Sendai Virus Replication:
An Ultrastructural Comparison of Productive and Abortive Infections in Avian Cells

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(Accepted 17 July 1970)

SUMMARY
Production of Sendai virus and virus-induced ultrastructural changes were compared in infected chick embryo lung epithelial cells and chick embryo fibroblast cells. By 48 hr after infection, chick embryo lung cells made 100 times more infectious virus, as measured by plaque assay, than chick embryo fibroblast cells. The chick embryo lung cells showed little cytopathology up to 48 hr, while chick embryo fibroblast cells began to show severe cytopathic changes at 24 hr. Both cell types contained abundant cytoplasmic aggregates of virus nucleocapsids, but budding virus particles were more common on the surfaces of chick embryo lung cells. The data indicate that late steps in virus maturation are relatively inefficient in chick embryo fibroblast cells.

INTRODUCTION
Sendai virus, a parainfluenza virus isolated from mammals (Chanock & Parrott, 1965), is classified with other parainfluenza viruses in the 'paramyxovirus' group (myxovirus subgroup II of Waterson, 1962).

Egg-adapted Sendai virus grows to high titre in ovo, and the embryos survive for several days (Jensen, Minuse & Ackermann, 1955; Blair & Robinson, 1968), but it does not grow as well in primary chick embryo fibroblasts (Blair & Robinson, 1968) and in many types of cultured mammalian cells (Gresser, 1961; Zhdanov, Bukrinskaya & Azadova, 1961; Bukrinskaya & Zhdanov, 1963; Northrop & Walker, 1965). Some types of cells yield disproportionate amounts of virus haemagglutinin relative to infectious virus (Zhadnov et al. 1961; Bukrinskaya & Zhdanov, 1963), and virus-induced cytopathology, sometimes including extensive cell fusion (Okada & Tadokoro, 1963), occurs soon after infection. These interactions of Sendai virus with cultured cells might be termed 'abortive', as in the interaction of SV 5 and BHK cells (Holmes & Choppin, 1966), while the interaction of Sendai virus with cells of the chorioallantoic endoderm might be considered 'productive', as with SV 5 and monkey kidneys cells (Compans et al. 1964; Holmes & Choppin, 1966).

We wished to study biochemical events in Sendai virus replication in a productive cell culture system and compare virus-induced changes with those occurring in an abortive system. Chorioallantoic endoderm in vitro (Barry, 1965) and cultured epithelial cells are about as productive for Sendai virus as the egg (Shimizu et al. 1955; Heath & Tyrrell, 1958; Mannweiler, 1960; Matsumoto & Maeno, 1961). Since recent biochemical work with Sendai virus was done with chick embryo fibroblast (CEF) cells (Blair & Robinson, 1968; Barry & Bukrinskaya, 1968), we chose epithelial cells from the same species, chick embryo
lung (CEL) cells (Franklin, Rubin & Davis, 1957), as the productive host for these comparisons. In this report we describe kinetics of virus growth and ultrastructural changes in both cell types.

METHODS

Virus. The strain of Sendai virus used in this work was obtained from Dr J. Enders, Harvard University (Gresser & Enders, 1961). Virus haemagglutinin was assayed by diluting virus in a 0.5% suspension of chicken erythrocytes. We passed the virus twice in the allantoic cavities of 12-day embryonated hens' eggs at 0.01 haemagglutinating units (HAU) per egg, and a third time at 1 HAU per egg. This stock contained incomplete virus (Kingsbury, Portner & Darlington, 1970; Kingsbury & Portner, 1970) which was eliminated by serial passages of the virus in chick embryo lung (CEL) cells at an input multiplicity of 0.01 p.f.u./cell with harvest at 48 hr after infection. Virus from the seventh such low multiplicity passage, containing only infectious virus particles, was used in the experiments reported here.

Cells. Primary chick embryo fibroblast (CEF) monolayer cultures were prepared by digestion of 12-day decapitated chick embryos with 0.25% pronase (Calbiochem). Plastic tissue culture plates (60 mm.) were seeded with cells suspended in Earle's saline containing 0.5% lactalbumin hydrolysate, 2% foetal calf serum, 500 units/ml penicillin, and 100 μg/ml of streptomycin (GM). After incubation for 48 hr at 37° in an atmosphere of 5% CO₂ in air, confluent monolayers were obtained. About 95% of the cells had fibroblastic morphology.

Chick embryo lung (CEL) cells were prepared by stirring whole lungs of 14-day chick embryos for 30 min. at room temperature with 0.25% pronase in phosphate-buffered saline lacking divalent cations. The cells were washed three times with the buffer saline by centrifugation at 3000g for 1 min. The washed cell pellet was suspended in GM and filtered through gauze. Since the lungs were not separated into single cells by pronase, cell concentrations were estimated by counting aggregates of three or more cells. About 7.5 x 10⁵ aggregates in 5 ml. of GM were added to each 60 mm. Petri dish. After incubation for 48 hr at 36° in 5% CO₂ and air, confluent monolayers had formed which consisted of 90 to 95% epithelial cells.

