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Properties of Influenza C Virus Grown in Cell Culture

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Influenza C virus was propagated successfully in primary chicken embryo lung (CEL) and fibroblast cells and in Madin-Darby canine kidney (MDCK) cells. In other cell lines, either no virus or only noninfectious hemagglutinin (HA) was produced. In productively infected cells (CEL), HA and infectious virus appeared by 24 h and reached a maximum by 36 to 48 h, cell-associated virus remaining at a constant low level. Infected Vero cells produced noninfective HA by 24 h which also remained predominantly cell associated until 60 to 72 h, when the cells disintegrated. Viral antigen was demonstrable on membranes of both CEL- and Vero-infected cells at 24 h; Vero cells yielded membrane vesicles containing HA, but none of the spherical or filamentous viral particles synthesized in CEL cells. Influenza C virus produced in cell culture or in eggs differed in several important respects from A and B viruses and from Newcastle disease virus. All influenza C preparations, regardless of infectivity or source, lacked detectable neuraminidase activity, yet retained the ability specifically to inactivate receptors only for influenza C. Influenza C HA was not inhibited by soluble glycoproteins highly active against HA of A virus. A rat serum glycoprotein uniquely inhibited influenza C by binding to the surface components of virions.

Group C influenza virus, a relatively infrequent cause of human influenza (6), has been classified as an orthomyxovirus on the basis of morphology, ability to agglutinate erythrocytes (1), destruction of receptors (11), and more recently its polypeptide composition and segmented genome (3, 14, 18).

Recent studies in this (M. Loughlin, D. Labat, R. J. O'Callaghan, and C. Howe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S306, p. 264) and other laboratories (14) have demonstrated that, unlike influenza A and B viruses, influenza C virus lacks neuraminidase and fails to bind to any of the sialoglycoproteins that represent receptor analogs for group A and B viruses. Although the chemical nature of the C receptor is still unknown, the interaction of C-virus hemagglutinin (HA) with cells and/or soluble HA inhibitors should lead to the identification of the receptor molecule and the definition of its relation to the membrane. Hitherto, all studies of attachment and elution have been done with virus grown in eggs. As has been pointed out elsewhere (14), virus grown in eggs, even after density gradient centrifugation, may still contain host-derived components. Comparative studies of viral growth in hosts other than eggs was therefore undertaken in order to define the chemical basis for viral interaction with receptors on erythrocytes, as well as those on cells in culture.

MATERIALS AND METHODS

Virus. Influenza C strain JJ, after 290 to 300 chicken amniotic passages, was used throughout these studies. Virus was routinely propagated in the amniotic cavity of 10-day embryos by direct inoculation of 0.2 ml of virus followed by incubation for 3 days at 32°C. Harvested amniotic fluid, assayed by hemagglutination of chicken erythrocytes, consistently contained 640 to 1,280 HA units (HAU)/ml. Influenza A (strains PR8 and A2/Japan), influenza B (Lee), and Newcastle disease virus were grown in the chorioallantoic (CA) membrane. Harvested infective chorioallantoic fluid (CAF) was stored at −70°C.

Cell and tissue cultures. Various cell cultures (approximately 10^6 cells) were inoculated with 0.2 to 0.4 ml of virus (256 to 512 HAU) and incubated at 32 or 37°C. Chicken embryo tracheal rings were prepared according to the method of Cherry and Taylor-Robinson (2). Chicken embryo fibroblast (CEF) cultures were prepared by trypsin treatment and maintained in Eagle minimal essential medium according to Lennette and Schmidt (15). Chicken embryo lung cells (CEL) were prepared according to the method of Darlington et al. (5). Vero (African green monkey kidney), Madin-Darby bovine kidney (MDBK), and Chang conjunctiva cells, 3T3 mouse fibroblasts, and L-929 cells were obtained from the American Type Culture Collection, and Madin-Darby canine kidney (MDCK) cells were obtained from Flow Laboratories, Inc., Rockville, Md. All cells were grown in minimal essential medium containing 10% fetal calf serum.

