.

Radiotherapy is now widely used for palliative treatment in symptomatic patients considered unsuitable for curative surgery and recently intensive treatment with combination chemotherapy has improved the survival of patients with small cell tumours (Smyth 1986). Despite these encouraging results, long lasting, disease free survival in a significant number of patients has not been achieved and the cytotoxic agents used are capable of producing considerable toxicity. Clearly new regimes and particularly more effective agents are needed to exert a substantial influence on the natural history of the disease.

1.2 Surgical Management of Bronchial Carcinoma

Although surgical resection currently offers the best hope of cure in patients with non-small cell lung cancer (NSCLC) only a small proportion (15-20%) of patients presenting with this disease are suitable candidates for operation. In the majority of patients clinical assessment and routine investigations will reveal evidence of spread of tumour from the primary site and in some, significant impairment of cardio-respiratory function precludes surgical exploration.

The results of many large series of patients undergoing successful resection suggest that approximately 25-35% will survive five years, 15% will live for ten years and 5% will be alive at twenty years (Belcher 1965, Paulson 1976, Mountain 1977, Wilkins 1978, Abbey Smith 1981, Bates 1981).

Improved post operative survival appears to be closely related to the stage of disease at operation, with involvement of mediastinal lymph nodes carrying a particularly poor prognosis (Mountain 1974). In a series of 3919 patients from the Mayo Clinic (Williams 1981), 16% were considered to have post surgical stage I disease. The five year survival for these patients was 69%. Higgins (1979) showed that the improvement in five year survival from 28% to 45% in two large Veterans Administration surgical trials was accounted for entirely by a higher proportion of more favourable TNM staged patients in the second study.

The role of surgery in the management of small cell lung cancer (SCLC) remains controversial. Most surgeons now agree that in this disease the incidence of dissemination at presentation is so high that in virtually all cases the rational therapeutic approach is the use of systemic chemotherapy (Mountain 1974, Osterlind 1985). The overall survival figures for surgery as primary treatment of small cell lung cancer are very poor with most centres reporting few patients alive after two years (Mountain 1977, Fox 1973, Wilkins 1978).

The rationale for surgical treatment of SCLC appears especially dubious as even the best centres report an operative mortality rate of 4-12% depending on whether a pneumonectomy or lobectomy is undertaken. However when a diagnosis of small cell cancer is made only after resection and glands are not involved, then prognosis compares favourably with other histological types (Shields 1975, Wright 1981).

1.3 Radiotherapy in the Management of Bronchial Carcinoma

The effectiveness of radiotherapy in the management of lung cancer depends on tumour histology, size and dissemination. SCLC is highly radiosensitive although as stated above is usually a generalised disease at presentation making curative treatment with radiation impossible in virtually all cases.

In seemingly 'operable' patients with small cell tumours, radical radiotherapy has been shown to be more effective in producing long term survivors than surgery (Fox 1973). However most workers would now agree that for the majority of patients with this disease the treatment of choice is combination chemotherapy.

Non-small cell lung cancer is less radiosensitive than SCLC and the results of radical radiotherapy compare poorly with surgical resection in operable patients. In a randomised comparison of these treatments in 58 operable patients (Morrison 1963) a marked survival advantage was seen in the surgically treated group (23% against 7% four year survival) particularly when the histology was squamous carcinoma. The place for radical radiotherapy in non-small cell tumours may be confined to Stage I patients in whom there is a medical contraindication to operation or to patients with Stage II disease where complete resection has not been achieved (Gregor 1984).

The results of many studies of radical radiation treatment in inoperable patients suggest that 35-50% will survive one year but only 5-7% will be alive after five (Deeley 1967, Caldwell 1968, Coy 1980). However of all the studies published, only one (Roswitt 1968) was conducted in a randomised fashion comparing the effect of radiation with no therapy. Only a marginally significant effect of treatment on survival was demonstrated although both the design of the study and methods of analysis used have been criticised. The rationale for treating inoperable patients with radical radiotherapy therefore is not supported by convincing data and more carefully controlled studies using modern radiation techniques are required.

This whole subject is well covered in an excellent critical review by Kjaer (1982).

No advantage in terms of patient survival has been demonstrated in combining radiation therapy with either chemotherapy (Gregor 1984) or surgery (Kjaer 1982) although there may be a place for pre-operative therapy in patients with superior sulcus (Pancoast) tumours (Paulson 1975). Perhaps the main indication for radiotherapy in lung cancer nowadays lies in its ability to palliate symptoms effectively. Haemoptysis, thoracic and bone pain, stridor and the effects of superior vena caval obstruction in general respond well to radiation therapy (Slawson 1979).

The exact place of radiation treatment in lung cancer is still uncertain. Until more carefully designed randomised studies of radical treatment are forthcoming it may be reasonable to reserve the use of radiotherapy for patients medically unfit for curative surgery and for palliation in symptomatic cases.

1.4 Chemotherapy in Bronchial Carcinoma

Autopsy studies indicate that all histological types of lung cancer can metastasise widely. This is especially true of small cell tumours where in one series (Matthews 1976) only 4% of cases had disease confined to the thorax. The same author (1973) performed post mortems on 202 patients dying within one month of an apparently "curative" resection from causes unrelated to lung cancer.

Persistent disease was found in a third of patients with squamous tumours, 16% of large cell cases and 40% of adenocarcinomas. Almost 70% of patients with small cell tumours had residual disease with 92% of these having distant metastases.

These figures may explain why with the current diagnostic techniques, local therapy i.e. "curative" surgery or radical radiotherapy will not eradicate this disease in the majority of cases. They provide further evidence to support the rationale of using systemic therapy especially in small cell cancers. These tumours are sensitive to many anti-cancer agents and most workers now agree that combination chemotherapy is the appropriate first line treatment. Non-small cell tumours are less chemosensitive and the use of drugs in this heterogeneous group is controversial and will be considered separately. It is for this reason that the importance of establishing an accurate histological diagnosis even in patients with widespread disease cannot be overstated.

1.4a Non-Small Cell Lung Cancer

Approximately 60% of patients developing NSCLC have evidence of metastases at presentation (Souhami 1985) and in the majority of the remaining patients surgery or radical radiotherapy will fail to eradicate the disease. Theoretically therefore it would seem appropriate to treat the majority of NSCLC patients with systemic chemotherapy. Unfortunately the relative insensitivity of these tumours to the currently available cytotoxic agents makes this form of management unsatisfactory especially as the toxicity of these drugs may be considerable.

The use of differing response criteria in small uncontrolled series of patients with varying histological types and prognosis has made the interpretation of drug activity in this disease difficult. Also, all results need to be interpreted in the knowledge that a small proportion of patients will survive for more than two years without treatment. Despite these difficulties, which can only be overcome by undertaking randomised comparisons between treated and untreated patients (Souhami 1985), many drugs have been shown to have activity in this disease.

(i) Single Agent Activity

Early trials (Selawry 1973, 1974) reported encouraging response rates to the available agents. More recently the results appear less optimistic perhaps due to the inclusion of heavily pre-treated patients. Of the many drugs assessed in this disease (Table 1.1) four deserve mention.

Ifosfamide, an alkylating agent related to cyclophosphamide, gave response rates of between 24% and 32% in three separate studies (Morgan 1983, Costanzi 1982, Harrison 1982) with the median survival in responding patients in one (Costanzi 1982) being 10 months.

Cis-platinum has been given in various schedules with the two largest studies (DeJager 1980, Vogl 1982) reporting response rates of 25% and 32%.

Vindesine has been assessed in nine different studies with response rates varying from 6% (Vogelzang 1982) to 31% (Furnas 1982) despite almost identical dosage regimes.

	MEAN RESPONSE RATE (%)
IFOSFAMIDE	26
CISPLATIN	20
MITOMYCIN C	20
VINDESINE	17
ADRIAMYCIN	13
ETOPOSIDE	11
METHOTREXATE	10
CYCLOPHOSPHAMIDE	8
CCNU	7

(TRIALS >3, PATIENTS >100)

(AFTER BAKOWSKI 1983)

TABLE 1.1 - SINGLE AGENT ACTIVITY IN NSCLC

Mitomycin C highlights the problems of assessing agents in this heterogeneous group of tumours. Response rates observed have ranged from 9% in an early study (Whittington 1970) to an impressive 40% in a small group of patients with squamous histology (Koons 1978).

One other single agent study is worthy of note in that it compared response rates and survival after two different doses of a drug (methotrexate) against placebo (Selawry 1977). Objective response was dose related with rates of 21% for 'high' dose, 11% for low dose, and 6% for placebo. Median survival was prolonged in responders compared with non responders (34 weeks versus 11 for high dose, 52 weeks versus 13 for low dose). The median survival in the placebo group (11 weeks) was similar to that of non responders in both treatment groups. Overall activity of single agents in NSCLC is shown in table 1.1

(ii) Combination Chemotherapy

Despite little evidence of significant single agent activity in this disease many combinations of cytotoxic drugs have been tried. Many of the shortcomings of the single agent studies noted above also apply to drugs given in combination. The various response rates of drug regimes containing one to four or more agents have been well summarised by Souhami (1984, 1985). In general there appears to be a tendency for combination therapy to produce higher response rates in squamous and adenocarcinoma subtypes but this may not hold for large cell tumours (Table 1.2). Overall response rates for drugs in combination are not much higher than with the best single agents and

in some studies (e.g. Klastersky 1983) the addition of a third agent only adds to the overall toxicity.

Gralla (1981) studied the combination of vindesine and two different does of cis-platinum (60mg/m^2 and 120mg/m^2) in a group of 81 patients with predominantly adenocarcinoma histology. Response rates for each regime were similar (46% and 40% with 10% complete responses) but the median duration of response (12 months versus 5.5 months) and median survival in responders (21.7 months versus 10 months) was prolonged in the high dose platinum group. These results are encouraging although the toxicity of the high dose regime was substantial. Other workers (Hansen 1976, Eagan 1979, Roberts 1980) have also noted the relative effectiveness of combination chemotherapy in adenocarcinoma.

Only one study of combination chemotherapy has compared treatment with an untreated control group (Cormier 1982) and few make a comparison with single agent therapy. Many authors unjustifiably point to increased survival in responding patients compared with non-responders as evidence of efficacy. There is no substitute for an appropriate untreated control group in any assessment of therapy in this disease as it may be possible that responders to therapy would have survived as long without treatment.

In the one study where combination chemotherapy (methotrexate, adriamycin, cyclophosphamide and CCNU) was compared in a prospective randomised fashion to placebo, the benefits of treatment were obvious (Cormier 1982). 35% of the treated group responded with no responses

	SQUAMOUS		ADENOCARCINOMA		LARGE CELL	
No of drugs	No of Studies	Response Rate (%)	No of Studies	Response Rate (%)	No of Studies	Response Rate (%)
1	18	11.1 (0-39)	14	9.6 (0-33)	6	8.2 (0-15)
2	5	10.1 (3-20)	6	14.3 (6-33)	3	11.5 (7-16)
3	7	22.4 (0-37.5)	9	26.2 (0-43)	3	14.6 (0-32)
4+	11	29.4 (9-87)	9	32.5 (6-64)	4	14.8 (0-35)

TABLE 1.2 Mean percentage response rate (with range) to various drug combinations (1 - 4+ drugs) in the three main histological subtypes of non-small cell lung cancer. (From Souhami 1984)

seen in the placebo group. Median survival for treated patients was 30.5 weeks compared to 8.5 weeks for untreated.

Combination chemotherapy is rarely used as palliative treatment in NSCLC as most effective regimes have concomitant toxicity. However the instillation of cytotoxic agents into the pleural space in patients with recurrent malignant effusions may prove useful palliative treatment.

In summary at the present time, the use of chemotherapy in patients with non-small cell lung cancer should be confined to carefully designed research studies in an attempt to find newer, more effective treatment regimes.

1.4b Small Cell Lung Cancer

The greatest advance in the management of bronchial carcinoma in recent years has been the improved survival in patients with small cell tumours treated with systemic combination chemotherapy. SCLC differs from the other types of lung cancer in the propensity for rapid growth and early dissemination. The TMN (Tumour, Nodes, Metastases) staging system which is widely used for most forms of cancer has been shown to have little relevance in the management of SCLC patients in whom the disease is usually disseminated at presentation (Mountain 1974). This led to the development of a specific staging system of "limited" (i.e. confined to one hemithorax including ipsilateral scalene nodes) and "extensive" disease. The median survival of placebo treated patients with limited disease has been shown to be 3 months, while patients with extensive disease have a median survival of 6 weeks (Zelen 1973). These figures emphasise the aggressive malignant nature of this disease.

In 1969 Green and colleagues showed that the lifespan of patients with extensive SCLC could be doubled with three courses of treatment with cyclophosphamide compared to placebo. Since then the activity of a variety of single agents in SCLC has been established (Table 1.3). Objective response rates of 30-40% are seen with many drugs but these responses are in general short lived with complete remissions being extremely unusual. In an attempt to improve on these results combination chemotherapy was tried with a marked improvement in the response rates and survival compared with single agent treatment (Alberto 1976, Edmonson 1976, Lowenbraun 1979).

Over the past 10 to 15 years a large number of patients with SCLC have undergone combination chemotherapy with many different agents in numerous studies. A number of facts have been clearly established:

(i) Using combination chemotherapy involving principally agents listed in Table 1.3, an objective response rate can be obtained in approximately 75% of patients in whom 15-25% will achieve a complete remission.

(ii) Survival in complete responders is significantly longer than that observed in patients with a partial response.

(iii) Patients with limited disease have higher complete response rates and more prolonged median survival (e.g. 60% versus 25%, 51 weeks versus 33 weeks, Bunn 1977) than patients with extensive disease.

Drug	No of Patients	Objective response (%)
Ifosfamide	52	63
Procarbazine	19	47
Nitrogen Mustard	55	44
Vincristine	43	42
Etoposide	213	40
Cyclophosphamide	363	38
Methotrexate	78	30
Adriamycin	53	30
Hexamethylmelamine	69	30
CCNU	76	14

TABLE 1.3 Objective response to single agent chemotherapy in small cell bronchogenic carcinoma (from Hansen 1980)

(iv) The survival of extensive disease patients who achieve a complete response is comparable to that of limited disease patients who achieve a complete response.

(v) Even with these encouraging responses 50% of patients with limited disease die within the first 12 months after diagnosis and 80-90% succumb within the first two years (Comis 1982). Many patients who respond to treatment and are disease free for two years, remain disease free and may even be considered cured (Ginsberg 1979).

1.5 Combined Radiotherapy and Chemotherapy in SCLC

The exact place of radiotherapy in the initial induction treatment remains uncertain. Most studies include radiation therapy to the primary tumour and to draining lymph nodes between courses of combination chemotherapy - so called "sandwich radiotherapy". The evidence that adding radiation therapy to standard chemotherapy is beneficial is supported by only one study (Oldham 1981) where the complete response rate was higher with a combined modality approach. In four studies (Fox 1980, Smyth 1980, Stevens 1979, Williams 1977) no such benefit was seen and indeed Hansen (1979) reported a significant shortening of median survival after chemotherapy from 14 to 11 months with the addition of radiotherapy.

The optimum sequence and timing of radiotherapy in relation to chemotherapy has not been established nor has the precise dose of radiation required to gain the maximum benefit without significant toxicity. Simultaneous treatment with drugs and radiotherapy may be

more effective in producing a remission than "sandwich" therapy (Comis 1982) but its toxicity appears unacceptable especially if adriamycin is included in the drug regime (Feld 1981).

Prophylactic whole brain irradiation has been shown to reduce the incidence of overt cerebral metastases but has not been shown to have any positive effect on survival (Maurer 1980).

Despite these unresolved questions which can only be answered by well planned clinical studies, radiotherapy continues to be included in most treatment regimes. It may be significant that virtually all the long term (more than 2 year) survivors in SCLC underwent combined modality therapy.

1.6 Methods of optimising chemotherapy in SCLC

Much of the clinical research performed in SCLC in the last few years has been aimed towards increasing the proportion of patients achieving a complete response and to improve the duration of such responses, as it is these patients who stand the best chance of long term survival.

(a) Intensification of treatment

In 1977 Cohen and colleagues using a well studied induction regime of CCNU, cyclophosphamide (CY) and methotrexate (MTX) showed that doubling the dosages of CCNU and CY and increasing the dose of MTX by half, doubled the objective response (45% to 96%) and median survival (20 to 42 weeks). Smyth (1981) improved on the complete response rate by increasing the dose of MTX from 15 mg/m^2 orally to 200 mg/m^2 as an i.v. infusion with folinic acid rescue.

Many other workers have since tried high dose induction therapy with a variety of drug combinations usually combined with sandwich radiotherapy. Impressive complete response rates of 70-80% in limited disease and up to 50% in extensive disease have been achieved. However the impact on long term survival has been extremely disappointing and many treatment regimes have been associated with significant toxicity.

Late intensification therapy has been tried in an attempt to prolong complete remissions. High dose drug treatment, often with total body irradiation and autologous bone marrow transplantation, has been given to patients achieving a complete remission with standard induction therapy (Stewart 1983). Unfortunately this aggressive treatment has failed to improve on the survival figures achieved with standard induction chemotherapy. Indeed recently the whole rationale for giving maintenance chemotherapy has been questioned and further trials are required to determine the optimum intensity and duration of treatment in SCLC.

(b) Non-Cross Resistant Drug Regimes

In an attempt to overcome the development of drug resistance after induction therapy, many centres have evaluated mutually non-cross resistant chemotherapy regimes. Cohen (1977) improved further on the results with MTX, CY and CCNU mentioned above by alternating courses of vincristine, adriamycin and procarbazine. The complete response rate obtained using alternating chemotherapy increased from 30% to 56% and median survival from 42 to 61 weeks. Many other workers (e.g. Aroney 1982, Dombernowsky 1979) have also

demonstrated that a second non-cross resistant drug combination can be shown to have some effect. However, they have failed to produce any real benefit in terms of patient survival.

1.7 Treatment of SCLC - the future

As stated above, in the last decade the outlook for patients presenting with SCLC has improved greatly. The use of aggressive combination chemotherapy has increased the median survival of responding patients with virtually all responses being associated with significant palliation. A small though important minority of patients are now alive and disease free at two years with a chance of long term survival. The challenge for the future is clearly to increase the percentage of patients achieving these long remissions. What is the way forward?

In clinical terms, it would seem important to continue to try to optimise chemotherapy by giving the most active drugs available in regimes which maximise cytotoxicity to the tumour without creating intolerable side effects. In the long run however it seems likely that major therapeutic advances will not be made unless more effective anti-cancer agents become available.

The chances of developing such agents have been greatly improved by the fact that many investigators are now able to grow human bronchial carcinomas either using special tissue culture techniques or in immunodeficient mice. This has greatly facilitated the study of the biological, immunulogical and cytogenetic characteristics of

this disease (Smyth 1985) thus allowing the development of more novel therapeutic strategies such as growth factor antagonists, immunomodulators and techniques involving monoclonal antibodies.

The efficacy of any new treatment regime can be rapidly assessed in the laboratory using either an in-vitro assay or xenografts established in immunodeficient rodents. It is to be hoped that over the next few years a greater understanding of the biology of the disease can be translated into encouraging therapeutic advances for the patient diagnosed as suffering from this terrible condition.

CHAPTER 2

LABORATORY ASSESSMENT OF ANTI CANCER AGENTS

2.1 Introduction

One of the major problems encountered by clinicians who treat cancer patients with chemotherapy is the fact that tumours which according to histological characteristics are identical nevertheless are highly individualistic in their sensitivity to anti-cancer agents. At present the decision to use or not to use a particular drug in a particular patient is based on the results of previous clinical trials in patients with the same type of tumour. Thus the knowledge that a particular drug has a low level of activity in a specific tumour may prevent the use of that drug in a few patients who might greatly benefit from it.

The success of the in vitro testing of the sensitivity of bacteria to antibiotics led to much interest in the development of a similar system where the sensitivity of human tumours to a given cytotoxic agent could be rapidly assessed in the laboratory. Clearly such a test would have two important applications. Firstly, it would be invaluable in the screening of new compounds or drug regimes that have potential use in treating cancer patients. Secondly, with

pretreatment testing of a patient's tumour to a wide range of chemotherapeutic agents it might be possible to 'individualise' drug regimes. This would allow treatment with agents known to be active in that particular tumour and prevent the use of inactive drugs with their associated toxicity.

Such a test would ideally be simple, cheap, rapid, easily standardised, statistically acceptable, capable of allowing for various methods of drug action and offer reasonable correlations with effects seen in the patient. Over the past three decades many different in vitro predictive assays and animal model systems have been developed (Figure 2.1) but at the present time no single technique has satisfied the above criteria as a reliable predictive test for cancer therapy.

Although the tailoring of individual patient therapy using laboratory systems has not been achieved, there now exist many different methods of assessing the activity of anti-cancer agents outwith the patient. Both in vitro and in vivo systems have proved invaluable in the development of all the currently available chemotherapeutic agents and these techniques will hopefully facilitate the evolution of newer, more effective treatment regimes.

2.2 In vitro methods of drug testing

Harrison (1907) and Carrel (1912) were the first researchers to demonstrate that animal cells could be taken from an organism and grown successfully in culture media in the laboratory. In the last

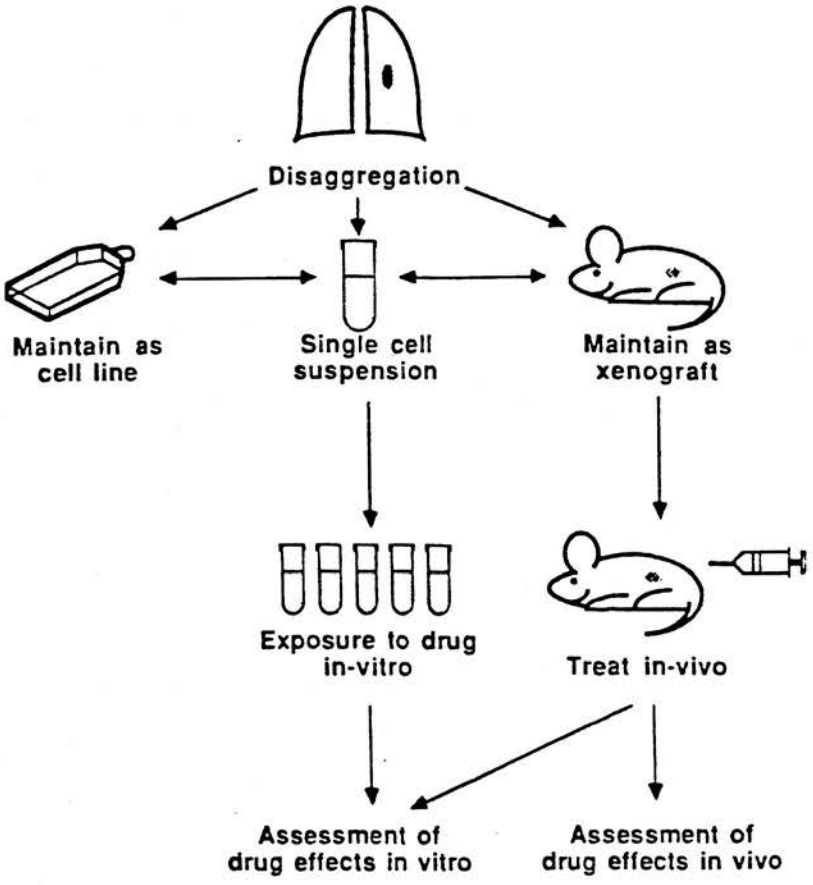


Figure 2.1: Laboratory methods of assessing anti-cancer agents

thirty years, tissue culture techniques have become extremely sophisticated and many workers have attempted to evolve systems to accurately test the activity of cytotoxic drugs in tumour cell preparation.

The majority of in vitro test systems involve the preparation of a single cell suspension of tumour cells which are then exposed to a fixed concentration of the cytotoxic agent being evaluated. Drug-induced cell damage can then be assessed using a number of different criteria and the activity of a number of different drugs in the one tumour cell type can then be compared. Several technical problems are encountered at all these steps.

2.2(a) Preparation of a single cell suspension

In general terms after surgical resection of a tumour, homogenates are prepared for drug testing by separation of the specimen into single cells or by dissection into small fragments. A single cell suspension may be produced mechanically with scissors or by the action of proteolytic enzymes such as trypsin, dispase or collagenase.

The viability and yield of cells depends directly on the method used to prepare the suspension as well as on the histological structure of the original tumour. Tumours having a dense stromal element requiring long mechanical processing give rise to cell suspensions of low viability. A reduced cellular vitality has also been associated with enzymatic treatment (Hofer 1974) and some workers (Barranco 1980) have reported a change in sensitivity of

cells to cytotoxic agents after exposure to trypsin. These problems may be overcome by using tumour fragments. However the disadvantage of this method lies in the inherent heterogeneous structure of most tumours with a marked regional variation in cellular metabolism observed within one tumour population (Siracky 1979).

2.2(b) Selection of appropriate drug concentration

The effectiveness of most anti-neoplastic agents corresponds directly to its serum concentration and to the amount of time an effective serum level can be maintained. For several agents a method of determining their ideal concentration in vivo remains to be developed. Numerous factors such as drug activation and catabolism, varied mechanisms of transport and dissimilar vascular and metabolic conditions in individual tumours prevent accurate estimation of 'effective' therapeutic drug concentrations in vivo and therefore makes appropriate drug monitoring in vitro impossible.

Different groups of workers select drug concentrations for sensitivity testing using various criteria e.g. based on the dose producing cell death in 50% of cultured cells (ID50), on the inhibition of growth of known cell lines (e.g. HeLa cells, Wright 1962), by using concentrations which respond to the serum therapeutic dosage converted to mg/ml of culture media, or merely on an arbitrary basis.

In most in vitro systems the contact time of the drug being assessed with the tumour cells is relatively long. Since the effective dose is calculated from a concentration x time

equation, the concentration of drug must initally be lower or the exposure time limited if a 'reasonable' drug exposure is to be achieved. In any test system it would seem reasonable to utilise several concentrations of a particular agent when attempting to define drug sensitivity.

2.2(c) Criteria for cell damage

When tumour cells are exposed to an anti-cancer agent, they do not exhibit an "all or nothing" reaction and a variety of influences on several measurable parameters are observed. The in vitro predictive test systems which have been developed over the past 25 years are in essence several different methods of measuring drug induced cytotoxicity using a variety of different parameters. Structural cell damage, viability, inhibition of cellular metabolism including radioactive precursor incorporation and measurement of cellular reproduction capacity (colony formation) have all been used as end points to measure the effectiveness of cytotoxic agents in vitro. These systems will now be considered.

2.3 Specific Predictive Systems

2.3(a) Morphological assessment of cellular damage

The earliest assessment of the effect of cytotoxic agents in vitro involved an evaluation of morphological cell damage. Wright and colleagues (1957) established fresh biopsy specimens as explant

cultures and then added drugs for 96 hours. Drug effects were evaluated microscopically by experienced observers and cell damage scored on a subjective scale. The initial report was encouraging with a good correlation demonstrated betweeen in-vitro tumour sensitivity and the subsequent clinical response. However, further studies by this group (Wright 1962) and others (Yarnell 1964) proved less encouraging with inconsistent responses from groups of cells from the same tumour, false positive in-vitro results, a lowish success rate in establishing biopsies in culture and a major subjective component involved in assessing drug-induced cell damage.

2.3(b) Measurement of cell viability

In an attempt to render morphological assessment of cytotoxicity less subjective, other assay systems measuring cell viability have been developed.

(i) Dye exclusion assays - The ability of viable cells to exclude supravital stains such as trypan blue, eosin and nigrosin has been used in assay systems for many years. Unfortunately most authors conclude that vital dyes are not reliable indicators of drug-induced cytotoxicity (Roper 1979), with a poor correlation between dye exclusion and long term reproductive ability (Rupniak 1983). However a recent report from Weisenthal and colleagues (1983) using a novel dye exclusion method demonstrated a better in vitro/in vivo correlation and using this assay, Bosanquet (1983) has shown valid results in determining the chemosensitivity of patients with chronic lymphatic leukaemia.

(ii) Chromium Release Assay - The degree of damage to cell membranes may be estimated by the release of chromium-51 in a previously labelled cell. Little experience has been reported with its use as an assay of chemotherapeutic drug effect. It shares the same weaknesses as dye exclusion tests in that although loss of cell membrane integrity may be a relatively early event in cell death, it is probably not a sensitive indicator of lethal drug-induced damage.
(iii) Loss of adherence - Holmes (1974), studying a small group of patients, reported an overall clinical correlation of 92% (100% true +ve, 88% true -ve) with cell kill assessed by loss of monolayer adherence. The period of in vitro exposure was relatively long (72 hours) and commenced only 24 hours after the cells were placed in culture. Only cells known to be viable by dye exclusion were used in the final adherence assay and only 13 patients were available for the clinical correlation. No other studies of cell adherence assays have been reported.

2.3(c) Measurement of Inhibition of cellular metabolism

Black (1954) in one of the earliest in vitro assays attempted to evaluate the response of human tumour cells to a variety of drugs by investigating inhibition of dehydrogenase activity by reduction of tetrazolium dyes. Although they demonstrated a positive correlation between in vitro effects and clinical response in the patient, other workers have been less enthusiastic, with many showing high (up to 50%) false positive in vitro results (Di Paoli 1961, Kondo 1971).

Despite this, reports have appeared recently describing colorimetric assays suitable for rapid testing of cancer cells with multiple drug combinations (Cole 1986).

Although fairly reliable at predicting non-response, assays that measure inhibition of cellular metabolism have not been actively pursued. Tests that measure changes in oxygen consumption have proved technically difficult with large variations observed in results from control cell populations. The other main problems with these assays are the qualitative nature of the results and the fact that the metabolism of both malignant and non-malignant cells is measured.

2.3(d)Measurement of radioactive precursor incorporation

The measurement of the incorporation of radiolabelled nucleosides (uridine, deoxyuridine, thymidine) into tumour cell preparations during in vitro drug exposure has been widely used as an indirect method of assessing cytotoxicity and remains an active area of investigation. Bickis (1966) provided the initial model for this form of assay by incubating slices of fresh human tumours with a number of anti-cancer drugs in the presence of radioactive tracers. Since then other labels such as ^{32}P (Yarnell 1964), radioactive leucine in long term culture (Morgan 1983) and tritiated thymidine or uridine in short term culture (Volm 1979) have been used.

Although positive correlations between depression of DNA synthesis in vitro and clinical responses in patients have been

observed (Mattern 1982), when compared to other in vitro tests, these measurements may not give an accurate assessment of cell survival (Roper 1976, Rupniak 1983). The problems with this assay system include:-

(i) The lack of incorporation of DNA precursors by tumours with a long cell cycle time or a large population of non-cycling cells.

(ii) Precursor incorporation into cells may be temporarily stopped by damage to cells in the isolation process.

(iii) Drug induced changes in nucleotide pools or nucleotide transport may cause underestimation of DNA synthesis.

(iv) Overestimation of DNA synthesis may arise from increased use of salvage pathways in the presence of drugs such as 5FU and methotrexate that affect the de novo pathway.

(v) The inability of the method to distinguish between DNA synthesis in tumour and non-tumour populations.

Attempts have been made to overcome these problems by using modified stem cell assays (Friedman 1982) and labelling index techniques (Livingston 1980) and much interest is still being shown in this form of assay.

2.3(e) Measurement of Reproductive Capacity (Clonogenic assays)

A major drawback of the systems described so far is the fact that they measure the effect of drugs on the entire cell population, which in most cases comprises a mixture of normal and neoplastic cells. Clearly measurements that include normal cells may give misleading results but also it has recently become apparent that even

the population of neoplastic cells in a given tumour is heterogeneous in its growth characteristics. Cell kinetic studies indicate that in most cases the bulk of tumour cells turn over slowly, die or terminally differentiate. Only a small percentage of the total population is made up of cells capable of repopulating the tumour - the so called "stem cells". These cells have been well characterised in bone marrow where they comprise approximately 1% of the total population (Pike 1970). Thus many of the in vitro assays already described measure the effect of drugs on cells that will probably not divide in vivo.

Stem cells at any particular time may be proliferating or non-proliferating. The large proportion of non-proliferating potential stem cells includes cells that can be stimulated to divide in vivo and it has been postulated that these cells may be responsible for the regrowth of tumours after treatment with cytostatic drugs. If such a hypothesis is correct then clearly the best in vitro assay system for predicting response to cytotoxic agents should selectively measure the effects of drugs on this small population of stem cells.

In 1971, Park and colleagues devised an in vitro system for cloning cells from transplantable murine myelomas and showed that the results obtained for drug assays in vitro mirrored in vivo responses. This work led to major developments in culturing stem cells from human tumours using a two layer soft agar system (Hamburger 1977).

In this assay (figure 2.2), a single cell suspension is prepared and mixed with culture medium and 0.3% agar (or agarose) at 40° C. One ml of the mixture is then plated in triplicate in 35mm plastic Petri dishes over a base layer of 0.5% agarose in culture medium that has hardened. Cultures are then incubated at 37° C in a well humidified atmosphere of 5-10% CO_2/90-95% air. Colonies (i.e. aggregates of 50 cells or more) are usually observed 7-21 days after plating. The number of colonies that grow is proportional to the total number of cells plated and thus a quantitative estimate of drug effects on colony forming cells can be made.

At the same time that Hamburger and Salmon were developing their clonogenic assay using myeloma cells, Courteney (1978) reported on a replenishable soft agar assay for solid tumours, where colonies grow in low oxygen tension in the presence of rat erythrocytes. Both systems take advantage of the observation that fibroblasts which normally 'contaminate' tumour cell cultures do not grow in soft agar. These two assays are now the most commonly used methods of assessing the activity of cytotoxic drugs in vitro.

2.4 Drug testing using the clonogenic assay

2.4(a) Methodology

Since the original description of the clonogenic assay, many investigators have attempted to grow most types of human cancers in this system. Approximately two thirds of the known tumour types have

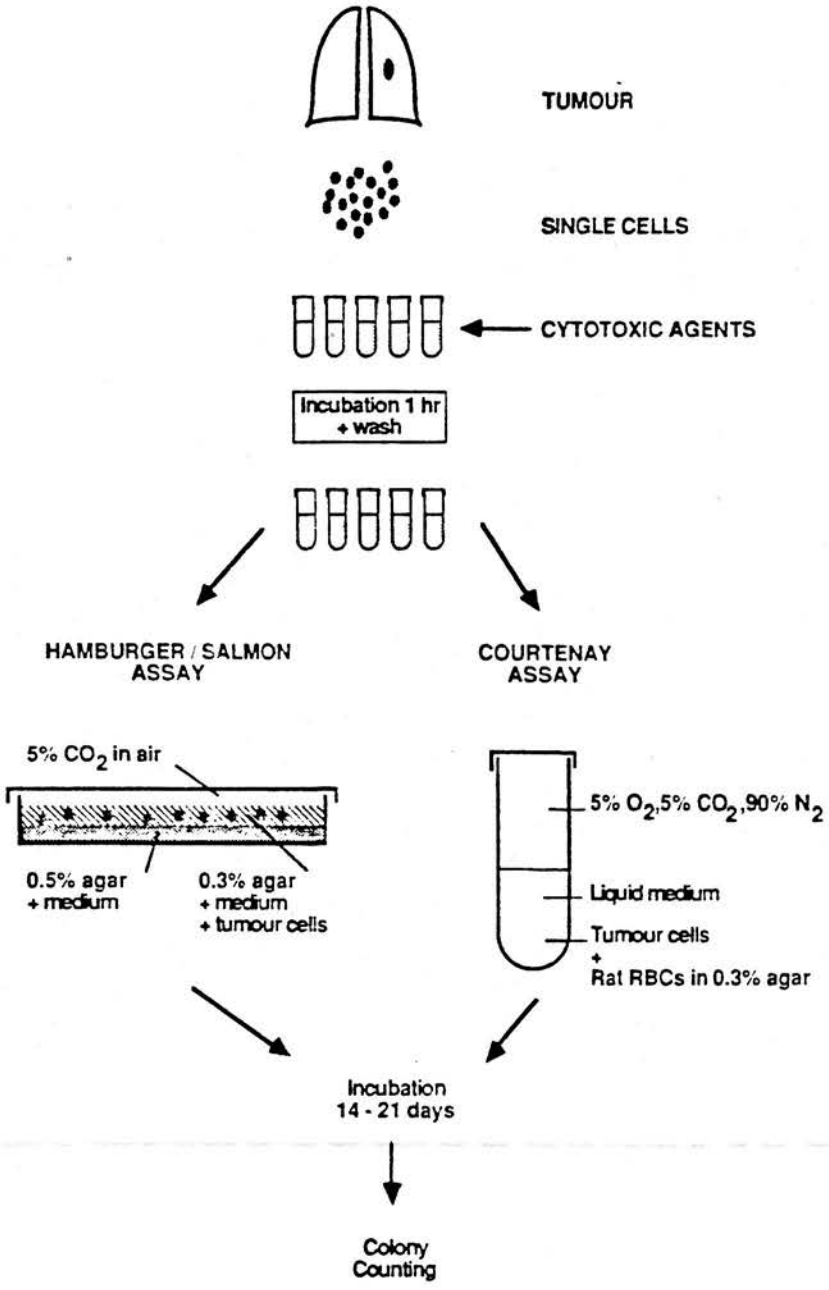


Figure 2.2: The clonogenic assays

been cultured with ovary, melanoma, lung and colon faring best (Salmon 1981, Von Hoff 1981). However, even in these examples only 50% of tumours tested were capable of producing enough colonies for drug testing (Mattern 1982). This disappointing value may be attributable to (i) reduced cell viability during isolation and preparation of single cell suspensions, (ii) nutritional or hormonal requirements specific to the tumour of origin, (iii) an expression of insufficient proliferation of the tumour.

In the standard assay system (Figure 2.2),(Hamburger 1977, Courteney 1978, Salmon 1981), cells are incubated for varying times (normally 1 hour) with different concentrations of a specific drug. Cells are then washed, unless continuous exposure is required, and plated out in agar. After 2-3 weeks of incubation, plates are counted and the activity of the drug expressed in terms of the colony survival in treated plates compared with controls. In most studies a mean colony count of at least 30 in control plates is required for the assay to be considered evaluable. In vitro sensitivity at a specific drug concentration is usually associated with a colony survival of 30% or less of controls, with colony survival in excess of 50% considered to indicate resistance (Von Hoff 1981).

2.4(b) Clinical correlations of the clonogenic assay

Salmon and colleagues at the University of Arizona reported the initial correlations of in vitro chemosensitivity and clinical response in 176 patients with myeloma, melanoma and ovarian cancer (Salmon 1983). The true positive rate was 67% and true negative rate

93%. Many other workers have published similar results in different tumours and using different assay methods. These results are well summarised by Carney (1985), who showed that in 715 direct in vitro/clinical correlations in 11 major studies, the overall true positive rate was 70% (range 42-92%) and the true negative rate was 92% (range 83-100%).

Can these in vitro correlations be used to improve the management of patients in the clinic? In a prospective trial, Von Hoff (1983) treated patients with single agent therapy based on in vitro assay results. He showed a higher objective response rate (25%) in patients whose treatement was based on clonogenic assay results than in those patients treated with the physician's choice of drug (11%) or where specimens failed to grow in the assay (14%). However this was not a randomised study and each group was extremely heterogeneous. In a small study of untreated patients with myeloma (Durie 1983), median survival was 48.5 months for 8 patients with in vitro sensitivity to melphalan but only 9 months for 8 patients resistant to melphalan in vitro.

Although these results appear encouraging, at the present time it is not feasible to use clonogenic assays to "tailor" chemotherapy for individual patients. The foremost problem continues to be the inability of many tumours to grow in the assay, with successful colony formation being achieved in only 30-40% in the best centres. In addition, pharmacological considerations including drug dosages, activation, duration of exposure and drug/drug interactions pose

difficulties in the clonogenic assay. Other problem areas exist such as the exact definition of a colony and the criteria used for defining sensitivity or resistance. A major drawback to using clonogenic assays as predictive tests has been highlighted by Von Hoff (1986) who showed a great variation in chemosensitivity between two tumour specimens from the same patient.

Until these technical problems are overcome, the in vitro tests of cytotoxicity should be considered as important research tools and not for widespread clinical application. Perhaps in the fullness of time they may prove most useful in identifying which drug should not be given as most studies show a predictive accuracy of in vivo resistance greater than 90%. However this figure may merely reflect the relative chemoresistance of most human tumours (Twentyman 1985).

2.5 Animal Tumour Models

Over the past three decades, many workers have attempted to develop animal models to assess the activity of anti-cancer agents in vivo. The advantages of such a system over in vitro methods are clear.

