EFFECTS OF VITAMIN A ON TUMOUR AND UNTRANSFORMED CELLS

by

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CHAPTER 1

INTRODUCTION

All trans-vitamin A alcohol or all trans-retinol (1), of molecular weight 286,46 Da, is an essential lipid soluble nutrient (2,3). Vitamin A was first discovered in eggs, milk, butter and fish liver oils (2-5), and in plants (leafy green vegetables, carrots, yellow or orange coloured fruits) in the form of the provitamin, β-carotene (1,3-7). The primary source of vitamin A is carotenoids, synthesised in plants and enzymatically converted to vitamin A in mammals (3,8). Vitamin A requirements are expressed in International Units (IU), and 1,0 IU of vitamin A is supplied by (1,5,7):

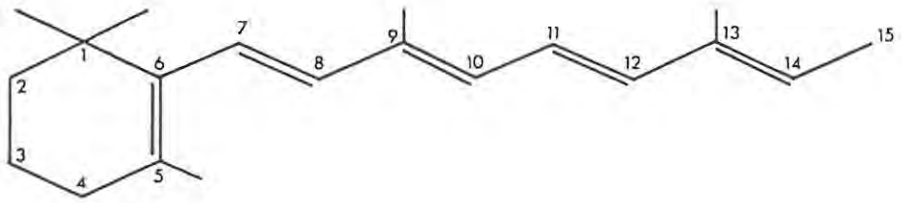
0,30 μg of all trans-vitamin A alcohol

0,344 μg of all trans-vitamin A acetate

0,55 μg of all trans-vitamin A palmitate

1,80 μg (in humans) (5) or 0,6 μg (in rats) (1,5) of all trans-β-carotene (based on the assumption that only one-third of dietary β-carotene is available for human use and only one-half of "usable" carotene is converted to vitamin A (5).)

Naturally occurring oxidation products of vitamin A alcohol are all trans-retinal (vitamin A aldehyde) and all trans-retinoic acid (vitamin A acid) (3,5). These three compounds have a common structure, (indicated in figure 1), which includes a cyclic end group (the trimethyl cyclohexenyl ring), a dimethyl-substituted all trans-tetraene chain and a polar hydroxyl, aldehyde or carboxyl end group (3,9,10).



cyclic end group

polyene chain

polar end group
$-CH_2OH$ = retinol
-CHO = retinal
-COOH = retinoic acid

FIGURE 1. Basic structure of retinoids and nomenclature of three natural vitamin A compounds (3).

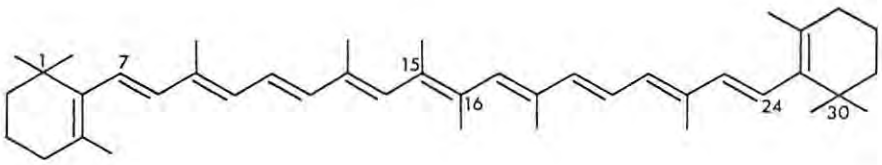


FIGURE 2. Structure of β-carotene (C30), the precursor of vitamin A (1).

Each of the three regions of the retinoid molecule can be chemically modified in a number of ways, resulting in an unlimited number of chemical analogues, known as retinoids (3,10-13). These synthetic analogues may share all or some of the biological activities of the naturally occurring vitamin A compounds (8,9,11). The analogues may be less toxic, yet more potent to biological systems than the natural compounds (9,10,12-16) while others have no vitamin A activity indicating certain specific structural requirements for activity (3,8,9).

Dietary vitamin A may be ingested in the form of long chain fatty acyl esters of retinol or as the provitamin β-carotene (3,8,11,17) (figure 2). The retinyl esters are hydrolysed to free retinol by pancreatic or brush border esterases before being absorbed by the intestinal mucosal epithelium (1,3,5,6,8,11,18). β-carotene, however, is absorbed and then enzymatically cleaved to yield two molecules of retinal, which are converted to retinol (1-3,11,17-19). Once inside the intestinal epithelial cell, the retinol is esterified with long chain fatty acids, primarily palmitate (1,3,5,8,11,18-20), to form retinyl esters which then pass into the lymphatic system, are transported as chylomicrons in the blood and are taken up by the liver for storage (1,3,8,11,18,20).

Mobilisation of the vitamin A from the liver stores requires hydrolysis of the retinyl esters and the conjugation of the free retinol with a retinol binding protein (zinc-dependent (21-24)) which is produced by the liver (21,25-29). This conjugate is then released into the blood where it binds to the protein transthyretin

(8,18,29,30) (prealbumin) to form a ternary complex (25,27-29) which transports retinol to target organs and cells as required. Normal nourished animals maintain a relatively constant plasma level of 30 - 60 µg/dl (2,11,22-24,31-35) (1 - 2 µM) retinol, by an unknown mechanism of regulation.

The alcohol group of retinol can be reversibly oxidised to retinal (33,36,37) by retinol dehydrogenase (18), and further oxidised (irreversibly) to retinoic acid (3,5,18), depending on the type of vitamin A compound required. Retinoic acid, however, may only replace the requirement for either retinol or retinal in certain specific functions (3,14,18).

Once inside the cell, retinol and retinoic acid can participate in a variety of metabolic reactions. These depend on the cell type. Some of these reactions occur in many cell types while others occur only in specific cells with retinol and retinoic acid associated with specific enzymes or proteins (3).

Both retinol and retinoic acid can be converted to glucuronides by the action of glucuronyl transferase, in the presence of UDP-glucuronic acid. The glucuronides are polar and water soluble, and form the majority of excreted metabolites of vitamin A compounds (1-3,17,18).

Retinol and retinoic acid form a complex with their respective cellular binding proteins (two distinct proteins, one specifically for each vitamin A compound (1,3,8,11,38,39) and differing from plasma

RBP (1,8,11,26,40)). The complex may then be translocated into the nucleus in the case of cellular retinoic acid binding protein (3,11,41), where the retinoic acid then binds to the chromatin (1,42), whereas cellular retinol binding protein may bind to the nucleus (11,42,43) and release the retinol for binding to specific binding sites on the chromatin (1,3,26,43-45). Modification or regulation of gene expression may result from this binding. In other words, the potential exists to activate or suppress the expression of genes by the interaction of vitamin A with the nucleus (10,26,41-43), and of particular interest is the interaction of vitamin A with the nucleus in neoplastic tissue.

Initial experimental approaches in the study of vitamin A metabolism were primarily concerned with experimental animals fed vitamin A deficient or excess diets. In 1925, Wolbach and Howe (cited in 14) reported that the columnar epithelium of many tissues was replaced by rapidly multiplying squamous keratinising epithelial cells in vitamin A-deficient rats. The restoration of a normal vitamin A-containing diet fully reversed these effects. In 1926, Fujimaki (cited in 46) found that rats on a vitamin A-deficient diet had a higher incidence of spontaneous gastric carcinomas. This work could not be repeated in succeeding years, due to the difficulty in working with spontaneous tumours that occur at very low frequency. This led to the consequent introduction of chemical carcinogens to produce tumours with a high frequency in experimental animals, thus enabling a relationship between vitamin A and cancer to be more clearly shown.

It appeared that vitamin A metabolism may be altered in tumours, as very little or no vitamin A was found in malignant tissue when compared to tissue of origin. The amount of retinol present also depended on the type of tumour and chemical carcinogen used for the induction of the tumour (11).

Contradictory results were obtained by early researchers when trying to relate different vitamin A levels (both deficiency and excess) to the incidence of various experimental tumours. More recently, there has been considerable evidence on the influence of various retinoids on the development of different epithelial tumours both in man and experimental animals.

In Table 1, experimental evidence indicating a relationship between vitamin A and cancer is summarised. The protective effects of the various retinoids against a number of epithelial tumours induced by a variety of carcinogens is emphasised by the data. A vitamin A deficiency, by comparison, results in increased growth of carcinogen-induced tumours. A number of studies have reported no substantial effect of retinoid supplementation and in some cases, adverse effects have been found (Table 1 and Table 2). This may be related to the dose of the carcinogen or of the retinoid, the type of retinoid, the dose schedule or the mode of administration of the retinoid. The reports may also indicate that certain types of tumour are refractory to the particular retinoid or to retinoids in general (13).

TABLE 1. The effect of different retinoids on the growth of epithelial tumours induced in experimental animals by a variety of carcinogens.

SITE	SPECIES	CARCINOGEN	RETINOID	EFFECT	REFERENCE
Skin	mouse	DMBA	R + anal	↓	cited in 11
	mouse	DMBA + TPA	RA + anal	↓	47
	rabbit	DMBA	RA	↓	cited in 11
	mouse	DMBA + croton oil	RA anal + aromatic Ret	↓	cited in 15
	rat	DMBA	R palm	↓	cited in 11
	mouse	DMBA	low vit A	↑	48
Lung	hamster	BP	R acet + anal	↓	cited in 2
	rat	3MC	R palm + R acet	↓	cited in 15
	rat	3MC	vit A def	↑	cited in 49
Mammary gland	rat	DMBA	R acet + Ret	↓	cited in 13
	rat	MNU	R acet + anal	↓	cited in 49
	rat	BP	R acet	↓	cited in 11
Bladder	rat	MNU	RA anal	↓	cited in 15
	rat	HO-BBN	RA anal	↓	cited in 49
	mouse	BBN	vit A + anal	↓	cited in 13
	rat	FANFT	RA	↓	cited in 11
	rat	FANFT	vit A def	↑	cited in 4
Stomach	hamster	DMBA	R palm	↓	cited in 2
	hamster	BP	R palm	↓	cited in 46
	rat	spontaneous	vit A def	↑	cited in 46
Colon	rat	DMH	RA	↓	cited in 49
	rat	aflatoxin	vit A def	↑	cited in 50
Cervix	hamster	DMBA	R palm	↓	cited in 2
	hamster	BP	R palm	↓	cited in 15
Liver	rat	DMAB	RA + anal	↓	48
	rat	aflatoxin	vit A def	↑	cited in 49
ADVERSE OR NO EFFECTS					
Colon	rat	MNU, DMH	vit A anal	N	cited in 11
Large bowel	rat	DMH	vit A anal	↑	cited in 4
Cheek pouch	hamster	DMBA	R acet + R palm	↑	cited in 2

↑ = increased growth, ↓ = decreased growth, N = no growth, R = retinol, RA = retinoic acid, Ret = retinoid, R acet = retinyl acetate, R palm = retinyl palmitate, anal = analogue, def = deficient.
DMBA = dimethylbenzanthracene
TPA = 12-O-tetradecanoylphorbol-13-acetate
BP = benzo(a)pyrene
3MC = 3-methylcholanthrene
MNU = N-methyl-nitrosourea
BBN = butyl-(4-hydroxy-butyl)nitrosamine
FANFT = N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide
DMH = 1,2-dimethylhydrazine
DMAB = 3'-methyl-4-dimethylaminoazobenzene

TABLE 2. The prophylaxis and therapy of human epithelial cancers by some retinoids.

SITE	RETINOID INTAKE	EFFECT	REFERENCE
Lung	incr vit A	↓	cited in 52
	low vit A	↑	cited in 51
Bladder	incr vit A	↓	cited in 53
	incr RA	↓	cited in 10
	low vit A	↑	cited in 7
Stomach	incr vit A	↓	cited in 4
	low vit A	↑	54
Cervix	incr vit A	↓	cited in 51
	low vit A	↑	cited in 11
Larynx	incr vit A	↓	cited in 52
	incr RA	↓	cited in 10
	low vit A	↑	cited in 7
Prostate	incr vit A	↓	cited in 7
	low vit A	↑	cited in 9
ADVERSE EFFECT			
Prostate	incr vit A	↑	cited in 52

↑ = increased growth, ↓ = decreased growth, RA = retinoic acid

Table 2 summarises the effects of retinoids on the prophylaxis and therapy of some human epithelial cancers. A low dietary vitamin A intake appears to result in increased tumour growth, while an increased dietary vitamin A intake or retinoic acid supplementation results in a decreased tumour growth. These natural retinoids, however, are limited in their usefulness as chemopreventative agents both in man and experimental animal due to inadequate tissue distribution or excessive toxicity (9,12,13,15,16). Deposition of excessive amounts of retinol and retinyl esters occurs in the liver, without a dose-response relationship in blood levels. Thus for chemoprevention of cancer, it is essential that research into synthetic modification of the retinoid molecule progresses, in order to overcome the limiting processes of the natural retinoids.

Significant advances in the study of vitamin A and cancer have occurred over the last few decades with the introduction of *in vitro* techniques. Initially, organ culture (prostate, trachea, mammary) was developed as an experimental tool, but this had a significant limitation in that mixed-cell populations were used, and thus replicate homogeneous samples were almost impossible to obtain. The introduction of cell culture methodology for the study of retinoid mechanism of action was a significant advancement, allowing molecular investigation into the role of retinoids in differentiation and carcinogenesis.

Continuous cell lines of both neoplastic and non-neoplastic (un-transformed) origin have been widely used and retinoids have been shown to suppress chemical carcinogenesis and control both cell

proliferation and cell differentiation in various cell culture systems. A more detailed discussion of the effects of retinoids on various cell lines is presented in chapter 2.

The aim of this study was to investigate the effect of vitamin A on the proliferation of untransformed and tumour cell lines cultured in our laboratory. In order to elucidate a possible site of action of vitamin A, ultrastructural examination of the cells was undertaken and in addition, the effect of vitamin A on a cell surface glycoprotein fibronectin was examined, since vitamin A is known to affect the structure and development of cell membranes.

CHAPTER 2

EFFECT OF VITAMIN A ON *IN VITRO* CELL PROLIFERATION

INTRODUCTION

The use of *in vitro* assay systems has, to a large extent, alleviated some of the problems associated with the study of retinoids in *in vivo* experimental systems. Such problems include the achievement of adequate tissue distribution, toxicity and the problem of distinguishing between specific and non-specific effects of retinoids (3,9). The external milieu in *in vitro* cell culture can be carefully controlled and hence effects due to specific additives in this environment can be determined with little non-specific interference.

In recent years, a number of different cell lines, both untransformed and malignant, have been employed in the study of the effects of various vitamin A compounds.

Lotan and Nicolson (55) undertook a study of the inhibition of proliferation of thirty-one different cell lines by retinoic acid and retinyl acetate, both at a concentration of 10^{-5}M. The cells (untransformed as well as chemically, virally and spontaneously transformed) included lymphoma, myeloma, sarcoma, neuroblastoma and carcinoma lines from a variety of species (rat, mouse, hamster and human). Retinoic acid was found to be more effective than retinyl

acetate in the growth inhibition of many of the cell lines, the degree of inhibition varying considerably from one cell line to another. Direct cytotoxicity was excluded as a means of inhibiting cell growth.

In another study, Lotan, Giotta, Nork and Nicolson (56) characterised the inhibitory effects of retinoic acid and retinyl acetate on the growth of two murine melanoma cell lines. They found that the inhibitory effects were dependent upon retinoid concentration (cytotoxicity excluded) and that both cell lines were more sensitive to retinoic acid than retinyl acetate, although one cell line was more sensitive than the other to both retinoids. The inhibitory effect of retinoid treatment could be reversed by replacing the retinoid-medium with control medium and the growth rate of the cells returned to that of the control cultures within 24 to 48 hours.

The inhibitory nature of these retinoids on murine melanomas (56) and rat mammary adenocarcinomas (55) prompted Lotan (57) to test the ability of retinoic acid to inhibit the growth of cell lines derived from human melanomas and breast carcinomas. Only two of six melanoma lines and two of four breast carcinoma lines (from human malignant tumours) showed inhibition due to retinoic acid addition. One non-malignant breast tissue cell line was not affected by retinoic acid while the other exhibited a slight (13%) inhibition. These results demonstrated a diverse response to retinoic acid amongst cell lines derived from tumours of similar histopathological type. Lotan suggested that each tumour may respond differently to retinoic acid therapy, and this could limit the use of such treatment to those tumours which are found to be sensitive to retinoic acid in vitro.

Dion, Blalock and Gifford (58) investigated the effect of retinoic acid on the growth of a transformed mouse L-cell line *in vitro* and found that the inhibition which resulted suggested the restoration of contact inhibition. Retinoic acid-treated L-cells ceased proliferation at cell densities which corresponded to those of confluent monolayers, while control cells continued proliferation to reach densities two to four times higher. Medium depletion was not a factor in the cessation of proliferation in the treated cultures as replenishment of medium failed to stimulate further proliferation. Control cells, however, continued proliferating exponentially on medium replenishment.

