# EFFECTS OF A PROSTAGLANDIN PRECURSOR, GAMMA-LINOLENIC ACID (GLA), ON MALIGNANT CELLS *IN VITRO* AND *IN VIVO*

by

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# PREFACE

The experimental work described in this thesis was carried out in the Departments of Pharmacology and Physiology, University of Natal, under the supervision of Dr J.H. Botha and Dr K.M. Robinson.

These studies represent original work by the author and have not been submitted in any form to another university. Where use was made of the work of others it has been duly acknowledged in the text.

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## ABSTRACT

Recent studies have shown that the proliferation of various human and murine tumour lines can be inhibited by the addition of gamma-linolenic acid (GLA) to the culture medium. These findings are consistent with the proposal put forward by Horrobin (1980) that malignant cells lack the enzyme, $\Delta 6$ desaturase, which is responsible for the conversion of linoleic acid (LA) to GLA. Since GLA is a prostaglandin (PG) precursor, inadequate conversion of LA to GLA would result in decreased production of PGs, particularly $PGE_1$, which has been shown to have an inhibitory effect on cell growth. Provision of GLA to enzyme deficient malignant cells should therefore bypass this blockade, increase $PGE_1$ synthesis and thus "normalise malignant cells".

This study was performed to examine further the effects of exogenous GLA on growth of malignant cells *in vitro* and *in vivo*. Cells of the continuous murine sarcoma (M52B) line and primary cultures of non malignant fibroblasts were used to investigate effects of GLA *in vitro*. Cultures were exposed to either single or multiple doses of a range of concentrations of GLA. Radioimmunoassay (RIA) was performed to compare the amounts of PGE and PGF released into the medium by GLA treated and control M52B cultures and thus determine whether the addition of GLA *in vitro* significantly affected production of these PGs.

Athymic BALB/c mice and immunocompetent BALB/c and Biozze mice as well as mice of the "Onderstepoort Strain" were used in various *in vivo* studies. Tumours were induced by the subcutaneous inoculation of approximately $1 \times 10^6$ cells of either the M52B line (into immunocompetent and athymic mice) or human breast carcinoma (NUB 1) line (into athymic mice). Take rates and latent periods were recorded. GLA treatment was initiated after tumours were established. In one study the fatty acid in hydrogenated coconut oil (HCO), which contains no PG precursors, was administered parenterally (100 ug/ml/day) to Biozze mice. Control mice were either untreated or injected with HCO only. In another study, BALB/c mice and mice of the "Onderstepoort Strain" had their diet supplemented with GLA (in the form of EPO) to an extent of 3.5%. Control mice consumed either standard laboratory chow only or, chow supplemented with either 35% sunflower seed oil (SSO) or 35% HCO, neither of which contain GLA. All diets were supplied *ad libitum*. Tumour sizes were measured every 48 hours and at the end of each experiment at which time tumours were excised and examined histologically.

GLA was found to produce inhibitory and toxic effects on growth of both M52B cells and non malignant fibroblasts *in vitro*, although the effect in the latter was observed only with high concentrations of the fatty acid. The inhibition of malignant cell growth was concentration dependant and was positively related to the duration of exposure to the fatty acid. Prior to death, cells treated with GLA accumulated

paranuclear granules which were shown histochemically to be lipid in nature. Electron microscopy confirmed the presence of large lipid deposits. Cultured M52B cells treated with GLA also released more PGE and PGF into the medium than did cells not exposed to the fatty acid. However, analysis of results using the Mann Whitney U test showed these differences to be statistically non significant for both PGE and PGF on two tailed tests.

In contrast to the inhibition of M52B cell growth observed in vitro, growth of solid M52B sarcomas and NUB 1 carcinoma xenografts in athymic mice was apparently unaffected by administration of dietary GLA. Analysis of data using an unpaired student's t-test showed that the differences in tumour volumes between control and treated groups were not statistically significant either before or at the end of the experiment.

While the inhibition of malignant cell growth caused by GLA in vitro was consistent with Horrobin's proposal that malignant cells may lack this PG precursor, whether or not these actions are mediated by the PGs remains obscure. Although an increase in PGE production by M52B cells was observed following GLA treatment, besides this increase being statistically non significant, it was not possible to determine whether this was due to $PGE_1$ (as suggested by Horrobin) or $PGE_2$.

It is possible that the effect produced in vitro was due to some factor other than raised PGE production, for example a non-specific

fat-overload effect or a change in cell membrane fluidity. The lack of effect of GLA on tumour growth *in vivo* may have been due to inadequate delivery of the fatty acid to the tumour site. However, whatever the mechanism of action of GLA *in vitro*, since oral GLA was supplemented to the maximum tolerated extent and produced no effect in immunodeficient mice *in vivo*, it would seem that in a similar clinical situation oral doses which would be practical may be ineffective.

LIST OF TABLES

LIST OF FIGURES

# CONTENTS

# CHAPTER 1

# INTRODUCTION

## 1.1 CANCER

Cancer, a disorderly, purposeless growth of cells, is currently one of the major causes of mortality (Endicott, 1972). The transformation of a normal cell to a cancer cell may be promoted by a variety of stimuli including viruses, radiation and chemicals (Honn et al., 1981). However, since the exact mechanism of transformation is unclear, much of cancer research is at present directed towards elucidating this mechanism and subsequently establishing a cure for the disease. Included amongst the many factors that are currently being investigated are the prostaglandins (PGs) which may play some role in regulation of cell division (Ames, 1983) and therefore be important in malignancy (Honn et al., 1981).

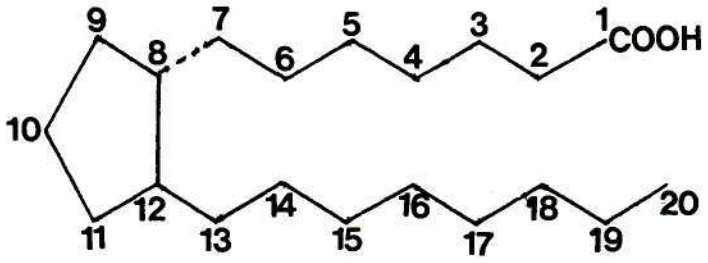
## 1.2 PROSTAGLANDINS AND THEIR PRECURSORS

The prostaglandins (PGs) are a group of naturally occurring unsaturated fatty acids which are widely distributed in both plant and animal kingdoms (Horrobin, 1978). They appear to be synthesised in virtually all mammalian tissues (Moncada and Vane, 1983), being produced "on demand" and then rapidly catabolised (Ziegler, 1982). PGs mediate a variety of biological effects and seem to play some role in almost all systems in the body (Karmali, 1980).

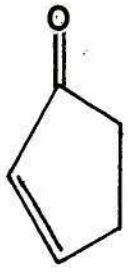
### 1.2.1 Chemistry, Biosynthesis and Metabolism

The PGs which are structurally related to prostanoic acid (Gränstrom, 1977)(Figure 1) may be subdivided into three main naturally occurring groups; the 1, 2 and 3 series having 1, 2 and 3 double bonds in their side chains respectively. PGs are named A, B, C, D, E and F according to substituents on the pentane ring (Figure 1). More recently, further related products of arachidonic acid (AA) metabolism, namely prostacyclin, thromboxanes, leukotrienes, hydroperoxy and hydroxy fatty acids have been discovered (Honn et al., 1981)(Figure 2). The PGs are derived from the essential fatty acids (EFAs) linoleic acid (LA) and alpha-linolenic acid (ALA), the former being the most important polyunsaturated fatty acid (PUFA) in the normal mammalian diet (Horrobin, 1978). Cis LA (cLA) is found in large quantities in many vegetable oils including evening primrose oil (EPO - which contains 72% cLA), safflower (73% cLA), corn (57% cLA) and sunflower seed oil (SSO which contains 58% cLA) (Schölkens et al., 1982), while smaller quantities are present in meat (Horrobin, 1978). The synthesis of PGs of the 1 and 2 series from cLA is shown in Figure 2. The immediate precursor of the 1 series is dihomogammalinolenic acid (DGLA; 8,11,14 - eicosatrienoic acid), and that of the 2 series is arachidonic acid (AA; 5,18,11,14 - eicosatetraenoic acid). The 3 series PGs are formed from alpha linolenic acid (ALA; 5,8,11,14,17 - eicosapentaenoic acid).

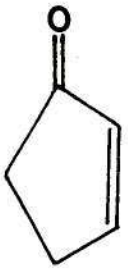
Figure 1: The structure of prostanoic acid and pentane rings of various prostaglandins



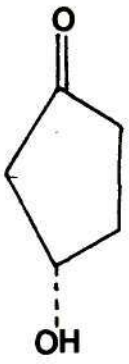

**PROSTANOIC ACID**



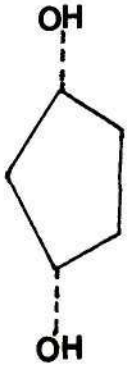

**PGA**




**PGB**




**PGE**




**PGF**

### 1.2.2 Postulated role of PGs

In addition to being ubiquitous, PGs have been found to play some role in most tissues of the body (Nakano, 1972). They appear to act locally, affecting only the activity of the tissue in which they are formed. Characterisation of their biological roles has been difficult because the effects of PGs vary according to the concentration and type of PG; and the site of action and species of animal (Ziegler, 1982). At present, there is no evidence to suggest that they are circulating hormones. However, these substances are known to play a regulatory role in the reproductive, renal, neural, immune and cardiovascular systems (Horrobin, 1978; Karmali, 1980). Furthermore there is also evidence that PGs are involved in regulation of cell growth (Ziegler, 1982).

#### 1.2.2.1 PGs and Cell Growth

The possible role of PGs in the control of cell proliferation was based originally on the observation that these substances induced accumulation of intracellular cyclic AMP (cAMP) which inhibited uncontrolled proliferation of slime moulds (Droller, 1981). More recent investigations using various malignant cell lines have since confirmed the finding that exogenous cAMP inhibits cell growth (Droller et al., 1981; Dubpernell and Gavurin, 1978; Honn et al., 1981; Karmali,

1980; Naseem and Hollander, 1973; Sheppard, 1972; Thomas et al., 1974). There is also considerable direct evidence to support a role for PGs in control of cell proliferation even though workers have produced different results with varying concentrations of these substances (Horrobin, 1978; Karmali, 1980). This could be expected if cAMP is involved as it stimulates and inhibits cell proliferation at low and high concentrations respectively (Sheppard, 1972; Karmali, 1980).

Exogenous $PGE_1$ (Johnson and Pastan, 1971; Kurtz et al., 1974; Pelus et al., 1979; Sheppard, 1972; Smith et al., 1984), $PGE_2$ (Delescluse et al., 1974; Pelus et al., 1979; Smith et al., 1984; Taylor and Polgar, 1977) and $PGB_1$ (Smith et al., 1984; Johnson and Pastan, 1971) have been reported to suppress growth and to simultaneously elevate cellular cAMP production (Johnson and Pastan, 1971; Polgar and Taylor, 1977) in various cultured normal cells.

In addition, the PG precursors LA, GLA, DGLA and AA generally have also been reported to affect growth of cultured non malignant cells, producing either inhibition or stimulation at low concentrations (Huttner et al., 1978; Wicha et al., 1979) and inhibition of cell proliferation at higher concentrations (Begin et al., 1985; Booyens et al., 1984b; Huttner et al., 1978; Petry et al., 1984). The ability of these PUFAs to affect cell growth appears to be specific for

## 2.1 CELL LINES

A primary culture is initiated from cells, tissues or organs taken directly from an organism. This culture develops into a cell line which, when it has developed the potential to be subcultured indefinitely *in vitro*, has been subcultured at least 70 times at intervals of 3 days and is capable of being frozen, successfully thawed and regrown is designated a continuous cell line (Paul, 1970).

Two continuous malignant cell lines, M52B derived from a murine sarcoma and NUB 1 derived from a human breast carcinoma, were used in this study which investigated the effects of GLA on malignant cells growing both *in vitro* and *in vivo*. In addition, primary cultures of non malignant fibroblasts were initiated from dorsal surface skin of adult BALB/c nude mice, in order to examine the effects of GLA on normal cells.

### 2.1.1 Murine Sarcoma M52B Line

No literature pertaining to the M52B line could be found. M52B cells were obtained from Dr D.W. Verwoerd, Veterinary Research Institute, Onderstepoort, who originally received the line from the National Cancer Institute, Bethesda, Maryland.

The only information available indicated that the line:

(i) was derived from a subcutaneous murine sarcoma

(ii) produced the murine sarcoma (MUS) and murine leukaemia (MUL) viruses and

(iii) transplanted efficiently in newborn BALB/c mice following subcutaneous inoculation of approximately $10^6$ cells.

### 2.1.2 Human Breast Carcinoma Line (NUB 1)

Cells of the NUB 1 line were obtained from Dr K.M.Robinson, University of Natal Medical School, Durban. The line was derived by passaging a poorly differentiated human breast carcinoma through nude mice. Well differentiated xenografts were removed and explants cultured. The resultant NUB 1 line consists of uniform tightly adherent epithelial cells lacking cytoplasmic oestrogen and progesterone receptors (R.Pegoraro, pers. comm.*).

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* Mrs.R.Pegoraro, Department of Chemical Pathology, University of Natal Medical School, Durban.

Figure 3: A schematic representation of the dry explant technique

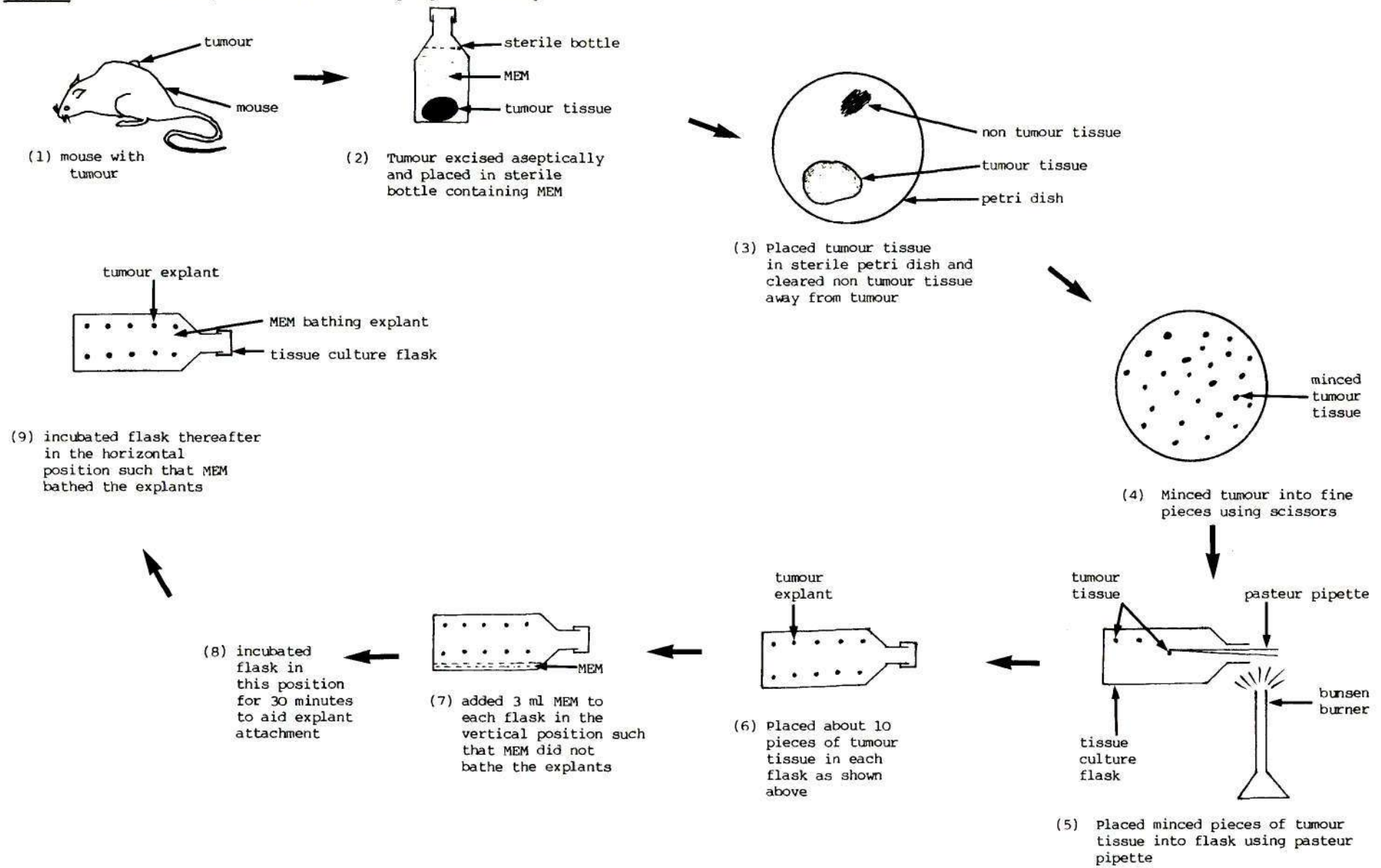
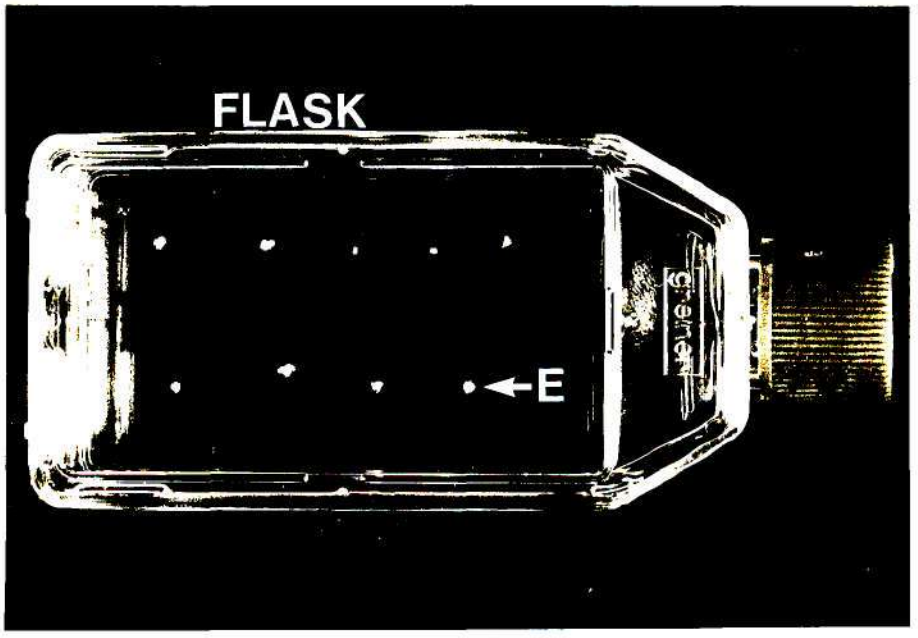


Figure 4: Figure showing tissue culture flask containing 9 explants of murine sarcoma tissue




E = explant

## 2.7 CELL STAINING

### 2.7.1 May-Grünwald / Giemsa Method

For observation of morphological features, cultured cells were stained by the May-Grünwald/Giemsa technique. The culture medium was decanted from the flask and 5 ml of 0.25% May-Grünwald (Gurr) in methanol added. After 6 minutes cells were rinsed twice with tap water and 5 ml of 5% Giemsa (Gurr) in water added for a further 6 minutes. Cells were again washed twice with tap water and air dried.

### 2.7.2 Trypan Blue

The dye exclusion test for cell viability (Philips, 1973) was performed using a 0,25% aqueous solution of trypan blue. Approximately 0.1 ml of the dye was added to 5 ml culture medium and the cells viewed after 10 minutes. Viable cells did not take up the stain.

## 2.8 CELL OBSERVATION

### 2.8.1 Macroscopic viewing

A rapid method of assessing cell density was to view culture

flasks against a light source. Cultures that exhibited a "white-mat" appearance were trypsinized. Stained cells were also viewed macroscopically.

### 2.8.2 Light Microscopy

Unstained cells were routinely viewed with an Olympus CK II inverted phase contrast light microscope at a magnification of X100 using green and neutral density grey filters. Viewing of stained cells did not necessitate the usage of a phase contrast microscope.

### 2.8.3 Photography of Cells

All photomicrographs of actively dividing cultured cells were taken on a Zeiss photomicroscope 3 with an interference filter and Ilford Pan F film (ASA 50) which was developed in 1:50 Rodinal.

### 2.8.4 Electron Microscopy

In order to examine cell morphology and detect virus production, cultured M52B cells (control and GLA treated) were prepared for electron microscopy according to the method of Robinson and Gregory (1978) as described below:

Semi-confluent cultures were rinsed in MEM, fixed in 1% glutaraldehyde in MEM, postfixed in 1% osmium tetroxide and embedded in araldite. Ultrathin sections (60-80 nm) were cut vertically on the Nova Ultratome with the araldite on the one side and the plastic of the flask on the other as described by Keen et al. (1973). These sections were then mounted on uncoated copper grids and stained with uranyl acetate and lead citrate (Reynolds, 1963; Watson, 1958).

Cultured M52B cells were viewed using a Zeiss EM 10B transmission electron microscope. All photographs were taken at 60 kV using Kodak electron image film 4489 which was developed in D19 (diluted 1+2 in water) and fixed in Amphix.

## 2.9 FREEZING OF CULTURED CELLS AND TUMOURS

### 2.9.1 Cultured Cells

For storage purposes, semi-confluent cultures were frozen according to the method of Bertoleni et al. (1976) in MEM supplemented with 20% foetal calf serum and 10% dimethyl sulphoxide (freezing medium). A measured 5 ml of freezing medium was added to cultures. Flasks were sealed, covered in aluminium foil and labelled. The procedure of freezing to -70°C was controlled over a period of 150 minutes as follows: Flasks were incubated at 4°C for the first half hour, at - 4°C

for the next 2 hours and finally at - 70°C in a Specht ultra deep freeze for the remaining storage period. Flasks were positioned horizontally during the freezing process.

Thawing of cells was performed rapidly at 37°C. The freezing medium was decanted and 5 ml fresh MEM containing 10% foetal calf serum, added. The pH of the culture medium was then adjusted and cells incubated at 37°C.

2.9.2 Explanted Tumours

Tumours were excised aseptically and placed in sterile, labelled vials which were then covered in aluminium foil and stored at -70°C.

# CHAPTER 3

# EXPERIMENTAL PROCEDURE

# IN VITRO

## 3.1 CHARACTERISATION OF THE M52B LINE

The continuous murine cell line was characterised in the following ways:

(i) by assessing tumorigenicity *in vivo* in BALB/c, Biozze and Swiss Albino ("Onderstepoort strain") mice (Section 4.3)

(ii) by histopathological examination of tumours (Section 4.6)

(iii) by assessing growth and behaviour of M52B cells *in vitro* (Section 2)

(iv) by examining the ultrastructure of M52B cells growing *in vitro* and *in vivo* (Section 2.8.4).

## 3.2 INVESTIGATION OF SOME OF THE EFFECTS OF GLA

*In vitro* studies were conducted on dense (approximately $2 - 4 \times 10^6$ cells per flask) and less dense (approximately $1.2 \times 10^6$ cells per flask) cultures of M52B cells as well as on primary cultures of non malignant fibroblasts (primary cultures

As the nude mouse is immunologically incompetent, it is highly susceptible to infection by foreign organisms. Therefore, in addition to the standard practices of mouse husbandry described for conventional systems, a specific pathogen free environment (SPF-4) was created for these animals.

## 4.2 MAINTENANCE OF MICE

### 4.2.1 Immunocompetent Mice

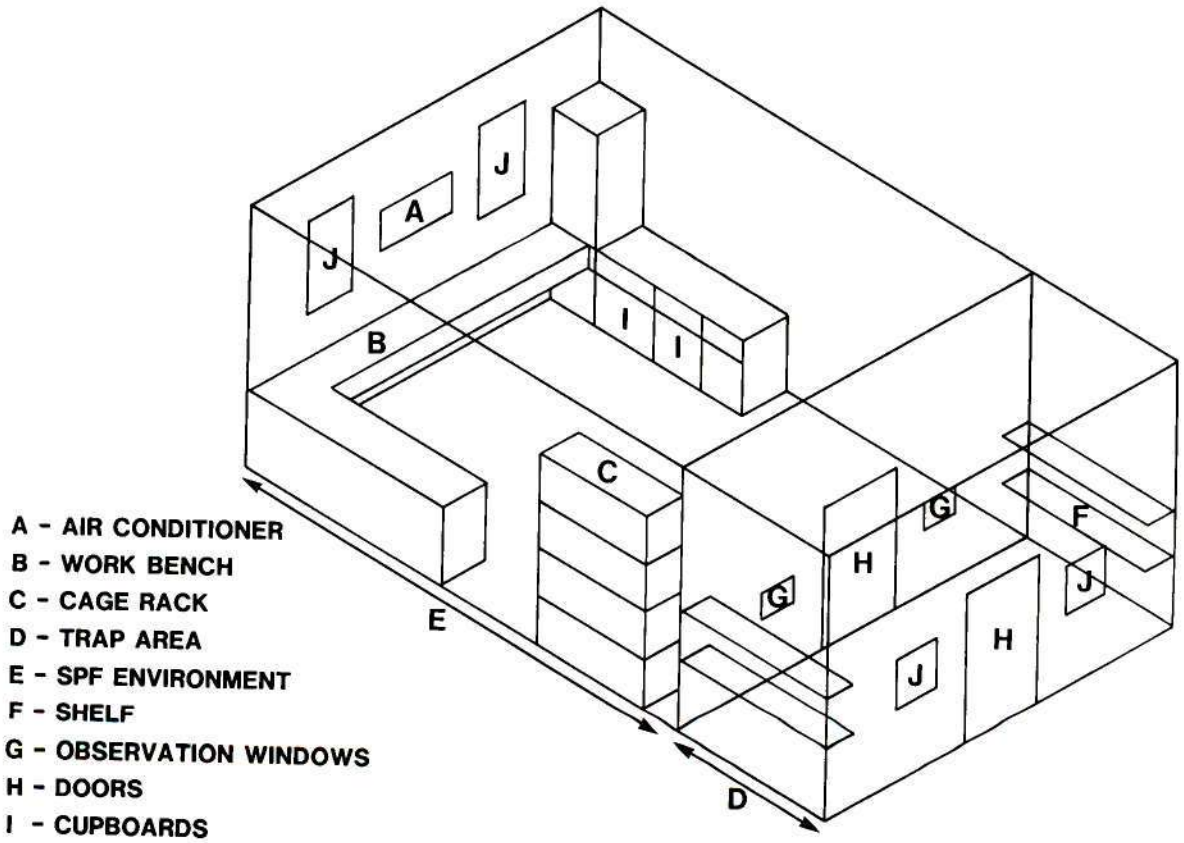
Immunocompetent mice were maintained under conventional temperature and humidity conditions.

### 4.2.2 Nude Mice

The nude mice were housed in a room designed with an entrance "trap" area as shown in Figure 5. The room was initially prepared by sealing off of all openings to the outside and thorough cleaning of all surfaces. A positive flow of filtered air through a thermo regulatory air conditioner (Homeaire) maintained a temperature of 25$^{o}$C.

The 9 m$^{3}$ room was sterilized with paraformaldehyde vapour produced by adding 300 ml 40% w/v formaldehyde to 100 g potassium permanganate.

Figure 5: The Nude Mouse Room



Following this procedure sterility was promoted by a continuously burning 30 watt ultraviolet germicidal lamp. Autoclaved supplies of food, water, sawdust, gowns, caps and shoes were stored in the "trap" area for a minimum of 24 hours before use.

The trap area was also used by personnel to dress in sterile cap, mask, gown and shoes (Kendon Laboratories) prior to entering SPF environment. Hands were washed thoroughly in a solution of 5% chlorhexidine gluconate (Hibitane).

### 4.2.3 Breeding of Mice

#### 4.2.3.1 Immunocompetent Mice

Litter sizes of BALB/c, Biozze and "Onderstepoort" mice ranged from 5 to 15 young. Mice were weaned at 21 days. Adult females were then paired with adult males of their respective strains to continue breeding.

#### 4.2.3.2 Nude Mice

The breeding system used was that of mating heterozygous (nu/+) females with homozygous recessive (nu/nu) males. An economical ratio which resulted in maximum nude litter production was mating of 1 male to 3 to 4 females. Litter

sizes ranged from 4 to 25 mice with approximately 50% being nudes. The nude mice grew slowly and were smaller in size than heterozygous littermates (Figure 6).

Within 3 to 5 days of birth, the homozygous nude mice were identified by either the absence of vibrissae or presence of crinkled, poorly developed vibrissae. Heterozygous males were immediately removed while the heterozygous females were left in the colony for continued breeding. The average weaning age was 28 days after which time the mother was removed and placed with a breeding mate.

Figure 6: Photograph of a BALB/c nude (nu/nu) and heterozygous immunocompetent (Nu/nu) mouse at the same age (17 days old) demonstrating size difference

N = nude mouse

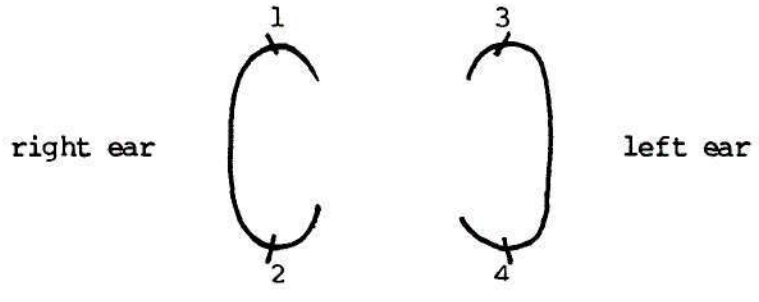
I = immunocompetent mouse

4.2.4 Caging

All mice were maintained in polypropylene boxes (depth 445 mm, width 280 mm, height 125 mm) with metal grills (Labotek). Each week cages for use in the SPF environment were washed and sterilized by soaking in 5% Hibitane (diluted 1 : 250) for 48 hours. Cages were then transferred to the "trap" area for 24 hours. A maximum of 20 mice were housed per cage.

4.2.4.1 Numbering of Mice

Mice were numbered by nicking the ears according to the scheme below




Higher numbers were indicated by more than one nick.

4.2.5 Bedding

Sawdust (pine and meranti wood), obtained from Baker Brothers,

was placed in each box to a depth of approximately 2 cm to serve as bedding. Sawdust for nude mice was sterilized by autoclaving for 20 minutes at 105 kPa. Bedding was replaced weekly.

### 4.2.6 Routine Diet

Pelleted laboratory chow (National Food Distributors), was provided in the food hoppers and drinking water was given by bottle. Both food and water were supplied ad libitum and changed thrice weekly.

Food and water supplied to nude mice were autoclaved at 105 kPa for 20 minutes. The composition of the Standard laboratory chow is shown in Table I.

Table I

Constituents of United Oil Epol Rat Cubes

| | | | |
|---|---|---|---|
| Protein | 18.71% | Vitamin A | 21 124 I.U/kg of feed |
| Fibre | 3.71% | Vitamin $D_3$ | 1 677 I.U. |
| Fat | 4.31% | E | 37.3 mg/kg feed |
| Sugar | 2.14% | $B_2$ Riboflavine | 5.7 mg/kg |
| Ca | 1.06% | Pantothenic acid | 14.8 mg/kg |
| NaCl | 0.63% | Niacin | 53.8 mg/kg |
| (Na= | 0.25% | Choline HCl | 1 117.8 mg/kg |
| Phosphates | 0.76% | | |
| Available Phosphates | 0.61% | $B_1$ Thiamine | 5.8 mg/kg |
| 70% from animal phosphates | | Folic acid | 2.1 mg/kg |
| Energy | 1 285.2 kJ/kg | (Folicia) | |
| | | K3 | 4.2 mg/kg |
| Amino Acids | | $B_6$ Pyrodoxine | 8.4 mg/kg |
| | | $B_{12}$ | trace |
| Arginine | 1.16% | Biotin | trace |
| Lysine | 0.93% | Amantioxidine | 178.2 mg/kg |
| Methionine | 0.38% | | |
| Cystine | 0.44% | Minerals | |
| Tryptophan | 0,16% | Calcium | 10.63 g/kg feed |
| Histidine | 0.33% | Phosphorus | 7.56 g/kg |
| Leucine | 1.44% | Magnesium | 1.42 g/kg |
| Isoleucine | 0.73% | Na | 2.5 mg/g |
| Phenylalanine | 0.74% | K | 4.43 mg/g |
| Tyrosine | 0.55% | | |
| Threonine | 0.90% | Mn | 17.6 mgm/kg |
| Valine | 0.95% | $MnSO_4$ | 104.8 mgm/kg |
| Glycine | 0.77% | $ZnSo_4$ | 429.6 mgm/kg (172 mg Zn/Kg) |
| | | $CuS_4$ | 828.2 mgm/kg |
| | 9.48% | Cobalt $SO_4$ | 10.5 mgm/kg |
| Available = | 8.66% | $FeSO_4$ | 471.4 mgm/kg |
| | | Ash | 4.5% |

## 4.3 GLA ADMINISTRATION

GLA was administered either orally or parenterally *in vivo*. For oral studies, oil enriched diets were prepared, while in parenteral studies GLA (Sigma) was mixed in hydrogenated coconut oil (HCO) and injected subcutaneously.

### 4.3.1 Preliminary Studies Conducted to Determine the Maximum Tolerated Oral Fat Supplementation

Pilot studies were conducted to determine the maximum dietary supplement of oil which mice would tolerate. Nude and immunocompetent BALB/c mice were fed standard chow supplemented with either 33%, 37% or 41% HCO over 42 days. HCO was used in these pilot studies because it was free of prostaglandin precursors.

Both nude and immunocompetent mice which received 41% HCO showed a loss of approximately 10% body weight. Supplementation to levels of 37% and 31% produced no changes in body weight. Histopathological investigations were therefore conducted only in the latter two groups. Results of examinations performed on skin, small bowel and liver indicated no abnormal fatty deposits in skin and small bowel of both nude and immunocompetent mice consuming 33% or 37% HCO. Liver of

nude and immunocompetent mice receiving 33% HCO were also devoid of abnormal fatty deposits while in the liver of both nude and immunocompetent mice consuming 37% HCO, mild fatty changes occurred with focal necrosis in the immunocompetent mice.

As pathological changes were noted only in mice consuming 37% HCO it was decided to use a slightly lower concentration, namely 35%, in the investigations.

4.3.1.1 Oil Enriched Experimental Diets

Evening primrose oil (EPO), used as a source of the prostaglandin precursor gamma linolenic acid (GLA) (Huang et al., 1982), was provided orally in the diet of some tumour bearing mice. However, since EPO also contained linoleic acid (LA), an additional prostaglandin precursor, isolation and interpretation of effects solely due to GLA were difficult. Sunflower seed oil (SSO) was therefore used as a control as it contained all fatty acids present in EPO with the exception of GLA (de Deckere and ten Hoor, 1979) (Table II). Furthermore, hydrogenated coconut oil (HCO) which consisted of saturated fats only (de Deckere and ten Hoor, 1979), was used as a second control (Table II).

TABLE II: The fatty acid composition of EPO, SSO and HCO

| Fatty Acid | | Percent Composition | | |
|---|---|---|---|---|
| Name | formula | EPO | SSO | HCO |
| caprylic acid | C 8 : 0 | 0 | 0 | 7 |
| capric acid | C 10 : 0 | 0 | 0 | 6 |
| lauric acid | C 12 : 0 | 0 | 0 | 4.5 |
| myristic acid | C 14 : 0 | 0.1 | 0 | 17 |
| palmitic acid | C 16 : 0 | 5.7 | 6 | 10 |
| stearic acid | C 18 : 0 | 0.9 | 4 | 14 |
| oleic acid | C 18 : 1(n-9) | 8.8 | 25 | 0 |
| cis-linoleic acid | C 18 : 2(n-6) | 74.2 | 62 | 0 |
| gamma linolenic acid | C 18 : 3(n-6) | 10.3 | 0 | 0 |

During the study both nude and immunocompetent mice were fed one of four diets.

(a) Standard laboratory chow or Standard chow supplemented with

(b) EPO

(c) SSO or

(d) HCO.

Diets were prepared as required, by the addition of 35 g

(43 ml) of the respective oils to 65 g crushed standard chow. The approximate rate of food consumption was 10 g per mouse per day of which 3.5 g was the oil.

Mixed diets were placed immediately in hoppers. In the SPF environment, food was protected with aluminium foil against oil degradation by the germicidal lamp. Oil stocks were protected from light in dark bottles covered with alumunium foil. Freshly mixed diets were provided on alternate days.

### 4.3.2 Parenteral Administration of GLA

HCO which served as a control in the oral study was used as a solvent for parenteral administration of GLA. GLA was administered subcutaneously in the region of the tumour using a 25 gauge needle (venoject).

Control mice were injected daily with 0.1 ml HCO only while mice in the experimental group were injected daily with 0.1 ml HCO containing 100 ug GLA.

## 4.4 TUMOUR INDUCTION

Tumours were induced in newborn immunocompetent mice, prior to full development of their immune systems, and in weaned (4-6 weeks old) nude mice by the subcutaneous inoculation of diced tumour suspensions or cultured cells. Mice were injected

1977).

$$\text{Volume } (mm^3) = \frac{\text{length (mm) x width}^2\text{(mm)}}{2}$$

Tumour growth rates were derived from volume measurements. The tumours were weighed immediately following excision on a top - pan balance and the mass recorded in grams.

## 4.6 TUMOUR EXCISION

Depending upon the condition of the animal, the size and degree of invasion of the tumours and the requirements of the particular study (Section 5), mice were either sacrificed or anaesthetised prior to tumour excision. Mice with aggressive invasive tumours were sacrificed by intraperitoneal injections of 12 mg sodium pentobarbitone (Sagatal, Maybaker). The remaining mice were anaesthetised with intraperitoneal injections of 0.6 mg sodium pentobarbitone and 0.125 mg ketamine hydrochloride (ketamine diluted 1:10 in 0.9% saline).

Following tumour removal, incisions in anaesthetised mice were sutured (3/8 circle 12 mm suture, Davis and Geck). Tumours were placed in weighed sterile vials containing 10% formol saline and weights recorded.

Tumours were processed for histopathological examination in the Department of Histopathology, University of Natal Medical School.

