https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

THE DISTRIBUTION OF INTERFERON-ALPHA IN NORMAL HUMAN TISSUES

By

NIZAM-UD-DIN KHAN

(M.B.B.S. UNIVERSITY OF PESHAWAR)

A thesis presented to the
Faculty of Medicine, University of Glasgow
for the degree of Ph.D

From

The University Division of Pathology,
Royal Infirmary,
Glasgow.

August 1990

© N.U.D. Khan 1990

ProQuest Number: 11007573

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 11007573

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

TABLE OF CONTENTS

CHAPTER FOUR

CHAPTER FIVE

CHAPTER SIX

LIST OF TABLES

LIST OF ILLUSTRATIONS

ACKNOWLEDGEMENTS

Firstly, I would like to thank the following doctors in the department for allowing me to use some of their collected tissues for study; McNicol A. M., Howatson A., Jackson R. and Stewart C.

I would also like to thank Dr. Angus Gibson for his advice and help in obtaining and assessing fetal tissues.

I am grateful to the staff of the labour ward at Glasgow Royal Maternity hospital, and the gynaecology department Glasgow Royal Infirmary for helping me to obtain fresh human placentas and amniotic fluids.

Although all the experimental work was carried out by me personally, I am indebted to Mrs. M.A. Farquharson for her advice and technical help in various immunocytochemical and *in situ* hybridization techniques and to Miss Alison Armstrong for her technical help in the radioimmunoassay techniques.

I would like to thank Dr. D. Carrington for his advice and supervision of the radioimmunoassay study, and Dr. P. Mackie for allowing me to carry out this study in his laboratory at Yorkhill hospital. The homogenization of tissues for the radioimmunoassay was carried out in the Institute of Biochemistry, Glasgow Royal Infirmary and I thank Dr. B. Cook for allowing me to work in his laboratory at the institute.

This work would have been impossible without the generous gift of commercially unavailable antisera. Dr. A. Meager (London) provided the H51 antiserum and Dr. K.A.F. Pulford (Oxford) the monoclonal KP1 antibody.

I thank Alfred Pauson for his help with statistical analysis of the data and Mrs. A. Lawrence for her advice on word processing which enabled me to type this thesis. I also thank Mrs. B. Burn for drawing some of the figures in this thesis.

I would like to express my gratitude to Dr. A.K. Foulis for his timely advice, dedicated supervision and constant encouragement that enabled me to complete this thesis.

I am also grateful to Professor Hugh Simpson for giving me the opportunity to work in his department.

Mrs. Isabel Main kindly typed the tables and legends of this thesis.

I thank the Ministry of Science & Technology Islamabad, Pakistan, for their financial support.

Special thanks to my mother and wife for their prayers, encouragement and support.

Finally I thank Allah for all that he has given me.

RELEVANT PUBLICATIONS

Khan N.U.D., Pulford K.A.F., Farquharson M.A., Howatson A., Stewart C., Jackson R., McNicol A.M. & Foulis A.K. (1989) The distribution of immunoreactive IFN-alpha in normal human tissues. *Immunology*, **66**, 201-206.

Khan N.U.D., Gibson A. & Foulis A.K. (1990) The distribution of immunoreactive interferon-alpha in formalin-fixed paraffin embedded normal human fetal and infant tissues. *Immunology*, (in press).

SUMMARY

The presence of interferon-alpha (IFN-alpha) in human tissues has been described extensively in viral infections. In the last decade many workers have also shown the presence of low levels of IFN-alpha in conditions other than viral infections. While the precise origin of the synthesis of low levels of IFN-alpha in these physiological conditions has not been clearly defined, some evidence has suggested that macrophages may be involved.

In an attempt to find the likely source of IFN-alpha in physiological conditions, an initial study was carried out in which the cellular distribution of immunoreactive IFN-alpha was studied in formalin fixed paraffin embedded normal adult human tissues from 38 different organs using various immunocytochemical techniques. These samples were drawn from over 300 individuals none of whom had evidence of viral infection. Tissue histiocytes from all organs in the body, with the exception of cerebral and cerebellar cortex in brain and renal cortex and medulla, stained positively for IFN-alpha. Kupffer cells, pulmonary alveolar macrophages and lymph node macrophages were also positive for IFN-alpha. Parenchymal cells in some other organs also contained immunoreactive IFN-alpha. These included syncytiotrophoblast in first, second and

third trimester placentas, cuboidal lining cells of the choroid plexus in the brain, thyroid follicullar cells, pituitary endocrine cells, adrenocortical cells and parathyroid endocrine cells. These findings are compatible with previous suggestions that IFN-alpha may be synthesized and released in the absence of viral infection and may have a role in normal physiology. The presence of IFN-alpha in most cells of the mononuclear phagocyte system suggests that these cells play a major role in the defence against viral infection. This speculation, however does not preclude other possible roles for IFN-alpha, such as modulation of cell growth, major histocompitability antigen expression etc.

The demonstration of immunoreactive IFN-alpha in formalin fixed paraffin embedded normal adult human tissues prompted other studies. In the first of these studies the cellular distribution of immunoreactive IFN-alpha was studied in formalin fixed paraffin embedded normal human autopsy tissues from 32 fetuses (7-42 weeks gestation) and 20 infants (aged from a few hours to 24 months). This study was performed to test the hypothesis that microbes have a role in switching on IFN-alpha synthesis. Fetal tissues are "germ free" while the infants had been exposed to a normal microbial flora. Immunoreactive IFN-alpha was first seen at 9 weeks gestation in macrophages in the fetal liver and thereafter was seen in macrophages in most

other organs except in kidneys and cerebral and cerebellar cortex. When infant lungs were compared with fetal lungs a statistically significant increase in the number of macrophages ($P<0.0001$, Mann-Whitney test) and the percentage of these cells expressing IFN-alpha ($P<0.0005$, Mann-Whitney test) was noted in infant lungs. No such changes were observed in spleen, liver and thymus following birth. These findings suggested that there is a basal level of IFN-alpha production by macrophages, which is not dependent on microbial products, but that such microbial products can enhance synthesis of this cytokine. Immunoreactive IFN-alpha was also demonstrated in parenchymal cells of thyroid gland, choroid plexus in brain, anterior pituitary gland and adrenal gland in the fetal and infant tissues. These findings were almost identical to those seen in adult tissues.

In a separate study an attempt was made to extract, detect and quantify IFN-alpha in human tissues using protein extraction and an immunoradiometric assay kit for the detection of IFN-alpha. 20 placentas (14-42 weeks gestation) obtained fresh within 1-2 hours of vaginal delivery, 4 specimens of amniotic fluid obtained at the time of caesarean section from 37-39 weeks gestation pregnancies, 10 samples of choroid plexus and cerebral cortex, 11 thyroid glands and 9 fetal adrenal glands from adult and fetal autopsies

performed within 10-24 hours of death were studied. IFN-alpha was detected in 9 placentas, 1 adult thyroid gland and all 4 amniotic fluids. However, this study failed to detect IFN-alpha in the remaining placentas and adult thyroid glands and in all choroid plexuses, cerebral cortex and fetal adrenal glands. Formalin fixed paraffin embedded tissues from these organs did show immunoreactive IFN-alpha in cells using the immunocytochemical techniques.

Finally an attempt was made to detect IFN-alpha messenger RNA (mRNA) in normal human tissues using an *in situ* hybridization method. Formalin fixed paraffin embedded normal human placenta (n=3), adult tonsil (5) and fetal spleen (3) were tested for the presence of IFN-alpha mRNA using two separate oligonucleotide probes to IFN-alpha mRNA. A poly-d-T probe (complementary to the poly-A-tail of mRNA molecules and which had been used previously by workers to demonstrate total mRNA in formalin fixed tissues) was used as a positive control in the *in situ* hybridization techniques. Although the study demonstrated mRNA in formalin fixed paraffin embedded normal human tissues using the poly-d-T probe, it failed to detect IFN-alpha mRNA with the two IFN-alpha oligonucleotide probes in the tissues studied. Some staining was observed with probe 1 in cells in the three fetal spleens and one tonsil. High power microscopy and various

immunocytochemical and immunofluorescence techniques helped to identify these cells as neutrophil polymorphs. This staining in neutrophil polymorphs was, however, inconsistent and ribonuclease enzyme treatment of tissue sections failed to abolish it and was therefore regarded as non-specific staining.

CHAPTER ONE

INTRODUCTION

1) GENERAL INTRODUCTION ON INTERFERON (IFN).

In 1957 Isaacs and Lindenmann identified a non-viral protein produced by cells in response to viral infection that could protect other cells of the same species from subsequent viral infection. They named this interfering protein **Interferon** (Isaacs & Lindenmann,1957).

Today interferon is identified as a group of proteins rather than a single protein. "To qualify as an interferon a factor must be a protein which exerts virus-nonspecific, antiviral activity at least in homologous cells through cellular metabolic processes involving synthesis of both RNA and protein" (Anonymous,1980).

In the current nomenclature interferons are classified into three distinct groups on the basis of antigenic specificities. They are interferon-alpha (IFN-alpha), interferon-beta (IFN-beta) and interferon-gamma (IFN-gamma).

In the old nomenclature interferons were also divided into three types, leucocyte interferon (IFN-alpha), fibroblast interferon (IFN-beta) and immune interferon (IFN-gamma). They were misnomers because both leucocytes and fibroblasts can produce IFN-alpha

and IFN-beta (Havell *et al.*,1978; & Hayes *et al.*,1979), while immune interferon is also induced by mitogens in addition to antigen induction. Another previous nomenclature divided interferons into only two types. a) Type 1, virus induced interferon (corresponding to IFN-alpha and IFN-beta). b) Type 2, antigen or mitogen induced interferon (IFN-gamma).

IFN-alpha.

A large number of genes coding for structurally different human IFN-alpha proteins have been identified. They are grouped into two subfamilies, IFN-alpha$_{I}$ and IFN-alpha$_{II}$. The IFN-alpha$_{I}$ subfamily has at least 14 potentially functional genes and one pseudogene, while the IFN-alpha$_{II}$ subfamily has only one functional gene, IFN-alpha$_{II}$ 1 and at least 7 pseudogenes. Two other pseudogenes IFN-alpha 11 and IFN-alpha 12 have also been described (Weissmann & Weber.,1986). The IFN-alpha$_{II}$ 1 gene is also called IFN-alpha omega 1 (Adolf,1990). The human IFN-alpha gene family is located on chromosome 9 (Slate *et al.*,1982). The IFN-alpha genes lack introns (non-coding sequences), they also lack N-glycosylation sites. IFN-alpha proteins are secreted as pre-IFNs containing a 23-amino acid residue leader sequence which is cleaved off during maturation. The mature IFN-alpha sequence of all subtypes contains 166 amino acids with the

exception of IFN-alpha 2, which contains only 165 aminoacids and IFN-alpha 1, which has 172 amino acids (Weissman & Weber.,1986). IFN-alpha molecules occur as pure proteins due to lack of N-glycosylation sites in the IFN-alpha genes as described above. It has a molecular weight of 19,000-24,000 daltons (Stanton *et al.*,1987). It is pH-2 (acid) stable except for one subtype which is pH-2 labile (Preble *et al.*,1982).

IFN-beta

Only a single gene coding for human IFN-beta(1) protein has been fully characterized. This is in sharp contrast to the many genes coding for IFN-alpha, but, like the IFN-alpha genes, this human IFN-beta gene is also located on the short arm of chromosome 9 and lacks introns (Trent, Olson & Lawn,1982). It codes for a 166 amino acid long peptide that is preceded by a 21 amino acid leader sequence. It has a molecular weight of 20,000 daltons. It is hydrophobic and occurs as a glycoprotein because the human IFN-beta gene contains an N-glycosylation site at position 180 (Derynck *et al.*,1980; & Houghton et al.,1980). The IFN-beta(1) has a 29% homology with IFN-alpha at the amino acid level and a 45% homology at the nucleotide level (Taniguchi *et al.*,1980).

A protein called IFN-beta(2) has also been described. The gene coding for this protein is not found on chromosome 9. It has a molecular weight of 23,700 daltons with 212 amino acids. It is neutralized by an antibody raised against IFN-beta(1) and shows antiviral activity that is considerably lower than IFN-beta(1) (Zilberstein *et al.*,1986; & Van-Damme *et al.*,1987). A gene coding for IFN-beta(2) has been located on chromosome 7 (Seghal *et al.*,1986). Its designation as an interferon is controversial (De-Maeyer & De-Maeyer Guignard,1988a).

IFN-gamma

IFN-gamma has also one gene, located on the long arm of chromosome 12 (Trent *et al.*,1982; Naylor *et al.*,1983). Unlike the IFN-alpha and IFN-beta genes, the IFN-gamma gene has 3 introns (Gray & Goeddel,1982). The molecular weight of IFN-gamma protein measured by molecular sieve chromatography ranges between 25,000-70,000 daltons (Falcoff,1972). However using sodium dodocyl sulphate polyacrylamide gel electrophoresis, Yip *et al* (1982) showed the presence of IFN-gamma molecules of two different molecular weights, 20,000 daltons & 25,000 daltons, respectively, and suggested that in native form the IFN-gamma protein may occur as a dimmer composed of 20,000 and 25,000 dalton subunits.

Caput *et al* (1986) identified a nucleotide base sequence TTATTTAT at the 3'non-coding region in the genes of various cytokines e.g tumour necrosis factor, interleukin 1, granulo-monocyte colony stimulating factor (GM-CSF), and most of the IFNs (including IFN-alpha, IFN-beta & IFN-gamma). It was also shown that introduction of a 51 nucleotide AT sequence (identical to that found in the human GM-CSF) into the 3'untranslated region of the rabbit beta-globulin gene, made the otherwise stable beta-globulin messenger RNA (mRNA) highly unstable. It was therefore suggested that this 3'non-coding TTATTTAT region of these genes contributes to the short half-life of cytokine mRNAs and serves as a logical point for post transcription regulation (Shaw & Kamen,1986).

Sources of IFNs.

IFN-alpha and IFN-beta.

Every cell in the vertebrate organism is said to be capable of producing IFN-alpha and IFN-beta. This has never actually been fully investigated for all different cell types that make up the vertebrate organism, but whenever differentiated cells like fibroblasts, macrophages, leucocytes and human and animal continuous cell lines have been examined, it has

been possible to induce IFN-alpha and IFN-beta production (De-Maeyer & De-Maeyer Guignard,1988b).

IFN-gamma.

IFN-gamma is produced by T lymphocytes activated by either antigens or mitogens. IFN-gamma is also probably produced by natural killer cells (Green, Cooperband & Kibrick,1969; Yip *et al.*,1982; Trinchieri & Perussia,1985).

Inducers of IFN synthesis.

IFN-alpha and IFN-beta.

1) Viruses. They were the first known inducers of IFN synthesis (Issacs & Lindenman,1967) and irrespective of their structure and mode of replication all viruses are capable of inducing IFN-alpha and IFN-beta synthesis.
2) Double stranded RNA (ds RNA). Both natural and synthetic dsRNAs induce IFN-alpha and IFN-beta with high efficiency (Field *et al.*,1967).
3) Micro-organisms. Bacteria; *Corynebacterium parvum, Brucella abortus* and *Listeria monocytogenes* can induce IFN-alpha and IFN-beta (Kirchner, Weyland & Storch,1986; Youngner & Stinebring,1964; Havell,1986). Lipopolysaccharides (endotoxins) derived from the walls of gram-ve bacteria can also induce IFN-alpha and beta (Gessani *et al.*,1987). Intracellular parasites like

rickettsiae induce acid stable type 1 interferons (Kohno *et al.*,1970). *Mycoplasma* under certain conditions can induce IFN-alpha (Birke *et al.*,1981). Detectable levels of IFN-alpha have been reported in the serum of children with acute *Plasmodium falciparum* infection (Ojo-Amaizi *et al.*,1981). *Toxoplasma gondii, Plasmodium berghei* and some strains of *Trypansoma cruzi* induce IFN-alpha and IFN-beta in mice (Freshman *et al.*,1966; Sonnenfeld and Kierszenbaum,1981).

4) Low molecular weight substances, such as cylcoheximides, kanamycin, toluidine blue and organic polymers such as pyran copolymers or polyvinylsulphate can also induce IFN-alpha and IFN-beta synthesis (Friedman & Vogal,1983; Stanton *et al.*,1987).

5) Cytokines. Interleukin 1 can stimulate the production of IFN-beta by fibroblasts (Billiau *et al.*,1986). It can also increase the production of interleukin 2 which in turn stimulates T lymphocytes' growth and IFN-gamma production (Vilcek *et al.*,1985). Interleukin 2 mainly regulates the synthesis of IFN-gamma (Kasahara *et al.*,1983), but can also induce IFN-alpha/beta in bone marrow cells (Reyes *et al.*,1986). IFN-gamma can also induce IFN-alpha synthesis (Hughes & Baron,1987). Other cytokines such as colony stimulating factor type 1, platlet derived growth factor and tumour necrosis factor can induce IFNs (Zullo *et al.*,1985; & Moore *et al.*,1984).

IFN-gamma.

The IFN-gamma inducers can be divided into two groups.

1) Mitogens.

2) Antigens.

1) Mitogens. Phytohemagglutinin and 12-0-tetradecanoylphorbol-13-acetate, potently induce T lymphocytes to produce IFN-gamma *in vitro* (Yip *et al.*,1982; Nathan *et al.*,1981; Falcoff,1972). Staphylococcal enterotoxin B also induces IFN-gamma synthesis in peripheral blood mononuclear cells *in vitro* (Lee *et al.*,1990).

Lipopolysaccharides (endotoxins) can stimulate IFN-gamma in murine T lymphocytes, if the T lymphocytes are first exposed to endogenously produced or exogenously applied interleukin 2 (Blanchard *et al.*,1986).

2) Antigens. IFN-gamma has been detected in the sera of immunized mice, after the administration of the immunizing antigen (Salvin, Youngner & Lederer,1973). It has also been detected in lymphoid cell cultures from immunized animals after exposure to the immunizing antigen (Gifford, Tibor & Peavy,1971). IFN-gamma synthesis by T lymphocytes in response to specific antigen stimulation is a clonally restricted process and only T lymphocytes previously sensitized to that antigen would produce IFN-gamma (Kiener &

Spitalny,1987). The antigen specific IFN-gamma synthesis by T lymphocytes is mainly dependent on antigen presenting or assessory cells, but can also take place even in the absence of these antigen presenting cells (De-Maeyer & De-Maeyer Guignard,1988c).

Actions of IFNs

1) The best known action of IFNs is their anti-viral activity (Isaacs & Lindenmann,1957). IFNs can inhibit attachment and uncoating of various viruses. They can also inhibit early viral transcription, viral translation, protein synthesis and budding from the cell surface (Balkwill,1989).

2) IFNs influence both cell growth and division (Paucker *et al.*,1962). The usual effect of IFNs is the inhibition of cellular replication and they act as mutual antagonists to growth factors (De-Maeyer & De-Maeyer Guignard,1988d). Tumour cell lines are more sensitive to the anti-proliferative effect of IFNs than normal fibroblasts (Paraf *et al.*,1983).

3) IFNs can reverse the phenotype of malignant fibroblast cells to a more normal phenotype (Sergiescu *et al.*,1986). They can also redirect other tumour cells to a more differentiated state (Exley *et al.*,1987) but occasionally high amounts of IFNs have the opposite

effect and block commitment to differentiation (Rossi,1985).

4) All three types of IFNs enhance the expression of class I major histocompatibility complex (MHC) antigens (Trinchieri & Perussia,1985). IFN-gamma induces or increases the expression of class II major histocompatibility complex antigens in a large number of cell types (Wong *et al.*,1983). IFN-alpha and IFN-beta mainly increase the expression of class I MHC antigens but can also stimulate the expression of class II MHC antigens on some lymphoid cells of B and T lineage (Capobianchi *et al.*,1985).

5) All interferons and especially IFN-gamma can cause stimulation of macrophages (Dean & Virelizier,1983; Nathan *et al.*,1983).

6) IFN-alpha and IFN-beta can also cause stimulation of natural killer cells (Gidlund *et al.*,1978).

Basis of action of IFNs.

IFNs interact with cells by binding to specific receptors present on the cell surface. Evidence for the existence of IFN receptors was first presented by Aguet(1980). IFN-alpha and beta share a common receptor (Branca & Baglioni,1981) while IFN-gamma uses a different receptor. Most studies so far have shown that IFN is internalized and degraded in much the same way as other polypeptide hormones. However internalization

is not an essential part of IFN's mechanism of action (Rubinstein & Orchansky,1986).

At least two enzymes have been discovered in humans whose levels are elevated by IFN treatment of cells: 1) (2'-5')A synthetase, which catalyses the synthesis of 2'-5' linked oligoadenylates and 2) A double-stranded, RNA dependent protein kinase. Detection of these enzymes has been used in the past to infer the presence of IFNs (Galabru *et al.*,1985). (2'-5')A synthetase can interact with double stranded RNA (dsRNA) to convert adenosine triose phosphate (ATP) into 2'-5'linked oligoadenylates. 2'-5'linked oligoadenylates can then activate an endonuclease that can cleave single stranded RNAs. This leads to degradation of messenger RNAs and ribosomal RNAs and causes irreversible translation inhibition. Activation of the dsRNA dependent protein kinase phosporylates the smallest subunit of the peptide chain initiation factor eIF-2 making it inactive. This results in inhibition of protein synthesis at the level of peptide chain initiation (Stanton *et al.*,1987).

Therapeutic uses of IFNs.

The high activity of several IFN preparations in treatment of hairy cell leukaemia has been confirmed in studies world-wide, with a response rate of 70-90%. IFN-alpha show some activity in multiple myeloma and

produces objective responses in patients with malignant endocrine pancreatic tumours, AIDS-related Kaposi's sarcoma, and in a small minority with renal cell carcinoma and melanoma (Balkwill,1989; Mandelli, Avvisati & Petrucci,1990). High activity of IFNs has also been reported in chronic myeloid leukaemia (Talpaz *et al.*,1987). IFN-alpha causes a rapid and selective lowering of platelet count in over 80% of patients with essential thrombocythaemia (Giles *et al.*,1988).

Due to their well known antiviral activity, IFNs have been tried clinically for treatment of the common cold. It has been shown that short term prophylaxis of family contacts of an infected individual reduced the risk of a rhinovirus cold by 78% but was not active against colds caused by other viruses (Douglas,1986) and that intranasal administration of IFN may control the spread of rhinovirus cold in some healthy contacts of ill family members (Hayden *et al.*,1986). However IFNs are ineffective in the treatment of symptomatic common cold patients (Balkwill,1989). It has been shown that administration of recombinant IFN-gamma prior to or at the time of lymphocytic choriomeningitis virus inoculation into mice, reduced significantly the virus titres in the spleens of the infected mice (Klavinskis, Geckeler & Oldstone,1989). Therapeutic injection of recombinant human IFN-alpha 2 directly into genital warts is an effective form of therapy for condyloma

acuminata (Eron *et al.*,1986). IFN-alpha has been shown to slow tumour growth rate in patients with laryngeal papillomatosis (Healy, Gelber & Trowbridge,1988). Clinical trials with IFNs in patients with chronic viral hepatitis B and C are currently been tried throughout the world (Ruiz-Moreno *et al.*,1990; Di-Bisceglie *et al.*,1989).

2) PHYSIOLOGICAL INTERFERON.

Since the discovery of IFN (Isaacs & Lindenmann,1957) much emphasis has been laid on the presence and role of IFN in viral infections. However in the last decade many workers have demonstrated the presence of IFNs in conditions other than viral infections and have used various words, such as physiological, preformed, spontaneous, endogenous, or constitutive IFN production to describe their findings.

The expression, "**physiological IFN response**" was first used by Bocci (1980). He put forward the idea that IFN was produced continuously in health in low physiological amounts which increased with an acute viral infection (Bocci,1981).

The most likely contenders for the production of low levels of physiological IFNs are cells of the mononuclear phagocyte system and lymphocytes. These cells may produce and release small amounts of IFNs into their surroundings (Bocci,1988). Evidence for this comes from some *in vitro* studies. It has been shown that normal rabbit and mouse peritoneal and alveolar macrophages (obtained from animals reared in conventional conditions but which were apparently pathogen free) immediately after isolation released low levels of IFNs in vitro (Smith & Wagner,1967; De-Maeyer, Fauve & De-Maeyer-Guignard,1971; Belardelli *et al.*,1987). De-Maeyer *et al* (1971) also showed that this

spontaneous release of IFN by macrophages in culture stops after 72 hours without further induction. The addition of lipopolysaccharides (endotoxin) or gram-ve bacteria to these macrophage cultures reinduced IFN production. Also, peritoneal macrophages obtained from mice reared in a germ free environment did not release detectable levels of IFN *in vitro*. Many animal viruses failed to multiply in fresh cultures of normal mouse peritoneal macrophages but the cells were rendered permissive by injection of mice with an antiserum directed against IFN-alpha and IFN-beta prior to harvesting, suggesting that peritoneal macrophages are maintained in an antiviral state by endogenous IFN *in vitro* (Belardelli *et al.*,1984). These findings raise the possibility that this spontaneous IFN production by macrophages is due to low levels of IFN inducers (bacteria and endotoxin) present under physiological conditions. Also, spontaneous production of IFN-gamma by T cells, newly isolated from the blood of adult and newborn humans, has been demonstrated without any antigen or mitogen stimulation *in vitro* (Martinez-Maza *et al.*,1984). This spontaneous release of IFN-gamma may be due to the T cells having been stimulated *in vivo*.

Human beings and animals are exposed to a variety of exogenous and endogenous microbial stimuli. A large number of bacteria colonize various parts of the human body such as the intestines, skin, genital tracts etc

to form the normal bacterial flora of the human body. Lipopolysaccharide (endotoxin), which is derived from the walls of gram-ve bacteria, is a good inducer of IFN-alpha and gamma (Kirchner *et al.*,1986; Blanchard *et al.*,1986). Man is also exposed to a large number of viruses and other substances in every day life. The human body exposed to these exogenous and endogenous stimuli may try to counteract them by producing various cytokines which help to maintain the animal in a state of health. Thus absorption of small amounts of endotoxin from the bacterial flora of the human body and exposure to low levels of viruses and other IFN inducers in the environment might be sufficient to provide a stimulus for low levels of IFN production by various cells like macrophages or lymphocytes. This IFN production would keep the cells in a primed state to combat any acute viral or bacterial infection and form the first line of defence. Certain sites of the human body have a high exposure to various exogenous and endogenous stimuli and are the most likely sites for physiological IFN production. These include the gut associated lymphoid tissue, duct associated lymphoid tissue, skin associated lymphoid tissue, immune associated lymphoid tissue (thymus, spleen & bone marrow), and the bronchoalveolar surface. Bocci *et al* (1984), showed the presence of IFN (IFN-gamma or an acid labile IFN-alpha or a mixture) in the lymph

draining the gastrointestinal tract of normal healthy rabbits. Some workers have reported low levels of IFN-alpha in normal human bone marrow (Zoumbas *et al.*,1985). An IFN-alpha like substance has been identified in the plasma of healthy individuals (Shiozawa *et al.*,1986) and IFN-alpha and IFN-gamma have been demonstrated in bronchoalveolar lavage fluid of healthy human volunteers (Prior & Haslam,1989).

In addition, recently, some conclusive evidence for the presence of immunoreactive-IFN-alpha in macrophages in conditions other than viral infections has emerged. Using an immunocytochemical technique in formalin fixed paraffin embedded normal human pancreas it was shown that cells with a similar morphology to macrophages contained immunoreactive-IFN-alpha (Foulis, Farquharson & Meager,1987). Using the above immunocytochemical method Howatson *et al* (1988) demonstrated immunoreactive-IFN-alpha in macrophages in human placenta and immunoreactive-IFN-alpha was shown in the Kupffer cells in normal human liver (Sutherland *et al.*,1989). These findings helped further to localize the likely source of physiological IFN synthesis.

Another important site where IFN-alpha has been demonstrated in physiological conditions is the placenta. Evidence for the presence of IFN in the placenta first emerged with the demonstration of IFN in mouse placenta (Fowler, Reed & Giron,1980). Later IFN

was demonstrated in the amniotic fluid in normal human pregnancy (Lebon *et al.*,1982), in human placenta, membranes and fetal blood (Duc-Goiran *et al.*,1985) and in perfused human term placentas (Bocci, Paulesu & Ricci,1985). Recently, IFN-alpha has been identified in normal fetal blood, fetal organs, placenta, membranes, amniotic fluid and decidua, using a specific two-site immunoradiometric assay (Chard *et al.*,1986). The ovine trophoblast antiluteolytic protein responsible for inhibiting corpus luteum regression in early pregnancy in sheep, shows a 30% sequence homology with human IFN-alpha protein (Stewart *et al.*,1987) and IFN-alpha 2 mimics some of the effects of ovine trophoblast antiluteolytic protein (Salamonsen *et al.*,1988). Finally immunoreactive-IFN-alpha has been localized to the syncytiotrophoblast of chorionic villi and to macrophages in human placentas (Howatson *et al.*,1988).

These studies, by showing the presence of immunoreactive-IFN-alpha in macrophages and other cells under physiological conditions, helped to pin-point the likely source of physiological IFN synthesis. However it is well known that cells, and especially macrophages, take up (phagocytoze) various proteins, foreign bodies, etc and thus the mere presence of IFN-alpha in cells does not provide conclusive evidence that these cells are actually producing IFN-alpha. It has been shown that IFN-alpha messenger RNA is present

constitutively in extracts of spleen, liver, kidney and peripheral blood leucocytes of normal human individuals (Tovey *et al.*,1987) proving that transcription of IFN-alpha genes is taking place under normal physiological conditions but this technique fails to define which cells are producing IFN-alpha at the time of the test.

Aims of the study

1) The first aim of the present study was to demonstrate cells containing immunoreactive-IFN-alpha in various normal human tissues. To achieve this aim an immunocytochemical technique using a sheep anti-human IFN-alpha antiserum (H51) was used to study the presence of immunoreactive IFN-alpha in a large number of formalin fixed paraffin embedded normal adult human tissues (Chapter Three). Alternative immunocytochemical techniques using a monoclonal anti-human IFN-alpha antibody and another polyclonal sheep anti-human IFN-alpha were also tried.
2) The second aim was to study the ontogeny of IFN-alpha in humans. This aim was achieved by studying the presence of IFN-alpha in formalin fixed normal human fetal tissues (Chapter four).
3) Also, as has been suggested above physiological IFN-alpha synthesis may be induced by the presence of low levels of various IFN inducers like

bacteria and viruses, present in the environment. Therefore the third aim of the study was to observe the effect of these physiological stimuli on IFN-alpha synthesis. To achieve this, the distributions of immunoreactive IFN-alpha containing cells in fetuses and infants was compared. It was expected that fetuses, which are essentially germ free, should have low levels of physiological IFN-alpha in their tissues compared to infants who are exposed to various exogenous stimuli. (Chapter four).

4) The fourth aim of the study was to show the presence of IFN-alpha in normal human tissues by a technique completely different from the immunocytochemical technique used by Foulis *et al* (1987). Chard *et al* (1986) had already demonstrated the presence of IFN-alpha in various fresh normal human fetal tissues, amniotic fluids and placentas, using a "Sucrosep" IFN-alpha two-site immunoradiometric assay. Therefore using the same "Sucrosep" IFN-alpha immunoradiometric assay an attempt was made to detect and quantitate IFN-alpha in some fresh normal human tissues (Chapter five). The detection of IFN-alpha in various fresh human tissues would provide additional support for previous findings of immunoreactive IFN-alpha in various formalin fixed normal human tissues and

strengthen the initial hypothesis of a low level synthesis of IFN-alpha under physiological conditions.

5) The final objective of the study was to demonstrate the presence of IFN-alpha messenger RNA (mRNA) in cells shown to contain immunoreactive IFN-alpha. This aim could be achieved by developing an *in situ* hybridization technique for the demonstration of IFN-alpha mRNA in formalin fixed normal human tissues. *In situ* hybridization is a technique used to demonstrate specific DNA or mRNA sequences in tissue sections or cytological preparations (Pardue,1985). It utilizes labelled nucleic acid probes which are complementary to the target DNA or mRNA sequences. These labelled probes can hybridize i.e form sequence specific, base paired duplexes with complementary DNA or mRNA sequence in tissue sections. The label is subsequently demonstrated, resulting in the precise cytological localization of the target sequence (Chapter six).

These studies would help define which cells normally produce IFN-alpha and give further insight into its physiological role in the human body.

CHAPTER TWO

MATERIALS AND METHODS.

MATERIALS.

They can be divided into 2 groups.

1) Human tissues.
2) Antibodies, immunoradiometric assay kit and probes used in the *in situ* hybridization study.

1) The human tissues studied can be further divided into three parts; a, b & c.

a) Normal human tissues from routine surgical specimens and autopsies collected over a period of 20 years were studied for the presence of immunoreactive IFN-alpha. As the study for the immunocytochemical localization of IFN-alpha in formalin fixed paraffin embedded normal human tissues was divided in 2 parts, described in chapter 3 and chapter 4, respectively, the tissues studied can also be divided into 2 groups.

i) Tissues used in chapter 3.

Normal adult human tissues studied for the immunocytochemical localization of IFN-alpha, described in chapter 3. These include tongue (n=3), salivary gland (6), oesophagus (11), stomach (11), duodenum (11), jejunum (10), appendix (10), large intestine

(10), liver (10), gallbladder (10), pancreas (8), adrenals (7), testes (10), ovaries of premenupausal non-pregnant women (10), ovaries of pregnant women (5), uterus (9), fallopian tubes (8), breast (10), cervix (10), skeletal muscle (10), myocardium (8), skin (8), bone marrow (10), spleen (9), lymph nodes (9), tonsil (8) kidney (6), ureters (8), renal pelvis (8), urinary bladder (3), prostate (9), bronchial tree (9), lungs & pleura (6), thyroid (8), pituitary (20), placenta (2 first timester, 5 second trimester and 5 third trimester), peripheral blood leucocytes (4), brain (5) and parathyroid (11). The peripheral blood leucocytes were studied in a clot formed from the buffy coats of 20 mls of blood from 4 human subjects, fixed in formol saline for 1 hour and embedded in paraffin wax. Normal human tissues collected from the routine surgical or autopsy specimens during this study period were fixed in formol saline for not more then 24-48 hours and embedded in paraffin wax. Cases from whom the specimens were collected did not have clinical evidence of any viral or bacterial infection.

ii) Tissues used in chapter 4.

Formalin fixed paraffin embedded normal human fetal tissues from 32 fetal autopsies performed within 24 hours of death were studied. These included 4 fetuses of 7-11 weeks gestation (removed accidently at curettage from women previously not known to be

pregnant), 20 fetuses of 12-25 weeks gestation (12 terminations of normal pregnancy, 5 hydrocephalics, 1 anencephalic and 2 fetuses with maternal placental complications) and 8 fetuses of 26-42 weeks gestation (stillbirths) with no evidence of viral or bacterial infection. Formalin fixed paraffin embedded normal tissues from autopsies on 20 infants aged from a few hours to 24 months were also studied for the immunocytochemical localization of IFN-alpha (chapter 4). These included 6 accidental deaths, 7 infants with congenital heart disease and 7 fatalities from sudden infant death syndrome. All showed no clinical or laboratory evidence of bacterial or viral infection.

b) Tissues used in chapter 5.

Various fresh human tissues and fluids were collected for the detection of IFN-alpha by the immunoradiometric assay. These included 15 placentas from selective terminations of pregnancy (14-18 weeks gestation), 5 placentas from normal vaginal deliveries (38-42 weeks gestation) and 4 specimens of amniotic fluid obtained at the time of caesarean section from 37-39 weeks gestation pregnancies. Also, 10 samples of choroid plexus and cerebral cortex, 11 thyroid glands and 9 fetal adrenal glands were obtained from adult and fetal autopsies performed within 10-24 hours of death. All 20 placentas were obtained fresh within 1-2 hours

of vaginal delivery. They were washed with running cold tap water to remove excess blood. The membranes were dissected off and small pieces of placental tissues were weighed and stored at -70°C. The choroid plexus and cerebral cortex samples, thyroid glands and fetal adrenal glands were snap frozen in liquid nitrogen and kept at -70°C. Small pieces of each such tissue from all cases were also fixed in formol saline for 24 hours and embedded in paraffin wax.

c) Tissues used in chapter 6.

Human tissues studied for the detection of IFN-alpha messenger RNA, using the *in situ* hybridization technique, included normal adult human tonsil (n=5), human placentas at 30-40 weeks gestation (3) and fetal spleen (3). The normal adult human tonsils were obtained fresh within half hour of surgical removal and the placentas were also obtained fresh within 1-2 hours of vaginal delivery. The fetal spleens were obtained from fetal autopsies performed within 24 hours of death. 2 spleens, 3 tonsils and 2 of the placentas studied, were fixed in buffered formalin for approximately 24 hours while 1 tonsil was fixed in buffered formalin for only 5 hours and embedded in paraffin wax. Finally 1 spleen, placenta and tonsil each, were fixed in formol saline instead of buffered formalin for 24 hours and embedded in paraffin wax.

2) Antibodies, immunoradiometric assay kit and probes used in the *in situ* hybridization study.

i) Antibodies.

Primary antibodies.

The polyclonal sheep anti-human IFN-alpha antiserum (H51). This antiserum was raised in sheep by Anthony Meager (National Institute for Biological Standards and Control, Potters Bar, Hertfordshire,UK) using human lymphoblastoid IFN-alpha Namalwa, "Wellferon" (Wellcome Research Laboratories, Beckenham,UK) as antigen. Wellferon was greater than 80% pure with respect to IFN protein. The sheep anti-human IFN-alpha antiserum (H51) neutralized all IFN-alpha preparations in a viral inhibition assay but also showed weak neutralization of purified human IFN-beta (Exley *et al.*,1984). The H51 antiserum did not neutralize IFN-gamma (Meager,1987).

Monoclonal anti-human IFN-alpha antibody (Code NO.0050). The Green Cross Corporation, Osaka 541, Japan.

Another polyclonal sheep anti-human IFN-alpha antiserum. Gift from Dr. Cantell, Helsinki.

KP1. This monoclonal antibody, raised against a lysosomal fraction of human lung macrophages, detected the monocyte/macrophages associated antigen CD68 in

routinely processed human tissue sections (tissues fixed in various fixatives, and embedded in paraffin wax). Gift from K.A.F Pulford, Oxford University, UK.