Patt, Itaya and Hakomori (59) reported that culturing hamster fibroblasts (NIL cells) and mouse fibroblasts (BALB/c 3T3 cells) in vitamin A-containing medium, enhanced contact orientation and cell-density dependent inhibition of cell growth. They also observed changes in the levels of a cell surface glycolipid and in LETS protein (large external transformation-sensitive protein, later called fibronectin - discussed in more detail in chapter 4).

Jetten, Jetten, Shapiro and Poon (60) characterised the action of retinoic acid on the mouse fibroblast cell lines MB4-7 and BALB/c 3T6 and 3T3, and the virus transformed derivative of 3T3 cells, 3T3SV. They showed that the retinoid addition inhibited growth, caused morphological changes and increased cell-to-substratum adhesiveness in the 3T6, 3T3 and MB4-7 cells, but that the 3T3SV cells remained unaffected. Their results also indicated an increase in LETS

glycoprotein (fibronectin) at the surface of the affected cells after retinoic acid treatment, which correlated well with the suggested role of this glycoprotein in increased adhesiveness. Treatment of the 3T6 and 3T3 cells with retinoic acid resulted in an increase in cell-associated glycosaminoglycan levels. There is a suggested relationship between glycosaminoglycans and external proteins such as the LETS protein, in that they form an extracellular complex responsible for cell-to-substratum adhesiveness. A retinoic acid binding protein was detected in the cytosol of 3T6 and 3T3 cells but not in 3T3SV cells, which suggested that the action of retinoic acid was mediated through this protein.

Shapiro and Poon (61) reported that retinoic acid induced alterations in cell growth, morphology and adhesion in a human intestinal epithelial line (407 cell line). Their observations, however, were uniquely different from those reported for non-epithelial cells. In this study, growth inhibition was dependent on cell density, with greatest inhibition at low cell density and diminished inhibition at confluency. Cells treated with the retinoid showed increased detachability, while control cells grew in tight clusters with maximum cell to cell contact, in direct contrast to retinoid treated cells. Glycosaminoglycan biosynthesis was inhibited by retinoic acid. The authors were unable to reconcile the differences between the intestinal 407 cells and the data on fibroblast cell lines, but suggested that an intrinsic difference exists in the response of the two cell types towards retinoids, possibly due in some way to epithelial cells being the "target" cells for retinoids <u>in vivo</u>.

With these studies providing the background information, an investigation of the effects of retinoid addition on the growth of tumour (human hepatoma and mouse melanoma) and untransformed (monkey kidney) cells was undertaken in this laboratory.

MATERIALS AND METHODS

REAGENTS

Sodium benzylpenicillin (Novo Industries (Pharmaceuticals) (Pty) Ltd. Johannesburg, South Africa) at 10^6 IU (600 mg) per vial was diluted to 100 ml with deionised water (together with streptomycin), to give a final concentration of 10 000 IU/ml.

Streptomycin sulphate (Novo Industries (Pharmaceuticals) (Pty) Ltd. Johannesburg, South Africa) at 10^6 µg/3 ml (per vial) was diluted to 100 ml with deionised water (together with penicillin), to give a final concentration of 10 000 µg/ml.

Trypsin subculturing solution, in a final volume of 1000 ml, contained : 8,0 g NaCl, 0,4 g KCl, 1,0 g D-glucose, 0,58 g $NaHCO_3$, 0,2 g EDTA, 0,0467 g trypsin (500 units/ml), 0,02 g phenol red, 10^5 IU penicillin, 10^5 µg streptomycin.

Phosphate buffered saline (Dulbecco's PBS without calcium or magnesium), in a final volume of 1000 ml, contained : 0,2 g KCl, 8,0 g NaCl, 1,15 g $Na_2HPO_4 \cdot 2H_2O$, 0,2 g KH_2PO_4.

Retinyl acetate stock solutions were prepared by diluting 380 µl retinyl acetate (Merck, Darmstadt, West Germany) (10^6 IU/g) to 10 ml with 95% ethanol to give a 100 mM solution. Further dilutions of this solution were prepared in 95% ethanol to give stock solutions of 10 mM, 5 mM and 1 mM retinyl acetate. 100 µl of stock solution was added to 100 ml culture medium to prepare final working solutions of 100 µM, 10 µM, 5 µM and 1 µM, each with a final ethanol concentration of 0,1% (vol:vol). The preparation of vitamin A solutions was carried out in the absence of artificial lighting and the solutions were maintained in dark brown bottles at 4°C. In each experiment, a control containing 100 µl 95% ethanol per 100 ml culture medium (0,1%) was prepared.

MEDIA

Eagle's basal medium (modified) (Flow Laboratories, Irvine, Scotland, United Kingdom) contained Hank's salts but no $NaHCO_3$. Each sachet was made up to a final volume of 10 l with Milli-Q water, to which 0,5 g ascorbic acid, 0,1 g serine, 0,06 g glycine and 7,5 g $NaHCO_3$ had been added. The media was sterile filtered through a millipore filtration unit (Millipore Corporation, Bedford, Massachusetts, USA) with a Sartorius prefilter and filters of pore sizes 0,45 µm and 0,22 µm (Millipore Corporation, Bedford, Massachusetts, USA) and collected in 100 ml, 250 ml and 500 ml sterile (autoclaved) media bottles (Schott Duran, Schott Glassworks Mainz, West Germany).

Foetal calf serum (filtered and ultraviolet irradiated) (State Vaccine Institute, Cape Town, South Africa) was sterile filtered into the culture medium through a 0,45 μm Millipore filter using a Swinnex-25 holder (Millipore Corporation, Bedford, Massachusetts, USA).

Growth medium was prepared by addition of 10% foetal calf serum and the antibiotics penicillin (500 IU/ml) and streptomycin (500 μg/ml) to the minimal essential medium. This medium was used for experimental purposes.

Maintenance medium containing 5% foetal calf serum and the two antibiotics (as above) was used to maintain cells between experimental periods at a slightly lower rate of proliferation.

Shelf culture medium containing 2% foetal calf serum and the antibiotics as above, was used to maintain the survival of shelf cultures (at room temperature) with very little growth occurring. These cultures were maintained as a "backup" for a period of two to three weeks before being re-incubated at 37°C.

CELL LINES

A human hepatoma cell line and an untransformed monkey kidney cell line (LLCMK) were obtained from the Department of Physiology, Medunsa, South Africa. A mouse melanoma cell line (BL-6) was obtained from Dr C. Albrecht, Department of Pharmacology, University of Stellenbosch Medical School, Tygerberg, South Africa. The monkey kidney cell line was used as the non-cancerous control, as this was the only

untransformed cell line of similar histopathological origin available to our laboratory at that time. The culturing of untransformed hepatocytes and melanocytes is a problematic undertaking and hence these cell lines were unavailable for our use.

METHODS

Experimental cell culture. Procedures were carried out on a laminar flow bench using aseptic techniques. Cells were maintained in 80 cm^2 Nunc tissue culture flasks (Weil Organisation, Durban, South Africa) while cells for experimental purposes were cultured in 25 cm^2 Nunc tissue culture flasks. The cells were maintained at 37°C in a Labcon forced circulation incubator Type FSIE.

Subculturing of cells. Cells were passaged once or twice per week, depending on their rate of proliferation, using a trypsin subculturing solution. Two methods of subculturing were used:

a) The cell medium was decanted, and 10 ml of trypsin solution was sterile filtered into the flask (containing either LLCMK, human hepatoma or BL-6 cells). The flask was incubated at 37°C until the cells were removed from the flask surface and were suspended in the trypsin solution. Excess cells were then decanted or the entire cell suspension was decanted into a sterile Nunc centrifuge tube (Weil Organisation, Durban, South Africa) and centrifuged at 2500 g in a Hettich Universal / K2S bench-top centrifuge for 10 minutes. The trypsin supernatant was decanted and the cell pellet was then

resuspended in either phosphate buffered saline (PBS) or cell medium. The pellet was dissociated by gentle passage through a pasteur pipette. This method was used mainly for routine culturing of cells.

b) The cell medium was decanted, and 10 ml of trypsin solution was sterile filtered into the flask (containing either LLCMK, human hepatoma or BL-6 cells). The flask was incubated at 37°C for 7 - 10 minutes (LLCMK) or 3 - 5 minutes (hepatoma or BL-6) and then most of the trypsin solution was decanted, leaving only a thin film of liquid covering the cells. The flask was then incubated at 37°C until the cells had lifted off the flask surface. The cell suspension could then be reduced by decanting off the excess cell population, or the entire population could be removed. This procedure was used mainly for experimental purposes.

Cell enumeration. Two methods were used for determining cell number:

a) Haemocytometry. The cells were trypsinised aseptically (at the start of an experiment) or under non-sterile conditions (when harvesting an experiment), and were then counted using a haemocytometer (Neubauer, West Germany).

b) Counting Plate. Cell number was determined using a perspex counting plate devised by a colleague (N.S. Skeef, personal communication). The plate was made to the dimensions of the undersurface of 25 cm^2 culture flask, with perforations drawn on the plate with a needle. The lines drawn were 1 mm and 0,5 mm apart as indicated in figure 3. A set counting pattern was followed to ensure

uniformity throughout. Six blocks could be counted (indicated in figure 3), either of size 0,5 mm X 0,5 mm (0,25 mm^2), or 1 mm X 1 mm (1 mm^2). The total surface area of a small flask is 2500 mm^2, hence counting six blocks of 0,25 mm^2 required a multiplication factor of 1666,67 and six blocks of 1 mm^2 required a factor of 416,67. This method was found to be advantageous, in that the results obtained in various experiments were consistent throughout the five replicate flasks, as indicated by their low standard error of the mean (SEM) values. A further advantage when following a daily growth curve was that the same flask could be counted successively for six days with no trypsinisation required.

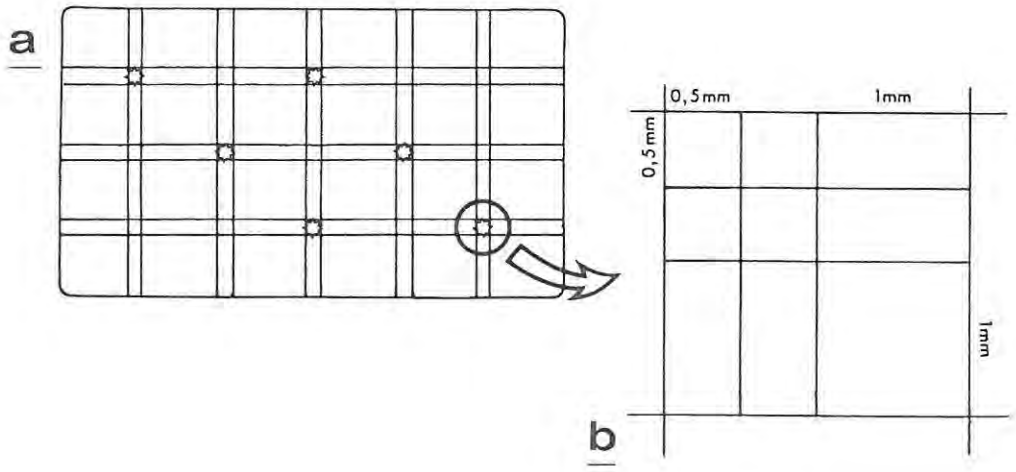


FIGURE 3. (a) Counting plate for cell enumeration (actual size). ☼ indicates areas counted. (b) Enlargement of counting area. (N.S. Skeef, personal communication).

Determination of the effect of vitamin A addition on the proliferation of LLCMK, BL-6 and human hepatoma cells.

a) Enumeration by haemocytometry after seven days of growth. Human hepatoma and BL-6 cells were seeded in 25 cm^2 culture flasks at a concentration of 2 X 10^5 cells per flask, while LLCMK cells were seeded at 2,5 X 10^5 cells per flask. Five replicates were prepared for each vitamin A concentration - 100 µM, 10 µM, 5 µM, 1 µM, control with ethanol (0,1%) and control without ethanol. 10 ml of control or vitamin A medium containing 10% foetal calf serum and the antibiotics, penicillin and streptomycin (ie. growth medium), was added to each flask at the start of the experiment. The flasks (30 per experiment) were incubated at 37°C. Media was replenished on day 6 and the cells were trypsinised on day 7. Once the cells were detached from the flasks, the trysin solution was diluted with phosphate buffered saline (PBS) to a final volume of 10 ml. The number of cells per flask was determined using the Neubauer haemocytometer. The percentage of growth inhibition by retinyl acetate was calculated from the equation:

$$100 - (R/C) \times 100$$

where R is the number of retinyl acetate-treated cells and C is the number of control cells (55,56,57).

b) Enumeration by counting plate on six consecutive days. LLCMK cells were seeded at 2,5 X 10^5 cells per 25 cm^2 culture flask and BL-6 cells at a concentration of 2 X 10^5 cells per flask. Five replicates at each vitamin A concentration (100 µM, 10 µM, 5 µM, 1 µM, control with ethanol, control without ethanol) were prepared for both LLCMK and BL-6 cells, while two replicates were prepared in a duplicate experiment with LLCMK cells only. 10 ml of control or

vitamin A-containing growth medium was added to each flask on day 0 (the start of the experiment). The cells were incubated at 37°C and after each successive 24 hour period the cell number per flask was determined using the counting plate. Media was replenished on day 4 (LLCMK and BL-6) and on day 5 (BL-6 only).

Statistical analysis. The statistical significance of difference was determined by the student's "t" test.

RESULTS

The effect of vitamin A addition on the proliferation of LLCMK, BL-6 and human hepatoma cells.

a) Enumeration by haemocytometry after seven days of growth. The effects of vitamin A supplementation on the proliferation of the different cell lines studied is shown in figure 4. There is clear evidence of a dose-response over the range 0 - 100 μM retinyl acetate, which is in accordance with work of Lotan and colleagues (3,55,56).

The untransformed LLCMK cells elicited no significant response to vitamin A addition, while the growth of the tumour cell lines was inhibited, as summarised in Table 3. The growth of the BL-6 cells in particular was significantly inhibited by vitamin A addition. The growth of all three cell lines appeared to be stimulated by the addition of the 0,1% ethanol (control) when compared to the control containing no ethanol.

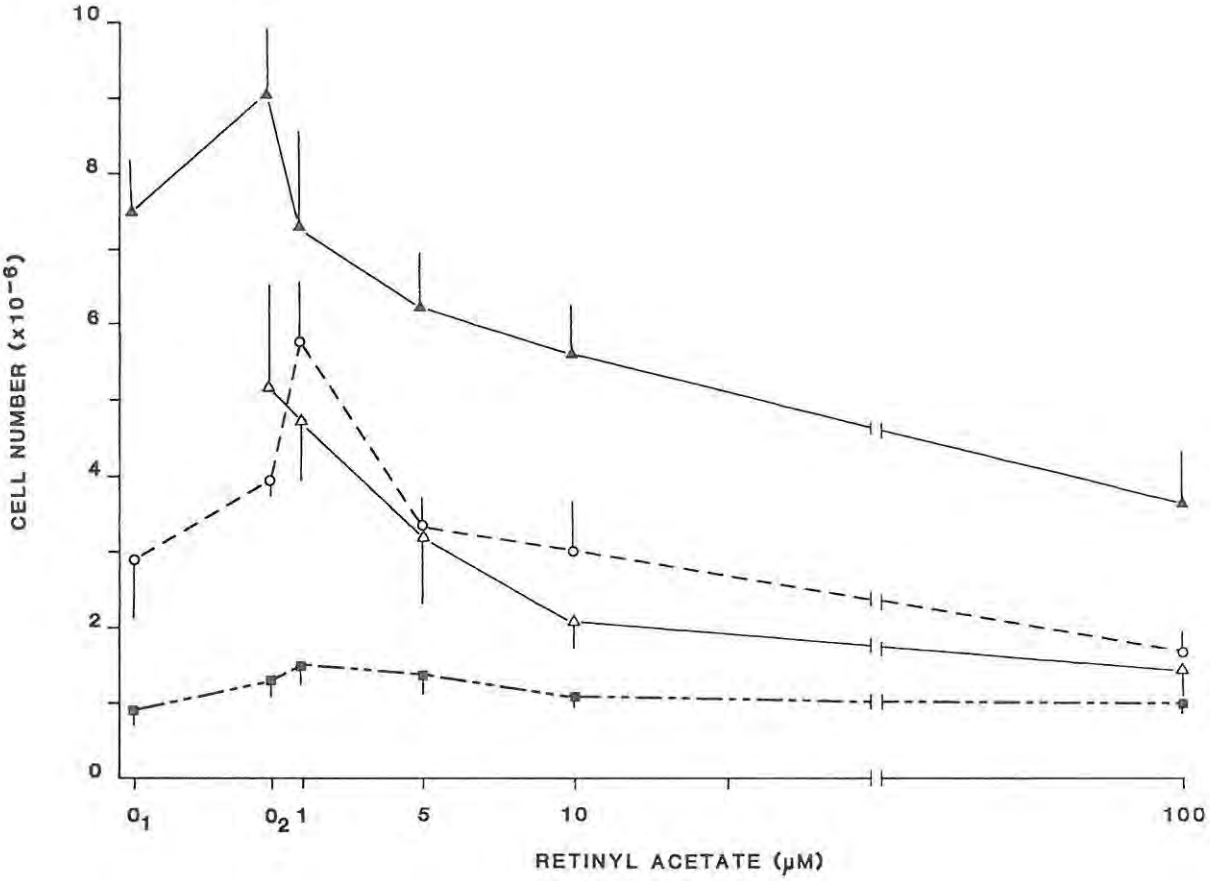


FIGURE 4. The effect of vitamin A supplementation on the proliferation of LLCMK, BL-6 and human hepatoma cells.

