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MAJOR ARTICLE

Intrauterine Growth Restriction Caused by Underlying Congenital Cytomegalovirus Infection

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(See the editorial commentary by Spector on pages 1497–9.)

Background. Human cytomegalovirus (HCMV) is the major viral etiology of congenital infection and birth defects. Fetal transmission is high (30%–40%) in primary maternal infection, and symptomatic babies have permanent neurological, hearing, and vision defects. Recurrent infection is infrequently transmitted (2%) and largely asymptomatic. Congenital infection is also associated with intrauterine growth restriction (IUGR).

Methods. To investigate possible underlying HCMV infection in cases of idiopathic IUGR, we studied maternal and cord sera and placentas from 19 pregnancies. Anti-HCMV antibodies, hypoxia-related factors, and cmvIL-10 were measured in sera. Placental biopsy specimens were examined for viral DNA, expression of infected cell proteins, and pathology.

Results. Among 7 IUGR cases, we identified 2 primary and 3 recurrent HCMV infections. Virus replicated in glandular epithelium and lymphatic endothelium in the decidua, cytotrophoblasts, and smooth muscle cells in blood vessels of floating villi and the chorion. Large fibrinoids with avascular villi, edema, and inflammation were significantly increased. Detection of viral proteins in the amniotic epithelium indicated transmission in 2 cases of IUGR with primary infection and 3 asymptomatic recurrent infections.

Conclusions. Congenital HCMV infection impairs placental development and functions and should be considered as an underlying cause of IUGR, regardless of virus transmission to the fetus.

Keywords. congenital; HCMV; IUGR; pregnancy; fetus; placenta; villi; chorion; amnion; blood vessels.

Human cytomegalovirus (HCMV) is the most common cause of congenital viral infection and permanent birth defects in the United States and occurs more frequently than other well-known disabilities, including Down syndrome, fetal alcohol syndrome, and neural tube defects [1]. Primary maternal infection in the first trimester of pregnancy poses a 30%–40% risk of virus transmission with birth defects that include mental retardation, neuromotor disabilities, intrauterine growth restriction (IUGR), and hearing loss [2–4]. Poor outcome is associated with viral replication, inflammation, edema, and fibrinoid development in the placenta [5,6]. In contrast, immune women have a low risk (0.2%–2.0%) of virus transmission, and infected babies are largely asymptomatic [2, 7]. Maternal neutralizing immunoglobulin G (IgG) suppresses HCMV replication in the placenta, and viral antigens are sequestered in syncytiotrophoblasts without infection of underlying cytotrophoblasts [8–12]. Recent studies revealed that

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the placental-fetal unit in congenital infection is hypoxic and that levels of a secreted form of the vascular endothelial growth factor (VEGF) receptor, fms-like tyrosine kinase 1 (sFlt1), are elevated in amniotic fluid [13]. In contrast, treatment of primary maternal infection after seroconversion with hyperimmune globulin enriched for HCMV IgG reduces transmission and improves outcome [14]. Analysis of these placentas revealed infection was suppressed and development of the syncytiotrophoblast surface and numbers of blood vessels in chorionic villi increased [13].

Infants with IUGR, birth weights less than 10th percentile, have a perinatal morbidity and mortality 5–30 times that of infants with higher weights [15]. In the present study, we focused on idiopathic IUGR, a manifestation of maternal and fetal disorders, to determine whether underlying congenital HCMV infection was involved. We found serological evidence of primary and recurrent maternal infection, viral replication in blood vessels of floating villi and the chorion, and viral proteins in the amniotic epithelium. Development of large fibrinoids with avascular villi, edema, and impaired cytotrophoblast differentiation reduced placental functions, resulting in hypoxia and IUGR.

MATERIALS AND METHODS

Study Groups

Approval for this pilot study was obtained from the Institutional Review Board of Cedars-Sinai Medical Center. Samples included maternal and cord blood and placentas at delivery from 9 uncomplicated deliveries (controls), 7 patients with IUGR, and 3 with preeclampsia. Subjects included only nonsmokers without diabetes or chronic hypertension. IUGR was diagnosed based on a predelivery clinical estimation of fetal weight, ultrasound evaluation [16], and birth weight below the 10th percentile [15] (http://www.who.int/reproductivehealth/topics/best_practices/weight_percentiles_calculator.xls. Accessed 24 January 2014.). Preeclampsia was defined as blood pressure >160/110, proteinuria, symptomatic with headaches and visual disturbance, epigastric tenderness, abnormal laboratory findings, or other organ system dysfunction. Mean age of controls was 32.8 ± 6.2 years; pregnancies with IUGR (33.3 ± 4.7 years) and preeclampsia (29.3 ± 5.7 years) were similar.

HCMV Serological Assays

HCMV immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA; Phoenix Pharmaceuticals), HCMV IgG avidity (Radim), and human IgG1 ELISA (eBioscience) were used to measure values in sera. HCMV IgG RecomBlot kit (Mikrogen) was used to characterize reactivity with viral proteins, including immediate-early 1 (IE1, UL123), p150 (UL32), CM2 (UL44, UL57/p52 DNA-binding proteins), pp65 (UL83), gB1, and gB2 (UL55). Immunoblot profiles of recombinant HCMV protein bands indicated infection was primary (IE1, CM2, p65), recurrent (IE1, p150, CM2, p65, gB1, gB2), or long past (p150, gB1, gB2). When maternal serum was unavailable (4 cases), determination of HCMV serostatus was based on reactivity of IgG in cord sera.

HCMV Neutralizing IgG Titers

Rapid neutralization assays were performed using the pathogenic clinical strain VR1814 propagated in human umbilical vein endothelial cells (HUVEC, Lonza) [17, 18]. HUVEC and human placental fibroblasts isolated from villous stroma [19] were grown on glass coverslips in 24-well plates. Heat-inactivated sera were mixed with 300–500 plaque-forming units (PFU) for 1 hour before infection. Cells were fixed 30 hour later and reacted with mouse mAb CH160 to HCMV IE1 and IE2 nuclear proteins [20], then goat anti-mouse IgG (Fab) conjugated with fluorescein isothiocyanate (Jackson ImmunoResearch), and IE-positive cells were counted. Neutralizing titer (IC_{50}) was defined as the serum dilution reducing the number of infected cells by 50%.

