THE REGULATION OF INTERFERON SYNTHESIS IN ANIMAL CELLS

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by

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CONTENTS

TABLES

FIGURES

INTRODUCTION

Interferons are proteins secreted by animal cells in response to infection with many kinds of viruses (Isaacs & Lindenmann, 1957; Ho, 1973a) or to treatment with synthetic inducer molecules such as double-stranded polyribonucleotides (Merigan, 1973). Interaction with the released interferon leads to the development of an anti-viral state in other cells of the same species.

A. The Chemistry of Interferons

The molecular weight of interferons generally falls between 20,000 and 40,000 but values of up to 160,000 have been found (Fantes, 1973). It has been demonstrated that human leucocyte interferon, induced by Newcastle Disease virus (NDV), can be separated into two components by hydrophobic affinity chromatography on alkyl-agarose and by ion-exchange chromatography (Chen et al. 1976). These components have the same molecular weight (26,000) as measured by molecular sieving on Sephadex G-75. Mouse interferon, induced in L cells by NDV, has also been shown to contain two different molecular species (Stewart, 1974).

The glycoprotein nature of interferons is indicated by their sensitivity to periodate treatment (Fantes, 1973) and the binding of mouse and human interferons to Concanavalin A, a plant lectin, having affinity for α-methyl-D-mannoside residues (Besancon & Bourgeade, 1974). Interferons often show microheterogeneity when separated by charge but Dorner, Scriba & Weil (1973) have shown that removal of sialic acid residues from rabbit interferon, by treatment with

neuraminidase, results in a homogenous preparation on subsequent isoelectric-focussing. This 'asialo-interferon' binds to the plant lectin phytohaemagglutin, which has an affinity for the oligosaccharide sequence galactose $\longrightarrow$ N-acetylglucosamine $\longrightarrow$ mannose.

The role of the carbohydrate moiety of interferon is not clear. Bose et al. (1976) labelled human interferon with D-[^{3}H]-glucosamine. Removal of the carbohydrate (up to 80%) led to a reduction in molecular weight of 4,000 but no loss of anti-viral activity. Messenger RNA (mRNA), isolated from cells induced for interferon synthesis, can be translated in heterologous cells, Xenopus oocytes or cell-free systems and the protein synthesized has anti-viral activity characteristic of the donor cells (De Maeyer-Guignard, De Maeyer & Montagnier, 1972; Reynolds, Premkumar & Pitha, 1975). Thus glycosylation may be unimportant for interferon secretion, anti-viral activity or species-specificity.

The high biological potency of interferons has made their purification by standard techniques of protein chemistry difficult (Fantes, 1973). The purest human interferon preparation yet obtained has 2.10^8 units per milligram of protein (Knight, 1976a). The activity of a particular interferon preparation is expressed as the reciprocal of that dilution of interferon at which virus cytopathic effect or plaque formation is reduced to 50% of control values. It can be calculated, assuming that one unit of interferon protects 10^6 cells, that a maximum of 100 molecules, of the above human interferon preparation, are needed to protect one cell. This assumes that all the protein is interferon.

B. The Biological Activity of Interferon

Treatment of cells with interferon inhibits subsequent virus replication. This anti-viral action depends on the presence of a nucleus in the treated cell (Radke et al. 1974; Young, Pringle & Follett, 1975) and on RNA and protein synthesis (Taylor, 1964; Sonnabend, Kerr & Martin, 1970).

It is not known if the anti-viral effect of interferon is mediated via the cell surface or if interferon first enters the cell. The anti-viral effect of human interferon in mouse-human cell hybrids is dependent on the presence of human chromosome 21 (Tan, Tischfield & Ruddle, 1973) and human cells trisomic for this chromosome are more sensitive to the anti-viral action of human interferon (Tan et al. 1974b). Antibodies to the cell surface of mouse-human hybrid cells containing human chromosome 21 inhibit the action of human interferon in human cells (Revel, Bash & Ruddle, 1976). This indicates that a gene for a cell-surface receptor might be carried by chromosome 21, however, this has been questioned by De Clercq, Edy & Cassiman (1976). The replication of all groups of viruses is inhibited in interferon-treated cells to some extent but the sensitivity of a virus may vary with the specific interferon being tested (Stewart, Scott & Sulkin, 1969). Studies of virus replication in interferon-treated cells have shown that translation of virus mRNA is inhibited (Repik, Flamand & Bishop, 1974; Wiebe & Joklik, 1975; Metz, Esteban & Danielescu, 1975) but for some viruses a primary effect on transcription or RNA turnover seems more likely (Metz, Levin & Oxman, 1976). A membrane-bound ribonuclease activity has been found in chick embryo cells treated with interferon (Marcus, Terry & Levine, 1975) but although Maenner

& Brandner (1976) could confirm this observation in chick cells they did not find induced ribonuclease activity in African Green monkey or hamster cells.

The mechanism of the interferon-mediated inhibition of translation has been studied extensively in cell-free systems. Samuel & Joklik (1974), using a cell-free system from interferon-treated mouse cells, demonstrated that translation of viral mRNAS, but not of cellular mRNAS, was inhibited. A ribosome-associated polypeptide (mol. wt. 48,000) was isolated from extracts of interferon-treated but not control cells. Samuel (1976) also showed that the inhibition of translation of viral mRNAS occurred at a step subsequent to the formation of the first dipeptide bond. The addition of transfer RNAs (tRNAs), from untreated or from interferon-treated cells, to the cell-free protein-synthesizing system partially removed the inhibition of viral mRNA translation.

Some cell-free systems from interferon-treated cells require the addition of double-stranded polyribonucleotides (dsRNAs) before an inhibition of protein synthesis can be demonstrated (Kerr, Brown & Ball, 1974). Pre-incubation of a post-ribosomal supernatant from interferon-treated cells with dsRNA and ATP before addition to a protein-synthesizing system enhanced the inhibitory effect 100-fold (Roberts, Clemens & Kerr, 1976a). A dsRNA dependent protein kinase activity was also found in extracts from interferon-treated cells (Roberts *et al.* 1976b) but neither of the two major proteins phosphorylated was related to the inhibitor of protein synthesis which had a relatively low molecular weight.

The addition of dsRNA has been found to increase endonuclease activity in extracts from interferon-treated but not

control cells (Sen et al. 1976). ATP and dsRNA are required during an activation phase but not during the degradation of viral mRNA. Lebleu et al. (1976) also showed that the addition of dsRNA to cell-free systems from interferon-treated cells promoted the phosphorylation of two proteins. The relationship of protein kinase activity to the inhibition of protein synthesis and increased endonuclease activity in extracts of interferon treated cells is not clear. Thus the precise in vivo mechanism(s) of interferon action has still to be elucidated.

The species specificity of interferon action is not absolute. Human and monkey interferons are active on both human and monkey cells (Bucknall, 1967) and hamster and mouse interferons also show reciprocal, heterologous activity (Stewart et al. 1969). Human interferon has also been found to have non-reciprocal anti-viral activity on a number of cell types including rabbit, cat and Chinese hamster cells (Desmyter, Rawls & Melnick, 1970; Desmyter & Stewart, 1976; Morgan, 1976).

Whereas virus macromolecular synthesis is inhibited in interferon-treated cells host RNA and protein synthesis does not seem to be grossly affected at the same interferon concentrations (Metz, 1975a). However, a number of non-anti-viral effects have been attributed to interferons. These include inhibition of the growth of normal and transformed cells (Gresser, 1972; Stewart et al. 1976; Knight, 1976b) and increased susceptibility to the toxic effects of double-stranded RNA (Stewart et al. 1972a). The induced synthesis of tyrosine aminotransferase (Beck et al. 1974) and of glutamine synthetase (Matsuno, Shirasawa & Kohno, 1976) is

inhibited by interferon treatment. However, dimethyl sulphoxide (DMSO)-induced haemoglobin synthesis in Friend erythroleukaemic cells is not inhibited by interferon treatment under conditions where virus production is inhibited (Swetly & Ostertag, 1974).

Interferon treatment can also alter the immune response e.g. treatment with mouse interferon usually inhibits the response of spleen cell cultures to antigen stimulation but under some conditions may enhance it (Gisler, Lindahl & Gresser, 1974). The non-anti-viral effects of interferon may reflect an incomplete discrimination between host and viral synthesis by the inhibitory mechanism.

C. The Synthesis of Interferon

Unlike other differentiated functions, for example the response to steroid hormones, interferon production is a property of most cell lines and of cell cultures derived from different tissues (Ho, 1973b) and is thus a promising model for the study of inducible protein synthesis in animal cells. In animals interferon production occurs in response to virus infection or to treatment with dsRNAs but a different response is found, involving only cells of the reticulo-endothelial system, when bacterial endotoxins are injected. The latter response does not involve new RNA synthesis and may represent the release of 'pre-formed' interferon (Merigan, 1973). Interferon production *in vivo* will not be discussed further.

Field *et al.* (1968) showed that the synthetic dsRNA polyriboinosinic-polyribocytidylic acid (poly(rI). poly (rC)), was able to induce interferon production in rabbit

kidney cells in culture. This showed that interferon is a cellular product and allowed the study of interferon synthesis without the complicating effects of virus replication.

Poly(rI). poly(rC) treatment at concentrations less than those required to produce detectable interferon in the medium can protect cells against virus infection (Colby & Morgan, 1971). This effect is considered to be mediated by interferon production (Vengris, Stollar & Pitha, 1975) because treatment of cells with antibody to interferon after induction with poly(rI). poly(rC) prevents the development of resistance to virus.

Undamaged cellular DNA, but not DNA synthesis, is necessary for interferon production in response to virus infection. Burke & Morrison (1965) found that only inhibitors of DNA synthesis which did not affect RNA or protein synthesis or the integrity of the DNA allowed virus induction of interferon synthesis in chick cells. The irradiation of cells with ultra-violet (UV) light, or growth in medium containing 5-bromo-2'-deoxyuridine (BUdR) followed by exposure to fluorescent light, also inhibited interferon production in response to virus infection (Burke, 1973). It was concluded that this was a result of damage to the cellular DNA.

Interferon production in response to induction with poly(rI). poly(rC) may be enhanced by prior UV-irradiation (Lindner-Frimmel, 1974; Mozes & Vilček, 1974, 1975). However, under optimal conditions for interferon production, the cellular response to poly(rI). poly(rC) is also sensitive to UV-irradiation (Mozes & Vilček, 1974).

Inhibitors of DNA-dependent RNA synthesis prevent interferon synthesis (Heller, 1963; Sehgal, Tamm & Vilček, 1976) and no detectable mRNA for interferon was found in uninduced cells in experiments on the synthesis of interferon in cell-free systems, Xenopus oocytes or heterologous cells (Reynolds et al. 1975; De Maeyer-Guignard et al. 1972; Kronenberg & Friedmann, 1975). Induction of interferon by viruses or poly(rI). poly(rC) must therefore be mediated, at least in part, by new mRNA synthesis. Experiments with inhibitors of protein synthesis have shown that new protein synthesis is also required (Burke, 1973).

The role of new protein synthesis after induction by poly(rI). poly(rC) is more complex (Vilček, 1970; Tan et al. 1970; Myers & Friedman, 1971). However, the presence of new mRNA for interferon in induced cells, as detected in heterologous cells or cell-free systems (De Maeyer-Guignard et al. 1972; Reynolds et al. 1975), implies that new protein synthesis must also occur.

No biologically active interferon has been detected in control cells in experiments in which interferon production or binding to cells was being investigated (Stewart, De Clercq & De Somer, 1972b; Ng, Berman & Vilček, 1972). It is possible, however, that some inactive, pre-formed interferon may be present in cells.

D. The Regulation of Interferon Production

1. Hyporeactivity

After induction, interferon production occurs for only a limited period of time. In rabbit kidney cells, for example, interferon production in response to NDV occurs

mostly between 12 and 24 hours after infection and between 2 and 4 hours from the commencement of poly(rI). poly(rC) induction (Tan et al. 1970). When interferon production has ceased cells may be hyporeactive (refractory) to further induction for a variable period of time (Paucker & Boxaca, 1967; Billiau, 1970). In mouse L cells the refractory state to reinduction was lost after two cell divisions but in non-dividing cells it did not diminish during the same period of time (Paucker & Boxaca, 1967).

2. Priming and Blocking

Interferon pretreatment can enhance (prime) or inhibit (block) interferon production in response to virus infection or synthetic inducers (Stewart, Gosser & Lockart, 1971a). Interferon production occurs earlier in cells pretreated with interferon (Levy, Buckler & Baron, 1966; Stewart et al. 1971a) and cells which normally do not produce interferon in response to certain viruses or to poly(rI). poly(rC) alone may do so when primed with interferon (Stewart et al. 1971a; De Clercq, Stewart & De Somer, 1973). Such primed cells contain interferon mRNA whereas non-primed, induced cells do not (Saito et al. 1976).

Neither the priming nor the blocking activity of interferon preparations was found to be separable from the antiviral activity by extensive purification (Stewart et al. 1973b). However, Borden, Prochownik & Carter (1975) claimed that interferon samples collected at 14 or 48h after NDV infection of mouse cells differed in their ability to inhibit interferon production in other cells.

Priming does not require protein synthesis during the interferon pretreatment (Stewart et al. 1971a), nor are

human cells trisomic for chromosome 21 more effectively primed than other cells (De Clercq, Edy & Cassiman, 1975) although such cells are more sensitive to the anti-viral action of interferon (Tan et al. 1974b). Thus priming is not directly related to the development of the anti-viral state.

Blocking of interferon production requires a longer period of interferon treatment at a higher concentration than does priming (Paucker & Boxaca, 1967). Inhibition of interferon synthesis by interferon pretreatment requires protein synthesis (Stewart, Gosser & Lockart, 1971b) and in general occurs if cells are optimally induced for interferon production. Blocking may be mediated by the anti-viral state.

3. Superinduction

If inhibitors of RNA or protein synthesis are added to cells at suitable times after induction the amount of interferon made is increased (Vilček, Rossman & Varacalli, 1969; Tan et al. 1970). This superinducing effect is analogous to the paradoxical effects of Actinomycin-D on rat hepatoma cells during the deinduction of tyrosine aminotransferase after steroid removal (Tomkins et al. 1969).

In rabbit kidney cell cultures, for example, treated for 60 minutes with poly(rI). poly(rC) interferon production normally occurs between 2 and $5\frac{1}{2}$ hours after induction commences. If Actinomycin-D (an inhibitor of DNA-dependent RNA synthesis) is added at the time of induction interferon production is inhibited. However, addition of Actinomycin-D for 30 minutes, $3\frac{1}{2}$ hours from the commencement of induction enhances interferon production (Vilček et al. 1969).

Similarly, Tan et al. (1970) found that treatment of rabbit kidney cells with cycloheximide (an inhibitor of protein synthesis) for 3 hours from the commencement of induction with poly(rI). poly(rC) led to increased yields of interferon after removal of the cycloheximide. Similar effects of inhibitors on interferon production have been reported to occur in human cells (Havell & Vilček, 1972).

E. A Model for the Regulation of Interferon Synthesis

Tan & Berthold (1977) proposed a model for the induction and regulation of interferon synthesis on the basis of their observations that inhibitors of RNA or protein synthesis alone could induce interferon production in sensitive human cell lines.

These inhibitors were chosen for study because viruses have been shown to inhibit RNA and protein synthesis in host cells (Metz, 1975b) and poly(rI). poly(rC) is a potent inhibitor of the initiation of protein synthesis in cell-free systems prepared from rabbit reticulocytes (Hunter et al. 1975). Interferon inducers, it was suggested, have a common action rather than a common structure.

Tan & Berthold (1977) postulated the existence of a labile repressor of interferon gene transcription, synthesized from a labile mRNA, to explain the need for induction (Fig. 1). The model proposes that inhibition of mRNA synthesis causes a decrease in the concentration of repressor mRNA and protein in the cell. On removal of inhibitor the interferon gene can be transcribed until the repressor concentration reaches its former level.

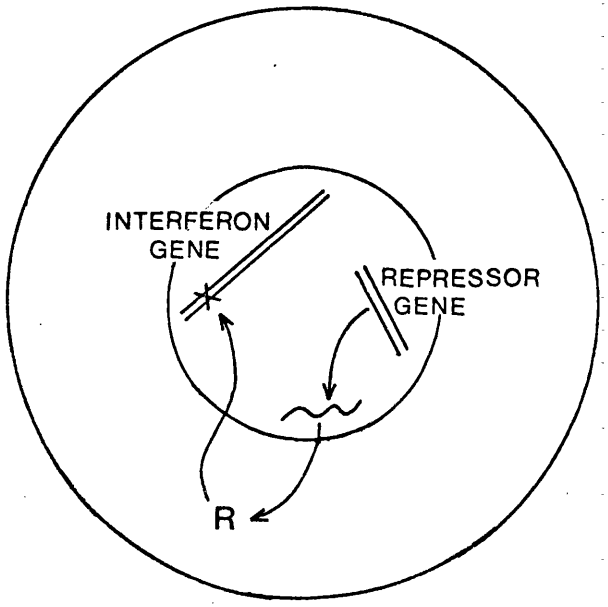
If protein synthesis is inhibited the concentration of

Figure 1 A model for the regulation of interferon synthesis

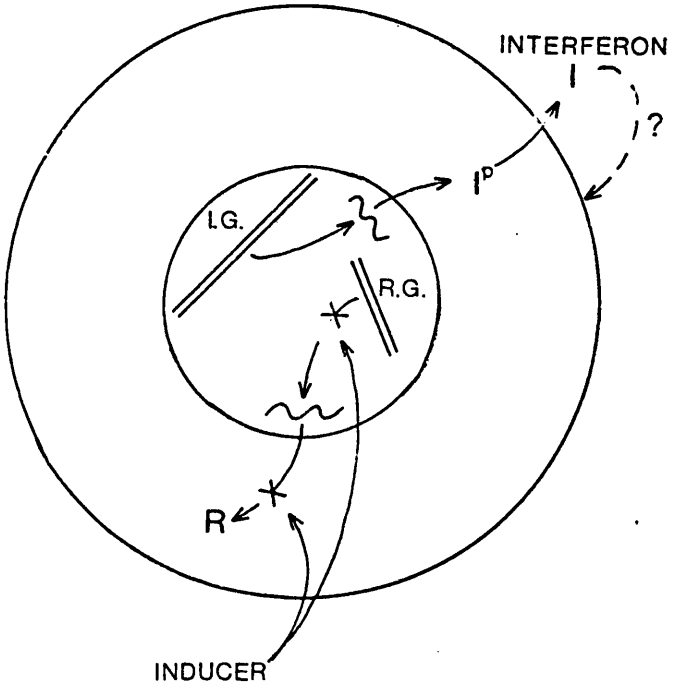
A. The repressor protein (R) prevents transcription of the interferon gene (I.G.) in the uninduced cell.

B. In the induced cell repressor gene (R.G.) expression is inhibited by the inducer. The interferon protein (Ip) can then be synthesized, glycosylated and secreted and may interact with the producing cell.

A. UNINDUCED CELL



B. INDUCED CELL



repressor decreases and interferon mRNA can be transcribed while inhibitor is present. After removal of the inhibitor, the accumulated, stable interferon mRNA can be translated and more mRNA made until the repressor again reaches a critical concentration.

This model does not explain the hyporeactivity of cells to a second induction nor the shut-off of interferon production while inducer is still present (Vilček & Havell, 1975).

A labile, post-transcriptional repressor of interferon synthesis, with a labile mRNA, has been proposed by Ng & Vilček (1972) to explain their observations on the super-induction of interferon production. This model proposes that during inhibition of protein synthesis in induced cells interferon mRNA synthesis proceeds but repressor protein concentrations fall. After removal of the inhibitor, translation of interferon mRNA can take place until the repressor concentration reaches its former levels.

Similarly the addition of Actinomycin-D, after sufficient stable interferon mRNA has accumulated, inhibits the synthesis of the labile repressor mRNA. Interferon translation continues as the amount of repressor mRNA and consequently repressor falls. After removal of the Actinomycin-D, repressor concentrations gradually increase and prevent interferon mRNA translation. An analogous model for the regulation of tyrosine aminotransferase production has since been shown to be an over-simplification (Steinberg, Levinson & Tomkins, 1975).

A feed-back inhibition of interferon on its own synthesis has been suggested previously (Colby & Morgan, 1971). Super-

induction may be explained, in part at least, by inhibitor treatment preventing the establishment of an anti-viral state in cells producing interferon.

That the development of the anti-viral state might inhibit interferon production and prevent its re-induction for a period of time is suggested by the observations of Chany & Vignal (1970). They isolated a line of mouse cells in the presence of interferon and examined its ability to respond to and make interferon. The cells were insensitive to the anti-viral action of interferon and produced much more interferon in response to UV-irradiated NDV (UV-NDV) than did the cells from which they were derived. On repeated induction with UV-NDV they were able to produce amounts of interferon comparable with control cultures induced for the first time. They still demonstrated the fall-off in interferon synthesis seen in normal cells after one induction.

On the basis of the model of Tan & Berthold (1977) these cells are still sensitive to the repressor of interferon production which necessitates induction and which causes interferon production to decline after inducer is removed or inactivated. They are not sensitive to the mediator of hyporeactivity and make more interferon than their parental cells. This strongly suggests that the antiviral state arising in cells by the action of the interferon which they produce may prevent further induction and limit interferon production. The possibility that a repressor of interferon production, other than that responsible for the anti-viral state, is released at the same time as interferon cannot be excluded. However, the variant cells of Chany & Vignal (1970) would have to be insensitive to this also.

Havell & Vilček (1975) have found that treatment of cells with Vinblastine (an alkaloid causing microtubule disaggregation) inhibited the secretion of interferon. These cells produced more interferon than the control cells. This observation confirms a role for secreted interferon in the control of its own synthesis but does not indicate that the anti-viral state is necessarily involved.

If the interferon induced anti-viral state is responsible for hyporeactivity to interferon inducers it would be expected that the resistance of cells to virus infection and the refractory period should be correlated. However, cell RNA or protein synthesis is likely to be less sensitive to interferon action than is virus replication. For example, significant inhibition of cell growth requires 200 units/ml of human fibroblast interferon (Knight, 1976b) whereas 1 unit of interferon is defined as being able to inhibit virus growth by 50%. In addition, the concentration of interferon at the surface of a cell after induction by poly(rI). poly(rC) is likely to be greater than that measurable in the medium. Therefore attempts to reproduce hyporeactivity with interferon pretreatment should involve high interferon concentrations and this is indeed found (see blocking above). Finally the measurement of virus resistance and hyporeactivity involves events occurring some time later and therefore correlation cannot be very precise.

These considerations explain the observation of Paucker & Boxaca (1967) that hyporeactivity was lost sooner in dividing cells than was virus resistance. Host synthetic functions (e.g. interferon production) would be restored before virus synthetic functions as the anti-viral state diminished.

Breinig, Armstrong & Ho (1975) found a correlation between hyporesponsiveness to reinduction and interferon production after a first induction with poly(rI). poly(rC) in rabbit kidney cell cultures. However they could not reproduce hyporeactivity with interferon pretreatment and stated that virus resistance persisted longer than hyporeactivity. Both these latter observations are not surprising given the above considerations on interferon concentrations at the cell surface and the differential sensitivity of host and virus synthesis.

Finally Billiau (1970) has shown in rabbit kidney cells that refractoryness to a second induction with poly(rI). poly(rC) only occurs if the first induction was carried out with sufficient poly(rI). poly(rC) to elicit detectable interferon production. Lower concentrations of poly(rI). poly(rC) stimulated the response to a second induction.

Superinduction of interferon production, would involve both inhibition of repressor synthesis and of the development of the anti-viral state if the model of Tan & Berthold (1977) is correct and if interferon action inhibits interferon production. Thus cells insensitive to the anti-viral action of interferon should still be superinducible though perhaps rather less so than control cells.

F. Genetics of the Interferon System

Models of the regulation of interferon induction and synthesis have relied heavily on experiments with inhibitors. In an attempt to find an approach which avoided inhibitor studies it was decided to look at naturally occurring variant cells which are unable to produce interferon but which are

sensitive to its anti-viral action. Two such variants are Chinese hamster ovary (CHO) cells (Puck, Cieciura & Robinson, 1958) and the VERO cell line derived from the African Green monkey (Yasumura & Kawakita, 1963). These cells are unable to make interferon (Morgan, 1976; Desmyter, Melnick & Rawls, 1968) possibly because of the synthesis of a repressor which is resistant to the action of interferon inducers. (Other possibilities for VERO cells are discussed in Section IV). The existence of such a repressor in these cells might be detected by the use of cell hybridisation techniques.

Cell hybridisation experiments have been used to study the expression of differentiated traits (Davidson, 1974) or of the transformed phenotype (Klein, 1976) and also, in situations where specific chromosome loss occurs, to allot genes to particular chromosomes (Ruddle & Creagan, 1975). Such experiments with human-mouse and human-Chinese hamster hybrid cells have allowed the allocation of the gene(s) for human interferon production to chromosomes 5 and possibly 2, (Tan, Creagan & Ruddle, 1974a; Morgan & Faik, 1977). The anti-viral effect of interferon in human-mouse hybrid cells depends on the presence of human chromosome 21 (Tan et al. 1973).

Cassingena et al. (1971) have isolated monkey-mouse hybrids and shown that the monkey genes for interferon production and sensitivity are on separate chromosomes. The hybrid cells were less sensitive to interferons than the parental cells and produced mouse, or mouse and monkey, interferons.

Mouse-hamster hybrids isolated by Carver, Seto & Migeon, (1968) produced ten-fold more hamster interferon than the

parental hamster cells and were eight-fold more sensitive to its action. The production of interferon therefore seems to be amenable to regulation in interspecific hybrid cells.

Chick interferon production can be 'switched-on' in hybrids of chick red blood cells with human cells but only if the human cells were originally able to produce interferon (Guggenheim, Friedman & Rabson, 1968). In Chinese hamster-human hybrid cells (Morgan & Faik, 1977) hamster interferon is made if human chromosome 18 is present but the parental Chinese hamster cells are not able to synthesize interferon. The requirement of human chromosome 21 for the action of interferon was also confirmed in these hybrids.

These experiments show that hybrid cells can make interferons of both parental types and can respond to both. The amounts of interferon made may differ from the parental cells and non-producing genomes may be activated.

The fusion of VERO cells with mouse cells which are able to produce interferon, and the selection of hybrid cells, thus promised to provide an insight into the reason for the failure of VERO cells to make interferon.

The characterisation of VERO cells and the properties of their hybrids with mouse cells are described in the following sections.

MATERIALS AND METHODS

A. MATERIALS

1. Cells

The VERO (Yasumura & Kawakita, 1963) and BSC.B (Hopps et al. 1963) established cell lines of African Green monkey (Cercopithecus aethiops) kidney were obtained from P. Marcus (University of Connecticut, Storrs, Connecticut) as was the LLC-MK_2 established cell line from the kidney of the Rhesus monkey (Macaca mulatta) (Hull, Cherry & Johnson, 1956).

Mouse L cells (Earle, 1943) and their derivatives lacking thymidine kinase (clD1) (Dubbs & Kit, 1964) and hypoxanthine-guanine phosphoribosyl transferase (clA9) (Littlefield, 1964b) were given by C. Colby (University of Connecticut) as were mouse 3T3 cells (Todaro & Green, 1963). L929 mouse cells, a clone of mouse L cells, were provided by W.E. Stewart II (Rega Institute for Medical Research, Leuven, Belgium).

Embryonated eggs, were a gift from Fisons Ltd., Loughborough (hybrids chicks of Rhode Island Red crossed with Light Sussex strains) or obtained from Golden Hatcheries, Kibworth, Leics. (Cobb strain).

Human foetuses, from therapeutic abortions, were obtained from a local hospital.

2. Viruses

The following viruses were obtained from C. Colby (University of Connecticut): Sindbis virus (Enders strain; Carver & Marcus, 1967) grown at 37 °C in monolayers of chick embryo cells (CEC); Sindbis virus temperature-sensitive mutant ts15 (Burge & Pfefferkorn, 1966) grown at 30 °C in monolayers

of CEC; Semliki Forest virus (strain 25639; Rockefeller Institute, New York) grown at 37 °C in CEC monolayers; Mengo virus (large plaque strain; Rockefeller Institute, New York) grown in mouse L929 cell monolayers at 37 °C; Newcastle Disease virus (NDV; California strain) grown at 39.5 °C in the allantoic cavity of 12-day-old embryonated eggs.

Encephalomyocarditis (EMC) virus, grown in Krebs II mouse ascites cells, was obtained from I. Kerr (National Institute for Medical Research, Mill Hill, London) and Sendai virus, grown in embryonated eggs, from S. Singh Sidhu (Royal Postgraduate Medical School, Hammersmith Hospital, London).

3. Interferons

i) Human leucocyte interferon

A reference preparation of Sendai virus-induced human leucocyte interferon (G-023-901-527) was obtained from the National Institutes of Health, Bethesda, Maryland and contained 20,000 reference units of interferon activity per ml.

ii) Human fibroblast interferon (1)

This was prepared, as described below, from the medium of NDV-infected human embryo cells. The titre relative to human leucocyte reference interferon (N.I.H. G-023-901-527) was 300 units per ml as assayed by the dye uptake method with Sindbis virus challenge. This preparation of human fibroblast interferon was insensitive to treatment at pH2.0, inactive on mouse D1 or L cells and protected human embryo cells against challenge with EMC or Sindbis viruses. It was also active on VERO, BSC.B and LLC-MK_2 monkey cells against Sindbis virus challenge and protected VERO cells from Mengo

virus plaque formation or EMC virus induced cell damage as measured by dye uptake. Human interferons have been reported previously to be active on other primate cells (Bucknall, 1967).

The titre of this human fibroblast interferon preparation was consistently lower on VERO cells (approximately 10-fold) as was the titre of the human leucocyte reference preparation (G-023-901-527) and also BSC.B and LLC-MK_2 cell interferons (Section I). Desmyter & Stewart (1976) have observed a similar, lower sensitivity of VERO cells to primate interferons. BSC.B and HES.A cells were similar in sensitivity to the action of human fibroblast interferon (1); LLC-MK_2 cells responded variably and were not as sensitive as human cells to the human leucocyte reference interferon.

iii) Mouse interferon (1)

This was prepared from the medium of Sindbis virus-infected mouse L cells as described below. The interferon titre was 125 units per ml as measured by the dye uptake method with Sindbis virus challenge.

This mouse interferon preparation was active on mouse L cells and D1 cells but inactive on human embryo cells or VERO cells and was insensitive to pH 2.0 treatment as shown by its activity after the virus inactivation treatment. The picornaviruses (EMC and Mengo) were found to be less susceptible to mouse interferon action.

Mouse interferon (2)

This Sindbis virus-induced interferon from mouse D1 cells (from M. Morgan) had a titre of 1,200 units per ml.

iv) Chick cell interferons

C1

A Sindbis virus-induced interferon from primary chick embryo cells (from M. Morgan) had a titre of 100 units per ml as assayed by the plaque reduction method.

C2

This preparation, induced by influenza virus in chick embryos, was generously provided by G. Bodo (Arzneimittel-forschung GmbH, Vienna) and had an activity of 225,000 MRC (A62/4) reference units per ml and a specific activity of 11,084 units per mg of protein. Its purification was described by Jungwirth et al. (1972).

v) LLC-MK_2 cell interferon

This was prepared from the medium of Sindbis virus-infected LLC-MK_2 cells as described below. Its anti-viral activity, as measured by the plaque reduction method in BSC.B cells, was 20 units per ml.

B. CHEMICALS

Polyethylene glycol (mol. wt. 6,000); sodium polyanethol sulphonate; protamine sulphate (herring) and cycloheximide; were obtained from Koch-Light Laboratories Ltd, Colnbrook, Bucks.

The Sigma Chemical Co., London, supplied testosterone propionate; sodium polyanethol sulphonate; chlortetracycline hydrochloride; kanamycin; cycloheximide; 6-thioguanine (2 amino-6-mercaptopurine); 5-bromo-2'-deoxyuridine; 8-azaguanine; vinblastine sulphate; ouabain octahydrate (Strophanthin-G) and trypsin (2-fold crystallised, type III from bovine pancreas).

Polyethylene glycol (mol. wt. 4,000); Giemsa powder; β-propiolactone; Nonidet P42 and water-soluble Phenol red were from B.D.H. Chemicals Ltd., Poole.

Neutral red chloride and Cristalite were from E. Gurr, London, and Searle, High Wycombe, Bucks. supplied Gurr's Giemsa Stain (R66) and pH 6.8 buffer tablets.

Polyriboinosinic-polyribocytidylic acid [poly(rI). poly(rC)] was obtained as a lyophilised powder with physiological salts containing 10% by weight of poly(rI). poly(rC) from P.L. Biochemicals Inc., Milwaukee, Wisconsin.

Diethylaminoethyl (DEAE) - dextran (mol. wt. 500,000) was from Pharmacia Fine Chemicals, Uppsala, Sweden, Cortisone acetate B.P. (Cortistab) from The Boots Co. Ltd., Nottingham and Gentamicin (Genticin) from British Schering Ltd., Slough, Bucks.

Newborn calf serum (gamma irradiated), foetal bovine serum (virus and mycoplasma screened) and sterile 2.5% trypsin solution were supplied by Gibco-Biocult, Glasgow.

Flow Laboratories, Irvine, Scotland, supplied the following media: Dulbecco's modification of Eagle's minimal essential medium (DME), RPMI 1640, 199 and Eagle's minimal essential suspension medium. Tylosin was also supplied by Flow Laboratories.

Mycostatin and Fungizone (Amphotericin B) were obtained from E.R. Squibb & Sons, London and penicillin (Cristapen) and streptomycin sulphate from Glaxo Laboratories Ltd., Greenford, Middx.

Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) was from Hopkin and Williams, Chadwell Heath, Essex.

Aminopterin was a gift from Serva Feinbiochemica GmbH

& Co. Heidelberg, Germany and Actinomycin-D was a gift from I.B. Holland of the Genetics Department, Leicester University.

Radiochemicals were obtained from the Radiochemical Centre, Amersham as follows:

Thymidine - (methyl-H3)	(23 Ci/mmol)
[2-14C]-thymidine	(59 mCi/mmol)
Hypoxanthine-8-C14	(60 mCi/mmol)
[5-3H]- uridine	(5 Ci/mmol)
[2-14C]-uracil	(61 and 59 mCi/mmol)

C. MEDIA AND SOLUTIONS

Dulbecco's Modification of Eagle's Medium (DME)

The growth medium used for all cell cultures (unless otherwise stated) was Dulbecco's modification of Eagle's minimal essential medium (Dulbecco & Freeman, 1959), supplemented with penicillin (100 units/ml), streptomycin sulphate (100µg/ml), mycostatin (25 units/ml) and Fungizone (1µg/ml) and with the addition of heat-inactivated calf serum (6%, D6; 10%, D10) or heat-inactivated calf serum (5%) and foetal bovine serum (5%, D55). RPMI 1640 (Moore, Gerner & Franklin, 1967) and 199 media (Morgan, Morton & Parker, 1950) were similarly supplemented.

Minimal essential medium (MEM) (suspension)

Minimal essential suspension medium (Eagle, 1959) was supplemented with antibiotics, 2% calf serum and 25mM Hepes buffer.

DME/Hepes/Glycerol

DME medium was supplemented with antibiotics as above and contained in addition 10% glycerol and 20mM Hepes buffer. Calf serum (5%) and foetal calf serum (5%) were also added.

HAT Medium (Littlefield, 1964a)

To D55 medium as formulated above were added hypoxanthine (10^{-4}M); thymidine ($1.6.10^{-5}$M); glycine (3.10^{-6}M) and aminopterin (4.10^{-7}M).

Medium for virus plaque assays

Overlay medium consisted of 1:1 (v/v) of 2-fold concentrated DME (with antibiotics and 12% calf serum) and 1.4% agarose.

Trypsin-Versene Salt Solution

This solution contained in one litre: 8g NaCl, 0.25g KCl, 0.25g anhydrous KH_2PO_4, 1.44g anhydrous Na_2HPO_4, 0.2g versene [EDTA (disodium salt)] and 10mg Phenol red. After sterilisation sterile trypsin solution was added to a final concentration of 0.05%.