## 4.7 TRANSMISSION ELECTRON MICROSCOPY

Following excision of tumours, samples of tumour tissue were fixed immediately in Karnovsky's fixative (Karnovsky, 1965) at pH 7.4 for 1 hour at 4°C and then postfixed in 1% osmuin tetroxide in cacodylate buffer at pH 7.4 for a further hour at 4° before dehydration through increasing concentrations of ethanol. Samples were embedded in Araldite resin (Glauert et al., 1965) and ultrathin sections were cut, stained and viewed as described in section 2.8.4.

## 4.8 STATISTICAL ANALYSIS OF RESULTS

Studies which consisted of one control and one experimental group with three or more mice per group were analysed statistically using the unpaired student's "t" test.*

Experiments with more than one control or experimental group were analysed according to the Kruskal-Wallis procedure (Siegel, 1956).

Statistical tests were used to compare

1. starting tumour volumes
2. final tumour volumes and
3. the difference between final and initial volumes of control and experimental group.

*(Tektronix 4051 computer using software written by a member of the Pharmacology department)

# CHAPTER 5

# EXPERIMENTAL PROCEDURE

# IN VIVO

## 5.1 M52B LINE

### 5.1.1 Determination of Some of the Effects of GLA

#### 5.1.1.1 Immunocompetent Mice

The effects of GLA on allografts in immunocompetent mice were examined in 4 experiments which varied in terms of strain of mice, route of fatty acid administration, control treatment and duration of study.

Tumours were established in all newborn mice as described in Section 4.4. The design of each of the experiments is shown in Table III.

Upon tumour development, mice were divided randomly into control and experimental groups. Control groups of BALB/c and "Onderstepoort strain" mice received standard laboratory chow while corresponding Biozze mice were either untreated or injected subcutaneously with 0.1 ml HCO. Treated groups of BALB/c and "Onderstepoort strain" mice received standard chow supplemented with 35% EPO while Biozze mice were injected with 0.1 ml HCO containing 100 ug GLA.

Table III: Summary of experiments conducted to investigate the effects of EPO and GLA on M52B tumours growing in immunocompetent mice

| | BALB/c randombred | | BALB/c inbred | Biozze inbred | "Onderstepoort" strain inbred |
|---|---|---|---|---|---|
| | Experiment 1 | Experiment 2 | | | |
| No. of mice inoculated | 64 | 19 | 70 | 43 | 47 |
| No. of mice with tumours | 9 | 8 | 36 | 19 | 12 |
| Take rate | 14% | 42% | 51% | 44% | 25% |
| Latent period | 35 days | 17 days | 12 days | 16 days | 19 days |
| No. of mice in control group | 2 | 4 | —— | 5 untreated 6 HCO | 4 |
| No. of mice in EPO group | 4 | 4 | —— | 8 (GLA in HCO) | 18 |
| Route of administration | oral | oral | —— | parenteral | oral |
| No. of days on experiment | 10 days | 13 days | —— | 10 days | 14 days |
| | | | No experiment conducted as all tumours regressed at 25 days | | |
| Tumour remission | 16% | 50% | 100% | 42% | 25% |

5.1.1.2 Nude BALB/c Mice

The effects of GLA on allografts in nude mice were assessed in 3 experiments which varied in terms of the number of animals per group, control diet and duration of study.

In all 3 experiments, weaned BALB/c nudes of both sexes were injected subcutaneously with approximately $10^6$ M52B cells as described (Section 4.4). Latent periods and take rates were recorded. Mice which developed tumours were randomised into control and experimental groups. Control groups received either standard laboratory chow or standard chow supplemented with 35% HCO or 35% SSO while all experimental groups received standard chow supplemented with 35% EPO (Table IV).

Table IV: Summary of the experiments conducted to investigate the effects of GLA on M52B tumours growing in nude BALB/c mice

| | Experiment I | Experiment II | Experiment III |
|---|---|---|---|
| No. of mice inoculated | 24 | 17 | 12 |
| No. of mice with tumours | 20 | 17 | 12 |
| Take rate | 83% | 100% | 100% |
| Latent period | 21 days | 21 days | 14 days |
| No. of mice in control group | 5 Standard chow<br>5 SSO<br>5 HCO | 8 Standard chow | 6 Standard chow |
| No. of mice in EPO group | 5 | 9 | 6 |
| No. of days on diet | 21 | 10 | 11 |

## 5.2 NUB 1 LINE

### 5.2.1 Investigation of Some of the Effects of GLA

#### 5.2.1.1 Nude BALB/c Mice

The effects of GLA on xenografts in nude mice were examined in 3 experiments which varied in terms of inoculum (NUB 1 cells or NUB 1 tissue), the number of animals per group, stage at which diets were started and duration of study. (Table V ).

Weaned nu/nu BALB/c mice of both sexes were injected subcutaneously with either NUB 1 tissue or cells as described in Section 4.4. Mice injected with NUB 1 cells were randomised 5 days after inoculation, prior to tumour development into control and experimental groups while mice inoculated with NUB 1 tissue were randomised into 2 groups subsequent to tumour establishment.

In addition to measuring tumour sizes, excising, weighing and examining histology of the tumours, incisions in nude mice of this study were sutured and the surviving animals observed for tumour recurrence.

Table V: Summary of the experiments conducted to investigate the effects of EPO on NUB 1 xenografts growing in nude BALB/c mice

| | Experiment I | Experiment II | Experiment III |
|---|---|---|---|
| Inoculum type | NUB 1 cells | NUB 1 tissue | NUB 1 tissue |
| No. of mice inoculated | 15 | 15 | 40 |
| No. of mice with tumours | 6 | 12 | 37 |
| Take rate | 40% | 80% | 92.5% |
| Latent period | 18 days | 19 days | 7 days |
| No. of mice in control group | 2 | 6 | 18 |
| No. of mice in EPO group | 4 | 6 | 19 |
| No. of days after inoculation when diet was started | 5 | 19 | 17 |
| No. of days on diet | 17 | 34 | 21 |

# CHAPTER 6

# RESULTS

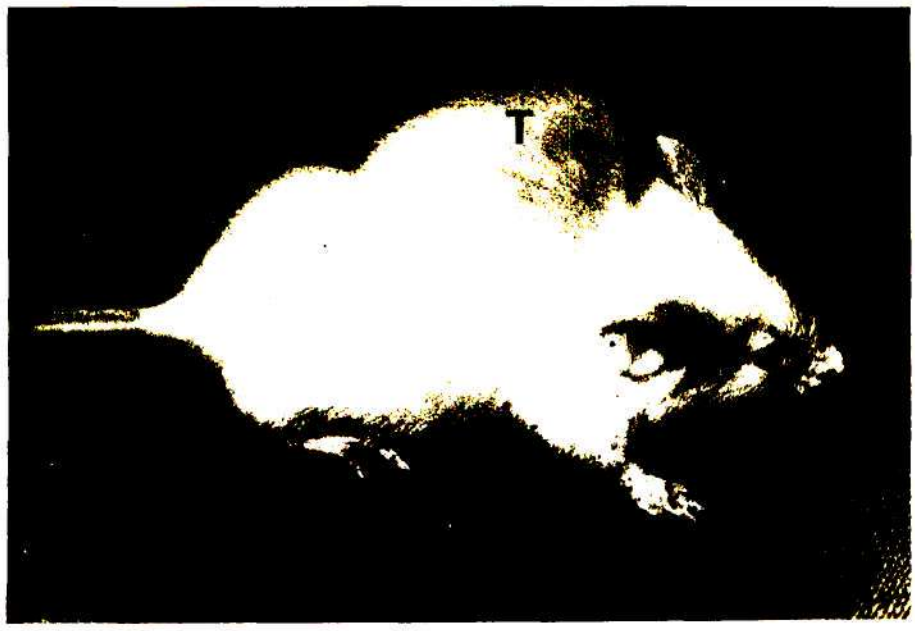
# IN VITRO

## 6.1 CHARACTERISATION OF THE M52B LINE

### 6.1.1 Tumorigenicity

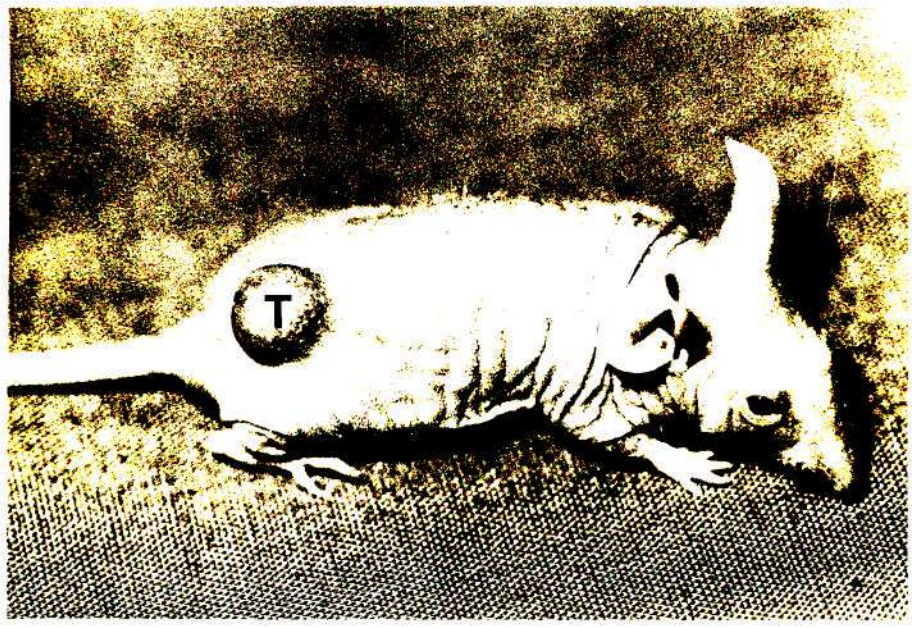
Cells of the M52B line were shown to be tumorigenic in immunocompetent BALB/c (randombred and inbred) (Figure 7), nude BALB/c (Figure 8), immunocompetent Biozze and immunocompetent outbred ("Onderstepoort strain") mice, following subcutaneous inoculation of approximately $10^6$ cells. Tumours appeared at the site of inoculation within 12-26 days as minute palpable nodules which enlarged rapidly and either regressed spontaneously or grew progressively to diameters of approximately 3.5 to 4 cm within 14 days of initial observation. Observed tumour latent periods, take rates and remissions in different strains of mice are recorded in Table VI. Take rate was apparently related to the immune status of the host, being highest in the nudes. Tumour remission was a frequent occurrence among immunocompetent mice, though not observed in nudes.

Figure 7: Immunocompetent inbred BALB/c mouse bearing an M52B tumour 30 days after inoculation




T = tumour

Figure 8: Nude BALB/c mouse bearing an M52B tumour 21 days after inoculation




T = tumour

Table VI: Tumour latent periods, take rates and remission following inoculation of $10^6$ M52B cells into BALB/c, Biozze and "Onderstepoort strain" mice

| | BALB/c | | | Biozze | "Onderstepoort" strain |
|---|---|---|---|---|---|
| | nu/nu | randombred immuno-competent | inbred immuno-competent | inbred immuno-competent | outbred immuno-competent |
| No. of mice injected | 53 | 83 | 70 | 52 | 47 |
| Latent period (days) | 18 | 26 | 12 | 15 | 19 |
| No. of mice developing tumours | 49 | 17 | 36 | 28 | 12 |
| Take rate (percent) | 92 | 20 | 51 | 53 | 25 |
| Tumour remission (percent) after 25 days | 0 | 35 | 100 | 28 | 25 |

### 6.1.2 Histopathology of Tumours

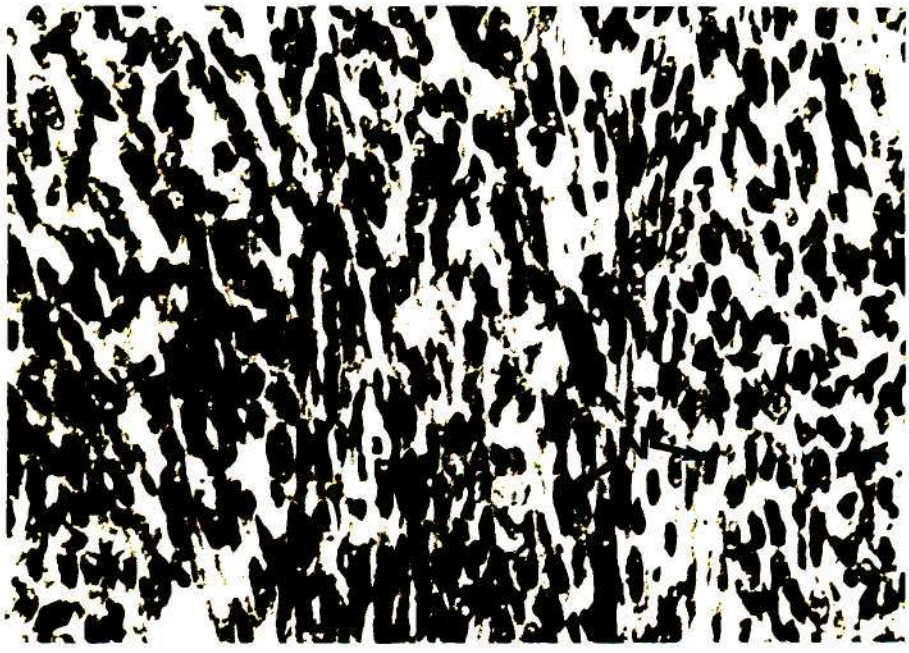
Tumours induced in all mice were solid and generally encapsulated. Small tumours (average maximum diameter of 2.5 cm) were subcutaneous and non invasive while the larger tumours (diameters exceeding 2.5 cm) appeared to lack a continuous capsule and were frequently attached to the dorsal skin or invaded the muscles of the abdominal wall.

Extensive central necrosis was common in large tumours while smaller nodules were less affected. Each tumour had its own peculiar vascular pattern which consisted of a main vascular trunk with smaller blood vessels radiating around the tumour.

Microscopic examination of the tumours revealed anaplastic sarcomas which consisted of pleomorphic, compactly arranged spindle cells (Figure 9) disposed in characteristic "herringbone" patterns (Figure 10).

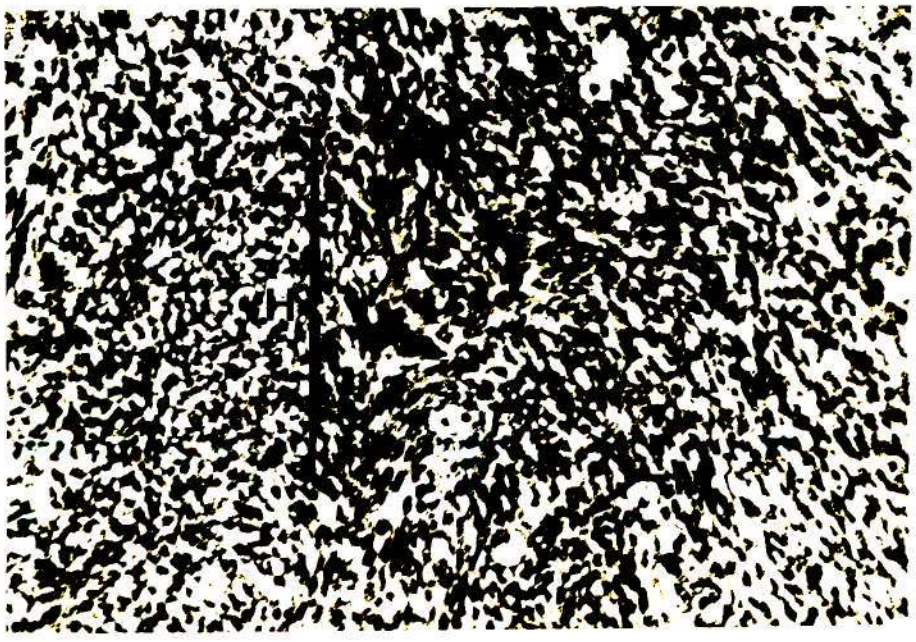
The identification of a poorly differentiated fibrosarcoma was favoured by the presence of pericellular reticulin (Figure 11). Argyrophilic fibres were evident intimately applied to the cytoplasmic borders of individual cells.

Figure 9: Photomicrograph of M52B tumour stained with haematoxylin and eosin to show spindle cells with large nuclei X320



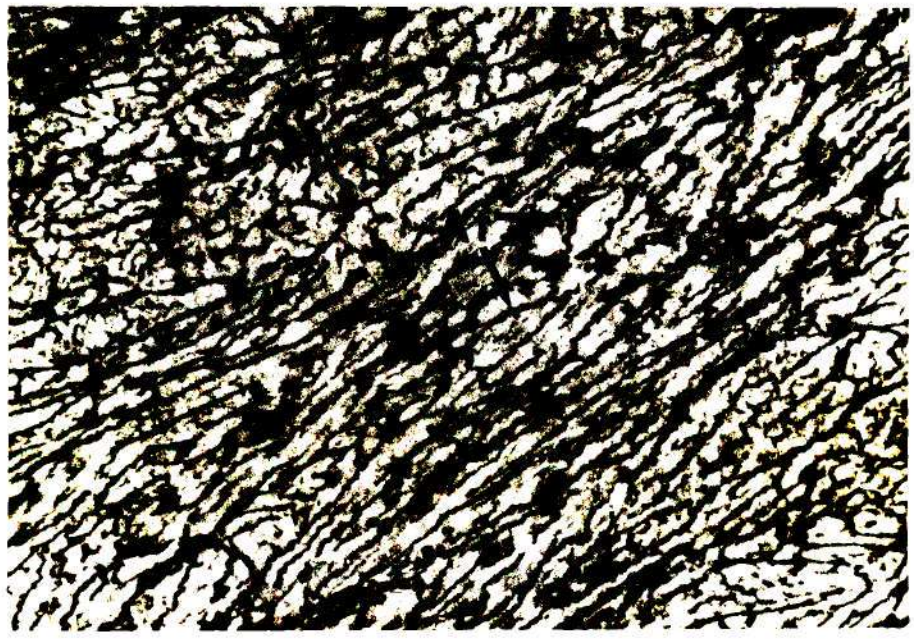
N = nuclei

Figure 10: Photomicrograph of M52B tumour stained with haematoxylin and eosin showing sarcoma cells arranged in a "herringbone" pattern X128



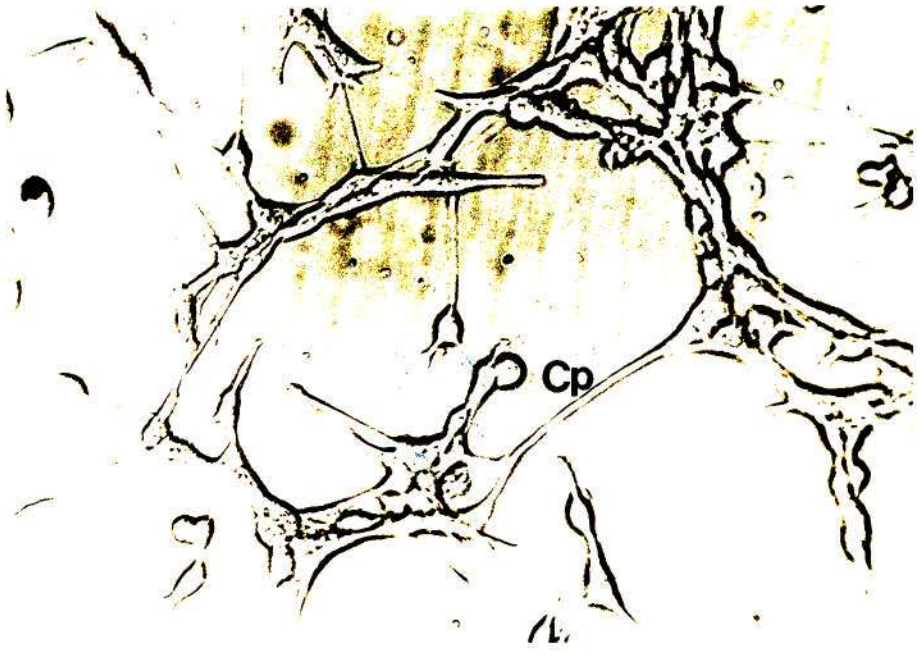
H = "herringbone" pattern
M = mitotic figures

Figure 11: Section of M52B tumour stained for reticulin X400



Af = argyrophilic fibres stained with silver nitrate

Figure 12: Interference photomicrograph of cultured M52B cells showing cytoplasmic processes X128




Cp = cytoplasmic process

### 6.1.4 Ultrastructural investigation of M52B cells growing in culture

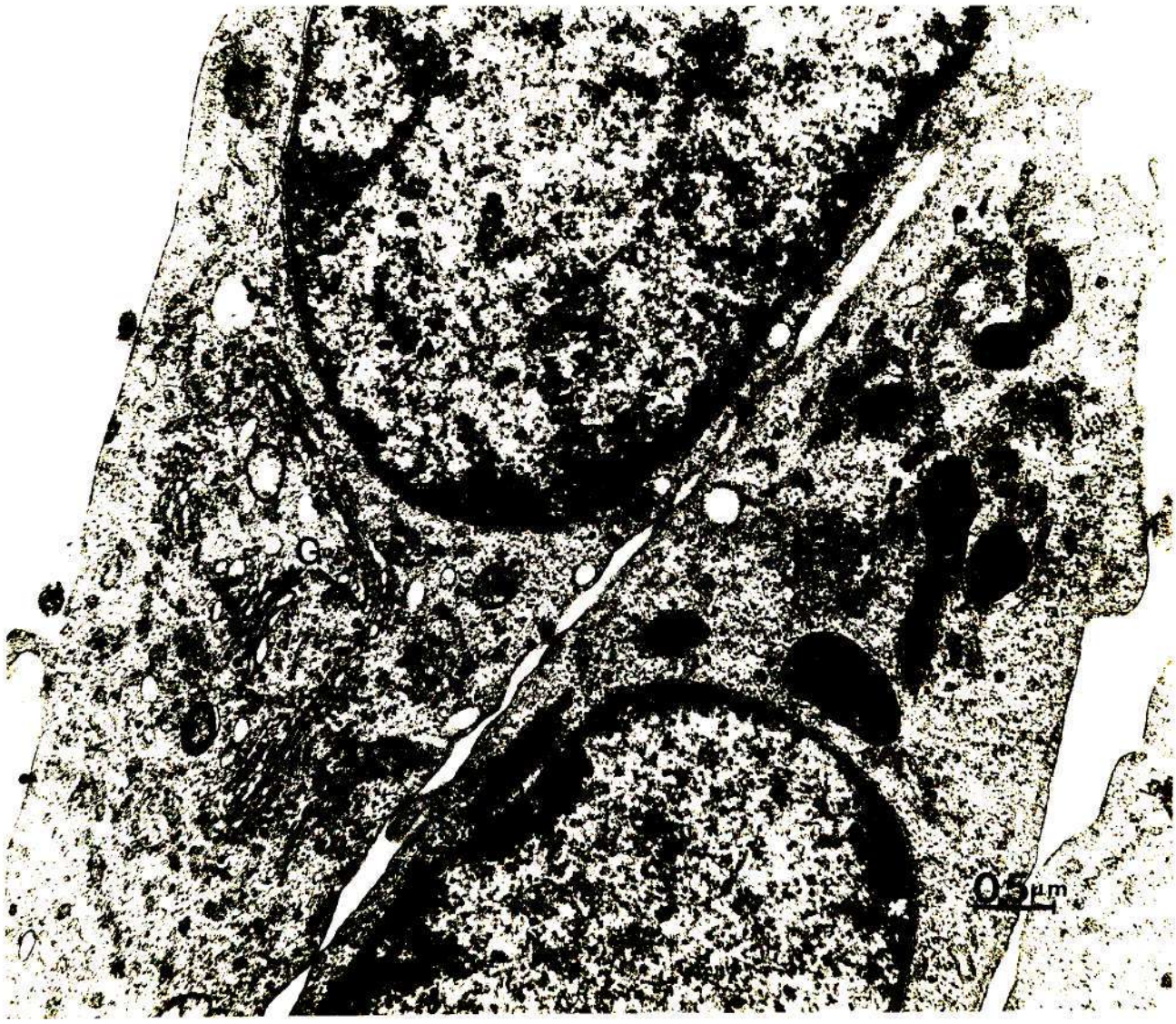
As examination of the murine sarcoma cells by light microscopy was limited, electron microscopic studies were carried out to further elucidate cell structure.

Ultrastructural investigation of the M52B cells revealed, in addition to the prominent nuclei and nucleoli observed by light microscopy, the presence of poorly developed mitochondria, well developed rough endoplasmic reticulum and golgi apparati, myelin bodies and viral particles (Figure 14).

Furthermore, the multilayered arrangement of cultured M52B cells was confirmed using transmission electron microscopy. The absence of desmosomes as deduced from ultrastructural observations supported the histological diagnosis of a sarcoma.

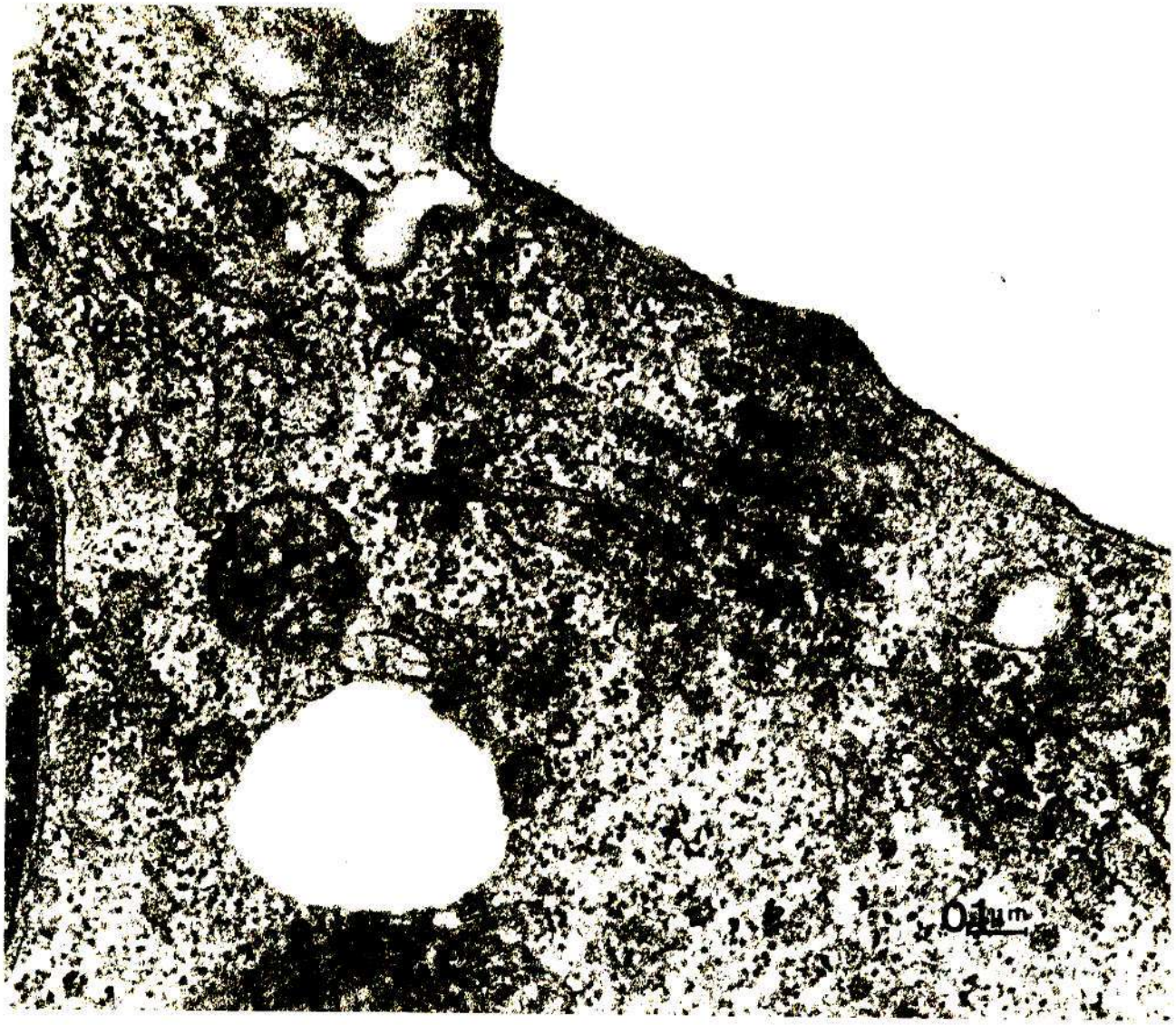
Figure 15: Electron micrographs of cultured M52B cells showing
(a) structure of mitochondria and golgi apparatus X29 670
(b) annulate lamellae and rough endoplasmic reticulum X72 450

(a)




G = golgi apparatus
M = mitochondrion

(b)




RER = rough endoplasmic reticuli
AL = annulate lamellae
F = fenestrae
P = polyribosomes

## Myelin Bodies

Numerous "onion bulb" type myelin figures of varying sizes were observed in the cytoplasm of M52B cells. Such whorled membranous bodies often contained cytoplasm or central inclusions which resembled viral particles or dense bodies (Figure 16).

## Cell Membrane

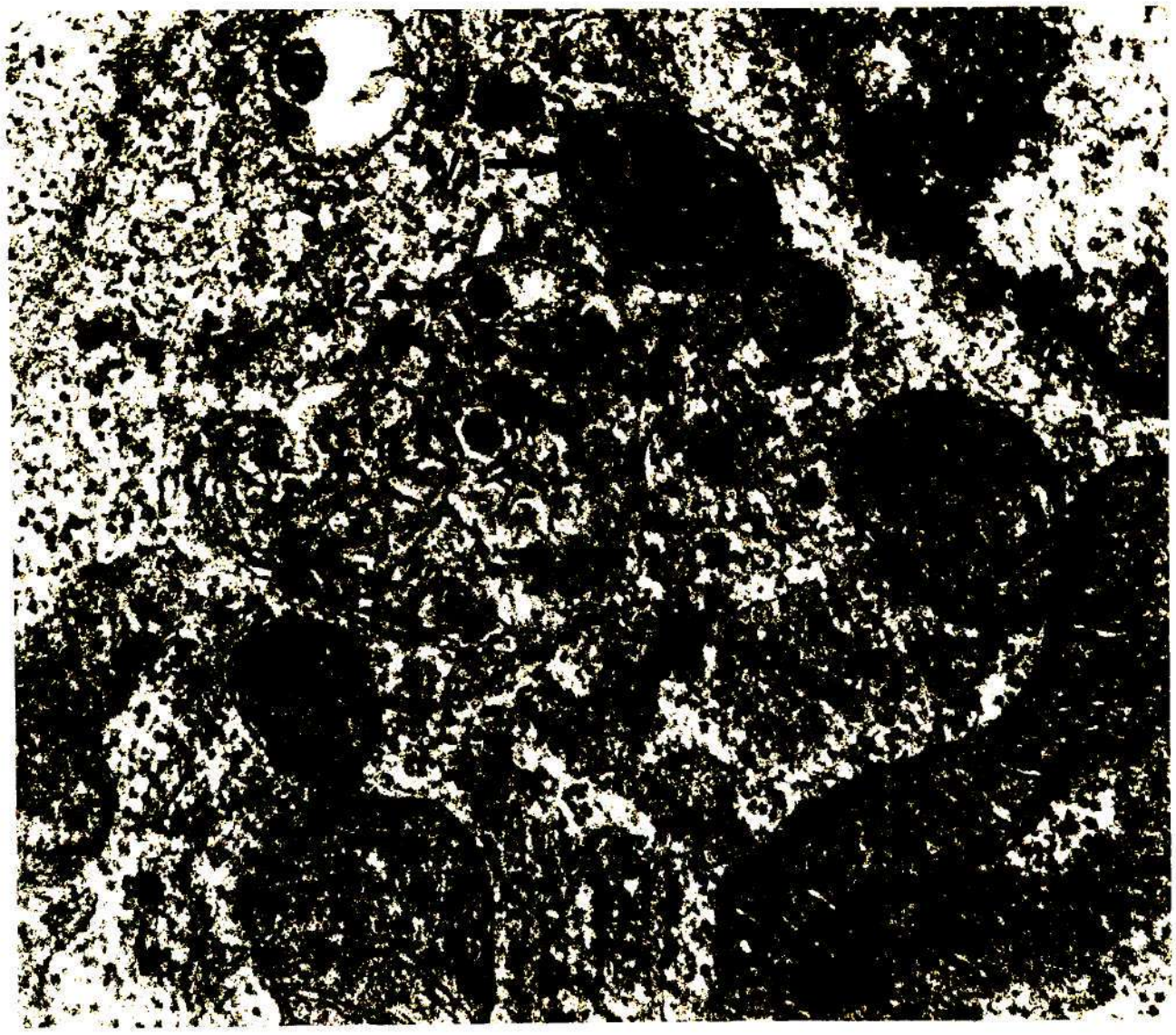
Activity occurred at the cell membrane as evidenced by phagocytosis and the formation of pinocytic vesicles. Short microvillous projections, most of which curved back to the surface were frequently observed on the cell membrane.

## Viral Particles

(a) Location

Viral particles associated with sarcoma cells were spherical and of two apparent sizes and morphologies (Figure 17). Particles were observed lying freely in the cytoplasm (Figure 16), associated with myelin figures (Figure 16), scattered in the intercellular (Figure 18a) and extracellular (Figure 18b) spaces, budding from the cell membrane (Figure 19) or apparently being liberated via microvilli (Figure 17 - V1).

