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Application of an Objective Biological Assay of Human Interferons to Clinical Specimens and a Survey of a Normal Population

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A rapid, quantitative, and nonsubjective method of interferon assay is described, which can be readily applied to clinical specimens. Automated data acquisition and data reduction allowed a significant increase in volume per unit of time over existing methodologies. Plasma always yielded higher (usually 2:1) interferon values than did serum obtained simultaneously. Ranges of interferon levels in plasma in normal control populations are reported as well as ranges for clinical virology laboratory technicians and patients with terminal malignancies or collagen vascular diseases.

The production and purification of human interferon (IFN) as well as the detection of IFNs in various biological samples are contingent upon a reliable, accurate, and rapid means of assay (4). IFN has historically been assayed by a plaque reduction method, standard to the discipline of virology. This method requires 5 to 7 days of culture in semimicrowells or petri dishes under an agar overlay to prevent random dispersion of de novo-synthesized virus. The method is time consuming, requires large quantities of reagents, and is subject to judgement errors in plaque counting. A decade ago, Finter (2) introduced the use of vital dye uptake as a method of estimation of inhibition of viral cytopathic effect (CPE) by IFN. This procedure was cumbersome and time consuming; consequently, it was adopted by only a relatively small number of laboratories. More recently, McManus (3) proposed vital dye uptake in microtiter plates with target cells in preformed monolayers as a quantitative measure of IFN titer. This procedure also was relatively time consuming, and the methods of sample dilution were laborious. Rubinstein et al. (6) have described recently a microtiter method for the rapid assay of IFN which relies on subjective analysis of viral CPE inhibition. We have combined the vital dye uptake assay of Finter (2) with the rapid microtiter assay of Rubinstein et al. (6) to obtain a rapid, qualitative, and nonsubjective microtiter assay of IFN antiviral activity. At present, we report the technique of this quantitative IFN assay and its application to identifying human IFN in 153 human biological specimens, including a large normal population. This method of IFN assay has the following advantages: (i) speed of performance with minimal physical manipulation, (ii) nonsubjective analysis of quantitative response with statistically generated confidence limits, (iii) assay interface with a dedicated laboratory computer, and (iv) timely application to the clinical laboratory on a cost-effective basis.

MATERIALS AND METHODS

Cell and virus culture. Primary human foreskin fibroblast cell cultures (SG-181) were established and maintained in Eagle minimal essential growth medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 50 μg of gentamicin sulfate per ml (MEM–10% FBS). Cells between passages 5 and 14 were used in this study. HEp-2 cells were obtained from S. Moyer, Vanderbilt University, Nashville, Tenn., and were grown in MEM–10% FBS. All cell cultures were grown in an atmosphere of 5% CO_2 in air at 100% humidity and 36°C. Vesicular stomatitis virus Indiana strain (VSV) and encephelomyocarditis virus (EMCV) were propagated in HeLa cells. Briefly, stock VSV (from S. Moyer, Vanderbilt University) or EMCV (from C. Baglionni, State University of New York at Albany, N.Y.) was adsorbed on HeLa cells in a minimal volume of serum-free MEM at a multiplicity of infection of 0.1 for 1 h. The culture was washed two times with phosphate-buffered saline (pH 7.4), MEM–10% FBS was added, and the culture was incubated for 18 h or until a distinct viral CPE was produced. The growth medium was harvested and centrifuged at 500 × *g* for 15 min, and the virus was titered by plaque assay under agar overlaid on SG-181 cells. Virus was stored at −70°C in small portions containing approximately 2.0×10^7 PFU of VSV or EMCV per ml.

IFNs. Human IFN-α (World Health Organization [WHO] Standard B, 69/19) and IFN-β (WHO no. G-023-902-527) reference standards were obtained from the National Institute of Allergy and Infectious Diseases, Bethesda, Md. Commercial preparations of human IFN-α and IFN-β were obtained from Interferon Sciences, Inc., New Brunswick, N.J., and HEM, Inc., Rockville, Md., respectively. These commercial IFNs were used daily as internal laboratory standards after being calibrated to WHO IFN standards.

