Hepatitis B Virus Translocates across a Trophoblastic Barrier

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Mother-infant transmission of hepatitis B virus (HBV) accounts for up to 30% of worldwide chronic infections. The mechanism and high-risk period of HBV transmission from mother to infant are unknown. Although largely prevented by neonatal vaccination, significant transmission continues to occur in high-risk populations. It is unclear whether HBV can traverse an intact epithelial barrier to infect a new host. Transplacental transmission of a number of viruses relies on transcytotic pathways across placental cells. We wished to determine whether infectious HBV can traverse a polarized trophoblast monolayer. We used a human placenta-derived cell line, BeWo, cultured on membranes as polarized monolayers, to model the maternal-fetal barrier. We assessed the effects of placental maturity and maternal immunoglobulin on viral transport. Intracellular viral trafficking pathways were investigated by confocal microscopy. Free HBV (and infectious duck hepatitis B virus) transcytosed across trophoblastic cells at a rate of 5% in 30 min. Viral transport occurred in microtubule-dependent endosomal vesicles. Additionally, confocal microscopy showed that the internalized virus traverses a monensin-sensitive endosomal compartment. Differentiation of the cytotrophoblasts to syncytiotrophoblasts resulted in a 25% reduction in viral transcytosis, suggesting that placental maturity may protect the fetus. Virus translocation was also reduced in the presence of HBV immunoglobulin. We show for the first time that transcytosis of infectious hepadnavirus can occur across a trophoblastic barrier early in gestation, with the risk of transmission being reduced by placental maturity and specific maternal antibody. This study suggests a mechanism by which mother-infant transmission may occur.

Over 350 million people worldwide are chronically infected with hepatitis B virus (HBV), with mother-infant transmission accounting for up to 30% of cases (19). Congenital infection results in chronic hepatitis in 90% of children and the risk of liver failure or hepatocellular carcinoma and death in early adult life. The incidence of in utero transmission of HBV is unknown, as mother-to-infant transmission may occur perinatally, during delivery, or early postpartum. HBV has been found in amniotic fluid, breast milk, and vaginal fluids, as well as cord blood and infant gastric contents (7, 24). Maternal HBsAg status is the most significant factor determining risk of perinatal transmission, although maternal HBcAb-negative status and high HBsAg titer have also been reported to increase the risk of transmission (6, 61).

Vaccination of newborn infants reduces the likelihood of perinatal transmission from HBsAg-positive mothers by 79 to 90% (2), and the likelihood is further reduced by concurrent administration of HBV immunoglobulin (HBIG). While this implies that most transmission probably occurs perinatally, a clinically significant proportion of neonatal viral infection occurs despite vaccination. Among children vaccinated at birth, a 1 to 5% viral transmission rate is reported (8, 9, 26, 45, 64). Figures of vaccination failure rates from China are even higher (51, 62), suggesting that in utero HBV transmission may be more significant among high-risk groups. Additionally, infection risk has been related to the presence of DNA in the placenta (61) and to maternal viremia (4, 38), supporting an association between the state of maternal HBV during pregnancy and the risk of transmission to the baby. It is unclear whether HBV can traverse intact epithelial barriers to infect the fetus during gestation. HBV DNA has been found in reducing of the concentrations from the maternal to the fetal side of the placenta, suggesting cell-to-cell transfer of virus in the placenta and a possible mechanism for in utero transmission (61, 63).

The placenta is made up of chorionic villi consisting of a fetal capillary, villous stroma, and a layer of trophoblast cells consisting of syncytiotrophoblasts and cytotrophoblasts. These cells constitute a tight polarized epithelial monolayer comprising tight junctions preventing lateral and paracellular diffusion of substrates. Their apical surfaces are in contact with maternal blood, while their basolateral surfaces are contiguous with the fetal circulation. By 20 weeks, these cells terminally differentiate and fuse to form multinucleated syncytiotrophoblasts (44). The syncytiotrophoblast constitutes the maternal-fetal barrier through which exchanges of substrates occur by transcytosis (15). Infection of the placenta and of the fetus depends on the permissiveness of these cells to the passage of pathogens. Maternal-fetal transmission of a number of viruses has been shown, with various consequences for the baby (17, 22, 23, 31).

We examined the ability of infectious HBV to cross the placenta in an in vitro system. Trophoblast-derived BeWo cells grown on semipermeable inserts form a polarized monolayer with tight junctions between cells as a model of the maternal-fetal barrier (5). We show that these cells transcytose cell-free HBV to a high degree in endosomes dependent on an intact cellular cytoskeleton. Utilizing duck hepatitis B virus (DHBV) as an infectious model, we confirmed that the transcytosed

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virus remains infectious. HBV transcytosis was reduced with syncytiotrophoblast formation, as well as by specific antibody. The findings of this study suggest that infectious HBV may be able to transcytose across the maternal-fetal barrier in the first trimester.

