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The Multiplication of an Influenza Virus Strain in a Continuous Line of Mammalian Cells*

(Brief Report)

By

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Strains of influenza A have been propagated in fertile eggs (1), in membranes from fertile eggs (2), and in a variety of primary cell cultures, including chick embryo fibroblasts (3) and hamster kidney cells (4). The virus has been grown in established cell lines: several strains of influenza A were propagated in the slow-growing 1-5C-4 line of *Chang*'s conjunctival cells (5, 6), and a strain of influenza A2 replicated to a low titer in KB cells (7).

Our interest in certain biochemical aspects of influenza virus prompted us to search for a cell host which would grow rapidly and support virus multiplication to high infectivity titers in a fluid as nearly chemically defined as possible.

The WSN strain of influenza A (3), which forms plaques on monolayers of chicken embryo fibroblast (CEF) and 1-5C-4 cells, was serially passaged in three hosts. After several passages the virus was given a designation indicating its particular host:

WSN_E virus was grown in 10-day old embryonated eggs using 0.005 to 0.1 hemagglutinating units of diluted allantoic fluid as inoculum and incubating 40 hours at 37° C. Hemagglutination (HA) titrations were performed as previously described (8).

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The WSN_C series was initiated from WSN_E by serial passage in primary CEF monolayers. CEF secondaries were used for plaque assay. Primary and secondary CEF cultures were prepared and maintained with supplemented medium 199 in the manner described by *Levinson* (9) except that the fluid and agar overlays which maintained the cells after infection were modified by omitting serum and adding bovine serum albumin to a concentration of 1 mg./ml. The other procedures for propagation and assay of WSN_C were similar to those reported by *Simpson* and *Hirst* (3).

The WSN_H series was initiated from WSN_C by serial passage in an established line of baby hamster kidney cells, BHK 21 (10).

Stock cultures of several established lines, BHK21, L (mouse), KB (human carcinoma), HeLa (human carcinoma) and A_2 (human amnion, ref. 11), were grown in *Eagle*'s minimal essential medium (MEM) containing 100 units/ml. sodium penicillin G and 100 mg./l. streptomycin sulfate and supplemented with 0.1 volume of fetal bovine serum. All cultures were incubated at 37° C in an humidified atmosphere of 5% CO_2 in air.

Cultures for virus propagation or plaque assay were prepared with the growth medium in 60 mm. plastic Petri plates 24 hours prior to infection. The confluent monolayers, containing about 5×10^6 cells, were washed once with *Dulbecco*'s phosphate buffered saline (PBS) and challenged with 0.2 ml. of virus diluted in PBS. Adsorption was at room temperature for 30 minutes (3).

The medium for maintenance of the cells after virus infection was MEM containing antibiotics, 1 mg./ml. BSA, and an increased amount of glucose (2 mg./ml.). Cultures for virus production received 5 ml. of maintenance medium, and the fluid was harvested after 48 hours incubation. Aliquots were stored at either —20° or +4° C.

Agar overlay contained 8 mg./ml. agar (Difco Noble) and 30 μg./ml. DEAE-dextran (Pharmacia) in maintenance medium. Cultures for plaque assay were overlaid with 2.5 ml. of agar medium which was permitted to gel for 15 minutes before the addition of 2.5 ml. of fluid maintenance medium. After 48 hours incubation the cell sheet was stained with 0.1 mg./ml. neutral red in a modified *Earle*'s balanced salt solution.

Under our conditions WSN_E multiplied in eggs to HA titers of 128 to 1024 and in CEF primary cultures to titers of 64 to 256, but it failed to produce either detectable HA or plaques in cultures of secondary CEF, HeLa, L or BHK21 cells.

In the WSN_C series the HA titers were low and plaque assays were negative during the first few passages, but the virus product of passage 6 (WSN_C6) regained the HA titer of 256 which had been obtained after the initial infection with WSN_E. WSN_C6 produced plaques on CEF

secondary monolayers, but the plating efficiency was low and erratic. Several modifications of the overlay did not improve the results. WSN_C5, 7, and 9 failed to produce detectable HA or plaques on monolayers of HeLa or L cells.

