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Serum-induced suppression of interferon (IFN) activity. Lack of evidence for the presence of specific autoantibodies to IFN-α in normal human sera

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SUMMARY

IgG antibodies binding to different IFN species have been described in sera of healthy and diseased individuals. Human serum immunoglobulins have also been shown to interfere with IFN bioactivity. To characterize these antibodies, human recombinant IFN-α2A (rIFN-α) was radioiodinated, and ligand binding studies were performed in human sera as well as on the human cell line A-549 in the presence of human serum. ^{125}I-rIFN-α bound to serum factors of healthy individuals. However, less than 3% of the binding was to IgG and the binding was non-saturable and therefore most likely non-specific. ^{125}I-rIFN-α bound to receptors on A-549 cells, and the ligand–receptor complexes appeared to internalize. However, both cell binding and internalization of ^{125}I-rIFN-α were independent of the presence of human serum. We conclude that normal human sera do not contain detectable autoantibodies to rIFN-α.

Keywords interferon-alpha human serum autoantibodies interferon receptor binding interferon activity

INTRODUCTION

Previous reports have described autoantibodies to IFN in a few patients with autoimmune and neoplastic diseases (reviewed by Panem [1]) as well as in patients undergoing treatment with IFN-α [2,3]. Several investigators have also reported the presence of anti-IFN antibodies in normal human sera [4–6]. By means of an IFN bioassay using the human lung carcinoma cell line A-549, neutralizing antibodies against all IFN types were recently found in serum from Danish blood donors as well as in normal human IgG prepared for therapeutic use [5]. However, these antibodies were not detectable if tested on bovine MDBK cells [3,5]. Immunoblotting and ELISA experiments have also been employed to show direct binding of human IgG to human recombinant-derived IFN (rIFN) species [4–6].

In order to characterize further the naturally occurring IFN-neutralizing antibodies, we investigated the direct binding of rIFN-α to A-549 cells in the presence of human serum, as well as to factors in human serum.

MATERIALS AND METHODS

Cell lines

Bovine MDBK cells were purchased from the American Type Culture Collection (Rockville, MD) and cultured in Nunclone tissue culture flasks (50 ml, 25 cm^2; Nunc, Roskilde, Denmark) in Eagle's minimum essential medium, supplemented with 7% fetal calf serum (FCS) (Gibco), 1% penicillin, 1% streptomycin, 1% L-glutamine, and 5% sodium hydrogen carbonate. Human A-549 cells were obtained from Dr K. Berg (University of Copenhagen, Denmark). The cells were cultured in Ham's F12K medium, supplemented with 8% FCS, 1% penicillin, 1% streptomycin, 3% L-glutamine, and 5% sodium hydrogen carbonate. Cells were passaged twice a week using trypsin.

Viruses

Vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) were obtained from Dr A. R. Thomsen (University of Copenhagen, Denmark). The viruses were propagated in murine L cells, and then kept at −70°C until use.

Interferon-alpha

Purified human rIFN-α2A (rIFN-α) (10^8 IU/mg) was a kind gift from Hoffmann-La Roche (Basel, Switzerland).

Radiolabelling of rIFN-α

^{125}I was from Amersham (Birkerød, Denmark). The chloramine T method was used as described elsewhere [7,8]. Briefly, 1 mg/ml of rIFN-α, corresponding to 10^5 IU/μl 0·01 M PBS, pH 7·4, was iodinated by adding 5 μl ^{125}I-Na, containing 6·4 μg chloramine T. After mixing and 10 min of incubation at room temperature,

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12·8 μg sodium metabisulphite were added. Separation of labelled IFN was done after 5 min on a Sephadex G-25 column (PD-10; Pharmacia, Uppsala, Sweden) equilibrated with 2% bovine serum albumin (BSA) (Sigma, St Louis, MO) in PBS. After iodination the total recovery of rIFN-α was calculated to be 35%. The specific activity was calculated to approximately 2×10^{14} ct/min per g. The biological activity of ^{125}I-rIFN-α was more than 70% of that of the unlabelled cytokine, judged by titration in the A-549/EMCV system using an IFN standard (G 032-901-527; National Institutes of Health, Bethesda, MD). To eliminate aggregates, ^{125}I-rIFN-α was chromatographed on a column of Sephadex G-75 superfine (Pharmacia) before use.

Human sera

Human sera from healthy blood donors were obtained from the Blood Bank of Rigshospitalet. Blood was drawn under sterile conditions and allowed to clot at room temperature. To avoid non-specific complement-mediated killing of the IFN target cells, one-half of each serum was heat inactivated (56°C, 30 min). Sera were aliquoted and stored at −70°C until analysed.

