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Infectivity of Human Coronavirus Strain 229E

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The replication of human coronavirus strain 229E was observed by using indirect immunofluorescence in infected monolayers of MRC continuous cells. By 8 h after infection, bright cytoplasmic fluorescence was detected in cells infected with human coronavirus 229E. Discrete foci of infection were observed from 8 to 16 h after infection in cells infected with high dilutions of human coronavirus 229E; each fluorescent focus corresponded to a single virus infection. A fluorescent focus assay is described, using indirect immunofluorescence, which is more sensitive than the established techniques of tube titration and plaque assay. Particle/infectivity ratios for unpurified and purified virus preparations revealed a considerable drop in infectivity on purification.

Human coronavirus strain 229E (HCV 229E) is a member of the family Coronaviridae (11, 14), which is composed of lipid-containing enveloped RNA viruses with distinctive widely spaced, club-shaped surface projections. The virus causes mild upper respiratory tract infections in man (11). The replication of HCV 229E in human cells has been studied by indirect immunofluorescence and thin-section electron microscopy (1, 2, 5). The first virus specific structures were observed by electron microscopy within 6 h after infection (2, 5), coinciding with the first specific immunofluorescence of infected cells stained with convalescent human serum (5). Virus particles were found within rough endoplasmic reticulum vesicles and beneath cell membranes within 12 h after infection (1, 5) and in cytoplasmic vesicles and in extracellular spaces within 24 to 36 h after infection (1), before the appearance of a cytopathic effect (5). Results presented in this paper from indirect immunofluorescence confirm and extend some of these observations.

At present the most common ways of quantifying infectious HCV 229E particles are by tube titration or plaque assay (2, 3, 6-8, 13). In this report, we describe a fluorescent focus assay for infectious HCV 229E particles using indirect immunofluorescence. We compare the sensitivity of this assay to those of tube titration and plaque assay.

MATERIALS AND METHODS

Virus culture. HCV 229E was grown in monolayer cultures of diploid cells of the MRC continuous (MRCc) line as described previously (10). The cell monolayers were infected at an input multiplicity of 0.1 50% tissue culture infective dose per cell and, after an adsorption period of 1 h at 33°C, were incubated at 33°C for 32 h in Eagle basal medium with 2% newborn calf serum. In some experiments, this unpurified virus suspension was clarified by centrifugation at 2,000 × *g* for 30 min at 4°C and then stored at −70°C.

Virus purification. For other experiments, virus was further purified at 0 to 4°C by pelleting at 75,000 × *g* followed by one or two cycles of isopycnic centrifugation in sucrose gradients as described previously (10). Virus of density 1.18 g/ml was used.

Tube titration. Virus was titrated in roller-tubes containing MRCc monolayers, with 3.16-fold ("half-log") dilutions with three tubes per dilution (2).

Plaque assay. The method used was based on that of Bradburne and Tyrrell (2). Cells were seeded into 50-mm petri dishes (Sterilin, Teddington, Middlesex, England) at 1.5×10^6 cells per dish in 5 ml of Leibovitz L15 medium and were used after 2 days of incubation at 37°C. The medium was then removed, and 0.2 ml of virus inoculum was added to each monolayer. Three plates were inoculated per virus dilution and adsorption was for 1 h at 33°C. Afterwards, the plates were drained and then covered with 5 ml of agar overlay medium. Plates were incubated for 5 days at 33°C and then fixed in Formol saline for 2 h at 25°C. The overlay was removed and the cells were stained with gentian violet.

Preparation of antisera. Immune serum against HCV 229E was prepared in New Zealand white rabbits as described previously (C. A. Kraaijeveld, M. H. Madge, and M. R. Macnaughton, J. Gen. Virol., in press). A 0.5-ml portion of purified virus was mixed with an equal volume of Freund complete adjuvant and then injected intracutaneously at up to 20 different places in the shaven back of a rabbit. Animals were bled before and 6 weeks after immunization, and the sera were stored at −20°C. Before use, sera were adsorbed with MRCc cells at 4°C for 16 h.

