

**A Model for the Modulation of Cancer Chemotherapy Using  
Human Tumour Xenografts**

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**APPENDIX 1, Page 393-**

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.

James Carmichael

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## INTRODUCTION

The outlook for the majority of patients who develop solid tumours remains bleak, with many having disseminated disease at the time of clinical presentation. Refinements in surgical and radiotherapeutic techniques have failed to make a significant impact on overall survival in these patients. Over the past two decades great progress has been made in the development of novel anti-cancer agents, and also the development of drug analogues with an improved therapeutic index compared with the parent compounds. However, apart from a few highly selected treatment areas, such as Hodgkin's and non-Hodgkin's lymphoma, leukaemia, choriocarcinoma and testicular teratoma, progress, in terms of cure, has been limited

Lung cancer has been the commonest cancer in men for many years and has recently 'achieved' a similar position in women. However, with changing smoking habits its incidence is no longer increasing in men, although it continues to do so in women. Lung cancer is a serious problem which accounted for 25,000 male and 8,000 female deaths in England and Wales in 1980. The chemotherapy of these diseases raises many interesting problems, particularly as the drug sensitivity of different cell types varies widely. Small cell lung cancer is

highly chemosensitive, although long term survival remains remarkably elusive, whereas the non small cell carcinomas give the best hope of cure with local treatments, but are normally chemoresistant should the disease become disseminated.

In addition to the generally poor response to chemotherapy, particularly in non-small cell carcinomas, one also has to consider the toxicity of the drugs used. Cytotoxic drugs generally have a low therapeutic index often causing alopecia, as well as haematological, gastrointestinal and neurological toxicities which are often dose-limiting.

The refractory nature of the majority of solid tumours to chemotherapeutic agents, in addition to the toxicities associated with these agents, have stimulated a great deal of research into experimental model systems as a means of assessing the activity and side effects of these drugs used singly or in combination.

Historically, rodent tumour systems were used but these have little resemblance to human solid tumours in terms of behaviour or chemotherapeutic response. Attention has therefore switched to the use of human tumour cells for both in vitro and in vivo studies. With regard to the latter, the

human xenograft model, growing in immuno-deficient animals has been used widely with the xenografts apparently maintaining human characteristics.

Cellular detoxication mechanisms are very important in the protection against cytotoxic drugs. A major detoxication system involves the tripeptide glutathione and its related enzymes. Alterations in glutathione levels have been shown to modify the cellular response to anti-cancer drugs. With the development of drugs that can manipulate glutathione homeostasis the potential exists for the development of more effective drug combinations and schedules.

The use of human xenografts gives the opportunity to assess the activity of new anti-cancer drugs, and also investigate the potential of altering the scheduling of drugs to improve the chemotherapy of solid tumours. In addition, by manipulating the glutathione-redox system in these xenografts a unique opportunity exists <sup>to modify</sup> tumours biochemically which could be of value in the development of rational scheduled chemotherapeutic regimens.

## SECTION 1 - REVIEW OF THE LITERATURE

### CHAPTER 1

#### CLINICAL MANAGEMENT OF LUNG CANCER

##### 1.1 Introduction

There are 3 main modalities of treatment in lung cancer namely:-

- (i) Surgery
- (ii) Radiotherapy
- (iii) Chemotherapy

The management of patients with lung cancer varies according to the histological type. In small cell carcinoma, the disease is almost invariably disseminated at the time of presentation, with systemic chemotherapy therefore playing a predominant role in management. However, other histological subtypes such as squamous cell carcinoma, adenocarcinoma and large cell carcinoma, generally grouped together as the non-small cell carcinomas, are more frequently limited to the primary site with consequently, an increased emphasis on local treatments.

## 1.2 Surgery

Surgery is generally considered the treatment of choice for the non-small cell carcinomas, assuming that the tumour is resectable, although patients require sufficient cardiopulmonary reserve to withstand pneumonectomy. However, Paulson (1968) from Californian figures, showed that in only 15% of cases was the tumour confined to the lung, 30% had evidence of regional dissemination and 55% had evidence of distant metastases. Le Roux (1968) reported similar figures from Edinburgh between 1949-1964.

Fewer patients with small cell carcinoma are suitable for resection in view of the high incidence of disseminated disease at presentation, leaving chemotherapy with or without radiotherapy, as the treatment of choice (Takita, et al 1973). However, in tumours with no evidence of dissemination which are found to be small cell in type and resectable at operation, prognosis is similar to other histological types (Shields, et al 1975). Surgery can therefore be recommended, probably with adjuvant chemotherapy, for this small minority of patients, as long term survival can result (Drakeley, et al 1979).

The results of surgery for lung cancer vary from centre to centre with staging differences a significant factor. Stanford, et al (1976) showed a 50% 5 year survival in a large

series of highly selected patients, with a 27% 5 year survival in surgically managed small cell carcinoma. However, most series give a 5 year survival of around 40% in patients without nodal involvement and 15% 5 year survival with nodes affected, as reported by Wilkins, et al in 1978.

### 1.3 Radiotherapy

There is great variation in the sensitivity of various sub-types of lung cancer to x-irradiation. Small cell carcinoma is highly sensitive, but is often widely disseminated at the time of presentation drastically limiting the effect of radiation when used alone (Cox, et al 1979). In small cell carcinoma radiation has been successfully employed as a palliative measure in the treatment of bony or cerebral metastases, although radiation does not influence survival in these patients.

In contrast non-small cell carcinomas, particularly squamous cell carcinomas, are more often limited to the primary site but are less radiosensitive. In a large unselected series, Deeley (1967) reported a 36% 1 year survival, with only 6% 5 year survival using 5000 cGy. Similar figures were obtained by Caldwell and Bagshaw (1968) using 6000 cGy. Palliatively radiation is used extensively with good

effect, particularly in the control of haemoptysis, cough and dyspnoea (Slawson and Scott, 1979).

Recently there has been great interest in the use of agents which potentiate or protect tissues against the effects of ionising radiation. Radiosensitisation was successful in vitro using misonidazole, although minimal clinical benefit resulted because of dose limiting neurotoxicity (Brown, 1984).

Many other radiosensitisers have been tested, some showing potential clinical applicability, such as bromodeoxyuridine (Mitchell, et al 1983); SR 2508 (Brown and Yu, 1984) and RSU 1069 (Adams et al, 1984). Chemotherapeutic agents have also been tested some showing apparent differential protection against alkylating agent injury in tumours compared with normal tissues (Phillips, et al 1983).

In non-small cell lung cancer combinations of radiotherapy and surgery have shown enhanced 3 year survival rates in patients with nodal involvement at the hilum or mediastinum (Cox, 1985). Improvement in long-term survival rates is the major contribution of radiation therapy when used in combination with chemotherapy (Salazar and Creech, 1980), although Bunn et al (1983) in a preliminary report, showed an increased complete response rate with combined modality therapy (81%) compared with chemotherapy alone (47%).

## 1.4 Chemotherapy

### 1.4a Small cell carcinoma - single agents

Chemotherapy is the treatment of choice for the vast majority of patients with small cell carcinoma. Many agents have been shown to have significant activity as shown in Table 1.1 with prolongation of survival using single agents shown on at least 3 occasions (Kokron et al, 1978; Green et al, 1969; Roswitt et al, 1986). In view of the widespread use of combination chemotherapy regimes in this disease, single agent data is no longer forthcoming, except in heavily pre-treated patients.

### 1.4b Small cell carcinoma - combination chemotherapy

Combination chemotherapy is clearly superior to single agent therapy both in terms of response rate and duration of survival (Bunn and Ihde, 1981).

In 1976 Hansen et al reported an increase in response rate and survival in patients treated with a 3 drug combination including CCNU when compared with a combination of methotrexate and cyclophosphamide. These 3 drugs have been used successfully at the National Cancer Institute - VA group where Cohen et al, (1977) reported a 96% objective response rate to a



combination of these agents, with a median survival of 42 weeks. In the same study inferior results were obtained with lower dosages of the same drugs. More recently, Smyth et al using the same drugs but with methotrexate at a much higher dose of 200 mg/m<sup>2</sup> with folinic acid rescue, reported a 58% response rate in 83 patients. The lower response rate in this study is partly explained by the use of stricter criteria in the assessment of response including repeat bronchoscopy. Despite the lower response rate, median survival of these patients was similar to that reported by Cohen et al (1977). Most other combinations have given response rates in the region of 80%, such as the 79% response rate reported by Gracia and Jimenez (1982) in 24 patients receiving a combination of cyclophosphamide, vincristine, adriamycin and etoposide. Similarly, Klastersky et al (1982) reported an 83% response rate to a combination of cis-platinum, adriamycin and etoposide with a median survival of 15 months in the complete responders and 10 months in the partial responders.

In an attempt to improve these results additional drugs have been incorporated into the regimes with limited effect, although in a controlled study of 105 patients Hansen et al (1978) did show an increase in median survival from 25 to 33 weeks when vincristine was added to the 3 drug combination of

| Drug             | No. Patients | Complete and Partial Response Rate |
|------------------|--------------|------------------------------------|
| Ifosfamide       | 52           | 63%                                |
| Procarbazine     | 19           | 47%                                |
| Nitrogen mustard | 55           | 44%                                |
| Vincristine      | 43           | 42%                                |
| VP-16            | 213          | 40%                                |
| Cyclophosphamide | 363          | 38%                                |
| Methotrexate     | 78           | 30%                                |
| Adriamycin       | 53           | 30%                                |

Table 1.1 Single agent chemotherapy in the treatment of small cell lung cancer (Hansen and Rorth 1979; 1980)

methotrexate, cyclophosphamide and CCNU. Despite the use of 4 and 5 drug combinations, the 2 year survival of patients with this disease remains abysmal with no significant improvement in response rate or survival over 3 drug combinations as shown in Table 1.2. A number of regimens have been used in the treatment of this disease, but comparison of results is difficult, as median survival of these patients can be affected by a number of factors, such as stage of disease, site of metastases and performance status. When allowances are made for these variables, the duration of response in a number of studies, utilising a wide variety of agents, is remarkably similar. Irrespective of the induction regime employed, a median survival of 6-10 months in extensive disease and 11-13 months in limited disease can be expected.

Salvage chemotherapy in general has not been successful, although Poplin et al (1982) reported a 31% response rate in 29 previously treated patients. These patients received a combination of CCNU, vincristine, methotrexate and procarbazine, with 5 complete responders, in whom a median survival of 11 months was achieved. Other centres have used alternating non cross-resistant schedules of drugs in an attempt to improve responses, Cohen et al (1979) showed a marked benefit in terms of response rate in patients treated with a regimen of metho-

trexate, cyclophosphamide and CCNU alternating with vincristine, adriamycin and procarbazine although survival was not affected. Others have failed to confirm this improvement including Vincent et al (1980) and Aisner et al (1982), the latter in a in a controlled study using cyclophosphamide, adriamycin and etoposide switching half of the patients to CCNU, vincristine, methotrexate and procarbazine at the time of maximal response.

More recent developments include the use of high dose chemotherapy with or without autologous bone marrow transplantation. Cyclophosphamide, melphalan and etoposide have all been used at very high dosages, but it is too early to comment on the duration of the responses and survival of patients in these studies.

#### 1.4c Non small cell carcinoma - single agents

In general non-small cell lung cancer is less sensitive than small cell carcinoma to cytotoxic drugs. Squamous and adenocarcinomas are considered more sensitive to the presently available agents than large cell carcinoma in which response rates of less than 10% are reported. Cyclophosphamide was the most widely used single agent giving response rates of approximately 20%, although recently many other agents have

Limited disease

Extensive disease

| No. of trials   | Limited disease |                   |                            | Extensive disease       |               |                 |                   |                            |                         |
|-----------------|-----------------|-------------------|----------------------------|-------------------------|---------------|-----------------|-------------------|----------------------------|-------------------------|
|                 | No. of patients | Mean % CR (range) | Mean med surv, mos (range) | Mean % 2 yr DFS (range) | No. of trials | No. of patients | Mean % CR (range) | Mean med surv, mos (range) | Mean % 2 yr DFS (range) |
| 2 drugs         |                 |                   |                            |                         | 5             | 193             | 21% (12-53)       | 7.5 (4.5-14.0)             | 0% (0)                  |
| 3 drugs         | 88              | 44% (44-44)       | 8.5 (5.0-11.5)             | 6% (6)                  | 11            | 364             | 27% (0-40)        | 7.0 (4.5-10.5)             | 1% (0-9)                |
| 4 drugs         | 84              | 27% (27)          | 10.0 (6.5-14.0)            | 0% (0)                  | 9             | 286             | 27% (0-42)        | 8.5 (4.0-11.0)             | 1% (0-8)                |
| 5 or more drugs | 74              | 59% (53-74)       | 11.5 (10.0-14.0)           | 9% (5-12)               | 4             | 147             | 34% (20-41)       | 9.0 (7.5-9.5)              | 7% (7)                  |
| Totals          | 246             | 52% (27-74)       | 10.0 (5.0-14.0)            | 7% (0-12)               | 29            | 990             | 26% (0-53)        | 8.0 (4.0-14.0)             | 2% (0-9)                |

CR = complete response; PR = partial response; med surv = median survival; DFS = disease free survival; mos = months; yr = year

Table 1.2 Results of combination chemotherapy in small cell lung cancer. Taken from Ihde, D.C. and Bunn, P.A. In: Recent Advances in Clinical Oncology, Williams, C.J. and Whitehouse, J.M.A. (eds). Chapter 24, Chemotherapy of Small Cell Bronchogenic Carcinoma, 305-323, 1982, Churchill Livingstone Publishers

been tested including vindesine, etoposide, cis-platinum and ifosfamide. The results of single agent data are summarised in Table 1.3. Although responses are observed to cytotoxic drugs in non small cell lung cancer, prolonged survival has never been achieved.

#### 1.4d Non small cell carcinoma - combination chemotherapy

Until recently combination chemotherapy gave disappointing results in non-small cell lung cancer with response rates no better than for the best single agents. However, significantly improved response rates have been reported by Gralla et al (1981) using combinations of vindesine and cis-platinum. He reported a 43% response rate to 2 separate combinations of these agents showing an increased duration of response and survival in responding patients treated with the higher dose regimen. The incorporation of additional drugs into this regime in an attempt to increase the response rate or duration of response have so far proven unsuccessful (Kelsen et al, 1982; Itri et al, 1983). The activity of the cis-platinum, vindesine combination has been confirmed in a randomised study carried out by the West of Scotland Lung Cancer Group (Elliot et al, 1984) who compared vindesine as a single agent with a cis-platinum and vindesine combination. The combination gave a

response rate of 39% compared with 5% from vindesine alone.

Klastersky et al (1982) used a combination of cis-platinum with etoposide reporting a 41% response rate in 87 patients.

There are few complete responders in these studies, however, and duration of response is therefore correspondingly short at 6-12 months. Although cytotoxic to the tumour, these combinations are likewise toxic to normal host tissues causing frequent alopecia, severe nausea and vomiting, myelosuppression and neurotoxicity. Thus for approximately 40% of patients who gain limited benefit from these combinations, a further 60% suffer significant toxicity for no benefit. As yet, no controlled study has shown increased duration of survival resulting from chemotherapy in non small cell lung cancer.

### 1.5 Conclusion

Surgery remains the treatment of choice in non-small cell carcinoma of limited extent, in addition to a minority of patients with small cell lung cancer in conjunction with adjuvant chemotherapy. Radiotherapy offers excellent palliation and occasional long term survival in all forms of lung cancer. It gives valuable local control in intensive regimens and when given prophylactically to the brain in small cell carcinoma. Chemotherapy is recommended for all patients with

|                  |                            |                       | <u>Pts</u> | <u>O.R.</u> |
|------------------|----------------------------|-----------------------|------------|-------------|
| Ifosfamide       | 1.2 mg/m <sup>2</sup>      | Morgan et al 1981     | 29         | 31%         |
| Cis-platinum     | 120 mg/m <sup>2</sup>      | De Jaeger et al 1980  | 59         | 24%         |
| VP-16            | 200 mg/day x 3             | Anderson et al 1981   | 19         | 21%         |
| Vindesine        | 3-4 mg/m <sup>2</sup> wkly | Gralla et al 1979     | 43         | 21%         |
| Vindesine        | 3 mg/m <sup>2</sup> wkly   | Mattson et al 1980    | 30         | 10%         |
| Adriamycin       | 60 mg/m <sup>2</sup>       | O'Bryan et al 1978    | 56         | 14%         |
| Cyclophosphamide | 1.1 g/m <sup>2</sup>       | Brugarolas et al 1978 | 30         | 10%         |

Table 1.3 Single agent chemotherapy of non-small cell lung cancer



small cell carcinoma, with or without additional local therapy. Although chemotherapy has had a significant impact on response rates and survival in small cell carcinoma, long-term survivors and cures remain infrequent. Despite improvements in response rates in non small cell carcinoma, the toxicity of the presently available chemotherapeutic regimens preclude the recommendation of their widespread use in clinical practice at this time. The precise role for combined modality treatment in these diseases remains undefined at present.

Despite these treatments, the majority of patients with lung cancer die within a year of diagnosis. In view of the relatively high incidence of disseminated disease at presentation, advances in systemic therapy would appear to offer the best chance of improved survival in these patients.

## CHAPTER 2

### EXPERIMENTAL MODELS

#### 2.1 Introduction

A number of experimental models have been developed in an attempt to investigate tumour biology, biochemistry and drug sensitivity. These include the development of cell culture techniques allowing for in vitro correlations with the patient response, and also many improvements in immunosuppressive techniques allowing for the growth of human tumours in animal models.

#### 2.2 Tissue Culture

For many years attempts have been made to culture malignant cells. In 1907 Harrison successfully explanted small fragments of tissue from the medullary tube region of frog embryos into clots of frog lymph, and Carrel (1912), using a surgically sterile technique maintained one cell strain in tissue culture for 34 years.

The establishment of malignant tumour lines in culture proved difficult, however, as reviewed in 1973 by Giard et al., showing only 6% success with 200 tumour samples. A number of

tumour lines were established however, including lung cancer lines (Gailleau 1960; Reed 1962; Giard et al, 1973).

Over the last 10 years a wide variety of tumour types have been established as cell lines, although some histological types have proven difficult to culture. A large number of cell types can be grown in tissue culture medium, such as Roswell Park Memorial Institute (RPMI) 1640 and Hams F12 medium, when supplemented with foetal calf serum. These media contain a wide variety of constituents, including essential amino acids. Recently it has become apparent that some cell lines grow more successfully in media supplemented with growth factors (Simms et al, 1980), often with no requirement for serum. A serum-free medium has been defined for the growth of small cell lung cancer. This consists of RPMI 1640 medium supplemented with hydrocortisone, insulin, transferrin, oestradiol and selenium - Hites medium (Simms et al, 1980). Similarly, a serum-free medium has been defined for the optimal growth of breast cancer cells (Calvo et al, 1984) and for non-small cell lung cancer, which has been designated ACL 3 (Brower et al, 1986). The latter comprises RPMI 1640 medium supplemented with hydrocortisone, insulin, transferrin, selenium, epidermal growth factor, ethanolamine, tri-iodothyronine, bovine serum albumin, glutamine, sodium pyruvate and hepes buffer.

With the use of selective media, a wide range of tumour cell types can now be grown successfully in culture. This allows for the production of large quantities of tumour tissue for the investigation of tumour cell biology and biochemistry, in addition to chemosensitivity and radiosensitivity studies. Improvements in chemosensitivity and radio-sensitivity testing have been aided by developments in cloning techniques using malignant cells, and these will be dealt with more fully in Chapter 2.3.

There are many criticisms of the experimental use of cell culture techniques to study the characteristics of human tumours. There are many differences between cells in culture and human solid tumours. Cultured cells are often growing in monolayers or as small floating aggregates, with few hypoxic cells, in contrast to the clinical situation. Likewise, drug access is not a problem in culture, although in large tumour masses drug accumulation can be impaired. To address this problem, a number of variations to basic culture techniques have enabled workers to study the effects of cytotoxic drug treatment on 3-dimensional tumour models.

### 2.3 3-Dimensional Tumour Models

#### 2.3a Millipore diffusion chambers

Millipore diffusion chambers can be used to culture tumour cells from which they can be harvested and directly inoculated intraperitoneally into a normal mouse host. Immune-suppressed animals are not required with this technique. Tumour cells can therefore be grown and treated in an animal host allowing the normal metabolism of cytotoxic drugs, avoiding effects of host immunity on tumour cell growth. Selby and Thomas (1980) reported on the use of these chambers for the clonal growth of malignant cells. Drug sensitivity testing using this system correlates well with human xenograft data. The model is highly labour intensive, however, which may preclude widespread experimental use.

#### 2.3b Multicellular spheroids

Using the method of Yuhas et al (1977), a variety of human tumours have been shown to form multicellular spheroids. Spheroids can be difficult to produce from primary tumours, however, with an intermediate phase in cell culture or xenografts often necessary (Yuhas, 1977). Tumour spheroids exhibit a number of features similar to solid tumour growth, including

hypoxic cells (Durand 1976), presence of diffusion gradients (Sutherland et al, 1979) and the ability to recover from potentially lethal damage (Twentyman 1980). The chemotherapeutic response of these spheroids generally correlates with clinical response and with results obtained from xenograft experiments (Jones et al, 1982).

### 2.3c Collagen matrix assay

This assay offers an alternative three dimensional system to the use of multicellular spheroids. Tumour or xenograft material can be grown as aggregates on a collagen matrix (Yang, et al, 1979). This assay system has also been adapted by Lawler et al (1983) using a mouse mammary tumour model, obtaining a high rate of growth. Briefly, this method uses a collagen gel base on which fragments of tumour are grown, and secured with further collagen gel. Tissue culture medium is added to this and the cells cultured as normal.

As with the spheroid system, measurement of response is highly labour intensive. Analysis of response is similarly achieved, either by external assesment of tumour growth, or by clonogenic cell survival.

The collagen matrix assay certainly appears to be a more permissive environment for the growth of breast carcinoma cells, but it remains to be seen whether similar results can be achieved with other cell types. However, as with spheroids, it is unlikely to be used widely as it is highly labour intensive.

#### 2.4 In vitro chemosensitivity assays

Modifications of cell culture methods, in particular cloning techniques, have made it possible to study the chemosensitivity and radio-sensitivity of human tumour cell lines (Puck and Marcus, 1955). Cloning in a semi-solid support such as agar was accomplished in the 1950's and 1960's utilising a wide variety of animal tumours. Bradley and Metcalf (1966) then developed colony forming assays for haematopoietic cell progenitors. Over the past 10 years a number of modifications have been made to this method, including the addition of a number of growth factors both by Hamburger and Salmon (1977) and Courtenay and Mills (1978) enabling the growth of human tumour clonogenic cells from primary neoplastic tissue. Predictive drug testing has frequently been performed on individual patients' tumours. A number of techniques have been

utilised although the clonogenic assay has been most widely used and generally its results are considered the standard by which other assays are compared.

Human tumours of a single histological type appear to have a pattern of response in vitro similar to their known clinical behaviour (Von Hoff, 1981). In addition, comparisons of in vitro responses have correlated well with subsequent clinical response in matched patients (Salmon et al, 1980). Good correlation has likewise been observed between clinical response and in vitro chemosensitivity testing using the Courtenay assay in melanoma (Tveit et al, 1980).

There are, however, a number of problems relating to the use of soft agar cloning in chemosensitivity testing. Firstly for the assay to be valid, a good single cell suspension has to be achieved, which can be difficult with certain tumour cell types. By producing a single cell suspension, normal cell to cell interactions are lost, which may be important in the response of these cells to chemotherapy and radiotherapy. Technically, there are problems with this assay, such as clumping artifacts which can potentially give false negative results. Often 2 to 3 weeks are necessary before an assay can be evaluated, and even then colonies can be difficult to count. Large numbers of cells are required for the assay, often



resulting in a limited number of drugs being tested with some tumours not evaluable at all. The main problem relates the low plating efficiency of many of these tumours, particularly primary tumours which commonly give plating efficiencies of approximately 1-5% using the Courtenay assay, and 0.001 - 1% with the Salmon technique (Courtenay et al 1978). Obviously a limited number of cells survive to produce colonies, and it remains questionable whether these clonogenic cells are representative of the tumour with regard to chemosensitivity, and whether they are in fact the true stem cell population.

In view of the many difficulties, particularly relating to the time consuming nature of clonogenic assays, a number of alternative assay methods have been described. These include assays of DNA precursor incorporation, (Bender et al, 1976) assays of membrane function, (Weisenthal et al, 1983) and also of cellular metabolism, (Black and Speer, 1954). Not surprisingly, many drawbacks have been encountered using these techniques, as with the clonogenic assay. The dye exclusion assay described by Weisenthal et al, (1983) is interesting, in that a high proportion of patients specimens can be analysed, using a limited number of cells, enabling a wide range of drugs to be tested. The assay is completed in 4 days, a distinct advantage over the clonogenic assay. However, there are

similar problems of clumping in the assay making results often difficult to interpret. Despite this, further comparative studies are warranted. Recently, an assay has been described which is based on the cellular reduction of a tetrazolium salt to a coloured formazan dye (Mossman, 1983). Formazan crystal formation is proportional to cell number and these crystals can be solubilised and measured using a spectrophotometer. This assay can be semi-automated using 96 well plates and a scanning multiwell spectrophotometer (ELISA reader). Comparable results have been obtained between this method, the clonogenic assay and the dye exclusion assay in the assessment of chemosensitivity (Carmichael et al, 1986) and radiosensitivity (Carmichael et al, 1986) of tumour cell lines.

In conclusion, the majority of human tumours can now be grown in culture, although on occasions defined media with specific additives are required for optimal growth. Despite the many problems associated with clonogenic assays, these remain the standard by which new chemosensitivity assays are compared. In vitro drug testing appears to give good predictability for clinical response, although limited data are available from single agents tested both in vivo and in vitro.

There are difficulties in the definition of sensitivity using these assays, however, with dosage and duration of exposure to drugs critical (Bateman, 1979).

Further clinical correlations are required for assessment of the validity of in vitro drug testing, particularly in patients treated with single agent therapy. Similarly, further evaluation is necessary using the 3-dimensional model systems (multicellular spheroids and collagen matrix assay) to assess their value, particularly in the study of drug resistance.

#### 2.5 Animal tumour models

Various animal models have been used over the past 30 years in the development and testing of new anti-cancer agents. They have given vital information regarding the cytotoxicity and side-effects of these drugs. By far the largest drug development programme was set up at the National Cancer Institute in Bethesda, Maryland in 1955 for the systematic screening of a wide variety of natural and synthetic substances to assess their cytotoxic potential. These compounds were tested against a range of transplantable rodent tumours which had arisen either spontaneously, or through viral, or chemical induction.

Each of these tumours had a different range of sensitivity, enabling the discovery of a wide variety of potentially valuable cytotoxic compounds. The variation in sensitivity of these tumours bears some relationship to their growth rate, which is a function of the growth fraction (the number of cells in mitotic cycle - rate of cell loss: Steel 1977), with high growth fraction tumours generally more sensitive to chemotherapeutic agents.

#### 2.5a Rodent tumours

A wide variety of rodent tumours are used in drug testing. The National Cancer Institute drug screening programme involved the use of a number of these, including L1210 leukaemia, P388 lymphocytic leukaemia, B16 melanoma and Lewis lung carcinoma. The leukaemias are both rapidly growing with a high growth fraction and were both instrumental in the discovery of a wide range of anti-cancer drugs. BCNU and cytosine arabinoside were found to be active using the L1210 system, and were later shown to have similar activity against P388. P388 leukaemia was, however, more sensitive to the vinca alkaloids and in addition, activity was noted with mithramycin in contrast to L1210.

B16 melanoma and Lewis lung carcinoma are more slowly growing with lower growth fractions than the rodent leukaemias (Zubrod, 1972). Although sensitive to the nitrosoureas they were found to be relatively resistant to anti-metabolites (Schepartz, 1977).

Animals used for cytotoxicity testing can also give vital information regarding drug toxicity. Although the maximum tolerated dose for each drug is evaluated in normal mice, it is also important to assess drug-related lethality when used in tumour-bearing animals. Drug toxicity and pharmacokinetics in tumour-bearing mice are often totally different to normal animals. This is related to a number of factors including decreased hepatic microsomal drug metabolising enzyme activity (Kato et al, 1982), and altered haematological indices (Piazza et al, 1981). These differences may lead to wide variations in lethality between normal and tumour-bearing mice with subsequent loss of tumour-bearing animals.

There are many other rodent tumours used experimentally. However relatively few active drugs have been 'discovered' using these models in the last 10 years, particularly for the treatment of human solid tumours. Therefore, there is now a trend towards the use of human tumours in drug screening programmes.

## 2.6 Human xenografts

Despite the use of rodent tumours with variable growth rates there are still marked differences in sensitivity between these and human solid tumours. This has resulted in failure of recognition of a number of agents with clinical activity, such as hexamethylmelamine (Griswold et al, 1963).

Many people have attempted therefore to heterotransplant a wide variety of human tumours into experimental animals. Initial attempts were unsuccessful, exemplified by Pezrilhe in 1793 xenografting a breast carcinoma into a dog (reported by Woglam in 1913) and by Leidy in 1851 transplanting breast carcinoma in frogs, although in the latter case vascularisation of the tumour was noted. Sailer (1900) considered human tumour heterotransplantation impossible without modification of the host animals and this has led to the use of various techniques to avoid the immunological rejection of the foreign tumour material. These techniques are listed below and will be discussed further in this chapter:-

- (a) Immune privileged sites
- (b) Skin islands
- (c) Millipore diffusion chambers
- (d) Immature hosts
- (e) Immune suppressed animals

(f) Immune deprived animals

(g) Congenitally immune deficient animals

#### 2.6a Immune privileged sites

##### (i) Anterior chamber of the eye

This work was developed by Greene (1938, 1940, 1952) who established a number of tumours using this model, although other workers were less successful (Russell and Johnson 1963). However, there are significant disadvantages in using this system, with a slow tumour growth rate, poor take rate and with a strictly limited time for maximal tumour growth.

##### (ii) Brain

Several difficulties are encountered when heterotransplanting to the brains of laboratory animals, but despite this a number of workers have been successful such as Greene (1951), Lumb (1954), Chesterman (1955), Epstein et al (1976) and Gazdar et al, (1982). However, the tumours are difficult to measure and premature host death common (Giovanella and Fogh 1978). Serial transplantation difficult with either of these models.

##### (iii) Hamster cheek pouch

Billingham et al (1960) and Cohen (1961) showed that grafts survived for long periods in the hamster cheek pouch in contrast to sites elsewhere in the trunk of the hamster. A

number of workers have since been able to heterotransplant human tumour at this site (Galton et al 1963; Yohn et al 1965; Goldenberg and Witte 1967; Smith 1969; Williams et al 1971; Adams et al 1972; Cobb 1974). Others have further immunosuppressed the animals by means of cortisone, cyclophosphamide or neonatal thymectomy (Handler et al 1956; Patterson et al 1957; Burt et al 1966; Goldenburg and Witte 1967; Kaufman and Lichtenauer 1967, 1968). Take rates are higher using this model with direct measurements possible, giving a more reliable assessment of growth and response to treatment.

There are limitations to this model, however, as hybridization between tumour and host cells has been shown by chromosome banding techniques (Lampert et al, 1968; Goldenberg et al, 1971, Adams et al 1972, Goldenberg et al, 1974). Unfortunately, there are marked differences in the handling of certain drugs by hamsters particularly podophyllotoxins (Goldenberg and Witte, 1967) which limits the usefulness of this model.

#### 2.6b Skin islands

It was noted in 1968 that skin allografts inlaid in skin discs could be sustained for long periods attached to a single vascular pedicle (Barker and Billingham 1968). Ziegler et al



(1972, 1973) studied skin islands in rats as a potential site of tumour transplantation but despite the fact that syngeneic and allogeneic tumours grew well in this system, skin xenografts survived only a few days.

#### 2.6c Millipore diffusion chambers

Human tumour cells were found to grow well in these diffusion chambers when implanted intraperitoneally into xenogeneic mice (Heckman 1967). More recently the system has been adapted by various workers to grow human tumours in soft agar, with the additional benefit of transplantability to an animal model if necessary (Smith et al 1976, Selby and Thomas, 1980). Chemosensitivity testing can be done either in vitro or in vivo using this model.

#### 2.6d Immature hosts

Immature immune-incompetent hosts have been used by numerous workers to grow human tumours. However, all of these models have a strictly limited timespan for observation of tumour growth to occur, and therefore chemotherapeutic experiments and serial transplantation are difficult to carry out (Vogel and Berry 1975). The chorioallantoic membrane has been used widely for this purpose (Murphy 1912; Hurst et al 1939;

Sommers et al 1952; Kaufman et al 1956; Leighton 1967).

Neonatal rats have been shown to produce tumours following injection of cells from a cancer cell line (Kutner and Southam 1960), although less success was obtained with the use of fresh tumour material (Southam 1966). Neonatal hamsters have also been used with variable results. However the effectiveness of immature model systems is hampered both by developing immunity causing tumour regression, and on occasions rapid death of the animals (Giovanella and Fogh 1978).

#### 2.6e Immunosuppression

Adult animals with a normal immune system can be immunosuppressed either with ionising radiation or with drugs. Murphy in 1926 showed that x-rays could temporarily eradicate lymphoid tissues allowing him to produce Ehrlich carcinoma transplants in rats. In 1951 Toulan showed that human tumour xenografts could be grown for a short period in x-irradiated animals and in the following year (Toulan 1952) reported increased survival of these xenografts when newly weaned rats were used. He later combined x-irradiation with cortisone pretreatment in these newly weaned rats reporting a 90% take rate (Toulan 1953). However, serial transplantation was difficult with only 0.5% of 1000 specimens producing tumour lines (Toulan 1958). Steroids

alone have been used to immunosuppress animals with variable success. In 1951 Billingham et al reported prolonged survival of skin homografts following cortisone therapy. In contrast, initial reports suggested that inhibition of tumour growth was occurring (Sugiura et al 1950; Higgins and Bennett, 1952). Foley (1952) showed that the effect of cortisone was variable with different tumours and in different strains of animal. Variability of growth with different tumour types can be pronounced with teratomas, gastrointestinal tumours, melanomas and cervical cancers (Patterson 1968; Williams et al 1971) producing higher take rates than bladder carcinoma (Handler et al 1956).

Floersheim (1982) showed that an immunosuppressive protocol of procarbazine, cyclophosphamide and anti-thymocyte serum allowed adequate tumour growth when compared with nude mice, but that cyclosporin A was relatively ineffective. This is in contrast to the report by Deeg et al (1982) who showed that cyclosporin A was effective as an immunosuppressive allowing for the growth of allogeneic tumours in dogs.

In conclusion, a number of methods of temporary immunosuppression have been used to allow the progressive growth of human tumours. These have had variable success, though serial transplantability is rare. One of the main problems with

temporary methods is that the treatment has to be continuous if immunosuppression is to be maintained for the duration of the experiment. The agents used may significantly affect the tumour with respect to growth characteristics and biochemistry. More important, when cytotoxic drugs are used to immunosuppress animals they could potentially induce cross-resistance in the tumour thereby altering the chemosensitivity profile of that tumour.

#### 2.6f Immune deprived animals

It is well known that the thymus is vital to cell mediated immunity and is important in the normal development of the paracortical region of lymph nodes. Selective depletion of this area is produced by neonatal thymectomy, with repletion following an intravenous injection of syngeneic thymic cells (Parrot, de Souza and East 1966). In 1962 Miller showed permanent impairment of immunity following thymectomy and x-irradiation and later Davies et al (1966) reported on the major role played by T-lymphocytes in xenograft rejection.

Reduction of the T-lymphocyte population can be achieved surgically (Davies and Lewis 1967, 1968) or by the administration of anti-lymphocyte serum (Lance and Medawar 1968) or anti-thymocyte serum (Woodruff and Anderson 1963).

The combination of thymectomy with either anti-lymphocyte or anti-thymocyte serum has been shown by a number of authors to be more effective than single modality treatment (Davies and Lewis 1967, Phillips and Gazet 1968, Cobb 1972). Mitchell et al (1974) combined thymectomy with low dose x-irradiation (600 cGy) in an attempt to grow human myeloma cells with limited success. A major improvement was reported by Davies et al (1969) who showed that the combination of thymectomy, lethal x-irradiation and syngeneic bone marrow reconstitution allowed for the progressive growth of human tumours which could destroy the host. This was confirmed by Cobb and Mitchley (1974) who showed that progressive tumour growth was obtained in 25% of xenografts using this method of immunosuppression. The addition of anti-thymocyte serum did not improve tumour take rates (Detre and Gazet 1973) although Berenbaum et al (1974) reported that 75% of tumours survived, or grew progressively from a series of 116 tumours transplanted into thymectomised, irradiated, mice treated with anti-lymphocyte serum.

In 1978 Steel et al showed that the practice of injecting  $5 \times 10^6$  bone marrow cells for reconstitution was inappropriately high. He achieved improved tumour take rates with injections of  $2 \times 10^5$  bone marrow cells with no increase in morbidity of the animals. At the same time, he reported that

the use of cytosine arabinoside injected intraperitoneally at a dose of 200 mg/kg 1-3 days before x-irradiation, protected against the lethal effects of the latter, enabling the bone marrow reconstitution to be omitted entirely. The take rates in these cytosine arabinoside treated animals were higher than with bone marrow reconstituted or with nude mice.

## 2.6g Congenitally immune deficient animals

### (i) Nude mice

The first hairless mutant mouse was discovered in Scotland in 1962 (Flanagan 1966) and was found to be athymic by Pantelouris (1968). Total absence of mature T cells was noted with normal B cell function. The initial infective problems of these mice were overcome by the use of strict pathogen free environments (Giovanella and Stehlin 1973). Nude mice are now available from a wide range of genetic backgrounds. The immune systems of these mice vary, with differences in tumour receptivity often observed. In particular, differences have been noted in natural-killer cell activity between strains, and also between different laboratories using the same strains. Natural killer cells are thought by many to play a significant role in tumour rejection although Fodstad et al (1984) showed lack of correlation between natural killer cell activity and tumour

growth control. Other factors are also important however, including host macrophage function as reported by Jacobovich et al (1984). Both nude mice (Gillis et al 1979) and thymectomised irradiated animals (Duprez et al 1984) have been shown to have normal T lymphocyte precursors but have defective lymphokine production.

Colonic tumours were first grown in 1969 (Rygaard and Povlsen) and subsequently numerous human tumour xenografts were successfully grown (Povlsen and Rygaard 1971; Epstein and Kaplan 1974; Giovanella et al 1974). However some cell types, including carcinoid tumours and seminoma, remain difficult to grow as xenografts.

(ii) Lasat mice

Festing (1980) reported on the discovery of an athymic, asplenic Lasat mouse. In these mice, tumour take rates were higher and the latent period prior to tumour growth shorter than with conventional nude mice, but infective problems were much greater, as may have been predicted.

(iii) Beige nude mice

There are now strains of mice available, termed beige mice, which are immunodeficient with regard to natural killer cell activity. From these animals a strain of nude mouse has been derived which has the characteristics of both strains

(Fodstad et al 1984). This, so called beige nude mouse, therefore has the immunological characteristics of a neonatal nude mouse, and is potentially a highly receptive host. As with lasat mice, however, infective problems may preclude its widespread use.

## 2.7 Conclusion

The vast majority of human cancers can now be grown either in cell culture or as xenografts in a wide variety of hosts. The choice of optimal model is dependent on the aims of the particular study. Cell culture techniques provide a rapid means of producing large numbers of tumour cells, and would seem ideal for use in drug screening projects. Three dimensional assay systems, such as multi-cellular spheroids, and collagen matrix assays address the problem of drug access and hypoxic areas of tumours, but the problems of cytotoxic drug pharmacokinetics remain. Human xenografts in immune-deficient animals are an improvement in this regard, although it is accepted that variability occurs in drug handling between species.



## CHAPTER 3

VALIDITY OF HUMAN TUMOUR XENOGRAFT MODELS3.1 Introduction

With the wide range of immunosuppressed animal models now available, the vast majority of human cancers have been established as xenografts. Most of this work has been carried out in nude mice, of varying genetic backgrounds, although some centres have used thymectomised, cytosine arabinoside primed, irradiated mice as described by Steel et al (1978).

3.2 Receptivity of xenografts

Comparisons of take rates between different human xenograft models have proven difficult, as the criteria for a positive take have varied between centres. In some models the continued presence of tumour material was sufficient for a positive take, (Berenbaum et al 1974), but it has since transpired that xenografted cells, in the absence of progressive growth, may not be serially transplantable (Sharkey et al 1978). It is now generally accepted that criteria for a positive take include progressive growth, maintenance of human characteristics and serial transplantability.

Wide variation in take rate occurs with different histological categories of tumours, with colorectal carcinomas, melanomas and lung cancers giving the best figures. Reports by Povlsen and Rygaard (1976) and Sharkey et al (1978) revealed impressively high take rates using melanoma and colorectal tumour samples. These figures have been confirmed by numerous other authors.

The first report of successful growth of bronchial carcinoma xenografts was by Greene (1952) who reported 13/13 positive takes using both the anterior chamber of the eye and guinea pig brain as models. Primary tumour samples were used in 3 cases, the remaining samples were obtained from secondary deposits. However, no information was given in this study regarding the number of transplants required from each specimen to produce a successful take. Excellent results were also reported by Shimosato et al (1976) and Sharkey et al (1978), with bronchial carcinoma generally transplanting well in the majority of model systems. Early reports showed high take rates of both squamous and small cell carcinomas, with little information on other histological categories. Shorthouse (Ch.M. thesis 1981) reported on the results of 49 lung cancer biopsy transplants, with a 78% success rate in establishment of

xenograft lines. However, a large number of these specimens were from metastatic deposits, with large cell and adenocarcinoma specimens giving the highest take rates.

In contrast, other tumours have been more difficult to establish. In breast carcinoma Povlsen and Rygaard (1976) were unsuccessful with 5 transplants and Sharkey et al (1978) achieved only 16% of positive takes from 87 specimens, with serial transplantability in only 7%. Giovanella et al (1978) were more successful with 67/127 (53%) positive takes, although no information was given regarding the number of grafts from each specimen showing progressive growth. However serial transplantation was achieved in 6/8 tumour lines attempted, far superior to results obtained by any other group.

Ovarian carcinoma has proven difficult to establish (Povlsen and Rygaard 1976; Sharkey et al 1978) although Kullander et al (1978) were more successful. Similar problems have been encountered in attempting to establish lymphomas and leukaemias (Povlsen and Rygaard 1974; Cobb 1974). Intracranial inoculation has improved these results (Epstein et al 1974, 1976) and recently a carcinoid tumour has been established for the first time in the anterior chamber of the eye (Nilsson et al 1984).

Despite the frequency of success with germ cell tumours, seminoma has proven very difficult to establish. Raghavan et al (1980) did report on the growth of a xenograft from a patient thought to have a seminoma, although the xenograft was shown to have germ cell features. There is no report on the establishment of a pure seminoma xenograft at present.

In summary, a large number of xenografted tumours have been established using various model systems. Take rates in the hamster cheek pouch and anterior chamber of the eye have been excellent but progressive growth is obviously limited and serial passage difficult. Of the models allowing for progressive growth, nude mice and TAR (thymectomy, cytosine arabinoside pretreatment and total body irradiation) mice give impressive take rates. Total body irradiation has been shown to temporarily impair natural killer cell function (Hastings - personal communication) with TAR mice giving marginally better take rates than nude mice (Rostom et al 1978). However, a significant number of these mice regain some or all of their immunity 4-6 weeks after total body irradiation. Although optimal for the establishment of many xenograft lines these TAR mice may be less than ideal for longer term chemotherapy

studies (Steel 1978). The establishment of some histological subtypes remains difficult, although with the use of lasat and beige nude mice these problems may well be overcome.

### 3.3 Factors Influencing the Successful Establishment of Xenografts

Successful takes are more common in tumours with a rapid doubling time, although it has to be stressed that this does not necessarily relate directly to the differentiation of the tumour. In fact, some non-transplantable tumours have been poorly differentiated (Houghton and Taylor 1978). Biopsies from metastatic deposits give higher take rates than primary tumour specimens (Sharkey et al 1978) and this may account in part for the very high take rate reported by Shorthouse (1981). Steel (1978) reported increased take rates on tumours first established in cell culture in agreement with other workers (Fogh et al 1977).

The strain of mouse can affect the take rate (Hanna et al 1982) as can the sex, with increased take rate in female animals (Pettengill et al 1980), both thought to be related to immunological factors. The site of implantation of the tumour fragment affected the take rate, with increased success observed when specimens were transplanted anteriorly on the

flank compared with posteriolateral implantation (Kyriazis and Kyriazis 1980; Dipersio 1981; Auerbach and Auberbach 1982). The enhanced growth observed with anterior implantation is thought to be related to improved vascularisation and decreased necrosis of the tumour. In addition, regional lymph node metastases were more frequent with anterior implantation. Similarly, Levy et al (1982) showed that the characteristics of the MCF-7 mammary adenocarcinoma cell line varied according to the site of inoculation.

In summary the tumour take rate is determined by a number of factors which can be loosely grouped into tumour related and host related. The tumour-related factors are the cell type, whether it is a primary or metastatic tumour sample, and its doubling time. Host-related factors consist of the strain of animal used, method of immunosuppression and site of implantation of the tumour.

### 3.4 Growth Kinetics

The growth kinetics of tumours can affect their response to cytotoxic drugs (Steel 1978). Cell proliferation in murine tumour systems is more rapid than in human solid tumours, with a significant difference in the duration of DNA synthesis

(Steel 1977). This may make a difference in the response of these tumours to certain anti-cancer drugs, particularly phase specific agents.

Similar growth kinetics have been observed in human solid tumours and in human xenografts. The labelling index in gastrointestinal tumour xenografts (Schmidt et al 1977) has been shown to be similar to previous reports on these neoplasms in patients. However, despite the similarity in labelling indices, tumour doubling times are significantly shorter in xenografts. This may be related to a number of factors, including decreased cell loss in xenografts (Lammerton and Steel 1975). Xenografted tumours are generally smaller and thus may have improved vascularisation in comparison with primary tumours and this may be important in the relative growth rates of these tumours. In addition, with successive passaging of xenografts, the more rapidly proliferating clones may be selected, which could alter their growth characteristics.

### 3.5 Maintenance of Human Characteristics

If human xenografts are to be used widely in the study of human cancer, the characteristics of the original tumour have to be maintained. This has been extensively studied by numerous authors showing that this is indeed the case for a number of parameters, as is shown in the following sections.

#### 3.5a Histopathology

In all models, maintenance of the histopathological features of the original human tumour are seen irrespective of the number of passages undergone (Povlson and Rygaard 1971; Houghton and Taylor 1978). However there is greater diversity of opinion regarding changes in differentiation of xenografted tumours. Sordat et al (1974) reported no change in differentiation of transplanted tumours, but many other authors have reported either increased or loss of differentiation. Selby et al (1979) showed loss of differentiation in a testicular teratoma xenograft using immunoperoxidase staining for  $\beta$ -HCG. Giovanella et al (1978) likewise observed loss of differentiation in a melanoma xenograft, whereas Sharkey et al (1978) showed increased differentiation in 25% of transplanted



tumours. Shorthouse (1981) reported frequent loss of differentiation although increased differentiation was noted in 6% of xenografted tumours.

### 3.5b Electron Microscopy

Similar electron microscopic features have been identified in xenografts when compared with original tumours, including the presence of keratin filaments and desmosomes in squamous cell carcinoma, neurosecretory granules in small cell lung cancer (Kameya et al 1977) and melanin granules and melanosomes as described by Selby et al (1979) in melanoma.

### 3.5c Histocytochemistry

In general the histocytochemical features of the donor tumour are maintained in xenografts. Houghton and Taylor (1978) showed no change in the epithelial mucin pattern of xenografts and Selby et al (1979) reported maintainance of pigmentation in 80% of melanoma xenografts, although in 2 loss of pigmentation occurred with loss of differentiation of the tumour. The latter finding was also reported by Shimosato et al in (1976) and Giovanella et al (1978).

### 3.5d Chromosomes

Many authors have shown that human karyotypes are maintained in xenografted tissue. When working with human xenografts it is important to establish frequently that the tumours have a human karyotype as spontaneous murine tumours have been known to arise in conjunction with xenograft tissue (Houghton and Taylor 1978; Goldenberg and Pavia 1982; Staab et al 1983). Maintenance of human characteristics has been shown by Whang-Peng et al (1982) who described a cytogenetic abnormality in small cell lung cancer, a 3 p deletion, which was shown to persist following multiple passages in tissue culture. However, chromosomal changes have been noted on passaging using banding techniques (Reeves and Houghton 1978).

### 3.5e Functional activity

It has been shown that species specific isoenzymes such as lactate dehydrogenase and glucose-6-phosphate dehydrogenase, maintain human patterns in xenografts (Houghton and Taylor 1978). Similarly the key APUD enzyme, L-dopa decarboxylase, has been demonstrated in both clinical specimens and in small cell carcinoma cell lines (Carney et al 1983). However, arylsulphatases have been shown to vary in transplanted tumours

compared with surgical specimens, although this may be related to alterations in the interstitial supportive tissue of these xenografts (Gasa 1981).

Surface antigens such as carcinoembryonic antigen and alphafoetoprotein which are associated with a variety of tumours in man, have been shown to persist in human xenografts. Carcinoembryonic antigen has been associated with colonic carcinoma in particular for many years and was noted in xenografts by Sordat et al in 1974. This finding has since been confirmed by many authors, with Miwa et al (1977) showing localisation of carcinoembryonic antigen by immunocytochemical techniques. Alphafoetoprotein has likewise been detected in teratomas (Raghaven et al 1980) and hepatoma xenografts (Hirohashi et al 1977) in nude mice. Similarly, in 1974 Mitchell et al induced myeloma in nude mice by the injection of bone marrow cells from affected patients. The characteristic immunoglobulin production of the original tumour was maintained in these animals with overproduction of IgG and IgA.

The growth of endocrine tissue in nude mice has proven difficult, but where this has been achieved normal hormone production has been maintained (Kameya et al 1977). Renal carcinoma xenografts have been shown to secrete erythropoietin

by a number of workers including Tamaoki in 1977. Ectopic hormone production, which is a feature of some human malignant neoplasms, has also been detected in nude mice bearing these tumours. Human chorionic gonadotrophin is often produced by testicular germ cell tumours and Selby et al (1979) illustrated the continued production of this hormone by immunohistochemical techniques and radioimmunoassay over a number of passages in xenografted mice. However, after 5 passages the production of hormone decreased, and this was associated with progressive loss of differentiation of the tumour.

Bronchial carcinomas are associated with a wide variety of ectopic hormone producing syndromes. Biochemical evidence for excessive production of these hormones is present in approximately 25% of small cell carcinoma patients, although the incidence of clinical syndromes is less than 10% (Greco 1981). A large number of hormones are produced by lung tumours including anti-diuretic hormone, adenocorticotrophic hormone, melanocytic-stimulating hormone and calcitonin (Becker et al 1983). These have all been shown to persist in xenografts (Kameya et al 1977; Shin et al 1977) and cell lines (Sorenson et al 1982). Recently, other tumour markers have been observed in small cell carcinomas, these include L-dopa decarboxylase (Baylin et al 1980), bombesin (Sorenson et al 1982; Moody et

al 1981, 1983), neuron-specific enolase (Marangos et al 1982) and the BB isoenzyme of creatine kinase (Carney et al 1985). These are relatively specific to this tumour type and have been shown to persist in cell culture (Sorenson et al 1982). Ectopic parathormone production has also been detected in lung cancer patients but as yet has not been identified in xenografted animals, although hypercalcaemia has been observed (Ohsawa et al 1977).

Of interest is the hormone-dependency of some tumours which can be grown in immunosuppressed animals. Breast carcinoma is the commonest of these and, likewise has been shown to be hormone dependent in some xenograft models (Hirohashi et al 1977; Levy et al 1982).

### 3.6 Metastatic disease

Initial reports suggested metastatic disease was a rare occurrence in human tumour xenograft models (Giovanella et al 1972; Detre and Gazet 1973; Sharkey et al 1978). With the passage of time, however, the number of reports of metastatic disease in xenografted animals has steadily increased. A number of important factors have come to light regarding this phenomenon.

The biology of the tumour cells implanted may well be important. It has been shown that there is enhanced metastatic potential in murine tumour cells obtained from metastatic sites when compared with the primary tumour samples (Talmadge and Fidler 1982; Poste et al 1982). A study reported by Kerbel et al (1984) showed similar findings using a human melanoma cell line transplanted into nude mice. In addition, it has become apparent that xenografts derived from established cell lines will frequently metastasise with systemic spread noted in both thymectomised, irradiated mice and in nude mice (Giovanella et al 1974; Franks et al 1975; Kier and Schroeder 1982). A new osteosarcoma strain has emerged that has a high metastatic potential (Kuroki 1984) and recently additional metastatic models have been observed. Gazdar (personal communication) has developed 2 metastatic models in nude mice, one a large cell carcinoma of bronchus and the other a pulmonary adenocarcinoma. Both tumours were originally established as cell lines, then inoculated intra-peritoneally into nude mice. The metastases appeared in multiple sites, including kidney, liver, heart, lungs and brain, similar to the pattern of distribution expected clinically.



Improved techniques of immunosuppression have also increased the incidence of metastases. Regional invasion is frequent in thymectomised, cytosine arabinoside pretreated, irradiated mice (Steel et al 1978) although metastases are more frequently seen with the use of neonatal nude mice (Sordat et al 1977). Natural killer cell activity is virtually absent in the neonatal animal, and this may well account for the difference in metastatic potential. Variations in natural killer cell activity may, likewise, explain strain dependent differences in metastatic potential of nude mice although others dispute this (Fodstad et al 1984). Reid et al (1981) showed that treatment with mouse interferon decreased natural killer cell activity allowing metastases to occur. The injection of xenogeneic human T-lymphocytes into tumour-bearing nude mice has also been shown to increase the incidence of metastases (Graham 1982), although the reason for this remains undefined.

On a more practical basis, one also has to consider the duration of survival of the host following transplantation, as premature death may preclude the appearance of micrometastases (Steel and Peckham 1980).

### 3.7 Cachexia

The cause of cachexia in humans remains in doubt, although abnormalities in glucose metabolism have been implicated. Other authors have suggested alterations in lipid metabolism involving "toxohormones" are related (Masuno et al 1981) although this remains speculative.

Despite frequent, and often marked weight loss in patients suffering from particular cancers, cachexia is rare in experimental animal models, even when the xenografts become very large (Povlsen and Rygaard 1971; Giovanella et al 1974). However, there are some reports of tumours causing weight loss, such as the murine sarcoma MCG101 (Eden et al 1983) in addition to human xenografts originating from a melanoma (Kondo et al 1977), and a hyperneophrroma (Strain et al 1980).

Cachexia could be caused by soluble human proteins, and lack of cachexia in mice may not necessarily infer loss of functional activity in xenografts, but may merely reflect lack of species cross-reactivity to these substances. However, it is likely that cachexia may be related to the production of cachetin. Cachetin, or tumour necrosis factor (Beutler and Cerami 1986), is produced by 'host' macrophages, with survival of 'host' macrophages limited following transplantation. In



addition, the immune reaction is different in these mice, with their macrophages not stimulated to produce this 'hormone'.

### 3.8 Chemotherapeutic response in xenografts

It is now generally accepted that the chemotherapeutic response in xenografts is similar to that of the tumour of origin.

In 1974 Ziegler showed complete regression in a Burkitts lymphoma xenograft treated with cyclophosphamide, similar to the expected clinical response. Kopper and Steel (1975) and Mitchley et al (1975) found similar correlation between clinical and xenograft response using a variety of tumour types.

However, it is known that there is variability in clinical response of any particular histological type of tumour to cytotoxic drugs. Similar variability was noted in xenografts by Osieka et al (1977) and Nowak et al (1978).

Of even more interest, is whether the xenograft maintains the same pattern of sensitivity, and therefore could predict the chemotherapeutic response of the 'source' tumour. A number of studies have addressed this problem, not without difficulty. Burt et al (1966) showed similarity of response in a bladder xenograft when compared with the donor patient, whereas other

authors have reported conflicting results. Hertz (1967) found poor correlation in methotrexate sensitivity comparing the response of choriocarcinoma in the patient and the xenograft growing in the hamster cheek pouch. However it should be emphasised that a relatively high dose of methotrexate was used in the animal, which was inappropriately sensitive, although this explanation does not account for the poor correlation for vinblastine sensitivity. This study, (Hertz, 1967), was far from ideal, however, as all patients had been treated before xenograft material was obtained, and the chemosensitivity tests were carried out after multiple tumour passages.

