JOURNAL OF VIROLOGY, Apr. 1995, p. 2700–2703 Vol. 69, No. 4
0022-538X/95/$04.00+0

Impairment of Multicycle Influenza Virus Growth in Vero (WHO) Cells by Loss of Trypsin Activity

NICOLAI V. KAVERIN[1,2] AND ROBERT G. WEBSTER[1,3]*

Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38101[1]; The D. I. Ivanovsky Institute of Virology, Moscow 123098, Russia[2]; and Department of Pathology, University of Tennessee, Memphis, Tennessee 38163[3]

Received 19 September 1994/Accepted 12 January 1995

We demonstrated that influenza virus replication in Vero (WHO) cells, a subline of African green monkey kidney cells, is impaired by rapid inactivation of trypsin in the culture fluids. Trypsin inactivation was caused by a factor secreted by Vero cells into the media. Repeated addition of trypsin to the culture medium of influenza virus-infected Vero cells restores the multicycle growth pattern of influenza A virus strains, allowing high yields to be obtained at a low multiplicity of infection. These findings may permit efficient use of Vero (WHO) cells in the production of influenza vaccines.

Hemagglutinin (HA), the major glycoprotein of influenza viruses, is synthesized in infected cells as a single polypeptide and undergoes a posttranslational cleavage that yields two subunits, HA1 and HA2, held together by a disulfide link. Such cleavage is mandatory for the production of infectious virus; virions containing uncleaved HA are noninfectious (14). The HAs of human and swine influenza virus strains, as well as those of the majority of avian influenza viruses, cannot be cleaved by ubiquitous intracellular proteases (7, 8). Thus, to ensure HA cleavage so that progeny virus is activated and infection proceeds in a multicycle fashion, it is necessary to add trypsin to the maintenance medium of cell cultures.

The biological system most widely used to produce influenza viruses in large quantities is the embryonated chicken egg. The preparations used as killed influenza vaccines are purified from virus-containing chicken embryo allantoic fluid. A large body of information obtained over the last decade (5, 6, 12) suggests that the embryonated chicken egg is not ideal for vaccine production, since even a single passage of a human influenza virus isolate in eggs results in selection of variants differing in their antigenic specificity from those of the original virus circulating in humans. The viruses isolated and passaged exclusively in cell cultures retain their antigenic characteristics, indicating that the use of cell culture-grown virus for vaccine production might prove highly advantageous. We therefore assessed the potential of a World Health Organization (WHO)-certified cell line, Vero (WHO), a subline of African green monkey kidney cells, as a system for high-yield production of influenza virus.

Inefficient multicycle replication of influenza A viruses in Vero (WHO) cells. While attempting to passage influenza A virus strains in Vero (WHO) cells, we encountered difficulties in obtaining high virus yields with low multiplicities of infection (MOI). The MOI had to be at least 0.005 of the 50% tissue culture infective dose ($TCID_{50}$) per cell to produce high yields. At lower doses, the titers were low or the virus failed to accumulate at all. In most instances, the accumulation of virus in cultures infected at a low MOI stopped after 48 h postinfection, as did the progress of the cytopathic effect. This pattern was strongly expressed in 50-cm^3 flasks, expressed less strongly in 6-well plates, expressed even less in 24-well plates, and expressed practically not at all in 96-well plates. Similar dependence of final virus yields on the input dose was not observed with MDCK cells, regardless of the plasticware used (data not shown).

Since the adverse effect of a low MOI was not apparent in the 96-well plates, we were able to perform infectivity titration in Vero (WHO) cells to assess the intrinsic sensitivity of the cells to influenza virus. The titrations were performed by using parallel limiting dilution techniques in Vero (WHO) and MDCK cells (8 wells per each 10-fold virus dilution). The susceptibility of Vero (WHO) cells to influenza virus infection was either of the same order as that of MDCK cells or only slightly (0.2 to 0.6 $\log_{10}$ $TCID_{50}$) lower (data not shown).

