The Placenta as a Site of Cytomegalovirus Infection in Guinea Pigs

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The development of cytomegalovirus (CMV) infection in the placenta was studied in Hartley guinea pigs inoculated at midgestation, and its role in determining the outcome of fetal CMV infection was assessed. A hematogenous spread of CMV from the mother to the placenta occurred early during the course of the infection. However, the virus remained present in placental tissues long after CMV had been cleared from maternal blood (i.e., 3 and 4 weeks postinoculation). At that time, the virus was able to replicate in placental tissues in the presence of specific maternal antibodies. Viral nucleocapsids were seen within nuclei of trophoblastic cells, and virions were present surrounding infected cells. In addition, typical CMV-induced histopathological lesions bearing CMV antigens were consistently localized at the transitional zone between the capillarized labyrinth and the noncapillarized interlobium. Whenever CMV infection of the fetus occurred, virus was isolated from the associated placenta. Among placental-fetal units with CMV-infected placentas, only 27% of the fetuses were found to be infected. In addition, there was a delay in the establishment of the infection in the fetus in relation to the placenta, although frequencies of virus isolation in placental and fetal tissues peaked at 3 weeks after CMV inoculation. These results suggest that during primary CMV infection of pregnant guinea pigs, the placenta not only serves as a reservoir for CMV but also acts to limit transmission of the virus to the fetus.

Cytomegalovirus (CMV) is the most frequent cause of congenital viral infection in humans (15). These infections are thought to be acquired via transplacental transfer of CMV and range in severity from an asymptomatic process with viremia and low birth weight to severe brain damage, neonatal cytomegalic inclusion disease, or death. Although CMV infections during pregnancy have been the subject of considerable study, little attention has been given to the associated placentas. Reports describing placental damage due to human CMV generally have not referred to specific areas or cells in the placenta, to the time course of appearance of the damage, or to its effect on the outcome of the fetus (1, 2, 4, 6, 22-26). Thus, knowledge of the events associated with placental CMV infection is fragmentary, and the mechanisms of transplacental transmission of CMV remain unclear.

Guinea pigs infected with guinea pig CMV have been used as a model for the study of human CMV infection (3). Transplacental transmission of CMV has been demonstrated to occur in the guinea pig, resulting in the birth of congenitally infected offspring (5, 13, 19, 21). Thus, this animal model is a unique tool for studying mechanisms of transmissions of virus from mother to infant. Furthermore, the structure of the guinea pig placenta also makes this species a promising subject for studies of in utero transfer of CMV. Placentation in the guinea pig occurs in a manner similar to that of humans, and both guinea pig and human placentas are classified as hemomonochorial (27). The guinea pig placenta shows very distinctive features at the light-microscope level, notably well-defined lobular organization, and the interface between the fetal and maternal circulation is somewhat similar to that of the mature human placenta (7, 20). In previous studies of primary CMV infection of pregnant

guinea pigs, CMV has been isolated from placental tissues (5, 12, 19). However, no report has systematically evaluated CMV infection at the placental level and its role in the development of fetal CMV infection.

In the present study, we investigated the development of primary acute CMV infection in the mature guinea pig placenta so as to gain insight into the mechanisms of transplacental transfer of CMV. We evaluated the time course of appearance of infectious virus and virus-induced lesions in the placenta in relation to maternal viremia and fetal CMV infection. We assessed the ability of guinea pig placenta to sustain CMV infection, and we determined the type of lesions induced by CMV and their localization in different areas of the placental tissues. Finally, we considered the role played by the CMV-infected placenta in modulating the outcome of CMV infection in the fetus.

MATERIALS AND METHODS

Virus and cell cultures. The prototype strain of guinea pig CMV (no. 22122, American Type Culture Collection, Rockville, Md.) was used. Salivary gland-passaged guinea pig CMV stocks were prepared as described previously (12). The virus stocks used throughout the study were at passage levels 26 to 28 and had a virus infectivity titer of 7.5 log₁₀ 50% tissue culture infective doses per ml.

Both primary and low-passage guinea pig embryo (GPE) cells were used for virus assay. Primary GPE cells were prepared from 30- to 40-day-old embryos of Hartley guinea pigs as described before (16). Passaged cells were grown and maintained in Eagle minimal essential medium containing Hanks balanced salt solution and 5% heat-inactivated newborn calf serum (Flow Laboratories, McLean, Va.).

