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THE BIOLOGY AND CHEMOSENSITIVITY OF OVARIAN CANCER *IN VIVO* AND *IN VITRO*

Thesis submitted for

Doctor of Medicine

by

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ABSTRACT

FACULTY OF MEDICINE
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Drug resistance to alkylating agents is known to be a major clinical problem in the management of patients with ovarian cancer. In a randomised clinical trial of 70 patients with advanced ovarian cancer the question was asked if this resistance could be overcome by a three drug combination, when compared to conventional management with a single alkylating agent. Cisplatin, adriamycin and cyclophosphamide given together achieved a complete clinical response rate of 67%, while oral chlorambucil alone gave a response rate of only 30%. The median survival was 17 months in the combination arm, and 11.8 months in the chlorambucil arm, but these differences were not significant ($p = 0.84$) and toxicity was considerably greater in the combination arm. More effective induction regimes are required to improve the outlook in ovarian cancer.

To examine the possible mechanisms of this drug resistance at the cellular level, a suitable tumour model system was required, and using the mouse M5076 ascites carcinoma, correlation was obtained between *in vitro* drug sensitivity curves assessed by clonogenic assay and the *in vivo* sensitivity in the mouse as well as the peak achievable plasma level in man. However, the M5076 was subsequently shown to have morphological and immunological features consistent with a histiocytic lymphoma. The use of primary cultures of human ovarian tumour specimens in soft agar was also assessed but these studies were not continued because of poor growth and low cloning efficiency.

The most satisfactory model for the study of cellular pharmacology was found to be human ovarian cancer cell lines. Four fold resistance was developed to the alkylating agent melphalan by stepwise incubation with increasing concentrations of the drug, and the degree of resistance achieved was found to be similar to that observed in the lines derived from clinically resistant patients. Cross resistance to cisplatin and thiotepa was observed, but not to adriamycin.

The uptake of melphalan by the sensitive cells and those in which resistance had been induced was found to be identical, and the resistant line dechlorinated as much melphalan to the inactive hydroxymetabolites as the sensitive line. Glutathione levels, however, were two fold higher in the resistant compared to the sensitive cells, and glutathione depletion either by omission of cysteine from the medium or preincubation with buthionine sulphoximine, a specific inhibitor of glutathione synthesis, was found to sensitise the resistant cells to melphalan. Buthionine sulphoximine at a dose of $50\mu M$ for 18 hours was minimally cytotoxic to the resistant cells when given alone, and reversed the degree of acquired resistance. It is concluded that the level of intracellular glutathione may be associated with resistance to melphalan, and that depletion of this thiol by the synthetic amino acid buthionine sulphoximine may have a role in the modulation of alkylating agent activity in human ovarian cancer.

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The results presented in this thesis are entirely the product of work performed personally by the author, except where otherwise stated in the text.

1. INTRODUCTION

1.1 Epidemiology

Ovarian cancer is the commonest fatal gynaecologic cancer and is the fourth leading cause of cancer death in women, with 3,500 deaths each year in the United Kingdom (1). The overall incidence is 26 per 100,000 female population between the ages of 35 and 64 years (2) and most of the younger patients die of the disease. With increasing age, other causes play a more major part. Ovarian cancer represents less than 20% of primary pelvic malignancies but because of the poor cure rate, there are as many deaths from ovarian cancer as from cancers of the cervix and endometrium combined. In the USA and in Western Europe the rise in incidence has been of the order of 1% per year over the last 30 years (3).

The aetiology is not known, but hormonal dysfunction and peritoneal irritation have been the two factors most frequently implicated (2). An environmental contribution is suggested by the low incidence in Japan, which rises after a generation to American levels in Japanese immigrants to Hawaii. There is epidemiological evidence that the incidence is higher in Caucasians, the upper socioeconomic groups, those who remain single or marry late, and in women with menstrual disorders or a history of subfertility (3). The risk of developing ovarian cancer after primary breast cancer is quadrupled and both cancers show a markedly increased incidence at the climacteric (1). These findings have suggested common aetiological factors of a hormonal nature in the two diseases. It is possible that pregnancy exerts a protective effect in both tumours by inhibiting ovulation and allowing the surface germinal epithelium and breast ductule prolonged rest periods.

There is some evidence that prior oestrogen therapy in postmenopausal women predisposes to ovarian cancer but the increased risk is not great (1, 4) and the contraceptive pill has not been found to predispose to ovarian tumours. About 50% of ovarian carcinomas exhibit oestrogen binding activity with properties suggesting moderate intracellular concentration of receptors (5), but it has

not been established whether this represents a vestigial function or a true role for sex hormones in the growth and maintenance of the neoplastic germinal epithelium (6). For example it has not yet been shown whether exogenous oestrogen can induce progesterone receptors or regulate human ovarian tissue growth in vitro.

As discussed more fully below, many ovarian tumours show features resembling those seen in mesothelioma, and peritoneal irritation principally with talc or asbestos has been put forward as a causative factor (7, 8, 9). The latter is a known carcinogen, associated primarily with pleural mesotheliomas, and to a lesser extent with peritoneal mesotheliomas. The evidence that talc is carcinogenic is less strong (10), but its frequent use as a perineal toilet preparation in Western societies, and its possible access to the peritoneal cavity via the Fallopian tubes has made the theory attractive if unproven.

Spontaneously arising animal tumours have been described in cattle, cats and particularly in dogs, but their occurrence is too rare to give a useful clue to their aetiology (11). Experimentally induced hormone imbalance can, however, give rise to ovarian epithelial tumours in the dog (12). Constant 12 hour lighting without seasonal rest periods produces ovarian adenocarcinomas in the domestic fowl, and this effect has been attributed to incessant ovulation and repair of the surface germinal epithelium (13). Chemical carcinogens generally give rise to granulosa cell tumours in laboratory animals rather than epithelial cancer, but there is one report of sutures impregnated with 7, 12 dimethylbenzanthracene inducing ovarian adenocarcinomas in the rat (14). Ovarian irradiation at birth in the mouse produces tubular adenomas in mice, and these bear some resemblance to the more benign forms of ovarian tumours in women (15). While the hormonal control of granulosa cell tumours has been studied in some detail, animal models of human epithelial ovarian cancer have not been of great value in the study of the preclinical pharmacology of cytotoxic chemotherapy.

Ovarian cancer in women is predominantly a disease of middle and old age, and the familial influence is slight. There are often few symptoms associated with the tumours and diagnosis is frequently late. Two thirds of tumours have spread beyond the pelvis at presentation. Pain is a rare feature unless the tumour twists on the fallopian tube, and vaginal bleeding is seldom seen. The symptoms observed may be lower abdominal discomfort, abdominal distension or lower limb oedema, and a mass may be present leading to a differential diagnosis of ovarian cyst, endometriosis, pelvic inflammatory disease, ectopic pregnancy, diverticular disease, colonic carcinoma and retroperitoneal tumours. The diagnosis of ovarian malignancy should be considered in any woman with persistent abdominal complaints, a long history of ovarian dysfunction and age above 40, but these guidelines are not sufficiently selective to be of great help to the general practitioner.

An increased index of suspicion is critical if earlier diagnosis is to be achieved in the absence of a simple screening test and the relative difficulty in biopsy in comparison with cervical and endometrial carcinoma. It is not yet clear what the contribution of newer imaging techniques such as ultrasound or computerised tomography will be towards improved diagnosis. Targeting of a radiolabelled monoclonal antibody to a putative ovarian surface antigen has been reported, but is invasive yet not sufficiently specific for routine use (16) even in high risk groups.

The extent of tumour at presentation as defined by the staging system of the International Federation of Gynaecology and Obstetrics (FIGO) correlates well with prognosis and is widely used (Figure 1). Survival varies from 60% at 5 years in Stage I/II disease to a median survival of 9 months in Stage IV disease (17). However, included in the largest group, classified as Stage III are those who have only small subdiaphragmatic deposits as well as those with massive omental and retroperitoneal disease, and this heterogeneity has given rise to much of the conflicting data in the literature. The group with tumour confined to the ovary, but with cytologically

Fig. 1.

UICC	**OVARY**	*FIGO*
T1	Limited to ovaries	I
T1a	One ovary. No ascites	Ia
T1b	Both ovaries. No ascites.	Ib
T1c	One or both ovaries. With ascites.	Ic
T2	With pelvic extension.	II
T2a	Uterus *and/or* tubes. No ascites	IIa
T2b	Other pelvic tissues. No ascites	IIb
T2c	Other pelvic tissues. With ascites.	IIc
T3	Extension to small bowel/omentum in true pelvis *or* intraperitoneal metastases/retroperitoneal nodes	III
M1	Distant organs.	IV

positive ascites (Figo Ic) are often thought to do worse than the more advanced Stages II and III and have a very high early mortality. It is not clear whether this is due to undetected more extensive disease or reflects a more rapid tumour proliferative rate. The relationship between bulk and survival is more difficult to establish because of the problems of measurement, but there is some evidence that it is important (18).

Markers for human ovarian cancer are at an early stage of development by comparison with testicular tumours or lymphomas. Carcinoembryonic antigen (CEA) can be detected by radioimmunoassay in the serum of 50% of patients with ovarian cancer, and in ascitic fluids of some ovarian cancer patients. CEA is also elevated in other gynaecological malignancies and in gastrointestinal tumours. This lack of specificity and the relatively low levels seen in ovarian tumour patients has limited the usefulness of CEA either in the diagnosis or monitoring of the disease (19). Low levels of human chorionic gonadotrophin (HCG) have been found in up to 30% of patients, and levels of alpha-foetoprotein (AFP) of borderline

significance are recorded in the occasional patient (20). Alkaline phosphatase isoenzymes, placental glycoproteins, and other glycoproteins associated with ovarian cancer have been studied by various groups, but none is truly specific or in common use.

Biology and histopathology of human ovarian cancer

The pathological classifications currently in use in ovarian cancer are based on present concepts of the development of the female urogenital tract. The ovaries develop from the bilateral urogenital ridges on the posterior wall of the coelomic cavity which give rise to a layer of cortical cells unfortunately termed "germinal epithelium", which plays the major role in the genesis of ovarian tumours. For the sake of clarity this layer will be referred to throughout as the 'surface germinal epithelium' to distinguish these cells from the true germ cells which are quite separate, and arise from the yolk sac endoderm near the hind gut, and migrate secondarily to the gonads where they localise to the cortical layer. These germ cells become closely associated with a single layer of spindle shaped cells, the granulosa cells, which proliferate under hormonal control and give rise to the primary follicle. Surrounding this is a basal lamina, and concentric layers of spindle shaped cells called theca cells. As the follicle matures, the number of granulosa cells increases and the theca cells differentiate and hypertrophy (Fig 2).

Ninety per cent of the malignant ovarian tumours arise from the 'surface germinal epithelium' whereas less than 60% of the benign tumours do so. As the urogenital ridge also gives rise to the fallopian tubes, uterus and cervix, tumours from these areas share a common origin from the Mullerian duct and often show morphological appearances similar to those of ovarian cancer.

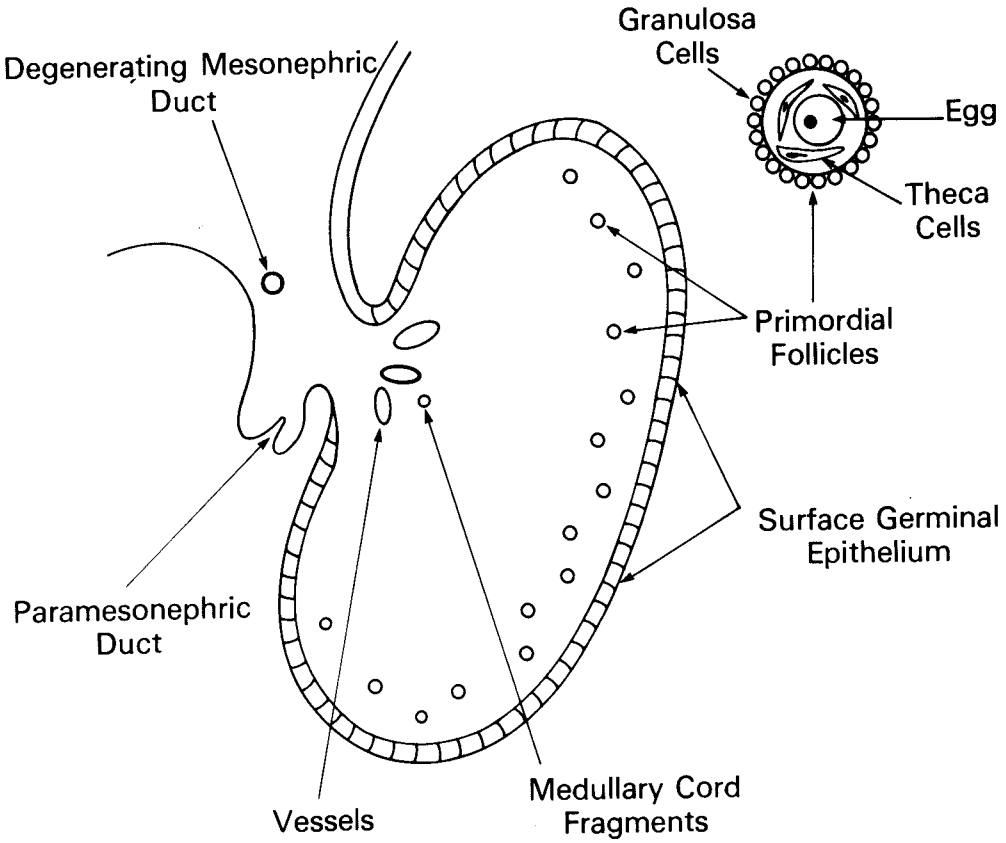


Fig. 2. Schematic diagram to show relationship of the surface germinal epithelium to other structures in the adult ovary.

Diagnosis of malignancy is based on the degree of cellular pleomorphism, the degree of structural abnormality with papillae formation, and the mitotic rate of the cells. Above all else, invasion of the stroma is required to confirm the diagnosis of malignancy. The WHO classification of ovarian tumours is the one most frequently used, and is shown in Table 1. The remainder of the discussion will be concerned solely with the tumours derived from the surface germinal epithelium which comprise the bulk of the ovarian cancers in women.

The morphological classifications of ovarian epithelial tumours rarely predict the natural history with sufficient accuracy to be clinically useful. In an attempt to refine this, a system based on cellular differentiation was proposed by Broders in 1926 (22). It divides the tumours into four grades depending on the proportion of undifferentiated cells (0 - 25% Grade I; 25 - 50% Grade II; 50 - 75% grade III; > 75% grade IV). When applied by one pathologist it is reproducible, and the grading system has been found to correlate with outcome in several series of patients treated by surgery and radiotherapy (23) as well as in more advanced disease treated with chemotherapy (24). Mucinous and endometrioid tumours tend to be low grade and low stage, while serous and solid adenocarcinomas tend to be high grade, high stage. High grade, low stage tumours have a worse prognosis than low grade, low stage lesions. Other workers have used a grading system based more on histological pattern than cytological differentiation (25).

In general, grading systems are more highly regarded by clinicians than by pathologists, who perhaps are more conscious of the intra-tumour variation in appearances, the selection involved in biopsy sampling, and the inter-observer variation inherent in a classification based on morphological criteria.

Table 1 WHO classification of malignant ovarian tumours (21)

1. Common 'epithelial' tumours

   A Serous
   B Mucinous
   C Endometrial
   D Clear cell (mesonephroid)
   E Brenner
   F Mixed epithelial
   G Undifferentiated carcinoma
   H Unclassified

II. Sex cord stromal tumours

   A Granulosa - theca cells
   B Androblastoma (Sertoli - Leydig)
   C Gynandroblastoma
   D Unclassified

III. Lipid cell tumours

IV. Germ cell tumours

   A Dysgerminoma
   B Endodermal sinus tumours
   C Embryonal carcinoma
   D Polyembryoma
   E Choriocarcinoma
   F Teratomas

1.2. Treatment of Ovarian Cancer

Surgery has traditionally been the mainstay of the treatment of ovarian cancer and the aim is to complete a bilateral salpingo - oophorectomy, hysterectomy and omentectomy. Maximum debulking is usually advised even if pelvic clearance is not technically feasible, and the evidence in favour of this rests on the finding that patients with residual tumour less than 2 cm in diameter after primary laparotomy have a better prognosis than those with larger volume disease (26, 27).

Surgery may also have a role in the reassessment of patients with advanced ovarian cancer after chemotherapy or combined chemo/ radiotherapy is completed. Opinions are divided on whether laparoscopy with biopsy or second look surgery with possible excision of residual disease is the better procedure (28, 29). Assessment is superior at operation as more areas can be examined, and the macroscopic appearances of a frozen pelvis frequently require one or more biopsies for clarification. Recent re ports have shown a marked discrepancy between the clinical reponse rate and the pathological response rate determined at second look laparotomy (30, 31, 32). The pathological complete remission rate in these studies has been of the order of 15-20%; approximately 50% of the clinical estimate. A major problem limiting the integration of second look procedures into current treatment strategies is the lack of non cross-resistant second line therapy, as relapses have been documented even after a pathological complete remission in some series (32).

Radiotherapy has been used in the treatment of ovarian cancer for the last 50 years, but analysis of the contribution of this technique has been hampered by variation in histological assessment, staging, selection of cases and dose administered. Doses of between 2500 and 5000 rad have been given, but the higher range requires sophisticated treatment planning to minimise normal tissue damage principally to the bowel. Early treatment plans avoided the diaphragm, but laparoscopy and closer examination at initial surgery has now shown this to be a frequent site of early metastases.

Several prospective randomised trials in patients with stages I, II and III ovarian cancer have demonstrated no significant benefit of radiotherapy over single agent melphalan (33, 34). One recent study from the Princess Margaret Hospital (35) has shown that patients with stages Ib, II and asymptomatic III disease who have undergone complete hysterectomy and bilateral salpingo-oophorectomy had a better 5 year relapse free survival if they were treated with whole abdominal irradiation plus pelvic boost, rather than less extensive radiotherapy with or without chlorambucil. While this is a study of a selected group, the results would indicate that radiotherapy may have a place in those patients with small volume disease - the majority of patients with stage III and IV disease will derive no benefit.

Hormone receptors for oestrogens and progestagens have been demonstrated in ovarian carcinomas, xenografts and human cell lines, but there is no evidence as yet to support their being functional (5). Clinical studies with oestrogens and the anti-oestrogen tamoxifen have not been encouraging (36) and cytotoxic drugs have been the treatment of choice in view of the apparent success of combination chemotherapy, though isolated responses to high dose progestagens have been reported (37). Modern receptor assays may improve the selection of patients and a case could be made for re-evaluating hormone therapy in advanced disease. Androgens and anti-androgens have not been fully tested to date, and may be worthy of further study in addition to the progestagens. New agents have not been very successful in phase II trials, and myelosuppression is frequently dose limiting (38). Hormone therapy has a major advantage over chemotherapy, in terms of lower toxicity, given a comparable response rate, but its role has yet to be defined.

Immunotherapy is theoretically attractive in ovarian carcinoma because of its intra-abdominal mode of spread. Either a non-specific or a targeted therapy could then be delivered by the intra-peritoneal route in high concentrations while minimising systemic toxicity. Clinical studies however have not been encouraging to date (38).

Chemotherapy in Advanced Ovarian Cancer

Ovarian cancer is moderately sensitive to cytotoxic drugs, and alkylating agents in particular have been used in this disease for the last 20 years, giving a clinical response rate of 35-65% when used as first line agents. Even with such treatment, the median survival in Stage III and IV disease is only of the order of 15 months (39). No one alkylating agent has emerged as superior, and patients who have received previous therapy with either cytotoxic drugs or radiation are less responsive (see Table 2a) than untreated patients.

More recently the non-alkylating agents cisplatin, hexamethylmelamine and doxorubicin have been used either alone or in combination, and give response rates almost equal to the alkylating agents in advanced disease (Table 2b). Of these, cisplatin is probably the most active (40) although its moderate to severe toxicity has limited more widespread application. At the time of writing, patients are generally (41) given single agent therapy with the alkylating agents cyclophosphamide, chlorambucil, melphalan or thiotepa or combinations of cyclophosphamide, cisplatin and adriamycin (PAC) with or without hexamethylmelamine or 5-FU (CHAD, Hexa-CAF, CHex UP).

Systemic chemotherapy is also being evaluated in the adjuvant setting, that is where all but microscopic disease has been excised, as in most stage I and II cases and the occasional stage III. Clearly rigorous control of the patient groups used for comparison in such studies is essential, as these patients have no measurable disease to follow. There is some evidence that radiotherapy is just as effective as chemotherapy in early disease, but problems of clinical assessment and patient accrual into trials has resulted in few studies of these 'early' cases of ovarian cancer, and they will not be discussed further in this review.

In advanced ovarian disease, one of the most important questions to be answered in clinical research is whether combination chemotherapy

Table 2a (38)

Alkylating Agent Chemotherapy in Stages III and IV ovarian cancer

Agent	No. of Patients	Response %
Melphalan	541	47
Cyclophosphamide	335	43
Cyclophosphamide (high dose)	36	61
Chlorambucil	388	51
Prednimustine	36	28
Thiotepa	337	48
Mustine	99	31
CCNU	74	19

Table 2b

Nonalkylating Agent Chemotherapy in Stages III and IV ovarian cancer

Agent	No. of Patients	Response %
Cisplatin	237	31
Hexamethylmelamine	142	25
Doxorubicin (adriamycin)	224	14
5-fluorouracil	92	18
Methotrexate	25	5
Methotrexate (high dose)	27	5
Vincristine	17	6
Vinblastine	16	13
Progestagens	50	10

gives any significant advantage over single alkylating agent treatment. Higher response rates are achieved with the combinations at the expense of some toxicity and this might be expected to predict for increased disease free survival, but only two have shown a survival benefit at two years (43, 44), and longer follow up is required for full evaluation. There have also been a number of inconclusive studies (39, 41, 42).

Apart from this overriding question of what form the initial induction therapy should take, the present dilemmas in advanced disease studies can be summarised as follows.

1. How many courses of drug are required to satisfy a clinical definition of resistance to chemotherapy in the absence of a complete response?
2. What additional treatment should be offered to the pathological complete responders (< 20% of all cases)?
3. How should the majority of patients who fail first line treatment be managed? In other words what are the conventional options available for dealing with the frequently encountered problem of clinical resistance?

The optimum duration of a chemotherapy course seems to be of the order of 4-6 months in the absence of evidence of disease progression. As ovarian cancer is only moderately chemosensitive, any objective evidence of improvement is usually taken as sufficient grounds for continuing with that regime. The question of consolidation treatment after complete remission is more difficult, but the requirement for further therapy has become clear over the last 3 years, as there remains a high probability of relapse (18). Some centres are evaluating abdomino-pelvic radiotherapy, some persist with further cycles of the induction drugs, and others use single agent maintenance. None of these treatment options has yet been shown to prolong survival, and toxicity poses formidable problems.

The treatment of relapse depends to some extent on the first

line drugs given. It is widely accepted that where a patient has failed on one alkylating agent given alone, little is to be gained from substituting a second drug with a similar mechanism of action, in keeping with a large body of data in animals demonstrating almost universal cross resistance among alkylating agents (45). A combination may produce some benefit, but a much lower response rate than observed in untreated patients and a median survival of only 4-6 months may be expected (46).

Cross resistance is not unique to ovarian cancer, but it is a major problem in this disease as only a restricted number of moderately active agents are available. However, as it is a reasonably common tumour and relatively easy to grow in vitro culture, it may be particularly suitable as a model to study the problem of alkylating agent resistance.

It should be pointed out that in a group of patients with advanced ovarian cancer, there are a number of causes of treatment failure in addition to primary drug resistance. The patient population is old and may have cardiac and other systemic degenerative disease problems, many of which may be covered by the clinical predictive variable performance status. Most patients have just been subjected to a major surgical procedure in the weeks before commencing the further insult of single or multiple chemotherapy. Absorption of the drug from the gastrointestinal tract may be relevant for orally administered drugs such as chlorambucil, and pelvic fibrosis as a result of prior surgery, radiotherapy or tumour induced desmoplasia may limit the access of drugs to the tumour. However, there is no doubt that intrinsic, acquired resistance and cross-resistance are major causes of treatment failure, and if they could be overcome this would be a significant contribution to improved survival in this disease.

One interesting new approach is to maximise the dose yet minimise the toxicity by giving a drug such as 5 FU, adriamycin or cisplatin by the intraperitoneal route using the large volume

continuous exchange method (47). This is only possible with large molecular weight drugs which have a slow peritoneal clearance, and is particularly appropriate for ovarian cancer with its tendency for intra-abdominal spread without distant metastases. This assumes that sensitivity and hence resistance is dose dependent over an achievable range, for which there is some in vitro evidence (18). The clinical value of dose escalation also depends on the extent to which toxicity is dose limiting. Even if a manoeuvre such as autologous bone marrow transplantation is employed to overcome myelotoxicity there is now evidence that the gastrointestinal or urinary tract toxicity becomes dose-limiting. Modulation of the activity is therefore theoretically more attractive, if knowledge of the mechanism of action of the drug can be applied to introduce an element of selectivity into the modulator if not the primary agent.

Prediction of biological behaviour - the concept of clinical resistance

Since the early days of medicine, doctors have been called upon to predict clinical outcome and have done so with varying degrees of success. More recently, an association has developed between the prognosis, which now is nearly always derived from the diagnosis, and the form of treatment applied. In the cancer field, the five year survival is the traditional end-point by which prognosis is assessed, although a shorter period may be more appropriate for some tumours. While absolute percentage survival figures have little meaning for the individual patient, for practical purposes the prognosis can be regarded as a spectrum, and the form of treatment given depends on the estimated position of the patient in that spectrum. Conventional wisdom states that well should be left alone at the 'good' end of this spectrum, and as the position shifts towards the poor prognosis end, the physician should intervene with progressively more intensive treatments. The final decision obviously depends on a matrix of the effectiveness and the toxicity of the proposed treatment, and the expectations of his patient.

Aware that cancer treatments have a low therapeutic ratio, doctors have historically been prepared to give "aggressive" treatments to the poor prognosis patients, and low toxicity regimens to the patients with a relatively good prognosis. This strategy depends firstly on the assumption that absolute cure is a rare event, as otherwise the good prognosis patient should be treated aggressively also, and secondly on there being a reasonable chance of tumour regression at clinically achievable doses. The assumption here is that a dose/response effect will at least partially overcome clinical resistance. This approach has been successfully challenged in a number of situations, such as Stage I Hodgkin's disease where radical radiotherapy given to a cancer with a relatively good prognosis is justified by a high chance of cure, or Stage II testicular teratoma where combination chemotherapy, previously considered too toxic, is now accepted management and achieves cure in the majority of cases.

In practice, the cost in long and short term toxicity has always to be weighed in each patient against the likely benefit in terms of increased lifespan of an acceptable quality. Clinicians are constantly looking for means of improving toxicity, defining the acceptable limits of side effects, and relating these to quality of life.

The next section reviews the various tumour and host factors that have been used to predict clinical outcome in ovarian cancer, and leads up to the rationale for the in vitro studies that are the basis of the laboratory work in this thesis. The justification for such studies rests on the assumption that a fuller understanding of the mechanisms of action and the mechanisms of resistance at the cellular or molecular level will eventually have an impact on clinical resistance.

Histology - light microscopy has proved useful in distinguishing 'benign' lesions with no stromal invasion from those that should be regarded as malignant, and for separating the tumours of germ cell and stromal origin. Within the broad group of common epithelial tumours, morphological classifications give little information on

likely behaviour, and the superior resolving power of electron microscopy has not made a significant contribution. Furthermore, a pathological diagnosis is a consensus rather than a quantifiable level and the categorisation often drifts with time, particularly in ovarian cancer where there is no universally accepted aetiological background to the classification.

Grade - Broders' grade is a refinement of histological analysis, and is based on a combination of tissue architecture and cellular differentiation. Correlation with survival is good (48), but ideally the assessment should be made by one pathologist owing to considerable inter observer variation. Grade is related to the extent of tumour at presentation, but the correlation with survival is independent of this association, and holds even when effective cytotoxic therapy is given (24).

Stage - this is a measure of the clinical extent of disease at presentation, and is severely limited by present imaging techniques. The FIGO system described above (Fig. 1) is based on the known common sites of metastases from ovarian tumours which are:

- the other ovary.
- direct and metastatic to the other pelvic tissues.
- transcoelomic spread to the peritoneum and diaphragm.
- peripheral nodes, principally the axillary and cervical chains.

Even in early localised pelvic disease, spread to one or more of these sites is likely, and 70% of patients present with metastases to the peritoneum or beyond. Unlike Hodgkin's disease, for example, accurate anatomical definition of disease is not helpful in prediction of the site of subsequent spread and the only group which can be isolated because of their excellent prognosis are the 10% of cases confined to one ovary. Furthermore, stage is only an observation at one point in time and it does not directly give a measure of the risk of subsequent spread, or the likely site of that spread, both important factors in devising a treatment strategy.

Ascites/Rupture - A theoretical distinction can be drawn between ascites formed because of lymphatic obstruction to the outflow tracts from the peritoneum, and that due to malignant cells in the fluid, but this separation is often marred in the published literature by failure to examine the fluid cytologically at surgery. There is, however, no firm evidence that grade for grade, and stage for stage, that those patients with ascites or rupture suffer any disadvantage (49).

Bulk - this may correlate with prognosis, but the effects in studies to date have been qualitative rather than quantitative. In the Gynecologic Oncology Group Study, the group treated with melphalan alone survived better if they did not have bulky disease after surgery (50), and there was a similar finding in the surgically assessed patients receiving combination chemotherapy (51).

Response - In very chemosensitive tumours such as lymphomas or testicular tumours a complete response correlates well with survival, but this relationship does not hold so well for the less sensitive tumours such as ovary. A partial response is of no prognostic value. The duration of complete response or disease free interval is a better guide, but the principal disadvantage of response as a predictor is that it can only be assessed retrospectively.

Prior treatment - this makes induction of a response very unlikely even if there was a response to the first line treatment, and the common clinical experience is that these patients do very badly.

Historical data banks - by pooling data from large numbers of patients treated in major centres in different parts of the world, an attempt has been made by computer analysis to provide a likely outcome of a patient with a particular set of clinical and pathological characteristics (52). In most cases, these would comprise those described above with the addition of performance status. This approach has been used primarily in breast cancer to date, and the technique is open to a number of objections particularly where it has

been substituted for concurrent untreated controls in clinical studies. However, the concept of correlating diverse clinical data by computer and assessing a prior probability of a future event is attractive, and may find a more concrete application in time.

In summary, morphological assessment of the tumour, and clinical measurement of disease position, bulk and extent give only a rough guide to likely biological behaviour. Advances in cell biology have recently been used in an attempt to refine present classifications into more prognostically relevant categories, and to take account of the effects of treatment, particularly cytotoxic drugs, on individual cells. A parallel could be drawn with the classification used for glomerulonephritis, which now comprises steroid sensitive and steroid insensitive groups. In the non-haematological malignancies, cell surface characterisation, and specific marker protein production has not contributed as much as in the lymphomas and leukaemias. There are few animal systems that provide good models for ovarian carcinoma. Techniques for culturing human cells from a variety of tumours have been developed over the last 10 years, and the hope is that they will provide a better model for studying the mechanism of action of drugs, and the means by which resistance is acquired. They may also allow more specific direction of management by drugs alone or in combination with radiotherapy.