■—- - -■ LLCMK cells

△———△ BL-6 cells (a)

▲———▲ BL-6 cells (b) - repeat experiment of BL-6 (a)

o- - - -o Human hepatoma cells

O_1 - control containing no ethanol

O_2 - control with ethanol (0,1%)

TABLE 3. Percentage growth inhibition of cell lines at different vitamin A concentrations.

CELL LINE	RETINYL ACETATE CONCENTRATION (μM)					
	0-EtOH	0+EtOH	1	5	10	100
BL-6 (a)	-	0,0	9,27	38,22	60,23	71,81*
BL-6 (b)	17,98	0,0	19,30	31,69*	38,49*	59,32*
Human hepatoma	27,71	0,0	-46,35*	16,88	24,18	57,43*
LLCMK	34,09	0,0	-13,64	-4,55	18,94	21,97

* $p < 0,05$ when compared to the control (0+EtOH). BL-6 (b) is a repeat experiment of BL-6 (a).

TABLE 4. Percentage growth inhibition of BL-6 cells at different vitamin A concentrations.

DAY	RETINYL ACETATE CONCENTRATION (μM)					
	0-EtOH	0+EtOH	1	5	10	100
3	15,47	0,0	3,23	27,71*	25,40*	52,42*
4	7,45	0,0	8,08	37,25*	39,14*	76,00*
5	15,38*	0,0	11,84	42,10*	33,19*	78,84*
6	11,94*	0,0	8,61*	33,39*	25,48*	76,68*
mean	12,56	0,0	7,94	35,11	30,80	70,99

* $p < 0,05$ when compared to the control (0+EtOH).

b) Enumeration by counting plate on six consecutive days. The LLCMK cell growth curve (figure 5 a and b) shows significant inhibition only at a vitamin A supplementation of 100 μM ($p < 0,05$). The growth curves for 1 μM, 5 μM and 10 μM closely follow those of the two controls, hence no significant inhibition occurred at these levels of vitamin A addition.

The BL-6 cells (figure 6), however, show a significant dose-response over the entire range of vitamin A supplementation. Marked inhibition of growth occurred with the 100 μM vitamin A-treated cells. The cells supplemented with 1 μM vitamin A also elicited a reduced rate of growth, as did the control cells without ethanol addition ($p < 0,05$). The rate of proliferation of the 5 μM and 10 μM vitamin A-treated cells was more significantly inhibited.

The growth inhibition of the BL-6 cells could be quantified from day 3 of the experiment, as indicated in Table 4.

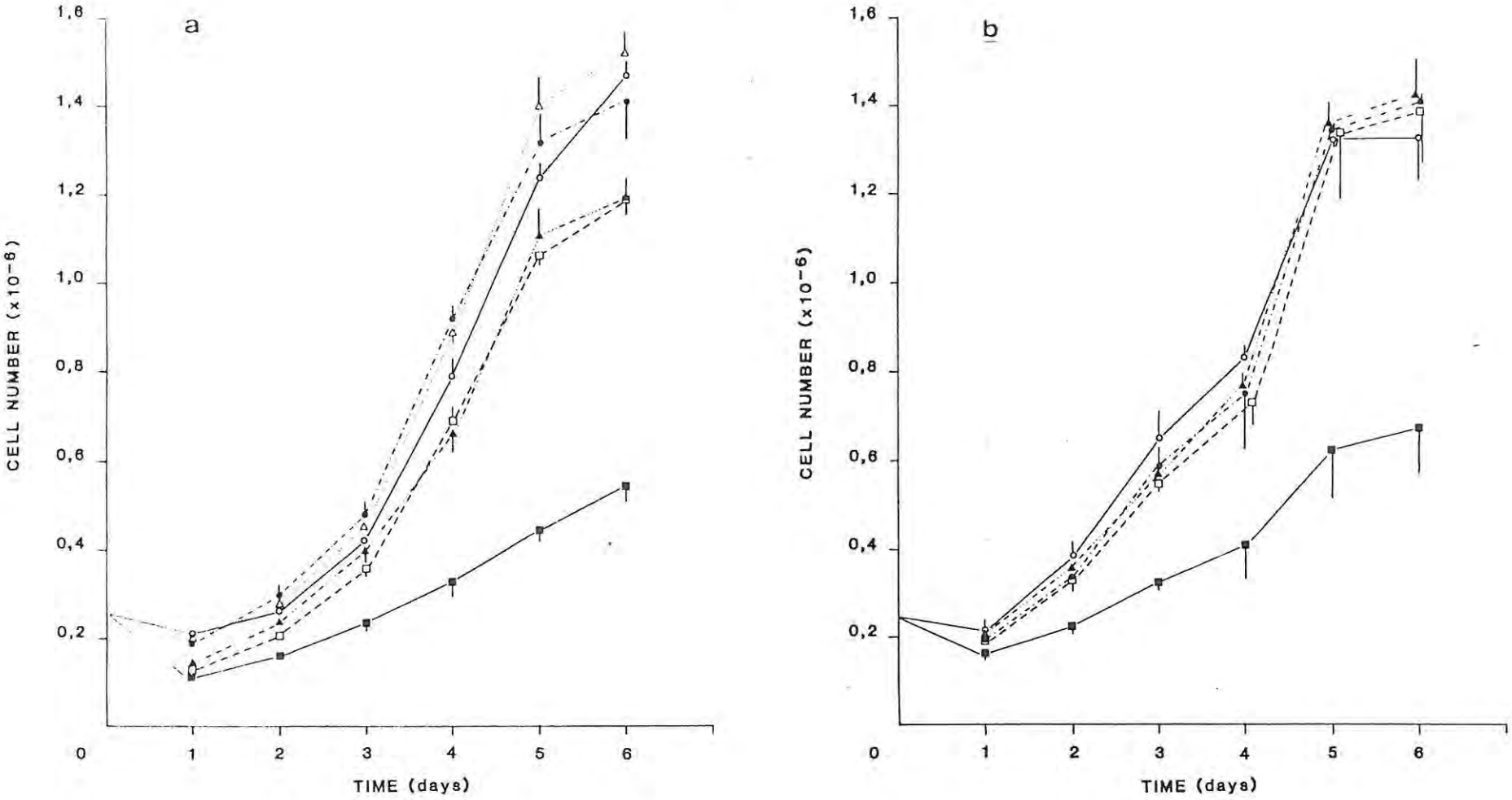


FIGURE 5. LLCMK cell growth curve at various levels of vitamin A supplementation.

(b) is a repeat experiment using two replicates, to confirm the results of (a).

△ ····· △ control (without ethanol), ○———○ control (with ethanol), ●·-·-·-·-● 1 μM vitamin A,

□- - - -□ 5 μM vitamin A, ▲- - -·····-▲ 10 μM vitamin A, ■———■ 100 μM vitamin A.

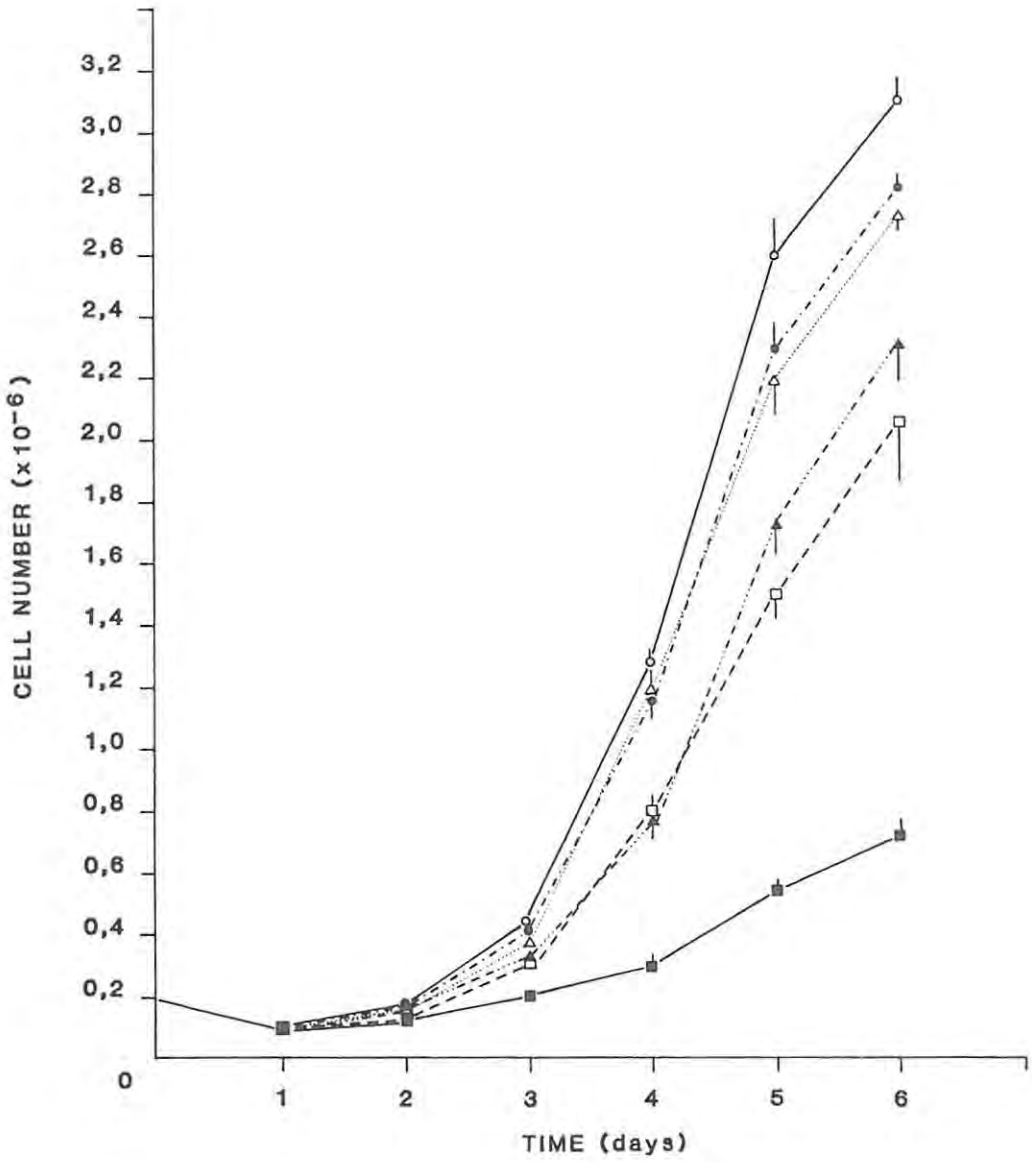


FIGURE 6. BL-6 cell growth curve at various levels of vitamin A supplementation.

△·····△ control (without ethanol), ○——○ control (with ethanol), ●-·-·-·-● 1 μM vitamin A, □- - - -□ 5 μM vitamin A, ▲·····▲ 10 μM vitamin A, ■——■ 100 μM vitamin A.

DISCUSSION

Enumeration of cell growth by haemocytometry showed a slight, but non-significant, inhibition of LLCMK growth at 10 μM vitamin A supplementation and in the control without ethanol, while 1 μM and 5 μM vitamin A supplementation appeared to elicit a slight, but non-significant, stimulation of LLCMK cell growth. Cell growth determination using the counting plate also showed no significant inhibition of LLCMK cell growth when treated with 1 μM, 5 μM and 10 μM vitamin A and in the control without ethanol. It is evident from these experiments that retinyl acetate appears to have little effect on the proliferation of LLCMK cells in vitro up to a concentration of 10 μM.

The LLCMK growth curve consisting of five replicates at each vitamin A concentration (figure 5 a) was not conclusive on its own, as the six concentrations were not set up on the same day. The two controls and the 1 μM vitamin A-treated cells were set up on a different day to the 5 μM, 10 μM and 100 μM vitamin A-treated cells, and hence the cells in the two parts of the experiment could easily have been at different stages of growth and exposed to slightly different conditions. The experiment was set up in this manner due to the difficulty in counting the cells from the entire experiment at one sitting. The slightly lower counts on day 1 for the latter half of the experiment indicated that these cells may have undergone slightly more harsh treatment at the start of the experiment, as all the LLCMK

cells were initially seeded (day 0) at the same starting concentration. The growth curves at different vitamin A treatments could thus not be conclusively compared.

This problem was not encountered with the BL-6 growth curve (figure 6) as the cells at all six vitamin A concentrations were seeded on the same day and hence under similar growth conditions. In this case, the experiment was set up in two halves, with approximately a two hour break in between. On counting the cells each day, this two hour break between the two halves was maintained in order to ensure uniformity. The two hour break enabled effective counting of all the replicates on the same day. The experiment was set up in this manner to eliminate the problem encountered in the initial experiment with the LLCMK cells above.

In order to eliminate the uncertainty of the data with the LLCMK cells, a duplicate experiment was set up. Here, all cells supplemented with the various concentrations of vitamin A were seeded on the same day and hence under similar growth conditions. Only two replicates were used at each vitamin A concentration, since this was a repeat experiment designed to confirm the previous experiment. The resulting growth curve (figure 5 b) was similar to that obtained in the first experiment and allowed a conclusive deduction to be made, namely that vitamin A supplementation up to a concentration of 10 μM appeared to have little effect on the proliferation of LLCMK cells.

The tumour cell lines BL-6 and human hepatoma, however, elicited a significant dose-response to retinyl acetate supplementation. The significant ($p < 0,05$) inhibition of BL-6 cell growth at 1 μM vitamin A supplementation was an important observation, as in in vivo systems the plasma concentration of vitamin A is approximately 1 μM (2,11,22-24,31-35). At higher (non-toxic) vitamin A concentrations (5 μM and 10 μM), there was an even greater inhibition of BL-6 cell growth. The difference between the effect of 5 μM and 10 μM vitamin A on inhibition of BL-6 cell growth was not significant.

The use of the haemocytometer (figure 4 and Table 3), however, did not produce results which were as statistically significant as those results determined by the counting plate (figure 6 and Table 4). Although the trends of tumour cell growth inhibition using the haemocytometer were clearly evident (figure 4), the differences between the cell numbers compared to the control were not always of statistical significance (Table 3). The inhibition of BL-6 cell proliferation is in accordance with work of Lotan and colleagues who found a 48% growth inhibition in BL-6 cells treated with 10 μM retinyl acetate (3,55,56). A greater inhibition of cell growth with increasing vitamin A supplementation has previously been reported by a number of researchers, working with a variety of different cell lines and different forms of vitamin A (55-60), as summarised in the introduction to this chapter.

The human hepatoma cells elicited a significant ($p < 0,05$) stimulation of growth when supplemented with 1 μM vitamin A. This level of vitamin A supplementation thus appeared to be the optimum for

proliferation of human hepatoma cells. At 5 μM and 10 μM vitamin A supplementation the inhibition of cell proliferation was not significant when compared to the control (with ethanol). However, when compared to the proliferation of the 1 μM vitamin A-supplemented cells, a substantial inhibition of growth was observed in both the 5 μM and 10 μM vitamin A-treated hepatoma cells. This indicates that the two transformed cell lines in this study elicit slightly different responses at low non-toxic levels of vitamin A supplementation.

The addition of 100 μM vitamin A to both the tumour and untransformed cells resulted in significant inhibition of proliferation ($p < 0,05$). This could be due to cytotoxicity, as the cells, when viewed under the optical microscope, did not appear to be in a normal healthy state. Many of the cells had detached from the flask surface, while others, which were still attached, appeared to have a "granular" appearance. This was one of the factors that prompted further investigation of these effects using ultrastructural techniques (chapter 3).