Phosphate buffered saline (PBS) (Dulbecco & Vogt, 1954)

PBS_A contained in one litre: 8g NaCl, 0.2g KCl, 1.15g Na_2HPO_4 and 0.2g KH_2PO_4. The pH was adjusted to 7.2 with 1N NaOH. PBS contained, in addition to the above, 100mg each per litre of anhydrous $CaCl_2$ and $MgCl_2.6H_2O$.

Hanks' Basal Salt Solution (Hanks' BSS) (Hanks & Wallace, 1949)

Hanks' BSS contained in one litre: 8g NaCl, 0.4g KCl, 0.14g $CaCl_2$, 0.1g $MgSO_4.7H_2O$, 0.1g $MgCl_2.6H_2O$, 0.06g $Na_2HPO_4.2H_2O$, 0.06g KH_2PO_4, 1g glucose, 0.02g Phenol red and 0.35g $NaHCO_3$.

Saline bicarbonate solution

1.68gm $NaHCO_3$; 0.5ml of 0.4% phenol red in 100ml of isotonic NaCl (0.9%).

Giemsa stain (1)

2gm of Giemsa were dissolved in 50ml glycerol for 24h

at 60 °C in a water bath. 50ml of methanol were added and the mixture filtered.

Giemsa stain (2)

0.5gm of Giemsa were dissolved in 33ml of glycerol by heating at 60 °C for 24h. 33ml methanol were added, the mixture stirred and filtered.

Phosphate buffer (0.1M pH 7.0)

6ml of Na_2HPO_4 (0.067M) and 4ml of KH_2PO_4 (0.067M) were diluted to 50ml with distilled water.

Toluene Scintillation Fluid

6g PPO (2,5-Diphenyloxazole) and 0.5g dimethyl POPOP (1,4-di-2-(4-methyl-5-phenyl oxazolyl) benzene) were dissolved in one litre of toluene.

Bray's Scintillation Fluid

60g napthalene, 4g PPO, 0.2g dimethyl POPOP, 100ml methanol and 20ml ethylene glycol were made to one litre with p-dioxane.

D. METHODS

1. Cell culture

i) Growth and sub-culture of cells

Cells were grown and maintained as monolayers on the surface of Petri dishes (Nunc; Falcon; Corning), flat glass bottles (250ml, 500ml medical flats or 1l Brockway bottles) or roller bottles (Bellco) as required at 37 °C.

Cells growing in Petri dishes were incubated at 37 °C in an humidified atmosphere of 10% CO_2 in air. Cells seeded into the larger glass bottles (1l or roller bottles) were gassed with 10% CO_2 in air for 60 s to prevent alkalinity of the medium and incubated at 37 °C. Roller bottles were

turned at approximately 0.25 rpm at 37 °C on a roller machine (Luckham Ltd). The growth medium used was D55 for primate cells and mouse L cells. Mouse D1 cells were cultured in D55 containing $3.25.10^{-4}$M 5 -bromo-2'-deoxyuridine. Chick embryo cells were grown in D6 and mouse 3T3 cells in D10. DME only, or 199 with 2% calf serum, was occasionally used as maintenance medium.

When confluent, cells were removed from the surface of containers by washing twice with PBS_A followed by treatment with a small volume of trypsin-versene solution. When rounding of cells was observed by microscopic examination or by changes in the opacity of the cell sheet the trypsin-versene was removed and the cell monolayers incubated at 37 °C to facilitate detachment. A suspension of the cells was made in growth medium and a suitable aliquot (usually one tenth) was replaced in the container with fresh growth medium as a stock culture. The remaining suspended cells were counted and diluted in growth medium to the cell density required for experiments. All procedures were carried out using standard aseptic techniques.

ii) Determination of cell numbers

A suspension of the cells to be counted was introduced beneath a cover-slip on an improved, Neubauer haemocytometer and the number in a volume of 10^{-4} ml. counted microscopically. A total of at least 200 cells was counted and clumped cells were resuspended.

iii) Cell cloning

Cells were suspended in medium and dispensed into 50mm Petri dishes at 10^2 to 10^3 cells per dish for cell cloning in growth medium, or up to 5.10^5 cells per dish for the

isolation of hybrid clones in selective medium. Colonies were allowed to develop during incubation at 37 °C for 14 days or longer with the minimum of disturbance.

Medium was removed from the Petri dishes and individual, well-separated colonies marked on the under-side of the dish with a felt-tip pen. Colonies were removed by gentle scraping with a sterile, wooden tooth-pick and transferred to 0.1ml of medium in wells of a microtitre tray (96 well disposo-tray; Linbro Chemical Co. Inc.; New Haven, Conn. U.S.A).

When these cells became confluent they were removed by trypsinization and transferred to 30mm Petri dishes.

iv) Giemsa staining of cell colonies

The medium was removed from colonies of cells in Petri dishes and the cells washed twice with 2ml PBS_A followed by fixation with 2ml of methanol for 2 min. This was removed and the staining solution of stock Giemsa (1) (diluted 20-fold in 0.2% Na_2CO_3) added.

After 10 to 30 min this was removed, the colonies washed with PBS_A and the Petri dishes drained by inversion on tissues. Colonies were visible as dark blue areas and were readily counted.

v) Storage of stock cell cultures

Cells were suspended at 2.10^6 per ml in Hepes buffered DME with 10% glycerol and 1ml aliquots dispensed to hard, plastic freezing tubes (38x12.5mm, Nunc). The cell suspension was kept at room temperature for 10 min for equilibration then cooled at 2-3 °C per min for 2h over liquid nitrogen. The samples were stored at less than -130 °C in a liquid nitrogen freezer (Union Carbide, LR40). When required, stock

cell cultures were thawed rapidly at 37 °C and seeded into 4ml growth medium in 50mm Petri dishes. Fresh medium was added after 24h and dead cells removed.

vi) Preparation of embryonic cell cultures

Chick embryo cells

Eleven-day old embryonated eggs were 'candled' to locate the air-sac. The shells were wiped with Wescodyne, the top removed and the membranes cut with scissors and forceps sterilised in absolute alcohol. The chick embryos were removed to a 100mm Petri dish containing PBS_A and the head, wings and legs removed. The torsos were placed in a dry Petri dish and chopped into very small fragments. The tissue was placed in a conical flask and washed twice with 100ml of PBS_A, to remove red blood cells, on a magnetic stirrer. The supernatant was removed by aspiration and the tissue incubated at 37 °C with stirring in 0.025% trypsin in PBS_A to separate cells. Detached cells were separated from tissue fragments by filtration through sterile muslin and the trypsin treatment repeated twice with the remaining tissue fragments. Collected cells were pelleted by centrifugation, resuspended in D6 and counted. The mixture of different chick cell types was seeded into Petri dishes etc. in D6 so as to be confluent after 24h and usually used, without sub-culture, as a primary culture.

Human embryo cells

Cells were obtained from aborted human foetuses essentially as described for chick cells. Cultures were established separately from skin, kidney, lung and liver tissue and the remaining cells pooled. Cultures contained only fibroblastic cells after a few sub-cultures. The first

sub-cultured cells were designated passage 1 and cells were used until passage 20; with 5-fold sub-culture as necessary. The cells from 2 different foetuses were designated HES.A and HEC.

2. Protein determination (Lowry et al. 1951)

An assay mixture containing $CuSO_4$ (0.008%), sodium/potassium tartrate (0.017%), Na_2CO_3 (1.67%), 0.08N NaOH and up to 16.7µg/ml of protein was left at room temperature for 10 min. Folin-Ciocalteau reagent was added, with rapid mixing to a final concentration of 0.4% and after 30 min at room temperature the E_{750} of the samples was recorded. A standard curve was prepared with bovine serum albumin.

3. Determination of radioactivity

The radioactivity of cooled samples in glass scintillation vials was determined in a Packard scintillation counter (model 3385) using the $[^3H]$ or $[^{14}C]$ automatic settings. For doubly labelled samples the $^3H[^{14}C]$ and $^{14}C[^3H]$ settings were used.

4. Detection and elimination of mycoplasma contamination

Mycoplasma contamination has been reported to have a number of adverse effects on cell cultures including effects on nucleotide metabolism, chromosome stability and interferon production (Stanbridge, 1971).

Mycoplasmas are prokaryotic organisms generally 0.35 to 0.8µm in size having a trilaminar membrane, (Teplitz & Revel, 1974). They grow in close association with the plasma membranes of cells in culture. Their independent growth only occurs on complex media and positive identification of mycoplasma colonies, on solid medium, from artifacts requires considerable experience.

Mycoplasmas are found in the respiratory tract of animals including man and the serum and trypsin used in cell culture may be contaminated unless measures are taken to avoid this. Contamination of cell cultures with mycoplasmas is thus a common problem. The detection of mycoplasma contamination of cell cultures by microbiological culture methods is difficult and it is sometimes necessary to include a 'feeder' layer of animal cells beneath the solid medium on which the mycoplasmas are to be isolated. Some species may become obligatory parasites on cells and cannot be grown independently (Furness & Whitescarver, 1975).

Alternative methods for the detection of contamination include electron microscopy, autoradiography of cells labelled with DNA or RNA precursors and biochemical methods e.g. arginase assays, measurement of nucleosidase action or the incorporation of uridine and uracil into RNA and electrophoresis of radioactively labelled RNAs.

The possibility of mycoplasma contamination of the present cultures was considered when the BSC.B cell line showed extensive vacuolation, lessened response to the action of poly(rI). poly(rC) and fragile chromosomes when fixed cells were prepared for karyotyping.

The inhibition of growth of VERO, BSC.B and LLC-MK_2 cells by HAT medium (Section IV) may also have been caused by lessened availability of exogenous thymidine because of mycoplasma nucleosidase activity. It has been reported (Hakala, Holland & Horoszewicz, 1963) that HeLa cells contaminated with mycoplasmas were unable to grow in medium containing amethopterin and thymidine.

Attempts were made to cultivate mycoplasmas aerobically and anaerobically from cell cultures or medium using mycoplasma broth and agar plates from a kit supplied by Flow Laboratories. The results were equivocal and so other detection methods were sought.

i) Uridine-Uracil incorporation ratios in cell cultures

Schneider, Stanbridge & Epstein (1974) showed that the ratio of the specific radioactivity of uridine to uracil incorporated into the RNA of human cell cultures was high (greater than 400) for uninfected cultures but less than 100 in cultures showing mycoplasma specific RNAs on polyacrylamide gels. It was not always possible to cultivate organisms even when mycoplasma RNAs were found to be present.

A modification of this method was used to test cells for contamination. Cells were seeded at 5.10^4 per sterile, glass scintillation vial in growth medium in quadriplicate. After 24h for cell attachment 1ml of medium containing 0.5 µC each of ^{3}H-uridine and ^{14}C-uracil (each adjusted to 61 mC/mmol) was added to each vial. The monolayers were incubated at 37 °C. 18h later the radioactive medium was removed and the monolayers washed four times with cold PBS_A. Macromolecules were precipitated with 10% trichloracetic acid (TCA) during 30 min at 4 °C. The precipitate was washed twice with cold 10% TCA and then twice with absolute ethanol. The samples were air-dried at 37 °C for 60 min. Extraction was with 0.2ml per vial of a 3-fold dilution in toluene of Koch-Light tissue solubiliser (TS-1) at 37 °C until microscopic examination showed completion of this process.

10ml of toluene scintillation fluid was then added and the radioactivity of the samples determined.

Treatments reported to eliminate mycoplasma infection (Stanbridge, 1971) include incubation at 41 °C passage of cells in the presence of antibiotics and treatment with sodium polyanethol sulphonate (Mårdh, 1975).

Treatment at 41 °C was carried out with confluent cell cultures in growth medium in small plastic bottles (~ 20 cm^2, Nunclon, Denmark) for 24h; cells were then replaced at 37 °C and passaged in bottles until ratios could be determined. Some cultures received two such treatments.

Other cultures were grown in the presence of 100 µg/ml of chlortetracycline, 200µg/ml of gentamicin, 200µg/ml of kanamycin and 10µg/ml of tylosin in growth medium for approximately 1 month before estimation of ratios.

Cells to be treated with sodium polyanethol sulphonate (SPS) were grown in plastic bottles and 10% SPS in growth medium added (1ml per bottle). The cell monolayers were incubated at 37 °C for 24h. A further 4ml of growth medium were then added and the cells incubated for a further 24h at 37 °C. Following this, fresh growth medium replaced the SPS and the cells were grown up for ratio determination. Mouse cells (D1) were affected rather more than primate cells by this treatment with about 75% cell killing.

Table 1 shows the ratios of incorporated ^{3}H-uridine to ^{14}C-uracil counts found for a number of cell types, before and after the different treatments, during a period of 9 months. All untreated cells, except human embryo cells, gave consistently low uridine-uracil ratios, even if newly cultured from stock cells kept in liquid nitrogen, indicating mycoplasma contamination when supplied. Treatment at 41 °C or labelling in the presence of antibiotics (see above) had

Table 1 Ratio of ^{3}H-uridine to ^{14}C-uracil incorporated into TCA-precipitable material

Treatment d	HEC	VERO	BSC.B	LLC-MK_2	D1	3T3
-	438; 119; 211	1;1;1;5a	2;1a;1c; <1	1	2; 3	1; 1
41 °C		3	1b			
Passage in antibiotics		1	1	2	18	
Antibiotics present during labelling		<1			3	
10% SPS		74; 104; 163; 132	66	<1	19; 21; 27; 23	
5% SPS					1	
2.5% SPS					1	
10% SPS; antibiotics present during labelling		121			23	

a. Stock cells from liquid N_2

b. 2 treatments

c. From cultures labelled separately with ^{3}H and ^{14}C

d. Described in text

no effect on the ratios found. Passage of D1 cells in antibiotics however did give a 6-fold higher ratio of uridine to uracil incorporated.

A striking difference of 100-fold in the ratios were found for VERO cells treated with 10% SPS; the ratios for BSC.B cells were increased 30 to 60-fold but LLC-MK_2 cell ratios were not affected. D1 cell incorporation ratios were increased 6 to 10-fold by 10% SPS as they were after passage in the presence of antibiotics. Treatment with 5% or 2.5% SPS was not effective. The high ratios for all the cells were maintained during sub-culture for a period of some months and probably indicated eradication of contamination.

The high ratios found with untreated human embryo cells may be characteristic of these cells or reflect the fact that they were less likely to have become contaminated as they were less long in culture.

The relatively low ratios found with SPS-treated mouse D1 cells may be because the basal cellular level of uracil incorporation is higher than for primate cells or because contamination was not completely eliminated. However the maintenance of a ratio of approximately 20 is unlikely if mycoplasmas grew up to their former numbers during sub-culture of the cells.

Untreated mouse 3T3 cells also had a low uridine-uracil incorporation ratio with considerable ^{14}C-uracil incorporation. These cells, when uncontaminated, are reported (Chan *et al.* 1973) to excrete uracil into the medium if labelled precursors are supplied and are therefore inefficient at utilisation of exogenous uracil. Thus poor uracil incorporation

is not unique to human embryo cells. It has also been shown (Hellung-Larsen & Frederikson, 1976) that incubation of contaminated cell cultures from a number of species with ^{3}H-uracil gives labelling only of mycoplasma rRNAs as detected by polyacrylamide gel electrophoresis. Uridine labelling is found in both host and mycoplasma rRNAs.

The use of uridine-uracil incorporation ratios is a convenient method for routine testing of cultures but should be supplemented with other techniques.

ii) Scanning electron microscopy of cells

A method for the visualisation of mycoplasma particles on individual cells was reported by Brown et al. (1974) using scanning electron microscopy. Infected cells had adherent particles, with a disc-like appearance, of 0.35 to 0.8um diameter.

Cell cultures were examined by a modification of the method of Brown et al. (1974). 30mm Petri dishes containing 18mm glass cover-slips were seeded with cells in growth medium. After 24h for cell attachment the cover-slips with adherent cells were washed in PBS_A, fixed in 4% glutaraldehyde in PBS_A and dehydrated in a series of ethanol concentrations from 25% to absolute ethanol. The cover-slips were then dipped in a 1:1 (v/v) mixture of amyl acetate and absolute ethanol and finally into amyl acetate. The cover-slips with the fixed cells were dried by the critical-point method, gold-shadowed and mounted with Durafix. The prepared cells were examined in a MINI SEM MSM-3 (International Scientific Instruments) at magnifications of 7.10^2 to 10^4.

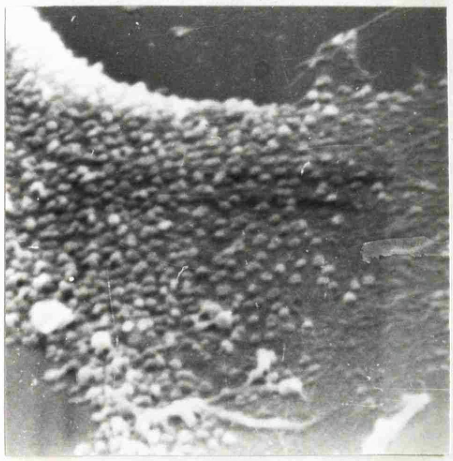
Particles having the appearance and dimensions of those shown by Brown et al. (1974) were found in untreated 3T3,

Figure 2 Scanning electron micrographs of untreated cells

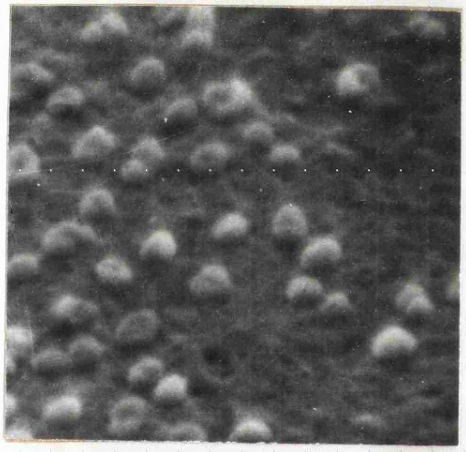
A. VERO cell (x 3,000)

B. VERO cell (x 10,000)

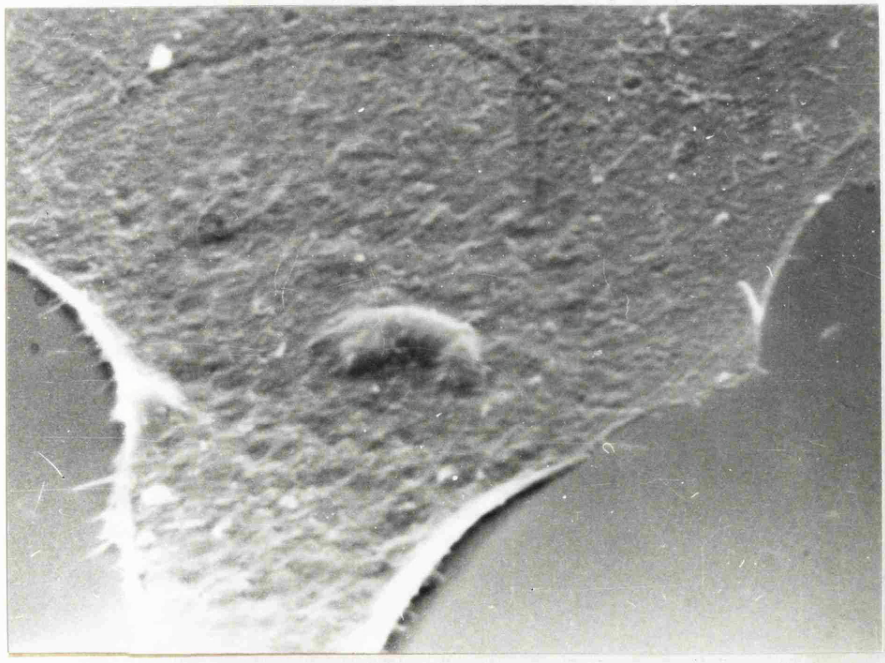
C. Human embryo cell (x 3,000)



A.



B.



C.

VERO, BSC.B and Mouse L cells but not in human embryo cell preparations. Examples of VERO cells and human embryo cells are shown in Fig. 2A,B & C.

iii) Autoradiography of cells labelled with precursors of RNA or DNA

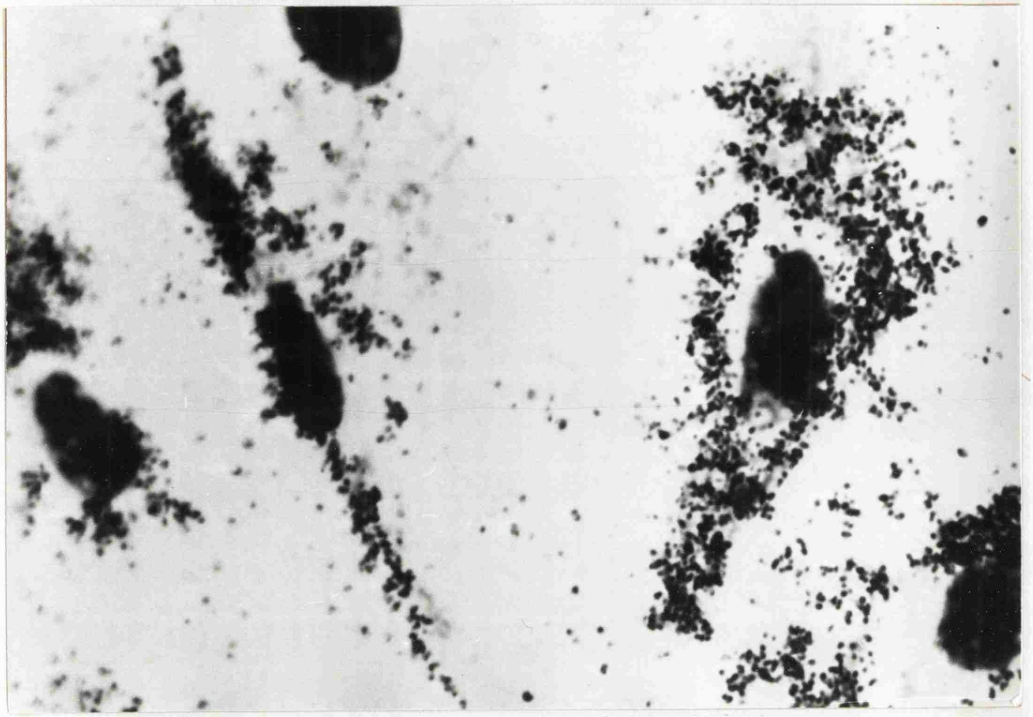
The close adherence of mycoplasma particles to cell membranes enables their detection by autoradiography with radioactively labelled RNA or DNA precursors followed by Giemsa staining of the cells. Contaminated cells show a characteristic concentration of developed silver grains over the cytoplasm (Schneider et al. 1973).

Cells were seeded in growth medium into 30mm Petri dishes containing sterile glass cover-slips which had been previously treated with 0.5% gelatin for 3h. After 24h for cell attachment ^{3}H-uridine or ^{3}H-thymidine was added and the cells incubated at 37 °C for the required time interval (see below). The medium was removed, the cells washed twice with PBS_A and fixed with 3:1 (v/v) glacial acetic acid and methanol at 0 °C for 10 min. Macromolecules were precipitated with 10% trichloracetic acid (TCA) at 0 °C for 10 min. The cells were then washed twice for 10 min with cold, distilled water followed by two changes of absolute ethanol at room temperature. After air-drying the cover-slips were mounted with Cristalite, cell-side uppermost, on slides previously acid-cleaned and treated with a solution of 0.1% gelatin and 0.01% chrome alum. (This treatment enhances the adherence of the film emulsion). The mountant was allowed to dry for 24h at room temperature. After this time the slides were warmed to 45 °C and dipped in Kodak NTB nuclear track emulsion (diluted 2-fold in distilled water), allowed to dry and

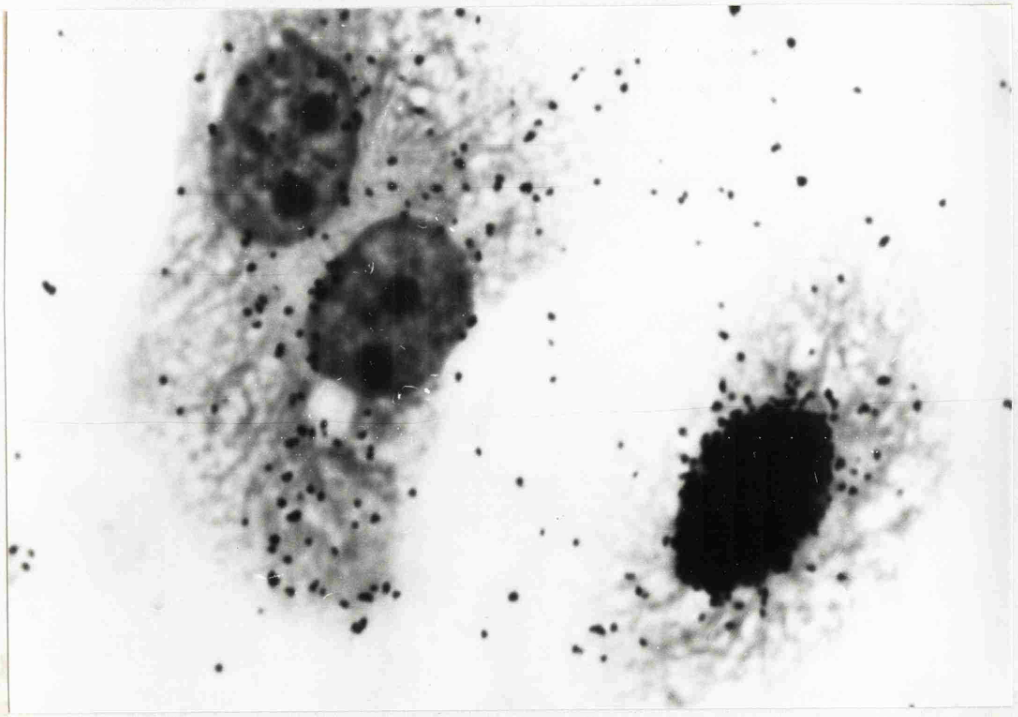
Figure 3 Autoradiographs of untreated and SPS-treated VERO cells

A. VERO cells labelled with 5-^{3}H uridine (5μC/ml) for 30 min and Giemsa stained.

B. SPS-treated VERO cells labelled with methyl ^{3}H-thymidine (25μC/ml) for 17h and Giemsa stained.



A.



B.

stored in light-proof boxes until sufficiently exposed (approximately 1 week). Development was with Kodak D19 developer (diluted 2-fold) for 2.5 min followed by distilled water washing and 5 min fixation with Metafix. Slides were then washed in distilled water and stained with Giemsa (2).

Fig. 3A shows untreated VERO cells labelled with 5μC/ml of 5-^{3}H uridine for 30 min. Fig. 3B shows VERO cells some months after SPS treatment labelled for 17h with methyl ^{3}H-thymidine (25μC/ml).

Silver grains were found extensively over the cytoplasm of the untreated cells after 30 min labelling with ^{3}H-uridine when it would be expected that only the nucleolus would be heavily labelled. The SPS-treated cells labelled with ^{3}H-thymidine showed heavily labelled nuclei but grains over the cytoplasm were not above background levels and these cells were probably not contaminated.

iv) Microbiological culture of mycoplasma from cell cultures

Confirmation of the presence of mycoplasmas in the medium of untreated VERO and BSC.B cells was made by R. Leach at the Central Public Health Mycoplasma Reference Laboratory, London. The organism was tentatively identified as M. hyorhinis.

v) The effects of SPS treatment on VERO and D1 cells

The response of VERO and D1 cells to their homologous interferons or to treatment with poly(rI). poly(rC) was not changed by SPS treatment. The growth of VERO cells in HAT medium was improved after SPS treatment as was virus plaque formation in these cells because the overlay medium became less acid.

5. Assay of Viruses

i) Plaque assay of virus preparations

The titre of infectious particles in a virus preparation may be measured, after suitable dilution, by estimation of the numbers of foci of dead cells under agarose produced by initial infections of single cells by single virus particles.

Virus preparations were diluted serially 10-fold in growth medium. Aliquots of 0.3ml were added to duplicate 50 mm Petri dishes containing confluent monolayers of the assay cells. After 30 min at 37 °C for virus adsorption, with intermittent swirling of the virus suspension over the cells, the diluent was removed and 4ml of an overlay of 1:1 (v/v) 1.4% agarose and 2-fold concentrated DME with 12% calf serum was added per plate. This was allowed to set and the cultures incubated at 37 °C (or 30 °C for temperature-sensitive viruses) until plaques were apparent by microscopic examination or by viewing plates obliquely. For Sindbis virus assayed on primate or mouse cells 48h incubation was necessary but for chick embryo assay cells and Sindbis virus only 24h was required. Plaques were visualised by staining with a liquid overlay of 2ml of 0.01% neutral red in PBS_A at 37 °C for 2 to 4h. Plaques were counted in Petri dishes having approximately 100 plaques and the number of plaque forming units (p.f.u.) per ml of the virus preparation calculated from the dilution factor and aliquot added per plate.

ii) Cloning of viruses

Virus preparations were diluted as for the plaque assay method but sterile neutral red solution was used to visualise plaques. Virus from a single plaque, preferably from a plate with only 1 plaque, was isolated by insertion of the tip of a

sterile Pasteur pipette through the agarose and cells were sucked up immediately beneath this area. The virus was suspended in 1ml of growth medium and used to infect a confluent monolayer of cells in a 50mm Petri dish. The medium was harvested when the cell monolayers showed a cytopathic effect (CPE) and stored at -20 ^{o}C for subsequent re-cloning or innoculation of cell monolayers for stock virus cultures.

iii) Haemagglutination assay of viruses

The virus preparation was diluted 10-fold in PBS. To a set of tubes was added 0.5ml PBS, and to the first tube 0.5ml of the 10-fold diluted virus. Serially 2-fold dilutions were then made by mixing the contents of the first tube, taking 0.5ml of this to the second tube and so on, the final 0.5ml was discarded. To each tube was then added 0.5 ml of 1% chick red blood cells in PBS and the tubes chilled for 1h in an ice bucket. The dilution point at which only 50% haemagglutination (H.A.) was seen was the virus titre (H.A. units). (Cells form a 'mesh' round the tube rather than a small pellet at the bottom).

6. Preparation of stock viruses

i) Sindbis virus

Chick embryo cell grown Sindbis virus was found to be less infective and gave variable sized plaques on primate cells. Consequently it was passaged in monkey cells by infection of monolayers of cells with small volumes of diluted virus (at approximately 1 p.f.u. per cell or less). The virus-containing medium was harvested when CPE was apparent and used to reinfect other cultures or stored at -20 ^{o}C. After 4 passages in monkey cells the Sindbis virus was twice cloned in these cells, as described above, and used for

plaque reduction and dye uptake experiments. Later, after SPS treatment of VERO cells, chick-grown Sindbis was again twice cloned in these cells to give 'mycoplasma-free' preparations. Cloning of Sindbis virus in monkey cells gave improved titres on human embryo cells, and less variable plaque morphology.

Mouse D1 cells were found to be variable in their response to the cytopathic effect of chick-grown Sindbis virus as were L929 cells but not mouse L cells.

Sindbis virus (chick cell grown) was passaged once in D1 cells and used for most dye uptake experiments with these cells and often with hybrid clones as these, too, showed variability in the cytopathic effect found after virus infection (Section V).

ii) Temperature-sensitive Sindbis virus (ts15)

This virus was grown at 30 °C in monolayers of chick embryo cells and assayed at 30 °C and 37 °C on experimental cells. D1 and L929 cells were insensitive to the cytopathic effect of this virus at 30 °C.

iii) Newcastle Disease Virus (NDV)

Stock, egg-grown NDV was diluted to 5.10^7 p.f.u. per ml in PBS_A. Air-sacs were marked on 11-day embryonated eggs, the shells wiped with 70% ethanol and small holes made to allow the introduction of 0.2ml per egg of diluted NDV, with a sterile syringe, beneath the embryonic membranes. The hole was covered with sticky tape and the eggs incubated at 37 °C for 48h in an egg incubator (Western (Curfew) Incubators Ltd; Chelmsford, Essex). The eggs were chilled at 4 °C for a few hours, the tops of the shells removed and the allantoic fluid surrounding the embryos harvested with sterile Pasteur

pipettes, taking care not to include red blood cells. The virus-containing fluids were stored at -70 °C until plaque assay of the preparation on chick embryo cells.

7. Cell Fusion

i) Inactivation of Sendai virus for cell fusion (Neff & Enders, 1968)

Concentrated, egg-grown Sendai virus in 5% sucrose with antibiotics, originally at 80,000 H.A.U. per ml, was treated with 0.1ml of a 10-fold dilution of β-propiolactone in saline-bicarbonate solution per 4ml of virus. After 10 min at 4 °C, with shaking, the virus was incubated at 37 °C for 2h, also with shaking. The preparation was then left at 4 °C for 24h to inactivate β-propiolactone, dispensed into 0.5ml aliquots and stored at -70 °C.

ii) Fusion of cells with inactivated Sendai virus

Confluent monolayers of cells in 50mm Petri dishes were washed once with growth medium and chilled at 4 °C for 5 min. 1ml per plate of chilled Sendai virus, diluted in growth medium as required, was added for 30 min at 4 °C with intermittent tilting of the plates. 2ml of growth medium were then added and the monolayers incubated at 37 °C for 2h. The virus was then removed, the cells washed twice with growth medium and fresh medium added. After 24h incubation at 37 °C cells were trypsinized and seeded in selective medium.

8. Interferon production and purification

i) Human cells

A confluent monolayer of early passage human embryo cells (HES.A) in a roller bottle ($\sim 1.10^8$ cells) was infected with Newcastle disease Virus (NDV) at 3 p.f.u. per

cell in 10ml of D6. After 30 min adsorption at 37 °C, 40ml of D6 was added and the cells incubated at 37 °C for 48h. The medium was then harvested and stored at -20 °C for acid precipitation, neutralisation and interferon assay.

ii) Mouse cells

Approximately 4.10^7 confluent mouse L cells in a 1l glass Brockway bottle were infected with 10^8 p.f.u. of Sindbis virus (as assayed on chick embryo cells) in 10ml of growth medium. After 30 min adsorption at 37 °C 25ml of growth medium were added and the cells incubated at 37 °C for 48h. The medium was harvested and stored at -20 °C until preparation and assay of interferon.

iii) $LLC\text{-}MK_2$ cells

A confluent monolayer of $LLC\text{-}MK_2$ cells in a 500ml bottle (approximately 2.10^7 cells) was infected with Sindbis virus at 10 p.f.u. per cell (as assayed on chick embryo cells). After 30 min adsorption at 37 °C 20ml of growth medium were added and the monolayers incubated at 37 °C for 24h. The medium was harvested and stored at -20 °C before interferon preparation.

iv) Partial purification of interferon preparations

Medium harvested from virus-infected cell monolayers (usually 50ml from approximately 4.10^7 cells growing in a 1l Brockway bottle) was acidified by the drop-wise addition of 2M perchloric acid, to a final concentration of 0.15M on ice. The preparation was left at 4 °C for 48h (Sindbis virus infected cells) or 5 days (NDV infected cells) to inactivate virus and precipitate some proteins. The precipitate was removed by low-speed centrifugation and the interferon - containing supernatant neutralised by the drop-

wise addition of 2M KOH on ice. The resulting precipitate of potassium perchlorate was allowed to settle for a minimum of 30 min in the cold and was finally removed by centrifugation. The supernatant was stored at -20 °C before assay of its interferon activity.

9. Plaque reduction assay of interferon and poly(rI). poly(rC)

i) Interferon

Duplicate monolayers of cells in 50mm Petri dishes were treated with 1ml of the interferon preparation to be tested diluted in growth medium. After 24h at 37 °C this was removed, the cells washed with growth medium and 100 p.f.u. per plate of Sindbis or Mengo virus added in 0.3ml of growth medium. Subsequent treatment was as for the plaque assay of viruses described above.

The interferon titre was taken as that dilution factor necessary to give 50% of control plaque numbers. (The results are plotted as percentage reduction of control plaque numbers versus dilution factor, with the latter on a logarithmic scale).

ii) Poly(rI). poly(rC)

Monolayers of cells in 50mm Petri dishes were treated in duplicate with a small volume (usually 1ml) of inducer diluted in PBS_A for 60 min. This was removed and the monolayers washed twice with PBS_A. Five ml of growth medium were added and the cells incubated at 37 °C for 24h.

Medium was removed and approximately 100 p.f.u. per plate of Sindbis virus added in 0.3ml of growth medium. Subsequent treatment of the cultures was as for the plaque assay of viruses described above.

