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The distribution of immunoreactive interferon-alpha in formalin-fixed paraffin-embedded normal human foetal and infant tissues

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SUMMARY

Human foetal and infant tissues were studied to test the hypothesis that microbes have a role in switching on interferon-alpha (IFN-α) synthesis. Foetal tissues were essentially 'germ free', while the infants had been exposed to a normal microbial environment in life. IFN-α was first seen at 9 weeks gestation in macrophages in the liver and thereafter was seen in macrophages in most other organs. When infant lungs were compared with foetal lungs, a statistically significant increase in the number of macrophages and the percentage of these cells expressing IFN-α was noted in the infant lungs. No such change was observed in spleen, liver and thymus following birth. These findings suggest that there is a basal production of IFN-α by macrophages that is not dependent on microbial products, but that such products can enhance synthesis of this cytokine.

INTRODUCTION

In a previous study we described the presence in normal human tissues of immunoreactive interferon-alpha (IFN-α) in tissue histiocytes and other cells of the mononuclear phagocyte system. Parenchymal cells in some organs also contained immunoreactive IFN-α (Khan *et al.*, 1989). Other workers have observed the presence of IFN-α in physiological conditions but they did not localize the cells which contained this product (Chard *et al.*, 1986; Ho-Yen & Carrington, 1987; Prior & Haslam, 1989; Tovey *et al.*, 1987; Bocci *et al.*, 1984; Zoumbos *et al.*, 1985; Shiozawa *et al.*, 1986).

Bocci (1988) has proposed that since bacteria and endotoxin are known inducers of IFN-α synthesis (DeMaeyer *et al.*, 1971; Havell, 1986), much of the physiological IFN-α response may be due to the presence of the normal microbial flora in the gut, skin, mouth and genital tract. This hypothesis is supported by *in vitro* studies, which have shown no spontaneous release of IFN-α by peritoneal macrophages derived from germ-free mice. This contrasts with release of low levels of IFN-α by peritoneal macrophages from mice kept in a normal environment (DeMaeyer *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-α 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, Cambryzynska-Nowak & Kwasniewska, 1984).

We attempted to test Bocci's (1988) hypothesis in man by comparing the frequency of IFN-α-containing tissue cells in foetuses with that found in infants. A normal foetus is germ-free and pathogen-free, but after birth it is exposed to a large variety of microbes. Therefore, if Bocci's hypothesis were true, low numbers of IFN-α-positive cells might be expected up until birth, after which a dramatic rise might be predicted.

The other objectives of the present study were (i) to establish whether the cellular distribution of IFN-α-containing cells in human foetal tissues was the same as that found in adults; and (ii) to find out at what stage of foetal life IFN-α-containing cells appeared.

MATERIAL AND METHODS

Tissues studied

Normal human tissues from 32 foetal autopsies performed within 24 hr of death were studied. These included four foetuses of 7–11 weeks gestation (removed at curretage from women previously not known to be pregnant), 20 foetuses of 12–25 weeks gestation (12 terminations of normal pregnancy, five hydrocephalics, one anencephalic and two foetuses with placental complications) and eight foetuses of 26–42 weeks gestation (stillbirths) with no evidence of viral or bacterial infection.

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Normal human tissues were also obtained from autopsies on 20 infants aged from a few hours to 24 months (six accidental deaths, seven infants with congenital heart disease and seven fatalities from sudden infant death syndrome). All showed no clinical or laboratory evidence of bacterial or viral infection.

Staining techniques

All foetal and infant tissues were fixed in formol saline and embedded in paraffin wax. Four-micron thick sections were cut from the paraffin-embedded blocks of all tissues and mounted on three aminopropyltriethoxysilane-coated glass slides. Respective serial sections from all tissues were stained with an indirect immunoperoxidase technique, in which diaminobenzidine was used as a substrate. The following primary polyclonal antisera and monoclonal antibodies (mAb) were used: polyclonal H51 (sheep anti-IFN-α, gift from Dr A. Meager (National Institute for Biological Standards and Control, Potters Bar, London, U.K.), polyclonal rabbit anti-muramidase, a macrophage marker (Dako, Roskilde, Denmark) and the mAb KP1 (a gift from Dr K. Pulford, University of Oxford, U.K.; Pulford *et al.*, 1989) and MAC 387 (Dako), both of which are also macrophage markers.