Indirect immunofluorescence technique. Cells were seeded on 12-mm cover slips and used after overnight incubation. The monolayers were infected with virus dilutions and fixed at appropriate times by

addition of methanol. The cover slips were halved and mounted, cell side uppermost, on microscope slides with Diatex, a fast-drying, nonfluorescent mountant. Positive serum to HCV 229E or a negative control serum was applied to each half of each cover slip at a dilution of 1:10 and also to uninfected control cells. Monolayers were incubated for 10 min at 37°C in a moist chamber, and the serum was then removed by two, 10-min washes in phosphate-buffered saline. After drying, the monolayers were treated with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulins, 1:10 (Wellcome Research Laboratories, Beckenham, England), for 10 min at 37°C, as described previously. Excess conjugate was removed by two 10-min washes in phosphate-buffered saline (the second containing a few drops of 2.5% trypan blue as counterstain); then the monolayers were rinsed in distilled water and were dried at 37°C. Fluorescence was observed under oil immersion with a Nikon SKE microscope, fitted with a Projectina epi-fluorescence attachment.

Scanning electron microscopy. Monolayers of cells on cover slips were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) containing 5% sucrose for 24 h at 4°C. After washing with 0.1 M cacodylate buffer, they were postfixed for 1 h on ice in 1% osmium tetroxide buffered with 0.1 M cacodylate buffer, pH 7.3. The fixed monolayers were dehydrated through a graded series of ethanol (25 to 100% in water) and subsequently dried at the critical point of liquid CO_2 with a Polaron Critical Point Drying Apparatus (Polaron Equipment Ltd., Watford, Herts, England). Dried cover slips were then mounted on aluminum stubs using Electrodag 915 (Acheson Colloids Company, Plymouth, England). A thin layer of gold was then evaporated onto the cells in a Polaron E5100 sputtering apparatus before examination in a Philips PSEM 500 scanning electron microscope.

Particle counts. Particle counts were performed on the virus preparations with the "loop-drop" method (15) which was modified due to the high salt concentration in the samples; grids were dipped four times in distilled water before final negative staining. This method (15) gives an error of about ±20%.

RESULTS

Infection of MRCc cells with HCV 229E. Cells in normal monolayer cultures of MRCc cells were typical fibroblasts, although the size of the cells varied considerably, with some particularly large, possibly multinucleate cells. As it was difficult to differentiate the cell edges by light microscopy, scanning electron microscopy was used for better resolution (Fig. 1). Cellular processes were observed connecting adjacent cells, with no apparent boundaries between the processes and the cells (Fig. 1a and b). Thus, there is probably continuity in the cytoplasm of these adjacent cells through the cytoplasmic processes. The morphology of MRCc cells, infected with a variety of HCV 229E dilutions, was unchanged up to 24 h after infection. After 24 h of infection, there was a cytopathic effect in the form of a general degeneration of the cells, but there was no sign of the syncytia described for many other coronaviruses (11).

Detection of virus antigen in infected cells by immunofluorescence. The appearance of virus antigen in infected monolayers of MRCc cells was observed by indirect immunofluorescence at hourly intervals from 4 h after infection. Within 8 h after infection a bright fluorescence was observed throughout the entire cytoplasm of the infected cells (Fig. 2), and this was particularly strong in the perinuclear region. Although some fluorescence appeared to be nuclear, it was shown by focusing the microscope through the complete cells to be cytoplasmic fluorescence above the nuclei.

From 8 to 16 h after infection, infected cells, detected by fluorescence, were found singly or in small clumps of up to eight (Fig. 2). Fig. 2a shows a single fluorescent cell and Fig. 2b shows a group of four fluorescent cells. An average of two to three fluorescent cells per group was observed for a number of virus dilutions from 8 h to 16 h after infection (Table 1), and the numbers of fluorescing cells followed a Poisson distribution. As many of the MRCc cells were multinucleate, being joined by cytoplasmic bridges, we suggest that the groups of fluorescent cells represent a single focus of infection and that the virus antigen synthesized was distributed throughout the continuous cytoplasm of these cells. Fluorescent foci on the monolayers showed Poisson distribution.

