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Rotavirus-Induced Fusion from Without in Tissue Culture Cells

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We present the first evidence of fusion from without induced in tissue culture cells by a nonenveloped virus. Electron micrographs of two strains of rotavirus, bovine rotavirus C486 and rhesus rotavirus, show that virally mediated cell-cell fusion occurs within 1 h postinfection. Trypsin activation is necessary for rotavirus to mediate cell-cell fusion. The extent of fusion is relative to the amount of virus used, and maximum fusion occurs between pHs 6.5 and 7.5. Fusion does not require virus-induced protein synthesis, as virus from both an empty capsid preparation and from an EDTA-treated preparation, which is noninfectious, can induce fusion. Incubation of rotavirus with neutralizing and nonneutralizing monoclonal antibodies before addition to cells indicates that viral protein 4 (VP4; in the form of VP5* and VP8*) and VP7 are involved in fusion. Light and electron micrographs document this fusion, including the formation of pores or channels between adjacent fused cells. These data support direct membrane penetration as a possible route of infection. Moreover, the assay should be useful in determining the mechanisms of cell entry by rotavirus.

Rotavirus, a member of the *Reoviridae* family, is a triple-layered, nonenveloped virus which is a major cause of severe gastroenteritis in mammalian young. Studies using MA104 monkey kidney cells to model in vivo events implicate the two outer capsid proteins, 37-kDa viral protein 7 (VP7) and 86.5-kDa VP4 in the initiation of infection. VP7 is the main protein of the outer shell, while VP4, which is probably present as a dimer, makes up the outer coat spike protein (37, 53).

In vivo, protease cleavage of rotavirus occurs in the lumen of the intestine. In vitro, this is simulated by controlled incubation with trypsin. Trypsin activation is necessary for rapid internalization and infection (6, 18); uncleaved rotavirus is internalized slowly and is not infectious (17, 25). Trypsin cleaves the VP4 spike protein at two highly conserved sites (Arg-241 and Arg-247) to yield two peptides, VP8* (28 kDa) and VP5* (65 kDa), which remain attached to the outer capsid (16, 29). VP8* contains the hemagglutination region and is probably responsible for viral attachment to receptors on the cell surface (24, 41). VP5* contains a conserved region (amino acids 382 to 405) with 72% sequence similarity to internal fusion proteins of Sindbis virus and Semliki Forest virus (30, 33). VP5* also has other conserved hydrophobic regions (31) which could be involved in membrane interactions. Although a function(s) for VP5* has not been established, these properties suggest that it is involved in cell entry.

The mechanism of infection is not fully understood, but it is probably a multiple-step event, including binding, protease cleavage, and entry, in which binding is independent of the other two steps and can occur without infection (11). Two modes of rotavirus entry into cells, direct membrane penetration and receptor-mediated endocytosis, have been suggested and may occur independently or simultaneously. Electron microscope studies show virions in coated vesicles, indicating that rotavirus entry occurs by receptor-mediated endocytosis (32, 38, 46). However, much evidence points to direct membrane penetration as a major route of entry by trypsin-cleaved virus.

Direct membrane penetration, with release of the inner capsid and viral core into the cytoplasm, is suggested by data indicating that the outer capsid protein VP7 localizes to the plasma membrane while the inner capsid and core proteins are present in the cytoplasm (17). Viral interaction with cell membranes is shown by release of fluorphores from vesicles of natural membranes (42) and from synthetic liposomes (35). Trypsin-cleaved, but not uncleaved, rotavirus also mediates the release of ^{51}Cr, [^{14}C]choline, and [^{3}H]inositol from prelabeled MA104 cells, indicating virus-induced disruption of the membrane (25). Release of such markers is inhibited by neutralizing monoclonal antibody (MAb) 2G4, which recognizes an epitope within the putative fusion peptide (33).

In enveloped viruses, fusion peptides are instrumental in syncytium formation and virus entry into the cell. The mechanism by which nonenveloped viruses are able to traverse the cell membrane during entry is very poorly understood. No syncytia have been demonstrated in group A rotavirus-infected MA104 cells; however, fusion from within has been documented in enterocytes of animals infected with group B porcine rotavirus (4, 49) and rat rotavirus (50).

In this study, we provide evidence of rotavirus-induced fusion from without, using two strains of group A rotavirus, bovine rotavirus (BRV) C486 and rhesus rotavirus (RRV). Fusion begins very rapidly and results in formation of binucleate cells and small syncytia. We hypothesize that the ability to detect and quantitate rotavirus-induced cell fusion will enable us and others to obtain more precise information concerning the early events of rotavirus infection, specifically, the factors that regulate plasma membrane penetration.

MATERIALS AND METHODS

Cells and virus preparation. Experiments were done with fetal African green monkey kidney cell line MA104 (no. 75-104), obtained from Biowhittaker (Walkersville, Md.), with passage numbers between 61 and 80. Cells were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g of glucose per liter and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; Gibco BRL, Burlington, Ontario, Canada) containing 10% fetal bovine serum (Gibco BRL), 100 IU of penicillin per ml, 100 IU of streptomycin per ml, and 0.25 μg of amphotericin B (Gibco BRL) per ml at 5% CO_2 and 37°C. Cells were passaged by using 1% trypsin (Gibco BRL) with 5 mM EDTA (Sigma, St. Louis, Mo.) when just confluent.

Two strains of plaque-purified rotavirus were used, BRV C486 (5) and RRV

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originally obtained from N. Schmidt. BRV stock was made by infecting MA104 monolayers at 10 PFU per cell with trypsin-activated (TA) BRV in DMEM without serum and without trypsin. Virus was harvested 8 h postinfection (p.i.), before significant cell lysis occurred, by freezing and thawing three times. The virus preparation was centrifuged at 1,100 × *g* and 4°C for 15 min and then centrifuged over a 40% sucrose cushion at 4°C at 80,000 × *g* for 4 h. The resulting virus pellet was resuspended in ice-cold DMEM and stored at −20°C. By plaque assay, the virus contained 10^{11} PFU/ml.

Plaque assays were done by a modification of the Sephadex overlay method of Aha and Sabara (2). MA104 cells were plated at 3.1×10^4/cm^2 in six-well tissue culture plates, grown for 48 h, and washed once with Dulbecco's phosphate-buffered saline (DPBS; Gibco BRL) containing 0.1 g of $CaCl_2$ per liter and once with DMEM before addition of virus. Ten-fold virus dilutions (1 ml per well) were added to monolayers for 1.5 h of adsorption at 4°C. The inoculum was removed, and the monolayer was washed and overlaid with 2 ml of 3% (wt/vol) Sephadex G-50 (Pharmacia, Uppsala, Sweden) per well in DMEM containing 0.5 μg of trypsin per ml, 20 mM L-glutamine, penicillin, streptomycin, and amphotericin B. After 72 h of incubation, cells were fixed and stained with 0.5% crystal violet (BDH, Toronto, Ontario, Canada) in 80% methanol, and plaques were counted.

For the focus-forming unit (FFU) assay, MA104 cells were grown in 96-well tissue culture dishes and gently washed twice with M199 medium (Gibco BRL) without serum. Ten-fold virus dilutions were made (with or without trypsin preactivation). A 100-μl volume of diluent was added, and cells were incubated overnight (15 to 18 h) at 37°C and 5% CO_2. The medium was gently aspirated, and the cells were fixed in cold methanol for 20 min at room temperature. Fixed cells were washed twice with 200 μl of PBS. The primary antibody (RRV hyperimmune serum R2) was diluted 1:2,000 in PBS plus 1% bovine serum albumin and incubated for 1 h at room temperature. The cells were washed three times with PBS. The secondary antibody, peroxidase-conjugated goat anti-rabbit immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), was diluted 1:2,000 in PBS plus 1% bovine serum albumin and added to cells for 1 h at room temperature. The cells were washed three times with PBS and stained with a Vectastain peroxidase-substrate DAB kit (Vector Laboratories, Burlingame, Calif.). Cells were examined microscopically, and stained cells were counted.