The anti-viral state developing was expressed as percentage reduction of virus-infected control plaque numbers.

10. Neutral red dye uptake assay for estimation of cytotoxicity

Damage to cells caused by viruses or anti-metabolites can decrease cell numbers by cell killing and also decrease metabolic activity in the remaining cells. Uptake of the vital dye, neutral red, by cells and its subsequent extraction and estimation by its extinction at 540 nm can be used to measure both cell numbers and the metabolic state of the cells and so the total damage induced can be estimated for a population of cells.

The method described for the assay of interferon or interferon inducers and for estimation of the effects of anti-metabolites (e.g. ouabain, mutagens) is a modification of that described by Finter (1969).

i) Measurement of cell damage by the dye uptake method

Monolayers of cells in glass scintillation vials or in wells of 25 well trays (Sterilin) (approximately 5.10^5 cells per vial or well when confluent) were stained with 1ml per well of a 0.01% solution of neutral red in PBS_A for 30 or 60 min at 37 °C. (The neutral red solution was a dilution of a filtered 0.1% stock solution). The dye solution was removed, the monolayers washed with PBS_A and 3ml per well of a 1:1 mixture of absolute ethanol and 0.1M NaH_2PO_4 added to extract the dye taken up by the cells. [It was found that industrial methylated spirits (I.M.S.) could be substituted for the absolute ethanol]. The extracted dye was mixed thoroughly with a small, hand-held electric stirrer (Minipack, Hammant and Morgan Ltd) and samples taken directly from the

Figure 4 The effect of cell number on neutral red dye uptake

Neutral red dye uptake after 30 min by VERO (●) and D1 (o) cells seeded at different densities 24h previously. (Each point is the average of 5 determinations).

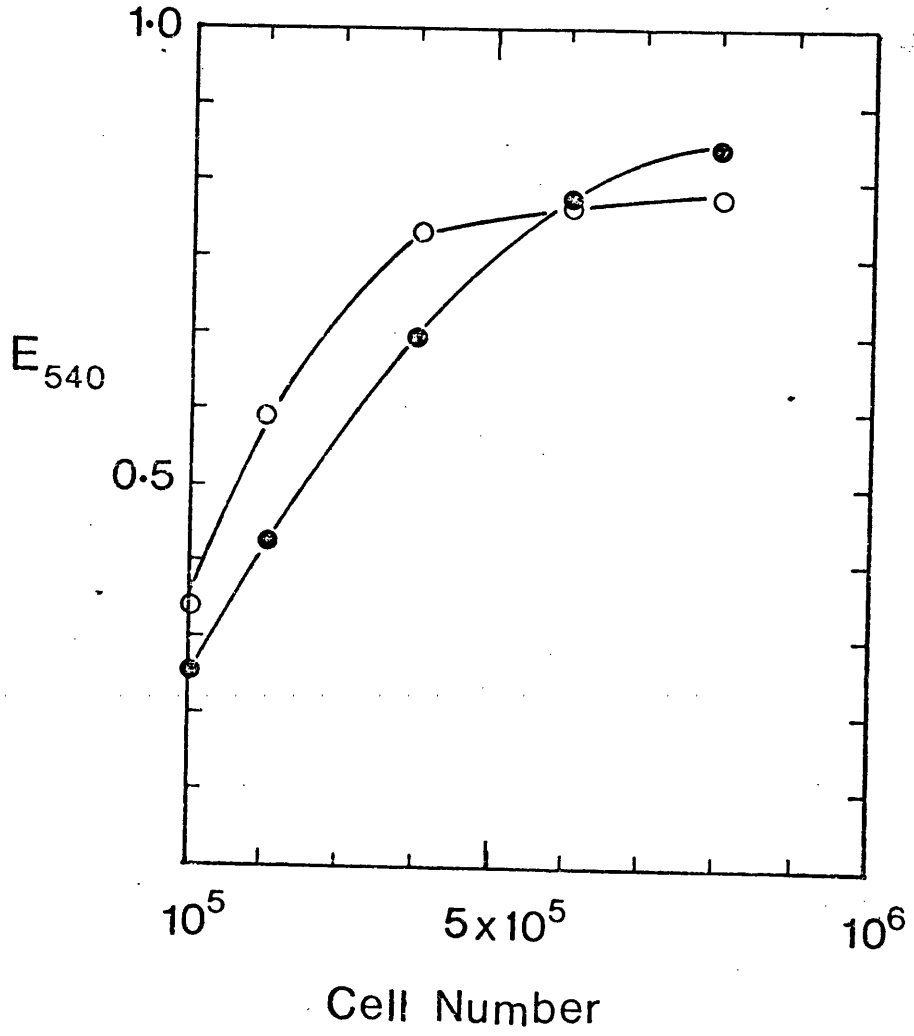
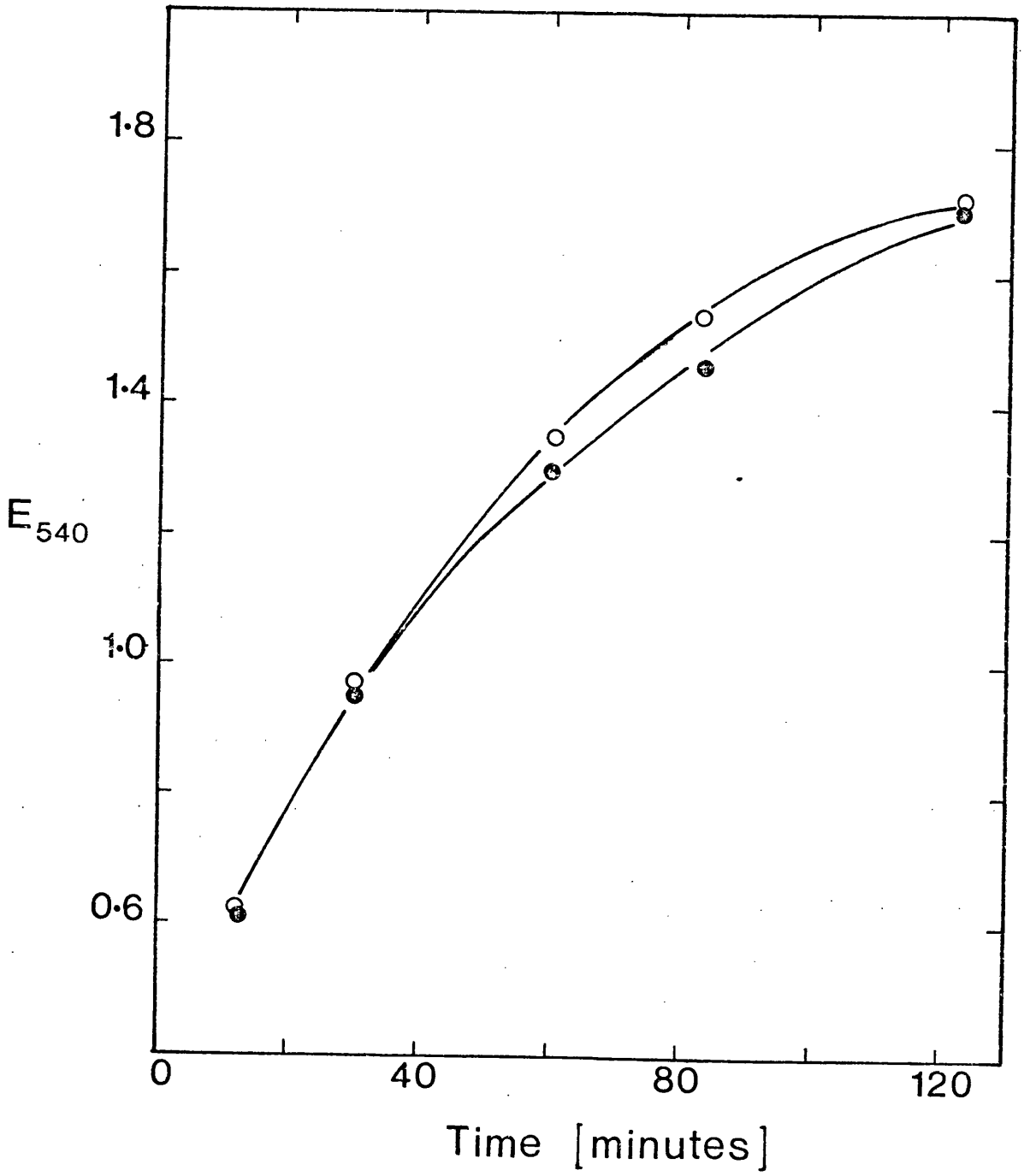

1·0
E_{540}
0·5
10^5
5×10^5
10^6
Cell Number

Figure 5 Neutral red dye uptake after varying incubation times

Neutral red dye uptake by confluent monolayers (5.10^5 cells) of VERO (●) and D1 (o) cells incubated for the indicated times at 37 °C. (Each point is the average of 5 determinations).


1·8
1·4
1·0
0·6
E_{540}
0
40
80
120
Time [minutes]

wells through a Pye-Unicam flow-through UV spectrophotometer (SP6-500) for measurement of the extinction at 540 nm. The blank was a 1:1 mixture of ethanol or I.M.S. and 0.1M NaH_2PO_4.

The effect of cell numbers on dye uptake

The amount of dye taken up by a culture is related to the number of cells surviving a given treatment and their metabolic condition. The effects of varying cell numbers were measured in the following experiment.

VERO and D1 cells were seeded at different cell densities in wells of 25 well trays. They were allowed to attach overnight at 37 ^{o}C and stained with neutral red, as described above, for 30 min at 37 ^{o}C. Uptake of dye was linear from 10^5 to 4.10^5 cells per well of each kind (Fig. 4). Confluent monolayers contain about 5.10^5 cells and the plateau region represents the removal by washing of excess seeded cells.

The effect of incubation time on dye uptake

The uptake of dye by different cell types varied; in particular VERO and D1 cells showed rapid uptake and high E_{540} values were obtained after 60 min staining time. The uptake of dye with time was therefore measured for VERO and D1 cells to check that,in experiments with a 60 min staining period,cell viability in untreated control monolayers was not underestimated relative to experimental monolayers of cells. Some departure from linearity of dye uptake with time was found after 60 min (Fig. 5) and consequently a 30 min staining time was adopted.

The relationship of E_{540} to neutral red concentration

The relationship of the concentration of neutral red to E_{540} should be linear if the E_{540} of a sample of extracted

Figure 6 The relationship of neutral red concentration to E_{540}

The E_{540} of neutral red for concentrations in I.M.S. representing up to 60% of total possible dye uptake by cells.

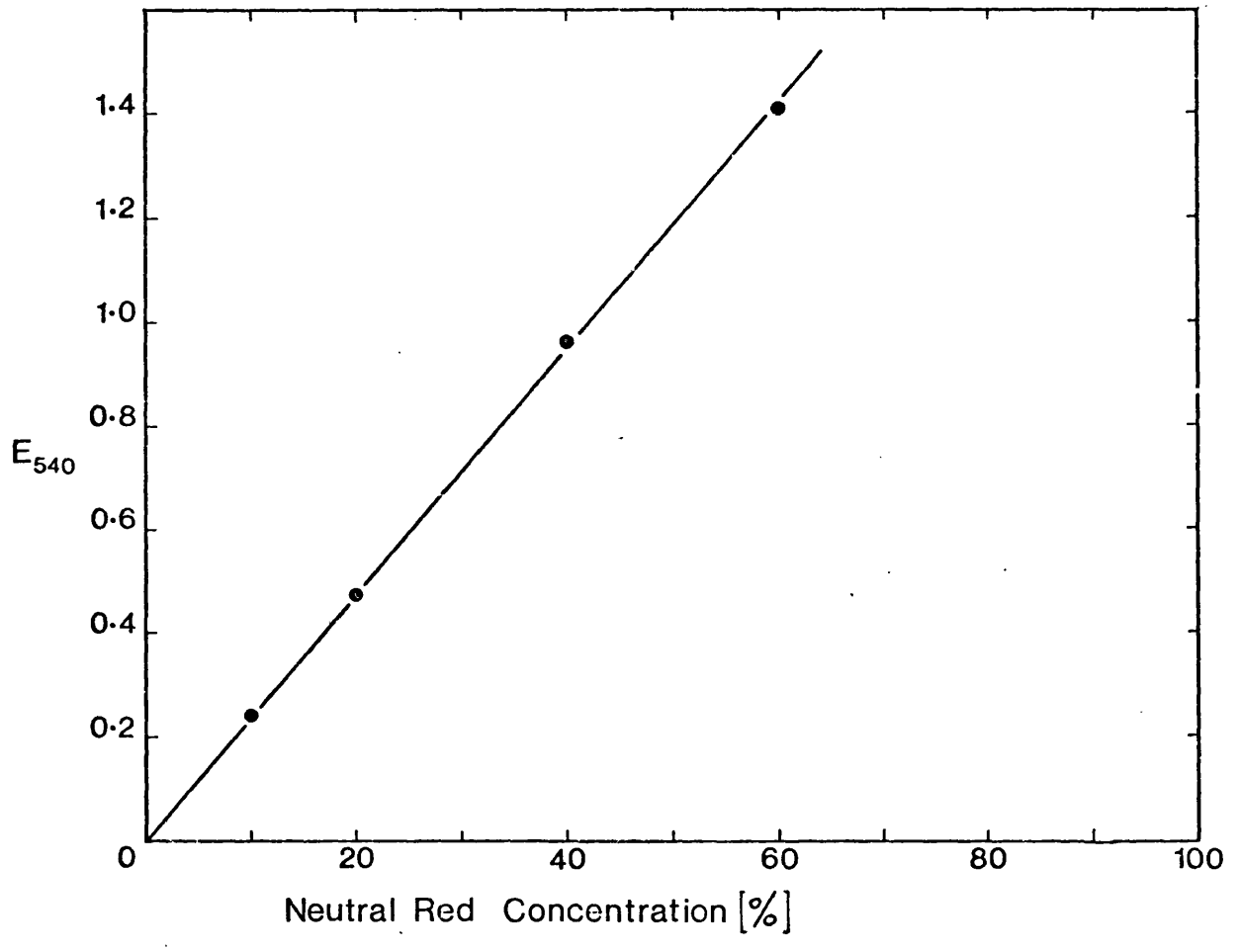
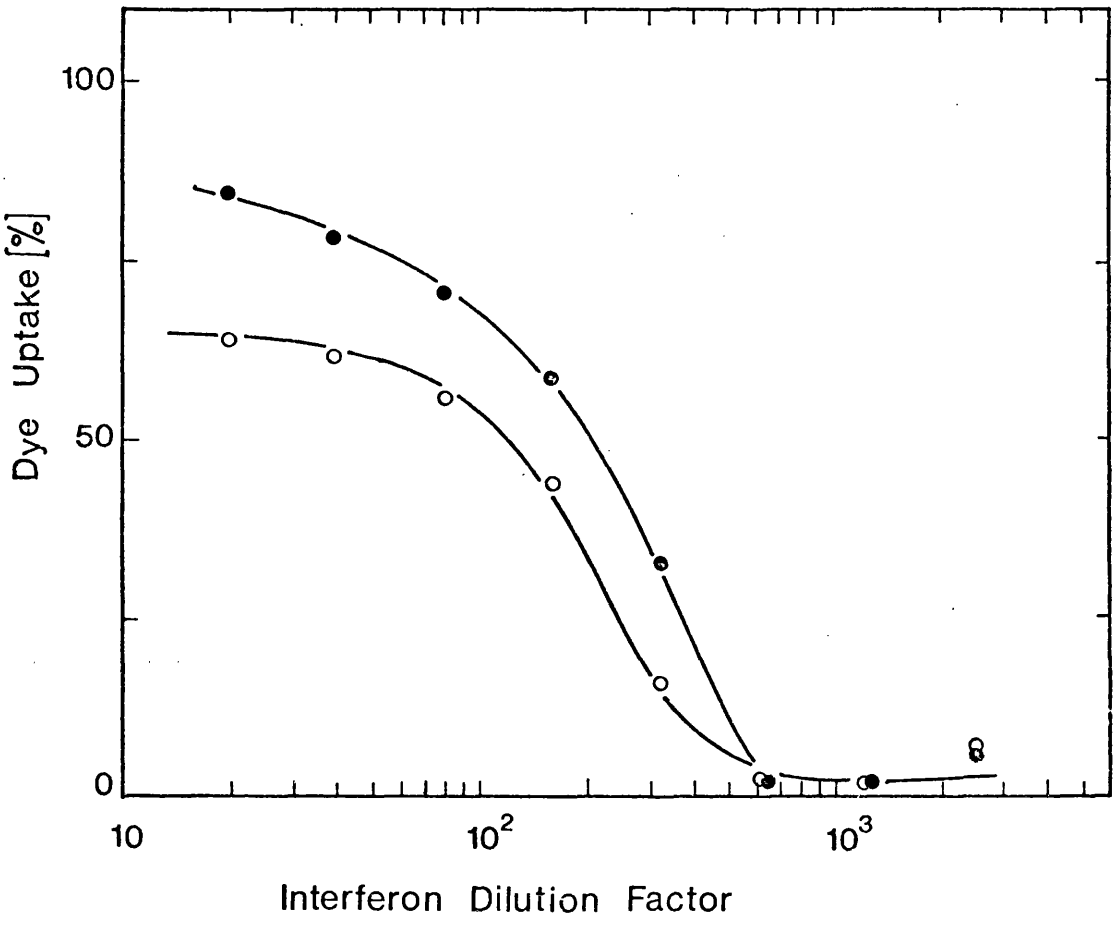

1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
E_{540}
20
40
60
80
100
Neutral Red Concentration [%]

Figure 7 Human interferon assay by neutral red dye uptake

Dose-response curves for human fibroblast interferon (●) and human leucocyte reference interferon (○, 200 units/ml) assayed on human embryo cells (HES.A) by neutral red dye uptake with Sindbis virus challenge.


100
50
0
Dye Uptake [%]
10
10^2
10^3
Interferon Dilution Factor

dye from cells is to be taken directly as a measure of cell viability.

A stock solution of 0.1% neutral red was diluted 30-fold in the extraction mixture, this being equivalent to a sample from cells showing total dye uptake, and diluted to give solutions representing 60% to 10% of total dye uptake. The relationship is linear to at least 60% uptake of total added dye (Fig. 6).

ii) Applications of the dye uptake method

Assay of interferon preparations

Interferon activity is measured by estimating the inhibition of virus replication in cells treated with interferon. Virus infection of cells often leads to cell destruction as well as to the production of progeny infectious virus particles and the former may be measured by the dye uptake method. In general monolayers of assay cells in vials or 25 well trays were treated with 0.4ml per well of interferon preparations diluted in growth medium in duplicate. After 24h incubation at 37 °C interferon was removed and the cells challenged with 0.2ml per well of Sindbis virus (1 to 5 p.f.u. per cell) or EMC virus (0.001 p.f.u. per cell). After 30 min adsorption at 37 °C unadsorbed virus was removed and growth medium replaced (1ml). After 48h incubation at 37 °C cells were stained with neutral red as described above. Occasionally cytopathic effect was determined visually after 24h on a scale from + (25%) to ++++ (100%) cell damage.

Fig. 7 shows the results of an assay of human fibroblast interferon (1) and National Institutes of Health human leucocyte reference interferon (G-023-901-527; 2,000 reference units/ml) carried out as described above on HES.A cells.

Cell protection after virus challenge is expressed as per cent dye uptake.

$$\% \text{ dye uptake} = \frac{E_{540} \text{ of sample} - E_{540} \text{ of virus-infected control}}{E_{540} \text{ of uninfected control} - E_{540} \text{ of virus-infected control}} \times 100$$

For interferon assays the percentage dye uptake is plotted versus the interferon dilution factor with the latter on a logarithmic scale. The interferon titre is taken as that dilution factor giving 50% dye uptake ($D.U._{50}$ units).

The titres of the two interferons were 140 $D.U._{50}$/0.4ml for the 100-fold diluted human leucocyte reference interferon and 210 $D.U._{50}$/0.4ml for human fibroblast interferon (1). The titre of human fibroblast interferon (1) in reference units is thus 300 units/ml.

The reason for the failure of dye uptake to reach 100% in interferon assays such as the one illustrated in Fig. 7 may have been that interferon or impurities in the more concentrated preparations had an inhibitory effect on cell metabolism. (However experiments on the cytotoxic effects of human fibroblast (1) on HES.A cells showed 96 and 98% dye uptake on treatment with 10-fold or 20-fold dilutions of the interferon preparation). The human leucocyte interferon preparation appeared to be particularly inhibitory of dye uptake. Such inhibition may result in an underestimation of anti-viral activity.

The estimation of the activity of an interferon preparation relative to a standard depends on the dose-response curves being parallel over their linear portion.

This was only approximately true for the two preparations assayed in Fig. 7. The validity of comparing the activity of human leucocyte and fibroblast interferons has been questioned (Edy, Billiau & De Somer, 1976).

Assay of the sensitivity of cells to the anti-viral action of poly(rI). poly(rC)

Cells treated with the synthetic inducer of interferon poly(rI). poly(rC) are able to develop an anti-viral state after treatment with concentrations of the inducer lower than those required to induce detectable interferon in the medium. This effect can be estimated by challenge with virus and the protection against cell damage measured by dye uptake as for an interferon assay.

Confluent monolayers of cells in 25 well trays or glass scintillation vials were treated with dilutions (0.5ml per well) of poly(rI). poly(rC) (with or without DEAE-dextran) in PBS_A for 60 min at 37 °C. This was removed, the monolayers washed twice with PBS_A and 1ml per well of growth medium added.

After 24h at 37 °C, to allow maximal development of an anti-viral state in the cells, Sindbis virus was added as described for the dye uptake interferon assay. On some assays challenge was with 0.001 p.f.u. per cell of EMC virus because of the variable cytopathic effect of Sindbis virus. This is indicated where appropriate.

The degree of protection against virus challenge was expressed as percentage dye uptake as for interferon assays.

Estimation of the effects of anti-metabolites on cell growth and viability

The inhibitory effects of a number of agents on cell growth and viability may be measured by direct counting of cells after treatment, formation of colonies or by measurement of cell damage by the dye uptake method after growth in the test medium. Colony formation depends on good plating efficiency in normal growth medium; if this is variable or poor, as it is for VERO cells, such experiments are not always informative.

The dye uptake method can be used to measure the effects of toxic agents on the growth of cells in a population after a number of generation times. It more closely estimates the effects on cells in normal culture conditions without the added variable of plating efficiency and takes less time than colony formation experiments.

Cells were seeded in 25 well trays in growth medium at 2.10^4 cells per well. After 24h for cell attachment the growth medium was replaced by medium containing the toxic agent to be tested. The cultures were incubated at 37 ^{o}C until control, untreated cells had reached confluence, (approximately 4 or 5 population doublings). The degree of growth inhibition or cytotoxicity was measured by dye uptake and results expressed as a percentage of control E_{540} values.

This method can be varied to measure the effects of agents having a cytotoxic effect on confluent cells or, by seeding fewer cells initially, can be used to measure cell growth over a longer period.

11. Karyotyping

i) Preparation

Cells were seeded in 100mm Petri dishes in growth medium, when semi-confluent, Vinblastine sulphate was added, at concentrations ranging from 0.05 to 10µg/ml, for short periods (4h) or for 24h at 37 °C to arrest cells in metaphase. (Light treatment of cells with trypsin or replacement of growth medium 24h before addition of Vinblastine sulphate was carried out if necessary to stimulate cell growth and division). After Vinblastine sulphate treatment the medium, containing rounded cells about to divide, was retained, the attached cells trypsinized and the cells and medium pooled, centrifuged and washed with PBS_A. The cell pellet after a further centrifugation was resuspended in hypotonic (0.6%) KCl and the cells allowed to swell for 15 to 30 min (depending on cell type) with microscopic examination. The cells were centrifuged from the hypotonic medium when sufficiently swollen and resuspended in fixative (3:1 v/v of methanol and glacial acetic acid) for 20 min. The fixed cells were stored at -20 °C if not used at once for slide preparation. Fixation was repeated if cells were stored for long periods at -20 °C.

Various methods of slide preparation were tried. An effective method was found to be the use of cleaned slides stored at 4 °C in absolute ethanol. These slides were flushed with 70% ethanol and fixed cells dropped onto them from a Pasteur pipette with vigorous blowing. Slides were dried on an Electrothermal slide-warmer at a setting of 3.

If cells were not to be treated by banding procedures they were dried by ignition of the alcohol in the pilot-light of a Bunsen burner.

ii) Giemsa staining of chromosomes

Method 1. Slides were hydrolysed in 1N HCl at 56 - 60 °C for 10 min, washed in running water for 10 min then stained in 0.05% Giemsa (1) in 0.2% Na_2CO_3 for 10 min. They were rinsed in distilled water and, if necessary, destained in very dilute HCl. After air-drying or blotting cover-slips were placed on the slides and attached with Cristalite for a permanent preparation.

Method 2. Slides were immersed in 50ml 0.1M phosphate buffer (pH 7.0) in a Coplin jar, 3ml of stock Giemsa (2) were added and stirred and the slides stained for 20 min. The Giemsa solution was flushed from the Coplin jar with distilled water to avoid a metallic deposit forming on the slides. Cover-slips were attached as in 1.

iii) Trypsin banding of chromosomes

Trypsin (2-fold crystallised) was prepared at 0.25% in triple distilled water. This solution was diluted 3-fold in distilled water before use. Slides were treated with the trypsin solution on ice for 10 to 60 s, washed with distilled water and stained in Giemsa (Method 2) for varying times (1 to 5 min). The best conditions for each cell type were determined by varying the concentration of trypsin and the exposure time.

iv) G11 staining of chromosomes

Slides were stained for 10 to 20 min in Gurr's Giemsa diluted 50-fold in distilled water at pH 11 (NaOH), rinsed in phosphate buffer (pH 6.8) and dried. Mouse chromosomes show a magenta colouring with pale blue centromeres whereas human chromosomes remain pale with red paracentromeric regions (Bobrow & Cross, 1974).

This method did not distinguish mouse D1 cell chromosomes from VERO monkey cell chromosomes; the latter staining as for mouse chromosomes even under varied times of staining. M. Bobrow (personal communication) has also been unable to show human-type staining with prepared VERO cell slides from this laboratory.

v) Photography of chromosomes

Well-spread preparations were photographed with a Zeiss photomicroscope using Ilford Pan F film. Processing was with Kodak D19 developer and Amfix.

Chromosomes were cut out from prints, arranged in order of size, and rephotographed.

12. Isozymes

i) Preparation of cell extracts

Confluent monolayers in 1l glass bottles were trypsinized and resuspended in growth medium. After centrifugation the cells were washed in PBS_A, re-centrifuged and resuspended in 1ml of a 10^{-5} dilution of Nonidet P42 in PBS_A. After a few minutes, with shaking, to solubilise cell proteins, the insoluble material was removed by centrifugation and the supernatant stored at -70 ^{o}C for use in cellulose acetate gel electrophoresis.

Confluent or semi-confluent cells from roller bottles or 1l bottles were trypsinized, washed in PBS_A and resuspended in a small volume of 0.02M TRIS (pH 8.0) containing 10^{-4} M dithiothreitol (Cleland's reagent) and $1.6.10^{-5}$ M thymidine. The cells and nuclei were broken by freezing in a mixture of acetone and solid carbon dioxide with thawing at 37 ^{o}C 3 times. The resultant broken-cell suspension was stored at -70 ^{o}C.

ii) Cellulose acetate gel electrophoresis

The term isozyme may be used to define different molecular forms of a single enzyme coded for by separate genes e.g. the A and B forms of lactate dehydrogenase, or different allelic forms of an enzyme from the same or a separate species e.g. glucose-6-phosphate dehydrogenase alleles A and B of humans.

Different molecular forms of enzymes within a species or from different species may be separated by their differing mobilities when subjected to electrophoresis in a buffer solution in a suitable supporting medium e.g. cellulose acetate or starch gels. Separation is by charge and in some gels (e.g. starch, polyacrylamide) also by size.

Bands of enzyme protein on gels may be detected by a variety of histochemical or autoradiographic methods. The most convenient method utilizes the reduction of nicotinamide adenine dinucleotide (or its phosphate) in the presence of enzyme substrate. This reduction is visualised by the reduction of nitro-blue tetrazolium or MTT tetrazolium [3-(4, 5-dimethyl thiazolyl-2) - 2, 5-diphenyl tetrazolium bromide] via the electron carrier phenazine methosulphate to form a blue colour as the tetrazolium dye is precipitated as a formazan.

In hybrid cells the presence of enzyme molecules with one or both parental cell mobilities may be detected by electrophoresis and, for enzymes with multiple sub-units, hybrid enzyme molecules may be formed generating a greater number of bands than is found for the parental cells alone.

Information about the chromosomes of a hybrid cell

population can be obtained if the chromosome assignment of the tested enzyme is known and new gene linkages can be determined if sufficient hybrid clones are tested and their karyotypes known.

Much work has been carried out on the human genome and enzyme markers are available for most chromosomes (Ruddle & Creagan, 1975). The karyotypes of primates show considerable simularities if allowance is made for translocation of chromosome arms and redistribution of heterochromatin (Ruddle & Creagan, 1975) and so the linkage of enzymes may be similar to that found in humans, at least for individual chromosome arms. This has been shown to be so for the galactokinase and thymidine kinase genes which are linked in man, the chimpanzee and the African green monkey (Orkwiszewski et al. 1976). Therefore to maximize the chances of detecting monkey enzymes in hybrids of mouse and monkey cells, having lost largely monkey chromosomes, it seemed practical to test for the marker enzymes for separate human chromosomes.

Lactate dehydrogenase (LDH)

This enzyme catalyses the oxidation of lactate to pyruvate with reduction of nicotinamide adenine dinucleotide (NAD). In humans the enzyme consists of four sub-units with two different polypeptide sequences (A and B). These may associate in any of the 5 possible combinations and so 5 bands may be found on gels. The two polypeptides are coded for by genes on chromosomes 11 and 12 of the human genome. Different tissues may have variable proportions, or only one, of the sub-units. The mouse also has the two LDH loci and therefore 5 possible bands on gels. However the mouse cell line A9 only shows the A form on starch gels (Nichols &

Ruddle, 1973). Human-mouse hybrid cells may show a large number of bands depending on the number of loci present.

The apparatus used for electrophoresis was that supplied by Millipore for use with their plastic-backed cellulose acetate 'Phoroslides'. The gels (7.6 x 2.5 cm) were wetted by placing edge-on in running buffer in a 100mm glass Petri dish, blotted and placed in the apparatus with the supplied, curved forceps such that each end of the gel rested in buffer. Samples of cell extract were supplied on a pencilled line half-way along the gel with applicators having a grooved metal tip holding 0.3µl. It was found that three different samples could be accommodated on one gel. (It is essential to include standards of both parental cell types). The lid of the apparatus was replaced and the unit attached to a Shandon-Southern power-pack. Electrophoresis was for 20 min at 100 volts (constant voltage setting). After the run the gel was removed, blotted and a small volume of staining solution (2ml) gently pipetted onto the surface. Excess stain was blotted off and the gel incubated in the dark until bands were apparent (5 to 10 min at room temperature). Their position was measured and recorded. Fig. 8A shows the positions of VERO and D1 cell LDH isozymes. The monkey cell line VERO was found to have a maximum of 5 LDH bands but only 1 band was seen with mouse D1 cell extracts.

Running buffer

1. Tris-HCl

   34.57 g/l of Tris adjusted to pH 8.3 with 1N HCl.

2. Barbital buffer (pH 8.6)

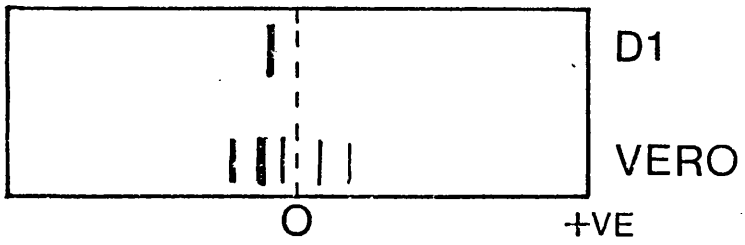
   2.76 g/l of diethyl barbituric acid with 15.4 g/l of sodium diethyl barbiturate.

Figure 8 Cellulose acetate gel electrophoresis of VERO and D1 cell isozymes

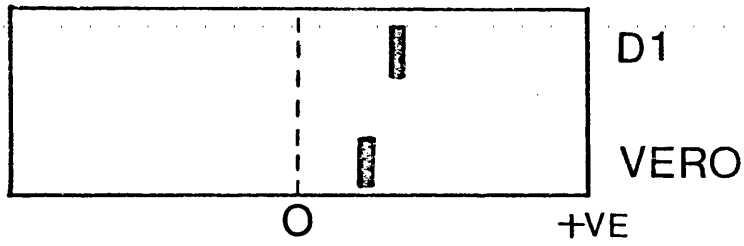
A. Lactate dehydrogenase (LDH) isozymes separated in TRIS/Barbital buffer for 20 min at 100 volts (constant voltage). Gels were stained with sodium lactate and NAD coupled with phenazine methosulphate and nitro-blue tetrazolium. Sample application was at the origin (O).

B. Glucose-6-phosphate dehydrogenase (G6PDH) isozymes separated in TRIS/EDTA/citric acid buffer (pH 7.5) for 40 min at 2 mA (constant current). Bands were visualised by staining with glucose-6-phosphate and NADP coupled with phenazine methosulphate and MTT-Tetrazolium.

A. LDH



B. G6PDH



For electrophoresis a 1:1 (v/v) mixture of 1) and 2) was used.

Staining solution

This contained 2mg/ml nitroblue tetrazolium, 0.2mg/ml phenazine methosulphate, 9.6mg/ml NAD and 47mg/ml sodium lactate.

Glucose-6-phosphate dehydrogenase

The enzyme catalyses the oxidation of glucose-6-phosphate to 6-phosphogluconate with the reduction of NADP.

The glucose-6-phosphate dehydrogenase (G6PDH) enzymes of mouse and man have different mobilities on cellulose acetate gels (Meera Khan, 1971) and bands of intermediate mobility are found when extracts of man-mouse hybrid cells are run but not with mixtures of the two parental extracts. The human gene for G6PDH is X-linked as is that for HGPRT. The difference in mobility of the monkey and mouse enzymes is less marked (Shannon & Macy, 1973) as determined on starch gels.

The gel was prepared as for LDH with the appropriate running buffer and electrophoresis was carried out at 2 mA (constant current setting) for 40 min with extracts of VERO and D1 cells as controls. Fig. 8B shows the mobilities of VERO and D1 cell enzymes.

Running buffer

1. 7.28 g of tris and 0.94 g of acid EDTA dissolved in 800ml of distilled water.
2. 3.15 g of citric acid and 0.3 g EDTA (disodium salt) dissolved in 200ml of distilled water.

Solution 1 was adjusted with solution 2 to pH 7.5 and the mixture made to a final volume of 1 litre.

Staining solution

1. Tris-HCl, EDTA buffer

121.1 g/l of tris and 1.49 g/l of EDTA (disodium salt) adjusted to pH 8.6 with 50% HCl.

2. 0.5ml of 1) with 1ml of a solution containing 0.8 mg/ml NADP (disodium salt), 4mg/ml glucose-6-phosphate, 0.4mg/ml MTT-tetrazolium, 0.08mg/ml phenazine methosulphate and 11.9mg/ml cobalt chloride.

This solution was used for staining the gels as for LDH.

13. Hypoxanthine and thymidine uptake and incorporation

i) Hypoxanthine

Monolayers of growing cells in 50mm Petri dishes (approximately 10^6 cells per dish) were incubated with 1ml of prewarmed D55 containing 0.1µC of ^{14}C-hypoxanthine. After 30 min at 37 °C this was removed and the cells washed 3 times with cold PBS_A followed by three washes with 1ml of 2M perchloric acid (PCA). The first two PCA washes were retained for determination of acid soluble radioactivity. The precipitated macromolecules were washed twice with 3ml of absolute alcohol, air-dried for 1h then solubilised with 2ml of 0.5N NaOH for 30 min. 1ml samples of the PCA soluble material and of the NaOH extracts were added to 9ml of Bray's scintillation fluid in glass scintillation vials and the radioactivity determined.

ii) Thymidine

Cells were seeded at 5.10^4 per sterile glass scintillation vial in D55. After 24h the medium was removed and 1ml of D55 containing 0.05µC of ^{14}C-thymidine added. The non-confluent monolayers were incubated at 37 °C. At

intervals medium was removed from duplicate cultures, the monolayers washed 4 times with cold PBS_A and treated with 5% trichloroacetic acid (TCA) for 30 min in the cold. The precipitate was washed twice with 5% TCA, twice with absolute ethanol and air-dried at room temperature for a minimum of 30 min.