As the retinyl acetate was made up in ethanol (final concentration in the cell media of 0,1%) the control cultures were those supplemented with 0,1% ethanol and hence the counts of the vitamin A-treated cells were compared to the counts of this control. It appears that the addition of 0,1% ethanol to the cell media resulted in a slight stimulation of cell growth, this effect being more pronounced in the tumour cells. This was in contrast to the results of Findley, Steuber, Ruymann, Culbert and Ragab (62) who found no effect of 0,04% ethanol on bone marrow and leukemia cells. The stimulation of cell

growth by the ethanol administration did not, however, mask the inhibitory effects of vitamin A on the tumour cells.

Figure 5 (a and b) and figure 6 show a similar phenomenon whereby the number of cells seeded per flask (day 0), whether untransformed or tumour cells, exceeded the number of cells determined on day 1. This indicates that a percentage of the cells did not withstand the harsh procedures of trypsinisation, centrifugation and the passage through a pasteur pipette (for dissociation of the cell pellet) associated with the preparation of the cell suspension. Using the counting plate technique, the cell number in a large (80 cm^2) culture flask (for seeding of an experiment) was determined prior to trypsinisation, and hence when these cells were removed from this flask a certain percentage of cells were obviously lost, thus contributing to the reduction in cell number. This did not pose a serious problem, however, as the most essential factor was ensuring that each flask in the experiment was seeded with a constant cell population.

The determination of the extent of inhibition using either haemocytometry on the seventh day of growth or enumeration by counting plate on six consecutive days, produced similar trends in BL-6 cell growth as can be seen by a comparison of results in Tables 3 and 4. It is evident by comparing figure 4 to figure 5 and figure 6 that results were more conclusive when cell number was determined by counting plate rather than by haemocytometry. The most striking difference between the results using the two techniques was the size of the SEM (standard error of the mean) bars, and hence results achieved by counting plate can be discussed with more conviction than

those determined by haemocytometry. A further limitation in the use of the haemocytometer was the determination of very low cell number. In particular, for example, was the case of the LLCMK cells treated with 100 μM vitamin A. The multiplication factor in haemocytometry is 10^4 (for the number of cells/ml), whereas the factor is 416,67 or 1666,67 (for total cell number) when using the counting plate, and this certainly influenced the results when only a few cells were enumerated. Hence, for the LLCMK cells, haemocytometry of 100 μM vitamin A supplementation showed a 21,97% (Table 3) growth inhibition (which was not statistically significantly different from the control), whereas the counting plate elicited a 49,17% growth inhibition (figure 5b) which was statistically significantly different ($p < 0,05$) from the control. It would appear, therefore, that the more reliable and consistent data was that obtained using the counting plate.

In conclusion, significant inhibition of malignant BL-6 but not of untransformed LLCMK cell growth was found with the addition of physiological (non-toxic) levels of vitamin A (1 - 10 μM) to *in vitro* cultures of these cells. Addition of 100 μM vitamin A resulted in significant inhibition of all three cell types (BL-6, human hepatoma and LLCMK), which may possibly be due to a cytotoxic effect, particularly since the cells appeared to have undergone certain ultrastructural changes as a result of this treatment. In order to investigate the possible effect of vitamin A addition on the ultrastructure of the cells, electron microscopy studies were undertaken.

CHAPTER 3

ULTRASTRUCTURAL OBSERVATIONS OF VITAMIN A-TREATED CELLS BY TRANSMISSION ELECTRON MICROSCOPY

INTRODUCTION

Vitamin A plays a key role in vision, reproduction, bone growth and epithelial development (1,3,5,6,18). A well established function of vitamin A is its role in the ultrastructural development of cells. Cell membrane integrity is known to be dependent on vitamin A (1,3) and of particular importance is the cell surface (plasma) membrane. Some of the effects of vitamin A, such as those related to cell adhesion and cell-cell interaction, appear to be exerted on the plasma membrane directly without nuclear interaction (1,63) while other effects are mediated through the nucleus (1,64) (discussed further in chapter 4).

An electron microscopy study was undertaken by Matter and Bollag (65) to investigate the effect of a retinoic acid analogue on chemically induced papillomas in mouse skin. They found a marked enhancement of proteoglycan production and secretion into the extracellular space, labilisation of the plasma membrane resulting in vacuolisation and loss of cytoplasmic constituents, an increase in lysosomal organelles and the formation of many necrotic cells. They proposed that cell necrosis was due mainly to labilisation of the plasma membrane and, together with the enhanced proteoglycan production and secretion, this

was responsible for the observed regression of the papillomas on treatment with the retinoid.

Prompted by this knowledge, together with the observation of a "granular" appearance of vitamin A-treated cells under the optical microscope (chapter 2), an ultrastructural study of the effects of vitamin A supplementation on both cancer and untransformed cells was undertaken. This was in an attempt to identify a possible mechanism of action of vitamin A, to account for the reduction in tumour cell growth observed earlier (chapter 2).

MATERIALS AND METHODS

REAGENTS

Retinyl acetate stock solutions as prepared in chapter 2.

Trypsin subculturing solution as prepared in chapter 2.

a) Transmission electron microscopy

Phosphate buffer (0,1 M at pH 7,3) (66) was prepared by mixing eight parts of solution 1 and two parts of solution 2:

Soln. 1: 35,814 g $Na_2HPO_4 \cdot 12H_2O$ / l

Soln. 2: 13,610 g KH_2PO_4 / l

Glutaraldehyde (5% solution) (66) was prepared by diluting 20 ml of 25% glutaraldehyde (ultrastructural grade) to 100 ml with phosphate buffer.

Osmium tetroxide (Millonig, 1% solution) (66) consisted of 0,5 g OsO_4, 45,0 ml solution D and 5,0 ml solution C, adjusted to pH 7,3 (if necessary, with 0,1 N HCl) and stored in tightly stoppered dark bottles at 4°C.

Soln. A: 2,26% $NaH_2PO_4 \cdot H_2O$

Soln. B: 2,52% NaOH

Soln. C: 5,40% glucose

Soln. D: 41,5 ml soln. A
8,5 ml soln. B

Ethanol (95%) (66) was diluted with distilled water as follows:

30%	160	ml	95%	EtOH	+	340	ml	H_2O	=	500	ml	30% EtOH
50%	260	ml	95%	EtOH	+	240	ml	H_2O	=	500	ml	50% EtOH
70%	370	ml	95%	EtOH	+	130	ml	H_2O	=	500	ml	70% EtOH
80%	420	ml	95%	EtOH	+	80	ml	H_2O	=	500	ml	80% EtOH
90%	475	ml	95%	EtOH	+	25	ml	H_2O	=	500	ml	90% EtOH

Embedding medium used was Taab 812/Araldite (R.H.M. Cross, personal communication).

Toluidine Blue stain (66) was prepared by dissolving 0,1 g toluidine blue powder in 10 ml sodium carbonate (2,5 g sodium carbonate in 100 ml distilled water).

Uranyl acetate stain (66) was prepared by dissolving 0,25 g uranyl acetate powder in 5 ml distilled water and mechanically shaking the mixture for 30 minutes (suitable for use for 24 hours).

Lead citrate stain (Reynolds) (66) was prepared by dissolving 0,44 g lead nitrate and 0,59 g sodium citrate in 10 ml distilled water. The solution was violently shaken for 1 minute and then mechanically shaken for 30 minutes. 2,6 ml fresh 1,0 M NaOH was added, the mixture was gently agitated until the cloudiness disappeared and then 4,0 ml distilled water was added.

b) Lipid determination

Ethanol (30% and 70%) were prepared as above.

Sudan IV (Scarlet R) (67) was prepared by dissolving 0,25 g Sudan IV powder in 50 ml 70% ethanol in a warm water bath and then filtering the solution.

Formalin (100% formaldehyde) was diluted to a 10% solution with 0,9% saline.

Mayer's Hematoxylin (Mallory) (68) was prepared by gently heating 1 g hematoxylin in 1000 ml distilled water. 0,2 g sodium iodate and 50 g potassium alum ($KAl(SO4)2 \cdot 12H2O$) were added and the solution was heated until dissolved. 1 g citric acid and 50 g chloral hydrate were added and the preparation was allowed to ripen for 6 - 8 weeks before use.

Farrant's medium containing glycerin, arsenious acid and gum arabic (exact composition unknown) was the water soluble mounting medium used.

METHODS

a) Transmission electron microscopy

Preparation of vitamin A-treated LLCMK, human hepatoma and BL-6 cells for transmission electron microscopy. LLCMK, human hepatoma and BL-6 cells were seeded at 2,5 X 10^5, 2,0 X 10^5 and 3,0 X 10^5 cells per 25 cm^2 flask, respectively. Vitamin A-containing growth medium was prepared at concentrations of 100 µM, 10 µM, 5 µM, 1 µM retinyl acetate and a control with ethanol (0,1%). The cells were incubated at 37°C with 10 ml of medium per flask. Medium was replenished on day 3 (BL-6 cells only) and day 6 (all cell lines) and the cells were trypsinised on day 7. The three cell lines were treated in the same manner in preparation for electron microscopy. The cell suspensions (in trypsin) were centrifuged in a Beckman microfuge B (California, USA) for 3 minutes. Each pellet was resuspended in media to rinse off the trypsin, and then microfuged again. Each cell pellet then underwent fixation and embedding (as below, R.H.M. Cross, personal communication).

Fixation and embedding protocol

		Minutes
Pre-fix:	5% glutaraldehyde	overnight
Wash:	Phosphate buffer (0,1 M, pH 7,3)	10 (X 2 washes)
Secondary fix:	OsO_4	90
Wash:	Phosphate buffer (0,1 M, pH 7,3)	10 (X 2 washes)
30% EtOH		5
50% EtOH		5
70% EtOH		5
80% EtOH		5
90% EtOH		5
abs EtOH		5
abs EtOH		5
propylene oxide		15
propylene oxide		15
75:25 pro:resin		60
50:50 pro:resin		75
25:75 pro:resin		75
pure resin		overnight
pure resin in moulds		36 hrs at 60°C

Preparation of glass knives was according to the procedure of R.H.M. Cross (66). These glass knives, however, were not satisfactory as the cell pellet appeared to contain some crystalline material which chipped the cutting edge of the glass knives, resulting in unsightly marks and the cutting of uneven sections. Hence, a diamond-edged knife was used on the LKB Ultrotome III ultramicrotome (Stockholm, Sweden) for cutting of the ultrathin sections.

Preparation of thick sections for optical microscopy and ultrathin sections for transmission electron microscopy. Thick sections (2 - 3 μm) were initially cut from the specimens and stained with toluidine blue for examination under the optical microscope. Ultrathin sections of the desired thickness (40 - 50 nm) were cut and appeared silver when reflected through the microscope. The sections were floated onto a water surface and collected on copper grids of 300 mesh (300 holes/inch). The sections on the grids were stained with the heavy metal stains uranyl acetate (30 minutes) and lead citrate (5 minutes). The LLCMK and human hepatoma cells were examined on the JEOL JEM 100 CX II transmission electron microscope (Tokyo, Japan) at an acceleration voltage of 80 KV, while the BL-6 cells were examined on the Hitachi HU 11B transmission electron microscope (Tokyo, Japan) at an acceleration voltage of 50 KV. (The JEOL JEM 100 CX II electron microscope was not operational at the time of the BL-6 study.) Photographs (using Ilford electron microscopy film) were taken of various sections and developed by the staff of the Electron Microscopy Unit, Rhodes University, Grahamstown, South Africa.

b) Lipid determination

Preparation of vitamin A-treated LLCMK and BL-6 cells for lipid determination. 80 cm^2 tissue culture flasks were used in the preparation of LLCMK and BL-6 cells for lipid determination. Both cell types were grown in media containing 100 µM retinyl acetate. The cells were maintained until confluency (retinoid-medium being replenished as required), and were then trysinised and centrifuged. The pellets were gently resuspended in 10% formalin (in 0,9% saline) and allowed to fix for 4 hours. The cell suspensions were again centrifuged and the formalin decanted.

Preparation and staining of microtomy sections for optical microscopy. The cell pellets were transferred onto a freezing microtome (Reichert, Austria) and 10 - 50 µm sections were cut and transferred onto glass microscope slides. The slides were allowed to dry in a 37°C oven. The sections were stained (67) in Sudan IV (0,5% in 70% ethanol) for 15 minutes and then rapidly washed in 70% and 30% ethanol (2 minutes in each solution). The sections were washed in distilled water, stained in Hematoxylin for 8 minutes and then washed well in distilled water. Farrant's medium was used as the water soluble mounting medium over which the glass coverslips were placed. The slides were allowed to air dry and the sections were then examined and photographed using a Zeiss photomicroscope (Zeiss, Germany).

RESULTS

a) Transmission electron microscopy

Ultrastructural observation indicated what appeared to be a few small lipid droplets located within the control human hepatoma cells (figure 7) and the control BL-6 cells (figure 8). A similar occurrence of a few small lipid droplets was found in the control LLCMK cells (figure 9). In both human hepatoma and LLCMK cells, treatment with 1 μM vitamin A did not appear to effect the ultrastructure of the cells, as their appearance was similar to that of the respective controls. Consequently, the electron micrographs at this level of vitamin A treatment are not shown.

The supplementation of 5 μM vitamin A to the LLCMK cells appeared to have no ultrastructural effect on these cells (figure 10), however, the same vitamin A supplementation to human hepatoma cells resulted in the increase in size of what were suspected to be lipid droplets, accompanied by vacuolation and the start of cell breakdown (figure 11). Problems were encountered with the sectioning of the 5 μM vitamin A-treated BL-6 cells and hence micrographs at this level of vitamin A are not shown. The addition of 10 μM vitamin A to the human hepatoma and LLCMK cells elicited similar ultrastructural responses to those observed in the 5 μM vitamin A-treated hepatoma and LLCMK cells respectively, and as a result these micrographs are again not shown.

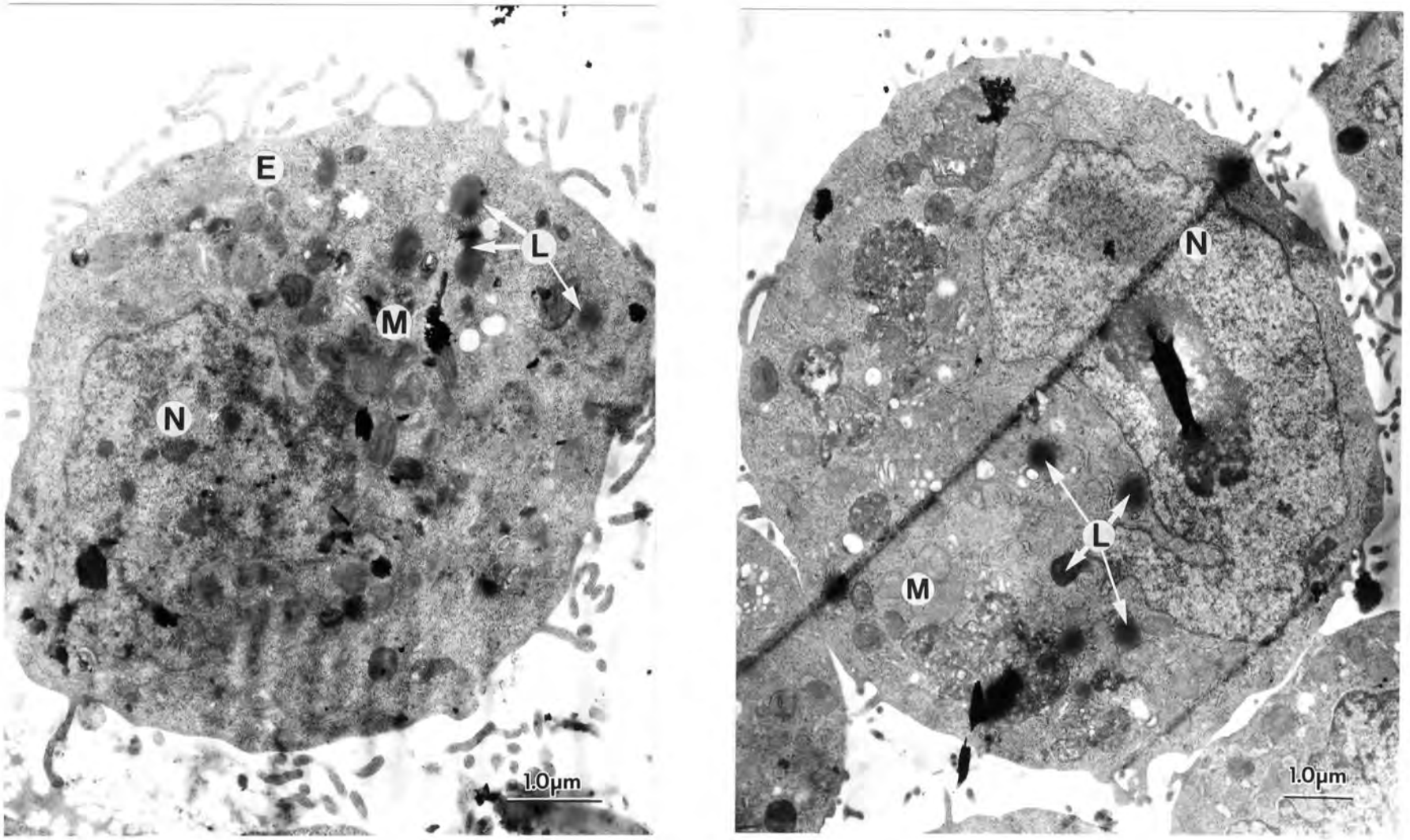


FIGURE 7. Transmission electron micrographs of control human hepatoma cells.