HCMV DNA Quantification

Biopsy specimens (5 each) were obtained from the placenta and frozen at −80°C. DNA was extracted (approximately 25 mg) using QIAamp DNA mini kit (QIAGEN). Quantitative PCR targeting the HCMV IE1 gene was performed using the Taqman Universal PCR Master Mix kit (Applied Biosystems). Forward (5′- GACTAGTGTGATGCTGGCCAAG) and reverse (5′-GCTACAATAGCCTCTTCCTCATCTG) primers were used with an internal probe (5′-AGCCTGAGGTTATCAGTGTAATGAAGCGCC) labeled at the 5′ end with the fluorescent reporter dye FAM and at the 3′ end with the quencher dye TAMRA. Assays were performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems). A 6-point standard curve and positive and negative controls were included. The numbers of HCMV IE genome copies were calculated as copies/g tissue. In additional experiments, nested PCR was done as reported [21].

Immunohistochemistry

Biopsy specimens (5 each) were obtained from placentas, fixed in formalin and paraffin embedded. For immunohistochemistry, serial 5 μm-thick tissue sections were deparaffinized using Clear-Rite 3 (Thermo Scientific), and antigen retrieval was performed (described below), followed by blocking with 1%–2% normal horse serum in PBS for 30 minutes to overnight. Sections were incubated with primary antibody overnight at 4°C, washed, and processed for color development using Vectastain ABC horseradish peroxidase (HRP) kits (mouse or rabbit). Briefly, slides were incubated with biotinylated secondary antibody for 1 hour, rinsed, and incubated with ABC complex (30 minutes). Slides were developed with a diaminobenzidine (DAB) substrate kit (Abcam) and counterstained with hematoxylin (Sigma). Primary antibodies were as follows: HCMV

infected cell proteins (ICP), cocktail of mouse monoclonal antibodies (Millipore MAB8121, containing clones 8B1.2, 1G5.2, 2D4.2), diluted 1/100; for cytokeratin 7, mouse monoclonal antibody (Dako clone OV-TL 12/30), diluted 1/100; for smooth muscle alpha-actin and smooth muscle myosin heavy chain, rabbit monoclonal antibodies (Abcam AB124964 and AB133567) diluted 1/1000 and 1/200, respectively. Antigen retrieval was performed as follows: HCMV ICP, tissue sections were incubated with 0.4% pepsin (Sigma-Aldrich, P-6887) in 0.01 N HCl for 30 minutes at 37°C then rinsed; cytokeratin 7, smooth muscle alpha-actin and smooth muscle myosin heavy chain, slides were heat treated (approximately 15 minutes) in 10 mM sodium citrate, pH 6.0, in a 2100-Retriever pressure cooker (Diatome), followed by depressurization and cooling for 2 hours. Images were taken on a Nikon TS100 inverted microscope equipped with a Nikon DS-F12 camera controlled by Nikon NIS-Elements F4.

Quantification of Secreted Cellular Proteins and cmvIL-10

Placental growth factor (PlGF), sFlt1, and soluble endoglin (sEng) were measured in sera using ELISA (Quantikine; R&D Systems). For in vitro assays, HUVEC were infected with VR1814 or mock infected. VEGF-A was depleted for 72 hours before harvesting conditioned medium (CM) at 2, 4, and 6 dpi, then stored at −80°C; cmvIL-10 was measured by ELISA. The cmvIL-10-specific IgG (1 μg/mL; affinity purified, polyclonal) and biotinylated cmvIL-10-specific IgG (0.1 μg/mL; affinity purified, polyclonal) were used for coating and detection, respectively. Protein concentrations were calculated from a standard curve using recombinant cmvIL-10 (R&D Systems).

RESULTS

Serological Diagnosis of Maternal HCMV Infection

All maternal sera lacked HCMV-specific IgM at delivery, which agrees with earlier reports that IgM rapidly declines after primary infection in pregnancy [22–24]. Subjects were grouped by HCMV IgG avidity and profiles of immunoblot reactive proteins as follows: controls (group A), asymptomatic infection (group B), IUGR (group C), and preeclampsia (group D; Table 1). Neonates with IUGR (group C) had significantly lower birth weights (2209 ± 446 g) than did controls (group A: 3443 ± 342 g, $P < .001$) or those with asymptomatic infection (group B: 3960 ± 476 g, $P < .001$) or preeclampsia (group D: 3207 ± 265 g, $P < .01$).

Summarized in Table 1, serological status was evaluated based on HCMV IgG avidity (Radim assay) and immunoblot profiles using recombinant HCMV proteins (Supplementary Figure 1). With regard to IgG avidity [21, 25, 26], infection was judged as long past (>6 months) with avidity above 45% and immunoblot reactivity with HCMV proteins p150, gB1, and gB2. Maternal IgG avidity in recurrent infection (groups B and C) was above 45%, and IgG reacted with proteins IE1, p150, CM2, p65, gB1, and/or gB2. Specific indicators of recurrent infection included IE1 and/or pp65. In primary infections (<90 days after onset), IgG avidity was below 45%, and proteins IE1, CM2, and p65 were detected. Additional reactivity with p150 and gB1 indicated late primary infection. HCMV IgG avidity in cord sera was higher than in maternal circulation, as reported elsewhere [21], except for IUGR cases 16 and 12 with primary infection and case 14 with past infection, suggesting impaired transport. Supplementary Figure 1 shows 5 mothers were seronegative (groups A, C, and D), and 5 had asymptomatic recurrent infection (group B). In IUGR group C, 3 had recurrent infection (cases 18, 2, and 3), and 2 had primary infection (cases 16 and 12). Infection was long past in 4 women (groups A, C, and D).

Neutralizing titers agreed with maternal serostatus (Table 1). Twelve seropositive sera had neutralizing activity in HUVEC (ID_{50} 1:512 to 1:1024); lower titers were obtained in placental fibroblasts (ID_{50} 1:16 to 1:256) [18]. Sera from IUGR case 12 lacked neutralizing activity in both cell types, suggesting seroconversion occurred late in gestation. The results indicated that of 7 mothers who delivered babies with IUGR, 3 had recurrent infection and 2 had primary infection that had not been diagnosed during gestation.