The following bridges were used for the indirect immunoperoxidase techniques: peroxidase-conjugated swine anti-sheep immunoglobulins (Serotec, Oxford, U.K.), peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako) and peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako).

The specificity of the H51 antiserum has been described previously (Khan *et al.*, 1989; Foulis, Farquharson & Meager, 1987), as has that of the KP1 mAb (Pulford *et al.*, 1989).

A normal placenta previously shown to have immunoreactive IFN-α in the syncitiotrophoblast (Howatson *et al.*, 1988) was used as a positive control throughout the studies.

'Specific staining' for IFN-α describes a situation where cells stained positively with the H51 antiserum in the indirect immunoperoxidase technique but negatively when the antiserum was substituted by normal sheep serum. The term is further defined by showing that preincubation of the H51 antiserum overnight at 4° with 'Wellferon' (human lymphoblastoid IFN-α Namalwa; Wellcome Research Laboratories, Beckenham, Kent, U.K.) prior to its use abolished all positive staining in the cells.

Determination of proportion of macrophages expressing IFN-α

To assess the proportion of macrophages expressing IFN-α in various tissues in infants and foetuses, cells positive with the mAb KP1 and those containing immunoreactive IFN-α were counted in the same 10 high power fields on consecutive serial sections (one higher power field = 62,500 square microns). The percentage of KP1-positive cells (macrophages) containing immunoreactive IFN-α was thus assessed.

Assessment of quantification of positive cells using the serial section technique

Two successive serial sections from nine blocks of tissue (four thymus, four liver, one spleen) were stained for IFN-α. 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 sections 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.

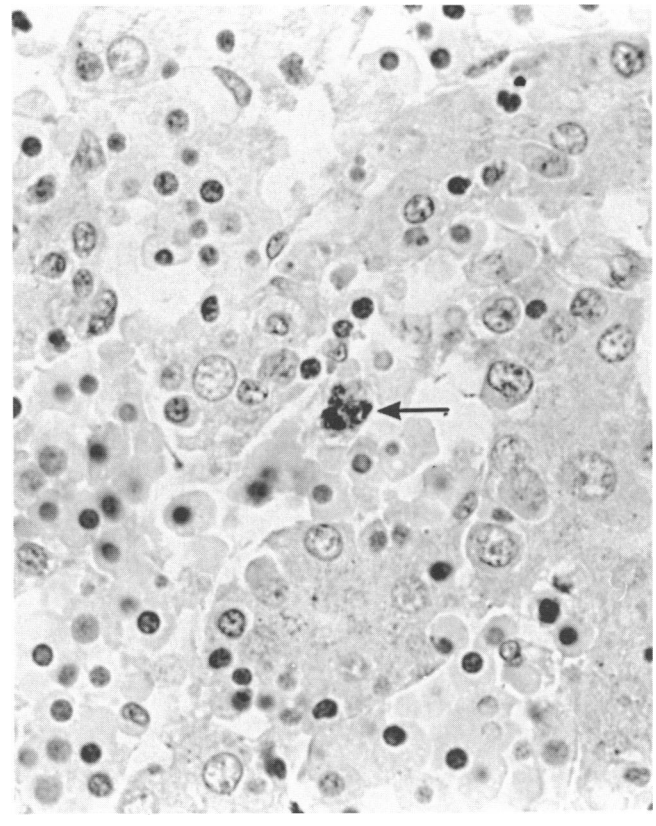

Figure 1. An intrasinusoidal cell (arrow) staining positively for IFN-α in the liver of a 9-week-old foetus. Indirect immunoperoxidase (IIP) for IFN-α (× 780).

