IMMUNOSPECIFIC ALBUMIN MICROSPHERES AS A DRUG DELIVERY SYSTEM FOR CISPLATIN AND 5-FLUOROURACIL FOR THE TREATMENT OF OVARIAN ADENOCARCINOMA

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Dedicated to Hanlie, Frances, Ernst and Cecilia

Certification of Supervisor

In terms of paragraph 9 of "General regulations for the degree of Ph D", I as supervisor of the candidate, Ernest John Truter, certify that I approve of the incoporation in this thesis of material that has already been published or submitted for publication.

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Contents

Abstract

Ovarian carcinoma is considered to be the most deadly of the gynaecological malignancies which in its earliest stages is usually asymptomatic. The unsatisfactory survival rates of patients on conventional chemotherapy regimens, necessitates vehicles capable of carrying cytotoxic agents directly to the malignant cells. This mode of targeted delivery allows for efficient tumour cell kill whilst sparing surrounding normal tissue and substantially reducing side-effects.

This project examined the possible therapeutic role of a targetable sustained drug delivery system, albumin immunomicrospheres containing the chemotherapeutic agents, cisplatin and 5-fluorouracil , for the treatment of ovarian adenocarcinoma. A rodent cell line, as a model, has proved to be similar to its human counterpart and also has shown to be transplantable from one animal to another. Such a model could therefore be useful for performing experiments relating to drug delivery targetability and therapeutic trials, as well as survival studies, in cases of ovarian adenocarcinoma.

In particular, this project examines the efficacy of the immunospecific microspheres containing the drugs in a highly concentrated form, administered intraperitoneally and targeted to an ovarian adenocarcinoma, in an attempt to enhance tumour cell kill whilst largely sparing surrounding normal tissue. It is widely recognized that the effectiveness of most chemotherapeutic drugs would be enhanced if they were to act selectively where they are needed. In order to achieve a therapeutically relevant dose in tumour cells, the amount of drug required usually proves also to be highly toxic to normal tissues. It was postulated that, to overcome the above, it may be feasible to develop a sustained immunospecific drug delivery system to optimize the action of cisplatin and 5-fluorouracil at the target site. With the attainment of the above, it was further postulated that higher doses of drugs could be delivered to the target area effecting higher tumour cell kill, that less normal tissue damage should occur and that toxic side effects of the drugs should be reduced. The rationale for selecting combination therapy of cisplatin and 5-fluorouracil is that, although it has been inferred that DNA intrastrand and interstrand cross-links produced by the cisplatin often repair, this repair can be blocked by 5-fluorouracil by inhibition of thymidylate synthetase, thus preventing DNA strand repair.

Albumin immunomicrospheres are relatively innocuous in terms of toxicity, non-antigenic and are capable of accommodating chemotherapeutic agents in a non-specific fashion. We

showed that they were capable of a 0.94% entrapment of 5-fluorouracil and 1.23% cisplatin. Delivery of these drugs at a target site, and at these concentrations, should effect extensive cell kill. As the microspheres are chemically stable and can be manipulated to offload the entrapped drugs satisfactorily, *in vitro* drug release profiles were performed employing immunospecific microspheres directed towards its target cells. Slow degradation of the drug- containing albumin immunomicrospheres showed that 0.283 µg cisplatin/ml plasma and 0.799 µg 5-fluorouracil/ml plasma could be made available at the target site over a 14 day period. These concentrations could be maintained over at least another 14 days and effect tumour cell kill satisfactorily. In order to assess the tumour cell kill, we performed clonogenic assays, cell survival growth curves, MTT cytotoxicity assays and assessed the induction of micronuclei in the tumour cells. The synergism between 5-fluorouracil and cisplatin showed a modulation of cisplatin cytotoxicity and total tumour cell kill was achieved at concentrations of 0.5 µg/ml 5-fluorouracil and 0.1 µg/ml cisplatin at the target site.

The above-mentioned evidence of effective targeting of the drugs was then investigated in female Wistar rats with ovarian adenocarcinoma to assess comparative survival times when treated with free drugs or immunospecific albumin microspheres containing the drugs. Animals given a free drug dose of 5 mg/kg cisplatin and 20mg/kg 5-fluorouracil, followed by a repeat dose at the same concentrations 7 days later showed that only 14% of the animals survived a 90 day trial period. Animals given an intraperitoneal bolus dose of immunomicrospheres at a dose of 10 mg/kg cisplatin and 40 mg/kg 5-fluorouracil showed that 60% of the animals survived the 90 day trial period. This data indicated to us that the survival probability of animals treated with drug-containing immunomicrospheres was substantially superior to other protocols employed in this study.

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Glossary of Abbreviations

5-FU	5-fluorouracil
Ab	antibody
ABTS	2,2' azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid)
BSA	bovine serum albumin
CAS	crystalline ammonium sulphate
CDDP	cisplatin
cm	centimeter
CO_2	carbon dioxide
di-sodium EDTA	di-sodium ethylenediaminetetraacetic acid
DTT	dithiothreitol
FCS	foetal calf serum
FITC	fluorescein-isothiocyanate-conjugated
g	gram
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HT	hypoxanthine
HUCS	human umbilical cord serum
Ig	immunoglobulin
KCl	potassium chloride
kg	kilogram
KH_2PO_4	potassium dihydrogen orthophosphate
kv	kilovolt
l	liter
LM	light microscopy
M	molar
mA	milliamp
mg	milligram
ml	millilitre
mm	millimeter
mM	millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
mV	millivolts
$Na_2HPO_4.2H_2O$	di-sodium hydrogen orthophosphate dihydrate
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate

NaOH	sodium hydroxide
nm	nanometer
OD	optical density
PBS	phosphate-buffered saline
PEG	polyethylene glycol
ppm	parts per million
ppm(l)	parts per million (liquid)
ppm(s)	parts per million (solid)
RP10	RPMI-1640 + 10% FCS
rpm	revolutions per minute
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TEM	transmission electron microscopy
TEMED	N,N,N',N'-Tetramethylethylenediamine
TISAB	Total Ionic Strength Adjustment Buffer
TST	Tris-saline-Tween
μg	microgram
μl	microlitre
μm	micrometer

Chapter 1

Introduction

1.1 General Introduction

Ovarian cancer, although less common than endometrial or cervical cancer, has been shown to have a low survival rate.

While the majority of women with advanced disease live 2 years with a reasonable quality of life, recurrent cancer eventually becomes symptomatic in most, and by 5 years only 35% still survive. Young and colleagues (1990) demonstrated that women with Stage I ovarian cancer may have survival rates as high as 90%, therefore, early diagnosis of ovarian cancer may offer a significant opportunity to improve survival rates in this deadly disease.

The treatment of ovarian cancer often involves one or more treatment modalities such as surgery, chemotherapy, radiotherapy and frequently treatment of recurrent and metastatic disease.

Most patients with ovarian cancer require post-operative chemotherapy. Cisplatin, the cornerstone of most regimens, is usually either given alone, or more commonly, in combination with other agents.Response rates of 60% to 80% are reported, but only approximately 20% to 30% of this treatment group experiences a complete resolution with normalization of clinical, radiological and immunological testing (Jones, 1993).

After extensive surgical cytoreduction, chemotherapy is the treatment of choice for patients with advanced ovarian cancer. A number of new treatments are currently being tested in clinical trials and among them are methods to overcome tumour resistance, the use of dose-intensive regimens and new drugs (Neijt, 1995). The dose of the drugs delivered seems to be important in clinical outcome, but new drugs and/or strategies are needed to improve the outcome in advanced disease.

In the design of chemotherapeutic regimens, all available data must be evaluated to ensure maximization of the therapeutic index of the regimen. While testing of anticancer

drugs in preclinical systems frequently fails to predict activity of a drug for a specific tumour (Schabel *et al.*, 1979), pharmacokinetic parameters from such studies are often much more helpful in defining the optimal dose and rate of infusion of the agent (Jones *et al.*, 1995). In reporting the results of standard-dose treatment trials, many observers have noted the importance of describing the actual delivered dose rather than the protocol-specified dose (Coppin, 1987). Because of the potential morbidity of high-dose therapy, strict adherence to protocol-described doses seems more common than in standard dose trials (Jones *et al.*, 1995).

Basic pharmacological and biological principles imply that the dose of chemotherapy delivered per unit of time is likely to be an important consideration in determining treatment outcome (Levin, 1993). This is implied because maximum cell kill is a function of adequate drug dose (Frei & Canellos, 1980) and the interval between treatments to prevent cell regrowth (Levin, 1993). The importance of dose density (defined as mg/m^2 /time period) in relation to clinical outcome in ovarian cancer has been reviewed by Levin and Hryniuk (1987). This concept has been applied to most chemotherapy regimens in which sequential courses are often administered as close together as drug-induced toxicity allows (Levin, 1993). Although drug dose is unquestionably important in determining the response to chemotherapy, the impact of dose intensity has only recently been analyzed for ovarian tumours (Levin & Hryniuk 1987).

Epithelial ovarian cancer is a highly drug-sensitive tumour. Approximately 60% to 80% of patients with advanced ovarian cancer will have objective responses to treatment with cisplatin-based regimens, and 50% of patients will achieve a complete clinical remission. (Ozols *et al.*, 1993). Unfortunately, a complete remission is not tantamount to a cure, and approximately 30% to 50% of patients will ultimately relapse. Patients who never achieve a complete remission, as well as those patients who relapse, are essentially incurable. Thus, even though ovarian cancer is highly responsive to chemotherapy, most patients are not cured. A multiplicity of pharmacological, biological and biochemical factors are likely to interact to limit the effectiveness of chemotherapy in the disease.

Unfortunately, all chemotherapeutic agents have side-effects. These can range from the production of minimal short-term interference with the patient's quality of life to severe effects which, in some cases, become life-threatening.

Ultimate success in the treatment of ovarian cancer should come when physicians are able to match given parameters concerning the tumour and its host with the most

appropriate options.

It is generally accepted that the principle of combination chemotherapy is normally extended to combined modality treatment for ovarian cancer, in which chemotherapy is used in conjunction with surgery or radiotherapy, or both, in an attempt to eradicate malignant neoplasms. Platinum-based therapy, either alone or in combination with other drugs, is the standard approach in the treatment of ovarian cancer. This is usually administered by intravenous infusion. Employing cisplatin by intraperitoneal infusion has, however, been demonstrated to be superior to the intravenous route, especially when administered together with another drug(s) (Ozols & Vermorken, 1997). Due to the fact that the systemic administration of most chemotherapeutic agents are limited by their resulting toxic side-effects, escalation of dose is confined within a relatively narrow range (Blackledge *et al.*, 1993). However, within the peritoneal cavity, very high concentrations of drugs may be achieved for long periods of time. This could possibly have an advantageous effect in the treatment of disease limited to that area. Drugs that appear to have theoretical advantages when given via the intraperitoneal route, include cisplatin and 5-fluorouracil (Markman, 1987).

For many years there has been a need to regulate and target drugs in the body where they are required, in order to reduce undesirable side-effects whilst still maintaining therapeutic levels of the drug at the target site. During the past 20 years, considerable progress has been made in this regard due to the development of a myriad of drug delivery systems. Many of these systems were tailored to carry all types of drugs including chemotherapeutic agents. One of the popular systems was microspheres containing therapeutic agents.

As mentioned earlier, to increase the efficacy of drugs, a drug delivery system should be designed to increase the amount of drug at the target site, while reducing the effect with non-target cells. Poste and Kirsch (1983) recognised that targeting of a drug should be established at three levels. Not only should the drug localise within the target organ, it should specifically interact with target cells and deliver its payload at therapeutic concentrations to subcellular compartments within these cells. It was also shown by Stella and Himmelstein (1980, 1985) that an improvement in therapeutic index depends, not only on successful site-specific delivery, but also on retention of the drug at the site of action.

The above-mentioned concepts formed the basis of our studies as it was postulated that

the use of particulate drug-containing microspheres, coupled to monoclonal antibodies, is a potential drug delivery system which can be targeted to specific sites or organs in the body. It was argued that an improvement in therapeutic efficacy of antineoplastic drugs such as cisplatin and 5-fluorouracil, by selective targeting to an ovarian adenocarcinoma, would be of clinical importance. It was also thought that the covalent linking of these drugs to albumin may impair functional activity, but can be circumvented by employing drug-loaded albumin microspheres coupled to monoclonal antibodies raised against the tumour. For this purpose, Santos (1996), recently raised two IgM monoclonal antibodies, anti-DMBA43 and anti-DMBA93, against a rodent ovarian adenocarcinoma cell line, DMBA-OC-1R, to couple to cisplatin- and 5-fluorouracil-containing albumin microspheres for the purpose of this study.

1.2 Principal aims of the study

The main aim of this study was to investigate the possible role of monoclonal antibody-conjugated albumin microspheres containing either cisplatin or 5-fluorouracil as a targeted drug delivery system against ovarian adenocarcinoma. In order to do this, we performed the following studies:
(i) we established whether albumin microspheres would entrap cisplatin and 5-fluorouracil in high enough concentrations to have a therapeutic effect on the tumour;
(ii) we determined whether the surface characteristics of the microspheres would allow the coupling of monoclonal antibodies;
(iii) we investigated the cytotoxic effects of cisplatin and 5-fluorouracil in combination in immunomicrospheres on a rodent ovarian adenocarcinoma cell line, DMBA-OC-1R. For this purpose, use was made of clonogenic assays, cell survival growth curves, MTT cytotoxicity assays, as well as assessment of nuclear damage by induction of micronuclei. It was assumed that these *in vitro* assays would give guidance as to our approach for *in vivo* therapeutic trials in an animal model;
(Iv) we thus acquired an animal model in which we could perform therapeutic trials.

In the rodent model we used, we first studied the drug distribution of cisplatin and 5-fluorouracil to the major organs to establish whether sufficient drugs would reach the ovary. The results showed that the disposition of the drugs in the ovary were of sufficient concentration to demonstrate a therapeutic effect at the target site (9,3% for cisplatin and 6,0% for 5-fluorouracil, respectively).

It was also important to establish the toxic side effects of the drugs, therefore

comparative studies employing free drugs and drug-loaded immunomicrospheres, on the blood chemistry and haematology, were performed. Only mild renal failure and hepatocellular toxicity was demonstrated which corrected itself without any observable clinical adverse reactions.

DMBA-OC-1R cells transplanted intraperitoneally into Wistar rats, rendered a transplantable ovarian adenocarcinoma as primary tumour, which was used as the model for evaluation of the drug delivery system. Four groups of tumour-bearing animals were used of which one group was not treated by any drugs. Another group was treated by free drugs and the remaining two groups by drug-containing immunomicrospheres of which the drug concentrations varied. The animal survival endpoints of all four groups were determined to establish the efficacy of the targetable drug delivery system.

CHAPTER 2

Literature Review

Ovarian carcinoma is considered to be the most deadly of the gynaecological malignancies due to the occult nature of this disease. While it is less common than cancer of the endometrium or cervical cancer, ovarian cancer has been shown to have an overall survival rate of only about 35%. It is further estimated that approximately 1 in 70 women, will develop at some stage of their lives, ovarian cancer.

2.1 Aetiology of epithelial ovarian cancer

The aetiology of ovarian cancer is unknown. Except for some relatively uncommon familial tendencies and some rare genetic clustering of breast, colon and ovary cancer, it has not been possible to identify any clinical useful high-risk groups for increased surveillance. Multiple pregnancies and the use of oral contraceptives may, to some extent, be protective because of decreased ovulation and hormonal influences. Whittemore (1994) found that a history of involuntary infertility, low parity, and a long time from menarche to menopause are associated with increased life time risk of ovarian cancer.

A review by Heintz *et al.* (1985) implies several factors which may play a role in the aetiology of ovarian cancer. Amongst these, an environmental factor might be of importance as suggested by the fact that the highest incidence of ovarian cancer occurs in industrialized countries where women with higher education, have higher incomes and different dietary habits. However, it is clear that not everyone is equally susceptible to these environmental factors. This variation in susceptibility may be dependant on genetically controlled factors.

2.2 Invasion and metastasis

It is known that more than 80% of epithelial ovarian cancers have metastasised by the time the disease is diagnosed. Most case studies have shown that tumour cell spread usually occurs over the surface of the peritoneal cavity, although haematogenous and lymphangitic metastases can occur. Many studies in cancer models have shown that tumour cell dissemination is a multistep process that requires loss of adherence to adjacent cells, increased motility, proteolytic degradation of basement membranes,

migration, adherence to vascular endothelium, proliferation, and tumour-induced angiogenesis (Elbendary *et al.*, 1992). Once successfully completed, this process can be repeated in a cyclical manner, resulting in further dissemination of tumour cells.

2.2.1 Intraperitoneal spread of ovarian cancer

The tendency of malignant epithelial ovarian tumours to develop papillary formation, often on the surface, lead to malignant epithelial desquamation and rapid spread over the surface of the peritoneum (Pickel *et al.*, 1992). It is not yet clear at which degree of proliferation and at what tumour size the exfoliated cells implant and grow to metastases on the peritoneum.

As ovarian cancers advance, mestastases are more frequent in the upper abdomen than in the pelvis. Using the universally accepted staging system of the International Federation of Gynaecology and Obstetrics (FIGO), Pickel *et al.* (1992) found metastases on the surface of the liver in only 15.7% of patients with Stage III disease, but in 54.5% of those with Stage IV. Peritoneal tumour deposits were similarly frequent in Stages III and IV, 71.6% and 68.2% respectively.

2.2.2 Retroperitoneal spread of ovarian cancer

Haematogenous dissemination of ovarian cancer is known to occur with very small primary tumours. This explains early extra-abdominal metastases in patients with Stage IV disease. However, lymphatic spread via the retroperitoneal lymphatics is much more common and always proceeds via the efferent lymphatic channels cranially into the regional lymph nodes (Pickel *et al.*, 1992).

Reports of the frequency of lymph node involvement by stage are conflicting, since they are not based on comparable surgical techniques. However, Tulusan *et al.* (1992) have found that the incidence of lymph node metastases is closely related to the surgical stage and extent of the histological examination. Many nodal metastases probably escape detection because enlarged nodes do not always contain tumour, while positive nodes are often macroscopically normal.

2.3 Diagnosis of epithelial ovarian cancer

In its earliest stages, ovarian cancer is usually asymptomatic. In approximately 60% of

cases, widespread intra-abdominal metastases are present by the time the diagnosis is made. Despite improvements in diagnosis and therapy, the long-term survival rate in patients treated for epithelial ovarian cancer is still disappointing. Because of the advanced stage of disease at the time of diagnosis, survival is poor. While the majority of women with advanced disease live 2 years with a reasonable quality of life, recurrent cancer eventually becomes symptomatic in most, and by 5 years only 35% still survive. In view of the significantly better survival with early-stage disease, efforts to develop methods for early detection of ovarian cancer should be emphasised. Young and colleagues (1990) demonstrated that women with Stage I ovarian cancer, after treatment, may have survival rates as high as 90%, therefore, early diagnosis of ovarian cancer may offer a significant opportunity to improve survival rates in this deadly disease. Serum levels of the tumour-associated antigen CA-125 (Zurawski *et al.*, 1990) and other tumour markers, as well as transvaginal sonography (Bourne *et al.*, 1991), have shown promise. However, as Creasman and Di Saia (1991) remark, the relative rarity of ovarian cancer, combined with the non-specific nature of the currently available screening tests, makes ovarian cancer screening unsatisfactory and not cost effective at the present time. Panza *et al.* (1988) showed that CA-125 is ineffectual as a marker in mucinous ovarian tumours, whereas Lloyd (1993) found that the carcinoembryonic antigen (CEA), expressed in the majority of mucinous ovarian tumours, is useful to distinguish between serous and mucinous cancers.

In 1974, Yamanaka and Deamer showed that superoxide dismutase activity in transformed cells is abnormal. The first *in vivo* observations of altered superoxide dismutase activities in malignant neoplasm were reported at nearly the same time by 2 different groups (Dionisi *et al.*, 1975; Sahu *et al.*, 1977). Oberley and Buettner (1979) reported that diminished amounts of manganese - containing superoxide dismutase have been found in all tumours which they have studied. However, Ishikawa and associates (1990) assessed a monoclonal antibody against manganese superoxide dismutase for use as a marker for epithelial ovarian cancer. An ELISA indicated that less than 1% of normal individuals had serum levels over 150 ng per ml of serum, whereas over 50% of patients with epithelial ovarian cancer showed amounts above 150 ng/ml. Because a high level of manganese superoxide dismutase is found in epithelial ovarian carcinoma cases, as compared with normal individuals and patients with non-epithelial ovarian carcinoma and other gynaecological malignancies, these authors suggested that the determination of this enzyme could provide a useful method for the detection and monitoring of responses to treatment in epithelial ovarian carcinomas.

For screening to thus be effective, the screening test must be able to detect cancers at a stage in their development when treatment can improve outcome more than treatment of symptoms. The ideal screening test for ovarian cancer should be able to detect the disease in a premalignant phase and hence provide a method for prevention of invasive disease. Unfortunately, there is as yet no well defined precancerous lesion of the ovary analogous to cervical intra-epithelial neoplasia or atypical endometrial hyperplasia (Jacobs & Oram, 1992).

Current efforts to improve survival rates for ovarian cancer by screening are, therefore, directed towards the detection of early stage disease. The rationale of screening for early stage ovarian cancer is the well documented observation that 5 year survival for this disease is closely correlated with stage at presentation (Kottmeier, 1982).

2.4 The histopathology of malignant ovarian tumours

The great variety of ovarian tumours makes a discussion of each type overwhelming. As mentioned previously, the most common type of malignant tumours is epithelial, accounting for 80% to 90% of the malignant tumours of the ovary. Discussion of other than epithelial tumours of the ovary is not in the interest of this study. For a wider review which includes amongst others, the sex-cord stromal tumours, germ cell tumours and tumours of uncertain histogenesis, see Saigo (1993).

According to the MD Anderson Cancer Centre (1996) there are basically 8 different major histological groups of epithelial ovarian carcinoma:

Histological Types	% of Ovarian Tumours	% Bilateral
Serous	46	73
Mucinous	36	47
Endometrioid	8	33
Clear cell	3	13
Transitional	2	-
Mixed	3	-
Undifferentiated		<2
Unclassified	<1	-

Ovarian carcinomas are histologically similar to those which originate in the uterine cervix, proximal vagina and endometrium (Saigo, 1993). The four most common histological subtypes are serous, mucinous, endometrioid and clear cell carcinomas. The most common of these is serous carcinoma, as compared to the approximately 8%-10% of endometrial carcinomas with a similar histological pattern (Hendrickson *et al.*, 1982). All ovarian tumours can show more than one histological pattern, however, they are generally classified according to the predominant type and are graded according to the degree of differentiation in the primary tumour in the ovary. The most differentiated tumours, deviating little from their benign counterparts, are the carcinomas of low malignant potential or borderline tumours. All other carcinomas are graded on the degree of differentiation: well-differentiated or Grade I, moderately differentiated or Grade 2; and poorly differentiated or Grade 3 carcinomas (Saigo, 1993).

2.5 Overview of the management of ovarian cancer

In general, the treatment of ovarian carcinoma usually involves one or more of the following treatment modalities: surgery, chemotherapy, radiation therapy, "second-look" surgery and the treatment of recurrent and metastatic disease.

2.5.1 Prognostic factors in ovarian cancer

The literature addressing prognostic factors in epithelial ovarian cancer has a number of recurring problems, including poor definition of measurement, missing data, restricted groups, differing endpoints, mixed pretreatment and post-treatment, and univariate analyses. These factors often create problems for the clinician, as prognostic factors essentially predict survival in epithelial ovarian cancer, and successful indices of prognosis usually reflect the growth potential of the tumour. Therefore, once factors are identified in univariate analysis, they must be placed in perspective with other known factors by multivariate analysis (Hakes, 1993).

Prediction of survival in epithelial ovarian cancer seems to remain a controversial subject and some reasons for this controversy have been mentioned. However, a review of a large number of multivariate analyses suggests that the most important traditional factors predicting survival are stage, residual disease, grade, and performance status. These factors overshadow other variables such as histology, mitosis, morphometrics, initial volume of disease, age, ascites, abdominal cytology, type of surgery, and others (Hakes, 1993). Ploidy and DNA index stand out as important

predictors among other variables which include S-phase fraction, oestrogen receptor, progesterone receptor, androgen receptor, CA-125, HER-2/*neu* proto-oncogene, P-glycoprotein, and others which are appearing in the literature at an accelerating pace. With such a variety of factors, along with the rapid appearance of new factors, it is not surprising that the relative importance of these factors is controversial.

2.5.2 The role of surgery in the management of ovarian epithelial cancer

Surgery plays a unique role in the treatment of ovarian cancer. It is generally considered to be the most important facet of therapy for this disease, but only rarely does it produce a cure without the aid of another modality. Because surgery must be combined with other types of therapy, the physician must be aware of the interactions between the various types of treatment.

Surgery for ovarian carcinoma is both diagnostic and therapeutic. If the pelvic mass in question turns out to be ovarian carcinoma, tumour debulking, including total abdominal hysterectomy and bilateral salpingo-oophorectomy, if possible, is usually performed. The therapeutic goal is thus to remove all tumour, if possible, to provide the greatest possibility of cure. Ovarian cancer is surgically staged and samples of tissues from various sites examined histologically for confirmation of the diagnosis, as well as grading of the tumour. Ascites or pelvic and abdominal washings are cytologically evaluated.

Accurate surgical staging is imperative as it provides the basis for the most appropriate post-operative treatment. It is still controversial as to whether extensive surgical resection actually improves 5 to 10-year survival rates. It is agreed however, that optimal tumour debulking (<1.0 cm residual) results in prolonged, good-quality survival (Williams & Hoskins, 1990). According to Hoskins (1994), surgical management of ovarian cancer involves: (a) establishment of diagnosis, (b) staging, (c) primary tumour cytoreduction, (d) interval tumour cytoreduction, (e) secondary tumour cytoreduction, (f) assessment of the status of the disease, and (g) palliation.

Whether diagnosing early cancer in a patient with an adnexal mass or confirming cell type and site of origin in a patient with ascites and carcinomatosis, the definitive diagnosis of ovarian cancer can only be made by surgical exploration (Hoskins, 1994). For patients with disease apparently confined to the ovary or pelvis, full surgical staging is required to confirm proper stage. This operation involves sampling of multiple peritoneal sites in the pelvis and abdomen.

Second-look surgery allows an opportunity to evaluate the effect of primary therapy and to remove any obvious residual tumour. However, until more effective therapy is available, second-look surgery in the asymptomatic patient with a normal physical examination is probably indicated only as a research procedure in patients on specified investigative protocols.

2.5.3 The role of radiotherapy in the management of ovarian epithelial cancer

Numerous reports published over the last two decades indicate that radiation is capable of permanently controlling ovarian cancer, however, its role in the management of ovarian carcinoma remains a controversial subject (Mychalzak & Fuks, 1993). These authors report that the long-term results of radiation therapy have, in general, been disappointing. For patients in early stage disease, 5-year and 10-year survival ranges from 60% to 80%, and for advanced stage patients, 5-year and 10-year survival ranges from only 7% to 20% (Dembo, 1985; Martinez *et al.*, 1985; Weiser *et al.*, 1988). Also, patients with macroscopic residual disease, after platinum chemotherapy, do not benefit from whole-abdomen radiotherapy (Schray *et al.*, 1988) which is thought to be equally as effective as chemotherapy (Sell *et al.,* 1990). The toxicity of radiation therapy, especially gastrointestinal effects, has usually been more severe than that associated with chemotherapy, and, for that reason, most oncologists prefer chemotherapy (Jones, 1993).

Intraperitoneal radioactive colloids have also been used, however, only patients with very early disease are considered to be good candidates for this therapy, which requires complete and uniform intraperitoneal distribution of the radioactive suspension (Jones, 1993).

2.5.4 The role of chemotherapy in the management of ovarian epithelial cancer

Most patients with ovarian cancer require surgery as well as post-operative chemotherapy. In the design of chemotherapeutic regimens, all available data must be evaluated to ensure maximization of the therapeutic index of the regimen. Of particular importance are the pharmacokinetic parameters obtained by the testing of anticancer drugs in preclinical systems. The information obtained is often helpful in defining the optimal dose and rate of infusion of a particular agent to be used (Jones *et al.*, 1995).

2.5.4.1 Chemotherapeutic regimens and epithelial ovarian cancer

It is generally accepted that combination chemotherapy is used in conjunction with surgery or radiotherapy, or both, in an attempt to eradicate malignant ovarian neoplasms.

When selecting agents for combination chemotherapy, it is important to realise that, where a combination requires a reduced dose because of additive toxicity, the advantage of the combination may be neutralized by the disadvantage of dose reduction (Frei, 1995). The effective combinations are those wherein qualitatively different toxicity allows for little or no dose reduction. Under these circumstances, a major additive and sometimes synergistic effect with a cure rate can be achieved (Einhorn & Donohue, 1977; Frei, 1985; Li *et al.*, 1980).

Another crucial issue for the use of agents in combination relates to cross-resistance. If cross-resistance exists among the agents, the effect of the agents might be no greater than that of a single agent (Frei, 1995). It must, however, be expected that at least some degree of cross-resistance, even among unrelated agents, will be frequently found.

The results of several studies have established that platinum-based combination chemotherapy is more effective than single agents or combination of drugs without a platinum analogue in ovarian cancer (Neijt, 1995). Of the platinum analogues, cisplatin is the most extensively studied compound that has clear-cut activity in patients with no prior chemotherapy, as well as in those who have received prior alkylating agents (Thigpen *et al.*, 1983; Wiltshaw & Kroner, 1976). It is usual in combination therapy that one or more of the following groups of drugs is used with cisplatin: alkylating agents, anthracylines, paclitaxel, hexamethylamine, ifosfamide and etoposide, all being effective in the treatment of ovarian cancer (Brandl *et al.*, 1997; Dimopoulos *et al.*, 1997; Dorval *et al.*, 1996; Miglietta *et al.*, 1997; Neijt, 1995; Thigpen, 1993; Veldhuis *et al.*, 1997). In the present study, this concept was employed, using cisplatin in combination with the antimetabolite, 5-fluorouracil.

In a recent study by Hoskins *et al.* (1996), twenty nine women with advanced ovarian carcinoma were treated with a developmental combination chemotherapy regimen consisting of mitomycin C, etoposide, cisplatin and carboplatin (MECCA). Prior to

chemotherapy, abdominal hysterectomy, salpingo-oophorectomy, omentectomy, washings for cytology and nodal and diaphragmatic palpation was the standard primary surgery. The protocol utilized the concepts of dose intensity, front-end loading, non-cross resistance and synergy. The rationale behind the development of the MECCA protocol was: (1) patients with epithelial ovarian cancer survived longer as the dose of the cisplatin increased. The intent was therefore to give as much chemotherapy as possible in as short a time as possible; (2) the Goldie-Coldman hypothesis (Goldie & Coldman, 1979) postulates that chemotherapy resistance is the cause of eventual treatment failure and emphasizes the importance of using non-cross resistance drugs. Previous experience of Hoskins and colleagues was that mitomycin C is occasionally active in platinum-resistant epithelial ovarian cancer and there has also been preliminary evidence of such activity for etoposide and, therefore, these drugs were included; (3) the efficacy of etoposide is known to be schedule-dependent with more protracted courses being more effective and so this was adopted; (4) the work of Skipper (1990) had suggested that the total dose of the drugs used was of paramount importance in improving the survival of patients with incurable cancers. Therefore, carboplatin was added to full-dose cisplatin to increase the total platinum dose without additional neurotoxicity or ototoxicity; (5) the cisplatin and etoposide were given together to make use of the potential synergy between them; (6) front-end loading (i.e. increasing the chemotherapy dose at the first cycle) would theoretically lead to a greater total cell kill because the number of cancer cells is then at its maximum. By decreasing the number of stem cells as quickly as possible, the chance of further development of resistant clones would be diminished.

Although the median overall survival using the MECCA protocol was 37 months with a 27% 5-year survival, the median failure-free survival was 15 months with a 14% 5-year failure-free rate. The predominant toxicity was haematologic and there was one toxic death. Hoskins and colleagues (1996) concluded that although the overall survival results are superior to their previous experience, the failure-free survivals were the same for their controls. They further concluded that the MECCA regimen has not demonstrated its apparent superiority in their randomized study and, at best, may only reflect selection differences and differences in relapse therapy.

The above study, as well as several other clinical trials, have conclusively shown that new strategies need to be generated in the treatment of ovarian cancer. The development of novel therapies has become increasingly multidisciplinary and translational in nature. The natural agents, camptothecins and taxoids, hormonal

therapy, photodynamic therapy, gene therapy, and targeted drug delivery systems are increasingly being investigated for the treatment of ovarian cancer.

The advent of paclitaxel for salvage therapy and, more recently, as a component of first-line treatment in advanced disease, has further improved response rates and prolonged survival (Dunton, 1997). Based on prospective trials by the Gynecologic Oncology Group, paclitaxel/cisplatin has become the new standard regimen in the United States (Ozols & Vermorken, 1997). In addition, it has now been demonstrated that peripheral blood stem cell support permits the administration of multiple cycles of high-dose chemotherapy. However, prospective randomized trials are needed to determine whether such dose intensive treatment is superior to standard-dose chemotherapy (Ozols & Vermorken, 1997).

A large number of combination chemotherapy regimens for various cancer types have been reported, most with higher response rates and toxicity than single agents (Bloss *et al.*, 1991; Forastiere *et al.*, 1992; Jacobs *et al.*, 1992; Kohno *et al.*, 1992). Several of the reported regimens includes combination therapy with cisplatin and 5-fluorouracil, most of them for the treatment of colorectal cancer and squamous cell carcinoma of the head and neck. 5-Fluorouracil (5-FU) and *cis*-diamminedichloroplatinum II (cisplatin), in combination, have been shown to have synergistic cytotoxicity against both murine and human neoplasms (Harstrick *et al.*, 1997; Rooney *et al.*, 1985; Schabel *et al.*, 1979). In all studies performed, higher response rate with combination therapy was found as compared to single agent treatment alone. Despite higher response rates achieved by combination therapy, overall survival did not improve in most cases.

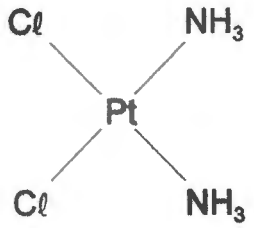
From the above, it is realised that chemotherapeutic drugs such as cisplatin and 5-fluorouracil, have to be given to the limits imposed by toxicity, on a definite schedule, which may have to be tailored around other methods of treatment such as surgery or radiotherapy or the use of sustained drug delivery systems.

The introduction of cisplatin-based chemotherapy has greatly improved the prognosis of patients with ovarian cancer (Van der Hoop *et al.*, 1990). Although complete recovery was very unlikely approximately 15 years ago, as many as 30% of the patients survive for 5 years or longer, despite the presence of tumour in International FIGO Stage III or IV disease at diagnosis (Van der Hoop *et al.*, 1990).

Cisplatin is a platinum co-ordination complex which demonstrates activity against a

variety of tumours, and is used as a chemotherapeutic agent alone or in combination with other antineoplastic drugs for the treatment of several types of genitourinary tumours. Platinum disposition in the body is biphasic, with a relatively rapid distribution phase in minutes and elimination half-lives which vary on average from 16,1 to 53,3 hours, with total doses ranging from 90 to 120 mg when infused intravenously at a rate of 360 to 480 mg/hr (Litterst *et al.*, 1976). Initial concentrations of platinum have been found to be highest in organs of excretion, gonads, spleen and adrenals, but remain significantly elevated only in the kidneys, liver, ovaries and uterus.

Cisplatin is an inorganic complex that contains a platinum atom surrounded in a plane by 2 chlorine atoms and 2 ammonia atoms in the cis-position (Lippard, 1982; Drug Information, 1986).



Neutrality of charge and the cis-configuration are necessary for the cisplatin complex to exert antineoplastic activity. In the relatively high chloride concentration in plasma, the complex is believed to be unionized, allowing passage of the drug through cell membranes. Intracellularly, in the presence of low chloride concentrations, the chloride ligands of the complex are displaced by water, resulting in the formation of positively charged platinum complexes that are toxic and probably act as the active form of the drug. Cisplatin binds to DNA and inhibits DNA synthesis by binding to guanine residues (Butour & Macquet, 1977); protein and RNA synthesis are also inhibited, but less extensively. The drug produces intrastrand and interstrand cross-links in DNA by binding at areas of specific base sequences; DNA-protein cross-links are also formed (Lippard, 1982; Drug Information, 1986).

Also, cisplatin can inhibit methionine uptake into tumour cells and cause perturbation of the methionine pools (Gross & Scanlon, 1986; Scanlon *et al.*, 1983). Cells may respond by increasing methionine biosynthesis and increasing the pools of folate cofactors (Krebs *et al.*, 1976).

It was also reported by Ormerod *et al.* (1994) and Gibb *et al.* (1997) that cisplatin kills ovarian tumour cells by inducing apoptosis and that apoptotic cell death does not require

degradation of DNA into nucleosomal-sized fragments.

5-Fluorouracil is an antimetabolite used alone, mostly for the palliative treatment of carcinoma of the colon, rectum, breast and pancreas that is not amenable to irradiation and/or surgery (Drug Information, 1986). Objective remissions, varying between 6 months to 5 years, have been reported in 10% to 35% of metastatic breast cancer patients who respond to 5-fluorouracil therapy (Drug Information, 1986).

5-Flourouracil *in vivo* is converted to 5-fluoro-2'-deoxyuridine-5' phosphate and 5-fluorouridine triphosphate which are incorporated into the RNA (Scanlon *et al.*, 1986). One or both of these 5-FU metabolites account for the antineoplastic activity of 5-FU in experimental models (Heidelberger *et al.*, 1982). In Drug Information, American Hospital Formulary Service (1986) these antimetabolites act by three different ways:

- it inhibits thymidylate synthetase thus interfering with thymidine production,
- it is incorporated in RNA to produce fraudulent RNA, and
- it inhibits the utilization of preformed uracil, required for RNA production, by blocking uracil phosphatase.

Any one of the above will block and prevent any action of the cell to repair the steady state of thymidylate synthetase and thus DNA synthesis.

Shirasaka *et al.* (1993) is of the opinion that the high response rate of cancer patients to 5-FU plus cisplatin may be due to a reciprocal relationship of the cytotoxic actions of both agents. In this connection, it is noteworthy that Scanlon *et al.* (1983 & 1986) reported that cisplatin inhibits the transport of neutral amino acids, including L-methionine, into L1210 murine leukaemia cells and also induces an elevation of the intracellular levels of reduced folates such as 5,10-methylenetetrahydrofolate and tetrahydrofolate, whereby the increased level of 5,10-methylenetetrahydrofolate results in a 2.5-fold increase in the binding of 5-fluoro-2'deoxyuridine-5'-monophosphate to thymidylate synthase.

Although various combination schedules have been reported as regards the potentiation of antitumour efficacy by the use of 5-fluorouracil plus cisplatin, it is suggested by Shirasaka *et al.* (1993) that the elevation of response rates in cancer patients is based on the mechanism as described in the above paragraph.

The present study is based on the synergistic anti-tumour activity of cisplatin and 5-fluorouracil as described by Shirasaka *et al.* (1993) and Scanlon *et al.* (1983 and 1986).

Another route being exploited in the treatment of ovarian cancer, is by intraperitoneal infusion. Intraperitoneal cisplatin has been shown to be superior to intravenous cisplatin when administered together with another drug(s) (Ozols & Vermorken, 1979).

2.5.4.2 Intraperitoneal chemotherapy

Since the late 1970's, investigators at a number of centres have actively investigated the intraperitoneal administration of cytotoxic agents as therapy for ovarian cancer (Braly *et al.*, 1995; Bruzzone *et al.*, 1997; Dedrick *et al.*, 1978; Markman *et al.*, 1989).

The metastatic spread of ovarian cancer is well defined and in the early stages is primarily intraperitoneal (Blackledge *et al.*, 1993). Intraperitoneal spread is invariably a major factor in the clinical presentation of a woman with ovarian cancer and also usually implicated in problems associated with progressing or relapsing disease. The importance of controlling intraperitoneal disease, therefore, is a major factor in the management of ovarian cancer.

All parts of the peritoneum interconnect with one another and therefore, fluid introduced in one area of the peritoneum should, in the absence of obstructions, reach all parts of the peritoneal surface (Ozols *et al.*, 1997).

Systemic administration of chemotherapeutic agents is limited by the resulting toxicity, and therefore, dose escalation is confined within a relatively narrow range (Blackledge *et al.*, 1993). Within the peritoneal cavity, however, high concentrations of cytotoxic agents may be achieved for long periods of time, which could have an effect on disease limited primarily to that area.

It has been demonstrated that drug uptake from the peritoneal cavity is principally through the portal circulation (Kraft *et al.*, 1968; Lukas *et al.*, 1971). Thus, cytotoxic agents to be rapidly metabolized in the liver during their first passage through the organ, would be expected to demonstrate the greatest pharmacokinetic advantage following regional delivery (Markman, 1993). Drugs such as 5-fluorouracil would fall into this category (Speyer *et al.*, 1980). When choosing agents for intraperitoneal administration, it is important to choose agents with slow clearance from the peritoneal cavity and rapid

clearance from the systemic circulation (Markman, 1993). An additional advantage of intraperitoneal administration, particularly as regards cisplatin, is that concentrations exceeding those required to overcome resistance, may be achievable within the peritoneal cavity following regional drug delivery.

After intraperitoneal therapy, drug uptake from the peritoneal cavity into tumour occurs by the mechanism of free surface diffusion, and it is critical that the drug-containing treatment volume come in direct contact with tumour tissue (Markman, 1993). A major factor defining the clinical situations in which intraperitoneal therapy is a rational therapeutic option, is the known limited penetration of antineoplastic agents directly into tumour or normal tissues (Markman, 1993). Unfortunately, the penetration of drug directly into tissue is quite limited and ranges from several cell layers to 1-3 mm (Los *et al.*, 1989; Nederman & Carlsson, 1984; West *et al.*, 1980; Ozols *et al.*, 1979). Certain agents, including cisplatin, however, enter the systemic compartment in significant concentration following intraperitoneal delivery. Thus, it is not surprising that cisplatin will be found deep within tissue of the peritoneal lining following intraperitoneal delivery because the agent reaches this area through capillary flow. However, near the surface of the peritoneal lining, cytotoxic drug concentrations are increased significantly following regional delivery (Los *et al.*, 1989).