One-step growth curves indicated that in Vero (WHO) cells, influenza virus replication did occur but at a somewhat lower rate than in MDCK cells (Fig. 1). However, neither the slightly lower susceptibility of Vero (WHO) cells to influenza nor the somewhat lower virus yield per cell could explain their failure to promote high titers of influenza virus at a low MOI in certain types of flasks. The kinetics of virus accumulation at low MOIs suggested that the defect was associated with impairment of multicycle growth.

Loss of trypsin activity in cell culture fluid. Trypsin is essential for multicycle growth of human influenza virus strains in most cell cultures (10). We therefore reasoned that inadequate levels of trypsin activity in the growth medium might account for the abrogation of virus accumulation at low input MOIs. To test this prediction, we subjected Vero (WHO) cell cultures to the same procedure as that used for infection; that is, the cells were washed three times with phosphate-buffered saline (PBS), overlaid with minimal essential medium-bovine serum albumin (MEM-BSA) containing 1.0 μg of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington) per ml, and incubated at 37°C and 5% CO_2. At different times, samples of the medium were taken and frozen at −70°C. All samples were thawed, twofold dilutions were prepared from each sample, and trypsin activity was determined (Fig. 2).

Measurement of trypsin activity in the culture fluids of Vero (WHO) cells in 50-cm^3 flasks, 6-well plates, and 24-well plates revealed a sharp decrease of activity, beginning at the first hours of incubation. The decrease was most apparent in the

* Corresponding author. Mailing address: Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, 332 N. Lauderdale, P.O. Box 318, Memphis, TN 38101. Phone: (901) 522-0400. Fax: (901) 523-2622. Electronic mail address: robert.webster@stjude.org.

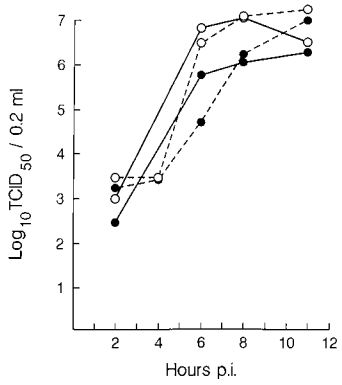

FIG. 1. One-step growth curve for X-31 and A/England/1/53 viruses in MDCK and Vero (WHO) cells. The Vero (WHO) cell line was obtained from the American Type Culture Collection at the level of the 154th passage. The cells were grown as monolayers in growth medium containing MEM supplemented with 10% unheated fetal calf serum. Influenza A/Fort Warren/1/50 (H1N1), and X-31 (H3N2) viruses were used. The viruses were passaged five times in Vero (WHO) cell cultures. The final stock virus preparations contained $10^{7.5}$ to $10^{8.25}$ $TCID_{50}$ per 0.2 ml and 32 to 128 hemagglutinating units. HA and infectivity titrations were performed essentially as described (11). Cells grown in 6-well plates were infected at an MOI of 50 $TCID_{50}$ per cell. After 1 h of adsorption, the inoculum was removed and the cells were overlaid with MEM-BSA without trypsin and incubated at 37°C. Samples were taken and frozen at −70°C. Infectivity titrations were performed by endpoint titration in MDCK cells grown in 96-well plates. ○, MDCK cells; ●, Vero (WHO) cells; ———, X-31; – – –, A/England/1/53.

50-cm^3 flasks, less apparent in the 6-well plates, and the least apparent in the 24-well plates. By 48 h of incubation, trypsin activity had disappeared altogether (Fig. 2A).

Unexpectedly, a loss of trypsin activity was also evident in other cell lines (rhesus monkey kidney LLC-MK_2, swine kidney [SwK], and MDCK) but was less pronounced than that in Vero (WHO) cell cultures (Fig. 2B and C). Substitution of acetyl-trypsin for TPCK-trypsin did not prevent the loss of activity, although it did result in a reduced rate of inactivation. There was virtually no loss of trypsin activity when either TPCK-trypsin or acetyl-trypsin was incubated at 37°C in maintenance medium in the absence of cells (Fig. 2B and C).