1.3 Predictive drug testing - the selection of a suitable model system to predict sensitivity and resistance in individual patients

A variety of methods have been developed over the last 30 years for predicting clinical response to anticancer drugs, following the successful techniques developed for antibiotic activity against bacteria. The methods differ in the way the drugs are exposed to the tissues under test, which may be *in vivo* (diffusion chamber, heterotransplantation in xenografts, subrenal capsule test), or *in vitro* (monolayer, organ, free floating slices and single cell suspension) and also in the end-points used to assess the effect of the drugs, which have been by morphological evaluation, cell counting,

enzyme activity, and tracer incorporation studies. The techniques have been well reviewed by Dendy (54) and Kaufmann (53).

The in vivo exposure methods are time consuming, slow and expensive, and have found favour in only a few special centres. Sufficient numbers of specimens have rarely been evaluated to assess the degree of clinical correlation (55-58). Experience with xenografts is increasing, but at present the proportion of ovarian tumours growing is low. Monolayer exposure has the advantage that the cells are intact and relatively free from mechanical or enzymatic trauma at the time of exposure, but cannot be established in each case, and the cells exposed may not be representative of the original tumour.

Organ and slice culture are difficult to quantitate, handle and reproduce, and most recent workers have favoured the single cell suspension, derived either by mechanical or enzymatic means from the parent tissues. The incubation used is commonly 1 hour at 37°C, largely for its convenience, and this has allowed some standardisation between groups. Continuous exposure may however, be more relevant to the in vivo situation for drugs with a long lalf life and a cell cycle specific mode of action, such as the antimetabolites and mitotic inhibitors.

The methods of assessing cytotoxicity have become more refined in recent years. Morphological assessment of damage induced by drugs to a monolayer gives a qualitative result, and while the subjectivity of the end-point can be improved by trypan blue or nigrosin dye exclusion, the accuracy remains poor. Absolute cell number can be counted by manual or mechanical means, and providing growth is continued for at least two doubling times before assessment, this provides a useful end-point for those assays employing monolayer growth. The counter will not distinguish between normal (e.g. fibroblasts) and malignant cells, or between viable and non viable cells and this can be a major problem in short term cultures of human tumours.

Incorporation of tritium labelled nucleic acid precursors (TdR, UdR, dUdR) or protein precursors (leucine) has been employed to assess the short term metabolic or replicative activity of the test cells, which may be either in suspension, monolayer or organ culture. The actual measurement can be performed by autoradiography or incorporation into trichloro-acetic acid precipitable materials. Rather than individualisation of one or more drugs for a particular tumour, Kaufmann (59) has used the in vitro sensitivity to adriamycin to predict a common sensitivity or resistance to a range of cytotoxic agents in the parent tumour.

The advantages of these techniques are that results should be obtainable within a short time (< 72 hours) which is convenient if clinical therapy is going to be based on the results. Many human tumour cells have long cell cycle times, however, and an unknown proportion of the cells in a tumour population may be non-cycling. Nucleotide pool sizes may be affected by mechanical trauma to the cells, and also by the antimetabolites, so that isotope incorporation may be only indirectly related to cytotoxicity.

All the above methods suffer from the disadvantages of separating effects on non-malignant cells from those on malignant cells, and from the fact that many of the cells counted may recover from the drug induced damage. In the tracer incorporation studes, some of the uptake may be due to cells which have a limited self-renewal capability. The cells under test in these systems may therefore not be relevant to the biological behaviour of the tumour after drug treatment as they are not true stem cells. In spite of these objections, reasonable clinical correlations have been achieved, and are summarised in Table 3.

Table 3 Growth and evaluation rates for human tumours in short term culture

Reference	No. of Patients	Method	Evaluation	In vitro In vivo correlation
Wright 1975 (60)	117	Monolayer	Morphology	64%
Hurley 1965 (61)	174	Organ culture	Morphology	65%
Tanneberger 1973 (62)	55	Organ culture	3H TdR 3 hr	65%
Wolberg 1962 (63)	19	Slices	Autoradiography	100%
Murphy 1975 (64)	45	Cell suspension	Autoradiography	100%
Kondo 1971 (65)	95	Cell suspension	Enzyme inhibition (succinic dehydrogenase)	75%
Wilson 1981 (66)	5	Monolayer	Leucine uptake	79%
KSST 1981 (67)	65	Cell suspension	3UdR uptake	75%
Kaufmann 1982 (53)	155	Cell suspension	3UdR uptake	67%

Assays for clonogenic tumour cells - the best assessment of in vitro cytotoxicity?

In proliferating cell populations, the inability to reproduce indefinitely is the only relevant criterion to assess cell lethality. Roper and Drewinko (68) showed that colony forming activity from a single cell suspension in soft agar was the most reliable, dose dependent index of cell lethality compared with doubling time, labelling index, dye exclusion, ^{51}Cr release and [^{3}H] thymidine incoporation. Such assays are purported to measure tumour "stem cells" which represent a concept of infinite self-renewal.

The hypothesis that such cells are relevant to tumour progression is largely based on studies on the haematological malignancies, and has been elegantly expounded by Nowell (69). At present tumour stem cells cannot be identified directly and the only reliable criterion available is the capacity of these cells to give rise to multiple progeny observed as colonies. The formation of colonies in culture was originally achieved only for tumour cells maintained as continuous lines in culture, and the generation of tumour cell colonies in the lung or spleen of experimental animals was used as an end point (70). The nature of the culture system precluded the study of many aspects of cytotoxic drug activity, and a major step forward came with a primary culture colony assay developed by Park in 1970 (71) using myeloma cells grown in soft agar.

More recently these studies have been extended to other solid tumours, and using a variety of experimental conditions, it is now possible to study the capacity of primary human tumours to form colonies in vitro. This raised the possibility of an assay to predict the in vivo tumour response or resistance using in vitro tests of cytotoxicity. It has not yet been resolved whether cells that are clonogenic under the test conditions are the same cells or a sub-group of the true stem cells in vivo (72).

Buick and Mackillop (73) have attempted to provide a mathematical

theory for the stem cell model of human tumour growth. They propose a differentiation hierarchy (Fig 3) from stem cells with an infinite capacity for self-renewal, through transitional cells in a phase of clonal expansion to end cells. The number of generations or divisions in the clonal expansion phase could vary considerably. Small colonies or clusters could be formed from these transitional cells, and they postulate that it should be possible to define a "cut off" size of colony at a given time in culture above which only a true stem cell colony should be recorded. Most assays at present use an arbitrary limit of between 20-50 cells after 10-21 days in culture, below which the term "cluster" is used, but this end-point may have to be refined.

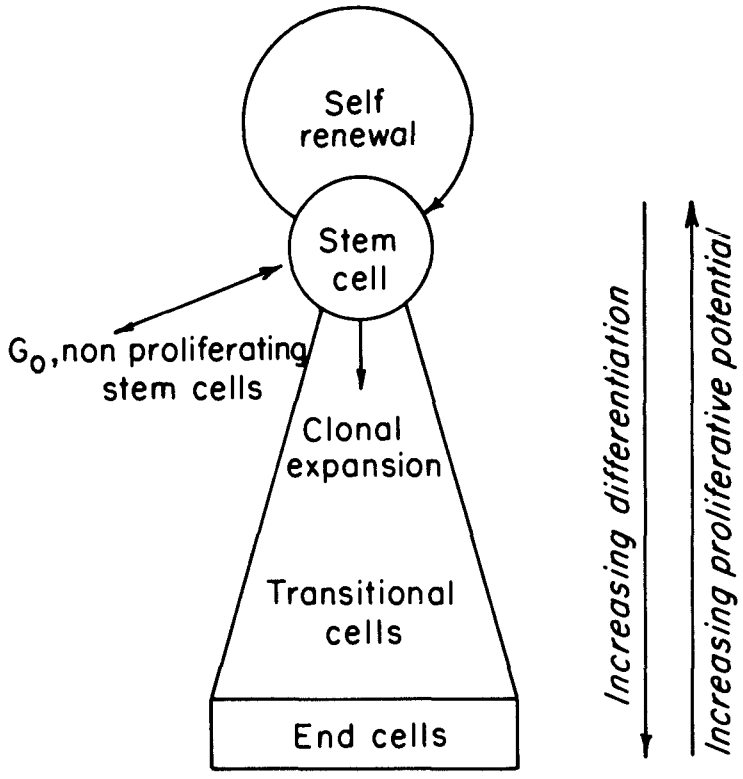


Fig. 3. Stem cell differentiation hierarchy proposed by Buick and Mackillop (73)

Clinical correlations between in vitro Colony Forming Activity and in vivo Drug Response in ovarian cancer

The recent publication of good clinical correlations between reduction in survival of colony forming units in soft agar derived from single cell suspensions of human tumours has resulted in widespread application of this technology. Taking the two largest series of all cancers assessed by these primary cloning (74, 75) methods, 23% of tumours form sufficient colonies in vitro to perform drug assays, and the clinical correlations average 60% for prediction of sensitivity and 97% for prediction of resistance, this data being based on 339 drug trials in 197 patients. Restricting the analysis to those patients with ovarian cancer reduces the numbers available for comparison, but Table 4 shows the correlation obtained in the 5 main centres. A consistent feature is the good correlation with resistance, a relatively commmon event in ovarian cancer, compared to the prediction of sensitivity which is only 60% accurate.

When taken together with the low proportion of all tumours which can be grown in primary culture at present, many workers have concluded that rather than the individualisation of treatment, the principal clinical application of the assay at present would be in the exclusion of certain patients from high dose, experimental or toxic drug regimes if resistance to these agents is shown in vitro.

Following from the above studies which suggested that soft agar colony formation was a good in vitro model of human ovarian cancer, some authors have explored the functional aspects of colony formation in agar and suggested that in vitro growth per se may be an indicator of the biological behaviour of the parent tumour (81). Attempts have also been made to use such systems to study the pharmacology of cytotoxic drugs at the cellular level. Preliminary studies of tumour resistance have been made by Alberts (78) who looked at 55 patients in relapse from ovarian cancer and found considerable evidence of in vitro cross resistance. Of relevance to the present study was the observation that melphalan resistance was almost always

Table 4 Clinical correlations with the stem cell assay in ovarian cancer (end-point tumour response)

Author	Method	No. of patients	Sensitivity correlation	Resistance correlation
Alberts (76)	Retrospective	40	62%	99%
Ozols (77)	"	79	64%	94%
Alberts (78)	Prospective	32	73%	100%
Natale (79)	"	28	77%	93%
Von Hoff (80)	"	25	44%	-

associated with *in vitro* adriamycin resistance. Cross resistance patterns may be useful for selecting second line treatment as well as in identifying potential mechanisms of resistance. The same author (82) has published some preliminary work on potential interactions showing *in vitro* drug synergism between vinblastine and bleomycin.

Further studies to elucidate the mechanisms of resistance may require the development of resistant cell lines which could be used to examine biochemical differences between sensitive and resistant populations. Xenografts prepared from human tumours with a range of known drug sensitivities are another alternative, but such systems are slow, open to a higher probability of phenotypic drift, and would require large numbers for evaluation.

Selection of a model system for the study of drug resistance in ovarian cancer

The principal aim of this project was to find a suitable system in which to study the phenomenon of cytotoxic drug resistance in ovarian epithelial cancer. As discussed above, the causes of clinical treatment failure are complex, and depend on toxicity, compliance, and general state of health as well as biochemical resistance, and tumour models are required in which the variables can be minimised and the questions answered quickly. Initially in 1980 primary human culture techniques looked promising, employing colony forming activity in a two layer agar culture as an end-point for the assay of cytotoxic drug activity (83). There was also evidence, in contrast to the previous experience with animal models of this disease, of a mouse model (M5076) for ovarian epithelial carcinoma which could be grown either subcutaneously or as an ascites tumour, and whose in vivo chemosensitivity pattern reflected that of ovarian carcinoma (84-86). The tumour had arisen spontaneously at the Southern Research Institute in C57 black mice and was maintained in the DB26F1 female hybrid.

The ideal system for study of resistance would have been the primary culture of human ovarian tumours, and it soon became clear that even if a regular supply of material could be obtained, there were major technical problems in the growth of colonies and in reproducibility of the assay. Some preliminary work was carried out on primary culture of human ovarian samples in conjunction with Dr. Morgan, University of Southampton, and at the same time arrangements were made to obtain mice bearing the M5076 carcinoma, to be used initially for further development of the cloning assay. Preliminary cytotoxic drug assays were performed using the M5076 once the technical problems of its growth in agar were overcome, but for reasons described later (p114) the in vitro studies of drug resistance were carried out in the human cell lines.

While the mechanism of action of many of the cytotoxic drugs had been studied intensively in animal cell lines, it was not

until 1982 that preliminary analysis of cytotoxicity studies on human ovarian cell lines,using a modification of the Salmon and Hamburger agar bilayer method became available (87). Few permanent ovarian carcinoma cell lines have been described (88-91) and none have been used to date in the study of resistance to cytotoxic drugs. Eight months of this project were spent in the laboratories of Dr. R.F. Ozols, National Cancer Institute, Bethesda, USA where several lines were undergoing development, one of which, 1847, was derived from a patient with ovarian carcinoma who had not been exposed to cytotoxic drugs. The cells retained their morphology and cytogenetic pattern after passage in culture 54 times, cloned in soft agar and formed tumours in nude mice (91). Some of these cells were grown in media in which the concentration of melphalan was increased in a stepwise fashion over a period of 6 months in an attempt to induce resistance. At the time of my joining the laboratory the degree of resistance had not been confirmed but the evidence suggested this would be a useful model system to study in vitro resistance to this particular alkylating agent.

1.4. Investigation of resistance to alkylating agents at the cellular level

Alkylating agents react with many molecules in the cell, but present evidence would suggest that the major target site is DNA, and hence any property of the cell which prevents alkylating agents from reaching this site and binding, or which leads to repair of alkylated DNA will lower the sensitivity of the cell to these compounds. The bifunctional alkylating agents mustine, melphalan, cyclophosphamide and chlorambucil have been the most clinically useful of this group. Alkylating reactions may be classed as either S_N1 (nucleophilic substitution, first order) or S_N2 (second order). In the S_N1 reaction, the target nucleophile X is replaced in a single step process by the alkylating carbonium ion Y^-. R represents the DNA or peptide chain. In the S_N2 reaction X, R and Y are associated in an ill-defined complex before the final reaction product is formed.

S_N1 reaction $RX \longrightarrow R^+ + X^- \xrightarrow{+Y^-} RY \times X^-$

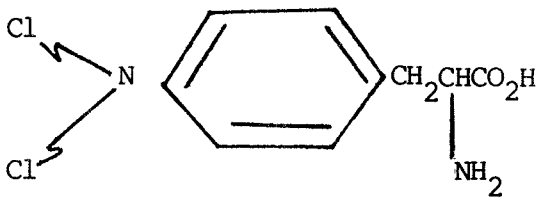
S_N2 reaction $RX + Y^- \longrightarrow (X..... R .....Y) \longrightarrow X^- + RY$

The principal difference is that as S_N1 reactions have a highly reactive intermediate, they will obey first order kinetics and the rate will depend only on the concentration of the alkylating agent, whereas the rate of S_N2 reactions depends on both the concentration of the alkylating agent and the target nucleophile. Virtually all the oxygen and nitrogen atoms on the purine and pyrimidine bases of DNA can be alkylated, but the N-7 of guanosine appears to be the main target site. The present hypothesis is that interstrand DNA cross-linking is the major factor in the anti-tumour effect of the alkylating drugs, whereas carcinogenicity and non-specific cytotoxicity may be related to monofunctional alkylations of DNA, nucleoprotein or cytosol protein of the cell (92).

Acquired resistance to alkylating agents is a major clinical problem, and there are a number of theoretical mechanisms by which this may occur:

- reduced uptake of the agent
- reduced activation of the agent
- increased breakdown of the agent
- increased production of target molecule or other nucleophile
- reduced sensitivity of the target site
- repair of alkylated DNA

Melphalan is a bifunctional alkylating agent widely used in myeloma and ovarian cancer with the following chemical structure:



It does not require activation, and at physiological pH it undergoes hydrolysis to the inactive mono-and dihydroxy- derivatives, resulting in a plasma half life of 1.8 hours. Using a number of in vitro animal tumour model systems, several possible mechanisms of resistance have been investigated. Initial studies concentrated on drug transport analyses as a defect at this level seemed the most likely explanation for the multiple cross-resistance observed in vivo.

Vistica showed that melphalan shared the leucine carrier system in L1210 cells (93), but comparison of the uptake and efflux of radiolabelled melphalan by the parent line and two resistant sublines was identical (94). However, Colvin (95) and Elliott (96) found less efficient melphalan transport in resistant variants of L1210 cells and Chinese hamster ovary cells respectively. Studies on human melanoma cells by Parsons (97) showed no difference in melphalan transport between sensitive and resistant cells, and suggested that resistance was due to reduced accessibility of DNA to the cross linking effect of melphalan. Using the alkaline elution technique, it has been shown that resistance to the nitrosoureas may be related to diminished formation of cross-links (98), and that enhanced excision repair synthesis is observed in the resistant cells.

Several workers have noted an increase in the non-protein thiol levels of resistant cells. Vistica extended these studies in L1210 cells using a specific assay for glutathione, a tripeptide which accounts for 90% of the intracellular non-protein thiol (99), and showed that L1210 cell sublines resistant to melphalan could be sensitised by depletion of the intracellular glutathione, whereas mouse bone marrow cells were not. The precise mechanism by which the glutathione renders the cells resistant to alkylation is not clear - thiol compounds are known to be important in detoxification of a wide range of drugs, but direct covalent interaction with melphalan at physiological pH is minimal (D. Vistica - personal communication). It is now thought that glutathione and related compounds have a central 'protective' role in maintaining the redox potential of the

cell and this could affect the reactivity of the oxygen and nitrogen radicals to alkylating agents. This role may not only be important for drug induced damage, as depletion of intracellular glutathione has recently been shown to sensitise hypoxic tumour cells to X-rays (100).

A number of mechanisms of resistance have been suggested by the experimental systems described above, and it may be that resistance to alkylating agents in vivo is due to the cumulative effect of several biochemical changes, rather than the single gene effects seen in resistance to the antimetabolite methotrexate (101). Changes in membrane permeability and thiol content have been consistent findings associated with the development of resistance to the alkylating agents in animal tumours. The former might be expected to be mediated through alteration of a carrier protein, while the latter could be modulated by synthetic or degradative enzymes, or membrane transport of the thiol itself. Either explanation would account for the wide, but not necessarily universal resistance observed to the alkylating agents. Identification of the mechanism of resistance should then allow development of a strategy by which it may be overcome.

1.5. Summary

Having selected a suitable model of human ovarian epithelial cancer, the plan was to examine the mechanisms of resistance originally described in animal tumour studies. In particular, it was decided to look for a difference between sensitive and resistant cells which could be clinically exploited, and the aims of the entire project can be succinctly stated as follows:

1. To compare the relative benefits of single alkylating agent therapy and combination chemotherapy in a randomised clinical trial in advanced ovarian cancer, and to study the degree of resistance and cross resistance encountered and compare this with the *in vitro* resistance found in primary culture of human ovarian tumour samples.

2. To characterise the *in vitro* growth of the M5076 mouse ovarian tumour in soft agar, and the possible role of the system as a model for the study of cytotoxicity in ovarian cancer by quantifying the drug induced reduction in colony forming activity.

3. To assess the suitability of human ovarian cell lines as a model system for the study of resistance to cytotoxic drugs.

4. To investigate possible mechanisms of resistance to the alkylating agent melphalan in the human ovarian cancer cell lines.

2.0. Methods

2.1. Clinical Trial - Advanced Ovarian Cancer

This trial was conducted under the auspices of the Wessex Regional Medical Oncology Unit and a complete protocol is included as appendix 4. The objective of the study was to evaluate conventional management with a single oral alkylating agent against intensive induction chemotherapy with a cisplatin containing combination with its associated toxicity. Patients with Figo Stages III and IV ovarian cancer, aged less than 70 years and with a Karnofsky performance status 4 or greater were eligible for randomisation, subject to informed consent, between long term single agent chlorambucil and five courses of combination therapy with cisplatin, adriamycin and cyclophosphamide (PACe) at monthly intervals. The chlorambucil was given at a dose of 5mg bd for 2 weeks out of 4, and the PACe as shown below.

Cisplatin	80mg/m^2	IV
Adriamycin	40mg/m^2	IV
Cyclophosphamide	1,000mg/m^2	IV

Cycle time 21 days

Adequate prehydration was given with the cisplatin to ensure a urine output of 150ml/hr. at the time of administration, and continued for 12-24 hrs afterwards. Patients were excluded if their creatinine clearance at entry was <50ml/min, to minimise the development of cisplatin nephrotoxicity. The doses of one or more drugs was modified in subsequent courses depending on the toxicity encountered, and the detailed dose reduction schedule is described in the protocol (appendix 4). The principal difference between the regimes is the lack of toxicity and ease of administration of the chlorambucil which is given as an outpatient, compared to the moderate to severe toxicity of PACe, which requires an in-patient stay of at least 24 hr, and closer monitoring of renal function and bone marrow reserve.

Patient data was extracted from the medical records initially by the medical staff, but later by the data collection staff of the Medical Oncology Unit, under medical staff supervision. Storage of data was on a Hewlett Packard HP9845T desk top computer with custom designed software to store and analyse the data.

Assessment was initially by physical examination, and repeat abdominopelvic ultrasound. For those patients in complete clinical remission, the preferred mode of assessment was a second look laparotomy at or around 6 months after the start of chemotherapy. This was subject to the consent both of the patient and the referring surgeon. The trial design incorporated a crossover in that patients who failed either arm were changed to the alternative arm if their general condition permitted further chemotherapy. Patient follow-up was continued indefinitely, monthly for the first 6 months, then 2 monthly for a further 6 months, and 3-4 monthly thereafter.

2.2. Human Ovarian Tumours

Samples of ovarian tumours 1-5 cm^3 taken at operation from patients throughout the Wessex Region between 1979 and 1982 were collected in tissue culture medium (McCoy's 5A with 10% foetal calf serum) and sent to the laboratory, the transport time varying from 1-48 hours. Gynaecologists were circularised with requests for clinical material, and given monthly supplies of medium to be stored at 4°C. Selection of the tissue was left to the individual surgeons who were asked to choose what appeared to be a cellular portion of the tumour which would not compromise the tissue diagnosis of the histopathologists. In addition samples of ascitic fluid removed from patients with known ovarian cancer were obtained from Winchester and Southampton hospitals within 3 hours of abdominal paracentesis and were sent in heparinised containers.

Primary culture technique - human tumours

The method used was that of Hamburger and Salmon (83), or with

occasional slight modifications as described below. A piece of tumour tissue weighing between 3-5 g was cut up in a 5cm petri dish containing McCoy's 5A medium with crossed scalpel blades into pieces no larger than $1mm^3$. Any remaining large pieces of tissue were teased apart with needles, and the cells released into the medium at this stage were termed the mechanically dissociated fraction (MDF). The cell suspension produced was aspirated once through a 23g neeale, then centrifuged at 300g for 5 min. The remaining fragments were transferred to a universal container and stirred in 10ml of an enzyme solution containing 0.2% collagenase III, 0.1% hyaluronidase and 0.05% deoxyribonuclease (Worthington) for 30' at 20°C. The cells released into the medium were aspirated through a 23g needle and then washed twice in McCoy's 5A enriched with 10% heat inactivated foetal calf serum (FCS) and 5% horse serum (HS). These cells were termed the enzymatically dissociated fraction (EDF).

An aliquot of each single cell suspension was then stained with trypan blue (0.1%) for 5 min. and the proportion of viable (dye excluding) nucleated cells determined in a haemocytometer. The mechanically dissociated fractions gave viabilities from 10-60% while the enzyme methods applied to the tissue remaining after the removal of the loose cells gave viabilities in the range 50-90%.

The cells were then spun down (300g, 5 min) and resuspended in CMRL 1066 with 15% heat inactivated horse serum (HS) asparagine (6.6 mg/ml) and mercaptoethanol (50uM) in 0.3% agar, and plated out in 3 x 1ml aliquots at concentrations of from 10^4 - 10^6 viable cells/ml in 35 mm petri dishes prepared for tissue culture. A feeder layer composed of 1ml of a mixture of McCoy's 5A, DEAE dextran 50ug/ml (Pharmacia), asparagine 6.6mg/ml 10% FCS 5% HS and 10% tryptic soy broth in 0.5% agar was found to be satisfactory. Incubation was then carried out in 6% CO_2 at 37°C and the petri dishes examined at 60x and 160x on an inverted microscope every 48 hr. Colony formation usually appeared between 6 and 10 days, and only aggregates of 30 or more cells were counted as colonies (Fig. 4).

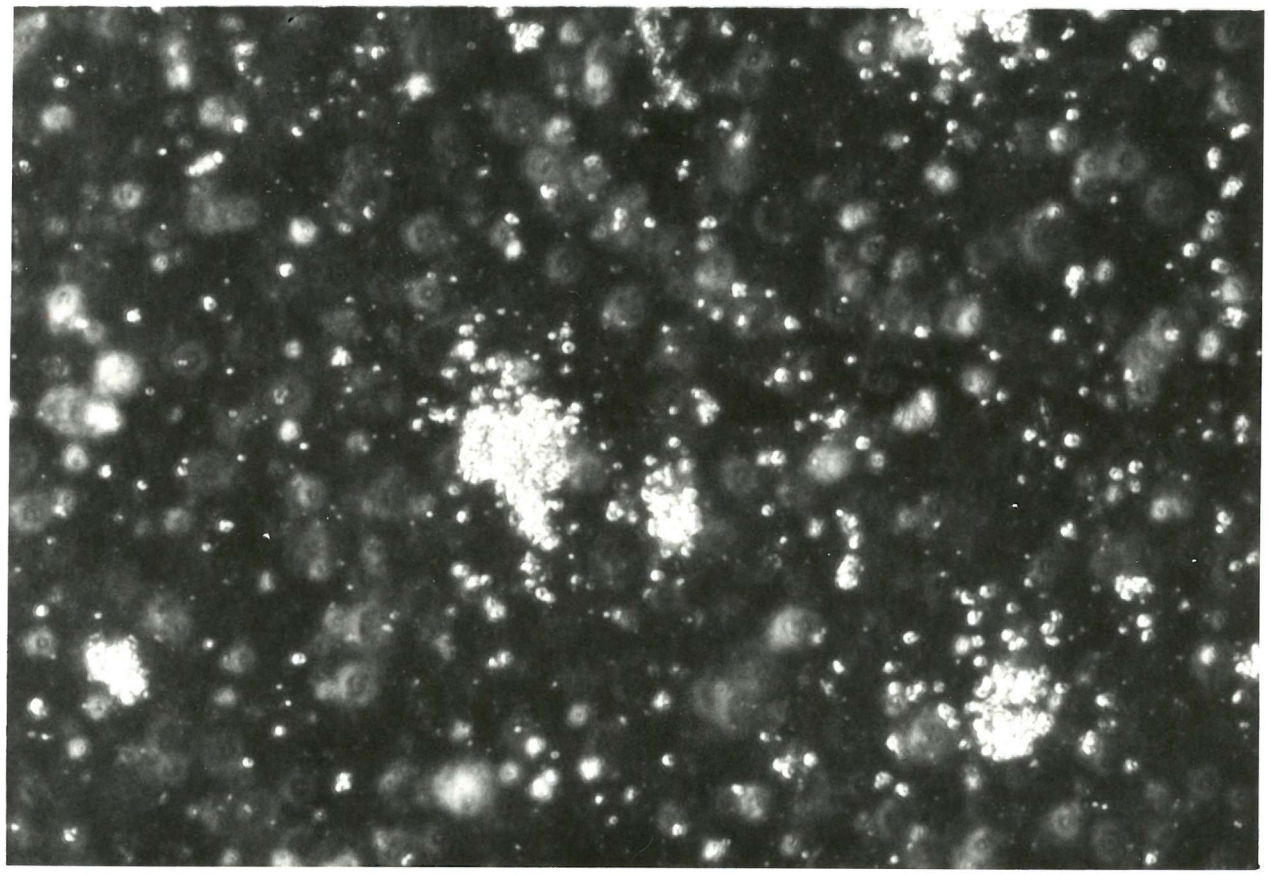


Fig. 4. Colonies comprising about 50 M5076 cells in 0.3% agar by Salmon technique at day 18. (Inverted microscope, unstained X125).

2.3. Mouse Ovarian Tumour - M5076

This tumour arose spontaneously in C57 black mice at the Southern Research Institute, Birmingham, Alabama 3520205 and can be maintained by serial passage subcutaneously as an ascites (85,86). The transplant has subsequently been maintained in the DB26F1 hybrid.

Subcutaneous implantation

A trocar and cannula was fashioned from a shortened pleural aspiration needle with internal diameter 1mm. A portion of solid tumour tissue was taken under aseptic conditions from a DB26F1 mouse and minced with crossed scalpels in 0.9% saline. One or two of the resulting small pieces were inserted into the cannula, and the tip inserted subcutaneously into the anterior part of the left flank. A small nodule appeared at 10 days and was approximately 1cm in diameter at 21 days. The tumour became necrotic if left much longer and was usually harvested at this time. The animals die with a large local tumour if left at a median time of 35 days, but occasionally metastases are noted, especially to the ovaries and peritoneum.

Growth as ascites

The M5076 tumour was disaggregated by the mechanical method described on page 37 and 10^6 cells, irrespective of viability, were injected intraperitoneally into 4 mice. After 6 weeks, large nodules appeared at the site of injection, together with marked ascites. Direct transfer of 0.2ml. of this ascites was found to produce malignant ascites reproducibly in 21 days. Usually the ascitic fluid was opaque but occasionally it was heavily bloodstained for reasons that are not clear.

Growth of M5076 in monolayer culture

Several attempts were made to establish the mechanically and enzymatically dissociated cells in monolayer culture in 60mm petri

dishes in a 6% CO_2 air incubator and in rectangular flasks in 20mM hepes buffered McCoy's 5A using either 15% FCS or HS. The cells initially adhered to the plastic and after 24-48 hr became detached, the total cell count rising by a maximum of 2 fold. It was noted that the M5076 cells during their period of attachment preferentially associated with adherent fibroblasts (Fig. 5). Thereafter the cells remained in suspension, but true exponential growth was not established, in contrast to the findings of Talmadge et al (102) with ascites grown M5076 cells. It was not possible to repeat their experiment with single cell suspensions derived from either solid or ascites grown M5076, using either McCoy's 5A, CMRL 1066 or RPMI 1640 medium. Coating the surface of the petri dish with Type 1 collagen (Sigma) had no effect on adherence.

Primary culture of tumour M5076 (Salmon and Hamburger method)

Initially attempts were made to apply the soft agar colony culture method of Salmon and Hamburger (83) to the subcutaneous M5076 tumour as described above under primary human tumour culture on page 30. Occasional colony formation was noted, but the medium rapidly turned acid, presumably due to active growth or repair metabolism, and this problem was not overcome by increasing the proportion of feeder layer to top layer.