Markman (1993) reported that the side effects associated with regional antineoplastic drug delivery may be systemic or local and occasionally both. For cisplatin, dose-limiting side effects are emesis, neurotoxicity and nephrotoxicity, particularly in the salvage setting, in which the patient may have pre-existing side effects from prior systemic cisplatin delivery. For 5-fluorouracil, the dose limiting side-effects are essentialy haemopoietic toxicity, gastrointestinal toxicity and cardiac toxicity, as reported by Keefe (1993). Drugs can also cause local irritation leading to abdominal pain. Perhaps an additional concern is the establishment of a convenient and safe method of drug delivery, especially when a peritoneal catheter is used as bowel perforation and/or infection may be a risk.

Cisplatin appears to be the drug with the greatest experience with intraperitoneal therapy of ovarian cancer. This drug is associated with limited adhesion formation and minimal or no abdominal pain following regional delivery (Markman, 1993). Markman (1986) further showed that approximately 25%-30% of patients with small-volume disease (largest tumour mass <0.5-1 mm in diameter) can achieve a significant response following intraperitoneal delivery of cisplatin. This finding was confirmed by Berek

(1990),Hacker *et al.* (1987), Howell *et al.* (1982), Markman *et al.* (1991), Pretorius *et al.* (1983) and ten Bokkel Huinink *et al.* (1990). Studies by Alberts *et al.* (1996) showed that cisplatin combined with cyclophosphamide, intraperitoneally administered, is associated with superior patient survival and less hearing loss and myelosuppression in patients with Stage III ovarian cancer.

Based on pharmacokinetic parameters, cisplatin and 5-fluorouracil are examples of drugs that have theoretical advantages when given by intraperitoneal routes (Markman, 1993).

Due to the advantages of intraperitoneal cisplatin delivery described by Ozols and Vermorken (1997), the present study exploited this route in order to, amongst others, reduce systemic toxicity. It was further decided to utilize microparticulate drug carriers as a vehicle to deliver the chemotherapeutic agents to the ovarian tumour cells.

2.6 The role of microparticulate drug carriers in ovarian epithelial cancer

Several other modalities for the treatment of ovarian carcinoma have recently been explored. These include immunotherapy, hormone therapy, cytokine therapy, photodynamic therapy and the use of microparticulate drug delivery systems.

Since the feasibility of artificial cells was first demonstrated by Chang in 1957, an increasing number of approaches in their preparation and application have become available. Artificial cell membranes can now be formed using a variety of synthetic or biological materials to produce desired variations in their permeability, surface properties and blood tissue compatibility. Almost any material can be included within artificial cells, which can then act as drug delivery systems. Since cells are the fundamental units of living organisms, it is not surprising that artificial cells can have a number of possible applications. This is especially so since these cells can be tailor-made to have specialized functions.

Medical science has long recognized the need to control, regulate and target the release of drugs in the body. In general, the aim has been to provide less frequent drug administration, constant and continuous therapeutic levels of drug in the systematic circulation or at a specific target site, and a reduction in undesirable drug side effects. During the past decade, considerable progress has been made in this regard.

Particulate drug delivery systems have received much attention as a means of targeting drugs to specific sites in, for example, cancer chemotherapy. Various particulate systems have been described, including emulsions, liposomes, microcapsules and microspheres, prepared from a variety of materials such as polysaccharides, lipids, alkylcyanoacrylates, polylactic acid and related polymers and proteins. Many of them are tailored to specifically carry chemotherapeutic agents, one of the more popular configurations being that of microspheres.

The basic rationale for controlled drug delivery is to alter the pharmacokinetics and pharmacodynamics of pharmacologically active moieties by using novel delivery systems, or by modifying the molecular structure and/or physiological parameters inherent in a selected route of administration (Li *et al.*, 1985). It is desirable that the duration of drug action become more a design property of a rate-controlled dosage form, and less, or not at all, a property of the drug molecule's inherent kinetic properties. Thus, optimal design of controlled release systems necessitates a thorough understanding of the pharmacokinetics and pharmacodynamics of the drug.

The way in which a drug is distributed within the body, will normally be determined by the intrinsic properties of the drug molecule itself (Illum, 1987). The final amount of drug reaching the target site in the body may normally only be a fraction of the administered dose. Some drugs are able to interact specifically with a few selected cell types due to the presence of relevant receptors present only on those cells. Most other drugs are less specific and will interact with receptors shared by a large number of cell types or will interact with cells by non-receptor mediated mechanisms. Thus, often the therapeutic effect of a drug will not be limited to an action on the target cell, but involve an action on non-target cells as well (Illum, 1987). This may lead to undesirable adverse reactions and in some cases, severe side effects.

In order to increase the efficacy and therapeutic index of drugs, it would be highly desirable to have delivery systems or techniques that can increase the amount of drug reaching the target site, while reducing the interaction with non-target cells. To obtain optimal site specificity, targeting at three levels should be established (Poste & Kirsh, 1983). The drug should not only localise within the target organ, but also interact specifically with target cells and deliver therapeutic concentrations of drug to specified subcellular compartments within the target cells. However, as shown by Stella and Himmelstein (1980, 1985), the retention of the drug at the site of action is also important if successful site specific delivery is to be followed by an improvement in therapeutic

index and clinical effect of the drug.

Various approaches, such as chemical modification of the drug carrier systems, have been considered in an attempt to achieve the goal of site specific delivery (Juliano, 1980). In the present context, the active targeting of colloidal particles refers to a change in the natural distribution pattern of a carrier particle by a deliberate modification of its size or surface characteristics, thereby directing it to specific cells, tissues or organs (Illum, 1987). These modifications include the use of hydrophilic agents to suppress opsonisation and particle adhesion to macrophages, together with cell-specific ligands and particle-monoclonal antibody conjugates.

A way to avoid uptake by the reticuloendothelial system is to change the surface properties of the colloidal carriers. The uptake of particles by the phagocytic cells is known to be determined mainly by the physicochemical characteristics of the particles, especially their size, surface charge and surface affinity (hydrophobicity/hydrophilicity) (Illum & Davis, 1982; Van Oss *et al.*, 1975). Van Oss *et al.* (1975) suggested that hydrophobic particles will be removed from the circulation rapidly, while more hydrophilic particles would be expected to remain in circulation for longer periods of time. It has also been suggested that coating of particles with negative or positive macromolecules can alter organ uptake considerably (Wilkens & Myers, 1966). Roerdink *et al.* (1983) examined whether surface charge can play a role in the phagocytosis of opsonised liposomes and found that the presence of negatively charged lipids within the liposome, profoundly suppressed the uptake by mouse peritoneal macrophages. Many advances in liposome formulation have resulted in a new generation of phospholipid vesicles with improved pharmacokinetic properties (Allen *et al.*, 1989; Allen *et al.*, 1992; Blume & Cevc, 1990; Gabizon & Papahadjopoulos, 1988; Klibanov *et al.*, 1990; Senior *et al.*, 1991; Papahadjopoulos *et al.*, 1991). Several studies by the groups of Illum & Davis (1983, 1984, 1986 and 1987) and Moghimi *et al.* (1991) have demonstrated that non-ionic hydrophilic coating agents, such as poloxamer and poloxamine block co-polymers, are able to dramatically diminish phagocytosis of intravenously injected microspheres in rats and rabbits, mainly due to the steric repulsive effect of the hydrophilic polyoxyethylene segments of the polymers to protein adsorption. The authors suggest that by coating the particles with the named block co-polymers, it has now been made possible to achieve a state of non-recognition by the reticuloendothelial system and a significantly prolonged circulation time. The lasting effect found for these block co-polymers has been attributed to the fact that they consist of a large hydrophobic group, which anchors the polymer to the surface of the microsphere, and large hydrophilic

groups that minimize both the uptake of plasma components (opsonization) and the adhesion between microspheres and macrophages by the so-called repulsion effect. Therefore, one of the major obstacles in drug targeting has now been overcome.

Although the major obstacle of particle phagocytes can now be overcome, an improvement in therapeutic efficacy of drugs by selective targeting to specific sites, would be of clinical importance. One method by which this may be achieved, is by antibody-mediated targeting of the drug (Rowland, 1983). Although drugs may be coupled directly to antibodies, only a small number of functional groups are available per antibody molecule, which can be successfully used without significant loss of antibody activity (Rowland, 1983). This effectively limits the molar drug-to-antibody ratio to 10:1.

To increase this ratio, drugs have been conjugated to carrier molecules such as dextran and human serum albumin (Tsukada *et al.*, 1982). However, the covalent linking of drugs to such complexes may also impair the functional activity of the drug. The use of drug-loaded microspheres coated with monoclonal antibody (immunomicrospheres) may circumvent these problems (Illum & Jones, 1985).

From the above discussion, it can be argued that the use of particulate sterically-stabilized microspheres coupled to monoclonal antibodies can be considered to be a potential system of drug delivery to specific organs in the body. A major aim in chemotherapeutic treatment of ovarian epithelial cancer, is to improve the efficiency of the cytostatic treatment, and the killing of tumour cells will be facilitated by exposing the tumour and its metastases to high concentrations of antineoplastic drugs. However, by conventional administration, it is not possible to use very high doses of drugs due to their toxic side-effects. Most current research employing drug delivery systems, employ the above-mentioned regimen for cancer therapy. The concept of using immunomicrospheres containing chemotherapeutic agents is therefore a feasible alternative approach in the treatment of ovarian epithelial cancer.

When developing an immunomicrosphere system containing substantial concentrations of drugs for targeting the following should be kept in mind. An effective system should:

- be relatively innocuous in terms of toxicity;
- be non-antigenic and also metabolizable within the body;
- possess high drug-entrapment properties;
- be capable of accommodating the selected chemotherapeutic agents;

- possess surface properties which do not lead to recognition as a foreign particle by the reticuloendothelial system;
- be chemically stable in solution;
- be amenable to preparation in large batches, and
- allow the attachment of monoclonal antibodies to its surface.

Human serum albumin immunomicrospheres are considered by many to be a potentially useful vehicle for carrying chemotherapeutic agents and, theoretically, complies satisfactorily with the above-mentioned criteria (Cheng *et al.*, 1993; Hagiwara *et al.*, 1997).

2.6.1 Immunospecific albumin microspheres

There has been considerable interest of late in using protein or polymer based microspheres as drug carriers. In 1972, Evans described methods for preparing heat cross-linked albumin microspheres which are free-flowing and non-agglomerated. Their size could be varied in the range from 0.5 μm to 1 mm and the rate of biodegradation *in vivo* could be adjusted by varying the time and temperature of cross-linking.

Many techniques of albumin microsphere preparation, as well as a myriad of potential uses are available today (Brown *et al.*, 1997; Burgess *et al.*, 1987; Burger *et al.*, 1985; Chen et al., 1994; Gallo *et al.*, 1984; Latha & Jayakrishnan, 1995; Liu & Leong, 1997; Luftensteiner *et al.*, 1997; Longo *et al.*, 1982; Pavenetto *et al.*, 1994; Schäfer *et al.*, 1994; Scheffel *at al.*, 1972; Yapel, 1985). Two basic methods of microsphere preparation have been described. One approach involves coacervation of an albumin solution and subsequent desolvation and hardening of the colloidal particles formed (Oppenheim 1984). A more widely used approach involves a phase separation emulsion technique. Here the albumin in water is emulsified by homogenization or/and sonication into an organic phase (usually cottonseed oil). The polymeric particles are either chemically crosslinked or hardened by heat treatment and the residual organic phase is removed (Gallo *et al.*, 1984). Also, heat-stabilized albumin microspheres are hydrophobic by nature, however, non-ionic surfactants from the poloxamine group are known to adsorb strongly to hydrophobic surfaces, rendering the microspheres hydrophilic. Water soluble drug molecules can be incorporated into the microspheres by including them in the albumin solution. Up to 10% w/w drug can sometimes be trapped in the microspheres in this way (Kramer, 1974; Widder & Senyei, 1983; Widder *et al.*, 1982). In contrast to the case of liposomes, there has been little concern about

the *in vitro* stability properties of albumin microspheres as they can be lyophilized and thus, should be reasonably stable during prolonged storage. Several techniques for the preparation of albumin microspheres containing various water-soluble chemotherapeutic agents have subsequently been developed (Goldberg *et al.*, 1984; Gupta *et al.*, 1986; Gupta *et al.*, 1988; Gupta & Hung, 1990; Kim *et al.*, 1993; Mehta *et al.*, 1988; Poznansky *et al.*, 1982; Tomlinson *et al.*, 1982; Tomlinson *et al.*, 1984). Of particular interest is the study by Nishioka *et al.* (1989) on the preparation of albumin microspheres with 9.2% cisplatin entrapment and lower drug concentrations over time in the peripheral blood when compared to a control group who received free-drug intravenous injections. Significant cisplatin levels were still present in the blood four weeks after administration of the cisplatin microspheres. This was thought to be of benefit as lower cisplatin concentrations in the circulation will reduce the side effects of the drug, while the sustained release of cisplatin over four weeks will maintain the therapeutic index. Another study by Truter (1995) on the preparation of albumin microspheres containing the antimetabolite, 5-fluourouracil, showed a drug entrapment of 1,5% and a sustained release over 30 days. These microspheres were incorporated intraperitoneally in Wistar rats and resulted in only mild toxic effects as shown by haematological and blood chemistry profiles, when compared to intravenous administration of the drug at the same concentrations.

As mentioned earlier, an improvement in therapeutic efficacy of anti-neoplastic drugs by selective targeting to specific sites would be of clinical importance. One method by which this may be achieved, is to conjugate drugs such as cisplatin and 5-fluorouracil, to human serum albumin. However, the covalent linking of these drugs to albumin may impair its functional activity. To circumvent these problems, the use of drug loaded, heat stabilized albumin microspheres coated with monoclonal antibodies, is proposed.

Monoclonal antibodies can be attached to microspheres by means of direct coupling if functional groups capable of covalently bonding with proteins, e.g. aldehyde groups are available on the surface of the microspheres (Illum & Jones, 1985). However, microspheres often carry a variety of groups on the surface such as carboxyl, hydroxyl, and/or amino groups, which either can be linked to monoclonal antibodies employing a coupling reagent, or can be modified to give reactive aldehyde groups. Albumin microspheres contain, due to their nature, functional groups on the surface, e.g. amino groups and carboxyl groups (Illum & Jones, 1985). These authors describe a technique for linking monoclonal antibodies to albumin microspheres by using a water-soluble carbodiimide derivative, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, to activate

carboxyl groups present on the surface of the microspheres, and thereby coupling the amino groups on the antibody molecule to the carboxyl group through an amide linkage (Goodfriend *et al.*, 1964).

2.7 Monoclonal antibodies against ovarian cancer

More than 50 monoclonal antibodies have been developed against ovarian cancer. However, only OC-125 has proved useful in the monitoring of disease as its antigen, CA-125, is expressed in the majority of endometrial adenocarcinomas, a small proportion of mucinous tumours and 80% of serous, clear cell and undifferentiated ovarian tumours (Bast *et al.*, 1990; Finkler *et al.*, 1988; Lloyd, 1993). Recently, Santos (1996) raised two IgM monoclonal antibodies, anti-DMBA-43 and anti-DMBA-93, against a rat ovarian cell line, DMBA-OC-1R, to link to cisplatin- and 5-fluorouracil albumin microspheres for the purpose of the present study.

2.8 Rodent tumour models in experimental chemotherapy

Animal cancer models have been used for many decades in the development of new cancer therapies and there is no doubt that they have contributed to some major breakthroughs. They have, for instance, been important in understanding dose response relationships and developing the concept of combination chemotherapy. However, many drugs that show activity in animal models, are inactive in humans (Martin *et al.*, 1986). This may be because of interspecies differences in anatomy, physiology and metabolism. In addition, transplantable tumours often come from highly selected tissue culture lines which can kill an animal within 2 to 3 weeks, bear little relationship to the original tissue of origin and may be immunogenic for the host. Moreover, tumours often grow at inappropriate sites. The activity of chemotherapeutic drugs may also be overestimated because criteria for anti-tumour activity in some animal experiments, for instance, reduction in the growth rate of tumours or an increase in lifespan, would not be recorded as a response in Phase I/II clinical trials (Balkwill *et al.*, 1990). Screening in animals may also fail to pick up important toxicities for human patients. Thus, while the use of murine tumour models has contributed to the general development of chemotherapy, it has not generally resulted in the development of therapies specific for ovarian cancers.

Rodent tumour models have been the basis for the majority of advances in human cancer biology over the last few decades, from the discovery of new anti-cancer drugs

to understanding the mechanism of action of other modalities to defining the biology of cancer - from oncogenes to studies of resistance, to cellular heterogeneity and to cell-kinetic principles (Valeriote, 1985). Thus, rodent tumours are useful in the study of human cancer to answer fundamental as well as clinically applicable questions on cancer.

2.8.1 Types of tumour models

Rockwell (1987) states that two processes are essential to the performance of successful and interpretable studies with transplantable rodent tumours. Firstly, the tumour/host system must be maintained as a stable biological model that will yield reproducible and meaningful data. Secondly, the techniques used to handle the animals and to treat the tumours must be chosen so as to minimise artefacts and allow meaningful extrapolation to clinical situations.

At present there are only a limited number of tumour models available which resemble human ovarian cancer (Schmäll & Zeller, 1992). A useful and reproducible model is, however, an essential prerequisite for preclinical studies, hence several models have been developed, for example, induction of ovarian tumours in Wistar rats by the administration of 7,12-dimethylbenz (a) anthracene (Kato *et al.*, 1974). This model was characterized *in vitro* and *in vivo* by Imaishi (1992), Kataoka *et al.* (1987) and Sugiyama *et al.* (1986). An autochthonous adenocarcinoma, which histologically indicated a similarity to high grade human serous tumour was raised. This tumour was found to be surgically transplantable serially in Wistar rats with relative stability over a number of transplant generations (Sugiyama *et al.*, 1986) and a transplantability rate of 87% (Imaishi, 1992).

The present study makes use of the above-mentioned cell line, which was supplied to our laboratory with the compliments of Dr Kataoka, Kurume University, Japan.

2.8.2 Maintenance of tumour models

Several techniques are used to maintain experimental rodent tumour lines (Hewitt, 1978; Hewitt & Blake, 1978; McCredie *et al.*, 1971; Rockwell, 1977; Twentyman *et al.*, 1980). Some are maintained by serial transplantation of small pieces of tumour or aliquots of tumour cell suspensions. Some malignant cell lines are maintained by continuous passage in cultures and tumours produced by injecting the cells into animals.

Sometimes tumours are stored frozen in liquid nitrogen, and aliquots of frozen stock can be thawed and inoculated into animals when tumours are required for experiments (Rockwell, 1987).

Each maintenance regimen has advantages and disadvantages. Frozen storage of aliquots of primary tumour is thought to have theoretical advantages over other maintenance regimens when it is desirable to maintain the phenotype of the primary tumour (Hewitt & Blake, 1978), as any form of serial passage will exert selection pressures on the tumour line. However, some selection will occur during freezing, storage, thawing and transplantation. Because of this, even maintenance in liquid nitrogen need not yield experimental tumours identical to the primary (Rockwell, 1987). A major problem encountered with frozen maintenance is the small amount of tumour available. Moreover, if the primary tumour is heterogeneous, different samples may contain subpopulations having different characteristics and therefore may yield tumours with different behaviours (Rockwell, 1987).

Maintenance of tumour cells *in vitro* will select for rapid growth under the culture conditions used for passage. As most cultures are maintained in an atmosphere of 95% air/1-5% $C0_2$, cells will generally be selected for an ability to withstand high oxygen concentrations, which are unphysiologic and may be toxic to unadapted, unselected cells (Richter *et al.*, 1972). Most cultures are initiated with small numbers of cells; this will select cells with an ability to proliferate rapidly without close contact with host cells or other tumour cells. Routine passaging of cultures to confluency will select those cells that experience the least growth inhibition under high density, acidic and nutrient-deprived conditions (Rockwell, 1987). *In vitro* maintenance will also select cells with adhesion characteristics suited to the culture system used for passaging. The selection pressures will be different for cells grown attached to a surface, cells grown in liquid media, cells grown suspended in agar and cells grown as spheroids (Rockwell, 1987). The nature and strength of the selection pressure will depend on the culture protocols.

Maintaining cells *in vitro* will also favour the selection of cells that are able to grow in that particular hormonal milieu. Frequently, conditioned media, feeder layers of radiation-sterilized cells, or red blood cells may be used to supply nutrients and stimulating factors, favouring proliferation of the tumour cells (Rockwell, 1987).
Thus, the media used to culture tumour cells are complex entities, which contain a variety of identified and unidentified factors that affect cell proliferation. Maintenance *in vitro* will select those cells that are best suited to the specific medium chosen to

culture them.

The characteristics of the tumour/host system will reflect changes in the hosts, as well as in the malignant cells. Genetic drift (Bailey & Scott, 1987) in the breeding stock may lead to animals, that are genetically different from the animal in which the tumour arose. Even changes in the microbiological status of the animals as well as changes in the husbandry practices used in the colony, may change the system. Bailey and Scott (1987) are of the opinion that movement of a colony from one institution to another creates special problems. One may inadvertently select nucleus stock not genetically representative of the original breeding colony and the genetic variant may become fixed in the new colony.

Unless the investigator has an unusual degree of control over the operation of the colony, he/she may find that the move results in changes in the breeding management protocols, husbandry practices and microbiological status of the colony, thereby changing the health and physiology of the animals and the behaviour of the tumour/host system (Rockwell, 1987).

2.8.3 Quality control of tumour models

The choice of tumour is critical. Of obvious importance is the appropriateness of the tumour line as a model for the subject being studied (Hewitt, 1978; Kallman, 1968; Kallman & Rockwell, 1977; Rockwell, 1977). One must also consider the genetic and immunological relationships between tumours and hosts.

One of the most serious problems that can occur in the use of transplantable tumour models is the presence, or the development, of genetic incompatibility between the tumour and the host (Corbett & Valeriote, 1987). In experimental chemotherapy, this can be a particularly treacherous occurrence if it goes unrecognized, because the immune system can markedly magnify a small tumour cell kill by a given agent, thereby resulting in a misleading set of experimental data.

Recognition of a developing incompatibility problem between host and tumour is sometimes difficult, unless one has extensive experience with that model and there are suitable quality controls (Corbett & Valeriote, 1987). These authors report the occurrence of genetic drift in their breeding stock which resulted in incompatibility of the host animals with several tumour models. They further report that it can also happen that

a particular subline of a tumour model can become less compatible with its usual host. They recommend the routine return to frozen storage of samples as a guard against change, but only if the material stored, is of proven quality. Changes in chemotherapy responsiveness can also be used as a simple test to evaluate the presence of minor tumour-host incompatibility.

It is well-known that the host immune response to tumours is an important parameter that can affect experiments intended to assess the effectiveness of anti-tumour therapies (Lord, 1987). Although it has often been recommended that non-immunogenic tumour models be chosen in order to avoid potential problems, this is not always possible. Also, although it may be ideal to use tumours of recent origin arising within a strictly inbred colony and to perform all experiments with animals from that same colony, that may also not always be possible.

Tumour models in which only one response endpoint can be assessed, usually tumour growth delay, are clearly of limited value (Siemann, 1987). It is nearly impossible in such models to evaluate mechanisms of action at either the cellular or molecular level. Models in which tumour growth delay, cure, or tumour growth delay and clonogenic cell survival can be evaluated, are more appropriate for evaluating biologically relevant questions, especially if supplemented by *in vitro* tumour cells grown in culture (Siemann, 1987). Such models can investigate mechanisms of action of different treatment modalities and assess the role in the overall tumour response of factors, such as cell-cycle redistribution, repopulation, reoxygenation, repair and drug uptake (McNally & De Ronde, 1980; Rockwell, 1977 and 1980). The ability to address and evaluate such factors using a variety of response assays is the advantage of transplanted tumour models. These tumours are well-characterized and highly reproducible (Siemann, 1987).

Because of the rapid growth of experimental neoplasms, tumours are generally implanted only 2-4 weeks before they are used for experiments (Rockwell, 1980). The tumour-host interactions may, therefore, be different from those of primary animal tumours, which require months or years to reach sizes at which they are detected. The relationships between the malignant cells and the various components of the haematopoietic system involved in specific and non-specific immune responses, may be quite different for tumours 2 weeks after implantation and for tumours which have grown and evolved in a host for months or years (Rockwell, 1980). Interactions between the malignant cells and the vascular and connective tissues may also differ greatly in rapidly

and slowly growing tumours. This might result, for example, in atypical vascular beds and larger hypoxic fractions in rapidly growing tumours. Rockwell (1980) is of the opinion that these limitations are not unique to *in vivo - in vitro* tumours, but are shared by all the rapidly growing tumours used in experimental therapeutics.

Animal survival endpoints can be quantified for solid tumours, but only for those tumours for which the median survival time is 25 days or less (Corbett & Valeriote, 1987). The size of the tumour inoculum must also be selected. According to these authors, the size must be at least one $\log_{10}$ higher than the number of cells required for 100% takes. This usually limits survival studies to solid tumours that grow rapidly and are also highly invasive. Furthermore, suitable implant sites must be selected for a specific tumour to be used. A mistake that is frequently made is the use of a number of cells that is only a small increment above the level that will produce takes in all the animals where quantification of survival time is essentially impossible.
Survival experiments require large numbers of animals. Many implants of tumour tissue are performed subcutaneously, making tumour measurement by calipers easy (Corbett *et al.*, 1984 and 1982; Skipper *et al.*, 1978). With a few simple precautions, tumour measurement endpoints can reliably be converted to cell kill by employing this method (Corbett & Valeriote, 1987). The tumour growth delay is based on the median tumour size of the group of animals inoculated.

Another consideration which should be kept in mind with an experimental chemotherapeutic model, is drug distribution factors. Essentially, there are three major drug distribution factors which must be taken into consideration (Corbett & Valeriote, 1987). The first, pharmacokinetics, deals with the change in activity of the agent as a function of time in the blood, and is related to drug activation and metabolism as well as drug excretion. Also of importance is the partitioning of the drug between the aqueous phase and serum components. The free drug is of major importance and is taken to reflect the concentration that bathes the tumour cells. Generally, this parameter is the one that investigators attempt to correlate with drug effect at a given tissue or cell level. The second factor is the distribution of drug at the level of the tumour cell. This depends upon the vasculature of the tumour and the subpopulations that make up the tumour. The former determines the concentration of the drug at the tumour cell level. The third factor relates to the concentration of drug or its active species within a cell. This can be modified by the environment of the cell, which affects the uptake and metabolism of the drug. Manipulation of the internal biochemistry of the cell itself can greatly influence the degree of sensitivity.

In experimental tumour models, the problem of drug resistance may be encountered. Therefore, care should be taken with both the dose and the schedule of drug exposure in developing resistant cell lines. One may consider to either study human tumour cells directly or at least employ treatment schedules in the model that parallel those used in the clinical setting (Corbett & Valeriote, 1987).

Factors seldom considered to have a direct effect on rodents in a chemotherapeutic model, are the effects of anaesthesia or restraint. Immobilization methods can have a variety of profound effects. Anaesthetics generally depress the respiration rate, decrease the heart rate and lower blood pressure (Pakes *et al.*, 1984). In tumours, anaesthetics may affect cell proliferation patterns (Kuramoto & Takahashi, 1977). Anaesthetics may also alter the response of tumours to chemotherapy (Brown, 1979; Pakes *et al.*, 1984; Peacock & Stevens, 1978). Anaesthetics may alter tumour and host drug sensitivity by a number of mechanisms. Changes in body temperature and blood flow through normal tissues may lead to changes in the pharmacokinetics and biodistribution of drugs (Brown, 1979; Hornsey *et al.*, 1977). Repeated anaesthetization can induce liver microsomes, leading to altered drug metabolism (Pakes *et al.*, 1984). Also, anaesthesia-induced changes in tumour blood flow, tumour oxygenation and tumour temperature may affect tumour cell proliferation, intratumour drug distributions and activation of drugs by tumour cells, thereby changing the antineoplastic effects of the drugs (Rockwell, 1987).

Care must be taken to minimize stress during experiments with laboratory animals. Even routine handling can be stressful, especially for rodents born and reared in a "clean" colony, and never manipulated by hand (Sedlacek & Mason, 1977).

2.8.4 Establishment of a transplantable rat ovarian adenocarcinoma model

In general, experimental ovarian carcinoma is difficult to study, probably due to its anatomical position which may create some difficulties. However, in 1974 Kato and associates successfully induced ovarian carcinoma in Wistar rats by embedding 7,12-dimethylbenz (a) anthracene (DMBA) intra-ovarially by the so-called "clipping" method. Morphologically, this tumour was found to be similar to human ovarian carcinoma, classified as a poorly differentiated adenocarcinoma. This finding was confirmed by Sekiya *et al.* (1979) who reported that adenocarcinoma is found in 39% of induced ovarian tumours when employing the clipping method. Nishida *et al.* (1982) is of the opinion that direct carcinogenesis to the ovarian superficial epithelium is caused by the

DMBA-saturated silk thread passed through it by piercing, rather than that the silk passed through the hilus ovarii, in an ovarian carcinoma model prepared by the same method.

A pure new cell line, DMBA-OC-1, was established from the above-mentioned primary ovarian carcinoma by Kataoka *et al.* (1987) by serial passaging. The authors observed that, although fibroblasts grew vigorously after starting the primary culture, the tumour cells formed colonies by using the fibroblasts as a feeder layer and became predominant at the 10^{th} passage and pure culture after 20 passages. Microscopically, the tumour cells showed a cobble-stone like arrangement and proliferated rapidly. Large nuclei with 1 to 2 prominent nucleoli, were also observed. Electron microsocopy showed well-developed cytoplasmic organellae with intercellular, desmosome-like gap junctions and microvilli on the cell surface. The doubling time of the cells was found to be approximately 32 hours.

Cells, inoculated subcutaneously in nude mice, showed marked tumorigenesis from a week later and died in approximately 6 weeks. Intraperitoneal inoculation led to carcinomatous peritonitis with marked haemo-ascites and metastases to the liver and spleen, and filling the entire abdomen with bloody ascitic fluid causing death in approximately 4 weeks.

Kataoka *et al.* (1987) concluded that the histological features of the DMBA-OC1 cells were very similar to the tumour and the cultured cell line originated from malignant tissue. Although both oestrogen and progesterone receptors were not found in the original tumour, Katabuchi (1983) noted the oestrogen receptor in 80% of DMBA-induced tumours and Kamura (1983) reported the presence of progesterone receptors in 30% of these tumours. These authors reported accelerative effects on proliferation *in vivo* and *in vitro* by means of oestrogen load and progesterone load, respectively. However, they further reported that since the same accelerative effect was noted in receptor-negative cases when loaded with progesterone, the method of measuring the progesterone receptor levels may possibly induce false negative results in comparison with the oestrogen receptor. They therefore recommend that these non-specific and accelerative effects requires further studies.

Sugiyama *et al.* (1986) serially transplanted DMBA-induced tumour fragments (4.0 mm^3) subcutaneously from rat to rat in order to characterize the tumour growth pattern. Following subcutaneous implantation, tumours exhibited two phases of growth: an initial

avascular phase characterized by slow growth, followed by a later phase of rapid growth in which the tumour became vascularised. During the first seven days, all the tumours maintained the same tumour volume as at the time of implantation. Thereafter, they began to grow gradually for about seven days and then grew rapidly. The tumours appeared spheroidal or ellipsoidal in shape and when they reached an approximate volume of 500 mm^3, the central portion of the tumour became necrotic, leaving a peripheral shell of living cells. All the tumour appeared well circumscribed by a capsule of connective tissue with obvious vascular development. Microscopically, the histological features of the transplanted tumours were similar to those of the primary tumours. They appeared less differentiated than the primary tumours and histologically they were classified as undifferentiated adenocarcinomas. In addition, the characteristics of the tumours were relatively stable over a number of transplant generations and no alteration of the histological pattern was observed.

Sugiyama *et al.* (1986) concluded that the morphological observations of the transplantable DMBA-tumour indicates potential value in the testing of anti-tumour agents due to its stability in growth pattern and overall characteristics.

Imaishi (1992) reported on the intraperitoneal implantation of ascitic cancer cells from the DMBA-tumour. He reported that in 87% of implants, the ascitic cells were successfully transplanted intraperitoneally in infant rats. The omentum was the first site at which the metastatic tumour appeared following the inoculation. Then the tumour disseminated throughout the peritoneal cavity and produced bloody ascites containing tumour cells by the 3rd week and caused death of the animals 34 ± 10 days. Histological examination of the omental tumour revealed an adenocarcinoma with solid and glandular structures among scant stroma, indicating a similarity to a high grade human serous tumour.

Imaishi (1992) concluded that the DMBA-model has clear-cut parameters to evaluate the anti-cancer effects of drugs, including changes in the survival period of the host. Thus, using this model, detailed observations on the mechanisms and efficacy of chemotherapeutic agents, as well as new modalities of treatment, should be possible.

It was further reported by Nakata *et al.* (1992) that manganese superoxide dismutase is highly expressed in primary and transplanted ovarian cancers in rats induced by DMBA, as judged by ELISA and Northern blot analysis. The serum levels of manganese superoxide dismutase in tumour-bearing rats were substantially higher than those in

normal control rats. Their data suggest that DMBA-induced ovarian cancer in rats is a good experimental model for human epithelial ovarian cancer, and that manganese superoxide dismutase is a reliable monitoring marker for disease in this type of animal model.

Chapter 3

Aims

This project originated as a continuation of our previous research on the application of human serum albumin microspheres as a sustained drug delivery system for chemotherapeutic agents. We have examined the entrapment capability of human serum albumin microspheres for the cytotoxic agent, cisplatin, as well as for the antimetabolite, 5-fluorouracil. The *in vitro* release behaviour of the microspheres of its payload and subsequent effects of the released drugs on ovarian epithelial cells, as well as the *in vivo* organ distribution of these drugs when administered via the intraperitoneal route in Wistar rats, were also studied.

Earlier studies by others have shown that the intraperitoneal delivery of chemotherapeutic agents in patients with ovarian cancers, is generally associated with drug uptake from the peritoneal cavity into tumour cells by the mechanism of free surface diffusion. Also, systemic administration of chemotherapeutic agents is limited by the resulting toxicity which confines dose escalation within a narrow range. Within the peritoneal cavity, however, high concentrations of drugs may be achieved for long periods of time, particularly if a targeted sustained drug delivery system is employed, which could have an effect on disease limited primarily to that area.

We decided to extend our studies to examine the capability of human serum albumin microspheres coupled to monoclonal antibodies against an ovarian epithelial carcinoma and containing cisplatin and 5-fluorouracil, as a potential drug delivery system for application in the treatment of ovarian epithelial cancer.

We obtained a transplantable rodent ovarian epithelial carcinoma model which was reported to be similar to its human counterpart. We managed to successfully raise two IgM monoclonal antibodies against this particular cell line. These monoclonal antibodies were coupled to drug-containing human serum albumin microspheres by employing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as a coupling agent, rendering immunomicrospheres.

The immunomicrospheres containing cisplatin and 5-fluorouracil respectively, were thought to be useful for *in vitro* release studies and it is postulated that the drugs should be effectively released from the immunomicrospheres after having attached itself to the receptor sites on the tumour cells, which are cultured in RPMI medium in a 5% $C0_2$

atmosphere, and affect cell kill. From our preliminary results it seems that human serum albumin immunomicrospheres containing cisplatin and 5-fluorouracil may be an ideal vehicle to obtain targeted drug delivery, maintaining therapeutic drug levels to cause tumour death, whilst having a reduction in the severe side effects caused by the drugs.

It is also hypothesized that sufficient doses of cisplatin and 5-fluorouracil can be delivered to tumour cells, effecting high tumour cell kill and less normal cell damage as the delivery system will be targeted by the attached monoclonal antibodies. The toxic side effects caused by the drugs should also be reduced. Thus, whilst possibly increasing the efficacy of the drugs, tolerance to the cisplatin and 5-fluorouracil should also be increased.

We further decided to perform *in vivo* studies to evaluate the drug delivery system. Primary transplantable rodent ovarian epithelial tumours are to be raised by intraperitoneal injection of $4x10^6$ DMBA-OC-1R cells in female Wistar rats. Tumour specimens, 5 mm in diameter, are to be aseptically excised after allowing 14 days for tumour development, and transplanted into several groups of female Wistar rats. The secondary tumours will be allowed 14 days to establish growth before treatment commences.

One group of tumour-bearing rats will be left untreated to establish average survival time whilst two other groups will be treated with cisplatin and 5-fluorouracil systemically and with intraperitoneally administered immunomicrospheres containing the drugs, respectively. Tumour growth or shrinkage will be monitored by x-rays using barium sulphate background contrasting to observe the efficacy of the drug delivery system regimen compared to the systemic administration of the drugs. Similar studies have been performed with other drug delivery systems such as liposomes, however, studies using human serum albumin immunomicrospheres as a combination drug therapy regimen with cisplatin and 5-fluorouracil appear to have not been performed to date.

This project may indicate that immunomicrospheres may be an acceptable modality for targetable drug delivery for the treatment of ovarian epithelial carcinoma which may enhance the advantages and usage of cisplatin and 5-fluorouracil combination chemotherapy. This type of delivery system may also reduce or possibly overcome some, if not the majority, of the severe side effects caused by these drugs.

CHAPTER 4

MATERIALS AND METHODS

Except where otherwise mentioned, all analytical reagents were of Analar grade. For making up of reagents, see Appendix.

4.1 Albumin microsphere preparation

The method used and modified was essentially that of Gallo *et al.* (1984) and Truter (1995), as this method complied with the variables which might effect the microsphere size and drug release characteristics. These variables were: albumin concentration, emulsion time, emulsification power, aqueous-to-oil phase volume ratio, stirring rate, emulsion drop rate, temperature of heat-stabilization and method of cooling the microsphere suspension. As a point of interest, Gallo's variables and microsphere sizes are shown in Table 1.

Order of evaluation	Variable	Level	Number of particles counted	Mean particle diameter ($\mu \pm$ SD)
First	Albumin concentration (mg/ml)	500	564	0,44 ± 0,30
Second	Emulsification time (min)	2	647	0,51 ± 0,28
Third	Power of emulsification (W)	125	584	0,54 ± 0,32
Fourth	Aqueous-to-non aqueous phase volume ratio (V/V)	1,0:30	231	0,61 ± 0,45
Fifth	Stirring rate (rpm)	1500	755	0,48 ± 0,29
Sixth	Miscellaneous	Standard*	346	0,58 ± 0,42
Seventh	Heat stabilization temperature (°C)	145	507	0,49 ± 0,37
Eighth	Non-aqueous phase	Cottonseed oil	462	0,56 ± 0,33

*Standard conditions were: emulsion added at 40 ± 10 drops/min, heat stabilized for 10 min and allowed to cool at room temperature

Table 1: Mean diameters and standard deviations for albumin microspheres (Gallo *et al.*, 1984)

4.2 Entrapment of drugs in albumin microspheres

4.2.1 5-Fluorouracil microsphere preparation

Albumin microspheres in the size range 1-5 μm in diameter, containing 5-fluorouracil (5-FU) (Farmitalia Carlo Erba), proved relatively simple to prepare by thermal denaturation at 120°C and by slowing the homogenate drop rate to 30 ± 10 drops/min rather than the normal 40 drops/min at 145°C.

- 170 ml of purified cottonseed oil (Needham Oil, RSA) was heated in a round bottom flask by a thermal mantle to 120°C, constantly being stirred by a propellor blade at 1700 rpm.

- 5 ml of an aqueous 50% human serum albumin (HSA) (Western Province Blood Transfusion Service, RSA) solution and 5 ml of an aqueous 5-FU solution (conc. 50 mg/ml) were added to 50 ml purified cottonseed oil at room temperature. The mixture was homogenized in a vortex mixer followed by sonication in a UMC ultrasonic bath (Instrulab) for 30 minutes at 125 W.

- The homogenate was injected into the heated cottonseed oil through a 16 gauge needle, cooled by an aqueous cooling mantle next to the propellor shaft, using a LKB 2132 microperpex peristaltic pump at a ratio of 30 drops/min. Steam, which developed in the reaction flask, was removed by vacuum.

- After the emulsion was added, heating and stirring was maintained for a further 10 minutes. The heating mantle was then switched off but the stirring continued until the emulsion had cooled to room temperature.

- Stirring was stopped and 200 ml diethyl ether added to the microsphere-cottonseed oil suspension. This was mixed well.

- The mixture was transferred to polypropylene centrifuge tubes and centrifuged at 4000 rpm in a Beckman L8-55 ultracentrifuge for 15 minutes at 10°C. After resuspending the microspheres in diethyl ether it was again centrifuged for 15 minutes at 10°C. This procedure was repeated 4 times to ensure complete removal of the oil. The ether was washed from the microspheres by suspending them in absolute alcohol and centrifuging at 4000 rpm for 15 minutes at 10°C. This procedure was repeated 3 times to ensure complete removal of the ether.

- After collection the microspheres were lyophilized using an Edwards Model VIF-03 freeze-drying apparatus. Upon freeze-drying, the glass sample collecting vials were sealed with Parafilm and stored at 4°C in the dark.

4.2.2 Cisplatin microsphere preparation

Albumin microspheres in the size range 1-5 μm in diameter, containing cisplatin (CDDP) (Farmitalia Carlo Erba), were prepared essentially in the same way as 5-FU microspheres. The CDDP concentration was 0,5 mg/ml H_20 and the cottonseed oil temperature was 100°C in order to allow satisfactory release of the drug as described by Yapel (1985).