Berenbaum et al (1974) experienced great difficulty in establishing tumours using mice prepared by thymectomy, irradiation and with anti-lymphocyte serum. Chemosensitivity tests were performed in only 4/104 tumours using histological damage as the end-point of assessment. Variable growth was obtained in these xenografts however, and therefore quantitation of the results was impossible. No direct patient-xenograft comparisons were achieved. However, in 1977 Giovanella et al reported good correlation with clinical response using breast carcinoma xenografts growing in nude mice with growth delay used as the end point of assessment.

Similar to Hertz (1967) xenografts were shown to be marginally more sensitive. Hayashi et al (1978) and Nowak et al (1978), using choriocarcinoma and colorectal xenografts respectively, have likewise claimed good correlation with clinical response.

Shorthouse (1980) reporting an excellent study, showed good correlation of response in 14 lung cancer xenografts when compared with clinical response in the respective donors. Five small cell carcinoma xenografts were established from previously untreated patients, all of whom had achieved complete remissions. All 5 xenografts completely regressed with identical therapy.

One other patient with small cell carcinoma had been treated prior to the establishment of the xenograft. This patient did not respond clinically and the xenograft was likewise resistant to chemotherapy. In the 8 non-small cell carcinomas, the poor patient response correlated very well with minimal response in the xenograft.

### 3.9 Conclusions

In general, human xenografts growing in immunosuppressed animal hosts retain human tumour cell characteristics. However, they do have shorter doubling times with a tendency to

lose differentiation over successive passages. The majority of cell types can now be successfully established as xenografts with these xenografts exhibiting a similar chemotherapeutic profile to the original tumours. Many human characteristics are maintained, including histological, electron microscopic and histochemical features. Functional activity is maintained and patterns of growth, including metastatic spread, have now been shown to be similar. Therefore human xenografts offer an excellent in vivo model for the study of the biology and biochemistry of human tumours.

## CHAPTER 4

ASSESSMENT OF CHEMOTHERAPEUTIC RESPONSE IN XENOGRAFTS4.1 Introduction

There have been prolonged debates regarding the validity of some xenograft models. One of the major problems relates to the end-point of assessment of response where the following methods can be used:

- (1) Histopathological assesment
- (2) Clonogenic cell survival assays
  - (a) following in vivo drug treatment
  - (b) following in vitro drug treatment
- (3) Tumour regression rate
- (4) Specific growth delay

4.2 Histopathological Assessment

This was first attempted by Berenbaum et al in 1974. He encountered many problems as great variability in growth was noted during the initial man to mouse passage. Of 44 tumours tested, only 3 showed extensive histological damage after treatment. Kyriazis et al (1983) carried out a similar pathological study but divided responses into mild, moderate

and severe. He showed good correlation between response determined by growth delay and pathological damage. He claimed that histopathological assessments were superior to tumour regression analysis as an indicator of chemosensitivity particularly when significant necrosis was present in the tumour.

#### 4.3 Clonogenic Cell Survival Analysis

Variability in host defence mechanisms could have a significant impact on chemotherapeutic response, particularly with long term experiments. Thymectomised, irradiated mice specifically, may regain some immunity 4-6 weeks after irradiation, with an increase in particular in natural killer cell activity (Hastings - personal communication). The problem of returning immunity can be avoided with the use of clonogenic assays (Courtenay and Mills 1978) to measure response following in vivo or in vitro drug exposure.

##### 4.3a Clonogenic Survival Following In Vivo Treatment

Tumour bearing animals are treated in vivo and the tumour resected 18-24 hours later, allowing full chemotherapeutic effect. Single cell suspensions are made from the excised tumour and incubated in soft agar using previously described

techniques. The proportion of tumour cells producing colonies from drug treated animals (plating efficiency) is compared with the plating efficiency from untreated control tumours. The surviving fraction of treated tumour cells is derived from the ratio of the respective plating efficiencies (Courtenay and Mills 1978).

#### 4.3b In Vitro Treatment

Tumours are excised and a single cell suspension obtained. The cells are then exposed to the drug(s) in vitro for a specific period, then plated out and incubated in soft agar. Bateman et al (1979) reported good correlation between this method and xenograft growth delay in pancreatic carcinoma.

#### 4.4 Xenograft Regression Rate

The rate of regression of a xenograft can be used as a assessment of chemotherapeutic response. This has been shown to be effective clinically in small cell carcinoma (Lenhard et al 1983) with regression in the first 28 days correlating well with survival. However, in xenografts regression rate is used less frequently than growth delay, although it may give important information in exquisitely sensitive tumours.

Kyriazis et al (1983) suggested that regression rates should always be calculated concurrently with histopathological assessment.

#### 4.5 Specific Growth Delay

This is the parameter most often used in the assessment of chemosensitivity in xenografts, and the one with which all new methods of response evaluation must be compared. It is derived from the tumour doubling ( $T_D$ ) times of control and treated xenografts by the following formula:-

$$\text{Specific Growth Delay} = \frac{T_D (\text{treated}) - T_D (\text{control}) (\text{Kopper 1975})}{T_D (\text{control})}$$

It is expressed in units of tumour doubling time, with the most sensitive tumours obviously having the highest value.

This is the most reliable method of assessing chemotherapeutic response at present, although there can be problems with its interpretation. Difficulties arise with tumours that regress completely and with experimental deaths prior to doubling of tumour volume. Estimation of tumour volume can also be problematical in view of the error involved in external



measurement, particularly in small tumours and cavitating tumours. Despite these problems, good correlation is found between xenograft growth delay and clinical response (Shorthouse 1981).

#### 4.6 Conclusion

There are a number of methods by which xenograft response can be measured. Survival would be the optimal parameter, but this is highly variable in human tumour models. Growth delay is, therefore, generally considered the optimal method for assessment of chemotherapeutic response.

## CHAPTER 5

### MODULATION OF CANCER CHEMOTHERAPY

#### 5.1 Introduction

Chemotherapy has been used to treat all types of human solid tumours with a variable degree of success. Apart from a few notable exceptions such as testicular teratoma, cure has remained remarkably elusive, with the narrow therapeutic index of the presently available cytotoxic drugs a major problem. The overall effect of chemotherapy remains a delicate balance between anti-tumour activity and toxicity. A number of factors may affect the response of tumours to cytotoxic drugs thereby causing drug resistance, and these can be categorised generally as host or tumour related:

## 5.2 Host Related Resistance Factors

### 5.2a Host Toxicity

The total dosage of chemotherapy administered to a patient is often attenuated by drug-related toxicity, particularly to the bone marrow. Variation in administered drug dosage could obviously affect the patients' clinical response.

### 5.2b Variations in Detoxication Systems

The majority of anti-cancer drugs are metabolised as foreign compounds mainly in the liver, and in the process may be activated or deactivated. There are a number of detoxication pathways for the handling of xenobiotics of which the following are the most relevant to cancer chemotherapy:

#### (i) Cytochrome P-450 system

This consists of a heterogenous group of enzymes with variable substrate specificity, essential in the metabolism of a wide range of xenobiotics.

#### (ii) Superoxide Dismutases

Anthracyclines have been associated with the production of several toxic species, including hydrogen peroxide, hydroxyl radicals and superoxide radicals (Sies et al 1983). Superoxide dismutases are involved in the detoxication of superoxide

radicals to form oxygen and hydrogen peroxide (Wefers and Sies 1983) which can then result in the production of hydroxyl radicals via Fenton-like reactions. Superoxide dismutases, catalase and glutathione peroxidase are involved in the detoxication of these species.

(iii) Glucuronidation

This represents one of the major conjugation reactions involved in the metabolic conversion of xenobiotics to polar water soluble metabolites, although glucuronidation may be less important than the glutathione redox system in the handling of cytotoxins (see below).

(iv) Glutathione Redox System

This system will be dealt with more comprehensively because of its importance in the prevention of alkylating agent toxicity and in view of the experimental data presented later in this text.

Glutathione is a tripeptide with the amino-acid sequence of  $\gamma$ -glu-cys-gly. Glutathione can either bind directly with electrophilic compounds to form glutathione conjugates or can be oxidised at the thiol group of the cysteine residue to form a disulphide, often denoted as GSSG. Glutathione is a major constituent of all cells and is normally the predominant non-protein thiol present. Thiols are in general very reactive

towards free radicals and are potentially very important in the prevention of oxidative damage by superoxide and hydroxyl radicals (Sies et al 1983).

Glutathione and glutathione-dependent enzymes such as glutathione peroxidase and the glutathione transferases, have been shown to participate in a large number of intracellular reactions involving the detoxication of drugs, carcinogens and their metabolites (Arias 1976; Meister 1981; Larsson 1984). Various toxins have been shown to deplete intracellular glutathione (Mitchell et al 1982) and glutathione transferase levels (Di Simplicio 1982) in the liver.

The glutathione transferases are a group of multi-functional isoenzymes involved in detoxication, with the capacity to bind an enormous number of hydrophobic compounds (Jakoby 1978). They are, in addition catalysts, for a wide variety of reactions involving glutathione, and may also directly act as scavengers by covalently binding alkylating radicals.

### 5.3 Tumour Related Resistance Factors

#### 5.3a Elevated Enzyme Levels

These can be particularly important in resistance to anti-metabolites. A good example is methotrexate, where high levels of dihydrofolate reductase can negate the effects of the drug. However, many other mechanisms are also important, such as impairment of polyglutamation.

#### 5.3b Alteration of Membrane Structure

The presence of a 170 kilodalton glycoprotein (P-glycoprotein), first described by Juliano and Ling (1976), has been shown to be associated with multi-drug resistance. It would appear to be particularly important in resistance to natural products such as adriamycin and the vinca alkaloids. This resistance is partly explained by enhanced drug efflux, resulting in diminished drug accumulation particularly in the nucleus of resistant cells.

#### 5.3c Kinetic Variations

Tumours with a low growth fraction are generally considered to be more resistant to chemotherapy, as many drugs are cell cycle or phase specific.

#### 5.4 Approaches to Manipulate Chemotherapy

With greater knowledge of the biochemical effects and pharmacokinetics of anti-cancer drugs, numerous attempts have been made to improve their therapeutic index. Arbitrarily, an improved therapeutic index can be achieved by either increasing the anti-tumour activity, or by protecting normal tissues from toxicity.

##### 5.4a Methods of Increasing Anti-Tumour Activity

Cytotoxic drugs can only be effective if they gain access to the neoplastic cells. Attempts have been made to improve drug uptake of neoplastic cells, by combining drugs with agents that are actively transported into cells. For instance, melphalan, a widely used alkylating agent, consists of nitrogen mustard attached to the amino-acid phenylalanine which is thought to promote incorporation of the drug into the cell by an active transport mechanism (Vistica et al 1977).

The pharmacokinetics of a drug are obviously affected by the route of administration. Variations in drug delivery can cause changes both in anti-tumour activity and in host toxicity. As an example of this, Evans et al in 1984, showed that higher doses of cyclophosphamide were tolerated by mice,

with no loss of anti-tumour activity when the cyclophosphamide was administered in divided doses in comparison to a single intra-peritoneal dose.

Many variations in drug administration have been attempted in order to increase the anti-tumour effect of cytotoxic drugs. These variations are listed below and discussed individually:

- i) timing of drug administration - circadian rhythm
- ii) combination with calcium channel blockers
- iii) combination with glucocorticoids
- iv) combination with electron-affinic radiosensitisers
- v) combination with buthionine sulfoximine
- vi) combination with biological response modifiers
- vii) combination chemotherapy
- viii) combined modality therapy

i) Timing of Drug Administration

The timing of administration of cytotoxic drugs may be important, as their metabolism appears to be affected by a circadian rhythm. This has been shown with methotrexate, (English et al 1982) cis-platinum, (Hrushesky et al 1982) and melphalan (Simpson and Stoney 1977). Both methotrexate and cis-platinum give maximal toxicity at 0600 hours with minimal toxicity at midnight, whereas melphalan toxicity was greatest



at 16.00 hours. However, a recent study by Focan (1979) stressed that far more tumour cells are in cycle in the early morning, and that approximately 30% more anti-tumour activity is found with the use of S-phase specific agents at this time. Therefore, although interesting, it remains debatable whether circadian rhythm differences can be utilised for any significant clinical benefit.

(ii) Combination with Calcium Channel Blockers

The vinca alkaloids, vincristine, vinblastine and vindesine are all known to interfere with calcium transport (Gietzen et al 1980). Tsuruo et al in 1981 first reported enhanced cytotoxicity both in vivo and in vitro with vinca alkaloids when combined with the calcium channel blocker verapamil. Tsuruo et al (May 1983) later reported that verapamil increased the cellular level of vincristine by blocking drug efflux particularly in resistant cell lines. He later showed (Tsuruo et al June 1983), that resistance to vinca alkaloids, or adriamycin which is often found to be cross-resistant, could be reduced with the use of calcium influx blockers.

However, a recent report by Rabkin et al (1983) pointed out that increased mortality could also occur through enhanced adriamycin cardiotoxicity when combined with verapamil.

Although there may be a place for the use of calcium channel blockers, particularly in the treatment of in tumours that have become resistant to multiple drugs, (Riordan and Ling 1985) a more detailed assessment of the toxicities associated with these combinations is warranted.

(iii) Combination with Glucocorticoids

Corticosteroids are active drugs in the treatment of lymphoid malignancies. In addition, glucocorticoid hormones have been shown to have significant effects on cytotoxic drug action. Prednisolone has been shown to enhance the anti-tumour activity of mustine, chlorambucil, melphalan and cyclophosphamide, with suppression of gastrointestinal toxicity in combination with the first two agents (Shepherd and Harrap 1982). Similar enhancement of activity has been observed with combinations of corticosteroids and platinum analogues (Goddard et al 1982). Prednimustine is a commercially available combination of chlorambucil and prednisolone which is presently undergoing clinical trial. However, in view of the mode of action of this combination, it is possible that scheduling may significantly affect the synergistic action.

(iv) Combination with Electron-Affinic Radiosensitisers

Nitroimidazoles such as misonidazole have been shown to increase the sensitivity of cells in vitro to the lethal effects of radiation, heat and many alkylating agents used in current cancer chemotherapy. Misonidazole has also been shown to be cytotoxic to hypoxic cells (Varnes et al 1980). Rose (1980) using the Lewis lung carcinoma model, showed improvement in the therapeutic index with an in vivo combination of misonidazole and melphalan. Enhanced activity has likewise been confirmed with combinations of misonidazole and other cytotoxic drugs as reported by many authors, including Sutherland et al (1980), using adriamycin in multicellular spheroids, and Stratford et al (1980) using cis-platinum.

The mechanisms of chemosensitisation by misonidazole have not been fully elucidated. Taylor et al (1982) showed that misonidazole both depleted intracellular glutathione and also exhibited enhanced cytotoxicity on glutathione depleted cells. However, using a glutathione depletor diethyl maleate, it was shown that glutathione depletion was not the only mechanism of action of misonidazole. It has been shown to enhance binding of cytotoxic drugs, increase DNA strand breaks and cross links,

in addition to altering the pharmacokinetics of some drugs (Tannock 1980). In general, misonidazole interacts best with bifunctional alkylating agents and nitrosoureas (McNally 1982).

Increased toxicity to the bone marrow and gut were also observed with combinations of misonidazole and cytotoxic drugs, requiring dosage modification of the latter (Rose 1980). However, there is normally a therapeutic gain with large doses of misonidazole, although it is ineffective at lower doses in mice (Rose 1980). Unfortunately, in man neurotoxicity is dose limiting, and it seems unlikely that sufficiently high serum levels will be achievable clinically (Brown 1984). Other radiosensitisers of the nitroimidazole class are still under investigation (Guichard and Malaise 1982; Adams et al 1984) which may not have dose-limiting neurotoxicity. This subject was recently excellently reviewed by Brown (1985).

(v) Combination with Buthionine Sulfoximine

Buthionine sulfoximine is one of a group of drugs that deplete intracellular glutathione (Griffith 1982). It is a competitive inhibitor of  $\gamma$  glutamyl-cysteine synthetase and has no other documented primary effects on the cell (Griffith and Meister 1979). It has been shown that buthionine sulfoximine will deplete glutathione in many cell lines, and when used in combination with radiation, heat or a number of cytotoxic drugs

will enhance cell kill (Roizin-Towle et al 1984; Mitchell and Russo 1983; Russo et al 1984; Russo and Mitchell 1985). Hamilton (personal communication) showed that buthionine sulfoximine treatment enhances the cytotoxic activity of melphalan in vivo, observing a similar anti-tumour effect with 5 mg melphalan in combination with buthionine sulfoximine to that normally achievable with 10 mg melphalan alone. No enhancement of toxicity was observed with the melphalan and buthionine sulfoximine combination in bone marrow cultures. Likewise, Russo et al (1986) has shown no enhancement of haematological toxicity either to the CFU-s population or the peripheral white cell count with the combination of buthionine sulfoximine and melphalan. SR 2508, a radiosensitiser under investigation at present, has been shown to be glutathione-dependent, with a 3-fold increase in radiosensitivity when used in combination with buthionine sulfoximine (Mitchell et al 1986).

Further studies with buthionine sulfoximine would seem indicated at this stage, to assess its potential to enhance the effects of radiation therapy and chemotherapy.

(vi) Combination with Biological Response Modifiers

A wide range of compounds have been used as biological response modifiers, but the most significant recent developments have been the production of large quantities of human interferons and interleukins, which now can be used either singly or in combination with cytotoxic drugs.

Interferons consist of a family of proteins having anti-viral, anti-polyreactive and immunomodulatory activities. Using recombinant DNA technology it has been possible to purify these in large quantities. Although human recombinant subtypes have maintained species specificity, some constructed hybrid proteins have gained the ability to cross-react with other species (Brunda and Rosenbaum 1984).

Interferons have many actions, including a direct inhibition of cell multiplication, modulation of the host immune system (Billiau 1981; Spina et al 1983) and alteration of cell surface antigens such as  $\beta$  microglobulin in small cell lung cancer (Funa et al 1986). Various interferons have been assessed using human xenografts as a model. Human fibroblast interferon has been shown to suppress the growth of human gliomas, melanomas and one lung cancer line when injected directly into the tumour, but no effect was seen with more distal injections (Ida et al 1982). Human leukocyte interferon

was shown to inhibit the growth of a human osteosarcoma xenograft in nude mice when injected intraperitoneally, 50,000 IU daily (Masuda et al 1983) and similarly, human lymphoblastoid interferon has been shown to inhibit the growth of human breast tumours in nude mice (Balkwill et al 1982).

In patients the results have been less successful, although there are some reports of activity in hairy cell leukaemia, nodular lymphoma, mycosis fungoides, breast cancer and melanoma (Quesada et al 1984). Stoopler et al (1980) found no activity in non-small cell lung cancer, and a similar result was reported by Jones et al (1983) using human lymphoblastoid interferon in small cell lung cancer. In addition to the disappointing clinical results, treatment was highly toxic, with anorexia, nausea and flu-like symptoms frequent.

More recently, attention has turned to the combination of human interferons with cytotoxic drugs. Inoue and Tan (1983) reported enhancement of actinomycin D and cis-platinum effects by human  $\beta$ -interferon and Balkwill and Moodie (1984) recently described potentiation of the cytotoxicity of sublethal doses of cyclophosphamide and adriamycin on human breast cancer xenografts using human lymphoblastoid interferon. In Balkwill's study mice were treated with 200,000 IU interferon daily which equilibrates to  $20 \times 10^6$  u/m<sup>2</sup> daily in man using

the calculations of Freireich et al (1966). This dosage is likely to be unacceptable to many patients, and further assessment is required of a more realistic schedule.

There is evidence that interferon may interfere with species specific drug metabolising enzymes (Singh et al 1982; Balkwill et al 1984), which will have to be taken into consideration in the construction of any clinical phase II studies using human interferons in combination with cytotoxic drugs. Decreased drug metabolising activity has been observed in tumour bearing animals (Kato et al 1982), and it would be of interest to assess the effect of interferon on this parameter, as altered drug metabolism could obviously influence the activity of a number of cytotoxic drugs.

(vii) Combination Chemotherapy

Very few human tumours are controlled with single agent chemotherapy and with the widespread use of drug combinations there are numerous ways in which interactions, both synergistic and antagonistic, can occur. For example, if drugs are administered simultaneously there may be competition both for protein binding sites and drug metabolising pathways. For instance, misonidazole has been shown to alter the pharmacokinetics of nitrosoureas, alkylating agents (Tannock 1980), and 5-fluorouracil (McDermott et al 1983), thereby increasing



the activity of these drugs. However, interactions can be antagonistic as reported by Tattersall et al (1973) using methotrexate and 5-fluorouracil both in vitro and in vivo.

It is known that the activity of a number of anti-cancer drugs can be affected by the phase distribution of cells in cycle. As several drugs have been shown to alter the phase distribution of cells as shown by continuous-flow cytophotometry (Revazova et al 1981; Yokomori et al 1983), there is potential for enhancing cytotoxicity of some drug combinations by scheduling techniques. This has been successful in cell culture with increased vincristine sensitivity following partial synchronisation with hydroxyurea (Mujagic et al 1983). Clinically, drug scheduling has likewise been shown to be effective with enhanced activity of vinblastine and cyclophosphamide following methotrexate and 5-fluorouracil (Focan 1979). However, although studies have shown beneficial effects of drug scheduling, the precise role for this modification of treatment remains unclear.

(viii) Combination of Modalities

Combined modality treatment offers significant advantages over combination chemotherapy in that the dose-limiting toxicities of different modalities often vary, with modification of treatment doses minimal. However, assessment of the value of these combinations remains preliminary.

(a) Surgery and Chemotherapy

In ovarian carcinoma surgical debulking in the pretreatment of stage III disease prior to chemotherapy has been shown to be beneficial. However the precise role of the combination of these modalities in lung cancer remains unclear although a combination of surgery and chemotherapy is occasionally used in patients found at thoracotomy to have small cell carcinoma.

(b) Radiotherapy and Chemotherapy

The combination of radiotherapy and chemotherapy can potentially improve treatment in a number of ways. Combination of these modalities tends to improve long-term survival rates although toxicity is increased (Salazar and Creech 1980). They are mutually supportive, in that chemotherapy is useful in treating disseminated disease but is often inadequate with bulky local disease in contrast to radiotherapy. In addition, enhancement of therapeutic effect can be stimulated in a number of ways (Steel and Peckham 1979):-

- (1) modification of radiation damage
- (2) inhibition of DNA repair
- (3) cell synchronisation
- (4) re-oxygenation following chemotherapy
- (5) improved drug access following radiotherapy
- (6) tumour shrinkage by radiotherapy leading to increased cell proliferation
- (7) tumour shrinkage by chemotherapy leading to smaller field sizes, allowing an increase in the maximum radiation dose to the tumour.

#### 5.4b Protection of Normal Tissues

The protective mechanisms can be divided into 2 main groups; exogenously administered agents and endogenous protective compounds:

##### (i) Exogenous Protection

##### (a) Mesna - sodium 2-mercaptoethane sulphate

Following an intravenous injection, mesna is oxidised to an inactive disulphide, and is subsequently regenerated in the kidney, and accumulates in the bladder. In its original form, mesna binds electrophilic reactive molecules including acrolein, a toxic product of cyclophosphamide and ifosfamide metabolism. The deactivation of acrolein prevents haemorrhagic

cystitis which can be dose-limiting. Mesna has also been shown to protect against cis-platinum related GI toxicity (Allan et al 1986). Of importance, it was shown that mesna did not affect the anti-tumour activity of cis-platinum using mouse L1210 leukaemia as a model (Allan et al 1986).

(b) WR-2721 - S-2(3-aminopropylamino) ethylphosphorothioic acid

Thiol groups have been shown for years to decrease the toxicity of alkylating agents, however therapeutic gain is only achieved if there is a differential response between host and tumour tissues. WR-2721 is reported to enter normal tissues by facilitated diffusion and tumour cells by passive diffusion, with intracellular reduction to the active sulphhydryl compound (Yuhas 1980). This mechanism is disputed by Millar et al (1982), although a differential effect between normal and tumour tissue was noted using mesna in combination with melphalan against melanoma xenografts. Millar proposed vascularity as the main cause of the differential effect. Yuhas (1980) confirmed the differential protective effect in vivo using a combination of WR-2721 and cis-platinum.

(c) Sodium diethyldithiocarbamate

This thiol has been shown to protect rats from cis-platinum renal toxicity. Renal function is preferentially protected, but there is also a significant decrease in gastrointestinal toxicity (Elliot et al 1983).

(d) Folinic acid

Methotrexate acts by inhibiting the enzyme dihydrofolate reductase, thereby preventing the production of the cofactor tetrahydrofolate which is essential in nucleic acid synthesis. Methotrexate toxicity is affected both by the dosage administered and the duration of infusion. Large doses of methotrexate can now be given as tetrahydrofolinic acid can be given and immediately reverse the effect, preventing excessive toxicity.

5.4b(ii) Endogenous Protection

(a) Detoxification systems

It is widely accepted that enzyme induction can occur in the liver. This has frequently been shown with the cytochrome P-450 system and with enzymes involved in conjugation. The cytochrome P-450 system can be induced in vivo with phenobarbital or 3-methylcholanthrene. However, effect of enzyme induction can be variable as metabolism of drugs via the

cytochrome P-450 system can result in activation of some agents and deactivation of others. Alternatively acrolein, a toxic metabolite of cyclophosphamide, has been shown to deplete cytochrome P-450 levels. As cyclophosphamide requires microsomal activation, acrolein could theoretically impair cyclophosphamide activity.

(b) Priming phenomenon

It is now well established that normal tissue toxicity of alkylating agents can be attenuated by the prior administration of a low dose of an alkylating agent (Millar et al 1976; 1978; 1980; 1981), allowing approximately 50% more drug to be administered.

This is not a new concept. In 1958 Smith showed that administration of a stathmokinetic agent, a colchicine derivative, protected against the lethal effects of x-irradiation given 48 hours later. He later reported on the protection afforded by vinblastine and vincristine on survival and haematopoiesis in irradiated mice (Smith and Wilson 1967). In 1968 Jeney and Connors reported decreased toxicity of merophan after pre-treatment with sub-toxic doses of the same compound. Millar and McElwain (1978) reported on a number of combinations of cytotoxic drugs with less than expected toxicity on normal tissues. They showed that cyclophosphamide

could protect against the lethal effects of busulphan or x-irradiation when given at different times prior to the administration of these toxic agents. This protective, 'priming', effect has been observed in the bone marrow, gut and urothelium (Millar and McElwain 1978). Haematopoietic stem cells have been shown to recover more rapidly in the pre-treated animals (Millar and McElwain 1978). The underlying mechanism of 'priming' remains unclear however, and although different tissues can be 'primed', the optimal time between priming and challenging with toxic doses for each tissue varies (Phelps and Blackett 1979). Particularly interesting is the fact that no experimental tumours have shown a priming effect, and, therefore, a therapeutic gain is possible. Priming doses of cyclophosphamide have been used clinically prior to high dose chemotherapy with cyclophosphamide or melphalan, with or without autologous bone marrow transplantation.

In summary, there are a number of ways in which we can potentially increase the therapeutic activity of the presently available anti cancer drugs. Although some of these methods are employed clinically at present, the majority require further evaluation, particularly with respect to their influence on anti-tumour activity.

## SECTION 2 - METHODS

### CHAPTER 6

#### MATERIALS AND METHODS - Human Tumour Xenograft Studies

##### 6.1 Introduction

The main aim in this study was to establish a variety of human lung cancer xenografts in an immunodeficient animal model. As it was important to carry out chemosensitivity tests on these xenografts, the choice of animal model was limited to those allowing progressive tumour growth. Animals suppressed by chemotherapeutic drugs were not used, in view of the potential problems relating to chemotherapeutic cross-resistance. The choice, therefore, lay between nude mice and the immune-deprived TAR (thymectomy, cytosine arabinoside treated, irradiated) mice, (see Table 6.1). As specific pathogen free facilities were not available in the animal unit at that time, TAR mice were used. Tumour explants were implanted subcutaneously into the flanks, and thereafter passaged at regular intervals. Chemotherapeutic responses in xenografts were assessed in vivo using growth delay as the



end-point. Histopathology was carried out on each tumour line at each passage with chromosomal analysis carried out following a minimum of 5 passages.

## 6.2 Source of Tumour Samples

Tumour material was obtained from two main sources. Patients from the Northern General Hospital with suspected bronchial carcinoma who were undergoing rigid bronchoscopy and biopsy for diagnostic purposes comprised one group, and the other consisted of patients at the City Hospital who were having surgical resections of previously diagnosed lesions. All specimens apart from one were obtained from these sources, with the last specimen obtained from a patient at the Western General Hospital. This was a skin nodule biopsy from a patient with recurrent small cell carcinoma of the lung, who was admitted for further chemotherapy.

The vast majority of specimens were therefore obtained from primary tumours. Surgical resection specimens were collected by the author directly from the operating theatre. Resected specimens were immediately stored on ice and then taken to the University Pathology Department. With a minimum of delay, sections of tumour tissue were taken for transplantation preferably from relatively non-necrotic areas, with a

biopsy from adjacent tissue for histological confirmation of malignancy. The xenograft specimen was immediately placed in Roswell Park Memorial Institute (RPMI) medium with added penicillin and streptomycin, and kept on ice. The second specimen was fixed in formal saline prior to histological staining. Bronchoscopy specimens were obtained directly from theatre and immediately divided in two parts, one being placed in RPMI medium as above and kept at 4°C, with the other half being fixed in formal saline for histology.

Specimens were transported by taxi where necessary assuring a minimal delay prior to implantation. The majority of samples were implanted within 4 hours.

### 6.3 Preparations of Biopsy Specimens

Specimens were prepared for implantation in the animal unit using sterile techniques under a laminar flow hood. Biopsy material was placed in a sterile Petri dish, in cold RPMI medium. As far as possible, all non-tumour and necrotic material was removed, and the remaining apparently viable tumour was dissected using cross-scalpels into approximately 8 mm<sup>3</sup> cubes ready for implantation.

#### 6.4 Preparation of Immune-Deprived Animals

Both male and female CBA/lac mice were used during this study. They were obtained from the University Animal Unit, George Square for the first 6 months of the study, but thereafter were supplied from a breeding colony at the Western General Hospital Animal Unit.

Thymectomy was carried out on newly weaned mice 21-28 days old, by the method described by Shorthouse (1981 ChM thesis) as shown in Table 6.1. Animals were anaesthetised using ether (May & Baker) for approximately 45 seconds. They were then laid in the supine position with each limb stretched by means of a bulldog clip and elastic band, and secured with a pin into cork matting. The neck was extended by means of a paper clip and elastic band which was likewise attached to the cork mat, with a cotton wool bolster placed between the scapulae. The skin was cleaned with methylated spirit, and a 1 cm vertical incision was made in the skin of the neck exposing the strap muscles of the neck. A midline line incision was then made in the upper sternum and the strap muscles divided by blunt dissection, thereby allowing access to the thoracic cavity. 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. The procedure lasted

CBA mice (3 - 4 weeks of age)

1) Neonatal thymectomy

↓

3 - 6 weeks

↓

2) Cytosine arabinoside 200 mg/kg I/P

↓

48 hours

↓

3) Total body irradiation 735 cGy (x-ray 250 kV source)

4) Prophylactic antibiotics for 14 days after irradiation

5) Tumour transplantation days 1 - 8 or days 15 - 22 after irradiation

Table 6.1 Preparation of immune-deprived animals for xenograft studies

approximately 90 seconds enabling 100 thymectomies to be completed at one session. Animals recovered within 2 minutes of the procedure with acute deaths accounting for approximately 5% of animals.

All immune-deprived animals were caged in a separate room and kept in a controlled environment with 12 hours light. They were given acidified water ad libitum to minimise the possibility of pseudomonas infection. Between 3-6 weeks following thymectomy animals were prepared for whole body irradiation. They were given a 'priming' dose of cytosine arabinoside (200 mg/kg) intraperitoneally 48 hours before irradiation, a procedure that has previously been shown to protect both the gastrointestinal tract and bone marrow from the lethal effects of radiation (Millar et al 1978).

X-irradiation was administered via a 250 KV, 15 mA source, using a Thaurus filter giving approximately 37 rads/minute. Mice were held in perspex chambers 30 cm from the x-ray source and were given 735 rads  $\pm$  5% total body irradiation. Allowing for the dose modification factor for x-rays, this corresponds well with the 900 rads given by Shorthouse (1981), using a  $^{60}\text{Co}$  source.

Following irradiation, animals were commenced on anti-biotic therapy of neomycin and terramycin administered in the drinking water for 14 days. This was to prevent septicaemia from commensal gut flora. Irradiation was tolerated well by the animals with few deaths, although transient diarrhoea was noted 7-10 days after treatment. Acute deaths within 28 days of thymectomy resulted in the loss of approximately 10% of animals.

#### 6.5 Tumour Implantation

Implantation of tumour fragments was carried out either in the first 7 days after irradiation or between 14-28 days following this procedure. Implantation between 7-14 days after irradiation resulted in increased mortality. For all of the chemotherapy studies, tumours were implanted the day following irradiation.

For tumour implantation animals were anaesthetised in ether then laid on their ventral surface. The skin was cleaned with methylated spirit and a 1 cm horizontal incision made in the mid-dorsal region. Using blunt forceps subcutaneous tracks were fashioned up the anterolateral aspect of the flanks and

8 mm<sup>3</sup> tumour fragments were implanted at this site. The skin was closed using a surgical clip, and the mouse identified using numbered ear tags 1-3,000 (Hauptner).

As stated previously the animals were housed in a room designated for the sole use of immune-deprived animals. As described by Shorthouse (1981), specific pathogen free facilities were deemed unnecessary.

#### 6.6 Tumour Passage

Following progressive growth of tumour xenografts to greater than 1 cm mean diameter, they were then suitable for transplantation to a subsequent generation. The host animals were sacrificed by cervical dislocation and the tumour(s) removed aseptically. These were then dissected, removing all necrotic material, and 8 mm<sup>3</sup> fragments produced using a cross-scalpel technique. These fragments were kept in ice-cold RPMI medium prior to subsequent implantation into freshly prepared immune-deprived animals. Sections of untreated tumour were taken for histological analysis at each passage with all of the above procedures carried out in a laminar flow hood.

### 6.7 Tumour Storage

Wherever possible tumour samples were stored from the original passage and each subsequent passage. Tumours were dissected as described previously into 8 mm<sup>3</sup> cubes, 10 of which were placed into vials containing 1 ml freezing mixture, which was composed of 10% dimethyl sulphoxide in new born calf serum. The vials were then placed in a -40°C freezer for 6 hours prior to transfer into liquid nitrogen.

When specimens were required for implantation they were removed from liquid nitrogen and rapidly thawed. The freezing mixture was aspirated and the tumour fragments washed repeatedly in RPMI medium with added penicillin and streptomycin. The fragments were then kept on ice until implantation which was carried out with minimal delay.

### 6.8 Tumour Measurement

Following implantation the animals were left undisturbed until removal of the surgical clip at 14 days. Tumour measurements were commenced 14 days after transplantation. The skin was shaved over the tumour area and perpendicular measurements were made using calipers, with the mean of 3 recordings for each measurement. Tumour volume was estimated using the formula  $\pi/6 Dd^2$ , where D = longest diameter and d = shortest



diameter, assuming the tumour to be ellipsoid. To assess the accuracy of these measurements a number of tumours from one cell line were excised and weighed following measurement, showing close correlation between actual and estimated tumour volumes, except at small tumour volumes.

#### 6.9 Tumour and Implant Take Rates

The tumour take rate relates to the number of original tumours which showed progressive growth and subsequent transplantability, thus enabling the establishment of xenograft lines. For a positive tumour take, animals were observed for a minimum of 3 months, following which they were sacrificed should there be no evidence of progressive tumour growth.

The implant take rate refers to the proportion of tumour implants that progressively grew in excess of  $0.3 \text{ cm}^3$ . This was assessed in all tumour samples following initial implantation and with each subsequent passage.

#### 6.10 Growth Rate

Tumours were assessed for growth twice weekly from 14 days after implantation. Tumours were measured as described using the formula  $\pi/6 Dd^2$  to estimate tumour volume. Following an initial lag period, progressive growth followed at a linear

rate. With each tumour xenograft line, the time taken for xenografts to double in volume from 0.2 cm<sup>3</sup> to 0.4 cm<sup>3</sup> (tumour doubling time-T<sub>D</sub>) was estimated to determine the growth rate. Tumour doubling time was estimated for all xenografts reaching this size, and mean values for each tumour line at every passage were determined.

#### 6.11 Specific Growth Delay

In all chemotherapy studies this parameter was used as the end point of assessment. It is calculated from the respective doubling times of control and treated animals by the following formula:-

$$\text{Specific growth delay} = \frac{T_D (\text{treated}) - T_D (\text{control})}{T_D \text{ control}} \quad (\text{Kopper \& Steel 1975})$$

The doubling times (T<sub>D</sub>) of each treatment group are based on the time taken for each xenograft to double its original volume. From these results mean doubling times were calculated for each group. The specific growth delay is expressed in units of control doubling time with the more sensitive tumours having higher values.

### 6.12 Drug Toxicity

End organ or specific drug toxicities can vary between species and also between strains of the same species. Therefore for all drugs used in the chemotherapeutic studies, an assessment of toxicity was made. A dose response curve was constructed for each drug from which a maximum tolerated dose could be calculated. The maximum tolerated dose used was marginally lower than the LD<sub>10</sub>, which is the dose of drug causing death in 10% of animals over a 30 day observation period. Normally, non-tumour-bearing immune-deprived animals were used for these toxicity studies.

In tumour bearing animals impairment of drug metabolising enzyme activity has been identified, therefore 90% of the LD<sub>10</sub> was used as the maximum tolerated dose in all experiments. This enabled the drugs to be effectively ranked based on their anti-tumour activity.

### 6.13 Chemotherapy Experiments

Following the establishment and characterisation of each xenograft line, a number of chemotherapeutic studies were carried out with xenografts in the 3rd to 7th passage.

For these experiments all animals, preferably of the same sex, were irradiated on the same day and transplanted 24 hours later. When a sufficient number of tumours had grown to volumes of 0.2 cm<sup>3</sup> to 0.7 cm<sup>3</sup>, they were divided into groups, stratified both for the sex of animals and for tumour size. Chemotherapy was then administered, based on the maximum tolerated dose, on a milligram per kilogram basis. The drugs were dissolved in water for injection, or normal saline, at a concentration enabling their administration in a volume of 0.1 ml per 10 g mouse body weight, and were all given intraperitoneally.

It was intended to have 10 tumours in each treatment group, but in view of the variability in take rates and growth rates, this was not always possible. Early treatment-related deaths were excluded from analysis, but in animals where regrowth or progressive growth was observed at the time of death, extrapolation of the growth curve was occasionally performed to estimate the tumour doubling time. Where permanent tumour regression occurred, mean growth delay could not be estimated and in this situation median growth delay was considered appropriate (Nowak et al 1978).

#### 6.14 Preparation of Single Cell Suspensions

The tumours were excised using an aseptic technique, and placed immediately in ice-cold RPMI medium with added antibiotics and transferred directly to the laboratory. They were dissected in a sterile Petri dish, in a sterile tissue culture hood, using a crossed-scalpel technique and rinsed 3 times in phosphate buffered saline. Enzymatic disaggregation was performed using collagenase 2 mg/ml in Ham's medium for 30 minutes at 37°C. The solution was centrifuged at 450 g, the supernatant discarded and the cell pellet resuspended in phosphate buffered saline with added trypsin (0.05%). The trypsin was removed after 5 minutes, the pellet resuspended in Ham's medium for 5 minutes and then into Ham's medium with added serum. The solution was then filtered through a sterile polyester mesh of pore size 25  $\mu\text{m}$ .

A sample of the filtered solution was taken and 6 drops of trypan blue added to differentiate dead from live cells and a cell count performed manually using a hemacytometer chamber.

This technique for the production of a single cell suspension was used for both chromosomal analysis and for clonogenic assays.

### 6.15 Chromosomal Analysis

Using a single cell suspension, metaphase spreads were produced by first incubating in colchicine 0.4 µg/ml in Ham's medium for 2 hours at 37°C. The solution was then centrifuged, the supernatant discarded and then resuspended in hypotonic potassium chloride solution for 8 minutes. Following this the cells were fixed by slowly adding a solution containing 3:1 methanol acetic acid. This solution was kept at 4°C for a minimum of 30 minutes, and was repeated on 3 occasions.

Drops of the suspension were heat dried on clean glass slides and Giemsa stained. Intact metaphase spreads were photographed and chromosomes counted where possible. This was done with the help of Sandra Lawrie (ICRF Medical Oncology Laboratory).

### 6.16 Cell Culture

Appropriate cell dilutions were made for each cell line in order that equal numbers of viable cells could be added to each tube. The cells were plated in 0.3% agar medium with added rat erythrocytes, foetal calf serum and Ham's F12 medium (Courtenay and Mills, 1978). One ml agar cultures were gassed with 5% O<sub>2</sub>,

5% CO<sub>2</sub> and 90% N<sub>2</sub> gas mixture. The plating efficiency, which is defined as the proportion of cells which proliferate and form colonies, was determined for each sample.

#### 6.17 Histopathological Techniques

Sections of original tumour tissue and specimens from xenograft tumours at each passage were assessed using standard histological techniques. All histopathology and electron microscopy work was done with the assistance of the Pathology Department, Western General Hospital. All samples were fixed in formalin, embedded in paraffin wax and sectioned at 4 µm. Sections were floated onto microscope slides, deparaffinised in xylene, treated with ethanol to remove xylene and brought to water. All specimens were examined using standard haematoxylin and eosin stain, with periodic-acid schift stain for the non-small cell carcinomas to distinguish adenocarcinomas.

#### 6.17a Haematoxylin and Eosin (H & E)

Sections were stained in standard Ham's haematoxylin for 2 minutes then blued in lithium carbonate. Following washing plates were placed in Putt's eosin for 2 minutes, then dehydrated, cleared and mounted in DPX.

#### 6.17b Periodic-acid Schiff (PAS)

Sections were stained in 1% aqueous periodic acid for 5 minutes and washed in water. They were then placed in Schiff reagent for 15 minutes, and washed in water for 30 minutes. The nuclei were stained in haematoxylin for 2 minutes, blued in lithium carbonate and washed in water. Sections were then dehydrated, cleared and mounted in DPX.

#### 6.18 Electron Microscopy

Wherever possible, specimens of original tumour tissue and freshly excised xenograft tissue were dissected into small fragments and fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and kept at 4°C. They were washed overnight in sodium cacodylate with 0.3 M sucrose, then fixed in 0.05 M sodium cacodylate and 1% osmium tetroxide for 1 hour at 4°C. The sections were then dehydrated and embedded in araldite. Sections of 30-50 nm were cut on a Reichert Om U2 ultratome stretched with chloroform and collected on uncoated copper grids. Grids were stained with uranyl acetate and lead citrate, 20 minutes in each solution, and then photographed.



## CHAPTER 7

MATERIALS AND METHODS - BIOCHEMICAL STUDIES7.1 Introduction

The second aim of this study was to investigate biochemical sequelae of cytotoxic drug administration, in particular the drug 'priming' phenomenon. In view of the association of glutathione and related enzymes in the detoxication of alkylating agents, it was decided to concentrate on this area with particular reference to the effect of cyclophosphamide on tumour cells and bone marrow cells. Commercially available male CBA mice from a solitary source were used for these experiments. This work was done in collaboration with Dr David Adams and Dr Roland Wolf, ICRF Medical Oncology Unit, Edinburgh.

7.2 Preparation of Animals

Male CBA mice 8-12 weeks of age, 22-30 g in weight, were used in these experiments. All cytotoxic drugs were administered intraperitoneally and x-irradiation was performed using the 250 kV source described previously.

### 7.3 Preparation of Tissue Samples

The effectiveness of cytotoxic chemotherapy is often a balance between anti-tumour activity and toxicity. In view of this a number of tissues were examined to investigate the biochemical effects of cytotoxins with all animals sacrificed by cervical dislocation.

#### 7.3a Tumour Tissue

Xenograft tumours were rapidly excised and all obvious necrotic debris removed. Individual samples were immediately placed in ice-cold phosphate buffer, and finely minced using a crossed scalpel technique. The phosphate buffer used for all these assays comprised of a 20 mM sodium phosphate buffer with 0.005 M ethylenediaminetetracetic acid (pH 7.7). They were then disaggregated using a polytron homogeniser, each undergoing 3 homogenisations of 10 seconds, taking appropriate precautions to keep the specimen cold and to minimise the possibility of aerosol spray contamination of the experimenter. Following this, the specimen was centrifuged at 40,000 g at 4°C for 60 minutes and the supernatant collected. This was then analysed immediately for reduced glutathione content or stored at -80°C for enzyme studies.

### 7.3b Liver

The livers were removed and immediately placed in ice-cold phosphate buffer. They were later removed from the buffer, filter paper dried and weighed. Following this phosphate buffer was added, 3 ml per mg wet weight of liver, and the livers were scissor-minced. They were homogenised using a Silverson Heavy Duty laboratory mixer emulsifier, then centrifuged at 30,000 g and the supernatant used for glutathione and glutathione transferase assays. All samples were stored at -80°C.

### 7.3c Bone Marrow

Animals were stretched out on their dorsal surface with the femurs of both hind limbs exposed. The bones were divided using scalpels and the marrow obtained by pushing through ice-cold phosphate buffer using a 23 gauge needle. Cells were disaggregated by passing through needles of increasing gauge using 1.5 ml buffer. 1.5 ml buffer was used for 6 femurs, giving a cell yield of approximately  $5 \times 10^7$ /ml. Cell counts were carried out using a haemocytometer taking 2 measurements for each sample. Cells were disrupted by sonication for 5 seconds on 3 occasions while ensuring the samples were kept at 4°C.

#### 7.3d Bladder

The bladders were removed, washed and then placed in ice-cold phosphate buffer. They were then divided by crossed-scalpels, resuspended in 5 ml phosphate buffer and disrupted using a Polytron homogeniser as previously described. The samples were then centrifuged at 30,000 g at 4°C for 30 minutes and the supernatant collected for assay purposes.

#### 7.4 Fluorescence Activated Cell Sorter (FACS)

Bone marrow and peripheral blood samples were sorted into constituent cell populations using a Becton-Dickinson FACS IV. These studies were carried out in collaboration with Dr John Ansell, Zoology Department, Edinburgh University, using equipment kindly supported by the Cancer Research Campaign. Peripheral blood and bone marrow specimens were separated using forward and 90° scatter with four distinct populations identified (Watt et al 1979; 1980):-

- 1) erythrocytes
- 2) lymphocytes
- 3) granulocytes
- 4) anomalous population - including blast cells and monocytes

The purity of these cell populations had previously been confirmed histologically, having been shown to be greater than 80% pure.

#### 7.5 Preparation of Granulocytes

Granulocytes were obtained for assay from three sources, bone marrow, peripheral blood and the peritoneum.

##### 7.5a Bone marrow

Samples were obtained as previously described. Bone marrow cells were suspended in ice-cold phosphate buffered saline containing 0.5% bovine serum albumin, azide and heparin. They were then separated into the 4 constituent cell populations using a fluorescence activated cell sorter by means of forward and 90° scatter (Watt et al 1980).

##### 7.5b Peripheral Blood

Animals were sacrificed in ether (May and Baker), placed on their dorsal surface and the inferior vena cava exposed. Using a 25 gauge needle approximately 0.5 ml blood was obtained from each animal. 1.5 ml blood was diluted in 20 ml ice-cold phosphate buffer containing azide, bovine serum albumin and heparin. This was spun at 450 g for 10 minutes at 4°C.

Erythrocytes were lysed using a distilled water shock technique adding 9 ml water to the resultant cell pellet. Hypotonicity was reversed using 1 ml of 10 times concentrated Eagles medium without added glutamine, and the solution was brought to 20 ml with phosphate buffer. The cell suspension was centrifuged at 1400 rpm for 10 minutes through 2 ml 10% foetal calf serum to remove red cell debris. The resultant cell pellet was resuspended in a few drops of phosphate buffer and separated by means of the FACS.

#### 7.5c Peritoneal Granulocytes

Peritoneal granulocytes were obtained using casein stimulation as previously described (Watt et al 1979). Animals were injected intraperitoneally with 2 ml of 0.2% (w/v) calcium caseinate and sacrificed 3 hours later using ether anaesthesia. 5 ml of ice-cold isotonic saline was injected intraperitoneally using a 25 gauge needle, then 10 minutes later the abdomen was lavaged using a Pasteur pipette, and the suspension washed in phosphate buffered saline three times at 4°C. The cells were resuspended in 10 ml 0.168 N ammonium chloride and left standing on ice for 10 minutes to lyse contaminating red blood cells. These cells were then later resuspended in 0.5 ml phosphate buffered saline containing bovine serum albumin 1%

W/V) and counted using a haemocytometer. The purity of all the above samples was assessed histopathologically using Giemsa stained preparations, and were found to be greater than 80% pure.

## 7.6 Glutathione Assays

Reduced glutathione was measured using two techniques.

### 7.6a Fluorimetric Assay

A fluorimetric assay using O-phthalaldehyde was used to determine glutathione levels as described by Hissin and Hilf (1976). O-phthalaldehyde 0.1 ml (1 mg/ml Sigma Chemicals Ltd) in absolute methanol was added to 1.89 ml phosphate buffer (20 mM sodium phosphate with 0.005 M ethylenediaminetetracetic acid [EDTA], pH 7.7).

Test solutions were prepared by mixing equivalent amounts of 10% TCA (trichloroacetic acid) and tissue homogenates and spinning at 2,000 g for 5 minutes in an Eppendorf centrifuge. Duplicate supernatant test solutions of 0.01 ml were added to the mixture of O-phthalaldehyde solution 0.1 ml (1 mg/ml Sigma Chemicals) and 1.89 ml phosphate buffer, as previously described. The resultant solution was mixed thoroughly and left to incubate at room temperature for 15 minutes. It was

then transferred to a quartz cuvette and fluorescence determined using a Perkin Elmer LS-3 fluorescence spectrometer (excitation/emission: 350/420 nm). These values were compared with glutathione control solutions and negative controls. The second method involved the measurement of cycle reduction of glutathione using glutathione reductase and NADPH as described by Tietze (1969).

#### 7.6b Cycling Dependent Glutathione Assay

Glutathione was estimated using a modification of the assay assessing cyclic reduction of Elman's reagent using NADPH (Sigma Chemicals Ltd) and glutathione reductase, as described by Tietze (1969). 1.6 ml of 200 mM sodium phosphate buffer with 10 mM EDTA, pH 7.3 as added to both test and standard cuvettes. 60  $\mu$ l of glutathione reductase 0.5 mg/ml (Boehringer Mannheim Ltd) was added to each cuvette with 100  $\mu$ l of DTNB Elman's Reagent - 5 mg/ml). Test samples were diluted in 0.6% sulphosalicylic acid and 200  $\mu$ l was added to the test cuvette, with 200  $\mu$ l 0.6 sulphosalicylic acid added to the standard. The reaction was started by adding 40  $\mu$ l NADPH (9 mg/ml) to each cuvette, with the samples read, following thorough mixing,



in a Hitachi 100-80 A spectrophotometer at a wavelength of 412 nm. Samples were assayed over 3 minutes and compared with glutathione standards.

### 7.7 Oxidised Glutathione

As glutathione is a labile compound that can be rapidly oxidised to its disulphide, estimations of the disulphide were occasionally made to assess the degree of autoxidation of samples. Reduction of the disulphates was achieved with glutathione reductase (1 mg/ml) and NADPH (9 mg/ml). Following incubation with these compounds, glutathione levels were determined fluorimetrically. Oxidised glutathione (disulphide) levels were estimated by subtracting glutathione levels in the untreated samples from levels measured in samples treated with glutathione reductase and NADPH.

### 7.8 Glutathione-Transferases

Glutathione transferase levels were measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig et al 1974).

The CDNB solution was prepared by adding 1 mM CDNB in 2% ethanol to an 0.1 M sodium phosphate buffer, pH 6.5. CDNB was added drop by drop to the buffer and heated to 50°C. A 20 mM glutathione test solution was also required.

One ml CDNB plus 0.05 ml phosphate buffer comprised the reference solution and when compared with 1 ml CDNB plus 0.05 ml glutathione test solution, gave the background value. Test samples were prepared in an identical manner to the glutathione assay samples and variable volumes of the resultant solution (0.01-0.05 ml) were added in an attempt to observe significant activity. An ideal sample comprised of 0.01 ml of 1 mg/ml cytosol. Samples were measured at 25°C using a Pye-Unicam SP8-500 uv/vis spectrophotometer at 340 nm. Protein estimations were carried out on the samples (Lowry et al 1951) and values expressed as enzyme activity = mol CDNB/minute/milligram cytosolic protein, except in the case of bone marrow samples where enzyme activity was expressed in terms of cell number.

#### 7.9 Glutathione Quench Curves

Interference with the glutathione assay could be caused by a number of tissue components (Mokrasch and Teschke 1984), and it was therefore important to assess the degree of quenching in all tissues.

A minimum of three samples from each tissue were assayed for glutathione as previously described. Known amounts of glutathione (25-800 mM) were added to identical tissue samples

and glutathione measured. Glutathione levels measured before and after the addition of known amounts of glutathione were compared and quench curves generated. Quench curves were constructed for liver, tumour and bladder tissues in addition to bone marrow cells and peripheral blood cells.

#### 7.10 Protein Measurements

All protein samples were analysed using the method described by Lowry et al (1951).

#### 7.11 Interleukin Preparations

##### 7.11a Interleukin I

Crude human interleukin I was isolated from human monocytes stimulated with concanavalin A. Activity was shown by pyrogen tests on CBA mice with no evidence of endotoxin contamination as shown by the Limulus amoebocyte lysate test. The supernatant was concentrated by and stored at  $-80^{\circ}\text{C}$ . Dr Gordon Duff, Ward 1, Northern General Hospital kindly supplied IL-1.

### 7.11b Interleukin 3

WEHI-3B, a mouse myelomonocytic cell line, is known to produce a number of substances including interleukin 3. Interleukin 3 is produced in relatively large amounts and can be isolated and purified from the supernatant of confluent cells (Ihle et al 1982; 1983). Crude conditioned media supernatant was used in this study, with no attempt made to purify or concentrate the substances. Similarly the Limulus amoebocyte lysate test for endotoxins was not carried out.

## SECTION 3 EXPERIMENTAL RESULTS

### CHAPTER 8

#### GROWTH CHARACTERISTICS OF HUMAN LUNG CANCER XENOGRAFTS

##### 8.1 Introduction

The main purpose of this study was to establish a valid tumour model for the study of drug sensitivity in human lung cancer. This included the study of methods to improve the therapeutic index of the presently available cytotoxic drugs. To address both these aims, it was decided to establish a human xenograft model. As 'specific pathogen free' facilities were not available at that time, TAR (thymectomy, cytosine arabinoside pretreatment and total body irradiation) mice were used.

##### 8.2 Xenograft Take Rate

Fifty-six specimens were obtained in all, the details listed in Table 8.1, with 51 of these containing tumour tissue. Progressive growth and serial transplantation were achieved in 16 xenografts giving a take rate of 31.3%. Successful tumour takes were achieved in squamous carcinoma 9/20 (45%), adenocarcinoma 5/16 (31%) and small cell carcinoma 1/6 (17%).

|   | No. of<br>biopsies | No. of<br>taken |
|---|--------------------|-----------------|
| All biopsies                              | 56                 |                 |
| All biopsies (with viable tumour)         | 51                 | 16 (31/3%)      |
| (a) Thoracotomy specimens                 |                    |                 |
| - total                                   | 43                 |                 |
| - no tumour                               | 1                  |                 |
| - viable tumour                           | 42                 | 13 (31%)        |
| Squamous carcinoma                        |                    |                 |
| - well diff.                              | 1                  | 0               |
| - moderately diff.                        | 9                  | 4 (43.8%)       |
| - poorly diff.                            | 6                  | 3               |
| Adenocarcinoma                            |                    |                 |
| - well diff.                              | 1                  | 0               |
| - moderately diff.                        | 8                  | 4 (33%)         |
| - poorly diff.                            | 6                  | 3               |
| Large cell carcinoma                      | 1                  | 0               |
| Undifferentiated carcinoma                | 2                  | 0               |
| Small cell carcinoma                      | 5                  | 0               |
| Adenosquamous carcinoma                   | 3                  | 1               |
| (b) Bronchoscopy specimens                |                    |                 |
| - total                                   | 12                 |                 |
| - no tumour                               | 4                  | -               |
| - viable tumour                           | 8                  | 2 (25%)         |
| - squamous                                | 4                  | 2               |
| - adenocarcinoma                          | 1                  | 0               |
| - small cell                              | 1                  | 0               |
| - undiff. carcinoma                       | 2                  | 0               |
| (c) Sub-cutaneous metastasis - small cell | 1                  | 1               |

Table 8.1 Establishment (tumour take rate) of human xenograft lines in immune-deprived CBA mice. Take rates of biopsies obtained from various sources.

