Expression of Human Decay-Accelerating Factor on Intestinal Epithelium of Transgenic Mice Does Not Facilitate Infection by the Enteral Route

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ABSTRACT

In vitro, infection of polarized human intestinal epithelial cells by coxsackievirus B3 (CVB3) depends on virus interaction with decay-accelerating factor (DAF), a receptor expressed on the apical cell surface. Although mice are highly susceptible to CVB3 infection when virus is delivered by intraperitoneal injection, infection by the enteral route is very inefficient. Murine DAF, unlike human DAF, does not bind virus, and we hypothesized that the absence of an accessible receptor on the intestinal surface is an important barrier to infection by the oral route. We generated transgenic mice that express human DAF specifically on intestinal epithelium and measured their susceptibility to infection by a DAF-binding CVB3 isolate. Human DAF permitted CVB3 to bind to the intestinal surface *ex vivo* and to infect polarized monolayers of small-intestinal epithelial cells derived from DAF transgenic mice. However, expression of human DAF did not facilitate infection by the enteral route either in immunocompetent animals or in animals deficient in the interferon alpha/beta receptor. These results indicate that the absence of an apical receptor on intestinal epithelium is not the major barrier to infection of mice by the oral route.

IMPORTANCE

CVB3 infection of human intestinal epithelial cells depends on DAF at the apical cell surface, and expression of human DAF on murine intestinal epithelial cells permits their infection *in vitro*. However, expression of human DAF on the intestinal surface of transgenic mice did not facilitate infection by the oral route. Although the role of intestinal DAF in human infection has not been directly examined, these results suggest that DAF is not the critical factor in mice.

Coxsackieviruses belong to the genus *Enterovirus* of the family *Picornaviridae*, a group of small, nonenveloped viruses with a single-strand, positive-sense RNA genome (1). Group B coxsackieviruses (CVBs) are important causes of viral myocarditis and meningitis, and they have been proposed to be a possible triggering agent for juvenile diabetes (1).

CVBs, like other enteroviruses, are transmitted by the fecal-oral route and are believed to initiate infection by crossing the intestinal mucosa, which is lined by polarized epithelial cells with distinct apical and basolateral surfaces. In polarized epithelial cells, the primary CVB receptor, the coxsackievirus and adenovirus receptor (CAR) (2–4), is not expressed on the apical cell surface but is instead confined to the basolateral surface, where it functions as part of the intercellular tight junction (5); CAR is thus inaccessible to virus approaching the apical cell surface from the intestinal lumen. Some CVB isolates bind to a second receptor, decay-accelerating factor (DAF) (6–8). Unlike CAR, DAF is highly expressed on the apical surface of polarized epithelial cells, and infection of polarized cells depends on virus attachment to DAF (9). In addition to providing a docking site for virus, DAF mediates a series of intracellular signals that permit virus to move across the cell surface, interact with CAR in the tight junction, and then enter the cell (10).

CVBs, when delivered by intraperitoneal (i.p.) injection, readily infect mice, causing myocarditis (11) and pancreatitis (12); infection of the heart and pancreas is mediated by murine CAR (13, 14). Although infection can be established by the enteral (oral [p.o.]) route in some murine models (15, 16), the gastrointestinal tract has been reported to act as a barrier to infection, particularly beyond the neonatal period (17). Consistent with the idea of an intestinal barrier, our own experience has been that infection by the enteral route requires virus doses much higher (10,000-fold or more) than those needed to infect mice by intraperitoneal injection (unpublished data; see Fig. 2 and 3).

Several elements are likely to contribute to the barrier, including both physical factors—such as gastric acidity and the impermeability of the intestinal epithelium—and the host's innate immune responses. Another factor may be the lack of an accessible CVB receptor on the intestinal mucosa: although murine CAR binds virus (3, 18), murine DAF does not (19). As is true of CAR expression in human intestinal epithelium, murine CAR is confined to intercellular junctions (20) and is absent from the apical surface of mouse intestinal cells (our unpublished data). To test the idea that access to a receptor on intestinal epithelium might facilitate infection by the enteral route, we generated transgenic

mice that express human DAF (hDAF) on intestinal epithelium and measured their susceptibility to infection by a DAF-binding coxsackievirus B3 (CVB3) isolate. Although intestinal epithelial cells isolated from the transgenic mice were susceptible to infection *in vitro*, expression of human DAF on intestinal epithelium did not facilitate infection by the enteral route.

MATERIALS AND METHODS

Viruses. Coxsackievirus B3 strains Nancy (21), RD (21), H3 (22), and H3-RD (H3-VP2-N138D) (21) were derived from full-length viral cDNA clones. Nancy and RD were produced by *in vitro* transcription with T7 RNA polymerase (RiboMax; Promega) and transfection of RNA into HeLa cells using DMRIE-C reagent (Invitrogen). H3 and H3-RD were generated by transfection of HeLa cells directly with plasmid DNA, using Lipofectamine 2000 (Invitrogen). Virus was harvested 4 days later.

