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Bioimmunoassays (BIAs) of Human Interferon (41764)

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Abstract. Simple, sensitive, and reproducible assay systems for measurement of the biological activity of interferon are described. The methods used are based on the quantification of cell membrane-bound viral and cellular antigens in interferon-treated cells by enzyme immunoassays. To measure the antiviral activity, samples of human interferon are titrated in microplates with human or bovine cells. After incubation with challenge virus (vesicular stomatitis or herpes simplex virus) the cells are fixed with glutaraldehyde and assayed for viral antigens by enzyme-labeled antibodies. This assay permits the detection of less than 0.1 unit of interferon per milliliter, after optimization of several factors, such as type of cell, multiplicity of infection, temperature, and period of incubation. The effect of interferon on cellular antigens is measured in a similar way, by using peroxidase-labeled antibodies directed against β_2-microglobulin. The two types of assays described appear suitable for kinetic experiments and for detection of interferons of different specificities in body fluids.

Interferons are proteins with ability to induce a multitude of biological activities. By their antiviral effect these hormone-like substances are able to influence the course of virus infections. Since interferon (IFN) also has regulatory functions on immune responses (1), it is possible that IFN is of importance in many other diseases besides virus infections. Recent observations indicate that several types of immunological diseases are associated with abnormalities in the IFN system (2–8). To detect IFN in serum and other body fluids and to elucidate pathogenic mechanisms where IFN is involved, it is of paramount importance to have access to simple and reliable assays of high sensitivity.

Earlier methods for assay of IFN have mainly been based on measurements of the antiviral effect of IFN, manifested as inhibition of cytopathic effect, plaque reduction, and inhibition of virus yields (9, 10). Purified IFN of different subtypes and monoclonal antibodies with specificity for these interferons have recently become available and this has made it possible to develop simple and rapid solid-phase immunoassays for the determination of IFN (11, 12). The sensitivity of these tests is still considerably lower, however, than the best bioassays, that are based on determination of the antiviral activity of IFN.

The present report describes the use of enzyme immunoassays for the measurement of two different biological activities of IFN. One of these assays determines the antiviral effect of IFN by measuring its ability to inhibit the expression of viral antigens on the surface of infected cells. The other bioimmunoassay (BIA) is based on the ability of IFN to increase the synthesis (13, 14) and/or the expression of histocompatibility antigens on the cell surface (15). The ligand used for the enzyme labeled antibodies was β_2-microglobulin in this type of BIA.

Materials and Methods. *Interferon preparations.* Human leukocyte interferon (IFN-α) was kindly supplied by the Red Cross Service, Helsinki, Finland. The specific activity of this preparation was 10^7 units/mg protein, as determined by repeated calibration against the international reference preparation 69/19. In the following all references to units of IFN pertain to the antiviral activity of the preparation as compared to the standard 69/19. IFN-β was prepared in our laboratory by stimulation of human embryonic lung fibroblasts with Sendai virus. Viral activity was removed by centrifugation at 100,000g for 2 hr and incubation at pH 2 overnight at 4°C. IFN-γ was induced in peripheral blood mononuclear cell cultures with phytohemagglutinin and phorbol myristic acid as described by Yip *et al.* (16). The preparations of IFN-α and IFN-β seemed to exclusively contain the homologous subtype of IFN, since the antiviral activity could be reduced more than 100-fold by incubation with specific antiserum.

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Antisera. Rabbit anti-HuIFN-α was a gift from Dr. Kurt Berg, Aarhus, Denmark. The antiserum had been obtained by immunization of rabbits with 90% pure human IFN-α, prepared as described by Berg and Heron (17) and neutralized 10 units of IFN-α at a dilution of 1:40,000.

Antiserum against HuIFN-β was a gift from the Rega Institute, Leuven, Belgium and had been prepared in goats by injections of purified (10^6 units/mg protein) IFN-β. The neutralization titer of the antiserum against 10 units of IFN-β was 1:40,000.

Monoclonal antibodies against herpes simplex virus (HSV) type 1 were produced in our laboratory by fusion of FO-cells with spleen cells from Balb/c mice, immunized with a solubilized membrane fraction of HSV-1 infected Balb/c embryonic cells. The antibodies were of isotype IgG1 and reacted specifically with glycoprotein gC of HSV-1.

