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The Neuraminidase Yield-Reduction Bioassay of Human and Other Interferons[1] (38822)

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The measure of reduction in yield of viral neuraminidase (Nase, EC 3.2.1.18) in chicken embryo fibroblast cell cultures infected with the recombinant influenza virus X7(F_1)(HON2) is a sensitive and reproducible bioassay for chicken interferon (1). The present investigation extends this bioassay to a number of other cell culture systems including human cell lines and strains. The number and varieties of interferon bioassays already available suggest a lack of satisfaction with current methods (2). A more precise and reproducible interferon bioassay than other methods is based on measurement of yields of the myxoviral enzyme neuraminidase (1). In addition, the application of a synthetic neuraminidase substrate (methyoxyphenyl-neuraminic acid) (3) greatly simplifies the neuraminidase yield-reduction assay of interferon obviating the time-consuming oxidation, heating, and solvent extraction steps of the commonly used thiobarbituric acid assay of neuraminic acid.

Materials and Methods. Cell cultures. The cell cultures, their origin, and the methods employed in their handling are described in Table I. For interferon bioassay, cells were cultured in upright 16 × 125-mm glass test tubes.

Viruses. The recombinant influenza virus X7(F_1) and vesicular stomatitis virus (VSV), Indiana strain, were prepared as previously described (1). Theiler's GDVII virus was obtained from Dr. Baron, National Institutes of Health, Bethesda, MD, and propagated in BHK-21 cells. Encephalomyocarditis virus (EMC) obtained from W. F. Scherer, University of Minnesota, was prepared from mouse L cell supernatant fluid.

Interferons. Human interferon from human leukocyte cultures induced with Sendai virus was provided by Dr. Cantell, Central Public Health Laboratory, Helsinki, Finland. Murine interferon was prepared from mouse L_{929} cell cultures or serum of mice inoculated with Newcastle disease virus (NDV), CG strain. Chicken interferon was induced *in ovo* with A/WS/33 (HON1) influenza virus and partially purified as previously described (4). Hamster interferon was prepared from brains of hamsters injected intracranially with West Nile virus; residual virus was inactivated by dialysis against pH 2 buffer for 24 hr.

Interferon bioassays. The GDVII hemagglutinin (HA) yield-reduction bioassay was performed as described by Oie *et al.* (5). The VSV plaque-reduction method was that described by Wagner (6). The EMC hemagglutinin yield-reduction technique was performed according to the method of Jameson *et al.* (unpublished studies).

The technique of the neuraminidase yield-reduction bioassay depended on the method of enzyme assay. (a) When fetuin was to be used as neuraminidase substrate, cells were treated with interferon as in the EMC HA yield-reduction assay. Attachment of X7(F_1) virus was allowed to occur for 60 min at 36°C with 0.25 ml of virus (diluted in medium) at a multiplicity of 5–10 based on plaque-forming units in primary hamster kidney cell cultures (7). Frozen and thawed extracts were assayed for neuraminidase by the thiobarbituric acid assay using fetuin as substrate (1). One unit is defined as 1 nmole of product released in 1 hr at 37°C. (b) When methoxyphenylneuraminic acid (MPN) (provided by the National Institutes of Health) was to be used as enzyme substrate, cultured cells in disposable tubes were treated with interferon and infected with X7(F_1) as above. After overnight incubation at 36°C, the medium was decanted, the cells washed three times with 1.0 ml of phosphate-buffered saline, pH 7.2, and then 0.2 ml of 2.5×10^{-3} *M* MPN in 0.1 *M* sodium phosphate buffer, pH

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TABLE I. CELL CULTURES.

