GLUCOCORTICOID-INDUCED CYTOLYSIS OF HUMAN LYMPHOBLASTOID CELLS

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DECLARATION

This thesis has been composed entirely by myself.

The work described was undertaken as part of the programme of a research team. The results are almost entirely my own work, and any results or information contributed by other members of the group are clearly acknowledged.

Edinburgh, January, 1977.

ACKNOWLEDGEMENTS

I am deeply indebted to the many people who have contributed in a variety of ways to this thesis. My supervisors have been Professor Alastair Currie and Professor Colin Bird. Professor Currie, as head of department, has made all the research facilities I have used available to me, and as grant-holder has been responsible for the overall strategy of the programme and the financial provisions with which to carry it out. He has given me very considerable encouragement and support throughout the three years, and I have been most grateful for his help in the selection of material for this thesis and his guidance in its construction. Professor Bird was my supervisor on a day-to-day basis for the first two years of the work before his departure to Leeds. He devoted many hours selflessly during the establishment of my project, and aided me with the innumerable problems which are associated with the establishment of a new experimental system. I owe a lot to his teaching and personal example, and am privileged to have had him and Professor Currie as my mentors in this work.

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in this thesis, and I thank her for making her data and electron micrographs available to me.

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SUMMARY

Glucocorticoids cause the regression of certain human leukaemias. As an *in vitro* model for this effect, I studied the action of methylprednisolone on human lymphoblastoid cells. The cell line mainly used was BLA_1, which was derived from a patient with acute lymphoblastic leukaemia.

At 500µg/ml, methylprednisolone causes lysis of BLA_1 cells after 48h exposure. The rôle of cytoplasmic glucocorticoid receptors in this cytolysis was investigated, and it was found that methylprednisolone-induced cytolysis is independent of receptor concentrations.

Methylprednisolone causes reduction in the uptake of L-pathway amino acids and/in the facilitated diffusion of uridine and thymidine, while stimulating A-pathway uptake of amino acids, deoxyglucose and simple diffusion of uridine and thymidine. Again, there is no evidence to implicate glucocorticoid receptors in these processes.

The mean volume of BLA_1 cell populations is reduced on exposure to methylprednisolone, and this appears to be the result of shrinkage of the cells and the appearance of small subcellular fragments. Alterations in the shape of cells are also found. There is an increase in the number of cells showing "blebs" on their surface which is maximal 2h after treatment./

treatment. There is a later increase in the number of enlarged dead cells in the population. HeLa cells show similar changes, though somewhat more slowly.

Lysis by methylprednisolone appears to be a widespread phenomenon in actively growing cell cultures. Attempts to grow cells resistant to 500µg/ml methylprednisolone were unsuccessful, although human red blood cells have increased stability *in vitro* at this concentration.

The results of this work indicate that after treatment morphological changes typical of at least some stages of apoptosis and coagulative necrosis may be detected. It would appear that the effects that have been observed are mediated by changes induced in the surface of the cells, and there is no evidence that the "steroid-receptor-gene activation" model of hormone action applies.

The relationship between the effects described and cell death by apoptosis is discussed.

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INTRODUCTION

There are three main factors involved in the determination of the growth rate of a tumour or tissue: (a) cell cycle time, (b) growth fraction and (c) death, removal or loss of cells. Of these cell death is the least extensively studied, and it is with this that I am primarily concerned.

The two main types of cell death that have been described are coagulative necrosis and apoptosis. Coagulative necrosis affects large numbers of cells and is usually the response of an area of tissue to a noxious stimulus resulting in the breakdown of homeostatic control mechanisms (Trump & Ginn, 1969). It is typified by the gradual swelling and dissolution of the cells. A second type of cell death involving the shrinkage of the cells and pyknosis of nuclei had been described, but its biological importance was poorly understood. It was not until Kerr, Wyllie & Currie (1972) investigated this phenomenon of shrinkage necrosis (christened "apoptosis" by them) that its range of occurrence and physiological importance was appreciated. They postulated that apoptosis was at least in many cases a physiological form of single cell death, and was intimately involved in the naturally occurring cell loss from normal and malignant tissues.

It is unnecessary to give a full description of apoptosis here/

here, but the most important aspects of the process are (1) it affects only random single cells in a tissue while the adjacent cells remain viable and (2) it occurs in normal tissue, untreated tumours, tumours undergoing regression after certain types of therapy, ontogenesis and teratogenesis. It therefore appears to be a widespread event involved in the control of cell population size, and its induction by certain anti-tumour agents indicates its probable importance in tumour regression.

Whilst the occurrence and morphology of apoptosis in vivo are now well established, little is known about the earliest events in the process or the intracellular mechanisms by which it is mediated and controlled. Our intention is to investigate the factors that initiate and control the extent of apoptosis and the biochemical changes in the cells involved while elucidating the exact sequence of ultrastructural changes. The frequency of apoptosis appears to be regulated in such a manner that it acts as a balance against mitosis to maintain cell population size. It is difficult to study the factors that cause individual cells to die in an apparently random manner. Equally, when investigating the biochemistry and morphology of the process it is desirable to be able to predictably induce apoptosis so that the earliest alterations in the cells may be observed. There are systems known for its induction in vivo, but/

but it is easier to define experimental conditions precisely in relation to concentrations of lethal agents and cellular interactions in a test tube than in an animal. We therefore set out to establish an *in vitro* system in which apoptosis could be predictably induced.

Glucocorticoids have lethal effects on lymphoid cells and tissues, especially in rodents (Claman, 1972). Two systems which have been examined in detail are rat thymus and certain mouse lymphomas (Dougherty, 1952; Harris, 1970). Most of the work on rodent lymphoid cells has been directed towards the elucidation of the biochemical action of steroids and the morphological and ultrastructural changes induced have received scant attention. As human lymphoid cells are fairly resistant to glucocorticoids (Claman, 1972) there has been little work on human material. However, certain human leukaemias and lymphomas may be successfully treated by glucocorticoids in combination with other drugs (Simone, 1974). As induction of apoptosis plays an important part in the regression of certain tumours (Kerr, Wyllie & Currie, 1972) it seemed possible to us that human leukaemic cells treated with glucocorticoids might prove a suitable model for the study of this form of cell death.

There are certain problems involved in the use of fresh human material. Cells may not be available regularly, and it especially difficult to obtain material that has not been exposed to drugs. There is a finite limit to the amount of material/

material that may be obtained from a single patient without causing stress or unnecessary discomfort, not to mention the ethical problems involved. Also, not withstanding how similar lymphoid tumours may appear on histological examination, they may vary widely in cell type (Habeshaw, Macaulay & Stuart, 1976), which causes problems in the interpretation of data obtained with cells from different patients.

It was essential to develop a system in which a population of human cells of uniform cell type could be readily and repeatedly obtained. Human lymphoblastoid cell lines (Nilsson & Pontèn, 1975) were used for this purpose. The cells came from the peripheral blood lymphocytes or lymphoid tissues of patients with various conditions, and were established in permanent culture either spontaneously or by co-cultivation with lethally irradiated cells containing Epstein-Barr virus (Pulvertaft, 1965; Steel, 1972). The sources and karyotypes of the lymphoblastoid cells used in this thesis are given in table I. All of the lines express B-cell characteristics: they synthesise immunoglobulins (Evans, Steel & Arthur, 1974), have C^3 receptors on their surfaces (Moore & Minowada, 1973), lack receptors for sheep red blood cells (Evans, Smith & Steel, 1975) and do not exhibit cytotoxic activity (Steel, Hardy, Ling & Lander, 1974). Some have already been shown to undergo a reduction in growth rate when/

Table I Origin and Karyotype of Human Lymphoblastoid Cell Lines

Cell line	Origin	Modal karyotype
RUS_1	Acute myeloblastic leukaemia	48XY 18p+
RUS_2	Acute myeloblastic leukaemia	48XY 3/8 translocation
BLA_1	Acute lymphoblastic leukaemia	46XY multiple breakages and recombinations
F89	Subacute lymphatic leukaemia	48XY multiple breakages and recombinations
GS_1	Chronic lymphatic leukaemia	48XX multiple breakages and recombinations
JIJOYE	Burkitt's lymphoma	Near tetraploid; many aberrations
EB_1	Burkitt's lymphoma	Polyploid; many aberrations
EB_4	Burkitt's lymphoma	47XX- many aberrations
PEN_2	Adult blood (Klinefelter's syndrome)	48XXY 14+
YAK_1	Cord blood	47XY partial trisomy 4

when exposed to glucocorticoids (Nilsson, 1971). We, therefore, investigated the effects of the glucocorticoid hormone, methylprednisolone sodium succinate (MPS) on human lymphoblastoid cells.

Little is known about the sequence of biochemical changes leading to cell death, and we are aware of no study in depth in which morphological and biochemical observations have been combined. Alison Robertson has established the ultrastructural changes induced by MPS while I have investigated some of the biochemical and cell surface effects. These are (1) the rôle of cytoplasmic glucocorticoid receptors in the process, (2) the effects of MPS on the entry of certain substrates into cells and (3) the effects of MPS on cell shape and volume.

STUDIES ON CYTOPLASMIC GLUCOCORTICOID RECEPTORS

Introduction

A model for steroid hormone action has been proposed by Jensen & De Sombre (1973) (figure 1). This involves the entry of the steroid into the cells (probably by passive diffusion in the case of glucocorticoids) and its binding to a specific cytoplasmic receptor protein. The newly-formed hormone-receptor complex then undergoes thermodynamic activation (which can only occur at 20°C or above in whole cells) and migrates to the nucleus where it causes alterations in gene activity. The altered gene products mediate the final steroid response. Thus, if this model is to apply to any particular system, hormone action will be totally dependent on the presence of cytoplasmic receptor molecules.

Work on rodent lymphoid cells has confirmed this as one mode of action of glucocorticoids. Cytoplasmic glucocorticoid binding proteins have been detected in rat thymocytes (Wira & Munck, 1970; Bell & Munck, 1973; Kaiser, Millholland & Rosen, 1973) and in steroid-sensitive mouse lymphomas (Baxter, Harris, Tomkins & Cohn, 1971). The nuclear acceptor site is chromatin, and almost certainly DNA since DNase destroys nuclear binding (Higgins, Rousseau, Baxter & Tomkins, 1973). In HTC (rat hepatoma) cells glucocorticoids/

Figure 1

Model of Steroid Hormone Action (Jensen & De Sombre, 1973)

Abbreviations:-

H	= Hormone (steroid)
R	= Receptor (soluble protein)
H-R	= Hormone - receptor complex
H-R'	= Acitvated hormone - receptor complex
hn RNA	= Heterogeneous nuclear RNA
m RNA	= Processed messenger RNA

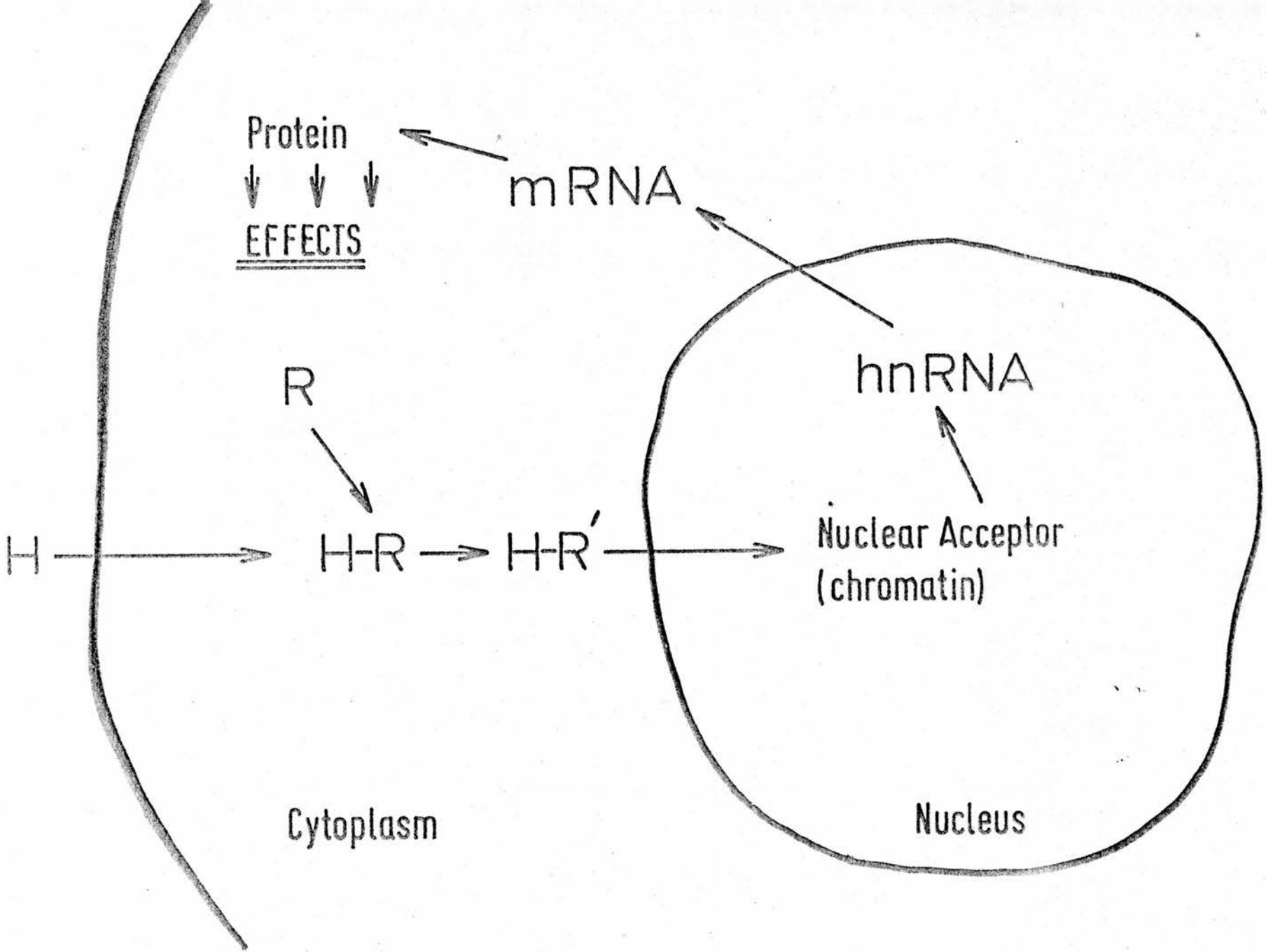


Figure 1 Model of Steroid Hormone Action

glucocorticoids induce tyrosine aminotransferase (Baxter & Tomkins, 1970) whereas in L (mouse fibroblast) cells they cause a general reduction in macromolecular synthesis (Pratt & Aronow, 1966). The glucocorticoid receptors from HTC cells bind to a different nuclear acceptor site from the receptors from L cells (Lippmann & Thompson, 1973) which suggests that there is a specific nuclear acceptor for each biological effect of steroids. Work by all of these authors indicates that there is only one species of cytoplasmic glucocorticoid receptors in each cell.

Sibley & Tomkins (1974) analysed several steroid-resistant lines cloned from the initially steroid-sensitive S49 mouse lymphoma. They found that most of the resistant lines either lacked cytoplasmic receptors or the receptors were present but were unable to bind glucocorticoids. A second smaller number of lines bound steroid to the receptor but the complex did not migrate and bind to the nucleus, probably either through defective thermodynamic activation of the complex or inactivated nuclear acceptor sites. In a third group the hormone was successfully translocated and bound in the nucleus but did not exert its final effect through some fault in gene activation or the gene products.

It was claimed by Lippmann et al (1973) that the presence of cytoplasmic receptor molecules could be related to the steroid responsiveness of human tissues. They found that patients with steroid-/

steroid-responsive acute lymphoblastic leukaemia had cytoplasmic receptors in their circulating leukaemic lymphocytes whereas there were none in lymphocytes from normal volunteers. When the patient's peripheral blood white cell count increased with a recurrence of the disease, the presence of cytoplasmic receptors correlated with steroid-induced remission, whilst if the leukaemic lymphocytes did not have receptor activity the disease did not respond to glucocorticoid therapy. In all cases glucocorticoids reduced incorporation of thymidine into DNA *in vitro* only in cells which had receptor activity. At the same time, Gailani *et al* (1973) working with both established lymphoblastoid cell lines and fresh human cells had mixed success in relating receptor activity to steroid sensitivity and commented that "measurement of steroid binding in human lymphoid tissues may not give an accurate assessment of glucocorticoid sensitivity". The following year Lippmann, Perry & Thompson (1974) were forced to modify their original findings, stating that "the presence of specific cytoplasmic receptor proteins for glucocorticoids in malignant tissues does not appear to guarantee steroid responsiveness". This followed their discovery of cytoplasmic receptors in three steroid-resistant lymphoblastoid cell lines - one murine and two human.

It was therefore of importance to investigate the role of cytoplasmic receptor proteins in the glucocorticoid-induced cytolysis of human lymphoblastoid cells. Since rat thymocytes were known/

known to undergo cytolysis at low concentrations of glucocorticoids *in vivo* and have cytoplasmic receptors, they were included as controls.

Materials and Methods

Cell culture

Cells were grown as suspensions in flasks or in roller culture in Eagle's minimum essential medium (MEM) or Ham's F10 medium with 10% tryptose phosphate broth, buffered with $NaHCO_3$ (20mM) and supplemented with 20% foetal calf serum (FCS) which had been inactivated by heating to 56°C for 1h. (This destroys any glucocorticoid binding capacity of globulins in the serum.) Cells were grown at 37°C in an atmosphere of 5% carbon dioxide 95% air. Maximum growth of cells was obtained by feeding every 2 - 3 days to maintain their concentration at between 0.2 and 1.0 x 10^6/ml.

All materials were obtained from Gibco-Biocult Ltd.

Cytotoxic assays

Duplicate samples of cells were fed with medium to a concentration of 0.2 x 10^6/ml. After 24h, when the cells were in the log phase of growth, methylprednisolone sodium succinate (MPS: Solumedrone, Upjohn) was added in aqueous solution at 1% v/v to give the appropriate final concentration. After incubation for a further 48h the cells were counted either by haemocytometer or on a Coulter counter, model ZF, and their viability assessed by their ability to exclude nigrosine (0.25%). Lysis was calculated as/

as a percentage of the viability of control cells receiving water instead of steroid.

Steroid binding by cytosol extracts

The method was based on the competitive binding assay of Baxter & Tomkins (1971). 3 - 5 x 10^8 cells were harvested by centrifugation (800 x g for 10min), washed twice in phosphate-buffered saline (PBS, Oxoid: 25mM KH_2PO_4, 100mM NaCl, pH7.4) at 0 - 4°C, resuspended in 1vol ice-cold tricine buffer (20mM tricine (N-tris(hydroxymethyl)methyl glycine), 2mM $CaCl_2$, 1mM $MgCl_2$, pH7.4: British Drug Houses) and homogenised. Rat thymuses were excised aseptically, blotted dry, chopped finely with scissors and homogenised in 1vol ice-cold tricine buffer. The cell homogenates were centrifuged at 105,000 x g for 1h at 4°C and duplicate 0.4ml samples of supernatant ("cytosol") were incubated at 0°C with various concentrations of (1,2(n)-^{3}H)-dexamethasone (Radiochemical Centre, Amersham: 19-29Ci/mmol) in the presence or absence of a 1,000 fold excess of unlabelled dexamethasone (Sigma, London). Unbound steroid was removed after 2h by the addition of 50µl activated charcoal (200mg/ml: British Drug Houses) which was vigorously agitated and centrifuged (600 x g for 1min). The supernatant was centrifuged (10,000 x g for 5min) to remove any residual charcoal, and 200µl samples of supernatant were assayed for radioactivity in toluene:triton X-100 scintillant (2:1 v/v,

5g/

5g/l butyl PBD) in a Beckman LS-250 liquid scintillation spectrometer. Efficiency corrections were made by the external standard ratio method. Specifically bound dexamethasone represents the difference between the amount of ^{3}H-dexamethasone bound in the presence and absence of a 1,000 fold excess of unlabelled dexamethasone. Protein concentrations were measured colorimetrically by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standards.

RESULTS

Cytotoxic assays

The extent of glucocorticoid-induced cytolysis was determined for a range of human lymphoblastoid cell lines in conjunction with other workers in the group (table II). Lysis at MPS concentrations below 500μg/ml was never greater than 20%, but all cell lines showed a marked increase in % lysis at 500μg/ml. The cell lines fall into three categories; those of high sensitivity (RUS_2, YAK_1, PEN_2, BLA_1 and RUS_1), those of intermediate sensitivity (F89 and GS_1) and JIJOYE which is less sensitive than any other cell line. Further work has shown that increasing the concentration of MPS above 500μg/ml causes increased lysis of JIJOYE cells, and the difference between cell lines reflects, in fact, different thresholds of sensitivity.

Cytoplasmic receptors

Assaying the amount of dexamthasone specifically bound with increasing dexamethasone concentration showed that the receptors from two representative cell lines and rat thymus saturated in the range 50 - 80nM (figure 2). Scatchard (1949) described a method of analysis for the interaction of small molecules with proteins, and the calculation of dissociation constants at equilibrium. Using this analysis, the linear relationship reveals that there is a single class of receptors, and that they have a uniform/

Table II

Cytotoxic Effect of MPS and Concentrations of Cytoplasmic Receptors

MPS(μg/ml)	0.05	0.5	5	50	500	Specific Binding (pmol/mg protein)
Cell Line	% Lysis					
RUS_1	7	17	15	18	74	0.82
RUS_2	12	10	9	10	88	0.66
BLA_1	0	5	1	2	76	0.62
F89	4	3	2	5	60	0.16
GS_1	8	7	6	6	54	0.71
JIJOYE	3	3	3	4	19	0.43
PEN_2	2	9	12	6	83	0.09
YAK_1	15	16	24	19	84	0.37
Rat thymus*						0.33

* Thymuses taken from female PCG/C rats, aged 88 days.

All assays were performed as in Materials and Methods (pages 13 - 15).

Results are the Means of at least two independent measurements in each case.

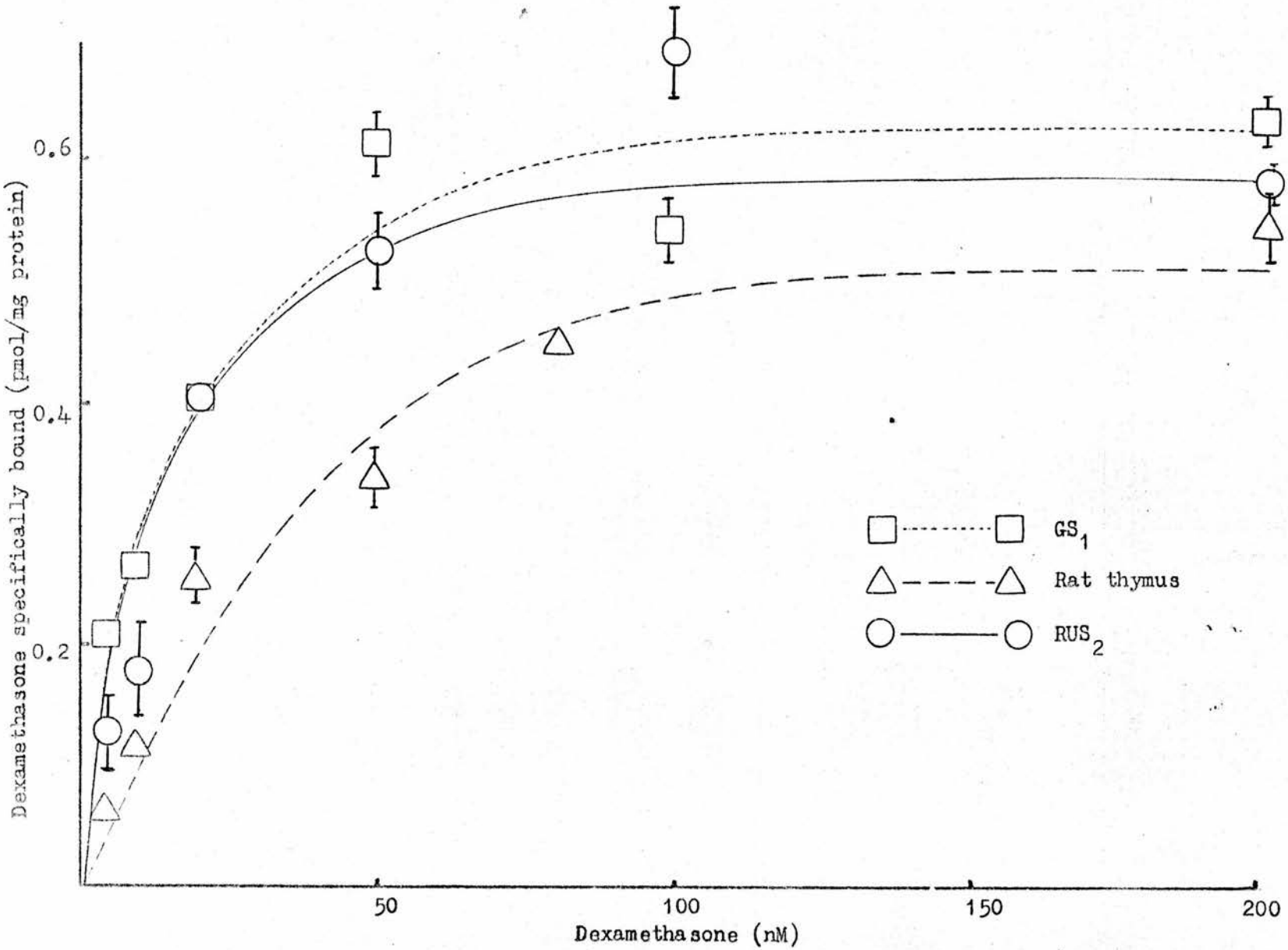


Figure 2 Specific Binding of Dexamethasone to Cytoplasmic Receptors

uniform steroid affinity (figure 3). The dissociation constants are 1.37 x 10^8 M/l for RUS_2, 5.04 x 10^8 M/l for GS_1 and 1.35 x 10^8 M/l for rat thymus.

