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EFFECT OF HUMAN INTERFERON PREPARATIONS ON *IN VITRO* ACTIVATION OF LYMPHOCYTES FROM NORMAL AND DOWN'S SYNDROME INDIVIDUALS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, the thesis entitled "EFFECT OF HUMAN INTERFERON PREPARATIONS ON *IN VITRO* ACTIVATION OF LYMPHOCYTES FROM NORMAL AND DOWN'S SYNDROME INDIVIDUALS" submitted by Claire G. Cupples in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Peripheral blood lymphocytes from people with Down's syndrome (trisomy of chromosome 21) have been compared with lymphocytes from normal (disomic 21) people for their sensitivity to human interferon preparations. The trisomic 21 lymphocytes, whether stimulated with phytohaemagglutinin (PHA) or by allogeneic cells, are more sensitive than the normal lymphocytes to inhibition of DNA synthesis by both fibroblast and leucocyte interferons. The presence of an extra chromosome does not affect the sensitivity of the trisomic 21 lymphocytes to the antigenic or mitogenic stimuli.

Kinetic studies show that maximum inhibition of DNA synthesis occurs when interferon is added just before, or at the same time as lymphocyte stimulation. Addition of interferon after stimulation results in less inhibition; the longer the period between stimulation and interferon addition, the smaller the final level of inhibition.

A new, more sensitive bioassay has been developed for measuring the inhibitory effect of interferon on PHA stimulated lymphocytes. The lymphocytes are grown in soft agar, so that each activated cell can produce a discrete clone of progeny. Preliminary results indicate that interferon inhibits initial lymphocyte activation rather than subsequent clonal proliferation.

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I. INTRODUCTION

1.01 Historical Background

In 1957, Isaacs and Lindenmann discovered that a substance released from influenza virus infected chick cells could protect uninfected cells of the same species from subsequent viral challenge (Isaacs and Lindenmann, 1957; Isaacs *et al.* 1957). They named the substance *INTERFERON* since it seemed to explain viral interference: the resistance of a virus infected cell to infection by other viruses. It was soon found that interferons are produced by virtually all virus infected animal cells, that they are not virus specific (they can protect cells from attack from a wide variety of viruses), and that they are usually species specific (interferon produced by one species of animal or by cells from that animal can only protect animals or cells of the same species); (for reviews see: Finter, 1973; Metz, 1975a; Burke, 1977). The lack of viral specificity combined with the fact that human interferon can be produced in tissue culture, and is non-toxic to uninfected cells, immediately lead to hopes that it would be of great value clinically in the treatment of many types of viral disease. However, because interferon has an extremely high specific activity (estimated at 10^9 antiviral units per mg of protein - Ng and Vilcek, 1972) and is produced by cells in very small amounts, it has not been possible in the past to produce enough human interferon for purification and clinical trials. It is only recently that a growing understanding of the genetics and regulation of interferon synthesis has made increased production possible. As larger amounts become available, therapeutic use of interferon will perhaps be feasible; limited clinical trials with partially purified interferon have already indicated that interferon can be used, with varying degrees of success, in the treatment of a number of virus diseases, e.g. Hodgkin's disease (Blomgren *et al.* 1976), hepatitis B (Greenberg *et al.* 1976), and dendritic keratitis (Sundmacher, 1976).

The discovery that interferons can also inhibit the growth of transplantable tumours in mice (Gresser *et al.* 1969) and the multiplication of tumour cells *in vitro* (Gresser *et al.* 1970), and can suppress the immune system (Braun and Levy, 1972) has lead to the first clinical use of interferon in the treatment of some types of human cancer (Strander *et al.* 1974). The main problem to be overcome if interferon is to be used in cancer therapy is the ability of some tumour cells to become resistant to interferon after prolonged exposure.

Interest in interferon extends far beyond its potential clinical applications. Research has been done on many aspects of the antiviral effect of interferon, for example: the action of interferon inducers, the genetics of interferon production and response, examination of the antiviral state in sensitive cells, and a search for the precise step at which interferon inhibits virus replication. Continuous effort has been made to purify interferon for chemical characterization. Recently, attention has been focussed on the cell multiplication inhibitory ("anticellular") action and the immunosuppressive effects of interferon.

1.02 Interferon Production and Characterization

Several human cell types are used for large scale production of human interferon. Most commonly, leucocytes (buffy coats) from whole blood are placed in culture and stimulated to produce interferon by exposing them to Sendai virus. The culture supernatant, when freed of contaminating cells and inducer virus particles, contains unpurified leucocyte interferon (Cantell *et al.* 1974). Transformed lymphoblastoid cell lines are also used to produce leucocyte interferon. Fibroblasts can be induced to produce interferon with either viruses or artificial inducers, the most common of which is polyriboinosinic: polyribocytidilic acid (poly I: poly C) a synthetic double stranded RNA. The highest levels of fibroblast interferon are obtained when the cells are induced in the presence of inhibitors of RNA and protein synthesis, e.g. actinomycin D and cyclohexamide (Ho *et al.* 1972). According to a recently proposed model (Tan and Berthold, 1977), inhibitors of protein synthesis interfere with the production of a labile regulator protein which normally suppresses the transcription of the interferon gene(s) allowing increased interferon production (superinduction). Since all commonly used inducers of interferon, including such diverse substances as mitogens, viruses, tilorone, and endotoxin, can inhibit protein synthesis, this model is put forward to explain interferon induction in general.

The interferons produced by virus induced human leucocytes and poly I: poly C induced fibroblasts differ in antigenicity, molecular weight, and biological activity. To determine whether the antigenic differences are due to the cell type or to the inducer, two lymphoid cell types and two fibroblast cell types were each induced with three interferon inducers (Newcastle disease virus, Sendai virus and poly I: poly C), (Vilcek *et al.* 1977). Antisera to one of the poly I: poly C induced fibroblast interferons neutralized the antiviral activity of all the fibroblast type interferons and none of the leucocyte interferons, indicating that different cell types produce different interferons. Antisera to Sendai induced leucocyte interferon, on the other hand, could partially neutralize fibroblast interferon, suggesting that leucocyte interferon contains two molecules, one of which is antigenically similar to fibroblast interferon. This idea was corroborated by chromatographing antiserum to leucocyte interferon on a column containing bound fibroblast interferon; the treated antiserum could no longer neutralize fibroblast interferon. The column-bound fraction was inactive against leucocyte interferon.

When fibroblast and leucocyte interferons are analyzed by polyacrylamide gel electrophoresis, leucocyte interferon separates into two peaks, one of 21,000 molecular weight and the other of 15,000. Fibroblast interferon shows one peak at 20,000 (Stewart and Desmyter, 1975).

Comparing the biological properties of human fibroblast and leucocyte interferon preparations, it was found that they show different dose response curves in assays for antiviral activity (Edy *et al.* 1976), and that leucocyte interferon has greater antiviral activity on cells from other animal species than fibroblast interferon (Gresser *et al.* 1974). Both interferons possess anticellular activity (Hilfenhaus *et al.* 1976).

Differences between fibroblast and leucocyte interferons could be due either to different primary amino acid sequences (i.e. to two separate structural genes) or to post translational modifications. To differentiate between these two possibilities, mRNA's were isolated from human lymphoblastoid cells and fibroblasts which had been induced to produce their respective interferons, and injected into *Xenopus laevis* oocytes; the oocytes can translate the mRNA's but are incapable of making specific post translational modifications (Cavalieri *et al.* 1977). The interferons produced by the oocytes showed the same differences in molecular weight and antigenic properties as the interferons produced directly by the leucocytes and fibroblasts from which the mRNA's were taken. These findings support the concept of separate genes for fibroblast and leucocyte interferons.

Interferons are also produced by lymphoid cells stimulated with antigens or mitogens (Wheelock, 1965). In mice, stimulants of thymus independent (B) lymphocytes induce an interferon antigenically similar to virus induced fibroblast interferon, while stimulants of thymus dependent (T) lymphocytes induce an antigenically dissimilar interferon, commonly designated immune or type II interferon (Wietzerbin *et al.* 1977). Type II interferon can be distinguished by its instability at pH 2 (Valle *et al.* 1975). Comparisons of mouse NDV and poly I: poly C induced interferons from T lymphocytes, B lymphocytes, macrophages, and primary embryonic cells have shown differences in antigenicity, heat and acid stability, and molecular weight depending on cell source and the mode of induction (Maehara *et al.* 1977).

Interferons may have some degree of tissue specificity (Einhorn and Strander, 1977). Lymphoblastoid cells in culture are more sensitive to the cell growth inhibitory effects of leucocyte interferon, while fibroblasts are more sensitive to fibroblast interferon. On the other hand, doubt has been cast on the idea of absolute species specificity with the discovery that human interferon has a pronounced antiviral activity on bovine and porcine cells (Gresser *et al.* 1974), bovine type II interferon shows its greatest cell growth inhibitory effect on porcine cells with some activity on human, rabbit, monkey, equine, and canine cells (Babuik and Rouse, 1977), and modified human interferon is active on cat cells but not on human cells (Desmyter, 1976).

There has been some debate over whether or not the antiviral and the anticell effects of interferon reside in the same molecule(s). Stewart *et al.* (1976) working with leucocyte interferon, and Knight (1976) working with fibroblast interferon, both preparations highly purified, have data which indicates that both activities reside in the same fraction of human interferon. Dahl and Degré (1975), on the other hand, report that they have separated the antiviral activity of leucocyte interferon from its anticellular activity, as assayed on monolayers of human fibroblasts. A difference has since been found in the response of lymphoblastoid cells to the two components: two of the three cell lines tested were inhibited by the antiviral fraction and not by the cell growth inhibitory fraction (Dahl and Strander, 1977).

The fact that interferon is not a single type of molecule but a collection of heterogenous molecules which differ according to the type of cell induced, the species of animal from which the cell was derived, and the inducer used makes it clear that low yields of interferon are not the only obstacle in the way of complete purification and characterization. Lack of assay systems that do not rely solely on its biological activities creates an additional obstacle.

1.03 Genetics of the Human Interferon System

Using Sendai virus fused mouse-human cell hybrids which preferentially lose human chromosomes, it has been shown that only those cells which retain human chromosome 5 can be induced to produce human interferon (Tan *et al.* 1974). The structural gene(s) for human interferon production have been localized on the longarm of chromosome 5 and possible regulator genes on the shortarm of the same chromosome (Tan *et al.* 1976). Fibroblast cell lines with a high ratio of chromosome

5 longarm to shortarm have been used to produce large amounts of human interferon on a regular basis; this interferon has recently been purified to homogeneity and labelled (Berthold and Tan, 1977).

Mouse-human cell hybrids which retained human chromosome 21 were the only cells which could be protected by human interferon from virus attack (Tan *et al.* 1973). Work with fibroblast cell lines containing varying numbers of copies of 21, or translocations of the longarm or shortarm of that chromosome, showed that increased sensitivity to human interferon is directly related to an increase in the number of copies of chromosome 21 longarm (Tan and Greene, 1976). Revel *et al.* (1976), have developed antisera to mouse-human hybrids containing human chromosome 21 which are capable of reversibly blocking the response of human cells to interferon. Since such antisera can only combine with cell surface components, they suggest that chromosome 21 codes for a cell surface receptor for interferon.

Cell growth inhibition by human interferon is also controlled by genes on human chromosome 21. Fibroblast cell lines which are monosomic, disomic, and trisomic for chromosome 21, as well as cells containing translocations of 21, have been used to show that an increased sensitivity to the anticell effects of human interferon is related to an increase in the number of copies of the longarm of chromosome 21 (Tan, 1976).

The fact that the anticell and antiviral effects of interferon both require chromosome 21 indicates that they operate at some point through a common pathway. the elucidation of which should help us to understand the mechanism of interferon action.

1.04 Antiviral Action of Interferon

Much effort has been devoted to understanding the mechanism by which interferon induces an antiviral state, but the explanation is still far from complete. One of the first steps involves the binding of interferon molecules to gangliosides in the cell membrane (Friedman, 1967; Kohn *et al.* 1976; and Vengris *et al.* 1976), but whether or not the molecules enter the cell is still being debated. Interferon covalently bound to Sepharose beads can induce an antiviral state (Ankel *et al.* 1973), indicating that it can act extracellularly. However, interferon has such a high specific activity that amounts sufficient to produce an antiviral response could be removed from the beads and ingested by the cell without the change in the concentration of bound interferon being detectable.

Interferon does not appear to prevent the adsorption of the virus particle to the plasma membrane or its subsequent decapsulation (Vilcek, 1969). Proof that interferon acts at a later stage in the viral replication cycle was provided by experiments that showed that interferon can block virus production in cells infected with purified virus RNA (Hermodsson and Philipson, 1963). Although interferon may have some effect on virus assembly and shedding (Chang *et al.* 1977), inhibition is most likely to occur at the level of viral RNA and/or protein synthesis.

Early experiments on vaccinia virus replication in interferon treated cells showed that, while transcription of virus mRNA was not affected, translation of the mRNA was prevented, with a resulting failure of DNA synthesis and assembly of virus progeny (Joklik and Merigan, 1966). Subsequent work with a number of different viruses strongly supports the idea that interferon blocks virus replication at the translational level (for reviews see: Metz, 1975b; Joklik, 1977;

Lewis *et al.* 1977).