**MATERIALS AND METHODS**

**Materials.** HBV was obtained from the supernatant of HepG2 cells transduced with Adeno-HBV and cultured for 14 days before cell supernatants were collected, clarified by low-speed centrifugation, and quantified for HBV by PCR. Adeno-HBV-transduced HepG2 cells produce infectious HBV virions via all viral replicative intermediates (50).

**Transcytosis studies.** BeWo cells were seeded to 70% confluence on six-well PET cell culture inserts, 1-μm pore size (BD Falcon), in a two-chambered system. In this system, BeWo cells form a polarized monolayer with tight junctions between cells, allowing access to both the apical and basolateral domains. Transmural resistance was measured daily at 37°C with a volthometer (Millipore, Australia), and the monolayer was determined to be polarized and contiguous if corrected transepithelial resistance measurements exceeded 100 Ω·cm². Blank inserts with media were used to zero between measurements. Cultures were not permitted to proceed for more than 5 days, to avoid overgrowth and cell layering. In addition, each transcytosis experiment included controls (50).

HBV translocation across a trophoblastic barrier

**HBV**

**RESULTS**

HBV transcytoses across BeWo monolayers. Confluent monolayers of BeWo cells were grown on semipermeable membranes, and medium containing cell-free HBV was added to the apical domains. Three 30-min basolateral medium collec-

The inoculum was left on the cells for 24 h and then removed and replaced with 2.5 M H2SO4 when color was visible. Absorbance was read at 450-nm wavelength.

**Indirect immunofluorescence.** BeWo cell monolayers used in the transcytosis studies described above were fixed in 4% paraformaldehyde with 0.01% Triton X-100 in phosphate-buffered saline after the final collection. Semipermeable membranes on which the cells were growing for transcytosis experiments were then excised from the inserts and adhered to glass slides to allow for immunodetection and microscopic visualization. Cells were stained with 1H1 monoclonal anti-pre-S DHBV antibody (42) and rabbit anti-ZO-1 antibody (against the cytoplasmic domain of ZO-1) and then visualized with Alexa 488 and propidium iodide before examination by confocal microscopy. The numbers of nuclei and cell boundaries were counted in multiple fields, and their ratios were used to determine the percentage of cell fusion.

**HBIG treatment.** BeWo monolayers were treated with purified HBIG for 72 h at 0, 100, or 1,000 mg/liter in both apical and basolateral supernatants. HBV was added to the apical domain, and basolateral supernatant was collected every 30 min and replaced with medium containing HBIG to maintain the concentration of HBIG throughout the experiment.

**IgG ELISA.** As most HBV hyperimmune globulin comprises immunoglobulin G (IgG), the rate of transcytosis of HBIG across BeWo monolayers was determined by total IgG enzyme-linked immunosorbent assay (ELISA). A standard curve was obtained by serial dilution of HBIG of known concentration. Immunosorbed plates (Nunc) were coated with 100 μl of 0.01% monomodal anti-human IgG Fc (Chemicon, Australia) in bicarbonate buffer. Plates were blocked in 3% skim milk powder in phosphate-buffered saline. Next, 100-μl samples were added to each well and incubated at 37°C for 2 h. Plates were then washed and conjugated with 100 μl of 0.02% sheep anti-human IgG horseradish peroxidase (Amersham) for 30 min at room temperature and detected with 100 μl of 1% 3',3,5'-tetramethylbenzidine buffer (Chemicon), with the reaction being terminated with 2.5 M H2SO4. The absorbance was read at 450 nm and 620 nm wavelengths.

**Microscopy.** Immunofluorescence examination of PDH was performed with an Olympus IX70 microscope. Images of BeWo monolayers were acquired with a Bio-Rad MRC 1024 Nikon scanning confocal microscope with Lasersharp software. An optimal X-100 image was obtained for visualization of the (apical) ZO-1 distribution while still detecting some signal from the (basolateral) very bright nuclear staining. Single X images in this confocal plane were used. Raw TIFF images were processed with Adobe Photoshop.