The plating efficiency was low (Mean 0.06% and range 0.006% to 0.1%), and was not enhanced by the addition of mouse embryo extract (103) to the feeder layer, or by deletion of mercaptoethanol or DEAE dextran from the upper layer. Filtering the cell suspension through a wad of sterile gauze or aspiration, through fine steel mesh of pore sizes 20, 60 and 100 as suggested by Tihon (104) reduced the yield and did not improve the resulting single cell suspension. Replacing the horse serum by foetal calf serum had no effect. Infection was a continual problem both with fungus and bacteria. The former (actinomyces) was related to sharing the incubator with samples from primary human tumours, which were often contaminated de novo. The mouse fur may also have harboured these organisms but immersion of

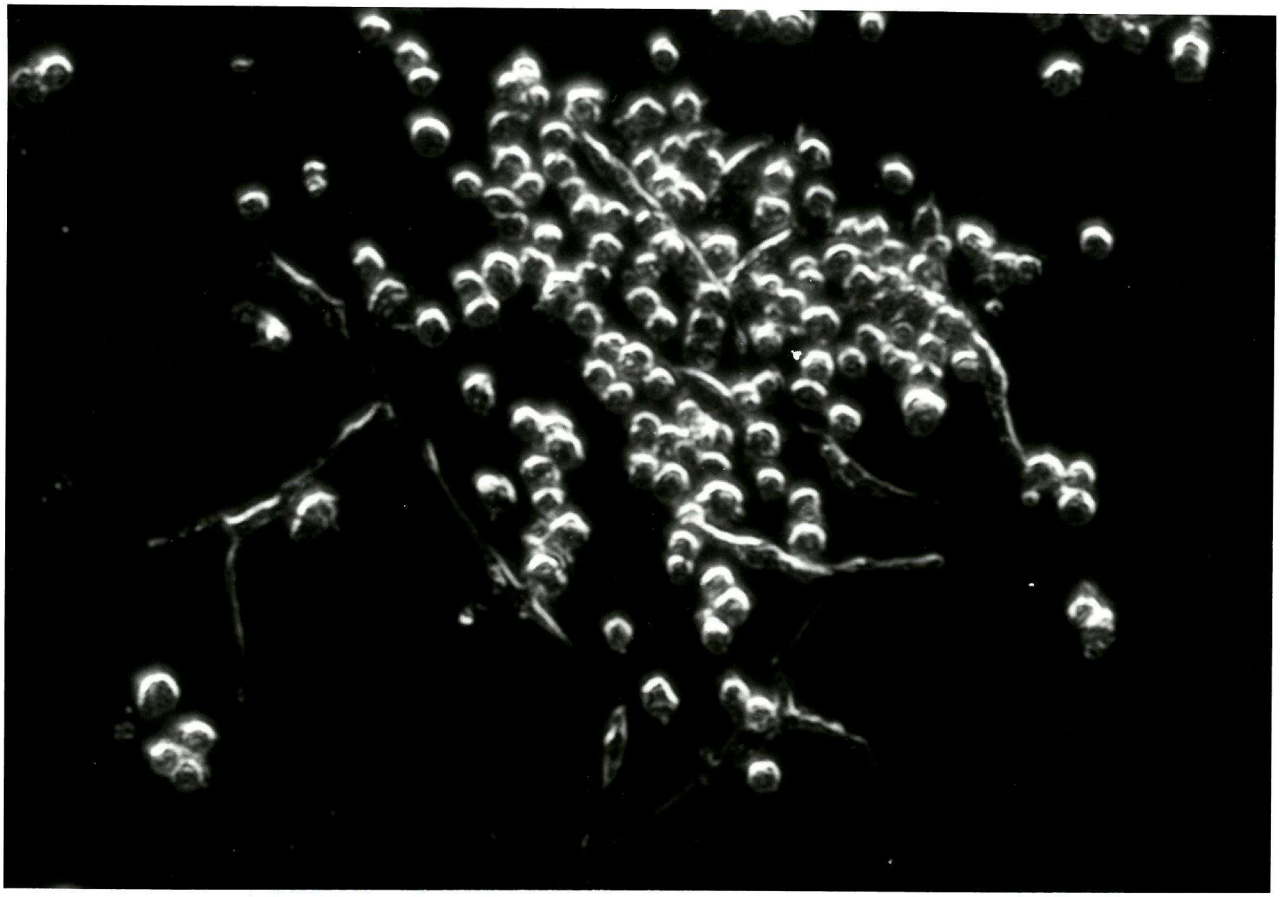


Fig. 5. M5076 cells adherent to plastic after 24hr growth in culture medium. (Inverted microscope, unstained X200)

the tumour in absolute alcohol had no effect. Pseudomonas aeruginosa isolated from the plates was traced to the water bath and adoption of a regular cleaning regime for the hardware and improved aseptic technique eventually led to a decline in this problem. A different soft agar colony culture method employing a single agar layer was described by Courtenay in 1978 (105) and evidence has been published demonstrating improved plating efficiency with this technique (106) compared to the method of Salmon et al.

Primary tumour culture method (Courtenay, 1978) M5076 mouse tumour

The tumour specimen was removed from the mouse under aseptic conditions and chopped into approximately 1mm cubes with crossed scalpels. Cells released into the medium were then aspirated through 19, 21 and 23 gauge needles and termed the mechanically disaggregated fraction (MDF). Where enzymes were used, the remaining tissue was incubated in 10-20ml. of 0.2% collagenase 111 and 0.05% deoxyribonuclease (Worthington) in serum free McCoy's 5A for 30' at 20°C with gentle mechanical stirring. The cells were then spun down at 300g for 5' and washed twice in McCoy's 5A with 10% FCS + 5% HS to remove traces of enzyme. These cells are termed the enzymatically disaggregated fraction (EDF). Where ascites specimens were processed the 3-5 ml. of fluid was suspended in 20 ml of McCoy's 5A containing 10 units/ml preservative free heparin, and then washed twice in McCoy's 5 A + 10% FCS + 5% HS.

The cells were then counted in a Neubauer chamber and appropriate dilutions made in McCoy's 5A with 10% horse serum at 5x the intended final concentration. To 1.2 ml of this cell suspension was added 1.2 ml. CMRL 1066 with 15% horse serum, 1.2 ml of a 1:8 dilution of twice washed August rat red cells made up in CMRL 1066 with 15% HS, and 3.6 ml of CMRL 1066 + 15% HS + agar which had been kept in a water bath at 44°C to give a final volume of 6ml and agar concentration 0.3%. One ml. of this solution was quickly transferred to ice for 10 minutes to solidify the agar and at least four replicate tubes were made of each sample.

The tubes were then gassed with 5% O_2, 5% CO_2 90% N_2 for 10 seconds, the cap sealed in place, and the tubes placed in rigid plastic lunch boxes which were gassed with the same mixture and sealed. Every 48-72 hr, the tubes were opened, 1-2 ml. of medium carefully layered on top of the agar, and the tubes and boxes regassed. Except for the first re-feed, some of the fluid was removed so that the total volume did not exceed 3ml. On days 12-18 the tubes were opened, the liquid decanted from the top and the agar plug placed on a glass slide, cut into three pieces and gently compressed with a 22 x 50 mm coverslip. They were then examined with an inverted microscope at a power of 80X.

The method as used above gave colonies between 30 and 200 cells in size (Fig. 6) and a linear plating efficency with M5076 ascites cells over the range of cell concentrations plated $2.5 \times 10^4 - 1 \times 10^6$ (Fig. 7). The addition of August rat red blood cells suggested by the original author, and found to be essential in melanoma cell colony formation by Tveit (106) did not affect cloning efficiency by the M5076. No significant difference was found in the number of colonies formed at 20% and 5% O_2, but the lower concentration was used for all subsequent experiments as in the original method. DEAE cellulose and 2-mercaptoethanol omission had no effect on colony formation, as was found with the Salmon method.

A summary of the comparison between the two techniques employed is shown in Fig. 8. The Salmon method is exactly as used by the authors, whereas both the published and modified Courtenay method are shown.

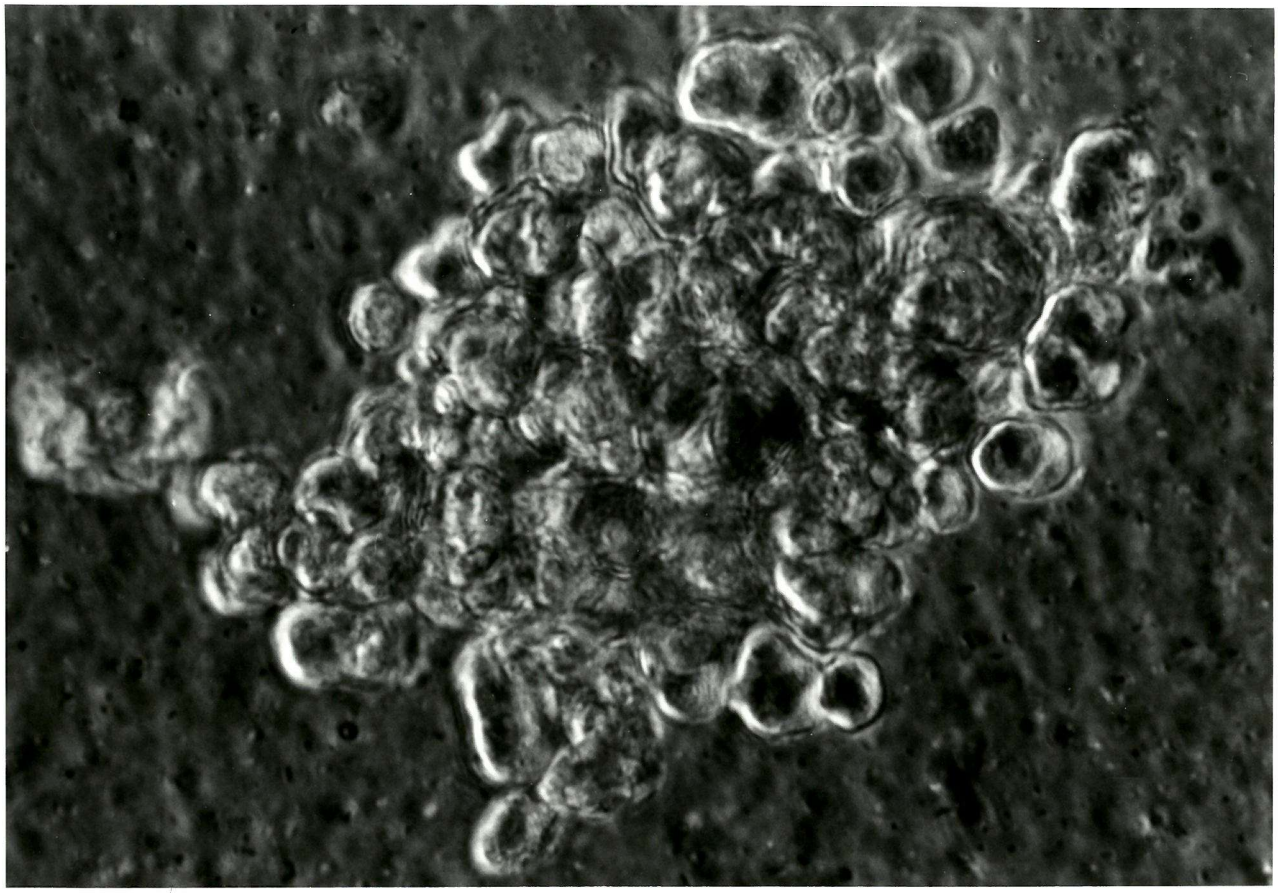


Fig. 6. M5076 colony in 0.3% agar by Courtenay technique (inverted microscope unstained X500)

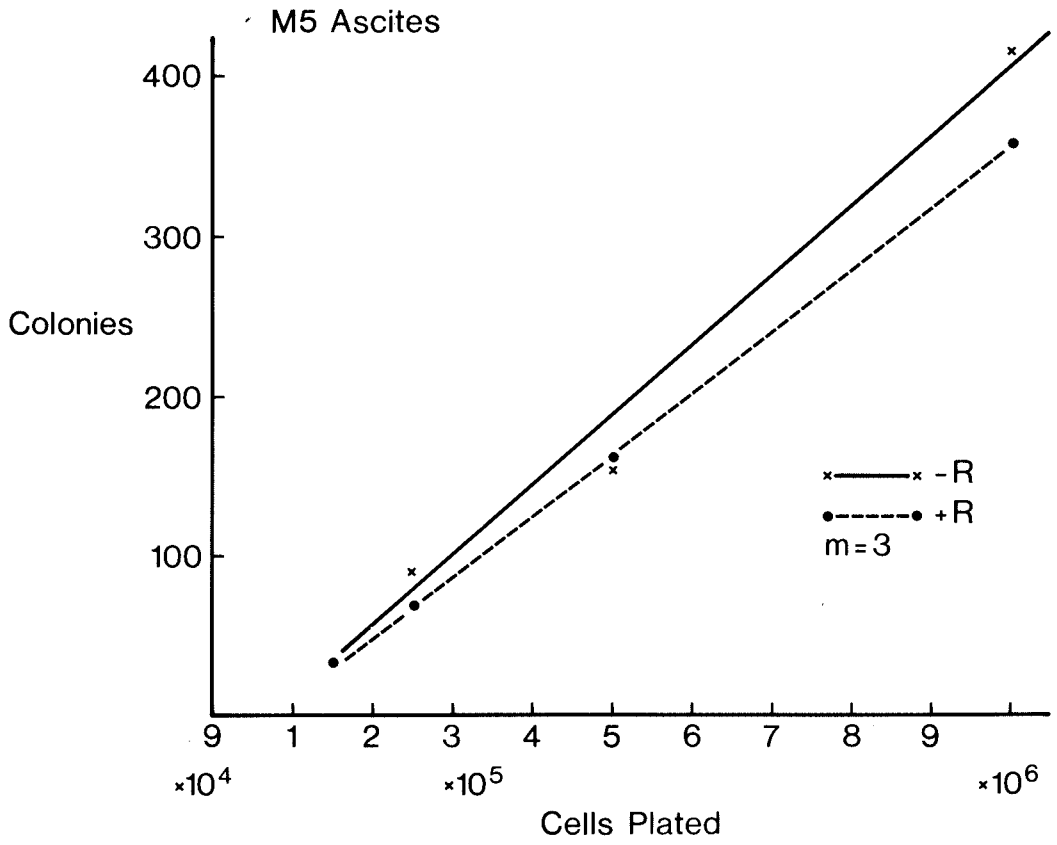
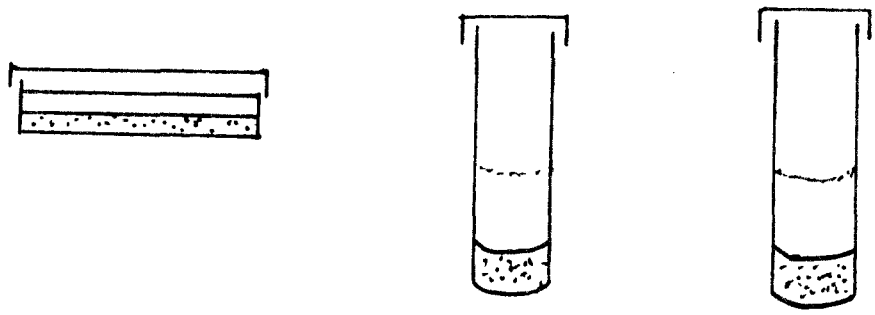


Fig. 7. Graph showing that the addition of August rat red blood cells to the 0.3% agar growth medium has no effect on colony formation by M5076 ascites employing the Courtenay technique.

-R no rat RBC

+R with rat RBC

Fig. 8. Methodological comparison of the Salmon (83), Courtenay (105) and modified Courtenay techniques of colony culture.



agar	feeder 0.5% top 0.3%	single 0.3%	single 0.17-0.3%
refeeding	-	+	+
O_2	20%	5%	5%
CO_2	5-7.5%	5%	5%
rat RBC	-	-	+
medium	CMRL 1066	CMRL 1066	Ham's F12
serum	13% horse serum	13% horse serum	15% newborn calf serum
incubation	14-21 days	14-21 days	14-28 days
infection rate	+++	+	+
complexity	++	+	+

Cytotoxic drug assays in soft agar - Courtenay colony culture technique M5076

The first prerequisite for a cytotoxic drug assay is a linear relationship between the number of viable cells plated and the number of colonies formed. For most purposes the drug incubation has been standardised at 1 hour at 37°C and a fixed concentration of cells are incubated with a range of drug concentrations. In each assay, a drug solvent and medium-only control was included and at least four replicate samples measured for each concentration point. In the standard assay, the cells at a concentration of 1.5×10^6/ml were preincubated with a minimum of 3 concentrations of drug extending over at least one log range. 0.5ml of medium containing 4.5×10^6 viable M5076 ascites cells/ml was added to 0.85 ml McCoys 5A + 10% FCS + 5% HS and 0.15 ml of a 10x drug solution in 0.9% saline added. The cell concentration during this incubation was 1.5×10^6 / ml. The cells were then washed twice in enriched McCoy's 5A with 10% FCS 5% HS and plated out as above. In some experiments, the drug was incorporated into the final plating medium to ensure continuous exposure to the drug throughout the period of colony formation. The number of colonies found in each dish was then plotted against the log of the drug concentration, and an estimate of sensitivity made based on the slope of the curve.

In vivo drug studies - increase in lifespan (ILS) M5076

The 1ml plug of agar, containing colonies exposed to drug concentrations of CCNU from 0.5 to 10µg/ml and a control was mixed with 3 volumes medium, and 0.2ml of the resulting suspension injected ip into each of 5 mice. The mean survival of each group was recorded as a ratio of the untreated control culture. Mice inoculated with non-drug treated cells are therefore assumed to have a lifespan of one, and any cytotoxic effect arising from preincubation of the cells with drug will be reflected in a fractional increase in the lifespan of the group.

These experiments were performed for two reasons:

a) to prove that the M5076 colonies after 14 days in culture were truly malignant and formed tumours when injected intraperitoneally (ip) into the DB2 6F1 mice.

b) to seek corroboration *in vivo* that the *in vitro* drug assay as described above, which assesses drug lethality by a reduction in colony formation at 14 days in agar culture, correlates with the ability of the remaining cells to produce malignant tumours on re-injection ip.

Labelling Index

These experiments were kindly carried out by Mr. J. Smallwood, Surgery Department, University of Southampton. M5076 cells were exposed for 1 hr to [^{3}H] thymidine (Amersham) and the uptake was measured in a cytocentrifuge preparation by autoradiography as described by Murphy (64). While this method can be quantitative by counting the proportion of labelled cells, for the purposes of this study a qualitative assessment of DNA synthesis was all that was required.

Characterisation of the M5076

The original tumour was fixed in formalin and wax embedded, cut and stained with haematoxylin and eosin and periodic acid Schiff (PAS) by standard methods for light microscopy. The electron microscopy samples were fixed in cold 2-5% glutaraldehyde, stained with osmium tetroxide, and examined on a Phillips electron microscope.

Immunological examination of the fresh cells was kindly performed on touch preparations by Dr. D. Jones, Department of Histopathology, University of Southampton. The techniques used to assess Fc γ receptors by rosetting with antibody coated sheep red cells, the acid esterase, non-specific esterase staining and fluoride inhibition were all carried out precisely as described by Yam et al (107). Immunoperoxidase staining for prekeratin, α 1 antitrypsin and lysozyme were performed by the method of Sternberger (108) on paraffin embedded material.

The agar embedded colonies were more difficult to handle. For light microsocpy staining the upper layer of agar containing the colonies was floated off the top layer in a water bath, after fixation overnight in cold 3% glutaraldehyde. The thin layer of agar was then dried on a glass slide using a filter paper wick on top, and stained by the Papanicolaou method (appendix 2). For electron microscopy the 1ml plug in the Courtenay assay was fixed in glutaraldehyde as above for 8 hr, then cut with a knife into 1mm portions and embedded in plastic for routine processing. In the immunological examination of the colonies, the unfixed plugs were mixed gently with phosphate buffered saline, spun in a cytocentrifuge and the tests performed on the resulting groups of cells, which at 14 days in culture would almost certainly have been derived from the colonies rather than from residual viable elements of those cells originally plated.

2.4. Human Ovarian Cancer Cell Lines

The parent line, 1847S, was obtained from an untreated patient with ovarian cancer in the laboratory of Dr. Aaronson, NCI (91). The cells formed colonies in nude mice and exhibited a stable cytogenetic pattern characteristic of human cells after more than 50 passages in culture. The cells were maintained in 75 cm^2 tissue culture flasks (Falcon) in growth medium comprising RPMI 1640 medium (Gibco) to which was added 5μg/ml insulin (Collaborative Research), 100 units/ml penicillin, 10μg/ml streptomycin, 48 mM sodium bicarbonate and 10% heat inactivated fetal calf serum (HIFCS). The doubling time of the cells was 23 hr.

By increasing the concentration of melphalan in the culture medium in a stepwise fashion from 10^{-8}M, cells were obtained after a period of 6 months which would grow in a concentration of 8 x 10^{-6}M, and were termed 1847 MER. An alternative method of isolating resistant cells by plucking individual colonies from agar cultures of 1847S containing up to 5 x 10^{-6}M melphalan did not produce a stable resistant cell population. The cells in the 1847 MER line were morphologically similar to, although slightly larger than, 1847S and had a doubling time of 28 hr.

Both cell lines formed 30-100 cell colonies in an agar bilayer, using a method modified from that of Salmon and Hamburger (83) in the following way:

- the plating (top) and feeder layers contained 0.3% and 0.5% agar, respectively, and were both made using the growth medium described above;
- 2-3 x 10^4 cells were plated in the top layer;
- 1ml RPMI 1640 + 10% HIFCS was added at 7 days; and
- the colonies were scored 12-14 days after plating.

Counting of the colonies was carried out by a Bausch and Lomb Omnicon FAS II image analysis system using a mechanical stage and custom software designed to count 35 fields from each 35mm petri dish, scoring a mean chord size of 60 μ as a colony. Each experimental point was represented by 3 replicate petri dishes.

Cytotoxic Drug Assays

In preliminary experiments, the 1847 cells were plated in monolayer in 6 cm petri dishes at a concentration of 3 x 10^4 per dish. Twenty-four hr later sets of 3 replicate dishes were exposed to melphalan in growth medium (containing 10% HIFCS) for 1 hr at 37°C, then fresh medium was added and growth continued for 4-5 days. This time was chosen to allow several doubling times to elapse without cell confluence being achieved in the control plates. The cells were harvested by the addition of 2ml 0.5 g/l trypsin in 0.2 g/l EDTA (Gibco), washed with two 2 ml changes of Isoton 11 and counted in a total volume of 10ml in a Coulter ZBI cell counter, set at aperture current 1/2 amp and amplification 32. The mean and standard deviation of the 3 counts was calculated, and each drug concentration point expressed as a percentage of the untreated control.

Drug-induced lethality was definitively assessed by reduction in colony forming activity in soft agar, by a technique similar to that described above for the M5076. Two different methods were used for drug exposure - suspension exposure for 1 hr for comparison with the M5076 studies using the Courtenay method, and monolayer exposure to minimise the chemical or mechanical effects of the detachment procedures. In the former method, incubations, including 1 control set for each 3 or 4 drug points were carried out in 15 ml Falcon tubes at 37°C for 1 hr in a total volume of 1.5 ml, and cell concentration 5-8 x 10^4/ml. Results were expressed as percent survival of control. The melphalan was initially dissolved at a concentration of 10mg/ml in 75% ethanol/water containing 0.32% HCl, and then diluted at least 1,000-fold to concentrations in the range 0.1-20 μM. At these dilutions, the solvent shows no *in vitro* cytotoxicity by comparison with a saline control.

To avoid a possible synergistic action between the effect of trypsin and the action of the drug, the incubation was carried out while the cells were adherent in monolayer and the cells were harvested and cloned in agar 24 hr later. The cells were plated in

monolayer in 6 cm petri dishes at 3 x 10^4 cells per dish, grown for 24 hr and then exposed to drug for 16 hr at 37°C. The medium was then changed, and growth allowed to continue for 24-28 hr. The entire contents of each petri dish were then harvested with trypsin/EDTA, and suspended in 3 ml of 0.3% agar in growth medium. One ml of this mixture was then plated in each of three 35 mm petri dishes and colony formation counted at 14 days as described above.

All experiments described with these cell lines were performed when the cells were at a subconfluent stage of growth, to minimize any differences in proliferative or metabolic capacity between log and nonlogarithmic growth cells.

Melphalan Transport (109)

Logarithmic phase cells growing in monolayer were washed in Dulbecco's phosphate buffered saline (PBS) containing 0.1 mM bovine serum albumin and 0.1% glucose. (^{14}C) melphalan was added at a concentration of 3.5 μM for 1.5,3,6,10,20,40 and 60 min at 37°C, and the uptake terminated by rinsing twice with ice cold PBS. The cells were harvested with trypsin and spinning the cells through Versilube F-50 silicone oil at 12,000g for 2 min in an Eppendorf microcentrifuge. Individual uptake estimates were performed in triplicate and cell recovery was found to be greater than 90%. The centrifuge tube tips containing the cell pellet were cut off and then the pellets solubilized in 0.2N sodium hydroxide prior to counting in a liquid scintillation counter.

The transport studies were carried out at 37°C apart from the cooling step at the end of incubation, and the whole procedure was completed within 90 min. Control populations were replated in 75 sq cm Falcon flasks with a 50% efficiency.

Dechlorination of L-PAM by Sensitive and Resistant Tumour Cells (109)

Radioactively labelled melphalan (1 mg/ml) was convered to its noncytotoxic derivative, 4-[bis(2-hydroxyethyl)amino]-L-phynylalanine-

(dihydroxy melphalan), by hydrolysis in 0.1 N NAOH for 60 min at 60°C and served as a standard for thin layer chromatographic studies. Optimal separation of melphalan (retention factor R_F = 0.93) from dihydroxy melphalan (R_F=0.6) was achieved on MN 300 cellulose in a solvent system consisting of isopropyl alcohol:formic acid:water (65:1:34, v/v).

Intracellular dihydroxy melphalan was identified utilizing a procedure which did not involve washing cells in aqueous buffers following exposure to radioactive melphalan. This was accomplished by layering 40 ml of cells (2.5 x 10^5 cells/ml) in medium with labelled melphalan onto a 10 ml cushion of Versilube F-50 silicone oil and pelleting the cells by centrifugation for 30 min at 12,000 x g. The radioactive supernatant and the silicone oil were removed by aspiration, the inside of the tubes swabbed to remove any residual radioactivity and the cell pellets lysed in 500 μl of distilled water. One hundred μl aliquots were applied to MN 300 cellulose and melphalan and dihydroxy melphalan were then separated by thin layer chromatography in isopropyl alcohol:formic acid:water 65:1:34, v/v). The plates were dried, and 1.0 cm sections removed and placed in scintillation vials for determination of radioactivity.

Glutathione Assay

Approximately 5 x 10^6 1847S and 1847 MER cells were harvested with trypsin and counted as above. The cells were washed once in PBS and spun at 300 g for 10 min in a 15 ml conical centrifuge tube. The supernatant was carefully removed, 0.9 ml double distilled water added to the precipitate which was then vortexed for 2-3 min to lyse the cells. 0.1ml 30% sulphosalicylic acid was added and the sample left on ice for 15 min to precipitate the proteins. The mixture was then spun at 12,000 g for 2 min in an Eppendorf centrifuge. The supernatant, amounting to about 0.8 ml was aspirated and stored at -20°C.

The standard assay for total glutathione was carried out under conditions similar to those described by Griffith (110). Three

working solutions were made up in stock buffer (125 mM sodium phosphate, 6.3 mM Na EDTA pH 7.5):

(i) 0.3 mM NADPH (Calbiochem);

(ii) 6 mM 5,5' dithiobis 2-nitrobenzoic acid (Sigma); and

(iii) 50 units of glutathione reductase/ml (Calbiochem)

700 μl of solution (i), 100 μl of solution (ii), 100 μl of solution (iii) were mixed in a test tube and 100 μl of the sample added, the mixture placed in a cuvette, and the absorption at 412 nm (A_{412}) monitored continuously until it exceeded 2.0, usually in 2-4 min. The glutathione content of the aliquot assayed was determined by comparison of the rate observed to a standard curve generated with known amounts of glutathione (112). The concentration is expressed as ng/10^6 cells and per mg total cell protein (Lowry).

To measure the oxidised glutathione (GSSG) subfraction, any glutathione (GSH) in the sample was first converted to a non-reactive form by the addition of 2 μl of neat 2-vinylpyridine per 100 μl solution, and after mixing vigorously for 1 min, allowed to stand at 25°C for 60 min. Any residual GSSG was measured exactly as described above in the GSH assay, although the amount present was generally less than 10% of the total cellular thiol content.

Glutathione Depletion

(i) Amino acid depletion

1847S and 1847 MER cells were grown in RPMI 1640 medium with identical additives to those in the growth medium described above, except that the amino acid cysteine was omitted. The base medium was prepared by the NIH media unit, and the supplements, including 10% HIFCS were added before 0.22μ filtration. This depleted medium was substituted for the standard growth medium while the cells were in logarithmic growth, and continued for a period of 24 hr, at which time the cells were harvested, and the total glutathione and GSSG levels per 10^6 cells and per mg protein (111) assayed. The sensitivity of these cells to melphalan was then assessed in the standard cloning assay.

(ii) Addition of specific inhibitor

The synethetic amino acid butatione sulphoximine is a specific inhibitor of γ-glutamylcysteine synthetase, the enzyme catalysing the last step in the synthesis of the tripeptide glutathione. A cystalline preparation was a gift from Dr. Vistica, National Institutes of Health, USA, and was dissolved in the standard growth medium (RPM1640 with cysteine) at a concentration of 50-100 μM. Drug cytotoxicity assays were performed on cells which had been pre-incubated with buthionine sulphoximine for 24 hr., and the inhibitor was also maintained throughout the period of exposure to the melphalan.

The cytotoxicity of buthionine sulphoximine alone at varying doses was assessed in a separate clonogenic assay.

RESULTS

3.1. Clinical Studies

A total of 70 patients have been entered on study up to the 28th March 1983 when the analysis was performed. 36 were randomised to the chlorambucil arm and 34 to the PACe arm, and the comparison of the distribution of the clinical features of the patients on the 2 arms is shown in Table 5. Three patients were excluded in total from the response evaluation, 2 for inappropriate treatment and one lost to follow up 6 months after diagnosis. The life table comparison of survival includes these patients entered on the study as they met the original selection criteria (Appendix 4).

The response data for the 64 evaluable patients is given in Table 6 and shows an overall response rate of 30% in the chlorambucil group compared to 67% in the PACe group. This includes data from the 24 patients in the study who had second look procedures (19 laparotomies, 5 laparoscopies) and therefore will underestimate the clinical complete response (CCR) and overestimate the true pathological complete response rate (PCR).

13 of these second look laparotomies were performed on patients in clinical complete remission, and of these 9(62%) were found to be in PCR, 2 had evidence of more than 90% reduction in tumour mass, one showed a 50% reduction in tumour bulk and one no significant change. The 9 patients in PCR had a median survival of 26 months (range 19-48) and of these 4 have died at 12,15,24 and 31 months from the date of the second laparotomy. By comparison the 15 patients found at second look procedure (out of 24 laparotomies and laparoscopies) to have residual disease had a median survival of 12 months (range 2-44). Those patients who had minimal residual disease (<2cm diam.) had a mean survival of 12 months compared to 5 months for those with more extensive disease. It is not possible to comment on the survival effect of surgical excision at second look laparotomy.

Fig. 9 shows the life table analysis of survival on the 2 arms

Table 5. Distribution of clinical features between the chlorambucil and combination chemotherapy (PACe) arms of the advanced ovarian trial.

		Chlorambucil	PACe
Number of patients		36	34
FIGO stage	III	22	26
	IV	14	8
Excision to	< 2cm	17	18
	> 2 cm	7	7
	not done	12	9
Age	50-70	26	24
	30-50	10	9
Performance	8-10	22	21
Status * (Karnofsky)	5-7	4	7
	1-4	1	0

* The data for performance status is incomplete as this was not always recorded.

Table 6. Response data - assessed 6 months after start of chemotherapy.

	OR	CR	PR	Fail	NE	Total
Chlorambucil	7(30%)	6(19%)	1	24	1	32
PACe	20(67%)	7(23%)	13	10	2	32

OR - overall response (CR+PR)

CR - complete response

PR - partial response

NE - not evaluable

of the study. The median survival of the chlorambucil group was calculated to be 11.8 months compared to 17 months for the PACe group, but there is no significant difference between the curves based on the log-rank test ($p=0.84$). The data is insufficient at present to compare disease free survival adequately, but 4 patients in each arm have relapsed but not died at approximately equal time intervals, and it is unlikely there will be a significant difference.