Hemadsorption assays. Infected and normal monolayers were washed free of medium and covered with 3 ml of 0.5% chicken or human erythrocytes.

Hemadsorption was allowed to take place at 4°C for 90 min. All flasks were then washed with cold phosphate-buffered saline (0.15 M, pH 7.2) until control monolayers showed no residual erythrocytes. Cultures showing single isolated hemadsorbing cells were scored as 1+. More widespread hemadsorption was scored on a scale up to 4+.

Inhibition studies. Four HAU of virus was mixed with various concentrations of nonspecific inhibitors, or with antisera in final volumes of 0.2 ml. After incubation for 30 min at 23 or 4°C, 0.2 ml of 0.5% chicken erythrocytes was added, and the agglutination patterns were allowed to form. Inhibitors tested included neuramine lactose (Calbiochem), Collocalia mucoid (13), fetuin (Colorado Serum Co.), rhesus and human glycoproteins derived from membranes solubilized with lithium diiodosalicylate-phenol (17) or chloroform-methanol (7), and rat serum extracted with chloroform-butanol (CB) (3:1, vol/vol). The CB-treated rat serum was concentrated by ultrafiltration through an Amicon XM-100 filter. Solutions of inhibitors were made from lyophilized preparations except for rat serum preparations, concentrations of which were quantitated on the basis of protein analyses by the Lowry method (16).

Neuraminidase assay. Approximately 25 HAU of virus in 0.1-ml quantities was mixed with 0.4 ml of substrate in phosphate buffer (pH 5.5) and incubated at 32, 35, or 37°C. After 2 or 18 h, samples were assayed for free *N*-acetylneuraminic acid (NANA) by the thiobarbituric acid method of Warren (21). Substrates were also hydrolyzed with 0.1 N H_2SO_4 for 1 h at 80°C to determine total NANA content. Substrates tested included neuramine lactose, fetuin, and Collocalia mucoid. Influenza virus (A2/57/Japan) served as a positive control for each assay.

Virus concentration and purification. Virus was pelleted by centrifugation at 105,000 × *g* at 4°C for 2 h in the 60 Ti Beckman rotor. Pellets were allowed to disperse with gentle agitation for 2 h at 4°C in phosphate-buffered saline. Pelleted virus (1.0 ml) was layered on a 20 to 50% (wt/wt) sucrose gradient and centrifuged for 3 h in an SW27 rotor at 24,000 rpm (113,000 × *g*). Virus-rich fractions were identified by HA titration. Sucrose was removed by passage through a Sephadex G-25 column (2.5 by 25 cm). This procedure resulted in negligible loss of viral infectivity and HA.

Preparation of antisera. Antibody to influenza C virus was prepared in roosters by intravenous injection (2 ml) of amniotic fluid containing 512 HAU of influenza C virus. Conjugation of these antisera with ferritin was kindly performed by K. C. Hsu, Columbia University. Antisera to influenza A, influenza B, Newcastle disease virus, and host factor were previously described (12). Antisera to normal Vero cells were prepared in rabbits by multiple intravenous injections of 10^6 cells each over a period of 2 weeks. Animals were bled 10 days after the final injection.

Electron microscopy. For the detection of viral antigens on cell surfaces, infected and control monolayers were treated with conjugated serum in situ, washed six times with phosphate buffer, fixed with glutaraldehyde (1% for 1 h at 4°C), and embedded in Epon as previously described (4). Thin sections and virus negatively stained with 2% phosphotungstic acid were examined in a Philips 201 or Hitachi HU11A electron microscope.

RESULTS

Host range. Attempts to grow influenza C in the CA membrane at 37°C failed repeatedly. At 32°C, only 30% of the eggs inoculated yielded CAF with titers in excess of 160 HAU/ml. Simultaneous inoculation of both the CA and amniotic cavities yielded virus which on subsequent CA inoculation consistently produced CAF with titers of 640 to 1,280 HAU/ml.