Double-staining technique

A double-staining technique was developed using H51 antiserum and a polyclonal rabbit anti-prekeratin antiserum (epithelial cytokeratin marker; Dako) to assess which cells contained IFN-α in human thymus. After overnight incubation of the sections with H51 antiserum at 4°, the sections were incubated for 30 min with peroxidase-conjugated swine anti-sheep immunoglobulins. The reaction was developed with diaminobenzidine as substrate. Double staining for epithelial cytokeratin was done by a subsequent 2-hr incubation of the sections with the polyclonal rabbit anti-prekeratin antiserum. This was followed by a 45-min incubation with rhodamine-labelled swine anti-rabbit immunoglobulins (Dako). Repeated washings were done between steps with Tris-buffered saline to avoid cross-reactions.

RESULTS

Specific positive staining for IFN-α was first observed at 9 weeks gestation in spindle-shaped or rounded cells scattered variably in the foetal liver (Fig. 1). Thereafter specific positive staining for IFN-α was seen in similar cells scattered variably in all foetal organs except kidney and cerebral and cerebellar cortex in brain. Most of these positive cells appeared to show a similar positive

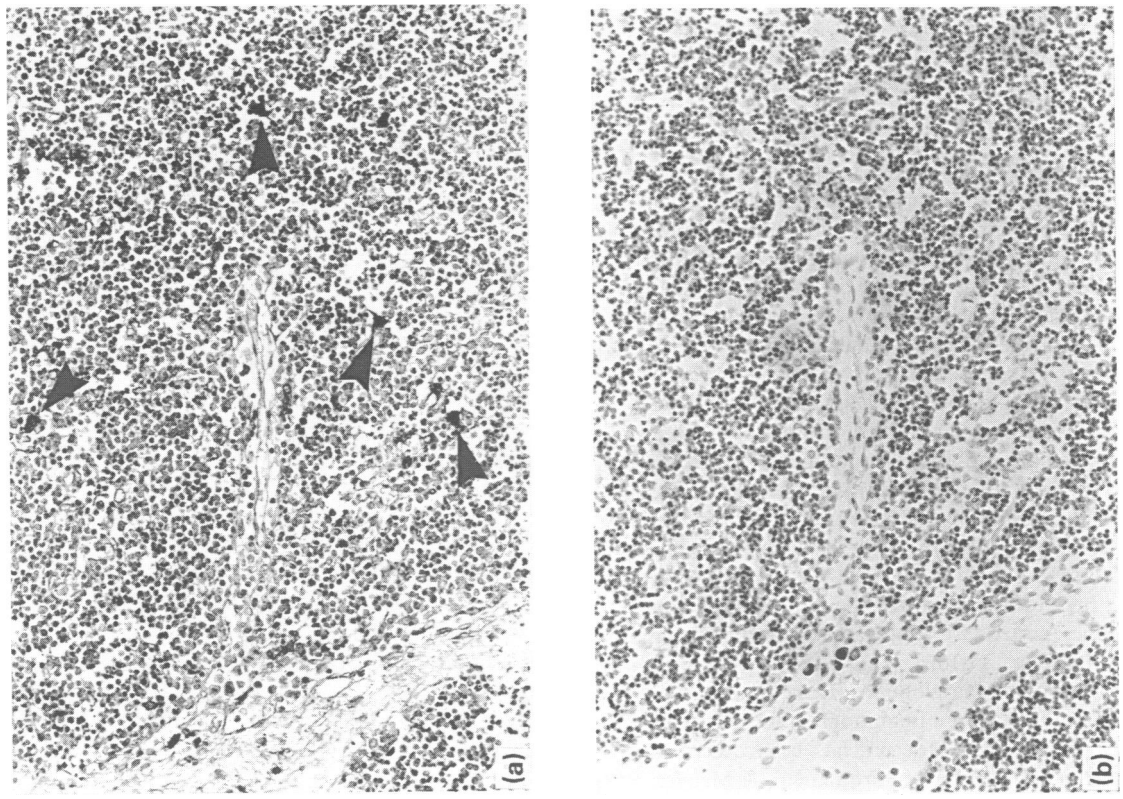

Figure 3. Cells staining positively for IFN-α (arrow heads) (a) often failed to stain positively for KP1 (b) in the foetal thymus ($\times$184).