The precipitate was solubilised in 0.2ml of Koch-Light tissue solubiliser (TS-1; 33% solution in toluene) for 30 min at 37 °C. 8ml of toluene scintillation fluid were added per vial and the radioactivity determined.

iii) Hypoxanthine and thymidine (suspended cells)

Cells were suspended at 10^6 or 2.10^6 per ml in Hepes-buffered MEM suspension medium and pre-warmed by shaking at 37 °C in a water-bath. ^{14}C-hypoxanthine or ^{3}H-thymidine was added to give a final activity of 0.1µC/ml. 250µl samples were taken at intervals into 5ml warm PBS_A in a glass funnel clamped over a glass fibre disc (Whatman GF/A; 2.5 cm) on a Millipore filter unit. A vacuum was applied and the cells washed 3 times with warm PBS_A. Similarly, other 250ul samples were taken into cold 5% TCA and filtered onto glass fibre discs. These were washed thoroughly with cold 5% TCA.

Both sets of discs were dried under a 250 Watt infra-red lamp (Phillips), inserted into flat-bottomed glass tubes ($1\frac{1}{2}$" x $\frac{1}{2}$"), and 2ml of toluene scintillation fluid were added. The tubes were capped with plastic closures, placed in glass scintillation vials and radioactivity determined.

14. Assay of hypoxanthine-guanine phosphoribosyl-transferase (HGPRT) activity

i) Preparation of cell extracts

Trypinsized cells were washed in PBS_A then suspended in 0.01M Tris (pH 8.0) at approximately 5.10^6 cells per ml. The cell suspension was left on ice for 15 to 20 min to allow the cells to swell. The cells were disrupted by homogenisation in a hand-held glass homogeniser with a Teflon pestle. Debris was removed bycentrifugation and the supernatant stored at -80 °C.

ii) HGPRT assay

HGPRT catalyses the formation of inosinic acid (IMP) from hypoxanthine and phosphoribosylpyrophosphate (PRPP). The IMP formed can be retained differentially on anion exchange discs (DEAE-cellulose). The assay method used was a modification of that of Sato, Slesinski & Littlefield (1972).

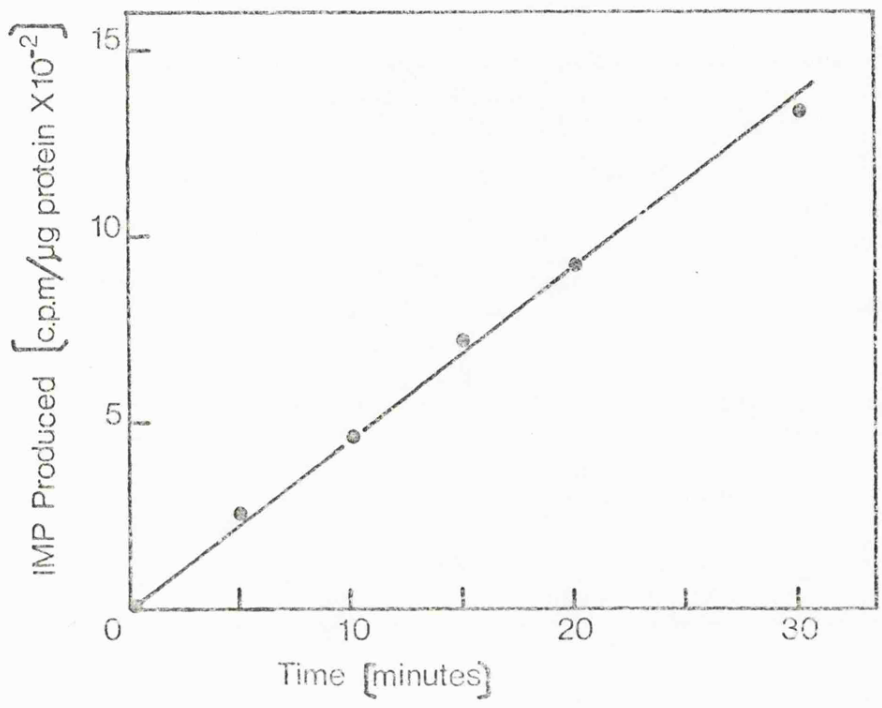
Reaction mixtures contained 6mM $MgCl_2$, 12mM phosphate buffer (pH 7.4), 12mM Tris-HCl (pH 7.4), 0.16mM ^{14}C-hypoxanthine (sp. act. 3.75mCi/mmol), 1mM PRPP and up to 60 µg of cell extract protein in a final volume of 250µl. This was prepared on ice in microfuge tubes (Jobling) and 0 time samples taken (10µl). Reaction mixtures were then incubated at 37 °C in a water-bath. Duplicate samples (10µl) were taken at intervals and spotted onto DEAE-cellulose discs (Whatman DE-81). After air-drying each disc was placed on 2 further DE-81 discs in a Millipore filter unit and washed 10 times with 4mM ammonium formate and 10 times with distilled water to remove excess ^{14}C-hypoxanthine. The discs were dried under an infra-red lamp and treated subsequently as

Figure 9 HGPRT assay

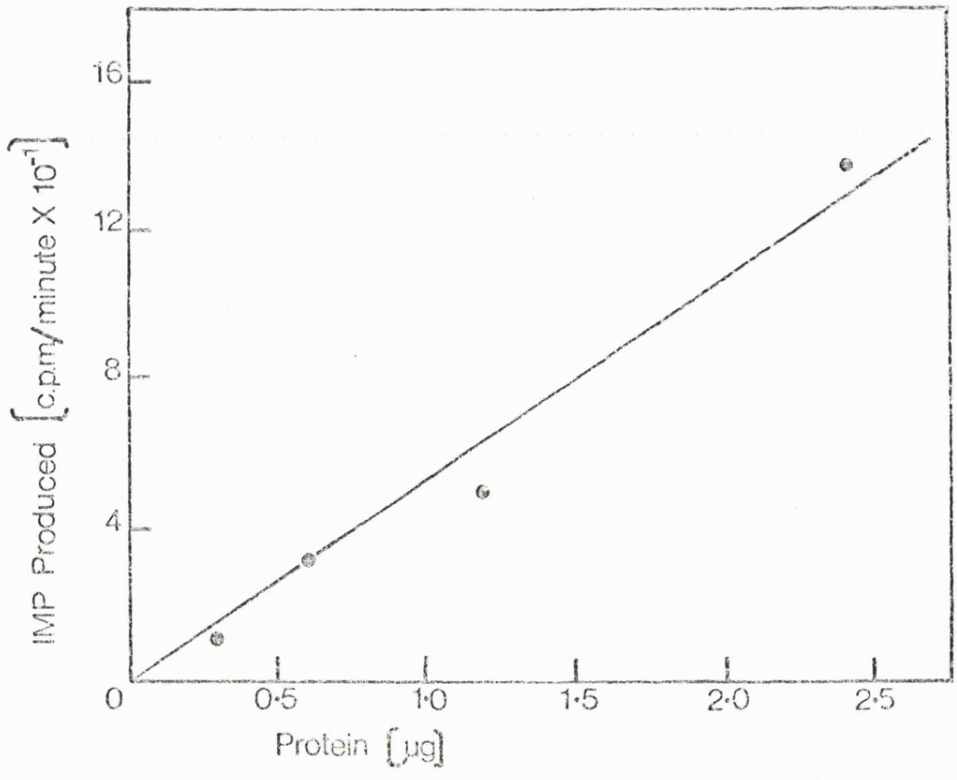
A. Time course of IMP formation by a VERO cell extract. Duplicate 10µl samples (1.6µg protein) were taken at the times indicated from a reaction mixture containing VERO cell extract incubated at 37 °C. The ^{14}C-IMP produced was separated from unused ^{14}C-hypoxanthine on DE 81 cellulose discs.

B. IMP formation with respect to protein concentration of VERO cell extracts. Duplicate 10µl samples (with the indicated amounts of protein)were taken from reaction mixtures incubated at 37 °C. (^{14}C-hypoxanthine concentration was 0.48mM). IMP formation was plotted against time and the rate determined over the linear portion of the graph. The rate of IMP formation is plotted against the amount of extract protein.

A.


IMP Produced [c.p.m/µg protein X10⁻²]
15
10
5
0
10
20
30
Time [minutes]

B.


IMP Produced [c.p.m/minute X 10⁻¹]
16
12
8
4
0
0·5
1·0
1·5
2·0
2·5
Protein [µg]

for samples on glass-fibre discs (see above). The reaction was linear with time and protein concentration (Fig. 9 A & B).

RESULTS

I. CHARACTERISATION OF THE INTERFERON RESPONSE OF VERO CELLS

Most experimental evidence supports the observation of Desmyter et al. (1968) that VERO cells are unable to synthesize interferon. However, Kohno et al. (1972) have published experimental results indicating that some strains may be sensitive to interferon inducers.

It was therefore necessary to test the interferon response of VERO cells before their use in cell hybridisation experiments. The production of interferon by virus-infected or poly(rI). poly(rC)—treated cells was examined and the anti-viral state induced by poly(rI). poly(rC) measured in primate and other cell types.

A. The production of interferon by virus-infected primate cells

Confluent monolayers of VERO, LLC-MK_2 and BSC.B cells in 100mm Petri dishes (approximately 10^7 cells) were infected with Sindbis virus at multiplicities of 5, 0.5 or 0.05 p.f.u. per cell in 1ml of growth medium. Unadsorbed virus was removed after 30 min and 8ml of fresh growth medium added. After 48h the virus-containing medium was harvested and stored at -20 ^{o}C.

The samples were prepared and assayed by dye uptake (Methods) on VERO and HES.A cells. It can be seen that no anti-viral activity was present in the samples from VERO cells (Table 2). LLC-MK_2 and BSC.B cells were able to produce interferon active on VERO and HES.A cells but uninfected cell

Table 2. Dye uptake assay of virus-induced interferon from BSC.B, LLC-MK_2 and VERO cells on VERO and HES.A cells

Cell line	Sindbis virus adsorbed (p.f.u./cell)	Anti-viral effect as % dye uptake [a, b]	
		VERO cells [d]	HES.A cells
VERO	0	1	0
	0.05	1	0
	0.5	1	0
	5	0	0
BSC.B	0	1	0
	0.05	4	24
	0.5	3	64
	5	13	75
LLC-MK_2	0	1	0
	0.05	72	76
	"	-	32 [c]
	0.5	79	74
	"	-	32 [c]
	5	77	68
	"	-	34 [c]

a. Methods

b. 5-fold sample dilution

c. 50-fold sample dilution

d. Human fibroblast interferon (1) had a titre of > 40 units/0.4ml on these cells

samples had no activity. The amount of interferon produced by BSC.B cells was related to the multiplicity of infection of the inducing virus but apparently this was not true for LLC-MK_2 cells. LLC-MK_2 cell interferon was as active on both of the cell types used for the assay but BSC.B cell interferon was more active on HES.A cells. The activity of the LLC-MK_2 preparations may be underestimated because, with the dye uptake assay, 100% protection of cells against virus was not always found, even with the lowest dilutions of interferon preparations. The dose-response curve often reached a plateau at about 80% dye uptake.

A range of dilutions of the LLC-MK_2 and BSC.B cell interferon samples was assayed on HES.A cells to investigate further the effects of the multiplicity of infection of inducing virus and the relative activities of the samples. The procedures used were as above and, for increasing multiplicity of inducing virus, interferon titres of 4, 11 and 25 units per 0.4ml were obtained for BSC.B cell samples and 45, 72 and 87 for samples from LLC-MK_2 cells. The multiplicity of infection of the inducing virus was now seen to be important in determining the yield of both LLC-MK_2 and BSC.B cell interferons. LLC-MK_2 cells produced higher titres of interferon than did BSC.B cells. The plateaux of the interferon dose-response curves were not at 100% dye uptake. This may be due to the presence of cell growth inhibitory factors at the low dilutions used or an effect of interferon itself on cell metabolism. An N.I.H. human leucocyte standard interferon preparation (G-023-901-527) behaved similarly when assayed on HES.A cells (Methods).

B. The induction of an anti-viral state in primate cells

i) Dye uptake assay

The synthetic inducer of interferon, poly(rI). poly(rC), can induce an anti-viral state in cells at concentrations much below those needed to induce interferon (Colby & Morgan, 1971). This effect is thought to be caused by the local production of small amounts of interferon by cells (Introduction) and is a more sensitive measure of a cell's ability to respond to this interferon inducer than is the assay of interferon released into the medium. Thus VERO, BSC.B and LLC-MK_2 cells were tested for their ability to respond with an anti-viral state on treatment with poly(rI). poly(rC) and the protection against virus-induced cytotoxicity was measured by the dye uptake method. The potentiating effect of diethylaminoethyl (DEAE)-dextran (Dianzani et al. 1968) on poly(rI). poly(rC) induction of an anti-viral state was also examined.

Confluent monolayers of cells in glass scintillation vials (approximately 5.10^5 cells) were treated in duplicate with inducer as described (Methods) except that 1.5ml of growth medium were added directly to the vials after the 60 min induction period.

No anti-viral state was induced in VERO cells by poly(rI). poly(rC) alone (Table 3) nor by any combination of poly(rI). poly(rC) and DEAE-dextran tested. BSC.B and LLC-MK_2 cells were not sensitive to treatment with poly(rI). poly(rC) only but did respond to this inducer in the presence of DEAE-dextran. Protection against the cytopathic effect of virus infection was seen in BSC.B and LLC-MK_2 cells treated with DEAE-dextran only. This substance was sometimes found

Table 3. Dye uptake assay of the anti-viral state induced by poly(rI). poly(rC) in BSC.B, LLC-MK_2 and VERO cells

Concentration of inducer		Anti-viral state as % dye uptake [a]				
Poly(rI). poly(rC) (µg/ml)	DEAE-dextran (µg/ml)	VERO	BSC.B		LLC-MK_2	
			Expt.I.	Expt.II	Expt.I.	Expt.II
2	-	-[b]	-	0	-	10
10	-	8	0	7	0	3
50	-	0	0	-	3	-
-	10	3	3	-	0	-
-	50	0	23	27	0	22
-	100	-	-	29	-	19
2	100	-	-	37	-	71
10	10	3	20	-	6	-
10	50	3	77	90	106	97
10	100	-	-	54	-	67

a. Methods b. Not tested

to enhance dye uptake in uninfected cells (not shown) and the protection seen in infected BSC.B and LLC-MK_2 cells was probably not a result of interferon production.

ii) Plaque reduction assay

As no cell line responded with an anti-viral state to poly(rI). poly(rC) treatment alone but only to combinations of poly(rI). poly(rC) and DEAE-dextran the plaque reduction assay was used to clarify the effects of DEAE-dextran on cells. Protamine sulphate (another potentiator of poly(rI). poly(rC) action) was also tested (Billiau et al. 1969).

Confluent monolayers of cells in 50mm Petri dishes (approximately 2.10^6 cells per dish) were treated with 1ml of inducer as described for the plaque assay of interferon inducers (Methods) with Sindbis virus challenge.

In these experiments BSC.B cells responded to treatment with poly(rI). poly(rC) alone (100µg/ml) but VERO cells did not (Table 4). However VERO cells were slightly sensitive to the action of high concentrations of poly(rI). poly(rC) in the presence of DEAE-dextran (or protamine sulphate). The viral plaque size was also diminished by inducer treatment of BSC.B cells. DEAE-dextran reduced plaque number but not plaque size in BSC.B cells. This was an unexpected result since DEAE-dextran is sometimes used to enhance plaque formation. The effect on plaque number but not on size may represent an effect on adsorption of virus and not a longer-lasting intra-cellular effect on virus multiplication. VERO cells were not affected by DEAE-dextran in this way nor did protamine sulphate alone have an effect on plaque formation.

Table 4. Plaque reduction assay of the anti-viral state induced by poly(rI). poly(rC) in BSC.B and VERO cells

Concentration of inducer			% reduction of control plaque numbers			
Poly(rI). poly(rC) (µg/ml)	DEAE-dextran (µg/ml)	Protamine sulphate (µg/ml)	VERO		BSC.B	
			Expt.I.	Expt.II.	Expt.I.	Expt.III.
2	-	-	- [a]	-	-	14
10	-	-	-	-	-	0
50	-	-	0	-	5	-
100	-	-	0	0	30	-
-	50	-	-	-	-	7
-	100	-	0	0	14	14
-	-	20	-	0	-	-
2	50	-	-	-	-	29 [b]
10	50	-	-	-	-	86 [b]
2	100	-	-	-	-	32 [b]
50	100	-	0	-	31	-
100	100	-	0	11	24	-
100	-	20	-	21	-	-

a. Not tested b. Smaller plaques

C. The response to poly(rI). poly(rC) and interferon by VERO and mouse D1 cells

VERO cells are relatively insensitive to synthetic interferon inducers in comparison with other monkey cell lines. To show that this insensitivity is unusual and as a preliminary to cell hybridisation experiments, VERO cells were compared with mouse D1 cells. Duplicate monolayers in 25 well trays (approximately 5.10^5 cells per well) were treated with poly(rI). poly(rC) as described (Methods). Challenge was with EMC virus (0.001 p.f.u. per cell) in Expt. I and with Sindbis virus (1 p.f.u. per cell) in Expt. II and the volume of added interferons was 0.5ml in Expt. I and 1ml in Expt. II. Dye uptake was estimated after 48h incubation at 37 ^{o}C (Methods).

Treatment with poly(rI). poly(rC) alone protected neither cell line against infection with EMC or Sindbis viruses (Table 5). D1 cells were completely protected by treatment with 10µg/ml of poly(rI). poly(rC) in the presence of 100 µg/ml of DEAE-dextran but VERO cells only responded with partial inhibition of EMC virus growth to 100µg/ml poly(rI). poly(rC) and 100µg/ml of DEAE-dextran. In other experiments (not shown) D1 cells showed 50% of control, uninfected cell dye uptake if treated with 2µg/ml poly(rI). poly(rC) in the presence of DEAE-dextran (50µg/ml) before Sindbis virus challenge. Neither cell line was protected against virus infection by DEAE-dextran treatment alone. No interferon activity was detected in the medium collected from Expt. II 24h after induction. (Part of the data for Expt. II is included in Tables 31 & 32 (Section V)).

Importantly for cell hybridisation experiments the cell

Table 5. Comparison of the response to poly(rI). poly(rC) and mouse and human interferons by VERO and D1 cells

Concentration of inducer		Anti-viral state as % dye uptake [a]			
Poly(rI). poly(rC) (μg/ml)	DEAE-dextran (μg/ml)	VERO		D1	
		Expt.I.	Expt.II.	Expt.I.	Expt.II.
10	-	- [b]	0	- [b]	3
100	-	0	0	0	6
-	100	-	0	-	0
10	100	-	0	-	102
100	100	21	7	99	105
Interferon	units/well				
Mouse fibroblast (2)	60	0	-	0	-
" " (1)	12.5	-	1	-	105
Human leucocyte (G-023-901-527)	20	75	-	0	-
" fibroblast (1)	40	-	21	-	0

a. Methods

b. Not tested

lines did not show cross-reactivity with the heterologous interferon. [The lack of response of D1 cells to mouse interferon in Experiment I reflects the insensitivity of EMC virus to the action of mouse interferon (Methods)].

D. The potency of synthetic interferon inducers

The relative insensitivity of the primate and mouse cell lines to the effects of poly(rI). poly(rC) without the potentiating effect of DEAE-dextran might reflect a low potency of the poly(rI). poly(rC) preparation used. Consequently human and chick embryo cells were tested by the dye uptake and plaque reduction assays for sensitivity to the anti-viral action of poly(rI). poly(rC).

1. Human embryo cells

i) Dye uptake assay

Approximately 2.10^5 human embryo cells (HEC) in wells of a 25-well tray were treated in triplicate with 1ml of inducer in maintenance medium. Subsequent treatment was as described (Methods) with Sindbis virus challenge.

Treatment of HEC with poly(rI). poly(rC) alone (10µg/ml) had a significant anti-viral effect (Table 6). In similar experiments with HES.A cells treatment with 2µg/ml of poly(rI). poly(rC) was effective against virus challenge giving 32% of control dye uptake.

Addition of DEAE-dextran or protamine sulphate to poly(rI). poly(rC) potentiated the anti-viral state induced in HEC but protamine sulphate was less effective than DEAE-dextran. A marginal anti-viral effect was seen when HEC were treated with DEAE-dextran or protamine sulphate alone.

In similar experiments with HES.A cells asay of medium

Table 6. Dye uptake assay of the anti-viral state induced by poly(rI). poly(rC) in human embryo cells (HEC)

Concentration of inducer (µg/ml)		Anti-viral state as % dye uptake [a]
Poly(rI). poly(rC)	DEAE-dextran	
1	-	5
10	-	14
100	-	55
-	10	9
1	10	86
10	10	91
	Protamine sulphate	
-	5	0
-	10	0
-	20	5
-	40	10
-	80	10
1	10	26
10	10	37
1	20	26
10	20	74
1	40	21
10	40	58

a. Methods

harvested 24h after induction with 100µg/ml poly(rI). poly (rC) alone or at 10 or 100µg/ml in combination with DEAE-dextran detected anti-viral activity. Five-fold dilutions of the samples gave 48%, 66% and 79% protection respectively of HES.A cells against Sindbis virus challenge. Untreated or DEAE-dextran treated cells produced no anti-viral activity.

HES.A cells were more sensitive to the action of poly(rI). poly(rC) than HEC (eg. treatment with 10µg/ml poly(rI). poly (rC) gave 54% and 89% of control dye uptake with HES.A cells in two experiments whereas only 14% dye uptake was observed with HEC, Table 6). The degree of response to interferon inducers may be determined by differences in genotype within a species. However, the passage levels of the cells used were different (10 for HEC and 16, 17 for HES.A cells) and so the *in vitro* age of the cells may be responsible for the differences observed. Tan, Chou & Lundh (1975) were unable to show differences in the response of human fibroblast cells to human interferon at different *in vitro* passage levels, but as the genes for interferon production and the ability to respond to interferon are on separate chromosomes in man (Tan *et al.* 1974a) their regulation may not be the same.

ii) Plaque reduction assay

Human embryo cells (HEC) in 50mm Petri dishes (approximately 10^6 cells per dish) were treated with 0.5ml of inducer diluted in PBS_A as described for the plaque reduction assay (Methods) and challenge was with Sindbis virus.

Again, treatment with poly(rI). poly(rC) alone (10µg/ml) induced a significant anti-viral state as measured by reduction in plaque numbers (Table 7). DEAE-dextran treatment alone (100µg/ml) also protected cells against virus infection (cf. BSC.B cells, Table 4). The addition of DEAE-

Table 7. Plaque reduction assay of the anti-viral state induced by poly(rI). poly(rC) in HEC

Concentration of inducer (µg/ml)		Anti-viral state as % reduction of control plaque numbers [a]
Poly(rI). poly(rC)	DEAE-dextran	
10	-	63
100	-	84
-	10	8
-	100	31
10	10	21 [b]
100	10	87 [b]

a. Methods

b. Smaller plaques

dextran did not potentiate the reduction in plaque numbers induced by poly(rI). poly(rC) treatment but plaque size was reduced.

The plaque reduction assay appeared to be more sensitive than the dye uptake assay when treatment of cells with poly(rI). poly(rC) alone was considered but this was not so with the addition of DEAE-dextran.

The preparation of poly(rI). poly(rC) used in testing the response of monkey and mouse cell lines was therefore able to induce a significant anti-viral state and anti-viral activity in sensitive cells in the absence of DEAE-dextran.

2. Chick embryo cells (CEC)

Similar experiments with CEC showed that a 94% reduction in plaque numbers resulted from treatment with poly(rI). poly(rC) (1µg/ml) in the presence of DEAE-dextran and 91% of control dye uptake was found after virus challenge of cells treated with poly(rI). poly(rC) (2µg/ml) and DEAE-dextran (50µg/ml). These cells are therefore more sensitive to the anti-viral action of poly(rI). poly(rC) than most monkey and mouse cell lines but appear to require the potentiating action of DEAE-dextran.

Summary

Sindbis virus infection of BSC.B and LLC-MK_2 cells, but not of VERO cells, resulted in the production of anti-viral activity. (Human embryo cells were more sensitive as assay cells than were VERO cells). Treatment of BSC.B cells and human embryo cells with poly(rI). poly(rC) alone induced an anti-viral state (Table 8) and as little as 2µg/ml protected the embryonic cells. In the presence of DEAE-dextran, poly

Table 8. Lowest concentration of interferon inducer giving significant protection against virus infection [a]

Cell line	Concentration of inducer (μg/ml)		Assay method [b]
	Poly(rI). poly(rC)	DEAE-dextran	
VERO	100	100	D.U.; P.R.
BSC.B	100	-	P.R.
"	10	10	D.U.
LLC-MK_2	2	50	D.U.
HEC	10	-	D.U.; P.R.
HES.A	2	-	D.U.
CEC	1	10	P.R.
"	2	50	D.U.
Mouse D1	2	50	D.U.

a. Greater than 10% dye uptake or reduction of control plaque numbers

b. D.U. = dye uptake; P.R. = plaque reduction

(rI). poly(rC) treatment induced an anti-viral state in all cell types tested but VERO cells were only weakly sensitive (Table 8). Anti-viral activity was detected in medium only from human embryo cells after induction with poly(rI). poly(rC) but not all cells were tested. [Later it was shown that D1 but not VERO cells could produce anti-viral activity after treatment with this synthetic interferon inducer (Section V, Table 33)].

Thus VERO cells were unable to produce anti-viral activity (interferon) in response to virus infection or treatment with high concentrations of poly(rI). poly(rC) and only a slight anti-viral state could be induced by the latter. These observations are in agreement with those of Desmyter et al. (1968).

DEAE-dextran at 100µg/ml induced no interferon activity in HES.A cells. However treatment of BSC.B, LLC-MK_2 and chick and human embryonic cells with DEAE-dextran often showed protection against cell damage as measured by dye uptake or reduction in plaque formation. This may have represented non-specific stimulation of dye uptake or an adverse effect on virus adsorption. Paradoxically, treatment of human cells with DEAE-dextran and poly(rI). poly(rC) (10µg/ml) reduced the inhibition of plaque formation found after treatment with poly(rI). poly(rC) alone but plaque size was reduced.

The plaque reduction and dye uptake assays for the anti-viral effects of synthetic interferon inducers were of similar sensitivity. BSC.B cells treated with 10µg/ml of poly(rI). poly(rC) and 50µg/ml DEAE-dextran gave 77% or 90% dye uptake on virus challenge in two experiments and 86% reduction of plaque numbers in a third experiment (but cf. HEC Tables 6, 7).

II. CELLULAR INTERACTION WITH POLY(rI). POLY(rC)

A. The enhancement of the poly(rI). poly(rC)-induced anti-viral state and interferon production

In Results, Section I it was shown that VERO cells were unable to produce interferon whereas BSC.B cells (derived from the same species) were able to do so (Table 2). However, treatment with poly(rI). poly(rC) at 100µg/ml in the presence of 100µg/ml of DEAE-dextran or 20µg/ml of protamine sulphate gave approximately 20% protection of VERO cells against Sindbis virus-induced cell damage or plaque formation (Tables 4 & 5). The anti-viral state induced by poly(rI). poly(rC) in the absence of detectable interferon production is believed to be mediated by the interferon system (Introduction).

It is possible that VERO cells are able to produce small amounts of interferon, sufficient to cause a detectable anti-viral state, in response to poly(rI). poly(rC) treatment. However, other effects of poly(rI). poly(rC) or DEAE-dextran cannot be excluded. Kjeldsberg & Flikke (1971) have shown that polio virus infection of the Detroit-6 human cell line can continue in the presence of 5µg/ml of Actinomycin-D whereas host RNA synthesis is blocked. Treatment of these cells with poly(rI). poly(rC) in addition to Actinomycin-D inhibits the synthesis of infectious polio virus RNA but not of other viral RNAs. This indicates that poly(rI). poly(rC) may be able to inhibit virus replication under conditions where interferon synthesis is unlikely.

Some cell types are partially protected against virus plaque formation or cytopathic effect by treatment with DEAE-dextran alone (Section I, Tables 3 & 4). This does not

seem to be true for VERO cells (Tables 3, 4 & 5).

A number of different treatments have been reported to enhance interferon production induced by poly(rI). poly(rC). These include pretreatment with homologous interferon (Havell & Vilček, 1972) or with poly(rI). poly(rC) (Billiau, Van den Berghe & De Somer, 1972b), often referred to as priming. High yields of interferon have been obtained from rabbit kidney cell cultures and from human fibroblasts by treatment after induction with cycloheximide and Actinomycin-D (Tan et al. 1970; Havell & Vilček, 1972) or by low doses of UV-irradiation of cells before induction (Lindner-Frimmel, 1974; Mozes & Vilček, 1974).

Treatment of VERO cells by the methods described above might possibly enhance the response of these cells to poly (rI). poly(rC) with the production of detectable interferon. This would show that the interferon gene in VERO cells was able to be expressed with the production of an active protein.

1. Priming of BSC.B and VERO cells with human interferon

The priming activity of interferon cannot be separated from its anti-viral action by extensive purification but it does not seem to depend on the development of the anti-viral state (Introduction). VERO cells are able to respond to the anti-viral effects of monkey and human interferons as expected, (Section I, Tables 2 & 5; Bucknall, 1967). It was therefore decided to test whether human interferon pre-treatment of VERO cells could stimulate a response to treatment with poly(rI). poly(rC) with BSC.B cells included as a control.

Confluent monolayers of VERO and BSC.B cells in 100mm Petri dishes (approximately 10^7 cells per dish) were treated with 5ml of human fibroblast interferon (1) at 75 or 37.5

reference units per ml for 24h. This was removed and 1ml per plate of inducer diluted in PBS added [poly(rI). poly(rC) 20µg/ml; DEAE-dextran 50µg/ml]. After 30 min incubation at 37 °C the inducer was removed, the cells washed twice with PBS and 10ml of growth medium added per plate. The plates were incubated at 37 °C for 24h (without development of cytotoxicity), when the medium was harvested for assay of anti-viral activity on BSC.B cells by the dye uptake method.

No anti-viral activity was detected in medium from the interferon-primed, poly(rI). poly(rC)-treated cells nor did poly(rI). poly(rC) treatment alone induce detectable anti-viral activity (Table 9). The resistance of the treated cells to virus infection was not tested.

BSC.B cells are able to produce small amounts of interferon in response to Sindbis virus infection and to develop an anti-viral state when treated with poly(rI). poly(rC) and DEAE-dextran (Section I, Tables 1, 2 & 3). However they are not sufficiently sensitive to the concentration of inducer used in this experiment to respond with interferon production. LLC-MK_2 cells (derived from the Rhesus monkey) are more sensitive to Sindbis virus induction of interferon and priming with monkey interferon of virus induced interferon production has been shown in these cells, though not in VERO cells (Stewart, Gosser & Lockart, 1971a; 1972c).

Desmyter & Stewart (1976) found that monkey cells were less sensitive than human cells to both human and monkey interferons, VERO cells being the least sensitive. BSC.B cells appeared to be as sensitive as human cells to human interferon but VERO cells were less sensitive to both human and monkey interferons (Methods; Section I, Tables 2 & 5).

Table 9 Interferon production by poly(rI). poly(rC)-treated VERO and BSC.B cells primed with human interferon

Human interferon pretreatment (units/ml) a	Poly(rI). poly(rC) + DEAE-dextran treatment b	Sample dilution	Anti-viral effect, as % dye uptake, in BSC.B cells c Samples from:	
			VERO	BSC.B
-	-	4	4	5
-	+	4	0	4
-	+	8	0	5
75	+	4	2	4
75	+	8	6	2
75	+	16	0	1
37.5	+	4	1	2
37.5	+	8	5	1

a. As assayed on HES.A cells (reference units)

b. 20µg/ml poly(rI). poly(rC) + 50µg/ml DEAE-dextran

c. See Methods

Monkey cells may therefore require more interferon for priming than cells which are very sensitive to the anti-viral action of interferon. Assay of the medium from primed and induced monkey cells on human embryo cells rather than on BSC.B cells may have led to the detection of small amounts of interferon if present.

The blocking effect of interferon pretreatment on subsequent interferon production is unlikely to be an explanation of the results in Table 9 as no interferon was produced in response to inducer alone.

The lack of response to priming in VERO cells may not be related to the defect in interferon production in these cells but a property of African Green monkey cells in general as priming was not found in BSC.B cells.

2. Priming with suboptimal concentrations of poly(rI). poly(rC) in human embryo cells

Treatment of human skin fibroblasts for 24h with concentrations of poly(rI). poly(rC) less than 1µg/ml, followed by induction with 50µg/ml poly(rI). poly(rC) for 2h, resulted in increased interferon production over controls receiving only 50µg/ml poly(rI). poly(rC) (Billiau et al. 1972b). Similar results were found in rabbit kidney cell cultures (Billiau, 1970) and in mouse L cells (Margolis, Oie & Levy, 1972). Interferon production occurred earlier in these primed cells (Margolis et al. 1972) and so the effects of poly(rI). poly(rC) priming are analogous to those of interferon pre-treatment (Introduction). It was decided to repeat the experiment of Billiau et al. (1972b) with human embryo cells before testing the effects of poly(rI). poly(rC) pretreatment of monkey cells since human embryo cells were found to be

more sensitive to the anti-viral effects of poly(rI). poly (rC) (Section I, Tables 6 & 7). The anti-viral state induced in the cells was measured rather than interferon production.

Confluent monolayers of human embryo cells (HEC, approximately 2.10^5 cells per well) were treated in triplicate with 1ml per well of poly(rI). poly(rC) diluted in growth medium (0.001 to 10µg/ml). These cells were incubated in the presence of the inducer for 24h at 37 °C.

Medium was removed from the primed cells and the monolayers washed with growth medium. A second induction was then carried out with 10 or 100µg/ml of poly(rI). poly(rC) diluted in PBS at 0.2ml per well for 60 min at 37 °C. This was removed, the cells washed with PBS, and 1ml of growth medium added per well. The monolayers were incubated for 24 h at 37 °C then challenged with Sindbis virus for estimation of the anti-viral state by dye uptake (Methods).

It can be seen (Table 10) that the cells were protected against virus infection by a single treatment with 100µg/ml of poly(rI). poly(rC) but not by 10µg/ml of inducer. The anti-viral response of these cells was not as great as that observed previously (Section I, Table 6).

Pretreatment of the cells with poly(rI). poly(rC) enhanced the anti-viral state induced by a second induction with 100µg/ml of poly(rI). poly(rC) but the maximum effect observed was only an increase in dye uptake of 7% over controls. No effect of pretreatment with inducer on the response to 10µg/ml of poly(rI). poly(rC) was found.

The effect of pretreatment with poly(rI). poly(rC) on a second induction in human embryo cells was not considered sufficiently encouraging and so was not repeated with VERO cells.

Table 10 The anti-viral state induced in human embryo cells pretreated with poly(rI). poly(rC)

Concentration of poly(rI). poly(rC) (µg/ml)		Anti-viral state as % dye uptake a
Priming	Induction	
0	10	3
0	100	30
0.001	10	3
"	100	37
0.01	10	5
"	100	37
0.1	10	5
"	100	34
1	10	0
"	100	33
10	10	5
"	100	33

a. Methods

3. Superinduction with Actinomycin-D and cycloheximide in human embryo cells

The paradoxical effect on interferon production of treatment with inhibitors of RNA and protein synthesis has been widely observed (Tan et al. 1970; Myers & Friedman, 1971; Havell & Vilcek, 1972). This superinduction, resulting in increased interferon yields, has been explained by the differential inhibition of the synthesis of a labile repressor of interferon transcription or translation (Introduction).

It was hoped that this phenomenon could be demonstrated in human embryo cells as these are likely to be more sensitive, and then examined in VERO cells in an attempt to show interferon production by these cells. The time course of treatment chosen was based on that of Havell & Vilcek (1972) for human cell cultures.