Binding of glucocorticoids was completely destroyed by incubation at 37°C for 30min, or by incubation with trypsin (1mg/ml) or protease (1mg/ml) at 20°C for 10min. Incubation under the same conditions with deoxyribonuclease (bovine pancreas; 100µg/ml) or ribonuclease (bovine pancreas; 100µg/ml) had no effect on binding. Thus, the glucocorticoid receptors in human lymphoblastoid cells are thermolabile proteins with very similar characteristics to those described by workers studying other systems (Hackney, Gross, Aronow & Pratt, 1970; Baxter & Tomkins, 1971; Munck & Wira, 1971).

Using saturating concentrations of dexamethasone (80nM), the cytoplasmic specific binding capacity of several cell lines was determined (table II). There is a large range of values with the highest (0.62 - 0.82 pmol/mg protein) in cells derived from patients with acute leukaemias (RUS_1, RUS_2 and BLA_1) and one chronic lymphatic leukaemia (GS_1). Intermediate values were found with the Burkitt's lymphoma derived cell line, JIJOYE, (0.43 pmol/mg protein) and in YAK_1 (0.37 pmol/mg protein) which was derived from normal cord blood. F89 (0.16 pmol/mg protein) from a subacute lymphatic leukaemia and PEN_2 (0.09 pmol/mg protein) from the blood of an adult with Klinefelter's syndrome, had the lowest levels.

Figure 3

Scatchard Analysis of Dexamethasone Binding

Scatchard (1949) used the Law of Mass Action expressed as:-

$$\frac{v}{c} = K(n - v)$$

where v = the concentration of small molecules bound, n = the maximum concentration of small molecules that can be bound, c = the concentration of free molecules and K = the dissociation constant. Plotting v/c (ratio of bound/free small molecules) against v, when v = 0, v/c = Kn, and when v/c = 0, v = n. Therefore, the maximum binding capacity is the intercept on the v axis and the dissociation constant is the intercept on the v/c axis divided by n.

Lines were fitted to the points by the "least squares" method. Correlation co-efficients are 0.89 (rat thymus), 0.91 (RUS_2) and 0.98 (GS_1)

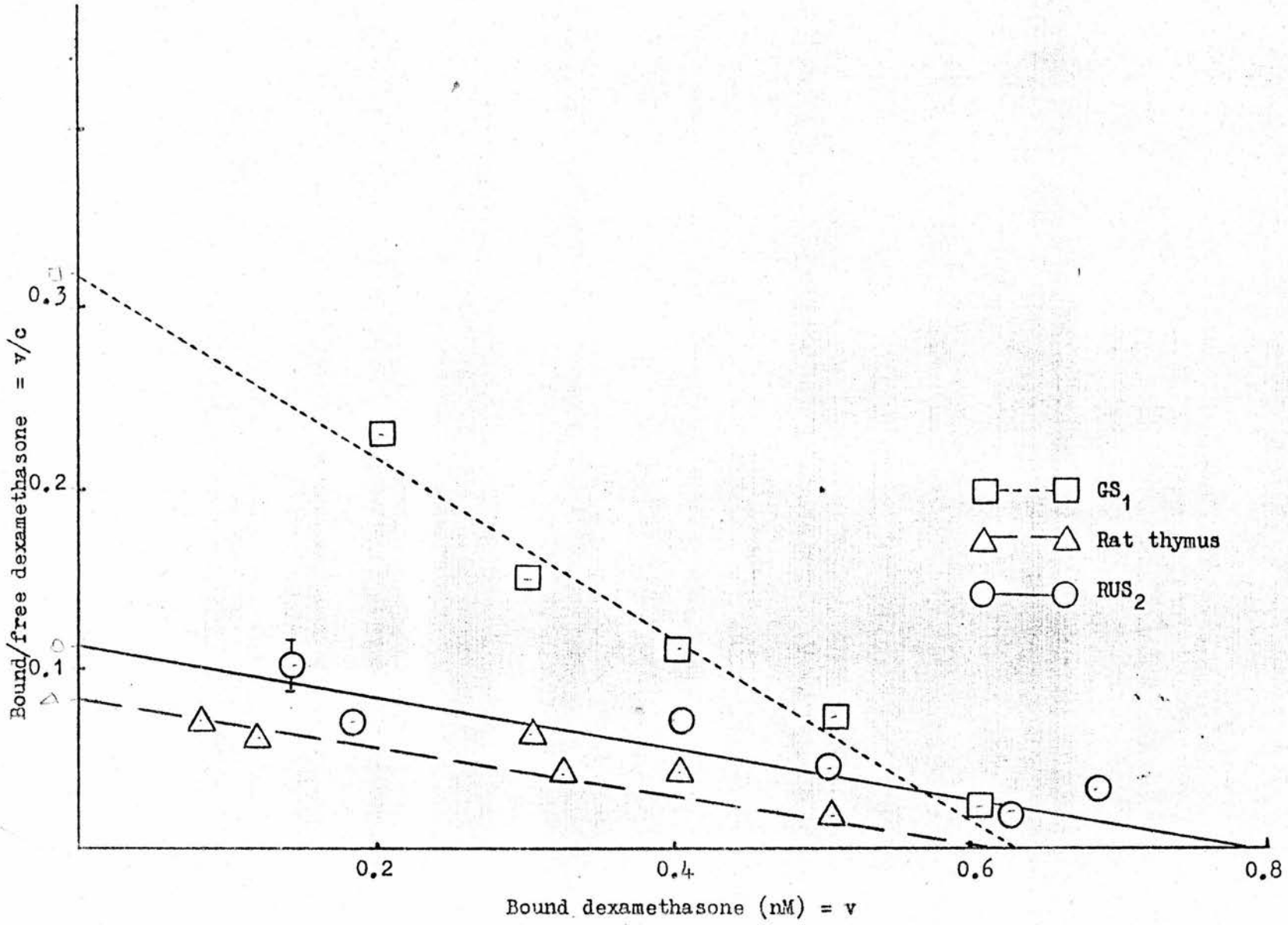


Figure 3 Scatchard Analysis of Dexamethasone Binding

COMMENT

Initially we found considerable variability in the results of the assay for glucocorticoid binding. Six assays performed on BLA_1 cells within a four week period gave results of 0.03, 0.01, 1.35, 0.74, 0.01 and 0.83 pmol/mg protein. Dexamethasone is dissolved in ethanol, and there could be up to 2% v/v ethanol in the final incubation mixture. Binding of dexamethasone was affected in an unpredictable manner if the ethanol concentration exceeded 1%, and it was only when we discovered this after several months' work that the assay worked reliably. All other factors were kept constant, and did not affect the results.

From examination of the data in table II, one central fact emerges: there is no quantitative relationship between the concentration of cytoplasmic receptors and the cytolethal effect. At 500 μg/ml MPS, PEN_2 cells with 0.09 pmol/mg bound show slightly greater cytolysis than RUS_1 with 0.82 pmol/mg. The concentration of steroid required for cytolysis is 10,000 times greater than that which saturates receptors. This could be accounted for by hindrance of access of steroid to cytoplasmic receptors in the intact cells, but further work has shown that receptors saturate at the same concentrations in both intact cells and cell-free extracts.

On this evidence, it is difficult to implicate cytoplasmic receptors in the cytolytic effect we observe <u>in vitro</u>.

EFFECTS OF MPS ON UPTAKE OF SUBSTRATES

Introduction

Inhibition of synthesis of macromolecules in lymphoid cells treated with glucocorticoids has been known for some time (White, Hoberman & Szego, 1948). Research has centred on the uptake and incorporation of carbohydrates, amino acids, nucleosides and lipids. Mainly rodent cells - in particular rat thymus (Makman, Dvorkin & White, 1966; 1968) and mouse lymphoma P 1798 (Rosen, Fina, Millholland & Rosen, 1972; Stevens, Stevens & Hollander, 1973; 1974) - have been studied.

There is an extensive literature on the effects of glucocorticoids on the entry of substrates into lymphoid cells. Rosen & Millholland (1975) have written a comprehensive review, and it would merely add to the complexity of this thesis without giving additional clarity if a detailed account of what is known were to be given here. However, the underlying findings are that amino acid, nucleoside and carbohydrate uptake into rodent lymphoid cells is inhibited by glucocorticoids at cytolethal concentrations. It has been reported that thymidine and leucine uptake (Rosen, Fina, Millholland & Rosen, 1972) and glucose uptake (Feldman, Kraetsch, Licthman & Peck, 1974) may be unaffected in certain cases, but these appear to be the exceptions to the general rule.

Since/

Since we had demonstrated the dose-dependent cytolytic action of MPS on human lymphoblastoid cells, it was of interest to investigate its effects at lytic concentrations on substrate uptake into these cells.

Materials and Methods

Materials

2-aminoisobutyric acid, 2-deoxy-D-glucose, L-leucine, thymidine, uridine (Sigma, London), 2-amino[1-^{14}C]isobutyric acid (59mCi/mmol), 2-deoxy-D-[1-^{3}H]glucose (19Ci/mmol), L-[4,5-^{3}H]leucine (60Ci/mmol), [6-^{3}H]-thymidine (26Ci/mmol) and [5-^{3}H]uridine (25Ci/mmol) were all prepared and added as aqueous solutions.

Uptake and incorporation of substrates

Assays were performed in two ways:-

1) Whole cell uptake. At the end of each assay, 8ml ice-cold PBS containing 1mM unlabelled substrate as a "chase" were added to each tube in ice. The cells were rapidly placed on to Millipore filters (HAWP, pore size 0.45μm) in a Millipore sampling manifold and washed with 40ml ice-cold PBS at a flow rate of 10ml/min. Filters were dried overnight and counted in 10ml toluene:butyl PBD (5g/l) scintillant.

2) Uptake into acid-soluble and -insoluble pools. When more detailed information on the distribution of substrate between acid-soluble and -insoluble pools was desired, cells were fractionated with ice-cold 5% trichloroacetic acid (TCA).

At/

At the end of each assay, the tubes were placed in ice and 8ml of ice-cold PBS added to each. Cells were washed by centrifugation at 400 x g for 3min followed by resuspension in 10ml ice-cold PBS and recentrifugation. The pellet was carefully drained, and resuspended in 1ml ice-cold 5% TCA. Insoluble material was sedimented by centrifugation at 2,500 x g for 10min at 4°C. 0.5ml of the supernatant was carefully removed and counted in 10ml toluene:triton scintillant. The entire acid-insoluble pellet was carefully drained and dissolved in 1ml water. It was counted in 10ml toluene:triton scintillant.

To minimise changes in pH during experiments, 20mM HEPES (2-[N-2-hydroxyethylpiperazine-N'-yl]ethanesulphonic acid, Gibco-Biocult) was substituted for sodium bicarbonate as a buffer in the media and Hank's balanced salts solutions.

Results

Effects of MPS on incorporation of uridine

The results (table III) show very similar inhibition of incorporation for all cell lines. This is quantitatively independent of the extent of cytolysis at the same concentrations of MPS or the intracellular concentrations of glucocorticoid receptors (see table II).

Site of action of MPS

The kinetics of appearance of labelled uridine in whole cells and in the acid-soluble and -insoluble pools was determined. None of the pools is saturated even after 120min exposure (figure 4a). The ratio of acid-soluble to acid-insoluble material shows that there is not a stable equilibrium between uptake and incorporation even after 120min, though the predominance of uptake over incorporation diminishes rapidly over the first 30min (figure 4b). The rate of uptake decays over the first 90min, whereas the rate of incorporation appears to be linear (figure 4c).

From these observations it was decided that in comparisons of uptake and incorporation cells should be pulsed with ^{3}H-uridine for 20min. This allows a significant amount of the label to be incorporated into acid-insoluble material whilst uptake is still the faster process and an equilibrium has not been established between uptake and incorporation.

When/

Table III

Effects of MPS on the Incorporation of Uridine into Human Lymphoblastoid Cells

2ml samples of cells (approximately 0.5×10^6/ml) were incubated with 20µl PBS or MPS for 1h. ^{3}H-uridine (1µCi/ml) was added for 20min, and the assay stopped by cooling the tubes in ice and adding 8ml ice-cold 5%TCA. The acid-insoluble precipitate was collected on Millipore GF (glass fibre) filters, washed twice with 10ml ice-cold 5%TCA and twice with ice-cold 70% ethanol. The filters were dried overnight and counted in 10ml toluene scintillant.

Results are the means from four separate observations in each case, and are expressed as the % inhibition of incorporation over controls receiving PBS instead of MPS.

Table III

Effect of MPS on Incorporation of Uridine into Human Lymphoblastoid Cells

Cell line	Control incorporation (CPM/10^6 cells)	MPS concentration (µg/ml) 0.5	5	50	500
		% inhibition			
RUS_1	2975	1	11	28	77
RUS_2	7254	10	19	44	85
BLA_1	13152	13	21	53	91
F89	17743	-2	14	42	87
GS_1	5539	13	16	37	81
JIJOYE	45620	12	17	32	84
PEN_2	12630	11	12	40	89
YAK_1	15571	9	14	36	85

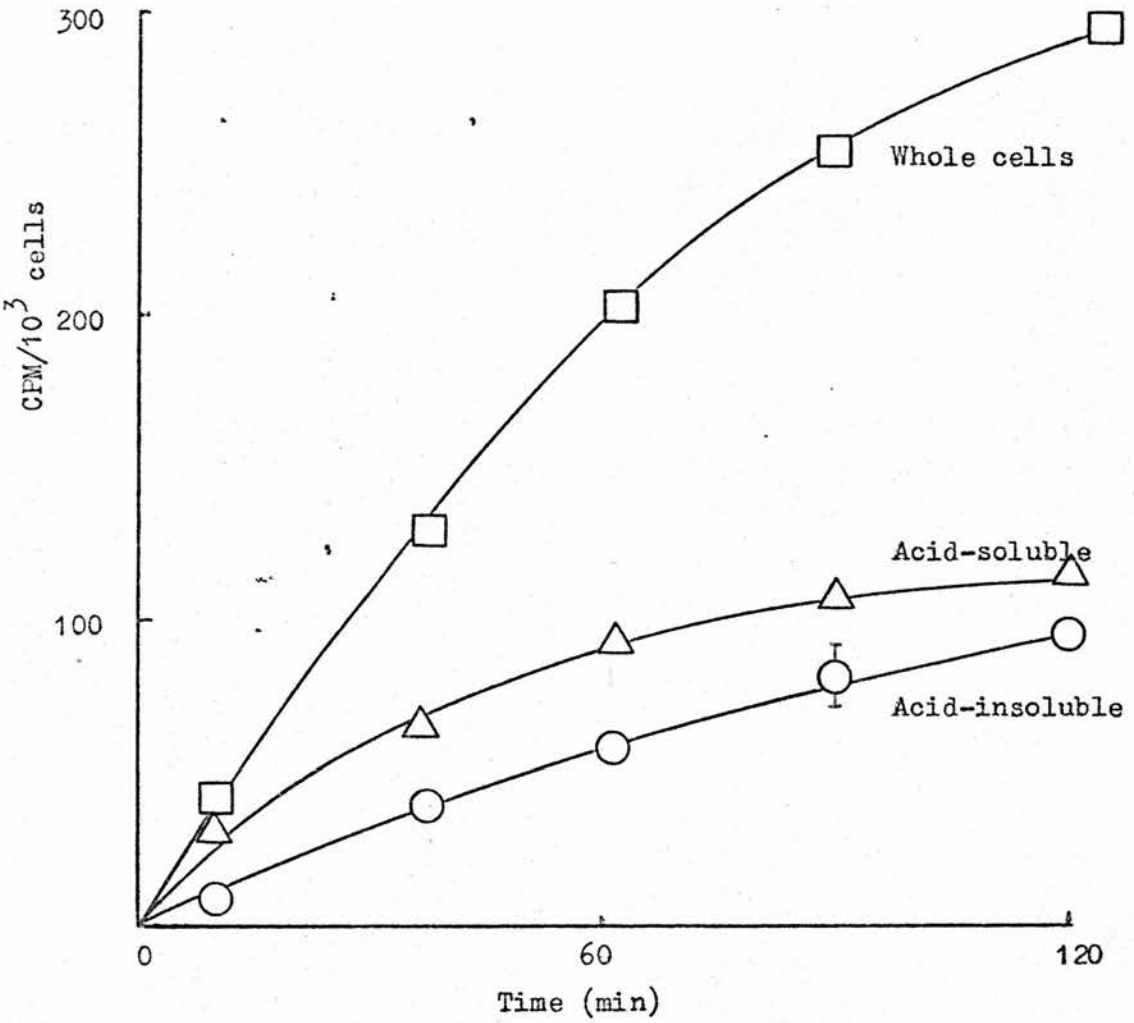


Figure 4a Uptake and Incorporation of Uridine into BLA_1 Cells

BLA_1 cells (approx. 0.5 x 10^6/ml) in MEM + HEPES were pulsed with ^{3}H-uridine for the times shown above at 1μCi/ml. 2ml samples were withdrawn and assayed either for uptake of uridine into whole cells or into acid-soluble and-insoluble pools, as detailed in Materials and Methods (pages 25 - 26).

Results are the means of two separate experiments.

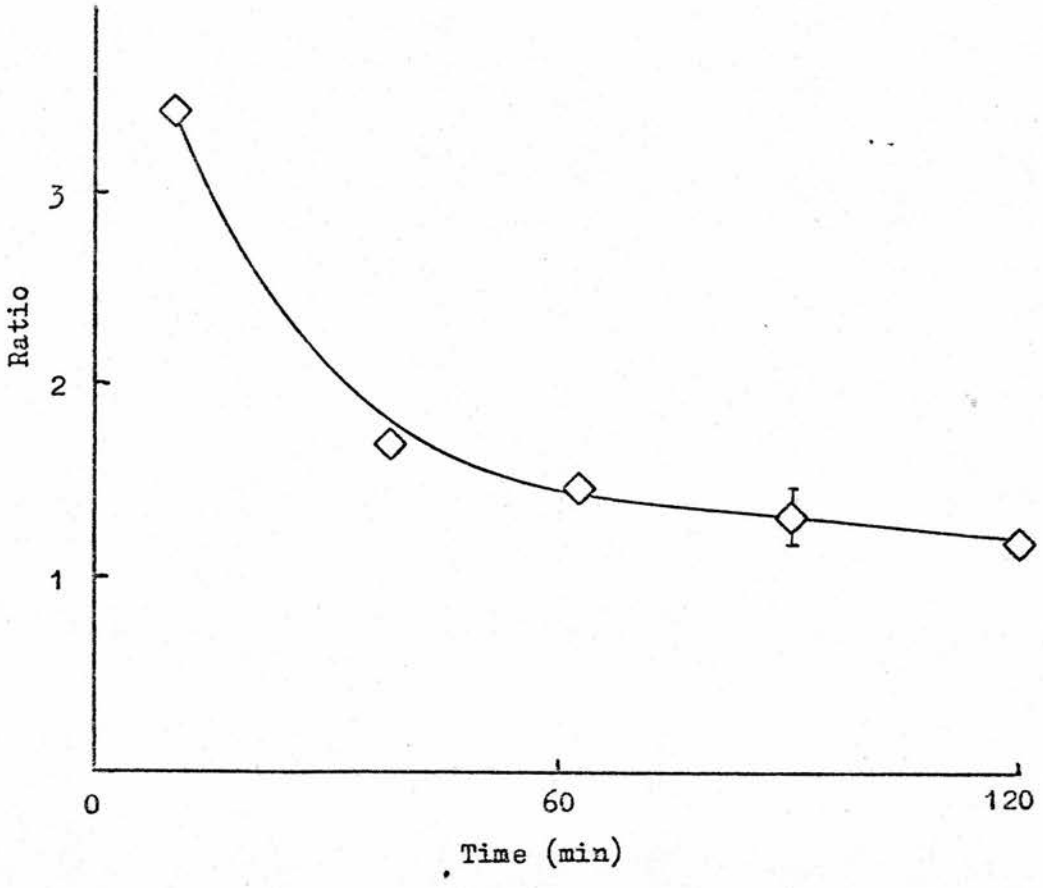


Figure 4b Ratio of Acid-soluble to Acid-insoluble Uridine Uptake into BLA_1 Cells

Experimental details are as in figure 4a, except that the results are expressed as the ratio of acid-soluble to acid-insoluble uridine.

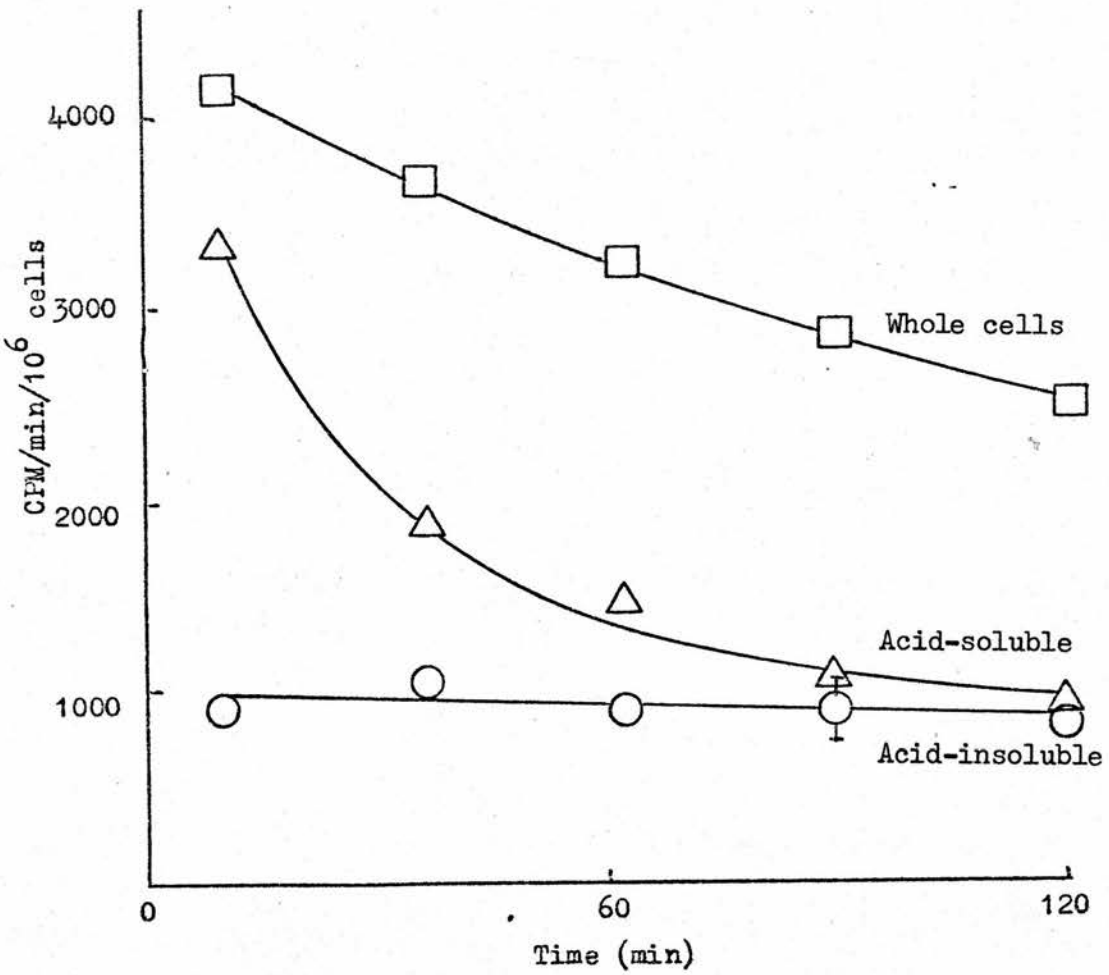


Figure 4c Rate of Uptake of Uridine into BLA_1 Cells

Experimental details are as given in figure 4a, except that the results are expressed as the rate of uptake of uridine in CPM/min.

When BLA_1 cells were treated with MPS at 50 or 500µg/ml, the extent of inhibition of uptake and incorporation was virtually the same (table IVa). With RUS_2 cells treated in the same way, the similarity in the extent of inhibition is even more marked (table IVb).

Mechanism of uptake of uridine

Plagemann (1970) has demonstrated two different uptake paths for uridine in rat hepatoma cells. One is saturable and predominates at low extracellular uridine concentrations (facilitated diffusion) whilst the other is non-saturable and operates at higher concentrations (simple diffusion). By employing the same experimental methods I have demonstrated the existence of these two pathways in human lymphoblastoid cells (figure 5). The facilitated diffusion pathway saturates at approx. 0.2mM whilst simple diffusion overtakes facilitated diffusion at concentrations greater than 0.8mM.

Effect of MPS on uridine uptake pathways

The initial velocities of each uptake pathway were assayed in the presence of MPS. 50µg/ml MPS reduces the velocity of facilitated diffusion uptake, especially at low extracellular uridine concentrations, and 500µg/ml at all concentrations (table V). In neither case is the rate of simple diffusion reduced, and, indeed/

Tables IV a & b

Effect of MPS on Uptake and Incorporation of Uridine

2ml samples of cells (approximately 10^6/ml) were treated with 20ul MPS or PBS to give the final concentrations desired. After 1h, ^{3}H-uridine (1μCi/ml) was added, and after a further 20min cells were assayed for acid-soluble and -insoluble ^{3}H-uridine as in Materials and Methods (pages 25 - 26).

Results are the means of two separate experiments in each case.

Table IV

Effect of MPS on Uptake and Incorporation of Uridine

(a) BLA_1	MPS (μg/ml)		
	0	50	500
Uptake (CPM/10^6 cells)	34490	20337	3718
Incorporation (CPM/10^6 cells)	8953	6031	997
Inhibition of uptake (%)	-	42	90
Inhibition of incorporation (%)	-	33	89

(b) RUS_2			
Uptake (CPM/10^6 cells)	19082	10050	2513
Incorporation (CPM/10^6 cells)	5754	2867	795
Inhibition of uptake (%)	-	47	87
Inhibition of incorporation (%)	-	50	87

Figure 5

Normal Uptake of Uridine by BLA_1 Cells

2ml samples of BLA_1 cells (approximately 10^6/ml) were treated with uridine in the range 10μM - 1mM. Each sample was pulsed with ^{3}H-uridine (1μCi/ml) for 20min and then assayed for whole cell uptake of uridine and acid-soluble and -insoluble uridine as in Materials and Methods (pages 25 - 26).