The ability of influenza C to replicate in a variety of cell systems was assessed by hemagglutination, hemadsorption, and infectivity assays in homologous systems and in chicken amnion (Table 1). In chicken tracheal ring organ cultures, primary inoculation and subculture passages of influenza C virus caused no ciliary inactivation, hemagglutination, or hemadsorption, and no infectious virus was produced. Limited levels of infection on primary inoculation

TABLE 1. *Replication of influenza C virus in cells of different hosts*

Cell[a]	1st passage		Infectivity on 2nd passage[b]	
	HAU/ml	HAD[c]	Homologous	Chicken embryo (AF)
Host				
AF	1,280	ND[d]	++++	–
CAF	640–1,280	ND	++++	++++
CTR	8	–	–	–
CEF	128	+	+	++
CEL	64–128	+	+	++
Vero	128–256	+	–	–
MDBK	64	+	–	–
MDCK	320	+++	++	+++
Chang	64	+	–	–
L-929	0–8	+	–	ND
3T3	0–8	+	–	ND

[a] Abbreviations: AF, Amniotic fluid; CAF, chorioallantoic fluid; CTR, chicken tracheal rings; CEF, chicken embryo fibroblast; CEL, chicken embryo lung; Vero, African green monkey kidney; MDBK, Madin-Darby bovine kidney; MDCK, Madin-Darby canine kidney; Chang, Chang conjunctiva; L-929, mouse fibroblast; 3T3, mouse cells.

[b] Infectivity in homologous systems and chicken amnion was measured by HAD and/or hemagglutination titers of extracellular fluids. Viral replication in vitro was judged by HAD on a scale of – (none) to 4+ (confluent) or in ovo by HA titers of 512 HAU/ml or greater.

[c] Hemadsorption (HAD) judged on a scale of – (none) to 4+ (confluent).

[d] ND, Not done.

of L-929 and 3T3 cells was evidenced by hemadsorption in rare isolated cells and the production of HA to low titer. Greater levels of infective virus were obtained in primary cultures of chicken embryo cells (CEF and CEL) and in cultures of MDBK, MDCK, Vero, and Chang conjunctiva cells as evidenced by HA titers of 64 HAU/ml or greater and 1+ to 4+ hemadsorption. Highest HA titers were consistently obtained in MDCK, Vero, and CEL cells. Hemagglutination particles produced in CEF, CEL, and MDCK cells initiated infection on inoculation in ovo (amniotic cavity) or in the homologous cell system. However, the hemagglutinating particles produced in Vero, MDBK, Chang conjunctiva, L-929, and 3T3 cells were never infectious in ovo or in the homologous cell system.

The abortive infection in Vero cells provided an accurate and reproducible measure of infectivity of virus samples. The quantity of infectious virus in an inoculum determined, up to a maximum, the number of cells in a Vero monolayer that became infected as evidenced by hemadsorption (Fig. 1). Virus in concentrations up to saturation multiplicity could be accurately quantitated in this system.

The identity of C virus produced in the several systems tested was confirmed by hemagglutination inhibition tests and inhibition of hemadsorption by specific antisera. Infectious viral HA from CEL, CEF, and MDCK cells and noninfective HA from Vero cells were inhibited up to a dilution of 512 by influenza C-specific antisera, including National Institutes of Health reference serum and our chicken antisera. Antisera to other influenza viruses failed to neutralize group C virus grown in any culture system. Rabbit antisera to chicken host factor inhibited HA of egg-grown C virus, but not of C virus grown in nonavian cells. In addition, antisera to normal Vero cells neutralized viral HA from infected Vero cells to a titer of 16 to 64.