Human embryo cells (HEC) in 50mm Petri dishes (approximately 10^6 cells per dish) were treated in duplicate with 0.5ml per plate of poly(rI). poly(rC) at 100µg/ml in PBS_A for 60 min at 37 °C. This was removed, the cells washed once with PBS_A, and 2ml of growth medium containing 10µg/ml of cycloheximide added (controls received growth medium only). At $5\frac{1}{2}$h from the commencement of induction Actinomycin-D was added to the experimental plates in 0.02ml to give a final concentration of 1µg/ml. After 30 min at 37 °C (6h after the commencement of induction) all inhibitors were removed, the cells washed twice with growth medium and 4ml of growth medium added to each plate. After 18h incubation at 37 °C the medium was harvested and assayed for anti-viral activity on HEC by the plaque reduction method, using 2ml of interferon dilution per assay plate and Sindbis virus challenge (Methods).

Medium from cultures having received Actinomycin-D only or cycloheximide and Actinomycin-D enhanced plaque formation in assay monolayers (up to 15%). The results were therefore expressed as percentage reduction of the plaque numbers on monolayers which had received the appropriate control medium.

As shown in Table 11 poly(rI). poly(rC) treatment alone did not induce anti-viral activity in medium from these cells. (Human interferon (1) at 60 units/ml gave 98% inhibition of control plaque numbers in this assay). A slight super-inducing effect of additional treatment with inhibitors was apparent, in particular, anti-viral activity was greatest in samples from cells treated with poly(rI). poly(rC) and Actinomycin-D. Dialysis of interferon samples, in experiments such as this, may be necessary to remove residual inhibitors which may affect interferon action on the assay monolayers.

Batches of human cells may vary in their sensitivity to interferon induction by viruses (Billiau, Joniau & de Somer, 1972a). The human cells used in this experiment were not as sensitive to poly(rI). poly(rC) as those of Havell & Vilcek (1972) and their temporal requirements for inhibitor treatment may also have been different.

Because of the problems of choosing optimal conditions for superinduction in human embryo cells it was decided not to carry out a similar experiment with VERO cells. A negative result might only mean that VERO cells differed widely from human embryo cells in response to the concentration of inhibitors used but in particular in the temporal relationship of inducer and inhibitor treatments.

Table 11 Interferon production by super-induced human embryo cells (HEC)

Cycloheximide a	Actinomycin-D b	Poly(rI). poly(rC) c	Interferon activity as % reduction of control plaque numbers d
-	-	+	0
-	+	+	39
+	-	+	12
+	+	+	17

a. 10µg/ml; 1 to 6h after commencement of induction

b. 1µg/ml; 5½ to 6h " " "

c. 100µg/ml from 0 to 1h

d. 10-fold dilutions of samples

4. The effect of steroid hormone treatment on interferon production and the anti-viral state induced by poly(rI). poly(rC)

Steroid hormones are believed to mediate their effects on target tissues by changes in transcription of certain genes. The hormones are transported via binding proteins to the nucleus where they bind to chromatin (Lewin 1974). Scolnick, Young & Parks (1976) have shown that glucocorticoid hormones can induce increased levels of mouse mammary tumour virus from mouse mammary carcinoma cells in cultures. This action appears to be at the level of transcription of viral RNA. It was considered that steroid hormone treatment of cells might affect inducible processes other than those involved in the normal target cell.

The effects of cortisol and testosterone on poly(rI). poly(rC) induction of an anti-viral state and of interferon production were first tested in human embryo cells (HES.A).

i) Human embryo cells

Human embryo cells (HES.A) in 25 well trays (approximately 2.10^5 cells per well) were treated in duplicate with 0.5ml per well of steroid hormones diluted in growth medium. Immediately 0.05ml per well of poly(rI). poly(rC) was added to give final concentrations of 10 or 100µg/ml. After 2h incubation at 37 ^{o}C inducer and hormones were removed, the cells washed twice with growth medium and 1ml per well of fresh medium added. The medium was harvested after 24h for assay of its anti-viral activity. The monolayers were then challenged with Sindbis virus for estimation of the anti-viral state by dye uptake (Methods).

Treatment with poly(rI). poly(rC) alone at 100µg/ml

(though not 10µg/ml) induced an anti-viral effect (Table 12). Cortisol treatment in addition to poly(rI). poly(rC) enhanced the anti-viral effect only at 4.10^{-8} M and diminished it at 5.10^{-6} M (the latter concentration was cytotoxic). Intermediate concentrations of cortisol had no significant effect.

Testosterone propionate, in addition to inducer (100 µg/ml) enhanced the anti-viral effect (except at 10^{-5} M in Expt. I). The maximum effect observed was an increase in dye uptake of 30% over controls receiving poly(rI). poly(rC) only.

No anti-viral state was found in cells treated with 10 µg/ml of poly(rI). poly(rC) alone. Hormone treatment of such cells did not increase dye uptake after virus challenge and so was not directly anti-viral, or stimulatory for cell metabolism. Slight anti-viral activity was detected in medium from cells treated with 100µg/ml of inducer and 10^{-6} M cortisol or 10^{-7} M testosterone from Expt. I. 10^{-7} and 10^{-8} M testosterone treatment of induced cells in Experiment II also produced slight anti-viral activity. (This anti-viral activity was measured by the dye uptake method on HES.A cells).

It was decided to examine whether the enhancing effects of hormone treatment could also be demonstrated in VERO cells.

ii) <u>VERO cells</u>

Monolayers of VERO cells in 25 well trays (approximately 5.10^5 cells per well) were treated in duplicate with 0.5ml per well of steroid hormones diluted in growth medium for 2h at 37 ^{o}C, as for human embryo cells, but without inducer.

Table 12 The effect of steroid hormone treatment on the anti-viral state induced by poly(rI). poly(rC) in human embryo cells (HES.A)

Cortisol (M)	Testosterone propionate (M)	Anti-viral state as % dye uptake c		
		Expt. I		Expt. II
		a	b	b
-	-	0	31	67
4.10^{-8}	-	- e	-	80
2.10^{-7}	-	0	22	69
1.10^{-6}	-	0	38	69
5.10^{-6} d	-	0	19	39
-	10^{-8}	-	-	87
-	10^{-7}	0	59	95
-	10^{-6}	0	38	97
-	10^{-5}	0	19	90

a. 10µg/ml poly(rI). poly(rC)

b. 100 " "

c. See Methods

d. Toxic in all experiments after 2h

e. Not tested

These cultures were washed twice with PBS_A, then 0.5ml per well of poly(rI). poly(rC) diluted in growth medium, in the presence of 100µg/ml of DEAE-dextran, was added. After 60 min at 37 °C this was removed and the cells washed twice with PBS_A before the addition of fresh growth medium (Expt. I).

In a second experiment duplicate monolayers were treated with hormones and inducer as for human embryo cells. 100 µg/ml of DEAE-dextran was added to half of these cultures in addition to poly(rI). poly(rC) (Expt. II).

All cultures were challenged after 24h with Sindbis virus for estimation of the anti-viral state by dye uptake (Methods).

VERO cells were protected against virus-induced cell damage by treatment with poly(rI). poly(rC) in the presence of DEAE-dextran at 100µg/ml though not by poly(rI). poly(rC) alone (Table 13). This confirms previous observations (Section I, Tables 4 & 5).

No hormone treatment enhanced the effect of poly(rI). poly(rC) and in most cases it was diminished. This lack of an enhancing effect in VERO cells is in accord with results found with a number of cell types in culture where steroid treatment reduced interferon production (Ho, 1973b). However interferon action can be stimulated by cortisol or testosterone under certain conditions (De Maeyer & De Maeyer, 1963) and as the anti-viral state induced by poly(rI). poly(rC) seems to result from interferon action this may explain the results with human embryo cells.

Table 13 The effect of steroid hormone treatment on the anti-viral state induced by poly(rI). poly(rC) in VERO cells

Concentration of steroid hormone		Anti-viral state as % dye uptake [a]			
Cortisol (M)	Testosterone propionate (M)	Expt. I		Expt. II	
		b	c	b	c
-	-	0	18	2	28
4.10^{-8}	-	-	-	1	10
2.10^{-7}	-	0	15	2	13
1.10^{-6}	-	1	16	1	9
5.10^{-6} [d]	-	1	13	0	13
	10^{-8}	-	-	0	11
	10^{-7}	0	18	1	8
	10^{-6}	3	18	3	14
	10^{-5}	1	18	3	4

a. Methods

b. Inducer concentration - 100µg/ml poly(rI). poly(rC)

c. " " " " "

with 100µg/ml DEAE-dextran

d. Toxic to cells

B. The cytotoxicity of poly(rI). poly(rC) for cells pretreated with homologous interferon

The inability of VERO cells to synthesize interferon after treatment with synthetic interferon inducers (Section V Table 33) may be a result of a failure of the inducer to interact efficiently with the cells; perhaps at the cell membrane. De Clercq & De Somer (1973) have shown that radioactive, cell-associated poly(rI). poly(rC) remained accessible to exogenous ribonuclease action for a shorter time in VERO cells than in responsive cells eg. human fibroblasts or mouse L929 cells. Cell-associated, radioactive poly(rI). poly(rC) was also more extensively degraded by VERO cells than by L929 cells (De Clercq & Stewart, 1974). These results indicate a difference in the fate of poly(rI). poly (rC) when associated with VERO cells than when associated with cells able to produce interferon.

Stewart et al. (1972a) found that interferon treatment of L929, mouse embryo or human embryo cells, followed by exposure to poly(rI). poly(rC) resulted in a marked cytotoxic effect. This was not unexpected as the interferon inducing ability of polynucleotides is related to their toxicity (Niblack & McCreary, 1971) and interferon pretreatment of cells can enhance their sensitivity to poly(rI). poly(rC) induction of interferon synthesis (Stewart et al. 1971a). Subsequent work has shown that the toxicity after interferon treatment only occurs if double-stranded ribonucleic acids are added (Stewart, De Clercq & De Somer, 1973a). The degree of cytotoxicity after interferon and poly(rI). poly(rC) treatment is related to the size of the poly(rI). strand of the synthetic inducer (Stewart & De Clercq, 1974) as is interferon production (Tytell et al. 1970).

However, the cytotoxic effect of interferon and poly (rI). poly(rC) treatment is not prevented by Actinomycin-D or cycloheximide unlike the production of interferon (De Clercq & De Somer, 1975). The latter authors postulated an interaction of poly(rI). poly(rC) with cells leading indirectly to interferon production and more directly to the toxic effect. It may therefore be possible for cells to exhibit the cytotoxic effect but not interferon production.

The response of VERO cells to treatment with interferon and poly(rI). poly(rC) might show at what level the response to inducers is blocked in these cells.

It was decided initially to attempt to demonstrate the cytotoxicity of poly(rI). poly(rC) in interferon-treated chick and human embryo cells.

1. Chick embryo cells

Confluent monolayers of chick embryo cells in glass scintillation vials (approximately 5.10^6 cells) were treated in duplicate with dilutions of chick interferon (C1; 1ml) in growth medium. After 24h at 37 °C this was removed and 0.5ml per vial of poly(rI). poly(rC) diluted in PBS added. 1.5ml of growth medium were added to the vials after 60 min without removal of the synthetic inducer. Cytotoxicity was measured by dye uptake (Methods) after 24h incubation at 37 °C.

A cytotoxic effect of interferon and poly(rI). poly(rC) treatment was observed in one experiment with relatively low amounts of both substances (Table 14). However, attempts to reproduce this effect were of limited success, even with increased amounts of chick interferon (25 units/vial) and poly(rI). poly(rC) (50µg/ml). The chick interferon used was

Table 14 The cytotoxicity of poly(rI). poly(rC) for chick embryo cells pretreated with chick interferon

Interferon (units/vial)	% dye uptake		
	Poly(rI). poly(rC) concentration (µg/ml)		
	0	2	10
0	100	100	84
1	95	82	77
3	100	74	65
5	101	79	59
10	94	73	56

biologically active as shown by its anti-viral action against Sindbis virus challenge in parallel cultures.

A marked cytotoxic effect of treatment with poly(rI). poly(rC) alone (10µg/ml) was found in another experiment. Low concentrations of interferon (3 units/vial) protected cells against this effect whereas higher concentrations enhanced it. This is analogous to the priming and blocking effects of different concentrations of interferon (Introduction).

A similar protection against the toxic effects of poly (rI). poly(rC) was found after interferon treatment of cells for 4h at 4 °C or 37 °C. In this experiment cells were pretreated with 5 or 10 units per vial of interferon followed by washing twice with Hanks' BSS. Duplicate cultures were then treated with 50µg/ml of poly(rI). poly(rC) as before, to determine toxicity, or challenged with Sindbis virus for estimation of the anti-viral effect of the interferon treatment by dye uptake (Methods).

Treatment with poly(rI). poly(rC) alone gave greater than 50% reduction in dye uptake and interferon pretreatment protected cells against this toxicity at both temperatures. The anti-viral effect of interferon was considerably diminished at 4 °C. Therefore the protective effect of the interferon preparation was not related to its anti-viral effect.

A partially purified chick interferon preparation (C2; 11,000 MRC reference units per mg protein) was used for a further study. Procedures were as previously described except that the cells were washed with Hanks' BSS, before addition of poly(rI). poly(rC). A reduction in dye uptake of only 20% of control values was found when chick cells were

Table 15 The cytotoxicity of poly(rI). poly(rC) for chick embryo cells pretreated with partially purified chick interferon

Interferon (MRC reference units/vial)	% dye uptake		
	poly(rI). poly(rC) concentration (µg/ml)		
	0	10	50
0	100	102	102
5	102	106	102
25	106	102	94
125	106	96	80

treated with 125 units per vial of chick interferon (C2) followed by 50µg/ml poly(rI). poly(rC) (Table 15). Treatment with poly(rI). poly(rC) alone was not toxic in this experiment.

It was concluded that chick embryo cells are relatively insensitive to the cytotoxic effects of treatment with interferon and poly(rI). poly(rC).

2. Human embryo cells

Stewart et al. (1972a) had observed a cytotoxic effect in human interferon treated human cells exposed to poly(rI). poly(rC). It was decided to attempt to reproduce this effect with human embryo fibroblasts.

Confluent monolayers of human embryo cells (HES.A) were treated in duplicate with human interferon as for the chick embryo cells. However poly(rI). poly(rC) treatment was for 60 min only, the inducer was removed and the cells washed with PBS, before 2ml of fresh growth medium were added per vial. Cytotoxicity was measured by dye uptake (Methods) after 72h (Expt. I) or 48h (Expt. II) incubation at 37 ^{o}C. Parallel cultures were treated with interferon or poly(rI). poly(rC) only and challenged with Sindbis virus for estimation of the anti-viral state by dye uptake (Methods).

No cytotoxic effect was observed in two experiments even with 75 units per vial of interferon and 50µg/ml of poly(rI). poly(rC). The concentrations of poly(rI). poly (rC) tested (2, 10 and 50µg/ml) and of interferon (30 and 60 units/vial) induced an anti-viral state and so these preparations were biologically active.

Human embryo cells do not appear to be particularly susceptible to the cytotoxicity of poly(rI). poly(rC) and interferon.

3. VERO, LLC-MK_2 and BSC.B cells

Confluent VERO, LLC-MK_2 or BSC.B cells in 30mm Petri dishes (approximately 10^6 cells) were treated in duplicate with 1ml of LLC-MK_2 cell interferon diluted in growth medium (2 units/ml) for 24h. Interferon was removed, the cell monolayers washed twice with PBS and 0.5ml of poly(rI). poly(rC) diluted in DME at 50µg/ml were added per plate. Control plates received 'mock' interferon or poly(rI). poly (rC) treatments. The monolayers were examined for cytotoxicity but none was observed in VERO and BSC.B monolayers after 48h (cf. priming of BSC.B and VERO cells with human interferon). After 72h all plates showed degeneration of the cell monolayers. LLC-MK_2 cells showed cytotoxicity in all plates after 48h. The lack of serum was probably responsible for this degeneration at 48 or 72h after treatment. No differential effect of interferon and poly(rI). poly(rC) treatment was found in any of the cultures.

The amount of interferon added was comparable with that used by Stewart *et al.* (1972a) in human embryo cells. However these authors found that human embryo cells required higher doses of poly(rI). poly(rC) than did L929 cells before the cytotoxic effect became pronounced. Continuous lines of primate cells may be even less sensitive and require larger amounts of both interferon and poly(rI). poly(rC) before cytotoxicity is seen.

An experiment similar to those described for human embryo cells was carried out with LLC-MK_2 monkey cells, using 30 or 60 units of human interferon for pretreatment and up to 50µg/ml of poly(rI). poly(rC). No cytotoxicity was observed after 48h.

4. Mouse L929 cells

To test that the methods used for cytotoxicity experiments were effective an experiment was carried out with mouse L929 cells in the same manner as described for human embryo cells. Monolayers were stained with neutral red (Methods) after 24h.

A cytotoxic effect was observed with as little as 2 µg/ml poly(rI). poly(rC) after treatment with 13 or 63 units of mouse interferon (1) (Table 16). After treatment with 10 µg/ml of poly(rI). poly(rC) and 63 units of interferon the cytotoxic effect was greater than 50%. These results are comparable with those of Stewart et al. (1972a).

It seems likely that L929 cells are unusual in their sensitivity to treatment with interferon and poly(rI). poly (rC). These cells are also sensitive to lysis by vaccinia virus if pre-treated with interferon. This effect is prevented by Actinomycin-D or cycloheximide treatment of the cells or heat treatment of the virus, (Stewart et al. 1973a) and is probably caused by the production of dsRNA during virus infection.

The cytotoxic effect of interferon and poly(rI). poly (rC) treatment of cells was not considered sufficiently reproducible or universal to give information on the interaction of synthetic inducers with VERO cells. This cytotoxicity effect may however be useful for the isolation of resistant L929 cells. Such mutants might be insensitive to interferon or to poly(rI). poly(rC) and possibly lacking in surface receptors for these molecules. Depending on the mechanism of action of the cytotoxic effect, it might be possible to isolate mutants insensitive to the anti-viral action of poly(rI). poly(rC) comparable with VERO cells.

Table 16 The cytotoxicity of poly(rI). poly(rC) treatment of mouse L929 cells pretreated with mouse interferon

Interferon (units/vial)	% dye uptake		
	poly(rI). poly(rC) concentration (µg/ml)		
	0	2	10
0	100	111	101
3	96	93	75
13	101	86	63
63	84	66	49

Summary

VERO and BSC.B cells were not primed in their response to poly(rI). poly(rC) by human interferon pretreatment under the conditions used.

A slight enhancement of the anti-viral effect induced in human embryo cells by poly(rI). poly(rC) was obtained if the cells were pretreated with suboptimal concentrations of inducer for 24h. Superinduction of interferon production in response to poly(rI). poly(rC) was also only slight in these cells. However steroid hormone treatment of human embryo cells enhanced the anti-viral action of poly(rI). poly (rC). This effect was not found with VERO cells.

No interferon production by VERO cells was observed nor was the anti-viral action of poly(rI). poly(rC) in these cells enhanced by treatments designed to do this.

The cytotoxic effect of interferon and poly(rI). poly (rC) treatment of cells was observed in L929 cells and to some extent in chick embryo cells. Human embryo cells and monkey cells, including VERO, were not sensitive to this effect. No information on the interaction of synthetic inducers with VERO cells as compared with other primate cells could therefore be obtained.

III. SELECTION OF VERO CELLS SENSITIVE TO THE ANTI-VIRAL ACTION OF POLY(rI). POLY(rC)

VERO cells are not able to synthesize interferon and respond poorly to the anti-viral action of poly(rI). poly(rC) in the presence of DEAE-dextran even after treatments designed to stimulate this response (Sections I, II & V).

The lack of interferon production by VERO cells may be caused by a defective interferon gene coding for an inactive protein or by the deletion of the gene in these cells. However a more interesting situation as regards the regulation of induced protein synthesis would be that VERO cells synthesize constitutively a super-repressor of interferon synthesis which is insensitive to interferon inducers.

If such a super-repressor is made by VERO cells it should be possible to isolate revertants of these cells with a less active repressor by suitable selection procedures. After treatment of a population of cells with poly(rI). poly(rC), those sensitive to its anti-viral action should be protected against virus infection for a time. The use of a temperature-sensitive virus mutant would enable the selection to be limited to one cycle of virus growth at the permissive temperature. Transfer of the cells to the non-permissive temperature before the poly(rI). poly(rC) induced anti-viral effect diminishes (with time or after cell division) would prevent further virus replication.

A. Selection

Approximately 2.10^7 VERO cells were treated with 2µg/ml of N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) in growth

medium for 24h at 37 °C. (This gave approximately 50% cell killing). The mutagen was removed and the cells washed twice with PBS_A. Fresh growth medium was added and the cells incubated for 6 days at 37 °C to allow growth of survivors and expression of mutations.

The cells were then seeded into small, plastic tissue culture bottles (Nunclon 20 cm^2) and allowed to form confluent monolayers (approximately 2.10^6 cells per bottle) during a further 7 days at 37 °C.

Inducer treatment was with 100 µg/ml of poly(rI). poly (rC) in growth medium at 2ml per bottle or 100µg/ml of poly (rI). poly(rC) in the presence of 20µg/ml protamine sulphate or 20µg/ml protamine sulphate alone, also at 2ml per bottle. Five bottles were used for each separate inducer treatment, a total of 10^7 cells for each inducer. After 24h incubation at 37 °C inducers were removed and 1ml per bottle of a temperature-sensitive (RNA^-) mutant of Sindbis virus, (ts15, Burge & Pfefferkorn, 1966) added at 20 p.f.u. per cell (as assayed at 30 °C on chick embryo cells). This virus is unable to grow at 37 °C on chick embryo cells. After 30 min adsorption at 37 °C residual virus was removed and growth medium replaced. The bottles of cells were transferred to a 30 °C hot room. Uninfected, and untreated, virus-infected cultures were also incubated at 30 °C to test the effects of lower temperatures on the cells and to determine the time necessary for viral cytopathic effect to develop.

After 72h at 30 °C cell damage was apparent in all cultures except uninfected controls. The growth medium was therefore replaced and the bottles of cells transferred to 37 °C.

Surviving cells were allowed to grow to confluence with fresh growth medium added as necessary and replicate cultures, having received the same inducer treatment, were pooled. VERO cells having received treatment with 100μg/ml poly(rI). poly(rC), VERO(IC), grew best and were used for most subsequent experiments. Populations of cells having received treatment with poly(rI). poly(rC) in the presence of protamine sulphate VERO(IC, PS), or with protamine sulphate alone, VERO (PS), did not grow well and were only tested for the presence of interferon activity or of infectious virus in their used growth medium.

B. The nature of the resistance of VERO(IC) cells to selection

An attempt to demonstrate, by the dye uptake method, the development of an anti-viral state after treatment of these cells with poly(rI). poly(rC) failed because virus did not give rise to a cytopathic effect in the infected, untreated control cell monolayers. Consequently the ability of these cells to support virus growth was compared with that of VERO cells at differing multiplicities of virus infection.

1. The yield of Sindbis virus from VERO and VERO(IC) cells

Confluent monolayers of cells in 50mm Petri dishes (approximately 2.10^6 cells per dish) were infected with 0.5 ml per plate, in quadruplicate, of wild-type Sindbis virus in growth medium at 10, 1 or 0.1 p.f.u. per cell. After 60 min adsorption of virus at 37 ^{o}C residual virus was removed and 5ml of growth medium added per dish. After 24h incubation at 37 ^{o}C cytopathic effect was recorded and medium harvested and pooled from 2 out of each 4 quadruplicate dishes. The virus yield from each cell type was determined by plaque assay

Table 17. Yield of Sindbis virus from VERO and VERO(IC) cells

Virus adsorbed	Cytopathic effect		Virus titre on VERO cells (p.f.u./ml)	
p.f.u. per cell	VERO	VERO(IC)	VERO	VERO(IC)
10	+++a	+++(+)	$6.5.10^8$	8.10^8
1	++(+)	++(+)	$1.2.10^9$	$1.4.10^9$
0.1	+	+(++)	8.10^8	1.10^9

a. + = 25% cell damage; ++ = 50%; +++ = 75%; ++++ = 100%. Brackets represent a different degree of killing in the 4 Petri dishes

on VERO cells (Methods).

The results of these assays are shown in Table 17. The virus titres were similar but with virus possibly growing slightly better on VERO(IC) cells. Thus, the VERO(IC) cells had not survived the selection procedure because of non-specific virus resistance or insensitivity to viral cytopathic effect.

2. Assay of temperature-sensitive Sindbis virus (ts15) on VERO and VERO(IC) cells

The possibility remained that VERO(IC) cells were resistant to ts15 Sindbis virus at 30 °C (the permissive temperature). A stock preparation of ts15 Sindbis virus was assayed by plaque formation on VERO and VERO(IC) cells and the titres obtained were 6.10^7 p.f.u. per ml at 30 °C for VERO(IC) cells and $5.5.10^7$ p.f.u. per ml for VERO cells at the same temperature. At 37 °C small plaques were found with the two cell types and this may have been a result of insufficiently accurate temperature control of incubation allowing virus growth.

VERO and VERO(IC) cells did not differ significantly in their ability to support plaque formation by ts15 Sindbis virus at 30 °C. Thus the survival of VERO(IC) cells during selection with poly(rI). poly(rC) and ts15 Sindbis virus was not because of resistance to the selecting virus alone. A second attempt to detect an anti-viral action of poly(rI). poly(rC) in these cells was therefore carried out by the plaque reduction method.

3. Plaque reduction assay of the anti-viral action of poly (rI). poly(rC) in VERO and VERO(IC) cells

Monolayers of cells in 50mm Petri dishes were treated for 60 min at 37 °C with 1ml per plate (in duplicate) of poly

(rI). poly(rC) at 100µg/ml in PBS_A alone or in the presence of 100µg/ml DEAE-dextran or 20µg/ml of protamine sulphate. This was removed, the cells washed with growth medium and fresh medium added. After 24h at 37 °C cell monolayers were challenged with 100 p.f.u. per dish of Sindbis virus and subsequent treatment was as for the plaque reduction assay of interferon inducers (Methods).

VERO(IC) cells appeared to be slightly sensitive to treatment with poly(rI). poly(rC) alone (Table 18) but this result was tentative because the plaque numbers of the two plates differed more than was usual. VERO cells were sensitive to the action of poly(rI). poly(rC) in combination with DEAE-dextran or protamine sulphate; VERO(IC) cells only responded if protamine sulphate and poly(rI). poly(rC) treatment was given. No anti-viral state was found with VERO(IC) cells which was significantly greater than that which could be induced in VERO cells. DEAE-dextran or protamine sulphate treatments were ineffective against virus challenge on both cell types. (The results for VERO cells are also included in Table 4, Section I).

4. <u>The production of interferon or infectious virus by the selected cell populations</u>

It was possible that the VERO(IC); VERO(IC, PS) and VERO(PS) cell populations had survived the selection procedure by establishing persistent, non-cytopathic virus infections or by the constitutive production of interferon. However assays of used medium from non-induced cell populations for interferon activity on HES.A cells were negative, as were assays for infectious virus on chick embryo cells at 30 °C and 37 °C.

Table 18. Assay of the anti-viral state induced by poly(rI). poly(rC) in VERO and VERO(IC) cells by the plaque reduction method

Concentration of inducer (µg/ml)			Anti-viral state as % reduction of control plaque numbers	
Poly(rI). poly(rC)	DEAE-dextran	Protamine sulphate	VERO	VERO(IC)
100	-	-	0	13
100	100	-	11	0
100	-	20	21	25
-	100	-	0	4
-	-	20	0	7

The selection of cells resistant to temperature-sensitive virus challenge after treatment with an interferon inducer raises a number of problems. Firstly, it must be possible to eliminate virus growth completely on transfer to non-permissive conditions and it may be necessary to add virus-specific anti-serum. Secondly, the virus used may be leaky for temperature-sensitivity on some host cells.

The anti-viral state induced in cells by poly(rI). poly (rC) is not permanent and, to avoid selection of unwanted virus-resistant cells, virus selection has to be for a limited period only. Repeated cycles of selection with virus, after inducer treatment, may be necessary to eliminate cells which have escaped infection by chance, or by their response to the action of interferon released from inducer-sensitive revertants. The addition of too high multiplicities of virus may destroy cells weakly sensitive to the anti-viral action of inducers.

The number of cells selected should be as large as is practicable and it is preferable to grow cells up from individual clones so that interesting properties of cells are not obscured by the response of a mixed population of cells. The number of cells selected in the experiment described (10^7 for treatment with poly(rI). poly(rC) alone) was not unreasonable in that the spontaneous mutation rate to ouabain resistance in VERO cells is $2.3.10^{-6}$ (Section IV) and 5 or more mutant clones might have been expected if all genes are able to be mutated at similar rates in these cells.

Summary

Mutagenized VERO cells, treated with poly(rI). poly(rC) were challenged with temperature-sensitive Sindbis virus to select for those which had developed an anti-viral state. Cells surviving this selection procedure were tested for their ability to support Sindbis virus growth, sensitivity to the anti-viral action of poly(rI). poly(rC) and the production of interferon or virus. No significant difference was found between VERO cells and cells surviving the selection procedure. Thus VERO cells appear to be unable to give rise to revertants sensitive to the action of interferon inducers.

IV. THE SELECTION OF SOMATIC CELL HYBRIDS OF VERO AND D1 CELLS

As shown above (Sections I-III) VERO cells are relatively insensitive to the anti-viral action of the synthetic interferon inducer poly(rI). poly(rC) even after treatment designed to enhance this action.

There are several possible reasons for the inability of VERO cells to make a detectable interferon. The gene coding for the interferon polypeptide may be deleted in VERO cells or mutated such that a defective protein is made. Mutation in a regulatory site, comparable with bacterial promoter or operator sites (Paul, 1975) could prevent transcription of the interferon gene. The presence of a repressor of interferon gene transcription or translation, insensitive to the action of inducer molecules, would also result in a lack of interferon production. This possibility is the most interesting for studies of the regulation of gene expression in hybrid cells. VERO cells may be better able to degrade inducer molecules than other cells or may lack a receptor site for such molecules. Finally, the processing of interferon by glycosylation or its secretion might be ineffective in VERO cells.

The production, by VERO cells, of a defective interferon molecule, able to compete with monkey interferon for binding sites, is unlikely. Desmyter *et al.* (1968) found no interference with the activity of a monkey interferon preparation when medium from induced VERO cells was present. Desmyter & Stewart (1976) found that human interferon, normally active on both human and cat cells, could be modified so as to be

active only on cat cells. No activity was found when medium from induced VERO cells was tested on cat cells and so the production of an aberrant interferon molecule by VERO cells is unlikely.

Mouse and monkey mRNAs from induced cells, could be translated in VERO cells and active mouse or monkey interferon was secreted (De Maeyer-Guignard et al. 1972; Kronenberg & Friedmann, 1975). This observation makes the presence of a post-transcriptional repressor in VERO cells unlikely, unless this acts only intraspecifically or is compartmentalized within the cell. That VERO cells can secrete, and if necessary glycosylate an interferon is also shown by these studies.

The degradation of synthetic inducers by VERO cells may explain their lack of sensitivity to the anti-viral action of these inducers (Section IIB). However several different viruses can replicate well in VERO cells (Macfarlane & Sommerville, 1969) and presumably their nucleic acids are not adversely affected by nuclease activity.

The most likely site for the defect in interferon production in VERO cells therefore seems to be at the level of the interferon gene itself, or at a related regulatory site on the DNA, or a mutation in the gene for a repressor of transcription.

Degradation of inducers cannot be completely ruled out as a mechanism for the inability of VERO cells to make interferon. However, it should be possible, with excess of inducer, to overcome such a defect. This has not been found as far as production of interferon is concerned although a slight anti-viral state can be attained after treatment of cells with high concentrations of poly(rI). poly(rC) in the

presence of DEAE-dextran (Section I, Tables 4 & 5). Because of the encouraging results obtained with the interferon system in cell hybrids (Introduction) it was decided to hybridise VERO cells with mouse cells which were able to synthesize interferon. The production of monkey interferon by a hybrid clone would eliminate the possibility that the VERO interferon gene or a regulatory site on the DNA was inactive because of mutation or deletion.

The finding of hybrid clones which were unable to produce mouse interferon (or which produced much less than the parental cells) would indicate the presence of VERO chromosomes causing repression of mouse interferon synthesis or resistance to inducer. These clones should also be insensitive to the anti-viral action of synthetic inducers. Because primate chromosomes are usually lost from mouse-primate hybrid cells (Weiss & Green, 1967; Cassingena et al. 1971; Brumback, 1973) it is unlikely that lack of mouse interferon production would be caused by mouse chromosome loss. Some sub-clones of hybrid cells unable to make mouse interferon might be expected to regain this ability after further loss of VERO chromosomes.

Hybrid clones able to produce only mouse interferon would be found if the VERO structural gene or a regulatory gene was defective or if regulation was species-specific. The same result would be obtained if the relevant VERO chromosomes were preferentially lost in hybrids, possibly because of the selective system used.

The sensitivity of cells to the anti-viral action of interferon is not linked to interferon production (at least in primates) (Tan et al. 1973; Tan et al. 1974a; Cassingena

et al. 1971). Thus any hybrid clone could be sensitive to either mouse or monkey interferons or to both.

Cell hybridisation studies are potentially able to show if VERO cells have a functional interferon gene or are able to repress synthesis of interferon by a heterologous genome.

A. Selective systems for the isolation of hybrids of VERO and D1 cells

The HAT selection system (Littlefield, 1964a) is widely used in the isolation of somatic cell hybrids. Parental cells are chosen lacking the enzymes thymidine kinase (TK) or hypoxanthine-guanine phosphoribosyltransferase (HGPRT). These enzymes are required for normal growth when the de novo synthesis of thymidylic acid (TMP) or inosinic acid (IMP) is prevented by the presence of the folate analogue aminopterin (Figs. 10 & 11). HAT medium (Methods) contains glycine in addition to hypoxanthine, aminopterin and thymidine because the conversion of serine to glycine by serine hydroxymethyltransferase is also inhibited by aminopterin.

Mouse D1 cells, selected for the absence of TK from mouse L cells, and unable to grow in HAT medium were available (Dubbs & Kit, 1964) as were $HGPRT^-$ mouse cells (c1A9of mouse L cells; Littlefield, 1964b). No mutants of VERO, or other monkey cells, lacking TK or HGPRT had been isolated (Sell & Krooth, 1972).

Mutant cells lacking HGPRT have been selected in the presence of the purine analogues 6-thioguanine (6TG) or 8-azaguanine (Thompson & Baker, 1973). The toxicity of 6TG seems to be a result of its incorporation into the DNA of cells (Nelson et al. 1975). Mutants with mechanisms of

Figure 10 HAT selection : thymidine biosynthesis

In the presence of aminopterin the production of thymidylic acid (dTMP) from deoxyuridylic acid (dUMP) catalysed by thymidylate synthetase (2) is prevented because of limiting amounts of folate derivatives. Cell growth depends on the formation of dTMP from thymidine by the salvage pathway catalysed by thymidine kinase (1).

