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Autoantibodies to crude human leucocyte interferon (IFN), native human IFN, recombinant human IFN-alpha 2b and human IFN-gamma in healthy blood donors

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SUMMARY

Recently, naturally occurring antibodies to IFN-α were discovered in a few systemic lupus erythematosus (SLE) and cancer patients; however, in most patients monitored for anti-IFN antibodies before treatment, no antibodies were found. In an attempt to explain the 'IFN-blocking effect' that we observed in all serum samples we investigated 200 sera from healthy blood donors. We isolated the globulin fraction, and used rabbit anti-human IgG and IgM columns, protein A columns and T-gel affinity chromatography to isolate human IgG and IgM. All sample fractions were tested in a biological IFN neutralization assay by means of a sensitive MTT-assay. We found that normal human serum contained autoantibodies to crude human leucocyte IFN, native human fibroblast IFN, recombinant human leucocyte IFN-α2b and recombinant human IFN-γ, and that these naturally occurring antibodies were biologically active immunoglobulins of IgG and IgM type. These anti-IFN antibodies were also present in purified human normal immunoglobulin pools. We conclude that all humans have naturally occurring anti-interferon antibodies in their serum, and it is a tempting theory that human cytokines and lymphokines are, at least partly, regulated by immunoglobulins.

Keywords interferon cytokines autoimmunity

INTRODUCTION

Many reports have described the presence of anti-IFN antibodies in patients treated with IFN (Vallbracht *et al.*, 1982; Steis *et al.*, 1988; Figlin *et al.*, 1988). In contrast, naturally occurring anti-IFN antibodies have only been described in a few patients with autoimmune diseases (Panem, 1982) or cancer (Trown *et al.*, 1983), while most patients and blood donors monitored for autoantibodies to IFNs have been found to be negative. Recently it was shown that antibodies to IFN-α and IFN-β are present in Lou/c rats (De Maeyer-Guinard *et al.*, 1984) and in a number of inbred mouse strains (De Maeyer-Guinard & De Maeyer, 1986).

Using an improved anti-viral neutralization bioassay combined with the MTT-method (Hansen, Nielsen & Berg, 1988; Hansen, Ross, Berg, 1990), affinity chromatography and radial immunodiffusion, we found that autoantibodies to native human IFN-α and IFN-β, and recombinant human IFN-α and IFN-γ were present in all healthy blood donors that we investigated.

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MATERIALS AND METHODS

Cell lines

A549 cells were obtained from Dr A. Meager, NISBC, London, and MDBK cells from American Type Culture Collection, Rockville, MD. MDBK and A549 cells were grown in Eagle's MEM with addition of 5% fetal calf serum (FCS), 1% each of penicillin and streptomycin, 1% L-glutamin, and 5% sodium hydrogen carbonate in Nunclone (Nunc, Roskilde, Denmark) plastic bottles. Cells were split twice weekly with trypsin at differenc cell density according to standard procedures. Cells were seeded in microtrays in 100–150 μl medium including 5–10% serum and incubated at 37°C for 48 h; cell viability reflected by the OD signals was determined by the MTT method (Hansen *et al.*, 1988).

Viruses

Vesicular stomatitis virus (VSV) was obtained from Dr D. Burke (Warwick University, UK); murine encephalo myocarditis virus (EMC) was kindly provided by Dr S. Grossberg, WI. The viruses were propagated in murine L cells, and supernatants were kept at −70°C until use. Virus batches were plaque titrated before being frozen.

Interferons

Crude human leucocyte IFN (CIF), generously provided by Dr K. Osther, Dallas, was obtained from buffy coat suspension to which Sendai virus had been added (Cantell *et al.*, 1974); specific IFN activity was 10^4 U/mg protein. Pure recombinant human leucocyte IFN-α2b, kindly provided by Essex-Pharma, Denmark, was produced in *Escherichia coli* (Biogen). Native human fibroblast IFN-β (specific activity 10^5 U/mg protein) was from Cytotech, Switzerland. Recombinant human IFN-γ (specific activity 10^7 U/mg protein) was a generous gift from Dr G. Adolf, Boehringer-Ingelheim.

The IFN assays described were calibrated using the 69/19 international reference preparation for human leucocyte IFN (from MRC, Mill Hill, UK). All IFN units are expressed in international units.