TABLE 1. Effects of dilution, heating, and Centricon ultrafiltration on trypsin-inactivating factor in Vero (WHO) cell culture fluid

Expt. no.	Vero (WHO) cell culture fluid[a]	Residual trypsin activity (%)[b]
1	Undiluted	5.3
	Diluted 1:2	25.6
	Diluted 1:4	65.1
	Diluted 1:8	80.1
	Diluted 1:16	80.5
2	Unheated	7.0
	Heated at 56°C for 30 min	10.5
3	Unfiltered	4.8
	Filtered through C-con 10	100.0
	Filtered through C-con 30	100.0
	Filtered through C-con 50	100.0
	Filtered through C-con 100	46.5

[a] Culture fluid of Vero (WHO) cells (MEM-BSA without trypsin) was collected after 72 h of incubation. Samples of the culture fluid (undilute, diluted in fresh medium, heated at 56°C, or filtered through Centricon filters) were mixed with TPCK-trypsin to make the final trypsin concentration 1.0 μg/ml and incubated for 1 h at 37°C. C-con, Centricon.

[b] The activity of 1.0 μg of trypsin per ml in fresh MEM-BSA medium was taken as 100%.

Differences in the rate of loss of trypsin activity in Vero (WHO) cell cultures grown in different kinds of plasticware (Fig. 2A) suggested that the volume of the maintenance medium per cell might be responsible. To test this prediction, we grew Vero (WHO) cells in 6-well plates and overlaid them with 2.0, 3.0, and 4.5 ml of maintenance medium; this step was followed by incubation at 37°C for 24 h. Measurement of trypsin activity demonstrated a clear dependence of trypsin inactivation on the volume of maintenance medium: the residual activities of trypsin were 9.1, 30.0, and 42.7% of the initial levels of 2.0, 3.0, and 4.5 ml of the medium, respectively.

Inhibition of trypsin activity is produced by a secreted factor. The loss of trypsin activity could result either from cellular secretion of an inhibitory factor into the medium or from direct contact of trypsin with the cells. The next series of