Figure 16: Electron micrograph of cultured M52B cell showing virus like particle in myelin figure and in the cytoplasm X93 150

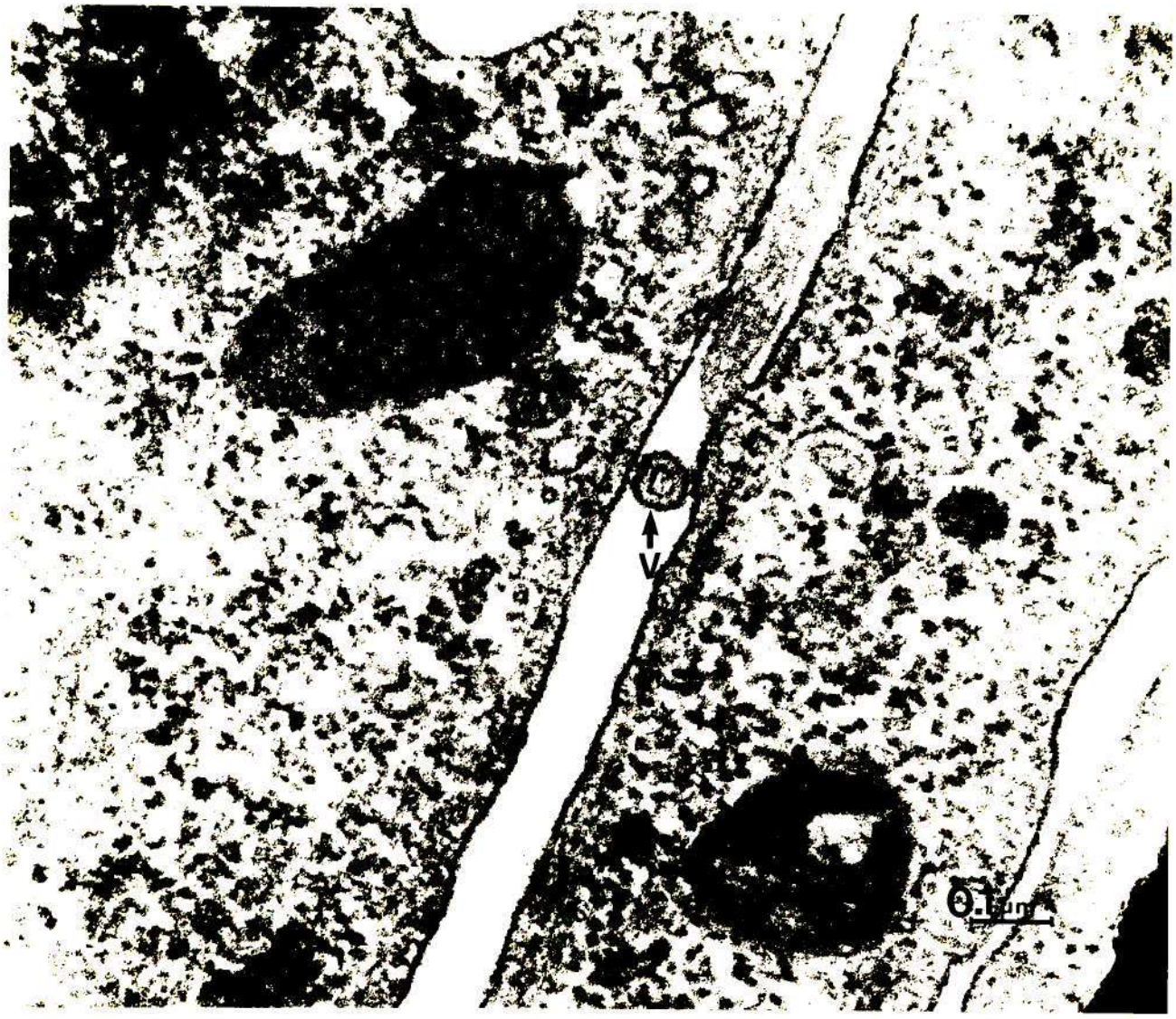


V 1 - virus like particle in myelin figure

V 2 - virus like particle free in cytoplasm

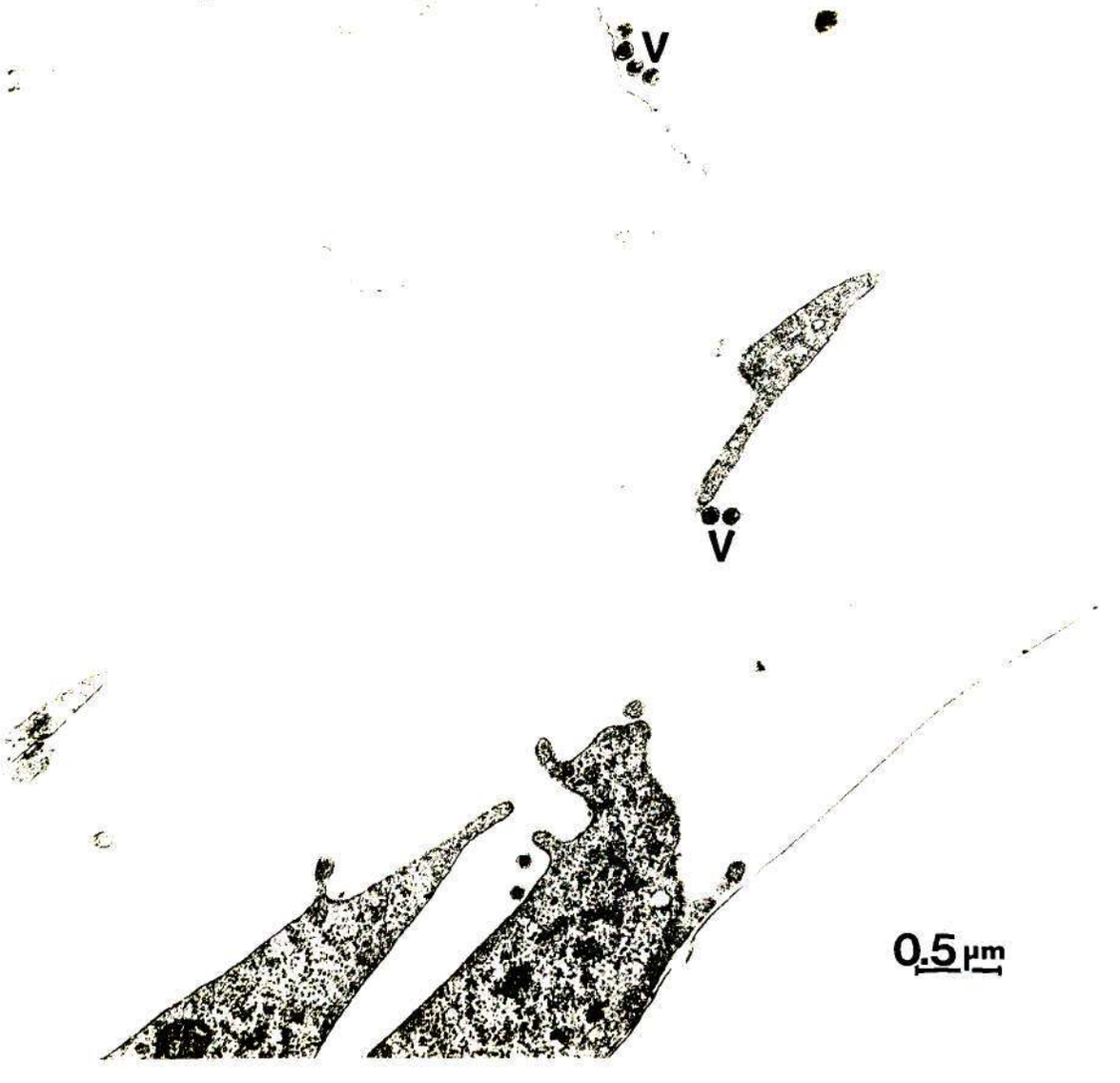
Figure 18: Electron micrographs showing cultured M52B cells with
(a) an intercellular (X100 000) and
(b) extracellular associated virus particles (X29 670)

(a)




V = intercellular virus particle

(b)

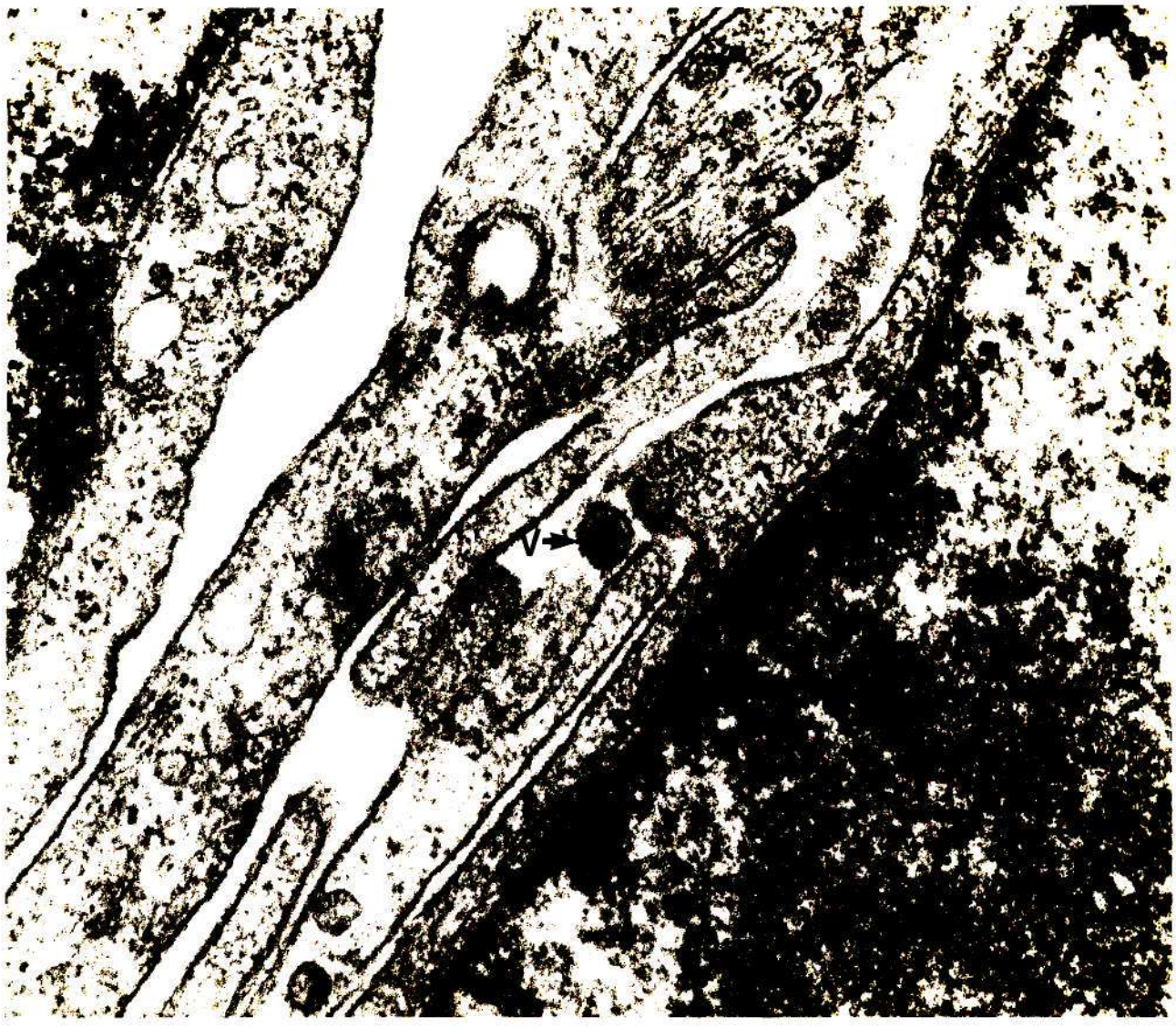



V = extracellular virus particles

v(b) Size and Morphology

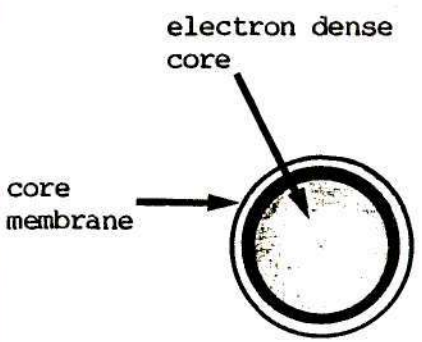
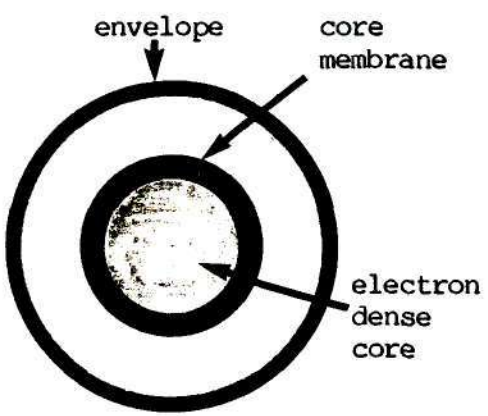
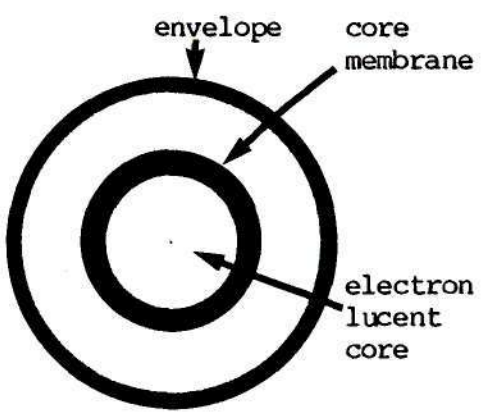
The approximate diameters of the putative viruses were 95 nm and 47 nm respectively with the former having core diameters of approximately 47 nm (refer to Figure 17). The cores of the latter particle could not be distinguished from their envelopes. Therefore, the viral particles were categorised into large enveloped particles and small virus particles which apparently lacked an envelope. However, differences in core density of the enveloped virus particles were observed. Figures 18a and 20 show large enveloped particles with electron lucent and electron dense cores respectively. Figure 21 was compiled to demonstrate the different sizes and morphologies of viral particles present in the M52B cell.

Figure 20: Electron micrograph of cultured M52B cells showing a large extracellular virus particle with an electron dense centre X93 150




V = virus with electron dense core

Figure 21: A representation of viral particles of varying sizes and morphologies found in M52B cells

| A | B | C |
|---|---|---|
|  |  |  |
| 1. small virus (47.6 nm)<br>2. liberated via mircrovillus<br>3. single membrane bound<br>4. electron dense core | 1. large virus (80-110 nm)<br>2. liberated by budding<br>3. double membrane bound<br>4. electron dense core | 1. large virus (80 nm)<br>2. liberated by budding<br>3. double membrane bound<br>4. electron lucent core |

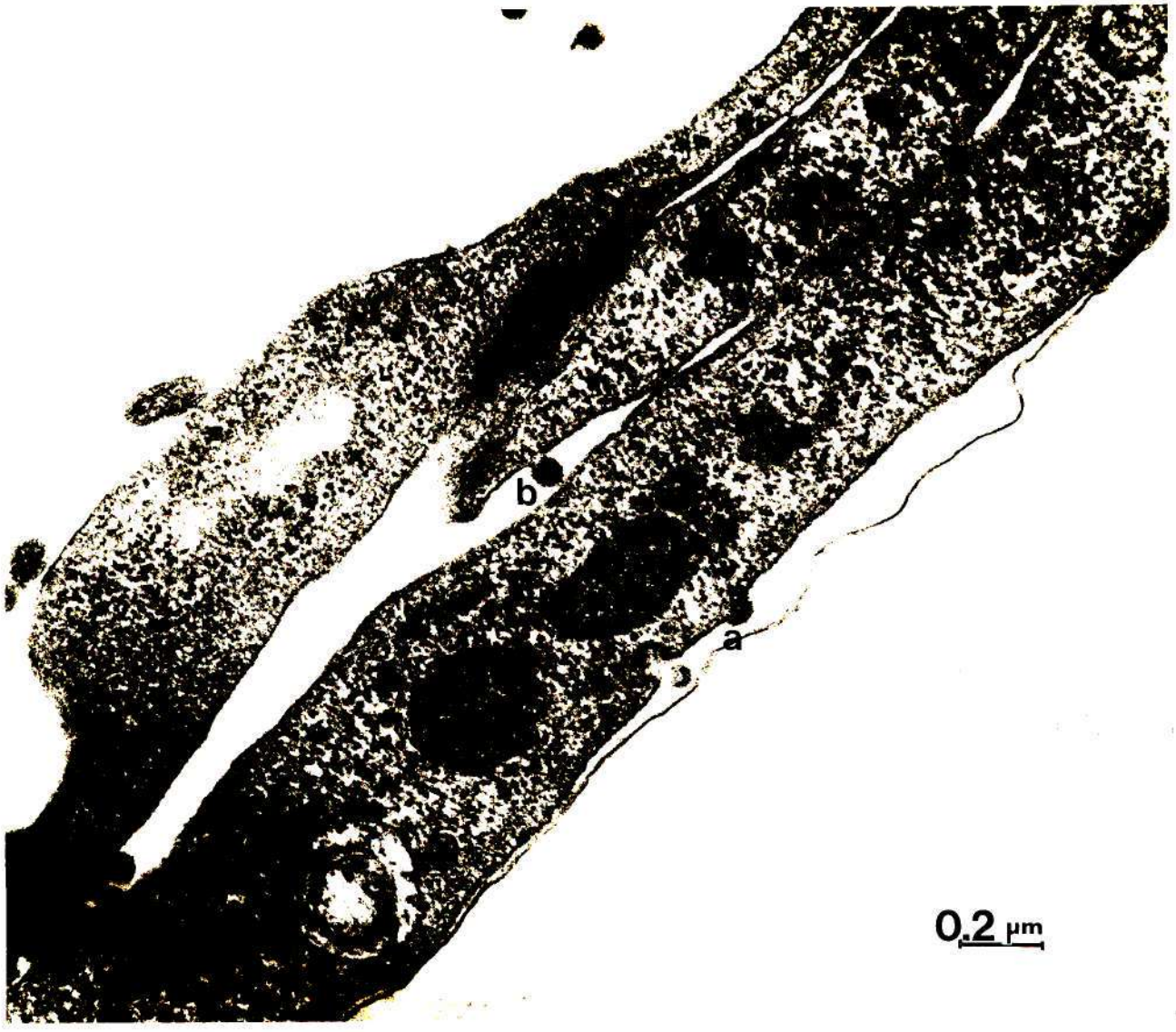
(c) The Budding Process

Numerous budding particles seen associated with the cell membrane led to the assumption that budding was involved in viral release.

Figure 16 represents the stage just prior to budding where single membrane bound virus particles occur either in the cytoplasm or myelin figures of M52B cells. In the postulated second stage (Figure 22a) these virus particles become associated with the cell membrane and thus acquire a second membrane. Figure 22b represents an advanced phase of (a).

Figure 19 shows that stage of budding just prior to the virus becoming an extracellular particle, while Figure 23 represents the product of budding - a double membrane bound virus particle outside the cell membrane.

Figure 22: Electron micrograph of cultured M52B cells showing an early stage (a) and late stage (b) of virus budding X57 500




a - in the early stages of budding the core of the virus may push against the cell membrane. The second membrane which encloses the virus particle may be attained during this stage.

b - in the advanced stage of a, more of the viral core is seen enclosed by a double membrane

Figure 23: An electron micrograph showing an extracellular virus particle (V) released by budding from an M52B cell. The virus particle consists of a core circumscribed by a double membrane X227 750

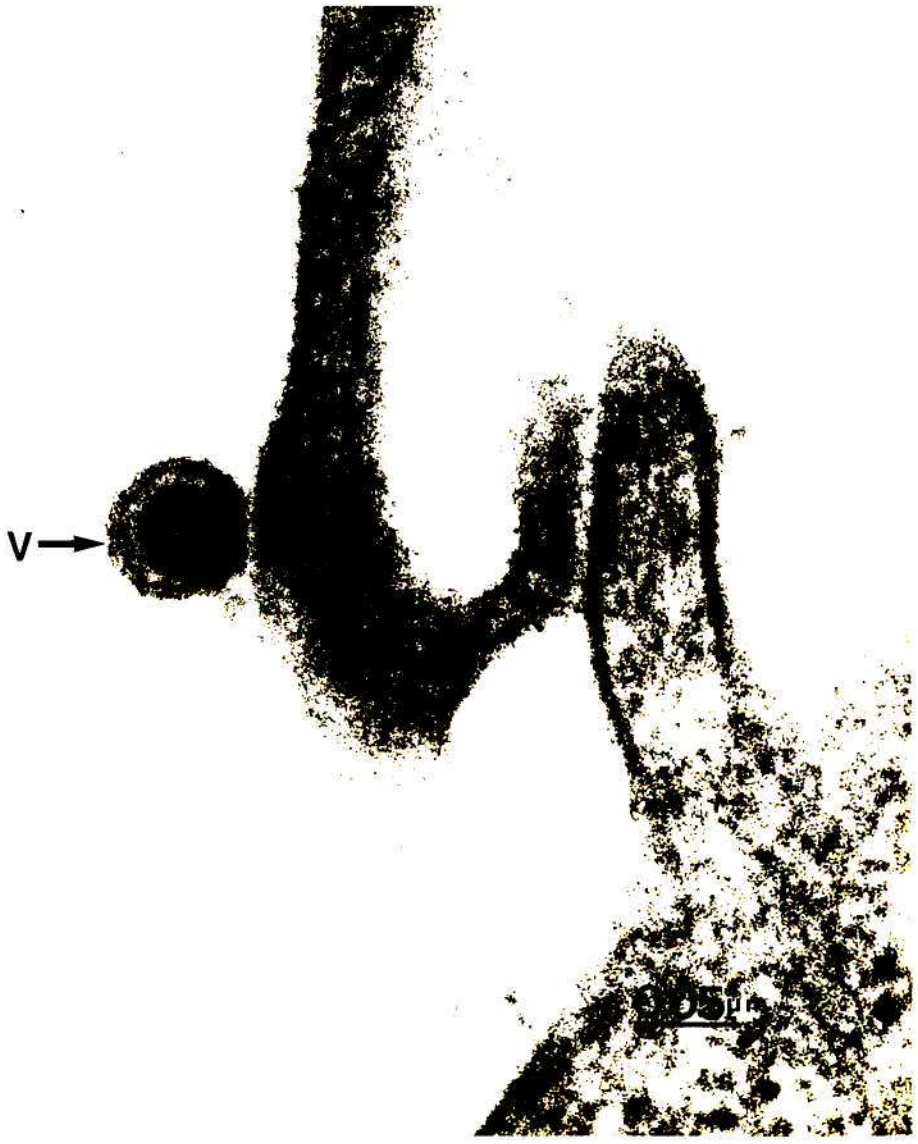


Figure 24: Appearance of dense cultures of M52B cells 48 hours after addition of a single dose of GLA (0 , 50 and 100ug/ml). May-Grünwald/Giemsa

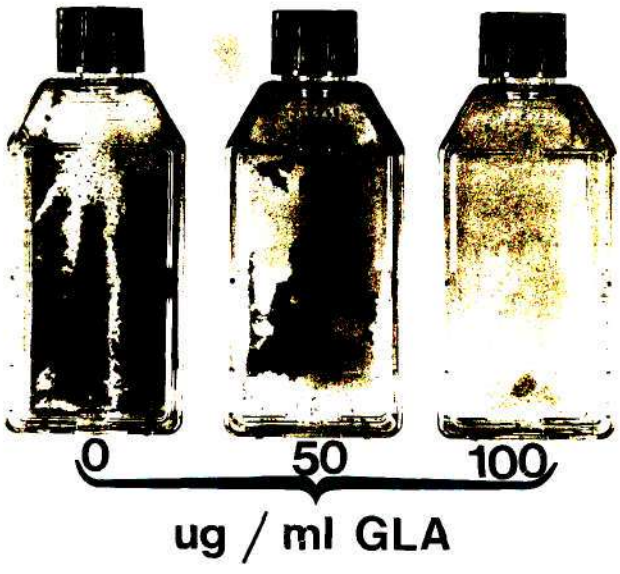


Figure 25: Appearance of dense cultures of M52B CELLS 48 hours after addition of multiple doses of GLA (0 - 50 ug/ml). May-Grünwald/Giemsa

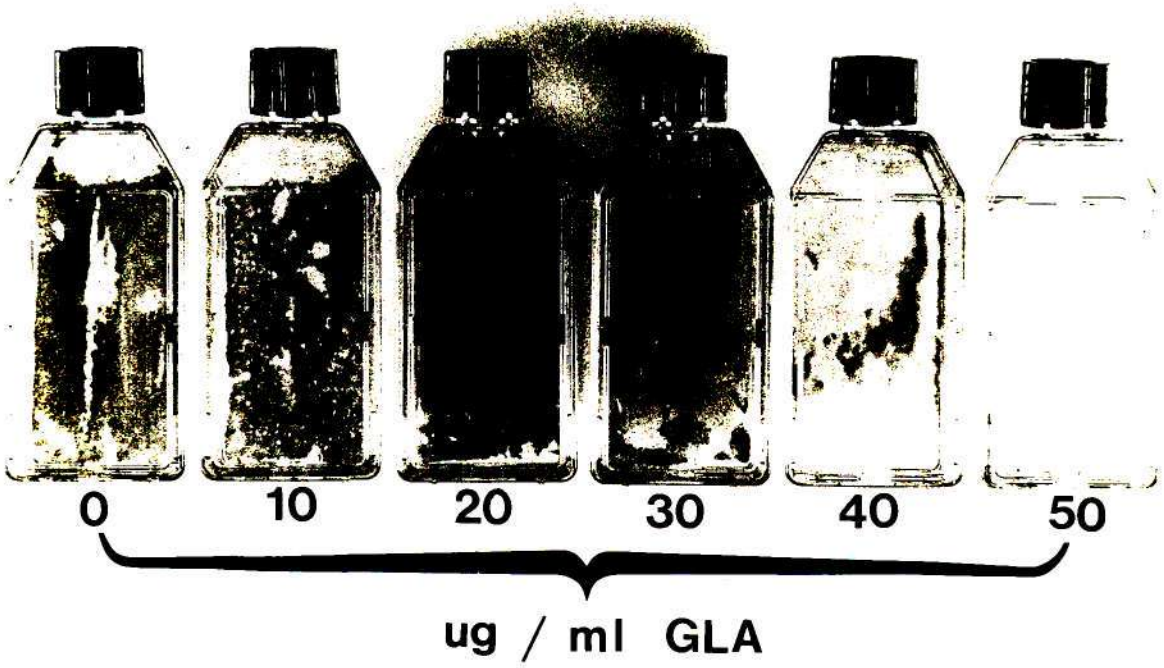
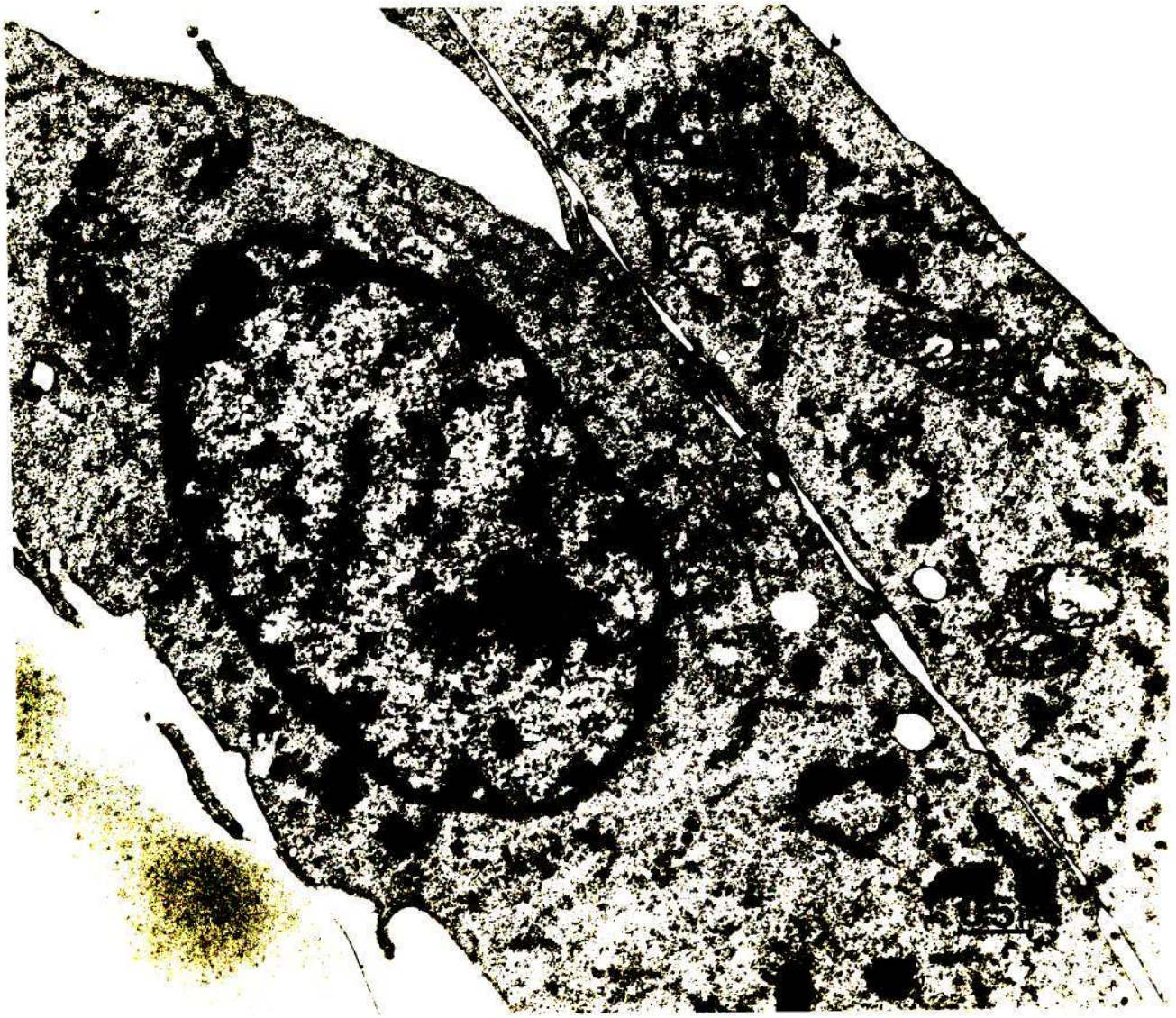


Figure 26: Electron micrograph of control M52B cells growing in culture X19 750



RER = Rough endoplasmic reticulum

## 6.3 EFFECTS OF GLA ON PROSTAGLANDIN SYNTHESIS

The amounts of PGE and PGF released into the medium by GLA treated and control M52B cultures are recorded in Table IX. Full data on PG calculations appear in Appendix 1. The results are reported as PGE and PGF and no distinction is drawn between the 1 and 2 series since anti $PGE_2$ antibody had a 20% cross-reactivity with $PGE_1$ at 50% inhibition of maximum binding and $PGF_{2\alpha}$ antibody had a 10% cross reactivity with $PGF_{1\alpha}$ (Norman et al., 1981; Norman and Joubert, 1982). Cells treated with GLA released more PGE and PGF into the medium than did controls. However, analysis of results using the Mann Whitney U test (Siegel, 1956) showed these differences to be statistically non significant for both PGE and PGF on two-tailed tests.

Table IX: Amounts of prostaglandins (PGs) released into the medium by control and GLA treated M52B cultures

| | M52B Cultures | |
|---|---|---|
| | Control | GLA treated |
| PGs (ug/mg protein/24hr) | | |
| PGE | | |
| median | 0.39 | 0.59 |
| range | 0.18 - 0.64 | 0.34 - 0.91 |
| *p | 0.08 | |
| PGF | | |
| median | 0.02 | 0.03 |
| range | 0.01 - 0.05 | 0.01 - 0.05 |
| *p | 0.56 | |

*p Mann Whitney U test (2 tailed)

# CHAPTER 7

# RESULTS

# IN VIVO

## 7.1 EFFECTS OF GLA ON IMMUNOCOMPETENT MICE BEARING M52B TUMOURS

### 7.1.1 Effects of Oral GLA on Randombred BALB/c Mice

Tumour take rates, latent periods and remissions observed in the two experiments in this study are recorded in Table VI (Section 5.1.1.1). Full data on tumour measurements appear in Appendix 2.1 and Appendix 2.2.

Due to the very small numbers of mice per group remaining at the end of the studies, results of the experiments were not statistically analysed and are therefore represented as tumour volumes only (Tables Xa and Xb). No conclusions can be drawn from these studies.

### 7.1.2 Effects of Oral GLA on Inbred BALB/c Mice

As inoculation of inbred immunocompetent BALB/c mice was followed by 100% tumour remission within 25 days, completion of this study was not possible (Section 5.1.1.1).

Table Xa: Mean volumes and SEM of M52B tumours in randombred, immunocompetent BALB/c mice consuming either normal or EPO supplemented chow

Experiment 1

| No. of days following treatment | Tumour Volume ($mm^3$) | Diet | |
|---|---|---|---|
| | | Control | Experimental |
| | | Normal chow (n=1) | EPO (n=4) |
| 0 | Mean | 2763.3 | 3466.3 |
| | SEM | - | 1374.5 |
| 4 | Mean | 4600.0 | 7499.8 |
| | SEM | - | 3256.0 |
| 7 | Mean | 6050.0 | 9164.0 |
| | SEM | - | 3988.2 |
| 10 | Mean | 6050.0 | 8385.9 |
| | SEM | - | 3242.9 |
| Increase in tumour volume over 10 days | Mean | 3286.7 | 4919.6 |
| | SEM | - | 2216.3 |

Table Xb: Mean volumes and SEM of M52B tumours in randombred, immunocompetent BALB/c mice consuming either normal or EPO supplemented chow

Experiment 2

| No. of days following treatment | Tumour Volume ($mm^3$) | Diet | |
|---|---|---|---|
| | | Control | Experimental |
| | | Normal chow (n=2) | EPO (n=2) |
| 0 | Mean | 5001.3 | 9069.3 |
| | SEM | 3226.8 | 717.3 |
| 3 | Mean | 7069.5 | 10616.0 |
| | SEM | 2452.5 | 616.0 |
| 6 | Mean | 8268.3 | 18753.8 |
| | SEM | 2311.8 | 303.8 |
| 8 | Mean | 7913.5 | 17056.0 |
| | SEM | 2137.5 | 1376.0 |
| Increase in tumour volume over 8 days | Mean | 2912.3 | 7986.8 |
| | SEM | 1089.3 | 2093.3 |

### 7.1.3 Effects of Parenteral GLA on Inbred Biozze mice

Results of the experiment in which mice were either untreated or injected subcutaneously with GLA in HCO or HCO only are included in Appendix 3, summarised in Table XI and graphically represented in Figure 28.

Due to high rates of tumour remission and subsequent small numbers of animals in this study, no statistical test could be used to analyse these results. Thus, it is not possible to make any inferences from this experiment on the effects of parenteral GLA on the growth of M52B tumours in inbred immunocompetent Biozze mice.

### 7.1.4 Effects of Oral GLA on Outbred mice of the "Onderstepoort strain"

The effects of oral GLA on M52B tumours in outbred mice are included in Appendix 4, summarised in Table XII and graphically represented in Figure 29. The results were not analysed statistically because of the small number of mice remaining in the control group at the end of the study.

Table XI: Summary of M52B tumour volumes prior to and following treatment of inbred, immunocompetent Biozze mice with parenteral GLA

| No. of days following treatment | Tumour volume ($mm^3$) | Control | | Experimental |
|---|---|---|---|---|
| | | Untreated (n=2) | Subcutaneous HCO (n=3) | Subcutaneous GLA + HCO (n=5) |
| 0 | Mean | 261.0 | 74.7 | 595.6 |
| | SEM | 117.0 | 31.3 | 422.1 |
| 3 | Mean | 470.8 | 129.0 | 1496.1 |
| | SEM | 86.8 | 27.2 | 823.5 |
| 5 | Mean | 610.5 | 543.5 | 2331.8 |
| | SEM | 10.5 | 414.1 | 1160.3 |
| 7 | Mean | 821.8 | 2001.0 | 3254.4 |
| | SEM | 85.8 | 668.6 | 951.0 |
| 10 | Mean | 2440.0 | 2676.7 | 4963.2 |
| | SEM | 1124.0 | 328.9 | 1157.6 |
| Increase in tumour volume over 10 days | Mean | 2179.0 | 2602.0 | 4367.6 |
| | SEM | 1241.0 | 333.4 | 1070.7 |

Figure 28: Volumes of M52B tumours in inbred, immunocompetent Biozze mice injected with nothing, GLA in HCO or HCO only

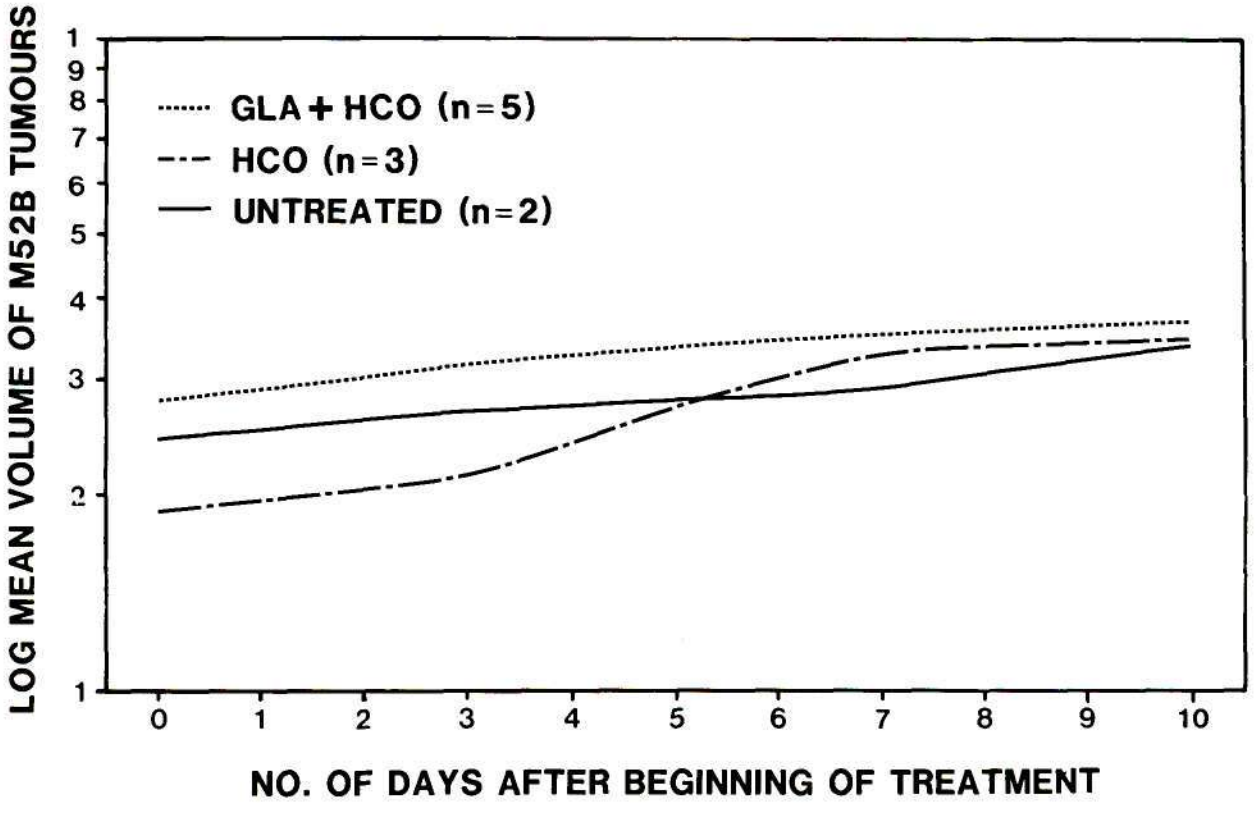


Figure 29: Volumes of M52B tumours in immunocompetent outbred mice of the "Onderstepoort strain" consuming either normal or EPO supplemented chow

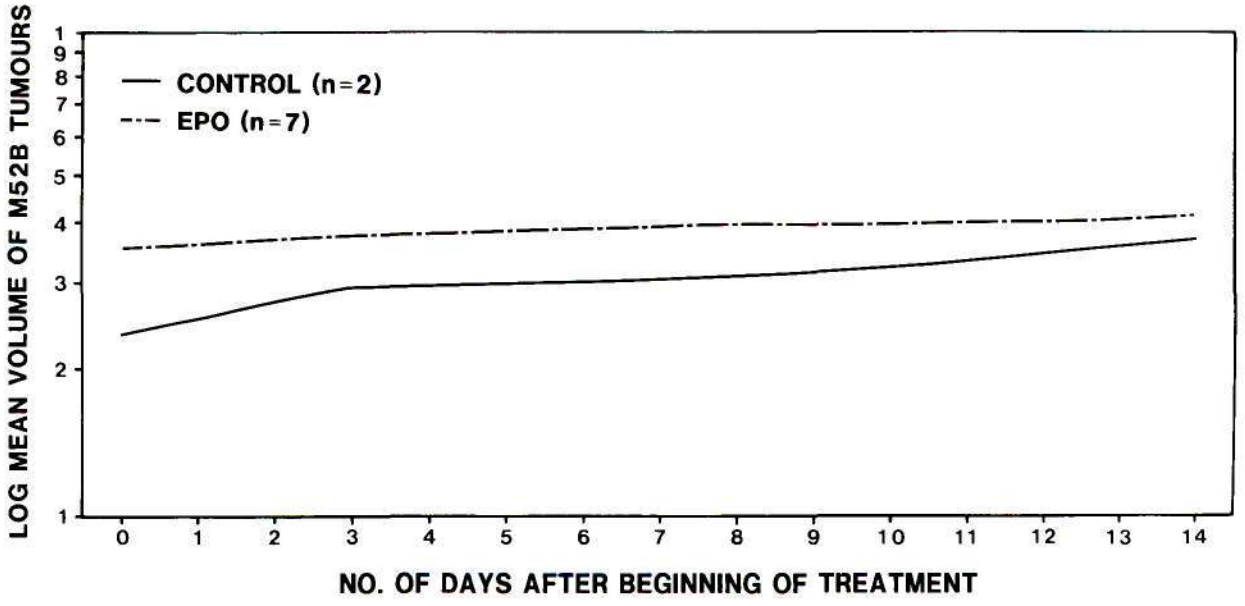


Figure 30a: Volumes of M52B tumours in nude BALB/c mice consuming either normal or; EPO, SSO or HCO supplemented chow

