Interferon Bioassay: Reduction in Yield of Myxovirus Neuraminidases

By J. J. SEDMAK AND S. E. GROSSBERG

Department of Microbiology, The Medical College of Wisconsin, Milwaukee, Wisconsin 53233, U.S.A.

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SUMMARY

Interferon reduced the production of virus neuraminidase during single-cycle growth of A2/HK/1/68 or recombinant X7(F1) influenza viruses in chicken embryo cell cultures. Neuraminidase activity, measurable within 6 h after infection, was reduced as much as 70 to 80% below control levels in interferon-treated cultures. X7(F1) neuraminidase yield was at least as sensitive a measure of interferon inhibition as vesicular stomatitis virus in the standard plaque-reduction assay. Interferon titre, expressed as the 30% neuraminidase reduction dose or NRD30, is derived from semi-logarithmic sigmoidal dose–response curves. The advantages that recommend the neuraminidase reduction bioassay for interferon assay include the precision of the enzyme assay (±1%o), the precision of measurement of replicate samples of interferon (±9%), the reproducibility of interferon titres obtained sequentially (±20%), as well as the rapidity and economy of the method.

INTRODUCTION

Neuraminidase (N-acetylneuraminic-acid-lactohydrolase, E.C. 3.2.1.18) is one of the important surface components of viruses of the myxovirus and paramyxovirus groups. This virus-specific enzyme can be readily detected in cellular plasma membranes within hours after infection (Kendal & Apostolov, 1970). Uninfected chicken embryo cell cultures, highly susceptible to infection with certain myxoviruses, have no detectable neuraminidase activity (Lipkind & Tsvetkova, 1967). The assay of the production of this influenza virus enzyme in cultured chicken embryo cells provides a highly precise, rapid, simple, and economical means to measure interferon action. To establish the validity of this bioassay of interferon, certain characteristics of the virus enzyme and several biological parameters were studied.

METHODS

Cell cultures. Primary chicken embryo cell cultures (CEC) were prepared from 10-day-old White Leghorn embryos. The cells were dispersed during a 3 h incubation at room temperature with 0.2% trypsin in complete Hanks' balanced salt solution (HBSS) without antibiotics, filtered through a funnel with four layers of gauze, and washed twice with complete HBSS containing 5% calf serum. Two ml of the cell suspension, containing 2.5 to 3.5 × 10⁶ cells/ml of growth medium, were dispensed on to a 12.5 cm² surface in soft-glass bottles and incubated at 36°C. Growth medium consisted of 5% heat-inactivated calf serum, 0.1% yeastolate (Difco), penicillin (100 units/ml), streptomycin (100 µg/ml), and mycostatin (50 units/ml) in HBSS.
Viruses. A2/Hong Kong/1/68 (H3N2) was obtained from M. T. Coleman, Center for Disease Control, Atlanta, Georgia. The X7(F1), or A0/NWS/37(HO)-A2/RI/5+/57(FI)(N2), recombinant (Kilbourne et al. 1967) was provided by E. Kilbourne, Mt. Sinai School of Medicine, New York, N.Y. Both viruses were prepared as allantoic fluid suspensions. Virus potency was determined by plaque titration in hamster kidney cell cultures (Grossberg, 1964) as well as by measurement of haemagglutinin and neuraminidase. Vesicular stomatitis virus (VSV) was prepared and titrated as previously described (Morahan & Grossberg, 1970a).

Interferon preparation. Interferon was prepared from the allantoic fluid of A0/WS/33 influenza virus-inoculated chicken embryos and partially purified as previously described (Morahan & Grossberg, 1970b).

