Persistent Infection of Continuous Line of Pig Kidney Cells with a Variant of the WSN Strain of Influenza A_0 Virus (36405)

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(Introduced by R. M. Chanock)

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In this report we describe the persistent infection of a continuous line of pig embryo kidney (RES) cells with a variant of the WSN strain of influenza A_0 virus. During the course of infection there were found in the culture fluids variants of influenza virus which differed from the original infecting strain in hemagglutinating activity, pathogenicity for cell cultures and virulence for mice.

In earlier studies (1, 2) we established a persistent infection of human embryo lung diploid fibroblasts with the PR8 strain of influenza A_0 virus which could be recovered from the cultures periodically for 208 days. In a similar study of human embryo skin-muscle fibroblasts infected with a variant of the same strain, virus also persisted in the cultures for a long time (235 days) after which they seem to have been spontaneously cured of infection (3). During these studies we observed that some of the variants of influenza A_0/PR8 virus recovered from the fluids of the persistently-infected cultures failed to induce detectable hemagglutination of chicken erythrocytes although they multiplied in embryonated eggs and were demonstrated in allantoic fluids in titers ordinarily sufficient to produce hemagglutination.

We then attempted to establish a persistent infection with the WSN strain of influenza A_0. WSN was chosen for study because it has the convenient properties of virulence for mice by intracerebral as well as intranasal inoculation, and cytopathogenicity for chick embryo fibroblast cultures.

Materials and Methods. Influenza virus strains. The WSN strain (4) was originally obtained from the American Type Culture Collection by Professor M. I. Sokolov, passaged in the allantoic cavities of embryonated hens' eggs, and stored lyophilized. Preliminary attempts to establish persistent infection of RES cultures with this strain have been described (5–7). In these experiments monolayers were exposed to uncloned influenza A_0/WSN virus at multiplicities of 0.0005 to 0.001 50% egg infectious doses (EID_{50}) per cell for 1 hr at 37°, inoculum removed, monolayers washed with medium and then maintained as usual. In three such experiments virus was recovered from the cultures for 6–13 days postinfection with no evidence of cytopathic effect (CPE) at any time. In a fourth experiment CPE appeared within 24 hr after inoculation, and for the following 100 days, during which 17 serial subcultures of the infected line were performed, variable degrees of CPE were always present. Virus was recovered from the cultures through day 98 of the experiment, by which time 16 subcultures had been performed. Cultures then became normal in appearance and were maintained for 116 days (15 additional subcultures); but virus was never again demonstrated in culture fluids, in cell homogenates or in cells cocultivated with uninfected RES or chick embryo fibroblast (CEF) cells, nor was viral antigen detectable by direct or indirect fluorescent-antibody techniques. The last variant of influenza A_0 virus obtained from these cultures, on day 98 of the experiment, was designated WSN/RES_{16}. This variant was found to differ in several respects from the original WSN strain; equal doses of

the original and variant viruses (by plaque titers on CEF) had about the same titers in mice by the intracerebral route, but WSN/RES_{16} had substantially higher titers in RES, human lung diploid cells and *Cercopithecus* monkey kidney cells. The WSN/RES_{16} variant was used to initiate the persistent infections described here.

Venezuelan encephalitis virus (VEE) used in interferon determinations was originally obtained from the Rockefeller Institute and passaged in CEF cultures.

Cell cultures. Swine cells were originally selected for study because swine are thought to be naturally subject to prolonged inapparent infection with influenza A_0 virus (8). A continuous line of pig embryo kidney cells, designated RES (9, 10) was used in attempts to initiate persistent infections. Cells were grown and maintained in Medium 199 containing 10% unheated beef serum and 100 units of penicillin and 100 μg streptomycin per ml. The same medium was used for the PS line of swine embryo kidney cells and the Vero line of *Cercopithecus* monkey kidney cells. Subcultures of continuous lines of cells were performed using a mixture of two parts 0.02% versene and one part 0.25% trypsin in phosphate-buffered physiologic saline.

Chick embryo fibroblast cultures were prepared from 9-day-old chick embryos using 0.25% Difco trypsin in Hanks' salt solution and grown in Hanks' solution with 0.5% lactalbumin hydrolysate, 5% beef serum and antibiotics. Primary trypsinized human embryo fibroblast (HEF) cultures were obtained from the Central Tissue Culture Laboratory of the Ivanovsky Institute of Virology and maintained in Eagle's basal medium with 5% fetal calf serum. Cultures were prepared in stationary glass tubes or flasks and were usually ready for use 2 to 3 days after seeding.