Experiment 1

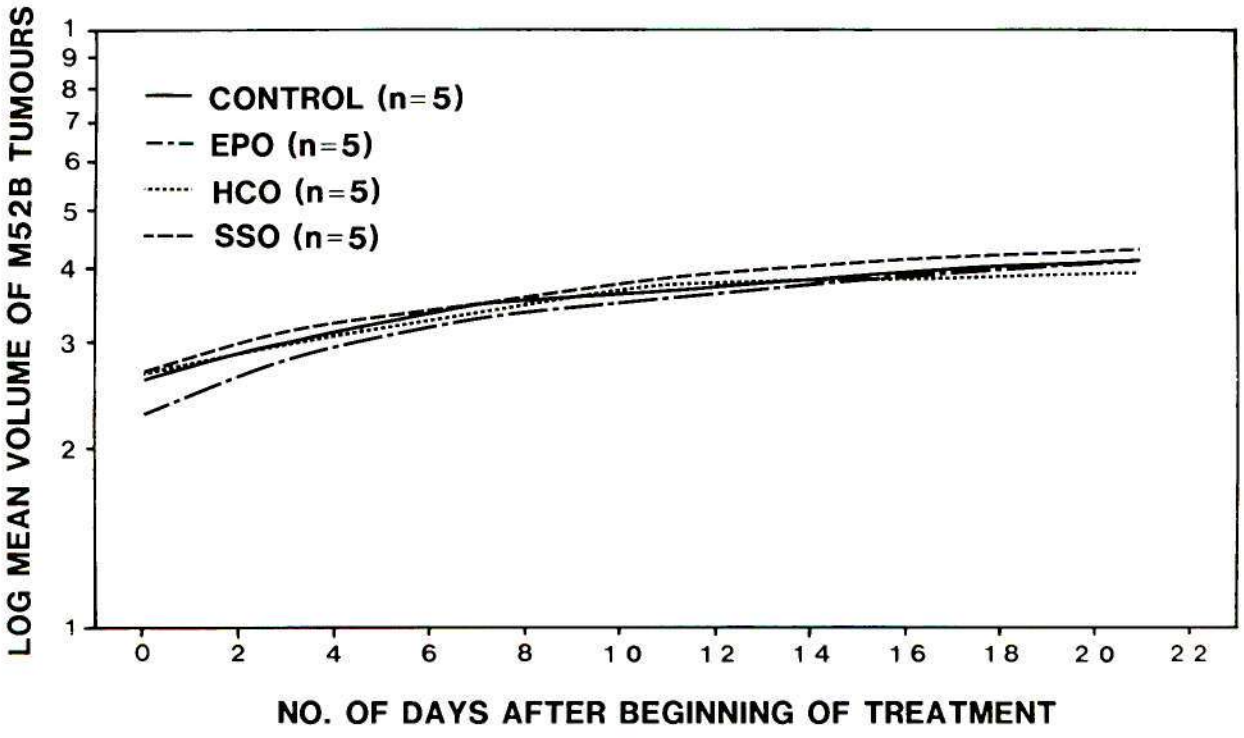


Table XIIIb: Summary of M52B tumour volumes in nude BALB/c mice prior to and following treatment with oral EPO

Experiment 2

| | Tumour Volume ($mm^3$) | Diet | | *p |
|---|---|---|---|---|
| | | Control | Experimental | |
| | | Normal chow (n=8) | EPO (n=9) | |
| Before treatment | Mean | 1066.7 | 1462.5 | 0.58 |
| | SEM | 377.1 | 524.8 | |
| 10 days after treatment | Mean | 10663.8 | 13136.0 | 0.62 |
| | SEM | 2660.4 | 3231.9 | |
| Increase in tumour volume over ten days | Mean | 9597.1 | 11673.5 | < 0.60 |
| | SEM | 2371.5 | 2863.9 | |

*Unpaired student's "t" test (2 tailed)

Figure 30b: Volumes of M52B tumours in nude BALB/c mice consuming either normal or EPO supplemented chow

Experiment 2

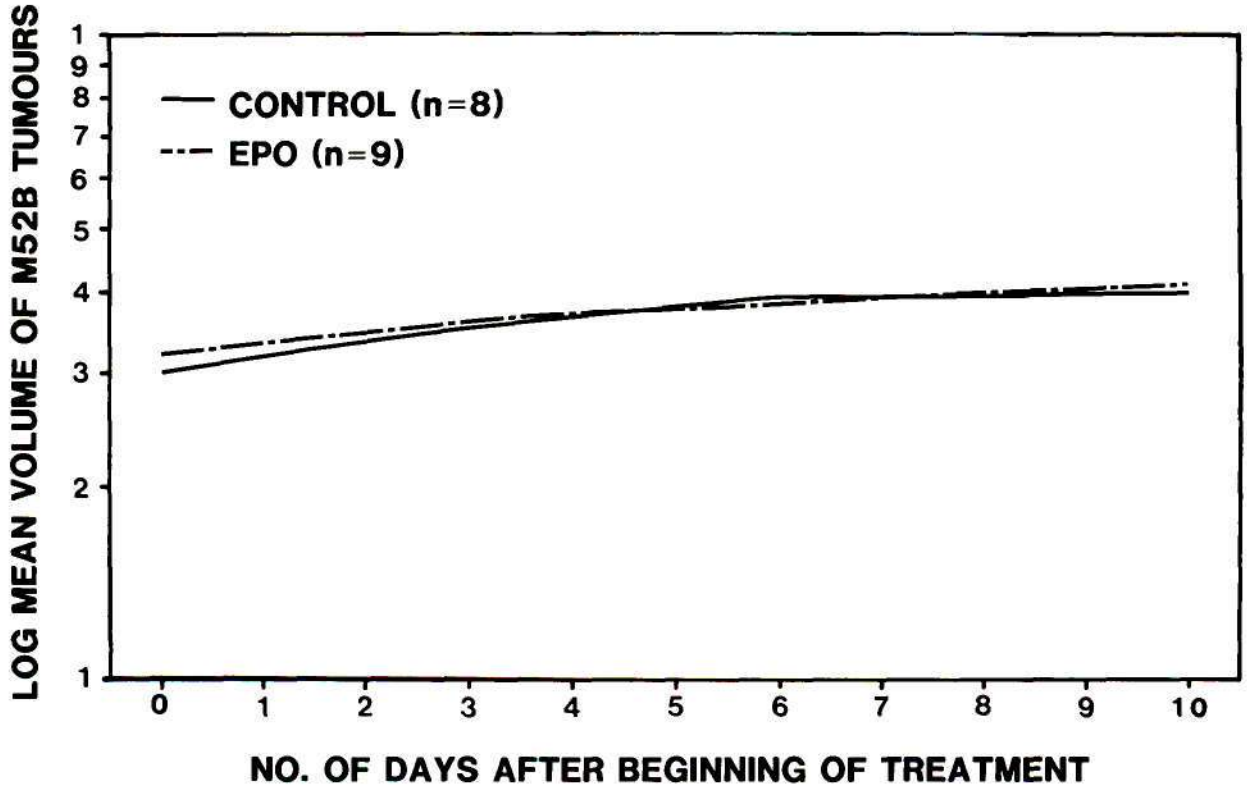


Table XIIIc: Summary of M52B tumour volumes in nude BALB/c mice prior to and following treatment with oral EPO

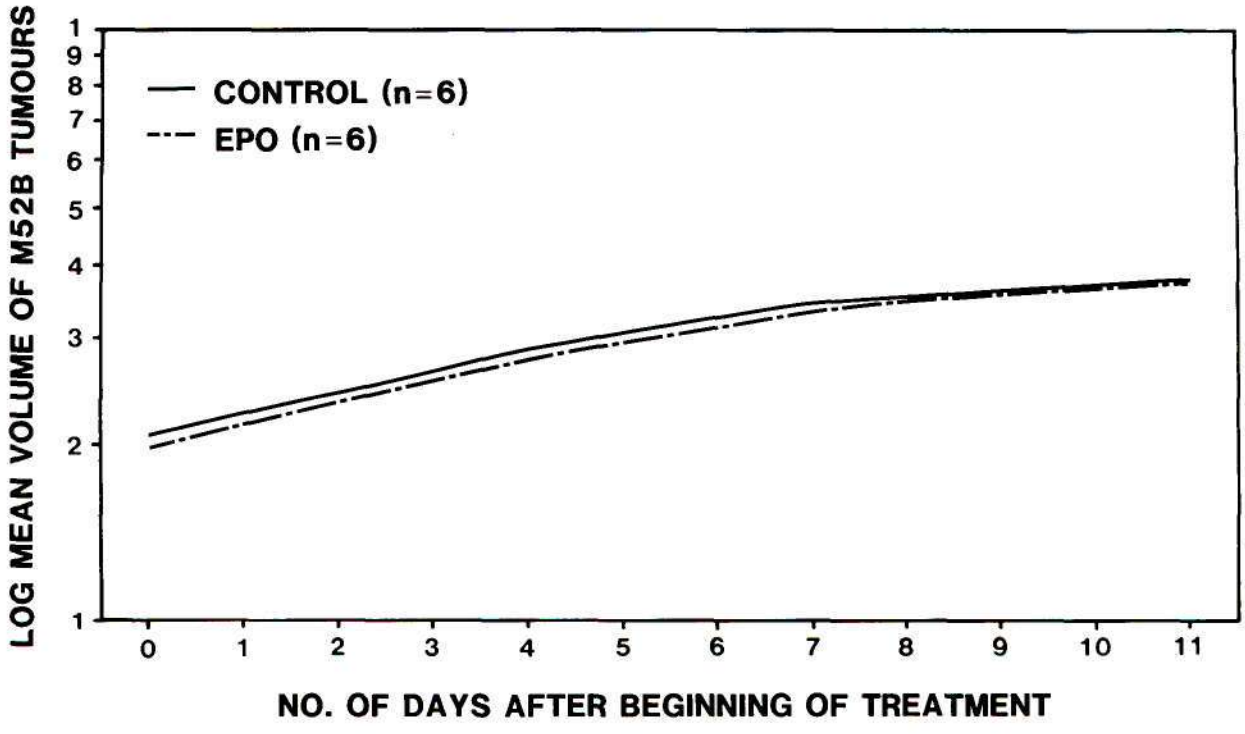
Experiment 3

| | Tumour Volume ($mm^3$) | Diet | | *p |
|---|---|---|---|---|
| | | Control | Experimental | |
| | | Normal chow (n=6) | EPO (n=6) | |
| Before treatment | Mean | 122.4 | 82.6 | 0.68 |
| | SEM | 50.2 | 25.8 | |
| 11 days after treatment | Mean | 5541.3 | 5358.9 | 0.74 |
| | SEM | 1292.5 | 1145.4 | |
| Increase in tumour volume over 11 days | Mean | 5418.9 | 5276.3 | < 0.95 |
| | SEM | 1294.4 | 1134.9 | |

*Unpaired student's "t" test (2 tailed)

Figure 30c: Volumes of M52B tumours in nude BALB/c mice consuming either normal or EPO supplemented chow

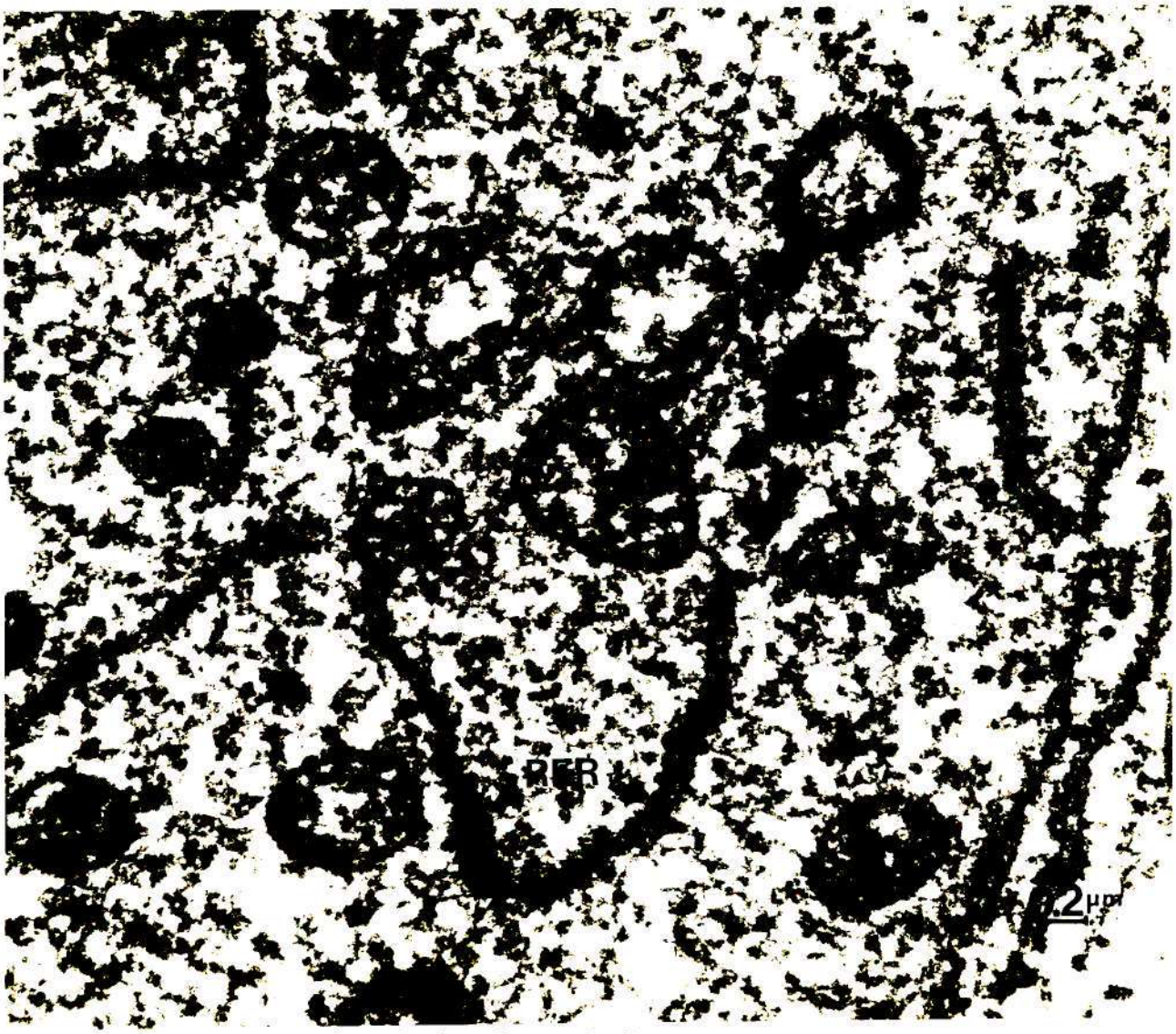
Experiment 3



## 7.3 TRANSMISSION ELECTRON MICROSCOPY OF CONTROL AND EPO TREATED MURINE SARCOMA TUMOURS

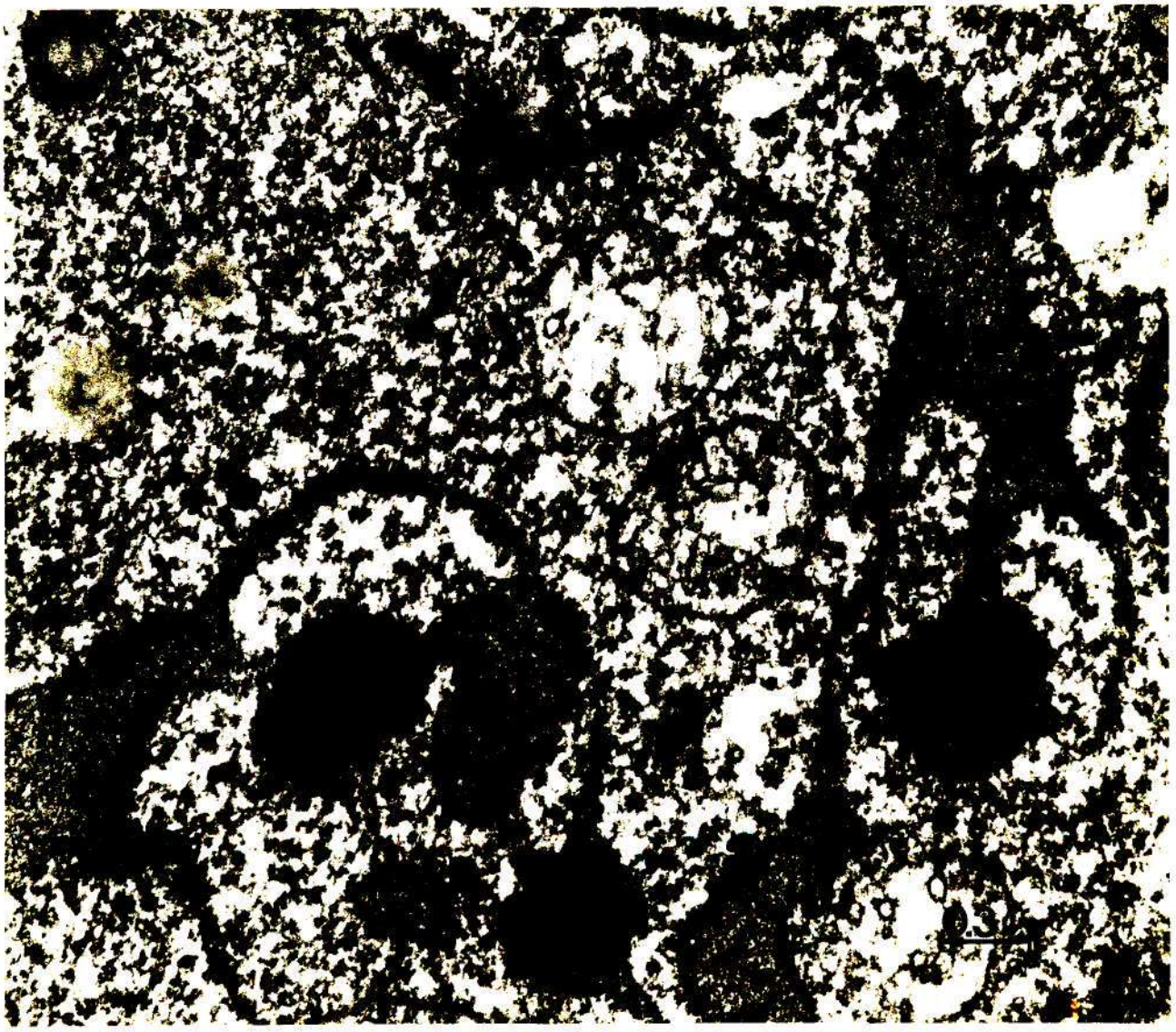
Although oral treatment with EPO did not significantly affect the growth of M52B tumours in nude mice, ultrastructural examinations were still performed on these tumours to assess for any morphological changes. Figure 31 is an electron micrograph of an untreated M52B tumour with no excess lipid accumulation and non distended endoplasmic reticulum. Electron microscopic examination of EPO treated M52B tumours revealed the presence of lipid droplets and numerous swollen endoplasmic reticulum in most cells (Figure 32a). As virus particles were observed in all samples the oil had no apparent effect on virus production (Figure 32b).

Figure 31: Electron micrograph of an untreated murine sarcoma growing in a nude mouse X41 250



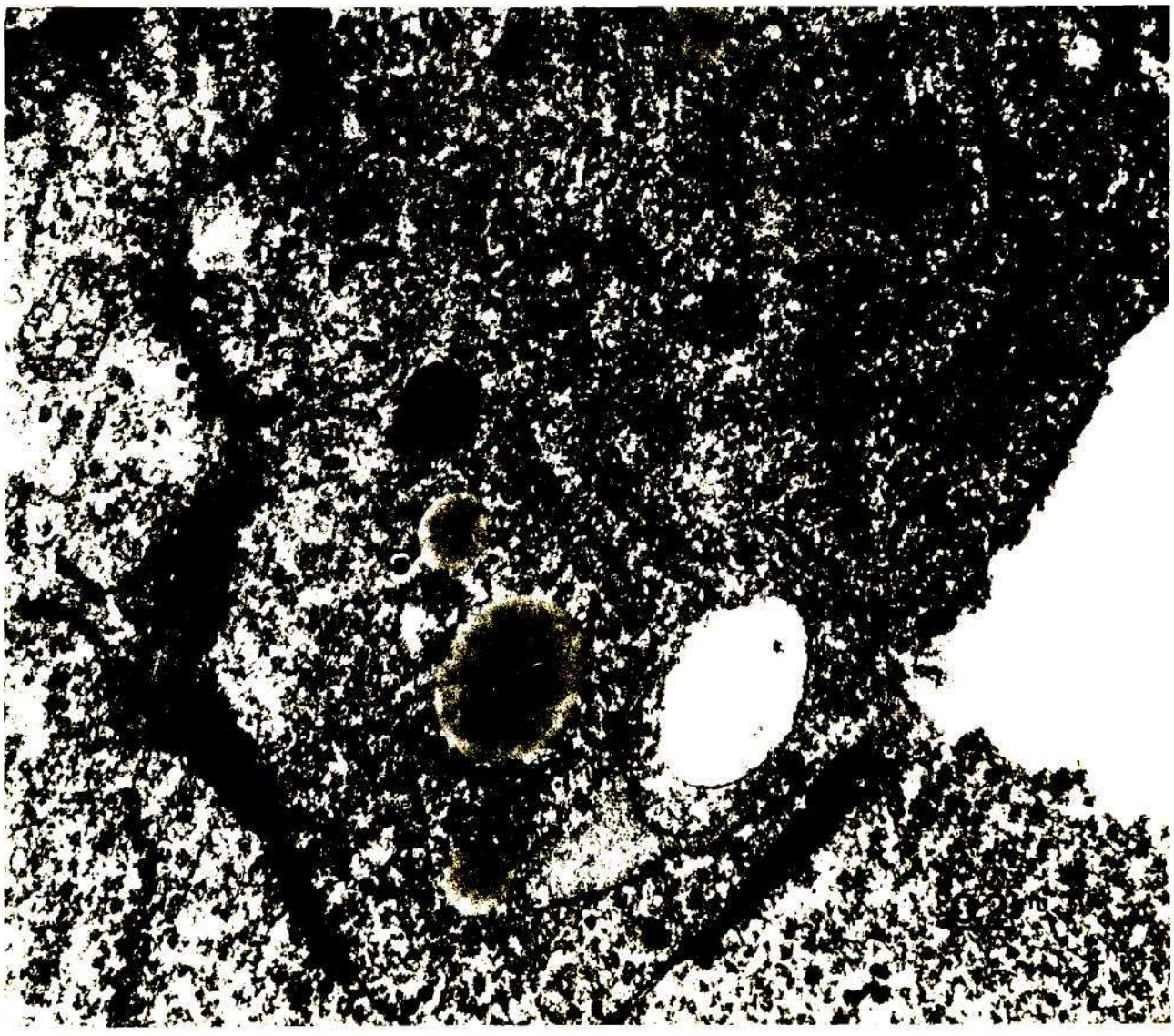

RER = rough endoplasmic reticulum

Figure 32a: Electron micrograph of a murine sarcoma growing in a nude mouse treated with oral EPO X40 600



RER = swollen rough endoplasmic reticulum
L = lipid

Figure 32b: Electron micrograph of a murine sarcoma growing in a nude mouse treated with oral EPO X41 250



V = virus particles
L = lipid
RER = swollen rough endoplasmic reticulum

## 7.4 EFFECTS OF ORAL GLA ON NUDE BALB/c MICE BEARING NUB 1 TUMOURS

Results of the three experiments conducted to assess the effects of oral GLA on nude BALB/c mice bearing NUB 1 xenografts are summarised in Table XIVa, b, c and d. The unpaired student's "t" test was used to compare the difference in initial tumour volumes between control and experimental groups.

Experiment 1 was a pilot study in which small numbers of animals were used. Results of this study were therefore not analysed statistically and thus no inferences can be made from this experiment (Table XIVa, Figure 33a and Appendix 6.1).

In experiment 2, although the study was concluded after 30 days, for statistical purposes, analysis of results was carried out after 20 days treatment as the number of mice per group decreased after this period due to animal deaths which resulted from massive tumour invasion. As the initial volumes in the control and experimental group differed significantly at the beginning of the experiment (Table XIVb), percent increase in volume over the experimental 20 days was assessed in this study (Table XlVc). Analysis of results using the unpaired student's "t" test showed the difference in percent increase in final tumour volumes between control and experimental groups to be statistically non significant (Table XIVc). Figure 33b is a

graphical representation of the growth of NUB 1 xenografts during the experiment (Appendix 6.2).

The third experiment, although large numbers of animals were used, statistical analysis of results showed that oral EPO had no signifcant influence on tumour growth (Table XIVd, Figure 33c and Appendix 6.3).

Figure 33a: Volumes of NUB 1 tumours in nude BALB/c mice consuming either normal or EPO supplemented chow

Experiment 1

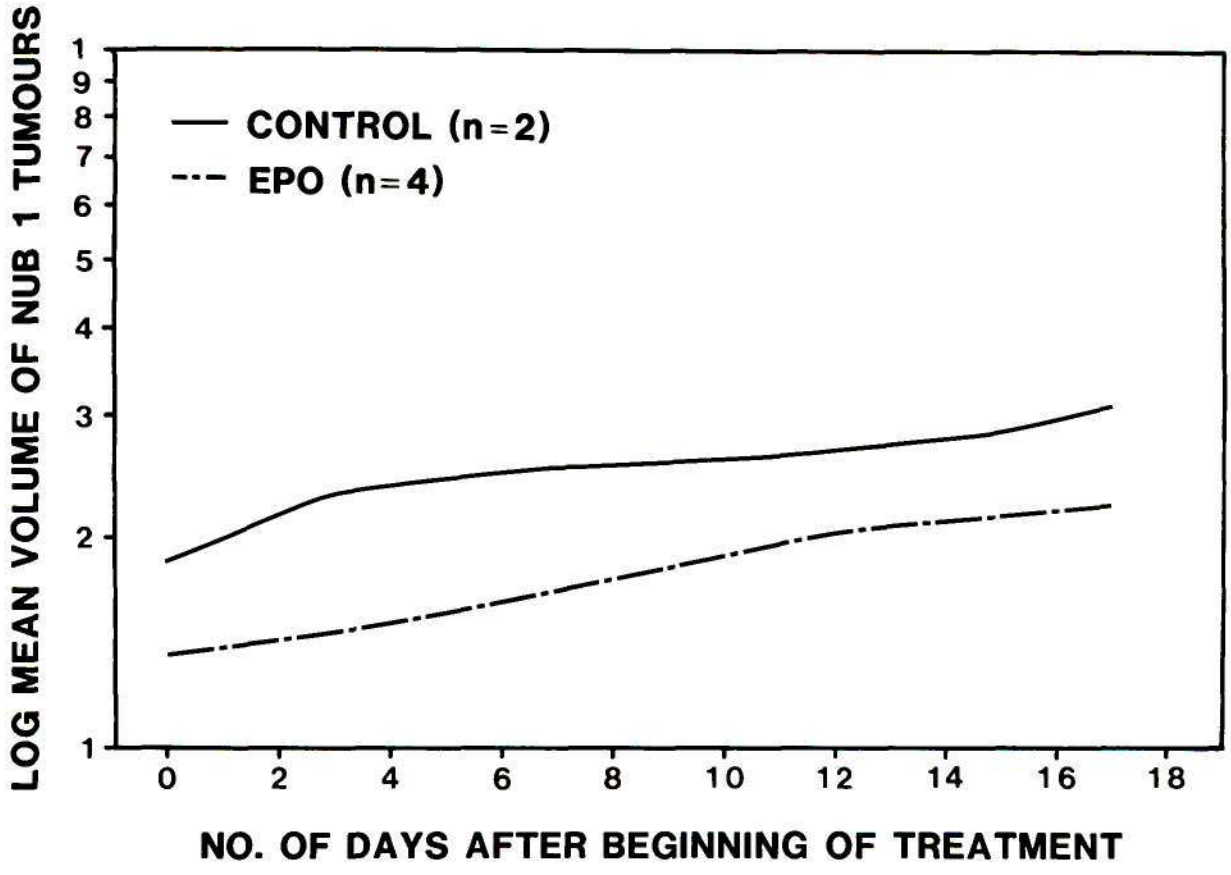


Table XIVb: Summary of NUB 1 tumour volumes in nude BALB/c mice prior to treatment with oral EPO

Experiment 2

| | Mean Tumour Volume ($mm^3$) | Diet | | *p |
|---|---|---|---|---|
| | | Control<br>Normal chow<br>(n=6) | Experimental<br>EPO<br>(n=6) | |
| Before treatment | Mean | 32.1 | 138.5 | 0.002 |
| | SEM | 10.4 | 43.4 | |

*Unpaired student's "t" test (2 tailed)

Table XIVc: Summary of percent increase in NUB 1 tumour volumes in nude BALB/c mice following treatment with oral EPO

Experiment 2

| | Mean Tumour Volume (mm ) | Diet | | *p |
|---|---|---|---|---|
| | | Control<br>Normal chow<br>(n=6) | Experimental<br>EPO<br>(n=6) | |
| Percent increase in tumour volume over 20 days | Mean<br>SEM | 2029.2<br>458.0 | 1762.0<br>330.5 | 0.646 |

*Unpaired student's "t" test (2 tailed)

Figure 33b: Volumes of NUB 1 tumours in nude BALB/c mice consuming either normal or EPO supplemented chow

Experiment 2

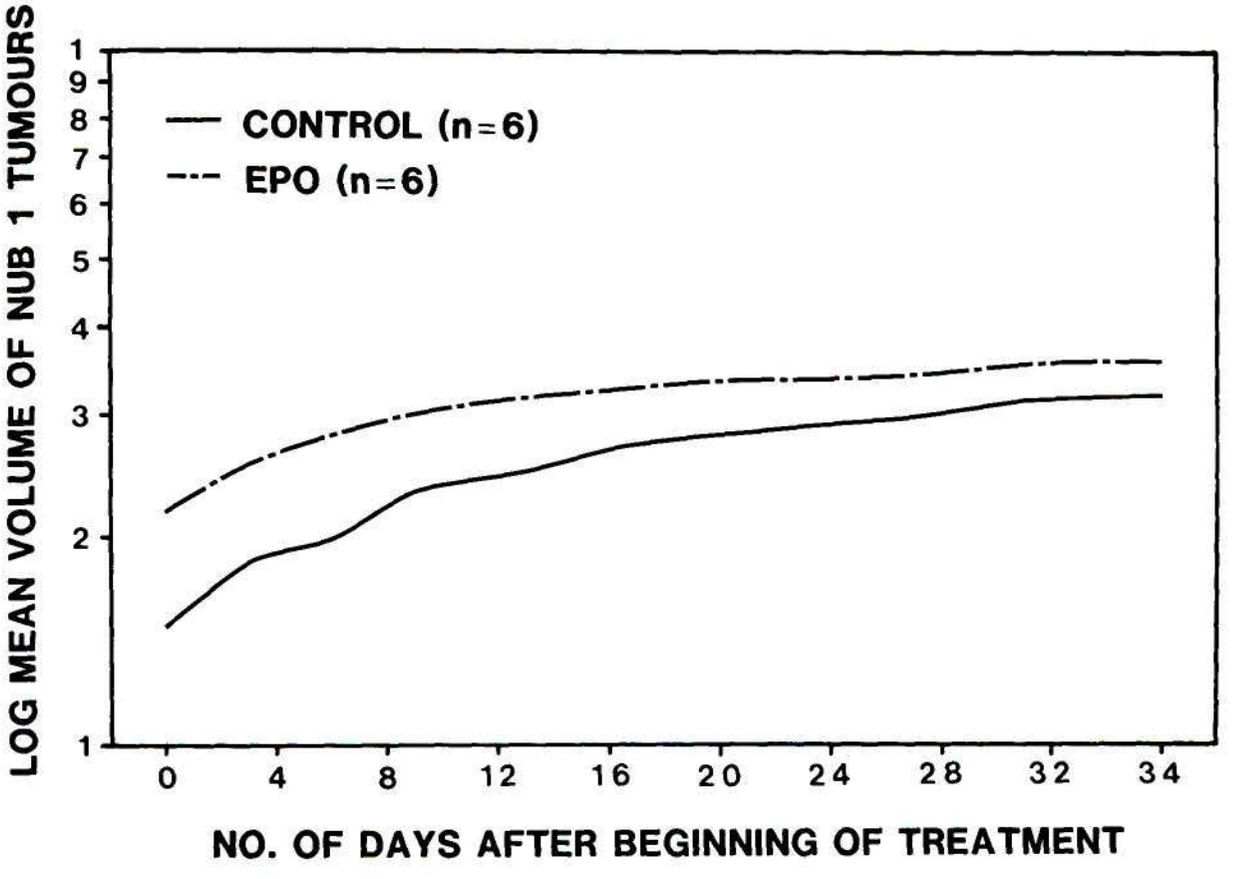


Table XIVd: Summary of NUB 1 tumour volumes in nude BALB/c mice prior to and following treatment with oral EPO

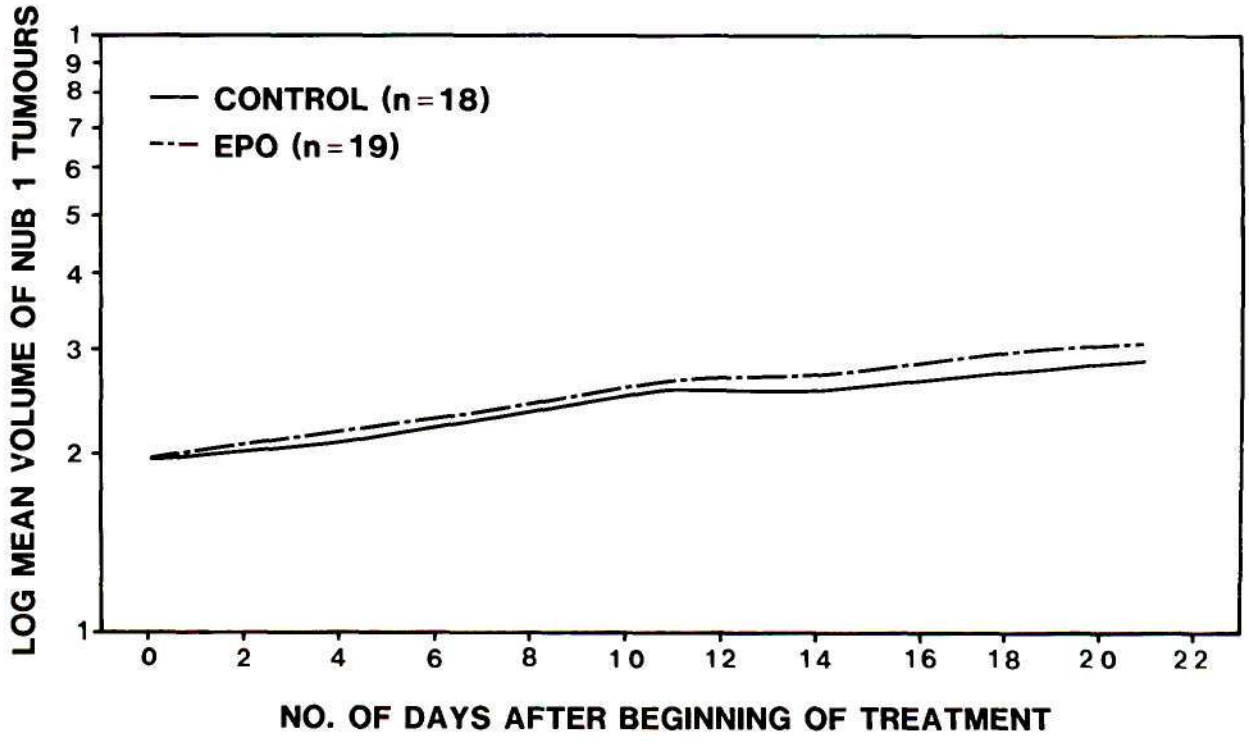
Experiment 3

| | Mean Tumour Volume ($mm^3$) | Diet | | *p |
|---|---|---|---|---|
| | | Control | Experimental | |
| | | Normal chow (n=18) | EPO (n=19) | |
| Before treatment | Mean | 94.0 | 89.4 | 0.66 |
| | SEM | 13.1 | 14.5 | |
| 21 days after treatment | Mean | 786.9 | 1094.0 | 0.50 |
| | SEM | 145.6 | 220.3 | |
| Increase in tumour volume over 21 days | Mean | 693.1 | 1004.5 | < 0.30 |
| | SEM | 141.6 | 213.1 | |

*Unpaired student's "t" test (2 tailed)

Figure 33c: Volumes of NUB 1 tumours in nude BALB/c mice consuming either normal or EPO supplemented chow

Experiment 3



### 7.4.1 Tumour Recurrence

Encapsulated NUB 1 xenografts were removed from control and treated mice in an attempt to investigate any differences in incidence of tumour recurrence. However, recurrence was noted in both control and treated animals; an observation which could be attributed to the fact that the entire tumour may not have been excised. Although recurrence occurred within two weeks of excision in 1 out of 6 control and 3 out of 6 EPO mice in Experiment 1 and in 1 out of 4 EPO mice in Experiment 2, this phenomenon was not observed in Experiment 3.

## 7.5 SUMMARY OF IN VIVO RESULTS

GLA, administered orally in the form of EPO was found to have

(1) no significant effect on the growth of M52B tumours in nude mice.
(2) no significant effect on NUB 1 tumours in nude mice.

Unfortunately, despite numerous attempts to grow the M52B cells in immunocompetent mice of various strains, most tumours regressed spontaneously. Consequently, the numbers of animals remaining in each group were too small to draw any real conclusions about the effect of GLA in these animals.

# CHAPTER 8

# DISCUSSION

Although growth of M52B cells *in vitro* was clearly inhibited by the addition of GLA to the culture medium, growth of solid M52B sarcomas *in vivo* in immunodeficient mice was apparently unaffected by dietary GLA in the form of EPO. Growth of NUB 1 xenografts in immunodeficient mice was also unaffected by dietary EPO.