4.3 Determination of drug encapsulation efficiency

4.3.1 Determination of 5-fluorouracil entrapment

The method to determine 5-FU concentrations were developed by the Department of Analytical Chemistry, Cape Technikon.

- To determine the entrapment of 5-FU in the HSA matrix, 10 mg of 5-FU microspheres were fused with 10 mg of sodium hydroxide pellets for 2 hours at 500°C. This mixture was dissolved in distilled water (made up to 25 ml), of which 10 ml was mixed with 10 ml Total Ionic Strength Adjustment Buffer (Merck). A calibration curve (Fig. 1) was constructed using standard aqueous sodium fluoride solutions in the following concentrations: 0,5 ppm, 1,0 ppm, 2,0 ppm and 5,0 ppm.

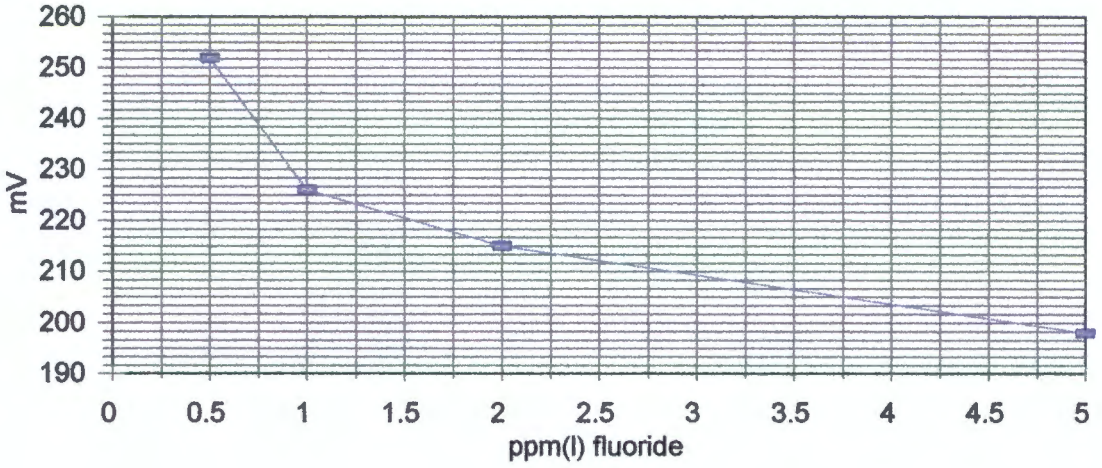


Fig. 1: Calibration curve for fluoride

The fluoride concentration was read with a millivolt meter fitted with a fluoride ion selective electrode (Metrohm Mod. 632). Millivolts were plotted on a graph against the concentration (in ppm) to calculate the fluoride concentration in a sample. The measured fluoride concentration (ppm(ℓ)) was converted to ppm(s) by the following formula:

$$\text{ppm(s)} = \frac{\text{ppm}(\ell) \times \text{ml}}{\text{g}}$$

The 5-FU concentration (ppm(s)) was calculated by the following formula:

$$\text{measured fluoride concentration X } \frac{\text{mol. mass of 5-FU}}{\text{mol. mass of } F^-}$$

= concentration of 5-FU in ppm (s)

= μg 5-FU/g microspheres or converted to mg 5-FU/g microspheres

4.3.2 Determination of cisplatin entrapment

- The method of Morazzoni *et al.* (1995) was used to determine the entrapment of CDDP in the HSA matrix. 10 mg of CDDP microspheres were wet ashed in 2 ml of a 3:1 mixture of 65% nitric acid and 70% perchloric acid in a glass beaker and heated on a hotplate until no nitrous oxide fumes were visible. The mixture was allowed to boil until dry on the hotplate. 3 drops of 1% hydrochloric acid was added to the flask and mixed, before the contents of the flask was transferred to a 100 ml volumetric flask and the volume made to 100 ml with distilled water. The digested sample was analyzed for its platinum content with a Varian Mod. AA1275 atomic absorption spectrophotometer fitted with a graphite tube atomizer (GTA95). 20 μl of sample was injected into the tube with a p20 Gilson Pipetteman automatic pipette.
- The temperature programme was as follows (McGahan & Tyczkowska, 1987):

	Temperature (°C)	Time (s)	Gas flow rate(l/min)	Read command
* Boiling off all volatiles	150	10	3	
	150	10	3	
* Burning the albumin	400	20	3	
	400	10	3	
* Ashing of the carbon	1500	20	3	
	1500	20	3	
* Atomizing the sample and reading Pt levels	2700	1.3	0	√
	2700	2	0	√
* Cleaning the system	3000	3	3	

A calibration curve (Fig. 2) was constructed using Titrisol platinum standard solutions (Merck) in the following concentrations: 0,0 ppm, 0,2 ppm, 0,5 ppm, 0,7 ppm and 1,0 ppm.

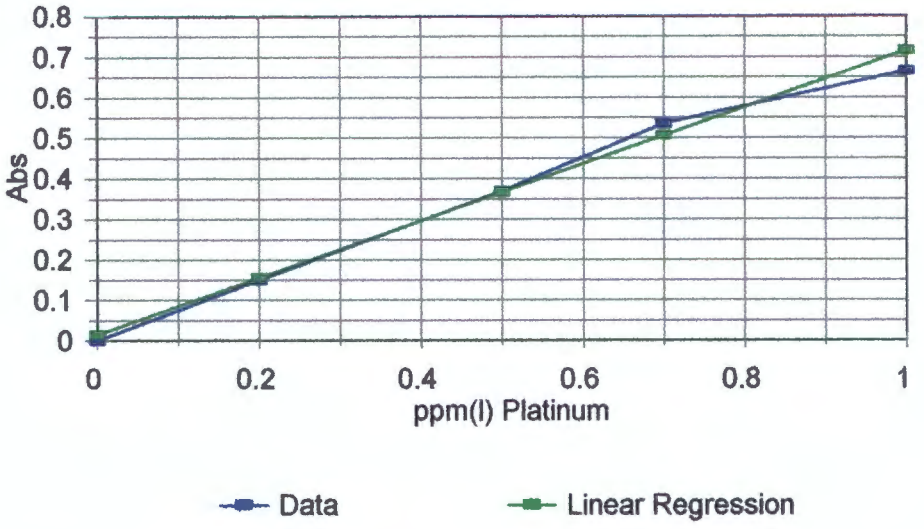


Fig. 2: Calibration curve for platinum

Absorbance was plotted on a graph against the concentration (in ppm). A linear regression was performed on the data to obtain the formula for the straight line (y = mx+c, where y = absorbance and x = concentration in ppm). The platinum concentrations were calculated from the measured absorbance values by using this regression line equation ($x = \frac{y}{m} - c$). The measured platinum concentration (ppm(ℓ)) was converted to ppm(s) by the following formula:

$$\text{ppm(s)} = \frac{\text{ppm}(\ell) \times \text{ml}}{\text{g}}$$

The CDDP concentration [ppm(s)] was calculated by the following formula:

$$\text{measured platinum concentration} \times \frac{\text{mol. mass of CDDP}}{\text{mol. mass of Pt}}$$

= concentration of CDDP in ppm(s)

= μg CDDP/g microspheres or converted to mg CDDP/g microspheres

Note: Steric stabilisation of HSA microspheres.

It is often deemed necessary to change the hydrophobic surface properties of the microspheres to avoid uptake by cells of the reticuloendothelial system, such as peritoneal macrophages (Illum & Davis, 1982; Van Oss *et al.*, 1975). Heat-stabilised albumin microspheres are hydrophobic by nature. However, if treated with a non-ionic surfactant, such as Poloxamine 908 (BASF), to be adsorbed to the hydrophobic surfaces of the microspheres, they can be made hydrophilic, thereby reducing macrophage uptake.

However, it is known that the adsorption of a monoclonal antibody molecule onto the surface of a microsphere may occur via either its antigen binding sites (Fab) or the Fc portion of the molecule. If the surface of the microsphere is hydrophilic, the specific antibodies tend to bind to the surface through the two Fab groups with the Fc portion protruding outwards, thus forming a more hydrophobic outer surface. In contrast, if the surface of a microsphere is hydrophobic, the Fc portions can bind to its surface thereby leaving the Fab binding sites free to bind with the antigenic cells (Illum *et al.*, 1983).

Hence, for the purpose of this project, the albumin microspheres were left untreated with surfactant as to allow for more Fab binding sites to bind with the antigenic tumour cells.

4.4 Monoclonal antibody coupling to albumin microspheres

4.4.1 Monoclonal Antibodies

A monoclonal antibody, specific for the tumour tissue, DMBA-OC-1R, was required for targeted drug delivery employing immunomicrospheres.

Two IgM monoclonal antibodies were raised against the DMBA-OC-1R cells as follows:

Three BALB/c mice were each immunized intraperitoneally with 100μl of versened mycoplasma-free DMBA-OC-1R cells at a concentration of 2×10^7 cells per ml. After 14 days, they were each boosted with 100 μl of DMBA-OC-1R cells at a concentration of 2×10^7 cells/ml. 14 days later, a small sample of blood was drawn from each mouse and tested for the production of antibody against DMBA-OC-1R cells in an indirect ELISA. One mouse was boosted 3 days later, while the other two were boosted 3 months later. Three to four days after the final booster, the mouse was sacrificed, its abdomen sterilised with 70% alcohol and the spleen carefully removed into sterile RPMI-

1640 medium. The hybridomas were prepared by fusing the spleen cells of the mouse with the SP2 myeloma cells as follows (Brown & Ling , 1988): The SP2 cells were versened off two or three 90 mm Petri dishes, washed in RPMI-1640 medium, centrifuged, resuspended in RPMI-1640 medium and counted. The viability of these cells were checked by the Trypan Blue exclusion method. The spleen cells were collected by squeezing the spleen through a sterile stainless steel sieve with the plunger of a 2 ml syringe. After further washing with RPMI-1640 medium, the cells were resuspended in 2 ml of RPMI-1640 medium and the cell number was determined by making a 1:100 dilution of the cells in white cell counting fluid. One tenth the number of SP2 cells was added to the 2 ml of spleen cells in a 50 ml centrifuge tube (Sterilin) and these cells were carefully pelleted by centrifugation at 1000 rpm for 5 min. The supernatant was discarded and the fusion of the plasma membranes of the cells was initiated by adding 1.5 ml of PEG 4000 (Merck) (warmed to 37°C), which permits closer contact of cells by reducing surface tension (Ritter, 1986), dropwise over a time period of 60 to 90 seconds with gentle mixing. A further 1 ml of warm RPMI-1640 medium was added over the next minute and then another 8 ml over 2 to 3 minutes. The tube was gently topped up to 45 ml with RPMI-1640 medium and the cells were centrifuged for 3 minutes at 700-1000 rpm. The pellet was gently resuspended in 50 ml of HAT-HUCS-RPMI-1640 medium. 500 μl of the cell suspension was added to each well of four 24-well plates (Corning). Another 500μl of the HAT-HUCS-RPMI-1640 medium was added to each well. The positive control, indicative of a successful fusion, was provided by SP2 cells grown in HAT-HUCS-RPMI-1640 medium. The basis of this is the SP2 cell's lack of the enzyme, hypoxanthine phosphoribosyl transferase, which is required for the *de novo* synthesis of nucleotides. The SP2 cell is unable to utilise the salvage pathway of nucleotide synthesis when grown in the presence of aminopterin and will therefore die (Kuby, 1992). The clones were fed with another 500 μl of HAT-HUCS-RPMI-1640 medium after 1 week and after another week, their supernatants were screened for antibody production using an ELISA. The positive clones were subjected to further screening by testing the reactivity of the antibody with living, unfixed DMBA-OC-1R, NRK and RESF cells in suspension, as well as with paraffin-embedded, formalin-fixed tissues by immunocytochemistry. The selected clones (anti-DMBA43 and anti-DMBA93) were subcloned twice by limiting dilution in HT-HUCS medium to ensure the monoclonality of the hybridomas. These two hybridomas were then expanded in RPMI-1640 medium with 10% FCS (RP10) and stocks stored frozen in liquid nitrogen.

Anti-DMBA43 and anti-DMBA93 were produced by the DMBA hybridomas grown in culture, either in RPMI-1640 medium with 10% FCS or in serum-free and protein-free

hybridoma medium (Sigma) and were harvested after 10-14 days, aliquoted and stored at -20°C. Anti-J138, an IgM antibody against *Mycoplasma pyrum*, anti-β-galactosidase, an IgG1 antibody, and anti-SM047, an IgM antibody against the SMO47 ovarian carcinoma cell line, were produced by hybridomas established in the laboratory of the Department of Clinical Science and Immunology, University of Cape Town. The antibodies were stored at 4°C in the presence of 0.02% sodium azide (Merck) once they were thawed. The rabbit anti-mouse immunoglobulin class- and subclass-specific antisera (IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA), used in the isotyping of anti-DMBA43 and anti-DMBA93, and rabbit anti-mouse immunoglobulins (Ig) were obtained from Serotec (UK). Fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins, peroxidase-conjugated goat anti-mouse immunoglobulins, biotinylated rabbit anti-mouse immunoglobulins and peroxidase-conjugated strepavidin were obtained from Dako (Denmark). Normal rabbit, goat and rat sera were obtained from the Animal Unit (University of Cape Town, RSA).

The hybridomas were screened (Brown & Ling, 1988) for immunoglobulin production by coating the wells of a Corning 96-well microtiter plate with 100 μl per well of DMBA-OC-1R cells at a concentration of $1x10^5$ cells per ml. The cells were incubated for 72 hours in a humidified 37°C incubator in the presence of 5% $C0_2$. After the removal of the conditioning medium, the cells were air-dried overnight. The cells were fixed with 0.1% glutaraldehyde (Merck) in 0.54 M PBS (pH 7.2) for 5 minutes. The plate was washed by gently submerging it in 0.54 M PBS 6 times after aspirating off the fixative. Any potential binding sites in the wells were then blocked with 200 μl per well of 0.54 M PBS containing 2% skim milk and 100 mM glycine (Merck) for 30 minutes at 37°C. The blocking buffer was discarded and 50 μl supernatant from each hybridoma clone was added to a well and allowed to bind for 90 minutes at room temperature. The mouse serum, diluted 1:1600 in ELISA diluent, provided the positive control, while anti-β-galactosidase served as the negative control. Unbound antibody was removed by washing the plate gently in Tris-saline-Tween (TST) (pH 8.0) 10 times. 100 μl of biotinylated sheep anti-mouse immunoglobulins, diluted to a concentration of 1 μg/ml in ELISA diluent, was added to each well for 60 minutes. The unbound antibody was washed away with two washes of TST. 100 μl of a 1:5000 dilution of peroxidase-conjugated strepavidin was added to each well for 30 minutes. After the last 10 washes with TST, 200 μl of the chromogenic substrate, ABTS, was added per well and the colour allowed to develop for 15-20 minutes. Absorbance was measured at an OD of 405 nm on a Organon Teknika ELISA reader.

As both extracellular and intracellular antigens are recognised in the ELISA, the supernatants of the positive clones were subjected to further screening. This was done to isolate those antibodies which only recognise surface antigens on DMBA-OC-1R cells, and not antigens on normal rat kidney or rat embryo skin fibroblasts. The reactivity of the antibodies was tested by means of immunofluorescence microscopy on living unfixed cells.

Cells were versened or trypsinised off confluent 100 mm Petri dishes, washed in RPMI-1640 medium, pelleted at 1500 rpm for 5 minutes and resuspended in RP10 medium. The cells were counted and diluted to 5×10^6 cells/ml. 50μl of the cell suspension was incubated together with 50 μl of antibody (undiluted harvest fluid) for 30 min on ice to prevent capping. 0.54 M PBS was added to dilute out the antibody and stop the reaction. The cells were then washed twice with 0.54 M PBS and pelleted at 1500 rpm for 5 minutes. The bound antibody was detected by incubating the cell pellet with 50 μl FITC-labelled goat anti-mouse immunoglobulins (diluted 1:4000 in RP10 medium) for 30 minutes on ice. After the cells were washed twice in 0.54 M PBS, the cells were mounted in 10 μl of 30% glycerol in 0.54 M PBS. The coverslips were sealed with nail varnish to prevent the slides from drying out. The slides were examined by fluorescence microscopy (Olympus-BH2 microsocope with blue dichromic filter, blue excitation filter, EY auxilliary excitation filter and 6435 barrier filter) (Johnson, 1989).

In order to select clones that reacted only with DMBA-OC-1R tissue and not with normal rat tissue, the supernatants were tested on normal rat tissue sections.

Normal rat tissues and DMBA-OC-1R tumour tissue from a nude mouse were fixed in formalin, dehydrated, cleared and embedded in paraffin wax, cut into sections 3 μm thick, mounted on albumin-coated glass slides and air-dried. Before the sections were stained, the slides were heated to 60°C to soften the wax and then the sections were deparaffinized in three changes of xylene (4 minutes each) and two changes of 100% ethanol (2 minutes each). The sections were brought to water through graded alcohols (10 dips each in 100% ethanol, 100% ethanol, 95% ethanol, 70% ethanol and distilled water). The sections were washed for 5 minutes in running tap water. Overfixed antigenic sites were revealed by incubating the sections in pre-warmed distilled water at 37°C for 10 minutes, followed by a 5 minute incubation in 0.1% trypsin (Difco) in 0.05 M Tris buffer (pH 7.6) (Isaacson & Wright, 1986). The digestion was stopped by placing the slides in cold running tap water for 5 minutes. After the slides were washed in distilled water for 5 minutes and in two changes of Tris-buffered saline (TBS), pH 7.6,

(5 minutes each), the sections were blocked with 10% normal goat serum in TBS for 30 minutes. The test or control (anti-β-galactosidase) monoclonal antibody (undiluted harvest fluid) was added for one hour after aspirating off the blocking buffer. The sections were washed for 9 minutes in 3 changes of TBS. The sections were incubated with the detecting antibody, FITC-labelled goat anti-mouse immunoglobulins, diluted 1:400 in RPMI-1640 medium with 10% normal rat serum. After washing the slides in 2 changes of TBS for 10 minutes, the cells were counterstained with 1 μg/ml of ethidium bromide (Sigma) and rinsed in water. The sections were mounted in 30% glycerol in PBS and examined by fluorescence microscopy at a wavelength of 495 nm (Van Noorden, 1986).

The specificity of the selected clones was further checked by testing their reactivity with a number of cell lines from various species and organ origins. Immunofluorescence microscopy was therefore performed on fixed cells.

25 μl of a versened cell suspension was added to each well of a Teflon-coated multi-well slide (Veenstra & Dowdle, 1992). The cells were air-dried overnight, fixed in acetone for 5 minutes and then washed in 2 changes of 0.54 M PBS. The slides were flooded with RP10 medium for 15 minutes before the primary antibody (undiluted harvest fluid of either the test antibodies, anti-DMBA43 or anti-DMBA93, or the control antibody, anti-J138) was reacted with the cells for one hour. Unbound antibody was removed by washing in 2 changes of PBS for a period of 10 minutes. The bound antibody was detected by incubation for 30 minutes with FITC-conjugated goat anti-mouse immunoglobulins (Dako) which were diluted 1:400 in RP10 medium. The cells were counterstained for 1 minute with ethidium bromide (1 μg/ml) (Sigma) after they had been washed in 2 changes of PBS. The slides were gently rinsed under running tap water, then mounted in 30% glycerol in PBS and examined with the fluorescence microscope at a wavelength of 495 nm.

The selected clones (anti-DMBA43 and anti-DMBA93) were subcloned twice by limiting dilution to ensure monoclonality of the hybridomas; the hybridomas were expanded in RPM1-1640 medium with 10% Foetal Calf Serum and stocks were frozen in liquid nitrogen. The class of the antibodies was determined by means of an ELISA.

A Greiner ELISA plate was coated with 50 μl per well of a 1:1000 dilution in 0.54 M PBS (pH 7.2) of rabbit anti-mouse immunoglobulin class-and subclass-specific antisera and incubated overnight at 4°C. The plate was washed three times with 0.54 M PBS and

then the wells were blocked for 50 minutes with 200 μl per well of 0.1% BSA (Boehringer Mannheim) in 0.54 M PBS. 50 μl of the harvest fluids containing the test antibodies (i.e. anti-DMBA93 and anti-DMBA43) or the control antibody (anti-SMO47), was added per well for 50 minutes after the blocking solution had been poured off. After washing the plate 3 times with TST, 50 μl of a 1:2000 dilution in blocking solution of peroxidase-conjugated goat anti-mouse immunoglobulins (Dako) was added per well for 30 minutes. The plate was washed 5 times with TST. Colour development was produced by adding 100 μl of ABTS substrate per well. The colour was allowed to develop for 25 minutes and the OD was read at 405 nm on the ELISA reader.

4.4.2 Purification of the monoclonal antibodies

Anti-DMBA43 and anti-DMBA93 antibodies were purified by means of hydroxyapatite chromatography (Harlow & Lane, 1988) from conditioned media of hybridomas grown for 10-14 days in serum-free and protein-free hybridoma medium (Sigma) or in RPM1-1640 medium with 10% Foetal Calf Serum or ascites from Balb/c mice.

The matrix of the column was prepared by hydrating 2 g of biogel HTP (Biorad) in 40 ml of 10 mM sodium phosphate buffer (pH 6.8) and tumbling it for 15 minutes at room temperature. The matrix was allowed to settle for 15 minutes, the supernatant was removed and the matrix was washed by tumbling with another 40 ml of 10 mM sodium phosphate buffer. After the matrix was washed for a third time, it was transferred to a 5 ml syringe column with a glass-sintered filter disc at its base. The matrix settled overnight and it was then washed with 5 column volumes (50 ml) of 10 mM sodium phosphate buffer. The harvest fluid or ascites was centrifuged at 10 000 rpm and loaded on the column. The ascites was diluted 1:10 with distilled water prior to centrifugation. The column was washed with 10 column volumes (100 ml) of 10 mM sodium phosphate buffer (pH 6.8). The antibodies were eluted by raising the concentration of the sodium phosphate buffer (pH 6.8). 5 ml of each of the following concentrations were used: 50 mM, 100 mM, 200 mM and 300 mM and 1 ml fractions were collected. The column was washed with 10 column volumes of 10 mM sodium phosphate buffer and stored. The absorbance of the fractions were determined at an OD of 280 nm on a Unicam SP1800 UV spectrophotometer to establish the peaks. These fractions containing the peaks were then electrophoresed on SDS-PAGE gels to determine the location and purity of the antibodies.

The proteins were subjected to one-dimensional denaturing gel electrophoresis on 11%

sodium dodecylsulphate (SDS)-polyacrylamide gels according to the method of Laemelli (Coligan *et al.*, 1995). The analysis was performed under reduced conditions. The protein samples to be analysed were diluted with an equal volume of double strength SDS/sample buffer containing the reducing agent, dithiothreitol (DTT) (Sigma). The final concentration of DTT in the sample/sample buffer mixture was 10 mg/ml. The samples were boiled for 2 minutes and 12 μl of the sample was loaded per well on a 15 cm x 9 cm gel. High and low molecular weight markers (BDH Electran) were also boiled for 2 minutes prior to loading. Electrophoresis was performed at room temperature. Initially a constant current of 10 mA was applied to the gel and then, once the bromophenol blue tracking dye had entered the separating gel, it was increased to 20 mA. After electrophoresing the samples, the gel was stained with 0.1% PAGE Blue stain for 1 hour and then destained with a 30% methanol: 10% acetic acid:60% distilled water mixture.

The protein concentration was determined by means of the BCA protein test (Pierce)as follows:
The working reagent (Pierce) was prepared by mixing 50 parts of Reagent A to 1 part of Reagent B. The standard curve was prepared by diluting the Bovine Serum Albumin (BSA) provided in the kit, in the same diluent as the sample to the following concentrations: 1.2 mg/ml, 1.0 mg/ml, 0.8 mg/ml, 0.6 mg/ml, 0.4 mg/ml and 0.2 mg/ml. 10 μl of the standard and samples were added to 200 μl of the working reagent in the wells of a microtiter plate. The plate was incubated at 37°C for 30 minutes and the OD was read at 405 nm on the ELISA reader. The concentrations were calculated by a programme for protein determinations in the ELISA reader which utilises the parameters obtained by fitting the linear regression curve to the standard curve.

Antibodies purified from ascites were found to be heavily contaminated with serum albumin and other proteins. An attempt was made to reduce excess protein contamination by precipitating the antibody with ammonium sulphate (Coligan *et. al.*, 1995; Harlow & Lane, 1988).

A predetermined volume of antibody was transferred to a beaker on a magnetic stirrer and stirred gently with a magnetic stirring bar. Ammonium sulphate crystals were added slowly to give a final concentration of 50% saturated ammonium sulphate solution so that the mouse antibodies would precipitate. The mass of crystalline ammonium sulphate (CAS) was calculated as follows:

$$CAS\ (g) = (0.5 \times \text{volume of antibody}) \times 76/100$$

Saturated ammonium sulphate = 760 g/l

The beaker was placed at 4°C overnight and the precipitate was microfuged at 10 000 rpm for 30 minutes. The supernatant was discarded and the pellet resuspended in a 0.1 to 0.3 of the original starting volume of the antibody in 0.54 M PBS (pH 7.2). The antibody solution was transferred to dialysis tubing or to an Eppendorf tube for dialysis. Dialysis tubing was placed over the mouth of the Eppendorf tube and secured with the lid of the tube in which a large hole had been made to allow dialysis. Dialysis took place overnight versus three changes of 0.54 M PBS. The antibody solution was microfuged to remove any remaining debris and subjected to SDS-polyacrylamide gel electrophoresis in order to check the purity. The concentration of the antibody solution was determined by the BCA protein test.

4.4.3 Attachment of monoclonal antibodies to human serum albumin microspheres

The carbodiimide method of Illum and Jones (1985) was employed to attach the monoclonal antibodies. By this method, a water-soluble carbodiimide derivative is used to activate carboxyl groups present on the surface of the microspheres and thereby couple the amino group on the antibody molecule to the carboxyl group through an amide linkage. Alternatively, an amino group on the microsphere can be coupled to a carboxylic acid group on the antibody via this method.

When microspheres do not carry groups which can link directly to the monoclonal antibodies, or when it is uncertain which groups they carry, it is possible to obtain antibody attachment by means of the carbodiimide method. The antibodies were dialysed versus 3 changes of 0,1 M sodium chloride solution at pH 7,0 at 4°C, prior to use. 0,5 mg/ml monoclonal antibodies (anti-DMBA43 and anti-DMBA93 at a 1:1 ratio) was added to each 50 mg of HSA microspheres. 10 mg of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (Sigma) was added and the coupling reaction of antibodies to microspheres allowed to occur for 2 hours at 4°C. The coupling reaction was then terminated by the addition of 0,03 g glycine for 2 hours at 4°C. The immunomicrospheres were then washed 3 times in PBS (pH 7.2) for 5 minutes with centrifuging between washings at 3000 rpm for 10 minutes to remove the glycine and uncoupled antibodies. The supernatant was discarded after the final spin and the immunomicrospheres lyophilized and stored dry at 4°C.

4.5 Drug release from the immunomicrospheres

In order to assess the availability of drug released from the immunomicrospheres, two separate experiments were conducted in which 150 mg 5-FU-loaded

immunomicrospheres and 150 mg CDDP-loaded immunomicrospheres were incubated at 37°C in 3 ml fresh, sterile plasma. Leakage of 5-FU and/or its metabolites and CDDP out of the immunomicrospheres were monitored at the first and every second day thereafter for a 14 day period.

For 5-FU assays, plasma was sampled and 150μl supernatant added to 450 μl distilled water and filtered through a Millipore filter (pore size = 0,22μm). 200 μl of the filtrate was fused with 20 mg sodium hydroxide pellets for 2 hours at 500°C before dissolving in 25 ml distilled water of which 10 ml was mixed with 10 ml Total Ionic Strength Adjustment Buffer (Merck). The fluoride concentration was read with a pH meter having an ion selective fluoride electrode (Metrohm Mod. 632). The total 5-FU concentration was calculated as in Section 4.3.1.

For CDDP assays, plasma was sampled and 150 μl supernatant added to 450μl distilled water and filtered through a Millipore filter (pore size = 0,22 μm). 20 μl of the filtrate was analyzed directly in a Varian Mod. AA1275 atomic absorption spectrophotometer fitted with a graphite tube atomizer (GTA95). The sample was injected into the tube with a p20 Gilson Pipetteman automatic pipette. The CDDP concentration was calculated as in Section 4.3.2.

4.6 Morphology of the immunomicrospheres

Using a Nikon Ultiphot light microscope, several fields of unstained immunomicrospheres were viewed. Eyepiece and stage micrometers were used to determine the size of the microspheres, as it was decided that they should be significantly smaller than the target cells as to possibly attach several microspheres to the cells and thus delivering higher concentrations of drugs.

Samples for scanning electron microscopy were placed on aluminium specimen stubs and dried in an oven at 37°C overnight. Coating with 30 nm of gold was performed, using a Balzers sputter coater. The samples were then examined and sized in a Cambridge Stereoscan S 180 M scanning electron microscope at an accelerating voltage of 25 KV at various magnifications using latex spheres (0,5 μm; Polaron) as controls for sizing (750 microspheres per batch were examined).

4.7 Maintenance of the cell lines

The rat ovarian adenocarcinoma cell line, DMBA-OC-1R, was a gift from Dr Akio Kataoka (Department of Obstetrics and Gynaecology, Kurume University, Kurume, Japan) and was established from an ovarian adenocarcinoma induced in rats by the carcinogen, 7,12-dimethylbenz(a)anthracene (Imaishi, 1992; Kataoka *et al.*, 1987; Sugiyama *et al.*, 1986). The cells were passaged by splitting them in a ratio of 1:10.

Normal rat kidney cells were obtained from Dr J de Larco of the National Institute of Health (USA). Rat embryo skin fibroblasts (RESF) and six other cells lines were established in the Department of Clinical Science and Immunology, University of Cape Town (RSA). Goliath, Kerchoff and Seafield cell lines were established from human ovarian carcinomas and Small, from a human melanoma. The B-Ov2 cell line was established from an ovarian metastatic deposit that had been excised from a nude mouse which had been inoculated subcutaneously with the human Bowes melanoma cell line. Rogina (UCT-Br 1) cell line was established from a human breast carcinoma. UW-OV-1, a human ovarian serous cystadenocarcinoma, was obtained from the University of the Witwatersrand Medical School. The following cell lines were obtained from the American Tissue Type Culture Collection (ATCC): MTW9/PL (a rat mammary gland carcinoma), SP2 (a mouse myeloma), SK-OV-3 (a human ovarian carcinoma), GH3 (a rat pituitary tumour), MCF7 and MDA-231 (human breast adenocarcinomas), T-47D (a human breast carcinoma) and K562 (a chronic myelogenous leukemic cell line).

The cell lines were maintained under sterile conditions in RPMI-1640 medium (Sigma) supplemented with 10% foetal calf serum (FCS) (State Vaccine, RSA), penicillin (Intramed, RSA) and streptomycin sulphate (Merck) at 37°C in a humidified 5% $C0_2$/95% air incubator (Hotpack). They were fed with medium and passaged twice weekly with versene or trypsin. Stocks were stored frozen in liquid nitrogen in RPMI-1640 medium with 10% FCS and 10% dimethyl sulphoxide (DMSO) (Freshney, 1983).

For use, a vial of cells was removed from the liquid nitrogen and the cells thawed immediately in a 37°C waterbath. Following thawing, the cells were quickly transferred to a centrifuge tube containing 10 ml RP10 medium to dilute out the DMSO. The cells were centrifuged at 1500 rpm for 5 minutes. The cell pellet was resuspended in 1 ml of RP10 medium and the cells transferred to a 60 mm Petri dish (Corning) and incubated at 37°C in a humidified 5% $C0_2$/95% air incubator (Hotpack).

Cells were routinely checked for mycoplasma infection (Freshney, 1983). Mycoplasma was detected by means of immunocytochemistry on multi-well test slides coated with DMBA-OC-1R cell monolayers (Veenstra & Dowdle, 1992). 25 μl of a cell suspension was added to each well of a Teflon-coated multi-well test slide (Highveld Biologicals, RSA) and the slide was left to air dry. The cells were fixed by flooding the slide with Carnoy's fixative (1 part acetic acid : 3 parts methanol) for 5 minutes. The fixative was removed using a Pasteur pipette and suction. After more fixative was added for a further 10 minutes, it was poured off and the slide was air-dried for 30 minutes. The slide was flooded with Hoechst 33258 stain which was either diluted 1:400 in 0.54 M phosphate-buffered saline (PBS) (pH 7.2) if fresh, or undiluted if old. It was left for 10-30 minutes in the dark. The stain was removed by suction and the slide washed in a Coplin jar with distilled water three times (1 minute per wash). The slide was drained, air-dried and mounted in 30% glycerol (Merck) in PBS. The nucleus fluoresced as expected when observed under a fluorescent microscope (Olympus BH-2). The appearance of granules of fluorescence in the cytoplasm indicated that the cells were contaminated with mycoplasma.

Mycoplasma-infected cells were treated as follows:
Human serum containing active complement was prepared from fresh blood by allowing the blood to clot for 45 min at 4°C and centrifuging the sample to remove the clot. The serum was sterile-filtered and stored at -80°C until required. The adherent monolayer of infected cells was rinsed three times with RPMI-1640 medium before the cells were versened off the dish. The cells were pelleted at 1500 rpm and washed once again with RPMI-1640 medium before they were resuspended in RPMI-1640 medium containing 10% human serum complement and incubated in a tube at 37°C in a 5% $C0_2$ humidified incubator. After 2 hours, the medium was changed and the cells were incubated for another 2 hours with RPMI-1640 medium containing 10% human serum complement. The cells were pelleted and incubated overnight with RPMI-1640 medium containing human serum complement in a Petri dish (Corning). An aliquot of treated cells was tested for mycoplasma after they had been washed three times with RPMI-1640 medium, versened off the dish, pelleted and resuspended in RP10 medium (RPMI-1640+10%FCS).

4.8 *In Vitro* Studies:

4.8.1 Drug treatment of DMBA-OC-1R cells for use in clonogenic assays and cell survival growth curves

The cytotoxic effects of either CDDP and 5-FU, either administered on their own or in combination, in either the free form or encapsulated in albumin microspheres, on DMBA-OC-1R cells were studied. The long term effects of the drug were evaluated by either studying the ability of cells to form clones in soft agar after treatment or by establishing cell survival growth curves *in vitro*. 1 x 10^5 DMBA-OC-1R cells were seeded in 2 ml of RP10 medium in 35 mm Petri dishes and allowed to adhere overnight in a 37°C humidified $C0_2$ incubator before exposing the cells to drugs. The cells were incubated for varying periods of time, ranging from 1 hour to 96 hours, with either free drug or encapsulated drug. Control samples consisted of cells that had not been exposed to drugs. The concentrations of CDDP drug administered varied from 0.01 μg/ml to 1 μg/ml in the free form and from 5 μg/ml to 25 μg/ml in the encapsulated form while that of 5-FU varied from 0.1 μg/ml to 1 μg/ml for the free form and from 5 μg/ml to 25 μg/ml when cells were treated with microspheres. After drug exposure, the cells were rinsed with RPMI-1640 medium and then detached with versene, centrifuged and the cell pellet washed 3 times with RPMI-1640 medium. After the final wash, the pellet was resuspended in 2 ml of RP10 medium and the cell number and viability was determined.

4.8.1.1 Clonogenic assay

After treatment, the cell number was adjusted to 2.5 x 10^3 cells per ml of RP10 medium. The cell/agar layer was prepared as follows: 10 ml of molten 1.32% agar (Bacto Difco agar from Difco) was mixed with 8 ml 2X RPMI-1640 medium and 2 ml FCS. This mixture was kept at 42°C until required. 1 ml of cells was mixed with 1 ml of the agar mixture. 0.5 ml of the cell/agar mixture was seeded on top of a feeder layer of 0.5 ml 0.5% agar in the wells of a 24-well plate (Corning) (i.e. 625 cells were seeded). The feeder layer of agar was prepared as follows: 10 ml of molten 1% agar was mixed with 8 ml 2X RPMI-1640 medium and 2 ml FCS. 0.5 ml of the agar (0.5%) was added to each well of a 24-well plate and allowed to set before the cell/agar layer was added. Each dosage sample was set up in triplicate. The plates were incubated for 14 days in a humidified 37°C incubator containing 5% $C0_2$. The colonies were fixed with 250 μl 3% glutaraldehyde in 0.54 M PBS and scored.

4.8.1.2 Cell survival growth curves

Cell survival growth curves were generated in order to study the recovery of cells after drug treatment. The cell number was adjusted to 1.25 x 10^4 cells per ml of RP10 medium. 2 ml of cells (2.5x10^4 cells) in RP10 medium was plated onto 35 mm Petri dishes (Corning). 10 Petri dishes per drug dosage was set up. Cell number was assessed on a daily basis over a time interval of 168 hours. 2 Petri dishes per drug dosage per time point were counted. The viability of the cells was also determined using trypan blue (Sigma). At each time point the remaining Petri dishes of cells that were still to be counted, were fed by removing the old culture medium and adding 2 ml of fresh RP10 medium.

4.8.2 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cytotoxicity assay

The *in vitro* cytotoxicity of CDDP and 5-fU, either alone or in combination, as well as in either the free drug or encapsulated form, was evaluated by using the MTT assay. This assay was used to assess the viabilty of cells after continuous exposure of the cells to drugs or to study the proliferation of cells after exposure to drugs (i.e. the long term effect).

The survival of cells after continuous exposure to drug was evaluated as follows: A single cell suspension of DMBA-OC-1R cells was obtained and the cell number adjusted to 5 x 10^4 cells per ml of RP10 medium. 100 μl of cells (5x10^3) were aliquoted into the wells of flat-bottom 96-well plates (one set of plates per time point). After allowing the cells to adhere overnight, the culture medium was removed and 200 μl of RP10 medium containing the appropriate amount of drug was added to each well. Cells were exposed to concentrations of 'free' CDDP ranging from 0.01 μg/ml to 1 μg/ml and 'free' 5-Fu ranging from 0.1 μg/ml to 1 μg/ml. The cells were also exposed to CDDP and 5-FU encapsulated in albumin microspheres in concentrations ranging from 5 μg/ml to 25 μg/ml. The control population was provided by cells that had not been exposed to drugs. Each dosage sample was set up in triplicate. The plates were incubated for varying lengths of time ranging from 24 to 120 hours and the surviving cells assayed for reduction of MTT.

The long term effect of the drugs after exposure was evaluated as follows:
1 x 10^5 DMBA-OC-1R cells were seeded per 35 mm Petri dish (Corning) containing 2

ml of RP10 medium. After allowing the cells to adhere overnight, the cells were exposed to 'free' drugs for 24 hours. The final concentration of CDDP in 2 ml of RP10 medium ranged from 0.01 μg/ml to 1 μg/ml and that of 5-FU from 0.1 μg/ml to 1 μg/ml. Cells were also exposed to concentrations of encapsulated CDDP or 5-FU ranging from 5 μg/ml to 25 μg/ml for 96 hours. The control consisted of cells that had not been exposed to drug. After drug exposure, the cells were rinsed with RPMI-1640 medium and then detached from the surface of the dishes with versene. They were washed three times with RPMI-1640 medium to remove any traces of drug. After the final wash, the cell pellet was resuspended in RP10 medium and then the cell number and viability was determined. The cell number was adjusted to 5×10^4 cells per ml. 5×10^3 cells in a 100 μl of RP10 medium were seeded in the wells of flat-bottom 96-well plates (Corning). The proliferation of drug-treated cells was assayed on a daily basis over a 168 hour interval using the MTT assay. One plate was set up for each time point. Each sample was set up in triplicate per time point.

A stock solution of 1 mg/ml MTT (Sigma) was prepared in phenol red-free RPMI medium (Sigma R-7509) and stored in the dark at 4°C for two weeks or frozen at -20°C. Four hours before the end of the appropriate incubation time at 37°C in a humidified 5% CO_2 incubator, the culture medium was removed by aspiration, 100 μl of MTT (1 mg/ml) was added to each well and the plate returned to the incubator to complete the incubation time. Untransformed MTT was removed by careful aspiration so as not to disturb the blue formazan crystals. 100 μl DMSO (Merck) was added to each well to dissolve the formazan crystals and the absorbance was measured at 540 nm on the ELISA reader. The amount of formazan present is proportional to the number of viable cells as only living cells will reduce MTT to blue formazan. Results were expressed as a % of the absorbance of the MTT reduced by the control cells. Wells containing no cells were used as blanks.

4.8.3 Assessment of nuclear damage caused by 5-fluorouracil and cisplatin (free drug and encapsulated) by induction of micronuclei

The presence of micronuclei in a cell is an indicator of chromosomal damage. In order to assess the cytotoxicity of CDDP and 5-FU at the molecular level in DMBA-OC-1R cells, micronuclei were induced. DMBA-OC-1R cells were detached from the surface of two 25 cm^2 culture flasks (Corning) with versene and centrifuged at 1500 rpm for 5 minutes. After discarding the supernatant, the cells were resuspended and washed in

RPMI-1640 medium and centrifuged at 1500 rpm for 5 minutes. The cell pellet was resuspended in 5 ml RP10 medium and the cell number was determined. The cell number was adjusted to 5 x 10^4 cells per ml. 2 ml of the cell suspension was seeded on top of sterile coverslips placed in 35 mm Petri dishes (Corning). The cells were allowed to adhere to the coverslips overnight in a 37°C humidified incubator containing 5% $C0_2$ before the drug treatment commenced. The culture medium was removed and to each Petri dish, 2 ml of RP10 medium containing either CDDP, 5-FU or a combination of both drugs (encapsulated or in the free form) at the appropriate concentration, was added. The amount of 'free' CDDP that was added ranged from a final concentration of 0.005 μg/ml to 0.1 μg/ml, while that of 'free' 5-FU ranged from 0.5 μg/ml to 10 μg/ml. The concentrations of drug that was encapsulated in albumin microspheres ranged from 10 μg/ml to 25 μg/ml for both CDDP and 5-FU. Each dose was set up in triplicate. The control consisted of cells which had not been exposed to drug. After exposing the cells to the drugs for either 1, 24 or 96 hours, the drug-containing medium was removed and the cells were rinsed three times with RPMI -1640 medium to remove any trace of drug that had not been taken up by the cell. 2 ml of RP10 medium was added to each Petri dish and then, in order to induce mitotic arrest in the cells, cytochalasin-B (Sigma) was added to each Petri dish to give a final concentration of 2 μg/ml for 24 hours. The cells were fixed with cold fixative consisting of 3 parts methanol to 1 part acetic acid. After air drying the cells, they were stained with 0,001% acridine orange in phosphate buffer (pH 6.8), a nuclear specific stain, for 1 minute and rinsed five times with distilled water. The coverslips containing the cells were mounted in buffer and the cells were examined using fluorescence microsocopy at 495 nm. The nucleation index in each sample was determined and the micronuclei in binucleated cells enumerated.