Results are the means of two separate experiments.

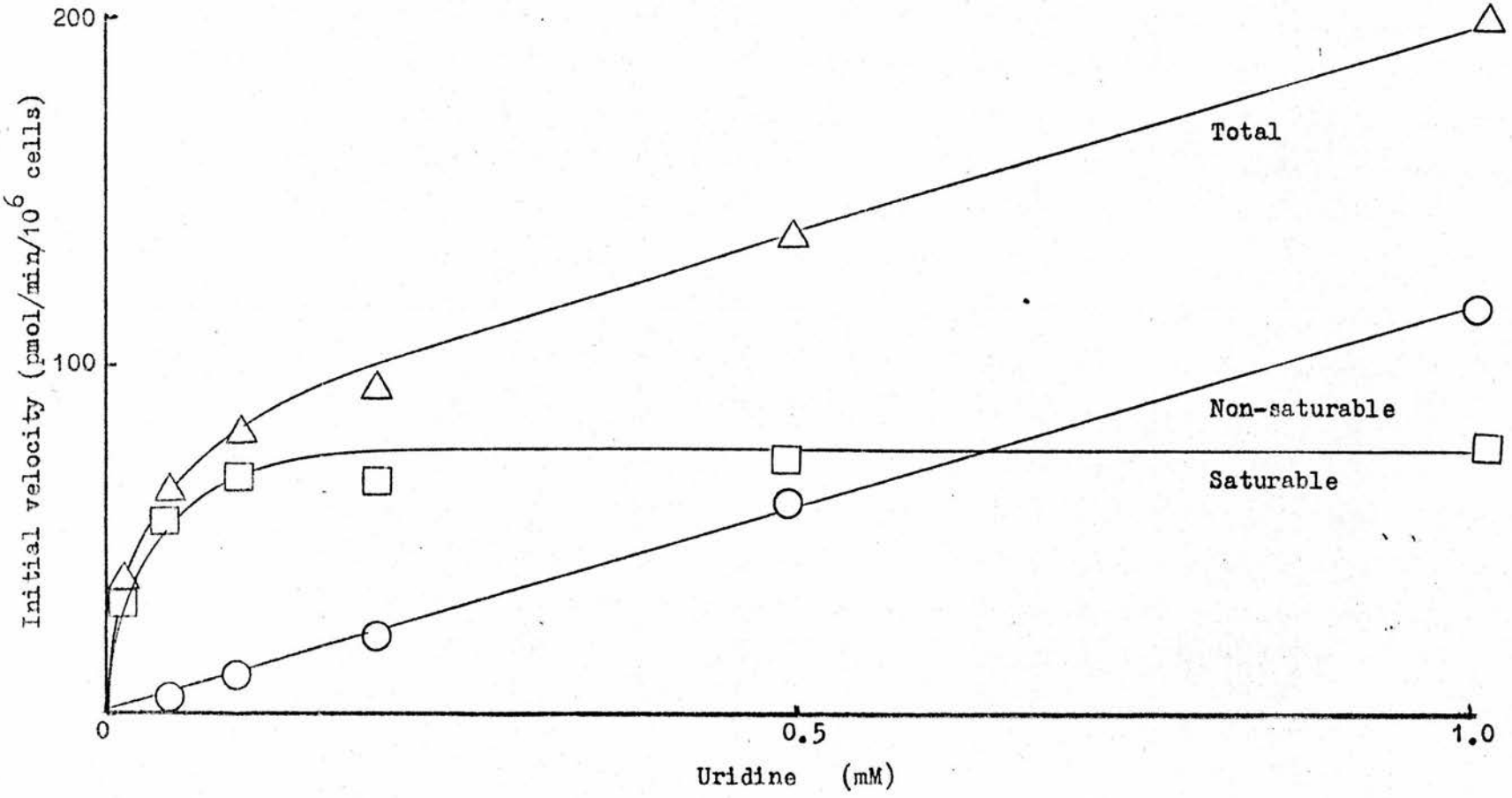


Figure 5 Normal Uptake of Uridine into BLA_1 Cells

Table V

Effect of MPS on Uridine Uptake Pathways in BLA_1 Cells

	Saturable Uptake			Non-Saturable Uptake		
	MPS (µg/ml)					
Uridine (mM)	0	50	500	0	50	500
0.01	33.6	20.6	2.8	1.3	1.5	1.4
0.05	59.9	55.9	15.9	6.4	7.6	7.2
0.1	67.1	52.2	25.0	12.8	15.2	14.4
0.5	72.0	55.0	40.2	64.0	76.0	72.0
1.0	71.9	62.4	40.1	128.0	152.0	144.0

Experiments were performed as described in figure 5, after 1h pre-incubation of the cells with 50 or 500µg/ml MPS for treated samples. Results are the means of two separate experiments, and are expressed as pmol/min/10^6 cells.

indeed, it would appear to be stimulated.

Effect of increased uridine concentrations on inhibition of uptake

Since MPS appears to act only against facilitated diffusion, it should be possible to reduce the percentage inhibition of uptake by increasing the extracellular uridine concentration. This is the case (table VI). The results given for EB_1 and EB_4 cells, which were derived from two patients with Burkitt's lymphoma, show the same pattern as BLA_1.

Kinetics of inhibition

The rate at which 50 and 500µg/ml MPS inhibited uridine uptake was investigated in two ways. First, cells were exposed to ^{3}H-uridine for 3min pulses before and after addition of MPS (figure 6). Secondly, cells were pulsed for 5min with ^{3}H-uridine and MPS was added at 1min intervals during the pulse (figure 7). The results indicate that the inhibition of uptake of uridine by MPS is instantaneous.

Normal uptake of thymidine

There is both simple and facilitated diffusion uptake of thymidine into BLA_1 cells (figure 8). Uptake by facilitated diffusion is much less important than in the case of uridine.

Effects/

Table VI

Effect of Uridine on Inhibition of Uridine Uptake by MPS

	Cell Line					
	BLA_1		EB_1		EB_4	
Uridine (mM)	0	1	0	1	0	1
Control						
Uptake (CPM/10^6 cells)	26019	1204	27756	868	47604	1420
50µg/ml MPS						
Uptake (CPM/10^6 cells)	12773	938	14873	615	16763	1191
Inhibition of uptake (%)	50	22	46	29	65	16
500µg/ml MPS						
Uptake (CPM/10^6 cells)	2320	848	2341	496	1093	958
Inhibition of uptake (%)	91	29	92	43	96	33

2ml samples of cells (approx. 10^6/ml) in MEM + HEPES were incubated with 20µl MPS or PBS to give the steroid concentration listed above. After 1h, ^{3}H-uridine (1µCi/ml) and, where appropriate, 1mM unlabelled uridine were added for a further 20min. Acid-soluble ^{3}H-uridine was then assayed as in Materials and Methods (pages 25 - 26).

Results are the means of two separate experiments.

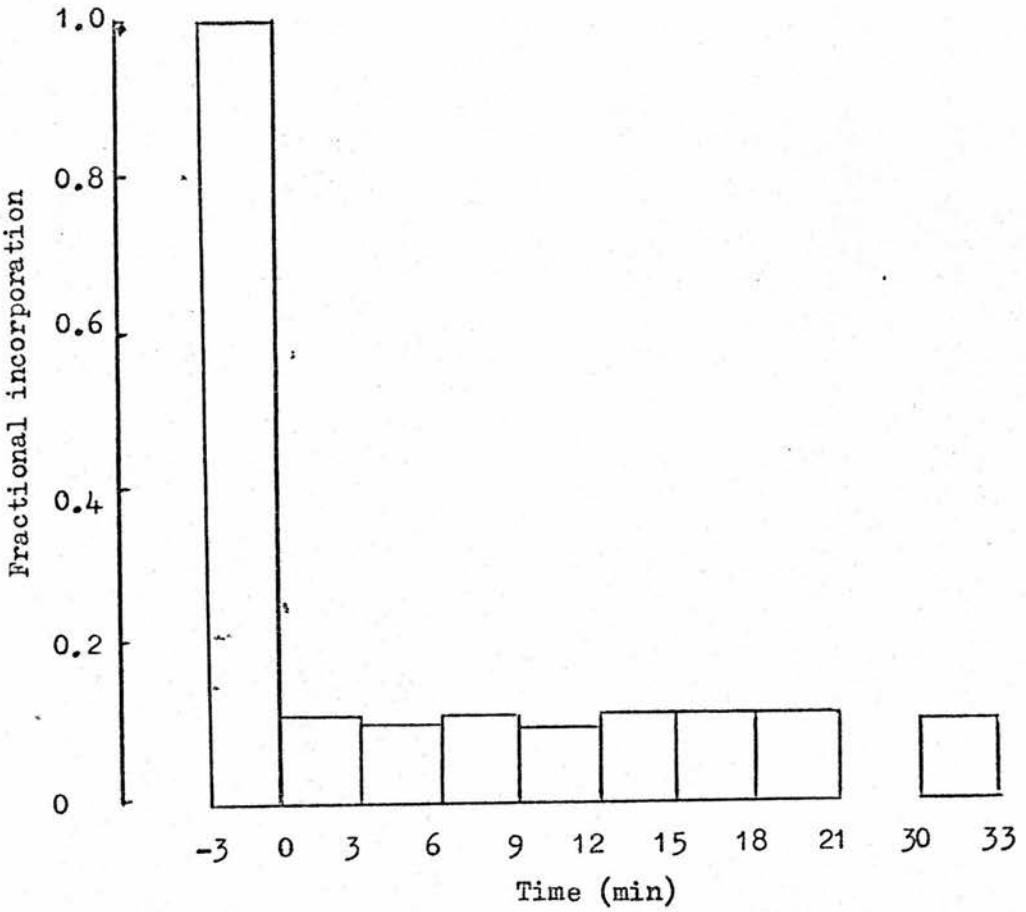


Figure 6 Kinetics of Inhibition of Uridine Uptake by MPS (I)

2ml samples of BLA_1 cells (approx. 10^6/ml) were treated with 500μg/ml MPS at 0min. During the 3min periods indicated above cells were pulsed with ^{3}H-uridine (10μCi/ml) and then assayed for acid-soluble ^{3}H-uridine as in Materials and Methods (pages 25 - 26).

The results are the means of two separate experiments.

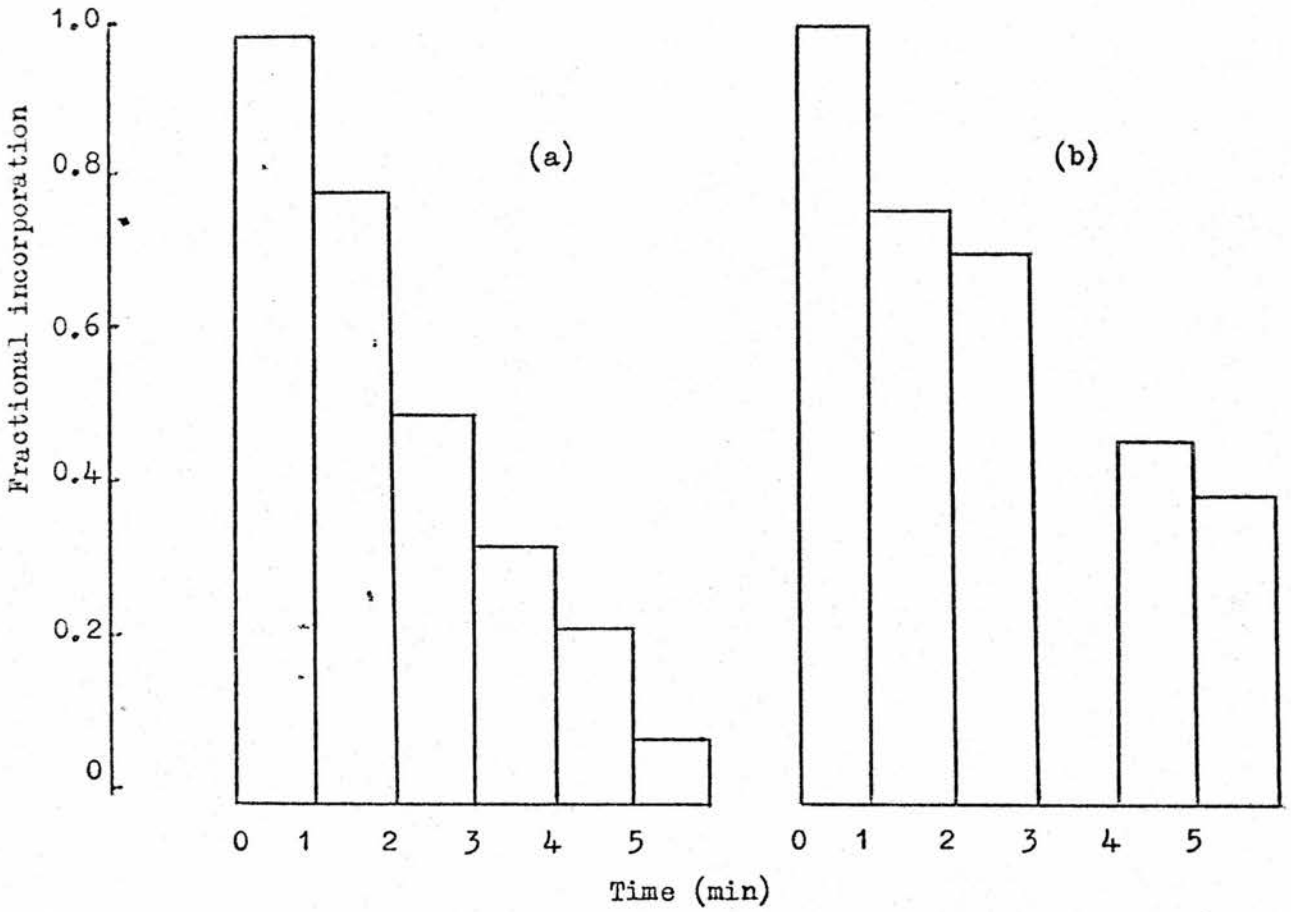


Figure 7 Kinetics of Inhibition of Uridine Uptake by MPS (II)

2ml samples of BLA_1 cells ($\sim 10^6$/ml approx.) were pulsed with ^{3}H-uridine (10μCi/ml) for 5min. During this pulse cells were exposed to (a) 500μg/ml MPS or (b) 50μg/ml MPS for 0,1,2,3,4 or 5min, as indicated on the "time" axis above. The cells were then assayed for acid-soluble ^{3}H-uridine as in Materials and Methods (pages 25 - 26).

Results are the means of two separate experiments.

Figure 8

Normal Uptake of Thymidine into BLA$_1$ Cells

Assays were performed as for uptake of uridine (figure 5, page 36) except that the appropriate concentrations of thymidine were substituted throughout.

Results are the means of two separate experiments.

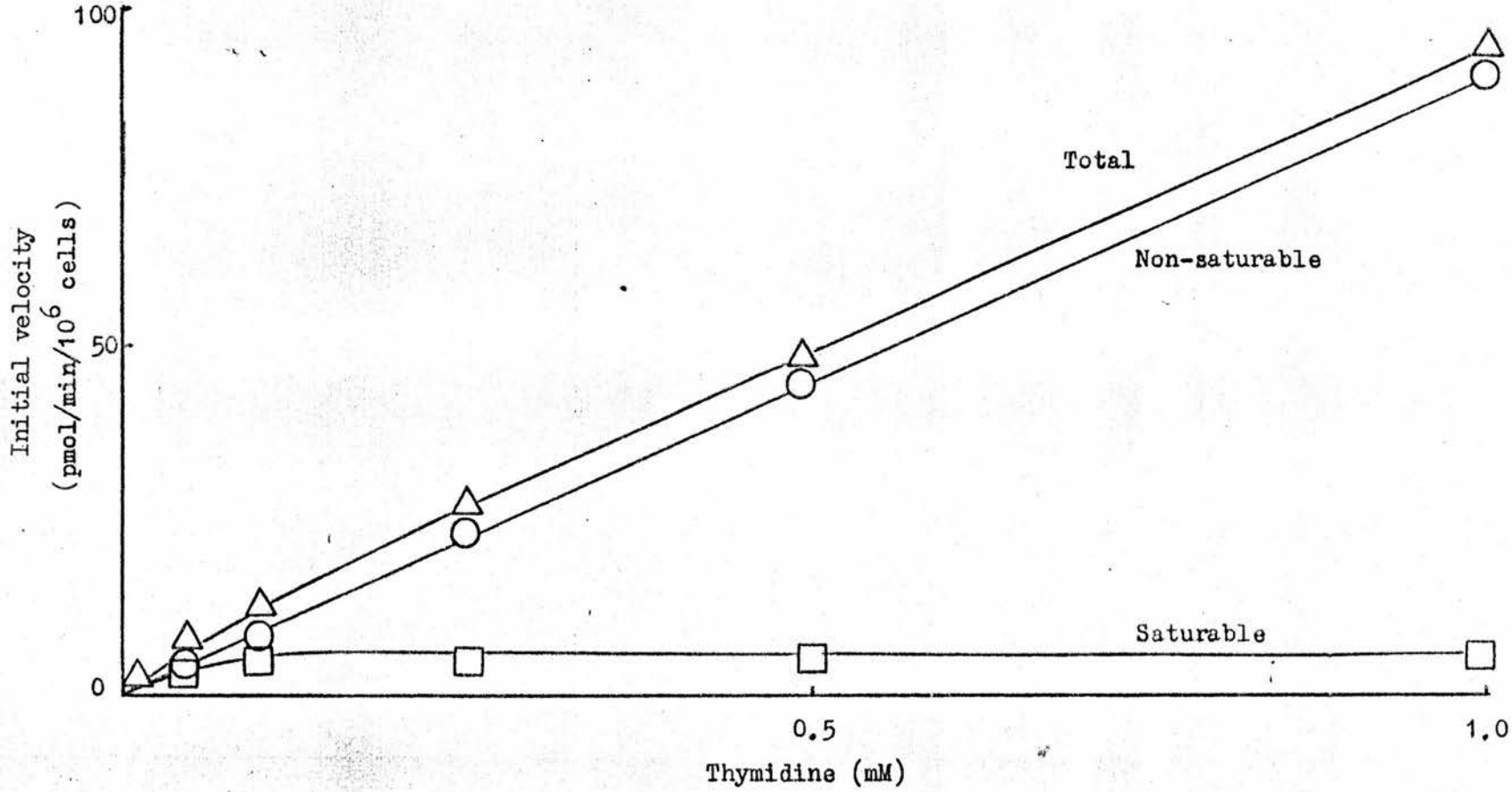


Figure 8 Normal Uptake of Thymidine into BLA_1 Cells

Effects of MPS on thymidine uptake and incorporation

Thymidine uptake is inhibited by MPS at 50 and 500µg/ml (table VII). Unlike uridine, there is a slight consistent additional inhibition of incorporation. Because facilitated diffusion uptake was proportionately small, it proved difficult to obtain accurate data for the effect of MPS on each pathway. However, as with uridine, if only the facilitated diffusion is inhibited, MPS should have a reduced inhibitory effect on thymidine uptake in the presence of 1mM thymidine - a concentration at which simple diffusion predominates. This is the case (table VIII).

Kinetics of inhibition of thymidine uptake

Pulsing cells for 3min with ^{3}H-thymidine before and after addition of 500µg/ml MPS revealed that inhibition of uptake was instantaneous (table IX). The slight increase of inhibition with exposure to MPS may again indicate an additional inhibition of incorporation.

Effect of MPS on 2-deoxy-D-glucose uptake

Having examined the effects of MPS on nucleoside uptake in detail, I then made a preliminary study of its effects on 2-deoxy-D-glucose uptake. 2-deoxy-D-glucose enters the cells by the same paths as glucose, and may be phosphorylated by hexokinase. It cannot, however, be converted to fructose-1-phosphate by phosphoglucoisomerase/

Table VII

Inhibition of Thymidine Uptake and Incorporation into BLA_1 Cells by MPS

MPS (µg/ml)	CPM/10^6 cells Acid-sol.	Acid-insol.	% inhibition Acid-sol.	Acid-insol.
0.5	29573	68683	0	0
5	26970	63607	9	8
50	13651	22926	54	67
500	3481	2363	88	97

2ml samples of BLA_1 cells (approximately 10^6/ml) were incubated for 1h with the appropriate concentration of MPS. The cells were then pulsed for 20min with ^{3}H-thymidine(1µCi/ml) and assayed for acid-soluble and -insoluble ^{3}H-thymidine as in Materials and Methods (pages 25 - 26).

Results are the means of two separate experiments.

Table VIII

Effect of Thymidine Concentration on Inhibition of Thymidine Uptake by MPS in BLA_1 Cells

MPS (µg/ml)	Thymidine (mM)	CPM/10^6 cells (% inhibition) Acid-sol.	Acid-insol.
0	0	27713	75035
0	1	603	56
500	0	3535 (88)	2643 (96)
500	1	490 (19)	61 (0)

2ml samples of BLA_1 cells (approximately 10^6/ml) incubated with the appropriate concentration of MPS were pulsed with ^{3}H-thymidine (1µCi/ml) for 20min and assayed for acid-soluble and -insoluble ^{3}H-thymidine as in Materials and Methods (pages 25 - 26).

Results are the means of two separate experiments.

Table IX

Kinetics of Inhibition of Thymidine Uptake into BLA_1 Cells by MPS

Exposure	CPM/10^6 cells	% inhibition
Control	66337	-
0min	19964	70
10min	18086	73
60min	16063	76

2ml samples of BLA_1 cells (approximately 10^6/ml) were pulsed for 3min with ^{3}H-thymidine (10µCi/ml), starting at the times shown above. MPS (500 µg/ml) was added at 0 min. At the end of each 3min pulse, whole cell uptake of ^{3}H-thymidine was assayed as in Materials and Methods (pages 25 - 26).

Results are the means of three separate experiments.

phosphoglucoisomerase.

Unlike uridine and thymidine, repeated attempts could not reveal any facilitated diffusion uptake of 2-deoxy-D-glucose into BLA_1 cells. Facilitated diffusion uptake of glucose is known in other systems (Renner, Plagemann & Bernlohr, 1972). Uptake assays were performed in the presence or absence of 1mM unlabelled 2-deoxy-D-glucose. The results indicate not only the lack of facilitated diffusion but also an apparent increase in uptake caused by MPS (table X).

Effects of MPS on amino acid uptake

There are at least four major pathways for amino acid uptake into cells (Tupper, Mills & Zorgniotti, 1976). The two most important pathways for neutral amino-acids are the A (or alanine-preferring) and the L (or leucine-preferring) pathways (Oxender & Christiansen, 1963). The A pathway has been reported to be facilitated diffusion and the L pathway simple diffusion (Christiansen, 1972). I studied the effects of MPS with and without a 1h pre-incubation on the uptake of leucine and 2-aminoisobutyric acid - an A pathway amino acid which is not metabolised by the cells. To assay uptake of both amino acids under identical conditions, cells were pulsed simultaneously with ^{3}H-leucine and ^{14}C-2-aminoisobutyric acid. The results (table XI) indicate that leucine uptake is inhibited by 500µg/ml MPS whereas aminoisobutyric acid uptake is stimulated.

Table X

Effect of MPS on 2-deoxy-D-glucose Uptake into BLA_1 Cells

Sample	CPM/10^6 cells	% change
10nM 2-deoxy-D-glucose		
Control	8802	-
500µg/ml MPS for 0h	10546	20
500µg/ml MPS for 1h	12850	46
1mM 2-deoxy-D-glucose		
Control	8426	-
500µg/ml MPS for 0h	9880	18
500µg/ml MPS for 1h	11354	37

Cells were washed free of glucose from the growth medium and resuspended in Hank's balanced salts solution buffered with Hepes (pH 7.2) at 10^6/ml. MPS was added as stated above, either at the same time as the label or 1h before its addition. Cells were pulsed with ^{3}H-2-deoxy-D-glucose (5µCi/ml) for 30min and assayed for whole cell uptake of label as in Materials and Methods (pages 25 - 26).

Results are the mean of three separate experiments.

Table XI

Effects of MPS on Amino Acid Uptake into BLA$_1$ Cells

Sample	CPM/10^6 cells	% change
Leucine		
Control	379316	-
500µg/ml MPS for 0h	434285	+14
500µg/ml MPS for 1h	155979	-59
Aminoisobutyric acid		
Control	162013	-
500µg/ml MPS for 0h	192978	+19
500µg/ml MPS for 1h	207388	+28

Cells were washed free of amino acids in the growth medium and resuspended in Hank's balanced salts buffere with Hepes (pH 7.2) at 10^6/ml. MPS was added as stated above, elther at the same time as the labels or 1h before their addition. Cells were pulsed simultaneously with ^{3}H-leucine (5µCi/ml) and ^{14}C-aminoisobutyric acid (1µCi/ml) for 30min. Cells were assayed for whole cell uptake of labels as in Materials and Methods (pages 25 - 26).

Results are the means of two separate experiments.

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Comment

MPS at 500µg/ml may decrease uptake of some substrates while increasing uptake of others. Uridine and thymidine facilitated diffusion uptake is inhibited instantaneously and probably competitively - although it is difficult to obtain accurate data on this because of the constant removal of nucleosides by phosphorylation. Simple diffusion uptake of nucleosides is slightly stimulated. Glucose uptake into BLA_1 cells is apparently only by simple diffusion, and is stimulated by MPS. Uptake of one amino acid (leucine) shows immediate slight stimulation followed within an hour by inhibition, whilst uptake of another amino acid (aminoisobutyric acid) is slightly stimulated. Thus MPS does not have a uniform effect on entry of substrates into BLA_1 cells, and the fact that it may stimulate uptake of one substrate whilst inhibiting uptake of another (or even stimulate uptake of a substrate by one pathway while inhibiting its uptake by another) argues against the possibility that MPS acts non-specifically against the cell surface to cause a generalised disorganisation of its function.