Mac 387. A monoclonal macrophage antibody (Dako, Roskilde, Denmark).

LN-26. A monoclonal B lymphocyte marker (Dako).

EMA. A monoclonal epithelial membrane antigen marker (Dako).

LeuM 1. A monoclonal antibody that detects Reed Sternberg cells and neutrophils (Dako).

Anti-muramidase. A rabbit polyclonal antiserum raised against the lysosomal fraction of human macrophages (Dako).

Anti-prekeratin. A rabbit polyclonal antiserum, directed against the cellular cytokeratin, called prekeratin (Dako).

The following secondary antisera were used in the study.

Peroxidase-conjugated swine anti-sheep immunoglobulin antiserum (Serotec, Oxford), peroxidase-conjugated swine anti-rabbit immunoglobulin antiserum (Dako), peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum (Dako), rhodamine-conjugated swine anti-rabbit immunoglobulin antiserum (Dako), rhodamine-conjugated rabbit anti-mouse immunoglobulin antiserum (Dako).

ii) The immunoradiometric assay kit.

A "Sucrosep" IFN-alpha immunoradiometric assay (Boots Celltech Diagnostics Ltd, Slough,UK) was used for the demonstration of IFN-alpha in human tissue homogenates and fluids. "Sucrosep" IFN-alpha assay is a two-site immunoradiometric assay which utilizes a ^{125}I labelled monoclonal antibody (designated Yok 5/19). The Yok 5/19 antibody recognizes at least 7 subtypes of IFN-alpha protein, namely IFN-alpha A(2), IFN-alpha C, IFN-alpha D(1), IFN-alpha G(5), IFN-alpha L, IFN-alpha J(7) and IFN-alpha K(6) (Data sheet, Celltech, culture product division,UK). The secondary antiserum in the "Sucrosep" IFN-alpha immunoradiometric assay kit was a sheep anti-human IFN-alpha antiserum attached to solid phase.

iii) Probes used in the *in situ* hybridization technique.

Probe 1.

Probe 1 was a 17 base oligonucleotide anti-sense IFN-alpha messenger RNA (mRNA) probe (Glasgow University, department of Biochemistry, Glasgow,UK). The nucleotide sequence of probe 1 was, 5'-C-C-T-C-C-C-A-G-G-C-A-C-A-A-G-G-G-3'. It was supplied as solution in ammonium hydroxide. The nucleotide sequence of probe

1 was based on selected conserved cDNA sequences of IFN-alpha genes A-H, position 481 to 497 (Goeddel *et al.*,1981). This probe had been used previously for the demonstration of the IFN-alpha WA gene (Torczynski, Fuke & Bollon,1984).

Probe 2.

Probe 2 was a 24 base oligonucleotide anti-sense IFN-alpha messenger RNA (mRNA) probe (Glasgow University, department of Biochemistry, Glasgow,UK). The nucleotide sequence of probe 2 was, 5'-G-A-T-C-T-C-A-T-G-A-T-T-T-C-T-G-C-T-C-T-G-A-C-A-3'. The nucleotide sequence of probe 2 was also based on selected conserved cDNA sequences of IFN-alpha genes A-H, position 499 to 502 (Goeddel *et al.*,1981). Probe 2 was supplied in pure solid form, with an optical density of 3.6.

Poly-d-T probe.

Poly-d-T (poly-deoxythymidylic acid) oligonucleotide probe (Pharmacia LKB Biotechnology, Central Milton Keynes,UK) was 20 deoxythymidylic acid bases (T-T-T etc) long. It is complementary to the poly-A tail (A-A-A etc) of mRNA molecules. It was supplied in pure form ready to use. The poly-d-T probe had been used by previous workers to demonstrate total mRNA in formalin fixed human tissues by *in situ*

hybridization (Pringle *et al.*,1989). In this study it was used as a control to demonstrate total mRNA in tissue sections from organs that were also used for the detection of IFN-alpha mRNA with probe 1 and probe 2 in the *in situ* hybridization technique.

METHODS

The methodology is divided into six main groups comprising various techniques used throughout the study. They are as follow.

A) Immunocytochemical techniques used for the detection of immunoreactive-IFN-alpha in formalin fixed paraffin embedded normal human tissues. They are again subgrouped into two.

i) Indirect immunoperoxidase technique used for the demonstration of immunoreactive-IFN-alpha using the polyclonal sheep anti-human IFN-alpha antiserum (H51).

ii) Immunocytochemical techniques used for the detection of IFN-alpha using a monoclonal antibody (Code NO.0050) and another polyclonal anti-human IFN-alpha antiserum beside H51 (gift from Dr. Cantell, Helsinki).

B) Indirect immunoperoxidase immunocytochemical techniques using various antibodies against macrophages, neutrophils and epithelial antigens.

C) Serial section technique. This method was used to study co-localization of different antigens in the same cell and thus helped to identify which cells contained immunoreactive-IFN-alpha.

D) Double staining technique. This technique was initially developed to identify which cells contained immunoreactive-IFN-alpha in formalin fixed normal human tissues. It was also used later to identify cells in the *in situ* hybridization technique. It utilized an indirect immunoperoxidase or an *in situ* hybridization technique in step one and an indirect immunofluorescence method in step two.

E) Immunoradiometric assay "Sucrosep" IFN-alpha (Boots Celltech,UK) for the detection of IFN-alpha in tissue homogenates and fluids.

F) *In situ* hybridization for the detection of IFN-alpha mRNA in formalin fixed paraffin embedded normal human tissues.

A) PRINCIPLE OF INDIRECT IMMUNOPEROXIDASE TECHNIQUE.

In this technique an unlabelled primary antibody binds to the antigen. The primary antibody is then identified by a horseradish peroxidase conjugated secondary antibody raised to the immunoglobulins of the species providing the primary antibody. The horseradish peroxidase enzyme is isolated from the root of the horseradish plant and is easily attached covalently or noncovalently to other proteins. It oxidizes several substances and can form coloured enzyme-substrate complexes. In this study 3'diaminobenzidine tetrahydrochloride (DAB) was used as a substrate (Appendix). It is converted by horseradish peroxidase into a brown product that is highly insoluble in alcohol and other organic solvents.

A(i) INDIRECT IMMUNOPEROXIDASE TECHNIQUE USED FOR THE DEMONSTRATION OF IMMUNOREACTIVE-IFN-ALPHA IN FORMALIN FIXED PARAFFIN EMBEDDED NORMAL HUMAN TISSUE USING THE POLYCLONAL SHEEP ANTI-HUMAN IFN-ALPHA ANTISERUM (H51).

The sheep anti-human IFN-alpha antiserum (H51) was supplied as neat serum. Normal sheep serum shows unacceptable nonspecific staining in immunocytochemical techniques on human tissues. Therefore prior to its use in the indirect immunoperoxidase technique the H51

antiserum had to be absorbed to avoid background or unwanted staining.

Protocol for the absorption of sheep anti-human IFN-alpha antiserum (H51).

1) A mixture of guinea pig and porcine liver powders (both from Sigma,UK) was used. Half an ml of each liver powder was put into a small test tube.
2) The powders were then washed with tris buffered saline (0.01M Tris HCL, pH 7.6, 0.15M sodium chloride). 3 mls of tris buffered saline was added to the tubes containing the powder and mixed well.
3) The tubes were then centrifuged at 1500g at room temperature for 5 minutes.
4) The supernatant fluid was discarded and the liver powder pellets resuspended in 3 mls of tris buffered saline and mixed well. The above procedure was repeated at least 3 times.
5) Then 1 ml of the sheep anti-human IFN-alpha antiserum (H51) (in 1/50 dilution, in tris buffered saline) was added to the washed liver powders and mixed well. It was left at $4^{o}C$ for 5 hours (occasionally mixed).
6) After 5 hours the tubes containing the mixture were centrifuged again at 1500g for 5 minutes and the supernatant antiserum was collected. It was

again added to freshly washed liver powders, mixed and left overnight at 4°C.

7) Next morning the tubes were spun at 1500g for 5 minutes, the supernatant antiserum was carefully collected and the liver powder pellets discarded. The H51 antiserum supernatant was again centrifuged for 5 minutes at 1500g to get rid of any traces of liver powder and finally the absorbed supernatant H51 antiserum obtained was ready for use in the indirect immunoperoxidase technique.

Coating of glass slides.

This was done to prevent cut sections floating off the slides during the immunocytochemical techniques.

Two methods were used

a) Poly-L-lysine (Sigma, Poole, Dorset,UK) coating.

b) 3-aminopropyltriethoxysilane (Sigma) coating.

a) Poly-L-lysine coating. A drop of poly-L-lyine was applied on the marked side of the prewashed glass slides and spread over the slide to from a thin film. It was left to dry for half an hour before use.

b) 3-aminopropyltriethoxysilane coating. Prewashed glass slides were put in 2% Decon 75 (Decon Lab;

Hove,UK) and left overnight. Next morning they were washed in running tap water for 1 hour. The slides were put first into acetone (BDH, Poole,UK) for 5 minutes and then into 2% 3-aminopropyltriethoxysilane in acetone for 5 minutes. This was followed by washing the slides in tap water for 1 hour and leaving them to dry. Once dried the slides could be stored for months before use.

Standard protocol for the indirect immunoperoxidase technique used for the demonstration of immunoreactive-IFN-alpha in formalin fixed paraffin embedded normal human tissue using the polyclonal sheep anti-human IFN-alpha antiserum (H51).

Steps.

1) 4 micron thick sections were cut from formol saline or buffered formalin fixed, paraffin wax embedded human tissues. The sections, mounted on poly-l-lysine or 3-aminopropyltriethoxysilane coated glass slides, were placed in two changes of xylene for 5 minutes each, to remove the paraffin wax. They were then placed in two changes of absolute alcohol and one change of methyl alcohol for 5 minutes each and rinsed in tap water.

2) The slides were washed in tris buffered saline for 10 minutes.

3) To remove the endogenous peroxidase activity in the tissue sections the slides were placed in 3% hydrogen peroxide (Evans Medical ltd, Langhurst, Horsham, UK) for 10 minutes.

4) Normal swine serum (Gibco,Paisley,UK) diluted 1/5 in tris buffered saline was applied to the tissue sections for 15 minutes.

5) Excess of normal swine serum was removed from the sections. The primary sheep anti-human IFN-alpha antiserum (H51), diluted 1/100 (in 50% normal human serum [Biogenesis, Bournemouth,UK] in tris buffered saline) was then applied on the tissue sections and left overnight at $4^{o}C$.

6) Next morning the slides were washed twice in tris buffered saline for 10 minutes.

7) Normal swine serum diluted 1/5 in tris buffered saline was then placed on the slides and left for 15 minutes (the secondary antiserum was a swine anti-sheep immunoglobulin antiserum and adding normal swine serum at this stage avoided non-specific sticking of the secondary antiserum to the tissue sections).

8) Excess of normal swine serum was removed and horseradish peroxidase conjugated swine anti-sheep immunoglobulin antiserum diluted 1/200 (in 20%

normal human serum in tris buffered saline), was added to the sections for 30 minutes.

9) The sections were washed twice in tris buffered saline for 10 minutes each time.

10) The slides were placed in DAB substrate solution (Appendix) for 10 minutes.

11) The sections were then washed in running tap water and counterstained with haematoxylin for 30 seconds. They were then rinsed in tap water and placed in acid alcohol (1% HCl in methyl alcohol) for a few seconds, rinsed again in tap water and placed in Scotts water (alkaline pH water) until blue. They were checked under a microscope at this stage for the degree of counterstaining and rinsed in water.

12) The slides were then placed in methyl alcohol for five minutes, in absolute alcohol (two changes) five minutes each and in xylene (two changes) for five minutes each. Finally they were mounted in a fixed mounting medium (Harleco synthetic resin, Kodak,UK) and visualized under a Leitz Laborlux light microscope.

Different batches of the secondary peroxidase conjugated swine anti-sheep immunoglobulin antiserum were used in the study. They showed a slight variation in staining and each time a new batch of the secondary

antiserum was used, the optimum dilution had to be worked out for that antiserum.

Evaluation of the specificity of the sheep anti-human IFN-alpha antiserum (H51).

a) Neutralization/blocking of the H51 antiserum with Wellferon (human lymphoblastoid Namalwa, IFN-alpha).

Protocol

1) The same procedure was used for the absorption of the H51 antiserum by the liver powders as that described earlier in section A (i), except that it was absorbed in a 1/20 dilution instead of a 1/50 dilution in tris buffered saline.
2) Two tubes were labelled A and B. To tube A was added 40 microlitres of the absorbed H51 antiserum in a 1/20 dilution + 30 microlitres of normal human serum + 130 microlitres (1300 mega units) of Wellferon.
3) To tube B was added 40 microlitres of the absorbed H51 antiserum in 1/20 dilution + 30 microlitres of normal human serum + 130 microlitres of tris buffered saline.
4) Both the tubes were left overnight at $4^{o}C$.

5) Next morning two sets of tissue sections on glass slides were set up for the indirect immunoperoxidase staining for IFN-alpha. On one set the neutralized H51 antiserum from tube A was used. The contents of tube B were used as a positive control on the other set.

6) The standard protocol for the indirect immunoperoxidase staining for IFN-alpha was then used.

The neutralization/blocking experiment was carried out on at least one section of all human tissues studied.

b) Negative Control.

Normal sheep serum (Scottish antibody production unit, Law hospital, Lanarkshire,Scotland), absorbed in the same way by liver powders as that described for H51 antiserum, was substituted for H51 antiserum in the neutralization/ blocking experiment with Wellferon as described above. The remaining procedure was the same as that for the H51 antiserum.

c) Positive Control.

Two tissues were used as positive controls.

1) Tissue sections from two human pancreases from patients with chronic pancreatitis, previously shown to have spindle shaped cells consistently

staining positively for immunoreactive IFN-alpha, were used as positive controls (Foulis *et al.*,1987).

2) Tissue sections from human placentas, previously shown to contain immunoreactive IFN-alpha in the syncytiotrophoblast and macrophages, were also used as positive controls (Howatson *et al.*,1988).

d) Assessment of cross reactivity of the H51 antiserum.

As stated before, in a viral inhibition assay the sheep anti-human IFN-alpha (H51) antiserum showed a weak neutralization of purified human IFN-beta (Exley *et al.*,1987). Therefore it was important to assess any cross reactivity of sheep anti-human IFN-alpha antiserum (H51) with IFN-beta before use in the indirect immunoperoxidase technique.

Two methods were adopted.

i) Neutralization/ blocking of the H51 antiserum with recombinant IFN-beta (Triton, Biosciences, CA,USA).

ii) Immunoblots.

i) Neutralization/ blocking of the H51 antiserum with recombinant IFN-beta.

The same procedure was used as that described earlier for the neutralization/ blocking experiment of the H51 antiserum with Wellferon except that the tris buffered saline in tube B in step 2 was replaced by 130 microlitres of recombinant IFN-beta (approximately 12,400 units).

ii) Immunoblots.

This technique was adopted as a quick means of excluding cross reactions of the H51 antiserum with IFN-beta and some other proteins and sera.

Protocol

1) A strip of nitrocellulose paper (Gelmen Sciences Inc, Ann Arbor, MI,USA) was marked into small sections and one edge cut as an identification mark.
2) The strip was washed in tris buffered saline and left to dry for a few minutes.
3) 2 microlitres of each of the following were spotted on the nitrocellulose paper strip in the centre of each section: normal sheep serum (Law hospital,Scotland), normal human serum (Biogenisis,UK), tumour necrosis factor (Genentech, San Francisco,USA), Wellferon (IFN-

alpha), IFN-beta (Triton, Biosciences, CA,USA) and interleukin 1-1 alpha and interleukin 1-1 beta (both from National Institute for Biological Standards and Control, Potters Bar,UK), tris HCL buffer,pH 7.6. The exact location of various substances on the nitrocellulose paper (the cut edge is used as a marker) was noted on a separate piece of paper.

4) The nitrocelluse paper was left to dry at room temperature for one hour.

5) The strip was treated with 3% bovine serum albumin in tris buffered saline for 2 hours. This helped to decrease the background staining.

6) The strip was washed twice in tris buffered saline for 10 minutes.

7) The nitrocellulose paper was then sealed in a plastic bag with 2 mls of absorbed H51 antiserum diluted 1/100 (in 50% normal human serum in tris buffered saline), and left overnight at $4^{o}C$.

8) Next morning the nitrocellulose paper was washed in 3 changes of tris buffered saline for 15 minutes.

9) It was then sealed in a fresh plastic bag containing 2 mls of the horseradish peroxidase conjugated swine anti-sheep immunoglobulin antiserum diluted 1/200 (in 20% normal human serum

in tris buffered saline). It was left at room temperature for 30 minutes.

10) It was then washed in tris buffered saline for 15 minutes, changing the fluid three times.

11) The nitrocelluse paper was then placed in the DAB substrate solution (Appendix) for 10 minutes.

12) It was finally washed well in tap water and left to dry at room temperature before being sealed in a plastic bag.

Aii) IMMUNOCYTOCHEMICAL TECHNIQUES USING OTHER PRIMARY ANTISERA TO IFN-ALPHA.

Two immunocytochemical technique were used.

1) Avidin-biotin immunocytochemical technique.

2) Indirect immunoperoxidase technique.

1) Protocol for the avidin-biotin immunocytochemical technique used for the demonstration of immunoreactive-IFN-alpha in formalin fixed paraffin embedded normal human tissues using a monoclonal anti-human IFN-alpha antibody (Code No.0050).

Steps.

1) The first 4 steps of the protocol were the same as in the protocol of the indirect immunoperoxidase

technique used for the demonstration of immunoreactive-IFN-alpha using the polyclonal sheep H51 antiserum.

2) Normal horse serum (Vector lab; Bretton, Peterborough,UK) [diluted 1 drop in 3 mls of tris buffered saline] was added to the tissue sections for 15 minutes, blocking non-specific sites on the tissue sections.

3) Excess of the normal horse serum was removed from the sections. The primary monoclonal anti-human IFN-alpha antibody (Code No.0050) diluted 1/100 in tris buffered saline, was left on the sections for 2 hours at room temperature.

4) The slides were washed in tris buffered saline for 10 minutes, changing twice.

5) The secondary antiserum, biotinylated horse anti-mouse immunoglobulins (Vector lab; UK), diluted 1/100 in 20% normal human serum in tris buffered saline, was placed on the sections for 30 minutes.

6) The sections were than washed in tris buffered saline for 10 minutes, changing twice.

7) A horseradish peroxidase conjugated Vectastain ABC reagent (Vector Lab. U.K) [Appendix] was applied to the sections for 1 hour at room temperature.

8) The sections were washed in tris buffered saline for 10 minutes, changing twice.

9) The slides were placed in a freshly prepared DAB substrate solution for 10 minutes.

10) The slides were washed, counterstained and mounted in a fixed mounting medium as described in the protocol of the indirect immunoperoxidase technique used for the demonstration of immunoreactive-IFN-alpha with the H51 antiserum.

Amendments to the above protocol.

i) Effect of trypsinization.

After step 4 the tissue sections were trypsinized (Appendix) for 10 minutes. The procedure was identical thereafter.

ii) Varying the primary antibody (Step 2).

Variation(a). The primary antibody diluted 1/100 was left overnight on the sections at 4^{o}C instead of 2 hours incubation at room temperature.

Variation(b). The primary antibody was used in 1/5, 1/10, 1/20, 1/50, 1/200 dilutions on consecutive serial sections of the same normal human tissue.

iii) Varying the secondary antiserum (Step 5).

Variation(a). The secondary antiserum, biotinylated horse anti-mouse immunoglobulins antiserum was left on for 1 hour and 2 hours on consecutive serial sections of the same normal human tissue at room temperature .

Variation(b). The secondary antiserum was used in 1/50 dilution.

iv) Varying the biotin detection system (Step 7).

Instead of the Vectastain ABC reagent, an alkaline phosphatase conjugated Streptavidin-biotin complex (Appendix) was applied to the sections for 1 hour. The sections were treated with an alkaline phosphatase substrate solution, Fast Red (Appendix), for 10 minutes. The sections were then counterstained in haematoxylin for 10 seconds, washed in tap water and mounted in Glycergel (water mounting medium) (Dako).

2) Indirect immunoperoxidase technique using the above monoclonal antibody.

Protocol

1) The first 4 steps of the protocol were the same as the protocol for the indirect immunoperoxidase technique used for the demonstration of immunoreactive-IFN-alpha using the H51 antiserum, described in section A(i).
2) The primary monoclonal anti-human IFN-alpha antibody (Code No.0050) diluted 1/100 in tris buffered saline was then applied to the sections and left for 2 hours at room temperature.
3) The slides were washed in tris buffered saline for 10 minutes.

4) A horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin antiserum diluted 1/50 (in 20% normal human serum in tris buffer saline) was applied to the sections and left for 30 minutes.
5) Sections were washed in tris buffered saline for 10 minutes, changing twice.
6) The slides were placed in DAB substrate solution for 10 minutes.
7) Sections were counterstained and mounted in a fixed mounting medium as described in the protocol of the indirect immunoperoxidase technique using H51 antiserum.

Negative Control.

Throughout the above avidin-biotin and indirect immunocytochemical techniques, a monolonal antibody, Clonab-LNC raised against an irrelevant antigen, (BIOTEST Serum Institute, West Germany) was applied on at least one section of each tissue as a negative control to exclude any non-specific staining of the monoclonal antibody.

Positive Control.

Two monoclonal antibodies, LN26 and EMA were used as positive controls. LN26 stains B lymphocytes and EMA antibody stains epithelial membrane antigens in formalin fixed human tissues. Sections from a human

lymph node (having abundant B lymphocytes) and breast tissue were used as control slides for LN26 and EMA antibody respectively. The protocol used for LN26 and EMA antibodies was the same in every respect to the protocols of the avidin-biotin immunocytochemical technique and the indirect immunoperoxidase technique using the monoclonal anti-human IFN-alpha antibody(Code No.0050).

The protocol used for the detection of immunoreactive IFN-alpha, using the polyclonal sheep anti-human IFN-alpha antiserum (Dr Cantell) was the same as the protocol for the polyclonal sheep anti-human IFN-alpha antiserum (H51).

B) INDIRECT IMMUNOPEROXIDASE IMMUNOCYTOCHEMICAL TECHNIQUES USING VARIOUS ANTIBODIES DIRECTED AGAINST MACROPHAGES, NEUTROPHILS AND EPITHELIAL ANTIGENS.

Two monoclonal antibodies, KP1 and Mac 387, and a polyclonal anti-muramidase antiserum were used throughout the study for the demonstration of macrophages. A neutrophil marker, LeuM 1 monoclonal antibody was used to identify cells in tissue sections during the *in situ* hybridization technique. A polyclonal anti-prekeratin antiserum (epithelial cell marker) was used to identify cells staining positively for immunoreactive IFN-alpha in human fetal thymus.

Protocol for the monoclonal antibodies, KP1, Mac 387 and LeuM 1 using an indirect immunoperoxidase technique.

The same protocol was used as that described for the monoclonal anti-human IFN-alpha antibody in section A(ii)2 with the following variations.

Trypsinization.

The tissue sections were trypsinized for 10 minutes prior to application of all three monoclonal antibodies.

Variation in primary antibodies.

The KP1 antibody was used at a 1/10 dilution and left on the sections overnight at $4^{o}C$.

The Mac 387 antibody was used at a 1/20 dilution and left on the sections for 2 hours at room temperature.

The LeuM 1 antibody was used at a 1/50 dilution and left on the sections for 2 hours at room temperature.

All the monoclonal antibodies were diluted in tris buffered saline.

Amendment to the staining protocol for KP1 antibody.

The tissue sections were trypsinized for 20 minutes instead of 10 minutes. The remaining procedure was the same.

Absorption of the KP1 antibody with Wellferon.

This procedure was performed to exclude the possibility of cross-reaction between KP1 and IFN-alpha.

Two tubes were taken marked A and B. 20 microlitres of KP1 antibody was put in tube A and 200 microlitres of Wellferon (2000 mega units) was added to the KP1 in tube A. In tube B, 20 microlitres of KP1 antibody was placed and 200 microlitres of tris buffered saline was added. The content of tube B were used as positive control. Both tubes A & B were left overnight at $4^{o}C$. Next morning the absorbed and the positive control unabsorbed KP1 antibody were used to stain slides as described above in the protocol for the KP1 antibody.

Protocol for the indirect immunoperoxidase technique using the polyclonal anti-muramidase and anti-prekeratin antisera.

The same protocol was used as that of the indirect immunoperoxidase technique using the H51 antiserum described in section A(i), with the following variations.

Trypsinization.

After step 3 the sections were trypsinized for 20 minutes.

Variation in primary antiserum.

The rabbit anti-muramidase antiserum was applied to the sections at a 1/200 dilution and left on at room temperature for 1 hour.

The rabbit anti-prekeratin antiserum was applied to the sections at a 1/200 dilution for 30 minutes at room temperature.

Both the antisera were diluted in tris buffered saline.

Variation in secondary antiserum.

Horseradish peroxidase conjugated swine anti-rabbit immunoglobulin antiserum was used at 1/50 dilution in 20% normal human serum in tris buffered saline, for 30 minutes.

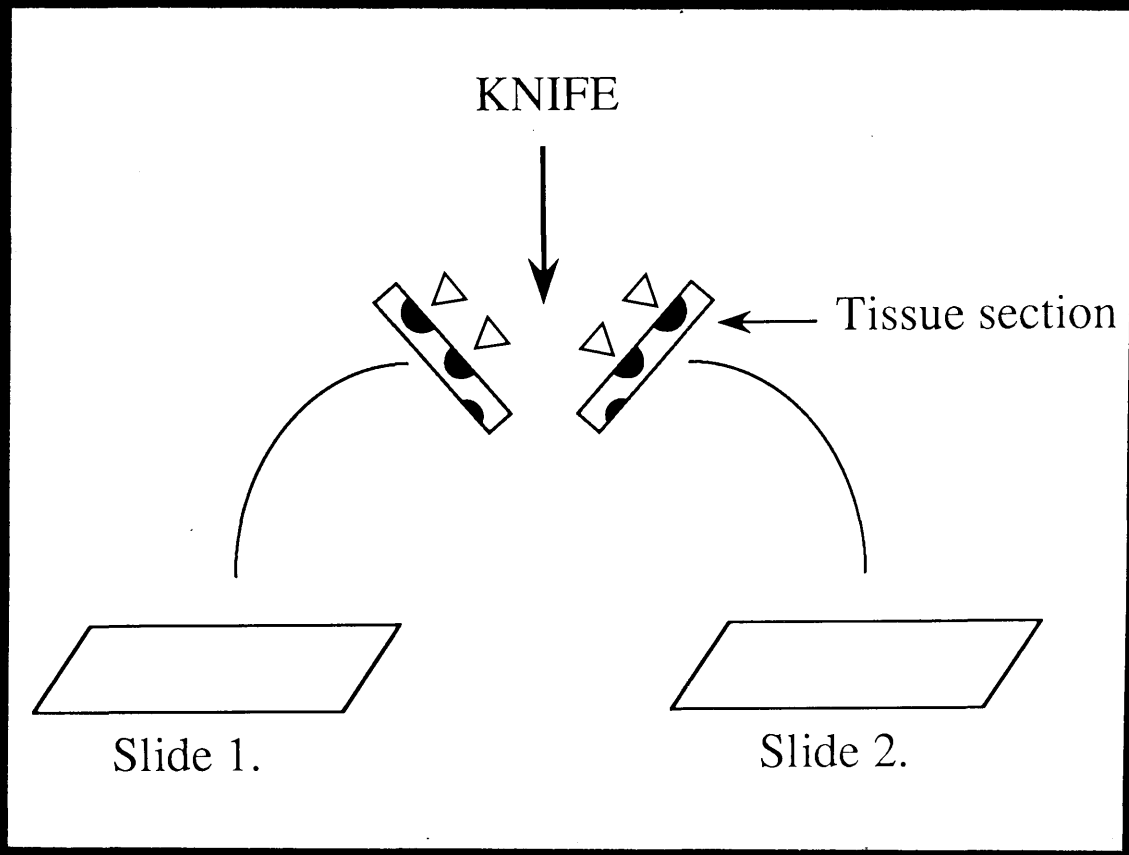
C) SERIAL SECTION TECHNIQUE.

Two serial section techniques were used.

1) Mirror image technique (Noordan,1986). A 3 microns thick section of formalin fixed paraffin embedded human tissue was cut and picked up on a poly-L-lysine or 3-aminopropyltriethoxysilane coated glass slide. The second serial section was turned upside down and picked up on another slide. Thus the superficial aspects of the two sections were those cut surfaces which had been separated by the knife (figure,2.1). One section was stained for

Figure 2.1

Mirror image serial section technique


KNIFE
Tissue section
Slide 1.
Slide 2.

IFN-alpha with the sheep anti-human IFN-alpha antiserum (H51) and the second section for KP1 (macrophage marker). For comparison the two sections were visualized together with two different microscopes but one diffuser(head). Specific fields were identified in each section and photographed. The photograph from one slide was printed in reverse so that the mirror image could be cancelled.

2) Two 3 micron thick sections were cut from formalin fixed paraffin embedded normal human tissues and both picked up on poly-L-lysine or 3-aminopropyltriethoxysilane coated glass slides in the usual way. The first serial section was stained for IFN-alpha with the sheep H51 antiserum and the second serial section for KP1. A similar field was identified in the two serial sections and photographed for comparison.

D) DOUBLE STAINING TECHNIQUE.

The technique can be divided into two parts.

a) Indirect immunoperoxidase technique used for the demonstration of immunoreactive-IFN-alpha using the polyclonal sheep H51 antiserum or *in situ* hybridization for the detection of IFN-alpha messenger RNA (mRNA).

b) Indirect immunofluorescence technique.

a) After the indirect immunoperoxidase technique used for the demonstration of immunoreactive-IFN-alpha or the *in situ* hybridization technique the slides were examined under the microscope to confirm positive staining of cells. The coverslips were then removed by placing the slides in xylene in the case of the indirect immunoperoxidase technique or in water in the case of the *in situ* hybridization technique. The same slides were then used in the indirect immunofluorescence technique. Slides stained days or months before for IFN-alpha by the above indirect immunoperoxidase technique could be used in this double staining technique.

b) The indirect immunofluorescence technique.

Principle.

The tissue sections are treated with an unlabelled primary antibody that binds to the antigen. The bound primary antibody is identified by a rhodamine labelled secondary antiserum raised to the immunoglobulins of the species providing the primary antibody. The slides are then veiwed by a fluorescence microscope.

Testing various antibodies suitability for immunofluorescence before use in the double staining.

Two monoclonal antibodies KP1 and Mac 387 and the rabbit polyclonal anti-muramidase and anti-prekeratin antisera were tested.

Protocol for the monoclonals KP1 and Mac 387.

The same protocol was used as that described for monoclonal anti-human IFN-alpha antibody in section B(2) with the following variations.

Trypsinization.

The tissue sections were trypsinized for 10 minutes prior to application of KP1 and MAC 387 monoclonal antibodies.

Variation in primary antibodies.

The KP1 antibody was used at a 1/10 dilution and left on the sections overnight at $4^{o}C$.

The Mac 387 antibody was used at a 1/20 dilution. It was left on two consecutive sections from the same tissue, for 2 hours at room temperature, and overnight at $4^{o}C$, respectively.

Both the monoclonal antibodies were diluted in tris buffered saline.

Variation in secondary antiserum.

A rhodamine conjugated rabbit anti-mouse immunoglobulin antiserum was used at a 1/20 dilution (in 20% normal human serum in tris buffered saline) for

45 minutes instead of the horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin antiserum. The slides were then washed in tris buffered saline for 10 minutes, counterstained in haematoxylin for 5 seconds, washed thoroughly in tap water and mounted in Glycergel (water mounting medium).

Control.

A section was treated with tris buffered saline instead of the primary antibody to avoid misinterpreting non-specific immunofluorescence due to the secondary antiserum.

Anti-muramidase and anti-prekeratin antisera.

The protocol was the same as the protocol of the indirect immunoperoxidase technique using the polyclonal sheep H51 antiserum described in section A(i) with the following Amendments.

Trypsinization.

After step 3 the sections were trypsinized for 20 minutes.

Variation in primary antiserum.

The rabbit anti-muramidase antiserum was applied to the sections at a 1/50 dilution and left on at room temperature for 2 hour.

The anti-prekeratin antiserum was applied to the sections at a 1/100 dilution for 2 hours at room temperature.

Both the antisera were diluted in tris buffered saline.

Variation in secondary antiserum.

A secondary rhodamine conjugated swine anti-rabbit immunoglobulin antiserum was used at a 1/20 dilution (in 20% normal human serum in tris buffered saline) for 45 minutes. The slides were then washed in tris buffered saline for 10 minutes, counterstained in haematoxylin for 5 seconds, washed thoroughly in tap water and mounted in Glycergel.

Control.

A section was treated with tris buffered saline instead of the primary antibody to avoid misinterpreting non-specific immunofluorescence due to the secondary antiserum.

Once the antibody tested was shown to detect antigen with strong immunofluorescence and little background it was then used in part (b) of the double staining technique, as described below.

The slides were taken to water after part (a) of the double staining technique and one of the above indirect immunoflourscence methods was applied to the same section. Double stained sections were viewed with a Leitz "Orthomat" fluorescence microscope.

Control for the double staining.

Tissue sections shown previously to have cells fluorescing strongly with the antibodies described in section (b) were used in the double staining technique as positive controls. These tissue sections were treated with tris buffered saline instead of the H51 antiserum in part (a) of the double staining technique. The remaining protocol was the same.

E) IMMUNORADIOMETRIC ASSAY "SUCROSEP" IFN-ALPHA (BOOTS CELLTECH,UK) FOR THE DETECTION OF IFN-ALPHA IN HUMAN TISSUE HOMOGENATES AND FLUIDS.

All human tissues had to be homogenized and the fluids cleared before use in the immunoradiometric assay.

Protocol for homogenization of human tissues.

1) All human tissues stored at -70^{o}C were defrosted and suspended in viral transport medium (Gibco,UK) at 4^{o}C. Approximately 1 gram of tissue was added per 2 mls of viral transport medium for all tissues except choroid plexus. As the choroid plexus tissue samples were very small in size, 1 gram of tissue was suspended in 4 mls of viral transport medium to obtain a reasonable volume for homogenization.

2) All the suspensions were homogenized for 60 seconds in an Ultra Turrax homogenizer (Janke & Kunkel).

3) The suspensions were then immediately centrifuged at 3500g for 30 minutes, at 4°C.

4) The clear supernatant was collected and stored in aliquots at -70°C.

During the entire process of homogenization the temperature of the suspensions was kept down by placing the suspension containers in ice. The homogenization bur was also cooled before use and during the homogenization procedures. This was done in order to avoid denaturation of proteins by a rise in temperature during processing.

The 4 amniotic fluids collected from caesarean section operations were also centrifuged at 3500g for 5 minutes at 4°C and the clear supernatants thus obtained were stored in aliquots at -70°C.

Amendment to the above protocol.

Pepstatin A (pepsin inhibitor) and Aprotinin A (trypsin inhibitor, 13.8 trypsin inhibitor units/milligram) (both were from Sigma, ST Louis,USA) were added to the homogenizing buffer just before the start of the homogenization procedure. They were used in a concentration of 1 microgram of Pepstatin A + 1

microgram of Aprotinin A/ ml of the homogenizing buffer.

The "Sucrosep" IFN-alpha immunoradiometric assay. Principle.

The principle of the "Sucrosep" IFN-alpha immunoradiometric assay is briefly outlined in figure,2.2. It shows that there are two sites of IFN-alpha capture, utilizing a ^{125}I monoclonal Yok 5/19 anti-IFN-alpha antibody in the first step and a polyclonal sheep anti-IFN-alpha antiserum (solid phase) in the second step.

Procedure for the "Sucrosep" IFN-alpha immunoradiometric assay, for the detection of IFN-alpha in human tissue homogenates and fluids.

Steps.

1) The freeze dried IFN-alpha standard provided with the "Sucrosep" IFN-alpha immunoradiometric assay kit (calibrated against the MRC 69/19 International reference preparation for human leucocyte IFN, obtained from the National Institute for Biological Standards and Control, Potters Bar, UK) was reconstituted with 3 mls of viral transport medium. The resulting stock solution contained 1024 international units/ml

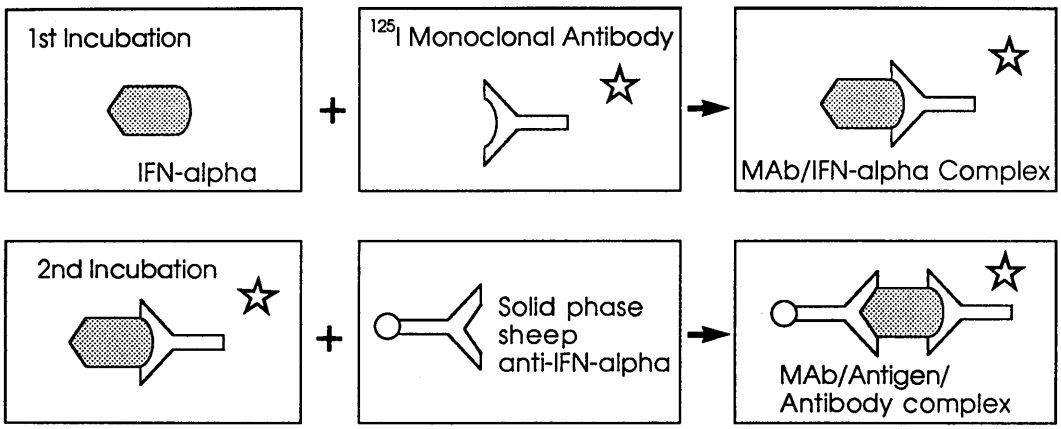


Figure 2 .2. Principle of the "Sucrosep" IFN-alpha immunoradiometric assays (IRMA)

(IU/ml) of IFN-alpha. A series of IFN-alpha standards; 0, 1, 4, 16, 64 and 256 IU/ml were prepared from the stock solution (1024 IU/ml of IFN-alpha) with viral transport medium as a diluent.

2) 200 microlitres of each standard and 200 microlitres of specimen were added in the test proper to each of 2 test tubes in a rack of 20 tubes.