4.9 The tumour host system

Note: Prior to the experimental procedures performed on the animals,approval by the Ethics Committee of the Faculty of Medicine, UCT was obtained.

All the experiments used female rats of the Wistar albino strain at age 8-10 weeks and weighing ± 200g . Very young rats were chosen as to minimise possible antigenic interaction between the implanted tumours and their hosts, often found in older animals. The original breeding pairs were obtained from the animal unit of the Medical Research Council (RSA) where the strain has been inbred for more than 20 years. In our laboratory, the strain was further inbred by brother-sister mating for over 4 years without selective sublining. No exogenous oncogenic virus or carcinogenic substances have

ever been introduced into the laboratories or animal house in which the rats were housed.

The environmental temperature of the animal house was kept constant at approximately 25°C. The cages in which they were kept, were cleaned in the same way each time by the same personnel and the bedding consisted of a mixture of sterile straw and corn cobs. Fresh water was readily at each animal's disposal, as well as rat and mouse breeder feed (Animal Specialities, RSA). The composition of the feed was as follows:

Composition	g/kg
Protein (min)	210
Fat (min)	40
Fibre (max)	60
Moisture (max)	120
Phosphorous (min)	8
Calcium (max)	18

The animals were always handled by the same personnel who checked them for pregnancy, transferring to other cages, anaesthetising, collection of blood, tumour inoculation, etc. as to keep stress levels as low as possible.

For blood collection, use was made of Halothane (Rhone-Poulec Rorer, RSA) inhalation to induce mild anaesthesia to restrain the animals for approximately 15-30 minutes. For surgical procedures, a mixture of Ketalar (Warner-Lambert, RSA) and Rumpon (Bayer, RSA) was used at a dose of 0,14 ml/kg. This dose was found to anaesthetise the animals for approximately 20 minutes to 4 hours. This anaesthetic mixture was preferred to pentobarbital anaesthesia, which frequently shows some serious side effects on vital regulatory processes in small laboratory animals.

Blood was collected for haematological and biochemical assays from the tail vein, after cleaning and disinfecting the skin with a 70% ethyl alcohol swab and restraint of the animal under mild Halothane anaesthesia. For haematological studies, 200 μl of blood was collected into Microtainer (Becton Dickinson, RSA) tubes containing EDTA anticoagulant. A further 500μl of blood was collected for blood chemistry and allowed to clot before separating the serum from the cells by centrifugation at 2500 rpm for 15 minutes. The serum was used for the assays.

As background contrast medium for taking X-ray images, approximately 1 ml 70% barium sulphate (X-ray grade, Kyranhebo, RSA) in egg albumin was slowly injected directly into the stomach via a 1,5 mm latex tube inserted into the stomach through the oral cavity and oesophagus. For this procedure mild Halothane anaesthesia was employed.
X-rays were performed under Ketalar/Rumpon anaesthesia in a Shimadzu condensor discharge mobile unit at 50 KV and 120 mAs.

4.9.1 Drug distribution studies

To determine the drug distribution to the major organs, 2 groups of 10 female Wistar rats were respectively injected with 20 mg/kg of 5-FU and 25 mg/kg of CDDP via the tail vein. The rats were sacrificed at 2 hours for 5-FU determination and at 4 hours for CDDP determination, respectively. After exsanguination, the major organs were removed and the drug levels (5-FU and CDDP) and/or its metabolites determined by the methods described in Section 4.3.1.

4.9.2 Blood chemistry and haematological profiles for the assessment of the effects of 5-fluorouracil and cisplatin (free drug and encapsulated) in female Wistar rats

To obtain blood chemistry and haematological profiles of the effects of 5-FU and CDDP in combination (free drug and encapsulated), 3 groups of 20 female Wistar rats, weighing ± 300 g, were used.

Group I rats *(free drug group)* were injected intraperitoneally with a combined bolus dose of chemotherapeutic agents containing 5 mg/kg CDDP and 20 mg/kg 5-FU.

Group II rats *(high dose immunomicrosphere group)* were injected intraperitoneally with a combined bolus dose of immunomicrospheres containing 10 mg/kg CDDP and 40 mg/kg 5-FU. The reason why the dose was double that of Group I was because of the slow fashion in which the drugs are released from the immunomicrospheres and to achieve optimum drug delivery at the target site.

Group III rats *(no drug group)* were the control animals. No drug treatment was given to this group.

From each rat of each group, 200 μl of blood was collected into Microtainer (Becton Dickinson, RSA) tubes containing EDTA anticoagulant for haematological studies. This included white blood cell count, platelet count, haemoglobin, hematocrit, red blood cell count, red blood cell indices and differential leucocyte count. Haematology counts and indices were performed on a Coulter T890 cell counter. The differential leucocyte count was performed on a methanol-fixed blood smear stained with Wright's stain (Merck) and counted on a Leitz Laborlux K light microscope utilizing a 50x oil immersion objective.

A further 500 μl of blood was collected from each rat for blood chemistry. The blood was allowed to clot before separating the serum from the cells by centrifugation at 2500 rpm for 15 min. The serum was used to quantitatively measure urea, creatinine, lactate dehydrogenase, gamma glutamyl transferase, alkaline phosphatases, alanine aminotransferase and aspartate aminotransferase. Blood chemistry was performed on a Ciba Corning Express Plus random access analyzer.

4.9.3 The tumour model

Primary, transplantable rat ovarian adenocarcinomas were induced in 10 female Wistar rats, 3-6 weeks old and weighing 40-100 g by intraperitoneal inoculation of $4x10^6$ DMBA-OC-1R cells, suspended in RPMI-1640 medium, using a 27 gauge needle. The tumours were allowed to establish themselves for 14 days in the animals. Abdominal distension due to the accumulation of ascitic fluid was considered to be evidence of reasonably sized tumour-formation. X-rays were also taken to observe for tumour mass formation. The rats were sacrificed by decapitation and the primary tumours immediately removed aseptically and placed in a sterile Petri dish containing RPMI-1640 medium at 37°C. After dicing the tumour into 3 mm^3 cubes, the tumour tissue was transplanted intraperitoneally into 4 groups of 20 female Wistar rats, 4-8 weeks old and weighing 80-120 gm. Secondary tumours were allowed to develop for 10 days in the peritoneal cavities of each animal before treatment commenced. Also, a few $3mm^3$ cubes of tumour tissue were placed in RP10 medium for cell counting. Any fat was trimmed off the tissue. The tissue was minced with a pair of scissors and then placed in a McCartney bottle containing 10 ml of a 0.25% trypsin solution (Difco 1:250) and a magnetic stirring bar. Digestion took place at 37°C for 20 minutes with slow stirring. Erythrocytes were then lysed with 0,83% ammonium chloride for 5 minutes. The cells were washed twice for 5 minutes per wash with RPMI-1640 medium, and pelleted by centrifugation at 1500 rpm for 5 minutes. A cell count of the tumour cells was performed using an Improved Neubauer haemocytometer and RP10 medium as the diluent.

Samples of the primary and secondary tumours were taken for electron microscopical examination and placed in cold 2,5% phosphate buffered glutaraldehyde for further processing. Samples were also taken for histological examination and placed in 10% formal-saline for further processing.

4.9.3.1 Tissue preparation for light (LM) and transmission microsocopy (TEM)

Tissue specimens for LM were collected in 10% formal-saline at room temperature. Representative samples were trimmed to a thickness of 3 mm and further fixed in 10% formal-saline for 24 hours at room temperature. The tissues were further processed in an automatic tissue processor (Shandon Duplex) for routine paraffin wax embedding i.e. fixation, dehydration, clearing and impregnation before final blocking of the tissue in Histosec embedding medium (Merck).

Tissue sections (3 μm thick) were cut on a Reichert OmE sliding microtome with a sharp steel knife, floated on the surface of a warm water bath and picked up on clean glass slides.

Deparaffinisation was done in xylene and the sections hydrated through graded ethanol solutions (100%, 96%, 70%) to distilled water before staining. The following staining methods were performed: (1) Haematoxylin and Eosin; (b) Alcian Blue PAS; (c) Reticulin stain; (d) Van Gieson and (e) Southgate's Mucicarmine, (see Appendix for methods). After coverslipping the sections, they were viewed at various magnifications with a light microscope.

Tissue specimens for TEM were collected in cold 2,5% phosphate buffered glutaraldehyde (Merck) at pH 7,4. After dicing into 1 mm^3 cubes, the tissue blocks were further fixed in fresh 2,5% phosphate buffered glutaraldehyde for 8 hours at 4°C. After 2 rinses of 15 minutes each in cold phosphate buffer (pH 7,4), secondary fixation of the tissue blocks occurred in cold 1% osmium tetroxide in veronal-acetate buffer (pH 7,4) for 1 hour. The tissue blocks were washed 3 times for 10 minutes each in distilled water and then dehydrated in graded ethanol solutions (70%, 96%, 96%+1% uranyl nitrate, 100%). The blocks were then impregnated with equal amounts of Spurr's resin and 100% ethanol for 90 minutes. Two changes of pure Spurr's resin were used for 60 minutes each at room temperature, then at 60°C for the same length of time.

Embedding was performed with fresh Spurr's resin in predried gelatine capsules (Elanco size 0). Polymerization took place at 60-70°C for 16-18 hours in an incubator. The capsules were trimmed in a Reichert TM 60 block trimmer with a steel cutter. 1μm survey sections were cut on an LKB Ultratome III with a glass knife, picked up and placed on a drop of water on a glass slide. The slide was placed on a hot plate until the water had evaporated and the sections adhered to the glass. The slides were stained with a heated solution (70°C) of 1% toluidine blue for 1 minute, washed in tap water, air-dried and mounted in DPX (BDH). The sections were then examined with a light microscope to select a suitable area for viewing with the electron microscope. The most suitable block was trimmed for thin sectioning with a diamond cutter in the block trimmer.

Thin sections (60-90 nm thick) were cut on the LKB ultramicrotome with a glass knife, stretched by chloroform fumes and the ribbons of sections were picked up on 150 or 200 mesh copper grids (Bio-Rad) and stored on filter paper in a small tissue culture dish (Greiner) until dry.

When the sections had dried, they were stained upside down on drops of uranyl acetate (Merck) in 50% ethanol on strips of dental wax for three minutes, rinsed by the dipping method, first in 50% ethanol, then in two changes of distilled water (30 dips each). The sections were then placed on drops of Reynolds' lead citrate stain for 5 minutes, rinsed in two changes of distilled water and placed in their respective dishes.
After drying, the sections were viewed in a Hitachi 600/2 transmission electron microscope at 75Kv, and relevant areas photographed.

4.9.3.2 Drug therapy protocols

The drug therapeutic trials were performed over a 90 day trial period before termination. Group IA rats *(free drug group)* were given an intraperitoneal combined bolus dose of chemotherapeutic agents containing 5 mg/kg CDDP and 20 mg/kg 5-FU on Day 10 after primary tumour transplantation. The same dose was repeated 7 days later.

Group IIA rats *(low dose immunomicrosphere group)* were injected intraperitoneally with a combined bolus dose of immunomicrospheres containing 1 mg/kg CDDP and 4 mg/kg 5-FU on Day 10 after primary tumour transplantation.

Group IIB rats *(high dose immunomicrosphere group)* were injected intraperitoneally with a combined bolus dose of immunomicrospheres containing 10 mg/kg CDDP and 40

mg/kg 5-FU on Day 10. The reason for doubling the dosage as a bolus dose as compared to the dosage of Group IA *(free drug group)*, was that the drug is released slower and in sustained fashion and can be tolerated well by the animals. This dosage was given to compare the survival rate of this group of rats with those of Group IIA *(low dose immunomicrosphere group)* .

Also, mild anaesthesia was required to inject the immunomicrospheres into the peritoneal cavity via a catheter and it was therefore performed once only to reduce stress levels in the animals.

Group IIIA rats *(no drug group)* were the control animals. No drug treatment was given to this group.

From each rat of each group, 500 μl of blood was collected at fortnightly intervals into heparinized tubes and centrifuged at 2500 rpm at 4°C for 5 minutes. The plasma was discarded and the erythrocyte pellet stored at -20°C before the superoxide dismutase levels were assayed.

No blood was taken for blood chemistry or haematological tests from the above groups of animals, as it was considered a risk factor which could affect the animal survival rate data after treatment with free drugs and immunomicrospheres respectively.

Note: Groups IA, IIA and IIIA animals could possibly be seen as control groups for Group IIB, which is in fact the immunomicrospheres evaluated for efficacy as a treatment modality. Group IIIA is the untreated control group.

4.9.3.3 Quantification of superoxide dismutase levels in tumour-bearing rats

In order to monitor disease progression or regression in all groups of the tumour-bearing rats, the levels of superoxide dismutase (SOD) enzymes were quantified using the BIOXYTECH SOD-525 assay kit (Oxis International, Inc.). This method, as mentioned in Chapter 2, Section 2.3, was preferred to CA-125, as also described by Yamanaka and Deamer (1974), Dionisi *et al.* (1975), Sahu *et al.* (1977), Oberley and Buettner (1979) and Ishikawa *et al.* (1990).

The erythrocyte pellet was resuspended in 4 packed cell-volumes of ice-cold water and thoroughly vortexed. 400 μl of ice-cold extraction reagent (absolute ethanol/chloroform,

62.5/37.5 (v/v) was added to 250 μl of lysate in a glass test tube. The mixture was vortexed for 30 seconds and then centrifuged at 3000xg and 4°C for 10 minutes. The upper aqueous layer was collected and kept at 4°C for the assay. The 50 mM 2-amino-2-methyl-1,3-propanediol/HCl buffer containing 0.11 mM diethylenetriaminepentaacetic acid, which was provided in the kit, was brought to 37°C. 40 μl of sample or water (control) was added to a tube containing 900 μl of the buffer. 30 μl of the mercaptan scavenger reagent was added, vortexed for 3-4 seconds and then incubated at 37°C for 1 minute. 30 μl of chromogen was added and the solution immediately vortexed for 3-4 seconds. The solution was transferred to a cuvette and the absorbance change was measured within 10-15 seconds at 525 nm for 1 minute. The spectrophotometer (Beckman) was calibrated against air. The SOD activity of each sample was determined from the ratio of auto-oxidation rates measured in the presence (test sample) and absence (control sample) of SOD.

4.10 Animal survival endpoints

After all procedures were performed, all the animals were placed back in their cages in the air-conditioned animal room where they had free access to water and food. They were observed on a daily basis and their status recorded.

Each death was recorded, an autopsy performed and tissue removed for macroscopical and/or microscopical evaluation.

After a 90 day interval all remaining living animals were killed as this was considered to be the termination of the study as no further information could be gained and natural deaths or deaths not necessarily related to the experiments may occur at this stage. Autopsies were performed on all the animals and the findings recorded.

4.11 Statistical methods

To obtain meaningful comparisons of the various treatment protocols, the significant data was analysed employing Corel Quatro Pro 8® software. An analysis of variance was performed on the data from the different test groups including *in vitro* assays as well as the rodent test groups at a significance level of 0,05 ($p<0.05$). From the results of the ANOVA, the p-values were compared in an attempt to prove variances between the test groups. The NCSS software was used to establish the trends for blood chemistry and haematological profiles within each group treated by the different therapy protocols.

CHAPTER 5

RESULTS

5.1 Human serum albumin microspheres

The preparation of human serum albumin microspheres by employing a heat-crosslinking method, proved to be suitable for this study and led to the production of well-defined microspheres. The incorporation of cisplatin (CDDP) and 5-fluorouracil (5-FU) in albumin microspheres by the method used, was found to be acceptable for the entrapment of these relatively heat-stable drugs. Microspheres containing 5-FU were prepared at 120°C, well below the decomposing temperature of 282°C for this drug. CDDP-containing microspheres were prepared at 100°C, also well below 270°C, which is the decomposing temperature of cisplatin.

The microspheres (Fig. 3) prepared by heat stabilization, were found to be almost perfect round beads, free-flowing and non-conglomerated, in the size range of 1-5 μm in diameter (Tables 2a & b). Although the microspheres were hydrophobic due to the preparation technique, they were found to be readily suspendable by vigorous shaking in physiological saline in high concentrations, rendering them ideal for intraperitoneal injection. Swelling of the microspheres in saline were negligible and considered to be insignificant.

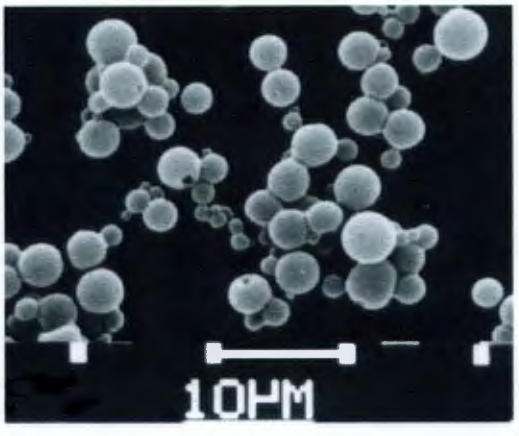


Fig. 3: Scanning electron micrograph of heat-stabilized human serum albumin microspheres containing 5-FU.

		Size (μm)						
		<1	1-2	2-3	3-4	4-5	5-6	>6
Batch	1	3	6	21	36	18	12	4
	2	2	2	15	27	39	14	1
	3	5	9	19	31	22	11	3
	4	4	2	24	31	16	18	5
	5	4	8	17	29	26	13	3
	Mean	3.6	5.4	19.2	30.8	24.2	13.6	3.2
	Std Dev	1.020	2.939	3.124	2.993	8.158	2.417	1.327

Table 2a Size distribution of heat-stabilized albumin microspheres containing 5-FU (expressed as a percentage after counting 750 microspheres per batch).

		Size (μm)						
		<1	1-2	2-3	3-4	4-5	5-6	>6
Batch	1	1	3	18	35	22	13	8
	2	2	4	13	29	27	18	7
	3	4	3	11	32	25	16	9
	4	2	2	19	30	24	15	8
	5	1	4	18	28	31	12	6
	Mean	2	3.2	15.8	30.8	25.8	14.8	7.6
	Std Dev	1.095	0.748	3.187	2.482	3.059	2.135	1.020

Table 2b: Size distribution of heat-stabilized albumin microspheres containing CDDP (expressed as a percentage after counting 750 microspheres per batch).

The reproducibility of microsphere preparation in large batches was easy to maintain and lyophilization and dry storage at 4°C appeared to have no detrimental effect on the quality and characteristics, even if stored for 7-10 days prior to use.

5.1.1 Drug incorporation in human serum albumin microspheres

To determine the entrapment concentration of 5-FU in the albumin microsphere matrix, the fluoride content was firstly determined by using a fluoride ion selective electrode (Metrohm Mod. 632) as described in Chapter 4, section 4.3.1. Using the constructed

calibration curve, the millivolt readings were plotted on a graph against the concentration [in ppm(ℓ)], in order to determine the fluoride concentration in the albumin microsphere sample (Table 3 and Fig 4).

5-FU conc.					
Batch	mV	ppm(l)	ppm(s)	5-FU ppm(s)	% 5-FU
1	247	0.6	1500	12063	1.03
2	248	0.57	1425	9750	0.98
3	249	0.55	1375	9408	0.94
4	251	0.52	1300	8895	0.89
5	252	0.5	1250	8553	0.85
Mean	249.4	0.548	1370	9374	0.94
Std Dev	1.855	0.035	88.600	606.212	0.061

Table 3: Data obtained from 5 batches of heat stabilized HSA microspheres with entrapped 5-FU.

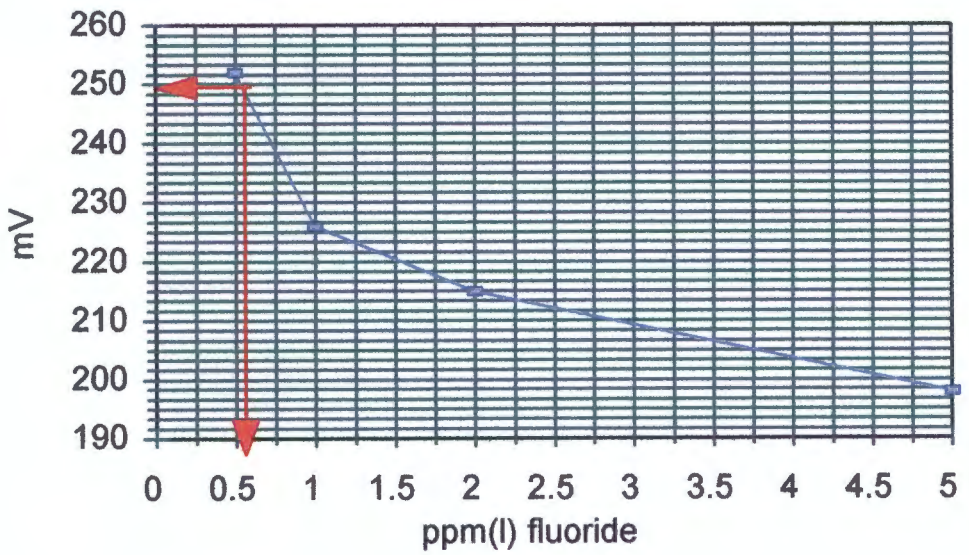


Fig. 4: Graph demonstrating the calibration curve (blue line) and millivolt readings (red line) to determine the average fluoride concentration in 5 samples of HSA microspheres prepared in separate batches.

To determine the average total 5-FU concentration of the HSA microspheres, the following calculation was employed to firstly determine fluoride concentration:

$$\text{ppm(s)F} = \frac{\text{ppm}(\ell) \times \text{dilution}}{\text{sample mass}}$$

$$\text{ppm(s)F} = \frac{0.548\ \text{ppm}(\ell) \times 25\ \text{ml}}{0.010\text{g}}$$

$$= 1370\ \text{ppm(s) fluoride}$$

The 5-FU content was then calculated:

$$\text{ppm(s) 5-FU} = \frac{\text{ppm(s)F} \times \text{mol. mass of 5-FU}}{\text{mol mass of F}}$$

$$= \frac{1370 \times 130}{19}$$

$$= 9373\ \text{ppm(s) 5-FU}$$

or

0.94% entrapment of 5-FU in the HSA microspheres

To determine the entrapment concentration of CDDP in the albumin microsphere matrix, the platinum content was firstly determined by using an atomic absorption spectrophotometer (Varian Mod. AA 1275) fitted with a graphite tube atomizer (GTA95) as described in Chapter 4, section 4.3.2. Using the constructed calibration curve, the absorbance was plotted on a graph against the concentration [in ppm(ℓ)]. A linear regression was performed and the equation for the best straight line was obtained. Using the equation, the absorbance readings were converted to ppm(ℓ), in order to determine the platinum concentration in the albumin microsphere sample (Table 4 and Fig. 5).

CDDP conc.					
Batch	abs	ppm(l)	ppm(s)	ppm(s) CDDP	% CDDP
1	0.554	0.7963	7963	12251	1.23
2	0.549	0.789	7890	12139	1.21
3	0.562	0.808	8080	12430	1.24
4	0.542	0.7788	7788	11982	1.20
5	0.565	0.8123	8123	12498	1.25
Mean	0.554	0.7969	7969	12260	1.23
Std Dev	0.008	0.012	112.5	188.44	0.019

Table 4: Data obtained from 5 batches of heat-stabilized HSA microspheres with entrapped CDDP.

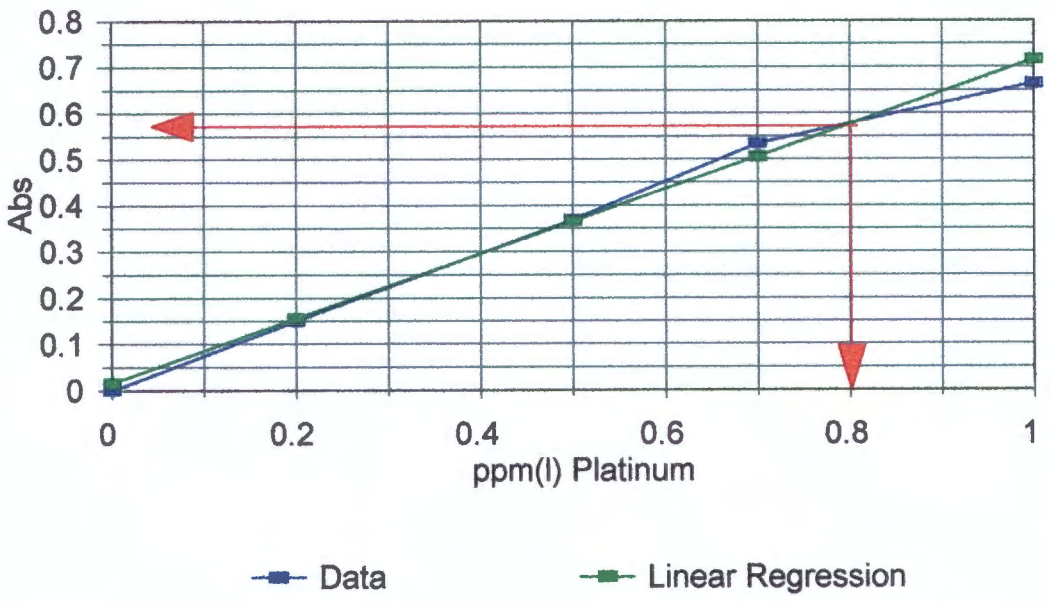


Fig. 5: Graph demonstrating the calibration curve (blue line), absorbance readings (red line) and linear regression line (green line) to determine the average platinum concentration in 5 samples of HSA microspheres prepared in separate batches.

To determine the average total CDDP concentration of the HSA microspheres, the following calculation was employed to firstly determine the platinum concentration:

$$X = \frac{Y}{M} - C$$

(X= Pt conc. in ppm(ℓ); Y = absorbance; M = X coefficient; C = constant)

$$X = \frac{0.5544}{0.6864} - 0.01085$$

$$= 0.79689 \text{ ppm}(\ell) \text{ Pt}$$

The ppm(ℓ) was then converted to ppm(s) using the following equation:

$$\text{ppm(s) Pt} = \frac{\text{ppm}(\ell) \times \text{dilution}}{\text{sample mass}}$$

$$\text{ppm(s) Pt} = \frac{0.797 \text{ppm}(\ell) \times 100 \text{ ml}}{0.010 \text{ g}}$$

$$= 7970 \text{ ppm(s) Platinum}$$

The CDDP content was then calculated:

$$\text{ppm(s) CDDP} = \frac{\text{ppm(s) Pt} \times \text{mol. mass of CDDP}}{\text{mol. mass of Pt}}$$

$$= \frac{7970 \times 300}{195}$$

$$= 12261 \text{ ppm(s) CDDP}$$

or

1.23% entrapment of CDDP in the HSA microspheres

From the above calculations, it was thus found that the concentrations of 5-fluorouracil and cisplatin entrapped in the crosslinked matrix of heat-stabilized human serum albumin microspheres was 0.94% and 1.23% respectively. These values were considered to be satisfactory for the purpose of this study, as extrusion for size distribution and centrifugation in ether and ethanol for removal of the cottonseed oil used during microsphere preparation, decreased the drug levels slightly.

5.2 The monoclonal antibodies

5.2.1 Establishment of hybridomas

The indirect ELISA on the test bleeds from all 3 BALB/c mice indicated that the mice had responded to the immunogen (DMBA-OC-1R cells) (Fig. 6). Three fusions were performed. Two assays were used to select hybridomas producing antibody against the immunogen.

The hybridoma culture supernatants were first screened in an indirect ELISA. The low absorbance readings, which were obtained when the supernatants of the first fusion were screened, improved when the multi-well plate was air-dried overnight, instead of only for 90 minutes, before the cells were fixed with glutaraldehyde. The positive supernatants of the first fusion were, however, also found to be non-selective as they also reacted with NRK and RESF cells in the indirect ELISA. However, since glutaraldehyde permeabilizes cells, the antibodies could have been binding not only to cell membrane antigens, but also to intracellular antigens.

With immunocytochemistry on live, unfixed cells in suspension, only membrane antigens will be bound by the antibodies and this assay confirmed that intracellular antigens were being bound in the ELISA. Whereas a strong signal was obtained in the ELISA, weak or no fluorescence was observed when the antibodies were reacted with NRK and RESF cells in suspension (Table 5).

	Absorbance in ELISA			Intensity of Membrane Fluoresence		
Clone Number	DMBA	NRK	RESF	DMBA	NRK	RESF
8	0.873	0.995	1.038	++	+/-	-
15	1.124	1.216	1.272	++	+	-
27	1.083	1.199	1.22	++	+	-
28	0.907	0.968	1.037	+	+	+
29	0.873	1.075	1.024	++	++	+/-
31	1.065	1.291	1.223	+	+	+
66	0.956	1.095	1.069	++	++	+
67	1.057	1.183	1.117	+++	+/-	+
77	1.103	1.202	1.265	++	++	+
79	1.154	1.2	1.229	+	+	+

Intensity of staining: +++ = strong, ++ = moderate, + = weak, +/- = borderline, - = absence of staining

Table 5: Comparison of results obtained for screening hybridomas using an ELISA with fixed cells and immunofluoresence on unfixed cells.

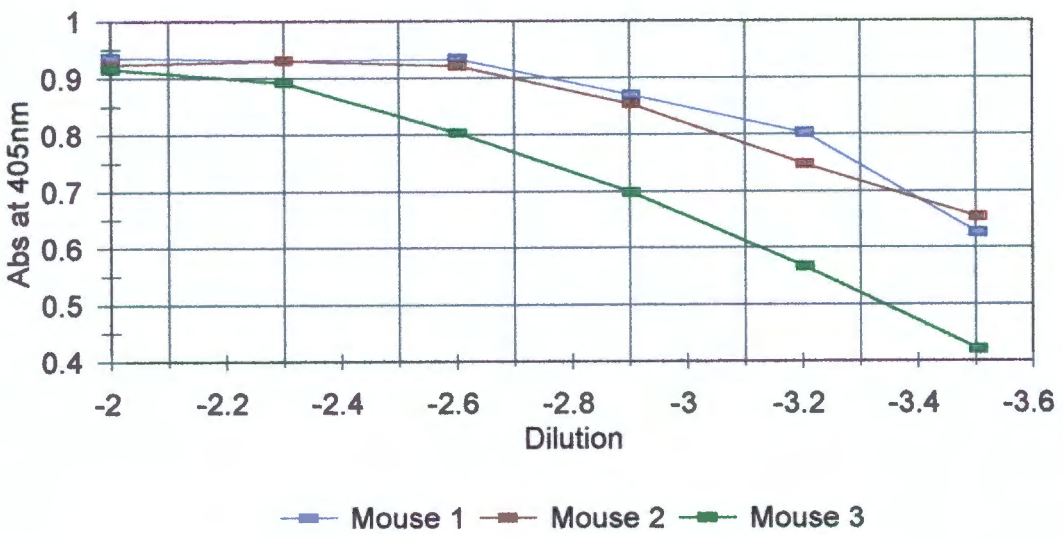


Fig. 6: Antibody titer in serum of BALB/c mice immunized with DMBA-OC-1R cells.

Fluorescence of varying intensities was observed when DMBA-OC-1R cells were reacted with the various supernatants. From the first fusion, 3 of the 22 hybridomas screened for antibody production by immunocytochemistry, were selected for further cloning by limiting dilution to establish a monoclonal antibody, since at this stage more than one type of antibody-secreting cell was present in the culture.

Only anti-DMBA56 gave rise to clones which did not react with NRK and RESF cells. One of these subclones was cloned further to ensure monoclonality. An average of 50% of the viable DMBA-OC-1R cells fluoresced when the supernatants were tested. It could be possible that this antibody is cell cycle-dependent.

The subsequent two fusions yielded 51 hybridomas of which 10 were selected by the ELISA for further screening with immunofluorescence membrane staining. These were highly specific for DMBA-OC-1R cell membrane antigens and did not bind to NRK and RESF cells. The reactivities of the 10 hybridomas were also tested on formalin-fixed paraffin-embedded wax sections of DMBA-OC-1R tumour grown subcutaneously in athymic nude mice (Fig. 7) and normal rat tissues. Trypsin digestion was included to unmask any antigenic sites which may have been obscured by the protein cross-linkages caused by formalin fixation. 2 of the 10 hybridomas (anti-DMBA11 and anti-DMBA61) did not react with the nude mice DMBA-OC-1R tumour sections (Table 6). The epitopes recognised by these antibodies are either sensitive to trypsin digestion or they have been altered by formalin fixation so that they can no longer bind to the antibodies. The antigenicity of these two epitopes may have been preserved had the supernatants been tested on cryostat sections of frozen tissue samples instead of paraffin-embedded sections. The non-specific staining which was found when the antibodies were tested on sections of normal ovary, spleen and liver (Table 6), was not reduced by preliminary blocking of Fc receptors with normal goat serum or by diluting the second antibody (FITC-goat anti-mouse) in RPMI medium with 10% FCS and 5% normal rat serum.

The monoclonality of the final two selected antibodies, designated anti-DMBA43 and anti-DMBA93, was ensured by further cloning of the hybridomas using the limiting dilution technique.

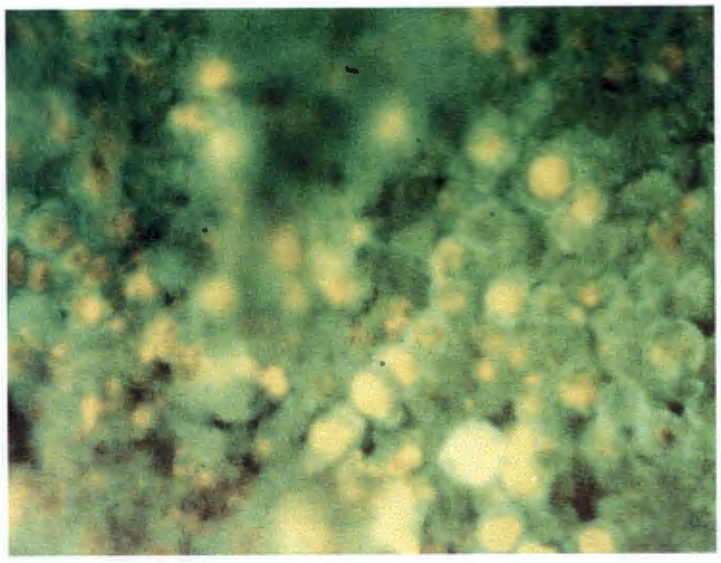


Fig. 7: Immunohistochemical localisation of anti-DMBA20 in a formalin fixed section of DMBA-OC-1R tumour grown in a nude mouse. The binding of anti-DMBA20 to the tumour was detected by staining the section with FITC-goat anti-mouse Ig and counterstained with ethidium bromide.(Mag x400)

Clone	Nude mouse tumour	Ovary	Kidney	Intestine	Liver	Spleen
anti-DMBA9	+++	+/-	-	-	+	+++
anti-DMBA11	-	N.D.	N.D.	N.D.	N.D.	N.D.
anti-DMBA20	+++	+/-	-	+/-	+	++
anti-DMBA43	+++	+/-	-	+/-	++	+++
anti-DMBA61	-	N.D.	N.D.	N.D.	N.D.	N.D.
anti-DMBA62	N.D.	+/-	-	+/-	+	++
anti-DMBA65	N.D.	+/-	-	+/-	++	++
anti-DMBA87	++	+/-	-	-	++	++
anti-DMBA93	+++	-	-	+/-	+++	+++
anti-DMBA94	+++	+/-	-	+/-	++	++
anti-β-galacto-sidase neg. control	-	+/-	-	+/-	+	++

Table 6: Immunohistochemical localisation of clones of anti-DMBA hybridomas in formalin-fixed, paraffin-embedded normal rat and nude mouse DMBA-OC-1R tumour tissues. (N.D. = not determined)

5.2.2 Purified monoclonal antibodies

Ascitic fluid titers of both anti-DMBA43 and anti-DMBA93 hybridomas showed maximal plateau binding at titers of $1:10^4$ (Fig. 8).

Anti-DMBA43 and anti-DMBA93 were purified from both serum-free and protein-free-conditioned media and from ascites by column chromatography on hydroxyapatite. The localisation and purity of the antibodies in the peaks were determined by SDS-PAGE (Fig 9 & 10). The presence of reduced antibodies is demonstrated by the presence of 2 bands on the gel - the one at 78 000 daltons (D) represents the heavy chain of IgM while the other at 25 000 D is the light chain (Fig 9C).

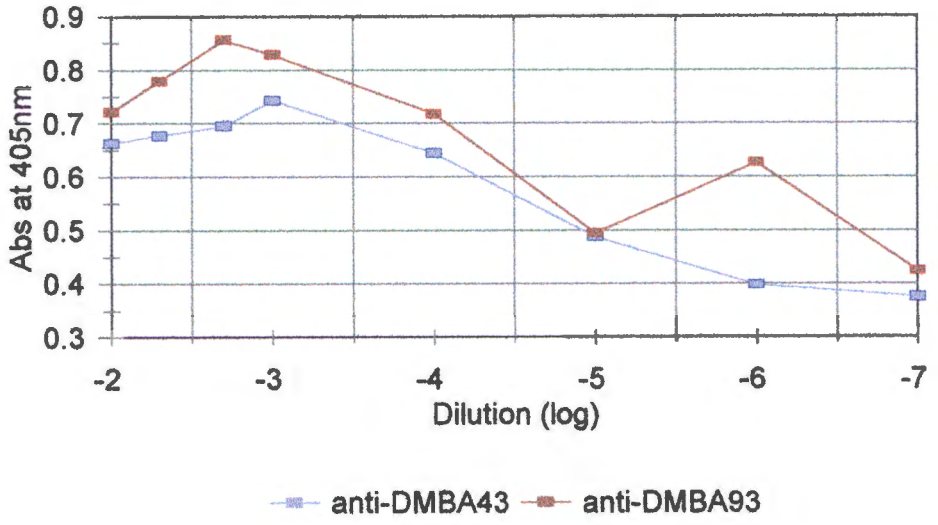


Fig. 8: Mean binding of anti-DMBA43 and anti-DMBA93 to DMBA-OC-1R tumour cells as a function of ascitic fluid dilution as determined by ELISA.

The antibodies were mainly eluted by 300 mM sodium phosphate buffer and a little was eluted with 200 mM (Fig 9 & 10). Antibodies purified from ascites were heavily contaminated with serum albumin (molecular weight of 66 000 D) and other proteins (Fig. 10C) and further purification by ammonium sulphate precipitation did not reduce the contamination significantly (Fig 10D). The dense band (66 000 - 76 000D) in lanes 6 and 9 in Fig. 10C and lanes 5 and 8 in Fig. 10D corresponds to the heavy chain of IgM contaminated with albumin.