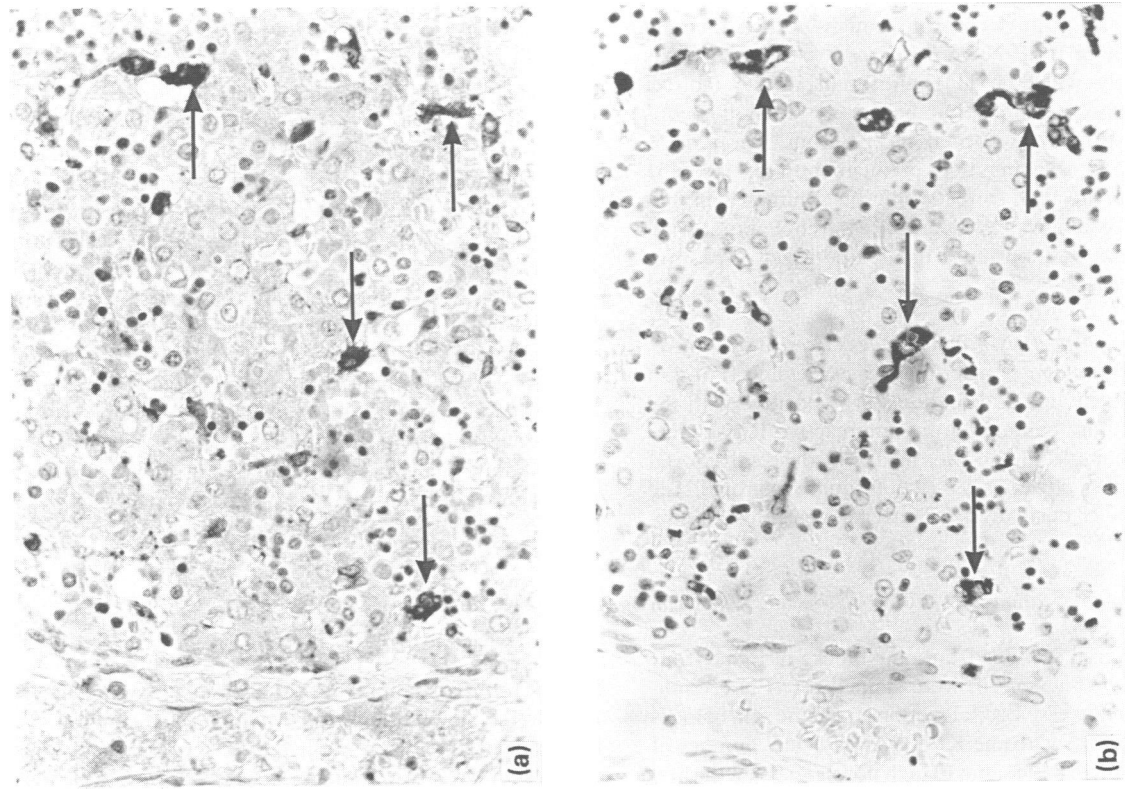

Figure 2. Liver of a 38-week gestation foetus. (a and b) Serial sections stained for IFN-α and KP1, respectively. The distribution of staining intrasinusoidal cells (arrows) is similar in both sections, showing that cells of the macrophage lineage contain IFN-α ($\times$360).

