INTERFERONS

PROPERTIES AND APPLICATIONS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF.DR.J.SPERNA WEILAND
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 14 MEI 1980 DES NAMIDDAGS
TE 3.00 UUR

DOOR

HUBERTUS SCHELLEKENS

GEBOREN TE 'S-HERTOGENBOSCH

1980
DRUKKERIJ J.H.PASMANS B.V., 'S-GRAVENHAGE

PROMOTOREN:	PROF.DR.N.MASUREL
	DR.L.D.F.LAMEIJER
CO-REFERENTEN:	PROF.DR.A.BILLIAU
	PROF.DR.M.FRENKEL

INTERFERONS

PROPERTIES AND APPLICATIONS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF.DR.J.SPERNA WEILAND
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 14 MEI 1980 DES NAMIDDAGS
TE 4.15 UUR

DOOR

WILLEM WEIMAR

GEBOREN TE 'S-GRAVENHAGE

1980
DRUKKERIJ J.H.PASMANS B.V., 'S-GRAVENHAGE

PROMOTOREN:	PROF.DR.N.MASUREL
	DR.L.D.F.LAMEIJER
CO-REFERENTEN:	PROF.DR.A.BILLIAU
	PROF.DR.M.FRENKEL

This thesis is based on:

Schellekens, H., De Wilde, G.A. & Weimar W. (1980). Production and initial characterization of rat interferon. *Journal of General Virology* 46, 243-247.

Stitz, L. & Schellekens, H. (1980). Influence of multiplicity of infection on the antiviral activity of interferon. *Journal of General Virology* 46, 205-210.

Van 't Hull, E., Schellekens, H., Löwenberg, B. & De Vries, M.J. (1978). The influence of interferon preparations on the proliferative capacity of human and mouse bone marrow cells *in vitro*. *Cancer Research* 38, 911-914

Schellekens, H. & Weimar, W. (1978). Cytotoxicity of interferon and viral inflammation. *International Virology* IV, 110.

Weimar, W., Heijtink, R.A., Schalm, S.W., Van Blankenstein, M., Schellekens, H., Masurel, N., Edy, V.G., Billiau, A. & De Somer, P. (1977). Fibroblast interferon in HBsAg positive chronic active hepatitis. *Lancet* II, 1282.

Weimar, W., Heijtink, R.A., Schalm S.W. & Schellekens, H. (1979). Differential effects of fibroblast and leucocyte interferon in HBsAg positive chronic active hepatitis. *European Journal of Clinical Investigation* 9, 151-154.

Weimar, W., Heijtink, R.A. Ten Kate, F.J.P., Schalm, S.W., Masurel, N., Schellekens, H. & Cantell, K. (1980). Double-blind study of leucocyte interferon administration in chronic HBsAg positive hepatitis. *Lancet* I, 336-338.

Weimar, W., Schellekens, H., Lameijer, L.D.F., Masurel, N., Edy, V.G., Billiau, A. & De Somer, P. (1978). Double-blind study of interferon administration in renal transplant recipients. *European Journal of Clinical Investigation* 8, 255-258.

Weimar, W., Stitz, L., Billiau, A., Cantell, K., & Schellekens, H. (1980). Prevention of vaccinia lesions in rhesus monkeys by human leucocyte and fibroblast interferon. *Journal of General Virology*, in press.

Schellekens, H., Weimar, W., Cantell, K. & Stitz, L. (1979). Antiviral effect of interferon *in vivo* may be mediated by the host. *Nature* 278, 742.

CONTENTS

Publication of this thesis was supported by the Netherlands Kidney Foundation.

CHAPTER 1

INTRODUCTION

Interactions that occur when organisms are infected at the same time by more than one type of virus are called interference. In general, this phenomenon manifests itself as tissue-immunity: tissue infected by one type of virus is resistant to infection by a second one. It has been assumed for a long time that a non-viral agent was responsible for this effect. It lasted until 1957 before this agent was discovered by Isaacs and Lindenmann, who called it interferon. Since 1957 a tremendous effort has been invested in this agent with as main goal the clinical application. Over the last 20 years more than 2000 papers on interferon have appeared. The main results of these studies are reviewed in this chapter.

The action of interferon is not virus-specific. In cells treated with interferon the replication of a wide spectrum of viruses is inhibited. In general, the activity of interferon is species-specific and restricted to the species in which it is induced. It has proven very difficult to purify interferon completely and this has only very recently been claimed for some interferons. So the physico-chemical characteristics of interferons have not yet been completely established. In Table 1 the general characteristics of interferons are listed. The major cause of the difficulties in purifying interferons is their tremendous biological activity. Current estimates of the dilution of interferon still biologically active are as low as 10^{-15} M. If this is true, interferon

Table 1. General characteristics of interferons

Glycoprotein
Molecular weight 15,000 to 100,000 dalton
Isoelectric point 6.5 to 7.5
Activity: Unaffected in acid(pH 2.0) and alkaline(pH 11.0) solutions
Species-specific
Not virus-specific

is 10^2 times more active than cobra venom, one of the most active biological agents. Because of the difficulties in purification, interferon has to be quantitated in a bioassay. Almost every laboratory has its own assay. A vast number of factors may influence the assay results. Therefore, international reference preparations are available of the most commonly studied types of interferon.

Under normal conditions the gene for interferon production is suppressed. When a cell is infected by a virus interferon production is probably triggered by double-stranded RNA (ds-RNA). It is generally held that both DNA and RNA viruses produce some ds-RNA during their replicative cycle. Also inactivated viruses will produce ds-RNA, provided their polymerase activity is retained. If the polymerase activity is inactivated, viruses will not induce interferon. Non-viral ds-RNA can also induce interferon. The most potent and widely used ds-RNA inducer of interferon is the synthetic polyinosinic-polycytidylic acid (poly(rI).poly(rC)).

Widely used non-viral inducers are listed in Table 2. Some substances as pokeweed mitogen induce interferon in lymphoid cells only. These cells will then produce another type of interferon called type II interferon (or 'immune' interferon). So the type of inducer can influence the type of interferon produced. Lymphoid cells challenged with virus will produce the 'classical' interferon or type I interferon.

Interferon does not inactivate viruses directly but its antiviral action is mediated by the cell. Interferon does not enter cells but interacts with a receptor on the cell surface. By this interaction an

Table 2. Non-viral inducers of interferons

	In vivo	*In vitro*
Polynucleotides	+	+
Anionic polymers	+	-
Tilorone	+	+
Acridine	+	-
Statolon	+	+
Bacteria and endotoxins	+	-
Mitogens	+	+(type II interferon)

intracellular protein is produced that exerts its antiviral effect on the molecular level. The level at which the viral replication is inhibited is dependent on the virus-cell system studied. Inhibition of transscription, translation and viral maturation have been reported. The suggested mechanisms include changes in viral m-RNA, in cellular m-RNA ribosomes, induction of nucleases, changes in the t-RNA pool, changes in the initiation factors, etc.

Interferon preparations not only exert antiviral but also other activities. These activities were generally considered to be caused by impurities, but recent studies with pure or almost pure preparations have shown that the interferon molecule itself is responsible for these 'non-antiviral ' activities. Cell division is delayed in interferon-treated cells. Small doses prime cells for enhanced interferon production after induction, while high doses inhibit production. Interferon treatment enhances the expression of transplantation antigens on the cell surface. Other changes in cell surface properties, such as inhibition of thymidine transport, increase of binding of concanavalin A and reduction of binding of thyroid-stimulating hormone and cholera toxin, are reported. Interferon can also enhance specialized cell functions, such as macrophage activity, natural killer (NK) cell activity and lymphocytic cytolysis of tumour cells. Interferon can modulate immune responses. It suppresses the delayed type hypersensitivity in mice. When administered before or together with antigen,

antibody production will be inhibited. Given several days after an antigen it will enhance antibody production.

When an organism is infected by a virus several host mechanisms are set to operate (Table 3). In general, the role of antibodies in viral

Table 3. Antiviral defence mechanisms

	Time needed for activation	Function
Inflammatory reactions	hours	inhibition of viral replication
Interferon	hours	inhibition of viral replication
Antibodies	several days	inhibition of viral spread
Cellular immunity	several days	inactivation and clearance of virus

infections is restricted to the inhibition of viral spread. During first infections with a virus antibodies are formed too late to prevent this transport. Some viruses spread without entering the extracellular space and avoid exposing themselves to antibodies. When a virus is replicating, cellular immunity and interferon are the main defences. Interferon production starts within hours after infection. The cellular immune system needs days to be activated. Decrease of viral replication seems more related to the interferon production than to activation of the immune system. Antibodies to interferon injected in experimentally infected animals can change a harmless infection into a lethal one. So interferon plays an important part in the host defences against viral infections. However, interferon is only virostatic and cannot clear viruses from the body like immune cells. The biological function of interferon seems to be to slow down an infection during the period necessary for activation of the immune system.

Soon after the discovery of interferon it was shown that exogenous

interferon, applied locally or systemically, can control viral infections. In general, more interferon is needed to prevent than to cure viral infections.

Interferon has been shown to be active against both virus-induced and non-virus-induced tumours in animals. Non-antiviral effects of interferon are supposed to play an important role in this antitumour effect, in particular macrophage activation and modulation of the immune system.

Use of interferon inducers is not yet feasible in man. Some inducers fail to induce interferon in man, others are toxic or antigenic. To evaluate its clinical usefulness, interferon has to be produced *in vitro*. The main sources for human interferon have been buffycoats induced with Sendai virus and fibroblasts induced with poly(rI).poly(rC). The buffycoats yield human leucocyte interferon (HLI) and the fibroblasts human fibroblast interferon (HFI). HLI and HFI differ in physico-chemical, antigenic and biological characteristics. Most studies with human interferons in man are anecdotical. The dose of interferon and frequency of injections vary widely. We know of one study only in which the effect of systemically administered interferon was unequivocally established (Merigan *et al.* 1978). In this study 500,000 units/kg/day, started within a day after diagnosis of herpes zoster, were able to control the spread of the lesions. Also the results with topically applied interferon in herpes keratitis are convincing. All other studies, including those on the alleged beneficial effect of interferon in hepatitis B virus infections are uncontrolled, inconclusive and involve just a few patients. There are also claims that interferon has a beneficial effect in tumour patients. Again, these studies are not properly controlled.

References

Finter, N.B. (ed.) (1973). *Interferons and Interferon Inducers. Frontiers of Biology*, vol. 2. Amsterdam, North Holland Publishing Company.

Friedman, R.M. (1977). Antiviral activities of interferon. *Bacteriological Reviews* 3, 543-567.

Gresser, I. (1977). On the varied biologic effects of interferon. *Cellular Immunology* 34, 406-415.

Isaacs, A. & Lindenmann, J. (1957). Viral interference. I. The interferon. *Proceedings of the Royal Society, series B* 147, 258-267.

Stewart, W.E. (1979). *The Interferon System*. Wien/New York, Springer Verlag.

Further references are listed in the general reference section.

Justification

The main theme of this thesis is the clinical evaluation of interferon. From the biology of the interferon system and animal experiments it can be expected that exogenous interferon will exert its optimum effect when used to prevent acute infections or to modulate chronic infections. Therefore, we administered interferon to patients with chronic hepatitis B virus infection (chapter 5) and to renal transplant recipients, in whom viral infections occur frequently in the first months after transplantation (chapter 6).

The other studies in this thesis are directly related to the problems we met in the clinical studies. We wanted to study interferon in an animal renal transplantation model. For us the most obvious choice was the rat. However, little was known about the production and characterization of rat interferon. Chapter 2 describes our experiences with rat interferon.

While we were well underway with the study in renal transplant recipients, we were contacted by Martin Hirsch, who was conducting a similar trial in Boston. Some of his patients receiving 3×10^6 U HLI every other day showed severe bone marrow depression. We had no such problem in our trial, but we used another type of interferon: HFI. For this reason we started a study on the toxicity of interferons for bone marrow *in vitro*. The results are presented in the first part of chapter 4. We extended this study to a more fundamental approach of

growth inhibition. In the second part of chapter 4 we present a simple method to measure growth inhibition of cells in monolayer and some results with this method.

Several possibilities arose to explain the negative results of the renal transplant trial, e.g. dosage, differential effects of HLI and HFI, and efficacy of interferon under circumstances of immunosuppression. We decided to study these problems in a monkey model. In the first part of chapter 7 we report our experiences with human interferon preparations in vaccinia virus infected rhesus monkeys. During this study we found that human interferon was able to protect monkeys *in vivo* against vaccinia virus, while the same virus was insensitive to interferon *in vitro*. These results are presented in the second part of chapter 7.

During the study of the *in vitro* sensitivity of viruses, we found that the effect of interferon was dependent on the amount of virus by which the cell cultures were infected. In chapter 2 the results are presented of an extensive study on the influence of multiplicity of infection on the antiviral effect of interferon in different cell-virus systems.

In this thesis the results of our studies have been arranged in such a way that the basic aspects of the interferon system, such as production, characteristics, antiviral and non-antiviral activities precede the clinical studies. Thereafter the experiments in rhesus monkeys are presented because they elucidate some of the problems that arose during the studies in man.

The clinical and basic studies have largely influenced each other. Therefore, we decided to present them in one thesis. We both feel responsible for the complete contents, although we have of course contributed from a different background. The greater part of this thesis has already been published in a number of publications (see page 7). Authorship and order of authors indicate the extent to which each of us has contributed to the various studies.

IN VITRO STUDIES

CHAPTER 2

PRODUCTION AND INITIAL CHARACTERIZATION OF RAT INTERFERON

The purpose of this study was to develop the technology for the production of rat interferon for *in vivo* studies.

This study was achieved by cooperation of the Departments of Virology and Internal Medicine, Erasmus University Rotterdam.

This chapter is based on:
'Production and initial characterization of rat interferon' by H.Schellekens, G.A. de Wilde & W.Weimar, published in *Journal of General Virology* 46, 1980, 243-247.

Introduction

Few and partly conflicting studies have been published on rat interferon. Differences exist between reports on the physico-chemical properties and the activity of rat interferon in cells of other species (Biernacka & Lobodzińska, 1973; Lobodzińska *et al.* 1973; Illinger *et al.* 1976). Interesting is the reported antiviral activity of human interferons in rat cells and the ability of human interferon to inhibit rat interferon activity (Duc-Goiran *et al.* 1971; Chany, 1976).

However, all these studies have been performed with rather impure interferon preparations. This study was designed to produce rat interferon of high activity for *in vivo* studies and to compare some of its physico-chemical and biological activities with other interferons.

Material and Methods

Viruses

The origin, propagation, and titration of herpes hominis type I, vaccinia, and vesicular stomatitis virus (VSV) are described in chapter 3. Newcastle disease virus (NDV), Komarow strain, was grown in the allantoic fluid of 10-day-old chicken embryos. The titre of this virus was expressed in plaque forming units (PFU), established in primary chicken embryo cell cultures.

Cells

Mouse L_{929} cells were originally obtained from Flow Laboratories (Irvine, Scotland). Chicken embryo cells were prepared by mincing and trypsinizing 10-day-old chicken embryos. Rat embryo cells (REC) were prepared by mincing and trypsinizing approximately 15-day-old embryos of WAG/Rij rats. The first passage of these cells was pooled and stored in liquid nitrogen using dimethylsulphoxide (DMSO) as a cryoprotective agent. REC used in these experiments were a subcultivation of the first passage. Normally, REC could be subcultivated up to the 30th passage. After the 40th passage we designated these cells Ratec cells. This Ratec cell line has now undergone more than 100 passages in our laboratory without degeneration.

XC cells (derived from a Rous sarcoma virus-induced rat tumour) and RR1022 cells (from a Schmidt-Ruppin sarcoma in a Rattus norvegicus) were both obtained from the American Type Culture Collection. Cells derived from a radiation-induced rat skin carcinoma (RSC cells), cells from rat urethral carcinomas (RUC cells), cells from a spontaneous rat rhabdomyosarcoma (RM cells) and cells from a rat osteosarcoma (ROS cells) were donated by Dr.G.W.Barendsen (Radiobiological Institute TNO, Rijswijk, The Netherlands). WIRA cells were donated by Dr.I.Gresser (Institut de Recherches Scientifiques sur le Cancer, Villejuif, France). Rous sarcoma virus-transformed human cells (RSb cells) were donated by Dr.T.Kuwata (Chiba University, Chiba, Japan).

All cells were routinely propagated in Brockway prescription bottles, in Dulbecco's modification of Eagle's minimal essential medium (DMEM), supplemented with 10% foetal calf serum (FCS) and antibiotics. When cells were grown in roller bottles, DMEM was supplemented with a buffer solution. This 100 x concentrated buffer solution (pH 7.2) consisted of 0.75 M-*N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulphonic acid (HEPES), 0.5 M-*N*-tris-(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES) and 0.5 M-morpholinopropane sulphonic acid (MOPS).

Interferons

Mouse interferon (MIF) was induced with NDV in L_{929} cells. The interferon preparation used had an activity of 10^6 units/mg protein. HLI was a kind gift from Dr.K.Cantell (Central Public Health Laboratory, Helsinki, Finland) and was prepared as described before (Cantell,1970). The activity was $10^{5.9}$ units/mg protein. HFI was prepared as described before (Billiau *et al.* 1973), and was obtained by the courtesy of Dr.A.Billiau (Rega Institute, University of Leuven, Leuven, Belgium). HFI had an activity of 10^6 units/mg protein.

To produce rat interferon (RIF) 2 x 10^7 Ratec cells in 100 ml DMEM plus 10% FCS were grown in 1.5 l roller bottles rotating at 0.5 rev/min. Usually within 4 days confluency was reached and the cells were refed with 100 ml DMEM plus 10% newborn calf serum (NCS). The next day the culture medium was discarded and the cells were challenged with NDV at a multiplicity of infection of 30 to 50 PFU/cell for 90 min.

After challenge the cells were washed twice with phosphate buffered saline (PBS) and 50 ml DMEM without serum added. After 24 h the medium was harvested and centrifugated for 2 h at 150,000 g to remove the bulk of NDV. The remaining NDV was either inactivated by pH 2.0 treatment for 5 days, or by adding perchloric acid to 0.15 M for 30 min. Perchloric acid treatment did not result in loss of antiviral activity. After adjusting the pH to 6.0 with 5.0 M-NaOH, the preparation was stored frozen at -20° C and designated 'crude interferon'. No replication of residual live NDV could be demonstrated after inoculation of the preparations in chicken embryos.

Interferon assay

Cells were grown in microtitre plates at a concentration of 2×10^4 cells per well in DMEM supplemented with 10% FCS. When a monolayer was formed, the supernatant was removed and the preparation to be tested added in twofold dilutions in DMEM with 1% FCS. After overnight incubation the supernatant was again removed, the cells were washed with PBS (Dulbecco's modification) and infected with VSV at a multiplicity of infection 10 tissue culture infective dose (50%) ($TCID_{50}$) per well. When the untreated infected control showed > 90% cytopathogenic effect, the incubation was terminated and the cells were stained with crystal violet. The interferon activity was expressed as the reciprocal of the maximum dilution protecting 50% of the cells. This 50% endpoint was either estimated visually or established by the dye elution method of Armstrong (Armstrong, 1971).

In every assay a laboratory standard was included to correct for variables between the different tests. One of the first rat interferon preparations produced in rat embryo cells challenged with NDV was diluted in DMEM supplemented with 10% FCS and stored in small ampoules at -70° C to serve as laboratory standard. The mean result of the first 10 tests of the antiviral activity of this standard was 96 units. This preparation was therefore considered to contain 100 laboratory reference units of RIF and all RIF activities in this study were titrated against this standard. This standard preparation has been tested until now on 28 different occasions. The results of all these tests were

normalized by a log transformation for further analysis. The log transformed results showed a normal distribution. The mean log titre was 1.85 (i.e. 71 units) with a standard deviation of 0.24. This resulted in a coefficient of variation of 13%. This indicates that our laboratory standard and method of testing of RIF activity are reliable. Research referencence preparation G-002-904-511 was used as a reference for the MIF assay in L_{929} cells, and preparation 69/19 as human interferon reference in RSb cells. Hence the activities of these interferons were expressed in international reference units.

Growth inhibition assay

The growth inhibitory activity of interferon was measured with a dye elution assay. In summary, approximately 10^5 cells were seeded in 6 cm petri dishes in 5 ml DMEM supplemented with 10% FCS and the appropriate interferon dilution was added. When the untreated controls reached semiconfluency, the incubation was terminated and the cells were stained with crystal violet. Because the uptake of crystal violet by cells was directly related to the number of cells, growth inhibition was estimated by extracting the dye with 2-methoxy-ethanol and comparing the E_{550} of the elutions from treated and control cultures.

Results

Factors influencing RIF production

The influence of the challenge dose of NDV on the interferon production by REC is shown in Table 4. The interferon production increased with increasing challenge doses. The highest production was obtained with the highest challenge dose tested ($10^{2.4}$ PFU/cell). Routinely, however, we used a challenge dose of 30 to 50 PFU/cell. Employing this challenge dose, NDV-containing allantoic fluid could be used without concentration.

The interferon-producing capacity of different rat cells challenged with NDV is shown in Table 5. The secondary REC and Ratec cells were the best producers. WIRA and RR1022 cells produced only moderate amounts of interferon. The same table shows that type and concentration

Table 4. Influence of challenge dose of NDV on interferon production by REC

Challenge dose (PFU/cell)	Interferon production (units/ml)
10^{0}	$10^{2.6}$
$10^{0.5}$	$10^{2.9}$
$10^{1.0}$	$10^{2.9}$
$10^{1.4}$	$10^{3.2}$
$10^{1.9}$	$10^{3.2}$
$10^{2.4}$	$10^{3.5}$

Table 5. Interferon production by rat cells challenged by NDV† at different serum concentrations

Type of cells	Serum concentration	Interferon production (units/ml)
REC		
Passage 7	10% FCS	$10^{3.5}$
Passage 10	10% NCS	$10^{3.2}$
	1% NCS	$10^{2.9}$
Passage 13	10% NCS	$10^{3.0}$
Ratec		
Passage 40	1% NCS	$10^{2.9}$
Passage 46	-	$10^{4.4}$
Passage 52	-	$10^{4.0}$
WIRA	10% NCS	$10^{2.0}$
RR1022	10% NCS	$10^{2.6}$

† Challenge dose: 30-50 PFU/cell

Table 6. Induction of interferon by poly(rI).poly(rC) in REC employing different schemes of induction

	Poly(rI).poly(rC) (100 µg/ml)	Poly(rI).poly(rC) (10 µg/ml) DEAE dextran (100 µg/ml)	Superinduction scheme A	Superinduction scheme B
Interferon production during 24 h after induction	≤50	150	<50	<50
Interferon production during 48 h after induction	50	≤50	<50	<50

Superinduction scheme A (Billiau *et al.* 1973)

Priming for 24 h with 3,000 units/ml. Induction with poly(rI).poly(rC) 50 µg/ml for 1 h. After induction treatment with cycloheximide 10 µg/ml for 5 h, the last 2 h combined with actinomycin D 1 µg/ml.

Superinduction scheme B (Wiranowska-Stewart *et al.* 1977)

Induction for 1 h with 100 µg/ml poly(rI).poly(rC) plus 50 µg/ml cycloheximide. Cycloheximide alone for 2 h. Thereafter, for 3 h cycloheximide combined with 50 µM-5.6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB). Finally, for 18 h treatment with 40 µM-DRB.

of serum and omitting serum from the culture medium did not influence production.

Poly(rI).poly(rC) is a potent inducer of interferon *in vitro*. In Table 6 the interferon production in rats cells challenged with poly(rI).poly(rC) is shown. Compared with NDV, poly(rI).poly(rC) alone proved a poor inducer of interferon in REC. The inducing capacity could only slightly be increased by adding diethylaminoethyl (DEAE) dextran or by priming. The superinduction schemes, successfully employed in human embryonic cells, actually inhibited interferon production in rat cells.