MTT assay

The MTT assay was carried out as described [9]. 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) was dissolved to 5 mg/ml in sterile PBS; 25 μl of this stock solution were added to each well of cells. After 2·5 h at 37°C, 100 μl of a lysing buffer (13% SDS, 50% dimethylformamide; pH 4·7) were added to each well, and the MTT–formazan was dissolved by means of an automatic plate mixer. Optical densities were measured at 570 nm in a SLT 210 ELISA scanner (SLT Lab. Instruments, Salzburg, Austria).

Interferon neutralization assay

This was performed as an antiviral neutralization assay in 96-well microtrays (Nunc), as detailed elsewhere [3,5]. Briefly, different concentrations of heat-inactivated sera were incubated in triplicate for 1 h at 37°C with 0·1–8 U/ml rIFN-α in culture medium supplemented with 5% newborn calf serum (Gibco). One hundred microlitres were then transferred to monolayers of cells. After 18 h at 37°C, the medium was changed to 150 μl culture medium, supplemented with 2% fetal calf serum and indicator virus. After further 22 h of incubation, cell viability was quantified by the MTT assay. The IFN-neutralizing titre was defined as the reciprocal of the serum dilution which significantly decreased (> 3 s.d.) the anti-viral activity of 0·5 U/ml of rIFN-α (final concentration).

^{125}I-rIFN-α binding to A-549 cells

A-549 cells were cultured to confluence (corresponding to $1·5 \times 10^6$ cells/well) in 6-well culture plates (Nunc). Before assay, the cells were carefully washed with RPMI 1640, supplemented with 1% BSA. Cells were incubated with 1 ml of RPMI 1640, supplemented with 1% BSA and ^{125}I-rIFN-α as well as human sera (untreated and heated) at different concentrations up to 20%. To determine specific cell binding of rIFN-α, control wells were treated in parallel with a 400 times molar excess of unlabelled rIFN-α. The plates were incubated under gentle agitation for 24 h at 4°C or 6 h at 22°C. Supernatants were removed and 'free' ^{125}I-rIFN-α was counted and analysed for decomposition by precipitation on ice using 5% (v/v) TCA. Cells were washed rapidly three times by dipping the plates in 10 *l* ice-cold PBS. 'Bound' rIFN-α was then solubilized in 2 M KOH. Samples were counted in a gamma counter (1272 CliniGamma LKB, Wallac, Finland) with an error less than 4%. Cell layers were microscopically examined before treatment with KOH [10]. All samples were run in duplicate.

^{125}I-rIFN-α binding to human serum factors

Binding of ^{125}I-rIFN-α to serum factors, as well as serum-induced degradation of the cytokine, were tested by Sephadex G-75 superfine column chromatography using 200-μl samples and 13 ml bed volume. The columns were eluted at 4°C with RPMI 1640, containing 0·15% BSA [8]. Total recovery of ^{125}I after the gel filtrations was always in excess of 95%. The amount of ^{125}I-rIFN-α that bound to IgG was investigated by second antibody precipitation of sera incubated with ^{125}I-rIFN-α. Serum, 10% in 100 μl RPMI 1640, supplemented with 0·15% BSA, was incubated with different concentrations of ^{125}I-rIFN-α for 20 h at 4°C and 37°C, respectively. Human serum IgG was then precipitated at 4°C with 100 μl of rabbit anti-human γ chain (A-424; Dako, Glostrup, Denmark) made 60% in RPMI 1640 medium, and IgG-bound ^{125}I-rIFN-α was separated by centrifugation at 2000 ***g*** for 15 min [11].

RESULTS

rIFN-α-neutralizing effect of human serum

As shown in Fig. 1 and Table 1, normal serum competitively neutralized rIFN-α when tested on human A-549 cells. In contrast, there was no effect of the sera when tested on the bovine cell line MDBK.

rIFN-α binding to serum factor(s)

Second antibody precipitation of sera pre-incubated with ^{125}I-rIFN-α revealed less than 3% binding to human IgG (data not shown). To investigate whether IFN-α bound to other serum factors, sera pre-incubated with ^{125}I-rIFN-α were chromatographed on Sephadex G-75 superfine. As shown in Table 1, there was no binding of ^{125}I-rIFN-α to serum factors of molecular weight above 5 kD. Protease-induced degradation of rIFN-α was negligible, because low molecular weight radioactivity was 2–4% and 4–7% when ^{125}I-rIFN-α was pre-incubated in sera for 20 h at 4°C and 37°C, respectively.

rIFN-α binding to A-549 cells and effect of serum

Maximum saturable binding of ^{125}I-rIFN-α to A-549 cells was reached after 18–22 h at 4°C and after 5–6 h at 22°C. Increased amounts of ligand associated with the cells at 22°C (Fig. 2), suggesting internalization of ligand–receptor complexes, receptor recycling, or increased receptor expression at 22°C [10,12]. To test whether human serum influences the binding of ^{125}I-rIFN-α to A-549 cells, binding was carried out at 22°C in the presence of 20% human serum. This, however, did not change the bound/free ratios of the tracer, indicating that the sera failed to affect cellular binding of ^{125}I-rIFN-α (Table 2). Identical results were obtained with normal and heat-treated sera. Tracer degradation was again negligible (Table 2).