Animal inoculation and evaluation. Randomly bred, pregnant Hartley guinea pigs were purchased from Camm Research Institute (Wayne, N.J.) and inoculated at 30 days of gestation. This time corresponds approximately to midpregnancy since the average length of pregnancy in the

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TABLE 1. Time course of CMV infection in placental tissue and maternal blood

No. of days post-maternal inoculation ^a	Mothers		Placentas			
	No. with viremia/no. tested	Antibody range ^b	No. tested	No. (%) with virus		Virus infectivity titer ^c in washed placental tissue (mean ± SE)
				Before washing	After washing	
8	3/4	<5	21	18 (86)	14 (67)	1.55 ± 0.53
14	3/6	<5–40	23	13 (57)	8 (35)	1.09 ± 0.45
21	0/4	5–20	12	9 (75)	12 (100)	2.60 ± 0.84
28	0/4	20–80	18	11 (61)	14 (78)	2.33 ± 0.65

^a Guinea pigs were inoculated at midgestation with 5.5 or 7.5 log₁₀ TCID₅₀ of virus.

^b Reciprocal value of neutralizing antibody titer.

^c Log₁₀ PFU per milliliter of washed placental suspension (10%, vol/vol).

guinea pig is 65 to 68 days (27). Serum samples were obtained from all animals before inoculation and were tested for the presence of neutralizing CMV antibodies. Only animals without preexisting antibodies were used for study. Experimental animals were inoculated subcutaneously in the left axilla with 7.5 or 5.5 log₁₀ 50% tissue culture infective doses of salivary gland-passaged guinea pig CMV in 1 ml. Control animals were uninoculated animals or guinea pigs inoculated with 1 ml of uninfected salivary gland suspension.

Pregnant guinea pigs were sacrificed 1, 2, 3, and 4 weeks after inoculation. Animals were anesthetized with diethyl ether. Maternal blood samples were obtained by cardiac puncture, and the uterus was removed aseptically. Each uterus was dissected in a sterile petri dish (150 by 25 mm). The amniotic fluid was removed with a 25 gauge needle and a syringe. Each placenta was then detached from its fetal membranes and fetus. The sera obtained from maternal blood samples taken before inoculation and at the time of sacrifice were tested for the presence of neutralizing antibody as described before (12).

Virus isolation. Virus isolations, titrations, or both were performed on the maternal blood, placental tissues, and fetal tissues. The maternal blood was allowed to clot at room temperature for 30 to 60 min. The serum was decanted, and the clot was briefly minced and used for virus isolation. The placenta was assayed for virus content before and after extensive washing to remove most of the blood. Infectivity titers were determined in the blood-containing and blood-free placental cell suspensions by serial dilutions of a 10% (vol/vol) suspension. The washed placental cell suspension was obtained as follows: using a 25 gauge needle and syringe, part of each placenta was perfused three times with 10 ml of Hanks balanced salt solution to remove most of the erythrocytes; the placenta was then minced into small pieces, washed three times with 50 ml of Hanks balanced salt solution, and finally homogenized with forceps. Fetal tissues were removed aseptically from each fetus, and the spleen, liver, lung, kidney, salivary gland, and thymus were pooled before virus assay. For each fetus, the brain and amniotic fluid were tested separately.

All samples were inoculated into three to six wells of confluent GPE cell monolayer grown in 24-well plates (Costar) and adsorbed for 1 h at 37°C in a 5% CO₂ incubator before culture medium was added. To avoid toxic effects from tissue debris, the culture medium was changed 24 h later. The plates were then incubated at 37°C in a 5% CO₂ incubator and examined weekly for 4 weeks for evidence of virus-induced cytopathic effect. For virus titrations, the number of plaques was also determined. All cultures which showed cytopathic effect were harvested and stored at

-70°C until needed for final identification. All virus isolates were identified by neutralization tests using type-specific antiserum.

Histology and electron microscopy. For histologic study, a portion of each placenta was fixed in 10% buffered Formalin for 24 h and placed in 20% ethyl alcohol until required for further processing. The fixed placentas were dehydrated through a graded series of ethyl alcohol to xylene, embedded in Paraplast II, sectioned, and stained with routine hematoxylin and eosin stain.

