Human Influenza A Viruses: Rapid Plaque Assay in Hamster Kidney Cells

Abstract. *The production of focal necrosis in hamster kidney cells maintained beneath an overlay of semisolid agar medium free of serum permits rapid, reproducible, quantitative assay for the three subtypes of influenza A viruses. Adaptation of virus to these cells was not required. A straight-line dose-response relationship was observed betweeen the number of plaques produced and the relative concentration of virus. Specificity was demonstrated by quantitative neutralization with immune serum.*

Generally, influenza type A viruses of human origin either cannot be propagated in mammalian or avian cells in vitro or else they grow only to low titer, with variable or slight cellular destruction (*1*, *2*). Formation of easily recognizable plaques, one important requisite for direct enumeration of infectious particles, has usually been induced by influenza virus strains previously adapted in the egg or in vitro to the same host species in which focal cellular destruction could be observed. Such focal necrosis had been reported with human influenza viruses in cultures of fibroblasts, lung epithelium, or kidney epithelium from the chicken embryo (*3*) as well as in kidney cells from the monkey (*4*) and calf (*5*). However, influenza strains unadapted to renal cells from the Syrian hamster (*Mesocricetus auratus*) both grow in and extensively destroy these cells maintained in serum-free fluid medium (*2*). A plaque assay in hamster kidney cells therefore seemed attainable for use in studies of influenza viral replication, pathogenesis, and epidemiology.

Primary cultures of hamster kidney cells were prepared (*2*), and 5-ml portions of a 1/200 or 1/300 dilution (weight/volume) of trypsin-dispersed cells in medium were dispensed in soft-glass, screw-cap, tablet bottles. The medium was composed of Hanks balanced salt solution (*6*), 10- to 20- percent heated calf serum, 0.25 percent lactalbumin hydrolysate, and 100 units of penicillin and 100 μg of streptomycin per milliliter. Cellular layers, 18 cm^2 in area, were usable after incubation at 37°C for 3 to 5 days.

The four virus strains used belonged to the three immunologic subtypes of type A: A/WS, A/NWS, A_1/FM-1, and A_2/Japan/305; their histories and preparation have been described (*2*, *7*). To infect hamster kidney cells, an influenza virus was diluted in GLB medium (composed of 1.0 percent gelatin and 0.5 percent lactalbumin hydrolysate in Hanks solution, and containing penicillin and streptomycin), and 0.2 ml of diluted virus was added to sheets of washed cells. The inoculated cell cultures were placed in an incubator at 37°C and rocked intermittently or continuously to distribute the inoculum and moisten the cells. After 1 or 2 hours the agar medium was added, and the cultures were incubated again at 37°C. Kinetic studies of adsorption of NWS virus showed that more than 90 percent of virus in the inoculum was adsorbed to cells in 120 minutes. The destruction of the cellular receptors for influenza virus by the specific enzyme obtained from *Cholera vibrio* adversely affected adsorption of virus, even when the receptor-destroying enzyme was added to cells 1 hour after virus adsorption had begun.

Serum was omitted from the semisolid medium used since it repressed or delayed the cytopathic effects of influenza virus (*2*, *8*). The integrity of cellular layers could be maintained under solid medium containing 1.5 percent agar. However, in the absence of serum, these cells tolerated staining with neutral red very poorly even when they were kept in the dark to obviate possibly lethal photosensitization. Staining with neutral red could be used for the isolation of virus clones but was unsatisfactory as a routine measure. Therefore, the more advantageous

Table 1. Concentrations of infectious influenza virus particles ($\log_{10}$ per ml) measured in chicken embryos and in hamster kidney (HK) cell cultures under fluid and solid media.

Influenza virus strain	Infectious particles*		Plaque-forming units (in HK cells under agar)
	In eggs	In HK cells under fluid	
NWS†	8.34	6.34	6.97
WS†	7.64	5.54	6.27
FM-1†	8.14	7.14	7.00
J/305†	6.94	2.34	3.47
NWS‡	8.64	5.34	6.95
NWS§	8.64	7.14	7.25

* Estimated (conversion factor from 50 percent infectivity titers = 0.69). † Egg-passaged, allantoic fluid virus preparation. ‡ Cell culture fluid preparation of egg-passaged virus that had been transferred two times in HK cells. § Cell culture fluid preparation of virus transferred six times in HK cells.