Drug testing in vivo can be performed on solid intact tumours which have a blood supply integrated with the vascular system of the host. Tumour disruption to form a suspension of cells can be avoided. The drug or combination of drugs being assessed can be administered in a similar manner to their clinical application. They are transported by normal pathways to the tumours and are subject to

the same metabolic and pharmacokinetic interactions seen in patients. The drugs can act through several cell cycles and it is possible to test compounds which must undergo metabolic activation (e.g. cyclophosphamide) to be fully active.

It is for these reasons that most workers agree that more relevant results can be obtained when anti-cancer agents are assessed using animal models compared with in vitro methods.

2.5(a) Transplantable rodent tumours

The first in vivo systems used for drug testing involved the use of transplantable rodent tumours which had arisen either spontaneously or through viral or chemical induction. The L1210 murine leukaemia, P388 leukaemia, B16 melanoma and Lewis lung carcinoma have been the most widely used murine tumours and formed the basis of a massive drug development programme set up over 30 years ago at the National Cancer Institute (Carter 1977).

Although the vast majority of anti-cancer agents in current clinical use were developed using rodent tumour systems, few similarities exist on a biological or biochemical basis betwen these tumours and solid human cancers. In an attempt to mimic more closely the behaviour of human neoplasms, many people have tried to heterotransplant human tumours into laboratory animals. Initial attempts using many different species as hosts were unsuccessful. This led to the development of various techniques to avoid the immunological rejection of the foreign tumour tissue by the host animal.

2.6 Human Tumour Xenografts

2.6(a) Immune-privileged sites

Implantation of human tumours to immune-privileged sites such as the anterior chamber of the eye, brain, subrenal capsule or cheek pouch has been successfully attempted (Green 1953). Unfortunately only limited growth of the xenograft is possible and progressive growth measurements are difficult. The techniques may prove valuable for establishing certain tumour types that fail to grow elsewhere or where therapy specific for that site is being investigated e.g. retinoblastoma will grow in the anterior chamber of the eye of Swiss nude mice but not in other species (Gallie 1977). Epstein (1967) has successfully grown human lymphomas in the brains of certain nude mice strains. Cobb (1974) found the hamster cheek pouch (+ATS adjuvant therapy) to be as receptive a host as artificially immune-suppressed CBA mice. However relatively few chemotherapy studies using these models have been reported.

2.6(b) Immune-suppressed mice

In 1914 Murphy showed that it was possible to grow and serially passage the Ehrlich mouse sarcoma in rats pre-treated with x-irradiation. Successful growth and transplantation of a variety of human tumours in irradiated rodents was first reported by Toolan in 1951. Two years later she demonstrated that take rates could be improved (90% of 101 tumours) if cortisone treatment was added and that cortisone treatment alone could adequately immunosuppress a mouse for xenografting.

The development of anti-lymphocyte serum (ALS) allowed laboratory animals to accept skin homografts (Woodruff 1963) and heterografts from other species (Lance 1968). The major problems associated with ALS or anti-thymocyte serum (ATS) are the production of sufficient quantities for routine use and the standardisation of batches. Prolonged administration of ATS may induce formation of anti-ATS antibodies which reduce immuno-suppressive activity and lead to immune complex formation. Tibbetts (1977) reported that mice given ATS for longer than one month tended to become unhealthy and tumour growth slowed after 30 days. This important point was highlighted in a study by Mitchley (1977) who showed that hexamethyl melamine could cure a human lung cancer xenograft grown in ATS treated mice but did not affect the same tumour grown in thymectomised, irradiated hosts.

Floersheim (1980) developed a short term immuno-suppressive regime involving ALS and pre-treatment with the cytotoxic agents procarbazine and cyclophosphamide and later compared it with other regimes including nude mice (1982). Animals pretreated with these agents survived longer than thymectomised or nude mice but progressive tumour growth was not observed beyond 30 days after implantation. Cyclosporin A was completely ineffective at producing sufficient immunosuppression to support xenograft growth.

It seems that at present pharmacological immunosuppression would appear to be inadequate to enable adequate tumour growth for effective drug assessment. If immunosuppression is to be prolonged, then these agents would have to be administered during drug testing making the validity of such tests questionable.

2.6(c) Immunodeficient mice

In 1961, Miller showed that the thymus played a central role in the development of the immune response to grafted foreign tissue in early life. He then demonstrated (1963) that the recovery of the immune mechanism in mice following total body irradiation was thymus dependent and if thymectomy was combined with irradiation then animals could be rendered immunodeficient for a prolonged period. Davies (1966) showed that irradiation doses could be increased to normally lethal levels (850 rads) if an injection of 5×10^6 syngeneic bone marrow cells was administered within three hours. Castro (1972) demonstrated that an animal prepared in this way would support the growth of a variety of human tumours.

Steel at the Institute of Cancer Research improved the receptivity of the model by giving only 2×10^6 marrow cells. He then developed an immunosuppressive regime involving neonatal thymectomy and total body irradiation (900 rads) after a priming dose of cytosine arabinoside (ArA-c) (Steel 1978). The rationale for using this method was based on the observation of Millar (1978) that a number of cytotoxic drugs could improve tolerance to irradiation. Such immune deprived mice could be housed under conventional conditions (Hay 1985) and support the growth of most human malignancies (Steel 1983).

Although there is some evidence that the immunity of the thymectomised/irradiated/ArA-c prepared animals may eventually return, significant immunosuppression has been recorded up to 6

months following irradiation (Steel 1980). This allows for progressive growth of xenografts over a prolonged period and provides a useful model for the testing of cytoxic agents.

2.6(d) Athymic "nude" mice

In 1966 Flanagan reported the genetic details of a hairless mutation occurring in a closed stock of albino mice in Ruchill Hospital, Glasgow. He called them "nude" mice (genetic symbol nu) and confirmed that the nu gene was a single autosomal recessive. He also noted a reduction in body growth of homozygotes and a high mortality from hepatic necrosis. Pantelouris (1968) discovered that the nude mouse lacked a thymus and along with heterozygotes (nu/+) had a low leucocyte count. A preliminary report of the immunobiology of the athymic mutant was published by Rygaard in 1969 and shortly afterwards (1969a) he described the successful hetero-transplantation of a human colonic tumour to a nude host.

Further immunological studies (de Sousa 1969, Jordan 1977) have demonstrated absence of lymphocytes in the thymus dependent areas of the spleen, a normal component of B cells, small numbers of lymphoid cells with T cell markers (+ve cells) of uncertain functional significance, a higher level of natural killer (NK) cells (Kiessling 1975) and an increase in macrophage activity. Other interesting immunodeficient rodent mutants discovered since include the Lasat mouse (Dh/nu) which is also asplenic (Lozzio 1976), the beige nude (bg/nu) which has lower NK cells activity (Lane 1972) and the athymic rat (rnu/rnu) (Festing 1978).

Since 1969 all the major human cancers have been successfully transplanted and serially grown in athymic mice in many laboratories. Receptivity for human tumours may be further improved by pre-treatment with ALS (Ohsugi 1980), x-irradiation (Ziegler 1982) or cyclophosphamide (Gallie 1977).

The major disadvantages in using a nude mice colony for drug testing lies in the cost of the animals and the fact that strict isolation procedures and specialised husbandry techniques are required to reduce the risk of infection and ensure a healthy colony (Giovanella 1973). However the improved receptivity for human tumours outweighs these problems and at present the nude mouse remains the most widely used host for in-vivo testing of cytotoxic agents against human tumours.

2.7 Characteristics of human tumour xenografts

2.7(a) Take rates

The vast majority of human cancers have now been grown as xenografts using a variety of immunodeficient murine hosts. Different centres have reported wide variations in take rates. This may be partly explained by the use of a variety of hosts of different receptivity, the size and form of inoculum, the technique and location of implantation and whether primary or metastatic tumour is used. Another major problem lies in the fact that the criteria for a positive take varies between centres. Some workers have taken histological evidence of 'viability' in a static nodule as proof of a

positive take, but it is now generally accepted that the criteria for a positive take should include progressive growth and serial transplantabilty.

Melanoma, bronchial and colorectal carcinoms are most easily grown with take rates of 50-80% (Steel 1983). Breast, ovarian and testicular tumours are generally more difficult to graft. Mattern (1985) attempted to establish 245 human bronchial tumours in nude mice and had a positive take rate of 44%. Like Shorthouse (1980b), he noted differences in take and growth rates with histological type, with squamous and adenocarcinoma being most easily grown. Fogh (1980) reported that metastases would grow more readily than primary tumours but Mattern's data does not support this.

2.7(b) Growth Rates

Since the size of xenografts can be easily measured with calipers, much is known about their growth rates (Steel 1977). Most centres report tumour doubling times of between 6-20 days (Rofstad 1982, Steel 1983, Mattern 1985). This represents an acceleration in growth rate after grafting since most clinical studies of tumour growth, usually based on easily measurable metastases, show doubling times in excess of 60 days (Steel 1983). Virtually all workers have demonstrated an acceleration in growth rate over the first 2-3 passages in mice with stable growth rates on succeeding passages (Houghton 1978, Steel 1983, Mattern 1985). It is not clear whether this is an adaptive change or a process of selection although there

is some evidence (Houghton 1983) that it is due to a decreasing cell loss factor rather than a change in phase duration or growth fraction.

2.7(c) Maintenance of human characteristics

Clearly it is essential for human xenografts growing in mice to maintain the characteristics of the original donor tumour if they are to provide a relevant model for the study of human cancer. The following features have been extensively studied:-

(i) Karyotyping:- The most basic requirement of human tumour xenografts is that they maintain a human karyotype. Reeves (1978) studied this extensively in colonic carcinoma xenografts and apart from small changes in the distribution of chromosomes, a human karyotype was always maintained through repeated passage. These results have been validated by other workers in histologically different tumours (Kopper 1975, Shorthouse 1980, Selby 1980). It remains important to continually check the karyotype of xenografts as there have been reports of murine sarcomas arising at the site of transplantation of human tumours (Houghton 1978a, Goldberg 1982, Beattie 1982). Karyotype analysis however is time consuming and requires considerable expertise. Some workers (Houghton 1983, Beattie 1982) have used an electrophoretic assay which separates the isoenzymes of lactate dehydrogenase into murine and human components as an alternative.

(ii) Histology:- Human tumours essentially retain the histological and morphological characteristics of the original tumour when transplanted into mice. Several workers have noted an increase in differentiation of xenografts after serial passage (Sharkey 1978, Tokita 1980), although others (Bailey 1981, Steel 1983) have reported increased nuclear pleomorphism with a more anaplastic appearance to many tumours. Shorthouse (1981) and Carmichael (1986) found the pattern of production of epithelial mucins well maintained in bronchial adenocarcinomas, a feature also noted in colonic tumours by Houghton (1978a).

(iii) Functional activity:- Ectopic hormone production is a feature of many human tumours and this property has been shown to persist when these tumours are grown in mice. Hayashi (1978) demonstrated that the production of human chorionic gonadotrophin (HCG) by a human choriocarcinoma continued when transplanted into nude mice. Shorthouse (1982) in an extensive study of human bronchial carcinoma xenografts found evidence of secretion of HCG, calcitonin and adrenocorticotrophic hormone (ACTH) in a significant number (13-78%). ACTH and B melanocyte stimulating hormone (MSH) have been shown to be produced by small cell lung cancer xenografts (Shimosato 1976). The presence of other key biological substances such as bombesin, neurone specific enolase and the APUD enzyme, L-dopa decarboxylase has also been confirmed in xenografts of this cell type (Carney 1983).

Expression of tumour related antigens such as carcino-embryonic antigen (CEA) has been observed in colonic carcinoma xenografts (Houghton 1978a). Raghavan (1980) studied the production of alpha fetoprotein (AFP) by teratoma xenografts and found good positive and negative correlations between the amounts produced in mice and in the original patient.

2.7(d) Cachexia and metastases

It is poorly understood why human malignant tumours, which frequently cause marked weight loss and cachexia in patients, are unable to produce these effects when transplanted into experimental animals, even when xenografts are allowed to grow to large sizes (Giovanella 1974).

It is now thought that the cachexia associated with "invasive" diseases is mediated by a multipotent protein, cachectin (tumour necrosis factor), produced by host macrophages (Beutler 1986). Considerable variation is observed among different species of mammals both in the sensitivity to this protein when administered exogenously and in the amounts of cachectin produced endogenously after challenge with endotoxin. The production and pathophysiological effects of this substance in immunocompromised rodents has not been studied.

Human tumour xenografts appear to lose their ability to metastasise when transplanted to experimental animals. The reasons for this are unclear although transplantation techniques may be important (Fidler 1973). Many workers have noted a relationship

between the degree of immunosuppression of the host rodent and the occurrence of metastases (Steel 1977, Moore 1977). It is possible that the high level of NK cell activity seen in nude mice prevents tumour spread as metastases are much more common in beige nude mice which have low NK cell activity (Talmadge 1980).

2.8 Drug testing using Human Tumour Xenografts

2.8(a) Methodology

It is unfortunate that as human tumour xenografts have become increasingly popular as a screening system for anti-cancer agents, no single effective methodology for assessing drug activity has evolved and become universally adopted. There is little standardisation with respect to time of initiation and scheduling of therapy, dosage levels and assessment of response between researchers and attempts to interrelate studies from different groups is often futile. Despite these problems, which can only be overcome by better communications and perhaps collaboration between groups, certain principles in the use of xenografts for drug testing have emerged.

Tumour fragments (3-5mm^3) or cell suspensions (approximately 5 x 10^6cells) are inoculated subcutaneously in the flanks of mice. The use of bilateral implants allows fewer animals to be used without influencing the degree of statistical certainty (Warenius 1980). The subcutaneous site offers the advantage that tumours may be readily palpated and easily measured with calipers. Tumour volumes are

calculated either by a geometrical formula or using a calibration curve technique. It is important for tumours to be well established at the initiation of therapy and most workers restrict drug testing to tumours that have attained a certain volume (usually 0.3 - 1.0 cm^3). Spontaneous regressions are rarely observed in tumours of this size.

Groups of 6-10 tumours are then allocated to either a control group or to treatment by one or more drugs, usually at different dosages. The choice of drug dose is difficult and clearly critical if the effectiveness of a series of drugs is being compared. Most workers would agree that this problem is best approached using the maximum tolerated dose of any drug. The rationale for this method relates to the work of Freireich (1966) who showed a linear relationship between the amount of cytotoxic drug that would kill 10% of experimental mice (lethal dose 10% or LD_{10}) and the maximum tolerated dose in man. However it is important to remember that this work correlated drug toxicity and not efficacy and that the pharmacokinetics of the same drug in man and mice may be vastly different.

After drug exposure caliper measurements are continued at regular intervals in both treated and untreated animals. The activity of the drug being tested can then be assessed using a number of different methods to compare treated and control groups.

2.8(b) Assessment of response to chemotherapy

Xenografts tend to grow as well circumscribed masses and do not readily metastasise. The lifespan of a tumour bearing animal therefore is not usually related to tumour burden and hence the use of death as an experimental endpoint is not practical.

(i) Histological changes:- Several studies have attempted to evaluate tumour response to treatment using histological criteria. Berenbaum (1974) showed minor changes in 3 out of 44 different human tumours treated with a variety of anti-cancer agents. Unfortunately tumour growth following treatment was not measured. A fundamental problem with this method of assessment was highlighted in a study of Hayashi (1978) who found necrosis, haemorrhage and degenerative changes in both treated and control tumours.

(ii) Growth Inhibition:- This widely used method compares the growth of treated and untreated tumours at a fixed point after drug exposure. The inhibition of growth due to treatment is generally expressed as a percentage of controls:

Growth Inhibition = [(1 - Treated vol/control vol) x 100]%

Since the measurement takes place at one time point, some workers excise tumours and express inhibition using tumour weights.

A major problem when comparing results with this method lies in deciding when the measurements should be taken. The T/C ratio constantly changes with time until the growth rates of treated tumours returns to that of controls. Most workers express drug activity at 'optimal' inhibition i.e. the greatest difference between

the two groups irrespective of time. Unfortunately if experiments are terminated at this 'optimum' time point without regrowth of tumours it may be impossible to distinguish between tumour shrinkage due to drug toxicity rather than efficacy at high dosages. In summary this method is suitable for detecting drug activity (usually defined as growth inhibition >50%) but does not allow accurate ranking of efficacy between active agents.

(iii) Specific Growth Delay (SGD):- This method was first described by Kopper and Steel in 1975. The 'actual' tumour growth delay represents the difference in time taken for a group of treated tumours to reach a specific size (usually double the original volume) compared with controls. If the activity of a drug is to be compared in tumours of different growth rates then the 'specific' growth delay can be calculated

$$SGD = \frac{\text{Doubling time (treated)} - \text{Doubling time (control)}}{\text{Doubling time (control)}}$$

The SGD therefore represents the number of doubling times spared by treatment.

The fact that tumours must regrow for the SGD to be determined overcomes the main problem of growth inhibition measurement where acute drug toxicity may contribute to tumour shrinkage. However there is some evidence that the tumour cells which survive cytotoxic treatment may regrow at a faster rate (Stephens 1977) and the SGD cannot be measured in tumours which regress completely. Despite this, the SGD is at present the most reliable method of assessing drug activity in xenografts.

(iv) Clonogenic cell survival:- Steel and colleagues have attempted to overcome the potential influence of host rejection responses in prolonged exposure experiments in xenografts by measuring the clonogenic cell survival in treated tumours. Tumour bearing mice are treated, the tumours are subsequently excised (usually after 24-48 hours), disaggregated into a single cell suspension and their ability to form colonies in semi-solid media is evaluated. The last step can be performed either in vitro or in diffusion chambers in vivo (Smith 1976). The main disadvantage of this method lies in the fact that only a minority of xenografts are able to form colonies reliably. Despite this, Steel (1983) has demonstrated a close correlation between clonogenic cell survival and SGD,

2.9 Validity of Human Xenografts as a chemosensitivity assay

The value of therapeutic studies on human tumour xenografts ultimately depends upon a close correlation between experimental and clinical results. The model must either reflect the known clinical sensitivity of the human disease or, more specifically, it should demonstrate sensitivity similar to that of the original donor tumour.

2.9(a) Broad characteristics of xenograft responses

It is now generally accepted that chemoresponsive human tumours give rise to xenografts which are relatively sensitive to standard chemotherapy. This data is well summarised by Steel (1983) who

studied both sensitive (teratoma, SCLC) and resistant tumours (breast, non-SCLC, colon and melanoma). He showed a close correlation between the average SGD in xenografts for drugs commonly used in these diseases and the complete response rates reported from large clinical studies.

(b) Direct comparison of responses with donor patients

These studies are difficult to perform because of the low success rate in establishing xenografts and the problems of obtaining an objectively measurable clinical response. Nowak (1978) studying colorectal cancers and Selby (1980) using melanomas both showed that the xenografts established from patients who subsequently showed some clinical response were more chemosensitive when compared to other tumours of the same histological type.

The largest study to compare directly the response to treatment in xenografts and donor patients was performed with bronchial carcinomas by Shorthouse (1980a, 1980b). In 16 direct comparisons (7 SCLC, 3 squamous, 5 adeno, 1 large cell) the clinical responsiveness of 5 small cell tumours was reflected in highly responsive xenografts. Two SCLC patients failed to respond clinically and their xenografts were similarly resistant. All donor patients with NSCLC failed to respond to treatment with the xenografts obtained from their tumours also being resistant.

These encouraging results help to validate the human tumour xenograft system as a reliable method of assessing chemotherapeutic agents. It now seems likely that any major advance in the chemotherapy of solid cancers will utilise this system for drug testing.

CHAPTER 3

INTERFERONS AND INTERFERON/DRUG COMBINATIONS AS ANTI-CANCER THERAPIES

3.1 Introduction

In 1957 Isaacs and Lindenmann discovered that living cells which were infected with a virus produced a factor which rendered them resistant to further infection. They showed that the immunity conferred by the release of this factor in response to the first "interfering" virus could also protect the cell from viruses of different antigenic and biological type. They called this factor Interferon (IFN). Over the next 20 years the biology and biochemistry of IFN was steadily unravelled.

Tyrrell (1959) showed that IFN made in calf kidney cells was inactive in chick cells and vice versa and it became clear that more than one IFN existed. Further work has shown that a whole family of IFNs exist depending on their cell of origin. They are complex proteins and glycoproteins, whose production can be induced by a wide range of stimuli and they are capable of producing profound effects on many aspects of cellular function (Burke 1985).

Three distinct antigenic types of IFNs are recognised:

(a) Alpha interferon (IFN-α) is induced by a variety of stimuli in many cells, the most common source being leucocytes or lymphoblastoid cell lines.

(b) Beta interferon (IFN-β) is produced by fibroblasts in response to viruses or synthetic polyribonucleotides.

(c) Gamma or 'immune' interferon (IFN-γ) is an exclusive product of T lymphocytes induced by stimulation with T cell mitogens or antigens.

IFN-α and β are stable in acid environments whereas treatment of IFN-γ at pH2 neutralises its activity. Only single genes have found coding for human IFNs β or γ. In contract up to twenty different genes coding for IFN-α subtypes have been defined and variations in biological activity between these subtypes have been described. IFN-α preparations produced by genetical engineering contain products of single IFN-α genes whereas those induced in human cells contain multiple IFN-α gene products.

The nomenclature of IFNs (especially alpha IFN) is complex although attempts have been made to standardise terminology (Stewart 1980, Daniels 1985). A prefix is used to designate the species of origin (e.g. Hu for human, MU for murine), IFN is the accepted abbreviation for interferon, followed by the designation α, β or γ to indicate the antigenic type. This is then followed by a numeric or letter designation to denote a specific subtype (e.g. IFN-α_2, IFN-α A). IFNs from particular cellular sources are similarly identified (e.g. alpha IFN from leucocytes, IFN-α[Le], alpha IFN from lymphoblastoid cell lines, IFN-α[Ly]). Recombinant DNA-derived IFNs are generally preceded by the prefix 'r' (e.g. rHuIFN-α_2).

Proposals for renaming the three most commonly used subtypes of IFN-α have recently been suggested (Daniels 1985):

(i) Interferon alpha-2a (IFN-α2a) for rIFN-αA(Roferon-A,Roche)

(ii) Interferon alpha-2b (IFN-α2b) for rIFN-α_2(Intron-A,Schering)

(iii) Interferon alpha-N1 (IFN-αN1) for HuIFN-α(Ly)(Wellferon, Burroughs-Wellcome).

Although differences exist, all classes of IFN have been shown to possess antiviral, immunoundulatory and antiproliferative activities. These observations led to much interest in the use of IFN in patients with cancer. Initial studies used crude preparations of IFN-α produced from human buffycoat leucocytes stimulated with Sendai viruses (Strander 1973). By 1981 the molecular cloning of human IFNs in E.coli permitted the large scale production of highly purified single molecular species of various IFN-αs as well as IFN-β and γ. This has allowed extensive evaluation of IFNs as anti-cancer agents.

3.2 Interferons as anti-cancer agents

3.2(a) Rationale for use

Although originally described as anti-viral proteins it soon became apparent that IFNs were capable of exerting profound effects on many aspects of cellular function. They have been referred to as "inducible inducers" (Krown 1986) since their production can be induced by a wide variety of agents (viruses, antigens, mitogens,

tumours etc) and their application to cells induces, in turn, the production of a wide range of biologically active substances. The rationale for using IFNs as anti-cancer agents is based on the following observations:

(i) Cell division in normal and malignant cells is slowed by exposure to IFNs (Taylor-Papadimitriou 1980, Balkwill 1977, 1978).

(ii) IFNs (especially IFN-γ) can amplify effects on immune reactions through stimulation of the different cell surface antigens involved in cellular interactions (Basham 1983, De Maeyer-Guignard 1985).

(iii) Maturation of cytolytic macrophages is stimulated by IFNs. This is especially true of IFN-γ which is also known as macrophage activating factor (Schultz 1978, Svedersky 1984).

(iv) IFNs enhance the activity of cytotoxic natural killer (NK) cells (Trinchieri 1978, Perussia 1980, Strander 1986).

(v) IFNs have been shown to have important modulatory effects on various aspects of cell differentiation in many tumour model systems (Rossi 1985).

(vi) The amplification of certain oncogenes can be suppressed by IFNs (Samid 1984, Jonak 1984).

(vii) The anti-viral properties of IFN may influence the growth of virus associated neoplasia.

(viii) Various IFNs have shown activity as single agents in laboratory models of human cancer (e.g. Bradley 1981, Borden 1982, Strander 1977, Balkwill 1977, 1978, 1982, Aapro 1983, Heston 1984, Hirabayashi 1985).

3.2(b) Phase I studies

All compounds which have potential use as anti-cancer agents must undergo extensive phase I studies before their clinical activity can be accurately assessed. The purpose of these studies is to define the toxicity and maximally tolerated dose (MTD) and perhaps to give an early indication of anti-tumour efficacy.

Extensive phase I studies with both "natural" and recombinant IFNs have now been performed in many patients (Strander 1986). Toxicity is primarily 'flu-like e.g. fever, chills, fatigue, myalgia and headache with some patients experiencing nausea and vomiting. These side effects are dose-related and rapidly reversible (Spiegel 1985). Chronic studies have demonstrated that these 'constitutional' side effects are non-cumulative and often improve with time. Early clinical studies have often ascribed these side effects to contaminants in impure IFN preparations. However, recent studies using IFN preparations purified to homogeneity have shown similar toxic effects (Tyrrell 1985, Krown 1986).

Other side effects such as renal, hepatic, haematological and cardiac toxicity, and CNS effects, although seen relatively infrequently may be severe. IFN induced depression of hepatic cytochrome P-450 activity has not been of major clinical importance so far. The ability of this enzyme system to detoxify or activate other drugs may prove more important as combinations of IFNs with other agents are assessed more intensively.

In general the MTD for alpha IFN has been highest for recombinant DNA produced IFNs compared with HuIFN-α (Ly) or HuIFN-α (Le). This may be related to the presence of multiple IFN subtypes or non-IFN-proteins in the latter two preparations. Differences in MTD have also been seen for different routes of administration (i.v. bolus, i.v. infusion, I.M. or S.C.), for different dosing schedules (e.g. daily -v- intermittent treatment) and when different criteria for dose-limiting toxicity were used. In a typical phase I study involving rHuIFN- 2b (Spiegel 1985) the MTDs were as follows: single doses up to 200 x 10^6IV/m^2 i.v. could be given, although on a daily dosing schedule 50 x 10^6IV/m^2 i.v. for 5 days was the MTD. For continuous dosing, 15 x 10^6IV/m^2 s.c. 3 x/week was the MTD.

Anticancer agents in general are thought to act by direct cytotoxicity to tumour cells and it is therefore assumed that the largest safely tolerated dose (the MTD) will also be the dose most likely to result in the greatest tumour cell destruction. This may not be true for IFNs and other so called "biological response modifiers" (BRMs) which may not exert their anti-cancer effect by a direct action but by modifying host responses. Clearly novel approaches are required when designing Phase I studies of IFNs and other BRMs since the MTD in terms of subjective and/or objective side effects may far exceed the dose required to influence a particular cell function. Indeed certain cell functions may actually be impaired if too low or too high a dose is administered.

Unfortunately it is unclear which, if any, of the many measurable effector cell functions influenced by IFN treatment are

directly related to tumour control.

To illustrate this problem many workers have assessed the effect of prolonged IFN administration on NK cell activity in cancer patients. No clear dose-response relationship has been demonstrated (Krown 1986) and indeed many reports have shown decreased NK cell cytotoxicity at higher doses (Maluish 1982, Edwards 1984). Most studies have also demonstrated marked fluctuations in activity with time (Golub 1982, Ernstoff 1982).

Therefore at present it is clearly impossible to define an 'optimal' dose level for IFNs from the currently reported phase I studies. Most laboratory studies suggest that sustained exposure to IFNs might be optimal to obtain maximum effects but until the exact mechanisms by which IFNs exert their anti-tumour effect can be elucidated, the scheduling of IFN therapy in patients will be based largely on guesswork.

3.2(c) Phase II studies

Various IFN preparations have now been administered to thousands of patients in phase II clinical studies. The activity of IFN-α in these studies is summarised in Table 3.1. Little activity has been seen in most adult solid tumours although it should be noted that most of these trials have been conducted in patients with advanced disease and may reflect inactivity against large, bulky tumours rather than particular tumour types.

Activity has been demonstrated in a variety of haemotological malignancies including multiple myelomas (Wagstaff 1985),

Active	Hairy-cell leukaemia
	Non-Hodgkin's lymphoma
	Renal cell carcinoma
	Kaposi's sarcoma
	Mycosis fungoides
	Superficial bladder cancer
	Chronic myelocytic leukaemia
	Multiple myeloma
Inactive	Colorectal carcinoma
	Non-small cell lung cancer
	Prostatic carcinoma
Conflicting or inadequate results (further studies required)	
	Breast cancer
	Osteogenic sarcoma
	Hodgkin's disease
	Malignant melanoma
	Small cell lung cancer
	Acute leukaemias
	Chronic lymphocytic leukaemia
	Ovarian carcinoma

Table 3.1: Summary of IFN-α activity in published phase II studies

non-Hodgkin's lymphoma (Leavitt 1985) and hairy-cell leukaemia where high response rates have been seen and IFN-α is now the treatment of choice (Quesada 1984).

In some of these studies, responses occurred after 4-6 months of treatment, so prolonged therapy may be beneficial in some cases. As stated above, optimal dosages have yet to be determined. However in Kaposi's sarcoma (Real 1984), renal cancer (Quesada 1983) and mycosis fungoides (Bunn 1984) a clear relationship was established between clinical response and IFN dose with low doses being relatively ineffective.

3.2(d) IFN and lung cancer

Clinical phase II studies of IFN in NSCLC have been negative. The results of the five published trials are summarised in Table 3.2. Both high and low dose regimes (all given by i.m. injection) using various dosing schedules failed to produce significant responses. Both natural human leucocyte and recombinant IFN-α were tested.

The reported series of IFN therapy in SCLC (summarised in Table 3.3) also shows few reports of disease response. Of the five studies, two failed to comment on response rates, with the largest series (Jones 1983) comprising only ten patients. High dose regimes (up to 200×10^6 i.u by i.v. infusion) were associated with significant toxicity with few patients tolerating the initial 'loading' doses. A larger study is required to ascertain the true activity of IFN in SCLC. However, the response rates reported in the few available patients would suggest that IFN therapy is inferior to standard chemotherapy in this chemosensitive disease.

Patients	Histology	IFN	Dosing Schedule	Response	Reference
11	9 adeno 2 squam	HuIFN-α(Le)	$3x10^6$i.u.,i.m daily for 30 days	None	Stoopler 1980
38	20 adeno 13 squam 5 large	HuIFN-α(Le)	$3x10^6$i.u.,i.m.x5/week	1P.R 5 stable	Figlin 1983
8	2 adeno 3 squam 2 large 1 alveolar	rIFN-αA	$20x10^6$i.u./m² i.m. daily for 12 weeks (no patient would tolerate this)	None	Leavitt 1984
15 (11 evaluable)	9 adeno 4 squam 1 adeno/squam	rIFN-αA	$50x10^6$i.u./m² i.m. x 3/week (with de-escalation to 50% then 10%)	None	Grumberg 1984
17	Squam SCLC	rIFN-α	$50x10^6$i.u./m²,i.m.x3/week	1P.R	Ernst 1984

Table 3.2: Clinical studies of IFN in NSCLC

Patients	IFN	Dosing Schedule	Response	Reference	Comments
8	HuIFN-α(Le)	100-200x10^6 i.u.,i.v. infusion daily for 5 days 6x10^6 i.u.,i.m. 3x week	Not reported	Mattson 1983	Untreated patients, severe CNS toxicity with infusions
10	HuIFN-α(Ly)	50-100x10^6 i.u./m^2 i.v. infusion daily for 5 days 3x10^6 i.u./m^2 i.m.x3/week for 1/12	None	Jones 1983	Untreated patients
3	rIFN-α_2	50x10^6 i.u./m^2 i.v. infusion daily for 5 days course repeated every 9-14 days	None	Jackson 1983	Small study (2 pts had only 1 course) severe toxicity
9	HuIFN-α(Le)	3x10^6 i.u.,i.m.daily for 1/12 6x10^6 i.u.,i.m.x3/week for 5/12	Not reported ?better survival than untreated	Mattson 1984	All patients had not responded to previous chemotherapy
4	HuIFN-α(Le)	3x10^6 i.u.,i.m.x 3/week	1P.R.	Strander (unpublished) in Strander 1984	Combined with radiotherapy

Table 3.3: clinical studies of IFN in SCLC

One study (Strander unpublished) combined IFN with radiation in 4 SCLC patients without much success. There have been no reports of studies combining IFN with chemotherapy in either SCLC or NSCLC.

3.3 IFN/cytotoxic drug combinations

The complexity of the anti-tumour actions of IFNs and their apparent failure as single agent treatment in most solid cancers has led to interest in developing their potential as adjuvants. It now seems likely that the most effective uses of IFN in cancer will come in combination with other treatment modalities (Borden 1986). The interactions between IFN and cytotoxic drugs have been assessed in laboratory models of human cancer and reports of clinical studies of these combinations are now appearing.

3.3(a) Pre-clinical studies

IFN/drug interactions have been extensively studied using in vitro systems. The results of these assays are summarised in Table 3.4.

Positive or synergistic interactions between various different IFNs and several different classes of anti-cancer agents have been described in various tumour types using many in vitro assay systems. The most striking synergy has been demonstrated with low doses of IFNs (Morris 1984, Welander 1985) which have little activity as a single agent but are able to augment the effectiveness of certain cytotoxic drugs.

Table 3.4: IFN/drug interaction using in-vitro systems

Reference	IFN	DRUG(S)	TUMOUR(S)	SYSTEM	RESULTS
Jelander 1985	rIFN-α_2	CDDP, CY + 6 others	Various esp. ovarian	Clonogenic Assay	Synergy and additive
Morris 1984	rIFN-α_2	ADR	Burkitt's lymphoma	Cell survival	Synergy (IFN low doses)
Aapro 1983	rIFN-α_2	Vinca alkaloids + 4 others	Myeloma, Breast, Colon, lymphomas	Clonogenic Assay	Synergy (lymphomas)
Kyle 1985	rIFN-αA	Nitrogen mustard 5-FU	Various (Colon, lymphomas)	Cell survival	Synergy (lymphomas)
Koehler 1985	rIFN-β	Vinblastine	Renal carcinomas	Growth inhibition	? Additive
Bregman 1985	rIFN-αA	DFMO Dexamethasone Retinoic Acid	Melanomas	Growth inhibition (in agar)	? Additive
Takahashi 1984	HuIFN-α(Ly)	various (7)	Lymphomas	Cell survival	Synergy
Bepler 1986	rIFN-αA	DFMO	SCLC cell lines	Clonogenic Assay	additive only
Ho 1985	IFN-β	Retinoic Acid	U937 lymphoma	cell survival	Synergy
Durie 1986	rIFN-α2b	CY melphalam	Melanoma	Clonogenic Assay	Synergy

Potentiation of chemotherapy by IFN using an in vivo model was first demonstrated in 1973 by Chirigos who showed that murine IFN could increase the cure rate of the LSTRA murine leukaemia by BCNU from 25% to 72% despite being ineffective as a single agent. The results of this and four other studies assessing the interactions between murine IFN and cytotoxic agents in murine tumours are summarised in Table 3.5. In 1984, two reports appeared of HuIFN/drug combinations in human tumour xenografts. Heston and colleagues treated a human renal adenocarcinoma with rHuIFN-α2b and α-difluoromethylormithine (DFMO) as single agents and in combination in an in vitro clonogenic assay and in vivo as xenografts in nude mice. Synergy was demonstrated in vitro using low dose IFN with additive effects being shown in vivo. The authors commented that the mechanism by which the drugs interacted to inhibit tumour growth was unclear but appeared to be a direct effect on tumour proliferation, perhaps related to modulation of polyamine metabolism.

Balkwill and Moodie at the ICRF (1984) showed that human lymphoblastoid IFN strongly increased the activity of sub optimal doses of cyclophosphamide and adriamycin against a human breast cancer xenograft grown in nude mice. Further studies confirmed that the activity of the combination of IFN and drugs was greatest when the two agents were administered simultaneously rather than sequentially.

3.3(b) Clinical studies

The success achieved in combining IFNs and cytotoxic drugs in tumour model systems stimulated many centres to launch pilot studies

Reference	IFN	Drug(s)	Tumour	Effect
Murine Tumours				
Chirigos 1973	Mu	BCNU	Murine leukaemia	synergy
Gresser 1978	Mu	CY	AKR lymphoma	additive
Tozawa 1982	Mu	CY	C1300 Neuroblastoma	? Synergy
Slater 1981	Mu	Various	L1210 leukaemia	Synergy with anti-metabolites
Sunkara 1983	Mu	DFMO	B16 melanoma	? additive
Human Tumours				
Heston	rHuIFN-α_2	DFMO	renal carcinoma in nude mice	additive
Balkwill 1984	HuIFN-α(Ly)	CY,ADR	breast carcinoma in nude mice	Synergy

Table 3.5: IFN/drug interactions using animal models

to assess the clinical activity of such combinations. At the present time at least 20 clinical trials of cytotoxic drug plus IFN combinations are currently ongoing (Green 1985). Few are large enough or have been reported in enough detail to conclusively support or refute the concept that interferons act synergistically with any cytotoxic agent.

Of the studies reported, only one has suggested that IFN enhances the toxicity of anti-cancer drugs (Lonberg 1985). However, few have reported increased activity. A major drawback to comparing studies is the fact that many different doses and schedules of IFN therapy are being tried varying from high dose, i.v. infusions at weekly intervals (Welander 1985a) to low dose, s.c. injections on alternate days (Ceto 1986).

Clearly the optimal scheduling of IFN/drug combinations in patients cannot be rationalised until more is understood about the exact mechanism by which IFNs may potentiate chemotherapy. Hopefully the answers to this problem will be obtained from carefully designed experiments using laboratory models of human cancer.

3.3(c) Possible mechanisms of IFN/drug synergy

Many of the laboratory studies of IFN/drug combinations have proved conclusively that the increased activity seen is not due to a simple additive effect but represents true synergy between the two agents (Balkwill 1984, Welander 1985). Few studies have attempted to explain the exact mechanisms involved.

Experiments using murine IFN in combination with drugs against murine tumour models may be misleading as it is possible that some

tumour inhibition may be due to the effects of Mu/IFN on host tissues. Several reports have appeared in the literature showing that MuIFN and IFN inducers can suppress hepatic cytochrome P-450 mono-oxygenase activity and thereby reduce drug metabolism (Renton 1976, Singh 1982). MuIFN has also been shown to enhance NK cell activity in the mouse (Djeu 1979). Both of these effects may influence tumour growth.

Since IFNs are species specific (Tyrrell 1959), by testing human IFNs against human tumours in a murine host it is possible to dissociate the direct and indirect anti-tumour actions of IFN. HuIFN has been shown to have no effect on the hepatic drug metabolising enzymes of the mouse (Balkwill 1984a) or murine NK cell activity (Balkwill 1982). Therefore the fact that HuIFN has been shown to potentiate the effect of drugs on human xenografts in nude mice (Balkwill 1984, Heston 1984) would suggest that HuIFN is exerting a direct effect on the tumour rather than the host. Also synergy has been well demonstrated in vitro (Table 3.4) although the precise mechanisms involved in these interactions have not been elucidated.

One study (Balkwill 1984a) has suggested that the augmentation of drug activity by IFN may be produced by changes in tumour cell cycle distribution. Treatment with HuIFN/CY combinations increased the number of cells in S phase which may have accounted for the synergy seen. These data have not yet been corroborated by others and would not explain why synergy has been seen between IFN and drugs whose activity is independent of cell cycle distribution (e.g. CDDP).

Further studies to investigate how IFNs interact with cytoxic agents are urgently required. Information gained from these experiments may facilitate the rational use of these promising IFN/drug combinations in patients with cancer.

AIMS OF THE STUDY

The main aim of this study was to investigate the activity of interferon/drug combinations in human lung cancer. The majority of the experimental work was performed on a series of human bronchial carcinoma xenografts established in immune deprived mice. Using this model the effectiveness of combining recombinant human alpha-2 IFN with a variety of cytotoxic agents was investigated in both SCLC and NSCLC tumours. Different doses and schedules of each component were assessed in order to determine the optimum method of administering these IFN/drug combinations.

Similar experiments were performed in vitro by establishing certain xenografts in continuous culture and using lung cancer cell lines developed in other centres. Preliminary studies to elucidate the precise mechanism by which IFNs can enhance cytoxic agents were also performed.

The results from the laboratory experiments were used to design a pilot study to assess the activity of human IFN and cis-platinum in combination in a group of patients with NSCLC.