Rapid, quantitative, and nonsubjective biological assay of human IFNs. The IFN assay described herein and briefly described in review elsewhere without documentation (3) was based on the quantitative dye uptake assay of Finter (2) and the rapid microtiter assay of Rubinstein et al. (6). In a 96-well microtiter plate, twofold dilutions of sample were made in 100 μl of MEM–10% FBS. SG-181 cells or HEp-2 cells were added in a single-cell suspension at a density of 2.5×10^4 to 3.0×10^4 cells per well in 100 μl of growth medium and at the indicated time were challenged with VSV or EMCV (50 μl of MEM containing 7×10^3 PFU per well). Each dilution was performed in triplicate at least. Viral CPE was observed in control wells within 15 to 20 h after viral infection (i.e., two viral replication cycles). Plates were incubated in an atmosphere of 5% CO_2 in air at 100%

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humidity and 36°C. The growth medium was removed by inversion, cell layers were washed two times with phosphate-buffered saline (pH 7.4), and the medium was replaced with 100 μl of MEM containing Finter stock 2% neutral red dye diluted 1:150 in MEM. After 2 h of incubation, the wells were inverted and washed two times with phosphate-buffered saline (pH 7.4), and the dye which was adsorbed in the viable cell layer was extracted into 100 μl of acidified ethanol (50% ethanol in 1% acetic acid). The extracted dye solution in the original microtiter plate was quantitated colorimetrically at 540 nm with a Flow Titertek colorimeter. Data reduction was carried out on an Apple II computer interfaced to a Titertek type 3100 microdensitometer with a module type 312B, both from Flow Laboratories. The Apple II microcomputer was configured with 64 kilobytes of memory, two floppy diskettes, and a printer (Paper Tiger model 560, Integral Data Systems). Assay data from the Titertek were analyzed in a two-step process. (i) Data from the Titertek were moved into specific computer memory through the instrument interface by a designated program which manipulated raw data and transferred assay results to the printer.

(ii) The data reduction program began by allowing optical density (OD) readings from specified wells to be deleted from the calculations. Such an operation, although used infrequently, allows flexibility in eliminating obviously aberrant data values. Eight cell control and eight virus-infected-cell control wells were each averaged, and an expected range of OD for the assay run was calculated as the difference of the two averages. The range was used to define the OD at which 50% protection by IFN was achieved for the assay. Each assay dilution was performed in triplicate, yielding three sets of OD values and their calculated means and standard deviations. The mean OD value of each dilution was used to calculate percent protection relative to the cell control- and virus-infected-cell control-derived range. Percent protection and percent standard deviation were printed together with the dilution ratio after each percent protection relative to dilution factor was printed, and the two values found to bracket the 50% OD level were used to linearly interpolate the dilution at which 50% protection was estimated to occur.

IFN assay of clinical specimens. Fresh serum or plasma (heparin) samples were obtained by venipuncture and frozen immediately at −70°C until time of assay. All assays were performed with SG-181 or HEp-2 cells as target cells, and each sample was preincubated with target cells for 2 h before infection with VSV or EMCV. A variety of human subjects were used, including (i) Red Cross blood donors, (ii) healthy specimen donors to a local commercial bloodbank, (iii) medical technologists employed at a Vanderbilt University Hospital virology laboratory (mean duration of employment, 1.1 years), (iv) patients identified as terminally ill with metastatic malignant disease, and (v) patients with either active or inactive rheumatoid arthritis or systemic lupus erythematosus.

RESULTS

Neutral red dye adsorption by human foreskin fibroblasts. Freshly trypsinized SG-181 cells in single-cell suspension distributed in plastic microtiter plates adhered firmly to the bottom of the wells in less than 1 h. Cells that were allowed to adhere to wells for 2 h and then subjected to multiple washes with phosphate-buffered saline (pH 7.4) adsorbed neutral red dye in a linear function dependent on cell density at cell concentrations of 0.5×10^3 to 0.4×10^4 cells per well (correlation coefficient, 0.985) (Fig. 1). Dye adsorption was complete within 2 h, and dye was readily extracted into acidified 50% ethanol.