Intestine-specific hDAF transgenic mice. All experiments with animals were performed under protocols approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia Research Institute. cDNA encoding full-length human DAF was cloned downstream of a 9-kb regulatory region of the mouse villin gene previously shown to direct gene expression within intestinal epithelium (23) and upstream of the bovine growth hormone polyadenylation signal (Fig. 1A). After linearization with the restriction enzyme AclI, the construct was injected into C57BL/6 murine embryos to generate transgenic mice in the Children's Hospital of Philadelphia Transgenic Mouse Core Facility. hDAF transgenic mice were maintained in a C57BL/6 background.

To detect the hDAF transgene, PCR analysis of tail DNA was performed with primers hDAF-transF (5′-GATAGAAGACGGGTAGTACC-3′) and hDAF-transR (5′-GATAGAGACGGGTAGTACC-3′) and internal control primers specific for the intestinal fatty acid binding protein gene, primers Fabpi-500-F (5′-CCTCCGGAGAGCAGCGATTAAAAGT GTCAG-3′) and Fabpi-500-R (5′-TAGAGCTTTGCCACATCACAGGT CATTCAG-3′) (http://mgc.wustl.edu/Protocols/PCRGenotypingPrimer Pairs/tabid/154/Default.aspx). Wild-type animals show a single control band (466 bp), and transgenic animals show a 229-bp hDAF band as well as the control band.

Wild-type and IFNAR-KO mice. Wild-type C57BL/6 mice were obtained from The Jackson Laboratory. Interferon alpha/beta receptor1 (IFNAR1)-knockout (IFNAR-KO) mice (C57BL/6 background) were provided by Stefania Gallucci of Temple University. Genotyping of IFNAR-KO mice was performed by PCR with primers IFNAR1-F (5′-AA AAGACGAGGCGAAGTGG-3′), IFNAR1-R (5′-CATTCCACGAAGAT GTGCTG-3′), and neo-R (5′-AATTCGCCAATGACAAGACGC-3′) as described previously (24). Wild-type animals show a 149-bp band (IFNAR1-F to IFNAR1-R), and knockout animals show a 249-bp band (IFNAR1-F to neo-R).

Immunoblots. Immunoblots to detect DAF protein were performed with rabbit anti-CD55 (H-319) antibody (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare).

Immunofluorescence. Tissue samples were fixed overnight in 4% paraformaldehyde (PFA) at 4°C and then dehydrated in 30% sucrose overnight at 4°C; samples were embedded in OCT medium and frozen in a dry ice-ethanol solution, and frozen tissue blocks were used to prepare cryosections 6 to 8 μm thick. Sections were fixed with 4% PFA (10 min at room temperature), washed with phosphate-buffered saline (PBS), and then stained with fluorescein isothiocyanate (FITC)-labeled anti-human CD55 (clone IA10; BD Pharmingen) and AF594 phalloidin (catalog number A12381; Life Technologies) for 1 h at room temperature. Samples were washed, mounted in Vectashield mounting medium containing DAPI (4′,6-diamidino-2-phenylindole), and examined in an Olympus BX51 fluorescence microscope. For detection of M cells, sections were stained with anti-CD55 and with a rat monoclonal antibody that recognizes an M cell-specific α-(1,2)-fucose-containing carbohydrate (25) (clone NKM 16-2-4; MBL International). Sections were examined with a

Zeiss LSM 510 confocal microscope, and z-stack images were obtained at 0.3-μm intervals.

Immunogold electron microscopy. Tissue was prepared for immune detection as follows: high-pressure freezing in an Abra HPM010 machine, freeze substitution in 100% acetone, 0.1% uranyl acetate, 0.1% glutaraldehyde for 3 days at −90°C, embedding in HM20 medium at −50°C, and polymerization under a 360-nm light for 48 h. Sections of HM20-embedded mouse intestinal tissue cut at a 60-nm thickness were labeled with anti-CD55 monoclonal antibody (MAb; MAb GTX113170; Genetex) and then secondarily detected with 15-nm gold-conjugated protein G (Electron Microscopy Science, Hatfield, PA). Images of labeled tissues were collected at 80 keV on an FEI Tecnai 12 microscope equipped with a Gatan US1000 2K charge-coupled-device camera.

Real-time RT-PCR. Mouse tissues were homogenized, and total RNA was isolated with TRIzol reagent (Invitrogen) and purified using a Qiagen RNeasy minikit. cDNA was produced with murine leukemia virus reverse transcriptase (RT; Promega), and real-time PCR was performed using Power SYBR green PCR master mix (Applied Biosystems) and the following primers: hDAF-transF and hDAF-transR (described above for detection of the hDAF transgene) to measure hDAF RNA; mDAF-F (5′-GTTG CTCCAGAAAGACTGAG-3′) and mDAF-R (5′-ATAATATGCCGGTT GGTATG-3′) to measure mouse DAF (mDAF); and mGapdhF (5′-AAA TGGTGAAGGTCGGTGTG-3′) and mGapdhR (5′-CATGTAGACCATG TAGTTGAG-3′) to measure GAPDH (glyceraldehyde-3-phosphate dehydrogenase). For each sample, copy numbers of mDAF and hDAF RNA were determined relative to the copy number for the GAPDH control.