Infectivity titrations. Titrations in tissue cultures employed four tube cultures for each tenfold dilution of virus in physiologic saline or Hanks' solution. Each tube was drained of medium, inoculated with a 0.1 ml aliquot and the inoculum left in contact with the cell sheet for 30 min at room temperature; 0.9 ml of maintenance medium was then added and the culture incubated at 37°. Cultures were examined daily for CPE and results scored 3–4 and 7 days postinoculation. At each dilution four embryonated eggs, 9- to 11-days-old, were inoculated in the allantoic cavity with 0.1 ml aliquots and incubated at 37°; allantoic fluids were collected at 48 hr and tested for hemagglutination of chicken erythrocytes. At each dilution three suckling mice were inoculated with 0.02 ml by the intracerebral route or 0.1 ml by the intranasal route and observed for 10 days for signs of encephalitis or respiratory illness. Blind passages of brain or lung tissues were performed 72 hr after inoculation. Plaque assays in CEF cultures used the method of Porterfield (11).

Hemagglutination (HA). HA and HA inhibition (HAI) tests were performed in clear plastic trays. To 0.5 ml volumes of antigen or antigen and serum diluted in physiologic saline were added equal volumes of 0.5% washed chick erythrocytes in the same diluent.

Serological tests. All viral isolates were identified as influenza A_0 by HAI and/or serum neutralization tests in eggs or tissue cultures. Standard equine typing sera used in HAI tests were provided by the All-union Research Institute of Vaccines and Sera, Leningrad, and the Viral Control Institute, Moscow. Sera for neutralization tests were prepared in rats immunized with influenza B, A_2 and A_0 viruses, including the original A_0/WSN strain and our RES_{16} variant of WSN (12). HAI tests employed 4 units of antigen and 2-fold dilutions of serum. Neutralization tests employed single serum dilutions with 100 and 1000 EID_{50} of virus.

Interferon. Undiluted culture fluids were heated to 60° for 30 min; 0.7 ml aliquots were added to washed 2-day-old RES tube cultures and incubated at 37° for 24 hr. Fluids were then removed, cultures washed and 0.2 ml of medium containing 10 plaque forming units (PFU) of VEE virus per cell added to each tube. The cultures were incubated at 37° for 30 min and the inocula removed, monolayers washed once, and then 1 ml aliquots of fresh Medium 199 with 2% beef serum added. Cultures were incubated at 37° for 24 hr, culture fluids removed and

TABLE I. Recovery of Virus from Culture Fluids of Five Lines of RES Cells Infected with Influenza A_0 Virus, Strain WSN, Variant RES_{16}.

Line No.	Total duration of infection from original inoculation until final degeneration of culture: Life span of line (Days)	Cycles of cell proliferation and degeneration	Period during which presence of virus demonstrable by hemagglutinin formation after intraallantoic inoculation of eggs: Duration Days	Cycles	Positive virus isolations/number of attempts	Virus titers (log_{10} EID_{50}/ml) Range	GMT[f]	Period during which presence of virus demonstrable only by CPE in tissue culture: Duration Days	Cycles	Virus islation attempts: Egg inoculation Positive/ Total No.	GMT (EID_{50})	Tissue culture Positive/ Total No.	GMT ($TCID_{50}$)
I	144	9	62	4	16/16	1.0–7.2	3.9	82	5	0/19	0	3/3	$\geqq 5.0$[c]
II	130[a]	4	105	3	16[b]/18	2.0–5.7	3.6	25[a]	1	0/9	0	ND[g]	—
III	126	5	62	3	11/11	1.0–5.0	3.4	64	2	0/16	0	3/3	$\geqq 5.0$[c]
IV	140	14	46	2	6/6	1.7–5.3	3.4	94	12	0/21	0	3/3	$\geqq 5.0$[c]
V	146	6	27	0	1/1	4.9	—	119	6	3/30	4.1	8/8	4.8[d] 3.6[e]

[a] Culture lost due to bacterial contamination.

[b] Two negative virus isolation attempts in eggs following which virus again demonstrable by egg inoculation for 50 days.