Rabbit antiserum against VSV was prepared by immunization of rabbits with partially purified virus, grown in baby hamster kidney cells. Serum was used without prior adsorption with cell suspensions, although background activity could be reduced by such treatment.

Peroxidase-conjugated sheep anti-rabbit and anti-mouse immunoglobulin, as well as peroxidase-conjugated sheep anti-β_2-microglobulin were purchased from Dakopatts AB, Sweden.

BIA based on measurement of viral antigen. The antiviral activity of IFN was assayed in two different cell lines. One of them, MDBK, (American Type Culture Collection, CCL 22) derived from bovine kidney was used, since it is known to be particularly sensitive to HuIFN-α. The other cell line, A549 (American type Culture Collection, CCL 185), derived from human lung carcinoma was sensitive to all three types of human IFN. The cells were grown in 96-well microtiter plates (Nunc, Denmark) with Eagle's minimal essential medium (MEM) supplemented with 4% fetal calf serum and antibiotics. After 3 days of incubation at 37°C, when a confluent monolayer of cells was obtained, the wells were washed twice and supplied with 0.1 ml of MEM supplemented with 2% fetal calf serum and antibiotics, or 0.1 ml of IFN specimens diluted in this maintenance medium. The plates were incubated overnight at 37°C. The wells were then washed three times with MEM and inoculated with 0.1 ml of either HSV-1 (F strain) or VSV (Indiana strain). The wells in the periphery of the microtiter plates were usually left uninoculated with IFN and virus as shown in Fig. 1, to avoid the influence of edge phenomena. After further incubation at 37°C the cells were fixed with 0.1 ml of 0.2% glutaraldehyde in phosphate-buffered saline (PBS). The fixative was removed after 1 hr at room temperature by washing three times with PBS. Dry microtiter plates were usually stored at −70°C before the enzyme immunoassay (EIA).

The EIA included the following steps: (a) washing with PBS containing 0.05% Tween 20; (b) addition of 0.1 ml of monoclonal anti-HSV antibodies or rabbit anti-VSV antibodies, diluted 1:100 and 1:800, respectively, in PBS–Tween; (c) incubation for 2 hr at room temperature followed by washing three times with PBS-Tween; (d) addition of 0.1 ml of peroxidase-conjugated anti-mouse immunoglobulin or anti-rabbit immunoglobulin, diluted 1:400 and 1:200, respectively, in PBS–Tween; (e) incubation for 2 hr at room temperature followed by washing three times with PBS–Tween; (f) addition of 0.1 ml substrate solution, containing 10 mg paraphenylenediamine, 1 ml methanol, 10 μl 30% H_2O_2 in 20 ml PBS; (g) addition of 0.1 ml of 1 *M* NaOH to each well to stop the enzyme–substrate reaction after 15 to 30 min at room temperature; and (h) measurement of optical density at 492 nm in a Titertek Multiscan spectrophotometer (Flow laboratories, Scotland). The IFN concentration was expressed in international units and was calculated by comparing the optical density of the specimens with that of an IFN standard.

BIA based on measurement of cellular antigen. The activity of IFN on the expression of cellular antigens on the cell surface was measured in A549 cells, grown in microtiter plates as described above. The IFN preparations were diluted in maintenance medium and each well was inoculated with 0.1 ml of IFN or medium only. After incubation for 24 hr at 37°C the medium was removed and the cells fixed by addition of 0.1 ml 0.2% glutaraldehyde. The fixation was interrupted after 1 hr by washing three times with PBS. The amounts of β_2-microglobulin exposed on the