At later stages of infection the number of fluorescent foci increased, and within 32 h after infection 20 to 30 fluorescent cells per focus were observed (Table 1). The increase in the number of fluorescent cells per focus after 16 h of infection was probably due to a release of infectious virus particles from the infected centers to the surrounding cells. Furthermore, within 24 h after infection the number of groups of fluorescent cells increased. This was presumably caused by released virus from the first cycle of replication infecting more cells. The time course of these later stages was variable.

Determination of virus infectivity by fluorescent focus assay. The titers of infectious virus from various HCV 229E preparations were calculated by counting the number of fluorescent foci per monolayer of HCV 229E-infected MRCc cells for known virus dilutions (Tables 1 and 2). In each case, the cell monolayers were infected with the smallest possible volume of virus suspension so that each virus particle had the shortest possible path to the cell surface.

A number of factors were important in obtain-

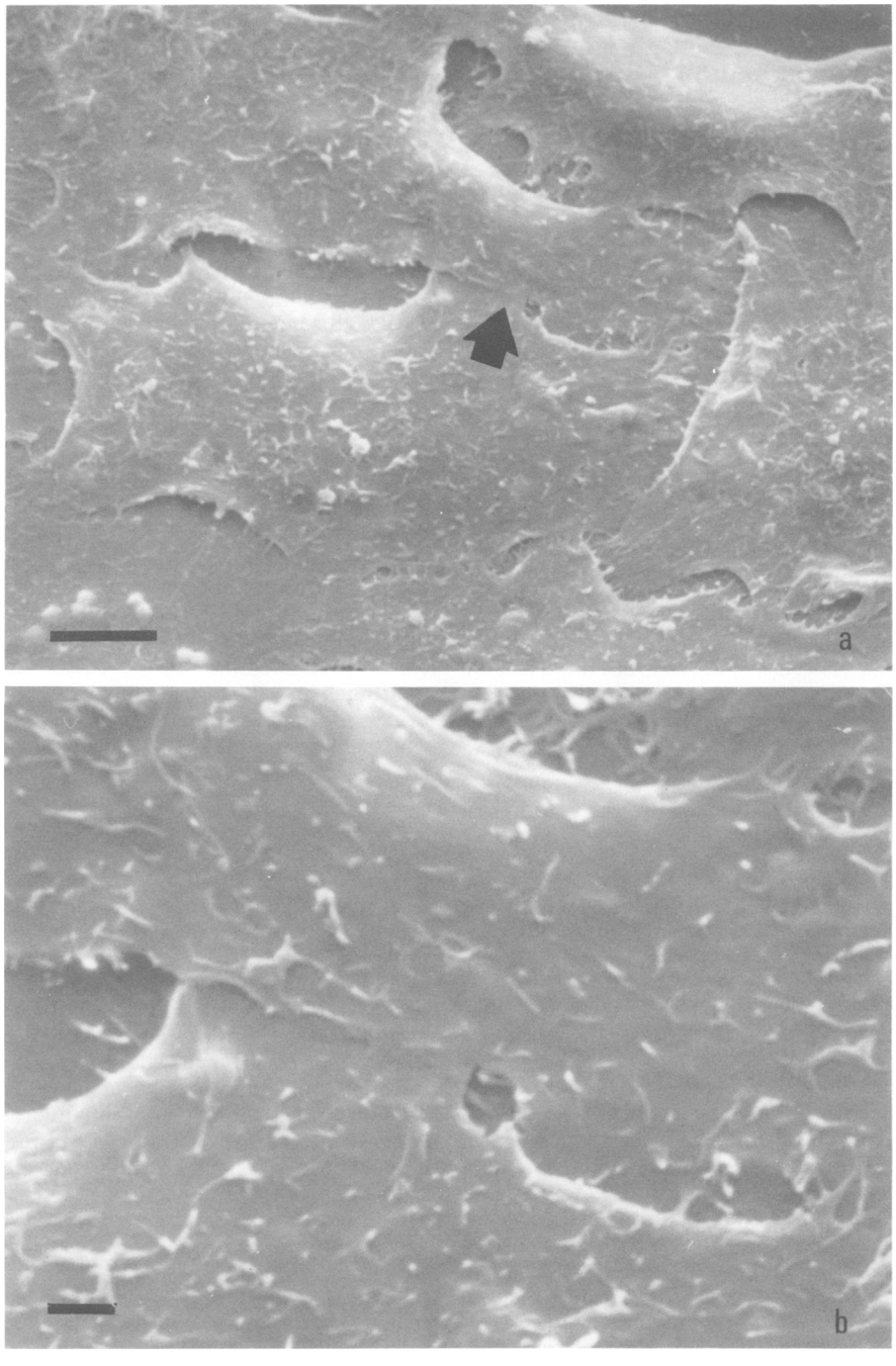