RRV was used as a crude lysate and in purified form. RRV lysate was prepared by the methods described for BRV and contained 2.5×10^8 PFU/ml. Purified RRV was prepared as follows. MA104 cells were grown in medium 199 with 10% fetal calf serum, L-glutamine, penicillin G, and streptomycin and innoculated with TA RRV. Infected cell cultures were harvested after 24 h by freeze-thawing two times, extracted with trichlorotrifluoroethane, and pelleted through a 30% (wt/vol) sucrose cushion. The virus was centrifuged and separated twice by CsCl (1.37 g/ml) gradients at 28,000 rpm (SW40 rotor; Beckman Instruments, Inc., Fullerton, Calif.) for 16 to 20 h. Bands containing either single-shell, double-shell or empty double-shell particles were collected by bottom puncture, and FFU per milliliter were determined. Double-shell RRV had 6×10^9 FFU/ml, singled-shell RRV had 1×10^3 FFU/ml, and empty capsid RRV had 2×10^7 FFU/ml.

To ensure that the same number of viral particles was added to each fusion assay, single-shell, double-shell, or empty double-shell preparations were analyzed by enzyme-linked immunosorbent assay (ELISA) for relative amounts of VP6 by using VP6-specific MAb 255/60 (47). Addition of the same number of each type of particle is important for comparison of the syncytium formation abilities of these viral particles. The VP6 ELISA was done in the presence and absence of EDTA. EDTA presumably removes the outer shell VP4 and VP7 proteins and allows detection of all VP6. This is shown by a significant increase in the amount of VP6 detected in this ELISA when the triple-layer viral particles are pretreated with EDTA. This increase in signal was not seen when purified double-layer virions were examined, as expected, since these particles had already had the outer proteins removed. The concentration of each particle type was normalized relative to the EDTA-ELISA values to ensure that equal numbers of viral particles of each type were used in the assay.

Virus-induced fusion from without. A total of 5×10^5 MA104 cells were seeded in 25-cm^2 flasks in 5 ml of DMEM–10% fetal bovine serum. After 24 h, the medium was replaced with 5 ml of DMEM–7% fetal bovine serum containing 10^{-4} M cholesterol (5-cholesten-3β-ol-3β-hydroxy-5-cholestene; Sigma no. C-3045), freshly made in 100% ethanol, and left on cells overnight. TA rotavirus was made by digesting BRV or RRV with 20 μg of type XIII trypsin (tolysulfonyl phenylalanyl chloromethyl ketone [TPCK] treated; Sigma) per ml for 30 min to 1 h at 37°C. Trypsin was inactivated by adding equimolar amounts of *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK; Sigma). In experiments with MAbs, the virus was incubated with the desired MAb for 2 h at 37°C or overnight at 4°C in DMEM without serum before continuing with the protocol.

The fusion procedure is a modified version of a method described by Hoekstra and Klappe (20). Cholesterol-treated cells were trypsinized, counted, centrifuged, and resuspended in DMEM at 10^6/ml, and 100 μl, containing 10^5 cells, was added to a 1.5-ml Eppendorf tube on ice. Cells were centrifuged at 400 × *g* (corresponding to 2,000 to 3,000 rpm in a microcentrifuge) for 25 s, the medium was removed without disturbing the cell pellet, and 100 μl of ice-cold virus at a dilution of 1×10^7 or 5×10^7 PFU/ml (i.e., 10 or 50 PFU per cell) was added to the cells. The pellet was gently resuspended, and the tube was placed on ice for 15 min to allow the virus to bind to the cells. Tubes were centrifuged at 400 × *g* for 10 s and put in a 37°C water bath for 15 min, and then the cells were resuspended by adding 400 μl of DMEM. Cells were then plated in six-well tissue culture plates containing 1 ml of DMEM–15% fetal bovine serum (pH 7.0) per well, incubated for 2 h, and then examined for fusion with an inverted phase-contrast microscope.

Fusion assay by phase-contrast microscopy. For each sample, 10 random fields were counted unless otherwise noted. Generally, 100 nuclei were examined in each field with either a 20× or 32× lens and assigned to a category of one, two, three, four, or more nuclei per cell. Cells containing two nuclei were considered to have undergone one fusion event; cells with three nuclei had undergone two fusion events; cells with four nuclei had undergone three fusions, etc. The fusion rate for a single count of 100 nuclei is the sum of all fusion events per 100 nuclei counted. The fusion rates for each of the 10 counts were averaged, and the standard error was calculated on the basis of the number of fields counted (10 fields unless otherwise noted).

Phase-contrast and electron microscopy of cell fusion. To document fusion, phase-contrast micrographs were taken 5 min and 2 to 3 h after the fusion assay with Kodak TMAX 400 film with automatic exposure. For electron microscopy, cells were fixed 1 h after undergoing the fusion procedure in 2% paraformaldehyde with 2.5% glutaraldehyde in 0.1 M phosphate buffer, postfixed in osmium tetroxide, embedded, sectioned, and stained with lead citrate and uranyl acetate.

Effect of pH on cell fusion. The fusion procedure described above was used with the following modifications. Using HCl and NaOH, the pH of DPBS containing $CaCl_2$ was adjusted to 5.0 to 9.0 at 0.5-U intervals. Suspensions of MA104 cells were added to Eppendorf tubes and centrifuged as already described. The cells were resuspended in 90 μl of DPBS at the appropriate pH and put on ice to cool. After 5 min, 10 μl of TA RRV at 5×10^7 PFU/ml, which had been diluted to this titer in DPBS (pH 6.5), was added to each tube except the control without virus, which received 10 μl of DPBS (pH 6.5) only. The virus was adsorbed on ice, and the cells were fused at 37°C, plated in tissue culture dishes, and assayed as described above.

MAbs. The MAbs used in these experiments, 2G4, 1A9, 7A12, 4C3, 60, 159, and 255/60, have been previously described (41, 44). Antibodies were tested at dilutions ranging from 1:10 to 1:500 and were used in the following experiments at a 1:500 dilution unless otherwise indicated.

RESULTS

Direct penetration of the cell membranes by rotavirus has been suggested as the probable route for productive infection. As further evidence of virus-membrane interaction, we demonstrate that rotavirus, a nonenveloped virus, is capable of mediating fusion from without in vitro.

Under standard culture conditions, rotavirus does not form syncytia in MA104 cells. We studied two strains of rotavirus, BRV C486 and RRV. Infection of MA104 cells grown under standard conditions with 0.1 to 10 PFU of TA BRV or TA RRV per cell resulted in cell lysis starting 10 to 12 h after infection. We did not detect syncytium formation during the course of infection, and phase-contrast microscopy showed minimal changes in cell morphology before 4 h p.i. (data not shown).

Addition of cholesterol to cell culture medium facilitates virus-mediated fusion from without. Formation of syncytia has been enhanced in several cell lines by changing the lipid composition of the plasma membrane (39, 40). On the premise that MA104 membrane lipids are not ideal for fusion, cell monolayers were incubated overnight with or without 10^{-4} M cholesterol in DMEM before proceeding with the fusion assay. Cells were trypsinized, pelleted, and resuspended as single cells. Either uncleaved RRV or TA RRV at 10 PFU per cell or an equal amount of medium without virus was added. Virus was allowed to bind to cells for 15 min at 4°C before the cells were briefly centrifuged at low speed, warmed to 37°C for 15 min, and plated in tissue culture dishes. Control cells incubated with cholesterol (but without added virus) showed only a slight increase in the amount of multinucleate cells compared with control cells which did not receive cholesterol (Fig. 1).