Tumours with features of both squamous and adenocarcinoma were implanted on 3 occasions with 1 successful tumour take (33%). Differences in take rates between histological categories were not statistically significant although numbers were small. All biopsies were from primary tumours except 2 samples, one a subcutaneous biopsy from a small cell carcinoma which was successfully grown, and the other a hilar lymph node biopsy from a squamous carcinoma which did not grow. Despite the limited amount of tissue, when viable tumour was obtained at bronchoscopy a take rate of 25% was achieved, similar to that observed with thoracotomy specimens (31%).

Sixteen xenograft lines were established, 9 squamous carcinoma, 5 adenocarcinoma, 1 small cell carcinoma and 1 adenosquamous tumour.

## 8.2 Tumour Implant Take Rates

In the original man to mouse passage of squamous carcinoma 26/180 (14.4%) of implants progressively grew in comparison to 10/159 (6.3%) in adenocarcinoma and 1/52 (2%) of small cell carcinoma implants. 5/30 adenosquamous carcinoma implants grew, but there were insufficient numbers in the other histological categories to analyse. Implant take rates are illustrated in Table 8.2. Once xenografts became established the

(a) Squamous Carcinoma

| <u>Xenograft</u> | <u>Take Rate</u> | <u>Xenograft</u>      | <u>Take Rate</u> |
|------------------|------------------|-----------------------|------------------|
| CX 104           | 1/10             | CX 108                | 5/10             |
| CX 109           | 8/10             | CX 112                | 1/10             |
| CX 121           | 2/10             | CX 133                | 3/8              |
| CX 140           | 2/6              | NX 002                | 3/10             |
| NX 004           | 1/5              | 11 biopsies no growth | 0/101            |

Implant take rate 26/180 = 14.4%

(b) Adenocarcinoma

|        |      |                       |       |
|--------|------|-----------------------|-------|
| CX 105 | 2/10 | CX 117                | 4/11  |
| CX 118 | 2/10 | CX 131                | 1/10  |
| CX 132 | 1/8  | 11 biopsies no growth | 0/110 |

Implant rake rate 10/159 = 6.3%

(c) Adenosquamous Carcinoma

|        |      |                      |      |
|--------|------|----------------------|------|
| CX 143 | 5/10 | 2 biopsies no growth | 0/20 |
|--------|------|----------------------|------|

Implant take rate 5/30 = 16.6%

(d) Small Cell Carcinoma

|        |      |                      |      |
|--------|------|----------------------|------|
| WX 310 | 1/10 | 5 biopsies no growth | 0/42 |
|--------|------|----------------------|------|

Implant take rate 1.52 = 2%

Table 8.2 Implant take rate in the first man to mouse passage, of various histological subtypes of human lung cancer. All tumours grown in immune-deprived CBA mice.



| Xenograft<br>Line | Histological<br>Type | Implant Take Rate (%) |             |             |              |             |             |             |
|-------------------|----------------------|-----------------------|-------------|-------------|--------------|-------------|-------------|-------------|
|                   |                      | Passage               |             |             |              |             |             |             |
|                   |                      | 1                     | 2           | 3           | 4            | 5           | 6           | 7           |
| NX 002            |                      | 30                    | 50          | 64          | 63           | 60          | 69          | 75          |
| CX 108            |                      | 50                    | 33          | 14          | 28           |             |             |             |
| CX 109            | Squamous             | 80                    | 58          | 76          | 80           | 69          | 77          |             |
| CX 133            |                      | 38                    | 21          | 55          | 63           |             |             |             |
|                   |                      | 33                    | 31          | 35          | 46           | 63          |             |             |
| CX 117            | Adenocarcinoma       | 36                    | 23          | 40          | 63           | 62          |             |             |
| CX 143            | Adenosquamous        | 50                    | 62          | 55          | 70           | 61          |             |             |
| WX 310            | Small cell           | 10                    | 23          | 41          | 50           |             |             |             |
| Totals            |                      | 31/<br>75             | 118/<br>289 | 286/<br>621 | 650/<br>1077 | 433/<br>685 | 326/<br>464 | 130/<br>174 |
|                   |                      | 43.3%                 | 40.8%       | 46%         | 60.4%        | 63.2%       | 70.25%      | 75%         |

Table 8.3 Implant take rate of human lung cancer xenografts in serial mouse to mouse passages in immune-deprived CBA mice.

implant take rate generally improved, as shown in Table 8.3, with implant take rates significantly better in passages 4-6 compared with passages 1-3 ( $p < 0.001 \chi^2$ ). In total 1408/2328 (60.5%) squamous carcinoma implants showed progressive growth, in comparison to 344/680 (50.6%) of adenocarcinomas and 109/261 (41.8%) of small cell carcinomas. This gave an overall implant take rate of 57% (1861/3269).

### 8.3 Effect of Delayed Implantation on Xenograft Take Rates

Using a well established xenograft (NX 002) at its fifth passage, the effect of delay on tumour implantation was assessed. Seventy mice were transplanted unilaterally at designated times following total body irradiation. All mice were transplanted on the same day and the results are illustrated in Table 8.4.

The best results are achieved by transplanting on Day 1 or Day 15 following total body irradiation. Successful tumour growth was also achieved with transplantation on Day 8, but the mice were less able to tolerate the procedure at this stage, resulting in a greater loss of animals. Radiation-related gastrointestinal toxicity was maximal at this time which may have resulted in these mice being less able to tolerate anaesthesia.

| Day after total body irradiation | Number of Mice |   |    |    |    |    |    |
|----------------------------------|----------------|---|----|----|----|----|----|
|                                  | 1              | 8 | 15 | 22 | 29 | 36 | 43 |
| Progressive growth               | 6              | 5 | 7  | 3  | 5  | 2  | 1  |
| No growth/regression             | 4              | 3 | 3  | 7  | 5  | 7  | 8  |
| Host death                       | 0              | 2 | 0  | 0  | 0  | 1  | 1  |

Table 8.4 Effect of timing of tumour implantation on implant take rates of human lung xenografts, using the squamous lung cancer xenograft NX002 at the 5th passage in immune deprived CBA mice.

#### 8.4 Effect of Liquid Nitrogen Storage on Xenograft Take Rates

Whenever possible tumour material from each passage was stored in liquid nitrogen at  $-196^{\circ}\text{C}$ . When specimens were recovered from liquid nitrogen implant take rates were very poor however, similar to the results achieved with the initial man to mouse passage. Therefore, this procedure was satisfactory for the maintenance of xenograft lines but the implant take rate was inadequate for the planning of chemotherapy experiments.

#### 8.5 Tumour Regression

It is known that a proportion of immune-deprived mice will regain some or all of their immunity within 6 weeks of total body irradiation. With returning immunity, stasis or even regression of tumour growth can be problematical. The incidence of tumour regression was assessed in all xenograft lines during the fourth passage, and was found to be extremely variable:-

|        |      |        |     |
|--------|------|--------|-----|
| CX 109 | 0%   | CX 143 | 10% |
| NX 002 | 3%   | CX 133 | 15% |
| CX 140 | 6%   | WX 310 | 15% |
| CX 117 | 7.8% | CX 108 | 20% |

Histopathological classification had no apparent effect on the incidence of tumour regression although regression was more common in slowly growing tumours.

#### 8.6 Incidence of Single and Double Takes in Xenografts

To assess the importance of returning host immunity on xenograft take rates, an analysis of implant take rate was carried out. All animals were implanted bilaterally and were categorised with respect to the number of implants showing progressive growth.

The distribution of implant takes was investigated in three separate tumours at the same passage. The tumours were histologically different and were analysed independently. The results are illustrated in Table 8.5, showing a tendency for each mouse to either accept or reject both implants in all three tumour groups. The results were analysed using the  $\chi^2$  goodness of fit test applied to a binominal distribution. These differences were highly significant ( $p < 0.001$ ), showing that returning host immunity was an important factor influencing implant take rates.

|  | No. tumours<br>per mouse | Observed no.<br>of mice | Expected no.<br>of mice |
|--|--------------------------|-------------------------|-------------------------|
| CX 117/3                               | 0                        | 31                      | 21.96                   |
| Adenocarcinoma                         | 1                        | 11                      | 29.28                   |
| (take probability 0.4)                 | 2                        | 19                      | 9.76                    |
| $X^2_{1df} = 21.52 \text{ } p < 0.001$ |                          |                         |                         |
| CX 143/3                               | 0                        | 36                      | 23.49                   |
| Mixed tumour                           | 1                        | 32                      | 57.42                   |
| (take probability 0.55)                | 2                        | 48                      | 35.09                   |
| $X^2_{1df} = 22.66 \text{ } p < 0.001$ |                          |                         |                         |
| NX 002/4                               | 0                        | 31                      | 14.99                   |
| Squamous                               | 1                        | 35                      | 66.70                   |
| (take probability 0.69)                | 2                        | 90                      | 74.27                   |
| $X^2_{1df} = 35.48 \text{ } p < 0.001$ |                          |                         |                         |

Table 8.5 Effect of host immunity on xenograft implant take rates. Incidence of progressive growth in one or both tumour implants using human non-small cell cancer xenografts at the 5th mouse to mouse passage.

## 8.7 Xenograft Growth

The pattern of xenograft growth is divided into a "lag phase" and a "growth phase". Growth rates can vary widely between xenografts and even within a xenograft line at the same passage, as illustrated in Figure 8.1.

### 8.7a Lag Phase

The lag phase encompasses initial cell death following transplantation, clearing of stromal debris and then tumour cell proliferation, until macroscopic growth is evident when the growth phase begins. The division of growth into two phases is somewhat arbitrary, and therefore in this study the lag phase was defined as the period lapsing until tumour volume reached  $0.05 \text{ cm}^2$ .

The lag phase was evaluated for all xenograft lines at every passage. The mean lag period for all xenografted tumours in the original man to mouse passage was 48.37 days (range 25-95 days) with no significant difference between squamous carcinomas (45.5 days) and adenocarcinoma xenografts (55.2 days). Variation in duration of the lag phase was seen between xenografts of the same histological type, between passages of the same xenograft and even within the same passage. Mean values for lag periods of tumours used in chemotherapy experiments are illustrated in Table 8.6.

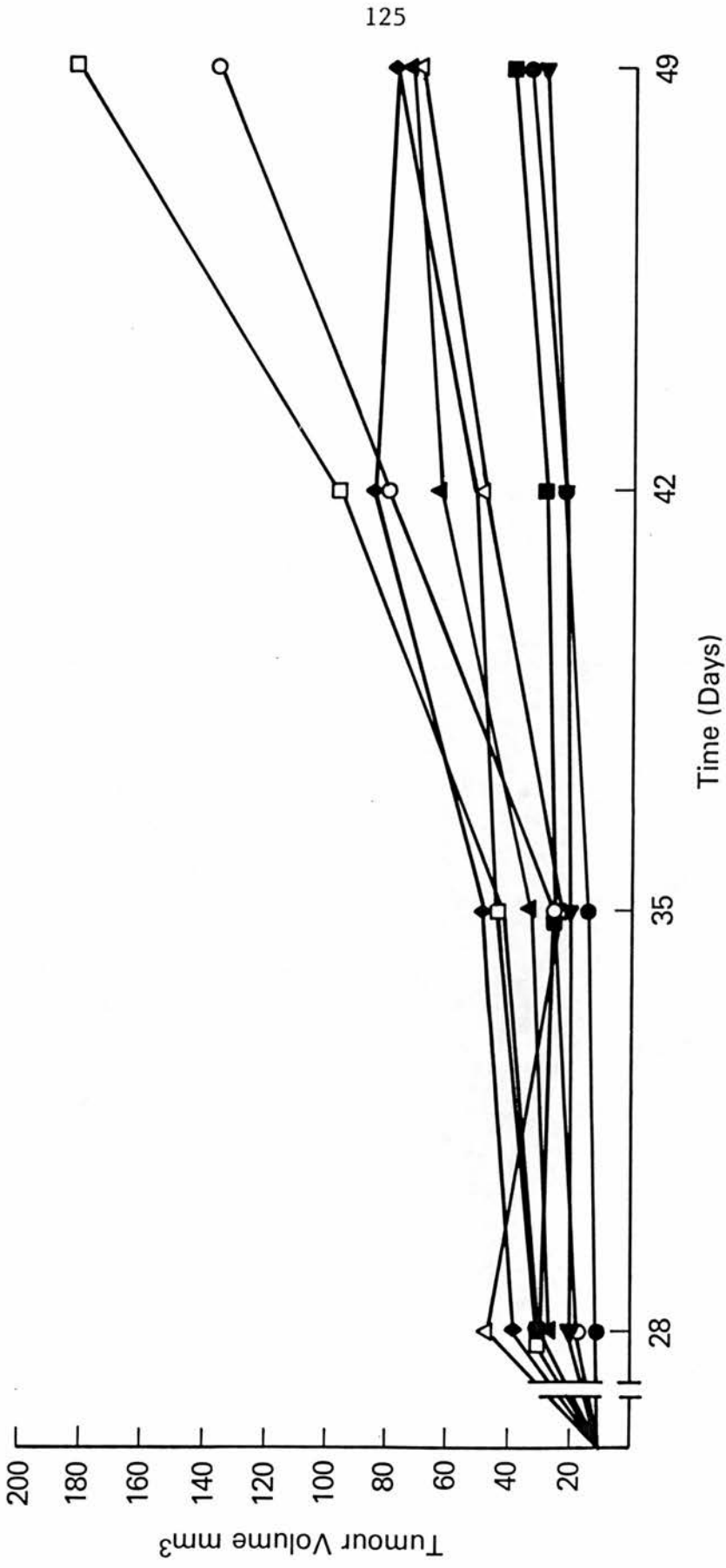


Fig. 8.1 Growth of human non-small cell lung cancer xenograft CX143-Adenosquamous carcinoma . 3rd passage; growth pattern of 8 individual tumours.



| Passage                         | Man to<br>Mouse<br>Passage | 1     | 2     | 3     | 4     | 5     | 6     |      |
|---------------------------------|----------------------------|-------|-------|-------|-------|-------|-------|------|
| NX 002                          | LP                         | 42    | 57    | 38    | 37.9  | 32.5  | 36.8  | 22.1 |
|                                 | T <sub>D</sub>             | 16.5  | 10.6  | 12.65 | 12.7  | 12.9  | 14.74 | 16.2 |
| CX 108                          | LP                         | 36.8  | 34.3  | 54    | 42    |       |       |      |
|                                 | T <sub>D</sub>             | 11.9  | 13.7  | 10.5  | 18.8  |       |       |      |
| CX 109                          | LP                         | 25    | 32    | 44.5  | 32.3  | 21    |       |      |
|                                 | T <sub>D</sub>             | 18.6  | 7.0   | 12.26 | 12.72 | 11.0  |       |      |
| CX 121                          | LP                         | 92.5  | 71.3  | 54    |       |       |       |      |
|                                 | T <sub>D</sub>             | 14.2  | 15.5  | 18.7  |       |       |       |      |
| CX 133                          | LP                         | 32    | 35.4  | 46.4  | 35.35 |       |       |      |
|                                 | T <sub>D</sub>             | 14.0  | 14.15 | 14.18 | 13.49 |       |       |      |
| CX 140                          | LP                         | 28.5  | 53.8  | 80.8  | 14.2  | 46.3  |       |      |
|                                 | T <sub>D</sub>             | 12.65 | 11.2  | 11.75 | 12.67 | 17.2  |       |      |
| CX 117<br>(adeno-<br>carcinoma) | LP                         | 39.3  | 87    | 37    | 29.6  | 34.1  |       |      |
|                                 | T <sub>D</sub>             | 11.25 | 10.7  | 11.82 | 10.9  | 11.04 |       |      |
| CX 143<br>(adeno-<br>squamous)  | LP                         | 56.8  | 46.3  | 34.5  | 37.8  |       |       |      |
|                                 | T <sub>D</sub>             | 19.13 | 18.66 | 15.64 | 16.83 | 11.9  |       |      |
| WX 130<br>(small<br>cell)       | LP                         | 38    | 70    | 52.3  | 45.4  |       |       |      |
|                                 | T <sub>D</sub>             | 14.9  | 17.2  | 14.25 | 18.1  |       |       |      |

Table 8.6 Mean lag periods (LP) and tumour volume doubling times (T<sub>D</sub>) in days of human lung cancer xenografts measured over successive passages in immune-deprived CBA mice. All xenografts are squamous carcinomas unless otherwise indicated.

### 8.7b Growth Phase

Growth in the majority of implants followed an exponential pattern. However stasis and regression of tumour growth did occasionally occur, this being more common in particular xenograft lines. This tends to suggest that in addition to returning host immunity there may be tumour related factors affecting xenograft take rate. Tumour volume doubling times varied from 11.25 days to 23.4 days in the original man to mouse passage, with similar variations amongst squamous and adenocarcinoma lines. Mean tumour doubling times for xenografts used in chemotherapy studies are shown in Table 8.6. However, it has to be appreciated that numbers in these groups were very small, with only one tumour to measure on occasions. Marginally less variation in volume doubling is seen in subsequent passages, both within the same tumour line and between xenografts of particular histological categories.

In the original man to mouse passage the mean volume doubling time for squamous carcinomas was  $15.53 \pm 5.02$  with a similar value  $15.64 \pm 4.86$  days in adenocarcinoma. At the third passage when tumours were relatively well established, the figures were  $13.71 \pm 4.86$  days in squamous and  $11.83 \pm 4.93$  days in adenocarcinoma xenografts.

Apart from a minimal general increase in growth rate, there was no obvious growth trend in individual xenograft lines, with some increasing growth rate with passage, some remaining static and others decreasing growth rate. The lag period varied from passage to passage but likewise, with no obvious trend. The lag period in these experiments did not decrease with increasing passage as has previously been reported. In addition, no correlation was noted between duration of the lag phase and tumour volume doubling time in the original man to mouse passage ( $r = 0.28$ ).

#### 8.8 Potential Error in Estimation of Xenograft Volume

Using the xenograft line NX002, a squamous carcinoma at the sixth passage, evaluation of the accuracy of caliper measurements in the estimation actual tumour volume was made. Twenty-four tumours were measured immediately prior to sacrifice and tumour volume estimated using the formula  $\pi Dd^2/6$  where D is the longest diameter and d the diameter perpendicular to this. This assumes the tumour to be ellipsoid. . The tumours which were of variable size were then excised, weighed and a graph constructed of estimated volume against actual tumour weight. A linear relationship was obtained above

0.2 cm<sup>3</sup> but below this the estimated volume was inappropriately high, with overlying skin thickness probably playing an important role in this measurement error.

## 8.9 Animal Deaths

### 8.9a Deaths Following Immune-deprivation

In all, approximately 10% of immune-deprived animals died prior to tumour transplantation. Acute deaths following thymectomy accounted for 5%, with an additional 3% of irradiated animals dying by day 14, the majority between days 7-10, thought to be due to radiation related gastrointestinal toxicity. Another 2% of animals progressively lost weight, although it was impossible to ascertain whether this related to the wasting syndrome associated with thymectomy, or chronic gut toxicity. Of the animals surviving immune-deprivation and successful transplantation, between 5-10% were unsuitable for chemotherapy studies as they had failed to gain sufficient weight. As stated previously, of the animals surviving these procedures, 57% would successfully grow tumours.

#### 8.9b Unexpected Deaths

On one occasion halfway through the project, a large number of animals became unwell, developing a wasting syndrome. Ten sick animals were sacrificed and underwent post-mortem examination. Macroscopically no abnormality was detected. Histologically the livers showed small zones of necrosis surrounded by mixed inflammatory cells, although the appearance of most of the organ was entirely normal. The spleens and kidneys were all normal. A periarteritis was noted in the lungs with oedema in the alveoli but no other abnormalities were detected in the lungs or other organs. Culture specimens were sent from all organs but no organisms were grown. Specimens were examined for common pyogenic organisms, anaerobic bacteria and tubercle bacilli with no growth. As pasturella infection had previously been a problem in the animal unit, specimens were examined specifically for this pathogen, but no organisms were detected and likewise, fungi were not isolated. In addition no viral particles were seen on electron microscopic examination of several organs. The CBA breeding colony was assessed for common murine viruses such as Sendai and Mouse Hepatitis Virus but these tests were all negative.

The illness affected both immune-deprived non-tumour bearing and tumour bearing animals, which were all housed in the one room. The breeding stock were not affected by the illness and there was no excess of neonatal deaths at that time.

At this stage there were a number of possibilities for these unexpected deaths, the most likely being infective, either atypical bacterial or viral. Another possibility was that the animals were receiving a higher dose of radiation than intended, but the dose-meter on the x-ray source was checked and found to be working normally. As there were large numbers of ill animals it was decided to sacrifice all, apart from a select few tumour bearing animals from each tumour line. These animals were treated with claforan (Roussel Ltd) and metronidazole (May & Baker Ltd) intraperitoneally 3 times daily for 1 week and arrangements were made to obtain mice from an outside source. The author is greatly indebted to Dr Gordon Steel at the Institute of Cancer Research, Sutton for kindly supplying 50 male, thymectomised CBA mice. These animals were kept in quarantine, irradiated and then 5 animals were transplanted with each of 10 xenograft lines. Each tumour was implanted bilaterally into 5 mice, and 9 xenograft lines were successfully salvaged. CX 104 a moderately differentiated

squamous carcinoma failed to take, however. Another 6 tumour lines were lost as there were no healthy animals suitable for transplantation. Following this procedure all of the original stock mice were sacrificed, resulting in the loss of 1057 mice, 453 of which were tumour bearing, 310 transplanted but non-tumour bearing and the remainder awaiting transplantation.

Over the next 2 months the mice were closely scrutinised but showed no evidence of the wasting disease. Another worry was that the infection could be transmitted by the tumour fragments, and also that any infiltration of the tumour itself by the infective agent could affect the physical characteristics of the tumour. Over the past 15 months however there has been no recurrence of this wasting disease, and the xenograft lines have remained remarkably consistent with respect to growth characteristics.

#### 8.10 Conclusions

The success rate in establishing xenograft lines was disappointing (31%) in comparison with other studies on lung cancer, particularly Shorthouse (1981), who used a similar tumour model, and reported a 78% take rate in tumour specimens from 49 patients.

There are a number of possible explanations for this discrepancy. Most important, the delay in tumour implantation was often much longer than desired in this study, in particular there was a delay of up to 1 hour on occasions before samples were transferred to a suitable medium, with implantation taking up to 4 hours on occasions. This compares very unfavourably with Shorthouse (1981), where all samples were transplanted within 2 hours, therefore viability of the samples used in this study may have been impaired. In addition, the majority of specimens obtained for this study were from primary tumours, whereas a large number of samples obtained by Shorthouse (1981) were from metastatic sites, the latter known to be associated with a higher take rate. Other factors, such as the level of natural killer cell activity in these animals was considered as a cause for the low take rate, but was considered unlikely as overall implant take rates were similar to those reported by Shorthouse (1981). Initial inexperience in the technique of thymectomy was also considered as a possible explanation for the poor take rate but was discounted as similar tumour take rates were obtained throughout in the study.

Implant take rates in squamous carcinoma and adenocarcinoma were similar, although only 1 adenocarcinoma line was passaged through many generations. Small cell carcinoma did



not take well, with numbers in the other histological categories too small for analysis. Implant take rates significantly improved in established lines, with the best results achieved by implantation in the first 7 days after irradiation, as previously reported (Shorthouse 1981). Storage of tumour tissue in liquid nitrogen was sufficient to maintain xenograft lines although implant take rates were poor. The disappointing take rates were probably due to the amount of necrotic material present in lung cancer xenografts, as 70% bladder cancer xenografts were successfully grown in these animals from samples stored in liquid nitrogen (tissue kindly supplied by Dr John Hay).

Tumour regressions were occasionally encountered, being variable between xenograft lines. Tumour related factors were certainly important, but returning host immunity was also a factor as regressions were more common in the slowly growing tumours. In addition by the analysis of the frequency of single and double implant takes in these animals, it was shown that host immunity was a statistically significant factor in determining the likelihood of successful transplantation.

Growth patterns in the xenograft were similar to previously reported studies, with a variable lag phase followed by an exponential growth phase. Duration of the lag phase varied

widely between and within tumour lines. However, in contrast with previous studies (Shorthouse 1981; Mattern et al 1981) the lag phase did not shorten with progressive passage. Similarly the growth rate did not progressively increase with passage as may have been expected from previous studies, although in this study the selection of tumours for transplantation may have been different. Tumours of 0.5-1.0 cm<sup>3</sup> were used wherever possible for transplantation, as these tumours had minimal necrosis. Tumours of variable growth rate were transplanted in the routine maintenance of xenograft lines to avoid selection of rapidly proliferating clones. Other studies frequently used the faster growing xenografts for transplantation, therefore often selecting the more rapidly growing cells. Every attempt was made to avoid this in this study.

Estimation of tumour volume by external caliper measurement was used for the assessment of growth and chemotherapeutic response. Analysis of tumour weight and estimated tumour volume showed that external measurement gave a valid estimation of tumour volume with tumours of greater than 0.2 cm<sup>3</sup>.

Loss of animals during immune-deprivation was a problem which obviously would have been diminished with the use of nude mice. The loss of animals from these procedures was similar to the experience of Shorthouse (1981). Despite these losses, the

use of immune-deprived mice was less expensive than a nude mouse colony would have been. Normally immune-deprived animals are considered more resilient to infection than nude mice, but unfortunately a major problem was encountered in this study. No cause was found for the many unexpected deaths, and although infection was the most likely aetiology, multiple attempts to isolate an organism were unsuccessful. Fortunately, there no longer appears to be a problem in the mouse colony. Obviously, infection can be a major stumbling block in any experiment using immune-deprived animals, and great care should be taken with these mice to minimise these risks. Following the loss of these mice, antibiotic therapy in the form of terramycin and neomycin in acidified drinking water has been instituted for 14 days following total body irradiation to reduce the risk of endogenous bacteraemias from gut flora.

## CHAPTER 9

### MAINTENANCE OF HUMAN CHARACTERISTICS BY XENOGRAFTS

#### 9.1 Introduction

Following the successful establishment of human lung cancer xenografts it was essential to show that the characteristics of the original tumour were maintained prior to chemotherapy studies.

#### 9.2 Histopathology

All tumours were classified according to the WHO histological classification (WHO 1981) as illustrated in Table 9.1, and graded using the Edinburgh Classification (1982) as shown in Table 9.2. Original sections from thoracotomy specimens were reviewed by Dr D Lamb (Department of Pathology, University of Edinburgh). All xenograft biopsies were reviewed and compared with the histology of the donor patient by Dr M A McIntyre (Department of Pathology, University of Edinburgh).

All tumours grew as discrete subcutaneous nodules in the flanks of the mice as shown in Figure 9.1. Some animals grew 3 or 4 nodules, the additional tumour material growing either along the implantation track or at the incision site. The

1. **Squamous cell carcinoma**  
variant (a) spindle cell carcinoma
  
2. **Small cell carcinoma**  
(a) oat cell carcinoma  
(b) intermediate cell type  
(c) combined oat cell carcinoma
  
3. **Adenocarcinoma**  
(a) acinar adenocarcinoma  
(b) papillary adenocarcinoma  
(c) bronchio-alveolar carcinoma  
(d) solid carcinoma with mucus formation
  
4. **Large cell carcinoma**  
Variants (a) giant cell carcinoma  
(b) clear cell carcinoma
  
5. **Adenosquamous carcinoma**
  
6. **Carcinoid tumour**
  
7. **Bronchial gland carcinomas**  
(a) adenoid cystic  
(b) mucoepidermoid  
(c) others
  
8. **Others**

Table 9.1 World Health Organisation Classification of Primary Lung Tumours 2nd Edition (WHO 1981)

**A. Squamous carcinoma**

1. Well differentiated - much keratinisation  
(up to 50% of islands of tumour).
2. Moderately differentiated - less obvious keratinisation.
3. Poorly differentiated - very occasional squamous pearl  
or single cell keratinisation or easily seen prickles.

**B. Adenocarcinoma**

1. Well differentiated - predominantly formed of obvious  
acinar formation some of which may be cribriform.
2. Moderately differentiated - less marked glandular  
formation including cribriform pattern and acinar  
structures in less than 50% of high power fields.
3. Poorly differentiated - scanty gland spaces, less than 1  
in 10 high power fields.  
  
plus
4. Bronchio-alveolar carcinoma.
5. Solid carcinoma with mucus formation.

Table 9.2 Edinburgh Classification (1982). Criteria for the differentiation of non-small cell lung cancer.



Figure 9.1 Human lung cancer xenograft growing subcutaneously in the flank of an immune-deprived CBA mouse.

majority of tumours had a variable degree of central necrosis particularly when the tumour volume exceeded 1 cm<sup>3</sup>, although two of the xenografts, both moderately differentiated squamous carcinomas, showed minimal necrosis, the tumours having a solid glistening white appearance on their cut surface. The small cell carcinoma xenograft (WX310) had a similar gross morphological appearance to that described by Shorthouse (1981), being invariably fragile and haemorrhagic.

All of the xenografts maintained the general histological characteristics of the original tumour, with occasional variations in differentiation. Examples are shown in Figures 9.2-9.11. Changes in differentiation from poorly differentiated to moderately well differentiated were fairly common as was the reverse. No well differentiated tumours were successfully grown. Xenograft CX 143, an adenosquamous carcinoma, was particularly interesting. The original tumour is illustrated in Figures 9.2 and 9.3, showing poorly differentiated mucin secreting adenocarcinoma (Figure 9.2) with areas of poorly differentiated squamous carcinoma (Figure 9.3). Over 6 passages the general adenosquamous pattern has been maintained as illustrated in Figures 9.4 and 9.5. However at the sixth passage more differentiation is seen in the squamous pattern, with similar areas of poorly differentiated adenocarcinoma.



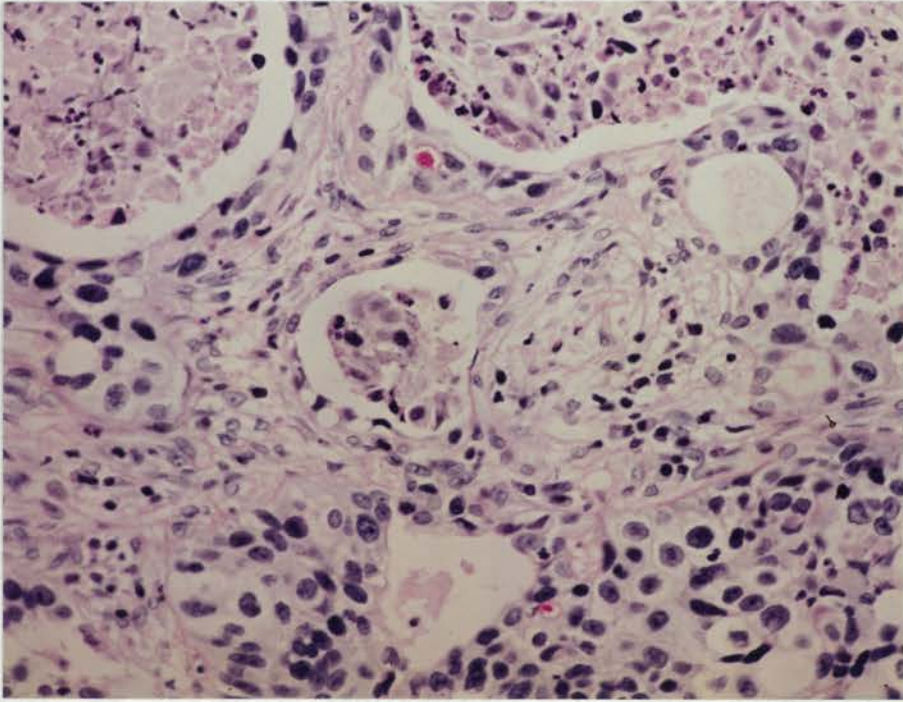


Figure 9.2 Histological appearance of donor patient tissue from the human lung cancer xenograft CX 143 - adenosquamous carcinoma showing PAS positive staining indicating adenocarcinoma, with glandular formation - poorly differentiated adenocarcinoma. (PAS; magnification 440 X approx).

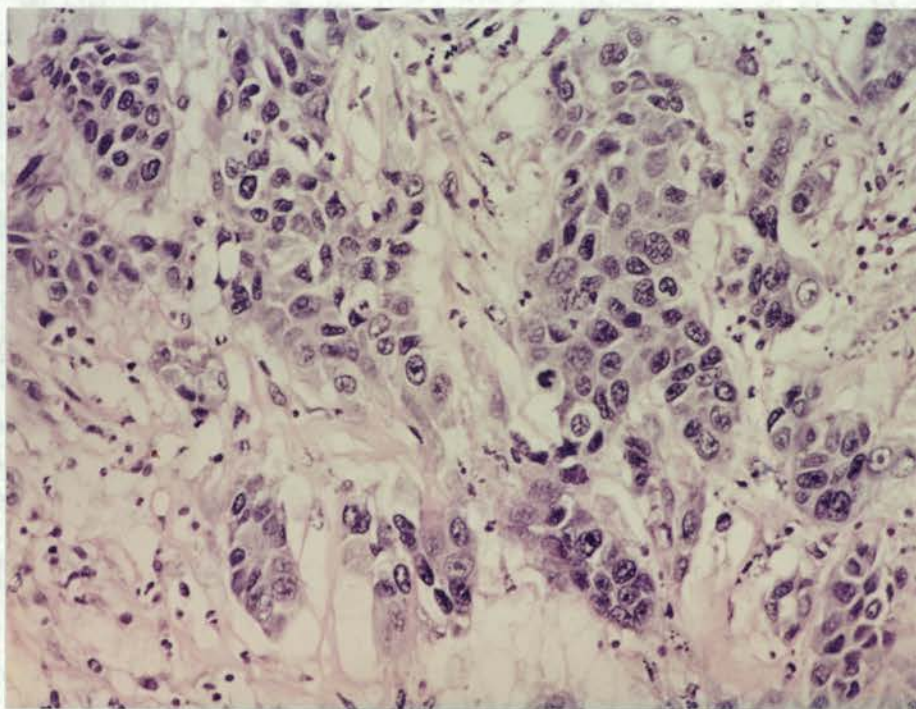


Figure 9.3 Histological appearance of donor patient tissue from the human lung cancer xenograft CX 143 -adenosquamous carcinoma. Clusters of poorly differentiated malignant cells with lack of glandular formation and no keratin formation. Appearances of poorly differentiated squamous carcinoma (H + E stain; magnification 440 X approx).

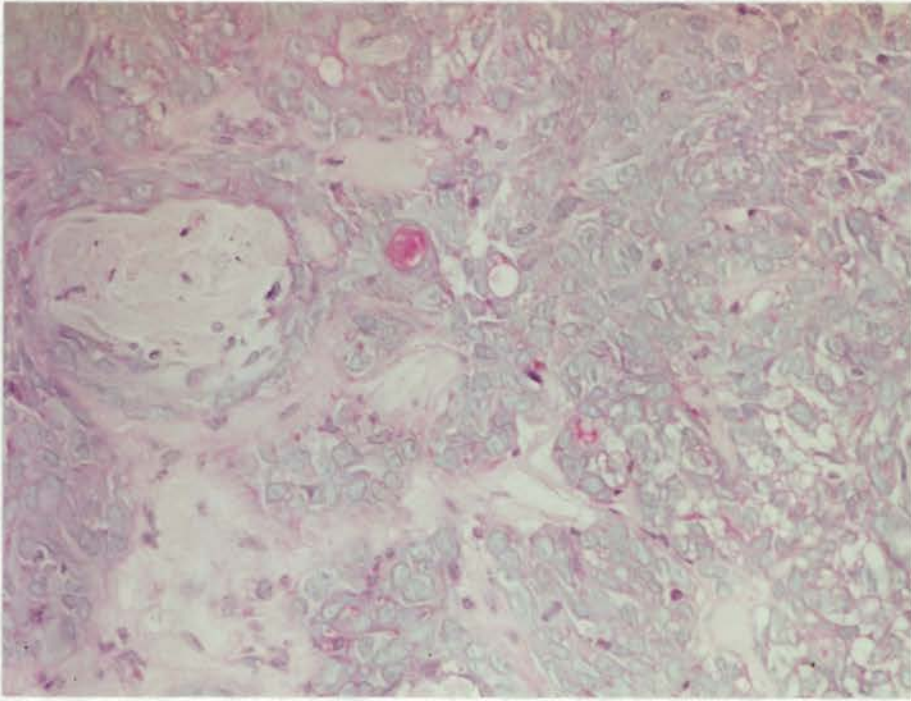


Figure 9.4 Human adenosquamous xenograft CX 143 at the 6th mouse to mouse passage. Minimal glandular formation with areas of PAS positivity. Appearances of poorly differentiated adenocarcinoma (PAS stain; magnification 440 X approx).

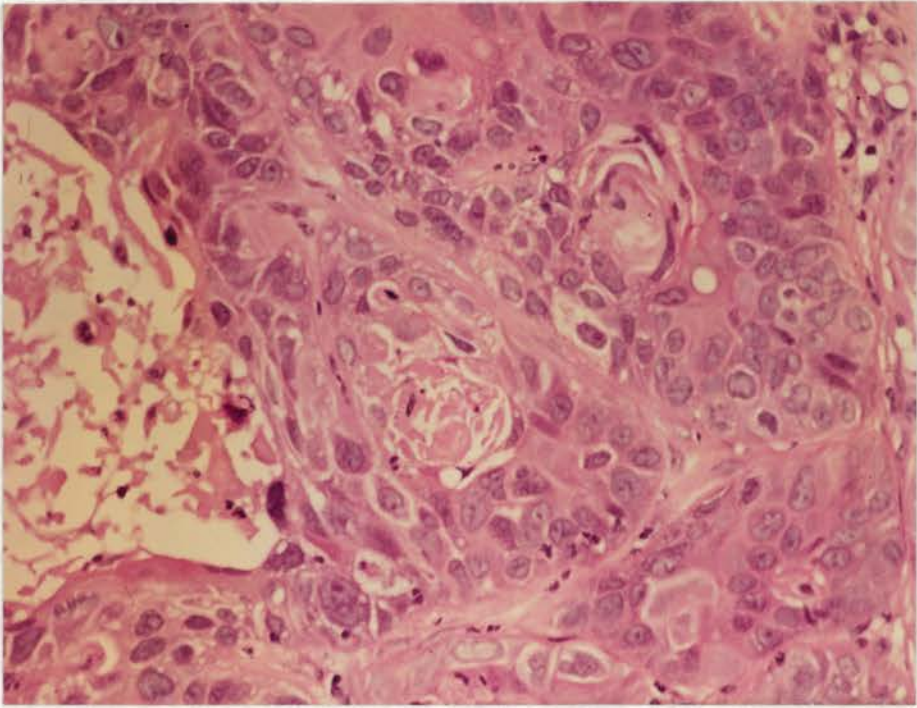


Figure 9.5 Human adenosquamous xenograft CX 143 at the 6th mouse to mouse passage. Areas of keratin formation in the absence of glandular formation. Appearances of moderately differentiated squamous carcinoma (H+E stain; magnification 440 X approx).

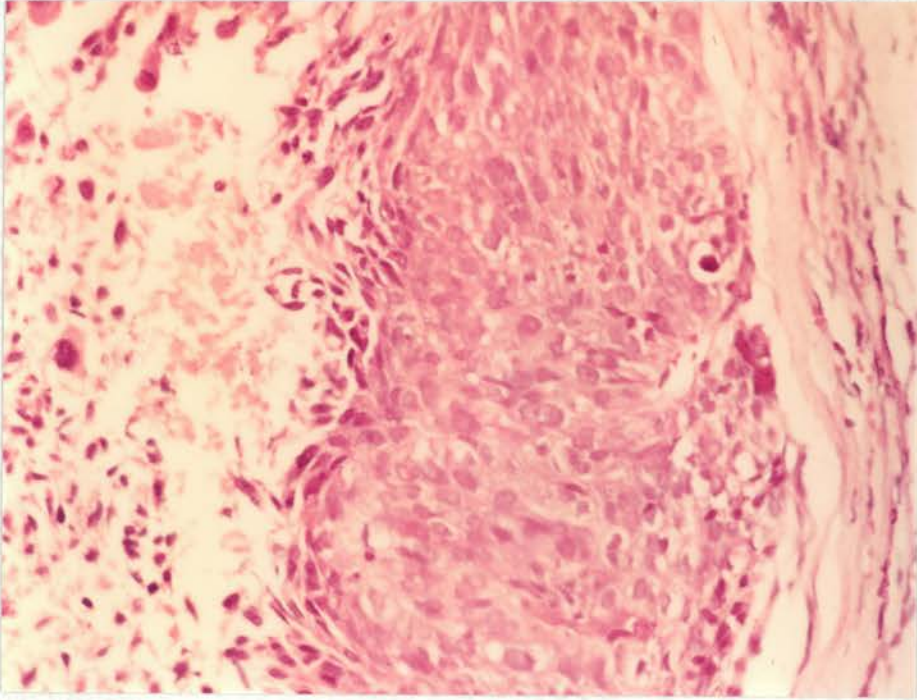


Figure 9.6 Original biopsy from donor patient of NX 002 xenograft. No evidence of glandular formation with moderate degree of keratinisation with some evidence of stratification. Appearance of moderately differentiated squamous carcinoma. (H+E stain; magnification 440 X approx).

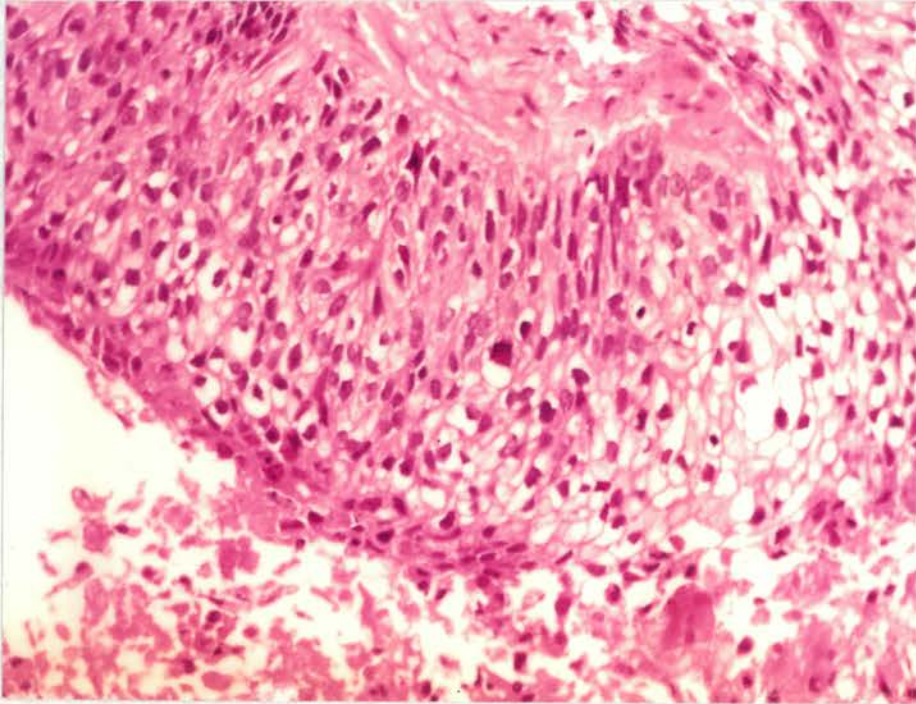


Figure 9.7 NX 002 human lung cancer xenograft at the 7th passage showing similar features to the original biopsy. (H+E stain; magnification 440 X approx).

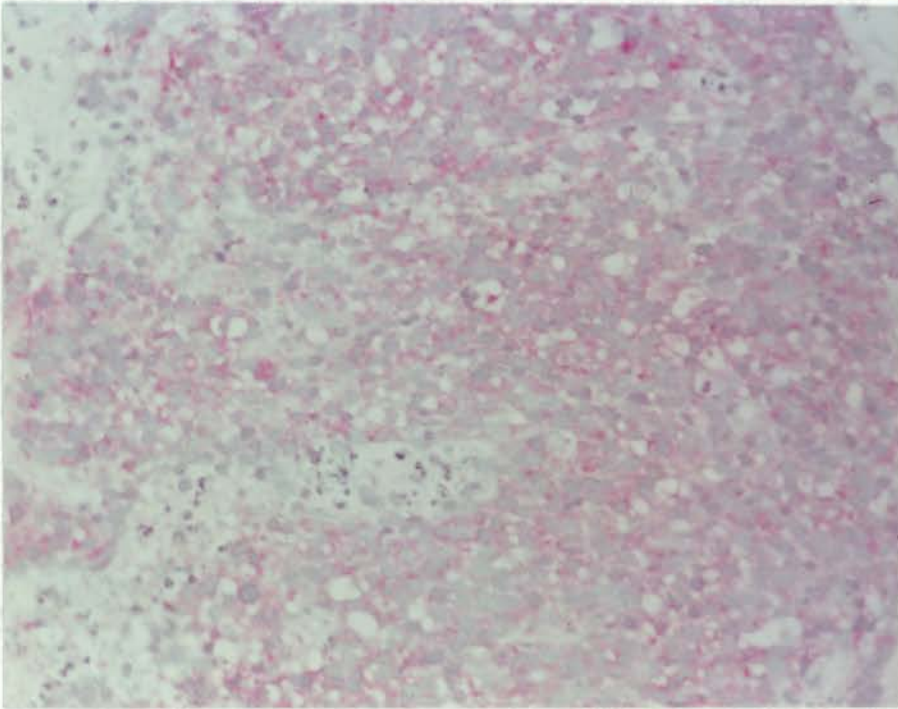


Figure 9.8 Original biopsy from donor patient of CX 117 xenograft. Large oval nuclei with PAS positivity in the cytoplasm, but with minimal glandular formation. Vacuolation of the cytoplasm. Appearances of poorly differentiated adenocarcinoma. (PAS stain; magnification 280 X approx).

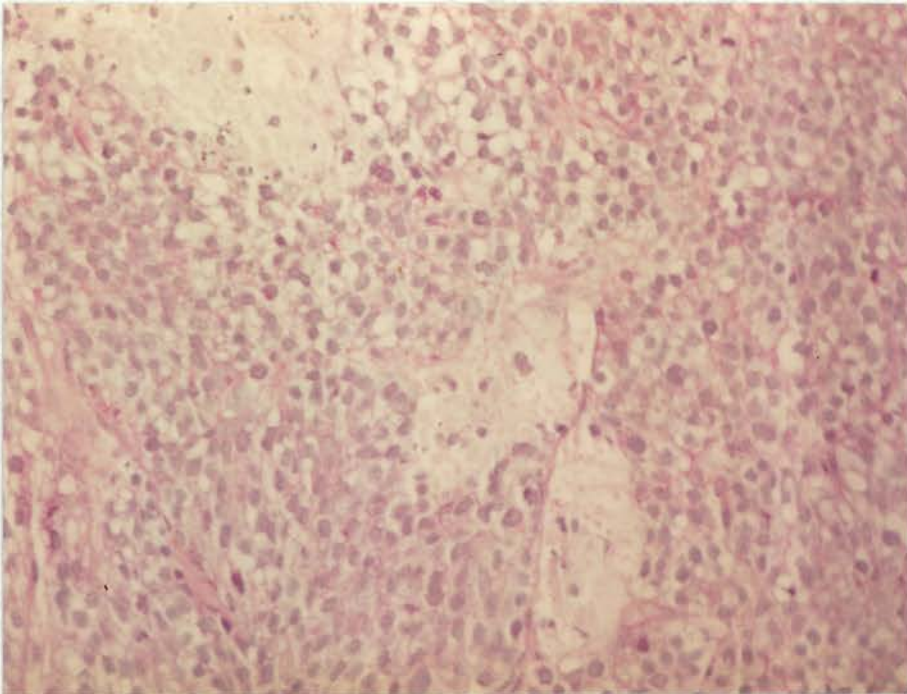


Figure 9.9 CX 117, a poorly differentiated adenocarcinoma xenograft at the 6th passage showing similar histological features to the donor tissue (PAS stain; magnification 280 X approx).



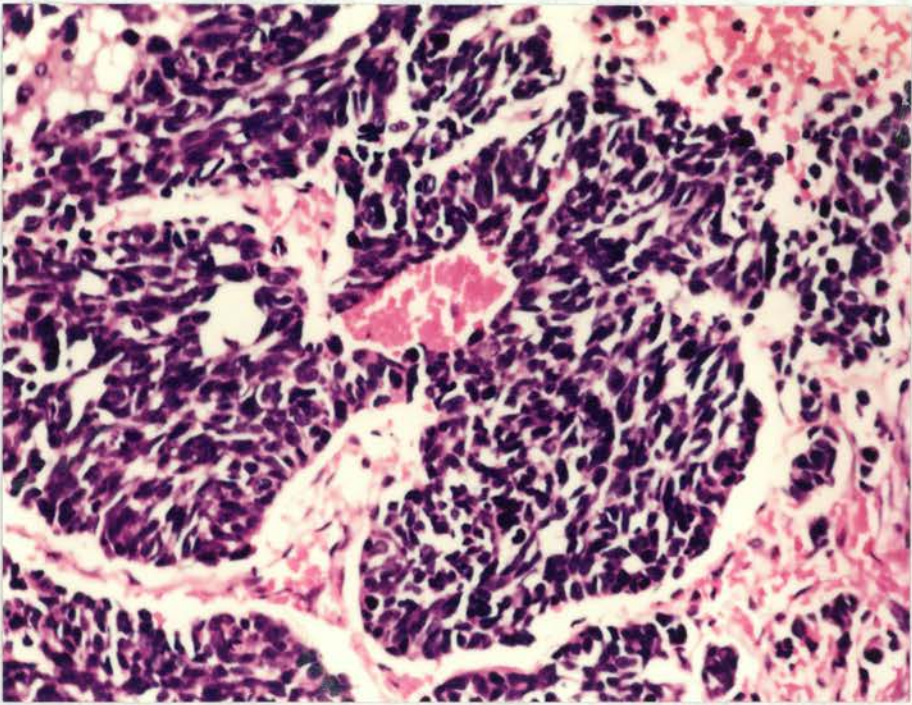


Figure 9.10 Original biopsy from the donor patient of WX 310. Small round cells with irregular darkly staining nuclei with minimal cytoplasm growing in sheets. Appearance of anaplastic small cell carcinoma. (H+E stain; magnification 440 X approx).

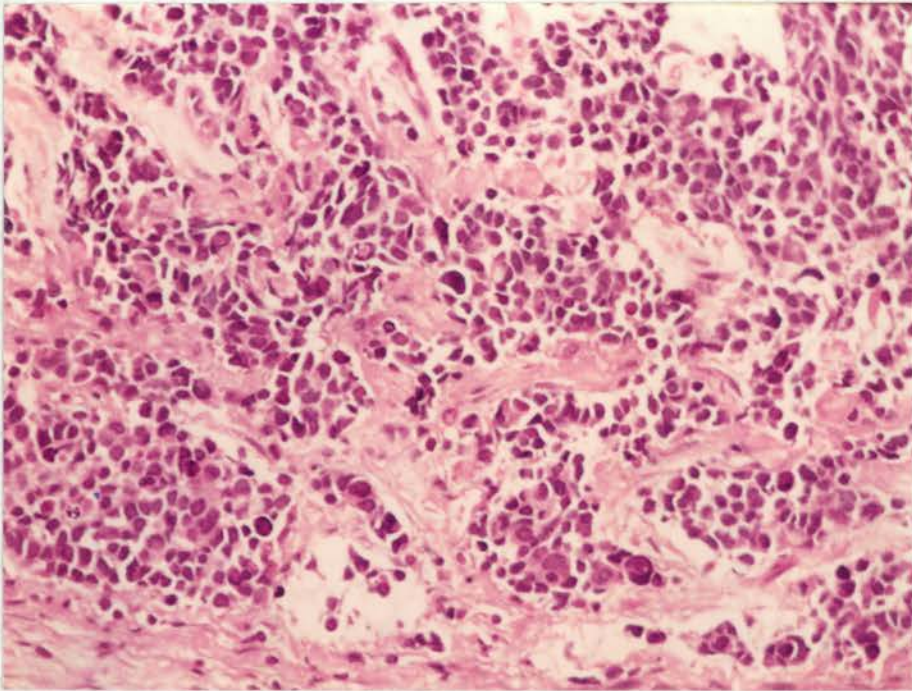


Figure 9.11 WX 310 xenograft at the 5th passage showing similar features to the original biopsy. (H+E stain; magnification 440 X approx).

NX 002 a moderately differentiated squamous carcinoma is illustrated in Figures 9.6 and 9.7 showing no significant change over 7 passages. Similarly, the appearance of CX 117 a poorly differentiated adenocarcinoma is essentially unchanged (Figures 9.8 and 9.9). The histological appearance of the one established small cell xenograft is shown in Figures 9.10 and 9.11.

Of the 9 tumours which underwent multiple passages, 5 retained similar differentiation characteristics, 3 increased differentiation (CX 108, CX 112, CX 140) whereas 1 became more anaplastic initially, although by passage 6 had regained the appearance of moderately differentiated squamous carcinoma. A number of reports have shown occasional increased differentiation of xenografts (Greene 1940; Helson et al 1975; Sharkey et al 1978) although only 6% showed increased differentiation in Shorthouse's study (1981). The 33% incidence of increased differentiation in this study is more akin to the 25% incidence described by Sharkey et al (1978).

## 9.2 Electron Microscopy

A number of xenograft lines were examined after a minimum of 4 passages in an attempt to identify ultrastructural features which would confirm the tumour cell type. Five tumour lines were examined, 1 poorly differentiated adenocarcinoma, 1 small cell carcinoma and 3 squamous carcinomas.

The features of the adenocarcinoma xenograft (CX 117) are shown in Figure 9.12. The cells were large mononuclear cells with profuse cytoplasm, which contained large granular structures and rough endoplasmic reticulum. No features of squamous differentiation were visible.