Cell free systems have been used recently to study the mechanism of interferon action more closely, but many of the results are controversial. Friedman *et al.* found that extracts from both interferon treated and control cells could translate viral mRNA equally efficiently *in vitro* unless the interferon treated cells had also been "infected" with virus (Friedman *et al.* 1972). They suggested that interferon potentiates an antiviral state which is only expressed following virus infection. However, other groups have found that non-infected interferon treated cells can also inhibit mRNA translation.

There is disagreement on whether the translation of host mRNA is also inhibited in interferon treated cells. Some researchers have found that extracts from interferon treated cells translate host mRNA as rapidly as extracts from control cells (Samuel and Joklik, 1974), while others have reported complete inhibition (Falcoff *et al.* 1973; Gupta *et al.* 1973). Lengyel's group later found that this inhibition could be largely overcome by adding tRNA from normal or interferon treated cells (Gupta *et al.* 1974).

Experiments showing that the development of an antiviral state requires the presence of the cell nucleus (Radke *et al.* 1974), and can be prevented by inhibitors of protein synthesis (Taylor, 1964), indicate that interferon has no direct antiviral activity. The most widely accepted hypothesis attributes antiviral action to an antiviral protein. There was very little direct evidence for the existence of such a protein until Samuel and Joklik (1974) reported a 48,000 Dalton protein present in the ribosomal washings from interferon treated cells, but not from untreated cells, which could inhibit viral mRNA translation in normal cells.

Treatment of a combined high density culture of mouse and human fibroblasts with mouse interferon results in the transfer of viral resistance from the mouse cells to the human cells, which are normally resistant to mouse interferon (Blalock and Baron, 1977). This suggests that interferon induces an antiviral substance which is not species specific, and which can be transferred to adjoining cells, perhaps by gap junctions. In addition to providing information on the characteristics of the antiviral substance, these experiments also help to explain how a very small number of interferon molecules can induce an antiviral state in many cells (i.e. the high specific activity of interferon).

1.05 Anticellular Action of Interferon

In 1962, Paucker and his coworkers reported that mouse interferon affected the growth rate of cultured mouse L-cells (Paucker *et al.* 1962). The findings were largely ignored until 1970 when Gresser found that mouse interferon had the same effect on the leukemic L-1210 strain of mouse cells (Gresser *et al.* 1970). Further work in the same laboratory showed that this "anti-cell" effect was not due to an increased cell mortality (Gresser *et al.* 1971); recent studies using time-lapse photography have shown that cell growth inhibition is due to a lengthening of the intermitotic interval (d'Hooghe *et al.* 1977). The changes that interferon produces on the cell membrane as shown by an increased binding of concanavalin A (Huet *et al.* 1974), decreased permeability to macromolecules (Degré and Hovig, 1976), increased electrophoretic mobility (Knight and Korant, 1977), and enhanced expression of histocompatibility antigens (Lindahl *et al.* 1976) may perhaps all be reflections of the increased time that interferon treated cells spend in interphase;

cellular interphase is characterized by a doubling in area of the plasma membrane and increased presentation of H-2 histocompatibility antigens (Graham *et al.* 1973).

Almost all human cells in culture which have not been previously exposed to interferon are sensitive to the anticellular effect whether they are primary, diploid, or continuous. Human lymphoblastoid cell lines which spontaneously produce interferon are insensitive to exogenously applied interferon (Adams *et al.* 1975). Tumour cell growth, both *in vivo* and *in vitro*, is inhibited (Gresser *et al.* 1969, 1970); observation of this "antitumour" effect has lead to preliminary clinical trials of human interferon in the treatment of human cancers (Strander *et al.* 1974).

Kuwata and his coworkers have established a subline of cells from transformed human embryonic cells which are resistant to the anticellular effects of interferon but which can still be protected against viral challenge (Kuwata *et al.* 1976). This was done by continuously culturing the cells in gradually increasing concentrations of interferon. The suggestion is put forward that interferon pretreated cells possess fewer interferon binding sites, and that the expression of the anticell effect requires more binding sites than the induction of an antiviral state. However, comparison of the two activities is still complicated by a shortage of pure, labelled interferon, and the difficulty of comparing antiviral and anticellular bioassays. Antiviral bioassays are done on confluent cells, and the interferon is usually present in the cultures for eight to eighteen hours. Formation of the antiviral state requires only about forty minutes, (Dianzani and Baron, 1975). Assays for cell growth inhibition use actively dividing cells, and the interferon is in contact with the cells for anywhere from three days to several weeks. The interferon is only replaced when the cells are split and fed. To produce cell growth inhibition, the interferon must be continuously in contact with the cells; the length of the bioassay increases the chances of non-specific inactivation with resultant error in measurement. In addition, it may not be valid to compare bioassays in which the cell populations differ so radically in their position in the cell cycle (Fuchsberger *et al.* 1975). There is still a need for an interferon assay that does not involve either its anticellular or antiviral properties.

The mechanism of the anticellular action of interferon is unknown. The only possible clue comes from the observation that, in cell-free protein synthesizing systems made from interferon treated cells, a dose of interferon sufficient to inhibit the synthesis of viral proteins by 95 to 100% will also inhibit synthesis of cellular proteins by 20 to 40% (Falcoff *et al.* 1973). Metz has hypothesized that selectivity in favour of the inhibition of viral protein synthesis may be due to the fact that interferon induces a selective block of the translation of mRNA's with high affinity ribosome binding sites; and that in general, viral mRNA's have higher affinities for ribosome binding sites than cellular mRNA's (Metz, 1975b).

1.06 Immune Modification by Interferon

1.06.1 *Production of Interferon by the Immune System*

Lymphocytes are divided on the basis of function into two major groups: T cells, which develop embryologically under the influence of the thymus, and are responsible for cellular immunity, and B cells, which are thymus independent, and are responsible for humoural immunity (antibody production). T and B cells remain quiescent in the blood and lymphoid tissue until stimulated by specific antigens or by non-specific mitogens. They then start to synthesize RNA and protein, followed 24 hours later by DNA synthesis and cell division. Mitosis continues for several generations accompanied by progressively increasing differentiation of the daughter cells until the mature cells capable of antibody synthesis or cell mediated immunity are produced (Ling and Kay, 1975). The final immune response to any given antigen is the result of complex interactions between T lymphocytes, B lymphocytes, and macrophages, governed, at least in part, by soluble effector molecules secreted by the activated lymphocytes. These molecules, collectively known as lymphokines, include: lymphocyte transforming factors, chemotactic factors for monocytes and polymorphonuclear leucocytes, cytostatic and cytotoxic factors affecting all types of white cell, factors for macrophage inhibition or activation, aggregation or spreading, and *interferon* (Granger, 1972). None of the lymphokines is purified or well characterized, and assay results are determined by the relative proportions of individual lymphokines present, many of which have opposing activities. For example, colony stimulating factor will promote *in vitro* growth of macrophage and granulocyte colonies from bone marrow precursor cells, while interferon decreases colony formation (McNeill and Gresser, 1973; Greenberg and Mosney, 1977).

Using fluorescence activated cell sorters to separate fluorescent antibody labelled B cells from unlabelled T cells, Epstein *et al.* have found that the T cell is the one responsible for mitogen and antigen stimulated interferon production (Epstein *et al.* 1974). (Virus induced leucocyte interferon production, on the other hand, seems to be confined to non-T lymphoid cells - Yamaguchi *et al.* 1977). The mitogen stimulated T lymphocyte is also the interferon producer in mice (Stobo *et al.* 1973). One particular T lymphocyte subpopulation, the suppressor cells which depress the cellular immune response *in vitro*, has been linked to interferon production. Johnson *et al.* have found that the ability of a particular mitogen to stimulate immune suppression by activating suppressor T cells is directly related to its ability to stimulate interferon production (Johnson *et al.* 1977). They concluded that it is the interferon produced by suppressor cells which is responsible for their suppressor activity. Further support comes from the finding that cyclic AMP and cyclic AMP inducers added to mitogen stimulated cells *in vitro* block both suppressor cell activity and interferon production (Johnson, 1977). It appears that the immune or type II interferon produced by mitogen or antigen activated lymphocytes may suppress the immune response either by inhibiting the proliferation and differentiation of leucocyte precursor cells, or by inhibiting other activated lymphocytes. Determination of the precise role of suppressor T cells is still an active field of research (Möller, 1975). The discovery that alpha fetoprotein induces suppressor T cell formation has lead to speculation that one function may be to suppress the maternal immune response to the fetus (Muigita and Gordi, 1977). Suppressor cells may also prevent the activation of clones of lymphocytes sensitized against "self" antigens; if this theory is correct then loss of suppressor cell function could lead to the development of autoimmune diseases.

1.06.2 *Effects of Exogenous Interferon on the Immune System*

a) In Vivo Experiments

Sheep red blood cells (S RBC's) injected into mice stimulate the formation of clones of B lymphocytes capable of producing antibodies to the foreign erythrocytes. If the spleen cells are then removed from the mouse and cultured in agar containing S RBC's, each antibody producing cell will become surrounded by a clear area where the red blood cells have lysed. This is known as Jerne's plaque forming cell (PFC) bioassay and is a measure of humoural immunity. Interferon preparations injected into mice will suppress the PFC response, with maximum suppression occurring when the interferon is administered two days prior to antigen injection (Chester *et al.* 1973; Brodeur and Merigan, 1974, 1975). Others have found that low doses of interferon cause mild enhancement of the PFC response (Braun and Levy, 1972), but this work has not been reproduced in any other laboratories. Immunoenhancement occurs if interferon is administered after antigenic stimulation (Brodeur and Merigan, 1975). This may be related to findings that, in virus infections that depress the humoural immune response, infection prior to administration of a challenge antigen leads to immunosuppression, while infection after antigen exposure is much less effective (Notkins *et al.* 1970).

Interferon and interferon inducers also inhibit the formation of delayed-type hypersensitivity (DTH) (DeMaeyer-Guignard *et al.* 1975). DTH can be quantified by injecting S RBC's into the footpads of mice on two separate occasions four days apart and measuring the swelling on the footpad 24 and 48 hours after the second injection. Interferon or Newcastle disease virus injected at various times from 48 hours before to 48 hours after the first S RBC injection decreased the S RBC response. Maximum decrease was observed when the interferon titre was brought to a maximum 24 hours before antigen administration. Diminished DTH is thought to be due to an antimitotic effect on lymphocyte precursors.

b) In Vitro Experiments

In vitro systems for measuring the suppression of antibody response by interferon have been developed in order to avoid the problems of maintaining high interferon titres in experimental animals. Lymphocytes can be sensitized to sheep erythrocytes in liquid culture; after five days of sensitization the PFC response can be measured by the standard Jerne PFC bioassay. So far, results obtained *in vitro* have been similar to the *in vivo* results. Interferon preparations added to lymphocyte cultures four hours before or up to forty hours after the addition of S RBC's resulted in a suppressed PFC response (Johnson and Baron, 1976). Interferon need only be present for four hours after the addition of antigen for maximum inhibition to occur (Johnson *et al.* 1975a). As the time between S RBC addition and the start of interferon treatment increases, the inhibition of the PFC response decreases. Interferon added after 48 hours produced no inhibition (Booth *et al.* 1976a) or mild enhancement (Gisler *et al.* 1974) of antibody response.

The use of spleen cell cultures from athymic (nude) mice, and the removal of macrophages from the lymphocyte cultures have shown that interferon has a direct effect on the antibody forming response of the B cell and does not require T cell and macrophage cooperation (Johnson *et al.* 1975b). Evidence that interferon is the substance responsible for decreased antibody response is indirect. The degree of PFC inhibition by various interferon preparations is directly proportional to their antiviral activity, and the activities cannot be separated by treatment with heat, trypsin, DNase, RNase, and periodate (Gisler *et al.* 1974). Both activities are acid stable. In the systems studied so far the immunosuppressive effect of interferon appears to be species specific.

The fact that interferon need only be present for four hours after the addition of antigen to give maximum suppression suggests that interferon does not act by preventing lymphocyte proliferation (mitosis does not start until 24 hours after stimulation). Booth and his coworkers have evidence to show that interferon inhibits B lymphocyte activation rather than clonal proliferation (Booth *et al.* 1976b; Finlay *et al.* 1977). Fractionated and unfractionated spleen cells stimulated with S RBC's and bacterial lipopolysaccharide were placed in poly-acrilymide vessels, the base of which contained 64 dimples. Each dimple contained one cell capable of forming an antibody producing clone. Interferon treatment was found to reduce the number of clones formed but to have no effect on the size of the clones.

Interferon can also suppress cell mediated immunity. The most commonly used indicator of cell mediated immunity is the ability of T lymphocytes to proliferate in culture in response to various T cell mitogens such as phytohaemagglutinin (PHA) and concanavalin A (Con A). Mitogens, unlike antigens, are non-specific and can stimulate a larger proportion of the cells in the culture to proliferate. The degree of stimulation is usually measured by the incorporation of labelled DNA precursors into cellular DNA. Interferon has been found to inhibit T cell activation in both mice (Lindahl-Magnussen *et al.* 1972) and man (Blomgren *et al.* 1974).

When lymphocytes from one person (or mouse) are placed in culture with lymphocytes from another unrelated person (or mouse), the lymphocytes are stimulated to proliferate due to differences in histocompatibility antigens. This reaction is known as the mixed lymphocyte reaction (MLR). In a two-way MLR, the lymphocytes from both donors are free to respond, in contrast to the one-way MLR in which the lymphocytes from one of the donors are prevented from responding by treatment with metabolic inhibitors (e.g. mitomycin C) or gamma rays. As in the case of mitogen stimulated cultures, the degree of stimulation can be measured by the macromolecular incorporation of labelled nucleotides. Interferon inhibits DNA synthesis in mixed lymphocyte cultures from both man (Blomgren *et al.* 1974) and mouse (Heron *et al.* 1976).