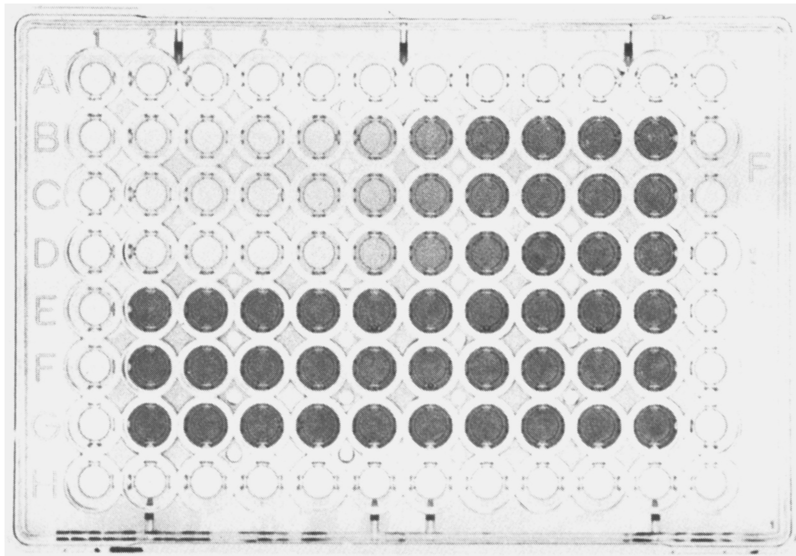

FIG. 1. BIA in microtiter plates with confluent monolayers of MDBK cells. Serial twofold dilutions of IFN-α were inoculated in triplicates into the wells in rows 2–4. After overnight incubation at 37°C the plates were washed three times and the wells in rows 2–7 infected with HSV-1. The wells in the periphery were left uninoculated to avoid the influence of edge phenomena. After further incubation for 18 hr the cells were fixed for 1 hr with 0.2% glutaraldehyde in PBS. The plates were then washed and examined by EIA.

cell surface was then determined by EIA. This procedure involved (a) incubation for 2 hr with 0.1 ml peroxidase-conjugated anti-β_2-microglobulin diluted 1:100 in PBS–Tween; (b) washing three times with PBS; (c) addition of 0.1 ml substrate solution to each well and incubation for 15 to 30 min at room temperature; (d) interruption of the enzyme–substrate reaction by addition of 0.1 ml 1 *M* NaOH; and (e) measurement of OD 492 nm in a spectrophotometer as described above.

Results. *Assay of antiviral activity in MDBK cells.* The use of EIA for assay of interferon was primarily tested in MDBK cells with HSV-1 as challenge virus. This cell line was selected since it has been considered to be highly sensitive to HuIFN-α and HSV was used since monoclonal antibodies with specificity to viral surface antigens of this virus were available in our laboratory.

Figure 2 shows the dose–response relationship in one experiment performed with a multiplicity of infection (m.o.i.) of 0.01 and a replication period of 18 hr. The results recorded are mean values obtained from six separate titrations of IFN-α with each dilution inoculated into six wells. Maximal inhibition of the synthesis of viral antigen was obtained with 25 units/ml and 50% inhibition with 4.4 units of IFN. Higher multiplicities and longer period of incubation with this virus reduced the sensitivity, whereas incubation at 39 or 40°C increased the sensitivity, in comparison to incubation at 37°C.

Similar experiments were performed with VSV as challenge virus. This virus was as expected more sensitive to the antiviral activity of IFN. Figure 3 shows an experiment in which IFN-α was titrated in three separate dilution series and each dilution inoculated into three wells. VSV was inoculated at a multiplicity of 10 and the plates were incubated at 37°C in 5% CO_2 for 9.5 hr, whereafter the replication of virus was stopped by fixation. Maximal inhibition was obtained with about 3 units of IFN and 50% inhibition with 0.43 units/ml. In comparison to monoclonal antibodies, the hyperimmune antiserum against VSV gave relatively high background activity with uninfected cultures. Varying the m.o.i. between 100 and 1 had limited effect on the sensitivity and lower multiplicities often lead to incon-

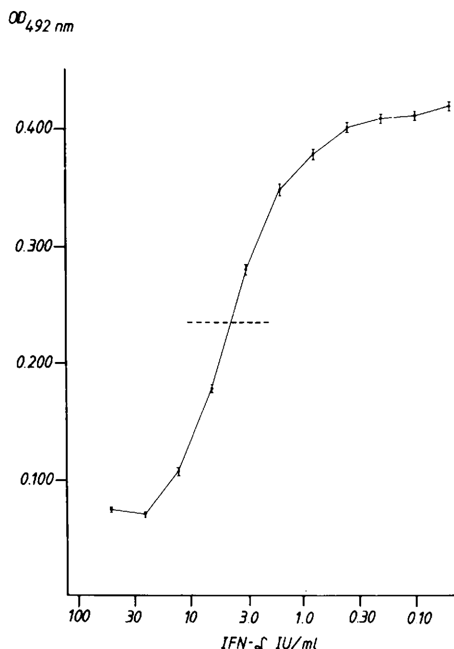

FIG. 2. BIA measuring antiviral activity in MDBK cells incubated overnight at 37°C with IFN-α and subsequently infected with HSV-1 at a m.o.i. of 0.01. The results show mean values and SEM from six dilution series of IFN, with each dilution inoculated into six cultures. The dashed line indicates 50% reduction of absorbance, compared to the virus-infected controls, not treated with IFN.

sistent results. Increasing the incubation temperature to 40°C enhanced the sensitivity, if the IFN titer was calculated from dilutions giving 50% inhibition of adsorbance.