WSN_C11 was inoculated onto monolayers of BHK21, L, KB, HeLa, and A_2 cells to test for incomplete cycles of replication. The cultures were examined for hemadsorbing centers (12) using chicken erythrocytes and hemoglobin stain (13). Hemadsorbing foci, though clearly visible

Table 1. Representative Serial Passages of WSN_H in BHK21 Cells

Experiment	Passage number	Inoculum HA units	Characteristics of product*		
			Log (HA/ml.)	Log (PFU/ml.)	Log (PFU/HA)
1	1	WSN_C14; 0.05	2.1	—	—
	2	WSN_H1; 26**	0.3	—	—
	3	WSN_H2; 0.4	2.4	—	—
	4	WSN_H3; 0.05	2.1	—	—
	5	WSN_H4; 0.025	1.2	7.4	6.2
	6	WSN_H5; 0.003	1.8	7.5	5.7
	8	WSN_H7; 0.025	2.7	8.6	5.9
	11	WSN_H10; 0.17	2.4	8.3	5.9
	14	WSN_H13; 0.005	2.4	8.6	6.2
	17	WSN_H16; 0.0025	2.1	8.5	6.4
2	1	WSN_C16; 0.025	1.8	8.3	6.5
	2	WSN_H 1; 0.0013	2.1	8.2	6.1
	3	WSN_H 2; 0.0025	2.0	8.4	6.4
	4	WSN_H 3; 0.0025	2.0	8.5	6.5

* The fluid overlay was harvested 72 hours after infection for passages 1 and 2 of experiment 1 and 48 hours after infection for the other passages.
** Smaller inocula of WSN_H1 produced no detectable HA.

on infected plates of all the cell lines, were far more numerous on BHK21 cells. WSN_C12 gave similar results.

The five cell lines were tested for virus production with WSN_C14, and the following HA titers were obtained: BHK21-128; L-32; KB-16; HeLa-4; and A_2-16. The BHK21 cells were therefore selected for further studies. These cells increased ten-fold in 48 hours after transfer, and the cell sheet could be maintained in good condition for 72 hours after virus infection.

The virus progeny from the BHK21 cells were serially passaged in BHK21 cells to initiate the WSN_H series. A partial record of this series is shown in Table 1, Experiment 1. A second and similar series, initiated with WSN_C16, is recorded in Table 1, Experiment 2. WSN_H forms small, regular, clear plaques on BHK21 monolayers. Some plaques, after

staining with neutral red, have red borders similar to those observed in 1-5C-4 cell cultures infected with the CAM influenza strain (6).

Several properties of WSN_E, WSN_C, and WSN_H were compared:

1. The three viruses and polyvalent influenza vaccine, all at 16 HA units/ml., were compared by the HA inhibition test. The vaccine served to test antiserum specificity. The highest dilutions of anti-WSN serum producing inhibition of HA were: 1:640-690 for WSN_E; 1:640 for WSN_C and WSN_H; and 1:80 for polyvalent vaccine.

2. The HA titers of the three viruses were from two- to four-fold lower when sheep erythrocytes were substituted for chicken erythrocytes.

3. WSN_E and WSN_H were compared for egg 50% infectious dose (EID_{50}) by standard techniques (14, 15). The log (EID_{50}/HA) for WSN_E was 6.5 and for $WSN_H 9$ was 5.8.

4. The infectivities of $WSN_C 16$ and $WSN_H 15$ were measured in plaque forming units (PFU) on BHK 21 monolayers. The log (PFU/HA) for $WSN_C 16$ was 5.3 and for $WSN_H 15$ was 6.1.

5. Examination by electron microscopy showed that the three viruses have similar morphologies (16).

These results indicate that WSN_E, WSN_C, and WSN_H are closely related, but not identical viruses.

The two most distinguishing properties of the WSN_H-BHK 21 system are the rapid growth rate of BHK 21 cells and the high specific infectivity of WSN_H virus. The PFU/EID_{50} ratio for WSN_H is greater than one, but that ratio is less than one for other influenza virus systems using either primary (3, 4) or established (6) cell hosts. The PFU/HA ratio for WSN_H and the EID_{50}/HA ratios of egg-adapted strains are of the same magnitude. We believe that virus from the WSN_H series differs sufficiently from the WSN strain with which this investigation was initiated to warrant the separate designation WSN_H. Studies are in progress on the nucleic acid of WSN_H and the nucleic acids produced upon infection.

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