After about six days in a one-way mixed lymphocyte reaction, the unblocked responder lymphocytes have developed into cytotoxic effector cells capable of destroying lymphocytes taken from the same donor as the blocked stimulator lymphocytes that they have been cultured with. The number of effector cells can be measured by putting them into culture with fresh target cells labelled with ^{51}Cr, and counting the amount of radioactivity released into the medium. When interferon is added to mixed human lymphocyte cultures during the sensitization phase, it causes a dose dependent increase in cytotoxicity; addition during the killing phase has no effect (Heron *et al.* 1976). Similar results have been obtained with mouse lymphocytes sensitized to L-1210 tumour cells (Lindahl *et al.* 1972).

Lindahl *et al.* found that interferon treatment of mice leads to increased expression of histocompatibility antigens on lymphoid cells as measured by increased adsorbtion of alloantiserum (Lindahl *et al.* 1976). There is also increased surface antigen expression in interferon treated L-cells (Lindahl *et al.* 1973). This may be due to the accumulation of cell membrane material in the extended interphase period which results from the anticellular effect of interferon (Knight and Korant, 1977). Further work has pinpointed enhanced antigen expression to the H-2K and H-2D antigens of the major histocompatibility locus in mice; the Ia antigen responsible for lymphocyte proliferation in a mixed lymphocyte reaction is unaffected (Vignaux and Gresser, 1977).

Another effect of interferon and interferon inducers on cell mediated immunity is prolongation of allograft survival in mice, accompanied by a marked lymphocytopenia (Mobraaten *et al.* 1973). High doses of rabbit interferon applied topically to corneal xenografts suppress rejection but low doses enhance it (Imanishi *et al.* 1977). Several Soviet researchers have also reported enhanced graft rejection following interferon treatment (Skurkovich *et al.* 1973). However, no attempts have been made to explain these findings or to correlate them with other parameters of cell mediated immunity.

Finally, interferon has been found to increase *in vitro* phagocytosis by both human and mouse macrophages (Imanishi *et al.* 1975; Donahoe and Huang, 1976; Rabinovitch *et al.* 1977). It seems likely that macrophage- T cell interactions are involved since macrophage activity is strongly regulated by T cell lymphokines (David, 1975).

1.07 Thesis Rationale

In cultured human fibroblasts, the antiviral effect of human interferon is regulated by genes on chromosome 21. Assignment of the genes to this particular chromosome was originally made using mouse-human cell hybrids which preferentially lose human chromosomes; only those hybrids which retained human chromsome 21 could still be protected by human interferon against virus challenge (Tan *et al.* 1973). Later, using human fibroblasts which were monosomic, disomic, or trisomic for chromosome 21, it was shown that sensitivity to the antiviral effects of human interferon is proportional to the number of copies of chromosome 21 in the cell (Tan, 1975). Inhibition of human fibroblast cell growth by human interferon preparations is also controlled by chromosome 21, and, again, the degree of inhibition is dependent on the number of copies of the chromosome present in the cell (Tan, 1976).

This thesis is primarily concerned with the relationship of chromosome 21 to the immunosuppressive action of human interferon. Specifically, I wished to determine whether lymphocytes from individuals with trisomy of chromosome 21 (Down's syndrome or mongolism) are more sensitive than lymphocytes from normal people to inhibition of cell mediated immunity by various human interferon preparations. The purpose was twofold: 1) to establish that genes on chromosome 21 control all of the responses of human cells to human interferon, so that future interferon assays can be based on genetic criteria, and 2) to further characterize the immunological defects associated with Down's syndrome. In addition, I have done preliminary experiments to determine whether the immunosuppressive effect of interferon is separate from its cell growth inhibitory effect.

Currently used interferon preparations all contain varying amounts of impurities, including the virus, mitogen, or antimetabolites used for interferon induction. In order to establish that an antiviral or anticellular effect being studied is due to interferon and not to contaminants, most people have relied on the criterion of species specificity. If the preparations used have no effect on cells from a different species, and if interferon preparations from other species have no effect on the bioassay system, it is assumed that the active ingredient is interferon. However, the recent reports that interferon is not always species specific (Gresser *et al.* 1974; Babiuk and Rouse, 1977; Desmyter, 1976) makes this test unreliable. Even if methods for large scale production of interferon are perfected in the near future, there will still be a need for a rapid interferon bioassay. The control of human interferon action by genes on chromosome 21 could provide just such an assay if it can be shown that it is common to all cell types and all interferon activities. This study of immunosuppression by interferon using fresh lymphocytes is a step in this direction.

Trisomy of chromosome 21 is the only human somatic trisomy compatible with long-term survival. However, it has been known for a long time that individuals with Down's syndrome (D.S.) are more susceptible than the normal population to infections (Siegel, 1948), and to solid tumours and leukemias (Holland *et al.* 1962). Numerous studies have been done to determine whether immunological defects are responsible, but most of the results are contradictory. Comparison of immunoglobulin levels in D.S. and normal people have shown either increased, decreased, or normal levels of IgA, IgM, and IgG in the mongoloids (for a summary of the published results from seven separate laboratories, see Rosner *et al.* 1973). Evaluation of the cell-mediated immune response as measured by levels of DNA synthesis in PHA stimulated lymphocytes has also yielded inconclusive results; several laboratories have found no difference between D.S. and normal populations (Fowler and Hollingsworth, 1973; Szigeti *et al.* 1974), while others have reported a decreased response in the mongoloids (Burgio and Nespoli, 1974; Rigas *et al.* 1970). The decreased response may be due to decreased numbers of T lymphocytes present in Down's syndrome (Levin *et al.* 1975). Leucocyte function is impaired in Down's syndrome as shown by decreased phagocytosis (Costello and Webber, 1976) and diminished adhesiveness (Rosner *et al.* 1973). Fibroblasts from D.S. patients appear to be two to three times more susceptible to transformation by SV 40 virus (Young, 1971), but the success ratio for establishing lymphoid cell lines, a process which involves an Epstein-Barr virus associated cell transformation, are no higher in trisomic cells (Woods *et al.* 1973). No previous studies have been done on the effect of interferon preparations on cell mediated immunity in people with Down's syndrome.

II. MATERIALS AND METHODS

2.01 Preparation of Cells

Venous blood was collected in sterile heparinized syringes from normal volunteers and from people institutionalized for Down's syndrome. The blood was immediately mixed with an equal volume of Hank's balanced salt solution (Ca^{2+} and Mg^{2+} free) (Gibco) containing 50 µg/ml chlortetracycline and 30 i.u./ml heparin. In order to obtain lymphocyte populations free of other blood cell types, up to 8 ml of blood/Hank's mixture was layered on top of 2.5 ml of Ficoll-Paque (Pharmacia) and spun for 30 min with a force of 400 g at the blood/Ficoll-Paque interface (Bøyum, 1976). Lymphocytes were removed from the interface and washed three times in Hank's. After the final wash, the cells were resuspended in RPMI-1640 medium (Gibco) containing 10% fetal calf serum (Flow), 50 µg/ml chlortetracycline and 1% 200 mM L-glutamine. Lymphocyte viability was determined by trypan blue exclusion.

2.02 Karyotyping

Lymphocytes from the people with Down's syndrome were karyotyped to insure that they contained three copies of chromosome 21, since in most cases the institutional diagnosis had been made on the basis of physical characteristics rather than chromosomal analysis. Eight drops of anticoagulated blood were added to 10 ml of complete RPMI-1640 medium containing a 1:200 dilution of stock PHA-P (Difco). After three days of incubation at 37°C in 5% CO_2, 2 µg of colcemid were added to the culture for three hours to arrest the cells in metaphase. Cells were then washed, incubated for 20 min in a hypotonic solution (0.075 M KCl) to cause them to swell, fixed in 3:1 methanol: glacial acetic acid, dropped onto clean chilled microscope slides, and flamed. The slides were fixed in methanol, stained with quinacrine, and examined under a fluorescence microscope. Suitable chromosome spreads were photographed.

2.03 PHA Stimulated Cultures

All culture work was done in Microtest plates (Falcon) consisting of 96 round bottomed wells, each with a capacity of 0.3 ml. Lyophylized PHA-P was reconstituted in 5 ml of sterile double distilled water, and then diluted to 1:500 with medium. Lymphocyte suspensions were adjusted to a cell concentration of 10^6 cells per ml. Each well contained 0.05 ml of cell suspension (final concentration: 2.5×10^5 cells per ml), 0.05 ml of an appropriate interferon dilution from 1:4 to 1:4000, and 0.1 ml of RPMI-1640 containing PHA (final concentration of PHA: 1:1000). This is the concentration of PHA that was found to give optimum stimulation, (See Appendix 1). Control cultures contained 0.05 ml of mock interferon or medium. Background stimulation due to the use of fetal calf serum instead of human serum was measured in cultures without PHA. All cultures were done in duplicate.

After the plates had been incubated for 72 h at 37°C in a humidified atmosphere of 5% CO_2 in air, 0.01 ml of ^{3}H-methyl thymidine (Schwartz Mann, specific activity 1.9 Ci/mmol, 0.1 mCi/ml) was added to each well. Four hours later, 20 μl of 20% sodium dodecyl sulfate (SDS) was added to lyse the cells. The contents of each well were placed on individual glass fibre filter papers, washed three times in ice cold 5% trichloracetic acid (TCA) to precipitate the DNA, and rinsed three times in cold 95% ethanol to remove the TCA. After drying, the papers were placed in liquid scintillation vials with Spectrafluor (6 g PPO and 75 mg POPOP per 1 ml toluene, Amersham Searle) and counted for 10 min or to 1% error on a Beckman LS-339 liquid scintillation system. To ensure that interferon was not altering the uptake of thymidine into the cell, the amount of radioactivity in the soluble fractions was also measured in one series of experiments.

2.03.1 *Effect of the Time of Addition of Interferon*

a) Pretreatment with Interferon

Purified lymphocytes (disomic 21) were resuspended in RPMI-1640 medium at a concentration of 10^6 cells per ml. Cultures were grown in Microtest plates. Each well contained 0.05 ml of cell suspension, 0.05 ml of an appropriate fibroblast interferon dilution (1/4 to 1/4096) and 0.1 ml of medium. All cultures were done in duplicate. After 1, 2, 4, 6 and 8 h, 0.05 ml of fluid was carefully removed from 12 wells and replaced with 0.05 ml of medium containing 0.25 µg of Wellcome purified PHA/ml and ¼ the original amount of interferon to replace the portion that was removed. ^{3}H-thymidine was added 72 h after the addition of PHA.

b) Addition of Interferon following PHA Stimulation

Each culture well contained 0.05 ml of purified lymphocyte suspension (disomic 21) (10^6 cells/ml), 0.05 ml of plain medium, and 0.1 ml of medium containing 0.25 µg PHA/ml. At various times, up to 48 h, 0.05 ml of culture fluid was removed from duplicate cultures and replaced with 0.05 ml of medium containing fibroblast interferon and replacement PHA. ^{3}H-thymidine was added 72 h after the lymophocytes had been placed in culture.

2.04 <u>One-way Mixed Lymphocyte Reactions (MLR's)</u>

After separation on Ficoll-Paque, lymphocytes to be used as stimulator cells were treated for 30 min at 37°C with 25 µg/ml mitomycin-C, washed twice in RPMI-1640 medium, and resuspended in medium at a concentration of 10^6 viable cells per ml. Viability was checked with 0.2% trypan blue. Cultures were grown in 96 well round bottomed Microtest plates, each well contained 0.05 ml of mitomycin-C treated stimulator cells (5×10^4 cells), 0.05 ml of untreated responder cells (5×10^4 cells), 0.05 ml of medium, and 0.05 ml of the appropriate interferon dilution. Controls contained 0.05 ml of mock interferon or medium in place of interferon. Two cultures were set up for each pair of donors: the cells which were used as the stimulators in one culture were used as the responders in the other. To measure background stimulation, stimulator and responder cells were taken from the same donor. All cultures were done in duplicate.

Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 96 h, 0.01 ml of ^{3}H-methyl thymidine was added to each well and incubation was continued for a further 24 h. Subsequent processing was identical to the PHA stimulated cultures.

2.05 Soft Agar Cultures

Ficoll-Paque purified lymphocytes (disomic 21) were resuspended at a concentration of 4×10^6 cells per ml in Eagle's minimal essential medium (MEM), supplemented with 20% fetal calf serum, 1% chlortetracyclene, 1% L-glutamine, and 3% sodium bicarbonate (7.5%). Two sets of cultures were set up in sterile plastic centrifuge tubes with screw tops, one set with plain induced fibroblast interferon and the other without. Those with interferon contained 0.5 ml of complete MEM containing 1 μg/ml of purified phytohaemagglutinin (Wellcome), 0.25 ml of cell suspension and 0.25 ml of an appropriate interferon dilution. Those without interferon contained 0.25 ml of complete MEM in place of the interferon dilution.