Reproducibility. The variability of the BIA was more closely examined in an experiment where eight twofold dilution series were assayed in microtiter plates with MDBK cells infected with HSV-1 at m.o.i. of 0.01. Each dilution was inoculated into six replicate cultures. The incubation period was 24 hr for IFN and 18 hr for HSV-1. In each microtiter plate were included eight wells with uninfected cells and eight wells with virus-infected cells, not previously exposed to IFN. The reduction of absorbance was calculated for each dilution of IFN. The dose of IFN reducing the absorbance by 50% was estimated from the results of two adjacent dilutions showing absorbance values close to the 50% level. Corresponding calculations were performed to determine interferon concentration giving 25% reduction of absorbance.

The variation of the IFN titers was in general found to be somewhat lower when the titer was measured at the 50% level than at the 25% level (Table I). The standard deviation (SD) was correlated to the number of replicates used for each dilution of IFN and with triplicates the SD was 7.0% (50% level) and 8.6% (25% level). By studying the variation of absorbance in cultures treated with very low concentrations of IFN it was found that a 20% reduction of absorbance was statistically significant ($P < 0.001$) if the titrations were performed in triplicates. The results further showed that the optical density (OD) was significantly higher in titrations performed in the periphery of the microtiter plates. This type of edge phenomenon also seemed to occur in the experiment depicted in Fig. 2, since values too high were obtained with the highest and lowest concentration of IFN.

Assay of IFN in A549 cells. Since MDBK cells are not suitable for assay of all three types of IFN, experiments were performed to obtain a cell–virus system sensitive to α-, β-, and γ-interferon. The sensitivity of a few different

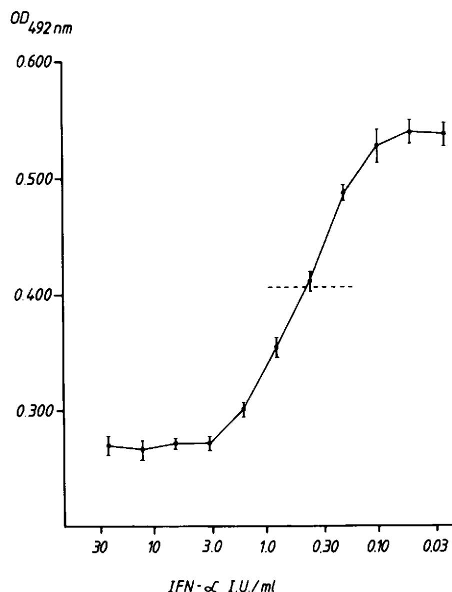

FIG. 3. BIA measuring antiviral activity of IFN-α in MDBK cells, challenged with VSV at a m.o.i. of 10. Each point on the curve shows the mean value and SEM from 12 assays. The period of incubation at 37°C was 18 hr for interferon and 9.5 hr for VSV. The dashed line indicates 50% reduction of absorbance, compared to the virus-infected controls, not treated with IFN.

TABLE I. REPRODUCIBILITY OF BIA STUDIED IN MICROTITER PLATES WITH MDBK CELLS

Number of titrations	Number of replicates	Mean IFN titer (units/ml ± SD)	
		50% Reduction	25% Reduction
48	0	4.32 ± 0.53	1.98 ± 0.40
24	2	4.33 ± 0.41	1.97 ± 0.26
16	3	4.31 ± 0.30	1.96 ± 0.17
8	6	4.30 ± 0.24	1.98 ± 0.085

Note. The cells were incubated with serial dilutions of IFN-α for 24 hr at 37°C, infected with HSV-1 at a m.o.i. of 0.01 and further incubated for 18 hr at 39°C. The concentrations of IFN, reducing the absorbance to 50% and 25%, respectively, were calculated with reference to the number of replicate wells inoculated with each dilution of IFN.

human and simian cell lines was tested. The most sensitive assay system for all three types of IFN was obtained with A549 cells and VSV as challenge virus.