The mechanism by which MPS causes these effects is unclear. Using similar concentrations to those which I have employed, Plagemann & Renner (1972) showed that prednisolone competitively inhibits facilitated diffusion of glucose into rat hepatoma cells. In the more extensive work with rodent tissues, Munck (1971) has suggested that decreases in glucose uptake cause decreases in intracellular/

intracellular ATP concentrations and that this mediates the inhibition of other uptake processes. Rosen, Fina, Millholland & Rosen (1972) demonstrated that uridine uptake is dependent on glucose utilisation in P 1798 cells. However, with the same cell line, Stevens, Stevens & Hollander (1974) found uridine uptake to be independent of glucose. Certainly, in BLA_1 cells, the effects cannot be mediated by lack of glucose since glucose uptake is stimulated rather than inhibited by MPS. It would seem that MPS is acting directly against certain components of the cell surface, and this will be discussed later.

THE NATURE OF MPS-INDUCED CYTOLYSIS

Introduction

I have earlier mentioned the cytolytic effect of MPS on human lymphoblastoid cells (table II). The method for assaying cytolysis by uptake of nigrosine has been described (pages 13 - 14). The relationship between this and other signs of cell death must now be examined in more detail.

Alison Robertson has been studying the kinetics of the morphological effects of MPS and the ultrastructural changes produced. When the kinetics of uptake of nigrosine are examined, there are three distinct phases (figure 9). There is a latent period during which the viability of the cells does not differ significantly from untreated cells. Then there is a phase during which there is a gradual increase in lysis followed by a plateau after the maximum extent of cytolysis has been achieved.

Alison Robertson has also investigated the ultrastructural changes in BLA_1 cells treated with MPS at lethal concentrations. Apart from the cells which show coagulative necrosis (figure 10, a & b) there appears to be another form of cell death occurring which shows at least some of the morphological features of apoptosis (figure 11, a - d). The major changes in apoptosis are/

Figure 9

Kinetics of Uptake of Nigrosine by BLA_1 Cells Treated with MPS

BLA_1 cells were set up at 0.2 x 10^6/ml in MEM and were treated at 0h with PBS or MPS (500µg/ml). At various times samples were removed and the %lysis of the cells determined as in Materials and Methods (pages 13 - 14).

Results are the means of two separate experiments, which were performed by Miss Margaret Gray.

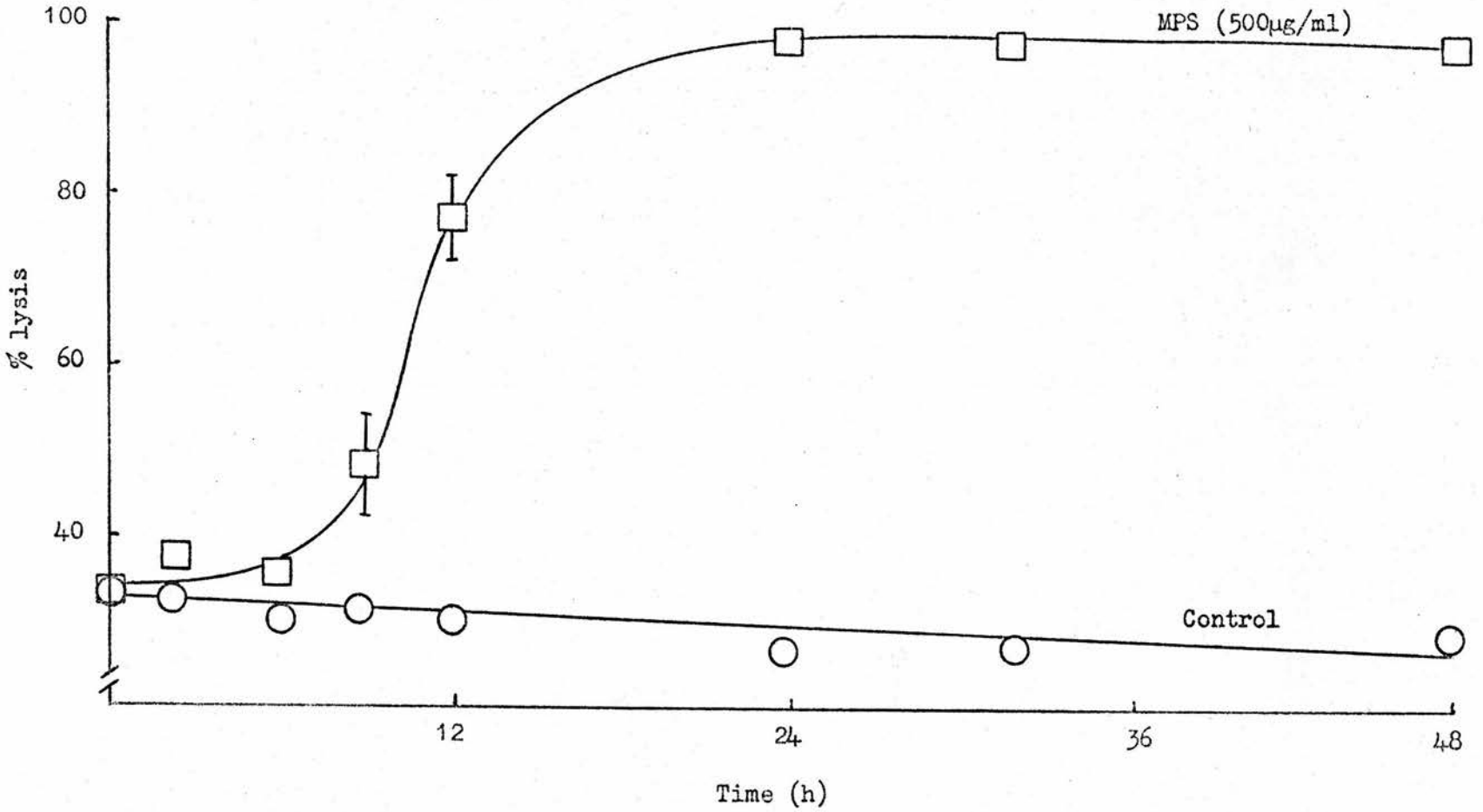


Figure 9 Kinetics of Uptake of Nigrosine by BLA_1 Cells Treated with MPS

Miss Alison Robertson has kindly provided the transmission electron micrographs used in figures 10 and 11.

Figure 10a BLA_1 cell treated with 500µg/ml MPS for 2h showing coagulative necrosis. The endoplasmic reticulum is swollen and the chromatin has condensed in the nucleus.
Magnification x 12,000

Figure 10b Late coagulative necrosis with the granular appearance of cytoplasmic debris. The plasma membrane has disappeared and there are several broken nuclear fragments.
Magnification x 9,500

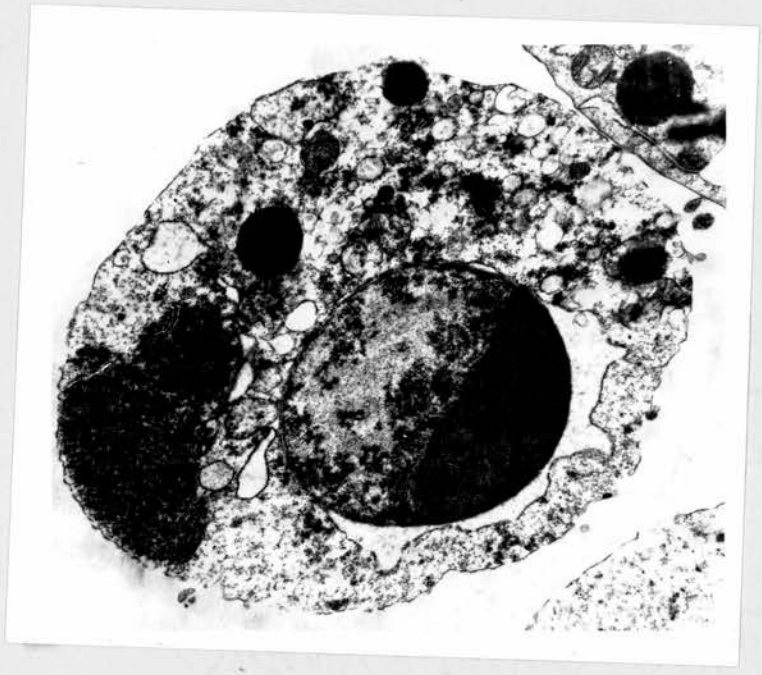


Figure 10a

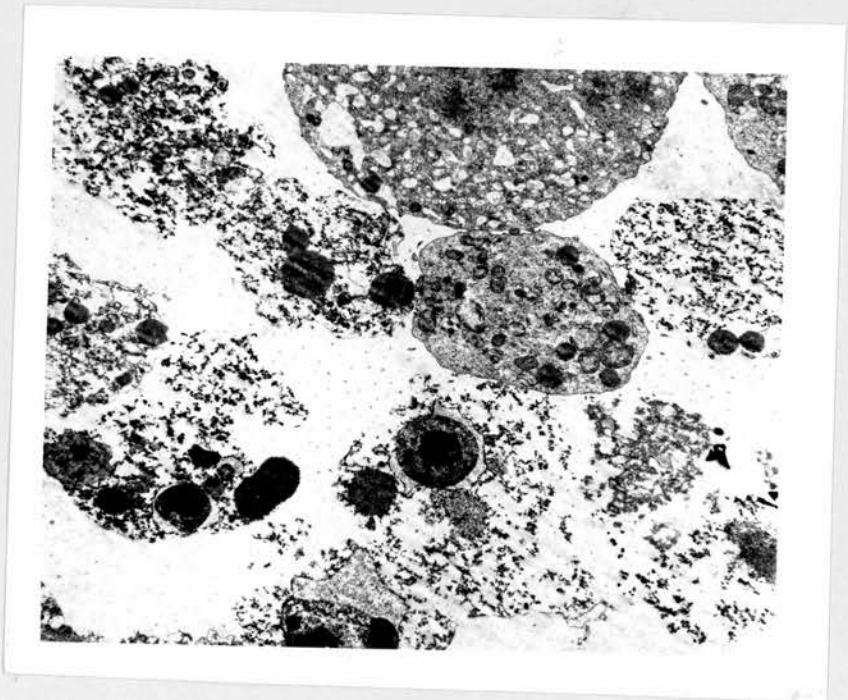


Figure 10b

Figure 11

All cells in these micrographs were treated with 500µg/ml MPS for 2h

Figure 11a Chromatin is clumped around the periphery of the nucleus. The mitochondria have localised in the cytoplasm but are still normal in appearance.
Magnification x 12,000

Figure 11b There is "blebbing" from the cell surface, but the nucleus and mitochondria still appear to be normal.
Magnification x 6,250

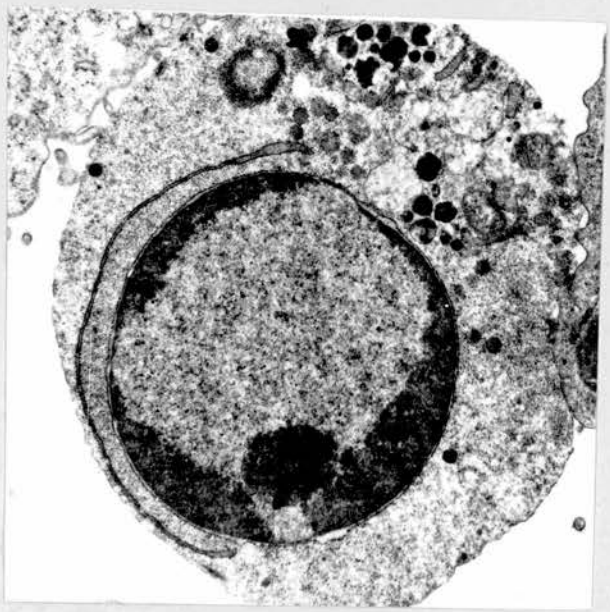


Figure 11a

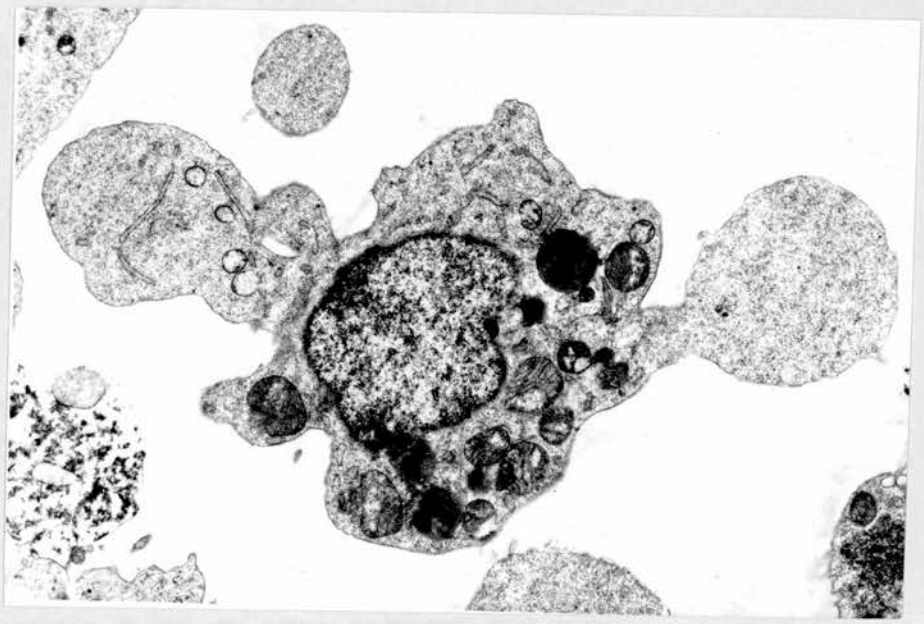


Figure 11b

Figure 11c There is"blebbing" from the cell surface, the organelles have condensed and the nucleus is possibly in the early stages of fragmenting.
Magnification x 8,200

Figure 11d The cell has fragmented. Note that the fragments are bounded by membranes and most do not appear to have any nuclear material.
Magnification x 7,300

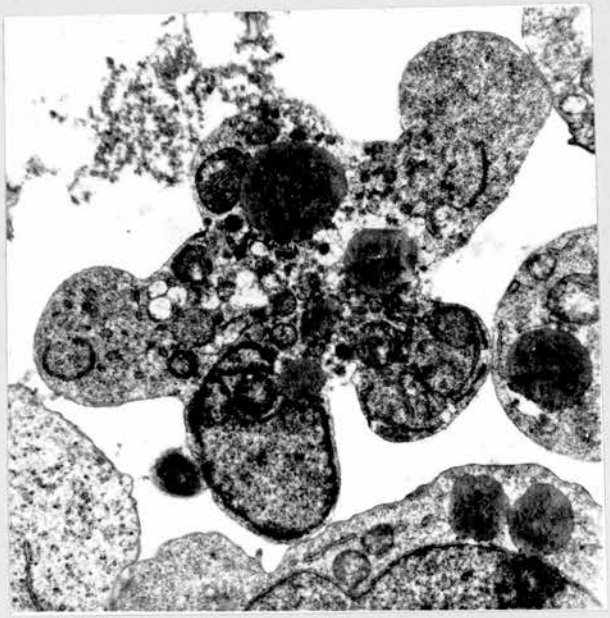


Figure 11c

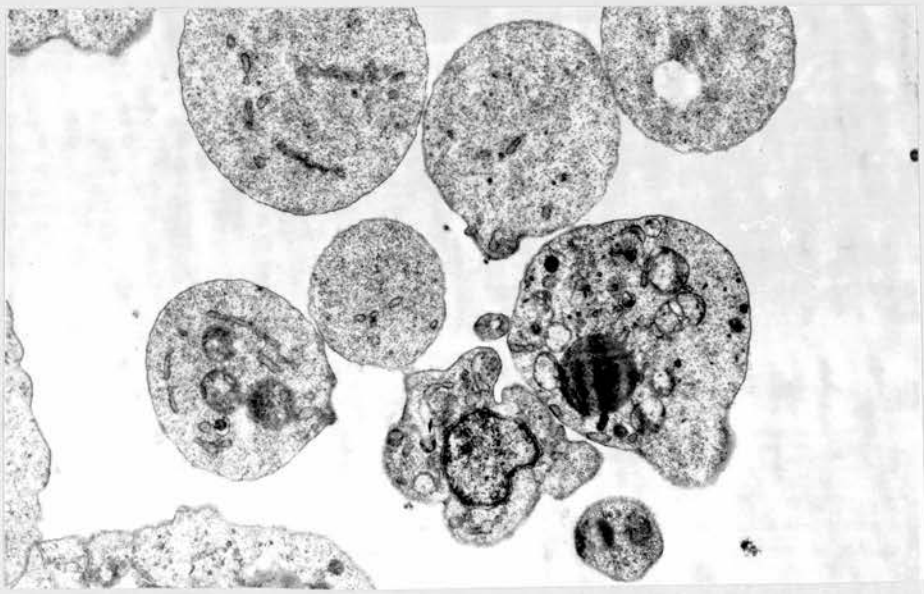


Figure 11d

are shrinkage of the cell, peripheral condensation of the chromatin in the nucleus, condensation of the cytoplasm and organelles, fragmentation of the nucleus and disruption of the cell into membrane-bounded fragments. This second form of cell death may be detected as early as 1h after treatment, and its incidence is maximal after 2 - 6h. It is possible that cells which show early changes consistent with apoptosis may later undergo coagulative necrosis. Uptake of nigrosine appears to be a late event in the changes, and ultrastructural signs of both forms of cell death may be seen before there is an increase in the uptake of nigrosine.

I have investigated the effects of MPS by two other means. First, since apoptosis involves cell shrinkage and coagulative necrosis is associated with cell expansion, I have measured the mean cell volume of treated and untreated samples. Secondly, apoptosis involves the formation of "blebs" from the cell surface, although this is not a specific "marker" for apoptosis. I have examined the shape of cells before and after treatment by scanning electron microscope.

Changes in Cell Volume

Introduction

The Coulter counter works on the principle that when a cell in a suspension of electrolyte passes through an orifice across which there is a potential difference it sets up an electrical resistance. The size of this resistance is proportional to the volume of the cell. Therefore, if one constructs a frequency histogram for the number of cells against the increasing resistance produced by them, one may estimate the mean cell volume of a population of cells.

Materials and methods

Cells were diluted 1:50 with Isoton (Coulter Electronics Ltd.) and counted over a resistance threshold range of 10 - 100 on a Coulter model ZF particle counter. By measuring the change in particle number between each threshold a frequency distribution of cell number against resistance may be compiled. From this the mean cell resistance may be calculated.

Employing a cell population of known volume, a factor relating resistance (measured as threshold) to volume may be calculated (Kt). As there is a linear relationship between resistance and cell volume, the mean cell volume of any sample is obtained by multiplying the mean cell threshold by the factor Kt for the particular attenuation. (Attenuation is used to produce an even/

even distribution of cell resistances over the threshold range of the Coulter counter) The counter was calibrated with 4C red blood cells (Coulter Electronics Ltd.; mean cell volume = 84fl) as standards. Since BLA_1 cells are almost ten times the volume of 4C cells, the Kt for 4C cells was calculated at attenuations (ATT) of 0.5, 0.707, 1.0 and 2.0. This confirmed that there is a linear relationship between ATT and Kt, and that at ATT = 4.0 - which was used for BLA_1 cells - Kt = 16.32 (figure 12).

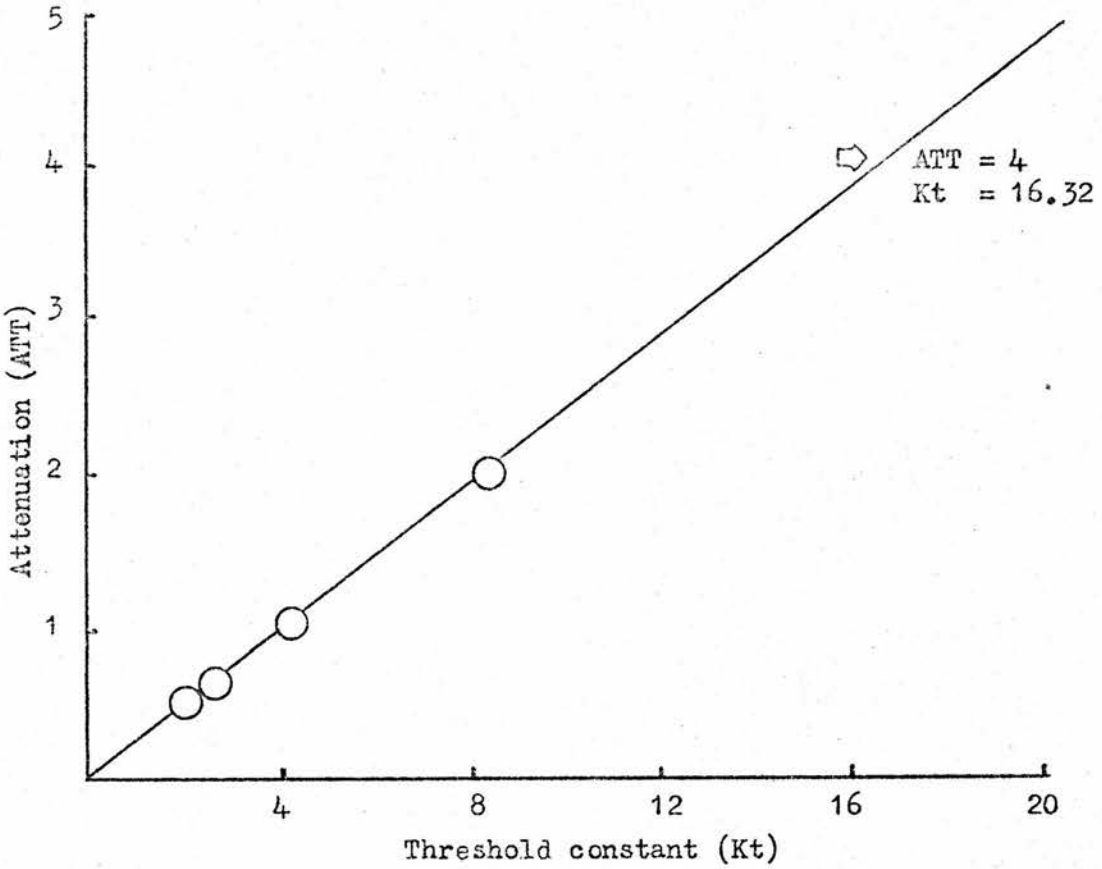
Results

BLA_1 cells were grown without the addition of fresh medium for 8 days. There was little change in the mean cell volume with changes in cell density or viability (table XII). When BLA_1 cells were treated with 500µg/ml MPS there was a latent period during which the mean cell volume did not change. This was followed by a gradual decrease in volume over a period of about 24h, after which the mean cell volume stabilised at a new lower value (figure 13). Examination of a histogram of cell volumes of treated and untreated samples shows that there is an increase in the heterogeneity of cell sizes with considerably more particles of around 500fl after treatment (figure 14).

Comment /

Figure 12

Calibration of the Coulter Counter with 4c Red Blood Cells



Threshold constants (Kt) were calculated for attenuations (ATT) 0.5, 0.707, 1.0 and 2.0. By extrapolation, at ATT = 4, Kt = 16.32. Extrapolation was by linear regression, correlation coefficient 0.99. Results are the means of four separate determinations for each attenuation, and the standard deviation was less than the symbol size in each case.

Table XII

Effect of Growth and Viability on the Volume of BLA_1 Cells

Day	% Viability	Volume (fl)
1	88	750
2	85	718
4	84	739
6	72	722
8	48	714

BLA_1 cells (0.2×10^6/ml) were grown without the addition of fresh growth medium. Viability was assessed by the nigrosine exclusion technique and volume by Coulter counter, as in Materials and Methods (pages 63 - 64).

Figure 13

Effects of MPS on the Mean Cell Volume of BLA_1 Cells

BLA_1 cells (0.4×10^6/ml) were treated with water or MPS (500µg/ml). At various times samples were withdrawn and the mean cell volume determined by Coulter counter, as in Materials and Methods (pages 63 - 64).

Results are the means of two separate experiments.

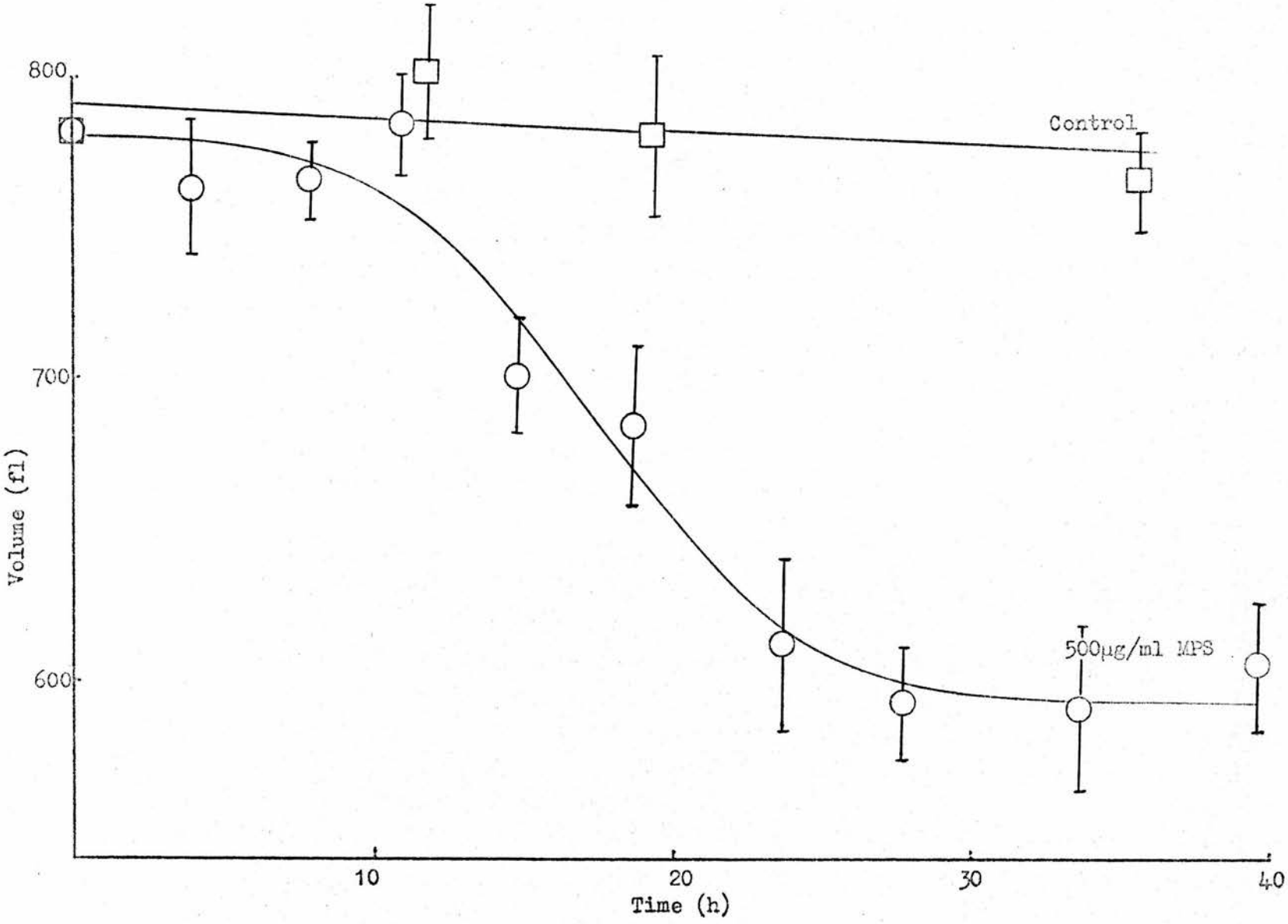


Figure 13 Effect of MPS on the Mean Cell Volume of BLA_1 Cells

Figure 14

Effect of MPS on the Distribution of BLA_1 Cell Volumes

BLA_1 cells (0.4×10^6/ml) were treated with water or MPS (500µg/ml). After 40h, the distribution of cell volumes was determined by Coulter counter, as in Materials and Methods (pages 63 - 64).