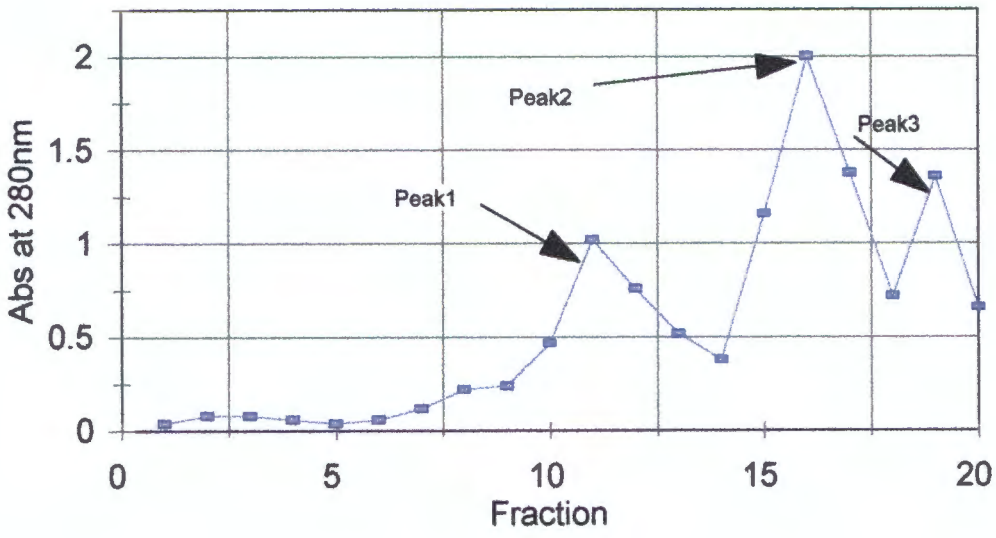


Fig. 9A: Elution of anti-DMBA43 from serum-free and protein-free harvest fluid - peak 1 eluted with 200 mM $NaPO_4$, peak 2 eluted with 300 mM $NaPO_4$.(peak 3 = non specific protien)

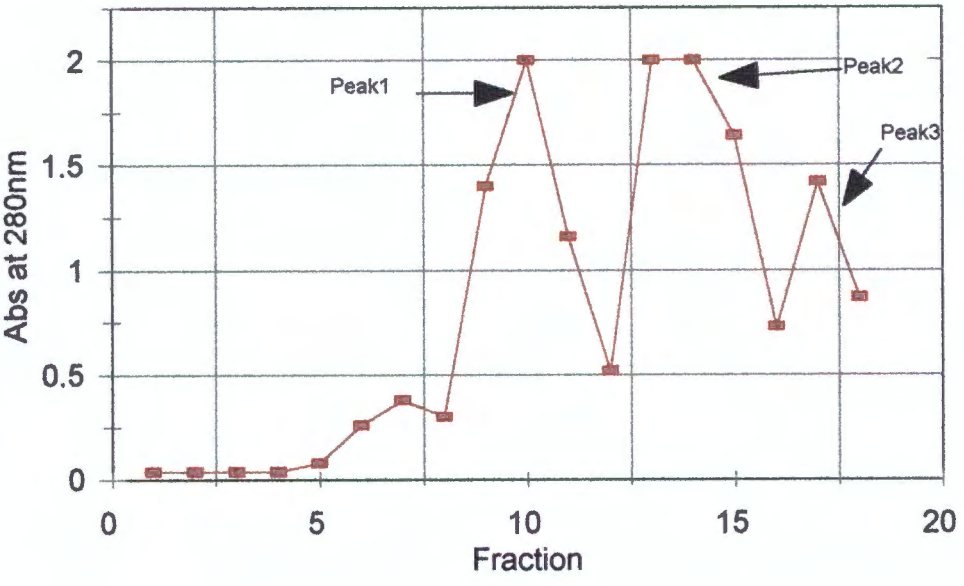


Fig. 9B: Elution of anti-DMBA93 from serum-free and protein-free harvest fluid - peak1 eluted with 200 mM $NaPO_4$, peak 2 eluted with 300 mM $NaPO_4$. (peak 3 = non specific protein)

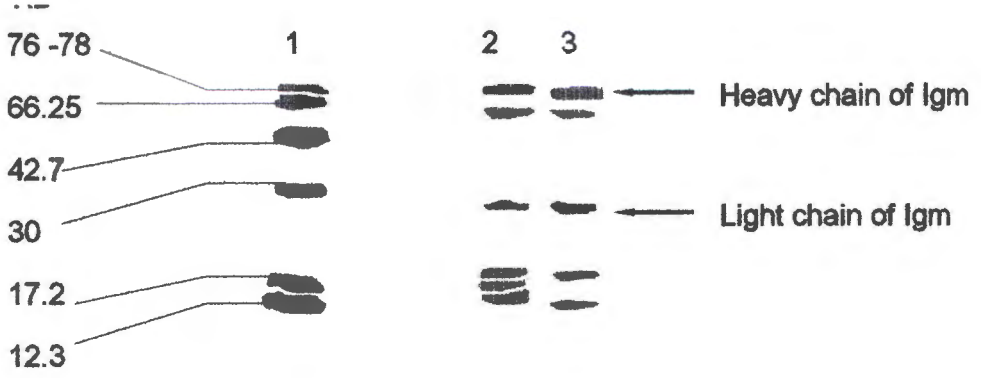


Fig. 9C: 11% SDS-polyacrylamide gel electrophoresis of reduced samples of anti-DMBA43(lane 2)and anti-DMBA93 (lane 3) purified by hydroxyapatite column chromatography from harvest fluid. Molecular weight markers (lane 1).

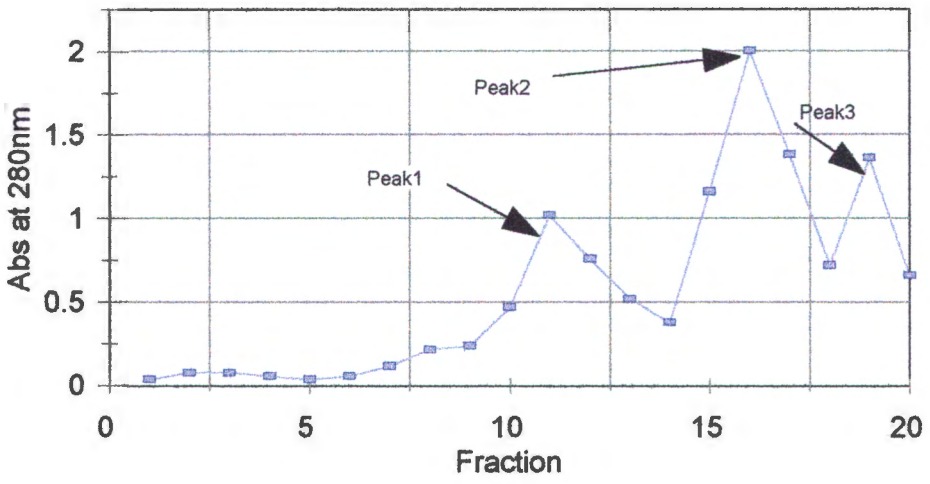


Fig. 10A: Elution of anti-DMBA43 from ascitic fluid - peak 1 eluted with 200 mM $NaPO_4$, peak2 eluted with 300 mM $NaPO_4$. (peak3 = non specific protein)

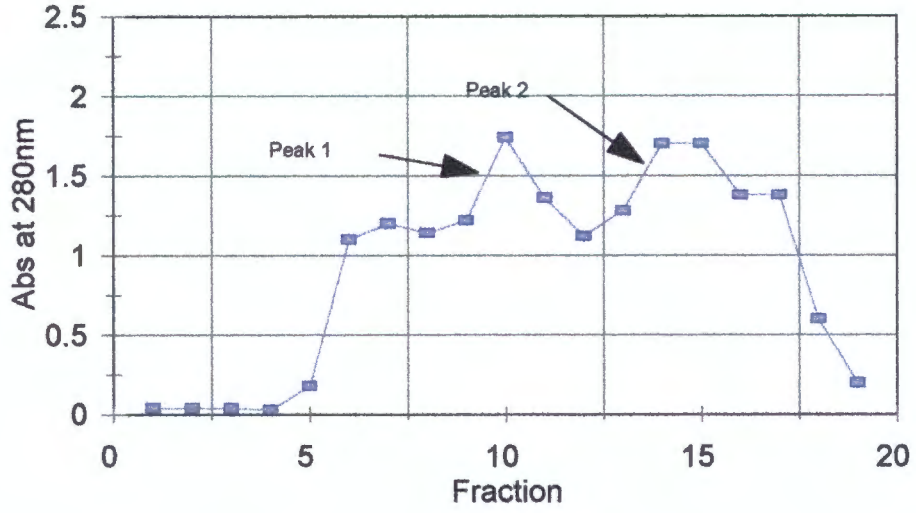


Fig. 10B: Elution of anti-DMBA93 from ascitic fluid -peak 1 eluted with 200 mM $NaPO_4$, peak 2 eluted with 300 mM $NaPO_4$.

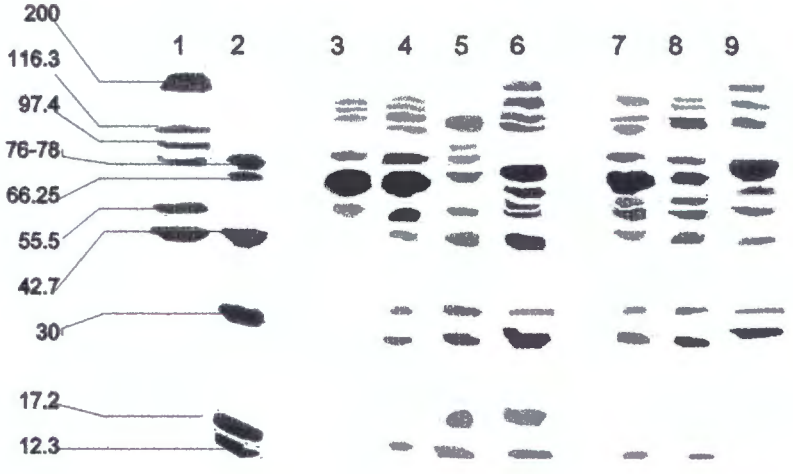


Fig. 10C: 11% SDS-polyacrylamide gel electrophoresis of reduced samples of fractions of antibodies eluted from ascites by hydroxyapatite column chromatography. Molecular weight markers (lanes 1 and 2); anti-DMBA43: fraction 7 (lane 3), fractions 8-12 (lane 4) corresponding to peak1 in Fig 10A, fractions 13 & 14 (lane 5), fractions 15-17 (lane 6) corresponding to peak 2 in Fig 10A; anti-DMBA93: fractions 6-11 (lane 7) corresponding to peak 1 in Fig. 10B, fraction 12 (lane 8) and fractions 13-17 (lane 9) corresponding to peak 2 in Fig 10B.

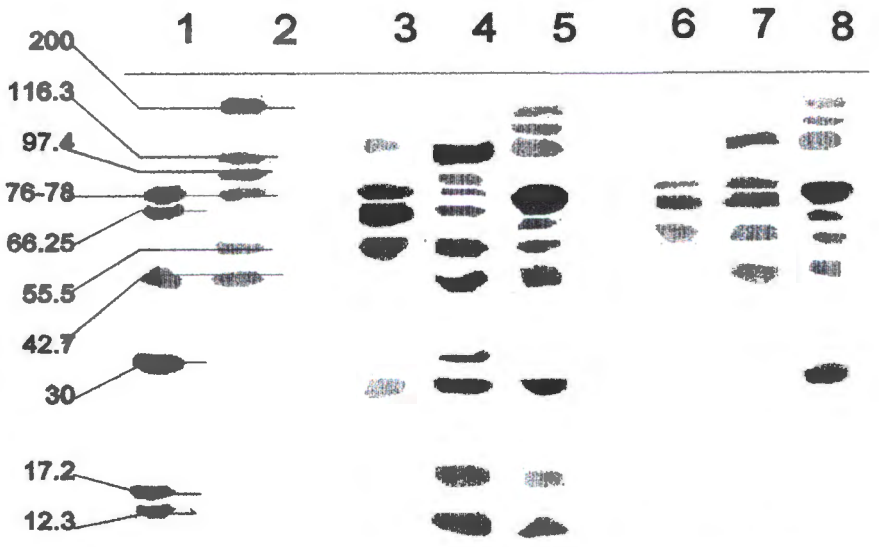


Fig 10D: 11% SDS-polyacrylamide gel electrophoresis of reduced samples of fractions of antibodies eluted from ascites by hydroxyapatite column chromatography and subjected to further purification with ammonium sulphate precipitation. Molecular weight markers (lanes 1 and 2); anti-DMBA43: fractions 8-12 (lane 3) corresponding to peak 1 in Fig.10A, fractions 13 & 14 (lane 4), fractions 15-17 (lane 5) corresponding to peak 2 in Fig. 10A; anti-DMBA93: fraction 6-11 (lane 6) corresponding to peak 1 in Fig. 10B, fraction 12 (lane 7) and fractions 13-17 (lane 8) corresponding to peak 2 in Fig 10B.

5.2.3 Further characterisation of purified anti-DMBA43 and anti-DMBA93 monoclonal antibodies

Both stable hybridoma lines produced antibodies of the IgM isotype. Immunocytochemistry on acetone-fixed DMBA-OC-1R cells demonstrated that the epitopes were resistant to acetone fixation (Fig. 11A & 11B). The resistance of the antigens to trypsin digestion was again confirmed. The monoclonal antibodies reacted equally strong with DMBA-OC-1R cells that had been trypsinised off the dishes (Fig. 12) as those that had been removed with versene (Fig. 11A and 11B).

The reactivities of anti-DMBA43 and anti-DMBA93 with cultured cell lines from various species were examined by immunofluorescent studies on acetone-fixed cells (Fig. 11A-H). It can be seen from Table 7 that both antibodies reacted strongly with only one out of sixteen cell lines (besides the one that they are naturally reactive against), namely a human melanoma cell line, Seafield (Fig. 11F). The 2 antibodies reacted weakly with the Goliath and MCF7 cell lines (Fig. 11G and 11H, respectively).

Table 7: Immunofluorescent reactivity of the test antibodies, anti-DMBA43 and anti-DMBA93, and the negative control antibody, anti-J138, with different cell lines.

Cell Line	Species and Type	Anti-DMBA43	Anti-DMBA93	Anti-J138
DMBA-OC-1R	Rat ovarian carcinoma	+++	+++	-
MTW9/PL	Rat mammary gland carcinoma	-	-	-
NRK	Normal rat kidney	-	-	-
RESF	Rat embryo skin fibroblasts	-	-	-
GH3	Rat pituitary gland tumour	-	-	-
B-OV2*	Human melanoma	-	-	-
GOLIATH	Human ovarian carcinoma	+ (only on a few cells)	+ (only on a few cells)	-
KERCHOFF	Human ovarian carcinoma	-	-	-
SK-OV-3	Human ovarian carcinoma	-	-	-
UW-OV-1	Human ovarian carcinoma	-	-	-
Seafield	Human ovarian carcinoma	+++	+++	+/-
MCF7	Human breast carcinoma	+ (only on a few cells)	+ (only on a few cells)	-
MDA231	Human breast carcinoma	-	-	-
Rogina	Human breast carcinoma	-	-	-
T47D	Human breast carcinoma	-	-	-
K562	Human chronic myelogenous leukemia	-	-	-
Small	Human melanoma	-	-	-

Intensity of staining: +++ = strong, ++ = moderate, + = weak, +/- = borderline, - = absence of staining.

* = This cell line was derived from the Bowes human melanoma cell line which was injected subcutaneously into athymic nude mice. The cells metastasised to the ovary in one of the mice. A cell line was established from this metastasis in culture.

Fig 11a-i: Specificity of anti-DMBA93 and anti-DMBA43 to various cell lines (arrows indicate fluoresence)

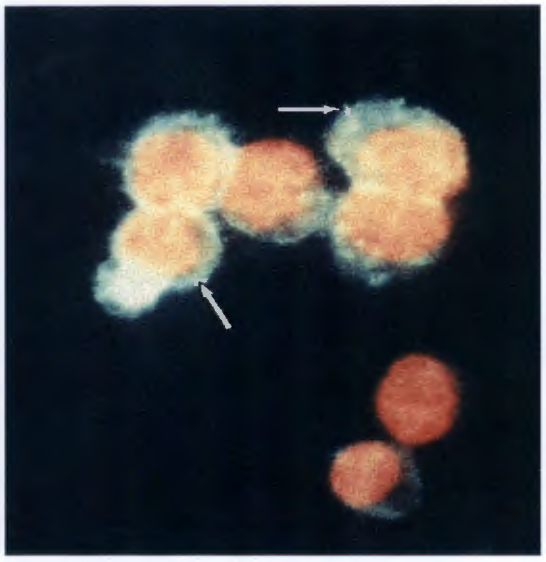


Fig. 11A: Versened DMBA-OC-1R cells with anti-DMBA43 (mag x400)

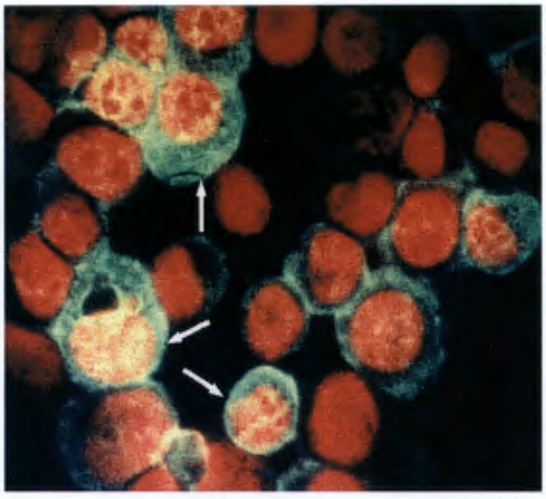


Fig.11B: Versened DMBA-OC-1R cells with anti-DMBA93 (Mag x400)

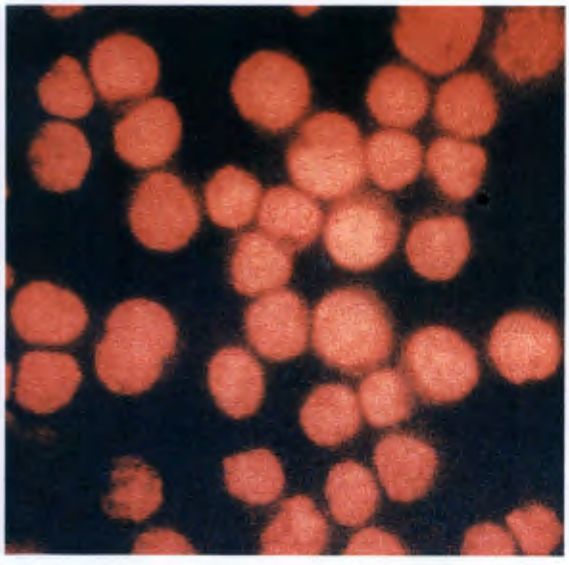


Fig. 11C: NRK cells with anti-DMBA93 (Mag x400)

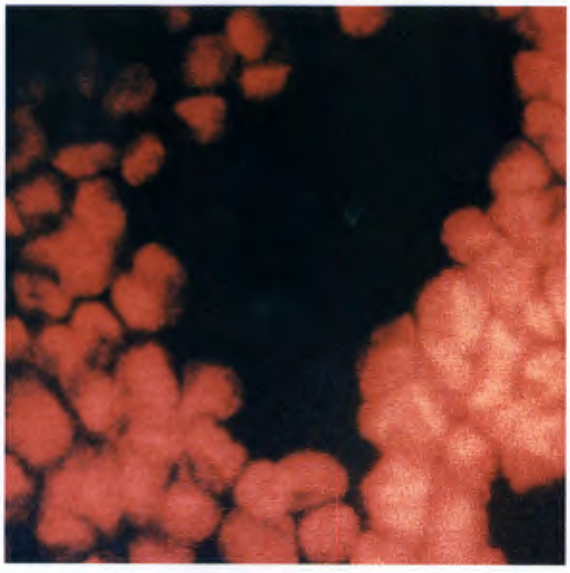


Fig. 11D: RESF cells with anti-DMBA43 (Mag x400)

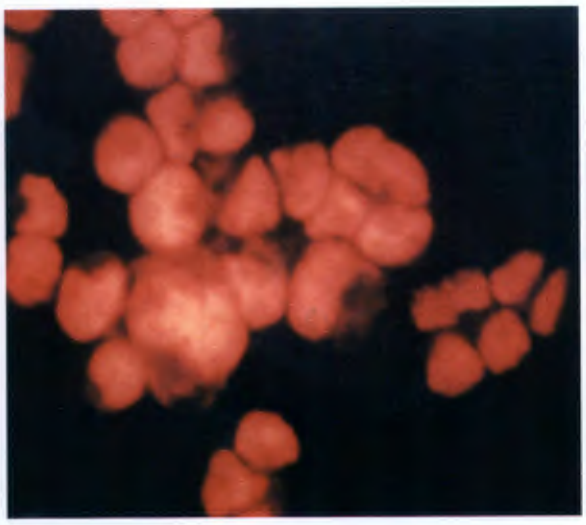


Fig. 11E: SK-OV-3 cells with anti-DMBA43 (Mag x400)

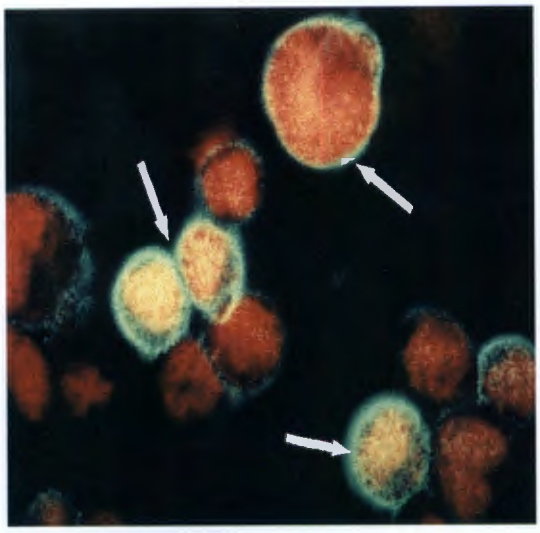


Fig. 11F: Seafield cells with anti-DMBA93 (Mag x400)

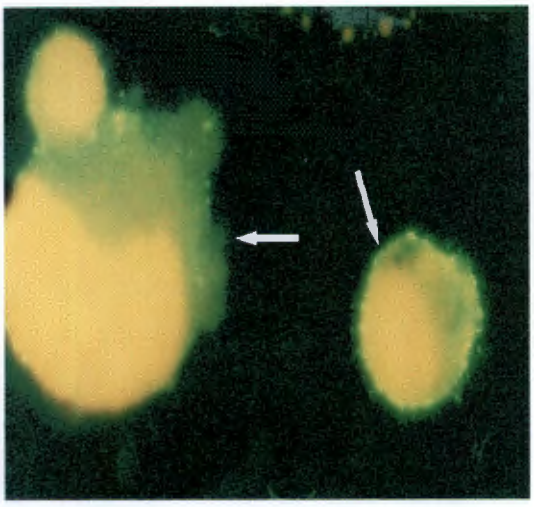


Fig. 11G: Goliath cells with anti-DMBA93 (Mag x400)

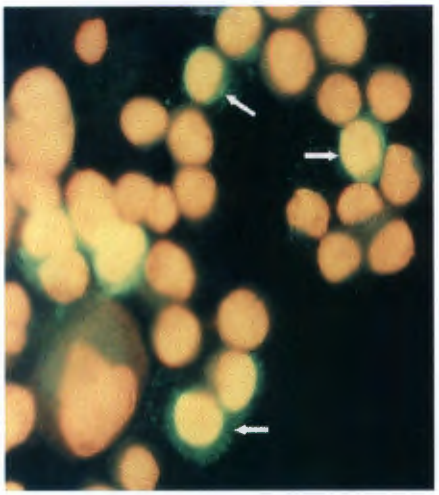


Fig. 11H: MCF7 cells with anti-DMBA43 (Mag x400)

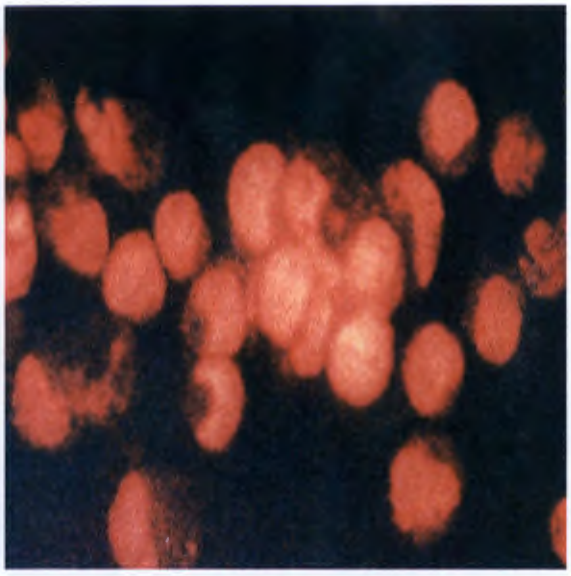


Fig. 11I: DMBA-OC-1R cells with anti-J138 (Mag x400)

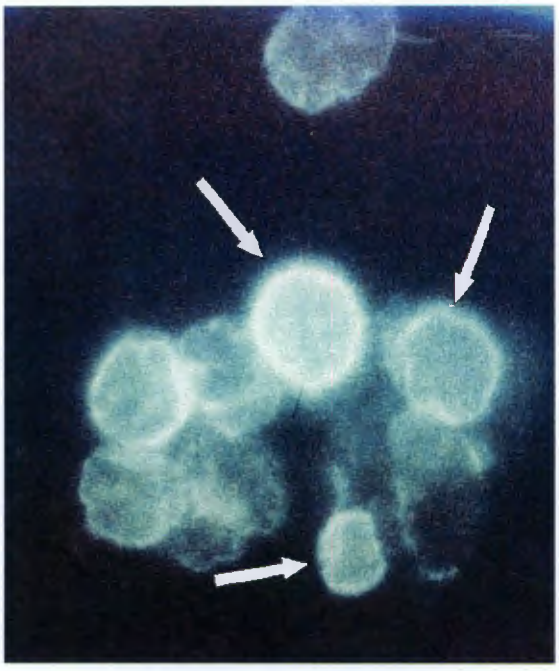


Fig. 12. Immunofluorescence reactivity as detected by FITC goat anti-mouse Ig of anti-DMBA93 binding to live unfixed cells trypsinized off the dish, shows that the epitope is not destroyed by trypsin treatment (Mag x400)

The reactivities of anti-DMBA43 and anti-DMBA93 with cultured cell lines from various species were examined by immunofluorescent studies on acetone-fixed cells (Fig. 11A-H).

5.3 Drug release from the immunomicrospheres

Drug release from human serum albumin microspheres can be sustained and controlled by various stabilization procedures, including heat, to facilitate cross-linking of the protein matrix. Previous experience showed that for the purpose of this project, heat cross-linked albumin microspheres were superior to chemically cross-linked albumin microspheres as regards stability and the leakage of CDDP and 5-FU, when incubated in plasma at 37°C.

Figure 13 shows the release behavior of 5-FU and/or its metabolites, as well as CDDP, from heat-stabilized albumin immunomicrospheres in plasma at 37°C.

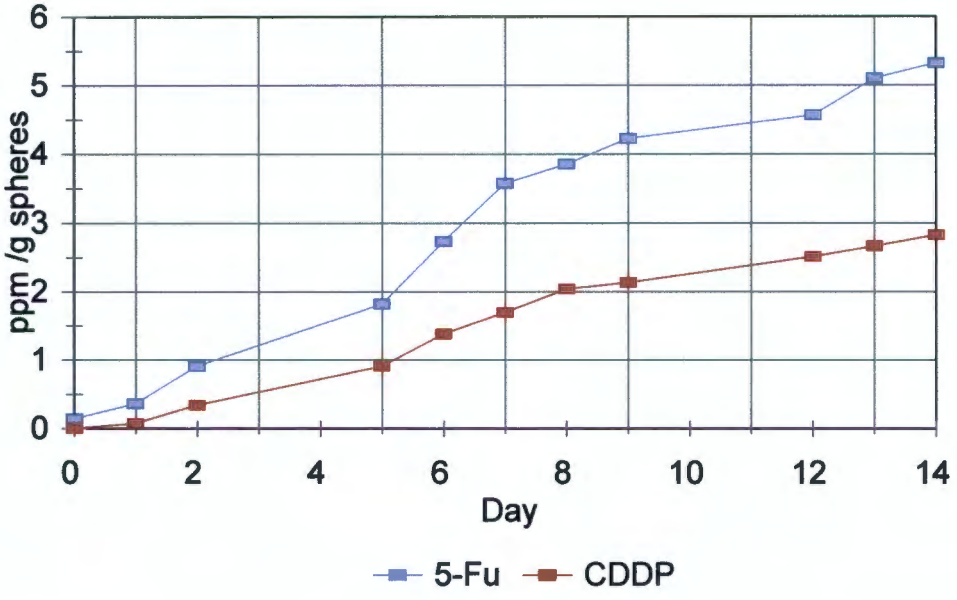


Fig. 13: Graph showing the trends of *in vitro* release of 5-FU and CDDP from albumin immunomicrospheres in plasma at 37°C. See Section 5.1.1 for quantitative analysis.

As shown in the above figure and as described in Section 5.1.1, the maximum levels of 5-FU in a fixed plasma volume of 3 ml, containing 150 mg 5-FU-loaded HSA immmunomicrospheres of 9373 μg/g (ppm) 5-FU concentration, 14 days after initiation of the experiment were 0.799 μg/ml plasma. The maximum levels of CDDP in the same volume of plasma, but containing 100 mg CDDP loaded HSA immunomicrospheres of 12261 μg/g (ppm) CDDP concentration, 14 days after initiation of the experiment, were 0.283 μg/ml plasma. These experiments demonstrated a slow degradation pattern of the protein microspheres.
As from Day 1 up to Day 5 of storage, the degradation rate showed only a slight increase when both 5-FU and CDDP leakage occured (0.273 μg 5-FU/ml plasma and 0.091 μg CDDP/ml plasma respectively). From Day 5 up to Day 9 of storage, the degradation rate increased substantially reaching levels of 0.635 μg 5-FU/ml plasma and 0.214 μg CDDP/ml plasma, respectively. At Day 14, when these studies were terminated, the drug levels were 0.799 μg 5-FU/ml plasma and 0.283 μg CDDP/ml plasma respectively. The degradation pattern appeared to continue until the microspheres had released their total payloads which took approximately 30 days for microspheres prepared by heat-stabilization procedures.

5.4 Drug Treatment of DMBA-OC-1R cells for use in clonogenic assays and cell survival growth curves.

The long term cytotoxic effects of a combination of CDDP at a fixed dose and 5-FU at varying concentrations, on DMBA-OC-1R cells were evaluated. The clonogenicity in soft agar of these cells was determined and cell survival growth curves were established to study the cytotoxic effects of the drugs after 24 hour exposure to free drugs and 96 hour exposure to the drugs encapsulated in albumin microspheres.

5.4.1 Clonogenic assays

DMBA-OC-1R cells, incubated for 14 days following drug treatment as described in Chapter 4, Section 4.8.1.1, were studied for colony formation from single cells and enumerated. The results were expressed as a percentage of the number of colonies arising from the control cells. The results , as demonstrated in Fig.14, suggests that 5-FU modulates the effect of the CDDP and there appears to be a dose-dependent decrease in the number of colonies when the 5-FU concentration is increased. This finding confirms the results as shown in the cell survival growth curves (Section 5.4.2).

As demonstrated in Fig. 14 and Table 8, the effects of low-dose CDDP on DMBA-OC-1R cells exposed for 24 hours, demonstrates 24.73% survival of the control cell growth ($p<0.05$). The addition of 5-FU in varying concentrations demonstrates a dose-dependent decrease in the ability of the cells to form clones in soft agar. The synergistic effect of CDDP and 5-FU is thus clearly demonstrated when cells were treated with 0.025 μg/ml CDDP and 1 μg/ml 5-FU ($p<0.05$).

It is thus clinically possible using a CDDP/5-FU free-drug infusion protocol to produce and maintain cisplatin serum concentrations of 0.025 μg/ml CDDP and 1 μg/ml 5-FU for 24 hours with acceptable host toxicity. Therefore the effects of prolonged high-dose CDDP/5-FU exposure on the same cell line, but from a targeted drug delivery system were examined. This was performed in order to examine if the cell sensitivity employing this modality, increased and fell within a clinically achievable range (also see the survival growth curves - Section 5.4.2).

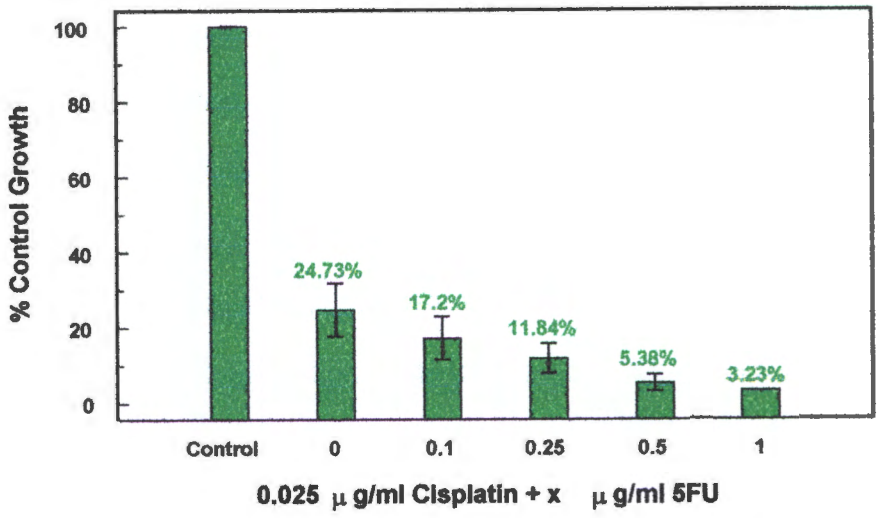


Fig. 14: Clonogenicity of DMBA-OC-1R cells cultured in soft agar for 14 days following 24 hr exposure to either no drugs (control) or 0.025 μg/ml CDDP together with varying concentrations of 5-FU. Results are expressed as a percentage of the number of colonies arising from untreated cells (mean +/-SEM).

First Parameter	Second Parameter	p-Value	Significant difference
Control	0.025 μg/ml CDDP	$p= 1.098x\ 10^{-2}$	yes
Control	0.025 μg/ml CDDP + 0.1 μg/ml 5-FU	$p = 7.040x10^{-3}$	yes
Control	0.025 μg/ml CDDP + 0.25 μg/m 5-FU	$p = 4.975x10^{-3}$	yes
Control	0.025 μg/ml CDDP + 0.5 μg/ml 5-FU	$p = 3.554x10^{-3}$	yes
Control	0.025 μg/ml CDDP + 1μg/ml 5-FU	$p = 3.155x10^{-5}$	yes
0.025 μg/ml CDDP	0.025 μg/ml CDDP + 0.1 μg/ml 5-FU	$p = 4.53 \times 10^{-1}$	no
0.025 μg/ml CDDP	0.025 μg/ml CDDP + 0.25 μg/ml 5-FU	$p =1.854\ x10^{-1}$	no
0.025 μg/ml CDDP	0.025 μg/ml CDDP + 0.5 μg/ml 5-FU	$p =5.845\ x10^{-2}$	no
0.025 μ g/ml CDDP	0.025 μg/ml CDDP + 1 μg/ml 5-FU	$p =3.803\ x10^{-2}$	yes

Table 8: Statistical analysis was performed on the results obtained in the clonogenic assay in which DMBA-OC-1R cells were treated with free drugs, to compare the effects of (a) 0.025 μg/ml CDDP + x μg/ml 5-FU against the control and (b) 0.025 μg/ml CDDP +x μg/ml 5-FU against 0.025 μg/ml CDDP.

Fig. 15 demonstrates the effect on the colony-forming ability of cells exposed for 96 hours to immunomicrospheres containing encapsulated drugs. As was observed in Fig. 14, the results as demonstrated in Fig. 15, also suggest that the addition of 5-FU modulates the effect of CDDP. The addition of 5-FU immunomicrospheres together with CDDP immunomicrospheres not only enhances, but emphasises the synergism between the two drugs. When comparing cells treated with 5-FU and CDDP with those treated only with CDDP, a significant difference is observed. However, a greater significance can be observed when cells treated with 5-FU and CDDP is compared to untreated cells (Table 9).

The comparative results between free drug and sustained drug release indicates the possibility of greater clinical outcome when employing immunomicrospheres containing drugs as compared to free drug alone. This is supported in the results observed by the cell survival growth studies in the following section.

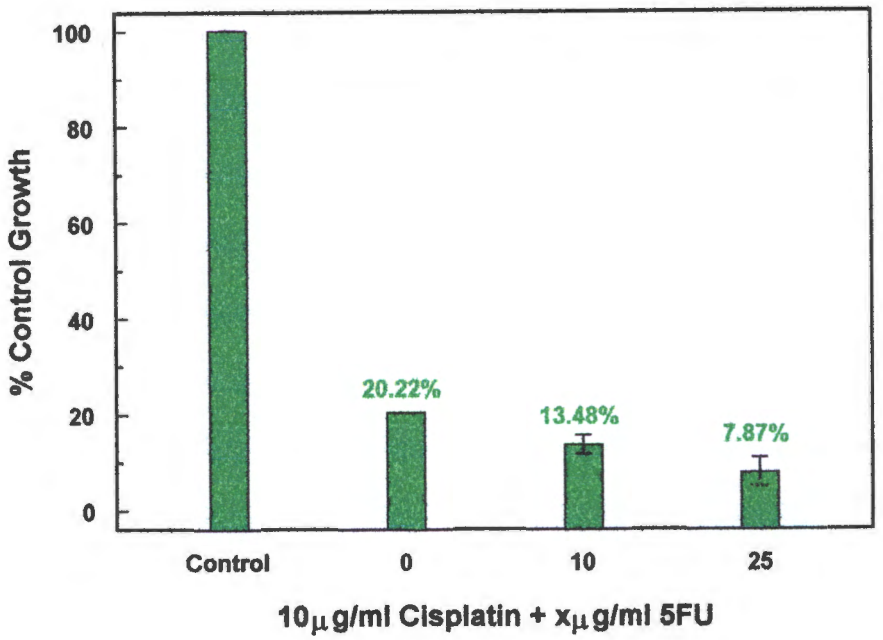


Fig. 15: Clonogenicity of DMBA-OC-1R cells cultured for 14 days in soft agar following 96 hour exposure to either no drugs (control), 10 μg/ml CDDP alone or 10 μg/ml CDDP in combination with either 10 μg/ml 5-FU or 25 μg/ml 5-FU. These drugs were encapsulated in albumin immunomicrospheres.

First parameter	Second parameter	p-Value	Significant difference
Control	10 μg/ml CDDP	$p = 1.146x10^{-5}$	yes
Control	10 μg/ml CDDP + 10 μg/ml 5-FU	$p = 1.688x10^{-5}$	yes
Control	10 μg/ml CDDP + 25 μg/ml 5-FU	$p = 2.565x10^{-5}$	yes
10 μg/ml CDDP	10 μg/ml CDDP + 10 μg/ml 5-FU	$p = 2.572x10^{-2}$	yes
10 μg/ml CDDP	10 μg/ml CDDP + 25 μg/ml 5-FU	$p = 1.417x10^{-2}$	yes

Table 9: Statistical analysis was performed on the results obtained in the clonogenic assay in which DMBA-OC-1R cells were treated with encapsulated drugs, to compare the effects of (a) 10 μg/ml CDDP + x μg/ml 5-FU against the control and (b) 10 μg/ml CDDP +x μg/ml 5-FU against 10 μg/ml CDDP.

5.4.2. Cell survival growth curves

Cell survival growth curves were generated for free drug and microspheres (non-antibody-linked and antibody-linked). Fig. 16(a) depicts the survival of cells over a 7 day period following 24hours exposure of exponentially growing DMBA-OC-1R cells to varying concentrations of 5-FU together with or without 0.025 μg/ml CDDP. A dose-dependent response is observed when the cells are treated with either 5-FU (Fig. 16 a & b) or CDDP (data not shown). The addition of increasing concentrations of 5-FU to 0.025 μg/ml CDDP significantly inhibits the survival of cells in a dose-dependent manner (Fig. 16(b) and Table 10). As observed in the clonogenic assays, the synergistic action between the two drugs, is once again evident. Cell survival is reduced to 21.95% (4.56-fold reduction) when cells are treated with 0.025 μg/ml CDDP only. Total cell kill is achieved when 1 μg/ml 5-FU is added together with 0.025 μg/ml CDDP. This represents a further 24,66-fold reduction in survival.

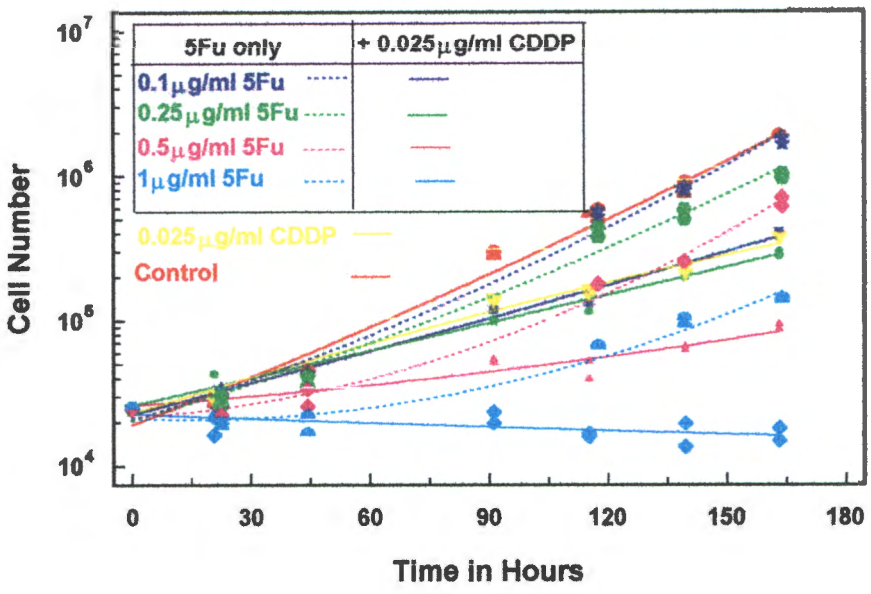


Fig. 16a: Cell survival growth curves for DMBA-OC-1R cells treated for 24hours with 0.1 μg/ml 5-FU (dark blue), 0.25 μg/ml 5-FU (green), 0.5 μg/ml 5-FU (pink), 1 μg/ml 5-FU (light blue) together with (unbroken line) or without (dotted line) 0.025 μg/ml CDDP. After the 24 hour treatment, cells were plated in 35 mm dishes and their growth was monitored. At each time point, cells from duplicate dishes were enumerated after they had been versened off the dishes.

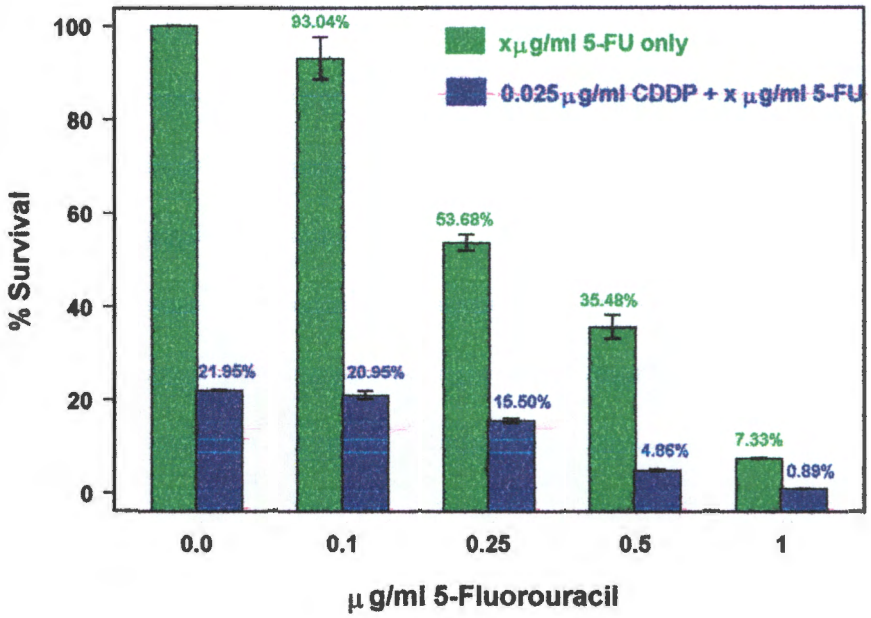


Fig. 16b: Dose-response curves for varying concentrations of 5-FU on its own (green bars) and 5-FU together with 0.025 μg/ml CDDP (blue bars) were generated using the data from the 168 hour time point in Fig. 16a. Cell survival was calculated as a percentage of the control cell growth.