The antiviral activity of IFN measured by BIA in this cell–virus system was highly dependent on the m.o.i. and temperature of incubation as demonstrated in Fig. 4. In this experiment the interferon-treated cells were infected with VSV at three different m.o.i. (10, 1, and 0.1) and subsequently incubated for 13 hr at either 37 or 40°C. The results presented in Table II shows that a 100-fold reduction of m.o.i. increased the sensitivity about 10-fold. A further 10-fold increase of the sensitivity of the assay was obtained if VSV was incubated at 40°C instead of 37°C. On the other hand, minor or no differences in sensitivity were obtained when the treatment of cells with interferon was performed at 40°C instead of 37°C. The results in Fig. 4 and Table II also show that this type of BIA with A549 cells infected with VSV at a m.o.i. of 0.1 and incubated at 40°C for 13 hr permits the detection of 0.1 units of IFN, when the detection limit is chosen as the amount of IFN giving a 50% reduction of absorbance.

Assay of IFN in A549 cells by measurement of β_2-microglobulin. All three types of IFN could be assayed in A549 cells by the demonstration of an increased concentration or activity of β_2-microglobulin on the surface of the cells. Figure 5 shows an experiment, in which the cells were incubated at 37°C for 24 hr with serial twofold dilutions of IFN-α. Six replicate cultures were used for each dilution of IFN. About 4 units of IFN could be detected

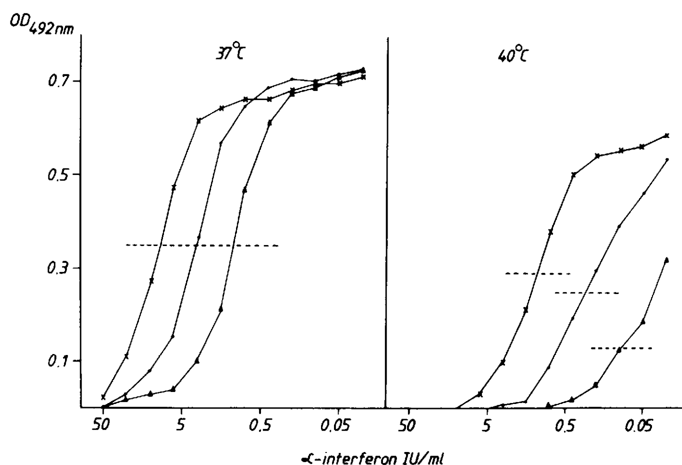

FIG. 4. Titration of IFN-α with BIA in A549 cells, exposed to VSV at different m.o.i. and incubated at different temperatures. The cells were first incubated overnight at 37°C with IFN and subsequently infected with VSV at a m.o.i. of 10 (×), 1.0 (○), and 0.1 (△), respectively. Infected cultures were either incubated at 37°C (left panel) or 40°C (right panel). Each point represents the mean absorbance of duplicate assays, and the measured OD values are corrected by subtraction of the background activity of uninfected controls. The dashed lines indicate 50% reduction of absorbance, compared to the virus-infected controls, not treated with IFN.

TABLE II. INFLUENCE OF INCUBATION TEMPERATURES AND MULTIPLICITY OF INFECTION ON THE SENSITIVITY OF BIA PERFORMED WITH A549 CELLS AND VSV

Incubation temperature for IFN (°C)	Multiplicity of infection	Detection limit (IFN units/ml[a]) in cultures incubated with VSV for 13 hr	
		37°C	40°C
37	10	15	2.0
37	1	4.7	0.24
37	0.1	1.4	0.043
40	10	0.5	1.2
40	1	3.2	0.25
40	0.1	1.1	0.10

[a] The IFN titer was calculated as the concentration of IFN-α which, in comparison to controls with medium, reduced the absorbance in infected cultures with 50%.

with this type of BIA. The results seemed to indicate that exposure of the cells to 500 units of IFN evoked an approximately twofold increase of the antigenic amount of β_2-microglobulin on the cells. The change of the cellular surface which could be recorded by this BIA developed relatively slowly after incubation with IFN. No change could be observed before 6 hr, and maximal increase of the absorbance was found after more than 24 hr of incubation.