Antiviral titration of standard IFN-α. The antiviral titration of WHO standard IFN-α with SG-181 cells was found to be dependent on the time of IFN preincubation before the infection of the culture with VSV (Fig. 2A). The range of antiviral activity of 100 IU of IFN-α per ml was titered between 76 U/ml at 0 h of preincubation and 420 U/ml at 6 h of preincubation of cells and IFN before viral infection. Maximal antiviral activity was consistently found to plateau after 6 h of IFN preincubation and gradually decreased through 24 h of preincubation. Expression of IFN antiviral activity was always demonstrated when the VSV or EMCV was added to the cultures simultaneously with the IFN. The titration curve of a serially twofold-diluted IFN standard was found to be constant at any one time of IFN preincubation before viral infection. When standard IFN-α (100 reference units per ml) was serially twofold diluted and allowed to incubate with SG-181 cells for 2 h before VSV infection, a titration curve (Fig. 2B) could be constructed. A plot of percent protection versus reciprocal dilution of the IFN sample produced a characteristic curve of IFN-induced antiviral protection. Identical titration curves were observed for both IFN-α and IFN-β (data not shown). When the titration curve was converted to a logarithmic scale, the log percent protection versus log reciprocal dilution plot revealed a linear titration curve (Fig. 2C). This plot was found to be linear over a range of five dilutions of standard IFN-α. For partially purified IFN internal standards, the log reciprocal of the dilution factor at which 50% protection occurred could be calculated by a linear regression. Clinical specimens frequently yield more complex log-log plots with

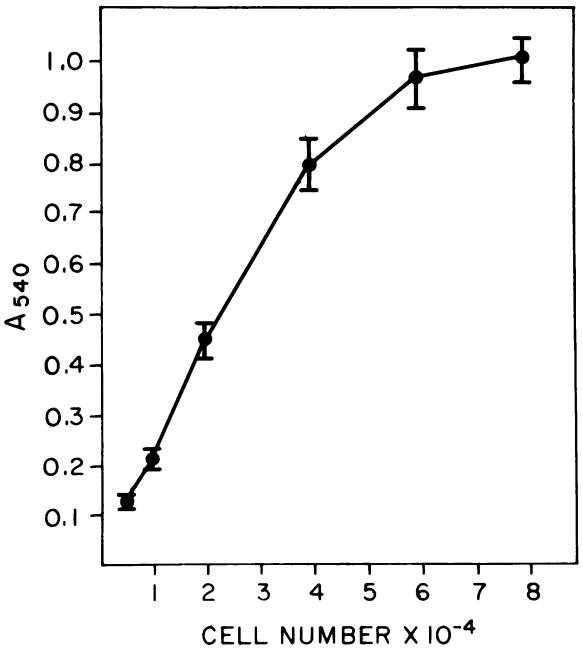

FIG. 1. Neutral red dye uptake by human foreskin fibroblast (SG-181) cells. Human foreskin fibroblasts were plated in microtiter plates at cell densities ranging from 0.5×10^4 to 8×10^4 and allowed to adhere to the plastic surface for 4 h. Cells were stained with neutral red dye as described in the text, and dye uptake was determined with a Flow Titertek colorimeter.

linearity found within a much smaller window around the 50% protection value. This led us to calculate the reciprocal dilution at which 50% protection occurred by linear extrapolation between the two points at which 50% protection was found. Statistical confidence limits were then constructed based on percent standard deviation of three replicate titrations.

Influence of IFN preincubation and target cell specificity on antiviral titration of IFN-α and IFN-β. As described above, the duration of IFN preincubation with SG-181 cells before viral infection of the cell culture had considerable influence on the observed titer of standard reference IFN-α and IFN-β. This time dependency was distinctly dependent on the target cell used (Fig. 3A and B). The development of the antiviral state in human foreskin fibroblasts followed a similar time-dependent relationship for IFN-α and IFN-β (Fig. 3A). This time dependency appeared to be biphasic. When IFN-α and IFN-β were preincubated with SG-181 cells for 0 to 2 h, an early plateau of antiviral activity was observed. This detected titer of IFN activity closely resembled the stated antiviral activity of National Institutes of Health reference standards. From 3 through 24 h of preincubation, the detected antiviral titer rose sharply and remained at levels of two to three times the reported National Institutes of Health standard level. When HEp-2 cells were used as the target cell for IFN-α and IFN-β assay and 100 reference units of IFN were titered, both IFNs demonstrated a significant rise in titered antiviral units during the first 2 h of IFN preincubation (Fig. 3B). At 2 h of IFN-α and IFN-β preincubation, a maximal laboratory titer of 3 to 3.5 times the reference National Institutes of Health titer was