SECTION II - XENOGRAFT STUDIES

This section deals with the assessment of the effectiveness of combining IFN with cytotoxic agents in a human tumour xenograft system. The activity of combinations of human alpha IFN and various drugs was compared to single agent treatment in a panel of human bronchial carcinomas maintained in immune deprived mice. Both NSCLC and SCLC xenografts were used for testing. Attempts were made to define the optimum dosage schedule for combination treatment in this model in the hope of designing an effective schedule for clinical testing.

CHAPTER 4: Materials and methods

4.1 Source of tumours

Specimens of human bronchial carcinoma tissue were obtained from three separate sources: (a) from surgically resected specimens from patients undergoing thoracotomy at the City Hospital, Edinburgh (b) from patients from the Northern General Hospital, Edinburgh undergoing rigid bronchoscopy and biopsy for suspected tumours and (c) from patients attending the Medical Oncology Unit at the Western General Hospital, Edinburgh for chemotherapy who had accessible subcutaneous metastases from small cell tumours. These lesions were biopsied under local anaesthetic. Xenografts implanted from these three sources were designated CX, NX and WX respectively and then numbered consecutively.

Resected specimens were collected directly from the operating theatre. Tumours were immediately stored on ice and then taken without delay directly to the University Department of Pathology. Sections of tumour tissue were taken for transplantation from non-necrotic areas of the specimen and placed immediately into cooled Roswell Park Memorial Institute medium (RPMI). Adjacent tissue was fixed in formal saline prior to histological staining to confirm malignancy.

Bronchoscopic biopsies and resected subcutaneous deposits were obtained directly from the procedure room and divided into two parts, one being placed in RPMI medium as above and the other being fixed

in formal saline and sent for pathological examination. Specimens were transported to the animal unit without delay, with the majority of samples being implanted within four hours.

Specimens were prepared for transplantation in the animal unit using sterile techniques under a laminar flow hood. Biopsies were placed in cooled RPMI medium in a sterile Petri dish. All stromal and necrotic material was removed and the remaining viable tumour tissue was dissected using cross-scalpels into small cubes (approximately 5-8cm^3) ready for implantation.

4.2 Preparation of immune-deprived animals

The majority of these experiments were performed using male CBA/lac mice bred in the animal unit at the Western General Hospital. In a few of the early studies and to maintain xenografts as stock, some female mice were used. On the few occasions when the health of the CBA breeding colony at the animal unit was poor, batches of newly weaned male CBA mice were purchased from Bantin and Kingman Ltd, Hull and used in complete experiments. The immunosuppressive regime used in these studies is outlined in Figure 4.1 and was developed by Steel (1978).

4.3 Anaesthesia

In the early part of the study, all surgical procedures were performed on animals anaesthetised with inhaled ether. Unfortunately

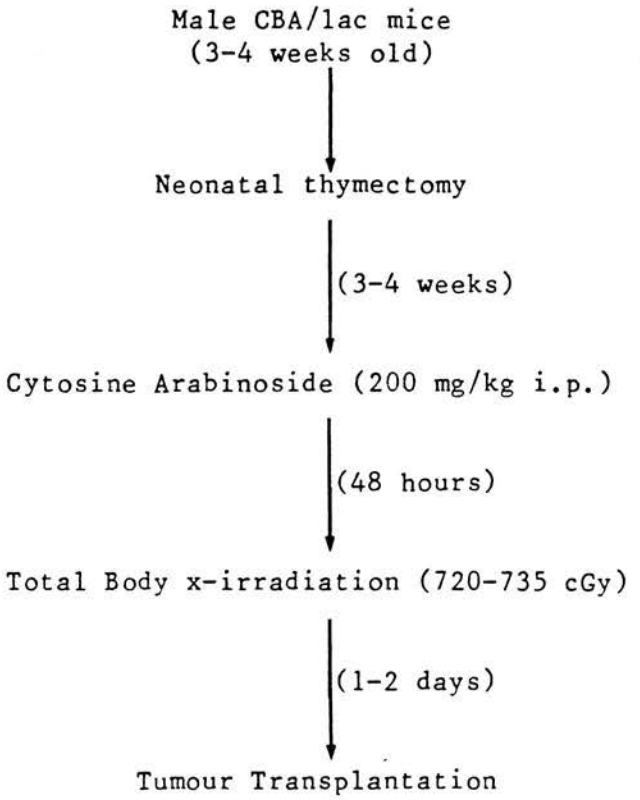


Figure 4.1 Immunosuppresive regime for xenograft studies

this method provided only a short period of deep anaesthesia (< 2 mins), was irritant to the animals' eyes, unpleasant for the operator and was impractical to use without assistance.

A change was therefore made to injectable agents using a combination of midazolam ("Hypnovel", Roche, Welwyn Garden City, Herts) 2 mg per ml and fentanyl/fluanisone ("Hypnorm", Janssen, Wantage, Oxon) 0.315mg per ml/10mg per ml. A single i.p. injection of 0.1 ml of a solution of 2 parts midazolam, 1 part hypnorm and 18 parts of water resulted in surgical anaesthesia after 5-10 minutes lasting 10-15 minutes with full recovery in 40-60 minutes in an adult mouse. This dose was reduced to 0.05-0.07 mls for neonatal animals undergoing thymectomy. Excessive respiratory depression could be reversed with naloxone (Du Pont, Stevenage, Herts) 0.1ml/kg i.p.

4.4 Thymectomy

Retrosternal thymectomy was performed on newly weaned (21-28 days old) mice. Animals were anaesthetised as outlined above and then laid in the supine position, each limb being secured by means of a padded bulldog clip and elastic band pinned into a cork board. The neck was extended using a paper clip and elastic band, and a cotton wool bolster was placed between the scapulae. The skin of the neck was cleaned with methylated spirit and a 0.5cm vertical skin incision was made with scissors to expose the strap muscles of the neck. Both salivary glands were displaced with forceps to expose the upper sternum. Access to the thoracic cavity was made through a short midline cut in the upper sternum and blunt dissection with the tips

of the scissor to divide the origins of the strap muscles.

Both lobes of the thymus were then aspirated using suction apparatus, the salivary glands apposed and the skin closed by means of a surgical clip (autoclip). The whole procedure could be performed in 2-3 minutes enabling 60-80 thymectomies to be completed at one session. The mortality rate for the procedure in experienced hands was approximately 5% with many deaths attributable to the anaesthetic.

4.5 Irradiation

3-4 weeks after thymectomy the mice were given a single i.p. injection of cytosine arabinoside (Upjohn, Crawley, W. Sussex) to protect the gastro-intestinal tract and bone marrow from the lethal effects of irradiation (Millar 1978). Two days later, they were irradiated using 250 kVp x-rays with Thoraeus Ii filtration. During irradiation the mice were placed in a 15cm diameter, 2.5cm high perspex jig. Ten mice could be accommodated at any one time and they were free to move about the jig during the period of irradiation. The jig was positioned in the centre of a 20x20 cm irradiation field at a focus-jig distance of 62cm. The mean radiation dose rate was 37cGy/min $\pm$ 2%.

In order to eliminate variations in dose due to varying dose rate, synchronous dosimetry was performed during all animal irradiation. A previous study reported by Hay (1985) using this equipment showed that the mean dose in the jig was 94.5% of that measured by the ionisation chamber (95% confidence limits $\pm$ 0.98%).

The dosimeter was calibrated for room temperature and atmospheric pressure for each run and ensured accurate x-ray dosing.

Previous studies using irradiated mice as hosts for human tumours (Davies 1969, Steel 1978, Kovnat 1982) have given doses of 850-1000 cGy using either ^{60}Co or ^{137}Cs sources. The Relative Biological Effectiveness (RBE) value of ^{60}Co γ-rays when compared to 250kVp x-rays for the whole body irradiation of mice is 0.8 (Green 1962). Therefore the dose of x-rays used in these studies (720-735cGy) was comparable with these earlier reports. Hay (1985) showed that the optimum x-ray dose with this system to ensure high tumour take rates and acceptable survival was 735cGy.

Following irradiation, animals were commenced on antibiotic therapy with oxytetracycline (Terramycin, Pfizer Ltd, Sandwich, Kent) 100mg and neomycin (Neobiotic, Upjohn, Crawley, W Sussex) 100mg added to each litre of drinking water for 14 days to prevent septicaemia from commensal gut flora. During this time animals were fed standard diet softened by soaking in acidified water to aid nutrition.

4.6 Tumour implantation

Tumour fragments were implanted the day following irradiation. When this procedure is delayed until 7-14 days it is associated with an increased mortality (Carmichael 1986). Animals were anaesthetised and secured on their ventral surface. The skin was cleaned with methylated spirit and a 1cm transverse incision made with scissors in the mid-dorsal region. Subcutaneous tracks were fashioned with blunt forceps along the lateral aspects of both flanks. Tumour fragments

(approximately $8mm^3$) were implanted subcutaneously near the axillae. The skin was closed with a surgical clip (autoclip) and the mouse identified using numbered ear tags (Hauptner).

4.7 Animal husbandry

After thymectomy mice were kept in a separate room reserved for immune suppressed animals. The room temperature was controlled by thermostat with 12 hours of light each day. Air was filtered when drawn into the building but specific pathogen free conditions were not required. Staff entering the room were kept to a minimum and wore gowns and overshoes.

The mice received a standard laboratory diet (CRM expanded, Labsure, Poole, Dorset), their drinking water being acidified to reduce the risk of pseudomonas infection. Standard animal boxes and bedding were used, both of which were autoclaved before use.

4.8 Tumour Passage

When tumour xenografts attained a volume of 0.5-1 cm^3 they were considered suitable for transplantation to a subsequent generation. The host animals were sacrificed by cervical dislocation and the tumours removed aseptically. All necrotic tissue was removed and small fragments (approximately 6-$8mm^3$) were produced using cross-scalpels. These fragments were kept in a petri dish of RPMI medium prior to subsequent implantation into freshly prepared animals. All procedures were carried out in a laminar flow hood.

At each passage, sections of tumour were taken for histological analysis and karyotyping was performed at regular intervals.

4.9 Storage of tumours

Wherever possible, tumour samples from the original specimen and early passages were stored in liquid nitrogen. Tumour tissue was dissected as previously described into small cubes. Ten cubes were placed into a plastic freezing vial containing 1ml of 10% dimethyl sulphoxide in foetal calf serum. The vials were placed in a $-40^{o}c$ freezer overnight prior to transfer into liquid nitrogen.

When specimens were required for implantation they were removed from the liquid nitrogen and rapidly thawed. The tumour fragments were aspirated with a pastette and washed repeatedly in RPMI medium at room temperature. Implantation was then carried out with minimal delay.

4.10 Histopathology

Sections of original tumour tissue and specimens from xenografts at each passage were assessed using standard histological techniques. Samples were fixed in formal saline, embedded in paraffin wax and sectioned at 4.4um. Sections were placed on microscopic slides, the paraffin removed by treatment with xylene which was further removed with ethanol. All specimens were stained with Ham's haematoxylin and Putt's eosin in the standard manner. Adenocarcinoma xenografts were stained with periodic-acid schiff (PAS) to assess mucin production.

This technical work was performed by the department of Pathology, Western General Hospital.

All slides were examined by Dr M McIntyre, Consultant Pathologist, Western General Hospital who made a histological diagnosis on the original tumour specimens and compared sections taken at subsequent passage for changes in the level of differentiation, amount of inflammatory infiltrate, mucin production and the presence of stromal tissue.

4.11 Chromosome analysis

The maintenance of a human karyotype by xenografts was checked by making chromosome preparations using the direct method described by Simoni (1983). Xenografts were removed using sterile techniques and placed in RPMI medium containing 5% foetal calf serum and glutamine. Non viable tumour tissue was removed by dissection and the remaining tumour was transferred to a fresh petri dish. The specimen was then chopped finely using dissecting scissors and scalpels until fragments of explant size were left. These pieces were then incubated in Colcemid (Gibco) at a final concentration of 0.04mg/ml in 10mls RPMI for 2 hours at 37°C. After centrifuging at 1700 rpm for 5 mins the medium was removed and replaced with 10mls 1% sodium citrate. After incubating at 37°C for 20 minutes the hypotonic solution was removed and 10mls of 3:1 methanol:acetic acid fixative was added. The preparation was left overnight in a -20°C freezer. The following day the fixative was removed by centrifrugation and replaced with 2-3mls of fresh 3:1 fixative. The xenograft pieces

were then removed and placed on a warm (40°C) clean slide. The fixative was then blotted dry with filter paper. A few drops of 60% acetic acid were added to the xenograft material and left for one minute. The xenograft pieces were then pulled slowly up and down the slide with the bent tip of a pasteur pipette until the acid had evaporated. Slides were then checked for mitoses and the best preparations were stained in 5% Giesma in Gurrs Buffer (pH 6.8) for 10 minutes. Human morphology of the chromosomes was then confirmed by direct microscopy.

4.12 Tumour measurement

Following tumour implantation, the animals were left undisturbed until a lump could be easily palpated or was clearly visible in the flank. The hair was then shaved over the xenografts and measurements made using calipers. Tumour volume was estimated assuming an ellipsoid shape. Any xenografts of awkward shape were not used in drug testing experiments. The longest and shortest perpendicular diameters were measured three times and the mean taken as the final measurement. Tumour volume was calculated using the equation:

$$\text{volume} = \frac{\pi}{6} \times D \times d^2$$

(D = longest diameter, d = shortest diameter)

The accuracy of these measurements was assessed by correlating the estimated volume using calipers with the weight of the excised tumour. This was performed for a range of tumour sizes and different histological types.

In general, tumours were measured three times per week and the time taken for the tumours to double from a specific starting volume (usually between 0.25 and 0.75cm^3) was estimated to determine the growth rate. The take rate for each tumour type was defined as the proportion of implants that progressively grew to a palpable size and was usually expressed as a percentage.

4.13 Assessment of chemotherapeutic agents

The majority of the work of this study involved assessing the activity of cytotoxic drugs administered as a single agent and in combination with IFN in the human lung tumour xenograft model.

For these experiments a group of animals of the same sex underwent thymectomy as one batch, the number of animals chosen depending on the take rate of the tumour being used. These animals were then irradiated at one session and tumour fragments were implanted the following day. When sufficient tumours had attained a volume of 0.25 - 0.75cm^3 they were divided into groups stratified for tumour size. Attempts were made to keep the number of tumours in each group at a reaonable level (8-10), groups with fewer than 6 tumours were considered unsuitable for drug testing.

In a hypothetical experiment where three treatment regimes were assessed in a tumour with a take rate of 70% (e.g. NX002), 50 animals would undergo thymectomy. Two would die post operatively, leaving 48 to undergo irradiation and transplantation with bilateral implants. A further 5 may be lost through irradiation toxicity and intercurrent infection, leaving 43 animals. With a take rate of 70%, this

represents 60 tumours. Of these, 15 would show insufficient growth and 5 would be of unsuitable shape, leaving 40 measureable tumours of useable size. These would be divided for assessment into four groups of ten tumours, a control group and three treatment groups, the allocation of groups to a specific treatment being randomised.

Chemotherapy was then administered often based on the maximum tolerated dose on a milligram per kilogram basis. All cytotoxic agents were dissolved in water for injection or normal saline and injected intraperitoneally except for the nitrosurea TCNU which was dissolved in polyetheylene glycol (PEG 400) and given orally by a gavage needle. All agents were prepared in a final concentration to allow their administration in a volume of 0.1ml per 10 grams body weight. Control animals were given injections of saline in an identical schedule to treated animals.

Caliper measurements were then continued three times weekly until the end point of the experiment which was usually when all tumours had doubled in volume or completely regressed. Wherever possible, the activity of one agent or combination of agents was expressed in terms of the specific growth delay (SGD). This figure was derived from the median doubling times (TD) of treated and control groups using the equation:

$$SGD = \frac{TD\ (treated) - TD\ (control)}{TD\ (control)}$$

The SGD represents the number of doubling times prolonged by treatment and allows comparison between tumours of different growth rates. A hypothetical experiment illustrating the use of SGD in shown in Fig 4.2.

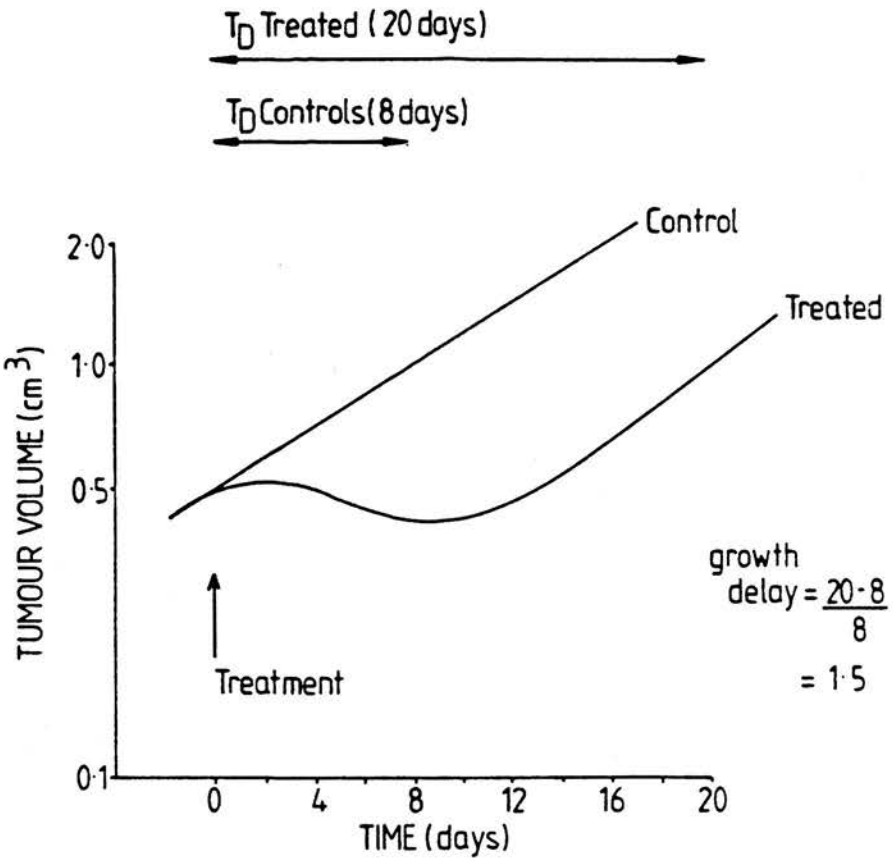


Fig 4.2: Hypothetical chemotherapeutic experiment showing calculation of the SGD

4.14 Statistical analysis

Expression of drug activity in terms of SGD allowed for comparison between tumours of differing growth rates but did not allow statistical comparisons between different agents and combinations of agents. Comparison of doubling times for groups of tumours receiving different treatments was difficult since the data was not normally distributed and non parametric tests were required. Also such tests could not include data from tumours which regressed completely or remained static in size.

Expert statistical advice was obtained and it was decided to compare the efficacy of treatments by calculating the slope of the growth curve for each tumour from the knowledge of its doubling time (or halving time in regressing tumours). This slope (D) was calculated as:

$$D = \frac{\log_e \text{size}}{\text{TD (days)}}$$

(size = 2 for doubling tumours, 1 for static and 0.5 for regressing tumours). For example, for a tumour doubling in 7 days:

$$D = \frac{\log_e 2}{7} = 0.1$$

A regressing tumour which halved in size in 25 days:

$$D = \frac{\log_e 0.5}{25} = -0.03$$

Static tumours would obviously have a slope of zero.

The mean slope for each treatment group was then calculated and differences between groups were assessed by analysis of variance. Interactions between IFN and drugs with combination treatment was assessed by 2-way analysis of variance using a factorial design. This allowed the nature of any interaction (additive, synergistic or antagonistic) to be determined.

4.15 Drug dosage and Toxicity

In most reported studies of drug testing using xenografts, agents are administered once at the maximum tolerated dose (MTD). This allows ranking of drugs based on their anti-tumour activity. To find the MTD of a drug, a full dose response experiment should be conducted and the dose of drug causing death in 10% of animals over a 30 day period (the LD_{10}) calculated. For most drugs, the MTD is considered to be approximately 80-90% of the LD_{10}.

In the studies of IFN/drug combinations reported here, an attempt was made to give the xenograft a chronic exposure to the cytotoxic agents and IFN and therefore many of the schedules described involve lower doses than the MTD given repeatedly e.g. 20% of MTD x 5 at weekly intervals or 50% of MTD x 2 at 10 day intervals. In some cases (e.g. NX004), full dose/response experiments were performed to ascertain the MTD for certain agents. Other workers (Steel 1983, Carmichael 1986) have published estimates of MTDs for drugs in thymectomised, irradiated, tumour bearing mice and these reports were used to plan the schedules of the other experiments.

4.16 Interferon

The interferon used in this study was the human recombinant alpha 2 subtype (rHuIFN-α2b). It was kindly supplied by the Schering Corporation, Bloomfield, New Jersey. It has a specific activity of 1.7×10^8 u/mg of protein. The purity of the product has been established by reversed phase HPLC to be greater than 98% pure (Nagabhushan 1985). Large scale production of this IFN has used the KMAC-43 strain of E.coli, the alpha-2 IFN gene being obtained from a cDNA clone, Hif-SN206 (Streuli 1980).

Freeze-dried IFN was thawed and dissolved in water for injection. It was administered daily in all schedules, injected subcutaneously diluted appropriately in a volume of 0.1ml, at a site distant to the tumours.

CHAPTER 5

Preliminary Experiments

This short section deals with the preliminary experiments pertaining to the establishment, characterisation and maintenance of the panel of human lung cancer xenografts used in drug testing.

5.1 Establishment of xenografts

A panel of eight bronchial carcinoma xenografts have been established in immunodeficient CBA mice using the techniques outlined in chapter 4 (Plate 5.1). Much of this work was performed by Dr J Carmichael and has been reported in detail in his MD thesis (1986).

The characteristics of these xenografts are summarised in Table 5.1. All the common histological types of lung cancer are represented with CX143 being of the rarer mixed adenocarcinoma and squamous type. The majority of tumours were grown from specimens resected at thoracotomy with two lines being established from endobronchial biopsies. The two small cell xenografts (NX004 and WX322) were grown from subcutaneous tumour deposits removed under local anaesthesia.

The take rate of specimens transferred from patient to mouse (passage 0) was approximately 20%. Once established as a xenograft, take rates varied with histological type and tended to increase with the number of times the tumour had been passaged (Table 5.1)

Plate 5.1: Immunodeficient CBA mice bearing human bronchial carcinoma xenograft.

Designation	Histology	Source	Passage No	Take rate (%)	Doubling time (days)
NX002	Squamous	Endobronchial biopsy	12	70	11-14
NX004	Small Cell	Skin metastasis	4	40	12-15
CX108	Squamous	1° resection	8	60	12-14
CX117	Adeno	1° resection	10	45	10-12
CX133	Squamous	1° resection	7	56	10-14
CX143	Adeno/Squamous	1° resection	9	75	14-19
WX 321	Large Cell	Endobronchial biopsy	2	25	12
WX 322	Small cell	Skin metastasis	3	40	12-15

Table 5.1: Characteristics of established xenografts (at the start of the study)

Growth rates, measured as the time taken for the tumours to double in volume after reaching a measurable size, were prolonged in early passages with some doubling times as long as 4-6 weeks. With serial passage these times shortened to 8-18 days and after the 4th or 5th passage remained fairly constant. Drug testing was not performed at these early passages.

5.2 Histological monitoring

Histological examination of the tumours was performed prior to the initial implantation and at each subsequent passage. This work was kindly performed by Dr M A McIntyre, Consultant Pathologist, Western General Hospital. In general, little change was seen in the histological apearance of the xenografts compared to the original donor tumour despite serial passage over many months (Plates 5.2 and 5.3). No significant changes were seen in the level of differentiation of the xenografts although some tumours tended to become slightly more differentiated with repeated passage. Mucin production in the adenocarcinoma (CX117) remained constant with time. The mixed adeno-squamous tumour (CX143) showed prominence of the squamous element after 24 months in mice, with only scant evidence of mucin producing elements after this time.

5.3 Karyotyping

Chromosome analysis was performed on all xenografts after the

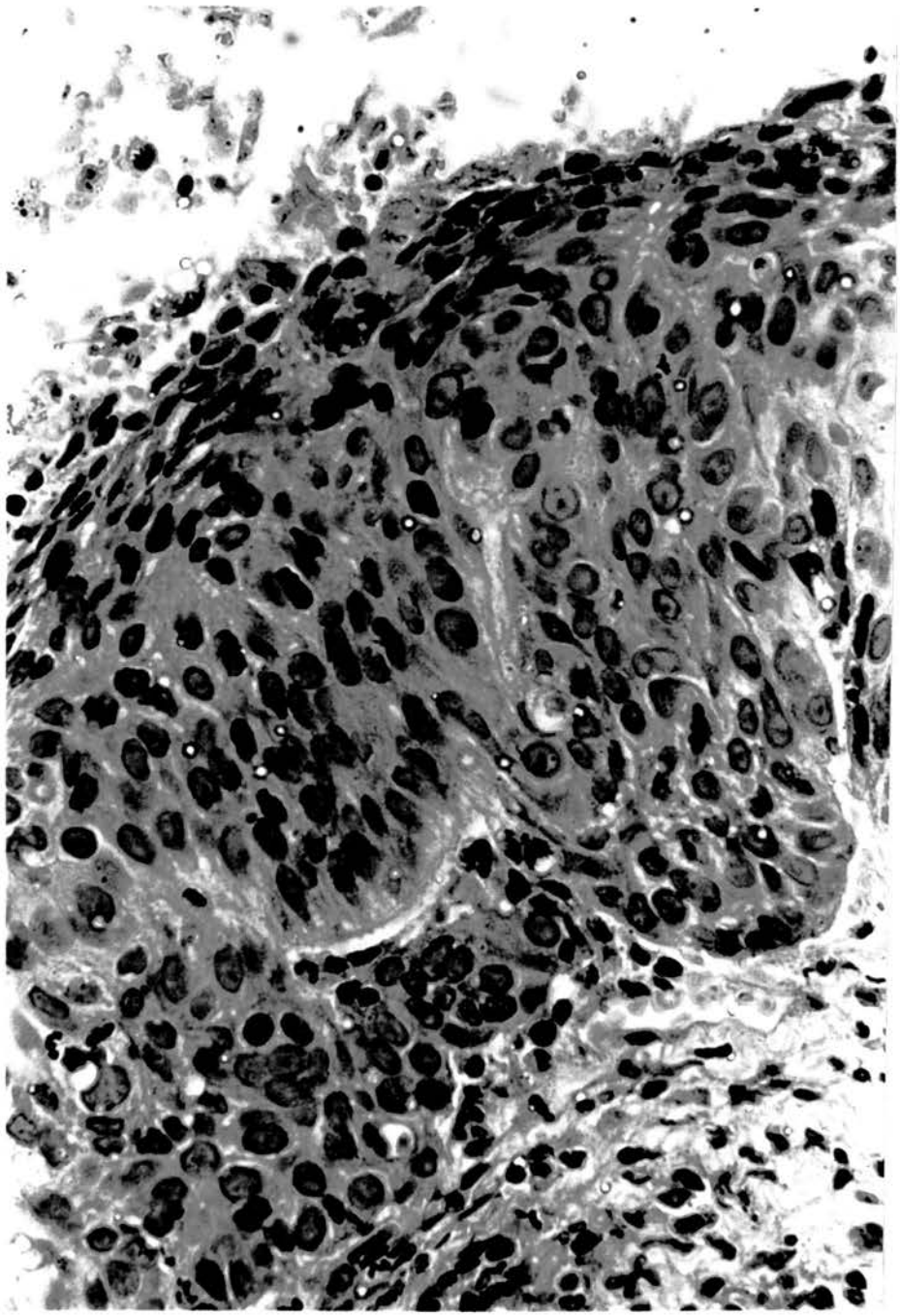


Plate 5.2: Xenograft NX002, moderately differentiated squamous carcinoma, original donor specimen (subcutaneous deposit), April 1985 (H and E, x 200)

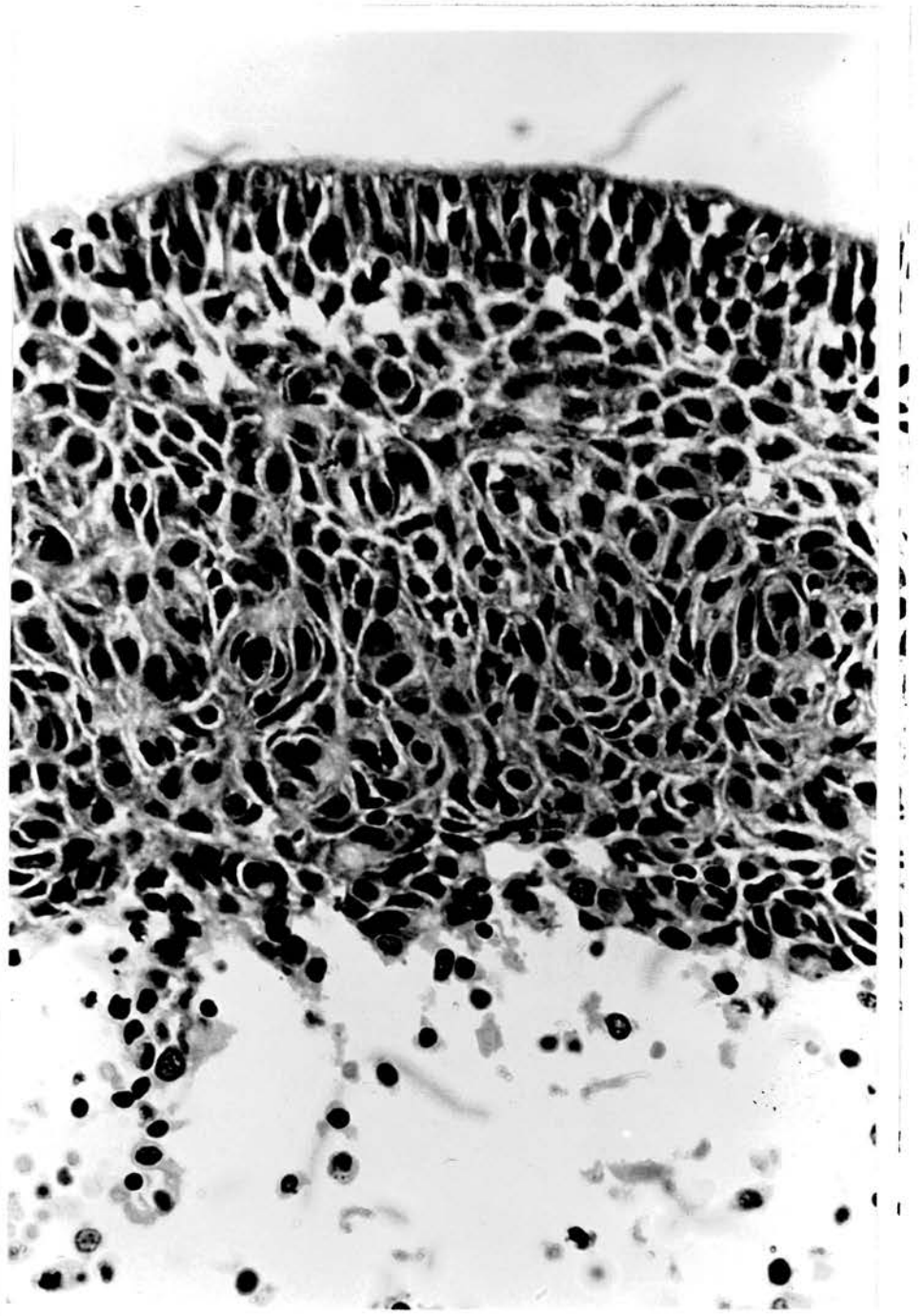


Plate 5.3: Xenograft NX002, 6th passage, June 1986, showing little change from original specimen shown in Plate 5.2 (H and E, x 200)

5th passage to ensure that a human karyotype was maintained. This work was kindly done by Ms Sandra Lawrie. Repeat analysis using the G-11 technique was performed on all tumours in January 1987 with no evidence of murine chromosomes being evident (Plate 5.4).

5.4 Animal health

The thymectomised, irradiated CBA mice used to bear xenografts were kept in a standard room in the animal unit without elaborate precautions to avoid pathogens. Antibiotics (terramycin and neomycin) were added to the drinking water for 14 days after irradiation in an attempt to reduce the incidence of septicaemia from commensal gut flora.

Despite this, a small number of mice (approximately 5%) would develop a wasting syndrome which in thymectomised mice is thought to be due to chronic infection with mouse hepatitis virus (MHV-1) (Sparrow 1980). Occasionally a more virulent infection would occur and large numbers of mice (up to 50% in any batch) were lost. These episodes were usually self-limiting and drug testing was not attempted with these mice.

In the summmer of 1986, a more serious infection occurred with whole batches of mice being lost. Affected animals had rapid breathing, wasting and occasionally paralysis of hind legs. These features were noted about 3-4 weeks after tumour transplantation. The infection did not appear to spread from cage to cage but became "endemic" in batches of mice which were thymectomised at the same time.

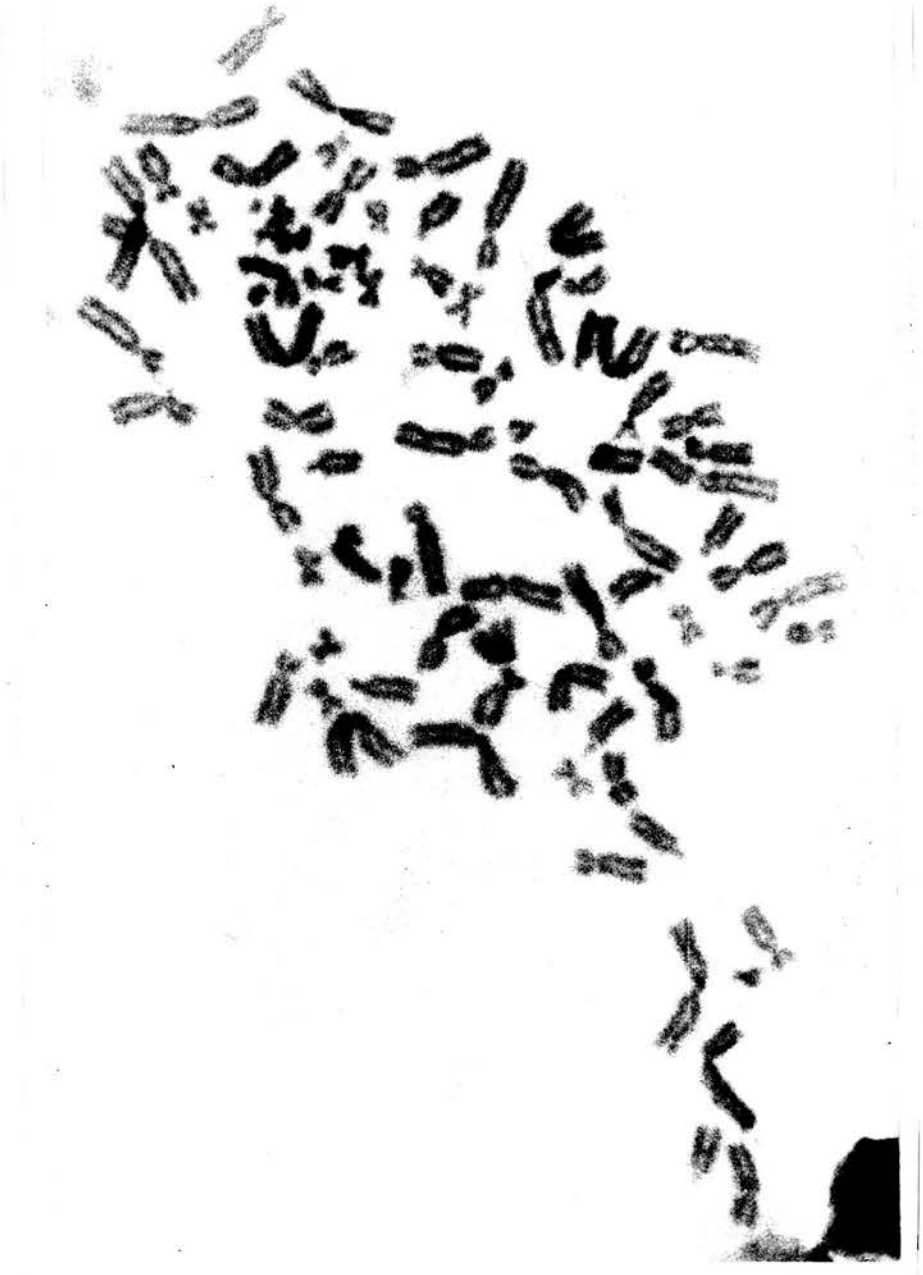


Plate 5.4: Chromosome preparation from xenograft NX004

Histology of the lungs from infected animals showed necrosis of bronchial epithelium and alveolar hyperplasia with haemorrhage and inflammatory infiltrate within the alveolar spaces. The slides were reviewed by Dr Philip Carthew of the MRC Experimental Embryology and Teratology Unit, Carshalton, Surrey and Andor Sebesteny, Veterinary Surgeon, ICRF Animal Unit, Clare Hall, South Mimms, Herts who both felt the likeliest cause of the changes was acute Sendai virus infection. However, raised titres of antibodies against Sendai virus were not detected in serum taken from affected animals.

It was noted that infection only occurred in batches of mice, was unusual in non-tumour bearing animals and very common in certain xenograft lines (NX004 and NX002). Dr Carthew speculated that the virus may have been spread by transplantation of the tumours since Sendai virus will grow in cells of many different species including man. An attempt was therefore made to assess the ability of the xenografts to transmit infection.

A large tumour (NX004) was excised from an affected animal and placed immediately into liquid nitrogen. The xenograft was sent frozen directly to Dr Carthew and Dr Auriol Hill at the MRC unit in Carshalton with specimens of the liver and lungs of the donor animal transported in formal saline. On arrival the tumour was frozen and thawed three times and then administered to 10 MF1 and 6 nu/nu mice by the intranasal and intraperitoneal route. After four weeks the mice all remained healthy. Serum from the MF1 mice showed no antibodies against mouse Hepatitis virus, Pneumonia virus of mice,

Sendai virus or reovirus III. No mycoplasma were isolated from any of the mice. Histological examination of the salivary glands, lungs, liver, spleen and gut showed no abnormalities, with no evidence of any virally transmitted disease. Histology of the donor animals' lungs showed the classical changes of acute viral infection described above. The liver was normal. Therefore it seemed clear that the infection was not being spread via the tumours.

Dr Hill felt that it was possible that the mice were infected with a human pathogen such as para-myxovirus or reovirus and held reservations about the accuracy of serological testing of serum from immunosuppressed animals. After 6-8 months the infection became less virulent and drug testing was restarted.

5.5 Accuracy of caliper measurements

The volume of tumour xenografts was calculated from two perpendicular diameters assuming an ellipsoid shape. To verify the accuracy of this technique, twenty xenografts of varying size and histological type were selected and their volume estimated with caliper measurement. The tumours were then excised and weighed. A good correlation between estimated volume and actual weight was obtained for all histological types (Figure 5.1), even for tumours considered too small or too large for drug testing.