Effect of trypsin activation of virus on fusion. Rotavirus is infectious only after trypsin cleavage of spike protein VP4. To see if both TA rotavirus and nonactivated rotavirus can induce fusion, cells were assayed for fusion by using uncleaved RRV

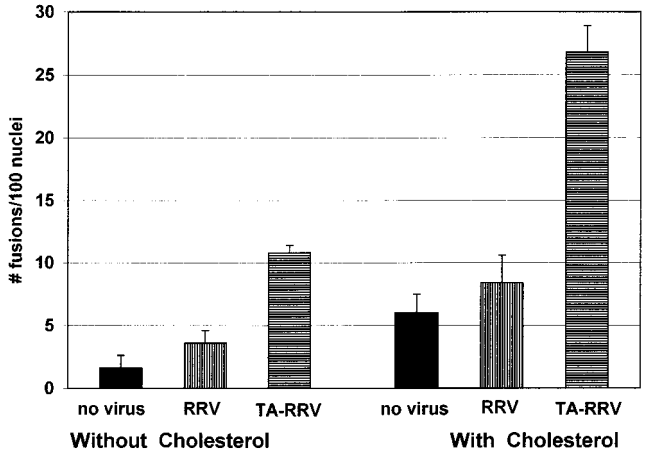


FIG. 1. Overnight incubation of MA104 cells with cholesterol increases the rate of fusion but does not itself induce fusion. Cells were grown with or without 10^{-4} M cholesterol as described in Materials and Methods. Uncleaved RRV or TA RRV, at 10 PFU per cell, was adsorbed to a single-cell suspension (with or without cholesterol treatment) for 15 min at 4°C. The cells were lightly centrifuged to form a pellet, incubated at 37°C for 15 min to induce fusion, and then plated in tissue culture plates. Fusion was assayed by light microscope 2 h after plating. Without virus, there was little fusion either with or without cholesterol treatment. RRV that had not been TA showed little fusion without cholesterol. TA RRV showed low levels of fusion without cholesterol and significantly higher levels with cholesterol-treated cells. These data are from one of three independent experiments with virtually identical results. Three independent experiments with BRV instead of RRV gave similar results (data not shown). Each error bar shows the standard error of the mean of five counted fields each containing 100 nuclei.

and TA RRV (Fig. 1). Only TA RRV in cholesterol-treated cells induced substantial levels of cell-cell fusion. Nonactivated RRV showed approximately the same level of fusion in cholesterol-treated cells as in control cells with no added virus (Fig. 1). Similar results were obtained when BRV and TA BRV were used (data not shown).

The amount of fusion varies between experiments. With TA virus, fusion can vary from a low of about 12% to a high of more than 40% of total cells. We do not know what causes this variation. To eliminate the possibility that the variation is due to changes in the amount of fusion that occurs without addition of virus (i.e., the background level of fusion), each experiment had controls which underwent the fusion procedure but did not contain added virus. Background levels of fusion also varied between experiments from about 1 to 6%, and increased levels of virus-induced fusion usually were accompanied by increased levels of background fusion.

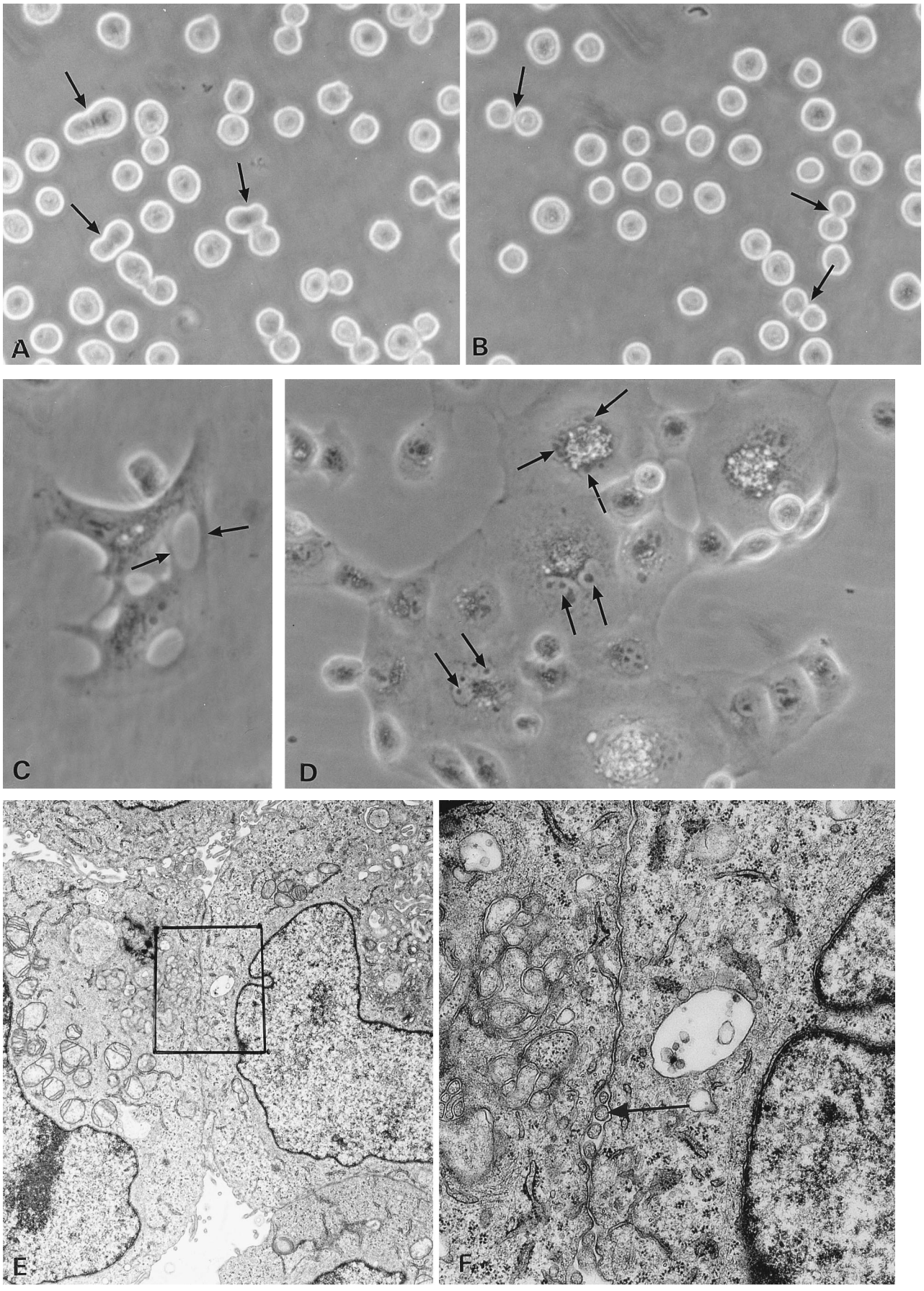
Light microscope observation of fused cells. Fusion can be seen when cells are examined directly after plating, as well as at later times. Immediately after fusion, cells with adsorbed TA rotavirus often appeared as doublets or triplets with poorly defined intervening plasma membranes (Fig. 2A). Control cells which had undergone the fusion procedure without added virus were generally seen as single cells (Fig. 2B). The few doublets that were present had separating membranes that were clearly visible by focusing on the region where the cells attached to each other.