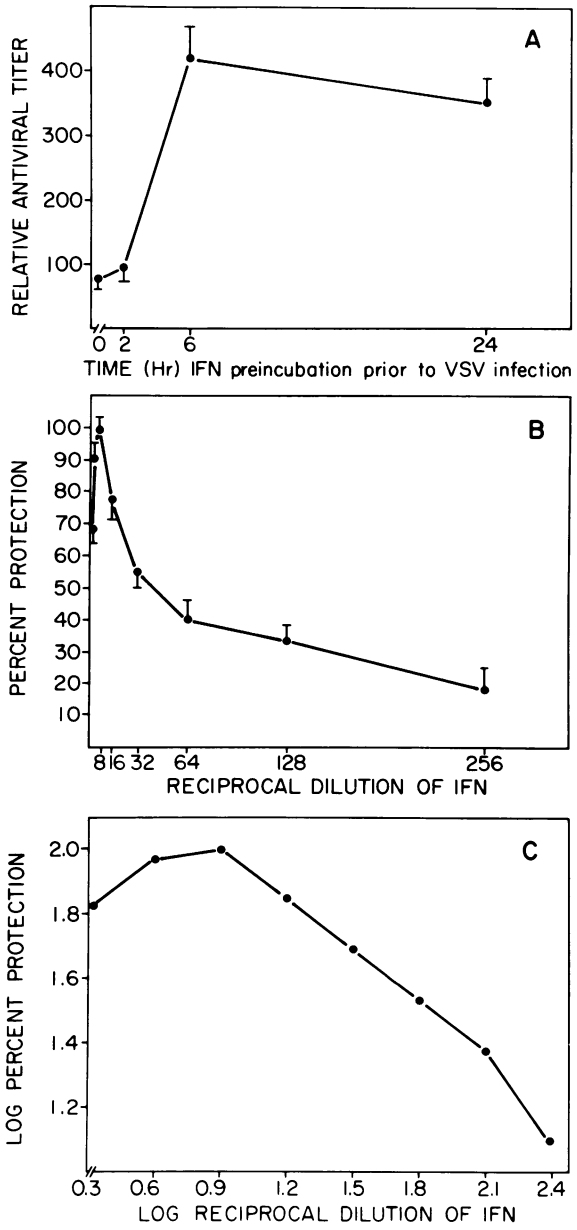

FIG. 2. IFN antiviral assay. A WHO standard IFN sample was assayed as described in the text. (A) SG-181 cells were incubated with IFN for 0 to 24 h before infection of the cell culture with VSV. The units of observed IFN activity are plotted relative to the time of IFN preincubation before viral infection. (B) Percent protection produced by each IFN dilution at 2 h of IFN preincubation. (C) When the data of panel B were converted to a log percent protection versus log reciprocal dilution of IFN, a linear relationship was revealed. The 50% protection point (i.e., 1 U of IFN activity) can be linearly extrapolated from such a point.