First parameter	Second parameter	p-Value	Significant difference
Control	0.025 μg/ml CDDP	$p = 3.693 \times 10^{-6}$	yes
0.1 μg/ml 5-FU	0.025 μg/ml CDDP + 0.1 μg/ml 5-FU	$p = 3.876 \times 10^{-3}$	yes
0.25 μg/ml 5-FU	0.025 μg/ml CDDP + 0.25 μg/ml 5-FU	$p = 2.265 \times 10^{-3}$	yes
0.5 μg/ml 5-FU	0.025 μg/ml CDDP + 0.5 μg/ml 5-FU	$p = 6.877 \times 10^{-3}$	yes
1 μg/ml 5-FU	0.025 μg/ml CDDP + 1 μg/ml 5-FU	$p = 9.339 \times 10^{-4}$	yes
Control	0.1 μg/ml 5-FU	$p = 2.56 \times 10^{-1}$	no
Control	0.25 μg/ml 5-FU	$p = 1.408 \times 10^{-3}$	yes
Control	0.5 μg/ml 5-FU	$p = 1.552 \times 10^{-3}$	yes
Control	1 μg/ml 5-FU	$p = 3.566 \times 10^{-6}$	yes
Control	0.025 μg/ml CDDP + 0.1 μg/ml 5-FU	$p = 1.156 \times 10^{-4}$	yes
Control	0.025 μg/ml CDDP + 0.25 μg/ml 5-FU	$p = 4.008 \times 10^{-5}$	yes
Control	0.025 μg/ml CDDP + 0.5 μg/ml 5-FU	$p = 3.988 \times 10^{-6}$	yes
Control	0.025 μg/ml CDDP + 1 μg/ml 5-FU	$p = 8.246 \times 10^{-7}$	yes

Table 10: Statistical analysis was performed on the results obtained in the cell survival growth curve assays after 168 hours (Fig. 16b) in which DMBA-OC-1R cells were treated with free drugs, to compare the effects of (a) 0.025 μg/ml CDDP + x μg/ml 5-FU against varying concentrations of 5-FU; (b) varying concentrations of 5-FU against the control and (c) 0.025 μg/ml CDDP +x μg/ml 5-FU against the control.

Exposure of cells to 400x CDDP (10 µg/ml) encapsulated in albumin microspheres for 96 hours (in comparison to 0.025 µg/ml in the free form for 1 hour), did not succeed in total cell kill (Fig. 17a & b) as CDDP leaks very slowly from the microspheres, as can be seen from the leak profile for CDDP (Fig. 13). Cell death, however, was enhanced by the addition of 5-FU (Fig. 17a & b). Despite the addition of 10 μg/ml 5-FU and 10 μg/ml CDDP, cells were able to recover (Fig. 17a), though at a slower rate than without 5-FU. Cell survival was reduced significantly by 42-fold to 1.2% when 25 μg/ml 5-FU was added together with CDDP (Table 11 & Fig. 17b).

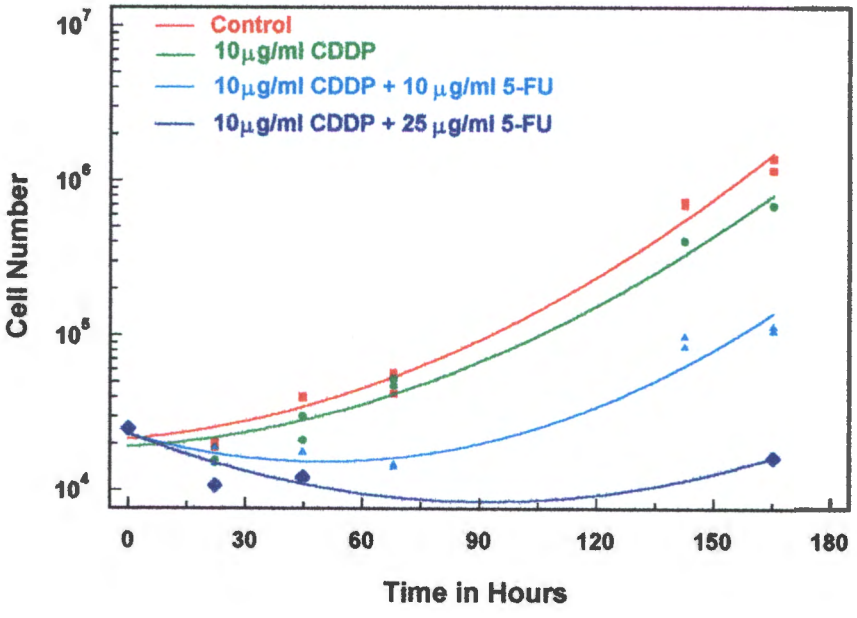


Fig. 17a: Effect of combination therapy with CDDP and 5-FU immunomicrospheres on the survival of DMBA-OC-1R cells. Cells were exposed for 96 hours to either 10 μg/ml CDDP (green), 10 μg/ml CDDP + 10 μg/ml 5-FU (light blue) or 10 μg/ml CDDP + 25 μg/ml 5-FU (dark blue). Recovery of the cells was monitored over a period of 7 days and compared to that of cells that were not exposed to drugs (red).

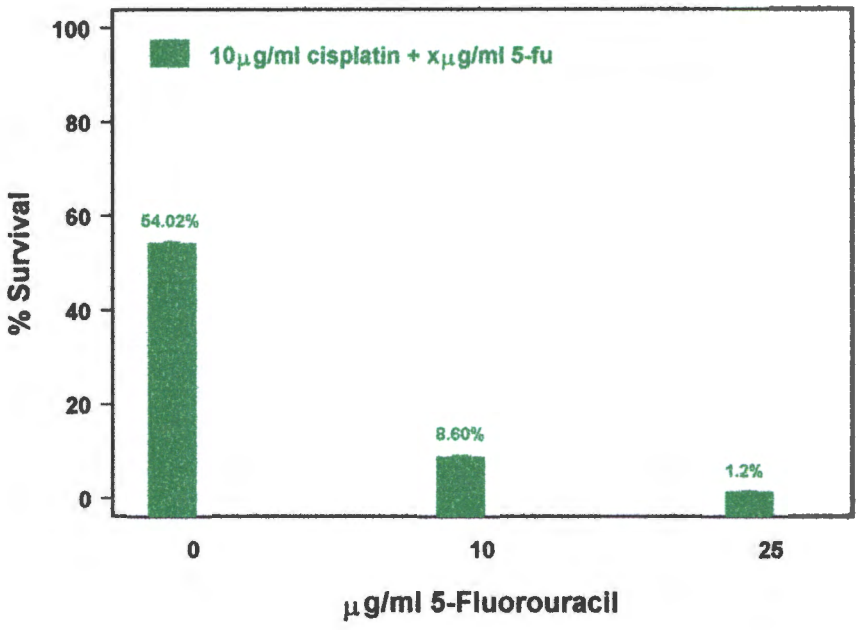


Fig. 17b: Effect of increasing the dosage of 5-FU on cells that were treated simultaneously with CDDP. This graph was generated by using the data from the 168 hour timepoint in Fig. 17a. Cell survival was calculated as a percentage of the survival of control cells.

First parameter	Second parameter	p-Value	Significant difference
10 μg/ml CDDP	10 μg/ml CDDP + 10 μg/ml 5-FU	$p = 5.378 \times 10^{-5}$	yes
10 μg/ml CDDP	10 μg/ml CDDP + 25 μg/ml 5-FU	$p = 1.367 \times 10^{-5}$	yes
10 μg/ml CDDP + 10 μg/ml 5-FU	10 μg/ml CDDP + 25 μg/ml 5-FU	$p = 1.358 \times 10^{-3}$	yes

Table 11: Statistical analysis was performed on the results obtained in the cell survival growth curve assays after 168 hours (Fig. 17b) in which DMBA-OC-1R cells were treated with encapsulated drugs, to compare the effects of (a) 10 μg/ml CDDP + x μg/ml 5-FU against 10 μg/ml CDDP and (b) 10 μg/ml CDDP + 25 μg/ml 5-FU against 10 μg/ml CDDP +10 μg/ml 5-FU.

The efficiency of targeted immunomicrospheres in comparison to untargeted microspheres in causing cell death was investigated. There was a significant decrease in cell survival to 4.03% when cells were exposed to antibody-targeted immunomicrospheres (Fig. 18b & Table 12). Untargeted microspheres had no growth inhibitory effect on the cells (Fig. 18a). These results demonstrate that immunomicrospheres are more effective in delivering drug directly to the cells than those which are not linked to cell-specific monoclonal antibodies.

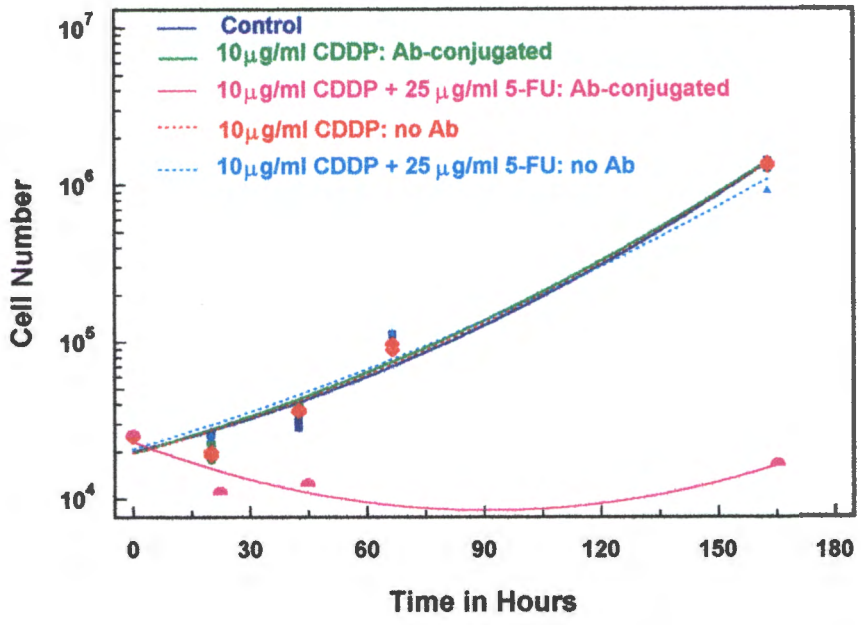


Fig. 18a: Survival profiles of cells exposed to untargeted and antibody-targeted microspheres. DMBA-OC-1R cells were exposed for 96 hours to either no drug (dark blue), 10 μg/ml CDDP + 25 μg/ml 5-FU encapsulated in either antibody-targeted microspheres (pink) or untargeted microspheres (light blue). Cell survival was observed over a period of 7 days. (Ab=antibody)

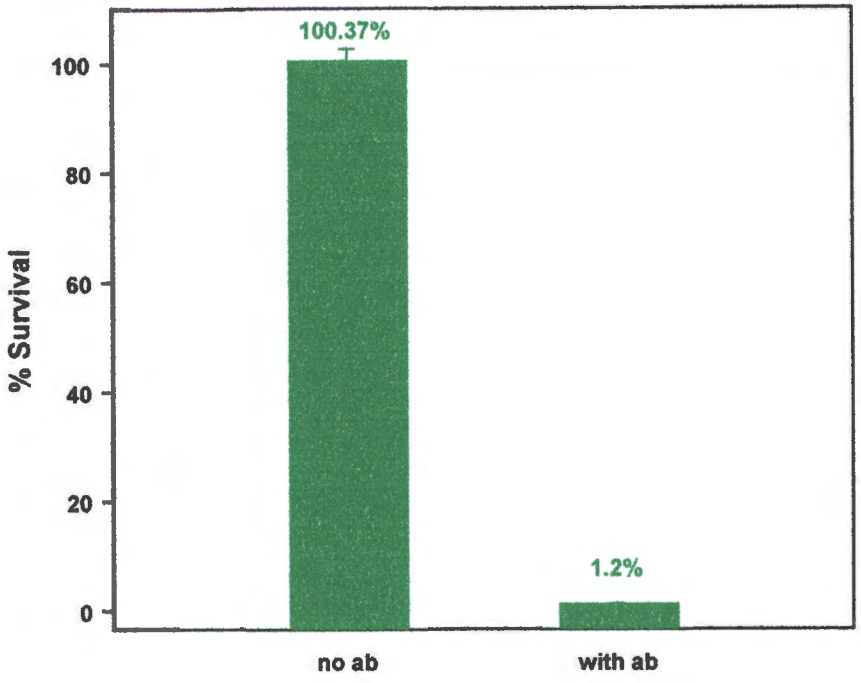


Fig. 18b: Inhibition of cell survival by immunomicrospheres. The recovery of cells treated with 10 μg/ml CDDP and 25 μg/ml 5-FU encapsulated in untargeted and antibody-targeted microspheres was determined by calculating the survival at 168 hours as a percentage of the control cell growth. (Ab = antibody)

First parameter	Second parameter	p-Value	Significant difference
Control	10 μg/ml CDDP + 25 μg/ml 5-FU (untargeted)	$p = 2.553 \times 10^{-1}$	no
Control	10 μg/ml CDDP + 25 μg/ml 5-FU (antibody-targeted)	$p = 2.402 \times 10^{-3}$	yes

Table 12: Statistical analysis was performed on the results obtained in the cell survival growth curve assays after 168 hours (Fig. 18b) in which DMBA-OC-1R cells were treated with encapsulated drugs, to compare the effects of untargeted and targeted microspheres against untreated cells (control)

5.4.3 MTT Assays

The *in vitro* cytotoxicity of CDDP and 5-FU, either alone or in combination, as well as either in the free drug or encapsulated form, was evaluated by using the MTT assay.

The viability of DMBA-OC-1R cells was assessed after continuous exposure to free drug for 24, 48 and 120 hours, as described in Section 4.8.2 in Chapter 4. The results were expressed as % cell survival of the control cells (untreated cells). The results, as demonstrated in Fig. 19 (free drug), showed that after 24 hours (Fig. 19a) no significant effect was observed. However, after 48 hours (Fig. 19b), a significant trend towards cell cytotoxicity was observed which was well established after 120 hours (Fig. 19c). Once again the observation is made that when the amount of 5-FU added to the cells is increased together with a constant amount of CDDP, cell survival decreases, reiterating the modulation of CDDP cytotoxic activity by 5-FU, as seen in the results of the clonogenic assays and cell survival growth curves. In Fig. 19c, we observed that total cell kill was achieved when 0.5 μg/ml 5-FU was added to the cell cultures, irrespective of the amount of CDDP that was added.

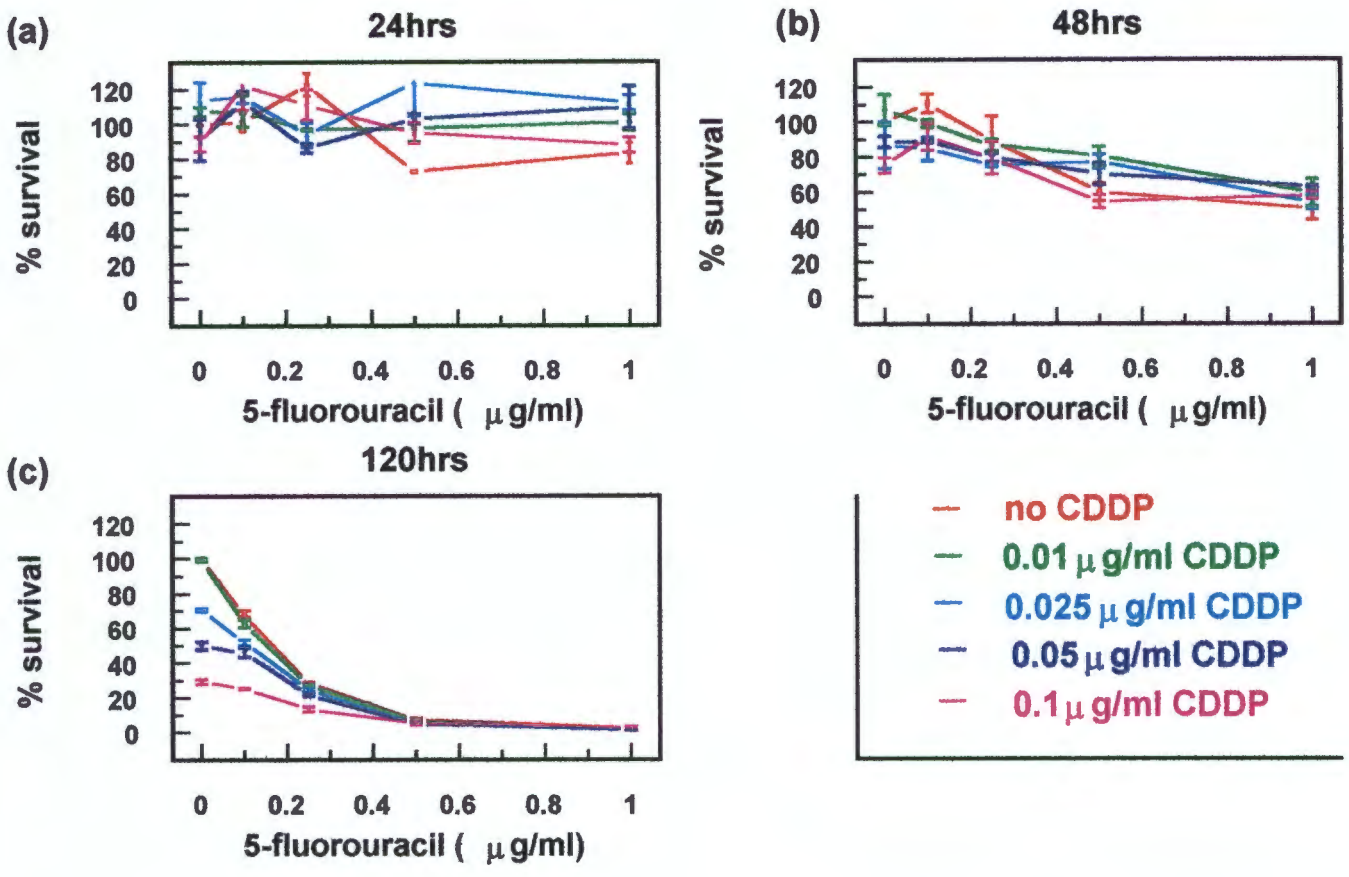


Fig. 19: Dose response curves for DMBA-OC-1R cells. The viabilty of DMBA-OC-1R cells was assesed by the MTT assay after continuous exposure to free drug for (a) 24 hours, (b) 48 hours and (c) 120 hours. Cells were exposed to no CDDP (red), 0.01 μg/ml (green), 0.025 μg/ml (light blue). 0.05 μg/ml (dark blue) and 0.1 μg/ml CDDP together with varying concentrations of 5-FU (error bars: SEM)

Fig. 20 illustrates the survival of DMBA-OC-1R cells for 168 hours after exposure for 96 hours to 10 μg/ml CDDP and varying concentrations of 5-FU contained in albumin microspheres which were either linked to antibody or not . Once again the modulation of CDDP by 5-FU is dose-dependent. This echoes the trend seen in the cell survival growth curves (Fig. 17a). Significant cell death was observed after 96 hours of treatment when antibody-linked immunomicrospheres were employed as compared to non-antibody-linked microspheres at all concentrations. The results further indicate that the use of immunomicrospheres show an enhanced effect of cell kill as compared to microspheres without antibody.

The comparative results between free drug and sustained drug release from immunomicrospheres indicates the possibility of greater clinical outcome as immunomicrospheres can be loaded with drugs at very high concentrations without exhibiting serious cytotoxicity as compared to free drug where dosage may be limited.

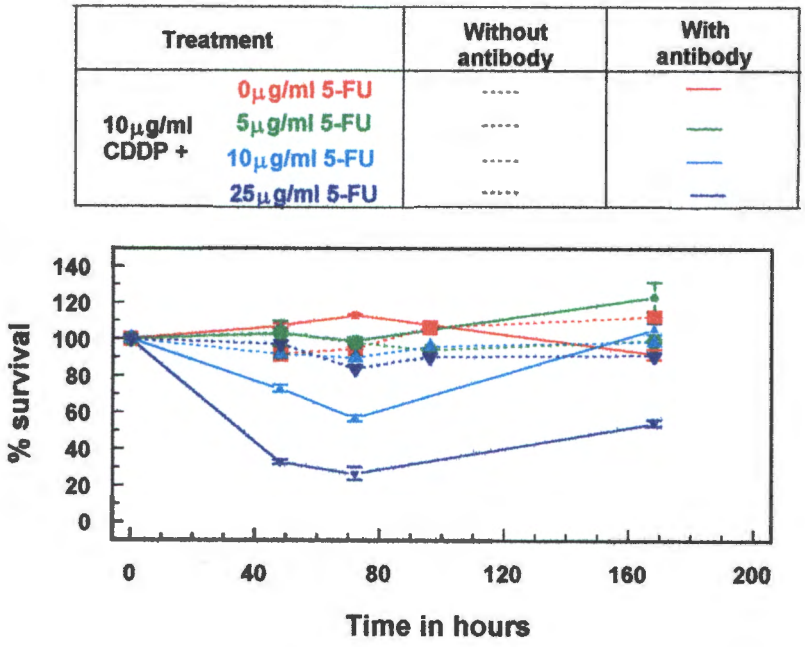


Fig. 20: Survival curves for DMBA-OC-1R cells as assessed by the MTT assay after exposure for 96 hours to encapsulated drugs. Cells were exposed to 10 μg/ml CDDP only (red), or 10 μg/ml CDDP + 5 μg/ml 5-FU (green), 10 μg/ml CDDP + 10 μg/ml 5-FU (light blue) or 10 μg/ml CDDP + 25 μg/ml 5-FU (dark blue) encapsulated in albumin microspheres which were either linked to antibody (unbroken lines) or not linked to antibody (dotted lines).

5.4.4 Micronuclei

The mode of action of CDDP is to form intrastrand cross-links in the DNA of cells (Lippard,1982). Cells are, however, capable of repairing this DNA damage. The inclusion of 5-FU in the treatment regimen is known to inhibit this repair and thereby enhances and ensures cell death. Micronuclei (Fig. 21), the presence of which is an indicator of chromosomal damage, have been induced in DMBA-OC-1R cells to assess the cytotoxicity of CDDP and 5-FU at a molecular level.

DMBA-OC-1R cells were exposed to either free drug for 1 hour or 24 hours or to

drugs encapsulated in albumin microspheres for 96 hours. After treatement they were arrested in their mitotic state by the addition of 2 µg/ml Cytochalasin B for 24 hours. Micronuclei were enumerated in binucleated cells after the cells had been fixed and stained with acridine orange (see Chapter 4, Section 4.8.3). Results were expressed as the number of micronuclei per 500 binucleated cells. The background level of micronuclei in untreated DMBA-OC-1R cells is 87.99 +/- 6.331 (SEM) as determined from results obtained from 15 experiments.

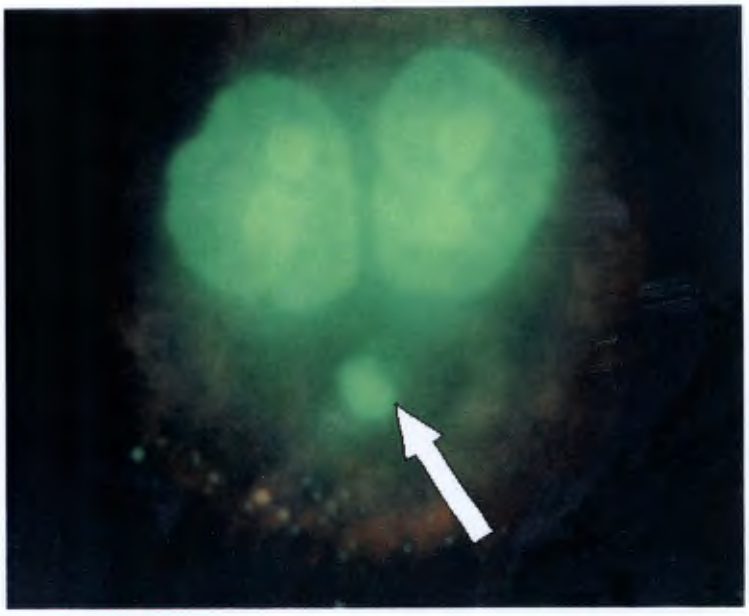


Fig. 21. The mutagenicity of CDDP and 5-FU is demonstrated by the induction of micronuclei (arrow) in binucleated DMBA-OC-1R cells.

Fig. 22 depicts the frequency of micronuclei in cells after exposure to either CDDP or 5-FU. Cells were exposed to the same concentrations of 5-FU for either 1 or 24 hours. A significant difference was observed with concentrations of 2.5-, 5- and 10 μg/ml 5-FU when the exposure time was increased (Table 13). Although significant differences were observed when cells were exposed for 24 hours to increasing dosages of 5-FU (Table 14), they were not as pronounced as those observed in the cell growth survival studies where cells were treated for 24 hours with lower concentrations of 5-FU ranging from 0 to 1 μg/ml in the free form (Fig. 16a & 16b), even when the dosage was increased to 10 μg/ml 5-FU in the micronucleus assay. The effect of 5-FU at the molecular level seems to reach a plateau at 5 μg/ml. This can be ascribed to the fact that 5-FU, unlike CDDP, does not cause DNA-strand breakage. A dose-dependent effect is observed with a 24 hour exposure to CDDP with significant increases in micronuclei at concentrations above 0.01 μg/ml (Table 15). CDDP doses above 0.1 μg/ml resulted in the production of degenerated cells

and increase in the number of micronuclei per cell, which made enumeration difficult.

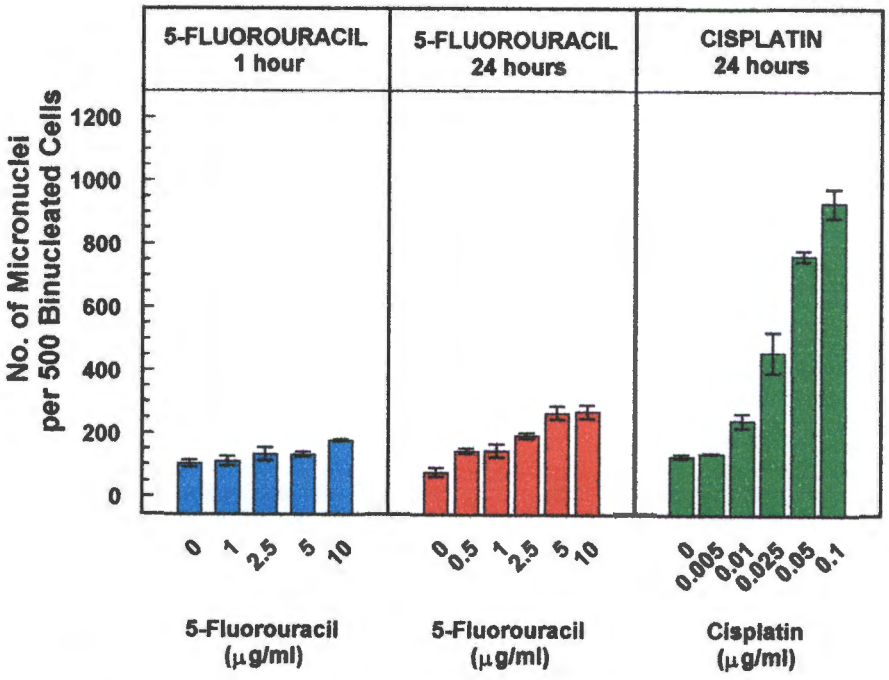


Fig. 22. Effect on the micronuclei frequency in DMBA-OC-1R cells when exposed to varying concentrations of 5-FU for (a) 1hour and (b) 24 hours and (c) varying concentrations CDDP for 24 hours. Bars indicate SEM.

First parameter	Second parameter	p-Value	Significant Difference
Control	Control	$p = 1.584 \times 10^{-1}$	no
1 μg/ml 5-FU	1 μg/ml 5-FU	$p = 7.283 \times 10^{-2}$	no
2.5 μg/ml 5-FU	2.5 μg/ml 5-FU	$p = 7.794 \times 10^{-3}$	yes
5 μg/ml 5-FU	5 μg/ml 5-FU	$p = 2.269 \times 10^{-4}$	yes
10 μg/ml 5-FU	10 μg/ml 5-FU	$p = 8.928 \times 10^{-4}$	yes

Table 13: Statistical analysis was performed on the results obtained in the micronuclei assay to compare the effects of treatment on the induction of micronuclei in DMBA-OC-1R cells with free drugs for 1 hour (first parameter) and for 24 hour exposure (second parameter).

First parameter	Second parameter	p-Value	Significant Difference
Control	0.5 μg/ml 5-FU	$p = 1.486 \times 10^{-2}$	yes
Control	1 μg/ml 5-FU	$p = 4.843 \times 10^{-2}$	yes
Control	2.5 μg/ml 5-FU	$p = 2.144 \times 10^{-3}$	yes
Control	5 μg/ml 5-FU	$p = 1.780 \times 10^{-3}$	yes
Control	10 μg/ml 5-FU	$p = 1.480 \times 10^{-3}$	yes

Table 14: Statistical analysis was performed on the results obtained in the micronuclei assay to compare the effects of 24 hour exposure to varying concentrations of 5-FU against no drug on the induction of micronuclei in DMBA-OC-1R cells.

First parameter	Second parameter	p-Value	Significant Difference
Control	0.005 μg/ml CDDP	$p = 2.404 \times 10^{-1}$	no
Control	0.01 μg/ml CDDP	$p = 9.099 \times 10^{-3}$	yes
Control	0.025 μg/ml CDDP	$p = 7.074 \times 10^{-3}$	yes
Control	0.05 μg/ml CDDP	$p = 4.619 \times 10^{-6}$	yes
Control	0.1 μg/ml CDDP	$p = 6.520 \times 10^{-5}$	yes

Table 15: Statistical analysis was performed on the results obtained in the micronuclei assay to compare the effects of 24 hour exposure to varying concentrations of CDDP against no drug on the induction of micronuclei in DMBA-OC-1R cells.

Cells were treated with varying concentrations of CDDP together with 1 μg/ml of 5-FU for 24 hours (Fig. 23). The modulatory effect of 5-FU, as observed in the previous assays (i.e clonogenic assay, cell growth survival assay and the MTT assay), was once again evident here as the frequency of micronuclei increased with the addition of 1 μg/ml 5-FU. This was especially evident at the lower concentrations of CDDP of 0.005 μg/ml and 0.01 μg/ml (Table 16).

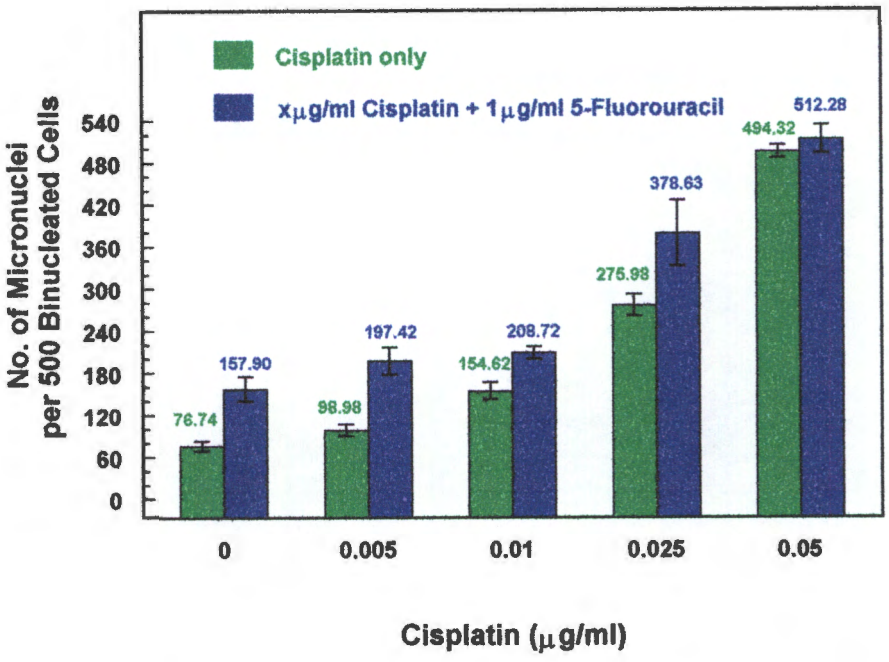


Fig. 23: Effect of 24 hours of exposure of DMBA-OC-1R cells to varying concentrations of CDDP (green bars) and varying concentrations of CDDP together with 1 μg/ml 5-FU (blue bars) on the frequency of micronuclei. Bars indicate SEM.

First parameter	Second parameter	p-Value	Significant Difference
Control	No CDDP + 1 μg/ml 5-FU	$p = 1.247 \times 10^{-2}$	yes
0.005 μg/ml CDDP	0.005 μg/ml CDDP + 1 μg/ml 5-FU	$p = 9.758 \times 10^{-2}$	yes
0.01 μg/ml CDDP	0.01 μg/ml CDDP + 1 μg/ml 5-FU	$p = 2.185 \times 10^{-2}$	yes
0.025 μg/ml CDDP	0.025 μg/ml CDDP + 1 μg/ml 5-FU	$p = 1.075 \times 10^{-1}$	no
0.05 μg/ml CDDP	0.05 μg/ml CDDP + 1 μg/ml 5-FU	$p = 4.611 \times 10^{-1}$	no

Table 16: Statistical analysis was performed on the results obtained in the micronuclei assay in which DMBA-OC-1R cells were treated with free drugs for 24 hours, to compare the effects of x μg/ml CDDP + 1 μg/ml 5-FU against varying concentrations of CDDP.

Fig. 24 illustrates a significant dose-dependent increase in the frequency of micronuclei when cells are exposed to10 μg/ml CDDP together with increasing amounts of 5-FU encapsulated in albumin microspheres for 96 hours. This trend was observed in the clonogenic assays (Fig.15), cell survival growth curves (Fig. 17a & b) and the MTT (Fig. 20) assays and further emphasises the modulatory effect of 5-FU.

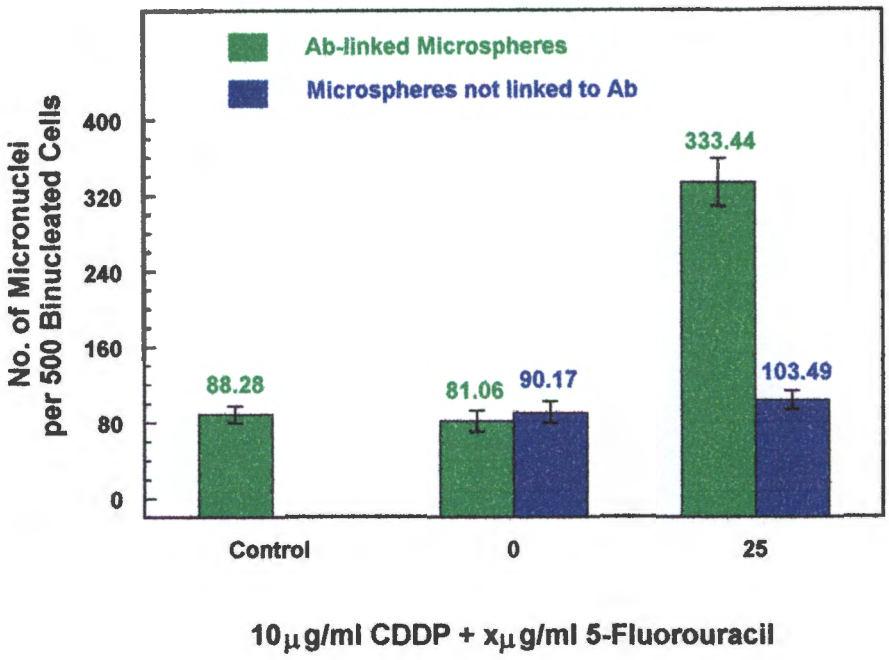


Fig. 24. Effect of 96 hour exposure of DMBA-OC-1R cells to 10 μg/ml CDDP together with varying concentrations of 5-FU, encapsulated in microspheres, on the frequency of micronuclei. Bars indicate SEM.

A significant 3.2-fold more micronuclei were found in cells that were treated for 96 hours with 10 μg/ml CDDP and 25 μg/ml 5-FU encapsulated in albumin microspheres that have been linked to antibodies and are therefore targeted to the cells. This is in comparison to the same amount of drugs in microspheres that have not been linked to antibodies (Fig. 24 and Table 17). This result once again emphasises the efficiency of targeted microspheres in achieving greater cell kill.

First parameter	Second parameter	p-Value	Significant Difference
Control	10 μg/ml CDDP (ab-linked)	$p = 6.440 \times 10^{-1}$	no
Control	10 μg/ml CDDP + 25 μg/ml 5-FU (ab-linked)	$p = 8.438 \times 10^{-4}$	yes
Control	10 μg/ml CDDP (non-linked)	$p = 3.267 \times 10^{-1}$	no
Control	10 μg/ml CDDP + 25 μg/ml 5-FU (non-linked)	$p = 9.053 \times 10^{-1}$	no
10 μg/ml CDDP (ab-linked)	10 μg/ml CDDP (non-linked)	$p = 2.077 \times 10^{-1}$	no
10 μg/ml CDDP + 25 μg/ml 5-FU (ab-linked)	10 μg/ml CDDP + 25 μg/ml 5-FU (non-linked)	$p = 9.867 \times 10^{-4}$	yes

Table 17: Statistical analysis was performed on the results obtained in the micronuclei assay in which DMBA-OC-1R cells were treated with encapsulated drugs, to compare the effects of (a) antibody-linked and non-linked microspheres against the control and (b) non-linked microspheres against linked microspheres.

5.5 The tumour host

5.5.1 Drug distribution studies

The time-concentration profiles of CDDP and 5-FU at concentrations of 20 mg/kg 5-FU and 25 mg/kg CDDP, administered intravenously as free drugs via the tail vein of a group of female Wistar rats, are presented in Fig. 25 (See Chapter 4, Section 4.9.1). As can be seen many organs of the reticuloendothelial system, as well as excreta (not shown due to difficulty in obtaining reliable samples), show high concentrations of both drugs. It was, however, observed that the disposition of the drugs in the ovary were of sufficient concentration to enable a therapeutic effect (9.3% for CDDP and 6.0% for 5-FU respectively)

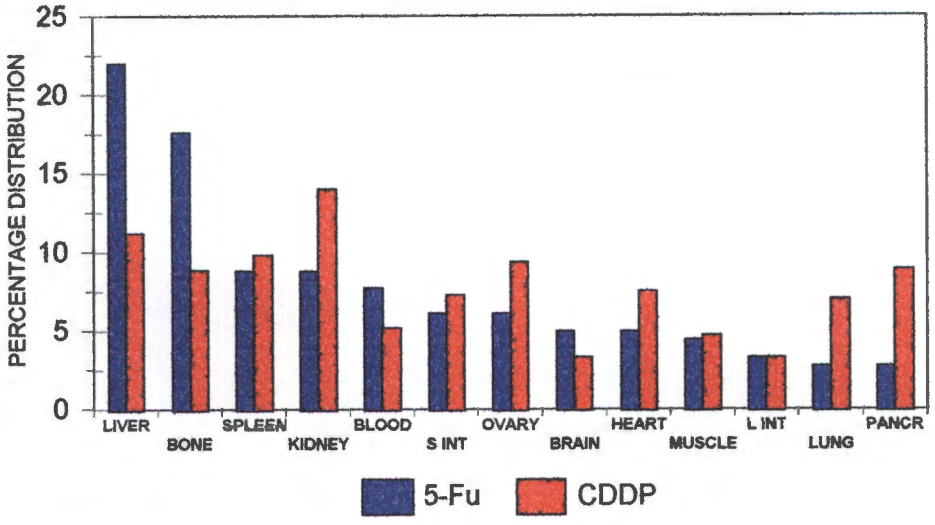


Fig. 25: Tissue distribution of 5-Fu and CDDP following intravenous drug administration (s int = small intestine; l int = large intestine; pancr = pancreas).

5.5.2 Blood chemistry profiles to assess the effects of 5-fluororuracil and cisplatin (free drugs and encapsulated) in female Wistar rats

As reported in Chapter 4, Section 4.9.2, three groups of female Wistar rats were used to obtain blood chemistry profiles in order to observe the toxic effects of the drugs on the organs. Group I rats were injected intraperitoneally with free drugs, Group II rats with immunomicrospheres containing the drugs and Group III rats were the control animals

who received no drug treatment.

Fig. 26a and Fig. 26b depict the serum urea and creatine levels in the three groups of animals. As demonstrated, these parameters indicated only mild renal toxicity which corrected itself within three weeks after drug administration. Fig. 26c, Fig. 26d and Fig. 26e shows the serum gamma glutamyl transferase, alanine aminotransferase and aspartate levels respectively in the three groups of animals. These parameters indicated mild hepatocellular toxicity which corrected itself three to four weeks after drug administration. Fig. 26f and Fig. 26g demonstrate serum alkaline phosphatases and lactate dehydrogenase levels in the three groups of rats. These parameters showed hepatocellular reactive changes which were sustained for a longer time period in the immunomicrosphere-inoculated group. Correction to normal levels after free drug administration took three to four weeks whereas in the case of drug-loaded immunomicrosphere administration, it took 8 to 9 weeks.

