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MHV-A59 Enters Polarized Murine Epithelial Cells through the Apical Surface but Is Released Basolaterally

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Coronaviruses have a marked tropism for epithelial cells. Entry and release of the porcine transmissible gastroenteritis virus (TGEV) is restricted to apical surfaces of polarized epithelial cells, as we have recently shown (J. W. A. Rossen, C. P. J. Bekker, W. F. Voorhout, G. J. A. M. Strous, A. van der Ende, and P. J. M. Rottier, 1994, *J. Virol.* 68, 7966–7973). In this paper we analyze the interactions of mouse hepatitis coronavirus A59 (MHV-A59) with polarized murine kidney cells (mTAL) grown on permeable supports. After inoculation from the apical or basolateral side, virus entry was found to take place only through the apical membrane. The virus utilized a protein of the carcinoembryonic antigen family as its receptor. In contrast to TGEV, MHV-A59 was released preferentially from the basolateral plasma membrane domain, as evidenced by the accumulation of viral proteins and infectivity in the basolateral culture fluid as well as by electron microscopical observations. In the mouse, MHV initially replicates in the nasal epithelium before being disseminated throughout the body; the basolateral release of MHV from epithelial cells into the animal's circulation may be the first step in the establishment of a systemic infection. © 1995 Academic Press, Inc.

INTRODUCTION

Coronaviruses infect humans and a variety of other mammals and birds, including livestock and companion animals. Most coronaviruses cause enteric and/or respiratory infections, but some spread systemically (Holmes, 1990; McIntosh, 1990). Mouse hepatitis virus (MHV) for instance infects other organs, and different MHV strains have different degrees of tropism for the liver, brain, and other tissues. Basically, they can be segregated into respiratory (polytropic) and enterotropic biotypes. All prototype strains including MHV-A59 and MHV-JHM infect the upper respiratory mucosa before infecting other organs. Viruses of the enterotropic biotype of MHV are restricted to intestinal mucosa, with minimal or no infection of other tissues (Compton *et al.*, 1993 and references therein).

Coronaviruses have a marked tropism for epithelial cells; thus, MHV replicates in the nasal epithelium before being disseminated to other organs (Compton *et al.*, 1993 and references therein). Epithelial cells form highly organized sheets, separating the external milieu from the organism's interior. In epithelial plasma membranes two domains can be distinguished, the apical and the basolateral, which are separated by tight junctions. Each domain has a distinct lipid and protein composition which results from a sorting process and from the function of the tight junctions that prevent mixing of membrane components (for reviews, see Gstraunthaler, 1988; Simons and van Meer, 1988; Cereijido *et al.*, 1989; Hubbard *et al.*, 1989; Rodriguez-Boulan and Nelson, 1989; Simons and Wandinger-Ness, 1990; Compans and Srinivas, 1991).

Epithelia form the first barrier to virus infections. Virus entry into and release from epithelial cells are polarized, i.e., restricted to the apical or basolateral membrane (for a recent review see Tucker and Compans, 1993). In general, entry and release of a particular virus occur within the same domain. Thus vesicular stomatitis virus (VSV) uses the basolateral membrane both for entry and release (Rodriguez-Boulan and Sabatini, 1978; Fuller *et al.*, 1984; Tucker and Compans, 1993 and references therein), whereas simian virus 40 exploits the apical membrane for both purposes (Clayson and Compans, 1988; Clayson *et al.*, 1989).

Though interactions between viruses and epithelial cells have been extensively investigated, little is known about the entry, release, and sorting of viruses that bud intracellularly. Coronaviruses are assembled at perinuclear membranes by budding into the intermediate com-

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partment (Tooze *et al.*, 1984; Klumperman *et al.*, 1994; Krijnse-Locker *et al.*, 1994). From there, the particles are transported in vesicles through the secretory pathway to the plasma membrane domain where they are released by exocytosis (Tooze *et al.*, 1987). Recently, we have shown that entry and release of the transmissible gastroenteritis coronavirus (TGEV) of swine are restricted to the apical surface of polarized epithelial cells (Rossen *et al.*, 1994). In contrast, entry of MHV-A59 into murine epithelial cells is restricted to the apical membrane, while release occurs preferentially from the basolateral side, as the present study shows.

MATERIALS AND METHODS

Cells, virus, and antisera

The origin of mouse kidney medullary thick ascending limb cells adapted to growth on a plastic support (M-mTAL-1p, shortly mTAL) has been previously described (Valentich and Stokols, 1986a,b). Cells were maintained at 37° and 5% CO_2 in plastic culture flasks (Nunc) in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO Laboratories), containing 10% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 μg/ml). The cells were passaged as follows. A confluent monolayer was rinsed twice with phosphate-buffered saline containing 0.02% EDTA (PBS-EDTA) and incubated for 15 min at 37° in PBS-EDTA. The detached cells were collected by centrifugation at 1500 *g* for 15 min, thoroughly resuspended in culture medium, and used to start new cultures. For the preparation of polarized cell monolayers, polycarbonate membrane filters attached to the bottom of plastic cups (Transwell inserts, 0.45 μm, 4.5 cm^2; Costar Corp., Cambridge, MA) were placed into six-well tissue culture plates. Cells from a confluent culture in a 80-cm^2 culture flask were obtained by trypsinization, suspended in 24 ml culture medium, and 1 ml was added per filter. Routinely, the tightness of the monolayer was checked by adding medium to the upper chamber up to a slightly higher level than in the lower chamber (Cerneus *et al.*, 1993; Rossen *et al.*, 1994).

The MHV strain A59 (MHV-A59) was propagated in Sac(−) cells as described earlier (Spaan *et al.*, 1981). Also, production of the rabbit polyclonal antiserum to MHV-A59 has been reported (Rottier *et al.*, 1981). Isolation and characterization of the monoclonal antibodies (mab) G43 against porcine aminopeptidase N and the mab CC1 against the MHV receptor have been previously described (Williams *et al.*, 1990; Dveksler *et al.*, 1991; Delmas *et al.*, 1992).

Virus infections

mTAL cells grown on filters were rinsed twice with infection medium (IM; DMEM containing 740 mg/liter $NaHCO_3$, 10 m*M* *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid and 10 m*M* 2-(*N*-morpholino)ethanesulfonic acid, pH 6.8) and inoculated with MHV-A59 diluted in IM at a multiplicity of infection (m.o.i.) of 10 at different times post seeding (ps). Basolateral inoculation was done by placing the filter on a 75-μl droplet of inoculum on parafilm. Apical inoculation was achieved by adding 400 μl of inoculum to the upper compartment. After 1 hr the inoculum was removed, the filters were rinsed three times with IM, and further incubated in IM containing 1% FCS or in DMEM containing 10% FCS.

Transepithelial resistance measurements

Transepithelial resistance (TER) was measured using a Millicell ERS apparatus (Millipore, Bedford, MA), according to the manufacturer's instructions. Measurements were performed at room temperature in culture medium. TERs were measured over monolayers of noninfected and MHV-A59-infected cells.

Indirect immunofluorescence assays

mTAL cells grown on permeable supports were inoculated with MHV-A59 from the apical or the basolateral side. At 8 hr postinfection (pi) cells were rinsed once with PBS and fixed with 3% paraformaldehyde overnight at 4°. Subsequently, the cells were washed three times with PBS containing 50 m*M* glycine (PBG). Filters were cut from their holders and divided into two or four equal parts. Next the cells were permeabilized with PBS containing 1% Triton X-100 for 10 min at room temperature, followed by three washes with PBG. Cells were then incubated for 30 min at room temperature with the polyclonal anti-MHV serum diluted in PBG (1:150), rinsed three times with PBG, and stained for 30 min with fluorescein-conjugated goat anti-rabbit IgG (Cappel) diluted in PBG (1:200). Finally, filters were washed extensively and mounted in FluorSave reagent (Calbiochem). Fluorescence was viewed with an Olympus BHS-F microscope.

Inhibition of infection by anti MHV-receptor antibodies

mTAL cells grown on glass coverslips were preincubated for 1.5 hr at 37° with serial dilutions of the mab CC1 against the MHV receptor (Williams *et al.*, 1990) or mab G43 directed against the TGEV receptor (Delmas *et al.*, 1992), the latter serving as a control. Cells grown on filter supports for 95 hr were preincubated, either from the apical or from the basolateral side, with the mab CC1 (diluted 1:25 in the culture medium) for 1 hr at 4° instead of 37° to prevent possible transcytosis of antibodies. Subsequently, cells were inoculated with MHV (m.o.i. = 10) from the apical or basolateral side at 37° (coverslips) or 4° (filters). At 9 hpi culture media were collected and used in an endpoint dilution infectivity assay on L-cells.

In addition, cells were fixed and processed for immunofluorescence as described above.