Of the 24 patients failing chlorambucil (Table 6), 12 were given second line treatment with PACe and there have been 2 partial responders, one of whom died at 7 months, the other is alive at 6 months after the crossover. Only one patient failing PACe received an adequate trial of chlorambucil and there was no response. Toxicity was not formally assessed in this analysis, but was without doubt greater in the combination chemotherapy arm, where nausea, vomiting, and pyrexial episodes requiring antibiotics, impairment of renal function and blood transfusion were more common. Two deaths in the combination arm were attributed to treatment related myelosuppression.

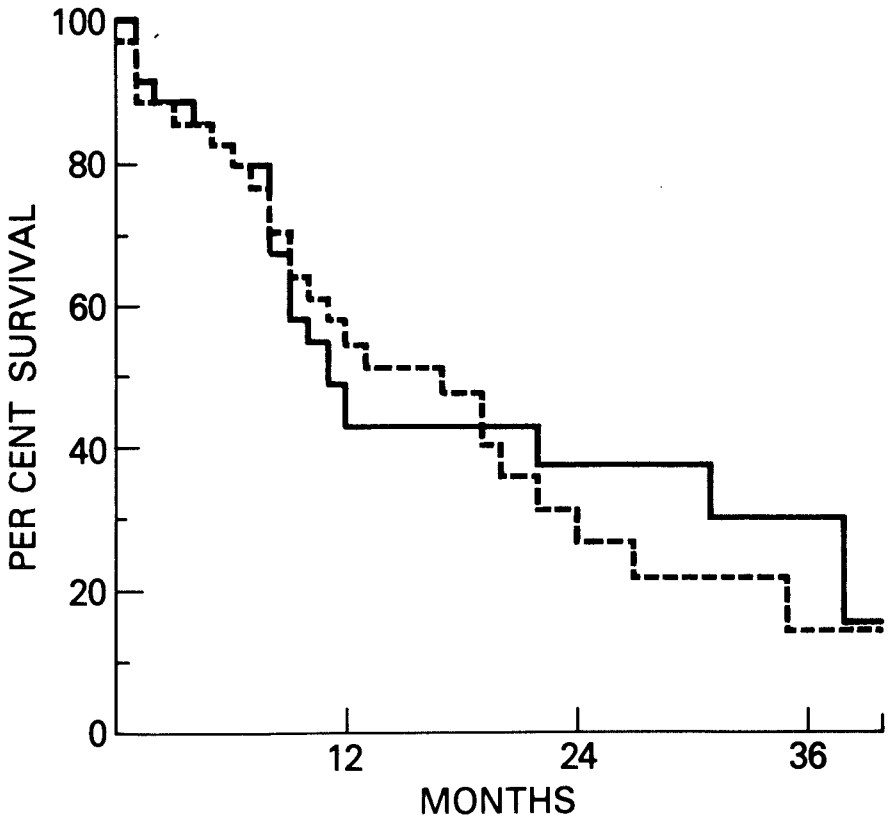


Fig. 9. Life table comparison of 70 patients with Figo stages III and IV ovarian carcinoma. There was no significant difference between the PACe group --- and those given chlorambucil ——— .

3.2. Cloning of Primary Human Ovarian Tumours

A total of 75 specimens was processed, comprising 47 solid tumours and 28 ascites specimens. As can be seen in Table 7 only a proportion of the tumours gave rise to more than 5 colonies per plate, and the overall plating efficiency was poor.

Table 7

Growth and plating efficiency of human ovarian tumours

Source	No. received	No. forming > 5 colonies (%)	Mean plating efficiency %	Range %
solid	47	19* (40)	0.03	0.002-0.08
ascites	28	11 (39)	0.02	0.001-0.07
total	75	30 (40)	0.03	0.001-0.08

* 17 (92%) of these were enzymatically dissociated

The ascites specimens gave no better results than the solid tumours after disaggregation, and the former were more likely to be contaminated or clumped, as heparin was frequently not added to the collection container by the ward staff. It appeared that the enzymatically disaggregated cells showed marginally improved colony formation, but the results were not satisfactory with either method. The viability as expressed by trypan blue exclusion was higher with the enzyme treated cells, as were the yields obtained, but the correlation between these 2 parameters and colony formation was not absolute (Fig. 10). There was a trend towards higher plating efficiency with increasing viability, but a significant proportion of cases showing high viability gave little or no colony formation. In view of the low growth, low plating efficiency and poor reproducibility, no satisfactory cytotoxic drug assays were performed by this method.

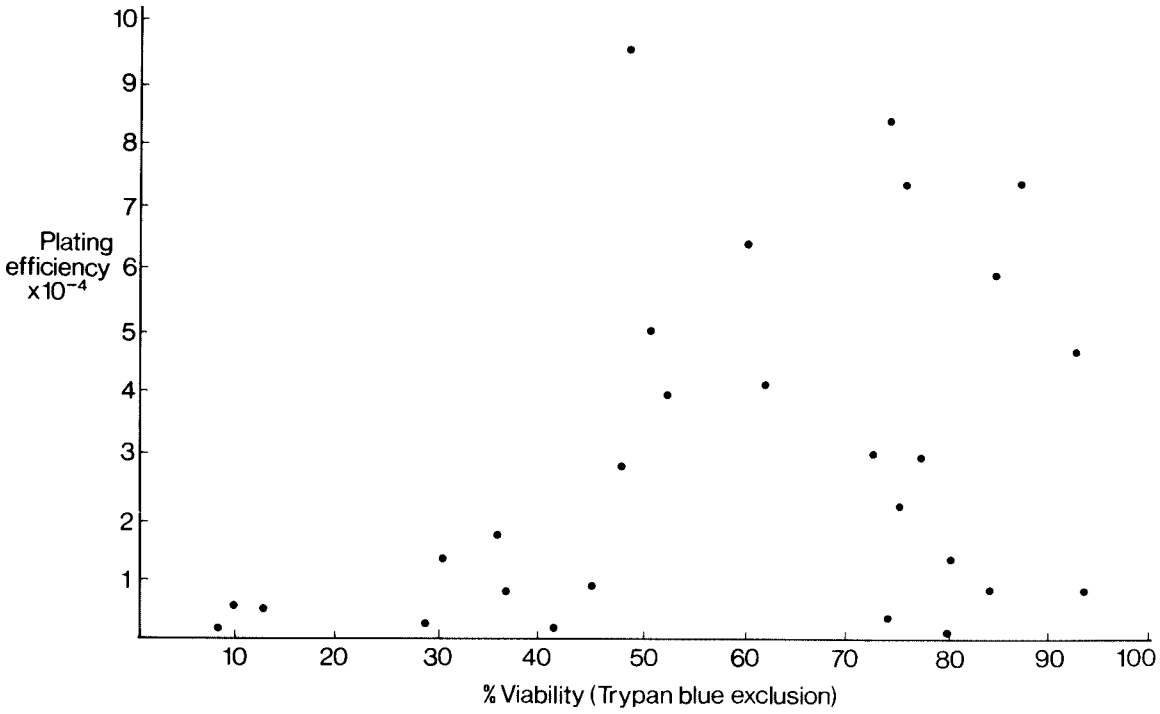


Fig. 10. The relationship between the viability of the single cell suspension by dye exclusion and the plating efficiency (colonies per total cells plated) in human primary ovarian tumour cultures.

3.3. M5076 - Primary agar culture results

Suspensions of M5076 cells were rarely found to contain clumps, irrespective of the disaggregation method employed, and an example of the single cell preparation obtained is shown in Fig. 11.

As with primary culture of human ovarian tumours, reproducibility with the Salmon method was poor, with colony formation achieved on only 6 out of 20 occasions on which the solid tumour was processed. Culture of the solid tumour was only slightly better with the Courtenay technique, with 2 out of 5 specimens giving colony growth. When ascites cells were grown, the reproducibility markedly improved, although a comparison of the plating efficiencies using the different techniques on the M5076 does not show a marked difference (Table 8). While a strict comparison is not possible with the data available in view of the poor growth, a higher proportion of viable (dye excluding) cells gave rise to colonies from mechanically dissociated samples than from enzyme treated tissues (Fig. 12) Using the TLX5, a mouse lymphoma which grows more rapidly as an ascites, there was a more clear cut advantage to the Courtenay method (Fig. 13), with a 5-fold higher plating efficiency compared to the Salmon technique. As the latter method is considerably simpler, and contamination much less of a problem, all later experiments used this method.

To test the hypothesis that the enzyme incubation damaged the surface of the cells and inhibited cloning, ascites M5076 cells were cloned with and without preincubation for 1 hour at 37°C with the standard enzyme mixture (0.2% collagenase III, 0.05% deoxyribonuclease). Fig. 14 shows that prior incubation with the enzymes inhibit colony formation. A labelling index experiment performed simultaneously on these two groups of cells confirmed much lower short term proliferation in the enzyme treated cells (Fig. 15). The non-enzyme treated cells showed diffuse intracellular uptake by about 10% of the cells (Fig. 15a), whereas the enzyme treated cells showed only non-specific adherence of the label (Fig. 15b).

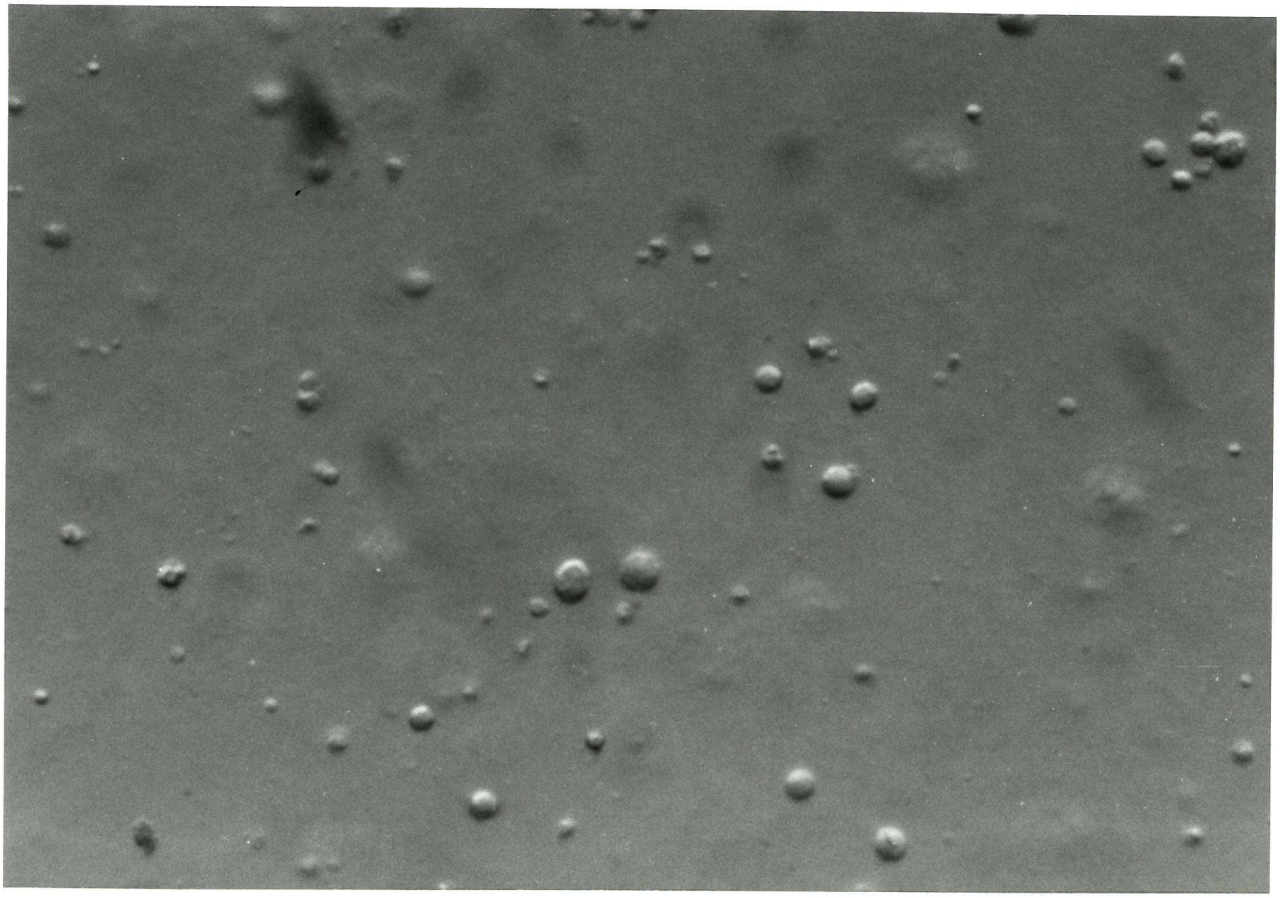


Fig. 11. Single cell suspension plated on 0.3% agar. M5076 solid tumour processed by enzymes and Salmon technique. Inverted microscope unstained X200.

Table 8 Cloning of M5076 murine tumour by plating technique (Courtenay vs Salmon) and dissociation method (mechanical vs enzymatic). P.E. : plating efficiency %.

Plating Method	Dissociation Method	No. growing	No. tested	P.E. (per total cell plated)	P.E. (per viable cell plated)
Salmon	Mechanical	6	20	0.06	0.3
Salmon	Enzymatic	5	20	0.06	0.2
Courtenay	Enzymatic	2	5	0.1	0.2
Courtenay	Mechanical	1	5	–	–
Courtenay	Ascites	6	7	0.25	0.3

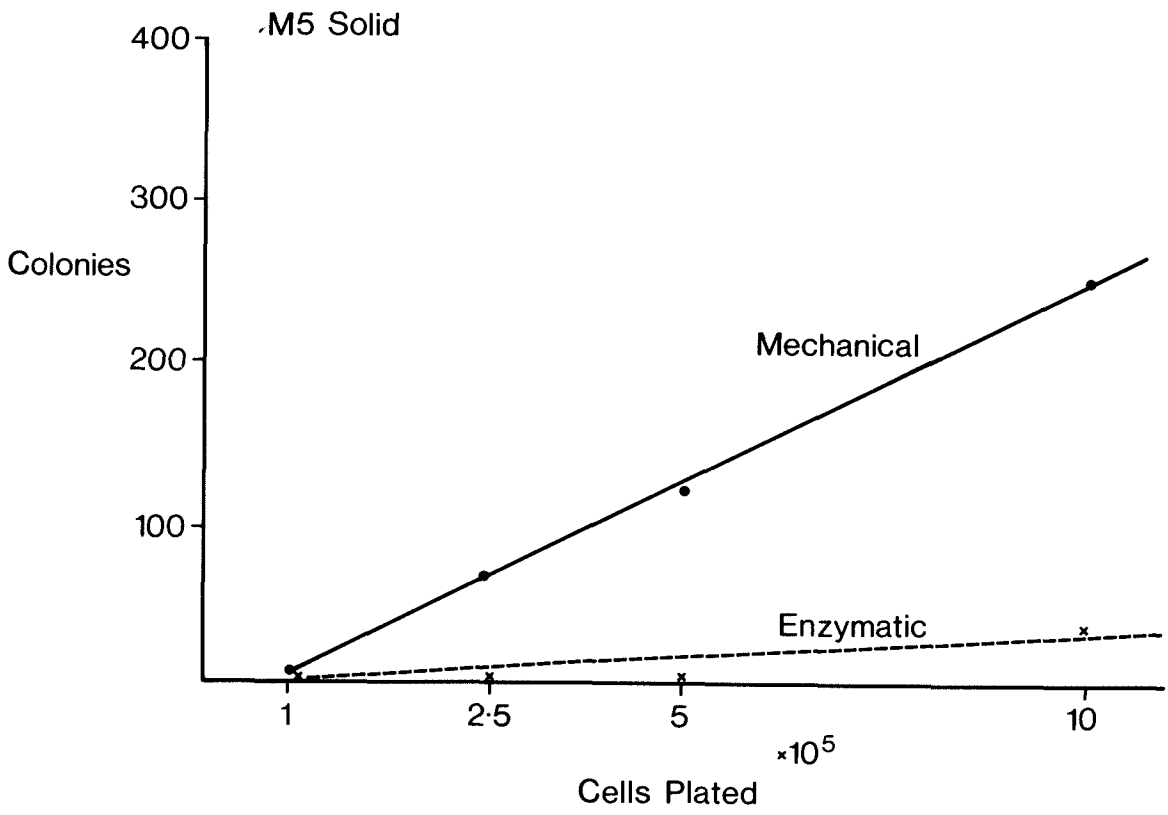


Fig. 12. Colony formation plotted against number of viable cells plated for enzymatic and mechanical dissociation. Salmon technique M5076.

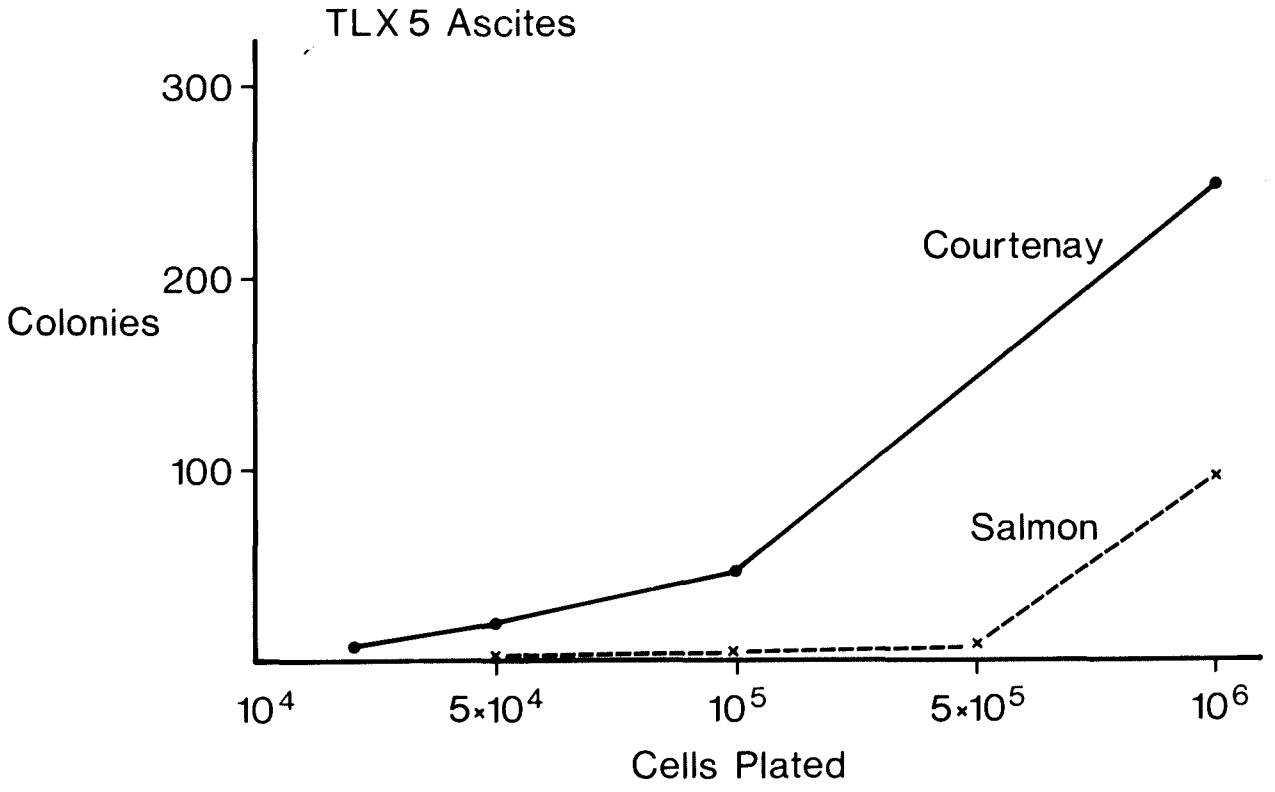


Fig. 13. Comparison of the colony formation by TLX5 ascites tumour grown by the Courtenay and Salmon techniques at day 14 in culture.

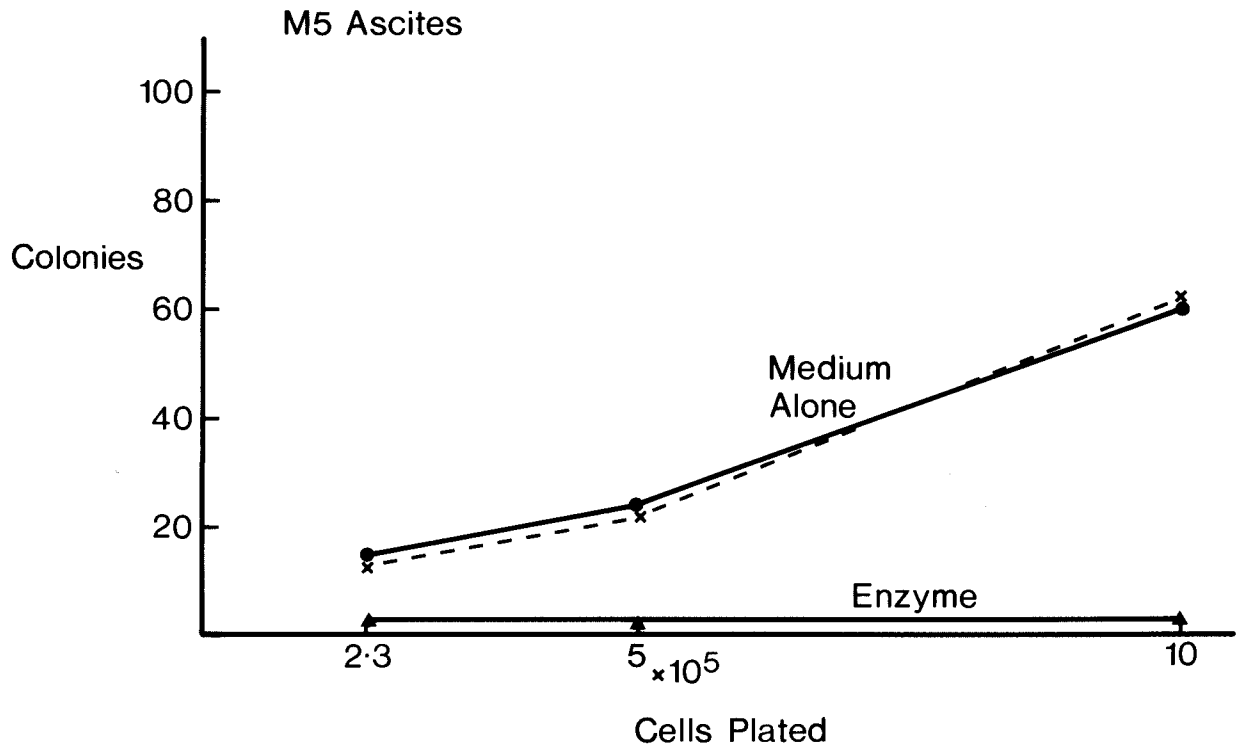
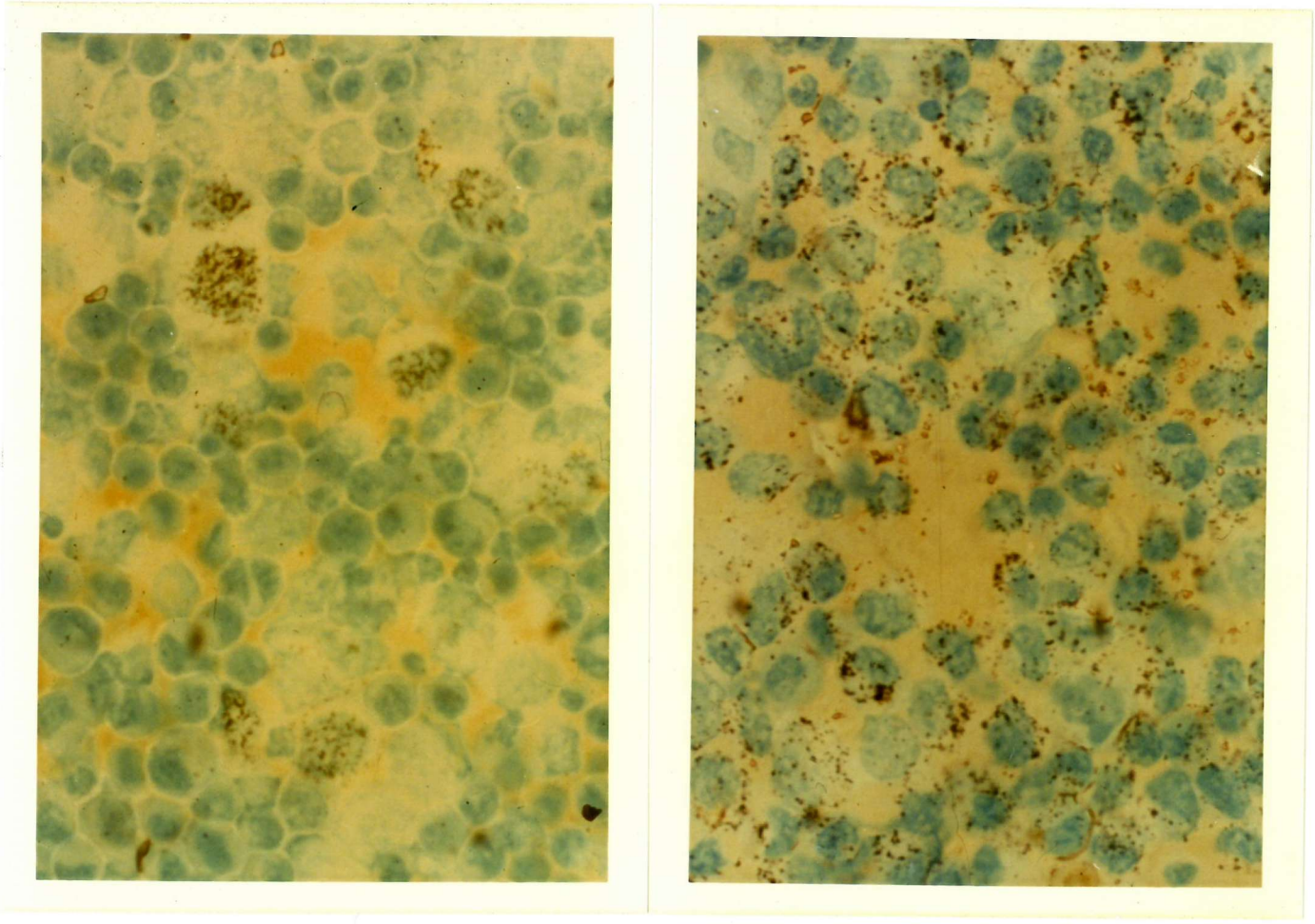


Fig. 14. Effect of prior incubation in 0.5% collagenase 111, 0.05% DNase for one hour at 37°C on colony formation by M5076 ascites tumour.



(a) (b)

Fig. 15. (^{3}H) labelling index comparison of non-enzyme treated M5076 cells (a) which show good uptake by those cells synthesising DNA during the 1 hr pulse label, and enzyme treated M5076 cells (b) which show little intracellular uptake.

3.4. Characterisation of the M5076 tumour

After subcutaneous inoculation, the tumour grows as a firm solid lump of tissue first appearing about 10 days, and increasing in size to about 1cm. across at 21 days. About day 28 the tumour becomes necrotic in the centre, and frequently ulcerates through the skin. Distant metastases rarely appear but have been noted in the liver, ovaries, bone and peritoneum. With transplantation by the intraperitoneal route, death usually supervenes about day 21, but in those mice which survive longer, distant metastases are more frequently seen in the liver, ovaries, lymph nodes and bone.

Histological appearances

With haematoxylin and eosin staining (Appendix 3) sections show an anaplastic tumour with a high mitotic rate (Figs. 16 and 17). The nuclei are ovoid with occasional nucleoli and there is no organisation into microglandular structures or evidence of mucin production.

No direct invasion of the lymphatics was seen, and in sections including liver metastases, the portal tracts were not infiltrated as would be expected in a lymphocytic lymphoma.

Electron microscopy showed sheets of uniform tumour cells with no extracellular matrix (Fig. 18 and 19) There were no tight junctions or desmosomes. The cytoplasm contained plentiful mitochondria and rough endoplasmic reticulum which was often dilated, moderate numbers of lysosomes, and sparse myelin figures. No secretory products such as mucin were seen. The nuclei were round, oval or indented without characteristic features. In summary the features were of an anaplastic tumour with no definite evidence to favour an epithelial, connective tissue or histiocytic origin.

The touch preparations showed large foamy cells (Fig. 20) with occasional evidence of erythrophagocytosis. Both the cells from the ascites and solid tumours showed rosette formation with antibody

coated sheep red cells (Fig. 20) indicating Fcγ receptors on their surface. Granular positive non-specific esterase staining was noted in most of the cells, and this was not inhibited by prior treatment with fluoride. Taken together, these features would indicate a macrophage/monocyte origin. Lysozyme was not found although the antiserum used was raised against the human rather than the mouse enzyme, and tests for antibody dependent cell cytotoxicity were unsuccessful. Immunoperoxidase staining for prekeratin, secretory component (epithelial markers) and α 1 antitrypsin (a macrophage marker) were all negative.

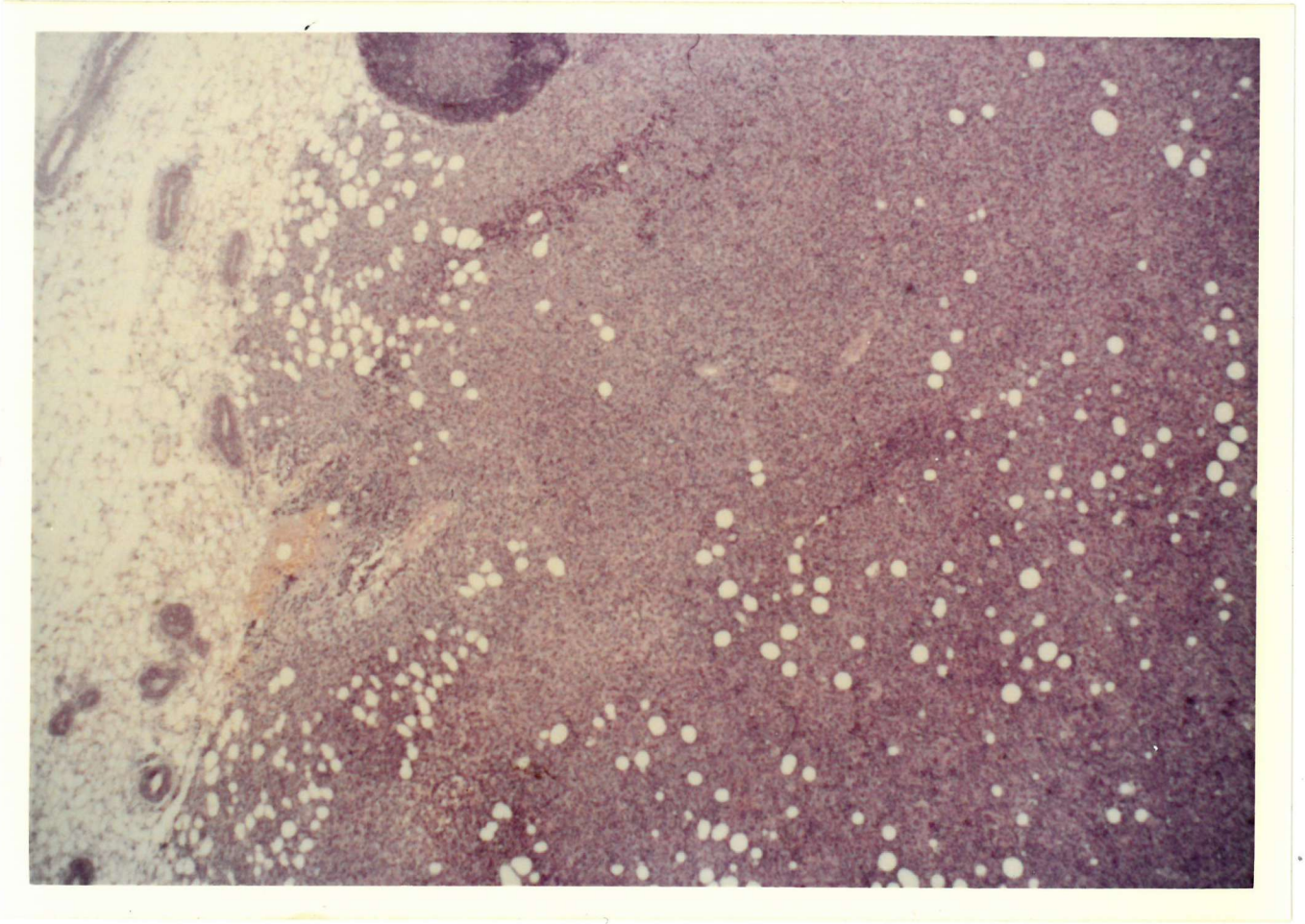


Fig. 16. Low power view of M5076 tumour (paraffin embedded, Haematoxylin and Eosin X80).