[c] Results of two titrations in RES cultures.

[d] Four titrations in RES cultures.

[e] Five titrations in PS cultures.

[f] GMT = geometric mean titer in log_{10}.

[g] ND = not done.

assayed for VEE virus by plaque assay in CEF cultures in duplicate or triplicate. Cultures preincubated with heated Medium 199 and with fluids from uninfected RES cultures served as controls. Reduction of titers of VEE by 1.0 $\log_{10}$ or more was considered evidence of interference.

Neuraminidase. The neuraminidase activity of infected allantoic and culture fluids was determined by a modification of the method of Aminoff (13). Neuraminidase activity was expressed as micrograms of sialic acid consumed in a standard reaction mixture.

Results. Initiation of persistently-infected cultures. Six flasks of RES monolayers were inoculated with allantoic fluid containing 0.0001 to 0.001 EID_{50} of uncloned WSN/RES_{16} virus per cell. Virus was recovered from the culture fluids in titers of 3.4 to 6.9 $\log_{10}$ EID_{50}/ml at 48 hr and 6.9 to 8.0 $\log_{10}$ EID_{50} at 72 hr postinfection, levels similar to those found after WSN infection. However CPE (rounding and increased cytoplasmic granularity of cells) appeared at 72 hr, and all cultures degenerated completely by the seventh day after infection. A seventh RES monolayer was inoculated with the WSN/RES_{16} variant at a multiplicity of 0.001, and the procedure modified in that the culture medium was changed at 24 hr after infection and daily thereafter. Cytopathic effects appeared at 48 hr and by the fourth day postinoculation the monolayer had degenerated almost completely; however, four small portions of the cell sheet, each one measuring 1–2 mm in diameter, remained intact.

The four isolated colonies which remained of the cell sheet began to increase in size; on day 21 after infection portions of two of the colonies were removed mechanically and transferred to individual culture tubes, and on day 25 the other two were similarly subcultured. Cells from each of the four subcultures attached to the glass promptly and within several days small colonies of cells were growing in each of the four tubes. The four colonies of cells remaining in the original flask culture also continued to grow, and new islands of cells appeared. The original flask culture was designated as Line I and the four sublines as Lines II–V. Throughout the rest of the experiment fluids of all cultures were changed three to five times each week.

Observations of cultures. Table I summarizes observations of each of the five cell lines. Lines I–IV behaved similarly. Colonies of cells continued to enlarge and new islands to appear; however, in each colony small areas of round, refractile cells and debris were always found among the normal cells. After periods of proliferation of cells, which never formed confluent monolayers and rarely covered as much as 20% of the surface of the glass, CPE became more widespread and most of the cells again degenerated. Degeneration of cells was followed by renewed proliferation so that there were repeated cycles of proliferation and degeneration, from 4 to 14 such cycles observed in the various lines. Lines I, III and IV finally degenerated completely 144, 126 and 140 days, respectively, after the initiation of the original infection. Line II proliferated more vigorously than the other three, and on day 29 after its subculture, cells were removed from the original culture tube with trypsin–versene and transferred to a 50 ml flask; this line underwent only four cycles of proliferation–degeneration (and was lost due to contamination 130 days after the initial infection).

Line V behaved somewhat differently; following three cycles of proliferation and degeneration as in the other four lines, cells began to proliferate with exceptional vigor, and by day 68 of the experiment a fully confluent cell sheet had formed, showing no CPE. Over the next 47 days 9–12 subcultures of various sublines were performed; but beginning with the ninth subculture all the cultures again showed CPE, and almost all cells degenerated. Several portions of the cell sheet remained in one flask, and these cells underwent three additional cycles of proliferation and degeneration, following which proliferation once again became more vigorous. By day 145 of the experiment an almost-confluent cell sheet had formed again; however, following trypsin–versene subculture all cells rapidly degenerated, and no viable cells remained after day 146.

TABLE II. Absence of Interferon Activity[a] in Fluids of RES Cultures Persistently Infected with Influenza Virus A_0, Strain WSN, Variant RES_{16}.