Concentration and partial purification of RIF

Ammonium sulphate has been widely used to precipitate interferons from different species. In Table 7 the effect of various ammonium sulphate concentrations on the precipitation of RIF is shown. Some activity

Table 7. Precipitation of RIF by ammonium sulphate

Saturation with ammonium sulphate(%)	Interferon in supernatant(units/100 ml)	Interferon in precipitate (units/10 ml)
0	$10^{2.3}$	-
25	$10^{2.6}$	$10^{3.0}$
40	$10^{1.8}$	$10^{3.8}$
55	<10	$10^{3.9}$
70	<10	$10^{4.0}$
85	<10	$10^{4.4}$
100	<10	$10^{4.4}$

precipitated at 25% and the precipitation was complete at 85% saturation. The precipitation of RIF at 85% saturation of ammonium sulphate appeared independent of pH in the range of pH 2.0 to pH 7.5 (results not shown). RIF activity could also be precipitated with zinc acetate at a concentration of 0.02 M. Zinc acetate precipitation of RIF resulted in a higher specific activity (Table 8). The preparations used in

Table 8. Flow sheet for partial purification of RIF

Initial: 100 ml crude rat interferon 120 units/ml activity 10^2 units/mg protein	
Ammonium sulphate precipitation method	Zinc acetate precipitation method
Precipitate with $(NH_4)_2SO_4$ added to 85% saturation	Precipitate with zinc acetate added to 0.02 M
Centrifuge 60 min at 10,000 rev/min	Centrifuge 60 min at 2,000 rev/min
Dissolve precipitate in 10 ml PBS	Dissolve precipitate in 10 ml 0.2 *N* HCl
Dialyse extensively against PBS	Dialyse first against saline, then against PBS
Centrifuge for 30 min at 10,000 rev/min	Centrifuge for 30 min at 10,000 rev/min
Sterilize by Millipore filtration	Sterilize by Millipore filtration
Final: 10 ml purified rat interferon	
68,000 units/ml Activity $10^{5.4}$ units/mg protein	68,000 units/ml Activity $10^{5.8}$ units/mg protein

the present study were concentrated and purified by zinc acetate precipitation. No loss in activity was encountered during this procedure. On the contrary, both methods of precipitation resulted in an unexpected increase in activity, as can be seen in Tables 7 and 8.

Stability of RIF

RIF appeared stable when stored at ~70° C. Our laboratory standard stored this way in the presence of 10% FCS showed no loss in antiviral activity during a 2-year period. Fig. I shows the stability in the presence of 1% FCS at other temperatures. At 0° C no loss in activity was observed during 24 h incubation. At a room temperature of 20° C activity started to decrease after 4 h. One hour at 37° C resulted in a 50% loss in activity. Heating at 100° C destroyed all activity after 15 min. Table 9 shows the effect of freezing and thawing on the anti-

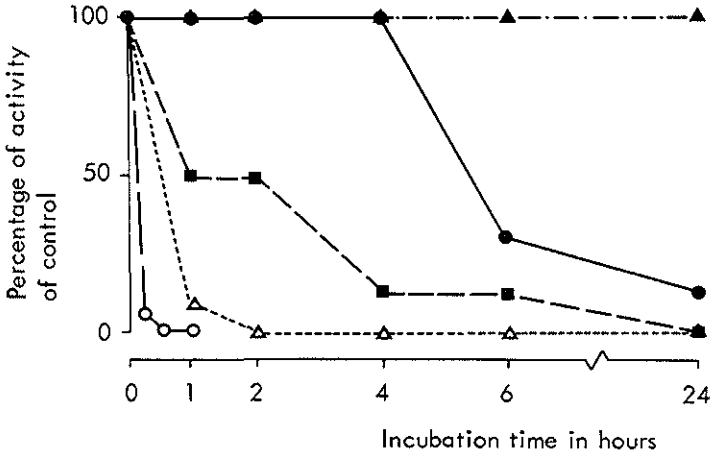

Fig. I. Influence of temperature on antiviral activity of RIF. RIF was incubated in DMEM supplemented with 1% FCS at the temperatures indicated: ▲= 0° C; ● = 20° C; ■ = 37° C; △ = 56° C; ○ = 100° C. The maximum incubation time at 100° C was 1 h, at the other temperatures 24 h. Samples were taken at the time-points indicated and immediately tested for interferon activity. Unincubated samples, which were kept frozen until immediately before testing, served as controls.

Table 9. Influence of freezing and thawing on antiviral activity of RIF

No. of times frozen and thawed	Interferon activity (units/ml)
1	$10^{2.2}$
3	$10^{2.2}$
5	$10^{1.9}$
7	$10^{1.9}$

The RIF preparation was diluted in DMEM supplemented with 1% FCS and frozen at -70° C. The sample was thawed in a waterbath at 37° C.

viral activity of interferon. Three cycles of freezing and thawing did not have any effect on activity. Seven cycles resulted in the loss of half of the activity.

Characterization and species-specificity of RIF

Table 10 shows that the antiviral activity in our preparations met the following criteria for an interferon. It was resistant to pH 2.0, sensitive to trypsin treatment, and inactive in heterologous mouse and human cells. Rat cells could be protected against both VSV and vaccinia virus.

Table 10. Characterization of RIF

Activity	Units/ml
Initial preparation†	$10^{4.5}$
Treated with trypsin¶	<10
Activity against vaccinia	$10^{4.0}$
Activity in L_{929} cells	<10
Activity in RSb cells	<10

† Treated at pH 2.0 for 5 days to inactivate NDV
¶ Treated for 1 h at 37° C with 0.25% trypsin

In Table 11 the antiviral activity of rat and human interferons in different rat cells is compared. The ability of these interferons to protect cells against the cytopathogenic effect of both VSV and herpes virus was tested. To protect Ratec cells against VSV, the least amount of RIF was necessary. The lowest protection against this virus was encountered in RUC II cells. ROS and RR1022 cells were equally well protected when the RIF activity was measured with herpes virus as challenge virus. Ratec cells were the least sensitive for the RIF protective effect against herpes virus. Apparently, there was no correlation between protection against VSV or herpes induced by RIF in these cells.

Table 11. Antiviral activity of rat and human interferons in rat cells of different origin

Type of interferon and challenge virus		Highest dilution giving 50% protection							
		Ratec	RUC II	RSC	ROS	RM	XC	RR 1022	WIRA
RIF†	VSV	$10^{4.8}$	$10^{2.8}$	$10^{3.4}$	$10^{3.9}$	$10^{3.4}$	$10^{4.0}$	$10^{4.0}$	$10^{3.6}$
RIF	Herpes	$10^{3.8}$	ns¶	ns	$10^{4.7}$	ns	ns	$10^{4.7}$	$10^{4.1}$
HLI	VSV and herpes	<10	<10	<10	<10	<10	<10	<10	<10
HFI	VSV and herpes	<10	<10	<10	<10	<10	<10	<10	<10

† $10^{4.8}$ units/ml
¶ not sensitive (virus does not produce cytopathogenic effects in these cells)

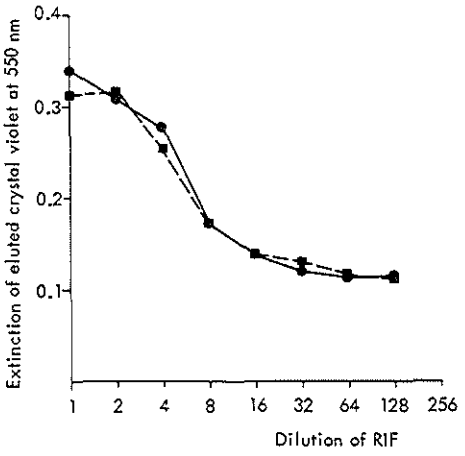

Fig. II. Antiviral effect of RIF in secondary REC pretreated with 10^3 units of HLI. The activity of an RIF preparation in REC pretreated with HLI was measured as described in Material and Methods. The extinction of untreated REC (■------■) is compared to that of REC treated with 10^3 units of HLI (●——●).

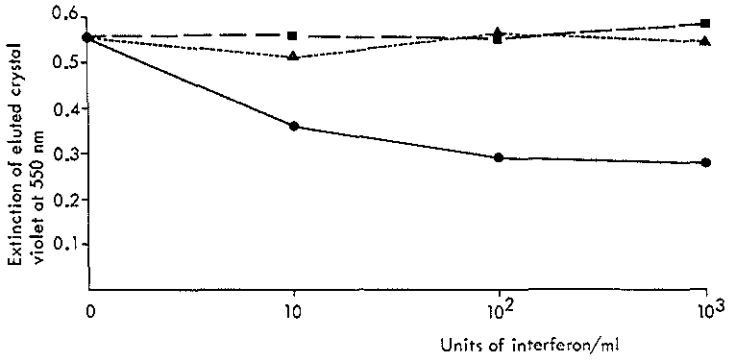

Fig. III. Influence of rat and human interferons on the growth of secondary REC. The inhibition of the growth of REC by RIF (●), HLI (■), and HFI (▲) was measured as described in Material and Methods.

HFI nor HLI had an antiviral effect in rat cells. In Fig. II the antiviral effect of RIF in REC pretreated with HLI is compared with the antiviral activity in untreated rat cells. There was no effect of HLI on the antiviral activity of RIF. Similar results were obtained when the cells were pretreated with HFI or when cells were incubated with RIF in the presence of either human interferon at a concentration of 10^3 units/ml. RIF also inhibited the growth of cells of the own species (Fig. III). 10^3 units of RIF/ml inhibited the growth of REC by 50%. Human interferons also lacked this activity in rat cells.

Discussion

This study shows that a high challenge dose of NDV is necessary to induce reasonable amounts of interferon in rat cells. When challenged with NDV, secondary REC and Ratec cells were the best producers of interferon of all cells studied.

Serum in the culture medium is a major source of impurities in interferon preparations produced *in vitro*. We demonstrated that the production of RIF was not affected when serum was omitted from the tissue culture medium. This made it possible to produce RIF of a high activity with the use of a single purification and concentration step.

Poly(rI).poly(rC) was a very poor inducer of RIF in REC. Addition of DEAE dextran only slightly enhanced the inducing capacity of poly(rI).poly(rC). Alone or combined with DEAE dextran it has also been reported to be a poor inducer of interferon when perfused through rat lungs (Tilles & Braun, 1973). It was not possible to enhance the interferon production by poly(rI).poly(rC) employing methods of superinduction successfully used in human cells (Billiau *et al.* 1973; Wiranowska-Stewart *et al.* 1977).

The properties of our RIF differed from those of the preparation of Schonne (1966), who found that the interferon activity was precipitated by ammonium sulphate at 100% saturation only, and that the interferon activity could not always be recovered when precipitated by 0.02 M-zinc acetate. He also reported a 50% loss of interferon activity after treatment with 0.15 M-perchloric acid and complete loss of activity

after 2 cycles of freezing and thawing. In the present study, RIF completely precipitated at 85% saturation of ammonium sulphate. Interferon activity could easily be recovered from the 0.02 M-zinc acetate precipitate. 0.15 M-perchloric acid could be used to inactivate NDV without any loss in interferon activity and the antiviral activity of interferon was not affected by 3 cycles of freezing and thawing.

There was a considerable increase in the total amount of RIF activity when our preparations were concentrated. This could not be explained by interferon induction by residual NDV in our preparations. Inoculation in embryonated eggs failed to eliminate live NDV and the preparation lacked activity in L_{929} cells, which are sensitive to interferon induction by both live and inactivated NDV. An antagonist of interferon activity produced in embryonic and other tissues has been described (Fournier *et al.* 1972). It is possible that during concentration this antagonist of interferon was lost. Another explanation of this increase in activity upon concentration can be that interferon molecules associate or combine with other substances, resulting in increased antiviral activity.

The influence of temperature on the antiviral activity of rat interferon reported here is in good agreement with other studies (Schonne, 1966; Billiau & Buckler, 1970). Activity of RIF in cells of other species, especially mouse cells, has been reported by several groups (Biernacka & Lobodzińska, 1973; Lobodzińska *et al.* 1973; Illinger *et al.* 1976). In the present study this cross protection could not be established. This difference may be explained by the comparatively high purity of our interferon preparation. There was also no activity of human interferon in rat cells, as reported by others (Duc-Goiran *et al.* 1971; Chany, 1976). Neither the antiviral activity of HFI nor the inhibition by HLI or RIF in rat cells was encountered. Probably, the purity of the interferon used plays a role. The purity of the human interferons employed in these studies was not always explicitly stated, but can be assumed to be comparatively low. RIF showed the same anticellular activities as reported for interferons of other species (Paucker *et al.* 1962). 10^3 units/ml of RIF inhibited the growth of rat cells by 50 %. From these results we conclude that the rat interferon system

does not differ from that of other species as has been concluded from earlier reports.

CHAPTER 3

INFLUENCE OF INPUT MULTIPLICITY OF INFECTION ON THE ANTIVIRAL ACTIVITY OF INTERFERON

The purpose of this study was to investigate the protective effect of interferon on the survival of virus-infected cells.

This study was achieved by cooperation of the Department of Virology, Erasmus University Rotterdam and the Virology Section of the Primate Centre TNO, Rijswijk.

This chapter is based on:
'Influence of multiplicity of infection on the antiviral activity of interferon' by L.Stitz & H.Schellekens, published in *Journal of General Virology* 46, 1980, 205-210.

Introduction

Early reports on the influence of the multiplicity of infection (MOI) on the antiviral state induced *in vitro* by interferon are conflicting. Protection of cells against vaccinia virus (Lindenmann & Gifford, 1963), Sendai virus (Finter, 1964), polio virus (Hallum & Youngner, 1966), Semliki forest virus (Finter, 1969) and fowl plague virus (Isaacs, 1959) as the result of interferon treatment was reported to be independent of the MOI. Sellers and Fitzpatrick (1962) established that the amount of interferon needed to protect rhesus monkey kidney cells against the cytopathogenic effect of bovine enteritis virus was directly related to the MOI. The same results were reported by Ho (1962) for VSV. Hallum and Youngner (1966) and Ke *et al.* (1970) failed to demonstrate this dependence for VSV.

The early results are, however, difficult to compare because it is not always clear what the exact MOI was and whether the antiviral activity was mediated by interferon. The methods used to measure the antiviral activity of interferon also differed widely.

More recent work by Oie *et al.* (1975) for murine cytomegalovirus, Postic and Dowling (1977) and Holmes *et al.* (1978) for varicella-zoster virus showed convincingly that inhibition of virus production or cytopathogenic effect by interferon was reduced when the MOI increased. Yamamoto *et al.* (1975) reported that the relative number of interferon-treated cells positive for T-antigen was greater when the MOI of SV40 was higher. These recent reports, however, relate only to DNA viruses. All previous studies were confined to one type of virus and interferon of one species.

This study was undertaken to determine the influence of the MOI on the protection of cells provided by interferon against both DNA and RNA virus infections.

Material and Methods

Cells

L_{929} cells, Vero cells and human embryonic lung (HEL) cells were purchased from Flow Laboratories (Irvine, Scotland) and had undergone an

unknown number of passages in our laboratory. Strains of rhesus monkey skin fibroblasts, obtained from biopsies taken from the chest and processed as described by Perrin *et al.* (1977), were designated as RSF 3314, 3504, 3544 and 3546 and were in their 30th to 40th passage. The continuous line of rat cells, derived from subcultivation of whole rat embryo cells, and designated as Ratec cells, is described in chapter 2; it was used in the experiments at about the 70th passage. All cell lines were grown as monolayers in 75 cm^2 Falcon plastic flasks at 37^0 C in a moist atmosphere containing 5% CO_2. The growth medium consisted of DMEM supplemented with 10% FCS heated at 56^0 C for 30 min, bicarbonate, 1% glutamine and antibiotics (penicillin, 75 units/ml^{-1}, streptomycin, 75 mg/ml^{-1}).

Interferons

An HLI preparation, kindly provided by Dr.K.Cantell (Central Public Health Laboratory, Helsinki, Finland) and prepared as previously described (Cantell & Hirvonen, 1978), had an activity of $10^{5.9}$ units/mg protein. All activity was expressed in international reference units in terms of the reference preparation 69/19.

MIF was prepared as described by Allen *et al.* (1976) and had an activity of $10^{6.0}$ units/mg protein. All activity was expressed in international reference units in terms of the reference standard G-002-904-511.

RIF was prepared in Ratec cells challenged with NDV and was partially purified. The preparation had an activity of $10^{5.7}$ units/mg protein when tested against an internal laboratory standard. Details of the production, purification and standardization of this interferon are described in chapter 2.

Viruses

The herpes virus strain used was isolated in our laboratory from a patient with a cold sore and was identified as herpes hominis type I by the immunofluorescence technique. The virus was propagated in L_{929} cells and the 3rd passage was used in this study. Two strains of vaccinia virus were used, the Dutch vaccination strain (Hekker *et al.* 1973)

(Rijksinstituut voor de Volksgezondheid (RIV), Bilthoven, The Netherlands) and the WR strain. The WR strain was also passaged in L_{929} cells. ECHO virus type 6 was obtained from the NIH, Bethesda Md., USA (Cat. no. V-039-001-20). This virus was routinely grown in Vero cells. VSV, Indiana strain, was propagated in L_{929} cells at a low MOI. Clones of VSV, designated 1 to 7, were prepared by the dilution technique. All virus titres given in the text were determined in cells of the same type as those used in that particular experiment, employing a microtitration technique.

Assessment of interferon activity

Except otherwise stated in the legends of the tables, the method used to determine the antiviral activity of interferon was as follows. The cells were grown in microtitre plates in DMEM + 10% FCS at a concentration of approximately 4×10^4 cells per well. When monolayers formed, the supernatant was removed and interferon added in twofold dilutions in DMEM + 1% FCS using 8 wells for every interferon dilution. After overnight incubation, the supernatant was removed and the cells were infected with different MOI, using 10 wells for each concentration.

The supernatant was removed after 48 h of incubation and the cells were stained with crystal violet. The interferon dilution protecting 50% of the cells at the different MOI used was assessed visually. When no protection was established, the amount of interferon necessary to protect 50% of the cells was assumed to be greater than the highest concentration used. When all cells infected with a certain MOI were protected, the amount of interferon needed was assumed to be less than the lowest concentration used.

Results

Influence of MOI on the protection of cells by interferon

To study whether the MOI has an effect on the protection of cells by interferon, experiments with different viruses and 8 different types of cells were carried out (Table 12). At an MOI of 100 $TCID_{50}$/cell, no protection was seen in any of the systems with the interferon concen-

Table 12. Units of interferon protecting 50% of cells of different species against the CPE of different MOI of VSV

Challenge dose (MOI)	Cell type							
	Mouse	Rat	Human	Monkey				
	L929	Ratec	HEL	Vero	RSF 3314	RSF 3544	RSF 3546	RSF 3504
100	> 200	> 200	> 2000	> 2000	> 2000	> 2000	> 2000	> 2000
10	> 200	3	250	> 2000	> 2000	> 2000	> 2000	125
1	> 200	1	30	2000	250	34	67	67
0.1	50	0.8	< 4	500	68	8	17	34

Cells were grown in microtitre plates and treated with interferon from the same species (except monkey cells which were treated with HLI);after overnight incubation, the cells were infected with VSV at the MOI indicated

trations used. When cells were infected with an MOI of 1, most cell types could be protected by interferon. At MOI < 1 much less units of interferon were needed to protect 50% of the cells.

In Table 13 the effect of the MOI of VSV, herpes, ECHO and vaccinia virus on the amount of interferon necessary to protect cells of different species is shown. The protection was challenge dose-dependent in

Table 13. Units of interferon protecting 50% of cells of different species against the CPE of VSV, herpes, ECHO and vaccinia virus

Challenge dose (MOI)	Cell type			
	L929	Ratec	Vero	RSF 3314
VSV				
100	> 200	> 200	> 2000	> 2000
10	> 200	3	> 2000	> 2000
1	50	1	2000	250
0.1	25	0.8	500	67
Herpes				
0.01	3	> 2000	> 2000	n.d.*
0.001	1	500	60	n.d.
0.0001	0.25	16	8	n.d.
0.00001	n.d.	4	< 4	n.d.
ECHO 6				
10	-†	-	250	> 2000
1	-	-	60	> 2000
0.1	-	-	8	1000
0.01	-	-	< 4	67
Vaccinia				
1	> 500	> 200	> 2000	> 2000
0.1	> 500	200	> 2000	> 2000
0.01	> 500	6	> 2000	> 2000
0.001	> 500	0.5	> 2000	> 2000

* n.d. = not done
† - = no CPE with this virus

all cells of the species studied, irrespective of the type of virus. Protection against ECHO virus and VSV by interferon was established when the MOI was $\leq$ 1 $TCID_{50}$/cell. The cytopathogenic effect (CPE) of vaccinia virus was not inhibited in human, monkey or mouse cells treated with an appropriate interferon, but was inhibited in interferon-treated rat cells at an MOI of < 1. The lack of protection of monkey and mouse cells against vaccinia virus was not because these cells are insensitive to interferon, as they were protected by interferon against VSV, herpes, and ECHO virus. Both vaccinia strains studied showed the same pattern of sensitivity.

The MOI-dependent protection by interferon might be explained if the virus populations studied were mixtures of interferon-sensitive and interferon-resistant viruses. However, when 7 different clones of VSV were compared, their sensitivity to interferon was similar (Table 14).

Table 14. Units of interferon protecting 50% of Lg29 cells against the CPE of different clones of VSV

Challenge dose (MOI)	Clones of VSV						
	1	2	3	4	5	6	7
100	n.d.	n.d.	> 500	n.d.	n.d.	> 500	n.d.
10	> 500	> 500	> 500	> 500	> 500	125	> 500
1	> 500	> 500	64	500	250	16	> 500
0.1	16	32	16	32	32	16	32
0.01	8	16	< 4	< 4	16	8	16
0.001	< 4	< 4	< 4	< 4	< 4	< 4	8
0.0001	< 4	< 4	< 4	< 4	< 4	< 4	8
0.00001	< 4	< 4	< 4	< 4	< 4	< 4	4

None were protected at an MOI of > 1, but all were protected when the MOI was $\leq$ 1. Another possibility is that interferon permits the selective growth of interferon-insensitive virus subtypes. Therefore, VSV was passaged at high MOI in interferon-treated cells and the interferon-sensitivity of this virus was compared with the parental strain.

Table 15. Units of interferon protecting 50% of L_{929} cells against VSV grown in interferon-treated cells

Challenge dose (MOI)	VSV passaged in untreated L_{929} cells	VSV passaged in interferon-treated cells*
10	128	512
1	64	32
0.1	16	8
0.01	8	4
0.001	4	< 2

*Before challenge with VSV at the MOI indicated, virus was passaged 3 times at an MOI of 10^2 $TCID_{50}$/cell in L_{929} cells with 500 units of interferon

Table 15 shows that the interferon-sensitivity was not influenced by this treatment.

Influence of time of incubation on the antiviral effect of interferon

The duration of the antiviral effect of interferon at different MOI in L_{929} cells is shown in Table 16. Again, there was no protection against the high MOI of VSV at the interferon concentrations studied. Protection could be induced at an MOI $\leq$ 1 $TCID_{50}$/cell, but this protection was only temporary. At an MOI of 0.1 $TCID_{50}$/cell, protection for 4 days required 20 times more interferon than protection for 2 days. It was not possible to protect cells at this MOI for more than 4 days with the interferon concentrations studied.

The need for more interferon for longer protection could be due to the fact that the antiviral state of cells decreases with time after the removal of interferon-containing medium. However, as shown in Table 17, there was no difference in the antiviral effect of interferon when cells treated only before viral challenge were compared with cells treated with interferon before, immediately after and again one day after infection. It has also been suggested that the enhanced antiviral effect of interferon at low MOI is caused by extra interferon induced by the infecting virus (Ho, 1962).

Table 16. Units of interferon protecting 50% of L929 cells at different times after infection with VSV

Challenge dose (MOI)	Hours after infection					
	24	48	72	96	120	144
1000	> 512	> 512	> 512	> 512	> 512	> 512
100	8	> 512	> 512	> 512	> 512	> 512
10	n.d.	> 512	> 512	> 512	> 512	> 512
1	n.d.	32	512	> 512	> 512	> 512
0.1	n.d.	16	32	512	> 512	> 512
0.01	n.d.	8	16	64	128	128
0.001	n.d.	1	1	16	16	< 1
0.0001	n.d.	1	1	1	1	< 1

Table 17. Effect of the presence of interferon during the replication of VSV on the units of interferon protecting 50% of L929 cells

Challenge dose (MOI)	No interferon present during viral growth	Interferon present during viral growth*
1000	> 200	> 200
100	> 200	> 200
10	200	100
1	50	50
0.1	25	25
0.01	13	13

* Interferon was additionally added to the cells after infection

To test this hypothesis, L929 cells were treated with interferon and 24 h later prechallenged with VSV at the low MOI of 0.001 $TCID_{50}$/cell. Prechallenged and control non-prechallenged cells were infected with different MOI of VSV 24 h after prechallenge. Table 18 shows the comparison of the antiviral state induced by interferon in prechallenged and non-prechallenged cells. There was no difference in the amount

of interferon needed to protect these cells against different MOI of VSV.

Table 18. Effect of prechallenge with VSV on the units of interferon protecting 50% of VSV-infected L929 cells

Challenge dose (MOI)	Non-prechallenged cells (units interferon)	Prechallenged cells* (units interferon)
100	> 500	> 500
10	> 500	> 500
1	30	30
0.1	1	1

* Before infection with the MOI indicated, the cells were prechallenged with VSV at an MOI of 0.001 $TCID_{50}$/cell

Discussion

This study shows that the amount of interferon needed to protect cells of different species against the cytopathogenic effect of both DNA and RNA viruses is directly related to the amount of virus the cells are challenged with. This MOI-dependent protection by interferon could not be explained in terms of differences in interferon sensitivity of clones within the population of viruses studied or in terms of selection of interferon-resistant types of viruses (see Tables 14 and 15). Viruses grown in cells treated with interferon had the same interferon sensitivity as the parental virus.