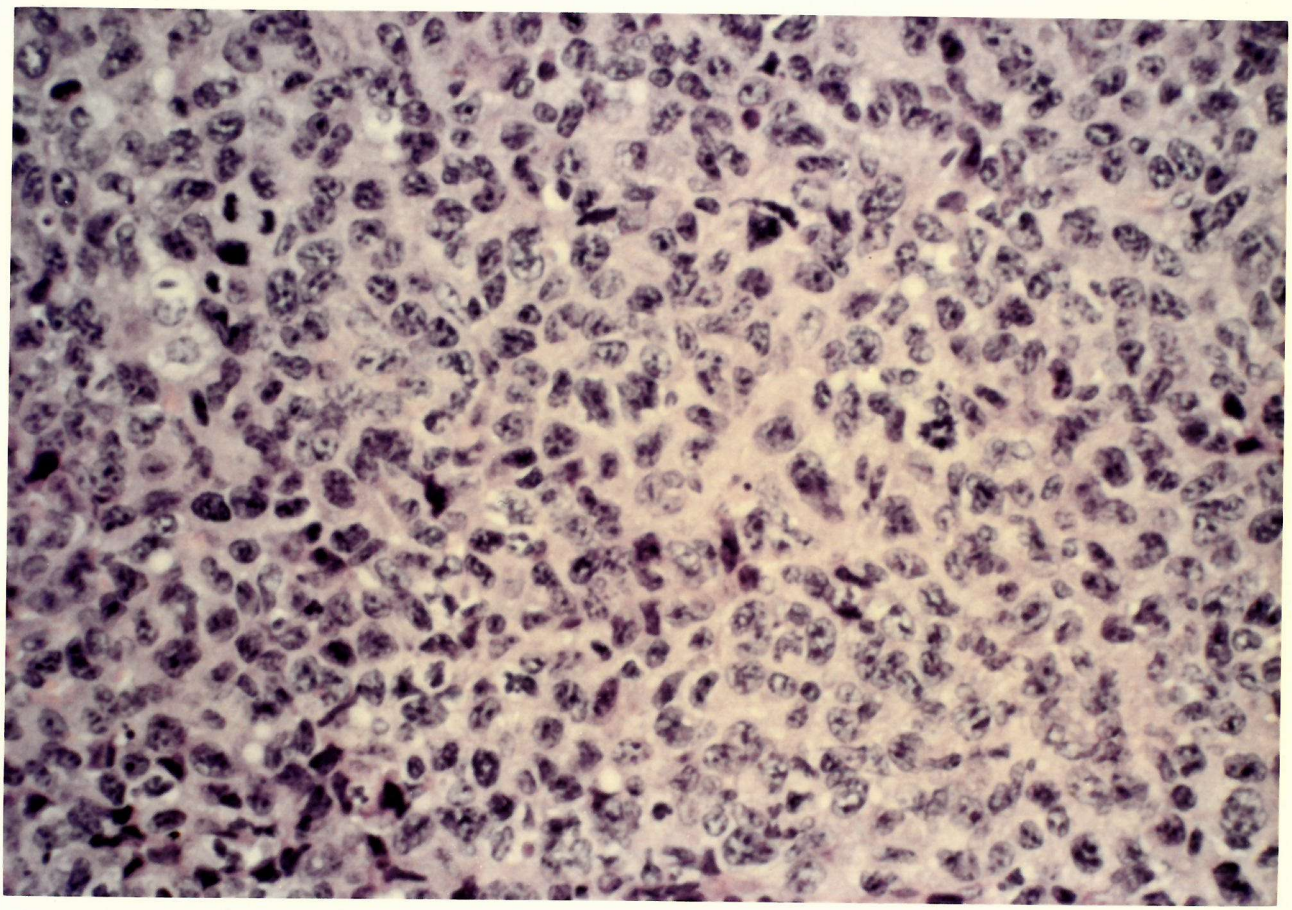


Fig. 17. High power view of M5076 subcutaneous tumour. Mitotic figures are indicated ▲ (paraffin embedded, Haematoxylin and Eosin X350).

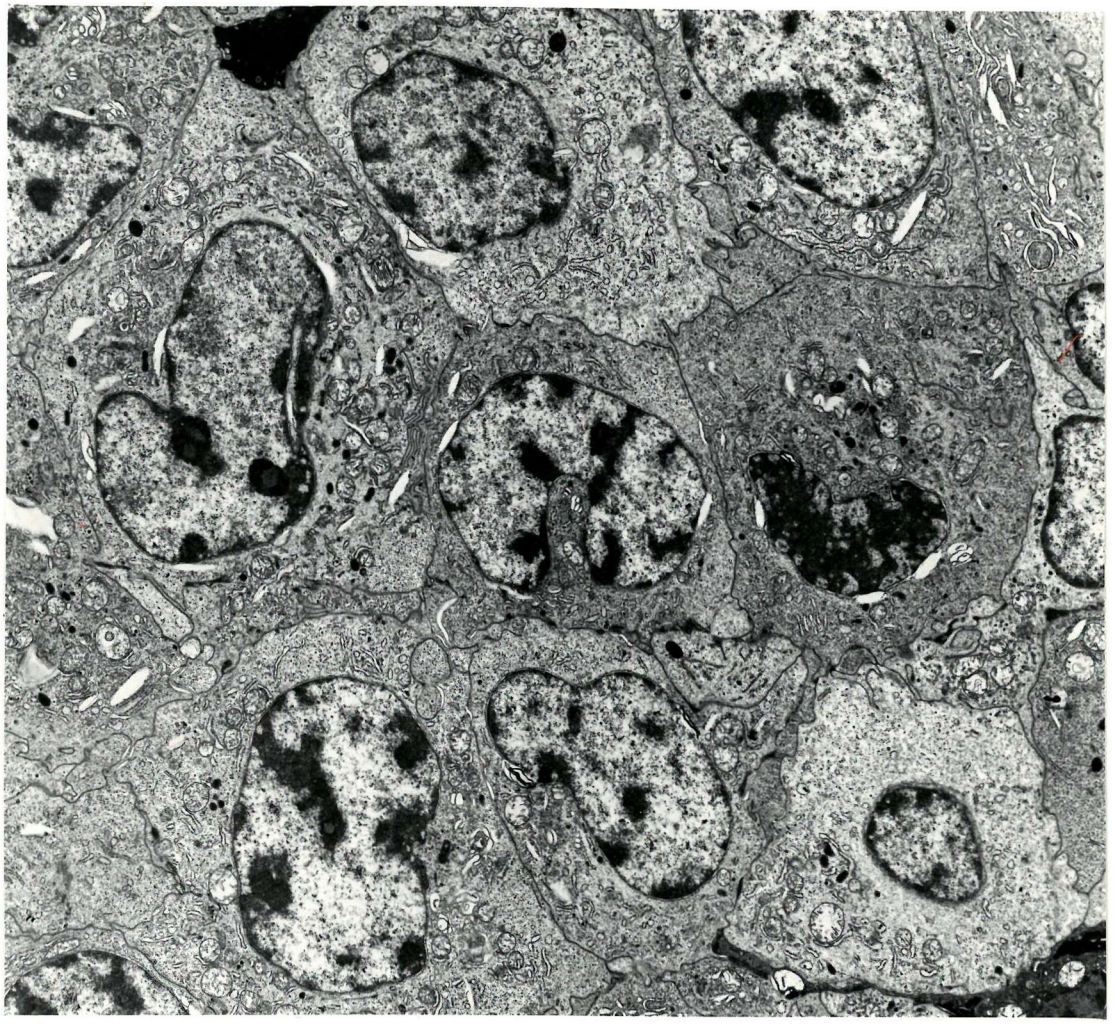


Fig. 18. Electron micrograph of the primary M5076 tumour. There are no microacini or other features to indicate differentiation. The nuclei are irregular with prominent nucleoli. X4000.

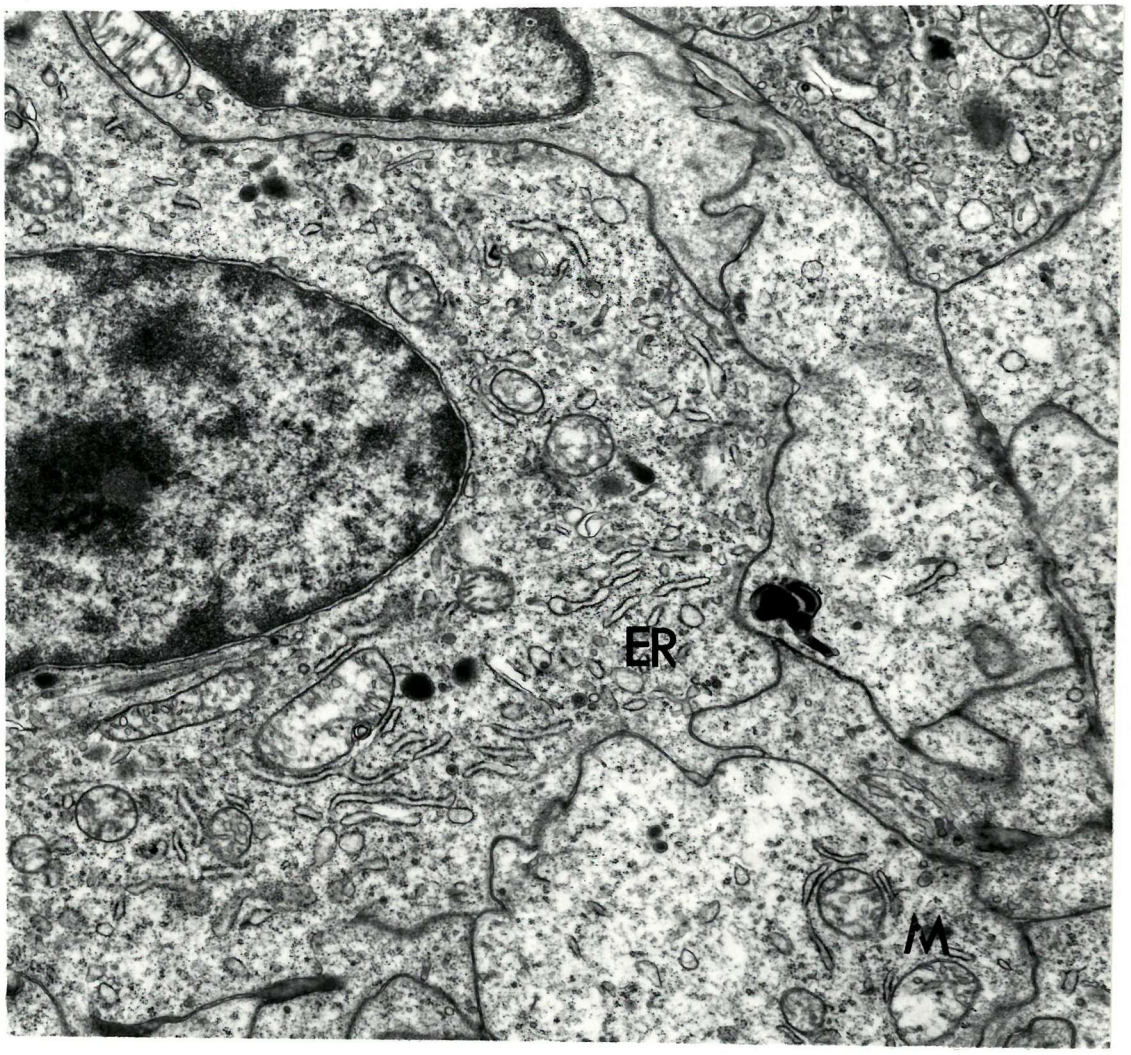


Fig. 19. Electron micrograph of primary M5076 tumour. No desmosomes or specialised intercellular junctions are seen. Rough endoplasmic reticulum (ER) is prominent, as are somewhat swollen mitochondria (M). X14,000.

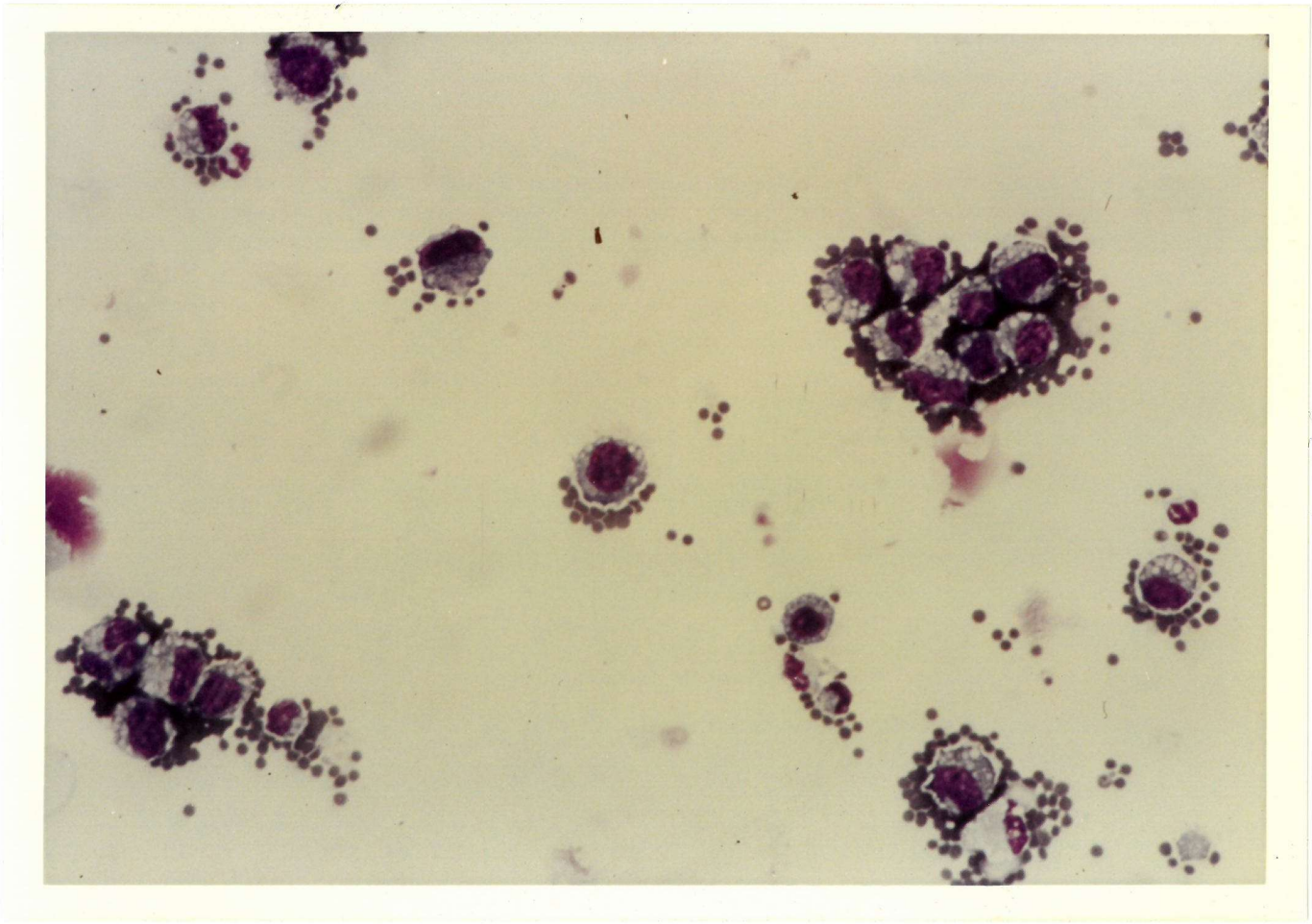


Fig. 20. Large foamy cells from M5076 ascites tumour incubated with antibody coated sheep red blood cells showing, rosette formation indicating Fc γ receptors. X350

Identification of colony forming cells in soft agar

The air dried agar embedded colonies are shown in Fig. 21 stained by the Papanicolaou method, which gave superior cytoplasmic and nuclear staining to the haematoxylin and essin technique. Electron micrographs (Fig. 22) of the cells in the colonies showed the cells to be slightly larger, but also without specific features indicating any form of differentiation as was the case with the primary M5076 tumour. Comparison with the original tumour at both the light and electron microscopic level is hampered by the suboptimal fixation and processing of the agar colonies resulting in loss of cytoplasmic detail, but the nuclear morphology is similar.

Immunological testing of the agar embedded cells confirmed the presence of Fcγ receptors and similar non-specific esterase staining to the parent cells (D. Jones, personal communication).

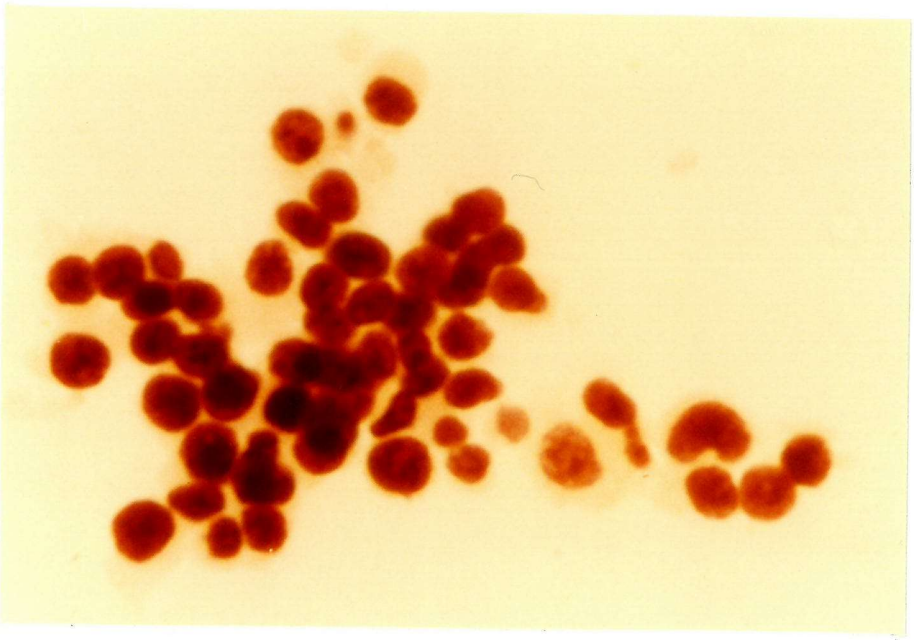


Fig. 21. M5076 colony in upper 0.3% agar by Salmon technique (Papanicolaou stain X400).

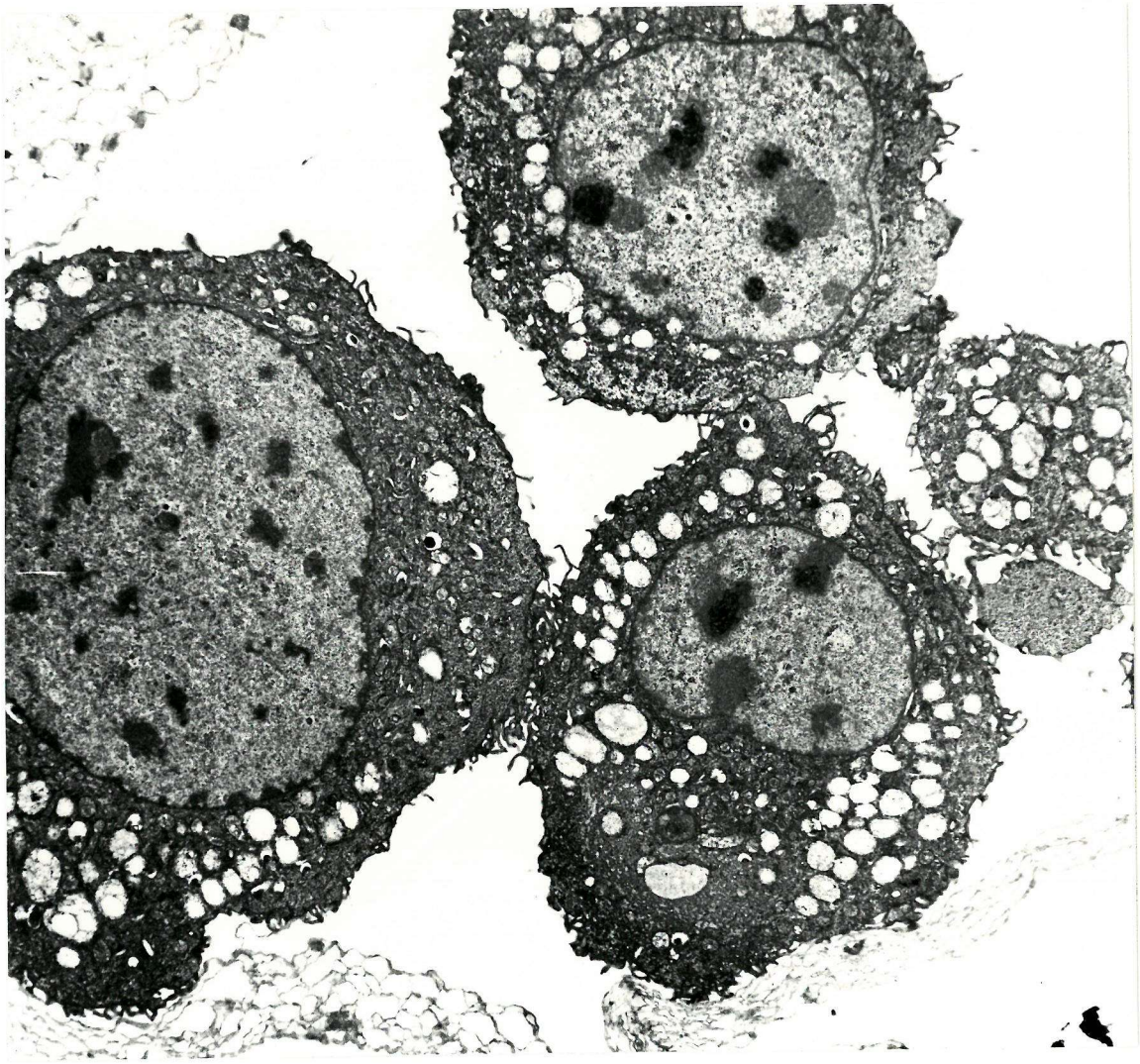


Fig. 22. Electron micrograph of M5076 cells derived from 14 day agar colonies. Fixation is suboptimal, but the cells have similar features to those from the primary tumour, as shown in Figs. 19-20. X 2,000.

As further proof of the malignancy of the cells comprising the colonies after 14 days in culture injection of colonies formed from M5076 cells not treated with cytotoxic drugs gave rise to tumours in 4 mice at 21 days. Two mice subsequently died of these malignancies, and the ascites cells from the remaining 2 again formed colonies on agar. As confirmation that the drug induced reduction in colony formation was proportional to the malignancy of the remaining cells, a fixed proportion of a 1ml agar plug at each concentration was injected into intact DB26F1 mice, and as shown in Table 9 the colonies formed from cells incubated at high drug concentration took longer to produce measurable tumour in intact mice, and those animals given the colonies grown from cells exposed to 10 μg/ml CCNU lived more than twice as long as those given non-drug treated cells.

Table 9. Increase in disease free survival and survival for DB26F1 mice injected with cells from colonies of preincubated M5076 cells. Each point is the mean of 5 mice, and is expressed as a fraction of the survival of the 'control' group of mice, injected with non-drug treated cells, who died at a mean of 30 days.

CCNU conc. (μg/ml)	Fractional increase in survival	Fractional increase in disease free survival
0.5	1.1	0.9
2	1.1	0.9
5	1.0	1.0
10	2.1	2.6

Cytotoxic drug assay results (M5076

Cytotoxic drug assays were carried out with the alkylating agents chlorambucil and CCNU, as well as cisplatin and adriamycin. The colony survival was expressed as a percentage of the untreated control cells following a 1 hr incubation with the drug in 15% serum at 37°C, and plotted against the drug concentration on a logarithmic scale, as shown in Figs. 23-26. The slope is linear over at least a one log concentration range for each drug, and comparison between the cytotoxicity of the drugs is best expressed by the dose at which there is a 50% reduction in colony formation (LD_{50}), which varies from 0.14 μg for adriamycin to 2.0 μg for cisplatin. With the dose ranges studied there was no evidence of a plateau at high drug concentrations.

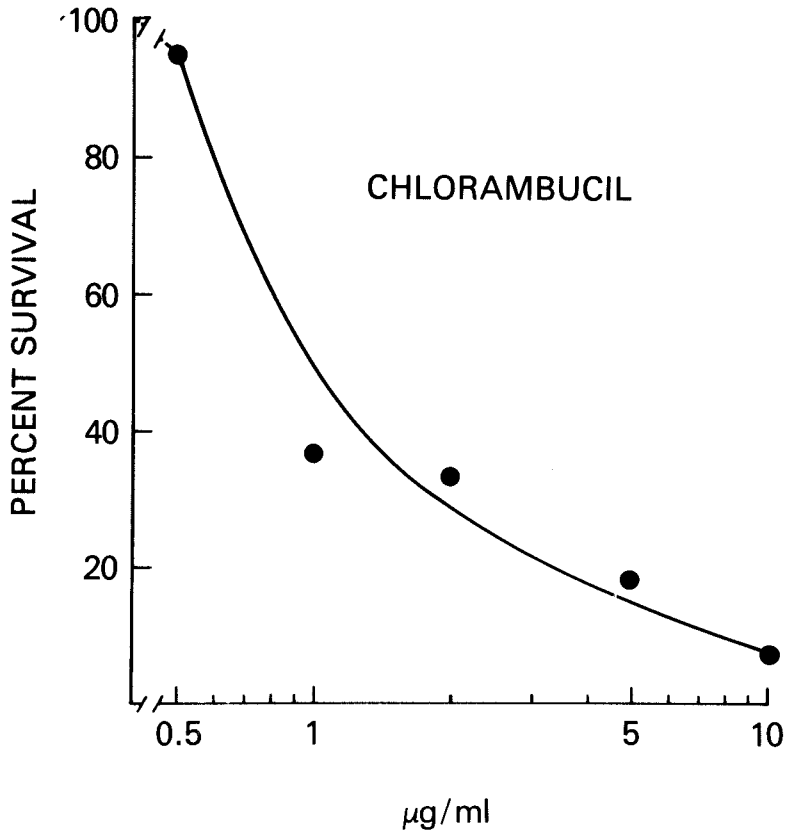


Fig. 23. Dose cytotoxicity curve for the M5076 ascites exposed to chlorambucil, for 1 hr at 37°C (Courtenay agar assay)

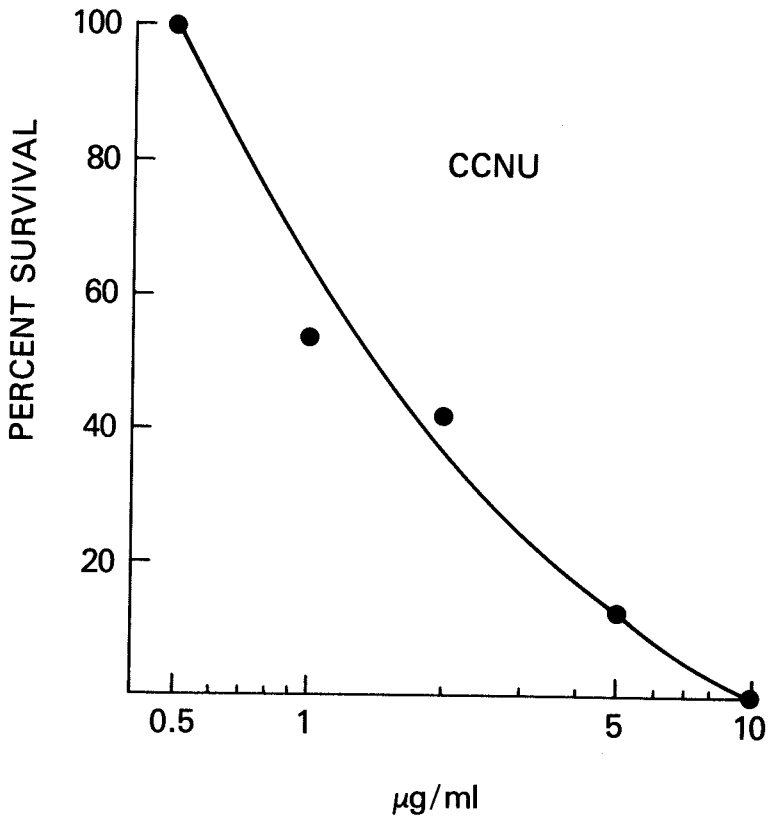


Fig. 24. Dose cytotoxicity curve for the M5076 ascites exposed to CCNU for 1 hr at 37°C (Courtenay agar assay)

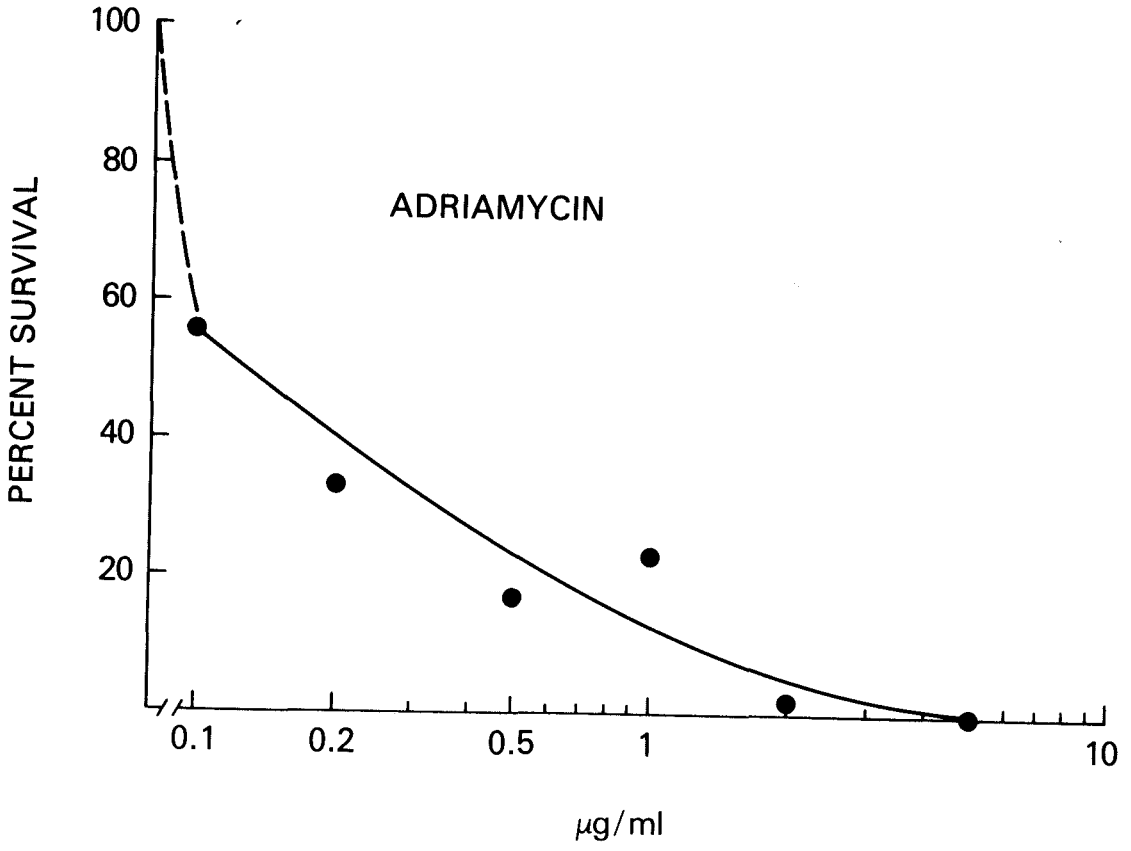


Fig. 25. Dose cytotoxicity curve for the M5076 ascites exposed to adriamycin for 1 hr at 37°C (Courtenay agar assay)