(a) Control

(b) Treated with 500µg/ml MPS for 40h

The mean cell volume of the samples is indicated by the arrow.

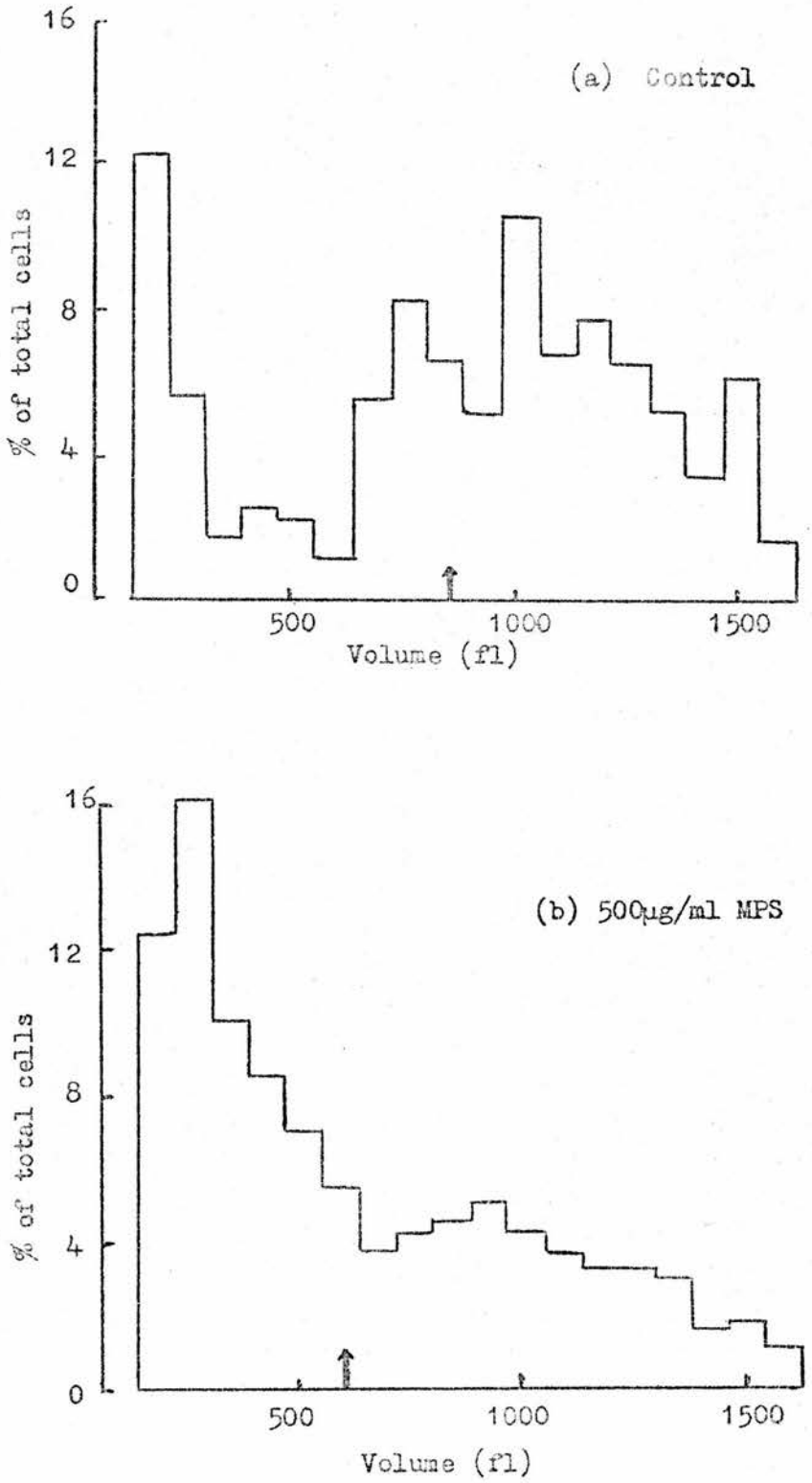


Figure 14 Effect of MPS on Distribution of BLA_1 Cell Volumes

Comment

Apoptosis involves the shrinkage of cells. If MPS induces apoptosis, a reduction in the mean cell volume of treated samples would be expected. This happens, and the rate at which the volume is reduced (figure 13) and cell lysis is increased (figure 9, page 56) are similar. Figure 14 shows a definite increase in the number of particles below 500fl after treatment with MPS. These are absent in untreated cultures, and may represent the genesis of membrane-bounded sub-cellular fragments by apoptosis.

Changes in Cell Shape

Introduction

The evidence from transmission electron microscopy is that when human lymphoblastoid cells are treated with MPS at 500μg/ml they produce processes ("blebs") which may separate from the cells to produce discrete membrane-bound fragments. Since scanning electron microscopy reveals the detailed structure of cell surfaces I used this to study the shape of cells before and after treatment with MPS.

Materials and methods

Cells were grown as described previously (pages 13 - 14), and MPS (500μg/ml) was added at the appropriate time. Cells were centrifuged gently (250 x g for 5min) and resuspended at 5×10^6/ml in the medium in which they were grown. 1 drop of cells was placed at either end of a 6 x 35mm cover slip and allowed to settle for 30min. Cells were fixed by addition of 1 drop of 1% osmium tetroxide, and after 30min were gradually dehydrated by passing through 10, 30, 50, 70, 90 and 95% acetone (2min in each) and stored in 100% acetone until critical-point drying. (Critical-point drying involves the substitution of liquid CO_2 at 800lb/in^2 and 20°C for the acetone. The temperature and pressure are gradually increased above the critical-point of CO_2 (1150lb/in^2, 31.5°C) at which the gas and liquid phases/

phases are in equilibrium and instant evaporation occurs. This avoids drying artefacts such as shrinkage and cracking.) The samples were coated with gold, which conducts away any charge induced on the surface by the electron beam, and viewed on the Cambridge Stereoscan 180 installed in the MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh.

Results

Initially, cells were prepared as a suspension in acetone, pressed on to Millipore filters and dried. This produces a basically "bald" cell with some long projections (figure 15a). When the cells were allowed to settle on to glass they exhibited a more ruffled surface (figure 15, b & c) with some processes adhering to the substrate. In both cases, cells were 6 - 10um in diameter.

½h after addition of MPS at 500µg/ml there was an increase in the number of cells showing "blebs" (figure 15, d - f). (Blebbing cells are always present in untreated samples. They represented less than 2% of the total cells, and this figure did not change with the % of cells taking up nigrosine.) These increased in number until they were most frequent 2 - 3h after treatment and became less frequent from 4h onwards. At the same time there was an increase in the number of small fragments, 1 - 3µm in diameter (figure 15g). These appeared to be/

Figure 15a Untreated BLA_1 cell on a Millipore filter. Note the smooth surface of the cell and the long processes extending outwards.
Magnification x 7600

Figure 15b Untreated BLA_1 cell on glass. The surface is convoluted and fine processes may be seen adhering to the substratum
Magnification x 7200

Figure 15c Untreated BLA_1 cell on glass. Note the lack of processes adhering to the substratum in this case.
Magnification x 3200

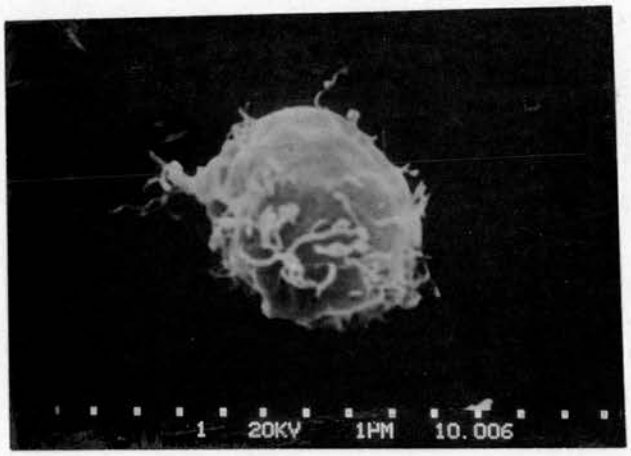


Figure 15a

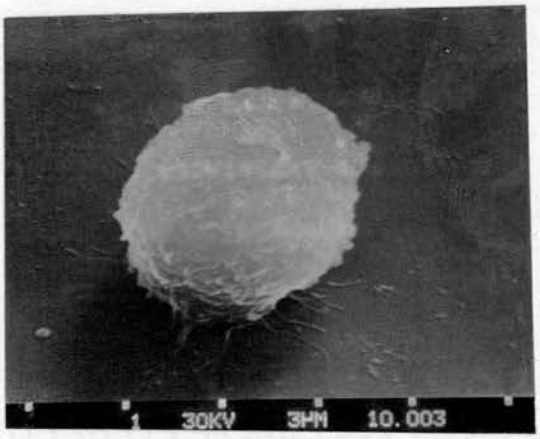


Figure 15b

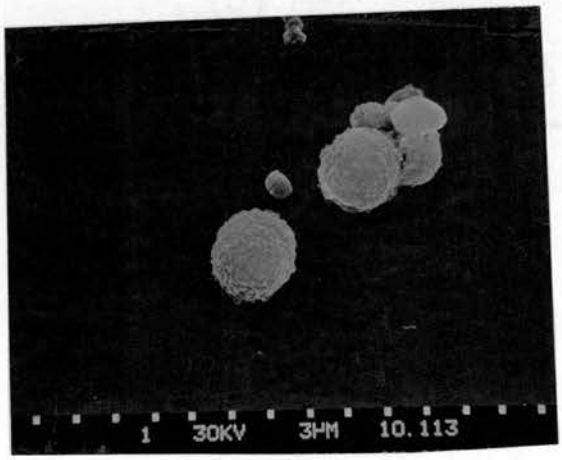


Figure 15c

Figure 15d BLA$_1$ cells treated with 500µg/ml MPS for 1h. Blebbing cells are indicated by arrows. Magnification x 3200

Figure 15e As for figure 15d

Figure 15f As for figure 15d

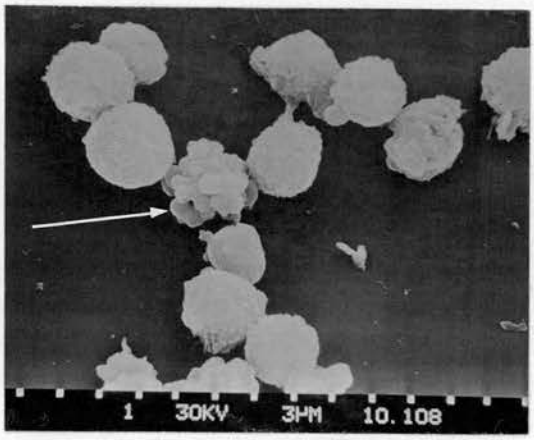


Figure 15d

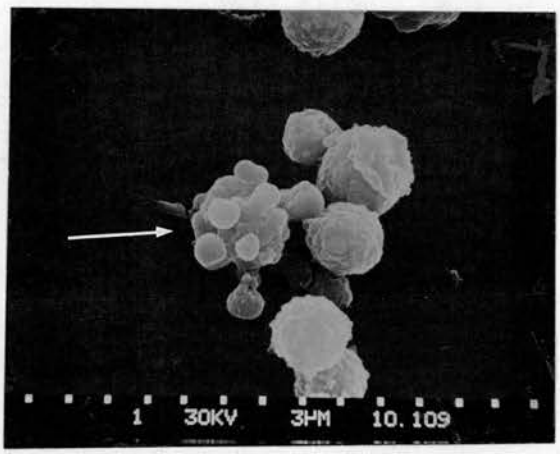


Figure 15e

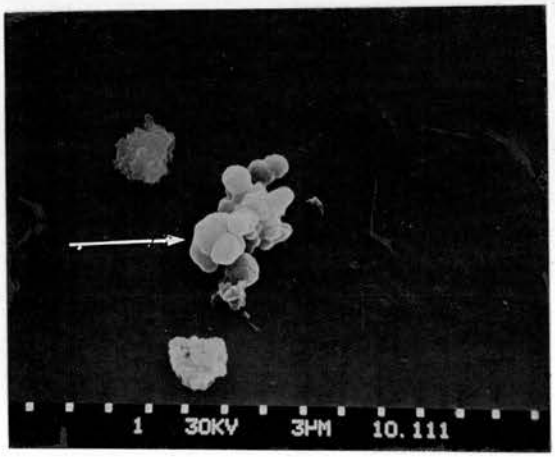


Figure 15f

Figure 15g A sub-cellular fragment from a BLA_1 culture treated with 500µg/ml MPS for 2h.
Magnification x 3200

Figure 15h Enlarged, irregular cell from a BLA_1 culture treated with 500µg/ml MPS for 4h.
Magnification x 3200

Figure 15i Enlarged, irregular cell from a BLA_1 culture treated with 500µg/ml MPS for 4h. Note that there is some evidence of blebbing on the cell surface, suggesting that this cell may be derived from an earlier blebbing cell.
Magnification x 3200

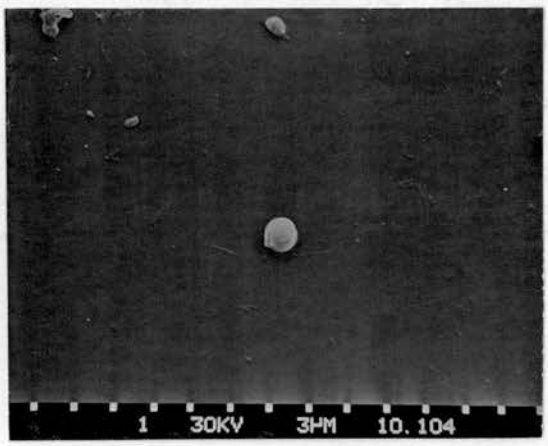


Figure 15g

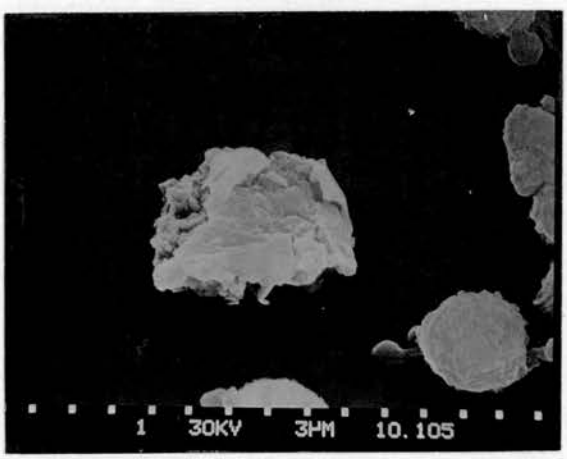


Figure 15h

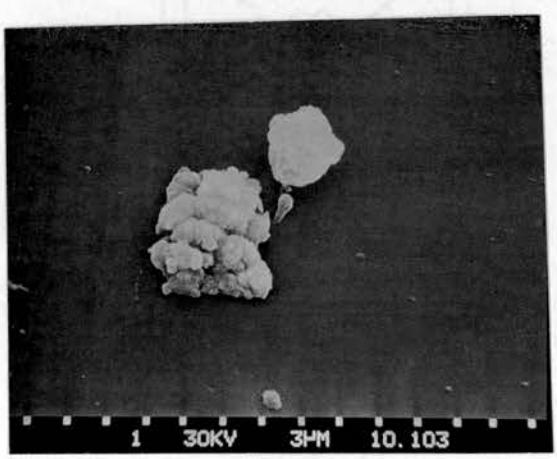


Figure 15i

be cellular in origin. At about 2h large, irregular cells, often 10 - 20μm in diameter, started to appear. These increased in number until about 12h, remaining constant from then until 48h (figure 15, h & i).

Comment

Alexander, Sanders & Brayalan (1976) have shown that the surface morphology of lymphocytes may be altered by the different methods of sample preparation for scanning electron microscopy. I have also found this with human lymphoblastoid cells. It was decided that allowing the cells to settle on to glass would give a fairer reflection of normal surface morphology than pressing them on to a filter.

The results agree with those of transmission electron microscopy, reported earlier in this section. Relating scanning and transmission electron microscope findings to what is known about cell death *in vivo*, we suggest that the blebbing cells may be apoptotic whereas the enlarged cells are undergoing coagulative necrosis.

Effects of 500μg/ml MPS on Non-lymphoblastoid Cells

Introduction

The lack of any lymphoblastoid cell lines that were resistant to MPS at 500μg/ml raised the possibility that this high concentration of steroid could be causing cytolysis in some "non-specific" manner by, for example, directly destroying the integrity of the plasma membrane. We therefore examined the effects of MPS on a number of cell lines. I examined in detail HeLa clone CS1, derived from a human cervical carcinoma, and 3T3 derived from Swiss mouse skin. To investigate the possibility that MPS acts only against cells that are actively dividing, its effects on fresh human red blood cells were examined.

HeLa CS1

Live HeLa cells form a dull, regular monolayer. Mitotic cells appear as bright, regular cells adhering less strongly to the substratum. Non-viable cells are also bright and adhere less strongly, but are irregular in shape (figure 16). Continuous observation of HeLa cells treated with 0.05, 5 and 500 μg/ml MPS revealed that dead cells appeared in treated and control cultures spontaneously at low frequency. After 6h, an increase in the number of dead cells in the culture receiving 500μg/ml MPS was observed, and the rate of appearance of these dead cells

was/

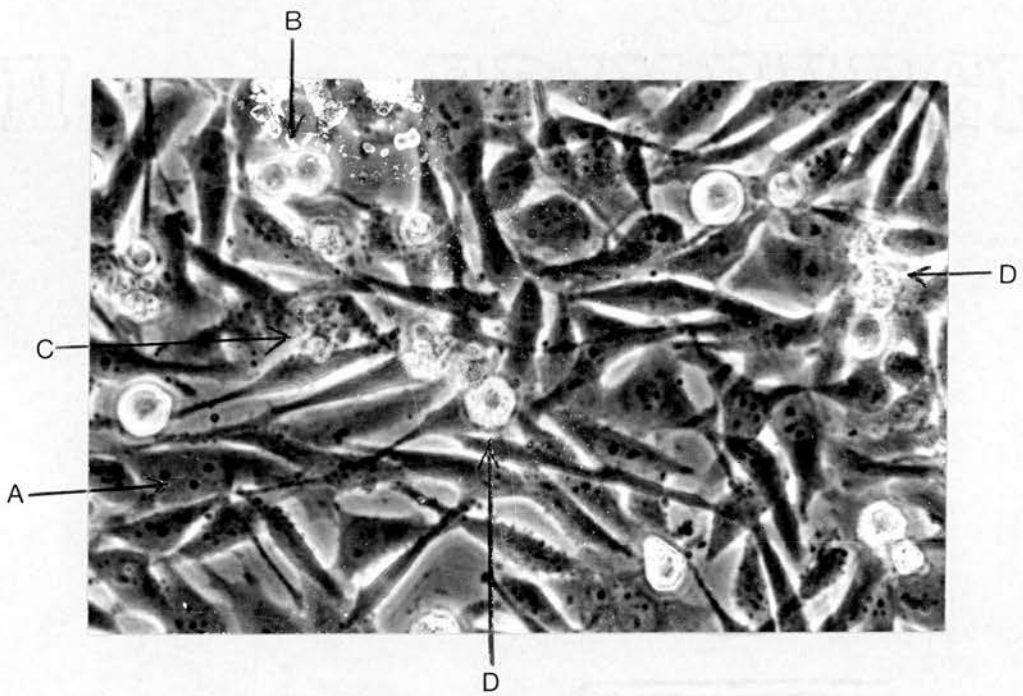


Figure 16

HeLa Cells (Phase Microscopy)

A = Normal cells

B = Mitotic cells

C = Dead cell (produced by fragmentation)

D = Dead cells (produced by "ballooning")

Magnification x900

was maximal 12 - 15h after treatment. Repeated observations of the same field under phase contrast at 37°C revealed two simultaneous forms of cell death. In some cases cells swelled gradually over a period of 2h, while in others there was a violent blebbing activity which lasted for 15 - 20min, and resulted in the fragmentation of the cell (figure 17, a - f). Russell, Rosemau & Lee (1972) observed the same type of phenomenon in HeLa cells treated with lymphotoxin, and called the processes "ballooning" and "popcorn" respectively. By analogy with our findings with human lymphoblastoid cells, we suggest that "ballooning" may represent coagulative necrosis and the "popcorn" effect apoptosis. Both forms of cell death appear simultaneously and apparently independently.

I also examined the effects of MPS on uridine uptake into HeLa CS1 cells. There was 44% inhibition of uptake at 50µg/ml and 90% at 500µg/ml (table XIII). These effects are very similar to those found with BLA_1 cells (table IV), with 42% inhibition at 50µg/ml and 90% at 500µg/ml.

3T3

3T3 cells are fibroblasts from mice. They were treated with MPS at 0.05 - 500µg/ml. After 48h the cells were scored for the extent of cytolysis (as defined for HeLa cells) by two independent/

Figure 17

"Popcorn" Cell Death in HeLa CS1 Cells

a) 0 min. The cell indicated has rounded up from the substratum as the first stage of cell death. In appearance it is very similar to early metaphase.

b) 15min. The cell has started to throw out blebs. It still appears to be intact at this stage.

c) 16½min. The cell appears to have divided into two parts. Blebbing is less rapid.

d) 18min. Blebbing continues to decrease, but the cell appears to be involuting.

e) 23min. There is a new violent outburst of blebbing. Both parts of the cell begin to fragment.

f) 24½min. The cell continues to disintegrate. The fragments start to disperse and there ceases to be any indication of the previous presence of a whole cell at this site.

Magnification in all cases x 600

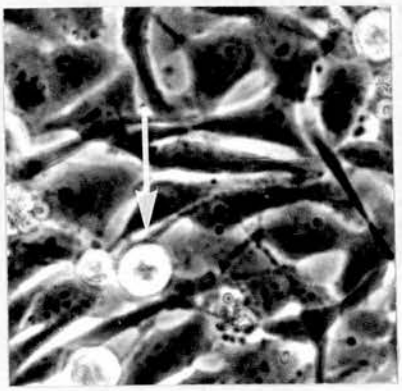


Figure 17a

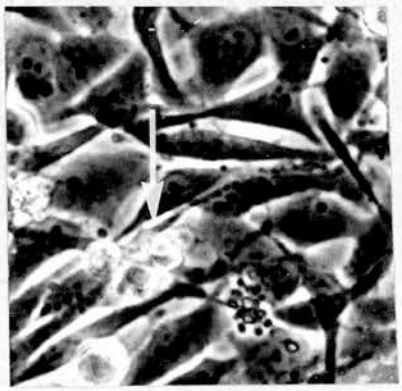


Figure 17b

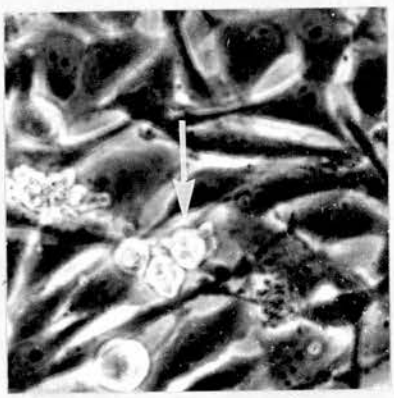


Figure 17c

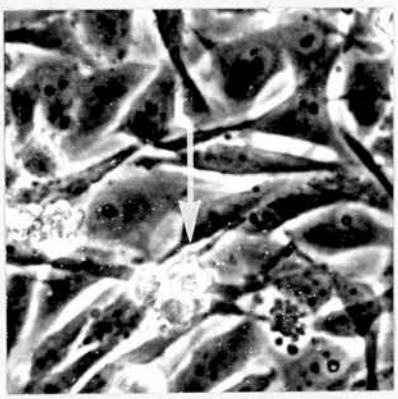


Figure 17d

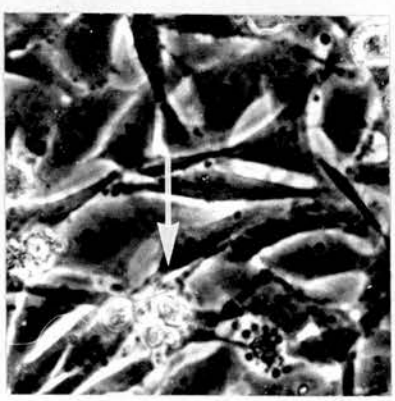


Figure 17e

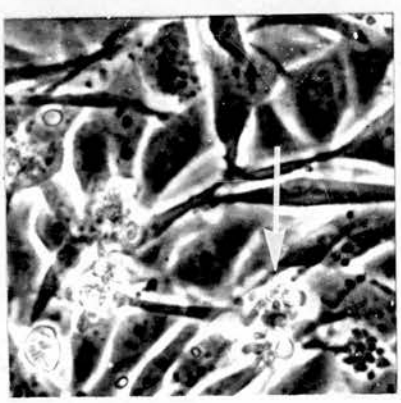


Figure 17f

Table XIII

Effect of MPS on Uridine Uptake into HeLa CS1 Cells

MPS (µg/ml)	CPM/10^6 cells	% inhibition
0	13716	-
5	12195	11
50	7656	44
500	1365	90

HeLa CS1 cells were suspended in MEM HEPES at 37°C by trypsinisation (final concentration - 10^6 cells/ml). ^{3}H-uridine (1µCi/ml) was added after 1h pre-incubation with the appropriate concentration of MPS or PBS. After 20min cells were assayed for whole cell uptake of ^{3}H-uridine, as in Materials and Methods (page 25).