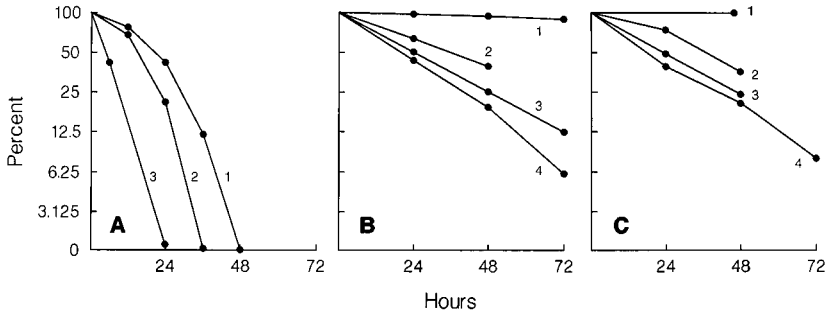

FIG. 2. Loss of trypsin activity in culture fluid. A highly sensitive assay of trypsin activity, based on a fluorogenic substrate, sodium benzoyl-L-arginine-7-amido-4-methylcoumarin hydrochloride (Sigma), was used (15). The substrate was dissolved to a final concentration of 0.2 mM in a buffer containing 50 mM of Tris-HCl (pH 8.0), 10 mM $CaCl_2$, and 1% dimethyl sulfoxide. An 0.1-ml sample of trypsin-containing cell culture fluid was added to 0.9 ml of the sodium benzoyl-L-arginine-7-amido-4-methylcoumarin hydrochloride solution and incubated at 37°C for 1 h. The samples were placed on ice and assayed in a Perkin-Elmer MPF-44B fluorescence spectrophotometer at activation and emission wavelengths of 380 and 460 nm, respectively. The initial concentrations of TPCK-trypsin and acetyl-trypsin were 1.0 μg/ml for Vero (WHO), MDCK, and LLC-MK_2 cells and 0.32 μg/ml for SwK cells. Ordinate, percent of initial trypsin activity; abscissa, time of incubation. For the growth of MDCK cells and rhesus monkey kidney LLC-MK_2 cells, we used MEM medium with 5% fetal calf serum heated for 30 min at 56°C. For the cultivation of a swine kidney cell line (SwK), RPMI 1640 medium was used with 5% heated fetal calf serum. For the experiments involving infection or mock infection, the cells were washed three times with phosphate-buffered saline and overlaid with maintenance medium. The latter had the same composition as the growth medium for each cell line, with the serum omitted and 0.3% BSA added. Unless otherwise stated, the maintenance medium contained 1.0 μg of TPCK-trypsin (Worthington) per ml. (A) Vero (WHO) cells, TPCK-trypsin. Curve 1, 24-well plates; curve 2, 6-well plates; curve 3, 50-cm^3 flasks. (B) Curve 1, TPCK-trypsin, no cells; curve 2, acetyl-trypsin, MDCK cells, 6-well plates; curve 3, TPCK-trypsin, MDCK cells, 6-well plates; curve 4, TPCK-trypsin, MDCK cells, 50-cm^3 flasks. (C) Curve 1, acetyl-trypsin, no cells; curve 2, acetyl-trypsin, LLC-MK_2 cells, 6-well plates; curve 3, TPCK-trypsin, LLC-MK_2 cells, 6-well plates; curve 4, TPCK-trypsin, SwK cells, 6-well plates.

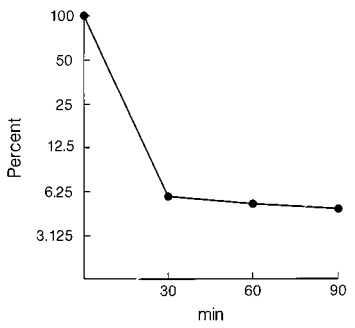

FIG. 3. Kinetics of inactivation of TPCK-trypsin with cell-free culture fluid of Vero (WHO) cells. Culture fluid (MEM-BSA without trypsin) was incubated with Vero (WHO) cells in 6-well plates for 72 h, collected, mixed with TPCK-trypsin to make the final concentration of the latter 1.0 μg/ml, and incubated at 37°C. Ordinate, percent of the initial activity; abscissa, time of incubation at 37°C.

experiments was designed to distinguish between these possibilities. Vero (WHO) cells were incubated for 72 h with the maintenance medium in the absence of trypsin. The culture fluid was collected and centrifuged at low speed to remove cells detached from the surface during incubation. The fluid was incubated with 1.0 μg of trypsin per ml. The results indicated that the cell-free culture fluid after cell growth inactivated trypsin and that this process was rapid, reaching completion after approximately 30 min of incubation at 37°C (Fig. 3). The effect could be titrated by diluting the fluid. The factor secreted into the fluid was stable to heating at 56°C for 30 min. It did not pass through Centricon filters detaining 10-, 30-, and 50-kDa macromolecules, but it did partially pass through those detaining 100-kDa macromolecules (Table 1).

Restoration of multicycle virus growth by repeated addition of trypsin. To verify that loss of trypsin activity was indeed responsible for the inability of influenza viruses to accumulate in Vero (WHO) cell cultures, we performed experiments in which the trypsin concentration was restored during infection by the repeated addition of trypsin to the culture medium. This procedure led to an increase in virus production in the cultures infected with low input doses, resulting in high final yields irrespective of the MOI (Table 2). The effect was especially striking in 6-well plates with dense confluent monolayers, that is, under conditions favoring a rapid loss of trypsin activity.

The discovery of the effect of trypsin-induced HA cleavage on influenza virus infectivity in 1975 (8, 10) was a major advance in the development of cell culture systems, allowing multicycle virus replication. Yet, to this day, cell cultures suitable for the accumulation of influenza virus are not numerous. In some cultures the growth of the virus is abortive or semiabortive due to specific deficiencies in the intracellular biosynthesis of the virus components (1, 2, 5). In many other cases, however, the factors responsible for the failure of cell cultures to support the growth of influenza viruses are still undefined.