Metabolic labeling and immunoprecipitation

MHV-infected mTAL cells and L-cells, both grown on filter supports, were labeled from 6–9 hpi by replacing the apical and basolateral medium with minimal essential medium lacking methionine (MEM-meth); the basolateral medium was supplemented with 200 μCi L-^{35}S-*in vitro* labeling mix (Amersham). In pulse–chase experiments cultures were incubated for 30 min with MEM-meth, before labeling the cells for 30 min with 200 μCi L-^{35}S-*in vitro* labeling mix. The pulse labeling period was followed by a chase with culture medium containing 4 m*M* methionine, 4 m*M* cysteine, and 10% FCS. After the labeling and chase periods, the cells were rinsed with ice-cold PBS and solubilized in 300 μl lysis buffer: TES (20 m*M* Tris hydrochloride, pH 7.5, 1 m*M* EDTA, 100 m*M* NaCl), containing 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 100 μg/ml phenylmethylsulfonyl fluoride. Nuclei were removed by centrifugation at 12,000 *g* for 10 min at 4°. For immunoprecipitation of intracellular proteins, a 100-μl aliquot of the lysate was taken and diluted further with 200 μl lysis buffer. For immunoprecipitation of released viral proteins media were collected and cleared by centrifugation for 10 min at 1,500 *g* at 4°. To the supernatants $\frac{1}{4}$ volume of a 5× concentrated stock solution of lysis buffer was added. To each sample 10 μl of the polyclonal MHV-A59 antiserum was added and the samples were incubated overnight at 4°. Immune complexes were adsorbed to formalin-fixed *Staphylococcus aureus* cells (BRL, Life Technologies, Inc.) using 75 μl of a 10% (w/v) suspension. After a 30-min incubation at 4° the immune complexes were collected by centrifugation at 12,000 *g* and washed three times with TES containing 0.1% Triton X-100. The final pellets were resuspended in 35 μl Laemmli sample buffer (62.5 m*M* Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% mercaptoethanol; Laemmli, 1970), incubated for 30 min at room temperature, and heated for 3 min at 95°. Samples were loaded onto a SDS–15%, 12.5%, or 10% polyacrylamide gel. Quantitation of radiolabeled proteins in the dried gels was carried out using a phosphorimager and Molecular Dynamics Imagequant Software (version 3.22). In control experiments, mTAL cells were infected at 16 hps with VSV (San Juan strain) or influenza (X-31 strain). Cells were inoculated from both the apical and basolateral side for 1 h (VSV) or from the apical side for 1.5 hr (influenza) at 37°. In the case of VSV all further incubations were done at 32°, to slow down the cytopathic effects (van Meer and Simons, 1982). Cells were labeled from 6–9 hpi with 300 μCi ^{35}S-labeling mix and media were collected. VSV and influenza proteins were immunoprecipitated as described above, using a rabbit anti-VSV serum and a rabbit anti-influenza serum, respectively.

Virus titration

Viral infectivity in culture media was determined at different times pi by a quantal assay on L-cells. L-cells grown in 96-well plates were inoculated with serial dilutions of medium samples from infected cells made in culture medium. $TCID_{50}$-values were determined using the Spearman/Kaerber formula (Kaerber, 1931).

Electron microscopy

mTAL cells grown on filters for 3 days were inoculated with MHV from the apical side. After 1 hr, the cells were washed three times with PBS to remove residual inoculum and culture medium was added. At 10 hpi cells were rinsed with PBS and fixed overnight in 2% paraformaldehyde, 0.5% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.2. Filters were then treated for 1 hr with 1% osmium tetroxide and for 1 hr with 2% uranyl acetate and cut from their holders. After dehydration in acetone they were embedded in Durcopan epoxy resin (Fluka). Ultrathin sections of 45 nm were cut on a Reichert UltracutS and stained for 2 min with Reynolds lead citrate (Reynolds, 1963). Sections were examined and photographed in a Philips CM10 electron microscope at 80 kV.

Estimation of the ratio between the surface areas of the apical and basolateral membrane domains

A method to estimate the ratio between membrane surface areas has been previously described (Weibel, 1979; Pfaller *et al.*, 1990). Shortly, relative values of surface areas S_v of the apical and basolateral membrane domains were estimated by point counting using a square lattice placed over electron microscopic graphs of mTAL cells, utilizing the relationship

$$S_v = I_i/P_r \times d,$$

where P_r is the number of lattice corners overlaying the reference structure (r), i.e., the cytoplasm area; d, the size of the square lattices used; and I_i, the number of intercepts of the lattice lines with the apical or basolateral membrane contour of mTAL cells. The ratio between the surface area of the basolateral and the apical membrane can be obtained from the equation I_i (basolateral)/I_i (apical).

RESULTS

Cells and virus

Several virus/epithelial cell combinations were tested to evaluate systems suitable for the study of coronaviruses in polarized cells; only a few combinations resulted in productive infections (Table 1). Here we focus on the MHV-A59 infection of a murine epithelial cell line (mTAL) derived from the kidney of a 15-day-old male ICR mouse (Valentich and Stokols, 1986a, b). Between 20 and 30% of these cells can

TABLE 1

The Infectability of Epithelial Cell Lines by Coronaviruses[a]

Cell line[c]	Virus[b]						
	BCV	CCV	FECV	FIPV	IBV	MHV	TGEV
BeWo	N.D.[d]	—	N.D.	—	N.D.	—	—
CaCo-2	N.D.	—	—	—	N.D.	—	—
FRT	N.D.	—	N.D.	—	N.D.	—	—
LLC-PK1	N.D.	—	N.D.	+/−[e]	N.D.	—	+
MDBK	N.D.	—	—	—	N.D.	—	—
MDCK I	+	—	—	—	N.D.	—	—
MDCK II	N.D.	—	—	—	N.D.	—	—
mTAL	N.D.	—	N.D.	—	N.D.	+	—
Vero C1008	—	N.D.	N.D.	N.D.	+	—	N.D.

[a] The cells were inoculated with the different viruses. At 8, 12, 24, and 48 hr pi the cells were fixed and prepared for an immunofluorescence assay.

[b] Abbreviations used for the viruses: BCV, bovine coronavirus (Mebus strain); CCV, canine coronavirus (strain I-71); FECV, feline coronavirus (strain 79-1683); FIPV, feline infectious peritonitis virus (strain 79-1146); IBV, infectious bronchitis virus (Beaudette strain); MHV, mouse hepatitis virus (strain A59); TGEV, transmissible gastroenteritis virus (Purdue strain).

[c] Abbreviations used for the epithelial cell lines: BeWo, a human choriocarcinoma cell line; CaCo-2, a human colon carcinoma cell line; FRT, Fisher rat thyroid cells; LLC-PK1, a porcine kidney cell line; MDBK, Madin–Darby bovine kidney cells; MDCK I and II, Madin–Darby canine kidney cells strain I and II; mTAL, mouse kidney medullary thick ascending limb cells; Vero C1008, a monkey kidney cell line.

[d] N.D., not determined

[e] Only a limited amount of cells could be infected.

be infected with MHV-A59 as judged by indirect immunofluorescence. Only few — if any — small syncytia (two to four cells) were observed. Virus started to be released from 6 hpi, reaching titers of 10^7-10^8 $TCID_{50}$ units/ml at 24 hpi. Furthermore, mTAL cells supported persistent MHV infection for at least 2 months (results not shown).

Establishment of tight monolayers

In order to establish cell polarity and independently gain access to the apical and basolateral membranes, mTAL cells were grown on permeable supports. To determine whether the cells formed tight monolayers the development of tight junctions was monitored by measuring the TER of the monolayer at different ps times. An appreciable TER was first recorded 16 hps, which increased thereafter and reached a plateau of about 450 Ωcm^2 between 80 and 120 hps (Fig. 1A). This value is consistent with previously reported data (Ecay and Valentich, 1992). The TER of mTAL cells is between that of MDCK I and MDCK II cells (Barker and Simmons, 1981; Cereijido *et al.*, 1978; Richardson *et al.*, 1981) and comparable to that of LLC-PK1 cells (Gstraunthaler, 1988 and references therein). Routinely, the integrity of the monolayer was