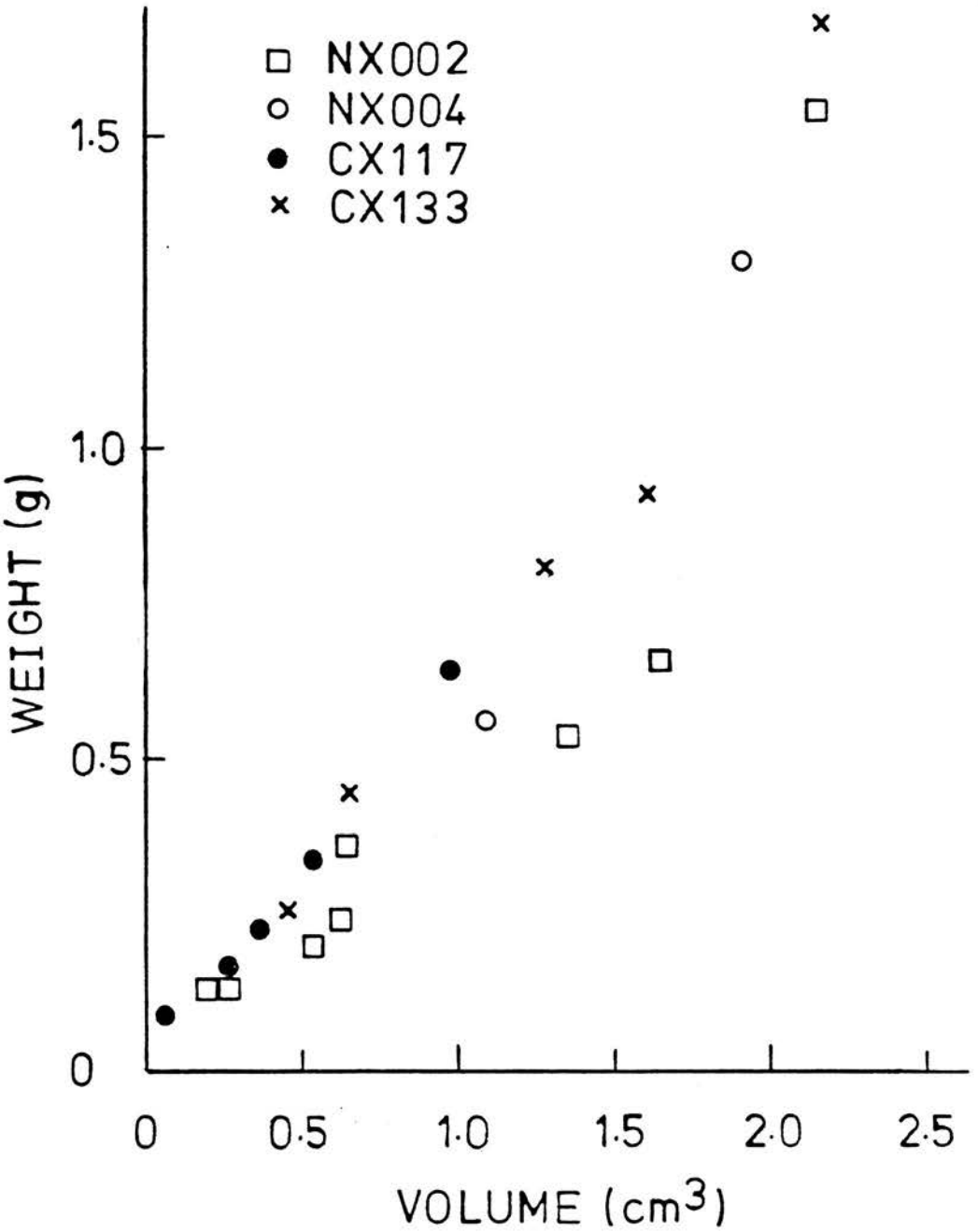


Figure 5.1: Correlation between estimated tumour volume by caliper measurement and actual tumour weight (n=20, t=12.77, r=0.949, $p<0.001$)

CHAPTER 6

Activity of IFN/drug combinations in NSCLC xenografts

6.1 Introduction

This section describes experiments performed to assess the anti-tumour activity of human alpha IFN and various cytotoxic drugs administered both as single agents and in combination in two human NSCLC xenografts.

Preliminary studies using these tumours (Carmichael 1986) had shown promising interactions between CDDP and human lymphoblastoid IFN, particularly in the adenocarcinoma, CX117. The dose of IFN used (2×10^5 units/mouse/day for 35 days) produced a significant cytotoxic effect when administered as a single agent. This dose of IFN is equivalent to approximately 20×10^6 units/m^2 daily in humans using the calculations of Freireich (1966) and would be unlikely to be tolerated by patients. The experiments reported here were designed to mirror more closely the clinical situation. A recombinant IFN preparation (rHu IFN-α2b) was used as we had clinical experience of its action and planned to use it in further clinical studies. This IFN contains only the α-2 subtype of IFN-α unlike human lymphoblastoid IFN which contains several IFN-α subtypes. Mice were treated with lower doses of IFN (2×10 units/day), the equivalent of which would probably be well tolerated by patients.

Since other studies investigating the interaction between IFN and drugs using xenografts (Balkwill 1984, Heston 1984) had shown evidence of synergy when chronic exposure to combinations was used, an attempt was made to administer the two agents repeatedly over a prolonged period rather than a single, maximally tolerated exposure. IFN was given subcutaneously on a daily basis while various cytotoxic agents were injected i.p. at 20% of their MTD x 5 at weekly intervals or at 50% of their MTD x 2 at a 10 day interval. Further studies investigating the optimum dosage schedules of IFN and drugs are outlined in Chapter 8.

The two NSCLC xenografts used in these studies were: (i) NX002, a moderately differentiated squamous carcinoma and (ii) CX117, a poorly differentiated adenocarcinoma. Drug testing was not performed before the 5th passage and the majority of experiments were performed between the 7th and 20th passages. Only tumours produced from one passage were included in any one assessment of an agent or combination and this passage number (e.g. NX004/12) is detailed for each experiment.

As detailed in Chapter 4, the activity of IFN/drug combinations was assessed by randomly allocating groups of 6-10 tumours to treatment with either the drug or IFN as a single agent, IFN/drug in combination, or to a control group. Tumours were measured three times weekly and an estimation of the tumour doubling time (TD) for each tumour was made. The median TD for each group was then calculated and the activity of any treatment expressed where possible in terms of the SGD compared to controls. Statistical comparisons were performed as outlined in Chapter 4. Complete regression or

stasis in growth of any xenograft or significant toxicity was noted. In the initial experiments (6.2-6.7) the activity of IFN-α2b in combination with three commonly used drugs (CDDP, ifosfamide and cyclophosphamide) was studied in both NX002 and CX117. In the subsequent experiments (6.8-6.12) other cytotoxic agents were assessed with IFN-α2b in NX002 only.

6.2 CDDP and IFN in NX002/10

CDDP was administered i.p. in a dosage of 1.4 mg/kg (20% of MTD) on days 0, 7, 14, 21 and 28. IFN was injected s.c. in a dose of 2 x 10^4 units/mouse/day for 35 days. The effect of this treatment on the growth of the xenografts is shown in Figure 6.1. The median TD for control tumours was 9 days. IFN at this dosage had no effect as a single agent (median TD = 10 days; SGD = 0.11). Treatment with CDDP alone caused a significant increase in TD compared to controls (median TD = 17 days; SGD = 0.88). The addition of IFN to CDDP increased the potency further (median TD = 27 days; SGD = 2.0). Three tumours (1 CDDP alone, 2 CDDP and IFN) regressed completely in this experiment. No significant toxicity from any treatment was noted and there were no treatment deaths. The interactin between CDDP and IFN was additive ($F = 0.224$, $p = 0.639$)

6.3 CDDP and IFN in CX117/10

CDDP and IFN were administered as single agents and in combination using the same dosage schedule used in 6.2. Control tumours had a median TD of 12 days. IFN alone had no effect

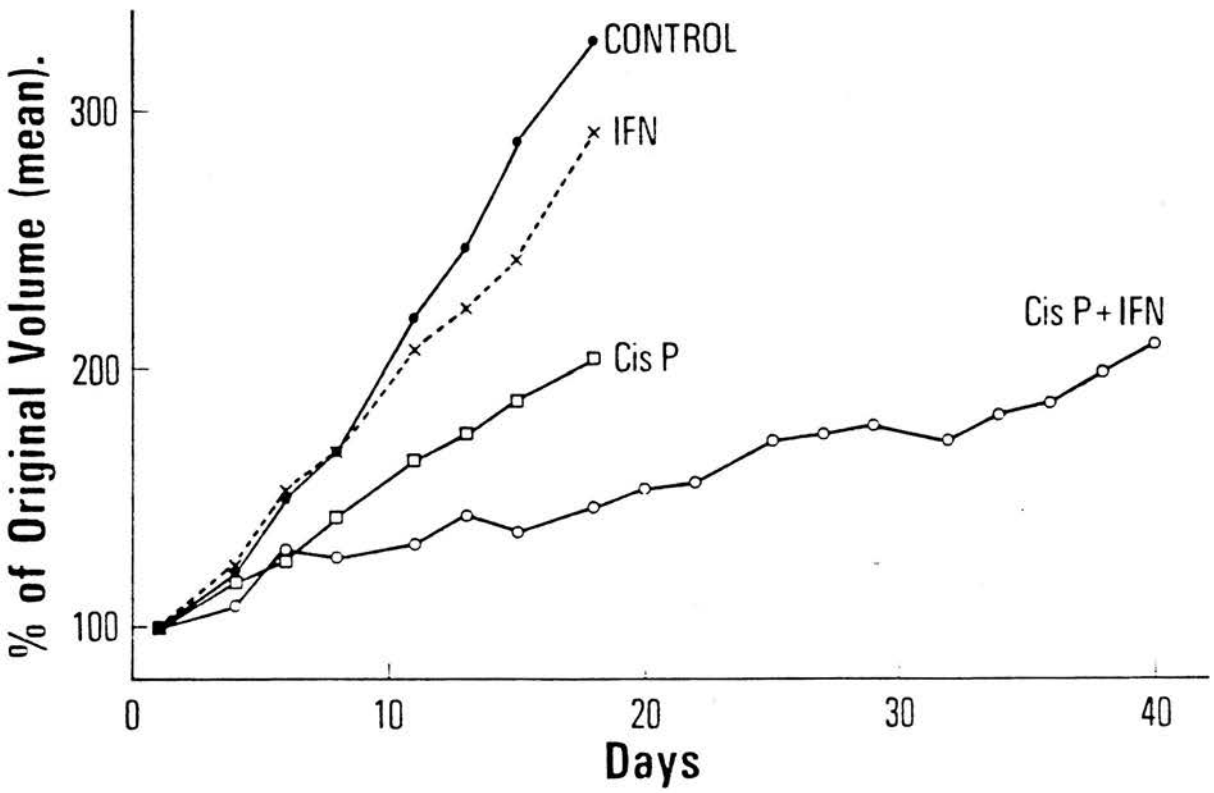


Fig 6.1: Inhibitory effect of ÇDDP/IFN combination on the growth of NX002/10 (growth expressed as the mean % of the original volume)

(median TD = 11 days). CDDP alone caused a small increase in the median TD to 14 days (SGD = 0.16). The combination of IFN and CDDP gave a significant prolongation of the mean TD (median TD = 18.5 days; SGD = 0.54). This interaction was synergistic ($F = 5.732$, $p = 0.025$)

6.4 IFOS and IFN in NX002/11

IFOS was given in a dosage of 60mg/kg (20% MTD) i.p. at weekly intervals x 5 in an identical schedule to CDDP. IFN (2×10^4 units) was given daily s.c. for 35 days. The inhibitory effect of this treatment on the growth of NX002/11 is shown in Figure 6.2. Control tumours doubled in a median of 9 days. IFN had no effect as a single agent (median TD = 10 days; SGD = 0.11). IFOS had little activity alone (median TD = 11.5 days; SGD = 0.27) but when IFN was added an increased effect was seen (median TD = 15 days; SGD = 0.66). However this interaction was not significantly synergistic ($F = 3.665$, $p = 0.06$).

6.5 IFOS and IFN in CX117/10

IFOS and IFN were administered in an identical schedule to that used in 6.4. Median TD for the control group was 12 days. IFN as a single agent had no effect (median TD = 11 days). IFOS alone had similar activity as CDDP alone in this xenograft (median TD = 14 days; SGD = 0.16). The addition of IFN to IFOS improved its potency

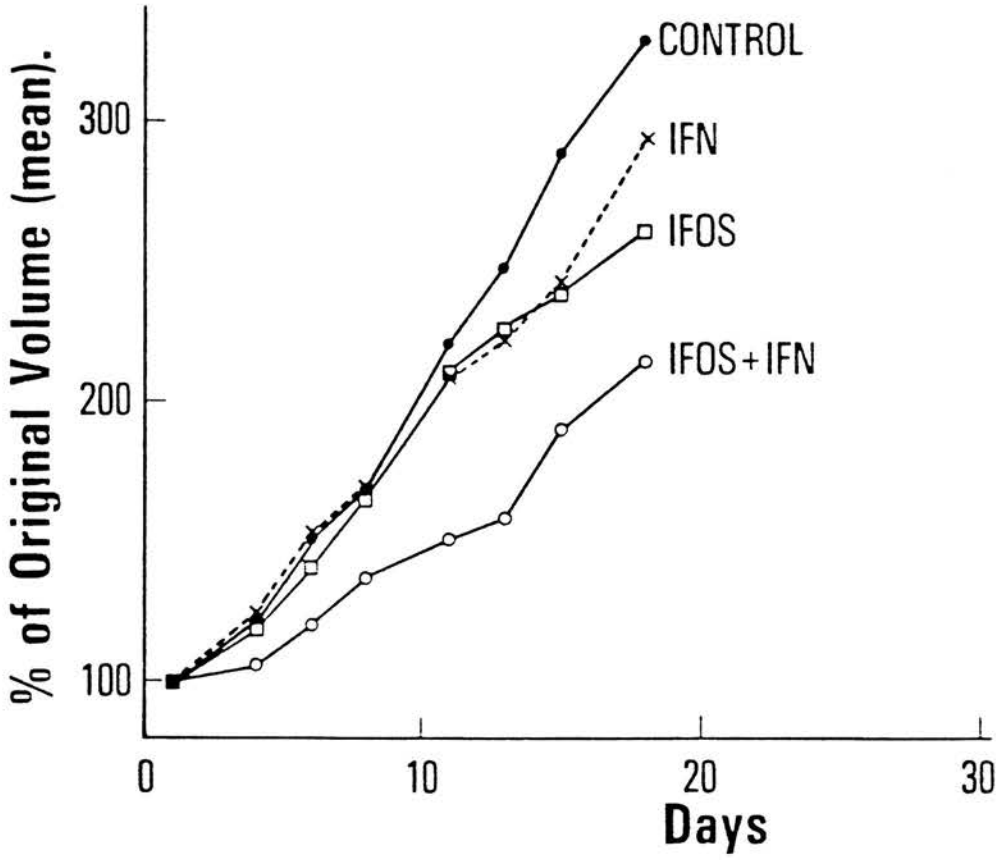


Figure 6.2: Inhibitory effect of IFN and IFOS on the growth of NX002/11. Results are recorded until the time of tumour doubling

(median TD = 16 days; SGD = 0.33) in an additive manner (F = 0.934, p = 0.34). One tumour in the IFOS alone group regressed completely.

Overall NX002 appeared to be a more chemosensitive xenograft line than CX117. The median TDs and SGDs for CDDP and IFOS alone and with IFN in NX002 and CX117 are shown as histograms in Figures 6.3 and 6.4.

6.6 Cyclophosphamide (CY) and IFN in NX002/15

CY was assessed in a similar manner to CDDP and IFOS above. It was injected in a dose of 40mg/kg i.p. at weekly intervals x 5. IFN was given as above (2×10^4 u/mouse/day). The effect of CY with and without IFN is shown in Figure 6.5. Control tumours had a median TD of 7 days. IFN had no effect as a single agent (median TD = 7 days). Treatment with CY alone was relatively ineffective (median TD = 8 days; SGD = 0.14) but was enhanced when IFN was added (median TD = 12 days; SGD = 0.71). This was an additive interaction (F = 0.648, p = 0.42). One tumour in this CY/IFN group regressed completely.

6.7 Cyclophosphamide and IFN in CX117/13

CY and IFN treatment was scheduled as in 6.6. The effect of the two treatments given singly and in combination is shown in Figure 6.6. Control tumours had a median TD of 11 days. Tumours treated with IFN alone grew quicker than controls (median TD = 9 days).

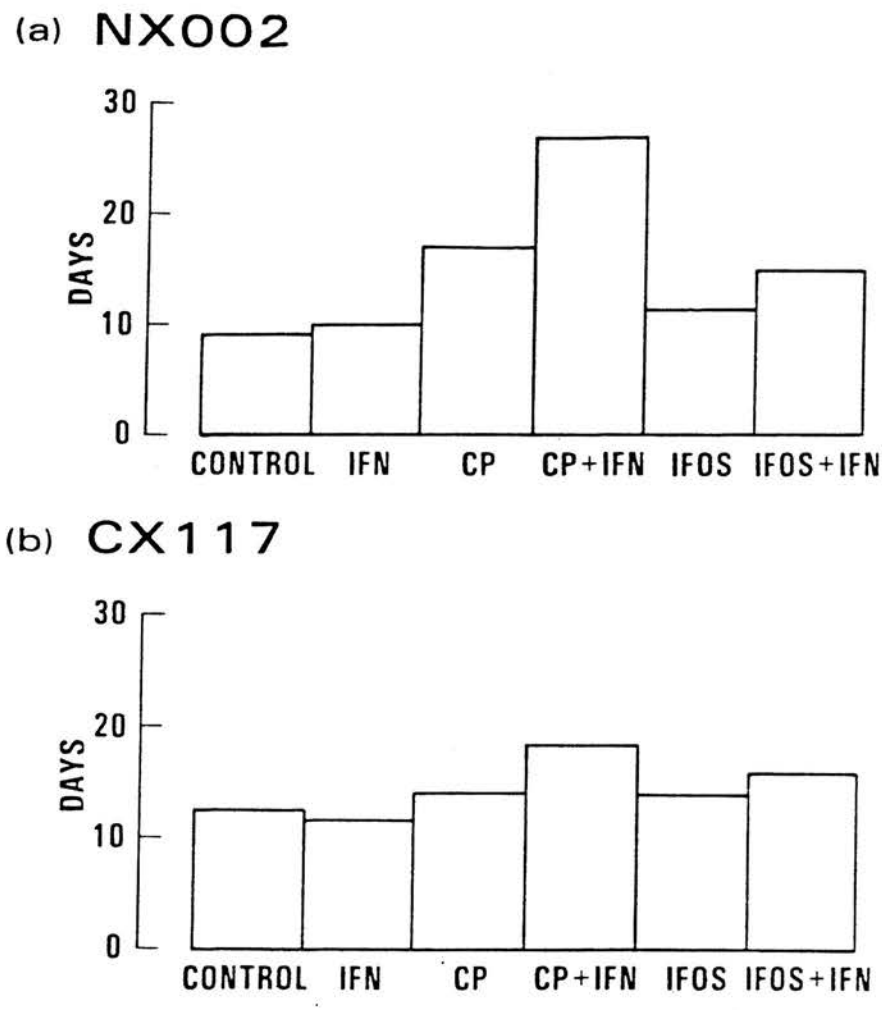


Figure 6.3: Effect of IFN, CDDP and IFOS alone and in combination on the median doubling time of the two NSCLC xenografts NX002 and CX117

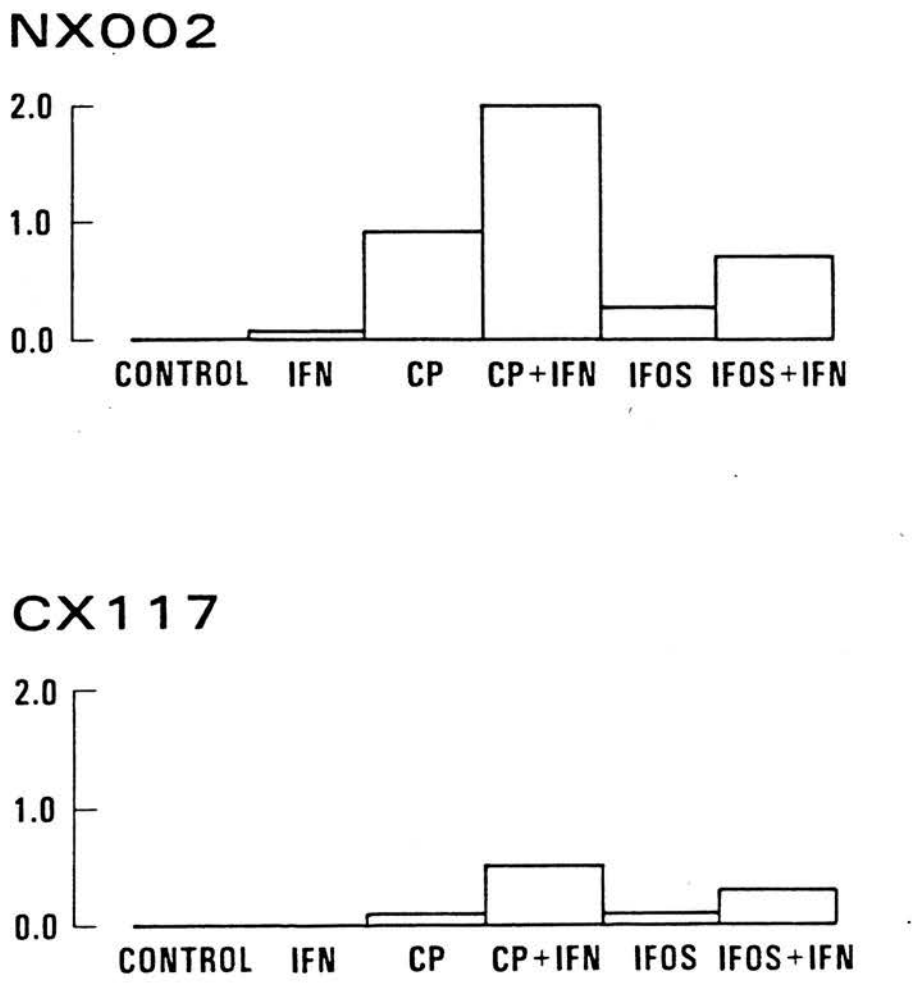


Fig 6.4: Effect of IFN, CDDP and IFOS alone and in combination on the SGD of the two NSCLC xenografts NX002 and CX117

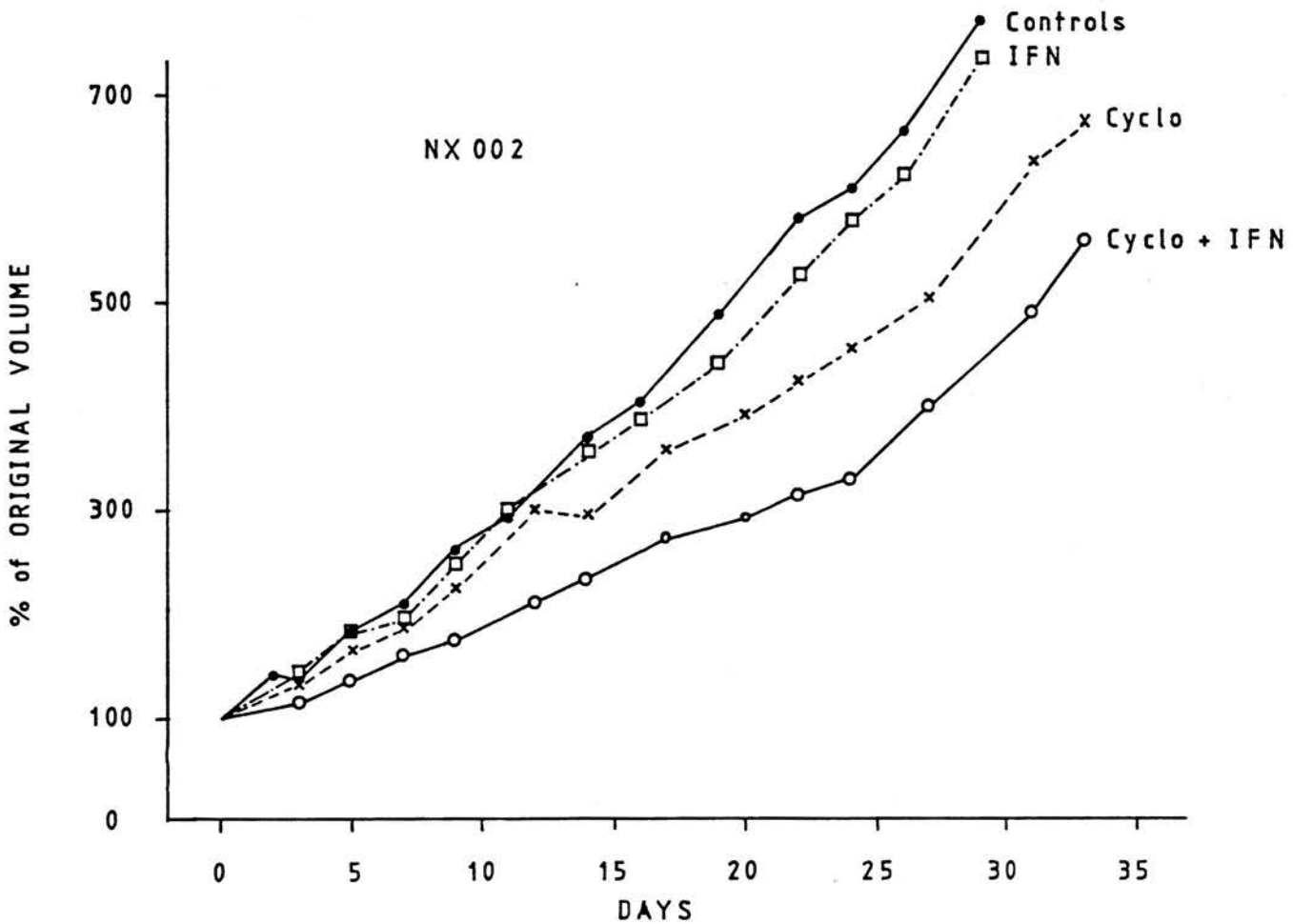


Fig 6.5: Effect of cyclophosphamide and IFN on NX002/15

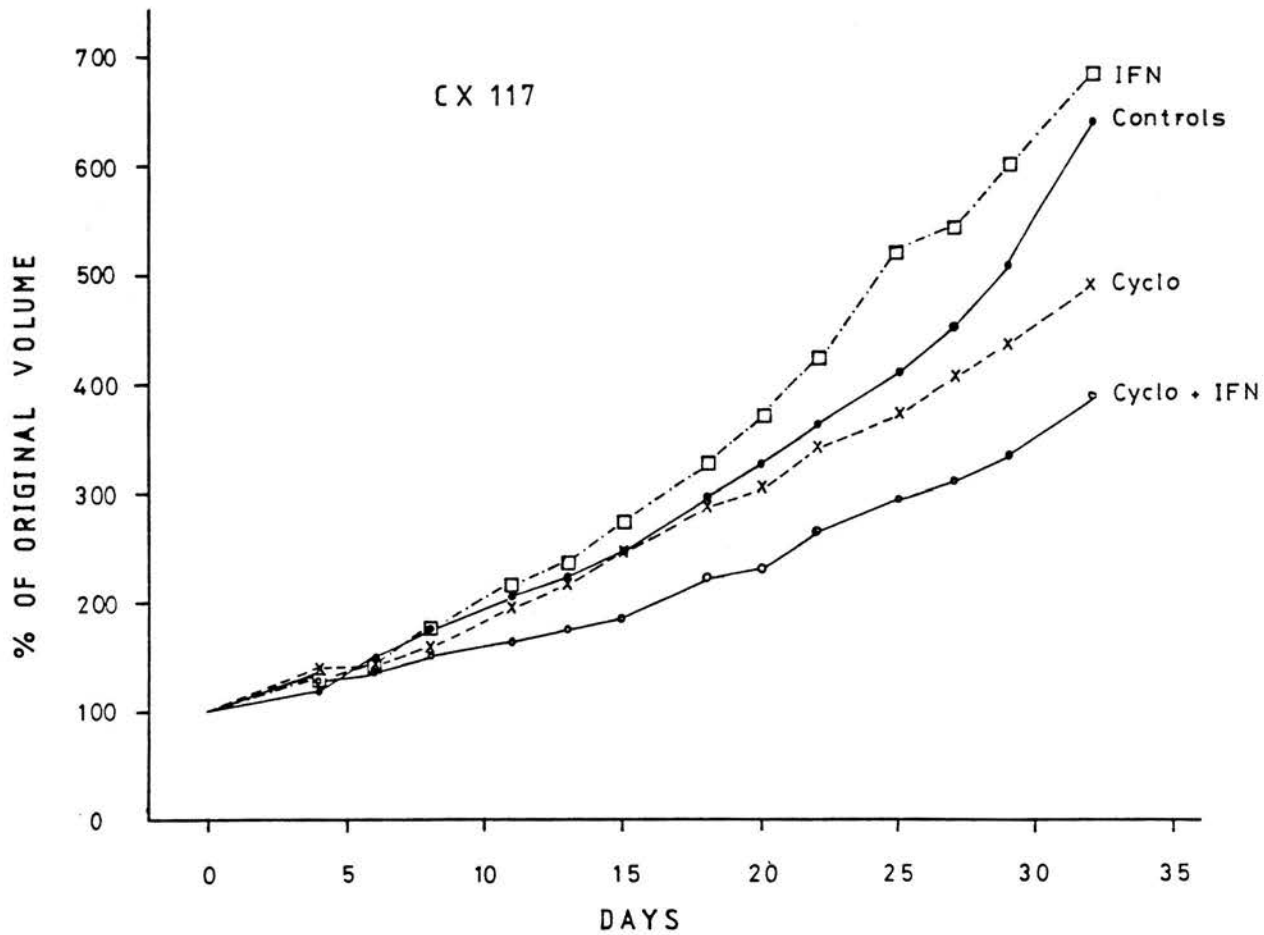


Fig 6.6: Effect of cyclophosphamide and IFN in CX117/13

CY alone had little activity (median TD = 12 days; SGD = 0.09). CY combined with IFN was more effective (median TD = 17 days; SGD = 0.54). However this interaction did not reach significance for synergy ($F = 3.0$, $p = 0.097$).

6.8 Platinum analogues and IFN in NX002/18

After demonstrating that IFN- 2b may enhance the activity of CDDP in NSCLC xenografts (sections 6.2 and 6.3) further studies were designed to ascertain whether a similar effect could be demonstrated using synthetic analogues of CDDP. Two compounds were tested: (i) CBDCA (carboplatin, JM8) and (ii) JM40 (melonato platinum). Both were given using a chronic dosing schedule with and without IFN in the squamous carcinoma xenograft NX002.

6.8 (a) CBDCA and IFN in NX002/18

CBDCA was administered at a dose of 15 mg/kg i.p. x 5 at weekly intervals. IFN was given in the usual schedule (2×10^4 u/mouse/day for 35 days). The effect of single agent and combination therapy in the growth of the xenograft is shown in Figure 6.7. The median doubling time for the control group was 8 days. IFN alone had no effect on tumour growth (median TD = 7 days). CBDA as a single agent had a profound effect. Of the seven tumours tested, three doubled in size (13, 18 and 23 days), two regressed completely and two (double tumours on one mouse) died after the first week of the experiment. Calculation of a median TD and SGD was therefore not possible.

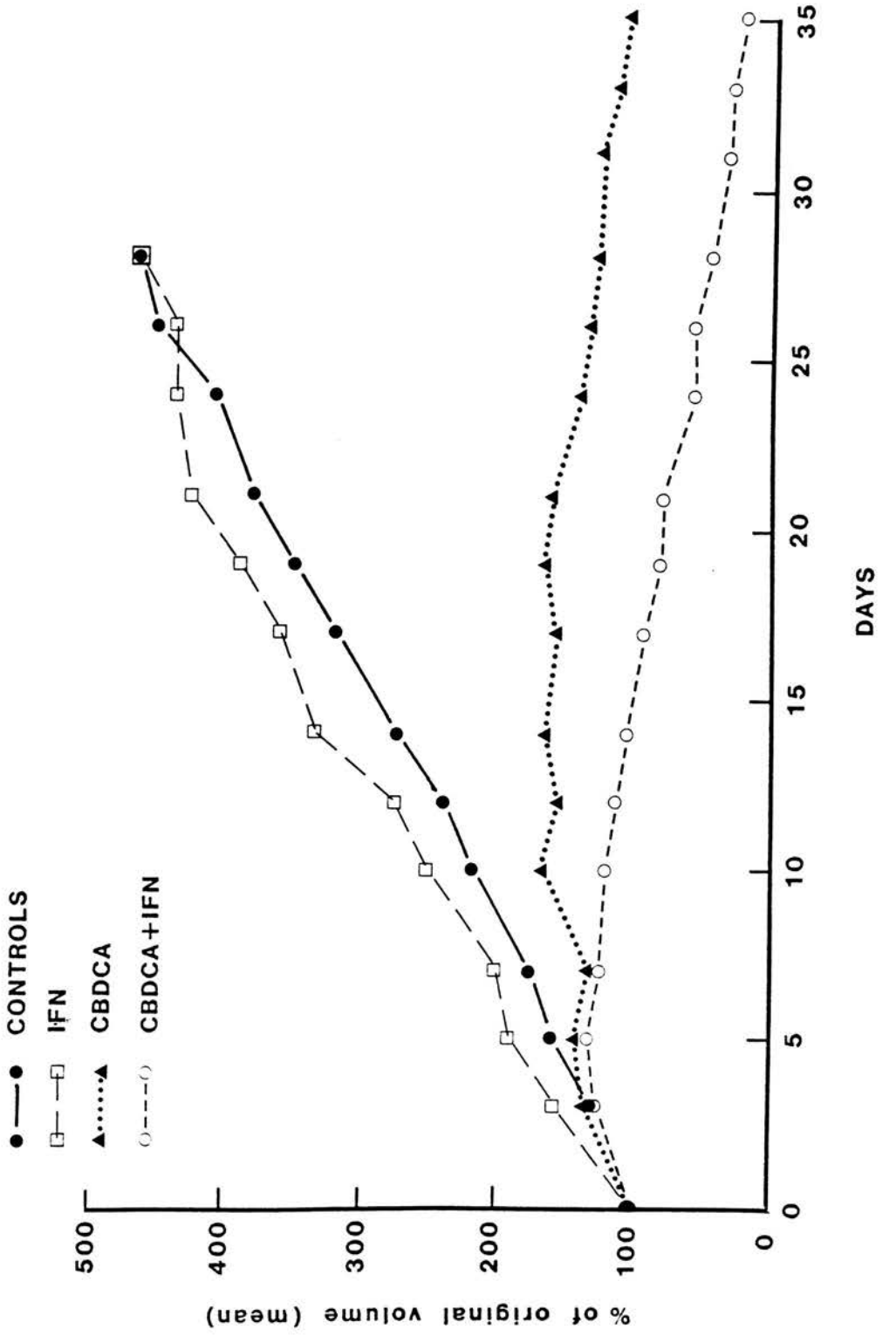


Figure 6.7: Activity of CBDCA and IFN in NX002/18

Enhanced activity was seen with the CBDCA/IFN combination group. Of the seven tumours tested no tumour doubled in size, two remained static at <50% of their original volume and five regressed completely. The interaction between IFN and CBDCA however was additive ($F = 3.448$, $p = 0.07$).

6.8 (b) JM40 and IFN in NX002/18

JM40 was given i.p. at 8mg/kg using an identical schedule to CBDCA. IFN was also administered as above. The effect of single agents and the combination of JM40 and IFN on tumour growth is shown in Figure 6.8. The control group was the same as used in 6.8(a) and doubled in 8 days. IFN alone had no effect (median TD = 7 days). JM40 alone also appeared to have no activity (median TD = 8 days). The JM40/IFN combination group had a median TD of 11 days (SGD = 0.37) with one tumour in the group remaining static. This interaction appeared additive ($F = 1.65$, $p = 0.21$).

The efficacy of the JM40/IFN combination treatment became more apparent after the first 10 days of the study (Figure 6.8). The calculation of the SGD from the first doubling time of the tumours would therefore tend to underestimate the activity of the combination group in a chronic experiment. If the time taken for the tumours to reach three times their starting volume (trebling time or Tt) is used, then possibly a more accurate estimate of the activity is perhaps made. Median Tt for the controls was 15 days, for IFN alone was 13 days and for JM40 alone was 18 days (SGD = 0.2) while only one of the seven tumours in the combination group reached three times its

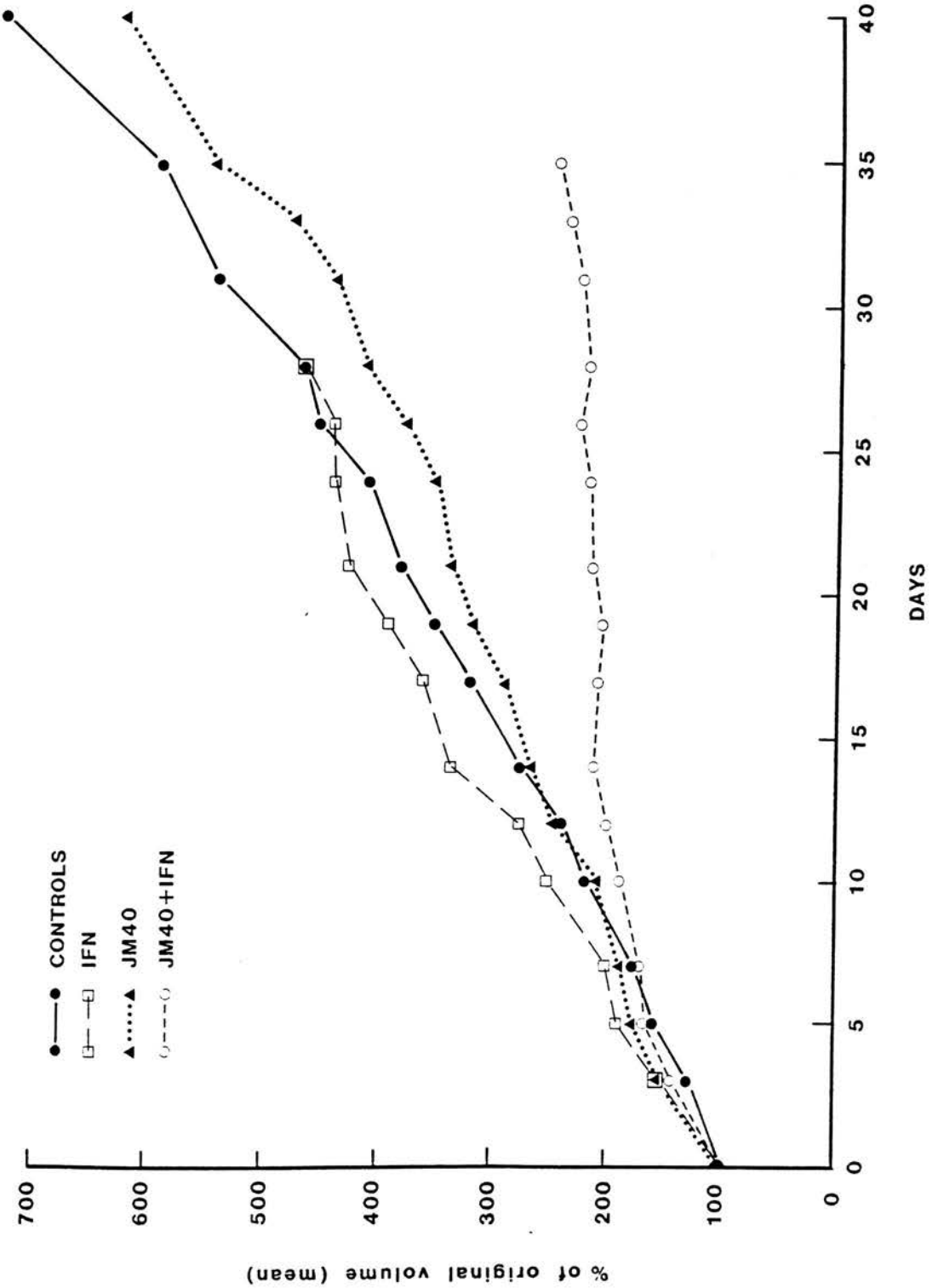


Figure 6.8: Activity of JM40 and IFN in NX002/18

original volume (in 23 days). The interaction between IFN and JM40 at this stage was strongly synergistic (F = 10.532, p = 0.003)

6.9 Adriamycin (ADR) and IFN in NX002/20

ADR was administered at a dose of 6mg/kg (50% of MTD) i.p. on days 0 and 10. IFN was given s.c. at 2×10^4 u/mouse daily for 20 days. Control tumours doubled in 10 days. The group treated with IFN alone had a median TD of 11 days (SGD = 0.1). ADR as a single agent had no effect on the tumour (median TD = 9 days). ADR in combination with IFN caused a small increase in median TD to 13 days (SGD = 0.3). Additivity was therefore seen (F = 0.31, p = 0.57).

6.10 Vindesine (VND) and IFN in NX002/21

VND was given in a similar schedule to ADR in 6.9 i.e. 50% of MTD (1mg/kg) i.p. on days 0 and 10. IFN was given s.c. 2×10^4 units/mouse daily for 20 days. The median TD for controls and IFN treated tumours was 11 days. VND as a single agent had no significant activity (median TD = 10 days). VND and IFN in combination caused an increase in median to 17 days (SGD = 0.55). Statistical analysis suggested additivity had occurred between VND and IFN (F = 0.74, p = 0.39).

6.11 Etoposide and IFN in NX002/21

This experiment was performed on the same batch of tumours as used in 6.10. Etoposide was given as 50% of the MTD (15mg/kg) i.p.

on days 0 and 10. The control group and IFN treated tumours both had a median TD of 11 days. The group of tumours treated with etoposide as a single agent had a median TD of 6 days and those treated with the combination of etoposide and IFN doubled in 9 days showing no activity for either treatment and no positive interaction occurred ($F = 0.33$, $p = 0.57$).