MTT assay

MTT, a tetrazolium salt (Sigma; cat. no. M2128), was dissolved in autoclaved PBS (5 mg/ml), sterile filtered, and stored at 4°C in a dark bottle; SDS practical grade was from Sigma and dimethylformamide (DMF) analytical grade was from Fluka (cat. no. 40250).

Briefly, Mosmann's method was modified as follows: 25 μl of a 5 mg/ml stock solution of MTT was added to each well after incubation with challenge virus; after incubation for 2 h at 37°C, 100 μl extraction buffer (comprising 12·5% SDS, 45% DMF, pH 4·7) were added to dissolve formazan–protein complexes; after overnight incubation at 37°C the ODs at 570 nm were measured in a Titer-Tech 96-well multi-scanner, employing the solubilizer as blank probe. No medium was removed prior to the addition of any ingredient.

Sera

Human serum samples donated by the Rigshospitalets Blood Bank were collected and allowed to clot at room temperature; the sera were separated by centrifugation at 500 rev/min for 10 min. Samples were stored at −70°C until analysed. Before assaying, the sera were heat-inactivated at 56°C for 30 min.

Neutralization test

This was made in 96-well tissue culture plates (Nunc). Different dilutions of sera (starting at 1/10), were incubated for 1 h at 37°C with 0·1–4·0 IU IFN/ml, in a total volume of 100 μl in Eagle's MEM, supplemented with 5% newborn calf serum or 2% Ultrosar (Gibco). The microtrays were transferred to A549 monolayers, 10000 cells were seeded in 100-μl Eagle's MEM + 5% newborn calf serum, and after overnight incubation at 37°C, EMC was added. After another 24-h incubation, 25 μl MTT were added to the wells and 2 h later, 100 μl lysis buffer, as described for the MTT assay.

The neutralizing titre was defined as the reciprocal value of the serum dilution which significantly decreases (> 3 s.d. of IFN control) the anti-viral effect of 1 IU/ml of IFN (final concentration).

Ammonium sulphate precipitation

Acetate buffer (2%) (40 mM, pH 5) and ammonium sulphate 250 mg/ml were added to serum which was left in the dark at room temperature for 24 h. Centrifugation was carried out for 30 min at 6000 rev/min.

The precipitate was washed with 1·75 M ammonium sulphate, and dialysis was carried out according to the following schedule: twice for 12 h against distilled water; 12 against acetate buffer pH 5; 12 h against distilled water; and 12 h against acetate buffer pH 5. The supernatant was loaded onto a DEAE-A sephadex column with a bed volume of 25%. The eluate was concentrated by repeated salting out with ammonium sulphate as stated above, and dialysis was carried out for 12 h against distilled water, followed by 24 h against PBS.

Affinity chromatography

Four affinity columns were used: (i) a combined rabbit anti-human (RAH) IgG + IgM immunoglobulin column (3010C + 3090y); (ii) a RAH-IgM column ('no-nonsense'); (iii) a T-gel column, all from Kem-en-tech, Copenhagen; and (iv) a protein A/Sepharose CL-4B column from Pharmacia. The RAH-IgG + IgM and RAH-IgM columns were constructed by means of CNBr-activated Sepharose 4B from Pharmacia according to the manufacturers' instructions. Affinity chromatography was carried out with serum globulin volumes ranging from 300 μl to 10 ml. The wash was collected in fractions of 900 μl at 8°C. Elution was carried out with 0·1 M CH_3 COOH pH 2·8 in tubes pre-wetted with 200 μl Tris buffer pH 9·0. T-gel, and serum was equilibrated with 0·75 M ammonium sulphate; 10 ml packed T-gel was used per 5 ml undiluted serum. After loading, the column was washed with 0·75 M ammonium sulphate until base line was reached. Elution was performed with 0·1 M NaCl.

By means of pressure dialysis the volume of the samples was kept constant in relation to the original serum samples.

Radial immunodiffusion in gel (Mancini method)

NOR-Partigen gels for radial immunodiffusion (Hoechst, Denmark) were used according to the manufacturer's instructions. Measurements were carried out as one-point calibration with internal serum pool, including the manufacturer's own standard (5 μl were added to each well). The final concentrations were determined by the NOR-Partigen reference table.