As in previous experiments, control cultures were included to measure maximum and minimum levels of lymphocyte stimulation. All tubes were incubated in a moist atmosphere of 5% CO_2 in air at 37°C. After 18 h, the tubes were centrifuged gently to sediment the cells and the supernatant was removed. Cells were washed once in Hank's balanced salt solution (Ca^{2+} and Mg^{2+} free) containing 5 mM EDTA. (The EDTA helped to dissociate the clumps of lymphocytes formed by the leucoagglutinating properties of PHA.) Cells which had been incubated with interferon were resuspended in 2 ml of 2:2:1 of double strength MEM: 0.75% agar (final concentration: 0.3%): fetal calf serum. Duplicate 1 ml aliquots were placed on top of previously prepared agar underlays (see below) in 35 mm tissue culture petri dishes (Nunc). Cells which were incubated without interferon were treated in the same way, except that interferon dilutions were added.

The agar underlays consisted of 2 ml of 2:2:1 2 x MEM: 1.25% agar: (final concentration: 0.5%): fetal calf serum containing 0.25 µg of purified PHA. The petri dishes were incubated for 5 days at 37°C in a humid 5% CO_2 atmosphere. Then 1 ml of 0.5 mg/ml 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) (Sigma) was added to half of the petri dishes and incubation was continued for a further 20 h (Schaeffer and Friend, 1976). The brick red colonies resulting from cellular uptake of the INT were examined with a Stereoscan microscope (X10). Colonies on the unstained plates were photographed under high power (X32).

2.06 Interferon Preparations

Fibroblast interferon was prepared from a high producing fibroblast cell line developed in this laboratory. Cells were grown in 75 cm Nunc tissue culture flasks until they were nearly confluent, then fed and allowed to reach confluency. For interferon induction, the growth medium was removed and 6.5 ml of new medium containing 0.125 mg/ml poly I:C (P-L Biochemicals Inc.) was added to the flask for three h. The induction medium was removed, the cell monolayer was washed three times in 25 ml of Hank's balanced salt solution, and 6 ml of new medium was added. The flask was incubated overnight (7 - 15 h) before harvesting the interferon containing medium. This crude interferon was spun at 28,000 rpm in a Beckman model L5-65 ultracentrifuge for one h to remove cells and any possible virus particles.

Human leucocyte interferon was prepared by Dr. K. Cantell (Cantell *et al.* 1974), and supplied by Dr. M. Krim. Superinduced fibroblast interferon was prepared in our laboratory on a routine basis as reported in previous publications (Tan and Berthold, 1977). These two interferon preparations were only used for the genetic studies; all other experiments used plain induced fibroblast interferon.

Mock interferon was prepared in the same way as the plain induced fibroblast interferon but without the addition of poly I: poly C.

To ensure that the effects studied in this thesis were due to interferon and not to contaminants, the interferon preparations were tested for their ability so suppress DNA synthesis in PHA stimulated mouse spleen cells; human interferons are known to have no effect on mouse cells. No species cross reactivity was observed.

N.B. Interferon preparations used in these experiments are referred to henceforth, for convenience, as interferon.

III RESULTS

3.01 Genetic Studies

Tables 1 to 12 show the results of six experiments designed to determine whether lymphocytes from individuals with Down's syndrome are more sensitive to the immunosuppressive effects of human interferon preparations than lymphocytes from normal individuals. In each experiment, with the exception of the first one, two mongoloids and one normal person were tested. PHA stimulated cultures were set up from all three people, and six dilutions of three interferon preparations, one leucocyte and two fibroblast, were added. Maximum attainable levels of stimulation were measured in cultures without interferon, and background levels in cultures without PHA. Mixed lymphocyte cultures were set up slightly differently: trisomic 21 lymphocytes were stimulated with disomic 21 white cells, inactivated with mitomycin C, while the cells from the disomic 21 individual were stimulated by inactivated cells from one of the mongoloids. Interferon treatment was the same as for the PHA cultures. Controls included all possible stimulator-responder combinations to see if the chromosomal complement had any effect on the ability of a cell to act as a stimulator or to respond to a stimulus. Background stimulation levels were measured in cultures where the stimulator and responder cells came from the same donor. The values in Tables 1 to 12 represent the amount of tritiated thymidine incorporated into the cellular DNA, expressed as counts per minute from duplicate cultures.

The average counts obtained for each interferon dilution in a particular culture type, treated with a particular interferon type, were then expressed as a percentage of the maximum stimulation level reached by lymphocytes from the same donor in cultures without interferon.

The percentages were plotted on log-probit paper to determine the dilution of interferon required for 50% inhibition of DNA synthesis. Table 13 represents the results of these calculations for each donor tested. To determine the interferon sensitivity of lymphocytes from trisomic 21 (T 21) donors relative to lymphocytes from disomic 21 (D 21) donors, the 50% endpoints from each T 21 donor were divided by the 50% endpoint for the D 21 lymphocytes tested on the same day, under the same conditions. These results are presented in Table 14.

It is clear that lymphocytes with three copies of chromosome 21 are more sensitive to interferon produced inhibition of DNA synthesis than lymphocytes with only two copies of the chromosome. Eighty-eight percent of the values presented in Table 14 are greater than 1. Of the seven less than or equal to 1, three belong to J.S., a girl classified by the institution in which she lives as being mongoloid on the basis of physical characteristics, and included in these experiments as a trisomic 21 subject. However, karyotyping done after these experiments showed that J.S. has only two copies of chromosome 21. All three types of interferon inhibit the trisomic cells to a greater degree than the disomic cells, whether measured in a mixed lymphocyte reaction or in a culture stimulated by PHA ($p >> 0.05$).

The data presented in Table 15 shows that the number of chromosomes present in a cell has no effect on the maximum levels of DNA synthesis attainable in PHA stimulated cells. There is also no significant difference ($p > 0.5$) in the maximum levels of DNA synthesis between the various donor combinations in the mixed lymphocyte cultures, that is, it made no difference which genotype, trisomic 21 or disomic 21 was the stimulator or responder (Table 16). Interferon had no effect on the

Tables 1 to 6

Phytohaemagglutinin Stimulated Cultures

Results of six experiments comparing suppression of DNA synthesis by interferon preparations in PHA stimulated lymphocytes from Down's syndrome and normal people. Figures are average counts per minute from duplicate cultures.

T-21: trisomic 21 D-21: disomic 21

TABLE 1

EXPERIMENT #1

Interferon Dilution (1:)	R.B. - D21 PHA = 1/160	R.B. - D21 PHA - 1/2560	C.R. - T21 PHA - 1/160	C.R. - T21 PHA - 1/2560
4	202	186	130	110
16	234	144	135	115
64	158	153	113	260
256	4462	3352	803	1294
1024	21762	8793	3772	4299
4096	23942	9594	6992	6862
Controls - no interferon				
maximum stimulation	21595	12881	8058	13188
Background	359	-	191	-

TABLE 2

EXPERIMENT #2

Interferon Type	Interferon Dilution (1:)	C.T. (D-21)	N.C. (T-21)	M.B. (T-21)
Superinduced Fibroblast	4	43	62	214
	16	81	86	256
	64	89	71	279
	256	1963	1373	1525
	1024	10024	5251	5125
	4096	6664	4235	12629
Plain Induced Fibroblast Interferon	4	4780	2493	4262
	16	11553	4449	4992
	64	12147	6335	10953
	256	20159	9996	23371
	1024	13622	6450	8559
	4096	-	-	-
Leucocyte Interferon	4	4695	1952	3669
	16	5425	2439	4222
	64	8298	2259	5269
	256	5880	3760	4006
	1024	10292	4219	8304
	4096	4647	2833	2303
Mock Interferon	4	18045	7312	12187
	16	18914	9670	15833
	64	15451	9931	17064
	256	23832	12202	31150
	1024	12708	6620	11085
	4096	-	-	-
	Average	19060	9779	19058
Controls	Max. stimulation	20369	13638	24284
(no interferon)	Background	94	197	114

TABLE 3

EXPERIMENT #3

Interferon Type	Interferon Dilution (1:)	W.C. (D-21)	D.B. (T-21)	R.D. (T-21)
Superinduced Fibroblast Interferon	80	833	320	198
	320	11900	12075	6906
	640	-	16530	12587
	1280	28882	15853	14125
	2560	25775	21505	19987
	-	-	-	-
Plain Induced Interferon	4	10275	12029	5257
	16	20173	15801	13395
	64	-	21851	14309
	256	24123	21047	16820
	1024	20120	21274	17541
	-	-	-	-
Leucocyte Interferon	4	5471	3560	2550
	16	21637	3039	1793
	64	27569	3796	1442
	256	21386	3892	2631
	1024	19130	6377	4307
	-	-	-	-
Mock Interferon	4	25242	24594	23915
	16	32726	23841	18895
	64	-	24081	20836
	256	26217	21388	12369
	1024	21928	18815	14717
	-	-	-	-
	Average	26528	22544	18147
Controls (No interferon)	Max Stimulation	32770	26997	20570
	Background			

TABLE 4

EXPERIMENT #4

Interferon Type	Interferon Dilution (1:)	L.P. (D-21)	H.C. (T-21)	J.S. (T-21)
Superinduced Fibroblast Interferon	80	836	262	1105
	160	5350	1177	4639
	320	12146	3843	14487
	640	16661	6076	14584
	1280	12742	8982	17613
	-	-	-	-
Plain Induced Fibroblast Interferon	4	13558	2643	13232
	16	16058	3844	17955
	64	17276	7159	20885
	256	15992	8933	23656
	1024	11609	10445	24785
Leucocyte Interferon	4	13057	2050	9647
	16	18748	1323	9445
	64	18770	1983	6334
	256	16217	1589	6435
	1024	30034	1895	13027
Mock Interferon	4	16907	16341	39807
	16	30511	13028	24285
	64	26885	14831	24343
	256	27568	12627	24302
	1024	24887	10713	24922
		-		
	Average	25362	13508	26188
Controls (no interferon)	Max. stim.	23241	14110	21176
	Background	491	203	410

TABLE 5

EXPERIMENT #5

Interferon Type	Interferon Dilution (1:)	B.A. (D-21)	S.N. (T-21)	L.G. (T-21)
Superinduced Fibroblast Interferon	16	120	78	72
	64	433	175	188
	256	9213	2172	2867
	1024	24028	6467	6539
	4096	25847	12788	9345
Plain Induced Fibroblast Interferon	4	3575	699	762
	12	17598	3022	1750
	36	30434	3020	3301
	108	24720	6210	7448
	324	25035	11113	13022
Leucocyte Interferon	12	37317	2526	7185
	36	27618	3483	5881
	108	24781	4585	4854
	324	21114	5449	6588
Mock Interferon	4	29850	1103	15592
	12	29527	20162	11714
	36	22143	19795	13197
	108	23372	14077	13677
	324	25879	16792	13051
	Average	26154	15186	12680
Controls (no interferon)	Max. Stim.			
	Background			

TABLE 6

EXPERIMENT #6

Interferon Type	Interferon Dilution (1:)	S.V. (D-21)	D.L. (T-21)	R.H. (T-21)
Superinduced Fibroblast Interferon	4	110	106	129
	16	129	107	119
	64	3083	413	333
	256	16882	5646	4291
	1024	34061	14724	11107
	4096	33527	21330	23187
Plain Induced Fibroblast Interferon	4	4699	1321	1131
	16	13383	4941	4289
	64	29823	14802	8655
	256	34108	18313	23687
	1024	31926	22363	27944
	4096	32824	31863	27834
Leucocyte Interferon	4	-	-	
	16	46987	4549	4397
	64	18087	6029	4352
	256	23973	8778	6334
	1024	27749	13408	8450
	1096	31746	14924	10607
Mock Interferon	4	-	26196	24004
	16	43919	30717	29395
	64	37906	27258	35245
	256	34317	26295	35667
	1024	36698	27315	33445
	4096	35384	25238	33008
	Average	37645	27171	31793
Controls (no interferon)	Max stimulation	35972	27314	35431
	Background	185	286	437

Tables 7 to 12

Mixed Lymphocyte Cultures

Results of six experiments comparing suppression of DNA synthesis by interferon preparations in mixed lymphocyte cultures from Down's syndrome and normal people. Figures are average counts per minute from duplicate cultures.