6.12 TCNU and IFN in NX002/20

In 1986 an opportunity arose to test a new nitrosourea in the xenograft system. TCNU is a unique water soluble dimethylamino-sulphonyl nitrosourea based on the endogenous aminoethane sulphonic acid, taurine. It has shown activity against experimental rodent tumours and the parent drug can be detected in the plasma in man up to 8 hours after a single oral dose.

TCNU was dissolved in polyethylene glycol and administered by oral gavage at 10 mg/kg (50% of MTD) on days 0 and 10. IFN was given daily in the usual dose for 20 days. Control tumours had a median TD of 10 days. IFN treated tumours doubled in 11 days (SGD = 0.1). Treatment with TCNU alone caused a significant delay in growth (median TD = 25, SGD = 1.5) with two of the ten tumours in the group remaining static. Only four tumours doubled in the TCNU/IFN combination group (8, 15, 20 and 22 days), four remained static and two animals died. Significant toxicity indicated by weight loss and diarrhoea was seen in both groups receiving TCNU but was not apparently worsened by the addition of IFN. This interaction was additive in nature ($F = 0.025$, $p = 0.87$).

6.13 Discussion

In all the experiments described above, the activity of small doses of cytotoxic drugs against the two NSCLC xenografts was enhanced by the addition of low doses of human alpha IFN. IFN alone at this dose had no significant effect on the tumours. The results are summarised in Table 6.1 with activity of each agent being expressed in terms of the SGD with and without IFN.

Unfortunately in some of the experiments measurement of the SGD gave a poor indication of the activity of a treatment regime. CBDCA was clearly an active agent in NX002 but measurement of the SGD was impossible, as fewer than 50% of the tumours doubled in size and therefore a median TD could not be calculated. The activity of the JM40/IFN combination was perhaps also underestimated by calculating the SGD from the first doubling of the tumour from the onset of treatment. Figure 6.8 illustrates that the effect of treatment with the combination compared to the other groups only became apparent afer the first 2 or 3 JM40 treatments (approximately 10 - 14 days) and the activity of the combination was therefore underestimated by measuring the first doubling time. It is perhaps unfortunate that in this model survival is not influenced by tumour growth and cannot therefore be used as an end point.

Although it has been demonstrated that the combination of IFN and chemotherapy would seem to be more effective than would be expected from the potency of each as a single agent, not all interactions proved to be statistically synergistic. However, antagonism was never seen and in many experiments the interactions

	DRUG	SGD (SINGLE AGENT)	SGD (WITH IFN)	SIGNIFICANCE OF INTERACTION
NX002				
	CDDP	0.88	2.0	0.639
	IFOS	0.27	0.66	0.066
	CY	0.14	0.71	0.428
	CBDCA	*	*	0.077
	JM40	0	0.37	0.003
	ADR	0	0.3	0.579
	VND	0	0.55	0.397
	VP16	0	0	0.570
	TCNU	1.5	*	0.875
CX117				
	CDDP	0.16	0.54	0.025
	IFOS	0.16	0.33	0.344
	CY	0.09	0.54	0.097

* Insufficient tumours doubled to calculate SGD (see text)

Table 6.1 Activity of various drugs in NSCLC xenografts expressed as SGD with and without IFN (SGD for IFN alone = 0.1). The values in the far right column denote the p value for the "significance of interaction" between the drug and IFN in combination ($p<0.05$ suggesting a positive effect). All experiments were performed once although IFN and CDDP in NX002 was repeated in Chapters 8 and 9.

fell just short of statistical significance. Perhaps repeat experiments with larger groups of tumours would be more helpful in defining the precise nature of any interaction between IFN and drugs.

The clinical studies of treatment with IFN discussed in Chapter 3 have not defined a dose-response effect and the optimal schedule of administration remains to be determined. In some tumours the duration of therapy seems to be at least as important as the dose administered. It was for these reasons that these combination experiments were designed to explore the use of IFN and cytotoxic drugs scheduled repeatedly over a period of weeks, rather than a single maximally tolerated exposure. IFN was administered daily and the cytotoxic agents given as 20% or 50% of the MTD in divided doses. Studies to define the optimum dose schedule for these IFN/drug combinations are described in Chapter 8.

The majority of cytotoxic drugs used in these experiments were alkylating agents although some enhanced activity was seen with adriamycin and vindesine. Statistically significant synergy was only seen with CDDP and the platinum analogue JM40 in combinaton with IFN. Improved activity with the addition of IFN was also seen with drugs which were both active or inactive in these two xenografts (Table 6.1). Although as one would expect both NSCLC tumours were resistant (SGD <1) to most agents. No attempt was made to explore the precise mechanism of IFN potentiation of chemotherapy in these experiments. This work is covered in Chapter 9.

The enhanced activity seen with the combinations of chemotherapy and IFN described here comfirms previous reports using this form of treatment in murine and human tumour models (Table 3.5). This effect

has not previously been reported in human non-small cell lung cancer. The following chapter outlines similar experiments using SCLC xenografts.

CHAPTER 7

Activity of IFN/drug combinations in SCLC xenografts

7.1 Introduction

This section is similar to Chapter 6 in that it deals with the activity of IFN/drug combinations in the xenograft model. After showing significant potentiation of the activity of certain agents by the addition of IFN in NSCLC tumours, it seemed important to try these combinations in SCLC xenografts. SCLC is a more chemosensitive disease than NSLC with chemotherapy being the treatment of choice in the majority of patients.

Two human SCLC xenografts were used for testing IFN/drug combinations, NX004 and WX322. Both were obtained from subcutaneous deposits of patients with metastatic SCLC. Similar experimental methods were employed for drug testing, with cytotoxic agents being given in sub-optimal doses at regular intervals and IFN being given daily at a low dosage level. Assessment of activity was as described previously. Drugs with known clinical activity in SCLC patients were assessed initially rather than merely repeating the same drugs used in Chapter 6.

As SCLC xenografts are generally more chemosensitive than NSCLC tumours it was considered to be important to perform full dose-response evaluations for each agent, including IFN, prior to testing combinations. This would enable more rational drug dosing

with combination therapy avoiding the use of a dose of drug that "cured" tumours as a single agent, leaving no room for improvement with combination therapy.

7.2 Dose-response effect of IFN in NX004/4

Three different doses of IFN-alpha were assessed in this tumour: 2×10^4 units/day, 5×10^4 units/day and 2×10^5 units/day. All were given subcutaneously for 35 days at a site distant to the tumours. The results of this treatment are shown graphically in Figure 7.1. Control tumours doubled in 7 days. No effect on tumour growth was seen with any dose of IFN. This experiment had to be terminated on day 26 as many of the tumours were considered to be too large for the host animal (6 x original volume).

7.3 Dose-response effect of CY in NX004/2

Four different doses of CY were assessed: 50mg/kg, 100mg/kg, 150mg/kg and 200mg/kg. Each was given i.p. divided into five equal doses (i.e. 10, 20, 30 and 40mg/kg) at weekly intervals on days 0, 7, 14, 21 and 28. The results are shown in Figure 7.2. Control tumours had a median doubling time of 9 days. A total dose of 50mg/kg and 100mg/kg had no effect (median TD 8.5 and 10 days respectively). The tumours in the groups treated with 150mg/kg and 200mg/kg remained static during the 35 days of treatment. 3 tumours doubled in the 150mg/kg group (at 29, 29 and 31 days) and 2 tumours doubled in the

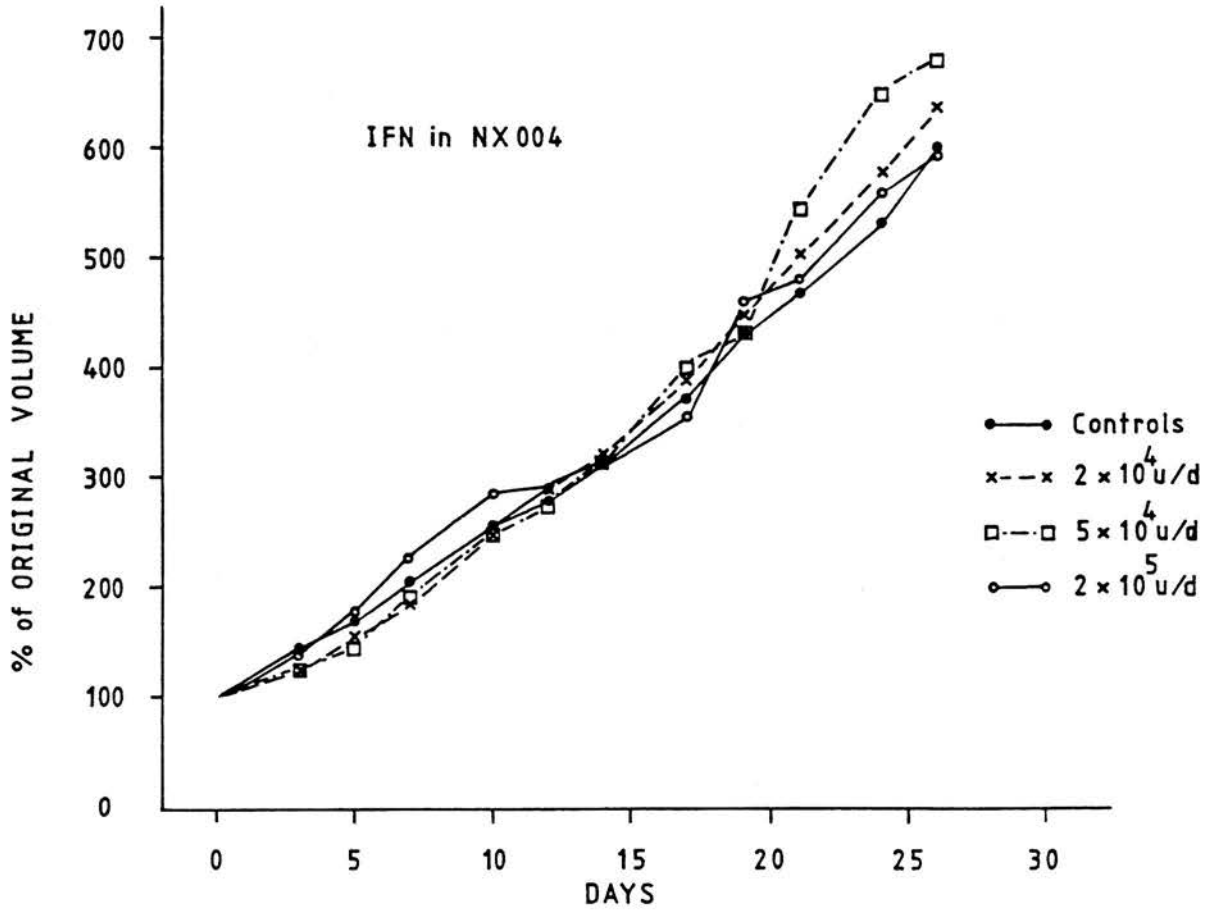


Figure 7.1: IFN dose/response experiment in NX004/4

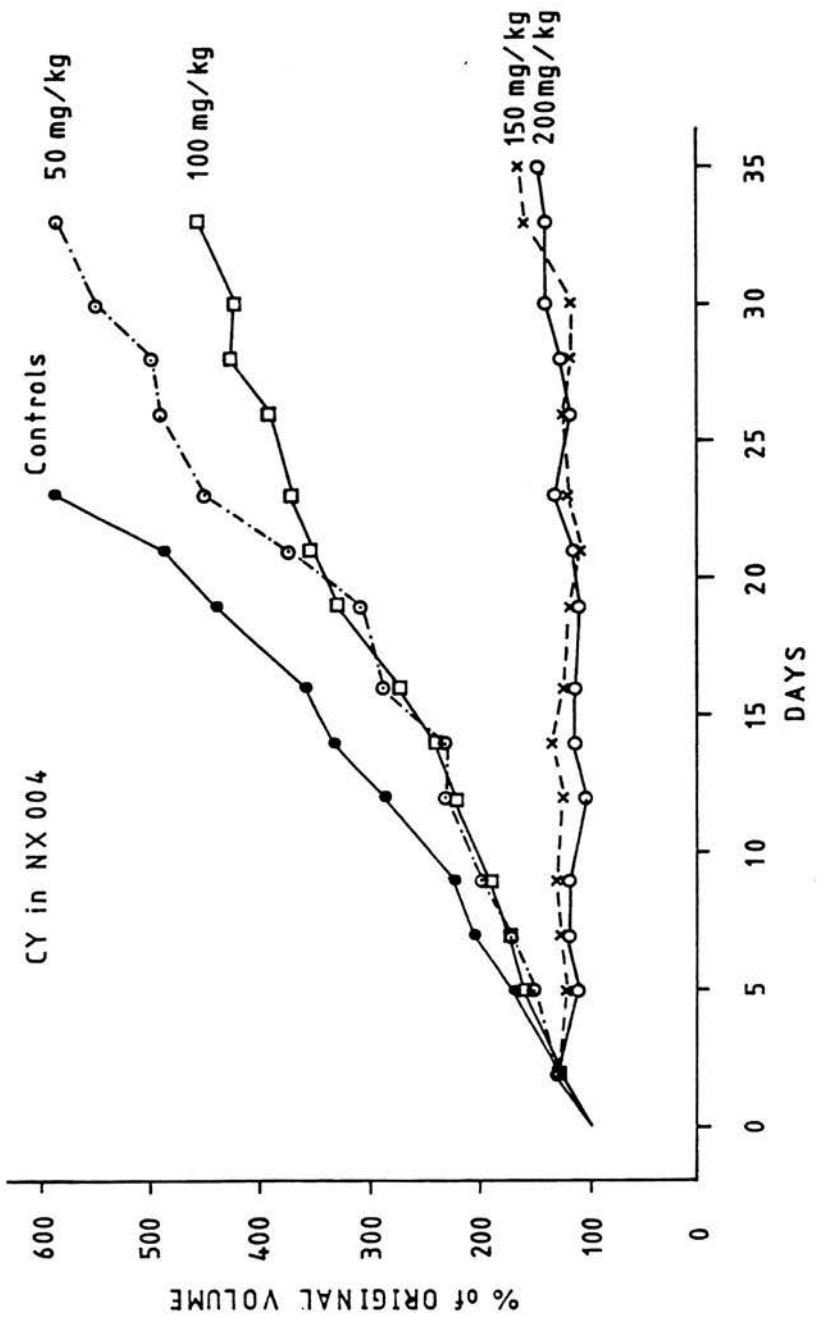


Figure 7.2: CY dose/response experiment in NX004/2

200mg/kg group (both at 27 days). The same number of tumours in each group regressed completely. No animals died in either group. There was no evidence of toxicity in the 200mg/kg group.

7.4 CY/IFN combination in NX004/4

CY was given as 30mg/kg i.p. at weekly intervals. IFN was given in the usual schedule. The growth of tumours in each group is outlined in Figure 7.3. Control tumours doubled in a median of 8 days. IFN alone had no effect. CY at this dose gave similar results to those seen in the dose/response experiment with most tumours remaining static. The addition of IFN did not influence the activity of CY ($p>0.1$).

7.5 Dose/response effect of ADR in NX004/4

As with CY, 4 different doses of ADR were assessed: 6, 8, 12 and 18 mg/kg. Each was given i.p in five divided doses at weekly intervals. The results are shown in Figure 7.4. Control tumours doubled in 9 days. Treatment with ADR at any dosage had no effect on the tumours. The group receiving 12mg/kg appeared to have some retardation of growth late in the experiment although this may have reflected drug toxicity. All animals in the 18mg/kg group died although this treatment had no effect on tumour growth. This tumour must be considered resistant to ADR therapy.

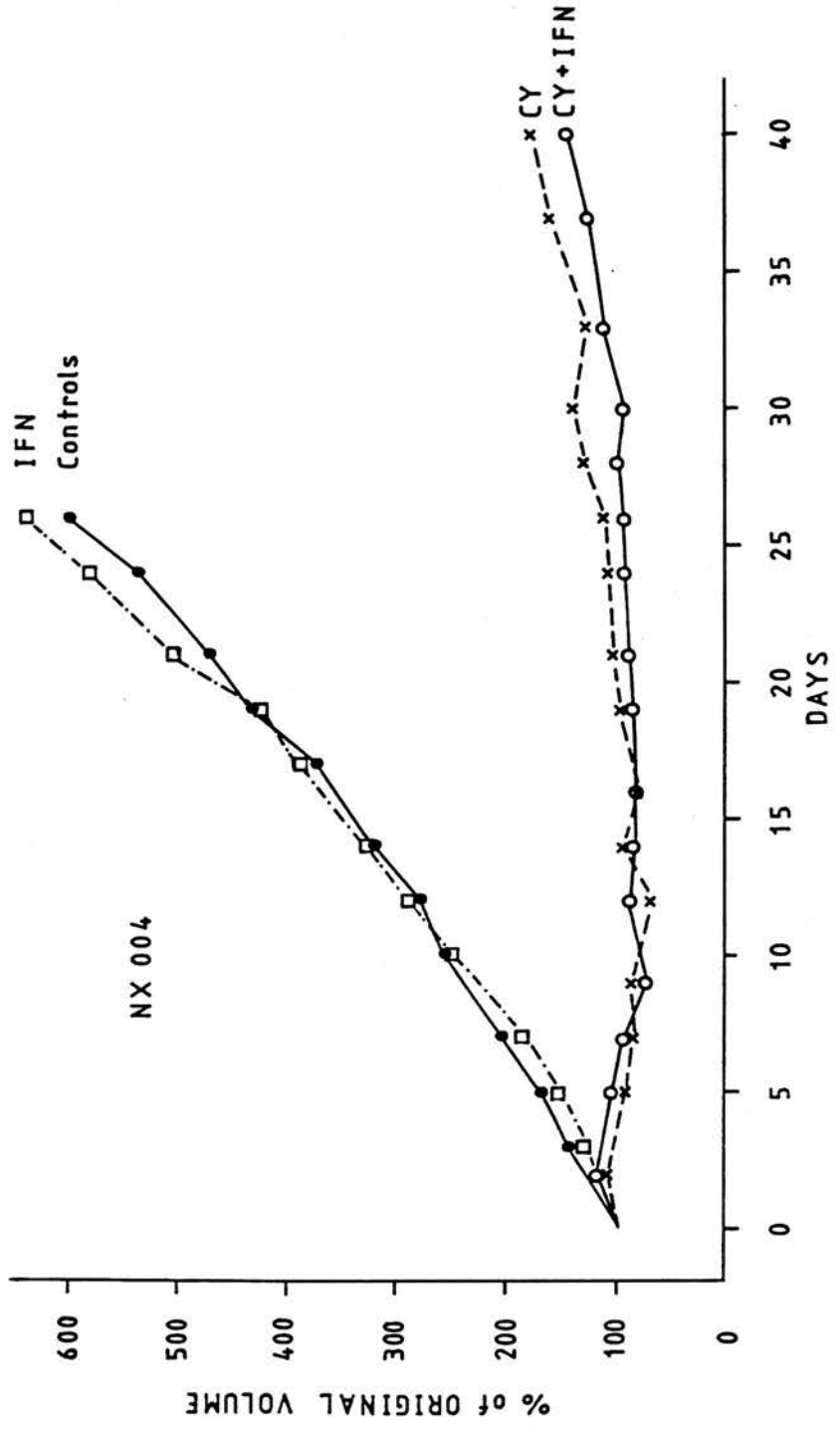


Figure 7.3: CY/IFN combination in NX004/4

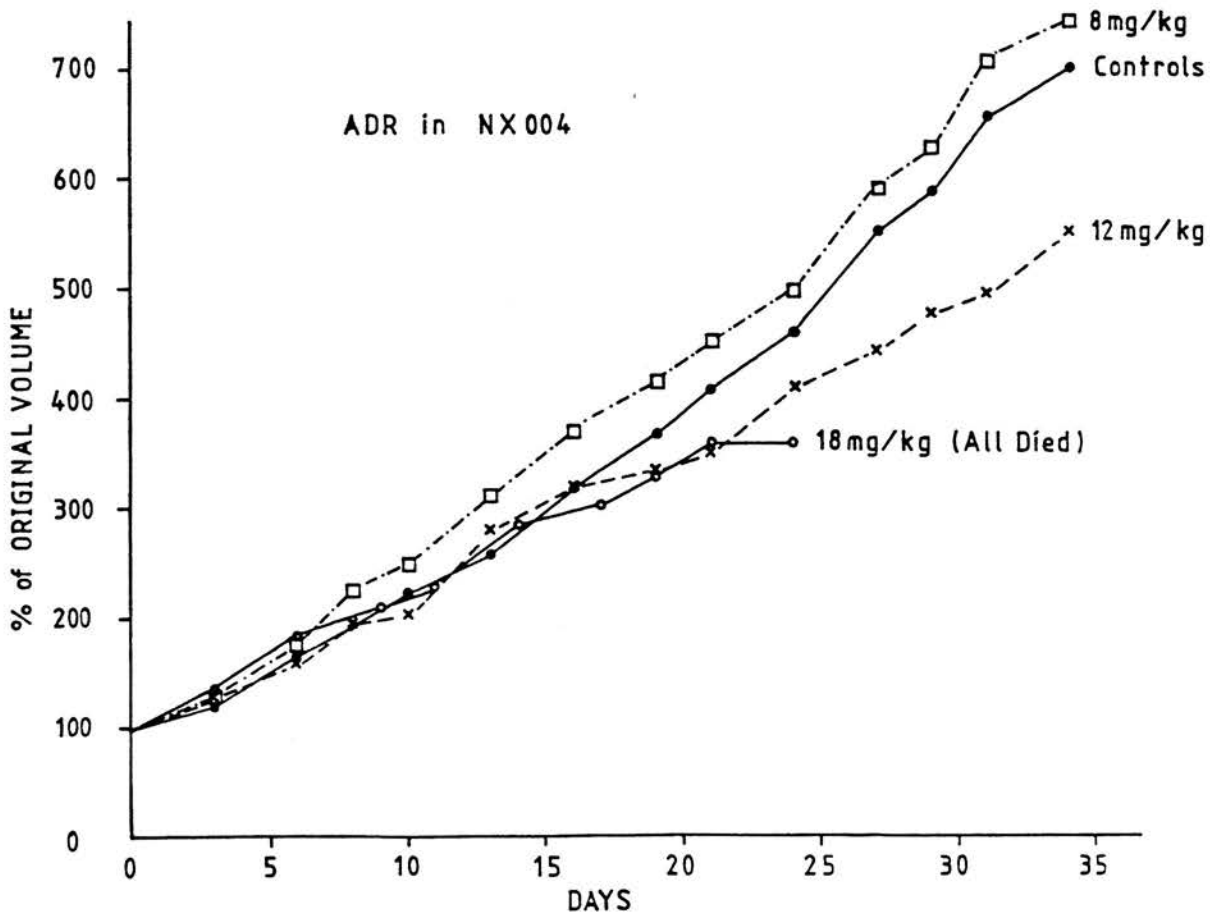


Figure 7.4: ADR dose/response experiment in NX004/4

7.6 ADR/IFN combination in NX004/5

ADR was given in a total dose of 12mg/kg. IFN was given in the usual schedule. The results of single agent and combination treatment are shown in Figure 7.5. Control tumours doubled in 7 days. ADR alone had similar activity to that seen in section 7.5 (median TD = 8 days). IFN alone had no effect. ADR in combination with IFN had no effect on this tumour (median TD = 7 days). An antagonistic effect was not seen ($p>0.1$).

7.7 Dose/response effect of Etoposide in NX004/2

Four different doses of etoposide were assessed (20, 35, 50 and 75mg/kg i.p. in five divided doses at weekly intervals). The results are shown in Figure 7.6. Control tumours doubled in 9 days. As with ADR, no dose of etoposide influenced the median doubling time of tumours from their original volume. The two groups receiving 50 and 75mg/kg appeared to demonstrate an effect after the first three doses (SGDs calculated from Tt = 0.23 and 0.3 respectively). However all animals receiving a total dose of 75mg/kg died. This tumour appeared to be resistant to etoposide.

7.8 Etoposide/IFN combination in NX004/5

Etoposide was given in a total dose of 45mg/kg. IFN was given in the usual schedule (2×10^4 units/mouse/day). The results are

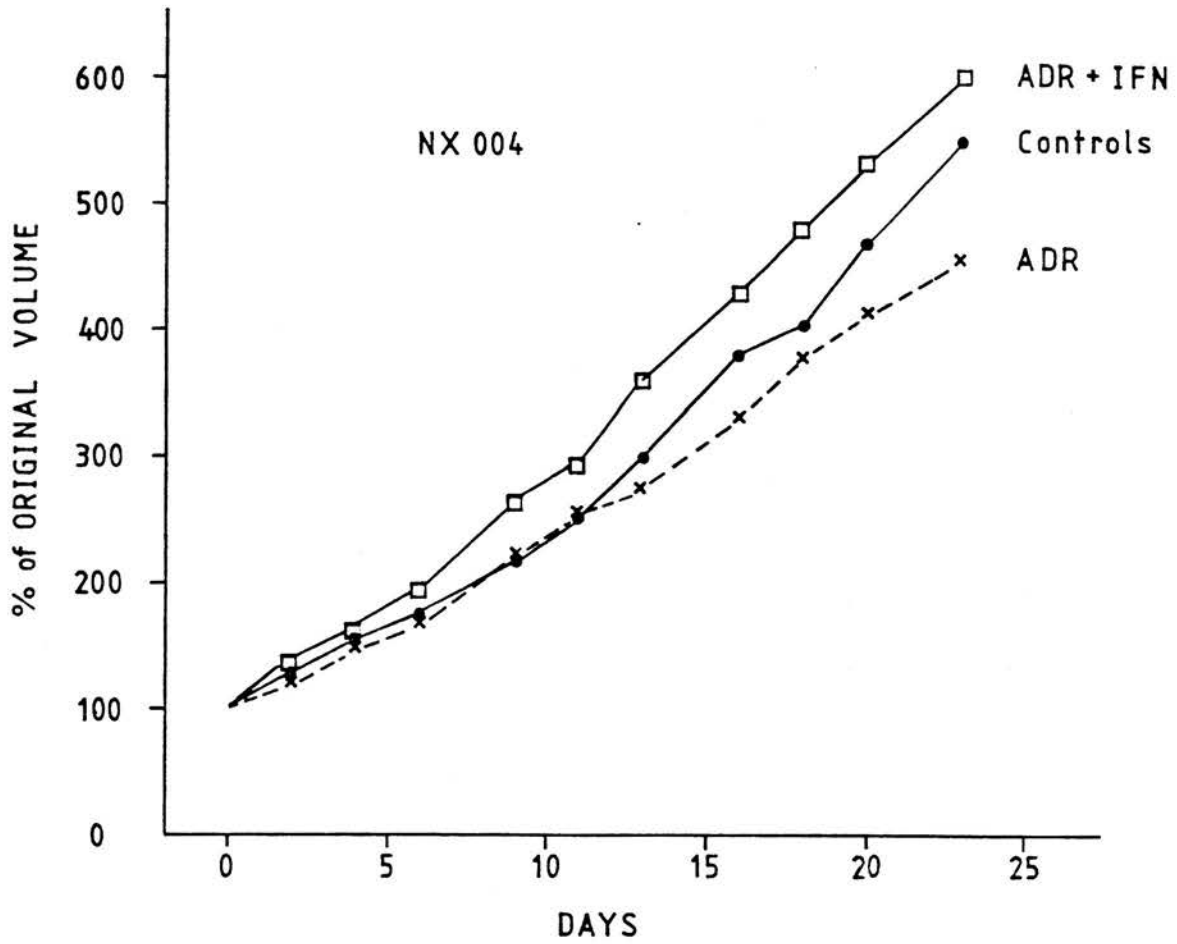


Figure 7.5: ADR/IFN combination in NX004/5

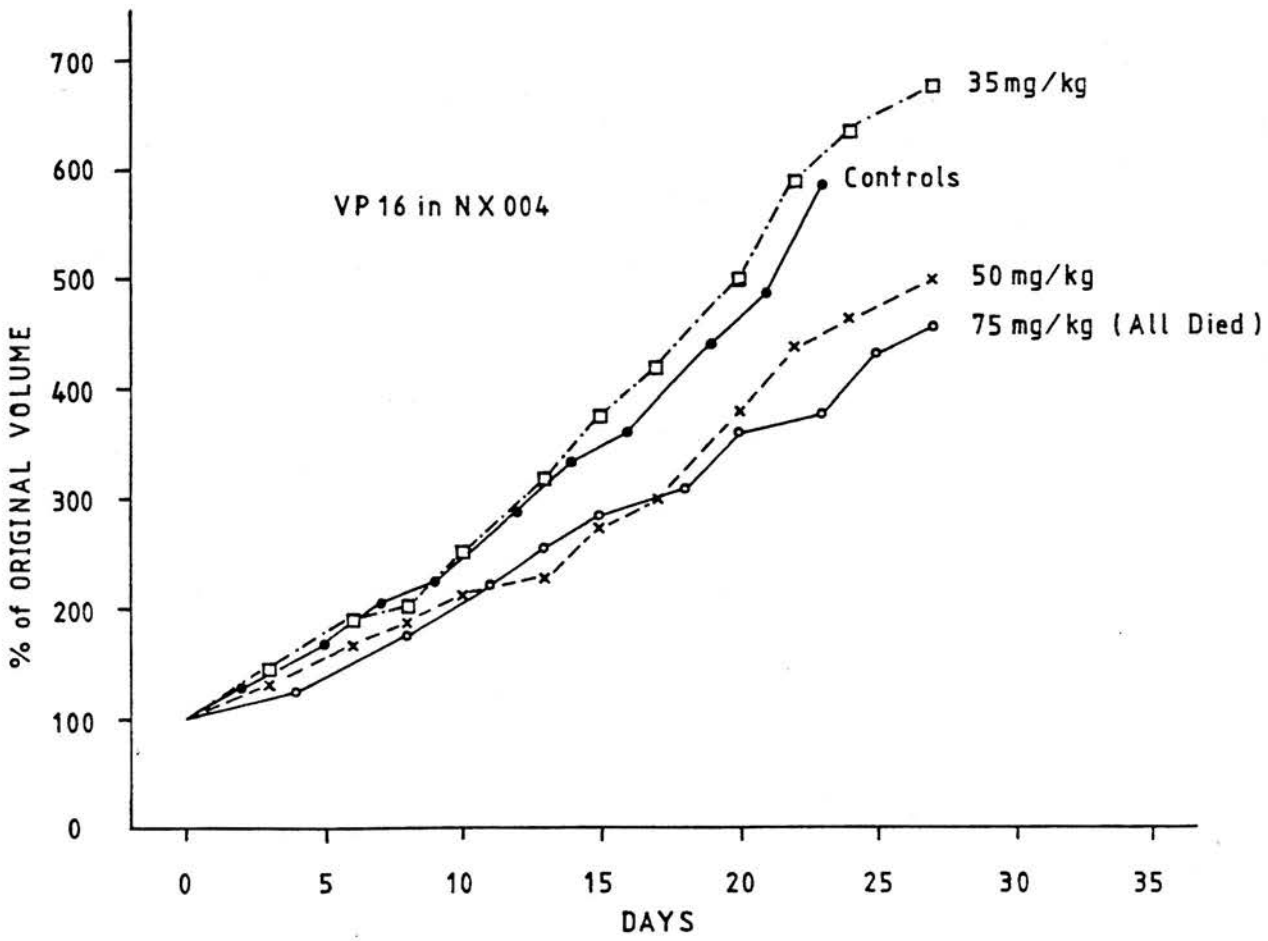


Figure 7.6: Dose-response effect of etoposide in NX004/2

shown in Figure 7.7. Control tumours doubled in 7 days. Etoposide as a single agent had no significant effect (median TD = 8 days, SGD = 0.14). The addition of IFN to etoposide did not influence its activity (median TD = 7 days), indeed tumours in the combination group appeared if anything to grow quicker than controls although significant antagonism was not seen ($p>0.1$).

7.9 CDDP/IFN combination in NX004/6

A full dose/response experiment was not performed for CDDP in this tumour. CDDP was given in the same schedule described for the NSCLC xenografts i.e. 1.4mg/kg x 5 at weekly intervals. Control tumours had a median TD of 7 days. IFN alone had no activity (median TD = 7 days). Of the seven tumours treated with CDDP alone, 3 regressed completely and four doubled in size (in 7, 9, 13 and 28 days). The median TD for the CDDP/IFN combination group was 7 days, the same as the controls and IFN as a single agent. This represented significant antagonism between IFN and CDDP ($F=5.1$, $p=0.03$)

7.10 CY/IFN combination in WX322/4

The dosage schedules for this combination experiment were identical to those outlined for NX004 in section 7.4. The control tumours doubled in 11 days. IFN alone had no effect. Tumours treated with either CY or CY and IFN remained fairly static in volume with no difference between the two groups. Unfortunately after approximately 28 days the majority of animals in these two groups developed signs of infection with wasting and rapid breathing. Equal

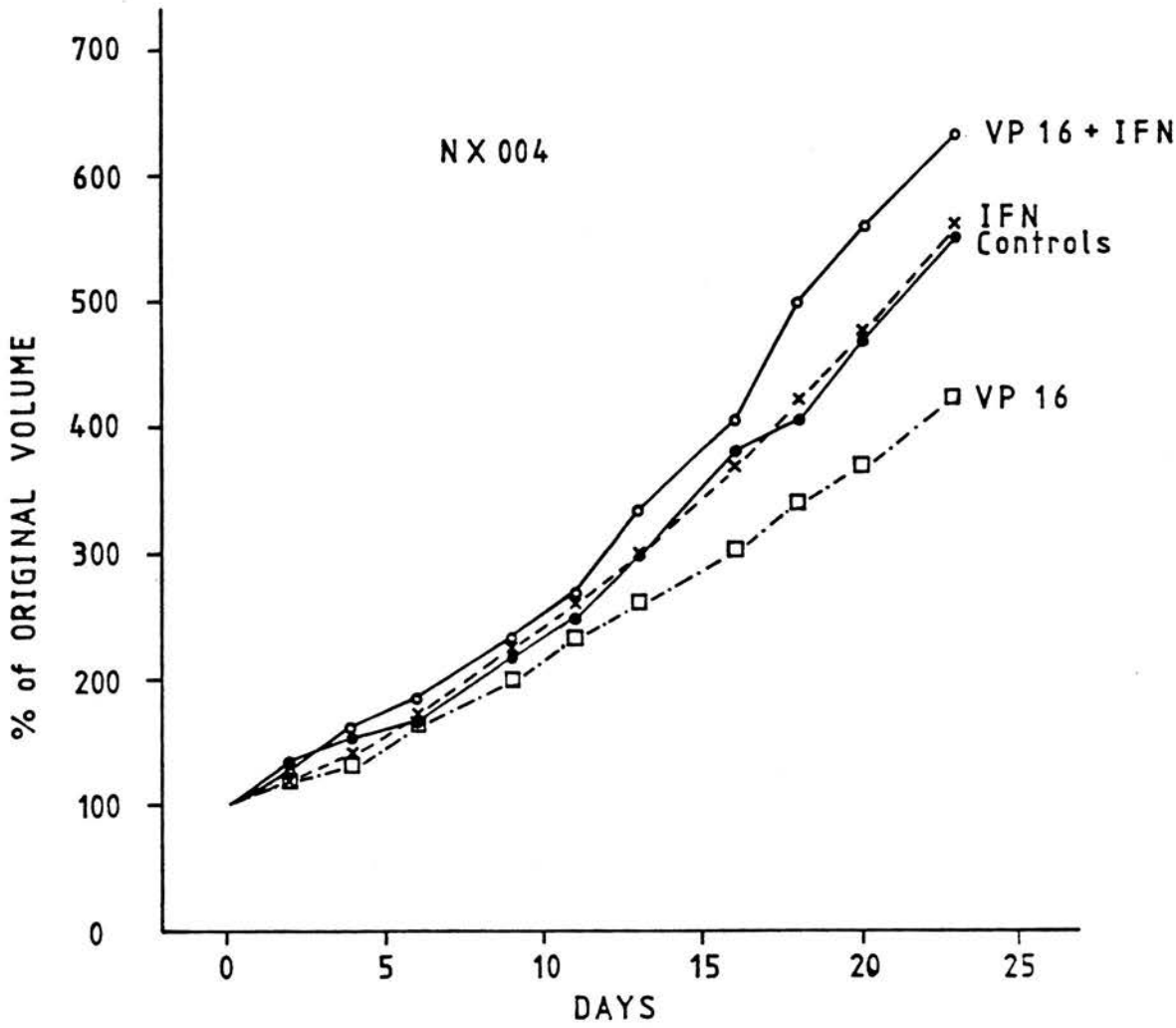


Figure 7.7 Etoposide/IFN combination in NX004/5

numbers of animals were affected in the two groups concerned. This was the last experiment performed (April 1986) prior to the serious infection described in section 5.4.

7.11 Etoposide/IFN combination in WX322/4

The dosage schedules for etoposide and IFN were as oulined for NX004 in section 7.8. Etoposide was given as 9mg/kg i.p. x 5. Control tumours doubled in 15 days. Etoposide alone had significant activity (median TD = 32 days, SGD = 1.13) with two tumours out of nine remaining static. When the drug was given in combination with IFN it appeared less potent (median TD = 20 days, SGD = 0.33) with one tumour remaining static. This was an additive result ($F=1.28$, $p = 0.27$)

7.12 Discussion

This section has assessed the effect of combining IFN with four different drugs (CY, ADR, Etoposide and CDDP) in two SCLC xenografts. IFN alone had no effect on the growth of either tumour. On no occasion was the activity of a drug enhanced by the addition of IFN, indeed in many cases the IFN/drug combination appeared less effective than the drug as a single agent, suggesting a negative interaction. However, only one combination (IFN and CDDP in NX004) showed significant antagonism. These data are in marked contrast to the experimental results described for the two NSCLC xenografts in chapter 6.

The two SCLC tumours used were more sensitive to certain agents (CY, etoposide in WX322 and CY, CDDP in NX004) than NX002 and CX117 with single agent treatment sometimes causing complete stasis in growth of the xenograft. However it should be noted that NX004 had a rather unusual sensitivity pattern, being sensitive to CY and CDDP but relatively resistant to ADR and etoposide. WX322 appeared a more chemosensitive tumour but overall in both tumours IFN was unable to improve on single agent drug activity whether the xenograft was sensitive or resistant to the drug in question.

It is unclear why IFN/drug combinations appear more active than single agents in NSCLC but not SCLC tumours. The dose and schedules of administration for each agent were identical and the xenografts were grown in the same type of host. Presumably therefore it must reflect a fundamental biological difference between the two tumour types.

It is not known why in the clinical situation small cell tumours are generally more sensitive to chemotherapy and irradiation than non small cell tumours. The fact that IFN can interact synergistically with certain drugs in NSCLC xenografts but antagonistically with the same agents in SCLC xenografts may help to shed light on important differences between the two histological subtypes of lung cancer. However this may not be possible until the exact mechanism by which thse IFN/drug interactions are mediated is fully elucidated.

CHAPTER 8

Optimising IFN/drug schedules

8.1 Introduction

The concept of combining IFNs with other anti-cancer treatment modalities has generated much interest both in the laboratory and clinic (Section 3.3). Many combinations of different IFNs and anti-cancer agents have been assessed using pre-clinical models and pilot studies are underway to test these treatments in patients. Unfortunately little attention has been paid to defining the optimum dosage schedules for administering these combinations and many different regimes are being assessed.

Laboratory data (Aapro 1983, Balkwill 1982, Welander 1985) suggest that IFNs have most anti-tumour activity when administered in a chronic schedule, but this method of treatment may not be optimal for cytotoxic drugs.

The experiments reported in this chapter were designed to try to establish the optimum method for administering IFN in combination with a cytotoxic agent using a combination studied in chapter 6 (IFN and CDDP in NX002 - Section 6.2). Both the influence of dose and schedule for each agent was assessed as well as the possible benefits of pretreating tumours with IFN prior to the administration of CDDP.

8.2 Methods

Drug testing was performed as described before. Groups of 8-10 tumours were stratified for size and randomly allocated to treatment or control groups. Treatment activity was expressed in terms of the specific growth delay where possible. Statistical analysis was as stated in chapter 4. These studies were performed between the 15th and 19th passages of NX002. The effect of different dosage schedules for each agents in the IFN-CDDP combination was assessed as follows:

(i) IFN-α2b (2×10^4 units/mouse/day) was given s.c. for 35 days in combination with the maximum tolerated dose of CDDP (7mg/kg) given i.p. as either 1.4mg/kg x 5 (on days 0,7,14,21 and 28) or 3.5mg/kg x 2 (on days 0 and 10) or as a single 7mg/kg dose on day 0.