3) 50 microlitres of radiolabelled monoclonal antibody was than added to each tube and the tube contents vortex mixed for a few seconds. 50 microlitres of radiolabelled monoclonal antibody was added to 2 tubes without specimens and left to the final counting procedure. These tubes were later referred to as "total count" tubes.

4) After an incubation period of 2 hours at room temperature, 50 microlitres of the polyclonal IFN-alpha antiserum (solid phase) suspension was added to the mixture and thoroughly mixed by repeated inversion of the capped bottle immediately before and during pipetting.

5) The tubes were then left for 2 hours on an orbital shaker. This helped to keep the polyclonal anti-human IFN-alpha antiserum (solid phase) in suspension while it reacted with IFN-alpha present in samples.

6) At the end of this period the tubes were removed from the shaker and any empty tube spaces in the racks were filled with spare tubes before commencing the separation procedure.

7) Labelled monoclonal antibody bound to IFN-alpha in the solid phase was washed and separated in a gravity dependent sucrose separation system "Sucrosep".

8) One ml of pre-wash buffer was added to each tube to wash the walls of the test tubes. The tubes were allowed to stand for 5 minutes.

9) Using a sucropette (reagent delivery device), 2 ml of "Sucrosep" reagent was delivered to the lower part of each tube. The dense "Sucrosep" solution displaces residual monoclonal antibody and the specimen to the upper part of the test tube. The sucropette was carefully removed when the sucrose solution had been layered below the incubate.

10) The tubes were then left for 15 minutes to allow the solid phase polyclonal antiserum to gravitate to the base of the tube, carrying with it any radiolabelled immune complex present.

11) Using an aspirator provided with the kit, the tube contents were aspirated, leaving approximately 0.3 ml in the bottom of each tube.

12) A second "Sucrosep" separation step was then carried out after which the tubes were sealed.

13) The radioactivity was counted in all the tubes with a gamma scintillation counter (Packard Auto Gamma 5650, United technologies, Packard), for a time sufficient to accumulate at least 50,000 counts in the "total count" tubes. These tubes with 50 microlitres of the radiolabelled monoclonal antibody were always added as a part of the assay in order to determine the relative radioactivity of the label.

14) Empty tubes were also used to check the background radiation.

15) Using a log/log plot, ^{125}I counts versus IFN-alpha standard concentrations were plotted. A standard curve was drawn from this data and used to interpolate IFN-alpha concentrations in the specimens studied.

In this study the "Sucrosep" IFN-alpha immunoradiometric assay was used to measure IFN-alpha in tissue homogenates diluted in viral transport medium (VTM). The "Sucrosep" IFN-alpha immunoradiometric assay was developed by Boots-Celltech Diagnostics,UK for the measurement of human IFN-alpha in serum or tissue culture fluids. As the assay was to be used on the extraction buffer (VTM) rather than serum buffer, it was considered important to determine the assay performance when VTM was used as a diluent. The following experiments were performed to test the

reproducibility and specificity of the assay with VTM as a diluent.

a) Intra-assay analysis.

To test the intra-assay precision of the "Sucrosep" IFN-alpha immunoradiometric assay, 8 samples of VTM with no IFN-alpha activity were analyzed on the same day by the "Sucrosep" IFN-alpha immunoradiometric assay, as described above. The mean of the total number of counts, standard deviation and coefficient of variation were determined for these 8 samples of VTM.

b) Inter-assay analysis.

The reproducibility of the assay was analysed by calculating the mean value for each standard concentration derived from 10 different "Sucrosep" IFN-alpha immunoradiometric assays (with VTM used as the diluent) performed over a period of several months. These mean values were used to plot a standard curve. Standard deviation, standard error and coefficient of variation were also calculated for each standard concentration.

c) Neutralization/ blocking experiment.

This experiment evaluated the specificity of the "Sucrosep" IFN-alpha immunoradiometric assay by removing IFN-alpha from the specimens before testing,

when performed in parallel to samples tested with no treatment.

Procedure of the neutralization/ blocking experiment.

1) IFN-alpha standards of 0, 1, 4, 16, 64, and 246 IU/ml were prepared from the standard IFN-alpha preparation of 1024 IU/ml.
2) 200 microlitres of each standard was added to a set of 4 tubes. Two tubes of each set were selected as the samples for the neutralization/ blocking and the remaining two were used as IFN-alpha standard controls.
3) 50 microlitres of the sheep anti-human IFN-alpha antiserum attached to solid phase was added to each of the tubes with the specimens to be neutralized.
4) The samples were placed in an orbital shaker and incubated at room temperature for two hours.
5) After this period, the samples were centrifuged at 3000g and 200 microlitres of the supernatant was collected.
6) The neutralized supernatants and the control IFN-alpha standards were assayed in duplicate for IFN-alpha activity following the standard procedure of the "Sucrosep" IFN-alpha immunoradiometric assay for the detection of IFN-alpha.

The solid phase in the third step of the above experiment was a sepharose micro-bead system to which the polyclonal sheep anti-human IFN-alpha antiserum was covalently attached. It was possible to capture IFN-alpha in the specimens onto the solid phase with incubation and remove it from the specimen by centrifugation. The supernatants could then be tested for residual unblocked IFN-alpha.

F) *IN SITU* HYBRIDIZATION TECHNIQUE FOR THE DETECTION OF IFN-ALPHA MESSENGER RNA (mRNA) IN FORMALIN FIXED PARAFFIN EMBEDDED NORMAL HUMAN TISSUES.

It is well known that messenger RNA (mRNA) can be easily destroyed by various ribonuclease enzymes found abundantly in the environment. Therefore certain precautions were always carried out during various *in situ* hybridization procedures.

Glassware.

All the glassware was placed in 2% RBS solution (Chemical Concentrates, Kent,UK) in water for 4-5 hours. The glassware was then washed thoroughly with tap water and dried before use in the *in situ* hybridization procedure.

Gloves.

Gloves were worn all the time while handling glassware and other reagents used in the *in situ*

hybridization technique to avoid contamination with the high concentrations of ribonuclease (RNase) enzymes found on the fingers.

Distilled water and buffers.

Distilled water and most buffers used in the *in situ* hybridization technique were treated with diethylpyrocarbonate (DEPC) (Sigma,UK). One ml of DEPC was added to 1000 mls of distilled water or buffer, mixed well by shaking and left overnight in capped bottles (DEPC is a potential carcinogen, therefore the above procedure was always performed in a fume chamber). Next morning the bottles containing the buffers and water were autoclaved at 140^{o}C. DEPC destroys ribonuclease enzymes in the solutions and autoclaving converts excess DEPC into ethanol and carbon dioxide (Arrand, 1985). After autoclaving the water and buffers were cooled before use in the *in situ* hybridization techniques. The word DEPC has been used throughout the text before buffers and water treated with DEPC, as described above.

Two oligonucleotide probes, probe 1 and probe 2, were selected for the demonstration of IFN-alpha mRNA in formalin fixed normal human tissue, as described earlier in the materials section. A poly-d-T probe demonstrating total mRNA in formalin fixed normal human tissues was also used as positive control for the *in situ* hybridization technique.

Probe 1 (17 base oligonucleotide probe) was supplied in ammonium hydroxide solution. To isolate the probe from the ammonium hydroxide solution a procedure called ethanol precipitation, was used.

Ethanol precipitation procedure.

1) 360 microlitres of oligonucleotide probe 1, supplied in ammonium hydroxide solution, was put in a microcentrifuge tube. 40 microlitres of 3 molar(M) sodium acetate (BDH,UK) in water was added to the probe and mixed to give 400 microlitres of 0.3M sodium acetate solution containing the oligonucleotide probe.
2) 1.2 mls of absolute ethanol (kept at -20^{o}C) was added to the 0.3M sodium acetate solution containing the oligonucleotide probe, mixed and left overnight at -70^{o}C.
3) Next morning the solution was centrifuged at 15,000g for 10 minutes at room temperature. A pellet was observed at the base of the tube.
4) The supernatant was removed with a glass pasteur pipette.
5) The pellet was rinsed with absolute ethanol, kept at -20^{o}C, by carefully pipetting down the side of the tube, opposite the pellet.
6) Next the above solution was centrifuged for 10 minutes at 15,000g.

7) Most of the ethanol was carefully removed with a glass pasteur pipette and the rest left to evaporate till the pellet was dry.

8) The pellet was dissolved in 20 microlitres of T.E buffer (Appendix).

9) 1 microlitre of the above solution was added to 500 microlitres of T.E buffer.

10) The optical density of this solution was measured with a spectrophotometer (PU 8620 series, Scientific & Analytical Equipment, Philips,UK). For oligonucleotide probes one optical density reading is equal to 20 micrograms of the probe per ml. With the help of the optical density reading from the spectrophotometer, it was shown that there was 94.4 micrograms of probe in the 20 microlitres of solution analysed. To obtain a probe concentration of 1 microgram/ microlitre, 74.4 microlitres of T.E buffer was added to this probe solution. The probe solution of 1 microgram/ microlitre in T.E buffer finally obtained was stored at -20^{o}C.

Probe 2 (24 base oligonucleotide) on the other hand was supplied in a pure solid form, ready to use (with an optical density reading of 3.6). T.E buffer was added to make a probe solution with a final

concentration of 1 microgram of the probe/ 1 microlitre of T.E buffer.

Poly-d-T probe was also obtained in a ready to use form and was diluted in T.E buffer to get a probe concentration of 1 microgram of the probe/ 1 microlitre of T.E buffer.

All three stock probes (1 microgram of the probe/ 1 microlitre of T.E buffer) were kept at -20^{o}C.

Labelling of the oligonucleotide probes.

The oligonucleotide probes were labelled with a nonradioactive labelling substance called digoxigenin.11.dUTP (Boehringer Mannheim Ltd, East Sussex, England).

Standard protocol for labelling of the IFN-alpha mRNA oligonucleotide probes with digoxigenin.11.dUTP.

It can be divided into two parts.

a) Labelling reaction.

b) Separation of the labelled probe.

a) Labelling reaction [end-labelling with terminal deoxynucleotidyl transferase(TdT) enzyme]

30 microlitres of diethylpyrocarbonate (DEPC) treated water and 10 microlitres of 5 x tailing buffer [Bethesda Research Lab; (BRL) Life Technologies Inc,USA] (100 mM potassium cacodylate, pH 7.2, 2mM

$CoCl_2$, 0.2 mM DTT) were put in a microcentrifuge tube and mixed well. 5 nanomoles of digoxigenin.11.dUTP and 1 nanomole of deoxy-cytidine-triphosphate (Pharmacia, UK) were then added to the above mixture. 1 microgram of the oligonucleotide probe was then added and mixed well. Finally approximately 44 units of the terminal deoxynucleotidyl transferase enzyme (BRL,USA) was added and mixed well. The mixture was spun in the microcentrifuge tube for a few seconds and incubated overnight at 37°C.

b) Separation of the labelled probe.

1) Next morning a vertical stand was taken and a ruler was placed in the horizontal position on the stand. A sephadex G-25 separation column (NAP-5, Pharmacia,UK) was attached to the ruler in upright position.

2) Equilibration of the sephadex G-25 separation column.

   A 0.1% standard saline citrate(SSC), 0.1 % sodium dodecyl sulphate(SDS) solution in water was placed on the top of the column till the column was full. The solution was allowed to drip down the lower end of the column till the column was empty. The process was repeated 3 times.

3) 10 microcentrifuge tubes were marked from 1-10 and placed in order in a rack.

4) A nitrocellulose paper strip (Gelmen Sciences,USA) was taken and divided into 10 small parts. A small edge was cut as an identification mark. The strip was first placed in DEPC water and then in DEPC 20xSSC solution (1xSSC= 0.15M sodium chloride, 0.015 M sodium citrate) for a few minutes. The nitrocellulose paper strip was taken out and dried between two filter papers (Whatman International, Maidstone,England).

5) The microcentrifuge tube (containing the probe + labelling reagents' solution incubated overnight at 37^{o}C) was centrifuged for a few seconds and placed on ice. 5 microlitres of 100mM ethylenediamine tetraacetic acid (Sigma) was added to the tube and mixed well to stop the labelling reaction. The solution was then placed on top of the sephadex separation column.

6) The microcentrifuge tube marked 1 was placed under the sephadex column. 200 microlitres of 0.1% SSC, 0.1% SDS solution was then placed on top of the sephadex column. Approximately 200 microlitres of fluid drips down the column which was collected in the microcentrifuge tube. The same procedure was repeated for all the 10 marked microcentrifuge tubes in an orderly way from 1 to 10.

7) One microlitre of the solution from each microcentrifuge tube was spotted on a separate

part of the nitrocellulose paper strip and dried in air. The nitrocellulose paper strip was then placed between two ordinary filter papers held by paper clips and baked at 80°C for two hours.

8) After baking at 80°C for two hours the nitrocellulose paper strip was washed briefly in buffer one (100mM tris HCL, 150mM sodium chloride).

9) The nitrocellulose paper strip was then sealed in a plastic bag with buffer 2 (3% bovine serum albumin [Sigma] in buffer one), for 30 minutes at 42°C.

10) The strip was washed briefly in buffer one.

11) The nitrocellulose paper strip was then sealed in a plastic bag containing alkaline phosphatase conjugated anti-digoxigenin antibody (Boehringer, Mannheim,UK) at 1/1000 dilution in buffer one and left for 30 minutes at room temperature, on a shaker.

12) The nitrocellulose paper strip was washed twice in buffer one for 15 minutes each, on a shaker, to remove the unbound anti-digoxigenin antibody.

13) The nitrocellulose paper strip was then washed with buffer three (100mM tris HCL, 100mM NaCl, 50mM magnesium chloride, pH 9.5) for 5 minutes on a shaker.

14) The nitrocellulose paper strip was incubated in a sealed plastic bag with an alkaline substrate solution, Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (both from Sigma) [Appendix] for 30 minutes. Light was excluded from the reaction by wrapping the plastic bag in aluminium foil.

15) The above reaction was stopped by placing the nitrocellulose paper strip in buffer four (10mM tris HCL, 1mM EDTA, pH 8) for 5 minutes. The nitrocellulose paper strip was then dried between two filter papers and sealed in a plastic bag.

16) The fractions of the separated solution which contained the labelled probe appeared as a blue dot on the nitrocellulose strip.

17) Those fractions containing labelled probe were pooled and placed in a freeze dryer for 16-18 hours. The freeze dried (lyophilized) labelled probe was stored at -20^{o}C. The labelled probe was reconstituted in 100 microlitres of TE buffer before use in *in situ* hybridization techniques.

Amendment to the protocol of the labelling reaction.

2 micrograms of probe was added to the labelling reaction reagents instead of 1 microgram. The remaining procedure was the same as above.

Labelling of the poly-d-T probe with digoxigen.11.dUTP.

The same labelling procedure was used as that described for the IFN-alpha mRNA oligonucleotide probes with the following amendments.

The labelling reaction.

4 microlitres of 5 x tailing buffer was added to 10 microlitres of DEPC water and mixed in a microcentrifuge tube. 2 nanomoles of digoxigenin.11.dUTP was then added to the above mixture, followed by the addition of 2 micrograms of the poly-d-T probe, and was mixed well. Finally, approximately 30 units of the terminal deoxynucleotidyl transferase enzyme was added to the mixture and incubated overnight at 37^{o}C. The separation procedure was the same as that described for IFN-alpha mRNA probes.

Protocol of *in situ* hybridization technique for the detection of IFN-alpha mRNA in formalin fixed paraffin embedded normal human tissues using the digoxigenin.11.dUTP labelled probe.

Procedure

1) 4 microns thick sections were cut from buffered formalin or formol saline fixed paraffin wax embedded normal human tissues and mounted on 3-aminopropyltriethoxysilane coated glass slides. A

set of 8 tissue sections from the same human tissues (cut and mounted as above) was taken and placed in two changes of xylene for 5 minutes each, to remove the paraffin wax. This was followed by placing the slides in two changes of absolute alcohol for 5 minutes and one change of methyl alcohol for 5 minutes and rinsed in DEPC water.

2) The slides were then washed in DEPC phosphate buffered saline, pH 7.3 (Oxoid Ltd,UK) for 5 minutes and placed in DEPC 0.2N HCL for 20 minutes at room temperature.

3) After rinsing the slides in DEPC phosphate buffered saline they were then placed in 0.3% Triton-X 100 (Sigma) in DEPC phosphate buffered saline for 15 minutes and again rinsed in DEPC phosphate buffered saline.

4) Proteinase-k treatment.

4 different concentrations of proteinase-k enzyme (Sigma) were prepared in proteinase-k buffer (0.1M tris HCL, 50mM ethylenediamine tetraacetic acid, pH 8) at 37^{o}C. These concentrations were 2, 5, 10 and 20 micrograms of the proteinase-k enzyme/ ml of proteinase-k buffer. 2 sections each, from the set of 8, were incubated at 37^{o}C, for 30 minutes, with 2, 5, 10 and 20 micrograms of proteinase-k enzyme/ ml of proteinase-k buffer, respectively.

5) The slides were washed for 1 minute in DEPC 0.2% glycine solution in water, to stop the proteinase-k reaction. They were then rinsed in DEPC phosphate buffered saline.

6) They were next placed in 4% paraformaldehyde solution in DEPC phosphate buffered saline for 5 minutes.

7) The slides were then washed in DEPC phosphate buffered saline for 5 minutes on a shaker and incubated with pre-hybridization buffer (50% formamide, DEPC 20xSSC) for 2 hours, at 37^{o}C.

8) Hybridization.

The digoxigenin labelled probe was added to the hybridization buffer, 50% formamide (Appendix), in a concentration of 5 microlitres of labelled probe/ 15 microlitres of hybridization buffer. One set of 4 tissue sections treated with 2, 5, 10 and 20 micrograms of proteinase-k enzyme/ ml of proteinase-k buffer in step 4, were treated with the above probe + hybridization buffer solution. The second set of tissue sections (treated with 2, 5, 10 and 20 micrograms of proteinase-k enzyme/ ml of proteinase-k buffer in step (4) was used as a negative control and treated with hybridization buffer only. All the sections were covered with dimethyldichlorosilane coated glass coverslips (Appendix). Next the sections were placed in a

humid box sealed in a plastic bag and incubated overnight at room temperature (approximately 20°C).

9) After 16-18 hours the coverslips were removed from the tissue sections by placing the slides in 4xSSC solution (1xSSC= 0.15M sodium chloride, 0.015 M sodium citrate) for 5 minutes.

10) Post-hybridization washes.
The slides were washed twice in 2xSSC solution at room temperature for 15 minutes and then in 0.1xSSC solution at room temperature for 20 minutes, followed by one wash in buffer one for 5 minutes. All these washes were performed on a shaker.

11) The alkaline phosphatase conjugated anti-digoxigenin antibody (1/500 dilution in buffer one) was applied on the sections for 2 hours at room temperature.

12) The slides were washed twice in buffer one for 15 minutes.

13) The slides were then washed once in buffer three for 5 minutes.

14) The slides were next placed in freshly prepared alkaline phosphatase substrate solution, NBT/BCIP (Appendix) in a coplin jar and light was excluded from the reaction. They were left overnight at room temperature.

15) After 16-18 hours the slides were removed from the substrate solution and washed in distilled water. They were then counterstained in haematoxylin for 5 seconds, washed thoroughly in distilled water, mounted in Glycergel (Dako), and veiwed under a Leitz Laborlux light microscope.

Working out the temperature of incubation of the oligonucleotide probes in the *in situ* hybridization technique.

The incubation temperature was calculated using a formula described for the oligonucleotide probes (Davis, Dibner & Battey,1986). The formula was as follows,

$$Tm = 16.6 \log(M) + 0.41(Pgc) + 81.5 - Pm - B/L - 0.65(Pf)$$

Tm (melting point). It is the temperature at which the strands of a DNA duplex or an RNA-DNA hybrid are half dissociated or denatured (Britten & Davidson,1987).

M is the molar concentration of sodium ions, to a maximum of 0.5M.

Pgc is the percentage of guanine or cytosine nucleotide bases in the oligonucleotide probe (between 30 and 70).

Pm is the percentage of mismatched nucleotide bases, if known.

Pf is the percentage of formamide in the hybridization buffer.

B is equal to 675 (synthetic oligonucleotide probes up to 100 nucleotide bases long).
L is the oligonucleotide probe length in nucleotide bases.

Once the *Tm* (melting point) for the oligonucleotide probe was calculated, the temperature of incubation (*Ti*) was then determined by using the following formula.

$Ti = Tm - 15^{o}C$

With 50% formamide (BDH,UK) in the hybridization buffer, the *Tm* (melting point) for probe one was $30.2^{o}C$. The incubation temperature (*Ti*) therefore worked out to be $15.2^{o}C$.

With 50% formamide in the hybridization buffer, the *Tm* (melting point) for probe two was $29.96^{o}C$. Therefore the incubation temperature (*Ti*) worked out to be $14.96^{o}C$.

Amendments to the protocol of the *in situ* hybridization technique for the demonstration of IFN-alpha mRNA using probe 1 and probe 2.

Varying the proteinase-k treatment.

a) The proteinase-k enzyme in the 4 different concentrations described in the standard protocol for *in situ* hybridization, was left on the sections for 20, 45 and 60 minutes at $37^{o}C$ respectively instead of the

standard 30 minutes. The remaining procedure was the same as above.

b) The proteinase-k enzyme was also used in a concentration of 40 micrograms of proteinase-k enzyme/ml of proteinase-k buffer, in addition to the above 4 different concentrations. The remaining procedure was the same as the standard hybridization protocol.

Varying the incubation temperature of hybridization and post-hybridization washes.

a) The tissue sections were hybridized overnight with the probe + hybridization buffer solution (step 12 of the standard hybridization protocol) at 37^{o}C. This was followed by the following post-hybridization washes. The slides were washed twice in 2xSSC (1xSSC= 0.15M sodium chloride, 0.015 M sodium citrate) at room temperature, for 15 minutes. They were then washed twice in 0.1XSSC solution at 37^{o}C for 20 minutes (the 0.1xSSC solution was made one day before the post-hybridization washes and incubated overnight at 37^{o}C). This was followed by one wash in buffer one for 5 minutes. All these washes were performed on a shaker.

b) The tissue sections were hybridized overnight with the probe + hybridization buffer solution (step 12 of the standard hybridization protocol) at 42^{o}C. This was followed by the following post-hybridization washes.

The slides were washed twice in 2xSSC solution at room temperature for 15 minutes. They were then washed once in 0.1xSSC solution at 42°C for 20 minutes (the 0.1xSSC solution was made one day before the washes and incubated overnight at 42°C), followed by one wash in 0.1xSSC solution for 20 minutes at room temperature. The slides were then washed once in buffer one for 5 minutes.

Negative control tissue sections (incubated with the hybridization buffer only) were also treated in exactly the same way as the sections incubated with the probe.

Varying the concentration of formamide in the hybridization buffer.

The concentration of formamide was reduced from 50% in the hybridization buffer to 20% (Appendix). The sections were then incubated overnight with the probe + hybridization buffer solution (step 12 of the standard hybridization protocol) at 37°C. The negative control sections incubated with the hybridization buffer only were treated in the same way. After 16-18 hours, the post hybridization washes done were as follow. The slides were washed twice in 2xSSC solution at room temperature for 20 minutes. They were then washed once in 2xSSC solution at room temperature for 15 minutes and in 0.1xSSC solution at 37°C for 15 minutes (the 0.1xSSC solution was made one day before the washes and

incubated at 37°C). This was followed by one wash in buffer one for 5 minutes. The remaining procedure was the same as the standard protocol.

Demonstration of total messenger RNA (mRNA) in formalin fixed normal human tissues using the poly-d-T probe.

All messenger RNA molecules have a poly A tail (a varying number of adenosine nucleotide bases) attached at the 3'end. It has been demonstrated that a probe directed against this part of the mRNA molecule, called a poly-d-T probe, can show the presence of mRNA in formalin fixed human tissues by *in situ* hybridization (Pringle *et al*,1989). This study provided an opportunity to demonstrate mRNA in formalin fixed human tissues and was used as a control for the *in situ* hybridization technique, developed to demonstrate IFN-alpha mRNA.

Protocol of the *in situ* hybridization technique adopted for the demonstration of total mRNA in formalin fixed normal human tissues using a digoxigenin.11.dUTP labelled poly-d-T probe.

The protocol adopted for the demonstration of total mRNA in formalin fixed normal human tissues using a digoxigenin.11.dUTP labelled poly-d-T probe was the same as the protocol used for the *in situ* demonstration

of IFN-alpha mRNA described earlier, with the following amendments.

Variation in proteinase-k treatment.

The proteinase-k enzyme in 4 different concentrations was left on the sections for 60 minutes instead of 30 minutes at 37°C.

Variation in step 7.

The slides were incubated in the pre-hybridization buffer for 1 hour at 37°C.

Variation in hybridization.

The labelled poly-d-T probe was used in a concentration of 3 microlitres/17 microlitres of the hybridization buffer and the sections were incubated overnight with the (probe + hybridization buffer) solution, at 37°C.

Variation in post-hybridization washes.

After the coverslips were removed, the sections were washed twice in 2xSSC buffer for 20 minutes at room temperature on a shaker. They were then washed twice in 2xSSC buffer, at 37°C for 20 minutes, followed by one wash in 2xSSC buffer, at room temperature for 5 minutes. They were finally washed in buffer one, at room temperature for 5 minutes.

Variation in step 14.

The slides were incubated in the alkaline phosphatase substrate solution (NBT/BCIP) for one hour, instead of using an overnight incubation.

Specificity of the *in situ* hybridization staining.

To demonstrate that positive staining obtained during the *in situ* hybridization procedures was specific, the tissue sections that showed positive staining in cells with the oligoprobes were treated with ribonuclease enzymes (RNase).

The RNase enzyme used in this study was Ribonuclease-A (Sigma). A stock solution of 10 milligrams of Ribonuclease-A/ml of water was made. The solution was then boiled for 10 minutes to get rid of deoxyribonuclease enzymes, aliquoted and stored at -20^{o}C until needed.

Protocol for RNase enzyme treatment of the sections.

The same hybridization protocol described earlier for the *in situ* localization of IFN-alpha mRNA or total mRNA was used, with the following addition.

After step 5 in the standard protocol for *in situ* hybridization, the sections were treated with Ribonuclease-A. The Ribonuclease-A stock solution prepared above was used in a concentration of 1 milligram of Ribonuclease-A/ml of 50mM tris HCL buffer, pH 7.5. It was left on the sections for 2 hours at 37^{o}C.

The sections were washed 4 times in DEPC phosphate buffered saline for 10 minutes. Both sets of tissue

sections described in the hybridization protocol (i.e the one incubated with the probe and the other "negative control", incubated only with the hybridization buffer) were treated with the Ribonuclease-A enzyme. The remaining procedure was the same as the hybridization protocol for the oligoprobes.

Positive control.

Sections incubated with 50mM of tris HCL buffer instead of the RNase enzyme in step 5 of the hybridization protocol were used as positive controls for the RNase procedure. These control sections were hybridized in exactly the same way as RNase treated sections with the probe and with the negative control (hybridization buffer alone).

Varying the Ribonuclease-A concentration.

The Ribonuclease-A enzyme was used in concentrations of 2, 4 and 8 milligrams/ml of 50mM tris HCL buffer, respectively.

CHAPTER THREE

THE DISTRIBUTION OF INTERFERON-ALPHA IN FORMALIN FIXED PARAFFIN EMBEDDED NORMAL ADULT HUMAN TISSUES.

INTRODUCTION

Since the discovery of IFN (Isaacs & Lindenmann,1957) much emphasis has been laid on the presence and role of IFN in viral infections. Recently, many workers have demonstrated the presence of IFN-alpha in conditions other than viral infections. IFN-alpha has been demonstrated in placenta by many workers (Chard *et al.*,1986; Duc-Goiran *et al.*,1985; Bocci *et al.*,1985). Some workers have reported low levels of IFN-alpha in normal human bone marrow (Zoumbas *et al.*,1985). Bocci *et al* (1984), showed the presence of IFN (IFN-gamma or an acid labile IFN-alpha or a mixture) in the lymph draining the gastrointestinal tract of normal healthy rabbits. An IFN-alpha like substance has been identified in the plasma of healthy individuals (Shiozawa *et al.*,1986) and IFN-alpha and IFN-gamma have been demonstrated in bronchoalveolar lavage fluid of healthy human volunteers (Prior & Haslam,1989). Traces of IFN-alpha have been demonstrated in the cerebrospinal fluid of normal individuals (Ho-Yen & Carrington,1987).

The expression, "physiological IFN response" was first used by Bocci (1980). He put forward the idea that IFN was produced continuously in health in low physiological amounts which increased with an acute viral infection (Bocci,1981).

The ability to demonstrate immunoreactive IFN-alpha in formalin fixed paraffin embedded human tissues using a polyclonal sheep anti-human IFN-alpha antiserum (H51) (Foulis *et al.*,1987) permitted an examination of the cellular distribution of IFN-alpha in a wide variety of formalin fixed paraffin embedded normal human tissues. Such a study was designed to help define which cells normally produce IFN-alpha and to hopefully give further insight into its physiological role.

In this study the polyclonal sheep anti-human IFN-alpha antiserum (H51), monoclonal anti-human IFN-alpha antibody (Code NO.0050) and another polyclonal sheep anti-human IFN-alpha antiserum (gift from Dr. Cantell, Helsinki) were used for the demonstration of immunoreactive-IFN-alpha in various formalin fixed paraffin embedded normal human tissues, as described in chapter 2, section A.

A new macrophage/ histiocytic marker, KP1 monoclonal antibody (developed by K.A.F. Pulford, Oxford), was used in this study in addition to the macrophage/ histiocytic marker, anti-muramidase antiserum.

OBSERVATIONS

Observations from assessment of the specificity of the H51 antiserum.

Non-specific and specific positive staining with the H51 antiserum.

Using the H51 antiserum in the indirect immunoperoxidase technique, positive staining for immunoreactive-IFN-alpha was observed in spindle shaped cells scattered variably in almost all formalin fixed paraffin embedded normal human tissues studied except cerebral and cerebellar cortex in brain, renal cortex and medulla. Positive staining was also observed in some other parenchymal cells in the human body. However a problem of non-specific staining with the H51 antiserum was encountered when studying the distribution of IFN-alpha in various formalin fixed paraffin embedded human tissues. This non-specific staining was mimicked by substituting normal sheep serum for the H51 antiserum in the indirect immunoperoxidase technique. (The normal sheep serum was used as a negative control for the sheep anti-human IFN-alpha antiserum [H51] in the indirect immunoperoxidase technique, described in chapter 2). Incubation of the H51 antiserum with Wellferon (IFN-

alpha) prior to its use in the indirect immunoperoxidase technique, as described in the neutralization/ blocking experiment in chapter two section A(a), failed to abolish this non-specific staining. Non-specific staining, as described above, was observed in the mucosal epithelial lining cells, oxyphil and chief cells of all stomach cases studied, in the mucosal epithelial lining cells of the 5 jejunal and 2 duodenal cases and in the epithelial lining cells of the bronchial tree in all cases studied. Non-specific staining was also seen in the tubular epithelial lining cells in kidney and in Leydig cells in the testis in all the cases studied and in the endocrine cells in 1 anterior pituitary gland and in 5 parathyroid glands.

The term "specific positive staining" was used in this study to describe a situation where cells stained positively when H51 antiserum was used as primary antiserum, but did not stain when the H51 antiserum was replaced by normal sheep serum in the indirect immunoperoxidase technique (figure 3.1). Figure 3.1(a), shows that spindle shaped or round cells scattered variably in the lamina propria of the jejunal mucosa are positive for IFN-alpha using the H51 antiserum. These cells stained negatively with the normal sheep serum shown in figure 3.1(b). However, mucosal epithelial lining cells staining positive in figure

3.1(a), with the H51 antiserum, also show similar staining with the normal sheep serum in figure 3.1(b), demonstrating that the epithelial staining was non-specfic. Specific positive staining was further defined by showing that positive staining of cells was abolished if the H51 antiserum was preincubated with Wellferon (IFN-alpha) prior to its use, as described in the neutralization/ blocking experiment in chapter two section A(a) [figure 3.1(c); & figure 3.6]. Figure 3.1(c), shows that staining in spindle shaped or round cells in lamina propria in the jejunum completely disappeared, while non-specific staining observed in epithelial cells was not abolished by preincubation of the H51 antiserum with Wellferon in the neutralization/ blocking experiment. The neutralization/ blocking experiment with Wellferon was carried out on at least one section of all human tissues studied.

The neutralization/ blocking experiment performed with recombinant IFN-beta.

In no instance did absorption of the H51 antiserum with IFN-beta alter the specific positive staining with the H51 antiserum [described in chapter two,section A(d,i)].

Immunoblots.

Immunoblot analysis [described in chapter two, section A(d,ii)] showed that the H51 antiserum reacted only with Wellferon (IFN-alpha) on the nitrocellulose paper strip (figure 3.2). The H51 antiserum did not recognize IFN-beta, tumour necrosis factor, interleukin 1-alpha and interleukin 1-beta, normal sheep serum, normal human serum and tris HCL buffer with the immunoblot technique.

Observations of specific positive staining in various formalin fixed normal human tissues.

Observations of specific positive staining for IFN-alpha with the H51 antiserum in various formalin fixed paraffin embedded normal human tissues are summarized in table 3.1.

It can be divided into 2 parts.

i) Specific positive staining observed in spindle shaped cells and other cells of the mononuclear phagocyte system.

ii) Specific positive staining observed in parenchymal cells.

TABLE 3.1

LOCALISATION OF IFN-ALPHA AND OTHER MACROPHAGE MARKERS IN FORMALIN FIXED NORMAL HUMAN TISSUES.

Organs	Type of cells +ve for IFN-alpha and their location in formalin fixed tissues	Relative numbers of cells positive with H5l	Relative numbers of cells positive with anti-muramidase	Relative numbers of cells positive with KPl
Gastrointestinal tract	spindle shaped (ss) cells in lamina propria, submucosa	+++	+++	+++
Liver	ss cells in the portal tracts	++	++	++
	Kupffer cells	+++	-ve	+++
Gall bladder	ss cells in submucosa	++	++	+++
Pancreas	ss cells in fibrous stroma	++	++	++
Bronchial tree	ss cells in submucosa	++	++	++
	ss cells in epithelium	+	+	+
Lungs	ss cells in interstitial tissue	+	+	+
	alveolar macrophages	+++	+++	+++
Kidney	ss cells in renal interstitium	-ve	+	+
	ss cells in glomeruli	+	+	++
Ureters and Renal pelvis	ss cells in submucosa	+	++	++

TABLE 3.1 continued /---

Urinary bladder	ss cells in submucosa	+	+	+
Prostate	ss cells in fibrous stroma	+	+	+
Testes	ss cells in fibrous stroma	+	+	++
Lymph nodes	ss cells in cortical and medullary sinuses	+++	+++	+++
	tingible body macrophages	+++	+++	+++
Tonsil	tingible body macrophages	+++	+++	+++
	ss cells around germinal centres	+++	+++	+++
Spleen	ss cells in red pulp	+++	+++	+++
Bone marrow	ss cells scattered between normal marrow cells	+	unsatisfactory staining	unsatisfactory staining
Blood	monocytes	-ve	+++	+++
Breast	ss cells in fibrous stroma	+	++	++
Fallopian tubes	ss cells in submucosa	+	+	+

TABLE 3.1 continued /----

Uterus endometrium	ss cells in the stroma	++	++	++
myometrium	ss cells between muscle fibres	+	+	+
Cervix	ss cells in submucosa, especially at squamo-columnar junction	++	+++	+++
Ovaries	ss cells in stroma, especially around corpus luteum	++	+	++
	ss cells in corpus luteum of menstruation	+++	-ve	+++
Heart	ss cells in fibrous stroma	+	+	+
Skeletal muscle	ss cells in fibrous stroma	+	+	+
Skin	ss cells in dermis	++	++	+
	ss cells in epidermis	+	-ve	-ve
Thyroid	ss cells in fibrous stroma	++	++	++
	parenchymal follicular cells	+++	-ve	-ve

TABLE 3.1 continued /---

Parathyroid	ss cells in fibrous stroma	++	++	++
	parenchymal endocrine cells	+++	-ve	-ve
Adrenal	ss cells in fibrous stroma	+++	-ve	+++
	cortical parenchyma	+++	-ve	-ve
Pituitary	endocrine cells in the anterior pituitary	+++	-ve	-ve
Brain	cuboidal epithelium of choroid plexus	+++	-ve	-ve
Placenta	syncytiotrophoblast	+++	-ve	-ve
	macrophages	++	++	++

HPF = High power field = 62,500 square microns
ss = Spindle shaped cells
\+ = Below 5 cells/HPF
++ = Between 5-15 cells/HPF
+++ = Above 15 cells/HPF.

i) Specific positive staining in spindle shaped cells and other cells of the mononuclear phagocyte system.

Table 3.1 shows that specific positive staining for IFN-alpha was observed in spindle shaped cells scattered variably in all human tissues studied except cerebral and cerebellar cortex in brain, renal cortex and medulla. A comparison between results with the H51 and anti-muramidase antisera and KP1 monoclonal antibody was made by counting positive cells in at least 10 high power fields (one high power field= 62,500 square microns) on consecutive serial sections, stained with the respective antisera. Most of the spindle shaped cells staining positively for IFN-alpha with the H51 antiserum also stained positively with the two macrophage/ histiocytic markers, anti-muramidase antiserum and the monoclonal antibody KP1 (figure 3.3). In bone marrow many spindle shaped cells showed positive staining with the H51 antiserum. The KP1 antibody and the anti-muramidase antiserum did not perform satisfactorily on these decalcified bone marrow sections, so comparisons were not possible here.

Other cells known to be components of the mononuclear phagocyte system also showed specific positive staining with H51 antiserum. These included Kupffer cells in the liver (figure 3.4), pulmonary alveolar macrophages in the lungs (figure 3.5),

tingible body macrophages and macrophage-like cells outside germinal centres in lymphoid tissue (figure 3.6). However osteoclasts in the bone marrow sections and monocytes in the buffy coats of the blood (both of which are components of the mononuclear phagocyte system) were negative for IFN-alpha. The alveolar macrophages, monocytes in the buffy coats of the blood, tingible body macrophages and macrophage-like cells outside the germinal centres in lymphoid tissues were also positive with KP1 and the anti-muramidase antiserum. Kupffer cells in the liver, however, were negative with anti-muramidase antiserum but strongly positive with KP1.