L = lipid droplet, N = nucleus, E = endoplasmic reticulum, M = mitochondria. Bar represents 1 μm.

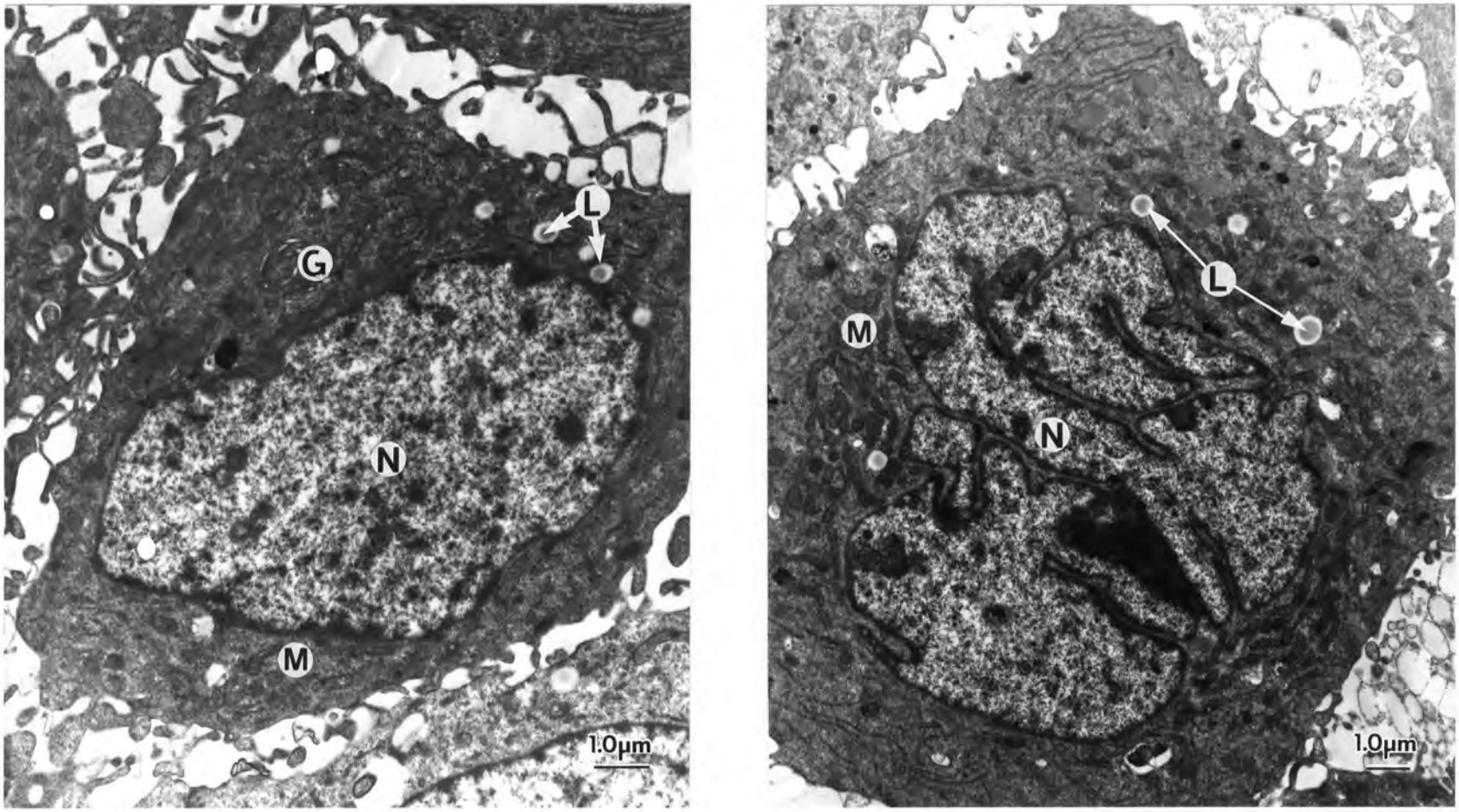


FIGURE 8. Transmission electron micrographs of control BL-6 cells.

L = lipid droplet, N = nucleus, M = mitochondria, G = Golgi body. Bar represents 1 µm.

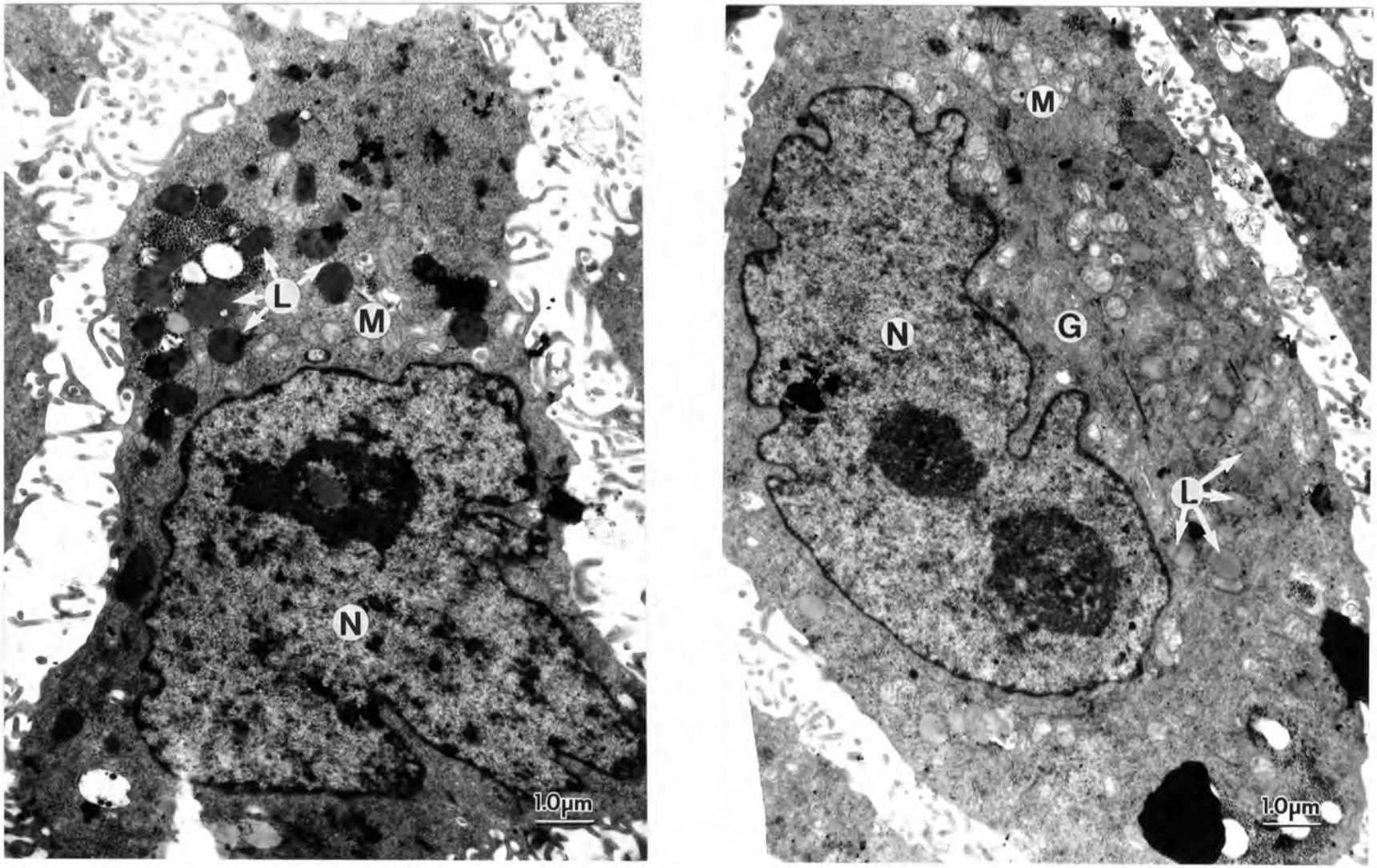


FIGURE 9. Transmission electron micrographs of control LLCMK cells.

L = lipid droplet, N = nucleus, M = mitochondria, G = Golgi body. Bar represents 1 μm.

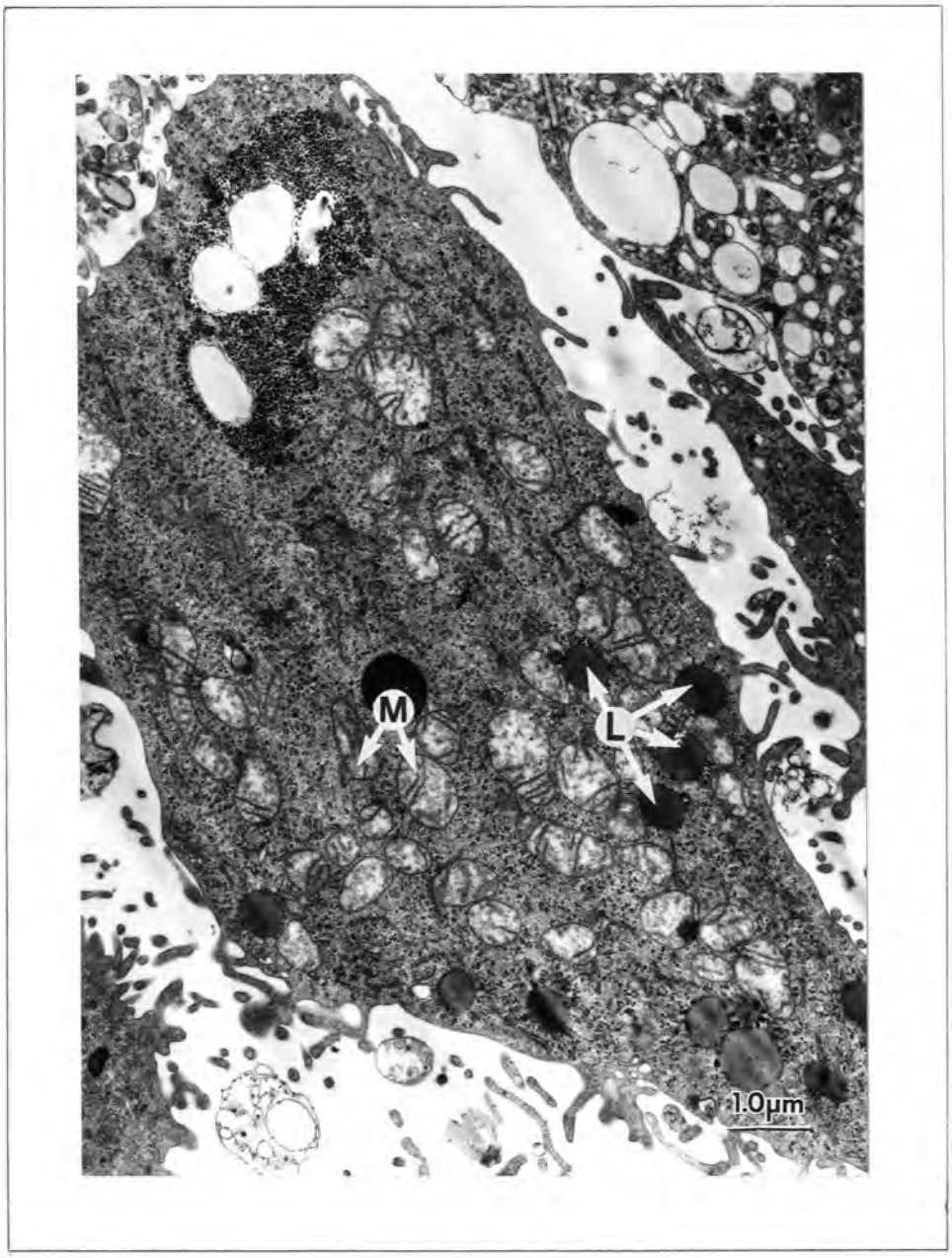


FIGURE 10. Transmission electron micrograph of an LLCMK cell supplemented with 5 µM vitamin A.

L = lipid droplet, M = mitochondria. Bar represents 1 µm.

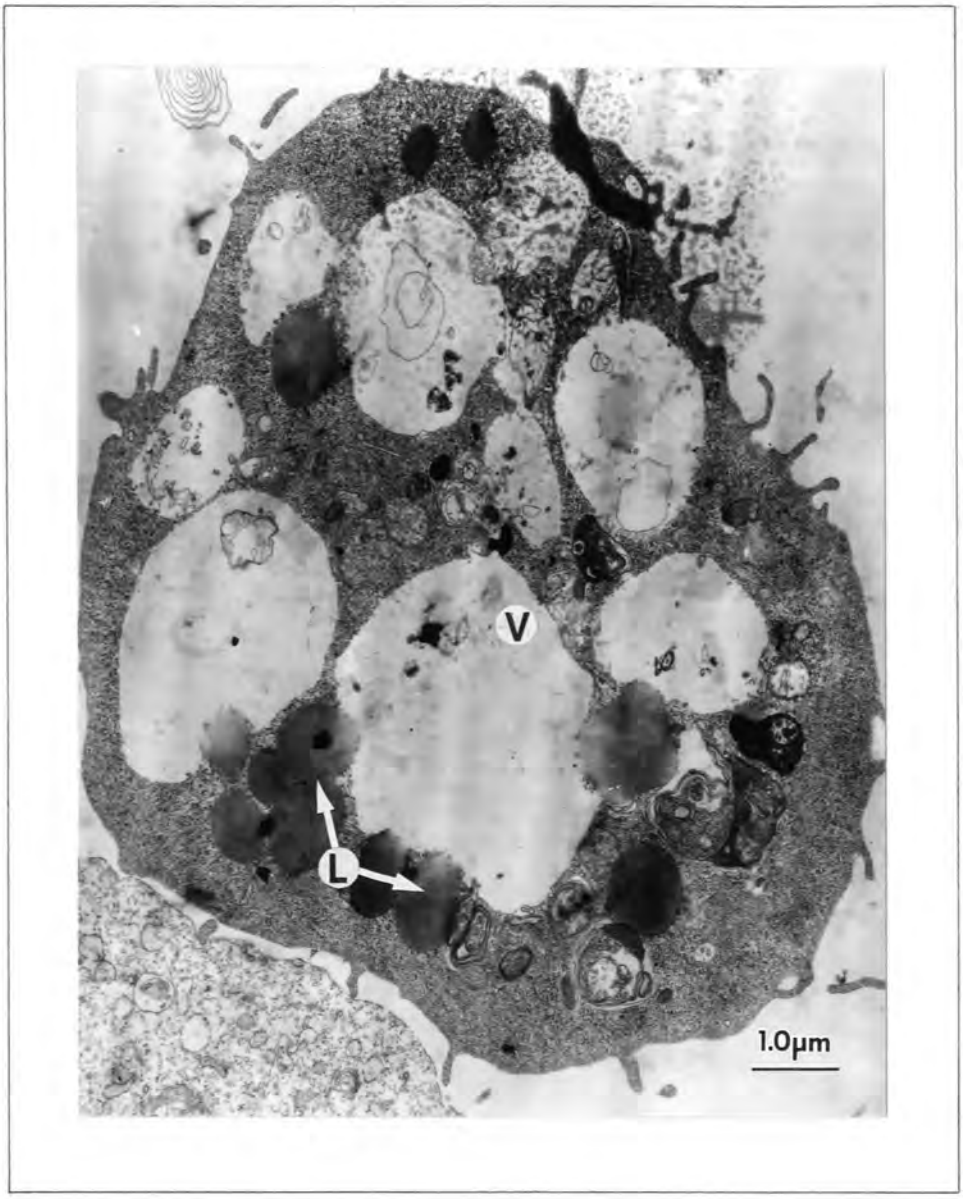


FIGURE 11. Transmission electron micrograph of a human hepatoma cell supplemented with 5 μM vitamin A.