Features of Pathology in Placentas From IUGR Cases

Examination of placental pathology revealed that IUGR cases 2, 3, 16, and 12 had evidence of fibrosis, inflammation, and hypoxia. These included large fibrinoids containing many necrotic, avascular villi (Figure 1*A*) and edematous villi (Figure 1*C*) absent in control placenta 8 (Figure 1*B* and 1*D*). Additional pathology included leukocytic infiltration (Figure 1*E*), dilated blood vessels (Figure 1*F*), and, in IUGR case 12, clusters of cytokeratin 7-positive cytotrophoblasts (termed cell islands), a pattern suggesting arrested differentiation (Figure 1*G*).

We subsequently quantified pathology, including (i) fibrinoids with embedded avascular villi (Figure 2*A*), (ii) edematous villi (Figure 2*B*), and (iii) leukocytic infiltration (inflammation) in the basal plate (Figure 2*C*). Placentas in the control group, including seronegative 8 and recurrent infection 4, 7, and 10, had less than 1 fibrinoid per field with 5 avascular villi (Figure 2*A*). IUGR with recurrent infection, case 2, had small and large fibrinoids with 25–50 avascular villi, whereas case 3 had small fibrinoids. In contrast, IUGR cases 12 and 16, with primary infection, had many small and large fibrinoids with 25–50 avascular villi. In addition, case 16 had many fibrinoids with 50 or more avascular villi, significantly larger than those in all the other placentas. Edematous villi were abundant in IUGR cases 2 and 3, with recurrent infection, and increased in asymptomatic recurrent infection 4 and 7 (Figure 2*B*). Leukocytic infiltration in the basal plate was most evident in IUGR cases 12 and 16 (Figure 2*C*). Considerable variability was found in IUGR

Table 1. Patient samples used in this study and HCMV serological status.

Group	PT	WT	GA	Sera	AV[a]	IgG1[b]	Neut[c] HUVEC	Neut HPF	HCMV[d] Immunoblot	HCMV Serostatus
A Controls	8	3310	39	M	Neg	4.76	<1:8	<1:8	Neg	Seronegative
				C	Neg	6.05	<1:8	<1:8	Neg	
	15	3360	37	M	Neg	4.40	<1:8	<1:8	Neg	Seronegative
				C	Neg	4.74	<1:8	<1:8	Neg	
	6	3160	39	M	67.3	2.28	>1:1024	1:128	p150, CM2, gB1, gB2	Long past
				C	73.7	4.76	>1:1024	1:128	p150, CM2, gB1, gB2	
	9	3940	39	M	58.5	3.40	1:512	1:64	p150, CM2, gB1, gB2	Long past
				C	69.3	8.36	1:512	1:64	p150, CM2, gB1, gB2	
B Asymptomatic Infection	1	3220	39	M	49.5	4.58	1:128	1:32	IE1, p150, p65, gB1	Recurrent
				C	62.5	1.14	1:128	<1:64	IE1, p150, p65, gB1	
	11	3810	41	M	NT	NT	NT	NT	NT	Recurrent
				C	59.2	6.16	1:512	NT	IE1, p150, CM2, gB1	
	4	4050	39	M	NT	NT	NT	NT	NT	Recurrent
				C	44.7	5.51	1:512	NT	IE1, p150, p65, gB1, gB2	
	7	4420	39	M	56.1	4.48	>1:1024	1:128	IE1, p150, CM2, p65, gB1, gB2	Recurrent
				C	71.2	6.63	>1:1024	1:256	IE1, p150, CM2, p65, gB1, gB2	
	10	4300	39	M	NT	NT	NT	NT	NT	Recurrent
				C	84.9	1.28	>1:1024	1:128	IE1, p150, CM2, p65, gB1, gB2	
C IUGR	20	2353	37	M	Neg	2.97	<1:8	<1:8	Neg	Seronegative
				C	Neg	6.07	<1:8	<1:8	Neg	
	14	2459	37	M	58.5	3.96	1:1024	1:256	p150, gB1, gB2	Long past
				C	55.1	9.24	1:1024	1:256	p150, gB1, gB2	
	18	2370	38	M	64.3	8.14	1:512	NT	IE1, p150, CM2, p65, gB1	Recurrent
				C	73.5	8.81	1:512	NT	IE1, p150, CM2, p65, gB1	
	2	2822	39	M	63.4	3.39	1:512	>1:128	p150, CM2, p65, gB1, gB2	Recurrent
				C	73.5	6.90	1:512	>1:64	p150, CM2, p65, gB1, gB2	
	3	1847	34	M	64.3	3.68	1:1024	1:32	IE1, p150, CM2, gB1, gB2	Recurrent
				C	67.1	3.82	1:1024	1:32	IE1, p150, CM2, gB1, gB2	
	16	2160	36	M	18.5	7.09	1:512	1:16	IE1, p150, CM2, p65, gB1	Primary
				C	11.1	7.72	1:512	>1:32	IE1, p150, CM2, p65, gB1	
	12	1450	32	M	36	4.54	<1:8	NT	IE1, CM2, p65	Primary
				C	33.5	1.12	<1:8	NT	IE1, CM2, p65	
D Preeclampsia	13	2920	38	M	Neg	3.36	<1:8	<1:8	Neg	Seronegative
				C	Neg	7.37	<1:8	<1:8	Neg	
	17	3442	40	M	Neg	1.47	<1:8	<1:8	Neg	Seronegative
				C	Neg	7.23	<1:8	<1:8	Neg	
	19	3260	38	M	NT	NT	NT	NT	NT	Long past
				C	50.7	7.52	NT	NT	p150, gB1, gB2	

Abbreviations: C, cord; GA, gestational age; HCMV, human cytomegalovirus; HPF, human placental fibroblasts; HUVEC, human umbilical vein endothelial cells; M, maternal; Neg, Negative; NT, not tested; PT, patient; WT, weight.

[a] Percent HCMV-specific avidity (Radim).

[b] IgG1 measured by ELISA (mg/mL).

[c] Neutralization titers ID50 in VR1814-infected HUVEC and HPF.

[d] HCMV Recomblot IgG (Mikrogen).

case 3 and asymptomatic recurrent infection 4 and 10. Together, the results suggest that pathology in the form of large fibrinoids with avascular villi, extensive edema, and inflammation, alone or in combination, could significantly reduce perfusion and transport of substances across the placenta, resulting in IUGR.