T-21: trisomic 21 D-21: disomic 21

TABLE 7

EXPERIMENT #1

Interferon Dilution (1:)	R.B. (D-21)	C.R. (T-21)
4	744	647
16	792	632
64	898	577
256	1643	780
1024	7099	2594
4096	12585	4342
Control: no interferon		
Maximum stimulation	9642	3747

TABLE 8

EXPERIMENT #2

Interferon Type	Interferon Dilution (1:)	Responder x Stimulator		
		D-21: C.T.xN.C.	T-21: N.C.xC.T.	T-21: M.B.xC.T.
Superinduced Fibroblast Interferon	4	65	232	59
	16	59	92	63
	64	69	79	69
	256	248	501	257
	1024	833	456	3826
	4096	2009	1187	1633
Plain Induced Fibroblast Interferon	4	1347	495	1207
	16	1868	463	2029
	64	2543	1645	2297
	256	3014	2052	2380
	1024	2568	1715	1159
	4096	-	-	-
Leucocyte Interferon	4	341	215	166
	16	324	209	367
	64	2915	172	310
	256	740	266	566
	1024	1240	509	940
	4096	1121	440	1860
Mock Interferon	4	3474	2045	2797
	16	3512	2432	4079
	64	3230	2358	4609
	256	2989	1921	3465
	1024	2308	1651	1585
	4096	-	-	-
	Average	3300	2188	3737

Controls No Interferon	Maximum Stimulation	C.T.xN.C.	N.C.xC.T.	M.B.xC.T.
		2858	4053	2659
	Background	C.T.xC.T.	N.C.xN.C.	M.B.xM.B.
		559	537	911
	Alternate	C.T.xM.B.	N.C.xM.B.	M.B.xN.C.
		4402	4227	4865

TABLE 9

EXPERIMENT #3

Interferon	Interferon Dilution (1:)	Responder x Stimulator D-21: W.C.xD.B.	T-21: D.B.xW.C.	T-21: R.D.xW.C.
Superinduced Fibroblast Interferon	80	146	174	312
	360	1366	531	1625
	640	1780	1381	3249
	1280	3538	1616	2723
	2560	3634	1252	3724
	-	-	-	-
Plain Induced Interferon	4	2420	1025	1893
	16	7959	1332	2731
	64	4441	1277	6108
	256	5118	1840	4189
	1024	4888	1414	3200
	-	-	-	-
Leucocyte Interferon	4	464	169	871
	16	464	144	650
	64	444	140	346
	256	993	182	436
	1024	1200	247	736
	-	-	-	-
Mock Interferon	4	5879	2038	4040
	16	5869	1864	4338
	64	5781	1839	-
	256	6616	2184	5168
	1024	6275	1576	3663
	-	-	-	-
	Average	6074	1900	4302

Controls No Interferon	Maximum Stimulation	W.C.xD.B.	D.B.xW.C.	R.D.xW.C.
		4244	2217	2679
	Background	W.C.xW.C.	D.B.xP.B.	R.D.xR.D.
		3138	1046	883
	Alternate	W.C.xR.D.	D.B.xR.D.	R.D.xD.B.
		5621	2667	2295

TABLE 10

EXPERIMENT #4

Interferon Type	Interferon Dilution (1:)	Responder x Stimulator D-21: L.P.xJ.S.	T-21: H.C.xL.P.	T-21: J.S.xL.P.
Superinduced Fibroblast Interferon	80 100 320 640 1280 -	512 1345 3146 5255 6133	144 878 1222 3103 2932	755 1527 3589 6084 8049
Plain Induced Interferon	4 16 64 256 1024 -	5581 7579 5475 6250 6368	1774 2668 3943 3441 4994	3181 5223 7738 5803 7322
Leucocyte Interferon	4 16 64 256 1020 -			
Mock Interferon	4 16 64 256 1024 -			
	Average	-	-	-

Controls No Interferon				
	Maximum Stimulation	L.P.xJ.S.	H.C.xL.P.	J.S.xL.P.
		6625	7156	7148
	Background	L.P.xL.P.	H.C.xH.C.	J.S.xJ.S.
		1209	2078	3341
	Alternate	L.P.xH.C.	H.C.xJ.S.	J.S.xH.C.
		8161	5485	9044

TABLE 11

EXPERIMENT #5

Interferon Type	Interferon Dilution (1:)	Responder x Stimulator		
		D-21: B.A.xL.G.	T-21: S.N.xB.A.	T-21: L.G.xB.A.
Superinduced Fibroblast Interferon	16	87	85	77
	64	165	108	144
	256	715	901	412
	1024	3137	2440	1335
	4096	4990	4774	2072
Plain Induced Interferon	4	339	260	188
	12	660	700	467
	36	1754	1579	945
	108	2811	3128	1452
	324	5112	4086	2462
Leucocyte Interferon	12	724	793	416
	36	1110	1501	355
	108	1975	2231	843
	324	2270	2702	1171
Mock Interferon	4	5586	5704	2800
	12	5899	5880	2639
	36	7190	6395	3299
	108	6610	6339	3380
	324	5905	6817	3342
	Average	6238	6227	3092

Controls No Interferon	Maximum Stimulation	B.A.xL.G.	S.N.xB.A.	L.G.xB.A.
		7539	6428	3212
	Background	B.A.xB.A.	S.N.xS.N.	L.G.xL.G.
		2202	918	613
	Alternate	B.A.xS.N.	S.N.xL.G.	L.G.xS.N.
		5151	3853	1914

TABLE 12

EXPERIMENT #6

Interferon Type	Interferon Dilution (1:)	Responder x Stimulator		
		D-21: S.V.xD.L.	T-21: D.L.xS.Y.	T-21: R.H.xS.V.
Superinduced Fibroblast Interferon	4	109	136	133
	16	107	114	107
	64	172	180	183
	256	727	742	582
	1024	4665	1477	1994
	4096	3136	2401	2529
Plain Induced Interferon	4	471	347	278
	16	1699	435	675
	64	3777	1129	1722
	256	4876	2658	2458
	1024	5723	2479	3169
	4096	3574	3355	3832
Leucocyte Interferon	16	331	248	311
	64	521	234	200
	256	1209	630	385
	1024	1912	712	703
	4096	1842	1157	931
Mock Interferon	4	3372	3183	3531
	16	4644	2887	3743
	64	3108	3483	3619
	256	3406	2652	4199
	1024	3991	2592	3748
	4096	3953	3431	3771
	Average	3745	3038	3768

Controls No Interferon	Maximum Stimulation	S.V.xD.L.	D.L.xS.V.	R.H.xS.V.
		4052	3237	4208
	Background	S.V.xS.V.	D.L.xD.L.	R.H.xR.H.
		3826	702	-
	Alternate	S.V.xD.L.	D.L.xR.H.	R.H.xD.L.
		5668	1850	1638

TABLE 13

DILUTION OF INTERFERON REQUIRED FOR 50% INHIBITION OF DNA SYNTHESIS

				Superinduced Fibroblast Interferon		Plain Induced Fibroblast Interferon		Leucocyte Interferon	
Subject	Karyotype	Age	Sex	PHA	MLR	PHA	MLR	PHA	MLR
R.B.	D-21	28	F	500	600	N.D.	N.D.	N.D.	N.D.
C.R.	T-21	17	F	2000	800	N.D.	N.D.	N.D.	N.D.
C.T.	D-21	34	M	1000	2500	12	10	1000	30
N.C.	T-21	17	F	4000	4000	24	30	4000	> 1000
M.B.	T-21	16	M	2000	6000	52	15	> 4000	260
W.C.	D-21	25	M	500	580	6	4	10	> 1000
D.B.	T-21	32	F	450	770	12	16	> 1000	> 4000
R.D.	T-21	32	M	700	530	12	16	> 1000	> 4000
L.P.	D-21	28	F	450	320	4	4	4	N.D.
H.C.	T-21	28	F	770	830	50	64	> 1000	N.D.
J.S.	See text	32	F	530	320	4	4	50	N.D.
B.A.	D-21	22	M	380	900	8	100	100	2000
S.N.	T-21	8	M	1500	1800	150	100	7000	4000
L.G.	T-21	9	F	1200	2000	90	100	3000	4000
S.V.	D-21	26	M	250	500	25	25	70	1000
D.L.	T-21	13	M	1000	1200	75	100	1600	4000
R.H.	T-21	14	M	2000	2000	125	100	> 4000	> 4000

N.D. = Not done

TABLE 14

SENSITIVITY OF TRISOMIC 21 LYMPHOCYTES TO INTERFERON PREPARATIONS

			Superinduced Fibroblast Interferon		Plain Induced Fibroblast Interferon		Leucocyte Interferon	
Subject	Age	Sex	PHA	MLR	PHA	MLR	PHA	MLR
C.R.	17	F	4	1.3	N.D.	N.D.	N.D.	N.D.
N.C.	17	F	4	1.6	2	3	4	> 30
M.B.	16	M	2	2.4	4.3	1.5	> 4	8.7
D.B.	32	F	0.9	1.3	2	4	∿ 100	> 4
R.D.	32	M	1.4	0.9	2	4	∿ 100	> 4
H.C.	28	F	1.7	2.6	12.5	4	∿ 250	N.D.
J.S.	32	F	1.2	1	1	1	12.5	N.D.
S.N.	8	M	4	2	19	1	70	4
L.G.	9	F	3.2	2.2	11	1	30	4
D.L.	13	M	4	2.4	3	4	23	4
R.H.	14	M	8	4	5	4	> 57	> 4

$$\text{Sensitivity} = \frac{\text{Trisomic 21 Lymphocyte Endpoint}}{\text{Disomic 21 Lymphocyte Endpoint}}$$

TABLE 15

DNA SYNTHESIS IN PHA STIMULATED LYMPHOCYTES AND UNSTIMULATED CONTROL CULTURES

Expt. No.	Disomic 21		Trisomic 21	
	PHA	Control	PHA	Control
2	20369	94	13638 24284	197 114
3	32770		26997 20570	
4	23241	491	21176 14110	410 203
5	26154		15186 12680	
6	35431	437	35973 27314	185 286

TABLE 16

DNA SYNTHESIS IN ONE-WAY MIXED LYMPHOCYTE CONTROL CULTURES

Expt. No.	Responder x Stimulator		
	T-21 x D-21	D-21 x T-21	T-21 x T-21
2	4053 2659	2858 4402	4227 4865
3	2217 2679	4244 5621	2667 2295
4	7156 7148	8161 6625	5484 9044
5	6428 3212	5151 7539	3853 1914
6	4208 3237	5668 4052	1638 1850

ability of lymphocytes to incorporate ^{3}H thymidine.

3.02 Interferon Kinetics

Figure 2 shows the results of experiments done to determine the kinetics of interferon action in PHA stimulated disomic 21 lymphocyte cultures. Kinetic curves are only shown for 1/4, 1/16, and 1/64 interferon dilutions since higher dilutions showed no inhibition. The titration curve in Figure 1 shows the action of interferon dilutions (1/4 to 1/16,000) added at the start of the culture period.

Two sets of conditions were explored: pretreatment of lymphocytes with interferon for varying periods of time prior to PHA stimulation, and addition of interferon at intervals after stimulation. Culture volumes were kept constant throughout the experiments; when interferon was added to cultures containing PHA, medium was first removed in an amount equal to the volume of interferon solution to be added. To compensate for the PHA removed with the medium, interferon dilutions contained the correct amount of replacement PHA. The same principle was followed when PHA was added to interferon pretreated cultures. This technique was found to be essential for accurate results when total culture volume was only 0.2 ml.

Examination of the kinetic curves shows that interferon treatment results in maximum suppression when added simultaneously with mitogen. Interferon added eight hours after PHA stimulation inhibited DNA synthesis by only one half. By forty hours, the most concentrated interferon preparation (1/4) still caused some inhibition, but the more dilute solutions (1/16 and 1/64) did not inhibit DNA synthesis. Pretreatment with interferon for one or two hours seems to produce less inhibition, or perhaps even enhancement, of DNA synthesis compared with interferon

Figure 1. Interferon Titration

Plain induced fibroblast interferon dilutions (1/4 to 1/16,000) added to lymphocyte cultures simultaneously with PHA. Average counts per minute from duplicate cultures.