Results are the means of two separate experiments, each performed in duplicate.

dependent observers, who did not know the steroid concentration in any culture. Cytolysis was recorded on a scale from 0 to ++++. Any results in which the observers did not independently reach the same assessment were disregarded. (This affected less than 10% of all observations.) The results (table XIV) indicate that 3T3 cells show acute cell death at 500µg/ml MPS and that this requires more than 24h to achieve its maximum. This is very similar to the situation with HeLa and the lymphoblastoid cell lines.

Human red blood cells

Human red blood cells differ from all the other cells we have studied in that they have no nucleus. Therefore, they will be resistant to any agent which requires gene activity or cell division for its effects. When fresh human red blood cells were treated with 500µg/ml MPS, not only were they not lysed, but they showed a 66% reduction in the rate of spontaneous haemolysis (figure 18). It would seem that MPS is having a protective rather than lethal effect on these cells.

Table XIV

Effect of MPS on 3T3 Cells

MPS (µg/ml)	Cell death 24h	48h
0	+	+
0.05	+	+
0.5	+	+
5	+	+
50	+	+
500	+++	++++

Experimental details are as given in the text (page 88).

The scale used is:- 0 = no dead cells

\+ = some dead cells

++ = about half cells dead

+++ = most cells dead

++++ = all cells dead

Figure 18

Effect of MPS on Autolysis of Human Red Blood Cells

Fresh human red blood cells were maintained in roller culture with only the addition of anti-coagulant. At various times samples were withdrawn for the measurement of haemolysis. Samples (200µl) were diluted with 5ml PBS and centrifuged (2000 x g for 10min) to remove all intact red blood cells. The extent of haemolysis was measured by assessing the amount of haemoglobin in the supernatant by measuring adsorption at 415nm on a Pye Unicam SP 1500 spectrophotometer.

This work was performed by Mr. J. Williamson under my supervision.

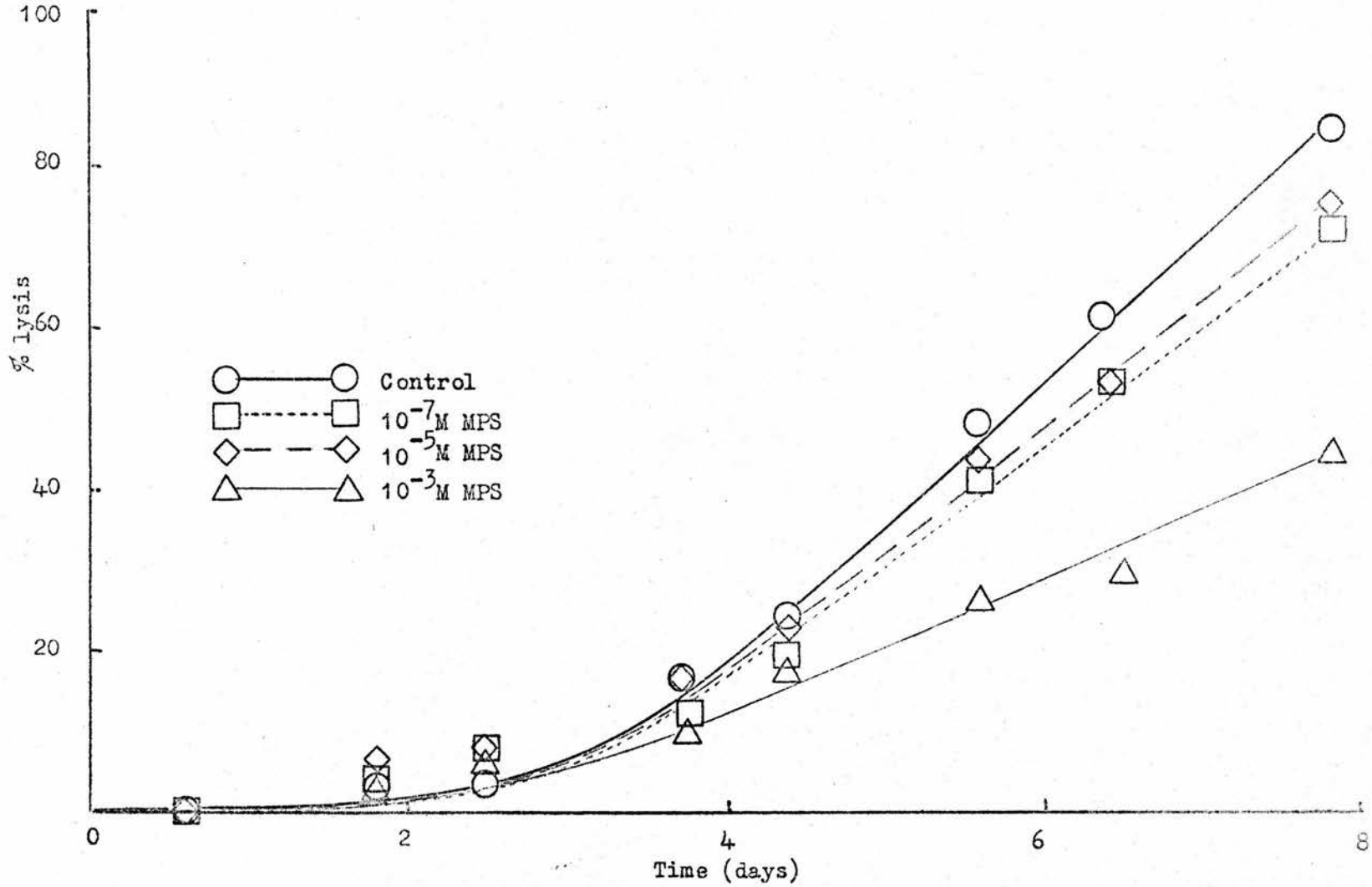


Figure 18 Effect of MPS on Autolysis of Red Blood Cells

Summary

The work on the nature of MPS-induced cytolysis which I have described may be summarised as follows:-

1] Uptake of nigrosine reveals only the latest stages in the lytic process.

2] There is a reduction in the mean cell volume of treated human lymphoblastoid cells.

3] Both transmission and scanning electron microscopy reveal two types of cell death in treated lymphoblastoid cells. One has at least some of the features of apoptosis, and occurs maximally from 2 - 6h after treatment; the other appears to be coagulative necrosis which increases continuously after treatment, but appears to follow the first form of cell death. Cells may progress to coagulative necrosis after undergoing some apoptotic changes.

4] HeLa cells show two forms of cell death which may be apoptosis and coagulative necrosis. The peak of apoptotic changes comes later than in lymphoblastoid cells.

5] All cells we have examined are lysed by MPS at 500µg/ml, with the exception of red blood cells.

Comment

MPS-induced cytolysis does not appear to be restricted to lymphoid cells, but it appears to be a phenomenon of all actively dividing cells. Mr. J. Drummond has demonstrated lysis of human lung, skin, amnion and muscle cells by MPS at 500µg/ml, and all his attempts to clone cells resistant to this concentration from sensitive cells have so far been unsuccessful. The fact that lysis does not occur in red blood cells would appear to indicate that we are not dealing with a totally non-specific effect of the type that would be expected with some agent which produced damage to the integrity of the plasma membrane.

IMPROVEMENT OF THE EXPERIMENTAL SYSTEM

Introduction

The "ideal" system

The aim of our work is to increase our knowledge of the mechanisms by which cells die. For this purpose a system is required in which cell death may be induced in a predictable and reproducible manner. Ideally, the cells should be readily synchronised so that any cell-cycle dependent effects may be studied, and they should be homogeneous so that there are no differences between effects on different cell types within a culture. They should be able to grow both in culture and in laboratory animals to enable us to compare the changes caused by cytolethal agents *in vivo* and *in vitro*. This conflicts to a certain extent with the importance of using human material for its clinical relevance, and while there are techniques such as xenografting, peritoneal diffusion and the use of immunoincempetent hosts for growing human cells in animals, it is more likely that two distinct systems - one human and one animal - will have to be used simultaneously.

Ideally, the cell death should be by apoptosis. We are particularly interested in this form of cell death because of its importance in the regulation of tumour growth. While

coagulative/

coagulative necrosis is often found in tumours, it is usually a consequence of the tumour outgrowing its blood supply and does not have the same homeostatic importance as apoptosis.

Problems associated with BLA_1 cells

BLA_1, in common with most human lymphoblastoid cell lines, is not homogeneous in that it has not been cloned from a single cell, and it tends to change its characteristics with time. At present, it has proved impossible to clone cells from existing cultures. We try to minimise these changes by culturing the cells for only a few months and then returning to the original bank of cells which is maintained frozen. It has also been impossible so far to synchronise BLA_1 cultures, and synchronisation itself may prove lethal to a proportion of the cells which poses problems when studying cell death. As with all human cell cultures, there are interpretative difficulties in relating *in vitro* findings directly to the clinical disease situation, especially when massive pharmacological doses of a drug must be used.

There are three possible methods for improving the present system:-

(1) manipulating the BLA_1 cells or other human lymphoblastoid/

lymphoblastoid cell lines to make the study of MPS effects more meaningful,

(2) looking for other cell lines which are more sensitive to glucocorticoids, and

(3) investigating the actions of other lethal agents.

Improvements to the human lymphoblastoid cell system

Human lymphocytes have been reported to be resistant to glucocorticoids, and this is thought to be species-dependent (Claman, 1972). It may be that all human cells are resistant, but the effects of glucocorticoids on certain human lymphoid tumours argues against this. As I have already remarked, all human cells we have examined seem to exhibit glucocorticoid-induced cytolysis only at 500µg/ml. There are three possible methods which might make human lymphoblastoid cells treated with glucocorticoids more suitable for study:-

(1) There may be a small population of cells which is sensitive to steroids at lower concentrations. This would explain the slight cytolytic effect seen at lower MPS concentrations (see table II). page 17 Cloning of lymphoblastoid cell lines (should this prove possible) may produce some clones with much lower sensitivity thresholds.

(2) It may be that apoptosis is dependent to a certain extent on cellular interactions for its existence. Such interactions are not as likely to occur in cell cultures as in tissues where there is a high degree/

degree of heterogeneity of cell types and a definite three-dimensional relationship between cells. Therefore, apoptosis may be initiated with reduced efficiency *in vitro* requiring higher concentrations of glucocorticoids, and it may yet prove possible to grow cells resistant to 500µg/ml MPS.

(3) There is increasing evidence from our own work and that of others that uptake of vital dyes may not be a reliable assay for cell death (Bhuyan, Loughman Fraser & Day, 1976; Roper & Drewinko, 1976), and it is possible that cytostasis (reduction in the growth rate of cells) *in vitro* may be a more reliable assay for cell death *in vivo*. Nilsson (1971) has shown cortisol-induced cytostasis of certain human lymphoblastoid cell lines, and we have some evidence to support this using as little as 0.05µg/ml MPS. It is possible that apoptosis may be involved in this cytostasis, and that further study of cytostatic effects may prove revealing. I shall deal with this more fully in the Discussion.

While these improvements do not overcome all the disadvantages, they would allow more meaningful information to be gained from BLA_1 or other human lymphoblastoid cell lines. So far, we have been unable to isolate BLA_1 cells which are sensitive to MPS at less than 500µg/ml or resistant to 500µg/ml.

We/

We have, therefore, made a preliminary study of some rodent lymphoid cell systems.

Rodent lymphoid cells

Cortisol causes the involution of the rat thymus, and we know that this is largely by induction of apoptosis (Shona Murray, personal communication). Rat thymus is, however, a heterogeneous cell system, and while thymic lymphocytes may be prepared *in vitro* in a relatively pure form, they do not remain viable long enough for experimental study.

Mouse lymphoma P 1798 is sensitive to glucocorticoids at about 0.1μM, but it does not grow as a cell line *in vitro*. We have been unsuccessful in our attempts to grow this tumour, perhaps because there is some difference between the BALB/C mice in which it has been maintained in the U.S.A. and the BALB/C mice in our laboratory.

L 5178 Y is a mouse lymphoma which grows efficiently as a suspension in culture or as a solid tumour in BALB/C mice. A cytotoxic assay revealed that it was slightly more sensitive to cytolysis by MPS than human lymphoblastoid cells (table XV). However, it proved impossible to induce any regression in the tumour *in vivo* with glucocorticoids, even at marginally sub-lethal concentrations, and thus this cell line is not sufficiently sensitive to work with.

Table XV

Effect of MPS on the Viability of L 5178 Y Cells

MPS (μg/ml)	% lysis
0	9
0.05	17
0.5	19
5	51
50	52
500	98

L 5178 Y cells were grown in Ham's f12 medium with 15% heat-inactivated horse serum. For each test, cells were adjusted to 0.2×10^6/ml, and a cytotoxic assay performed as in Materials and Methods (pages 13 - 14).

Other Lethal Stimuli (i) Prednisolone

Rather than finding a cell line sensitive to a given stimulus, it is also possible to investigate which agents are lethal for a given cell population. While methylprednisolone is assumed to have the same biological properties as prednisolone but with increased stability, it was important to check that cells were not more sensitive to prednisolone than methyl-prednisolone. This was done in two ways: [1] comparing the cytolytic effects, and [2] comparing the effects on uridine uptake of prednisolone and methylprednisolone.

Materials and methods

Prednisolone sodium succinate (PS; Sigma) was prepared and administered as an aqueous solution of no more than 1% of the final volume. Cytotoxic and uridine uptake assays were performed as in Materials and Methods (pages 13, 14 and 25).

Results

MPS is more efficient at inducing cytolysis than PS at 1 - 2 mM* (figure 19). Over the same concentration range, PS is/

* In order to compare PS with MPS, concentrations are given in molarities in this section. For comparison, 500µg/ml MPS is 1.39mM

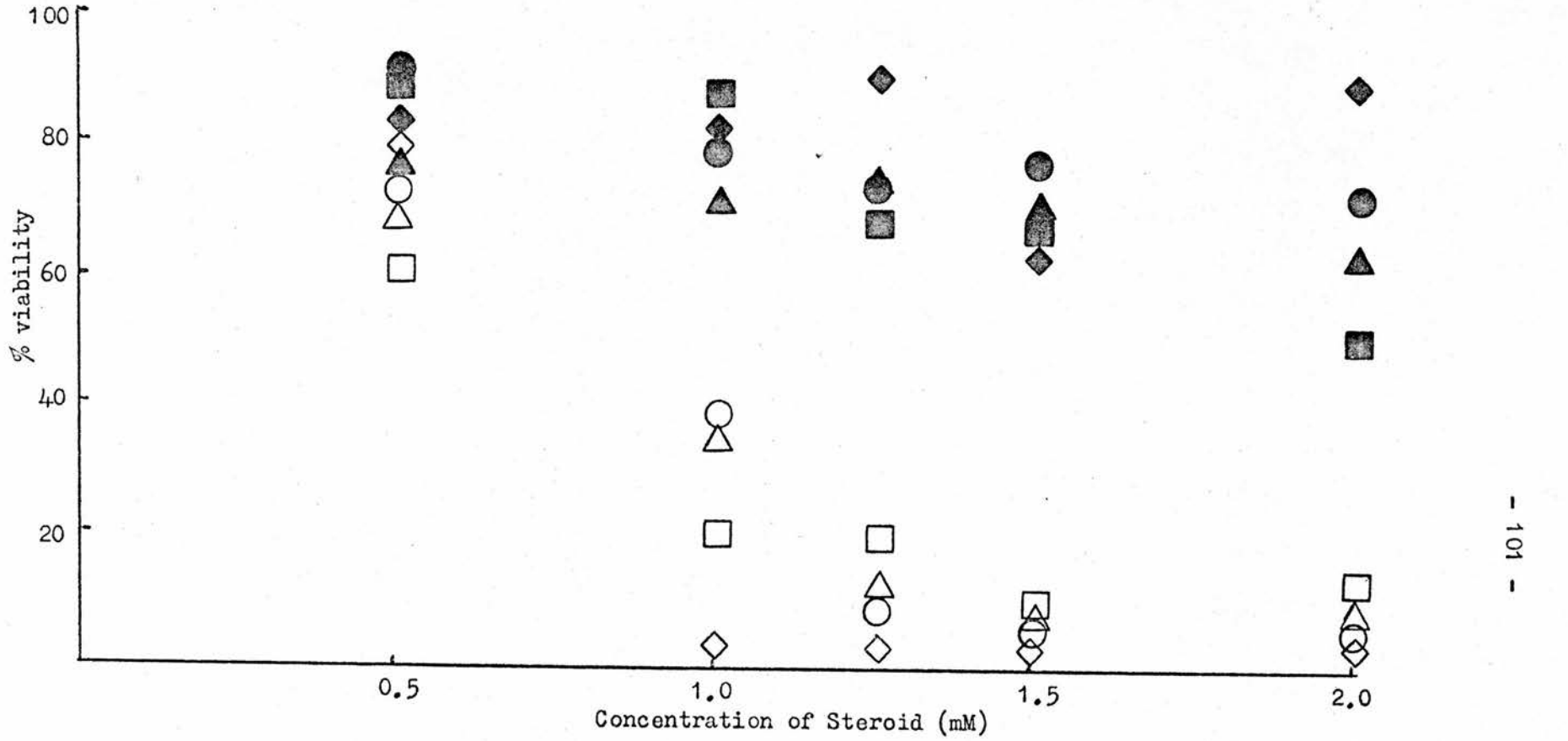


Figure 19 Effects of PS and MPS on Cell Viabilities

Cells were exposed to the appropriate concentration of PS or MPS for 48h, and their viabilities assessed, as in Materials and Methods (pages 13 - 14).

is less efficient than MPS at reducing the rate of cell growth (figure 20). PS also does not inhibit the uptake of uridine into BLA_1 cells as much as MPS (table XVI).

Comment

The reduced efficiency of PS compared with MPS may be explained in two ways; the methyl group may aid the interaction of MPS with the cellular target through which it expresses its effects, or MPS may have increased effects through prolonged activity compared with the less stable PS. The logic behind the methylation of prednisolone (at the 16 position) is to protect the steroid from the 16-hydroxylase found in some cells. If the decreased stability of PS was responsible for its decreased efficiency, then it would become increasingly less effective than MPS with the length of the experiment. Cytolysis is a long term process (48h) whilst uridine uptake inhibition is a short term effect (less than 1h). At 1mM MPS appears to be about 50% more efficient than PS for both long term and short term effects. This would suggest that it is the physical interaction of the steroid with its cellular target that is the factor affecting the relative potency of these two steroids.

PS certainly has no advantages over MPS as a lethal stimulus, and using PS would not improve the experimental system in any way.

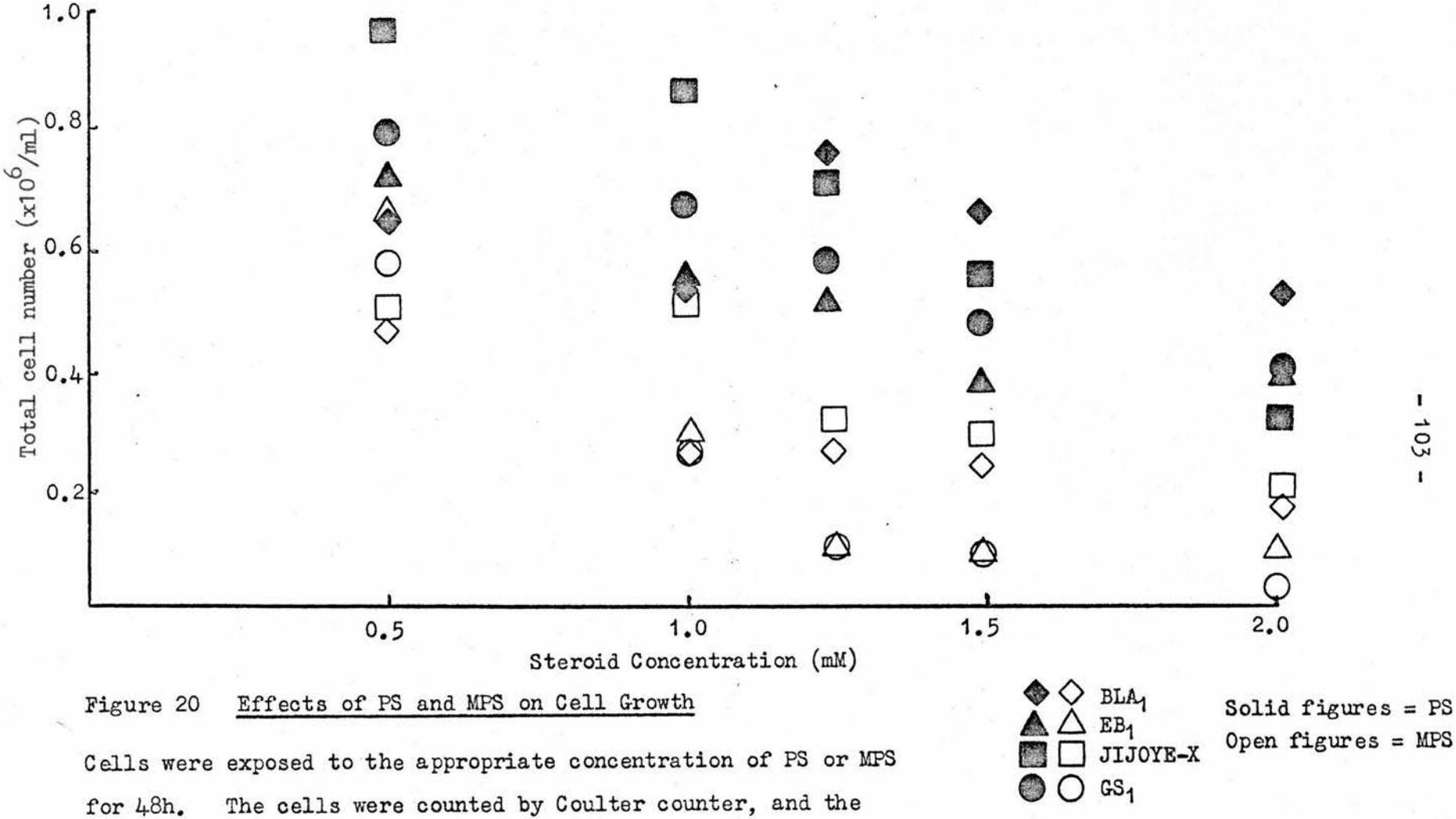


Figure 20 Effects of PS and MPS on Cell Growth

Cells were exposed to the appropriate concentration of PS or MPS for 48h. The cells were counted by Coulter counter, and the results expressed as the number of cells in treated cultures compared with control cultures receiving only water.

Table XVI

Effects of PS and MPS on Uridine Uptake into BLA_1 Cells

MPS	PS	CPM	% inhibition
0	0	50397	-
1mM	0	6419	88
0	1mM	15951	69

2ml BLA_1 cells (10^6/ml) in MEM + HEPES were pulsed for 20 min with ^{3}H-uridine (1μCi/ml) after incubation with 1mM MPS, 1mM PS or PBS for 1h. Cells were assayed for total cellular uptake of ^{3}H-uridine, as in Materials and Methods (page 25).

Results are the means of two separate experiments, each performed in duplicate.

Other Lethal Stimuli (ii) Ouabain

Ouabain is known to kill cells (Macdonald, Sachs & Orr, 1972). It binds to and inhibits Na^+-K^+ ATPase on the cell surface (Whittam & Chiperfield, 1975). Since this ATPase regulates the water and ionic balance in the cell, and since it is virtually certain that the extrusion of water accounts for the shrinkage of the apoptotic cell, ouabain might prove a suitable lethal stimulus for the study of apoptosis.

Materials and methods

Ouabain (Sigma, London) was prepared and administered as an aqueous solution of no more than 1% of the total volume. Cytotoxic assays were performed as in Materials and Methods (page 25).

Results

As a preliminary investigation I examined the effects of ouabain on the growth and lysis of BLA_1 cells. The cells show acute lysis and no growth at 50, 5 and 0.5μg/ml, and there was a marked cytostatic effect at 0.05μg/ml (table XVII). The effects were detectable at 24h, but took 72h to maximise. The same extent of cytolysis was observed when the cells were exposed briefly to ouabain (as short as 1h) (table XVIII).

Comment/

Table XVII

Effect of Ouabain on BLA_1 Cells - Continuous Exposure

Ouabain (µg/ml)	24h	48h	72h
	% lysis		
0	22	19	17
0.005	20	14	12
0.05	18	23	18
0.5	32	79	90
5	33	93	93
50	32	76	97
	Total cells ($x10^6$/ml)		
0	.37	.54	1.05
0.005	.41	.61	.98
0.05	.28	.45	.53
0.5	.30	.26	.16
5	.29	.24	.16
50	.32	.26	.16

BLA_1 cells (0.2×10^6/ml) were incubated with the appropriate concentration of ouabain for up to 72h. Total cell counts were assayed by Coulter counter and %lysis as in Materials and Methods (pages 13 - 14).