Plaque titration. Sendai virus has been titrated most commonly in embryonated eggs by the end-point dilution method (Gresser, 1961). A haemadsorption technique using monolayer cultures of Chang's human conjunctival cells (Northrop & Walker, 1965) has also been described. We discovered that Sendai virus formed plaques in CEL monolayers and devised a plaque method which is as sensitive as infectivity titrations in embryonated eggs. Cell monolayers were washed with phosphate-buffered saline (PBS) containing divalent cations and inoculated with 0.1 ml. of virus suspension. After adsorption for 30 min. at 37°, cultures were overlaid with 5 ml. of 1% agar in Earle’s saline containing 0.5% lactalbumin hydrolysate, 0.1% yeast extract, 1.0% gelatin, 5% foetal calf serum and antibiotics. After 5 days, a second 5 ml. nutrient overlay containing neutral red was added to the monolayers. Foci of infection were first seen as 1 mm. zones of apparent hyperplasia on the fourth day after infection. The cells began to disintegrate thereafter and the foci became plaques 0.5 to 1.5 mm. in diameter by the tenth day.

There was a linear relationship between virus concentration and plaque number. A single stock of Sendai virus, titrated in CEL monolayers on six different occasions, gave plaque titres ranging from 2.4 x 10⁸ to 3.6 x 10⁸/ml. Comparison of the plaque assay with egg infectivity titration (EID 50 as estimated by the Reed & Muench (1938) method, eight eggs/virus dilution), revealed approximately equal sensitivities: 3.3 x 10⁸ p.f.u./ml. versus 2.5 x 10⁸ EID 50/ml.
Our strain of Sendai virus did not produce plaques in CEF cells, although infected cultures showed severe cytopathic effects when maintained under a liquid medium.

*Infection of cells.* Monolayers of CEF or CEL cells were inoculated with 5 to 10 p.f.u./cell. After 30 min. at 37°, the inocula were removed, and the monolayers washed three times with PBS. Five ml. of GM were added and the cultures were incubated at 37°. At intervals after infection, the medium was removed from the monolayers and assayed for virus haemagglutinin and infectivity. At all times after infection, over 90% of the progeny virus was extracellular, as determined by comparing virus titres in the medium with titres in cells disrupted by three cycles of freezing and thawing.

*Electron microscopy.* Infected and control cells collected at 24 or 48 hr after infection were scraped from the plates and washed by suspension in PBS and sedimentation at 1000g. The washed cells were fixed in 1% glutaraldehyde, postfixed in 1% osmic acid, and dehydrated in graded alcohols. The fixed cells were then embedded in epoxy resin 812 and sectioned. Sections were stained with uranyl acetate (Stempak & Ward, 1964) and lead citrate (Reynolds, 1963). Negatively stained specimens were prepared as previously described (Kingsbury & Darlington, 1968). Specimens were examined in a Siemens 1A electron microscope.

**RESULTS**

*Virus growth*

Chick embryo lung cells were productive for Sendai virus; as shown in Fig. 1a, they made about 100 times more virus per cell than chick embryo fibroblast cells (Fig. 1b). In cells, virus HA production paralleled infective virus production, and it was only by about 40 hr that p.f.u.: HAU ratios decreased much below 10^6. This late relative decline in virus infectivity probably reflects thermal inactivation of the virus, since we found the half-life of Sendai virus infectivity in growth medium to be 6 hr at 37°. CEF cells not only made less infective virus than CEL cells in the first 15 hr, but there was little increase in infective virus titre thereafter and p.f.u.: HAU

![Fig. 1. Single-step growth of Sendai virus (a) in chick embryo lung (CEL) cells and (b) in chick embryo fibroblast (CEF) cells. ○——○, p.f.u./cell; •——•, HAU/10^6 cells.](image)
ratios decreased below 10^6 from 24 hr onwards. Evidently, Sendai virus replication in CEF cells was abortive when compared to growth in CEL cells. There did not appear to be any block in adsorption or penetration of virus into CEF cells, since haemadsorption tests showed cell-associated virus haemagglutinin in 95% of cells by 12 hr after infection, indicating successful infection, though each cell had only a few erythrocytes attached. In contrast, the surfaces of the more productive CEL cells were completely covered by erythrocytes when they were examined in the same way.

Light microscopic examination of infected CEL cultures under fluid medium revealed little cytopathic effect up to 48 hr after infection. Thereafter, cells became progressively more refractile and rounded, but only a small minority had detached from the monolayer by 72 hr after infection. On the other hand, CEF cells began to round up and detach from the monolayer by 24 hr after infection, and by 48 hr after infection few cells remained attached. We saw polykaryons only rarely in infected CEL or CEF cultures.

Intracellular development of Sendai virus

The morphological aspects of the intracellular development of Sendai virus were essentially the same regardless of the cell type (CEL or CEF). There was extensive accumulation of fibrils (Fig. 2, 3) in the cell cytoplasm at 24 hr after infection, but this was more marked by 48 hr. In some instances these fibrils filled almost the entire cytoplasm of the cells. The fibrils measured from 160 to 180 Å in diameter, and when cut in cross-section appeared hollow (dashed arrow, Fig. 4).