L = lipid droplet, V = vacuole. Bar represents 1 μm.

The human hepatoma and BL-6 cells treated with 100 μM vitamin A were found to have accumulated a great number of large lipid droplets which appeared to extend throughout the cell interior (figure 12 and 13 respectively). A further observation was that numerous tumour cells treated with this level of vitamin A appeared to be in various stages of degeneration (figure 14).

The effect of 100 μM vitamin A supplementation on the LLCMK cells (figure 15) was an observed increase in the number and size of the lipid droplets (compared to control cells), as well as a number of cells at various stages of cell degeneration. However, the general effect was not as marked as that found with the tumour cells.

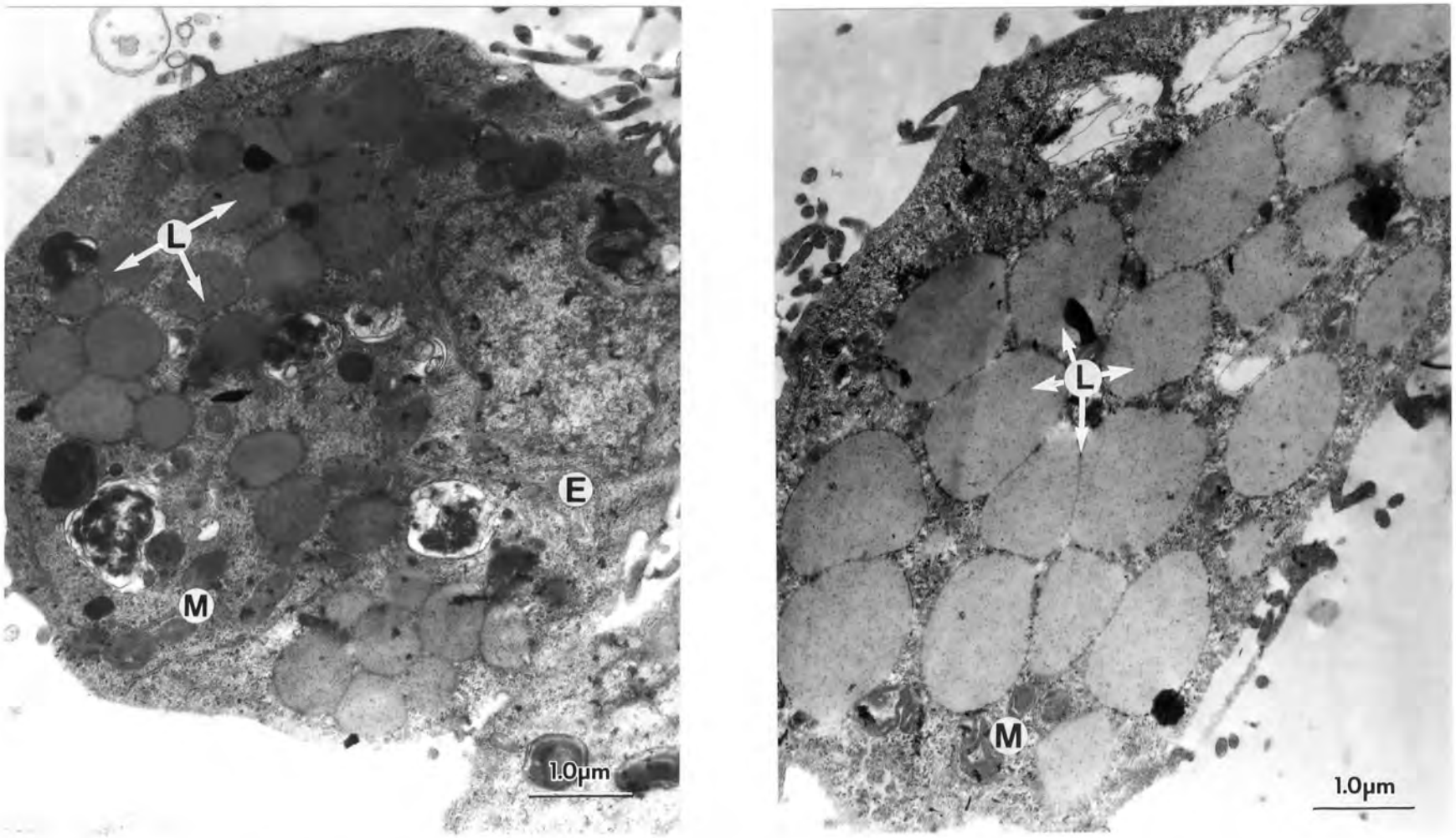


FIGURE 12. Transmission electron micrographs of human hepatoma cells supplemented with 100 μM vitamin A. L = lipid droplet, M = mitochondria, E = endoplasmic reticulum. Bar represents 1 μm.

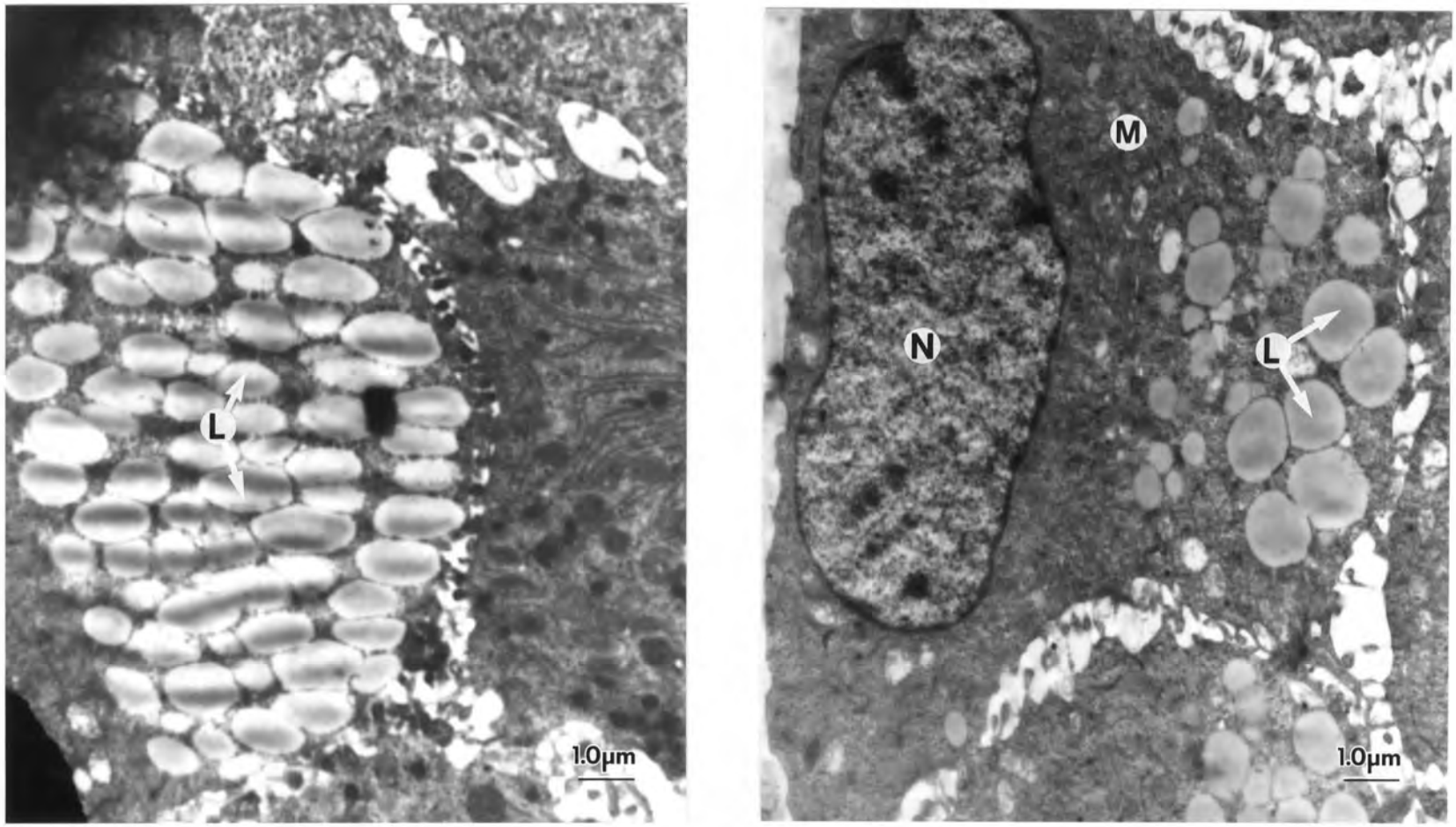


FIGURE 13. Transmission electron micrographs of BL-6 cells supplemented with 100 μM vitamin A. L = lipid droplet, N = nucleus, M = mitochondria. Bar represents 1μm.

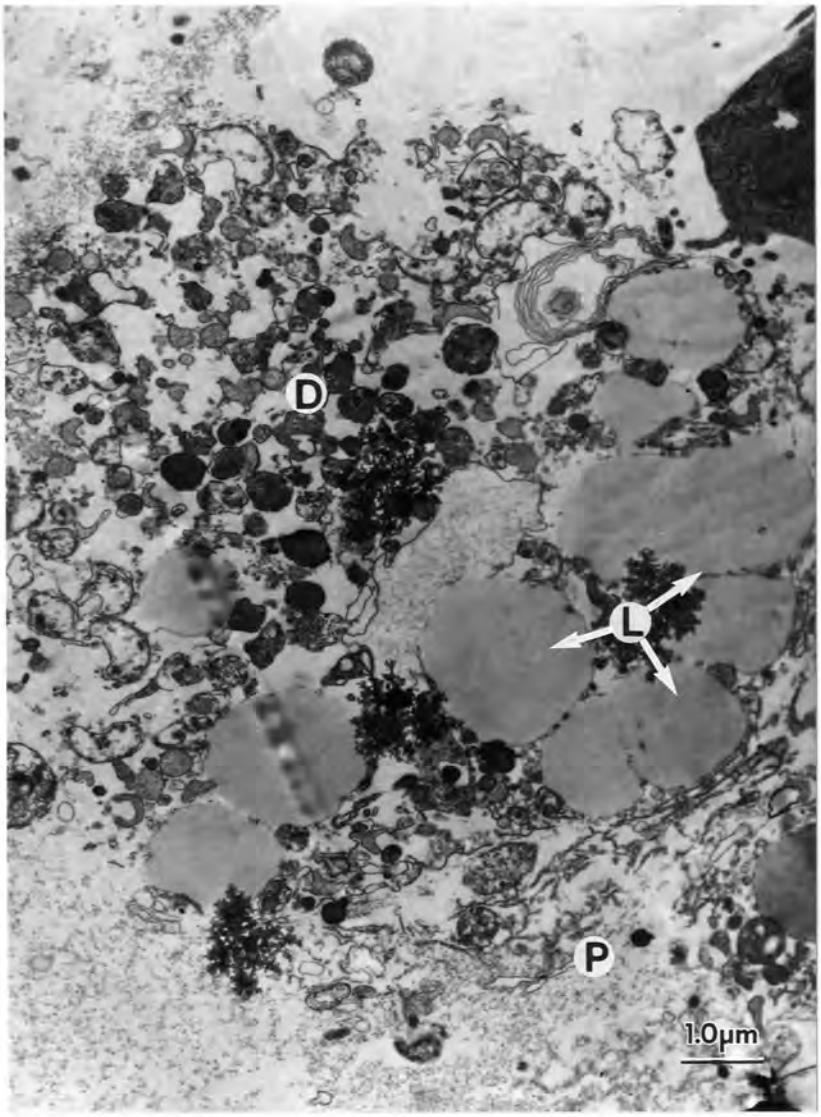


FIGURE 14. Transmission electron micrograph of cell degeneration in a human hepatoma cell supplemented with 100 µM vitamin A. L = lipid droplet, D = degeneration of cellular constituents, P = periphery of former cell. Bar represents 1 µm.

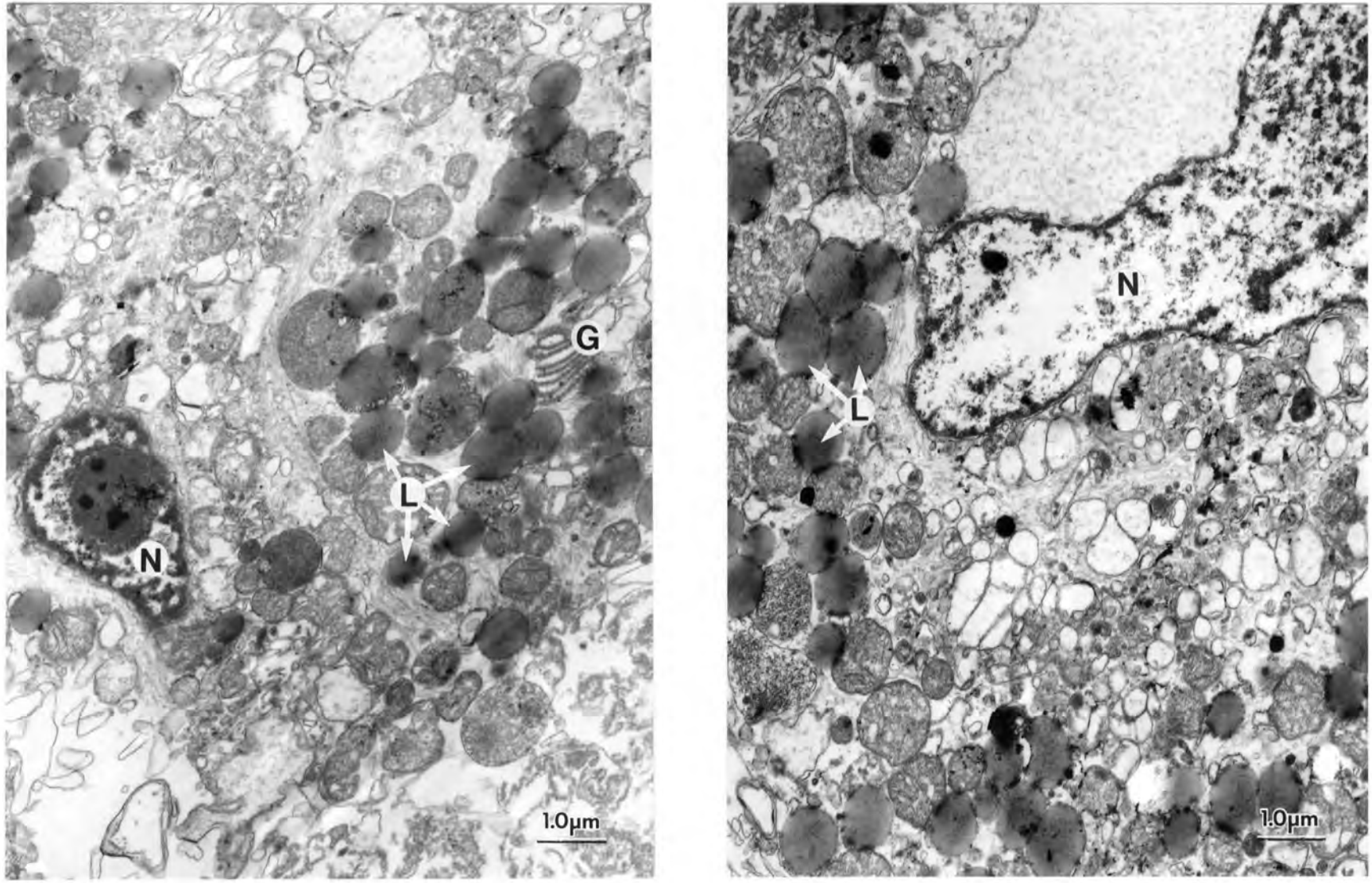


FIGURE 15. Transmission electron micrographs of LLCMK cells supplemented with 100 µM vitamin A. Cellular degeneration is apparent in these micrographs. L = lipid droplet, N = nucleus, G = Golgi body. Bar represents 1 µm.

b) Lipid determination

Optical microscopy of the LLCMK and BL-6 cells which were treated with 100 μM vitamin A, sectioned and then stained with Sudan IV, revealed that the red lipid stain had been taken up by both cell types. The two cell types were indistinguishable under the optical microscope and thus only the stained BL-6 cell sections are shown (figure 16). This indicated that the droplets observed under transmission electron microscopy were indeed of a lipid nature.