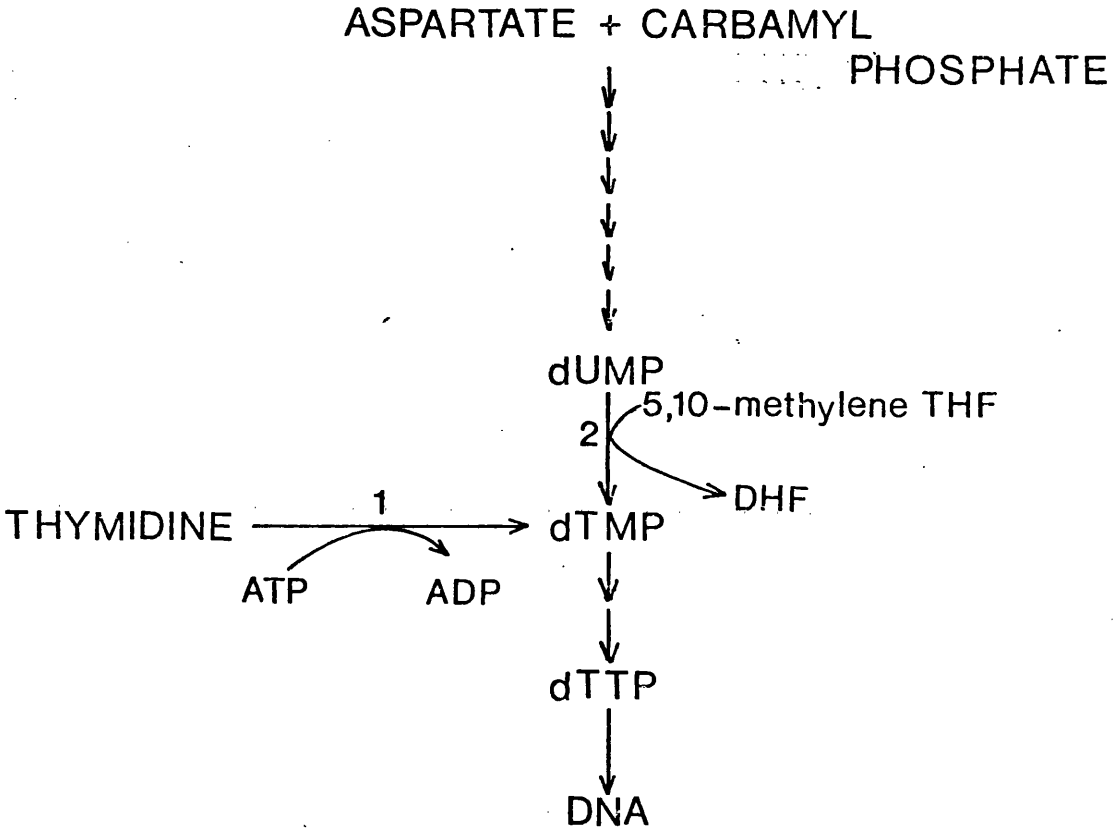
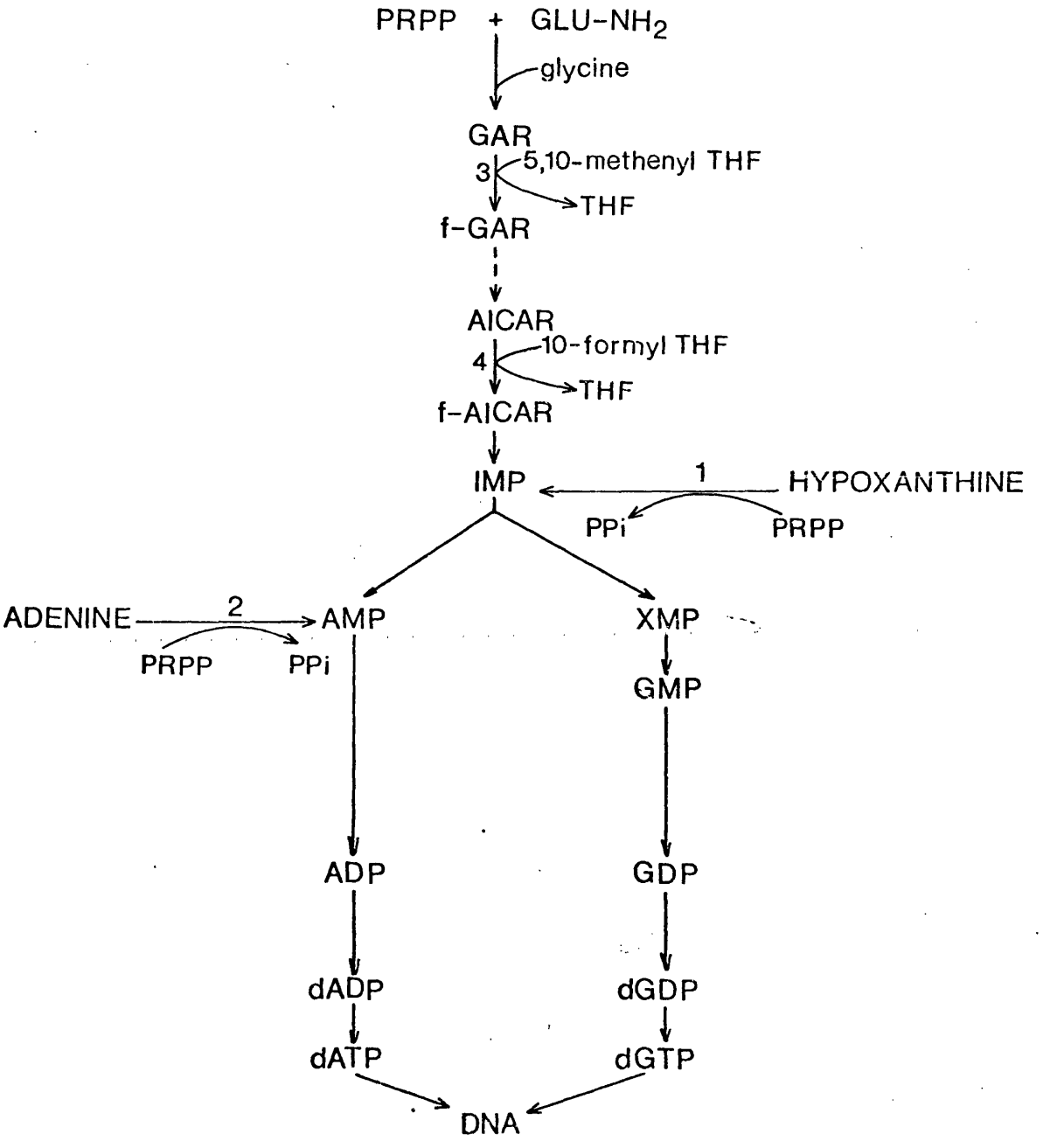

ASPARTATE + CARBAMYL PHOSPHATE
dUMP
5,10-methylene THF
2
DHF
THYMIDINE
1
dTMP
ATP
ADP
dTTP
DNA

Figure 11 HAT selection : purine biosynthesis

Formylation reactions catalysed by phosphoribosylglycinamide formyl transferase (3) and phosphoribosylaminoimidazole carboxamide formyl transferase (4) are inhibited in the presence of aminopterin because of limiting amounts of folate derivatives. The activities of the hypoxanthine and adenine salvage enzymes, HGPRT (1) and adenine phosphoribosyl transferase (2), are essential for cell growth.


PRPP + GLU-NH2
glycine
GAR
3
5,10-methenyl THF
THF
f-GAR
AICAR
4
10-formyl THF
THF
f-AICAR
IMP
1
HYPOXANTHINE
PPi
PRPP
ADENINE
2
AMP
PRPP
PPi
XMP
GMP
ADP
GDP
dADP
dGDP
dATP
dGTP
DNA

resistance to 6TG other than complete loss of HGPRT activity have been isolated (Gillin et al. 1972).

Cells resistant to the pyrimidine analogue 5-bromo-2'-deoxyuridine (BUdR) and lacking TK, have also been isolated (Kit et al. 1963). Growth of cells in BUdR results in an incorporation of this analogue into DNA with chromosomal aberrations occurring because of the sensitisation of the cells to shortwave visible light (Thompson & Baker, 1973). The selection of cells lacking TK is more difficult than that of $HGPRT^-$ cells because a step-wise accumulation of mutations seems to be necessary (Freed & Mezger-Freed, 1973; Roufa, Sadow & Caskey, 1973). Other than those lacking TK, mutants include transport defective and BUdR-dependent cell lines (Davidson & Bick, 1973).

The mutation rates found in animal cells in culture (10^{-4} to 10^{-8} mutations per cell per generation) are higher than those found in bacteria although bacteria are haploid (Thompson & Baker, 1973). The frequency with which mutants, resistant to various agents, arise has not always been found to vary as expected with the ploidy of the cells tested (Harris, 1971; Mezger-Freed, 1972) and the true mutational nature of variants of cultured cells has been questioned (Thompson & Baker, 1973).

A number of criteria must be satisfied before BUdR or 6TG-resistant cell variants can be used for hybridisation studies. These are, the retention of the phenotype in the absence of selection, loss of TK or HGPRT activity, sensitivity to HAT medium, and low reversion rate in HAT medium after treatment to enhance cell fusion.

Practical problems complicate the isolation of suitable mutants for cell hybridisation studies. Metabolic co-operation (Subak-Sharpe, Burk & Pitts, 1969) can render cells sensitive to 6TG or BUdR by the transfer of toxic substances from sensitive to resistant cells at high cell densities.

The relatively low frequency of mutant cells in a population necessitates seeding cells at fairly high densities in selective medium. The low plating efficiency of monkey cells (5% for 10^2 VERO cells in normal growth medium in 50mm Petri dishes) also requires that cells are seeded at high densities.

It was decided to attempt to isolate mutant cells by mutagen treatment of monkey cells followed by mass selection of populations with increasing concentrations of 6TG or BUdR. Resistant cell populations would then be cloned and tested for HGPRT or TK activity and sensitivity to HAT medium.

1. <u>The isolation of populations of monkey cells resistant to 6TG or BUdR</u>

Mutagen treatment of cells [eg. by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or by ethyl methane sulphonate (EMS)] increases the frequency of mutation under various selective conditions (Thompson & Baker, 1973). It is most effective if treated cells are allowed to grow for a few generations so that variant phenotypes may be expressed.

Usually mutagen toxicity is quantitated in terms of percentage inhibition of plating efficiency. A treatment is chosen giving 30% cell killing if multiple mutations are to be avoided or up to 90% cell killing if maximal mutant production is required. Because of the low plating efficiencies

of monkey cells toxicity was estimated from the killing of cell populations.

i) The toxicity of MNNG for monkey cells

VERO, BSC.B and LLC-MK_2 cells in 100mm Petri dishes were treated with 0-10µg/ml of MNNG in PBS for 2h. The mutagen was removed, the cells washed twice with PBS_A, and fresh growth medium added. After 24h at 37 ^{o}C the cells were examined for cytotoxicity. It was found that treatment with 2µg/ml of MNNG gave approximately 50% cell killing.

VERO and LLC-MK_2 cells showed greater cell killing if treated with 2µg/ml of MNNG and subcultured in growth medium for 9 days. LLC-MK_2 cell numbers were reduced to 5% of control values and VERO cell numbers to 14% of controls.

Because treatment of cells with 2µg/ml of MNNG gave substantial cell killing after subculture this concentration was chosen for the isolation of TK^- and $HGPRT^-$ mutants.

ii) The toxicity of 6TG and BUdR for monkey cells

Initial, qualitative observations on the effects of BUdR in the growth medium showed that BSC.B and LLC-MK_2 cells were inhibited by concentrations of BUdR of 1.10^{-5}M and 2.10^{-5}M respectively. VERO cells were similar in sensitivity to BUdR.

6TG at 4.10^{-6}M was found to be toxic for growing BSC.B and LLC-MK_2 cells but VERO cells were more sensitive to this purine analogoue.

A comparison of the relative toxicity of 6TG and BUdR for monkey cells and their resistant derivatives, as measured by dye uptake, is shown in Tables 20 & 21.

iii) Isolation of 6TG-resistant variants of VERO and BSC.B cells

Approximately 4.10^7 confluent VERO cells were treated with 2µg/ml of MNNG in growth medium for 24h. This was removed, the cells washed twice with PBS, and subcultured in fresh growth medium. After 8 days expression time the cells were transferred to growth medium with 6.10^{-7}M 6TG. Over a period of months the cells were subcultured in increasing amounts of 6TG. During this time cells were seeded at 10^2 per 50mm Petri dish in growth medium with 3.10^{-6}M 6TG. (50% conditioned medium, in which normal VERO cells had grown, was included in this medium.) A normal colony was observed in one plate and this was grown up to give VERO ($6TG^R$ 1,2 and 3) cells. These were resistant to 3.10^{-6}M, $7.5.10^{-5}$M and 6.10^{-5}M 6TG respectively.

Similarly, BSC.B cells were treated with 2µg/ml of MNNG but for 1h only in PBS. These cells were subcultured to allow expression of mutant phenotypes then selected with concentrations of 6TG from 2.10^{-7}M to 6.10^{-4}M in growth medium over a period of some months. The resulting 6TG-resistant cell populations were designated BSC.B ($6TG^R$ 1 and 2), resistant to 1.10^{-6}M and 6.10^{-4}M 6TG respectively.

iv) Characterisation of the 6TG resistant populations of VERO and BSC.B cells

The incorporation and uptake of ^{14}C-hypoxanthine was measured in VERO and BSC.B cells and their 6TG-resistant derivatives as 6TG resistance may be mediated by a defect in transport of this analogue. VERO cells were tested in monolayer culture and BSC.B cells both in monolayers and in suspension (Methods).

A reduction in acid precipitable radioactivity was found for all $6TG^R$ cell populations (Table 19). However acid soluble radioactivity was reduced by greater than 70% of control values. It is likely that an inability to transport hypoxanthine into the cells can explain their reduced incorporation of this purine into macromolecules and their resistance to 6TG.

The presence of HGPRT activity in extracts of VERO and VERO ($6TG^R$ 3) cells was determined as described (Methods).

Extracts of VERO ($6TG^R$ 3) cells converted ^{14}C-hypoxanthine to inosinic acid at 42% of the rate found with VERO cells. In a similar experiment BSC.B ($6TG^R$ 2) cell extracts showed only 6% of the HGPRT activity of a control VERO cell extract.

VERO ($6TG^R$) cells appear to be primarily deficient in the transport of hypoxanthine and this explains their resistance to low levels of 6TG.

BSC.B ($6TG^R$) cells appear to have negligible HGPRT activity but this does not correlate with the 35% of control incorporation of ^{14}C-hypoxanthine into PCA-precipitable material shown in Table 19. BSC.B ($6TG^R$) cell extracts may be degrading IMP formed during the HGPRT assay because of increased nucleotidase activity.

During attempts to clone the population of BSC.B ($6TG^R$ 2) cells it was observed that their plating efficiency in conditioned medium (in which normal BSC.B cells had been growing) with added 6TG at 1.10^{-5}M was 0.3% (100 cells were seeded per 50mm Petri dish). In conditioned medium in which BSC.B ($6TG^R$ 2) cells had been growing, originally with 1.10^{-5}M 6TG, the plating efficiency of these cells was 64%.

Table 19. ^{14}C-hypoxanthine uptake and incorporation by 6TG-resistant populations of VERO and BSC.B cells

Cells	^{14}C cpm per 10^6 cells c				
	PCA soluble	PCA precipitable		Total	
	I a	I a	II b	II b	III b
VERO	4,586	680	- e	-	-
VERO(6TG^{R}1)	1,228(27)	286(42)$_d$	-	-	-
VERO(6TG^{R}2)	794(17)	244(36)	-	-	-
BSC.B	1,564	774	940	2,580	3,500
BSC.B(6TG^{R}2)	396(25)	272(35)	360(38)	1,120(43)	1,360(39)

a Monolayer cultures

b Suspended cells

c After 30 min incubation

d % of control values

e Not tested

These cell populations may therefore be capable of degrading exogenous 6TG or 6TG may be unstable in growth medium at 37 °C.

The purpose of isolating 6TG-resistant cells was for use in cell hybridisation experiments. It is essential that such cells should be unable to survive in HAT medium. Therefore VERO ($6TG^R$ 1) cells were compared with VERO cells by the dye uptake method for their growth in HAT medium and in growth medium containing 6TG or BUdR. VERO ($6TG^R$ 1) cells were more sensitive to HAT medium with the normal concentrations of hypoxanthine, thymidine and glycine (Table 20, line 4) than were VERO cells. Increasing the concentration (4-fold) of thymidine alone minimized this difference as did increasing the concentrations of hypoxanthine, thymidine and glycine.

As expected VERO cells were sensitive to $1.5.10^{-6}$M 6TG and VERO ($6TG^R$ 1) cells were appreciably resistant. At higher 6TG concentrations no difference was seen in dye uptake between the two cell types.

No significant difference was found in the sensitivity of VERO and VERO ($6TG^R$ 1) to medium containing BUdR and so a non-specific membrane change is unlikely to be responsible for the 6TG resistance.

In a similar experiment BSC.B and BSC.B ($6TG^R$ 1) cells did not differ significantly in their growth in HAT medium nor in growth medium containing 1.10^{-6}M 6TG or 3.10^{-4}M BUdR. However BSC.B ($6TG^R$ 2) cells, grown in 6.10^{-4}M 6TG over a period of time were observed to be more sensitive to HAT medium, even with 8-fold hypoxanthine, thymidine and glycine, than were BSC.B cells (not shown).

Table 20. The sensitivity of VERO and VERO ($6TG^R1$) cells to HAT medium and to 6TG and BUdR

Concentration (M) in HAT medium a			% dye uptake c	
Hypoxanthine	Thymidine	Glycine	VERO	VERO ($6TG^R1$)
8.10^{-4}	$1.28.10^{-4}$	$2.4.10^{-5}$	65	78
4.10^{-4}	$6.4 .10^{-5}$	$1.2.10^{-5}$	48	49
2.10^{-4}	$3.2 .10^{-5}$	6.10^{-6}	47	35
10^{-4}	$1.6 .10^{-5}$	3.10^{-6}	45	29
"	$1.28.10^{-4}$	"	52	61
"	$6.4 .10^{-5}$	"	51	49
"	$3.2 .10^{-5}$	"	48	35
Concentration of 6TG (M) b				
$1.5.10^{-6}$			8	67
3.10^{-5}			5	11
6.10^{-4}			10	9
Concentration of BUdR (M) b				
1.10^{-5}			71	74
7.10^{-4}			54	57
3.10^{-4}			57	46

a Aminopterin at 4.10^{-7}M

b In growth medium

c Methods

After cloning of the populations of VERO ($6TG^R$) and BSC.B ($6TG^R$) cells it is possible that cell lines suitable for use in hybridisation studies might be obtained.

v) Isolation of populations of monkey cells resistant to BUdR

VERO, BSC.B and LLC-MK_2 cells were treated for 24h with 2µg/ml MNNG in growth medium. This was removed, the cells washed with PBS_A and after a period in growth medium, for expression of mutations, selected with increasing concentrations of BUdR. Intermittently cells were exposed, for approximately 30 min, to a Kodak Cold light illuminator (Model 3), to kill those which had incorporated BUdR into their DNA.

VERO ($BUdR^R$) and BSC.B ($BUdR^R$) cell populations were derived, able to grow in the presence of 1.10^{-5}M BUdR.

LLC-MK_2 ($BUdR^R1$) cells, able to grow in 4.10^{-4}M BUdR, were also isolated.

Cells grown in the presence of BUdR, were not killed by exposure to a Kodak Cold light illuminator. Therefore LLC-MK_2 cells, treated with 2µg/ml of MNNG for 4h, followed by 72h in growth medium were selected in growth medium containing 8.10^{-4}M BUdR and at intervals were exposed to UV light from a Hanovia bactericidal lamp at 30 cm for 5 to 60 s. After recovery from the UV treatment the cells were cultured in growth medium containing 8.10^{-4}M BUdR and designated LLC-MK_2 ($BUdR^R2$).

vi) Characterisation of the BUdR-resistant populations of VERO, BSC.B and LLC-MK_2 cells

BSC.B ($BUdR^R$) cells were tested for their ability to take up ^{3}H-thymidine while in suspension (Methods). After 30 min incubation at 37 ^oC BSC.B ($BUdR^R$) cells had accumulated

less than 1% of the ^{3}H-thymidine found in control BSC.B cells. These BUdR-resistant cells were therefore totally defective in thymidine uptake and this was probably the reason for their ability to grow in medium containing BUdR.

However the plating efficiency of BSC.B ($BUdR^{R}$) cells in BSC.B cell 'conditioned medium' with 1.10^{-5}M BUdR added was 0%. In medium originally containing 1.10^{-5}M BUdR in which BSC.B ($BUdR^{R}$) cells had grown the plating efficiency of these cells was 91%. BSC.B ($BUdR^{R}$) cells may be able to break down BUdR because of nucleosidase action by the cells or from contaminating mycoplasma or BUdR may be relatively unstable in growth medium at 37 °C.

LLC-MK_2 ($BUdR^{R}2$) cells were grown in monolayers and their ability to incorporate ^{14}C-thymidine into acid-precipitable material was examined, (Methods). After 30 min incubation at 37 °C ^{14}C-thymidine incorporated by LLC-MK_2 ($BUdR^{R}2$) cells was 114% of that of control LLC-MK_2 cells. The mechanism of resistance of LLC-MK_2 ($BUdR^{R}2$) cells to BUdR is unlikely to be defective transport of thymidine nor lack of thymidine kinase as both are necessary for thymidine incorporation.

Finally all BUdR-resistant cells were tested for their ability to grow in HAT medium and thus their suitability for use in cell hybridisation studies. All BUdR-resistant cells were found to grow in HAT medium as well as, or better than, control cells (Table 21).

LLC-MK_2 ($BUdR^{R}1$) cells were as sensitive to 6TG, at all concentrations tested, as LLC-MK_2 cells. However BSC.B ($BUdR^{R}$) cells were more sensitive than BSC.B cells to 3.10^{-5}M

Table 21. The sensitivity of mouse D1 cells and of monkey cells and their BUdR-resistant derivatives to HAT medium and to 6TG and BUdR

Concentration (M) in HAT medium [a]			% dye uptake [c, d]						
Hypoxanthine	Thymidine	Glycine	$D1_e$	$VERO_e$	$VERO(BUdR^R)_e$	BSC.B	BSC.B($BUdR^R$)	$LLC\text{-}MK_2$	$LLC\text{-}MK_2(BUdR^R_1)$
8.10^{-4}	$1.28.10^{-4}$	$2.4.10^{-5}$	5	80	79	58	72	79	82
4.10^{-4}	$6.4\ .10^{-5}$	$1.2.10^{-5}$	5	75	79	52	64	49	54
2.10^{-4}	$3.2\ .10^{-5}$	6.10^{-6}	5	65	76	52	57	42	46
10^{-4}	$1.6\ .10^{-5}$	3.10^{-6}	5	55	64	48	55	37	41
"	$1.28.10^{-4}$	"				63	74	58	62
"	$6.4\ .10^{-5}$	"				37	62	58	59
"	$3.2\ .10^{-5}$	"				44	57	44	46
Concentration of 6TG (M) [b]									
	$1.5\ .10^{-6}$					94	85	25	25
	$3\ .10^{-5}$					42	16	20	22
	$6\ .10^{-4}$					15	16	20	19

Concentration of BUdR (M) b				
$1 .10^{-5}$	91	93	93	97
$7 .10^{-4}$	79	89	95	106
$3 .10^{-4}$	68	77	100	102

a Aminopterin at 4.10^{-7}M

b In growth medium

c Methods

d Average of three determinations

e " " five "

6TG. The reason for this is not known.

The dye uptake method does not seem to be adequate for measuring the relative toxicity of BUdR for cells as this requires several generations of cell growth and exposure to a more intense dose of short-wave visible light than that given by a Kodak Cold light illuminator (P. Faik, personal communication).

However BSC.B ($BUdR^R$) cells were able to grow better than BSC.B cells in medium containing up to 3.10^{-4}M BUdR. No difference in growth in BUdR-containing medium was found between LLC-MK_2 and LLC-MK_2 ($BUdR^R_1$) cells in this experiment.

In a similar experiment (not shown) LLC-MK_2 ($BUdR^R_2$) cells were found to be similar in sensitivity to HAT medium but better able to grow in medium containing 8.10^{-4}M BUdR than LLC-MK_2 cells.

None of the BUdR-resistant cell populations isolated appeared to be useful for cell hybridisation because of their growth in HAT medium. The selection procedure used was not sufficiently stringent because of inadequate exposure to shortwave visible light and UV-light was not an effective substitute.

2. <u>The use of HAT medium containing ouabain as a selective agent for mouse-primate hybrid cells</u>

During the course of this work it was reported that mutants of mouse L cells and CHO cells could be isolated which were resistant to ouabain (Baker <u>et al</u>. 1974). This resistance was co-dominant in hybrid cells.

Ouabain inhibits the ATPase associated with sodium and potassium transport across the plasma membrane of cells. Mouse

cells are sensitive to ouabain at a concentration of 10^{-3}M whereas human cells are killed in the presence of 10^{-7}M ouabain (Baker et al. 1974; Kucherlapati, Baker & Ruddle, 1975).

Colonies of cells able to grow in HAT medium with 10^{-6}M ouabain, have been obtained from mixtures of HGPRT$^-$ mouse cells with human skin fibroblasts after treatment with inactivated Sendai virus (Kucherlapati et al. 1975). Assuming that VERO cells were very sensitive to ouabain, the combination of HAT medium and ouabain seemed promising for the selection of hybrid cells from mixtures of VERO cells and mouse TK$^-$ or HGPRT$^-$ cells.

The directed loss of particular monkey chromosomes could be a problem with such a selective system and so the use of intermediate ouabain concentrations is important. It is not known which primate chromosome carries the gene for the ouabain-sensitive ATPase.

i) The sensitivity of monkey and mouse D1 (TK$^-$) cells to HAT medium

Monkey cells and mouse D1 cells were tested for their sensitivity to HAT medium by dye uptake (Methods). The growth of monkey cells in normal HAT medium (Table 21, line 4) was less than 50% of that of control cells. Increasing the concentration of thymidine alone or of hypoxanthine, thymidine and glycine allowed more cell growth.

Mouse D1 cells were killed in HAT medium and dye uptake was never greater than 5% of that of control cells (Table 21). No increase in viability was found with an 8-fold increase in the concentration of hypoxanthine, thymidine and glycine.

ii) The sensitivity of VERO and mouse D1 cells to ouabain

The sensitivity of VERO and D1 cells to concentrations of ouabain from 10^{-8}M to 10^{-3}M was determined by dye uptake (Methods). VERO cells, as expected, were killed in the presence of 10^{-7}M ouabain but mouse D1 cells were only sensitive to the toxic effects of ouabain at 10^{-3}M (Table 30, Section V).

iii) The effect of treatment of VERO and D1 cells with sodium polyanethol sulphonate on their growth in HAT medium containing ouabain

Growth of cells in HAT medium depends on the ability of cells to utilize exogenous thymidine and hypoxanthine. Contamination of cell cultures with mycoplasmas may lead to break down of thymidine by the action of nucleosidase and thus limit the amount available to the cells (Hakala et al. 1963).

The problems of detection and eradication of mycoplasma contamination of cell cultures have been discussed (Methods). Sodium polyanethol sulphonate (SPS) was found to be the most effective agent for eliminating mycoplasmas. SPS was also found to be cytotoxic although VERO cells were less sensitive than mouse D1 cells to this agent.

VERO and D1 cells, treated with SPS, were tested for their ability to grow in HAT medium alone, or in the presence of 10^{-5}M or 10^{-6}M ouabain, by dye uptake (Methods). Growth of SPS-treated VERO cells in HAT medium alone (Table 22) was greater than that observed for untreated cells in previous experiments (Table 21 row 4). D1 cells were still killed in HAT medium after SPS treatment as was expected for this TK^- cell line.

Table 22. The growth of SPS-treated VERO and D1 cells in HAT medium or ouabain - containing medium

Medium	Concentration of ouabain (M)	% dye uptake a, b	
		VERO	D1
HAT	-	90	6
"	10^{-5}	4	5
"	10^{-6}	3	6
D55	10^{-6}	3	98

a Methods

b Average of five determinations

The presence of 10^{-6}M ouabain in growth medium was toxic for VERO cells but not for D1 cells. HAT medium containing 10^{-5}M or 10^{-6}M ouabain was toxic for both VERO and D1 cells. Consequently SPS-treated VERO and D1 cells were used for cell hybridisation studies and the selective medium chosen was HAT medium with 10^{-5}M or 10^{-6}M ouabain.

B. The enhancement of cell fusion by treatment with Sendai virus or polyethylene glycol

The first hybrid cells to be isolated were from spontaneous fusions of mouse cells (Barski, Sorieul & Cornefert, 1960). Treatment of cells with inactivated Sendai virus, to increase the frequency of cell fusion, was introduced by Harris & Watkins (1965) and it was shown that cells of different species could be fused.

Virus-induced cell fusion is temperature and energy dependent and requires the presence of calcium ions (Giles & Ruddle, 1973). It has been shown that Sendai virus can cause aggregation of membrane proteins and Poste & Allison (1973) have suggested that such protein clustering on adjacent membranes can initiate fusion. Sendai virus grown in eggs is likely to be contaminated with avian leukosis viruses and so its complete inactivation is important. β.propiolactone treatment is more effective for this purpose than UV-irradiation (Neff & Enders, 1968; Klebe, Chen & Ruddle, 1970).

A number of chemical agents have been shown to possess fusogenic activity including lysolecithin (Howell & Lucy, 1969); glyceryl mono-oleate (Ahkong et al. 1973) and polyethylene glycol (PEG) (Pontecorvo, 1975). Polyethylene

glycol, originally used for the fusion of plant protoplasts, has been successfully used to fuse human fibroblasts and lymphocytes as well as Chinese hamster and mouse cells (Pontecorvo, 1975).

The use of chemical agents eliminates the possibility of introducing unwanted, foreign genetic material into hybrid cells or of establishing a persistent virus infection. Polyethylene glycol promises to be the most useful fusogen currently available as lysolecithin is cytotoxic and glyceryl mono-oleate has to be used as a fresh emulsion.

The effects of Sendai virus and polyethylene glycol treatment of cells on the formation of colonies in selective medium were therefore tested.

1. β.propiolactone-inactivated Sendai virus

The frequency of colony formation after Sendai treatment was compared with the spontaneous frequency in mixtures of VERO and D1 cells. Two 100mm Petri dishes were seeded with 5.10^6 each of VERO and D1 cells in growth medium. After 24h at 37 °C the medium was removed and the monolayer of cells in 1 plate treated with inactivated Sendai virus. 1ml per plate was added of a 4-fold dilution of inactivated Sendai virus, originally at 80,000 H.A.U. per ml, as described in Methods. The second 100mm Petri dish served as a control and was treated similarly but with cold growth medium. After these treatments the monolayers were trypsinized and seeded in selective medium at densities ranging from 10^2 to 5.10^5 cells per 50mm Petri dish. No significant difference was found in the cell numbers for each 100mm Petri dish. After 35 days incubation at 37 °C with frequent changes of selective medium colonies were counted by viewing the Petri

dishes in oblique light.

Colonies were only found in Petri dishes seeded with 2.10^5 or more cells (Table 23). Sendai virus-treatment increased the average number of colonies per plate 3-fold.

The preparation of Sendai virus used originally had a titre of 80,000 H.A.U. per ml but on re-assay this was found to have fallen to 2,000 H.A.U. per ml. The experiment described was carried out approximately six months after use of this virus preparation for two cell hybridisation experiments and presumably the virus had deteriorated on storage during this time.

The number of cells seeded in selective medium affects the number of colonies obtained. A 3-fold difference in frequency was found between Petri dishes seeded with 2.10^5 or 5.10^5 cells even if the cells were Sendai virus-treated. Under these conditions hybrid clones with a low plating efficiency may be at a disadvantage. VERO cells have plating efficiencies of 1 to 5% if 100 cells are seeded per 50mm Petri dish in growth medium. A plating efficiency of 17% was found if 5.10^2 VERO cells were seeded. In RPMI 1640 medium with hypoxanthine, thymidine, glycine and aminopterin the plating efficiency of VERO cells was 10-fold less than that found in RPMI 1640 medium alone.

D1 cells have a high plating efficiency and hybrid clones with mouse-like morphology and growth properties may therefore predominate amongst those clones isolated.

Table 23. The frequency of colony formation in selective medium after Sendai virus treatment of mixed monolayers of VERO and D1 cells

Number of cells seeded per 50mm Petri dish	Average colony number per Petri dish		Frequency of colony formation [b]	
	Sendai virus treated	Control	Sendai virus treated	Control
5.10^5	56 (14) [a]	19 (5)	$8.9.10^{-3}$	$2.6.10^{-4}$
2.10^5	7 (15)	2.5(2)	$2.9.10^{-4}$	8.10^{-4}
2.10^4	0 (3)	0 (2)	0	0
10^4	0 (10)	0 (3)	0	0

a The numbers of replicate plates are given in parentheses

b Relative to the number of both cell types seeded

2. Polyethylene glycol

i) The effect of polyethylene glycol treatment on the frequency of colony formation in selective medium by mixtures of VERO and D1 cells

Because of the low enhancement of colony formation by Sendai virus treatment seen above (Table 23) and the objections to the use of viruses as cell fusing agents it was decided to test polyethylene glycol for its ability to fuse VERO and D1 cells.

The method used was that described by J. Lucy (personal communication). 10^7 cells each of VERO and D1 cells were suspended in 1ml of 40% polyethylene glycol (mol. wt. 4,000) in DME or in 1ml of DME only. After incubation for 50 min at 37 °C in a water-bath 9ml of DME were added slowly dropwise and the diluted cells incubated for a further 30 min at 37 °C. The cells were then diluted in selective medium (HAT medium with 10^{-6}M ouabain) and seeded at 4.10^5, 2.10^5 or 10^5 cells per 50mm Petri dish. After incubation for 34 days at 37 °C colonies were visualised by Giemsa staining (Methods) and counted.

The frequency of colony formation after PEG treatment (Table 24) was not significantly different from that found with Sendai virus-treated cultures (Table 23). An increase in colony numbers for PEG treated cells as compared with control cells of 2 to 5-fold was found.

Colony formation by PEG-treated cells was less affected by low cell density than was colony formation by Sendai virus-treated cells. Seeding of 2.10^5 or 4.10^5 PEG-treated cells in selective medium gave a frequency of colony formation which was essentially the same (Table 24). However, for

Table 24. The frequency of colony formation in selective medium of VERO and D1 cells treated in suspension with polyethylene glycol (PEG)

Number of cells seeded per 50mm Petri dish	Average colony number per Petri dish		Frequency of colony formation [b]	
	PEG-treated	Control	PEG-treated	Control
4.10^5	52 (15)[a]	18 (5)	$7.7.10^{-3}$	$2.2.10^{-4}$
2.10^5	24 (15)	5 (5)	$8.3.10^{-3}$	4.10^{-4}
10^5	6 (14)	3 (5)	$1.7.10^{-4}$	$3.3.10^{-4}$

a The numbers of replicate plates are given in parentheses

b Relative to the number of both cell types seeded

Sendai virus-treated cells, the frequency of colony formation in selective medium was reduced 3-fold when 2.10^5 as compared with 5.10^5 cells were seeded (Table 23).

ii) The effect of PEG treatment on colony formation in selective medium by VERO and D1 cells alone

To determine the expected proportion of TK^+ revertants of D1 cells or ouabain-resistant mutants of VERO cells among presumptive hybrid clones these cells were treated separately with PEG. Colony numbers in selective medium were counted for mixtures of the two cell types and for VERO or D1 cells alone.

PEG treatment was as previously described with 2.10^7 per ml of VERO or D1 cells alone or with mixtures containing 10^7 cells of each type. Colonies were visualised by Giemsa staining after 24 days at 37 ^oC with frequent changes of HAT medium containing 10^{-5} or 10^{-6}M ouabain.

The frequency of colony formation in HAT medium with 10^{-6}M ouabain in this experiment (Table 25a) was less than in the previous experiment (Table 24) for mixtures of PEG-treated VERO and D1 cells. This probably reflects the variation in plating efficiency seen with different batches of medium and serum for the same cell type.

Selection of PEG-treated mixtures of cells in HAT medium containing 10^{-5}M ouabain reduces colony formation approximately 2-fold (Table 25b). Because ouabain resistance is only co-dominant in cell hybrids (Baker et al. 1974) it is likely that the mouse ATPase cannot properly reverse the effects of inhibited monkey ATPase at high ouabain concentrations.

The frequency of mutation of PEG-treated VERO cells to presumed ouabain resistance was found to be $2.3.10^{-6}$ (3

Table 25. The frequency of colony formation in selective medium of PEG-treated VERO or D1 cells alone

a) Selection with HAT medium and 10^{-6}M ouabain

Number of cells seeded per 50mm Petri dish	Average colony number per Petri dish			Frequency of colony formation		
	VERO+D1	VERO	D1	VERO+D1	VERO	D1
5.10^5	19(4)[a]	0.25(4)	0(4)	$2.6.10^{-4}$	2.10^{-6}	0
$2.5.10^5$	10(4)	0(4)	0(2)	$2.5.10^{-4}$	0	0
10^5	0.5(4)	0(4)	-[c]	2.10^{-5}	0	-

b) Selection with HAT medium and 10^{-5}M ouabain

Number of cells seeded per 50mm Petri dish	Average colony number per Petri dish			Frequency of colony formation		
	VERO+D1	VERO	D1	VERO+D1	VERO	D1
5.10^5	12(8)	0(4)	0(4)	$4.2.10^{-4}$	0	0
$2.5.10^5$	5(12)	0.5(4)	0(4)	5.10^{-4}	5.10^{-5}	0
10^5	0.4(8)	0(4)	0(4)	$2.5.10^{-5}$	0	0

a The numbers of replicate plates are given in parentheses

b Relative to the number of both cell types seeded

c Not tested

colonies arising from a total of $6.8.10^6$ cells seeded in HAT medium containing 10^{-5} or 10^{-6}M ouabain). Baker et al (1974) found a mutation frequency of 1.10^{-5} for untreated CHO cells selected for 10-fold wild-type resistance to ouabain.