## 8.1 EFFECTS OF GLA IN VITRO

The inhibitory and toxic effects of GLA on growth of the sarcoma cells and non malignant fibroblasts *in vitro* were similar to those of other studies using human oesophageal carcinoma (Booyens *et al.*, 1984a; Leary *et al.*, 1982), breast carcinoma (Begin *et al.*, 1985; Robinson and Botha, 1985; Robinson *et al.*, 1985), lung carcinoma (Begin *et al.*, 1985), osteogenic sarcoma (Booyens *et al.*, 1984a; 1984c), hepatoma (Booyens *et al.*, 1984b; Dippenaar *et al.*, 1982b), mouse melanoma (Dippenaar *et al.*, 1982a); and benign LLCMK monkey kidney cells (Booyens *et al.*, 1984b). The concentration dependant and time related inhibition of growth reported in all these studies was also confirmed in the present study using M52B cells. Furthermore, the effects of GLA on cell growth were related to the absolute cell number as the fatty acid was found to produce more pronounced effects in less dense

cultures, presumably as more GLA was available per cell. Despite this, each cell line investigated has been reported to respond to a slightly different extent with respect to changes in morphology and growth inhibition, to equivalent doses of GLA. These apparent differences could be related to factors such as structure and function of the cells, culture environment, the mitotic rate, number and size of cells (Leary et al., 1982).

### 8.1.1 Proposed mechanism/s of action of GLA in vitro

Although exogenous GLA, in doses ranging from 0,05 to 200 ug/ml has been reported to consistently inhibit the growth of malignant cells in vitro, the exact mechanism/s of action is unknown. However, many workers (Booyens et al., 1984a,1984b, 1984c; Dippenaar et al., 1982a, 1982b; Leary et al., 1982) have assumed that the observed effects of GLA may be due to the fatty acid bypassing a deficient $\Delta 6$ desaturase and consequently serving as substrate for $PGE_1$ synthesis, as previously proposed (Horrobin, 1980a). If the effects of GLA were mediated mainly in this way, then non malignant cells which are presumed to have a functional $\Delta 6$ desaturase and therefore an endogenous source of GLA, might be unaffected or respond differently to the addition of exogenous GLA. While GLA at a concentration of 10 ug/ml has been reported to be without effect on the growth of cultured normal bovine kidney

MDBK cells (Dippenaar et al., 1982a), in another study using the same concentration of the fatty acid, it was found to inhibit proliferation of cultured benign LLCMK cells (Booyens et al., 1984b). In the present study using non malignant fibroblasts, exposure to high concentrations of GLA (> 50 ug/ml) also inhibited cell growth. The inhibitory effects of GLA on growth of non malignant cells may imply that the actions of the fatty acid are not exclusively due to bypassing a deficient $\Delta 6$ desaturase. In fact, other polyunsaturated as well as saturated fatty acids have also been reported to inhibit growth of non malignant human foreskin fibroblasts and guinea pig smooth muscle (Huttner et al., 1977), rabbit smooth muscle (Smith et al., 1984) and epithelial cells of rat jejunum (Petry et al., 1984). A finding which casts further doubt upon Horrobin's proposal is the reported effect of LA on growth of malignant cells. Since such cells are presumed to be deficient in $\Delta 6$ desaturase and therefore unable to metabolise LA, this fatty acid would be expected to have no effect on malignant cell proliferation. With the exception of a single study in which 10 ug/ml LA was found to be without effect on growth of cultured human hepatoma cells (Booyens et al., 1984b), in other studies using different concentrations of the fatty acid, results to the contrary have been reported. For example, LA at concentrations of 1 uq/ml and 5 ug/ml has been found to augment growth of cultured mammary (Kidwell et al., 1978; Wicha et al., 1979) and ascites tumour XS 63.5 (Holley et al.,

1974) cells respectively while at 20 ug/ml and 50 ug/ml it has been shown to inhibit proliferation of cultured human osteogenic sarcoma (Booyens et al., 1984a, 1984c), breast and lung carcinoma (Begin et al., 1985) and; human breast and oesophageal carcinoma (Robinson et al.,1985) respectively.

Thus, since LA and GLA have generally been reported to produce effects inconsistent with the deficient $\Delta 6$ desaturase theory, it is possible that effects observed with both these fatty acids might have been via a non-specific fat-effect. In fact, prior to cell death, both malignant and non malignant cells treated with GLA in the present study accumulated paranuclear granules which were lipid in nature. The cell death may therefore have been secondary to a fat overload. In addition, the accumulation of lipid by these cells increased with increasing concentrations of the fatty acid. However, other workers who reported lipid accumulation in GLA treated human mammary (Robinson and Botha, 1985) and oesophageal (Robinson et al., 1985) carcinoma cells, found equivalent doses of LA and DGLA to produce fewer or no obvious lipid deposits and to affect growth of these cells to a lesser extent. Thus, inhibition of growth of these fatty acid treated cells cannot be solely attributed to cellular lipid accumulation. It is possible, however, that the non-specific inhibition of growth by the fatty acids may have been due to altered metabolism or cell membrane structure brought about by exposure of neoplastic

cells to excess exogenous unsaturated fatty acids (Carroll, 1981: Vitale and Broitman, 1981; Welsch and Aylsworth, 1983). Incorporation of cis-unsaturated fatty acids into the plasma membranes of malignant cells may render these structures more susceptible to changes in fluidity (Abbas et al., 1982). Alternatively, the inhibition of cell growth by GLA in this study, and by DGLA and LA in other studies (Robinson and Botha, 1985; Robinson et al., 1985) may have resulted from a detergent effect, as these fatty acids were dissolved in $Na_2CO_3$ hence forming soaps. However, the fact that the oleic acid-soap, which is known to lower surface tension more than the palmitic and stearic acid soaps (Singleton, 1960), stimulated cell proliferation in non malignant smooth muscle cultures at high concentrations of 90 uM while equivalent concentrations of the latter two fatty acids persistently inhibited growth, casts doubt upon the assumption that these and other fatty acids are influencing cell growth purely via a detergent effect (Huttner et al., 1978). More recently, workers (Begin et al., 1985; Smith et al., 1984) also indicated that the inhibitory effects of polyunsaturated fats on growth of malignant cells in vitro may be related to lipid peroxide production.

Despite the many possible suggestions that have been offered to explain the mechanism of action of GLA on cell growth, there appears to be much support for the proposal that provision of GLA to malignant cells increases PGE synthesis and subsequently

"normalises" cell growth.

Indeed, there is considerable direct and indirect evidence, involving the use of PG synthetase inhibitors, and cAMP, that exogenous $PGE_1$ inhibits the growth of malignant cells (Section 1.2.2.1). However, exogenous $PGE_2$ has also been reported to retard malignant cell growth (Section 1.2.2.1). Thus, since GLA is a precursor of both $PGE_1$ and $PGE_2$ (Horrobin, 1980a), it is possible that $PGE_2$ may also have contributed to the effect of GLA upon cell growth. The PG assay performed in the present study showed that although PGE levels rose after exposure of M52B cells to GLA, this increase was not statistically significant. In addition, it was not possible to determine whether the elevated PGE levels represented $PGE_1$ or $PGE_2$ since anti $PGE_2$ and anti $PGF_{2\alpha}$ antibodies cross reacted significantly with $PGE_1$ and $PGF_{2\alpha}$ respectively (Norman and Joubert, 1982; Norman et al., 1981). However, the small increase in PGE recorded in this study may imply that the actions of GLA are partially mediated by PGE, but the extent to which $PGE_1$ and $PGE_2$ may have been involved in inhibiting the growth of M52B cells could not be determined. If the actions of GLA were in fact mediated by increased synthesis of $PGE_1$ then DGLA which is a metabolite of GLA and the immediate precursor to $PGE_1$ (Horrobin, 1980b), would also be expected to inhibit cell proliferation. While DGLA at concentrations of 100 and 200 ug/ml has been reported to produce such an effect,

though to a lesser extent than equal doses of GLA, concentrations of 20 to 50 ug/ml DGLA stimulated growth of malignant cells (Robinson and Botha, 1985). These findings therefore do not support the suggestion that $PGE_1$ is the sole mediator of the effects observed with GLA.

In summary, there is considerable evidence that exogenous fatty acids may generally exert a non-specific fat-effect on a wide range of non malignant and malignant cells in vitro. While this is particularly so at high doses (> 50 ug/ml), the exact mechanism/s of the effects observed with low concentrations (< 50 ug/ml) of certain of the fatty acids remain obscure. In particular, while effects of GLA may in part be mediated via a fat overload, it is possible that provision of deficient precursor for PGE synthesis may also play a role.

Since GLA inhibited the growth of M52B cells in vitro, further studies were conducted to determine whether such an effect could be reproduced in vivo. However, in vivo models are clearly different from in vitro systems in many ways. Cells in solid tumours exist in a variety of environments with respect to $O_2$ and $CO_2$ concentrations, nutrient availability and pH, while such factors are controlled for optimum growth in culture (Rockwell, 1977). Furthermore, the growth of tumours in vivo is influenced by the host's immune system (Ziegler, 1982), while cultured cells are free from such influence. The effects

of drugs *in vivo* also depend on the vascularity of the tumour, transport of the drug to and within the tumour and metabolism of the drug by the host. Though the influence of these factors *in vivo* does not permit direct comparison between *in vitro* and *in vivo* studies, it is essential to assess the effects of drugs in *in vivo* animal models prior to any use in humans to determine not only efficacy but also safety of the drug.

## 8.2 EFFECTS OF GLA IN VIVO

Findings of the present *in vivo* investigation using M52B and NUB 1 tumours were generally consistent with those of other workers who failed to reproduce the *in vitro* effects of GLA *in vivo*. For example, the growth of human tumour xenografts in immunocompetent rats (Booyens and Koenig, 1984) and immunodeficient nude mice (Botha *et al.*, 1983) has been reported to be unaffected by dietary EPO and subcutaneous administration of GLA respectively. It is possible that in studies with nude mice, the immune status of the animal may have influenced the results (Botha *et al.*, 1983). There is indeed both *in vitro* and *in vivo* evidence which indicates that exogenous saturated and polyunsaturated fatty acids, in particular LA, linolenic acid and AA are immunosuppressive and consequently promote tumour growth (Section 1.2.2.1). Many workers have further indicated that since LA, GLA and AA are PG precursors, the immunosuppression produced by these fatty acids

may be mediated by synthesis of PGs (Section 1.2.2.1). Thus, since the oil containing diets used in the present study were rich in either polyunsaturated fat and PG precursors (EPO and SSO) or saturated fat (HCO), it was possible that these oils which are known to affect the immune system might have produced variable effects in animals of differing immune status. Unfortunately, only results obtained using immunodeficient mice were analysed statistically in the present study and these indicate that neither EPO, SSO nor HCO had any significant effect on growth of tumours in vivo. Although studies were conducted on various strains of immunocompetent mice, little can be inferred from these investigations as results were based on data collected from small numbers of animals. This was unavoidable due to the occurrence of spontaneous tumour remission in large numbers of animals.

In addition to the obvious differences between in vitro and in vivo environments, other possible factors which may contribute to the difference in effects of GLA observed in these two systems include the following. While GLA was administered in its pure form in vitro, in most of the in vivo experiments the fatty acid was administered in the form of EPO. Since the other fatty acids present in EPO e.g. LA, oleic, myristic, palmitic and stearic acid have been reported to affect the endocrine system and the fluidity of cell membranes, it is possible that these additional fatty acids may have masked the

effects of GLA (Section 1.2.2.1).

Furthermore, many workers have reported a positive association between dietary LA and the growth of mammary (Carroll and Khor, 1971; Hopkins et al., 1981; Rao and Aylsworth, 1976; Tinsley et al., 1981), colon, skin, hepatic, pulmonary and pancreatic cancers. However, such an association has not been reported in sarcoma bearing animals (Birt et al., 1981; Carroll and Khor, 1975; Hopkins et al., 1978; Reddy et al., 1977; Watson and Mellanby, 1930). If dietary LA does in fact promote growth of tumours in vivo, then the apparent lack of effect of EPO on both the carcinomas and sarcomas in this study may be explained by a neutralization of the effects of GLA by LA. In addition, Booyens and Katzeff (1985) have recently reported that oleic acid promotes growth of malignant cells in vitro. They have therefore suggested that the lack of effect of GLA containing diets in vivo may be due to the influence of oleic acid. It seems unlikely, however, that this fatty acid would have exerted a significant effect on tumour growth in the present study as EPO only contains 8.8% oleic acid.

Thirdly, the lack of effect of GLA on tumour growth in vivo may have been related to the fact that treatment was initiated on well established tumours. However, the influence of tumour size on the effects of the fatty acid would not appear to be significant as prophylactic administration of GLA has also been

reported to be without effect on tumour growth (Botha et al., 1983).

A fourth possibility is that the direct administration of GLA to M52B cells in vitro which resulted in intracellular lipid accumulation may have been the cause of cell death. In vivo however, the supplemented fatty acid may have been metabolised prior to reaching the tumour site. Although subcutaneous administration of GLA could be expected to deliver more of the fatty acid to the site of the tumour than oral administration and consequently be more likely to inhibit tumour growth, such results were not obtained in the study of Botha et al. (1983) using this route. However, Ghayur and Horrobin (1981) showed that tumour growth was inhibited by subcutaneous administration of EPO to rats bearing mammary allografts. In both the present study and in that of Botha et al. (1983) in which GLA was administered parenterally, fat deposits were observed at the site of injection of the fatty acid. This complicated assessment of tumour sizes. Presumably, Ghayur and Horrobin (1981) did not encounter this problem as smaller volumes were administered to larger hosts i.e. rats. Furthermore, rats have been reported to have a more active $\Delta 5$ desaturase than mice (Stone et al., 1979) which allows them to convert exogenous GLA preferentially to $PGE_2$. Since $PGE_2$ has been shown to inhibit tumour growth in vivo (Section 1.2.2.1), it is possible that the inhibition of growth reported by Ghayur and Horrobin

following administration of EPO may be related to $PGE_2$ synthesis. Their findings may therefore reinforce the argument put forward earlier that $PGE_1$ alone is probably not responsible for the inhibitory effects of GLA upon the growth of malignant cells.

In addition, as already discussed, it is possible that the effects of EPO on allograft growth observed by Ghayur and Horrobin may have been related to the fact that the rats used in their study were immunocompetent while the mice used in the present study and in that of Botha et al., (1983) were immunodeficient.

Another possible reason for the difference in responses of allografts to subcutaneous GLA may be related to the fact that the allograft in the present study was a sarcoma while that used by Ghayur and Horrobin (1981) was a hormone dependant (Bradley, 1976) mammary carcinoma. Thus, the inhibition of tumour growth produced by GLA in the latter study may have been due to alterations in the hormonal environment. In fact, there is evidence which indicates that fatty acids increase prolactin secretion (Chan et al., 1975, 1977; Welsch and Aylsworth, 1983; Welsch and Nagasawa, 1977) and hypersecretion of this hormone has been reported to inhibit the growth of many mammary tumours (Welsch and Aylsworth, 1983; Welsch and Nagasawa, 1977) including the R3230AC rat mammary tumour used by Ghayur and

## REFERENCES

Abbas, M.K., Yoo, T.J. & Viles, J. (1982). Ultrastructure and fatty acid composition of fatty acid - modified Morris 7777 Hepatoma cells. Cancer Research 42: 4639-4644.

Adam, O., Wolfram, G. & Zollner, N. (1982). Prostaglandin formation in man during intake of different amounts of linoleic acid. Annals of Nutrition Metabolism 26: 315-323.

Ames, B.N. (1983). Dietary carcinogens and anticarcinogens. Science 221: 1256-1264.

Anderson, D.W. & Crowle, A.J. (1982). Regression and differentiation of Neuroblastoma tumours in mice treated with differentiating agents prostaglandin $E_1$, and a phosphodiesterase inhibitor, RO20-1724. Cancer Letters 16: 287-295.

Barnett, R.E., Scott, R.E., Furcht, L.T. & Kersey, J.H. (1974). Evidence that mitogenic lectins induce changes in lymphocyte membrane fluidity. Nature (London) 249: 465-466.

Begin, M.E., Das, U.N., Ells, G. & Horrobin, D.F. (1985). Selective killing of human cancer cells by polyunsaturated fatty acids. Prostaglandins Leukotrienes and Medicine 19:177-186.

Bennett, A., Charlier, E.M., McDonald, A.M., Simpson, J.S., Stamford, I.F. & Zebro, T. (1977). Prostaglandins and breast cancer. The Lancet 2:624-626.

Berlinger, N.T. (1984). Deficient immunity in head and neck cancer due to excessive monocyte production of prostaglandins. Laryngoscope 94 (11 I): 1407-1410.

Bertoleni, L. & Diamond, L. (1976). Preservation of cells in monolayer culture by feezing in-situ. Tissue Culture Association Manual 2: 487-488.

Birt, D.F. & Pour, P.M. (1983a). Increased tumorigenesis induced by N-nitrosobis (2-oxopropyl) amine in Syrian Golden Hamsters fed high fat diets. Journal of the National Cancer Institute 70(6): 1135-1138

Birt, D.F. & Pour, P.M. (1983b). Influence of dietary fat on spontaneous lesions of Syrian Golden Hamsters. Journal of the National Cancer Institute 71(2): 401-406.

Birt, D.F., Salmasi, S. & Pour, P.M. (1981). Enhancement of experimental pancreatic cancer in Syrian Golden Hamsters by dietary fat. Journal of the National Cancer Institute 67(6): 1327-1332.

Birt, D.F., Stepan, K.R. & Pour, P.M. (1983). Interaction of dietary fat and protein on pancreatic carcinogenesis in Syrian Golden Hamsters. Journal of the National Cancer Institute 71(2): 355-360.

Booyens, J., Dippenaar, N., Fabbri, D., Engelbrecht, P. & Katzeff, I.E. (1984a). The effect of gamma-linolenic acid on the growth of human Osteogenic Sarcoma and Oesophageal Carcinoma cells in culture. South African Medical Journal 65: 240-242.

Booyens, J., Dippenaar, N., Fabbri, D., Engelbrecht, P., Louwrens,

C.C., & Katzeff, I.E. (1984b). Some effects of linoleic acid and gamma-linolenic acid on the proliferation of human Hepatoma cells in culture. South African Medical Journal 65: 607-612.

Booyens, J., Engelbrecht, P., le Roux, S., van der Merwe, C.F., Katzeff, I.E. & Louwrens, C.C. (1984c). Some effects of the essential fatty acids linoleic acid and alpha-linolenic acid and of their metabolites gamma-linolenic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, and of prostaglandins $A_1$ and $E_1$ on sarcoma cells in culture. Prostaglandins Leukotrienes and Medicine 15(1): 15-33.

Booyens, J. & Katzeff, I.E. (1986). Dietary fats and Cancer. South African Journal of Science. In Press.

Booyens, J. & Koenig, L. (1984). The effect of dietary supplementation with gamma-linolenic acid on the growth of induced tumours in rats. South African Medical Journal 65: 660-661.

Booyens, J. & van der Merwe, C.F. (1985). Chronic arachidonic acid eicosanoid imbalance: a common feature in coronary artery disease, hypercholesterolemia, cancer and other important diseases. Significance of desaturase enzyme inhibition and of the arachidonic acid desaturase-independant pathway. Medical Hypotheses 18(1): 53-60.

Botha, J.H., Robinson, K.M. & Leary, W.P. (1983). Parenteral gamma-linolenic acid administration in nude mice bearing a range of human tumour xenografts. South African Medical Journal 64: 11-12.

Bradley, C.J., Kledzik, G.S. & Meites, J. (1976). Prolactin and oestrogen dependancy of rat mammary cancers at early and late stages of development. Cancer Research 36: 319-324.

Bregman, M.D. & Meyskens, F.L. (Jr.)(1983). Inhibition of human malignant melanoma colony forming cells in vitro by prostaglandin $A_1$. Cancer Research 43: 1642-1645.

Bregman, M.D., Peters, E., Sander, D. & Meyskens, F.L. (Jr.)(1983). Dexamethasone, Prostaglandin A and retinoic acid modulation of murine and human melanoma cells grown in soft agar. Journal of The National Cancer Institute 71(5): 927-932.

Bregman, M.D., Sander, D. & Meyskens, F.L. (Jr.)(1982). Prostaglandin Al and El inhibit the plating efficiency and proliferation of murine melanoma cells (Cloudman S-91) in soft agar. Biochemical and Biophysical Research Communications 104(3): 1080-1086.

Buttke, T.M. (1984). Inhibition of lymphocyte proliferation by free fatty acids. I. Differential effects on mouse B and T lymphocytes. Immunology 53(2): 235-242.

Carroll, K.K. (1981). Neutral fats and Cancer. Cancer Research 41: 3695-3699.

Carroll, K.K. & Khor, H.T. (1971). Effects of level and type of dietary fat on incidence of mammary tumours induced in female Sprague-Dawley rats by 7,12 - dimethylbenz (a) anthracene. Lipids 6: 415-420.

Carroll, K.K. & Khor, H.T. (1975 . Dietary fat in relation to tumorigenesis. Progress in Biochemical Pharmacology 10: 308-353.

Carter, C.A., Milholland, R.J., Shea, W. & Ip, M.M. (1983). Effect of the prostaglandin synthetase inhibitor indomethacin on 7,12-dimethylbenz(a)anthracene-induced mammary tumorigenesis in rats fed different levels of fat. Cancer Research 43: 3559-3562.

Cave, W.T. (Jr.) & Erickson-Lucas, M.J. (1982). Effects of dietary lipids on lactogenic hormone receptor binding in rat mammary tumours. Journal of the National Cancer Institute 68(2): 319-324.

Chan, P-C., Didato, F. & Cohen, L.A. (1975). High dietary fat, elevation of rat serum prolactin and mammary cancer. Proceedings of the Society for Experimental Biology and Medicine 149: 133-135.

Chan, P-C., Ferguson, K.A. & Dao, T.L. (1983). Effects of different dietary fats on mammary carcinogenesis. Cancer Research 43: 1079-1083.

Chan, P-C., Head, J.F., Cohen, L.A. & Wynder, E.L. (1977). Influence of dietary fat on the induction of mammary tumours by N-nitrosomethylurea: associated hormone changes and differences between Sprague-Dawley and F344 rats. Journal of the National Cancer Institute 59(4): 1279-1283.

Cohen, F. & Jaffe, B.M. (1973). Production of prostaglandins by cells in vitro: Radioimmunoassay measurement of the conversion of arachidonic acid to $PGE_2$ and $PGF_{2\alpha}$. Biochemical and Biophysical Research Communications 55(3): 724-729.

Cohen, N.D. & Hilf, R. (1975). Influence of insulin on

oestrogen-induced responses in the R3230AC mammary carcinoma. Cancer Research 35: 560-567.

Crawford, M.A. (1983). Prostacyclin, Thromboxane and Leukotrienes: Background to essential fatty acids and their prostanoid derivatives. British Medical Bulletin 39(3): 210-213.

Das, U.N. (1981). Cell membrane fluidity and prostaglandins. Medical Hypotheses 7: 549-553.

de Deckere, E.A.M. & ten Hoor, F. (1979). Effects of dietary fats on the coronary flow rate and the left ventricular function of the isolated rat heart. Nutrition and Metabolism 23: 88-97.

Delescluse, C., Colburn, N.H., Duell, E.A. & Voorhees, J.J. (1974). Cyclic-AMP elevating agents inhibit proliferation of keratinizing guinea pig epidermal cells. Differentiation 2: 343-350.

Dippenaar, N., Booyens, J., Fabbri, D. & Katzeff, I.E. (1982a). The reversibility of cancer: evidence that malignancy in melanoma cells is gamma-linolenic acid deficiency - dependant. South African Medical Journal 62: 505-509.

Dippenaar, N., Booyens, J., Fabbri, D., Engelbrecht, P. & Katzeff, I.E. (1982b). The reversibility of cancer: evidence that malignancy in human hepatoma cells is gamma-linolenic acid deficiency - dependant. South African Medical Journal 62: 683-685.

Droller, M.J. (1981). Prostaglandins and neoplasia. Review article. The Journal of Urology 125: 757-760.

Droller, M.J., Schneider, M.U. & Perlmann, P. (1978). A possible role of prostaglandins in the inhibition of natural and antibody

dependant cell - mediated cytotoxicity against tumour cells. Cellular Immunology 39: 165-177.

Dubpernell, S.A. & Gavurin, L. (1978). The effect of cyclic AMP on the growth and morphology of a normal human fibroblast parent strain and its transformed progeny line. Cell Differentiation 7: 375-386

Eisenbarth, G.S., Wellman, D.K. & Lebovitz, H.E. (1974). Prostaglandin $A_1$ inhibition of Chondrosarcoma growth. Biochemical and Biophysical Research Communications 60(4): 1302-1308.

Endicott, K.M. (1972). Cancer. In The World Book Encyclopedia Vol.3. p 135.USA: Field Enterprises Educational Corporation.

Erickson, K.L. (1984). Influence of dietary fatty acid concentration and geometric configuration on murine mammary tumorigenesis and experimental metastasis. Journal of Nutrition 114(10): 1834-1842.

Favalli, C., Garaci, E., Etheredge, E., Santoro, M.G. & Jaffe, B.M. (1980). Influence of PGE on the immune response in melanoma bearing mice. Journal of Immunology 125: 897-902.

Fukushima, M., Kato, T., Ueda, R., Ota, K., Narumiya, S. & Hayaishi, O. (1982). Prostaglandin $D_2$, a potential antineoplastic agent. Biochemical and Biophysical Research Communications 105(3): 956-964.

Fulton, A.M. (1984). Effects of indomethacin on the growth of cultured mammary tumours. International Journal of Cancer 33: 375-379.

Fulton, A.M. & Levy, J.G. (1980). Inhibition of murine tumour growth

and prostaglandin synthesis by indomethacin. International Journal of Cancer 26: 669-673.

Gabor, H., Hillyard, L.A. & Abraham, S. (1985). Effect of dietary fat on growth kinetics of transplantable mammary adenocarcinoma. Journal of the National Cancer Institute 76(6): 1299-1305.

Gammal, E.B., Carroll, K.K. & Plunkett, E.R. (1967). Effects of dietary fat on mammary carcinogenesis by 7,12-dimethylbenz (a) anthracene in rats. Cancer Research 27: 1737-1742.

Gemsa, D., Leser, H.G., Diemann, W. & Resch, K. (1982). Suppression of T lymphocyte proliferation during Lymphoma growth in mice: role of $PGE_2$ - producing suppressor macrophages. Immunobiology 161(3-4): 385-391.

Ghayur, T. & Horrobin, D.F. (1981). Effects of essential fatty acids in the form of evening primrose oil on the growth of the rat R3230AC transplantable mammary tumour. IRCS Medical Science 9: 582.

Glauert, A., Rogers, G.E. & Glauert. R.H. (1956). A new embedding medium for electron Microscopy. Nature 178: 803

Goodwin, J.S., Husby, G. & Williams, R.C. (Jr.)(1980). Prostaglandin E and cancer growth. Review. Cancer Immunology and Immunotherapy 8: 3-7.

Granström, E. (1977). Chemistry of prostaglandins and thromboxanes. In Prostaglandins and Thromboxanes, ed. Berti, F., Samuelsson, B. & Velo, G.P. Vol. 13, pp 1-8. New York and London: Plenum Press.

Grinwich, K.D. & Plescia, O.J. (1977). Tumour mediated immuno-

suppression: prevention by inhibitors of prostaglandin synthesis. Prostaglandins 14(6): 1175-1182.

Gross, L. (1982). Oncogenic Viruses. Vol. 1. Great Britain: Pergammon Press.

Hammarström, S. (1977). Prostaglandin production by normal and transformed 3T3 fibroblasts in cell culture. European Journal of Biochemistry 74: 7-12.

Hial, V., Horakova, Z., Shaff, R.E. & Beaven, M.A. (1976). Alteration of tumour growth by aspirin and indomethacin; studies with two transplantable tumours in mouse. European Journal of Pharmacology 37: 367-376.

Hilf, R., Bell, C., Goldenberg, H. & Michel, I. (1971). Effect of fluphenazine HCl on R3230AC mammary carcinoma and mammary glands of the rat. Cancer Research 31: 1111-1117.

Hilf, R., Bell, C. & Michel, I. (1967). Influence of the mammotropic tumour $MtTF_4$ on the growth and biochemistry of the R3230AC mammary carcinoma and mammary glands. Cancer Research 27: 482-489.

Hofer, D., Dubitsky, A.M., Reilly, P., Santoro, M.G. & Jaffe, B.M. (1980). The interactions between indomethacin and cytotoxic drugs in mice bearing B-16 melanomas. Prostaglandins 20(6): 1033-1038.

Holley, R.W., Baldwin, J.H. & Kiernan, J.A. (1974). Control of growth of a tumour cell by linoleic acid. Proceedings of the National Academy of Sciences USA 71(10): 3976-3978.

Hong, S.L., Polsky-Cynkin, R. & Levine, L. (1976). Stimulation of

prostaglandin biosynthesis by vasoactive substances in methylcholanthrene transformed mouse BALB/3T3. Journal of Biological Chemistry 251: 776-780.

Hong, S.L., Wheless, C.M. & Levine, L. (1977). Elevated prostaglandin synthetase activity in methyl-cholanthrene-transformed mouse BALB/3T3. Prostaglandins 13(2): 271-279.

Honn, K.V., Bockman, R.S. & Marnett, L.J. (1981). Prostaglandins and Cancer: A review of tumour initiation through tumour metastasis. Prostaglandins 21(5): 833-864.

Hopkins, G.J., Hard, G.C. & West, C.E. (1978). Carcinogenesis induced by 7,12-dimethylbenz(a)anthracene in C3H-$A^{VY}$fB mice: influence of different dietary fats. Journal of the National Cancer Institute 60(4): 849-853.

Hopkins, G.J., Kennedy, T.G. & Carroll, K.K. (1981). Polyunsaturated fatty acids as promoters of mammary carcinogenesis induced in Sprague-Dawley rats by 7,12-dimethylbenz (a) anthracene. Journal of the National Cancer Institute 66(3): 517-522.

Hopkins, G.J. & West, C.E. (1976). Possible roles of dietary fats in carcinogenesis. Life Sciences 19: 1103-1116.

Horrobin, D.F. (1978). Prostaglandins. Physiology, Pharmacology and Clinical Significance. Great Britain: Churchill Livingstone.

Horrobin, D.F. (1980a). The reversibility of cancer: the relevance of cyclic AMP, calcium, essential fatty acids and prostaglandin E1. Medical Hypotheses 6: 469-486.

Horrobin, D.F. (1980b). The regulation of prostaglandin biosynthesis: negative feedback mechanisms and the selective control of

formation of 1 and 2 series prostaglandins: relevance to inflammation and immunity. Medical Hypotheses 6: 687-709.

Huang, Y.S., Cunnane, S.C., Horrobin, D.F. & Darignon, J. (1982). Most biological effects of zinc deficiency corrected by $\gamma$linolenic acid (18:3$\omega$6) but not by linoleic acid (18:2$\omega$6) Atherosclerosis 41: 193-207.

Hughes, D., Caspary, E.A. & Wisniewski, H.M. (1975). Immunosuppression by linoleic acid. Lancet 2: 501-502.

Humes, J.L., Cupo, J.J. & Strausser, H.R. (1974). Effects of indomethacin on Moloney Sarcoma Virus - induced tumours. Prostaglandins 6: 463-473.

Humes, J.L. & Strausser, H.R. (1974). Prostaglandins and cyclic nucleotides in Moloney Sarcoma tumours. Prostaglandins 5(2): 183-196.

Huttner, J.J., Milo, G.E., Panganamala, R.V. & Cornwell, D.G. (1978). Fatty acids and the selective alteration of in vitro proliferation in human fibroblast and guinea-pig smooth muscle cells. In Vitro 14(10): 854-859.

Huttner, J.J., Panganamala, R.V., Cornwell, D.G., Sharma, H.M., Gweby, E.T. & Geer, J.C. (1977). Fatty acids and their prostaglandin derivatives: inhibitors of proliferation of aortic smooth muscle cells. Science 197: 289-291.

Inbar, M. & Shinitsky, M. (1974). Cholesterol as a bioregulator in the development and inhibition of Leukemia. Proceedings of the National Academy of Sciences USA 71: 4229-4231.

Ingerman-Wojenski, C., Silver, M.J., Smith, B., Nissenbaum, M. &

Sedar, A.W. (1981). Prostacyclin production in rabbit arteries in situ: inhibition of arachidonic acid induced endothelial cell damage. Prostaglandins 21: 655-664.

Ip, C., Carter, C.A. & Ip, M.M. (1985). Requirements of essential fatty acid for mammary tumorigenesis in the rat. Cancer Research 45: 1997-2001.

Jacobson, H.I. (1974). Oncolytic action of prostaglandins. Cancer Chemotherapy Reports 58: 503-511.

Jaffe, B.M. (1974). Prostaglandins and Cancer: an update. Prostaglandins 6(6): 453-461.

Johnson, G.S. & Pastan, I. (1971). Change in growth and morphology of fibroblasts by prostaglandins. Journal of the National Cancer Institute 47(6): 1357-1364.

Jubiz, W., Frailey, J., & Smith, J.B. (1979). Inhibitory effect of prostaglandin $F_{2\alpha}$ on the growth of a hormone-dependant rat mammary tumour. Cancer Research 39: 998-1000.

Karmali, R.A. (1980). Review: Prostaglandins and Cancer. Prostaglandins and Medicine 5: 11-28.

Karnovsky, M.J. (1965). A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. Journal of Cell Biology 27: 127-139.

Keen, L.N., Reynolds, R., Whittle, W.L. & Kruse, P.F. (Jr.)(1973). Vertical sectioning. B. Cells in plastic flasks. In Tissue Culture - Methods and Applications, ed. Kruse, P.F.(Jr.) & Patterson, M.K.(Jr.) pp. 448-451. New York and London: Academic Press.

Kidwell, W.R., Monaco, M.E., Wicha, M.S. & Smith, G.S. (1978). Unsaturated fatty acid requirements for growth and survival of a rat mammary tumour cell line. Cancer Research 38: 4091-4100.

Kollmorgen, G.M., King, M.M., Kosanke, S.D. & Do, C. (1983). Influence of dietary fat and indomethacin on the growth of transplantable mammary tumours in rats. Cancer Research 43: 4714-4719.

Kurtz, M.J., Polgar, P., Taylor, L. & Rutenburg, A.M. (1974). The role of adenosine 3',5' - cyclic monophosphate in the division of Wl 38 cells. Biochemical Journal 142: 339-344.

Leary, W.P., Robinson, K.M., Booyens, J. & Dippenaar, N. (1982). Some effects of gamma-linolenic acid on cultured human Oesophageal Carcinoma cells. South African Medical Journal 62: 681-683.

Lee, E.C., Tu, R.S., Fantone, J.C. & Varani, J. (1984). Functional responses of tumour cells to phorbol esters: role of prostaglandins. Oncology 41(3): 210-216.

Levine, L., Hinkle, P.M., Voelkel, E.F. & Tashjian, A.H. (Jr.)(1972). Prostaglandin production by mouse fibrosarcoma cells in culture: inhibition by indomethacin and aspirin. Biochemical and Biophysical Research Communications 47: 888-896.

Lewis, G.P. (1983). Prostacyclin, Thromboxanes and Leukotrienes. Immunoregulatory activity of metabolites of arachidonic acid and their role in inflammation. British Medical Bulletin 39(3): 243-248.

Lichtenstein, L.M., Gillespie, E., Bourne, H.R. & Henney, C.S. (1972). The effects of a series of prostaglandins on in vitro models of

the allergic response and cellular immunity. Prostaglandins 2: 519-528.

Longnecker, D.S., Roebuck, B.D. & Kuhlmann, E.T. (1985). Enhancement of pancreatic carcinogenesis by a dietary unsaturated fat in rats treated with saline or N-nitoso (2-hydroxypropyl)(2-oxopropyl) amine. Journal of the National Cancer Institute 74(1): 219-222.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with the Folin Phenol reagent. Journal of Biological Chemistry 193: 265-275.

Lupulescu, A. (1978). Enhancement of carcinogenesis by prostaglandins in male Albino Swiss mice. Journal of the National Cancer Institute 61(1): 97-106.

Lupulescu, A. (1980). Effects of prostaglandins on tumour transplantation. Oncology 37: 418-423.

Lynch, N.R., Castes, M., Astoin, M. & Salomon, J-C. (1978). Mechanism of inhibition of tumour growth by aspirin and indomethacin. British Journal of Cancer 38: 503-512.

Lynch, N.R. & Salomon, J-C. (1979). Tumour growth inhibition and potentiation of immunotherapy by indomethacin in mice. Journal of the National Cancer Institute 62(1): 117-121.

MacManus, J.P. & Whitfield, J.F. (1974). Cyclic AMP, prostaglandins and the control of cell proliferation. Prostaglandins 6(6): 475-487.

Malachi, T., Chaimoff, Ch., Feller, N. & Halbrecht, I. (1981). Prostaglandin $E_2$ and cyclic AMP in tumour and plasma of breast cancer patients. Journal of Cancer Research and Clinical

Oncology 102: 71-79.

Manku, M.S., Horrobin, D.F., Karmazyn, M. & Cunnane, S.C. (1979). Prolactin and zinc effects on rat vascular reactivity: possible relationship to dihomo-$\gamma$-linolenic acid and to prostaglandin synthesis. Endocrinology 104: 774-779.

Marx, J.L. (1983). Do tumour promoters affect DNA after all? Science 219: 158-159.

McCormick, J.N., Neill, W.A. & Sim, A.K. (1977). Immunosuppressive effect of linoleic acid. The Lancet 2: 508.

McHugh, M.I., Wilkinson, R., Elliott, R.W., Field, E.J., Dewar, P., Hall, R.R., Taylor, R.M.R. & Uldall, P.R. (1977). Immunosuppression with polyunsaturated fatty acids in renal transplantation. Transplantation 24: 263-267.

Meade, C.J. & Mertin, J. (1978). Fatty acids and immunity. Advances in Lipid Research 16: 127-165.

Mertin, J. & Hunt, R. (1976). Influence of polyunsaturated fatty acids on survival of skin allografts and tumour incidence in mice. Proceedings of the National Academy of Sciences USA 73(3): 928-931.

Mihas, A.A., Gibson, R.G. & Hirchowitz, B.I. (1975). Suppression of lymphocyte transformation by 16,(16) dimethyl prostaglandin $E_2$ and unsaturated fatty acids (38949). Proceedings of the Society for Experimental Biology and Medicine 149: 1026-1028.

Moncada, S. & Vane, J.R. (1983). Prostacyclin, Thromboxane and Leukotrienes: Introduction. British Medical Bulletin 39(3): 209.