Persistently infected RES cultures: line number	50 Days[b] Postinfection		80 Days[c] Postinfection	
	Titer of challenge virus[d]	HA on primary egg passage	Titer of challenge virus[d]	HA on primary egg passage
I	6.6	+	5.7	−
II	5.7	+	6.4	+
III	6.7	+	6.1	−
IV	6.9	+	6.4	−
V	6.8	−	ND	ND
Controls:	Titer of challenge virus			
Uninfected RES cultures	6.7			
Medium 199	6.8			

[a] Reduction of titers of VEE challenge virus by ≥ 1 $\log_{10}$PFU by preincubation of RES with fluids inactivated 60° for 30 min.

[b] I, II, III = 53 days; IV and V = 56 days postinfection.

[c] 82 days postinfection.

[d] RES cultures incubated with fluids from persistently-infected cultures, washed, and then challenged with VEE at MOI 10PFU, and VEE in fluids titered in CEF 24 hr later.

ND = not tested.

Interferon activity in infected cultures. Nine culture fluids from the five lines were examined for the presence of interferon-like activity (Table II). Although two fluids were found to contain interferon-like factors, seven did not.

Virus isolations. Culture fluids from each line were examined for the presence of virus once or twice each week. In fluids from Lines I through IV virus was easily demonstrated until 62, 105, 62 and 46 days after initial infection, respectively, using the routine method of intraallantoic inoculation of eggs with hemagglutination by the allantoic fluid as the indicator of presence of virus. Following these variable initial periods virus could no longer be demonstrated in the four lines by routine egg inoculation. Fluids from Lines I through IV were studied on 19, 9, 16 and 21 occasions, respectively, by inoculation of eggs and three serial blind passages in eggs without the appearance of hemagglutinins in the allantoic fluids. However the continued presence of CPE in the cell lines, with recurring cycles of cell destruction and proliferation, suggested that virus might still be present. Nine of the allantoic fluids harvested after initial inoculations of eggs were passaged into normal RES cell cultures; typical CPE was produced in all cases. Six of the nine fluids were titrated in PS cell cultures and they were found to contain >5.0 $\log_{10}$ $TCID_{50}$ per ml of virus. One variant (VL-IV-106)[1] from Line IV, one of the viruses so demonstrated, was selected for further study (see below) and was subsequently confirmed serologically as influenza A_0 virus.

Line V was studied in greater detail. Culture fluid yielded hemagglutinating virus by routine inoculation of eggs only until day 27 after infection (two days after subculture of the line from Line I). For the next 119 days culture fluids were studied on 30 occasions by intraallantoic inoculation followed by three serial blind passages in eggs, and, as in the first four lines, hemagglutinins were not demonstrated. Eight of the 27 culture fluids failing to produce hemagglutinins on routine inoculation of eggs were tested in RES and PS cell cultures, and all were found to contain virus cytopathogenic for these cultures, in titers of 3.0 to 6.8 $\log_{10}$ $TCID_{50}$/ml of original culture fluid. Neutralization test of

[1] The variant is designated VL-IV-106 to show that it was isolated from Line IV on day 106 after initial infection; other variants were designated by the same convention.

TABLE III. Comparison of Selected Biological Properties of the WSN Strain of Influenza A_0 Virus with Variants Recovered from Persistently-Infected Cultures.

	Biological Properties[b]								
	Pathogenicity for cell cultures		Pathogenicity for embryonated hens' eggs		Pathogenicity for suckling white mice				
Virus Variant[a]	PS $\log_{10}TCID_{50}$/ml	CEF $\log_{10}PFU$/ml	Infectivity $\log_{10}EID_{50}$/ml	Hemagglutinin formation in allantoic fluid	Intracerebral route $\log_{10}LD_{50}$	Intranasal route $\log_{10}LD_{50}$	Neuraminidase activity[d] μg/ml	$\mu g/TCID_{50}$	$\mu g/EID_{50}$
WSN	3.7	5.4	6.6	+	5.8	ND	65	17.6	9.9
WSN/RES_{16}	5.5	5.0	7.0	+	5.9	ND	57	10.4	8.1
VL-V-63 (3rd egg pass.)	3.5	—[c]	$\geqq 1.0$	—	—	—	25	7.2	ND
VL-V-63 (5th egg pass.)	4.0	8.3	9.2	+	8.2	2.3	31	7.7	3.4
VL-V-146	3.5	7.5	8.8	+	7.5	1.0	32	9.2	3.6

[a] All variants prepared in allantoic fluid.