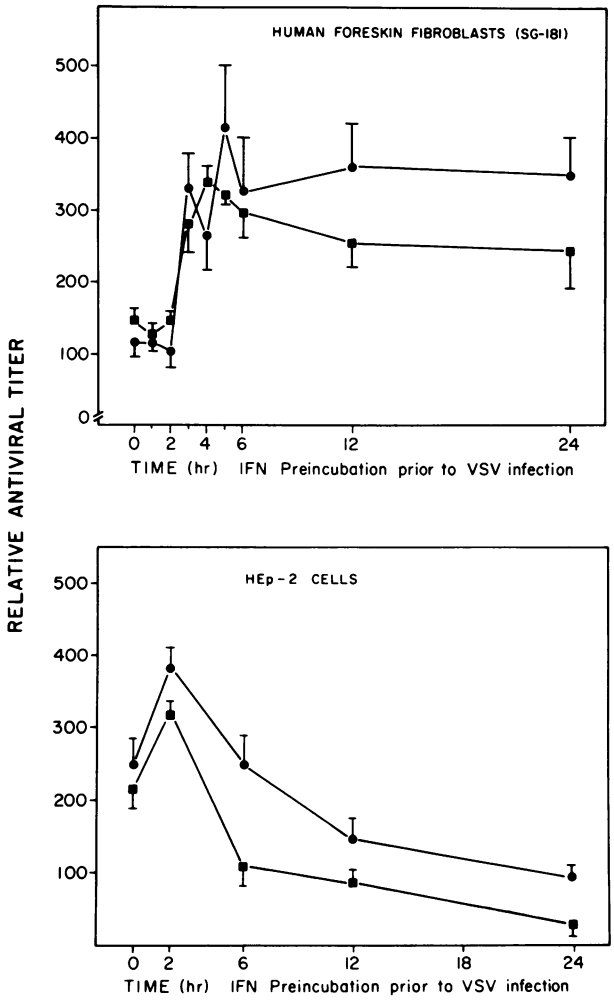

FIG. 3. Antiviral titration of internal standard human IFN-α and IFN-β with SG-181 and HEp-2 cells as target. Either SG-181 or HEp-2 cells were incubated with the IFN samples for 0 to 24 h before infection of the cultures with VSV. These IFN antiviral assays were performed as described in the text. Symbols: ●, IFN-α; ■, IFN-β).

TABLE 1. Comparison of IFN levels in serum and in heparinized plasma from individuals with high endogenous levels of IFN

IFN level (U/ml)		Ratio of IFN in plasma to IFN in serum
In serum	In plasma	
150 ± 10	348 ± 20	2.38
157 ± 5	339 ± 5	2.15
695 ± 24	873 ± 45	1.25
117 ± 11	222 ± 5	1.89
181 ± 7	332 ± 9	1.83
527 ± 28	638 ± 61	1.21
458 ± 71	881 ± 60	1.77
124 ± 31	217 ± 15	1.75

TABLE 2. Comparative human IFN titers in plasma

Plasma source	*n*	Mean IFN titer (U/ml ± SD)[a]	Range
Red Cross blood donor volunteers	104	69 ± 323	≤10–3,296
Normal control subjects (non-hospital blood bank)	24	23 ± 29	≤10–111
Clinical virology lab technicians	5	133 ± 12	90–159
Terminal malignancy patients	15	313 ± 240	63–850
Collagen-vascular disease patients (i.e., four lupus and rheumatoid arthritis)	5	513 ± 236	332–873

[a] All reported values were normalized to standard control for each individual assay.

obtained. Similar laboratory titer decay curves were observed for each IFN.

Antiviral assay of clinical blood samples. Venipuncture specimens were obtained from several patients suspected of having elevated IFN levels. Both unpreserved serum and plasma heparin samples were collected simultaneously. All IFN assays were performed on the same day or on consecutive days. Internal IFN standards were assayed at the ratio of approximately one standard per six clinical samples (Table 1). It was consistently observed that antiviral activity could be detected in both plasma and serum samples. IFN levels in plasma were always found to be greater than levels in serum, usually by a factor of two.

Venipuncture specimens from a large pool of healthy blood donors were collected from two independent sources, the first being the Nashville Chapter of the American Red Cross and the second being a commercial immunohematology laboratory (Table 2). The latter sample pool, consisting of 24 independent samples, had a mean titer of 23 ± 29 IU of IFN per ml (range, ≤10 to 111 IU of IFN per ml), whereas the American Red Cross sample pool of 104 independent samples had a mean titer of 69 ± 324 IU of IFN per ml (range, ≤10 to 3296 IU of IFN per ml. The distribution of IFN titers in the ARC pool is expressed in Fig. 4. The average titer of the American Red Cross samples was decreased to 38 ± 52 IU/ml if 1 sample out of the 104, with a titer of 3,296 IU/ml, is deleted. Normal, healthy controls obtained from a clinical virology laboratory had an elevated mean titer of 133 ± 12 IU/ml (range, 90 to 159 IU/ml). Patients with collagen vascular disease had the highest mean titer (513 ± 236 IU/ml) confirming previous reports of elevated IFN levels in this disease class (5). To our surprise, patients with a variety of terminal neoplastic diseases had significantly elevated IFN levels in plasma, an observation which has not been previously reported to our knowledge.