Name of cell	Source	Growth medium	Maintenance medium
Human foreskin 7 strain	Dr. B. Brown	BME, 10% fetal bovine serum	MEM (Earle's), 2% fetal bovine serum
BUD-8 human skin strain	Dr. S. Baron	MEM (Earle's) with nonessential amino acids, 10% fetal bovine serum	MEM (Earle's) with 2% fetal bovine serum
Embryo kidney human strain	Abbott Laboratories	BME containing 200 mg/100 ml glucose, 10% fetal bovine serum	BME with 2% fetal bovine serum
Chang conjunctiva human line	Dr. E. Kilbourne	Medium 199 with 10% calf serum	Waymouth's with 1% gelatin or MEM (Earle's) with 2% fetal bovine serum
FL human amnion line	Dr. B. Brown	Medium 199 with 10% fetal bovine serum	Waymouth's with 1% gelatin or MEM (Earle's) with 2% fetal bovine serum
African green monkey kidney line	Microbiological Associates	—	BME with 0.5% calf serum
RK-13 rabbit line	Flow Laboratory	MEM (Earle's) containing nonessential amino acids, 10% fetal bovine serum	MEM (Earle's) with 2% fetal bovine serum
Hamster kidney primary[a]	—	Hanks' salt solution, 15% calf serum, 0.5% lactalbumin hydrolysate	Hanks' salt solution, 0.5% lactalbumin hydrolysate, 1% gelatin, 0.15% yeast extract
L_{929} mouse line	Microbiological Associates	MEM with 10% fetal bovine serum	MEM (Hanks') with 2% fetal bovine serum
Chicken embryo[b] primary	—	Hanks' salt solution, 0.1% yeast extract, 5% calf serum	Hanks' salt solution, 0.25% lactalbumin hydrolysate, 0.5% gelatin

[a] Prepared as described (7).
[b] Prepared as described (1).
Abbreviations: MEM = minimal essential medium of Eagle; BME = basal medium of Eagle.

5.9, added directly onto the cells. Tubes were incubated at 37°C long enough to obtain an absorbancy at 580 nm of 0.4–0.6 for virus-infected cultures not receiving interferon. The enzyme reaction was stopped by immersion of the tubes in an ice-bath, 0.2 ml of the diazonium salt of 4-amino-2,5-dimethoxy-4′-nitroazobenzene (prepared as a solution of 6.0 mg/ml in 0.4 *M* sodium phosphate at pH 7.0) was added, and the mixtures kept at 0°C for 15–20 min for development of the chromagen. The chromagen was solubilized by the addition of 4.0 ml of 0.5 *N* NaOH. After vortex mixing the absorbance was determined at 580 nm in reference to an MPN reagent blank. The absorbancy can be determined directly in the culture tube, if an appropriate adaptor for the spectrophotometer is available, or after transfer into cuvettes. The absorbancy of the cell control receiving neither virus nor interferon was subtracted and the data plotted as the reciprocal of the interferon dilution on a log axis versus the neuraminidase yield expressed as percentage of the virus control (infected culture without interferon) on the arithmetic axis. Interferon potency is expressed as the NRD_{30} or 30% inhibition of enzyme yield, i.e., 70% of virus control (1).

Results. Production of hemagglutinin, neuraminidase, and virions in human cells. Infection of human BUD-8 cell cultures with the

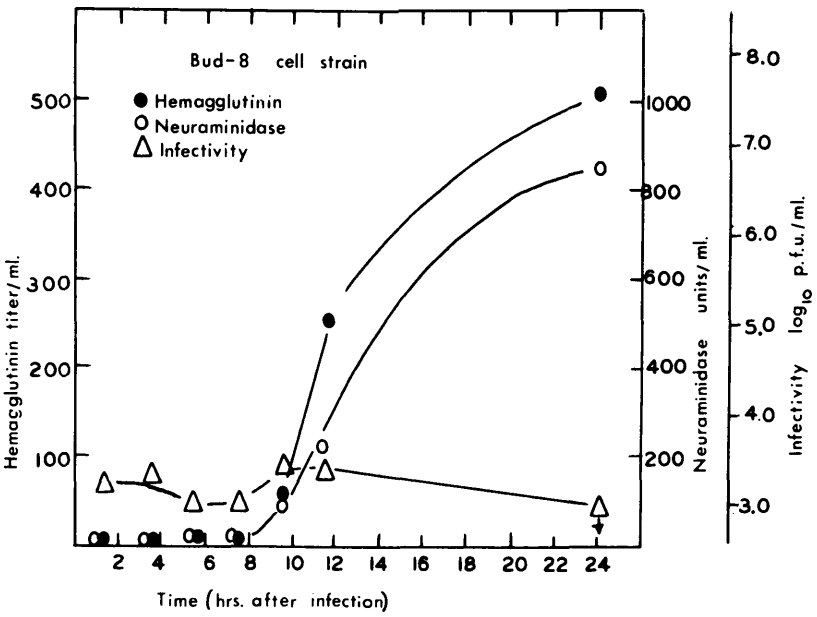

FIG. 1. Kinetics of production of X7(F_1) viral hemagglutinin, neuraminidase and infectious virus in BUD-8 human skin cell strain.