Neuraminidase assay. Neuraminidase was assayed by the thiobarbituric acid (TBA) method of Warren (1959) as modified by Webster & Pereira (1968). Fetuin (Grand Island Biological Company, Grand Island, N.Y.), 6 mg/ml, was used as enzyme substrate without further purification, and mixtures of twice-frozen-and-thawed cultured cells and supernatant fluids were the source of virus enzyme. The double cycle of freezing and thawing was necessary to release cytoplasmic and membrane-bound neuraminidase. The quantity of enzyme used was 0.1 ml either of the culture mixture or of a dilution that would give an \( E_{550} \) of 0.5 to 0.7 in 1 h. One unit of enzyme activity is defined as the amount of enzyme necessary to release one n-mol of \( N \)-acetylneuraminic acid (NANA) from fetuin substrate per h, at 37 °C. The TBA derivative of the product of neuraminidase activity had an absorption spectrum identical to that of the TBA derivative of 99 % pure NANA (General Biochemicals, Chagrin Falls, Ohio).

Haemagglutination. The fluids from twice-frozen-and-thawed cultures were titrated at 4 °C for yield of haemagglutinin (H.A.) with 0.5 % washed human 'O' erythrocytes. The culture fluids and erythrocytes were diluted in phosphate-buffered saline (pH 7.1) containing 0.1 % gelatin. A unit of haemagglutinin is defined as the highest dilution of virus showing agglutination.

Interferon inhibition of yields of neuraminidase, haemagglutinin and virus. Growth medium was decanted from confluent CEC, and cultures were overlaid with 1.0 ml of either GLM (1 % gelatin, 0.5 % lactalbumin hydrolysate, penicillin, streptomycin, and mycostatin in Scherer's maintenance medium in which parenamine was replaced by its constituent amino acids), (Microbiological Associates, Inc., Bethesda, Maryland) or interferon diluted in GLM. After 16 to 18 h the supernatant fluid was decanted, the CEC washed twice with 1.0 ml of GLM and then infected with 0.1 ml of virus diluted in GLB (0.5 % gelatin and 0.25 % lactalbumin hydrolysate in complete HBSS) to give an input multiplicity of between 1 and 10 p.f.u./cell. Virus was allowed to attach to gently oscillating cultures for 1 h at 36 °C. Infected cells were then washed three times with GLM and overlaid with 0.1 ml of GLM for incubation at 36 °C in closed bottles. The cell cultures were harvested at intervals and frozen at −70 °C. After thawing in a 37 °C water bath, the cultures were subjected to a second freeze–thaw cycle before determination of neuraminidase activity, H.A., and p.f.u. (Grossberg, 1964). Interferon titres were expressed as the 30 % neuraminidase reduction dose (NRD30) (see Results).

Plaque-reduction assay for interferon. The assay determined the dilution of interferon which reduced the number of plaques formed by VSV on 10-day chicken embryo cell cultures (Wagner, 1960). Titres are expressed either as median plaque-depressing dose (PDD50) or as the 27 % plaque development or survival dose (PSD27) (Jordan, 1972).
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![Graphs and diagrams](image)

Fig. 1. Enzyme kinetics of A₂/Hong Kong/1/68 neuraminidase. (a) Dependence of the rate of the reaction upon fetuin concentration. (b) Lineweaver & Burk plot of data given in (a). (c, d) The dependence of the rate of the enzyme reaction upon the enzyme concentration.

**RESULTS**

**Kinetics of myxovirus neuraminidase**

The assay of neuraminidase was validated in the following experiments with the A₂/HK/1/68 enzyme and commercial fetuin as substrate. Sufficient enzyme was used to release 40 n-mol of NANA in 50 min as measured with the standard assay. The rate of formation of free NANA from fetuin exhibited saturation kinetics with a Michaelis constant of 2.6 mg/ml (Fig. 1 (a) and 1 (b)). The rate of formation of NANA with non-limiting substrate was linear up to 60 min at the concentrations of enzymes tested (Fig. 1 (c)) and the rate of formation of NANA was linearly dependent on the enzyme concentration (Fig. 1 (d)). Similar kinetics were obtained for the X₇(F₁) enzyme with a Michaelis constant of 5.1 mg/ml.