Virus infection. For parenteral (i.p.) infection, virus (100 μl, diluted in PBS) was injected into the peritoneum of 8- to 10-week-old mice; for enteral (p.o.) infection, an animal feeding needle (20 gauge, 1.5 inch; Fisher) was used to administer virus by gavage (200 μl diluted in PBS buffered with 3% NaHCO₃). Preliminary experiments indicated that peak tissue titers were achieved at 2 days after i.p. infection, and we expected that p.o. infection would be somewhat slower. Mice were therefore euthanized at 2 days after i.p. infection and 3 days after p.o. infection, and tissues were collected and stored at −80°C. The tissues were homogenized in PBS, homogenates were centrifuged to remove any debris, and the supernatant containing the virus was subjected to a plaque assay. Results were analyzed by 2-tailed Student's *t* test for unpaired samples.

Virus binding to mouse intestine *ex vivo*. Freshly harvested intestines were fixed in 4% PFA for 1 h at 4°C and then washed with binding buffer (minimal essential medium, 20 mM HEPES buffer). Intestines were cut into 1-cm-long segments, slit lengthwise to expose the mucosal surface, and then incubated in binding buffer with or without anti-DAF MAb IF7 (ascitic fluid diluted 1:50) for 1 h at room temperature. Intestinal segments were then incubated with ³⁵S-labeled CVB3 RD-H3 (40,000 cpm, approximately 550 PFU/cpm) in binding buffer for 1 h at room temperature. After three washes in binding buffer, the intestine-bound radioactivity was measured in a scintillation counter. Results were analyzed by 2-tailed Student's *t* test for unpaired samples.

Polarized intestinal epithelial cell monolayers from transgenic mice. Intestinal crypts were isolated from the duodenum as described previously (26) and suspended in a 24-well plate in 50 μl of Matrigel containing recombinant murine epidermal growth factor (EGF; 50 ng/ml; PeproTech), murine Noggin (100 ng/ml; PeproTech), human R-spondin-1 (250 ng/ml); and Y27632 (10 μM; R&D Systems), an inhibitor of Rho-associated protein kinase. Matrigel was overlaid with 50% WRN medium (conditioned medium from L cells expressing Wnt, R-spondin, and Noggin, as described in reference 27). The medium was replaced on the next day with 50% WRN medium supplemented with 10 μM Y27632 (50% WRN-Y) and then daily with 50% WRN-Y to permit growth of stem cell-enriched spheroids. Spheroids were trypsinized, split 1:3, and resuspended in fresh Matrigel approximately every 3 days.