Two to three hours after incubation at 37°C, two types of fused cells were apparent in cells which had attached and spread on tissue culture surfaces. Cells could appear joined together by tubes of cytoplasm (Fig. 2C), or cells could be completely fused to form small syncytia (Fig. 2D). In all cases, no discernible plasma membrane separated the nuclei. Most fused cells contained two to four nuclei, but occasionally five or more nuclei were present.

Electron microscopy shows that fused regions have no intervening membranes. Cholesterol-treated cells, with and without added TA BRV or TA RRV, were pelleted and fixed for electron microscopy 1 h after the temperature was raised to 37°C to initiate fusion. Control cells which had undergone the fusion procedure without rotavirus addition had distinct and continuous plasma membranes (Fig. 2E and F). Cells fused with TA BRV or TA RRV had regions where there was no visible bilayered membrane between adjacent cells even when examined at high-power magnification (Fig. 2G). In many cases, plasma membrane projections from adjacent fused cells were interleaved, making it impossible to determine cell boundaries (Fig. 2H and I). Most cells had large vacuoles, presumably containing cholesterol (Fig. 2H). Occasionally, virus particles were seen closely associated with adjacent cells, apparently in the process of forming a broad pore or bridge between the cells (Fig. 2J). This type of connection was not found in closely packed cells without virus (Fig. 2E and F).

Extent of fusion is related to amount of virus added. To determine if the amount of fusion is dependent upon the amount of virus per cell, the number of fusion events was examined with either no virus or 1, 10, 50, or 100 PFU per cell. In six separate experiments with both TA RRV (Fig. 3) and