Double staining technique observations.

The monoclonal antibodies KP1 and Mac 387 (another macrophage marker) did not show sufficient immunofluorescence in the indirect immunofluorescene technique to be used in the double staining technique described in chapter two section(D). Only the anti-muramidase antiserum used in the indirect immunofluorescence technique showed strong immunofluorescence in macrophages, with little background, and it was therefore used in part(b) of the double staining technique. It was shown by using the double staining technique that some tingible body macrophages in human tonsil staining positively with

the H51 antiserum i.e containing immunoreactive IFN-alpha, also showed immunofluorescence with the anti-muramidase antiserum (macrophage marker) on the same tissue section (figure, 3.7).

When this study was carried out the antigen recognized by the KP1 monoclonal antibody was not known. Therefore it was important to assess the possibility of cross reaction between the KP1 antibody and the H51 antiserum. The experiment of absorption of KP1 antibody with Wellferon prior to its use in the indirect immunoperoxidase technique, as described in chapter 2, was thus performed to assess if the KP1 antibody recognized IFN-alpha in tissue sections.

Observations from experiment of absorption of the KP1 antibody with Wellferon.

Absorption of KP1 with Wellferon had no effect on staining using this antibody, demonstrating that KP1 antibody did not recognize IFN-alpha in tissue sections.

The KP1 antibody, as shown in table 3.1, stained all cells of the mononuclear phagocyte system except osteoclasts in the bone marrow. Comparison of KP1 antibody and the anti-muramidase antiserum showed that KP1 was a better macrophage/ histiocytic marker. The KP1 antibody showed positive staining in Kupffer cells in the liver and spindle shaped cells in the ovaries,

which stained negatively with the anti-muramidase antiserum. The KP1 monoclonal antibody also showed less background staining, as compared to the polyclonal anti-muramidase antiserum in tissue sections. Trypsinization of tissue sections for 20 minutes instead of the usual 10 minutes trypsinization described in the standard protocol for KP1 antibody staining procedure, resulted in a background staining in neutrophils which was not observed with 10 minutes trypsinization.

ii) Observations of specific positive staining with the H51 antiserum in parenchymal cells in various formalin fixed normal human tissues.

Specific positive staining for IFN-alpha with the H51 antiserum was seen in adrenocortical cells in the adrenal gland (figure, 3.8), cuboidal epithelium of the choroid plexus in brain (figure, 3.9), follicular epithelium in thyroid gland (figure, 3.10) and endocrine cells in the parathyroid and anterior pituitary gland (figure, 3.11 & 3.12). Specific positive staining for IFN-alpha was also seen in the syncytiotrophoblast in human placenta (figure, 3.13).

In the adrenal glands specific positive staining for IFN-alpha was observed in the adrenocortical cells of the zona fasciculata and zona glomerulosa. In the zona reticularis interpretation of the staining was

difficult because of the presence of brown staining lipofuscin, but some positive staining was probably present there also. In the 20 pituitary glands stained for IFN-alpha in this study, 19 showed specific staining in some of the endocrine cells of the anterior pituitary. Only one of the 20 pituitary glands showed non-specific staining. No attempt was made to define which pituitary endocrine cells contained IFN-alpha. In the 11 cases of parathyroid studied, 5 showed specific positive staining for IFN-alpha in endocrine cells. 6 showed only non-specific staining. The cuboidal cells of the choroid plexus in brain showed specific positive staining in all cases studied. The staining observed in the cuboidal epithelial cells of the choroid plexuses was uniformly distributed throughout the entire epithelial lining. Thyroid follicular cells also showed specific positive staining in all cases studied, however the intensity of the staining in the follicular cells varied from case to case. Specific positive staining was observed in the syncytiotrophoblast and in macrophages in all first, second and third trimester placentas studied. In no instance did the KP1 antibody or the anti-muramidase antiserum stain the parenchymal cells in these tissues.

Observations with the monoclonal anti-human IFN-alpha antibody (Code NO.0050).

The monoclonal antibody (Code No.0050) showed weak positive staining for IFN-alpha in the syncytiotrophoblast of formalin fixed paraffin embedded normal human placentas (n=5), when applied to the sections at a 1/100 dilution, with an overnight incubation at 4^{o}C and when using the avidin-biotin immunocytochemical technique [section A(ii),1] (figure, **3**.14). Trysinization of tissue sections or using the monoclonal antibody (Code.No.0050) in lower dilutions did not increase the positive staining in syncytiotrophoblast of the placenta [section A(ii),1,i]. However the positive staining in syncytiotrophoblast of the placenta improved slightly, when the biotinylated horse anti-mouse immunoglobulin antiserum was left on the sections for 2 hours with a 1/50 dilution [section A(ii),1,iii]. The staining was also slightly improved when the Vectastain ABC reagent was replaced by streptavidin-biotin complex in the biotin detection system [section A(ii),1,iv] (figure, **3**.15). However, no staining for IFN-alpha was observed with the monoclonal antibody (Code No:0050) in cuboidal epithelial cells of choroid plexus, endocrine cells or macrophages in other formalin fixed paraffin embedded normal human tissues.

In no instance did the negative control monoclonal antibody Clonab-LNC showed positive staining in the syncytiotrophoblast of placenta. When using the same staining protocol as the monoclonal anti-human IFN-alpha antibody, the positive control monoclonal antibodies, LN26 and EMA, showed positive staining in B lymphocytes and epithelial cells respectively (figure, **3**.16).

The second polyclonal sheep anti-human IFN-alpha antiserum (Dr.Cantell) also showed no positive staining for IFN-alpha in various formalin fixed human tissues studied.

Figure 3.1

Specific positive staining

In a) the section is stained by an indirect immunoperoxidase (IIP) technique using H5l as the primary antiserum. Many cells in the lamina propria of the jejunum stain positively. These cells do not stain when normal sheep serum is substituted for H5l in the technique (b), or when H5l is preincubated with Wellferon prior to use in the technique (c) (x l80)

Non-specific staining
The mucosal lining cells, by contrast, are stained in all three preparations.

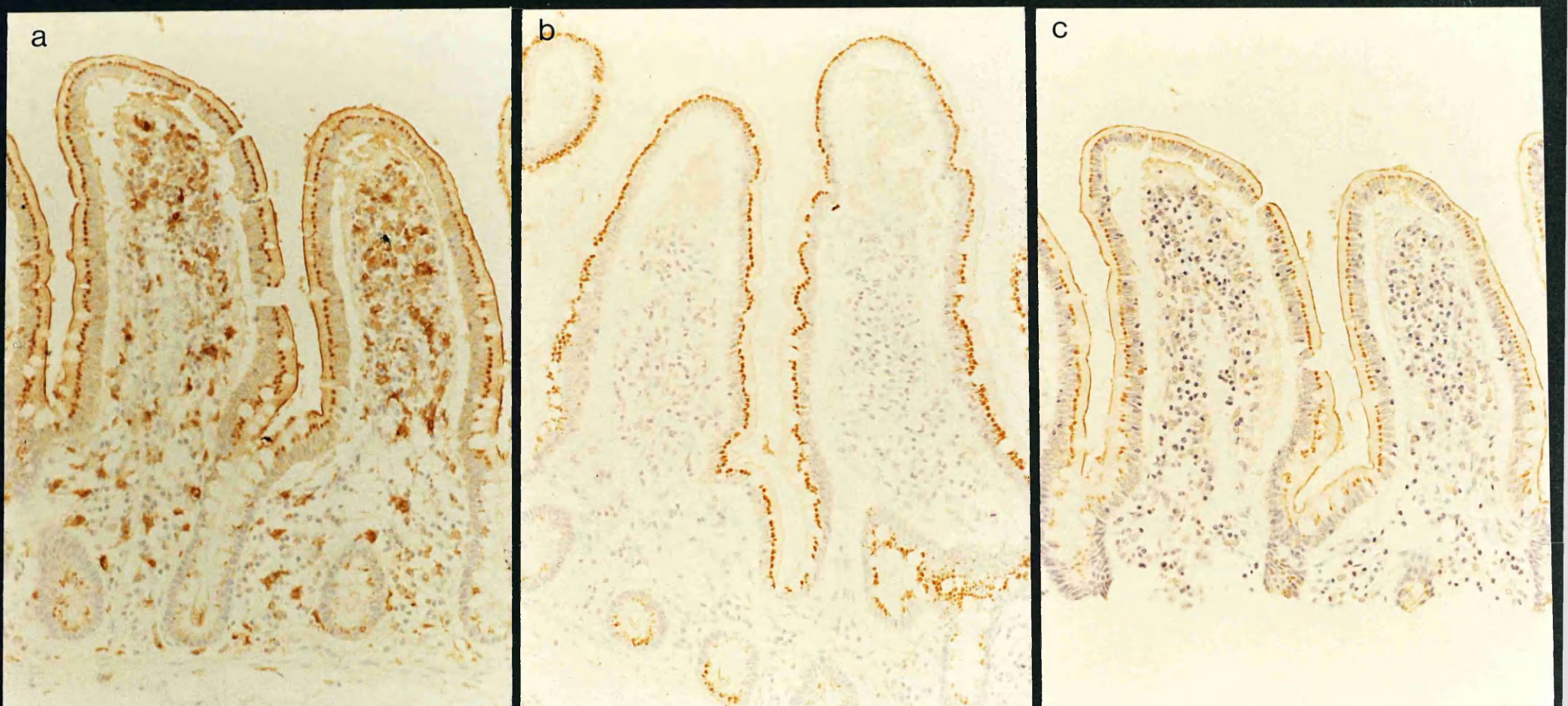

a
b
c

Figure 3.2

Immunoblot

This figure shows that the H5l antiserum recognised only Wellferon in the immunoblot technique. The materials spotted on the nitrocellulose paper strip from left to right (cut edge) are as follows: Normal human serum, normal sheep serum, tumour necrosis factor, Wellferon (IFN-alpha), IFN-beta, interleukin l-alpha, interleukin l-beta and tris HCL buffer.

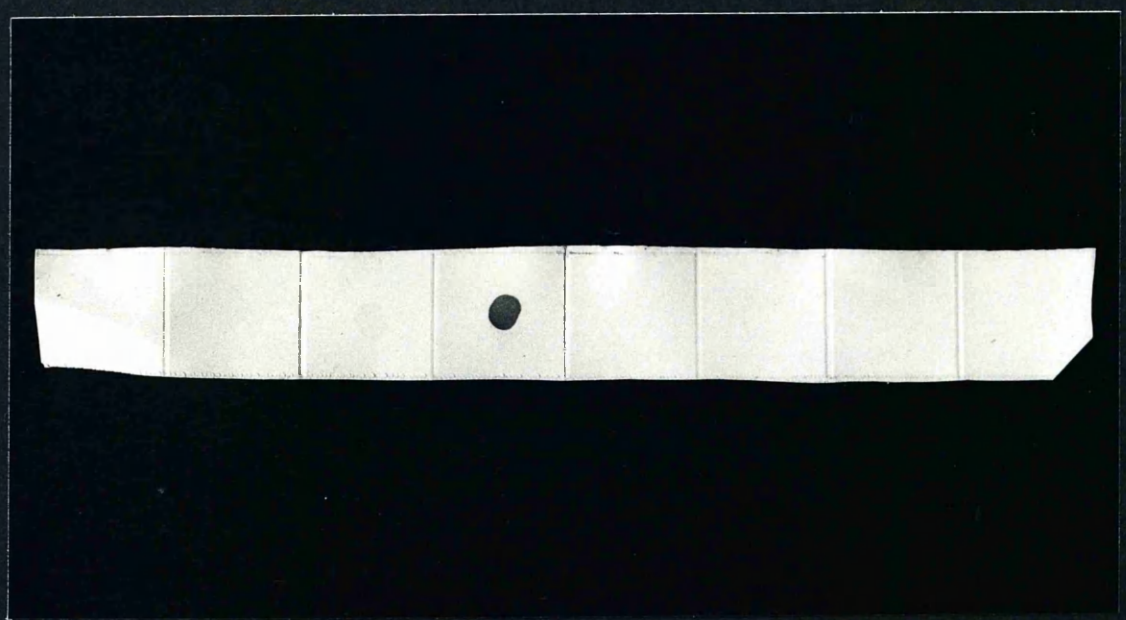
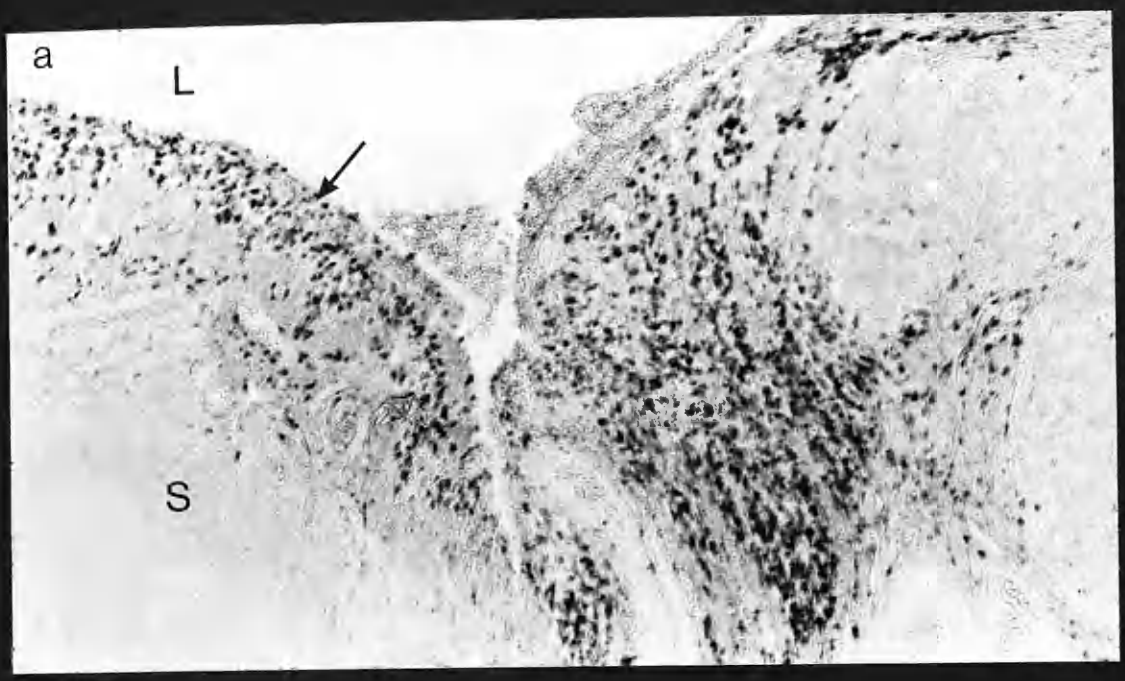


Figure 3.3

Involuting corpus luteum in ovary.

L represents the lumen of the corpus luteum and S the ovarian stroma. In between lies the wall of the corpus luteum which is heavily infiltrated by cells which express both IFN-alpha (a) and KP1 (b) (arrowed). This shows that cells expressing IFN-alpha in this corpus luteum are macrophages.

IIP for IFN-alpha in (a) and KP1 (b) (x 75).


a
L
S

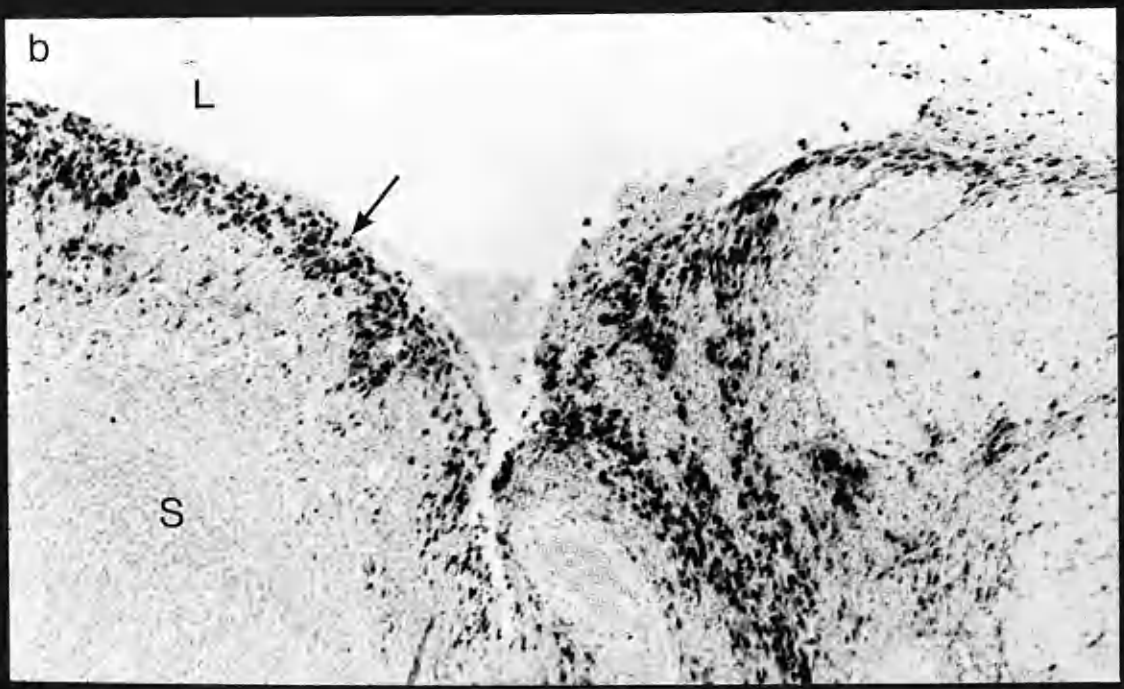

b
L
S

Figure 3.4

IFN-alpha in liver

Kupffer cells in the liver show positive staining for IFN-alpha.

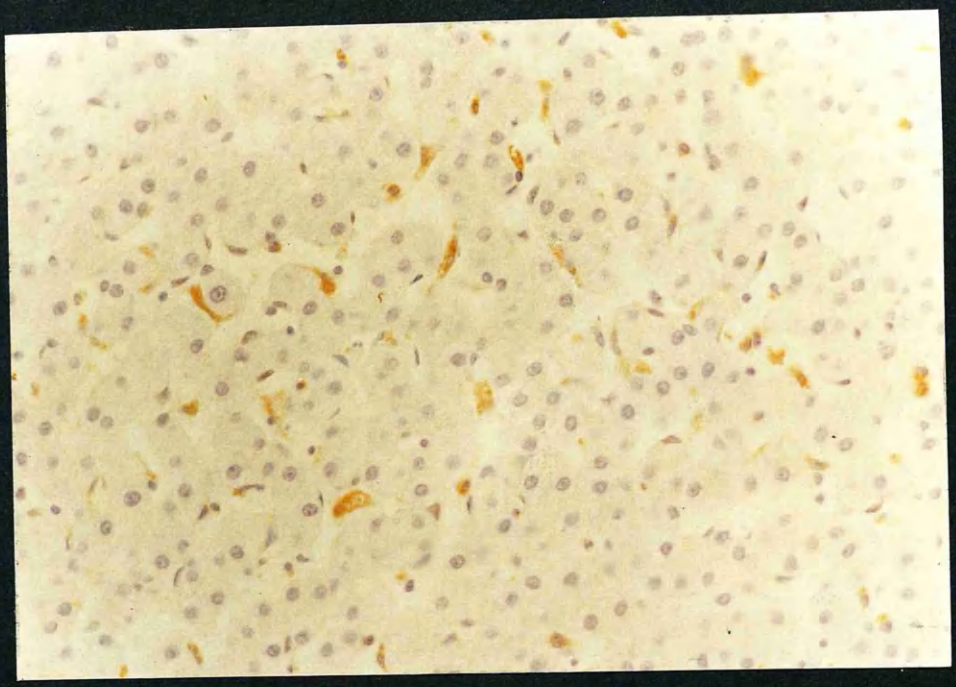
IIP for IFN-alpha (x 300).

Figure 3.5

IFN-alpha in lung

Pulmonary alveolar macrophages in the lungs show positive staining for IFN-alpha.

IIP for IFN-alpha (x 190)



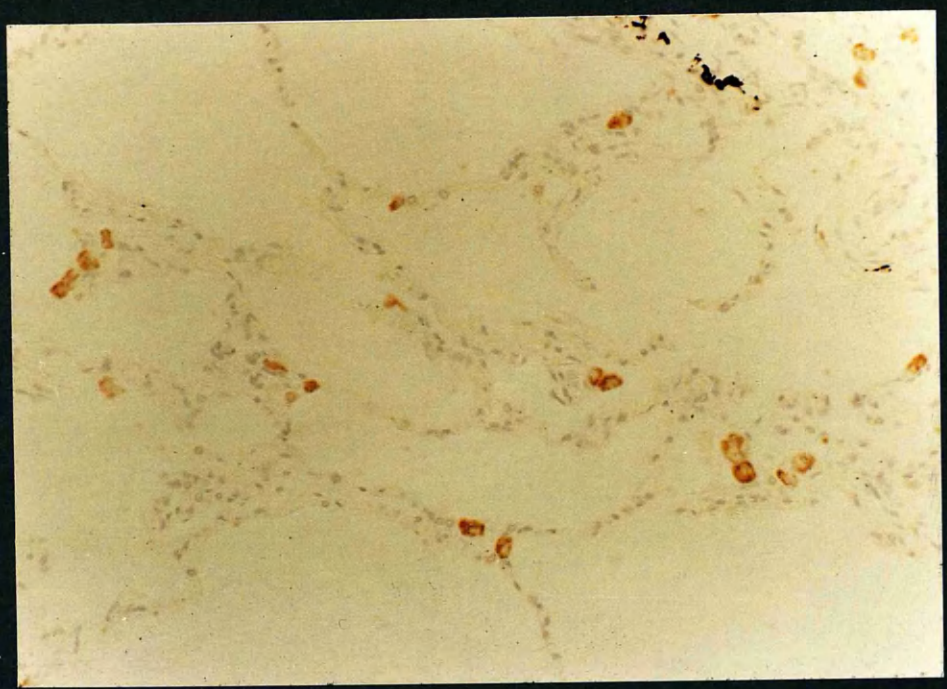
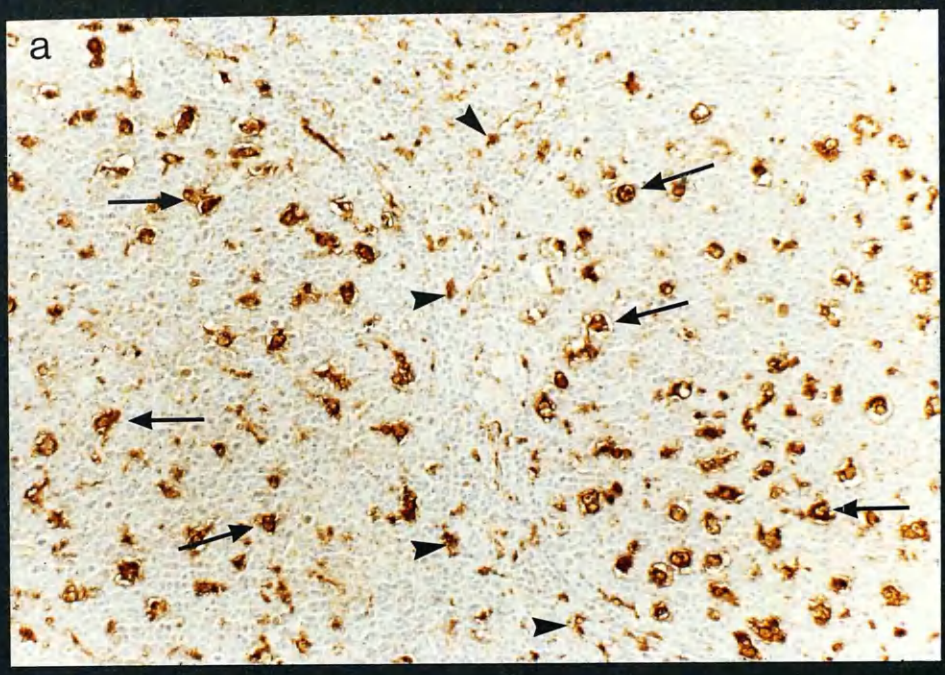


Figure 3.6

Absorption with Wellferon

Tingible body macrophages within germinal centres (arrows) in a lymph node are strongly positive for IFN-alpha as are some cells outwith the germinal centres (arrow heads) IIP for IFN-alpha (x 150) (a). (b) shows that all positive staining in these cells is completely abolished by preincubation of the H51 antiserum with Wellferon.


a

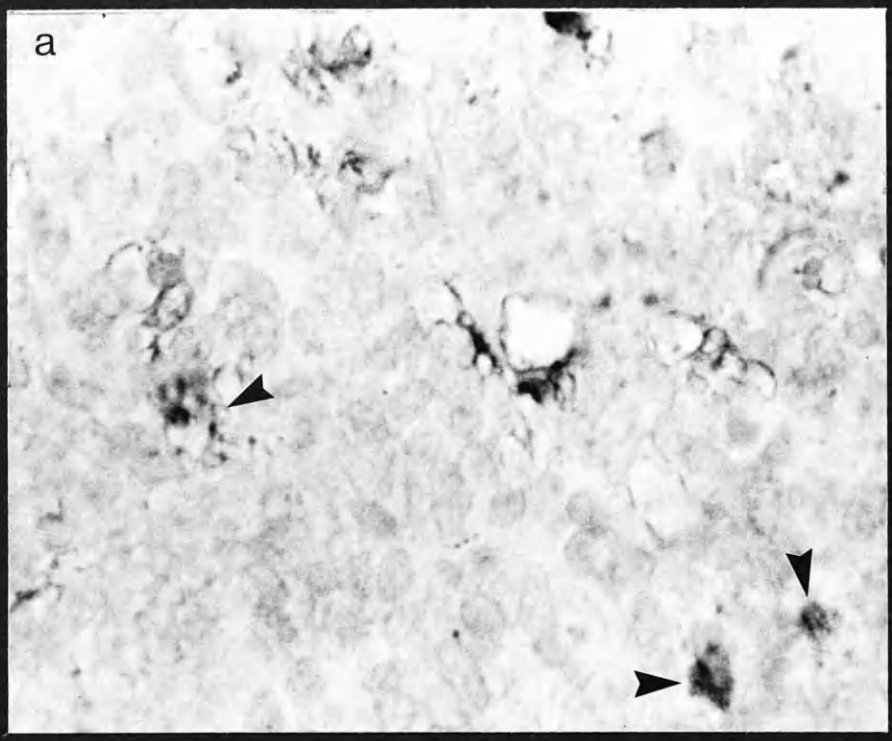

b

Figure 3.7

Adult tonsil.

Some tingible body macrophages staining positively for IFN-alpha (arrow heads), in (a), also fluoresce with anti-muramidase (macrophage marker) in (b). This shows that cells of the macrophage lineage contain IFN-alpha.

IIP for IFN-alpha (a) and immunofluorescence using anti-muramidase (b) on the same section (x 780).


a

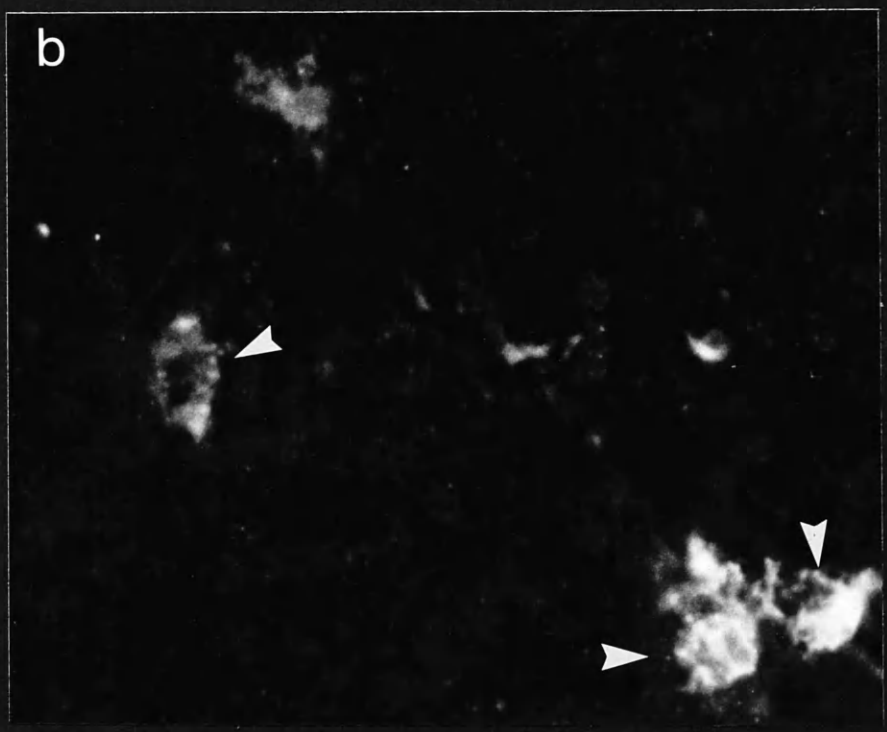

b

Figure 3.8

IFN-alpha in adrenal cortex

Adrenocortical cells in the adrenal gland show positive staining for IFN-alpha (arrows).

IIP for IFN-alpha (x 380).

Figure 3.9

IFN-alpha in choroid plexus

The cuboidal lining epithelium of choroid plexus (arrow) in the brain is strongly positive for IFN-alpha.

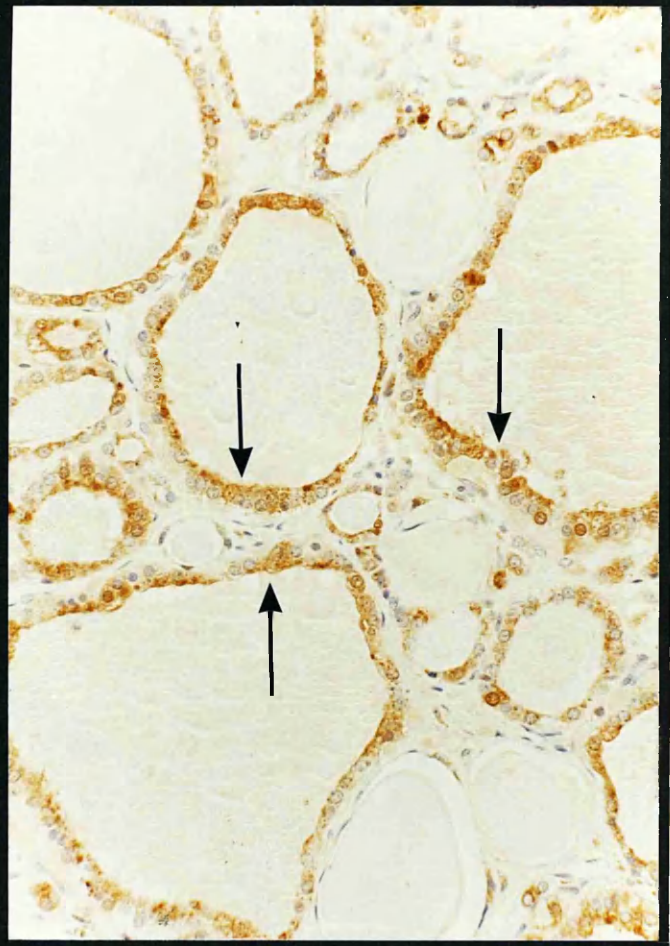
IIP for IFN-alpha (x 150).

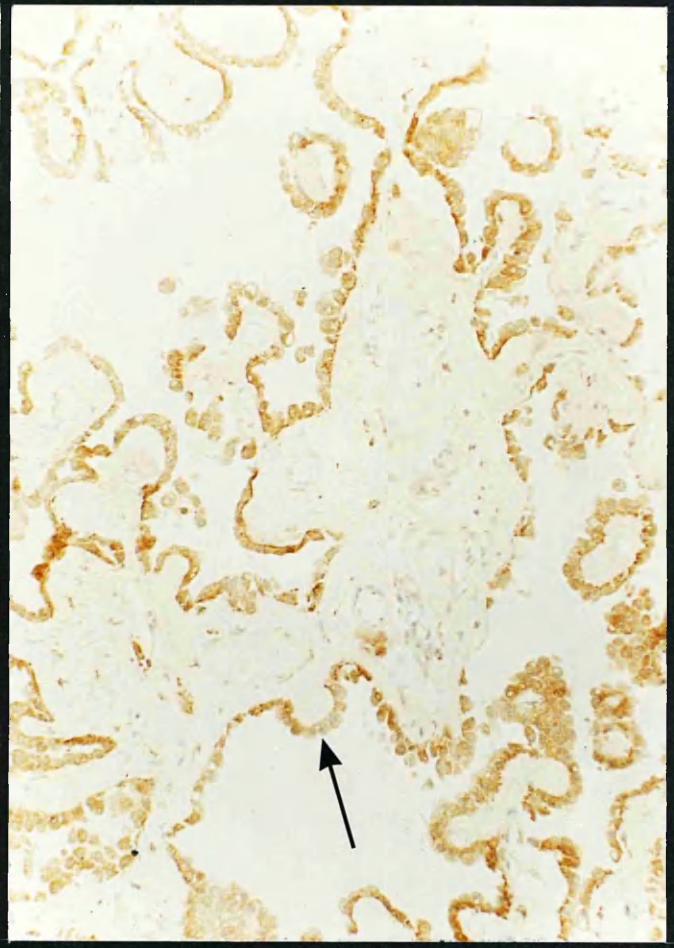
Figure 3.10

IFN-alpha in thyroid gland

Thyroid follicular cells (arrows) show positive staining for IFN-alpha.

IIP for IFN-alpha (x 270).





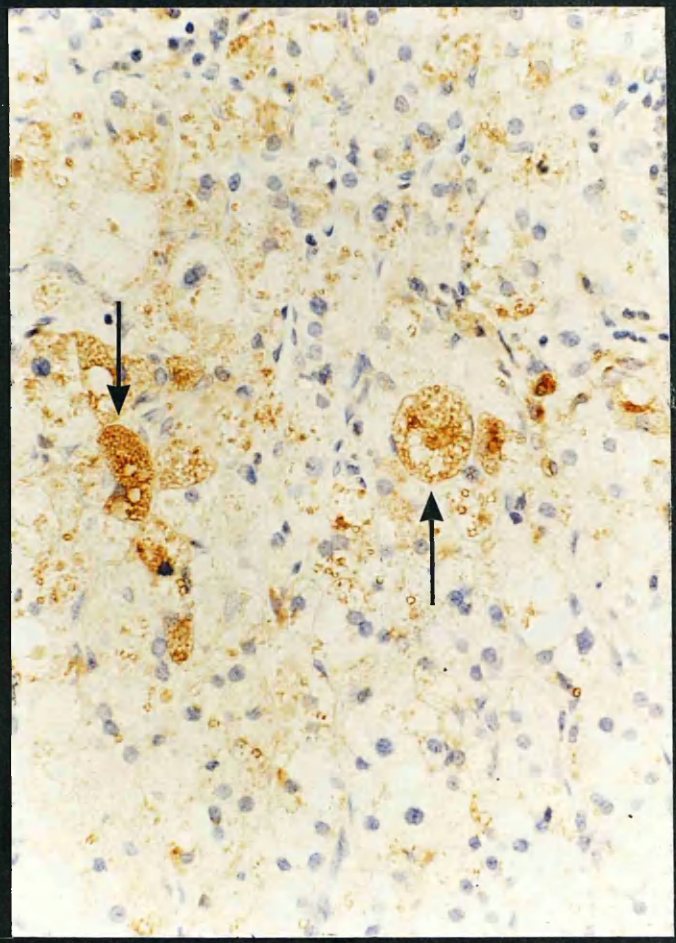


Figure 3.11

IFN-alpha in parathyroid gland

Endocrine cells in the parathyroid gland show positive staining for IFN-alpha.

IIP for IFN-alpha (x 300).

Figure 3.12

IFN-alpha in anterior pituitary

Some endocrine cells in the anterior pituitary gland show positive staining for IFN-alpha.

IIP for IFN-alpha (x 300).

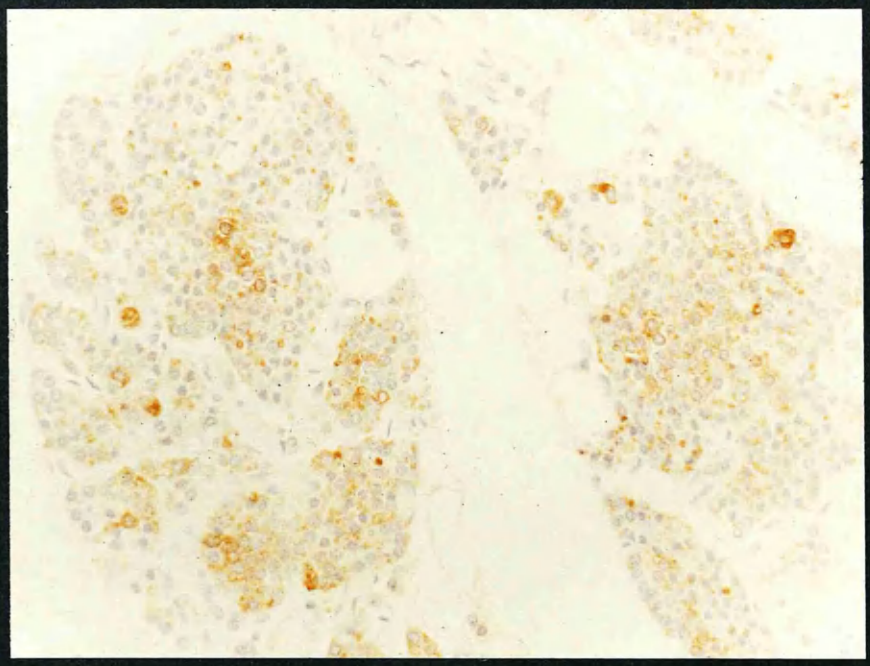


Figure 3.13

IFN-alpha in placenta (H51)

Syncytiotrophoblast in the placenta show positive staining for IFN-alpha.

IIP for IFN-alpha with the polyclonal sheep anti-human IFN-alpha antiserum (H51) (x 380).