FIG. 1. *Scanning electron micrographs of uninfected MRCc cells. (a) Low power micrograph, arrow indicates a cellular process connecting adjacent cells. Bar represents 5 μm. (b) Higher power micrograph of the connecting cellular process shown in (a). Bar represents 1 μm.*

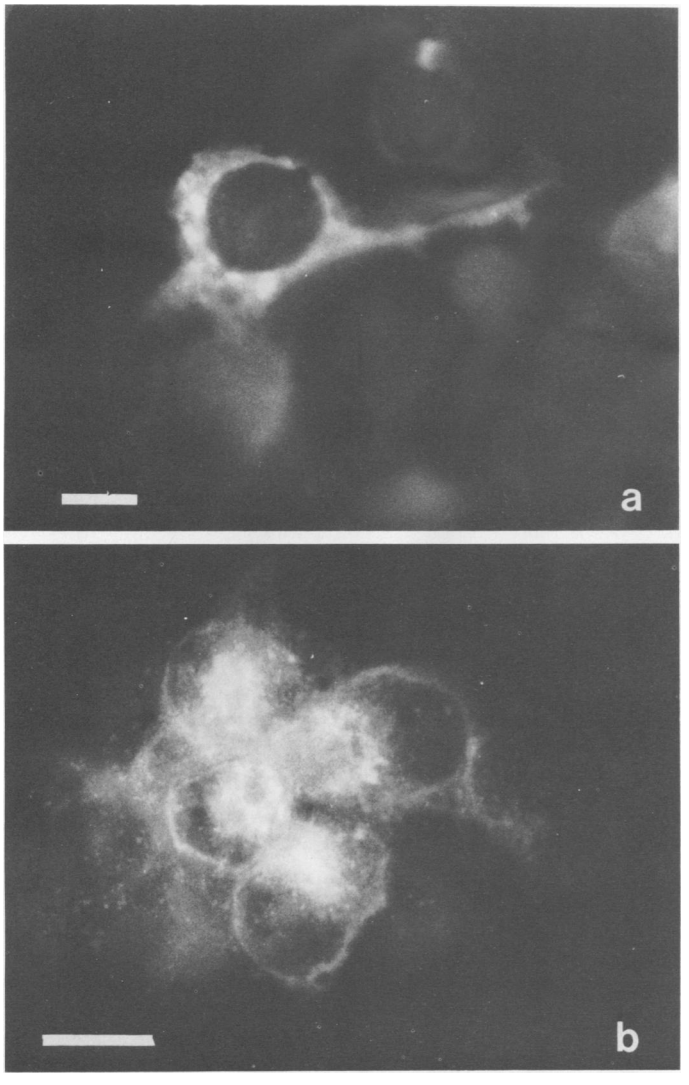

FIG. 2. *Micrographs of fluorescent foci from HCV 229E-infected MRCc cells for 8 h at 33°C. (a) One cell; (b) a group of cells. Bar represents 10 μm.*

ing maximum titers of infectious virus. As virus antigen did not appear at the same time in all cells, not all of the infected cells fluoresced in monolayers stained in less than 8 h after infection, and cells from a second cycle of infection fluoresced when staining was done later than 16 h after infection. The age of the MRCc monolayers also influenced the susceptibility of the cells to HCV 229E. Lightly seeded, 1-day-old monolayers were more susceptible than were older, more heavily seeded monolayers. The virus dilution in the inoculum influenced the titer of infectious virus (Table 2). Higher titers of infectious virus were obtained with MRCc monolayers infected with low HCV 229E concentrations. This was probably related to the difficulty in counting large numbers of fluorescent cells, and to the infection of cells with more than one virus particle in monolayers infected with high virus concentrations.