It has been suggested (Ho, 1962) that interferon is more active at a low MOI because 'extra' interferon is then induced by the infecting virus. Using double challenge experiments, we were unable to establish such 'extra' protection.

A decrease in the antiviral state of the cells with time cannot be responsible for the failure of interferon to protect against the cytopathogenic effect of the viruses. Additional interferon administration during the incubation period did not influence the results. At the

concentrations studied, interferon protected against viral cytopathogenicity only when cells were challenged with an MOI $\leq$ 1 $TCID_{50}$/cell. This protection lasted for a certain time only, dependent on the MOI, and this indicates that, even when cells are infected with a single virus particle only, interferon is not capable of preventing cell death. The only effect of interferon seems to be a reduction of virus production. This only postpones the moment at which all cells will be infected.

The decrease in the production of progeny virus by interferon is caused by inhibition of viral replication at the transcriptional or translational level, depending on the virus-cell system used (Friedman, 1977). Apparently, inhibition of viral replication by interferon does not lead to protection of those cells. Various explanations can be considered: (1) The production of mature virus particles may be suppressed by interferon, while the functions interfering with the cellular metabolism that lead to cell death are unaffected (Marcus & Sekellick, 1976); (2) There is an overall reduction in viral replication by interferon which leads to diminished production of virus, but remaining viral production of virus is sufficient to interfere with cellular metabolism to such an extent that the cells cannot survive; (3) The infecting virus directly inhibits the antiviral activity of interferon (Lindenmann, 1959). Experiments are in progress to test these hypotheses.

Finally, this *in vitro* influence of the MOI on the antiviral activity of interferon is likely to play a role *in vivo* too. Interferon *in vivo* is more effective in preventing than in curing viral infections and has little effect when administered late (Finter, 1973). During well-established viral infections *in vivo*, the virus challenge to cells is probably so high that interferon cannot prevent cell death. One should, however, be cautious in extrapolating *in vitro* effects of interferon to *in vivo* effects. Rhesus monkeys can be protected against vaccinia virus infection by interferon, while the same interferon preparation is not effective in inhibiting the same virus *in vitro* (This thesis, chapter 7).

CHAPTER 4

CELL GROWTH INHIBITION BY INTERFERONS

The purpose of this study was to investigate the inhibitive activity of interferon on cell division of haemopoietic tissue and to observe this anticellular effect during conditions of viral inflammation.

This study was achieved by cooperation of the Departments of Pathology, Virology, Haematology and Internal Medicine, Erasmus University Rotterdam.

This chapter is based on:
'The influence of interferon preparations on the proliferative capacity of human and mouse bone marrow cells *in vitro* by E. van 't Hull, H.Schellekens, B.Löwenberg & M.J.de Vries, published in *Cancer Research* 38, 1978, 911-914;
'Cytotoxicity of interferon and viral inflammation' by H.Schellekens & W.Weimar, published in *International Virology* IV, 1978, 110.

General Introduction

The first 'non-antiviral' activity of interferon to be reported was the inhibition of cell multiplication (Paucker *et al.* 1962). It has been heavily disputed in the past whether interferon itself or impurities were responsible for this anticellular effect. Now that pure interferons are available, it has become clear that the anticellular factor shares all biological and physico-chemical properties with the antiviral factor and, apparently, is interferon (Knight, 1976).

This chapter contains two studies on the inhibition of cell multiplication by interferon. An obvious application of interferon in man is the prevention of viral infections in the immunocompromised host. However, in these patients the bone marrow potential may be restricted and further suppression of the bone marrow by interferon is undesirable. The first part of this chapter deals with the *in vitro* toxicity for bone marrow cells of interferon prepared for clinical use. In the second part aspects of the biological significance of the anticellular effect are studied.

INFLUENCE OF INTERFERON PREPARATIONS ON THE PROLIFERATIVE CAPACITY OF HUMAN AND MOUSE BONE MARROW CELLS *IN VITRO*

Introduction

Viral infections present a major problem in primates (humans and rhesus monkeys) with primary immune deficiencies and those subjected to immunosuppressive therapy. It has become evident that the severity and incidence of viral infections are particularly prominent in patients who have been treated with immunosuppressive regimens following allogeneic bone marrow transplants (Balner *et al.* 1968; van Bekkum *et al.* 1978). The further development of bone marrow transplantation in humans is largely dependent on the possibility for control or prevention of these complications (Thomas *et al.* 1977). Recently, the successful application of interferon as an antiviral drug in humans has been reported (Desmyter *et al.* 1976; Greenberg *et al.* 1976; Jones *et al.* 1976).

Interferon preparations in high concentrations, however, inhibit cell division. Therefore, one must be aware of possible bone marrow toxicity (Cheeseman *et al.* 1976) because the bone marrow reserve may be severely limited, particularly in patients treated with bone marrow transplantation and cytotoxic agents. Testing of the clinically applied interferon preparations for bone marrow toxicity prior to clinical use will be important. This can hardly be achieved *in vivo* in man. Therefore, one must rely on *in vitro* assays. Colony-forming cultures, in which progenitor cells are induced to proliferate and differentiate may meet the criteria of a convenient assay.

HLI and HFI and their effects on haemopoietic myeloid and erythroid progenitors in culture were studied. The inhibition of the formation of granulocytic-macrophage colonies and erythroid colonies during exposure of marrow cells to interferon was investigated. The effect of mouse L-cell interferon on the formation of erythroid as well as granulocytic colonies in mouse bone marrow was also included in the study in order to compare the species specificity of the interferon preparations.

Material and Methods

Interferon preparations

HLI was kindly supplied by Dr.K.Cantell (Central Public Health Laboratory, Helsinki, Finland). The interferon was induced and semipurified as described before (Cantell, 1970). The batch used had an activity of 6×10^6 units/ml and a protein concentration of 7 mg/ml. HFI was kindly supplied by Dr.V.G.Edy (Rega Institute, University of Leuven, Leuven, Belgium), was induced and semipurified as described before (Billiau *et al.* 1973) and had an activity of 10^6 units/20 mg of protein. A control preparation was prepared from human plasma protein fraction V, following the same procedures as used for the manufacture of the interferon preparations. This preparation was also used as a control in *in vivo* studies in patients. Mouse interferon was induced with NDV in mouse L_{929} cells and prepared as described before (Allen *et al.* 1976). It had an activity of 10^6 units/mg of protein. Control preparations were prepared from L_{929} cell culture supernatant following the same procedures as those used to prepare the interferon preparations. The interferon preparations were diluted to the desired concentration in DMEM and were added to the cell suspensions as a part of the culture medium wherein the cells were cultured.

Preparation of cells

Human bone marrow was obtained aseptically from patients requiring a diagnostic puncture. Patients were suffering from a variety of diseases, predominantly malignant tumours and infections; some patients manifested a mild anaemia. Blood was collected by venipuncture. The erythrocytes were removed from the nucleated bone marrow and blood cells by sedimentation at unit gravity in 0.1% methyl cellulose (Methocel McDow Chemical Co., Midland,Michigan, U.S.A.) in DMEM (Flow Laboratories, Irvine, Scotland).

Mouse bone marrow was obtained by dissecting the femurs of 6- to 8-week-old female BALB/c mice. One of the ends was cut with scissors, and the marrow was flushed from the shafts. All cells were carefully washed with Hank's balanced salt solution after collection.

Myeloid colony cultures for human cells

To determine the number of colony-forming units (CFU_c) we adopted the culture method of Pike and Robinson (1970). The underlayers were prepared by mixing 10^6 leucocytes with 1 ml of culture medium (0.5% agar, 20% serum, and DMEM). The serum component contained equal parts of horse serum, FCS, and 3% Trypticase Soy Broth (Difco Laboratories, Detroit, Mich., U.S.A.). Thereafter, an overlayer containing the marrow cells in 0.25% agar in DMEM, supplemented with 20% serum, was pipeted on top of the leucocyte underlayers. After incubation for 14 days in a humidified atmosphere of 5% CO_2 - 95% air, the colonies were counted with an inverted microscope.

Three petri dishes were used for each experimental point.

Erythroid colony cultures from human cells

Colony formation in plasma clots was determined according to the method of McLeod *et al.* (1974). Nucleated bone marrow cells in a concentration of 2×10^3/ml were suspended in medium containing DMEM, 20% FCS, 1% bovine albumin (fraction V; Sigma Chemical Co., St.Louis, Mo., U.S.A.), 2% asparagine, 0.25 unit erythroprotein (step III; Connaught Medical Laboratories, Willowdale, Ontario, Canada), and 10% bovine citrated plasma (Grand Island Biological Co., Grand Island, N.Y., U.S.A.). The cells were cultured in microtitre plates (C.A.Greiner und Söhne, Nürtingen, B.R.D.), and 0.1 ml of the cell suspension was incubated per well.

The cultures were placed in an incubator under the same conditions as those used for the myeloid colony method. After 2.5 days of incubation, the clots were removed from the microtitre plates and stained with benzidine after fixation with 5% glutaraldehyde in phosphate-buffered saline (0.01 M isotonic NA_2HPO_4-KH_2PO_4 buffer, pH 7.0 to 7.2).

Cell aggregates containing more than 8 cells were counted with the light microscope. The aggregates consisted of differentiated erythroblasts. Each experimental point represents the average colony counts of at least 10 whole clots.

Granulocytic-macrophage colony cultures of mouse cells

The system was originally described by Bradley and Metcalf (1966). Nucleated bone marrow cells were suspended in an agar solution (0.3%) in DMEM supplemented with 20% serum (consisting of equal parts of horse serum, FCS, and 3% Trypticase Soy Broth solution).

One ml of this mixture containing 10^5 cells was plated per plastic petri dish (diameter 3.5 cm). As a stimulator 0.1 ml of mouse fibroblast-conditioned medium was added to each plate. The dishes were incubated for 6 days under the same conditions as those for the other cultures. Thereafter, colonies were counted.

Results

HLI and HFI inhibited the formation of human erythroid colonies (CFU_e). The extent of inhibition was linearly related to the dosage of interferon (Fig. IV).

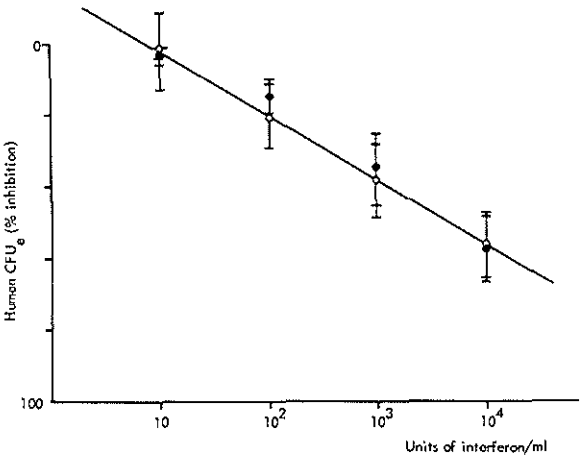

Fig. IV. Cytotoxicity of HFI (o) and HLI (●) for human CFU_e. Nine experiments; bars: SD.

HLI and HFI preparations also suppressed the formation of CFU_c (Fig. V). Inhibition of CFU_e and CFU_c by HFI was similar. Colony growth from both cell populations was reduced for 50% by 10^4 units/ml.

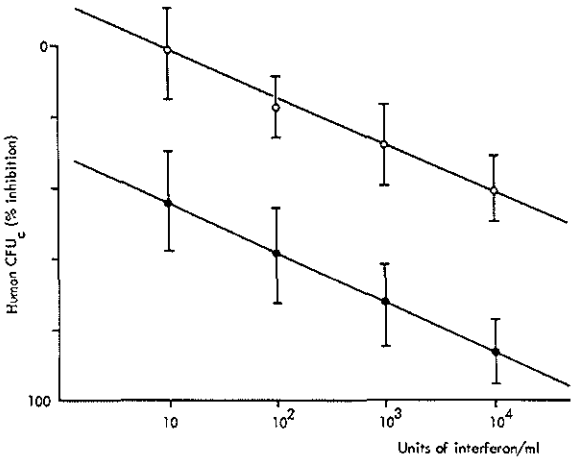

Fig. V. Cytotoxicity of HFI (o) and HLI (●) for human CFU$_c$. Nine experiments; bars: SD.

However, HLI differed clearly from HFI: (a) it was significantly more toxic to human CFU$_c$ than to CFU$_e$; (b) this interferon showed no or only minor inhibitive effect on murine CFU$_c$ and CFU$_e$ (Table 19).

Table 19. Specificity of the toxicity of interferon preparations

	CFU$_c$ and CFU$_e$ of mouse bone marrow cells†			
	HFI		HLI	
	CFU$_e$/10^5 cells	CFU$_c$/10^5 cells	CFU$_e$/10^5 cells	CFU$_c$/10^5 cells
Control	202.3±24.7¶	100.0±10.0	202.3±24.7	100.0±10.0
1 x 10^4 U	194.7±23.7	94.4± 7.4	131.3±20.1	58.8± 9.6
1 x 10^3 U	203.4±26.6	98.6±11.4	177.1±25.9	91.5± 9.1
1 x 10^2 U	209.9±24.4	101.5± 7.4	191.2±21.9	102.9±10.4
1 x 10 U	198.7±26.6	99.6±11.3	208.3±35.5	102.7±10.3
Mock interf.	197.1±20.1	102.4± 8.2		

† Human bone marrow cells were not inhibited by mouse L-cell interferon

¶ Mean ± SD

A third interferon preparation, produced in mouse L_{929} cells, was also investigated for its effect on human and mouse progenitor cells. Whereas mouse interferon showed inhibition of the colony growth of mouse CFU_e and CFU_c in a linear dose-effect relationship (Figs. VI and VII), it was not toxic for bone marrow (results not shown).

This indicates that the toxicity of human interferon may be species-dependent.

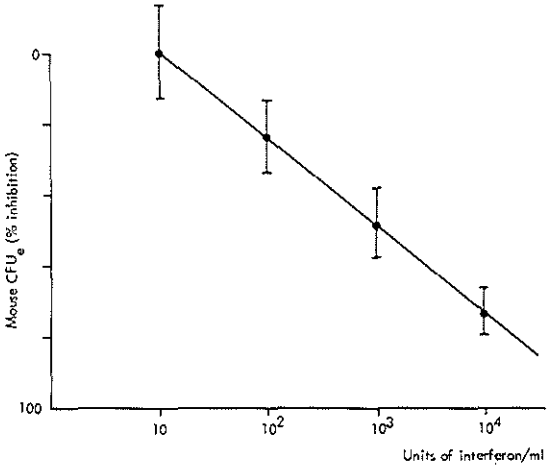

Fig. VI. Cytotoxicity of mouse L-cell interferon for mouse CFU_e. Five experiments; bars: SD.

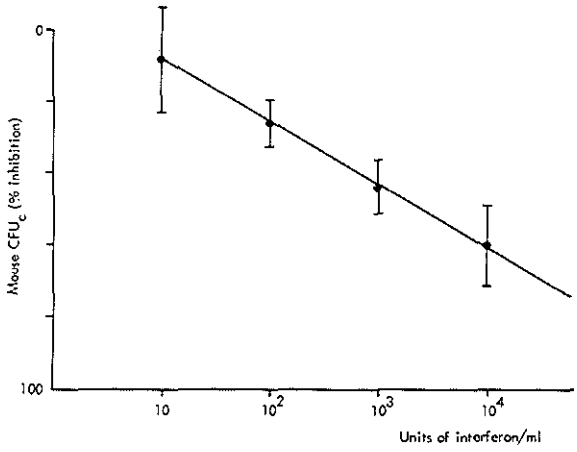

Fig. VII. Cytotoxicity of mouse L-cell interferon for mouse CFU_c. Five experiments; bars: SD.

Discussion

Interferon preparations inhibit CFU_e and CFU_c *in vitro*. HFI inhibits the formation of CFU_c and CFU_e *in vitro* at concentrations much larger than those needed for the establishment of the antiviral state of cells *in vitro*. The toxic concentration of HFI also exceeds the serum titre of interferon in patients treated with high doses of HFI (20×10^6 units/ml fibroblast interferon daily, a serum titre rise of 30 units/ml (V.G.Edy, personal communication)). The effect of HLI on CFU_e is similar to that of HFI, but HLI is comparatively more toxic to CFU_c. Only 10 units of HLI suffice to inhibit the growth of CFU_c by approximately 40%, whereas as much as 10^4 units of HFI are needed to produce a similar reduction. Using a similar assay system, other investigators have reported that HLI preparations may be extremely toxic for CFU_c in human bone marrow cultures (Greenberg & Mosny, 1977; Nissen *et al.* 1977). To our knowledge, the effect of erythroid progenitors has never been investigated.

Others have demonstrated in a different experimental system that the cytotoxic and antiviral activities of interferon preparations share many physico-chemical and biological characteristics (McNeill *et al.* 1975; Knight, 1976). This may suggest that interferon itself is responsible for the cytotoxic effects. However, in view of the nonavailability of completely purified interferon, one cannot be certain whether the cytotoxicity is due to impurities in the preparation. Comparison of the cytotoxic effect of HLI on mouse and human CFU_c, respectively, shows that this interferon is less toxic for mouse bone marrow, indicating a (cell- and species-)specific effect and not toxicity due to impurities. Therefore, the cytotoxic effect could be closely related to the antiviral effect of interferon (compare Fig. V and Table 19).

The toxicity of HLI could pose a problem in clinical application because it is already apparent at lower than pharmacological concentrations.

The finding that HLI is more toxic for CFU_c than for CFU_e is of interest in view of previous work of Einhorn and Strander (1977). They suggest that the inhibiting effects of interferon are specific for those target cells that are prevalent in the tissue in which the inter-

feron is induced.

The effect of mouse interferon on murine CFU_e and CFU_c is essentially similar to that of HFI on human bone marrow. The adverse effect of L-cell interferon on the colony formation has been reported to be less prominent than the effect of interferons from other murine sources. McNeill and Gresser (1973) explained this effect by a contamination of L-cell interferon with colony-stimulating activity, but in our material no significant colony-stimulating activity was present.

No doubt, it will be necessary in the future to test the interferon toxicity on bone marrow for *in vivo* relevance. This should be done in direct comparison with clinical and haematological parameters, and this should finally establish the value of the method. Until the toxicity ratio of interferon has been more thoroughly established in *in vivo* studies, one must rely largely on extrapolation from *in vitro* tests.

The above data imply that, in terms of marrow toxicity, HFI might represent a preferable choice over HLI, especially in patients with marrow insufficiency.

CELL GROWTH INHIBITION BY INTERFERON DURING CONDITIONS OF VIRAL INFLAMMATION, ESTABLISHED WITH A SIMPLE METHOD

Introduction

Apart from antiviral activity, interferon preparations show other activities as well, such as alteration of the immune response *in vitro* and *in vivo* (Gresser, 1977; Finlay *et al.* 1977; Sonnenfeld *et al.*1977), activation of macrophages (Schultz *et al.*1977; Stebbing *et al.*1978) and inhibition of cell growth (Paucker *et al.*1962; Gresser *et al.* 1970; Gresser, 1977; this chapter). Because in these studies interferon preparations certainly were not pure, it is uncertain whether interferon is responsible for these activities. However, it is now well established that one glycoprotein causes both the antiviral activity and the inhibition of cell growth (Knight, 1976; Stewart *et al.* 1976; Buffett *et al.* 1978).

In vitro interferon inhibits cell growth in comparatively high concentrations only (Paucker *et al.* 1962; Gresser *et al.* 1970; this chapter). *In vivo* these concentrations are attained at this site of the viral infection only (Finter, 1973). One aim of this study was to mimick some of the local conditions of a viral infection *in vitro* and study their effect on growth inhibition. The commonly used techniques to quantitate inhibition of cell growth are cell counting and measuring ^{3}H-TdR incorporation. Especially when growth inhibition in monolayers is tested, these methods are time-consuming. We have studied growth inhibition quantitatively by measuring the dye uptake by monolayers *in vitro*.

Material and Methods

Cells

RSb cells were a kind gift from Dr.T.Kuwata (Chiba University, Chiba, Japan). The origin and characteristics of these transformed human cells have been described elsewhere (Kuwata *et al.* 1976).

Interferon and interferon titration

HLI was provided by Dr.K.Cantell (Central Public Health Laboratory, Helsinki, Finland). This partially purified interferon had an activity of $10^{5.9}$ units/mg protein. HFI was kindly supplied by Dr.A.Billiau (Rega Institute, University of Leuven, Leuven, Belgium) and had an activity of 10^{6} units/mg protein. Interferon was titrated as described before, using RSb cells (Schellekens *et al.* 1975). Interferon activity was expressed in international reference units (U/ml).

Quantitation of cell growth inhibition

The cell growth inhibition was measured by a modification of a method described by Armstrong (1971) to quantitate the antiviral activity of interferon by dye uptake and elution.

RSb cells were seeded in 6 cm petri dishes and interferon was added in appropriate concentrations. Cells were incubated until the control cultures had reached semiconfluency (in general after 3 to 4 days). After incubation, the dishes were drained and the cells were washed 3 times with phosphate buffered saline. The cells were stained for 20 min with a dye solution (5 g crystal violet (Merck), 8 g NaCl, 50 ml formaldehyde solution (Merck), 500 ml ethanol, water to 1000 ml). After staining, the dishes were washed extensively with tap water and allowed to dry. 5 ml 2-methoxy-ethanol were added to extract the crystal violet. Up to 24 h were needed to extract all dye. When the elution was completed, the absorbance of the extracted dye was measured at 550 nm. Petri dishes without cells showed minimal absorption of crystal violet, so all crystal violet eluted was considered as eluted from cells. The anticellular activity of interferon preparations was also assayed by measuring the ^{3}H-TdR uptake, according to Fuse and Kuwata (1976).

Virus

VSV, Indiana strain, was routinely grown in our laboratory in monolayer cultures of L_{929} cells. VSV was stored frozen at -70° C. Titres were expressed as $TCID_{50}$, established in RSb cells using standard techniques.

Results

Inhibition of cell growth as measured by cell counts, dye uptake and ^{3}H-TdR incorporation

Fig. VIII shows that the amount of eluted dye is correlated to the number of cells. The correlation coefficient between cell number and eluted dye was 0.95. As little as 10,000 cells in a 6 cm dish can be measured accurately. In our experimental conditions a difference in E_{550} of 0.1 corresponded with 7,500 RSb cells.

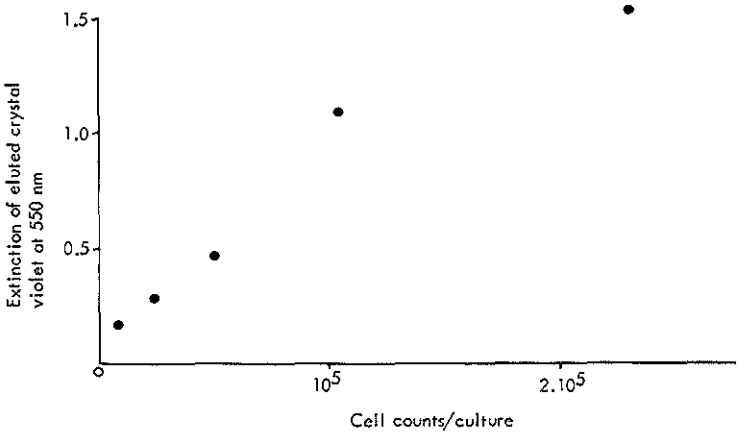

Fig. VIII. Correlation between number of cells and E_{550} in dye elution assay.

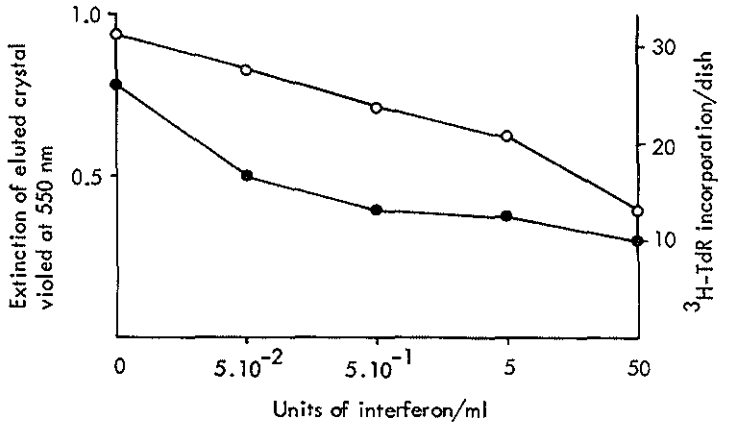

Fig. IX Inhibition of growth of RSb cells by HFI.