(ii) CDDP (1.4mg/kg x 5) was given in combination with three different doses of IFN (5×10^4, 10^5 or 2×10^5 units/day) given s.c. for 35 days.

(iii) The effectiveness of pretreatment with IFN followed by treatment with CDDP alone was compared with simultaneous treatment with both agents. The design of the treatment groups is shown in Figure 8.1. One group received IFN (2 x 10 units/day) for 3 days before a single 5mg/kg dose of CDDP. A second group was given the two treatments simultaneously from day 0 to day 10. A third group received the same combination treatment after 3 days pretreatment with IFN. Treatment activity was assessed by comparing the mean tumour volumes and percentage increase in volume at days 0 and 10 rather than measurement of the SGD. It was felt that measurement of tumours for the initial 10 days would most accurately reflect the effect of pretreatment.

	DAY	-3	0	10
(i)	Group 1	IFN	CDDP (5mg/kg)	
(ii)	Group 2		CDDP + IFN	
(iii)	Group 3	IFN	CDDP + IFN	
(iv)	Group 4		CONTROL	

Figure 8.1: Design of treatment groups to assess the effectiveness of pretreatment with IFN

8.3 Results

(i) Different doses and schedules of CDDP The results of these experiments are summarised in Table 8.1. Control tumours had a median doubling time of 8-12 days. IFN alone at this dose again had no activity (SGD<0.2). The addition of IFN enhanced the activity of all three doses of CDDP with the maximum effect being seen with the middle dose (3.5mg/kg x 2). In this group no tumour doubled in size (SGD for CDDP alone = 4.25). A single high dose of CDDP was least effective (SGD for combination = 3.1, for CDDP alone = 2.8). The interactions with CDDP 1.4mg/kgx5 and 7mg/kgx1 appeared additive ($F<2$, $p>0.1$) with the middle dose showing a positive interaction ($F=5.28$, $p=0.03$). However meaningful comparisons between these groups were impossible as the experiments were performed at different times.

(ii) Different doses of IFN The results of these experiments are summarised in Table 8.2. Control tumours had a median doubling time of 8 days. CDDP alone (1.4mg/kg x 5) caused a prolongation of median doubling time to 14 days (SGD = 0.75). This effect was enhanced by the addition of all three doses of IFN. However statistically significant interactions were not seen. The middle dose of IFN (10^5 units/mouse/day) appeared to be the most effective when combined with this dose of CDDP (SGD increasing from 0.75 to 7.0). The activity of IFN alone at these doses was not assessed but from previous work (Carmichael 1986), the highest dose given here (2×10^5 units/mouse/day) resulted in a SGD of 0.34.

		Median doubling Times (days)	SGD	Regressions	Static
	Controls	9	-	-	-
CDDP 1.4 mg/kg x 5	IFN	10	0.11	-	-
	CDDP	17	0.88	-	-
	CDDP + IFN	27	2.00	2	-
	Controls	8	-	-	-
CDDP 3.5 mg/kg x 2	IFN	9	0.12	-	-
	CDDP	42	4.25	2	-
	CDDP + IFN	*	-	2	-
	Controls	12	-	-	-
CDDP 7 mg/kg x 1	IFN	12	0	-	-
	CDDP	46	2.8	3	-
	CDDP + IFN	49.5	3.1	3	-

* Calculation of doubling time and SGD impossible in this group as no tumour doubled

TABLE 8.1: The effect of three different schedules of CDDP alone and in combination with a fixed dose of IFN (2×10^4 unit/mouse/day for 35 days). Experiments performed once on separate occasions.

	Median Doubling Time (days)	SGD	Regressions	Static
Controls	8	-	-	-
CDDP alone	14	0.75	2	-
CDDP + IFN (5×10^4u/day)	43	4.3	1	-
CDDP + IFN (10^5u/day)	64	7.0	2	2
CDDP + IFN (2×10^5u/day)	48	5.0	-	-

Table 8.2: The effect of a fixed dose of CDDP (1.4mg/kg x 5 at weekly intervals) alone and in combination with three different doses of IFN. Experiment performed once.

		Mean Tumour Volume (cm^3)		
		DAY -3	DAY 0	DAY 10
IFN	CDDP	.45	.58 (129)	.60 (134)
	CDDP + IFN	.42	.60 (140)	.58 (136)
IFN	CDDP + IFN	.44	.62 (138)	.55 (124)
Control		.48	.63 (129)	1.27 (261)

Table 8.3: Influence of IFN pretreatment on IFN/CDDP combinations in NX002/20. Figures in brackets represent % increase from starting volume on day -3. Experiment performed once.

(iii) Pretreatment with IFN The results of these experiments are summarised in Table 8.3. Control tumours grew steadily reaching 261% of their original starting volume at the end of the study (14 days). Pretreatment with IFN did not influence tumour growth from days -3 to 0. Sequential treatment with 3 days of IFN followed by 10 days of CDDP was as effective in suppressing growth as 10 days of combination treatment. Indeed no two groups were significantly different at the $p<0.05$ level at any stage apart from at day 10 when all treated groups were significantly different from controls ($F=10.33, p<0.0001$) but not from each other.

8.4 Discussion

The results of these experiments indicate that both dose and schedule can influence the activity of combination therapy with IFN and CDDP in this tumour. Statistically significant interactions were only seen once and the overall design of the experiments made valid comparisons impossible. Larger experiments performed repeatedly are required before firm conclusions can be reached. This may be best done using an in-vitro model. The effectiveness of pretreatment with IFN remains unclear.

Previous studies performed using animal models of human cancer have shown that the administration of larger doses of IFN results in greater inhibition of tumour growth (Balkwill 1982, 1984, Welander 1985, Carmichael 1986). A similar dose-response relationship has been demonstrated in phase II studies of IFN therapy in patients with Kaposi's sarcoma (Real 1984), renal cancer (Quesada

1983) and mycosis fungoides (Bunn 1984). This effect presumably reflects the direct cytotoxic actions of IFNs on tumour cells and may not be relevant to IFN/drug combinations. Of the few reported laboratory studies of IFN/drug combinations which investigated the effect of dose of either agent, most found increased synergy at higher dosage levels (Balkwill 1984, Durie 1986, Welander 1985). However one group (Morris 1984), was only able to demonstrate potentiation if low doses of IFN were used.

No in vivo studies have been reported which specifically evaluate the importance of scheduling on the activity of IFN/drug combinations. The results from in vitro testing suggest that exposure to both agents over a prolonged time interval is important (Aapro 1983, Welander 1985) and many of the clinical studies currently assessing these combinations have attempted to achieve this (Durie 1986, Green 1985). Certainly IFN and other biological response modifiers as single agents are most effective both in the laboratory and the clinic when given in a chronic schedule (Balkwill 1982, Spiegel 1985, Vaage 1987, Welander 1985).

Whether IFN and drugs in combination are best administered concurrently or sequentially is unclear. Welander (1985) studied this scheduling effect in vitro with a variety of drugs and cell lines. Although positive interactions were observed when the two agents were given sequentially, the most potent synergy was demonstrated with combined continuous exposure. In the one experiment reported here, no difference was seen between sequential treatment with IFN and CDDP (i.e. 3 days IFN followed by 10 days CDDP) and combined therapy (i.e. 10 days CDDP + IFN). Unfortunately

it was not possible to include a group treated with CDDP alone to ensure that a positive interaction between CDDP and IFN had occurred. If no such interaction had occurred, then the activity seen with both forms of treatment would merely reflect 10 days of CDDP treatment (since IFN alone has no effect) and would obviously cause similar delay in growth with each group. Data from experiments in Chapter 6 would suggest that it may take at least 10 days of combined treatment with IFN and drugs before significant synergy can be appreciated. Clearly this makes the results of these experiments investigating the effect of three days pretreatment with IFN difficult to interpret.

What is the relevance of this work to the design of clinical trials? A major factor preventing the prescribing of IFN/drug combinations on a rational basis lies in the fact that the precise mechanisms of synergy between the two agents is poorly understood. Many of the cellular effects of IFN have a complex dose-response relationship and some (e.g. NK cell activity) may even be inhibited in patients at higher doses (Edwards 1985). Another potential problem in the design of IFN/drug combinations may lie in the fact that individual patients have been shown to exhibit totally different degrees of tissue responses (e.g. modulation of 2-5A synthetase) to the same dose of IFN (Merritt 1986). Until more is known about the precise mechanisms of the interactions between IFN and cytotoxic drugs, the design of relevant clinical studies will remain difficult.

CHAPTER 9

Possible Mechanisms of Synergy

9.1 Introduction

The results obtained in Chapter 6 have suggested that an interesting interaction occurs between interferon and certain cytotoxic agents when tested against human NSCLC xenografts in mice. Whilst other workers have reported similar results in other test systems (Chapter 3), little attention has been paid to explaining why such synergy may occur. Most reports comment on the many different actions of IFNs on tumour cells and host responses and are eager to point out the possible clinical potential of combining IFN and cytotoxic agents. In the scope of this project it was not felt possible to investigate these mechanisms in detail. However two sets of experiments were performed.

One possible explanation for the apparent increase in the activity of cytotoxic drugs in animal experiments may be that IFN potentiated the toxicity of the drugs to the host and subsequent tumour shrinkage was due to ill health in combination treated groups. To assess this, we have measured the body weight of mice undergoing combination treatment for 35 days as an assessment of animal health and compared these mice with control and single agent groups.

Secondly we have assessed the possible influence of cell cycle effects in IFN/drug combinations as in the one reported study which looked at possible mechanisms of interaction (Balkwill 1984a), these effects appeared to be important.

9.2 Body weight study

(i) Methods Clearly it was important to investigate a combination where positive interactions were seen. Treatment with IFN and cyclophosphamide in CX117/10 (section 6.6) was selected. Groups of 6-8 tumours (5-6 animals) were randomly allocated to either a control group, to receive cyclophosphamide 40mg/kg i.p. at weekly intervals, IFN 2×10^4 units/mouse/day s.c. daily for 35 days, or to a combination group. Animals were weighed each week and the lean body weight was calculated by subtracting the weight of the tumour(s). This was estimated using the correlation between tumour size and weight shown in figure 5.1. This correction prevented an overestimation of body weight in control animals with growing tumours and a possible false weight loss in animals whose tumours were smaller because of treatment.

(ii) Results These are shown in figure 9.1. The mean weekly weight of control animals increased steadily through the four week period. No significant difference was seen in the mean weekly weight of the other three groups. Treatment with IFN and cyclophosphamide was certainly not associated with weight loss when compared to cyclophosphamide alone. The animals in both these groups appeared healthy throughout the study. Although sporadic deaths (probably

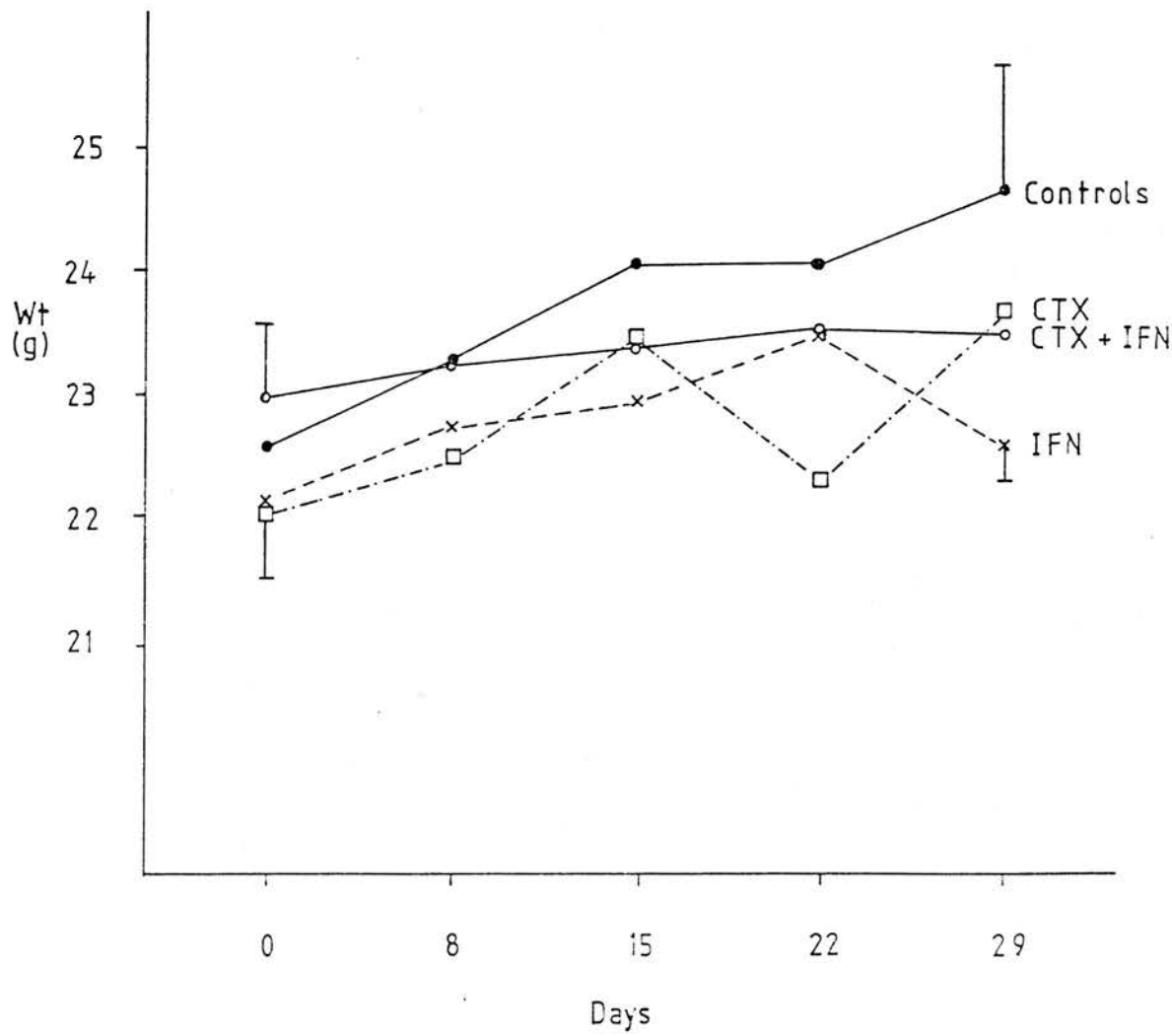


Figure 9.1: The effect of single agent and combination treatment with cyclophosphamide (CTX) and IFN on mean body weight in CX117/10

related to intercurrent infections) did occur in all the xenograft experiments (see section 5.4) an excess of deaths or increased ill health was not seen in groups of animals treated with combination therapy compared to single agent treatment. It can be concluded that any enhancement of cytotoxic drug activity by IFN in xenografts is not mediated through increased toxicity to the host animal.

9.3 Cell cycle effects

In vitro studies have demonstrated that IFNs are capable of increasing the length of all phases of the cell cycle in malignant cells (Balkwill 1978). Many cytotoxic agents are known to be more effective in specific phases of the cell cycle and therefore it is possible that IFNs may potentiate a cycle-specific drug by increasing the number of cells in a certain phase. We have studied the effect of drug and IFN treatment on the cell cycle of tumour cells taken from xenografts treated with two combinations, one where a positive interaction occurred and one where no such interaction was seen.

(i) Methods

Two drug/IFN combinations were assessed: CDDP and IFN in NX002 (section 6.2) and CYCLO and IFN in NX004 (section 7.4). The doses used were identical to those used in the original experiments. Ten animals were included in each group. Two animals were sacrificed on day 0 (pretreatment) and then each day until day 4 after treatment. IFN was given daily to the surviving animals in the IFN and combination groups in the study. The drugs were given once on day 0.

Cell suspensions were prepared using the method described by Martens (1980). Briefly, the tumours were removed and chopped finely with crossed scalpels in RPMI 1640 medium. The suspension was shaken and gently pipetted through a 100 um^2 nylon mesh. The resulting suspension was spun down at 1000rpm for 5 mins and then resuspended in Hanks' buffered salt solution containing 10mM HEPES. The cells were then centrifuged for 10 mins at 400g and resuspended in 0.9%NaCl. They were then added dropwise to twice the volume of 96% ethyl alcohol at $-20^{o}C$. The samples were then stored at $4^{o}C$ for later use.

DNA staining was performed on the entire batch of samples prior to fluorescence measurement. Cells were taken from the pellet, adjusted to a concentration of 1×10^6 cells/ml and spun down at 1000rpm for 5 minutes. Cells were resuspended in 1 ml of staining mixture comprising 0.005% propidium iodide in 0.1% sodium citrate in the presence of 0.1% triton X. This stain was stable for 2-3 hours.

Cells were analysed with a FACS-II cell sorter (Becton Dickinson, Sunnyvale, Ca) using a 488mm argon laser. The percentage of cells in each phase of the cell cycle was analysed from the resulting histogram printout using the mathematical model of Dean (1980). The mean % values of different groups at any one time were compared by analysis of variance.

(ii) Results

The changes in cell cycle distribution during the first four days of each treatment regime is illustrated in Figures 9.2 and 9.3. In NX002 where a positive interaction occurred between IFN and CDDP little change in cell cycle distribution was seen in any group. IFN certainly did not affect the proportion of cells in each phase of the

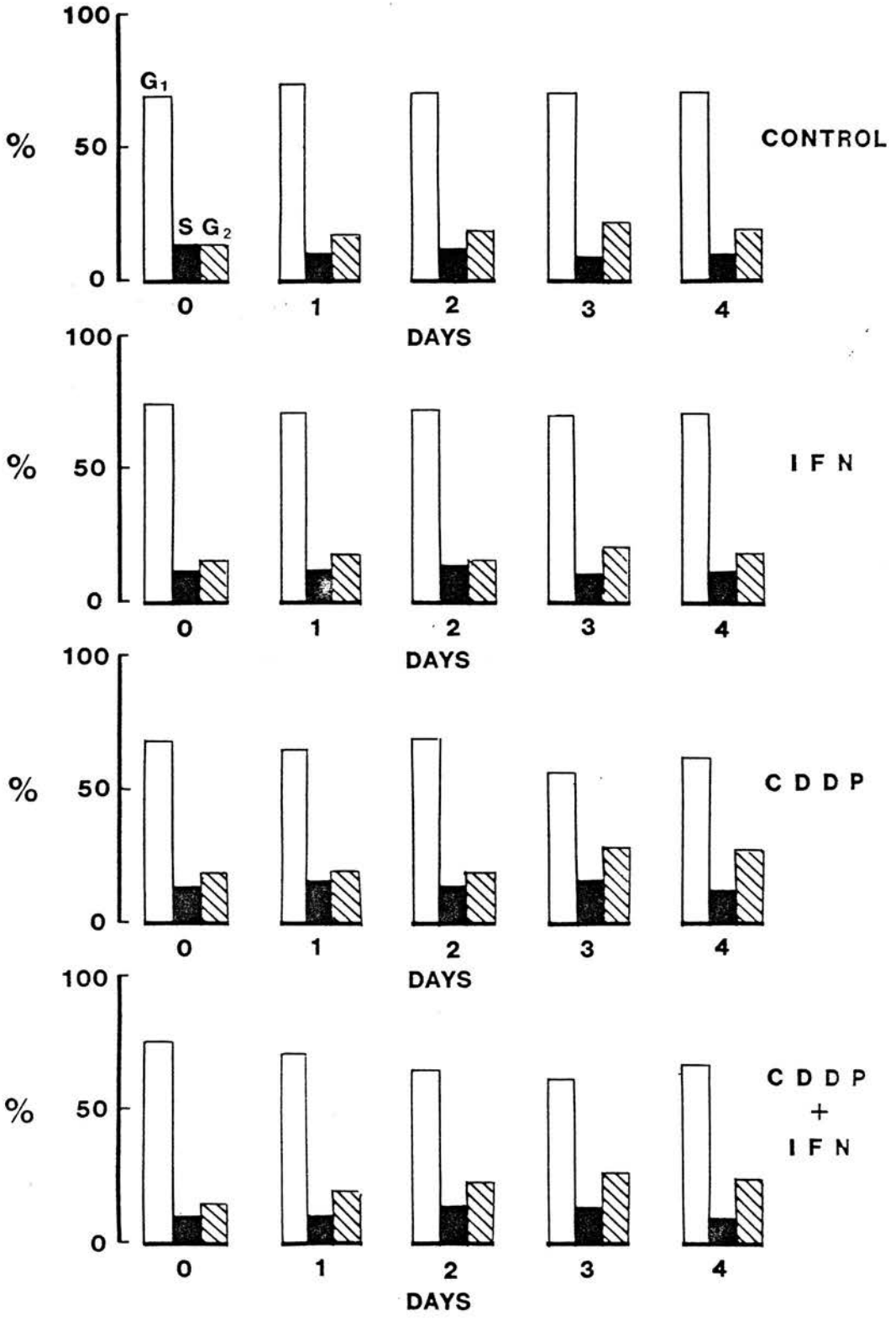


Figure 9.2: Changes in cell cycle distribution in NX002 after IFN/CDDP therapy

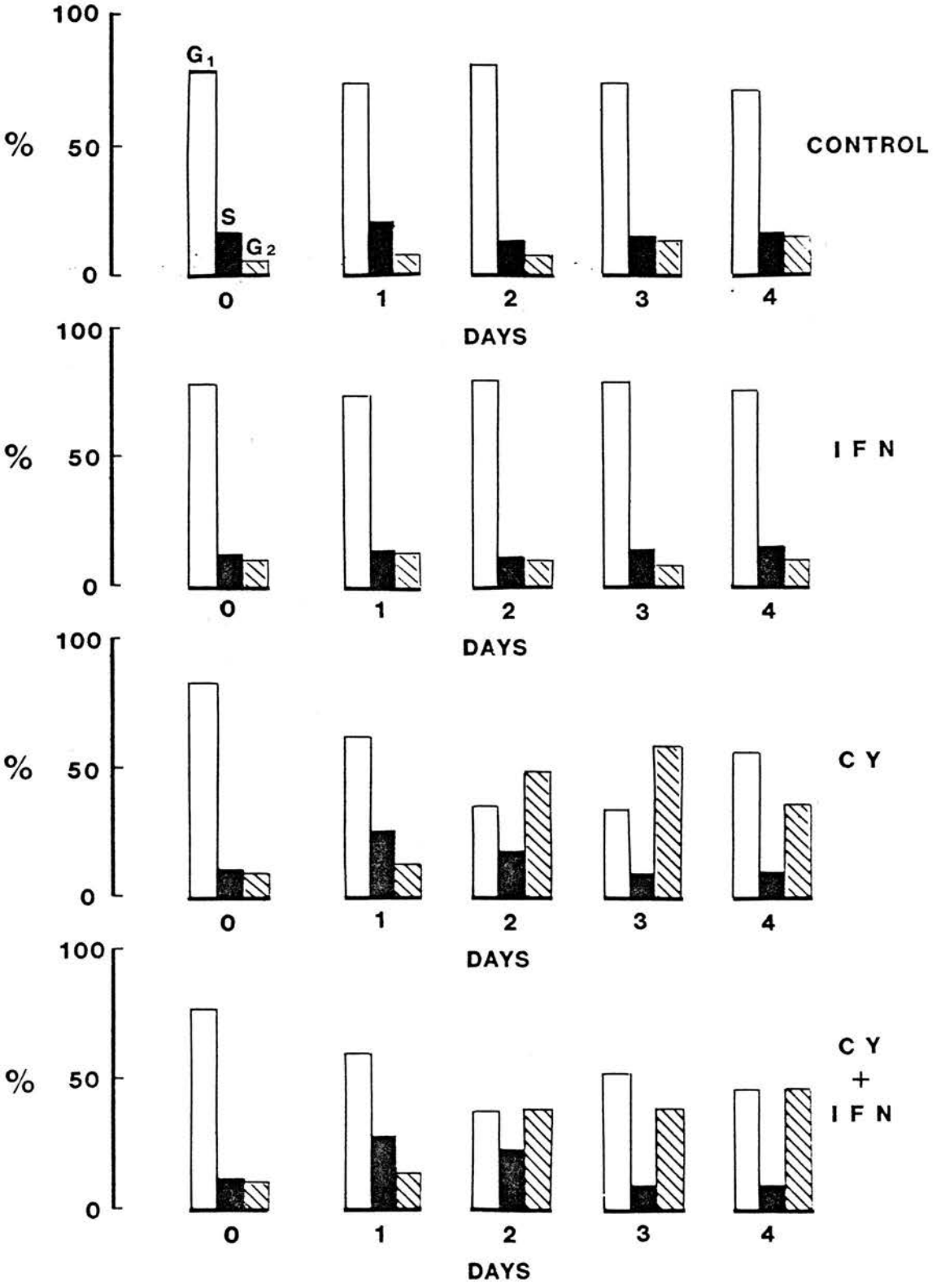


Figure 9.3: Changes in cell cycle distribution in NX004 after IFN/CY therapy

cycle. In both the CDDP and the CDDP/IFN groups there was a small rise in the percentage of cells in G2 on days 3 and 4. There was no difference between these two groups.

In NX004 where no interaction between CY and IFN was seen, more marked changes were seen reflecting the influence of CY on the cell cycle distribution. IFN alone had no effect compared to controls. In the CY and CY/IFN groups an immediate increase in the percentage of cells in the S phase was seen. This was followed 24 hours later by a sharp rise in the proportion of cells in G2. Although this effect was perhaps more marked in the CY alone group, no significant differences between these groups were seen. There was certainly no early and sustained rise in S phase cells in the combination group which has been reported by Balkwill (1984a).

9.4 Discussion

It is likely that interactions between IFN and chemotherapeutic agents are complex. There may be direct interactions between the drugs and IFN on tumour cells or indirect effects of IFN on drug metabolism or host defences. All these mechanisms have been postulated in previous studies of IFN/drug combinations.

Aapro (1983) showed synergistic effects between the vinca alkaloids and leucocyte interferon in a clonogenic assay and hypothesised that this may have been the result of the known effects of vinblastine on tubulin and the action of IFN on the cyto-skeleton. As well as suggesting that phase I-II clinical trials should be started, they also conceded that "mechanistic studies would be needed

to delineate the nature of the potentiating drug interaction".

Saito (1987) studied IFN gamma and various drugs in a similar assay system. By introducing macrophages from malignant effusions they showed a potentiation of the activity of IFN gamma alone as well as in combination with doxorubicin. However the efficacy of the two agents was never more than additive. The role of macrophages in the efficacy of cytotoxic drugs is poorly understood. Colotta (1986) has shown that the combination of cytotoxic agents and macrophages enhances the susceptibility of tumour cells to macrophage killing. The demonstration of this effect required direct contact of the macrophages with the tumour cells. As it is well known that IFNs (especially IFN-gamma) are capable of activating macrophages for tumour cell killing (Pace 1983, Schultz 1978, Svedersky 1984) this is a possible mechanism by which IFNs and drugs could interact synergistically. However, IFN gamma has shown little such potential in assay systems compared to IFN alpha and it would not explain why positive interactions have been seen in-vitro in the absence of tumour associated macrophages.

It seems more likely that IFNs and drugs interact directly on tumour cells. This was certainly the conclusion of the one elegant study (Balkwill 1984a) which has investigated this effect in some detail. The authors had shown previously (Balkwill 1984) that human IFN potentiated the effect of adriamycin and cyclophosphamide in a human breast carcinoma in nude mice. They then showed that human IFN did not affect hepatic levels of key drug metabolising enzymes whereas murine IFN did. The addition of murine IFN to the human

IFN//drug combinations had no effect on the final result and did not increase the toxicity of the combination therapy. They concluded that the increased activity of the combination was due to effects on the tumour rather than the host. Additional studies investigating the effect of this treatment on cell cycle distributions suggested that IFN, which alone had little effect on the cell cycle, appeared to increase the number of cells in S phase when given with cyclophosphamide or adriamycin. This did not happen with CY or ADR alone. They hypothesised that since both drugs would be more active against cells actively synthesising DNA, the S phase accumulation seen in the combination therapy groups was responsible for the enhanced cytotoxicity.

In our brief studies in this section, we have shown that any potentiation of alkylating agents by IFN is not mediated through increased toxicity to the host animal. This would certainly explain why synergy has been clearly demonstrated in vitro. Like Balkwill we have shown that IFN alone has little effect on cell cycle kinetics. We have shown like others (Balkwill 1984a, Barlogie 1978) that treatment with CY is associated with an induction of G2 phase arrest. Unlike Balkwill, we have not shown that the addition of IFN delayed this G2 block and increased the number of cells in S phase. However since this combination was not synergistic in this tumour we cannot rule out this effect as a possible mechanism of interaction in other systems.

CDDP is thought to exert its cytotoxicity by forming interstrand and intrastrand crosslinks in tumour cell DNA and although the specificity of CDDP with regard to the phase of the cell cycle appears to differ among cell types (Zwelling 1982), the effects on

cross-linking are most pronounced during the S phase. In our studies neither CDDP, IFN or the combination had any significant influence on cell cycle distribution of a tumour where positive interactions were seen. We must therefore conclude that although it is likely that these effects are mediated directly on the cell, modulation of cell cycle distribution is not the sole mechanism involved.

Summary

The experiments reported in this section have demonstrated that potentially important interactions between IFN and cytotoxic agents can occur. Synergy was seen between various cytotoxic agents and low doses of alpha IFN in NSCLC xenografts with the most marked effect being seen with CDDP and platinum analogues and IFN. No such effect was seen with various drugs and IFN in two SCLC tumours and indeed there was some evidence that antagonistic interactions had occurred. The section on dosage and scheduling was unhelpful and requires repeating perhaps using an in-vitro model. More studies investigating the precise mechanisms of these interactions may be required before these combinations can be properly exploited in a clinical setting.

SECTION III : IN VITRO STUDIES

CHAPTER 10

Methods

10.1 Introduction

In Section II the activity of combination therapy with interferon alpha and various cytotoxic agents was studied in vivo using human lung cancer xenografts. This system allows for drug testing on solid tumours in a physiological 'environment' but complex variables such as tumour vascularity, host rejection responses and drug penetration and kinetics can make interpretation of drug activity difficult. This is especially true when biological response modifiers are being assessed. In this section an attempt was made to measure direct cytotoxic effects by testing these combinations against lung cancer cells in vitro.

In order to try and make direct in vivo/in vitro comparisons assays were attempted on cells taken from the xenografts tested in Section II. In vitro assessments of drug/IFN combinations were also performed using human lung cancer cell lines established in other centres. In one of these cell lines (A549) a xenograft line was established in the thymectomised, irradiated mouse model to allow further in vitro/in vivo comparisons.

Two types of clonogenic assay were performed to assess treatment activity. In the cell lines which grew as floating aggregates and

for cell suspensions prepared from xenografts, a soft agar (Courtney) assay was used. In the cell lines which grew as an adherent monolayer, a simple 'plastic' cloning assay was used.

10.2 The Courtney Assay

This assay system measures the effect of treatment with cytotoxic agents on the cells within a tumour which are capable of forming a clone and repopulating the tumour (the 'stem cells'). The percentage of these cells within a tumour may be low and the cloning efficiency may be further reduced by cell damage during disaggregation and the inadequacy of culture conditions. The Courtney soft agar system represents an attempt to improve the plating efficiency of tumour cells by simulating more closely growth conditions in vivo.

Cells are cultured in a low oxygen concentration to provide a more physiological oxygen tension in the medium and the addition of rat red blood cells supplies labile growth factors lacking in standard culture media. The detailed methodology is as follows:

10.2 (a) Preparation of cell suspensions

Xenografts were removed using sterile techniques and placed in chilled RPMI medium prior to transfer to the laboratory. Non-viable tissue was then removed by dissection and the remaining tumour fragments transferred to a fresh petri dish. The tumour tissue was then disaggregated using crossed scalpels into a broth. This was

then passed through a N100 nylon mesh filter, transferred to a universal container and then spun at 2,000 rpm for 6 minutes. The supernatant was removed and the cell pellet resuspended in fresh medium. A single cell suspension was then made by flushing this preparation five times through a 19G needle. Cell numbers were then counted using a haemocytometer with viability being estimated by nigrosin exclusion. Suspensions were diluted with culture medium to 5 x the required final concentration.

When this assay was used in cell lines which grew as floating aggregates (H69 and WX322) less disaggregation was required. A small aliquot (2-5mls) of medium and cells was taken from the culture flask. A single cell suspension was obtained by flushing through a 21G needle. Cell numbers were counted using a haemocytometer and viability assessed using nigrosin exclusion. The suspension was diluted to 5 x the required final concentration with culture medium.

10.2 (b) Preparation of rat red cells (RBCs)

Adult male and female August rats were obtained from Bantin and Kingman (Hull). Animals were anaesthetised with ether and laid supine. The fur on the anterior chest wall was soaked with ethanol. Blood was withdrawn by cardiac puncture into a sterile syringe containing a small quantity of preservative-free heparin. The blood was then ejected into a universal container and the level of the meniscus marked. 10mls of PBS was then added. The blood was spun at 3,000 rpm for 15 minutes. The buffy coat was removed with the supernatant and discarded. The RBCs were rinsed twice with PBS and

then resuspended to the original blood volume in RPMI medium. The resultant sample was then heat inactivated for 1 hour at 44°c. RBCs were stored for up to 1 month at 4°c.

10.2 (c) Assay procedure

RPMI 1640 medium supplemented with 10% foetal calf serum and 2.5mg/ml bovine insulin, 1mM sodium pyruvate and MOPs were used in all cultures. Tumour cell cultures were diluted to 5 x the required final concentration. Rat RBCs were diluted 1:8. Drugs were made up in PBS and diluted to 10 x the required final concentration. 5% agar solution was sterilised in a pressure cooker and then diluted with warmed medium to make a 0.5% agar solution (2mls of 5% agar and 18mls medium).

To set up 5 replicate tubes for a single drug concentration the following was measured out into a glass universal container and kept at 37°:

1.2mls of tumour cell suspension
0.6mls of rat RBCs
0.6mls of drug or control (PBS)

3.6mls of freshly prepared 0.5% agar medium was quickly added to make up to 6mls (0.3% agar). This was rapidly mixed by inversion and then using a pastette 1ml aliquots were pipetted into 5 Falcon tubes before the agar could set. The tubes were then placed in ice for 5 minutes to allow the agar plug to form.

Treated and control tubes were then placed in a rack and incubated in a Hereans incubator at 37°c, 100% humidity, 5% Co_2, 5% O_2 and 90% N_2. After 7 days, 1 ml of culture medium was gentle

pipetted on top of the agar plug. This was repeated at 14 days. At 21 days, 1 ml of "old" medium was carefully withdrawn and replaced with 1 ml of fresh medium.

Colony counting was performed at approximately 21-28 days. Medium was discarded using a pastette. The agar plug was decanted onto a 5cm petri dish lid and squashed flat with a grided dish to facilitate counting. Colonies of more than 50 cells were counted under an inverted microscope. Drug activity was calculated from the number of colonies surviving treatment compared to controls and expressed as a percentage.

10.3 The plastic cloning assay

Drug activity could be rapidly assessed in cell lines which grew as adherent monolayers (A549, H125, H23) by using a plastic cloning assay. In principle the assay is similar to the Courtenay assay but agar support and extra nutrients are not required.

A major advantage of this system is that since the cells grow adherent to plastic it is possible to change the culture medium at any time. This allows for cells to be exposed to cytotoxic agents for set time periods (e.g. 1 hour, 24 hours, 5 days etc.) and for scheduling of drug combinations into sequential or concurrent exposure.

Cells were harvested from culture flasks by a short exposure to 0.2% trypsin and 0.02% versin. A single cell suspension was made by flushing the suspension through a 19G needle. Cell numbers and viability were assessed with a haemocytometer using nigrosin exclusion. The cells were then carefully pipetted into 6 well

costar plates (2mls/well), the final concentration of the suspension being adjusted in light of the plating efficiency to allow approximately 100 colonies to grow in each well. The plates were then incubated at 37^{o} for 24 hours to allow the cells to attach. The following day all medium was carefully removed from each well with a pastette and replaced with 2ml of fresh medium containing the drug being tested in the required concentration. Control plates were treated with fresh medium alone. Each drug concentration was performed in triplicate.

Cells were returned to the incubator and left undisturbed. The medium was changed and drug removed at the appropriate time if limited exposure was required. In general, colony counts were performed after 5-7 days. Medium was removed by pastette and the remaining cells were washed in PBS, fixed with methanol/acetone (1:1) and stained with haematoxylin. Colonies of >32 cells were counted using an inverted microscope and drug activity expressed as the percentage colony survival compared to controls. All assays were performed in triplicate to ensure uniformity of results.

10.4 Drug/IFN scheduling

The cell lines used for combination testing in vitro are shown in Table 10.1. To enable the correct interpretation of the interactions between agents, full dose-response experiments for each agent were performed prior to combination therapy. This allowed selection of an appropriate dose for each agent in the combination.

Designation	Cell Type	Source
Courtenay Assay		
NCI-H69	Small cell	Prof. A Harris (Newcastle)
A549	Adenocarcinoma	Prof. A Harris (Newcastle)
WX322	Small cell	Xenograft
NX002	Squamous	Xenograft
Plastic Assay		
A549	Adenocarcinoma	Prof. A Harris (Newcastle)
NCI-H125	Adenocarcinoma	Dr. S Merry (Glasgow)
NCI-H23	Adenocarcinoma	Dr. S Merry (Glasgow)

Table 10.1 Cell lines used in in vitro assay

Human recombinant IFN- 2b (Schering Corporation, Bloomfield N.J.) was diluted in culture medium and tested over a range of concentrations (1-10^6 units/ml). Two cytotoxic agents, CDDP (Bristol Myers, Slough) and adriamycin (Farmitalia Carlo Erba, St. Albans), were used as single agents and in combination with IFN. Drug concentrations over the range of 10^8 to $4x10^{-6}$M(CDDP) and $5x10^{-9}$to $2x10^{-7}$M(ADR) were used.

In the Courtenay assays CDDP only was used in combination with IFN. Cells were treated continuously with each agent. No attempt was made to pretreat with IFN and drugs and then remove these agents prior to plating out in agar. In the plastic assays, combinations of IFN and both drugs were tested. Exposure of cells to agents for 24 hours and 5 days were performed.

10.5 Statistical analysis

Single agent dose response experiments showed that both ADR and CDDP had non linear curves in most experiments. The validity of merely multiplying the effects of each agent in a combination to get the expected interaction result would appear to be questionable as discussed by Steel (1979). He suggested a complex "isobole" method of assessing interactions between two agents with non-linear dose/response curves and this type of analysis was therefore undertaken. Unfortunately this method does not allow for any statistical consideration of the data in terms of assessing the significance of any interations seen.

Expert statistical advice was obtained and it was decided to test the significance of any effect of different agents both singly and in combination by analysis of variance with the data transformed to $\log_e$.

The Student-Newman-Keuls procedure was used for single agent activity, with interactions between combinations of IFN and drugs being assessed by 2-way analysis of variance using a factorial design. The null hypothesis was that interactions were additive and this allowed for separations into significant synergy or antagonisms (supra- or sub-additivity as described by Steel).

10.6 In vitro/In vivo comparisons

Direct in vitro/in vitro comparisons were attempted when drug/IFN combinations were studied in cells taken from the xenografts NX002 and WX322. The adenocarcinoma cell line A549 was established as a xenograft by injecting a cell suspension ($>10^7$ cells in 0.5ml) s.c. into the flank of a thymectomised, irradiated CBA mouse. This allowed drug testing in vivo. CDDP (3.5mg/Kg x 2) and IFN (2×10^4 units/day) were given as single agents and in combination in groups of tumour bearing mice using the typical schedules described in Chapter 4. Treatment activity was assessed using the specific growth delay and compared to the results of the same treatment applied to the cell line in vitro.

CHAPTER 11

In vitro results

11.1 Courtenay Assays

Attemps made to perform assays on xenografts taken directly from mice were unsuccessful. This was in part due to an extremely low plating efficiency and presumably due to special cultural requirements, since the small number of colonies that would form did not show progressive growth. Assays were performed on the SCLC xenograft WX322 after it was established as a cell line. The majority of Courtenay assays were thus performed on the established cell lines A549 and H69.

(i) A549 This cell line had a plating efficiency of approximately 1% in agar which allowed for easy testing. The effect of IFN and CDDP as single agents is shown in Figure 11.1. Combination experiments were performed on separate days from the single agent experiments. Marked inter experimental errors were seen (10^2u/ml of IFN caused 65% and 22% on separate days). When the two agents were given in combination a sub-additive interaction was seen. (Appendix - isobologram 1). However this was not statistically significant ($F=2.3$, $p=0.15$).