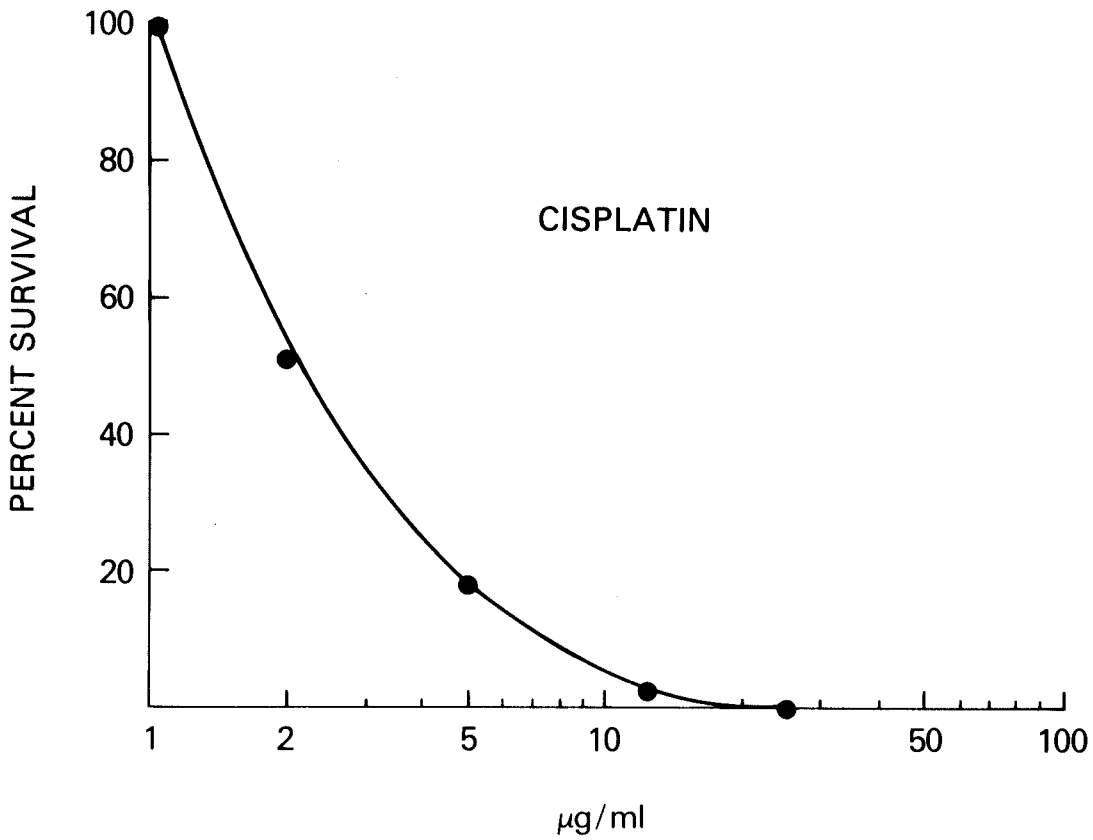


Fig. 26. Dose cytotoxicity curve for the M5076 ascites exposed to cisplatin for 1 hr at 37°C (Courtenay agar assay)

Table 10 shows the comparative effect of chlorambucil when incorporated into the upper layer. This is referred to as "continuous incubation" in the literature, although the extent of exposure will depend on the half-life of the drug being used, which is about 90 minutes in the case of chlorambucil. It is clear from the data given that the colony survival is reduced by continuous exposure to about one third of the value obtained by a 1 hr exposure.

Table 10 Continuous incubation of M5076 cells plated in soft agar with chlorambucil (cell concentration 2 x 10^5/ml)

Chlorambucil concentration µg/ml	Mean no. of colonies/ml ± SD	% control
Control	1964	100
0.2	700 ± 159	35.6
2	165 ± 18	8.4

Table 11 (overleaf) shows pharmacological data obtained principally from unpublished observations on the M5076 by the Southern Research Institute, Alabama, and the Pharmacy Department, Aston University. Either the peak plasma concentration (PPC) or the concentration X time product (CXT) derived from the area under the drug concentration/ time curve has been used as a standard for defining sensitivity (74,75). A plot of the in vitro LD_{50} values for the M5076 against the PPC in man is approximately linear (Fig. 27), and suggests that the in vitro mouse tumour may have a role as a model for prediction of in vivo cytotoxicity in man.

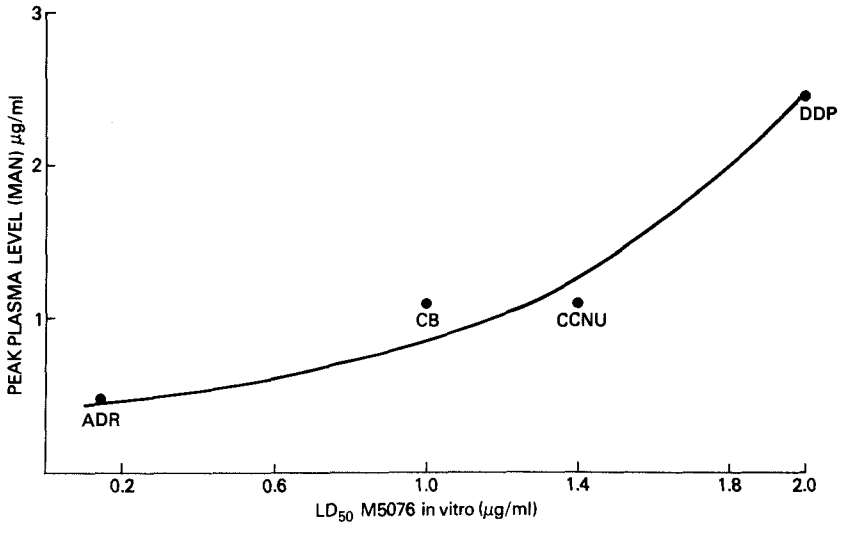


Fig. 27. Relationship between the LD_{50} assessed by M5076 agar colony assay and the peak plasma level for each drug in man.

Table 11. Collated *in vitro* and *in vivo* pharmacological data for cytotoxic drugs in man and the mouse M5076 tumour.

	MAN				MOUSE(M5076)					
					in vitro	*in vivo* cytotoxicity tests (1) - (4)				
Drug	Dose/ m^2	Peak conc µg/ml	Half life hr	CXT µg/hr/ml	LD50 µg/ml	(1) LD50 mg/kg	(1) ID90 mg/kg	(1) Ther index	(2) 3M572	(3) 3M532	(4) ILS
Adriamycin	45	0.48	30	3.5	0.14	5.6	-	-	+	+	++
CCNU	30-100	1.1	0.1	ND	1.4	57	13	4.4	++	++	++
Cisplatin	100	2.49	0.5	1.94	2	7.5	3.6	2.1	+	++	+
Chlorambucil	06	1.10	1.5	2.38	1	46	-	-	-	++	+

Key:
- CXT — concentration x time
- \- — no activity
- Ther. index — therapeutic index (LD_{50} : ID_{90})
- \+ — moderate antitumour activity
- ++ — significanct antitumour activity
- MD — not done

(1) J Hickman personal communication
(2) A Bogden " "
(3) A Bogden " "
(4) M D'Incalci " "

Also shown in Table 11 are the results of 4 independent tests of drug activity on the subcutaneously implanted M5076 tumour in intact mice, performed in 3 centres (Birmingham UK; Mario Negri, Milan; Southern Research Institute, Alabama). The first employs a "therapeutic index" derived from the ratio of the LD_{50} to the ID_{90} (the dose that inhibits tumour formation in 90% of the animals). This combines a toxicity and a response assessment. The second is an estimate of tumour growth delay. The last two, designated (3) and (4) in Table 11 give a qualitative assessment of activity by measuring the increase in survival time of the animals inoculated with both the tumour and the drug, and may overestimate the true activity of a drug.

3.5. Human ovarian cell lines

Characterisation

The monolayer growth curve of each cell line is shown in Fig. 28 and demonstrates a doubling time of 23 hr for 1847S and 28 hr for 1847 MER. The cells in 1847 MER were morphologically similar to those of 1847S apart from being slightly larger. Both lines showed loss of contact inhibition when grown on plastic. The resistant line had a lower plating efficiency than the sensitive line, and when plated on tissue culture plastic, 35% of 1847 MER were adherent at 24 hr compared to 50% for 1847S. The plating efficiency of 1847 MER in soft agar was 5% and of 1847S was 8%. Representative colonies are shown in Fig. 29. These are well defined and easily counted manually or by an image analysis system.

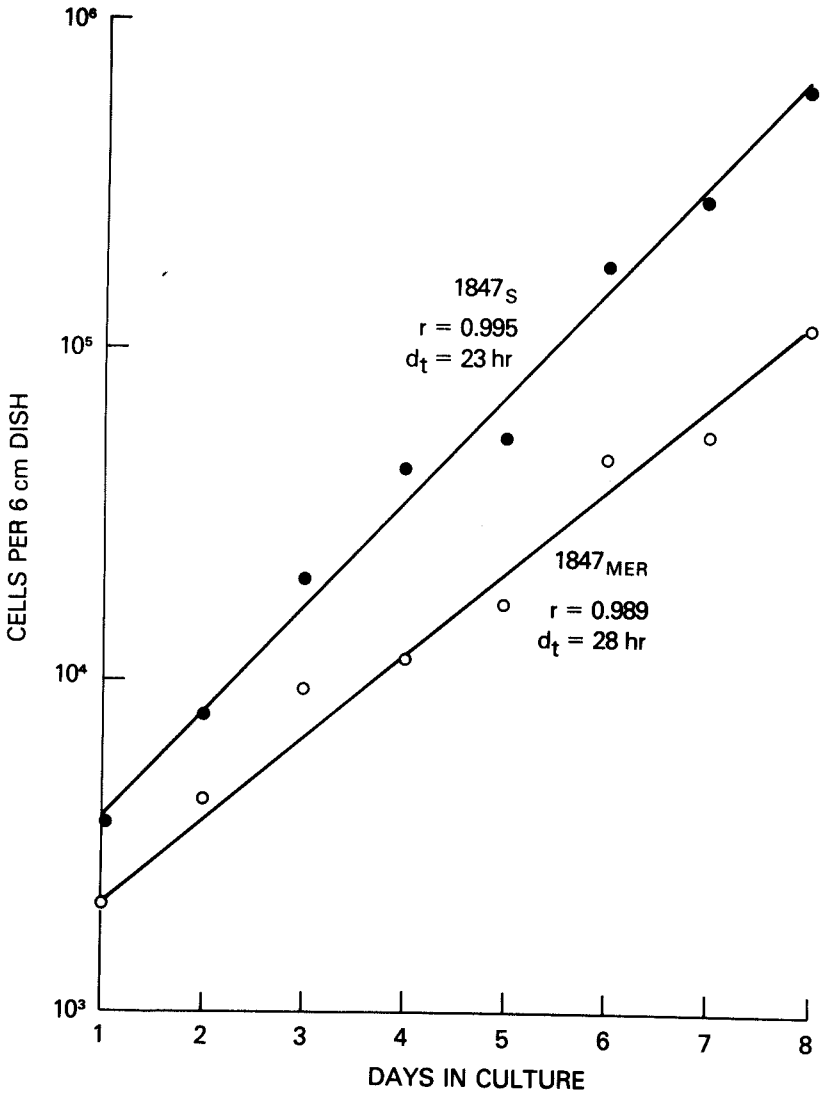


Fig. 28. Monolayer growth of 1847S and 1847 MER in RPMI 1640 + 10% FCS. 5 X 10^3 cells were plated 18 hr before the first time period.

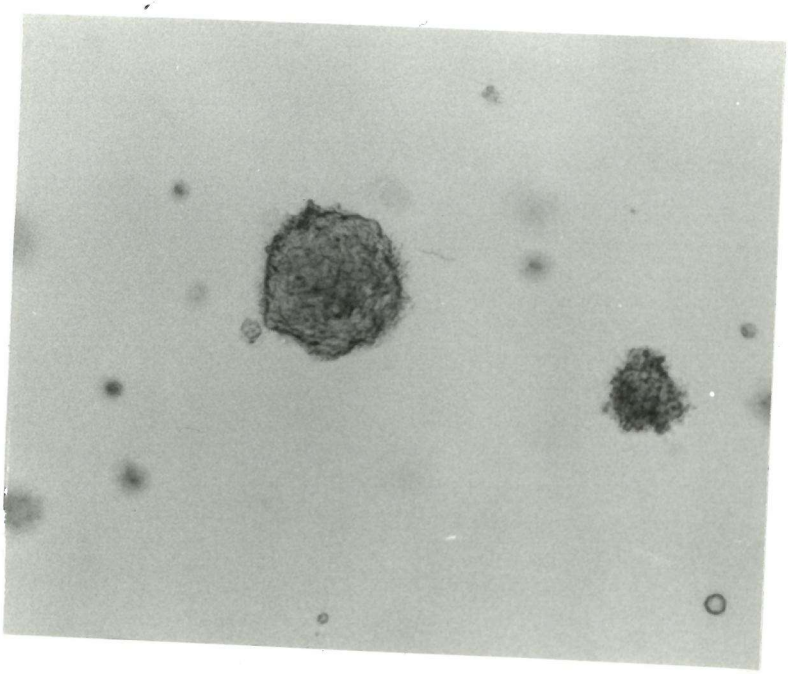


Fig. 29. A colony derived from 1847S after 14 days growth in soft agar. ×80

Fig. 30 shows the melphalan dose response curves for 1847S and 1847 MER, where the end point used is reduction in adherent cells after 5 days growth after exposure to melphalan for 18 hr. The concentration of melphalan reducing survival to 50% of the non-drug-treated control (LD_{50}) was calculated to be 0.95 μM for 1847S and 3.2 μM for 1847 MER in this experiment, giving a relative resistance of 3.3:1. Also shown are the melphalan dose response curves for 2 additional cell lines derived from patients clinically resistant to alkylating agents, NIH : OVCAR-2 and NIH : OVCAR-3. The dose response curves for these two cell lines were similar to that of 1847 MER, demonstrating that the degree of resistance produced by stepwise incubation of the sensitive line 1847S was of the same order as that in the lines established from clinically resistant patients.

Experiments where reduction in colony formation was used as the end-point for melphalan induced lethality gave similar values for the relative resistance of 1847 MER to 1847S. Where reduction in adherent cells was used, the LD_{50} ratio (resistant : sensitive) was 3.6 (range 3.5 - 3.8), where drug incubation was carried out in suspension and agar cloning used as the end-point for cytotoxicity, the ratio was 3.8 (range 2.8 - 4.5) and when the cells were exposed in monolayer and then trypsinised and cloned in soft agar the ratio was 3.4 (range 2.8 - 4.2). Representative curves showing comparisons, between 1847S and 1847 MER, of the dose cytotoxicity relationship for the two cloning techniques are shown in Figures 35 and 36.

1847S and 1847 MER were also exposed to a range of concentrations of adriamycin, cisplatin and thiotepa. Fig. 31 shows that there is no evidence that 1847 MER is cross-resistant to adriamycin. In contrast, a subline of 1847S in which resistance was induced to adriamycin (1847 ADR) shows approximately four-fold resistance when assessed under identical conditions at the same time as the lines 1847S and 1847 MER. The curve for thiotepa at concentrations 1-10 μM is identical to that for adriamycin and shows no cross-resistance. When exposed to cisplatin at concentrations of 0.5 - 5 μM, the melphalan resistant line shows a similar degree of resistance to a further subline of 1847 in which resistance had been induced to cisplatin (1847 CP) (Fig. 32).

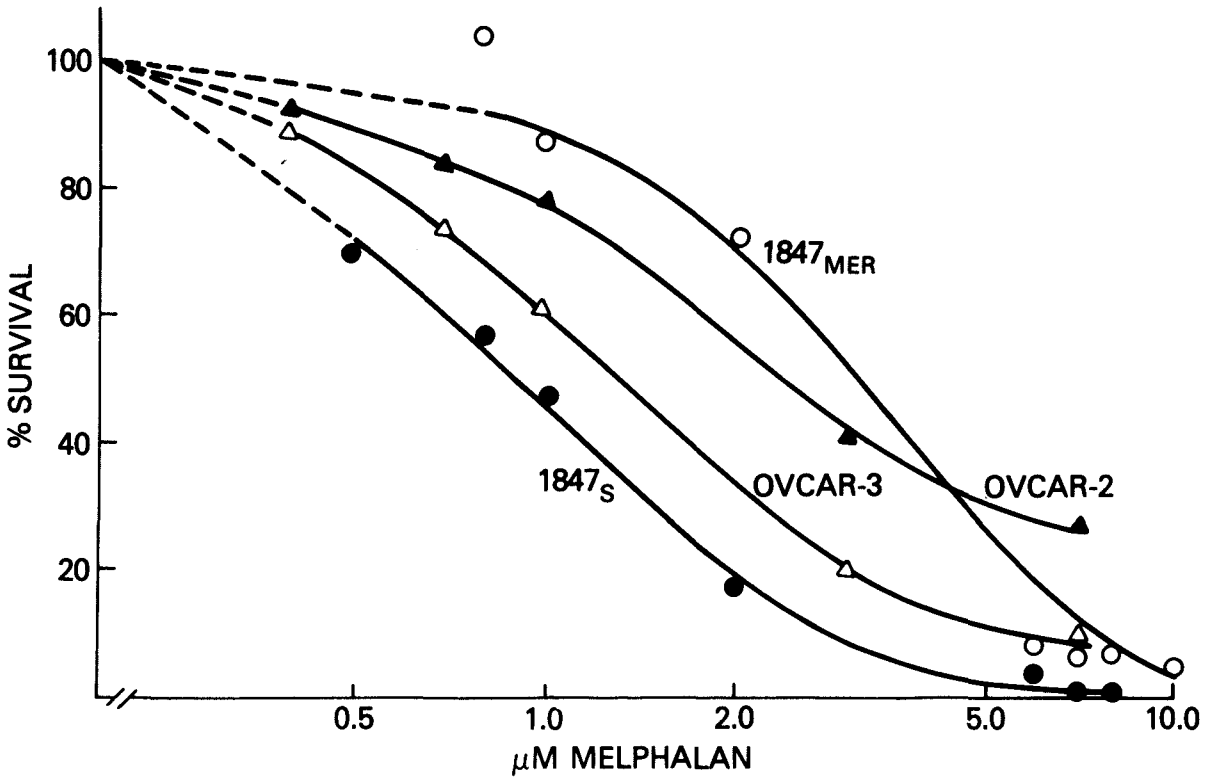


Fig. 30. Dose cytotoxicity curve for melphalan. The 1847S and 1847 MER cells were exposed for 18 hr in monolayer to the drug at 37°C, and the end-point is the number of cells remaining adherent to plastic 5 days later (approx 5 cell doubling times). Cell lines derived from patients clinically resistant to alkylating agents are shown for comparison (OVCAR-2 and OVCAR-3).

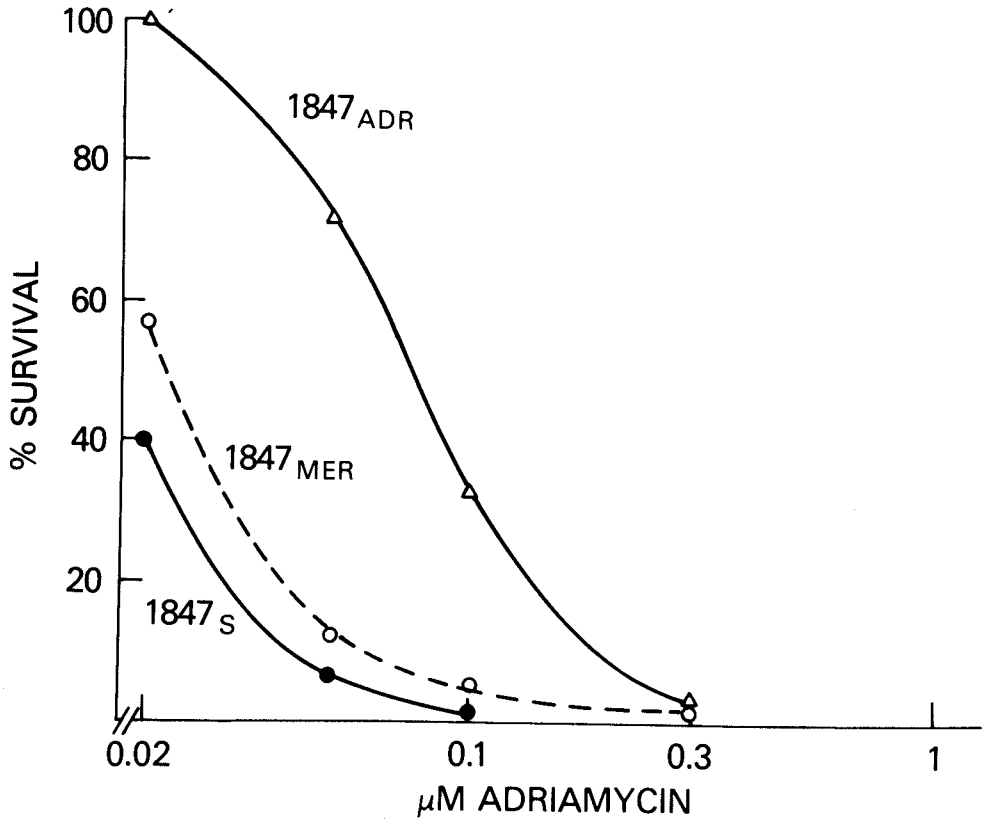


Fig. 31. Cross resistance study. 1847S and 1847 MER cells were exposed in monolayer for 18 hr to adriamycin. End point was number of cells remaining attached to plastic at 5 days post drug. A line with induced resistance to adriamycin 1847 ADR is shown for comparison.

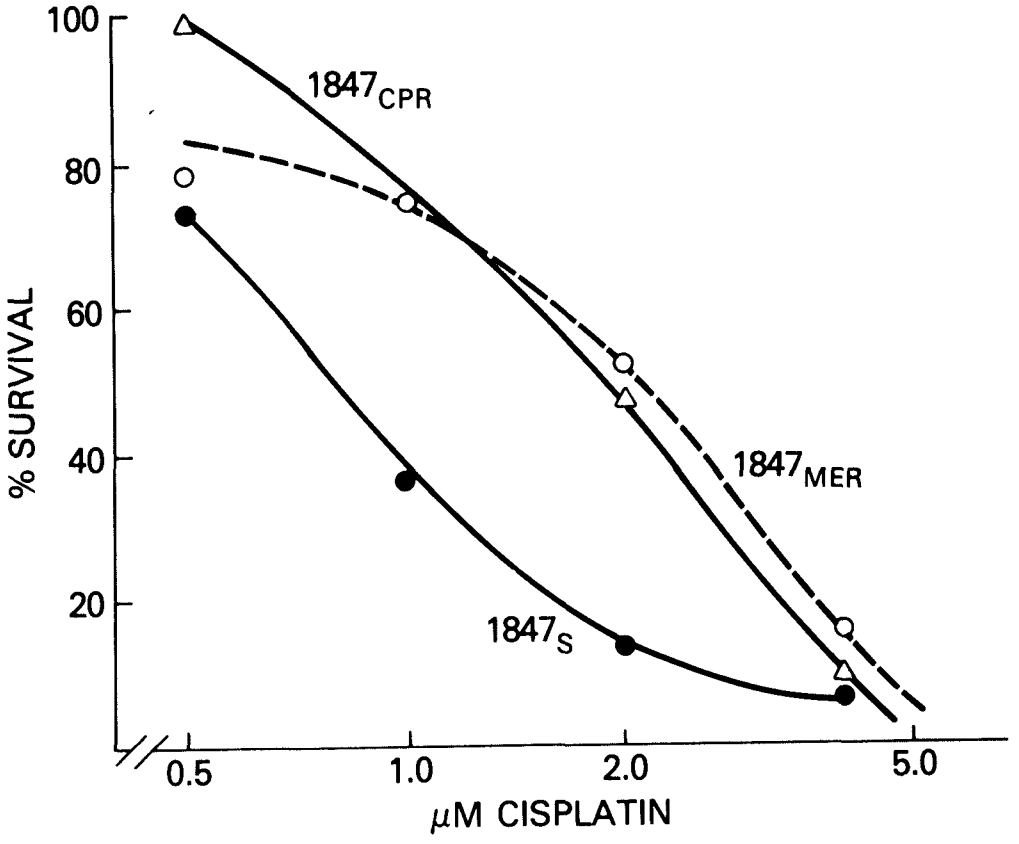


Fig. 32. Cross resistance study. 1847S and 1847 MER cells were exposed in monolayer for 18 hr to cisplatin, and the percentage survival calculated from the number of untreated cells adherent to plastic 5 days later. A cell line with induced resistance to cisplatin (1847 CPR) is shown for comparison.

3.6. Biochemical studies on mechanism of resistance

Transport of Melphalan

Fig. 33 compares the uptake of melphalan in 1847S and 1847 MER. In both cell lines there was a rapid uptake over the first 10 min and a plateau phase was reached within 30 min. There was no significant difference in melphalan accumulation between the melphalan sensitive and melphalan resistant cell lines.

Dechlorination of Melphalan

Fig. 34 compares the rate of hydrolysis of melphalan to the mono- and di-hydroxy derivatives, after incubation with radiolabelled melphalan for 90 minutes. The curve after incubation for 3 hrs is similar and demonstrates that there is no significant difference in the rate of dechlorination of melphalan between 1847S and 1847 MER.

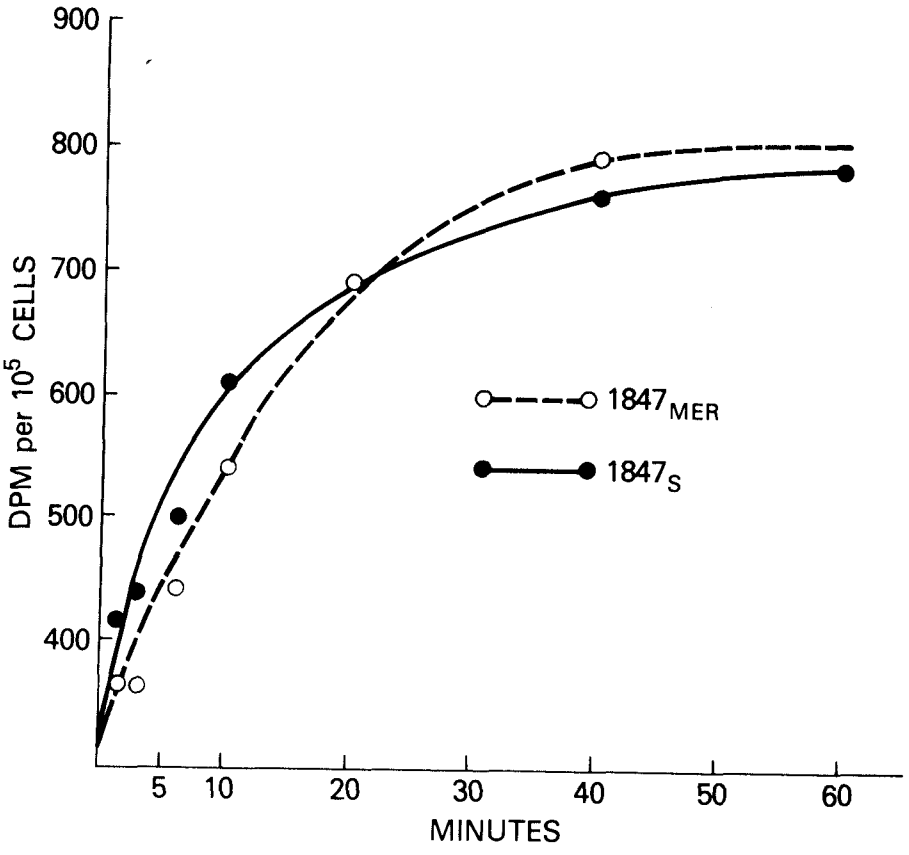


Fig. 33. Uptake of [^{3}H] melphalan (6.4 mCu/mg) when added at 37°C and pH 7.5 to 1847S and 1847 MER in monolayer in RPMI 1640 + 10% FCS.

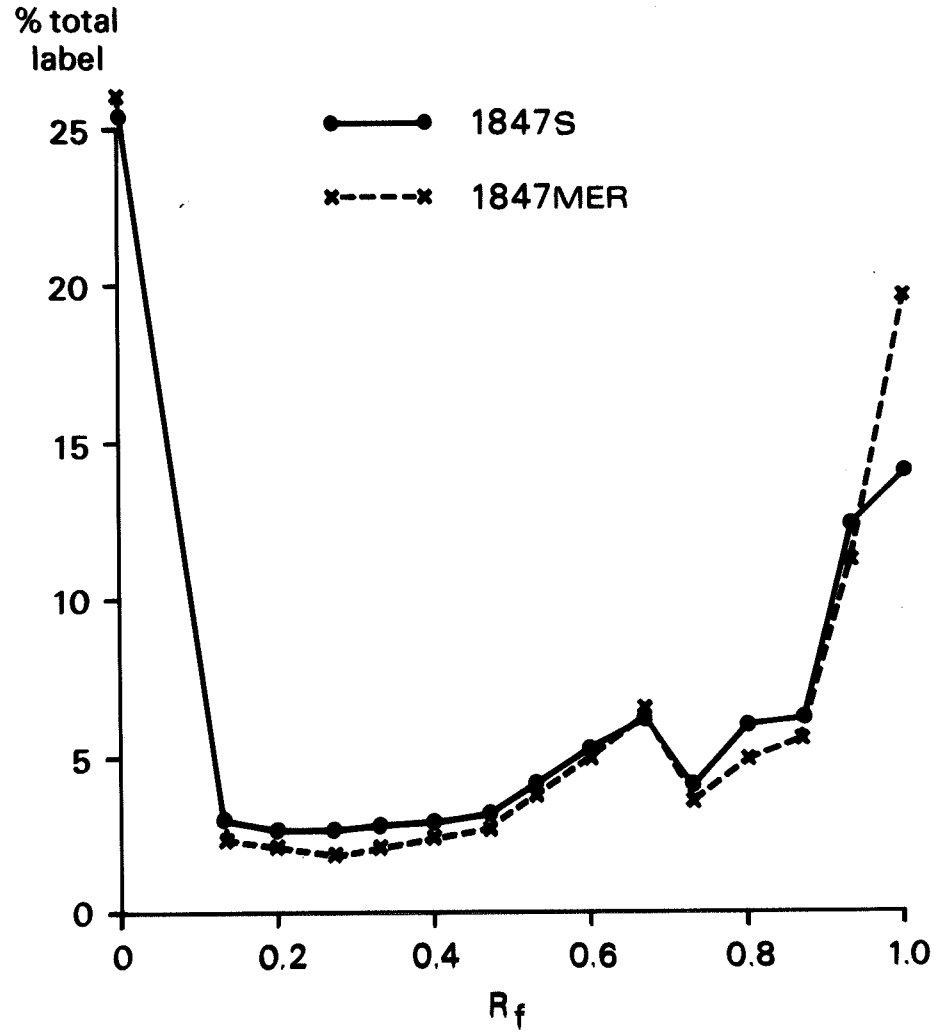


Fig. 34. Dechlorination of intracellular [^{3}H] melphalan 30 minutes after uptake assessed by thin layer chromatography.