Fig. IX gives the results with the dye elution method and ^{3}H-TdR incorporation in RSb cultures incubated with HFI. The two methods give similar results. 50 units of HFI reduced both the dye-uptake and the ^{3}H-TdR incorporation by 50%.

Cell growth inhibition by interferon during conditions of viral inflammation

Effect of viral challenge. The cells at the site of a viral infection are not only challenged with interferon, but also by the infecting virus. Table 20 shows the cell growth of RSb cells protected by interferon and challenged by VSV. Viral challenge seemed to have no effect on the anticellular activity of interferon.

Table 20. Influence of viral challenge on the inhibition of cell growth by HFI

	E_{550} of crystal violet eluted (SD)
Control	1.688 (0.148)
40 units HFI/ml	0.736 (0.069)
40 units HFI/ml + 10^4 CPE D_{50} VSV	0.825 (0.082)
10^4 CPE D_{50} VSV	0.052 (0.002)

30 petri dishes were seeded with 125,000 cells/dish. In 20 dishes 40 units of HFI were added. After 24 h in 10 dishes treated with HFI 10^4 CPE D_{50} VSV/dish were added. After 72 h of further incubation cell growth was measured with the dye elution technique.

Effect of mixtures of different types of interferon. During viral inflammation *in vivo* different types of cells may produce interferon. Cells will thus be challenged by different types of interferon. As it has been reported that interferon of one species can inhibit the action of interferon of another species, possibly by interaction at the cell receptor site (Chany, 1976), we tested the anticellular activity of different types of interferon added simultaneously. The results are shown in Table 21. Cell growth was inhibited for 40% by HLI (100 U)

and for 65% by HFI (100 U). When the interferons were added simultaneously, the growth inhibition was 80%. Therefore, any addition that may occur is only slight.

Influence of temperature. Viral infections can cause a rise in body temperature and in local temperature by provoking an inflammatory reaction. Table 22 shows that the anticellular activity of HFI and especially HLI was enhanced by a rise in temperature. At 37° C the inhibition by 40 U of HLI was negligible, but at 39° C cell growth was reduced by more than 60%.

Table 21. Growth inhibitory activity of HFI-HLI mixture

	E_{550} of crystal violet eluted (SD)
Control (n = 5)	0.417 (0.090)
100 units HLI/ml (n = 5)	0.258 (0.035)
100 units HFI/ml (n =10)	0.148 (0.036)
100 units HLI/ml + 100 units HFI/ml (n =10)	0.074 (0.026)

100,000 RSb cells were seeded in each petri dish and incubated with the indicated additions for 72 h. After incubation, cell growth was measured with the dye elution technique

Table 22. Effect of temperature on the inhibition of cell growth by HFI and HLI

Temperature	Control	Treatment	
		40 U/ml HFI	40 U/ml HLI
37° C	0.294	0.138 (52%)	0.243 (16%)
38° C	0.307	0.126 (59%)	0.216 (31%)
39° C	0.244	0.037 (85%)	0.093 (61%)

RSb cells were seeded into 6 cm petri dishes (100,000 cells/dish) and incubated for 72 h at 37°, 38° or 39° C (18 dishes at each temperature). 6 dishes of each group of 18 were treated with 40 U of HFI/ml, 6 dishes with 40 U of HLI/ml and 6 dishes were left untreated. After incubation, cell growth was measured with the dye elution technique. The mean extinction at 500 nm is shown with the percentage of growth inhibition in parentheses.

Discussion

The measurement of cell growth inhibition of cells in monolayer by dye uptake and elution has a number of advantages:
(1) it is not necessary to detach the cells from the surface on which they are cultured, a necessary step in other methods;
(2) compared with cell counting, the dye elution method is quicker, more convenient and less sensitive to variables in and between technicians;
(3) the dye elution method is also faster and more economical than the ^{3}H-TdR uptake technique; no special laboratory equipment or training in handling radioactive materials is needed.

This study shows that the anticellular activity of interferon is not influenced by viral challenge or by simultaneous action of different types of interferon. Raising the temperature to 38^{o} and 39^{o} C even enhances the cytotoxicity. The biological significance of the cytotoxic effect of interferon is still obscure. It might be interpreted in several ways. Together with the specific inhibition by interferon of viral replication, inhibition of cell growth would result in an amplified antiviral effect. Another suggestion is that both the inhibition of viral replication and of cell growth are the result of the same molecular biological alteration in the cell.

The primary function of interferon can be regulation of macromolecular processes and viral replication probably has some characteristics that make it very sensitive to this regulation. Therefore, the antiviral activity could just be a side-effect of the interferon system and can be an explanation for recently published studies of interferon in other conditions than viral infections (Gresser, 1977; Johnson, 1978; Trinchieri & Santoli, 1978; Trinchieri *et al.* 1978).

IN VIVO STUDIES

CHAPTER 5

INTERFERON ADMINISTRATION IN CHRONIC HBsAg POSITIVE HEPATITIS

The purpose of this study was to evaluate the efficacy of interferon in an otherwise untreatable disease.

This study was achieved by cooperation of the Departments of Internal Medicine, Virology, and Pathology, Erasmus University Rotterdam, the Rega Institute, University of Leuven, Leuven, Belgium, the Central Public Health Laboratory, Helsinki, Finland, the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the London School of Hygiene and Tropical Medicine, London, England, and Organon Scientific Development Group, Oss.

This chapter is based on:
'Fibroblast interferon in HBsAg positive chronic active hepatitis' by W.Weimar, R.A.Heijtink, S.W.Schalm, M.van Blankenstein, H.Schellekens, N.Masurel, V.G.Edy, A.Billiau & P. De Somer, published in *Lancet* II, 1977, 1282;
'Differential effects of fibroblast and leucocyte interferon in HBsAg positive chronic active hepatitis' by W.Weimar, R.A.Heijtink, S.W.Schalm & H.Schellekens, published in *European Journal of Clinical Investigation* 9, 1979, 151-154;
'Double-blind study of leucocyte interferon administration in chronic HBsAg-positive hepatitis' by W.Weimar, R.A.Heijtink, F.J.P.ten Kate, S.W.Schalm, N.Masurel, H.Schellekens & K.Cantell, published in *Lancet* I, 1980, 336-338.

Introduction

Liver cell damage in chronic hepatitis B surface antigen (HBsAg) positive hepatitis is probably the result of immunological action against hepatitis B virus-infected hepatocytes. Therefore, attempts have been made to treat this disease with immunosuppressive agents. Unfortunately, results of prednisone-azathioprine therapy were not encouraging (Schalm *et al.* 1976; De Groote *et al.* 1978). Another way of treatment is the elimination of the hepatitis B virus. In fact, eradication of the pathogenic organism is essential in all infectious diseases. However, until now this has not been accomplished in chronic HBsAg positive hepatitis with antiviral nor with immunoactivating agents as we have pointed out elsewhere (Weimar & Schellekens, 1979).

In this chapter we describe the attempts to affect hepatitis B virus with interferon. To clarify our line of thinking we discuss these studies in chronological order.

Initial Studies

In 1976 it was reported that daily administration of 4×10^5 - 1×10^7 reference units of HLI induced a rapid fall in hepatitis B core antigen (HBcAg) associated DNA polymerase (DNAP) in the sera of 3 patients (Greenberg *et al.* 1976). This enzyme activity is generally assumed to reflect the number of circulating Dane particles (the proposed hepatitis B virus). The suppressive effect on DNAP was transient when the interferon was given for 10 days or less, but appeared sustained for weeks after therapy in the 2 patients when treated for more than 1 month. In addition, these 2 patients showed a drop in HBcAg titre and disappearance of hepatitis B 'e' antigen (HBeAg). One of them became permanently negative for HBsAg, HBeAg and DNAP (Greenberg *et al.*1979).

Also in 1976 an effect of HFI was claimed in chronic HBsAg positive hepatitis (Desmyter *et al.* 1976). In the patient studied a decrease in the percentage of liver cell nuclei positive for HBcAg was observed after 7 injections of 10^7 reference units of HFI given on alternate days. HBsAg was not affected.

We studied the effect of HFI (Rega Institute, University of Leuven, Leuven, Belgium) and found no effect on hepatitis B virus indices. However, in 1 of our control patients a dramatic drop in DNAP was observed.

HFI IN HBsAg POSITIVE CHRONIC HEPATITIS

Promising results of the effect of interferon therapy on indices of hepatitis B virus in HBsAg positive chronic hepatitis patients have been published (Desmyter *et al.* 1976; Greenberg *et al.* 1976) and editorials have suggested a possible breakthrough in antiviral therapy (Editorial Lancet,1976; Ho, 1976; Zuckerman, 1976). Before attempting to eradicate hepatitis B virus by long-term interferon treatment and to determine the effect of elimination of the virus on the course of liver disease, we made dose-response studies with HFI under controlled conditions.

4 patients (HBsAg positive for at least 6 months, aspartate amino transferase (AST) consistently more than twice normal, HBeAg positive, measurable DNAP activity) participated in our first study. 2 patients received HFI intramuscularly twice weekly, starting with 3 times 2×10^6 reference units, followed by 3 times 4×10^6 reference units, and finally 3 times 8×10^6 reference units. No placebo preparations were administered in the 2 control patients. DNAP activity, HBeAg, HBsAg titre ('Hepatest'), AST and leucocyte counts were determined several times before, twice weekly during, and several times after treatment. No effect was observed on indices of virus activity in the interferon-treated patients; 1 control showed a striking decline of DNAP activity; 1 patient treated with interferon became leucopenic (Fig. X).

To investigate the possibility that daily administration might be effective, 3 patients were given 3×10^6 reference units of HFI every day for 2 weeks. 3 other patients served as controls. Again, there was no effect on hepatitis B virus indices.

It is possible that HLI is more effective than HFI or that individuals differ in responsiveness to interferon. However, evidence for the efficacy of interferon presented by others has come from uncontrol-

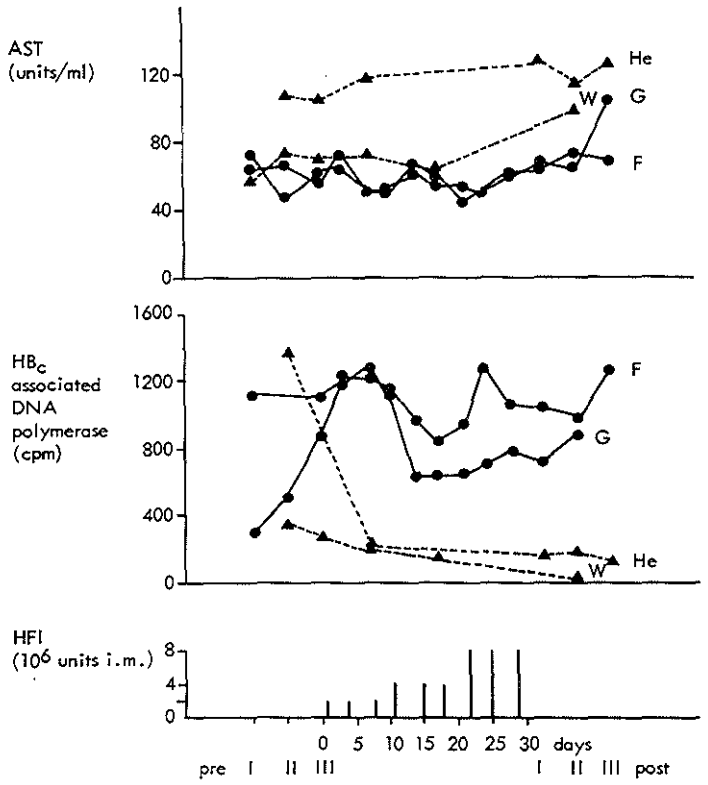

Fig. X. HFI administration in HBsAg positive chronic hepatitis patients; ●——● treated patients; ▲——▲ control patients.

led observations- a criticism made by Chalmers (1977) and with good reason, since we found that DNAP activity fell in a control patient. More evidence from dose-response studies in individual patients is needed before long-term treatment with interferon in a clinical trial is warranted.

From this study we drew 2 conclusions: (a) we had to compare HFI with HLI and (b) all our studies should be properly controlled. Therefore, we decided to give HLI to the same patients who had received daily injections of HFI. The same dosage schedule was used and the same patients served as controls.

DIFFERENTIAL EFFECTS OF HFI AND HLI IN HBsAg POSITIVE CHRONIC HEPATITIS

Introduction

The therapeutic management of chronic hepatitis has improved markedly following the use of steroids and antimetabolites (Summerskill *et al.* 1975). However, the benefits of this therapy are not striking in chronic hepatitis associated with hepatitis B virus infection (Schalm *et al.* 1976). Recently, exogenous interferon has been claimed to modify indices of hepatitis B virus replication in patients with CAH (Desmyter *et al.* 1976; Greenberg *et al.* 1976).

We have shown previously that twice-weekly administration of HFI did not have consistent effects on the indices of hepatitis B virus replication in chronic hepatitis. The possibilities remained, however, that daily injections could affect viral replication, or that HLI would be more effective than HFI. Therefore, we have evaluated the effects of daily HFI and HLI administration in patients with HBsAg positive chronic hepatitis.

Patients, Material and Methods

We studied 6 patients who fulfilled the following criteria: HBsAg positive for at least 1 year, positive for HBeAg and DNAP activity, increased AST activity, normal α_1 fetoprotein concentrations, liver biopsy showing chronic active hepatitis, and no previous immunosuppressive therapy. Drs.M.van Blankenstein, R.A.Heijtink and S.W.Schalm helped us in selecting the patients and following their course.

HFI was prepared at the Rega Insitute, University of Leuven, Leuven, Belgium, by a method described by Billiau *et al.*(1973). HLI was produced at the Central Public Health Laboratory, Helsinki, Finland, as described by Cantell *et al.* (1974). Both interferon preparations contained approximately 10^6 reference units/mg of protein, titrated in primary human fibroblasts with VSV as challenge virus and calibrated against the Medical Research Council 69/19 standard of HLI.

In the first study 3 of 6 patients received 3×10^6 reference units HFI intramuscularly(i.m.) daily for 2 weeks. In the second study 10 weeks later, the same 3 patients received 14 i.m. injections of 3×10^6 refe-

rence units HLI; 2 patients daily for 2 weeks, the third patient within 20 days. Control patients did not receive placebo injections.

Blood samples were taken twice weekly for 2 weeks before and for 2 weeks after interferon administration, and 3 times weekly during interferon administration. Control patients were bled approximately weekly on several occasions before, during, and after the treatment periods.

HBsAg was measured by radioimmunoassay with Ausria II (Abbott Laboratories) and positive sera were titrated by a reversed haemagglutination inhibition test (Hepatest, Burroughs Wellcome). HBeAg and anti-HBeAg were detected by double immunodiffusion. Reference sera were kindly provided by Dr.E.H.van Elven (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam). Identical results were obtained in 50 sera tested both in Amsterdam and in our laboratory. DNAP activity was determined as incorporation of ^{3}H(dTMP) into an acid-insoluble product by the method of Kaplan *et al.* (1973). Registered counts/min were only corrected for background radiation (approximately 30 cpm). All sera from one patient were tested simultaneously; one serum of a healthy control was included in the test. For confirmation of our results, 15 different samples of concentrated serum were tested simultaneously by Dr.C.R.Howard (London School of Hygiene and Tropical Medicine, London, England) and by our laboratory with comparable results ($r = 0.996$; $p < 0.001$ Spearman rank correlation test). To test the specificity of the reaction for DNAP activity, a limited number of concentrated sera from all patients and controls were absorbed with sheep anti-HBsAg or normal sheep serum. After another absorption with rabbit anti-sheep serum, enzyme activities were not modified if the first absorption was performed with normal serum; in the case of absorption with anti-HBsAg serum, enzyme activities were lowered to the level of our normal control sera. Leucocytes (n: 3-10 x 10^{9}/l), platelets (n: 150-450 x 10^{9}/l) and AST (n: 5-30 U/l) were measured by conventional methods. AST (enzyme commission number: EC 2.6.1.1.) was measured using UV kinetic measurement technique at 340 nm in an LKB8600 Reaction Rate Analyser at 35^{o} C with l-aspartic acid 125 mmol/l in Tris buffer 40 mmol/l as substrate at pH 8.0 (test combination Baker Laboratories, Deventer, The Netherlands).

Results

Viral indices

DNAP (Fig. XI). During HFI administration no patient showed a consistent reduction of DNAP activity to less than pretreatment values (mean of 3 samples taken within 2 weeks before injections). During HLI administration, a continued fall in DNAP activity was observed in all 3 patients (-63%, -66%, -64%); after treatment DNAP activity rose to initial values in the 2 patients, but remained persistently (> 9 months) negative in 1 individual; DNAP activity remained high throughout the study in control patients.

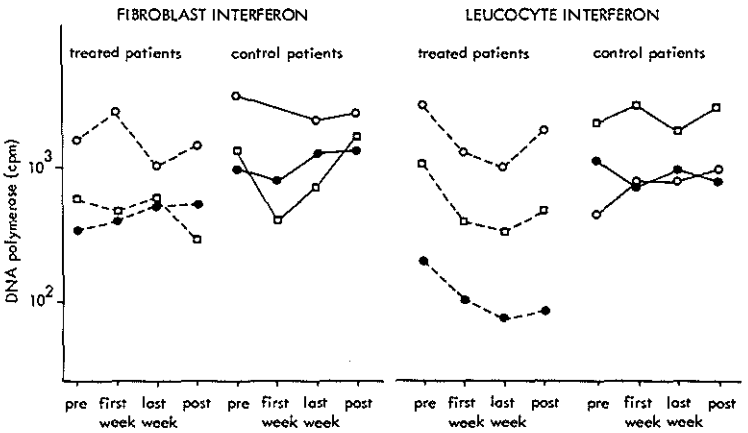

Fig. XI. Mean DNAP activities 2 weeks prior to, during and 2 weeks after treatment with HFI and HLI preparations in HBsAg positive chronic hepatitis patients (upper limit of normal= 100 cpm). All patients were HBeAg positive during both study periods, except for patient ● who became HBeAg negative 7 weeks after HFI administration.

HBsAg. HBsAg titres remained stable in all 6 patients during both HFI and HLI administration.

HBeAg. During HFI administration all patients remained HBeAg positive. In 1 of these patients, however, the HBeAg became undetectable 7 weeks after the first study. During HLI administration, the other 2 patients remained HBeAg positive. Control patients remained HBeAg positive during both study periods.

Liver function (Fig. XII)

During HFI administration AST activities declined in all 3 patients (-29%, -37%, -45%) and returned to pretreatment values in the follow-up period.

During HLI administration AST activities declined in 1 patient from 177 U/l to 78 U/l and declined further in the follow-up period to 30 U/l; no effect on AST activity was noted in the other 2 treated patients.

Control patients showed no change in AST activities during both study periods.

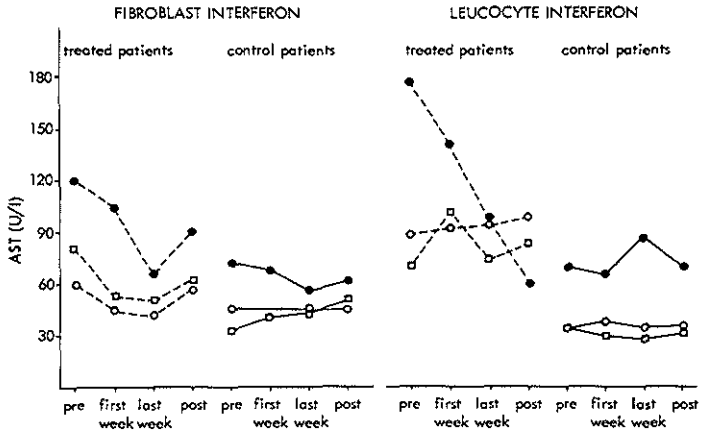

Fig. XII. Mean AST activities 2 weeks prior to, during, and 2 weeks after treatment with HFI and HLI preparations in HBsAg positive chronic hepatitis patients (upper limit of normal = 30 U/l).

Side effects

In 1 patient a transient fall in leucocytes to 2.5 x 10^9/l was observed during HFI administration; in the same patient HLI injections had to be stopped 5 times for 1 or 2 days because of leucopenia (1.4 x 10^9/l), mainly due to granulocytopenia (0.6 x 10^9/l). In another patient leucopenia was observed during HLI injections only (Fig. XIII).

Chills and fever were noted in 1 patient during both studies and in another patient during HLI administration; promethazine was effective in prevention. No other side effects were observed.

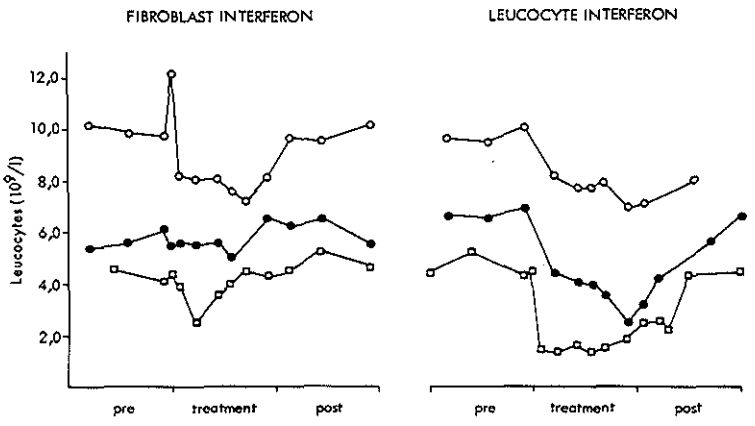

Fig. XIII. Leucocytes 2 weeks prior to, during, and 2 weeks after treatment with HFI and HLI preparations in HBsAg positive chronic hepatitis patients (normal = 3-10 x 10^9/l).

Discussion

Our results indicate that the effects of HFI and HLI in patients with HBsAg positive chronic hepatitis are different. HFI influences serum transaminase levels, an indicator of liver cell damage, while HLI seems mainly to affect DNAP activity, assumed to be a marker of circulating Dane particles.

The effect of HLI on DNAP is in agreement with the results of Greenberg *et al.* (1976). However, the small number of patients, the fluctuating levels of the enzyme activity, and its spontaneous decline in 1 of our patients reported earlier must make us cautious in our interpretation. The mechanism by which HLI affects only DNAP activity and not HBsAg titre is not clear. In most virus-cell systems, interferon acts at the post-transcriptional level (Friedman, 1977), where selective inhibition of translation may occur (Wiebe & Joklik, 1975). This could explain the discrepancy of the effect of interferon and DNAP activity and HBsAg in our patients.

Immunoreactive mechanisms have been implicated in the cell destruction leading to elevated serum transaminase activities in chronic

hepatitis B infection (Eddleston, 1974). The decrease in transaminase levels by interferon in our patients could thus be explained by a direct immunosuppressive effect of interferon or by reduced expression of viral antigens on the liver cell membrane leading to a smaller number of targets for the immune apparatus.

In this study the leucocyte count was affected more by HLI than by HFI. The rapid decrease (within 24 h) of leucocyte numbers, mainly granulocytes, in 1 of the patients makes a direct effect on bone marrow unlikely, and suggests redistribution or cytotoxicity for peripheral leucocytes.

HFI and HLI are not identical. They differ not only in physicochemical properties (Cesario *et al.* 1977) and antigenic composition (Havell *et al.* 1975), but also in antiviral activity and cytotoxicity in certain cell systems (Edy *et al.* 1976; this thesis, chapter 4). Molecules of human interferon may contain multiple active sites, and the number and distribution of these determinants are different for HLI and HFI (Paucker *et al.* 1975). Therefore, a different biological activity can be expected; this may account for the differential effects of HFI and HLI in this study.

At present, only a limited amount of human interferon is available for small clinical trials. It is important to report observations such as these, so that the need for further production of both types of interferon for future studies may be assessed.

Entracte

We partially confirmed the results of Greenberg *et al.*(1976). HLI induced a fall in DNAP levels in the same patients in whom HFI proved ineffective. In 2 patients the effect on DNAP was transient; 1 patient remained DNAP-negative after the study. In these patients HBeAg became undetectable 7 weeks after HFI injections and 3 weeks before HLI administration. Therefore, we did not know which (if any) interferon preparation had induced the complete disappearance of HBeAg and DNAP. In the other 2 patients HFI and HLI affected HBsAg titre and HBeAg.

Possibly, the dose of interferon was too small or the period of injections was too short.

In the meantime other groups reported their experience with interferon in chronic HBsAg positive hepatitis.

Kingham *et al.* (1978) treated 2 patients with 1 x 10^7 units HFI daily for 2 weeks. HBsAg titres did not fall; DNAP was not measured. An immediate drop in anti-HBcAg titre was noted in both patients. This was difficult to interpret in view of the half-life of antibodies. It could be explained by an effect of interferon on the complement fixation reaction that was used to determine the anti-HBcAg titre. Such an effect has been described for HLI (Aho *et al.* 1976). In 1 patient with raised AST levels an initial increase in AST was seen followed by a return to pretreatment levels at the end of the second week of injections and normalization in the follow-up period. We did not find this effect in our patients.