To establish polarized epithelial cell monolayers, spheroids were
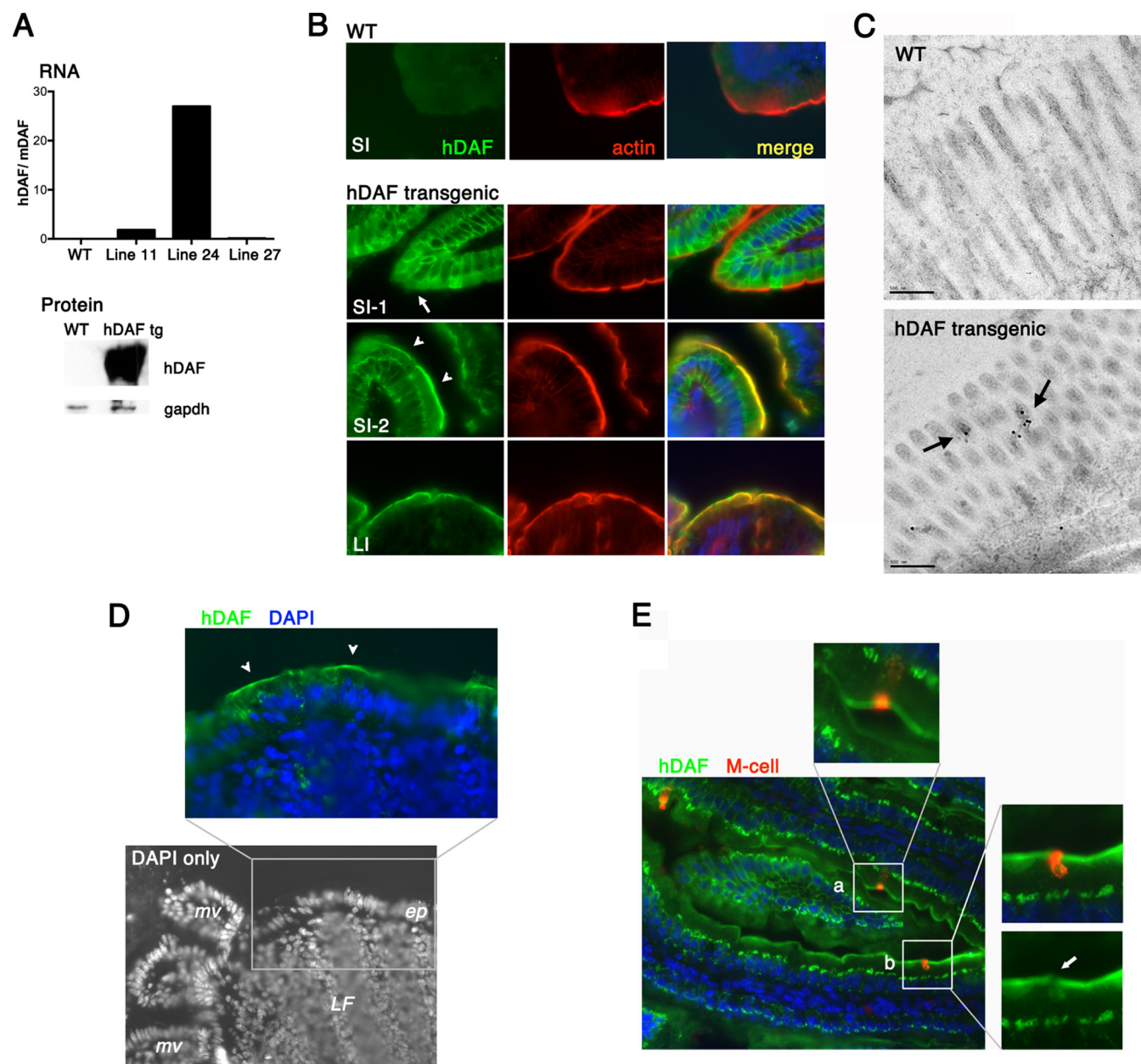
FIG 1 hDAF expressed on the epithelium of transgenic mice. (A) hDAF RNA and protein expression. (Top) RNA was measured by real-time PCR to assess hDAF RNA expression in the intestines of wild-type (WT) and transgenic mice (lines 11, 24, and 27). Data are shown as the ratio of the hDAF RNA copy number to the copy number for endogenous murine DAF RNA. (Bottom) Immunoblot to measure hDAF protein expression in intestines of wild-type and transgenic (tg) animals (line 24). (B) Immunofluorescence detection of hDAF in the intestines of wild-type and transgenic animals (line 24). In the small intestine (SI), hDAF (green) was detected both within cells (SI-1, arrow) and on the apical cell surface (SI-2, arrowheads), marked by phalloidin staining of the cortical actin layer (red). In the large intestine (LI), hDAF was expressed on the apical cell surface. (C) Immunogold electron microcopy to detect DAF in the small intestine. Gold label (arrows) is associated with the microvilli in transgenic but not wild-type mice. (D) Black-and-white image of DAPI-stained distal small intestine, showing a lymphoid follicle (LF) with overlying epithelium (ep) and adjacent microvilli (mv). The expanded color panel shows follicle-associated epithelium stained for hDAF (green); arrowheads indicate DAF at the apical epithelial cell surface. (E) Small intestine stained for hDAF (green) and an M cell marker (red). Some M cells appear to stain for surface DAF (inset a); however, the red M cell staining is associated with a neighboring cell in a different focal plane (inset b, arrow).

treated with TrypLE enzyme (Life Technologies) to obtain a single-cell suspension, and cells were plated in 50% WRN-Y on Transwell filters precoated with placental type IV collagen; medium was replaced daily with 50% WRN (without Y27632) until transepithelial electrical resistance (measured with an epithelial volt ohmmeter [World Precision Instruments]) was appreciable (typically, $>500 \ \Omega \ cm^2$).

To examine DAF and CAR expression, polarized monolayers were fixed with 4% PFA, excised, stained with FITC-labeled anti-DAF IA10, and then permeabilized and stained with CAR-specific rabbit antiserum (28) and AF594-labeled antirabbit secondary antibody. To measure susceptibility to infection, polarized monolayers were exposed to CVB3 H3-RD (5 PFU/cell) for 1 h at 16°C and then cultured for 8 h at 37°C. Infected cells were identified by staining with antibody specific for enteroviral VP1 (NCL-Entero; Leica), as described previously (10).

RESULTS

Transgenic mice expressing hDAF on intestinal epithelium. We generated mice expressing human DAF under the control of the murine villin promoter, which drives gene expression specifically in intestinal epithelium. Three transgenic lines were established. Real-time RT-PCR analysis, using endogenous murine DAF as the internal standard (Fig. 1A), and immunoblot analysis of intestinal tissue demonstrated that the highest levels of hDAF RNA and protein were expressed in mice of line 24, and all further experiments were performed with these mice. Immunofluorescence staining of sections of small and large intestine with a monoclonal anti-hDAF antibody demonstrated that hDAF was expressed in intestinal epithelial cells (Fig. 1B); only faint background staining was detected in wild-type mice.

Throughout the large intestine, intense staining was evident on the apical cell surface, just above the cortical actin layer that directly underlies the plasma membrane. In the small intestine, two interspersed patterns of staining were observed. In approximately two-thirds of the small-intestinal epithelium, the predominant staining was intracellular, below the cortical actin. In patches comprising approximately one-third of the epithelial cell surface, strong expression was observed on the epithelial cell surface, with hDAF being detectable on intestinal microvilli by immunogold electron microscopy (Fig. 1C). Human DAF was also present on the follicle-associated epithelium overlying lymphoid follicles in the small intestine (Fig. 1D); as expected with the villin promoter, transgenic hDAF was not expressed on lymphoid cells.