FIG. 2. (A) MA104 cells 15 min after fusion by TA BRV. Fused cells were visible as doublets and triplets (arrows), with apparently no separating membranes. For fusion, TA BRV was adsorbed to a single-cell suspension of cholesterol-treated MA104 cells at 4°C for 15 min. Cells were pelleted, incubated at 37°C for 15 min to allow fusion, plated in six-well tissue culture dishes, and immediately photographed. (B) Control MA104 cells which had undergone the fusion procedure but without added virus and were photographed at the same time point as those in panel A. Most cells were singlets, and when they were present as doublets, a clear plasma membrane was visible between the two cells (arrow). Magnification in panels A and B, ×218. (C and D) Two types of cell-cell fusion were visible in TA BRV-fused MA104 cells photographed 3 h postfusion, after the cells had attached and spread on a tissue culture plate. In panel C, fused cells have connecting tubes of cytoplasm (arrows) with no obvious intervening membrane separating the cells. Panel D shows small syncytia with multiple nuclei (arrows). Magnification in panels C and D, ×218. (E and F) High- and low-power magnifications of control MA104 cells which underwent the fusion procedure but without added virus. Cells were fixed as a pellet 1 h after being brought to 37°C to induce fusion. Panel E shows clear separation between adjacent cells. Magnification, ×5,000. Panel F, a higher-power magnification of the boxed area in E, shows a distinct bilayered plasma membrane. Small circles between cells are cross-sections of microvilli (arrow). Magnification, ×20,000. (G) High-power magnification (×75,000) of the region where two MA104 cells have fused. Osmophilic areas (dark patches) of unknown origin often are present at cell-cell boundaries. Here the osmophilic areas are still visible but no plasma membrane is detectable. Note the scattered pieces of membrane embedded within the cytoplasm (arrows), which probably are remnants of the cell membrane. The inset shows a lower-power magnification (×7,500) of the same area. Cells were fixed as a pellet 1 h after undergoing TA RRV-induced fusion from without. (H) Two MA104 cells after fusion from without by TA RRV showing interleaved projections of cell membrane. Note that a third cell at the bottom of the micrograph did not fuse with the cell above it and the cell boundaries remained distinct. Also note the microvilli within large, membrane-bound vesicles (arrow), a feature of fusion from without in continuous cell lines. Magnification, ×7,500. (I) Higher-power magnification (×25,000) of the boxed area in panel H. Although it is difficult to trace the outline of either cell, there are regions with no interposing membrane (arrows). Cells were fixed as a pellet 1 h after undergoing the fusion procedure. (J) Induction of cell-cell fusion by TA BRV. Arrows show regions where virus is in close contact with the cell and no membrane can be seen immediately adjacent to the virus. The arrowhead shows the formation of a cell-cell connection (a channel or bridge) in the making. The curved arrow points to a region where the cells have fused and two cytoplasms of different densities touch. No membrane is visible between the cells at this point. Although no virus was found in association with this feature, it is possible that the numerous virions within the cytoplasm of the upper cell were involved in making this connection. When cells similar to this attach and spread on tissue culture surfaces, fused regions like this may become the channels depicted in panel C. Magnification, ×25,000.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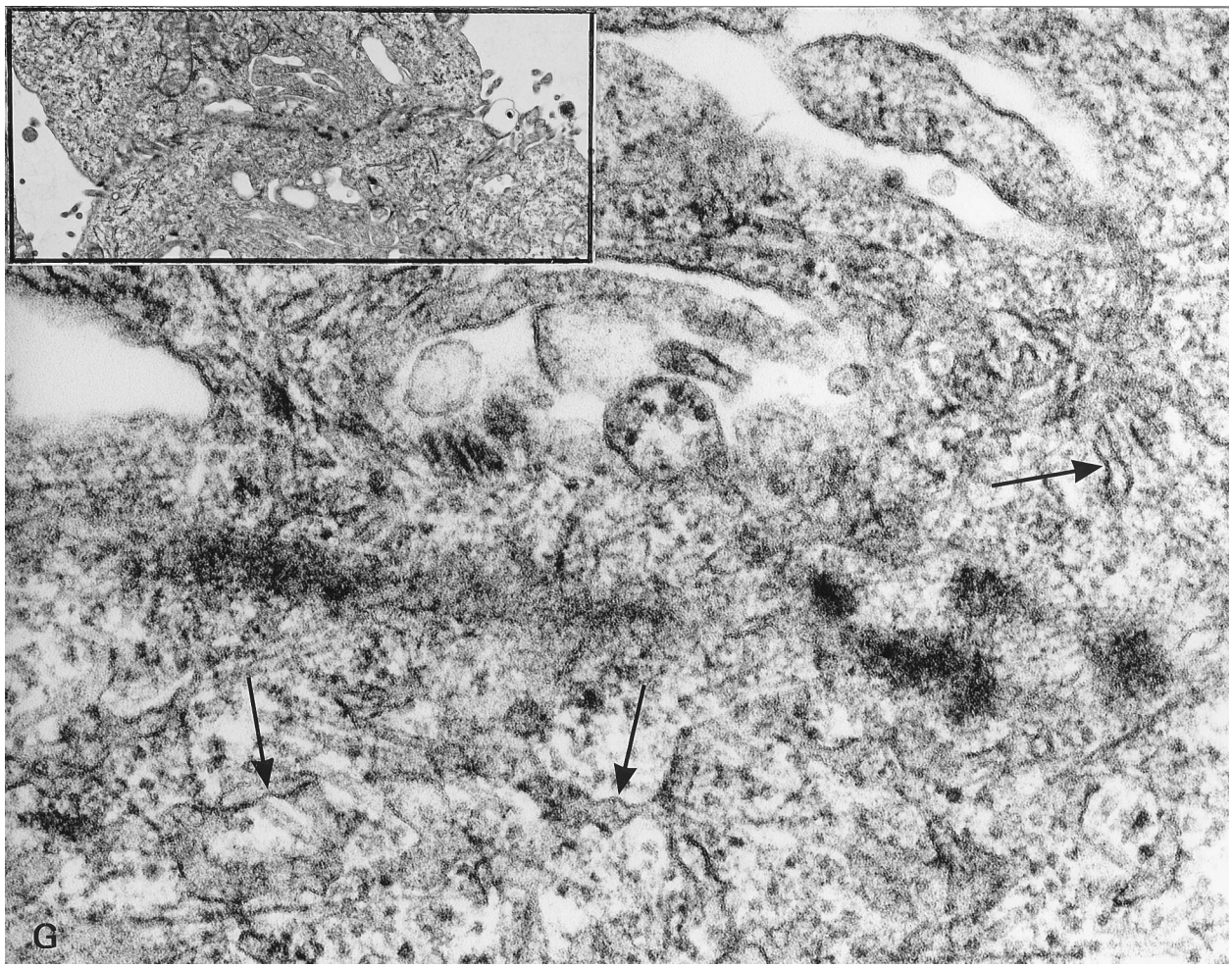
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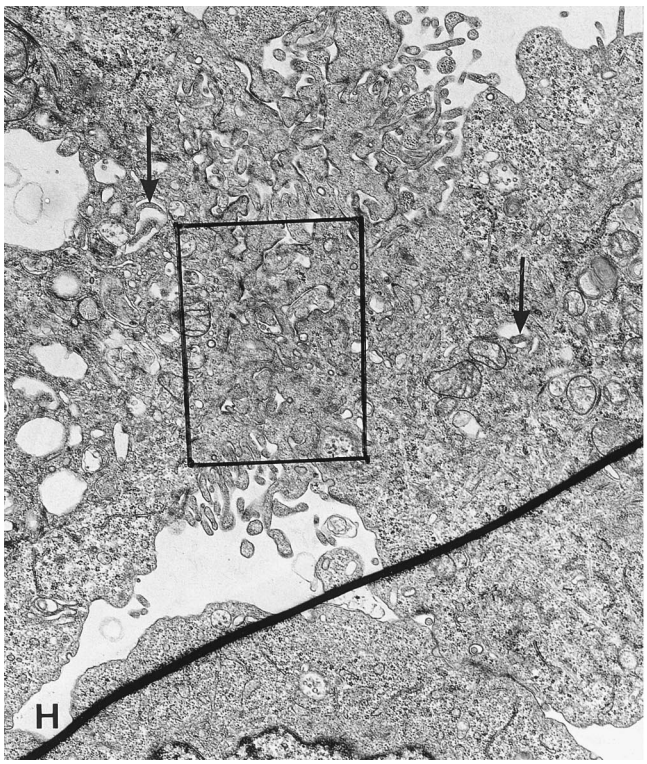
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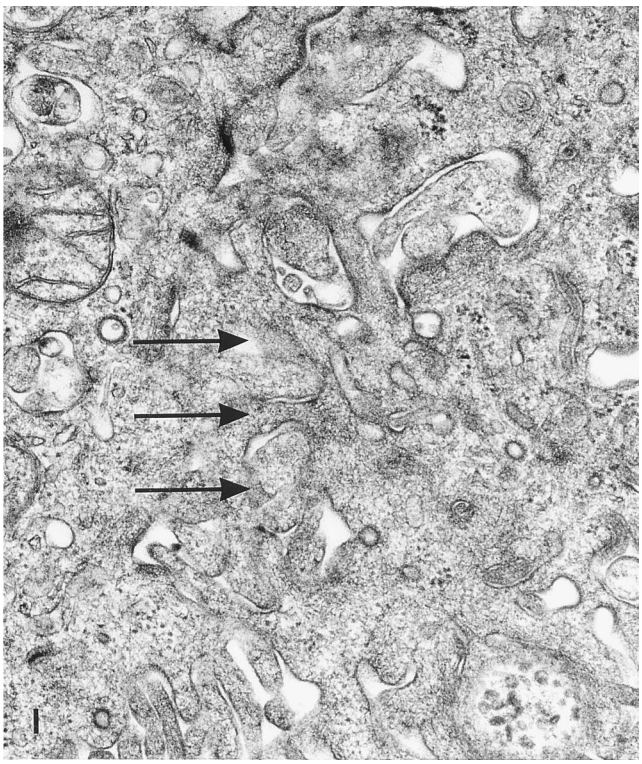
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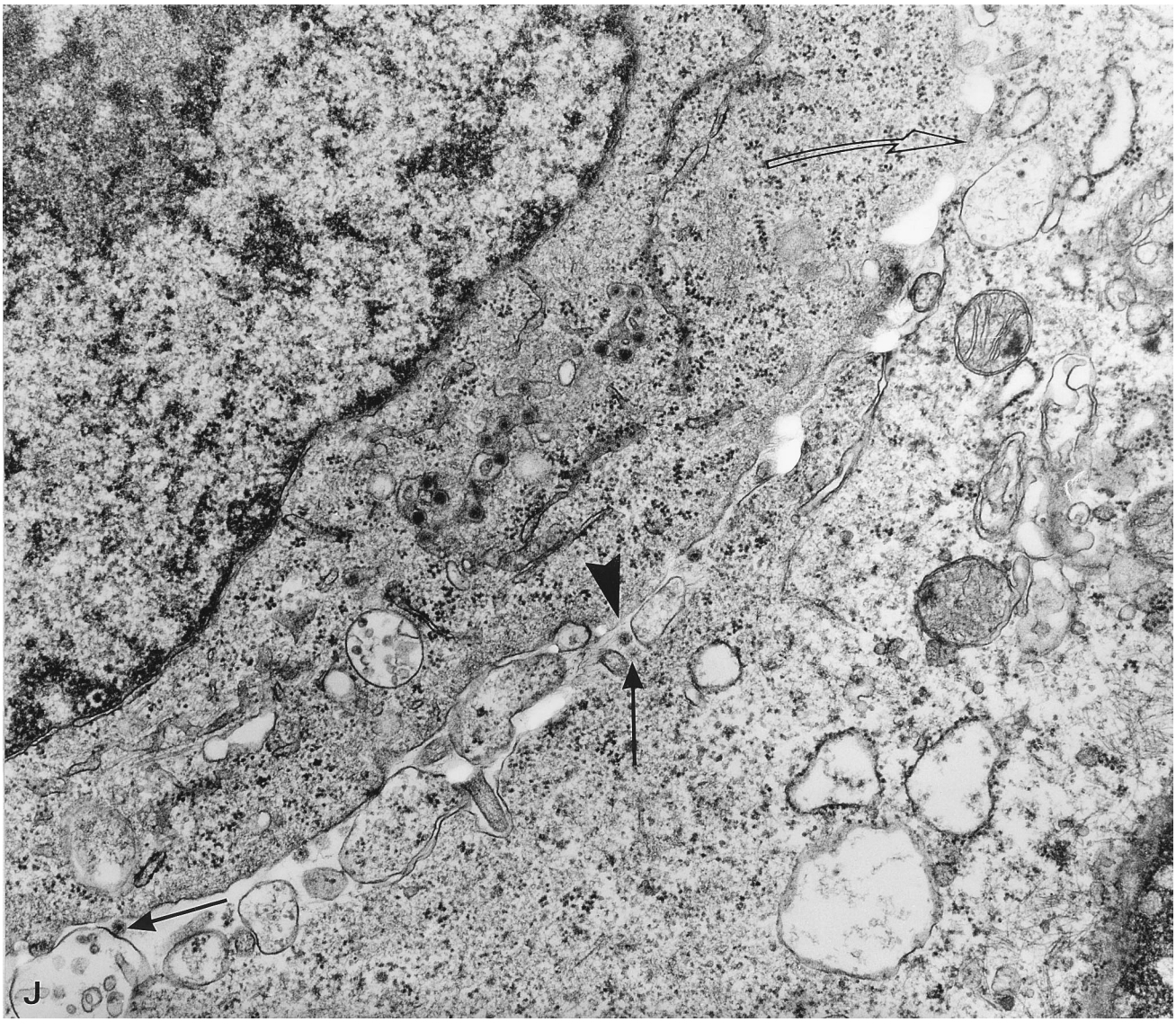

FIG. 2—*Continued.*

TA BRV (data not shown), we found increasing rates of fusion with increasing levels of virus.