Discussion. The standard procedures used for assay of IFN have previously been based on determinations of the antiviral effect, usually measured by reduction of plaque formation, inhibition of virus yield, or cytopathic effect (9). Several types of modifications of these bioassays have been described. The viral cytopathogenicity may be determined by microscopic examination or by dye-uptake methods (18, 19) and the inhibition of viral replication may be assessed, e.g., by measuring the yield of infectious virus (20), viral hemagglutinin (21), or neuraminidase (22). One of the BIAs described in this report is also based on determination of the antiviral activity of IFN and utilizes as a first step, titration of IFN in microtiter plates similarly as described by Dahl and Degree (23) and Havell and Vilcek (24). In order to improve the test we have employed as a second step an enzyme immunoassay, which measures viral replication by the demonstration of viral antigens on the cell surface. With this combination of methods, a highly sensitive and reproducible assay of IFN is obtained. Thus, when triplicate determinations were performed, the coefficient of variation was consistently below 10%. After optimization of various parameters the BIA allowed the detection of 0.1 units of IFN/ml, if the titer was calculated as the dose giving 50% reduction of absorbance values. Because of the high reproducibility of the test a 20%

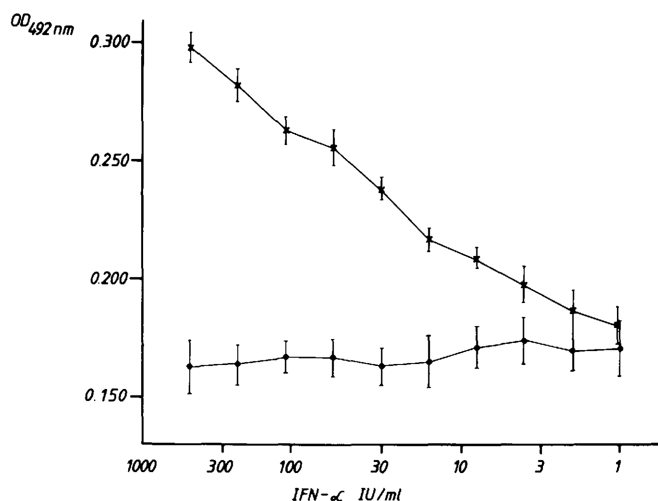

FIG. 5. Assay of IFN-α in A549 cells by demonstration of increased concentration of β_2-microglobulin on fixed cells with EIA. The results show mean values and SEM obtained from cultures in two microtiter plates, where each dilution of IFN was inoculated into six wells and incubated for 24 hr at 37° (×). A corresponding number of wells were incubated with medium only (O).

reduction of absorbance could be used as a detection limit, indicating that it is possible to detect even lower amounts of IFN.

The BIA described in this report seems to have several advantages over conventional techniques used for assay of the antiviral activity of IFN. Because of its sensitivity and reproducibility the test allows exact comparisons between different IFN preparations and the assay should permit determination of small quantities of IFN, e.g., in serum, liquor cerebrospinalis, and other body fluids. Furthermore, the test is easy to perform and the use of microplates facilitates washing procedures and automatization of other procedures, as reading and calculation of titers. A technical detail which may be favorable is that the immunoassay can be performed on microplates stored at −70°C.

The use of enzyme- and radioimmunoassays for measurement of the antiviral activity of IFN have recently been described by Julkunen *et al.* (25), Salonen and Salmi (26), and Lyons *et al.* (27). The enzyme immunoassays were more sensitive and had a detection limit of 0.25 to 1.0 units of IFN-α. When the same cell–virus system was used the enzyme immunoassays were found to be two- to eightfold more sensitive than the standard methods based on inhibition of cytopathic effect and plaque reduction. (25, 26). These types of assays therefore seems to provide useful tools for studying the role of IFN in virus infections and immunological diseases. We have used the BIA for assaying IFN both in supernatants from leukocyte cell cultures and in sera from patients with systemic lupus erythematosus (4) and immunodeficiency diseases.

IFN is a heterogenous group of proteins and may exhibit different types of actions on different cells. An example of this was recently described by Wallach *et al.* (13) who showed that the synthesis of HLA antigens is much more stimulated by IFN-γ than by IFN-α and IFNβ. For studies of the physiological and pathological role of IFN it may therefore be important to have access to methods that detects other effects of IFN than the antiviral activity. In this report we describe a BIA, which is based on the demonstration of β_2-microglobulin on the cell surface. This interferon assay is rapid and very simple to perform and the sensitivity is high enough to permit detection of about 4 units of IFN. It seems probable that this BIA can be applied for studies of cell membrane alterations, e.g., in tumor and immunological diseases.

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