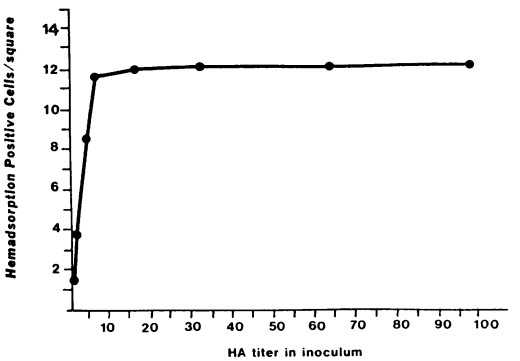


FIG. 1. *Titration of influenza C infectivity in Vero cells. Vero cells in 35-mm plates with 2-mm² grids seeded to give approximately 500 cells/square were infected with influenza C virus at predetermined HA titers. At 48 h p.i., the number of hemadsorbing cells was counted in 10 random squares by two independent observers.*

Kinetics of viral growth. The growth kinetics of virus was measured in a cell system (CEL) that produced infectious virus as well as in one that produced defective particles (Vero). Virus production in CEL cells began at about 20 h postinfection (p.i.), the concentration of extracellular virus increasing to a maximum by about 36 h, measurable as either HA or infectivity (Fig. 2). For the latter, hemadsorption end points in Vero cells were used as the indexes of infection. The increase in HA titer closely paralleled the increase in infectivity, indicating that infectious particles were being made at all times postinfection. The titer of cell-associated virus released by freeze-thawing remained relatively low throughout the period of observation. Cell-associated virus failed to exceed a titer of 128 HAU/ml, representing only 25% of the total HA in the culture. Virus released from cells by three cycles of freezing and thawing had an infectivity/HA ratio equivalent to or exceeding that of extracellular virus.

Hemagglutinating virus first appeared in Vero cells 12 h after infection. In contrast to the CEL

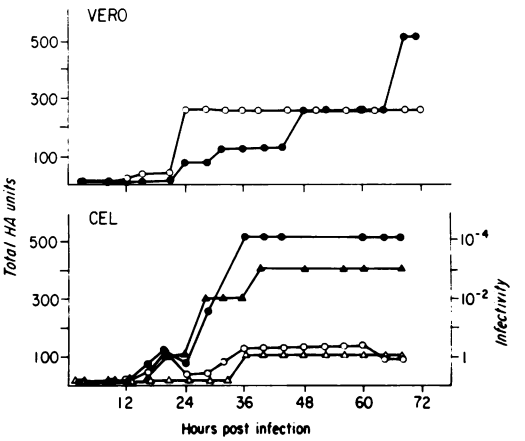


FIG. 2. *Kinetics of influenza C growth in Vero and CEL cells. Monolayers of Vero and CEL cells were infected with 256 HAU of virus and incubated at 32°C. Extracellular fluid and extracts of infected cells, prepared by three cycles of freeze-thawing, were obtained at 3-h intervals and frozen at −70°C for subsequent titrations. The infectivity titers of CEL samples were calculated from hemadsorption endpoint determinations as described in the text. Symbols: (●) Cell-free HA titer cell; (○) cell-associated HA titer; (▲) cell-free virus infectivity; (△) cell-associated virus infectivity.*

system, viral HA produced 24 to 48 h p.i. remained predominantly cell associated; at 48 to 72 h, extracellular HA equaled or exceeded cell-associated HA. At 72 h, there was a sudden increase in extracellular virus, probably due to nonspecific cellular disintegration noted in infected monolayers and simultaneously in control cultures. Even at this stage, however, cells from infected monolayers could be passaged, the infection continuing in subcultures as evidenced by hemadsorption and production of HA to low titer. By the fourth to sixth passage, no more hemadsorbing cells could be found.

Morphology of virus and infected cells. Progeny of both productive and abortive infections were studied by electron microscopy, using chicken antibody labeled with ferritin. Labeled antisera to virus were unreactive with control uninfected monolayers of CEF or Vero cells (Fig. 3A). At 24 h p.i., viral antigen appeared predominantly as budding viral particles, and only limited amounts of membrane-associated antigen were detected (Fig. 3B). In contrast, cells examined at 48 h p.i. contained viral antigen along linear or projecting regions of the cell membrane and in what appeared to be membranous bodies (Fig. 3C). These changes were limited to about 2% of the Vero cells examined, consistent with the occurrence of hemadsorption in only a few cells in each confluent monolayer (Fig. 1). At no time could nucleocapsids be identified morphologically in either budding particles or cell cytoplasm.