Figure 3.14

IFN-alpha in placenta (monoclonal antibody)

Syncytiotrophoblast in the placenta show weak positive staining for IFN-alpha.

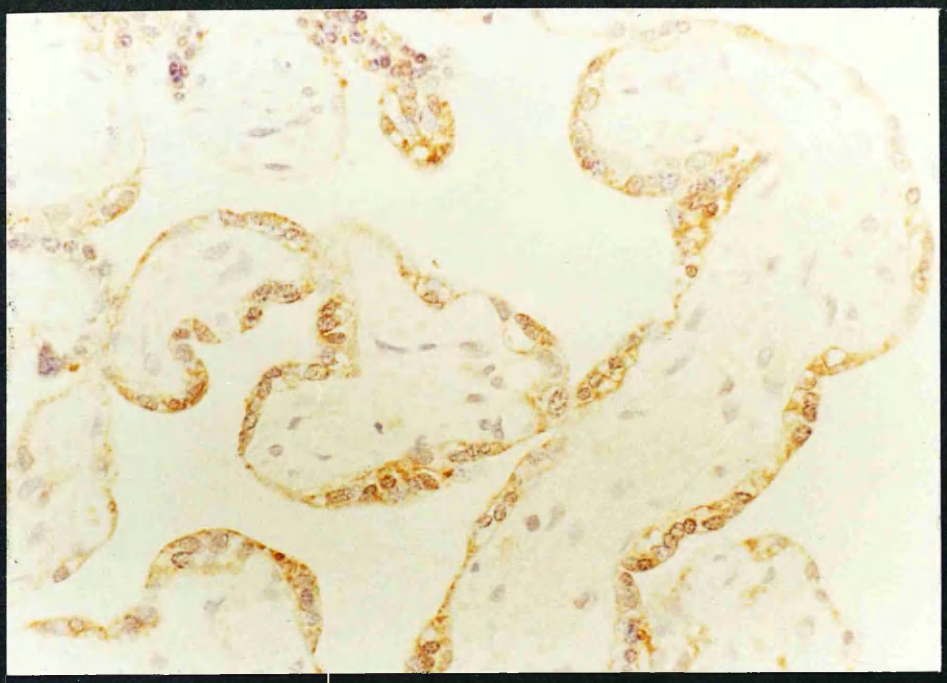
Avidin-biotin for IFN-alpha with the monoclonal anti-human IFN-alpha antibody (Code No. 0050) (x 210).

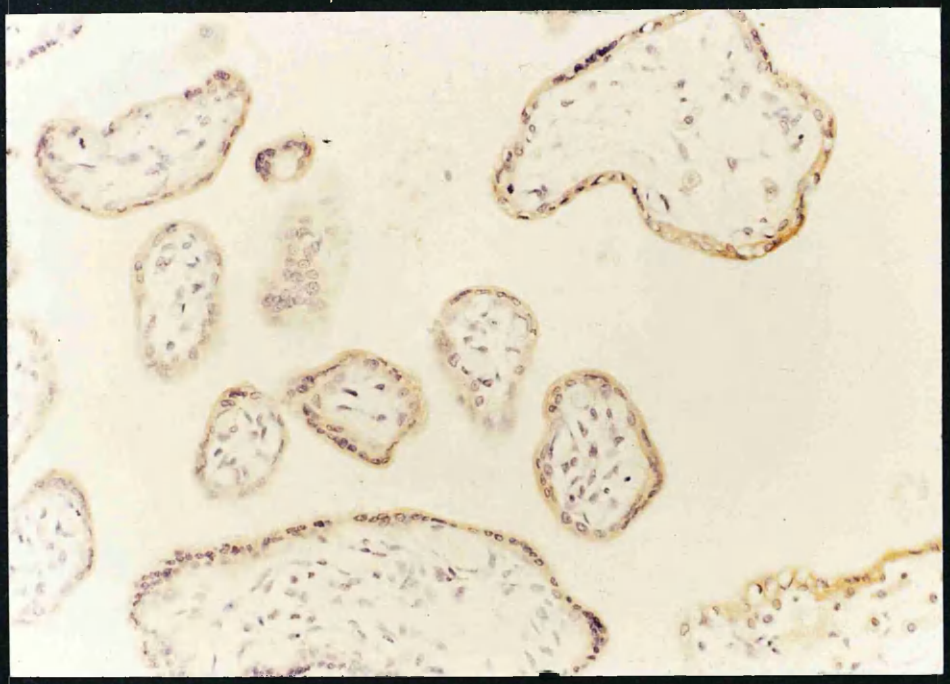
Figure 3.15

IFN-alpha in placenta (monoclonal antibody)

Syncytiotrophoblast in the placenta show slightly strong positive staining for IFN-alpha than in figure 3.14.

Streptavidin-biotin for IFN-alpha with the monoclonal anti-human IFN-alpha antibody (Code No. 0050) (x 390).





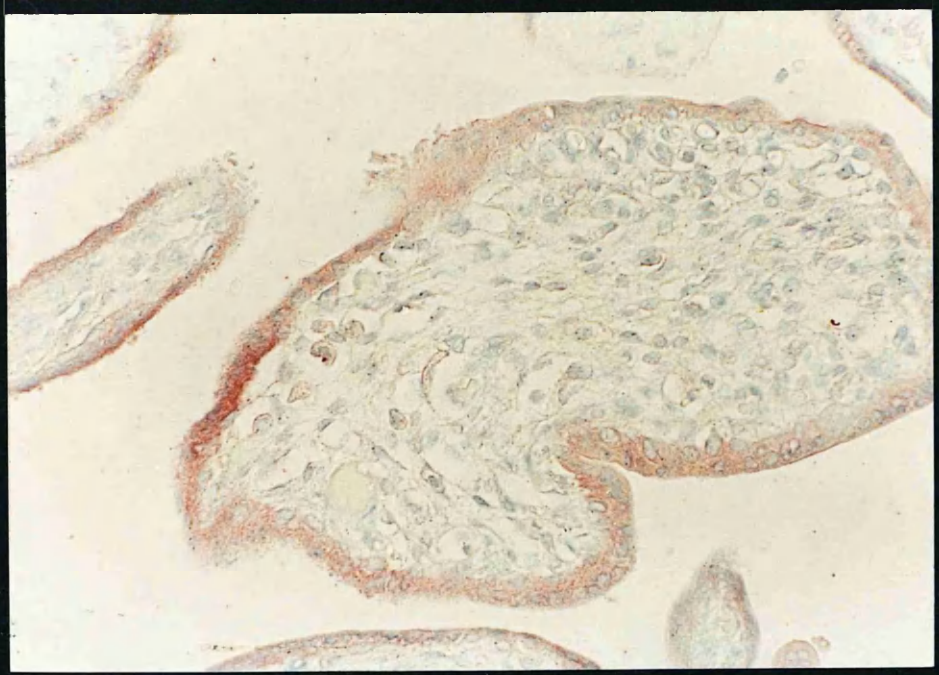


Figure 3.16

Female breast (EMA)

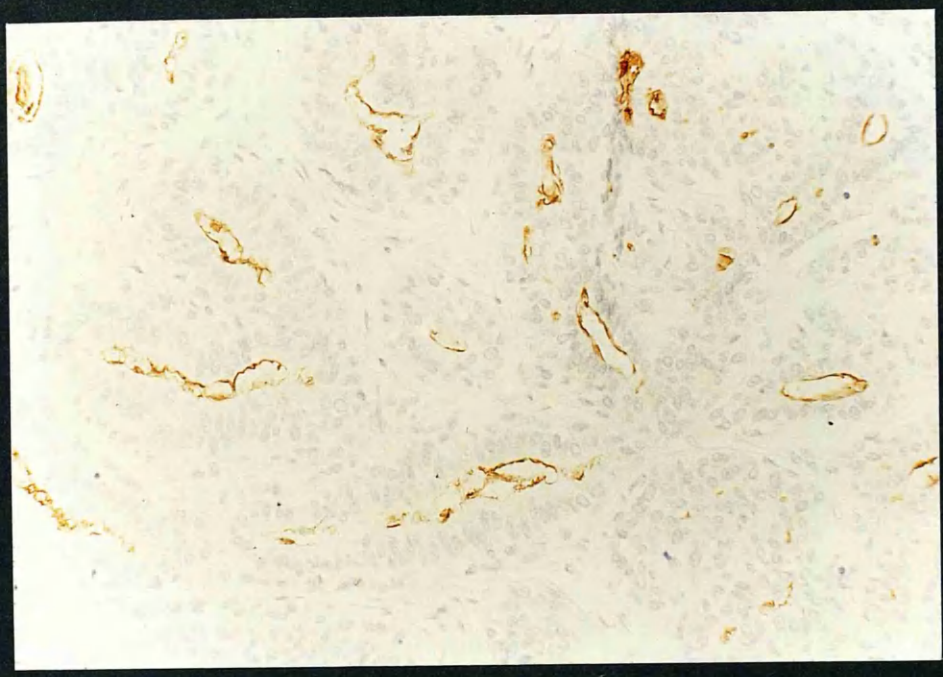
Epithelial cells in the breast are staining positively for epithelial membrane antigen (EMA)

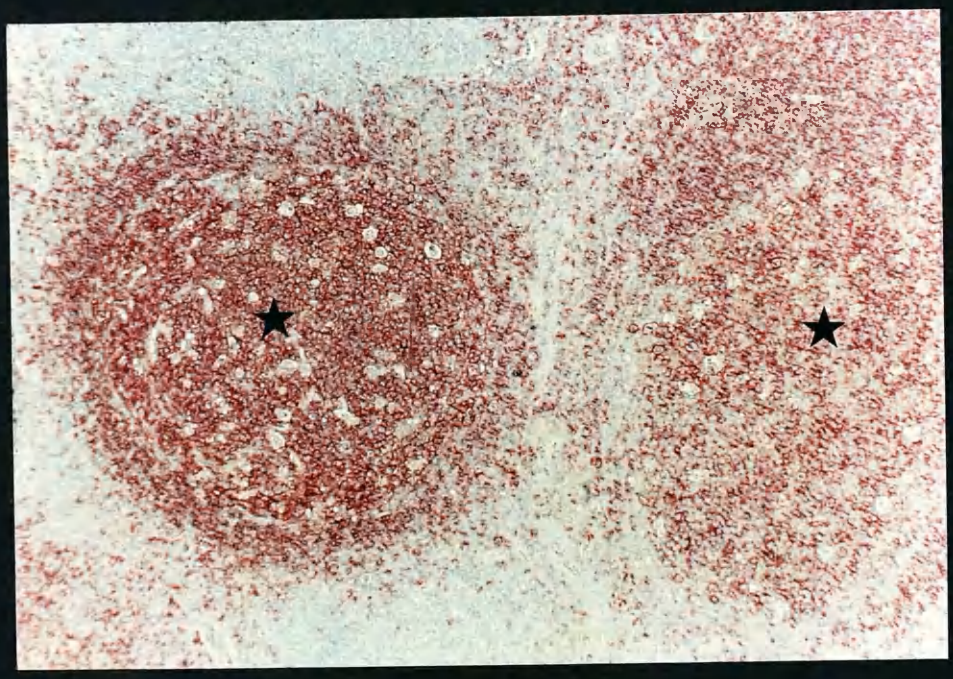
Avidin-biotin for EMA (x 200)

Lymph node (LN26)

Two germinal centres are marked (✱) in this section. B lymphocytes mainly present in these germinal centres are positive for LN26 (B cell marker) as are some B lymphocytes outwith the germinal centres.

Streptavidin-biotin for LN26 (x 80)





DISCUSSION

This study demonstrated that spindle-shaped cells, positive for immunoreactive IFN-alpha with the H51 antiserum, were present in nearly all human tissues except cerebral and cerebellar cortex in brain and renal cortex and medulla. These cells also showed a similar staining with the two macrophage/histiocytic markers, KP1 antibody and anti-muramidase antiserum, suggesting that the cells are a part of the mononuclear phagocyte system and probably are tissue histiocytes, normally present in human tissues. Other cells known to be part of the mononuclear phagocyte system, such as tingible body macrophages, Kupffer cells and pulmonary alveolar macrophages were also positive for IFN-alpha with the H51 antiserum. The finding that monocytes in the peripheral blood were negative for IFN-alpha is confirmation of previous work (Saksela *et al.*,1984).

While it is conceded that the demonstration of immunoreactive IFN-alpha in tissue histiocytes does not provide direct evidence that they are actually producing IFN-alpha, its presence in these and other cells known to be part of the mononuclear phagocyte system, such as tingible body macrophages, Kupffer cells and pulmonary alveolar macrophages in over 300 hundred individuals with no clinical evidence of viral infection, leads to the conclusion that most cells of

the mononuclear phagocyte system probably produce IFN-alpha under normal circumstances.

One cause of the synthesis of IFN-alpha by such cells may be the presence of low levels of inducers of IFN-alpha synthesis in normal human tissues (Bocci,1981). Endotoxin released from gut bacteria may be one such inducer (Bocci,1988). The human body is also exposed to a variety of microbes (viruses, bacteria, etc) under physiological conditions. These microbes do not produce any evident clinical infection because they are efficiently counteracted by the host defences. This stimulus may, however, be sufficient to activate cells of the mononuclear phagocyte system (which are usually the first line of defence against infections and other exogenous stimuli) to produce IFN-alpha and other cytokines. Many workers have suggested that there may be production of low levels of endogenous IFN-alpha under physiological conditions (Gresser *et al.*,1985; Belardelli *et al.*,1984; Galabru *et al.*,1985; Zoumbos *et al.*,1985; Gessani *et al.*,1987; Bocci *et al.*,1984; Bocci,1988). While the precise origin of this IFN-alpha production has not been clearly defined, some evidence has suggested that macrophages may be involved. It has been shown that normal rabbit and mouse peritoneal macrophages release low levels of IFN *in vitro* (Smith & Wagner,1967; De-Maeyer *et al.*,1971). Gresser *et al* (1985) showed that

injection of mice with antibody to mouse interferon-alpha/beta decreased the level of 2'-5' oligoadenylate synthetase (marker of IFN action) *in vitro* in peritoneal macrophages, and rendered them permissive for vesicular stomatitis virus infection. Similarly, in another study, many animal viruses failed to multiply in fresh cultures of normal mouse peritoneal macrophages but they were rendered permissive by injecting mice with an antiserum directed against IFN-alpha and beta prior to harvesting, suggesting that peritoneal macrophages are maintained in an anti-viral state by endogenous IFN-alpha in vivo (Belardelli *et al.*,1987).

It is therefore tempting to suggest that the cells of the mononuclear phagocyte system, shown positive for immunoreactive IFN-alpha in our study, might be the main source of the low levels of endogenous IFN-alpha found in physiological conditions. It could also be inferred that these cells play a front line role in the defence against viral infection.

It is of course possible that mononuclear phagocyte cells may produce IFN-alpha in the absence of known inducers of its synthesis, and speculation that IFN-alpha produced by these cells may have a role in defence against viral infection does not preclude other possible roles for this substance, such as modulation of cell growth and the immune response.

The finding of immunoreactive IFN-alpha in the cuboidal epithelium of choroid plexus is in keeping with the demonstration of detectable levels of IFN-alpha in the cerebrospinal fluid of normal individuals (Ho-Yen & Carrington,1987). The brain contains neurons which are permanent cells (permanent cells once destroyed cannot be replaced). Viral infection of brain thus has disastrous effects but these viral infections are remarkably infrequent. The choroid plexus produces cerebrospinal fluid which bathes the whole brain. It is therefore suggested here that IFN-alpha may be synthesized by cuboidal epithelial cells of choroid plexus and released into the cerebrospinal fluid, where it may have a role in preventing viral infections in the central nervous system.

Using the H51 antiserum immunoreactive IFN-alpha was demonstrated in the syncytiotrophoblast and macrophages in normal human placenta. When this study was conducted the findings of Howatson *et al* (1988) of the presence of immunoreactive IFN-alpha in the syncytiotrophoblast and macrophages in normal human placenta were not yet published. Immunoreactive IFN-alpha was also demonstrated in the syncytiotrophoblast in human placentas using the monoclonal anti-human IFN-alpha antibody (Code No.0050). This was confirmation of the findings by other workers that IFN-alpha was present in the human placenta (Chard *et al.*,1986; Bocci

et al.,1985; Duc-Goiran *et al.*,1985). However these authors did not establish the site of IFN-alpha synthesis. It is of interest that sheep corpus luteal regression is prevented by release of the hormone ovine trophoblastic anti-luteolytic protein (oTP-1) from sheep trophoblast. This hormone has been shown to have significant nucleotide and amino acid sequence homology with IFN-alpha (Imakawa *et al.*,1987; Stewart *et al.*,1987). Furthermore human IFN-alpha and oTP-1 have similar effects on cultured sheep endometrial cells (Salamonsen *et al.*,1988). It has been demonstrated recently that IFN-alpha enhances the ectopic production of the beta-subunit of human chorionic gonadotrophin by bladder tumour cells (Iles & Chard,1989). Whether or not IFN-alpha has a hormonal effect in human pregnancy awaits investigation.

The stimulus for the production of IFN-alpha in human pregnancy is not known. Inducers of IFN-alpha synthesis such as viruses, bacteria, endotoxins, etc can be excluded as a possible source of stimulus for induction of IFN-alpha synthesis in the placenta, since IFN-alpha production seems to be a common phenomenon, demonstrated by many workers, in normal human pregnancy with no evidence of microbial infections. Viral infections in fetuses are relatively rare in spite of a poorly developed fetal immune system. It is possible that IFN-alpha synthesis by the placenta and fetal

tissues may be partly responsible for this low incidence of viral infections in fetuses. IFN can also affect cell growth and differentiation (Paucker *et al.*,1962). The fetal tissues are being constantly remodelled and therefore it is equally possible that IFN-alpha is produced as a normal cytokine in human pregnancy along with other factors that control or influence cell growth and differentiation of fetal tissues.

The demonstration of immunoreactive IFN-alpha in endocrine cells of thyroid follicles, parathyroid, anterior pituitary and adrenal cortex with the H51 antiserum was completely unexpected. However the possibility of various hormonal effects of IFN-alpha in man awaits further investigation.

The monoclonal anti-human IFN-alpha antibody (Code No.0050) also demonstrated immunoreactive IFN-alpha in the syncytiotrophoblast in formalin fixed paraffin embedded normal human placentas. However, it failed to stain any macrophage type cells or other endocrine cells in the human body. It is possible that the monoclonal anti-human IFN-alpha antibody (Code No.0050) antibody recognises a specific IFN-alpha protein antigen that is found only in the placenta. Also, in contrast to the polyclonal antiserum, the monoclonal antibody recognises only one specific epitope. If that particular epitope is not exposed in fixed tissues or

is denatured or is not present in adequate amounts for the antibody to react with, no positive reaction would be obtained.

The fact that the second sheep polyclonal anti-human IFN-alpha antiserum did not detect IFN-alpha in formalin fixed paraffin embedded human tissues, but the H51 did, suggests that relatively few epitopes of the interferon molecule are unchanged by formalin fixation.

KP1 was a new macrophage/ histiocytic monoclonal antibody developed by K.A.F. Pulford,Oxford. When the present study was carried out the antigen recognised by KP1 monoclonal antibody was not known. The KP1 antibody was shown to recognize macrophages in nearly all human tissues studied with little background problems and proved better as a macrophage marker than the anti-muramidase antiserum. This study (published in Khan *et al.*,1989) was the first to show the specificity of the KP1 antibody as a macrophage/ histiocytic marker. Results similar to these with the KP1 antibody were confirmed in a subsequent study (Pulford *et al.*,1989).

CHAPTER FOUR

THE DISTRIBUTION OF INTERFERON-ALPHA IN FORMALIN FIXED PARAFFIN EMBEDDED NORMAL FETAL AND INFANT HUMAN TISSUES.

INTRODUCTION

In the previous chapter the cellular distribution of immunoreactive IFN-alpha was studied in formalin fixed paraffin embedded normal adult human tissues. The indirect immunoperoxidase technique using the H51 antiserum showed that tissue histiocytes, Kupffer cells, pulmonary alveolar macrophages and lymph node macrophages contained immunoreactive IFN-alpha. Parenchymal cells in some other organs also contained immunoreactive IFN-alpha. It was suggested that the presence of IFN-alpha in most cells of the mononuclear phagocyte system may play a major part in defence against viral infection.

Bocci (1988) has proposed that since bacteria and endotoxin are known inducers of IFN-alpha synthesis (Havell *et al.*,1986; Gessani *et al.*,1987) much of the physiological IFN-alpha response may be due to the presence of the normal bacterial flora in the gut, skin, mouth and genital tract. This hypothesis is supported by *in vitro* studies which have shown spontaneous release of IFN-alpha by peritoneal

macrophages from mice kept in a normal environment (De-Maeyer *et al.*,1971). It has also been observed that mice kept in a germ free environment, or those treated with broad spectrum antibiotics, which destroy the normal microbial flora, have lower levels of the IFN mediated enzymes, 2-5A synthetase and protein kinase, than mice kept under normal environmental conditions (Galabru *et al.*,1985). Maximal production of IFN-alpha in mice occurs at 8 weeks of age, shortly after weaning, when the animal would be expected to experience maximal novel microbial challenge (Black-Olszewska, Cambzynska-Nowak and Kwasniewska, 1984).

In this study an attempt was made to test Bocci's hypothesis (1988) of physiological IFN synthesis in man by comparing the frequency of IFN-alpha containing tissue cells in fetuses with that found in infants. A normal fetus is germ free but after birth it is exposed to a large variety of microbes. Therefore, if Bocci's hypothesis were true, low numbers of IFN-alpha positive cells might be expected up until birth, after which a dramatic rise might be predicted.

The other objectives of the present study were 1) to establish whether the cellular distribution of IFN-alpha containing cells in human fetal tissues was the same as that found in adults and 2) to find out at what stage of fetal life IFN-alpha containing cells appeared.

Determination of proportion of macrophages expressing IFN-alpha.

To assess the proportion of macrophages expressing IFN-alpha in various tissues in infants and fetuses, cells positive with the monoclonal antibody KP1 and those containing immunoreactive IFN-alpha were counted in the same 10 high power fields on consecutive serial sections (one high power field = 62,500 square microns).

Assessment of quantitation of positive cells using the serial section technique.

Two successive serial sections from 9 blocks of tissue (4 thymus, 4 liver, 1 spleen) were stained for IFN-alpha. Similarly two further successive serial sections from the same blocks of tissue were stained with the KP1 antibody. The number of positive cells counted in the same 10 high power fields on the first and second serial section was counted for each antibody technique. A linear regression analysis was then performed to assess how closely the results on the second serial section correlated with those on the first serial section.

OBSERVATIONS

Like the study described in chapter 3, a similar problem of non-specific staining was seen using the H51 antiserum in formalin fixed paraffin embedded normal

fetal and infant tissues. This non-specific staining was mimicked by substituting normal sheep serum for the H51 antiserum in the indirect immunoperoxidase technique. Non-specific staining was observed in the epithelial lining cells of the bronchial tree and in the tubular epithelial lining cells in kidneys in all cases studied. Non-specific staining was also seen in Hassall's corpuscles in the fetal and infant thymus.

The term "specific positive staining" as defined in chapter 3 was used in this study to describe a situation where cells stained positively when H51 antiserum was used as primary antiserum, but did not stain when the H51 antiserum was replaced by normal sheep serum in the indirect immunoperoxidase technique. Specific positive staining was further defined by showing that positive staining of cells was abolished if the H51 antiserum was preincubated with Wellferon (IFN-alpha) prior to its use, as described in the neutralization/ blocking experiment with Wellferon, chapter 2, section A(a). This neutralization/ blocking experiment was carried out on at least one section of all fetal and infant tissues studied (figure 4.10).

i) Ontogeny.

Specific positive staining for IFN-alpha was first observed at 9 weeks gestation in spindle shaped or somewhat rounded cells scattered variably in the fetal

liver (figure 4.1). Thereafter, specific positive staining for IFN-alpha was seen in similar cells scattered in all fetal and infant organs except kidney and cerebral and cerebellar cortex in brain. As described in chapter 2 section C, a special serial section "mirror image" technique was used to study co-localization of different antigens in the same cell. This helped to identify which cells contained immunoreactive-IFN-alpha. In this study the first serial section was stained with the H51 antiserum for IFN-alpha and the second mirror image serial section was stained for KP1 (macrophage marker), as described in chapter 2. Most of the spindle shaped or somewhat rounded cells containing immunoreactive IFN-alpha appeared to show a similar positive staining with the macrophage/ histiocytic marker, KP1 monoclonal antibody (figure 4.2). Thymus, in both fetuses and infants, proved to be an exception in this regard (figure 4.3).

ii) Observations from assessment of quantitation of positive cells using the serial section technique.

Quantitation of positively staining cells on successive serial sections appeared to be reliable. When cells were counted on 2 adjacent serial sections stained for IFN-alpha, as described earlier, and results subjected to regression analysis the *r* value

was 0.997. The equivalent figure for KP1 staining was 0.999.

iii) Determination of proportion of macrophages expressing IFN-alpha.

Figure **4**.4 illustrates the number of cells positive with KP1 antibody in 10 high power fields (as previously defined) in the thymus, spleen, liver and lungs of infants and fetuses (12 weeks gestation and above). It demonstrates a statistically significant increase in the number of KP1 positive cells in infant lungs when compared to fetal lungs ($P<0.0001$, Mann-Whitney test). The number of KP1 positive cells in fetal and infant thymus, spleen and liver did not show any statistically significant difference between the 2 groups.

The number of spindle shaped or rounded cells positive for IFN-alpha in 10 high power fields in the thymus, spleen, liver and lungs in infants and fetuses is illustrated in figure **4**.5. It also shows a statistically significant increase in the number of immunoreactive IFN-alpha positive cells in the infant lungs when compared to fetal lungs ($P<0.0001$, Mann-Whitney test). There was no statistical difference between the numbers of IFN-alpha positive cells in spleen, thymus and liver found in fetuses compared to the numbers found in infants.

Figure **4**.6 illustrates the percentage of KP1 positive cells containing immunoreactive IFN-alpha in 10 high power fields in the spleen, liver and lungs of infants and fetuses. The percentage of macrophages expressing IFN-alpha was significantly greater in the infant lungs compared to fetal lungs ($P<0.0005$, Mann-Whitney test). Such a difference was not observed in the other organs examined. Alveolar macrophages shown positive for IFN-alpha in the lungs of an infant (aged 5 months) are shown in figure **4**.7.

In the thymus, as shown in figure **4**.3(a) many cells containing immunoreactive IFN-alpha failed to stain positively with the KP1 monoclonal antibody (macrophage/ histiocytic marker) figure **4**.3(b). Tissue sections from these thymus cases were also stained with two other macrophage markers, the monoclonal antibody MAC 387 and the polyclonal anti-muramidase antiserum, using an indirect immunoperoxidase technique described in chapter 2. This showed that most IFN-alpha containing cells in the thymus stained negatively with the monoclonal antibody MAC 387 and the polyclonal anti-muramidase antiserum, demonstrating that these cells were probably not macrophages. A double staining technique was used to establish whether cells in the thymus containing immunoreactive IFN-alpha were thymic epithelial cells. The double staining technique utilized the H51 antiserum in part(a) and a polyclonal

anti-prekeratin antibody (epithelial cytokeratin marker) in part(b), as described in chapter 2, section(D). It showed that cells positive for immunoreactive IFN-alpha failed to fluoresce for prekeratin and were therefore probably not epithelial cells (figure **4**.8). The control sections (adult human thyroid), used in the double staining technique, double stained for IFN-alpha and prekeratin, showing that there was no problem in identifying both these markers within the one cell (figure **4**.9).

Unfortunately the number of spindle shaped cells positive for IFN-alpha in the gastrointestinal mucosa of fetuses could not be counted and compared with that in infants because of post mortem autolysis in the fetal gastrointestinal mucosa.

iv) IFN-alpha in parenchymal cells of various human tissues.

The findings of IFN-alpha in parenchymal cells of various human fetal tissues are summarized in table **4**.1.

It shows that specific positive staining for immunoreactive IFN-alpha was first observed in the fetal zone of the adrenal cortex at 10 weeks gestation (figure **4**.10). This zone is a major part of the fetal adrenal gland and produces a steroid, dehydroepiandrosterone sulphate which is transported to

the placenta and used as a precursor for the production of estrogens, necessary for the maintenance of pregnancy. This fetal zone disappears rapidly after birth. All fetal adrenal glands above 10 weeks gestation showed a similar specific positive staining for IFN-alpha. Staining observed in cells in the fetal zone of adrenal glands was very strong compared to staining seen in the adrenocortical cells in adult adrenal glands, described in chapter 3. Cuboidal epithelium of choroid plexuses in the brain showed specific positive staining for IFN-alpha at 11 weeks gestation (figure **4**.11). The cuboidal epithelium of all choroid plexuses from fetuses above 11 weeks gestation and from infants were positive for immunoreactive IFN-alpha. The specific positive staining observed in the cuboidal epithelium of choroid plexus in some cases was patchy and not as uniform as that observed in the adult choroid plexus described in chapter 3. Specific positive staining for IFN-alpha was demonstrated in thyroid follicular cells at 13 weeks gestation (figure **4**.12). All thyroid glands of fetuses above 13 weeks gestation and of infants showed specific positive staining for IFN-alpha in the thyroid follicular cells. Some endocrine cells in the anterior pituitary glands showed specific positive staining for IFN-alpha at 13 weeks gestation and onwards.

TABLE 4.1

TITLE: LOCALIZATION OF IFN-ALPHA IN PARENCHYMAL CELLS OF FORMALIN FIXED NORMAL HUMAN FETAL AND INFANT TISSUES.

ORGANS	TYPE OF CELLS +VE WITH H5l	GESTATIONAL AGE AT WHICH IFN-ALPHA APPEARS	FREQUENCY OF H5l +VE CELLS IN FETUSES. 7-ll WEEKS G (n)	FREQUENCY OF H5l +VE CELLS IN FETUSES. l2-25 WEEKS G (n)	FREQUENCY OF H5l +VE CELLS IN FETUSES. 26-42 WEEKS G (n)	FREQUENCY OF H5l +VE CELLS IN INFANTS (n)
ADRENAL GLAND	Fetal zone cortical cells	l0 weeks	++ (l)	+++ (20)	+ (8)	+ (5)
	Definitive zone cortical cells	l2 weeks	(0)	+ (20)	+ (8)	+ (8)
THYROID GLAND	Thyroid follicular cells	l3 weeks	(0)	+++ (23)	+++ (9)	+++ (9)
CHOROID PLEXUS IN BRAIN	Cuboidal epithelium	ll weeks	+ (l)	+ (20)	+++ (9)	+++ (8)
ANTERIOR PITUITARY GLAND	Occasional endocrine cells	l3 weeks	(0)	+ (20)	+ (5)	+ (6)

G = Gestational age
HPF = High Power field = 62,500 square microns.
\+ = 5 cells/HPF
++ = between 5-l5 cells/HPF
+++ = above l5 cells/HPF

Figure 4.1

IFN-alpha in fetal liver

An intrasinusoidal cell (arrow) staining positively for IFN-alpha in the liver of a 9 week old fetus.

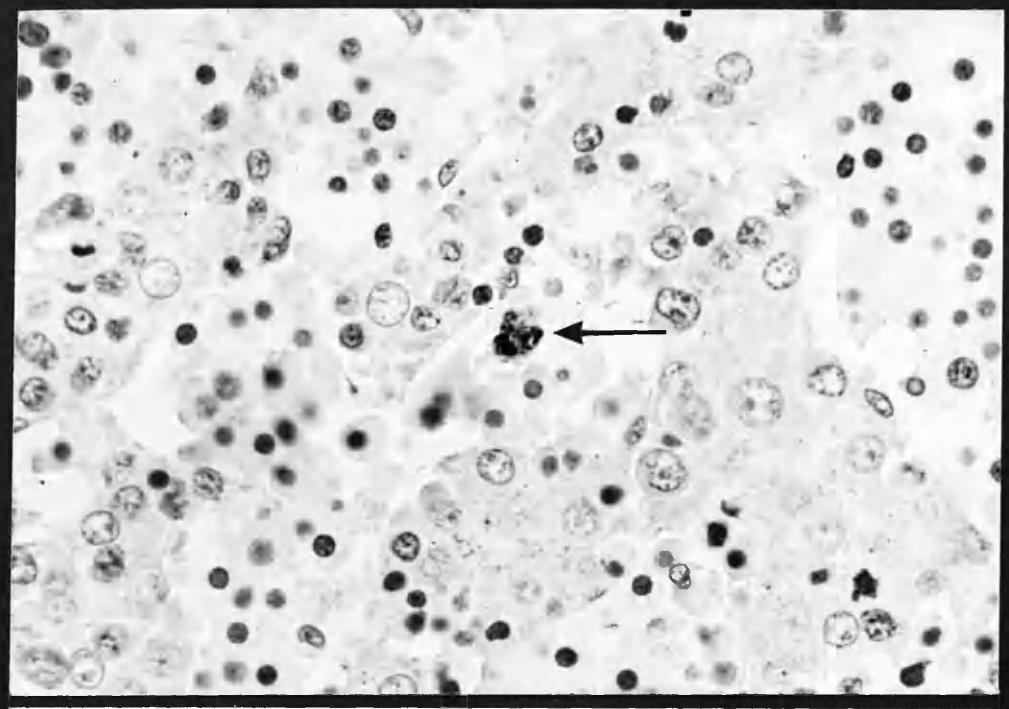
Indirect immunoperoxidase (IIP) for IFN-alpha (x 780).

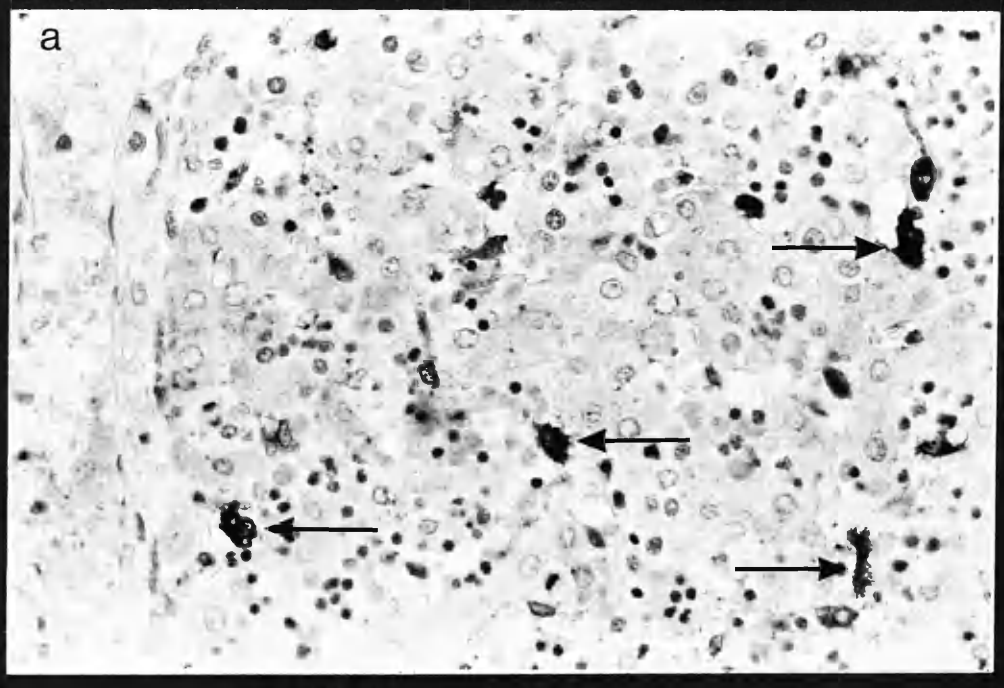
Figure 4.2

Macrophages in fetal liver contain IFN-alpha

Liver of a 38 week gestation fetus. Figure (a) and (b) are serial sections (mirror image technique) stained for IFN-alpha and KP1 respectively. The distribution of staining of intrasinusoidal cells (arrows) is similar in both sections showing that cells of the macrophage lineage contain IFN-alpha.