Intestinal M cells are specialized epithelial cells that transport antigens and microorganisms from the intestinal lumen to the gut-associated lymphoid tissue (29). Because M cells have been implicated in the transcytosis of viruses across the intestinal mucosa (30–34), we determined whether hDAF was expressed on M cells by costaining with antibody specific for an M cell marker (25). Although there appeared to be some overlap of hDAF and the M cell marker in some sections (Fig. 1E, inset a), careful analysis of serial z-sections revealed that hDAF was expressed on the surface of neighboring cells rather than on the M cells themselves (Fig. 1E, inset b).

DAF-binding CVB3 RD is infectious in the mouse model. The prototype DAF-binding CVB3 isolate is CVB3 RD, a derivative of CVB3 Nancy isolated after passage in RD rhabdomyosarcoma cells (35). In comparison to CVB3 Nancy, CVB3 RD was reported to be avirulent in mice (35), and we found that it replicated poorly in mice after intraperitoneal injection (Fig. 2A). For experiments with DAF transgenic mice, we wanted to use a DAF-binding isolate that replicated efficiently in mice. We therefore tested CVB3 H3-RD, a DAF-binding derivative of the cardiovirulent isolate CVB3 H3 which differs from CVB3 H3 in only a single capsid residue (21). After inoculation with 10^5 PFU by the intraperitoneal route, CVB3 H3-RD replicated to high titers in the pancreas, liver, and heart (Fig. 2A). Virus titers were the same in wild-type mice and in DAF transgenic mice (Fig. 2B; $P > 0.1$), indicating that expression of hDAF on intestinal epithelium did not prevent or enhance virus replication in other tissues.

Polarized epithelial cell monolayers from hDAF transgenic mice are susceptible to infection *in vitro*. Because a complex set of DAF-dependent intracellular signals and cytoskeletal rearrangements is required for CVB3 infection of polarized human intestinal epithelium (10, 36), we wanted to be sure that human
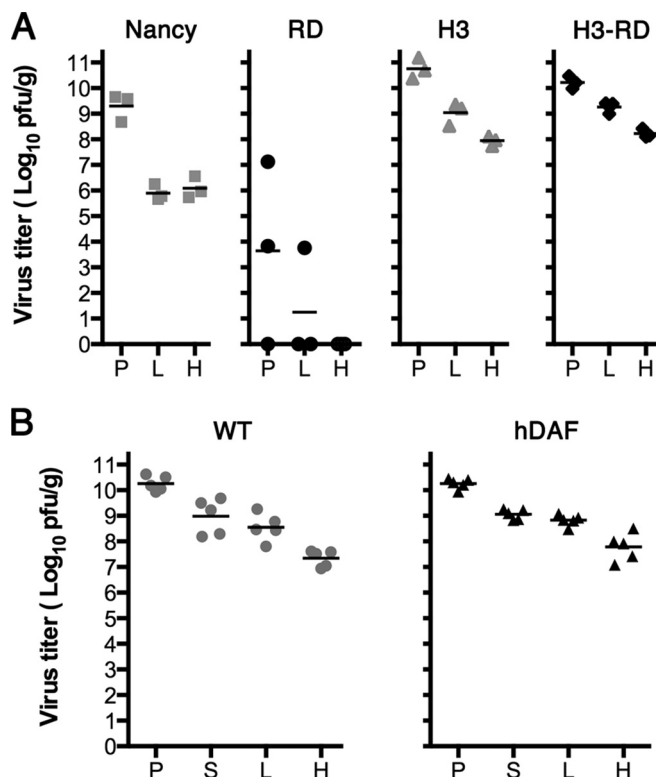
FIG 2 Wild-type and hDAF transgenic mice are equally susceptible to intraperitoneal infection by a DAF-binding virus isolate, CVB3 H3-RD. (A) Wild-type C57BL/6 mice were injected intraperitoneally with 10^5 PFU of CVB3 Nancy, RD, H3, and H3-RD, and virus titers in tissues were measured after 2 days. Titers obtained in individual animals are shown, with mean titers indicated as bars. (B) Wild-type (WT) and hDAF transgenic mice were injected intraperitoneally with 10^5 PFU of CVB3 H3-RD, and virus titers in tissues were measured as described above. P, pancreas; S, spleen; L, liver; H, heart.

DAF functions to permit infection of murine cells. To test this, we made use of recent technical advances in the culture and maintenance of nontransformed primary epithelial cells (27, 37, 38). We isolated intestinal stem cells from the duodenum of a DAF transgenic mouse and from a wild-type littermate and used these to produce polarized epithelial cell monolayers. Monolayers from the DAF transgenic (but not the wild-type) mouse expressed human DAF on the apical surface (Fig. 3A); monolayers from both the transgenic and wild-type mice expressed endogenous CAR, primarily within tight junctions and along the lateral cell membrane. When exposed to CVB3 H3-RD, the DAF transgenic monolayer, but not the wild-type monolayer, became infected, as demonstrated by expression of newly synthesized viral capsid protein (seen at 8 h but not at 0 h postinfection; Fig. 3B). These results demonstrate that human DAF expressed on the surface of primary murine intestinal epithelial cells facilitates infection by CVB3.