Biological assay of normal human immunoglobulins

Fraction no. IgG-10-1-890213 (stabilized with glycine 16% W/v HNI and 2% W/v glycine), from a pool of healthy donors was isolated according to standard procedures at the State Serum Institute, Copenhagen, using ethanol precipitation procedure for immunoglobulins (purity above 98%). The preparation was a generous gift from Dr M. Dam, Department of Blood Fractionation (Copenhagen). The experiments were carried out as neutralization tests *versus* all types of IFNs with immunoglobulin concentrations varying from 15 mg/ml to 0·1 mg/ml.

Dot-blot analysis

Pure recombinant IFN-α (5 μl; concentration 10 mg/ml) was applied onto nitrocellulose paper (NCP); NCP was blocked by 2% Tween/Tris pH 7·4 (for 10 min) and subsequently by 1% BSA/0·3% Tween/Tris, pH 7·4 (for 10 min). Serum was diluted 1:5 in 0·5% BSA/Tween/Tris, pH 7·4, and incubated overnight with NCP. NCP was then washed in 0·3% Tween/Tris, pH 7·4, three times for 10 min. NCP was incubated with conjugate (Dako P 212, 1:50 in 0·5% BSA/0·3% Tween/Tris, pH 7·4), for 4 h at room temperature. Washing was carried out for 10 min in Tris, pH 7·4, three times, and the NCP was washed for 10 min in

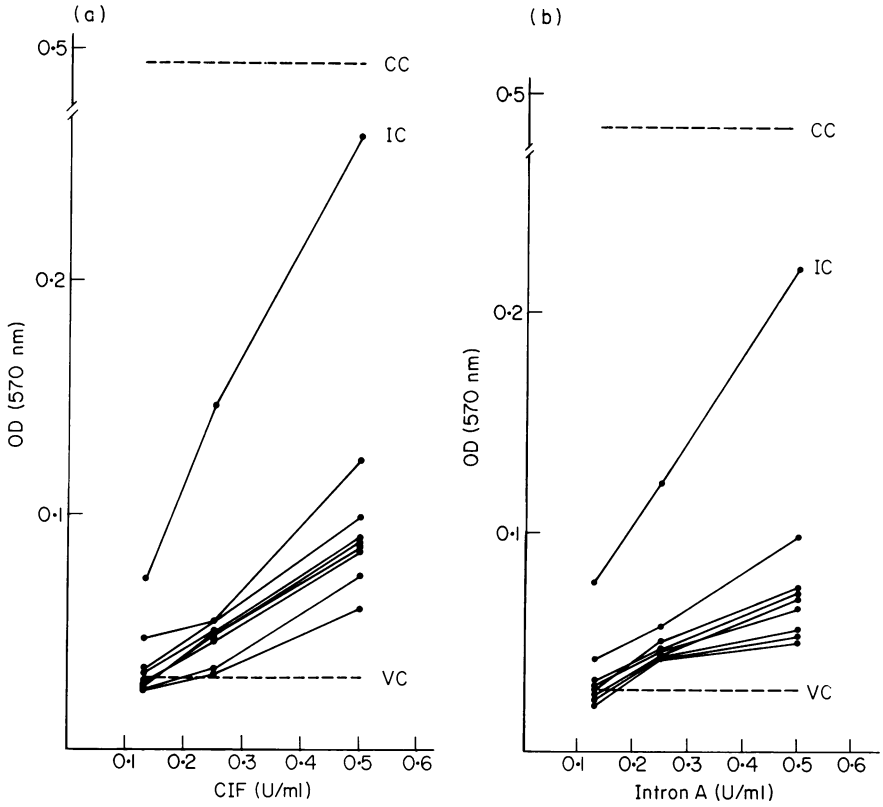

Fig. 1. Interferon neutralization assay of sera from eight donors: (a) against human leucocyte IFN (CIF); and (b) against recombinant human IFN-α2b (Intron A). Sera at 1:20 dilutions were incubated with 0·13, 0·24 and 0·5 IU IFN/ml, respectively, for 1 h, at 37°C, and the remaining active IFN was determined by the MTT assay. CC, cell control; IC, IFN + virus control; VC, virus control.

Tris, pH 7·4, diluted 1:5 in distilled water. Finally, the NCP was dyed with naphtol in methanol Tris pH 7·4 (1:5 in distilled water). The reaction was stopped by washing five times in distilled water.