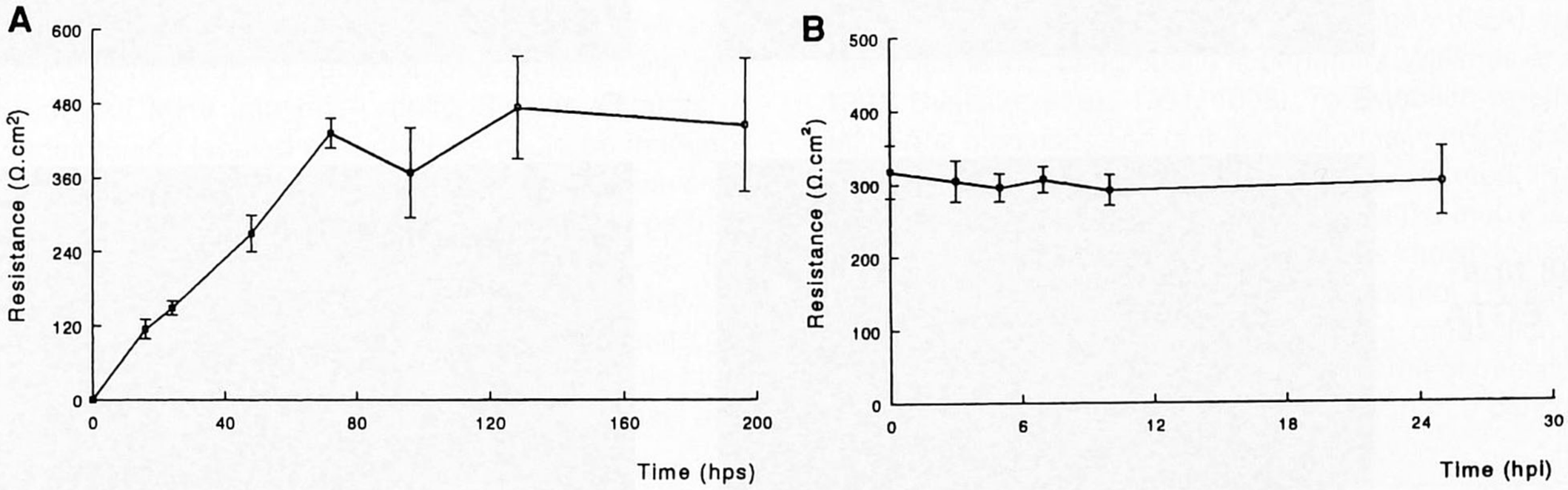
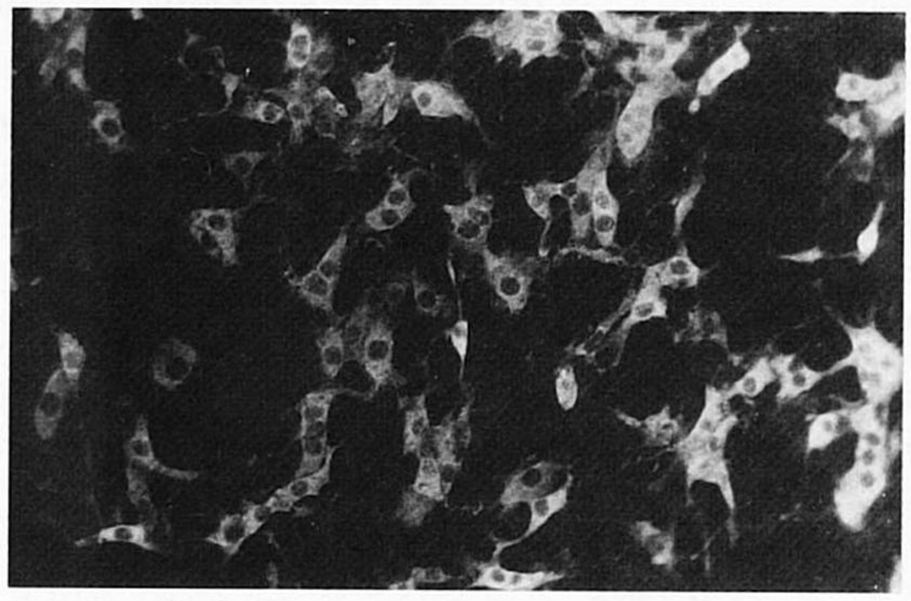


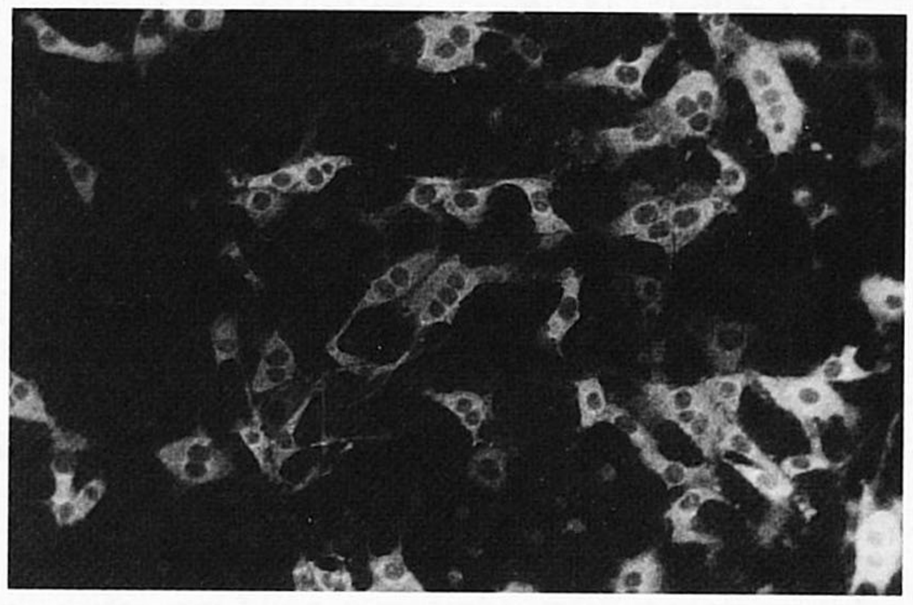
FIG. 1. Transepithelial resistance measurements of mTAL cells. Measurements were done at room temperature with a Millicell ERS apparatus. (A) The development of resistance over time after plating the cells on filter supports (time in hours post seeding (hps)). (B) The course of the transepithelial resistance during infection with MHV (time in hours post infection (hpi)). Cells were infected from the apical side at 48 hps.