TABLE II. COMPARISON OF INTERFERON TITERS OBTAINED BY THE X7(F_1) NEURAMINIDASE YIELD-REDUCTION METHOD AND OTHER BIOASSAYS IN A VARIETY OF CULTURED CELLS.

Species	Cell Culture	Enzyme[a] yield (units/ml)	Mean interferon[b] titer (NRD_{30})	Mean interferon titers by other assays
Human	Foreskin-7 strain	610	365,000	260,000 (EMC $HYRD_{0.5\ log_{10}}$)
	BUD-8 skin strain	940	222,000	125,000 (EMC $HYRD_{0.5\ log_{10}}$)
	Embryo kidney strain	1600	17,500	27,500 (EMC $HYRD_{0.5\ log_{10}}$)
	Chang conjunctiva line	320	25,600	17,500 (EMC $HYRD_{0.5\ log_{10}}$)
	FL amnion line	90	22,000	26,000 (EMC $HYRD_{0.5\ log_{10}}$)
Monkey	AGMK line	1150	7,000	12,000 (EMC $HYRD_{0.5\ log_{10}}$)
Rabbit	RK-13 line	245	1,800	1,300 (EMC $HYRD_{0.5\ log_{10}}$)
Hamster	Kidney primary	720	27	52 (SFV $HYRD_{0.5\ log_{10}}$)
Mouse	L_{929} line	350	5,360	6,150 (GDVII $HYRD_{0.5\ log_{10}}$)
Chicken	Embryo primary	6500	1,000	950 (VSV PDD_{50})

[a] Neuraminidase determined by the thiobarbituric acid assay with fetuin as substrate.

[b] Homologous species interferons were used except for AGMK and RK-13 in which a human interferon preparation was used.

Abbreviations: EMC = encephalomyocarditis virus; SFV = Semliki Forest virus; VSV = vesicular stomatitis virus.

recombinant influenza virus X7(F_1) resulted in production of both hemagglutinin and neuraminidase without infectious virus progeny (Fig. 1). Similar results indicating incomplete replication cycles were also obtained with FL human cell line and with mouse L_{929} cell line. The ratio of enzyme units to hemagglutinin was 1.0–2.0 for the BUD-8 cells, approximately 4.0 for the FL cells, and approximately 10 for both mouse L cell and chicken embryo culture systems. Maximum production of hemagglutinin and neuraminidase

TABLE III. TITRATIONS OF MURINE INTERFERON BY THE GDVII HEMAGGLUTININ AND X7(F_1) NEURAMINIDASE YIELD-REDUCTION METHODS.

	Mouse serum NIH G002-903-026		Mouse serum MCW 052970[c]		Mouse serum MCW 062671	
	HA[a]	Nase[b]	HA	Nase	HA	Nase
Number of titrations[d]	8	8	9	9	9	9
Mean titer	6800	7650	1890	1980	1460	1580
S/D	5490	2800	957	342	905	368
SD % of Mean	87.5	36.5	50.5	17.5	62.0	23.2

[a] GDVII HA $YRD_{0.5\ log_{10}}$.

[b] X7(F_1) Nase YRD_{30} determined using the TBA neuraminidase assay with fetuin as substrate.

[c] Medical College of Wisconsin (MCW) laboratory standard interferon preparations.

[d] Simultaneous titrations of the interferon potency of a particular interferon preparation by both assays.

occurred at 24 hr in the BUD-8 cells. The magnitude but not the time-course of neuraminidase and hemagglutinin production was generally different for interferon-treated cells (1).