Thus, in order to record maximum infectious virus, lightly seeded 1-day-old MRCc monolayers were infected with the smallest possible volume of virus suspension for between 8 and 16 h. The highest virus dilution producing at least one fluorescent focus per 10^3 cells was used to calculate the virus titer.

Comparison of different virus assays. Co-

TABLE 1. *Titers of infectious virus obtained at different times after infection in HCV 229E-infected MRCc cells*

Time after infection (h)	Virus dilution[a]	No. of fluorescent foci per monolayer × 10^{-2} [b]	Avg no. of fluorescent cells per focus[b]	Estimated virus (titer/ml) of undiluted inoculum
8	10^{-3}	40	2.0	4.0×10^7
16	10^{-3}	40	2.8	4.0×10^7
24	10^{-4}	7	5.9	7.0×10^7
32	10^{-4}	29	27.0	2.9×10^8

[a] Lowest dilution producing a readable number of fluorescent cells.

[b] Determined from counting 50 fields: 107 cells per field, 2×10^5 cells per vial, 0.1 ml of inoculum of virus.

TABLE 2. *Titers of infectious virus obtained after 15 h from MRCc monolayers infected with various HCV 229E dilutions*

Virus dilution	No. of fluorescent foci per monolayer × 10^{-2} [a]	Estimated virus (titer/ml) of undiluted inoculum
10^{-2}	170	5.6×10^6
10^{-3}	32	1.1×10^7
10^{-4}	4	1.3×10^7

[a] Determined from counting 50 fields: 107 cells per field, 1.0×10^6 cells per petri dish, 0.3 ml of virus inoculum.

ronaviruses are unstable and readily lose infectivity and their structural integrity on purification (11). Thus, in the following experiments to compare virus infectivity and particle/infectivity ratios for different assay methods, HCV 229E preparations were used that had undergone minimum purification. The virus preparations were samples of supernatant fluids from HCV 229E-infected MRCc cells that had been clarified by centrifugation at $2,000 \times g$ for 30 min at 4°C and then stored at −70°C. In the electron microscope, virus particles from these preparations appeared as typical coronavirus particles, with little or no clumping.

Table 3 shows the results of a typical experiment comparing the titers of infectious virus and particle/infectivity ratios of an HCV 229E preparation by tube titration, plaque assay, and fluorescent focus assay. Titers of infectious virus determined by fluorescent focus and plaque assays were about 15 times higher than those determined by tube titration. It is interesting to note that in all experiments, higher titers of infectious virus were obtained by fluorescent focus assay rather than by plaque assay, and that the particle/infectivity ratios obtained by both fluorescent focus and plaque assays were less than 50.

TABLE 3. *Comparison of different virus assays*

Virus assay	Virus (titer/ml)	Particle/infectivity ratio[a]
Tube titration	10^6 ($10^{5.5}$ to $10^{6.5}$)[b]	540
Plaque assay	1.5 (1.4 to 1.7) $\times 10^7$ [c]	36
Fluorescent focus assay	4.0 (3.6 to 4.2) $\times 10^7$ [c]	13

[a] Particle count by electron microscopy, 5.4×10^8/ml.

[b] Average of 10 experiments (range of virus titers in parentheses).

[c] Average of 5 experiments (range of virus titers in parentheses).

TABLE 4. *Comparison of particle/infectivity ratios of different virus preparations by fluorescent focus assay*

Virus purification	Virus (titer/ml)	Particle count per ml	Particle/infectivity ratio
Unpurified[a]	1.25×10^7	5.40×10^8	4.3×10^1
Purified[b]	5.60×10^6	7.04×10^{10}	1.3×10^4

[a] Clarified supernatant fluid from infected MRCc monolayers (incubated for 32 h at 33°C).