HCMV Replicates in Blood Vessels of Placentas From IUGR With Primary Infection

Quantitative PCR of frozen biopsy specimens showed that IUGR case 16 with primary HCMV infection contained 5×10^6 genome copies/g placenta. Using nested PCR, viral

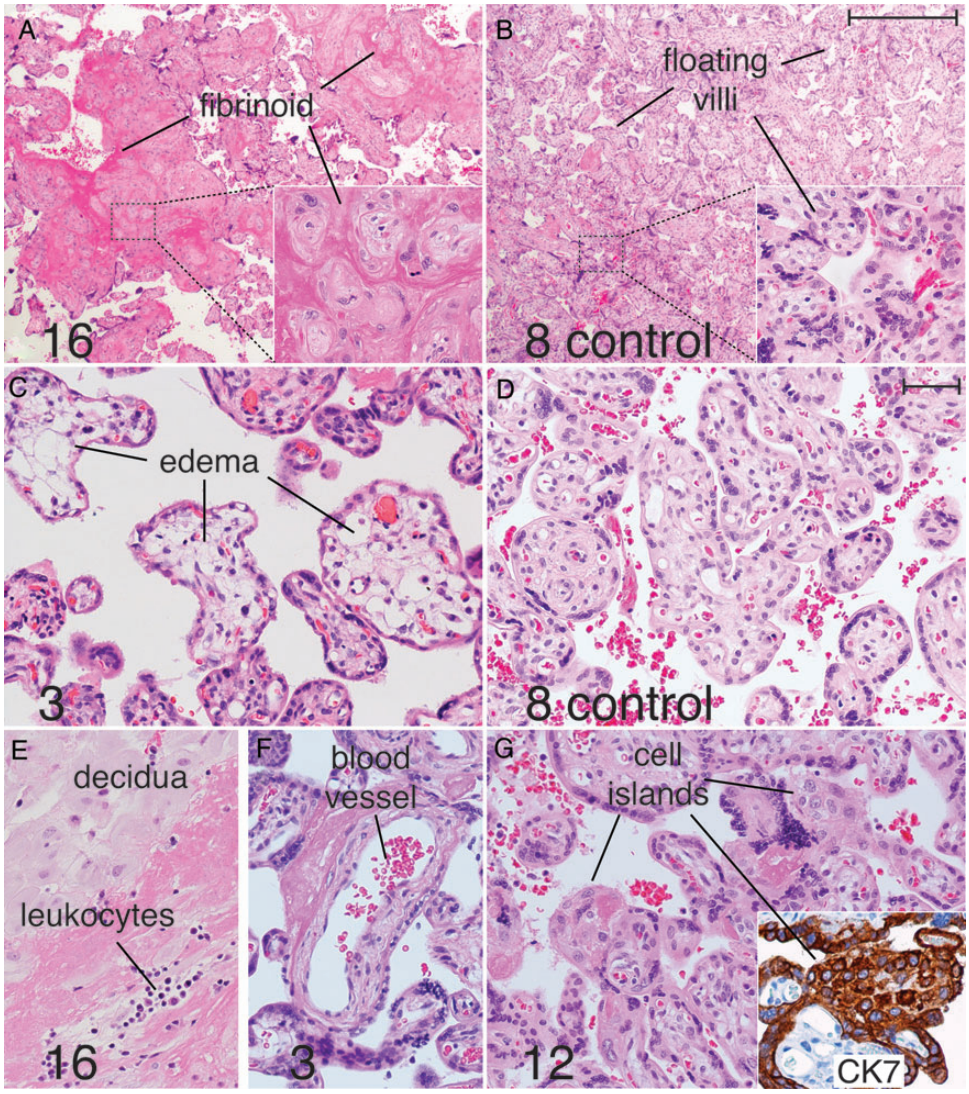

Figure 1. Pathology in placentas from cases of IUGR. *A*, Necrotic, avascular villi embedded in large fibrinoids, case 16. *B*, No large fibrinoids in seronegative control. *C*, Edematous villi, case 3. *D*, Absence of edematous villi in seronegative control. *E*, Leukocytic infiltration in basal plate and decidua, case 16. *F*, Increased diameter of villous blood vessel, case 3. *G*, Accumulation of cytotrophoblasts (cell islands), case 12. Inset, cytokeratin 7 (CK7). Scale bars *A* and *B* = 500 μm, *C*–*G* = 50 μm. Abbreviation: IUGR, intrauterine growth restriction.

DNA was also detected in one biopsy specimen each from IUGR cases 2 and 3 with recurrent infection (Table 2). Viral DNA was not found in any other placentas or any sera. Next, we investigated HCMV infection using immunohistochemistry to localize infected cell proteins in specialized cell types. In the basal plate, interstitial cytotrophoblasts contained viral antigens (Figure 3*A*), and endothelial cells in lymphatic vessels contained viral proteins in nuclei and cytoplasmic vesicles (Figure 3*B*, insets). Viral proteins were present in a comparable replication pattern in glandular epithelial cells (Figure 3*C*, insets) that expressed CK7 (Figure 3*D*). In addition, IUGR cases 2, 18, 3, 16, and 12 with congenital infection expressed viral proteins in cells of the basal plate to a variable degree (not shown).

Detailed analysis of placentas from IUGR cases with primary HCMV infection revealed that infected cell proteins were expressed in blood vessels in chorionic (floating) villi and the chorion. In case 16, virus replicated in smooth muscle (SM) cells in the media (middle layer) of arteries (Figure 4*A*) and veins in the chorion (Figure 4*E*) that expressed SM myosin (Figure 4*B* and 4*F*). In contrast, neither arteries nor veins expressing SM myosin (Figure 4*D* and 4*H*) in the chorion from IUGR cases 3 and 2 with recurrent infection expressed viral proteins (Figure 4*C* and 4*G*). For IUGR case 12 with primary infection, blood vessels in some intermediate villi expressed HCMV proteins and SM myosin (Supplementary Figure 2*A* and 2*B*), whereas other blood vessels lacked viral proteins (Supplementary Figure 2*C* and 2*D*).