IIP for IFN-alpha (a) and KP1 (b) (x 450).




a

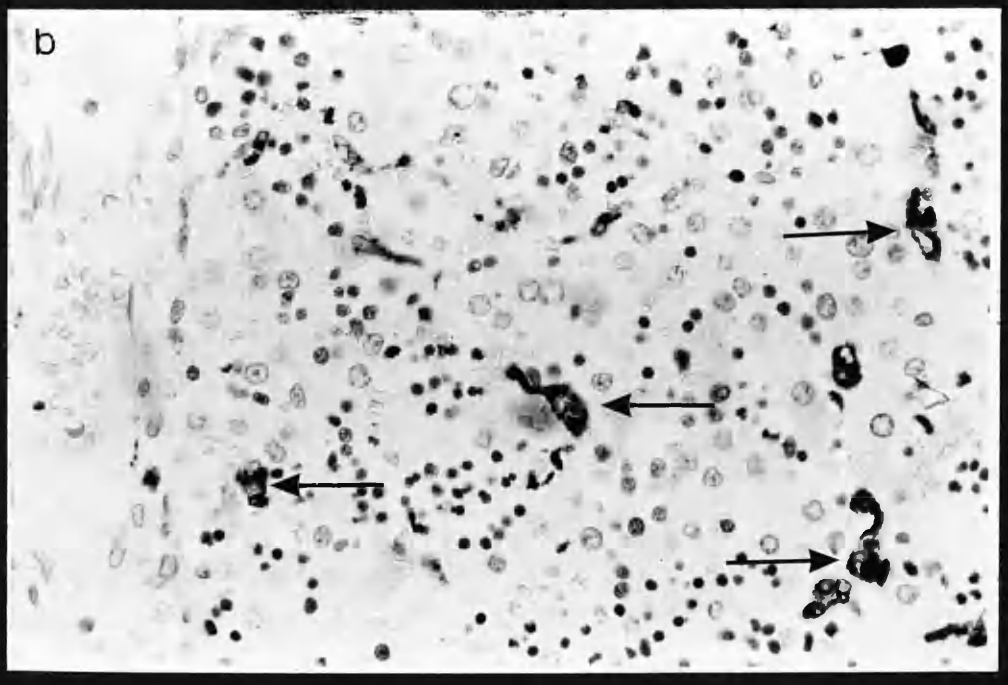
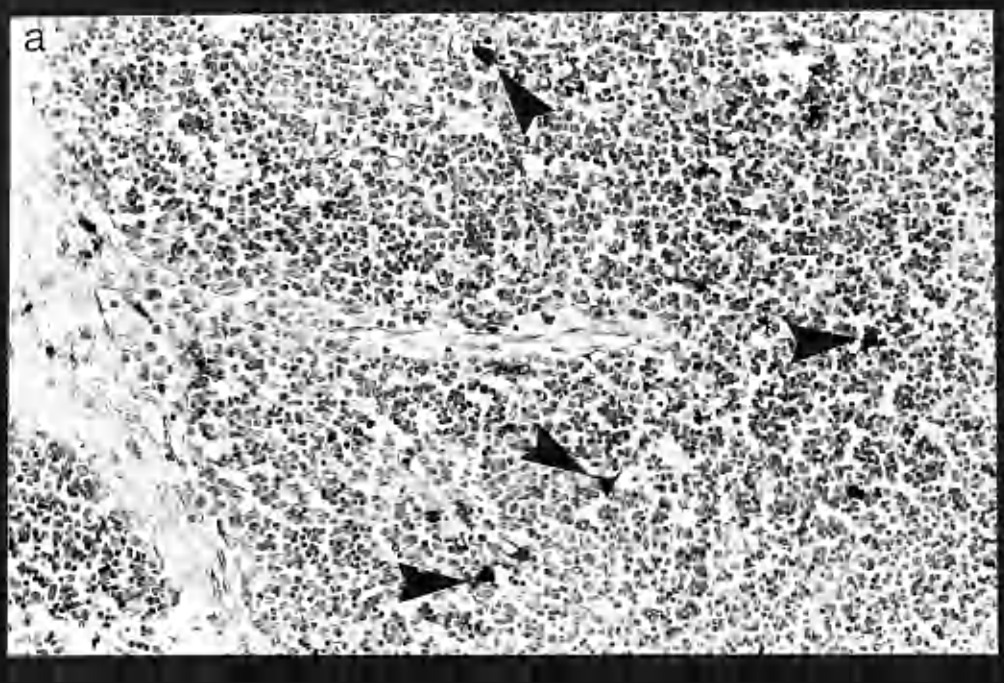

b

Figure 4.3

IFN-alpha in fetal thymus

Cells staining positively for IFN-alpha (arrow heads) (a), often failed to stain positively for KP1 (b) in the fetal thymus (mirror image serial section technique).

IIP for IFN-alpha (a) and KP1 (b) (x 230)


a

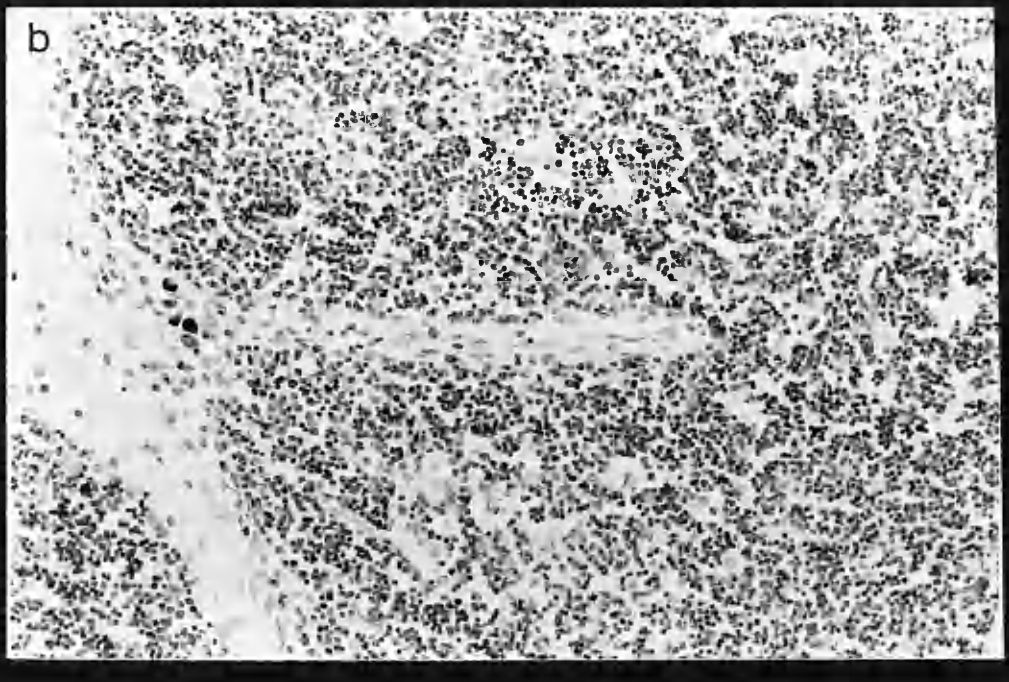

b

Figure 4.4

Distribution of macrophages in thymus, spleen, liver and lungs.

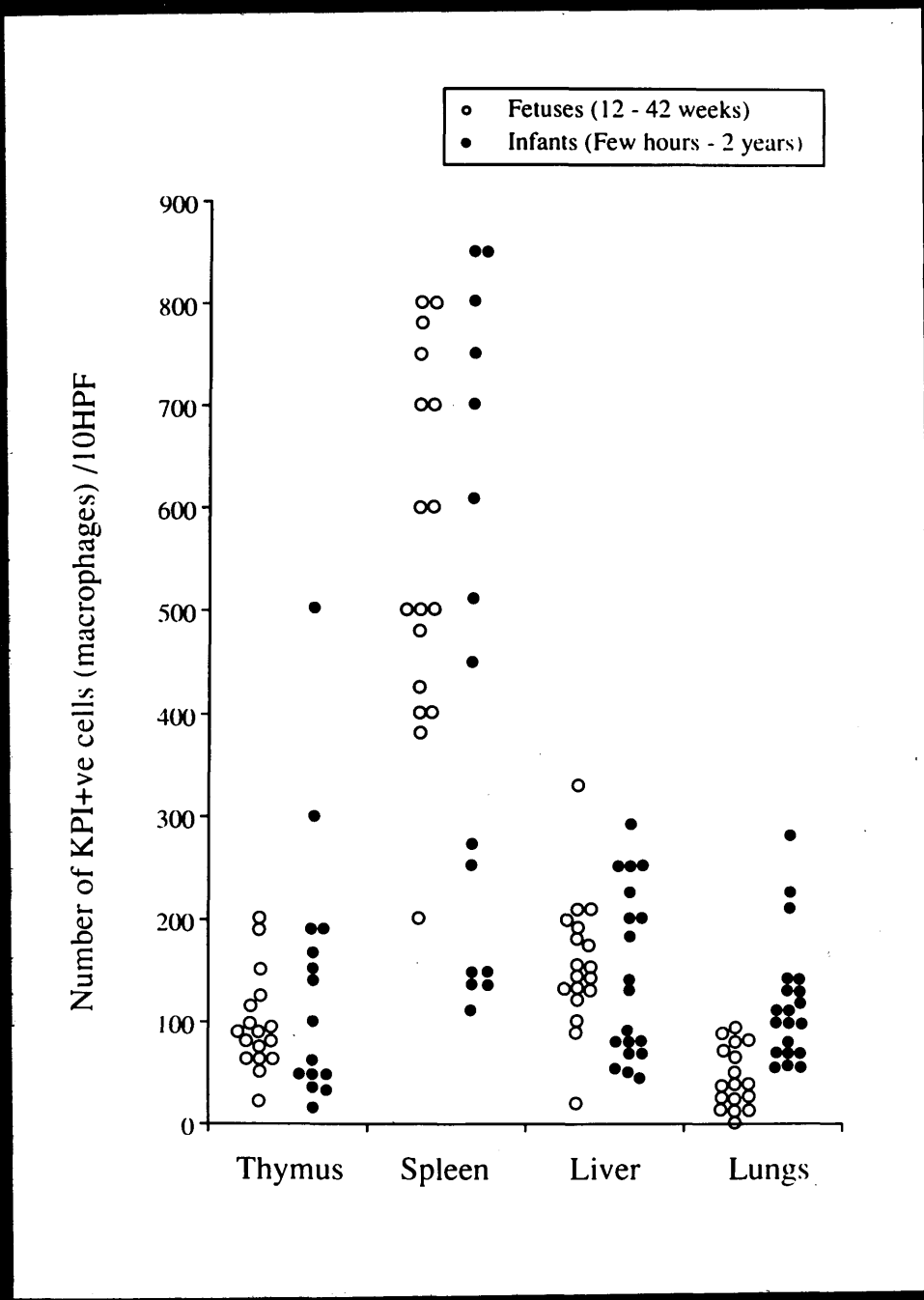

Fetuses (12 - 42 weeks)
Infants (Few hours - 2 years)
Number of KPI+ve cells (macrophages) /10HPF
900
800
700
600
500
400
300
200
100
0
Thymus
Spleen
Liver
Lungs

Figure 4.5

Distribution of immunoreactive IFN-alpha (IR-IFN-alpha) positive cells in thymus, spleen, liver and lungs.

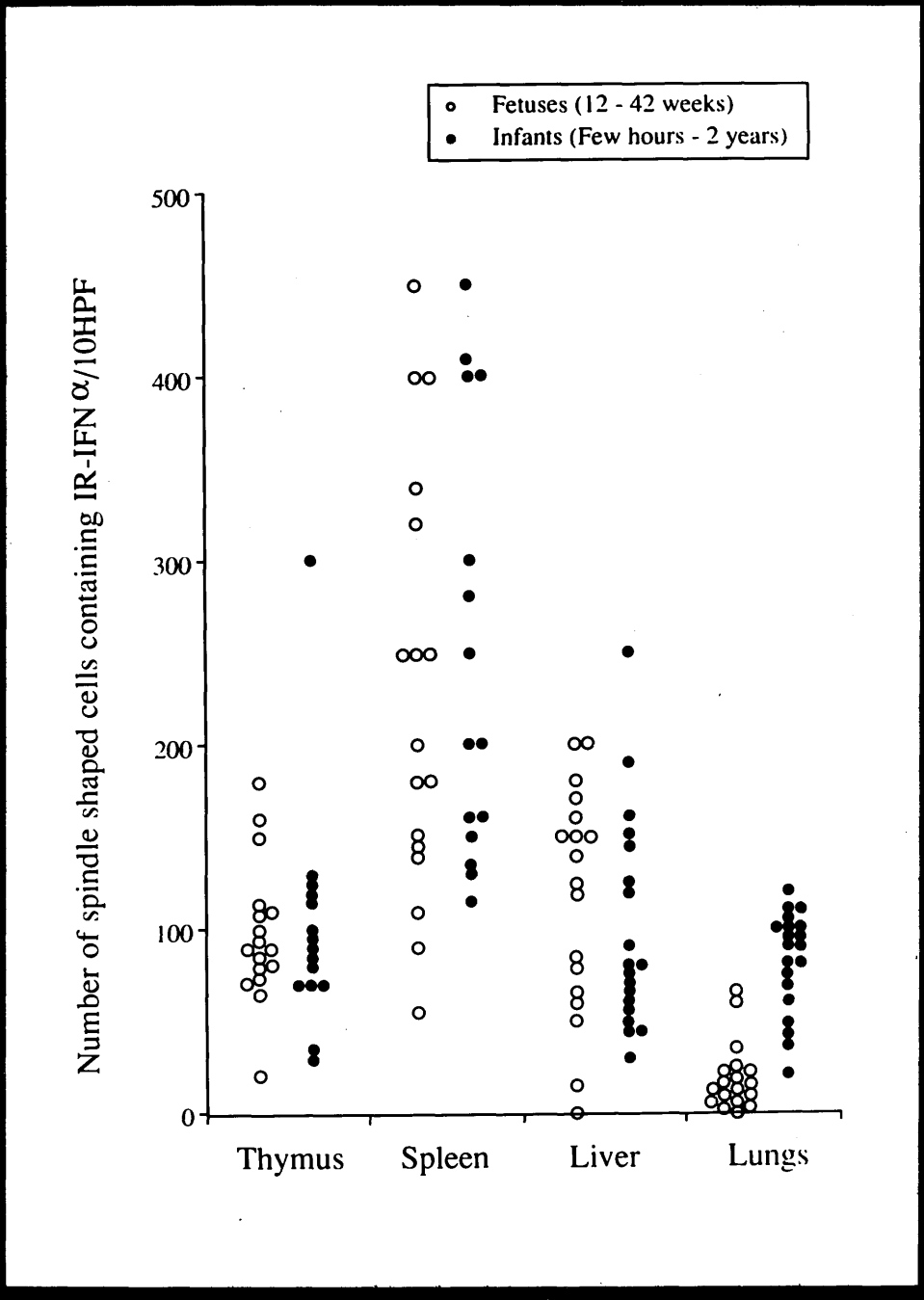

Fetuses (12 - 42 weeks)
Infants (Few hours - 2 years)
Number of spindle shaped cells containing IR-IFN α/10HPF
500
400
300
200
100
0
Thymus
Spleen
Liver
Lungs

Figure 4.6

Percentage of macrophages containing IR-IFN-alpha in spleen, liver and lungs.

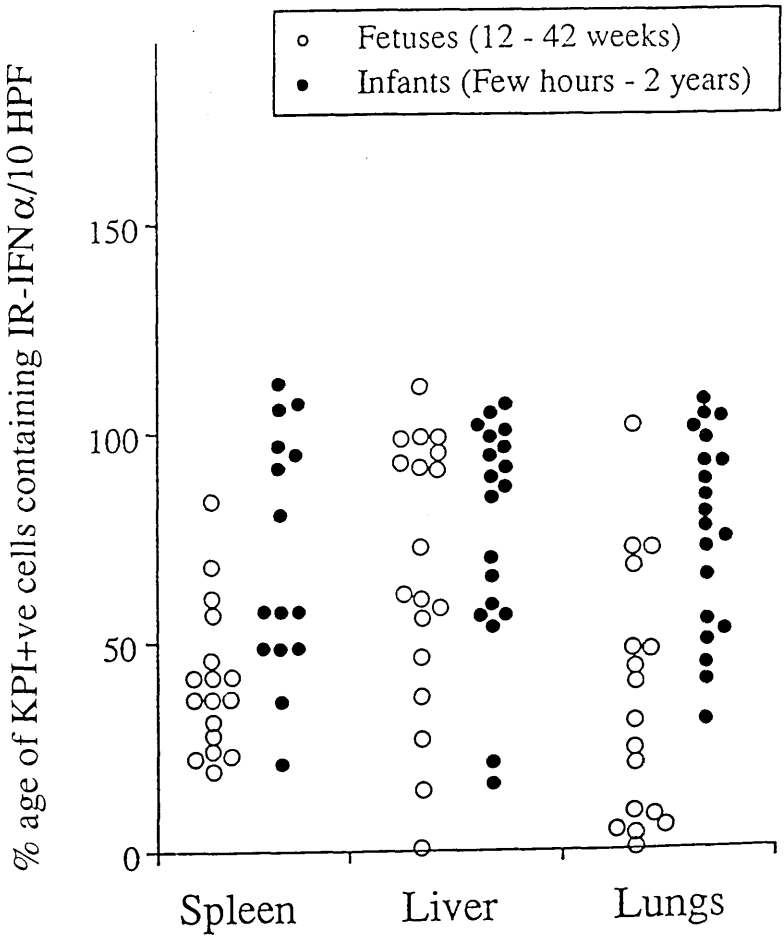

Fetuses (12 - 42 weeks)
Infants (Few hours - 2 years)
% age of KPI+ve cells containing IR-IFN α/10 HPF
150
100
50
0
Spleen
Liver
Lungs

Figure 4.7

IFN-alpha in infantile lung

Pulmonary alveolar macrophages in the lungs of an infant (aged 5 months) show positive staining for IFN-alpha.

IIP for IFN-alpha (x 350).

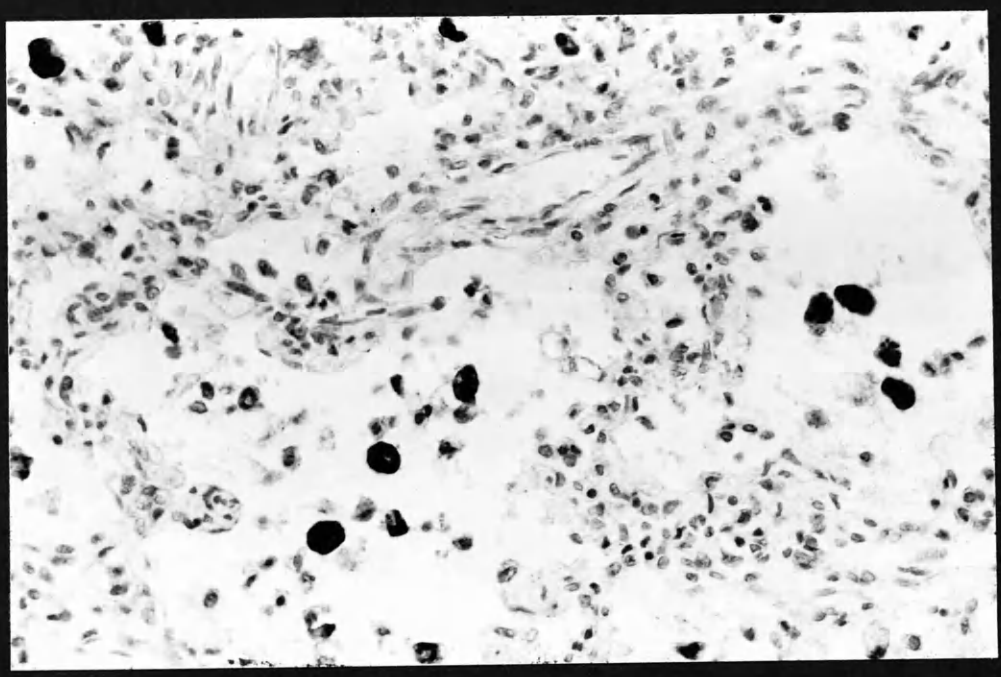
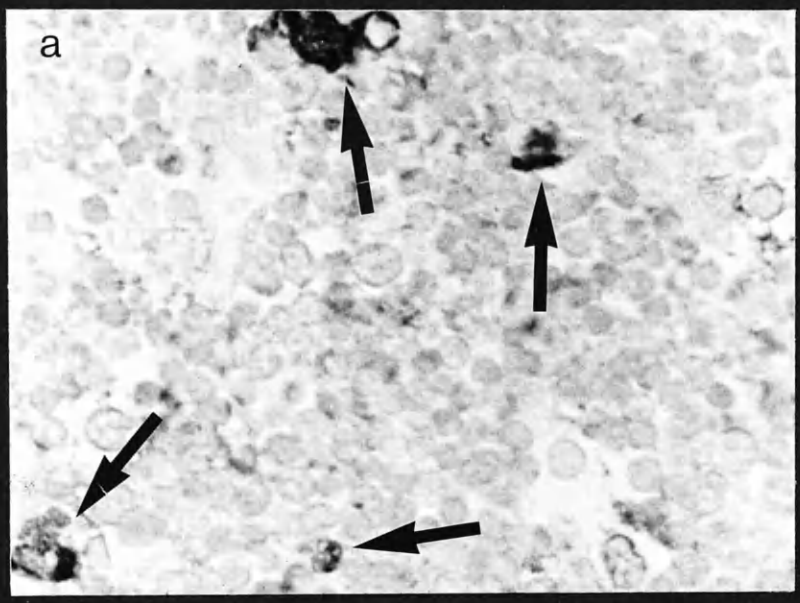


Figure 4.8

IFN-alpha in fetal thymus.

Cells positive for IFN-alpha (arrows) (a) failed to fluoresce for prekeratin (epithelial cytokeratin) in (b) (arrows). This shows that they are probably not epithelial cells.

Double staining technique; IIP for IFN-alpha (a) and immunofluorescence for prekeratin (b) on the same section (x 780).


a

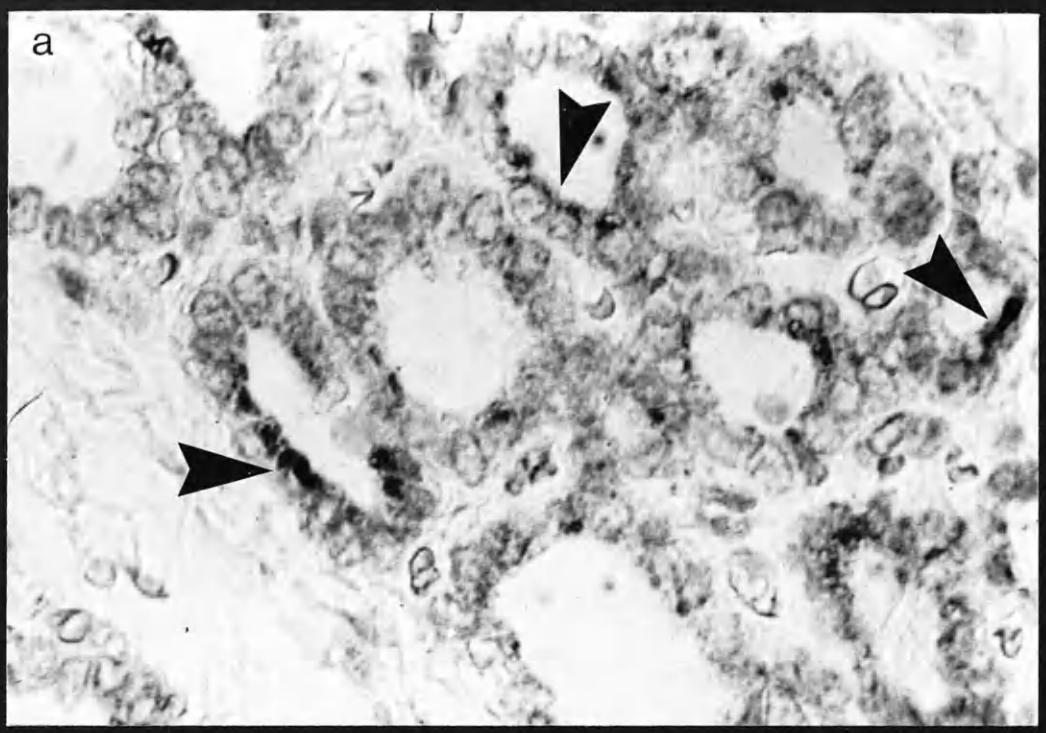

b

Figure 4.9

Double staining technique for IFN-alpha and prekeratin.

Thyroid follicular cells positive for IFN-alpha (arrow heads) (a), fluoresce strongly for prekeratin (b). This demonstrates that there is no problem in identifying both these markers within one cell.

Double staining technique: IIP for IFN-alpha (a) and immuno-fluorescence for prekeratin (b) on the same section (x 780).


a

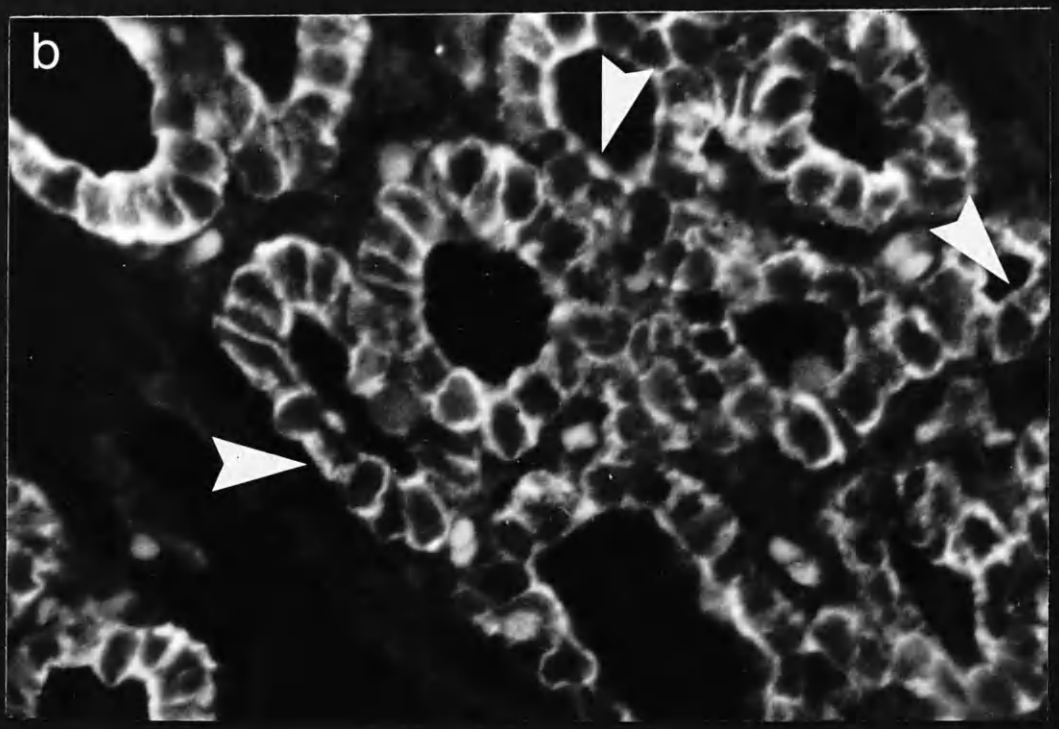
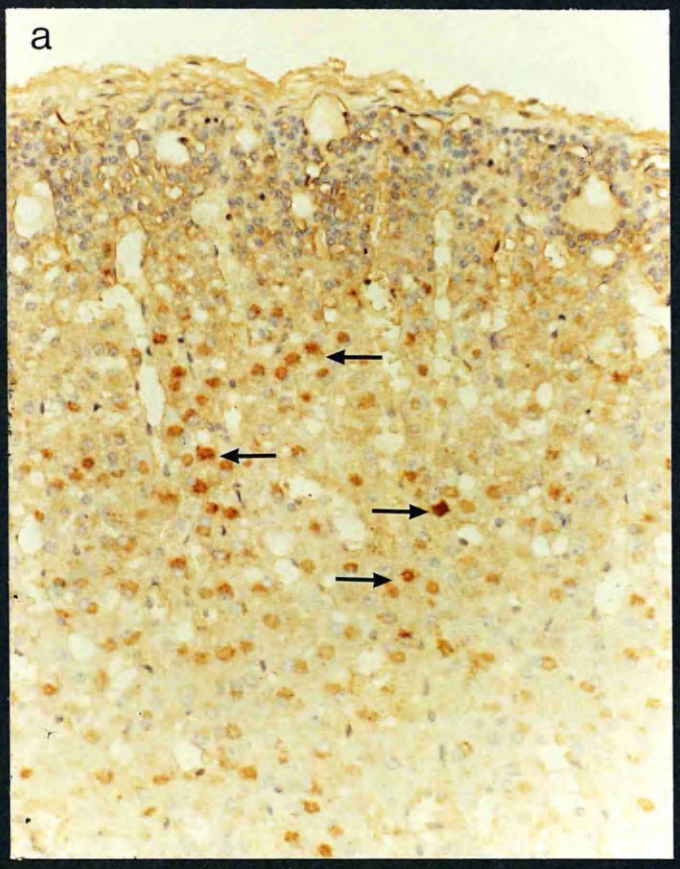

b

Figure 4.10

Specificity of staining for IFN-alpha in fetal adrenal.

Most cells in the fetal zone of fetal adrenal gland show specific positive staining for IFN-alpha (arrows) (a). The positive staining is completely abolished by absorption of the H5l antiserum with Wellferon prior to its use in the IIP technique (neutralization/blocking experiment) (b).

IIP for IFN-alpha with unabsorbed H5l antiserum (a) and absorbed with Wellferon (b) (x 200)


a


b

Figure 4.11

IFN-alpha in fetal choroid plexus

The cuboidal lining epithelium of choroid plexus in brain (arrow) is positive for IFN-alpha.

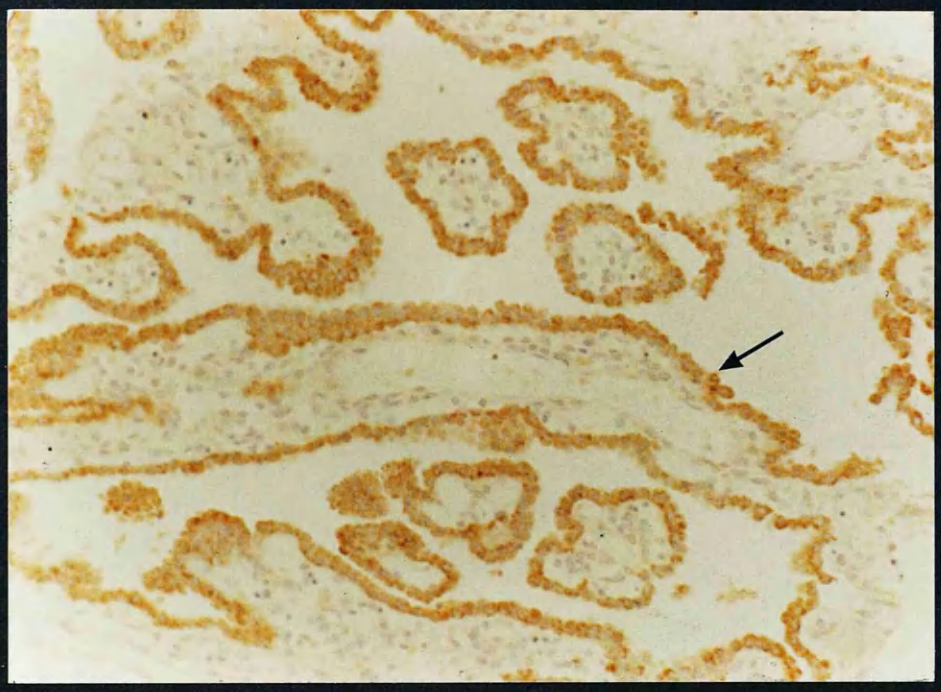
IIP for IFN-alpha (x 200).

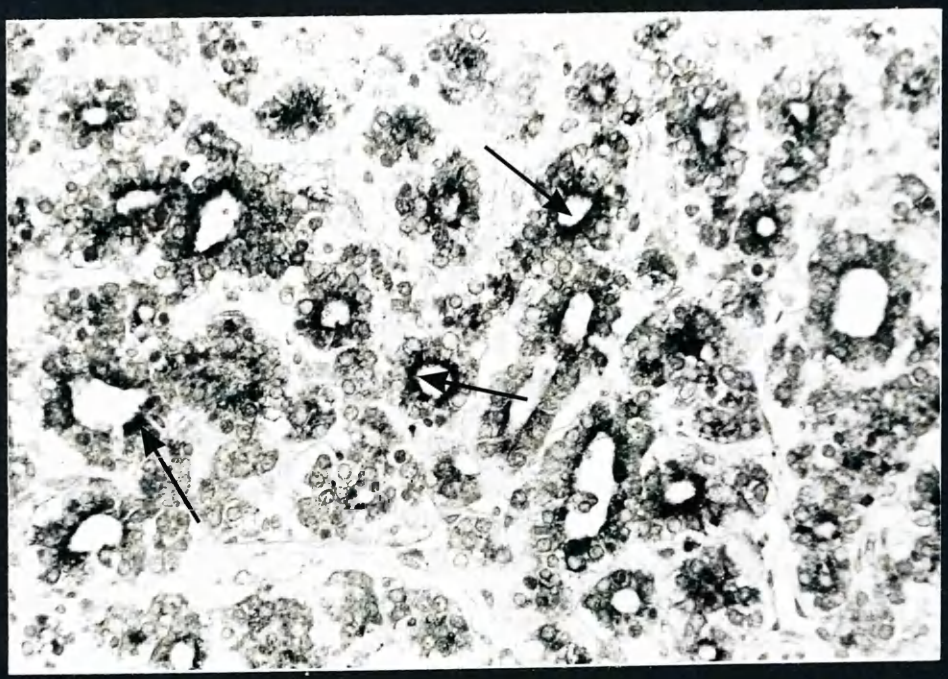
Figure 4.12

IFN-alpha in fetal thyroid

Thyroid follicular cells (arrows) show positive staining for IFN-alpha.

IIP for IFN-alpha (x 350).





DISCUSSION

This study shows that immunoreactive IFN-alpha is present in very early fetal life in spindle shaped cells that are scattered variably in nearly all fetal tissues except in kidneys and cerebral and cerebellar cortex in brain. Most of these cells showed similar positive staining with the macrophage/ histiocytic marker, KP1 monoclonal antibody, and were therefore probably tissue histiocytes (macrophages).

The demonstration of immunoreactive IFN-alpha in macrophages in germ free fetuses suggests that bacteria, endotoxin or viruses are not necessary for the initial synthesis of this product in man.

There does remain the possibility that endotoxin from the mother could cross the placenta into the fetus. When administered in large quantities to experimental animals endotoxin can cross the placental barrier (Dzvonyar *et al.*,1970). Traces of endotoxin can be found in the portal blood under physiological conditions but this is taken up by the reticuloendothelial system in the liver so that the peripheral blood is practically endotoxin free (Jacob *et al.*,1977). Thus exposure of the fetus to endotoxin from the mother in a normal pregnancy is likely to be negligible.

After birth, with exposure to a normal microbial flora, there was a significant increase in the number

of macrophages in the infant lungs, and the percentage of these cells containing IFN-alpha also increased. This finding provides some support for Bocci's hypothesis. While the finding that IFN-alpha positive macrophages were present in germ free fetuses suggests that there is a basal level of IFN-alpha production, not dependent on microbial products, the increased expression of IFN-alpha in infant lungs suggests that such products do have a local role in enhancing IFN-alpha synthesis. This enhancing effect was not seen in organs not directly exposed to a microbial flora.

Unfortunately the effect of a normal microbial flora on macrophages in the gastrointestinal tract, proposed by Bocci (1988) as an important site for physiological IFN-alpha synthesis, could not be assessed because of post mortem autolysis in the gastrointestinal mucosa of fetuses.

In fetal and infant thymuses many cells present in the cortical regions of the thymus which contained immunoreactive IFN-alpha did not stain positively with any of the macrophage/ histiocytic markers used in this study. Double staining for IFN-alpha and prekeratin showed that IFN containing cells were not thymic epithelial cells either. At present the exact nature of these cells cannot be determined. They could be a population of macrophages not recognised by any of the macrophage markers used in this study. Alternatively,

they may represent a completely different cell type which could only be marked using fresh frozen tissue sections and appropriate monoclonal antibodies.

The findings of immunoreactive IFN-alpha in parenchymal cells of various organs were almost identical to those seen in the adult human tissues (chapter three). It was proposed in the previous chapter that secretion of IFN-alpha by cells in the choroid plexus could be a defence mechanism against viral infection in the central nervous system but the function of IFN-alpha in the other organs remains unknown.

Previous work had suggested that IFN-alpha may be present in fetal tissues. The presence of IFN-alpha had been reported in a variety of fetal tissues using a specific two-site immunoradiometric assay (Chard *et al.*,1986). In the study by Chard *et al* (1986) approximately 60% of the fetal blood samples and fetal organ homogenates showed positive IFN-alpha levels. However the study did not localize the source of IFN-alpha in these tissues and also failed to mention which fetal tissues had positive levels of IFN-alpha. Equally the gestation age at which IFN-alpha was first found was not studied.

The demonstration of immunoreactive IFN-alpha in various normal human fetal tissues has potentially opened up a new area for future research. It provides

an opportunity to apply the same techniques as used here to study the tissue distribution and role of other cytokines, such as IFN-gamma, tumour necrosis factor and the interleukins in fetal development. Tumour necrosis factor and interleukin 2 like material have already been demonstrated in human placenta and amniotic fluid (Jaatela, Kiuusela & Saksela,1988; Soubiran, Zapitelli & Schaffer,1987) and IFN-gamma has been demonstrated in the umbilical cord blood of newborns (Martinez-Maza *et al.*,1984).

CHAPTER FIVE

EXTRACTION, DETECTION AND QUANTITATION OF INTERFERON-ALPHA IN HUMAN TISSUES AND FLUIDS.

INTRODUCTION

In the previous two chapters the presence and distribution of immunoreactive IFN-alpha in various formalin fixed paraffin embedded adult, infant and fetal human tissues was assessed using immunocytochemical techniques. In this chapter an attempt is described to detect and quantify IFN-alpha in fresh human tissues. The detection of IFN-alpha protein in various fresh human tissues would provide additional support for findings of immunoreactive IFN-alpha in formalin fixed normal human tissues and strengthen the hypothesis that low levels of IFN-alpha are synthesized under physiological conditions.

In order to achieve these objectives, homogenates from various human tissues were made, as described in chapter two section(E), and tested for the presence of IFN-alpha using a "Sucrosep" IFN-alpha immunoradiometric assay. The "Sucrosep" IFN-alpha immunoradiometric assay utilizes a non-cenrifugation system-the sucrose layering separation system, described by Wright & Hunter(1983). The "Sucrosep" IFN-alpha assay is a two-site immunoradiometric assay which

utilizes a ^{125}I labelled monoclonal antibody (designated Yok 5/19). The Yok 5/19 antibody recognizes at least 7 subtypes of IFN-alpha protein, namely IFN-alpha A(2), IFN-alpha C, IFN-alpha D(1), IFN-alpha G(5), IFN-alpha L, IFN-alpha J(7) and IFN-alpha K(6). The secondary antiserum in the "Sucrosep" IFN-alpha immunoradiometric assay kit was a sheep anti-human IFN-alpha antiserum attached to sepharose as the solid phase.

The "Sucrosep" IFN-alpha immunoradiometric assay has been used previously to detect IFN-alpha in human serum (Abbott *et al.*,1984), cerebrospinal fluid (Ho-Yen & Carrington,1987), nasopharyngeal secretions (Salas,1987) and tissue homogenates and amniotic fluids (Chard *et al.*,1986). The assay can detect as little as 0.5 IU/ml of IFN-alpha and showed negligible cross reactivity with IFN-beta and IFN-gamma (Abbott *et al.*,1984). In another study Salas (1987) showed that the "Sucrosep" IFN-alpha immunoradiometric assay can detect as little as 0.2 IU/ml of IFN-alpha in nasopharyngeal secretions, diluted in viral transport medium.

Various biological assays have also been used in the past to detect IFN-alpha. The biological assays are laborious, involving cell culture and challenge viruses which require special handling. They are time consuming and less sensitive when compared to solid phase radioimmunoassays. Several radioimmunoassays have been

described and all perform favourably compared to biological assays. Therefore in the present study no attempt was made to use biological assays for detecting IFN-alpha.