DISCUSSION

The method of IFN bioassay described allows a rapid, quantitative measurement of circulating IFN levels in patient samples as well as an accurate research tool capable of yielding IFN titers with statistical confidence limits of analysis. When a microtiter colorimeter interfaced with a dedicated microcomputer is used, a relatively large volume of IFN assays can be performed by a single operator per day with a turnaround time of 24 h or less.

As expected, normal controls had low levels of IFN activity, and minimal levels that would not have been detected in serum were detected in plasma samples. Our results corroborate reports (5) that patients with collagen-

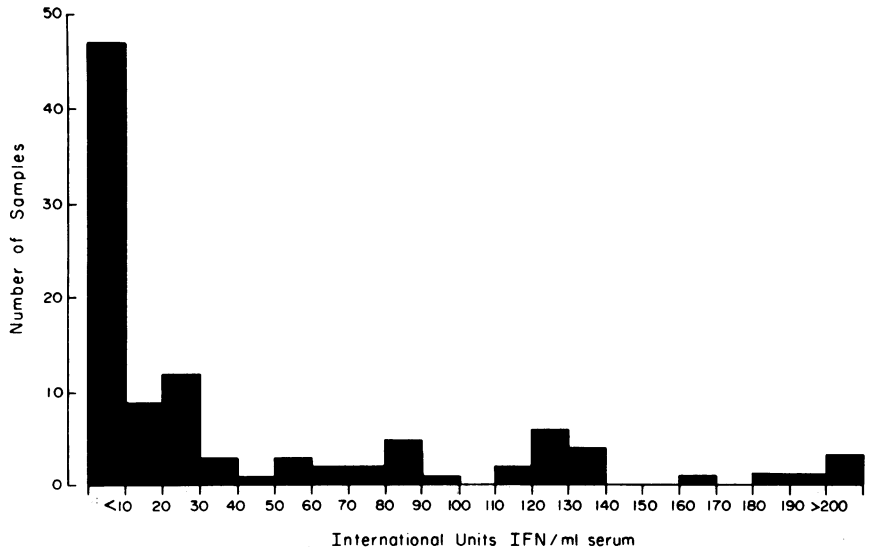

FIG. 4. Distribution of individual plasma IFN titers from American Red Cross pool. Assay on HEp-2 cells with EMCV indicator.

vascular disease have high levels of circulating IFN. However, we have not investigated as yet the physicochemical or the antibody neutralization properties of this IFN avidity. Moreover, all of the patients (5) in our limited group of collagen-vascular disease subjects had significantly elevated IFN levels in contrast to the 50% of patients previously reported as having an elevated-pH-labile IFN-α (5). Surprisingly, some of our patients with terminal malignancies had high levels of endogenous IFN activity in plasma. Although subclinical viral infection cannot be ruled out, we believe this observation needs to be confirmed with age-matched hospital controls. Although there is no direct evidence, the lower levels of IFN found in serum versus in plasma from paired samples drawn simultaneously may be due to adsorption of IFN on the fibrin clot. Since it is known that IFN-β binds to tissues more avidly than does IFN-α (1), the lack of an ~2:1 titer of plasma versus serum in two of our paired patient samples (Table 1) may be a reflection of different ratios of circulating IFN-α and IFN-β in these two subjects. Alternatively, proteolytic enzymes activated in the clotting process may differentially inactivate the IFNs.

Although any IFN-responsive cell line should be suitable, our results demonstrate that care must be taken in the length of IFN preincubation with the target cells before virus addition, since induction and decay curves are target cell dependent. For standard reporting purposes, all laboratory titers should be corrected by adjustment of observed titers to those of reference standards run simultaneously under identical conditions.

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