Enzyme Immunoassay of Interferon with Peroxidase-labelled Virus-specific Monoclonal Antibodies

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SUMMARY

To quantify the antiviral effect of interferon (IFN) we applied a mixture of two horseradish peroxidase-labelled monoclonal antibodies, specific for the E1 glycoprotein of Semliki Forest virus, in a direct enzyme immunoassay. This assay is suitable for detection of virus replication in L-cells, seeded as monolayers in 96-well plates. Inhibition of absorbance values caused by IFN was determined in a Flow Titertek Multiskan. Three IFN samples from different sources were titrated simultaneously in the enzyme immunoassay and in the vesicular stomatitis virus plaque reduction test in five consecutive experiments. Titres were calculated as the inverse value of the dilution of IFN causing 25% inhibition of absorbance values and 50% reduction of plaque counts respectively. The results show equality of precision and reproducibility between and within the two assays. However, the enzyme immunoassay is more convenient and objective than the plaque reduction assay.

Many methods for assessing the antiviral effects of interferon (IFN) have been described (Wagner, 1961; Isaacs, 1963; Finter, 1969; Jameson et al., 1977; McNeill, 1981) based on the reduction of virus effects or of virus yields. The latter can be measured by classical virological methods like plaque reduction or more recently with rather elaborate immunoassays using conventional antisera against vesicular stomatitis virus (VSV) or its proteins (Wallach, 1983; Shoham et al., 1984).

Monoclonal antibodies (MA) with specificity for the glycoproteins of Semliki Forest virus (SFV) proved useful for the detection of SFV in cell culture by enzyme immunoassay (Van Tiel et al., 1984). This assay was adapted for assessing IFN and compared well with the VSV plaque reduction method.

The avirulent strain MRS MP 192/7 of SFV (Henderson et al., 1970) was inoculated at a m.o.i. of about 1 onto monolayers of L-cells in Roux flasks. After 20 h at 37 °C and centrifugation at low speed the supernatant with $2 \times 10^9$ p.f.u./ml was stored in small portions at $-70^\circ$C. Further virological methods have been described previously (Kraaijeveld et al., 1979). L-cells, a continuous line of mouse fibroblasts, grown in Dulbecco’s minimal essential medium (DMEM) with 0.01 M-HEPES buffer, supplemented with 10% calf serum and antibiotics, were used throughout. The same medium with 5% calf serum was used for diluting virus or cells.

The production, purification and characterization of MA against the E1 and E2 glycoproteins of SFV have been described elsewhere (Boere et al., 1983, 1984). For this study purified MA 8.139 (IgG2a, neutralizing, protective, but without haemagglutination inhibition activity) and MA 8.64 (IgG1, inhibiting haemagglutination but non-neutralizing or protective) both directed against E1 glycoprotein but not competing with each other were used in a mixture, since this has been shown to be better for antigen detection than a single MA (Van Tiel et al., 1984). Both MA were conjugated to horseradish peroxidase (HRPO) by the periodate method (Nakane &
Kawaoi, 1974). The two freshly prepared conjugates were added in volumes of 0.1 ml to 4.8 ml of phosphate-buffered saline (PBS) pH 7.2, and the mixture was passed through a 0.45 μm membrane filter (Schleicher & Schüll). The bacteria-free conjugate, diluted 1:50, was stored in 0.5 ml volumes in sterile siliconized glass tubes at 4 °C. Immediately before use the conjugate was diluted 1:5000 in PBS. No loss of activity was observed over a 6 month period.

Three sources of IFN were used: firstly, cell culture IFN induced in L-cells by u.v.-inactivated Newcastle disease virus (NDV); secondly, mouse serum IFN induced by intraperitoneal injection of BALB/c mice with poly(rI)-poly(rC); thirdly, samples of a standard batch of mouse IFN kindly provided by the NIH (code no. G-002-904-511). The IFN-containing fluids were stored in small portions at -20 °C. Before use IFN-containing samples and control samples (normal mouse serum and tissue culture fluid from uninfected cells) were diluted with DMEM.

IFN activity was determined by a modified VSV plaque reduction test (Kraaijeveld et al., 1982). The sample dilution causing 50% plaque reduction was found by interpolation. The IFN titre is the inverse value of this dilution.

Growth curves of SFV in cell culture determined by direct enzyme immunoassay with these labelled MA have been described (Van Tiel et al., 1984). We adapted this assay to quantify the antiviral effect of IFN by measuring the inhibition of virus replication. The immunoassay was performed in 96-well plates (cat. no. 3596, Costar). Into each well, 5 × 10^4 L-cells (0.05 ml) were seeded out to form monolayers. The cells were incubated overnight at 37 °C in DMEM supplemented with 10% heat-inactivated calf serum. After the supernatant was discarded, the monolayers of L-cells were incubated with 0.1 ml of 10^-3, 10^-3.5, 10^-4 and 10^-4.5 dilutions (in quadruplicate) of IFN, using four wells for each dilution. Control wells received 0.1 ml DMEM.