The ultrastructural appearance of the small cell xenograft after 5 passages (WX 310) is shown in Figure 9.13. It shows a large granular nucleus with no evidence of intracytoplasmic secretory granules. Secretory granules may have been expected as the patient showed clinical evidence of inappropriate anti-diuretic hormone secretion. As electrolyte studies were not performed on these mice it is not known whether the mice were affected by a similar syndrome, although ectopic hormone production has frequently been shown to persist in xenografts (Ruddon et al 1980; Shorthouse et al 1982). However, there are limitations to the measurement of ectopic hormone production as an assessment of tumour burden, particularly when

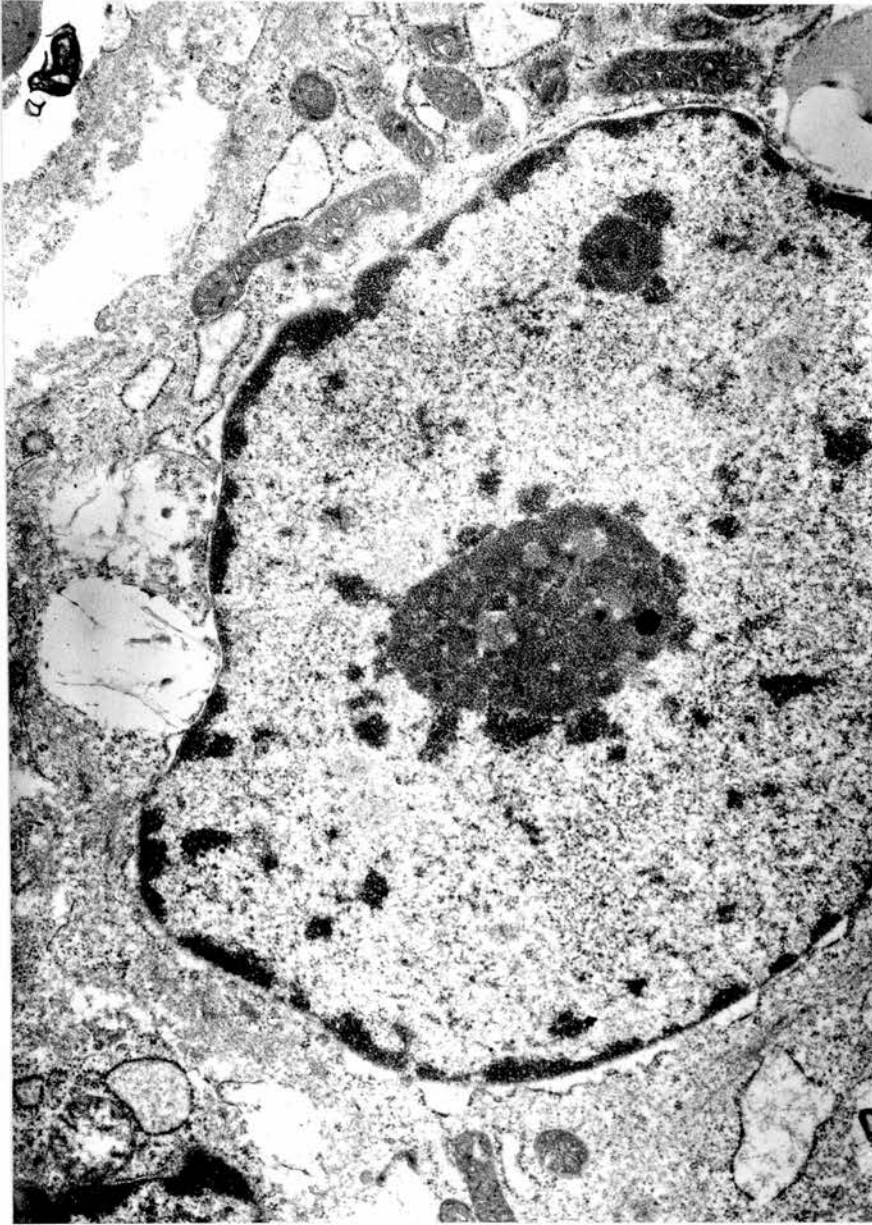


Figure 9.12 Electron microscopic appearance of CX 117, an adenocarcinoma xenograft at the 5th passage. Typical large mononuclear cells with profuse cytoplasm containing mitochondria and dilated endoplasmic reticulum. Large nucleus with prominent nucleolus. No visible features of squamous differentiation. Magnification 13,4000 X .

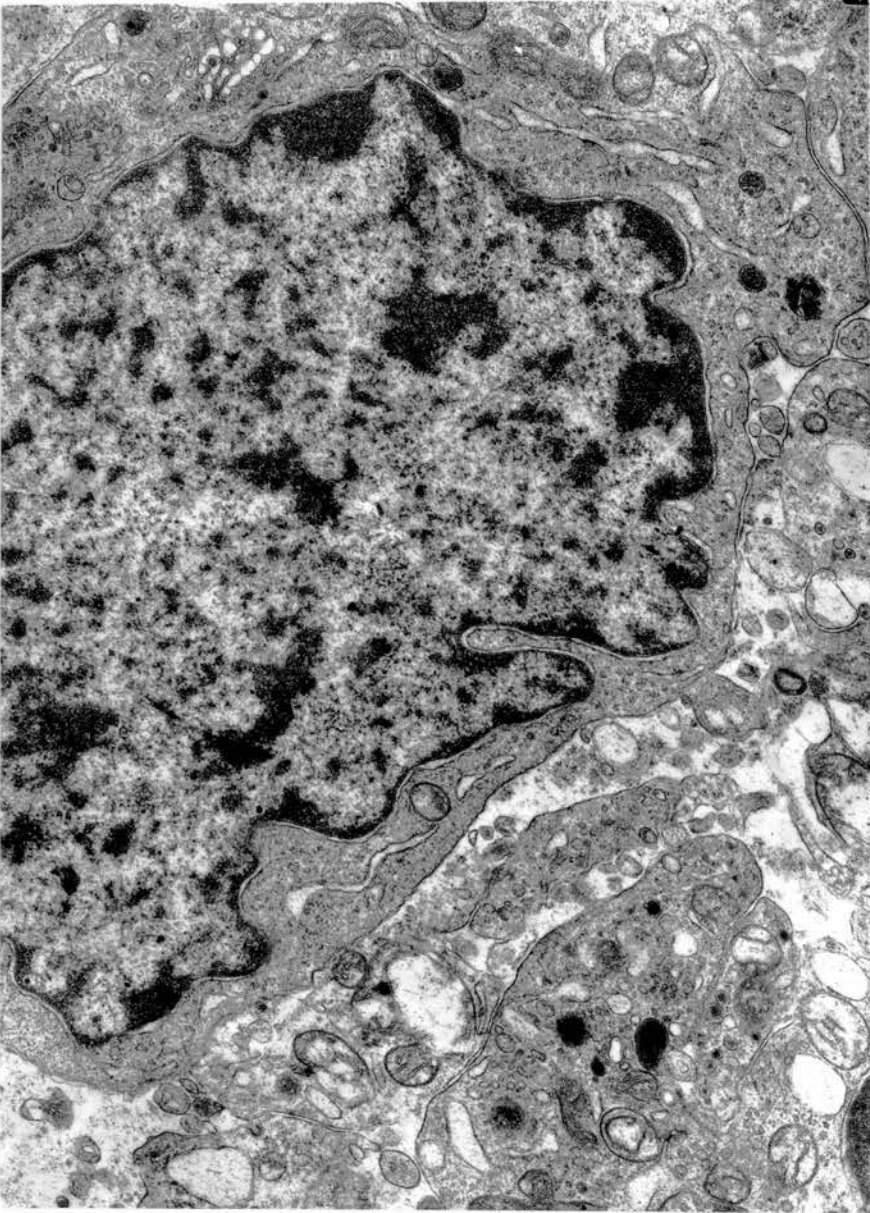


Figure 9.13 Electron microscopic appearance of WX 310, a small cell carcinoma xenograft after 5 passages. A large irregular nucleus, without a nucleolus but with chromatin clumping. Few organelles and no intracytoplasmic secretory granules. Magnification 13,400 X.

central necrosis is present (Quayle 1982). The lack of secretory granules is consistent with the development of the more resistant variant phenotype (Gazdar 1985). Variant small cell lines have been shown to lose secretory granules in addition to exhibiting decreased production of dopa decarboxylase and creatinine phosphokinase. These changes are often associated with amplification and increased expression of the c-myc and related oncogenes. The variant sub-type is also associated with a poor prognosis, and is generally associated with resistance both to cytotoxic drugs and to radiation.

Electron microscopy was performed on three squamous carcinomas at the fourth passage, namely NX 002, CX 133 and CX 140. The classical appearances of tonofilaments and desmosomes were present in all specimens, and examples are shown in Figures 9.14 and 9.15.

### 9.3 Chromosome Studies

All tumours were examined at a late stage to ensure that a human karyotype was maintained (Warenius 1980). Karyotypically all tumours examined were of human origin with metacentric and submetacentric and acrocentric chromosomes with a number of these chromosomes showing break points. No murine karyotypes were

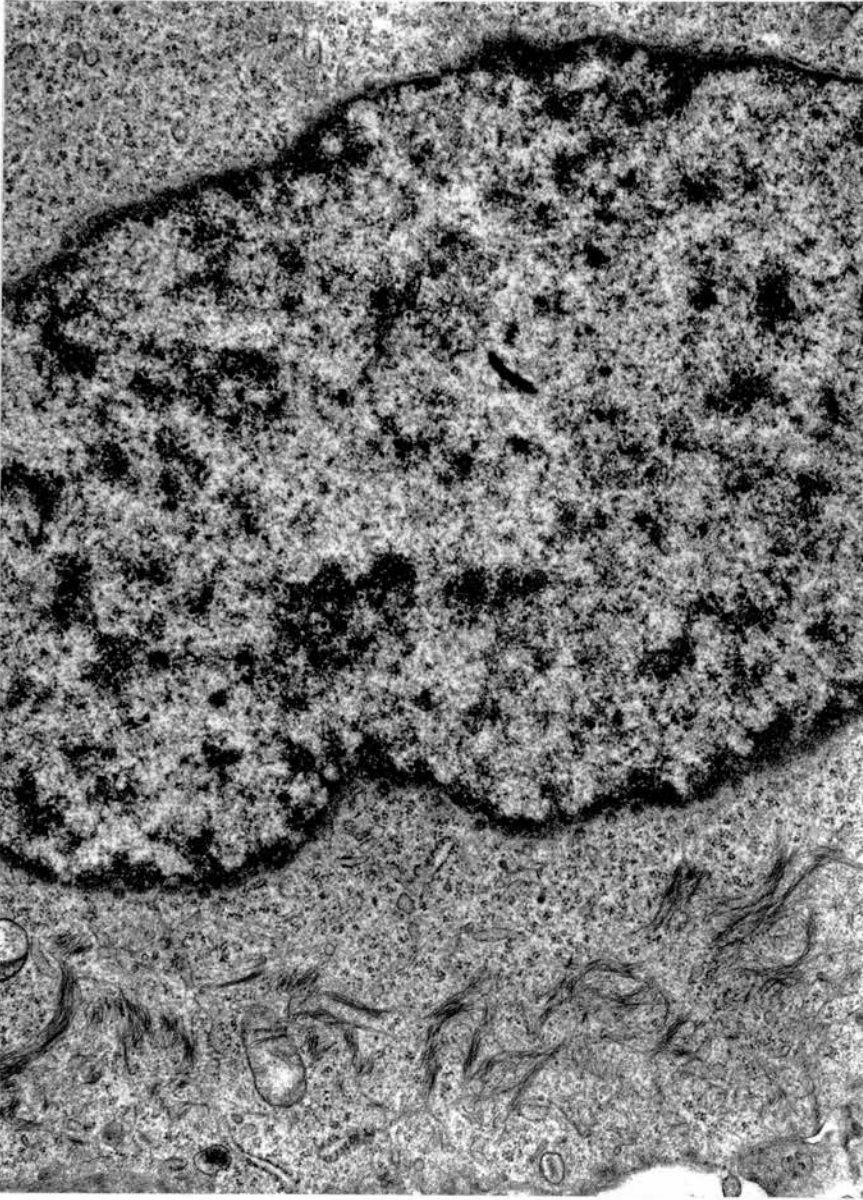


Figure 9.14 Electron microscopic appearance of moderately differentiated squamous carcinoma showing intracytoplasmic keratin filaments - tonofilaments. Taken from NX002 xenograft at a magnification of 13,400 X.



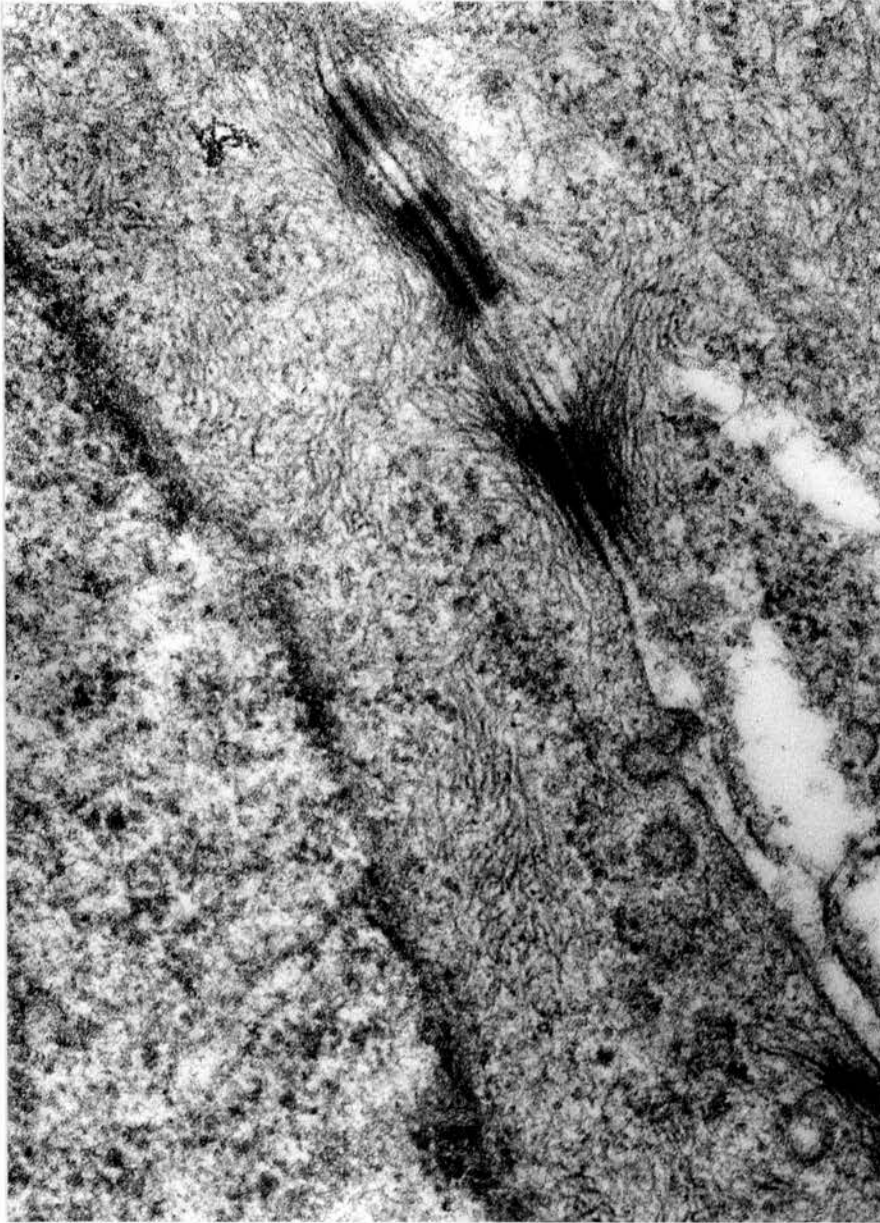


Figure 9.15 Electron microscopic appearance of moderately differentiated squamous carcinoma exhibiting intercellular bridges - desmosomes and tonofilaments. Taken from xenograft at 54,000 X magnification.

isolated from any of the tumour samples examined. CX 140, more extensively studied, was found to have 36 chromosomes and is illustrated in Figure 9.16.

Relatively few metaphase spreads were examined from other xenografts but of those examined, chromosome number was depleted with 36 chromosomes in CX 140, 31 chromosomes in CX 117 and 28 chromosomes in NX 002. In addition to alteration of chromosome number, a number of break points were also visualised.

#### 9.4 Metastases

There was no macroscopic evidence of metastatic disease in any of the tumour bearing animals examined. With larger tumours there was evidence of local invasion but no distant spread was observed. This is consistent with other reported xenograft series (Detre and Gazet 1973; Giovanella et al 1972, 1973; Sharkey et al 1978; Shorthouse 1981).

#### 9.5 Cachexia

Although weight loss was observed in a number of mice, no evidence of cachexia was detected in any particular xenograft line. The finding is in keeping with previous studies (Povlsen and Rygaard 1971; Giovanella et al 1974). Cachexia has been



Figure 9.16 Metaphase spread of CX 140 xenograft (5th passage) showing typical human chromosomes (n=36) with metacentrics, submetacentrics and acrocentrics.

observed in a malignant melanoma xenograft (Kondo et al 1977) and rarely in other tumours. No cachexia was detected in Shorthouse's study (1981) using similar tumour bearing animals.

#### 9.6 Conclusion

This study confirms that many human characteristics are maintained in xenografts when grown in immune-deprived animals. Their histopathological appearances showed no change in classification, although alteration in differentiation was frequently noted. Increased and decreased differentiation of xenografts were encountered, as has previously been described. Ultrastructurally, xenografts were shown to exhibit the features of their particular histological type, even after many passages. Typical features of squamous carcinoma, adenocarcinoma and small cell carcinoma were seen by electron microscopy after a minimum of 4 passages in immune-deprived mice.

Karyotypically all tumours were of human origin with typical human chromosomes showing metacentrics, submetacentrics and acrocentrics. The typical murine karyotype ( $n = 42$ ) contains exclusively acrocentric chromosomes, and no murine karyotype was observed in these samples. Chromosome number was decreased in all xenograft lines examined, including

CX 140 (n = 36); CX 117 (n = 31) and NX 002 (n = 28), although insufficient metaphase spreads were obtained for definitive karyotyping or banding studies.

Previously, the lack of metastases in xenografts questioned the validity of these model systems, although the short duration of animal survival following tumour transplantation was considered a possible explanation for this discrepancy. There is now increasing evidence that metastases are, in fact, more common than originally thought. The first metastatic models were seen in osteosarcomas when the tumour was inoculated intravenously. Over the past year however, many more models have been described, including a number of intraperitoneal human tumour models such as ovarian adenocarcinoma (Hamilton et al, 1984), colonic adenocarcinoma and non-small cell carcinoma (Gazdar - personal communication). In the latter 2 models, documented histological spread was detected in numerous sites, including the liver, lungs, pericardium and brain. The metastases were all detected by day 52 following tumour transplantation although in these cases the tumours were initially established as cell lines. The possibility is raised that the site of tumour inoculation may be important in the development of metastatic disease in these

animals. These metastatic models may be a significant advance in the development of xenografts for further experimental studies.

In conclusion, xenografts in general maintain the characteristics of the donor tumour , although minimal variation in histological differentiation and functional activity may occur. In addition to their value as models for chemosensitivity testing, xenografts are also valuable as tools for the study of the biology and biochemistry of human tumours.

## CHAPTER 10

### CLINICAL STUDIES

#### 10.1 Non-small Cell Lung Cancer

In this section details of the clinical studies carried out in the ICRF Medical Oncology Unit at the Western General Hospital will be discussed.

##### 10.1a Introduction

The prognosis of unresectable non-small cell lung cancer remains extremely poor despite encouraging recent results using a chemotherapy schedule employed by Gralla et al (1981). A similar combination of drugs was used in this study in an attempt to confirm the activity and assess the treatment related toxicity of a cis-platinum-vindesine combination.

##### 10.1b Patients and Methods

Sixty-three patients with histologically proven, inoperable non-small cell carcinoma of the bronchus were treated between March 1981 and May 1983. All patients had evaluable disease in previously non-irradiated sites and were of good performance status; none had received any prior chemotherapy.

They had normal bone marrow function and no hepatic or renal disease. All the histology was reviewed by one pathologist (Dr M A McIntyre, Pathology Department, Western General Hospital, Edinburgh). Patient details are summarised in Table 10.1. Thirty-three patients had extensive disease with metastatic spread as described in Table 10.2. Patients with cerebral metastases at presentation were not included although 5 patients subsequently developed evidence of central nervous system involvement.

Prior to entry into the study all patients had a full clinical examination, chest x-ray, barium swallow, diaphragmatic screening and audiometry. Blood tests included full haematological indices, electrolytes, liver function tests and creatinine clearance. All patients underwent bronchoscopy for diagnosis and staging even though in some the diagnosis was established by lymph node biopsy. Histological details are shown in Table 10.1. Liver ultrasound and isotope bone scans were only performed when metastatic disease was suspected clinically, or because of abnormal biochemical results.

The treatment schedule consisted of vindesine 3 mg/m<sup>2</sup> intravenously on days 0, 7, 14 and 21 and cis-platinum 100 mg/m<sup>2</sup> on Days 0 and 21. The cis-platinum was infused in 500 ml 10%



|                           |          |
|---------------------------|----------|
| No. of patients           | 63       |
| Sex M:F                   | 43:22    |
| Age (yr)                  |          |
| Median                    | 58.5     |
| Range                     | 38-70    |
| Performance status (ECOG) |          |
| 0                         | 26 (41%) |
| 1                         | 15 (24%) |
| 2                         | 5 (8%)   |
| Cell type                 |          |
| Squamous carcinoma        | 30 (48%) |
| Adenocarcinoma            | 15 (24%) |
| Large cell carcinoma      | 5 (8%)   |
| Disease extent            |          |
| Intrathoracic             | 30 (48%) |
| Metastatic                | 33 (52%) |

Table 10.1 Details of patients entered into the trial of cis-platinum and vindesine in the treatment of non-small cell lung cancer.

| Site of metastatic disease | No. of patients |
|----------------------------|-----------------|
| Liver                      | 9               |
| Distal lymphatics*         | 9               |
| Bone                       | 7               |
| Skin                       | 5               |
| Intrapulmonary             | 4               |
| Pleural effusion           | 3               |

\*including cervical node

Table 10.2 Sites of metastatic disease in patients treated with cis-platinum and vindesine in the non-small cell lung cancer study.

mannitol over 30 minutes following 6 hours pre-hydration with one litre dextrose/saline. A further 1.5 litres dextrose/saline was administered in the 18 hours after the cis-platinum infusion. Cycles of chemotherapy were repeated every 42 days. Full haematological and biochemical profiles were carried out prior to each cycle. In addition a full clinical examination and radiological assessment was performed, as well as an appraisal of treatment-related toxicity. Response was assessed following 3 courses of treatment unless progressive disease or unacceptable toxicity made earlier cessation of treatment necessary.

All pre-treatment investigations were repeated at this stage except bronchoscopy which was restricted to patients in whom there was a clinical response. Clinical responses were defined as follows:- complete remission (CR) was the complete disappearance of all evaluable disease including a negative bronchoscopy. Partial remission (PR) was defined as greater than 50% reduction in measurable tumour diameters, with no new lesions appearing.

#### 10.1c Results

All 63 patients were evaluable for response. The results are outlined in Table 10.3. The overall response rate was 33%

| Group              | No. | CR | PR | OR | %  |        |
|--------------------|-----|----|----|----|----|--------|
| All                | 63  | 5  | 16 | 21 | 33 |        |
| Squamous           | 43  | 1  | 13 | 14 | 22 | N.S.   |
| Adenocarcinoma     | 15  | 3  | 12 | 15 | 33 |        |
| Large cell         | 5   | 1  | 1  | 2  | 40 |        |
| Male               | 43  |    |    | 13 | 30 | N.S.   |
| Female             | 20  |    |    | 8  | 40 |        |
| Limited disease    | 30  | 3  | 11 | 14 | 47 | P<0.05 |
| Extensive disease  | 33  | 2  | 5  | 7  | 21 |        |
| Performance status |     |    |    |    |    |        |
| 0                  | 26  |    |    | 11 | 42 | N.S.   |
| 1                  | 24  |    |    | 6  | 25 |        |
| 2                  | 13  |    |    | 4  | 30 |        |

Table 10.3 Response of non-small cell lung cancer patients to cis-platinum and vindesine in combination.  
 CR = complete response; PR = partial response;  
 OR = objective response

with 5 patients (8%) achieving a complete remission. Similar response rates were noted for all cell types. Patients with limited disease on entry responded significantly better than those with extensive disease (Chi squared analysis  $p < 0.05$ ). There was no significant sex difference and performance status on entry into the study had no relationship to response.

Median duration of response was 4 months (range 2-15 months), although 2 patients remain in remission following observation periods of 6 and 8 months respectively.

Median survival of responding patients was 14 months compared with 4.8 months for non-responding and 6.5 months in the overall group. Survival curves are shown in Figure 10.1. Ten patients remain alive 6-25+ months after entry into the study.

All 63 patients were evaluated for toxicity, and the results are listed in Table 10.4 using WHO criteria. Side-effects were common and often severe. Alopecia was universal and gastrointestinal toxicity severe, although in 40% vomiting was controlled with high dose metoclopramide (Allan et al 1984). Haematological toxicity was moderate, although blood transfusion was required in 20 patients following at least 2 cycles of treatment. Peripheral neuropathy was common, and although occasionally severe, was reversible. High tone

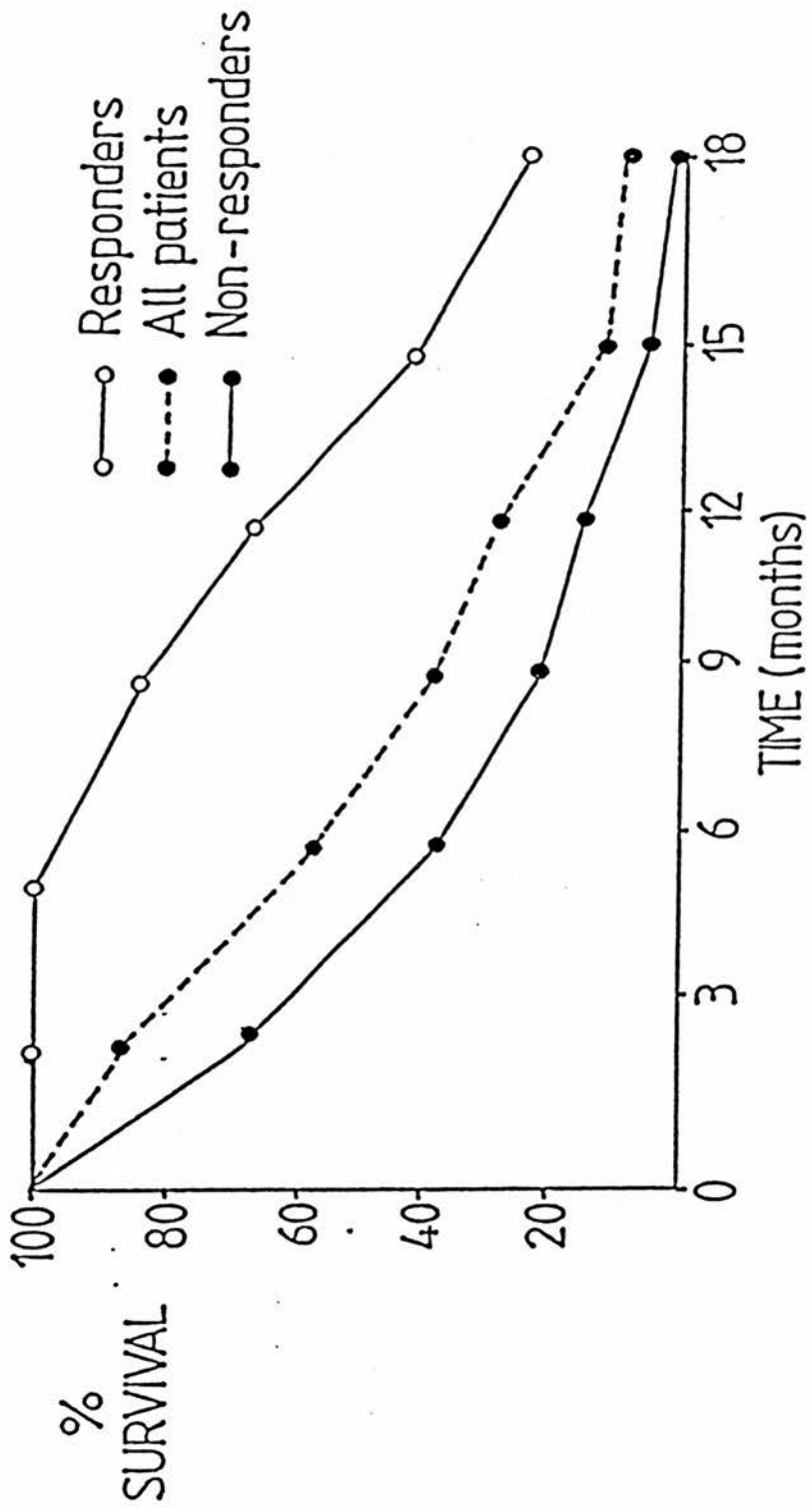


Figure 10.1 Cis-platinum and vindesine in the treatment of non-small cell lung cancer. Survival of patients by response to chemotherapy.

|                         |    |      |
|-------------------------|----|------|
| <b>Alopecia</b>         |    |      |
| Grade 2/3               | 63 | 100% |
| <b>Gastrointestinal</b> |    |      |
| Grade 2                 | 15 |      |
| Grade 3                 | 39 |      |
| Total                   | 54 | 86%  |
| <b>Neurotoxicity</b>    |    |      |
| Peripheral              |    |      |
| Grade 1                 | 15 | 24%  |
| Grade 2                 | 15 | 24%  |
| Grade 2                 | 9  | 14%  |
| Deafness                | 16 | 24%  |
| Constipation            | 10 | 16%  |
| <b>Haematological</b>   |    |      |
| WBC                     |    |      |
| Grade 2                 | 33 | 52%  |
| Grade 3                 | 7  | 11%  |
| RCB                     |    |      |
| Grade 2                 | 20 | 32%  |

Table 10.4 Toxicity of vindesine and cis-platinum in combination in the treatment of non-small cell lung cancer. All toxicities graded according to WHO criteria.

hearing loss was detected in 16 patients and was symptomatic in 10 cases. Renal impairment was not a clinical problem although it was demonstrated in 2 patients biochemically. Regardless of response, performance status deteriorated in 20 patients with improvement in only 4 cases.

#### 10.1d Discussion

Until recently the results of chemotherapy in non-small cell lung cancer were extremely disappointing. Single agents demonstrated minimal activity and little improvement was seen with combination chemotherapy (Livingston et al 1977). One of the best studied regimes was a 5 drug combination (BACON) reported by Livingston et al in 1976. Despite encouraging initial responses seen initially with this combination, these were not confirmed when larger numbers of patients were treated, with only a 20% response rate in 116 patients, in whom considerable toxicity was observed.

Recent reports by Gralla and co-workers (Gralla et al 1981; Kelsen et al 1982; Itri et al 1983) have shown response rates of 30-50% with combinations of vindesine and cis-platinum. One study compared the effects of low dose cis-platinum (60 mg/m<sup>2</sup> every 6 weeks) with high dose (120 mg/m<sup>2</sup> every 4 weeks), when used in combination with vindesine. The



vindesine was administered at a dosage of 3 mg/m<sup>2</sup> weekly for 6 weeks, then alternate weekly. Response rates were similar in both groups but duration of response was significantly longer with the higher cis-platinum dose regimen. The addition of cyclophosphamide, adriamycin or bleomycin to the high dose cis-platinum regimens failed to improve on these response rates however.

A similar response rate of 33% was achieved in 63 patients of good performance status. The patients studied included more patients with squamous carcinoma than in Gralla's group 1981, but these results, in agreement with his, show no difference in response rates in different histological categories as shown in Figure 10.2.

In contrast, however, duration of response and survival was disappointingly short in this study compared with those reported by Gralla. Despite a relatively large dose of cis-platinum 100 mg/m<sup>2</sup> 3 weekly x 6, the median duration of response was only 4 months. However, treatment was discontinued at 18 weeks, whereas in Gralla's study drugs were continued until disease progression or unacceptable toxicity supervened. Median survival of responders was 14 months, with a 1 year survival of 67%. Non-responders had a median survival

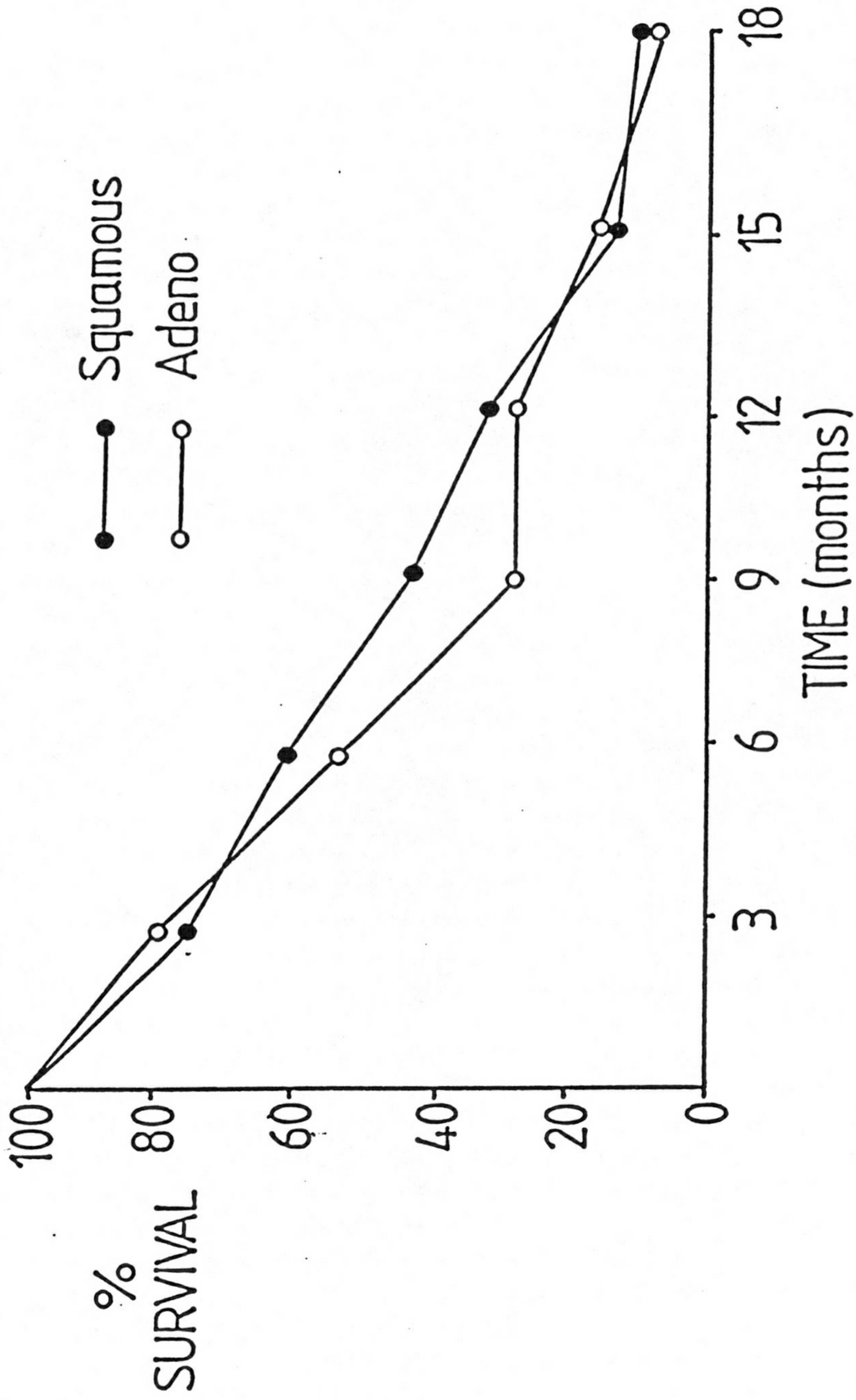


Figure 10.2 Effect of histology on survival of patients with non-small cell lung cancer treated with cis-platinum and vindesine in combination.

of 4.8 months, with a 1 year survival of 15%, whilst in the whole group of 63 patients the corresponding figures were 6.5 months and 29% respectively.

The toxicity of this drug combination was considerable, with deterioration in performance status common following treatment. Alopecia and gastrointestinal toxicity were almost universal, the latter being controlled in only 40% with high dose metoclopramide (Allan et al, 1984). Weight loss was almost invariable with a mean loss of 4.1 kg.

This study confirms the activity of vindesine and cis-platinum in combination in the treatment of non-small cell carcinoma of the bronchus. However, in view of the relatively short duration of response and considerable toxicity, this regimen is likely only to be of benefit to a selected few patients.

## 10.2 Small Cell Lung Cancer

### 10.2a Introduction

For many years it has been accepted that small cell carcinoma is a chemosensitive disease, with combination chemotherapy more effective than single agent treatment. Results of the many chemotherapy studies reported in this

disease are well summarised in 2 excellent reviews (Bunn & Ihde 1981; Ihde & Bunn 1982). Unfortunately the majority of the published trials report uncontrolled studies, although it is accepted that controlled studies are difficult in a disease offering median survival of only 6-17 weeks in untreated patients. The object of this study was to assess the activity of combination chemotherapy using methotrexate, cyclophosphamide and the nitrosourea, CCNU, in patients with small cell lung cancer and to compare these results retrospectively with results observed in a similar patient population treated between 1972 and 1978

#### 10.2b Patients and Methods

##### (1) Patients treated between 1971-1978

This group comprised of 193 patients with small cell lung cancer who were admitted to hospitals in the North Lothian District of the Lothian Health Board. The names of these patients were obtained from the National Cancer Registry. The case records of 43 patients were unobtainable, and in a further 10 the histological diagnosis could not be confirmed, leaving 140 assessable patients. Treatment comprised of radiotherapy alone in 37 patients, radiotherapy and single

agent chemotherapy (cyclophosphamide) in 9, cyclophosphamide alone in 9 and surgical resection in 11 patients. The remaining 75 patients were treated symptomatically.

(ii) Patients treated between 1979-1981

All patients less than 80 years of age with histologically confirmed small cell lung cancer were entered in the study, if their performance status was less than 4 (Eastern Co-operative Oncology Group Scale). Of 108 patients presenting over this time period with small cell lung cancer, 13 were excluded on the grounds of age, performance status or questionable histology. A further 12 patients had no evaluable disease leaving 83 patients who were entered on study. Patients were staged by clinical examination, chest x-rays, barium swallow, diaphragmatic screening, full blood count and assessment of electrolytes, urea, liver function tests and serum calcium. Isotope scans of liver, bone or brain, liver ultrasound or bone marrow aspiration were only performed if there was overt clinical suspicion of metastatic disease involving any of these sites. Results were analysed using the conventional categories of limited and extensive disease, the former referring to disease apparently limited to the lung, mediastinum or ipsilateral supraclavicular nodes. The patients all received the following chemotherapeutic regime:

Methotrexate 200 mg/m<sup>2</sup> given as a 24 hour infusion  
followed by 48 hours folinic acid rescue  
(9 mg/m<sup>2</sup> 6 hourly)

Cyclophosphamide 1 g/m<sup>2</sup> i.v.

Both drugs were given every 3 weeks for 4 courses. In addition, CCNU was administered orally at a dosage of 100 mg/m<sup>2</sup> with course 1 and 50 mg/m<sup>2</sup> with course 3.

At 12 weeks all patients were assessed for response, using standard criteria.

Patients achieving a complete or partial response were then randomised to receive no immediate treatment or to receive 6 months maintenance therapy with vincristine (1.4 mg/m<sup>2</sup> intravenously days 1 and 8) plus procarbazine (100 mg/m<sup>2</sup> orally days 1-14), alternating each month with methotrexate (40 mg/m<sup>2</sup>) and cyclophosphamide (500 mg/m<sup>2</sup>) both given intravenously on days 1 and 8.

#### 10.2c Results

Patient details of both groups are illustrated in Table 10.5. Ninety-six of the patients in the 1971 group were male (69%) and 44 female. Their median age was 65 years (range 39-83).

|                               | 1971-1978          |            | 1979-1981  |
|-------------------------------|--------------------|------------|------------|
|                               | Supportive<br>Care | Treated    | MCC Regime |
| Number                        | 75                 | 65         | 83         |
| Median Age (range)            | 67 (44-86)         | 65 (39-83) | 61 (28-75) |
| Sex M:F                       | 51:24              | 45:20      | 50:33      |
| Limited/<br>extensive disease | 25:50              | 43:22      | 45:38      |

Table 10.5 Characteristics of patients presenting with small cell lung cancer in the North Lothian District, Lothian Health Board, 1971-1981

Disease was apparently confined to the lung in 21%, mediastinal or local invasion of ribs in 27% and widely metastatic in 52%. Patient treated between 1979-1981 comprised of 50 men (60.2%) and 33 women, with a median age of 60 years (range 28-75). Forty-five patients (54%) had limited disease and 38 (46%) extensive disease at presentation. Performance status (P.S.) was assessed in the 1979-1981 patient group only, with 21 having a P.S. of 0, 35 P.S. of 1, 22 P.S. of 2 and 5 a P.S. of 3.

Patients treated with chemotherapy between 1979 and 1981 were assessed for response at 12 weeks from the start of treatment. Fifty-eight per cent of patients had achieved an objective response (partial or complete remission) at this stage). Following bronchoscopy 25% of patients were considered to be in complete remission. Survival of all groups is shown in Figure 10.3. Median survival was 2 months in the patients treated between 1971-1978, actively treated patients surviving a median of 5 months as opposed to 3 weeks for patients receiving supportive care only. Fifteen per cent of the actively treated patients survived 1 year and 2 patients survived more than 3 years (3 years 8 months and 5 years 1 month). Median survival for the 1979-1981 group was 9 months,



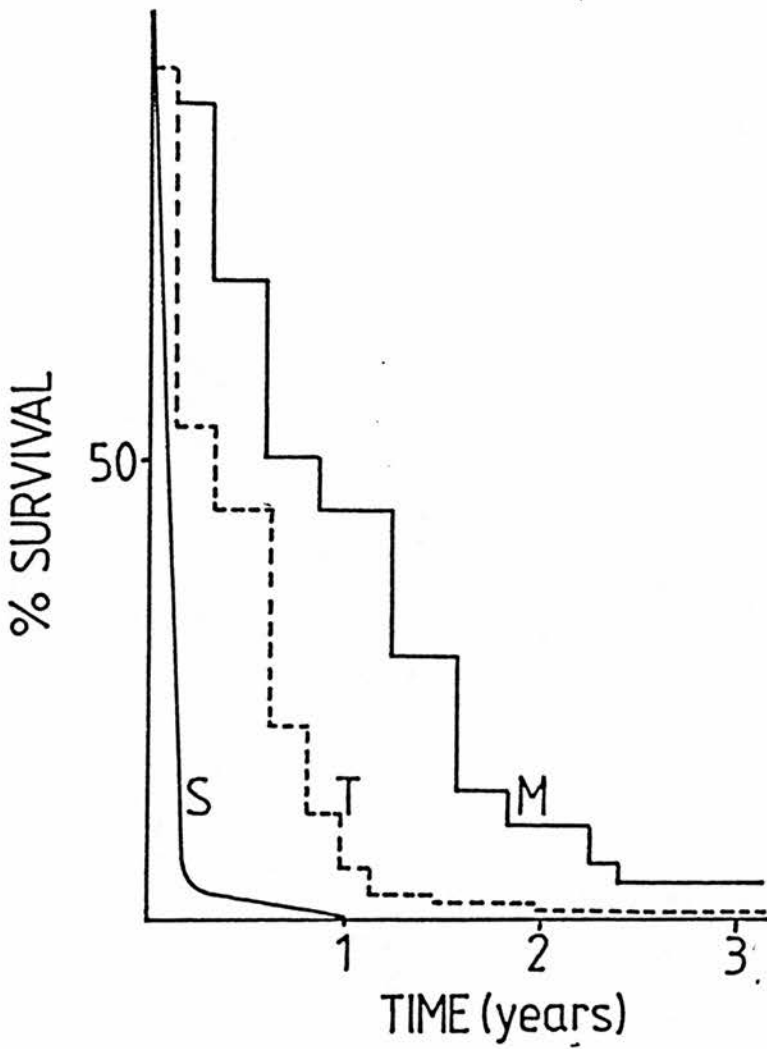


Figure 10.3 Impact of combination chemotherapy on survival of patients with small cell lung cancer.  
 M = patients treated with combination chemotherapy 1979-1981 (n = 93); T = actively treated patients 1971-1978 (n = 65); S = supportive care only 1971-1978 (n = 75).

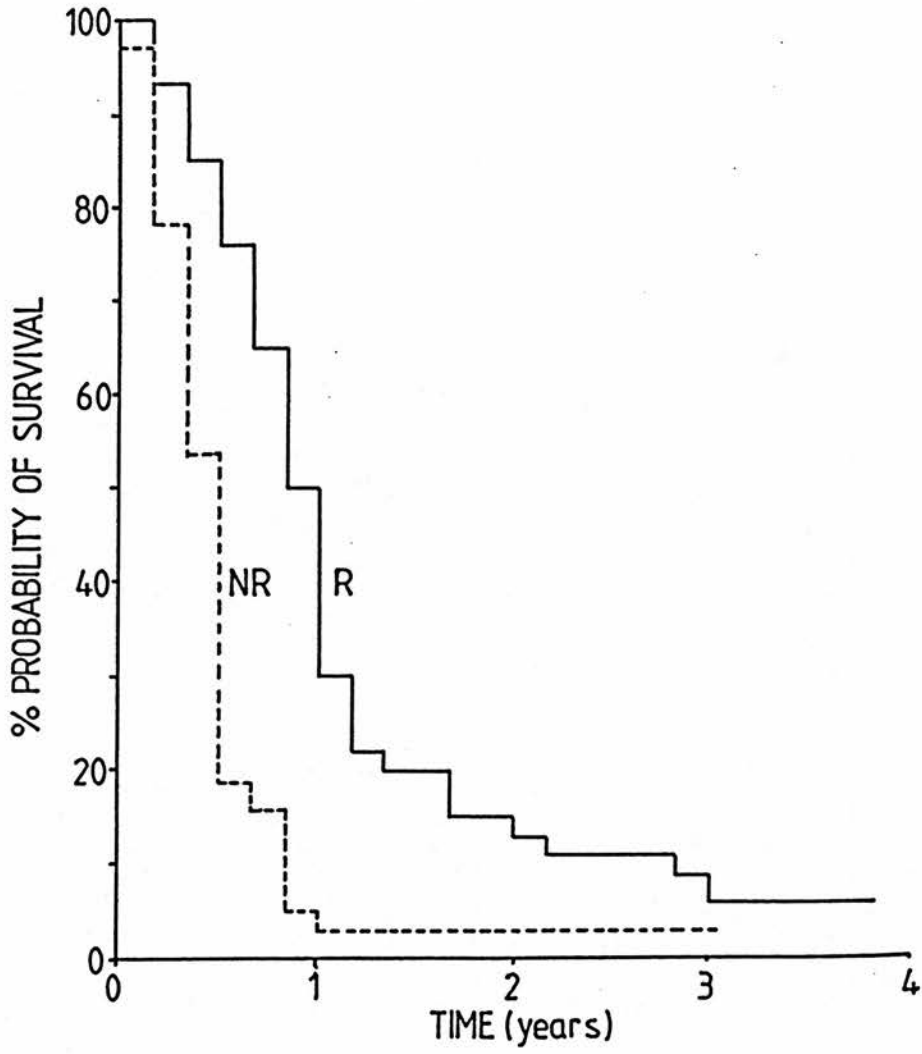


Figure 10.4 Effect of response to chemotherapy on survival of small cell lung cancer patients treated with combination chemotherapy; Edinburgh 1979-1981

with 33% alive at 1 year and 13% at 2 years. Three patients remain alive and in remission at 3 years and 9 months, 4 years, and 4 years 4 months from diagnosis.

Positive factors associated with prolonged survival included performance status at presentation and response to chemotherapy. Figure 10.4 illustrates that responding patients survived longer than patients not achieving a partial response ( $p < 0.001$ ). Of patients achieving a complete response, 26% were alive at 2 years and 14% at 3 years. The influence of performance status is illustrated in Figure 10.5, showing that survival of patients with a performance status of 0-1 was significantly longer than patients with a performance status of 2-3 ( $p < 0.001$ ). The survival of patients receiving further chemotherapy immediately was no different to patients treated on relapse. Therefore, no apparent benefit was derived from the use of maintenance chemotherapy, as illustrated in Figure 10.6. Patients relapsing locally with intrathoracic disease were treated with radiotherapy (4500 cGy in 20 fractions), whereas patients relapsing systemically were considered for alternative chemotherapy. Four patients relapsed in the brain, and were treated with cranial irradiation. Drug toxicity was observed but was considered tolerable by the majority of patients. The majority of patients had alopecia of minor

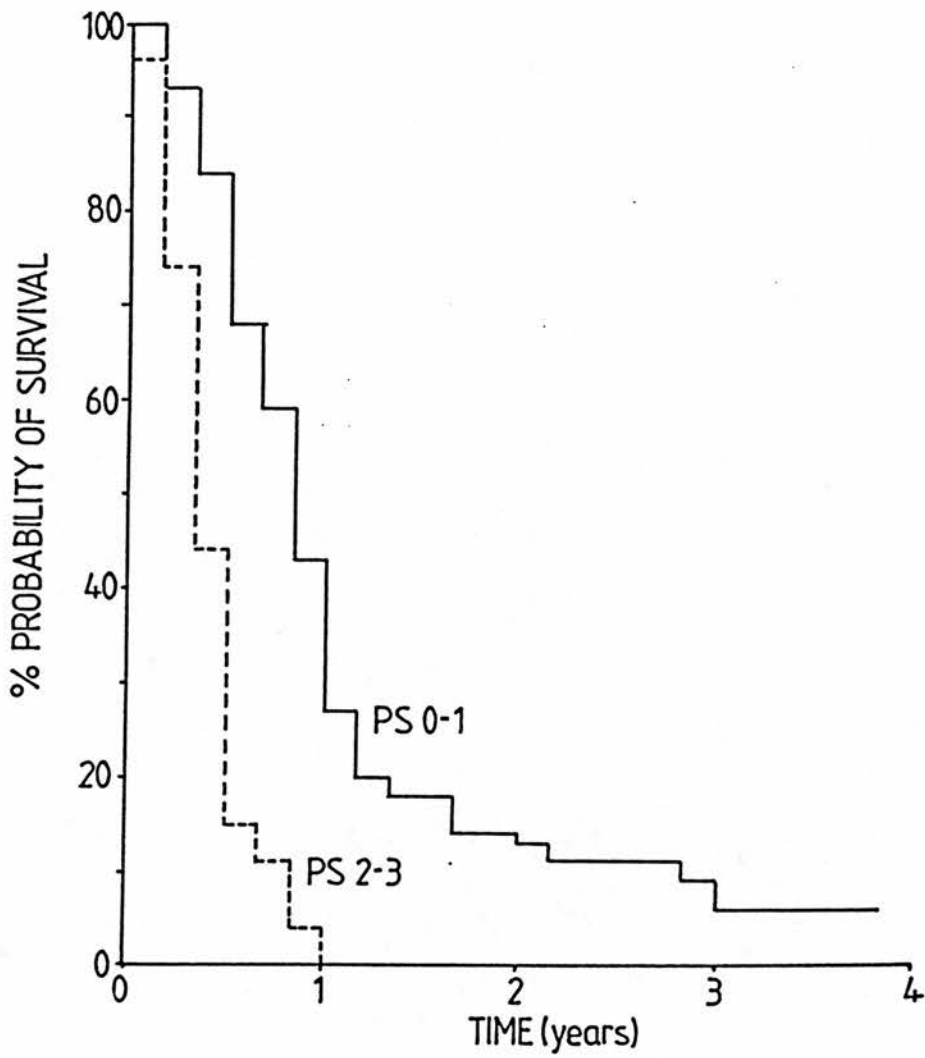


Figure 10.5 Effect of performance status on survival of patients with small cell carcinoma treated with combination chemotherapy

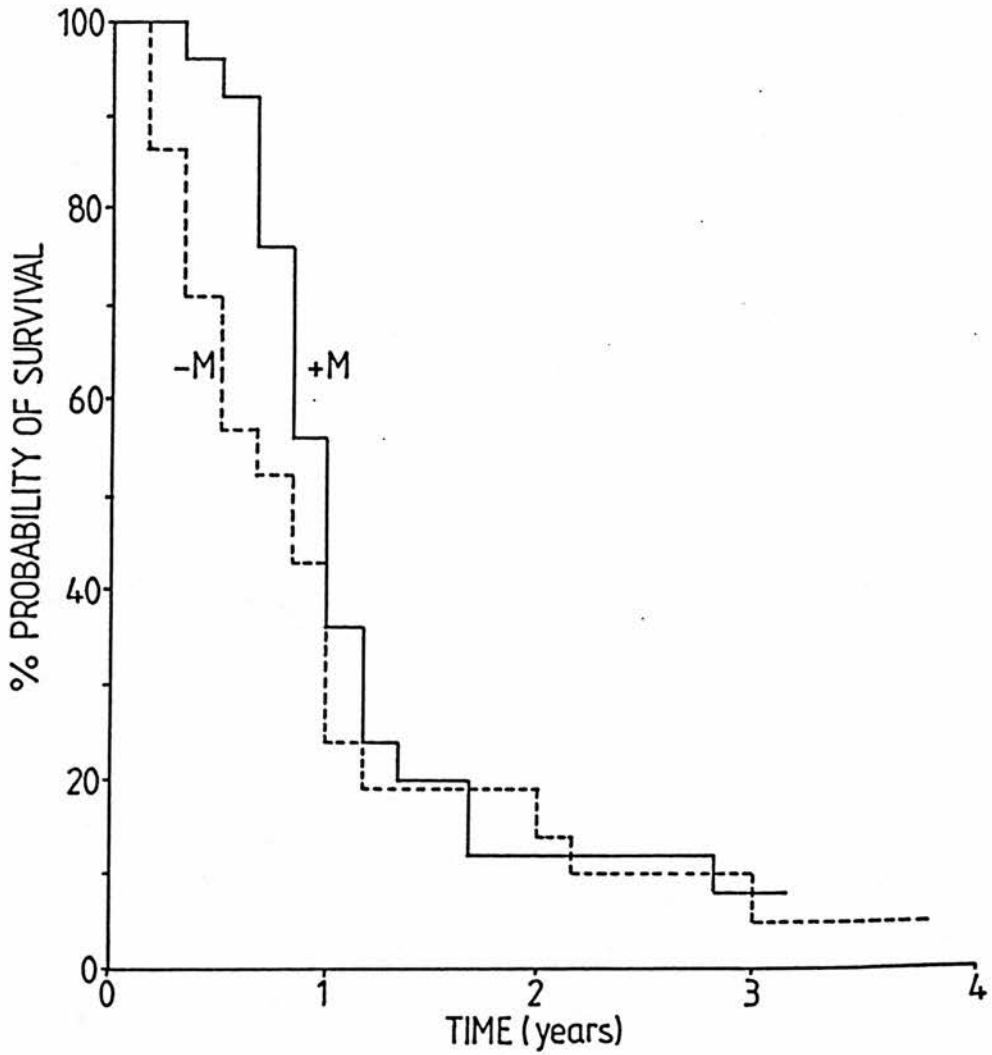


Figure 10.6 Effect of 6 months additional maintenance chemotherapy on survival of patients with small cell lung cancer treated with chemotherapy. M+ = maintenance chemotherapy; M- = observation alone.

degree which was reversible with moderate gastrointestinal toxicity in 29% (WHO grade 2). Eight patients had haematological toxicity requiring active intervention but there were no treatment-related deaths.

#### 10.2d Discussion

Prior to 1979, patients with small cell lung cancer in the North Lothian District had a median survival of only 8 weeks, and even selected patients treated with surgery, radiation or single agent chemotherapy survived a median of only 5 months. The introduction of combination chemotherapy in 1979 increased the median survival of patients to 9 months. Despite the extensive literature on the responsiveness of small cell lung cancer to chemotherapy, no one regime has emerged as standard therapy. The results reported in this study are very similar to many other trials using different drug combinations. Maintenance therapy had no apparent benefit, similar to results published from other studies exploring the same concept (Vincent et al 1980; Daniels et al 1980; Young et al 1981).

Combination chemotherapy has certainly improved prognosis for the majority of patients with small cell lung cancer with long-term survival possible (Johnson et al 1985). However, despite this, the majority of patients relapse and their disease is then frequently resistant to further therapy.

### 10.3 Summary

There remains a wide variation in the response of the different histological categories of lung cancer. Small cell carcinoma remains the most chemosensitive of the cell types, although long-term survival and cure remain remarkable elusive. More recently attempts have been made to increase the cure rate with the use of induction regimes similar to the one used in this study, followed by high dose chemotherapy and autologous bone marrow transplantation. The results of these studies are awaited with interest but even if successful these regimes are likely to be of value to a limited number of patients, and further developments in the therapy of this disease are necessary.

Unresectable non-small cell carcinoma of the lung remains a common and difficult problem. Response rates to chemotherapy are lower, and the duration of these responses are disappointingly short. Combinations of cis-platinum and vindesine

have given the best results with chemotherapy but severe toxicity precludes the widespread use of these combinations. The development of new platinum analogues may reduce the toxicity of these combinations but are unlikely to dramatically improve the response rate in these tumours. New drugs are required and greater understanding of the mechanism of inherent resistance in these tumours may help in the development of a more effective therapeutic approach.



**CHAPTER 11****CHEMOTHERAPY STUDIES IN XENOGRAFTS****11.1 Introduction**

Prior to commencing chemotherapy studies in xenografts, experiments were performed to identify maximal tolerated doses of each individual drug. Drug toxicity was primarily assessed by animal survival experiments although limited studies were carried out to determine degrees of haematological and renal toxicity.