Intestinal hDAF binds virus *ex vivo* but is not sufficient for infection by the enteral route. To determine whether hDAF was expressed on intestine at levels sufficient to permit virus attachment, we incubated sections of small and large intestine with radiolabeled CVB3 H3-RD and measured DAF-specific binding. Significantly more virus bound to sections of both the small and large intestines from transgenic mice than to sections from wild-type mice (Fig. 3C; $P < 0.005$). Virus attachment to transgenic
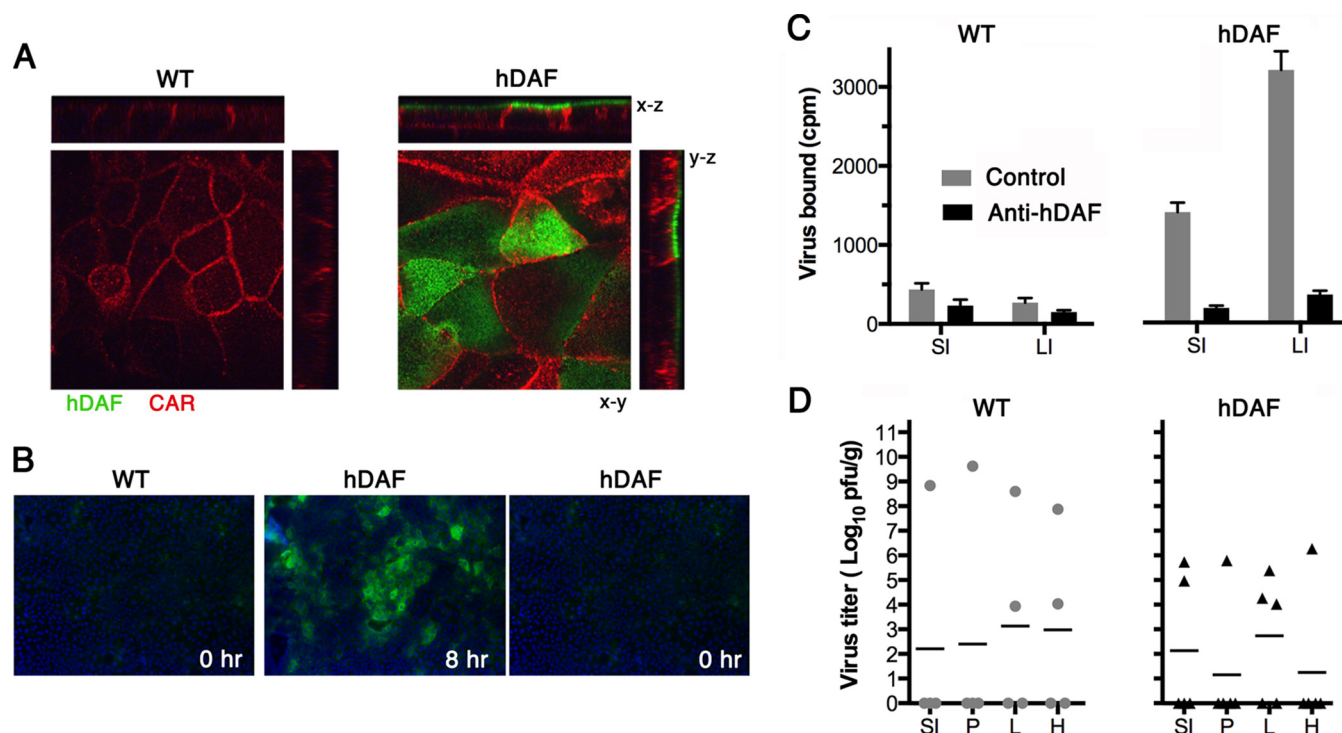
FIG 3 Human DAF permits infection of primary murine intestinal cells *in vitro*, but hDAF transgenic mice resist infection by the oral route, despite virus attachment to hDAF on intestinal epithelium. (A) Primary intestinal epithelial cells from hDAF transgenic or wild-type (WT) mice were cultured as polarized monolayers on Transwell filters and stained with anti-DAF (green) and anti-CAR (red) antibodies, as described in Materials and Methods. DAF is present on the apical surface of monolayers from hDAF but not WT mice. (B) Polarized monolayers were exposed to CVB3 H3-RD (5 PFU per cell) for 1 h at 16°C, and then monolayers were washed and incubated at 37°C to permit infection to proceed. At 8 h, monolayers were stained to detect newly synthesized viral capsid protein VP1; input virus (0 h) was not detectable. (C) Intestinal segments (1 cm long) were treated with medium containing anti-hDAF MAb IF7 or with medium alone (Control) and then exposed to ^{35}S-labeled CVB3 H3-RD, and binding was determined as described in Materials and Methods. Results are indicated as the mean cpm bound + SD for triplicate samples. (D) Wild-type mice ($n = 4$) and hDAF transgenic mice ($n = 5$) were exposed to 1×10^9 PFU of CVB3 H3-RD by gavage, and virus titers in tissues were determined at 3 days postinfection. SI, small intestine; P, pancreas; L, liver; H, heart; LI, large intestine.