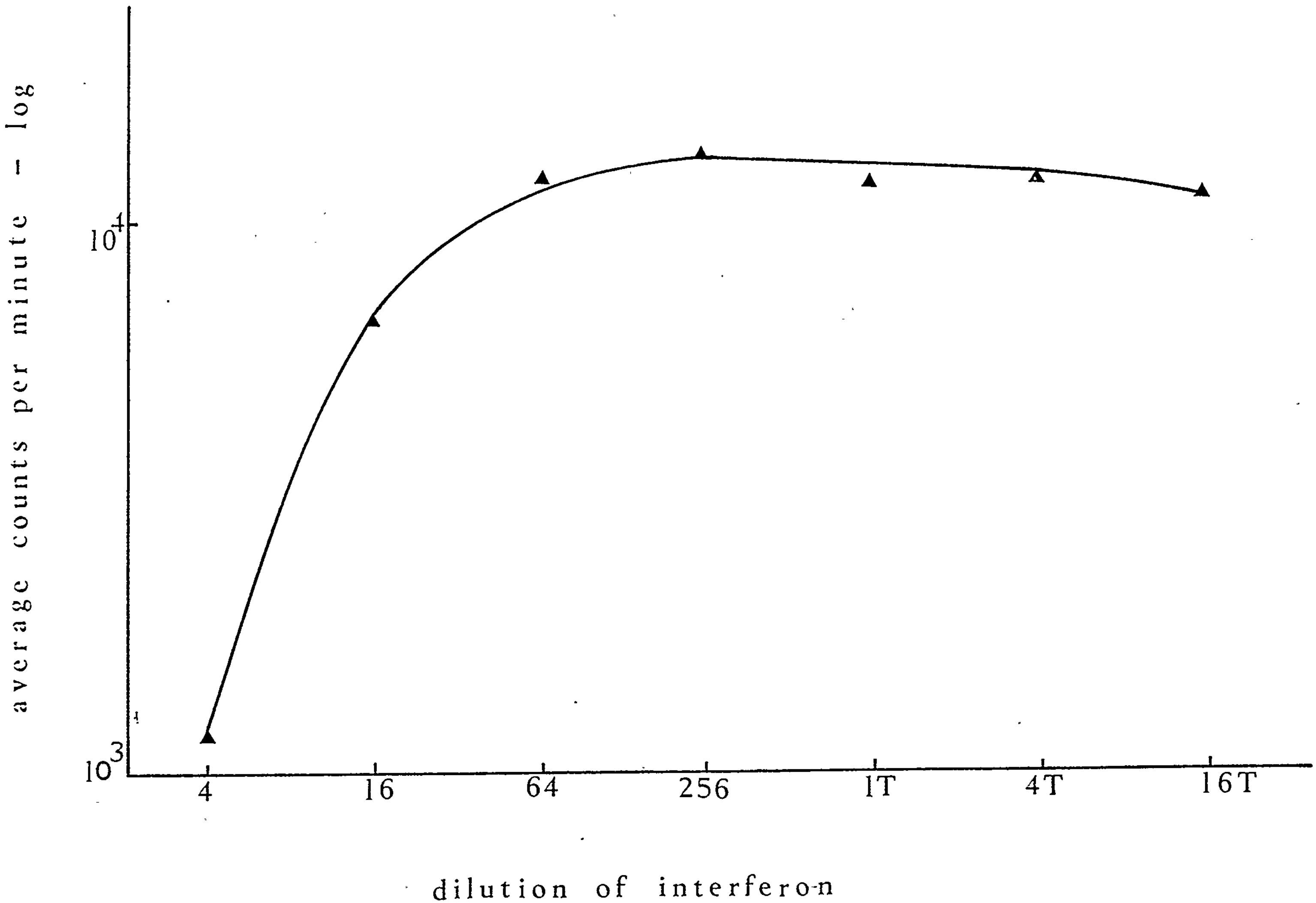
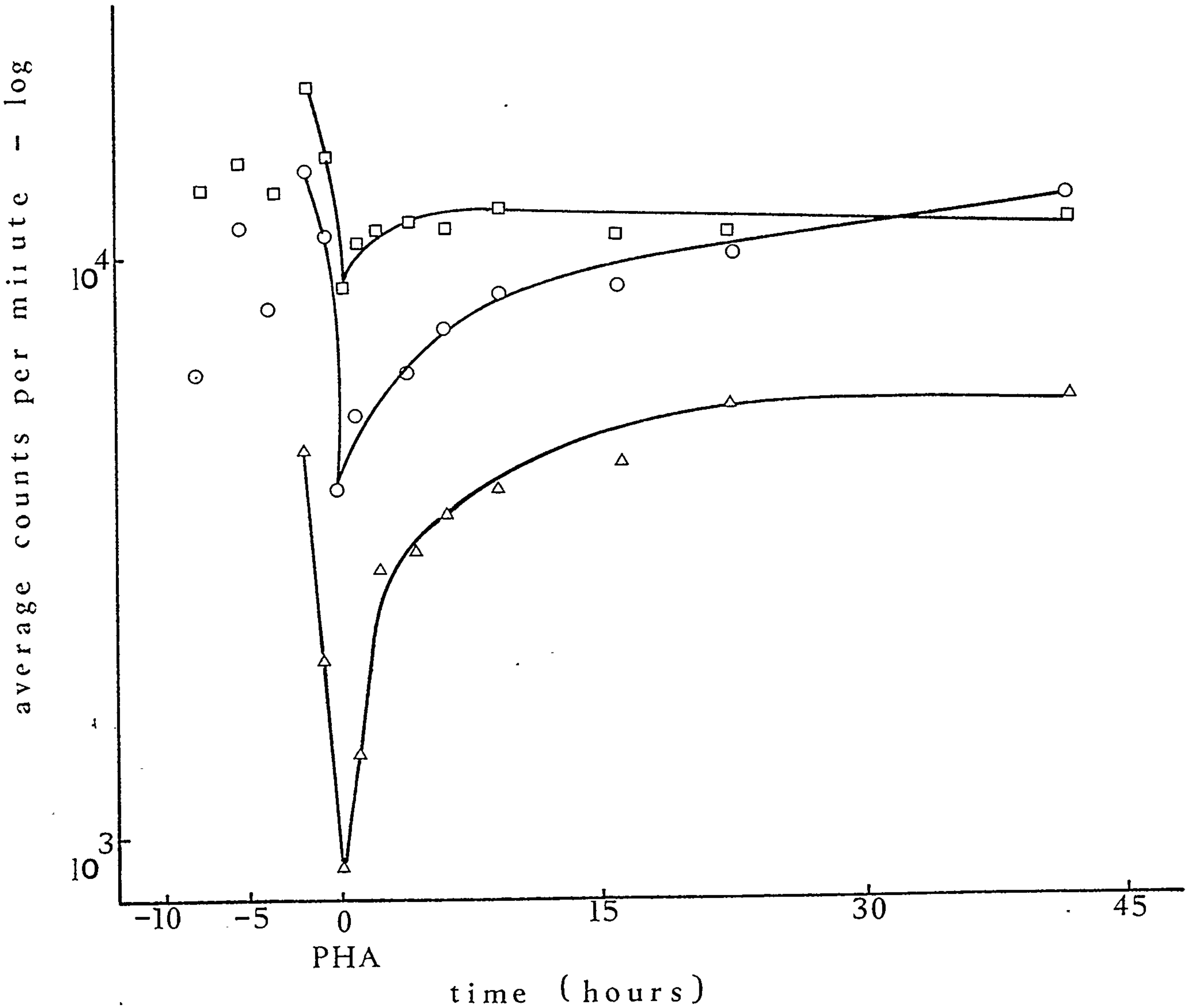

average counts per minute - log
10^4
10^3
4
16
64
256
1T
4T
16T
dilution of interferon

Figure 2. Interferon Kinetics

Plain induced fibroblast interferon dilutions (1/4 to 1/64) added to lymphocyte cultures from eight hours before to forty-two hours after lymphocyte stimulation. Average counts per minute from duplicate cultures.

- △ 1/4 dilution of interferon
- ○ 1/16 dilution of interferon
- □ 1/64 dilution of interferon


average counts per minute - log
10^4
10^3
-10
-5
0
15
30
45
PHA
time (hours)

added at zero time.

3.03 Soft Agar Cultures

Use was made of a new technique developed for growing PHA stimulated lymphocytes in soft agar, so that each dividing lymphocyte gives rise to a clone of cells (Rozenszajn *et al.* 1975). Lymphocytes (disomic 21) were first incubated in liquid medium for eighteen hours, with or without interferon, then placed in soft agar for five days. Both phases contained PHA. Cells which had not seen interferon in the liquid cultures were exposed to it in the agar phase, eighteen hours later. The purpose of these experiments was to determine whether interferon inhibits lymphocyte activation (i.e. a true immunosuppressive effect) or clonal proliferation (i.e. a cell growth inhibition effect). In theory, if the mechanism is immunosuppression, interferon treatment should decrease the number of colonies formed without affecting the final size of the colonies. If the mechanism is purely cell growth inhibition, interferon should decrease the size of the colonies but have no effect on the number of lymphocytes activated, and, therefore, no effect on the number of colonies formed. Interferon added to the agar phase should only be able to inhibit clonal proliferation since the lymphocytes are already committed to the first cycle of cell division by eighteen hours.

Results of the soft agar culture experiments are shown in Figures 3 to 8. Each photograph shows unstained cells after five days in culture. Cells in Figure 3 were not exposed to PHA at any time and as a result have not been stimulated to divide. Figure 4 shows PHA stimulated lymphocytes which have not been treated with interferon. In contrast to those in Figure 3, the stimulated cells have become actively dividing blast cells with abundant cytoplasm and visible nucleoli. Each group

Figure 3. Unstimulated human peripheral blood lymphocytes in soft agar culture (X32).

Figure 4. Phytohaemagglutinin stimulated lymphocytes in soft agar culture (X32).

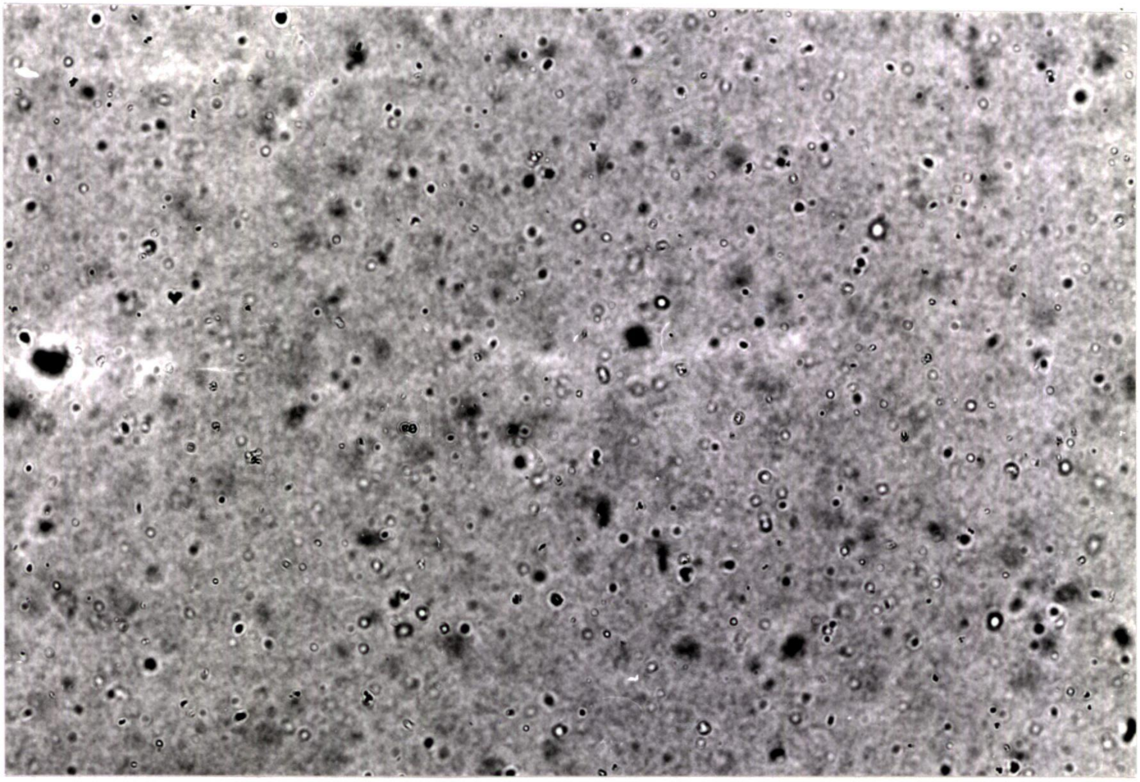


FIGURE 3

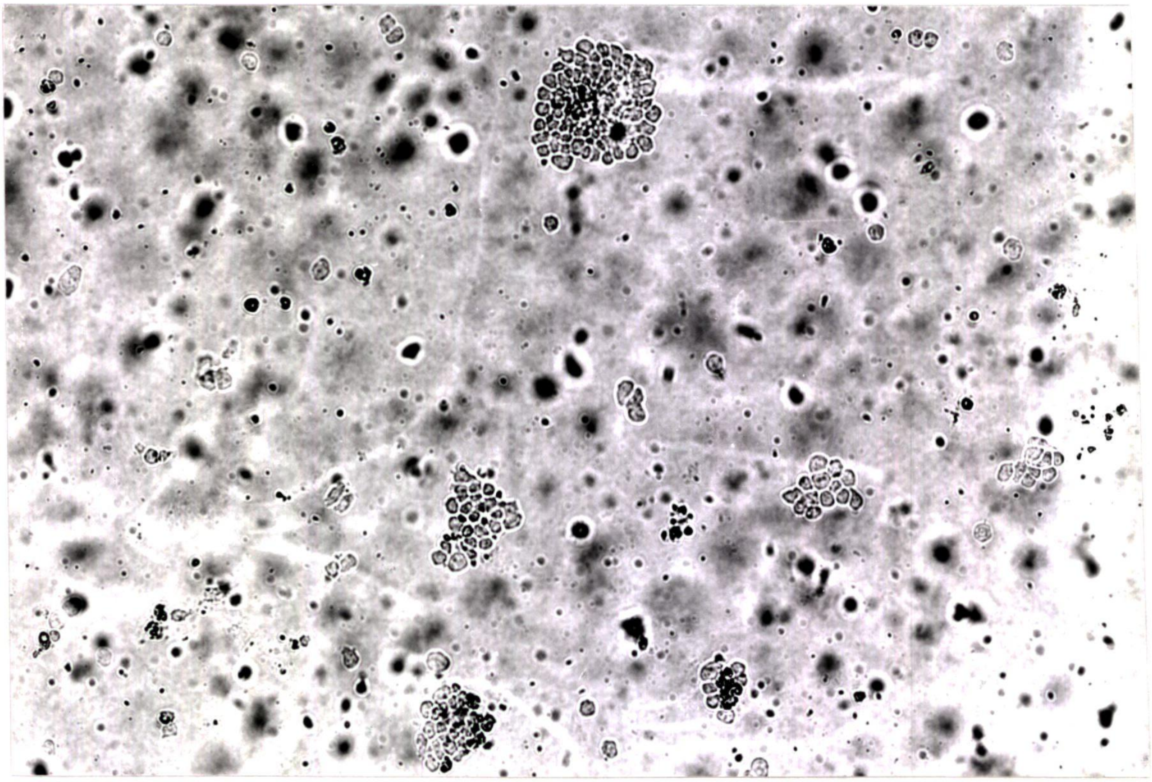


FIGURE 4

Figure 5. PHA stimulated lymphocytes in agar culture following treatment in liquid culture (18 hours) with a 1/4 dilution of interferon (X32).

Figure 6. PHA stimulated lymphocytes exposed to a 1/4 dilution of interferon in agar culture only (X32).