**11.2a Drug Toxicity Studies**

All drugs used in growth delay chemotherapy experiments underwent acute toxicity studies to determine their maximum tolerated dose. Using a minimum of 8 animals per group, survival curves were constructed for each agent. From these survival curves, the LD<sub>10's</sub> (dosage of drug in mice causing 10% lethality at 30 days) were calculated for each drug. The survival curve for cis-platinum is illustrated in Figure 11.1. The maximum tolerated dose of drugs can vary in immunosuppressed animals as is shown with cis-platinum (Figure 11.1), and therefore, wherever possible immune-suppressed or immune-deprived animals were used. A dosage of drug slightly less

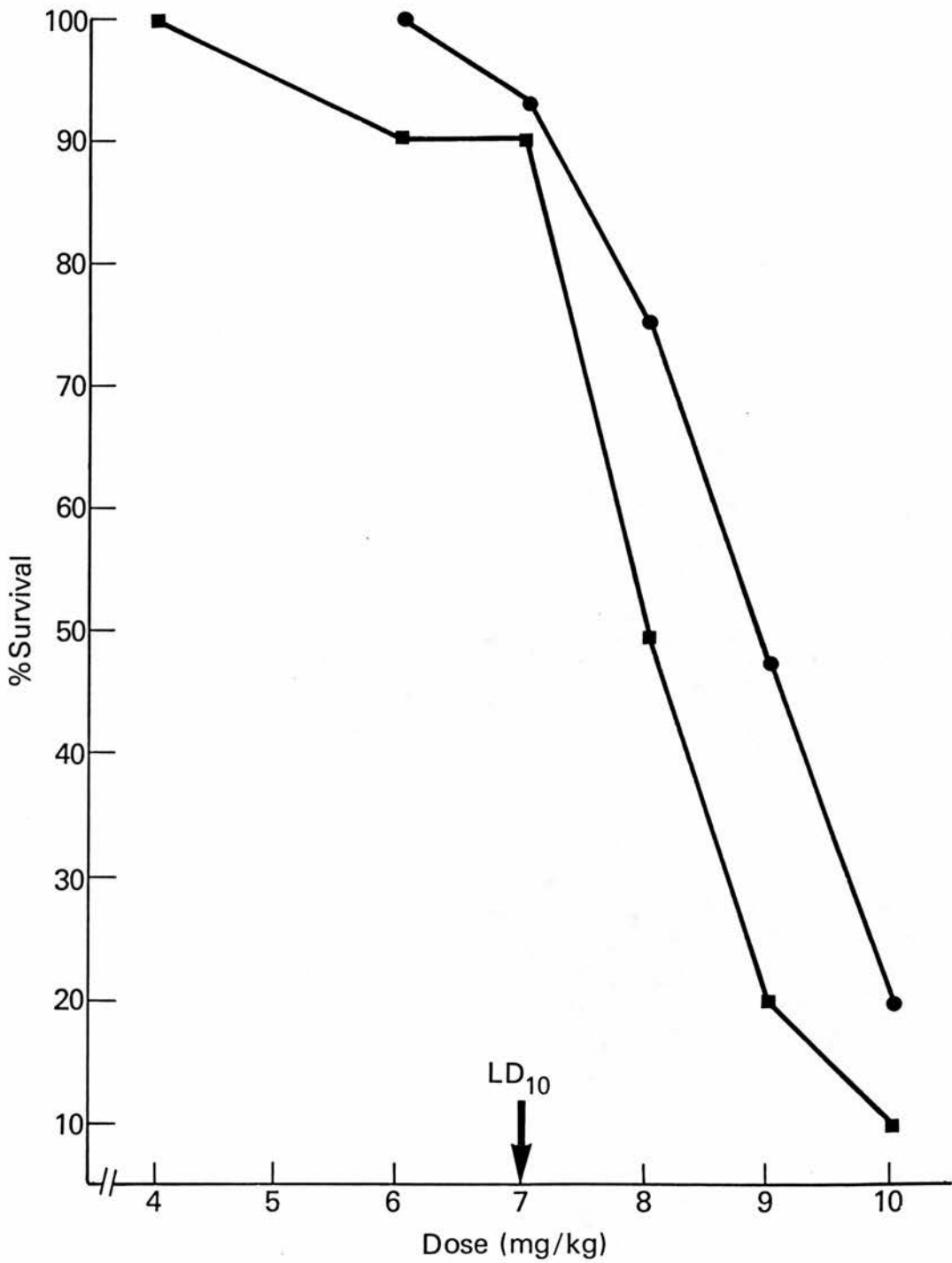


Fig. 11.1 Determination of LD<sub>10</sub> of cis-platinum in CBA mice:  
●—● normal CBA ■—■ immunosuppressed.

than the LD<sub>10</sub> was used as the maximum tolerated dose with the survival data for the drugs used in chemotherapy experiments shown in Table 11.1. The maximum tolerated doses of drugs used in these experiments were lower than those used by other investigators as illustrated in Table 11.2. The reduction in maximum tolerated doses of these drugs was not entirely related to the immune status of these animals. Stock CBA mice, although able to tolerate higher drug doses than immune-deprived animals were only able to take approximately 70% of the dose tolerated by similar strains of mice as reported by other laboratories.

#### 11.2b Bone Marrow Toxicity

All agents used in chemotherapy studies were administered at the maximum tolerated dose to groups of 10 animals, and white cell counts were measured using a tail vein bleeding technique. 20  $\mu$ l of whole blood was collected by capillary attraction and made up to 10 ml with isotonic saline. Red cells were lysed using Zaponin (Coulter Products Ltd) and white cell counts measured using a coulter counter. Each measurement was the mean of the values from 3 animals and was estimated as a percentage of control, and the results are illustrated in Table 11.3.

| Drug                     | Dose (mg/kg) | No. of mice | Survival % |
|--------------------------|--------------|-------------|------------|
| Cis-platinum             | 4            | 8           | 100        |
|                          | 6            | 16          | 94         |
|                          | 7            | 30          | 93         |
|                          | 8            | 30          | 70         |
| Vindesine                | 0.5          | 10          | 100        |
|                          | 1            | 10          | 90         |
|                          | 1.5          | 10          | 90         |
|                          | 2            | 20          | 90         |
|                          | 2.5          | 10          | 70         |
|                          | 3            | 10          | 40         |
| Etoposide<br>(VP-16 x 1) | 30           | 10          | 10         |
|                          | 40           | 10          | 70         |
|                          | 50           | 10          | 60         |
| Etoposide (x 3)          | 15           | 8           | 100        |
|                          | 20           | 8           | 75         |
|                          | 25           | 8           | 62.5       |
| JM40                     | 30           | 10          | 100        |
|                          | 40           | 10          | 90         |
|                          | 50           | 10          | 70         |
| JM8                      | 50           | 8           | 100        |
|                          | 70           | 8           | 100        |
|                          | 80           | 8           | 75         |
|                          | 100          | 8           | 62.5       |
| Cyclophosphamide         | 150          | 10          | 100        |
|                          | 200          | 10          | 80         |
|                          | 250          | 8           | 0          |
|                          | 300          | 10          | 10         |
| Vincristine x 2          | 0.6          | 8           | 100        |
|                          | 0.7          | 8           | 62.5       |
|                          | 0.8          | 8           | 50         |

Table 11.1 30 day survival of CBA mice treated with varying doses of cytotoxic drugs intraperitoneally.

Maximum Tolerated Doses of Cytotoxic Drugs (mg/kg)

|                  | <u>Previous studies</u> | <u>Western General<br/>Hospital</u> |
|------------------|-------------------------|-------------------------------------|
| Cis-platinum     | 10 <sup>a</sup>         | 7                                   |
| Carboplatin      | 160 <sup>a</sup>        | 75                                  |
| JM40             | -                       | 40                                  |
| Vindesine        | 3 <sup>b</sup>          | 2                                   |
| Vincristine      | 0.8 x 2 <sup>c</sup>    | 0.6 mg x 2                          |
| Cyclophosphamide | 200 <sup>c</sup>        | 150                                 |
| Ifosfamide       | 233 x 3 <sup>c</sup>    | 300 x 1                             |
| VP-16            | 20 x 3 <sup>c</sup>     | 15 x 3                              |

<sup>a</sup>Steel G.G. et al. Br J Cancer (1983) 47: 1-13

<sup>b</sup>Evans B.D. et al. Br J Cancer (1982) 45: 466-468

<sup>c</sup>Shorthouse A.J.. ChM Thesis, University of London 1981

Table 11.2 Maximum tolerated doses of drugs as measured in CBA mice in 2 different laboratories.

| Drug  | Time After Injection (days) |    |     |     |     |     |
|---|-----------------------------|----|-----|-----|-----|-----|
|   | 2                           | 4  | 6   | 8   | 10  | 14  |
| Cis-platinum                                    | 83                          | 78 | 78  | 118 | 78  | 102 |
| Vindesine                                       | 85                          | 70 | 81  | 101 | 97  | 123 |
| Cis-platinum + vindesine<br>(100% of each drug) | 83                          | 36 | -   | -   | -   | -   |
| Cis-platinum + vindesine<br>(75% of each drug)  | 87                          | 42 | 61  | 102 | 176 | 129 |
| JM40  | 64                          | 45 | 92  | 78  | 130 | 124 |
| Cyclophosphamide                                | 51                          | 26 | 70  | 86  | 133 | 94  |
| Ifosfamide                                      | 64                          | 36 | 48  | 87  | 135 | 124 |
| Ifosfamide + mesna                              | 77                          | 33 | 49  | 99  | 142 | 96  |
| Etoposide (35 mg/kg)                            | 88                          | 32 | 115 | 154 | 168 | 119 |
| Etoposide (15 mg/kg x 3)                        | 91                          | 41 | 43  | 107 | 192 | 113 |

Table 11.3 White cell counts expressed as a percentage of control, as measured in CBA mice following maximum tolerated doses of various drugs.

Cis-platinum and vindesine had minimal bone marrow toxicity although enhanced effect was noted with the drug combination. Complete results are not available for the full dose cis-platinum and vindesine combination following which all animals had died by day 6. The platinum analogue JM40 was more myelotoxic, as was etoposide (VP16). The alkylating agents cyclophosphamide and ifosfamide were also myelotoxic, although ifosfamide was more myelotoxic than expected, with urotoxicity generally considered dose-limiting.

#### 11.2c Renal Toxicity

One of the main problems arising from cis-platinum administration is renal toxicity. Blood urea estimations were therefore carried out on groups of 4 mice on alternate days following the administration of cis-platinum 7 mg/kg, or the platinum analogue JM40 at a dosage of 40 mg/kg. Animals were killed by ether anaesthetics and blood obtained from the inferior vena cava, with two samples pooled together. The samples were spun at 450 g plasma removed, and specimens assayed in duplicate. The assays were kindly performed by Dr D Horn (Department of Clinical Chemistry, Western General Hospital). The results are shown in Table 11.4. As the LD<sub>10</sub> of cis-platinum was slightly lower in immune-deprived mice, the possibility that irradiation may have affected renal function

|  | Time (days) |      |      |       |       |       |       |
|--|-------------|------|------|-------|-------|-------|-------|
|  | 0           | 2    | 4    | 6     | 8     | 10    | 14    |
| Cis-platinum<br>(7 mg/kg)                      | 9.9         | 8.45 | 17.9 | 9.0   | 18.65 | 11.65 | 10.15 |
| JM40<br>(40 mg/kg)                             | 9.65        | 8.9  | 10.1 | 10.35 | 11.15 | 11.13 | 10.1  |
| Vindesine 2 mg/kg<br>+<br>Cis-platinum 7 mg/kg | 8.6         | 6.4  | 20.0 | -     | -     | -     | -     |

Table 11.4 Effect of maximum tolerated doses of platinum compounds on blood urea measurements in CBA mice.



was considered. However, no difference was noted histologically in any of the animals treated with cis-platinum with or without total body irradiation. Likewise, a few untreated irradiated mice were sacrificed and blood urea estimations performed. In none of these mice was the blood urea found to be elevated.

### 11.3a Chemotherapeutic Response

Of the 9 xenograft lines available for experimental work, 6 were chosen for chemotherapy studies as they were relatively non-necrotic and had good implant take rates. All 6 were non-small cell carcinoma xenograft lines comprising of 1 adenosquamous, 1 adenocarcinoma and 4 squamous carcinoma lines. In addition, limited studies were carried out on the 1 small cell carcinoma xenograft (WX 310). Of the 6 non-small cell xenografts 5 were derived from patients who underwent thoracotomy at the City Hospital, Edinburgh. The other xenograft was obtained from a patient investigated at the Northern General Hospital. This patient later underwent "curative" resection at the City Hospital and remains disease-free 2 years later. No direct comparison of patient and xenograft response was possible as these patients did not receive chemotherapy.

| Xenograft           | Cell Type                 | <u>Treatment</u> |              |             |
|---------------------|---------------------------|------------------|--------------|-------------|
|                     |                           | Vindesine        | Cis-platinum | Combination |
| Median Growth Delay |                           |                  |              |             |
| NX 002              | squamous                  | <0.2             | 2.0          | 1.9         |
| CX 109              | squamous                  | 0.31             | 0.73         | 1.00        |
| CX 133              | squamous                  | 0.26             | 1.23         | 0.61        |
| CX 140              | squamous                  | <0.2             | 1.45         | 0.77        |
| CX 143              | adenosquamous             | 0.21             | 0.29         | <0.2        |
| CX 117              | adenocarcinoma            | <0.2             | 0.87         | 0.74        |
|                     | growth delay<br>(average) | <u>0.2</u>       | <u>1.09</u>  | <u>0.87</u> |

Table 11.5 Chemotherapeutic response of 6 human non-small cell lung cancer xenografts to maximum tolerated doses of vindesine, cis-platinum and a combination of these drugs (75% of MTD of each drug).

| Xenograft           | Cell Type                 | <u>Treatment</u> |             |      |
|---------------------|---------------------------|------------------|-------------|------|
|                     |                           | Vindesine        | Carboplatin | JM4  |
| Median Growth Delay |                           |                  |             |      |
| NX 002              | squamous                  | 2.0              | 0.90        | 0.33 |
| CX 109              | squamous                  | 0.73             | 0.73        | 0.36 |
| CX 133              | squamous                  | 1.23             | 1.16        | 0.71 |
| CX 140              | squamous                  | 1.45             | -           | 1.33 |
| CX 143              | adenosquamous             | 0.29             | 0.48        | 0.48 |
| CX 117              | adenocarcinoma            | 0.87             | 0.95        | 0.76 |
|                     | growth delay<br>(average) | 1.09             | 0.84        | 0.66 |

Table 11.6 Chemotherapeutic response of 6 human non-small cell lung cancer xenografts to maximum tolerated doses of cis-platinum and 2 platinum analogues.

| Xenograft           | Cell Type                 | <u>Treatment</u> |            |           |
|---------------------|---------------------------|------------------|------------|-----------|
|                     |                           | Cyclophosphamide | Ifosfamide | Etoposide |
| Median Growth Delay |                           |                  |            |           |
| NX 002              | squamous                  | 0.36             | -          | 0.2       |
| CX 133              | squamous                  | 0.41             | 0.47       | <0.2      |
| CX 140              | squamous                  | -                | 0.58       | <0.2      |
| CX 143              | mixed                     | 0.63             | 1.38       | 0.38      |
| CX 117              | adenocarcinoma            | 0.52             | 1.40       | 0.91      |
|                     | growth delay<br>(average) | 0.48             | 0.96       | 0.36      |

Table 11.7 Chemotherapeutic response of 5 human non-small cell lung cancer xenografts to maximum tolerated doses of cyclophosphamide, ifosfamide and etoposide.

The results are presented in groups according to the drug treatment, and are illustrated in Tables 11.5, 11.6 and 11.7. The response of the xenografts to cis-platinum, vindesine and a combination of the 2 drugs was of interest in view of the clinical trial in non-small cell lung cancer described in Chapter 10. In view of the severe toxicities often associated with cis-platinum therapy, an assessment was made of the activity of the platinum analogues carboplatin and JM40 which are generally considered less toxic. Other agents were tested to confirm the validity of this model as an indicator of chemosensitivity and also in view of their potential use in future chemotherapeutic schedules.

#### 11.3b Xenograft Response to Cis-platinum

The results are listed in Table 11.5. The sensitivity of these xenografts varied with median growth delays ranging from 0.73 to 2.0 doubling times in squamous carcinoma lines with NX 002, a moderately differentiated squamous carcinoma, the most sensitive. The adenosquamous carcinoma (CX 143) responded poorly with a growth delay of only 0.29, with the adenocarcinoma (CX 117) giving a growth delay of 0.87 doubling times. This gave an average growth delay of 1.09 doubling times in these xenograft lines.

### 11.3c Xenograft Response to Vindesine

The results are listed in Table 11.5. Vindesine showed minimal activity in these xenografts, with the best response, a growth delay of 0.31 doubling times, obtained in CX 109, a moderately differentiated squamous carcinoma xenograft..

### 11.3d Xenograft Response to Vindesine and Cis-platinum

#### Combination

The results are shown in Table 11.5. Both drugs were administered at 75% of their individual maximum tolerated doses. Lethality was variable with maximum tolerated doses of both drugs in combination giving a mortality of approximately 40%. Seventy-five per cent of the maximum tolerated doses of both drugs given in combination gave variable lethality, on average 10%. At these doses the combination showed similar activity to cis-platinum as a single agent being less effective against the CX 140 xenograft but more active against CX 109. This combination was ineffective against the adenosquamous line CX 143, although some activity was detected against the adenocarcinoma xenograft CX 117.

### 11.3e Platinum Analogues

Chemosensitivity studies were performed using both carboplatin and JM40. The growth delay results were compared with the activity exhibited by cis-platinum, with the data illustrated in Table 11.6. Cis-platinum tended to be more active than carboplatin with this drug marginally more active than JM40. However, differences were not statistically significant (Kruskal-Wallis, One Way Analysis of Variance).

### 11.3f Miscellaneous Agents

Cyclophosphamide was used against 4 tumour lines, two squamous, 1 adenosquamous and 1 adenocarcinoma, giving an average growth delay of 0.48 doubling times. Ifosfamide, an alkylating agent of similar structure but with a different spectrum of activity and toxicity, was more effective than cyclophosphamide giving an average growth delay of 0.96 tumour doubling times in 4 tumours. Ifosfamide was particularly active in two of the tumours tested, the adenosquamous carcinoma xenograft CX143 and the adenocarcinoma xenograft CX117. Etoposide (VP16) gave disappointing results with an average growth delay of only 0.36 doubling times in 5 xenograft lines. The results are illustrated in Table 11.7.

| Single Agents                                    | Median Growth Delay |
|--|---------------------|
| Cis-platinum                                     | 2.0                 |
| Carboplatin                                      | 0.9                 |
| Cyclophosphamide                                 | 0.36                |
| JM40   | 0.33                |
| Etoposide  | 0.2                 |
| Vindesine  | <0.2                |
| Combination chemotherapy (75% MTD of each agent) |                     |
| Cis-platinum/vindesine                           | 1.9                 |
| Cis-platinum/VP-16                               | 0.75                |
| Carboplatin/vindesine                            | 1.65                |

Table 11.8 Chemotherapeutic response of the human squamous lung cancer xenograft, NX 002, to maximum tolerated doses of a number of single agents, and a limited number of drug combinations. Drugs were administered at 75% of their maximum tolerated dose in all combination chemotherapy regimes.



### 11.3g Combination Chemotherapy

Using the moderately differentiated squamous carcinoma xenograft NX 002, three drug combinations were tested and the results are illustrated in Table 11.8. Individual drugs in the combination chemotherapy regimes were administered at 75% of their maximal tolerated dose. Cis-platinum was combined with etoposide giving a disappointing overall result, showing a growth delay of only 0.75 tumour doubling times using 8 tumours per group. The combination of carboplatin and vindesine gave a growth delay of 1.65 doubling times in the same tumour. Cis-platinum and vindesine was marginally the most effective combination giving a growth delay of 1.9 doubling times. However this was no more effective than cis-platinum as a single agent.

### 11.3h Small Cell Carcinoma

One small cell xenograft, WX 310, was serially transplantable. It was tested in one chemotherapy experiment using cyclophosphamide 150 mg/kg with 10 tumours in each treatment group. A growth delay of 1.25 doubling times was observed with cyclophosphamide at this dosage which is less than may have been expected. Shorthouse (1981) reported growth delays of greater than 3 doubling times for this drug used as a single agent against small cell carcinoma xenografts. However, the donor patient had previously been treated with methotrexate, cyclophosphamide and CCNU. Originally the patient had achieved a complete response but on relapse re-treatment was much less

successful using the same drugs. As the xenograft line was established following the failure of re-induction chemotherapy this may explain the relative resistance of this xenograft to cyclophosphamide. No further chemotherapy studies were performed with this xenograft line and as this was the only small cell xenograft available it was decided to concentrate on the non-small cell xenografts.

#### 11.4 Discussion

The estimation of maximum tolerated doses for each drug were somewhat imprecise in view of relatively small animal numbers used in these experiments. However, it is important to strike a balance between accuracy of this parameter and the unnecessary killing of animals. The likelihood of wide variation from the true maximum tolerated doses are unlikely using these numbers. Intra-peritoneal injections of vindesine and vincristine, which can cause a sclerotic reaction at the injection site, occasionally resulted in random deaths even at low doses, making estimations of maximum tolerated dose somewhat imprecise. When drugs were administered in a divided dose schedule, higher cumulative drug doses were tolerated by the mice than with single injections. Potentially, there are a number of possible explanations for the increased drug tolerance such as changes in the distribution of cells in cell cycle and alterations in drug metabolising enzyme activity. These factors will be dealt with in a later section dealing with the protective effect of 'priming' doses of drugs (Chapter 13).

However, the lethality of a drug can be related to many other factors, such as the solvent used for the drug. For example, the formulation of etoposide includes polysorbate 80, a surface active agent which has been associated with allergic reactions (O'Dwyer and Weiss, 1984). Some mice died within 24 hours of receiving etoposide although it remains pure conjecture whether an allergic reaction was involved in these deaths.

Myelotoxicity, as measured by peripheral white cell counts, was minimal with single doses of cis-platinum and vindesine, but more severe with the alkylating agents and etoposide. Renal toxicity, as illustrated by the blood urea, was greater in cis-platinum treated animals than in JM40 or carboplatin treated, and was also severe in animals treated with both cis-platinum and vindesine. However, from these studies the aetiology of the increased lethality from the combination remains in doubt.

The platinum analogues are interesting compounds, particularly carboplatin, which has shown significant clinical activity in a number of studies (Alberts et al, 1985; Muggia et al, 1985; Smith and Evans 1985), with minimal renal toxicity and greatly reduced gastrointestinal symptoms. This compound and another platinum analogue JM9 (CHIP) have been shown to be the most active of the platinum compounds, so far evaluated, and it seems likely that one of these two drugs will be used widely in clinical practice in the near future.

Clinical response data shows ifosfamide (Morgan et al 1981) and cis-platinum (De Jager et al 1980) having the greatest single agent activity. Clinical response to vindesine has been variable, with Gralla et al (1979) reporting a 21% response rate but this was not confirmed by either Mattson et al (1980) with a 10% response rate or Elliot et al (1984) reporting a 0% response rate, with the xenograft response to vindesine correlating better with the latter two studies. Cyclophosphamide had minimal activity in xenografts, as predicted, with etoposide likewise giving disappointing results. Cis-platinum, carboplatin and ifosfamide were the most effective agents in the xenografts tested. Cis-platinum had variable activity in xenografts similar to that observed clinically.

The results of combination chemotherapy were disappointing in xenograft experiments with the cis-platinum-vindesine combination no more active than cis-platinum alone, although it must be appreciated that with the combination only 75% of the maximum tolerated doses of both drugs were given. Cis-platinum with etoposide was less active than the cis-platinum-vindesine combination using the moderately differentiated squamous carcinoma xenograft NX 002. In the same tumour, carboplatin with vindesine showed similar activity to the cis-platinum and vindesine combination, although again was no more effective than cis-platinum alone.

It is appreciated that the use of maximum tolerated doses of drugs to compare their activity, in this setting, is an imprecise method of assessing relative chemosensitivity. However, it is likewise inappropriate to administer the agents in doses proportionate to those used clinically, as the handling of cytotoxic drugs can vary widely between species. Non-small cell lung cancer xenografts therefore exhibit a similar chemotherapeutic profile to that observed clinically. As may have been expected, no dramatic responses were seen although variation in response between xenograft lines of similar histology was observed. Exquisite drug sensitivity has been observed in small cell lung cancer xenografts (Shorthouse 1981) but responses of this magnitude were not seen in these non-small cell lung cancer xenografts.

There is a need for active, new agents, particularly for the treatment of non-small cell lung cancer, although variations in scheduling techniques and different combinations of the presently available drugs may prove beneficial. The human xenograft model offers a unique opportunity to investigate both the activity of new chemotherapeutic agents, and the effect of manipulations of scheduling techniques on anti-tumour activity.

## CHAPTER 12

### INTERFERON AND CYTOTOXIC DRUG COMBINATIONS

#### 12.1 Introduction

As stated in Chapter 5, interest has grown recently in the potential use of human interferons in cancer therapy. Balkwill et al (1984) have previously shown potentiation of cyclophosphamide in breast carcinoma xenografts, with other studies also showing potentiation of cytotoxic drug activity in combination with interferon (Aapro et al 1983; Inoue and Tan 1983; Welander et al 1985).

In view of the poor response of non-small cell lung cancer to chemotherapy, both in vivo and in vitro, it was decided to investigate the effects of combinations of cytotoxic drugs with human interferon, using the human xenograft model.

#### 12.2 Materials and Methods

##### 12.2a Interferon

Human lymphoblastoid interferon ( $\alpha$ 2-Namawla) was obtained as previously described (Fantes et al 1980). It was prepared and kindly provided by Dr F R Balkwill (Imperial Cancer

Research Fund, London). It was tested for activity and aliquoted as previously described (Balkwill et al 1983). This interferon was shown not to stimulate natural-killer cell activity in nude mice, this work carried out by Dr Balkwill. It had a specific activity of  $1-2 \times 10^8$  U/mg and was essentially pure. Animals were injected subcutaneously at the base of the neck with  $2 \times 10^5$  U of interferon daily with control animals receiving subcutaneous saline. This interferon dose is roughly equivalent to  $20 \times 10^6$  U/m<sup>2</sup> daily in man.

#### 12.2b Cytotoxic drugs

Two drugs were used in combination with interferon. Cyclophosphamide (Farmitalia Carlo Erba Ltd, St Albans, UK) was chosen in view of its previously reported potentiation by interferon (Balkwill et al 1984). Cis-platinum (Bristol-Myers Ltd, Slough, UK) was used as it was the most active single agent in these xenografts, and because of previously reported potentiation by  $\beta$ -interferon (Inoue and Tan 1983). Drugs were administered intraperitoneally at 20% of their maximum tolerated dose with a maximum of 5 weekly injections being given. Total cytotoxic drug dosage in these animals was roughly equal to the maximum tolerated dose i.e. cis-platinum 7 mg/kg and cyclophosphamide 200 mg/kg.

### 12.2c Study Design

Three lung xenograft lines were studied: NX 002, a moderately differentiated squamous carcinoma, CX 117, an adenocarcinoma, and CX 143, an adenosquamous carcinoma. All animals were irradiated on the same day and transplanted with tumour the following day. When sufficient numbers of tumours were greater than  $0.3 \text{ cm}^3$ , mice were stratified according to tumour size then randomly allocated to one of 4 treatment groups; cytotoxic drug alone, interferon alone, cytotoxic drug plus interferon in combination, or a control group.

Interferon was administered subcutaneously daily for 35 days, with cytotoxic drugs given intraperitoneally weekly for 5 weeks. Tumour volume was estimated from thrice weekly measurements performed with calipers.

### 12.3 Results

Experimental results from each of the xenograft lines are illustrated in Tables 12.1; 12.2 and 12.3.

#### 12.3a NX 002 - moderately differentiated squamous carcinoma

The results are shown in Table 12.1. The median control tumour doubling time ( $T_D$ ) was 16 days, with a mean tumour volume increase of 436.9% in control mice over the 35 day



experiment. Interferon alone and cyclophosphamide were minimally active with  $T_D$ 's of 21.5 days, a specific growth delay of 0.34 doubling times. Cis-platinum treatment resulted in an increased median tumour doubling time of 35 days; a growth delay of 1.18 doubling times. The combination of cyclophosphamide and interferon caused a minimal increase in  $T_D$  to 32 days; specific growth delay of 1.0 doubling times. The cis-platinum and interferon combination was the most active, resulting in an increase of median tumour doubling time to 52 days, a growth delay of 2.25 doubling times. Using the Kruskal-Wallis one way analysis of variance this treatment was statistically significantly superior to other treatments ( $p < 0.05$ ). Using the mean increase in tumour volume at 35 days as the parameter of response, the combination of cis-platinum and interferon was significantly more active than other treatment groups ( $p < 0.005$ , Kruskal-Wallis one-way analysis of variance). The tumour volume doubling time was increased, with less of an increase in mean tumour volume at 35 days in the cyclophosphamide plus interferon group. However, these differences did not reach statistical significance.

Table 12.1a

| Treatment                     | No. tumours | T <sub>D</sub> (median) days | Specific growth delay | 35 day tumour volume (mean) % control |
|-------------------------------|-------------|------------------------------|-----------------------|---------------------------------------|
| Control                       | 10          | 16                           | -                     | 436.9                                 |
| Interferon                    | 6           | 21.5                         | 0.34                  | 284.4                                 |
| Cis-platinum<br>1.4 mg/kg x 5 | 6           | 35                           | 1.18                  | 169.1                                 |
| IFN + cis-platinum            | 8           | 52                           | 2.25<br>(p<0.05)      | 144.1<br>(p<0.005)                    |
| Cis-platinum<br>7 mg/kg       | 8           |                              | 2.0                   |                                       |

Table 12.1b

| Treatment                        | No. tumours | T <sub>D</sub> (median) days | Specific growth delay | 35 day tumour volume (mean) % control |
|----------------------------------|-------------|------------------------------|-----------------------|---------------------------------------|
| Control                          | 10          | 16                           | -                     | 436.9                                 |
| Interferon                       | 6           | 21.5                         | 0.34                  | 284.4                                 |
| Cyclophosphamide<br>40 mg/kg x 5 | 8           | 21.5                         | 0.34                  | 335.75                                |
| IFN +<br>cyclophosphamide        | 6           | 32                           | 1.0 (NS)              | 220.2 (NS)                            |
| Cyclophosphamide<br>150 mg/kg    | 8           |                              | 0.36                  |                                       |

Tables 12.1a and 12.1b Effect of human alpha interferon and cytotoxic drugs administered singly and in combination in the treatment of the human lung cancer xenograft NX002.

### 12.3b CX 143 - Adenosquamous Carcinoma

The results using this xenograft line are shown in Table 12.2. Median control  $T_D$  was 16 days, with interferon alone resulting in minimal growth delay ( $T_D = 23$  days, specific growth delay 0.44). Cyclophosphamide and cis-platinum were marginally more active giving specific growth delays of 0.68 and 0.81 doubling times respectively. Interferon combinations were the most active, with the cis-platinum combination superior. A median  $T_D$  of 41 days was achieved with this combination compared with 35 days in the cyclophosphamide and interferon group with specific growth delays of 1.56 and 1.18 doubling times respectively. The cis-platinum and interferon combination was significantly superior to control or either agent alone using both specific growth delay ( $p < 0.05$ ), and 35 day tumour volume increase ( $p < 0.05$ ) as parameters of response (Kruskal-Wallis one-way analysis of variance).

### 12.3c CX 117 - Adenocarcinoma

The results are illustrated in Table 12.3. CX 117 was the most rapidly dividing tumour in this study, with a median control  $T_D$  of 13 days. Cyclophosphamide treatment resulted in an increased median  $T_D$  to 19.5 days (specific growth delay 0.5 doubling times) while cis-platinum treatment caused an increase

Table 12.2a

| Treatment                     | No. tumours | T <sub>D</sub> (median) days | Specific growth delay | 35 day tumour volume (mean) % control |
|-------------------------------|-------------|------------------------------|-----------------------|---------------------------------------|
| Control                       | 8           | 16                           | -                     | 425.9                                 |
| Interferon                    | 6           | 23                           | 0.44                  | 227.0                                 |
| Cis-platinum<br>1.4 mg/kg x 5 | 6           | 29                           | 0.81                  | 325.4                                 |
| IFN + cis-platinum            | 6           | 41                           | 1.56<br>(p<0.05)      | 134.5<br>(p<0.05)                     |
| Cis-platinum<br>7 mg/kg       | 8           |                              | 0.29                  |                                       |

Table 12.2b

| Treatment                        | No. tumours | T <sub>D</sub> (median) days | Specific growth delay | 35 day tumour volume (mean) % control |
|----------------------------------|-------------|------------------------------|-----------------------|---------------------------------------|
| Control                          | 8           | 16                           | -                     | 425.9                                 |
| Interferon                       | 6           | 23                           | 0.44                  | 227.0                                 |
| Cyclophosphamide<br>40 mg/kg x 5 | 8           | 27                           | 0.68                  | 325.3                                 |
| IFN +<br>cyclophosphamide        | 6           | 35                           | 1.35 NS               | 143.0<br>(p<0.02)                     |
| Cyclophosphamide<br>150 mg/kg    | 8           |                              | 0.63                  |                                       |

Table 12.2a & 12.2b Effect of human  $\alpha$  interferon and cytotoxic drugs administered singly and in combination in the treatment of the human lung cancer xenograft CX143.

in  $T_D$  to 22 days (specific growth delay 0.69 doubling times). Interferon alone had activity with a  $T_D$  of 23 days (specific growth delay 0.77 doubling times). The cyclophosphamide and interferon combination was marginally more active than single agent therapy, with a  $T_D$  of 27 days (specific growth delay 1.07 doubling times), but these were not statistically significant. The cis-platinum-interferon combination was by far the most active, causing an increase in  $T_D$  to 51 days; a specific growth delay of 2.92 doubling times ( $p < 0.01$ ). Similarly, tumour volume increase at 35 days was limited to 136.8% in this group, by far the most active of the groups ( $p < 0.01$ , Kruskal-Wallis one-way analysis of variance).

#### 12.3d Toxicity

No significant difference was noted in the white cell counts in any of the groups compared to control, using blood obtained from the tail veins of 3 animals in each group at day 35. Significant weight loss was not observed in any of the animals, with no mice losing more than 10% of body weight. However, control mice did gain more weight than the other groups, with this increase being attributed to increasing tumour burden.

Table 12.3a

| Treatment                     | No. tumours | T <sub>D</sub> (median) days | Specific growth delay | 35 day tumour volume (mean) % control |
|-------------------------------|-------------|------------------------------|-----------------------|---------------------------------------|
| Control                       | 6           | 13                           | -                     | 792.6                                 |
| Interferon                    | 6           | 23                           | 0.77                  | 303.8                                 |
| Cis-platinum<br>1.4 mg/kg x 5 | 4           | 22                           | 0.69                  | 257.4                                 |
| IFN + cis-platinum            | 6           | 51                           | 2.92<br>(p<0.01)      | 136.8<br>(p<001)                      |
| Cis-platinum<br>7 mg/kg       | 8           |                              | 0.87                  |                                       |

Table 12.3b

| Treatment                        | No. tumours | T <sub>D</sub> (median) days | Specific growth delay | 35 day tumour volume (mean) % control |
|----------------------------------|-------------|------------------------------|-----------------------|---------------------------------------|
| Control                          | 6           | 13                           | -                     | 792.6                                 |
| Interferon                       | 6           | 23                           | 0.77                  | 303.8                                 |
| Cyclophosphamide<br>40 mg/kg x 5 | 6           | 19.5                         | 0.5                   | 384.2                                 |
| IFN +<br>cyclophosphamide        | 6           | 27                           | 1.07                  | 322.4 NS                              |
| Cyclophosphamide<br>150 mg/kg    | 8           |                              | 0.52                  |                                       |

Table 12.3a & 12.3b Effect of human  $\alpha$  interferon and cytotoxic drugs administered both singly and in combination in the treatment of human lung cancer xenograft CX 117.

#### 12.4 Discussion

Interferon as a single agent has been shown to have minimal activity in non-small cell lung cancer (Grundberg et al 1985). Using weekly injections of cis-platinum and cyclophosphamide at 20% of the maximum tolerated dose, minimal effects on tumour growth were observed, as may have been expected. The combination of interferon and cyclophosphamide in all 3 lines showed greater activity than that observed with either agent alone, although at best showed additive effects. The cis-platinum-interferon combination was by far the most effective in all 3 xenograft lines. The combination was particularly active in the adenocarcinoma line (CX117), giving a specific growth delay of 2.92 doubling times. This is in comparison to growth delays of 0.77 and 0.22 doubling times respectively for interferon and cis-platinum alone. The response to this combination is also significantly superior to a maximum tolerated dose of cis-platinum given as a single intraperitoneal injection, when a growth delay of only 0.87 doubling times was observed.

It remains pure conjecture as to the mechanism of this increased cytotoxicity. It has been shown that human lymphoblastoid interferon does not increase natural killer cell activity in the host, and it has also been shown that human

interferon does not affect drug metabolising activity in the mouse (Balkwill et al). Recently, it was reported that interferon increased free radical formation in vivo, and if this claim is substantiated it could certainly help to explain the enhanced toxicities observed with interferons in combination with cytotoxic agents such as cyclophosphamide, adriamycin and cis-platinum. It does not explain the relative difference in potentiation between cis-platinum and cyclophosphamide combinations. However it would appear from published reports that the greatest enhancement is observed with the use of interferon in combination with the most active cytotoxic drug against that particular tumour.

As previously indicated, there are a number of problems to be addressed before this therapy can be introduced into the clinic. Firstly, in this model human interferon has no effect on any of the host tissues, whereas in patients effects on numerous tissues could be expected. Mouse interferon has been shown to affect the activity of drug metabolising enzymes in mice (Singh et al 1982), with similar effects likely to be observed in patients when human interferon is used. How much this will interfere with the beneficial effects of these combinations remains an open question. However a reasonable indication could be obtained using this model system with a



hybrid interferon which expresses inter-species cross-reactivity. The use of hybrid interferons in this model could give an indication of the likely clinical effects of altered drug metabolism on interferon-cytotoxic drug combinations.

Secondly, the schedule used in this study, delivering the equivalent of 20 mega units per day to humans, would not be tolerated by many patients and therefore, more realistic schedules require to be tested in this model. New schedules require to be assessed both in terms of dosage and frequency of administration of the interferon. Recently, further work on this model has been performed by Dr R J Fergusson (ICRF Medical Oncology Unit) using a much lower dose of interferon. Using doses of interferon at approximately 10% of those used in this study, similar potentiation was observed with combinations of interferon and cis-platinum, with interferon alone having no effect on xenograft growth (Fergusson - personal communication).

Thirdly, in view of the toxicity of cis-platinum, it would seem worthwhile testing more drugs, particularly platinum analogues, in combination with interferon, in an attempt to achieve similar results.

Finally, it has to be remembered that the cytotoxic drug dosages used in this study were much lower than those normally incorporated into cytotoxic drug combinations. Further studies

should be done using higher cis-platinum doses in an attempt to improve the cytotoxicity of this combination. In this study no significant bone marrow toxicity was observed. Similarly, weight loss, a good general indicator of toxicity in these mice, was not seen in treated animals, therefore higher cytotoxic drug doses may well be tolerated.

In conclusion, the combination of interferon with cytotoxic drugs, particularly cis-platinum, gives a greater anti-tumour effect than can be achieved with either agent used singly in the treatment of non-small cell lung cancer xenografts. Likewise, this cytotoxicity is greater than that observed with maximum tolerated doses of these agents given as a single intra-peritoneal injection. Although further studies are required, these results are very encouraging and it is hoped that such combinations may improve on the rather dismal results currently achieved by cancer chemotherapy in non-small cell lung cancer.

## CHAPTER 13

### MODULATION OF CHEMOTHERAPY: - EFFECT OF CALCIUM INFLUX BLOCKERS

#### 13.1 Introduction

As stated in Chapter 5, the use of calcium channel blockers has been shown to increase the activity of vinca alkaloids, adriamycin, and the epidophyllotoxin etoposide (Yalowich and Ross 1984). This increase in activity is more pronounced in cell lines exhibiting pleiotropic drug resistance, and is felt to be due to a membrane effect primarily inhibiting drug efflux from the cell. Vindesine, a vinca alkaloid, was found to have minimal activity against these non-small cell lung cancer xenografts. This drug was therefore combined with verapamil, a calcium channel blocker, in an attempt to enhance its cytotoxicity.

## 13.2 Materials and Methods

### 13.2a Study design

Two separate experiments were carried out. The first experiment was designed to assess the cytotoxicity of vindesine and verapamil in combination, and the second to investigate the bone marrow toxicity associated with this combination.

### 13.3 Effect of verapamil on the cytotoxicity of vindesine

The cytotoxicity of vindesine and verapamil in combination was assessed using tumour-bearing CBA mice carrying either NX 002, a moderately differentiated squamous carcinoma xenograft, or CX 140, a moderately differentiated squamous carcinoma xenograft. For each xenograft line, the following groups were studied using a minimum of 5 animals per group:

- (a) saline control 0.2 ml/day x 10 i.p.
- (b) vindesine 0.2 mg/kg/day x 10 i.p.
- (c) verapamil 50 mg/kg/day x 10 i.p.
- (d) vindesine 0.2 mg/kg/day x 10 i.p.  
+  
verapamil 50 mg/kg/day x 10 i.p.

### 13.3b Results

#### (i) CX 140

The control group consisted of 11 tumours with a mean tumour doubling time of 24.3 days. Groups treated with verapamil and vindesine alone comprised of 5 tumours per group with tumour doubling times of 21 days and 24 days respectively. The group given the combination of both drugs tolerated treatment very poorly. All 7 animals, carrying 11 tumours, died shortly after injections were discontinued at 7 days with no animal surviving more than 10 days.

#### (ii) NX 002

A mean tumour doubling time of 16.6 days was observed in the control group of 8 tumours. This compared with 18.8 days in the verapamil treated group of 5 tumours and 21.4 days in an identical number of vindesine treated animals. Once again, all animals in the group receiving a combination of both drugs died, with mice treated with a maximum of 7 days chemotherapy.

### 13.4 Effect of verapamil on the bone marrow toxicity of vindesine

Bone marrow toxicity of vindesine, verapamil and a combination of these drugs was assessed using female non-tumour bearing CBA mice, with 10 animals per group. The same groups were used as for the previous experiment.

#### 13.4a Drugs

Vindesine (Eli-Lilly Ltd) was diluted in sterile normal saline to a concentration of 0.02 mg/ml. CBA mice received 0.1 ml/10 g body weight (0.2 mg/kg) intraperitoneally daily for 10 days, a maximum dose of 2 mg/kg. Verapamil was administered at 50 mg/kg, the lowest dose used by Tsuruo et al (1981; 1983). Verapamil was diluted to a concentration of 2 mg/ml, animals therefore receiving 0.25 ml/10 g body weight, intraperitoneally.

#### 13.4b Haematological toxicity

White cell counts were carried out every second day, with blood collected using a tail vein technique. 20  $\mu$ l of whole blood was collected in capillary tubes from 3 animals in each group. The blood was directly added to 9.98 ml normal saline to which 3 drops Zaponin (Coulter Chemicals Ltd) had been added. The latter was used to lyse red blood cells, and white cell counts were then performed using a Coulter Counter.

#### 13.4c Results

White cell counts for all treatment groups, expressed as a percentage of control, are shown in Table 13.1. In addition, the effect of cyclophosphamide 150 mg/kg was used as a positive control. Vindesine alone caused a depletion in the white cell

count to 52% of control at day 8, with a recovery by day 10. Verapamil alone caused no significant alteration in the white cell count. The combination of drugs caused an earlier depletion in the white cell count but this had fully recovered by day 10. However, the latter sample consisted of only one specimen, as there was only one survivor in this group at this stage.

### 13.5 Discussion

The combination of vindesine (0.2 mg/kg/day) and verapamil (50 mg/kg/day) was exceedingly toxic both to tumour-bearing and non tumour-bearing CBA mice. These animals did not die a haematological death as the white cell count was normal in these animals at the time of death. Therefore, the two most likely aetiologies are enhanced neurological toxicity or enhanced peritoneal toxicity, although neither was experimentally substantiated.

These results are in keeping with the previous reports showing no enhancement of adriamycin toxicity on bone marrow cultures when used in combination with verapamil. Whether enhancement of vindesine activity can be achieved with smaller doses of these drugs remains an open question. However, these studies do suggest that a more detailed assessment of host

|        | DVA | VERAP | DVA + VER | CYCLO (150 mg/kg) |
|--------|-----|-------|-----------|-------------------|
| Day 2  | 99  | 106   | 89        | 90                |
| Day 4  | 96  | 93    | 48        | 51                |
| Day 6  | 81  | 106   | 91        | 26                |
| Day 8  | 52  | 86    | 91        | 69                |
| Day 10 | 118 | 86    | 140*      | 86                |
| Day 14 | 79  | 106   | -         | 133               |
| Day 16 | 156 | 200   | -         |                   |
| Day 20 | 88  | 84    | -         |                   |

Table 13.1 Effect of verapamil (VER) and vindesine (DVA) on the white cell count in CBA mice. Verapamil 50 mg/kg i.p. daily x 10 and vindesine 0.2 mg/kg i.p. daily x 10. White cell count expressed as a percentage of control with all results a mean of 3 specimens.



toxicities is warranted prior to the use of verapamil-cytotoxic drug combinations in clinical protocols.

Recently, further studies have been performed by the author to identify the mode of potentiation of calcium channel blockers in pleiotropically resistant cells. Using two pleiotropic mutant pairs, the CHO cell lines Aux B<sub>1</sub> and its pleiotropic mutant CHR<sup>C5</sup>, made resistant to increasing doses of colchicine, were kindly provided by Dr V Ling (Toronto, Canada) and 2 MCF-7 cell lines, the wild-type, (WT) and its pleiotropic mutant ADR<sup>10</sup>, made resistant to increasing doses of adriamycin, were kindly provided by Dr K Cowan (National Cancer Institute, USA). It was found that both pleiotropically resistant cell lines were collaterally sensitive to the calcium channel blockers diltiazem and verapamil, compared with wild-type cells. In addition, an increase was noted in surface transfer receptor number in both resistant cell lines, although treatment with calcium channel blockers did not affect the ratio of surface transfer receptors in resistant and sensitive cells. Further studies are underway in an attempt to identify factors important in reversibility of drug resistance using calcium channel blockers.

**CHAPTER 14****CYCLOPHOSPHAMIDE PRIMING: EFFECT ON ANIMAL SURVIVAL****14.1 Introduction**

The results of the biochemical experiments will be presented in the following chapters. Initially, data are presented showing the protective effects on survival of small 'priming' doses of cyclophosphamide given prior to a normally lethal dose of the same compound.

**14.2 Materials and Methods**

Male CBA mice were used to establish a dose of cyclophosphamide that was lethal to 100% of animals when administered as a single intraperitoneal injection. Similar mice were then used to identify the most effective 'priming' or protective dose of cyclophosphamide and the optimal day of administration of this priming dose prior to the lethal dose. For all of these survival experiments a minimum of 10 animals per group were used.

### 14.3 Results

#### 14.3a Identification of a Lethal Dose of Cyclophosphamide

Groups of mice were treated with doses of cyclophosphamide from 250 mg/kg to 500 mg/kg in 50 mg/kg increments. A single dose of 350 mg/kg was the minimum dose causing death of all animals and therefore was used for all of the biochemical experiments. At this cyclophosphamide dose, death is attributed to either haematological or urotoxicity with pulmonary toxicity dose-limiting at doses between 400-500 mg/kg. Death occurs much earlier following doses of cyclophosphamide over 500 mg/kg with cardiac toxicity the most likely aetiological factor.

#### 14.3b Protective Effect of Cyclophosphamide 'Priming'

Using lethal cyclophosphamide doses of 350 mg/kg and 400 mg/kg, the protective effects of variable 'priming' doses of the same drug were studied. Survival following a lethal cyclophosphamide dose was assessed in both pre-treated and control animals. The results are illustrated in Figure 14.1. All priming doses were given 5 days prior to the lethal dose in this part of the experiment. A priming dose of 75 mg/kg cyclophosphamide was found to be the most effective,

particularly against a lethal dose of 350 mg/kg, when 90% survival was achieved. This experiment was repeated 3 times with identical results.

With regard to the optimal timing of this priming dose of cyclophosphamide, a similar experiment was carried out using 350 mg/kg as the lethal dose and 75 mg/kg as the priming dose. The priming dose was administered to groups of mice at variable times prior to the lethal dose. The results are illustrated in Figure 14.2. Maximal protection was observed 5-7 days after the priming dose of cyclophosphamide using these particular doses.

#### Discussion

These results confirm previous reports of protection against the lethal effects of alkylating agents by pre-treatment with the same or similar compounds (Jeney and Connors 1968; Millar et al 1978). The 'priming' phenomenon is not limited to alkylating agents, however, with the protection observed against a number of toxic insults. Ozone, for instance, has been shown to protect against the lethal pulmonary toxicity of subsequently administered high concentrations of this compound (Chow and Tappel 1972). Likewise, tolerance to heat can be observed on exposure to thermal stress (Mitchell and Russo 1983).

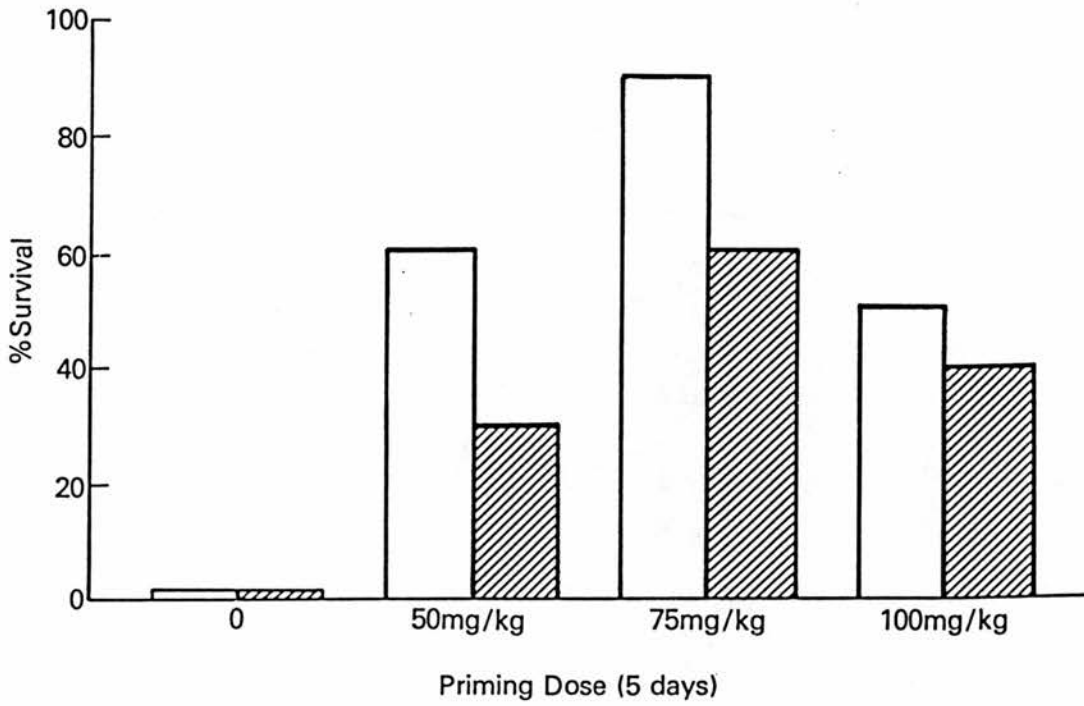


Fig. 14.1 Effect of variable priming doses of cyclophosphamide on 14 day survival following:  350mg/kg and  400 mg/kg in CBA mice.

Similarly, numerous agents have been shown to protect against the lethal effects of x-irradiation. Colchicine was one of the first compounds shown to be protective against x-irradiation (Smith 1958), followed by the vinca alkaloids (Smith and Wilson 1967). More recently, Millar (1978) and colleagues at the Institute of Cancer Research in London have shown radioprotection by numerous agents including methotrexate, cyclophosphamide and cytosine arabinoside, with the latter compound extensively studied. If given 2-3 days prior to irradiation, cytosine arabinoside offers maximal protection in terms of animal survival. However, by altering the interval between exposure to the drug and irradiation, maximal protection can be seen in different tissues, such as the bone marrow or gastrointestinal tract (Phelps and Blackett 1979). The mechanism of cytosine arabinoside protection may well relate to the recruitment of cells into a relatively radio-resistant phase of the cell cycle, late S-phase. In agreement with this hypothesis, a second dose of cytosine arabinoside administered at the time of maximal radioprotection is highly toxic, suggesting redistribution of cells in cycle. It seems likely that the strathmokinetic agents work in a similar way.

In contrast, in the development of thermotolerance, production of heat shock proteins and elevation in glutathione

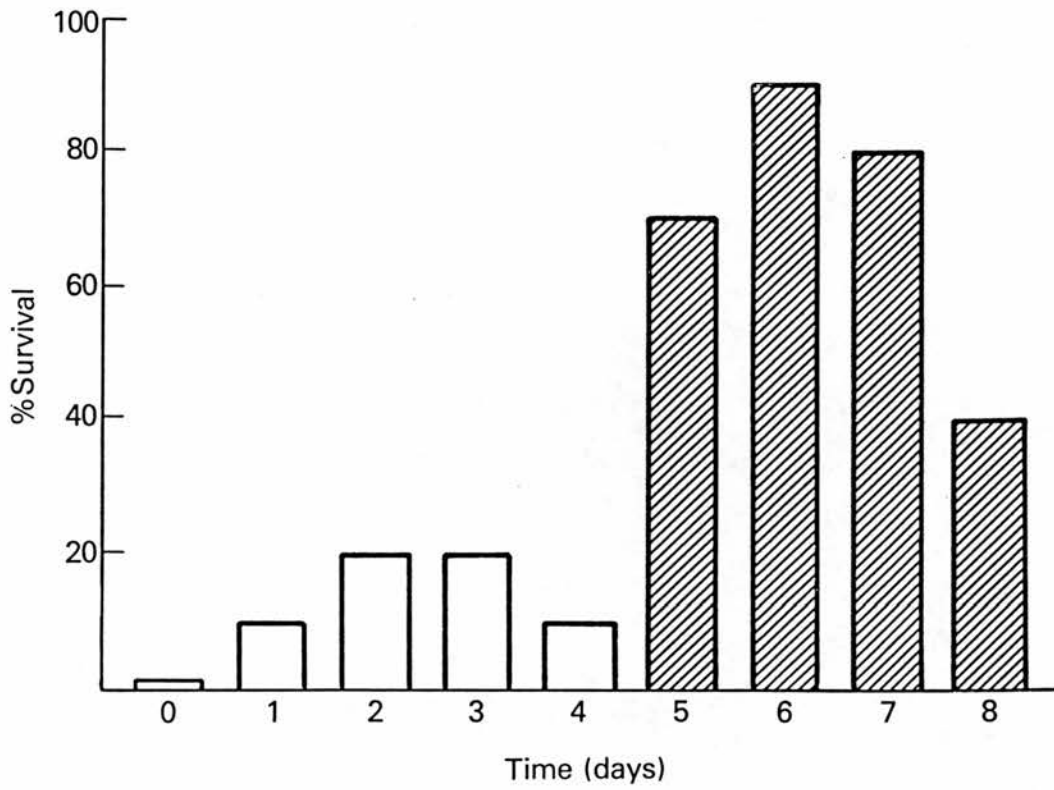


Fig. 14.2 Effect of varying time between priming (75mg/kg) and lethal dose (350mg/kg) of cyclophosphamide on 14 day survival in CBA mice:

▨ maximal protection.

levels are considered important (Mitchell and Russo 1983). However, the mechanism of protection afforded by alkylating agents against similar compounds and x-irradiation remains unknown, although a number of typical features have become apparent. Alkylating agent 'priming' has been shown to increase the rate of recovery of the CFU-S, CFU-GM and peripheral granulocyte populations following the subsequently administered high dose of chemotherapy. However, the enhanced recovery of blood elements is not due to diminished sensitivity of the stem cell population to the subsequent toxic insult. The exact mechanism of protection afforded by alkylating agents against similar compounds and irradiation remains far from clear.