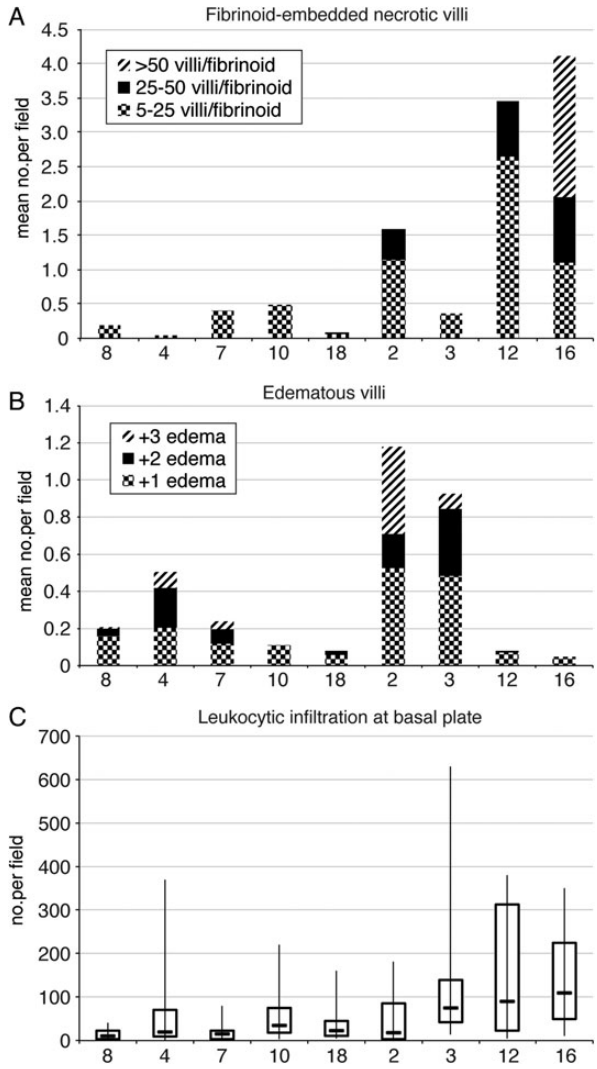

Figure 2. Quantification of pathological features in cases of IUGR. *A*, Avascular villi embedded in fibrinoids were counted, and distribution among fibrinoids of various sizes (defined as 5–25, 25–50, or >50 villi) determined. Results shown as average number of villi in fibrinoids of each size class per field (10× objective, approximately 1 mm^2 area per field). At least four biopsies and more than 100 fields were examined per placenta. *B*, Edematous villi apparent in IUGR were counted according to severity (3 = most severe, representing a bloated villus with sparse mesenchyme and few visible blood vessels). Average numbers of edematous villi in each class indicated by fill patterns. At least four biopsies and more than 100 fields (10× objective, representing approximately 1 mm^2) were examined. *C*, Leukocytic cell infiltrates in basal plate were counted and the distribution per field presented in a box and whisker format that marks the four quartiles. Median count is indicated by the solid central bar, the second and third quartiles within the boxes below and above, respectively. First quartile is represented by vertical line below the box and fourth (highest) quartile by line above the box. For each placenta, at least four biopsies and between 14 and 37 fields (10× objective, representing approximately 1 mm^2) were examined. Abbreviation: IUGR, intrauterine growth restriction.

Unexpectedly, evidence of HCMV transmission was found in the amniotic membranes, which are composed of polarized epithelial cells facing the fetus bathed in amniotic fluid (Figure 5). IUGR cases 12 and 16, with primary infection, contained cytoplasmic vesicles filled with HCMV antigens in accord with fetal infection and virion uptake at the apical membrane (Figure 5*A* and 5*C*). Amniotic epithelial cells in the membranes of group B placentas 4, 10, and 7, with asymptomatic recurrent infection, also contained cytoplasmic vesicles with virion proteins (Figure 5*E, G, B, D,* and *F*), which were clearly visualized in grazing sections (ie, cross-section of concave surface; Figure 5*F*). Virion proteins were not detected in the amniotic epithelium from a group A seronegative control (Figure 5*H*), group B recurrent infection patients 1 and 11, and group C recurrent infection with IUGR cases 2, 18, and 3, indicating these babies were spared (data not shown).

Elevated Anti-angiogenic Factors and cmvIL-10 in IUGR Cases With HCMV Infection

We next measured the hypoxia-related factors sFlt1 and sEng, which increase in parallel [27], and cmvIL-10, a viral immunosuppressive cytokine made late in infection [28]. As shown in Table 2, levels of sFlt1 in IUGR cases 2 and 3 were extremely elevated (180 992 pg/mL and 250 306 pg/mL, respectively) and increased in case 16 (16 823 pg/mL). Quantification of sEng showed that IUGR case 3 was highest (20.4 pg/mL), followed by case 2 (11.3 pg/mL) and case 16 (10.7 pg/mL). In maternal sera from case 12, with primary HCMV infection and preeclampsia, sFlt1 and sEng were elevated (18 107 pg/mL and 19.4 pg/mL, respectively). Maternal sera from recurrent infection 7 had elevated sFlt1 (14 816 pg/mL) and sEng (20.3 pg/mL). Lastly, cmvIL-10 was detected in maternal and cord sera from IUGR cases 2, 3, and 16, in accord with viral replication.

Next, we considered whether HCMV-infected endothelial cells from the umbilical cord could secrete the cytokines detected in utero. For these experiments, HUVEC were infected with VR1814, and sFlt1, PlGF, and cmvIL-10 levels were measured in CM; sFlt1 increased modestly, whereas PlGF (Supplementary Figure 3) and sEng declined (data not shown), in accord with decreased surface expression of Eng in infected HUVEC [29]. Viral cytokine cmvIL-10 increased throughout the course of viral replication, reaching the highest level at 6 days (Supplementary Figure 3).

DISCUSSION

Although recognized as a viral cause of IUGR, congenital HCMV infection is seldom diagnosed in affected newborns without other clinical symptoms [2]. Here we assessed the serological status of women who delivered infants with idiopathic IUGR (group C) and found 5 cases with underlying primary or recurrent infection with placental pathology, including impaired development (Tables 1 and 2). Immunostaining for

Table 2. Hypoxia status, pathological features, and presence of infected cell proteins.