**Data analysis.** Data were imported from the primary sources into Microsoft Excel for analysis. Duplicates were sampled at random. The two-tailed Student’s t-test was used to determine the significance of differences between values. We accepted P values of <0.05 as significant.
tions were taken before and after the addition of drugs that inhibit specific transcytotic pathways (see “Transcytosis studies,” above). To each culture, brefeldin A, monensin, colchicine, or no drug was added. Transepithelial resistance of the BeWo monolayers did not vary with the addition of virus or drug (data not shown). Virus titer in the supernatants was determined by quantitative PCR. Results are given as means of pre- and posttreatment collections from duplicate wells. Percent transcytosis was calculated for each time point, allowing for progressive loss of virus from the apical to the basolateral domain.

Five percent of apical HBV was efficiently transcytosed by polarized trophoblasts in 30 min (Fig. 1). Transcytosis of cell-free virus varied little in the no-treatment groups. The addition of brefeldin A, an agent that disrupts both the Golgi and endosomes (20, 30), markedly inhibited HBV transcytosis. Likewise, monensin, a Na+/H+ ionophore that inhibits endosomal acidification (36), inhibited viral transcytosis from pretreatment levels of over 4% transcytosis to 1.7% in 30 min. Disruption of the cellular cytoskeleton with colchicine (32) resulted in a similar degree of marked inhibition of transcytosis. The degrees of inhibition from all of these agents were very similar, suggesting that they all inhibit different aspects of a single pathway. It appears from these data that cell-free HBV is transported from the apical to the basolateral surface of BeWo cells by transcytosis in endosomes and that these are reliant upon cytoskeletal elements for traffic.

Viral transcytosis is inhibited at 4°C. To determine whether virus was being transported by an active cellular process, the temperature dependence of viral translocation was examined. Monolayers of BeWo cells on membranes were incubated at 4°C for 1 h. At this temperature, energy-dependent cellular functions are largely inhibited, but passive diffusion of molecules should remain unaffected. DHBV and [3H]inulin were added to the apical supernatant of the BeWo monolayers. Basolateral collections of media were taken every 2 h, and cells were then rapidly warmed to 37°C and further collections were taken.

Figure 2A shows that at 4°C, with all cellular activity halted but diffusion minimally affected, there was very little virus detected in the basolateral supernatant. These cells, when warmed to 37°C, transcytosed virus effectively, indicating the energy dependence of viral transport. In addition, the rapid rate of transcytosis upon warming suggests that the virus had bound to cell surface molecules at 4°C, which are then internalized almost immediately upon cell warming. By contrast, [3H]inulin diffusion was not temperature dependent and remained at about 0.9% throughout the 4 h. In addition, the markedly higher rate of transport of the much larger DHBV virion than of the small, 5-kDa [3H]inulin molecule is indicative of active transport of virus rather than diffusion. Transcytosis of hepadnavirus in BeWo cells appears to occur through a process that requires cellular metabolism and perhaps through a receptor-mediated mechanism.
Transcytosed virus is infectious. The internal pH of transcytotic endosomes is acidic (around pH 5), promoting fusion of the viral and endosomal membranes and thus releasing the virus into the cytosol. HBV is an enveloped virus, and envelopment is necessary for its infectivity (57); thus, it was possible that endocytosis of the HBV in BeWo cells would inactivate the virus. In order to determine whether viral DNA transcytosed through the BeWo monolayer consisted of infectious virus particles, we utilized DHBV as an infectious model of HBV. DHBV is very similar to HBV in structure and protein functions (49) and has almost identical replication cycles (34). Like all members of the hepadnavirus family, it is a hepatitisotropic, species-specific virus. In the absence of a readily available infectible cell culture system for HBV, DHBV serves as an excellent and well-characterized infectious model (47).

Polarized BeWo cell monolayers grown on semipermeable membranes were examined for DHBV transcytosis by PCR and by infectious virus titer determined on virus-naive primary duck hepatocytes. As described above, virus was added to cells apically, and each monolayer was treated with trafficking inhibitors or with no drug.

DHBV transcytosed across the BeWo monolayer with efficiency similar to that of HBV, and its passage was likewise affected by transcytosis inhibitors. Virus transcytosis was inhibited by monensin and colchicine similarly, suggesting that the endosomes inhibited by monensin were reliant on microtubular function (Fig. 2A). We thus determined that DHBV was a suitable model for examining the transcytosis of infectious HBV in BeWo cells.

Infection of PDH with basolateral supernatant from BeWo monolayers showed that transcytosis of HBV through trophoblasts does not affect its infectivity. Infectious hepadnavirus transcytosed across BeWo monolayers at 3 to 5% per 30 min (Fig. 3A). The infective viral titer was about 10-fold less than total DNA by PCR, but the percentage of transcytosis remained the same, indicating that the virus had not been altered by its passage through the BeWo cells (Fig. 3A). As both human and duck HBVs were transported across human trophoblastic cells, it is likely that the pathway used by the viruses is not host species or cell type specific.