No TK^+ colonies of D1 cells, able to grow in selective medium, were found from a total of $5.9.10^6$ cells seeded. This is in agreement with the findings of Migeon, Smith & Leddy (1969) who stated that "despite extensive use of these cells, there has never been a proven reversion to wild-type enzyme activity". The loss of TK by D1 cells is presumed to result from gene or chromosome deletion.

If 5.10^5 PEG-treated VERO and D1 cells are seeded in selective medium at a ratio of 1:1 the frequency of colony formation is $1.3.10^{-4}$ relative to the number of VERO cells seeded (Table 25). The frequency of mutation of VERO cells to ouabain resistance is $2.3.10^{-6}$. Therefore 0.56% of colonies growing in selective medium from mixtures of VERO and D1 cells seeded would be expected to be mutants of VERO cells.

iii) The effect of PEG treatment of monolayers of VERO and D1 cells on colony formation in selective medium

A method for fusing cells in monolayers with PEG was described by G. Pontecorvo (personal communication) as a variation of that published (Pontecorvo, 1975).

VERO and D1 cells were seeded at 10^6 cells each per 50 mm Petri dish in growth medium. After 24h at 37 ^{o}C medium was removed and 4ml of a 100% solution of PEG (mol. wt. 6,000) in DME was added to the side of one Petri dish; a control Petri dish received DME only.

Table 26. Colony formation in selective medium by mixtures of VERO and D1 cells treated in monolayers with polyethylene glycol

Number of cells seeded per 50mm Petri dish	Mean colony numbers per plate		Frequency of colony formation [b]	
	PEG treated	Controls	PEG treated	Controls
2.10^5	88(3)	0.6(3)	$2.3.10^{-3}$	$3.3.10^{-5}$
10^5	29(10)	0(5)	$3.4.10^{-3}$	0
5.10^4	9(10)	1(5)	$5.6.10^{-3}$	5.10^{-4}
$2.5.10^4$	1.2(9)	0.2(5)	$2.1.10^{-4}$	$1.25.10^{-5}$
10^4	0.1(10)	0(5)	1.10^{-5}	0

a The number of replicate plates is given in parentheses

b Relative to total cell numbers seeded

After gentle rocking for 1 min the PEG was removed and replaced by 5ml of a 33% solution of PEG in DME. This procedure was repeated with 14% and 6.7% PEG solutions. Finally the monolayers were washed twice with growth medium and 5ml of growth medium added. After 24h at 37 ^{o}C the cells were trypsinized and seeded in selective medium. The PEG-treated monolayer contained $3.5.10^{6}$ cells whereas the control monolayer contained $5.3.10^{6}$ cells. This may reflect the cell fusion which had occurred among the PEG-treated cells. Selective medium was replaced frequently and after 36 days at 37 ^{o}C colonies were counted by viewing the plates in oblique light.

This method of PEG treatment gave the highest frequency of colony formation in selective medium found (Table 26). The enhancement of colony formation by PEG treatment of cells over the spontaneous rate was 150-fold but fewer colonies arose from untreated cell mixtures than was previously found.

The frequency of colony formation for a number of different selective systems and fusogens is shown in Table 27. Sendai virus or PEG treatment of VERO and D1 cells gave a similar frequency of colony formation in selective medium to that found by a number of workers. The spontaneous rate of colony formation by VERO and D1 cells was generally higher than in mouse/mouse cell fusions or in mouse/Chinese hamster cell fusions (Klebe *et al.* 1970; Croce *et al.* 1971; Pontecorvo, 1975) but was similar to that found for mouse/human cell fusions (Croce, Koprowski & Eagle, 1972).

The frequency of hybrid colonies found using resistance to ouabain as part of the selective system depended on the other selective marker used (Baker *et al.* 1974).

Table 27. The frequency of hybrid colony formation in selective media after Sendai virus, lysolecithin or PEG treatment of cells

Reference	Fusogen	Cells	Selective system	Cell density	Frequency of hybrid formation
Baker et al. (1974)	Sendai	CHO/CHO[a]	AuxB1$^+$;Pro$^+$	1-2.10^5/78.5 cm^2	5.8.10^{-5}
"	"	" a	AuxB1$^+$;Pro$^+$ Oua^R	"	8.9.10^{-5}
"	"	" a	Oua^R;Pro$^+$	"	3.10^{-4}
"	"	" b	Oua^R;HAT	"	1.9.10^{-5}
"	"	" b	Temp^R;HAT	"	7.10^{-4}
Klebe et al. (1970)	"	Mouse HGPRT$^-$ x Mouse TK$^-$	HAT	2.10^6/25cm^2	1.05.10^{-4}
	-	"	"	"	$< 4.10^{-5}$
Croce et al. (1971)	Lysolecithin	Mouse HGPRT$^-$	"	10^6/28cm^2	7.5.10^{-4}
	Sendai	x	"	"	4.1.10^{-4}
	-	Mouse TK$^-$	"	"	5.6.10^{-5}

Croce et al. (1972)	Lysolecithin	Human HGPRT$^-$	"	$10^6/5.5cm^2$	$3.4.10^{-4}$
	Sendai	x	"	"	$1.7.10^{-4}$
	-	Mouse TK$^-$	"	"	6.10^{-4}
Pontecorvo (1975)	PEG	Chinese	"	$10^5$to$10^6/25cm^2$	$4.5.10^{-3}$
	-	Hamster HGPRT$^-$ x Mouse TK$^-$	"	"	$2.6.10^{-5}$

a Oua^R; AuxB1$^+$; Pro$^-$ X Oua^S; AuxB1$^-$; Pro$^+$

b Oua^R; AG^R; Temp^R X Oua^S; AG^S; tsH1

c Relative to cell numbers of both parents seeded (1:1 ratio)

Summary

Attempts to isolate HGPRT$^-$ or TK$^-$ cell lines, unable to grow in HAT medium, from monkey cell lines were not successful. However VERO cells were found to be as sensitive to the toxic effects of ouabain as are human cells. A selective system using HAT medium with 10^{-6}M ouabain was found to be suitable for the isolation of VERO-D1 cell hybrids.

PEG was tested for its usefulness as a fusing agent because of the undesirability of using Sendai virus when virus-induced functions were to be studied in the hybrid cells. Treatment of cell monolayers with PEG was found to be an effective method of cell fusion.

V. THE DERIVATION AND PROPERTIES OF HYBRID CLONES FORMED BY THE FUSION OF VERO AND D1 CELLS

VERO and D1 cells were fused by treatment of monolayers with β-propiolactone-inactivated Sendai virus (1,000 H.A.U. per 2.10^6 cells) as described in Methods, or as in section IV (500 H.A.U. per 10^7 cells). Other mixed cultures were fused in suspension with 40% polyethylene glycol (mol. wt. 4,000) or treated in monolayers with 100% polyethylene glycol (mol. wt. 6,000) as described in section IV.

The fused cells and control cultures were placed in HAT medium containing ouabain (10^{-5} or 10^{-6}M) to select hybrid colonies. Colonies were transferred to wells of microtitre trays, containing selective medium, using sterile, wooden toothpicks (Methods). Survival was often poor for a number of reasons including mechanical damage during transfer, differences in the ability of different batches of medium to support clonal growth, necrosis of colony centres caused by over-long incubation before transfer and low plating efficiency of cells during chromosomal loss.

Generally, colonies growing up in selective medium were pooled and recloned. The plating efficiency of cells in selective medium, after polyethylene glycol fusion (Table 26), was 0.04% for 2.10^5 mixed cells seeded per 50mm Petri dish. When colonies from this fusion were pooled and reseeded at 200 cells per 50mm Petri dish in selective medium the plating efficiency was 2.5%. This indicates that the original, pooled colonies were derived from hybrid cells and not from cells escaping selection by metabolic co-operation (Subak-Sharpe et al. 1969) or by metabolising break-down products of dying cells.

Table 28. The derivation of hybrid clones from the fusion of VERO and D1 cells

Group	Clones	Fusing agent	Concentration of ouabain (M) in HAT medium	Ratio of VERO to D1 cells during fusion
I	1-11	Inactivated Sendai virus [a]	10^{-6}	3:1
II	1-3	"	"	"
III	1-2	"	10^{-5}	"
IV	1-5	"	"	1:1
V	1-5	"	10^{-6}	"
VI	1-4	-	"	"
VII	1-3	-	"	"
VIII	1	Inactivated Sendai virus [b]	"	"
IX	1	-	"	"
X	1	PEG (suspension)	"	"
XI	1-8	" (monolayer)	"	"

a 1,000 H.A.U. per 2.10^6 cells

b 500 H.A.U. per 10^7 cells

The fusing agent and selective medium used for the isolation of hybrid clones are listed in Table 28. Clones in separate Groups (I to XI) were of independent origin; clones within a group may not have been but did differ in properties, e.g. clone XI4 was found to be sensitive to both mouse and human interferons whereas clone XI2 was sensitive only to mouse interferon. Such differences could have arisen from the loss of different chromosomes in clones derived from the same original hybrid cell or because the recloned cells arose from different hybrid cells.

The hybrid clones were grown in selective medium until sufficient cells were available for their characterisation. Microscopic examination of these clones revealed that I4, I5, VII1, VII2, VII3 and XI4 had a morphology intermediate between that of the parental cells. Clone X1 cells were similar to VERO cells in appearance whereas most other clones resembled D1 cells.

A. Growth in selective media

1. HAT medium and BUdR-containing medium

Cells are only able to grow in HAT medium if they contain active thymidine kinase. Such cells are able to incorporate the thymidine analogue BUdR into their DNA thus becoming susceptible to DNA damage when exposed to short-wave visible light (Section IV). The growth of VERO and D1 cells and clones VII 1-3 and II1 was compared in BUdR-containing medium (after exposure to fluorescent light) by dye uptake (Methods), and also in HAT medium and in selective medium.

VERO cells were inhibited at BUdR concentrations of

3.10^{-4} and 3.10^{-5}M as were cells of clones VII 1-3 and II1 (Table 29) and thus they possess an active thymidine kinase. D1 cells (TK^-) were insensitive to the presence of BUdR in growth medium.

Cells of clones VII 1-3 and II1 were able to grow in HAT medium containing ouabain (10^{-6}M) but HAT medium alone was inhibitory for those of Group VII. The chromosomal constitution of these cells had presumably become adapted for growth in selective medium.

VERO cells were able to grow in HAT medium alone but not in selective medium. D1 cells were unable to grow in HAT medium or selective medium. These observations confirm those made previously (Section IV).

The growth of cells of clones VIII 1, IX1 and X1* was also inhibited by BUdR at a concentration of 3.10^{-4}M. (Clone X1 was redesignated clone X1* as it had lost the ability to grow in selective medium after a number of sub-cultures). These clones therefore possess thymidine kinase activity which must have been derived from the VERO parental cells as D1 cells have not been known to revert to a TK^+ phenotype (Migeon et al. 1969).

Clone X1* was able to grow in HAT medium only in the absence of ouabain (10^{-6}M). Its loss of ability to grow in selective medium was therefore a result of regained ouabain sensitivity.

2. Ouabain-containing medium

The selective medium used for the isolation of hybrid clones contained a concentration of ouabain intermediate between that toxic for D1 cells and VERO cells. It might be predicted that some hybrid clones would be more sensitive to

Table 29. Growth of cells in medium containing BUdR or in selective medium

Ouabain (10^{-6}M)	HAT medium	% dye uptake a					
		VERO	D1	VII1	VII2	VII3	II1
-	+	71	6	49	39	43	77
+	+	5	4	77	64	65	74
Concentration of BUdR (M) in growth medium							
3.10^{-5}		33	104	36	36	32	31
3.10^{-4}		12	95	18	15	16	21

a Methods

ouabain than are D1 cells although there would be a strong selective pressure for the loss, from hybrid cells, of the VERO chromosomes carrying the gene for the ouabain-sensitive ATPase. Cells were therefore tested for ouabain sensitivity by dye uptake (Methods).

VERO cells were killed in the presence of ouabain concentrations greater than 10^{-7}M whereas D1 cells could grow in concentrations less than 10^{-3}M (Table 30). All of the hybrid clones tested, except X1*, were as resistant to the effects of 10^{-5}M ouabain as D1 cells. However some growth inhibition of clones VII 1-3, VIII1, IX1 and in particular of XI4 was observed in the presence of 10^{-4}M ouabain. It is possible that these cells contain a ouabain-sensitive ATPase.

The growth of clone X1* cells was inhibited by the presence of ouabain (except at 10^{-8}M). This clone resembled VERO cells in its sensitivity to the toxic effects of ouabain except that at each concentration tested some residual growth of clone X1* was observed. It is possible that this clone is a VERO mutant with an altered ATPase.

B. Lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PDH) isozymes

The different mobilities in an electric field of the equivalent enzymes from different species can be used to detect the presence of active genes from different parental cells in hybrid clones (Giles & Ruddle, 1973). Extracts from hybrid clones were therefore tested for the presence of LDH or G6PDH isozymes with the mobilities characteristic of the enzymes of D1 or VERO cells (Methods).

Table 30. Cell growth in medium containing ouabain

Ouabain concentration (M)	% dye uptake a									
	Expt. I		Expt. II				Expt. III		Expt. IV	Expt. V
	VERO	D1	II1	VII1	VII2	VII3	VIII1	IX1	X1*	XI4
10^{-3}	4	40	15	5	6	6	3	3	17	4
10^{-4}	2	99	100	83	90	81	92	85	15	47
10^{-5}	2	98	99	100	102	103	96	98	22	100
10^{-6}	2	102	103	104	103	105	98	101	18	95
10^{-7}	7	99	101	104	110	108	-b	-	28	103
10^{-8}	103	100	101	101	103	104	-	-	98	92

a Methods

b Not tested

The monkey isozymes of LDH were found in clone X1 extracts but the presence in addition of the mouse LDH isozyme could not be ascertained (Table 34). All other clones examined had LDH or G6PDH with the mobilities of the mouse enzymes except for clone I1 where an isozyme with possible 'hybrid' mobility was found in addition to mouse-like G6PDH.

Extracts of VERO and clone X1 cells also contained enzyme activity which could be detected on cellulose acetate gels in the absence of the substrate for G6PDH.

The bands of activity were found on the cathodal side of the origin and had identical mobilities. A similar phenomenon, attributed to alcohol dehydrogenase, has been observed by others (Wilkinson, 1970).

Clone I5, which was found to be insensitive to the cytopathic effect of EMC virus (see later), had the mouse isozymes of both G6PDH and LDH.

Hybrid cells would not be expected to contain many monkey chromosomes and so the detection of monkey isozymes is a matter of chance. That clone I5 had neither monkey LDH nor G6PDH activity is not significant. The detection of three different monkey isozymes in clone X1 cells supports the idea that this clone is a mutant of the VERO cell line.

C. Chromosomes

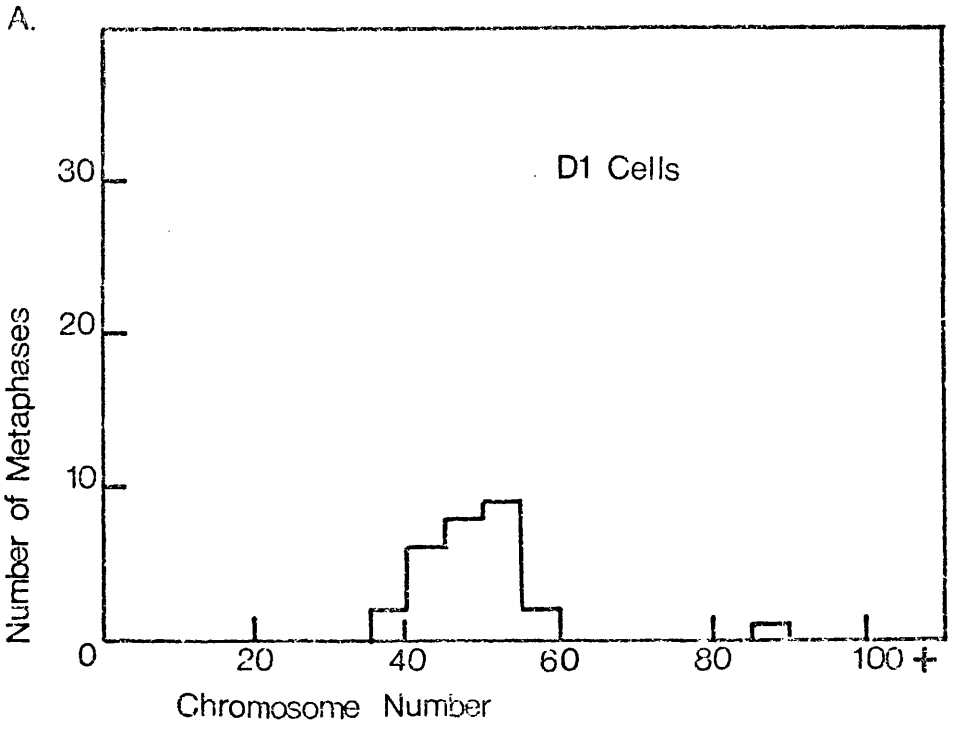
Chromosome preparations of VERO and D1 cells were prepared and Giemsa stained (with or without trypsin banding) as described (Methods). The distribution of chromosome numbers for these cells is shown in Fig. 12A, B. The modal chromosome number for D1 cells was 5 less than for VERO cells. A proportion of VERO cells were pseudo-tetraploid (approximately 20%).

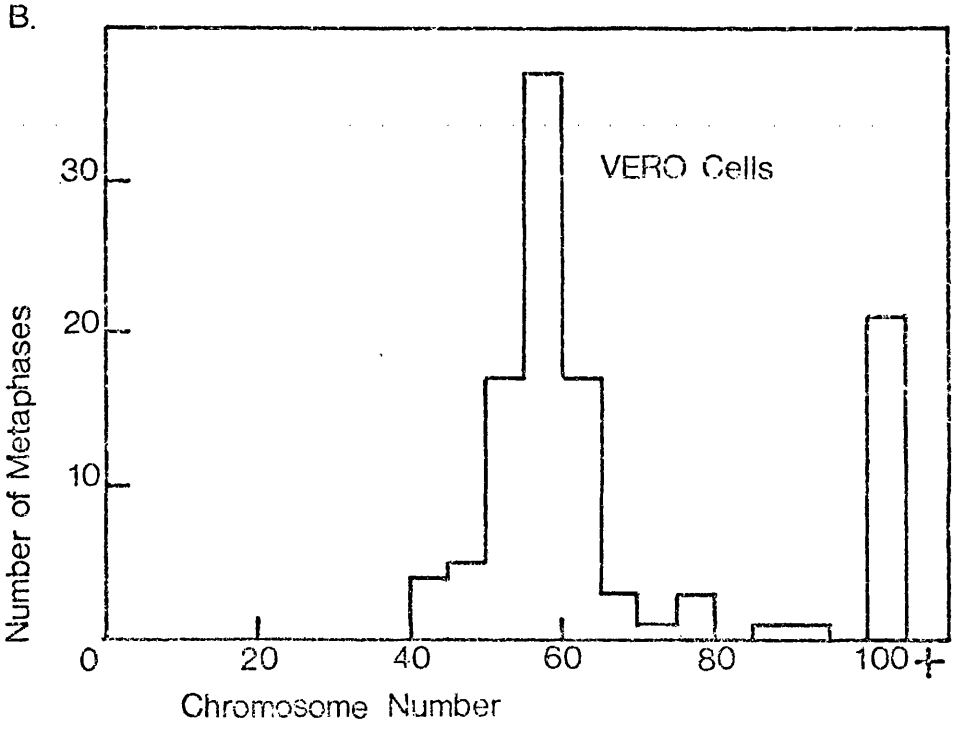
Figure 12 Frequency distribution of chromosome numbers

The chromosomes of well-spread, Giemsa-stained metaphases were counted.

A. D1 cells (28 determinations)

B. VERO cells (110 determinations)


A.
D1 Cells
Number of Metaphases
30
20
10
0
20
40
60
80
100+
Chromosome Number


B.
VERO Cells
Number of Metaphases
30
20
10
0
20
40
60
80
100+
Chromosome Number

The chromosomes of VERO and D1 cells are shown in Figs. 13A, B & 14A, B. VERO cell chromosomes are mostly metacentric or submetacentric and small marker chromosomes with a non-staining region can be seen. The chromosomes of D1 cells are generally telocentric but about nine large metacentric chromosomes are present. Two of these large chromosomes have secondary constrictions.

Examination of the chromosomes of clone X1 showed that they were mostly metacentric or sub-metacentric. The chromosome numbers of seven out of ten metaphase spreads counted fell between 52 and 58. Clone X1 chromosomes were re-examined after this clone had lost the ability to grow in selective medium. A similar chromosome number was found and metacentric and sub-metacentric chromosomes predominated. VERO-like marker chromosomes could be observed in well-spread metaphases. It was concluded that clone X1 was a mutant of VERO cells although the presence of a small amount of mouse chromosomal material could not be ruled out except by differential staining of chromosomes.

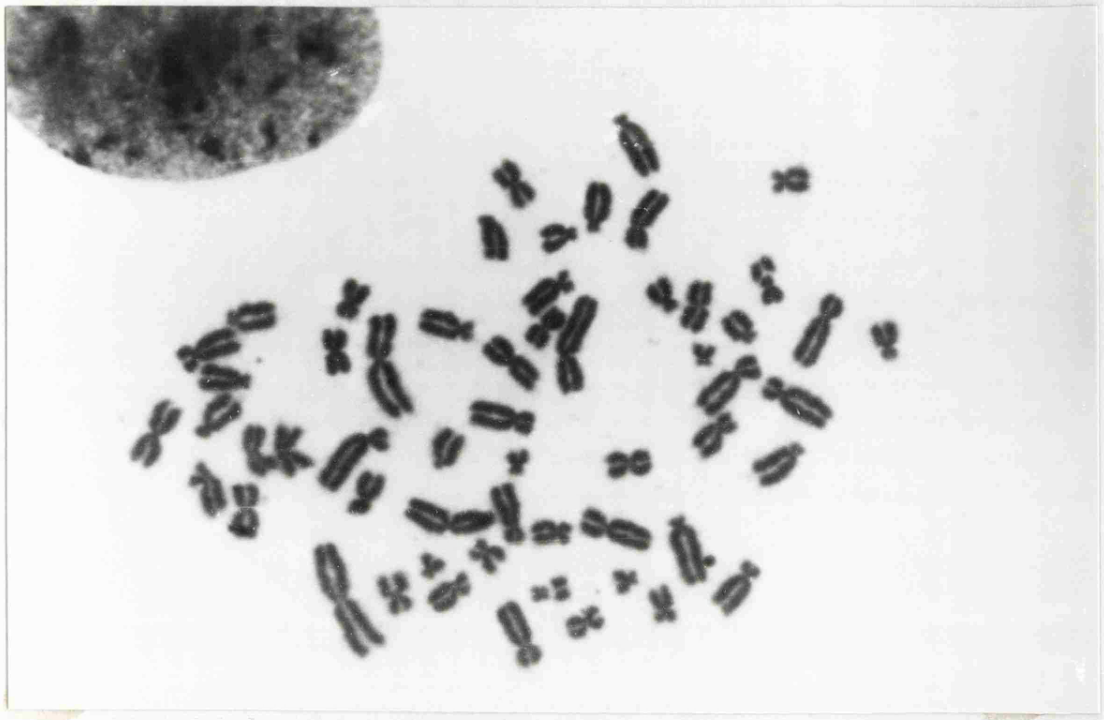
Fig. 15A, B shows the chromosomes of clone XI4. There are fifteen non-telocentric chromosomes, two of these being small and metacentric and probably of monkey origin. At least one large chromosome has a secondary constriction and is therefore from the mouse parent. Chromosome numbers between 50 and 55 predominated in this clone but in some metaphase spreads 70 to 90 chromosomes were counted.

Precise determination of the origin of the chromosomes in hybrid clones requires analysis using differential staining. The G11 technique, used to discriminate between mouse and human chromosomes in hybrid cells (Bobrow & Cross,

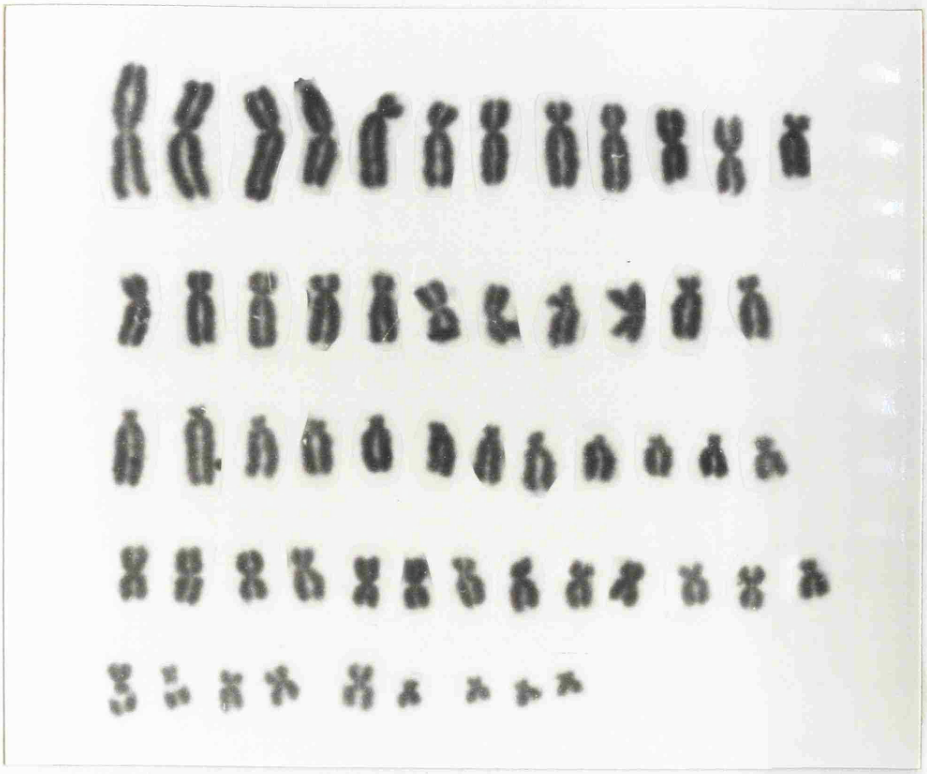
Figure 13 Chromosomes of VERO cells

A. Giemsa-stained metaphase spread

B. Idiogram of metaphase spread in A



A.

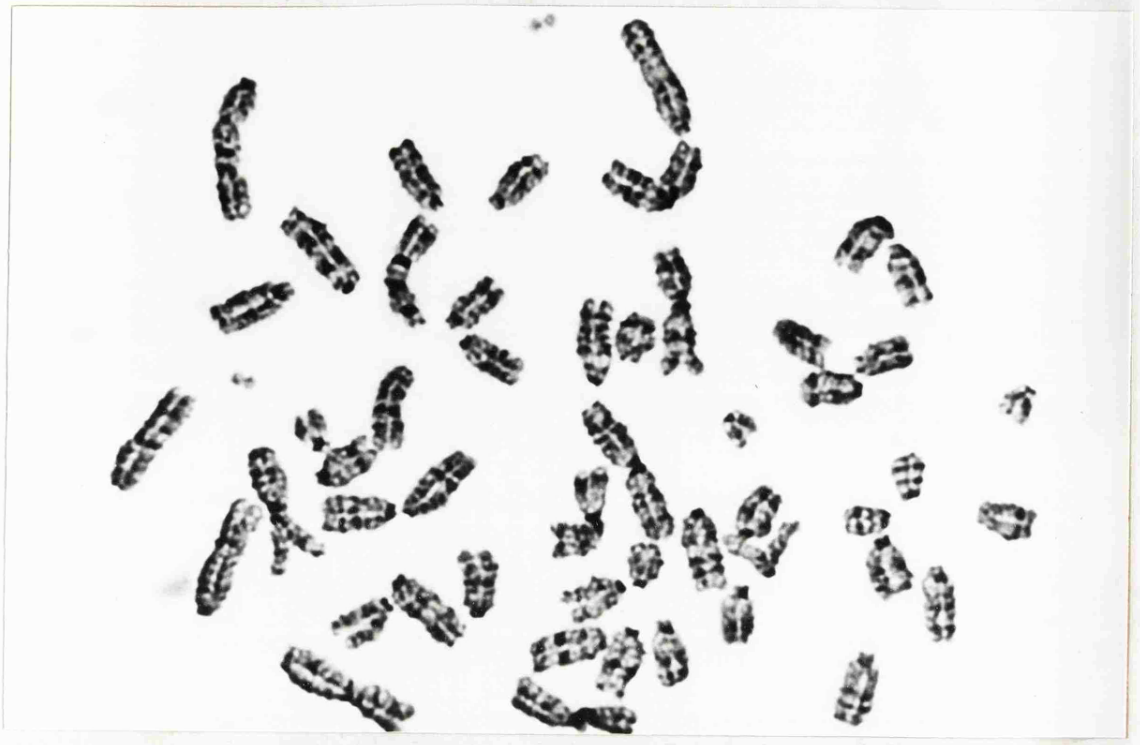


B.

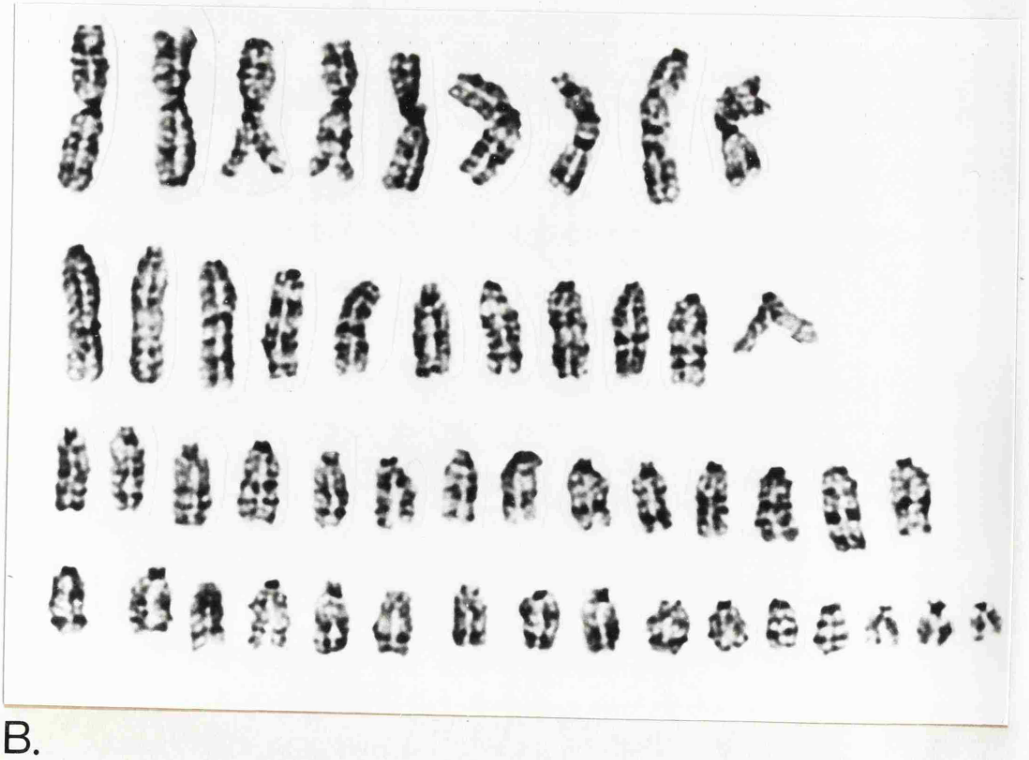
Figure 14 Chromosomes of D1 cells

A. Trypsin-banded, Giemsa-stained metaphase spread

B. Idiogram of metaphase spread in A



A.

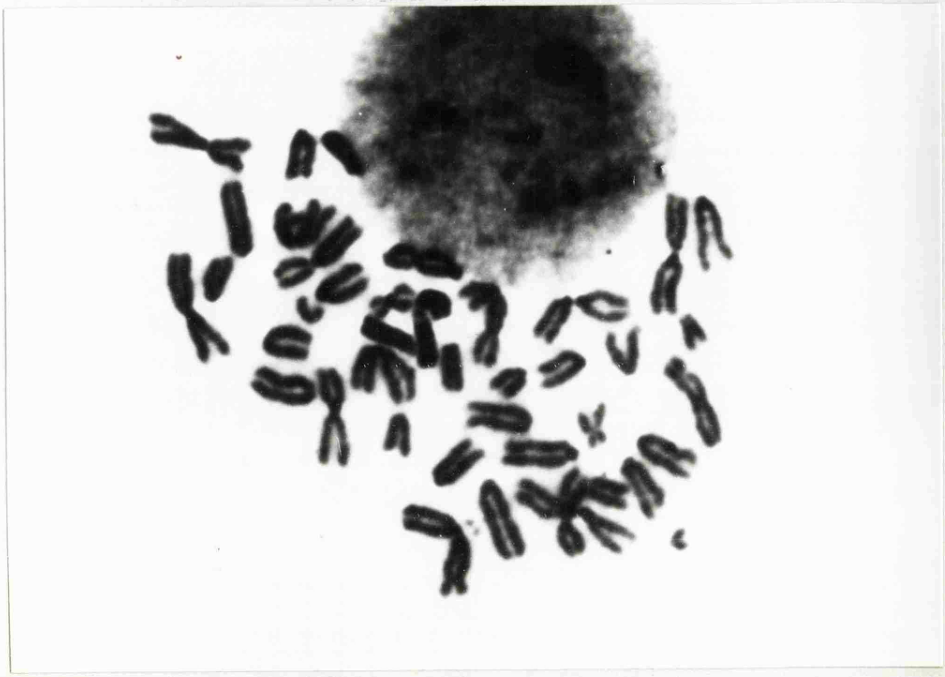


B.

Figure 15 Chromosomes of clone XI4 cells

A. Giemsa-stained metaphase spread

B. Idiogram of a second metaphase spread



A.

B.

1974) did not distinguish VERO chromosomes from those of mouse cells (Methods and M. Bobrow, personal communication).

D. The action of interferons and poly(rI). poly(rC)

1. Mouse and human interferons

The sensitivity of a hybrid clone to the anti-viral effect of a particular parental interferon is not related to its ability to produce interferon of that species-specificity. These functions have been shown to be directed by separate chromosomes in human and monkey cells (Cassingena et al. 1971; Tan et al. 1974a; Tan et al. 1973).

In addition, primate chromosomes are usually preferentially lost from mouse-primate hybrids (Weiss & Green, 1967; Cassingena et al. 1971; Brumback, 1973) and so it would be expected that most hybrid clones would be sensitive to the anti-viral effect of mouse interferon but not necessarily to that of primate interferon.

Cells of hybrid clones were seeded in 25 well trays in growth medium so as to be confluent after 24 to 48h incubation. [Selective medium was not used because of the possible inhibition of the anti-viral effect of interferon by ouabain (Lebon et al. 1975)]. Monolayers were treated for 24h with 0.4ml of interferons diluted in growth medium (a minimum of 5 units of mouse interferon or 12 units of human interferon per well). The anti-viral effect was measured by challenge with Sindbis virus and the dye uptake method as described (Methods). EMC virus challenge was sometimes used and the degree of cytopathic effect was estimated visually after 24h (Methods). The virus used for challenge is indicated for each hybrid clone (Tables 31 & 34).