The infectious progeny produced in CEL cells and released into the medium were pleomorphic, including both spherical and filamentous forms (Fig. 4A–D). The spherical particles were covered with spikes and had an approximate diameter of 100 to 120 nm. The filaments, measured along segments with parallel sides, were 75 nm wide and enlarged terminally to a diameter of 120 nm. The noninfectious hemagglutinating particles produced in Vero cells were also pleomorphic but, unlike CEL virus, did not include spherical particles. The predominant forms were much larger (500 to 750 nm) membrane vesicles covered with spikes, presumably of viral origin (Fig. 4E).

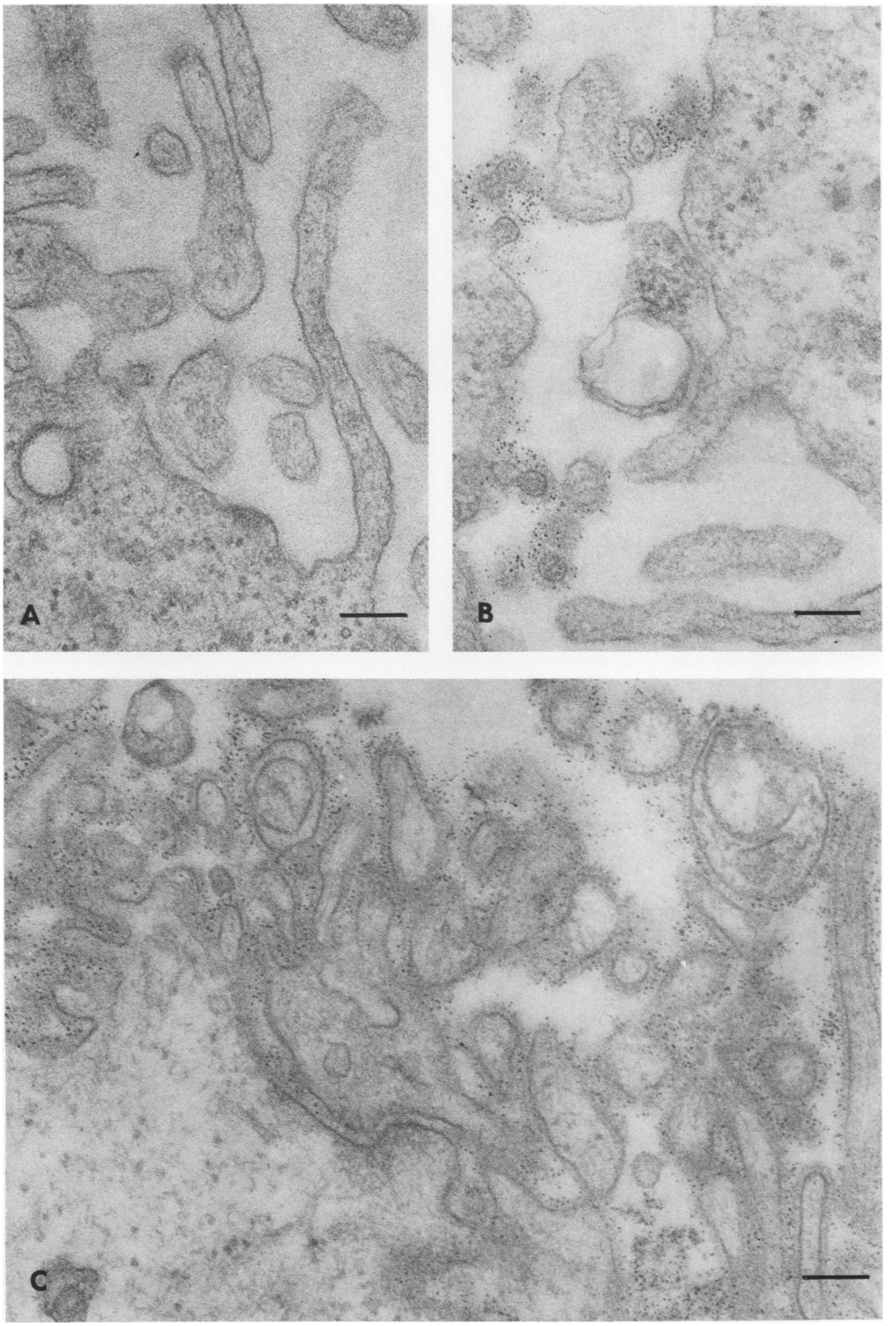
Attachment and elution. Defective and infectious particles were compared with respect to attachment to and elution from erythrocytes and glycoproteins. Influenza C grown in chicken embryos eluted from erythrocytes within 60 to 90 min at 37°C, causing complete destruction of receptors for fresh influenza C virus. Samples of virus grown in Chang, Vero, MDBK, CEL, and MDCK cells eluted from chicken erythrocytes at the same rate, as judged by the conversion of positive hemagglutinin patterns to negative buttons and concomitant complete destruction of receptors for influenza C. Receptors for influenza A and B viruses were unaffected by prior attachment and elution of influenza C.