**Kinetics of virus growth and inhibition by interferon**

A₂/HK/1/68 and X₇(F₁) were selected as most suitable from a number of myxoviruses tested for neuraminidase production and sensitivity to interferon. Duplicate cultures treated for 18 h with either maintenance medium or 200 PDD₉₀ of interferon were assayed for neuraminidase and H.A. at intervals after A₂/HK/1/68 infection. An appreciable increase of neuraminidase in control cultures was detected 6 h after infection (Fig. 2(a)). Interferon treatment reduced neuraminidase activity by 75% at 6 h and by 60% at 24 h. The results of a similar experiment with X₇(F₁) shown in Fig. 2(b) correlate the depression by interferon of X₇(F₁) neuraminidase, H.A., and p.f.u. X₇(F₁) neuraminidase production in cul-
Fig. 2. (a) Effect of interferon on kinetics of production of A₂/HK/1/68 neuraminidase and haemagglutinin. (b) Effect of interferon on kinetics of formation of X₇(F₃) neuraminidase, haemagglutinin, and virus particles. Haemagglutinin titre: ○—○, interferon absent; •••, interferon present. Neuraminidase titre: △—△, interferon absent; ▲—▲, interferon present. Infectivity (p.f.u.) titre: ◻, interferon absent; ☻, interferon present.

Cultures treated with 200 PDD₉₀ of interferon was inhibited 72% at 6 h and 80% at 24 h after challenge. Whereas the ratio of total (free and cell-associated) neuraminidase to HA production in CEC for A₂/HK/1/68 virus was 2, the ratio for X₇(F₃) was 10; these ratios did not significantly change as a result of inhibition by interferon.

Development of antivirus resistance as a function of time after addition of interferon

Pre-treatment with 100 PDD₉₀ interferon for a minimum of about 6 h was necessary for the development of maximum resistance to challenge with either virus. However, when 100 PDD₉₀ units of interferon were added simultaneously with the A₂/HK/1/68 virus and neither subsequently removed, the same degree of reduction of neuraminidase yield (59%) was observed as in cultures pretreated for 18 h.

Dose–response relationships: interferon concentrations and neuraminidase production

Neuraminidase activity was measured 6 h after infection in sextuplicate cultures treated with four dilutions of interferon before challenge with A₂/HK/1/68 (input multiplicity of 1.0 p.f.u./cell). The results in Table 1 illustrate the high precision of graded antivirus
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Table 1. Effect of interferon pre-treatment of chicken embryo cell cultures on neuraminidase yield 6 h after infection with A2/HK/1/68

<table>
<thead>
<tr>
<th>Reciprocal of interferon dilution</th>
<th>Mean extinction*</th>
<th>Standard deviation of extinction (or as % of mean)</th>
<th>Neuraminidase units</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.196</td>
<td>0.006 (3%)</td>
<td>146</td>
<td>70-8</td>
</tr>
<tr>
<td>32</td>
<td>0.260</td>
<td>0.048 (18%)</td>
<td>195</td>
<td>60-8</td>
</tr>
<tr>
<td>128</td>
<td>0.461</td>
<td>0.025 (6%)</td>
<td>345</td>
<td>30-8</td>
</tr>
<tr>
<td>1024</td>
<td>0.639</td>
<td>0.068 (11%)</td>
<td>478</td>
<td>4-0</td>
</tr>
<tr>
<td>No interferon†</td>
<td>0.665</td>
<td>0.034 (5%)</td>
<td>498</td>
<td>0-0</td>
</tr>
</tbody>
</table>

* Mean of six samples.
† E<sub>550</sub> of other controls: uninfected cultures, 0.040; infected cultures with no fetuin, 0.050; NANA (30 n-mol), 0.440; and NANA (30 n-mol) plus infected cell extract, 0.430.

Fig. 3. Relationship between interferon concentration and neuraminidase production. Cultures were treated with interferon for 18 h before challenge with A2/HK/1/68. The mean (●) and standard deviation (vertical bars) of neuraminidase activity of four replicate cultures are shown at each dilution of interferon.