Group	PT	Sera	sFlt (pg/mL)	PlGF (pg/mL)	sEng (pg/mL)	Cmv IL-10	HCMV Infection, Hypoxia and Pathology
B Asymptomatic Recurrent Infection	4	M	NT	NT	NT		Infected cell proteins in amniotic epithelium.
		C	608	NT	4.4		
	7	M	14 816	193	20.3		Infected cell proteins in syncytiotrophoblasts and amniotic epithelium. Edema and dilated blood vessels.
		C	895	1.4	3.3		
	10	M	NT	NT	NT		Infected cell proteins in decidual glands and amniotic epithelium. Some leukocytic infiltration.
		C	705	9.4	3.7		
C IUGR with Primary or Recurrent Infection	18	M	524	5.3	5.3		Infected cell proteins in decidual glands.
		C	281	8.0	6.2		
	2	M	2494	888	5.9	+	Infected cell proteins in interstitial CTBs and decidual glands. Considerable edema, fibrinoid-embedded avascular villi, leukocytic infiltration, T-P changes[a]. HCMV DNA detected by nested PCR.
		C	180 992	572	11.3	+	
	3	M	2163	376	9.6	+	Considerable edema, variable leukocytic infiltration, few fibrinoid-embedded avascular villi, dilated blood vessels. HCMV DNA detected by nested PCR.
		C	250 306	103	20.4	+	
	16	M	15 157	50.3	3.3	+	HCMV DNA quantified in biopsies (5.0×10^6 genome copies/g). Infected cell proteins in decidual glands, blood vessels in floating villi, arteries and veins in chorion and amniotic epithelium. Many large-size fibrinoid-embedded avascular villi. T-P changes[a], cell islands[b]. Considerable leukocytic infiltration. Infected interstitial CTBs.
		C	16 823	7.8	10.7	+	
	12	M	18 107	68.6	19.4		Infected cell proteins in decidual glands, blood vessels in floating villi and amniotic epithelium. Moderate-size fibrinoid-embedded avascular villi, dilated blood vessels, T-P changes[a], cell islands[b]. Leukocytic infiltration. Infected interstitial CTBs. Preeclampsia.
		C	1033	10.7	3.3		

Abbreviations: C, cord; HCMV, human cytomegalovirus; M, maternal; NT, not tested; PlGF, placental growth factor; PT, patient.

[a] Tenney-Parker (T-P) changes.

[b] Cytotrophoblast (CTB), cell islands.

infected cell proteins revealed that virus replicates in smooth muscle cells of arteries and veins in floating villi and the chorion. HCMV proteins in vesicles of amniotic epithelial cells were taken as evidence of transmission and fetal infection. Nonetheless, accumulation of viral proteins in cytoplasmic vesicles suggested virions were cleared from amniotic fluid and replication was suppressed, which could reduce inflammation.

Considering pathology, large fibrinoids with many avascular villi and edematous villi, which could impair transport functions, and leukocytic infiltration at the basal plate, suggesting inflammation, were the most prominent features in IUGR placentas (Table 2). For primary infection case 16 with transmission, HCMV DNA was detected in the placenta, viral replication was sustained in blood vessels of villi and chorion, and cmvIL-10 was present in circulation. For primary infection case 12 with transmission, impaired cytotrophoblast differentiation (cell islands), hypoxia (Tenney-Parker changes), and dilated blood vessels suggested primary infection exacerbated by maternal preeclampsia with elevated sFlt1 and sEng contribute to dysfunction [27, 30]. Extensive edema, also associated with IUGR, was evident in recurrent infection cases 2 and 3. In addition, cord sera contained extremely elevated sFlt1, which inhibits functions of VEGF and PlGF and is associated with hypoxia, as reported for amniotic fluid from untreated (ie, without HIG therapy) primary congenital HCMV infection [13]. In contrast, placentas 4, 7, and 10 from asymptomatic recurrent infection did not differ from seronegative controls in prevalence of fibrinoids, inflammation, edema, or levels of anti-angiogenic factors in sera (data not shown). The results indicate that high-avidity IgG with neutralizing activity has the potential to reduce infection-associated pathology in the placenta. However, understanding the mechanisms by which recurrent infection leads to pathology could benefit from knowing whether reinfection occurred.

In cases of stillbirth associated with congenital HCMV infection, decreased exchange capacity of placentas with progressive fetal thrombotic vasculopathy was identified as the prominent histological abnormality [31–33]. Avascular villi increase the occurrence of IUGR [34] and can lead to major thrombotic events and death in fetuses older than 34 weeks. In cases of acute HCMV infection, arterial and venous thrombosis occur independently from other risk factors [35]. Albeit infrequently, viral antigens and DNA have been detected in adult human arterial smooth muscle cells [36] and vessels from coronary artery