RESULTS

IFN and virus titrations were carried out to ensure that minute IFN concentrations could be quantified by the MTT method. Initial experiments demonstrated distinct IFN dose–response curves within the range of 0·1–2·0 IU IFN/ml. Furthermore, the range of IFN concentrations yielding the most pronounced change in OD signals as a function of IFN neutralization (high slope in the dose–response curve) was defined for each type of IFN (0·5–1·0 IU/ml for native human IFN-α and IFNβ and recombinant IFN-α2b; and 1·0–2·0 IU/ml for recombinant human IFN-γ.

Anti-IFN activity in sera from healthy blood donors

In preliminary experiments we observed an IFN-neutralizing effect in heat-inactivated sera from healthy donors. The neutralizing effect *versus* native human IFN-α and IFN-β as well as recombinant human IFN-α and IFN-γ was found in each of the 200 healthy donors tested. The donors were equally distributed as to sex and uniformly distributed throughout the age range of 15–65 years. The individual variation in the IFN-neutralizing activity of the sera was less than 45% from the mean value. Figure 1 illustrates eight donors chosen at random, tested against native human IFN-α (CIF) and recombinant human IFN-α2b (Intron A), show that all donor sera significantly inhibited the added amount of IFN in a dose–response related manner. Similar results were obtained with native human IFN-β whereas the general neutralization of recombinant human IFN-γ whereas the general neutralization of recombinant human IFN-γ seemed lesser, but the difference was not significant.

Purification of human immunoglobulin on antibody columns

More than 90% of the IFN neutralizing activity could be recovered in ammonium sulphate-precipitated globulin fractions from the same donor sera. Sera from donors were pooled into three groups (a mixed pool of male/female; equal age distribution; a female pool; and a male pool) and affinity chromatographies of the dialysed preparations by means of specific RAH immunoglobulin columns were performed. Examination of the eluates and washes was performed in our MTT anti-viral neutralization bioassay (Hansen *et al.*, 1990).

Using the combined RAH-IgG + IgM column (Fig. 2a), the neutralizing activity was not present in the wash, but could be recovered from eluates, and in most cases the inhibition corresponded to 100% as compared with the original input. Radial immunodiffusion confirmed the efficiency of the immunoadsorption experiments (Table 1).

Eluates and washes from RAH-IgM columns both possessed an interferon-inhibiting effect (data not shown). The eluate from the protein A column (Fig. 2b) showed a distinct IFN-inhibiting effect, and the wash showed some effect corresponding to about 30% inhibition as compared with the IFN control of CIF, about 40% of Intron A, 30% of IFN-β and 20%