mouse samples was inhibited by the anti-DAF monoclonal antibody IF7, which blocks CVB3 attachment to human DAF (7), indicating that the binding was DAF dependent ($P < 0.001$); attachment to wild-type samples was not significantly inhibited by IF7 ($P > 0.1$). Significantly more virus bound to sections of large intestine than to sections of small intestine ($P < 0.005$), consistent with the more extensive expression of hDAF on the surface of the large intestine (Fig. 1B). Nonetheless, virus binding to the small intestine was substantial. Given the specific radioactivity of the virus used in this experiment (550 PFU/cpm), approximately 8×10^5 PFU bound per cm of intestinal segment, suggesting that the small intestine (which is 30 to 40 cm long [39]) had the capacity to bind more than 2×10^7 PFU, at least under *ex vivo* conditions.

However, when hDAF transgenic mice were exposed to CVB3 H3-RD delivered by gavage, little or no systemic infection was noted. Results for hDAF transgenic mice were the same as those obtained for wild-type mice: virus replication at 72 h was detected in only a few animals exposed by gavage to 10^9 PFU of CVB3 H3-RD (Fig. 3D); virus titers in the small intestine, heart, lung, and pancreas were not significantly different in transgenic versus wild-type mice ($P > 0.1$ for all comparisons). Similar results were obtained whether or not virus was administered in bicarbonate buffer (data not shown), which has been reported to protect poliovirus from neutralization by gastric acid (40). We also conducted experiments in suckling mice, in which intestinal barriers

to infection are reportedly less effective (17); however, we observed no differences in the susceptibility of wild-type and transgenic animals to virus delivered by gavage (not shown). Taken together, the results indicated that although human DAF permits infection of murine polarized intestinal epithelial cells *in vitro*, and although virus binds to human DAF on the surface of the intestinal epithelium of DAF transgenic mice, the mice were nonetheless not susceptible to oral infection.

Interferon type I signaling inhibits infection by the enteral route, but intestinal hDAF does not enhance susceptibility in interferon receptor-knockout mice. Work with poliovirus receptor (PVR) transgenic mice has demonstrated that the innate immune system is a powerful barrier to infection by the enteral route; infection does not occur, even when the receptor is expressed on intestinal epithelium (41), unless the type I interferon response has been ablated by deletion of interferon alpha/beta receptor 1 (40). We therefore tested whether expression of hDAF on intestinal epithelium would permit enteral infection in the absence of an effective interferon response by crossing hDAF transgenic mice with mice deficient in IFNAR1 (IFNAR-KO mice). IFNAR-KO mice were much more susceptible to infection than were wild-type mice, with robust virus replication being found in animals exposed to oral doses of 10^7 and 10^8 PFU (Fig. 4B and C). However, expression of hDAF in these immunodeficient mice—which we confirmed by immunostaining, as in Fig. 1B (data not
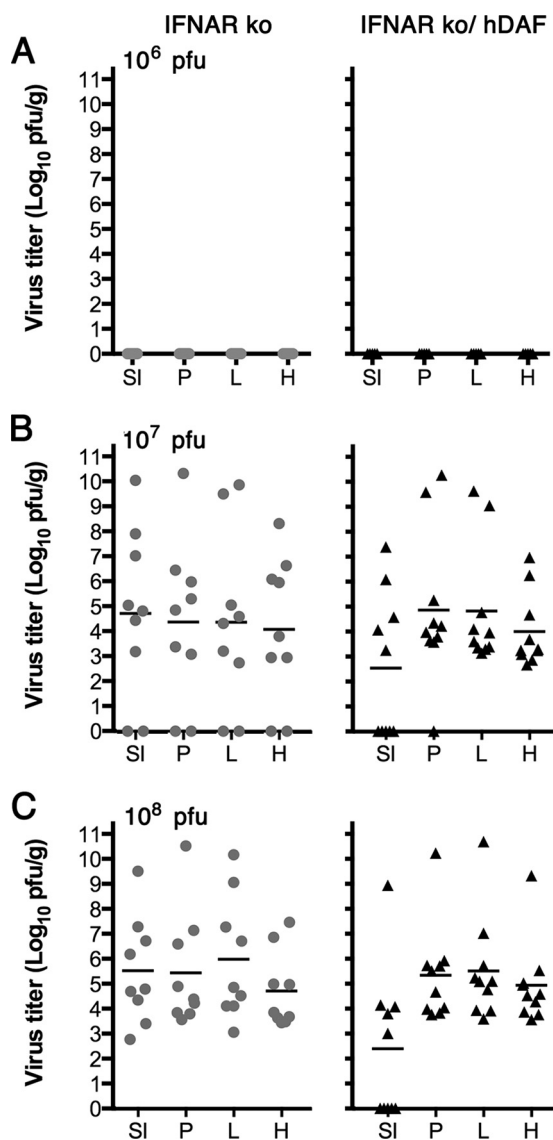
FIG 4 Human DAF on intestinal epithelium does not enhance the susceptibility of IFNAR-KO mice to oral infection. IFNAR-KO mice and IFNAR-KO mice expressing the hDAF transgene were exposed to CVB3 H3-RD by gavage (10^6 to 10^8 PFU), and virus titers in tissues were determined at 3 days postinfection. (A) 1×10^6 PFU; (B) 1×10^7 PFU; (C) 1×10^8 PFU. Titers obtained in individual animals are shown, with mean titers indicated as bars. SI, small intestine; P, pancreas; L, liver; H, heart.