Scullard *et al.* (see Dunnick & Galasso, 1979) treated 8 patients with daily injections of 6 x 10^5 - 3 x 10^6 reference units HLI for periods from 5 to 9 weeks. 5 of these patients had measurable DNAP activity and all showed a fall during treatment. These 5 patients also showed a fall in Dane particle numbers. HBeAg disappeared in 2 of the 3 patients in whom it was present. However, none of these individuals developed a permanent change in Dane particle markers or HBsAg. In fact, these indices reverted to pretreatment levels in some patients while still on interferon.

At an NIH meeting on clinical trials with interferon (April, 1978) Robinson showed the results of the Stanford group (see Dunnick & Galasso, 1979).They had treated 7 patients, including the 2 already reported (Greenberg *et al.* 1976) with daily injections of 4.2 x 10^5 - 1.2 x 10^7 reference units HLI, for at least 1 month. In 2 patients all hepatitis B virus markers including HBsAg completely disappeared. In another DNAP and HBeAg became undetectable, while HBsAg remained present. In the other 4 patients partial reductions in circulating Dane particle markers were seen, but these changes reverted when the interferon injections were stopped.

Desmyter reported at the same meeting that 3 patients were treated with 3×10^6 reference units HFI daily for 1 week: 1 patient with 1×10^7 reference units HFI daily for 1 week and 1 patient with daily injections of variable doses (1×10^5 - 1×10^6 units) HFI during 1 month. No consistent changes in viral indices were found (see Dunnick & Galasso, 1979).

Finally there was a report on 1 patient in whom DNAP, HBcAg and HBeAg disappeared during a 3-month course with daily injections of 1×10^6 reference units HFI (Dolen *et al.* 1979); HBsAg remained detectable.

In summary, data were available of 29 patients with chronic HBsAg positive hepatitis who had received interferon preparations. Only 1 of the 13 patients given HFI showed a permanent change of virus markers. A transient decrease in DNAP activity was the most consistent finding in the 19 patients treated with HLI. In 4 of them DNAP remained negative after treatment and 2 patients became in addition HBsAg negative. No control patients were involved in the studies by others. 1 of our control patients became DNAP negative. Dosage schedules varied considerably within and between the different studies. Exact data on dose and duration of administration were not always obtainable. Nevertheless, we knew that 14 doses of 3×10^6 reference units HLI were not sufficient to affect DNAP activity definitely in all patients and we had the impression that interferon should be administered for at least 1 month.

We decided to perform a study in which we injected high doses HLI for a period longer than 1 month. We chose to start with 12×10^6 reference units daily for 1 week and halved this dose every week until discontinuation after week 6. This dosage schedule was of course a long shot into the dark, but we were not aware of chronic HBsAg positive hepatitis patients who had been treated with higher dosages interferon for a longer time.

Moreover, we had to be careful in view of the decrease in leucocyte counts that could be induced even with daily doses of 3×10^6 reference units HLI.

DOUBLE-BLIND STUDY OF HLI ADMINISTRATION IN CHRONIC HBsAg POSITIVE HEPATITIS

Introduction

In recent years human interferon preparations have been reported to affect indices of hepatitis B virus infections. In chronic HBsAg positive hepatitis, interferon was found to reduce HBcAg associated DNAP activity and HBeAg (Greenberg *et al.* 1976; Scullard *et al.*, see Dunnick & Galasso, 1979; this chapter), HBcAg titre (Greenberg *et al.* 1976), anti-HBcAg titre (Kingham *et al.* 1978), Dane particle number (Scullard *et al.*, see Dunnick & Galasso, 1979) and percentage of HBcAg-containing liver cell nuclei (Desmyter *et al.* 1976). In 2 patients all indices of hepatitis B virus infection, including HBsAg disappeared during interferon treatment (Greenberg *et al.* 1979). However, none of these studies was properly controlled and the number of patients was small. Because indices of hepatitis B virus infection fluctuate, the only way to establish the value of interferon administration in chronic HBsAg positive hepatitis is a double-blind study. We report here the results of such a study, in which interferon was injected in higher dosages than previously reported (Greenberg *et al.* 1976; Desmyter *et al.* 1976; Kingham *et al.* 1978; Scullard *et al.*, see Dunnick & Galasso, 1979; this chapter).

Patients, Material and Methods

16 patients (1 woman, 15 men) were studied who fulfilled the following criteria: HBsAg positive for at least 12 months, positive for HBeAg and HBc specific DNAP activity, abnormal levels of AST, but normal α_1 fetoprotein, α_1 anti-trypsin, caeruloplasmin and ferritin, and a biopsy showing chronic active hepatitis with positive HBcAg immunofluorescence. Patients were randomized to receive either HLI or placebo (human albumin).

3 patients were on immunosuppressive therapy (prednisone and/or azathioprine). The regimens were followed from 3 months before to 3 months after interferon administration.

HLI was prepared as described elsewhere (Cantell *et al.* 1974). It had an activity of $10^{6.4}$ reference units/mg protein. On an outpatient basis patients received 12 x 10^6 reference units i.m. during 1 week. Thereafter, the dose was halved every week until discontinuation after week 6, in an attempt to find an optimum dose for long-term therapy. On day 2 all 16 patients were vaccinated with an inactivated non-human influenza virus (HeqlNeql) to allow the effect of exogenous interferon administration on a primary humoral immune response to be studied. None of the patients had detectable antibodies against this virus. Promethazine, 25 mg 3 times a day, was given to patients with fever.

HBsAg titre was determined in a reverse passive haemagglutination test (Auscell, Abbott Laboratories). Anti-HBcAg was assayed with RIA (Corab, Abbott Laboratories) in serial dilutions of serum according to the manufacturer's directions. HBeAg was determined by the enzyme-linked immunosorbent assay (ELISA) technique (Organon, Oss, The Netherlands) as described before (Van der Waart *et al.* 1978). DNAP activity was determined as incorporation of ^{3}H(dTMP) into an acid-insoluble product as described before (Howard, 1978). Activity in negative control sera was 220 $\pm$ 50 cpm (mean $\pm$ SD). The specificity of the reaction was confirmed by neutralization as described before (this chapter). HBcAg was tested by immunofluorescence technique on liver tissue from a biopsy specimen taken on the day after the last injection. Leucocyte and thrombocyte numbers and AST levels were determined with routine methods. For statistical analysis the Mann Whitney U test was used.

Results

After randomization no statistically significant difference was found between interferon and placego groups for age, estimated duration of illness, HBsAg titre, HBeAg titre, DNAP activity, AST levels, leucocyte or thrombocyte counts (Table 23).

2 patients in the interferon group were on immunosuppressive therapy (1 patient 20 mg prednisone daily, the other 50 mg azathioprine daily). 1 patient in the placebo group received 10 mg prednisone and 50 mg azathioprine daily. The only female patient in the study was in the interferon group.

Table 23. Clinical and virological indices of patients after randomization

	Interferon group (n=8) median (range)	Placebo group (n=8) median (range)	z value
Age	38 (25-60)	36 (20-53)	0.47*
Duration of illness (years)	2.3 (1-5.5)	2.3 (1-6)	0.00*
AST (U/l)	52 (24-76)	53 (29-138)	0.37*
Leucocytes (x 10^9/l)	5.0 (3.9-7.6)	6.1 (4.7-11.2)	1.21*
Thrombocytes (x 10^9/l)	186 (155-267)	172 (139-271)	0.95*
HBsAg titre^{-1}	12800 (1600-51200)	12800 (3200-51200)	0.74*
HBeAg titre (P/N ratio)	5.8 (4.5-11.1)	7.4 (5.0-14.4)	1.37*
DNAP (cpm)	2375 (284-3272)	725 (271-3759)	1.58*

* $p > 0.1$ (Mann Whitney U test)

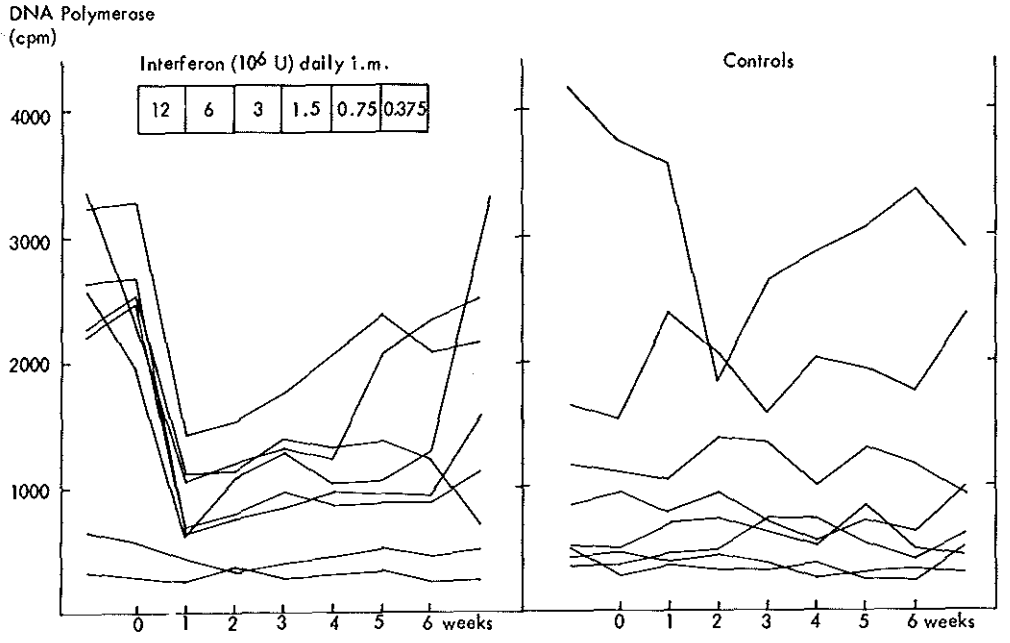

Fig. XIV. DNAP levels in interferon and control groups

Fig. XIV shows the DNAP activity in all patients. In the interferon-treated group DNAP activity fell in 6 of 8 patients in the first week. The main drop in DNAP activity (median 65%, range 53-75%) occurred in these patients after the first 2 injections of 12 x 10^6 reference units (Fig. XV). Despite continuation of daily injections of this dose, no further fall was observed after day 4. In the placebo-treated patients DNAP activity fell between day 2 and day 4 (mean 32%, range 10-60%) and returned to initial values on day 7.

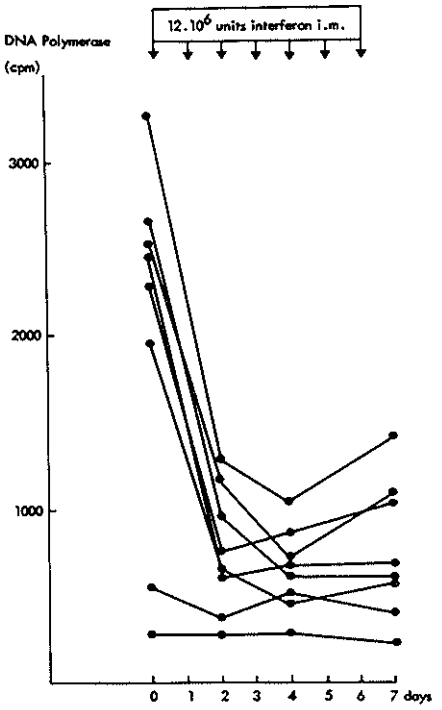

Fig. XV. DNAP levels during administration of 12 x 10^6 reference units of HLI.

In 2 patients HBeAg titre paralleled the initial drop in DNAP activity. In the other 6 patients HBeAg titre was not influenced by interferon administration. There was no effect on HBsAg titre or anti-HBcAg titre. Liver biopsy specimens taken on the day after the last injections showed positive HBcAg immunofluorescence in 30-70% (median 50%)

Table 24. Changes in AST levels and leucocyte and thrombocyte counts in interferon and placebo groups.

	Initial† values	Δ day 7¶	Δ day 14¶	Δ day 21¶	Δ day 28¶	Δ day 35¶	Δ day 42¶
AST (U/l)							
Interferon	52 (24-76)	+13 (-16-+62)	+ 1 (-21-+46)	-11-+80 (+4)	+25 (-2-+178)	+27 (0-+209)	+44 (-9-+183)
Placebo	53 (29-138)	-11 (-16-+29)	+ 3 (-16-+29)	- 3-+31 (+11)	+19 (+1- +38)	+20 (-3- +39)	+20 (-1-+102)
Leucocytes (10^9/l)							
Interferon	5.0 (3.9- 7.6)	-3.0+ (-4.3--1.9)	-2.7+ (-3.5--1.6)	-2.7+ (-3.8--0.3)	-1.9* (-3.0--0.9)	-1.6 (-2.8-+0.1)	-1.4 (-3.9-+1.0)
Placebo	6.1 (4.7-11.2)	-0.3 (-4.5- 0)	-0.8 (-3.7--0.2)	-0.4 (-2.7-+0.9)	+0.1 (-3.5-+1.4)	-0.6 (-4.3-+0.7)	-0.3 (-4.5-+0.3)
Thrombocytes (10^9/l)							
Interferon	186 (155-267)	-63+ (-92--38)	-49* (-71-+3)	-18* (-94-+28)	-30* (-95-+31)	-22* (-48- +1)	+11 (-30- +68)
Placebo	172 (139-271)	- 5 (-19-+31)	+42 (0-161)	+12 (-12-+69)	+48 (-9-+138)	+28 (-12-+69)	+78 (+10-+175)

† mean of 3 determinations between day -14 and day 0; n = 8; median (range)
¶ n = 8; median (range) vs initial value
\+ $p < 0.01$ vs initial value and placebo
* $p < 0.01$ vs placebo

of liver cell nuclei in patients of the interferon group and in 35-85% (median 55%) in control patients. No statistically significant difference was observed in AST levels between treated and control patients (Table 24).

The vaccination procedure induced fever (38-39^{0} C) in all 16 patients. No interferon was detected in the sera from the control patients after vaccination. All patients responded with a rise in antibody titre to Heq1Neq1 virus. No difference was found in antibody levels between the 2 groups after weeks 1, 2, 3 and 4.

Late effects

During the 3-month follow-up period all DNAP levels returned to initial values. In 1 patient of the placebo group, DNAP activity became undetectable 3 weeks after the last injection. This was associated with a rise in anti-HBsAg and AST and a drop in HBeAg titre.

Side effects

Chills and/or fever (38-40^{0} C) were noted in all 8 interferon-treated patients after the first injection only. 1 interferon patient had increasing hair loss. A significant drop in leucocytes was seen during the first 3 weeks of interferon treatment. 6 out of 8 patients in the interferon group developed leucopenia ($\leq$ 2.5 x 10^{9}/l) during the second week. Thrombocytes were significantly lower than initial values after the first treatment week (Table 24). In none of the patients thrombocytes dropped to values below 100 x 10^{9}/l.

Discussion

Several studies have shown an effect of interferon administration on indices of hepatitis B virus infection in CAH. The most consisting finding has been a reduction in DNAP activity, which could be induced by injections of between 4 x 10^{5} and 3 x 10^{6} reference units of HLI (Dunnick & Galasso, 1979).

Also in this study, we found a drop in DNAP activity after the first injections of interferon. Thereafter, no further fall was observed, despite continuation of daily injections of 12 x 10^{6} reference units

during the first week. The results of interferon administration in the 2 patients on immunosuppressive therapy (1 woman, 1 man) were the same as in the other patients. In this randomized study we could not confirm effects of interferon on other indices of hepatitis B virus infection that have been reported before, such as HBsAg titre (Greenberg *et al.* 1976), HBeAg (Greenberg *et al.* 1976; Scullard *et al.*, see Dunnick & Galasso, 1979; this chapter) and anti-HBcAg titre (Kingham *et al.*1978). Interferon administration had no effect on the number of liver cell nuclei positive for HBcAg. AST was not affected. Interferon had no effect on the primary immune response to an inactivated non-human influenza virus (HeqlNeql).

In an earlier report on interferon administration in HBsAg positive chronic hepatitis we described a control patient who became DNAP-negative during the study. In the present study, too, DNAP activity became undetectable in 1 patient after placebo treatment. This makes uncontrolled observations of the efficacy of interferon in individual patients with HBsAg positive chronic hepatitis difficult to interpret.

The effects of interferon are complex and poorly understood (Gresser, 1977; Friedman, 1977; this thesis). It can inhibit viral multiplication *in vivo* both directly and through the host. The drop in DNAP activity in the beginning of interferon administration could be mediated by the host, e.g. by the raised body temperature on the synthesis of complete Dane particles. The vaccination-induced fever was also associated with a fall in DNAP activity in our control patients. No further fall in DNAP was noted after the temperature had returned to normal at the end of the first week of interferon treatment.

We used higher dosages of interferon than reported before (see Dunnick & Galasso, 1979). This resulted in leucopenia in 6 of the 8 patients during the first 2 weeks. Therefore, continuation of daily injections of high doses of interferon or increasing the dose might not be possible.

In conclusion, the only effect of interferon administration in chronic HBsAg positive hepatitis was a transient reduction in DNAP activity, without apparent clinical significance. On the basis of these findings, long-term treatment of this disease with HLI alone does not

look promising. However, the other dosage schedules might perhaps be effective e.g., intermittent interferon administration, a gradual increase of the dose, or a combination with other therapies.

Conclusion

In this chapter we have reviewed the available literature on interferon administration in chronic HBsAg associated hepatitis. We have shown our attempts to eradicate the hepatitis B virus with different dosages HFI and HLI.

In our opinion, curing patients with chronic HBsAg positive hepatitis with interferon alone is not possible.

CHAPTER 6

DOUBLE-BLIND STUDY OF INTERFERON ADMINISTRATION IN RENAL TRANSPLANT RECIPIENTS

The purpose of this study was to evaluate the prophylactic activity of interferon in a patient group highly susceptible to viral infections.

This study was achieved by cooperation of the Departments of Internal Medicine, Virology, and Surgery, Erasmus University Rotterdam, and the Rega Institute, University of Leuven, Leuven, Belgium.

This chapter is based on:
'Double-blind study of interferon administration in renal transplant recipients' by W.Weimar, H.Schellekens, L.D.F.Lameijer, N.Masurel, V.G.Edy, A.Billiau & P. De Somer, published in *European Journal of Clinical Investigation* 8, 1978, 255-258.

Introduction

In animal models interferon exerts its clearest effect when given to prevent viral infections (Finter, 1973). For this reason human interferon might be expected to be useful when acute viral infections are likely to occur. This is the case after renal allograft transplantation.

Table 25 shows the viral infections diagnosed in renal transplant recipients in Rotterdam from June 1973 until July 1975. In this period 48 patients received a renal allograft and 43 of them were followed for at least 3 months. Neither attempts to isolate viruses nor serological tests were performed on a regular basis. In 34 patients adequately screened for viral infections, 59 positive diagnoses were made in 32 patients.

Table 25. Number of viral infections in renal transplant recipients in Rotterdam, June 1973 to July 1975.

	< 3 months after transplantation	> 3 months after transplantation	Total
HSV	17	4	21
CMV	15	2	17
EBV	1	1	2
	33	7	40
Rubella	2	3	5
Parainfluenza	0	4	4
Influenza A	1	2	3
Influenza B	0	1	1
RSV	2	2	4
Adenovirus	1	1	2
	6	13	29
Total	39	20	59

Table 26. Viral infections in renal allograft recipients

			Herpetoviridae*			
Reference		No.of patients	CMV	HSV	VZV	EBV
Craighead *et al.*	(1967)	41	73			
Simmons *et al.*	(1974)	132	74	26	5	
Balfour *et al.*	(1977)	28	64	54	18	
Pass *et al.*	(1978)	40	92	70		
Fiala *et al.*	(1975)	35	96	35	24	0
Koranda *et al.*	(1974)	200		35	13	
Armstrong *et al.*	(1976)	23	43	38		32
Andersen & Spencer	(1969)	36	91			
Rytel & Balay	(1976)	41	90			
Strauch *et al.*	(1974)	21				56
Pien *et al.*	(1973)	17	59	53	35	
Betts *et al.*	(1975)	54	63			

			Papovaviridae*	
Reference		No.of patients	BK	Papilloma (warts)
Coleman *et al.*	(1973)	74	38	
Koranda *et al.*	(1974)	200		43
Shah *et al.*	(1974)	17	18	
Lecatsas *et al.*	(1973)	17		

* numbers denote % of patients

Table 26 shows the incidence of viral infections in renal transplant recipients in different centres. Most of these infections occurred in the first 3 months after transplantation and were caused by members of 2 groups of DNA viruses: Herpetoviridae : herpes simplex (HSV), cytomegalo virus (CMV), varicella zoster virus (VZV) and Epstein-Barr virus (EBV); and Papovaviridae: BK virus, papilloma (warts) virus.

Apart from producing clinical viral disease, viral infections can modulate the immune system. Further immunosuppression can predispose to superinfections (Rubin *et al.* 1977), while immunostimulation may

lead to graft rejection (Simmons *et al.* 1974). Moreover, viruses may be involved in oncogenesis, leading to the high incidence of malignant tumours in renal allograft recipients (Matas *et al.* 1975; Penn, 1975).

In this chapter we describe our attempt to control viral infections in renal allograft recipients with HFI. We injected a dose of HFI comparable to the amount of HLI claimed to be effective in reducing viral infections (Strander *et al.* 1976).

Patients, Material and Methods

Interferon preparations

Interferon preparations were produced in human embryo fibroblasts by the method described by Billiau *et al.* (1973), modified by Edy (Edy *et al.* 1976; Edy, 1977). The interferon was titrated by inhibition of VSV replication in human diploid cells using a dye uptake method, and was calibrated against the Medical Research Council 69/19 standard of HLI. The approximate activity, before addition of human serum albumin as a stabilizer, was $10^{4.7}$ units/mg of protein. Placebo preparations were dilutions of human plasma protein fraction (Belgian Red Cross), concentrated and fractionated with the same techniques as the crude interferon preparations.

Patients

The study group consisted of 18 patients who received their renal transplants in the University Hospital Rotterdam-Dijkzigt from February 1976 to April 1977. All non-diabetic, HBsAg-negative patients between 18 and 45 years of age were included. The immunosuppressive regimen consisted of prednisone (35 mg daily) and azathioprine (1-2 mg/kg daily). During acute rejection episodes the dose of prednisone was increased.

The study was set up in a double-blind, placebo-controlled fashion, in consecutive pairs to avoid seasonal influences, and to assist in early detection of possible side-effects of interferon therapy. In each pair the patients received either placebo or interferon (3×10^6 U) i.m. twice weekly for 3 months, starting 1-2 h before transplantation.

Blood samples for haematology were taken daily during the first month after transplantation and twice weekly for the following 2 months. Blood samples for virus serology and liver function parameters were taken weekly during the first 3 months and twice monthly in a 3-month follow-up period.

Viral diagnosis

Antibodies to influenza virus A and B, adenovirus, respiratory syncytial virus (RSV), measles virus, CMV (AD-169 strain), HSV, VZV, parainfluenza viruses I, II and III, polio viruses I, II and III, *Mycoplasma pneumoniae* and *Chlamydia psitacci* were determined by the complement fixation (CF) test by a microtitre technique according to Lennette (1969). Antibodies to rubella virus were measured by the haemagglutination inhibition (HI) test, heterophile antibodies by the method of Paul-Bunnell , and HBsAg and anti-HBsAg by radioimmunoassay. Serial specimens from each patient were tested simultaneously. A fourfold or greater increase in antibody titre within 2 weeks was considered to be serological evidence of infection. As prevention of clinical illness was our main interest, viral isolations were performed only if a viral infection was suspected on clinical grounds.

Results

Table 27 shows the incidence of viral infections in both the interferon and placebo groups. Significant rises of CF antibody titres for CMV were seen in 9 of the 18 patients studied (50%). 5 were in the interferon group, 4 in the control group. 5 of these 9 patients were initially CF seronegative for CMV. In 2 patients of the interferon group and 1 of the control group this seroconversion was related to an episode of fever ($> 38^{0}$ C, for more than 2 days) and thrombocytopenia ($< 100 \times 10^{9}/l$). In one patient of the interferon group this episode was accompanied by hepatitis, mononucleosis, leucopenia ($< 2.5 \times 10^{9}/l$), and CMV shedding in the urine, and was considered life-threatening.