(ii) H69 The plating efficiency in agar of H69 cells was approximately 5%. The effects of IFN and CDDP as single agents are shown in figure 11.2. When compared to A549 cells, CDDP had similar activity (IC_{50} 0.3uM) but IFN was less effective (IC_{50} approx 10^5units/ml) with a flatter dose/response curve. The isobologram showing the interaction between IFN and CDDP is shown in figure 11.3.

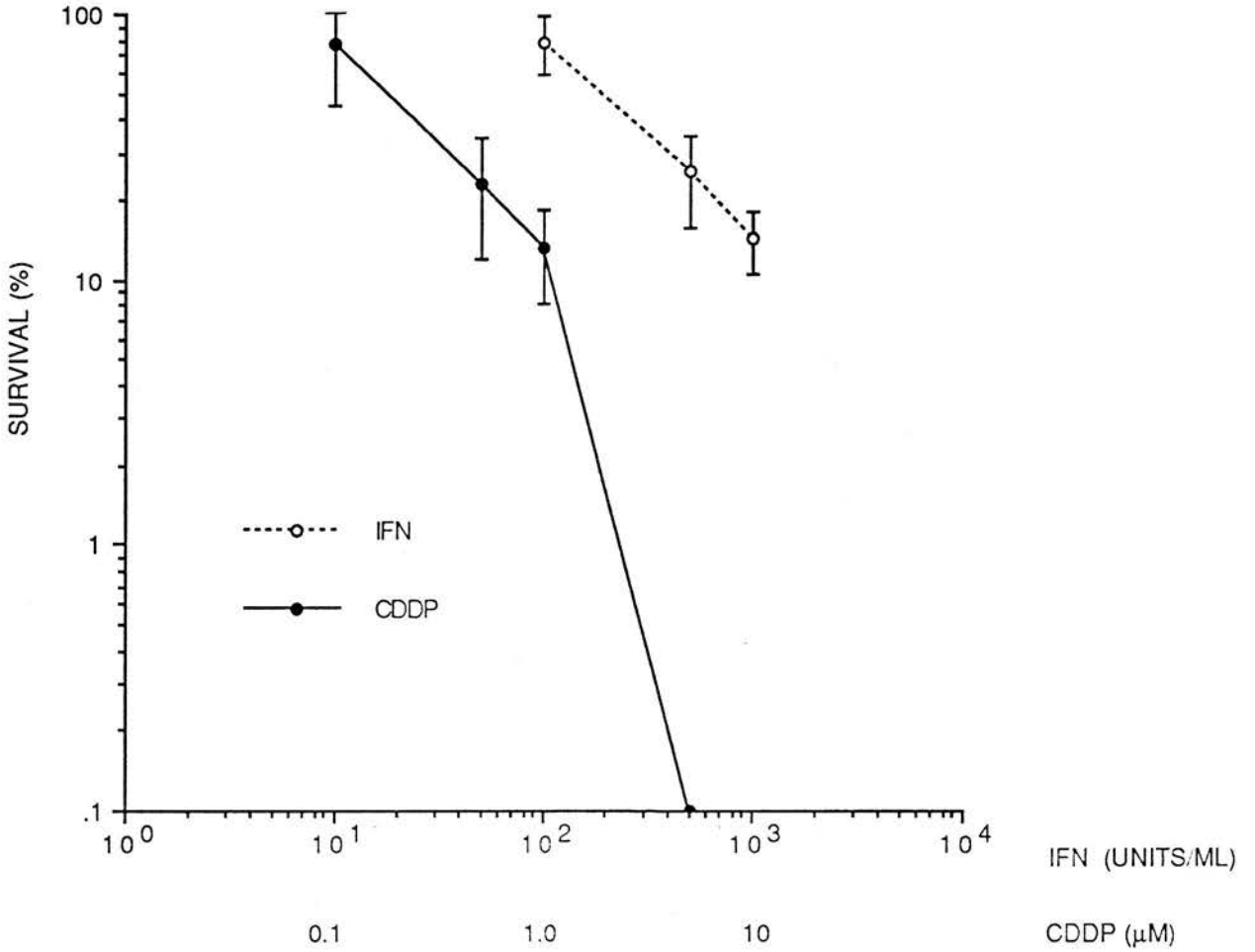


Figure 11.1 Effect of IFN and CDDP on A549

(Courtenay Assay)

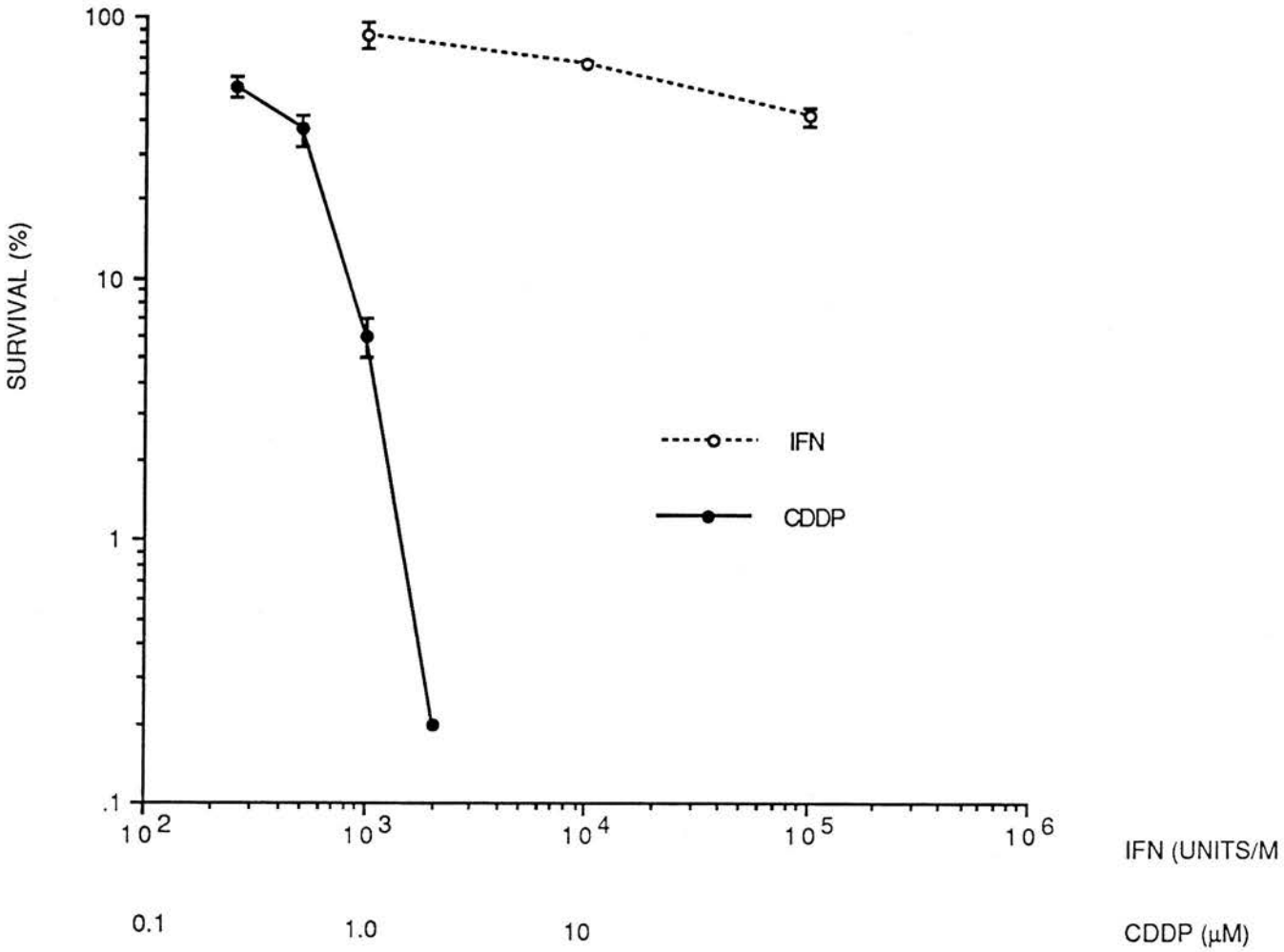


Figure 11.2 Effect of IFN and CDDP on H69 (Courtenay Assay)

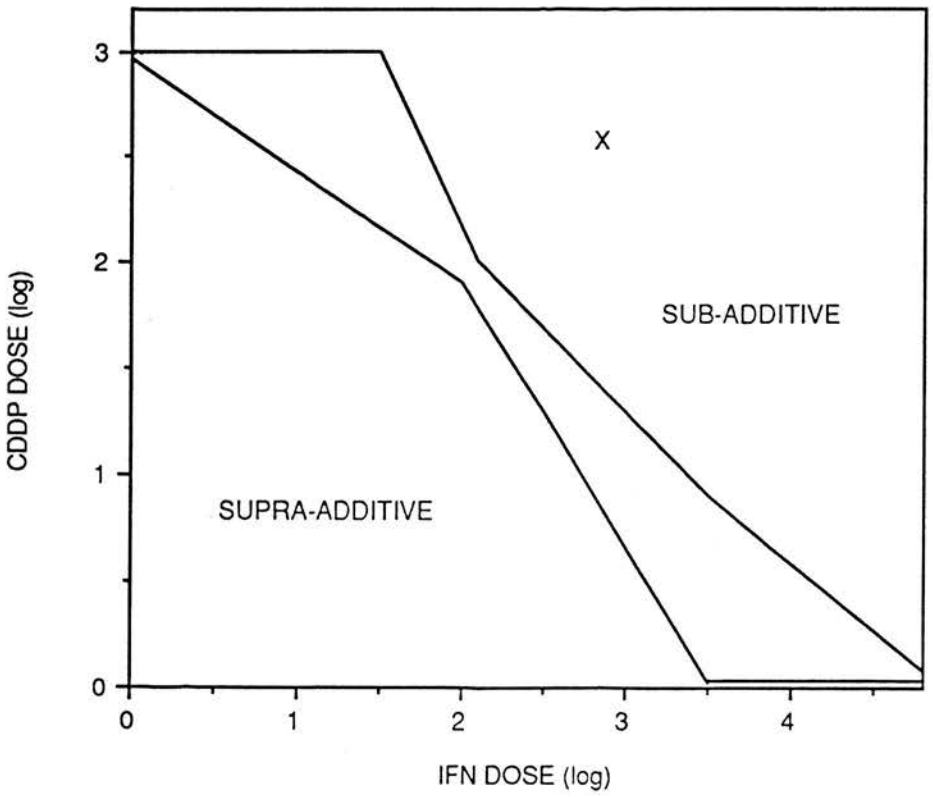


Figure 11.3 Isobologram showing effect of IFN and CDDP in combination on H69 (Courtenay Assay). Doses of each agent converted to logarithms. The isoeffect plot is drawn from single dose/response curves (for methods see Steel 1979). The cross marks the observed effect from IFN/CDDP combination.

Two different doses of IFN (10^4 and 10^3 units/ml) and CDDP (1uM and 0.5uM) were tested. Although the isobologram of their combinations suggests subadditivity, statistical analysis shows that an additive interaction occurred ($F = 0.4$, $p = 0.64$).

(iii) WX 322 As with the other xenografts, attempts to perform Courtenay assays with WX322 tumours direct from mice were unsuccessful. A continuous cell line of WX322 growing as floating aggregates was eventually established by Dr S Langdon. Courtenay assays have been performed with these cells using both IFN and CDDP. Surprisingly the WX322 cell line appears to be acutely sensitive to IFN with 10 units/ml causing a 90% inhibition of colony formation and even 1 unit/ml having a significant effect (16% inhibition). This sensitivity to IFN is not seen in vivo (see section 7.10) and presumably represents an adaption to growing in vitro as a cell line. This change in sensitivity precluded direct in vitro/in vivo comparisons using WX322 and no in vitro combination studies were attempted. The extreme sensitivity of this cell line to IFN is being further investigated by Dr S Langdon.

11.2 Plastic Assays

The three adenocarcinoma cell lines A549, H125 and H23 were used to assess the activity of two different schedules (24 hour and 5 day exposure) of two drugs CDDP and ADR alone and with IFN in these assays. The plating efficiencies of these well established lines were high (approx 30%).

(i) 24 hour exposures

Single agent activity of 24 hour exposures of IFN, CDDP and ADR expressed as the percentage survival for the three cell lines is illustrated in Figures 11.4 to 11.6. The concentration of drug resulting in 50% inhibition (IC_{50}) was similar for all three cell lines although H125 was generally more sensitive to ADR and CDDP that the other two lines. The IC_{30}values for IFN were also within a similar range. These figures are shown in table 11.1. IC_{50} values could not be calculated for IFN since no concentration tested resulted in a 50% inhibition of colony formation. The effect of combining either drug and IFN for 24 hours is shown as isobolograms in Figures 11.7 to 11.9.

A549

Figure 11.7 shows the effect of combination treatment for 24 hours in A549. The addition of IFN 10^4units/ml made little effect on three different doses of CDDP and ADR. These interactions appeared sub-additive on the isobologram but statistically were additive ($F = 1.0$, $p = 0.4$ for CDDP and $F = 0.05$, $p = 0.76$ for ADR). Similar results were seen with a smaller dose of IFN (10^3units/ml) which had little activity as a single agent. For simplicity, these results have been recorded in the Appendix.

H125

Figure 11.8 shows the effect of combination treatment for 24 hours in H125. The results were similar to A549. In all doses the addition of IFN (10^4) to both drugs gave more activity than seen with the drug alone but the interactions seen although appearing subadditive on the isobologram, were no more than additive ($F = 0.0$, $p = 0.99$ for CDDP and $F = 1.9$, $p = 0.17$ for ADR). A similar effect was seen with a lower dose of IFN (10^3). For simplicity, these results have been recorded in the Appendix.

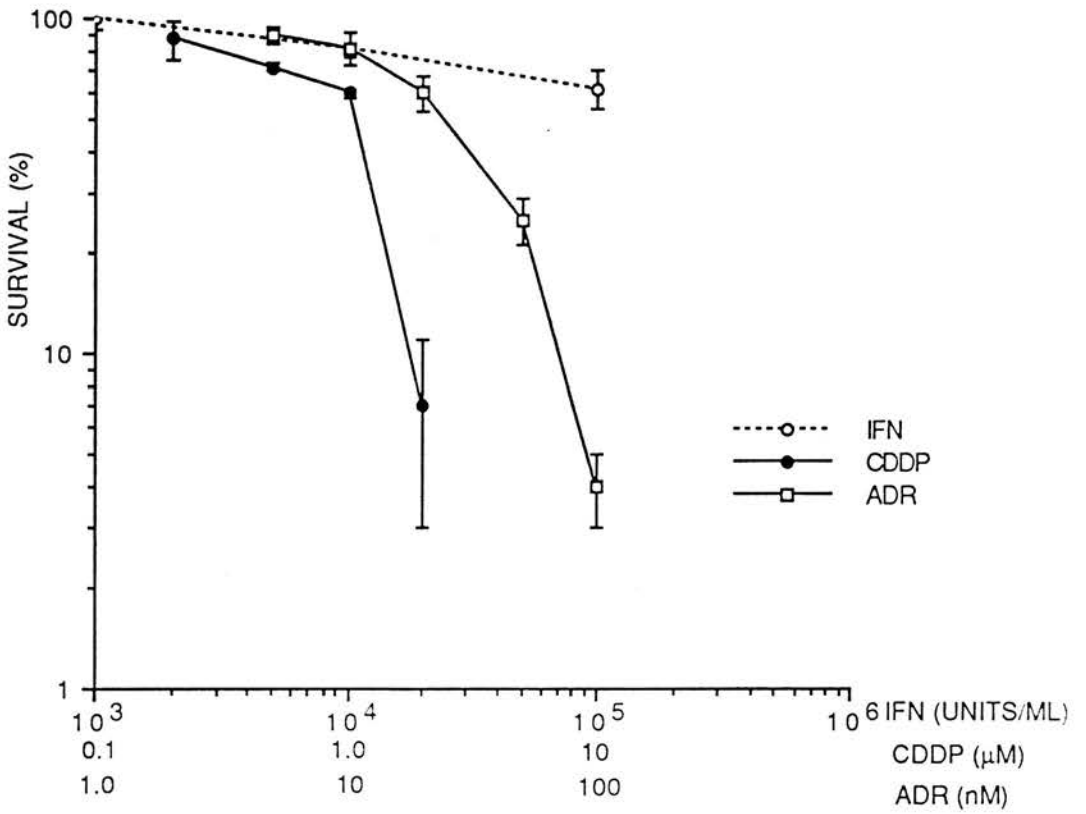


Figure 11.4: Single agent activity (24 hour exposure) in A549 (plastic assay)

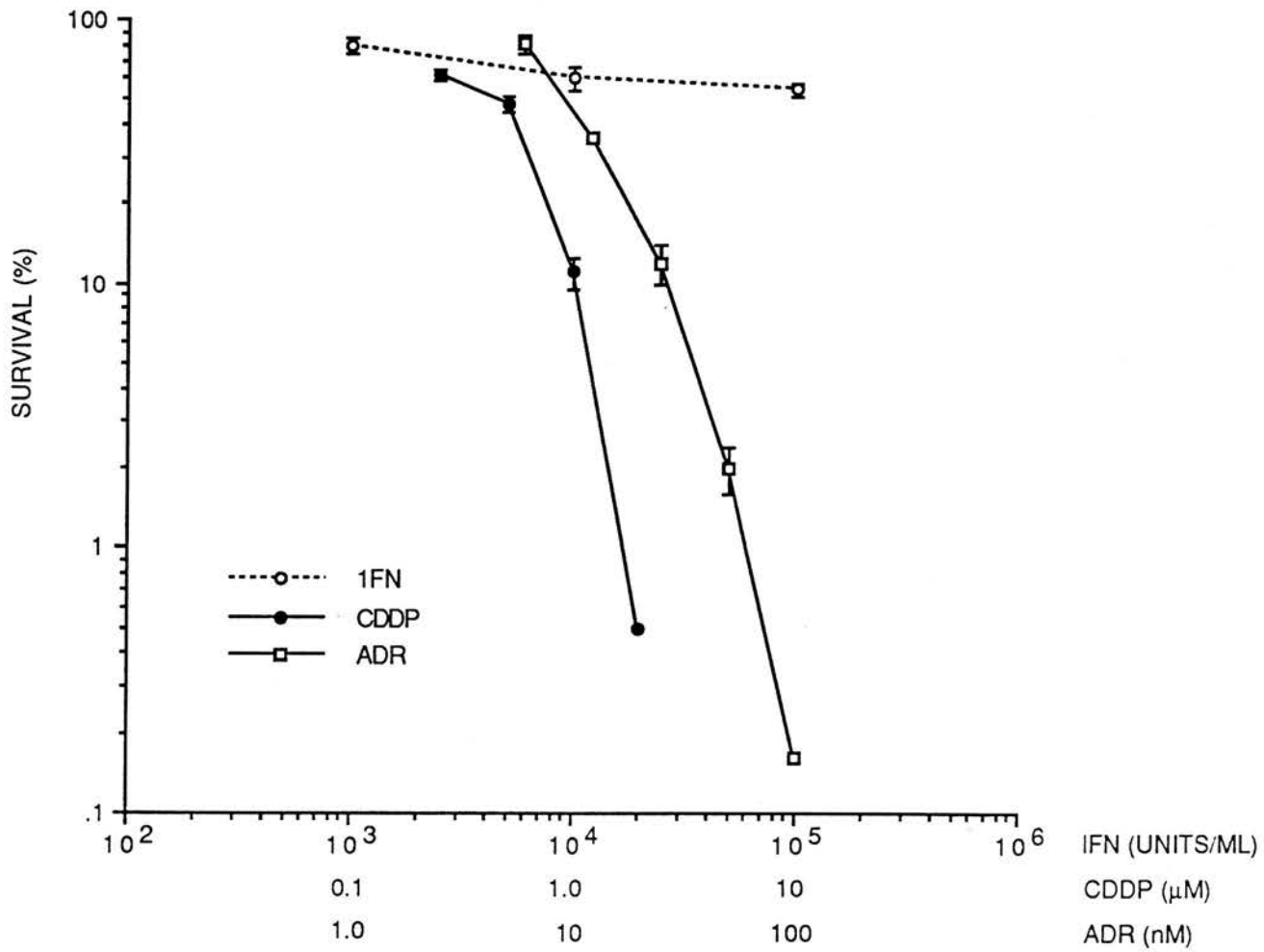


Figure 11.5: Single agent activity (24 hour exposure) in H125 (plastic assay)

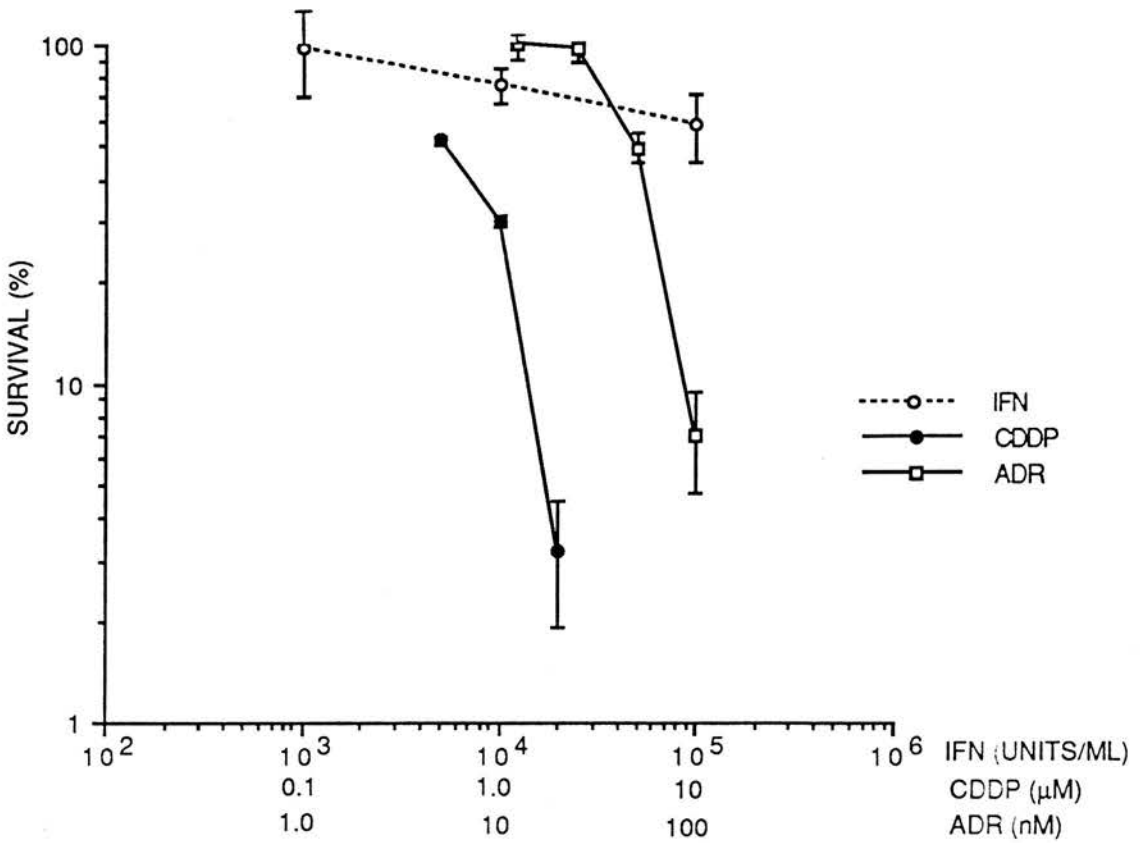


Figure 11.6: Single agent activity (24 hour exposure) in H23 (plastic assay)

	IFN* (units/ml)	CDDP (uM)	ADR (uM)
A549	8×10^4	1.2	24
H125	7×10^3	0.48	10
H123	6×10^4	1.1	49

Table 11.1: IC_{50} values for single agents (24 hour exposure) (plastic assay)

* IC_{30} for IFN

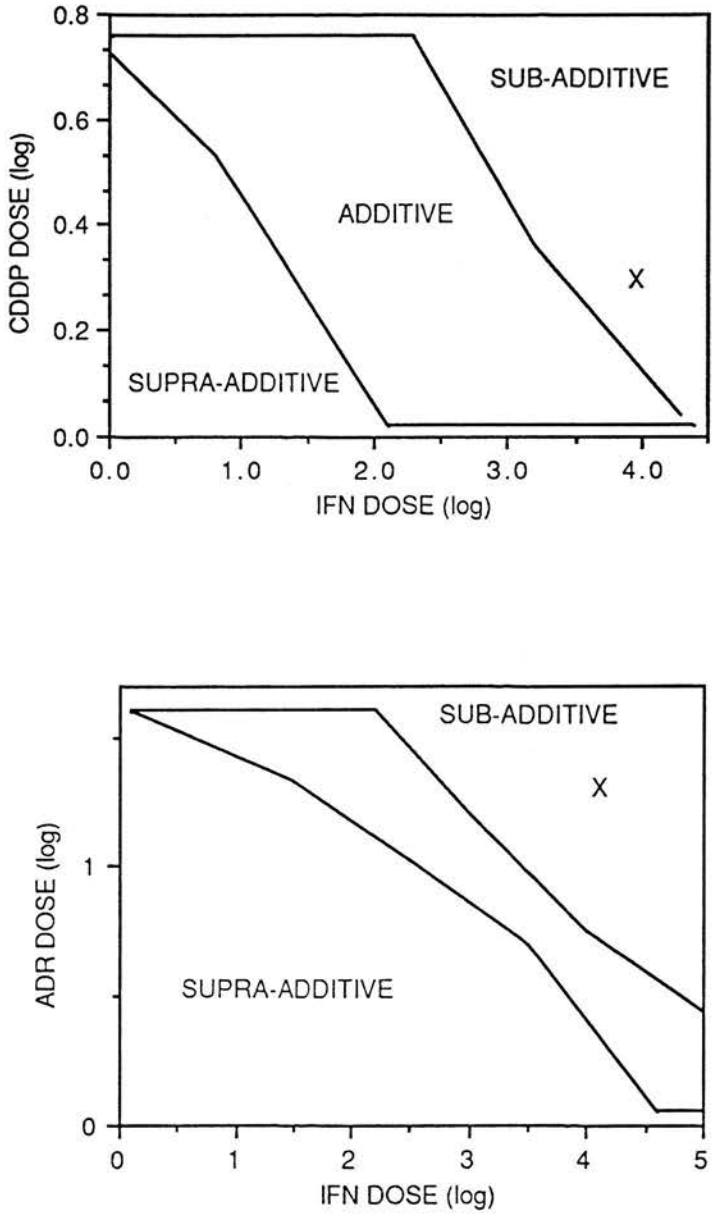


Figure 11.7: Effect of CDDP/IFN and ADR/IFN treatment on A549 (24 hour exposure)

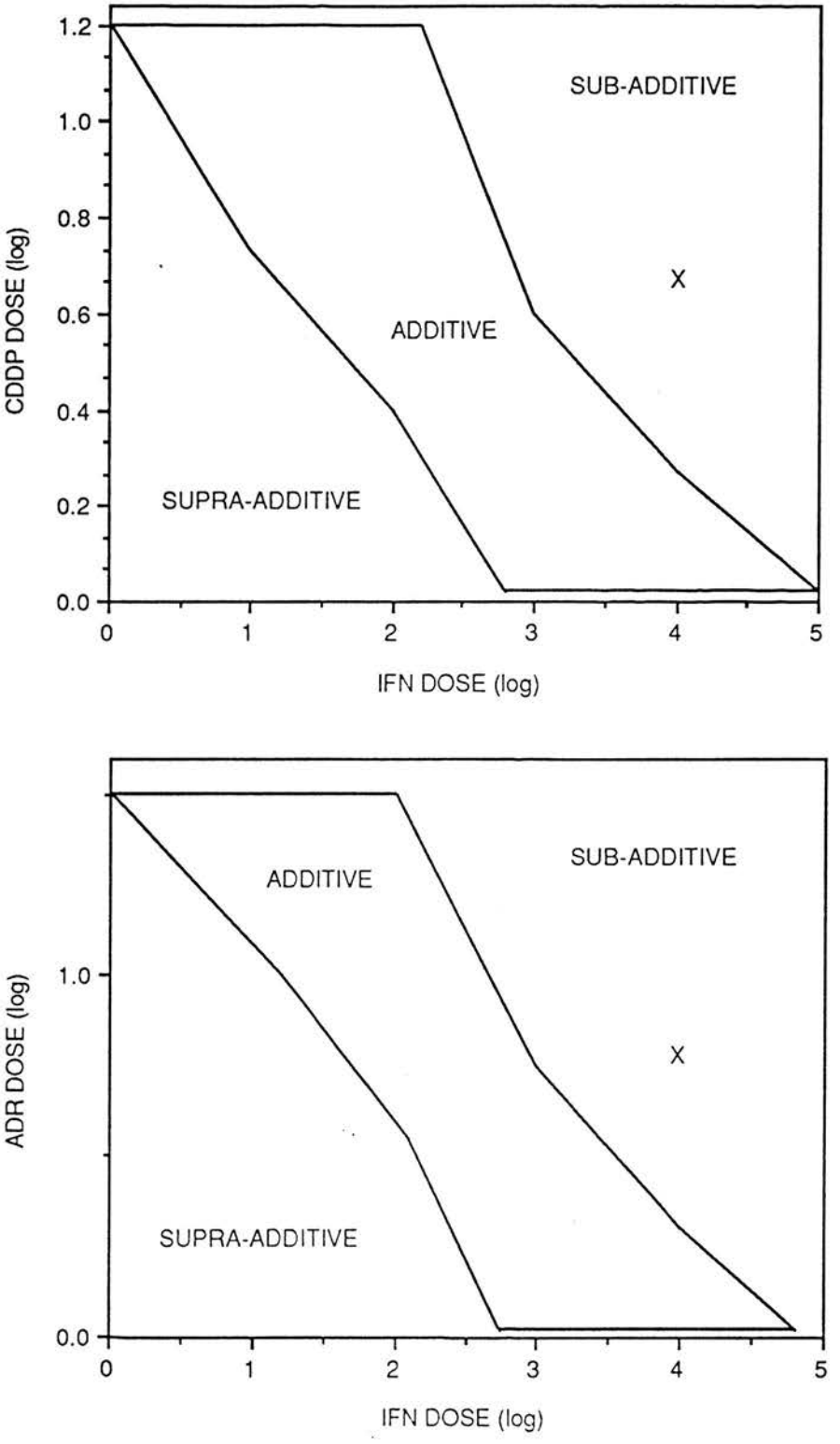


Figure 11.8: Effect of CDDP/IFN and ADR/IFN on H125 (24 hour exposure)

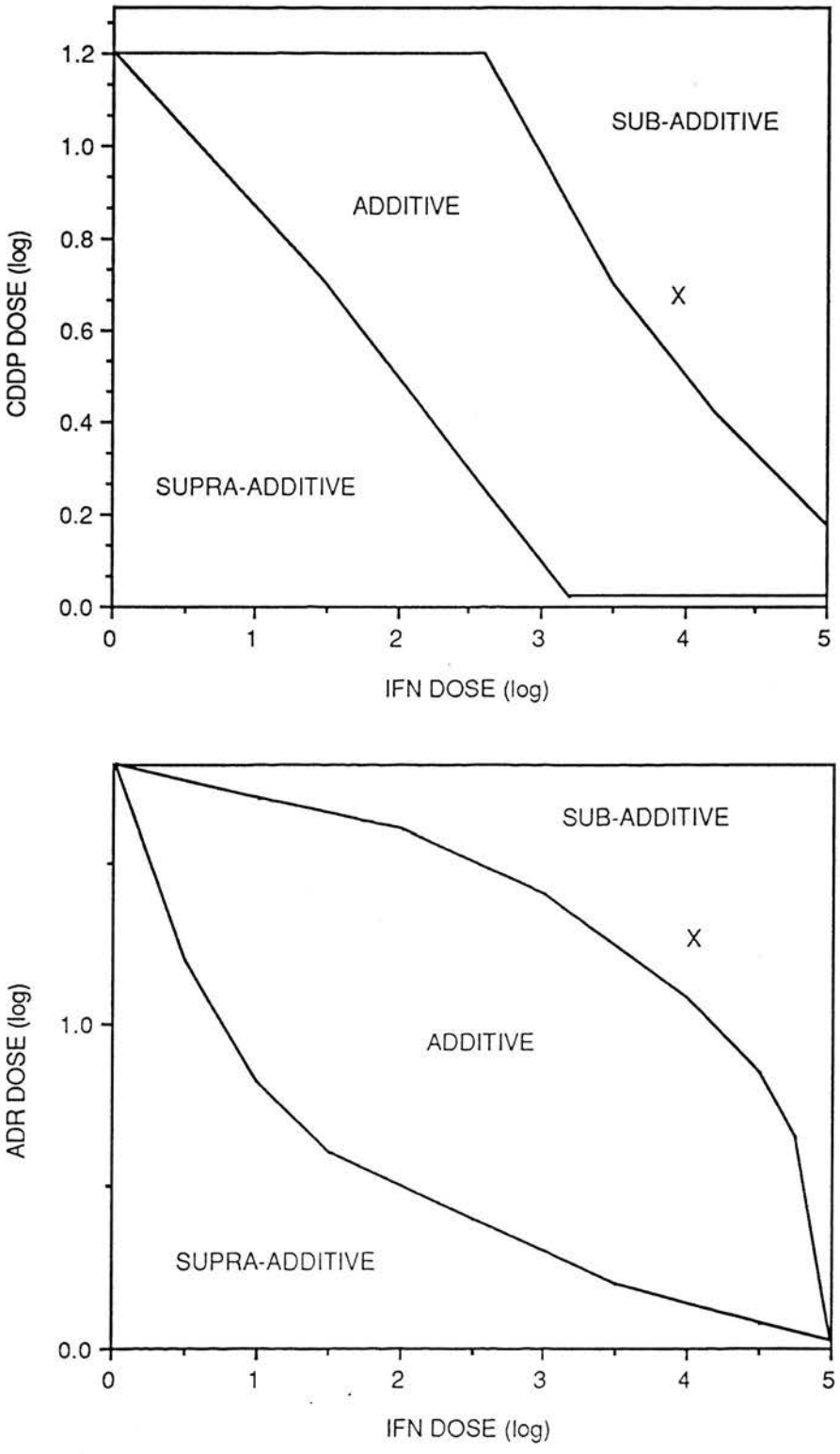


Figure 11.9: Effect of CDDP/IFN and ADR/IFN treatment on H23 (24 hour exposure)

H23

Figure 11.9 shows the effect of combination treatment with a 24 hour exposure in this cell line. Once again no positive interactions were seen. The activity of CDDP was virtually unaffected by the addition of IFN. The interaction was additive in nature (F=0.8, p=0.48). The results with ADR were interesting with significant sub-additivity being seen (F=3.9, p=0.045). Combinations using IFN at 10^3 units/ml were not assessed in this cell line.

ii) 5 day exposures

Single agent activity of 5 days exposure to IFN, CDDP and ADR for the three cell lines is illustrated in Figures 11.10 to 11.12. The IC_{50} (IC_{30} for IFN) values are shown in table 11.2. As expected the longer exposure resulted in a greater inhibition of colony formation and lower IC_{50} values than the 24 hour exposure. This was especially true for IFN. Once again H125 was the most sensitive line to IFN whilst H23 was relatively resistant to ADR compared to the other two lines. The effect of combining either drug with IFN for a 5 day exposure is shown in figures 11.13 to 11.15.

A549

Figure 11.13 shows the effect of combination treatment for 5 days in A549. Similar results to the 24 hour exposures were obtained. Statistically, the interactions betwen the agents were additive (F =2.3, p=0.12 for CDDP and F=0.6, p=0.454 for ADR). Further combinations with a higher dose of IFN (10^3units /ml) were also additive. This data is presented in the Appendix for simplicity. The ADR/IFN combination appeared less promising in a 5 day schedule although unfortunately only one dose of ADR and two doses of IFN were assessed.