Hepadnaviral traffic in BeWo cells. The inhibition of viral transport with the various agents described above suggested that a specific transcytotic route was taken by HBV in BeWo cells. In order to differentiate the patterns of intracellular virus distribution, cell monolayers used in the experiments described above were fixed and stained for ZO-1 and DHBV for examination by confocal microscopy.

The BeWo cells were a single cell layer, with no overgrowth and with tight junctions between the cells in a honeycomb pattern characteristic of polarized epithelia (Fig. 4A). DHBV immunostaining was visible at the level of the tight junctions, indicating the presence of the virus at the apical surface. The cytoplasmic distribution of the virus suggested accumulation in vesicular structures (Fig. 3B). We notably did not see a perinuclear Golgi pattern of distribution of virus in brefeldin A-treated cells, indicating that viral transport in BeWo cells occurs independently of the Golgi and is likely via the transcytotic pathway (Fig. 3B) (27, 60). Monensin treatment led to virus localizing to an area close to the apical surface of the cells (Fig. 3B). This is probably the subapical compartment (SAC), described for other epithelial cells as a sorting center directing newly endocytosed substances to their plasma membrane destinations (53). Also of note in the monensin-treated cells is that there seems to have been minimal viral entry after the addition of the drug, as very little cytoplasmic virus is evident. Colchicine treatment led to cytoplasmic, widely distributed accumulation of virus intracellularly (Fig. 3B). These results suggest that hepadnavirus traffics from the apical surface to the SAC of BeWo cells in microtubule-dependent endosomal vesicles before traveling to the basolateral surface and without recourse to the Golgi, strongly suggestive of transcytosis as the mechanism of transcellular transport.

HBV transcytosis is inhibited by cellular differentiation. With placental development, trophoblasts differentiate and fuse into multinucleated cells that form the syncytiotrophoblast. The formation of this continuous barrier has been associated with reduced viral transmission in some in vitro studies (40, 41). We stimulated differentiation of BeWo cells with forskolin, a potent inducer of cAMP (48). With differentiation, cells fuse as they divide; however, terminally differentiated cells will not further divide. Balancing these effects of cell maturation, we generated cell monolayers that remained contiguous but contained a significant proportion of terminally differentiated syncytiotrophoblasts. We then added HBV to...
the apical medium, and basolateral collections were taken at 30-min intervals over several hours. [3H]inulin diffusion studies ascertained the integrity of the monolayers. Cells were then fixed, and intracellular tight junctions were stained with ZO-1 before examination by confocal microscopy to determine the percentage of cell fusion.

Confocal microscopy revealed fusion of 3 to 10 cells within a single cell boundary in cultures treated with forskolin (Fig. 5A). We determined a fusion rate of 21.0% ($\pm$ 0.7%) in treated cells. That is, on average, more than a fifth of the cells were multinucleated. These monolayers did not transcytose virus as effectively as untreated cells, resulting in a small but statistically significant inhibition of transcytosis compared to the untreated BeWo cells. Monolayers displaying differentiation transcytosed only 4.4% of apically applied virus, compared to 5.9% in undifferentiated cells ($P = 0.0168$) (Fig. 5B). This is a reduction in the rate of transcytosis of around 24%, close to the cell fusion rate of 21%. The reciprocity between reduced viral transport and cell fusion suggests that they are related. Differentiation of the BeWo monolayer from mononuclear cells with tight junctions to partial syncytiotrophoblast formation resulted in significant and proportional reduction in transcytosis.

HBIG prevents HBV transcytosis. Antibodies against HBV surface antigens are protective against disease. The presence of high levels of HBV-specific antibody in infected mothers may affect fetal infection. The effect of HBIG on the transcytosis of HBV was studied. We used doses of HBIG based on minimum target serum concentrations of antibody in liver transplant patients receiving HBIG: approximately 100 mg/liter (10, 21, 29). HBIG treatment at both 100 mg/liter and 1,000 mg/liter reduced HBV transcytosis by a modest but significant degree compared to untreated cells and antibody-negative serum ($P = 0.024$) (Fig. 6). The addition of 10 times more HBIG, however, caused little additional reduction in transcytosis ($P = 0.71$). The specificity of the action of the antibody was determined by examining DHBV transcytosis in the presence of HBIG. HBV, but not DHBV, translocation was inhibited by HBIG (Fig. 6).