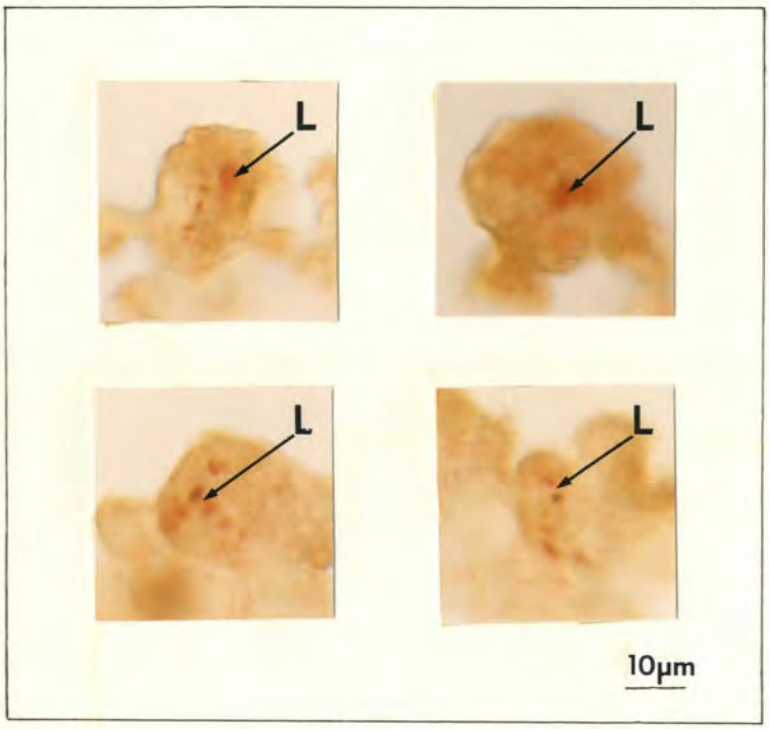


FIGURE 16. Sudan IV lipid staining of cell sections.
L = lipid droplet. Bar represents 10 μm.

DISCUSSION

The results obtained from the electron microscopy study correlate well with the cell growth results in chapter 2.

There appeared to be little ultrastructural difference between LLCMK control cells (figure 9) and the LLCMK cells treated with 1 μM, 5 μM (figure 10) and 10 μM vitamin A. This correlates with the cell growth results where the cell counts (determined both by haemocytometry and counting plate) for 1 μM, 5 μM and 10 μM vitamin A-treated cells were not significantly different from those of the control LLCMK cells.

The difference between the human hepatoma control cells (figure 7) and those cells treated with 5 μM vitamin A (figure 11) is clearly evident. The size of the lipid droplets has increased markedly as has the extent of vacuolation, preceeding the breakdown of the cell. The differences indicated here reflect the earlier differences obtained in cell counts between the control and 5 μM vitamin A-treated hepatoma cells.

The supplementation of 100 μM vitamin A to LLCMK (figure 15), human hepatoma (figure 12) and BL-6 (figure 13) cells has marked ultrastructural effects. All three cell types showed an increase in the number and size of the lipid droplets compared to the respective controls. The effect was far greater in the tumour cells, where the cell interior consisted mainly of the large lipid droplets. The ratio of lipid droplet size to cell size was very much lower in the LLCMK cells than the tumour cells. Extensive cell degeneration was apparent

in all three cell types treated with this high vitamin A dose. This is in agreement with the results in chapter 2 (figure 4, 5 and 6) where all three cell types showed marked inhibition of growth at this level of vitamin A treatment. It appears that at this level, the vitamin A has a cytotoxic effect on both untransformed and tumour cells.

It has previously been suggested that possible mechanisms of inhibition of tumour cell growth by vitamin A supplementation may be through a disturbance of metabolic functioning of the cell (69,70) or through a disruption of the cell membrane (1,3,65). A possible disruption of cell function through lipid accumulation was observed in this study. It would appear that the fat-soluble vitamin A, since it can readily pass through the lipid membrane of the cell (1), may be accumulating in the lipid droplets described above. The lipid nature of these droplets was confirmed by Sudan IV staining (figure 16). A similar observation of lipid accumulation was found by Robinson, Bux and Botha (69,70), when human oesophageal and breast cancer cell lines were treated with the fatty acid, gamma-linolenic acid.

At low vitamin A concentrations the cells may effectively metabolise part or all of the lipid-soluble vitamin. This capacity for metabolism of the fat-soluble vitamin A may vary between different cell lines and may be higher in untransformed cells than in tumour cells. This may explain the difference in lipid accumulation between the untransformed cells (figure 10) and the tumour cells (figure 11), at a level of vitamin A supplementation of less than 10 μM in this

study. This in turn may partly explain the difference in inhibition of growth of the tumour but not the untransformed cells at this level of vitamin A supplementation. At a higher level of vitamin A supplementation, however, the amount of vitamin A and lipid accumulation may far exceed the metabolic capacity of the cells. This may more drastically disrupt the metabolic functioning of the cells, resulting in a decreased proliferation of both tumour and untransformed cells. More detailed studies will, however, be required to substantiate this proposal.

The cell degeneration observed to a limited extent at low levels of vitamin A supplementation but very apparent, particularly in the tumour cells, at the cytotoxic level (100 μM) of vitamin A, may be due to a disruption of metabolic functioning of the cells, as a result of the lipid accumulation, or may be the result of a more direct effect of vitamin A supplementation on cell membrane structure and function. This cell degeneration would also account for the more marked effect of higher levels of vitamin A supplementation on the rate of proliferation of the different cell types.

The possible effect of vitamin A supplementation on cell membrane structure and function, and in particular, its involvement in fibronectin synthesis and release, will be examined in the next chapter.

CHAPTER 4

EFFECT OF VITAMIN A ADDITION ON THE RELEASE OF FIBRONECTIN BY CULTURED CELLS USING AN ELISA SYSTEM

INTRODUCTION

Fibronectins are high molecular weight glycoproteins (71-74) that are present on many cell surfaces, in extracellular fluids, in connective tissue matrices and in most basement membranes (72-75). The various forms of fibronectin have previously been known by a number of different terms, including LETS protein (large external transformation-sensitive protein), CSP (cell surface protein), CIg (cold-insoluble globulin), CAF (cell adhesion factor) and various others (74-77). Fibronectins have been implicated in a variety of cell properties, such as cell adhesion, morphology, cytoskeletal organisation, migration, differentiation and oncogenic transformation (73).

Fibronectins are composed of similar polypeptide subunits of 220 000 - 250 000 Da that are linked by disulphide bridges into dimers and multimers (71-74,78,79). The cellular and plasma forms of fibronectin are similar but not identical in structure and function. Plasma fibronectin appears to be a dimer while cellular fibronectin occurs both as dimers and multimers (72,74,78,80).

The various domains of fibronectin contain the different binding functions of the molecule, including heparin, fibrin, collagen and cell binding regions. These domains are protease resistant and are linked by flexible protease susceptible polypeptide regions (73,78,79). Fibronectin contains approximately 5% carbohydrate, consisting mainly of complex oligosaccharides linked to asparagine residues (73,75,78). The carbohydrate moiety was suggested to have no major function in secretion of fibronectin, and no detectable effect on a number of the biological activities of the molecule (78). The primary function of the carbohydrate moiety appears to be the stabilisation of specific regions against proteolytic attack (73,75,77,78).

A characteristic common to many transformed cells is a decrease in the amount of surface-associated fibronectin (75,80-82). A number of studies have demonstrated the role of fibronectins in restoring normal morphology, adhesion and cytoskeletal arrangement of cultured cells *in vitro*.

Yamada, Yamada and Pastan (83) isolated CSP (cell surface protein) from chick embryo cells by urea extraction. They found that the protein had agglutinating activity, was sensitive to protease digestion and required divalent cations for activity. They therefore suggested that the molecule played a role in cell adhesion.

In another study, Yamada, Yamada and Pastan (84) added the isolated CSP to a variety of transformed cells *in vitro* (SVT2, NRK and L929 cells). The cells became elongated and flattened and these

morphological changes were antagonised by addition of an antibody to CSP. The cells showed increased adhesion to the substratum and a restoration of contact inhibition on addition of CSP.

Ali, Mautner, Lanza and Hynes (85) added LETS glycoprotein, which was isolated from normal (NIL) cells, to transformed cells (HSV-transformed NIL). They found that certain morphological features and adhesive properties were restored in the transformed cells. In addition, a fibrillar network on the cell surface and the arrangement of actin in cables was also observed in the LETS-treated transformed cells.

Ali and Hynes (86) observed an increase in migration of both normal and transformed cells on the addition of LETS glycoprotein. The glycoprotein attached to the cells in a fibrillar network and binding was greater to normal than to transformed cells.

Yamada, Olden and Pastan (77) isolated and purified CSP from chick embryo fibroblasts and added the protein to 14 transformed cell lines from several different species (chicken, hamster, mouse, rat and human). The results indicated a reversion to a more normal fibroblastic morphology, adhesiveness, cell surface architecture, microfilament bundle organisation, motility and alignment at confluence. The authors suggested that the effects of CSP appear to be due to at least two actions, namely increased cell-substratum adhesion and altered cell-cell interactions.

Smith, Riggs and Mosesson (87) examined various human epithelial cell lines for their expression of fibronectin. In the cell lines derived from nonmalignant tissues or from primary carcinomas, fibronectin was found predominantly in an extracellular matrix, while fibronectin was markedly reduced or absent from extracellular matrices in all cell lines derived from metastatic carcinomas.

Hassell, Pennypacker, Kleinman, Pratt and Yamada (88) investigated the mechanism of fibronectin accumulation by control and vitamin A-treated chick sternal chondrocytes. They found that control chondrocytes, which lack fibronectin as a surface protein, synthesised almost as much fibronectin as vitamin A-treated chondrocytes (which contained cell surface fibronectin), but that it was secreted primarily into the culture medium. Although the fibronectin of the control chondrocytes had a slightly lower molecular weight than that synthesised by the vitamin A-treated cells, it bound to the cell surface of both normal and treated cells. The vitamin A-treated cells, however, were more effective in binding fibronectin synthesised by either control or treated cells. The authors thus proposed that in chondrocytes, vitamin A appears to regulate the cellular accumulation of fibronectin by increasing the ability of the cell surface to bind fibronectin rather than by altering its synthesis. It appears that the synthesis or availability of a specific receptor for fibronectin, which is lacking in chondrocytes, is stimulated by the addition of vitamin A. The authors suggested that the smaller molecular size of the fibronectin in the control chondrocytes may be due to incomplete processing which results in an immature fibronectin. They suggested that retinyl phosphate (a vitamin A derivative) may act as a lipid

intermediate in glycosylation of fibronectin, thus producing a mature fibronectin of proper molecular size in the vitamin A-treated chondrocytes.

Vitamin A is known to play a role in glycoprotein synthesis (1-3,89). Both retinol and a hydroxylated retinoic acid metabolite can be phosphorylated and then reacted with a monosaccharide such as mannose to form a glycosyl (mannosyl) retinyl phosphate (1-3,14). This conjugate is then able to transfer the monosaccharide via a glycosyltransferase to a macromolecular glycoprotein acceptor (such as fibronectin). This reaction takes place in the cell membrane thus allowing the glycosylation of membrane glycoproteins in situ (3).

Tumour promoters such as 12-tetradecanoylphorbol-13-acetate (TPA) elicit a range of biochemical responses in cells in vitro, many of which mimic those observed in cancer or transformed cells. Such responses include decreased cell-substratum adhesion, changes in cell surface glycoproteins and loss of cell surface fibronectin (90,91). Retinoids are known to inhibit many of these TPA-induced effects (90,91). This prompted Bolmer and Wolf (63) to study the effects of TPA and retinoid addition on the loss of fibronectin from cultured mouse 3T3 cells. They found that addition of TPA induced the release of fibronectin from 3T3 and enucleated 3T3 cells into the media. This TPA-induced fibronectin release was inhibited by retinoic acid and retinoic acid derivatives in a dose-dependent manner in both 3T3 and enucleated 3T3 cells. They concluded that at least one aspect of tumour promotion induced by phorbol esters, namely the loss of

fibronectin, does not require the presence of the cell nucleus and further that retinoids can inhibit this aspect of tumour promotion without nuclear involvement.

A study by Kim and Wolf (64) was undertaken to determine whether a nuclear or extranuclear mechanism was involved in the interaction of vitamin A and fibronectin. They found that vitamin A appears to regulate the synthesis of fibronectin through its action on fibronectin mRNA transcription, and propose this to be the first reported observation of an action of vitamin A at the genomic level on the synthesis of a specific protein. Thus, from the same laboratory, we have evidence of vitamin A affecting fibronectin metabolism through both a nuclear and an extranuclear mechanism.

It is thus clear that the absence or presence of vitamin A in varying amounts could have a direct effect on membrane glycoproteins. Of particular importance in this study was the effect of vitamin A on the specific glycoprotein, fibronectin, the synthesis and secretion of which, as described above, is an important factor in the development and growth of transformed cells. The effect of vitamin A on the release of fibronectin was investigated using only the LLCMK and BL-6 cells, as the human hepatoma cell line became contaminated and was unavoidably lost. A further supply of the cell line was unavailable.

MATERIALS AND METHODS

REAGENTS

Retinyl acetate stock solutions as prepared in chapter 2.

Sodium carbonate buffer (0,05 M, pH 9,6) containing 0,02% sodium azide was prepared by adding 66,6 ml Na_2CO_3 (5,3 g/l) to 133,4 ml $NaHCO_3$ (4,2 g/l).

Albumin fraction V (Bovine serum) (Boehringer Mannheim, West Germany) was prepared as a 5% solution in 0,9% saline. The fibronectin content was removed using affinity chromatography (see under methods below). The fibronectin-free albumin was diluted one in ten with 0,9% saline, giving a 5 mg/ml solution, and a further dilution of 12 µl/ml (of this solution) in 0,9% saline was carried out to prepare a 60 µg/ml solution.

Fibronectin standards were prepared from rat fibronectin (Calbiochem-Behring Corporation, La Jolla, California, USA) provided at 0,6 mg/ml. A stock solution of 1000 ng/ml was prepared by diluting 5 µl fibronectin (Fn) to 3 ml with filtered sodium carbonate buffer (SCB), containing 60 µg/ml fibronectin-free bovine serum albumin (Fn-free BSA). A 100 ng/ml stock solution was prepared by a one in ten dilution with filtered SCB (containing 60 µg/ml Fn-free BSA). Fn standards of 5 ng/ml to 60 ng/ml were freshly prepared in SCB (with Fn-free BSA) on the day of use.

PBS-Tween. Phosphate buffer (0,2 M, pH 7,0) containing 9 g/l NaCl and 1 ml/l Tween 20 (polyoxyethylene sorbitan monolaurate, Merck, Schuchardt, West Germany) was prepared by adding 305 ml Na_2HPO_4 (28,39 g/l) and 195 ml $NaH_2PO_4 \cdot 2H_2O$ (31,20 g/l) to 500 ml deionised water.

Goat antiserum to rat fibronectin (Calbiochem-Behring Corporation, La Jolla, California, USA) at 1mg/ml was diluted one in one thousand with PBS-Tween.

Rabbit anti-goat IgG (Sigma, St. Louis, USA) was diluted one in one thousand with PBS-Tween.

Goat anti-rabbit IgG peroxidase conjugate (Sigma, St. Louis, USA) with enzyme activity of 38 purpurogallin units/ml was diluted one in one thousand with PBS-Tween.

Citrate phosphate buffer (0,1 M, pH 4,2) was prepared by adding 58,6 ml $C_6H_8O_7 \cdot H_2O$ (35,85 g/l) to 41,6 ml Na_2HPO_4 (28,39 g/l).

2,2'-Azinobis(3-ethylbenzthiazoline sulfonic acid) (ABTS 0,2 mM) (Sigma, St. Louis, USA) contained 109,74 mg/l ABTS in citrate phosphate buffer, pH 4,2.

Hydrogen peroxide (H_2O_2) (Unilab, Saarchem, Krugersdorp, South Africa) was used at a final concentration of 2 mM.

Preparation of fibronectin-free bovine serum albumin. A 5% solution of BSA fraction V in 0,9% saline was passed through a 9,5 cm X 0,8 cm (internal diameter) affinity column of gelatin-sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The Fn-free BSA was collected and the Fn was eluted from the column with 10 ml of 2,0 M urea.