APICAL **BASOLATERAL**

16 hps

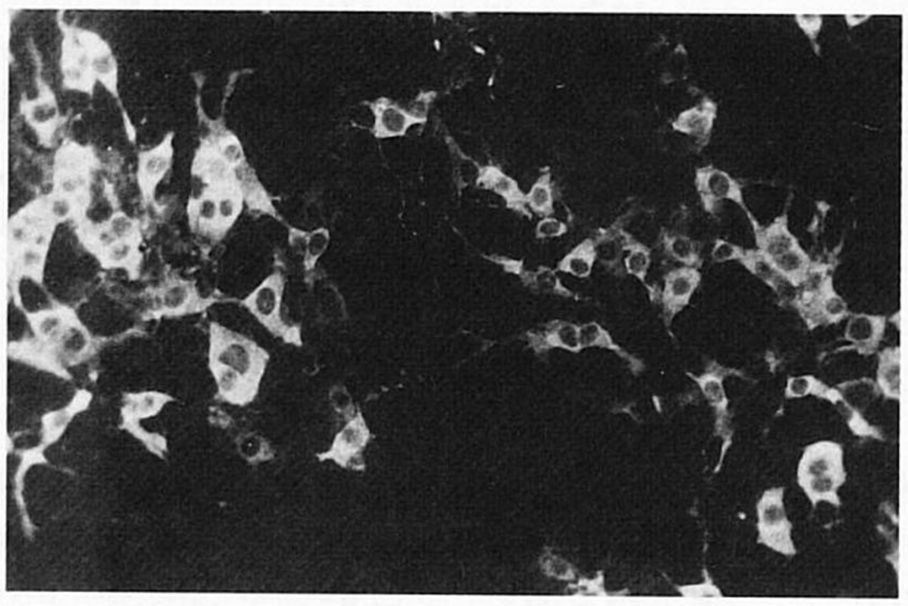


A

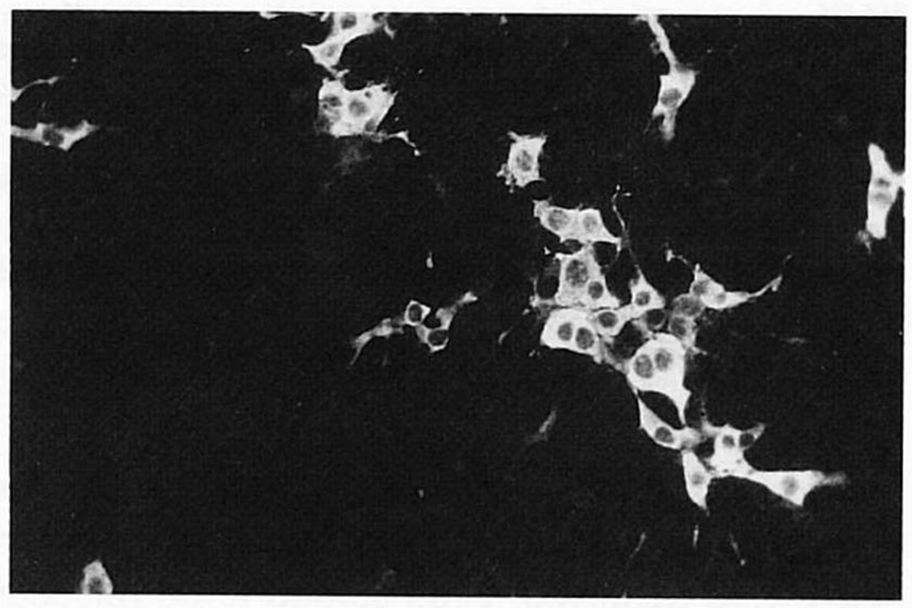


B

38 hps

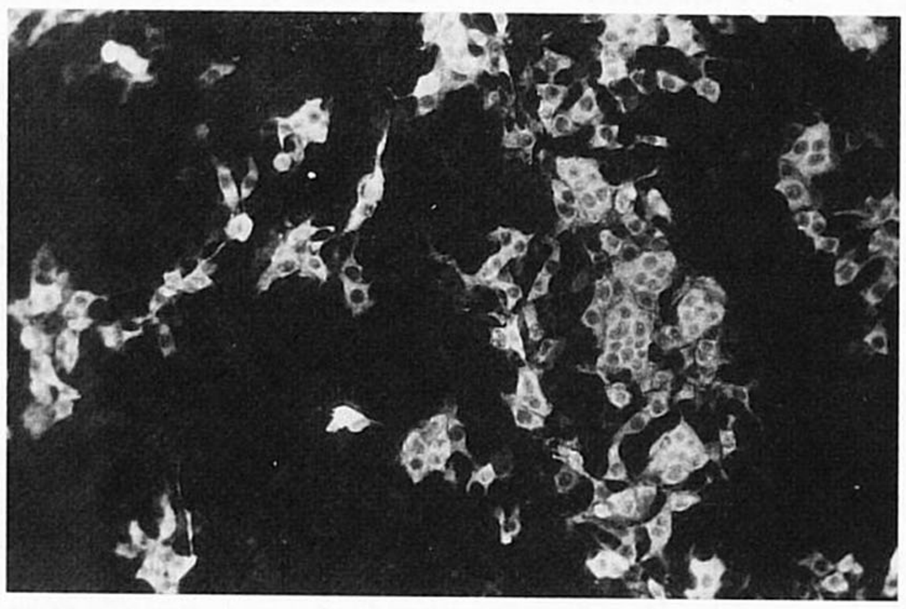


C

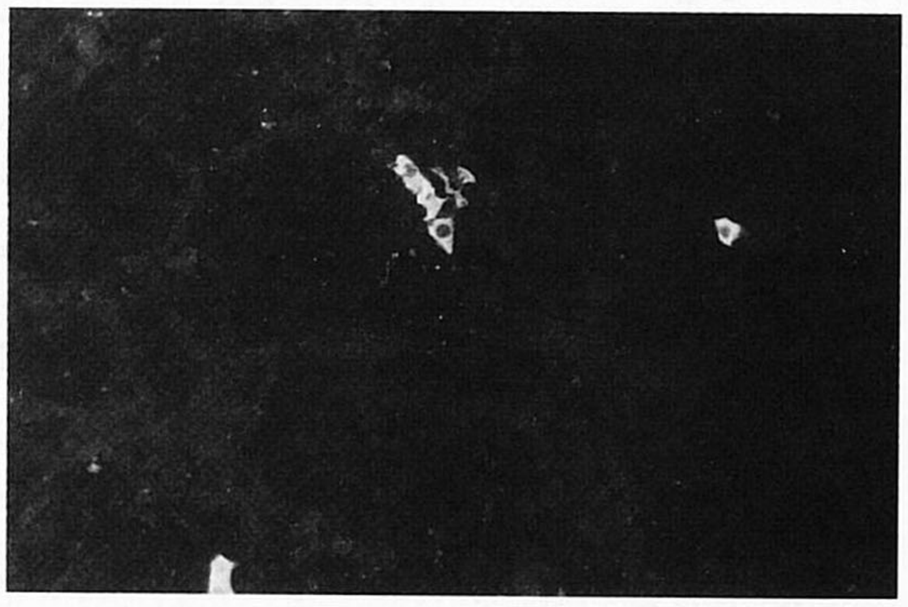


D

88 hps — EGTA

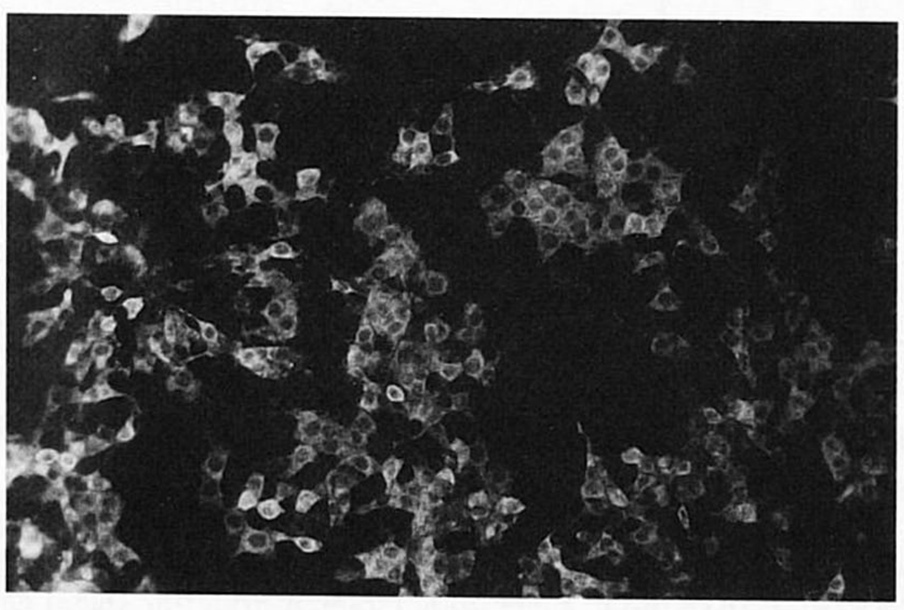


E

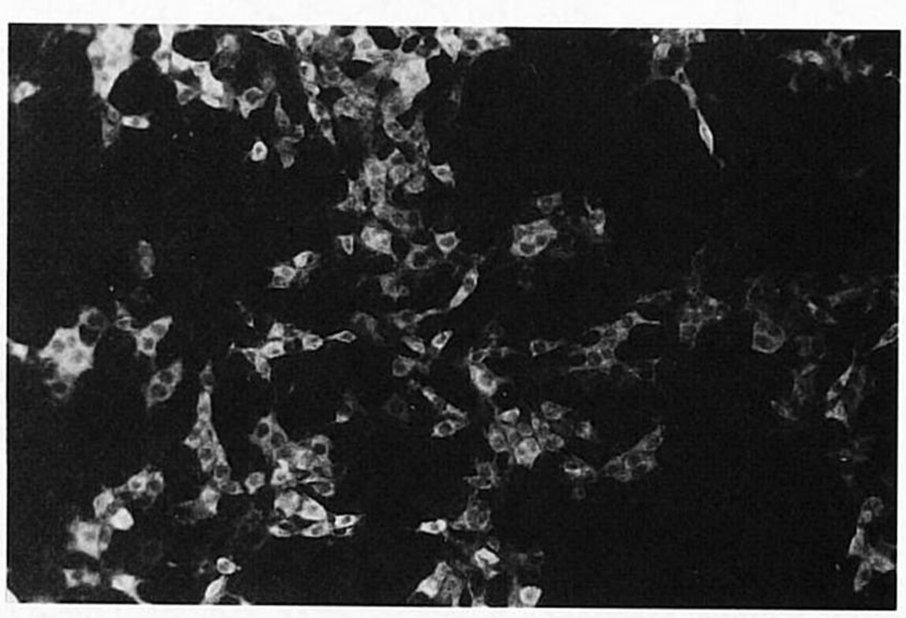


F

88 hps + EGTA



G



H

checked by adding culture medium to the apical compartment up to a slightly higher level than in the basolateral compartment (Cerneus *et al.*, 1993; Rossen *et al.*, 1994). No leakage of culture medium occurred from 12 to 16 hps, indicating that by then the cells have formed a completely tight monolayer. The TER was still increasing at this time similar to observations in other epithelial cells (Herzlinger and Ojakian, 1984; Rossen *et al.*, 1994). To be sure that the integrity of the monolayer was not lost during infection experiments with MHV, the TER over infected monolayers was measured (Fig. 1B). No change in resistance was observed until at least 24 hpi, consistent with the absence of leakage of culture medium from the apical to the basolateral compartment even after another 24 hr. These results indicate that the monolayers maintain their integrity during infection with MHV.

Polarity of virus entry

Immunofluorescence studies. To identify the polarity of MHV-A59 entry into mTAL cells monolayers were inoculated from either side at different ps times. The efficiency of infection was monitored using an indirect immunofluorescence assay. As shown in Fig. 2, MHV-A59 could infect mTAL cells from the apical side at all times. Also, the basolateral side initially allowed infection — as efficiently as the apical side — but infectability through this domain gradually decreased (Fig. 2B). At 38 hps, a substantial drop had occurred, and at 88 hps only a few cells were infectable (Figs. 2D and 2F).

These latter observations may have a trivial reason: the inability of the virus to pass the filters and reach the cells late after plating. To exclude this possibility, cells grown for 88 hr were treated with EGTA before inoculation. This treatment opens the tight junctions between the cells. As a consequence, intercellular transport of viruses becomes possible and plasma membrane proteins, e.g., viral receptors, are redistributed over the cell surface. Figures 2G and 2H show that the cells could now be infected from both sides with equal efficiency.