OBSERVATIONS

Observations from experiments on the performance characteristics of the "Sucrosep" IFN-alpha immunoradiometric assay.

i) The intra-assay analysis.

8 viral transport medium samples with no IFN-alpha activity were analysed by the "Sucrosep" IFN-alpha immunoradiometric assay on the same day. The mean of the total number of counts was 117, with a standard deviation of 7.33 and a coefficient of variation of 6.26% (table 5.1).

ii) The inter-assay analysis.

The mean value for each standard concentration derived from 10 different standard curves (with viral transport medium as a diluent) performed over several months, was used to plot a standard curve for "Sucrosep" IFN-alpha immunoradiometric assay (figure 5.1). The standard deviation, standard error and coefficient of variation for each point of the standard curve is given in table 5.2. In these inter-assay tests

the coefficient of variation varied from 11% to 18% which was considered to be acceptable.

iii) Neutralization/ blocking experiment (chapter 2, section E(c).

It was possible to neutralize 100% of the IFN-alpha activity in the 1-16 IU/ml of IFN-alpha standards. 1.5% and 8% residual IFN-alpha activity remained in the 64 and 256 IU/ml of IFN-alpha standards respectively (figure 5.2 & table 5.3).

Observations obtained from the "Sucrosep" IFN-alpha immunoradiometric assay analysis of amniotic fluids and tissue homogenates.

i) Amniotic fluids

All 4 amniotic fluids analyzed by the "Sucrosep" IFN-alpha immunoradiometric assay had detectable levels of IFN-alpha, with a mean level of 10 IU/ml of IFN-alpha (table 5.4).

Neutralization/ blocking experiments, described in chapter two section E(c), showed that all detectable IFN-alpha activity in the 4 amniotic fluid samples could be removed (i.e completely neutralized) demonstrating physiological IFN-alpha activity as a natural feature of the amniotic fluids.

ii) Tissue homogenates.

The results of IFN-alpha testing in tissue homogenates are given in table 5.5. It shows that detectable levels of IFN-alpha were observed only in 9 placentas and 1 thyroid gland. All detectable IFN-alpha activity in these placental and thyroid samples was completely neutralized by the neutralization/ blocking experiment. This demonstrated that the IFN-alpha levels detected in the tested samples were specific.

Amendment to the homogenizations protocol, i.e addition of anti-protease enzymes to the homogenization buffer before homogenization of the tissues, had no effect on the detectable levels of IFN-alpha in these tissues.

A direct comparison between the "Sucrosep" IFN-alpha immunoradiometric assay and the indirect immunoperoxidase technique using the H51 antiserum was made (table 5.5). A correlation was found between the "Sucrosep" IFN-alpha immunoradiometric assay and the indirect immunoperoxidase technique using the H51 antiserum for placenta and cerebral cortex, but not for choroid plexus, thyroid and adrenal glands (cerebral cortex, which was negative for immunoreactive IFN-alpha by the indirect immunoperoxidase technique described in chapter three, was included in this study as a negative control specimen for the "Sucrosep" IFN-alpha immunoradiometric assay). These findings suggest that

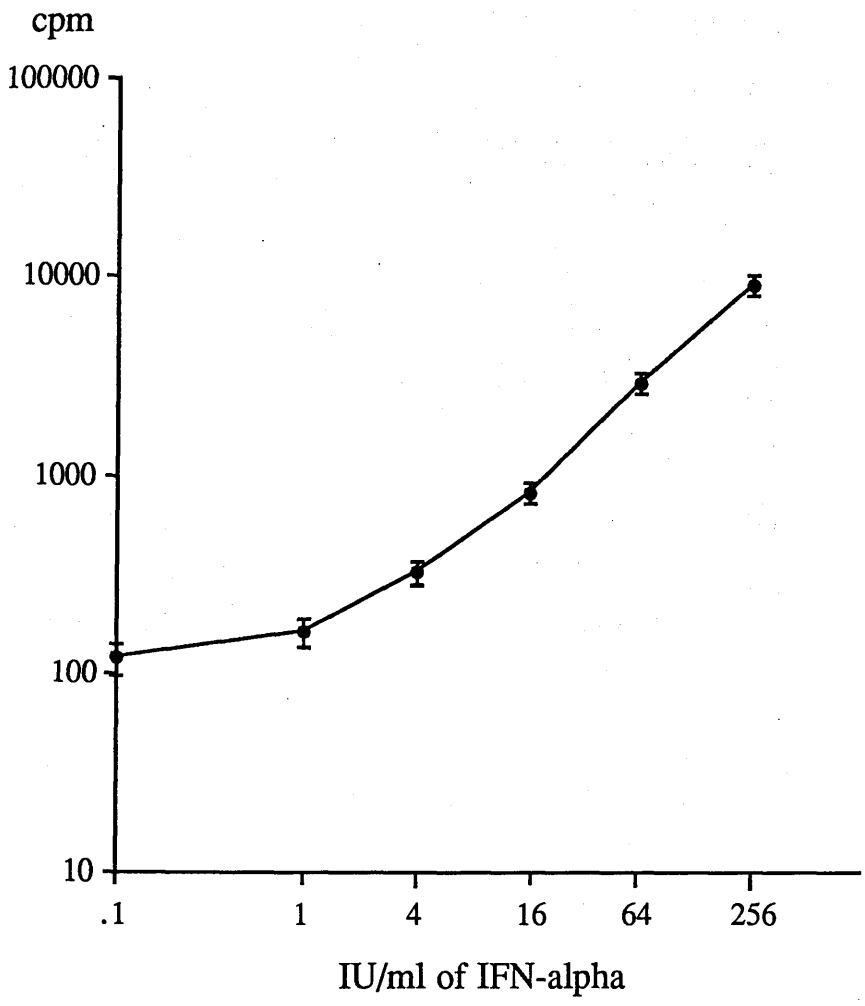


Figure **5** .1. VMT standard curve for IFN-alpha values (0-256IU/ml) over 10 consecutive assays using "Sucrosep" IFN-alpha IRMA. (A line drawn through the mean with bars indicating ± 2 standard deviations.)

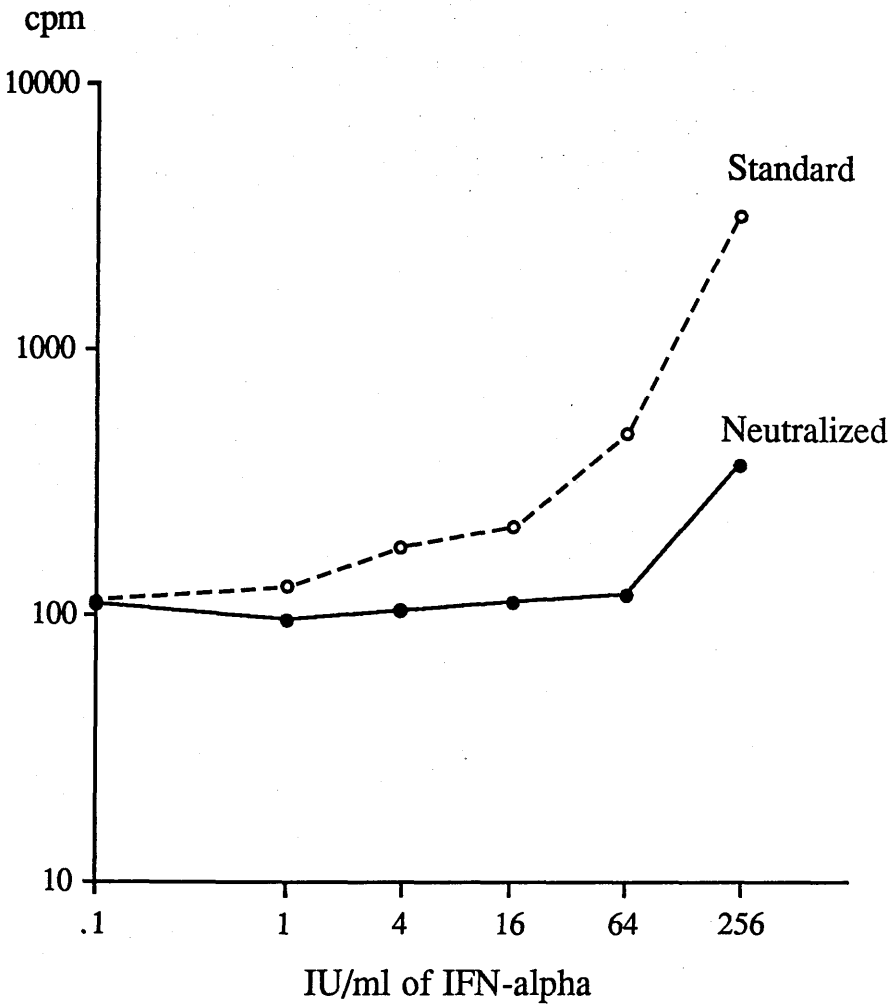


Figure 5 .2. Neutralization/blocking of IFN-alpha activity in IFN-alpha standards diluted in VTM assayed by "Sucrosep" IFN-alpha immunoradiometric assay.

TABLE 5.1

VIRAL TRANSPORT MEDIUM (VTM) SAMPLES WITH NO IFN-ALPHA ASSAYED ON THE SAME DAY BY "SUCROSEP" IFN-ALPHA IRMA.

NUMBER OF SAMPLES	VTM (cpm)
1	104
2	110
3	121
4	125
5	120
6	124
7	113
8	119

Mean = 117
Standard deviation = 7.3
Coefficient of variation = 6.3%

IRMA = immunoradiometric assay
cpm = counts/minute

TABLE 5.2

STATISTICAL ANALYSIS OF A SERIES OF IFN-ALPHA STANDARD LEVELS (0-256 IU/ml) IN VTM MEASURED BY "SUCROSEP" IFN-ALPHA IRMA.

IFN-alpha standard levels in VTM (IU/ml)	mean (cpm)	S.D.	S.E.	C.V(%)
0	118.7	21.8	2	18
1	160	25.9	2	16
4	323.8	41.6	4	13
16	822	96.1	9	11
64	2972	362.7	36	12
256	9417.7	1134	113	12

I.U. = International units
S.D. = Standard deviation
S.E. = Standard error
C.V(%) = Coefficient of variation
VTM. = Viral transport medium

TABLE 5.3

NEUTRALIZATION/BLOCKING OF IFN-ALPHA ACTIVITY IN IFN-ALPHA STANDARDS IN VTM ASSAYED BY "SUCROSEP" IFN-ALPHA IRMA.

IFN-ALPHA STANDARDS IN VTM (IU/ml)	%AGE OF REDUCTION OF IFN-ALPHA ACTIVITY IN IFN-ALPHA STANDARDS BY N/B EXPERIMENT
1	100%
4	100%
16	100%
64	98.5%
256	92%

N/B = Neutralization/blocking

TABLE 5.4

DETECTION OF IFN-ALPHA IN AMNIOTIC FLUIDS ASSAYED BY "SUCROSEP" IFN-ALPHA IRMA.

NUMBER OF AMNIOTIC FLUID SAMPLES	DETECTABLE LEVELS OF IFN-ALPHA (IU/ml)
1	9
2	17
3	14
4	10

Mean = 12.5 IU/ml of IFN-alpha.

TABLE 5.5

TITLE: THE DEMONSTRATION OF IFN-ALPHA IN HUMAN TISSUES BY IRMA AND ITS COMPARISON WITH THE IMMUNOCYTOCHEMICAL FINDINGS USING THE H51 ANTISERUM.

ORGANS	TOTAL NUMBER OF CASES (n)	NUMBER OF CASES WITH DETECTABLE IFN-ALPHA BY IRMA (n)	RANGE OF DETECTABLE IFN-ALPHA BY IRMA. (IU/ml)	MEDIAN OF DETECTABLE IFN-ALPHA BY IRMA. (IU/ml)	NUMBER OF CASES +VE BY IIP TECHNIQUE USING H51 ANTISERUM (n)	
PLACENTA 2nd Trimester	15	7	0.5 - 7	2.44	15	
PLACENTA 3rd Trimester	5	2	0.5 - 2	1.25	5	
THYROID GLAND	11	1	9	9	11	
FETAL ADRENAL GLAND	9	0	0	0	9	
CHOROID PLEXUS IN BRAIND	10	0	0	0	10	
CEREBRAL CORTEX IN BRAIN	10	0	0	0	0	

IRMA = Immunoradiometric assay

IIP = Indirect immunoperoxidase

there may be a quantitative or qualitative difference between the tissues studied.

DISCUSSION

Many workers in the past had demonstrated the presence of IFN-alpha in human placenta and amniotic fluids, using various techniques (Chard *et al.*,1986; Bocci *et al.*,1985; Duc-Goiran *et al.*,1985; Lebon *et al.*,1982). The observations of the present study confirm the presence of detectable levels of IFN-alpha in placenta and amniotic fluids, using the "Sucrosep" IFN-alpha immunoradiometric assay. The same assay system was also used by Chard *et al* (1986) to demonstrate IFN-alpha in placenta, amniotic fluid and fetal tissues. In the present study approximately 50% of the placentas contained detectable IFN-alpha, which was not as high as the 96% positive result observed by Chard *et al* (1986). The range and median of the detectable IFN-alpha levels obtained in the present study was, however, somewhat similar to that observed by Chard *et al* (1986). All amniotic fluids studied had detectable levels of IFN-alpha, which also confirmed the observations of previous workers. The detectable IFN-alpha levels observed in 9 placentas, 1 thyroid gland and all 4 amniotic fluid samples in the present study were completely neutralized by the neutralization/ blocking experiment and therefore can

be regarded specific positive observations. Chard *et al* (1986) did not confirm the specificity of IFN-alpha in the positive samples in his study and therefore it is impossible to compare the apparent discrepancy between the two studies.

The negative observations in adult thyroid glands, choroid plexuses in brain and fetal adrenal glands were not consistent with the immunocytochemical findings using the H51 antiserum described earlier. There are at least three possible explanations for these negative observations. Firstly, the monoclonal antibody (Yok 5/19) used in the "Sucrosep" IFN-alpha immunoradiometric assay is known to recognise only 8 of more than 20 IFN-alpha subtypes. Therefore it is a possibility that those organs which did not show any detectable levels of IFN-alpha by the immunoradiometric assay, but were positive for immunoreactive IFN-alpha by the H51 antiserum in the indirect immunoperoxidase technique, contained an IFN-alpha protein not recognized by the monoclonal antibody Yok 5/19, used in the immunoradiometric assay. Secondly, all the placenta and amniotic fluid samples studied were obtained fresh within 1-2 hours of normal vaginal delivery or caesarean section. The adult thyroid glands, choroid plexuses in brain and fetal adrenal glands, on the other hand, were obtained from adult and fetal autopsies within 10-24 hours of death. It is possible

that degradation of IFN-alpha proteins took place in these autopsy specimens resulting in failure to detect this protein by the "Sucrosep" IFN-alpha immunoradiometric assay. Thirdly, all tissues were homogenized using viral transport medium (VTM) as the extraction buffer. One gram of the tissue was added to 2-4 mls of VTM. This means a dilution factor of 2-4 times. It is suggested that these organs may contain such low levels of IFN-alpha proteins that the dilution factor makes it impossible to detect these in tissue homogenates using extraction buffer.

One line of future research could be to obtain fresh human tissues from transplant organ donors or from surgical operations and test them for IFN-alpha, instead of using tissues from autopsies.

CHAPTER SIX

AN ATTEMPT AT DEMONSTRATING INTERFERON-ALPHA mRNA BY *IN SITU* HYBRIDIZATION.

INTRODUCTION

As described in chapter 5, IFN-alpha was detected in fresh human placentas using a specific two-site immunoradiometric assay. Immunoreactive IFN-alpha was also demonstrated in macrophages, syncytiotrophoblast of the placenta and in parenchymal cells in various normal human tissues (chapter 3 & 4). However it is well known that many cells, and especially macrophages, phagocytose various proteins, foreign bodies, etc and thus the mere presence of IFN-alpha in cells does not provide conclusive evidence that these cells are actually producing IFN. To provide further evidence for IFN-alpha synthesis, it was important to demonstrate IFN-alpha messenger RNA (mRNA) in cells that stained for immunoreactive-IFN-alpha by the immunocytochemical methods. The presence of IFN-alpha mRNA would demonstrate that transcription of IFN-alpha genes and synthesis of IFN-alpha protein was taking place in these cells.

Various techniques for the demonstration of nucleic acid sequences are available. They include *in situ* hybridization and southern, northern and dot blot

hybridization techniques. An *In situ* hybridization technique was first described by Gall & Pardue (1969) for the detection of ribosomal gene sequences. Since then a large number of workers have used the same principle for the demonstration of cellular mRNA. *In situ* hybridization techniques differ from the other three techniques in that the nucleic acid investigated is not extracted from the tissues. Labelled nucleic acid probes are hybridized with cellular DNA or RNA in fixed tissue sections or cytological preparations, and the reaction visualized by a label detection system. This allows precise cytological localization of the nucleic acid sequence in the histological sections.

In the present study two oligonucleotide (synthetic) probes, based on selected conserved cDNA sequences of IFN-alpha genes, were used in the *in situ* hybridization techniques for the demonstration of IFN-alpha mRNA. Probe 1 had been previously used for the demonstration of the IFN-alpha WA gene (Torczynski *et al.*, 1984). A poly-d-T oligonucleotide probe (complementary to the poly-A tail of mRNA molecules) used previously to detect total mRNA in formalin fixed tissues (Pringle *et al.*,1989), was used as a positive control for the *in situ* hybridization techniques used in this study. Oligonucleotide probes are synthetic, short single stranded probes which can be easily synthesized in abundance using automated DNA

synthesizers. They do not require lengthy procedures of isolation and can be stored very conveniently at -20°C. They also have the advantage of better penetration of tissue sections than longer cDNA or RNA probes (Moench *et al.*,1985). Most workers have used radioactive isotopes to label probes for *in situ* hybridization techniques. However, nonradioactive labelling methods using biotin, and more recently digoxigenen, have been described (Pringle *et al.*,1987; Brigati *et al.*,1983; Farquharson, Harvie & McNicol,1990). Digoxigenen is a derivative of the cardiac glycoside digoxin and has been used successfully to label oligonucleotide probes at the 3' end using terminal deoxynucleotidyl transferase (Farquharson *et al.*,1990). Digoxigenen can be identified by immunocytochemical techniques using an anti-digoxigenen antibody. Digoxigenen (a plant derivative) has not been found in human tissues, unlike biotin which is present in abundance in some human tissues. Farquharson *et al* (1990) suggested that 3' end labelling of oligonucleotide probes with digoxigenen should be useful for use in *in situ* hybridization procedures, particularly for the detection of mRNAs in tissues with high endogenous biotin activity.

In this study all the three oligonucleotide probes were labelled at the 3'end with digoxigenin.11.dUTP, using the terminal deoxynucleotidyl transferase enzyme. These labelled probes, hybridized to cellular mRNA i.e

forming sequence specific, base paired duplexes with complementary mRNA sequences in tissue sections, can be identified with an alkaline phosphatase conjugated anti-digoxigenen antibody and appropriate chromogen.

OBSERVATIONS

i) Labelling procedure observations.

Labelling of probes 1 & 2 with digoxigenen.11.dUTP.

Both the probes were efficiently labelled with digoxigenen.11.dUTP by the 3' end labelling reaction using the terminal deoxynucleotydal enzyme, as described in chapter 2, section (F). There was no difference between labelling reactions performed with 1 microgram and 2 micrograms of probes 1 & 2. The labelled probes, separated from unincorporated material by the separation procedure described in chapter 2, were always obtained in fractions 4 & 5 (figure, 6.1).

Labelling the poly-d-T probe.

The poly-d-T probe was also labelled efficiently with digoxigenen.11.dUTP, and the labelled probe, separated from unincorporated material, was obtained in fractions 4 & 5.

ii) *In situ* hybridization procedure observations.

Probe 1.

Using probe 1 staining was observed in small round cells in three fetal spleens (two fixed in buffered formalin and one in formol saline for 24 hours) and one adult tonsil (fixed in buffered formalin for 24 hours) using the standard protocol for *in situ* hybridization described in chapter 2. In all 3 spleens, staining of these rounded cells was seen in tissue sections treated with 10-20 micrograms of proteinase-k enzyme/ml of proteinase-k buffer and in the tonsil in sections treated with 5-10 micrograms of proteinase-k enzyme/ml of proteinase-k buffer. These cells were small and round and the nuclei appeared multilobated on high power oil emulsion microscopy (figure, **6**.2). Sections from the above tissues stained with the monoclonal antibody, LeuM 1, using an immunocytochemical technique described in chapter 2, showed a staining pattern similar to that observed in the *in situ* hybridization technique with probe 1 i.e cells identical to those positive with probe 1 by *in situ* hybridization technique also stained positively for LeuM 1 (figure,**6**.3). In addition to staining Reed-Sternberg cells in Hodgkin's disease, LeuM 1 is a recognized marker for polymorphonuclear neutrophils. The double staining technique using *in situ* hybridization with

probe 1 in the part(a) and immunofluorescence with anti-muramidase antiserum in the part(b), as described in chapter 2, showed that cells fluorescing with the anti-muramidase antiserum were not stained with probe 1 (figure,6.4). There was no correlation between the staining patterns obtained by *in situ* hybridization with probe 1 and immunperoxidase with the monoclonal antibody KP1 in any tissues examined.

The positive staining observed in cells described above with probe 1 was not uniformly distributed throughout the same tissue section, being often confined to certain parts of the tissue section.

No staining was observed in the *in situ* hybridization technique in the other tonsils or placentas tested in this study.

The staining observed in the cells described above was seen only when 1 microgram of probe 1 was used in the 3' end labelling reaction. No staining was seen when 2 micrograms of probe 1 was used in the labelling reaction.

Observations of amendments in the *in situ* hybridization protocol for probe 1.

a) Varying the proteinase-k treatment.

Varying the proteinase-k treatment of the sections had little effect on the above staining pattern. There

was an overall increase in the background staining with increased incubation times of the proteinase-k enzyme. Increasing the proteinase-k enzyme concentration to 40 micrograms/ml of proteinase-k buffer showed increased staining of the rounded cells in splenic tissue sections. It did not affect the staining in tonsil.

b) Varying the incubation temperature of the hybridization procedure and post-hybridization washes.

Instead of incubating the tissue sections at room temperature, as in the hybridization procedure of the standard protocol, the tissue sections were incubated with the probe + hybridization buffer solution at 37^{o}C, followed by post-hybridization washes, as described in chapter 2. It produced a similar staining pattern to that observed with the standard protocol. Also hybridization of tissue sections with the probe + hybridization buffer solution at 42^{o}C, followed by post-hybridization washes, as described in chapter 2, produced a similar staining pattern to that observed with the standard protocol.

c) Varying the concentration of formamide.

Reducing the concentration of formamide from 50% in the hybridization buffer to 20%, followed by the hybridization procedure and post hybridization washes,

described in chapter 2, produced negative staining in all tissues.

RNase treatment.

RNase treatment of spleen and tonsil sections (1-4 milligram/ml of ribonuclease-A), as described in chapter 2, failed to abolish the positive staining observed in polymorphs, seen in certain tissues with probe 1.

Probe 2.

No positive staining was observed with probe 2 in any of the tissues studied using the standard protocol and applying all the amendments described in chapter 2 for the hybridization protocol.

Poly-d-T probe.

Observations of the hybridization procedure.

The poly-d-T probe showed positive staining in the cytoplasm of most cells in the tissues studied (figure, **6**.5). The staining was best, with little background in tissue sections treated with 2 or 5 micrograms of proteinase-k enzyme/ml of the proteinase-k buffer. Increasing the proteinase-k enzyme concentration, although increasing the staining, caused very high background. The staining with the poly-d-T probe was consistently reproducible.

Figure 6.1

Labelling of probes 1 and 2

The labelled oligonucleotide probes 1 and 2, separated from unincorporated material by the separation procedure, are obtained in fractions 4 and 5 shown as dark spots on the nitrocellulose paper strip.

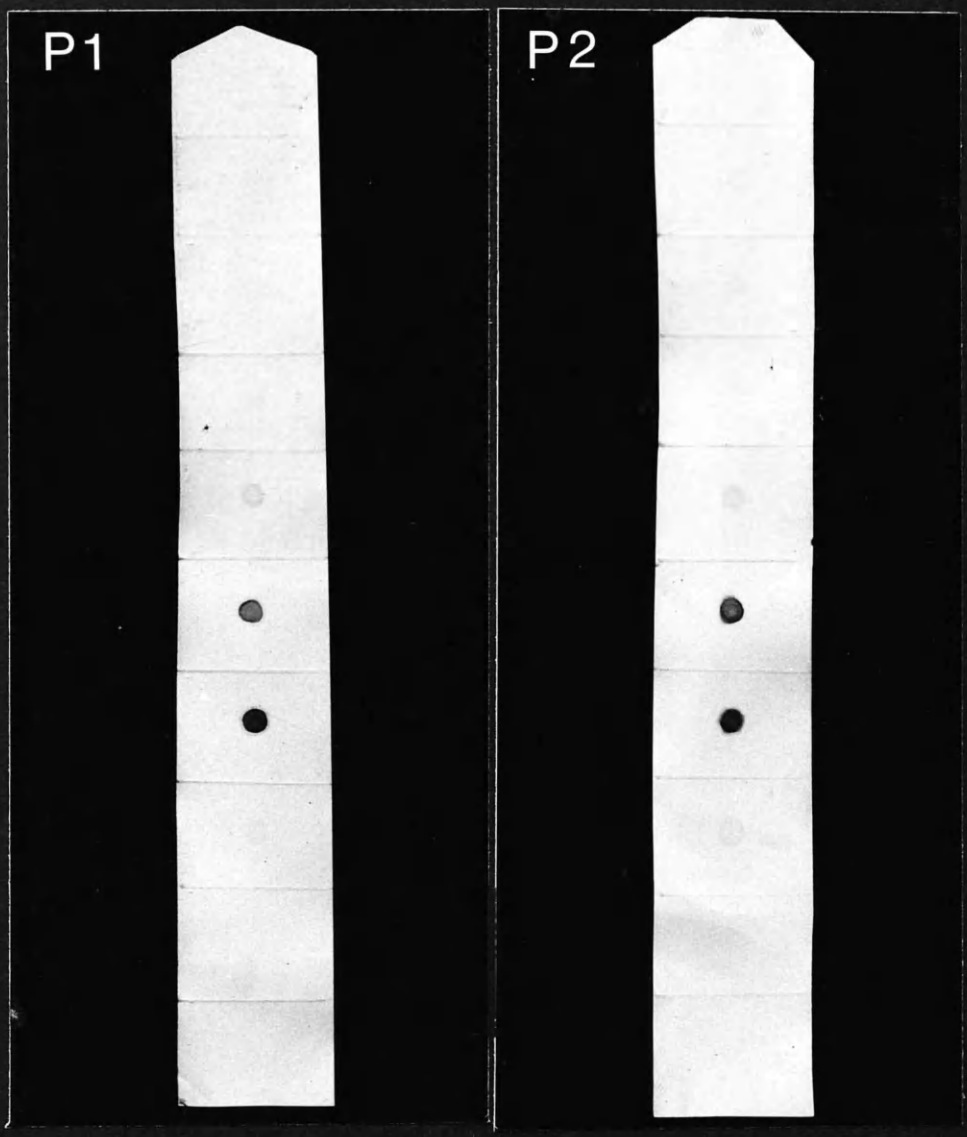

P1
P2

Figure 6.2

Probable non-specific hybridization with probe 1

Round shaped cells with multilobated nuclei are shown staining with probe 1 in spleen.

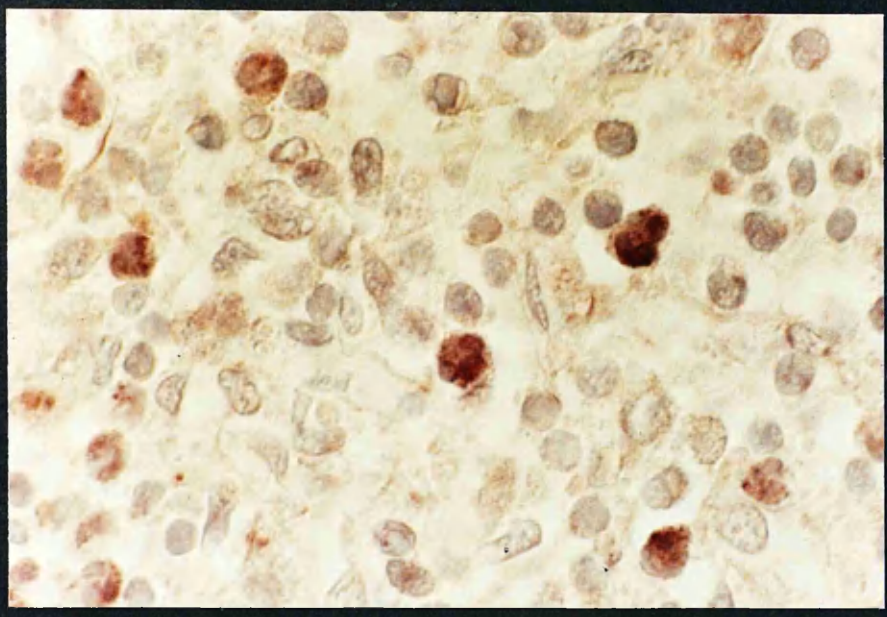
In situ hybridization for IFN-alpha mRNA with probe 1 (x 880).

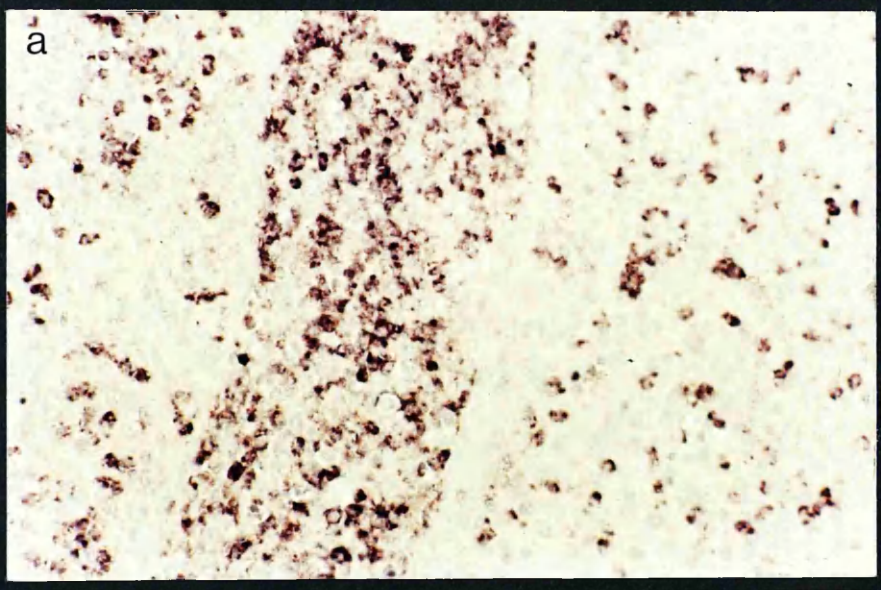
Figure 6.3

In situ hybridisation using probe 1 in tonsil

Figure (a) and (b) are sections stained for IFN-alpha mRNA and LeuM1, respectively (LeuM1 antibody, in addition to staining Reed-Sternberg cells in Hodgkin's disease, is a recognized marker for polymorphonuclear neutrophils). The distribution of staining cells is similar in both sections, showing that cells stained for IFN-alpha mRNA are probably polymorphonuclear neutrophils.

In situ hybridization for IFN-alpha mRNA with probe 1 (a) and IIP for LeuM1 (b) (x 350).




a

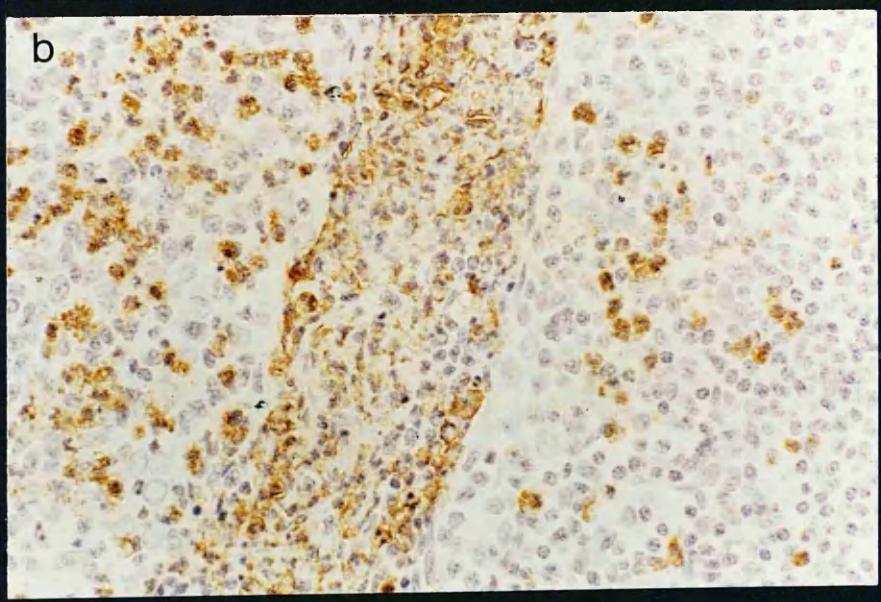

b

Figure 6.4

Cells in spleen showing positive in situ hybridization with probe 1 are not macrophages.

Cells fluorescing with anti-muramidase antiserum (macrophage marker) in (b) are not staining with probe 1 (a) (arrow heads).

In situ hybridization for IFN-alpha mRNA with probe 1 (a) and immunofluorescence using anti-muramidase antiserum (b) on the same section (x 890).

a

b

Figure 6.5

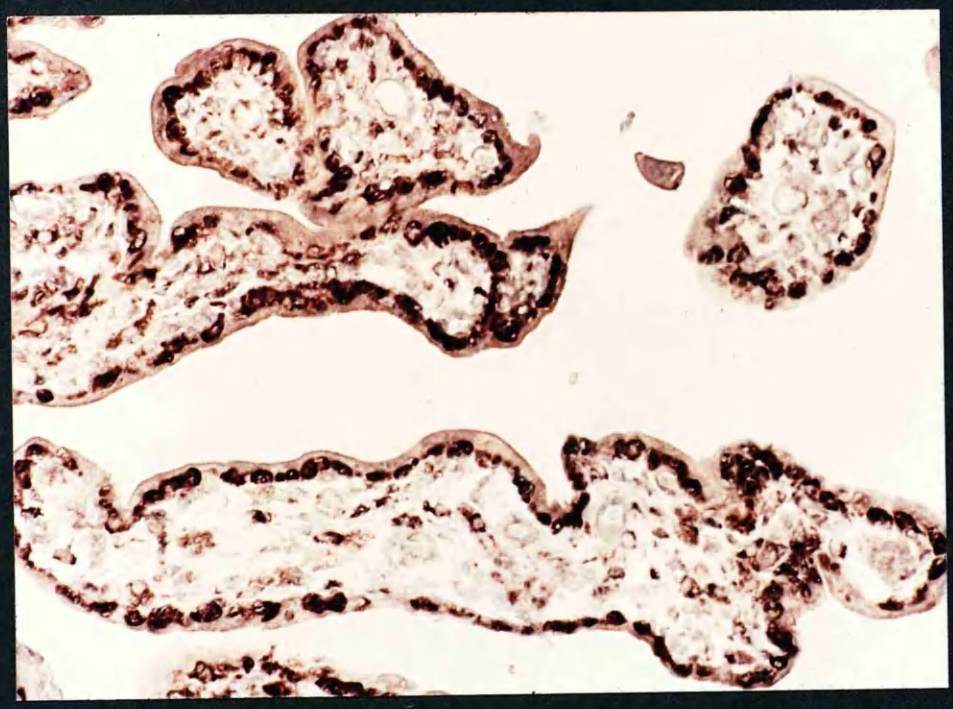
In situ hybridistion for total mRNA

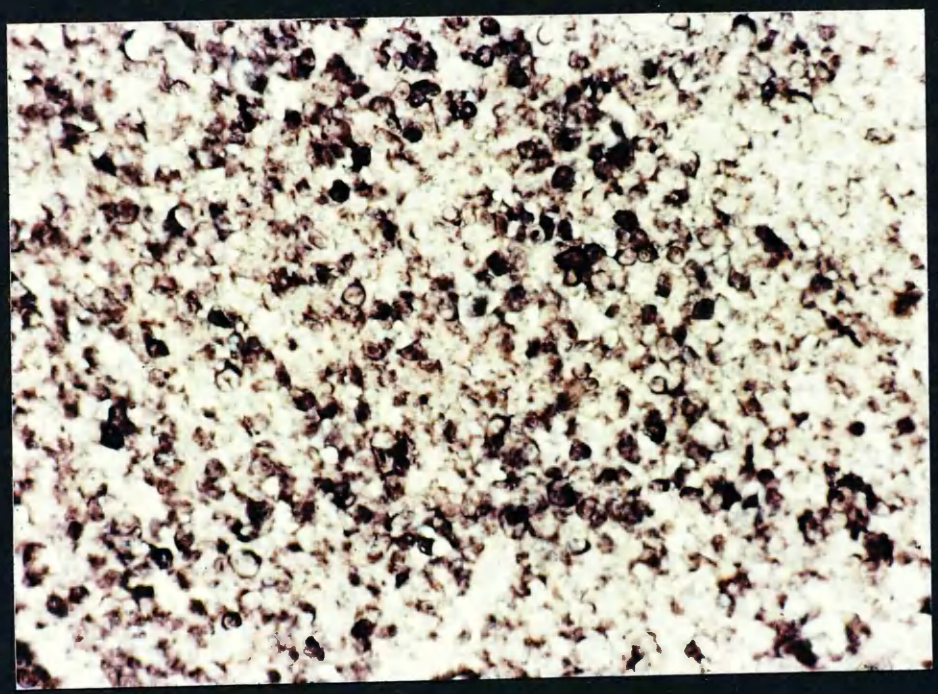
This shows mRNA in syncytiotrophoblast in placenta.