[b] Clarified supernatant fluid from infected MRCc monolayers (incubated for 32 h at 33°C) was further purified by pelleting at $75,000 \times g$ followed by one cycle of isopycnic centrifugation in sucrose gradients. Complete virus particles of density 1.18 g/ml (9) were used.

Effect of purification of yield of infectious virus. The results above describe the assay of infectious virus in relatively impure virus preparations, namely culture medium from HCV 229E-infected MRCc cells that had been clarified once. Any substantial purification of the virus, such as pelleting at $75,000 \times g$ followed by one or two cycles of isopycnic centrifugation in sucrose gradients, resulted in a marked drop in virus infectivity. Table 4 shows the results of a typical experiment using fluorescent focus assay for the measurement of infectious virus in purified and unpurified virus preparations. Similar results were obtained with plaque assay and tube titration. The increase in particle/infectivity ratio with purification varied considerably for different purification procedures and virus preparations, although the increase in this ratio was between 10^2 and 10^4 times for all assay methods.

Examination of unpurified and purified HCV 229E preparations by negative staining showed essentially similar virus particles (4). In both cases, more or less spherical particles were observed with almost complete coronas of surface projections, although there was a tendency for purified virus particles to disrupt with the release of ribonucleoprotein.

DISCUSSION

We confirm reports (5, 12) that have shown only cytoplasmic fluorescence in HCV 229E-infected cells. Fluorescence has been observed 6 h after infection (5), considerably before maximum virus production or detectable cytopathic effect. In this study fluorescence was usually seen within 8 h after infection, whereas released infectious virus was not observed until 12 h after infection and cytopathic effect not until 24 h after infection (Macnaughton, unpublished data). Previous fluorescent antibody studies on HCV 229E-infected cells have used relatively large quantities of virus in the inoculum so that most of the cells became infected. However, upon infecting MRCc monolayers with high virus dilutions, we observed fluorescent foci of up to eight cells. The number of fluorescent foci and the distribution of fluorescent cells within them remained constant up to at least 16 h after infection. We have concluded that each fluorescent focus was due to a single virus infection. Furthermore, we suggest that from 8 to 16 h after infection, the number of fluorescent foci produced can be used to determine the titer of infectious virus.

This report also compares the sensitivity of this fluorescent focus assay with that of other assays for infectious virus. Although tube titration and plaque assay methods have been described previously for HCV 229E (2, 3, 6–8, 13), there are no reports of a fluorescent focus assay for HCV 229E or any other coronavirus. We confirm reports showing that plaque assays for infectious HCV 229E are more sensitive than tube titration (7, 13). Furthermore, in our hands fluorescent focus and plaque assays produced virus titers that were about 15 times higher than those obtained by tube titration.

Particle/infectivity ratios have not, to our knowledge, been previously reported for coronaviruses with any infectivity assay method. Results with fluorescent focus and plaque assays show a particle/infectivity ratio of under 50 for unpurified HCV 229E preparations. Virus purification, involving pelleting and one or two cycles of isopycnic centrifugation in sucrose gradients, led to considerably increased particle/infectivity ratios with all the assay methods used. This increase varied with different virus preparations and purification procedures. Although the morphology of unpurified and purified virus preparations appeared to be similar by electron microscopy and only complete virus particles containing ribonucleoprotein were used (10), some surface changes may have occurred during purification, such as the removal or damaging of some of the surface projections. Certainly in some purified preparations there was a loss of some ribonucleoprotein. Such changes may explain the large decrease in infectivity of the purified virus particles.

In this paper we have described a fluorescent focus assay for HCV 229E infectivity that is quicker and gives higher titers of infectious virus than either plaque assay or tube titration. Furthermore, we have shown particle/infectivity ratios of less than 50 for all our fluorescent focus assays and that the proportion of infectious particles drops considerably upon purification. Further studies are in progress to adapt our fluorescent focus assay for HCV 229E infectivity to other coronaviruses and to compare its efficiency with other titration methods for these viruses.

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