[b] All titers are geometric means of two to seven determinations.

[c] — = negative at all dilutions tested including undiluted allantoic fluid.

[d] Modification of the method of Aminoff (1961), neuraminidase activity expressed as mcg sialic acid consumed from standard reaction mixture.

[e] ND = not tested.

[f] + = positive in undiluted fluid; not titered.

one of these variants (VL-V-63) in PS cultures showed it to be influenza A_0 virus.

On day 125 of the experiment primary allantoic inoculation of culture fluid from Line V again resulted in hemagglutinin formation, and hemagglutinating virus was also detected on days 132 and 146 (titers 3.1, 1.3 and 8.1 $\log_{10}$ EID_{50}/ml in each of the three culture fluids, respectively).

Reversion of variants to hemagglutinin production. When the variant VL-V-63 was passaged through the allantoic cavities of embryonated eggs, hemagglutinins were not detected for four passages. On the fifth passage hemagglutinins were detected in allantoic fluid, and hemagglutinins continued to be produced through four additional passages, with infectivity titers in the allantoic fluids of 8.1–9.5 $\log_{10}$ EID_{50}/ml, and HA titers of 1:640 to 1:1024. The reappearance of detectable hemagglutinins was also found with another "nonhemagglutinating" variant from Line IV (VL-IV-106) after four passages in eggs followed by three in PS cells.

Biological properties of variants of virus isolated from infected RES cultures. Preliminary studies were undertaken to see if the biological properties of the influenza virus had changed during prolonged infections of RES cells. One such study of two variants obtained from Line V is summarized in Table III. Of greatest interest is the variant VL-V-63, which did not elicit detectable hemagglutinins in allantoic fluid on the first through fourth passages, but did so on the fifth and all subsequent passages. Using PS cells as the indicator, the third egg passage of VL-V-63 was found to contain 3.5 $\log_{10}$ $TCID_{50}$/ml of allantoic fluid; the original WSN and the variant VL-V-146 at about the same titers contained readily detectable hemagglutinins.

Unlike the original WSN and WSN/RES_{16} strains, the "nonhemagglutinating" third egg passage of VL-V-63 did not produce plaques in CEF monolayers under agar overlay; on the fifth egg passage this property had returned. Further studies of third egg passage VL-V-63 in tissue culture are shown in Table IV; it proved to be noncytopathogenic for both primary CEF and human embryo fibroblast monolayers in fluid cultures while retaining cytopathogenicity for two continuous lines of pig embryo kidney (PS and RES) and the Vero line of *Cercopithecus* monkey kidney cells. The third egg passage VL-V-63 also appeared to lack pathogenicity for suckling mice by either intracerebral or intranasal routes at the titer of virus present, whereas the fifth egg passage of the same variant was virulent for mice by both routes.

No significant differences in neuraminidase activity of the different variants were detected.

Discussion. Although persistent infections of various animal cell cultures with many viruses have been described (14), cultures persistently infected with influenza virus have been reported infrequently (15). Tyrell (16) found that cultures of bovine kidney cells infected with the WS strain of influenza A_0 virus continued to release virus for three months, and that an interferon-like substance could be demonstrated in the culture fluids. Sykes *et al.* (17) reported persistent infection of a continuous line of human carcinoma cells with several strains of influenza A_0 and with one of A_2 virus. Vyalushkina and Gavrilov (18) described an infection of guinea pig lung cells with A_0/WSN, and Timakov *et al.* (19), a probable persistent infection of L-cells with the same strain.

Walker (14) has suggested criteria for classifying persistently-infected animal cell cultures according to differences in the probable mechanisms by which infections are sustained. The peculiar behavior of the RES cultures infected with WSN/RES_{16} frustrated our efforts to complete analysis by Walker's criteria; when we attempted to clone infected cells with and without antiserum in the medium, to superinfect with influenza and other viruses and to prepare cells for fluorescent-antibody staining of antigen, the cultures rapidly degenerated. However, it is clear that no antiserum or other antiviral substances were required to sustain persistent infection, and no interferon-like activity could be consistently detected in the culture fluids.

The disappearance of the property of hemagglutination is puzzling and not explained

TABLE IV. Cytopathogenicity[a] of the WSN Strain of Influenza A_0 Virus and Two Variants for Several Types of Cell Culture.