Maximum fusion occurs between pHs 6.5 and 7.5. In cell-cell fusion mediated by enveloped viruses, the pH at which fusion is maximal is an indicator of the viral route of entry. Viruses which induce fusion at neutral pH are thought to fuse directly with the plasma membrane, while viruses which require an acidic pH for fusion enter the cell by the endocytotic pathway. In rotavirus-induced fusion, maximum fusion occurred between pHs 6.5 and 7.5 (Fig. 4).

EDTA-treated RRV is noninfectious but can induce fusion. Infectivity of rotavirus depends upon the presence of Ca^{2+} ions to maintain an intact outer capsid (43). Addition of sufficient EDTA renders the virus noninfectious, presumably by removing outer shell proteins VP4 and VP7 (10, 12). To see if noninfectious virions are capable of inducing fusion, we treated TA RRV with 0.1, 1, and 10 mM EDTA for 30 min at 37°C before using these preparations in the fusion assay. Subsequent analysis indicated that the virus treated with 10 mM EDTA was no longer infectious although it was competent to induce fusion in MA104 cells. Virus treated with 0.1 and 1 mM EDTA retained infectivity and also induced fusion (Fig. 5).

Reduction in infectivity after EDTA treatment of TA RRV was scored by counting the number of live cells in a 100-μl sample at 24 and 48 h p.i. by using trypan blue staining. In the sample taken from the fusion done without virus, the number of live cells was taken as 100%. The sample of cells fused with TA RRV (without added EDTA) showed 100% death 24 h p.i. Cells fused with TA RRV treated with 0.1 or 1 mM EDTA showed 90% death at 24 h p.i. and 100% death at 48 h p.i. Cells fused with TA RRV treated with 10 mM EDTA showed 90% survival at 24 and 48 h p.i.

Empty-shell rotavirus capsids, but not single-shell virions, can induce fusion. Single-shell RRV virions have only the inner VP6 shell, a core of double-stranded RNA, and associated structural proteins VP1, VP2, and VP3. Without the outer coat proteins VP4 and VP7, single-shell viral particles are not infectious. Empty-shell RRV particles have VP1, VP2, and VP3 and the VP6 inner shell surrounded by the outer shell of VP7 and VP4 but contain no RNA and are not infectious.

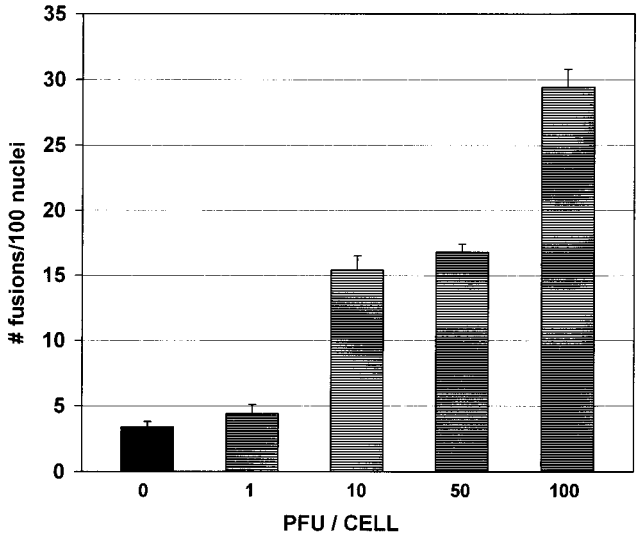

FIG. 3. Fusion depends upon the amount of virus added per cell. Cholesterol-treated MA104 cells were fused as described in Materials and Methods, with TA RRV at 1, 10, 50, and 100 PFU per cell. Control cells had no virus but underwent the fusion procedure. The levels of fusion increased with higher numbers of TA virions per cell. These data are from one of four replicate experiments which gave similar results. Three replicate experiments with TA BRV gave similar results (data not shown). Each error bar indicates the standard error of the mean of 10 counted fields each containing 100 nuclei.

Double-shell RRV particles are complete virions containing the RNA core, the inner shell proteins, and the outer VP7 shell with the VP4 spikes.

Separation of double-shell, single-shell, and empty-shell virions by centrifugation as described in Materials and Methods provided only relative separation. Some infectious particles remained both in the single-shell fraction after two bandings and in the empty-shell fraction after a single isopycnic separation. The purified double-shell RRV fraction contained 6×10^9 FFU/ml, while single-shell RRV had 1×10^3 FFU/ml and empty-shell RRV had 2×10^7 FFU/ml. To compare fusion rates among the three types of preparations, it was necessary to standardize them by VP6 ELISA. As described in Materials and Methods, this provided an indirect way of quantifying the number of viral particles in each preparation and ensured that

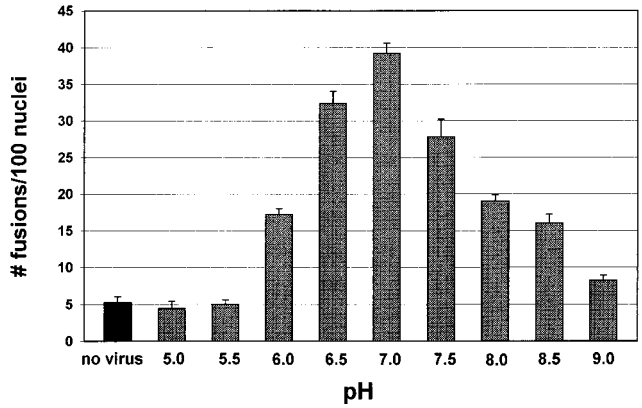

FIG. 4. pH dependence of RRV-induced fusion from without. When the fusion procedure was carried out in DPBS with a pH of 5.0 to 9.0, the maximum amount of fusion occurred between pHs 6.5 and 7.5. These results are representative of six independent experiments. Each error bar indicates the standard error of the mean of five counted fields each containing 100 nuclei.

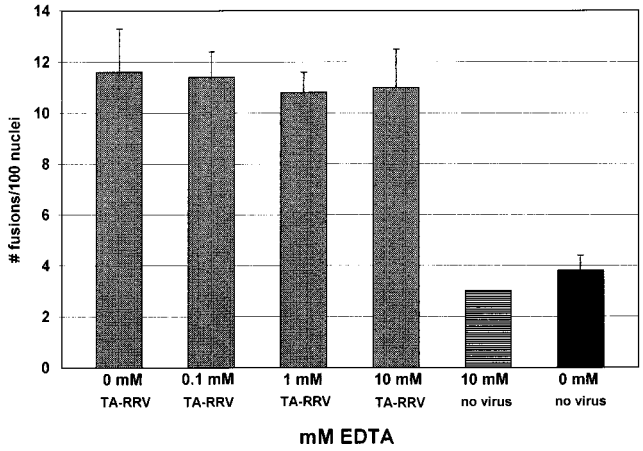

FIG. 5. Fusion does not depend upon infection or upon production of a virus-induced cell protein. TA RRV at 10 PFU per cell was incubated with the indicated levels of EDTA for 30 min at 37°C before use in the standard fusion procedure. A subsequent assay showed that treatment of TA RRV with 10 mM EDTA rendered the virus noninfectious. Fusion was unaffected by all levels of EDTA. These data are from one of two replicates with essentially identical results. Each error bar indicates the standard error of the mean of five counted fields each containing 100 nuclei.

the same number of each type of virus particle was available to interact with the MA104 cells.