Meanwhile, the need for wider use of cell cultures in the preparation of diagnostic reagents and vaccines has been underscored by the discovery of antigenic changes in the HA molecule after a single passage in chicken embryos (6, 12, 13). These concerns prompted us to assess the Vero (WHO) cell line as an alternative to eggs for the production of influenza virus vaccines. Difficulties encountered in our attempts to propagate influenza virus strains in these cells suggested that trypsin activity might be lost during prolonged incubation of the cultures at 37°C in the course of multicycle virus replication. Indeed, the measurement of trypsin activity by a sensitive fluorogenic technique demonstrated its rapid loss in the maintenance medium of Vero (WHO) cell cultures. Unexpectedly, a slower but still substantial loss of trypsin activity was observed in other cell lines, including MDCK cells, which are widely used for influenza virus studies, including those involving multicycle replication of the virus.

The adverse effect of the loss of trypsin activity in Vero (WHO) cell cultures could be overcome by the repeated addition of trypsin during the course of infection. A higher volume of maintenance medium per area of cell monolayer also led to improved multicycle growth at low input doses, most likely because of a lower concentration of trypsin-inactivating factor in the medium.

The nature of the factor(s) responsible for trypsin inactivation in cell cultures remains to be characterized; it is secreted into the medium and rapidly inactivates trypsin (Fig. 3). Apparently, the curves for trypsin inactivation in cell cultures (Fig. 2) reflect the kinetics of the accumulation of the inhibitory factor rather than the kinetics of its interaction with trypsin.

TABLE 2. Effect of multiple trypsin additions on the growth of influenza virus in Vero (WHO) cells in plates

Expt. no.	Virus[a]	Plate (no. of wells)	State of the monolayer	Time of trypsin addition p.i. (h)[b]	Result[c] with input dose ($TCID_{50}$/well) at indicated time							
					10^4		10^3		10^2		10^1	
					48[d]	72	48	72	48	72	48	72
1	FW/50	6	Dense	0	64	64	32	64	2	2	1	1
	FW/50	6	Dense	0, 24, 48	64	64	32	64	16	32	8	32
	X-31	6	Dense	0	64	64	16	16	1	2	0	1
	X-31	6	Dense	0, 24, 48	32	64	32	64	16	64	4	32
2	FW/50	6	Nonconfluent	0	16	16	16	32	16	64	2	8
	FW/50	6	Nonconfluent	0, 24, 48	16	16	16	32	16	64	4	32
3	FW/50	24	Nonconfluent	0	32	32	32	32	16	64	4	32
	FW/50	24	Nonconfluent	0, 24, 48	32	32	32	32	16	32	8	32
	X-31	24	Nonconfluent	0	64	64	32	64	16	64	4	32
	X-31	24	Nonconfluent	0, 24, 48	64	64	32	64	16	64	8	64

[a] FW/50, A/FW/1/50.
[b] TPCK-trypsin was added at the postinfection (p.i.) times (in hours) to a final concentration of 1.0 μg/ml.
[c] Values are reciprocals of HA titers.
[d] Hours p.i.

Results of passage of the trypsin-inactivating factor through graded filters were consistent with a molecular mass of close to 100 kDa; alternatively, the factor may be heterogeneous in size, containing a high-molecular-mass fraction (>100 kDa) and a smaller fraction with a molecular mass of between 50 and 100 kDa (Table 1). Among the many inhibitors of serine proteinases able to inhibit the cleavage of low-molecular-weight substrates by trypsin, only a few possess molecular masses as high as those of our putative factor (9). Inter-alpha-trypsin inhibitor of human plasma is represented by a native molecule of 180 kDa as well as lower-molecular-mass species that retain inhibiting activity. Related inhibitors were detected in baboon plasma (3). If the trypsin-inactivating factor we detected belongs to the group of protein inhibitors of proteinases, as suggested by our rough estimates of molecular mass, the findings reported here could provide additional insight into serine proteinase inhibitors. Although numerous and extensively studied, such inhibitors are mostly derived from plants, bovine pancreas, human and animal plasma, and tissues of invertebrate species (9), so that their structure and enzymological properties have been defined far better than their biosynthesis, intracellular transport, and mechanism of secretion. In this regard, the availability of inhibitors produced by cultured cells may prove to be a valuable asset.