Preparation of a fibronectin standard curve using an ELISA (Enzyme Linked Immunosorbent Assay) system (92,93). Plastic 96-well flat bottom sterile microtiter plates (with lids) (Dynatech, Laboratory and Scientific Equipment Company, Johannesburg, South Africa) were used for the ELISA assay. Wells were coated with 100 µl of Fn standards (5 ng/ml - 60 ng/ml), with 100 µl of SCB (containing 60 µg/ml Fn-free BSA) as a blank, or with 100 µl of Fn-free BSA (60 µg/ml), incubated at 37°C for 30 minutes and then washed ten times with PBS-Tween. 100 µl of diluted (1/1000) goat anti-Fn was added to the wells and the plate was incubated at 37°C for one hour. Following three washes with PBS-Tween, 100 µl of diluted (1/1000) rabbit anti-goat IgG was added to the wells and the plate again incubated at 37°C for 30 minutes. After ten washes with PBS-Tween, 100 µl of diluted (1/1000) goat anti-rabbit IgG peroxidase conjugate was added to the wells and the plate was incubated for 30 minutes at 37°C. Following a final wash (ten times with PBS-Tween), 100 µl of substrate (0,2 mM ABTS, 2 mM H_2O_2 in citrate phosphate buffer, pH 4,2) was added to the

wells. The enzyme activity was measured after 30 minutes incubation at 37°C by reading absorbance at 410 nm on a Dynatech Mini-reader II (Dynatech Laboratories Inc., Alexandria, Virginia, USA).

Preparation of cells for determination of fibronectin release. LLCMK and BL-6 cells were seeded at 3 X 10^5 cells per 25 cm^2 flask. Growth medium was prepared containing 1 µM, 5 µM, 10 µM and 100 µM vitamin A, as well as a control without ethanol and a control containing ethanol (0,1%). The cells were incubated at 37°C in 10 ml of control or vitamin A-containing medium. The medium was replenished on day 4 and was collected in glass vials on day 6. The culture medium collected from both LLCMK and BL-6 cells treated with the varying concentrations of vitamin A was diluted 1 in 1000 with SCB (containing 60 µg/ml Fn-free BSA). The medium was analysed for fibronectin content (released by the cells) using the ELISA system described above for preparation of a fibronectin standard curve.

RESULTS

The fibronectin standard curve over the range of 5 ng/ml to 60 ng/ml (0,5 ng/well to 6 ng/well) is shown in figure 17. Each point is the mean of six replicate experiments, where each experiment was set up in triplicate (ie 3 wells), and the absorbance of each well was read twice. The standard error of the mean (SEM) bars for the six experiments are indicated in figure 17.

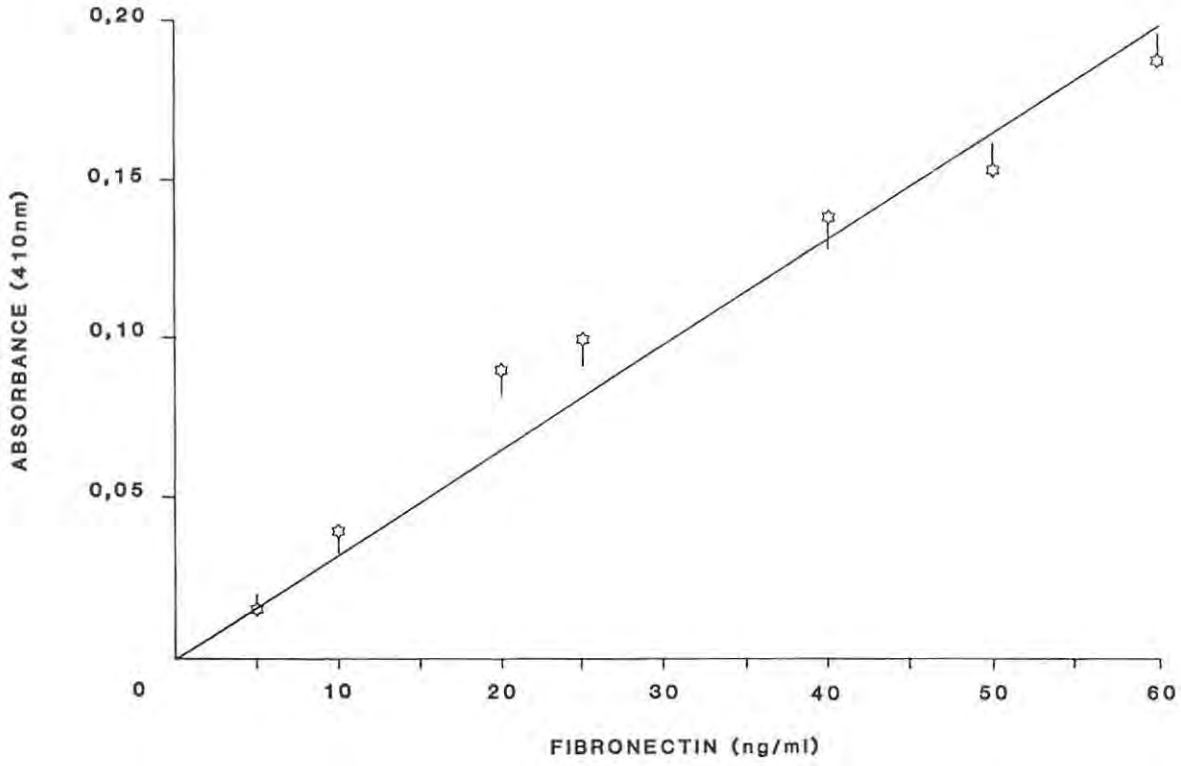


FIGURE 17. Fibronectin standard curve.

The analyses of the fibronectin content of LLCMK and BL-6 cell media, supplemented with varying concentrations of vitamin A, are indicated in Table 5.

TABLE 5. Fibronectin content of LLCMK and BL-6 cell media supplemented with different levels of vitamin A.

	FIBRONECTIN CONCENTRATION (µg/ml)					
VIT. A	0-EtOH	0+EtOH	1µM	5µM	10µM	100µM
LLCMK	34,1	36,4	50,0	39,4	37,5	45,5
BL-6	39,8	52,7	49,6	44,3	39,4	48,1

There appears to be no substantial difference in fibronectin content of the LLCMK cell media or the BL-6 cell media at the various levels of vitamin A supplementation when compared to the controls. A comparison of the fibronectin content of the LLCMK cell media to that of the BL-6 cell media again indicates no substantial differences.

DISCUSSION

Results from this study indicate that vitamin A supplementation to the two cell lines used did not lead to an increased secretion of fibronectin into the cell media. This, however, does not rule out the possibility that vitamin A supplementation may result in increased production of fibronectin in LLCMK and BL-6 cells. Such increased production could result in an increase in fibronectin bound to the cell surface, as found by Hassell, Pennypacker, Kleinman, Pratt and Yamada (88), using *in vitro* cultured chondrocytes. They proposed that

vitamin A appears to regulate cellular accumulation of fibronectin in chondrocytes by increasing the ability of the cell surface to bind fibronectin. They also proposed that the addition of vitamin A resulted in a stimulation of the synthesis or availability of a specific receptor for fibronectin. Further investigations would, however, have to be undertaken with the cell lines used in this study to quantify the fibronectin present on the cell surface before and after vitamin A supplementation, before such proposals could be substantiated for BL-6 and LLCMK cells.

Numerous studies have indicated that fibronectin is lost from the cell surface of many, but not all, transformed cell lines (77,80,82,85,87). This may be due to a lack of fibronectin synthesis, incomplete processing of the glycoprotein molecule or possibly that the fibronectin (whether immature or mature) is secreted into the cell media and does not have the ability to bind to the cell surface (80).

One of the difficulties experienced in assaying for fibronectin in this study was that the fibronectin standard curve has a sensitivity range of 5 ng/ml to 60 ng/ml, which may be too sensitive for the quantification of larger amounts of fibronectin, such as those measured in this study (ranging from 34 μg/ml to 53 μg/ml). Possibly a less sensitive assay system, requiring less dilution of the sample, may be more beneficial in quantifying this level of fibronectin.

In conclusion, different levels of vitamin A supplementation to both LLCMK and BL-6 cells did not appear to have a substantial effect on

the release of fibronectin into the cell media, and there appeared to be no difference in release of fibronectin into the media between the untransformed and tumour cell lines. This would suggest that the effect of vitamin A supplementation on cell proliferation and cellular ultrastructure observed earlier, particularly in the BL-6 cells, did not arise through any effect of vitamin A on fibronectin secretion by these cells. The possibility that such effects could still arise through an effect of vitamin A on fibronectin synthesis and fibronectin binding to the cell surface cannot be excluded. This would, however, require experimentation to quantify such extracellular-bound fibronectin, possibly using some form of urea treatment as suggested by Yamada and colleagues (75,83,84).

CHAPTER 5

DISCUSSION AND CONCLUSIONS

There is a tendency, when developing a hypothesis for a mechanism of action of a metabolic substance, to postulate a single mechanism. However, when surveying the vast amount of literature available on the metabolic and biochemical reactions of vitamin A, several mechanisms of action suggest themselves. With respect to the function of vitamin A in cell growth and differentiation, it has been suggested that its effect is probably exerted through an association with the cell nucleus and mRNA, and/or an effect on the cell surface, possibly mediated through an involvement of vitamin A in glycoprotein synthesis (1).

The effect of vitamin A addition on cell growth in the different cell lines used in this study, clearly showed a substantial inhibition of tumour cell growth at low non-toxic levels of vitamin A. This effect was particularly evident in the BL-6 cell study when using the counting plate technique. Here, significant inhibition of cell growth on addition of 5 μM and 10 μM vitamin A was observed from day 3. Unfortunately, the human hepatoma cell line was lost prior to studies on cell growth using the counting plate, and thus the inhibition of growth of this cell line on addition of vitamin A could not be similarly quantified. The use of the haemocytometer in the study of human hepatoma cell growth inhibition resulted in large standard error of the mean (SEM) values, hence reducing somewhat the importance of the inhibition observed. However, the substantial reduction in cell

growth of the human hepatoma cell line when compared to the optimum growth of this cell line at 1 μM added vitamin A, showed that, indeed, the inhibition of growth was substantial, even if not of statistical significance.

The BL-6 cell growth inhibition study (experiment (a) of Table 3) using the haemocytometer showed a higher percentage of growth inhibition at the concentrations of 5 μM, 10 μM and 100 μM vitamin A supplementation than the duplicate experiment ((b) of Table 3). However, these differences in cell growth, when compared to the control, although substantial, were not statistically significant, due again to the very large SEM values obtained using the haemocytometer. The inhibition at 10 μM vitamin A supplementation (60% in experiment (a), Table 3) showed a statistical significance of difference at the level of $p < 0,1$ but this was not reported as being statistically significant, as the rest of the studies were quantified at the level of $p < 0,05$. By comparison to the effect on the two tumour cell lines, addition of vitamin A to the untransformed LLCMK cells elicited only a minor effect on cell growth.

The addition of 100 μM vitamin A, however, had a marked effect on the growth of all three cell lines, both tumour and untransformed. This level of vitamin A supplementation appeared to be cytotoxic to the cell lines in this study. This was verified by the transmission electron microscopy undertaken to examine the ultrastructural effects of the supplemented vitamin A on both the tumour and untransformed cells. The large lipid droplets which accumulated in all three cell

lines at this high level of vitamin A supplementation were thought to disrupt the metabolic functioning of these cells, possibly due to a "lipid overload". In mammalian systems, the fat-soluble vitamin A tends not to exist in the free state, but is found both extracellularly and intracellularly bound to a transport protein (1). This protein serves to protect the cells from the destructive effect of the fat-soluble vitamin and helps to solubilise the vitamin. Thus in the cells in this study, the intracellular retinol binding protein was either not produced, or was produced in amounts far too low to cope with the excessive amount of vitamin A administered to the cell media. Hence, the destructive effects observed at the high cytotoxic level of vitamin A supplementation.

At the low non-toxic levels of vitamin A supplementation, the tumour cells were found to be more susceptible to lipid accumulation than the untransformed cells, an effect which correlated well with the cell growth studies. In control (unsupplemented) cells, both tumour and untransformed, small lipid droplets were observed. These droplets may contain other fat-soluble components, but may also contain small amounts of vitamin A, possibly present in the foetal calf serum in the growth medium. These small lipid droplets may play a vital role in maintaining normal metabolic functioning of the cells, possibly even exerting their effects through the cell nucleus, and then subsequently being metabolised. However, a critical level may be reached, where the cell can no longer cope if excess lipid, for example vitamin A, enters the cell. This may result in accumulation of lipid as lipid droplets as was observed in the tumour cells at low non-toxic levels of vitamin A supplementation. This may result in an effect on general

cell metabolism and/or on the cell nucleus and thus on transcription and translation, leading possibly to an effect on further production of retinol binding protein, for example. The cumulative effects may thus contribute to cell degeneration, as seen at the cytotoxic levels of vitamin A in this study. Obviously a great deal of further experimentation is required to substantiate these proposals.

Kim and Wolf (64) have suggested that at low physiological levels, vitamin A may exert an effect on the regulation of synthesis of various proteins, such as fibronectin. An increase in vitamin A supplementation may thus increase the synthesis of fibronectin or the availability of receptors for fibronectin binding on the cell surface (as discussed by Hassell, Pennypacker, Kleinman, Pratt and Yamada (88)), thus resulting in increased adhesiveness of cells and a restoration of density dependent growth in tumour cells - in other words, a reduction in the number of tumour cells, such as that found in this study.

The vitamin A supplementation, at the non-toxic levels, may also exert an extranuclear effect, such as the glycosylation of cell surface glycoproteins, for example fibronectin. Many tumour cells are characterised by the absence of cell surface fibronectin, which may either be due to a lack of synthesis or possibly due to secretion into the cell media. While this study was inconclusive concerning the effects of vitamin A on fibronectin release in cultures of tumour and

untransformed cells, the possibility cannot be ruled out that an effect may have occurred at the cell surface level, (for example on fibronectin receptors), which was not detected by the assay system used.

In conclusion, while vitamin A supplementation to tumour cells in particular was found to have a substantial effect on cell growth, no mechanism of action of vitamin A on the inhibition of this cell growth was unequivocally determined. Various mechanisms were proposed, based on the results of this and other studies, but these would require further substantiation. However, the suggestion remains that vitamin A probably has no single mechanism of action on cell growth, but at least two mechanisms, one involving an extranuclear effect and one through an effect on the cell nucleus and protein synthesis.

SUMMARY

Vitamin A and its chemical analogues (retinoids) are known to play a role in the maintenance and differentiation of epithelial tissue. Retinoids have been shown to inhibit carcinogenesis in a number of tissues in experimental animals and to inhibit the growth of various untransformed and cancer cell lines *in vitro*.

This study investigated the effect of retinyl acetate supplemented at concentrations of 1 μM, 5 μM, 10 μM and 100 μM to *in vitro* cultured untransformed LLCMK cells, and transformed BL-6 melanoma and human hepatoma cell lines. A small but non-significant effect of vitamin A addition on the growth of the untransformed cells was observed, while substantial inhibition of proliferation of the two tumour cell lines was found. At the cytotoxic level of 100 μM supplemented vitamin A, all three cell lines showed marked inhibition of growth. This led to an electron microscopy study to examine the ultrastructural effect of the vitamin A addition.

At the low non-toxic levels of vitamin A addition (1 - 10 μM), no ultrastructural changes were observed in the untransformed cells. However, at a level of 5 μM and 10 μM vitamin A addition in the tumour cells, an increase in the size of suspected lipid droplets was observed. At the cytotoxic level of 100 μM supplemented vitamin A, large lipid droplets were very apparent, as was much cellular degeneration. This effect was more marked in the tumour cells than in the untransformed cells. The lipid nature of the droplets was confirmed by using the lipid stain, Sudan IV.

In order to investigate the effect of added vitamin A at the cell surface level, an ELISA system was used to quantify the level of the cell surface glycoprotein, fibronectin, in the culture media. Vitamin A plays an important role in the production of mature fibronectin by participating in the glycosylation of the molecule. This study showed no major effect of added vitamin A on the release of fibronectin into the culture media. This did not, however, exclude the possibility that the vitamin A was involved in the production and enhanced binding of fibronectin to the cell surface, and was possibly also exerting an effect on the availability of fibronectin receptors. Further studies would, however, be required to substantiate such effects of vitamin A supplementation.

No single mechanism of action of vitamin A on tumour cell growth inhibition was identified, but the possibility that at least two mechanisms exist, was suggested.

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