oral route, and that expression of human DAF on murine intestinal epithelium would enhance the susceptibility of transgenic mice to oral infection. We found that expression of human DAF permitted virus to bind to the intestinal mucosa of DAF transgenic mice *ex vivo* and permitted infection *in vitro* of polarized intestinal epithelium derived from these mice. Nonetheless, expression of human DAF on intestinal epithelium did not permit infection by the enteral route.

We cannot exclude the possibility that, despite the results of *in vitro* and *ex vivo* control experiments, the levels of DAF expression on the apical surface were inadequate to permit virus to attach to intestinal epithelium in living animals, in which factors such as the mucus layer or rapid peristalsis may have interfered with virus access to DAF. We do not know why, in some areas of the small intestine, DAF was retained within cells rather than expressed at the apical surface; because apical sorting depends on DAF's glyco-sylphosphatidylinositol (GPI)-linked membrane anchor (42), one possible explanation is that some small-intestinal cells may lack the machinery required for synthesis and attachment of the GPI moiety, as has been reported for some murine cell lines (43).

In a common view of enterovirus pathogenesis—largely based on studies of poliovirus infection in nonhuman primates—ingested virus first replicates in the alimentary tract, moves to draining lymph nodes, and spreads through the bloodstream (or by neuronal transport) to other organs (44). There has been controversy about whether primary poliovirus replication occurs in mucosal epithelium itself (45) or whether virus moves directly to mucosal lymphoid tissues (46) by transcytosis through overlying M cells (31, 32, 34). In humans, the poliovirus receptor (PVR) is expressed on epithelial cells, on M cells, and in mucosal lymphoid follicles, and it has been suggested that infection by the oral route may involve expression of the receptor on all these cell types within the intestine (47). Although we consider it unlikely, it remains possible that expression of human DAF on M cells or intestinal lymphoid cells might have permitted establishment of an oral model of CVB3 infection.

Studies of poliovirus infection in mouse models indicate that multiple factors influence infection by the enteral route, including PVR expression, innate immunity, mucosal integrity, and interactions with intestinal flora (40, 48, 49). A major difference between the mouse models of poliovirus and CVB3 infection is that, unlike poliovirus, CVB3 has a natural receptor in mice, murine CAR; once CVB3 enters the bloodstream, infection can spread readily in the absence of any transgenic receptor. We found that IFNAR-knockout mice were susceptible to relatively low oral doses of CVB3, whether or not they expressed human DAF on the intestinal epithelium.

Poliovirus replicates efficiently in the intestines of PVR transgenic mice (49), presumably either in epithelium or in mucosal lymphoid tissue, but systemic spread occurs only when IFNAR1 is deleted (40); these results suggest that interferon signaling is a major barrier to viral dissemination from the intestine rather than to local replication. We did not directly trace the passage of CVB3 from the intestinal lumen to target organs, and we do not know specifically where infection is blocked in wild-type and hDAF transgenic mice, or how the block(s) is overcome in IFNAR1-knockout mice. How IFNAR1 controls poliovirus or CVB3 spread from the intestine is not well understood. The simplest explanation is that type I interferon, acting through IFNAR1, suppresses viral replication. However, it is conceivable that indirect effects of

shown)—did not enhance their susceptibility to enteral infection across a range of virus doses (Fig. 4A to C). A lower virus titer was detected in the small intestines of hDAF transgenic mice exposed to 10^8 PFU ($P < 0.05$), but in no case was significantly more virus detected in DAF transgenic mouse tissues ($P > 0.1$ for all other comparisons).

DISCUSSION

Because murine DAF does not bind CVB3 (19) and because attachment to DAF on the apical cell surface is important for infection of polarized human epithelial cells *in vitro* (9, 10), we hypothesized that the lack of an accessible apical receptor on intestinal epithelium might be a major barrier to infection of mice by the

IFNAR deletion—changes in intestinal flora, or alterations in mucosal structure or permeability that permit virus access to mouse CAR—may also be important.

The results presented here do not support our initial idea that the presence or absence of a receptor on intestinal epithelium determines whether mice are susceptible to oral infection. They suggest that, at least in mice, infection of polarized epithelium may be neither necessary nor sufficient for infection to occur. However, we believe that the results leave open the question of whether intestinal DAF is important for the pathogenesis of CVB3 infection in humans. CVB3 and other enteroviruses have evolved to infect humans by the enteral route, and although infection in mice depends on IFNAR1 deletion, immunocompetent humans are highly susceptible. Given that the barriers to infection may be very different in humans and mice, we do not believe that the current data exclude a possible role for DAF in the transmission of DAF-binding CVB3 across human intestinal mucosa.

ACKNOWLEDGMENTS

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