## CHAPTER 15

EFFECT OF TOXIC COMPOUNDS ON MOUSE TISSUE GLUTATHIONE AND  
GLUTATHIONE TRANSFERASE LEVELS15.1 Introduction

Glutathione, an intracellular tripeptide, is involved in cellular defence mechanisms against reactive molecules. Many xenobiotics have been shown to reduce hepatic glutathione levels (Di Simplicio 1982), and conversely, alterations in glutathione levels have been shown to affect the response of cells to cytotoxic drugs (Roizin-Toile et al 1984). Likewise, glutathione transferases have been shown to be protective to the liver (Di Simplicio 1982) and tumour cells in vitro (Batist et al 1985). In addition, elevations in glutathione and glutathione-S-transferase levels have been associated with the development of drug resistance both in vivo (Wolf et al 1985) and in vitro (Hamilton et al 1985).

The following experiments were carried out to elicit the role, if any, of glutathione and glutathione transferase levels in the protection afforded by low dose alkylating agents. This work was carried out in collaboration with David Adams and Roland Wolf in the Imperial Cancer Research Fund laboratories.

## 15.2 Cyclophosphamide as a Priming Agent

### Methods

Male CBA mice 8-12 weeks of age were used for these studies. Animals were injected with varying doses of cyclophosphamide intraperitoneally. They were then sacrificed at different times and samples prepared for glutathione and glutathione transferase assays. Hepatic and bone marrow samples, collected in the manner described in Chapter 7, were assayed at these time points. Specimens were collected for 10 days following the priming dose with all samples assayed in triplicate. For these experiments glutathione was measured using the fluorimetric method described by Hissin and Hilf (1977) with glutathione transferases measured as described in Chapter 7 (Habig et al 1976) using 1-chloro-2,4-dinitrobenzene as substrate.

## 15.3 Results

### 15.3a Hepatic Glutathione Levels

The effect of varying doses of cyclophosphamide on hepatic glutathione levels was assessed. Three cyclophosphamide doses were used, two priming doses 75 mg/kg and 100 mg/kg, in addition to a lethal dose of 500 mg/kg. The

results are illustrated in Figure 15.1. As is shown initially, there was a depletion in glutathione levels following all three doses of cyclophosphamide, with recovery and subsequent minimal elevation of levels in surviving animals, three days after drug exposure. Depletion was minimal, with the lower doses (less than 30% decrease), whereas 500 mg/kg resulted in a 60% reduction of liver glutathione levels which did not recover prior to the death of all the animals in that group.

#### 15.3b Hepatic Glutathione Transferase Levels

Aliquots of samples from the above experiments were assayed for glutathione transferase as described in Chapter 7. The results were virtually identical to the glutathione data, showing an initial depletion of transferase levels following the priming doses with subsequent recovery and minimal overshoot of levels in surviving animals. With the lethal dose, glutathione transferase levels were depleted to a similar degree as glutathione and failed to recover prior to death of all the animals.

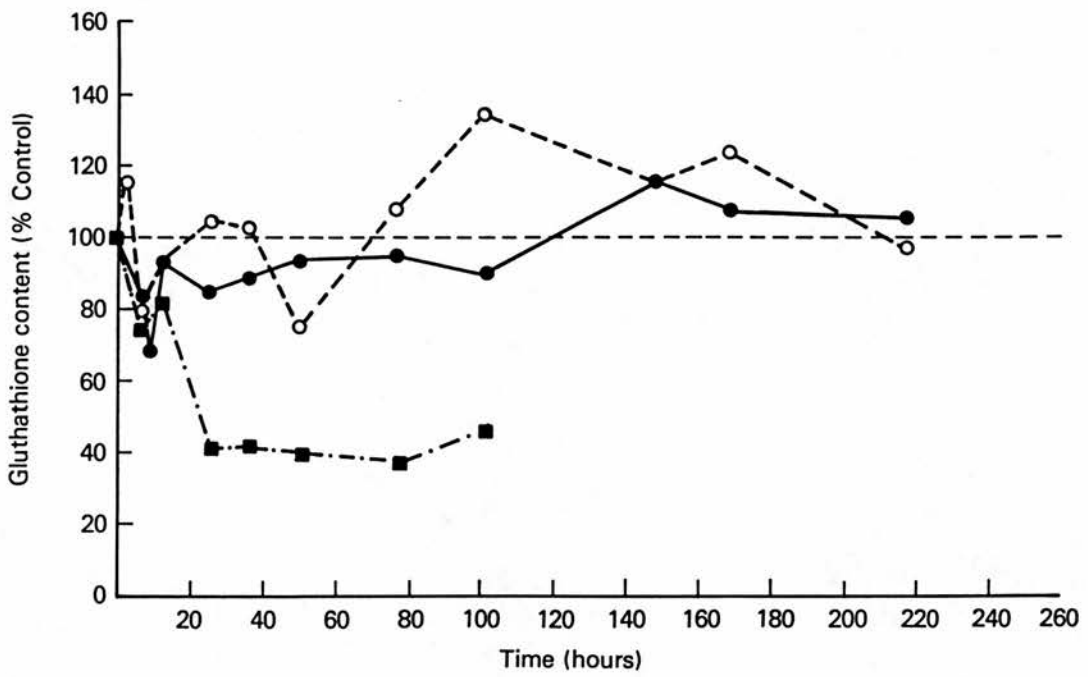


Fig. 15.1 Effect of varying doses of cyclophosphamide on mouse liver glutathione levels: ●—● 75mg/kg ○—○ 100mg/kg and ■—■ 500mg/kg.

### 15.3c Bone Marrow Glutathione Levels Following Cyclophosphamide Priming

Bone marrow glutathione levels were measured using samples obtained from the femurs of animals used in the above study. As can be seen in Figure 15.2 greater variation in bone marrow glutathione levels was observed, in comparison with hepatic values. All 3 cyclophosphamide doses caused a profound fall in bone marrow glutathione levels, dropping to <30% of control values at 36 hours. Glutathione levels recovered by day 4 with subsequent overshoot of values to approximately 180% control by 120 hours in the animals treated with priming doses. However, in the animals treated with the lethal dose of cyclophosphamide, levels fell to <5% of control by 35 hours with minimal recovery prior to death. Of interest, was a transient fall noted in glutathione levels between days 5-6, noted also for glutathione transferase levels. This would not appear to be a totally random event, as this experiment was repeated three times with a similar result obtained on each occasion. The mechanism behind this remains unexplained, although it is possible that circadian rhythm effects played a role.

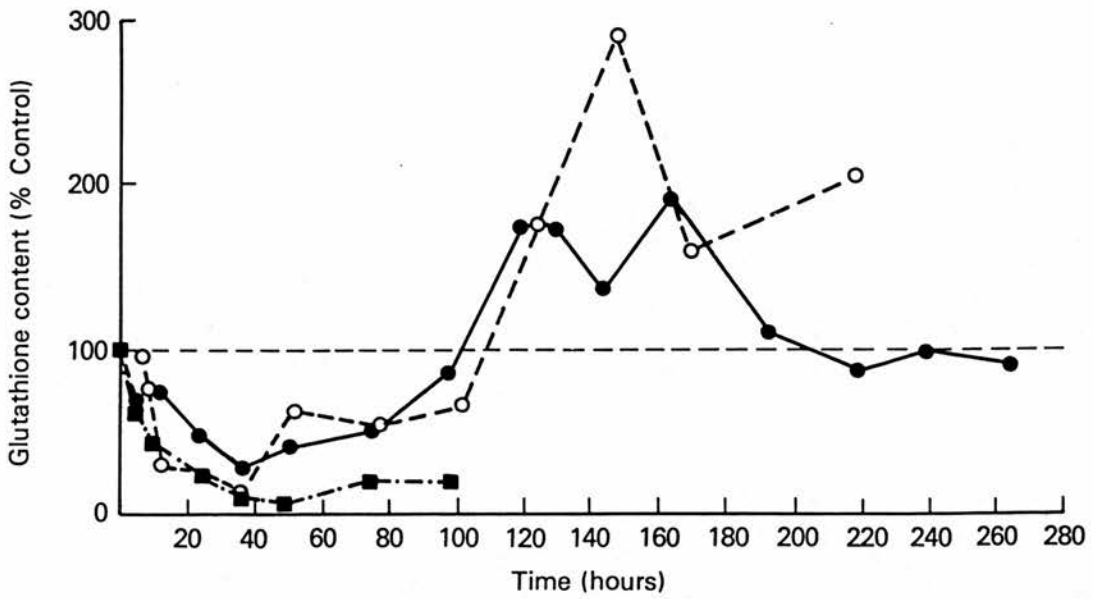


Fig. 15.2 Effect of varying doses of cyclophosphamide on mouse bone marrow glutathione (GSH-mol/ $10^6$  cells) expressed as a percentage of control; ●—● 75mg/kg ○--○ 100mg/kg ■-.-■ 500 mg/kg.

### 15.3d Bone Marrow Glutathione Transferase Levels

Glutathione transferases were measured using the same bone marrow samples. Once again, depletion to 30% of control with subsequent overshoot of glutathione transferase values was observed following the priming doses of cyclophosphamide. The lethal dose of cyclophosphamide resulted in depletion of values to less than 5% of control with no recovery of glutathione transferase levels noted prior to death of all the animals in the group.

### 15.4 Effect of Cyclophosphamide Priming Doses on Tissue Glutathione Levels Following Subsequent Lethal Dose Cyclophosphamide Treatment

#### Methods

Male CBA mice 8-12 weeks of age were used. Animals were treated with a lethal dose of cyclophosphamide (350 mg/kg) 5 days after pre-treatment with either saline, or cyclophosphamide (75 mg/kg). Bone marrow glutathione and glutathione transferase levels in treated mice were compared with control untreated mice at each time point.

## 15.5 Results

### 15.5a Bone Marrow Glutathione Levels

The results are shown in Figure 15.3. Primed animals once again have elevated bone marrow glutathione levels at day 5. Following the lethal dose, however, levels fall dramatically in both groups. There was recovery in primed animals which survived the lethal dose, with overshoot of glutathione levels to >300% of control values.

### 15.5b Bone Marrow Glutathione Transferase Levels

Bone marrow glutathione transferase levels are illustrated in Figure 15.4, with a similar trend animals, glutathione transferase levels of approximately 800% of control were observed following recovery from the 'lethal' dose.

## 15.6 Effect of Cyclophosphamide, Cytosine Arabinoside and X-Irradiation on Mouse Bone Marrow Glutathione and Glutathione Transferase Levels

### Methods

Male CBA mice 8-12 weeks of age were used. Animals were treated with either cyclophosphamide 75 mg/kg, cytosine



arabinoside 200 mg/kg (both used as priming doses in mice), or X-irradiation 200 cGy. Mouse liver and femurs were excised and specimens processed as previously described.

## 15.7 Results

### 15.7a Glutathione Levels

Minimal effect on hepatic glutathione content is observed following cyclophosphamide, cytosine arabinoside or x-irradiation at these doses with the results shown in Figure 15.5. The results for all 3 treatment modalities on bone marrow glutathione levels are shown in Figure 15.6. All 3 treatments caused a fall in bone marrow glutathione levels, with subsequent recovery and overshoot. The timing of the maximal increase in glutathione levels varied, although the degree of overshoot was similar.

### 15.7b Glutathione Transferase Levels

The results are illustrated in Figure 15.7, showing an identical trend to that shown for glutathione levels, with an initial depletion, followed by overshoot of glutathione transferase levels to supranormal levels. Glutathione transferase values of approximately 200% were observed with each treatment, although the timing of this increase varied.

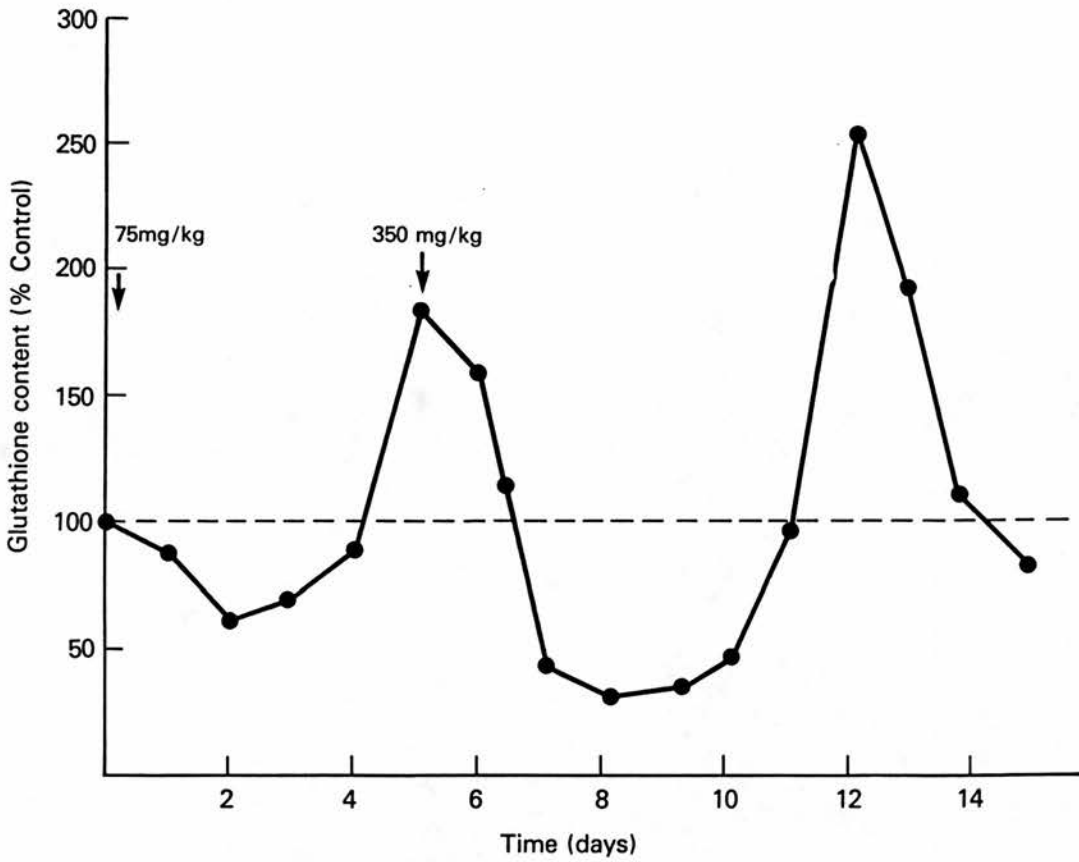


Fig. 15.3 Bone marrow glutathione levels in CBA mice treated with a priming dose of cyclophosphamide (75 mg/kg) followed by a lethal dose (350 mg/kg) five days later.

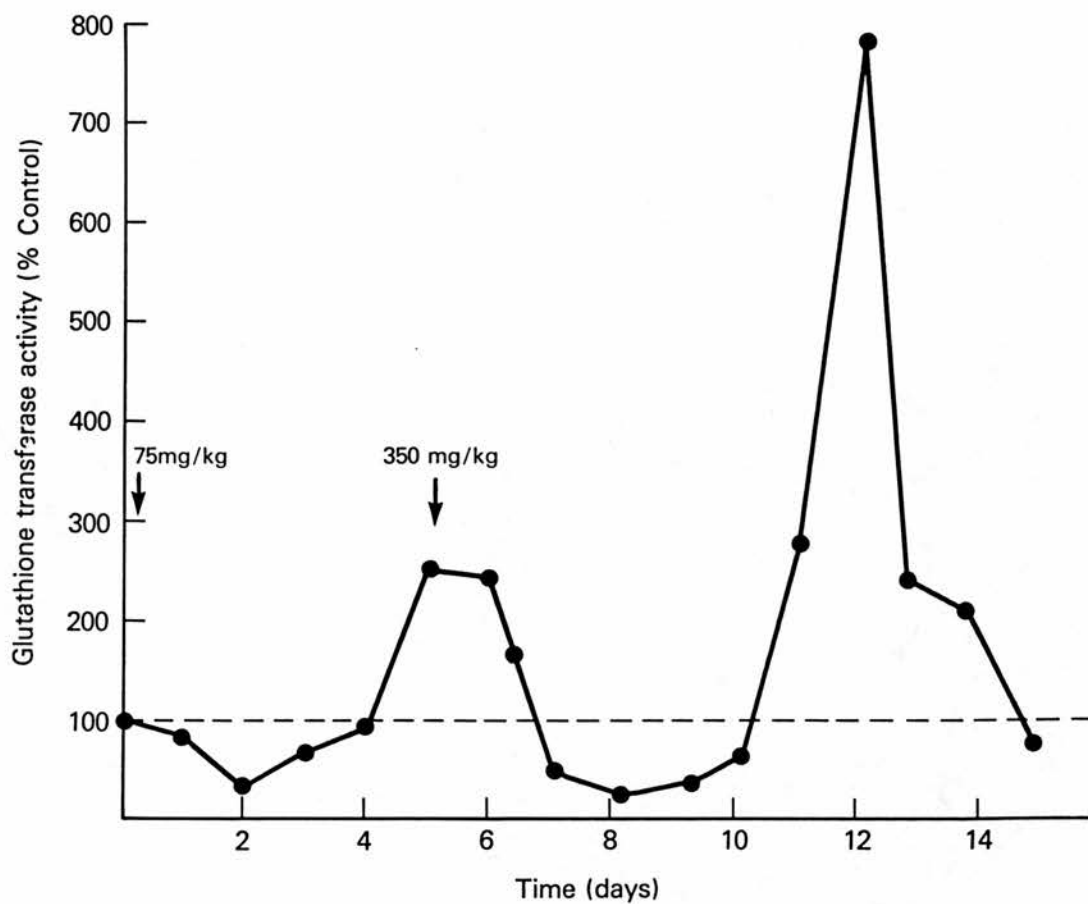


Fig. 15.4 Bone marrow glutathione transferase levels in CBA mice treated with a priming dose of cyclophosphamide (75 mg/kg) followed by a lethal dose (350 mg/kg) five days later.

### 15.8 Quenching Effect of Tissue Homogenates

Although non-specific fluorescence of histidine-containing peptides can interfere with the fluorescent assay (Mokrasch and Teschke 1984) this is minimal at pH 8.0. Another problem relates to the non-specific quenching of glutathione by different tissues. A number of factors are involved in this, including protein content. This problem was addressed by adding known amounts of glutathione to extracts from various tissues with known glutathione content.

### 15.9 Results

The relative quenching of different tissue homogenates is shown in Figures 15.8 and 15.9. In these figures the control curve represents the fluorescence of different amounts of glutathione. For the tissues, a known concentration of glutathione is added to each sample so that the increase in fluorescence of tissue samples should run perpendicular to the control curve. As can be seen in these two figures, absorbance is less than expected from the control curves. As can be seen in Figure 15.8, greatest quenching of glutathione is observed with liver and tumour tissue. In the tumour tissue proteins from necrotic cells may be important, due to the formation of protein-sulphydryl complexes, thereby reducing the measurable

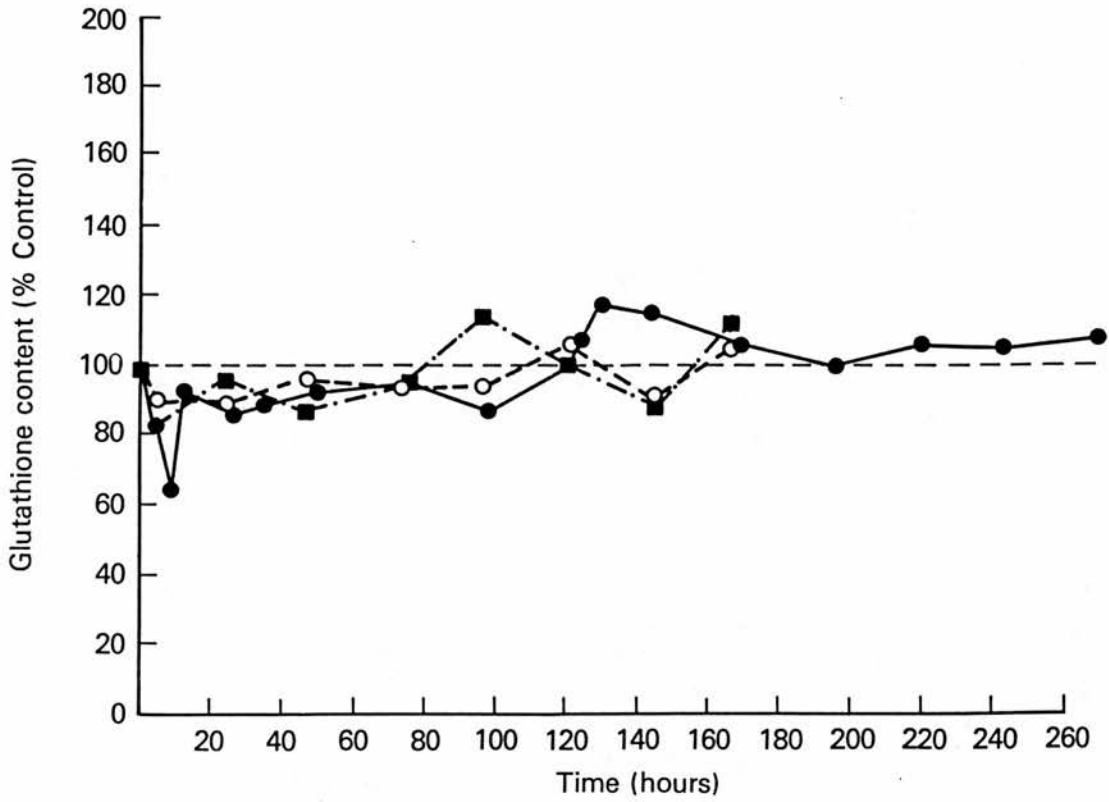


Fig. 15.5 Effect of cyclophosphamide, cytosine arabinoside and X-irradiation on mouse liver glutathione levels (GSH-mol/g wet weight). ○—○ cyclophosphamide 75mg/kg, ○—○ cytosine arabinoside 200 mg/kg □—□ whole body irradiation 200 cGy.

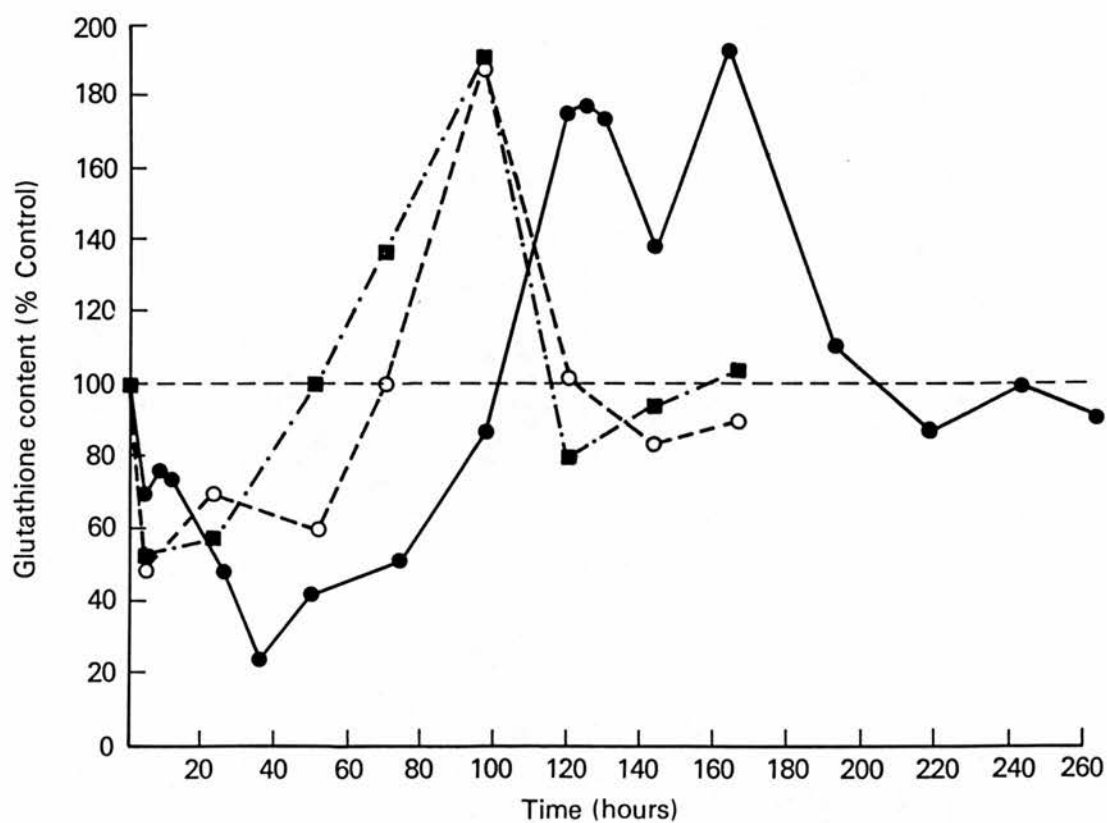


Fig. 15.6 Effect of cyclophosphamide, cytosine arabinoside and X-irradiation on mouse bone marrow glutathione levels (GSH-moles/ $10^6$  cells) expressed as a percentage of control:   
 ○—○ cyclophosphamide 75 mg/kg; ○--○ cytosine arabinoside 200 mg/kg, □-·-□ XRT 200 cGy.

non-protein thiol concentration. Various detoxifying enzymes from liver tissue, from lysosomes for example, may be an important cause of degradation of the glutathione. For the determination of absolute glutathione values in tissue, the observed values should be corrected using these graphs.

#### 15.10 Discussion

These studies show that exposure of various mouse tissues to cytotoxic agents or x-irradiation causes depletion of glutathione and glutathione transferase levels. This is not entirely unexpected as there is a vast literature on the effects of toxic chemicals on glutathione levels, particularly in the liver. In the liver, following non-lethal doses, there is subsequent recovery of these levels by 72 hours with minimal overshoot of values above control levels. Recovery was not observed following lethal doses.

In the bone marrow a different picture emerged with a more dramatic fall observed in both glutathione and glutathione transferase levels with subsequent recovery and overshoot of values to approximately 180% of control levels. This overshoot occurred at a different time following exposure to cyclophosphamide than with cytosine arabinoside or x-irradiation.

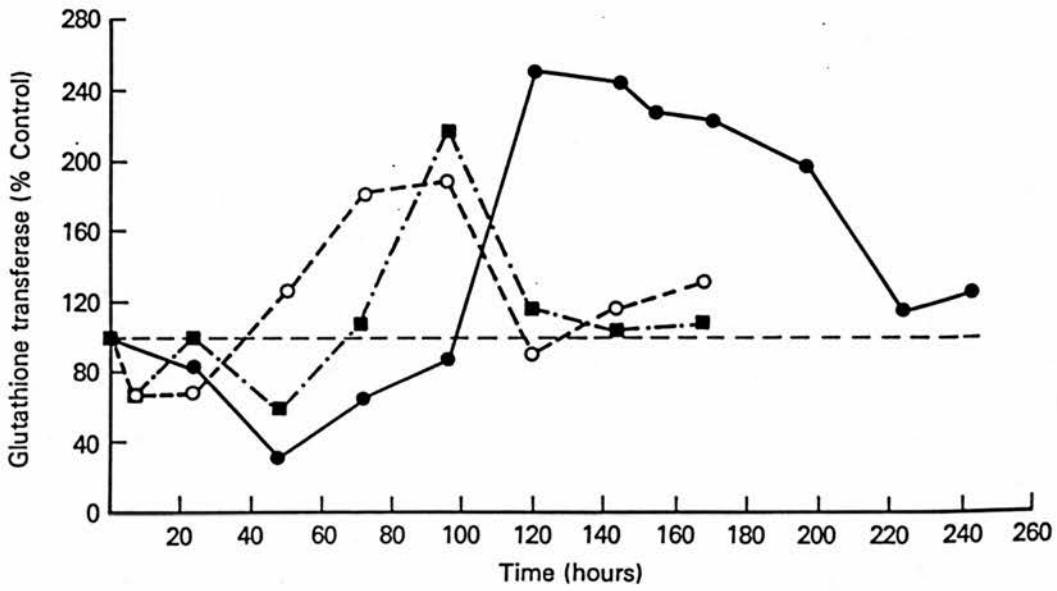


Fig. 15.7 Effect of cyclophosphamide, cytosine arabinoside and total body irradiation on mouse bone marrow glutathione-S-transferase levels (activity = mol DNCB/min/ $10^6$  cells)  
 ●—● cyclophosphamide 75 mg/kg; ○—○ cytosine arabinoside 200 mg/kg; ■—■ total body irradiation 200 cGy.



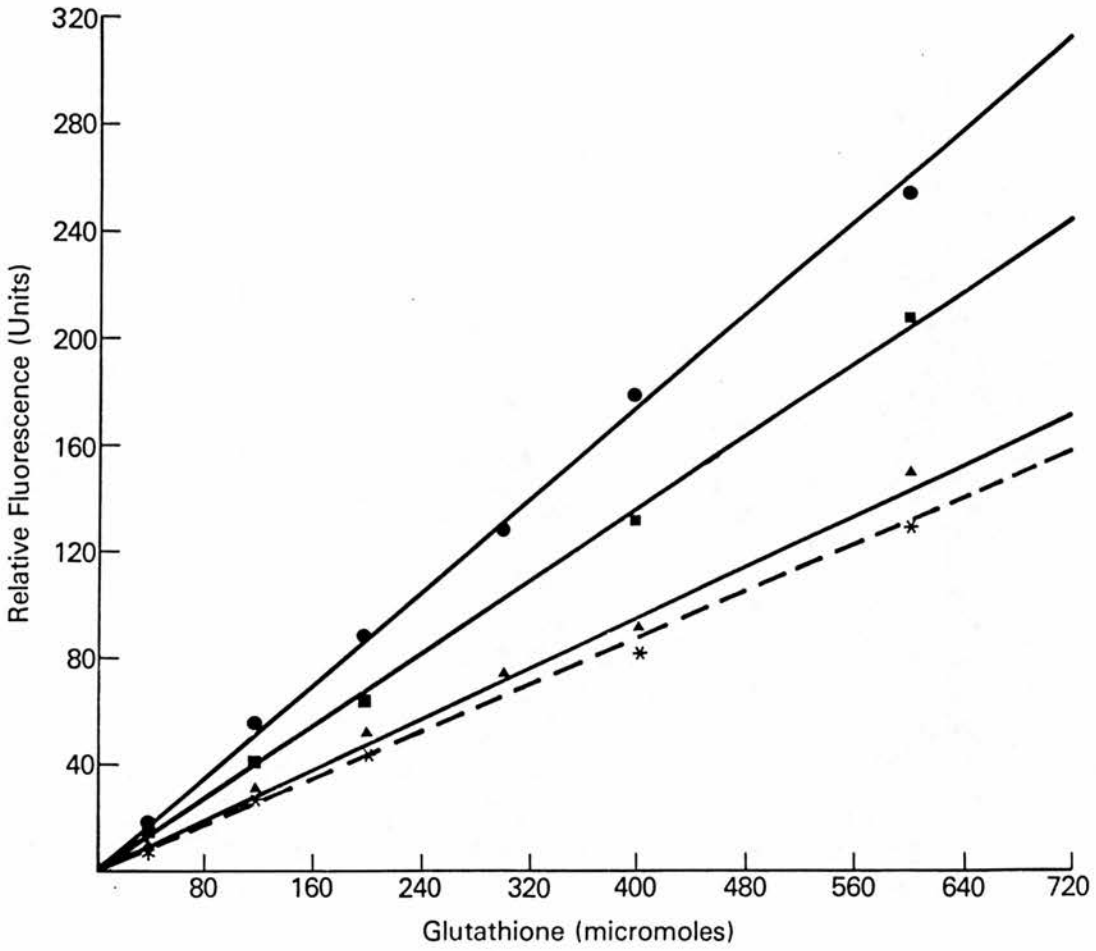


Fig. 15.8 Quenching effect of tissue homogenates on the glutathione assay: ●—● control; ■—■ bladder; ▲—▲ liver; \*—\* tumour (NX002).

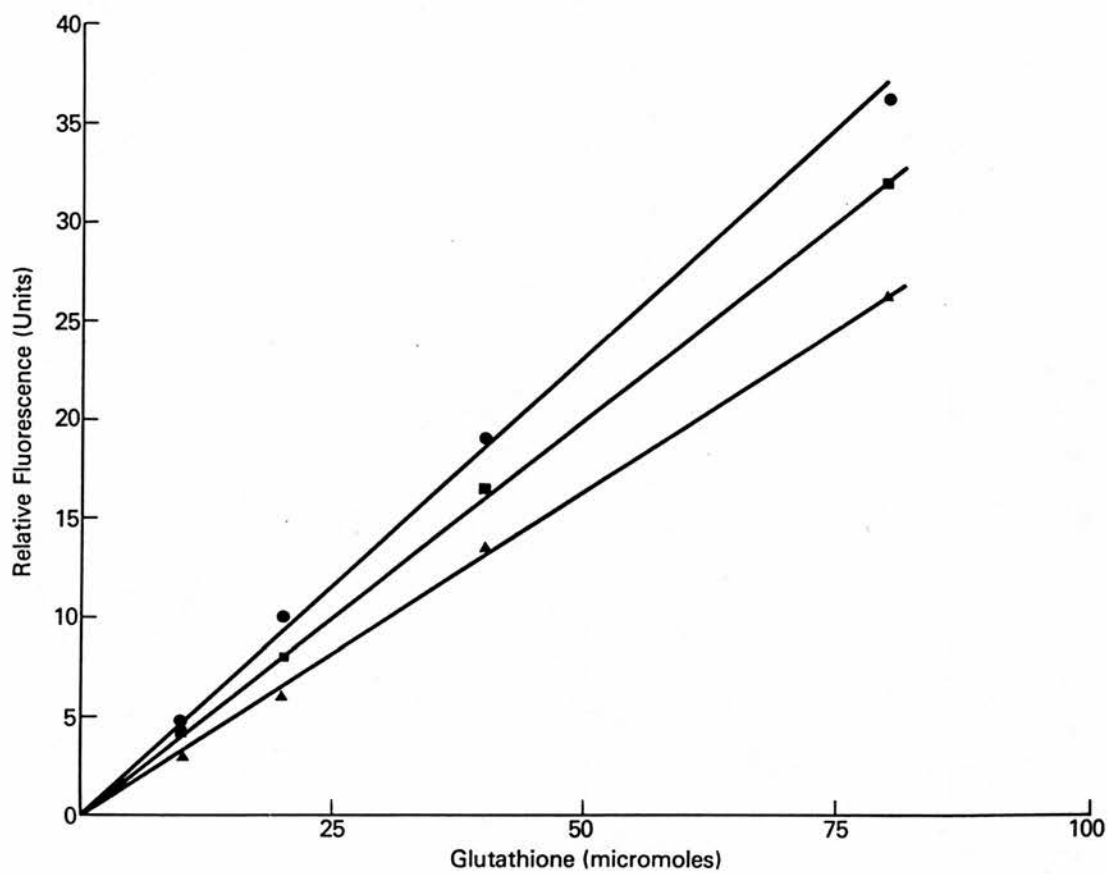


Fig. 15.9 Quenching effect of whole blood and bone marrow on the glutathione assay: ●—● control; ■—■ bone marrow; ▲—▲ whole blood.

It is of great interest that in the case of cyclophosphamide this overshoot occurred at the time of maximal protection against the lethal cyclophosphamide dose. The elevation in glutathione and glutathione transferase levels following cytosine arabinoside occurs between 72-96 hours after a 200 mg/kg dose with 48-72 hours the time at which maximal protection is afforded against the lethal effects of radiation. This was the dosage and schedule of cytosine arabinoside treatment as used in the preparation of immune-deprived animals for the xenograft studies.

However, on the negative side, priming with cytosine arabinoside did not protect against the lethal effects of cyclophosphamide despite the elevations in glutathione and glutathione transferase levels. Similarly, no survival protection was afforded by pretreatment with radiation (200 cGy) prior to cyclophosphamide lethal dose administration, given at the time of maximal glutathione elevation. For these studies CBA mice were primed with either 200 mg/kg cytosine arabinoside intraperitoneally or with 200 cGy whole body irradiation. At the time of maximal overshoot in glutathione levels, 96 hours, the mice were then challenged with a lethal dose (350 mg/kg of cyclophosphamide) intra-peritoneally. No survival protection was observed in these studies.

Cytosine arabinoside treatment blocks cell cycle progression. The protection produced by this compound against the lethal effects of radiation almost certainly results from the recruitment of cells into late S-phase, a relatively resistant phase of the cell cycle. Variation in cell cycle distribution also occurs following x-irradiation and cyclophosphamide treatment. In fact, the alterations in glutathione levels observed at these times could be partially explained by cell cycle recruitment. Harris and Teng (1973) showed variations in glutathione levels throughout the cell cycle, with late S-phase cells having the highest glutathione although these findings have not been reproducible. However, changes in cell cycle distribution would seem unlikely to fully explain the alterations in glutathione and glutathione transferase homeostasis observed in these studies.

Glutathione and glutathione transferase levels, compounds known to be protective to cells by detoxication mechanisms, were found to be elevated at the time of maximal protection against a lethal dose of cyclophosphamide. However, prior to making any direct correlations, a number of very important questions required to be addressed. Firstly, the bone marrow is a dynamic and extremely heterogenous population. Did the changes in bone marrow glutathione levels merely represent

alterations in bone marrow cell populations? Certainly Fried et al (1973) studying the effects of cyclophosphamide on mouse bone marrow, showed dramatic alterations in overall cellularity, and also an increase in the relative proportions of granulocyte and granulocyte precursors from 25% to approximately 50% in treated mice. Secondly, the metabolism of cyclophosphamide is interesting in that the cytotoxicity and haematological toxicity are attributed to one metabolite, phosphoramidate mustard, with another metabolite, acrolein, implicated in the toxicity, particularly to the urogenital tract.

Therefore, it was of interest to evaluate which metabolite was responsible for the protective effect on survival provided by priming doses of cyclophosphamide. In addition, was the same metabolite responsible for the observed alterations in glutathione homeostasis?

Thirdly, it has been shown in erythrocytes that there is a feedback control of glutathione synthesis. It was of interest to ascertain whether alterations in glutathione and glutathione transferase levels represented a compensatory synthetic process or merely preferential survival of cells with higher initial levels of these two compounds. Finally, it is known that drug priming can protect the bone marrow, gastrointestinal tract and

the bladder against subsequent toxic insult. What remains far from clear is whether tumour tissue can be protected in a similar manner. Obviously, if a similar phenomenon occurs in tumour tissue, this would negate the protective effects of priming on the bone marrow, and would result in an unchanged therapeutic index of cyclophosphamide. These questions will be addressed individually in the following chapters.

## CHAPTER 16

### EFFECT OF CYTOTOXIC DRUGS ON THE BONE MARROW

#### 16.1 Introduction

The effect of cytotoxic drugs on the bone marrow was studied both biologically, in terms of overall cellularity, and biochemically by measurement of glutathione and glutathione transferase levels in individual bone marrow cell populations.

#### 16.2 Bone Marrow Cell Number

The effect of various toxic agents on bone marrow cell number was studied.

##### 16.2a Methods

Bone marrow cell counts were carried out during each glutathione time course experiment with the cells counted using a haemocytometer. The marrow was obtained from the femurs of 3 mice, with the effect on bone marrow cellularity of cyclophosphamide 75 mg/kg, cytosine arabinoside 200 mg/kg and whole body irradiation 200 cGy measured over 7 days.

### 16.3 Results

Bone marrow cellularity was assessed at different times following cyclophosphamide 75 mg/kg, cytosine arabinoside 200 mg/kg and x-irradiation 200 cGy with the results illustrated in Figure 16.1. With all three compounds there was an initial increase over the first 24 hours, followed by a progressive fall in cell number, maximal at day 5, with recovery by day 7. In comparison, acrolein, a metabolite of cyclophosphamide, when given intra-peritoneally, caused minimal depletion in bone marrow cellularity as shown in Figure 16.2.

### 16.4 Discussion

The results from this study confirm a previous report (Fried et al 1973) showing depletion of nucleated cell number 4 days after 2 mg cyclophosphamide intra-peritoneally. In the same study, they showed that the relative of proportion granulocytes and granulocyte precursors in the bone marrow increased from 25 to 50% following cyclophosphamide. Thus, from these results, it seems possible that the changes in glutathione and glutathione transferase levels, observed in these studies, could be explained by changes in bone marrow cell populations. Therefore, it was essential to isolate the individual cell populations for further investigation.



### 16.5 Bone Marrow - Individual Cell Populations

As a means of isolating respective cell populations, a fluorescent activated cell sorter (FACS) was used. Various cell types from bone marrow and peripheral blood suspensions were separated using a Becton-Dickinson FACS IV, on the basis of their differential forward and right angle light scattering properties (Watt et al 1980; Ritchie et al 1985). The 488 nanometre line of an argon ion laser was used for the excitation of both the forward and right angle scatter signals, with samples run at approximately 1000 cells per second. This work was performed in collaboration with Dr John Ansell, Department of Zoology, University of Edinburgh. Using these techniques, the bone marrow was divided into 4 distinct cell populations as shown in figure 16.3. This figure illustrates the respective cell populations from a control untreated sample, but following a cyclophosphamide priming dose there were dramatic changes observed in these cell populations as shown in figure 16.4. By gating off appropriate segments, different populations were sorted, and these were collected in aliquots of 300,000 cells in ice-cooled Eppendorf centrifuge tubes. During the procedure all samples were kept at 4°C, to minimise autoxidation of glutathione. Following collection, cells were immediately centrifuged at 250 g, the supernatant aspirated, and the cell

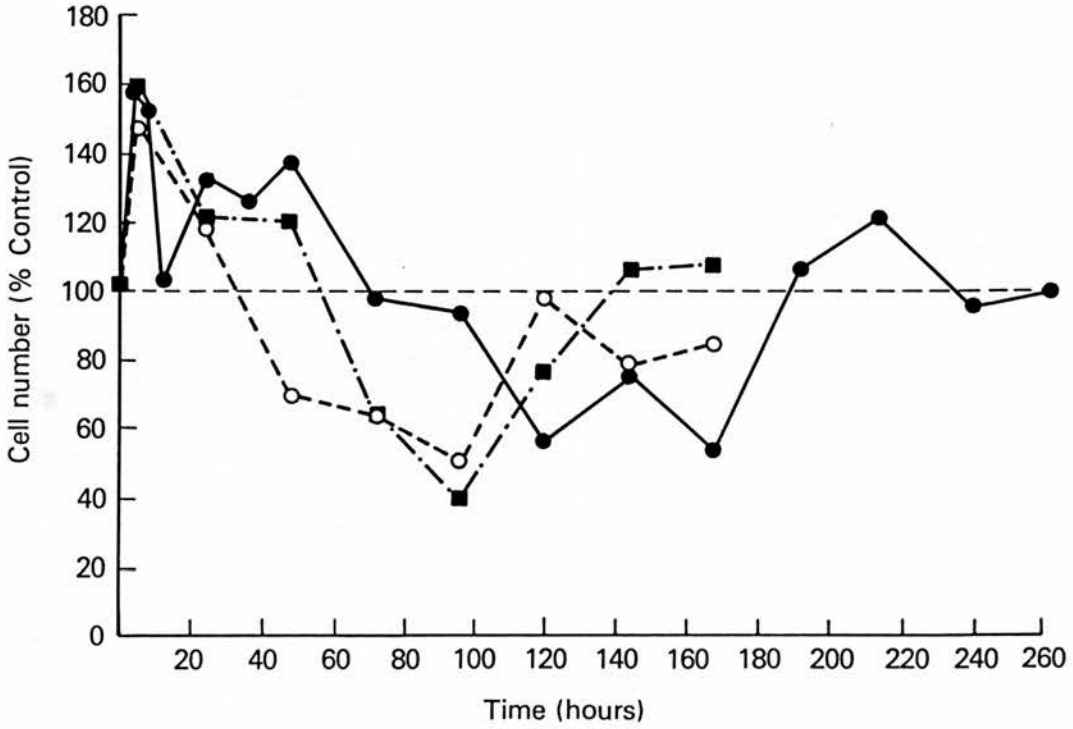


Fig.16.1 Effect of cyclophosphamide, cytosine arabinoside and X-irradiation on mouse bone marrow cell number: ●—● cyclophosphamide 75 mg/kg; ○—○ cytosine arabinoside 200 mg/kg; ■—■ total body irradiation 200 cGy.

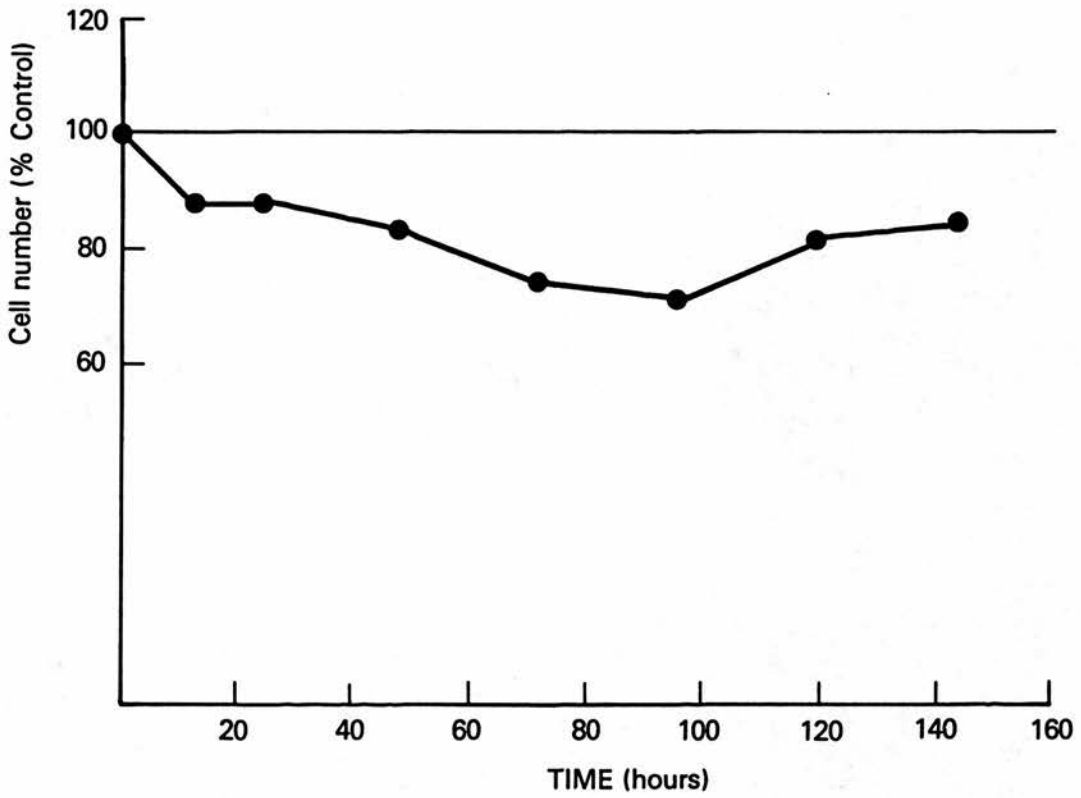


Fig. 16.2 Effect of Acrolein (6.2mg/kg) on bone marrow cell number.

pellet frozen in dry ice. Numerous samples of all 4 sub-groups were collected: granulocytes, lymphocytes, erythrocytes and an anomalous population consisting mainly of monocytes and stem cells. The majority of samples were used for biochemical analyses, although aliquots from each sub-group were histologically analysed, to confirm purity of the samples.

Glutathione was estimated using O-phthalaldehyde as previously described (Hissin and Hilf 1976). Glutathione transferase levels were virtually undetectable using 300,000 cells aliquots, therefore 3 samples were pooled and used for each estimation. All glutathione and glutathione transferase values were expressed per  $10^6$  cells.

## 16.6 Results

Glutathione levels in the different bone marrow cell populations are shown in table 16.1 with comparisons of control and cyclophosphamide primed samples. For this experiment animals were treated with 100 mg/kg cyclophosphamide as a priming dose. Erythrocytes had the lowest glutathione level, with no difference noted between primed samples and control. Both the lymphocyte and anomalous cell fractions had higher levels of glutathione but no significant change in level was detected following cyclophosphamide priming. The granulocyte

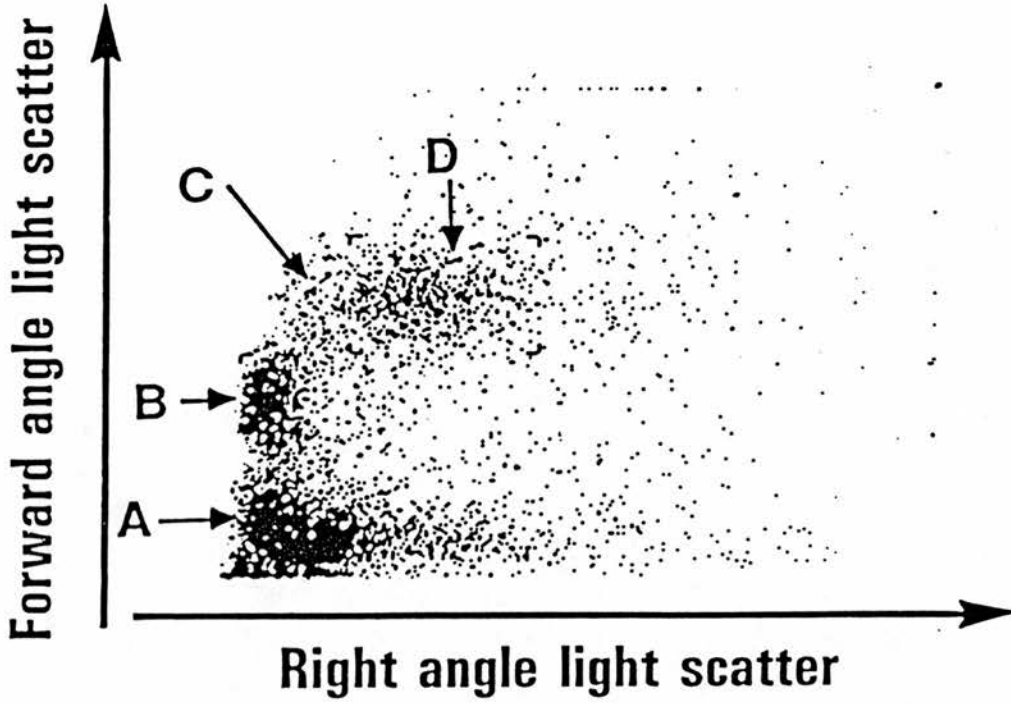


Figure 16.3 Becton-Dickinson FACS 'dot plots' of mouse bone marrow from untreated animals. Four populations observed; A-erythrocytes, B-lymphocytes, C-anomalous population, D-granulocytes.

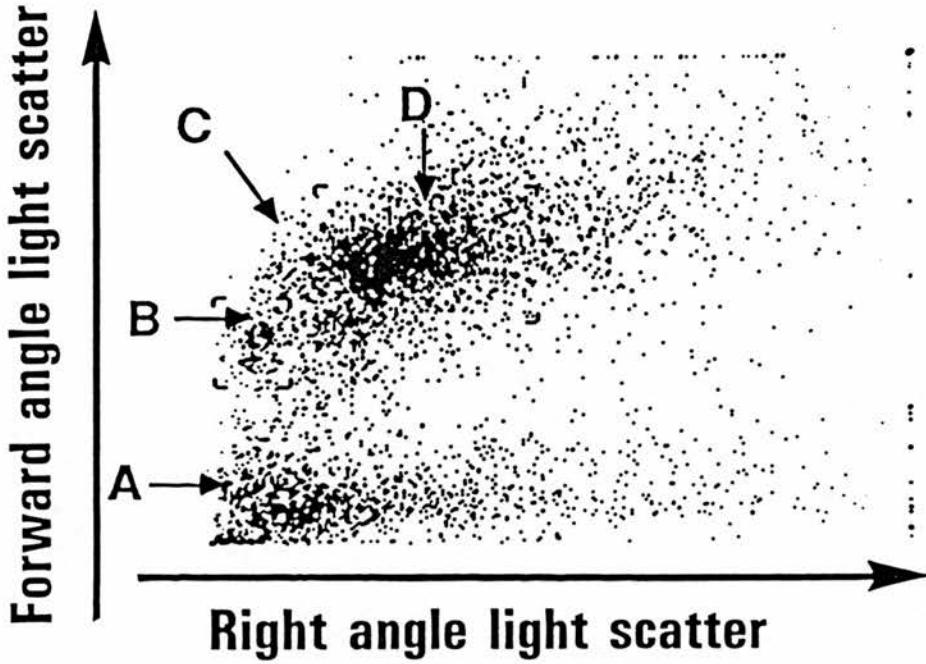


Figure 16.4 Becton-Dickinson FACS 'dot plots' of mouse bone marrow following treatment with cyclophosphamide 100 mg/kg 5 days previously. Groups A-D as in Figure 16.3

population had, by far, the highest level of glutathione, with a significant increase in levels following cyclophosphamide treatment. Increases of greater than 50% were observed in repeated experiments with the results of the first 3 experiments illustrated in table 16.1. Glutathione transferase levels were carried out on the granulocyte samples, with increases of the same degree detected, as shown in table 16.2.

#### 16.7 Glutathione and Glutathione Transferase in Peripheral Blood Granulocytes

If glutathione levels in granulocytes prove to be a good indicator of 'drug priming', it would be extremely valuable if these measurements could be performed on a peripheral blood specimen rather than bone marrow specimens. Peripheral blood specimens were therefore obtained from mice treated in an identical manner. Animals were given a cyclophosphamide priming dose of 100 mg/kg with blood collected from the inferior cava following sacrifice of the animal by ether anaesthesia. Erythrocytes were lysed using a distilled water shock technique and remaining cells separated on the FACS as previously described for the bone marrow cells. Cellular distribution of peripheral blood samples are shown in figures 16.5 - control sample - and figure 16.6 - cyclophosphamide

| Cell Type        | Control | Cyclophosphamide<br>100 mg/kg |
|------------------|---------|-------------------------------|
| Erythrocytes     | 6.9     | 6.96                          |
| Lymphocytes      | 11.66   | 10.23                         |
| Anom. population | 14.5    | 14.16                         |
| Granulocytes i)  | 36.0    | 41.33                         |
| ii)              | 25.43   | 41.23                         |
| iii)             | 25.56   | 44.5                          |

Table 16.1 Glutathione levels in individual bone marrow cell populations separated using a Becton-Dickinson FACS. Values are expressed as emission units per  $10^6$  cells in both control and cyclophosphamide treated cells.



treated sample. Granulocytic glutathione levels are shown in table 16.2. Levels of glutathione measured in peripheral blood samples were lower than those detected in bone marrow granulocytes, although the degree of overshoot in levels from primed samples was similar. Unfortunately insufficient cells were obtained to measure granulocytic glutathione transferase levels from peripheral blood specimens.

#### 16.8 Glutathione in Peritoneal Granulocytes

Glutathione is a labile compound with autoxidation a serious problem in measurement. Separation using the FACS can take a long time, with autoxidation of glutathione potentially problematical. A method was utilised therefore to obtain large numbers of granulocytes rapidly for measurement of glutathione and glutathione transferase levels. Approximately  $10^7$  cells per mouse were obtained using this technique (Watt et al 1979), with a relatively high purity of granulocytes. Normal CBA mice were injected with 2 ml of 2% casein intraperitoneally. Animals were sacrificed 3 hours later and peritoneal lavage performed using ice-cold phosphate buffered saline. The cells were spun down at 250 g for 5 minutes and washed 3 times in

| Specimen                            | Control | Prime |
|-------------------------------------|---------|-------|
| 1) Marrow - (FACS)                  |         |       |
| glutathione                         | 27.0    | 39.0  |
| glutathione transferases            | 0.014   | 0.036 |
| 2) Peripheral blood - (FACS)        |         |       |
| glutathione                         | 22.77   | 32.5  |
| 3) Peritoneal - (Casein stimulated) |         |       |
| glutathione                         | 38.4    | 62.33 |
| glutathione transferases            | 0.020   | 0.029 |

Table 16.2 Effect of cyclophosphamide 100 mg/kg on murine granulocytic glutathione and glutathione transferase levels. Glutathione expressed as emission units per  $10^6$  cells, and glutathione transferase levels expressed as absorption per  $9 \times 10^6$  cells.