5 patients (31%) showed serological evidence of HSV infection. 2 of them were originally CF seronegative. 4 were in the placebo group and 1

Table 27. Viral infections

Viral infections		Interferon group									Placebo group								
		1[†]	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
CMV	Serological	-	+	-	-	+	-	+	+	+	-	+	-	+	-	+	-	+	-
	Clinical[¶]	-	-	-	-	+++	-	-	-	+	-	-	-	-	-	+	-	-	-
HSV	Serological	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	+	+	-
	Clinical	-	-	-	-	-	-	+	-	-	++	++	-	-	-	-	-	++	-
VZV	Serological	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
	Clinical	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Influenza A	Serological	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Clinical	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rubella	Serological	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-
	Clinical	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RSV	Serological	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
	Clinical	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Viral infections serological + clinical						5									4				
Viral infections serological only						6									5				
No. of patients with viral infections:																			
Serological + clinical						5									4				
Serological only						2									2				

[†] Numbers denote patient pairs
[¶] Clinical infections graded as + : minor; ++ : serious; +++ : very serious

in the interferon group. An extensive and painful eruption involving the facial skin and/or oral mucosa was seen in 3 patients of the placebo group. The patient in the interferon group showed only a minor herpes labialis. Virus isolations were positive in all 4 cases of herpes eruptions.

3 patients with positive antibody titres for rubella before transplantation showed significant HI titre rises. 2 of them were in the interferon group, 1 in the placebo group. In 1 patient of the interferon group this seroconversion was accompanied by a meningo-encephalitis that was considered on clinical grounds to be of viral origin.

1 clinical case of influenza-like illness was seen in the interferon group. It was associated with seroconversion to influenza virus. In another interferon-treated patient we found serological evidence of rubella virus, RSV, CMV and VZV infections. These seroconversions were not related in time and no clinical viral infections were evident. No significant changes in CF antibody titres were noted in any patient for the other viruses that were tested for. All viral infections occurred in the 3 months after transplantation. No viral infections were diagnosed in a 3-month follow-up period.

In summary, a total of 5 clinical viral infections were observed in 5 patients of the interferon group. In the placebo group 4 patients with clinical viral infections were seen. On serological grounds 6 subclinical infections occurred in the interferon group and 5 in the placebo group. No difference was found in the number of patients for whom only serological evidence was found for viral infection (2 in each group).

Table 28 shows the number of episodes ($\geq$ 2 days) of leucopenia, thrombocytopenia, elevated transaminases as well as acute rejection for both the interferon and placebo group. Patients in the interferon group had slightly fewer episodes of leucopenia, thrombocytopenia, and acute rejection. No difference was found in the number of episodes in which serum transaminase levels were raised. Pain at the injection site was not reported. In 2 patients febrile reactions were noticed after intramuscular administration of interferon, with temperature rises up to 38^{0} C.

Table 28. Haematology, liver function parameters, and acute rejection episodes

	Interferon group	Placebo group
Leucopenia† ($\leq 2.5 \times 10^9$/l)	2	3
Thrombocytopenia† ($\leq 100 \times 10^9$/l)	3	5
Raised AST† ($\geq$ 35 IU/l)	4	4
Raised ALT†¶ ($\geq$ 35 IU/l)	6	6
Acute rejection	13	16

† numbers denote episodes of $\geq$ 2 days
¶ ALT = alanine aminotransferase

Discussion

Systemic administration of HLI has been used in clinical trials for the therapy of herpes zoster (Strander *et al.* 1973; Emödi *et al.* 1975; Merigan *et al.* 1978), serious CMV infections (Arvin *et al.* 1976; Emödi *et al.* 1976; O'Reilly *et al.*1976) and HBsAg-positive chronic hepatitis (Greenberg *et al.* 1976; Desmyter *et al.* 1976; this thesis, chapter 5). Only in herpes zoster suggestive evidence for successful treatment has been reported. In animal studies interferon appeared to be effective when given prophylactically or early in the course of viral infection. Evidence for the prophylactic value of systemic HLI administration in man was suggested by Strander *et al.* (1976) and Ahström *et al.* (1974), who reported a significant lower incidence of symptoms of common viral infections in patients given interferon in the treatment of osteosarcoma and leukaemia.

In the present study we have evaluated the prophylactic effectiveness of HFI in a double-blind trial in renal transplant recipients. HFI injections were tolerated well by our patients. No evidence was found for an adverse effect on bone marrow, liver or renal transplant. Our results indicate that HFI given twice weekly i.m. in a dose of 3×10^6U is not capable of reducing the number of clinical and subclinical viral

infections in renal allograft recipients. Severe herpes simplex infections occurred only in the placebo group, but the difference from the interferon group was not statistically significant. Clinical symptoms of CMV and rubella virus infections were more severe in individual patients in the interferon group.

Several possibilities arise to explain the apparent lack of effect of interferon in our trial. It could be due to the immunodepressed state of our patients, although it has been reported that interferon might be effective under these circumstances (Neumann-Haefelin *et al.* 1976).

Alternatively, the dosage of interferon we used could have been insufficient, although Strander *et al.* (1976) suggested that a comparable dosage of HLI can protect against viral infections.

Finally, HFI could be less active than HLI. Recently it has been shown that these two interferon preparations are equally effective in the treatment of herpetic keratitis (Neumann-Haefelin *et al.* 1977).

However, nothing is known about the comparative efficacy of systemic administration of these interferons. In conclusion, it would seem that the possible slight benefits that may accrue from administration of this dosage of HFI in renal transplant recipients are outweighed by the technical and economic problems of interferon production for larger trials. It remains to be demonstrated whether other types of interferon may be more effective in this group of patients.

CHAPTER 7

THE EFFECT OF HUMAN INTERFERON ON VACCINIA VIRUS INDUCED SKIN LESIONS IN RHESUS MONKEYS

The purpose of this study was to find an optimum dosage schedule for systemic administration of human interferon by use of a monkey model.

This study was achieved by cooperation of the Departments of Virology and Internal Medicine, Erasmus University Rotterdam, the Virology Section of the Primate Centre TNO, Rijswijk, the Rega Institute, University of Leuven, Leuven, Belgium, and the Central Public Health Laboratory, Helsinki, Finland.

This chapter is based on:
'Antiviral effects of interferon *in vivo* may be mediated by the host' by H.Schellekens, W.Weimar, K.Cantell & L.Stitz, published in *Nature* 278, 1979, 742;
'Prevention of vaccinia lesions in rhesus monkeys by human leucocyte and fibroblast interferon' by W.Weimar, L.Stitz, A.Billiau, K.Cantell & H.Schellekens, to be published in *Journal of General Virology*, 1980.

General Introduction

Interferon is being evaluated clinically in a number of institutes and in a wide variety of diseases. Dosage schedules vary along with the institutes and the diseases and seem more related to the amount of interferon the clinicians can get hold of than to sound experimental data. To provide these experimental data we studied the effect of human interferons in rhesus monkeys on the development of skin lesions after intradermal infections with vaccinia virus. If human interferon could influence the development of these lesions it would mean a non-mutilating, easy-to-score, primate model to study problems like dose-response relations, timing of interferon administration, relation of *in vitro* and *in vivo* activities, toxicity, etc.

The intradermal vaccinia infection has a longstanding reputation in interferon research. The first *in vivo* experiments both in animals and in man studied the effect of *local* interferon administration on the subsequent take of intradermal vaccinia infections. They showed that exogenous interferon had an antiviral effect *in vivo*.

In the first part of this chapter we describe the effect of *systemic* interferon administration on vaccinia infection. It concerns the dose-response effect of HLI, the relative efficacy of HLI and HFI and the influence of route of administration.

In the second part the *in vitro* activity of HLI against vaccinia virus is compared with the *in vivo* effect.

PREVENTION OF VACCINIA LESIONS IN RHESUS MONKEYS BY HLI AND HFI

Introduction

In recent years evidence has been accumulating for the effectiveness of systemic interferon administration in the treatment of both viral and neoplastic diseases in man (Dunnick & Galasso, 1979). Treatment schedules in different studies vary considerably: clearly the optimum dose and frequency and route of administration are not known. High doses of interferon are found to be necessary to influence the course of an established viral infection (Merigan *et al.* 1978). Less interferon may be needed for prevention of viral disease. However, results of studies on the prophylactic efficacy are conflicting (Strander *et al.* 1976; Cheeseman *et al.* 1979; this thesis, chapter 6).

The interferon preparations used so far for clinical studies have been derived from two sources: human leucocytes induced with Sendai virus (HLI) and human diploid fibroblasts induced with double-stranded RNA (HFI). Little is known of the comparative efficacy of these preparations. When applied topically, no difference was found in the effect on herpes keratitis (Sundmacher *et al.* 1978). Used systemically in HBsAg-positive chronic hepatitis, differential effects of these interferons were reported (This thesis, chapter 5).

In an attempt to determine an optimum treatment regimen and to clarify possible differences in the efficacy of these interferon preparations, we have performed dose-response studies with HLI and compared the activity of HLI and HFI in rhesus monkeys inoculated intradermally with vaccinia virus. It has been reported that relatively crude interferon preparations can suppress vaccinia lesions in monkeys, whether injected intradermally or intravenously (Andrews, 1961; Pinto *et al.* 1970).

Material and Methods

Virus

The source, propagation, and titration of vaccinia virus (RIV strain) have been described elsewhere (Hekker *et al.* 1973).

Animals

Rhesus monkeys (*Macaca mulatta*) bred at the Primate Centre TNO (Rijswijk, The Netherlands) and weighing 1.5 to 3 kg were employed. Only animals lacking antibodies to vaccinia virus, as tested by a serum neutralisation test, were used.

Interferons

HLI was prepared as described before (Cantell *et al.* 1974) and had an activity of $10^{6.2}$ units/mg protein. HFI, prepared and partially purified as described elsewhere (Billiau *et al.* 1979), had an activity of $10^{6.0}$ units/mg protein.

Interferon titration

Interferon activity was measured with a dye uptake method, employing diploid skin fibroblasts and VSV as a challenge virus (Finter, 1969); units refer to the standard of HFI (G-023-902-527) provided by the National Institutes of Health (Bethesda, Md., USA). Interferon activity was also determined by a cytopathic effect inhibitory assay, employing RSb cells and VSV as a challenge virus; units refer to the standard of HLI (Medical Research Council 69/19).

Experimental design

Animals were kept in quarantine from 2 weeks before the start until 2 weeks after the experiments. Interferon was injected daily, starting on the day before vaccination until 7 days after vaccination. Each monkey was inoculated on the chest by intradermal injection of 0.05 ml aliquots of live vaccinia virus at different concentrations (10^7, 10^6 and 10^5 $TCID_{50}/ml^{-1}$), UV- inactivated and heat-inactivated virus (10^7 $TCID_{50}/ml^{-1}$ before inactivation) and saline.

Each virus dilution and all controls were injected at 3 sites. Animals were kept under general anaesthesia during vaccination. The monkeys were examined daily and the skin lesions were scored by 2 independent observers on an arbitrary scale from 0 to 4, based on appearance and severity of papules and pustules. Each day one third of the animals in turn were anaesthesized to allow photographic recording of the lesions

as well as blood sampling for various tests. Blood samples were taken 3 to 4 hours after the first interferon injection.

Results

In the first experiment groups of 3 monkeys received i.m. injections of either saline or 5×10^5 units/kg HFI or HLI. Typical vaccinia skin lesions developed in all untreated monkeys: pustules appeared between day 4 and day 7 after infections. All virus dilutions induced these lesions, while no lesions were produced by inactivated virus or saline. All 3 monkeys treated with HLI and 1 monkey treated with HFI were completely protected against vaccinia virus. In the 2 other animals injected with HFI, the pustules were smaller than in the control animals. In the protected monkeys, no vaccinia lesions developed after discontinuation of interferon treatment during the observation period of 42 days.

In the second experiment 3 groups of 3 animals were given different dose schedules of HLI i.m. (5×10^5, 1.25×10^5 and 0.5×10^5 units/kg, respectively); a control group received i.m. injections of saline. All control animals developed vaccinia lesions. The highest dose of HLI protected 2 monkeys completely; the other two dosage regimens inhibited formation of pustules in 1 monkey of each group. Lesion size appeared to be influenced by the dosage of interferon. Fig. XVI shows the inhibition of lesion scores in monkeys treated with different doses of HLI.

In the third experiment 2 groups of 3 monkeys received HLI (5×10^5 units/kg) i.m. or intravenously (i.v.); 2 other groups received HFI (5×10^5 units/kg) i.m. or i.v.; again a control group received i.m. injections of saline. The 3 control monkeys developed pustules. All animals treated with HLI, whether by i.m. or i.v. route, were protected. All monkeys treated with HFI i.m. were equally well protected. I.v. given HFI depressed lesion size in 2 monkeys, while 1 monkey was completely protected. Fig. XVII shows the inhibition of skin lesions in monkeys treated with HFI given by different routes.

Serum interferon levels 3 h after i.m. injections are shown in Table 29. Similar levels were found after injections of HLI, whether the sera were assayed on diploid skin fibroblasts against the HFI standard or on

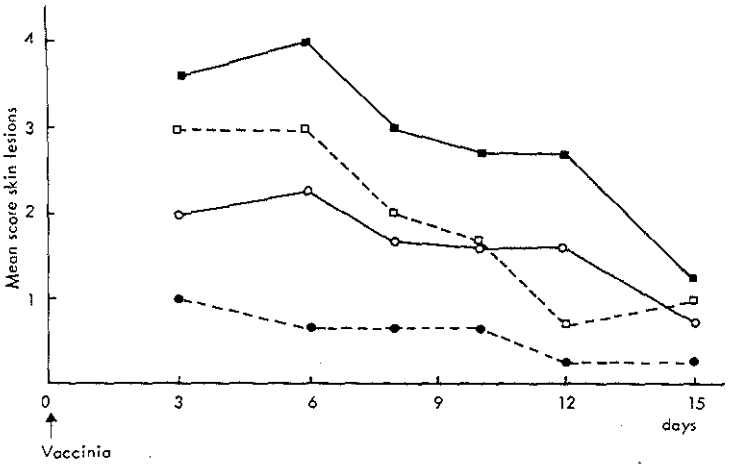

Fig. XVI. Skin lesion scores in rhesus monkeys treated i.m. with HLI: ■——■ controls (n = 6); □——□ daily dose 0.5 x 10^5 units/kg (n = 3); ○——○ daily dose 1.25 x 10^5 units/kg (n=3); ●——● daily dose 5 x 10^5 units/kg (n = 6). Interferon was injected from 1 day before to 7 days after vaccinia infection.

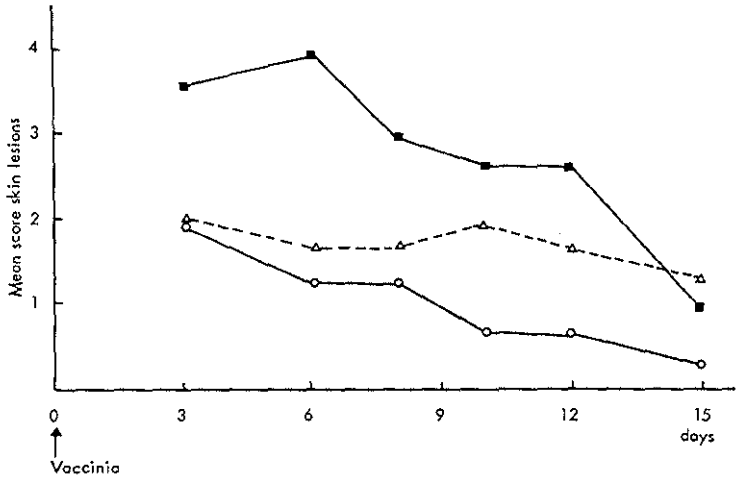

Fig. XVII. Skin lesion scores in rhesus monkeys treated with 5 x 10^5 units/kg HFI: ■——■ controls (n = 6); △——△ i.v. route (n = 3); ○——○ i.m. route (n = 6). Interferon was injected from 1 day before to 7 days after vaccinia infection.

Table 29. Levels of serum interferon and vaccinia lesion score in rhesus monkeys treated with human interferons

Type	Dose (units/kg)	Serum interferon† (units/ml)	Mean lesion score on day 6
HLI	5×10^5	250	0.7
	1.25×10^5	120	2.2
	0.5×10^5	80	3.0
HFI	5×10^5	140	1.3
Control	-	< 25	4.0

† Blood samples taken 3 h after i.m. injection; average values of 3 animals; interferon activity assayed on diploid fibroblasts against HFI standard

a continuous cell line (RSb cells) against the HLI standard. After i.m. injections of HFI, interferon activity in the sera was detected only when the assay on diploid fibroblasts was used. Table 29 shows that injections of 5×10^5 units/kg of HFI resulted in serum levels comparable with those after HLI given in a dose of 1.25×10^5 units/kg. Furthermore, these doses were about equally effective in reducing lesion scores.

Discussion

The present study shows that i.m. administration of either HLI or HFI can protect rhesus monkeys against vaccinia virus induced skin lesions. With HLI, the development of lesions was suppressed with all 3 dose regimens used: 5×10^5, 1.25×10^5 and 0.5×10^5 units/kg. However, only the highest dose was capable of completely suppressing lesions. From this it would appear that the corresponding dose for man would be approximately 30×10^6 units daily.

From the studies described in the second part of this chapter it would seem that the direct antiviral effect of interferon on cells does

not play the main role in the *in vivo* protection of monkeys against vaccinia virus. *In vitro*, the monkey cell/vaccinia virus system is virtually insensitive to both HFI and HLI. Therefore, it can be proposed that the effect against vaccinia virus in monkeys results from the activation of host defence mechanisms other than interferon, such as NK-cells or macrophages. It may well be that lower doses would be sufficient to provide *in vivo* protection in those host/virus systems where the direct effect of interferon on viral replication does play an important role in limiting initiation or progression of disease. In patients with malignancies treated with 3×10^6 units of HLI 3 times a week, the incidence of symptoms of viral infections was reported to be reduced (Strander *et al.* 1976). In general, higher dosages are probably needed if therapy is started after appearance of symptoms of viral infections. The dose of HLI necessary to suppress lesions completely in our vaccinia/monkey model roughly corresponded with that necessary to influence the course of varicella or herpes zoster in patients (Arvin *et al.* 1978; Merigan *et al.* 1978).

One can hypothesize that in a therapeutic, as opposed to prophylactic situation, the direct effect of interferon on viral replication is of less importance. Therapeutic effects may result mainly from activation of other host-defence mechanisms which require higher doses of interferon. The prevention of vaccinia virus induced skin lesions was not dependent on the amount of virus inoculated. Lesions appeared at several inoculation sites with different virus doses or not at all; lesion sizes correlated inversely with the dose of interferon. This is in line with our hypothesis that interferon does not exert its action by inhibiting virus replication, but rather by activating host-defence mechanisms. This hypothesis could explain why interferon is not fully effective in immunocompromised patients (Cheeseman *et al.* 1979; this thesis, chapter 6).

HFI inhibited lesion development in much the same way as HLI. Quantitative comparisons between the effects of these 2 interferons must take into account that they are 2 different molecules with different host ranges and different dose response curves *in vitro* (Edy *et al.* 1976). In the vaccinia/monkey model the effect of 5×10^5 units/kg of

HFI was about the same as that of 1.25 x 10^5 units/kg of HLI. Thus, on the basis of nominal units, HFI was about 4 times less effective than HLI. One could explain this difference by the fact that i.m. injected HFI results in lower blood titres than HLI (Edy *et al.* 1978). Recent evidence indicates that HFI is inactivated at the i.m. injection site (W.E.Stewart, personal communication). In our experiments, the group of HLI-treated and HFI-treated monkeys with comparable protection also had comparable blood titres. Thus, our experiments indicate that quite high serum levels are a requirement for interferon to be active against vaccinia virus *in vivo*.

In order to circumvent the problem of insufficient absorption of HFI, 1 group of monkeys was given HFI by the intravenous route. These monkeys were also partially protected, but less so than monkeys given the same dose by the i.m. route. It is known that i.v. injected interferon is rapidly cleared from the circulation. Therefore, while this procedure ensures that all interferon reaches the blood stream, it also may allow too little time for HFI to activate host-mediated mechanisms.

The Scientific Committee (1970) reported that the insensitivity of vaccinia virus to interferon *in vitro* discouraged their plans to modify this infection by systemic interferon administration. In our opinion, this insensitivity provides an interesting model to study the antiviral mechanisms by which interferon acts *in vivo*. Treatment regimens inferred from this model may also give some guidelines for the treatment of patients with interferon. However, the limitations of this model must also be kept in mind, and the crucial comparisons between HLI and HFI will have to be done in clinical trials.

ANTIVIRAL EFFECT OF INTERFERON *IN VIVO* MAY BE MEDIATED BY THE HOST

Introduction

Interferon has antiviral effects both *in vivo* and *in vitro*. Viral replication is inhibited in interferon-treated cells *in vitro* because the reproductive cycle of the virus is inhibited at the transcriptional or translational levels, depending on the virus-cell system studied (Friedman, 1977). This direct inhibition of viral replication has been assumed to also be the mechanism by which interferon exerts its antiviral effect *in vivo*. We report here results that indicate protection by interferon against viral infection *in vivo* without inhibition of the viral replication of the same virus *in vitro*.

Material and Methods

To test the antiviral effect of HLI *in vivo*, 6 rhesus monkeys were infected intradermally with vaccinia virus, strain of the RIV, described before by Hekker *et al.* (1973). They were vaccinated on day 0 with the following preparations: 10^7 $TCID_{50}ml^{-1}$ vaccinia virus; 10^6 $TCID_{50}ml^{-1}$ vaccinia virus; 10^5 $TCID_{50}ml^{-1}$ vaccinia virus; 10^7 $TCID_{50}ml^{-1}$ vaccinia virus, subjected to UV-inactivation and heat-inactivation; NaCl. For each dilution, 0.05 ml were injected at 3 sites. 3 monkeys were treated daily with HLI from day -1 to day 7 at 5 x 10^5 units/kg^{-1} i.m. The HLI was prepared as described previously (Cantell & Hirvonen, 1978). The preparation used in these experiments had an activity of 2.1 x 10^6 units/mg protein.

RSb cells, described in chapter 2 of this thesis, or rhesus monkey skin fibroblasts, described in chapter 3, were plated in microtitre plates, grown to confluency (4 x 10^4 cells/well) and treated with serial dilutions of HLI containing 10^4 units/ml of rhesus monkey sera (before and after injection of 5 x 10^5 units/kg^{-1} HLI). After overnight incubation, the supernatant was removed and cells were infected with VSV or vaccinia virus (multiplicity of infection 0.25 $TCID_{50}$/cell). The incubation was terminated when untreated infected control cells showed more than 90% CPE. The cells were stained with crystal violet. Both viruses

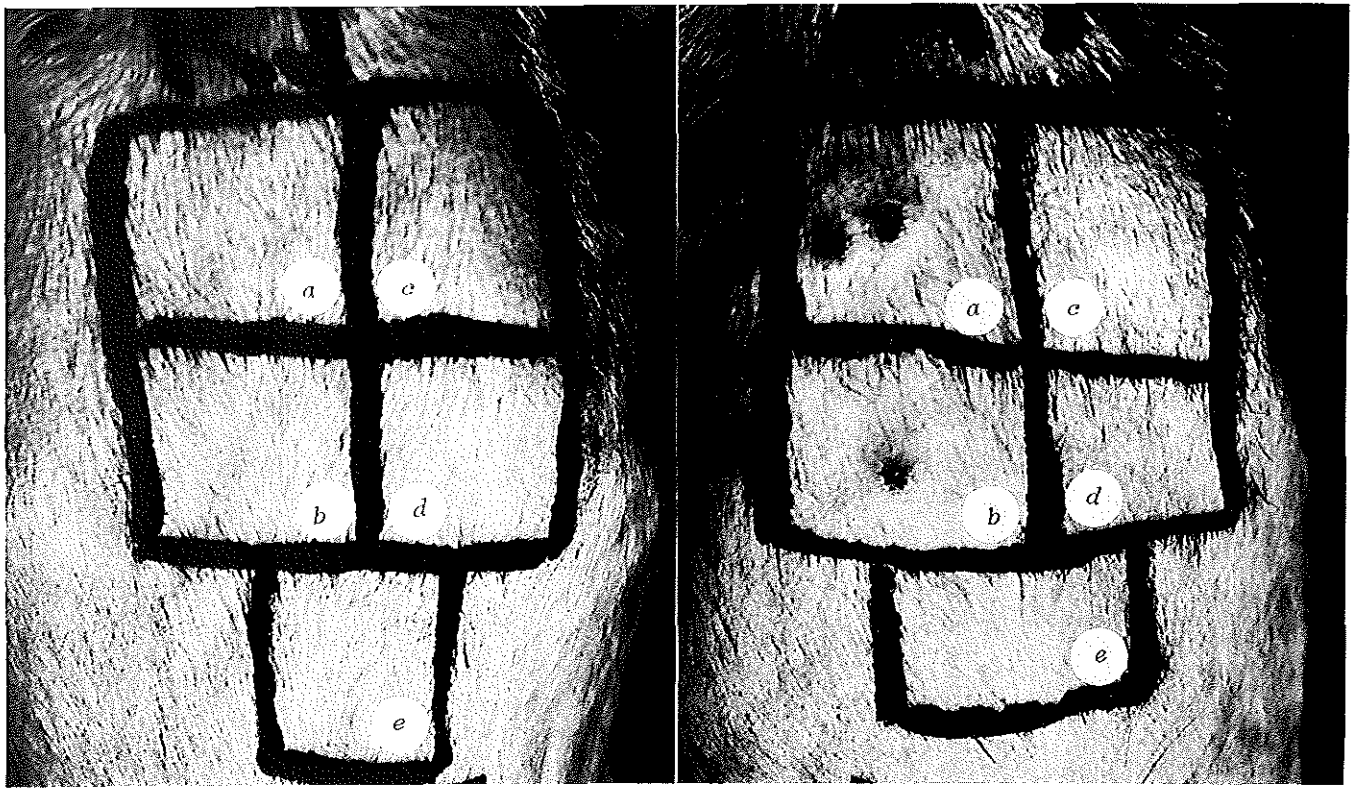

Fig. XVIII. In vivo *effect of HLI in rhesus monkeys infected intradermally with vaccinia virus. The vaccination sites 12 days after infection are shown in a rhesus monkey treated with HLI (left) and in a control monkey (right); (a)* 10^7 $TCID_{50}ml^{-1}$ *vaccinia virus; (b)* 10^6 $TCID_{50}ml^{-1}$ *vaccinia virus; (c)* 10^5 $TCID_{50}ml^{-1}$ *vaccinia virus; (d)* 10^7 $TCID_{50}ml^{-1}$ *vaccinia virus, subjected to UV-inactivation and heat inactivation; (e) NaCl.*

were always tested simultaneously in the same microtitre plate and an interferon standard preparation was included. The activity of interferon is expressed as the reciprocal of the highest dilution giving 50% protection. The titres are given as reference units (tested against standard preparation 69/19 of the Medical Research Council).