Nakano, J. (1972). General pharmacology of prostaglandins. In The Prostaglandins: Pharmacological and Therapeutic advances, ed. Cuthbert, M.F. pp 23-124. London: The Whitefriars Press.

Naseem, S.M. & Hollander, V.P. (1973). Insulin reversal of growth inhibition of Plasma Cell Tumour by prostaglandin or adenosine 3',5'-monophosphate. Cancer Research 33: 2909-2912.

Newberne, P.M. (1981). Dietary fat, immunological response and cancer in rats. Cancer Research 41: 3783-3785.

Norman, R.J., Bredenkamp, B.L.F., Joubert, S.M. & Beetar, C. (1981). Fetal prostaglandin levels in twin pregnancies. Prostaglandins and Medicine 6: 309-316.

Norman, R.J. & Joubert, S.M. (1982). Fetal urinary prostaglandins. Prostaglandins Leukotrienes and Medicine 8: 171-172.

Offner, H. & Clausen, J. (1974). Inhibition of lymphocyte responses to stimulants induced by unsaturated fatty acids and prostaglandins. Lancet 2: 400-401.

Overjera, A.A., Houchens, D.P. & Barker, A.D. (1977). Sensitivity of a human tumour xenograft in nude (athymic) mice to various chemically active drugs. In Proceedings of the second International workshop on nude mice, ed. Normura, T., Ohsawa, N., Tamoski, N. & Fujiwara, K. pp. 451-460. Tokyo: University of Tokyo Press/Stuttgart: Gustave Fischer. Verlag.

Owen, K., Gomolka, D. & Droller, M.J. (1980). Production of prostaglandin $E_2$ by tumour cells in vitro. Cancer Research 40: 3167-3171.

Pantelouris, E.M. (1968). Absence of thymus in a mouse mutant.

Nature 217: 370-371.

Paul, J. (1970). Cell and tissue culture. Fourth edition. Edinburgh & London: E & S Livingstone.

Pelus, L.M., Broxmeyer, H.E., Kurland, J.I. & Moore, M.A.S. (1979). Regulation of macrophage and granulocyte proliferation: Specificities of prostaglandin E and lactoferrin. Journal of Experimental Medicine 150: 277-292.

Pelus, L.M. & Strausser, H.R. (1977). Prostaglandins and the immune response. Life Sciences 20: 903-913.

Peterson, H.I. (1983). Effects of prostaglandin synthetase inhibitors on tumour growth and vascularization. Experimental studies in the rat. Invasion and Metastasis 3(3): 151-159.

Petry, F.M., Tutton, P.J.M. & Barkla, D.H. (1984). The influence of arachidonic acid metabolites on cell division in the intestinal epithelium and in colonic tumours. Prostaglandins Leukotrienes and Medicine 15: 349-358.

Philips, H.J. (1973). Dye exclusion tests for cell viability. In Tissue Culture: Methods and Applications ed. Kruse, P.F.(Jr.) & Patterson, M.K.(Jr.) pp.406-408. New York and London: Academic Press.

Plescia, O.J., Smith, A.H. & Grinwich, K. (1975). Subversion of immune system by tumour cells and role of prostaglandins. Proceedings of the National Academy of Sciences USA 72(5): 1848-1851.

Polgar, P. & Taylor, L. (1977). Effects of prostaglandin on substrate uptake and cell division in human diploid fibroblasts.

Biochemical Journal 162: 1-8.

Pollard, M. & Luckert, P.H. (1983). Prolonged antitumour effect of indomethacin on autochthonous intestinal tumours in rats. Journal of the National Cancer Institute 70(6): 1103-1105.

Prasad, K.N. (1972). Morphological differentiation induced by prostaglandin in mouse neuroblastoma cells in culture. Nature New Biology 236: 49-52.

Rao, G.A. & Abraham, S. (1976). Brief communication: enhanced growth rate of transplanted mammary adenocarcinoma induced in C3H mice by dietary linoleate. Journal of the National Cancer Institute 56(2): 431-432.

Reddy, B.S., Watanabe, K. & Weisburger, J.H. (1977). Effect of high fat diet on colon carcinogenesis in F344 rats treated with 1,2 dimethylhydrazine, methylazoxymethanol acetate or methylnitrosurea. Cancer Research 37: 4156-4159.

Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. Journal of Cell Biology 17: 208-212.

Ring, J., Seifert, J., Mertin, J. & Brendel, W. (1974). Prolongation of skin allografts in rats by treatment with linoleic acid. Lancet 2: 1331.

Robinson, K.M. & Botha, J.H. (1985). Effects of gamma-linolenic acid, dihomo-gamma-linolenic acid and ethanol on cultured human mammary carcinoma cells. Prostaglandins Leukotrienes and Medicine. In press.

Robinson, K.M., Bux, S. & Botha, J.H. (1985). Ultrastructural

alterations induced in cultured carcinoma cells by exogenous prostanoid precursors. Proceedings of the Electron Microscopy Society of Southern Africa 15: In press.

Robinson, K.M. & Gregory, M.A. (1978). Virus-like-particles in carcinoma of the oesophagus cell lines. Proceedings of the Electron microscopy Society of Southern Africa 8: 43-44.

Robinson, K.M., Haffejee, A.A. & Angorn, I.B. (1980). Tissue culture and prognosis in carcinoma of the oesophagus. Clinical Oncology 6: 125-136.

Rockwell, S. (1977). In vivo - in vitro tumour systems: New models for studying the response of tumours to therapy. Laboratory Animal Science 27(5): 831-851.

Sakaguchi, M., Hiramatsu, Y., Takada, H., Yamamura, M., Hioki, K., Saito, K. & Yamamoto, M. (1984). Effect of dietary unsaturated and saturated fats on azoxy methane induced colon carcinogenesis in rats. Cancer Research 44: 1472-1477.

Santoro, M.G., Philpott, G.W. & Jaffe, B.M. (1976). Inhibition of tumour growth in vivo and in vitro by prostaglandin E. Nature 263: 777-779.

Santoro, M.G., Philpott, G.W. & Jaffe, B.M. (1977a). Inhibition of B-16 melanoma growth in vivo by a synthetic analog of prostaglandin $E_2$. Cancer Research 37: 3774-3779.

Santoro, M.G., Philpott, G.W. & Jaffee, B.M. (1977b). Dose dependant inhibition of B-16 melanoma growth in vivo by a synthetic analogue of prostaglandin $E_2$. Prostaglandins 14(4): 645-651.

Sato, M., Narisawa, T., Sano, M., Takahashi, T. & Goto, A. (1983).

Growth inhibition of transplantable murine colon adenocarcinoma 38 by indomethacin. Journal of Cancer Research and Clinical Oncology 106(1): 21-26.

Schölkens, B.A., Gehring, D., Schlotte, V. & Weithmann, U. (1982). Evening primrose oil, a dietary prostaglandin precursor, diminishes vascular reactivity to renin and angiotensin II in rats. Prostaglandins Leukotrienes and Medicine 8: 273-285.

Seyberth, H.W., Bonsch, G., Müller, H., Minne, H.W., Erlenmaier, T., Strein, K., Imbeck, H. & Mrongovius, R. (1980). Prostaglandin E production and hypercalcemia in rats bearing the Walker Carcinosarcoma. British Journal of Cancer 42: 455-461.

Shahabi, N.A., Safa, A.R. & Wittliff, J.L. (1984). Biochemical and morphological characterization of MCF-7 human breast tumour cells after treatment with prostaglandins PGA and PGF (Meeting Abstract). Journal of Cell Biology 99(4): 92a.

Sheppard, J.R. (1972). Difference in the cyclic adenosine 3',5'-monophosphate levels in normal and transformed cells. Nature New Biology 236: 14-16.

Shinitsky, M. & Barenholz, Y. (1978). Fluidity parameters of lipid regions determined by fluorescence polarization. Biochimica et Biophysica Acta 515: 367-394.

Shipman, C. (Jr.) (1973). Trypsin. In Tissue Culture: Methods and Applications, ed. Kruse, P.F. and Patterson, M.K. pp 5-8. New York and London: Academic Press.

Siegel, S. (1956). Non parametric statistics for the behavioural Sciences, pp 116-117. New York: McGraw Hill.

Simon, S.R. & Ershler, W.B. (1985). Hormonal influences on growth of B16 Murine Melanoma. Journal of the National Cancer Institute 74(5): 1085-1088.

Singleton, W.S. (1960). Properties of the liquid state. In Fatty Acids, ed. Markley, K.S. Second edition, pp 579-581. New York: Interscience Publishers.

Smart, J. & Ralph, R. (1983). Properties of the cyclic AMP-dependant protein kinases in mouse Mastocytoma cells. Molecular and Cellular Biochemistry 50(2): 107-113.

Smith, B.J., Wills, M.R. & Savory, J. (1983). Prostaglandins and cancer. Annals of Clinical and Laboratory Science 13(5): 359-365.

Smith, D.L., Willis, A.L. & Muhmad, I. (1984). Eicosanoid effects on cell proliferation in vitro: relevance to atherosclerosis. Prostaglandins Leukotrienes and Medicine 16: 1-10.

Stein-Werblosky, R. (1974). Prostaglandin and cancer. Oncology 30: 169-176.

Stone, K.J., Willis, A.L., Hart, M., Kirtland, S.J., Kernoff, P.B.A. & McNicol, G.P. (1979). The metabolism of dihomo-$\gamma$-linolenic acid in man. Lipids 14(2): 174-180.

Strausser, H. & Humes, J. (1975). Prostaglandin synthesis inhibition: effect on bone changes and sarcoma tumour induction in BALB/c mice. International Journal of Cancer 15: 724-730.

Sykes, J.A.C. & Maddox, I.S. (1972). Prostaglandin production by experimental tumours and effects of anti-inflammatory compounds. Nature New Biology 237: 59-61.

Tannenbaum, A. & Silverstone, H. (1953). Nutrition in relation to cancer. In Advances in Cancer Research, ed. Greenstein, J.P. and Haddow, A. Vol.1, pp 451-501. New York: Academic Press.

Tashjian, A.H. (Jr.)(1975). Editorial. Prostaglandins, Hypercalcemia and Cancer. New England Journal of Medicine 293: 1317-1318.

Tashjian, A.H. (Jr.), Voelkel, E.F., Goldhaber, P. & Levine, L. (1973). Successful treatment of hypercalcemia by indomethacin in mice bearing a prostaglandin-producing fibrosarcoma. Prostaglandins 3: 515-524.

Taylor, L., Polgar, P., McAteer, J.A. & Douglas, W.H.J. (1979). Prostaglandin production by type II alveolar epithelial cells. Biochimica et Biophysica Acta 572: 502-509.

Thomas, D.R., Philpott, G.W. & Jaffe, B.M. (1974). The relationship between concentration of prostaglandin E and rates of cell replication. Experimental Cell Research 84: 40-46.

Ting, C-C. & Hargrove, M.E. (1982). Tumour cell triggered macrophage mediated suppression of the T-cell cytotoxic response to tumour associated antigens. II. Mechanisms for induction of suppression. Journal of the National Cancer Institute 69(4): 873-878.

Tinsley, I.J., Schmitz, J.A. & Pierce, D.A. (1981). Influence of dietary fatty acids on the incidence of mammary tumours in the C3H mouse. Cancer Research 41: 1460-1465.

Trevisani, A., Ferretti, E., Capuzzo, A. & Tomasi, V. (1980). Elevated levels of prostaglandin $E_2$ in Yoshida Hepatoma and the inhibition of tumour growth by non-steroidal anti-inflammatory drugs. British Journal of Cancer 41: 341-347.

Tutton, P.J.M. & Barkla, D.H. (1980). Influence of prostaglandin analogues on epithelial cell proliferation and xenograft growth. British Journal of Cancer 41: 47-51.

Vitale, J.J. & Broitman, S.A. (1981). Lipids and immune function. Cancer Research 41: 3706-3710.

Watson, A.F. & Mellanby, E. (1930). Tar cancer in mice. II. The condition of the skin when modified by external treatment or diet, as a factor in influencing the cancerous reaction. British Journal of Experimental Pathology 11: 311-322.

Watson, D.M.A., Kelly, R.W., Hawkins, R.A. & Miller, W.R. (1984). Prostaglandins in human mammary cancer. British Journal of cancer 49: 459-464.

Watson, M.L. (1958). Staining tissue sections for electron microscopy with heavy metals. Journal of Biophysical and Biochemical Cytology 4: 475-478.

Welsch, C.W. & Aylsworth, C.F. (1983). Enhancement of murine mammary tumorigenesis by feeding high levels of dietary fat: A Hormonal Mechanism ? Journal of the National Cancer Institute 70(2): 215-221.

Welsch, C.W. & Nagasawa, H. (1977). Prolactin and murine mammary tumorigenesis: A Review. Cancer Research 37: 951-963.

Wicha, M.S., Lance, A.L. & Kidwell, W.R. (1979). Effects of free fatty acids on the growth of normal and neoplastic rat mammary epithelial cells. Cancer Research 39: 426-433.

Yang, T.J., Dale, J.B. & Machanoff, R. (1976). Effects of prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$ on the growth of leukaemia cells

in culture. Journal of Cell Science 20: 199-206.

Yoneda, T., Kitamura, M., Ogawa, T., Aya, S. & Sakuda, M. (1985). Control of $VX_2$ carcinoma cell growth in culture by calcium, calmodulin and prostaglandins. Cancer Research 45: 398-405.

Young, M.R. & Knies, S. (1984). Prostaglandin E production by Lewis Lung carcinoma: Mechanism for tumour establishment in vivo. Journal of the National Cancer Institute 72(4): 919-922.

Ziegler, J.L. (1982). Current Concepts: Cancer and the prostaglandins. A Scope Publication: The Upjohn Company.

# APPENDIX 1

PG PRODUCTION (CORRECTED FOR PROTEIN CONTENT OF CELLS)

BY CONTROL AND GLA TREATED M52B CELLS

| | PG (ng/mg protein/24 hours) | |
|---|---|---|
| | Control | GLA treated |
| PGE | 404 090 | 388 385 |
| | 495 040 | > 743 110 |
| | 233 711 | 718 813 |
| | 179 446 | 860 580 |
| | 525 140 | 360 040 |
| | 318 529 | 340 000 |
| | 379 060 | 592 609 |
| | 639 481 | 909 241 |
| | | 410 970 |
| PGF | 38 833 | 12 802 |
| | 22 173 | 37 033 |
| | 9 762 | 28 500 |
| | 11 257 | 32 032 |
| | 22 035 | 17 418 |
| | 18 301 | 17 234 |
| | 22 241 | 35 453 |
| | 35 204 | 51 821 |
| | 49 345 | 34 558 |

# APPENDIX 2.1

MEASUREMENTS OF M52B TUMOURS IN RANDOMBRED IMMUNOCOMPETENT BALB/c MICE CONSUMING EITHER NORMAL CHOW OR EPO SUPPLEMENTED CHOW

Experiment 1

| No. of days following treatment | Tumour Measurements | | | | | |
|---|---|---|---|---|---|---|
| | Control (n=2) | | | | | |
| | 1 | | | 2 | | |
| | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) |
| 0 | 20.3 | 16.5 | 2763.3 | 12.0 | 12.0 | 864.0 |
| 4 | 23.0 | 20.0 | 4600.0 | 14.0 | 14.0 | 1372.0 |
| 7 | 25.0 | 22.0 | 6050.0 | 16.0 | 16.0 | 2048.0 |
| 10 | 25.0 | 22.0 | 6050.0 | Spontaneous regression | | Spontaneous regression |

MEASUREMENTS OF M52B TUMOURS IN RANDOMBRED IMMUNOCOMPETENT BALB/c

MICE CONSUMING EITHER NORMAL CHOW OR EPO SUPPLEMENTED CHOW

Experiment 1

| No. of days following treatment | Tumour measurements | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=4) | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | |
| | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) |
| 0 | 6.3 | 6.3 | 125.1 | 20.6 | 15.4 | 2442.7 | 24.1 | 20.3 | 4965.7 | 24.7<br>10.0<br>30.0 | 20.7<br>10.0<br>6.0 | 6331.9 |
| 4 | 9.4 | 9.4 | 415.3 | 24.0 | 23.4 | 6570.7 | 25.1 | 23.3 | 6813.3 | 36.3 | 30.0 | 16200.0 |
| 7 | 12.6 | 12.6 | 1000.2 | 24.9 | 24.4 | 7412.2 | 26.2 | 24.9 | 8122.1 | 39.3 | 32.2 | 20121.6 |
| 10 | 19.5 | 14.5 | 1966.0 | 25.0 | 24.9 | 7750.1 | 26.9 | 21.9 | 6450.8 | 41.9 | 28.8 | 17376.8 |

# APPENDIX 2.2

MEASUREMENTS OF M52B TUMOURS IN RANDOMBRED IMMUNOCOMPETENT BALB/c MICE CONSUMING EITHER NORMAL CHOW OR EPO SUPPLEMENTED CHOW

Experiment 2

| No. of days following treatment | Tumour measurements | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control (n=4) | | | | | | | |
| | 1 | | 2 | | 3 | | 4 | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) |
| 0 | 22.0 | 20.0 | 4400.0 | 34.0 | 22.0 | 8228.0 | 21.0 | 13.0 | 1774.5 | 11.0 | 11.0 | 665.5 |
| 3 | 29.0 | 22.0 | 7018.0 | 36.0 | 23.0 | 9522.0 | 28.5 | 18.0 | 4617.0 | 12.0 | 11.0 | 726.0 |
| 6 | mouse died* | | | 40.0 | 23.0 | 10580.0 | 33.0 | 19.0 | 5956.5 | spontaneous regression | | |
| 8 | ——— | | | 38.0 | 23.0 | 10051.0 | 32.0 | 19.0 | 5776.0 | ——— | | |

* unknown cause

MEASUREMENTS OF M52B TUMOURS IN RANDOMBRED IMMUNOCOMPETENT BALB/c MICE CONSUMING EITHER NORMAL CHOW OR EPO SUPPLEMENTED CHOW

Experiment 2

| No. of days following treatment | Tumour measurements | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=4) | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | |
| | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) |
| 0 | 11.0 | 11.0 | 665.5 | 37.0 | 23.0 | 9786.5 | 29.0 | 24.0 | 8352.0 | 10.0 | 10.0 | 500.0 |
| 3 | 11.0 | 11.0 | 665.5 | 39.0 | 24.0 | 11232.0 | 32.0 | 25.0 | 10000.0 | spontaneous regression | | |
| 6 | spontaneous regression | | | 41.0 | 30.0 | 18450.0 | 35.0 | 33.0 | 19057.5 | ——— | | |
| 8 | ——— | | | 40.0 | 28.0 | 15680.0 | 36.0 | 32.0 | 18432.0 | ——— | | |

# APPENDIX 3

MEASUREMENTS OF M52B TUMOURS IN INBRED IMMUNOCOMPETENT BIOZZE MICE

INJECTED WITH NOTHING, GLA IN HCO OR HCO ONLY

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Untreated control (n=5) | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) |
| 0 | 4.0 | 2.5 | 12.5 | 3.5 | 3.5 | 21.4 | 12.0<br>9.0 | 6.0<br>6.0 | 378.0 | 4.0 | 3.5 | 24.5 | 8.0 | 6.0 | 144.0 |
| 3 | 9.0 | 7.0 | 220.5 | 6.0 | 5.0 | 75.0 | 11.0<br>9.0 | 7.0<br>8.0 | 557.5 | 3.5 | 3.5 | 21.4 | 12.0 | 8.0 | 384.0 |
| 5 | spontaneous regression | | | 6.0 | 5.0 | 75.0 | 12.0<br>10.0 | 6.0<br>9.0 | 621.0 | 4.0 | 4.0 | 32.0 | 12.0 | 10.0 | 600.0 |
| 7 | | | | 6.0 | 4.0 | 48.0 | 12.0<br>11.0 | 8.0<br>8.0 | 736.0 | spontaneous regression | | | 15.0 | 11.0 | 907.5 |
| 10 | | | | spontaneous regression | | | 13.0<br>18.0 | 8.0<br>10.0 | 1316.0 | | | | 22.0 | 18.0 | 3564.0 |

MEASUREMENTS OF M52B TUMOURS IN INBRED IMMUNOCOMPETENT BIOZZE MICE

INJECTED WITH NOTHING, GLA IN HCO OR HCO ONLY

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treated control (HCO) n=6 | | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) |
| 0 | 10.0 | 4.0 | 80.0 | 4.0 | 4.0 | 32.0 | 3.0 | 3.0 | 13.5 | 7.0 | 6.0 | 126.0 | 4.0 | 3.0 | 18.0 | 6.0 | 6.0 | 108.0 |
| 3 | 12.0 | 5.0 | 150.0 | spontaneous regression | | | spontaneous regression | | | 9.0 | 6.0 | 162.0 | 6.0 | 5.0 | 75.0 | spontaneous regression | | |
| 5 | 16.0 | 5.0 | 200.0 | ——— | | | ——— | | | 19.0 | 12.0 | 1368.0 | 5.0 | 5.0 | 62.5 | ——— | | |
| 7 | 22.0 | 17.0 | 3179.0 | ——— | | | ——— | | | 20.0 | 14.0 | 1960.0 | 12.0 | 12.0 | 864.0 | ——— | | |
| 10 | 23.0 | 17.0 | 3323.5 | ——— | | | ——— | | | 20.0 | 15.0 | 2250.0 | 17.0 | 17.0 | 2456.5 | ——— | | |

MEASUREMENTS OF M52B TUMOURS IN INBRED IMMUNOCOMPETENT BIOZZE MICE

INJECTED WITH NOTHING, GLA IN HCO OR HCO ONLY

| No. of days after treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Experimental (GLA+HCO) n=8 | | | | | | | | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | | 7 | | | 8 | | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) |
| 0 | 3.5 | 3.0 | 15.8 | 20.0 | 15.0 | 2250.0 | 5.0 | 5.0 | 62.5 | 4.5 | 4.5 | 45.6 | 10.0 | 10.0 | 500.0 | 6.0 | 5.0 | 75.0 | 6.5 | 6.5 | 137.3 | 2.0 | 2.0 | 4.0 |
| 3 | 18.0 | 8.0 | 576.0 | 23.0 | 20.0 | 4600.0 | spontaneous regression | | | spontaneous regression | | | 18.0 | 18.0 | 1764.0 | 9.0 | 7.0 | 220.5 | 10.0 | 8.0 | 320.0 | spontaneous regression | | |
| 5 | 18.0 | 8.0 | 576.0 | 25.0 | 21.0 | 5512.5 | ——— | | | ——— | | | 24.0 | 20.0 | 4800.0 | 9.0 | 7.0 | 220.5 | 11.0 | 10.0 | 550.0 | ——— | | |
| 7 | 19.0 | 8.0 | 608.0 | 25.0 | 21.0 | 5512.5 | ——— | | | ——— | | | 24.0 | 21.0 | 5292.0 | 18.0 | 18.0 | 2916.0 | 23.0 | 13.0 | 1943.5 | ——— | | |
| 10 | 19.0 | 8.0 | 608.0 | 25.0 | 22.0 | 6050.0 | ——— | | | ——— | | | 26.0 | 24.0 | 7488.0 | 22.0 | 22.0 | 5324.0 | 33.0 | 18.0 | 5346.0 | ——— | | |

# APPENDIX 4

MEASUREMENTS OF M52B TUMOURS IN IMMUNOCOMPETENT OUTBRED "ONDERSTEPOORT STRAIN" MICE CONSUMING EITHER NORMAL OR EPO SUPPLEMENTED DIETS

| No. of days following treatment | Tumour measurements | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control (n=4) | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) |
| 0 | 6.0<br>1.0 | 6.0<br>1.0 | 108.5 | 15.0 | 7.0 | 367.5 | 10.0 | 10.0 | 500.0 | 13.0 | 10.0 | 650.0 |
| 3 | 8.0<br>3.0 | 8.0<br>3.0 | 269.5 | 20.0 | 12.0 | 1440.0 | spontaneous regression | | | spontaneous regression | | |
| 7 | 10.0<br>5.0 | 10.0<br>5.0 | 562.5 | 25.0 | 14.0 | 2450.0 | ——— | | | ——— | | |
| 10 | 12.0<br>8.0 | 12.0<br>8.0 | 1120.0 | 34.0 | 12.0 | 2448.0 | ——— | | | ——— | | |
| 14 | 16.0<br>12.0 | 15.0<br>10.0 | 2400.0 | 36.0 | 22.0 | 8712.0 | ——— | | | ——— | | |

MEASUREMENTS OF M52B TUMOURS IN IMMUNOCOMPETENT OUTBRED "ONDERSTEPOORT STRAIN" MICE CONSUMING EITHER NORMAL OR EPO SUPPLEMENTED DIETS

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=8) | | | | | | | | | | | | | | | |
| | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | | 7 | | 8 | |
| | L θ (mm) | Vol. ($mm^3$) | L θ (mm) | Vol. ($mm^3$) | L θ (mm) | Vol. ($mm^3$) | L θ (mm) | Vol. ($mm^3$) | L θ (mm) | Vol. ($mm^3$) | L θ (mm) | Vol. ($mm^3$) | L θ (mm) | Vol. ($mm^3$) | L θ (mm) | Vol. ($mm^3$) |
| 0 | 29.0 15.0 | 3262.5 | 19.0 17.0<br>8.0 7.0 | 2941.5 | 15.0 13.0 | 1267.5 | 36.0 16.0 | 4608.0 | 30.0 24.0 | 8640.0 | 26.0 15.0 | 1625.0 | 22.0 15.0 | 2475.0 | 12.0 10.0<br>13.0 10.0 | 1250.0 |
| 3 | 36.0 22.0 | 8712.0 | 25.0 17.0<br>8.0 7.0 | 3808.5 | 25.0 17.0 | 3612.5 | 41.0 19.0 | 7400.0 | 31.0 24.0 | 8928.0 | 28.5 13.5 | 2597.1 | 28.0 18.0 | 6156.0 | ——— | |
| 7 | 39.0 26.0 | 13182.0 | 29.0 22.0<br>10.0 8.0 | 7338.0 | 27.0 20.0 | 5400.0 | 44.0 22.0 | 10648.0 | 29.0 25.0 | 9062.5 | 32.0 21.0 | 7056.0 | 36.0 21.0 | 7938.0 | ——— | |
| 10 | 40.0 28.0 | 15680.0 | 44.0 23.0 | 11638.0 | 31.0 23.0 | 8199.5 | 46.0 21.0 | 10143.0 | 28.0 22.0 | 9196.0 | 35.0 18.0 | 5670.0 | 37.0 24.0 | 10656.0 | ——— | |
| 14 | 43.0 29.0 | 18081.5 | 46.0 29.0 | 19343.0 | 35.0 25.0 | 10937.5 | 46.0 22.0 | 11132.0 | 34.0 26.0 | 11492.0 | 40.0 23.0 | 10580.0 | 42.0 25.0 | 13125.0 | ——— | |

# APPENDIX 5.1

MEASUREMENTS OF M52B TUMOURS IN NUDE BALB/c MICE CONSUMING

EPO, SSO OR HCO SUPPLEMENTED CHOW; OR NORMAL CHOW

Experiment 1

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normal Chow (n=5) | | | | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | Mean vol. ($mm^3$) n=5 | SEM | | |
| | L (mm) | θ (mm) | $Vol_3$ ($mm^3$) | L (mm) | θ (mm) | $Vol_3$ ($mm^3$) | L (mm) | θ (mm) | $Vol_3$ ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | | | | | |
| 0 | 6.5<br>5.4<br>20.0 | 6.5<br>5.2<br>7.5 | 772.8 | 9.0 | 8.0 | 288.0 | 12.0 | 9.5 | 541.5 | 10.0 | 10.0 | 500.0 | 1.0 | 1.0 | 0.5 | 420.6 | 130.2 | | | |
| 3 | 20.0<br>24.5 | 9.0<br>10.0 | 2035.0 | 11.0 | 11.0 | 665.5 | 14.7<br>9.0<br>5.5 | 13.0<br>7.0<br>5.5 | 1545.8 | 13.0 | 11.0 | 786.5 | 4.5 | 4.5 | 45.6 | 1015.7 | 349.0 | | | |
| 7 | 24.0<br>13.8<br>26.8 | 12.0<br>13.0<br>13.0 | 5158.7 | 17.0 | 14.5 | 1787.2 | 18.3<br>26.8 | 16.0<br>22.0 | 8828.0 | 14.7 | 10.0 | 735.0 | 4.5 | 4.5 | 45.6 | 3310.9 | 1635.1 | | | |
| 11 | 24.6<br>33.0 | 15.7<br>14.0 | 6265.8 | 17.7 | 17.5 | 2710.3 | 21.5<br>28.5 | 19.0<br>25.0 | 12787.0 | 12.5 | 11.2 | 784.0 | 7.0 | 6.4 | 143.4 | 4538.1 | 2321.4 | | | |
| 15 | 27.5<br>33.0 | 21.5<br>17.0 | 11124.4 | 21.8 | 20.5 | 4580.7 | 26.5<br>38.5 | 22.0<br>26.5 | 19931.3 | 18.2 | 12.7 | 1467.7 | 8.6 | 8.6 | 318.0 | 7484.4 | 3634.3 | | | |
| 18 | 44.0 | 31.0 | 21142.0 | 26.5 | 26.5 | 9304.8 | 27.5<br>38.5 | 27.5<br>29.0 | 26587.7 | 15.5 | 11.0 | 937.8 | 9.5 | 9.5 | 428.7 | 11680.2 | 5288.7 | | | |
| 21 | 49.0 | 34.0 | 28322.0 | 30.0 | 30.0 | 13500.0 | 27.5<br>38.5 | 27.5<br>30.0 | 27723.4 | 16.0 | 12.0 | 1152.0 | 10.0 | 10.0 | 500.0 | 14239.5 | 6085.8 | | | |
| Mass of tumours (g) | 15.17 | | | 6.42 | | | 10.83 | | | 2.05 | | | 0.27 | | | | | | | |

MEASUREMENTS OF M52B TUMOURS IN NUDE BALB/c MICE CONSUMING EPO,

SSO OR HCO SUPPLEMENTED CHOW; OR NORMAL CHOW

Experiment 1

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HCO (n=5) | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | Mean vol. ($mm^3$) n=5 | SEM |
| | L (mm) | θ (mm) | Vol ($mm^3$) | L (mm) | θ (mm) | Vol ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | | |
| 0 | 8.3 | 6.3 | 164.7 | 10.0 | 10.0 | 500.0 | 4.5 | 4.5 | 45.6 | 15.5<br>14.0 | 6.5<br>8.0 | 775.4 | 14.5 | 11.5 | 958.8 | 488.9 | 173.9 |
| 3 | 11.5<br>8.6 | 7.2<br>7.5 | 540.0 | 12.7 | 11.5 | 839.8 | 6.6 | 6.6 | 143.7 | 16.4<br>14.8 | 8.0<br>9.0 | 1124.2 | 20.4 | 15.1 | 2325.7 | 994.7 | 370.4 |
| 7 | 13.5<br>12.3 | 11.0<br>11.5 | 1630.1 | 16.0<br>5.6 | 16.0<br>4.1 | 2095.1 | 8.0 | 8.0 | 256.0 | 33.7 | 14.4 | 3494.0 | 25.7 | 20.0 | 5140.0 | 2523.0 | 834.0 |
| 11 | 28.0 | 19.0 | 5054.0 | 19.5<br>6.0 | 18.5<br>5.7 | 3434.4 | 9.5 | 9.5 | 428.7 | 39.5 | 27.8 | 15263.6 | 29.0 | 23.0 | 7670.5 | 6370.2 | 2514.3 |
| 15 | 32.5 | 21.0 | 7166.3 | 24.0<br>9.0 | 19.0<br>9.0 | 4696.5 | 11.5 | 11.5 | 760.4 | 42.2 | 21.5 | 9753.5 | 30.3 | 21.5 | 7003.1 | 5876.0 | 1508.8 |
| 18 | 36.5 | 25.0 | 11406.3 | 24.0<br>12.0 | 21.0<br>11.0 | 6018.0 | 13.0 | 13.0 | 1098.5 | 46.5 | 23.7 | 13059.3 | 32.0 | 25.0 | 10000.0 | 8316.4 | 2147.8 |
| 21 | 38.0 | 26.0 | 12844.0 | 25.0<br>13.0 | 13.0<br>12.0 | 3048.5 | 14.0 | 14.0 | 1372.0 | 48.0 | 24.0 | 13824.0 | 34.0 | 26.0 | 11492.0 | 8516.1 | 2614.3 |
| Tumour mass (g) | 13.50 | | | 8.00 | | | 1.30 | | | 10.41 | | | 9.50 | | | | |

MEASUREMENTS OF M52B TUMOURS IN NUDE BALB/c MICE CONSUMING

EPO, SSO OR HCO SUPPLEMENTED CHOW; OR NORMAL CHOW

Experiment 1

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SSO (n=5) | | | | | | | | | | | | | | | | | | Mean vol. ($mm^3$) n=5 | SEM |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | | | | | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | | | | | |
| 0 | 11.0<br>6.0 | 11.0<br>6.0 | 773.5 | 5.5<br>5.5 | 4.6<br>5.5 | 141.4 | 9.2<br>11.0<br>9.5 | 6.5<br>10.0<br>8.5 | 1087.5 | 8.0<br>8.0<br>4.7 | 8.0<br>6.0<br>4.7 | 451.9 | 5.0 | 4.0 | 40.0 | | | | 498.9 | 195.3 |
| 3 | 15.0 | 8.8 | 580.8 | 7.7<br>9.5<br>4.8 | 7.5<br>8.8<br>4.8 | 639.7 | 18.0<br>13.5 | 17.0<br>13.0 | 3699.5 | 8.5<br>19.0 | 8.5<br>9.0 | 1076.6 | 6.0 | 6.0 | 108.0 | | | | 1220.9 | 638.4 |
| 7 | 23.5 | 20.0 | 4700.0 | 21.0<br>11.2 | 11.8<br>10.0 | 2022.0 | 32.7 | 16.8 | 4614.6 | 34.8 | 16.5 | 4737.2 | 10.0 | 8.5 | 361.2 | | | | 3287.0 | 895.1 |
| 11 | 27.5 | 21.5 | 6355.9 | 23.5<br>12.0 | 15.3<br>9.5 | 3292.1 | 47.0 | 28.0 | 18424.0 | 40.7 | 17.3 | 6090.6 | 10.7 | 10.0 | 535.0 | | | | 6939.5 | 3060.0 |
| 15 | 34.0 | 29.5 | 14794.3 | 32.5<br>12.5 | 16.5<br>10.0 | 5049.1 | 40.0 | 36.5 | 26645.0 | 47.0 | 21.3 | 10661.7 | 14.5<br>7.0 | 12.5<br>5.5 | 1238.7 | | | | 11677.8 | 4402.7 |
| 18 | 40.0 | 33.0 | 21780.0 | 39.0 | 29.0 | 16399.5 | 43.0 | 39.0 | 32701.5 | 48.5 | 23.5 | 13392.1 | 15.7<br>6.0 | 14.0<br>6.0 | 1646.6 | | | | 17183.9 | 5090.8 |
| 21 | 41.0 | 33.0 | 22324.5 | 40.0 | 30.0 | 18000.0 | 44.0 | 40.0 | 35200.0 | 49.0 | 24.0 | 14112.0 | 21.0<br>23.0 | 17.0<br>18.0 | 6760.5 | | | | 19279.4 | 4731.8 |
| Tumour mass (g) | 11.80 | | | 9.10 | | | 11.80 | | | 14.20 | | | 1.64 | | | | | | | |

MEASUREMENTS OF M52B TUMOURS IN NUDE BALB/c MICE CONSUMING

EPO, SSO OR HCO SUPPLEMENTED CHOW: OR NORMAL CHOW

Experiment 1

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=5) | | | | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | Mean vol. ($mm^3$) n=5 | SEM | | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | | | | | |
| 0 | 8.0 | 6.0 | 144.0 | 8.0 | 6.5 | 169.0 | 8.5 | 8.5 | 307.1 | 7.3 | 7.3 | 194.5 | 10.0 | 5.0 | 125.0 | 187.9 | 32.0 | | | |
| 3 | 6.5<br>12.0 | 6.5<br>10.0 | 737.3 | 10.0 | 9.0 | 405.0 | 11.0<br>12.0 | 10.8<br>8.5 | 1075.0 | 12.0<br>9.5 | 10.0<br>9.5 | 1028.7 | 4.5<br>6.5 | 4.5<br>5.8 | 154.9 | 680.2 | 177.8 | | | |
| 7 | 15.0<br>16.8 | 13.0<br>13.0 | 2687.1 | 10.5<br>12.0 | 8.3<br>12.0 | 1225.7 | 12.5<br>18.2 | 12.5<br>12.0 | 2287.0 | 27.0 | 17.0 | 3901.5 | 10.0 | 10.0 | 500.0 | 2120.3 | 589.4 | | | |
| 11 | 17.5<br>19.8 | 17.0<br>14.3 | 4553.2 | 17.6 | 17.3 | 2633.8 | 14.7<br>20.6 | 13.2<br>13.5 | 3157.8 | 30.7 | 18.3 | 5140.6 | 14.5<br>6.5 | 13.0<br>6.5 | 1362.6 | 3369.6 | 676.5 | | | |
| 15 | 21.5<br>23.7 | 19.0<br>22.0 | 9616.2 | 31.5 | 21.5 | 7280.4 | 15.5<br>22.8<br>30.0 | 13.2<br>14.1<br>7.7 | 4506.1 | 33.8 | 23.0 | 8940. 1 | 19.5 | 18.8 | 3446.0 | 6757.8 | 1209.2 | | | |
| 18 | 23.5<br>27.0 | 21.0<br>24.0 | 12957.8 | 36.0 | 26.0 | 12168.0 | 16.5<br>24.0 | 14.5<br>16.0 | 4806.6 | 36.0 | 25.0 | 11250.0 | 29.0 | 23.5 | 8007.6 | 9838.0 | 1513.6 | | | |
| 21 | 47.0 | 31.0 | 22583.5 | 37.0 | 27.0 | 13486.5 | 24.0<br>17.0 | 16.0<br>16.0 | 5248.0 | 42.0 | 30.0 | 18900.0 | 33.0 | 28.0 | 12936.0 | 14630.8 | 2947.0 | | | |
| Mass of tumours (g) | 10.00 | | | 6.29 | | | 4.18 | | | 9.20 | | | 6.50 | | | | | | | |