Synthesis of viral proteins. Studies of MHV protein synthesis confirmed our observation of a preferentially apical entry of MHV into mTAL cells. Similar amounts of [^{35}S]methionine-labeled viral proteins could be immunoprecipitated using polyclonal anti-MHV serum when cells had been inoculated from the apical side at any ps time (Fig. 3). After basolateral inoculation, however, the amount of precipitable proteins drastically decreased as a function of time. At 14 (Fig. 3) and 24 hps (results not shown) cells could be infected efficiently from this side; later, the efficiency of infection started to decrease, and

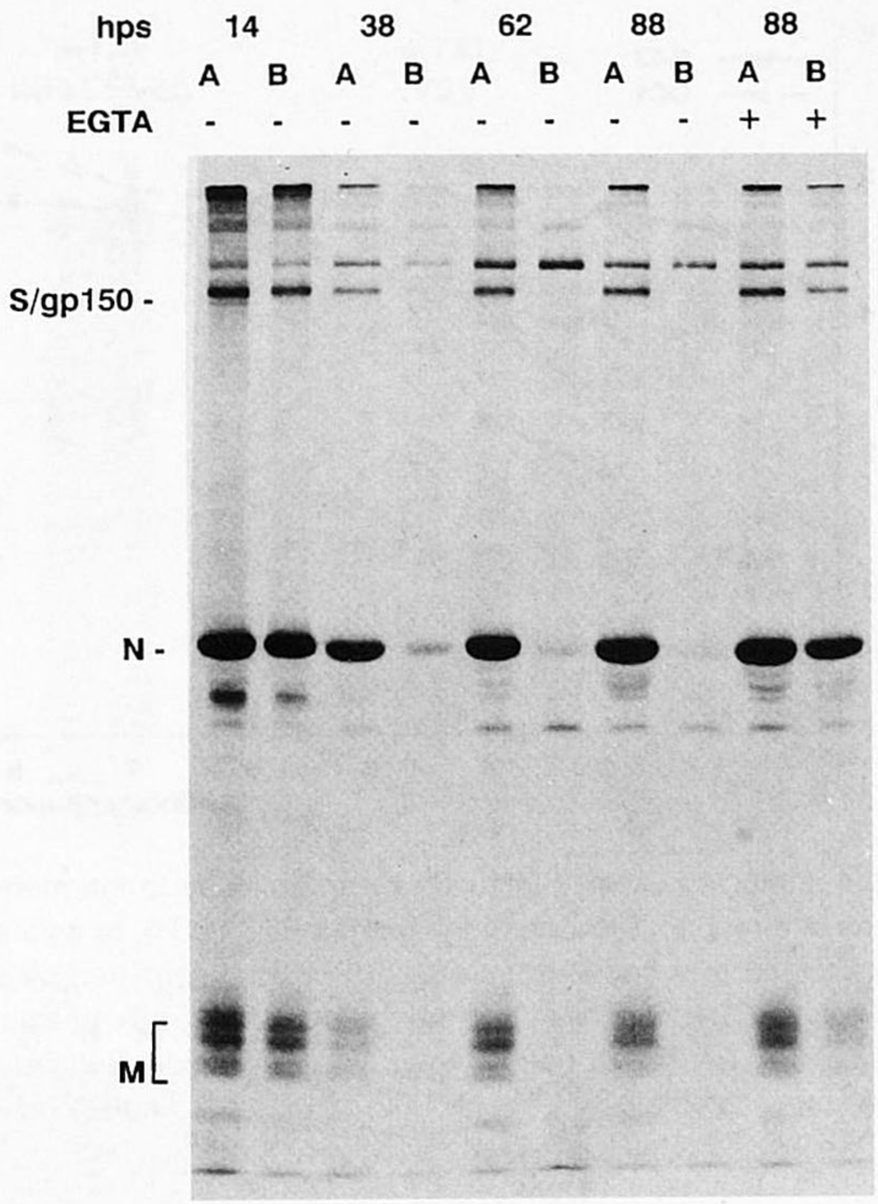


FIG. 3. Side of virus entry into mTAL cells: a radio-immunoprecipitation assay. mTAL cells were inoculated with MHV from the basolateral (B) or apical (A) side at different hours post seeding (hps). Some monolayers were treated with EGTA before infection as indicated. Cells were labeled with [^{35}S]methionine (7–10 hpi) and viral proteins were immunoprecipitated from cell lysates using a MHV-specific antiserum. Proteins were separated in a SDS–10% polyacrylamide gel. Indicated are the 150-kDa form of the MHV spike protein (S/gp150), the nucleocapsid protein (N), and the membrane protein (M).

by 88 hps virus entry had become restricted to the apical side almost completely. Again, treatment with EGTA before inoculation at 88 hps rendered the cells susceptible to infection from the basolateral membrane domain.

Inhibition of MHV infection by receptor antibodies. MHV-A59 binds to receptors on the plasma membrane that are isoforms of the murine biliary glycoprotein (BGP), a member of the carcinoembryonic antigen (CEA) subfamily in the immunoglobulin superfamily (Williams *et al.*, 1991; Dveksler *et al.*, 1991, 1993). To establish whether MHV-A59 also uses one of these isoforms in mTAL cells, an infection inhibition experiment was performed. Prior to inoculation cells were preincubated with a mab (CC1) directed against the murine BGP, which is known to block the interaction of the virus with this receptor (Williams *et al.*, 1990; Dveksler *et al.*, 1991). The production of progeny virus was determined as a measure of infection. The antibody indeed inhibited the infection in a concen-

FIG. 2. Side of virus entry into mTAL cells: an immunofluorescence assay. Filter-grown mTAL cells were inoculated with MHV from the apical (A, C, E, G) or basolateral (B, D, F, H) side at different ps times. Cells were fixed at 10 hpi and prepared for intracellular immunofluorescence using a MHV-specific antiserum. Some monolayers were treated with EGTA before inoculation (G, H). hps, hours post seeding.

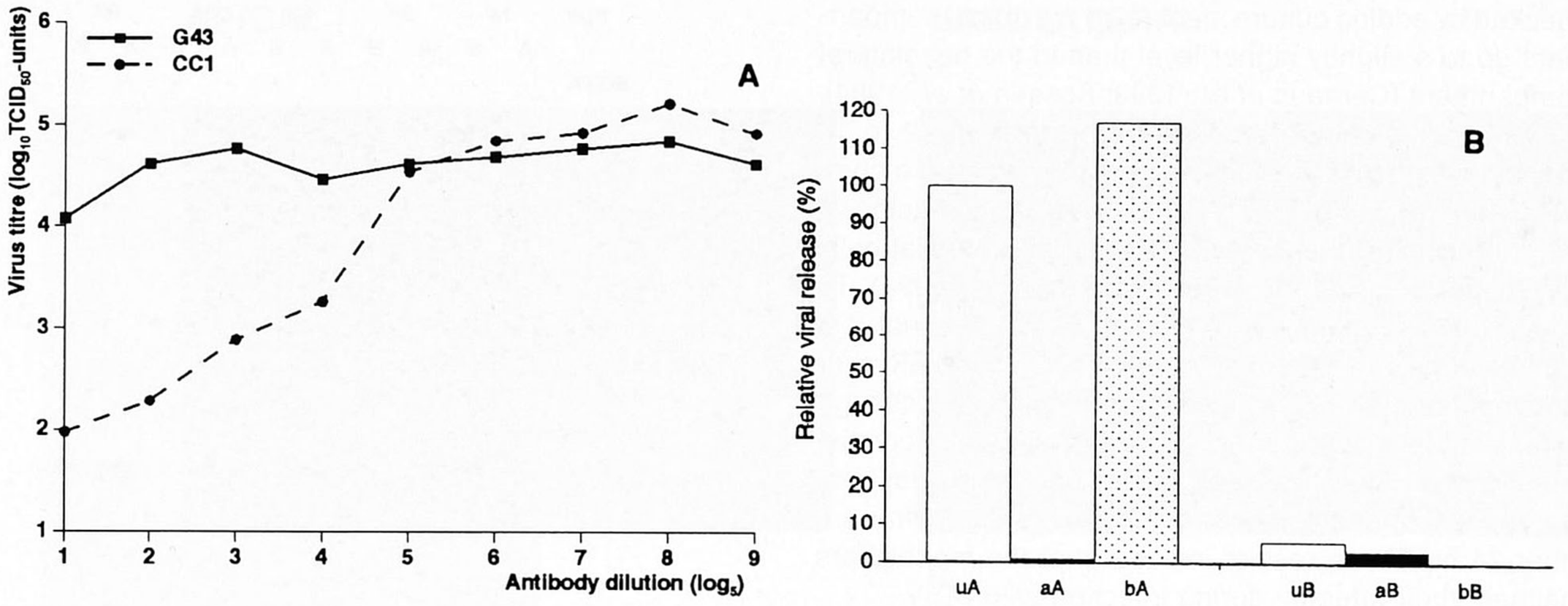


FIG. 4. Inhibition of MHV infection by an antibody to the murine biliary glycoprotein (BGP). (A) Cells were incubated for 1.5 hr at 37° with serial dilutions of a mab to isoforms of the murine BGP (CC1), or as a control with a mab to the TGEV receptor (G43), before inoculation with MHV. Media were collected at 9 hpi, and the infectivity was determined by an endpoint dilution assay on L-cells. (B) Filter-grown 95-hr-old cells were treated with the mab CC1 for 1 hr at 4° from the apical (aA, aB), basolateral (bA, bB) or neither (uA, uB) side and inoculated with MHV for 1 hr at 4° from the apical (uA, aA, bA) or basolateral (uB, aB, bB) side. Subsequently, cells were incubated at 37° for 9 hr and media were collected to determine the amount of infectious viral particles. $TCID_{50}$, 50% tissue culture infectious dose.

tration-dependent manner (Fig. 4A). No inhibition was found when cells were preincubated with a mab directed against the TGEV receptor (G43, Delmas *et al.*, 1992). These results were confirmed using an indirect immunofluorescence assay (results not shown). Clearly, the receptor used by MHV to enter the mTAL cells belongs to the BGP subfamily of molecules. In another experiment we studied whether the polarity of MHV entry correlated with the distribution of the receptor. Filter-grown fully differentiated cells were now pretreated from the basolateral or apical side with the CC1 mab and subsequently inoculated from either side. A productive infection could only be achieved by inoculation from the apical side and this infection was blocked completely and specifically by the receptor antibodies (Fig. 4B). Treatment with mab CC1 from the basolateral side had no effect on infection through the apical membrane. Consistently, the low level of infection that could be achieved by basolateral inoculation was completely abolished by the antibody treatment from this side. These results indicate that the polarized entry correlates with a polarized distribution of the MHV receptor.