Results and Discussion

In control monkeys the lesions reached their maximum on day 7. Daily i.m. injections of 5 x 10^5 units HLI/kg body weight during 8 days completely inhibited the development of the typical vaccinia-induced skin lesions (Fig. XVIII). No skin lesions were detected during the observation period of 4 weeks following infections. However, we were unable to correlate this distinct *in vivo* effect of HLI with a corresponding inhibition of the CPE of the same virus on diploid rhesus monkey skin fibroblasts or on RSb cells. Up to 10^4 units of HLI failed to protect the cells against the CPE of vaccinia virus (RIV strain), although the CPE of VSV was effectively inhibited (Table 30).

Table 30. In vitro *inhibition of the CPE of vaccinia virus by HLI and serum from treated rhesus monkeys*

Preparation	RSb cells		Skin fibroblasts from rhesus monkeys	
	VSV	Vaccinia virus	VSV	Vaccinia virus
HLI, 10^4 units/ml^{-1}	10,000	< 1	10,000	< 1
Pre-serum†	< $10^{¶}$	<10	nd*	nd
Post-serum†	350¶	<10	nd	nd

† Before and after 4 h after injection of rhesus monkeys with 5 x 10^5 units/kg HLI
¶ Mean titre of 3 monkeys
* nd = not determined

Surprisingly, vaccinia virus was resistant to interferon in our *in vitro* system, but the same results were obtained *in vitro* with the vaccinia virus strain WR and with smaller challenge doses (10^{-3} $TCID_{50}$; this thesis, chapter 3). Also no anti-vaccinia activity could be detected in the sera of the monkeys 4 h after the first injection with HLI. At this time, the sera showed the maximum titre of antiviral activity when tested against VSV. Similar experiments performed with human diploid skin fibroblasts and rhesus monkey kidney cells showed identical results. There was also no inhibition of the production of infectious vaccinia virus.

These findings indicate that interferon can be effective *in vivo* against a virus which is insensitive to its antiviral action in various cell types. Likewise, it has been shown by others (Gresser *et al.*1972) that interferon can inhibit *in vivo* the growth of tumour cells which are resistant to its growth inhibitory action *in vitro*. Interferon may activate several defence systems of the host, for example, the cytotoxicity of the NK cells and the macrophages (Gidlund *et al.* 1978; Gresser & Tovey, 1978). Perhaps such 'aggressive' cells can selectively destroy virus-infected cells *in vivo*.

SUMMARY

This thesis describes our three-year wandering through the interferon field from 1976 to 1979. It is introduced by a chapter (1) reviewing the present state of knowledge of the interferon system. The next three chapters (2, 3 and 4) are concerned with production, antiviral activity and cell growth inhibition of interferon *in vitro*. Thereafter, we present in chapters 5, 6 and 7 our *in vivo* studies on human interferon administration in therapeutic and prophylactic settings.

In chapter 2 we describe the production of rat interferon of relatively high activity (about 10^6 units/mg protein) in embryonic rat cells treated with Newcastle disease virus at a high multiplicity of infection. The cells were cultured in serum-free medium and the interferon was precipitated and concentrated with 0.02 M-zinc acetate or with ammonium sulphate with 85% saturation. With both methods the increase in interferon activity was greater than the concentration factor. The rat interferon activity was stable on treatment with 0.15 M-perchloric acid and after 3 cycles of freezing and thawing, but incubation at 37° C for 1 h resulted in a 50% loss in activity. It had no cross activity in human or mouse cells. The sensitivity of different types of rat cells for interferon differed widely and was dependent on the challenge virus. Human interferons had no detectable antiviral activity on rat cells and did not block the activity of rat interferon.

In chapter 3 the dependence of antiviral activity of interferon on multiplicity of infection (MOI) is shown. Cells could not be protected against the cytopathogenic effect of vaccinia, herpes, ECHO or vesicular stomatitis virus at an MOI > 1. At an MOI $\leq$ 1, cells could be protected. The amount of protecting interferon was inversely related to the MOI. When protection was afforded, it was only transient. The duration of the antiviral effect of interferon was also inversely related to the MOI.

The dependence of the antiviral effect on the MOI could not be explained by assuming the viruses to be mixtures of subtypes with different interferon sensitivity. Also selection by interferon treatment of interferon-insensitive subtypes could not be shown. The greater anti-

viral effect of interferon at low MOI was not caused by induction of interferon by the infecting virus. A direct inactivation by the virus of the antiviral effect of interferon could not be demonstrated. These results indicate that when interferon-treated cells are infected, they will not survive the infection. The only result of the interferon treatment will be inhibition of viral replication, to some extent leading only to a delay in cell death.

Chapter 4 contains 2 studies on the inhibition of cell multiplication by interferon. The first part describes a study of the toxicity of interferon to bone marrow by the use of *in vitro* colony-forming assays for haemopoietic cells. The relative inhibitory effects of 2 interferon preparations, HLI and HFI, were compared with regard to their effect on both myeloid (CFU_c) and erythroid (CFU_e) progenitor cells. CFU_e formation in human bone marrow cells *in vitro* appeared to be fairly resistant to both interferons. Only high doses of both interferons gave a marked inhibition of CFU_e. However, the toxicity of HLI and HFI was divergent for CFU_c formation in bone marrow. HLI appeared to be considerably more inhibitory for CFU_c than HFI. The effects of mouse interferon, induced in L_{929} cells, on the growth of CFU_c and CFU_e in murine bone marrow cells were comparable with those of HFI on human cells. The toxicity of human and murine interferons was species-specific. Except for the toxicity of HLI to CFU_c in human bone marrow, the toxicity of interferon was marked only with concentrations of interferon far exceeding the amount necessary to produce an antiviral state *in vitro*.

In the second part of chapter 4, the cell growth inhibition of interferon during conditions of viral inflammation is described. This anticellular activity was not affected by viral challenge nor by simultaneous treatment of cells with different types of interferon. Elevated temperatures enhanced the cell growth inhibitory activity of both HLI and HFI. Cell growth inhibition was measured by a dye uptake and elution technique. Results with this technique were similar to those obtained by cell counting and measurement of ^{3}H-TdR incorporation.

In chapter 5 we present 3 studies on the effects of HFI and HLI in chronic HBsAg positive hepatitis. HFI had no effect on hepatitis B virus indices, while HLI induced a fall in HBc-associated DNA polyme-

rase activity, This enzyme activity is supposed to reflect the number of circulating full Dane particles (the proposed hepatitis B virus). In a double-blind long-term study with high doses of HLI we found no effect of interferon on other indices of hepatitis B virus than DNA polymerase activity. The fall in DNA polymerase activity can readily be explained by fever that was associated with HLI administration. In our opinion, treatment of chronic HBsAg-positive hepatitis with interferon alone has no beneficial effect.

In chapter 6 we report our data on a double-blind study of HFI administration in renal transplant recipients. Because interferon is especially effective in animal models when given prophylactically, human interferon preparations can be expected to be useful when acute viral infections can be predicted. This occurs after renal allograft transplantation. However, intramuscular administration of 3×10^6 units HFI given twice weekly for 3 months did not control viral infections. In view of these results we developed a primate model in an attempt to find an effective dosage schedule for human interferon administration.

In chapter 7 we describe an experimental vaccinia virus infection in rhesus monkeys and the prophylactic antiviral activity of systemically administered human interferon. Daily injections of HLI (5×10^5 units/kg) given from day -1 to day 7 after vaccination protected the monkeys completely against vaccinia virus. No skin lesions developed after discontinuation of therapy. Lower dosages decreased the severity of these lesions. Intramuscular gifts of 5×10^5 reference units/kg of HFI resulted in similar serum levels and were equally effective in reducing skin lesion score as 1.25×10^5 reference units/kg HLI.

We showed that systemically administered human interferon preparations had an antiviral activity *in vivo* against vaccinia virus. However, vaccinia virus proved to be resistant to these interferon preparations in our *in vitro* systems. Also no anti-vaccinia activity could be detected in the sera of the monkeys 3 to 4 h after the first interferon injection. These findings indicate that interferon can be effective *in vivo* against a virus that is insensitive to its antiviral action *in vitro*. We concluded that the antiviral effect of interferon *in vivo* may be mediated by the host.

SAMENVATTING (SUMMARY IN DUTCH)

In dit proefschrift worden enkele aspecten van het interferon systeem beschreven, waaraan wij van 1976 tot 1979 gewerkt hebben.

Hoofdstuk 1 geeft een overzicht van de huidige kennis over interferon. Hoofdstukken 2, 3 en 4 handelen over productie, antivirale activiteit en celgroeiremming *in vitro*. In de hoofdstukken 5, 6 en 7 komen de *in vivo* studies aan de orde, waarin humaan interferon therapeutisch en profylactisch werd toegediend.

In hoofdstuk 2 wordt een methode beschreven om op grote schaal ratte-interferon te produceren met Newcastle disease virus als inductor. Er kon gebruik worden gemaakt van serum-vrij medium, waardoor slechts één zuiverings- en concentratieprocedure nodig was om interferon van hoge activiteit te verkrijgen. Na precipitatie en concentratie met 0.02 M-zink acetaat of met 85% verzadigd ammonium sulfaat bleek de interferon activiteit boven de concentratiefactor toe te nemen. Ratte-interferon was bestand tegen 0.15 M-perchloorzuur en tegen driemaal invriezen en ontdooien. Incubatie bij 37^{o} C gedurende één uur resulteerde in een 50% verlies van activiteit. De gevoeligheid voor interferon van verschillende typen rattecellen bleek sterk te variëren en was bovendien virus-afhankelijk. Ratte-interferon was niet actief in menselijke en muizecellen. Humane interferonen toonden geen antivirale activiteit in rattecellen en konden de activiteit van ratte-interferon op die cellen niet blokkeren. Wij concludeerden dat het ratte-interferonsysteem niet verschilt van dat van andere species.

In hoofdstuk 3 wordt aangetoond dat de antivirale activiteit van interferon afhankelijk is van de hoeveelheid infecterend virus (multiplicity of infection, MOI). Cellen konden door interferon niet worden beschermd tegen vaccinia, herpes, ECHO en vesicular stomatitis virus wanneer de MOI > 1 was. Bij een MOI $\leq$ 1 werd wel bescherming gevonden. De hiervoor benodigde hoeveelheid interferon was omgekeerd afhankelijk van de MOI. Bescherming bleek een tijdelijk fenomeen te zijn: hoe hoger de MOI was, des te korter de bescherming aanhield. Verschillende mogelijkheden werden nagegaan om de MOI afhankelijkheid van interferon te verklaren. Uitgesloten werd dat dit verschijnsel wordt veroorzaakt door

de aanwezigheid van subtypen virussen met verschillende interferon gevoeligheden. Ook werd geen selectie van interferon ongevoelige subtypen gevonden door interferon behandeling. Het antivirale effect van interferon bij lage MOI werd niet veroorzaakt door inductie van interferon door het infecterende virus. Evenmin werd directe inactivatie van het antivirale effect van interferon door virus gevonden. Wij concludeerden dat ook met interferon behandelde cellen een virale infectie niet kunnen overleven. Door interferon wordt de virale replicatie slechts geremd, zodat celdood wordt uitgesteld.

Hoofdstuk 4 bevat twee studies over de celgroei-remmende werking van interferon. In het eerste gedeelte werd dit effect bestudeerd met behulp van beenmergkweken in agar. De remmende werking van humaan leucocyten interferon (HLI) en humaan fibroblasten interferon (HFI) op voorlopercellen van de myeloïde (CFU_c) en erythroïde (CFU_e) reeks werden met elkaar vergeleken. Interferon bleek CFU_e formatie nauwelijks te beïnvloeden. HLI remde CFU_c vorming veel sterker dan HFI. Muize-interferon, geïnduceerd in L929 cellen, remde de groei van CFU_c en CFU_e in muizebeenmerg op dezelfde wijze als HFI dit in menselijke cellen deed. Celgroeiremming van de onderzochte interferonen was species-specifiek.

In het tweede gedeelte van hoofdstuk 4 werd de celgroeiremmende eigenschap van interferon onderzocht onder omstandigheden van een virale infectie. Het anticellulaire effect werd niet beïnvloed door virus, noch door het tegelijk aanwezig zijn van verschillende typen interferon. Hogere temperaturen (38-39^{o} C) deden de cytotoxiciteit van zowel HLI als HFI toenemen. De groei van cellen in monolayer werd gemeten met een kleur- en extractiemethode. Resultaten met deze snelle techniek bleken identiek te zijn aan die verkregen met celtelling en met de bepaling van ^{3}H-TdR incorporatie.

In hoofdstuk 5 komen onze pogingen aan de orde om chronische HBsAg positieve hepatitis therapeutisch te beïnvloeden met interferon. HFI had geen effect op hepatitis B virus indices, terwijl HLI een daling van de HBc-'associated' DNA polymerase (DNAP)-activiteit veroorzaakte. Deze enzymactiviteit is een maat voor de hoeveelheid circulerende Dane partikels (het veronderstelde hepatitis B virus).

In een dubbelblinde studie met hoge doses HLI vonden wij echter geen ander effect op hepatitis B virus indices dan de al genoemde daling van het DNAP. Deze daling zou het gevolg kunnen zijn van de door HLI geïnduceerde koorts en hoeft niet te berusten op replicatieremming van het hepatitis B virus. Naar ons inzicht is toediening van uitsluitend interferon aan patiënten met chronische HBsAg positieve hepatitis niet zinvol.

In hoofdstuk 6 wordt een dubbelblinde studie met HFI in niertransplantatiepatiënten beschreven. In dierexperimenteel werk komt de antivirale activiteit van interferon voornamelijk tot uiting wanneer het profylactisch wordt toegepast. Daarom kan men veronderstellen dat menselijke interferonpreparaten klinisch waardevol zullen zijn in die situatie, waar men virale infecties kan verwachten. Dit is het geval na niertransplantaties. Intramusculaire toediening van 3×10^6 units HFI tweemaal per week gedurende 3 maanden was echter niet in staat virale infecties bij niertransplantatiepatiënten te voorkomen. Om deze reden ontwikkelden wij een primatenmodel ten einde tot een effectief antiviraal doseringsschema voor humaan interferon te komen.

In hoofdstuk 7 wordt een experimentele vaccinia virus infectie in de rhesusaap beschreven. Dagelijkse injecties van 5×10^5 units/kg HLI van dag -1 tot dag 7 na vaccinatie beschermden de apen volledig tegen vaccinia virus. Er ontstonden ook geen huidlaesies na dag 7. Lagere doses HLI verminderden de ernst van deze huidlaesies. Intramusculaire toediening van 5×10^5 units/kg HFI resulteerde in gelijke bloedspiegels en was ook even effectief als 1.25×10^5 units/kg HLI.

Systemisch toegediend humaan interferon kon dus een antivirale activiteit *in vivo* uitoefenen, in dit geval bij vaccinia virus. Het bleek echter dat vaccinia virussen resistent waren tegen interferon *in vitro*. In de sera van de apen was ook geen anti-vaccinia activiteit aantoonbaar 3-4 uur na toediening van interferon. Interferon kan dus *in vivo* effectief zijn tegen een virus dat ongevoelig is voor de antivirale werking van interferon *in vitro*.

Wij concludeerden dat het antivirale effect van interferon *in vivo* niet op replicatieremming van het virus hoeft te berusten maar via gastheermechanismen kan verlopen.

ACKNOWLEDGEMENTS

This thesis is the result of an intensive cooperation between preclinical and clinical institutes. The authors wish to thank everyone who joined them on their journey through the interferon world. We needed their help, advice, permissiveness, and inspiring opposition.

More than a hundred persons were involved in our studies. To mention by name only a few of them would be ungrateful to the others. Therefore, we should like to express our thanks to the following institutes that made this thesis possible:

Department of Internal Medicine II (Gastroenterology)
Department of Pathology II
Primate Centre TNO, Rijswijk
Renal Transplantation Unit (Departments of Surgery and Internal Medicine I)
Department of Virology

'Audiovisuele Dienst'
Department of Biostatistics
Central Clinical Chemistry Laboratory
Department of Haematology
Department of Internal Medicine III
Department of Medical Microbiology
Department of Pathology I

Central Public Health Laboratory, Helsinki, Finland
Finnish Red Cross, Helsinki, Finland
Rega Institute, University of Leuven, Leuven, Belgium

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam
Scientific Development Group, Organon, Oss
London School of Hygiene and Tropical Medicine, London, England

Netherlands Kidney Foundation
'Koningin Wilhelmina Fonds' of the Netherlands National Cancer League
Belgian Department of the Economy (Prototype Projects)
Belgian ASLK (General Savings and Retirement Fund)

LIST OF ABBREVIATIONS

ALT	alanine aminotransferase
AST	amino aspartate transferase
CAH	chronic active hepatitis
CF test	complement fixation test
CFU_c	human myeloid colonies (colony forming units)
CFU_e	human erythroid colonies (colony forming units)
CMV	cytomegalo virus
CPE	cytopathogenic effect
cpm	counts per minute
DEAE	diethylaminoethyl
DMEM	Dulbecco's modification of Eagle's minimal essential medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNAP	DNA polymerase
DRB	dichloro-1-β-D-ribofuranosyl benzimidazole
ds-RNA	double-stranded RNA
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B 'e' antigen
HBsAg	hepatitis B surface antigen
HEL cells	human embryo lung cells
HEPES	hydroxyethylpiperazine-*N*'-2-ethane sulphonic acid
HFI	human fibroblast interferon
HI test	haemagglutination inhibition test
HLI	human leucocyte interferon
HSV	herpes simplex virus
i.m.	intramuscular(ly)
i.v.	intravenous(ly)
MIF	mouse interferon
MOI	multiplicity of infection
MOPS	morpholinopropane sulphonic acid

NCS	newborn calf serum
nd	not done (determined)
NDV	Newcastle disease virus
NK cell	natural killer cell
PBS	phosphate buffered saline
PFU	plaque forming units
P/N ratio	positive/negative ratio
poly(rI).poly(rC)	polyinosinic-polycytidylic acid
REC	rat embryo cells
RIF	rat interferon
RIV	Rijksinstituut voor de Volksgezondheid
RM cells	cells derived from a spontaneous rat rhabdomyosarcoma
RNA	ribonucleic acid
ROS cells	cells from a rat osteosarcoma
RSb cells	Rous sarcoma virus-transformed human cells
RSC cells	cells from a radiation-induced rat skin carcinoma
RSV	respiratory syncytial virus
RUC cells	cells from rat urethral carcinomas
SD	standard deviation
$TCID_{50}$	tissue culture infective dose (50%)
TES	tris-(hydroxymethyl)methyl-2-aminoethane sulphonic acid
U	units
vs	versus
VSV	vesicular stomatitis virus
VZV	varicella zoster virus
XC cells	cells derived from a Rous sarcoma virus-induced rat tumour

REFERENCES

Aho, K., Strander, H., Leikola, J., Kleemola, M., Carlström, G. & Cantell, K. (1976). Inhibitor of complement-fixation reaction associated with interferon treatment. *Clinical Immunology and Immunopathology* 5, 399-403.

Åhström, L., Dohlwitz, A., Strander, H., Carlström, G. & Cantell. K. (1974). Interferon in acute leukaemia in children. *Lancet* I, 166-167.

Allen, P.T., Schellekens, H., Van Griensven, L.J.L.D. & Billiau, A. (1976). Differential sensitivity of Rauscher murine leukaemia virus (MuLV-R) to interferons in two interferon-responsive cells lines. *Journal of General Virology* 31, 429-435.

Andersen, H.K. & Spencer, E.S. (1969). Cytomegalovirus infection among renal allograft recipients. *Acta Medica Scandinavica* 186, 7-19.

Andrews, R.D. (1961). Specificity of interferon. *British Medical Journal* 1, 1728-1730.

Armstrong, J.A. (1971). Semi-micro, dye-binding assay for rabbit interferon. *Applied Microbiology* 21, 723-725.

Armstrong, J.A., Evans, A.S., Rao, N. & Ho, M. (1976). Viral infections in renal transplant recipients. *Infection and Immunity* 14, 970-975.

Arvin, A.M., Feldman, S. & Merigan, T.C. (1978). Human leukocyte interferon in the treatment of varicella in children with cancer: a preliminary controlled trial. *Antimicrobial Agents and Chemotherapy* 13, 605-607.

Arvin, A.M., Yeager, A.S. & Merigan, T.C. (1976). Effect of leukocyte interferon on urinary excretion of cytomegalovirus by infants. *Journal of Infectious Diseases* 133 (suppl.), A205-210.

Balfour, H.H., Slade, M.S., Kalis, J.M., Howard, R.J., Simmons, R.L. & Najarian, J.S. (1977). Viral infections in renal transplant donors and their recipients: A prospective study. *Surgery* 81, 487-492.

Balner, H., Van Bekkum, D.W., De Vries, M.J., Dersjant, H. & Van Putten, L.M. (1968). Effect of anti-lymphocyte sera on homograft reactivity and G-v-H reactions in rhesus monkeys. In: *Advance in Transplantation. Proceedings of the First International Congress of the Transplantation Society, Paris, June 1967,* ed. by J.Dausset,

J.Hamburger & G.Mathé. Copenhagen, Munksgaard, pp.449-456.

Van Bekkum, D.W., Löwenberg, B. & Vriesendorp, H.M. (1978). Bone marrow transplantation. In: *Immunological Engineering*, ed. by D.W.Jirsch. Lancaster, MTP Press, pp.179-227.

Betts, R.F., Freeman, R.B., Douglas, R.G., Talley, T.E. & Rundell, B. (1975). Transmission of cytomegalovirus infection with renal allograft. *Kidney International* 8, 387-394.

Biernacka, I.& Lobodzińska, M. (1973). Rat interferons. II. Induction and properties of an interferon-like inhibitor from rat embryonic cell cultures. *Archivum Immunologiae et Therapiae Experimentalis* 21, 471-475.

Billiau, A. & Buckler, C.E. (1970). Production and assay of rat interferon. In: *Proceedings of the International Symposium on Standardization of Interferon and Interferon Inducers, London, 1969 (Symposium Series on Immunobiological Standardization, vol. 14)* Basel/ New York, Karger, pp. 37-44.

Billiau, A., Van Damme, J., Van Leuven, F., Edy, V.G., De Ley, M., Cassiman, J.-J., Van den Berghe, H. & De Somer, P. (1979). Human fibroblast interferon for clinical trials: production, partial purification and characterization. *Antimicrobial Agents and Chemotherapy* 16, 49-55.

Billiau, A., Joniau, M. & De Somer, P. (1973). Mass production of human interferon in diploid cells stimulated by poly-I:C. *Journal of General Virology* 19, 1-8.

Bradley, T.R. & Metcalf, D. (1966). The growth of bone marrow cells *in vitro*. *Australian Journal of Experimental Biology and Medical Science* 44, 287-300.

Buffett, R.F., Ito, M., Cairo, A.M. & Carter, W.A. (1978). Antiproliferative activity of highly purified mouse interferon: brief communication. *Journal of the National Cancer Institute* 60, 243-246.

Cantell, K. (1970). Preparation of human leukocyte interferon. In: *Proceedings of the International Symposium on Standardization of Interferon and Interferon Inducers, London, 1969 (Symposium Series on Immunobiological Standardization, vol. 14)*. Basel/New York, Karger, pp. 6-8.