Glutathione levels in ovarian cancer cell lines

The glutathione concentration of 1847S and 1847 MER cells is shown in Table 12. Levels were two-fold higher (1.9 ± 0.4) in the resistant compared to the sensitive line. The values given for total glutathione are the mean of 5 replicate experiments, whereas the oxidized subfraction, (GSSG) which comprises less than 5% of the sample, was assayed only once. Incubation of the cells in a cysteine free medium for 3, 6, 9 and 24 hr reduced the total glutathione to the percentages of those measured in the complete medium shown in Table 13. All cytotoxicity experiments employing glutathione depletion by this method were performed after 24 hr culture in cysteine-free medium at which time the total glutathione concentration is reduced to less than 15% of its original level.

Table 14 shows the effect of the inhibitor buthionine sulphoximine (BSO) given for 18 hr to 1847S and 1847 MER at doses of 10-100 μM, on the total intracellular glutathione levels. The levels in both cell lines are reduced, but even at 100 μM concentrations of BSO, the glutathione level after 18 hr was considerably higher in the resistant line (3.7 nM for 1847 MER vs 2.7 nM/10^6 cells in 1847S), though the percentage reduction was the same (82% vs 81% respectively).

Table 12. Intracellular concentrations of total glutathione and reduced glutatione (GSSG) in melphalan sensitive (1847S) and resistant (1847 MER) cell lines.

Cell Line	1847S	1847 MER
Total GSH ($nM\ 10^6$ cells)	10.7 ± 3.6	20.3 ± 8.5
GSSG ($pM\ 10^6$ cells)	196	359

Table 13. Effect of incubation in cysteine free media (RPMI 1640 + 10% FCS) on glutathione levels.

Cell Line	1847S	1847 MER
Time in cysteine-free medium		Glutathione level: percent of control
3 hr	47%	57%
6 hr	35%	60%
9 hr	34%	48%
24 hr	14%	12%

Table 14. Total intracellular glutathione levels after 18 hr incubation with BSO in the melphalan sensitive (1847S) and resistant (1847 MER) cell line. Values are expressed as nM per 10^6 cells and as a percentage of the GSH concentration in complete medium without BSO. Effect of BSO upon glutathione levels in melphalan sensitive (1847S) and resistant (1847 MER) ovarian cancer cell lines.

Cell Line	1847S		1847 MER	
Dose BSO (μM)[1]	Glutathione levels[2] (percent of control)			
10	4.9 μM	45.5%	9.8 μM	38.2%
25	3.4	31.6%	8.5	33.2%
50	2.7	25.4%	6.5	25.3%
100	1.6	16.7%	3.7	19.4%

[1] Incubation with BSO for 18 hr

[2] nM GSH per 10^6 cells.

Effect of nutritonal glutathione reduction on melphalan cytotoxicity in 1847 MER

Fig. 35 shows that reversal of melphalan resistance occurs with a 24 hr preincubation of 1847 MER cells in a cysteine free medium, corresponding to an 85% reduction in total glutathione levels. The dose response curve for 1847 MER cells after nutritional deprivation of cysteine was not significantly different to the dose response curve for 1847S cells grown in standard medium. At doses of 1-5 μM melphalan, there was no colony formation by 1847S cells preincubated in the cysteine-free medium.

Effect of Buthionine sulfoximine on melphalan cytotoxicity in 1847 MER

Fig. 36 shows the effect of prior incubation for 18 hr with 100 μM BSO on the sensitivity of 1847S and 1847 MER to melphalan applied for 2 hr to the cells in suspension before cloning in agar. The degree of enhancement of sensitivity produced by the glutathione inhibitor is of the order of 3.5 fold, and renders the LD_{50} of the sensitized 1847 MER cells similar to that of 1847S grown in complete medium without the inhibitor. An even greater degree of enhancement was produced by 18 hr preincubation of the cells in 50 μM BSO, followed by the addition of melphalan together with the inhibitor for a further 18 hr (Fig. 37). Under these conditions, the enhancement factor was 10 fold.

A dose response curve for BSO given alone to 1847 MER cells for 18 hr showed a colony survival of 70% at 100 μM, 80% at 50 μM and only minimal cytotoxicity at doses below 50 μM. (Fig. 38). Employing the method of Momparler (109) for quantitating the degree of interaction between the melphalan and the BSO by comparison of the surviving fraction (S_F) when given alone and together (S_F BSO and melphalan: S_F BSO x S_F melphalan) gives a synergy ratio of 30 for the particular case of 100 μM BSO for 18 hr and 3 μM melphalan.

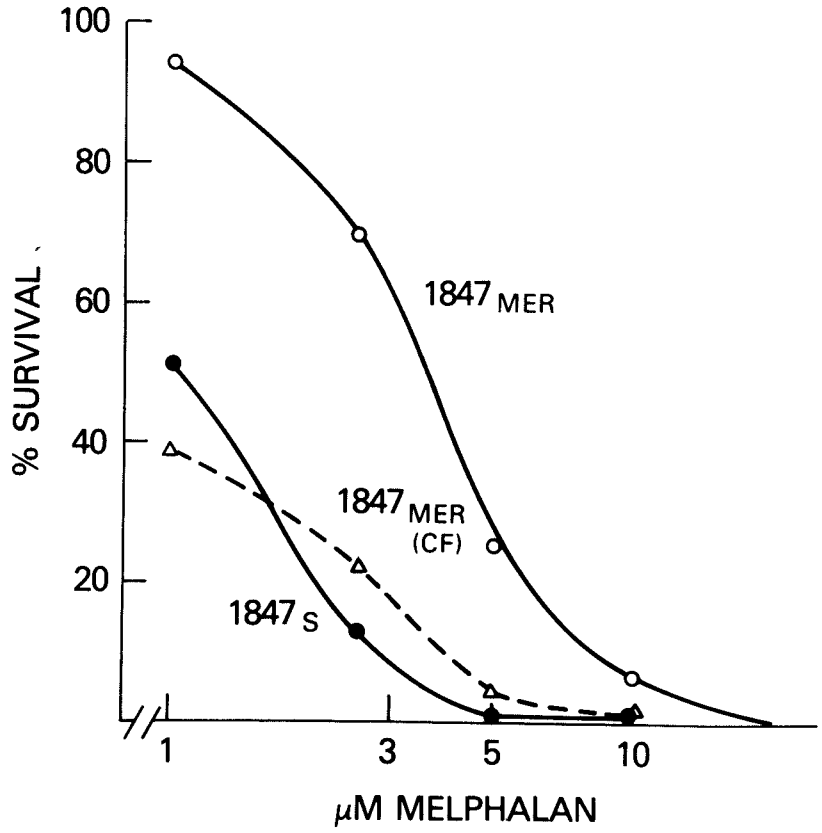


Fig. 35. Melphalan dose response curves for 1847S and 1847 MER were determined using cloning as an end-point following growth of the cells in complete media and in cysteine free media assay. Exposure to melphalan was in monolayer culture for 18 hr following which the cells were cloned in agar. The dashed line (Δ---Δ) represents the resistant line 1847 MER preincubated in cysteine free medium for 24 hr. At the concentrations of melphalan used there was no cloning of 1847 S cells after a 24 hr exposure to cysteine free media.

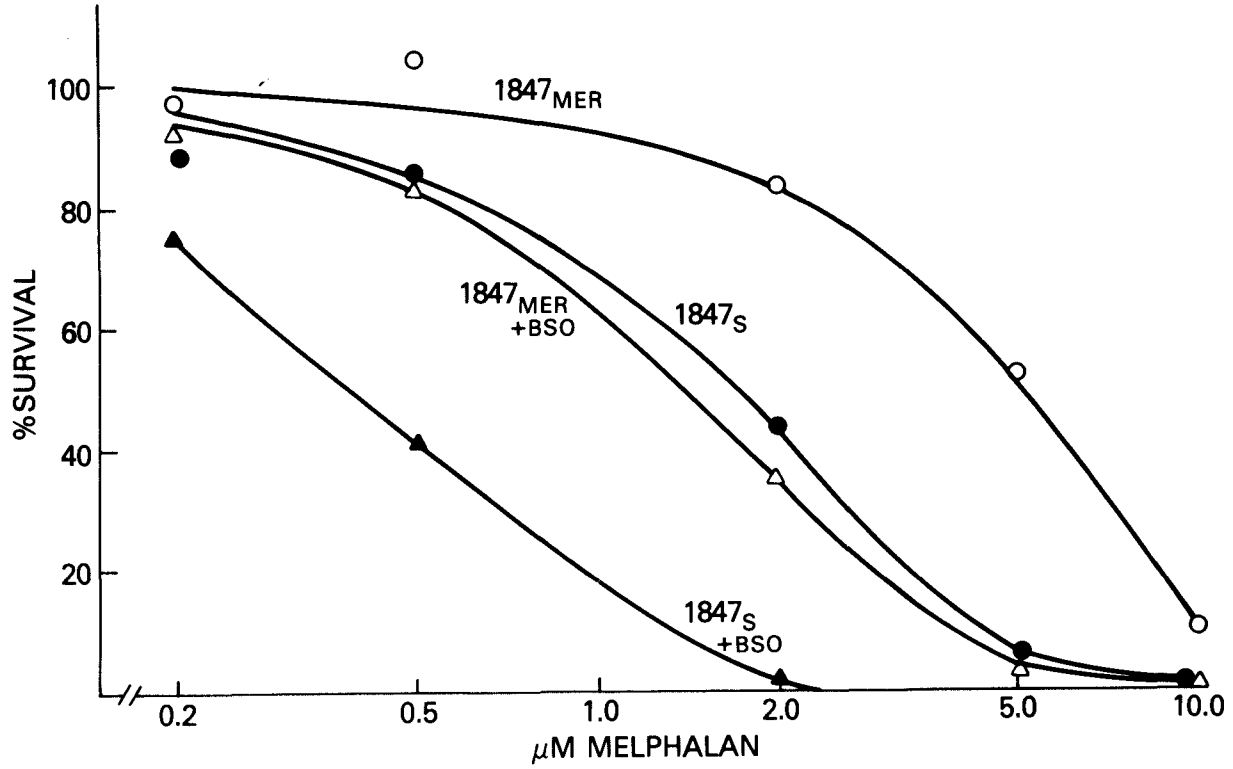


Fig. 36. 1847S and 1847 MER cells were grown in complete media or for 18 hr in media with added BSO (100 μM) after which the cells were harvested, exposed to melphalan for 2 hr and cloned in agar.

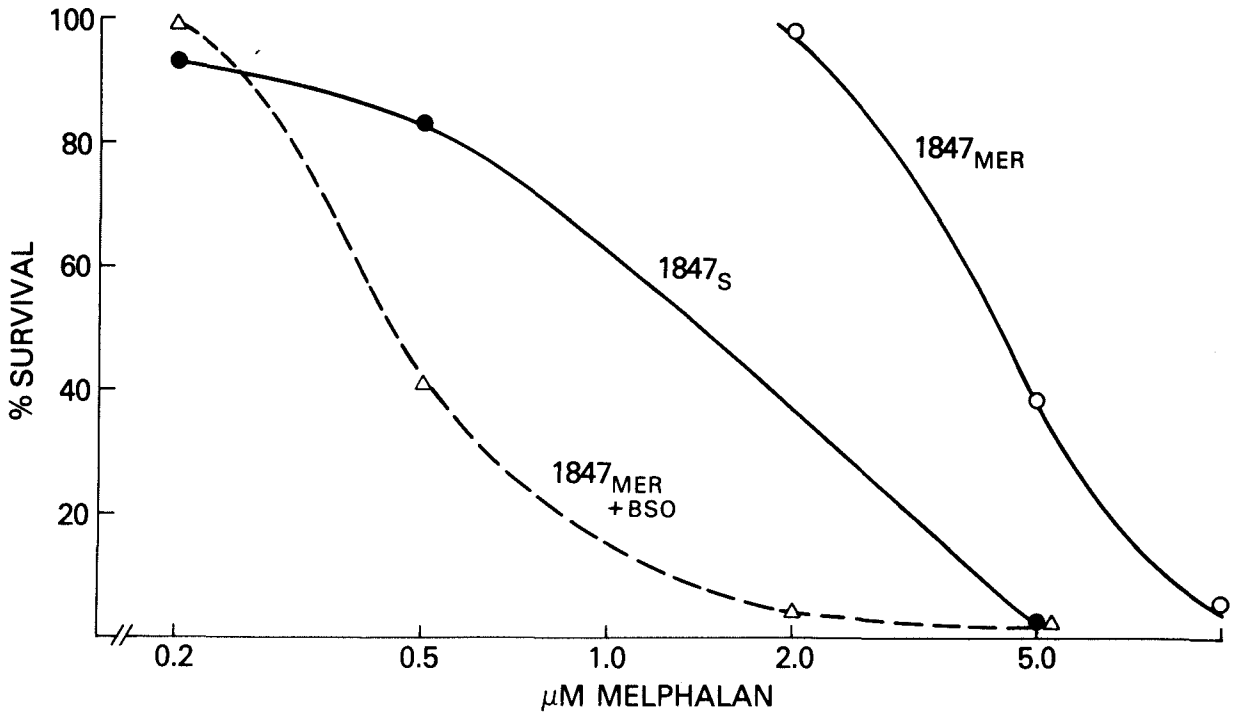


Fig. 37. 1847S and 1847 MER were grown in complete media or in media with added BSO (50μM) for 18 hr prior to the addition of melphalan. After an additional 18 hr the cells were harvested and cloned in agar.

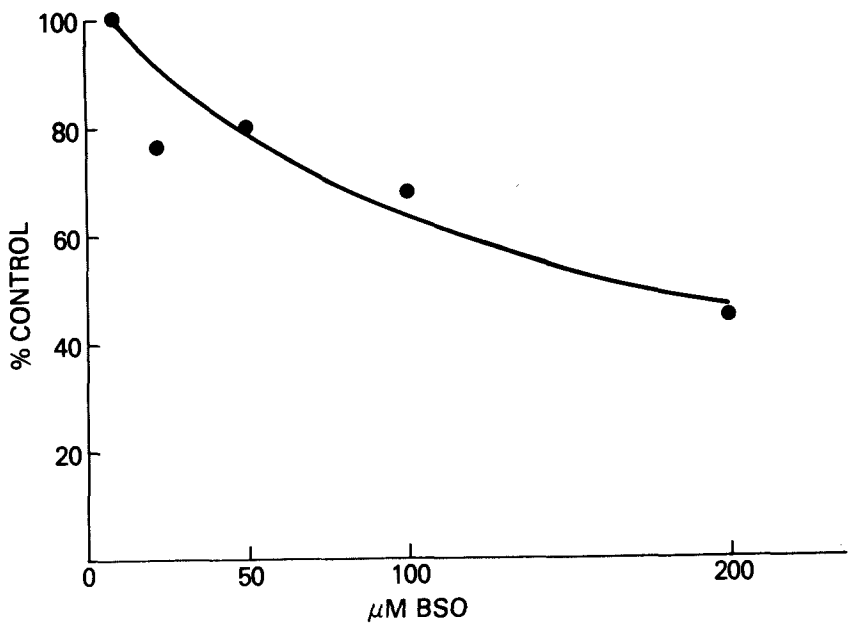


Fig. 38. Cytotoxicity of buthionine sulphoximine. Cells were preincubated at the concentrations indicated in monolayer for 24 hr and the cytotoxicity assessed by colony formation in soft agar at 14 days.

DISCUSSION

4.1. Clinical studies

The results demonstrate from the analysis of the 64 evaluable patients (table 6) that the response rate was higher in the group given the combination PACe (67%) compared to those given single agent chlorambucil (30%). Survival was however not significantly increased in the combination group. This is comparable to the results achieved in a number of trials where combination chemotherapy has increased the response rate but shown only a slight improvement in projected survival (113-115). Two trials (43-44) have shown a significant survival benefit from combination chemotherapy over single alkylating agent therapy, but these results need further evaluation with longer follow-up. Intrinsic drug resistance therefore continues to be a major problem in advanced ovarian cancer.

As expected, the clinical assessment of complete response (CCR) overestimated the pathological complete response (PCR) assessment made at laparotomy as only 62% of those in CCR were found to be in PCR. A pathologically documented complete response holds the best probability for long term survival (26 months for those in PCR compared to 12 months for those patients clinically free of disease found to have residual tumour at second look laparotomy). However, even in those patients with a surgically documented complete remission there is evidence of a steady rate of relapse. It is reasonable to assume that these relapses are due to cells, resistant or partially resistant to the drugs used as first line treatment, which are not detectable at the time of response evaluation. Obviously no current or envisaged imaging technique can improve the response assessment obtained at a second look surgical procedure. The implication for management is that drugs with an effective cell kill several-fold greater than achieved with present combinations are required, and a serum or urine marker sensitive enough to predict microscopic residual disease would be a significant advance in the management of this disease.

While these results would indicate no clear benefit for the PACe combination, there is evidence from the literature that combination

chemotherapy increases the probability of achieving a surgically documented complete remission, and that this in turn is the single most important factor associated with prolonged survival in ovarian cancer. No one combination has emerged as superior to the others in achieving this aim. There is no doubt that toxicity is greater with the combination regimes used, as in addition to one or more alkylating agents, cisplatin is frequently included.

The response rate of the patients crossed over to second line therapy on relapse was poor, with an overall rate of 15% and very short survival. The ability to tolerate second line therapy was variable, and only 46% of patients received adequate second line treatment, which was arbitrarily defined as 2 courses or 2 months. Dose reductions were frequently required due to myelosuppression, and while this would diminish the apparent effectiveness of second line treatment, a considerable proportion of these treatment failures can be attributed to cross-resistance. Given this high degree of myelosuppression, treatment strategies based on the addition of further cytotoxic therapy (chemotherapy or radiotherapy) at doses which are likely to be effective may not be clinically achievable. This holds even in the selected troup of pathological complete remitters, and either this end point must be recognised earlier and non-cross resistant treatment added before marrow reserve is exhausted, or the initial induction policy must be refined to obtain more durable remissions.

For the particular case of the patients failing chlorambucil, there is evidence that the alkylating agent resistance is associated with cross-resistance to cisplatin. This drug cross-links DNA bases in much the same way as an alkylating agent (116). The anthracycline adriamycin is also included in the PACe regime, and while this compound has been shown to intercalate with DNA (117) its precise mechanism of action is not clear. This study would indicate there is cross-resistance to this agent also.

The phenomenon of cross-resistance has been observed in a number of clinical studies in human ovarian cancer (118) and in several animal models (92). To examine the extent and possible mechanisms of

this resistance an in vitro model was required. Ideally, this should provide a finite end-point of cytotoxicity as well as allow manipulation of the dose and possibly scheduling of the individual agents under test.

4.2.1. Human ovarian cancer - in vitro studies

Attempts to establish human ovarian tumours in primary agar culture met with little success. Even after resolution of the problem of bacterial contamination the low proportion of tumours giving rise to colony formation (<40%) and the small numbers of colonies formed (never more than 100 per plate), made the development of a tumour drug assay logistically impractical. At least 3 drugs have to be tested if the assay is to be clinically useful.

When the precise role such as assay might have in ovarian cancer is considered more fully, its clinical application can be seen to be further limited. The clinical response rate is moderately high with present combinations ($\sim$ 70%), and the three most effective drugs can be given as first line therapy without any requirement for a predictive test. The most useful place for such an assay would therefore be at the time of first relapse where it could be used to predict resistance and possibly sensitivity. However, as shown in the present clinical trial and discussed in a review of several recent clinical studies (46) the response rate (<25%) and remission duration (<8 months) of second line therapy is very poor, and out of 100 potential patients tested at first relapse, less than 5% would derive clinical benefit (119). Many of the treatments chosen would have been selected empirically in any case.

The applicability of such an assay would change if the effectiveness of second line therapy were to improve, or if the proportion of tumours giving rise to colonies in agar could be raised. Growth factors in tissue culture are presently the subject of considerable research effort and of these the most intensively studied has been epithelial growth factor (EGF), a mitogenic peptide derived from the mouse submaxillary gland (120) which has been shown to increase colony formation in up to 50% of cases (121). Insulin, transferrin,

follicle stimulating hormone, the steroid hormones, triiodothyronine, aminoethanol, and mercaptoethanol have all been found to have growth promoting effects in certain systems (122), and need to be evaluated for their effect on primary ovarian tumour culture. Other factors which could be varied are the amino acid content, the serum content, and possibly the trace metal levels of the culture media. Given the present low colony growth rate, this would be a major undertaking.

4.2.2. Mouse M5076 tumour

The decision was taken to look for an *in vitro* model system with reproducible colony growth characteristics, and use this to study the mechanisms of resistance to cytotoxic drugs. The M5076 mouse tumour was selected for further study primarily because of its *in vivo* sensitivity pattern to a number of cytotoxic drugs (84-86), which corresponded to that of human ovarian carcinoma. The tumour is slow growing for a mouse tumour, and remains localised to the peritoneal cavity or at the subcutaneous injection site, only metastasising late to the ovaries, liver or lymph nodes, but rarely the lung. It is presently being used for screening by the Experimental Cancer Chemotherapy Unit at Aston University, Birmingham, U.K., and is on the reserve panel of tumours employed by the National Cancer Institute, U.S.A., new drug screening program. It was originally thought to be a carcinoma, and was described as having arisen spontaneously in a mouse ovary (85). These groups regard histology as of secondary importance and rely principally on statistical correlations between activity against animal tumours and those cancers in man of a type known to be responsive to existing drugs. The relevance of this screening evaluation has at times been criticised as a major part of the data is derived from the study of the mouse P388, L1210 and TLX5 lymphoma/leukaemia tumours, which constitute a very restricted range of tumour type.

Initial experiments were directed towards establishing soft agar colony formation by the tumour, and correlating the *in vitro* drug data with the known *in vivo* responses to the same drugs. *In vitro* screening for new cytotoxic agents would be feasible if an *in vitro* cloning technique could be shown to give a good correlation with the results achieved on whole animals, or prediction of sensitivity and/or resistance in man.

In the present study, the morphological features of the M5076 showed an anaplastic tumour without differentiating features. The foamy appearance on the touch preparations, the phagocytosis, the enzyme histochemistry and the presence of Fcγ receptors (Fig 20) strongly suggest a macrophage origin for this tumour, on the basis of the criteria established by Yam et al (107). While it is known that some animal tumours may contain up to 60% macrophages, the cells described above constituted the majority of the population in both the solid and ascitic form of the tumour, and it is unlikely these results are based on contaminating peritoneal macrophages. These findings are corroborated by the work of Talmadge et al (102).

Colony formation in agar by single cell suspensions derived from the M5076 solid tumours was achieved with some difficulty, but in contrast to the experiments of Talmadge, suspension cultures could not be established from either the solid or ascitic tumours. The immunological surface markers and intracellular enzymes of the tumour allowed identification of the cells in the colonies and the results are compatible with their origin from the parent tumour. The malignancy of the cells in the colonies was also confirmed when these cells formed tumours on re-injection into the peritoneal cavities of DB26F1 mice.

Damage sustained during the disaggregation procedures was considered a possible reason for the poor clonogenic growth observed. While the mechanical methods released large numbers of cells into the medium, the majority of these were non-viable by trypan blue exclusion, and unlikely to give rise to colonies. Enzyme methods produced greater cell yields, and improved viability, but no better clonogenicity. The problem was eventually overcome by using M5076 ascites cells with the addition of preservative free heparin to prevent clumping.

Incubation of these ascites cells with the same enzyme cocktail under the same conditions, impaired both their labelling index and clonogenic growth (Fig 14,15). It would appear that the enzymes

collagenase III and deoxyribonuclease, or impurities contained in them, damaged the cell membranes of these cells sufficiently to prevent growth in agar, but not enough to permit them to take up trypan blue.. Other workers have obtained improved clonogenic growth after enzyme disaggregation of solid tumours employing various concentrations and mixtures and durations of exposure up to 48 hr in some cases (122). The conclusion that enzyme procedures are contraindicated should be restricted to this cell type (M5076) and the particular enzyme preparations used.

The choice of agar culture system is dictated principally by the requirements of the tumour to be cultured. While it did not prove possible to modify the Salmon double agar technique and achieve satisfactory results with the M5076 the human ovarian cell lines grew well on a simplified version of it. The technical differences between the methods are summarised in Fig. 8. The Salmon method, while more complicated, requires no refeeding with the attendant risk of contamination, and allows for examination at regular intervals so that mechanical or automated counting methods can be employed. The method of colony counting described by Courtenay (compression of the agar between coverslip and slide) is cumbersome and irreversibly damages the sample. The viewing time must therefore be fixed in advance, and a larger number (4/5 vs 3) of replicate samples used to compensate for the varying thickness of the agar gel at the time of counting. The compromise solution of using identical reagents to the Courtenay assay but growing the cells in 35mm Petri dishes did not succeed, and the drug cytotoxicity studies on the M5076 were carried out using the Courtenay technique in 15 ml Falcon tubes.

Tveit et al (106) demonstrated improved growth, plating efficiency and dose/survival curves in xenografts cultured in agar by the method of Courtenay in comparison to Salmon's technique. In their study only part of this differnece was due to the use of August rat red blood cells, and low (5%) O_2. These factors were not found to be of significant benefit in promoting colony formation by the M5076 cells, and the precise reasons for the differences in the methods remains unclear.

The drug dose/cytotoxicity curves (Figs. 23-26) show a linear dose response over a 1-2 log dose range. There is no significant plateau at high drug concentrations, which has been observed in drug assays performed with the Salmon method (74). This observation implied that resistant clones could easily be isolated from primary clonogenic cultures, but a critical review by Selby et al (119) concluded that much of this resistance was artefactual. In a recent report, D'Incalci (123) has reported the development of a M5076 tumour resistant in vivo to cyclophosphamide, and it would be of great interest to assess the in vitro sensitivity of this resistant tumour by clonogenic assay by comparison with the parent cells. There are insufficient dose points on the curves to determine if a 'shoulder' exists at low drug doses, a phenomenon seen in radiation response curves (119) and attributed to the effect of sublethal damage. This shoulder has also been described in some in vitro cytotoxic drug dose response curves (124).

The major difficulty lies in the interpretation of these in vitro dose response curves. It would be expected that an in vitro assay would overestimate the activity of certain drugs, as the problems of access to the tumour are much diminished. This may explain the observation that adriamycin has only moderate activity in vivo in the M5076 while it appears active over a wide range in vitro. It became clear however that sensitivity and resistance are not firm end points in vivo in the M5076, and that even if resistance were to be developed in vivo or in vitro, there would be formidable problems in comparison of the in vitro and in vivo pharmacology (119, 123, 125). As a model for human ovarian cancer, the subcutaneous M5076 grew little better in vitro than the primary human cultures, and the studies suggested that the disaggregation procedures were the principal cause of the failure of colony formation in agar, an observation confirmed by Steel et al in attempts to clone cells from xenografts (125). It was not found possible to improve the growth in vitro of either the human or the murine tumours by limited manipulation of the culture conditions but it was possible to conclude from the deletion studies performed that none of the additives to the media were toxic at the levels employed.

Compared to *in vivo* studies whether in animal tumours or xenografts, clonogenic assays do offer a controlled environment where there are no host defences to contend with. Development of serum free media, which has been achieved for human small cell and breast tumours in culture (122, 126) would permit even more rigorously defined conditions for the study of hormones and other growth factors. Clonogenic assays are also very sensitive and relatively rapid and inexpensive by comparison with *in vivo* techniques, and are capable of giving a more quantitative result (127). It was therefore decided to build on the experience of *in vitro* cloning studies as an end-point for cytotoxicity, and apply them to the human ovarian cell lines which became available at the NCI.

In addition to being more easily and rapidly tested, these lines had the advantage of a sensitive and resistant subline of the parent tumour available for comparison. They were also of the same histogenetic origin as human ovarian cancer, although there was a degree of selection for proliferative capacity inherent in the development of the cell line.

4.2.3. Human ovarian cancer cell lines

The development of these cell lines enabled a comparison to be made between the effects of melphalan in lines derived from previously untreated patients, lines derived from patients who relapsed with progressive disease while on therapy with alkylating agents, and in lines made resistant *in vitro* by stepwise incubation of a melphalan sensitive line (1845S) with increasing concentrations of melphalan (1847MER). The induced resistance in 1847MER is similar to that in cell lines established from patients refractory to therapy (the OVCAR lines), suggesting that this model is of clinical relevance for the subsequent studies of resistance modulation.

It has been demonstrated by the three related assays of *in vitro* cytotoxicity that the resistant line 1847MER is about 4 times more resistant than the parent line 1847S to melphalan, by comparison of the LD_{50} values obtained. It was also apparent that the best

agreement between the techniques and the reproducibility within a particular method was obtained with the longer drug incubation times (e.g 12-16 hr). This is in keeping with the half life of melphalan (60-90 min) as well as the time required for complete binding of melphalan to DNA, which has been shown to be of the order of 9-12 hr (128). While 1 hr at 37°C has been widely employed as a convenient standard, this period may not be sufficient to allow for the variables of temperature, pH and binding. In all the comparisons between sensitive and resistant lines, or between different culture media, the analyses were carried out at the same time under identical conditions on the same subculture of cells. The third method described for assessing cytotoxicity, in which the cells were exposed in monolayer, and then harvested with trypsin only after allowing adequate time for transport and drug binding, is the least open to theoretical objection (Fig. 35) as the cell membranes remain intact during exposure. Colony formation, by assessing considerable if not necessarily infinite capacity for self renewal, is also preferred as an end point to cell number measurement (68).

In vitro cross resistance was demonstrated in 1847MER to cis-platin and thiotepa, but not to adriamycin. The cross resistance is therefore less than universal, and similar to that observed in the clinical study (Section 4.1). The principal difference is that adriamycin cross-resistance is a common clinical finding in alkylating agent resistance (78). These findings are similar to those observed for induced melphalan resistance in L1210 mouse leukaemia cells where there is cross resistance to cisplatin but not to adriamycin (129) and in CCNU resistant Walker 256 carcinoma cells where cross resistance has been shown to several bifunctional alkylating agents (130). It may be relevant that both of these systems also show an induced resistance 4-5 times that of the parent cell line.

4.3. Mechanism of resistance to melphalan in human ovarian cell lines

The transport studies showed no difference in the uptake of melphalan up to 60 min between 1847S and 1847MER. As the experiment could not be carried out with the cells in suspension culture, uptake studies within the first 120 sec were not performed, as the design

required termination by two cold washes with PBS, rather than simply a rapid spin through oil to separate the extracellular from intracellular label. Prior trypsinisation of the 1847S cells or mechanical detachment would obviously introduce further artefact. These results are in agreement with studies in L1210 resistant lines (94) and in a melphalan resistant human melanoma cell line (97).