In situ hybridization for total mRNA with poly-d-T probe (x 380).

This shows mRNA in various cells in lymphoid tissue in adult tonsil.

In situ hybridization for total mRNA with poly-d-T probe (x 380).





RNase treatment

8 milligrams of Ribonuclease-A/ml of buffer, applied to the sections as described earlier in chapter 2, significantly reduced the positive staining with the poly-d-T probe in tissue sections.

DISCUSSION

When using probe 1 some staining was seen in cells in 3 spleens and 1 tonsil with the *in situ* hybridization technique for the demonstration of IFN-alpha mRNA in formalin fixed paraffin embedded normal human tissues. The cells staining with probe 1 were very round and had multilobated nuclei. Tissue sections from these spleen and tonsil cases, stained with the monoclonal antibody LeuM 1, using an immunocytochemical technique, showed that cells with similar morphology to that seen in the *in situ* hybridization technique were also stained positively. (The LeuM 1 monoclonal antibody recognises an epitope on neutrophils and Reed-Sternberg cells). These cells therefore appeared probably to be neutrophil polymorphs. Cytological detail obtained with the *in situ* hybridization technique was far from excellent and thus cells could not always be readily identified by morphological means alone. Double staining of tissue sections with probe 1 and anti-muramidase antiserum (macrophage marker) showed that cells fluorescing with the anti-muramidase

antiserum were not stained with probe 1. These cells also failed to stain for KP1, the macrophage monoclonal antibody, demonstrating that they were probably not macrophages. The staining observed was inconsistent, not uniformly distributed in the same tissue section and it also failed to disappear with RNase treatment. Therefore this staining must be regarded as non-specific.

No detectable staining was obtained with probe 2. The poly-d-T probe, on the other hand, showed specific positive staining in the cytoplasm of cells in all tissues studied. This demonstrated that mRNA was detectable in these formalin fixed paraffin embedded normal human tissues and was confirmation of previous work (Pringle *et al.*,1989).

A study using biotinylated oligonucleotide probes has shown calcitonin, adrenocorticotrophic hormone, prolactin and growth hormone mRNAs in formalin fixed paraffin embedded tissue sections from medullary thyroid carcinoma cases and pituitary glands (Hankin & Lloyd,1989). Using a digoxigenen labelled oligonucleotide probe, Farquharson *et al* (1990) demonstrated mRNA for the adrenocorticotrophin precursor, pro-opiomelanocortin (POMC), in rat pituitary gland tissues fixed in buffered formalin or 4% paraformaldehyde. However, in all these studies mRNA of various hormones was detected in endocrine cells,

where the number of copies of mRNA molecules may be very high. In a study performed for the detection of human papillomavirus in various human lesions it was estimated that at least 800 copies of the viral genome/cell were required to obtain a positive result in an *in situ* hybridization technique, using a biotin labelled probe (Crum *et al.*,1986).

As described above, total cellular mRNA was demonstrated in this study in formalin fixed paraffin embedded human tissues using the poly-d-T probe by an *in situ* hybridization technique. The poly-d-T probe can hybridize with any mRNA molecule having a poly-A tail. However the study failed to show IFN-alpha mRNA in placenta or in macrophages in tonsil and spleen with the two oligonucleotide probes, 1 and 2. It is well known that cells of the mononuclear phagocyte system perform many functions, such as phagocytosis and antigen processing and presentation, and that they produce many cytokines such as interleukin 1, tumour necrosis factor etc. Therefore if these cells do produce IFN-alpha, perhaps only a very small proportion of the total cellular mRNA pool would be IFN-alpha mRNA. As already described by Crum *et al* (1986), a certain number of nucleic acid molecules are required to be present in cells to be detected by the biotin labelled *in situ* hybridization detection systems. It is suggested that the number of the IFN-alpha mRNA

molecules may be below the threshold level for detection by the digoxigenen labelled probes used in the *in situ* hybridization technique. It could be argued that failure to detect a positive signal in the *in situ* hybridization technique may be due to the absence of the IFN-alpha mRNA molecules. However mRNA for IFN-alpha has been detected in normal human spleen and lymph nodes using dot blot hybridization technique (Tovey *et al.*,1987), so this explanation is unlikely.

Another possible cause of a false negative result in the system used here would be the fact that only oligonucleotide probes were used. During formalin fixation the target nucleic acid sequence may be broken and thus not detected. The use of longer probes or additional oligonucleotide probes may provide an advantage in this situation.

REFERENCES

Abbott, S.R., Buimovici-Klein, E., Cooper, L.Z., Briggs, M. & Weller, I.V.D. (1984) Rapid detection of immunoreactive interferon-alpha in AIDS. *The Lancet*, **i**, 564-566.

Adolf, G.R. (1990) Monoclonal antibodies and enzyme immunoassays specific for human interferon-omega 1: Evidence that IFN-omega 1 is a component of human leukocyte IFN. *Virology*, **175**, 410-417.

Aguet, M. (1980) High-affinity binding of ^{125}I-labelled mouse interferon to a specific cell surface receptor. *Nature*, **284**, 459-61.

Anonymous, (1980) Interferon nomenclature. *Nature*, **286**, 110.

Arrand, J.E. (1985) Preparation of nucleic acid probes. In *Nucleic acid hybridization-a practical approach*, eds. Hames, B.D. & Higgens, S.J. pp 17-45. IRL Press, Oxford.

Balkwill, F.R. (1989) Interferons. *The Lancet*, **i**, 1060-1063.

Belardelli, F., Gessani, S., Proietti, E., Locardi, C., Borghi, P., Watanabe, Y., Kawade, Y. & Gresser, I. (1987) Studies on the expression of spontaneous and induced interferons in mouse peritoneal macrophages by means of monoclonal antibodies to

mouse interferons. *Journal of General Virology*, **68**, 2203-2212.

Belardelli, F., Vignaux, F., Proietti, E. & Gresser, I. (1984) Injection of mice with antibody to interferon renders peritoneal macorphages permissive to vesicular stomatitis virus and encephalomyocarditis virus. *Proceedings of the National Academy of Sciences of the U.S.A.* **81**, 602-606.

Billiau, A., Van-Damme, J., Opdenakker, G., Fibbe, W.E., Flakenburg, J.H.F. & Content, J. (1986) Interleukin 1 as a cytokine inducer. *Immunobiology*, **172**, 323-335.

Birke, C., Peter, H.H., Langenberg, U., Muller-Hermes, W.J.P., Peters, J.H., Heitmann, J., Leiblod, W., Dalluge, H., Krapf, E. Kirchner, H. (1981) Mycoplasma contamination in human tumour cell lines: Effect on interferon induction and susceptibility to natural killing. *Journal of Immunology*, **127**, 94-98.

Blach-Olszewska, Z., Cambrzynska-Nowak, M. & Kwasniewska, E. (1984) Age related synthesis in BALB/c mice. In *Physiology and pathology of interferon systems*, eds. Borecky, L. & Lackovic, V. pp 224. Karger Basel.

Blanchard, D.K., Djeu, J.Y., Klein, T.W., Friedman, H. & Stewart II, W.E. (1986) Interferon-gamma

induction by lipopolysaccharide: Dependence on interleukin 2 and macrophages. *Journal of Immunology*, **136**, 963-970.

Bocci, V. (1980) Is interferon produced in physiological conditions? *Medical Hypotheses*, **6**, 735-745.

Bocci, V. (1981) Production and role of interferon in physiological conditions. *Biological Reviews of the Cambridge Philosophical Society*, **56**, 49-85.

Bocci, V. (1988) Roles of interferon produced in physiological conditions. A speculative review. *Immunology*, **64**, 1-9.

Bocci, V., Muscettola, M., Paulesu, L. & Grasso, G. (1984) The physiological interferon response II. Interferon is present in lymph but not in plasma of healthy rabbits. *Journal of General Virology*, **65**, 101-108.

Bocci, V., Paulesu, L. & Ricci, M.G (1985) The physiological interferon response: IV. Production of interferon by the perfused human placenta at term. *Proceedings of the Society for Experimental Biology and Medicine*, **180**, 137-143.

Branca, A.A. & Baglioni, C. (1981) Evidence that type I and II interferons have different receptors. *Nature*, **294**, 768-770.

Britten, R.J. & Davidson, E.H. (1985) Hybridization strategy. In *Nucleic acid hybridization-a*

practical approach, eds. Hames B.D. & Higgens, S.J. pp 3-15. IRL Press, Oxford.

Brigati, D.J., Myerson, D., Leary, J.J., Spalholz, B., Travis, S.Z., Fong, C.K.Y., Hsiung, G.D. & Ward, D.C. (1983) Detection of viral genomes in cultured cells and paraffin embedded tissue sections using biotin-labeled hybridization probes. *Virology*, **126**, 32-50.

Capobianchi, M.R., Ameglio, F., Tosi, R. & Dolei, A. (1985) Differences in the expression and release of DR, BR and DQ molecules in human cells treated with recombinant interferon-gamma: Comparison to other interferons. *Human Immunology*, **13**, 1-11.

Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S. & Cerami, A. (1986) Identification of a common nucleotide sequence in the 3' untranslated region of mRNA molecules specifying inflammatory mediators. *Proceedings of the National Academy of Sciences of the U.S.A.* **83**, 1670-1674.

Chard, T., Craig, P.H., Menabawey, M. & Lee, C. (1986) Alpha-interferon in human pregnancy. *British Journal of Obstetrics and Gynaecology*, **93**, 1145-1149.

Crum, C.C., Nagai, N., Levine, R.U. & Silverstein, S. (1984) *In situ* hybridization analysis of HPV 16 DNA sequences in early neoplasia. *American Journal of Pathology*, **123**, 174-182.

Davis, L.G., Dibner, M.D. & Battey, J.F. (1986) Hybridization with synthetic ^{32}P end-labeled probe. In *Basic methods in molecular biology*, pp 75-78. Elsevier, New York.

Dean, R.T. & Virelizer, J.L. (1983) Interferon as a macrophage activating factor. I. Enhancement of cytoxicity by fresh and matured human monocytes in the absence of other soluble signals. *Clinical and Experimental Immunology*, **51**, 501-510.

De-Maeyer, E. & De-Maeyer-Guignard, J. (1988a) Production of IFN-gamma by T cells & modulation of T cell, B cell, and macrophage activity by interferons. In *Interferons and other regulatory cytokines*, pp 221-273. John Wiley & Sons, Chichester.

De-Maeyer, E. & De-Maeyer-Guignard, J. (1988b) Induction of IFN-alpha and IFN-beta. In *Interferons and other regulatory cytokines*, pp 39-66. John Wiley & Sons, Chichester.

De-Maeyer, E. & De-Maeyer-Guignard, J. (1988c) Production of IFN-gamma by T cells & modulation of T cell, B cell, and macrophage activity by interferons. In *Interferons and other regulatory cytokines*, pp 221-273. John Wiley & Sons, Chichester.

De-Maeyer, E. & De-Maeyer-Guignard, J. (1988d) The effects of interferons on cell growth and

division. In *Interferons and other regulatory cytokines*, pp 134-153. John Wiley & Sons, Chichester.

De-Maeyer, E., Fauve R.M. & De-Maeyer-Guignard, J. (1971) Production d'interferon au niveau du macrophage. *Annales Institut Pasteur*, **120**, 438-446.

Derynck, R., Content, J., De-Clercq, E., Volckaert, G., Tavernier, J., Devos, R. & Fiers, W. (1980) Isolation and structure of a human fibroblast interferon gene. *Nature*, **285**, 542-547.

Di-Bisceglie, A.M., Martin, P., Kassianides, C., Lisker-Melman, M., Murray, L., Waggoner, J., Goodman, Z., Banks, S.M. & Hoofnagle, J.H. (1989) Recombinant interferon-alfa therapy for chronic hepatitis C. *New England Journal of Medicine*, **321**, 1506-1510.

Douglas, R.G. Jr (1986) The common cold-relief at last? *New England Journal of Medicine*, **314**, 114-115.

Duc-Goiran, P., Robert-Galliot, B., Lopez, J. & Chany, C. (1985) Unusual apparently constitutive interferons and antagonists in the human placental blood. *Proceedings of the National Academy of Sciences of the U.S.A.* **82**, 5010-5014.

Dzvonyar, J., Ruzicska, G., Desio, G. & Csaba, B. (1970) Distribution of p^{32} labelled coli endotoxin in the tissues of pregnant rabbit and of their

fetuses. *American Journal of Obstetrics and Gynaecology*, **106**, 721-725.

Eron, L.J., Judson, F., Tucker, S., Prawer, S., Mills, J., Murphy, K., Hickey, K., Rogers, M., Flannigan, S., Hien, N., Katz, H.I., Goldman, S., Gottlieb, A., Adams, K., Burton, P., Tanner, D., Taylor, E. & Peets, E. (1986) Interferon therapy for condylomata acuminata. *New England Journal of Medicine*, **315**, 1059-1064.

Exley, R., Nathan, P., Walker, L., Gordon, J. & Clemens, M.J. (1987) Anti-proliferative effect of interferons on Daudi Burkitt-lymphoma cells: Induction of cell differentiation and loss of response to autocrine growth factors. *International Journal of Cancer*, **40**, 53-57.

Exley, T., Parti, S., Barwick, S. & Meager, A. (1984) A comparison of the neutralizing properties of monoclonal and polyclonal antibodies to human interferon alpha. *Journal of General Virology*, **65**, 2277-2280.

Falcoff, R. (1972) Some properties of virus and immune-induced human lymphocyte interferon. *Journal of General Virology*, **16**, 251-253.

Farquharson, M., Harvie, R. & McNicol, A.M. (1990) Detection of messenger RNA using a digoxigenen end labelled oligonucleotide probe. *Journal of Clinical Pathology*, **43**, 424-428.

Field, A.K., Tytell, A.A., Lampson, G.P. & Hilleman, M.R. (1967) Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. *Proceedings of the National Academy of Sciences of the U.S.A.* **58**, 1004-1010.

Foulis, A.K., Farquharson, M.A. & Meager, A. (1987) Immunoreactive alpha-interferon in insulin secreting beta cells in type 1 diabetes mellitus. *The Lancet*, **ii**, 1423-1427.

Fowler, A.K., Reed, C.D. & Giron, D.J. (1980) Identification of an interferon in murine placenta. *Nature*, **286**, 266-267.

Freshman, M.M., Merigan, T.C., Remington, J.S. & Brownlee, I.E. (1966) *In vitro* and *in vivo* antiviral action of an interferon-like substance induced by toxoplasma gondii. *Proceedings of the Society for Experimental Biology and Medicine*, **123**, 862-866.

Friedman, R.M. & Vogel, S.N. (1983) *Advances in Immunology*, eds. Dixon, F.J. & Kunkel, H.G. Vol. 34, pp 97-140. Academic Press, New York & London.

Galabru, T., Robert N., Buffet-Janvresse, C., Riviere, Y. & Hovanessian, A.G. (1985) Continuous production of interferon in normal mice: Effect of anti-interferon globulin, sex, age, strain and environment on the levels of 2-5A synthetase and

p67K kinase. *Journal of General Virology*, **66**, 711-718.

Gall, J.G. & Pardue, M.L. (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparation. *Proceedings of the National Academy of Sciences of the U.S.A.* **63**, 378-383.

Gessani, S., Belardalli, F., Borghi, D. & Gresser, I. (1987) Correlation between the lipopolysaccharide response of mice and the capacity of mouse peritoneal cells to transfer an antiviral state. Role of endogenous interferon. *Journal of Immunology*, **139**, 1991-1998.

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.

Gifford, G.E., Tibor, A. & Peavy, D.L. (1971) Interferon production in mixed lymphocyte cell cultures. *Infection and Immunity*, **3**, 164-166.

Giles, F.J., Singer, C.R.J., Gray, A.G., Yong, K.L., Brozovic, M., Davies, S.C., Grant, I.R., Hoffbrand, A.V., Machin, S.J., Mehta, A.B., Richard, J.D.M., Thomas, M.J.G., Venutas, S. & Goldstone, A.H. (1988) Alpha-interferon therapy for essential thrombocythaemia. *The Lancet*, **ii**, 70-72.

Goeddel, D.V., Leung, D.W., Dull, T.J., Gross, M., Lawn, R.M., McCandliss, R., Seeburg, P.H., Ullrich, A., Yelverton, E. & Gray, P. W. (1981) The structure of eight distinct cloned human leukocyte interferon cDNAs. *Nature*, **290**, 20-26.

Gray, P.W. & Goeddel, D.V. (1982) Structure of the human immune interferon gene. *Nature*, **298**, 859-863.

Green, J.A., Cooperband, S.R. & Kibrick, S. (1969) Immune specific induction of interferon production in cultures of blood lymphocytes. *Science*, **164**, 1415-1417.

Gresser, I., Vignaux, F., Belardelli, F., Tovey, M.G. & Maunoury, M.T. (1985) Injection of mice with antibody to mouse interferon-alpha/ beta decreases the level of 2'-5' oligoadenylate synthetase in peritoneal macrophages. *Journal of Virology*, **53**, 221-227.

Hankin, R.C. & Lloyd, R.V. (1989) Detection of messenger RNA in routinely processed tissue sections with biotinylated oligonucleotide probes. *American Journal of Clinical Pathology*, **92**, 166-171.

Havell, E.A. (1986) Augmented induction of interferons during *Lesteria monocytogenes* infection. *Journal of Infectious Diseases*, **153**, 960-969.

Havell, E.A., Hayes, T.G. & Vilcek, J. (1978) Synthesis of two distinct interferons by human fibroblasts. *Virology*, **89**, 330-334.

Hayes, T.S., Yip, Y.K. & Vilcek, J. (1979) Le interferon production by human fibroblasts. *Virology*, **98**, 351-363.

Hayden, F.G., Albrecht, J.K., Kaiser, D.L. & Gwaltney, J.M (1986) Prevention of natural colds by contact prophylaxis with intranasal alpha 2-interferon. *New England Journal of Medicine*, **314**, 71-75.

Healy, G.B., Gelber, R.D. & Trowbridge, A.L. (1988) Treatment of recurrent respiratory papillomatosis with human leukocyte interferon. *New England Journal of Medicine*, **319**, 401-407.

Houghton, M., Eaton, M.A.W., Stewart, A.G., Smith, J.C., Doel, S.M., Catlin, G.H., Lewis, H.M., Patel, T.P., Emstage, J.S., Carey, N.H. & Porter, A.G. (1980) The complete amino acid sequence of human fibroblast interferon as deduced using synthetic oligodeoxyribonucleotide primers of reverse transcriptase. *Nucleic Acids Research*, **8**, 2885-2894.

Ho-Yen, D.O. & Carrington, D. (1987) Alpha-interferon responses in the cerebrospinal fluid of patients with suspected meningitis. *Journal of Clinical Pathology*, **40**, 83-86.

Howatson, A.G., Farquharson, M.A., Meager, A., McNicol, A.M. & Foulis, A.K. (1988) Localization of alpha-interferon in the human fetoplacental unit. *Journal of Endocrinology*, **119**. 531-534.

Hughes, T.K. & Baron, S. (1987) A large component of the antiviral activity of mouse interferon-gamma may be due to its induction of interferon-alpha. *Journal of Biological Regulators and Homoeostatic Agents*, **1**, 29-32.

Iles, R.K. & Chard, T. (1989) Enhancement of ectopic beta-human chorionic gonadotrophin expression by interferon-alpha. *Journal of Endocrinology*, **123**, 501-507.

Imakawa, K., Anthony, R.V., Kazemi, M., Marotti, K.R., Polites, H.G. & Roberts, R.M. (1987) Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. *Nature*, **330**, 377-379.

Issacs, A. & Lindenmann, J. (1957) Virus interference. I. The interferon. *Proceedings of the Royal Society of London (Biological Sciences)*, **147**, 258-267.

Jaatela, M., Kiuusela, P. & Saksela, E. (1988) Demonstration of tumour necrosis factor in human amniotic fluids and supernatants of placental and decidual tissues. *Laboratory Investigation*, **58**, 48-52.

Jacob, A.I., Goldberg, P.K., Bloom, N., Degenshein, G.A. & Kozim, P.J. (1977) Endotoxin and bacteria in portal blood. *Gastroenterology*, **72**, 1268-1270.

Kasahara, T., Hooks, J.J., Dougherty, S.F. & Oppenheim, J.J. (1983) Interleukin-2 mediated immune interferon (IFN-gamma) production by human T cells and T cell subsets. *Journal of Immunology*, **130**, 1784-1789.

Kiener, P.A. & Spitalny, G.L. (1987) In *Lymphokines and Interferons*, eds. Clemens, M.J., Morris A.G. & Gearing, A.J.H. pp 17. IRL Press, Oxford.

Kirchner, H., Weyland, A. & Storch, C. (1986) Local interferon induction by bacterial lipopolysaccharide in mice after pretreatment with *corynebacterium parvum*. *Journal of Interferon Research*, **6**, 483-487.

Klavinskis, L.S., Geckeler, R. & Oldstone, M.B.A. (1989) Cytotoxic T lymphocytes control of acute lymphocytic choriomeningitis virus infection: IFN-gamma, but not TNF-alpha displays antiviral activity *in vivo*. *Journal of General Virology*, **70**, 3317-3325.

Kohno, S., Kohase, M., Sakata, H., Shimizu, Y., Hikita, M. & Shishido, A. (1970) Production of interferon in primary chick embryonic cells infected with *Rickettsia Mooseri*. *Journal of Immunology*, **105**, 1553-1558.

Lebon, P., Girard, S., Thepot, F. & Chany, C. (1982) The presence of alpha-interferon in human amniotic fluid. *Journal of General Virology*, **59**, 393-396.

Lee, C.L.Y., Lee, S.H.E., Jay, F.T. & Rozee, K.R. (1990) Induction of Interferon-gamma synthesis by staphylococcal enterotoxin B (SEB) in peripheral blood mononuclear cells. *Immunology*, **70**, 94-99.

Maeger, A. (1987) Antibodies against interferons. Characterization of interferons and immunoassays. In *lymphokines and Interferons-A Practical Approach*, eds. Clemens, M.J., Morris A.G. & Gearing, A.J.H. pp 105-127. IRL Press, Oxford.

Mandelli, F., Avvisati, G. & Petrucci, M.T. (1990) A new strategy in the treatment of multiple myeloma. *European Journal of Haematology (Supplementum)*, **51**, 129-134.

Martinez-Maza, O., Andersson, U., Andersson, J., Britton, S. & Ley, M.D. (1984) Spontaneous production of interferon-gamma in adult and newborn humans. *Journal of Immunology*, **132**, 251-255.

Moench, T.R., Gendelman, H.E., Clements, J.E., Narayan, O. & Griffin, D.E. (1985) Efficiency of *in situ* hybridization as a function of probe size and fixation technique. *Journal of Virology Methods*, **11**, 119-130.

Moore, R.N., Larsen, H.S., Horohov, D.W. & Rouse, B.T. (1984) Endogenous regulation of macrophage proliferative expansion by colony-stimulating factor induced interferon. *Science*, **223**, 178-181.

Nathan, C.F., Murray, H.W., Wiebe, M.E. & Rubin, B.Y. (1983) Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *Journal of Experimental Medcine*, **158**, 670-689.

Nathan, I., Groopman, J.E., Quan, S.G., Bersch, N. & Golde, D.W. (1981) Immune (gamma) interferon produced by a human T-lymphoblast cell line. *Nature*, **292**, 842-844.

Naylor, S.L., Sakaguchi, A.Y., Shows, T.B., Law, M.L., Goeddel, D.V. & Gray, P.W. (1983) Human immune interferon gene is located on chromosome 12. *Journal of Experimental Medcine*, **57**, 1020-1027.

Noordan, S.V. (1986) Tissue preparation and immunostaining techniques for light microscopy. In *Immunocytochemistry. Modern methods and applications*, eds. Polak, J.M. & Noordan, S.V. pp 26-53. John Wright & Sons Ltd. Bristol.

Ojo-Amaize, E.A., Salimonu, L.S., Williams, A.I.O., Akinwolere, O.A.O., Shabo, R., Alm, G. & Wigzell, H. (1981) Positive correlation between degree of parasitemia, interferon titres, and natural killer

cell activity in *plasmodium falciparum*-infected children. *Journal of Immunology*, **127**, 2296-2300.

Pardue, M.L. (1985) *In situ* hybridisation. In *Nucleic acid hybridisation-a practical approach*, eds. Hames, B.D. & Higgens, S.J. pp 179-202. IRL Press, Oxford.

Paraf, A., Philips, N., Simonin, G., De Maeyer-Guignard, J. & De Maeyer, E. (1983) Differential cytostatic effect of interferon on murine BALB/c B and T-cell tumors. *Journal of Interferon Research*, **3**, 253-260.

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

Preble, O.T., Black, R.J., Friedman, R.M., Klippel, J.H. & Vilcek, J. (1982) Systemic lupus erythematosus: presence in human serum of an unusual acid-labile leukocyte interferon. *Science*, **216**, 429-431.

Pringle, J.H., Primrose, L., Kind, C.N., Talbot, I.C. & Lander, I. (1989) *In situ* hybridization demonstration of poly-adenylated R.N.A sequences in formalin-fixed paraffin sections using a biotinylated oligoprobe. *Journal of Pathology*, **158**, 279-286.

Prior, C. & Haslam, P.L. (1989) Interferon in lungs. *The Lancet*, **i**, 1333.

Pulford, K.A.F., Rigney, E., Micklem, K.J., Jones, M., Stross, P., Gatter, K.C. & Mason, D.Y. (1989) KP1: a new monoclonal antibody that detects a monocyte/ macrophage associated antigen in routinely processed tissue sections. *Journal of Clinical Pathology*, **42**, 414-421.

Reyes, V.E., Ballas, Z.K., Singh, H. & Klimpel, G.R. (1986) Interleukin 2 induces interferon alpha/beta production in mouse bone marrow cells. *Cellular Immunology*, **102**, 374-385.

Rossi, G.B. (1985) Interferons and cell differentiation. In *Interferon 6*, ed. Gresser, I. pp 31-68. Academic Press, London.

Rubinstein M. & Orchansky P. (1986) The interferon receptors. *CRC Critical Reviews in Biochemistry*, **21**, 249-275.

Ruiz-Moreno, M., Jimenez, J., Porres, J.C., Bartolome, J., Moreno, A. & Carreno, V. (1990) A controlled trial of recombinant IFN-alpha in caucasian children with chronic hepatitis B. *Digestion*, **45**, 26-33.

Saksela, E., Virtanen, I., Hovi, T., Secher, D.S. & Cantell, K. (1984) Monocytes is the main producer of human leucocyte alpha-interferons following

Sendai virus induction. *Progress in Medical Virology*, **30**, 78-86.

Salamonsen, L.A., Stuchbery, S.J., O'Grady, C.M., Godkin, J.D. & Findlay, J.K. (1988) Interferon-alpha mimics effects of ovine trophoblast protein 1 on prostaglandin and protein secretion by ovine endometrial cells *in vitro*. *Journal of Endocrinology*, **117**, R1-R4.

Salas, M.D.P. (1987) Alpha-interferon responses in children with respiratory syncytial virus associated bronchiolitis. *MSc thesis, University of Glasgow*.

Salvin, S.B., Youngner, J.S. & Lederer, W.H. (1973) Migration inhibitory factor and interferon in the circulation of mice with delayed hypersensitivity. *Infection and Immunity*, **7**, 68-75.

Sehgal, P.B., Zilberstein, A., Ruggieri, R.M., May, L.T., Ferguson-Smith, A., Slate, D.L., Revel, L. & Ruddle, F.H. (1986) Human chromosome 7 carries the beta 2 interferon gene. *Proceedings of the National Academy of Sciences of the U.S.A.* **83**, 5219-5222.

Sergiescu, D., Gerfaux, J., Joret, A.M. & Chany, C. (1986) Persistent expression of v-*mos* oncogene in transformed cells that revert to nonmalignancy after prolonged treatment with interferon.

Proceedings of the National Acadmey of Sciences of the U.S.A. **83**, 5764-5768.

Shaw, G. & Kamen, R. (1986) A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA-degradation. *Cell*, **46**, 659-667.

Shiozawa, S., Chihara, K., Shiozawa, K., Fujita, T., Ikegami, H., Koyama, S. & Kurimoto, M. (1986) A sensitive radioimmunoassay for alpha-interferon: circulating alpha-interferon like substance in the plasma of healthy individuals and rheumatoid arthritis patients. *Clinical and Experimental Immunology*, **66**, 77-87.

Slate, D.L., D'Eustachio, P., Pravtcheva, D., Cunningham, A.C., Nagata, S., Weissmann, C. & Ruddle, F.H. (1982) Chromosomal location of a human alpha interferon gene family. *Journal of Experimental Medcine*, **155**, 1019-1024.

Smith, T.J. & Wagner, R.R. (1967) Rabbit macrophage interferons. Conditions for biosynthesis by virus-infected and uninfected cells. *Journal of Experimental Medcine*, **125**, 559-577.

Sonnenfeld, G. & Kierszenbaum, F. (1981) Increased serum levels of an interferon like activity during the acute period of experimental infection with different strains of *Trypanosoma cruzi*. *American*

Journal of Tropical Medicine and Hygiene, **30**, 1189-1191.

Soubiran, P., Zapitelli, J.P. & Schaffer, L. (1987) IL-2 like material is present in human placenta and amnion. *Journal of Reproductive Immunology*, **12**, 225-234.

Stewart, H.J., McCann, S.H.E., Barker, P.J., Lee, K.E., Lamming, G.E. & Flint, A.P.F. (1987) Interferon sequence homology and receptor binding activity of ovine trophoblast antiluteolytic protein. *Journal of Endocrinology*, **115**, R13-R15.

Stanton, G.J., Weigent, D.A., Fleischmann, W.R. Jr., Dianzani, F. & Baron, S. (1987) Interferon review. *Investigative Radiology*, **22**, 259-273.

Sutherland, C.G.G., Al-Badri, A.M.T., Howatson, A.G., Farquharson, M.A. & Foulis, A.K. (1989) Immunlocalisation of alpha-interferon in liver diseases. *Journal of Clinical Pathology*, **42**, 1065-1069.

Talpaz, M., Kantarjian, H.M., McCredie, K.B., Keating, M.J., Trujillo, J. & Gutterman, J. (1987) Clinical investigation of human alpha interferon in chronic myelogenous leukemia. *Blood*, **65**, 1280-1288.

Taniguchi, T., Mantei, N., Schwarzstein, M., Nagata, S., Muramatsu, M. & Weissmann, C. (1980) Human leukocyte and fibroblast interferons are structurally related. *Nature*, **285**, 547-549.

Torczynski, R.M., Fuke, M. & Bollon, A.P. (1984) Human genomic library screened with 17-base oligonucleotide probes yields a novel interferon gene. *Proceedings of the National Academy of Sciences of the U.S.A.* **81**, 6451-6455.

Tovey, M.G., Streuli, M., Gresser, I., Gugenheim, J., Blanchard, B., Guymarho, J., Vignaux, F. & Gigou, M. (1987) Interferon messenger RNA is produced constitutively in the organs of normal individuals. *Proceedings of the National Academy of Sciences of the U.S.A.* **84**, 5038-5042.

Trent, J.M., Olson, S. & Lawn, R.M. (1982) Chromosomal localization of human leukocyte, fibroblast and immune interferon genes by means of *in situ* hybridization. *Proceedings of the National Academy of Sciences of the U.S.A.* **79**, 7809-7813.

Trinchieri, G. & Perussia, B. (1985) Immune interferon: a pleiotropic lymphokine with multiple effects. *Immunology Today*, **6**, 131-136.

Van Damme, J., Opdenakker, G., Simpson, R.J., Rubira, M.R., Cayphas, S., Vink, A., Billiau, A. & Van Snick, J. (1987) Identification of the human 26-kD protein, interferon beta2 as a B cell hybridoma/ plasmacytoma growth factor induced by interleukin 1 and tumour necrosis factor. *Journal of Experimental Medcine*, **165**, 914-919.

Vilcek, J., Henriksen-Destefano, D., Siege,l D., Klion, A., Robb, R.J. & Le, J. (1985) Regulation of IFN-gamma induction in human peripheral blood cells by exogenous and endogenously produced interleukin 2. *Journal of Immunology,* **135**, 1851-1856.

Weissmenn, C. & Weber, H. (1986) The interferon genes. *Progress in Nucleic Acid Research and Molecular Biology,* **33**, 251-302.

Wright, J.F. & Hunter, W.M. (1983) The sucrose layering separation: a non-centrifugation system. In *Immunoassays for Clinical Chemistry,* eds. Hunter, W.M & Corrie, J.E.T. Second edition, pp 170-177. Churchill Livingstone, Edinburgh.

Wong, G.H.W., Clark-Lewis, I., McKimm-Breschkin, J.L., Harris, A.W. & Schrader, J.W. (1983) Interferon-gamma induces enhanced expression of Ia and H-2 antigens on B lymphoid, macrophage, and myeloid cell lines. *Journal of Immunology,* **131**, 788-793.

Yip, Y.K., Barrowclough, B.S., Urban, C. Vilcek, J. (1982) Purification of two species of human gamma (immune) interferon. *Proceedings of the National Academy of Sciences of the U.S.A.* **79**, 1820-1824.

Youngner, J.S & Stinebring, W.R. (1964) Interferon production in chickens injected with *Brucella abortus*. *Science,* **144**, 1022-1023.

Zilberstein, A., Ruggieri, R., Korn, J.H. & Revel, M. (1986) Structure and expression of cDNA and genes

for human interferon-beta 2, a distinct species inducible by growth-stimulatory cytokines. *The EMBO Journal*. **5**, 2529-2537.

Zullo, J.N., Cochran, B.H., Huang, A.S. & Stiles, C.D. (1985) Platlet derived growth factor and double-stranded ribonucleic acids stimulate expression of the same gene in 3T3 cells. *Cell*, **43**, 773-800.

Zoumbos, N.C., Gascon, P., Djeu, J.Y. & Young, N.S. (1985) Interferon is a mediator of hematopoietic suppression in aplastic anaemia *in vitro* and possibly *in vivo*. *Proceedings of the National Academy of Sciences of the U.S.A.* **82**, 188-192.

APPENDIX

1) Procedure for use of 3'diaminobenzidine tetrahydrochloride (DAB).

i) Reconstitute 21 mls of stock DAB (Sigma,UK) in 300 mls of water to make a 0.03% DAB solution which is then aliquoted in 21 mls containers and frozen. DAB is a potential carcinogen therefore the entire procedure is performed in a fume chamber.

ii) Add 21 mls of the frozen DAB solution to 300 mls of tris HCL buffer, pH 7.6 and allow to thaw.

iii) 6 drops of 100 vols (30%) hydrogen peroxide (BDH,UK) are then added to the above solution.

iv) The solution is filtered and then used to incubate slides. On expiry of the working life of the DAB solution i.e approximately 2 hours, the solution is poured into a waste DAB container. This contains 1 pellet of hypochlorite bleach that inactivates the DAB, which can than be poured down the sink. All the above procedures are performed in a designated covered area and gloves and protective clothing are worn all the time when using DAB.

2) Trypsinization

Preheat a water bath to 37^{o}C. Place two staining dishes each containing 300 mls of 0.05M tris HCL buffer, pH 7.6. In it add 100 milligrams of

trypsin (Sigma) and 100 milligrams of calcium chloride to one dish. Make sure all solids are dissolved. Sections are first placed into the dish with tris HCL buffer for 5 minutes and then transferred to the trypsin solution.

3) **Alkaline phosphatase substrates.**

Fast Red.

Add 2 drops of dimethyl formamide to 5 milligrams of naphthal AS BI phosphoric acid (Sigma) in a tube. In a separate tube dissolve Fast Red TR salt in veronal acetate buffer (0.9715 gm sodium acetate, 1.4715 gms sodium barbitone (both from BDH,UK), 247.5 ml distiled water and N/10 HCL, pH 9.2). Mix the contents of the two tubes and add 200 micrograms of 1mM levamisole (Sigma). Mix well and filter before use.

Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (both from Sigma).
To 7.5 mls of buffer three add 33 microlitres of NBT and 25 microlitres of BCIP. No levamisole was added to the NBT/BCIP solution used in the labelling reaction described in chapter 2, section(F). In the *in situ* hybridization technique, however, 1.8 milligrams of levamisole was added to 7.5 mls of buffer three, to block

endogenous alkaline phosphatase activity in the tissue sections.

4) **Vectastain ABC reagents (Vector, UK).**

Add exactly two 2 drops of reagent A (Avidin DH) to 10 mls of tris buffered saline (0.01M tris HCL, pH 7.6, 0.15M NaCl). Then add exactly 3 drops of reagent B (Biotinylated horseradish peroxidase H) to the above solution, mix and allow this solution to stand for about 30 minutes before use.

5) **Streptavidin-biotin complex (DAKO).**

One drop of reagent A (streptavidin in 0.01M phosphate buffer, 0.15M NaCl, 15mM NaN_3, pH 7.2) and one drop of reagent B (biotinylated alkaline phosphatase in 0.05M tris HCL, 0.1M NaCl, 1mM $MgCl_2$, 15mM NaN_3, pH 7.2) are added to 5 ml of 0.01M tris HCL buffer, pH 7.6. They are mixed well and allowed to stand for 30 minutes before use (1 drop is equal to approximately 45 microlitres).

6) **T.E buffer.**

Dissolve 0.605 gms of 10mM tris HCL and 0.186 gms of ethylenediamine tetraacetic acid in 500 mls of water, pH 8.

7) **Hybridization buffer.**

It consisted of the following materials.

i) 0.01M tris HCL (pH 7.5).

ii) 12.5% Denhardt's solution (0.02% Ficoll 400, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone in DEPC water) (All were from Sigma).

iii) DEPC 2xSSC solution.

iv) 20% & 50% formamide (BDH).

v) 0.5% sodium dodecyl sulphate (BDH).

vi) 250 micrograms/ ml of salmon sperm DNA (denatured and sonicated, from Sigma).

vii) 50% dextran sulphate (Sigma).

viii) DEPC Water.

8) Coating of coverslips for use in the *in situ* hybridization techniques.

Place the slides in 5% dimethyldichlorosilane in chloroform (Sigma) for 30 minutes. This substance is highly toxic and should be handled with maximum care in a fume chamber. Wash with plenty of water and spread to dry. Once dried they can be stored for future use.

GLASGOW UNIVERSITY LIBRARY