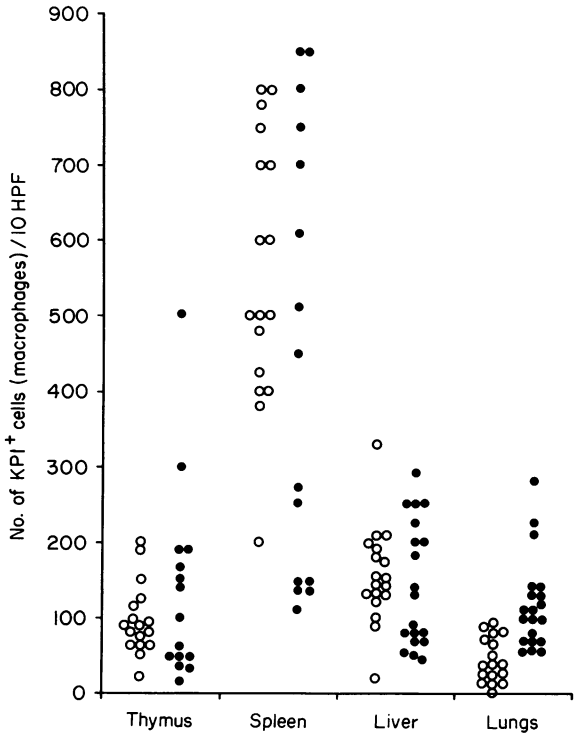

Figure 4. Distribution of macrophages in thymus, spleen, liver and lungs. (●) Infants (few hours to 2 years); (○) foetuses (12–42 weeks). HPF = high power field; one HPF = 62,500 square microns.

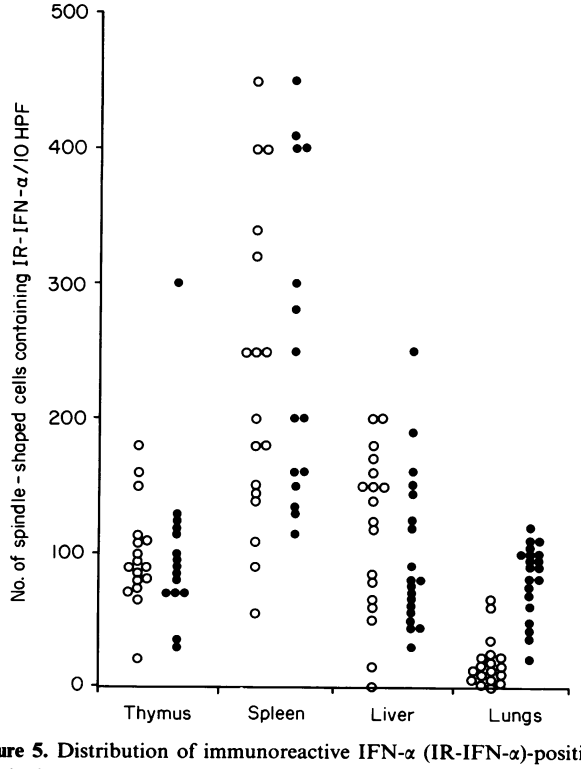

Figure 5. Distribution of immunoreactive IFN-α (IR-IFN-α)-positive cells in thymus, spleen, liver and lungs. (●) Infants (few hours to 2 years), (○) foetuses (12–42 weeks).

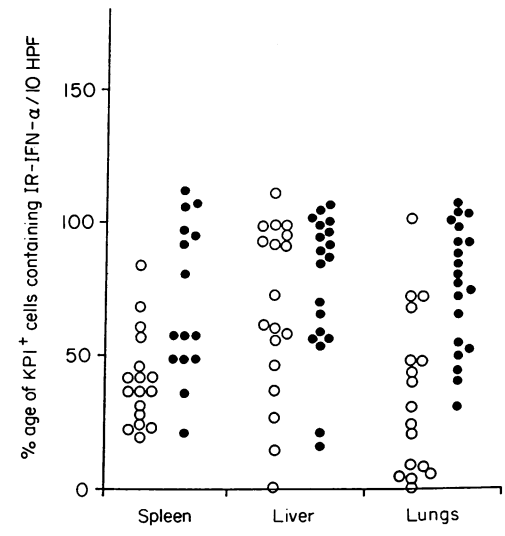

Figure 6. Percentage of macrophages containing immunoreactive IFN-α in spleen, liver and lungs. (●) Infants (few hours to 2 years); (○) foetuses (12–42 weeks).

staining with the macrophage/histiocytic marker KP1 (Fig. 2). Thymus proved to be an exception in this regard (Fig. 3).