Release of MHV from mTAL cells

Titration of released virus infectivity. The epithelial cell surface from which viruses are released may be important in pathogenesis (Tashiro *et al.*, 1988, 1990). We therefore attempted to identify the site on mTAL cells from which MHV is shed. First experiments had shown that release of MHV was indeed polar when cells had been infected as early as 4 hps and medium collected at 9 hpi (results not shown). A compact value for polarity was determined by calculating the infectivity ratios in the apical and basolateral medium ($Ri_{B/A}$) of six cultures inoculated in parallel at 16 hps and harvested 9 hr later. An average $Ri_{B/A}$ value of 10.7 was obtained indicating that 91% of the infectivity measured was present in the basolateral medium. When following the temporal appearance of virus in both media we found that throughout the productive phase of the infection the infectivity re-

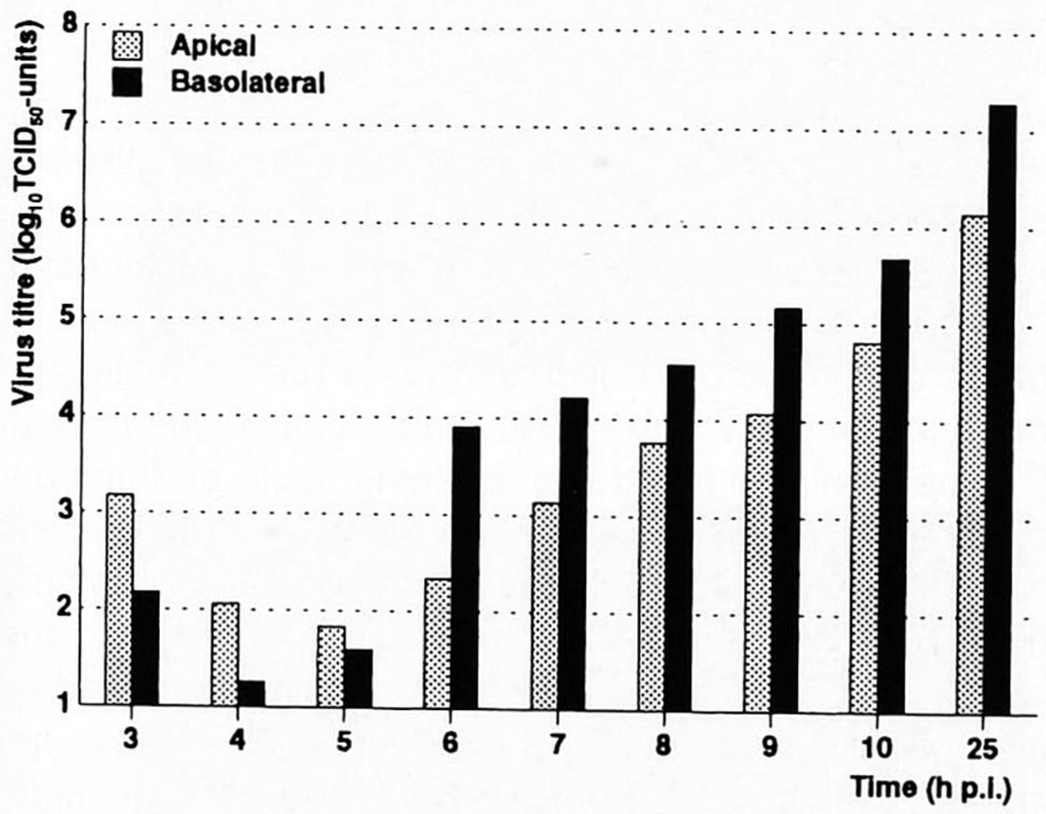
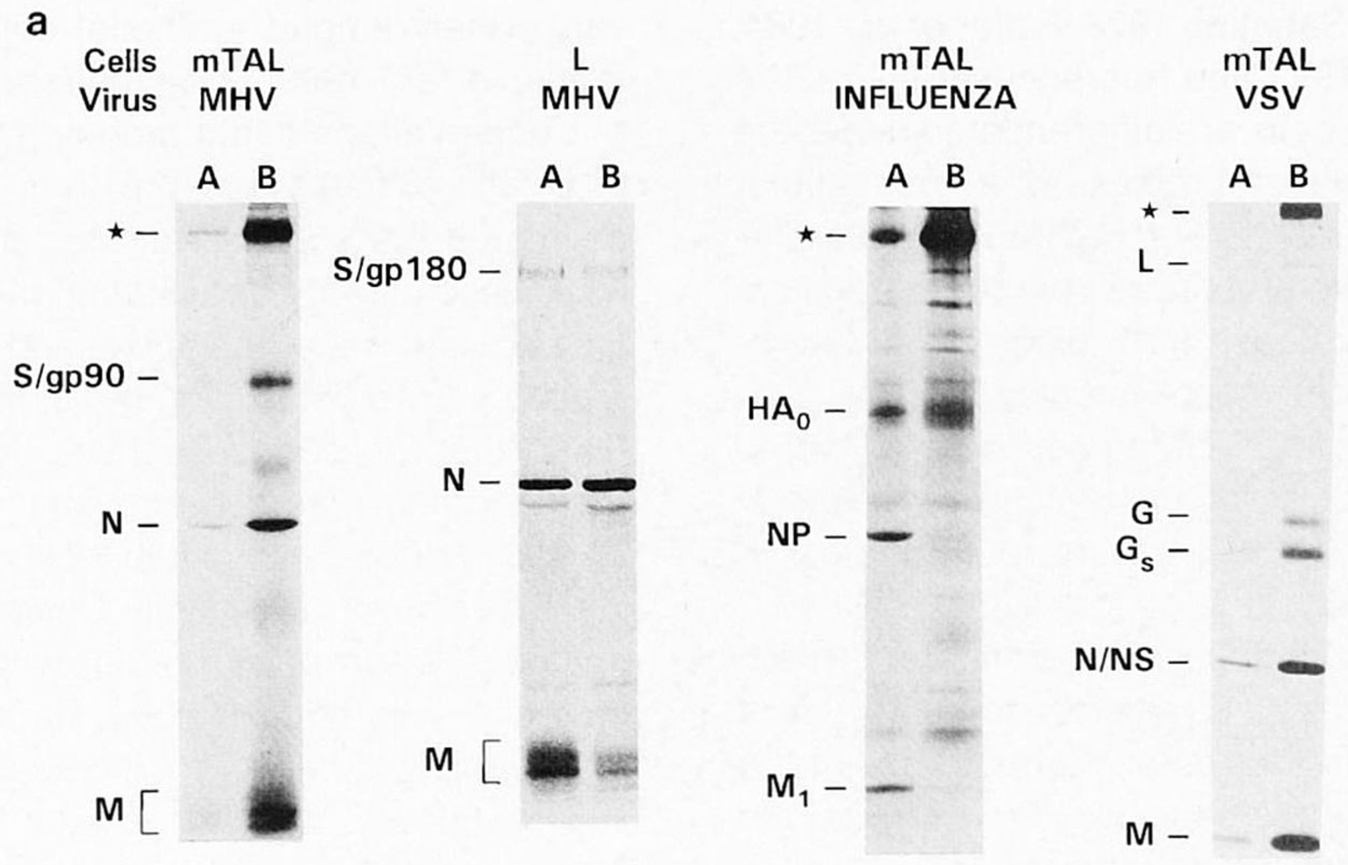
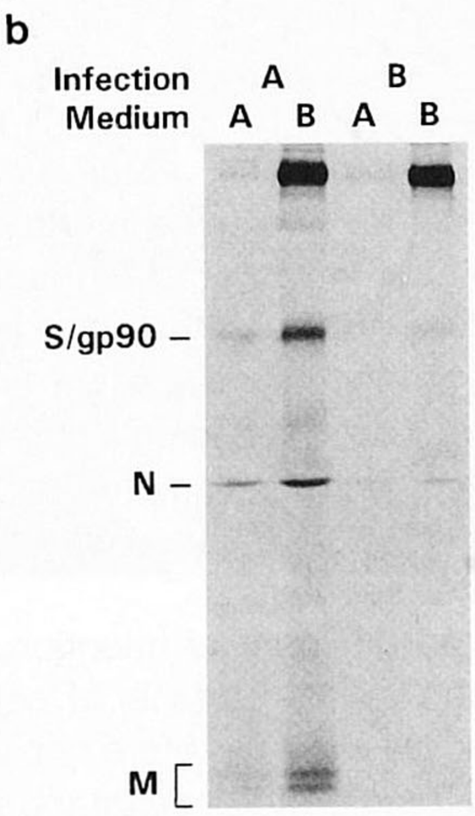


FIG. 5. Time course of infectivity released from infected mTAL cells. Filter-grown cells were infected apically at 16 hps with MHV. Media were collected at different times pi and replaced by fresh infection medium (IM) containing 1% FCS. The amount of infectious viral particles in the apical and basolateral medium was determined in an endpoint dilution assay on L-cells. $TCID_{50}$, 50% tissue culture infectious dose.