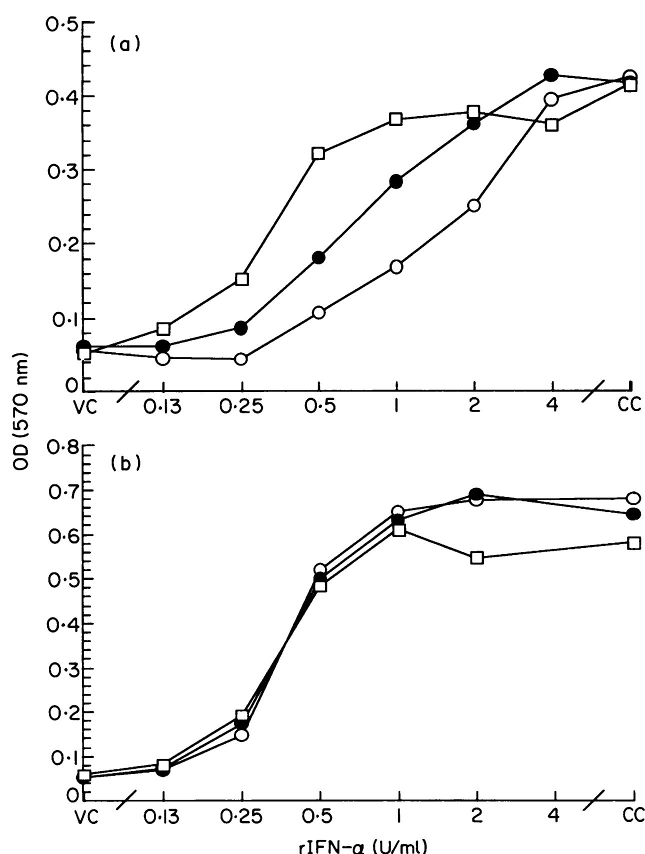

Fig. 1. Serum-induced suppression of rIFN-α activity. Culture media were conditioned with 5% (●) or 10% (○) of a heat-inactivated normal human serum pool ($n=22$), or with 10% newborn calf serum (□). rIFN-α, at various concentrations, was added and incubated at 37°C for 1 h. IFN activity was measured in triplicate using A-549 cells challenged with EMCV (a) or MDBK cells challenged with VSV (b). Results are shown as mean values (s.d. < 12%). VC, virus controls, no rIFN-α; CC, cell controls, no rIFN-α and no virus.

Table 1. Effect of sera on antiviral assays and corresponding serum binding of rIFN-α

	Anti-rIFN-α bioassay†		rIFN-α binding study‡	
Serum*	A-549	MDBK	4°C	37°C
B.B1 pool ($n=22$)	160	10	9 (10)	11 (10)
26/11 pool ($n=22$)	80	ND	6 (12)	8 (11)
M 1-16	80	ND	−2 (2)	1 (ND)
Q-2	160	0	ND	ND
Q-3	160	0	0 (ND)	3 (ND)
SUS-13	40	10	−6 (0)	2 (ND)
MBH 8	160	10	−8 (ND)	−5 (ND)
J-11	80	0	ND	ND
MBH 20	40	ND	−4 (ND)	−3 (ND)

* Heat-inactivated sera were investigated; similar results were obtained with non-heated sera.

† These results are shown as the maximum serum dilution which inhibited the anti-viral effect of 0·5 U/ml of rIFN-α.

‡ ^{125}I-rIFN-α (6000–10000 ct/min) was added to 200 μl 50% serum and incubated overnight at 4°C and 37°C. The binding of IFN-α to serum factors was then determined by gel filtration. These results are shown as the amount of tracer (in %) recovered in the high-molecular-weight fractions (HMW) corrected for the amount of tracer found in HMW when the tracer was processed without serum. Values in parentheses are from parallel experiments with 400 molar excess of unlabelled rIFN-α.

ND, not determined.