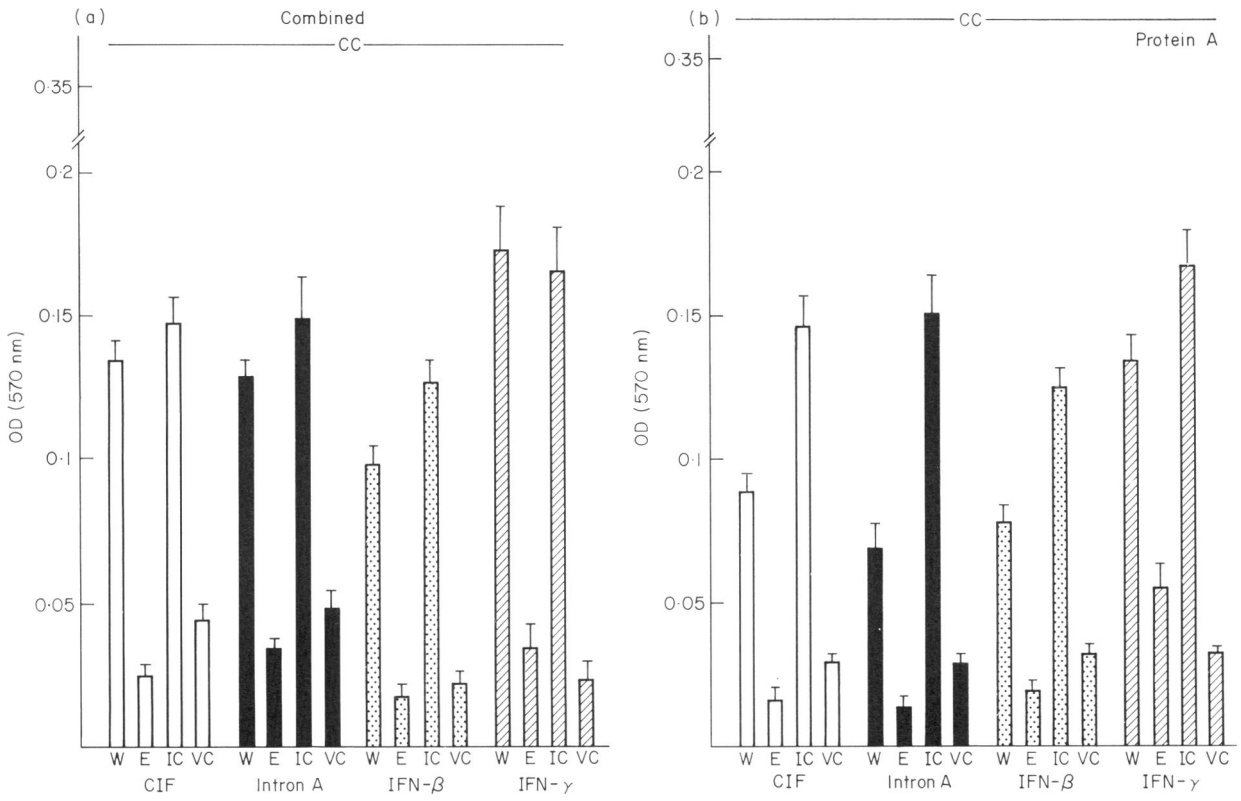

Fig. 2. Interferon neutralization of donor serum purified on (a) rabbit anti-human IgG + IgM sepharose columns; and (b) protein A–Sepharose columns. Donor pool was purified on a combined anti-human IgG + IgM Sepharose column and the wash (W) and eluate (E) were examined in a MTT/anti-viral neutralization bioassay against 1·0 IU of human leucocyte IFN (CIF), recombinant human IFN-α2b (Intron A), native human IFN-β and recombinant human IFN-γ, respectively. Input was about equal to E in all four experiments. IC, IFN control; VC, virus control; CC, cell control.

Table 1. Radial immunodiffusion of affinity chromatography purified fractions (Mancini method)

Test	IgG	IgM
Control serum and (serum-pool)	+	+
RAH-IgG + IgM (wash)	Trace	−
RAH-IgG + IgM (eluate)	+	+
RAH-IgM (wash)	+	−
RAH-IgM (eluate)	Trace	+
Protein A (wash)	−	+
Protein A (eluate)	+	Trace
T-gel (wash)	−	−
T-gel (eluate)	+	+
Ammonium sulphate-precipitated globulin fraction	+	+

Control serum Ig determined from Mancini reference table from Hoechst: IgG, 12·3 g/l IgM, 1·6 g/l.
RAH, rabbit anti-human.

of IFN-γ). The immunodiffusion control (Mancini method) showed IgG in eluates (Table 1).

Comparison of the results from the protein A column and the RAH-IgM column seems to indicate that IgG produces the greater effect.

Protein fractionation by T-gel

The IFN-neutralizing effect in the globulin fraction was further characterized by means of T-gel, which is a commonly used method for isolating antibodies (Porath, Maisano & Belew, 1985; Porath, 1986; Hutchens & Porath, 1986). After the affinity chromatography all the IFN-inhibiting effect had disappeared from the wash but was recovered in the eluate (data not shown). The Mancini control showed that there were IgG and IgM in the eluate, but not in the wash (Table 1). Thus the method confirms the presence of IgG and IgM autoantibodies with neutralizing effect against IFNs in the serum from healthy donors. All eluates and washings were tested for cell toxicity and anti-viral effect and found to be negative at a dilution of 1:20.

A dot-blot analysis (Towbin & Gordon, 1984) using recombinant human IFN-α2b as antigen showed precipitation when using the serum pool. In contrast, the wash from the T-gel showed no reaction.