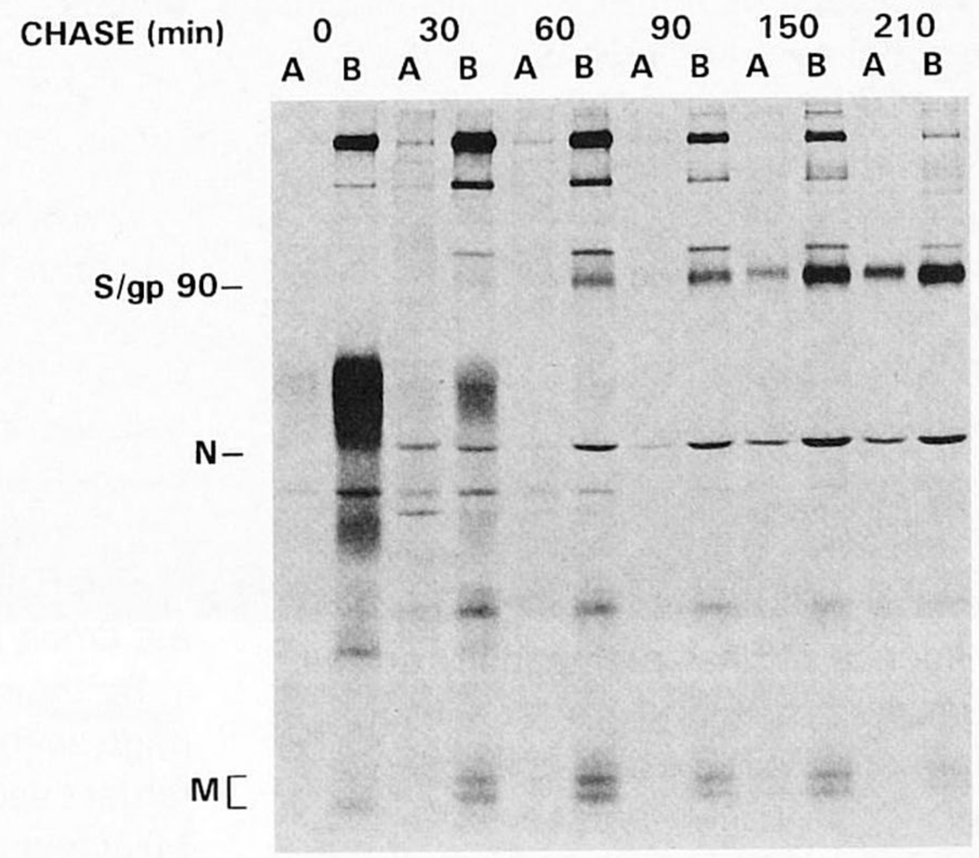


FIG. 6. Release of radiolabeled MHV proteins from infected cells. Filter-grown mTAL cells and L-cells were infected apically with MHV, VSV, or influenza virus at 16 hps (a, c) or inoculated apically or basolaterally with MHV at 40 hps (c). Cells were labeled with [^{35}S]methionine from 6–9 hpi (a, b) or pulse labeled from 6–6.5 hpi with [^{35}S]methionine and chased for the indicated times (c). Subsequently, viral proteins were precipitated from the apical (A) and basolateral (B) media using MHV-, VSV-, or influenza virus-specific antiserum. Proteins were separated in a SDS–10% (VSV), 12.5% (influenza/MHV in mTAL cells), or 15% (MHV in L-cells) polyacrylamide gel. The positions of the viral structural proteins are indicated. Note that the MHV S protein in virus released from L-cells remains largely uncleaved and that the bands representing influenza NP and M_1 may contain also some cleaved HA_0. The asterisk indicates a host cellular protein released preferentially into the basolateral medium by mTAL cells.

leased into the basolateral fluid largely exceeded that found in the apical fluid (Fig. 5). At 25 hpi, for instance, the $Ri_{B/A}$ value was 13.5, corresponding to 93% of the infectious MHV particles released basolaterally.

Radioimmunoprecipitation of released virus. To study the exit of MHV-A59 from mTAL cells in more detail, [^{35}S]methionine-labeled viral proteins released into the medium were analyzed. Influenza virus and VSV in mTAL cells and MHV in unpolarized L-cells served as controls. Monolayers were infected apically at 16 hps, labeled with [^{35}S]methionine from 6–9 hpi, the media collected, and released viral proteins immunoprecipitated using corresponding antisera. As shown in Fig. 6a the MHV proteins were predominantly present in the basolateral medium of MHV-infected mTAL cells. In contrast, L-cells released MHV proteins both in the "apical" and "basolateral" media, with a slight preference for the former, as judged from the distribution of the M protein. This result suggests that the filter support may hamper the basolateral appearance of MHV to some extent, resulting in an underestimate of the amount of MHV proteins released basolaterally. The two control viruses behaved as expected: influenza virus was released preferentially from the apical membrane and VSV from the basolateral membrane

(Rodriguez-Boulan and Sabatini, 1978; Fuller *et al.*, 1984; Tucker and Compans, 1993 and references therein). We conclude that the mTAL cells are differentiated under the conditions used; the polarized release of a host cellular protein (~250 kDa; asterisk in Fig. 6a) that appears predominantly on the basolateral side supports this notion. We also studied MHV release from older mTAL monolayers (Fig. 6b), where cells were infected at 40 hps. At this time the cells are fully differentiated as reflected by the inability to infect them through the basolateral membrane. Again, MHV proteins were found mainly in the basolateral medium.

One might argue that the observed basolateral release of MHV might be the result of efficient transcytosis of apically released virus rather than of direct basolateral secretion. To investigate this possibility we analyzed the effects of MHV antiserum added either to the apical or the basolateral medium on infectivity appearing on the opposite side. If transcytosis would occur, virus would likely be neutralized and/or captured on one side leading to a decrease in infectivity detectable on the other. No such effects were observed. We also carried out a pulse–chase experiment to assess whether radiolabeled virus after an initial apical appearance would be redirected to the basolateral side. Figure 6c shows that this is not the case as labeled viral proteins accumulate in the basolateral medium without a transient apical presence.

Electron microscopic analysis

To further analyze the polarity of virus release and to investigate the influence of filter permeability on our results, an electron microscopic study of MHV-infected mTAL cells was performed. Figure 7A shows an overview of a cell infected apically at 3 days ps and fixed at 10 hpi. Part of the apical membrane and of the intercellular space is shown enlarged in Fig. 7B. Numerous viral particles were observed in the intercellular spaces. They accumulated below the tight junctions and were apparently secreted from the basolateral side. Only few virions were seen attached to the apical membrane. Figure 7C shows that viral particles accumulated not only between adjacent cells, but also between the basal membrane and the filter on which the cells were grown. Since these particles are not detected in our immunoprecipitations and titrations, these assays inherently result in an underestimate of the actual amount of basolaterally released virus.

The surface ratio between basolateral and apical membranes

The results presented above suggest a preferential release of MHV from the basolateral membrane domain of mTAL cells. However, the relative surface areas of basolateral and apical membrane domains are known to vary widely among epithelial cells (Parton *et al.*, 1989; Pfaller *et al.*, 1990; van Genderen *et al.*, 1991). Hence, our observations might merely reflect the particular surface ratio of these domains in mTAL cells. We therefore estimated the surface ratio for our mTAL cells electron microscopically by quantitating the relative values of surface densities S_v of the apical and basolateral membrane domains of 15 cells using the point counting method. We found that the surface density of the basolateral membrane domain was 1.7 times that of the apical membrane domain. For comparison, in MDCK I cells the basolateral surface is approximately five times as large as the apical domain (Parton *et al.*, 1989), whereas in LLC-PK1 and MDCK II cells both domains have an almost equal surface density (Pfaller *et al.*, 1990; van Genderen *et al.*, 1991). The ratio of 1.7 is at least five times smaller than the ratio of virus released into the basolateral versus apical compartments; this is consistent with a selective process.

DISCUSSION

We have extended our studies on coronaviral entry and release by examining the interactions between MHV-A59 and cells of a polarized murine epithelial line. The virus infects mTAL cells from the apical side, but this polarity of entry becomes evident only some time after the cells have been seeded on a permeable filter support. In contrast, virus is released preferentially from the basolateral side, which was observed already from the earliest times after seeding.