TA single-shell RRV did not induce any fusion above the background level present without virus. However, the TA empty capsid preparation induced the same level of fusion as trypsin-activated whole RRV despite the fact that it was over 100-fold less infectious (Fig. 6).

MAb 2G4 recognizes an epitope on VP5* that is important for infection and fusion. Neutralizing MAb 2G4 recognizes a region surrounding Gln-393 within the putative fusion region

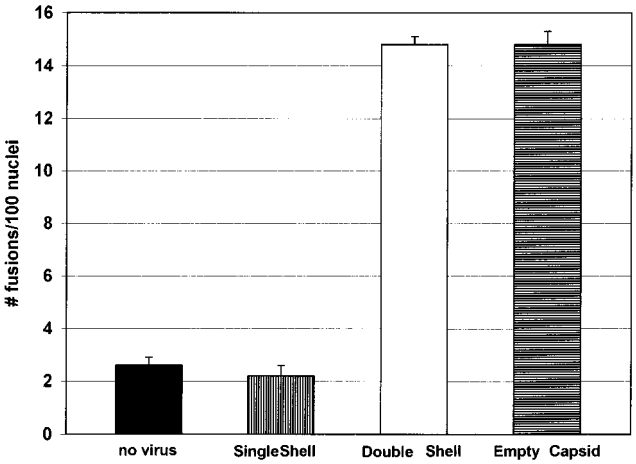

FIG. 6. Single-shell, double-shell (complete virions), and empty double-shell RRV particles were made and TA as described in Materials and Methods. Since single-shell and empty-shell particles are not infectious, it was not possible to standardize to PFU per cell. Instead, the preparations were standardized by VP6 ELISA to have equal numbers of particles (as measured indirectly by VP6 content) before use in the fusion assay. Single-shell particles, lacking VP7 and VP4, did not induce fusion. Virus from an empty-shell viral preparation and intact double-shell virions induced identical levels of fusion, indicating that infection is not necessary for fusion and that VP7 and VP4, but not VP6, are involved in fusion. These data are from one of five experiments with essentially identical results. Each error bar indicates the standard error of the mean of 10 counted fields each containing 100 nuclei.

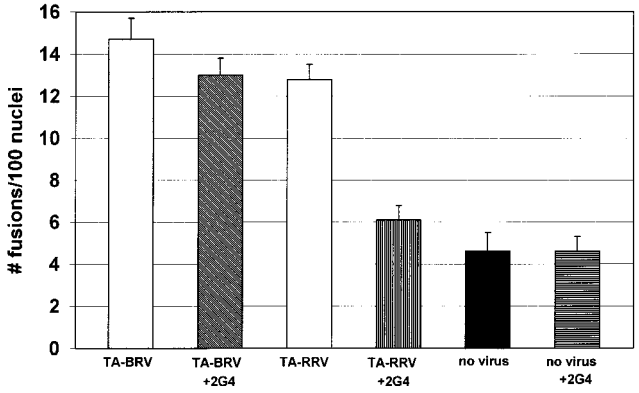

FIG. 7. The VP5* fusion peptide is involved in cell-cell fusion. MAb 2G4 is a conformationally dependent antibody which recognizes an epitope which includes Gln-393 within the fusion peptide of VP5* of RRV. TA RRV (10 PFU per cell), when incubated with a 1:50 dilution of 2G4 and used in the standard fusion procedure, did not induce fusion. MAb 2G4 did not bind to BRV, which has a single amino acid difference (Asn-393). When TA BRV, at 10 PFU per cell, was incubated with 2G4 and used in the standard fusion assay, it did not inhibit BRV-mediated fusion. These data are from one of five replicate experiments with identical results. Each error bar indicates the standard error of the mean of 10 counted fields each containing 100 nuclei.

of VP5* (34). To see if incubation of rotavirus with MAb 2G4 would interfere with fusion, 2G4, at dilutions from 1:10 to 1:500, was incubated with TA RRV and with TA BRV before testing in the fusion procedure. Incubation of 2G4 completely neutralized TA RRV and prevented fusion at these dilutions (Fig. 7). BRV has Asn instead of Gln at amino acid 393. Incubation of 2G4 with TA BRV did not inhibit fusion and did not neutralize infection at dilutions between 1:10 and 1:500 (Fig. 7).

Fusion and neutralization are independent events. To see if other regions of VP4 or other viral structural proteins are involved in fusion, we tested the effects of incubating TA RRV with a battery of MAbs. Incubation with MAbs 4C3, 60, and 159, which recognize epitopes on VP7, inhibited fusion, although only 4C3 and 159 neutralized TA RRV (Fig. 8). As before, 2G4 inhibited fusion and neutralized infection. MAbs 1A9 and 7A12 recognize epitopes on VP8* (which contains the viral hemagglutinin). Fusion was inhibited partially by 1A9 and not at all by 7A12, although both MAbs neutralized infection. Finally, 255/60, a MAb which recognizes the subgroup 1 determinant on VP6, the major inner capsid protein, neither inhibited fusion nor neutralized infection.

DISCUSSION

We present the first evidence of induction of multinucleate cells and small syncytia in MA104 cells by fusion from without with BRV and RRV. Multinucleate cells were observed by electron microscopy within 1 h p.i. and could be produced by noninfectious, EDTA-treated virus or by empty capsids. Syncytium formation by fusion from within that occurs at least 10 h p.i. has been reported for several nonenveloped viruses, including avian reovirus (26), Nelson Bay virus (52), and group B rotavirus (4, 49, 50). These fusions presumably require viral protein synthesis. Direct fusion of baculovirus with the membrane of brush border vesicles has also been reported, although no baculovirus-induced cell-cell fusion was mentioned (21).

The morphology of rotavirus-fused MA104 is identical to that reported for tissue culture cells fused from without by enveloped viruses. At the light microscope level, fused MA104 cells usually have two to five nuclei with no discrete intervening membrane (Fig. 2D) and appear morphologically similar to HeLa cells which have undergone fusion from without induced by Sendai virus. In both Ma104 and HeLa cells, microvilli are found in large membrane-bound structures within the cytoplasm of cells (28). Electron microscopic observation of fused MA104 cells shows numerous interwoven processes or enlarged villi which appear contiguous in some regions. This is quite similar to the syncytium morphology induced by Nelson Bay virus (52). In addition, microvilli were found in large, membrane-bound structures within the cytoplasm of fused MA104 cells (Fig. 2G and H), matching a morphological feature of Sendai virus-fused HeLa cells (27).