The plot of data (Fig. 3) as the percentage of control neuraminidase activity against the logarithm of the reciprocal of the interferon dilution gives a sigmoidal dose–response curve with a rectilinear region extending from 40 to 85% of control activity, corresponding to a reduction of 15 to 50%. The 30% neuraminidase reduction end-point (NRD<sub>30</sub>) was measured in relation to interferon concentration. The standard deviation of the samples ranged from 3% of the mean value at one dilution of interferon to 18% at another. Higher input multiplicity increased neuraminidase production but did not alter the graded response to interferon. The presence of 2-deoxyribose is believed to account for the small background E<sub>550</sub> in uninfected controls and in infected cell extracts without fetuin substrate. The extinction was essentially the same for a standard preparation of NANA as for NANA incubated in the presence of infected cell extract for 1 h. These observations suggest that NANA aldolase or other interfering enzymes were not present in the cell extracts.

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selected because it fell near the midpoint of the linear portion of the majority of dose–response curves. The sigmoidal dose–response curves obtained are typical of other plots of interferon titrations (Gifford & Koch, 1969; Hallum, Thacore & Youngner, 1970; Jordan, 1972).

Interferon titres obtained in six sequential titrations were highly reproducible in cultures harvested 6 or 24 h after infection. The rectilinear portions of all dose–response curves were parallel. One preparation assayed with X7(F1) as challenge and tested 24 h after infection had a mean titre of 100 with a standard deviation of 19.5 %. Such reproducibility compares well to a highly reproducible method based on the incorporation of radioactively labelled uridine into virus RNA (Allen & Giron, 1970; Koblet, Kohler & Wyler, 1972).

Another interferon preparation measured at 6 h had a mean titre of 175 with a standard deviation of 25 % of the mean when A2/HK/1/68 was used as a challenge; titres measured at 24 h were consistently lower (mean NRD30 of 49) with a standard deviation of 25 %. Thus, the apparent titre of interferon, reflecting sensitivity of a given virus to interferon, diminished during a single cycle of virus growth of A2/HK/1/68 but not of X7(F1) virus. Whether an inhibitor of interferon (Ghendon, 1965; Grossberg & Morahan, 1971) is induced during the replication of A2/HK/1/68 virus remains undetermined.

Comparative sensitivity of neuraminidase yield-reduction test

When the potency of an interferon preparation (81771) was measured simultaneously by the X7(F1) neuraminidase-reduction and VSV plaque-reduction methods, the NRD30 titres were at least equivalent to the 50 % VSV plaque-reduction titre. If the plaque-reduction titre is instead calculated as a 73 % reduction, or PSD27, as recently recommended (Jordan, 1972), the X7(F1) NRD30 titre (1300) is threefold higher than the VSV plaque-reduction titre (450). The NRD30 titre of the Medical Research Council interferon standard (67/18) was 100, slightly higher than the 79 units assigned to it. The bioassay is thus suitably sensitive.

DISCUSSION

Myxoviruses are generally considered to be sensitive to interferon action. Reduction in the yield of influenza virus haemagglutinin was used for the bioassay of interferon in the earliest studies of Isaacs & Lindenmann (1957). In the studies reported here neuraminidase characteristically paralleled haemagglutinin production, and interferon reduced the yield of both structural components as well as infectious virus. The high ratio of total neuraminidase to haemagglutinin observed after infection of chicken embryo cells with X7(F1) virus and the sensitivity of this recombinant to interferon action recommend its use in the neuraminidase bioassay.

This single-cycle, yield-reduction method may overcome the several disadvantages of current interferon bioassays (Grossberg, 1972) in precision, reproducibility, and the relatively brief period of virus growth needed prior to assay of the enzyme. The method is also economical, requiring inexpensive glycoprotein substrates and relatively simple equipment, features which have made it possible to automate the neuraminidase assay (Kendal & Madeley, 1969), providing a means to measure numerous interferon preparations.

The extension of this technique to other cultured cells requires the demonstration of low background levels of cellular neuraminidase, since such enzymes are known to occur normally in certain tissues (Lipkind & Tsvetkova, 1967). We have recently been able to utilize this interferon bioassay in a variety of mammalian cell cultures, including human cells.
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REFERENCES


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