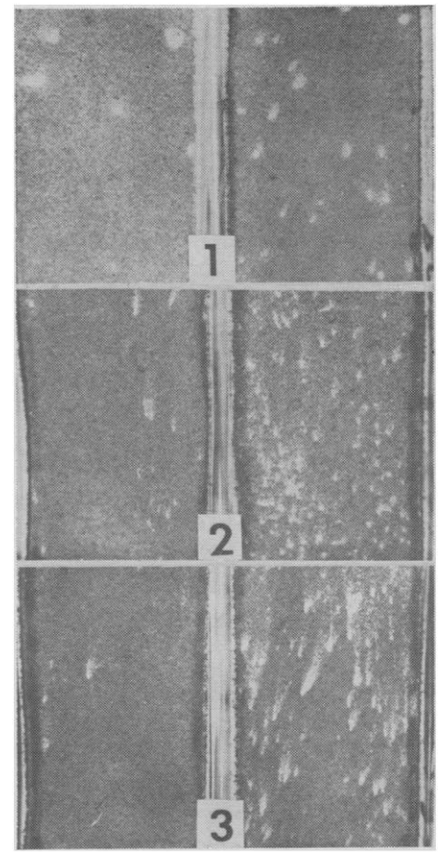

Fig. 1. Plaques produced by influenza A viruses as allantoic fluid preparations in hamster kidney cells under semisolid agar. Cells were stained with crystal violet 42 to 48 hours after infection (× 1). *1*, A/NWS virus, $10^{-5.8}$ dilution of virus on left, $10^{-4.7}$ on right. *2*, A_1/FM-1 virus, $10^{-5.0}$ dilution of virus on left, $10^{-4.0}$ on right. *3*, A_2/Japan/305 virus $10^{-2.0}$ dilution of virus on left, $10^{-1.0}$ on right.

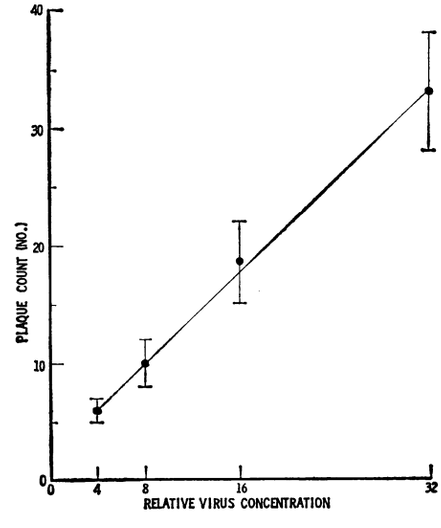

Fig. 2. Dose-response relationship between number of plaques produced by NWS virus in hamster kidney cell cultures and the relative concentration of virus.

method of Holland and McLaren (*9*) was used. After the semisolid agar medium was gently decanted, the cells were fixed and stained for plaque counts by adding 0.5 percent crystal violet solution in 20 percent aqueous ethanol. The semisolid medium contained 1 percent gelatin, 0.5 percent lactalbumin hydrolysate, 0.1 percent yeast extract, 100 units of penicillin per milliliter, 100 μg of streptomycin per milliliter, sufficient molten Bacto-agar (Difco) to make its final concentration 0.5 percent, and enough 1.4 percent $NaHCO_3$ so that the final concentration was 4m*M* (*10*).

Figure 1 shows virus plaques produced in 2 days. Strain NWS produced the clearest plaques, which measured 2 to 4 mm in diameter. Strain FM-1 produced clear 1- to 2-mm plaques, whereas WS and Japan/305 produced similar but somewhat less distinct plaques. Comet-like plaques measuring 0.5 or 1 by 4 or 5 mm sometimes occurred. Virus plaques had microscopically irregular borders. In central areas of plaques, scattered, viable cells were sometimes seen and appeared to be either in various stages of membrane retraction or completely unaffected. Plaques were produced as readily by virus strains that had undergone many passages as by those that had no more than two passages since isolation from man. Formation of plaques was more than 90 percent complete by the end of day 2, although their size and clarity increased on day 3.

To help establish the validity of this assay, and its relationship to other means of measurement, the numbers of influenza virus particles infectious for the allantois of chicken embryos were compared with those for hamster kidney cells. Table 1 shows that infectivity measured in hamster kidney cells was somewhat lower than that measurable in chicken embryos even for virus with limited passage in hamster cells. Inasmuch as these strains of virus had either been isolated in eggs or had been serially propagated in them, one possible explanation for these disparities is that selection adapted these virus populations to grow in chicken embryos. Titers of infectious particles by plaque assay were similar to or inexplicably higher than those in tube cultures. There is a straight-line relationship between the relative concentration of influenza virus particles infectious for hamster kidney cells and the number of plaques (Figs. 1 and 2). Whether the fate of the particles that are non-

Table 2. Comparison of virus neutralization tests performed by plaque-reduction method and by abolition of infectivity for eggs and tube cell cultures.

Serum dilution	Plaque number* 60 PFU vs.		Cultures destroyed§ 80 $TCID_{50}$ vs.		Eggs infected‖ 50 EID_{50} vs.	
	NWS-rs†	nrs‡	NWS-rs	nrs	NWS-rs	nrs
1/9	0	30	0/3	3/3	0/5	5/5
1/27	0	28	0/3	3/3	0/5	5/5
1/81	0	33	0/3	3/3	0/5	
1/243	0		0/3		0/5	
1/729	4		0/3		4/5	
1/2187	8		3/3		4/5	
1/6561	24		3/3		5/5	

* Average of counts in three cultures of hamster kidney cells. † Rabbit serum containing antibody against NSW virus. ‡ Antibody-free, normal rabbit serum. § Numerator is the number of hamster kidney tube cultures destroyed; denominator is the number inoculated. ‖ Numerator is the number of eggs yielding hemagglutinin; denominator is the number inoculated.

infectious for a heterologous species system depends upon failure to be adsorbed to cells, ineffective reception and penetration into cells, or unfavorable intracellular milieu is still not known.