The present studies show that by the fifth passage of H1N1 and H3N2 influenza viruses in Vero (WHO) cells, the yields of virus are slightly lower than in MDCK cells, even when trypsin is repeatedly added. However, by the tenth passage in Vero (WHO) cells, the yields of infectious virus in Vero (WHO) cells was as high as in MDCK cells and in chicken embryos (4). The repeated addition of trypsin to the medium of Vero (WHO) cells infected with influenza viruses has the potential for producing sufficient virus for human vaccine production and for circumventing the selection of variants that can occur in chicken eggs (6, 12).

We thank Christopher Clark for excellent technical assistance and Dayna Anderson for typing the manuscript.

This work was supported by Pasteur Merieux Serums and Vaccines, Cancer Center Support (CORE) grant CA-21765, and American Lebanese Syrian Associated Charities (ALSAC).

REFERENCES

1. **Almond, J. W.** 1977. A single gene determines the host range of influenza virus. Nature (London) **270:**617–618.
2. **Conti, G., P. Valcavi, A. Natali, and G. C. Schito.** 1980. Different patterns of replication in influenza virus-infected KB cells. Arch. Virol. **66:**309–320.
3. **Gebhardt, W., and K. Hochstrasser.** 1986. Inter-α-trypsin inhibitor and its close relatives, p. 389–402. *In* A. J. Barrett and G. Salvesen (ed.), Proteinase inhibitors. Elsevier, Amsterdam.
4. **Govorkova, E. A.** Unpublished data.
5. **Israel, A.** 1980. Genotypic and phenotypic characterization of a mammalian cell-adapted mutant of fowl plague virus (FPV). J. Gen. Virol. **51:**33–40.
6. **Katz, J. M., C. W. Naeve, and R. G. Webster.** 1987. Host-cell mediated variation in H3N2 influenza viruses. Virology **156:**386–395.
7. **Kawaoka, Y., and R. G. Webster.** 1988. Sequence requirements for cleavage activation of influenza virus hemagglutinins in mammalian cells. Proc. Natl. Acad. Sci. USA **85:**324–328.
8. **Klenk, H.-D., R. Rott, M. Orlich, and J. Blodorn.** 1975. Activation of influenza A viruses by trypsin treatment. Virology **68:**426–439.
9. **Laskowski, M., and I. Kato.** 1980. Protein inhibitors of proteinases. Annu. Rev. Biochem. **49:**593–626.
10. **Lazarowitz, S. G., and R. W. Choppin.** 1975. Enhancement of infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology **68:**440–454.
11. **Palmer, D. F., M. T. Colman, W. R. Dowdle, and G. C. Schild.** 1975. Advanced laboratory techniques for influenza virus diagnosis. Immunol. Ser. **6:**51–57.
12. **Robertson, J. S., J. S. Bootman, R. Newman, J. S. Oxford, R. S. Daniels, R. G. Webster, and G. C. Schild.** 1987. Structural changes in the hemagglutinin which accompany egg adaptation of an influenza A (H1N1) virus. Virology **160:**31–37.
13. **Schild, G. C., J. S. Oxford, J. C. de Jong, and R. G. Webster.** 1983. Evidence for host-cell selection of influenza virus antigenic variants. Nature (London) **303:**706–709.
14. **Webster, R. G., and R. Rott.** 1987. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. Cell **50:**656–666.
15. **Zimmerman, M., B. Asche, E. C. Yurewicz, and G. Patel.** 1977. Sensitive assays for trypsin, elastase, and chymotrypsin using new fluorogenic substrates. Anal. Biochem. **78:**47–51.