Quantification of positively staining cells on successive serial sections appeared to be reliable. When cells were counted on two adjacent serial sections stained for IFN-α, as described in the Materials and Methods, and results subjected to regression analysis, the r value was 0·997. The equivalent figure for KP1 staining was 0·999.

Figure 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 foetuses (12 weeks gestation and above). It demonstrates a statistically significant increase in the number of KP1-positive cells in infant lungs compared to foetal lungs ($P < 0·001$, Mann–Whitney test). The number of KP1-positive cells in foetal and infant thymus, spleen and liver did not show any statistically significant difference between the two groups.

The number of spindle-shaped or rounded cells positive for immunoreactive IFN-α in 10 high power fields in the thymus, spleen, liver and lungs in infants and foetuses is illustrated in Fig. 5. It also shows a statistically-significant increase in the number of immunoreactive IFN-α-positive cells in the infant lungs compared to foetal lungs ($P < 0·0001$, Mann–Whitney test). There was no statistical difference between the numbers of IFN-α-positive cells in spleen, thymus and liver found in foetuses compared to the numbers found in infants.

Figure 6 illustrates the percentage of KP1-positive cells containing immunoreactive IFN-α in 10 high power fields in the spleen, liver and lungs of infants and foetuses. The percentage of macrophages expressing IFN-α was significantly greater in infant lungs compared to foetal lungs ($P < 0·0005$, Mann–Whitney test). Such a difference was not observed in the other organs examined.

In the thymus, as shown in Fig. 3, many cells containing immunoreactive IFN-α failed to stain positively with the macrophage/histiocytic marker, KP1. These IFN-containing cells also stained negatively with the other macrophage mAb MAC 387 and the polyclonal anti-muramidase antiserum.