Neuraminidase yield-reduction interferon bioassay in various animal cells. A wide range of neuraminidase yield was observed in different cell culture systems with a range from 90 units in the FL human cell line to 6500 in primary chicken embryo cell cultures (Table II). However, one advantage of an enzyme bioassay for interferon is that initially low enzyme yields, as indicated by low absorbancies, can be amplified simply by prolonging the incubation of enzyme with substrate until an acceptable absorbancy is obtained for the virus-infected control not treated with interferon.

The sensitivity of a variety of cultured cells to homologous species interferons by the X7(F_1) neuraminidase yield-reduction assay was compared to another sensitive assay for each culture system. The titers shown are the means of at least two simultaneous titrations with both the X7(F_1) neuraminidase yield-reduction assay and the other indicated interferon assay. The human cell strains, foreskin-7 and BUD-8 skin, were much more sensitive to interferon by either the X7(F_1) neuraminidase yield-reduction assay or the EMC-HA yield-reduction assay than were the embryo kidney strain or any of the human cell lines. The sensitivity of the neuraminidase yield-reduction bioassay at least equaled that of the EMC hemagglutinin yield-reduction bioassay for human interferon. The neuraminidase yield-reduction assay in the BUD-8 cells measured 220,000 units for the National Institutes of Health reference standard G023 901 527 to which had been assigned a value of 20,000 units. The neuraminidase yield-reduction assay was also at least as sensitive as the other bioassays tested in other strains of cells.

Comparison of the X7(F_1) neuraminidase yield-reduction and the GDVII hemagglutinin yield-reduction methods for murine interferons. The potency of three mouse serum interferon preparations was tested repeatedly over a period of 9 mo simultaneously by the GDVII hemagglutinin and the X7(F_1) neuraminidase yield-reduction techniques (Table III). The mean titers obtained by the two methods were essentially equivalent for each of the three interferon preparations. However, the standard deviation of the titer expressed as percentage of mean titer was significantly lower with the X7(F_1) enzyme method than the GDVII-HA bioassay for all three serum preparations, suggesting that the X7(F_1) bioassay is more precise than the GDVII-HA bioassay.

Interferon bioassay using a synthetic substrate, methoxyphenylneuraminic acid, for assay of neuraminidase. A neuraminidase enzyme assay method based on the solubilization of the diazonium salt chromagen of methoxyphenol (MP) released from the methoxyphenylneuraminic acid (MPN) (2) by

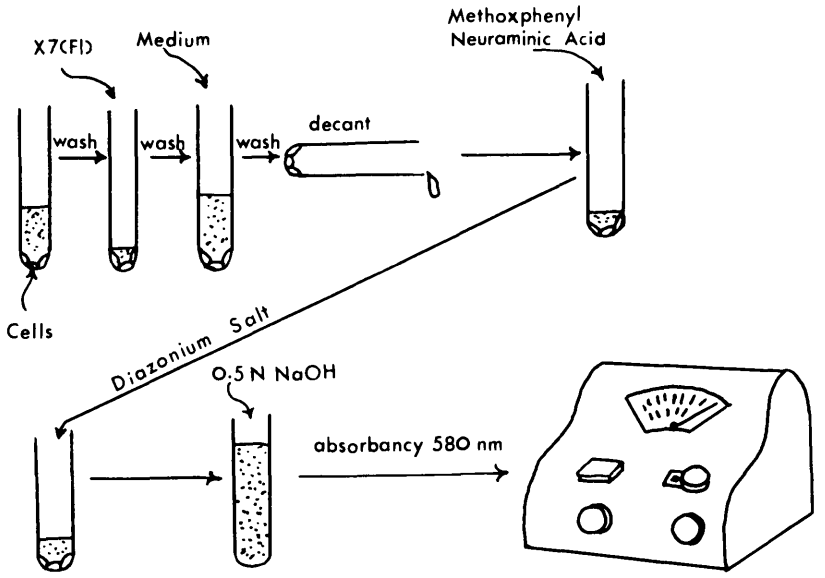

FIG. 2. Methoxyphenylneuraminic acid neuraminidase assay as applied to cultured cells in tubes.