Infectious and defective influenza C particles were compared with influenza A and B viruses with respect to susceptibility to inhibition by glycoprotein receptor substances and analogs (Table 2). Collacolia mucoid and glycoproteins obtained from human erythrocyte membranes treated with lithium diiodosalicylate-phenol or chloroform-methanol were potent inhibitors of influenza A HA. The same virus was inhibited also by fetuin in somewhat higher concentrations. None of these substances were active against influenza C, even at 50- to 500-fold-higher concentrations. Analogous glycoproteins of rhesus erythrocytes, active against measles viral HA, lacked inhibitory activity for any of the influenza viruses tested, thus reflecting the differences in hemagglutinin receptor specificity between myxoviruses and measles virus.

In contrast, CB-extracted rat serum showed significant inhibition of influenza C virus from all sources, but had no effect against influenza A virus. CB-extracted rat serum also completely blocked hemadsorption by Vero cells infected with influenza C virus, repeated washing of which failed to restore hemadsorption.

Influenza C virus grown in cell culture or in the amniotic cavity had no detectable neuraminidase activity when tested with Collocalia mucoid, fetuin, CB-extracted rat serum, or neuramine lactose as substrates, whether incubated at 37, 35, or 32°C. The chicken embryo virus had been partially purified and concentrated by sucrose density gradient centrifugation to remove chromogens, which are normally found in amniotic fluid and which interfere with the thiobarbituric acid measurement of free NANA. Under the conditions used, influenza A/Japan released 53 to 93% of the NANA available on the various substrates tested.