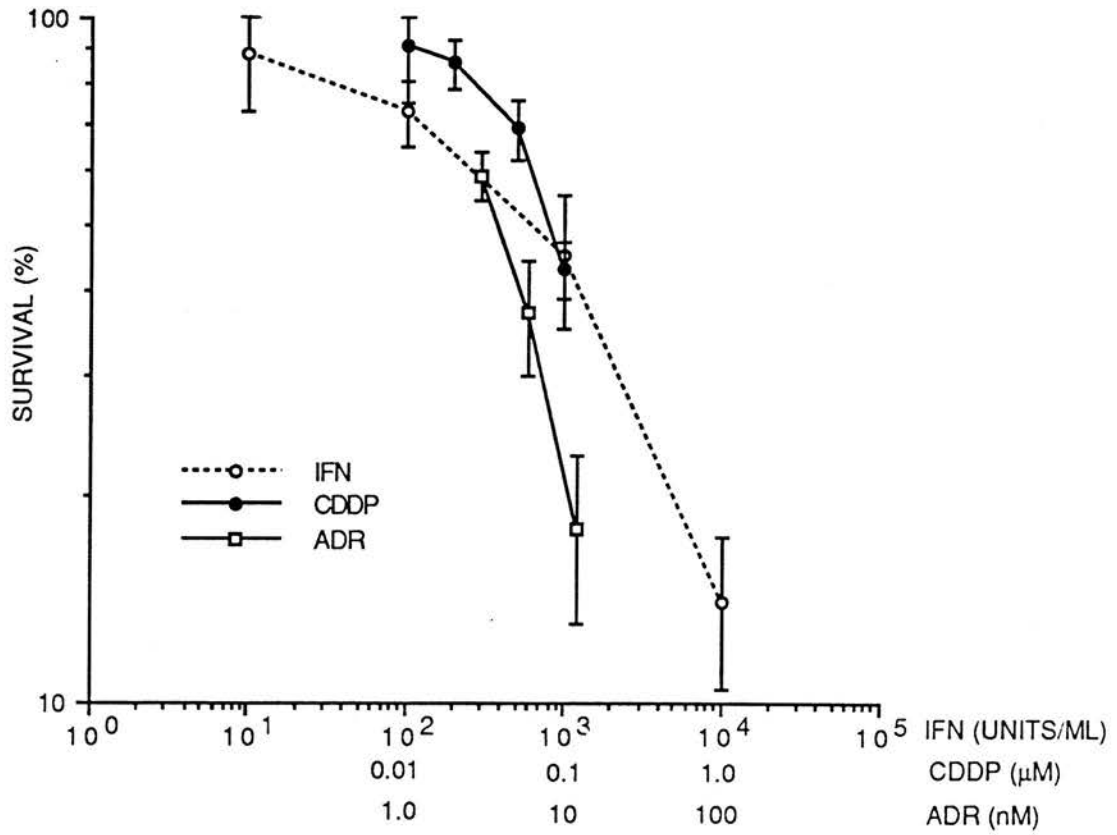
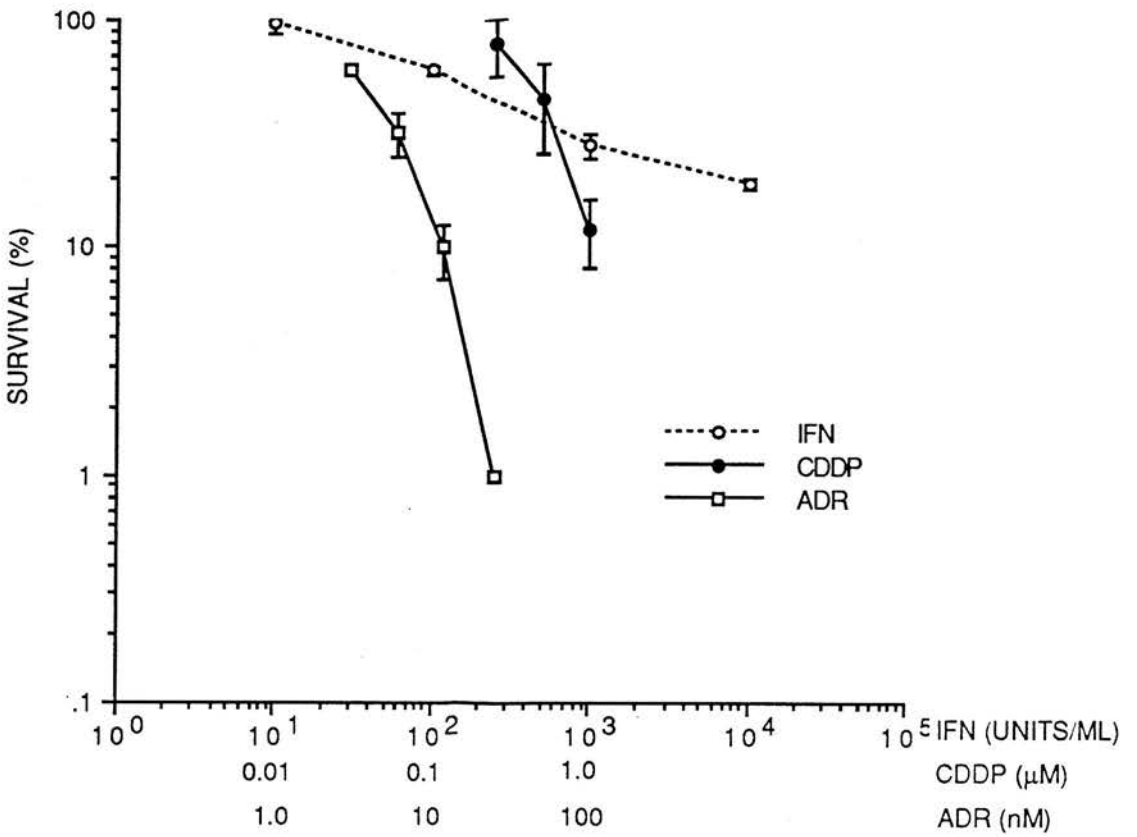


Figure 11.10: Single agent activity of IFN, CDDP and ADR on A549 (5 day exposure)



Figue 11.11: Single agent activity of IFN, CDDP and ADR in H125

(5 day exposure)

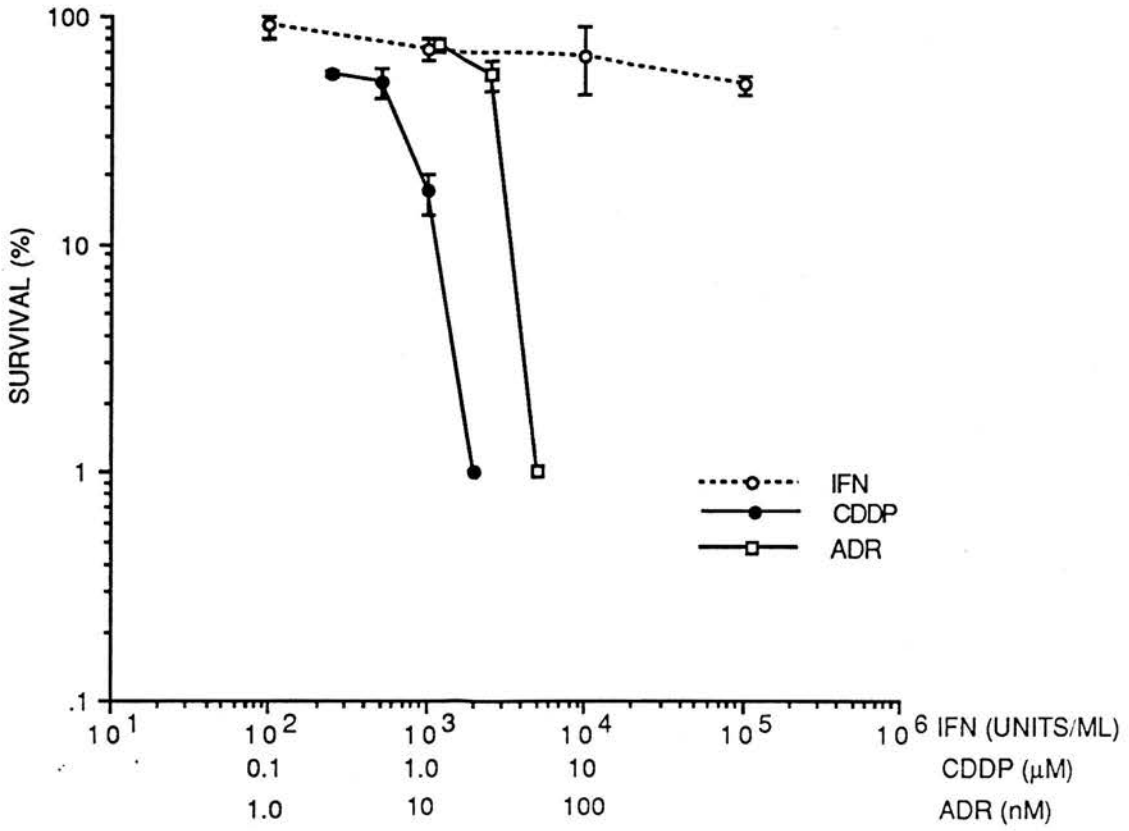


Figure 11.12: Single agent activity of IFN, CDDP and ADR in H23

(5 day exposure)

	IFN* (units/ml)	CDDP (uM)	ADR (uM)
A549	2×10^2	0.08	5.0
H125	7×10^1	0.45	4.0
H23	8×10^2	0.54	27.0

Table 11.2: IC_{50} values for single agents (5 day exposure)

(*IC_{30} for IFN)

H125

The effect of combination treatment with IFN and both ADR and CDDP for 5 days in H125 is shown as isobolograms in Figure 11.14. Again increased activity for two agents was seen (especially at low drug doses) when compared to the drug alone. This was an additive effect (F=2.64, p=0.06 for CDDP and F=0.8, p=0.5 for ADR). These results are similar to the 24 hour exposures.

H23

Figure 11.15 shows the results of 5 days combination therapy in this cell line. Overall the results were similar to the other cell lines. Additive interactions were seen between IFN and ADR (F=0.2, p=0.788). In one combination (CDDP 0.25uM and IFN 10^3units/1) there was an antagonistic effect (F=4.1, p=0.02) but this was not repeated at higher doses of CDDP.

11.3 In vitro/in vivo comparisons

As stated above, attempts at establishing the xenografts as cell lines or testing cells from xenografts directly in vitro have to date been unsuccessful. WX322 has been grown as a cell line but its sensitivity to IFN appears to have dramatically increased in the process making direct in vitro/in vivo comparisons meaningless.

Successful growth of the adenocarcinoma cell line A549 as a subcutaneous xenograft was achieved and combination therapy was assessed after the third passage. Control tumours had a median TD of 13 days. The group treated with interferon alone had a median TD of 19 days (SGD 0.46). CDDP as a single agent had identical activity (median TD 19 days, SGD 0.46). Combination treatment resulted in median TD of 29 days (SGD 1.2) with one of the seven tumours in the group being cured. Statistically this

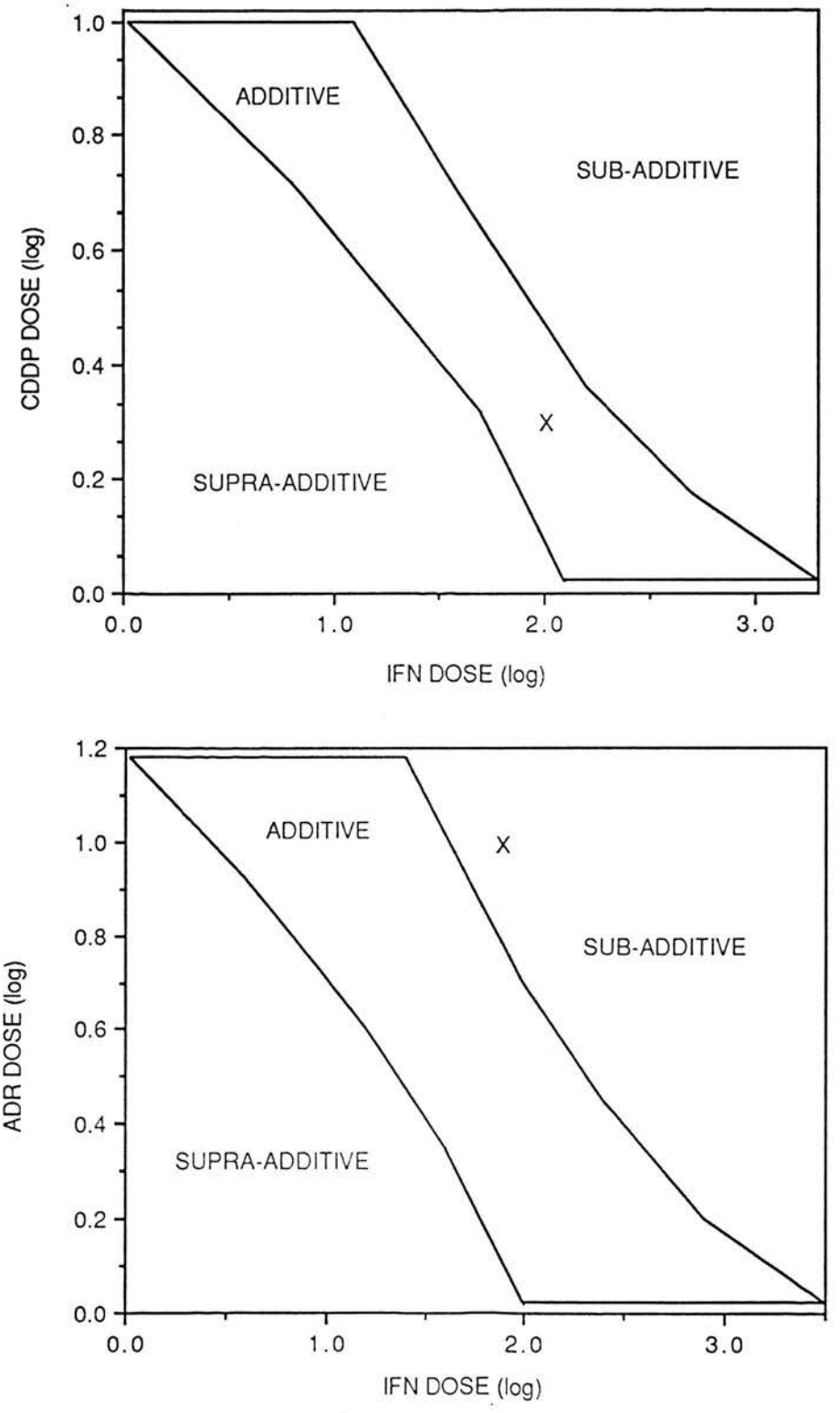


Figure 11.13: Effect of CDDP/IFN and ADR/IFN treatment on A549 (5 day exposure)

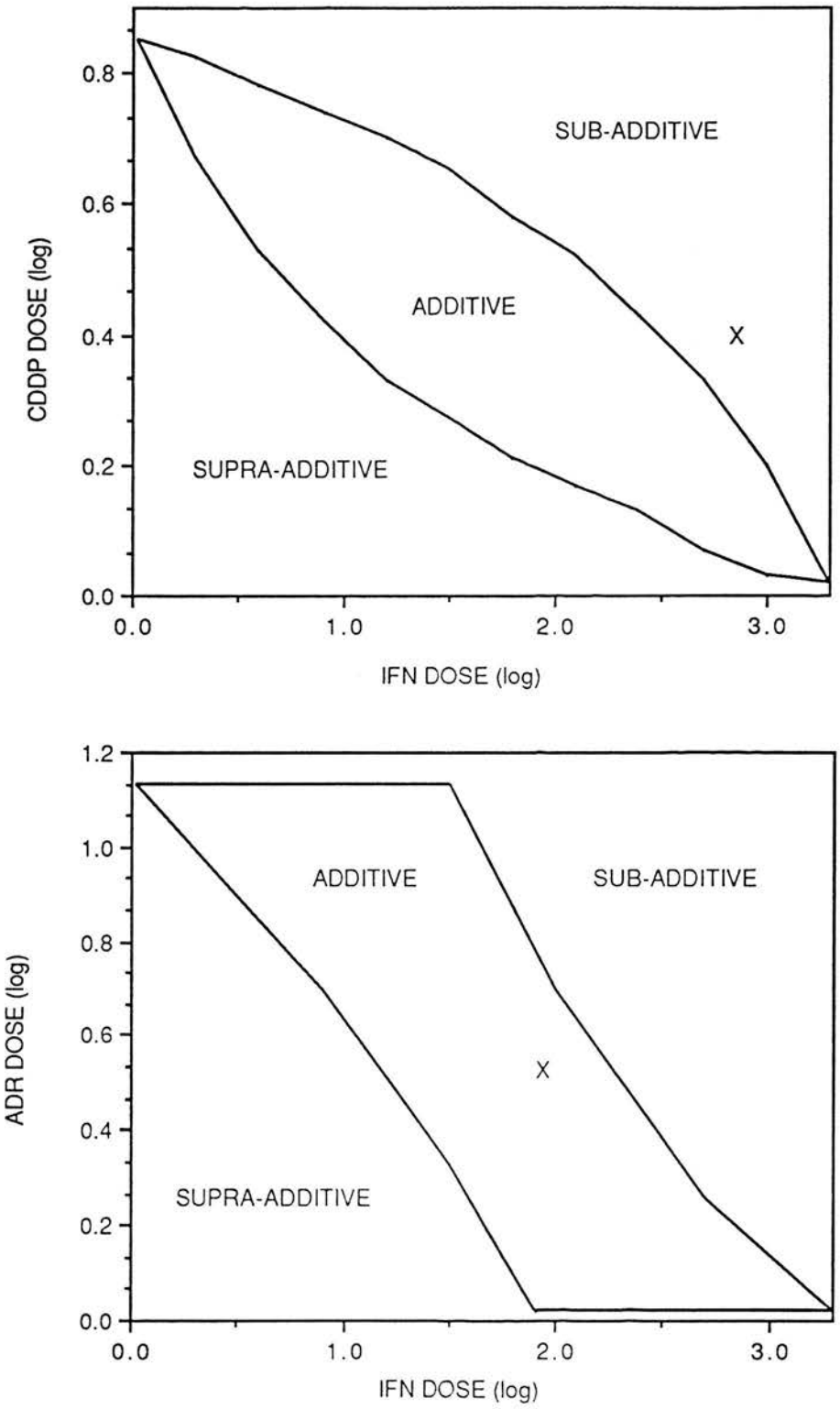


Figure 11.14: Effect of CDDP/IFN and ADR/IFN treatment on H125 (5 day exposure)

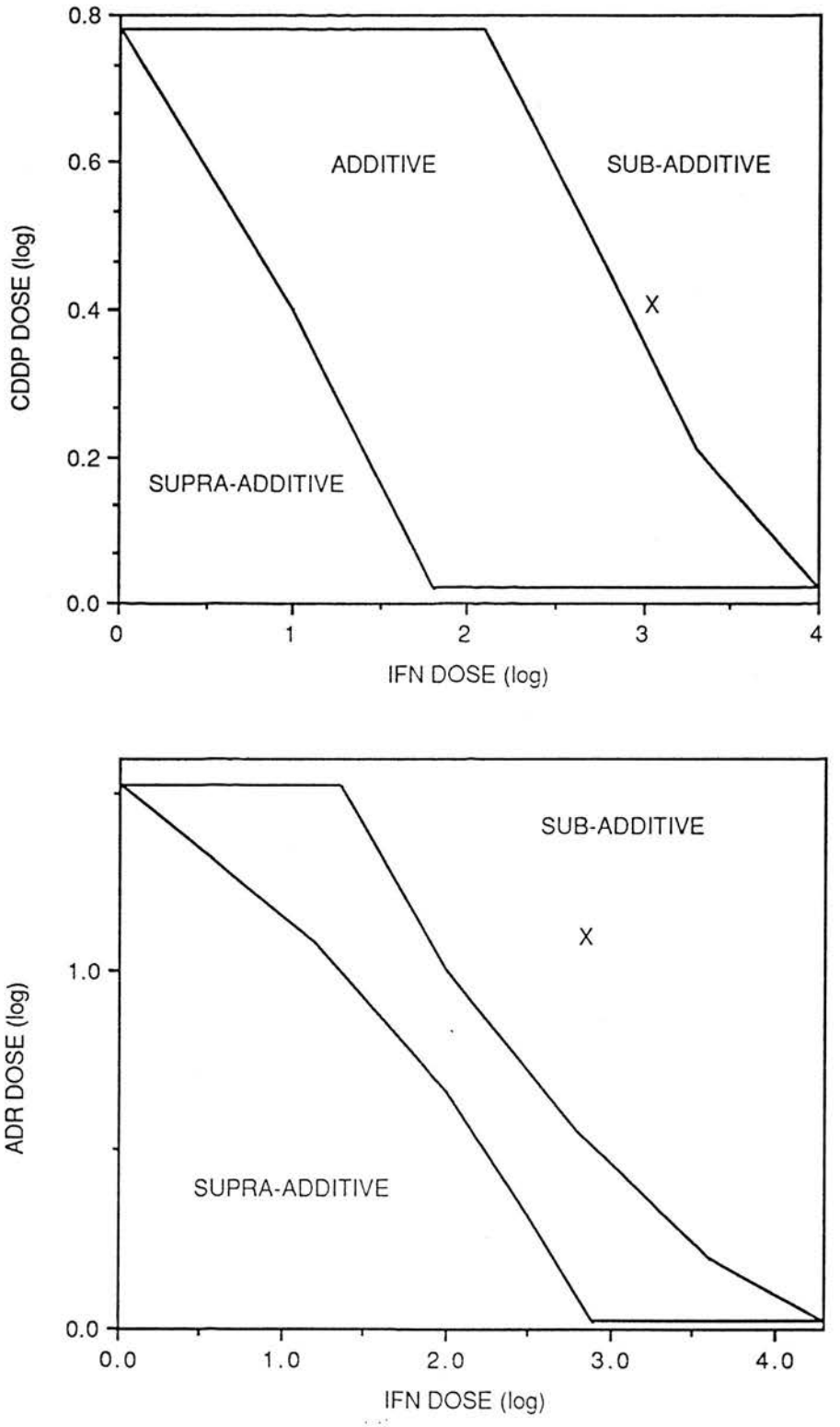


Figure 11.15: Effect of CDDP/IFN and ADR/IFN treatment on H23 (5 day exposure)

appeared to show additivty ($F = 1.36$, $p = 0.4$).

These results would appear to be comparable with the interactions seen between CDDP and IFN against A549 cells in vitro for both the Courtenay assay (Figure 11.1) and the plastic assays (Figures 11.7 and 11.13). These short exposure in vitro experiments (24 hours) are less comparable to the xenograft tests than the longer exposures on plastic or the Courtenay assay. In vivo chronic exposure was achieved by divided doses of CDDP ten days apart and daily injections of IFN.

11.4 Discussion

The experiments in this section have assessed the effects of combining IFN with two drugs against human lung cancer cell lines using two in vitro assay techniques. Two different exposure times were used in the plastic assay and various doses of both drugs and IFN were tested. In all cases, the interactions between the two agents were no more than additive. Statistically significant sub-additivity was seen twice in the H23 cells with CDDP over 5 days and ADR over 24 hours. However as multiple statistical tests were performed (3 cell lines, 2 exposure times, 3 doses of 2 different drugs = 36 comparisons) one might expect two apparently significant results to appear by chance ($p>0.05$) when in fact no actual effect was present.

Positive interactions have been shown in vitro by other workers (Welander 1985, Heston 1984, Gohji 1986, Rosenblum 1984, Ho 1985, Hirabayashi 1985, Aapro 1983) using a variety of assay systems and different cell lines. Most workers have tested different drugs with different IFN subtypes and a variety of different types of interaction from obvious synergy to frank antagonism have been seen. Some (Saito 1987)

have, like us, only shown additive effects. No pattern seems to be emerging. No single drug appears to cause synergy consistently although DFMO and the alkylating agents appear the most successful. Clearly not all drug types are suitable for testing in vitro. In addition, no dosing schedule appears more successful than others although chronic exposure seems to be important.

Some of the studies above could be criticised in the method by which the nature of any interaction was assessed. Many used the term "synergy" to describe a response where the level of cell survival for the combination was less than the product of the separate surviving fractions for each agent alone. As pointed out by Steel and Peckham (1979) this multiplicative method only holds if the dose-response curves for each agent are linear (i.e. the agent is "additive with itself"). Most dose-response curves are not linear. Only one other study (Gohji 1986) apart from ourselves uses Steel's method of calculating additivity. They showed definite evidence of synergy. Unfortunately this method does not allow for measurement of the significance of any observed interaction and it was for this reason that the data here was subjected to an alternative statistical analysis.

Only one study (Heston 1984) has attempted to make a direct in vitro/in vivo comparison. They showed evidence of synergy in vitro but just additive effects in vivo between DFMO and IFN-γ in a renal cancer cell line. We showed similar additive interactions in vitro and in vivo between CDDP and IFN in the lung adenocarcinoma cell line A549. It was unfortunate that we were unable to assess an interaction known in be synergistic in vivo using an in vitro assay. This would have enabled us to assess if the interaction was mediated through a direct effect on the tumour cells. Attempts to establish these xenografts as cell lines are continuing.

SECTION IV - CLINICAL STUDY

Chapter 12 - Evaluation of cisplatinum combined with interferon-alpha in patients with NSCLC

12.1 Introduction

The aim of testing novel therapeutic strategies in laboratory tumour models is to ultimately provide more effective treatment for patients with cancer. It is surely only of academic interest if a certain treatment is shown to be extremely effective at killing tumour cells in culture or in laboratory animals but is inactive in patients with similar tumours. All promising advances in the laboratory must eventually be assessed in a clinical setting both to validate the pre-clinical testing system used and improve the outlook of patients with hitherto untreatable conditions.

After obtaining the promising results described in Section II of combination treatment with IFN and cytotoxic drugs in NSCLC xenografts it was felt important to design a pilot clinical study to assess this form of treatment in patients with lung cancer. Since the most impressive synergy between the two components of the combination was seen when cisplatinum was used, it was decided to evaluate this combination initially. Information gained from the scheduling experiments (Chapter 8) was used in the design of the study. The objectives of the study could be summarised as follows:

1. To examine the anti-tumour activity of human recombinant interferon alpha combined with CDDP in treating squamous, large cell and adenocarcinoma of the bronchus.
2. To identify any factors associated with a response to the treatment.
3. To document the toxicity associated with this treatment.

12.2 Selection of patients

Patients with histologically proven or cytologically confirmed non-small cell carcinoma of the bronchus were considered for the study. All patients had to have inoperable but evaluable disease. The age limit for entry was 75 years. All entrants required a performance status of 2 or less and a life expectancy in excess of 12 weeks. Adequate renal and hepatic function (serum creatinine <0.11mmol/l and bilirubin <14mmol/l) were required as well as evidence of adequate bone marrow reserve with white cell count greater than $3.5 \times 10^9/l$ and a platelet count in excess of $150 \times 10^9/l$.

A previous history of malignant disease apart from treated basal cell and squamous carcinoma of the skin excluded patients from the study. Previous surgery or radictherapy made patients ineligible unless there was documented evidence of progressive disease at an untreated site. Previous chemotherapy excluded patients from the study.

12.3 Investigations and assessments

Standard UICC criteria were used to assess responses. The investigations performed before entry into the study and prior to each treatment course are outlined in table 12.1. Optional investigations such as liver or bone scans, respiratory function tests, bone marrow examination and mediastinal imaging (with barium, gallium or CT scans) were only performed routinely when they proved essential for judging tumour response. Investigations and assessments were recorded on standard EORTC clinical trial forms.

12.4 Design of Treatment

Human recombinant interferon alpha-2 (Schering Plough, Kenilworth, NJ) was given on three days of each week at a dose of 3MU subcutaneously. Cis-platinum (Bristol-Myers, Slough) was given at 100mg/m^2 intravenously one week after the commencement of the initial dose of IFN and thereafter at four weekly intervals. Standard anti-emetic treatment (I.V. metoclopramide infusion) was given with cis-platinum treatment.

The first assessment of response was therefore three weeks after the second dose of CDDP. The minimum number of treatment courses assigned for a full assessment of response to treatment was two. Patients were only taken off chemotherapy under the following circumstances: (i) unacceptable toxicity at any time, (ii) the presence of disease progression or lack of response to

	Before entry to study	Prior to each treatment course
Full blood count	+	+
Urea and electrolytes	+	+
Creatinine clearance	+	+
Liver function tests	+	+
Calcium/phosphate/magnesium	+	-
Total protein/albumin	+	+
Chest radiograph	+	+
Clinical measurement (nodes, skin deposits etc)	+	+
Bronchoscopy	+	-
Performance status	+	+
Life expectancy	+	-
Body weight	+	+
Clinical toxicity	-	+

Table 12.1: Investigations performed during the study

treatment (iii) complete regression of disease. Radiotherapy was allowed at any time for the palliation of symptoms.

Patients were fully informed of the experimental nature of the treatment and its potential benefits and side effects. They were also informed of their right to withdraw from the protocol therapy at any time if they wished. The study protocol was approved by the local ethics committee. At the outset it was anticipated that between 30-40 patients would be treated but that patient accrual would be carefully reviewed. A minimum of 14 fully evaluable patients would be required before the study could be closed.

12.5 Results

To date, 32 patients have been recruited into the study. Their characteristics are summarised in table 12.2. As a group of patients with inoperable NSCLC their mean age was comparatively low (57.9 years). The distribution of histological types of NSCLC was as usually encountered. The majority of patients had a performance status of 1 on entry to the study.

At the time of reporting of the 32 patients entered, 26 have completed the study and 6 are still being treated. Of the 26 completed, 1 patient was not considered evaluable, leaving 25 completed evaluable patients. Four had a marked deterioration in their condition due to progression of their disease prior to receiving their first dose of CDDP. Since they did not receive

Total number	32
Sex ratio (M:F)	7:1
Mean age (years)	57.9
Histology:- Squamous	14
Adeno	11*
Large cell	7
Performance status on entry	0:9
	1:20
	2:3

* one patient in fact had incorrect histology (lymphoma)

Table 12.2: Characteristics of patients entered into study

combination therapy, these patients are not included in evaluation of response to treatment. They have been included in the analysis of results on an 'intention to treat' basis and are considered elsewhere. The unevaluable patient was a 42 year old man who had an abnormal chest radiograph and clear evidence of bone metastases. The diagnosis of an adenocarcinoma was made on a needle aspirate of his pulmonary lesion. The patient made a dramatic response to the first course of treatment but subsequently died in fulminant hepatic failure. At autopsy, his pulmonary and bone lesions had completely regressed but his liver was replaced by a large cell lymphoma. It was assumed that lymphoma was the initial diagnosis (despite this unusual differential response) and he was not deemed evaluable for the study.

The toxicity recorded in 25 patients receiving at least one course of CDDP is shown in table 12.3. Nausea and vomiting (WHO grade 2-3) was seen in virtually all patients but only one patient experienced grade 4 symptoms. In two patients this side effect led to withdrawal from the study. 'Flu like symptoms related to the IFN injections were common but only prevented one patient from completing the study. Treatment delay (up to 1 week) due to a fall in creatinine clearance was necessary in three patients. No significant myelosuppression was observed.

The majority of patients completing the study received two courses of CDDP (Table 12.4). The four patients who only received one course of CDDP had significant progressive disease after this course. This table also shows the reasons for withdrawal of patients from the protocol.

Nausea and vomiting	24	(Grade 1:3 Grade 2:10 Grade 3:10 Grade 4:1)
Fever	17	
Myalgia	9	
Rigors	7	
Anaemia	5	(3 required transfusion)
Tinnitus	5	
Diarrhoea	3	
Renal (rise in creatinine clearance)	3	
Alopecia	2	
Angina	1	
Dizziness after IFN	1	
Peripheral neuropathy	1	

Table 12.3: Toxicity seen in 25 patients receiving at least one course of CDDP and IFN

No of courses of CDDP	1 :	4
	2 :	12
	3 :	4
	4 :	1
		21

Reason for withdrawal	
No response	6
Progessive disease	16
Toxicity	4
	26
Still on study	6
TOTAL	32

Table 12.4: No of courses of CDDP received and reasons for withdrawal of patients in study

Age	Histology	Site of response	Status
57	Squamous	Chest	Off study
61	Squamous	Nodes	Off study
61	Squamous	Chest	Off study
38	Adeno	Nodes	On study
51	Squamous	Nodes	On study
56	Squamous	Chest	On study

Table 12.5: Characteristics of responding patients

Partial responses were seen in 6 male patients. 3 are still being treated and 3 have been withdrawn due to significant toxicity (Grade 3-4 nausea and vomiting, debilitating 'flu like symptoms). The characteristics of these patients is shown in table 12.5. Five of the six patients had squamous histology. Half the patients had responses in neck nodes. They were not significantly younger than the group of patients as a whole. With 27 evaluable patients this represents a response rate of 22% with three patients still on treatment without evidence of response as yet. The response rate on an 'intention to treat' basis (i.e. including the four patients who were withdrawn before receiving CDDP) is 19%. It is too early at this stage to comment on survival figures for any groups of patients.

12.6 Discussion

This ongoing study has attempted to assess the potential of combination therapy with CDDP and IFN in patients with NSCLC. Low doses of IFN were given in a chronic schedule as this seemed important in the xenograft studies which prompted the trial. CDDP was administered at four weekly intervals rather than the conventional three weekly, in an attempt to avoid any enhancement of toxicity. Nausea and vomiting were seen in virtually all patients but were appropriate to the amount of CDDP given. Many patients experienced 'flu like symptoms from IFN but these were rarely severe enough to limit treatment. Toxicity from the combination was therefore predictable but rarely dose limiting.

Four patients were withdrawn from the study with progressive disease between receiving their first dose of IFN and commencing CDDP. All were elderly (aged 68-74) and had poorer performance status (2 grade 1, 2 grade2) than the group as a whole. Clearly the possibility that one week's treatment of IFN accelerated their disease should be considered. No reports of this effect have previously been recorded despite the fact that thousands of patients with advanced cancers have been treated with IFNs. Also these patients continued to deteriorate after the IFN was discontinued. A more likely explanation of the findings is that in retrospect these patients did not have a life expectancy of 12 weeks at entry and should not have been included in the study. Although they did not receive combination therapy and cannot be included in an analysis of response to this treatment, they are included in the calculation of overall response rates as it was our intention to treat them at the outset of the trial.

Our toxicity data are very similar to those reported by Walsh et al (1987) who performed a phase I study of the combination of alpha IFN and CDDP in eighteen patients (6 with NSCLC). They used a slightly higher dose of IFN (5×10^6 units/m^2) in an identical schedule with escalating doses of CDDP up to 30mg/m^2 given weekly.

Six partial responses have been seen so far. All but one occurring in patients with squamous histology. This represents a response rate of 22%. How does this compare with single agent activity of CDDP in this type of patient? Five reports have

been published on the activity of CDDP in NSCLC. Doses have ranged from 75 to 120mg/m^2 every three to six weeks. Response rates have shown considerable variation and do not appear to relate to dosage. The two largest studies (DeJager 1980, Vogel 1982) reported response rates of 25% and 32% respectively. The response duration was around 3 months and median survival time 5 months for both trials. Three smaller studies (Britell 1978, Casper 1979, Rossof 1976) reported response rates below 10%. Survival data were not recorded for these studies. So although no direct comparison can be made to a single agent study using an identical dosage schedule, our results appear encouraging and worthy of further study. The rational development of this treatment would seem to be a randomised comparison between CDDP and CDDP plus IFN in NSCLC patients.

Clinical responses were seen in both primary tumours and in lymph node metastases. It is of interest that five of the six responders to the treatment had squamous histology. This phenomenon has not been seen in single agent studies of CDDP in NSCLC where responses were seen evenly in adenocarcinoma and squamous tumours. In reported studies few responses were seen in large cell lesions (Souhami 1985). Potentiation of CDDP by IFN was seen in both adenocarcinoma and squamous xenografts but not in adenocarcinoma cell lines. The reason why patients with squamous histology seemed to respond more readily to the combination therefore remains unclear. A larger study is required to determine whether certain tumour types are more sensitive.

CONCLUSIONS AND FUTURE DIRECTIONS

This thesis has investigated the potential of a new form of treatment in human lung cancer. This tumour is a major killer of both men and women throughout the world and is currently remarkably resistant to existing therapies. Some progress has been made in the last two decades in prolonging the survival of patients with small cell lung cancer using combination chemotherapy. However, long term survival is uncommon. Non small cell lung cancer, which is three times more common than SCLC does not respond to standard chemotherapy. If effective, this would seem the logical form of treatment since the vast majority of patients present with widespread disease. Clearly newer agents and more effective regimes are required if an impact on this condition is to be made.

The development of new anticancer agents has been facilitated by the emergence of laboratory methods that allow for testing of cytotoxic activity outwith the patient. Although no perfect system exists it is possible to evaluate new treatments rapidly and to avoid unnecessary toxicity to patients from the use of inactive regimes.

Interferons have burst onto the clinical scene in the last decade without such rigorous pre-clinical testing. The results of phase II clinical studies have been disappointing with significant activity only demonstrated in certain haematological malignancies. Few responses have been seen in the common cancers. Single agent studies in patients with lung cancer were certainly negative and there is no place for this form of treatment in these patients.

The concept of combining interferons with anti-cancer agents is not new, the first report of the combinationin a laboratory model appearing in

1974 (Chirigos). Recently much interest has been shown in this form of treatment. Different workers using different laboratory test systems have published results which have on the whole looked very encouraging. We were the first to report on these interactions in human lung cancer.

The bulk of this thesis has described the interactions between IFN alpha and cytotoxic agents in human lung cancer xenografts. In summary we have shown promising interactions between a variety of agents and IFN in NSCLC tumours but have been unable to produce similar results using the same agents in SCLC xenografts. The reasons for this are unclear. However in some aspects this is reassuring. If the increased activity was seen with all drugs in all tumours then it would be likely that the effect was due to some 'trick' of the system and not to a true interaction. The effect seen is certainly more complex than this.

There is evidence from both animal and clinical studies to show that the administration of larger doses of IFN results in greater inhibition of tumour growth, presumably reflecting the direct cytotoxic action of IFN. This does not seem to be the case for IFN/drug combinations. Despite the fact that few studies investigating the effect of dose and schedule on IFN/drug interactions have been performed, many clinical trials using this form of treatment are ongoing. Most studies appear to be using the maximum tolerated dose of each agent which may not be optimal. It is important if these studies are negative to investigate other doses and schedules of promising combinations before concluding that no interaction occurs.

A major hurdle in designing relevant clinical studies in this field is to be the lack of understanding concerning the possible mechanisms by which

drugs may interact with interferons. The scope of this project did not allow for detailed investigation of this problem. However it was shown that the results obtained in the xenograft model were not mediated through increased toxicity to the host animal. We have been unable to show that the modulation of cell cycle distribution is a factor in drug/IFN interactions and must conclude that other mechanisms are involved.

The results from the in vitro experiments described in this thesis are disappointing. On no occasion was a significant synergistic interaction seen. Technical problems prevented the direct in vitro/in vivo comparison of a treatment regime which was promising in vivo but the one comparison which was made showed similar results in each system. Other workers have clearly demonstrated synergy in vitro and it must be concluded that these interactions represent a direct action on tumour cells.

The results of our clinical study merit further investigation. The toxicity from the combination was predictable and rarely dose limiting. The high frequency of squamous histology in responding patients was unexpected although it is interesting that in the xenograft studies NX002, a squamous carcinoma, was the most sensitive tumour to the CDDP/IFN regime.

What are the possible future directions of this research? In the laboratory it is important to continue assessing different test systems. However, the possible permutations are virtually infinite and the purpose of this kind of work now must be to look for the emergence of a pattern of interactions. The main thrust of preclinical work in this field must now involve the investigation of the possible mechanisms by which synergy can occur.

Clearly the systems used for these experiments should involve tumours common to clinical practice and be capable of producing relevant responses. Work should only be done using agents where there is no doubt that a positive interaction occurs and using dosage regimes which could be converted to a clinical setting. Although it may be technically easier to use an in vitro model it may be more relevant in the final outcome to involve an animal system despite the problems of species specificity.

Perhaps the most fruitful avenue of research to follow initially would be to study the effect of IFNs on drug metabolism. Techniques are now evolving to monitor drug kinetics and effects at the cellular and even nuclear level and it would seem relevant to study drug metabolism with and without the influence of IFNs where positive interactions are known to occur. Such studies are currently ongoing in our laboratory. In the future it is hoped that groups interested in IFN/drug interactions will collaborate in this form of research rather than pursuing individual ideas on models which do not show clear synergy.

Another possible fruitful area for research would be to increase the understanding of IFN on host responses. Many earlier clinical studies have shown that the same dose of IFN can cause quite different amounts of stimulation of the immune system in different individuals. This may be important when interpreting data from groups of patients given the same dose of IFN in a clinical study. However at present it seems improbable that these effects are important in IFN/drug interactions.

Further clinical studies are certainly needed to confirm that these interactions are clinically important and not just an artefact of laboratory tumour model systems. The best way to investigate this is to perform a direct comparison of the activity of CDDP alone and with IFN in a

group of patients with NSCLC. As discussed earlier, careful consideration will have to be given to dosage regimes and schedules. If the addition of IFN confers only marginal benefit then a large group of patients will have to be recruited and the trial will take a long time to produce results. However at the end of the day, this form of assessment has to be undertaken to prove whether this interesting idea of combining IFN with cytotoxic drugs will have any benefit in the management of patients with this terrible disease.

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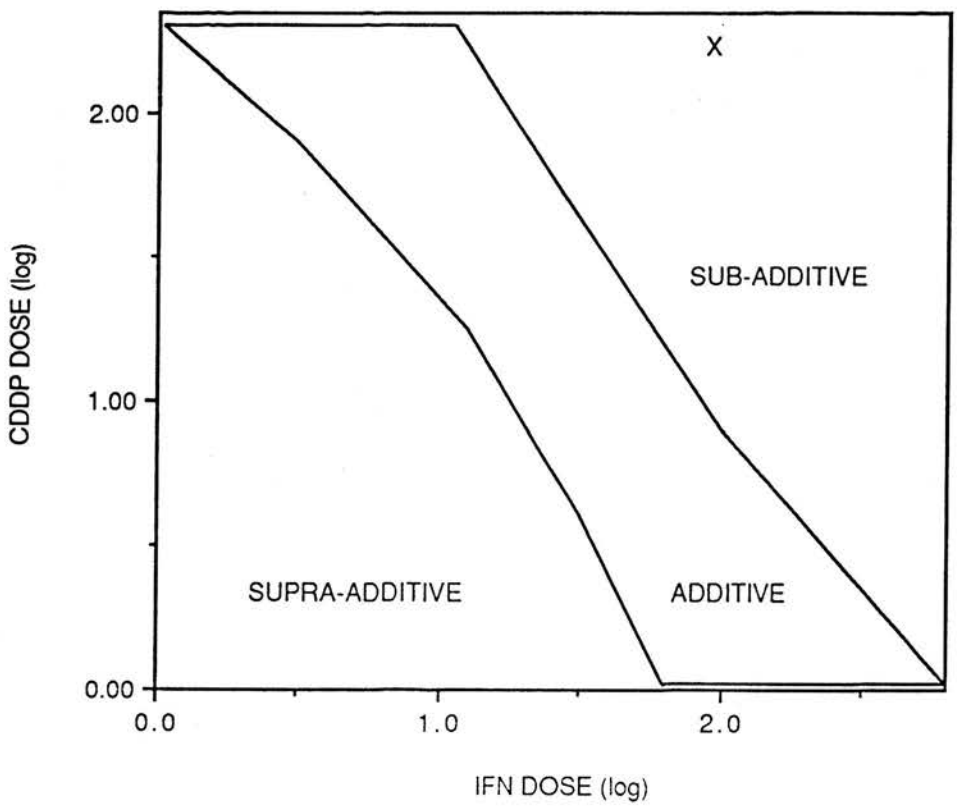
APPENDIX I



Isobologram showing interaction between IFN and CDDP in A549 (Courtney Assay). The cross marks the observed effect with IFN/CDDP combination. This would appear to be sub-additive.

APPENDIX II

In vitro plastic assays

1. A549 (24 hour exposure)

	% inhibition of colony formation	
ADR dose (nM)	ADR alone	ADR + IFN (10^3units/ml)
5	8	-
10	19	28
20	40	46
50	75	80
100	97	96
CDDP dose (nM)	CDDP alone	CDDP + IFN (10^3units/ml)
0.2	13	5
0.5	28	25
1.0	40	60

(IFN 10^3units/ml as single agent caused 1% inhibition)

2. H125 (24 hour exposure)

	% inhibition of colony formation	
ADR dose (nM)	ADR alone	ADR + IFN (10^3units/ml)
6	19	57
12	64	79
25	88	94
CDDP dose (nM)	CDDP alone	CDDP + IFN (10^3units/ml)
0.5	52	62
1.0	89	92
2.0	99	100

(IFN 10^3units/ml as a single agent caused 20% inhibition)

3. A549 (5 day exposure)

	% inhibition of colony formation	
ADR dose (nM)	ADR alone	ADR + IFN (10^3units/ml)
12	82	93
CDDP dose (nM)	CDDP alone	CDDP + IFN (10^3units/ml)
0.02	13	88
0.05	31	89
0.10	57	95

(IFN 10^3units/ml as a single agent caused 67% inhibition)