Anti-IFN effect in human normal immunoglobulin preparation

In order to document further that the inhibitory effect of serum was due to immunoglobulins we investigated a human normal purified IgG immunoglobulin fraction (HNI-IgG) from the State Serum Institute which proved also to contain autoantibodies with neutralizing activity *versus* native human IFN-α (Fig. 3a), IFN-β, and also recombinant human IFN-α2b (Fig. 3b) and IFN-γ. The results yielded a dose–response curve, in which the highest HNI-IgG concentrations completely

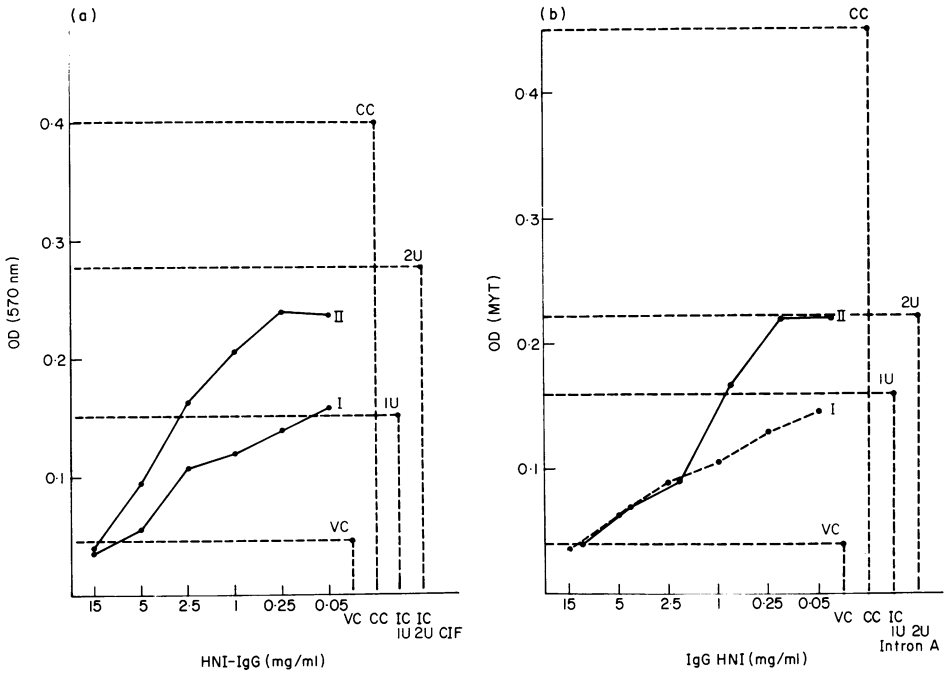

Fig. 3. Neutralization of human normal immunoglobulins (IgG) from the state serum institute, *versus* (a) human leucocyte IFN (CIF); and (b) recombinant human IFN-α2b (Intron A). Dilutions of purified IgG from healthy donors were incubated with CIF and Intron A, 1 and 2 IU/ml, respectively, and the MTT anti-viral neutralization bioassay was performed. Curve I, 1 U/ml; curve II, 2 U/ml. VC, virus control, CC, cell control; 1 U and 2 U (IFN control), OD signal from cells treated with 1 or 2 IU IFN/ml + virus. ——, Intron A 1 U; - - -, intro A 2 U.

neutralized the IFN effect, whereas the lower concentrations were close to the IFN controls. In neutralization experiments employing recombinant human IFN-α2b, CIF and native human IFN-β, we could observe a neutralizing effect down to a concentration of 0·05 mg IgG/ml, whereas neutralizing effect towards recombinant human IFN-γ was seen until a concentration of 2·5 mg IgG/ml (data not shown). The highest HNI-IgG concentration (15 mg/ml), however, was weakly toxic in the biological assay (8·5% inhibition of cell growth compared with the controls as measured in the MTT assay). All lower HNI-IgG concentrations were not toxic in the biological assay.

Anti-IFN titre measured by the MTT-ANB

We determined the titer of the IFN-neutralizing effect of our three serum pools *versus* native human IFN-α (CIF), recombinant human IFN-α2b (Intron A), native human IFN-β and recombinant human IFN-γ (Table 2). Pool 1 (men + women) had a neutralizing titre towards native human IFN-α and Intron A of 160, native human IFN-β 320 and recombinant human IFN-γ 80. The female pool had a significantly lower neutralizing titre to recombinant human IFN-γ.