FIG. 3. *Immunoelectron microscopy of influenza C-infected Vero cells. Monolayers of Vero cells infected with 256 HAU of influenza C and monolayers of mock-infected Vero cells were incubated at 32°C for 24 or 48 h. At these times, monolayers were washed, covered with ferritin-conjugated viral antisera, incubated at room temperature for 1 h, washed, and prepared for sectioning. (A) Control, mock-infected cells at 48 h; (B) influenza C-infected cells at 24 h p.i.; (C) infected cells at 48 h p.i. Magnification is ×70,200, and bars represent 150 nm.*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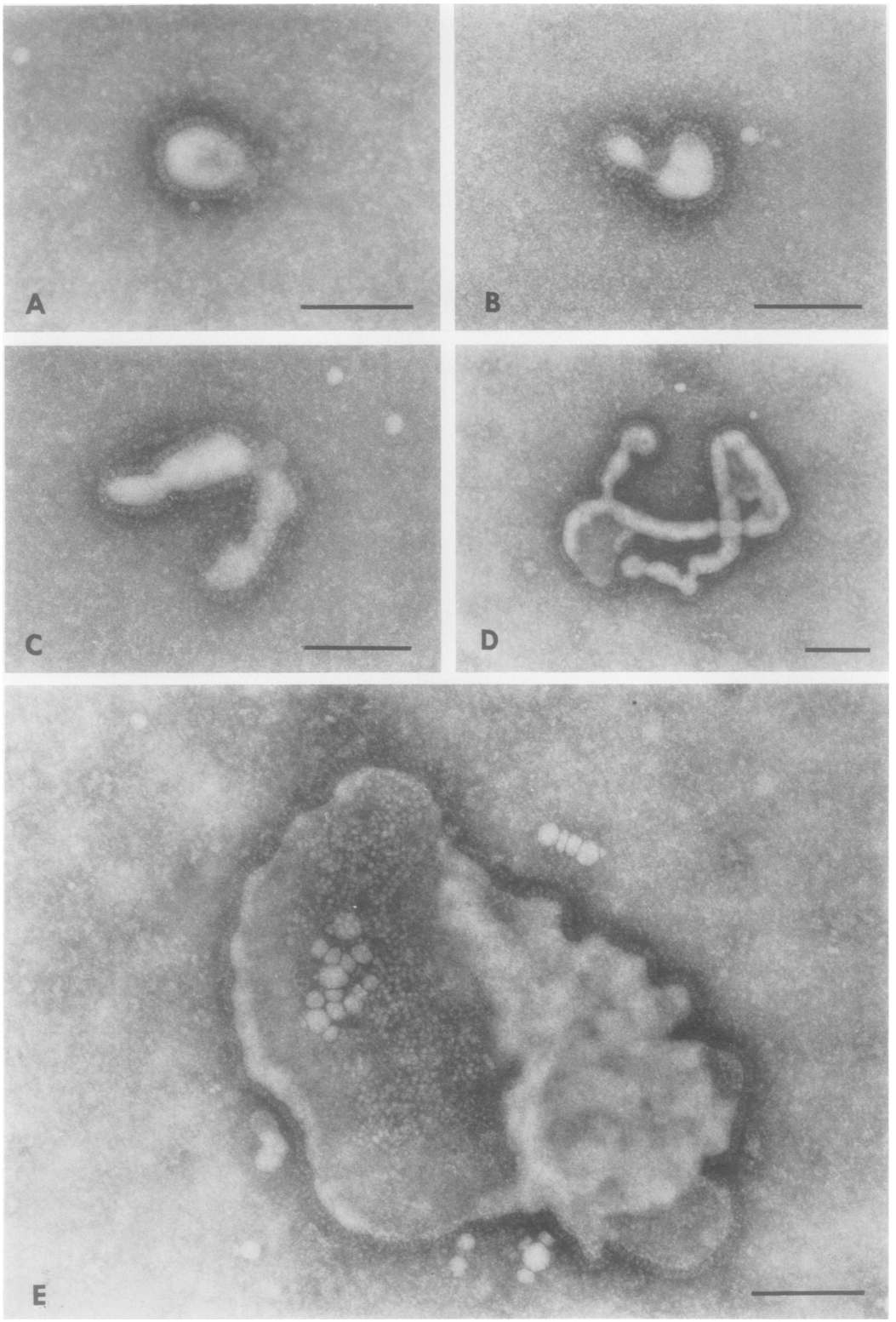
A
B
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D
E

DISCUSSION

We have shown that infection with influenza C virus could be initiated in a variety of cell lines, among which only MDCK cells supported synthesis of infectious virus. Particles released after infection of Vero cells contained HA but were noninfectious, suggesting a defect(s) in some structural component of the virus or faulty viral assembly. Deficiencies in RNA, nucleocapsid protein, matrix protein, or elution enzyme are all possible reasons for this observation, viral elution being the only one of these factors that could be readily tested. In this respect, the otherwise defective particles produced by Vero cells were comparable to fully infectious virus from other sources in destroying influenza C receptors on chicken erythrocytes. The actual defect(s) responsible for the abortive infections in Vero and other cell lines (L-929, 3T3, MDBK) remains to be identified.