# APPENDIX 5.2

MEASUREMENTS OF M52B TUMOURS IN NUDE BALB/c MICE

CONSUMING EITHER NORMAL OR EPO SUPPLEMENTED CHOW

Experiment 2

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control (n=8) | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) |
| 0 | 14.7 | 14.7 | 1588.3 | 22.0 | 17.3 | 3292.2 | 8.6<br>5.0 | 8.6<br>5.0 | 380.5 | 11.5 | 10.5 | 633.9 | 7.7<br>8.2 | 7.0<br>7.2 | 401.2 |
| 3 | 24.0 | 19.0 | 4332.0 | 31.0 | 20.0 | 6200.0 | 15.0<br>9.0<br>7.0 | 14.0<br>6.8<br>6.0 | 1804.1 | 38.0<br>17.0 | 16.0<br>8.5 | 5478.1 | 12.2<br>9.8 | 9.7<br>9.5 | 1016.2 |
| 6 | 25.5 | 19.5 | 4848.2 | 39.5 | 35.0 | 24193.8 | 27.0<br>11.2 | 19.5<br>9.0 | 5587.0 | 33.0 | 23.7 | 9267.9 | 25.3 | 15.5 | 3036.2 |
| 10 | 35.0 | 21.0 | 7715.5 | 38.0 | 36.5 | 25312.8 | 39.4 | 22.0 | 9534.8 | 37.2 | 27.0 | 13559.4 | 27.5 | 17.5 | 4210.9 |
| Tumour mass (g) | 4.00 Invasive tumour | | | 8.50 Invasive tumour | | | 4.00 Invasive tumour | | | 6.44 Invasive tumour | | | 8.20 Invasive tumour | | |

Experiment 2

| No. of days following treatment | Tumour measurements | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control (n=8) | | | | | | | | | |
| | 6 | | | 7 | | | 8 | | Mean vol. ($mm^3$) n=8 | SEM |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | | |
| 0 | 16.5 | 14.0 | 1617.0 | 6.5<br>8.0<br>12.5 | 6.0<br>7.0<br>7.0 | 619.3 | 2.0 | 1.0 | 1.0 | 1066.7 | 377.1 |
| 3 | 27.0 | 22.5 | 6834.4 | 10.5<br>18.0<br>23.0 | 9.3<br>11.0<br>19.0 | 5694.6 | 4.0 | 3.5 | 24.5 | 3923.0 | 921.2 |
| 6 | 29.3 | 27.0 | 10679.9 | 15.0<br>15.7<br>29.0 | 13.5<br>12.3<br>25.0 | 11617.0 | 7.5 | 5.7 | 121.8 | 8669.0 | 2651.2 |
| 10 | 33.3 | 25.5 | 10826.7 | 18.7<br>19.0<br>27.2 | 13.6<br>16.0<br>26.9 | 14002.5 | 8.2 | 6.0 | 147.6 | 10663.8 | 2660.4 |
| Mass of tumour (g) | 8.20 Invasive tumour | | | 5.16 Invasive tumour | | | 0.10 Invasive tumour | | | | |

MEASUREMENTS OF M52B TUMOURS IN NUDE BALB/c MICE

CONSUMING EITHER NORMAL OR EPO SUPPLEMENTED CHOW

Experiment 2

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=9) | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol ($mm^3$) | L (mm) | θ (mm) | Vol ($mm^3$) |
| 0 | 10.0<br>10.0<br>9.5 | 8.0<br>7.0<br>7.5 | 832.2 | 9.5 | 8.0 | 304.0 | 16.5<br>19.5 | 10.0<br>11.0 | 2004.8 | 18.0<br>36.0 | 13.0<br>14.0 | 5049.0 | 7.0 | 5.0 | 87.5 |
| 3 | 39.0<br>14.5 | 13.0<br>10.5 | 4094.8 | 18.0<br>15.0 | 10.5<br>12.0 | 2072.3 | 22.0<br>22.0 | 14.0<br>16.5 | 5150.8 | 39.0 | 26.0 | 13182.0 | 10.0<br>16.0<br>4.5 | 9.5<br>7.0<br>4.0 | 879.3 |
| 6 | 42.5<br>17.5 | 17.5<br>11.5 | 7665.0 | 39.0 | 22.0 | 9438.0 | 29.5<br>28.5 | 20.5<br>18.5 | 11075.8 | 40.0 | 30.0 | 18000.0 | 13.2<br>20.5<br>6.0 | 13.0<br>11.5<br>5.0 | 2546.0 |
| 10 | 48.5<br>20.5 | 20.5<br>12.0 | 11659.9 | 45.0 | 24.5 | 13505.6 | 44.0 | 37.0 | 30118.0 | 45.0 | 35.0 | 27562.5 | 16.0<br>25.0<br>11.0 | 13.0<br>18.5<br>7.5 | 5939.5 |
| Tumour mass(g) | 5.80 Invasive Tumour | | | 7.00 Invasive Tumour | | | 4.30 Invasive Tumour | | | 14.20 Invasive Tumour | | | 2.00 Invasive Tumour | | |

Experiment 2

| No. of days following treatment | Tumour measurements | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=9) | | | | | | | | | | | |
| | 6 | | | 7 | | | 8 | | | 9 | | | Mean vol. ($mm^3$) n=9 | SEM |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | | |
| 0 | 18.0 | 10.0 | 900.0 | 25.0 | 13.0 | 2112.5 | 1.0 | 1.0 | 0.5 | 21.0 | 12.0 | 1872.0 | 1462.5 | 524.8 |
| | | | | | | | | | | 20.0 | 6.0 | | | |
| 3 | 22.0 | 11.0 | 1331.0 | 29.5 | 16.5 | 4015.7 | 3.5 | 3.5 | 64.3 | 27.0 | 15.0 | 4731.5 | 3946.8 | 1302.9 |
| | | | | | | | 3.5 | 3.5 | | 28.0 | 11.0 | | | |
| | | | | | | | 3.5 | 3.5 | | | | | | |
| 6 | 29.0 | 18.0 | 4698.0 | 34.0 | 20.0 | 6800.0 | 10.0 | 5.0 | 354.9 | 39.5 | 15.5 | 7219.9 | 7533.1 | 1711.2 |
| | | | | | | | 8.0 | 3.5 | | 22.0 | 15.0 | | | |
| | | | | | | | 6.2 | 5.0 | | | | | | |
| | | | | | | | 5.5 | 5.5 | | | | | | |
| | | | | | | | 4.5 | 3.0 | | | | | | |
| 10 | 28.0 | 20.0 | 5600.0 | 39.0 | 24.0 | 11232.0 | 11.5 | 6.0 | 1422.0 | 45.0 | 19.0 | 11185.7 | 13136.0 | 3231.9 |
| | | | | | | | 19.0 | 9.0 | | 25.5 | 15.5 | | | |
| | | | | | | | 11.0 | 9.0 | | | | | | |
| Mass of tumour (g) | 0.28 Invasive tumour | | | 5.60 Invasive tumour | | | 0.57 Invasive tumour | | | 7.90 Invasive tumour | | | | |

# APPENDIX 5.3

MEASUREMENTS OF M52B TUMOURS IN NUDE BALB/c MICE

CONSUMING EITHER NORMAL OR EPO SUPPLEMENTED DIETS

Experiment 3

| No of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control (n=6) | | | | | | | | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | | Mean vol. ($mm^3$) n=6 | SEM | | | | |
| | L (mm) | | Vol. ($mm^3$) | L (mm) | | Vol. ($mm^3$) | L (mm) | | Vol. ($mm^3$) | L (mm) | | Vol. ($mm^3$) | L (mm) | | Vol. ($mm^3$) | L (mm) | | Vol. ($mm^3$) | | | | | | |
| 0 | 6.0 | 6.0 | 108.0 | 13.5 | 7.0 | 330.8 | 2.0 | 1.0 | 1.0 | 12.0 | 4.5 | 121.5 | 11.4 | 5.5 | 172.4 | 1.0 | 1.0 | 0.5 | 122.4 | 50.2 | | | | |
| 4 | 12.0<br>13.5 | 8.5<br>9.0 | 980.3 | 22.5 | 9.7 | 1058.5 | 12.4 | 10.5 | 683.6 | 20.0 | 10.5 | 1102.5 | 14.5<br>7.4 | 9.0<br>4.7 | 669.0 | 7.5<br>5.7 | 6.5<br>5.7 | 251.0 | 790.8 | 132.0 | | | | |
| 7 | 29.0 | 21.5 | 6702.6 | 29.7 | 11.5 | 1963.9 | 18.0 | 14.9 | 1998.1 | 25.0 | 11.0 | 1512.5 | 23.4 | 17.5 | 3583.1 | 10.0<br>11.0 | 6.8<br>6.8 | 485.5 | 2707.5 | 897.2 | | | | |
| 11 | 35.0 | 26.0 | 11830.0 | 33.5 | 15.0 | 3768.8 | 24.5 | 18.5 | 4192.6 | 33.0 | 17.5 | 5053.12 | 28.5 | 19.0 | 5144.3 | 31.0 | 14.5 | 3258.9 | 5541.3 | 1292.5 | | | | |
| Tumour mass (g) | 4.54 invasive tumour | | | 2.28 non invasive tumour | | | 2.00 non invasive tumour | | | 2.30 invasive tumour | | | 3.10 invasive tumour | | | 0.41 non invasive tumour | | | | | | | | |

MEASUREMENTS OF M52B TUMOURS IN NUDE BALB/c MICE

CONSUMING EITHER NORMAL OR EPO SUPPLEMENTED CHOW

Experiment 3

| No of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=6) | | | | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | | Mean vol. ($mm^3$) n=6 | SEM |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | | |
| 0 | 7.0 | 6.0 | 126.0 | 5.5 | 4.0 | 44.0 | 7.0 | 7.0 | 171.5 | 6.0 | 6.0 | 108.0 | 4.5 | 4.5 | 45.6 | 1.0 | 1.0 | 0.5 | 82.6 | 25.8 |
| 4 | 17.0 | 14.2 | 1713.9 | 12.5 | 7.5 | 351.6 | 11.7 | 11.7 | 800.8 | 11.0 | 9.5 | 496.4 | 8.7 | 7.2 | 225.5 | 8.0 | 6.0 | 144.0 | 622.0 | 238.0 |
| 7 | 23.7 | 16.1 | 3071.6 | 17.4 | 10.9 | 1033.6 | 22.0 | 16.4 | 2958.6 | 22.5 | 13.0 | 1901.3 | 24.0 | 17.5 | 367.5 | 11.5 | 11.5 | 760.4 | 1682.2 | 469.3 |
| 11 | 25.0 | 21.5 | 5778.1 | 19.0 | 15.5 | 2282.4 | 29.2 | 21.0 | 6438.6 | 32.0 | 19.5 | 6084.0 | 33.0 | 24.0 | 9504.0 | 17.2 | 15.5 | 2066.2 | 5358.9 | 1145.4 |
| Tumour mass (g) | 3.00 invasive tumour | | | 1.30 non invasive tumour | | | 2.50 invasive tumour | | | 2.30 non invasive tumour | | | 2.80 non invasive tumour | | | 1.20 invasive tumour | | | | |

# APPENDIX 6.1

MEASUREMENTS OF NUB 1 TUMOURS IN NUDE BALB/c MICE

CONSUMING EITHER NORMAL OR EPO SUPPLEMENTED CHOW

Experiment 1

| No. of days following treatment | Tumour measurements | | | | | |
|---|---|---|---|---|---|---|
| | Control (n=2) | | | | | |
| | 1 | | | 2 | | |
| | L (mm) | Θ (mm) | Vol. ($mm^3$) | L (mm) | Θ (mm) | Vol. ($mm^3$) |
| 0 | 4.5 | 4.5 | 45.6 | 5.0 | 5.0 | 62.5 |
| 3 | 8.0 | 6.5 | 169.0 | 6.0 | 5.0 | 75.0 |
| 7 | 11.8 | 10.0 | 590.0 | 6.1 | 5.5 | 92.3 |
| 12 | 12.0 | 10.0 | 600.0 | 11.0<br>5.5 | 7.0<br>5.5 | 352.7 |
| 15 | 13.7 | 11.5 | 905.9 | 12.0<br>6.8 | 7.4<br>6.8 | 485.8 |
| 17 | 15.5 | 15.5 | 1861.9 | 12.5<br>8.8 | 8.6<br>7.8 | 729.9 |
| Tumour mass (g) | 1.00 | | | 0.30 | | |

MEASUREMENTS OF NUB 1 TUMOURS IN NUDE BALB/c MICE

CONSUMING EITHER NORMAL OR EPO SUPPLEMENTED DIETS

Experiment 1

| No. of days following treatment | Tumour measurements | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=4) | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) |
| 0 | 3.5 | 2.0 | 7.0 | 6.5 | 4.8 | 74.9 | 1.5 | 1.5 | 1.7 | 2.5<br>2.0 | 2.5<br>2.0 | 11.8 |
| 3 | 3.5 | 2.0 | 7.0 | 6.5 | 5.0 | 81.3 | 3.0 | 3.0 | 13.5 | 3.0<br>2.0 | 3.0<br>2.0 | 17.5 |
| 7 | 3.5 | 2.0 | 7.0 | 8.0 | 6.0 | 144.0 | 3.0 | 3.0 | 13.5 | 3.5<br>2.0 | 3.5<br>2.0 | 25.4 |
| 12 | 8.0 | 4.5 | 81.0 | 9.0 | 8.0 | 288.0 | 4.0 | 3.0 | 18.0 | 5.0<br>2.0 | 5.0<br>2.0 | 66.5 |
| 15 | 8.0 | 5.9 | 139.2 | 10.5 | 7.9 | 327.7 | 5.5 | 3.5 | 33.6 | 5.7<br>2.3 | 5.0<br>2.3 | 77.3 |
| 17 | 10.4<br>5.0 | 6.0<br>5.0 | 249.7 | 10.8 | 8.1 | 354.3 | 5.0 | 4.5 | 50.6 | 6.3<br>2.3 | 5.1<br>2.3 | 88.0 |
| Tumour mass (g) | 0.02 | | | 0.10 | | | 0.01 | | | 0.01 | | |

APPENDIX 6.2

MEASUREMENTS OF NUB 1 XENOGRAFTS IN NUDE BALB/c MICE

CONSUMING EITHER NORMAL CHOW OR EPO SUPPLEMENTED CHOW

Experiment 2

| No of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control (n=6) | | | | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | | Mean vol. ($mm^3$) n=6 | SEM |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | | |
| 0 | 6.5 | 5.0 | 81.3 | 4.0 | 3.0 | 18.0 | 6.4 | 3.0 | 28.8 | 3.2 | 3.2 | 16.3 | 3.0<br>3.0<br>2.5 | 3.0<br>3.0<br>2.5 | 34.8 | 3.0 | 3.0 | 13.5 | 32.1 | 10.4 |
| 3 | 8.2 | 6.4 | 167.9 | 4.8 | 3.6 | 31.1 | 6.6 | 3.5 | 40.4 | 6.6 | 4.1 | 55.5 | 4.6<br>4.1<br>4.6 | 4.4<br>2.7<br>4.3 | 102.0 | 3.8 | 3.8 | 27.4 | 70.7 | 22.5 |
| 6 | 8.2 | 7.0 | 200.9 | 6.2 | 5.5 | 93.8 | 6.4 | 3.6 | 41.5 | 7.1 | 4.6 | 75.1 | 5.6<br>4.1<br>5.2 | 4.8<br>3.1<br>4.6 | 139.2 | 4.3 | 4.3 | 39.8 | 98.4 | 25.5 |
| 9 | 12.1 | 9.5 | 546.0 | 7.2 | 5.8 | 121.1 | 7.8 | 3.7 | 53.3 | 8.5 | 6.3 | 168.7 | 9.0<br>6.9 | 6.0<br>6.8 | 321.5 | 5.5 | 5.4 | 80.2 | 215.2 | 76.9 |
| 13 | 12.5 | 11.5 | 825.6 | 8.8<br>3.0 | 6.5<br>3.0 | 199.4 | 6.5 | 3.5 | 39.8 | 9.0 | 6.0 | 162.0 | 8.9<br>8.4 | 6.0<br>8.4 | 456.6 | 8.3 | 6.5 | 175.3 | 309.9 | 117.9 |
| 16 | 14.0 | 13.0 | 1183.0 | 10.0<br>4.5 | 8.0<br>4.5 | 365.6 | 8.5 | 4.0 | 68.0 | 9.0 | 6.5 | 190.1 | 12.0<br>10.0 | 7.0<br>9.2 | 717.2 | 10.5 | 6.6 | 228.7 | 458.8 | 171.7 |

Experiment 2 (Control cont.)

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control (n=6) | | | | | | | | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | | Mean vol. ($mm^3$) n=6 | SEM | | | | |
| | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | | | | | | |
| 20 | 15.0 | 15.0 | 1687.5 | 11.5 | 10.0 | 575.0 | 5.5 | 4.3 | 50.8 | 10.5<br>4.0 | 7.0<br>4.0 | 289.2 | 13.0<br>11.3 | 6.2<br>10.0 | 814.9 | 12.0 | 8.5 | 433.5 | 641.9 | 235.1 | | | | |
| 23 | 17.0 | 15.5 | 2042.1 | 12.5 | 11.5 | 826.6 | 7.0 | 4.0 | 56.0 | 9.5<br>4.7 | 7.0<br>4.7 | 284.7 | Sacrificed | | | 12.0 | 9.0 | 486.0 | 739.1 | 350.4 | | | | |
| 27 | 17.0 | 16.0 | 2176.1 | 14.5 | 12.5 | 1132.8 | 8.5 | 5.0 | 106.3 | 11.0<br>4.5 | 7.5<br>4.5 | 354.9 | | | | 14.0 | 12.0 | 1008.0 | 955.5 | 361.9 | | | | |
| 31 | 21.0 | 17.0 | 3034.5 | 16.5 | 16.0 | 2112.0 | 8.0 | 5.0 | 100.0 | 12.0<br>7.0 | 8.5<br>6.5 | 581.3 | | | | 16.5 | 12.0 | 1188.0 | 1403.2 | 529.8 | | | | |
| 34 | 21.5 | 18.0 | 3483.0 | 17.7 | 17.0 | 2557.7 | 6.4 | 4.3 | 59.2 | 12.5<br>7.2<br>4.5 | 8.2<br>6.1<br>4.5 | 599.8 | | | | 16.0 | 12.0 | 1152.0 | 1570.3 | 635.5 | | | | |
| Tumour mass (g) | 1.63 | | | 1.56 | | | 0.15 | | | 0.46 | | | 1.10 | | | 0.30 | | | | | | | | |

MEASUREMENTS OF NUB 1 XENOGRAFTS IN NUDE BALB/c

CONSUMING EITHER NORMAL OR EPO SUPPLEMENTED CHOW

Experiment 2

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=6) | | | | | | | | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | | Mean vol. ($mm^3$) n=6 | SEM | | | | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | | | | | | |
| 0 | 15.5 | 6.5 | 327.4 | 7.0 | 4.1 | 58.8 | 5.5<br>6.5<br>5.5 | 4.0<br>4.0<br>5.5 | 179.2 | 6.6<br>4.5 | 5.5<br>4.5 | 145.4 | 10.0 | 3.9 | 76.1 | 5.0 | 4.2 | 44.1 | 138.5 | 47.4 | | | | |
| 3 | 20.9 | 9.2 | 884.5 | 11.3 | 6.0 | 203.4 | 6.8<br>7.8<br>6.6 | 5.5<br>5.3<br>6.1 | 335.2 | 8.3<br>6.3 | 7.3<br>6.3 | 346.2 | 11.9 | 5.7 | 193.3 | 7.3 | 5.8 | 122.7 | 347.4 | 123.6 | | | | |
| 6 | 24.0 | 10.0 | 1200.0 | 12.4 | 10.0 | 620.0 | 8.2<br>10.5<br>8.5 | 7.6<br>7.0<br>7.6 | 739.5 | 12.1<br>7.8 | 9.6<br>7.8 | 794.8 | 13.5 | 7.0 | 330.8 | 9.8 | 6.2 | 188.3 | 645.6 | 160.6 | | | | |
| 9 | 29.3 | 12.8 | 2400.3 | 13.5 | 10.4 | 730.1 | 9.5<br>11.5<br>11.0 | 9.0<br>7.0<br>8.7 | 1082.8 | 13.5<br>8.7 | 11.5<br>8.7 | 1221.9 | 16.4 | 8.1 | 538.0 | 8.5 | 6.3 | 168.7 | 1023.6 | 345.3 | | | | |
| 13 | 30.5 | 13.5 | 2779.3 | 15.5 | 12.5 | 1210.9 | 23.3<br>10.0 | 10.5<br>9.7 | 1754.9 | 23.4 | 13.3 | 2069.6 | 18.5 | 9.0 | 749.3 | 11.5 | 8.0 | 368.0 | 1488.7 | 397.0 | | | | |
| 16 | 32.0 | 15.0 | 3600.0 | 15.5 | 15.0 | 1743.8 | 27.0<br>11.0 | 11.5<br>11.0 | 2450.9 | 24.5 | 12.5 | 1914.1 | 20.0 | 10.5 | 1102.5 | 12.0 | 8.0 | 384.0 | 1865.9 | 494.9 | | | | |

(cont.):

Experiment 2

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=6) | | | | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | 5 | | | 6 | | | Mean vol. ($mm^3$) n=6 | SEM |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | | |
| 20 | 33.5 | 15.5 | 4024.2 | 17.0 | 15.0 | 1912.5 | 29.0<br>13.0 | 13.0<br>13.0 | 3549.0 | 27.5 | 14.0 | 2695.0 | 22.5 | 11.5 | 1487.1 | 11.0 | 8.5 | 397.3 | 2344.2 | 602.5 |
| 23 | Sacrificed | | | 21.8 | 13.5 | 1986.5 | 31.8<br>13.9 | 14.2<br>12.0 | 4206.9 | 26.0 | 14.0 | 2548.0 | 25.0 | 13.5 | 2278.1 | 10.8 | 9.3 | 467.0 | 2297.3 | 599.8 |
| 27 | ——— | | | 23.5 | 13.7 | 2205.3 | 30.0<br>14.5 | 16.0<br>12.0 | 4884.0 | 26.5 | 15.0 | 2981.3 | 25.5 | 14.0 | 2499.0 | 12.0 | 9.0 | 486.0 | 2611.1 | 708.9 |
| 31 | ——— | | | 27.5 | 16.5 | 3743.4 | 15.0<br>30.5 | 13.2<br>18.0 | 6247.8 | 28.5 | 14.5 | 2996.1 | 26.5 | 15.5 | 3183.3 | 12.5<br>5.8 | 8.7<br>5.8 | 570.6 | 3348.2 | 908.8 |
| 34 | ——— | | | 30.5 | 15.5 | 3663.8 | 15.5<br>37.0 | 12.5<br>19.0 | 7889.4 | 27.5 | 13.5 | 2505.9 | 28.5 | 18.0 | 4617.0 | 14.0<br>5.5 | 8.2<br>5.5 | 553.9 | 3846.0 | 1220.1 |
| Tumour mass (g) | 3.10 | | | 1.60 | | | 4.73 | | | 0.34 | | 2.16 | | | | 3.50 | | | | |

PERCENT INCREASE IN VOLUME OF NUB 1 XENOGRAFTS IN NUDE BALB/c MICE

CONSUMING EITHER NORMAL CHOW OR EPO SUPPLEMENTED CHOW

Experiment 2

| No. of days following treatment | Control (n=6) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Percent increase in tumour volume relative to day 0 | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean | SEM |
| 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | - |
| 3 | 106.6 | 72.8 | 40.3 | 240.3 | 193.0 | 1.0 | 109.0 | 37.4 |
| 6 | 147.3 | 420.9 | 44.0 | 360.8 | 300.0 | 194.4 | 244.7 | 57.7 |
| 9 | 572.0 | 572.7 | 85.4 | 934.8 | 823.9 | 494.0 | 580.5 | 120.8 |
| 13 | 916.1 | 1007.8 | 38.2 | 893.9 | 1211.9 | 1198.7 | 877.8 | 176.8 |
| 16 | 1356.0 | 1930.9 | 136.1 | 1066.4 | 1960.9 | 1594.0 | 1340.7 | 278.3 |
| 20 | 1976.9 | 3094.4 | 76.5 | 1647.5 | 2241.6 | 3111.1 | 2029.2 | 458.0 |

PERCENT INCREASE IN VOLUME OF NUB 1 XENOGRAFTS IN NUDE BALB/c MICE

CONSUMING EITHER NORMAL CHOW OR EPO SUPPLEMENTED CHOW

Experiment 2

| No. of days following treatment | EPO (n=6) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Percent increase in tumour volume relative to day 0 | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean vol. | SEM |
| 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | - |
| 3 | 170.1 | 245.7 | 87.1 | 138.1 | 154.2 | 178.4 | 162.3 | 21.3 |
| 6 | 266.5 | 953.9 | 312.7 | 446.7 | 334.9 | 327.1 | 440.3 | 105.6 |
| 9 | 633.1 | 1141.0 | 504.3 | 740.5 | 607.4 | 282.5 | 651.5 | 116.6 |
| 13 | 748.8 | 1958.4 | 879.4 | 1323.6 | 885.2 | 734.5 | 1088.3 | 194.7 |
| 16 | 999.5 | 2864.0 | 1267.8 | 1216.6 | 1349.7 | 770.7 | 1411.4 | 302.9 |
| 20 | 1129.0 | 3150.9 | 1880.7 | 1753.8 | 1856.4 | 800.9 | 1762.0 | 330.5 |

APPENDIX 6.3

MEASUREMENTS OF NUB 1 TUMOURS IN NUDE BALB/c MICE

CONSUMING EITHER NORMAL OR EPO SUPPLEMENTED DIETS

Experiment 3

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control (n=18) | | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | |
| | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) |
| 0 | 10.0 | 4.3 | 92.5 | 6.0 | 4.9 | 72.0 | 3.7<br>4.9<br>4.6 | 3.5<br>4.9<br>4.6 | 130.1 | 4.3<br>6.3 | 3.8<br>5.8 | 137.0 | 4.9 | 4.9 | 58.8 | 7.2 | 4.0 | 57.6 |
| 4 | 10.0 | 5.3 | 140.5 | 9.0 | 5.8 | 151.4 | 4.8<br>5.8 | 4.2<br>4.5 | 101.0 | 11.0 | 4.5 | 111.4 | 7.0 | 5.3 | 98.3 | 7.5 | 4.0 | 60.0 |
| 7 | 9.0 | 5.0 | 112.5 | 10.5 | 7.2 | 272.2 | 6.2<br>6.7<br>4.8 | 5.0<br>5.5<br>4.8 | 234.1 | 12.8 | 4.5 | 129.6 | 7.2 | 6.5 | 152.1 | 7.4 | 4.0 | 59.2 |
| 11 | 8.2 | 4.7 | 90.6 | 12.7 | 11.0 | 768.4 | 12.8<br>5.3 | 7.0<br>5.0 | 379.9 | 17.7 | 6.7 | 397.2 | 7.5 | 7.0 | 183.8 | 6.0 | 4.5 | 60.7 |
| 14 | 9.8 | 4.7 | 108.2 | 14.0 | 10.9 | 831.7 | 14.0 | 6.4 | 286.7 | 18.5 | 7.3 | 492.9 | 7.3 | 6.5 | 154.2 | 6.5 | 4.5 | 65.8 |
| 18 | 8.5 | 4.0 | 68.0 | 16.4 | 12.5 | 1281.3 | 17.0 | 7.0 | 416.5 | 20.0 | 8.2 | 672.4 | 7.3 | 6.5 | 154.2 | 4.5 | 4.2 | 39.7 |
| 21 | 9.2 | 4.0 | 73.6 | 18.4 | 15.6 | 2238.9 | 18.1 | 8.7 | 685.0 | 20.5 | 10.0 | 1025.0 | 7.3 | 6.6 | 159.0 | 4.5 | 4.2 | 39.7 |
| Tumour mass (g) | 0.01 | | | 0.79 | | | 0.49 | | | 0.47 | | | 0.02 | | | 0.001 | | |

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control (n=18) | | | | | | | | | | | | | | | | | | | |
| | 7 | | | 8 | | | 9 | | | 10 | | | 11 | | | 12 | | | 13 | | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol ($mm^3$) |
| 0 | 7.5 | 4.6 | 79.4 | 6.9 | 4.3 | 63.8 | 5.2 | 3.5 | 31.9 | 4.4 | 3.8 | 31.8 | 8.0<br>5.1 | 5.1<br>3.7 | 138.9 | 5.4 | 4.2 | 47.6 | 8.5<br>5.9 | 7.0<br>4.0 | 255.4 |
| 4 | 7.6 | 5.2 | 102.8 | 9.2 | 4.5 | 93.2 | 7.0 | 5.0 | 87.5 | 5.5 | 5.5 | 83.2 | 8.5<br>5.7 | 5.8<br>5.7 | 235.6 | 5.4 | 4.5 | 54.7 | 11.0<br>6.3 | 6.9<br>4.0 | 312.2 |
| 7 | 9.7 | 5.9 | 168.8 | 10.8 | 5.0 | 135.0 | 14.5 | 6.8 | 335.2 | 6.3 | 6.3 | 125.0 | 12.5 | 7.0 | 306.3 | 5.5 | 4.8 | 63.4 | 11.8<br>7.7 | 7.8<br>4.7 | 444.4 |
| 11 | 11.3 | 7.0 | 276.9 | 10.4 | 5.8 | 174.9 | 13.8 | 10.0 | 690.0 | 9.5 | 9.5 | 428.7 | 16.5 | 7.5 | 464.1 | 6.2 | 5.8 | 104.3 | 20.5 | 8.8 | 793.7 |
| 14 | 13.3 | 7.0 | 325.9 | 10.8 | 6.3 | 214.3 | 14.3 | 8.4 | 504.5 | 9.5 | 8.8 | 367.8 | 16.9 | 8.2 | 568.2 | 8.0 | 5.8 | 135.6 | 20.6 | 9.3 | 890.8 |
| 18 | 15.1 | 7.5 | 424.7 | 12.7 | 6.5 | 268.3 | 17.7 | 10.0 | 885.0 | 12.1 | 9.6 | 557.6 | 17.7 | 9.5 | 798.7 | 9.5 | 6.2 | 185.6 | 24.8 | 10.8 | 1446.3 |
| 21 | 16.1 | 8.5 | 581.6 | 13.2 | 6.6 | 287.5 | 19.5 | 11.5 | 1289.4 | 14.7 | 12.0 | 1058.4 | 19.0 | 10.0 | 950.0 | 12.0 | 7.0 | 294.0 | 27.5 | 12.0 | 1980.0 |
| Tumour mass (g) | 0.28 | | | 0.14 | | | 0.65 | | | 0.50 | | | 0.90 | | | 0.01 | | | 1.11 | | |

(Control cont.):

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control (n=18) | | | | | | | | | | | | | | | | | |
| | 14 | | | 15 | | | 16 | | | 17 | | | 18 | | | Mean vol. ($mm^3$) n=18 | SEM |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | | |
| 0 | 6.5 | 6.3 | 129.0 | 7.0 | 6.0 | 126.0 | 5.1 | 4.2 | 45.0 | 5.3 | 4.9 | 63.6 | 7.3 | 6.0 | 131.4 | 89.4 | 14.5 |
| 4 | 7.5 | 6.3 | 148.9 | 6.6 | 5.6 | 103.5 | 9.0 | 4.5 | 91.1 | 8.0 | 5.5 | 121.0 | 9.5 | 6.2 | 182.6 | 141.8 | 23.6 |
| 7 | 9.3 | 7.4 | 254.6 | 6.4 | 5.3 | 89.9 | 9.0 | 5.0 | 112.5 | 8.5 | 6.5 | 179.6 | 9.7<br>3.5 | 6.2<br>3.5 | 207.9 | 208.5 | 40.5 |
| 11 | 10.7 | 9.0 | 433.4 | 6.0<br>4.8<br>4.5 | 4.8<br>4.3<br>3.8 | 146.0 | 11.5 | 7.7 | 340.9 | 11.8 | 8.0 | 377.6 | 12.0<br>3.5 | 6.4<br>3.5 | 267.2 | 416.8 | 72.8 |
| 14 | 11.0 | 9.5 | 496.4 | 5.5<br>4.3<br>3.5 | 4.2<br>3.6<br>3.5 | 97.8 | 12.0 | 7.0 | 294.0 | 12.0 | 8.0 | 384.0 | 12.7<br>3.5 | 6.4<br>3.5 | 281.5 | 499.7 | 97.8 |
| 18 | 11.4 | 9.5 | 514.4 | 5.0<br>5.0<br>3.7 | 4.7<br>4.2<br>3.7 | 124.6 | 13.2 | 7.7 | 391.3 | 14.3 | 9.4 | 631.8 | 18.7 | 7.0 | 458.2 | 836.2 | 185.0 |
| 21 | 12.0 | 10.3 | 636.5 | 11.0 | 6.3 | 218.3 | 15.0 | 8.7 | 567.7 | 17.5 | 11.3 | 1117.3 | 21.4 | 9.5 | 965.7 | 1094.0 | 220.7 |
| Tumour mass (g) | 0.27 | | | 0.01 | | | 0.19 | | | 0.63 | | | 0.50 | | | | |

MEASUREMENTS OF NUB 1 TUMOURS IN NUDE BALB/c MICE

CONSUMING EITHER NORMAL OR EPO SUPPLEMENTED CHOW

Experiment 3

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=19) | | | | | | | | | | | | | | | | | | | | | |
| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | | 7 | | |
| | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) | L (mm) | θ (mm) | Vol. ($mm^3$) |
| 0 | 6.0 | 5.0 | 75.0 | 9.8<br>6.5 | 5.9<br>5.3 | 230.7 | 10.7 | 4.8 | 123.3 | 2.7 | 2.7 | 9.8 | 4.3<br>3.7 | 4.3<br>3.7 | 65.1 | 5.1 | 4.4 | 49.4 | 5.7 | 5.0 | 71.3 |
| 4 | 7.8 | 5.2 | 105.4 | 17.5 | 7.5 | 492.2 | 10.7 | 6 | 192.6 | 5.5 | 4.5 | 55.7 | 10.5 | 6.5 | 221.8 | 6.3 | 5.5 | 95.3 | 7.9<br>5.5 | 5.5<br>5.5 | 202.7 |
| 7 | 8.7 | 5.2 | 117.6 | 19.9 | 8.8 | 770.5 | 11.3 | 6.8 | 261.3 | 6.0 | 5.5 | 90.8 | 10.8 | 6.7 | 242.4 | 6.7 | 5.7 | 108.8 | 8.2<br>8.7 | 7.0<br>5.5 | 332.5 |
| 11 | 13.5 | 6.5 | 278.9 | 26.0 | 9.7 | 1223.2 | 14.8 | 9.3 | 640.0 | 9.0 | 7.0 | 220.5 | 14.0 | 8.0 | 448.0 | 9.0 | 8.0 | 288.0 | 18.7 | 9.0 | 757.4 |
| 14 | 13.7 | 6.8 | 316.7 | 29.6 | 10.5 | 1631.7 | 16.8 | 9.3 | 726.5 | 10.4 | 7.5 | 292.5 | 15.4 | 8.8 | 596.3 | 10.0 | 6.5 | 211.3 | 18.7 | 10.0 | 935.0 |
| 18 | 16.4 | 7.6 | 473.6 | 30.5 | 14.5 | 3206.3 | 22.2 | 10.0 | 1110.0 | 11.5 | 8.0 | 368.0 | 18.0 | 9.5 | 812.3 | 12.6 | 7.0 | 308.7 | 21.7 | 12.7 | 1750.0 |
| 21 | 16.7 | 8.5 | 603.3 | 32.2 | 13.6 | 2977.9 | 24.0 | 13.3 | 2122.7 | 13.5 | 8.3 | 465.0 | 18.4 | 10.4 | 995.1 | 14.1 | 7.0 | 345.5 | 25.3 | 14.6 | 2696.5 |
| Tumour mass (g) | 0.37 | | | 1.90 | | | 1.10 | | | 0.23 | | | 0.70 | | | 0.21 | | | 1.10 | | |

EPO cont.:

Experiment 3

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=19) | | | | | | | | | | | | | | | | | | | | | |
| | 8 | | | 9 | | | 10 | | | 11 | | | 12 | | | 13 | | | 14 | | | 15 | | |
| | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) | L (mm) | θ | Vol. ($mm^3$) |
| 0 | 8.0 | 4.4 | 77.4 | 6.0 | 3.7 | 41.1 | 8.7 | 7.0 | 213.2 | 7.2 | 5.5 | 108.9 | 7.0 | 5.0 | 87.5 | 8.6<br>5.4 | 5.7<br>3.4 | 170.9 | 5.8 | 2.7 | 21.1 | 3.5 | 3.5 | 21.4 |
| 4 | 8.8 | 5.9 | 153.2 | 6.5 | 3.8 | 46.9 | 8.5 | 6.3 | 168.7 | 7.5 | 5.7 | 121.8 | 8.8 | 5.0 | 110.0 | 8.7<br>5.5 | 5.6<br>4.5 | 192.1 | 5.8<br>4.2 | 2.3<br>3.0 | 45.0 | 4.8<br>4.1 | 3.8<br>3.0 | 53.1 |
| 7 | 8.9<br>3.0 | 6.0<br>3.0 | 173.2 | 6.5 | 3.8 | 46.9 | 8.8 | 6.5 | 185.9 | 10.0 | 6.3 | 198.4 | 8.4 | 5.7 | 136.5 | 10.5<br>5.5 | 7.2<br>5.2 | 346.5 | 4.5<br>4.2 | 3.5<br>3.4 | 51.8 | 4.8<br>4.0 | 4.5<br>3.5 | 73.1 |
| 11 | 12.0 | 8.5 | 433.5 | 6.5 | 3.8 | 46.9 | 10.2 | 8.4 | 359.9 | 11.2 | 7.5 | 315.0 | 9.5 | 7.0 | 232.8 | 19.2 | 18.5 | 693.6 | 5.5 | 5.5 | 83.2 | 5.2<br>4.8 | 4.6<br>4.5 | 103.6 |
| 14 | 15.5 | 8.8 | 600.2 | 7.0 | 3.5 | 42.9 | 11.4 | 9.0 | 461.7 | 11.8 | 7.3 | 314.4 | 9.6 | 7.0 | 232.2 | 19.7 | 8.5 | 711.7 | 4.7 | 3.9 | 35.7 | 4.5<br>4.5 | 4.5<br>4.5 | 91.1 |
| 18 | 18.3 | 10.0 | 915.0 | 5.3<br>2.5 | 3.3<br>2.5 | 36.7 | 13.5 | 10.5 | 744.2 | 14.3 | 7.5 | 402.2 | 13.5 | 7.5 | 385.3 | 20.0 | 11.0 | 1210.0 | 5.5 | 4.5 | 55.7 | 5.5<br>5.0 | 5.2<br>3.5 | 105.0 |
| 21 | 20.9 | 11.7 | 1430.5 | 5.6<br>3.2 | 3.3<br>2.5 | 40.5 | 14.5 | 10.8 | 845.6 | 16.4 | 8.8 | 635.0 | 13.8 | 7.6 | 398.5 | 21.0 | 11.3 | 1340.7 | 6.1 | 5.1 | 79.3 | 11.0 | 6.0 | 198.0 |
| Tumour mass (g) | 0.66 | | | 0.01 | | | 0.62 | | | 0.30 | | | 0.15 | | | 1.00 | | | 0.01 | | | 0.03 | | |

EPO cont.:

Experiment 3

| No. of days following treatment | Tumour measurements | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPO (n=19) | | | | | | | | | | | | | | | | | |
| | 16 | | | 17 | | | 18 | | | 19 | | | Mean vol. ($mm^3$) n=19 | SEM |
| | L (mm) | θ (mm) | Vol ($mm^3$) | L (mm) | θ (mm) | Vol ($mm^3$) | L (mm) | θ (mm) | Vol ($mm^3$) | L (mm) | θ (mm) | Vol ($mm^3$) | | |
| 0 | 6.3 | 4.3 | 58.2 | 4.5 | 4.5 | 45.6 | 5.8 | 5.0 | 72.5 | 7.9 | 6.3 | 156.8 | 89.4 | 14.5 |
| 4 | 7.5 | 5.3 | 103.9 | 4.4 | 4.3 | 40.7 | 6.3<br>4.4 | 5.0<br>4.4 | 121.3 | 9.5 | 6.0 | 171.0 | 141.8 | 23.6 |
| 7 | 7.8 | 5.7 | 126.7 | 6.0 | 5.0 | 75.0 | 6.0<br>4.5 | 6.0<br>4.5 | 153.6 | 13.0 | 8.5 | 469.6 | 208.5 | 40.5 |
| 11 | 14.5<br>3.0 | 9.5<br>3.0 | 667.8 | 7.5 | 6.0 | 135.0 | 6.5 | 6.0 | 117.0 | 17.5 | 10.0 | 875.0 | 416.8 | 72.8 |
| 14 | 15.9<br>3.0 | 11.0<br>3.0 | 975.5 | 8.6 | 5.5 | 130.1 | 7.8 | 5.8 | 131.1 | 19.2 | 10.5 | 1058.4 | 499.7 | 97.8 |
| 18 | 18.5 | 13 0 | 1563.3 | 11.0 | 6.5 | 232.4 | 9.7 | 8.5 | 350.4 | 22.0 | 13.0 | 1859.0 | 836.2 | 185.0 |
| 21 | 21.4 | 16.1 | 2773.5 | 12.4 | 8.0 | 396.8 | 11.4 | 9.5 | 514.4 | 22.8 | 13.0 | 1926.6 | 1094.0 | 220.7 |
| Tumour mass (g) | 1.10 | | | 0.24 | | | 0.23 | | | 1.38 | | | | |

## PUBLICATIONS EMANATING FROM THIS STUDY

Ramchurren, N., Botha, J.H. & Leary, W.P. (1985). An investigation into the effects of gamma linolenic acid on murine sarcoma M52B. South African Journal of Science 81:331.