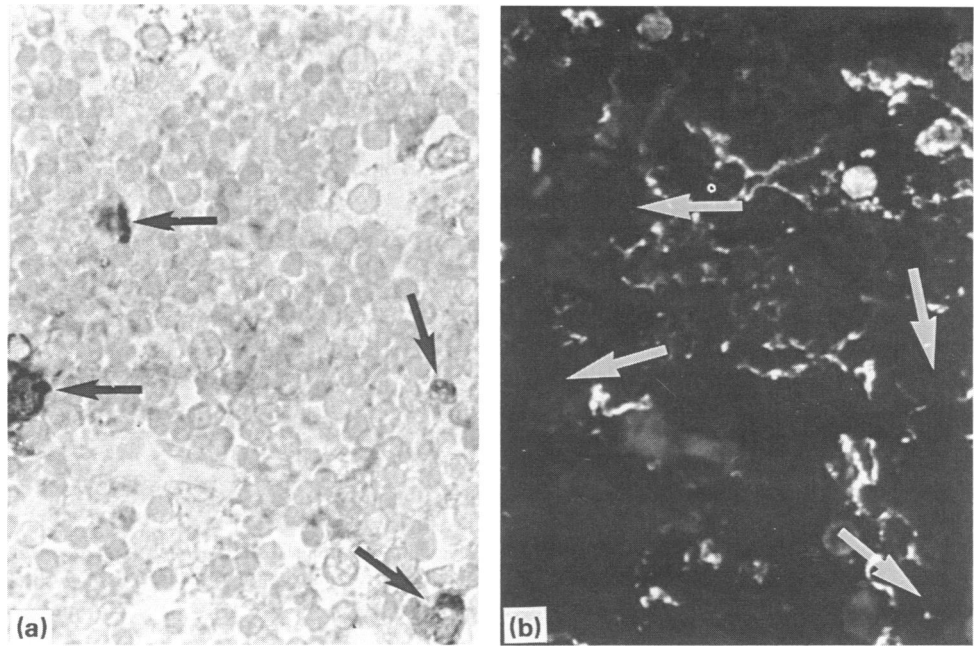

Figure 7. Foetal thymus. Cells positive for IFN-α (arrows) (a) failed to fluoresce for prekeratin (epithelial cytokeratin) in (b) (arrows). This shows that they are probably not epithelial cells. IIP for IFN-α (a) and immunofluorescence for prekeratin (b) on the same section (×624).

Table 1. Localization of IFN-α in parenchymal cells of normal formalin-fixed human foetal and infant tissues

Organs	Type of cells +ve with H51	Gestational age at which IFN-α appears (weeks)	Frequency of H51 +ve cells in foetuses 7–11 weeks G (*n*)	Frequency of H51 +ve cells in foetuses 12–25 weeks G (*n*)	Frequency of H51 +ve cells in foetuses 26–42 weeks G (*n*)	Frequency of H51 +ve cells in infants (*n*)
Adrenal gland	Foetal zone cortical cells	10	++ (1)	+++ (20)	+ (8)	+ (5)
	Definitive zone cortical cells	12	(0)	+ (20)	+ (8)	+ (8)
Thyroid gland	Thyroid follicular cells	13	(0)	+++ (23)	+++ (9)	+++ (9)
Choroid plexus in brain	Cuboidal epithelium	11	+ (1)	+ (20)	+++ (9)	+++ (8)
Anterior pituitary gland	Occasional endocrine cells	13	(0)	+ (20)	+ (5)	+ (6)

G, Gestational age; HPF, high power field = 62,500 square microns.
+, 5 cells/HPF; ++, between 5 and 15 cells/HPF; +++, 15 cells/HPF.

Double-staining with sheep anti-IFN-α (H51) and a prekeratin antibody (epithelial cytokeratin marker) showed that cells positive for immunoreactive IFN-α failed to fluoresce and were therefore probably not epithelial cells (Fig. 7).

IFN-α in parenchymal cells of various human tissues

Immunoreactive IFN-α was also observed in cells of the foetal zone of the adrenal cortex at 10 weeks of gestation age. This zone is a major part of the foetal adrenal gland which completely disappears after birth, to be replaced by the permanent adult adrenal cortex. Cuboidal epithelium of choroid plexuses in brain became positive at 11 weeks gestation and thyroid follicular cells and some endocrine cells in the anterior pituitary gland at 13 weeks gestation (Table 1).

DISCUSSION

This study has shown that immunoreactive IFN-α is present in very early foetal life in spindle-shaped or somewhat rounded cells that are scattered variably in nearly all foetal tissues except in kidneys and cerebral and cerebellar cortex in brain. Most of

these cells showed a similar positive staining with the macrophage/histiocytic marker KP1 and were therefore probably macrophages.

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

There does remain the possibility that endotoxins from the mother could cross the placenta into the foetus. 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 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 foetus 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 infant lungs and the percentage of these cells containing IFN-α also increased. This finding provides some support for Bocci's hypothesis. While the finding that IFN-α-positive macrophages were present in germ-free foetuses suggests that there is a basal level of IFN-α production, not dependent on microbial products, the increased expression of IFN-α in infant lungs suggests that such products do have a local role in *enhancing* IFN-α 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 could not be assessed because of post-mortem autolysis in the gastrointestinal mucosa of the foetuses.

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

The finding of immunoreactive IFN-α in parenchymal cells of thyroid, choroid plexus in brain, anterior pituitary gland and the adrenal gland was almost identical to that seen in adult human tissues (Khan *et al.*, 1989). We have proposed that secretion of IFN-α by cells in the choroid plexuses could be a defence mechanism against viral infection in the central nervous system, but the function of IFN-α in the other organs remains unknown.

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