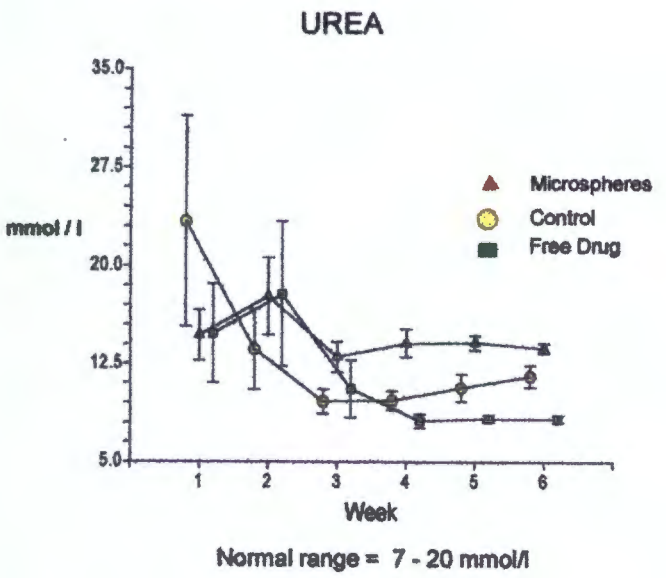


Fig. 26a

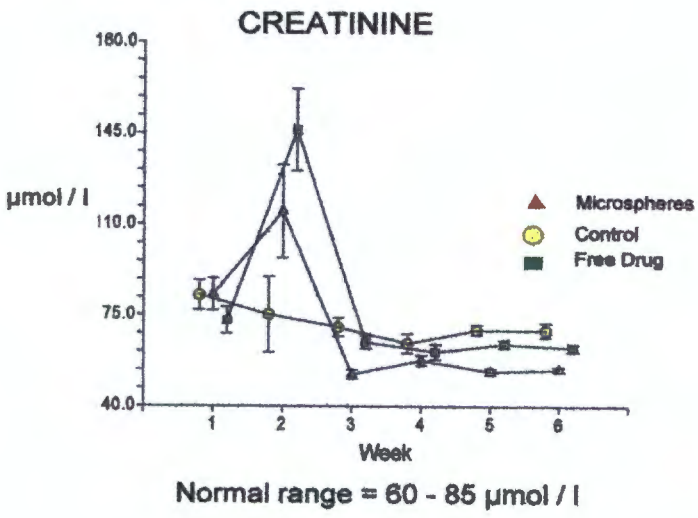
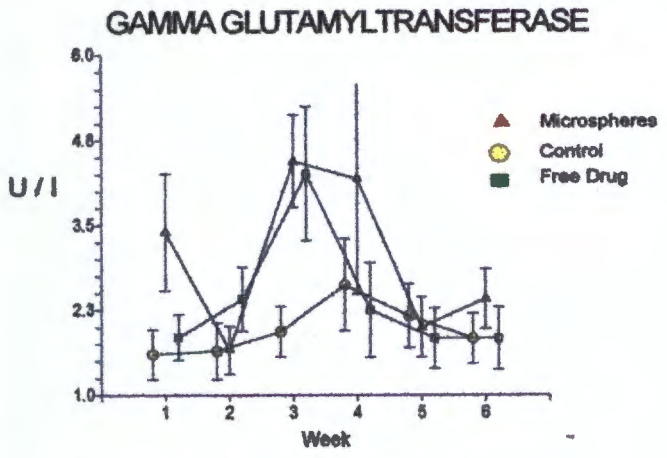


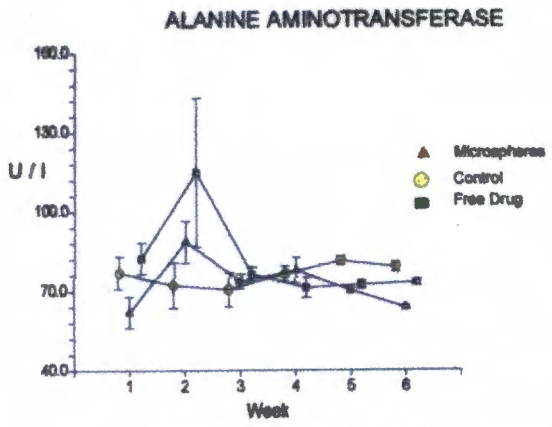
Fig. 26b

Figs. 26a & b, Serum urea and creatinine status after administration of 5-Fu and CDDP (as free drug and drug-loaded immunomicrospheres).



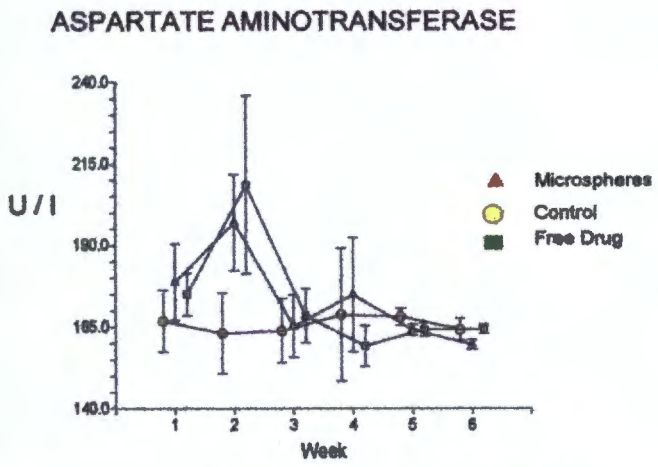
Normal range = 1.2 -3.1 U / l

Fig 26c



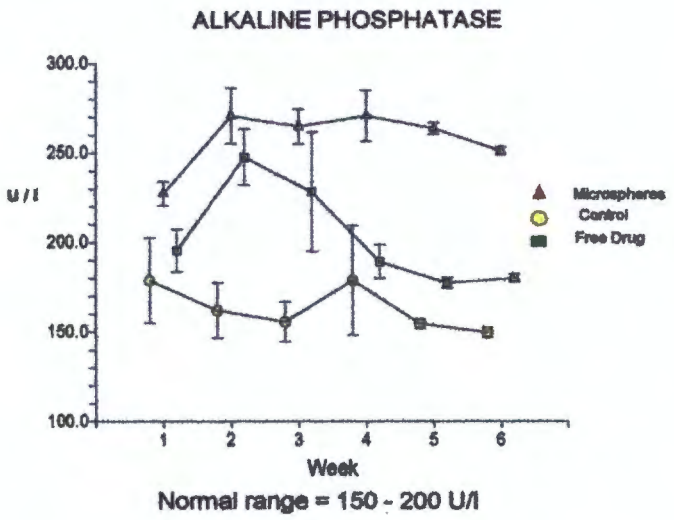
Normal range = 65 - 85 U /l

Fig. 26d



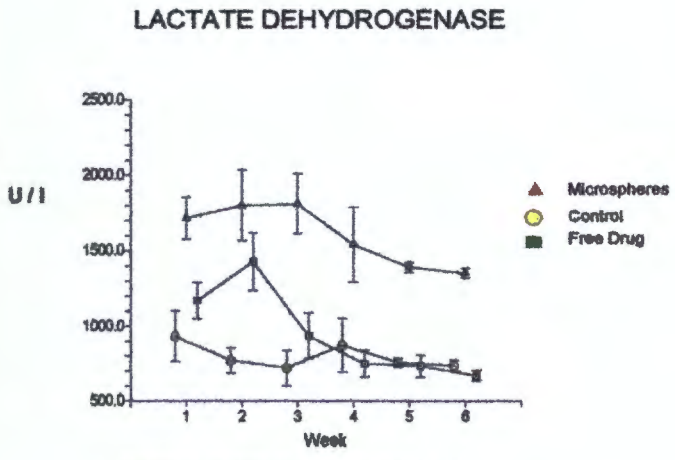
Normal range = 150 -185 U/l

Fig. 26e



Normal range = 150 - 200 U/l

Fig. 26f



Normal range = 650 -1000 U / l

Fig. 26g

Figs. 26 c, d, e, f & g. Serum gamma glutamyltransferase, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase status after administration of 5-Fu and CDDP (as free drug and drug-loaded immunomicrospheres).

5.5.3. Haematological profiles to assess the effects of 5-fluorouracil and cisplatin (free drugs and encapsulated) in female Wistar rats

As in the previous section, the same group of female Wistar rats were used to obtain haematological profiles in order to assess the toxic effects of the drugs on the bone marow. Fig. 27a, Fig. 27b and Fig. 27c depicts the erythrocyte count, haemoglobin and haematocrit levels which indicated mild erythropenia in the free drug and encapsulated drug groups. Haemoglobin levels returned to normal, 4 weeks after drug administration. Fig. 27d shows the total leucocyte count. Mild leukopenia was observed in the free drug group. Fig. 27e shows the platelet count which remained normal throughout the drug administration period. No thrombocytopenia was observed in any of the three groups of rats.

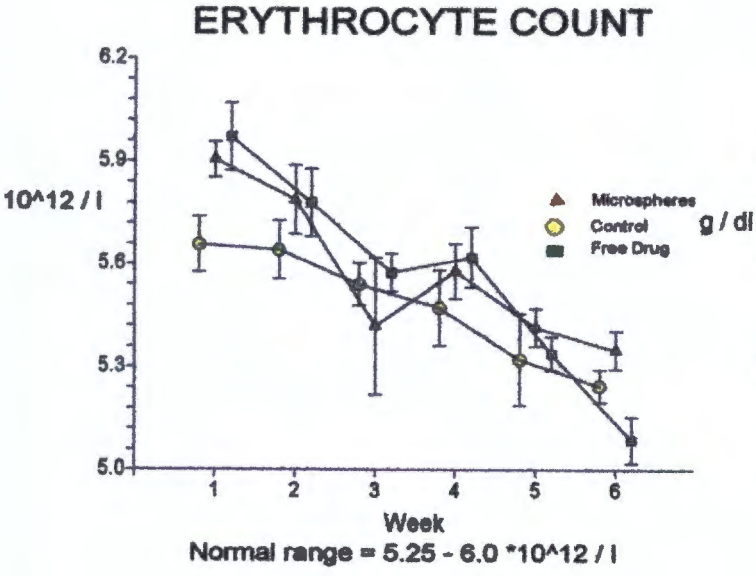


Fig. 27a

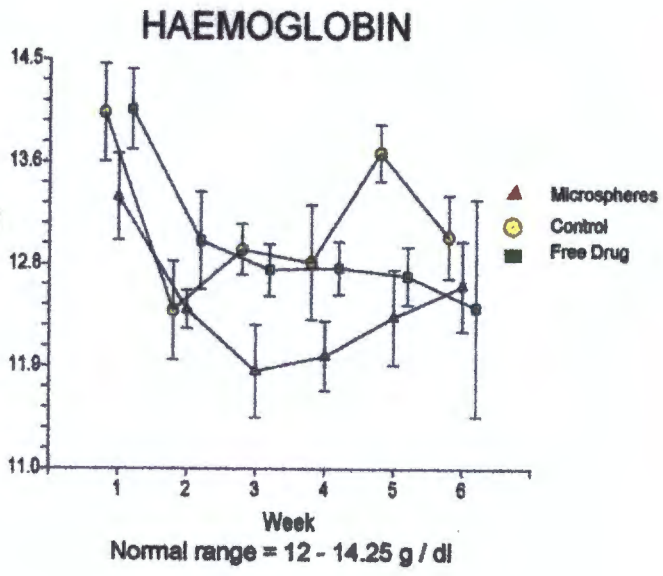


Fig. 27b

Figs 27 a& b. Erythrocyte count and haemoglobin levels after administration of 5-Fu and CDDP(as free drug and drug-loaded immunomicrospheres).

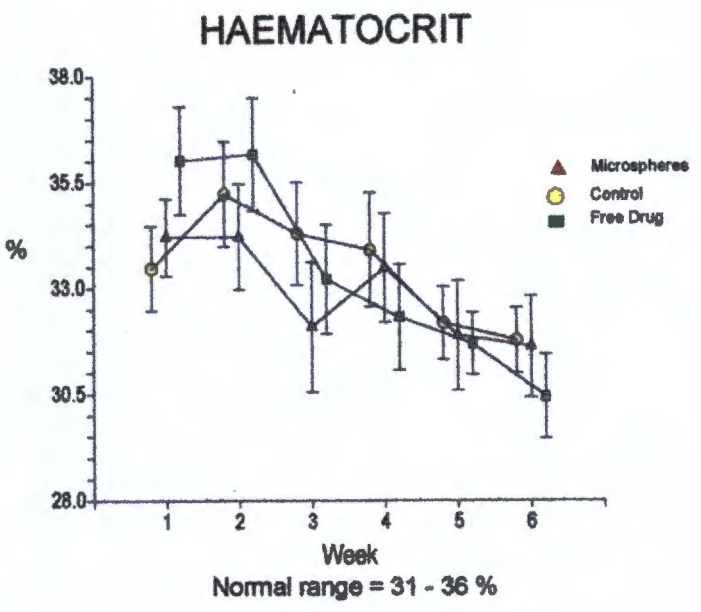


Fig. 27c

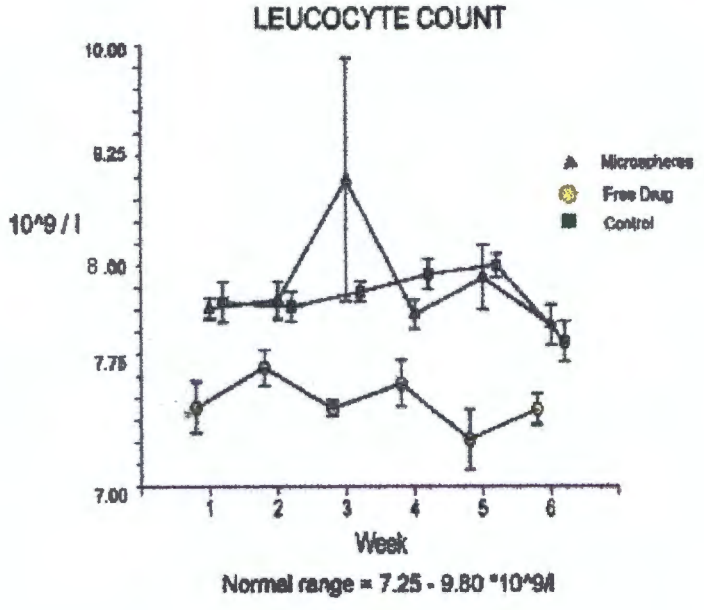


Fig. 27d

(Please note. The coded markers of the leucocyte count are different to all other parameters)

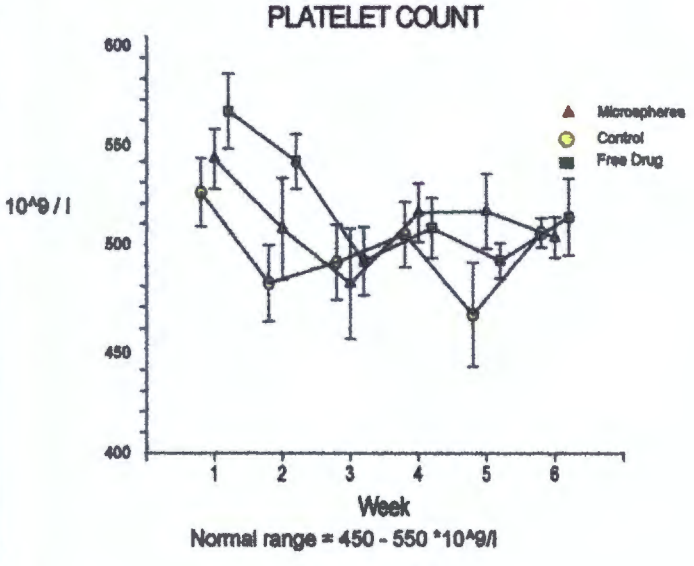


Fig. 27e

Figs. 27c, d & e: Haematocrit levels, leucocyte and platelet counts after administration of 5-Fu and CDDP (as free drug and drug-loaded immunomicrospheres).

5.6 The tumour model

Primary transplantable adenocarcinomas were induced in female Wistar rats as described in Chapter 4, Section 4.9.3. Abdominal distention due to retention of bloody ascitic fluid was noted within 10 days following the intraperitoneal inoculation of 4×10^6 DMBA-OC-1R cells. The ascites contained tumour cells, as well as neutrophils, lymphocytes, histiocytes and mesothelial cells (Fig. 28).

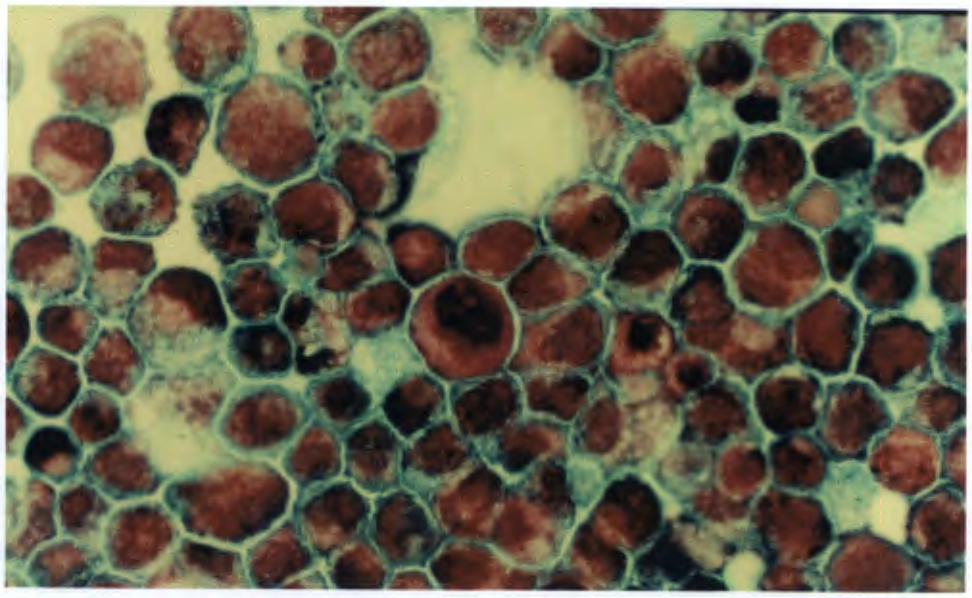


Fig. 28: General overview of neoplastic cells, lymphocytes and histiocytes in the ascitic fluid (Papanicolaou stain, original mag x40)

The primary tumours (Fig. 29) were allowed to establish themselves for 14 days before removal for transplantation. The omentum was the first location in which the tumour appeared following inoculation, after which the tumour disseminated throughout the peritoneal cavity.

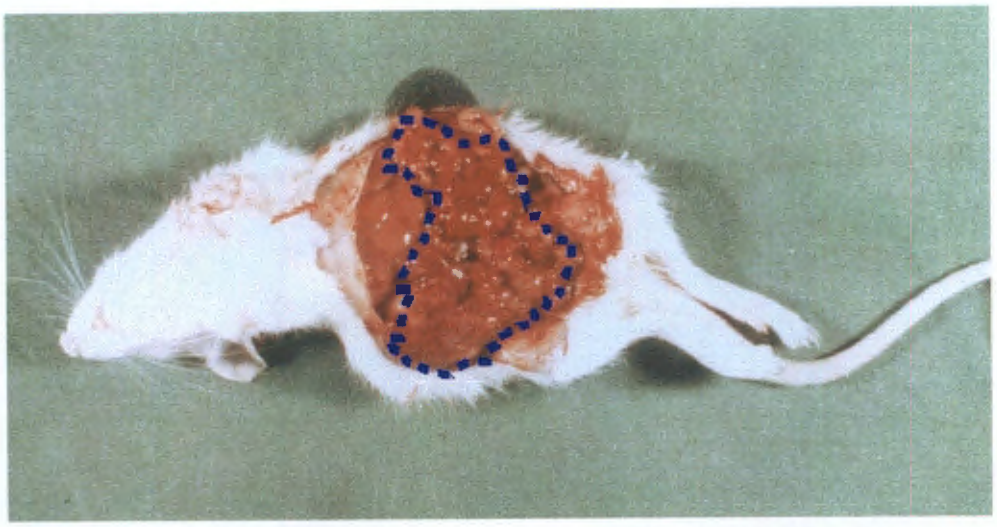


Fig. 29: Bulky tumour (demarcated) in the peritoneal cavity of a female Wistar rat ten days after primary inoculation with $4x10^6$ DMBA-OC-1R cells

Intraperitoneally transplanted 3 mm tumour cubes were allowed to develop into secondary tumours for 10 days before treatment commenced. Tumour development was monitored by X-rays to detect secondary tumour mass formation (Fig. 30) before treatment commenced.

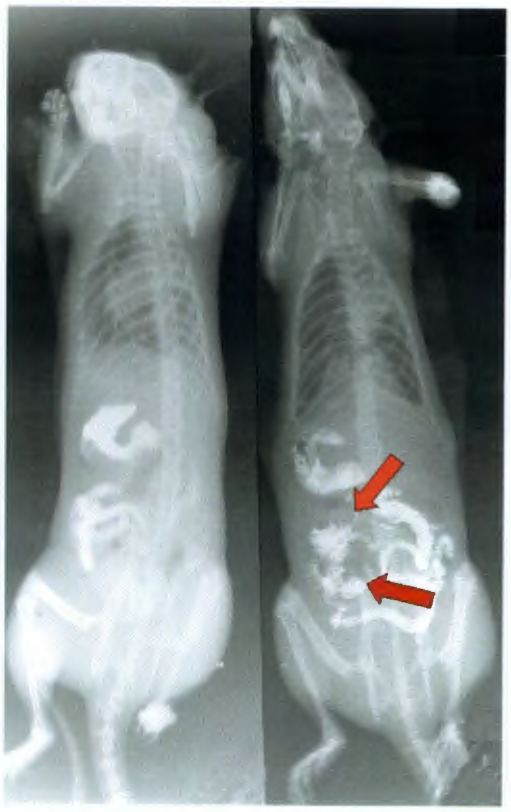


Fig. 30: X-rays of control rat(left) without tumour and test rat(right) with secondary tumour mass(arrows) following intraperitoneal transplantation of 3mm^3 primary tumour fragment after 10 days (barium contrasted).

5.6.1 Histology of the tumour

Tissue specimens taken for light- and transmission electron microscopy of both primary and secondary tumours revealed identical histological features. Generally, they were undifferentiated neoplasms characterised by high mitotic activity. Definitive evidence of a solid pattern was noted and glandular structures were scarcely seen. However, the transmission electron microscopy showed microvilli on the cell surface which suggests an adenocarcinoma.

Neoplastic cells were arranged mostly in sheets or cords supported by a scant delicate connective tissue stroma with a fine reticular appearance with obvious vascular development (Fig. 31 & 32). The neoplastic cells were round or oval, becoming elongated in certain areas with large pleomorphic, vesicular nuclei, prominent eosinophilic nucleoli and abundant eosinophilic cytoplasm (Fig. 33). Occasional histiocytes were also observed in the neoplasms with occasional areas of tissue necroses and haemorrhages. Mucin was negative in the tumour cells as demonstrated

by the Alcian blue PAS and Mucicarmine stains (Data not shown). Neovascularization of the tumour was also found to be well-developed(Fig. 32).

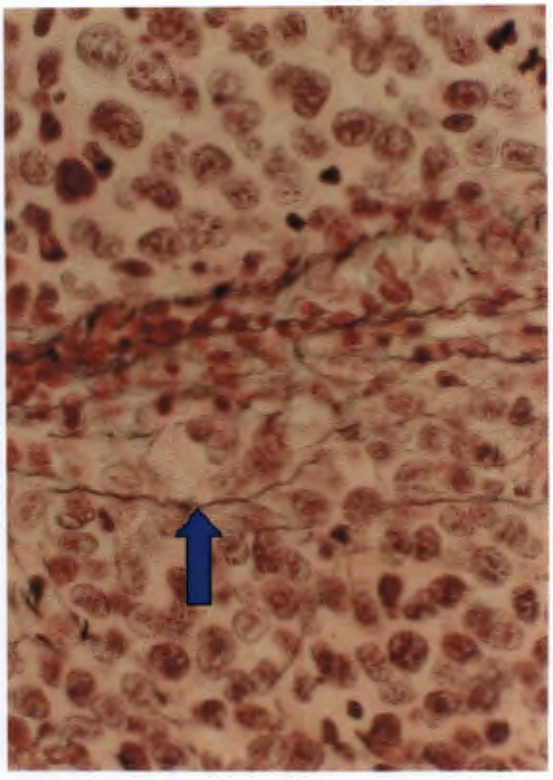


Fig. 31: Histological section showing reticular fibres(arrow) in tumour tissue stroma (Gordon & Sweet's stain, original mag x40)

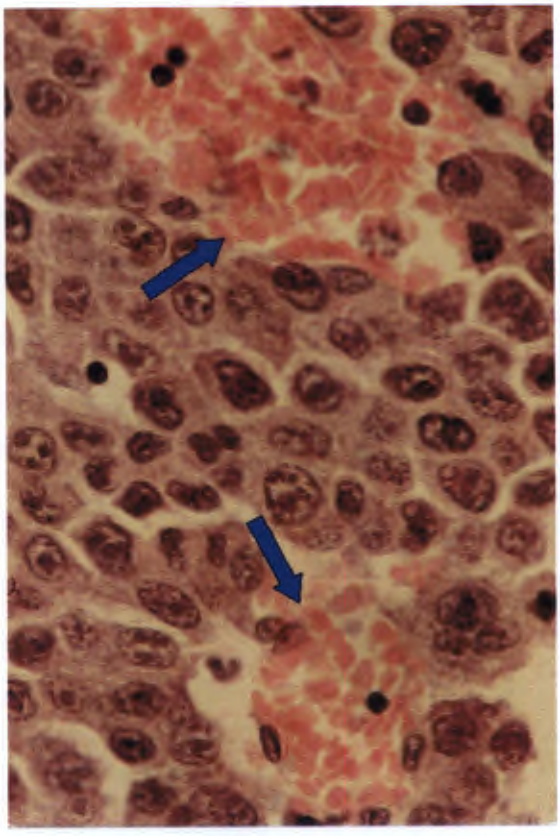


Fig. 32: Capillary sprouts(arrows) in tumour tissue stroma (Haematoxylin & Eosin, original mag x40)

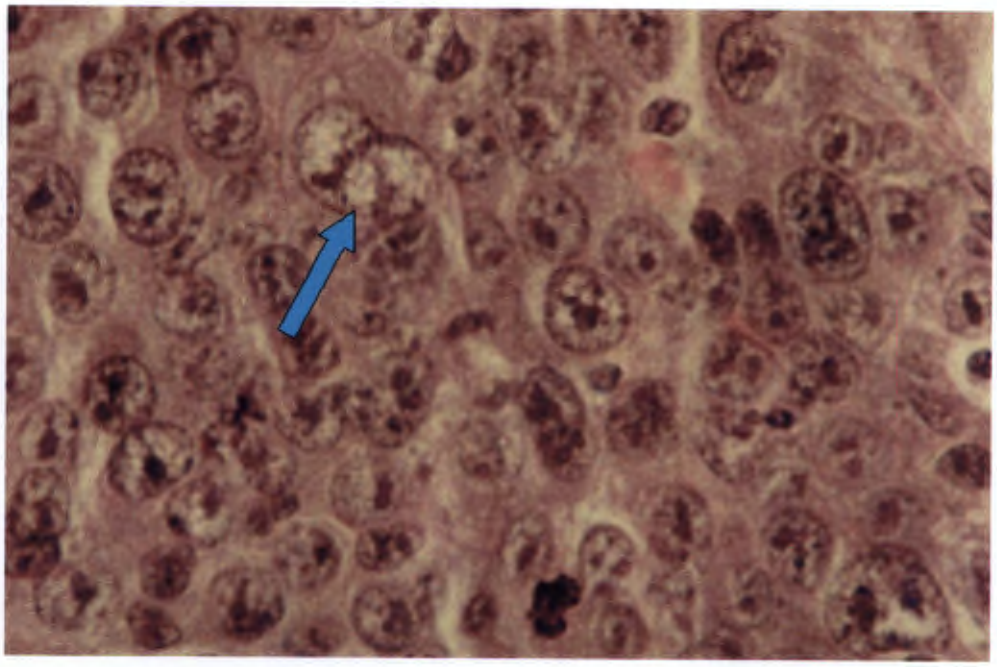


Fig. 33: Histology of transplanted, DMBA-induced ovarian tumour. Tumour cells proliferate in solid growth pattern. Tumour cells show large, pleomorphic nuclei, prominent eosinophilic nuclei and eosinophilic cytoplasm. Occasional histiocytes(arrow) were observed (Haematoxylin & Eosin, original mag x40)

Electron microscopically, tumour cells had a nucleus in the middle of the cells. The nuclei with prominent nucleoli were mostly round in shape, however, some of them were markedly deformed (Fig. 34). Microvilli were noted on the cell surface. In the cytoplasm, rough endoplasmic reticulum, mitochondria and other cell organelles were well-developed (Fig. 35). Intercellularly, desmosome-like junctions were frequently seen, however in some areas contact with obscure intercellular junctions were observed (Fig. 36).

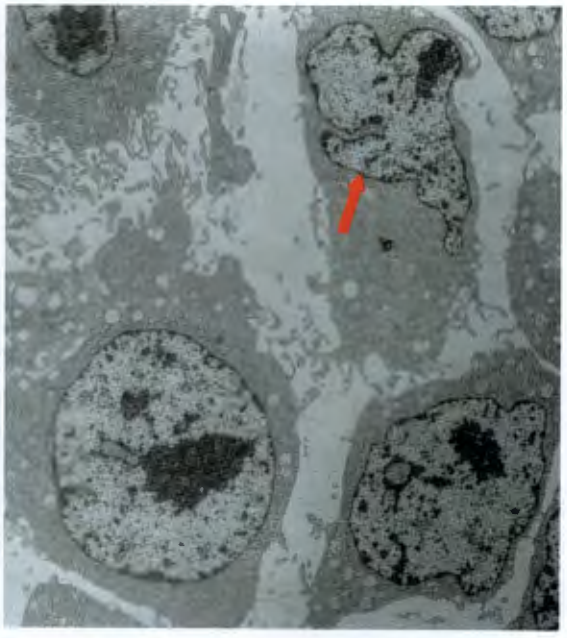


Fig. 34: Tumour cells showing large, centrally positioned nuclei, however, some were markedly deformed (arrow) (original mag x7500)

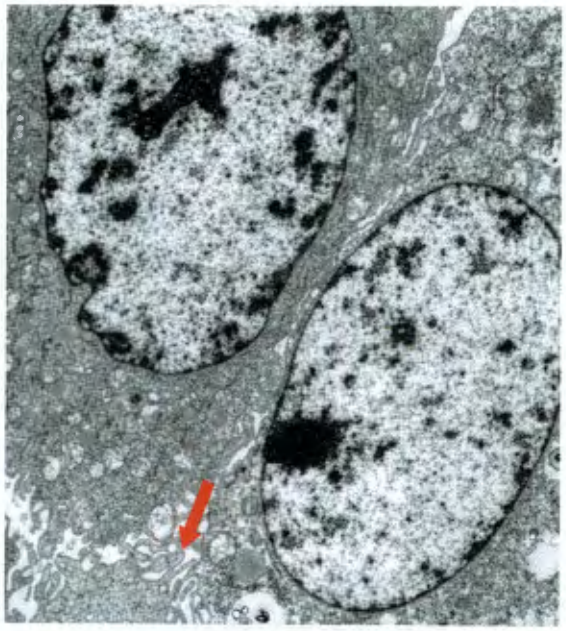


Fig. 35: Microvilli (arrow) on some tumour cell surfaces. Well developed cytoplasmic organelles are evident in this micrograph (original mag x12000)

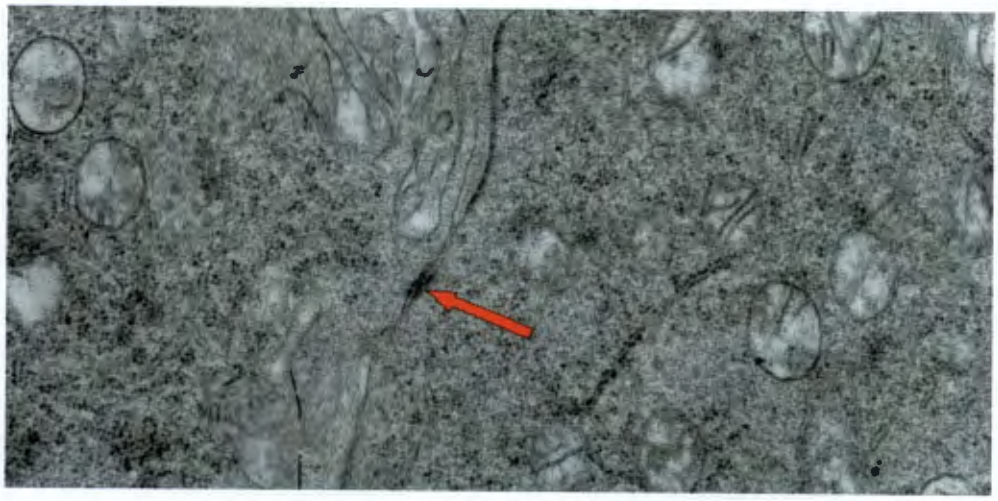


Fig. 36: Tumour cells showing intercellular desmosome-like structures(arrow) (original mag x36000)

5.6.2 Therapy studies

As described in Chapter 4, Section 4.9.3, primary tumour fragments, 3 mm^3 in size, were intraperitoneally transplanted into 4 groups of female Wistar rats. Secondary tumours were allowed to develop over a 10 day period before chemotherapeutic treatment commenced. No surgical debulkings were performed prior to treatment.

In an attempt to monitor disease progression or regression in the therapeutic groups of the tumour-bearing rats, superoxide dismutase (SOD) enzymes were quantified for each group as described in Chapter 4, Section 4.9.3.3. Fig. 37 shows the profiles obtained for each group monitored over a 90 day period.

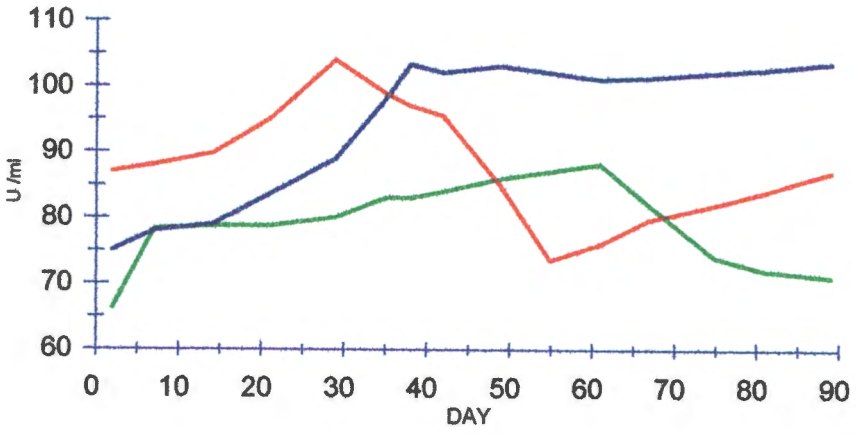


Fig. 37: Mean superoxide dismutase trends for 3 groups of animals:
a)Group IA = free drug group,
b) Group IIA = low dose immunomicrosphere group,
c)Group IIB = high dose immunomicrosphere group

As demonstrated in Fig. 38, the animal survival studies indicate the various outcomes of the three different treatment protocols employed and studied over the 90 day trial period, as well as the group of animals (control group) which received no treatment after primary tumour transplantation.

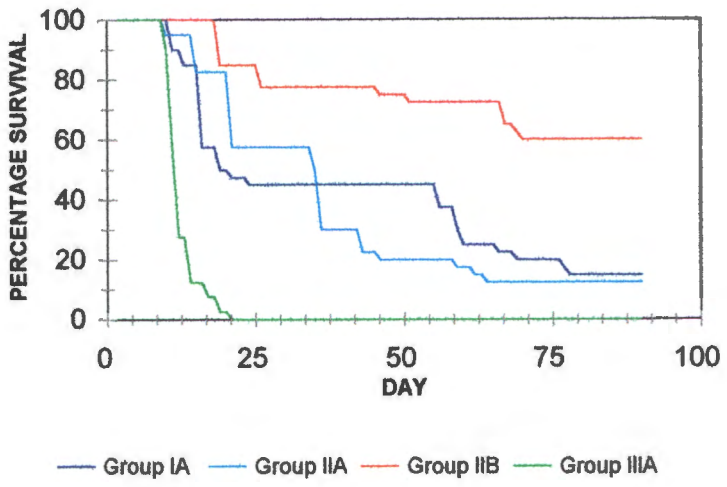


Fig. 38: Mean animal survival curves of animals with transplanted tumours followed by treatment:

a)Group IA = free drug group,
b) Group IIA = low dose immunomicrosphere group,
c)Group IIB = high dose immunomicrosphere group
d)Group IIIA = no treatment group

The maximum survival time for Group IIIA rats (no treatment group) who received no treatment, was 19 days. Gross examination of the peritoneal cavities of the animals who died showed extensive intraperitoneal disease, demonstrating massive hemoascites and peritoneal carcinomatosis with metastases to the liver and spleen. Diffuse studding of tumour deposits on the peritoneal surfaces, viscera and diaphragm was also noted.

Group IA rats (free drug group) were given an intraperitoneal combined bolus dose as free drugs at a dose of 5 mg/kg CDDP and 20 mg/kg 5-FU, followed by a repeat dose at the same concentrations 7 days later. The two separate doses spaced 7 days apart, was performed to reduce the effects of free drug toxicity. Survival studies of this group showed that only 14% of the animals survived the 90 day trial period. All animals who died showed the same peritoneal carcinomatosis as the untreated animals (Group IIIA).

Group IIA rats (low dose immunomicrosphere group) were given an intraperitoneal bolus dose of immunomicrospheres at a dose of 1 mg/kg CDDP and 4 mg/kg 5-FU. This group also indicated that only 14% of the animals survived the 90 day trial period. At post mortem, animals who died showed peritoneal carcinomatosis.

Group IIB rats (high dose immunomicrosphere group) were given an intraperitoneal bolus dose of immunomicrospheres at a dose of 10 mg/kg CDDP and 40 mg/kg 5-FU. Fig. 39 shows drug-containing immunomicrospheres attached to the tumour tissue in the peritoneal cavity. This group of animals showed a markedly improved survival period when compared with the previous treatment protocols with 60% of all animals surviving the 90 day trial period. Again the animals who died showed extensive peritoneal carcinomatosis.

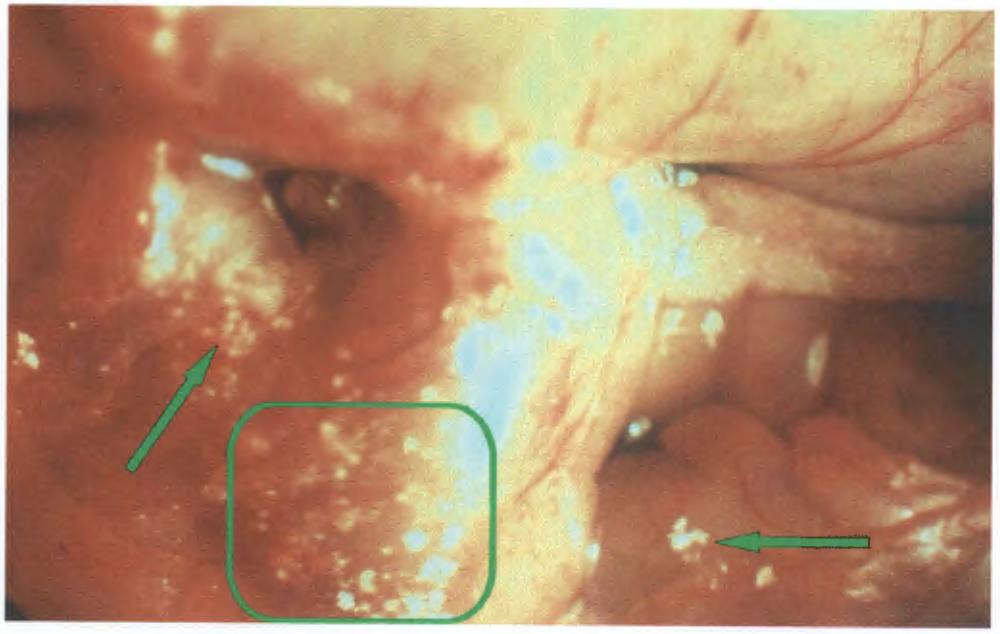


Fig. 39: Immunomicrospheres(blocked and arrows) attached to DMBA-OC-1R tumour tissue in the peritoneal cavity of a female Wistar rat

Chapter 6

Discussion

6.1 Feasibility of Human Serum Albumin microspheres as a drug delivery system for cisplatin and 5-fluorouracil

While drugs are available to combat a wide range of malignant diseases, the therapeutic efficacy of these agents is often diminished by their inability to gain access to the diseased site at the appropriate dosage.

In order to achieve a therapeutically relevant dose in tumour cells, the amount of drug required usually proves to be highly toxic to normal tissues and cells. Therefore, what is needed are vehicles capable of carrying the cytotoxic agent in a highly concentrated form directly to the tumour target, thus allowing for more efficient tumour cell kill while largely sparing surrounding normal tissues and cells.

Since the feasibility of artificial cells was first demonstrated by Chang in 1957, an increasing number of approaches in their preparation and application have become available. By employing an immunospecific drug delivery system such as monoclonal antibody conjugated albumin microspheres containing the cytotoxic agent, cisplatin, and separately, a system containing the antimetabolite, 5-fluorouracil, we demonstrated that the carriers can be targeted directly to tumour cells where the drugs were delivered and caused tumour cell death by interference with DNA synthesis (Butour & Macquet, 1977).