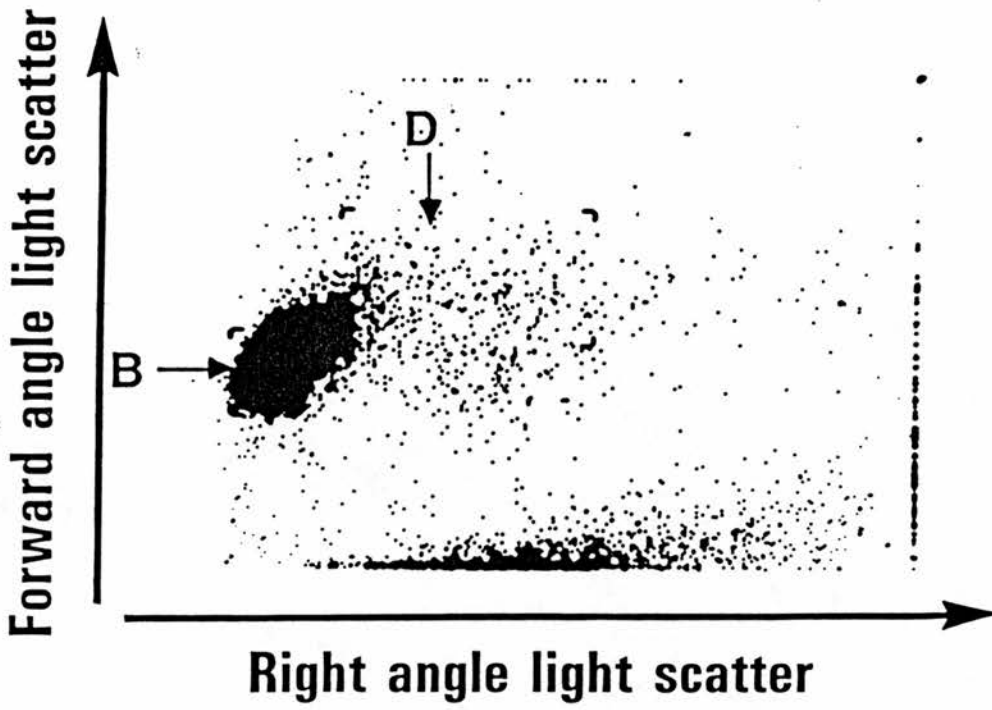


Figure 16.5 Becton-Dickinson FACS 'dot plots' of mouse peripheral blood from control animals. Group B - lymphocytes, Group D - granulocytes.

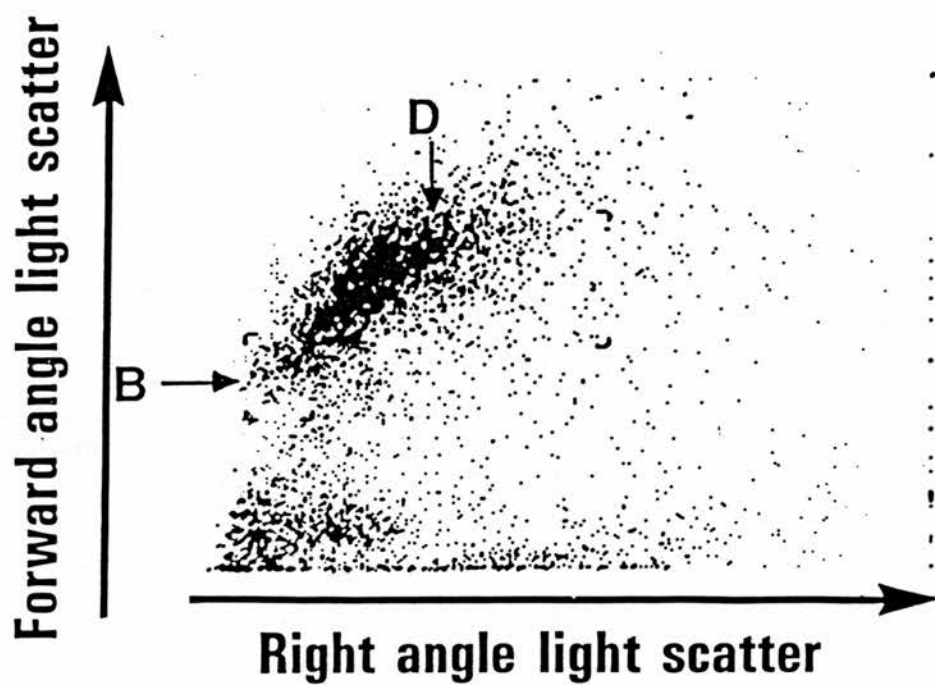


Figure 16.6 Becton-Dickinson FACS 'dot plots' of mouse peripheral blood from animals treated with cyclophosphamide 5 days previously.

ice-cold 0.168 M ammonium chloride. Cells were allowed to stand for 10 minutes in ammonium chloride to lyse erythrocytes. Samples were centrifuged at 250 g for 5 minutes, the resultant pellet resuspended in the phosphate buffer used for glutathione estimations, and the cells counted using a haemocytometer. The results are illustrated in table 16.2. Glutathione and glutathione transferase levels were higher using this technique, but whether this relates to the more rapid collection of these cells with minimal autoxidation of glutathione, or to the casein stimulation itself remains a matter of speculation. Despite this, increases in glutathione and glutathione transferase levels were detected in cyclophosphamide treated animals. These increases were proportionately similar to those observed using granulocytes obtained by FACS separation.

#### 16.9 Conclusions

Several methods have been described to separate bone marrow and peripheral blood specimens into their constituent cell types. This was achieved in these studies, with a high degree of purity, in that greater than 80% purity of cell type was estimated by histological examination of these samples.

On separating the bone marrow into 4 sub-populations, it was found that the highest glutathione and glutathione transferase levels were found in the granulocyte population. This is in keeping with previous reports on glutathione levels in haematopoietic cells. Following a cyclophosphamide priming dose, elevation of bone marrow glutathione levels can be attributed both to an increase of cells (granulocyte and granulocyte precursor cells) having high initial glutathione levels, and also to a true increase in glutathione levels in these cells. This is of great interest as it is the enhanced recovery of the granulocytic population which is the predominant factor associated with improved survival in primed animals.

Higher levels of glutathione were detected in the peritoneal casein stimulated granulocytes although it remains unresolved whether this increase relates to the casein stimulation itself, or whether this is merely a more valid representation of granulocytic glutathione levels. FACS separation can take up to 30 minutes to collect 300,000 cells, dependent on the cell concentration and it is possible that this delay results in the autoxidation of glutathione, with subsequent loss of activity. It is certainly suggestive, as peripheral blood granulocytes, which take the longest to collect, have the

lowest glutathione levels. Despite the loss of overall activity in peripheral blood samples, relatively similar increases in glutathione levels were detected in cyclophosphamide treated cells.

The measurement of glutathione or glutathione transferase levels in peripheral blood this could be of great importance clinically should drug priming and high dose chemotherapy become more widely used. Potentially, there could be a relatively simple assay on peripheral blood specimens from drug 'primed' patients to predict whether they are maximally protected against the toxic effects of high dose chemotherapy.

## CHAPTER 17

EFFECT OF INTERLEUKINS ON DRUG PRIMING17.1 Introduction

Over the past 30 years considerable interest has focussed on host response to infection. Phagocytic cells have been shown to secrete soluble products which have distinct biological properties. The term interleukin-1 (IL-1) is now used to describe a single molecule or family of molecules having these properties, including lymphocyte activating factor, endogenous pyrogen and leukocyte endogenous mediator. IL-1 has been shown to stimulate a granulocytic reaction in vivo, due to accelerated release of mature neutrophils from the bone marrow (Craddock et al 1956). Similarly, increased colony stimulating activity is noted with IL-1 whilst interleukin-3 (IL-3) has been shown to enhance differentiation of granulocytes.

One constant finding with the priming phenomenon is the more rapid recovery of peripheral blood elements, particularly granulocytes, in pre-treated animals. In view of the biological activities of these interleukins, it was considered



appropriate to assess whether IL-1 or IL-3 could protect mice by enhancing bone marrow recovery following a lethal dose of cyclophosphamide.

## 17.2 Materials and Methods

### 17.2a Animals

Male CBA mice 8-12 weeks of age were used in this study. Interleukins were injected subcutaneously at the appropriate times with saline being given to control animals.

### 17.2b Interleukins

Crude human IL-1 was obtained as described in Chapter 7.11 from concavalin A stimulated human monocytes. This was kindly provided by Dr Gordon Duff, Department of Rheumatology, Northern General Hospital, Edinburgh. Human IL-1 was used, as this substance has previously been shown to possess inter-species cross-reactivity. It was shown to be active in these animals by pyrogen testing.

WEHI-3B cells were used as a source of IL-3, as previously described (Ihle et al 1983), from conditioned media. This was kindly provided by Dr V. van Heynigen, Medical Research Council, Clinical Population & Cytogenetics Unit, Western General Hospital, Edinburgh

### 17.2c Experimental Design

For all experiments a minimum of 8 animals were used in each group. All interleukins were assessed for protection against a lethal dose of cyclophosphamide (350 mg/kg).

Four experiments were carried out using crude human IL-1. Various schedules for administration of the IL-1 were assessed. Timing of administration ranged from 48 hours before the cyclophosphamide injection to 48 hours after injection. Interleukin 3 was administered 3 times daily for 1-3 days prior to cyclophosphamide or for 24-48 hours after the cyclophosphamide injection.

### 17.3 Results

The results of these experiments are illustrated in Tables 17.1 and 17.2. Duration of survival was unaffected by any of the interleukin schedules used in these experiments, and there were no long-term survivors in any experiment.

#### 17.4 Discussion

The protection afforded to animals treated with a priming dose of cyclophosphamide is attributed, at least in part, to a more rapid recovery of peripheral granulocytes. Interleukins have been shown to increase peripheral granulocyte numbers, and in particular, the crude human IL-1 used in this study has been shown to produce a pyrexia and a granulocytic reaction, in the CBA mice used in this study some 12 hours following IL-1 administration.

Although it is accepted that by no means all potentially beneficial schedules were studied, this experiment suggests, that the enhanced granulocytic proliferation detected in primed animals is a secondary phenomenon and not the primary aetiology of this protection.

| Exp. No. | Group                 | Timing of IL-1 administration | Mean survival (days) |
|----------|-----------------------|-------------------------------|----------------------|
| 1        | Control               |                               | 6.6                  |
|          | IL-1 0.25 mls s/c     | Time 0 hrs                    | 9.0                  |
|          | IL-1 0.25 mls s/c     | Time -12 hrs                  | 7.125 NS             |
|          | IL-1 0.25 mls s/c     | Time -24 hrs                  | 7.25                 |
| 2        | Control               |                               | 8.9                  |
|          | IL-1 0.25 mls s/c x 2 | Time -24 hrs                  | 7.5 NS               |
|          | IL-1 0.25 mls s/c x 2 | Time -48 hrs                  | 6.5                  |
| 3        | IL-1 0.25 mls s/c x 2 | Time -24 hrs                  | 9.6 NS               |
|          | IL-1 0.25 mls s/c x 2 | Time -48 hrs                  | 7.0                  |
| 4        | Control               |                               | 9.4                  |
|          | IL-1 0.25 mls s/c x 2 | Time +24 hrs                  | 10.1 NS              |
|          | IL-1 0.25 mls s/c x 2 | Time +48 hrs                  | 7.5                  |

Table 17.1 Effect of crude human IL-1 on the survival of CBA mice following a lethal dose of cyclophosphamide (350 mg/kg).

| Exp. No. | Group                        | Timing of IL-1 administration | Mean survival (days) |    |
|----------|------------------------------|-------------------------------|----------------------|----|
| 1        | Control                      |                               | 6.6                  |    |
|          | IL-3 0.25 mls tds for 1 day  | -24 hrs                       | 10                   |    |
|          | IL-3 0.25 mls tds for 2 days | -48 hrs                       | 7                    | NS |
|          | IL-3 0.25 mls tds for 3 days | -72 hrs                       | 11                   |    |
| 2        | Control                      |                               | 8.9                  |    |
|          | IL-3 0.25 mls bd for 1 day   | +24 hrs                       | 9.8                  | NS |
|          | IL-3 0.25 mls bd for 2 days  | +48hrs                        | 7.25                 |    |

Table 17.2 Effect of conditioned media from WeH, 3b cells IL-3 on the survival of CBA mice following a lethal dose of cyclophosphamide (350 mg/kg).

## CHAPTER 18

EFFECT OF ACROLEIN ON PROTECTION AFFORDED BY LOW DOSE CYCLOPHOSPHAMIDE18.1 Introduction

Cyclophosphamide is activated in the liver to 4-hydroxycyclophosphamide. Thereafter, it is further metabolised with the most active cytotoxic metabolite considered to be phosphoramidate mustard. With the production of this metabolite, equimolar amounts of the toxic metabolite acrolein are produced, the latter responsible for the bladder toxicity associated with cyclophosphamide. Acrolein is a highly reactive molecule which has a high affinity for glutathione, and its toxicity can be prevented by treatment with sulphhydryl containing compounds such as mesna and N-acetyl-cysteine.

It was, therefore, of great interest to ascertain whether acrolein or phosphoramidate mustard was responsible for the increase in glutathione levels observed following drug priming, and similarly, which metabolite was responsible for the protective effect on survival following the lethal cyclophosphamide dose.

## 18.2 Protective Effect of Acrolein Pretreatment

### 18.2a Materials and Methods

Groups of 10 male CBA mice were used for lethality studies, as previously described. A lethal dose of cyclophosphamide was used (350 mg/kg), with or without acrolein pretreatment, at a dose of either 3.1 mg/kg or 6.2 mg/kg, with acrolein given intraperitoneally 5 days before cyclophosphamide. An acrolein dose of 7.4 mg/kg was lethal to 50% of the mice but no animals died following doses of 3.1 mg/kg or 6.2 mg/kg.

### 18.2b Results

The effect of acrolein pre-treatment on animal survival following a lethal dose of cyclophosphamide is shown in figure 18.1. Acrolein 3.1 mg/kg was inactive, but significant protection was afforded by the higher dose of 6.2 mg/kg ( $p < 0.05$ ). However, neither dose was as effective as 75 mg/kg cyclophosphamide.

### 18.3 Effect of Acrolein on Glutathione and Glutathione Transferase Levels

#### 18.3a Materials and Methods

Glutathione and glutathione transferase levels were measured in murine tissues following the administration of acrolein 6.2 mg/kg. The experiment was performed in an identical fashion to the cyclophosphamide time course experiment described in Chapter 15. Animals were sacrificed and tissues excised at the following time points; 13 hours, 24 hours, 48 hours, 72 hours, 96 hours, 120 hours and 144 hours. At these time points, livers, femurs and bladders were excised from these animals and collected in groups of 3. Liver and bone marrow samples were prepared as previously described (Chapter 7). Complete bladders were excised, scissor minced in ice-cold phosphate buffer and then homogenised using a Polytron homogeniser. Specimens were then centrifuged at 30,000 g for 30 minutes at 4<sup>0</sup>C with the cytosolic fraction used for glutathione and glutathione transferase assays.

The effect of acrolein 6.2 mg/kg on glutathione and glutathione transferases levels was assessed in control and cyclophosphamide primed animals. The following groups were studied:



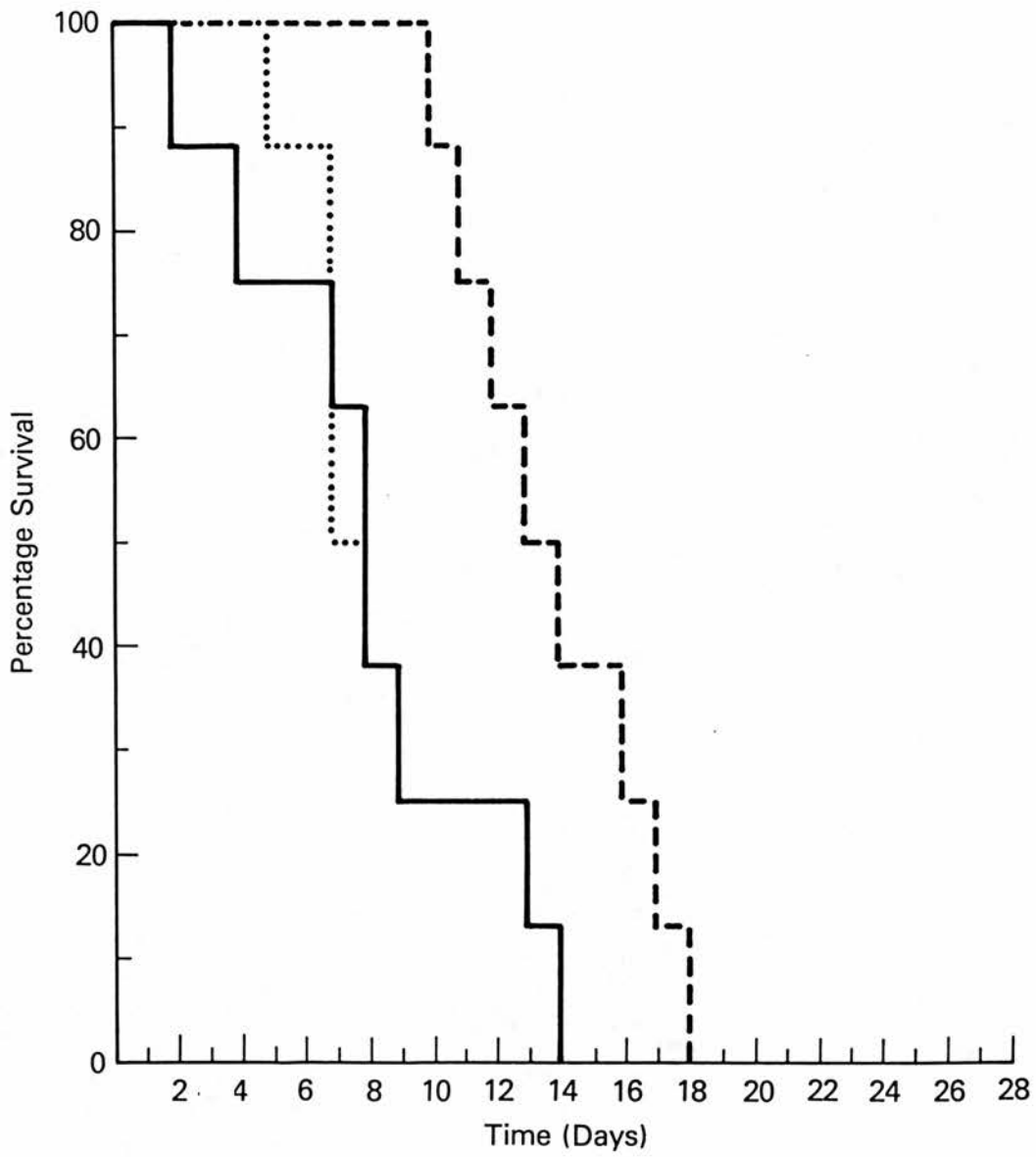


Figure 18.1 Effect of acrolein as a priming agent given 5 days before a lethal dose of cyclophosphamide (350mg/kg) in CBA mice: — control;..... Acrolein 3.1 mg/kg; --- Acrolein 6.2 mg/kg.

- (i) Control - saline 0.2 ml i.p.
- (ii) Acrolein - 6.2 mg/kg i.p.
- (iii) Cyclophosphamide 75 mg/kg i.p. 5 days before acrolein  
6.2 mg/kg i.p.
- (iv) Cyclophosphamide 75 mg/kg i.p., 5 days before saline  
0.2 ml i.p.

### 18.3b Results

Figure 18.2 shows the effect of acrolein 6.2 mg/kg on bone marrow glutathione levels. It can be seen that the glutathione level is initially depleted, with recovery and subsequent overshoot by day 5, similar to the results obtained following a cyclophosphamide priming dose as described in Chapter 15. Cyclophosphamide priming prevented the initial depletion of glutathione seen with acrolein alone. Cyclophosphamide priming, in addition to N-acetylcysteine and mesna treatment, all impaired the subsequent overshoot of glutathione levels observed in bone marrow samples treated with acrolein alone. . Similar trends were observed with glutathione transferase levels, the results being illustrated in figure 18.3. Single agent treatment with N-acetyl-cysteine or mesna, did not significantly affect glutathione or glutathione transferase levels in these animals.

N-acetyl cysteine and mesna were both administered on two occasions at doses of 200 mg/kg. N-acetyl cysteine was given 30 minutes before and 30 minutes after acrolein treatment, whereas mesna was given 30 minutes before and 6 hours after acrolein. Both of these compounds prevented the initial depletion of glutathione and glutathione transferase levels in acrolein treated mice, and likewise the subsequent overshoot in values was impaired.

#### 18.4 Conclusions

Although the protective effect of acrolein was inferior to cyclophosphamide at priming concentrations, it remains very interesting that this toxic compound can protect at all. In addition, it is possible that greater protection could have been achieved with a different dosage, or perhaps with a slow infusion of acrolein. Certainly, the acrolein produced in the metabolism of cyclophosphamide is assimilated less rapidly. Bladder glutathione levels are shown in figure 18.4. An increase in glutathione level is noted at day 6, following acrolein treatment although this was the only time point at which a significant elevation was observed. A similar increase was seen in glutathione transferase levels, although great variation was seen from day to day in this experiment. This

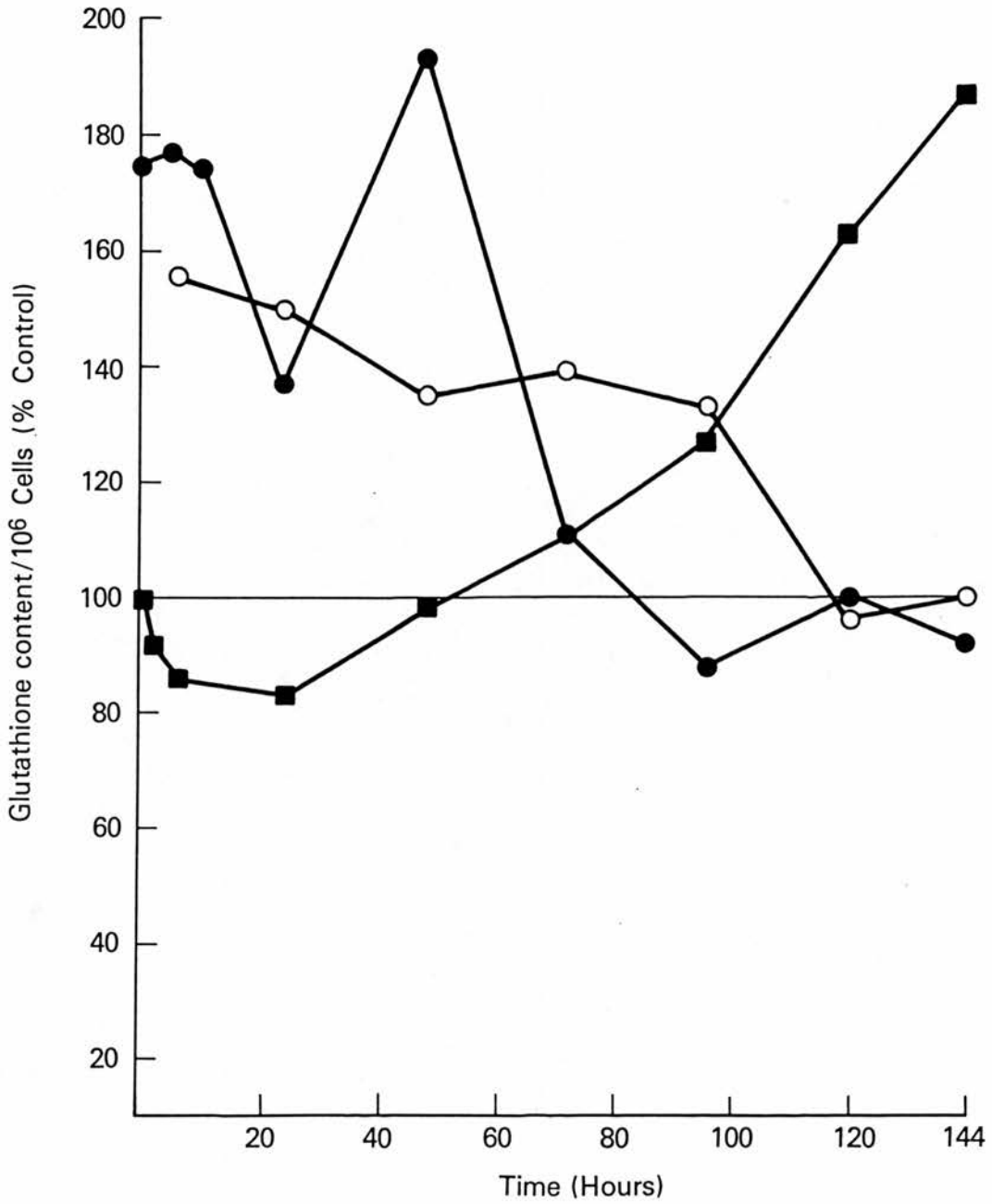


Fig. 18.2 Effect of acrolein treatment on bone marrow glutathione content in CBA mice: ●—● cyclophosphamide 75mg/kg + acrolein 6.2mg/kg ○—○ cyclophosphamide 75mg/kg (-5 days) ■—■ Acrolein 6.2 mg/kg.

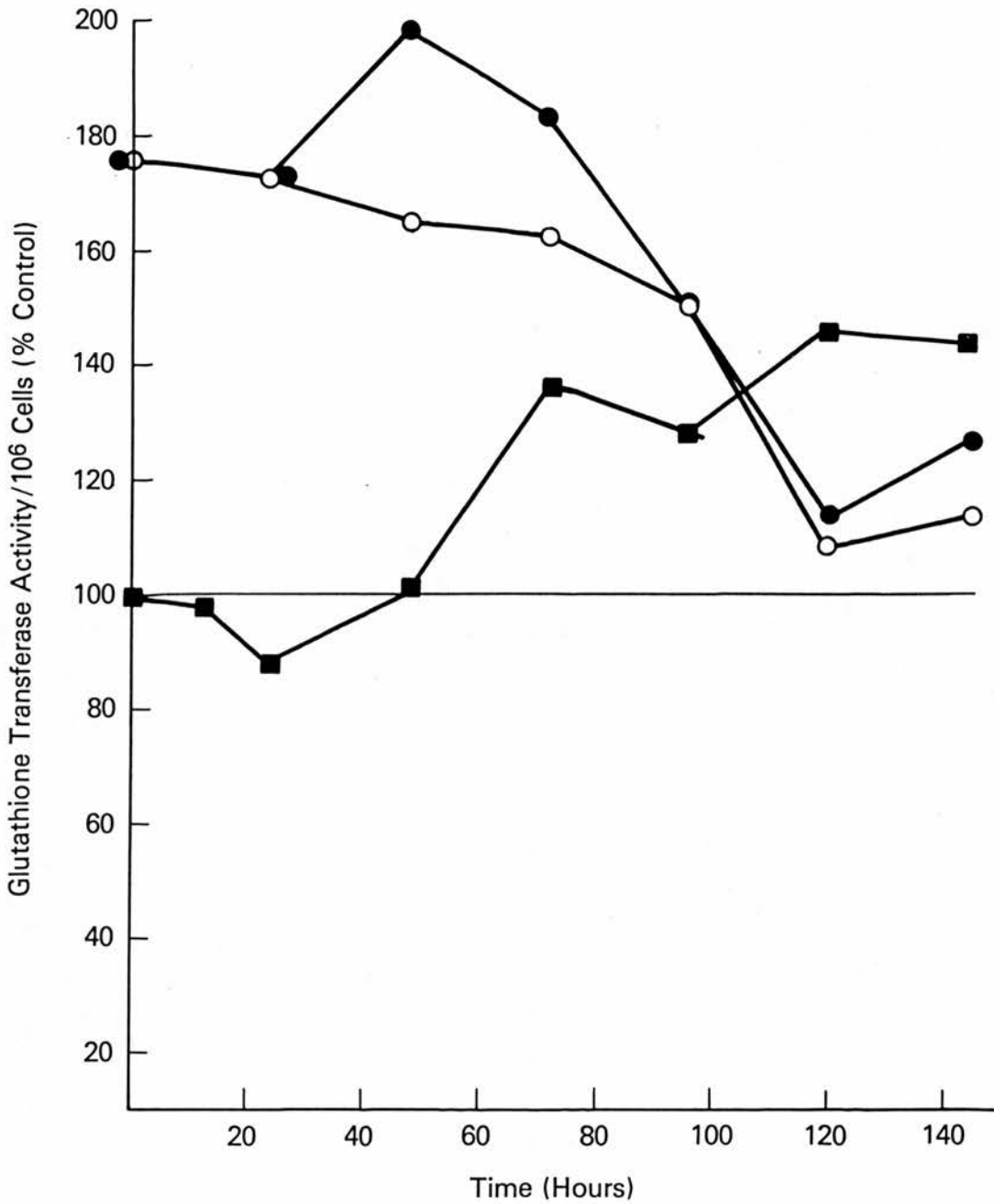


Fig. 18.3 Effect of acrolein treatment on bone marrow glutathione-S-transferase activity in CBA mice: ●—● cyclophosphamide 75mg/kg -5 days + acrolein 6.2mg/kg ○—○ cyclophosphamide 75mg/kg -5 days ■—■ acrolein 6.2mg/kg.

experiment has been repeated and a qualitatively similar result achieved. Improvements are necessary in the preparation of bladder samples for this assay, before reliable results can be achieved or any worthwhile conclusions drawn. However consideration has to be given to the noxious nature of acrolein which precludes the frequent repetition of this experiment.

Changes in bone marrow glutathione and glutathione transferase levels are seen following acrolein therapy. These changes are virtually identical to those seen after cyclophosphamide priming, both in terms of degree and timing of the response. It seems likely that the change in glutathione level observed following cyclophosphamide treatment relates, at least in part, to acrolein production in view of the high affinity of this compound for glutathione. It would have been interesting to confirm this hypothesis by performing a similar experiment with phosphoramidate mustard, but unfortunately this compound is extremely unstable.

Elevations in bladder glutathione and glutathione transferase levels were seen on day 6 following acrolein treatment, but this experiment should be repeated when techniques have been improved for the preparation of bladder specimens. Any changes in bladder glutathione or glutathione transferase levels would be of great interest in view of the observed

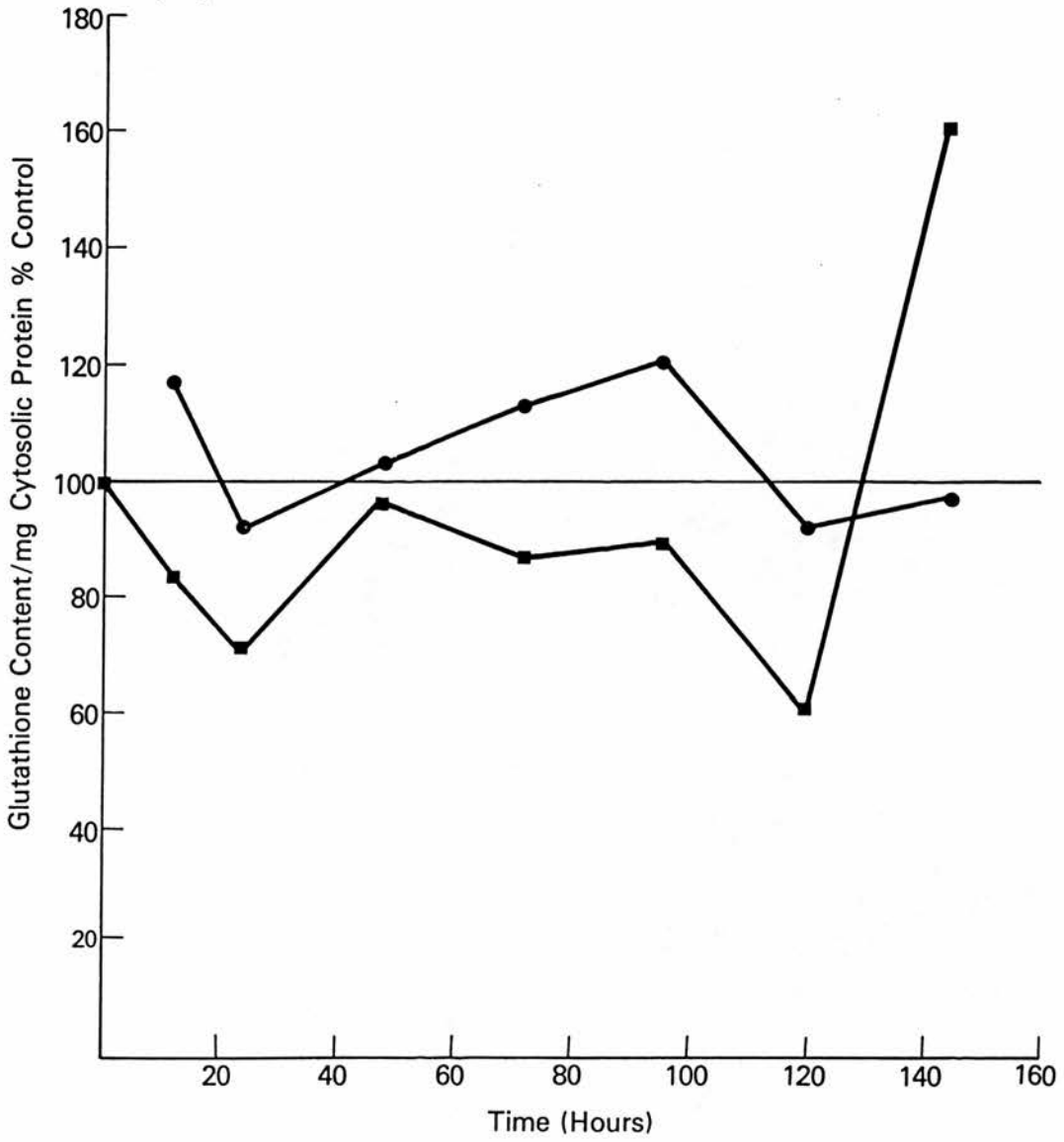


Fig. 18.4 Effect of acrolein treatment on bladder glutathione content in CBA mice:  
■ acrolein 6.2mg/kg ● cyclophosphamide 75mg/kg-5 days + acrolein 6.2 mg/kg.

improvement in the pathological appearances of bladder tissue in cyclophosphamide primed animals subsequently challenged with a high dose of the same compound.



**CHAPTER 19****TUMOUR GLUTATHIONE AND GLUTATHIONE TRANSFERASE LEVELS****19.1 Introduction**

Modulation of cytotoxic drug toxicity could prove to be a very important development in cancer chemotherapy. However, the clinical role for drug priming remains undetermined until the effect of this on tumour tissue is fully evaluated. Obviously, the potential for 'priming' tumour tissue is theoretically possible. Should this be substantiated then the beneficial effects of drug priming would be negated, resulting in an unchanged therapeutic index. Therefore the effect of cytotoxic drugs on tumour glutathione and glutathione transferase levels was investigated in a number of experiments.

Initial experiments were performed with human lung cancer lines, to determine the effect of cell type on glutathione and glutathione transferase levels. Experiments were then performed to determine whether glutathione levels in liver or bone marrow were affected by immunosuppressive procedures. Following this, experiments were carried out to measure glutathione levels in human lung cancer xenografts of different histologies. The effect of maximum tolerated doses

of various cytotoxic drugs on tumour glutathione levels was then assessed and finally, experiments were performed to determine whether tumour tissue can be primed by low doses of alkylating agents.

## 19.2 Glutathione and Glutathione Levels in Human Tumour Cell Lines

### 19.2a Materials and Methods

The human tumour cell lines were kindly provided by Dr A. F. Gazdar, NCI-Navy Medical Oncology Branch, National Cancer Institute, Bethesda, Maryland, USA. Thirty-one lung cancer cell lines were used, 16 small cell lung cancer cell lines and 15 non-small cell lung cancer cell lines. Of the small cell lines, 6 were derived from patients who had received no chemotherapy, prior to establishment of the cell line, whereas the remainder were derived from previously treated patients. Three cell lines exhibited characteristics of the variant phenotype (Gazdar et al 1985). The fifteen non-small cell lines comprised of 4 adenocarcinoma, 3 large cell carcinoma, 2 adenosquamous, 2 squamous, 2 bronchiolo-alveolar and 2 mesothelioma cell lines.

All cell lines, apart from 1 small cell line NCI-H510, were grown in Roswell Park Memorial Institute (RPMI) medium (Gibco) supplemented with 10% (v/v) foetal calf serum with added penicillin and streptomycin. NCI-H510 cells were grown in the serum-free defined medium - HITES (Simms et al 1980). All cell lines were shown to be mycoplasma free and were maintained in exponential growth phase. Cells were disaggregated and passaged 1 week before and fresh medium added 48 hours before harvesting of the cells for all assays.

All small cell lines, apart from NCI-H841, grew as floating aggregates. They were harvested on the day of the experiment, centrifuged at 250 g for 5 minutes, washed 3 times in ice-cold phosphate buffered saline, then resuspended in 2 ml of the appropriate solution. Glutathione assays were performed using a modification of the Tietze assay (1969). Following washing in ice-cold phosphate buffered saline, cells were resuspended in 2 ml of 0.6% sulphosalicylic acid, sonicated for 5 seconds, then centrifuged at 650 g for 5 minutes at 4°C. The supernatant was assayed for glutathione content, and the residual cell pellet assayed for protein. (Lowry et al 1951). For glutathione transferase estimations, cells were resuspended in phosphate buffer, and stored at -70°C awaiting completion of the assay.

All non-small cell lung cancer lines grew as monolayer cultures. Cells were detached from the plastic and disaggregated using a 5 minute trypsin wash at 37<sup>0</sup>C. Trypsin was then inactivated by adding an equal volume of serum containing medium. Thereafter, cells were treated in a similar manner to the small cells described above.

#### 19.2b Results

Glutathione levels for the small cell lines are listed in Table 19.1 with duplicate values for each cell line. Duplicate assays were performed on cells from different tissue culture flasks. Table 19.2 lists glutathione levels for the non-small cell lung cancer cell lines. Glutathione levels were found to be higher in the more resistant non-small cell lines, with particularly high values in squamous carcinoma and mesothelioma cell lines. Glutathione transferase levels for all 31 cell lines are illustrated in Table 19.3.

### 19.3 Effect of Immune-Deprivation on Tissue Glutathione Levels

#### 19.3a Materials and Methods

Hepatic and bone marrow glutathione levels were measured in stock CBA mice using the fluorometric assay of Hissin and Hilf (1976). Four mice per group were used in the following groups;

- (1) Control untreated mice
- (2) Thymectomised, irradiated mice
- (3) Tumour-bearing, thymectomised, irradiated mice.

Animals were sacrificed by cervical dislocation, and specimens collected, as described in Chapter 7.

#### 19.3b Results

Hepatic and bone marrow glutathione levels are shown in Table 19.4 for all 3 groups. No difference was observed between control mice and mice immune-deprived by means of thymectomy and total body irradiation. However, significantly depleted hepatic glutathione levels were detected in tumour bearing immune-deprived mice, although normal bone marrow values were observed in these mice.

| <u>Cell Line</u> | <u>Cell Type</u>   | <u>Glutathione</u><br>(nanomoles/mg protein) |               |
|------------------|--------------------|--|---------------|
|                  |                    | <u>Exp. 1</u>                                | <u>Exp. 2</u> |
| NCI-H187         | classic, untreated | 43.62  | 53.46         |
| NCI-H209         | classic, untreated | 48.2   | 51.8          |
| NCI-H526         | variant, untreated | 59.7   | 70.85         |
| NCI-H678         | classic, untreated | 37.92  | 40.34         |
| NCI-H719         | classic, untreated | 77.4   | 62.61         |
| NCI-H889         | classic, untreated | 41.43  | 75.6          |
| NCI-H60          | classic, treated   | 67.57  | 65.27         |
| NCI-H69          | classic, treated   | 67.9   | 47.56         |
| NCI-H82          | variant, treated   | 26.24  | 25.39         |
| NCI-H128         | classic, treated   | 34.44  | 34.47         |
| NCI-H146         | classic, treated   | 32.08  | 44.48         |
| NCI-H249         | classic, treated   | 27.26  | 44.41         |
| NCI-H345         | classic, treated   | 50.35  | 58.38         |
| NCI-H510         | classic, treated   | 49.86  | 93.15         |
| NCI-H524         | variant, treated   | 11.48  | 39.03         |
| NCI-H841         | variant, treated   | 56.8   | 58.8          |

Table 19.1 Glutathione levels measured in human small cell lung cancer cell lines.

| <u>Cell Line</u> | <u>Histology</u>   | <u>Glutathione</u><br>(nanomoles/mg protein) |               |
|------------------|--------------------|--|---------------|
|                  |                    | <u>Exp. 1</u>                                | <u>Exp. 2</u> |
| NCI-H23          | adenocarcinoma     | 116.4  | 120.7         |
| NCI-H125         | adenocarcinoma     | 130.9  | 126.6         |
| NCI-H522         | adenocarcinoma     | 82.0   | 29.52         |
| A549             | adenocarcinoma     | 130.9  | 85.28         |
| NCI-H157         | large cell         | 92.36  | 70.19         |
| NCI-H460         | large cell         | 157.3  | 203.4         |
| NCI-H661         | large cell         | 62.3   | 98.1          |
| NCI-H596         | adenosquamous      | 144.0  | 230.9         |
| NCI-H647         | adenosquamous      | 162.0  | 192.2         |
| NCI-H322         | bronchioloalveolar | 176.5  | 177.4         |
| NCI-H358         | bronchioloalveolar | 145.6  | 151.5         |
| NCI-H226         | squamous           | 181.4  | 210.2         |
| NCI-H520         | squamous           | 203.4  | 225.33        |
| NCI-H290         | mesothelioma       | 172.2  | 263.7         |
| JMN              | mesothelioma       | 288.0  | 97.0          |

Table 19.2 Glutathione levels measured in human non-small cell lung cancer cell lines.

| <u>Cell Line</u> | <u>Cell Type</u> | <u>Glutathione Transferase</u><br>(Act/min/mg/protein) |                     |
|------------------|------------------|--|---------------------|
|                  |                  | <u>Experiment 1</u>                                    | <u>Experiment 2</u> |
| NCI-H187         |                  | 0.894  | 0.36                |
| NCI-H209         | Small cell       | 1.02   | 1.402               |
| NCI-H526         | lung cancer      | 3.81   | 3.48                |
| NCI-H678         | (untreated)      | 1.09   | 1.56                |
| NCI-H719         |                  | 0.971  | 1.06                |
| NCI-889          |                  | 0.34   | 0.3                 |
| NCI-H60          |                  | 0.495  | 0.25                |
| NCI-H69          | Small cell       | 1.15   | 1.522               |
| NCI-H82          | lung cancer      | 0.645  | 0.745               |
| NCI-H128         | (treated)        | 1.75   | 1.85                |
| NCI-H146         |                  | 0.933  | 0.966               |
| NCI-H249         |                  | 0.49   | 0.909               |
| NCI-H345         |                  | -  | -                   |
| NCI-H510         |                  | 2.82   | 3.52                |
| NCI-H524         |                  | -  | -                   |
| NCI-H23          | adeno-           | 6.44   | -                   |
| NCI-H125         | carcinoma        | 1.66   | -                   |
| NCI-522          |                  | 3.76   | 2.835               |
| A549             |                  | 0.74   | -                   |
| NCI-H157         | large cell       | 1.47   | -                   |
| NCI-H460         |                  | 1.51   | 1.301               |
| NCI-H661         |                  | 3.06   | -                   |
| NCI-H596         | adeno-           | 0.5  | -                   |
| NCI-H647         | squamous         | 3.75   | 2.29                |
| NCI-H322         | bronchiolo-      | 0.64   | 1.15                |
| NCI-H358         | alveolar         | 2.11   | 1.2                 |
| NCI-H226         | squamous         | 1.52   | -                   |
| NCI-H520         |                  | 7.47   | 5.81                |
| NCI-H290         | mesothelioma     | 1.04   | 1.11                |
| JMN              |                  | 0.712  | -                   |

Table 19.3 Glutathione transferase levels in human lung cancer cell lines.



#### 19.4 Tumour Glutathione Levels

Glutathione levels were found to vary between tumours. This variation was evident between tumours of the same histological type, and also between different tumour types. Glutathione levels of approximately 16 nanomoles/mg protein were detected, although variation between tumours was approximately 25%. Mean glutathione levels were determined in 4 tumour lines with the highest value detected in NX 002 xenografts, 24.2 nanomoles/mg protein, with CX 140 16.2 nanomoles/mg protein, CX 143 10.73 nanomoles/mg protein and CX 117, 6.66 nanomoles/mg protein. However, a number of factors can affect these levels, however. The size and growth rate of the tumours are important, in addition to the nutritional status of the host animal.

#### 19.5 Effect of Maximum Tolerated Doses of Cytotoxic Drugs on Tumour Glutathione Levels

##### 19.5a Materials and Methods

Three human non-small cell lung cancer xenograft lines were used for these studies; NX 002, and CX 140, both moderately differentiated squamous carcinomas and CX 143, an

| <u>Group</u>                          | <u>Liver</u>      | <u>Bone Marrow</u>                                    |
|---------------------------------------|-------------------|---|
| 1. Control                            | 2.28 mM $\pm$ 8%  | 3.87 x 10 <sup>-10</sup> micromoles/cell<br>$\pm$ 10% |
| 2. Thymectomised/<br>irradiated       | 2.04 mM $\pm$ 12% | 4.7 x 10 <sup>-10</sup> micromoles/cell<br>$\pm$ 8%   |
| 3. Tumour-bearing/<br>Immune-deprived | 1.36 mM $\pm$ 15% | 4.8 x 10 <sup>-10</sup> micromoles/cell<br>$\pm$ 10%  |

Table 19.4 Hepatic and bone marrow glutathione levels in; (1) control CBA mice; (2) immune-deprived CBA; (3) tumour-bearing, immune-deprived mice.

adenosquamous carcinoma. Mice were stratified into groups according to sex and to tumour size. For each xenograft line the following groups were studied:-

- (i) Control
- (ii) Cyclophosphamide 200 mg/kg i.p.
- (iii) Vindesine 2 mg/kg i.p.
- (iv) Cis-platinum 7 mg/kg i.p.

Using 4 animals per group, maximum tolerated doses of cytotoxic drugs were administered intra-peritoneally 48 hours prior to sacrifice of the mice. Tumours were prepared for analysis as described in Chapter 7, with bone marrow samples obtained simultaneously. Glutathione levels were performed using the method described by Hissin and Hilf (1976).

#### 19.5b Results

Glutathione levels, expressed as a percentage of control are illustrated in Table 19.5. Bone marrow glutathione levels were depleted by all drugs, although less depletion was observed with cis-platinum. A slightly different picture emerged on analysis of tumour glutathione levels. Tumour glutathione levels were unaffected by vindesine, which was

found to be inactive in these xenografts. Likewise, cis-platinum was found to be inactive against the adenosquamous xenograft CX 143, with no depletion of glutathione observed in these tumours. In contrast, cis-platinum, which had the highest activity of any single agent in the xenograft line, NX 002, causing a growth delay of 2.0 doubling times depleted glutathione levels to 56.2% of control in this line.

However, no detectable change in glutathione levels was observed in the CX 140 xenograft line, despite marginal activity with cis-platinum. Conversely, cyclophosphamide, which had minimal effect on the growth of NX 002 xenografts, was shown to deplete glutathione levels to 68% of control in these tumours.

#### 19.6 Effect of Priming Doses of Cyclophosphamide on Tumour Glutathione and Glutathione Transferase Levels

The effect of cyclophosphamide (75 mg/kg) on tumour glutathione and glutathione transferase levels was studied in three non-small cell lung cancer xenografts, and in small cell lung cancer xenografts kindly supplied by Dr A F Gazdar, Navy Medical Oncology Branch, National Cancer Institute, Bethesda, USA.

| <u>Xenograft</u> | <u>Treatment</u>           | <u>Tumour<br/>Glutathione<br/>(% control)</u> | <u>Bone Marrow<br/>Glutathione<br/>(% control)</u> |
|------------------|----------------------------|---|--|
| NX 002           | control                    | 100   | 100  |
|                  | cyclophosphamide 200 mg/kg | 68.3  | 25.3   |
|                  | vindesine 2 mg/kg          | 89.5  | 56.0   |
|                  | cis-platinum 7 mg/kg       | 56.2  | 49.3   |
| CX 143           | control                    | 100   | 100  |
|                  | cyclophosphamide 200 mg/kg | ND  | ND   |
|                  | vindesine 2 mg/kg          | 82.9  | 50.6   |
|                  | cis-platinum 7 mg/kg       | 83.0  | 79.5   |
| CX 140           | control                    | 100   | 100  |
|                  | cyclophosphamide 200 mg/kg | 86.8  | 47.0   |
|                  | vindesine 2 mg/kg          | 119.0   | -  |
|                  | cis-platinum 7 mg/kg       | 92.9  | 76.0   |

Table 19.5 Effect of maximum tolerated doses of cytotoxic drugs on tumour and bone marrow glutathione levels in tumour-bearing immune-deprived CBA mice.

## 19.6a Materials and Methods

### (i) Non-small cell lung cancer xenografts

Immune-deprived CBA mice, as previously described for the xenograft studies, were used. Three xenograft lines were studied, NX 002, CX 117 and CX 143. For each part of this experiment animals of the same age and sex were used, although both male and female mice were used at different stages. The mice were immune-deprived and transplanted with tumour on the same day. Tumours of 0.3 cm<sup>3</sup> or greater were included with equivalent numbers of tumours included in both treated and control groups. Groups were stratified according to tumour size with a minimum of 4 tumours included per group. The mice received either cyclophosphamide 75 mg/kg or saline i.p. For the pilot study using the xenograft line CX 117, animals were sacrificed on days 2, 4 and 5 after injection. Thereafter definitive studies were performed sacrificing mice on days 2, 6 or 10 following cyclophosphamide administration. Tumour samples were prepared for glutathione and glutathione transferase estimations with assays performed as described in Chapter 7. Simultaneously, femurs from these mice were removed and the bone marrow cells assayed for glutathione and glutathione transferase levels. Tumour levels were evaluated on a per milligram protein basis (Lowry et al 1951), with bone

marrow values expressed per  $10^6$  cells as before. All results were subsequently expressed as a percentage of control values.

(ii) Small Cell Lung Cancer Xenografts

This experiment was performed in an identical fashion to the previous experiment. Two small cell lung cancer cell lines were grown as xenografts in nude mice. NCI-H209 a cell line showing classical small cell features, and NCI-H82 a line exhibiting variant small cell morphology (Gazdar et al 1985; Carney et al 1985) were used for this study. NCI-H209 was derived originally from an untreated patient with NCI-H82 derived from a patient who had relapsed following treatment with cyclophosphamide, adriamycin, vincristine and procarbazine. The nude mice were housed in specific pathogen free conditions. Cyclophosphamide 75 mg/kg or saline, was administered intraperitoneally and mice subsequently sacrificed on days 2, 6 and 10 thereafter. Bone marrow and tumour specimens were prepared as previously described (Chapter 7).

19.6b Results

(i) Non-Small Cell Lung Cancer Xenografts

The pilot experiment, using CX 117, tumours confirmed the overshoot in bone marrow glutathione levels following low dose cyclophosphamide, with observed values relative to control of

64% on day 2, 110% on day 4, 140% on day 5 and 129% on day 6. However, no overshoot in glutathione levels was observed in tumour tissue. Initially, depletion of glutathione levels was observed (56% on day 2, 58% on day 4), although these returned to normal by day 5 (95% of control).

Bone marrow and tumour glutathione estimations from experiments using the xenograft lines NX 002 and CX 143 are shown in Figure 19.1, with levels illustrated in each group as a percentage of control. A decrease in bone marrow glutathione content was observed on day 2, followed by recovery and overshoot by day 6, with values returning to normal by day 10. This trend was similar for both lines, although absolute glutathione values differed. The observed glutathione overshoot (147%) was less than that previously seen in non-tumour bearing animals. A similar trend in glutathione transferase levels was seen, with the results from the NX 002 xenograft illustrated in Figure 19.2

Tumour glutathione levels show a different trend, however, with no depletion observed in these non-small cell xenografts on day 2. By day 6 tumour glutathione levels in the NX 002 group remained less than control values, with minimal



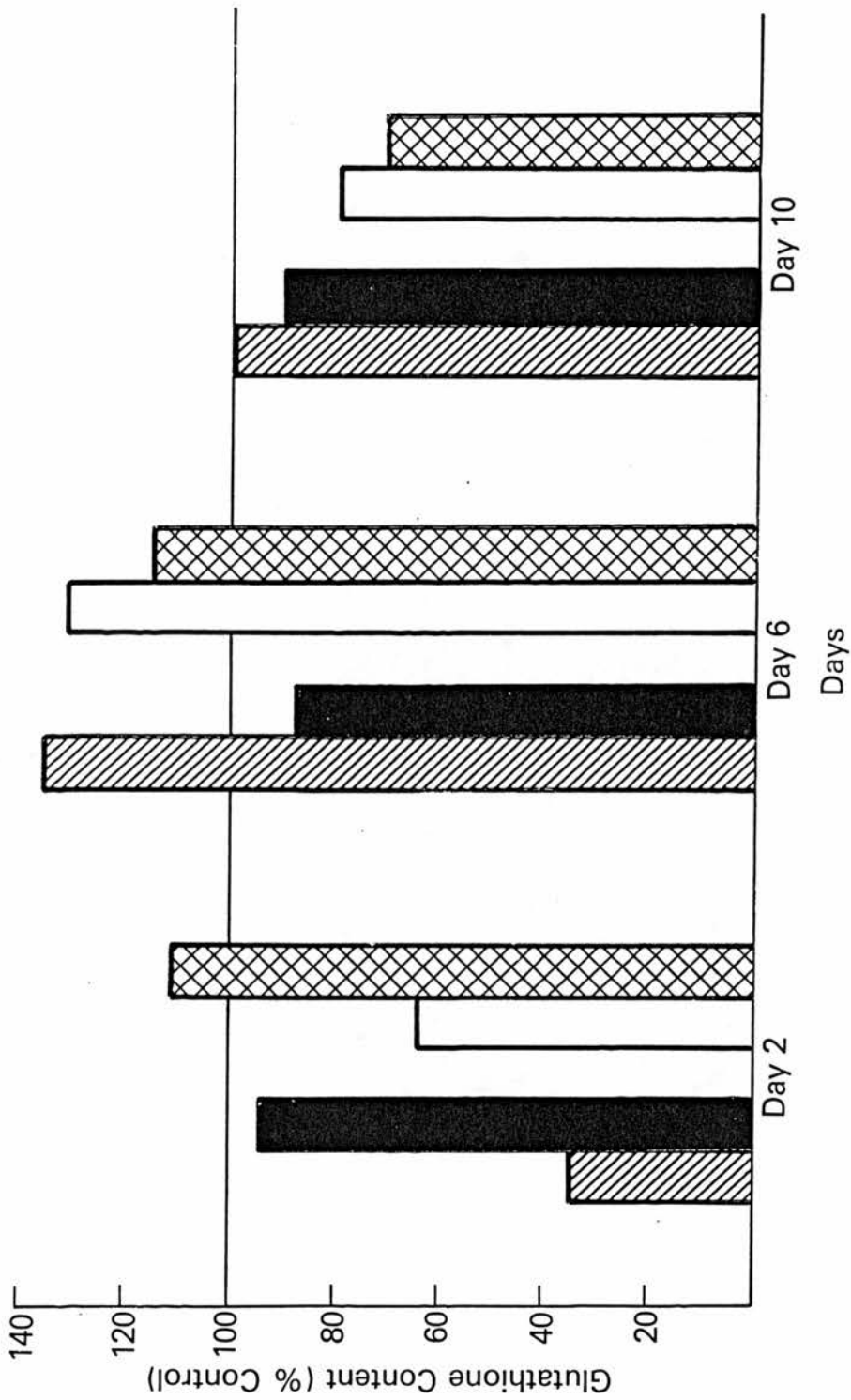


Fig. 19.1 Effect of cyclophosphamide priming on tumour and bone marrow glutathione levels in immune suppressed CBA mice bearing non-small cell lung cancer xenografts. ■ NX002 bone marrow ▨ NX002 tumour □ CX143 bone marrow ▩ CX143 tumour.

elevation observed in the CX 143 group (115% of control). Likewise, at day 10 no overshoot was observed, with no statistically significant difference in tumour glutathione levels noted between cyclophosphamide treated and control mice at any of the time points. Corresponding glutathione transferase values are illustrated in Figure 19.2. Once again no elevation of glutathione transferase level was observed at any of these time points.