Ramchurren, N. & Botha, J.H. (1985). Spontaneous remission of M52B sarcomas growing in immunocompetent mice. ARK 7 (2):22-24.

Ramchurren, N., Bux, S. & Robinson, K.M. (1985). Morphological characterization of a continuous murine sarcoma line. Proceedings of the Electron Microscopy Society of Southern Africa. 15: In Press.

Ramchurren, N., Botha, J.H., Robinson, K.M. & Leary, W.P. (1985). Effects of gamma linolenic acid on murine cells in vitro and in vivo. South African Medical Journal 68: In Press.

## An investigation into the effects of gamma linolenic acid on murine sarcoma M52B

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Addition of certain fatty acids to cultured tumour cells has been shown to influence growth and morphology significantly [Abbas, Yoo and Viles (1982). *Cancer Res.* 42, 4639 – 4649; Gaspar, Alaniz and Brenner (1975). *Lipids* 10, 726 – 731]. The present study tested the effects of gamma linolenic acid (GLA) on the murine sarcoma M52B.

Initially, this sarcoma, about which little is known, was characterised. The cell line was found to be tumourigenic in newborn BALB/c mice. Histology revealed a spindle cell fibrosarcoma with a herringbone pattern. For *in vitro* studies, cells ($2 \times 10^6$ per flask) were cultured in Eagle's minimum essential medium supplemented with 10% foetal calf serum. The effects of GLA in sodium carbonate were assessed in two ways. In the first experiment single doses of either 50 or 100 $\mu$g/ml GLA were administered. In the second study each of five flasks of cells were exposed daily to fresh medium and 10, 20, 30, 40 or 50 $\mu$g/ml GLA respectively. Control flasks for both experiments received $Na_2CO_3$ only.

All cells which received a dose of 100 $\mu$g/ml GLA had died by 48 hours. A single exposure to 50 $\mu$g/ml produced a similar effect after 96 hours, while at 48 hours these cells had stopped dividing and had accumulated dense paranuclear granules. When GLA was administered daily its influence on cells increased with time. These time dependent changes appeared to be dose related, the most pronounced effects being observed with the higher concentrations of the fatty acid. Growth rates and morphology were consistently unaffected by the addition of $Na_2CO_3$.

An *in vivo* study paralleling the *in vitro* investigation is at present in progress. Two groups of BALB/c mice with tumours of the M52B line are receiving normal and GLA-supplemented diets. Preliminary results appear to conflict with those of the *in vitro* study.

ARK 7 (2), 22-24, August 1985.

# SPONTANEOUS REMISSION OF M52B SARCOMAS GROWING IN IMMUNOCOMPETENT MICE

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## INTRODUCTION

While the availability of athymic mice has made the study of tumor xenografts from a range of different hosts possible, the defective cell mediated immunity and altered B lymphocyte function of these mice (1) preclude investigations into the importance of the immune system in the malignant condition and its relationship to invasion and metastasis. Immunocompetent hosts would therefore be more suitable for such studies and for others involving manipulation of the immune system. In fact, virus producing allografts have been grown in immunocompetent chickens (2),guinea fowls (3),turkeys (3) and mice (4,5,6,7) but, in all these studies a significant incidence of spontaneous tumour remission has been reported. It has however been proposed that spontaneous remission of murine allografts growing in immunocompetent mice could be minimised by the use of suitable highly inbred strains (8).

The present study therefore aimed to establish such a system with the murine sarcoma (MuS) and leukemia (MuL) virus producing line, M52B. This line has previously been grown in an outbred Swizz altino (Onderstepoort strain) and athymic BALB/c mice (8). Although approximately 10% - 20% of tumours induced in the former group regressed spontaneously the number of animals remaining was still large enough to make further investigations feasible (8). Attempts were made to reproduce a similar model locally using inbred BALB/c mice obtained from the Natal Institute of Immunology (Pinetown). In addition, inbred Boizze, randombred BALB/c and outbred mice of the Onderstepoort strain strain were included in the study to compare the growth of M52B cells in mice of these genetically differing backgrounds.

## MATERIALS AND METHODS

Immunocompetent mice of different strains and breeding were used to test the tumorigenicity of M52B cells in vivo. Randombred, pregnant BALB/c mice were obtained from the animal colony, University of Natal Medical School. Inbred, pregnant BALB/c and Biozze mice were purchased from the Natal Institute of Immunology, Pinetown, while outbred Swizz mice of the Onderstepoort strain strain were obtained from Dr. D. W. Verwoerd, Veterinary Research Institute, Onderstepoort. In addition, athymic BALB/c mice obtained from Dr. K. Robinson, University of Natal, were used so that tumorigenic properties of M52B cells in mice of different immune status could be compared.

Immunocompetent mice were maintained under conventional temperature and humidity conditions while athymic mice were housed in an SPF-4 environment.

Tumours were induced in newborn immunocompetent mice, prior to full development of their immune systems, and in weaned (4-6 weeks old) nude mice by the subcutaneous inoculation of approximately $1 \times 10^6$ (6) M52B cells in 0.1mℓ minimum essential medium (MEM,FLOW). Mice were injected in the flank region with a 25 gauge needle.

Lengths and widths of tumours were measured with Vernier calipers at 2 or 3 day intervals. Tumours were palpated regularly to assess spontaneous remission.

## RESULTS

Results of this investigation are summarised in Table 1. As anticipated, take rates were highest in immunodeficient nude mice. In immunocompetent mice, take rates were highest in the inbred strains which were also characterised by the shortest latent periods. Spontaneous tumour remission, though not observed in nude mice, was a frequent occurrence in all strains of immunocompetent mice, being greatest in inbred mice. Although take rates and latent periods were similar in inbred BALB/c and Biozze mice, remission rates differed greatly between the two, being 66% and 32% respectively. Therefore, in immunocompetent mice used in this study, M52B tumours grew most satisfactorily in the Biozze strain i.e. high take rate, short latent period and relatively low percent remission.

## DISCUSSION

Attempts to establish persistant M52B sarcomas in different strains of random-bred, inbred and outbred immunocompetent mice were not entirely successful as approximately 38% of all induced tumours regressed spontaneously within two weeks of first appearance. Highest take rates and shortest latent periods were observed in inbred strains, but unexpectedly, tumour remission was greatest in these mice. Therefore this finding suggests that tumour remission may be unrelated to both take rate and latent period, although such an association has been previously demonstrated in chickens (2). Furthermore, the use of certain inbred strains did not preclude the occurrence of remission although there was some association between genetic background and remission rates as fewest tumours underwent remission in the inbred Biozze strain. Therefore, of all the immunocompetent mice used in this investigation, the Biozze were found to be the most suitable hosts for further studies involving the M52B sarcoma.

In contrast to the findings with immunocompetent mice, all M52B sarcomas induced in athymic nude mice grew progressively thus confirming an association between inhibition of tumour growth and cell mediated immunity.

In conclusion, in studies where immunologically compromised mice would be unsuitable hosts for M52B tumours, it seems that the inbred Biozze strain from the Natal Institute of Immunology would be the most suitable hosts for investigations into behaviour and biological properties of these tumours.

## REFERENCES


1. Pantelouris EM (1968). Nature 217 : 370.

2. Dinowitz M, Ratin H (1966). J.Natl.Cancer Inst. 36: 189.

3. Andrews C. (1970). Viruses and Cancer. Weidenfeld and Nicolson (London) Great Britain, Chaucer Press.

4. Fefer A, McCoy JL, Glynn JP (1967). Cancer Res 27,1626.

5. Law LW, Ting RC, Stanton MF (1968). J.Natl.Cancer Inst. 40: 1101.

6. Siegler R (1970). J.Natl.Cancer Inst. 45: 135.

7. Rockwell S. (1980). Br. J. Cancer 41: 118.

8. Verwoerd D.W. Veterinary Research Institute, Onderstepoort, Personal Communication.



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TABLE 1 : TUMORIGENICITY OF M52B CELLS IN IMMUNOCOMPETENT AND NUDE MICE

| | IMMUNOCOMPETENT MICE | | | NUDE MICE | |
|---|---|---|---|---|---|
| | BALB/c | | BIOZZE | ONDERSTE-POORT STRAIN | BALB/c |
| | randombred | inbred | inbred | outbred | inbred |
| No. of mice inoculated | 83 | 70 | 52 | 47 | 53 |
| No. of mice with tumours | 17 | 36 | 28 | 12 | 49 |
| Take rate (percent) | 20 | 51 | 53 | 25 | 92 |
| Latent period (days) | 26 | 12 | 15 | 19 | 18 |
| Tumour remission: | | | | | |
| x week after latent period: | | | | | |
| Week 1 x = 1 | 28% | 38% | 25% | 25% | 0% |
| Week 2 x = 2 | 28% | 66% | 32% | 25% | 0% |
| Week 3 x = 3 | 35% | 100% | * | * | 0% |

* Study terminated before three weeks

*Proceedings of the Electron Microscopy Society of Southern Africa 1985, In Press.*

# MORPHOLOGICAL CHARACTERISATION OF A CONTINUOUS MURINE SARCOMA LINE

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Cells of the continous M52B murine sarcoma line, which produces both murine sarcoma (MuS) and leukaemia (MuL) viruses and transplants efficiently in BALB/c mice, were obtained from Dr. D. Verwoerd (Onderstepoort). As relatively little was known about the M52B cells, this study was undertaken to characterise the line with respect to tumorigenicity and growth and morphology in vivo and in vitro.

All M52B cultures were initiated, using a dry explant technique[1], from subcutaneous solid sarcomas growing in immunocompetent BALB/c mice. Cells were maintained in Eagle's minimum essential medium (containing 10% foetal calf serum and antibiotics) and were inoculated into mice of different genetic backgrounds to assess tumorigenicity and examine histology. Cultured cells were viewed daily by phase-contrast microscopy. Semi-confluent cultures were prepared for transmission electron microscopy according to the method of Robinson and Gregory[2].

Tumours (induced in all mice) were anaplastic sarcomas which consisted of pleomorphic compactly arranged spindle cells disposed in herringbone patterns. Cultured cells were spindle shaped with long cytoplasmic processes. Ultrastructural investigation of M52B cells revealed, in addition to the prominent nuclei and nucleoli observed by phase-contrast microscopy, the presence of short microvilli, sparse mitochondria, well developed endoplasmic reticulum with annulate lamellae, golgi apparatus, myelin bodies and viral particles (Fig. 1).

Spherical enveloped virus particles with diameters of approximately 95nm (Fig. 2) were frequently observed budding from the cell surface (Fig. 3). Two different enveloped forms were observed, having either electron lucent (20% occurrence) or dense (80% occurrence) cores (Fig. 4). Non-enveloped, dense structures differing in size and morphology were seen in association with microvilli (Fig. 2). Approximate diameter of these particles was 47nm. Since core diameters of the larger putative viruses were similar to diameters of the non-enveloped particles, the latter were tentatively identified as cores of the larger viruses. All non-enveloped particles were dense and therefore thought to be related to the larger dense virus particles.

Although the MuS and MuL viruses have been reported to be morphologically indistinguishable, the identification of 2 types of particles in this study makes it tempting to assume that these forms represent the sarcoma and leukaemia viruses. However, positive identification of each type of particle can only be achieved by more direct experimentation, possibly involving biochemical assay[3].

References:


1. Robinson, K.M., Haffejee, A. and Angorn, I.B. (1980) Clinc. Oncol. 6, 125.
2. Robinson, K.M. and Gregory, M.A. (1977) Proc. Electron Microsc. Soc. South Afr. 7, 125.
3. Gross, L. (1983) Oncogenic viruses, Great Britain, Pergamon Press, 561.



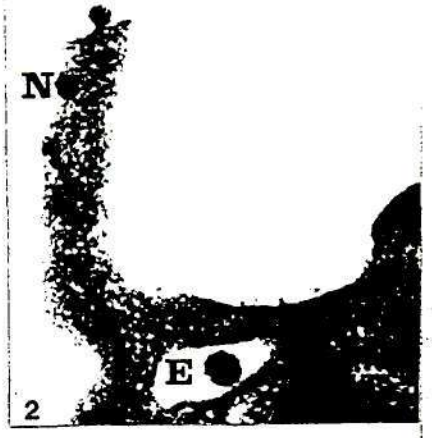





Fig. 1 Cultured M52B cells showing nuclei (N) endoplasmic reticulum (ER), mitochondria (M) and viral particles (V).
Fig. 2 Enveloped (E) and non-enveloped (N) viral particles.
Fig. 3 Enveloped viral particles (E) budding from cell surface.
Fig. 4a Electron lucent enveloped virus particle.
Fig. 4b Electron dense enveloped virus particle.

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# EFFECTS OF GAMMA LINOLENIC ACID ON MURINE CELLS IN VITRO AND IN VIVO

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SUMMARY

The effects of gamma-linolenic acid (GLA) on growth of cells of the continuous murine sarcoma line M52B were investigated *in vitro*. Prostaglandin (PG) production by these cells following GLA treatment was also measured. GLA inhibited the growth of M52B cells and became overtly toxic at high doses or after long periods of exposure to lower doses. The inhibitory effects of GLA were accompanied by an increase in PGE production by M52B cells. However, the rise in PGE was not statistically significant. Accordingly the extent to which PGE may contribute to the inhibition observed with GLA remains unclear. In order to establish whether these *in vitro* effects could be reproduced *in vivo*, athymic nude mice bearing murine sarcoma allografts were fed either standard laboratory chow or chow supplemented with 35% evening primrose oil (EPO) which contains 10% GLA. As there was no significant difference in tumour volumes between the two groups at the end of the treatment period, the oil enriched diet was concluded to be without effect on tumour growth in this *in*

vivo model.

## INTRODUCTION

Recent studies have shown that the proliferation of various human and murine tumour lines in vitro can be inhibited by the addition of gamma-linolenic acid (GLA) to the culture medium (1,2,3,4). Attempts by other workers to reproduce these effects in nude mice bearing human tumour xenografts (5) have been unsuccessful, though GLA in the form of evening primrose oil (EPO) has been reported to suppress growth of allografts in rat hosts (6). It has been suggested that the enzyme $\Delta$-6-desaturase which is responsible for the conversion of linoleic acid (LA) to GLA might be deficient in malignant cells (Fig. 1). Since GLA is a prostaglandin (PG) precursor, inadequate conversion of LA to GLA would result in decreased PG production, particularly $PGE_1$. Provision of GLA to enzyme deficient malignant cells should bypass this blockade, increase $PGE_1$ synthesis and reverse cancer growth (7). This study was undertaken to: (1) investigate the in vitro effects of exogenous GLA on M52B

sarcoma cells;

(2) determine whether the addition of GLA in vitro significantly affects PGE production by M52B cells;

(3) assess the in vivo effects of dietary GLA in the form of EPO on the growth of M52B sarcomas in athymic nude (immunologically compromised) (8) and immunocompetent BALB/c mice and thus determine any influence of the immune status on host response to GLA.

## MATERIALS AND METHODS

### Murine sarcoma cells.

The murine sarcoma and leukemia virus producing M52B line was originally obtained from Dr. D. Verwoerd (Ondestepoort). Cells were grown in 25cm$^2$ Greiner tissue culture flasks in Eagle's mimimum essential medium (Flow) supplemented with 10% foetal calf serum (Flow) and antibiotics (MEM). Culture medium was replaced at 3-4 day intervals.

Cells of the M52B line were tumorigenic upon subcutaneous inoculation of newborn immunocompetent and weaned 4-6 week old

nude BALB/c mice. The sarcomatous nature of the tumours was confirmed by light and transmission electron microscopy.

Effects of GLA in vitro.

In vitro studies were conducted on dense (approximately 2-4 x $10^6$ cells per flask) and less dense (approximately 1,2 x $10^6$ cells per flask) cultures of M52B cells. The effects of GLA (Sigma, L2378) in 0.1M sodium carbonate ($Na_2CO_3$)(9) were assessed in two ways.

In the first experiment 4 groups of 4 flasks of cells at each density were exposed to a single dose of 5ml MEM containing either:

a) no additives

b) 0.1 ml $Na_2CO_3$

c) 50 or 100 ug/ml GLA

In the second experiment for cells at both densities, 7 flasks were exposed daily for 3 days to fresh MEM containing either:

a) no additives

b) 0.1 ml $Na_2CO_3$

c) 10, 20, 30, 40 or 50 ug/ml GLA

The entire experiment was performed twice. All cultures were viewed daily by phase contrast microscopy and observations recorded at the end of each experiment. Persisting cells were stained with 0.25% May-Grunwald (Gurr) in methanol followed by 5% Giemsa (Gurr) in water.

Radioimmunoassay (RIA) was performed to compare the amounts of PGE and PGF released into the medium by GLA treated and control cultures. The following procedure was repeated twice. Two groups of 3 flasks each were exposed once to 5ml MEM containing either 50ug/ml GLA or 0.1ml $Na_2CO_3$. Twenty-four hours later, 2ml samples of MEM from each flask (as well as control MEM which had not been in contact with cells) were adjusted with 0.1N citric acid to pH 3.5 and extracted 3 times into ethyl acetate. After drying under vacuum, samples were subjected to chromatography on silicic acid columns according to a modification (10) of the method of Mitchell et al (11). RIA of PGE and PGF was performed as described by Norman et al(12,13).

To calculate the amount of PGs released into the medium by cells, the control result obtained for MEM not exposed to cells was subtracted from all values. Recoveries were monitored with tritiated PGFM and were always greater than 75%.

Cells in all flasks, from which the medium was analysed for PGs were lysed osmotically in 0.1% KCl and assayed for protein content according to the method of Lowry et al(14). PG production was then expressed as ug/mg protein/24 hours. Since anti-$PGE_2$ and anti-$PGF_{2\alpha}$ antibodies cross-reacted significantly with $PGE_1$ and $PGF_{1\alpha}$ respectively (11), results are reported as PGE and PGF and no distinction is drawn between the 1 and 2 series.

Effects of GLA in vivo.

Tumours were induced by inoculating weaned nude and newborn immunocompetent BALB/c mice subcutaneously with approximately $10^6$ M52B cells in 0.1ml medium. Details of tumours established in BALB/c mice are represented in Table 1. Since tumour remission occurred repeatedly in immunocompetent BALB/c mice, it was only possible to determine effects of oral GLA on M52B cells in vivo

in nude BALB/c mice. EPO which contains 10% GLA (6) was used as a source of the fatty acid. Mice in the control group consumed standard laboratory chow (National Food Distributors) whereas that received by the experimental group was supplemented with 35% EPO (3.5% GLA). The approximate rate of food consumption was 10g per mouse per day. Diets were supplied ad libitum over 10 days. Tumour volumes were measured every 48hrs and at the end of the experiment after which tumours were excised and examined histologically.

RESULTS

In vitro Study

Results of the experiment in which M52B cultures were exposed to a single dose of either 50 or 100ug/ml GLA are summarised in Table II. Observed changes were related to both duration of exposure and concentration of the fatty acid. Prior to death, cells often accumulated refractile paranuclear granules the lipid nature of which were confirmed by staining with Scharlach R. The inhibitory and toxic effects of GLA on

growth of M52B cells in vitro were more pronounced in less dense cultures.

Results of the experiment in which M52B cultures were exposed to multiple doses of GLA are summarised in Table III. Once again, effects were more pronounced in less dense cultures and were also dose and time related in all cultures.

The amounts of PGE and PGF released into the medium by GLA treated and control M52B cultures are recorded in Table IV. Cells treated with GLA released more PGE and PGF into the medium than did controls. However, analysis of results using the Mann Whitney U test showed these differences to be statistically non-significant for both PGE and PGF on two-tailed tests.

In vivo Study

Results of the in vivo study investigating effects of oral GLA on M52B tumours are summarised in Table V and Fig. 2. Analysis of data using an unpaired Students t test showed differences in tumour volumes between control and treated groups to be statistically non-significant both prior to and at the end of the experiment.

DISCUSSION

Although growth of M52B cells *in vitro* was significantly influenced by GLA, growth of solid M52B sarcomas *in vivo* in athymic mice was apparently unaffected by the addition of GLA to the diet.

The observed effects of GLA on cell growth *in vitro* were concentration and time dependent and were more pronounced in less dense cultures, presumably as more GLA was available per cell. These findings are consistent with results of similar studies using human oesophageal carcinoma (1), hepatoma (2) osteogenic sarcoma (3) and mouse melanoma (4) cells. However, the mechanism by which GLA exerts its action remains obscure. Although the present study showed a rise in PGE production by M52B cells following GLA treatment this increase was found to be statistically non-significant and in addition it was not possible to determine whether it was due to $PGE_1$ or $PGE_2$. Since other workers have shown that addition of exogenous PGEs to cells in culture inhibits growth (15,16) the increase in PGE recorded in

the present study suggests that the actions of GLA may be partially mediated by PGE. However, the extent to which $PGE_1$ may be involved remains unclear.

It is possible that the effects of GLA on growth observed *in vitro* may also be related to a non-specific fat overload as most cells demonstrated excessive lipid accumulation prior to death. Such an effect would obviously manifest more markedly at higher concentrations of the fatty acid.

While GLA has been found to consistently affect the growth of malignant cells *in vitro*, these effects have generally not been reproducible *in vivo* (5,17), although Ghayur *et al* (6) showed that tumour growth was inhibited by subcutaneous administration of EPO to rats bearing mammary allografts. Such results were not obtained in the present study using allograft bearing nude mice. Possible reasons for the conflicting findings may include the following. It is known that the rat has a more active $\Delta$-5-desaturase than the mouse (18) and hence would convert exogenous GLA preferentially to $PGE_2$ (Fig. 1). The findings of Ghayur *et al*(6) would then further reinforce the argument that

$PGE_1$ alone is not responsible for the inhibitory effect of GLA upon the growth of malignant cells. Since nude mice have defective cell mediated immunity (8) and prostaglandins are known to affect the function of T cells (19), it is not unlikely that GLA would produce different effects in animals of different immune status. In addition, metabolic differences between the allografts, i.e. a sarcoma and a mammary carcinoma (6) may influence the effects of the fatty acid. Furthermore, administration of dietary rather than parenteral EPO might have resulted in inadequate delivery of GLA to the tumour site as fats undergo metabolism after oral administration. However, since oral EPO was supplemented to the maximum tolerated extent and produced no effect, it would seem that in a clinical situation oral doses which would be practical may be ineffective.

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REFERENCES


1. Leary WPP, Robinson KM, Booyens J, Dippenaar N. Some effects of gamma-linolenic acid on cultured human oesophageal carcinoma cells. S Afr Med J 1982; 62: 681-683.
2. Dippenaar N, Booyens J, Fabbri D, Engelbrecht P, Katzeff IE. The reversibility of cancer : Evidence that malignancy in human hepatoma cells is gamma-linolenic acid deficiency-dependant. S Afr Med J 1982; 62: 683-685.
3. Booyens J, Dippenaar N, Fabbri D, Engelbrecht P. The effect of gamma-linolenic acid on the rate of proliferation of human osteogenic sarcoma and oesophageal carcinoma cells in culture. S Afr Med J 1984; 65: 240-242.
4. Dippenaar N, Booyens J, Fabbri D, Katzeff IE. The reversibility of cancer : evidence that malignancy in melanoma cells is gamma-linolenic acid deficiency-dependant. S Afr Med J 1982; 62: 505-509.
5. Botha JH, Robinson KM, Leary WPP. Parenteral gamma-linolenic acid administration in nude mice bearing a range of human tumour xenografts. S Afr Med J 1983; 64: 11-12.
6. Ghayur T, Horrobin DF. Effects of essential fatty acids in the form of evening primrose oil on the growth of the rat R3230 AC transplantable mammary tumour. IRCS Med Sci 1981; 9: 581.
7. Horrobin DF. The reversibility of cancer : the relevance of cyclic AMP, calcium, essential fatty acid and prostaglandin $E_1$. Med Hypothesis 1980; 6: 469-486.

8. Pantelouris EM. Absence of thymus in a mouse mutant. Nature 1968; 217: 370.

9. Ingerman-Wojenski C, Silver MJ, Smith B, Nisserbawm M, Sedar AW. Prostacyclin productions in rabbit arteries in situ: inhibition by arachidonic acid induced endothelial cell damage. Prostaglandins 1981; 21: 655-664.

10. Botha JH, Robinson KM, Ramchurren N, Reddy K, Norman RJ. Human esophageal carcinoma cell lines: Prostaglandin production, biological properties and behaviour in nude mice. In press.

11. Mitchell MD, Flint AP, Bibby J, Brunt J, Arnold JM, Anderson ABM, Turnbull AC. Plasma concentrations of prostaglandins during late human labour : influence of normal and preterm labour. J Clin Endocrinol Metab 1978; 46: 947-951.

12. Norman RJ, Bredenkamp BLF, Joubert SM, Beetar C. Fetal prostaglandin levels in twin pregnancies. Prostaglandins and Med 1981; 6: 309-316.

13. Norman RJ, Joubert SM. Fetal urinary prostaglandins. Prostaglandins, Leukotrienes and Med 1982; 8 (2): 171-172.

14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin Phenol reagent. J Biol Chem 1951; 193: 265-275.

15. Santoro MG, Philpott GW, Jaffe BM. Inhibition of tumour growth in vivo and in vitro by prostaglandin E. Nature 1976; 263: 777-781.

16. Thomas DR, Philpott GW, Jaffe BM. The relationship between concentrations of prostaglandin E and rates of cell replication. Exp Cell Res 1974; 84: 40-46.

17. Booyens J, Koenig L. The effect of dietary supplementation with gamma-linolenic acid on the growth of induced tumours in rats. S Afr Med J 1984; 65: 660-661.

18. Stone KJ, Willis AL, Hart M, Kirtland SJ, Kernoff PBA, McNicol GP. The metabolism of dihomo- -linolenic acid in man. Lipids 1979; 14(2): 174-180.

19. Horrobin D F. The regulation of prostaglandin biosynthesis: Negative Feedback mechanisms and the selective control of formation of 1 and 2 series prostaglandins: Relevance to inflammation and immunity. Med Hypotheses 1980; 6: 687-709.

LIST OF FIGURES:

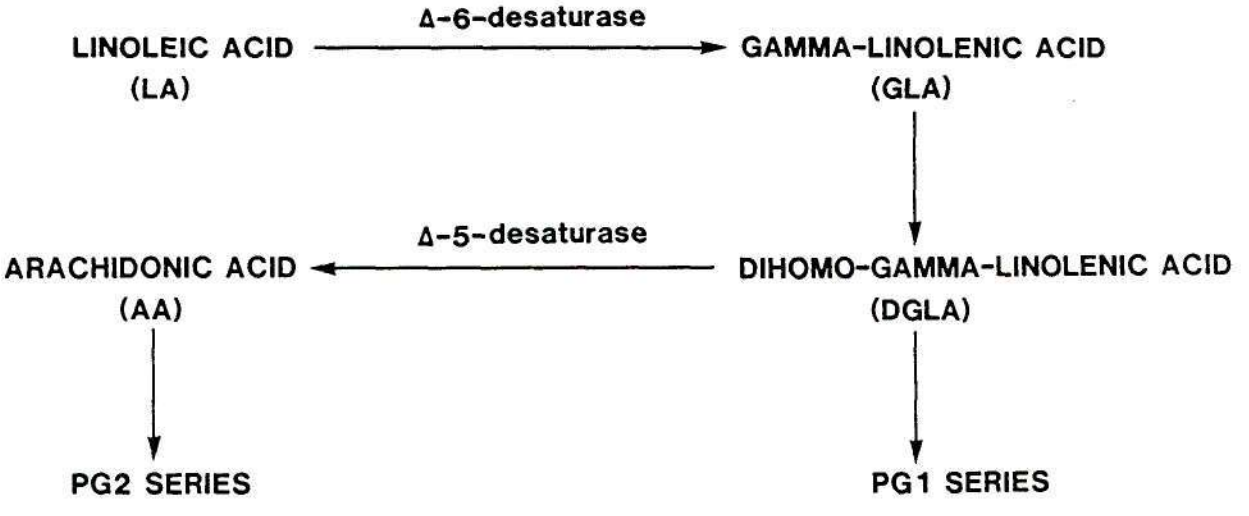


Fig. 1 : Formation of prostaglandins from linoleic acid.

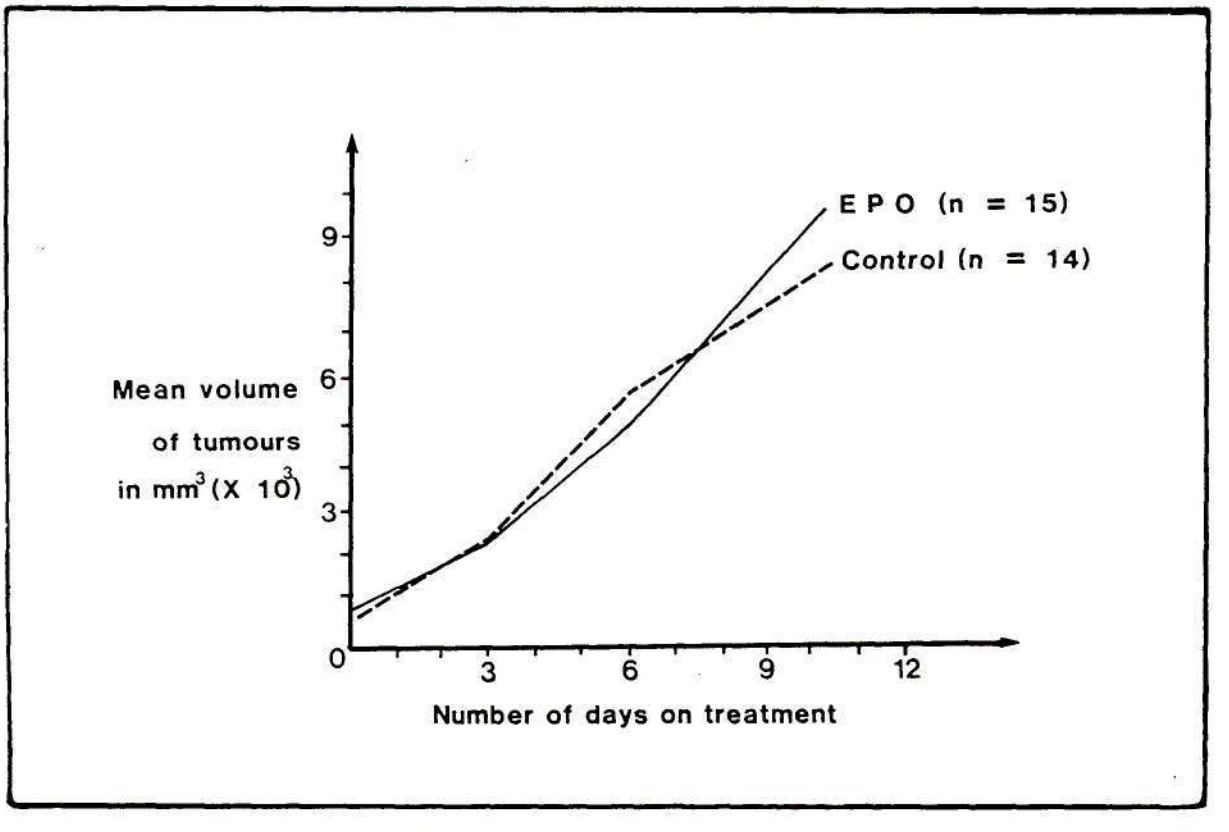


Fig. 2 : Tumour volumes in nude BALB/c mice fed control or EPO supplemented diets.

LIST OF TABLES :

Table 1: Tumorigenicity of M52B cells in BALB/c mice

| | BALB/c mice | |
|---|---|---|
| | Athymic | Immunocompetent |
| Number of mice injected | 53 | 153 |
| Latent period (days) | 17 | 15 |
| Take rate (%) | 92 | 33 |
| Remission of tumours by 25 days (%) | 0 | 100 |

Table II : Effects of a single dose of GLA on M52B cultures

| Time after addition of GLA | Density of cultures | Single doses of GLA 50ug/ml | 100ug/ml |
|---|---|---|---|
| 24 hours | Dense | *nmc | +png in approximately 50% cells |
| | Less dense | approximately 90% cells dead | 100% cells dead |
| 48 hours | Dense | +png in approximately 50% cells | 100% cells dead |
| | Less dense | 100% cells dead | 100% cells dead |

Control cultures were morphologically unaltered at the end of the experiment.

*nmc = no obvious morphological changes

+png = accumulation of paranuclear granules

Table III : Effects of multiple doses of GLA on M52B cultures

| Time after addition of GLA | Density of cultures | Multiple doses of GLA 10ug/ml | 20ug/ml | 30ug/ml | 40ug/ml | 50ug/ml |
|---|---|---|---|---|---|---|
| 24 hours | Dense | *nmc | *nmc | *nmc | *nmc | *nmc |
| | Less dense | +png in approximately 35% cells | +png in approximately 60% cells | +png in approximately 85% cells | +png in approximately 90% cells | +png in approximately 90% cells |
| 48 hours | Dense | +png in approximately 40% cells | +png in approximately 50% cells | +png in approximately 70% cells | approximately 80% dead cells | 100% cells dead |
| | Less dense | approximately 60% cells dead | approximately 80% cells dead | 100% cells dead | 100% cells dead | 100% cells dead |
| 72 hours | Dense | approximately 60% cells dead | approximately 80% cells dead | 100% cells dead | 100% cells dead | 100% cells dead |
| | Less dense | 100% cells dead | 100% cells dead | 100% cells dead | 100% cells dead | 100% cells dead |

Control cultures were morphologically unaltered at the end of the experiment.

*nmc = no obvious morphological changes

+png = accumulation of paranuclear granules

Table IV: Amounts of prostaglandins (PGs) released into the medium by control and GLA treated M52B cultures

| | M52B Cultures | |
|---|---|---|
| | Control | GLA treated |
| PGs (ug/mg protein/24hr) | | |
| PGE | | |
| median | 0,39 | 0,59 |
| range | 0,18 - 0,64 | 0,34 - 0,91 |
| PGF | | |
| median | 0,02 | 0,03 |
| range | 0,01 - 0,05 | 0,01 - 0,05 |

Table V: M52B tumour volumes in nude BALB/c mice receiving EPO and control diets before and after the treatment period

| | Tumour Volume Mean ± SEM ($mm^3$) | |
|---|---|---|
| | Before treatment | After treatment |
| Control (n=14) | 662 ± 737 | 8464 ± 1718 |
| EPO (n=15) | 911 ± 357 | 10025 ± 2194 |