We also demonstrated that albumin microspheres containing therapeutic relevant concentrations of cisplatin and 5-fluorouracil, can readily be prepared by a heat-stabilization technique. Not only did we achieve to prepare microspheres in the size range of 1-5µm, but also found that they released the drugs in a sustained fashion. By employing the heat-stabilization technique, the reproducibility of the technique was confirmed, in that stable 1-5µm albumin microspheres were readily prepared in large batches by the method described by Truter (1995). We demonstrated that therapeutically relevant doses of cisplatin and 5-fluorouracil were released in sustained fashion and reached maximum levels in 14 days after incorporation in a plasma milieu and maintaining therapeutic levels for at least another 14 days. However, as Stella and Himmelstein (1980, 1985) showed, the retention of drugs at the site where they are

needed, is important if successful site specific delivery is to be followed by an improvement in therapeutic index and clinical effect of the drug.

Various approaches, such as chemical modulation of the drug carrier system, have been considered to achieve the above. In our studies, we opted to apply the concept of employing heat-stabilised albumin immunomicrospheres containing cisplatin and 5-fluorouracil as an alternative approach in the treatment of an ovarian adenocarcinoma model, DMBA-OC-1R, on which we performed a series of *in vitro* and *in vivo* studies.

We found heat-stabilised albumin immunomicrospheres containing cisplatin and 5-fluorouracil to:

* be relatively innocuous in terms of toxicity;
* be relatively non-antigenic within the body;
* be metabolizable within the body;
* possess high drug-entrapment properties;
* be capable of accommodating cisplatin and 5-fluorouracil in relatively high concentrations;
* be chemically stable in solution;
* be amenable to preparation in large batches , and
* allow the attachment of monoclonal antibodies to its surface.

It was thus possible for us to demonstrate the potential use of heat-stabilised human serum albumin immunomicrospheres for carrying the chemotherapeutic agents as they complied satisfactorily with the above-mentioned criteria as was also demonstrated by Cheng *et al.* (1993) and Hagiwara *et al.*(1997).

6.2 Monoclonal antibodies against DMBA-OC-1R ovarian adenocarcinoma

A rat ovarian adenocarcinoma cell line, DMBA-OC-1R, was obtained from the Department of Obstetrics and Gynaecology, Kurume University, Japan. This cell line was originally established in Wistar rats by employing the carcinogen, 7,12-dimethylbenz(a)anthracene by Kato *et al.*(1974), and characterised by others (Imaishi, 1992; Kataoka *et al.*, 1987; Sugiyama *et al.*, 1986). The cell line was found by these authors to resemble its human counterpart and also to be transplantable from one animal to another within the species with little or no change in the heterogeneity of the tumour. Our studies confirmed these findings and made it possible for us to raise two IgM monoclonal antibodies against the DMBA-OC-1R cell line in order to successfully

link to drug-containing albumin microspheres rendering immunomicrospheres.

6.2.1 Target specificity of anti-DMBA43 and anti-DMBA93

The answer to the success of employing immunomicrospheres in ovarian cancer therapy lies in the identification of ovarian tumour-specific antigens. A number of tumour-associated antigens have been identified by mouse monoclonal antibodies against ovarian cancer in humans and are used in clinical studies. Examples are CA-125, epithelial surface antigen (ESA), folate-binding protein, carcinoembryonic antigen (CEA), human milk fat globule (HMFG) and placental-type alkaline phosphatase (PLAP) (Bast *et al.,* 1990; Finkler *et al.,* 1988; Kuby, 1992; Tucker & Ward, 1988). These antigens are, however, also found in normal tissues and are therefore generally not suitable for use as targets for immunoconjugates. Attempts have been made to produce monoclonal antibodies with tumour-restricted specificity. MOV16, MOV18 and MOV20 were raised against an ovarian carcinoma which had not reacted with existing monoclonal antibodies (Isaacson & Wright, 1986).

We developed and partially characterised two stable hybridomas obtained from the fusion of spleen cells of BALB/c mice injected with the rat ovarian cell line, DMBA-OC-1R, and the mouse myeloma cell line, SP2. Two IgM antibodies, designated anti-DMBA43 and anti-DMBA93, were selected for their ability to bind membrane antigens on DMBA-OC-1R but not on NRK and RESF cells by means of screening using immunofluorescence techniques on live, unfixed cells. The indirect ELISA was found to be unsuitable for screening as intracellular antigens were also detected due to the permeabilisation by glutaraldehyde.
We were unsuccessful in our attempts to establish whether the two antibodies recognised different antigenic determinants by means of a reciprocal blocking inhibition test. This involved the detection of a FITC-conjugated antibody binding to the cells which had been previously blocked with the other antibody in a direct immunofluorescence assay on cells in suspension. This assay was previously used to establish that three monoclonal antibodies raised against a human osteosarcoma recognised different epitopes (Hosoi *et al.,* 1982).

The factors determining the clinical success of an immunoconjugate include the Ig class of the monoclonal antibody and the degree of its cross-reaction with normal tissue. Ideally the antibody should be tumour-specific and bind to its epitope with a high affinity (Gregoriadis, 1977; Hellstrom *et al.,* 1987). By immunofluorescence staining of acetone-

fixed cell lines and formalin-fixed, paraffin-embedded tissues of rats, we demonstrated that the two anti-DMBA monoclonal antibodies reacted with only DMBA-OC-1R rat ovarian carcinoma cells and not with normal rat tissues or cell lines. The antibodies reacted strongly with only one out of the five human ovarian cell lines and not at all with any of the other cell lines established from different origins and species. These results bode well for their use in coupling to albumin microspheres.

The anti-DMBA antibodies localised specifically in the paraffin wax sections of DMBA-OC-1R tumour were obtained from nude mice. Both antibodies were found to be IgM and may be thought to be less effective for antigenic coupling as IgM antibodies have lower affinities than the preferred IgG antibodies which also localise better in a tumour than IgM, due to their smaller molecular size. The pentameric IgM antibodies could have been fragmented into bivalent monomer IgMs in order to overcome this disadvantage. However, a loss of avidity and possibly also affinity may have ensued.

IgM antibodies are generally generated by carbohydrate antigens (Ritter, 1986). We were unable to determine whether the specificity was associated with carbohydrate residues by labelling for carbohydrate on a Western blot using the ECL method. Most of the antigens associated with ovarian carcinomas have been identified as high molecular weight glycoproteins with a carbohydrate content of more than 50% and are known as mucins (Burchell & Taylor-Papadimitriou; 1990; Graham *et al.;* 1996; Lloyd, 1993; Shimizu & Yamauchi, 1982). The immunogenicity of these molecules, due to their large size, in rodents has resulted in the generation of a number of monoclonal antibodies which have the potential in being developed into immunoconjugates. Immunoblot analysis has revealed that the molecular weight of molecules such as polymorphic epithelial mucins containing the epitopes, differs amongst different mucinous tumours. The variation in molecular weight can be explained by the fact that the mucins contain large domains of tandem repeats of amino acids rich in threonine, serine and proline (Burchell & Taylor-Papadimitriou, 1990; Graham *et al.*; 1996) containing five potential O-glycosylation sites and antibodies generally recognise epitopes of 4-5 amino acids. The polymorphic epithelial mucin which is encoded by the MUC1 gene, is expressed abundantly in carcinomas but is not a true mucin as it has a transmembrane domain. As a result of aberrant glycosylation, it has shorter sugar side chains, probably owing to the lack of B1-6GlcNAc transferase and a concurrent increase in α-2-3-sialyl transferase (Graham *et al.,* 1996). This results in the exposure of epitopes which are masked on normal mucin as well as the creation of novel carbohydrate epitopes.

However, Alcian Blue PAS and Mucicarmine stains performed on paraffin wax embedded sections of the DMBA-OC-1R tumour, were negative. These results suggest that the antibodies raised were not against carbohydrate antigens.

6.3 Effects of drug-containing immunomicrospheres on the DMBA-OC-1R cell line

The IgM monoclonal antibodies, anti-DMBA43 and anti-DMBA93, raised against the DMBA-OC-1R cell line were shown to readily attach itself to the cisplatin- and 5-fluorouracil-containing albumin microspheres when the carbodiimide method of Illum and Jones (1985) was employed.

Clonogenic assays performed by us demonstrated the synergistic effect of cisplatin and 5-fluorouracil and also that the 5-fluorouracil modulated the effect of cisplatin (Harstrick *et al.*, 1997 and Rooney *et al.*, 1985). Our results demonstrated that sustained drug release from targeted immunomicrospheres inhibited colony formation of DMBA-OC-1R cells in culture, to a much greater extent than when the drugs (in free form) were added to the cells. This finding was considered to be of major importance, as the possibility of a greater clinical outcome could possibly be expected. Also, these results were supported by the cell survival growth studies and micronuclei assays. Comparative free-drug and drug-containing immunomicrosphere and no antibody-linked microsphere profiles indicated that antibody-free microspheres had no growth inhibitory effect on the DMBA-OC-1R cells in culture. However, only about 1.2% of cells survived when they were exposed to the antibody-targeted immunomicrospheres. Cell survival was reduced significantly by 42 fold to 1,2% when the drug concentration was at 25µg/ml 5-fluorouracil and 10 µg/ml cisplatin.

These results thus demonstrated that the immunomicrospheres containing 5-fluorouracil and cisplatin are more effective in delivering drug directly to the tumour cells than those which are not linked to cell-specific monoclonal antibodies. When using free drugs, we demonstrated that total cell kill could be achieved when drug concentrations were 1 µg/ml 5-fluorouracil and 0.025 µg/ml cisplatin. However, these results may be misleading in the clinical setting as the toxicity to normal cells in the body should seriously be considered when applying this type of protocol.

In order to demonstrate the toxicity of cisplatin and 5-fluorouracil as free drug or

contained in immunomicrospheres, we performed the MTT assay which demonstrated an enhanced effect of cell kill by the drug-containing immunomicrospheres as compared to drug-containing microspheres without antibody. We further showed comparative results between free drugs and sustained drug release from immunomicrospheres which indicated the possibility of greater clinical outcome as microspheres can be loaded with drugs at very high concentrations, without causing serious cytotoxicity to normal cells and tissues as compared to free drugs where dosage may be limited. The cytotoxic effects to all major organs may be extrapolated from the drug distribution studies which we performed in Wistar rats, as well as the blood chemistry and haematology profiles obtained.

As regards the drug distribution studies when using free drugs, only 9.3% cisplatin and 6% 5-fluorouracil respectively were found in the ovaries, 2 hours after intravenous administration of a bolus dose of 20 mg/kg 5-fluorouracil and 2,5 mg/kg cisplatin. The highest levels most of drugs were found to have been deposited in vital organs of the haematopoeitic system and kidneys. Although only mild renal toxicity was observed when observing urea and creatinine status in the free drug and immunomicrosphere groups, correction to normal levels after free drug administration occured at a faster rate compared to the immunomicrosphere groups. However, it should be remembered that drugs were released in sustained fashion by the immunomicrospheres. Liver function tests showed the same phenomenon with these parameters indicating mild hepatocellular toxicity which corrected itself three to four weeks after administration of free drugs and eight to nine weeks in the case of immunomicrosphere administration. As regards the haematology, mild erythropenia and leukopenia were observed in both groups. Other parameters were normal.

It should, however, be kept in mind, that, in the clinical setting, repeated administration of free drugs as used in routine treatment protocols, will result in severe side-effects on the blood chemistry and haematology. This will also perpetuate the problem, as a reduction in dose due the toxicity of the drugs used, will neutralize any advantage of the therapy due to the disadvantage of dose reduction (Frei, 1995). In the case of an immunomicrosphere protocol, the procedure may be only a "once-off " bolus dose or at the most, fewer doses at longer time-intervals due to a sustained supply of drugs at the target site. In this situation, less long-term side-effects can be expected.

Therefore, pertaining to the above discussion, toxic side-effects should be weighed against survival and thus therapy protocols were investigated in the rodent tumour

model.

6.4 The Tumour Model

We studied the potential role of tumour-targeted albumin immunomicrospheres containing cisplatin and 5-fluorouracil in female Wistar rats with peritoneal ovarian adenocarcinoma. Tumour mass was observed by X-rays before treatment commenced. Histology of the tumour confirmed an ovarian adenocarcinoma.

Animal survival curves, as demonstrated in Chapter 5, Section 5.6.2, clearly showed that immunomicrosphere protocols had superior survival times as compared to those treated by a free drug protocol. Comparative results of animals treated intraperitoneally by a free drug protocol at a dose of 5 mg/kg cisplatin and 20 mg/kg 5-fluorouracil, followed by a repeat dose at the same concentrations showed a survival rate of 14% over a 90 day trail period. Animals treated with an intraperitoneal bolus dose of immunomicrospheres at a dose of 10 mg/kg cisplatin and 40 mg/kg 5-fluorouracil showed a survival rate of 60 % over the 90 day trial period.

Superoxide dismutase assays performed over the 90 day trial period indicated a substantial decrease in levels in the high dose immunomicrosphere group (Group IIB) when compared with the free drug group (Group IA). These results further accentuate the superiority of drug-loaded immunomicrosphere protocols over those employing free drugs at the same concentrations.

From the above results it was thus possible to extrapolate mortality probability profiles (Fig. 40) for all 4 groups of animals used in the survival studies. The results revealed a marked difference in the possible prediction of death of the grouped animals. The untreated control group (Group IIIA) indicate the probability of death of the surviving animals to be 1 in 3,8 at Day 14 after primary tumour transplantation. For animals of the free drug treated group (Group IA), death probability is 1 in 2,1 at Day 72, for animals of the low drug dose immunomicrosphere group (Group IIA), it is 1 in 2,1 at Day 60 and for animals of the high drug dose immunomicrosphere group (Group IIB) it is 1 in 0.45 at Day 60.

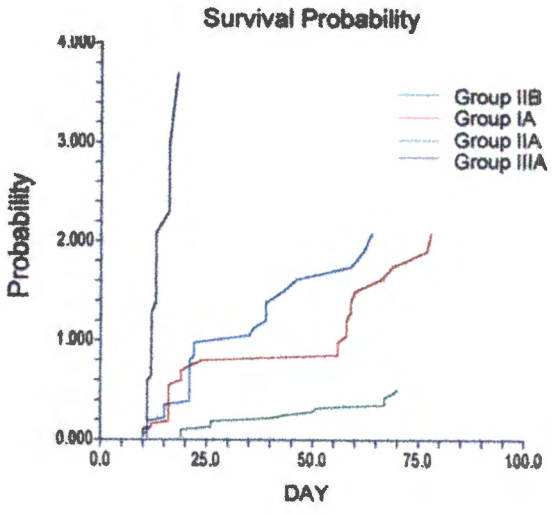


Fig. 40: The mortality probability profiles for Group of Wistar rats of which 3 groups recieved different regimes:

a)Group IA = free drug group,

b) Group IIA = low dose immunomicrosphere group,

c)Group IIB = high dose immunomicrosphere group

d)Group IIIA = no treatment group(control)

The above data indicates that the survival probability of animals from Group IIB (high dose immunomicrosphere group) is substantially superior to the other protocols employed in this study.

Chapter 7

Conclusion

We have clearly demonstrated that a targetable sustained drug delivery system can be developed and implemented as a treatment modality for ovarian adenocarcinoma.

We were able to demonstrate that the concept of employing immunomicrospheres containing chemotherapeutic agents may be a feasible alternative approach in the treatment of intraperitoneal cancers such as ovarian adenocarcinoma as compared to conventional chemotherapeutic regimens. This modality is dependent on the physical and chemical characteristics of the drug carrier to be used. Our results demonstrated that heat-stabilised albumin microspheres are not only capable of entrapping cisplatin and 5-fluorouracil in high concentrations, but also capable of delivering the drugs in a sustained fashion in a plasma milieu. These albumin microspheres were further capable of being coupled quite readily to monoclonal antibodies by a carbodiimide technique, thus rendering targetable immunomicrospheres. By employing this method, carboxyl groups present on the surface of the microspheres can be coupled to the amino group on the antibody molecule through an amido linkage.

Our results reiterated the synergistic effect of cisplatin and 5-fluorouracil whether they were employed as free drug or released from the immunomicrospheres. *In vitro* assays such as clonogenic assays, cell growth survival studies, MTT assays and micronuclei assays demonstrated that greater cell kill was achieved when cisplatin and 5-fluorouracil were released in sustained fashion by immunomicrospheres as compared to cell exposure to free drug. We could thus conclude that a drug delivery system is more effective in delivering its payload to the target site than free drugs.

As regards cytotoxicity studies, we demonstrated that drugs delivered to the target site by immunomicrospheres had no greater side-effects than drugs delivered in free form. The blood chemistry and haematology profiles supported these findings. However, the cytotoxic effects to the targeted tumour cells, were greatly enhanced by employing target-directed immunomicrospheres containing cisplatin and 5-fluorouracil. Animal survival studies demonstrated this hypothesis as 60% of animals treated with drug containing immunomicrospheres survived a 90 day trial period as compared to 14% of animals treated by a free drug protocol at the same dosage. These results were further

supported by decreasing superoxide dismutase levels in immunomicrosphere treated animals. Hence, we could postulate mortality probability profiles which greatly favoured survival of animals treated by drug-containing immunomicrospheres as shown in Chapter 6, Section 6.4.

Further work will include studies to quantify the amounts of cisplatin and 5-fluorouracil entering the tumour cells to cause DNA damage. This is considered essential as successful treatment depends on drugs entering the tumour cell.

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APPENDIX 1

50% Human serum albumin

50 g human serum albumin was obtained in the lyophilised form from the Western Province Blood Transfusion Services. It was reconstituted by the addition of 100 ml of distilled water.

Total Ionic Strength Adjustment Buffer (TISAB)

Dissolve 58 g NaCl and 3.6 g trans-1,2-diaminocyclohexane-N,N-N',N'-tetraacecetic acid in 900 ml distilled water. Adjust to pH 5 and the volume to 1000 ml with distilled water.

Sodium Fluoride Solutions

Prepare a 1000 ppm stock solution of NaFl by dissolving 2.21 g NaFl in 1000 ml of distilled water. Dilute to the required concentrations with distilled water.

1% Hydrochloric Acid

Add 3 ml of concentrated (10.18 M, 34%) hydrochloric acid (Saarchem) to 99 ml distilled water.

5x Versene (EDTA in buffered saline)

0.65 g di-sodium EDTA
0.5 g KCl
20 g NaCl
2.85 g $Na_2HPO_4.2H_2O$
0.5 g KH_2PO_4

Dissolve the reagents in 450 ml of distilled water and adjust the pH to 7.2 - 7.4. Make up to 500 ml in a volumetric flask. Dilute 1 part versene with 4 parts distilled water for use. Filter-sterilise through a 0.22 μm filter.

RPMI-1640 Medium

Add to 900 ml of triple-distilled water :

10.41 g RPMI-1640 (Sigma, cat no. R 6504)

2 g $NaHCO_3$ (Merck)

20 mg Streptomycin sulphate (Merck) (final concentration of 20 μg/ml)

0.1 ml Penicillin at 500 000 units/ml (final concentration of 50 units/ml)

Stir the reagents until they have dissolved. Adjust pH to 7.1 with concentrated HCl. Make up to 1000 ml in a volumetric flask and filter-sterilise through a 0.22 μm filter. Store at 4°C.

Trypan Blue Viability Test

Add 0.1 ml trypan blue (Sigma) stain to 0.9 ml of cells. Only non-viable cells will take up the blue stain.

White Cell Counting Fluid

Dissolve 10 mg crystal violet in 2% acetic acid.

PEG 4000 (for fusion of lymphocytes with myeloma cells)

2 g of polyethylene glycol (PEG), molecular weight 4000 daltons, is weighed out and autoclaved. 2 ml of warm (37°C) RPMI-1640 is added to the PEG while it is still molten. Store at 4°C. Discard if not used within 2 weeks of preparation.

HAT-HUCS-RPMI-1640 Medium

Add to 88 ml of RPMI: 10 ml HUCS

1 ml of 100x aminopterin stock solution

1 ml of 100x hypoxanthine (HT) solution

Store at 4°C.

HUCS (Human Umbilical Cord Serum)

Human umbilical cord serum was pooled from different patients and then decomplemented by heat inactivation at 56°C for 30 minutes. It was filter-sterilized and stored frozen at -20°C in 50 ml aliquots.

100x Aminopterin Stock Solution (4×10^{-7} M)

Add 25 ml of distilled water and 0.5 ml of 5 M NaOH to 1.76 mg of aminopterin. Neutralize with 5 M HCl and adjust the volume to 100 ml with distilled water. Filter-sterilize and store at -20°C. Keep away from light.

10 M NaOH

Dissolve 40 g NaOH pellets in 100 ml of distilled water.
Dilute as required with distilled water.

5 M HCl

Add 49 ml of concentrated HCl (10.18 M, 34%) to 51 ml of distilled water.
Dilute to required concentrations with distilled water.

100x HT (Hypoxanthine) Stock Solution

0.34 g hypoxanthine (1×10^{-3} M)
0.0968 g thymidine (1.6×10^{-3} M)
0.0055 g glycine (2.93×10^{-4} M)
2.7515 g sodium pyruvate (0.1 M)

Add 125 ml of distilled water to the hypoxanthine and add up to 2.5 ml of 10 M NaOH dropwise until the hypoxanthine has dissolved. Add the remaining compounds and stir until they have dissolved. Adjust the final volume to 250 ml with distilled water, filter-sterilize and store in aliquots at -20°C.

HT-HUCS-RPMI-1640 Medium

Add to 89 ml of RPMI-1640, 10 ml HUCS and 1 ml of 100x HT solution. Store at 4°C.

FCS (Foetal Calf Serum)

This was obtained from the State Vaccine Institute (Pinelands, South Africa), thawed at 37°C and decomplemented by heat inactivation for 30 minutes at 56°C. It was filter-sterilized by passing the serum through a pre-filter, a 8 μm filter and finally through a 0.22 μm filter. 50 ml aliquots were stored at -20°C.

Serum-free and Protein-free Hybridoma Medium

Add to 900 ml of triple-distilled water:

18.5 g Serum-free and protein-free hybridoma medium (Sigma Cat no. S2772)

2.25 g $NaHCO_3$ (Merck)

20 mg Streptomycin sulphate (Merck) (final concentration of 20 μg/ml)

0.1 ml Penicillin at 500 000 units/ml (final concentration of 50 units/ml)

Stir the contents until they are dissolved. Adjust the pH to 7.1 with concentrated HCl. Make up to 1000 ml in a volumetric flask and filter-sterilise through a 0.22 μm filter. Store at 4°C.

1% Sodium azide in 0.54 M PBS

Dissolve 100 mg in 10 ml of 0.54 M PBS, pH 7.2.

For storage of antibodies, add 20 μl of 1% sodium azide per 1 ml of antibodies.

0.54 M Phosphate-buffered Saline (PBS), pH 7.2

8 g NaCl

0.2 g KCl

1.442 g $Na_2HPO_4.2H_20$

0.2 g KH_2PO_4

Add 800 ml of distilled water to the above reagents and stir until dissolved. Adjust the pH to 7.2 with concentrated HCl . Adjust the volume to 1000 ml in a volumetric flask and autoclave.

0.1% Glutaraldehyde in 0.54 M Phosphate-buffered Saline

Add 0.4 ml of a 25% glutaraldehyde solution (Merck) to 99.6 ml of PBS, pH 7.2.

ELISA Blocking Buffer (2% skim milk, 100 mM glycine in 0.54 M PBS)

2 g of skim milk powder and 0.75 g of glycine were dissolved in 90 ml of 0.54 M PBS, pH 7.2 and the volume was adjusted to 100 ml.

ELISA Diluent

29.22 g NaCl (0.5 M)

1.799 g Na_2HPO_4 (0.01 M)

5 ml Triton X-100 (0.5%)

Dissolve the reagents in distilled water and adjust the volume to 1000 ml.

Tris-Saline-Tween (TST), pH 8

5.844 g NaCl (0.1 M)

6.055 g Tris (0.05 M)

0.5 ml Tween 20 (0.5%)

Dissolve the NaCl and Tris in 800 ml of distilled water. Adjust the pH to 8.0 with concentrated HCl. Add Tween and adjust the volume to 1000 ml.

ABTS Substrate

Add 100 μl ABTS and 10 μl of 30% H_2O_2 (Merck) to 10 ml 0.1 M citrate buffer. Store in the dark until required.

0.1M Citrate Buffer

Dissolve 525 mg citric acid monohydrate in 40 ml distilled water. Adjust the pH to 4 with 3 M NaOH. Adjust the volume to 50 ml.

40 mM ABTS (2,2' azino-di(3-ethyl-benzthiazoline) sulfonic acid)

219 mg of ABTS is dissolved in 10 ml of distilled water and frozen at -20°C in 120 μl aliquots.

0.25%Trypsin for Tissue Culture

A 0.5% stock solution is prepared by dissolving 2.5 g of 1:250 Difco-certified trypsin in 500 ml of 0.001 M HCl. Stir at room temperature for 90 minutes to 2 hours. Filter through Whatman filter paper No. 541. Add 1 ml of 0.5% phenol red (for tissue culture) and filter-sterilize through a pre-filter and a 0.45 μm filter. Store frozen until required. In order to prepare EDTA-trypsin for trypsinising cells, add an equal volume of 2x TD to the thawed trypsin and add 1 ml of 2% EDTA in TD per 100 ml of the diluted trypsin. Store at -20°C.

0.001 M HCl

Add 0.5 ml of 1 M HCl to 500 ml distilled water.

2xTD

Dissolve each of the following reagents in order in 900 ml distilled water, ensuring that each one has dissolved before the following one is added:

16 g NaCl
0.76 g KCl
0.25 g $Na_2HPO_4.2H_2O$
6 g Tris base

Allow the solution to be exposed to the air for 30 minutes at room temperature. Adjust the pH to 7.4 - 7.5 with 3 M HCl and leave for another 30 minutes. Check the pH and make up to 1000 ml, aliquot and autoclave. For 1X TD, add 1part 2X TD to 1part distilled water and filter-sterilise.

2% EDTA in 1X TD

Dissolve 2 g of EDTA in 100 ml of 1X TD and filter-sterilise.

30% glycerol in 0.54 M PBS

Add 30 ml of glycerol to 70 ml PBS.

0.1% Trypsin in 0.05 M Tris

Add 0.1 g of 1:250 Difco certified trypsin to 100 ml of 0.05 M acidified Tris buffer, adjust the pH to 7.6 and stir at 37°C.

0.05 M acidified Tris-buffer, pH 7.6

Dissolve 6.1 g of Tris (Boehringer Mannheim) in 50 ml distilled water and add 31 ml of 1 M HCl. Adjust to pH 7.6 and make up the volume to 1000 ml in a volumetric flask. Store at 4°C.

Tris-buffered Saline (TBS), pH 7.6

Dissolve 8.77 g NaCl (0.15 M) and 6.05 g Tris base (25 mM) in 900 ml of distilled water. Adjust the pH to 7.6 with concentrated HCl and make the volume up to 1000 ml in a volumetric flask.

100 μg/ml Ethidium Bromide

Dissolve 1 mg ethidium bromide in 10 ml distilled water. Dilute to required concentration. Store at 4°C.

0.1% Bovine Serum Albumin (BSA) in 0.54 M PBS

Dissolve 0.1 g BSA in 100 ml of PBS, pH7.2.

0.3 M Sodium Phosphate Buffer, pH 6.8

Prepare stock solutions of 0.3 M $Na_2HPO_4.2H_2O$ (dissolve 13.349 g in 250 ml distilled water) and 0.3 M $NaH_2PO_4.2H_2O$ (dissolve 10.349 g in 250 ml distilled water). Mix the two solutions to obtain a pH of 6.8. 10 mM, 50 mM, 100 mM and 200 mM sodium phosphate solutions can be obtained by diluting the 0.3 M sodium phosphate solution with distilled water to the required molarities.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (11% Running Gel)

Assemble the clean glass plates with the spacers aligned between the two plates and clamp them. Seal the sides of the plates with 1.5% sealing agar. Seal the bottom end by pouring sealing agar onto a spare glass plate and place the assembled plates upright on the agar.

Prepare the running gel solution by mixing 3.3 ml acrylamide, 2.25 ml running gel buffer and 3.45 ml distilled water. Finally add the catalysts, 25 μl ammonium persulphate and 25 μl TEMED (N,N,N',N'-Tetramethylethylenediamine). Immediately pipette this mixture between the two glass plates so that the level is about two comb lengths away from the top of the glass plates. Gently overlay with isopropanol so as to prevent oxygen from inhibiting polymerisation and to get rid of air bubbles. Allow to set. Once the running gel has set, remove the isopropanol and make up the 4% stacking gel by combining 1.1 ml acrylamide, 2 ml stacking gel buffer, 4.9 ml water, 25 μl ammonium persulphate and 25 μl TEMED. Pipette this mixture on top of the running gel. Slide in the comb and allow to set. Once the stacking gel has set, clamp the plates into the electrophoresis apparatus and seal with 1.5% sealing agar. Add the reservoir buffer to the tanks and check for any leakages.

1.5% Sealing Agar

Dissolve 1.5 g agar in 100 ml distilled water by heating in a microwave oven.

30% Acrylamide

Dissolve 30 g Acrylamide and 0.8 g *bis*-acrylamide (N,N'-Methylene-*bis*-acrylamide) in 50 ml distilled water. Adjust the volume to 100 ml and filter the solution through Whatman's No. 1 filter paper. Store the solution in a dark bottle at 4^0C.

Running Gel Buffer, pH 8.8

(1.5 M Tris-HCl; 0.4% SDS)

Dissolve 18.17 g Tris and 0.4 g SDS in 80 ml distilled water. Adjust the pH to 8.8 with concentrated HCl and adjust the volume to 100 ml.

Ammonium Persulphate

Dissolve 0.1 g ammonium persulphate in 0.9 ml distilled water. This solution is stable for 1 week when stored at 4°C.

Stacking Gel Buffer, pH 6.8

(0.5 M Tris-HCl; 0.4% SDS)

Dissolve 6.06 g Tris and 0.4 g SDS in 80 ml distilled water and adjust the pH to 6.8 with concentrated HCl. Adjust the volume to 100ml.

Reservoir buffer, pH 8.5

(0.025 M Tris; 0.192 M Glycine; 0.1% SDS)

Dissolve 3.03 g Tris, 14.41 g glycine and 1 g SDS in 950 ml distilled water. Adjust the volume to 1000 ml and check that the pH is 8.5. Do not adjust the pH if it is incorrect but discard the solution and start again.

2X SDS/Sample Buffer

Combine 2.5 ml stacking gel buffer, 2 g sucrose, 0.4 ml of a 0.04% solution of bromophenol blue, 4.6 ml of a 10% solution of SDS and 0.5 ml distilled water. Store frozen at -20°C in 200 μl aliquots. In order to reduce the samples, add 10 μl of a 400 mg/ml DTT to 200 μl of the thawed 2X SDS/sample buffer.

0.04% Bromophenol Blue

Dissolve 40 mg bromophenol blue in 100 ml distilled water and filter through Whatman's filter paper No. 541.

10% SDS

Dissolve 10 g SDS in 100 ml distilled water.

400 mg/ml DTT (Dithiothreitol)

Dissolve 400 mg DTT in 1 ml distilled water. Store frozen at -20°C in 20 μl aliquots.

Destain for SDS-PAGE gels

Mix 3 parts of methanol, 1 part of acetic acid and 6 parts distilled water.

1% PAGE Blue (for the staining of SDS-PAGE gels)

Dissolve 1g PAGE Blue 83 (BDH) in 100 ml of destain for SDS-PAGE gels.
Dilute 1:10 in destain for use.

0.1 M Sodium Chloride, pH 7

Dissolve 5.844g NaCl in 1l distilled water and autoclave.

Hoechst Stain

Dissolve 50 mg Hoechst compound 33258 (or bis-benzamide) and 10 mg thiomersal in 100 ml 0.54 M PBS, adjust the pH to7.2 and store in a dark bottle away from light.

0.56% Potassium Chloride

Dissolve 0.56 g of potassium chloride in 100 ml of distilled water and filter-sterilise.

1.32% Agar

Dissolve 1.32 g agar (Bacto Difco) in 100 ml distilled water by heating in a microwave oven.

1% Agar

Dissolve 1g agar (Bacto Difco) in 100 ml distilled water by heating in a microwave oven.

2X RPMI-1640

Add to 450 ml of triple-distilled water :

10.41 g RPMI-1640 (Sigma, cat no. R 6504)
2 g $NaHCO_3$ (Merck)
20 mg Streptomycin sulphate (Merck) (final concentration of 20 μg/ml)
0.1 ml Penicillin at 500 000 units/ml (final concentration of 50 units/ml)

Stir the reagents until they have dissolved. Adjust the pH to 7.1 with concentrated HCl. Make up to 500 ml in a volumetric flask and filter-sterilise through a 0.22 μm filter. Store at 4°C.

3% Glutaraldehyde in 0.54 M PBS

Add 12 ml of 25% glutaraldehyde (Merck) to 88 ml of 0.54 M PBS, pH 7.2.

1 mg/ml MTT

Dissolve 100 mg of MTT in 100 ml of phenol-red free RPMI and filter-sterilise through a 0.22 μm millipore-filter. Store at 4°C in the dark for up to 2 weeks or store frozen at -20°C.

100 ug/ml Cytochalasin B

Dissolve 1 mg Cytochalasin B in 10 ml distilled water. Store in 10 ul aliquots at -20°C.

0.001% Acridine Orange

0.1g acridine orange is dissolved in 100 ml distilled water (i.e. 0.1% solution). This is diluted further to 0.001% for staining by adding 0.4 ml of the 0.1% acridine orange solution to 40 ml phosphate buffer (pH 6.8) (Merck).

Ketalar/Rumpon

Mix 5 parts Rumpon (Bayer) to 9 parts Ketalar (Warner-Lambert)

70% Barium Sulphate in Egg Albumin

Make a paste by adding 70 g barium sulphate to 100ml of egg albumin

0.83% Ammonium Chloride

Dissolve 0.83 g ammonium chloride in 100ml of distilled water. Filter sterilise through a 0.22 μm filter.

2.5% Phosphate-buffered glutaraldehyde

Add 10 ml of a 25% stock solution of glutaraldehyde to 90 ml of phosphate buffer, pH 7.4

10% Formal Saline

Add 10 ml formalin and 1 g sodium chloride to 85 ml distilled water. Adjust the volume to 100 ml with distilled water.

Haematoxylin and Eosin Stain

Dewax the sections in xylene.

Hydrate the sections through graded alcohols to water:

- absolute alcohol
- 90% alcohol
- 70% alcohol
- tap water

Stain in Mayer's Haematoxylin for 10 minutes.

Wash well in running tap water.

Briefly, differentiate the sections in 1% acid alcohol

"Blue" in Scott's tap water

Wash in tap water

Stain in 0.1% aqueous eosin for 3 minutes

rinse the sections briefly in water

Dehydrate the sections in alcohols:

- 70% alcohol
- 90% alcohol
- absolute alcohol
- absolute alcohol

Clear the sections in Xylene

Mount the sections in DPX

Mayer's haematoxylin

Dissolve 1 g haematoxylin, 50 g potassium or iron alum and 0.2 g sodium iodate in 1000 ml distilled warm water. Add 50 g chloral hydrate and 1 g citric acid and boil the mixture for 5 minutes. Allow the mixture to cool before it is filtered.

Alcian Blue PAS

Reagents: 1) 0.3% alcian blue in 3% acetic acid
Dissolve 0.3 g alcian blue in 100 ml of 3% acetic acid.

2) 3% acetic acid
Add 3 ml glacial acetic acid to 97 ml distilled water.

3) 0.1% nuclear fast red in 5% aluminium sulphate
Dissolve 0.1 g nuclear fast red in 5% aluminium sulphate.

4) 5% aluminium sulphate
Dissolve 5 g aluminium sulphate in 100 ml distilled water.

5) Periodic acid solution
Dissolve 1 g periodic acid in 200 ml distilled water.

6) Schiff's reagent (Merck)

Method: Dewax sections in xylene and hydrate through a series of alcohols to water. Stain sections in alcian blue solution for 20 minutes. Rinse well in distilled water. Stain for 10 minutes in nuclear fast red. Rinse the sections in distilled water, dehydrate rapidly in a series of alcohols, clear in xylene and mount in DPX.

Reticulin Stain (Gordon & Sweet's Method):

Reagents: 1) 10.2% aqueous silver nitrate solution

Dissolve 10.2 g silver nitrate in 100 ml distilled water.

2) Silver solution

Add strong ammonia solution drop by drop to 5 ml of 10.2% aqueous silver nitrate solution until the precipitate which it forms, just dissolves. Add 5 ml of a 3.1% solution of NaOH. Add strong ammonia drop by drop until the resulting precipitate just dissolves (not completely clear).
Make up the solution to 50 ml with distilled water.

3) 3.1% NaOH

Dissolve 3.1 g NaOH pellets in 100ml distilled water.

4) Acid-$KMnO_4$

Mix 95 ml of 0.5% $KMnO_4$ and 5 ml of 3% H_2SO_4.

5) 0.5% $KmnO_4$

Dissolve 0.5 g $KmnO_4$ in 100 ml distilled water.

6) 1% Oxalic acid

Dissolve 1 g oxalic acid in 100 ml distilled water.

7) 2.5% iron alum

Dissolve 2.5 g iron alum (ammonium iron (III) sulphate dodecahydrate) in 100 ml distilled water.

8) 10% aqueous formalin

9) 0.2% gold chloride

Dissolve 0.2 g gold chloride in 100 ml distilled water.

10) 5% sodium thiosulphate

Dissolve 5 g sodium thiosulphate in 100 ml distilled water.

Method: Dewax sections in xylene and hydrate through a series of alcohols to water. Oxidise the sections in acidified $KmnO_4$ for 1-5

minutes. Wash the sections in water and bleach in 1% oxalic acid for 3-5 minutes. Rinse in distilled water, wash again in tap water and 2-3 changes of distilled water. The sections are mordanted in 2.5% iron alum for 10 minutes to 2 hours and washed in 2-3 changes of distilled water. They are then treated with silver solution until transparent (about 30 seconds) and washed well in distilled water. The sections are reduced in 10% aqueous formalin, washed in tap water and rinsed in distilled water. Sections are toned in 0.2% gold chloride for 10-15 minutes, rinsed in distilled water and treated with 5% sodium thiosulphate for 5 minutes. After washing the sections in water, they are dehydrated in a series of alcohols, cleared in xylene and mounted in DPX.

Von Gieson Stain

Reagents: 1) Von Gieson's reagent

Add 10 ml 1% acid fuchsin to 100 ml saturated picric acid solution.

Oxidise this solution by boiling it for 3 minutes

2) Weigert's haematoxylin

Solution A: Dissolve 1 g haematoxylin in 100 ml of absolute ethanol.

Solution B: Add 4 ml 30% ferric chloride and 1 ml conc. HCl to 100 ml distilled water. Allow the solution to ripen for 1 week.

Mix equal parts of solutions A & B just before use.

Method: Dewax sections in xylene and hydrate through a series of alcohols to water. Stain the sections in Weigert's haematoxylin for 15 minutes and wash well in running tap water. Differentiate in 1% acid alcohol, rinse in water, blue in Scott's tap water, rinse well in water and counterstain in Von Gieson's reagent for 3 minutes. Rapidly rinse the sections in water so as not to wash the fuschin out of the tissues. Rapidly dehydrate the sections through a series of alcohols as picric acid is removed by alcohol. Clear the sections in xylene and mount in DPX.

Southgate's Mucicarmine Method

Reagents: 1) Carmine solution

Add 1 g carmine and 1 g aluminium hydroxide to 100 ml 50% ethanol. Mix well and then add 0.5 g anhydrous aluminium chloride and boil for 3 minutes. Allow the solution to cool and make up the volume to 100 ml with 50% ethanol.

2) Mayer's haematoxylin

Method: Dewax sections in xylene and hydrate through a series of alcohols to water. Stain the nuclei with Mayer's haematoxylin and differentiate with 1% acid alcohol. Wash the sections with water, blue them in Scott's tap water and wash again. Stain the sections in carmine solution for 30 minutes, rinse in distilled water, dehydrate through an alcohol series, clear in xylene and mount in DPX.

1% Osmium Tetroxide in Veronal-Acetate Buffer pH 7.4

Mix 12.5 ml of 2% OsO_4, 5 ml veronal -acetate buffer and 2.5 ml distilled water in a glass-stoppered bottle. pH to 7.4 with 0.1M HCl (+/- 5 ml).

Veronal-acetate buffer

Dissolve 2.89 g barbitone sodium (sodium veronal) and 1.15 g sodium acetate (anhydrous) in 100 ml distilled water. Store at 4°C.

2% Osmium Tetroxide

Dissolve 1 g OsO_4 in 50 ml distilled water in a clean glass beaker. Store at 4°C.

1% uranyl acetate in 96% ethanol

Dissolve 1 g uranyl acetate in 100 ml 96% ethanol.

Spurr's Embedding Resin

Weigh the following reagents into a polythene beaker sequentially: 10 g ERL 4206 (a cycloaliphatic diepoxide), 6 g DER 736 (a flexibiliser) and 26 g nonenyl succinic anhydride (NSA). Mix thoroughly with a magnetic stirrer and then, whlie stirring, add 400 ul S-1 accelerator dropwise.

1% Toluidine Blue in 1% Borax

Dissolve 1 g Toluidine Blue in 100 ml of 1% Borax.

1% Borax

Dissolve 1 g borax in 100 ml distilled water.

Reynold's Lead Citrate Stain

Add 1.33 g lead nitrate ($Pb(NO_3)_2$), 1.76 g sodium citrate ($Na_3(C_6H_5O_7).2H_2O$and 30 ml distilled water to a 50 ml volumetric flask and shake the suspension vigorously for 1 minute. Allow the suspension to stand for 30 minutes with intermittent shaking to ensure complete conversion of the lead nitrate to lead citrate. Add 8 ml 1 M NaOH and make the volume up to 50 ml with distilled water. Mix by inversion. If any faint turbidity is present, centrifuge the solution. The pH should be 12+/- 0.1.