Table XVIII

Effect of Pulses of Ouabain (50μg/ml) on BLA_1 Cells

Pulse (h)	24h	48h	72h
		% lysis	
0	9	6	9
1	56	83	98
3	64	81	100
6	43	82	87
24	51	80	85
Continuous	40	65	82
		Total cells ($x10^6$/ml)	
0	.37	.63	.87
1	.12	.09	.08
3	.10	.08	.05
6	.13	.12	.09
24	.13	.11	.09
Continuous	.20	.17	.16

Separate samples of BLA_1 cells (0.2×10^6/ml) were exposed to 50μg/ml ouabain for the stated times, after which they were washed three times and resuspended in ouabain-free medium at 37°C. At 24, 48 and 72h after the initiation of exposure to ouabain, the samples were assessed for % lysis, defined as the % of cells taking up nigrosine (0.25%), and for total cell count by Coulter counter.

Results are the means of four separate experiments.

Comment

This system has two advantages over the MPS system. First, the fact that cells may be exposed to ouabain for brief periods and be irreversibly injured suggests that certain critical lethal biochemical events take place within the first hour of exposure to ouabain. In contrast, MPS must be present continuously for maximal effects on human lymphoblastoid cells (Bird et al (1977). in preparation). It is obviously easier to investigate critical changes when they occur within a short, defined time-span. Secondly, if cell lysis is a programmed event, it will depend on certain biochemical processes taking place in an ordered manner. It should be possible, therefore, to obtain mutants which are defective in these processes and thus resistant to the agent. This would certainly be true if cytolysis were a gene regulated event. For the induction of new gene products there are recognition and control sites which may be altered, and the induced gene products may themselves be defective in some way. The S49 mouse lymphoma system analysed by Sibley & Tomkins (1974) is a good example of this. The possible reasons for our failure to obtain cells resistant to 500µg/ml MPS could be that cytolysis is a result of a general breakdown in cell control (Trump & Ginn, 1969) probably involving many sites and requiring multiple mutations in the cell for resistance, or any mutation which confers resistance may itself be lethal. Ouabain resistant cells/

cells are known (Rosenberg, 1974) and Dr. Arthur (MRC Clinical and Population Cytogenetics Unit) has a clone of resistant JIJOYE cells. By identifying the nature of the mutations that initiate resistance, it will be easier to identify the biochemical processes involved in cell death.

In conclusion, ouabain may prove a more satisfactory stimulus for study than MPS because of the increased possibilities for biochemical analysis. However, the criteria for apoptosis are ultrastructural, and the effects of ouabain on cellular ultrastructure must be examined before we can draw any conclusions about its suitability.

DISCUSSION

The rôle of cytoplasmic glucocorticoid receptors

Perhaps the most fundamental finding of my work has been that cytoplasmic glucocorticoid receptors do not appear to be involved in the morphological or biochemical effects of MPS on human lymphoblastoid cell lines which I have described. The conclusions I have reached concerning cytoplasmic glucocorticoid receptors are:-

1) They cannot be implicated in cytolysis because (a) there is no quantitative relationship between the intracellular concentration of these receptors and the extent of cytolysis, (b) the concentration of glucocorticoid required for cytolysis is 10,000 times that which saturates receptors and(c) steroid must be present throughout the whole time for maximal effects (Bird et al (1977) in preparation). This does not appear to be true for receptor-mediated effects.

2) They cannot be implicated in the early effects on the uptake of precursors because (a) there is no quantitative relationship between the intracellular concentrations of these receptors and the extent of inhibition of uptake, (b) the concentration of glucocorticoid required for at least 50% inhibition of uptake is more than 1,000 times that/

that which saturates receptors and (c) the effects on facilitated diffusion uptake of nucleosides are far too rapid to allow alterations in transcription and translation to take place.

This contrasts with what has already been found in other extensively studied non-human systems. I have already mentioned the rôle of cytoplasmic glucocorticoid receptors in the cytolysis of rodent cells (pages 7-11). The Jensen & De Sombre model has been shown to apply to at least one glucocorticoid-sensitive mouse lymphoma, S49, (Sibley & Tomkins, 1974) in that functioning cytoplasmic receptors are necessary for glucocorticoid effects. When the biochemical effects of glucocorticoids on sensitive rodent cells are considered, there is considerable evidence to implicate specific gene activation in the effects (Mosher, Young & Munck, 1971; Young, Barnard, Mendelsohn & Giddings, 1974; Stevens & Stevens, 1975; Borthwick & Bell, 1976):

1) There is a latent period of 15 - 30min before the earliest effects are seen. This period allows for alterations in transcription and translation to take place.
2) RNA synthesis and hormone presence are required for at least the first 15min after addition of the hormone. After this there is only a requirement for continuing protein synthesis.

3)/

3) There is an increase in RNA synthesis 10 - 15min after addition of the hormone, and this is not caused by induction or activation of RNA polymerase but by increased activity of the gene template.

There still remains the finding that administration of actinomycin D (which inhibits RNA synthesis) at the same time as cortisol to rats does not prevent involution of the thymus (Hechter & Halkerston, 1965). There is no conclusive proof that biochemical and morphological effects of steroids are in any way related, and I am aware of no study which indicates that they are. It is quite possible that the morphological and biochemical effects are mediated by totally independent mechanisms, and claims that early steroid-induced biochemical effects are markers for cell death must therefore be treated with caution.

The possibility remains that cytoplasmic receptors may mediate some effects on the growth of human lymphoblastoid cells. Nilsson (1971) has reported retardation of growth in human lymphoblastoid cells treated with cortisol at 1μM, and similar concentrations of glucocorticoids may reduce the growth rates of fibroblasts (Pratt & Aronow, 1966; Nacht & Garzon, 1974). We are currently investigating the relationship between the nuclear binding of glucocorticoids and the cytostatic/

cytostatic effect in human lymphoblastoid cell lines, and it appears that cytostasis may be receptor mediated. As cytostasis *in vitro* may reflect cell death *in vivo*, it is still possible that the Jensen & De Sombre model may apply to human lymphoid tumours regressing after treatment with glucocorticoids, although much more work on the relationship between nuclear binding of steroid and tumour regression must be undertaken to clarify this.

Site of action of MPS

If, as I suggest, cytoplasmic receptors and alterations in gene activity are not involved in the effects I have described, we must examine other possible cellular sites for the action of MPS. MPS causes inhibition of uptake of certain precursors, alterations in the shape and volume of cells and ultimately cell lysis. The common factor between these three effects is that they all involve the cell surface in some way. There is increasing evidence for the cell surface as one possible site of action of glucocorticoids, and it has been reported that direct interaction between glucocorticoids and the cell surface causes changes in cell shape (Dell 'Orco & Melnykovitch, 1970), phospholipid metabolism (Fiskin & Melnykovitch, 1972) and glucose uptake (Plagemann & Renner, 1972). It is also known that glucocorticoids may interact with the lysosomal membrane to/

to stabilise it (see Nacht & Garzon, 1974). I suggest that the effects I have observed may be mediated at the level of the cell surface. It is not possible to say exactly in what manner MPS may affect the cell surface to bring about the range of changes observed. One theory involves decreased intracellular ATP concentrations caused by reduced uptake of glucose (Munck, 1971). Since MPS stimulates rather than inhibits the uptake of glucose into BLA_1 cells, this is clearly not the mechanism in the case of these cells. On the information available it is not possible to suggest any alternative, and we must agree with Stevens' comment (1974) that glucocorticoids "alter some undefined key membrane property".

The specificity of MPS effects

A possible criticism of the studies I have made is that extremely high concentrations of glucocorticoid are employed, and that this may be causing non-specific cell death. We have attempted to find cell lines that are insensitive to the high concentrations of MPS we have used. HeLa, 3T3 and a range of human primary cell strains were examined, but they all lysed at 500µg/ml MPS. Nevertheless, it cannot be dismissed as non-specific cell death of the type that would occur if the cells were exposed to strong acids or other highly noxious agents./

agents. We do detect at least some morphological changes typical of apoptosis, and since apoptosis may be a programmed form of cell death in some circumstances this suggests that there may be specificity in the changes we observed. The uptake of glucose and A-pathway amino acids is stimulated by MPS, which argues that there is not a general interference with cell surface functions. Although cells resistant to MPS have not been grown, it is very unusual to find a culture in which there are no viable cells even after 168h exposure to 500µg/ml.

The only cells we have found which are "resistant" to 500µg/ml MPS are human red blood cells which, in fact, show increased survival in vitro at this concentration. Cells may require to be actively dividing before they are affected by MPS, or cytolysis may be cell cycle dependent, although we have no direct evidence for this at present. Even allowing for differences in plasma membrane sturcture and properties between red blood cells and other cells, this suggests that MPS is not merely destroying the integrity of the plasma membrane.

Despite the fact that we can find no evidence of gene involvement, cytolysis may still be programmed. Cyclic nucleotides, especially cyclic AMP are known to act as regulators of cell functions (Pastan, Johnston & Anderson, 1975) and there are many other molecules which act as activators, inhibitors or regulators of cell metabolism (Tomkins, 1975). Turnell, Clarke & Burton/

Burton (1973) have suggested that raised intracellular free fatty acid concentrations may be the lethal stimulus, and MPS could affect free fatty acid concentrations without the nucleus being involved merely by interacting with the enzymes of triglyceride metabolism. The possible rôle of Ca^{++} in the initiation of mitosis and the inactivation of the chick erythrocyte nucleus (Harris, 1968) demonstrates how something as simple as ionic concentrations can alter cell metabolism drastically. (It is well known how the simplest ion, H^{+}, must be carefully regulated for almost any cellular function to occur properly.) Thus, while the concentrations of MPS we have used are unphysiological, this need not detract from the importance of the results we have obtained, and MPS may still be acting as the stimulus for some form of programmed cell death.

The nature of cell death

I have referred throughout this thesis to cytolysis and cell death, and it is important now to consider precisely what these terms mean. "Cytolysis" may be defined as the state in which a cell loses the integrity of its plasma membrane to the extent that it admits molecules such as nigrosine. It is not itself a morphologically distinctive form of cell death, and its relationship to the ultrastructural changes of apoptosis and coagulative necrosis is unknown. Clearly, since a cell depends on the stability of its cytoplasmic microenvironment/

environment, which is maintained by the semi-permeable plasma membrane, for its viability, a cell becomes dead when the integrity of its plasma membrane is destroyed. Nevertheless, a cell may be dead without showing any morphological damage to its plasma membrane. Indeed, apoptosis involves the fragmenting of the cell into membrane bound particles, and the membranes surrounding these particles appear to be intact by electron microscopy. It has been suggested by others that the uptake of vital dyes is not an accurate assay for cell death in vitro (Bhuyan, Loughman, Fraser & Day, 1976; Roper & Drewinko, 1976), and our work tends to support this idea. Nigrosine uptake is at best a late event in cell death and there is not any reason why a cell which is functionally dead need lose the integrity of its plasma membrane to the extent that it admits nigrosine.

"Cell death" is much more difficult to define than "cytolysis". It is a term which is often used, though few workers have ever attempted to specify precisely what it means. Bessis (1964) as paraphrased by Cooper, Bedford & Kenney (1975) states that "the moment of death coincides with the loss of a certain percentage of the cell organisation or the interaction between vital cell organelles". Trump & Ginn (1969) have proposed a very similar definition in which a reversible injury becomes irreversible when it causes a breakdown in intracellular homeostasis/

homeostasis which exceeds a certain critical threshold. Both of these definitions suffer from the faults that they refer to cell organisation or homeostasis (themselves undefined) and a certain level (again undefined) beyond which organisation or homeostasis must be disturbed for an injury to become irreversible and cell death occur. Cell organisation (and cell homeostasis) seem to me to be similar concepts to entropy; we are aware they exist but are incapable of measuring them directly. "How much organisation does a cell have?" is as difficult a question to answer as "how much entropy does a litre of water have?". The only way in which the organisation of a cell can be measured is by assessing the effects that alterations in organisation cause to be expressed in other cell parameters. Which merely begs the question, "which parameters?".

I have already stated the drawbacks in measuring membrane integrity as an assay for death of cells. By measuring dye exclusion we reveal dead cells, but not necessarily all of the dead cells in a population. The other parameters often used to assess the viability of cell populations relate to the ability of cells to reproduce themselves. The commonest are growth rate (reduction in which is termed "cytostasis"), DNA synthesis and colony forming efficiency. These suffer from the opposite drawbacks from nigrosine uptake; they may fail to reveal live cells. Any agent which causes cells to

go/

go into a resting state will cause reductions in all three parameters which could be taken as indicating cell death. High concentrations of thymidine, for example, will block cells on entry into S phase, but this is reversible and removal of thymidine will allow normal growth to recommence. Equally, BLA_1 cells have proved impossible to clone, which gives them a colony forming efficiency of zero.

There is much more work to be done in defining to a greater degree the critical events in cell death and in establishing reliable in vitro assays for the process. Much of our work will be directed towards these ends.

Future developments

To undertake further study of cell death it will be necessary to have a system which is predictable and manageable. I have already discussed the characteristics of such a system (pages 94 - 95) and in an attempt to satisfy these conditions we will investigate (i) a glucocorticoid-sensitive mouse lymphoma, (ii) ouabain-induced cell death and (iii) cytostatic effects in human or murine cells treated with glucocorticoids.

Mouse lymphoma P 1798 is reported to be sensitive to cytolysis by prednisolone at less than 1μM. Resistant cells are/

are known, and the cells may be grown *in vivo* or maintained for a few days *in vitro*. This may prove a satisfactory system for study.

Ouabain kills human cells, and cells resistant to ouabain may be isolated. We do not, however, know the form of cell death induced by ouabain, and this would have to be established before we could confidently use ouabain as a lethal stimulus for apoptosis. Ouabain inhibits $Na^+ K^+$ ATPase, which is involved in the ionic regulation of cells, and it is possible that ouabain merely lyses the cells through loss of osmotic control. It may be, however, that the ionic balance inside the cell can initiate apoptosis, and I shall discuss this later in this section. Ultrastructural studies on ouabain-induced cytolysis will establish its suitability or otherwise.

Cytostatic effects induced by glucocorticoids may be gene-regulated in BLA_1 and other lymphoblastoid cell lines. It would appear that some cells are resistant to glucocorticoid-induced cytostasis. We do not, however, know the relationship between cytostasis *in vitro* and cell death *in vivo* in detail.

There is then the question of what to do with a suitable system once one has been established. I have suggested, based on the remarks of Bessis (1964) and Trump & Ginn (1969) that the loss of a certain amount of cell organisation is responsible for/

for initiating cell death. The moment when cellular organisation is disrupted to the point that irreversible changes leading to cell death (as we can at present detect it) I shall call the "critical point".

Since changes in cell organisation cannot be measured directly but can only be observed through their effects on cellular precesses, we must choose and observe certain critical parameters in dying cells. These may be morphological or biochemical, and it is vital that both types of observations are related to each other at all times. We know some of the morphological changes in cells undergoing apoptosis, and, indeed, the process remains defined only in ultrastructural terms. Cells show clumping of the chromatin on the periphery of the nucleus, shrinkage of the whole cell and its organelles, blebbing from the cell surface, fragmentation of the nucleus and ultimately disruption of the cell into membrane bound particles. These are morphological responses to biochemical changes following attainment of the critical point, and we must select for study certain biochemical parameters in cells, alterations in which may lead to the morphological changes.

Since apoptosis involves shrinkage of the cells, presumably through loss of water, we will examine the effects of lethal agents/

agents on the intracellular concentrations of ions ions involved in cellular water regulation. These are Na^+ and K^+, and their intracellular concentrations will be measured in two ways: on bulk samples by atomic adsorption and in single cells by X-ray emission. Atomic adsorption is a widely used technique, but it has the same disadvantage as we encountered when measuring cell volume by Coulter counter - it reveals only changes in cell populations and not in individual cells. When a cell is under an electron beam, as in an electron microscope, its atoms become excited and fluoresce X-rays. These may be collected and their energies analysed to measure the concentrations of atoms inside single cells. Such a system is fitted to the scanning electron microscope in the MRC Clinical and Population Cytogenetics Unit. It should prove possible using this to measure intracellular Na^+ and K^+ in single cells before and after treatment with lethal agents and relate these directly to morphological changes. This has not yet been possible, because sample preparation involves the dehydration of the specimen and this affects the distribution of diffusible ions. Apparatus for viewing frozen hydrated specimens is at present undergoing evaluation, and will overcome this problem.

There are other biochemical measurements which may reflect/

reflect the degree of organisation in the cell. ATP and cyclic AMP concentrations, and the rate of synthesis and degradation of DNA, RNA and protein all seem reasonable possibilities, and we shall study these. This may give us some idea of the changes in cells after the critical point has been reached. At the same time, we will investigate different lethal stimuli that produce the same biochemical and morphological changes in cells. From any mode of action common to the different stimuli it may be possible to deduce the changes in cell organisation associated with the critical point. It is also important to isolate cells resistant to lethal stimuli, as determination of the biochemical change which confers resistance will give an indication of the biochemical processes necessary for cell death.

When we know more about the mechanisms and control of cell death at the level of the single cell, and the processes which render certain types of cells sensitive to lethal stimuli while others remain insensitive, then we will be much nearer a method for inducing the specific deletion of tumour cells.

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PUBLICATIONS

The following work connected with this thesis has been published or is at present in preparation for publication:-

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CYTOPLASMIC RECEPTOR LEVELS AND GLUCOCORTICOID RESPONSE IN HUMAN LYMPHOBLASTOID CELL LINES

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Summary.—The cytolethal response to treatment with prednisolone was investigated *in vitro* in eight human lymphoblastoid cell lines containing varying concentrations of specific cytoplasmic glucocorticoid receptors. A similar response was observed in seven of the lines irrespective of their concentration of cytoplasmic receptors, and pharmacological doses of steroid, well above those required to saturate receptors in cell-free extracts, were required for a massive lethal response. One cell line derived from Burkitt's lymphoma was refractory to lethal effects even with pharmacological doses of steroid.

A similar unresponsiveness to the cytolethal effect of prednisolone *in vitro* was observed in fresh lymphoblasts derived from patients with acute lymphoblastic leukaemia despite evidence of a satisfactory clinical response to therapy which included steroid. The resistance of human lymphoblastoid cells to treatment with glucocorticoids *in vitro* may result from a defect in activation subsequent to the binding of steroid to cytoplasmic receptors.

THE cytolethal effects of glucocorticoid hormones on normal and neoplastic lymphoid cells are well established (Dougherty, 1952; Harris, 1970; Rosenau *et al.*, 1972). Moreover, in combination with other drugs, glucocorticoid hormones are highly effective in the treatment of acute lymphoblastic leukaemia (ALL) of man (Simone, 1974). At the molecular level, however, the precise mode of action of glucocorticoid hormones on lymphoid cells has still to be resolved. It is generally held that binding of steroid to specific protein receptor molecules in the cytoplasm is the first step in the cytolytic process in sensitive cells. Subsequently, steroid-receptor complexes are believed to undergo a temperature-dependent conformational change and migrate to the nucleus, where they influence transcriptional activity in such a way that cell lysis results (Munck *et al.*, 1972; Higgins *et al.*, 1973; Thompson and Lippman, 1974).

However, much of the current state of knowledge concerning the mechanism of glucocorticoid hormone action is based on experiments with rodent tissues, including thymocytes and various cultured cell lines. Little is known of these events in human lymphoid cells and, in particular, the role of cytoplasmic receptors in the initiation of hormone effects appears uncertain. In one study (Lippman *et al.*, 1973) with freshly isolated lymphoblasts from patients with ALL, a close correlation was found between hormone responsiveness *in vivo* and the concentration of cytoplasmic receptors. However, other studies (Gailani *et al.*, 1973; Lippman, Perry and Thompson, 1974) with 3 lymphoblastoid cell lines *in vitro*, failed to reveal such an association and the role of cytoplasmic receptors in the initiation of cytolethal effects by glucocorticoids in human cells remains to be established.

To investigate this problem, we have

studied the relationship of cytoplasmic receptor levels and glucocorticoid cytolethal effects in a series of human lymphoblastoid cell lines derived from patients with leukaemia or lymphoma, or without malignant disease.

MATERIALS AND METHODS

Cell lines.—The cell lines were derived from freshly isolated human lymphoid cells of lymph glands, lymphoid tumours or peripheral blood. They were established as permanent cell lines in suspension culture, either spontaneously or by a process of co-cultivation with lethally irradiated cells containing Epstein–Barr virus (EBV) as described previously (Pulvertaft, 1965; Jensen *et al.*, 1967; Steel and Edmond, 1971; Steel, 1972). Previous studies have shown these cells to have the characteristics of B lymphocytes by their ability to synthesize immunoglobulins (Evans, Steel and Arthur, 1974), to have $C^{1}3$ receptors on their surface membranes (Moore and Minowada, 1973), to lack receptors for sheep red blood cells (Evans, Smith and Steel, 1975) and to be devoid of cytotoxic activity (Steel *et al.*, 1974).

Cell culture.—Cells were grown in suspension in conical glass flasks or roller culture bottles in Eagle's minimum essential medium (MEM, Gibco Biocult), or Ham's F10 medium with 10% tryptose phosphate broth (Gibco Biocult), supplemented with 20% heat-inactivated (56°C for 1 h) foetal calf serum (FCS, Gibco Biocult), at 37°C in a humidified atmosphere of 5% CO_2 in air. They were maintained at densities between 3 and 10×10^5/ml by feeding with fresh medium every 3–4 days.

Chromosome analysis.—Approximately 2×10^6 viable cells were resuspended in 5 ml of fresh growth medium. After 24 h, a drop of 0·02% dimethylcolchicine was added to the culture and the incubation continued at 37°C for a further 60–90 min. The cells were harvested by centrifugation, exposed to 0·0075 mol/l KCl for 10 min and fixed in 3 changes of methanol : glacial acetic acid (3 : 1, v/v). Drops of the fixed suspension were allowed to dry on clean slides, stained for 8 min in 0·5% quinacrine dihydrochloride, washed for 5 min in running water, mounted in distilled water under a sealed coverslip and examined with a Leitz Ortholux microscope with Ploem's vertical illumination using an HBO 200 u.v. source. Most cell lines have been examined repeatedly at intervals of a few months, and from 6 to 30 metaphase spreads photographed and fully analysed on each occasion.

Steroid binding by cell extracts.—The binding of glucocorticoid hormones to specific high affinity cytoplasmic receptors was studied by the competitive binding assay developed by Baxter and Tomkins (1971) using radioactively labelled and unlabelled dexamethasone. $3–5 \times 10^8$ cells were harvested by centrifugation (800 *g* for 10 min), washed twice in phosphate buffered saline (PBS; 0·025 mol/l KH_2PO_4, 0·1 mol/l NaCl, pH 7·4) at 0–4°C, recentrifuged and homogenized in ice-cold tricine buffer (0·02 mol/l tricine, 0·002 mol/l $CaCl_2$, 0·001 mol/l $MgCl_2$, pH 7·4). Rat thymuses were excised aseptically, rinsed in ice-cold PBS, blotted dry and chopped finely with scissors in 1 vol of ice-cold tricine buffer and homogenized. The cell and thymic homogenates were centrifuged at 105,000 *g* at 4°C for 1 h and duplicate aliquots of cytosol (0·4 ml) incubated at 0°C with varying concentrations of [1, 2(n)-^{3}H]-dexamethasone (19–29 Ci/mmol; Radiochemical Centre, Amersham) in the presence or absence of a 1000-fold excess of non-radioactive dexamethasone (Sigma). Unbound steroid was removed after 2 h by addition of 50–100 μl activated charcoal (200 mg/ml; BDH Chemicals), which was vigorously agitated for 5 sec and centrifuged (600 *g* for 1 min). The supernatant was recentrifuged (10,000 *g* for 5 min) and aliquots (200 μl) of supernatant assayed for radioactivity in a toluene-based scintillant containing Triton X-100 (33% v/v; Intertechnique) and butyl-PBD (5 g/l; Intertechnique) in a Beckman LS-250 liquid scintillation spectrometer (efficiency ~30%). Specifically bound dexamethasone represents the difference in amount of ^{3}H-dexamethasone bound to cytosol in the absence and presence of 1000-fold excess of non-radioactive steroid. Protein concentration was measured by the technique of Lowry *et al.* (1951) using bovine serum albumin as standard.

Cytolethal tests.—Duplicate cultures of cells ($3–5 \times 10^5$/ml) were grown in MEM supplemented with 20% heat-inactivated FCS at 37°C in a humidified atmosphere of 5% CO_2 in air. After 48 h when cells were in log phase of growth, methyl predni-

solone sodium succinate (Solumedrone, Upjohn) was added in aqueous solution at concentrations between 10^{-7} and 10^{-3} mol/l (final volume 1%). After incubation for a further 48 h, the total number of cells was enumerated with a haemacytometer and the viability assessed by exclusion of nigrosine (0·25%). Per cent lysis was calculated by comparison with control cultures which received no steroid.

RNA synthesis.—The effect of prednisolone on the incorporation of (5-^{3}H)-uridine (^{3}HU; 27 Ci/mmol; Radiochemical Centre, Amersham) into the acid-insoluble fraction of cells was estimated. Duplicate cultures of cells ($3–5 \times 10^5$/ml) were grown as described above. Solumedrone was added at concentrations between 10^{-6} and 10^{-3} mol/l to duplicate 1·0 ml aliquots of cells and after 1 h these were pulsed with 1·0 μCi/ml ^{3}HU for 20 min. The cells were collected in microfibre glass filters in a sampling manifold (Millipore), precipitated with ice-cold 5% trichloroacetic acid (3×10 ml) and washed with ice-cold 70% ethanol (3×10 ml). Filters were dried at 37°C and assayed for radioactivity in a toluene-based scintillant containing butyl-PBD (5·0 g/l) in a Beckman LS-250 liquid scintillation spectrometer (efficiency $\sim 30\%$). Results are expressed as incorporation of ^{3}HU into the acid insoluble fraction/10^6 viable cells.

RESULTS

Origin and karyotype of lymphoblastoid cell lines

The origin, karyotype and age *in vitro* of the 8 cell lines used in our studies are shown in Table I. Whereas there was some variation in chromosome constitution within each line, there was always a clear modal karyotype. Four lines—RUS_1, RUS_2, PEN_2 and YAK_1—had only minor alterations to the normal diploid human complement, but the others had multiple breakages and recombinations, including fragments and abnormal chromosomes the precise origin of which could not be established.