The mechanism by which rotavirus interacts with membranes to cause fusion is unknown. Enveloped viruses frequently undergo conformational changes in their fusion glycoproteins following protease cleavage and a secondary event which can include binding to a receptor and/or a pH change. These conformational changes appear to mediate fusion, and without them fusion does not occur (reviewed in reference 19). Trypsin cleavage and other factors, such as loss of Ca^{2+}, may trigger similar conformational changes in rotavirus and may be involved in fusion (14). It is possible that trypsin cleavage of VP4 initiates conformational changes to expose hydrophobic regions that interact with the cell membrane. Both the putative fusion peptide and a region around amino acid 510 (predicted to form an α-helical coiled coil with a hydrophobic face capable of interacting with a membrane [31]) are candidates for such interaction. The fact that rotavirus-induced fusion is maximal at pH 7.0 suggests that the virus may enter directly through the plasma membrane (51). However, substantial amounts of fusion also occur with a pH as low 6.0, which suggests that fusion is not inhibited by acidic pH and therefore rotavirus also could use the endocytotic pathway as a means to enter the cell.

Reduction and rearrangement of disulfide bridges result in conformational changes and subsequent membrane penetration by corona and Sindbis viruses (1, 45). There is no evidence

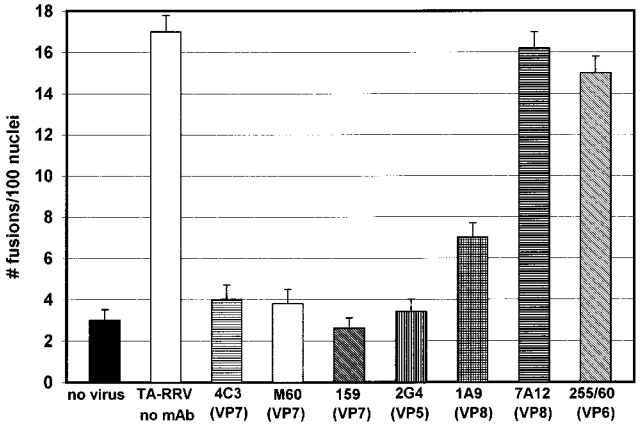

FIG. 8. Incubation of TA RRV at 10 PFU per cell with a battery of various antibodies to rotavirus capsid proteins (all diluted 1:500), followed by use in the standard fusion procedure, indicates that fusion can be inhibited by antibodies to VP7, as well as by antibodies to VP4. All MAbs to VP7 completely inhibited fusion. MAb 2G4, which recognizes a region in VP5*, also inhibited fusion. One MAb to VP8* (7A12) had no effect on fusion, although another MAb to VP8* (1A9) consistently showed significant but not complete inhibition. The MAb to VP6, 255/60, also did not inhibit fusion. These data are from one of three replicate experiments with identical results. Each error bar indicates the standard error of the means of 10 counted fields each containing 100 nuclei.

for a disulfide bridge(s) between VP8* and VP5*, but animal strains of rotavirus have a disulfide bridge connecting Cys-203 and Cys-216 in VP8* and another connecting two highly conserved cysteines, Cys-318 and Cys-380, in VP5*. The latter disulfide bridge brings the fusion peptide and the trypsin cleavage site into close proximity near the end of the outer capsid spike protein (36). Trypsin cleavage does not induce changes in disulfide linkage (36). Recently, the effect of reducing agents on rotavirus in cell culture showed that rotavirus infectivity was not inhibited by dithiothreitol, probably because the disulfide bonds in assembled virus are very resistant to reduction (47). Nevertheless, it would be interesting to see if reducing agents affect rotavirus-induced fusion.

Rotavirus binding to cells is not dependent upon trypsin cleavage (7, 25). However trypsin cleavage generates interactions with the plasma membrane resulting in viral penetration and productive infection (25). Cryoelectron microscopy shows that the putative fusion peptide is near the head of the VP4 spike, where it can easily interact with the plasma membrane (37). The inhibition of fusion by 2G4 supports a role for VP4 and, more specifically, for the VP5 segment in the induction of cell-cell fusion.

Addition of 10 mM EDTA rendered RRV noninfectious, presumably by a conformational change in VP7 and concomitant loss of VP4 (10, 12, 13). Since EDTA-treated virus was not infectious, it is probable that newly synthesized viral protein does not mediate the fusion event. We found that the 10 mM EDTA treatment did not inhibit cell fusion. Presumably, VP4 (as VP5*-VP8* and/or as VP4-VP7) remains in solution after being released from the outer capsid by calcium chelation and therefore can continue to mediate cell-cell fusion. Alternatively, hydrophobic domains of VP7 may be exposed and act alone or in conjunction with VP4. It is interesting that EDTA-treated virus maintained the abilities to permeabilize artificial and brush border-derived membrane vesicles and to induce fusion (35, 42).

Both TA double-shell RRV and TA empty capsids present outer shell proteins VP7 and VP4 to MA104 cells, and both can induce fusion. The empty capsids do not contain the RNA viral genome and are more than 100-fold less infectious than full viral particles; these facts support the notion that virus-induced protein synthesis is not involved in fusion. Single-shell particles present VP6 to MA104 cells and do not induce fusion, indicating that VP6, in the absence of VP4 and VP7, cannot mediate cell-cell fusion.

VP5* is associated with release of fluorphores from artificial and enterocyte-derived liposomes and with the release of radiolabeled isotopes from prelabeled cells. This release is prevented by neutralizing MAb 2G4, which recognizes the putative fusion peptide and does not interfere efficiently with virus binding to cells (41). Moreover, this release also occurs in the presence of EDTA but not without cleavage of VP4. These data support the hypothesis that rotavirus can interact with and disrupt the cell membrane to form cytoplasmic connections between cells at an early stage of cell-cell fusion. We can see the formation of such connections at the electron microscopic level (Fig. 2J). These cytoplasmic channels may widen as the cell attaches and spreads, eventually resulting in complete fusion of the adjacent cells.

The inhibition of rotavirus-induced fusion by various antibodies confirms the specificity of the assay and points to a role for VP5*, VP8*, and VP7 in fusion (Fig. 7 and 8). All VP7 antibodies (4C3, m60, and 159) and the 2G4 antibody to VP5* inhibited fusion. One neutralizing antibody to VP8* (1A9) partially inhibited fusion, but another neutralizing antibody (7A12) did not. Whether the antibodies to VP7, VP5*, and VP8* have a direct effect or inhibit syncytium formation indirectly remains to be determined. The fact that virus incubated with MAb 7A12 can mediate fusion but is not infectious indicates that neutralization and fusion are distinct events, as they are in respiratory syncytial virus (48).

Cooperation between the fusion and hemagglutination proteins is necessary for syncytium formation in Newcastle disease virus and parainfluenza virus (22, 23). In rotavirus, there is clear evidence for interactions between VP4 and VP7. One indication of such interactions is the difficulty with which VP4 spikes are removed from the outer capsid in the presence of intact VP7 (3). Moreover, the plaque phenotype is known to be affected by the parental origin of both VP4 and VP7 (8). VP7 plays a role in the presentation of the 2G4 epitope in the putative fusion region (9), while VP4 can affect the conformation of VP7 (15). Recent evidence shows that neutralization-positive epitope 1 (NP1), located within the hemagglutination domain of VP8*, is in a region critical for interaction of VP4 and VP7 (54). The observation that antibodies to both surface proteins inhibit syncytium formation provides further evidence for the interaction of VP4 and VP7 in viral entry into cells. It will be interesting to determine if individually expressed rotavirus proteins or groups of proteins can also mediate this effect and if so, what specific regions are responsible.

Although considerable progress has been made in understanding the mechanism by which enveloped viruses enter cells, the same cannot be said for icosahedral viruses. It is our hypothesis that the ability of rotaviruses to induce syncytium formation in cells is intimately related to the mechanism by which these viruses enter cells. Future studies will determine which rotavirus protein(s) and which regions of this protein(s) are directly capable of mediating cell fusion and how proteolytic cleavage of VP4 activates this event.

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