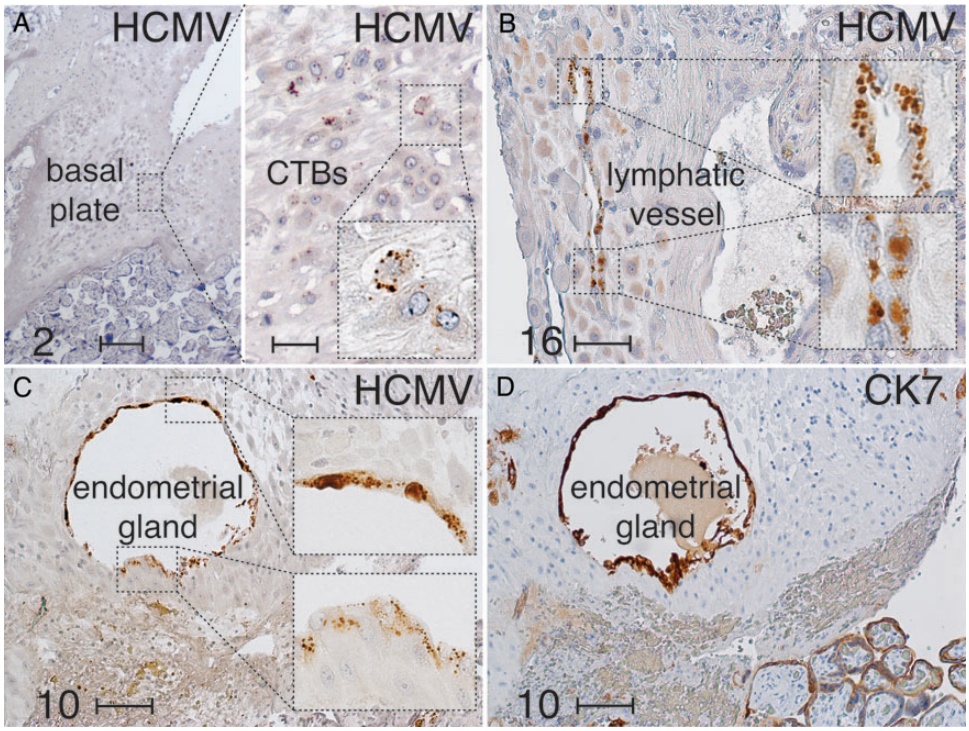

Figure 3. HCMV infected cells in the basal plate of primary and recurrent infections detected by immunohistochemistry. *A*, (left panel) Low power image of a site within the basal plate containing CTBs that express HCMV infected cell proteins. (right panel and inset) High power image of area indicated in panel *A* (rectangle) showing infected cell proteins in the cytoplasm of invasive CTBs. *B*, Infected cells in a lymphatic vessel in the decidua. Signal was detected in cytoplasm (upper inset) and nuclei (lower inset) of infected cells at the luminal surface. *C*, HCMV proteins in cells at the luminal face of an endometrial gland detected in cytoplasm and nuclei. *D*, Section adjacent to that in *C* shows cytokeratin 7 (CK7). Scale bars *A*(left panel) = 200 μm, *A*(right panel) = 20 μm, *B* = 50 μm, *C*–*D* = 100 μm. Abbreviations: CTB, cytotrophoblast; HCMV, human cytomegalovirus.

disease [37, 38]. Moreover, HCMV replicates in smooth muscle cells from human umbilical arteries in vitro [39]. Here we discovered a pattern of viral replication in smooth muscle cells of arteries and veins in cases of primary infection, which indicates stepwise transmission from infected cytotrophoblasts to villous blood vessels, the chorion, and fetal circulation [8, 12]. Although transmission occurred in 3 of 5 fetuses from group B asymptomatic recurrent infection, the placental vasculature was not affected. Transmission rates are 30% and 38% when seroconversion occurs in first and second trimester, respectively, and frequently leads to disease [4, 40, 41]. Although transmission is higher (72%) in third trimester, infected babies are usually asymptomatic. Unlike IUGR cases 16 and 12, with primary infection, viral DNA was not detected in placentas from asymptomatic recurrent infection 4, 7, and 10, suggesting transmission may have occurred late in gestation. These findings show that high-avidity, neutralizing IgG reduces HCMV replication and subsequently protects the developing placenta from inflammation and associated damage [9, 10, 12].

Amniotic epithelial cells of fetal membranes represent the first line of defense against intra-amniotic bacteria and respond to pathogens through the function of toll-like receptors (TLRs) [42], key regulators of innate immune defense [43] that inhibit bacterial growth [44]. In spontaneous labor at term and in preterm parturition associated with chorioamnionitis, TLR2 and TLR4 are upregulated in the amniotic epithelium [45]. In primary and recurrent HCMV infection, we observed vesicles containing viral antigens near the apical membranes of epithelial cells, suggesting virion uptake from amniotic fluid (Figure 5). We recently discovered similar patterns by immunostaining the amnion from 2 cases of primary maternal infection with HCMV DNA-positive amniotic fluid that confirms virus transmission (unpublished observations). With regard to innate immunity, HCMV gB and gH display determinants recognized by TLR2 with which they directly interact, inhibiting inflammatory cytokine responses to infection in vitro [46]. Whether this pathway protects the amniotic epithelium from virus replication remains to be determined. Infection with bacterial pathogens could cause additional complications leading to inflammation [10, 11]. Amniotic epithelial cells could also express IgG receptors that could internalize antibody-virion complexes [47]. In this regard, we measured HCMV-specific IgG in amniotic fluid from seropositive mothers and confirmed that amniotic epithelial cells express the neonatal Fc receptor,