Cantell, K. & Hirvonen, S. (1978). Large scale production of human leucocyte interferon containing 10^8 units/ml. *Journal of General Virology* 39, 541-543.

Cantell, K., Hirvonen, S., Mogensen, K.E. & Pyhälä, L. (1974). Human leukocyte interferon:production, purification, stability, and animal experiments. In: *In Vitro. Proceedings of a Tissue Culture Association Workshop, 1973*, ed. by C.Waymouth. Rockville, Md., Tissue Culture Association, pp. 35-38.

Cesario, T.C., Schryer, P.J. & Tilles, J.G. (1977). Relationship between the physicochemical nature of human interferon, the cell induced, and the inducing agent. *Antimicrobial Agents and Chemotherapy* 11, 291-298.

Chalmers, T.C. (1977). Randomize the first patient ! *New England Journal of Medicine* 296, 107.

Chany, C. (1976). Membrane-bound interferon specific cell receptor system: role in the establishment and amplification of the antiviral state. *Biomedicine* 24, 148-157.

Cheeseman, S.H., Rubin, R.H., Black, P.H., Cantell, K. & Hirsch, M.S. (1976). *Interferon Scientific Memoranda*. Buffalo, N.Y., Calspan Corp., November 1976.

Cheeseman, S.H., Rubin, R.H., Stewart, J.A., Tolkoff-Rubin, N.E., Cosimi, A.B., Cantell, K., Gilbert, J., Winkle, S., Herrin, J.T., Black, P.H., Russell, P.S. & Hirsch, M.S. (1979). Controlled clinical trial of prophylactic human-leukocyte interferon in renal transplantation. Effects on cytomegalovirus and herpes simplex virus infections. *New England Journal of Medicine* 300, 1345-1349.

Coleman, D.V., Gardner, S.D. & Field, A.M. (1973). Human polyomavirus infection in renal allograft recipients. *British Medical Journal* 3, 371-375.

Craighead, J.E., Hanshaw, J.B. & Carpenter, C.B. (1967). Cytomegalovirus infection after renal allotransplantation. *Journal of the American Medical Association* 201, 725-728.

Desmijter, J., De Groote, J., Desmet, V.J., Billiau, A., Ray, M.B., Bradburne, A.F., Edy, V.G., De Somer, P. & Mortelmans, J. (1976). Administration of human fibroblast interferon in chronic hepatitis-B

infection. *Lancet* 2, 645-647.

Dolen, J.G., Carter, W.A., Horoszewicz, J.S., Vladutiu, A.O., Leibowitz, A.I. & Nolan,J.P.(1979). Fibroblast interferon treatment of a patient with chronic active hepatitis. *American Journal of Medicine* 67, 127-131.

Duc-Goiran, P., Galliot, B. & Chany, C. (1971). Studies on virus-induced interferons produced by the human amniotic membrane and white blood cells. *Archiv für die gesamte Virusforschung* 34, 232-243.

Dunnick, J.K. & Galasso, G.J. (1979). Clinical trials with exogenous interferon: summary of a meeting. *Journal of Infectious Diseases* 139, 109-123.

Eddleston, A.L.W.F. (1974). The role of immunological mechanisms in acute and chronic hepatitis. *Postgraduate Medical Journal* 50,348-353.

Editorial (1976) Interferon therapy in chronic hepatitis B. *Lancet* II, 1122-1124.

Edy, V.G. (1977) Large scale production of human interferon in monolayer cell cultures. *Texas Reports on Biology and Medicine* 32, 132-137.

Edy, V.G., Billiau, A. & De Somer, P. (1976). Human fibroblast and leucocyte interferons show different dose-response curves in assay of cell protection. *Journal of General Virology* 31, 251-255.

Edy, V.G., Billiau, A. & De Somer, P. (1978). Non-appearance of injected fibroblast interferon in circulation. *Lancet* I, 451-452.

Edy, V.G., Braude, I.A., De Clercq, E., Billiau, A. & De Somer, P. (1976). Purification of interferon by adsorption chromatography on controlled pore glass. *Journal of General Virology* 33, 517-520.

Einhorn, S. & Strander, H. (1977). Is interferon tissue specific? Effect of human leucocyte and fibroblast interferons on the growth of lymphoblastoid and osteosarcoma cell lines. *Journal of General Virology* 35, 573-577.

Emödi, G., O'Reilly, R., Müller, A., Everson, L.K., Binschwanger, U. & Just, M. (1976). Effect of human exogenous leukocyte interferon in cytomegalovirus infections. *Journal of Infectious Diseases* 133 (suppl.), A199-204.

Emödi, G., Rufli, T., Just, M. & Hernandez, R. (1975). Human interferon

therapy for herpes zoster in adults. *Scandinavian Journal of Infectious Diseases* 7, 1-7.

Fiala, M., Payne, J.E., Berne, T.V., Moore, T.C., Henle, W., Montgomerie, J.Z., Chatterjee, S.N. & Guze, L.B. (1975). Epidemiology of cytomegalovirus infection after transplantation and immunosuppression. *Journal of Infectious Diseases* 132, 421-433.

Finlay, G.J., Booth, R.J. & Marbrook, J. (1977). Interferon-induced antibody suppression: a selective effect on high density, late responding precursor cells. *European Journal of Immunology* 7, 123-126.

Finter, N.B. (1964). Quantitative hemadsorption, a new assay technique. I. Assay of interferon. *Virology* 24, 589-597.

Finter, N.B. (1969). Dye uptake methods for assessing viral cytopathogenicity and their application to interferon assays. *Journal of General Virology* 5, 419-427.

Finter, N.B. (1973). Interferon and inducers *in vivo*: I. Antiviral effects in experimental animals. In: *Interferons and Interferon Inducers (Frontiers of Biology, vol. 2)*, ed. by N.B.Finter. Amsterdam, North-Holland Publishing Company, pp. 295-361.

Fournier, F., Chany, C. & Sarragne, M. (1972). Origin and possible mode of action of a tissue antagonist of interferon. *Nature* 235, 47-48.

Friedman, R.M. (1977). Antiviral activities of interferon. *Bacteriological Reviews* 3, 543-567.

Fuse, A. & Kuwata, T. (1976). Effects of interferon on the human clonal cell line, RSa: inhibition of macromolecular synthesis. *Journal of General Virology* 33, 17-24.

Gidlund, M., Orn, A., Wigzell, H., Senik, A. & Gresser, I. (1978). Enhanced NK cell activity in mice injected with interferon and interferon inducers. *Nature* 273, 759-761.

Greenberg, H.B., Pollard, R.B., Lutwick, L.I., Gregory, P.B., Robinson, W.B. & Merigan, T.C. (1976). Effect of human leucocyte interferon on hepatitis B virus infection in patients with chronic active hepatitis. *New England Journal of Medicine* 295, 517-522.

Greenberg, H.B., Robinson, W.S. & Merigan, T.C. (1979). Management of chronic hepatitis B: the use of antiviral agents. In: *Problems in Liver Diseases*, ed. by C.S.Davidson. Stuttgart, Thieme, pp.232-239.

Greenberg, P.L. & Mosny, S.A. (1977). Cytotoxic effects of interferon *in vitro* on granulocytic progenitor cells. *Cancer Research* 37, 1794-1799.

Gresser, I. (1977). On the varied biologic effects of interferon. *Cellular Immunology* 34, 406-415.

Gresser, I., Brouty-Boyé, D., Thomas, M.-T. & Macieira-Coelho, A. (1970). Interferon and cell division. I. Inhibition of the multiplication of mouse leukemia L1210 cells *in vitro* by interferon preparations. *Proceedings of the National Academy of Sciences* 66, 1052-1058.

Gresser, I., Maury, C. & Brouty-Boyé, D. (1972). Mechanism of anti-tumour effect of interferon in mice. *Nature* 239, 167-168.

Gresser, I. & Tovey, M.G. (1978). Antitumor effects of interferon. *Biochimica Biophysica Acta* 516, 231-247.

De Groote, J., Fevery, J. & Lepoutre, L. (1978). Long-term follow-up of chronic active hepatitis of moderate severity. *Gut* 19, 510-513.

Hallum, J.V. & Youngner, J.S. (1966). Quantitative aspects of inhibition of virus replication by interferon in chick embryo cell cultures. *Journal of Bacteriology* 92, 1047-1050.

Havell, E.A., Berman, B., Ogburn, C.A., Berg, K., Paucker, K. & Vilček, J. (1975). Two antigenically distinct species of human interferon. *Proceedings of the National Academy of Sciences* 72, 2185-2187.

Hekker, A.C., Bos, J.M. & Smith, L. (1973). A stable freeze-dried smallpox vaccine made in monolayer cultures of primary rabbit kidney cells. *Journal of Biological Standardization* 1, 21-32.

Ho, M. (1962). Kinetic considerations of the inhibitory action of an interferon produced in chick cultures infected with Sindbis virus. *Virology* 17, 262-275.

Ho, M. (1976). Interferon and hepatitis B virus. *New England Journal of Medicine* 295, 562-563.

Holmes, A.R., Rasmussen, L. & Merigan, T.C. (1978). Factors effecting the interferon sensitivity of human cytomegalovirus. *Intervirology* 9, 48-55.

Howard, C.R. (1978). The detection of DNA polymerase in the diagnosis of HBV infection. *Journal of Medical Virology* 3, 81-86.

Illinger, D., Coupin, G., Richards, M.& Poindron, P. (1976).Rat interferon inhibits steroid-inducible glycerol 3-phosphate dehydrogenase synthesis in a rat glial cell line. *FEBS Letters* 64, 391-395.

Isaacs, A. (1959). Action of interferon on the growth of sublethally irradiated virus. *Virology* 9, 56-61.

Isaacs, A. & Lindenmann, J. (1957). Viral interference. I. The interferon. *Proceedings of the Royal Society Series B* 147, 258-267.

Johnson, H.M. (1978). Differentiation of the immunosuppressive and antiviral effects of interferon. *Cellular Immunology* 36, 220-230.

Jones, B.R., Coster, D.J., Falcon, M.G. & Cantell, K. (1976). Topical therapy of ulcerative herpetic keratitis with human interferon. *Lancet* II, 128.

Kaplan, P.M., Greenman, R.L., Gerin, J.L., Purcell, R.H. & Robinson, W.S. (1973). DNA polymerase associated with human hepatitis B antigen. *Journal of Virology* 12, 995-1005.

Ke, Y.H., Armstrong, J.A., Breining, M.K., Ople, L., Postic, B. & Ho, M. (1970). The assay and standardization of rabbit interferon. In: *Proceedings of the International Symposium on Standardization of Interferon and Interferon Inducers, London, 1969 (Symposium Series in Immunobiological Standardization, vol. 14)*. Basel/New York, Karger, pp. 131-144.

Kingham, J.G.C., Ganguly, N.K., Shaari, Z.D., Mendelson, R., McGuire, M.J., Holgate, S.J., Cartwright, T., Scott, G.M., Richards, B.M. & Wright, R. (1978). Treatment of HBsAg-positive chronic active hepatitis with human fibroblast interferon. *Gut* 19, 91-94.

Knight, E. (1976). Antiviral and cell growth inhibitory activities reside in the same glycoprotein of human fibroblast interferon. *Nature* 262, 302-303.

Koranda, F.C., Dehmel, E.M., Kahn, G. & Penn, I. (1974). Cutaneous complications in immunosuppressed renal homograft recipients. *Journal of the American Medical Association* 229, 419-424.

Kuwata, T., Oda, T., Sekiya, S. & Morinaga, N. (1976). Characteristics of a human cell line successively transformed by Rous sarcoma virus and simian virus 40. *Journal of the National Cancer Institute* 56, 919-923.

Lecatsas, G., Prozesky, O.W., Van Wijk, J. & Elt, H.J. (1973). Papova virus in urine after renal transplantation. *Nature* 241, 343-344.

Lennette, E.H. (1969). Complement fixation test. In: *Diagnostic Procedures for Viral and Rickettsial Infections*, ed. by. E.H.Lennette & N.J.Schmidt. New York, American Public Health Association, p. 52.

Lindenmann, J. (1959). Interferon und inverse Interferenz. *Zeitschrift für Hygiene und Infektionskrankheiten* 146, 287-309.

Lindenmann, J. & Gifford, G.E. (1963). Studies on vaccinia virus plaque formation and its inhibition by interferon. *Virology* 19, 302-309.

Lobodzińska, M., Biernacka, I. & Skurska, Z. (1973). Rat interferons. I. Biological properties of interferons in rat serum. *Archivum Immunologiae et Therapiae Experimentalis* 21, 461-469.

Marcus, P.I. & Sekellick, M.J. (1976). Cell killing by viruses. III. The interferon system and inhibition of cell killing by vesicular stomatitis virus. *Virology* 69, 378-393.

Matas, A.J., Simmons, R.L. & Najarian, J.S. (1975). Chronic antigenic stimulation, herpesvirus infection, and cancer in transplant recipients. *Lancet* I, 1277-1279.

McLeod, D.L., Shreeve, M.M. & Axerad, A.A. (1974). Improved plasma culture system for production of erythrocytic colonies *in vitro*: quantitative assay method for CFU_e. *Blood* 44, 517-534.

McNeill, T.A. & Gresser, I. (1973). Inhibition of hemopoietic colony growth of interferon preparations from different sources. *Nature* 244, 173-174.

McNeill, T.A., Havredaki, M. & Fleming, W.A. (1975). Interferon and bone marrow colony stimulating factor. In: *Proceedings of a Meeting held at the Gubbendam Institute of Science, Oeivas, Portugal*, ed. by A.Geraldes. New York, Academic Press, pp.437-445.

Merigan, T.C., Rand, K.H., Pollard, R.B., Abdallah, P.S., Jordan, G.W. & Fried, R.P. (1978). Human leukocyte interferon for the treatment of herpes zoster in patients with cancer. *New England Journal of Medicine* 298, 981-987.

Neumann-Haefelin, D., Shrestha, B. & Manthey, K.F. (1976). Effective antiviral prophylaxis and therapy by systemic application of human interferon in immunosuppressed monkeys. *Journal of Infectious*

Diseases 133 (suppl.), A211-216.

Neumann-Haefelin, D., Sundmacher, R., Skoda, R. & Cantell, K. (1977). Comparative evaluation of human leukocyte and fibroblast interferon in the prevention of herpes simplex virus keratitis in a monkey model. *Infection and Immunity* 17, 468-470.

Nissen, C., Speck, B., Emödi, G. & Iscove, N.N. (1977). Toxicity of human interferon preparations in human bone marrow cultures. *Lancet* II, 203-204.

Oie, H.K., Easton, J.M., Abjasjo, D.V. & Baron, S. (1975). Murine cytomegalovirus: induction of and sensitivity to interferon *in vitro*. *Infection and Immunity* 12, 1012-1017.

O'Reilly, R.J., Everson, L.K., Emödi, G., Hansen, J., Smithwick, E.M., Grimes, E., Pahwa, S., Pahwa, R., Schwartz, S., Armstrong, D., Siegal, F.P., Gupta, S., Dupont, B. & Good, R.A. (1976). Effects of exogenous interferon in cytomegalovirus infections complicating bone marrow transplantation. *Clinical Immunology and Immunopathology* 6, 51-61.

Pass, R.F., Long, W.K., Whitley, R.J., Soong, S.J., Diethelm, A.G., Reynolds, D.W. & Alford, C.A. (1978). Productive infection with cytomegalovirus and herpes simplex virus in renal transplant recipients: role of source of kidney. *Journal of Infectious Diseases* 137, 556-563.

Paucker, K., Cantell, K. & Henle, W. (1962). Quantitative studies on viral interference in suspended L cells. III. Effects of interfering viruses and interferon on the growth rate of cells. *Virology* 17, 324-334.

Paucker, K., Dalton, B.J., Ogburn, C.A. & Törmä, E. (1975). Multiple active sites on human interferons. *Proceedings of the National Academy of Sciences* 72, 4587-4591.

Penn, I. (1975). The incidence of malignancies in transplant recipients. *Transplantation Proceedings* 7, 323-326.

Perrin, L.H., Tishon, A.& Oldstone, M.B.A. (1977). Immunologic injury in measles virus infection. III. Presence and characterization of human cytotoxic lymphocytes. *Journal of Immunology* 118, 282-290.

Pien, F.D., Smith, T.F., Andersen, C.F., Webel, M.L. & Taswell, H.F.

(1973). Herpesvirus in renal transplant recipients. *Transplantation* 16, 489-495.

Pike, B.L. & Robinson, W.A. (1970). Human bone marrow colony growth in agar gel. *Journal of Cellular Physiology* 76, 77-84.

Pinto, C.A., Haff, R.F., Valenta, J.R., Pagano, J.F., Dicuollo, C.J. & Ferlauto, R.J. (1970). Human interferon. II. Biological activity. In: *Proceedings of the International Symposium on Standardization of Interferon and Interferon Inducers, London, 1969 (Symposium Series in Immunobiological Standardization, vol. 14)*. Basel/New York, Karger, pp. 105-112.

Postic, B. & Dowling, J.N. (1977). Susceptibility of clinical isolates of cytomegalovirus to human interferon. *Antimicrobial Agents and Chemotherapy* 11, 656-660.

Rubin, R.H., Cosimi, A.B., Tolkoff-Rubin, N.E., Russell, P.S. & Hirsch, M.S. (1977). Infectious disease syndromes attributable to cytomegalovirus and their significance among renal transplant recipients. *Transplantation* 24, 458-465.

Rytel, M.W. & Balay, J. (1976). Cytomegalovirus infection in renal allograft recipients: assessment of the competence of humoral immunity. *Infection and Immunity* 13, 1633-1637.

Schalm, S.W., Summerskill, W.H.J., Gitnick, G.L. & Elveback, L.R. (1976). Contrasting features and responses to treatment of severe chronic active liver disease with and without hepatitis B_S antigen. *Gut* 17, 781-786.

Schellekens, H., Huffmeyer, J.H.P.M. & Van Griensven, L.J.L.D. (1975). The influence of emetine on the induction of interferon by poly-I: poly-C in Swiss mice. *Journal of General Virology* 26, 197-200.

Schonne, E. (1966). Properties of rat-tumor interferon. *Biochimica Biophysica Acta* 115, 429-439.

Schultz, R.M., Papamatheakis, J.D. & Chirigos, M.A. (1977). Interferon: an inducer of macrophage activation by polyanions. *Science* 197, 674-676.

Scientific Committee on Interferon (1970). Progress towards trials of human interferon in man. *Annals of the New York Academy of Sciences* 173, 770-781.

Sellers, R.F. & Fitzpatrick, M. (1962). An assay of interferon produced in rhesus monkey and calf kidney tissue culture using bovine enterovirus M6 as challenge. *British Journal of Experimental Pathology* 43, 674-683.

Shah, K.V., Daniel, R.W., Zeigel, R.F. & Murphy, G.P. (1974). Search for BK and SV40 virus reactivation in renal transplant recipients. *Transplantation* 17, 131-134.

Simmons, R.L., Lopez, C., Balfour, H. Jr., Kalis, J., Rattazzi, I., Najarian, J.S. (1974). Cytomegalovirus: clinical virological correlations in renal transplant recipients. *Annals of Surgery* 180, 623-632.

Sonnenfeld, G., Mandel, A.D. & Merigan, T.C. (1977). The immunosuppressive effect of type II mouse interferon preparations on antibody production. *Cellular Immunology* 34, 193-206.

Stebbing, N., Dawson, K.M. & Lindley, I.J.D. (1978). Requirements for macrophages for interferon to be effective against encephalomyocarditis virus infection of mice. *Infection and Immunity* 19, 5-11.

Stewart, W.E. (1979). *The Interferon System*. Wien/New York, Springer Verlag.

Stewart, W.E., Gresser, I., Tovey, M.G., Bandu, M. & Le Goff, S. (1976). Identification of the cell multiplication inhibitory factors in interferon preparations as interferons. *Nature* 262, 300-302.

Strander, H., Cantell, K., Carlström, G., Ingimarsson, S., Jakobsson, P. & Nilsonne, U. (1976). Acute infections in interferon-treated patients with osteosarcoma: preliminary report of a comparative study. *Journal of Infectious Diseases* 133 (suppl.), A245-248.

Strander, H., Cantell, K., Carlström, G. & Jakobsson, P. (1973). Clinical and laboratory investigations on man: systemic administration of potent interferon to man. *Journal of the National Cancer Institute* 51, 733-742.

Strauch, B., Siegel, N., Andrews, L.L. & Miller G. (1974). Oropharyngeal excretion of Epstein-Barr virus by renal transplant recipients and other patients treated with immunosuppressive drugs. *Lancet* I, 234-237.

Summerskill, W.H.J., Korman, M.G., Ammon, H.V. & Baggenstoss, A.H.

(1975). Prednisone for chronic active liver disease: dose titration, standard dose, and combination with azathioprine compared. *Gut* 16, 876-883.

Sundmacher, R., Cantell, K., Skoda, R., Hallermann, C. & Neumann-Haefelin, D. (1978). Human leukocyte and fibroblast interferon in a combination therapy of dendritic keratitis. *Albrecht von Graefes Archiv für klinische und experimentelle Ophthalmologie* 208, 229-233.

Thomas, E.D., Buckner, C.D., Banaji, M., Clift, R.A., Fefer, A., Flournoy, N., Goodell, B.W., Hickman, R.O., Lerner, K.G., Neiman, P.E., Sale, G.E., Sanders, J.E., Singer, J., Stevens, M., Storl, R. & Weiden, P.L. (1977). One hundred patients with acute leucemia treated by chemotherapy, total body irradiation and allogeneic marrow transplantation. *Blood* 49, 511-533.

Tilles, J.G. & Braun, P. (1973). Induction of interferon in perfused whole rat-lung cultures. *Proceedings of the Society for Experimental Biology and Medicine* 144, 460-463.

Trinchieri, G. & Santoli, D. (1978). Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. *Journal of Experimental Medicine* 147, 1314-1333.

Trinchieri, G., Santoli, D., Dee, R.R. & Knowles, B.B. (1978). Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Identification of the anti-viral activity as interferon and characterization of the human effector lymphocyte subpopulation. *Journal of Experimental Medicine* 147, 1299-1313.

Van der Waart, M., Snelting, A., Cichy, J., Wolters, G.& Schuurs, A. (1978). Enzyme-immunoassay in the diagnosis of hepatitis with emphasis on the detection of 'e' antigen (HBeAg). *Journal of Medical Virology* 3, 43-49.

Weimar, W. & Schellekens, H. (1979). Prophylaxis and therapy of HBsAg positive hepatitis. *Biomedicine* 30, 135-138.

Wiebe, M.E. & Joklik, W.K. (1975). The mechanism of inhibition of reovirus replication by interferon. *Virology* 66, 229-240.

Wiranowska-Stewart, M., Chudzio, T. & Stewart, W.E. (1977). Repeated 'superinduction' of interferon in human diploid fibroblast cultures. *Journal of General Virology* 37, 221-223.

Yamamoto, K.N., Yamaguchi, N. & Oda, K. (1975). Mechanism of interferon-induced inhibition of early simian virus 40 (SV 40) functions. *Virology* 68, 58-70.

Zuckerman, A.J. (1976). Interferon therapy for hepatitis. *Nature* 263, 640.

CURRICULUM VITAE H.SCHELLEKENS

H.Schellekens werd op 25 februari 1949 geboren te 's-Hertogenbosch. Hij behaalde in 1967 het Gymnasium B diploma aan het St. Janslyceum te 's-Hertogenbosch. Hij verrichte de medische studie aan de Medische Faculteit Rotterdam, later de Erasmus Universiteit Rotterdam. Hij was vanaf 1973 tot en met 1976 wetenschappelijk medewerker van de afdeling Pathologische Anatomie II van de Erasmus Universiteit. Hij volgde vanaf 1977 tot 1980 de opleiding tot specialist in de microbiologie in het Academisch Ziekenhuis Rotterdam-Dijkzigt. Hij is vanaf 1 februari 1980 als viroloog verbonden aan het Primatencentrum TNO te Rijswijk.

CURRICULUM VITAE W.WEIMAR

W.Weimar werd in 1947 in 's-Gravenhage geboren. In 1966 behaalde hij het Gymnasium B diploma aan het Gymnasium Haganum. Aansluitend studeerde hij medicijnen aan de Medische Faculteit Rotterdam, alwaar in 1972 het artsexamen werd afgelegd.

Na het vervullen van de militaire dienstplicht volgde de opleiding tot internist op de afdeling Inwendige Geneeskunde III (hoofd: Prof.Dr.J.C.Birkenhäger) van het Academisch Ziekenhuis Rotterdam-Dijkzigt. Vanaf 1979 is hij in dit ziekenhuis als internist werkzaam op de afdeling Nefrologie (hoofd: Dr.L.D.F.Lameijer).