Table 2. Anti-IFN titre

Serum pool	Final titre (1 U)			
	Anti-human IFN-α (CIF)	Anti-Intron A	Anti-human IFN-β	Anti-human IFN-γ
Male + female	160	160	320	80
Female	80	160	80	20
Male	80	160	160	160

DISCUSSION

The presence of autoantibodies to IFN-α has been discussed previously. In 1982, Panem (1982) examined 200 patients with different autoimmune diseases for neutralizing autoantibodies to IFN. Even though none of the patients had earlier been treated with IFN, one patient was found to be positive. Mogensen *et al.*, (1981) described a 77-year-old woman with a herpes-zoster infection who had circulating antibodies in the blood to native human IFN-α before, during and after IFN treatment. Trown *et al.*, (1983) investigated groups of patients in a sensitive RIA for autoantibodies to recombinant human IFN-α and found two out of 76 cancer patients to be antibody positive; 60 diabetic patients and 200 healthy blood donors were all antibody negative. Panem *et al.*, (1982) reported that out of six SLE patients only one was found to be antibody positive. Thus, to this date autoantibodies to human IFN-α have been found in only a few patients, and no autoantibodies have been reported in healthy donors.

We have developed an objective and very sensitive biological IFN test which have used in our neutralization tests, and which has made it possible to measure very small amounts of antibodies. In our studies we have used A549 cells, which is a human cell line sensitive to all types of human IFN. The use of these cells proved to be of great importance since for reasons not understood it has been almost impossible for us to trace the naturally occurring autoantibodies to interferons by the use of, for example, bovine cells (MDBK).

The IFN-neutralizing effect which was found in human serum and which could be recovered in the ammonium sulphate-precipitated immunoglobulin fraction, proved by affinity chromatography and radial immunodiffusion to be due (at least partly) to autoantibodies of IgG and IgM classes. Using a combined RAH-IgG+IgG affinity chromatography column and the T-gel we could remove the IFN-neutralizing effect from serum and recover it in the eluate. The wash from the protein A column had a weak IFN-neutralizing effect which might be due to the anti-IFN-IgM or -IgG_3 antibodies that were only weakly bound to the column (Table 1), whereas the eluate had a strong effect, caused by anti-IFN-IgG. Using a RAH-IgM affinity chromatography column, we found an IFN-neutralizing effect both in eluate and wash. This could be attributed to the presence of IgG antibodies in the wash and IgM antibodies in the eluate, both having IFN-neutralizing activity. A common feature was that in the biological assay the OD signals from eluates were slightly below those from the virus controls. This could perhaps be explained if it is assumed that the small amount of A549 produced IFN which is induced by challenge virus is also neutralized by human autoantibodies. This would cause an enhanced viral attack on the target cells. Control experiments showed that this effect was not due to toxicity from the samples, as control cultures had a normal OD signal. The fact that IFN-neutralizing activity was present in human normal IgG immunoglobulin preparations from the State Serum Institute with a purity or more than 98% strongly supports our findings.

In connection with the titre investigations, we were surprised that the neutralizing titre against IFN-γ was not higher in relation to the other IFNs investigated, considering the fact that IFN-γ is known to disappear very rapidly from the blood stream and that it is very difficult to measure IFN levels comparable to the amount injected. If this disappearance was caused by autoantibodies alone, then we could have expected a titre significantly higher than that found. It seems that other mechanisms such as, for example, specific enzymes or complement factors may also be acting. It is interesting to note that women have a significantly lower titre of autoantibodies to IFN-γ than do men (Table 1).

De Maeyer-Guignard & de Maeyer (1986) observed the presence of autoantibodies to IFN-α and IFN-β in inbred mice after affinity chromatography separations. This activity was connected with the IgG and, to a lesser degree, the IgM fraction; the investigators were surprised at this phenomenon and pointed out the fact that no naturally occurring antibodies to IFN had been demonstrated in humans.

However, our experiments have now demonstrated that humans have an anti-IFN effect in serum which can be connected with the IgG and IgM fraction, and we find it therefore justified to conclude that humans have autoantibodies to the IFN system as was originally shown by de Maeyer-Guinard de Maeyer (1986) to be the case in the mouse.

It may seem paradoxical that humans have autoantibodies against IFN, since these would inhibit their general anti-viral activity, making a virus attack more severe. The question as to what role the autoantibodies play in the human body remains to be answered. Our results together with those showing that autoantibodies to IL-1α are present in healthy blood donors (Svensson *et al.*, 1989) might suggest that naturally occurring antibodies to other cytokines will be found in the future.

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