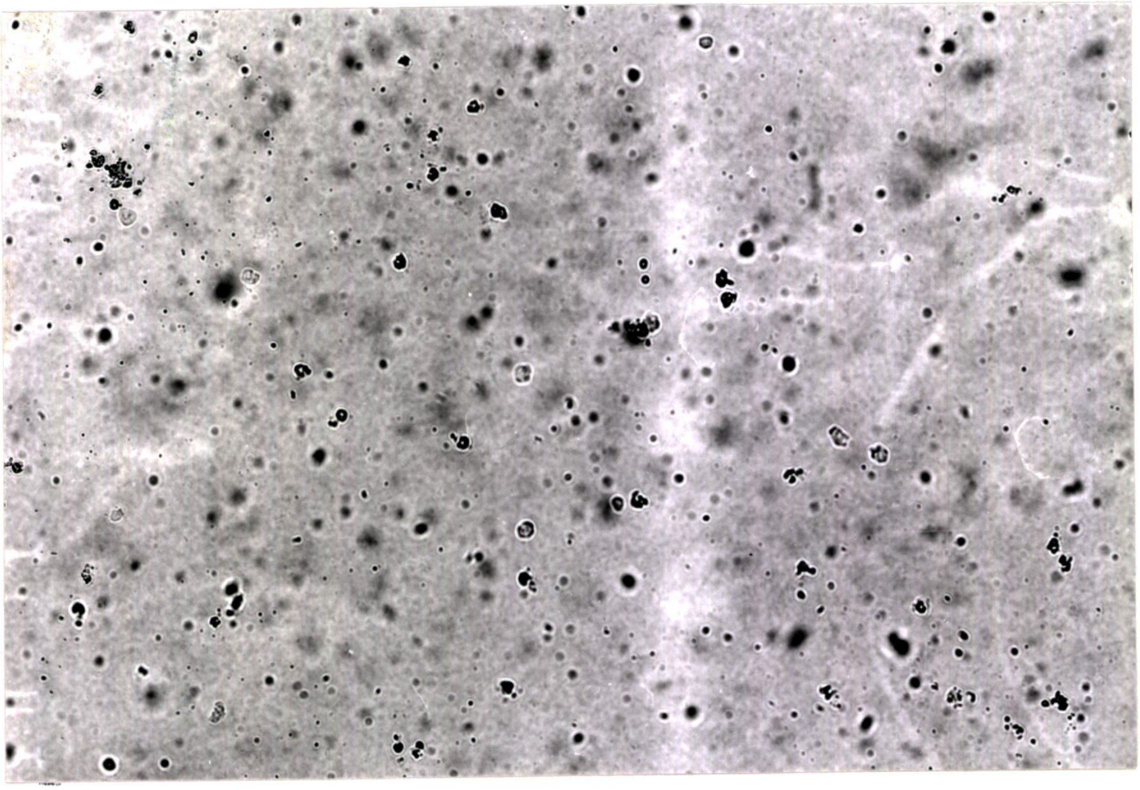


FIGURE 5

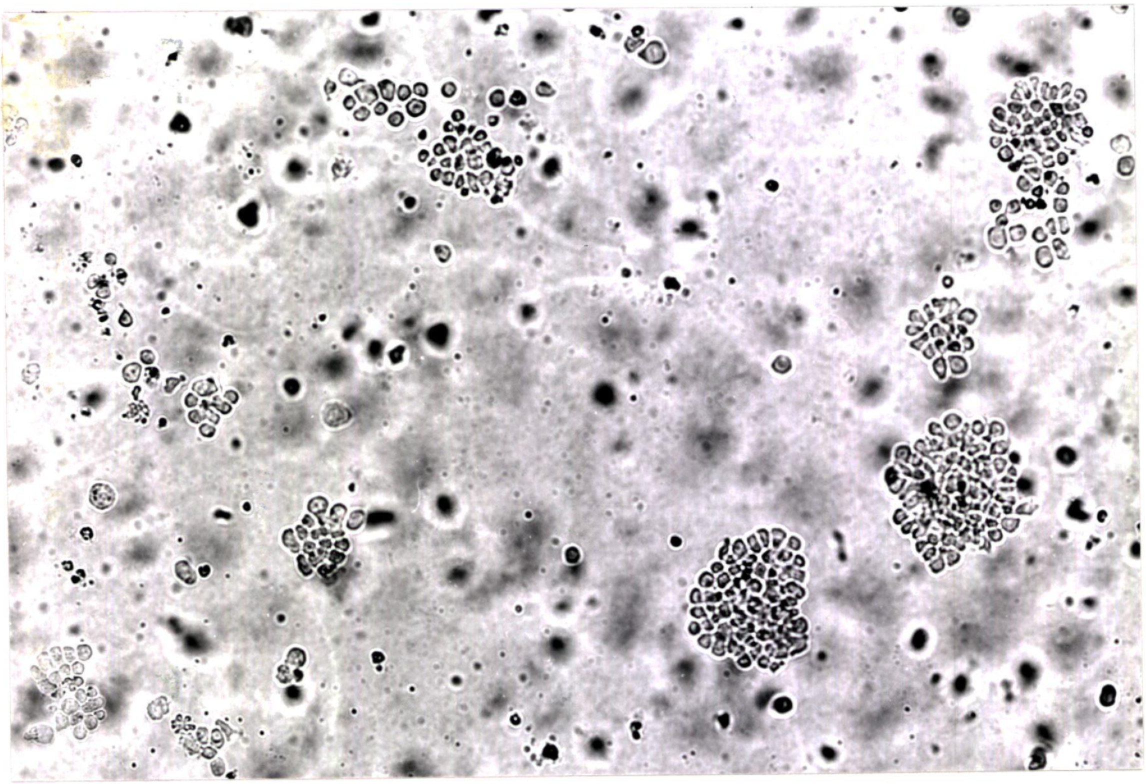


FIGURE 6

Figure 7. PHA stimulated lymphocytes in agar culture following treatment with a 1/1000 dilution of interferon in liquid culture (X32).

Figure 8. PHA stimulated lymphocytes in agar culture following treatment with a 1/4000 dilution of interferon in liquid culture (X32).

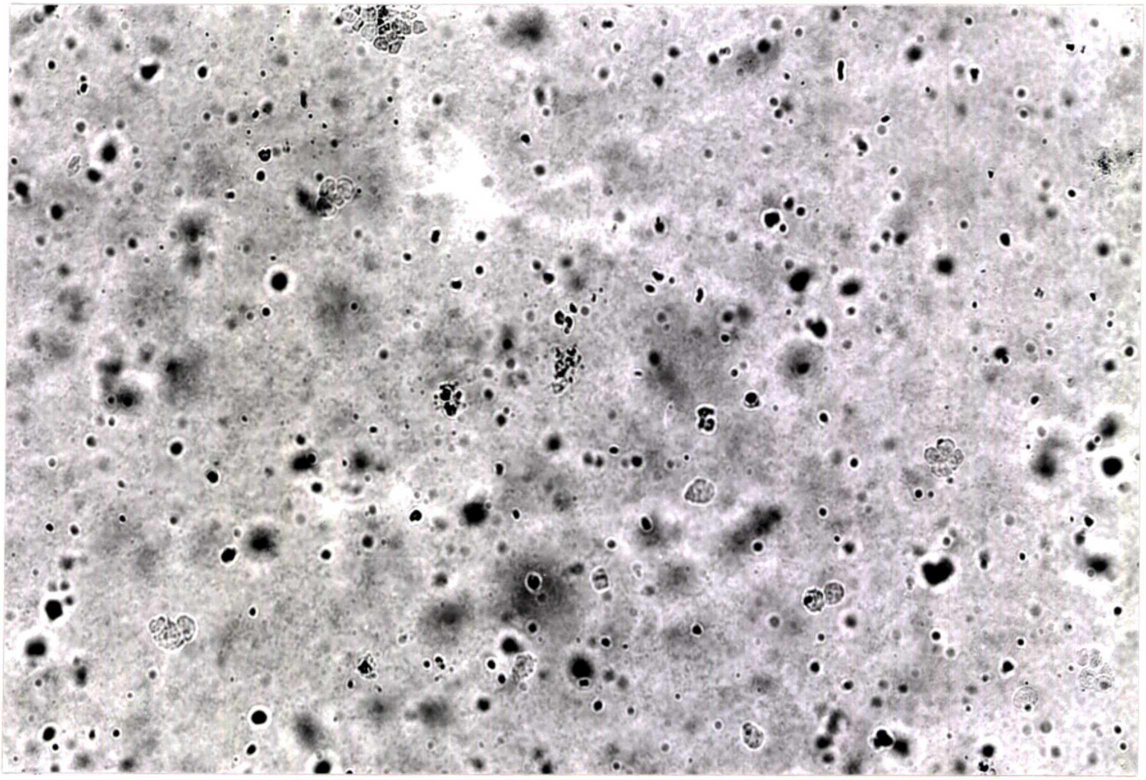


FIGURE 7

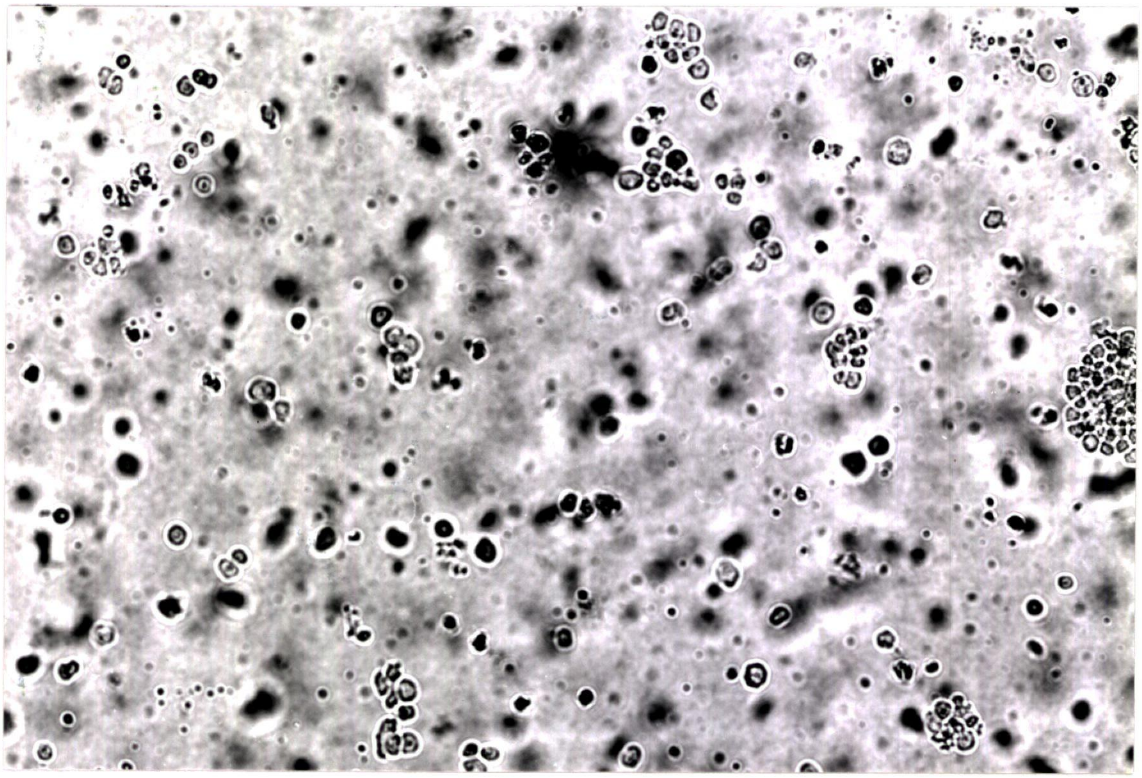


FIGURE 8

of cells is a clone which developed from a single activated lymphocyte. The cells shown in Figure 5 were treated with a 1/4 dilution of interferon in liquid culture, at the time of their initial contact with PHA. Some enlarged blast-like cells are visible, but no colonies have formed. Cells treated with the same concentration of interferon after they were transferred to the agar phase, eighteen hours after PHA stimulation (Figure 6) formed colonies which appear to be the same size as those formed in the control cultures (Figure 4). Examination of the plates "stained" with INT showed that control and interferon treated cultures produced the same number of colonies.

Figures 7 and 8 show cells which were exposed in liquid culture to interferon dilutions of 1/1000 and 1/4000 respectively. The 1/1000 dilution was the lowest dilution which would permit the development of colonies; the colonies were the same size as in the control cultures (Figure 4) but examination of the INT "stained" plates showed that they were far fewer in number. A 1/4000 dilution of interferon allowed maximum colony formation.

DISCUSSION

4.01 Genetic Studies

The response of cultured human fibroblasts to the antiviral and cell growth inhibitory activities of human interferon is controlled by a gene or genes on chromosome 21; fibroblasts with an extra copy of the chromosome are more sensitive to interferon than normal cells (Tan, 1975, 1976). In this project, comparison of fresh peripheral blood lymphocytes taken from normal and Down's syndrome people has shown that suppression of DNA synthesis by interferon is greater in trisomic 21 lymphocytes.

Cultured fibroblasts are cells which have lost most of the characteristics of cells in the tissue from which they were derived. In contrast, mitogen or antigen stimulated lymphocytes differentiate from quiescent cells into blast cells capable of antibody and lymphokine production, and specific cell killing. The end result of this project is to show that chromosome 21 is involved in the response of a normal, differentiated cell to interferon, and that the immunosuppressive action of interferon is, like the antiviral and anticellular activities, mediated through chromsome 21.

Trisomic 21 fibroblasts are already being used routinely in some laboratories to provide increased sensitivity for antiviral bioassays. It would not be practical to use fresh peripheral blood lymphocytes from people with Down's syndrome for routine bioassay of interferon's immunosuppressive activity, but trisomic 21 lymphocytes and macrophages could be used to differentiate interferon from other lymphokines with similar functions, such as lymphotoxin, macrophage inhibition factor, and macrophage spreading inhibition factor. Comparison of the effects of crude and fractionated lymphokine preparations on disomic and trisomic cells, may show whether interferon is a separate substance, or whether lymphokines are actually one substance whose action depends on the bioassay system in which it is tested.