Glucocorticoid cytoplasmic receptors in lymphoblastoid cell lines

In steroid binding studies specific receptors in the cytoplasmic extracts (cytosol) of lymphoblastoid cells became saturated with dexamethasone at concentrations above $5–8 \times 10^{-8}$ mol/l as illustrated in Fig. 1. Scatchard (1949) analysis of the data, shown in the insert of Fig. 1, yields a straight line consistent with a single class of receptor molecules

TABLE I.—*Origin, Karyotype, Age in Culture and Cytoplasmic Receptor Concentration of Human Lymphoblastoid Cell Lines*

Cell line	Origin	Modal karyotype	Age in culture (mth)	Specifically bound dexamethasone (pmol/mg protein)
RUS_1	Acute myeloblastic leukaemia	46 XY 18p+	27	0·82
RUS_2	Acute myeloblastic leukaemia	46 XY 3/8 Translocation	27	0·66
BLA_1	Acute lymphoblastic leukaemia	46 XY Multiple breakages and recombinations	34	0·62
F89	Subacute lymphatic leukaemia	48 XY Multiple breakages and recombinations	94	0·16
GS_1	Chronic lymphatic leukaemia	48 XX Multiple breakages and recombinations	73	0·71
J1JOYE	Burkitt's lymphoma	Near tetraploid. Multiple breakages and recombinations	96	0·43
PEN_2	Adult blood*	48 XXY 14+	24	0·09
YAK_1	Cord blood	47 XY Partial trisomy 4	17	0·37
Fresh thymus	Rat+		—	0·33

The results shown are the mean of 2 separate determinations.
* Klinefelter's syndrome. + Female PVG/C rats aged 88 days.

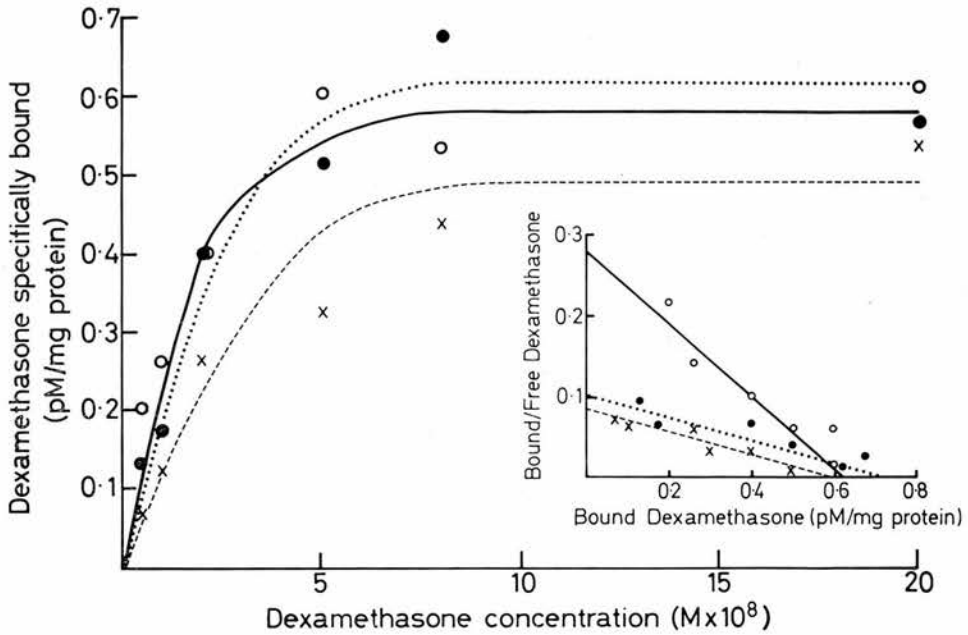


FIG. 1.—Specific binding of dexamethasone to cell-free extracts of human lymphoblastoid cell lines and rat thymus. Each point represents the mean of 3 separate experiments. The insert shows Scatchard plots of the data. ○———○, RUS_2 cells; ○– – –○, GS_1 cells; ×–·–·–×, rat thymus.

of uniform steroid affinity. The equilibrium (dissociation) constants for the 2 examples shown, calculated from the intercepts of the reciprocal plots, were $1{\cdot}0 \times 10^{-8}$ mol/l (RUS_2) and $2{\cdot}3 \times 10^{-8}$ mol/l (GS_1). For comparison, Fig. 1 shows also the binding of dexamethasone to cytoplasmic receptors of fresh rat thymus, a tissue of known high sensitivity to the cytolytic effects of glucocorticoid hormones *in vivo* (Dougherty and White, 1945); saturation occurred at similar concentrations of steroid, and the dissociation constant ($3{\cdot}7 \times 10^{-8}$ mol/l) was of similar magnitude.

Further characterization of lymphoblastoid cell receptors revealed that they were thermolabile and completely inactivated by 30 min pre-incubation at 37°C. Similarly, incubation for 10 min at 20°C with trypsin (1 mg/ml) and protease (1 mg/ml) destroyed the binding capacity of cytosol. Incubation with deoxyribonuclease (bovine pancreas, 100 μg/ml) and ribonuclease (bovine pancreas, 100 μg/ml) had no significant effect on the binding characteristics. Thus, the cytoplasmic glucocorticoid receptors of human lymphoblastoid cells appear to be of a protein nature similar to those described in other glucocorticoid sensitive tissues (Hackney *et al.*, 1970; Munck and Wira, 1971; Baxter and Tomkins, 1971).

Using the competitive binding assay at saturating concentrations of dexamethasone (8×10^{-8} mol/l), the relative concentration of receptors in the cytosols of the various cell lines was determined. As shown in Table I a gradation in receptor concentration was found. The highest levels (0·62–0·82 pmol/mg protein) occurred in cell lines derived from patients with acute leukaemia and from one case of chronic lymphatic leukaemia, whilst intermediate concentrations (0·37–0·43 pmol/mg protein) were found in lines derived from a Burkitt's lymphoma and a healthy placental cord blood. The lowest levels (0·09–0·16 pmol/mg protein)

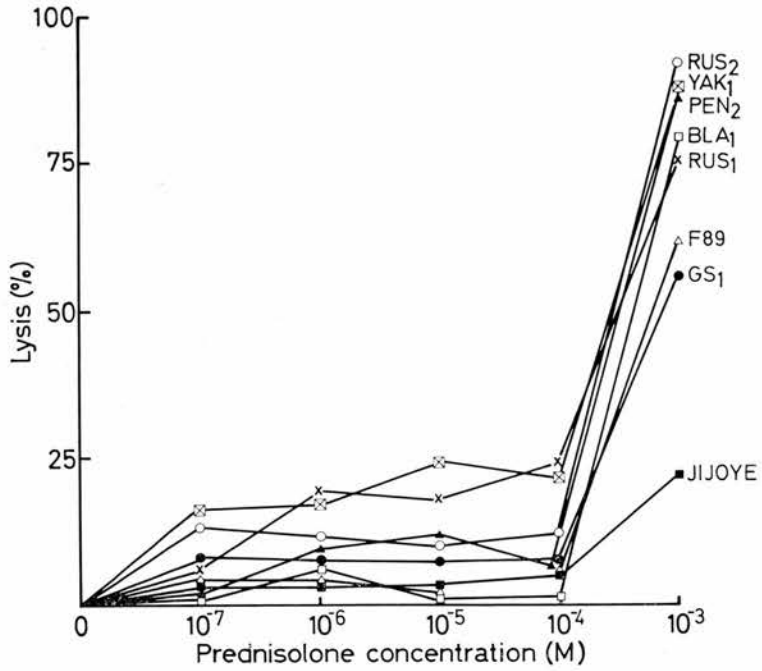


FIG. 2.—Cytolytic effect of prednisolone on human lymphoblastoid cell lines. Per cent lysis was calculated by comparison with control cultures which received no steroid. Each point represents the mean of 2 separate experiments.

were found in the lines derived from a patient with subacute lymphatic leukaemia and from the peripheral blood of a non-leukaemic adult patient. The concentration of receptors (0·33 pmol/mg protein) in the fresh rat thymus corresponded to the intermediate values obtained in the cell lines.

Glucocorticoid cytolethal response

The lethal response was assessed morphologically by the ability of cells to exclude the dye nigrosine, following incubation with aqueous preparations of steroid for 48 h. As shown in Fig. 2, a mild lethal response (10–15% of cells) was observed with prednisolone at concentrations of 10^{-7}–10^{-4} mol/l although these effects were apparently not in direct proportion to absolute concentrations of steroid. A marked increase in the cytolethal effect was observed, however, when the steroid concentration was increased to 10^{-3} mol/l and in some instances more than 85% of cells were killed. The magnitude of this enhanced lethal response, however, did not correlate with the measured levels of specific cytoplasmic hormone receptors, and some of the cell lines with low receptor concentration appeared to be as sensitive as those with high receptor levels (compare Fig. 2 and Table I). The cell line derived from Burkitt's lymphoma, however, was notably resistant to lethal effects even with high doses of steroid. Table I and Fig. 2 show also that no correlation could be established between steroid receptor levels or sensitivity to cytolytic effects and criteria which may be related to the malignant " potential " of lymphoblastoid cells *in vivo*, namely the origin of the cells (from malignant or non-malignant conditions), the degree of abnormality of modal karyotype or the age of cells *in vitro*.

The concentration of prednisolone

(10^{-3} mol/l) required to achieve severe lethal effects exceeds physiological plasma levels of steroid (10^{-6}–10^{-7} mol/l) by several orders of magnitude. Moreover, as can be seen in Fig. 1, it is considerably in excess of steroid concentrations required to saturate receptors in cytoplasmic extracts. However, when other glucocorticoid hormones such as cortisol and dexamethasone were tested over the same concentration range virtually the same, or in some cases somewhat reduced, lethal effects were obtained, and no significant differences were observed when steroids soluble in ethanol or dimethylsulphoxide were substituted for aqueous preparations. Furthermore, destruction of transcortin binding activity of serum with heat (56°C for 1 h) did not reduce the lethal response obtained with cortisol or prednisolone.

Ultrastructural studies of cultures treated with 10^{-3} mol/l prednisolone showed that less than 3% of steroid-treated cells contained EBV particles and the cytolethal effects could not be attributed to induction of virus lytic cycle.

Cytolethal tests were also performed with lymphoblasts isolated from the peripheral blood of 6 patients with ALL before commencement of therapy. Despite an apparent satisfactory clinical response to chemotherapy which included prednisolone, these cells did not show any greater sensitivity to the lethal effects of glucocorticoids *in vitro* than the cultured lymphoblasts. Insufficient material was available, however, to estimate the receptor levels in these cells.

Glucocorticoid effect on RNA synthesis

The effect of prednisolone on the incorporation of ^{3}HU into the cold acid-insoluble fraction of lymphoblastoid cells was studied as an earlier and more sensitive index of cell damage than nigrosine. Preliminary investigations showed that significant inhibition of ^{3}HU incorporation could be detected within 1 h of addition of prednisolone. Similar results were observed in all the cell lines studied, including the Burkitt's lymphoma cell line, as shown in Table II, irrespective of their specific cytoplasmic receptor concentration: thus, 1 h after addition of 10^{-5} and 10^{-6} mol/l steroid there was a slight reduction ($\leqslant$20%) in ^{3}HU incorporation; with 10^{-4} mol/l prednisolone moderate reductions (30–50%) were observed whilst addition of 10^{-3} mol/l steroid produced a marked inhibition ($>$75%) of ^{3}HU incorporation in all cell lines.

TABLE II.—*Effect of Prednisolone on Incorporation of ^{3}H-uridine into Human Lymphoblastoid Cell Lines*

Cell line	Control incorporation (ct/min/10^6 viable cells)	Fractional incorporation of control: Prednisolone concentration (mol/l) 10^{-6}	10^{-5}	10^{-4}	10^{-3}
RUS_1	2975	0·99	0·89	0·72	0·23
RUS_2	7254	0·90	0·81	0·56	0·15
BLA_1	13152	0·87	0·79	0·47	0·09
F89	17743	1·02	0·86	0·58	0·13
GS_1	5539	0·87	0·84	0·63	0·19
JIJOYE	45620	0·88	0·83	0·68	0·16
PEN_2	12630	0·89	0·88	0·60	0·11
YAK_1	15571	0·91	0·86	0·64	0·15

The results shown are the mean of two separate determinations and represent incorporation of ^{3}H-uridine into the acid-insoluble fraction/10^6 viable cells.

DISCUSSION

In contrast to the findings *in vivo* with lymphoblastoid cells of ALL patients (Lippman *et al.*, 1973), our results clearly show that the level of specific cytoplasmic receptors in human lymphoblastoid cells cannot be used to predict their responsiveness to glucocorticoid treatment *in vitro*. Similar responses to steroid treatment were obtained with all but one of the cell lines despite widely varying levels of cytoplasmic receptors: the exception was a cell line derived from Burkitt's lymphoma, although it showed a similar response to inhibition of RNA synthesis as the other cell lines. It is noteworthy that in our studies significant lethal effects were observed only with doses of steroid which produced a severe reduction ($>$75%) in incorporation of RNA

precursors. Other workers (Rosen *et al.*, 1972; Stevens, Stevens and Hollander, 1974) have claimed that smaller reductions in RNA synthesis are associated with impending lethal effects, although their experiments did not include morphological observations of cell death.

Although failure to exclude nigrosine is a rather insensitive test of cytolethal damage since it occurs late in the process of cell death, other techniques which employ release of specific radiolabels from damaged cells measure similar late phenomena and are associated with inherent interpretative difficulties due to "spontaneous" release of label (^{51}chromium) or internal radiation effects (^{125} iododeoxyuridine).

When compared with rodent lymphoma cell lines, human lymphoblastoid cells appear relatively insensitive to the lethal effects of glucocorticoids *in vitro*. Rodent lymphoma cell lines (Harris, 1970; Rosenau *et al.*, 1972; Turnell, Clarke and Burton, 1973; Kondo, Kikuta and Noumura, 1975), nearly always show marked lethal responses to concentrations of glucocorticoids in the physiological range (10^{-6}–10^{-7} mol/l) and thus may differ fundamentally in their biological responsiveness to steroid hormones.

The failure to correlate cytoplasmic receptor levels with glucocorticoid responses, and the requirement of pharmacological doses of steroid for substantial cytolethal effects, suggest that cytoplasmic receptors may not be responsible for initiation of the lethal glucocorticoid effects we have observed in human lymphoblastoid cells. Alternatively, some form of steroid resistance may have developed during the long period of cultivation of cells *in vitro*. However, in our hands freshly isolated lymphoblasts from ALL patients showed a similar resistance to lethal glucocorticoid effects *in vitro*. It is possible, therefore, that defects in activation of glucocorticoid cytolethal methanisms may occur in lymphoblastoid cells cultured *in vitro* for short or long periods of time, rendering cells insensitive to all but massive doses of steroid.

Until recently, the emergence of resistance to steroid effects has been attributed to quantitative reductions in cytoplasmic receptor levels (Rosenau *et al.*, 1972; Lippman *et al.*, 1973). Clearly, in our cell lines this cannot account for steroid resistance if present. However, Sibley and Tomkins (1974) have recently shown in studies with steroid-resistant clones of mouse lymphoma cells that whilst resistance to steroid effects results predominantly from quantitative deficiencies in steroid receptors, other more subtle defects in hormone activation may occur. Thus, resistance may result from qualitative defects in cytoplasmic receptor molecules or reduction in the capacity for transfer of formed steroid receptor complexes to the nucleus. Rarely, defects in the specific localization of complexes within the nucleus appear to occur since nuclear binding of steroid receptor complexes did not provoke a lethal response in some clones.

It is evident, therefore, that the binding of steroids to cytoplasmic receptors represents only one stage of a complex series of events leading to expression of hormone effects. It remains to be seen whether the activation of steroids in human lymphoblastoid cells *in vitro* differs fundamentally from that occurring *in vivo*. It seems likely, however, that analysis of each step in the activation process will be required before the potential responsiveness of cells to glucocorticoid hormones can be predicted accurately.

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for other proteins (Gregoriadis, 1974), and it suggests an interaction of the enzyme with the liposomal lipids. Entrapment of proteins in liposomes that can be tolerated by man can be achieved under sterile conditions (Gregoriadis *et al.*, 1974).

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Precocious Development of Uridine Diphosphate Glucuronyltransferase Activity in Chick-Embryo Liver after Administration of 11β-Hydroxy Steroids with and without Thyroxine

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UDP-Glucuronyltransferase (EC 2.4.1.17) activity is low in or absent from fresh homogenates or microsomal preparations of chick-embryo liver until hatching at 21 days, when it rapidly increases to adult values (Dutton & Ko, 1966). Precocious development of adult activity can occur in embryo liver of 15 days or later, after grafting, 3–4 days earlier, of the cephalic region of the anterior-pituitary gland from a hatching or hatched bird on to the chorioallantoic membrane (Wishart & Dutton, 1974, 1975*a*). Injection of adrenocorticotrophic, follicle-stimulating or thyrotrophic hormone, known to be secreted by this region of the gland (Brasch & Betz, 1971), had no reproducible effect on the transferase activity (Leakey & Dutton, 1975).

To mimic natural release of hormone from endogenous or grafted tissue we developed a new technique of hormone administration, involving continuous flow down a paper strip from a reservoir on to the chorioallantoic membrane. Rate of flow is controllable. By this means we have demonstrated that adrenocorticotrophic hormone and corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione) provoke precocious appearance of the transferase on day 17 when applied from day 13 (Leakey & Dutton, 1975).

We summarize here further effects on the enzyme after treatment with corticosteroids of the embryo *in ovo* and of liver in organ culture.

Corticosterone, cortisol (11β,17,21-trihydroxy-4-pregene-3,20-dione) and aldosterone (11β,21-dihydroxy-3,20-dioxo-4-pregnene-18-al) all stimulated precocious activity, but 11-deoxycorticosterone, progesterone (4-pregnene-3,20-dione), pregnenolone (3β-hydroxy-5-pregnen-20-one), tetracortisol (3α,17α,21-trihydroxy-5β-pregnane-11,20-dione), testosterone (17β-hydroxy-4-androsten-3-one) and oestriol [1,3,5(10)-estratriene-13,16α,17β-triol] had no effect. Ability to stimulate appeared to require the 11β-hydroxy group; cortisone (17α,21-dihydroxy-4-pregnene-3,11,20-trione) was much less effective than cortisol, requiring 5 times its concentration for stimulation.

Although lower doses occasionally stimulated, the minimal amount of corticosterone or cortisol for reproducible effect was 0.18 μmol per egg administered over the 4 days. Maximal stimulation of transferase was 30-fold, i.e. to some 3–4 times adult activity.

Minimal time of administration required for evident enzyme stimulation 4 days after onset of administration of high doses of corticosteroid was 2–4 h. The minimal time for

transferase to respond depended on steroid dosage. High doses (0.5 μmol/egg per day), which are lethal if applied for 96h, stimulated activity in 48h, whereas 0.2 μmol/egg per day (non-lethal) needed 72h. However, thyroxine (10–15nmol/day) added to the lower dose of steroid brought activity up in 48h. Under these conditions thyroxine alone had no effect. As the pituitary graft requires only 48h for stimulation it may exert its effect through both the adrenocorticotrophic and thyrotrophic hormone.

When applied at 0.05 μmol/egg per day over 4 days (higher doses were lethal), corticosterone had no effect on liver transferase of embryos aged 9 or 10 days at the onset of treatment. Application of this dose from day 11, however, gave the same stimulation as when applied from day 13. As 72h are needed for stimulation to be apparent, it seems that competence of chick-embryo liver transferase to respond to corticosterone application by this method begins on day 13–14. This is the time of competence after pituitary grafts (Wishart & Dutton, 1975*a*), and suggests again a linkage between the two effects. Both corticosterone application and grafting if carried out before day 11, provoke liver necrosis. The late onset of competence with these physiological stimulators of the transferase contrasts with the ability of phenobarbital to induce the enzyme within a few hours of incubation from day 0 (Wishart & Dutton, 1975*b*).

To determine if 11β-hydroxy steroids acted directly on embryo liver, they were added to the medium of organ cultures. Although the transferase is spontaneously developed under these conditions (Ko *et al.*, 1967; Skea & Nemeth, 1969), an increased rate of development is measurable when phenobarbital is added (Burchell *et al.*, 1972). When corticosterone was added at various concentrations, no obvious increase in the rate of the transferase induction was noted in cultures from 15-day embryo liver.

If thyroxine was also present, however, UDP-glucuronyltransferase activity was twofold that of controls after 3 days. Although phenobarbital on injection *in ovo* at that age raises the enzyme activity 50-fold, its maximum increase of rate of development in cultures then is still only twofold. The doubling observed with corticosterone plus thyroxine may therefore be considered significant. Optimal concentrations of the two hormones in the medium under these conditions were 10–15 μmol and 0.75–1.25 μmol respectively.

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Inhibition of Uridine Uptake by Methylprednisolone in Human Lymphoblastoid Cells

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Glucocorticoids have been shown both *in vivo* and *in vitro* to cause the death of sensitive lymphoid cells, and, in combination with other drugs, they are used in the treatment of human lymphoid leukaemias (Simone, 1974). Much work has been directed towards

Table 1. *Effect of prednisolone on uridine-uptake pathways*

Samples (2ml) of BLA_1 cells (approx. 1×10^6/ml) in modified Eagle's medium+20mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid]+20% foetal calf serum were incubated with 20μl of methyl prednisolone, sodium succinate or 20μl of water for 1h. [^{3}H]Uridine (1μCi/ml) was added for 20min at the same time as the appropriate amount of unlabelled uridine. Cells were then cooled, washed twice with ice-cold phosphate-buffered saline, and resuspended in 1ml of 5% (w/v) trichloroacetic acid. Results are expressed as uptake into acid-soluble material in pmol/min per 10^6 cells.

	Saturable uptake		
Prednisolone* (μg/ml) ...	0	50	500
Uridine (μM)			
10	33.6	20.6	2.8
50	59.9	55.9	15.9
100	67.1	52.2	25.0
500	7.20	55.0	40.2
1000	71.9	62.4	40.1
	Non-saturable uptake		
Prednisolone* (μg/ml) ...	0	50	500
Uridine (μM)			
10	1.3	1.5	1.4
50	6.4	7.6	7.2
100	12.8	15.2	14.4
500	64.0	76.0	72.0
1000	128.0	152.0	144.0

* Prednisolone was administered as methylprednisolone sodium succinate, concentrations equivalent to prednisolone being given as μg/ml.

Table 2. *Rate of inhibition of uridine uptake by prednisolone*

Samples (2ml) of cells (as in Table 1) were pulsed for 3min with [^{3}H]uridine (10μCi/ml) at intervals before and after adding prednisolone (500μg/ml) (at 0min). Results are expressed as percentage inhibition of uptake into acid-soluble material.

Time of pulse (min)	Percentage inhibition of uptake
−3–0	0
0–3	89
3–6	91
6–9	89
30–33	90

the elucidation of the biochemical effects of these steroids on sensitive cells, using mainly mouse lymphoma cells or rat thymocytes. It has been established that in these cells among the earliest effects of lethal doses of glucocorticoids are the inhibition of uptake of carbohydrates, nucleotides and amino acids, and that these actions depend on sequential transcriptional and translational activity (Young *et al.*, 1974; Stevens & Stevens, 1975).

In an attempt to investigate the effects of glucocorticoids on human lymphoid cells, we have studied their action on human lymphoblastoid cells in culture. We have already

shown that these cells have a range of cytoplasmic steroid-receptor concentrations, show marked, but slightly different, cytolethal effects after culture in 1 mM-prednisolone for 48 h, and all demonstrate a similar decrease in incorporation of [^{3}H]uridine into acid-insoluble material at 0.1 mM- or 1 mM-prednisolone. This last effect is independent of the concentration of cytoplasmic receptors, and cannot be correlated directly with the ultimate extent of cytolysis (Bird *et al.*, 1975).

We have now investigated this inhibition of uridine incorporation in further detail using one cell line, BLA_1, which was derived from the peripheral blood of a patient with acute lymphoblastic leukaemia. Both the uptake of labelled uridine into the acid-soluble pools of the cells and its incorporation into acid-insoluble material have been assayed. It can be shown that any inhibition of incorporation of uridine is accompanied by an identical decrease in its uptake, and this suggests that the prime action of prednisolone is against the uptake of uridine into the cell. We assayed the uptake of uridine into the cells at various extracellular uridine concentrations, and found that uridine uptake consists of the same two-component system demonstrated by Plagemann (1970). Facilitated diffusion was the dominant path up to extracellular uridine concentrations of 10 μM, after which simple diffusion assumed greater importance. When the same assays were performed in the presence of 50- or 500-prednisolone/ml, it was found that the inhibition was greatest at the lowest uridine concentrations, and that it was only the facilitated-diffusion pathway that was inhibited (Table 1). The rate with which this inhibition occurred was also measured, and appears to be virtually instantaneous (Table 2).

Jensen & De Sombre (1973) have demonstrated a mechanism for steroid-hormone action, which involves the binding of the hormone to a cytoplasmic receptor, the activation of the hormone–receptor complex and its subsequent transfer to the nucleus where it alters transcriptional activity. This model applies to the inhibition of substrate uptake in glucocorticoid-sensitive rodent lymphoid cells (Sibley & Tomkins, 1974; Stevens & Stevens, 1975). Thus there is a latent period, during which alterations in transcription and translation take place, between the initial challenge with the steroid and the earliest detectable effects. The rapidity of the effects in our human lymphoblastoid cell system suggests that there is an alternative mechanism for steroid-hormone action. Although the biochemical effects obtained are more striking than those in rodent cells, we must use much higher doses of steroid to obtain significant cell death. The fact that this concentration of prednisolone (500 μg/ml) is > 10000 times the concentration of dexamethasone that saturates the cytoplasmic receptors (assayed by both cell-free and whole-cell binding systems), and the equal inhibition of uridine uptake in cells with high or extremely low concentrations of receptors make it difficult to implicate these receptors in the steroid action. We suggest, therefore, that prednisolone is acting directly against the facilitated diffusion uptake at the level of the cell surface.

It remains to be shown whether this effect is specific to lymphoid cells, and whether prednisolone is exerting a specific effect against uridine uptake or altering some undefined membrane property in a manner that will interfere with the facilitated diffusion uptake of other substances.

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