Intracellular dechlorination of melphalan 1.5 and 3 hr after exposure to the radiolabelled drug was identical in the sensitive and resistant lines. These results differ from those reported by Vistica (94) in his studies on L1210 cells, but are in keeping with the pharmacologic studies of Alberts et al (131) who showed that the majority of in vivo metabolism of melphalan to the inactive hydroxy derivatives occurred by passive hydrolysis.

The demonstration of higher GSH levels in the 1847 MER line suggests a role for this tripeptide in the development of acquired resistance to melphalan. In particular, depletion of GSH to less than 15% of its original value by the cysteine free medium markedly sensitized the cells (Fig. 35 and Table 13) to the alkylating agent. Cysteine depletion alone for 24 hr was associated with minimal cytotoxicity. In vivo sensitisation by cysteine depletion has been carried out in the mouse (129) and of interest was that in this animal GSH depletion and sensitisation to melphalan cytotoxicity were much greater in the tumour cells than in the bone marrow granulocyte/monocyte stem cells.

Equivalent sensitization and equivalent reduction in GSH was achieved by preincubation with the γ-glutamylcysteine synthetase inhibitor buthionine sulfoximine (BSO). This agent produced only slight toxicity when used alone on 1847 MER at the doses and incubation times employed, which suggests that clinically important sensitization may be feasible. However, prolonged glutathione depletion has been shown to result in excessive oxidative damage to the cell even in the absence of an alkylating agent (132, 133). In vivo experiments have been carried out in mice with the L1210 leukaemia (134) using nutritional depletion and BSO, and a moderate degree of sensitization related to glutathione depletion demonstrated.

Unfortunately, there is no toxicology data available on the effects of BSO in large animals and in humans as would be required if this agent were to be selected for clinical trials as a chemosensitizer.

Connors' (135) in a review of protection against alkylating agent damage by a variety of thiol compounds distinguished between freely diffusible compounds such as cysteine, from those which do not enter cells such as thiosulphate. The latter will only be effective if the rate of formation of the reactive alkylating intermediate is the limiting step (see S_N1 reaction, p 25). In the case of a diffusible protective agent (such as cysteamine or GSH), the compound must be distributed close to the DNA or other target site if it is to have a high probability of interfering with the alkylation process. In these studies, thiol depletion has been used to induce a considerable degree of sensitisation, and it is possible that a differential rate of depletion either at the tissue or cellular level could be used to therapeutic advantage to protect normal tissues.

The precise mechanism of the protective effect of glutathione against melphalan cytotoxicity is unknown. Glutathione has been shown to inhibit the alkylation of DNA by the nitrosamines (136) in mouse liver cells. An association has been demonstrated between the magnitude of the chemosensitisation effect in animals of the nitro-imadazoles and intracellular thiol levels (137). There is also some evidence that the chemosensitisation to melphalan by these agents results from increased numbers of DNA cross-links being formed (138, 139). Direct covalent reaction between melphalan and glutathione at physiological pH is precluded by the high PKa of the latter (140), although GSH does combine with more reactive metabolites of alkylating agents such as acrolein (141). There is also evidence that a significant proportion of cisplatin administered to rats is bound *in vivo* to glutathione, and that glutathione transferase levels in liver and kidney are altered for several days after a single intraperitoneal dose (142). The mechanism by which the cell changes its total glutathione levels in response to a drug challenge has not been established, and non-specific toxic effects have to be distinuished from a programmed response such as enzyme induction or repression which could lead to the emergence of resistant clones.

While glutathione is the principal intracellular thiol, a correlation has been demonstrated between the cytoplasmic metallothionein concentration and resistance to the alkylating agent chlorambucil (143). Metallothionein is a low-molecular weight protein rich in cysteine, and further evidence of its protective role is based on the observation that 30% of the radioactivity from labelled chlorambucil can be extracted at the same molecular weight as the metallothionein fraction.

Several intracellular roles have been assigned to GSH and it has been shown to function as part of a redox cycle in which GSH is continually regenerated from GSSG by the enzyme glutathione reductase, which obtains the proton from $NADPH^+$ (144). A variety of organic peroxides can, mediated by glutathione peroxidase, reoxidise the GSH to complete the cycle. Damage to these enzymes or the glutathione transferase system could also profoundly affect the intracellular glutathione levels (145), and thus modify alkylating agent cytotoxicity by decreasing the ability of the cell to protect the DNA from melphalan induced damage. These other possibilities for the modulating effect of GSH on alkylating agent activity need to be investigated, but it is reasonable to conclude that glutathione protects critical sites on DNA, RNA or protein by altering the nature of the DNA or DNA-protein cross-links, and hence the three-dimensional structure of the nucleoprotein matrix.

These studies demonstrate that glutathione levels correlated with melphalan resistance in human ovarian cancer cell lines. The observation that reduction in glutathione levels is able to restore sensitivity to melphalan has potential clinical significance since the development of resistance to alkylating agents is a major factor which limits the usefulness of alkylating agent chemotherapy in patients with advanced ovarian cancer. It remains to be seen if any of the *in vitro* methods of glutathione reduction (145) are applicable in the clinical situation.

4.4. Summary

The studies reported in this thesis were stimulated by the observation that cytotoxic drug resistance is a major problem in the management of advanced ovarian cancer. The disease is moderately chemosensitive, but relapse is almost inevitable within 2 years. Recently available drugs therefore work but do not succeed. The alkylating agents comprise the backbone of treatment, and intrinsic resistance, acquired resistance and cross-resistance to other alkylating agents and non-alkylating agents are frequent clinical observations. The problem has been well described over the last 15 years, but the conclusions reached from animal studies on the possible mechanisms of this resistance have suggested a number of biochemical differences between sensitive and resistant tumour cells.

An interim analysis of an ongoing randomised clinical trial in advanced ovarian cancer between combination chemotherapy and a single oral alkylating agent showed a marked improvement in response for the combination (67% vs 30%), but overall survival was not significantly different in the two arms. Toxicity was greater in the combination arm and two treatment related deaths were recorded. The crossover design confirmed that response to second line therapy was poor and survival short. The conclusion was that more effective induction regimes are required in ovarian cancer to overcome this resistance to therapy.

In an attempt to improve on the empirical basis for the selection of drugs, primary culture of human ovarian cancer cells was attempted in a two-layer soft agar system. Only a small proportion were established in culture, and this lack of success was attributed to the disaggregation procedures employed. Mechanical methods gave low yields of viable cells, and enzyme methods of tissue dissociation, while giving improved yields, were found to inhibit colony formation in vitro.

Studies on a mouse carcinoma (M5076) grown as a subcutaneous tumour encountered similar difficulties, but when the same tumour was grown as an ascites, colony growth improved and drug assays were

performed with chlorambucil, CCNU, cisplatin and adriamycin employing reduction in colony formation as an end-point. A limited degree of correlation was obtained between the in vitro sensitivity and published data for the in vivo tumour in the mouse. It has also been suggested by other authors that this tumour is a good animal model of the drug responsiveness of human ovarian carcinoma, and there was an association between the clonogenic assay data and the peak achievable plasma level in man. This suggests that this tumour may be useful as part of an in vitro phase II screening programme for new cytotoxic agents. The in vivo tumour is at present of interest to preclinical pharmacologists as it shows a pattern of response to cytotoxic drugs which differs from that of the existing lymphoma/leukaemia models, which have been criticised for selecting drugs that are likely to be effective against only a restricted range of human cancers in subsequent clinical studies. Other experiments performed as part of this project showed that the M5076 has in fact the features of a histiocytic lymphoma rather than a carcinoma. This is not necessarily a disadvantage, as the value of a drug screening system is related to the accuracy of its prediction for clinical studies, and historically this has not been shown to correlate with the histology of the animal tumour screen.

In the course of the project, the opportunity became available to work in the laboratories of the National Cancer Institute, Bethesda, USA, where human ovarian cancer cell lines had been developed. Lines were available from untreated ovarian cancer patients, from which sublines were obtained by stepwise incubation in increasing melphalan concentrations, that were four fold resistant to this drug. A further line was derived from a patient clinically resistant to alkylating agents. The degree of resistance to melphalan in this intrinsically resistant line was of the same order of magnitude as in the line with acquired resistance. Cross resistance to cisplatin and thiotepa was observed in the melphalan resistant line, but the sensitivity to adriamycin was similar to that observed in the parent line. Further studies were then carried out to look at the possible mechanisms of this resistance.

The uptake of melphalan by the sensitive cells and those in

which resistance had been induced was found to be identical, with a plateau reached after 30 min in both cell lines. Dechlorination of melphalan to the non-cytotoxic metabolites was also similar in the sensitive and alkylating agent resistant cells. Exogenous thiols have long been used as protective agents against alkylating agent toxicity, and following on some studies in the L1210 mouse leukaemia, it was found that levels of the tripeptide glutathione, the principal intracellular thiol, were two fold higher in the resistant compared to the sensitive cells. Furthermore, depletion of glutathione to between 10 and 25% of its original level resulted in marked sensitisation of the cells to alkylating agent cytotoxicity, and in the resistant cells was able to reverse the degree of acquired resistance. Glutathione depletion was achieved either by omission of cysteine from the medium, or preincubation in the presence of buthionine sulphoximine, a specific inhibitor of glutathione synthesis. The latter method seemed particularly attractive, as buthionine sulphoximine at a dose of 50μM for 18 hr was minimally cytotoxic to the resistant cells when given alone, yet completely reversed the degree of acquired resitance to melphalan.

The success of an alkylating agent sensitiser will obviously depend on it being possible to selectively enhance alkylating agent cytoxicity in tumour cells without achieving a similar degree of sensitisation in the dose limiting normal tissues such as bone marrow or gastro-intestinal tract. Sensitisation to chemotherapeutic agents remains an attractive alternative to high dose regimes now being evaluated in a variety of tumours.

If the raised glutathione levels in the resistant cells can be shown to be a specific effect related to acquired resistance, then such sensitisation may have a particular role in second line ('salvage') therapy, as treatment failure is related to drug resistance in a high proportion of these cases. Sensitisation could also be employed as part of a strategy where late intensification was given. Further studies could concentrate on the effect of glutathione depletion on the binding of alkylating agents to subcellular fractions, particularly those in the nucleosome, and look for differences in a range of resistant and sensitive human tumour cell lines, as well as

in normal bone marrow stem cells. Myelosuppression is the principal dose limiting toxicity for alkylating agents, and the kinetics of glutathione depletion in the bone marrow relative to that achieved in the tumour cells may determine the degree of sensitisation that is clinically achievable. In addition, the mechanism of the raised glutathione level could be explored, as this may be related to amplification or repression of a gene coding for an enzyme involved in glutathione metabolism.

The aetiology of resistance may ultimately prove to be multi-factorial in many cases, and in addition to the studies described here on alkylating agent interaction and binding to macromolecules leading up to crosslink formation, there is increasing evidence that the ability of the cell to repair such damage may be related to the degree of resistance. The development of these human ovarian cancer cell lines has allowed acquired resistance to be studied in detail, and should provide a more rational basis for the modulation of cytotoxic drug activity.

Conclusions

1. Combination chemotherapy improves the clinically and surgically documented complete remission rate in advanced ovarian cancer, but has no significant effect on survival.

2. Cross-resistance between chlorambucil, cisplatin and adriamycin is a significant problem in advanced ovarian cancer.

3. Primary culture of human ovarian tumours in soft agar as a basis for *in vitro* cytotoxicity testing is not sufficiently reliable a procedure to improve clinical management by selecting patients likely to be sensitive or resistant to one or more drugs.

4. The mouse M5076 tumour when grown as an ascites gave rise to clonogenic growth in soft agar. Preliminary *in vitro* cytotoxicity data suggested that there was some correlation with *in vivo* data. The tumour was shown to be a histiocytic lymphoma rather than a carcinoma.

5. Human ovarian cancer cell lines were found to be the best model for the study of *in vitro* drug resistance. Cell lines in which resitance was induced to melphalan by stepwise incubation with increasing drug concentration were compared to the parent untreated cell line, and lines derived from patients clinically resistant to alkylating agents. There was no difference in melphalan uptake or dechlorination to the inactive metabolites between the parent and resistant lines.

6. Glutathione levels were two fold higher in the resistant lines. Depletion of glutathione either by cysteine depletion or incubation with the inhibitor buthionine sulphoximine (BSO) produced marked sensitisation to the cytotoxic effects of melphalan. BSO itself was only minimally cytotoxic and may have a role as a potentiator of alkylating agent activity.

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Appendix 1 Source of Reagents and Animals

Tissue culture reagents

McCoy's 5A medium (liquid) CMRL 1066 medium with glutamine (liquid) L-glutamine Penicillin/streptomycin solution	Gibco Europe Ltd 3 Washington Road, Paisley, PA3 4EF.
Sodium pyruvate L-serine Asparagine Mercaptoethanol	Sigma Chemical Co., Fancy Road, Poole, Dorset
Foetal calf serum Horse serum L-ascorbic acid	Sera Lab. Ltd., Crawley Down, Sussex RH10 4FF
Bacto agar	Difco laboratories Detroit, Michigan
DEAE dextran	Pharmacia Fine Chemicals Uppsala, Sweden

Drugs

Insulin 100 units/ml	Eli Lilly
Heparin without preservative 1000 units/ml	Weddel Pharmaceuticals Ltd, London EC1A 9HY
Adriamycin	Farmitalia Carlo Erba, Barnet, Herts
Chlorambucil	Sigma Chemical Co. Ltd., P.O. Box 14508, St. Louis, U.S.A.
CCNU	Lundbeck

Animals

DB26F1 C57 black mice female	OSLAC 76 Ltd., Shaws Farm, Blackthorne, Near Bicester, Oxfordshire

Appendix 2

Papanicolaou staining technique

Taken from Manual of Histological Demonstration Techniques.
H.C. Cook. Butterworths 1974.

1. 95% alcohol for 1 min.
2. 70% alcohol for 1 min.
3. Distilled water 3 minutes.
4. Harris's haematoxylin for 2 min.
5. Tap water 2 min.
6. 1% acid alcohol for 1-2 min.
7. Tap water for 2 min.
8. Ammoniated water (distilled water to which are added a few drops of concentrated amonia) for 1 min.
9. Tap water 3 min.
10. 70% alcohol for 2 min.
11. 95% alcohol for 2 min.
12. OG 6 solution (Raymond A. Lamb) for 2 min.
13. 95% alcohol for 0.5 min.
14. 95% alcohol for 0.5 min.
15. EA36 solution for 3 min.
16. 95% alcohol for 0.5 min.
17. Absolute alcohol 1 min.
18. Xylene (2 changes) for 2 minutes.
19. Mount in DPX under a glass coverslip.

Appendix 3

Haematoxylin and Eosin (H and E) stain.

1. Wash in tap water
2. Harris's haematoxylin 4 min.
3. Wash in tap water.
4. Differentiate in 1% HCl in 70% alcohol
5. Wash in tap water
6. Essin 1% for 5 mins.
7. Wash in tap water
8. Dehydrate in increasing ethanol concentrations
9. Clear in xylene and mount in DPX

LATE (FIGO III or IV) OVARIAN CANCER CHEMOTHERAPY SECOND STUDY (SO2)

INTRODUCTION

Poor results with surgery and radiotherapy in the management of ovarian carcinoma have led to the use of chemotherapy in the advanced stages of this disease. It was shown as early as the 1950's that alkylating agents could produce objective regression of disease. Evaluation of drug therapy has, to some extent, been limited by indiscriminate use of these agents. The alkylating agents have become the most widely used group of antineoplastic drugs in ovarian cancer chemotherapy, and they produce a 40-50% response rate. Conventional doses of cyclophosphamide resulted in 144 responses in 335 patients, a response rate of 43%, while intensive therapy with high dose cyclophosphamide (120mg/Kg) gave a 61% response rate, 22 out of 36 patients responding.

Fewer studies have been undertaken using non-alkylating agents. In a review of adriamycin in ovarian carcinoma, Blum showed a median response rate of 16% in patients who had failed on previous chemotherapy. In a randomised trial of adriamycin or melphalan in untreated ovarian carcinoma, de Palo et al reported a response in 8 out of 19 patients treated with adriamycin and 4 out of 20 patients given melphalan (the difference was not significant). No cross-resistance was observed between the two drugs on cross-over of therapy, and it was concluded that on the evidence available adriamycin was comparable with the most effective of the conventional treatments.

Cis diamminedichloroplatinum (DDP) was initially shown to be a useful agent in ovarian cancer by Wiltshaw. She achieved a 25% response rate in patients who had been previously treated with an alkylating agent. A similar response rate (33%) was found in 12 previously treated patients in the first Southampton late ovarian study.

Combinations of drugs have been investigated and combining adriamycin with cyclophosphamide appears encouraging. Parker et al have obtained excellent results in patients without prior radiotherapy or chemotherapy, using adriamycin and cyclophosphamide on a dosage schedule of 40 mg/m^2 and 500 mg/m^2 respectively every 3 weeks. They report 8 objective responses in 10 untreated patients and a further 6 responses

in the subsequent 8 patients, giving an overall response rate of 78%. Lloyd et al reported an 80% response rate using a dosage schedule of adriamycin (40mg/m^2, day 1) and cyclophosphamide (200 mg/m^2, days 3-6). Twelve of 15 patients with no prior chemotherapy had objective responses with 5 patients (33%) achieving complete remission from which no patient has relapsed after 7 - 12 + months observation. Of the 8 patients previously treated with cytotoxic chemotherapy, 2 achieved partial remission (25%). The difference in response between patients with prior chemotherapy is significant (p= 0.02) and Lloyd et al conclude that in previously untreated patients, this combination of adriamycin and cyclophosphamide is superior to single agent chemotherapy. They also advocate its use as adjuvant therapy for stages 1 and 2 ovarian carcinoma after surgical treatment and radiotherapy.

Evidence supporting the place of combination chemotherapy in the management of advanced ovarian carcinoma has come from the study of Young et al. These clinicians showed in a prospective randomised trial, that a significantly greater number of patients achieved remission with a multiple drug combination (CR + PR = 75%, CR = 33%) when compared with single agent chemotherapy (CR + PR = 54%, CR = 16%). Complete remission was also an important factor in survival - those achieving a documented CR having a median survival exceeding three years.

In the first Southampton late ovarian study (SO1) a combination of DDP, adriamycin and cyclophosphamide was used for previously untreated patients. Eleven of 16 patients achieved a clinical complete remission, 3 patients a partial remission and 2 patients refused further therapy after one cycle (1 nausea and vomiting and 1 fit due to hyponatraemia). It must be emphasised that these remissions in this pilot study were not confirmed surgically and that the high complete remission rate (69%) may be artificial.

Pharmacokinetic and toxicity studies in SO1 suggest that there was no reduction in renal or G.I. toxicity when DDP was given by a 24 or 48 hour infusion. The response rate for infusions compared with bolus DDP was not assessed due to the small numbers in this pilot study, though complete response did occur in patients receiving DDP by bolus or infusion.

This study combines three drugs with significant single agent activity in carcinoma of the ovary, cyclophosphamide, adriamycin and cis diamminedichloroplatinum, and will evaluate the overall response and toxicity in patients with previously untreated ovarian carcinoma. The design of the protocol compares initial intensive chemotherapy with a three drug combination with standard single agent chemotherapy. Drug information on DDP, adriamycin and cyclophosphamide is given in Appendix 5, 6, and 7.

I. OBJECTIVES

a) To compare the relative effectiveness of initial intensive chemotherapy using a three drug regimen with standard single agent chemotherapy. b) To compare the toxicities of the two regimens and the quality of life and survival of patients in each arm.

c) To assess the usefulness of maintenance chemotherapy in patients who achieve a pathologically documented complete remission.

d) To assess the usefulness of laparoscopy and second look laparotomy in patients in apparent clinical remission following chemotherapy.

II. SELECTION

Patients with primary ovarian cancer between the ages of 15 and 69 who have been found at laparotomy to have disease which is incurable by operation and is extending into the upper abdomen and beyond (FIGO Classification Stages III and IV). Initial assessment is shown in Appendix.

Exclusions - Patients with:-

i. pleural effusion is the only sign of extra-pelvic disease, unless there is unequivocal histological proof of malignancy;

ii. any prior non-surgical anti-cancer therapy, other than instillation of cytotoxic agents at surgery;

iii. other cancers, within the preceeding 5 years except skin cancer;

iv. bone marrow depression (leuco-erythroblastic anemia with thrombocytopenia less than 100,000/u.l);

iv. bone marrow depression (leuco-erythroblastic anemia with thrombocytopenia less than 100,000/u.l);

v. impaired renal function - creatinine clearance < 70 L/24 hr, serum creatinine > 150 u.mol/l (1.5 mgs %), blood urea > 8 u.mol/l (50 mgs %);

vi. impaired liver function - serum bilirubin > 30 u.mol/l (2 mgs %), alkaline phosphatase >300 i.u. AST >50 I.U./l.

vii. Karnofsky status of less than 5 (Appendix II).

Patients with recurrent ovarian cancer after primary surgery may be included if prior chemotherapy or radiotherapy has not been given.

b) Pathological

All common forms of primary malignant epithelial tumours as listed unde the World Health Organisastion Classification would be included in the study:

A: Serous Tumours
- adenocarcinoma
- papillary adenocarcinoma
- papillary cystadenocarcinoma;
- surface papillary carcinoma;
- malignant adenofibroma
- cystadenofibroma.

B: Mucinous Tumours
- adenocarcinoma
- cystadenocarcinoma;
- malignant adenofibroma
- cystadenofibroma

C: Endometrioid Tumours
- a) carcinoma;
  - i) adenocarcinoma,
  - ii) adenocanthoma
  - iii) malignant adenofibroma and cystadenofibroma.

D: Clear Cell (Mesonephroid) Tumours
- carcinoma
- adenocarcinoma.

E: Combinations of A to D

G: Undifferentiated Carcinoma

Exclusions - on pathological grounds:

Epithelial tumours of borderline or low potential malignancy;
Germ cell tumours, including malignant teratomata;
Brenner and mixed mesodermal tumours;
Sex cord-stromal tumours such as granulosa cell tumours;
Sertoli Leydig cell tumours;
Suspected secondary cancers;
Sarcoma, carcino-sarcoma.

The initial primary diagnosis upon which patients would be admitted to the study would be that of the local pathologist in the district where the operative treatment has been undertaken. It is appreciated that frequently, biopsy material only will be available, but where possible, sections from four blocks would be desirable. To ensure uniformity, sections wil be reviewed by the study pathologist, and the Broder grade assessed.

III DEVIATIONS FROM PROTOCOL TREATMENT

All patients will continue follow-up for five years irrespective of recurrence, or any deviation from protocol treatment.

A decision to interrupt or postpone treatment must be taken by the clinician concerned on an individual basis. Dose modifications will be used as shown in appendix 4.

IV PROGRESSIVE ASSESSMENT

Follow-up assessment of toxicity, Karnofsky performance status, response and survival will be carried out monthly.

V CRITERIA FOR ASSESSMENT

a) Measurable change in tumour bulk by:

i. Clinical or radiological measurement including IVP
ii. Ultrasonic scanning.
iii. Isotope scanning
iv. CAT scanning
v. Other techniques
vi. Second Look laparotomy.

VI MODE AND DURATION OF CHEMOTHERAPY (Fig. 1)

Following staging, patients will be randomised to receive either 5 cycles of PACe or intermittent chlorambucil for an initial period of 6 months. Patients will be restaged at the completion of induction therapy and those in complete remission will proceed to laparoscopy or laparotomy.

In the PACe arm, a patient in pathological complete remission will be randomised to receive maintenance chlorambucil or no maintenance. Where a second look laparotomy is carried out and resection is possible 2 further cycles of PACe will begin. A patient showing a partial response or static disease will be given chlorambucil as secondary treatment. In the case of progressive disease, treatment is at the discretion of the referring clinician.

Patients receiving chlorambucil who progress will be crossed over to combination chemotherapy with PACe. At six months, these patients will be restage and a second look laparotomy considered if they are in clinical complete remission. If there is static disease, unresectable or partially resectable disease, the chlorambucil will be continued until evidence of progression occurs. In the case of a complete remission, chlorambucil will be given as

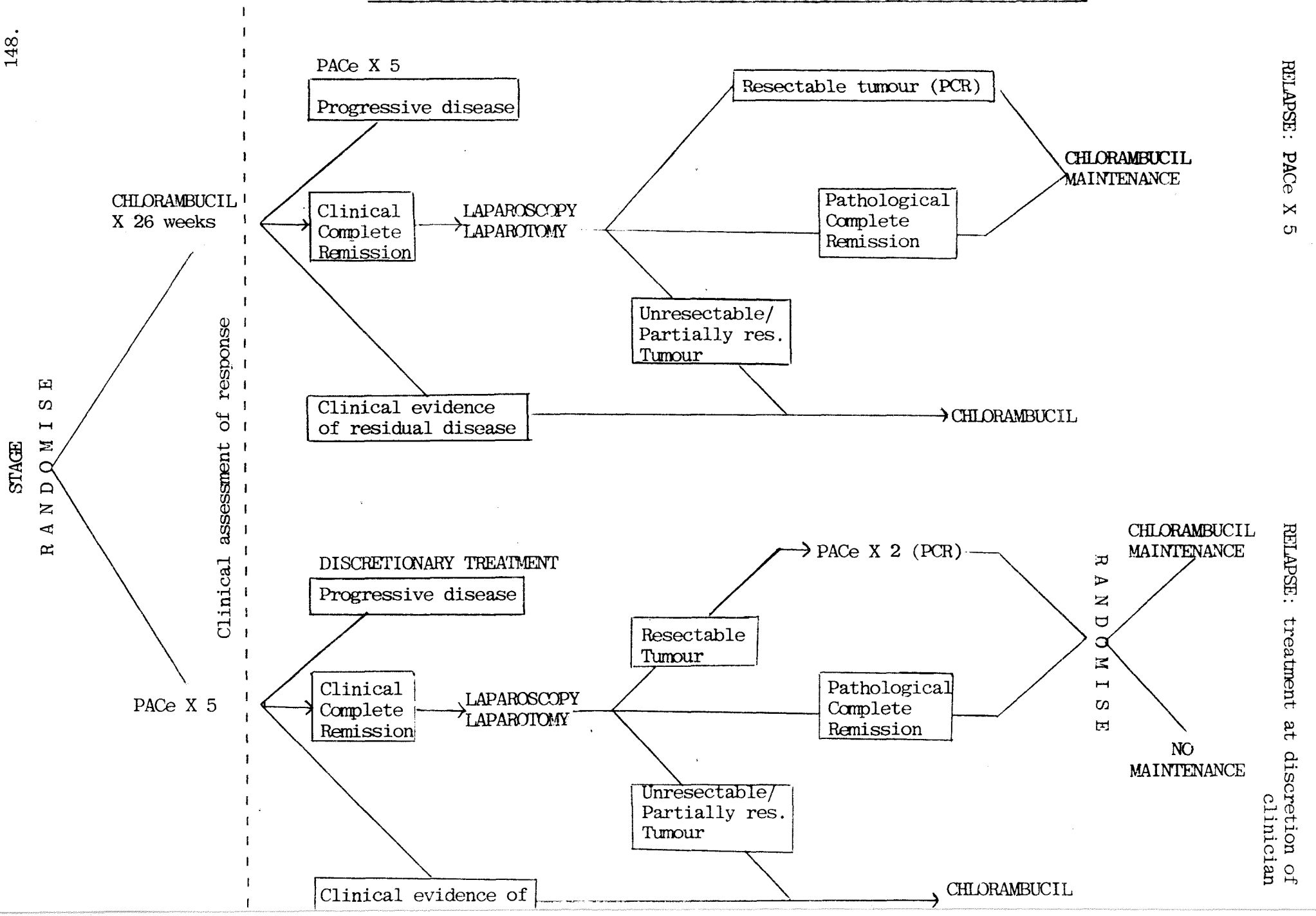
MAINTENANCE THERAPY

APPENDIX I

Initial Assessment on Entry

a) History and physical examination
b) Review histology
c) Review laparotomy findings
d) Full blood count
e) Urea and electrolytes, creatinine clearance, liver function tests.
f) Audiograms
g) ECG
h) Chest X-ray
i) IVP lymphangiogram (optional)
j) CAT abdominal scan or
k) Liver and abdominal ultrasound.
l) Collection of ascites and tumour nodules for in vitro Human ovarian tumour clonogenic assay.

FLOW CHART showing treatment for ovarian tumours Figo Stages 111 & 1V



APPENDIX II

Karnofsky Scale

	10. Normal, no complaints, no evidence of disease.
Able to carry on normal activity: no special care is needed	9. Able to carry on normal activity, minor signs or symptons of disease.
	8. Normal activity with effort, some signs or symptons of disease.
	7. Cares for self, unable to carry on normal activity, or do active work.
Unable to work, able to live at home, cares for most personal needs, a varying amount of assistance is needed	6. Requires occasional assistance, but is able to care for most of his needs.
	5. Requires considerable assistance and frequent medical care.
	4. Disabled, required special care and assistance.
Unable to care for self, required equivalent of institutional or hospital care; disease may be progressing rapidly	3. Severly disabled, hospitalisation is indicated, although death not imminer
	2. Very sick, hospitalisation necessary, active supportive treatment necessary
	1. Moribund, fatal processes progressing rapidly.
	0. Dead.

APPENDIX III
Assessment of Toxicity

	Haematological		Dose reduction
Grade 0	WBC	>3,5000/ul	100% of dose
	Platelets	>150,000/ul	
Grade 1	WBC	3,000 - 3,499/ul	75% of dose
	Platelets	100,000 - 149,999/ul	
Grade 2	WBC	2,500 - 2,999/ul	50% of dose
	Platelets	75,000 - 99,999/ul	
Grade 3	WBC	<2,500/ul	0% of dose - defer 1 week
	Platelets	<75,000/ul	

	Renal		
Grade 0	creatinine clearance	>70 L/24 hr	100% of dose
Grade 1	creatinine clearance	>50 L/24 hr	50% of dose
Grade 2	creatinine clearance	<50 L/24 hr	0% of dose - defer 1 week

Ototoxicity

Grade 0	no demonstrable hearing loss
Grade 1	< 10 Db loss in frequency range, 4,000 - 8,000 H_3 + tinnitus
Grade 2	> 10 Db loss in frequency range, 4,000 - 8,000 H_3 + tinnitus (no clinical hearing loss)
Grade 3	clinically demonstrable hearing loss

Allergy

Anaphylactic reactions with cis DDP have been reported in patients who had received prior cis DDP. although the majority occurred in patients who were receiving other chemotherapeutic agents as well.

Grade 0	no reaction
Grade 1	transient wheezing and tachycardia, no therapy required.
Grade 2	facial oedema, wheezing, tachycardia, hypotension, requiring adrenaline steroids or antihistamines.
Grade 3	anaphylactic shock.

Infections

Grade 0	fever during leucopenia
Grade 1	localised infection, organism isolated
Grade 2	bacteraemia
Grade 3	septicaemia and septic shock

Figure 2

Schema for Chemotherapy

PACe

Cis Diamminedichloro Platinum 80 mg/m^2 i.v. Day 1. Hydration with dextrose/saline 500 ml/hr. is given for at least 2 hours until the uring output exceeds 150 ml/hr. DDP is then given by IV bolus and infusion with N Saline (+ potassium) is continued at 250 ml/hr for at least 6 hr or until vomiting stops.

Adriamycin 40 mg/m^2 i.v. Day 1.

Cyclophosphamide 1 gram/m^2 i.v. Day 1.

Chlorambucil

Chlorambucil 10 mg/day daily with alternate 2 weeks on therapy then 2 weeks off therapy.