Trophoblasts and BeWo cells are known to actively transport IgG via the Fc receptor (12). In order to confirm that BeWo cells were able to transcytose HBIG in this model, we added HBIG to the apical domain of membrane-grown BeWo cells and assayed the apical and basolateral supernatants for IgG by ELISA. We found that BeWo cells transport 8% of apically applied HBIG every 30 min (Fig. 6). This is a very high rate of transcytosis and probably represents both active trans-

![FIG. 4. Hepadnavirus traffic in BeWo cells. BeWo monolayers used in the experiments reported in Fig. 2A were fixed in paraformaldehyde and stained for DHBV pre-S antigen (red) and ZO-1 (green). Nuclei are red. (A) 1x images show BeWo nuclei in a single layer. (B) In untreated BeWo cells (No Drug), intracellular DHBV is in vesicular structures. Treatment with brefeldin A (Bref) led to virus accumulation in a typical Golgi pattern. Monensin (Mon) treatment led to virus accumulation in a discrete, subapical, non-Golgi area of the cell, most likely the SAC. Little virus is seen elsewhere in the cell. Endocytosis of further virus appears to have been inhibited by monensin. Colchicine (Col)-induced disruption of microtubules inhibits endosomal transport, resulting in scattered virus throughout the cell. Bar, 10 µm.](http://jvi.asm.org/)
This represents free virus and whether it remains infectious. It is likely that HBV formed complexes with Ig that were saturated at 100 mg/liter, resulting in no further effect from 1,000 mg/liter of HBIG. The resultant virus-Ig complex may saturate at 100 mg/liter, resulting in no further effect from v ing substrates. The dependence on microtubules is characteristic of SAC-dependent, Golgi-independent trafficking (18). Infectious virus is subsequently released from the basolateral surface of trophoblasts into the fetal circulation. At around 12 weeks of gestation, trophoblasts fuse and differentiate into multinucleated syncytiotrophoblasts. We found that a mixed population of cells did not transcytose virus as effectively as a monolayer of undifferentiated cells. This may be due to changes in BeWo cellular protein expression accompanying maturation that reduced virus attachment and transport (33, 35, 39, 43). As transport of molecules across the maternal-fetal barrier must continue to take place throughout pregnancy, it is likely that specific cell factors play a role in HBV transport and that these change with cellular differentiation.

Maternal IgG protects the infant by its active transcytosis across the placenta (11, 12). In some viral infections, such as CMV and herpes simplex virus, the presence of maternal antibodies is significant in protecting the fetus (14, 46). In others, such as HIV, no identifiable protective antibodies have been isolated to date. In Epstein-Barr virus infection, transmission to the fetus may actually be enhanced by antibody-mediated viral entry (16). The presence of maternal a-Hbc has been associated with lower levels of perinatal transmission of HBV (6). In addition, HBIG treatment of newborn infants helps prevent transmission, underlying the role of passive immunity in preventing disease acquisition. In our study, HBIG transcytosed actively in BeWo cells. HBIG treatment of cells also significantly and specifically reduced the rate of HBV transcytosis and may have reduced its infectivity. The presence of protective maternal antibodies transcytosing across the maternal-fetal barrier with infectious virus may be one mechanism limiting viral transmission in utero. Conversely, insufficient maternal antibody levels may predispose to HBV transmission during pregnancy.

In determining the factors that contribute to mother-infant HBV transmission, the role of fetal development should be considered. The fetus does not begin to develop a liver until around 12 weeks of gestation (37). It is not known at what stage the development of the functional enhancers and promoters that allow the exclusively hepatotropic HBV to infect hepatocytes takes place. We have shown that while HBV can cross the pre-12-week maternal-fetal barrier, it appears that this is less likely with trophoblast differentiation in later gestation. Fetal hepatic immaturity may be how the fetus remains protected from HBV; this possibility goes somewhat toward
explaining the efficacy of HBIG and HBV immunization at birth in preventing infection.

This study addresses some key points regarding maternal-fetal transmission of HBV. The virus is able to cross trophoblastic cells using the endosomal transport system and to remain infectious. The high permeability of undifferentiated trophoblasts to viral transcytosis suggests that early pregnancy is a potentially high-risk period for viral transmission. Finally, these studies suggest that some vaccine failures may represent infections acquired in utero through transcytosis of HBV across the placenta, and they propose one mechanism for mother-infant transmission.

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We thank Elizabeth Grgacic for assistance with primary duck hepatitis B virus infections using the endosomal transport system and to remain infectious. The high permeability of undifferentiated trophoblasts to viral transcytosis suggests that early pregnancy is a potentially high-risk period for viral transmission. Finally, these studies suggest that some vaccine failures may represent infections acquired in utero through transcytosis of HBV across the placenta, and they propose one mechanism for mother-infant transmission.

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