Table 31. Responsiveness to the anti-viral action of interferons

Expt.	Cells	Mouse interferon (1)		Human interferon (1)	
		Units/well	% dye uptake a	Reference units/well	% dye uptake a
1	III1	12.5	90	30	4
	" 2	"	62	"	2
	V2	"	65	"	3
2	II1	"	22	"	0
	VII1	"	78	"	0
	" 2	"	83	"	0
	" 3	"	81	"	0
	VERO	"	1	"	21
	D1	"	105	"	0
3	X1	10	13	24	63
4	XI4	5	86	12	28
	VERO	"	0	"	43
5	X12	"	61	"	5
6	X1*	10	0	24	9
	XI4	10	61	"	0

a Methods; Sindbis virus challenge

Most clones, like the parental mouse D1 cells, were sensitive to the anti-viral action of mouse but not of human interferon (Tables 31 & 34). However one clone, X1, was sensitive to the action of human interferon but only very weakly sensitive, if at all, to mouse interferon. This clone lost the ability to grow in selective medium and was subsequently cultured in growth medium. When it was retested for its sensitivity to interferons it responded to human interferon, albeit poorly, but not to mouse interferon.

Clone XI4 responded to the anti-viral action of both interferons when first tested but after a number of passages it had lost the ability to respond to human interferon but was still able to respond to mouse interferon. Thus, during subculture clone XI4 may have lost the monkey chromosome which governs the ability to respond to monkey interferon.

2. Induction of an anti-viral state by poly(rI). poly(rC)

Cells may be sensitive to the anti-viral action of poly (rI). poly(rC) at concentrations much below that at which interferon production can be detected (Colby & Morgan, 1971). However, the anti-viral effect is thought to be mediated by interferon (Vengris et al. 1975).

VERO cells respond poorly to the anti-viral action of poly(rI). poly(rC) in the presence of DEAE-dextran while D1 cells respond well and also produce interferon (Tables 32 & 33). VERO-D1 hybrids might be expected to show a reduced sensitivity to poly(rI). poly(rC) compared with D1 cells if the VERO chromosomes present include one coding for a repressor of interferon production.

Hybrid clones and parental cells were seeded in 25 well trays in growth medium as for the interferon sensitivity

tests. Poly(rI). poly(rC) treatment was as described (Methods) and the monolayers were challenged with EMC or Sindbis virus. The cytopathic effect of EMC virus infection was estimated visually after 24h incubation but all dye uptake assays were carried out 48h after addition of virus (Methods).

The cytopathic effect of EMC virus was prevented more effectively by poly(rI). poly(rC) treatment than by interferon treatment and monolayers could be incubated for 48h without cell damage occurring. The concentration of interferon at the cell surface after poly(rI). poly(rC) treatment is presumably very high.

Table 34 shows the anti-viral response of all clones to treatment with 100µg/ml of poly(rI). poly(rC) in the presence of DEAE-dextran (100µg/ml). The data for a number of clones is given in detail in Table 32.

All clones were found to be more responsive to treatment with poly(rI). poly(rC) than VERO cells except clone X1. This clone was insensitive both before and after its loss of ability to grow in selective medium and this data taken together with information on the chromosomes, isozymes and interferon sensitivity of clone X1 supports the idea that it is a mutant of VERO cells.

Clones II1, and VII1 and 3 were less sensitive to the anti-viral action of 10µg/ml of poly(rI). poly(rC) than D1 cells. This might be because of the presence in these cells of a monkey chromosome carrying a gene for the repression of interferon synthesis and hence of the poly(rI). poly(rC) induced anti-viral state.

The ability of a hybrid clone to respond to the anti-viral action of poly(rI). poly(rC) was not related to human

Table 32. The induction of an anti-viral state by poly(rI). poly(rC)

Expt.	Cells	% dye uptake a	
		Concentration of poly(rI). poly(rC) (μg/ml)b	
		10	100
1	III1	- d	86
	" 2	-	82
	V2	-	81
2	II1	50	67
	VII1	65	82
	" 2	90	79
	" 3	60	92
	VERO	0	7
	D1	102	105
3	X1	-	6
4	XI4	-	81
	VERO	-	11
5	X1*	-	5
	XI4 c	-	43

a Methods; Sindbis virus challenge

b In the presence of 100 μg/ml of DEAE-dextran

c After loss of response to the anti-viral action of human interferon

d Not tested

interferon sensitivity. Clone XI4 was able to respond to poly(rI). poly(rC) before and after loss of sensitivity to human interferon, although the response in the latter case was less.

3. Interferon production after treatment with poly(rI). poly(rC)

VERO cells are unable to produce interferon in response to virus infection (Section I, Table 2), or to treatment with poly(rI). poly(rC) (Table 33). D1 cells, on the contrary, produce anti-viral activity in response to poly(rI). poly(rC) treatment and to virus infection (Methods & Table 33).

Medium was harvested 24h after treatment of cells with 100µg/ml poly(rI). poly(rC) in the presence of DEAE-dextran (from experiments to determine the anti-viral state) and assayed for anti-viral activity on mouse and primate cells. The dye uptake method was used with Sindbis virus challenge (Methods).

Initially, undiluted samples were assayed on D1 and VERO cells. However, it was found that medium from uninduced control cells protected D1 cells against the cytopathic effect of Sindbis virus. Results were calculated relative to cultures treated with control medium but are tentative only (Table 34, results in parentheses). Mouse L cells were used subsequently as these were not protected by control samples. This problem was not found with VERO cells but as these cells are less sensitive to primate interferon than are human embryo cells (Section I, Tables 2 & 5; Desmyter & Stewart, 1976) it was decided to use the latter cells so that small amounts of primate interferon could be detected.

The data for samples assayed on Mouse L cells and human

Table 33. Interferon production in response to poly(rI). poly(rC)

Cells	% dye uptake d			
	Assay cells			
	Assay	Mouse L	Assay	Human Embryo
	1 b		2 b	
XI1		86		0
"3		78		0
"4		64		0
"5		82		0
"6		44		0
"7		74		0
D1		67		0
VERO		0		0
VII1	3 c	39	3 c	4
" 2		25		0
" 3		25		0
VIII1		71		0
IX1		76		0
X1		0		10
X14 a		67		0
"5 a		88		0
X1*	4 b	0	4 b	15
XI4		54		2

a Repeat assay of samples from Expt. 1

b 5-fold sample dilutions

c 4-fold sample dilutions

d Methods; Sindbis virus challenge

embryo cells is given in detail in Table 33. All samples assayed showed anti-viral activity on mouse cells except for those from VERO cells and clone X1 (Tables 33 & 34). Clone X1 cells therefore resemble VERO cells in yet another property. Samples from clones VII1 and VII3 had less anti-viral activity on mouse L cells than samples from other cells. These clones were also less sensitive to the anti-viral action of 10µg/ml of poly(rI). poly(rC) than were D1 cells (Table 32), and it is suggested that they might contain a monkey chromosome carrying a gene for the repression of interferon synthesis. No firm conclusions can be drawn until the relative titres of the samples have been determined.

Assays carried out on human embryo cells revealed slight anti-viral activity in samples from clone X1 cells. No anti-viral activity on primate cells was detected in any other sample, including that from VERO cells. Thus it appears that in contrast to VERO cells, clone X1 cells do synthesise a primate interferon-like substance. However, this finding does not correlate with the lack of anti-viral resistance found after treatment of these cells with poly(rI). poly(rC). The anti-viral activity is unlikely to result from the action of residual inducer on the human embryo cells as such activity was not found in samples from other clones.

Clone XI4 cells did not produce anti-viral activity detectable on human cells when tested at a time when they were sensitive to human interferon. This is consistent with the assignment of the genes responsible for interferon production and action to separate monkey chromosomes (Cassingena et al. 1971), but does not confirm it as clone XI4 may have contained the chromosome governing monkey interferon production but with the interferon gene inactive.

For some clones of group X1 the production of anti-viral activity is shown but not the anti-viral effect produced in response to poly(rI). poly(rC) (Table 34). The cytopathic effect of Sindbis virus was variable in these cells and no conclusions could be drawn as to virus resistance.

E. The cytopathic effect of viruses

1. Picornaviruses

Both VERO and D1 cells are sensitive to the cytopathic effects of Mengo and EMC viruses. However plaque formation by Mengo virus on VERO cells required 72h incubation as compared with 48h on D1 cells. Dubois & Chany (1976) showed that the CV1 line of African Green monkey cells produced less infectious EMC virus than did mouse kidney cells. Thus primate cells may be less susceptible than mouse cells to picornavirus infection.

All of the hybrid clones tested were sensitive to EMC virus cytopathic effect except clone I5. This clone was resistant to 0.3 p.f.u. per cell of EMC virus. Interestingly clone I5 cells, treated with 100µg/ml each of poly(rI). poly (rC) and DEAE-dextran showed a toxic effect when subsequently infected with EMC virus and examined 48h later for estimation of the induced virus resistance. (Dye uptake was reduced to 71% of that of control cells).

This effect may be analogous to the cytotoxic effect of interferon and double stranded RNA treatment of cells observed by Stewart *et al*. (1972a). However treatment of clone I5 cells with mouse and human interferons (in an attempt to measure the anti-viral effect) did not result in a toxic effect after infection with EMC virus.

2. Togaviruses

VERO cells were sensitive to the cytopathic effect of Semliki Forest virus, and of Sindbis virus grown in chick, VERO and mouse cells. Only infection with D1 cell-grown Sindbis virus lead to a cytopathic effect in D1 cells. (Mouse L cells were susceptible to the cytopathic effects of all Sindbis virus preparations).

Most hybrid clones resembled D1 cells in their susceptibility to infection with Sindbis virus. However, clone X1, XI2 and XI4 cells were sensitive to the cytopathic effects of mouse L cell-grown Sindbis virus and clones I4 and I10 cells to VERO and chick cell-grown Sindbis virus respectively. Clone I1 cells differed from D1 cells in not being susceptible to the cytopathic effect of D1 cell-grown Sindbis virus but were killed by EMC virus infection (Table 34).

The membrane composition of togaviruses varies with the cells on which they are grown (Pfefferkorn & Shapiro, 1974). This may explain the differing abilities of Sindbis virus preparations to infect and cause a cytopathic effect in D1 cells and in the hybrid clones.

Summary

A number of clones have been isolated in HAT medium containing ouabain (10^{-5} or 10^{-6}M) from mixtures of VERO and D1 cells treated with inactivated Sendai virus or polyethylene glycol. All but one of these clones were presumed to be hybrids of VERO and D1 cells because they continued to grow in selective medium and were killed by fluorescent light treatment after growth in BUdR-containing medium.

Clone X1 lost its ability to grow in selective medium and

Table 34. Summary of hybrid clone properties

Interferon sensitivity				Poly(rI). poly(rC) sensitivity	Interferon production Assay cells			Isozymes		Virus sensitivity
Clone	a Human		Mouse	a	b	Human	VERO	Mouse	LDH	G6PDH	
I1				E/DU +						Hybrid? + Mouse	S (D1) Insens. c
2,3				" +						Mouse	
4				" +						Mouse	S (VERO) Sens. c
5									Mouse	Mouse	EMC Insens. c
7	E/CPE	-	+	E/DU +							
8	"	-	(+)	" +							
9	"	-	-	" +							
10	"	-	(+)	" +							
11	"	-	+	" +							S (CEC) Sens. c
II1	S/DU	-	+	S/DU +	0		-	(+)	Mouse	Mouse	
III1,2	"	-	+	" +	0		-	(+)		Mouse	
IV2,3	E/CPE	-	(+)	E/CPE +	0		-	(+)			

4									Mouse	
5	E/CPE	-	(+)	E/CPE	+	0		-	(+)	
6	"	-	+	"	+	0		-	(+)	
V1	"	(+)	+	"	+	0		-	(+)	
2	S/DU	-	+	S/DU	+	0		-	(+)	
VI3	E/CPE	(+)	+	E/CPE	+	0		-	(+)	
VII1,2	S/DU	-	+	S/DU	+	4	-		+	Mouse
3	"	-	+	"	+	4	-		+	
VIII1	"	-	+	"	+	4	-		+	Mouse
IX1	"	-	+	"	+	4	-		+	Mouse
X1	"	+	(-)	"	-	4	(+)		-	Monkey
1*	"	(-)	-	"	-	5	(+)		-	
XI1						5	-	-	+	
2	S/DU	-	+							
3						5	-	-	+	
4	S/DU	+/-	+	S/DU	+	5	-	-	+	
6-8						5	-	-	+	

a Method; E = EMC virus challenge; S = Sindbis virus challenge; CPE = cyto
DU = dye uptake

b Sample dilution factor

c Sens. = sensitive; Insens. = insensitive; S = Sindbis virus, host cells i

its response to the anti-viral action of interferons, and of poly(rI). poly(rC), resembled that of VERO cells. Examination of the chromosomes and isozymes of this clone indicated that it was a derivative of VERO cells as did its VERO cell-like morphology. It was slightly more resistant to the toxic effect of ouabain, than VERO cells and may thus be a mutant of the VERO cell line with an altered ATPase.

All other clones were sensitive to the anti-viral action of poly(rI). poly(rC) and of mouse interferon. In response to poly(rI). poly(rC) treatment a mouse-specific anti-viral substance was produced but no anti-viral activity could be detected on primate cells. Only clone XI4 (other than clone X1) was sensitive to the action of human interferon. Preliminary chromosomal analysis revealed the presence of at least two putative monkey chromosomes in cells of this clone.

Certain clones (II1, VII1 and VII3) were found to be less sensitive to the anti-viral action of poly(rI). poly(rC) than D1 cells, and appeared to produce less mouse-specific anti-viral activity. The possibility that a VERO chromosome, carrying a gene coding for a repressor of interferon synthesis, is present in these clones should be examined further.

Susceptibility to the cytopathic effect of Sindbis virus preparations grown on cells of different species varied among the clones. Clone I5 was unusual in being insensitive to the cytopathic effect of EMC virus unlike both VERO and D1 cells. The reason for this should be determined as should the reason for the toxicity observed in cells of this clone after treatment with poly(rI). poly(rC) and DEAE-dextran followed by infection with EMC virus.

All hybrid clones retained an ability to synthesize mouse interferon and to establish an anti-viral state after treatment with poly(rI). poly(rC) and no clones were able to produce detectable primate interferon. However, more detailed analysis of the anti-viral response of clones to different concentrations of poly(rI). poly(rC) and assay of the titre of anti-viral activity produced on the most sensitive cells available might reveal the presence of a monkey repressor of interferon synthesis.

DISCUSSION

A. Interferon Induction in VERO cells

The VERO cell line was chosen for study because it is unable to make interferon (Desmyter et al. 1968) and may represent a regulatory mutant (Introduction). Several workers have confirmed this observation (Schafer & Lockart, 1970; De Clercq & De Somer, 1973; Rodgers & Merigan, 1974) but Kohno et al. (1972) found that two strains of VERO cells, out of three tested, were able to produce small amounts of interferon. Assuming that cross-contamination had not occurred it is possible that variant, inducible sub-strains had arisen during passage in culture.

1. Virus infection

VERO cells were shown to be unable to produce detectable interferon in response to different multiplicities of infecting Sindbis virus (Section I, Table 2). However, infection with this virus induced interferon synthesis by BSC.B and LLC-MK_2 cells though rather less in the former. Human embryo cells (HES.A) were more sensitive to the anti-viral action of monkey interferon preparations than were VERO cells but their use as assay cells did not allow the detection of anti-viral activity in samples from VERO cells (Section I, Table 2).

2. Poly(rI). poly(rC)

VERO cells may be unable to synthesize interferon in response to virus infection because the inducing molecule is degraded. However, treatment with poly(rI). poly(rC) in the presence of DEAE-dextran, which is thought to give protection against degradation (Pitha & Carter, 1971), resulted in only

a slight anti-viral effect and no interferon production (Section I, Tables 3-5; Section V, Tables 32 & 33). The strain of VERO cells used therefore resembles the non-inducible strains reported by most workers.

The toxic interaction of poly(rI). poly(rC) with interferon-treated L929 cells (Stewart et al. 1972a) was not found to be a general phenomenon and could not be used to investigate the effects of poly(rI). poly(rC) on VERO cells (Section IIB).

De Clercq & De Somer (1973) reported that BSC.1 cells (equivalent to BSC.B cells) were sensitive to the anti-viral action of 10µg/ml of poly(rI). poly(rC) and human skin fibroblasts could be protected against virus infection by treatment with as little as 0.004µg/ml. However treatment of BSC.B cells with poly(rI). poly(rC) alone was only significantly effective at 100µg/ml and human embryo cells required treatment with a minimum of 2µg/ml (Section I, Table 8). Thus the poly(rI). poly(rC) preparation used for interferon induction may have been of low potency but sub-clones of a cell line and different batches of embryonic cells may vary in ability to produce interferon (Ho, 1973b).

Mycoplasma contamination has been reported to increase and to decrease interferon production by cultured cells (Stanbridge, 1971) but the number of different reports that VERO cells are non-inducible make it unlikely that such contamination would be the explanation for the defect. Furthermore, BSC.B cells responded to poly(rI). poly(rC) treatment with an anti-viral state while contaminated and SPS-treated VERO cells (Methods) were no more sensitive to the action of interferon inducers than untreated cells. The

effects of possible latent virus infection cannot be excluded.

Treatment with DEAE-dextran alone enhanced dye uptake by some cells, possibly by stimulating cell metabolism (Section I B & D). In other cases the presence of DEAE-dextran, in addition to poly(rI). poly(rC), reduced the inhibition of plaque formation which resulted from poly(rI). poly(rC) treatment alone but plaque size was reduced (Section I, Table 7). These inconsistent effects were not observed with VERO and D1 cells and do not complicate the interpretation of their response to interferon inducers.

B. Enhancement of Interferon Production

The slight anti-viral response of VERO cells to treatment with high inducer concentrations might be unrelated to interferon production (Section IIA). Alternatively it could be an indication of an altered regulatory molecule eg. an inducer-insensitive 'repressor' or ineffective 'activator' of interferon gene expression. A similar situation is found with the Chinese hamster cell line, CHO, which does not produce interferon but does develop slight virus resistance when treated with high concentrations of poly(rI). poly(rC) in the presence of DEAE-dextran (Morgan, 1976). CHO-human hybrid cells which contain human chromosome 18 are able to produce hamster interferon, suggesting that CHO cells are regulatory variants (Morgan & Faik, 1977).

A number of treatments can enhance interferon production and it was hoped that their application to VERO cells might amplify the slight anti-viral response (Section IIA).

1. Interferon pretreatment

Pretreatment of cells with interferon can lead to increased interferon production in response to induction (Stewart et al. 1971a) but this could not be demonstrated with VERO and BSC.B cells (Section II, Table 9). Because of the failure of BSC.B cells to synthesize interferon no conclusions could be drawn about the state of the interferon gene in VERO cells. In response to Sindbis virus infection BSC.B cells produced less anti-viral activity than LLC-MK_2 cells (Section I, Table 2) but Desmyter et al. (1968) found that NDV infection resulted in similar yields of interferon from BSC.1 cells and human skin fibroblasts. Cell lines derived from the African Green monkey are not therefore inherently poor producers of interferon.

2. Superinduction and poly(rI). poly(rC) pretreatment

The failure to demonstrate appreciable enhancement of the anti-viral state or of interferon production by human embryo cells after pretreatment with poly(rI). poly(rC) or superinduction by anti-metabolites (Section II, Tables 10 & 11) shows the difficulties of using treatments whose mechanism of action is not known. A negative result can only be taken as meaningful when a wide range of conditions have been tested.

3. Steroid hormones

A slight enhancement of the anti-viral state induced by poly(rI). poly(rC) in human embryo cells (HES.A) was found after steroid hormone treatment (Section II, Table 12). De Maeyer & De Maeyer (1963) showed that Sindbis virus-induced interferon production in a rat tumour cell line was diminished by treatment with comparable amounts of cortisol

or a testosterone derivative. Steroid hormones, in particular androgens, can enhance overall macromolecular synthesis in target organs (Karlson et al. 1975) whereas interferon production is favoured by the slowing down of such synthesis (eg. in 'aged' cells, Carver & Marcus, 1967).

The anti-viral action of interferon in the rat cells was, however, enhanced by steroid hormone treatment (De Maeyer & De Maeyer, 1963). The development of an anti-viral state in poly(rI). poly(rC) treated cells apparently depends on both interferon production and action (Introduction) and the slight stimulatory effect of steroid hormone treatment on the anti-viral state induced in HES.A cells (Section II, Table 12) was probably a balance of the effects on these two processes.

The lack of an effect in VERO cells is not informative. These cells were isolated from kidney, a target organ for androgens (Karlson et al. 1975) but may have 'dedifferentiated' during passage in vitro or have arisen from connective tissue cells. The human embryo cultures were of mixed origin and it is likely that steroid hormone-sensitive cells would be present.

C. Isolation of Inducible VERO Revertants

The reason for the absence of interferon production by VERO cells is a matter of conjecture. It is unlikely that the animal from which the cells were isolated was unable to produce interferon as it would probably have succumbed easily to infection. If the lack of interferon production is a result of changes in the interferon structural genes two mutations would have had to occur or loss of the relevant

chromosomes. Such multiple events are more likely to occur during *in vitro* passage as cultured cells undergo karyotypic changes during establishment as continuous cell lines (Terzi, 1974). Early stock cultures of VERO cells might contain 'wild-type' inducible cells which would be valuable as controls for the non-inducible strains in genetic and biochemical experiments.

A single change in a regulatory locus or an epigenetic change would explain more simply the loss of function of two structural genes. The selection procedure described (Section IIIA) was devised to isolate inducible revertants of the VERO cell line. Because the anti-viral state induced by poly(rI). poly(rC) is not permanent infection with a temperature-sensitive virus was necessary to allow the removal of the selective pressure by changing the incubation temperature.

MNNG, the mutagen used, causes transitional changes in DNA (Legator & Flamm, 1973). The failure to isolate revertants of VERO cells suggests that the mutational event necessary to restore inducibility is not a transition or that genetic material, perhaps the interferon structural genes, is deleted from these cells. A number of practical problems also complicated the selection of inducible revertants of the VERO cell line (Section III).

D. Somatic Cell Genetics

The information which could be gained by the study of somatic cell hybrids of VERO cells and cells able to produce interferon has been discussed (Introduction). Briefly, such experiments would show if the monkey interferon genes

in VERO cells could be activated or if an altered regulatory molecule could inhibit the expression of interferon genes in a heterologous genome.

Because of the close evolutionary relationship of the monkey and man it seemed likely that monkey-mouse hybrid cells would share the advantages of human-mouse hybrid cells such as isozyme and karyotype differences and the preferential loss of the primate chromosomes (Ruddle & Creagan, 1975). Also mouse and primate interferons did not show heterologous activity on VERO and D1 cells (Section I, Table 5) thus allowing differentiation of the products of the two genomes.

1. Selective systems

Mouse D1 cells lacked TK and could be used directly for hybrid selection in HAT medium. Suitable $HGPRT^-$ or TK^- mutants of VERO, BSC.B or LLC-MK_2 cells could not be isolated (Section IV). Such mutants of BSC.B and LLC-MK_2 cells would have allowed control fusions to be carried out.

The difference in sensitivity of primate and mouse cells to ouabain (Kucherlapati et al. 1975; Sections IV & V) enabled intermediate concentrations of this drug to be used with HAT medium for the isolation of VERO-D1 hybrid cells. Because exposure to ouabain kills cells containing the sensitive monkey ATPase hybrid cells having lost both genes for this enzyme would probably have a selective advantage in medium containing intermediate ouabain concentrations. Should this gene be linked to that coding for monkey interferon hybrid cells re-expressing the VERO interferon gene might be lost because of their selective disadvantage.

A system which selects for the presence of certain

chromosomes is preferable but such a system often requires mutant parental cells (eg. amino acid auxotrophs, Ruddle & Creagan, 1975). Treatment of VERO cells with mutagens was to be avoided to prevent possible additional mutations occurring in the genes of the interferon system.

2. Cell Fusion

PEG-induced cell fusion was as effective as Sendai virus treatment in increasing the yield of colonies growing in selective medium over the spontaneous frequency (Section IV B). PEG fusion of monolayer cultures gave the greatest increase over the spontaneous rate of fusion but for practical purposes PEG treatment of suspended cells was more convenient.

The frequency of colony formation in selective medium was comparable with that found by other workers using rodent and primate parental cells and selection with HAT medium (Section IV, Table 27).

Colony formation in selective medium after Sendai virus-induced fusion of VERO and D1 cells at a ratio of 3:1 respectively was not quantitated. Hybrid clones derived from such fusions did not differ markedly from others except for clone I5 which was resistant to the cytopathic effect of EMC virus (Section V E). Increasing the ratio of VERO to D1 cells during fusion would be expected to give rise to some hybrid cells initially containing more than one VERO genome. Such hybrid cells might have a phenotype more closely resembling that of VERO cells as differentiated functions are often not extinguished when tetraploid differentiated cells are fused with diploid control cells (Davidson, 1974).

E. Characterisation of hybrid clones

1. Chromosomes

Parental continuous cell lines with their karyotypic variability complicate chromosomal analysis of hybrid cells. Differential staining of human and mouse chromosomes by the G11 technique allowed the detection of a small number of human chromosomes in hybrid cells (Bobrow & Cross, 1974) but such differences in staining could not be demonstrated in chromosome preparations from VERO and D1 cells (Methods; M. Bobrow, personal communication). The differential staining of mouse and chimpanzee chromosomes was reported by Friend, Chen & Ruddle (1976) and suggests that suitable conditions were not determined for the mouse and monkey chromosome preparations. A preliminary indication of the number of monkey chromosomes in a hybrid cell can be gained by normal Giemsa staining as the chromosomes of mouse D1 cells are largely telocentric and those of VERO cells metacentric or submetacentric (Section V, Figs. 13 & 14). At least two chromosomes similar to those of VERO cells were found in clone XI 4 cells (Section V, Fig. 15).

2. Isozymes

The apparently random segregation of primate chromosomes from rodent-primate hybrid cells (Ruddle & Creagan, 1975) makes the detection of primate isozymes a matter of chance. The search for monkey isozymes in such hybrid cells may be aided by a knowledge of the gene markers for human chromosomes assuming that the monkey equivalents can be distinguished from those of the mouse. It is not unlikely that gene linkage is broadly similar in different primates (Ruddle & Creagan, 1975; Orkwiszewski *et al*. 1976). The

presence of monkey TK in some hybrid clones was inferred from their regained sensitivity to BUdR-containing medium (Section V, Table 29). Other isozymes tested had the electrophoretic mobility characteristic of the mouse enzymes except for those of clone X1 and G6PDH of clone I1.

Other distinguishing features of primate cells which could be used to characterise hybrid cells are diptheria toxin sensitivity, surface antigens and polio virus susceptibility (Ruddle & Creagan, 1975). Preliminary experiments (not shown) demonstrated that VERO cells were more sensitive to diptheria toxin than mouse D1 cells. Diptheria toxin sensitivity is determined by a gene on human chromosome 5 (Ruddle & Creagan) as is human interferon production (Tan et al. 1974a; Morgan & Faik, 1977). If these traits are linked in the African Green monkey VERO cells may contain the chromosomes normally carrying the monkey interferon genes.

3. Interferon sensitivity

Only one clone, other than the suspected VERO cell mutant X1, responded to human interferon (Section V, Table 31). Because of the preferential but random loss of primate chromosomes from rodent-primate hybrid cells it would not be expected that many clones would have this property. In addition hybrid cells containing only one copy of the necessary gene(s) might be less sensitive than the parental VERO cell line to human interferon and VERO cells respond less well to the anti-viral action of primate interferons than do human embryo cells (Section I, Table 2 ; Desmyter & Stewart, 1976). Chany (1976) reported that a mouse-human hybrid clone containing only human chromosome 21 was

insensitive to the action of human interferon. This conflicts with the assignment of sensitivity to human interferon to this chromosome (Tan et al. 1973). However, the presence of any other human chromosome in hybrid cells, in addition to chromosome 21, allowed an anti-viral state to develop. Chany (1976) suggested that the presence of additional human surface antigens allowed the expression of a hypothetical interferon receptor. Such surface effects might obscure the expression of a similar gene in VERO-mouse hybrid cells.

4. Poly(rI). poly(rC)

The ability of all clones except X1 to develop an anti-viral state in response to treatment with poly(rI). poly(rC) must be a result of their ability to make and respond to mouse interferon (Section V, Tables 31-33). Certain clones, treated with 10μg/ml of poly(rI). poly(rC), developed a lesser anti-viral state than control D1 cells perhaps indicating inhibition of mouse interferon gene expression by the presence of certain VERO cell chromosomes. No examples of the extinction of interferon production were found and so such cells may be the only evidence for the existence of a monkey repressor of interferon gene expression. Other clones should be tested for their ability to respond to low concentrations of poly(rI). poly(rC).

The haploid chromosome number of the African Green monkey is 30 (Stock & Hsu, 1973) and since on average seven primate chromosomes are retained in rodent-primate hybrid cells (Ruddle & Creagan, 1975) it might be expected that at least one hybrid clone with the monkey chromosome carrying the interferon gene would have been isolated. However, this assumes random chromosome loss and does not allow for the

possible directed chromosome loss induced by growth in ouabain-containing medium.

No conclusive evidence for monkey interferon production by any hybrid cells was obtained and the results support the view that the interferon gene is inactive or deleted in VERO cells.

5. Virus susceptibility

The lack of a cytopathic effect (CPE) in cells exposed to virus may be a result of a failure of the virus to infect the cells or cellular resistance to the mechanism of cell killing. The variable susceptibility of D1 cells to Sindbis virus preparations was probably a result of resistance to killing as a Sindbis virus preparation grown in these cells without noticeable CPE was found to kill mouse L cells (not shown). However, the lipid composition of Togaviruses varies with the host cell (Pfefferkorn & Shapiro, 1974) and inefficient adsorption may play a part in the Sindbis virus-resistance of D1 cells. The selection of D1 cells from mouse L cells by growth in BUdR-containing medium (Dubbs & Kit, 1964) resulted in chromosomal loss (Migeon et al. 1969) and possibly mutations which could have contributed to the resistance to Sindbis virus CPE. The variable susceptibility to Sindbis virus CPE of hybrid clones derived from D1 cells is not surprising (Section V, Table 34).

The resistance of clone I5 to EMC virus CPE may be a result of the loss of a surface receptor for this virus (Levintow, 1974) or resistance to CPE. Cellular RNA and protein synthesis are inhibited during EMC virus infection and virus-specific dsRNA has been implicated in the latter (Levintow, 1974). However, cellular degradation can occur

without the formation of dsRNA and other factors must therefore be involved. The fortuitous isolation of a cell line expressing constitutively the anti-viral state would be very interesting. It remains to be determined whether clone I5 is resistant to infection by other viruses and able to produce interferon.

F. Interferon Gene Expression-Further Approaches

The relatively high proportion of tetraploid VERO cells present in a normal population (Section V, Fig. 12) suggests that it might be possible to isolate a tetraploid sub-clone and to test hybrids of such cells with mouse cells for inducibility of interferon production. Increased dosage of the VERO genes coding for the hypothetical repressor would be expected to prevent interferon induction.

If hybrids of CHO cells with monkey cells (eg. BSC.B) are found to have regained inducibility for hamster interferon production, as have certain CHO-human hybrid cells (Morgan & Faik, 1977), the response of VERO-CHO cell hybrids would be interesting. Activation of the CHO cell interferon genes would suggest, but not prove, that the structural genes for monkey interferon were inactive in VERO cells.

Two-dimensional electrophoresis has been used to separate the chromosomal proteins of cells expressing the globin genes and of non-induced cells with significant differences being found in only six such proteins (Peterson & McConkey, 1976). Similarly chromatin from interferon-synthesizing cells and non-induced cells could be analysed for the presence of regulatory proteins.

Purification of interferon mRNA is now theoretically

possible as fractions can be assayed in cell-free systems for the ability to direct interferon synthesis (Reynolds et al. 1975). DNA complementary to interferon mRNA (cDNA) could then be synthesized by the use of 'reverse transcriptase' (Temin & Baltimore, 1972) and used as a 'probe' to detect interferon-specific DNA or RNA sequences in non-inducible (eg. VERO) or non-induced cells. Deisseroth et al. (1976) hybridised globin cDNA with the DNA and RNA of mouse-human hybrid cells in which mouse globin gene expression was no longer inducible and showed that although globin-specific DNA sequences were present no globin mRNA was made after induction.

The presence of 'pre-formed', inactive interferon in non-induced cells or the synthesis by VERO cells of a defective protein cannot yet be tested. However, progress in the preparation of antibodies to purified interferon (Lockart, 1973) should allow the detection of cross-reacting material lacking the ability to protect cells against virus infection.

G. Regulation of Interferon Gene Expression

The available evidence supports the idea that interferon induction results from new interferon mRNA synthesis (Introduction). It was suggested that a labile repressor protein prevented transcription from the interferon gene unless inducer was present and that the action of the inducer was to inhibit repressor production (Tan & Berthold, 1977).

An analysis of E.coli mutants, constitutive or non-inducible for the synthesis of the lactose catabolism enzymes, led to the proposal of the operon hypothesis (Jacob

& Monod, 1961) and the subsequent biochemical analysis of the regulatory molecules and genetic loci involved (Goldberger, Deeley & Mullinix, 1976). Similar mutants (eg. of interferon production) would allow this approach to be extended to animal cells.

Mouse cells selected for virus-resistance (Morgan, Colby & Hulse, 1973) may express constitutively the anti-viral state though not interferon production. The VERO and CHO cell lines are non-inducible for interferon production (Desmyter et al. 1968; Morgan, 1976).

Cell hybridisation studies showed that human chromosome 18 coded for a function which restored inducibility to the CHO cell genome (Morgan & Faik, 1977) and this chromosome may carry a gene for an activator of interferon gene expression or a molecule involved in processing or transport of the inducer.

Monkey interferon production by VERO cells or by VERO-mouse hybrid cells could not be demonstrated (Sections I-III & V) and this suggests the absence of the monkey interferon genes. Slight evidence for a super repressor in VERO cells was the lessened anti-viral state induced in some VERO-mouse hybrid cells when a lower concentration of inducer was tested (Section V, Table 32) and the slight anti-viral state which developed in VERO cells in response to treatment with high concentrations of inducer (Section I, Tables 4 & 5).

The selection of cells resistant to the toxicity of interferon and poly(rI). poly(rC) treatments (Stewart et al. 1972a) or to treatment with poly(rI). poly(rC) alone (Billiau et al. 1972b) from cell lines which are known to possess active interferon genes might result in the isolation

of different classes of non-inducible variant cells. Somatic cell genetic analysis of such variants should lead to an increased understanding of the regulation of eukaryotic gene expression.

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THE REGULATION OF INTERFERON SYNTHESIS IN ANIMAL CELLS

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ABSTRACT

The inability of the VERO cell line to produce interferon suggested that it might represent a regulatory variant which would be useful for somatic cell genetic studies of the regulation of interferon production.

It was confirmed that VERO cells are unable to produce interferon in response to virus infection whereas BSC.B cells (similarly derived from the African Green monkey) were able to do so. Treatment of a number of cell lines and embryonic cells with polyriboinosinic. polyribocytidylic acid (poly(rI). poly(rC)) (usually in the presence of diethylaminoethyl (DEAE)-dextran) resulted in the development of an anti-viral state but VERO cells were found to be only weakly responsive. No effective method for significantly enhancing the anti-viral action of poly(rI). poly(rC) or interferon production was found.

The cytotoxic effect of interferon and poly(rI). poly(rC) treatment was found to be limited to L929 cells and thus could give no information on the non-inducibility of VERO cells.

Mutagenized VERO cells surviving selection by infection with temperature-sensitive Sindbis virus after treatment with poly(rI). poly(rC) were similar to VERO cells in their ability to support the growth of wild-type or temperature-sensitive Sindbis virus or to respond to poly(rI). poly(rC) treatment and they did not release virus or interferon into the medium, VERO cells cannot therefore give rise to inducible revertants.

HAT medium containing ouabain was found to be suitable

for the selection of hybrids of VERO cells with mouse D1 cells lacking thymidine kinase. Mouse D1 cells are able to produce interferon. Polyethylene glycol treatment effectively replaced treatment with inactivated Sendai virus for the promotion of cell fusion.

All VERO-mouse hybrid clones examined were sensitive to the anti-viral action of poly(rI). poly(rC) treatment and many were shown to produce mouse interferon, however, none were able to produce monkey interferon. Certain clones were less sensitive than mouse D1 cells to the anti-viral action of poly(rI). poly(rC) and this may have been an indication of the activity of a VERO cell-coded repressor of interferon production. Thus there is no evidence that VERO cells are able to produce interferon and only slight evidence for an altered regulatory function in these cells.