Some of the many defects that accompany Down's syndrome, such as increased incidence of infections (Siegel, 1948) and tumours (Holland *et al.* 1962), may be due to disorders of the immune system. Results of *in vitro* tests for immunocompetence have not been conclusive. Elevated, normal, and depressed immunoglobulin levels have been reported (for summary of results see: Rosner *et al.* 1973), as well as normal (Fowler and Hollingsworth, 1973; Szigeti *et al.* 1974) and decreased (Burgio and Nespoli, 1974; Rigas *et al.* 1970) T cell function. My data agrees with previous reports that T lymphocyte response to mitogens is no different in Down's syndrome from that in normal people. Unlike Burgio and Nespoli (1974) I do not find a decrease in mitogen response in mongoloids with increasing age. There is also no indication in the data that trisomic 21 lymphocytes are hypersensitive in mixed lymphocyte culture as reported by Sasaki and Obara (1969).

Since it is possible that interferon is produced by activated suppressor T lymphocytes as a lymphokine for modifying cell mediated immunity (Johnson *et al.* 1977), the increased interferon sensitivity of lymphoid tissue found in Down's syndrome may be one of the factors contributing to the higher incidence of virus diseases and neoplasms in these people. Interferon produced by mitogen stimulated lymphocytes may be responsible for the decreased T cell function observed in mongoloids by some investigators. However, since it is possible that depressed cellular immunity may be due to factors other than increased interferon sensitivity, it would be advisable to measure the inhibition of trisomic 21 lymphocyte activity by substances other than interferon, perhaps by gonadotropin (Morse *et al.* 1976).

The results of these experiments cannot be used to show whether there is a difference between the effects of leucocyte and fibroblast interferons on lymphocyte activity. None of the interferon preparations used was purified; the leucocyte interferon may have contained inducer virus, and the superinduced fibroblast interferon probably contained small amounts of protein synthesis inhibitors. The only mock interferon preparation available was prepared for the plain induced fibroblast interferon. Future comparisons of the three interferons should include appropriate mock interferon solutions, which are difficult to concoct, or purified interferons.

4.02 Kinetics of Interferon Action

My experiments with PHA stimulated human lymphocytes, and previous experiments with concanavalin A stimulated mouse spleen cells (Lindahl-Magnussen *et al.* 1972; Rozee *et al.* 1973) have shown that maximum inhibition of DNA synthesis occurs when interferon is added at the time of mitogen stimulation. Increasing the interval between PHA stimulation and interferon addition results in a corresponding decrease in inhibition.

PHA stimulated lymphocytes cultured in soft agar showed similar results. High concentrations of interferon added at the start of the liquid culture period inhibited the formation of colonies, whereas the same concentration added eighteen hours later, when the lymphocytes were transferred to agar phase, allowed maximum colony formation. When more dilute interferon solutions were added to the liquid cultures, the colonies which developed in the agar were the same size as those in the control cultures, but there were fewer of them. This agrees with the results of experiments in which mouse B lymphocytes stimulated with sheep red blood cells were grown in individual wells so that the cells in each well at the end of the culture period were the progeny of a single activated lymphocyte. Interferon treatment was found to reduce the number of cells that could proliferate to form clones, but to have no effect on the final number of cells in the clone (Booth *et al.* 1976b).

Mitogen stimulation of lymphocytes results in alterations in the activities of almost every cellular metabolic pathway investigated (Ling and Kay, 1975). Synthesis of all types of RNA is increased, the rate of protein synthesis rises, carbohydrate metabolism is affected, and polyamine synthesis is increased. All of these changes begin within several hours of activation, although maximum levels may not be reached until several days later. DNA synthesis and the first mitoses occur twenty-four hours after mitogen stimulation. Since interferon has its main inhibitory effect during the first eighteen hours following lymphocyte stimulation, it seems probable that interferon blocks lymphocyte activation by interferring with one or more of the metabolic changes which accompany mitogen stimulation. Subsequent mitosis is much less susceptible to inhibition.

It is significant that viruses will not replicate in lymphocytes until six to eighteen hours after mitogen stimulation (Edelman and Wheelock, 1968), perhaps due to a lack of sufficient cellular machinery. Maximum virus replication, accompanied by inhibition of cellular DNA synthesis occurs between twenty-four and forty-eight hours following mitogen addition (Sullivan *et al.* 1975). The events which precede the first wave of mitosis in a lymphocyte culture are reflected by levels of

DNA synthesis measured several days later.

Cells which are not inhibited by interferon during activation may belong to a different subclass of lymphocytes. Booth and his coworkers separated mouse spleen cells on density gradients and found that they could be divided into three populations on the basis of response to stimulation by sheep red blood cells and inhibition by interferon (Finlay *et al.* 1977). Interferon had a selective effect on a population of high density lymphocytes which responded later than the other cell fractions to SRBC stimulation. It is possible that the interferon sensitive cells are the less differentiated precursor cells. A similar explanation may apply to selective inhibition of PHA stimulated T lymphocytes.

4.03 Evaluation of the Soft Agar Bioassay

A number of technical problems had to be overcome before the agar system produced any useful results. PHA, an extract of the kidney bean, *Phaseolus vulgaris*, contains leucoagglutinating and haemagglutinating activity in addition to its mitogenic activity. During the incubation with PHA in liquid culture which precedes agar culture the lymphocytes form clumps which, in my experience, cannot be dispersed mechanically. (Stimulated cells cannot be added directly to the agar phase because clumping and cellular interaction are necessary for lymphocyte activation - Peters, 1972.) To obtain the single cell suspension essential for cloning experiments, I found it necessary to resuspend the cells in 5 mM EDTA and mix vigorously on a vortex type mixer. The addition of N-acetyl-D-galactosamine, a sugar which can block PHA action by displacing it from its binding sites on the cell surface (Borberg *et al.* 1966), was less effective than EDTA. It was also necessary to use a more purified PHA preparation from Wellcome, rather than the cruder PHA from Difco used in earlier experiments. (Titration curves of the two preparations are shown in appendix 1.)

Problems with leucoagglutination lead me to mistrust published work on lymphocytes cultured in soft agar systems (Rozenszajn *et al.* 1975; Fibach *et al.* 1976) where it is claimed that mechanical disruption of lymphocytes stimulated with crude Difco PHA is sufficient for obtaining single cell suspensions. A study which shows a difference between the ability of PHA stimulated lymphocytes from healthy people and cancer patients to form colonies in soft agar (Wilson and Dalton, 1976), may be an artifact due to differences in their sensitivity to the leucoagglutinating properties of the mitogen; lectins are widely used for their ability to preferentially agglutinate malignant cells (for review see Sharon, 1977).

A further problem with the soft agar culture system was the determination of an optimum cell number. Sufficient cells must be used to allow lymphocyte activation in liquid culture, but too many cells forming clones in agar culture lead to counting difficulties and close apposition of clones. In the experiments reported here, 10^6 cells were added to each 1 ml liquid culture, well above the 10^5 cells per ml considered minimum for lymphocyte growth (Ling and Kay, 1975). Each tube was used to set up two agar cultures with 5×10^5 cells each. If one cell in 2.5×10^3 can give rise to a colony (Rozenszajn *et al.* 1975) each plate should have 200 colonies. This number of colonies is very difficult to count in a petri dish with a diameter of 35 mm. However, experiments with an initial cell number of 5×10^5 in the liquid cultures failed to produce clones in agar. Each experimental system has its own unique characteristics; more investigation is necessary to determine optimum cell concentrations for both liquid and agar cultures.

The soft agar bioassay does have some advantages over the conventional liquid culture bioassays. In my experiments, interferon preparations titrated in Microtest plates had endpoints (the dilution of interferon required for 50% inhibition) of 1/16 to 1/256 (Table 13), while agar assays gave endpoints closer to 1/500 to 1/1000. This increased sensitivity would be very useful for assaying solutions with very low levels of interferon activity. In addition, agar cultures do not rely on the use of radioisotopes which can cause cellular damage (Marz *et al.* 1977), and which increase the work involved in obtaining results. However, the system's most obvious advantage is that it can be used to analyze the events involved in the suppression of cell mediated immunity by interferon.

4.04 Summary

These experiments have shown that PHA stimulated primary lymphocytes derived from the blood of people with Down's syndrome are more sensitive than normal disomic 21 lymphocytes to suppression of DNA synthesis by human interferon preparations. Preliminary experiments using PHA stimulated lymphocytes grown in soft agar cultures indicate that interferon effects cellular events occurring within the first eighteen hours following stimulation. The more mature cells formed later in the culture period are much less sensitive to interferon, suggesting that interferon selectively inhibits undifferentiated lymphocytes. The immunosuppressive effect of interferon on T lymphocytes appears to be separate from the cell growth inhibitory effects, although the fact that chromosome 21 regulates both responses implies the involvement of a common cellular pathway.

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Figure 9. PHA TITRATION CURVES

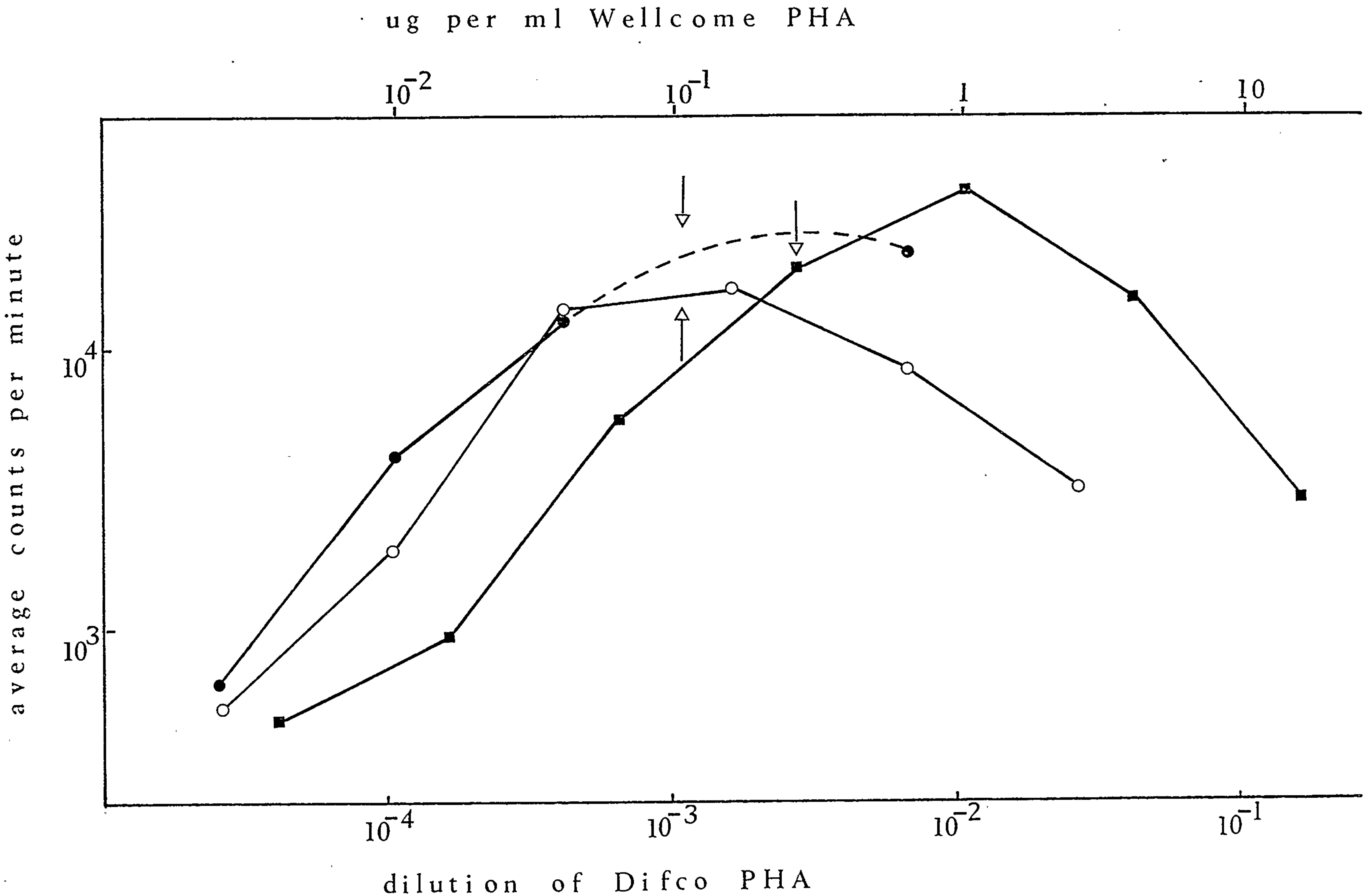
Titration curves for Difco unpurified and Wellcome purified PHA, showing the concentrations used to stimulate 5×10^4 cells in assays performed in Microtest plates.

● Difco PHA - disomic 21 lymphocytes

○ Difco PHA - trisomic 21 lymphocytes

■ Wellcome PHA - disomic 21 lymphocytes

↑ Concentration used in experiments


ug per ml Wellcome PHA
10^{-2}
10^{-1}
1
10
average counts per minute
10^4
10^3
10^{-4}
10^{-3}
10^{-2}
10^{-1}
dilution of Difco PHA