Specific antibody reduced the plaque count (Table 2). Dilutions of serum, heated at 60°C for 20 minutes, were mixed with equal volumes of virus diluted appropriately in GLB medium, kept at 22° to 24°C for 1 hour, and added to cells (0.2 ml for each 18-cm^2 layer) which were incubated at 37°C for 1 hour. The inoculated cells were then covered with semisolid agar medium and incubated at 37°C. Within 2 days the plaques were counted, and the antibody concentration was determined. Comparison with one other type of direct test in tube cultures of hamster kidney cells, requiring 7 to 10 days for completion, or with an indirect test in embryonated eggs, for hemagglutinin production after 2 days, demonstrated that the method utilizing an endpoint where the plaque reduction was more than or equal to 50 percent appeared somewhat more sensitive. Addition of the rabbit antiserum to the semisolid agar shortly after it was overlaid prevented development of NWS virus plaques whereas antibody-free rabbit serum did not do so.

Cell culture systems which can sustain growth of members of the *Myxovirus influenzae* group without overt cellular destruction usually require indirect detection of virus, for example, by hemadsorption (*11*), since these viruses may multiply even in cells that look completely normal or healthy (*12*). Why kidney cells of the Syrian hamster are destroyed by influenza viruses without prior adaptation to this species remains undetermined. That these viruses can cause focal necrosis of hamster-kidney cells under semisolid medium provides a rapid means for quantitative measurement of infectious virus particles and of corresponding neutralizing antibody (*13*).

SIDNEY E. GROSSBERG
Department of Microbiology, Cornell University Medical College, New York

References and Notes

1. D. L. Walker, in *Ann. Rev. Microbiol.*, C. E. Clifton, Ed. (Annual Reviews, Palo Alto, Calif., 1960), p. 177.
2. S. E. Grossberg, *Proc. Soc. Exptl. Biol. Med.* **113**, 546 (1963); ———, *Federation Proc.* **22**, 557 (1963).
3. N. Ledinko, *Nature* **175**, 999 (1955); A. Granoff, *Virology* **1**, 252 (1955); B. S. Wright and B. P. Sagik, *ibid.* **5**, 573 (1958); R. W. Simpson and G. K. Hirst, *ibid.* **15**, 436 (1961).
4. C. Henry and J. S. Youngner, *J. Immunol.* **78**, 273 (1957); P. W. Choppin, *Virology* **18**, 332 (1962).
5. G. Negroni and D. A. J. Tyrrell, *J. Pathol. Bacteriol.* **77**, 497 (1959); F. Lehmann-Grube, *Virology* **21**, 520 (1963).
6. J. H. Hanks, *Proc. Soc. Exptl. Biol. Med.* **71**, 196 (1949).
7. The A_2/Japan/305/57 strain used was in second chicken embryo passage after its isolation from man by S. E. Grossberg and J. M. McCown at the U.S. Army 406th Med. Gen. Laboratory, Japan.
8. Monolayers could not maintain their integrity under serum-free medium if they were formed in growth medium containing less than 10 percent serum.
9. J. J. Holland and L. C. McClaren, *J. Bacteriol.* **78**, 596 (1959).
10. Concentrated stock solutions for use in semisolid medium were prepared individually in unconcentrated Hanks solution without bicarbonate. Several different serum-free, agar-containing media failed to sustain hamster kidney cells which often quickly rounded up and detached themselves from glass; lactalbumin hydrolysate and yeast extract were essential ingredients, and Hanks solution was comparable, or perhaps slightly superior, to Scherer's maintenance solution [W. F. Scherer, *Am. J. Pathol.* **29**, 113 (1953)].
11. J. Vogel and A. Shelokov, *Science* **126**, 358 (1957); J. E. Hotchin, R. Deibel, L. M. Benson, *Virology* **10**, 275 (1960).
12. C. Morgan and H. M. Rose in *Virus Growth and Variation, Ninth Symposium Soc. Gen. Microbiol.*, A. Isaacs and B. W. Lacey, Eds. (Cambridge Univ. Press, London, 1959), p. 256.
13. The technical assistance of C. D. Cobb and S. C. Rosen is gratefully acknowledged. This work was supported by the New York City Health Research Council and by the Surgeon General, U.S. Army, under sponsorship of the Commission on Viral Infections, Armed Forces Epidemiological Board, and was performed during the tenure of a research career development award from NIH.

24 March 1964