	Primary cell cultures		Continuous cell lines		
	CEF	HEF	RES	PS	Vero
WSN	+[b]	+	+	+	+
WSN/RES_{16}	+	+	+	+	+
VL-V-63 (3rd egg passage)	−[c]	−	+	+	+

[a] Three serial passages at 5 day intervals.

[b] + = complete destruction of cell sheet at each passage.

[c] − = no cytopathic changes in any passage, and negative results of inoculation into RES indicator cultures of culture fluids and cell homogenates at each passage level.

by our preliminary observations. We first supposed that virus was simply not multiplying in eggs to titers sufficient to cause detectable hemagglutination. This seems unlikely because the original WSN and WSN/RES_{16} strains at the same infectivity titers readily hemagglutinate. However the nonhemagglutinating variants were never recovered in titers higher than 6.5 $\log_{10}$ $TCID_{50}$/ml, and it would have been desirable to examine concentrated higher-titered preparations both by hemagglutination and by electron microscopy. Unfortunately such concentrates were not prepared successfully.

"Nonhemagglutinating" strains of influenza virus have been described several times (20–22); one such strain was believed to have lost its V antigen (21); another apparently retained its ability to provoke HAI antibodies, and it was concluded that its hemagglutinin must be "masked" (22). The appearance of such altered strains of influenza virus has not previously been reported in association with persistent infections.

The linkage of nonhemagglutination with changes in other biological properties resembles that recently demonstrated for four laboratory characteristics associated with neurovirulence of the WSN and NWS strains by Hobson *et al.* (23), who speculated that the simultaneous change of several such markers might represent a repression or deletion of adjacent genes. The simultaneous reversion to the original state by all the altered properties of our variants seems inconsistent with a deletion or other genotypic change.

It is not yet clear which, if any, of the various mechanisms which maintain persistent viral infections of cell cultures play important roles in chronic infections of intact organisms. Persistent infections of animals with influenza virus have been described (8, 24). It has been suggested that latent infections of animals or man might constitute the reservoir of influenza virus between epidemics (25–27) and that the mechanism sustaining such infections might be analogous to that of a carrier culture (28). We speculate that transient alterations in the biological properties of influenza viruses similar to those observed during experimental persistent infections of tissue cultures may take place in natural latent infections.

Summary. RES cells were infected with the RES_{16} variant of the WSN strain of influenza A_0 virus and the culture medium changed at frequent intervals; five lines of cells were established in which infection was sustained for periods of 126 to 146 days. Each line of persistently-infected cells showed recurring cycles of cell degeneration and repopulation; these cultures did not remain in equilibrium with the virus indefinitely, however, and ultimately degenerated.

The presence of virus in the RES culture fluids was easily demonstrated for periods of 27 to 105 days in various cell lines using as the indicator intraallantoic inoculation of eggs with hemagglutination by the allantoic fluids. Subsequently this method failed to demonstrate virus, although the continued presence of CPE suggested that the cultures were still infected. By using as indicator of the presence of virus the production of CPE in normal RES or PS cultures inoculated with fluids from the infected lines, it was found that influenza A_0 virus was still present in the fluids, and that it was able to propagate in eggs without producing detectable hemagglutinins. Only modest infectivity titers were found in the nonhemagglutinating allantoic fluids; however the original WSN and WSN/RES_{16} variants at the same infec-

tivity titers readily produced detectable hemagglutination. This fact suggests that the virus had not simply lost its ability to multiply in eggs to titers sufficient to produce hemagglutination, but that at least part of the infectious virus did not hemagglutinate. Spontaneous reversion to the original hemagglutinating state was noted in one infected line (VL-V) during the last days of infection, just before complete degeneration of the culture, and in two cases reversion followed serial passages in eggs (VL-V-63) and in eggs and PS cells (VL-IV-106).

In preliminary studies it appeared that "nonhemagglutinating" variants also differed from WSN, WSN/RES_{16}, and other "hemagglutinating" variants in several other biological properties: nonhemagglutinating variants were not virulent for mice by the intracerebral or intranasal routes and they were not cytopathogenic for primary human embryo fibroblast or CEF monolayer cultures either under fluid medium or agar, but they retained cytopathogenicity for RES, PS and Vero cells. Reversion to hemagglutination was accompanied by the return of these other lost properties.

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