After 24 h at 37 °C 5000 p.f.u. (m.o.i. 0.10) or 15 000 p.f.u. (m.o.i. 0.33) of SFV in 0.025 ml was added to each well, and the plates were incubated at 37 °C. Infected and uninfected cells without preincubation with IFN served as controls. After 4, 10, 16 and 22 h the supernatants were discarded and the monolayers fixed with 0.05 ml 0.05% glutaraldehyde (Merck) for 10 min at 37 °C. The plates were then washed carefully with tap water, rinsed with PBS pH 7.2 and shaken dry. The mixture of labelled MA was added in portions of 0.05 ml/well and the plates were incubated at 37 °C for 1 h, washed three times with PBS and shaken dry. The amount of bound HRPO was visualized by incubation with 0.05 ml of substrate solution for 30 min at room temperature. The substrate solution was prepared by adding 0.2 ml of a stock solution of 60 mg 3,3',5,5'-tetramethylbenzidine (TMB; Sigma) in 10 ml DMSO and 0.1 ml of a stock solution consisting of one tablet of urea peroxide (UP; Organon Teknika, Boxtel, The Netherlands) dissolved in 7.5 ml distilled water to 12 ml 0.11 M sodium acetate/citric acid buffer pH 5.5. After 30 min at room temperature the reaction was stopped by adding 0.2 ml of 1 M H₂SO₄ per well. Peroxidase activity was quantified by measuring the A₄₅₀ with a Titertek Multiskan photometer (Flow Laboratories). All extinction values shown are the means of quadruplicates. The IFN titre was defined as the inverse value of the dilution of IFN causing 25% reduction of absorbance values measured on infected control cells without IFN.

In Fig. 1 the results are given as growth curves of the mean absorbance values (n = 4) for virus controls, IFN-preincubated wells and cell controls. At 4 h after infection the absorbance values were below 0.100, like those for uninfected cells, showing that virus replication was still negligible, as observed before (Van Tiel et al., 1984). Six h later the absorbance of virus controls had increased considerably, especially at the higher m.o.i. The inhibition by IFN was already obvious and clearly related to the amount of IFN added. At a 10^-4 dilution of IFN, the inhibition persisted until at least 16 h after infection. The test was highly reproducible as shown by the small standard deviation of the mean absorbance values. Standard IFN still showed about 20% inhibition at 16 h in a 10^-4 dilution at the lower m.o.i.

Samples of the international standard IFN (NIH), NDV-induced L-cell IFN and poly(rI)-poly(rC)-induced mouse IFN were simultaneously titrated in the enzyme immunoassay and VSV plaque reduction test (PRT) in five consecutive experiments. For each of these tests, trypsinized L-cells from the same Roux flask were seeded out at the same time in 96-well plates. Furthermore, the two fold dilutions of IFN used were identical and simultaneously pipetted onto the monolayers. Also, the cells were infected at the same time with either VSV (40 p.f.u./well) or
Fig. 1. Titration of IFN after various times of infection with SFV. Cell control wells (●) contained neither IFN nor virus. Virus control wells (■) contained only virus, added in 0.025 ml portions at a m.o.i. of either 0.10 (a) or 0.33 (b). International standard interferon was added in 0.1 ml portions of dilutions 10^{-3} (●), 10^{-3.5} (□), 10^{-4} (▲) and 10^{-4.5} (∆).

Fig. 2. Titration of IFN by enzyme immunoassay and by plaque reduction test. International standard IFN (a), NDV-induced L-cell IFN (b) and poly(rI)-poly(rC)-induced mouse IFN (c) were titrated simultaneously by enzyme immunoassay and plaque reduction assay in five consecutive tests. Points plotted are the means of fourfold observations in the first test, representing absorbance values at 450 nm (—) and % plaques of virus control plaque count (—) respectively. Titration curves of both tests are presented for international standard IFN (●), NDV-induced L-cell IFN (□), control L cell supernatant (■) and poly(rI)-poly(rC)-induced mouse IFN (▲), control normal mouse serum (●), with their virus control (☆) and cell control (★). Vertical bars represent the standard deviation of the mean of fourfold results.

SFV (5000 p.f.u./well). After 22 h the VSV plaques were developed with neutral red. In the enzyme immunoassay the cells were fixed at 22 h with glutaraldehyde and the cells were tested with the HRPO-labelled MA. The results of experiment no. 1 are presented in Fig. 2. It can be seen that the slope of the VSV PRT curve was somewhat steeper than that of the enzyme
immunoassay, but with the latter test the standard deviations of the mean were generally smaller; this fact allows an arbitrary definition of the enzyme immunoassay titre (EIA$_{25}$) as the inverse value of the dilution of IFN associated with a 25% reduction of the absorbance value measured on infected control cells without IFN. The mean titres, VSV-PRT$_{50}$ ± s.d. and EIA$_{25}$ ± s.d., of five consecutive experiments for international standard IFN were 3694 ± 1036 and 2753 ± 1363 respectively. Simultaneously performed experiments for NDV-induced L-cell IFN and poly(rI): poly(rC)-induced mouse serum IFN gave similar results. These results indicate that both tests are comparable with regard to sensitivity, reproducibility and reliability.

The correspondence of mean titres for both tests and their small standard deviations indicate that the enzyme immunoassay is as accurate and reproducible as the VSV plaque reduction test. It can detect inhibition by IFN at 10 h after infection, is easier to perform, less prone to subjective reading than other IFN assays and less time-consuming than the plaque reduction test. This enzyme immunoassay seems especially suited for IFN titration of large numbers of samples and may be adapted to the assay of human IFN with suitable virus–cell systems.

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REFERENCES


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