(ii) Small cell lung cancer xenografts

Following the administration of cyclophosphamide 75 mg/kg, glutathione and glutathione transferase levels were measured in bone marrow cells and tumour tissue from nude mice bearing small cell lung cancer xenografts. The results are illustrated in Figure 19.3. Glutathione levels were measured using the method of Hissin & Hilf (1976) as previously described. As the specificity of this assay has been questioned, these results were validated using a more specific assay (Tietze 1969), measuring total glutathione by cyclic reduction of Elmans reagent glutathione reductase and NADPH. A similar trend was observed with both assays.

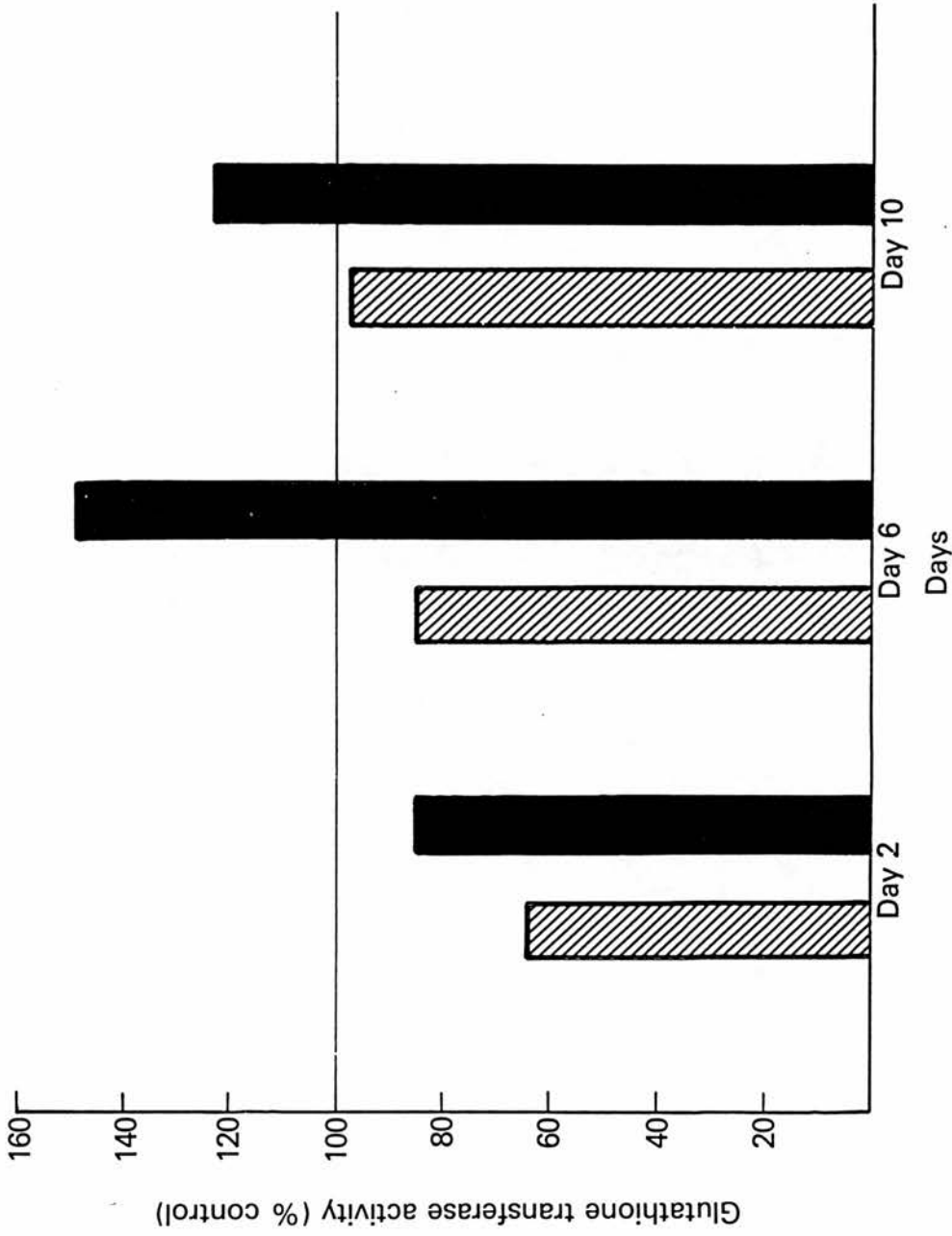


Fig. 19.2 Effect of cyclophosphamide priming on tumour and bone marrow glutathione transferase activity in immune suppressed CBA mice bearing non-small cell lung cancer xenografts. ■ Bone marrow Nx 002 ▨ Tumour

As is shown in Figure 19.3, using the assay described by Hissin and Hilf (1976), depletion of glutathione levels is observed in the bone marrow on day 2 with elevation of glutathione levels seen by day 6, reaching 122% in NCI-H82 and 146% in NCI-H209. These values had returned to normal by day 10. In tumour tissue, however, glutathione levels were essentially unchanged on day 2, with depletion of levels observed on days 6 and 10 in NCI-H209 and on day 10 with NCI-H82. No significant overshoot in glutathione levels was observed on day 6 in either xenograft line. Using the Tietze assay (1969), similar trends were observed. In bone marrow samples glutathione depletion was observed on day 2, in mice bearing NCI-H82 xenografts levels fell to 55%, and with NCI-H209 to 80% of control values. On day 6, elevation of glutathione to 142% and 127% of control levels were seen in NCI-H82 and NCI-H209 respectively although these values had returned to normal by day 10.

In tumour tissue from NCI-H82, glutathione levels were minimally depleted; day 2 88.7%, day 6 84.7% and day 10 88.7% of control. With NCI-H209, a more chemosensitive tumour, values of 72.7%, 65.6% and 95.2% of control were observed on days 2, 6 and 10 after cyclophosphamide 75 mg/kg.

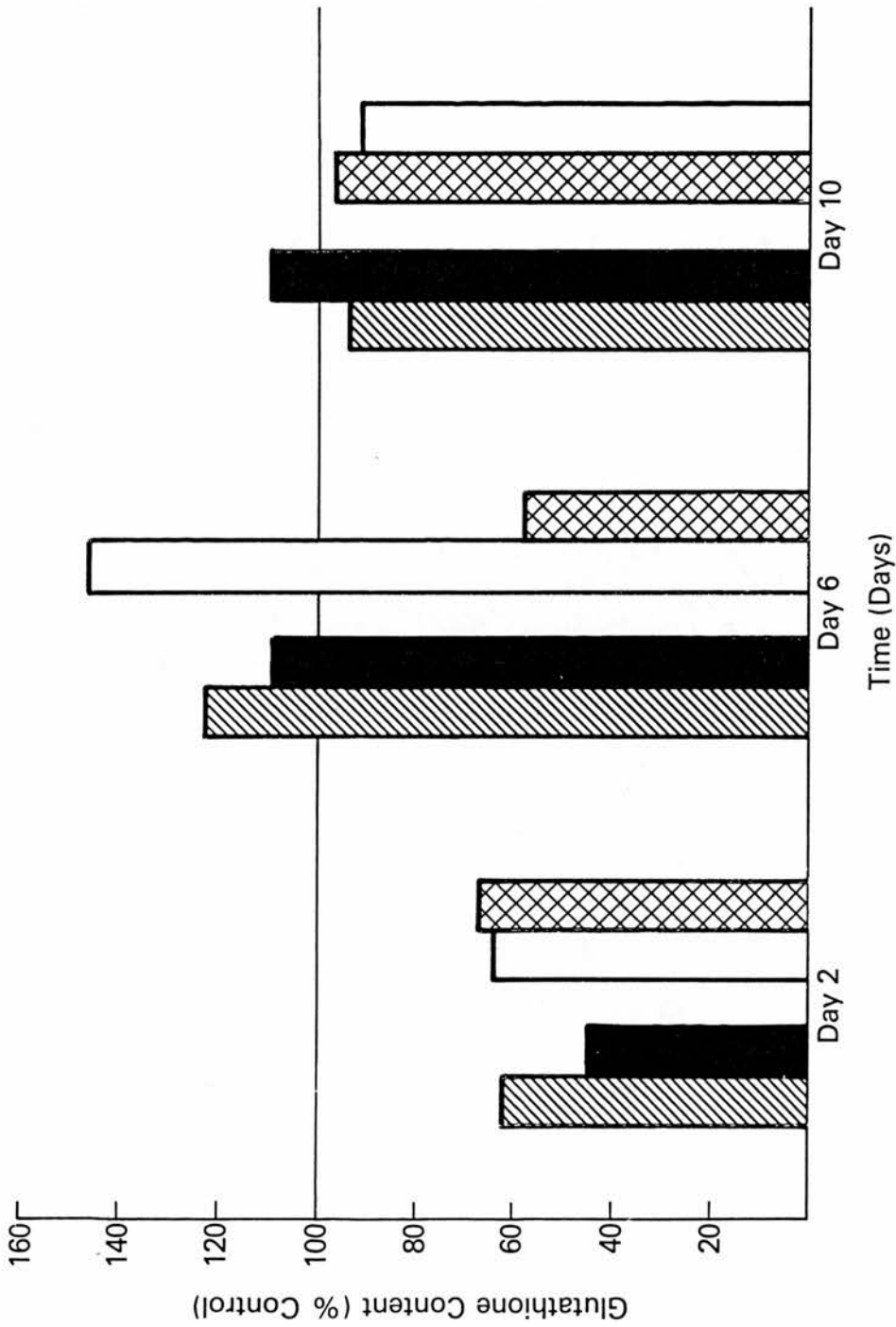


Fig. 19.3 Effect of cyclophosphamide priming on tumour and bone marrow glutathione levels in athymic nude mice bearing small cell lung cancer xenografts. ▨ NCI-H209 bone marrow ■ NCI-H209 tumour □ NCI-H82 bone marrow ▩ NCI-482 tumour.

Glutathione transferase levels were measured in the same samples. Depletion of bone marrow glutathione transferase levels were seen on day 2 following cyclophosphamide, 54% of control in NCI-H82 and 62% in NCI-H209. No significant overshoot in bone marrow or tumour glutathione transferase levels were observed during this experiment.

#### 19.7 Discussion

A number of interesting findings are presented in this chapter. The difference in basal glutathione levels between small cell and non-small cell lung cancer cell lines is extremely interesting. Non-small cell lung cancer is markedly more resistant to chemotherapy and radiotherapy than small cell lung cancer, with glutathione levels in the non-small cell lines almost universally higher. Of particular interest, mesothelioma cell lines had the highest glutathione levels, with this cell type highly resistant clinically. It was hoped that a similar difference would have been observed between small cell lines derived from treated and untreated patients, as the former are frequently resistant to chemotherapy. However, no obvious difference in glutathione levels was detected between these groups. It is possible, that when no longer stressed by cytotoxic drugs, that glutathione levels in

small cell lines derived from previously treated patients returned towards basal values. Alternatively, it is conceivable that clones of cells low in glutathione content grow out in culture. Certainly, in recent experiments performed by the author, using a human ovarian cancer cell line it was found that by second passage the glutathione level had fallen to 30% of the level detected in the original tumour tissue. Although glutathione transferase levels tended to be slightly higher in non-small cell lines, no obvious difference was observed between groups.

Immune-deprivation had minimal effect on hepatic glutathione but tumour bearing animals had significantly depleted values. No difference was noted in bone marrow glutathione levels in any of these groups, however. Glutathione levels varied between xenograft lines with also a significant variation in levels between tumours of the same xenograft line. Standard derivations of  $\pm 25\%$  were observed in these measurements. To obtain valid results, using tumour tissue, a number of factors require to be addressed. In particular, attention should be paid to the size and growth pattern of the tumour. Maximum tolerated doses of cytotoxic drugs caused depletion in tumour glutathione levels at 48 hours. This depletion bore some relationship to the chemo-

sensitivity of the tumour to that agent, but was not directly related as may have been predicted. Other factors, such as the affinity of the drug for glutathione, are also likely to be important.

In both the cyclophosphamide priming experiments elevation in bone marrow glutathione and glutathione transferase levels were observed in tumour-bearing animals 6 days after priming dose of cyclophosphamide. The magnitude of this overshoot was reduced in comparison with the studies carried out in normal CBA mice. It remains to be elucidated whether this reduction is a true difference, relating to alterations in the response normal tissues of tumour-bearing animals to cytotoxic drugs. Alternatively, in view of the time taken to process tumour samples this difference could merely indicate increased autooxidation of glutathione. Processing of the tumour samples caused a delay in the preparation of bone marrow samples which may have resulted in diminished glutathione levels. Further studies are indicated to resolve this question.

Depletion of tumour glutathione and glutathione transferase levels were seen on day 2 after the priming dose of cyclophosphamide (75 mg/kg) but no significant overshoot of



these values were observed on day 6 in tumour tissue, either in the relatively resistant non-small cell xenografts or in small cell lung cancer xenografts.

We know that we can protect animals from the lethal effects of cyclophosphamide by pre-treatment with a smaller priming dose. This protection is accompanied by elevation in glutathione and glutathione transferase levels in granulocytes, which recover more rapidly in pre-treated animals. From these studies, no significant elevation of glutathione or glutathione transferase levels are observed in tumour tissue at the time of maximal protection from the lethal effects of cyclophosphamide. Thus, from this aspect no priming effect is observed in tumour tissue. The potential remains therefore, to enhance the therapeutic index of cyclophosphamide by pretreatment with low doses.

Previously Jeney and Connors (1968) showed diminished activity of melphalan by pretreatment with low dose alkylating agents with activity assessed by measurement of DNA cross-links. The study suggested that tumour protection could occur with priming doses of alkylating agents. In contrast, Evans et al (1982) showed that much higher doses of cyclophosphamide were tolerated by mice when administered by divided dose schedule, with minimal loss of anti-tumour activity. The

findings in these experiments are more in keeping with the latter study. However, it has previously been recognised that not all alkylating agents can 'prime', and that molecular structure may be important. This could well be a significant factor in the protection afforded by cyclophosphamide with its unusual metabolism and toxicities.

**CHAPTER 20****MODULATION OF GLUTATHIONE LEVELS**20.1 Introduction

Glutathione levels have been shown to be important in the response of various tissues to both radiation and certain cytotoxic drugs. In general, reduction of glutathione levels will enhance the effect of many cytotoxic drugs, including adriamycin and cis-platinum, and also radiation, particularly in anaerobic conditions. There are a number of methods of depleting intracellular glutathione. Many toxic compounds will cause a non-specific decrease in glutathione level, although several agents are now available which will significantly deplete glutathione levels with a degree of selectivity. N-ethyl-maleamide depletes glutathione by forming covalent adducts with cellular thiols, including protein sulfhydryls. However, the high reactivity of N-ethyl-maleamide affords minimal specificity and considerable toxicity. Diethylmaleate is less reactive, and as it is a substrate for glutathione transferase, can more specifically deplete glutathione levels. Ethacrynic acid is converted to a substrate for glutathione transferase in hepatocytes, and has been used to deplete

glutathione in cell culture (Habig et al 1974; Klassen and Fitzgerald 1974). Another approach is to reduce glutathione to the disulphide with diamide the most popular reagent (Harris et al 1971), although this compound has been shown to exhibit non-specific reactivity (Harris and Biaglow 1972). Glutathione levels can also be reduced by limiting synthesis. This can be achieved by impairing the reductive capability of the cell either directly by inhibition of glutathione reductase with nitrosoureas (Babson et al 1981; Smith and Boyd 1984; Paraidathathu et al 1985) or indirectly by decreasing NADPH generation through inhibition of glucose-6-phosphate dehydrogenase with dihydroepiandrosterone (Lopes-S and Rene 1973). On the other hand, a more specific glutathione depletion is afforded by sulfoximine derivatives such as methionine and buthionine sulfoximine (Rowe et al 1969). These compounds directly inhibit  $\gamma$ -glutamyl cysteine synthetase with buthionine sulfoximine the drug of choice, as it has no other documented primary effects on the cell.

In contrast, fewer methods of increasing intracellular glutathione levels are available. Elevations of glutathione levels are observed following treatment with 2-oxothiazolidine-4-carboxylate (Boettcher and Meister 1984) which stimulates glutathione synthesis by providing high levels of intracellular

cysteine following cleavage with oxoprolinase. Unfortunately glutathione itself is a tripeptide that does not readily enter into the cell because of exofacial membrane non-permeability. It is known that the anionic function of the carboxyl group restricts the flow of glutathione into the cell. Removal of this anion can be accomplished by the production of a glutathione ester, which is permeable, and is cleaved intracellularly to glutathione. Monoethyl and diethylesters of glutathione were synthesized and supplied by Dr Angelo Russo of the National Cancer Institute, USA. These esters have been shown to be capable of increasing intracellular glutathione levels (Puri and Meister 1983; Wellner et al 1984).

In view of their relative specificity, two compounds were selected for further investigation. Buthionine sulfoximine was used to deplete glutathione levels, and the monoethylester of glutathione was used in an attempt to elevate intracellular glutathione levels.

The first experiment was designed to address the question of whether the increase in glutathione and glutathione transferase levels observed 5 days after a cyclophosphamide priming dose, related to a compensatory synthetic process, or merely represented survival of cells originally high in glutathione content. This was achieved with the use of

buthionine sulfoximine following the priming dose of cyclophosphamide. Further studies were then performed with buthionine sulfoximine to assess the degree and kinetics of glutathione depletion in different tissues.

A number of studies were carried out using the monoethyl ester of glutathione. Initial studies concentrated on validation of the structure of the ester. Thereafter, assessments were made of the activity of the ester both in vitro and in vivo. In addition, the potential of this compound as a means of rescue following treatment with buthionine sulfoximine was studied.

## 20.2 Effect of Buthionine Sulfoximine on Cyclophosphamide Priming

### 20.2a Materials and Methods

C3H mice were supplied by Frederick Animal Centre, Maryland, USA with all animals 8-12 weeks of age. Survival studies were carried out on groups of 8 mice with biochemical experiments performed on separate groups of 3 mice. Cyclophosphamide was administered at a dose of 75 mg/kg as a priming dose, with toxic doses of 350 mg/kg or 500 mg/kg. In CBA mice 350 mg/kg was lethal to all controls, but caused no lethality

in control C3H mice, with 500 mg/kg found to be lethal to all C3H animals. Buthionine sulfoximine was given intraperitoneally at a dosage of 26.5 mg/mouse on either one or two occasions at 6 hour intervals prior to the lethal doses of cyclophosphamide for the survival studies, or prior to sacrifice in the biochemical study. Six treatment groups were investigated, as described in Table 20.1. Animal survival was assessed at 14 days with glutathione assayed as previously described (Tietze 1969). Glutathione levels were expressed as micromoles per  $10^6$  cells and micromoles per milligram protein. Glutathione transferases were measured as previously described using chlorodinitrobenzene as a substrate. Studies were limited to bone marrow, with samples prepared as described in Chapter 7.

#### 20.2b Results

The results of lethality experiments are summarised in Figures 20.1a and 20.1b. Fifty per cent survival was observed in animals pretreated with cyclophosphamide 75 mg/kg 5 days before a lethal dose of 500 mg/kg. The protective effect of a cyclophosphamide priming dose is completely lost in animals subsequently treated with buthionine sulfoximine. In fact, enhanced toxicity is observed in buthionine sulfoximine treated

| Group | Priming Therapy              | Subsequent Therapy                            |
|-------|------------------------------|---|
| 1     | saline                       | saline  |
| 2     | saline                       | buthionine sulfoximine x 1<br>6 hrs before    |
| 3     | saline                       | buthionine sulfoximine x 2<br>12+6 hrs before |
| 4     | cyclophosphamide<br>75 mg/kg | saline  |
| 5     | cyclophosphamide<br>75 mg/kg | buthionine sulfoximine x 1<br>6 hrs before    |
| 6     | cyclophosphamide<br>75 mg/kg | buthionine sulfoximine x 2<br>12+6 hrs before |

Table 20.1 Treatment groups for the study of buthionine sulfoximine treatment on the response of C3H mice to a cyclophosphamide priming dose



animals receiving cyclophosphamide 350 mg/kg, irrespective of previous treatment. In contrast, cyclophosphamide 350 mg/kg was not lethal in control or primed animals not receiving buthionine sulfoximine.

Glutathione and glutathione transferase levels are shown in Table 20.2. Elevations are observed in both measurements following low dose cyclophosphamide. Buthionine sulfoximine treatment resulted in lowering of the bone marrow glutathione level in untreated mice to 68% of control. Cyclophosphamide pretreatment resulted in elevation of glutathione levels to 143% of control. However, in cyclophosphamide pre-treated mice who were subsequently treated with buthionine sulfoximine, glutathione levels fall to 47% of control values. Similar figures were obtained following 1 or 2 injections of buthionine sulfoximine. Glutathione transferase levels were elevated in animals pre-treated with cyclophosphamide, irrespective of subsequent therapy. Buthionine sulfoximine therapy had no effect on glutathione transferase levels. These experiments were repeated on 3 occasions and qualitatively similar results obtained.

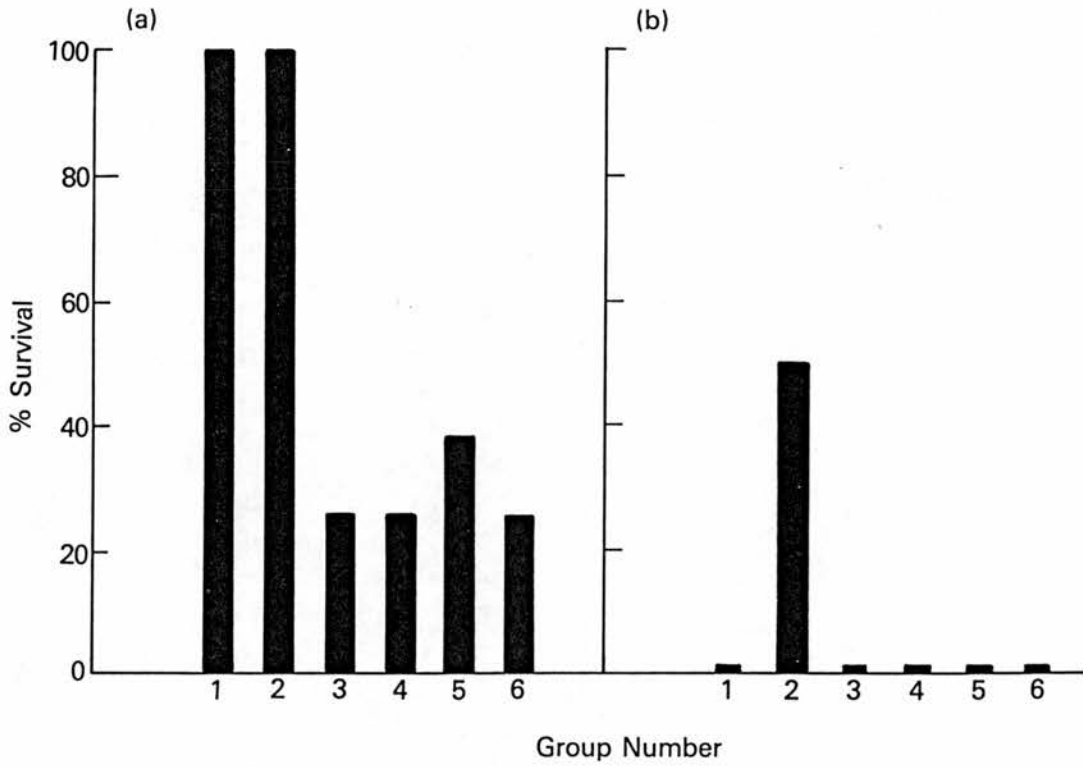


Fig. 20.1 14 day lethality in C3H mice following (a) cyclophosphamide 350mg/kg or (b) cyclophosphamide 500mg/kg. Group 1 control; Group 2 cyclophosphamide 75mg/kg 5 days before; Group 3 buthionine sulfoximine 6 hours before; Group 4 buthionine sulfoximine X 2, 6 and 12 hours before; Group 5 cyclophosphamide 75mg/kg 5 days before and buthionine sulfoximine X1; Group 6 cyclophosphamide 75mg/kg 5 days before and buthionine sulfoximine X2.

| Group                        | *GSH          | % Control | +GST         | % Control |
|------------------------------|---------------|-----------|--------------|-----------|
| 1. Control                   | 0.81 ± 0.108  | 100.0     | 6.87 ± 2.24  | 100.0     |
| 2. CTX 75 mg/kg              | 1.15 ± 0.09   | 143.0     | 13.38 ± 1.18 | 194.8     |
| 3. BSO x 1                   | 0.544 ± 0.124 | 67.2      | 6.55 ± 2.89  | 95.3      |
| 4. BSO x 2                   | 0.564 ± 0.078 | 69.6      | 7.55 ± 1.9   | 109.8     |
| 5. CTX 75 mg/kg +<br>BSO x 1 | 0.403 ± 0.118 | 49.8      | 13.36 ± 3.14 | 194.5     |
| 6. CTX 75 mg/kg +<br>BSO x 2 | 0.361 ± 0.101 | 44.8      | 15.70 ± 1.96 | 228.5     |

Table 20.2 Glutathione and glutathione transferase levels in murine bone marrow. Effect of buthionine sulfoximine on the response to low dose cyclophosphamide (75 mg/kg).

\*Glutathione content expressed in nanomoles per  $10^6$  cells ± S.D.

+Glutathione-S-transferase specific activity ( $\times 10^{-4}$ ) per  $10^6$  cells ± S.D.

### 20.3 In Vitro Depletion of Glutathione Levels Using Buthionine Sulfoximine

Many cell lines have been shown to decrease intracellular glutathione levels in response to buthionine sulfoximine. Ten millimolar buthionine sulfoximine has been shown to deplete glutathione to undetectable levels within 4 hours using Chinese hamster lung V79 fibroblast cells. However, no decrease in viability of these cells is observed until cells are incubated for 16 hours or greater at this concentration. Using the same concentration of buthionine sulfoximine it takes 16 hours to deplete glutathione levels in the adenocarcinoma cell line A549 to less than 1% of control making the drug less effective as a sensitiser to drugs or radiation in this cell line. The length of time taken for any particular cell to deplete its glutathione is variable and dependent on numerous factors.

### 20.4 In Vivo Depletion of Tissue Glutathione Levels Using Buthionine Sulfoximine

Buthionine sulfoximine depleted glutathione in all of the cell lines tested, although the rate of depletion varied with the cell line. In these experiments buthionine sulfoximine was

used to deplete glutathione in vivo. Different tissues were studied to assess the degree of depletion obtainable and the rate of this depletion.

#### 20.4a Materials and Methods

C3H mice 8-12 weeks of age (approximately 20 g weight) were injected with buthionine sulfoximine 26.5 mg intraperitoneally every 6 hours. Four animals were used per group, with mice sacrificed by decapitation and organs removed and processed rapidly. The following tissues were studied, bone marrow, liver, kidney, heart, muscle and spleen. Bone marrow and liver specimens were prepared as described in Chapter 7. The remaining tissues were dissected using crossed scalpels and homogenised using a Polytron homogeniser. Thereafter, specimens were spun at 30,000 g for 30 minutes and supernatants assayed for glutathione content. Protein estimations were performed using the method of Lowry (1951).

#### 20.4b Results

The effect of buthionine sulfoximine, given as 2 injections of 26.5 mg per mouse, on tissue glutathione levels is shown in Table 20.3. Hepatic levels were reduced to 76% of control with control livers having values of 89 nanomoles

glutathione per mg protein. Cardiac glutathione was reduced to 21% of control with these levels remaining low 12 hours after the last buthionine sulfoximine injection. Skeletal muscle exhibited a fall in glutathione level to 16% of control values. Control cardiac muscle had a glutathione level of 31.8 nanomoles per mg protein and skeletal muscle 27.8 nanomoles glutathione per mg protein.

Further studies were performed with buthionine sulfoximine administered for 4 days, and organs assayed for glutathione 24 hours after the last dose of buthionine sulfoximine. The results are illustrated in Table 20.4. The more prolonged treatment with buthionine sulfoximine caused a greater depletion of bone marrow glutathione to 52% of control with a similar depletion observed in skeletal muscle (18% of control). Once again elevation of renal glutathione was seen (153% of control).

The final study was performed to obtain a more detailed assessment of response of renal glutathione levels to buthionine sulfoximine. The results are illustrated in Table 20.5

## 20.5 Studies with the Glutathione Monoethyl Ester

The monoethyl ester of glutathione was synthesised by Dr Angelo Russo (Radiation Oncology Branch, National Cancer Institute, Bethesda, Maryland, USA) according to the method of Anderson et al (1985) and separated by column chromatography. Using 220 megahertz nuclear magnetic resonance spectroscopy, performed by Dr Russo, it was shown that the glycyl portion of the glutathione was esterified, as shown in Figure 20.2. By utilising different pH conditions, the deprotonation of carboxyl groups is apparent. It can be seen that the singlet at 3.8 parts per million does not shift with varying pHs; therefore establishing that the glycyl alpha proton must be associated with the ester carboxyl group. Further studies were then performed to assess the functional activity of this ester by measurement of glutathione levels both in vitro and in vivo.

|            |        | Control | Buthionine<br>Sulfoximine<br>2 x 26.5<br>6 hourly | Glutathione<br>Ester<br>0 hrs | Buthionine<br>Sulfoximine<br>+<br>Glutathione<br>Ester |
|------------|--------|---------|---|-------------------------------|--|
| Time (hrs) | Tissue |         |   |                               |  |
| 0          | Heart  | 31.78   |   |                               |  |
|            | Kidney | 1.597   |   |                               |  |
|            | Liver  | 89.2    |   |                               |  |
|            | Muscle | 27.84   |   |                               |  |
| +1         | Heart  |         |   |                               | 11.71  |
|            | Kidney |         |   |                               | 30.63  |
|            | Liver  |         |   |                               | 43.62  |
|            | Muscle |         |   |                               | 10.72  |
| +2         | Heart  |         |   | 18.4                          | 8.04   |
|            | Kidney |         |   | 1.82                          | 3.54   |
|            | Liver  |         |   | 61.66                         | 49.5   |
|            | Muscle |         |   | 15.78                         | 6.49   |
| +3         | Heart  |         | 6.95  | 15.22                         | 7.60   |
|            | Kidney |         | 1.74  | 1.36                          | 2.01   |
|            | Liver  |         | 67.57   | 91.5                          | 55.1   |
|            | Muscle |         | 5.08  | 16.26                         | 5.97   |
| +4         | Heart  |         |   |                               | 7.15   |
|            | Kidney |         |   |                               | 2.266  |
|            | Liver  |         |   |                               | 55.4   |
|            | Muscle |         |   |                               | 6.86   |
| +6         | Heart  |         | 6.75  | 17.8                          | 6.00   |
|            | Kidney |         | 1.70  | 1.029                         | 1.97   |
|            | Liver  |         | 67.57   | 78.72                         | 52.15  |
|            | Muscle |         | 4.56  | 10.46                         | 4.30   |

Table 20.3 Effect of buthionine sulfoximine and glutathione monoethyl-ester on mouse tissue glutathione levels. Each glutathione measurement represents a mean of 3 samples expressed in nanomoles/mg protein.



| Tissue      | Glutathione<br>(nanomoles/mg protein) |                               |           |
|-------------|---------------------------------------|-------------------------------|-----------|
|             | Control                               | Buthionine sulfoximine (x 16) | % Control |
| Liver       | 78.72                                 |                               |           |
| Kidney      | 0.918                                 | 1.41                          | 153       |
| Bone marrow | 41.56                                 | 21.68                         | 52.2      |
| Spleen      | 26.94                                 | 18.2                          | 67.2      |
| Muscle      | 27.87                                 | 1.41                          | 17.9      |

Table 20.4 Effect of buthionine sulfoximine given for 4 days on tissue glutathione. Glutathione measured 24 hours after final injection of buthionine sulfoximine.

#### 20.5a In Vitro Studies with a Glutathione Monoethyl Ester

A549, a human lung adenocarcinoma cell line, was used for the in vitro studies. Cells were incubated with the ester in culture medium for varying lengths of time. For processing the cells were centrifuged at 250 g for 5 minutes, then washed three times with phosphate buffered saline. The cells were resuspended in 0.6% sulphosalicylic acid and glutathione measured using the method described by Tietze (1969). With A549 cells minimal elevation in glutathione levels was observed, reaching 120% of control values at 30 minutes. With more prolonged exposure to ester no further increase in glutathione was observed. However, using Chinese hamster lung V79 cells, values of greater than 200% of control were achieved at 1 hour in 3 separate experiments using the same monoethyl ester.

#### 20.5b In Vivo Studies with a Glutathione Monoethyl Ester

Studies were performed to assess the degree of uptake of the monoethyl ester in different tissues. The following tissues were studied; heart, kidney, liver and muscle. The effect of the ester alone was studied and in addition the ability of the ester to rescue tissues from the effects of buthionine sulfoximine was assessed. The following 4 groups were studied.

| Group  | No. of BSO injections | Time of sacrifice after final BSO injection | Glutathione nanomoles/mg protein | % Control    |      |
|--------|-----------------------|---|----------------------------------|--------------|------|
| Kindey | Control               | -   | 1.28 ± 0.13                      | -            |      |
|        | 1                     | +2  | 1.34 ± 0.16                      | 105          |      |
|        |                       | +5  | 1.84 ± 0.29                      | 144          |      |
|        |                       |   |                                  |              |      |
|        | 5                     | +2  | 2.76 ± 0.36                      | 215          |      |
|        |                       | +5  | 1.87 ± 0.19                      | 146          |      |
|        |                       | +9  | 3.20 ± 0.09                      | 179          |      |
|        | 9                     | +1  | 2.75 ± 0.29                      | 215          |      |
|        |                       | +2  | 2.20 ± 0.13                      | 172          |      |
|        |                       | +5  | 2.98 ± 0.19                      | 233          |      |
|        | Bone marrow           | Control                                     | -                                | 23.94 ± 3.40 | -    |
|        |                       | 9   | +2                               | 4.92 ± 0.63  | 20.5 |
| Liver  | Control               | -   | 70.50 ± 10.1                     | -            |      |
|        | 1                     | +2  | 49.80 ± 11.3                     | 9.0          |      |
|        |                       | +5  | 6.23 ± 0.92                      |              |      |
|        |                       |   |                                  |              |      |
|        | 5                     | +5  | 7.81 ± 1.21                      | 11.0         |      |
|        |                       | +9  | 25.35 ± 4.39                     | 36.0         |      |
|        | 9                     | +1  | 18.60 ± 6.92                     | 26.1         |      |
|        |                       | +2  | 12.85 ± 4.06                     | 18.2         |      |
|        |                       | +5  | 9.25 ± 2.64                      | 13.0         |      |
|        |                       | +9  | 27.6 ± 3.57                      | 39.0         |      |

Table 20.5 Effect of buthionine sulfoximine on renal, hepatic and bone marrow glutathione levels.

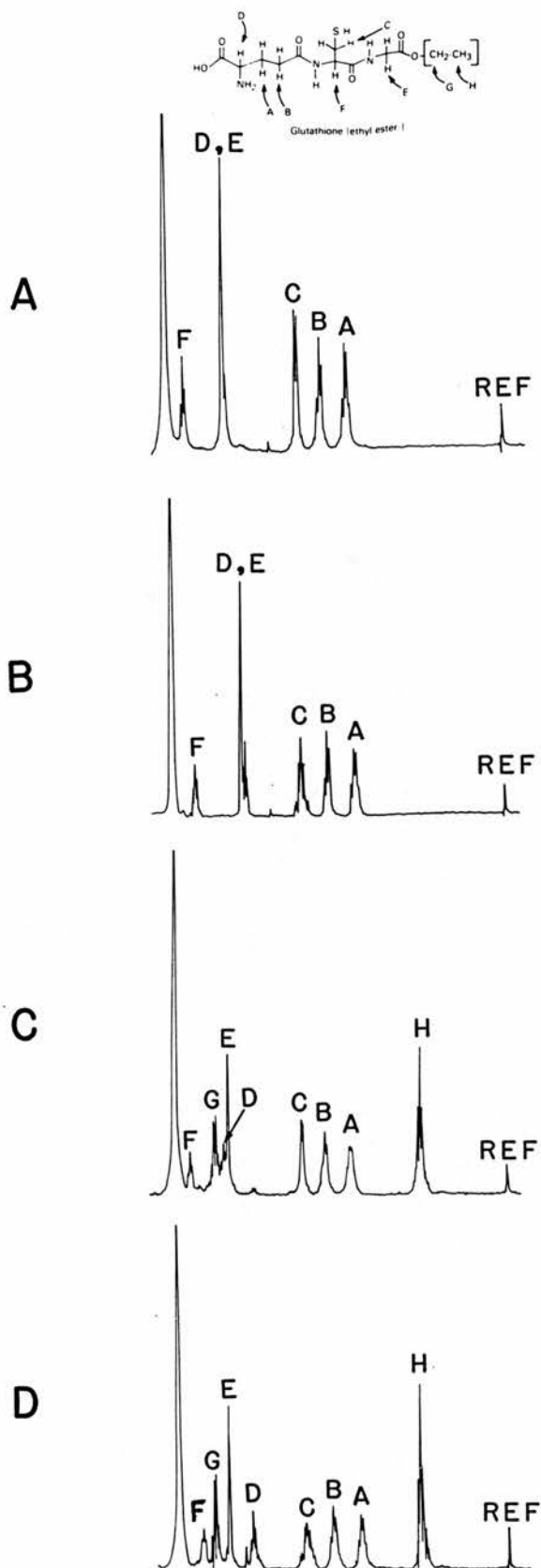


Fig. 20.2 220 mega-Hertz proton nuclear magnetic resonance spectra of glutathione and its monoethyl ester, performed in  $D_2O$  using trimethylsilyl propionate as reference. The protons are labelled alphabetically on the chemical structure, and corresponding labelling is seen on the spectra. (A) Glutathione pH 1.5, (B) Glutathione pH 10.0, (C) Glutathione monoethyl ester pH 1.5, (D) Glutathione monoethyl ester pH 10.0.

- (1) control
- (2) buthionine sulfoximine (26.5 mg i.p. x 4, 6 hourly)
- (3) glutathione monoethylester (40 mg i.p. at Time 0)
- (4) buthionine sulfoximine + glutathione monoethylester

Buthionine sulfoximine 26.5 mg was administered intraperitoneally every 6 hours for 24 hours prior to commencement of the study, with the glutathione ester (40 mg per mouse) administered intraperitoneally at time 0. At specified times thereafter, groups of 3 mice were sacrificed and prepared for glutathione estimations. Mice were decapitated, and deblooded organs were removed and weighed. Tissue was then transferred to test tubes and 2 ml of phosphate buffered saline with 1 millimolar EDTA was added. Specimens were minced, homogenised and then centrifuged at 30,000 g for 30 minutes at 4°C. The supernatant was removed and frozen at -70°C prior to assay. Glutathione was assayed according to the method described by Tietze (1969). The results are summarised in Table 20.3. Buthionine sulfoximine caused a fall in glutathione values in heart, liver and muscle, although kidney values were above control levels. Ester treatment caused no elevation in glutathione levels in any of the tissues investigated apart from minimal increase in kidney levels at 2 hours. If anything, levels were lower in mice treated with the ester

compared with control. The use of the ester in an attempt to rescue tissues following buthionine sulfoximine treatment gave interesting results. Ester treatment had minimal effect on the tissues apart from kidney samples. Very high glutathione levels (1900%) were observed in the kidney 1 hour after injection of the ester in buthionine sulfoximine treated mice, with levels remaining slightly elevated for 6 hours.

#### 20.6 Effect of Buthionine Sulfoximine and Glutathione Ester on Tumour Glutathione Levels

Glutathione levels were measured in 2 small cell lung cancer xenografts, NCI-H209 and NCI-H82, the details of which were described in Chapter 19. The xenografts were grown in nude mice and were treated with either buthionine sulfoximine 26.5 mg intraperitoneally on 2 occasions, 6 and 12 hours before sacrifice, or glutathione ester 40 mg intraperitoneally 2 hours before killing. Tumour tissue was excised immediately and homogenised using a polytron homogeniser. Glutathione levels were assayed using both methods described previously. The results are summarised in Table 20.6. Buthionine sulfoximine significantly depleted glutathione levels in both xenograft lines (approximately 30% of control) using both assays. However, ester treatment resulted in no signifi-

cant elevation in glutathione levels, levels ranging from 76-116% of control values in both assays. Experiments to ascertain whether buthionine sulfoximine-treated tumour tissue can be rescued with a glutathione ester have yet to be performed.

## 20.7 Discussion

Buthionine sulfoximine treatment was shown to significantly deplete glutathione levels in many tissues including cardiac muscle, liver, skeletal muscle, and tumour tissue. Levels in tumour tissue fell to **30%** of control values following 2 injections of buthionine sulfoximine over 12 hours. In contrast, minimal depletion of glutathione values (67% of control) are observed in bone marrow specimens. Interestingly, minimal haematological toxicity has been observed using buthionine sulfoximine in combination with melphalan (Hamilton - personal communication; Russo et al 1986). In the case of melphalan, significantly enhanced anti-tumour activity is observed in combination with buthionine sulfoximine with minimal, if any, increase in toxicity. No enhancement of bone marrow toxicity is observed in these studies with minimal depletion of glutathione seen in this tissue. Substantial

## Glutathione Content (% control)

| Xenograft Line | Fluorimetric Assay |      | Tietze Assay |     |
|----------------|--------------------|------|--------------|-----|
|                | BSO                | GE   | BSO          | GE  |
| NCI-H82        | 29.4               | 96.9 | 37.8         | 116 |
| NCI-H209       | 24.9               | 76.1 | 30.3         | 77  |

Table 20.6 Effect of buthionine sulfoximine (BSO) and glutathione monoethyl ester (GE) on small cell lung cancer xenograft glutathione levels. Glutathione measured using 2 methods; fluorimetric assay and Tietze assay.



overshoot in glutathione levels is normally observed in the bone marrow following cyclophosphamide priming. However, in mice subsequently challenged with buthionine sulfoximine a more profound fall in glutathione content is seen. Levels of 47% of control are observed in contrast to the 180% normally seen after drug priming. This result is highly suggestive that a compensatory synthetic process is being interrupted by buthionine sulfoximine treatment. The protective effect of low dose cyclophosphamide (75 mg/kg) on animal survival is completely lost in these mice subsequently treated with buthionine sulfoximine with, in fact, enhanced toxicity observed compared with control mice. However, it should be stressed that buthionine sulfoximine could be depleting glutathione in other organs which could account for some of the increase in mortality. The fact that the protective effect of cyclophosphamide is lost with subsequent buthionine sulfoximine treatment is very interesting. This treatment prevents the overshoot in glutathione levels without impairing the overshoot in glutathione transferase levels. This suggests that glutathione is more important in this protection, and that the glutathione transferase increase is relatively impotent in the presence of diminished substrate (glutathione) concentrations.

However, whether increased levels of glutathione transferase would be protective if the glutathione level could be maintained at 100% of control remains a matter for conjecture.

The structure of the glutathione ester was confirmed using proton nuclear magnetic resonance spectroscopy with the ester shown to be attached to the glycyl portion of the compound. However, studies with the ester have been relatively disappointing. In vitro studies with V79 cells have shown good uptake at 30 minutes to 2 hours with glutathione values reaching 200% of control levels. With A549 cells however, minimal elevation of glutathione was detected over this period. Perhaps consideration should be given to basal glutathione levels in these cell lines, however as A549 cells have very high levels in comparison to V79 cells. Further studies are required using a range of cell lines to ascertain whether ester uptake is affected by the basal glutathione level. In vivo studies have been particularly disappointing with no increase in glutathione detected in cardiac muscle, liver or striated muscle. Renal glutathione levels were minimally elevated following ester administration. Surprisingly, renal glutathione levels were not depleted by buthionine sulfoximine, in contrast slight elevation in levels were detected. In mice treated with both buthionine sulfoximine and the glutathione

ester however, there was a dramatic increase in glutathione level at 1 hour with values of 1800% of control observed. Glutathione levels remained elevated for 6 hours following injection of the ester in buthionine sulfoximine treated animals. The high renal glutathione values could be related to high renal excretion of the ester. Alternative methods of ester administration such as continuous low dose infusion are now being studied to assess whether higher, potentially protective, glutathione levels can be achieved in other tissues.

A major problem was encountered with the studies performed on renal tissue. Although actual values for other tissues correlate well with reported glutathione values, renal glutathione levels reported in these experiments were much lower than in previously reported studies. It is possible that glutathione was unstable in the preparations used, as tissues high in  $\gamma$ -glutamyl transpeptidase have previously been shown to interfere with glutathione levels. Further studies are now being performed to address this problem

In the 2 small cell lung cancer xenografts, buthionine sulfoximine caused a significant fall in glutathione levels. Although the degree of this depletion was similar to some other tissues, such as cardiac and striated muscle, it was much

greater than the depletion observed in bone marrow cells. Potentially, this could be of great value clinically by increasing the therapeutic index of drugs that are detoxified by the glutathione redox system, if their dose-limiting toxicity is haematological. Certainly, buthionine sulfoximine can enhance the cytotoxicity of melphalan without significantly altering the haematological toxicity or survival in these mice. It should be stressed that caution is still recommended in the use of buthionine sulfoximine with drugs that have dose-limiting toxicities other than haematological. Caution should also be exercised in the presence of a regenerating bone marrow as a greater depletion in glutathione levels may be observed with consequently increased toxicity. In addition, one should remain alert to the possibility of unexpected major organ toxicities with combinations of buthionine sulfoximine with myelotoxic cytotoxic drugs.

The use of the glutathione ester to rescue cells from buthionine sulfoximine toxicity was not successful, although optimal scheduling of administration of the ester remains undefined. Encouraging results were obtained with a combination of buthionine sulfoximine and ester in the kidney, with consistent elevation in renal glutathione levels. This

certainly justifies an in vivo experiment using these two agents in an attempt to reduce the renal toxicity of cis-platinum which is often dose-limiting.

Further in vivo experiments are certainly indicated using buthionine sulfoximine in combination with various cytotoxic drugs to ascertain whether the therapeutic index of these drugs can be improved. Esters of glutathione potentially offer interesting possibilities of rescue although the optimal ester and schedule of administration of these esters remain unclear.

## CHAPTER 21

GENERAL CONCLUSIONS AND FURTHER STUDIES

Human tumour xenografts were established from lung cancer specimens using CBA mice immune-deprived by means of thymectomy, cytosine arabinoside priming and total body irradiation. Tumour tissue was obtained primarily at thoracotomy although a few specimens obtained at bronchoscopy were included. Xenograft lines were successfully established in 31% of samples with similar take rates observed using both squamous and adenocarcinoma samples. Lower take rates were achieved using anaplastic carcinomas, both small cell and large cell, although numbers were small. Proportionately fewer continuous xenograft lines were established in this study than in the previous study reported by Shorthouse (1981). Delay in transplantation of tumour tissue is likely to have been the predominant reason for this discrepancy. These delays occurred both at the time of excision of the tumour, prior to placement into tissue culture medium, and also before implantation into the immune-deprived host. Following growth in the primary man to mouse passage, subsequent take rates were similar to previously reported

studies. A range of 16 lung cancer xenografts were established, including squamous carcinoma, adenocarcinoma, adenosquamous carcinoma and small cell carcinoma lines. Generally, moderately to poorly differentiated tumours were grown more successfully, with no well differentiated tumours established. Following a presumably infective outbreak in the mouse colony, 9 xenograft lines were salvaged of which 6 non-small xenografts cell and 1 small cell xenograft were selected for chemotherapy studies, with the growth characteristics of these xenografts similar to previously described studies. Tumour volume doubling times varied from passage to passage, and also within the same passage, although no obvious growth pattern was evident. Histologically, the xenografts retained similar morphological features to the original donor tissue. However, minor changes in differentiation were seen with different passages, although no reliable pattern was evident with both increased and decreased differentiation observed.

The six non-small cell xenografts used for the chemotherapy studies included 1 adenocarcinoma and 1 adenosquamous carcinoma and 4 squamous cell lines. As may have been expected, all xenografts were generally resistant to single agent chemotherapy. Of the single agents tested, cis-platinum

was the most active, with ifosfamide also exhibiting some activity. The platinum analogues carboplatin (JM8) and JM40 were more active than cyclophosphamide, vindesine or etoposide but were less active than cis-platinum. However, carboplatin is certainly worthy of further study in view of its slightly different spectrum of activity and diminished toxicity in comparison to cis-platinum. A limited number of drug combinations were tested with none of these combinations showing greater activity than maximum tolerated doses of the most active single agent.

The interferon experiments were extremely interesting. Certainly supra-additive results were achieved with combinations of human lymphoblastoid interferon and cis-platinum. Recently these results have been confirmed by Dr R Fergusson in the ICRF Medical Oncology Unit using recombinant human alpha interferon in combination with cis-platinum or ifosfamide. However, prior to the incorporation of interferon combinations in to clinical trials, a number of important questions require to be addressed. The testing of schedules that would realistically be acceptable to the majority of patients is a priority. In addition, the testing of a wide range of cytotoxic drugs both as single agents and in combination should be carried out with human interferon in an attempt to define the optimal



therapy. Likewise, it would appear important to assess the effect of interferon on drug metabolism. The use of a hybrid interferon that affects both human and mouse tissues could be used in this model to help answer this question. An understanding of the aetiology of this potentiation could obviously be of great value. Following optimisation of conditions, the use of interferon in combination with cytotoxic drugs could prove to be a significant advance in the treatment of many solid tumours refractory to standard chemotherapy, such as non-small cell lung cancer.

The use of the calcium channel blocker verapamil in combination with vindesine was singularly unsuccessful in these xenografts. Vindesine itself was inactive in all xenografts tested, and when used in combination with verapamil, toxicity was severe with all animals dead by day 10. Death was not related to haematological toxicity, with enhanced local peritoneal toxicity the likely aetiology. However, the addition of verapamil was unlikely to have significantly altered the cytotoxicity of vindesine in these xenografts, as calcium channel blockers preferentially enhance the activity of certain cytotoxic drugs in pleiotropically resistant cells. Pleiotropic resistance is often associated with the presence of p-glycoprotein, a 180,000 dalton molecular weight membrane

glyco protein (Riordan and Ling 1985). In recent studies we have shown that pleiotropically resistant cells are 'collaterally sensitive' to calcium channel blockers. There are many unanswered questions relating to the aetiology of pleiotropic drug resistance. Answers to these questions may well improve the chemotherapy of solid tumours.

Another potential means of modulating cytotoxic drug action is by the use of low doses or priming doses of cytotoxic drugs. In these studies, it was confirmed that low dose cyclophosphamide could protect against the lethal effects of a subsequently administered high dose of the same compound. The protection is maximal 5 to 7 days after the priming dose, the time of maximal overshoot in glutathione levels in the bone marrow. The overshoot in glutathione level is found predominantly in the granulocytic fraction, with granulocytes the bone marrow cells which recover most rapidly following the 'lethal' dose. In a similar manner, the bladder can be protected against potentially lethal doses of cyclophosphamide. From these studies it remains unclear whether the increased survival in primed mice is due to reduced bone marrow toxicity, reduced bladder toxicity, or both. Limited studies on bladder glutathione have suggested that a similar overshoot in glutathione level is observed following a priming dose of

cyclophosphamide. It is far from clear which metabolite of cyclophosphamide is responsible for this protection, or which metabolite caused the increase in glutathione and glutathione transferase levels. It seems likely that many metabolites are involved, as acrolein, phosphoramidate mustard and 4-hydroxycyclophosphamide have all been shown to form adducts with glutathione. Acrolein was also shown to be capable of protecting animals from the lethal effects of high dose cyclophosphamide, although was less effective than low dose cyclophosphamide. As revealed by the experiments using buthionine sulfoximine, it seems likely that the overshoot in glutathione levels seen following drug priming results from a compensatory synthesis process. Of great importance is the lack of apparent priming in tumour tissue which is certainly gratifying from the clinical point of view, as it had previously been suggested that sulphhydryl-containing compounds were unlikely to be protective in view of their lack of specificity (Connors 1966). However, this is probably true for many exogenously administered sulphhydryl-containing compounds.

The studies with buthionine sulfoximine and the monoethyl ester of glutathione were extremely interesting. Certainly, in vitro these compounds are able to selectively alter intracellular glutathione levels and, as a result, could potentially modify cytotoxic activity of numerous drugs.

Modulation of glutathione levels can significantly affect the activity of drugs that are either activated (De Graff et al 1985) or detoxified by the glutathione redox system. However, the results from in vivo studies are less clear cut. Buthionine sulfoximine can reduce tumour glutathione levels to approximately 25% of control over 12 hours. This offers the potential to increase the therapeutic index of drugs detoxified by glutathione, particularly if haematological toxicity is dose-limiting, as bone marrow glutathione levels are minimally affected by buthionine sulfoximine. Using 1 injection of the glutathione ester at a dose of 40 mg per mouse intra-peritoneally, tumour glutathione levels were unaffected although a small increase in renal glutathione was observed. However, buthionine sulfoximine treatment, followed by administration of the glutathione ester resulted in a dramatic increase (1800%) in renal glutathione levels. Potentially, the increase in renal glutathione levels could be exploited clinically by protection from cis-platinum-induced renal damage, although

further in vivo studies are indicated to validate the renal glutathione experiments, and examine this hypothesis. Another important question to address is whether increased glutathione levels can be achieved in other tissues by administration of the ester, perhaps in alternative schedules.

In conclusion, a panel of human lung cancer xenografts have been established which are being maintained in immune-deprived mice. Experimental work has predominantly centred on the non-small cell lung cancers. These xenografts maintained human characteristics and exhibited a similar chemosensitivity profile to that observed clinically. Responses were poor, both to single agent and combination chemotherapy, although encouraging results were obtained with interferon-cytotoxic drug combination. Further studies are indicated for the development and testing of more realistic schedule for interferon-drug combinations. In addition, this human tumour xenograft model could be used for the study of novel drug schedules and other methods of modulating chemotherapy, apart from the mechanisms discussed in this text.

It seems likely that increased glutathione levels, either in the bone marrow or bladder, may play a role in the survival protection afforded by low dose alkylating agents such as cyclophosphamide. Bone marrow and bladder tissue can be

protected against the toxic effects of high dose cyclophosphamide, but in these studies no protection, in terms of increased glutathione or glutathione transferase levels, was observed in tumour tissue.

A more detailed in vivo assessment of the effects of buthionine sulfoximine on different tissues would be worthwhile. Data using the glutathione monoethyl ester remains preliminary, although the potential remains to rescue tissues from buthionine sulfoximine related toxicity.

There have been many exciting advances in our understanding of the development and treatment of cancer over the past 10 years. There is increasing awareness of the prevalence and importance of various proto oncogenes (Little et al 1983), and increasing understanding of the effects of various growth factors on tumour cell growth (Cuttita et al 1985). Developments in the production, and the use of monoclonal antibodies both diagnostically and therapeutically, may be used over the next 10 years to increase our understanding of tumour biology. Likewise, increased awareness of the effects of many biological response modifiers may alter our therapeutic approach to the treatment of many forms of cancer. However, there is likely to be a considerable delay before any radical changes are made in our approach to the treatment of solid tumours. In the

interim, a large number of patients with poorly responsive solid tumours will be treated with combination chemotherapy as described in the clinical studies. A number of the strategies discussed in this volume could, potentially, be of value by reduction of the toxicity associated with combination chemotherapy. However, whether these strategies could result in higher response rates clinically remains less clear. Priming doses of cyclophosphamide have been used clinically to reduce the toxicity of high dose chemotherapy with cyclophosphamide or melphalan. It would be interesting to ascertain whether a similar reduction in 'host' toxicity could be achieved with standard doses of chemotherapy using this technique.

There are many potential ways of modulating the response to cytotoxic drugs. Alteration of glutathione homeostasis is just one method, although the human xenograft model is an excellent model for the study of a number of these strategies. The long-term results of chemotherapy of lung cancer remains disappointing, even in small cell lung cancer where initial response rates are high. It is hoped that modulation of the chemotherapy of these tumours may improve this rather dismal picture.

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