By their natural route of infection, coronaviruses engage with the apical surface of cells in their primary target tissue. We were therefore not surprised to find that MHV has a strong preference for the apical membrane of polarized mTAL cells, like TGEV, another coronavirus, has for porcine epithelial cells (Rossen *et al.*, 1994). The polar entry of viruses into epithelial cells appears to reflect the distribution of their receptors (Fuller *et al.*, 1984, 1985; Clayson and Compans, 1988; Rossen *et al.*, 1994). In mTAL cells the receptor used by MHV is a member of the CEA subfamily, similar to the situation in other cells (Williams *et al.*, 1991; Dveksler *et al.*, 1991, 1993). Attempts to determine its distribution at the plasma membrane by immunofluorescence and confocal microscopy were unsuccessful due to the low expression level of this protein in mTAL cells. Our infection inhibition experiments, however, indicate that the receptor occurs predominantly at the apical membrane when cells are polarized. CEA proteins have been recognized in the apical plasma membrane surface of differentiated epithelial cells, in native tissue, and in cell lines (Shirota *et al.*, 1988; Sugiyama *et al.*, 1988; Lisanti *et al.*, 1990; Baghdiguian *et al.*, 1991). The development of polarity of MHV entry into mTAL cells with time most likely reflects the course at

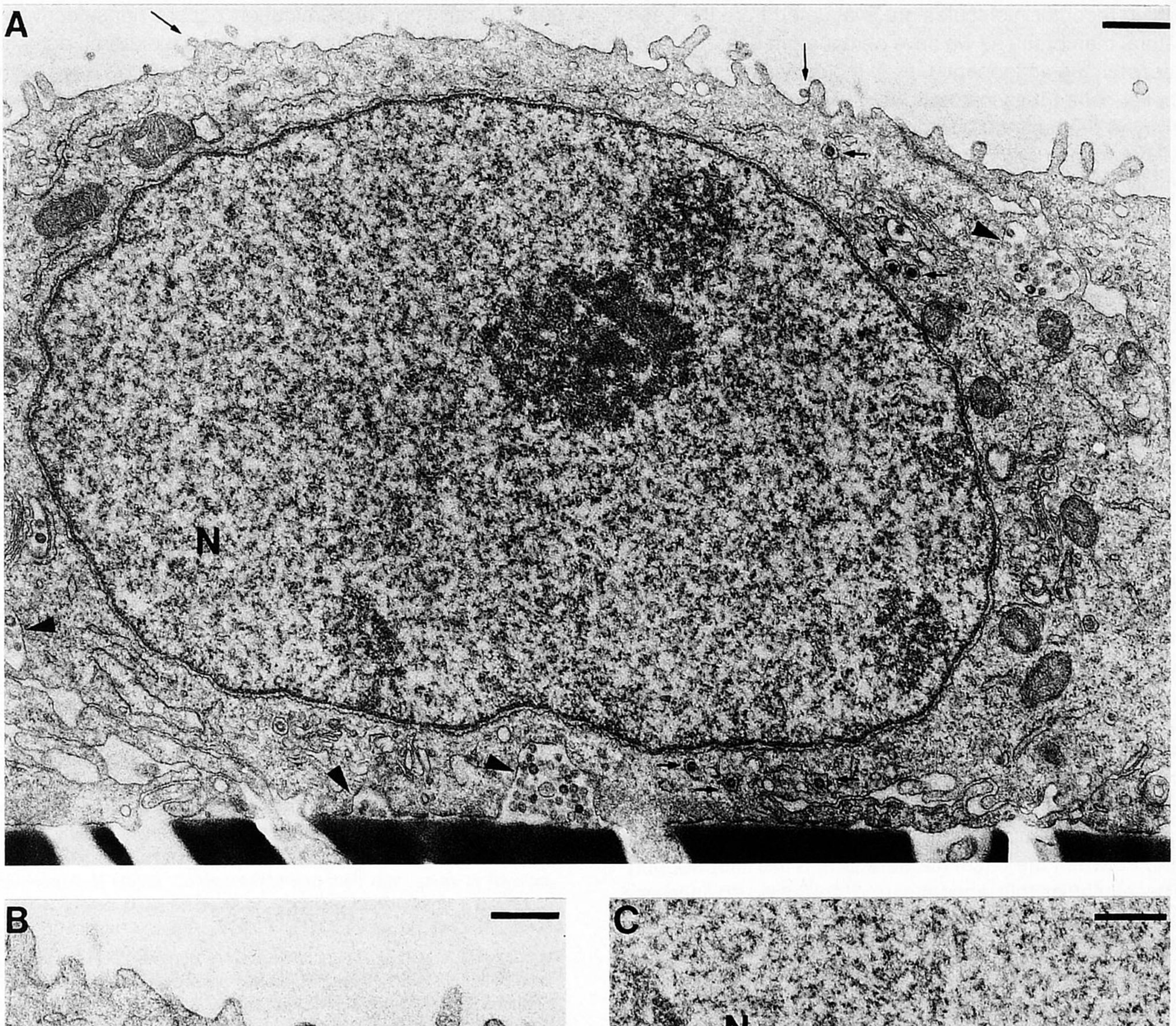


FIG. 7. Electron microscopical analysis of MHV-infected mTAL cells. Filter-grown mTAL cells were inoculated apically at 3 days postseeding. At 10 hpi cells were fixed, embedded, and sections were cut for observation in the electron microscope. (A) Overview of a cell with very few viral particles bound to the apical membrane domain (large arrows) and a large amount of viruses accumulating in intercellular spaces and between cells and the filter (arrowheads). The filter can be seen on the bottom. Indicated are also some of the intracellular viral particles (small arrows) and the nucleus (N). (B) Enlargement of a part of the apical membrane and the intercellular space filled with viral particles. (C) Enlargement of a part of the basolateral membrane. Between the filter (bottom) and the basal membrane accumulating viral particles are observed. Calibration bars 0.53 (A), 0.30 (B), and 0.38 μm (C).

which receptor molecules are evacuated from the basolateral membrane as we have demonstrated for the TGEV receptor, the aminopeptidase N molecule, in porcine epithelial cells (Rossen *et al.,* 1994). Hence, the polarity of entry is probably due to the polarized distribution of the MHV-receptor, although also other entry factors may be involved.

Polar release of viruses from their host cells has been repeatedly demonstrated, but entry and release generally happen both at the same membrane (Tucker and Compans, 1993). This is not true for MHV, as shown in this study. The polarity of release developed rapidly and was already evident before the entry of the virus had become polarized. At that time also the release of VSV (basolateral) and influenza virus (apical) were already polar. Clearly, both cellular transport pathways are established rapidly. These findings are consistent with electron microscopic observations which showed that the exit of influenza virus and VSV from MDCK cells becomes polarized as soon as cell–cell contacts or cell-substrate contacts are established (Rodriguez-Boulan *et al.,* 1983). Also the release of TGEV from porcine epithelial cells was polarized long before entry had become restricted to the apical side (Rossen *et al.,* 1994).

The polarity in MHV release from mTAL cells was less pronounced than that of TGEV from LLC-PK1 cells (Rossen *et al.,* 1994). However, we may have underestimated the extent of polarity under our experimental conditions. First, electron microscopy revealed large numbers of viral particles trapped in the lateral spaces between cells and between the basal membrane and the filter support. The extent of this accumulation appeared to increase with the age of the monolayer. Second, individual cells were seen to grow on top of the monolayer as well as on the (plastic) sides of the growth chambers as has also been observed by others (Zurzolo and Rodriguez-Boulan, 1993). Virus released from these cells has obviously been scored apically. This effect is not negligible: the extent of polarity, expressed as the ratio of virus released basolaterally and apically ($R_{B/A}$), was always significantly higher when monolayers had been infected from the basolateral side. The observations are also consistent with the general decrease in the $R_{B/A}$ values found as older monolayers were used.

Murine coronaviruses not only infect epithelial cells, they are also neurotropic and replicate in astrocytes, oligodendrocytes, and neurons (Sorensen and Dales, 1985; Parham *et al.,* 1986; Matsubara *et al.,* 1991). In cultured hippocampal neurons, viral proteins are sorted in a polarized fashion, and axons and dendrites correspond with the apical and basolateral plasma membrane domains, respectively, of epithelial cells (Dotti *et al.,* 1988, 1990). Recently, it has been reported that MHV (JHM strain) particles are present both in axons and in dendrites of cultured rat hippocampal neurons, suggesting that virus dissemination occurs nonselectively, i.e., both from the apical and the basolateral domain equivalent (Pasick *et al.,* 1994). This is at variance with results obtained in our MHV-A59 system (with virus exit selectively from the basolateral membrane) and TGEV system (with preferential virus release from the apical side; Rossen *et al.,* 1994).

The polarized release of viruses can determine viral spread (Tashiro *et al.,* 1988, 1990); it may help to explain the elementary pathogenetic differences between MHV and TGEV after infection of epithelial surfaces. The latter causes an enteric disease, and it spreads among epithelial cells of the porcine intestine (Doyle and Hutchings, 1946; Pensaert *et al.,* 1970a,b). In contrast, MHV-A59 infects the upper respiratory mucosa and then disseminates to other organs (Compton *et al.,* 1993 and references therein). The occurrence of MHV strains causing intestinal infections associated with lateral spread and, conversely, of TGEV-related viruses lacking this pathogenic potential should allow us to verify the role of directional release from epithelial cells in viral pathogenesis.

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