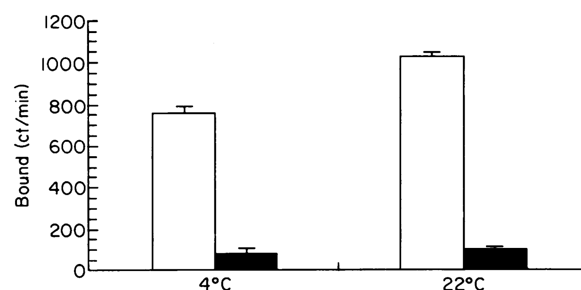

Fig. 2. Temperature-dependent association of ^{125}I-rIFN-α to A-549 cells. ^{125}I-rIFN-α, 23000 ct/min, was added to triplicate wells with (■) or without (□) 400 molar excess unlabelled rIFN-α. After 22 h at 4°C and 6 h at 22°C, respectively, cell-bound ^{125}I-rIFN-α was measured. The experiment was repeated once with similar results. The data are shown as mean values + s.d. of triplicate determinations.

DISCUSSION

By definition, an autoantibody must bind the antigen through the Fab fragment(s); the binding should be specific and saturable.

In humans, cytokine autoantibodies may emerge as a result of cytokine therapy, or they may occur without apparent exogenous stimulation [13]. Thus, nanomolar concentrations of naturally occurring, high affinity and neutralizing autoantibodies to IL-1α and IL-6 are frequently found in sera of healthy and diseased individuals [8,11,14–19]. The Fab fragments of these autoantibodies exhibit specific and saturable binding IL-1α and IL-6, respectively [11,15] (and unpublished data).

IgG antibodies that interfere with IFN bioactivity and/or bind IFN have been reported in sera of healthy as well as diseased individuals [3–6]. However, it is important to realize that antibody interference in an IFN bioassay does not by itself prove that this is caused by a specific autoantibody to IFN. Also, the binding of antibodies to immobilized (and possibly denatured) IFN, for example by the use of immunoblotting techniques, direct ELISA, or affinity chromatography [4–6,20,21], does not necessarily mean that these immunoglobulins are autoantibodies. Therefore, to avoid false conclusions, cytokine-binding immunoglobulins should also be evaluated by quantitative saturation-binding analyses.

These considerations prompted us to perform direct ligand-binding studies using ^{125}I-rIFN-α. We chose the chloramine T method of iodination of human rIFN-α, because this procedure has been shown to conserve the receptor binding property and bioactivity of the cytokine [7]. This was verified by the fact that the resulting ^{125}I-rIFN-α retained bioactivity and bound with high avidity to receptors on human A-549 cells.

We confirmed previous findings that normal human serum suppressed rIFN-α bioactivity in a concentration dependent

Table 2. Effect of sera on the binding of ^{125}I-rIFN-α to A-549 cells at 22°C

Serum*	Bound (ct/min)	Bound/free (%)	TCA precipitation (% ^{125}I in pellet)
BSA medium†	1023	5·2 (0·5)	92
26/11	1191	5·6 (0·6)	93
M 1-16	1240	5·3 (0·5)	94
Q-3	1191	5·1 (0·4)	94
SUS-13	1138	5·2 (ND)	93
MBH 8	1246	5·5 (0·7)	93
J-11	1231	5·3 (ND)	95
MBH 20	1057	5·4 (0·6)	93

* Heat-inactivated sera were used at a 20% final concentration. Similar results were obtained with 20% non-heated sera. ^{125}I-rIFNα, 23000 ct/min, was added to each well.

† Assay medium without human serum. Values in parentheses are from parallel experiments with 400 molar excess of unlabelled rIFN-α.

ND, not determined.

manner when using A-549, but not MDBK cells [5]. We also found that serum factors, including IgG, bound to rIFN-α. However, apart from being quantitatively insignificant, the binding of ^{125}I-rIFN-α to IgG was non-saturable, indicating that the binding was non-specific. There was no significant saturable binding to serum factors when sera were pre-incubated with biologically relevant doses of ^{125}I-rIFN-α and, subsequently, gelfiltrated.

The discrepancy between serum-induced suppression of IFN-α bioactivity and lack of specific binding of ^{125}I-rIFN-α to serum factors might be explained by the presence in serum of IFN-α receptor antagonists. However, receptor antagonist activity was not found in direct binding experiments using ^{125}I-rIFN-α and A-549 cells.

We conclude that normal serum does not contain detectable high-avidity autoantibodies to IFN-α. The data suggest that serum immunoglobulin inhibits IFN-α bioactivity *in vitro* by interference with post-receptor processes in some (A-549 cells) but not all target cells.

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