Specific hemadsorption developed in a consistently low percentage of cells in monolayers inoculated with influenza C virus over a wide range of input multiplicities. This finding implies that some physiological characteristic of a small segment of the cell population is required for even abortive infection to occur, or that only a small subpopulation of cells with a stable trait or in a specific period of cell division were susceptible at the time of infection. Experiments with synchronized cultures now underway may provide answers to some of these questions.

The results of our studies of the attachment and elution of virus grown in various cell types agree with earlier observations on egg-grown virus made by ourselves and others. We have confirmed that the influenza C virion lacks neuraminidase, yet destroys receptors in the course of elution from erythrocytes. This holds true for virus obtained from any host system thus far examined, making it unlikely that the putative enzyme might be a host-derived contaminant in preparations of virus grown in chicken embryos.

Chicken host antigen is present in influenza C virus grown in either amniotic or chorioallantoic sites, as shown by inhibition of viral HA by rabbit antibody to normal chicken tissue. Antibody to normal Vero cells inhibited the noninfective HA which is produced in Vero cells after inoculation with influenza C and which comprises cell membrane bearing viral envelope constituents. These particles undoubtedly still contain normal membrane antigens, antibody to which may inhibit adjacent HA by steric hindrance.

The receptor for influenza C virus probably does not include NANA. Sialoglycoprotein receptor substances and analogs, although show-

TABLE 2. *Inhibition of measles and influenza A and C viral HA by soluble glycoprotein receptor substances and analogs*

Soluble receptor substance or analog	μg[a] required to inhibit 4 HAU of:				
	Influenza A (FML)	Influenza C			Measles
		AF	CEL	Vero	
Collocalia mucoid	1	>60	>60	>60	ND[b]
Fetuin	20	>50	>50	>50	ND
Glycoprotein from:					
Human erythrocyte ghosts					
LIS-phenol extracted[c]	0.1	>60	>60	>60	>100
CM extracted[d]	1.0	>50	>50	>60	>50
Rhesus erythrocyte ghosts					
LIS-phenol extracted	>60	>60	>60	>60	2
CM extracted	>60	>50	>50	>50	6
Neuramine lactose	>50	>50	>50	>50	ND
Rat serum (CB extracted)[e]	>250	10	10	10	>250

[a] Glycoproteins were quantitated in terms of dry weight except for CB-extracted rat serum, which was quantitated by protein content.
[b] ND, Not done.
[c] LIS, Lithium diiodosalicylate.
[d] CM, Chloroform-methanol.
[e] CB, Chloroform-butanol.

FIG. 4. *Morphology of influenza C virus propagated in cell culture. Extracellular fluids of infected cultures were centrifuged to pellet the virus, and the resulting pellets were negatively stained with phosphotungstic acid (2%, wt/vol). (A–D) Virus obtained from CEL cells; (E) particle obtained from Vero cells. Bars represent 150 nm. (A), (B), (C), and (E) are magnified ×120,000, and (D) is magnified ×67,500.*

ing potent inhibitory activity against HA of influenza A viruses, were inactive against influenza C HA. On the other hand, CB-extracted rat serum inhibited only influenza C and had no effect against influenza A as originally reported by Styk (19). The nature of this C-specific inhibitor has not been fully elucidated (8–10, 20). We have established that its activity is due to direct blockage of viral HA, rather than to any interaction with the erythrocyte. For this reason, rat serum inhibitor appears to contain a molecule(s) representing a specific receptor analog for influenza C virus. Purification and analysis of the active protein, its binding to virus, and its modification by viral elution enzyme will be the subject of a subsequent report.

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