neuraminidase greatly simplified the bioassay of interferon. Since phenol red reacts with the diazonium salt reagent used in this assay, infected cells are washed with phosphate-buffered saline to remove any traces of phenol red, and the MPN substrate applied directly to the cells (Fig. 2). Since approximately 70% of the neuraminidase produced in the cell culture systems studied remains cell associated, this procedure serves to concentrate the enzyme thereby accelerating the neuraminidase assay. The X7(F_1) virus caused little or no cytopathic effect in human and mouse cell culture systems, allowing the monolayers of cells to be washed without loss of cells. Thus, enzyme-substrate incubation time could be shortened as much as 10-fold relative to the assay using frozen and thawed cell extracts and fetuin as the substrate. A three-way comparison of interferon titers obtained simultaneously with the MPN and TBA neuraminidase methods as well as with the GDVII-HA assay is presented in Table IV. The mean titer obtained with the GDVII method was comparable to that obtained with the neuraminidase assays. However, again the variability of the GDVII bioassay was greater than that observed with the neuraminidase methods.

TABLE IV. SIMULTANEOUS TITRATION OF AN L-CELL INTERFERON PREPARATION BY VARIOUS METHODS.

	Assay		
Experiment	X7(F_1) MPN[a] NRD_{30}	X7(F_1) TBA[b] NRD_{30}	GDVII[c] $YRD_{0.5\log_{10}}$
1	17,000		26,000
2	16,170		32,000
3	20,000	11,000	9,200
4	20,000	9,500	20,000
5	11,000		9,600
6	8,500	9,500	11,000
7	13,600	17,500	20,000
Mean titer	15,180	11,875	18,200
SD	4,070	3,810	8,800
SD % of Mean	26.8	32.0	48.4

[a] X7(F_1) neuraminidase yield-reduction assay using methoxyphenylneuraminic acid as substrate for determination of enzyme yield.

[b] X7(F_1) neuraminidase yield-reduction assay using the thiobarbituric acid determination of neuraminidase yield with fetuin as substrate.

[c] GDVII virus hemagglutinin yield-reduction assay.

Discussion. The neuraminidase yield-reduction bioassay for interferon is sensitive and precise and can be used in a variety of animal cell culture systems without the production of infectious virus. Usable yields of

neuraminidase were obtained with a number of human cell lines and strains; the best yields were measured in cells from embryo kidney and BUD-8 strains. Cultures that produced low yields of neuraminidase could also be used in a neuraminidase yield-reduction bioassay merely by prolonging the incubation time for the enzyme reaction with substrate. Usable yields of X7(F_1) neuraminidase were also obtained with monkey, rabbit, hamster, mouse, and chicken cell cultures. The X7(F_1) neuraminidase yield-reduction assay was at least of equal sensitivity to another highly sensitive interferon assay in each of the cell culture systems examined. The sensitivity and reproducibility of the neuraminidase yield-reduction assay indicates that it may be the assay of choice for several species of interferon. The wide host range for the X7(F_1) virus and the sensitivity of its ability to produce neuraminidase to inhibition by different species of interferon suggests its possible use as a universal interferon assay.

A new enzyme assay for neuraminidase based on the solubilization of a methoxyphenol-diazonium salt chromagen is simple and rapid and has been applied to neuraminidase yield-reduction interferon assays. The sensitivity and low variation of such interferon measurements are equivalent to those obtained using the thiobarbituric acid neuraminidase assay with fetuin as substrate. This technique permits the colorimetric measurements of enzyme yield in the same tubes in which the cells were grown, treated with interferon, and challenged with virus.

Summary. The production of neuraminidase by the recombinant influenza virus X7(F_1) in human, monkey, rabbit, hamster, mouse, and chicken cell cultures is inhibited by interferon. Described is a new enzyme assay for neuraminidase that can be applied to the bioassay of interferons. The advantages of this interferon bioassay are its sensitivity, reproducibility, rapidity, and convenience.

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Note added in proof: Experiments subsequent to this manuscript have revealed that the production of neuraminidase in the RK-13 cell line is variable and at times too low for use in an interferon assay.

Note added in proof: Low yields of neuraminidase have recently been obtained in the RK-13 rabbit cell line, an observation which makes this line unsuitable for the neuraminidase bioassay. However, high neuraminidase yields are obtained in another interferon-sensitive line of rabbit skin (RS) cells.

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