In order to determine whether or not the masses of fibrils seen in infected cells were virus nucleocapsids, infected CEL cells were washed three times in PBS, suspended in 0.01 M-KCl, 0.0015 M-MgCl2, 0.01 M-tris-HCl (pH 7.4) and disrupted with a Dounce homogenizer. Extracts were clarified by low speed centrifugation, negatively stained with phosphotungstate and examined in the electron microscope. Deoxycholate-treated Sendai virus particles were used as controls. The cytoplasmic extracts contained numerous rigid nucleocapsid fragments, 170 Å in diameter (Fig. 5) which were identical in appearance to the nucleocapsids released from Sendai virus particles by sodium deoxycholate treatment (Fig. 6).

Virus release

Numerous budding virus particles were seen in Sendai virus-infected CEL cultures (Fig. 7). The particles were quite pleomorphic and many filamentous forms were seen. However, no filamentous forms were seen in negatively stained preparations of virus from such cultures. This probably reflects sensitivity of these forms to changes in osmolarity and/or surface tension during negative staining.

In Sendai virus-infected CEF cultures, budding forms were occasionally present but were much less frequent than in CEL cells. Many CEF cells contained nucleocapsids arrayed just under the plasma membrane (Fig. 8). That portion of the membrane overlaying these filaments had the fuzzy appearance characteristic of virus haemagglutinin in thin section.

Discussion

Although chick embryo lung cells produced more Sendai virus than chick embryo fibroblast cells, virus nucleocapsids accumulated abundantly in both cell types. Thus, even in the productive Sendai virus infection, synthesis of nucleocapsid subunits and nucleocapsid assembly seemed to proceed faster than packaging of nucleocapsids into virus particles. This contrasts with observations of Compans et al. (1964) on growth of the related paramyxovirus,
Fig. 2. CEL cell 48 hr after infection with Sendai virus. Nucleocapsid filaments occupy much of the cell cytoplasm.

Fig. 3. CEF cell 48 hr after infection with Sendai virus. The cytoplasm of this cell contains abundant nucleocapsid filaments.
Fig. 4. CEF cell 48 hr after infection with Sendai virus. Higher magnification showing nucleocapsids cut transversely (dashed arrow) and longitudinally (solid arrows).

Fig. 5. Negatively stained (PTA) nucleocapsids from a cytoplasmic extract of Sendai virus-infected CEL cells, 48 hr after infection.

Fig. 6. Negatively stained (PTA) nucleocapsids released from Sendai virus particles by 0.5% sodium deoxycholate.
SV 5. These workers found that virus nucleocapsids accumulated markedly in relatively unproductive BHK cells, but not in productive monkey kidney cells. Another difference is that BHK cells fused extensively after SV 5 infection, while we saw little cell fusion in either CEF or CEL cells. Still, the factor which determines whether infection by a paramyxovirus is cytocidal or moderate may be a function of the cell membrane, as Compans et al. (1964) suggested. We saw microvilli and budding viruses in much higher concentrations at the surfaces of CEL cells than in CEF cells, indicating greater biosynthetic activity of the epithelial cell plasma membranes and suggesting a relationship between membrane biosynthesis and virus envelopment.

Accumulation of abundant nucleocapsids in CEF cells indicates that many steps in Sendai virus replication before virus assembly occur normally in these cells. Thus, CEF cells, which are more conveniently obtained in large quantities than CEL cells, may be suitable for examinations of virus-specific macromolecules synthesized after infection (Barry & Bukrinskaya, 1968; Blair & Robinson, 1968). However, it is possible that productive cells differ quantitatively or even qualitatively from unproductive cells with respect to such macromolecule syntheses. Full understanding of Sendai virus replication and the molecular basis of abortive infection may require comparative studies of both cell types. CEL cells are useful in another way: for studies on virus RNA and other virus
constituents, where radioisotopically labelled virus particles may be required, CEL cells make abundant particles relatively uncontaminated by cellular debris (Kingsbury et al. 1970; Kingsbury & Portner, 1970).

In other papers (Kingsbury et al. 1970; Kingsbury & Portner, 1970), we describe incomplete virus particles and subgenomic RNAs of Sendai virus. When CEF or CEL cells were infected with virus populations containing mixtures of incomplete and infectious Sendai virus, in contrast to complete virus used in this study, the time course of virus replication and ultrastructural features of infected cells were similar to what we have reported here, but even less infectious virus was produced by CEF cells (Portner & Kingsbury, 1969; Darlington, Portner & Kingsbury, 1969).

Miss E. Morgan and Miss R. A. Scroggs provided skilled technical assistance. Supported by Research Grants AI-05343 and AI-05765 from the National Institute of Allergy and Infectious Diseases, by Training Grant CA-05176 from the National Cancer Institute, and by ALSAC. D. W. Kingsbury received Career Development Award HD-14,491 from the National Institute of Child Health and Human Development.

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(Received 10 July 1970)