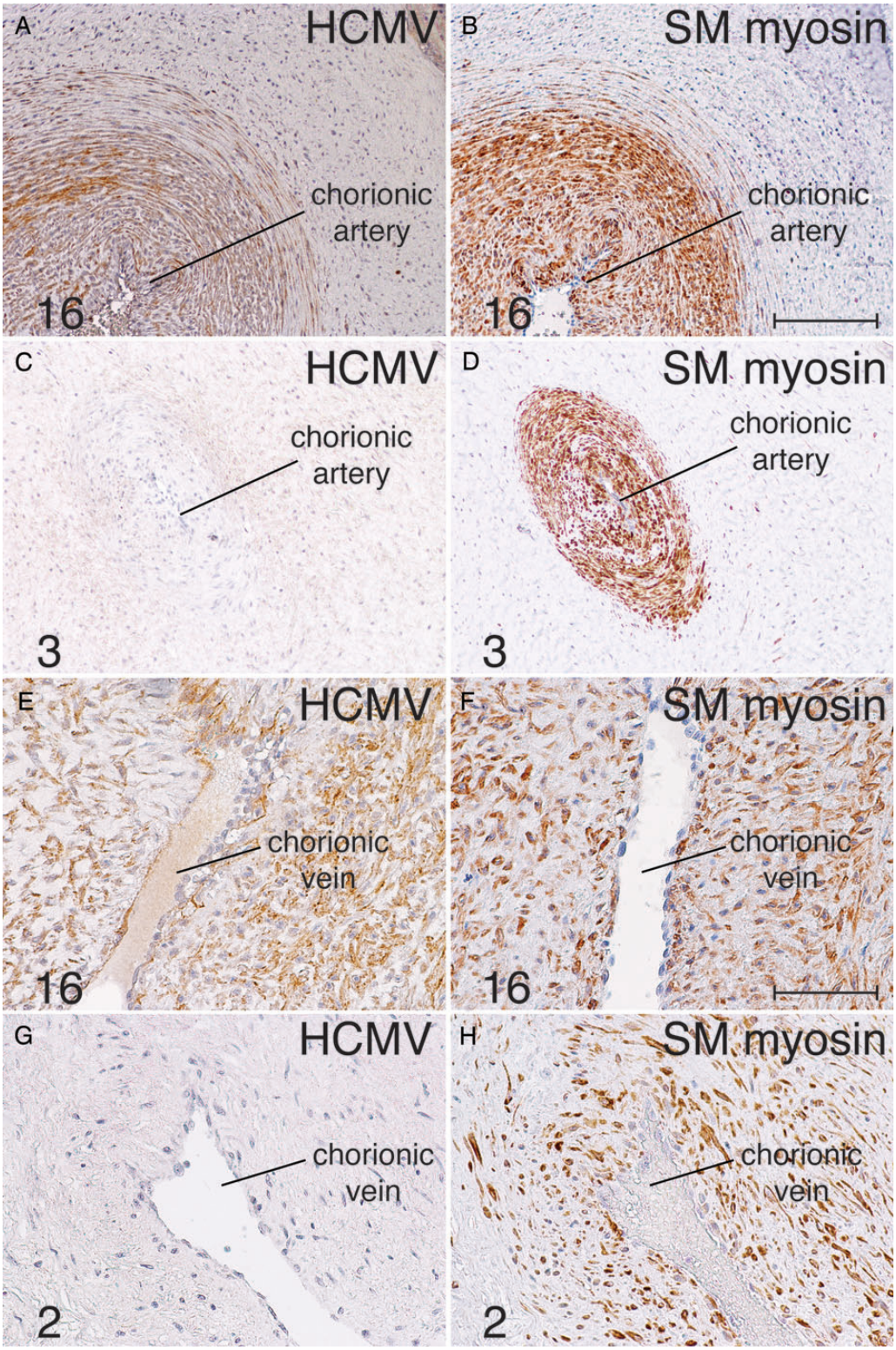

Figure 4. HCMV infected cells in blood vessels of chorion detected by immunohistochemistry in a case of IUGR with primary infection. HCMV proteins (left panels) and smooth muscle (SM) myosin heavy chain (right panels) stained in parallel sections. *A* and *B*, Adjacent sections showing HCMV proteins in smooth muscle cells surrounding an artery in the chorion of placenta of IUGR case 16. *C* and *D*, Adjacent sections showing absence of viral proteins in smooth muscle cells surrounding an artery in the chorionic plate of placenta #3. *E* and *F*, Parallel sections showing HCMV proteins in smooth muscle cells surrounding a vein in the chorionic plate of placenta #16. *G* and *H*, Adjacent sections showing absence of viral proteins in smooth muscle cells surrounding a vein in the chorionic plate of placenta #2. Scale bars in *B* = 200 μm for (*A–D*). Scale bar in *F* = 100 μm for *E–H*. Abbreviations: HCMV, human cytomegalovirus; IUGR, intrauterine growth restriction.

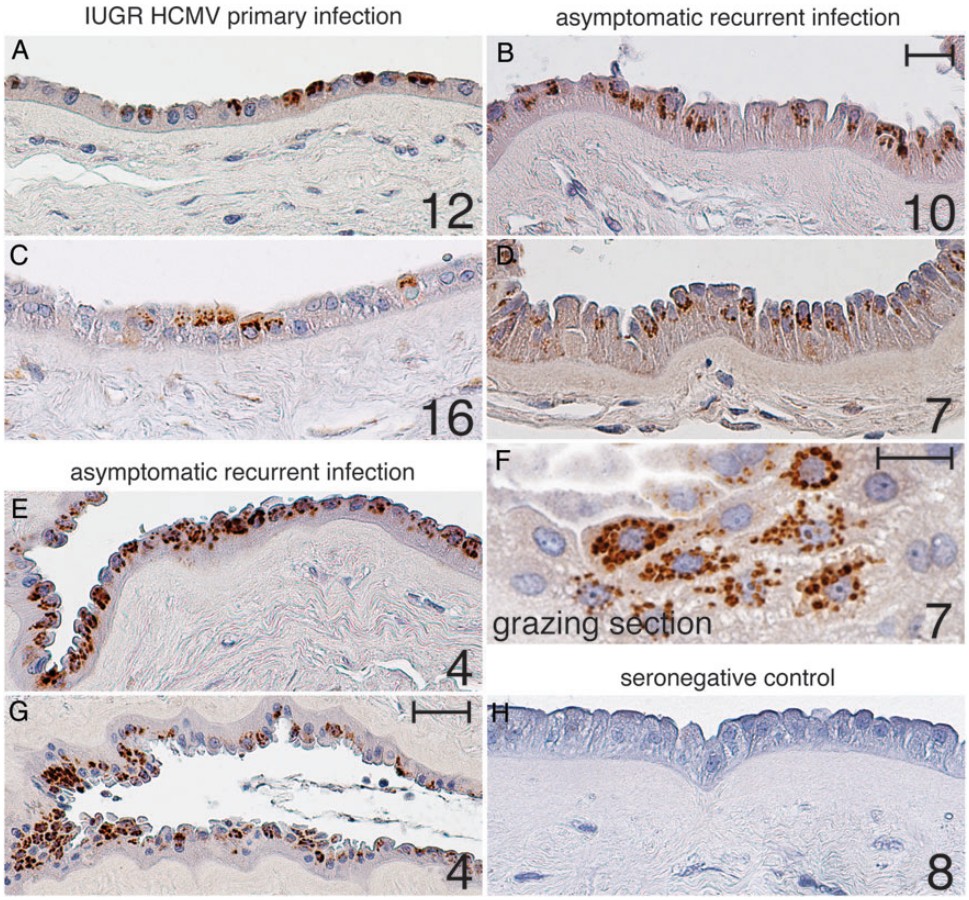

Figure 5. Immunohistochemical detection of HCMV proteins in epithelial cells of amnion. Viral proteins accumulated in cytoplasmic vesicles of amniotic epithelial cells in placentas from primary and recurrent infections (*A–G*) but not in a seronegative control (*H*). *F*, Grazing section across amniotic epithelial cells revealing the highly vesicular nature of the signal. Scale bar in *B* = 25 μm for *A–E* and *H*. Scale bar in *G* = 25 μm. Scale bar in *F* = 15 μm. Abbreviations: HCMV, human cytomegalovirus; IUGR, intrauterine growth restriction.

TLR2, and TLR4 in vitro (unpublished observations), suggesting these molecules could contribute to virion clearance, reduce inflammation, and delay the rupture of fetal membranes.

In conclusion, analysis of biopsy specimens revealed crucial information about congenital HCMV infection, involvement of placental blood vessels in transmission, and viral proteins in amniotic epithelial cells, providing further evidence of fetal infection. Our findings (2 cases) indicate that primary infection impairs placental development and leads to IUGR and virus transmission (Tables 1 and 2). In recurrent infection (8 cases), 5 babies were asymptomatic, even though virus transmission occurred (3 cases) (Table 1, group B). However, 3 others had IUGR, a placental defect, without virus transmission (Tables 1 and 2, group C). Our detailed analysis shows that high-avidity, HCMV-neutralizing IgG reduces viral replication in the placental-fetal unit but may not preclude transmission [13, 14, 48–50] and suggests antibody treatment merits consideration in the clinical management of primary maternal infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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