For ultrastructural studies, portions of placentas were minced and fixed overnight in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After fixation, specimens were washed overnight in cacodylate buffer and postfixed in 1.33% osmium tetroxide in S-collidine buffer for 1 h. This was followed by en bloc staining with 0.5% uranyl acetate for 4 h, dehydration in ethanol, and embedding in Epon (10). Thick sections (1 μ m) were stained with toluidine blue and examined with a light microscope. Sections with areas of pathology were further cut into thin sections with an LKB Ultratome III and stained with uranyl acetate and lead citrate. Thin sections were examined under a Philips EM 300 electron microscope.

Immunoperoxidase staining procedure for detection of CMV antigens. For immunoperoxidase staining, paraffin-embedded tissue sections were placed on gelatin-coated slides and baked for 1 h in a 58°C oven. The avidin-biotinylated horseradish peroxidase complex (ABC) method (Vectastain ABC kit for antibodies produced in guinea pigs; Vector Laboratories, Burlingame, Calif.) was utilized to localize CMV antigens in the histological sections (17). After deparaffinization in xylene and dehydration with absolute ethanol, the slides were treated with 6% H₂O₂ in methanol for 30 min to quench the endogenous peroxidase activity. Slides were then progressively hydrated, treated with a 10% trypsin solution for 30 min, and exposed to 0.4% normal guinea pig serum for an additional 30 min. The tissues were then exposed to guinea pig serum containing antibody to guinea pig CMV for 2 h. This primary antiserum, which had a neutralizing antibody titer of 1:320, gave an optimal response for immunoperoxidase staining at a dilution of 1:400. Control slides with normal guinea pig serum or anti-herpeslike virus serum were included in each experiment. The primary antisera, anti-CMV and controls, had all been adsorbed twice on GPE cells for 1 h at 37°C and twice on guinea pig placental tissue for 1 h at 37°C before use. The ABC reaction was obtained by successive treatments with diluted biotinylated anti-guinea pig immunoglobulin G produced in goats and the ABC reagent. Sections were then incubated in a peroxidase substrate consisting of

diaminobenzidine tetrahydrochloride solution and H_2O_2 and counterstained with Harris hematoxylin for 5 s.

RESULTS

Time course of placental CMV infection and maternal viremia. It is known that CMV can be isolated from the blood and placentas of pregnant Hartley guinea pigs early during the course of primary infection (5, 12). At the time of maternal viremia, isolation of CMV from placentas might simply reflect virus infection of the maternal blood filling the placental blood spaces. These experiments were designed to determine whether CMV could persist in placental tissues after virus had been cleared from maternal blood and to assess the development of CMV infection in the placenta. Groups of pregnant guinea pigs were inoculated with CMV at midgestation and sacrificed on days 8, 14, 21 and 28 post-maternal inoculation. Mothers were evaluated for the presence of virus and neutralizing antibody in their blood. In addition, virus recovery from placental tissues was compared before and after extensive washing of the placental tissues to remove most of the blood contained in the placenta, and virus infectivity titers were determined in the washed placental tissue suspension (Table 1).

Virus was present in the blood from 6 of 10 mother animals tested on days 8 and 14 postinoculation, and 2 of the 10 mothers showed neutralizing antibodies. On days 21 and 28 postinoculation, all mothers had detectable levels of neutralizing antibodies, and none of the eight mothers tested was viremic. Virus was isolated from blood-free and blood-containing placental tissues throughout the study period. Early during the course of maternal infection, at the time when virus was also detected in maternal blood, the frequency of virus recovery from blood-containing placental tissue was higher than that of blood-free placental tissue. In contrast, at weeks 3 and 4 postinoculation, a larger percentage of washed placentas showed virus as compared to blood-containing placentas. The frequency of virus recovery from washed placental tissues dropped from 67% on day 8 to 35% on day 14 postinoculation. On days 21 and 28 postinoculation, the frequency of virus recovery from washed placental tissues increased to 100 and 78%, respectively. Although there was a considerable variation from placenta to placenta, at each time point examined, virus infectivity titers of the washed placental suspension showed a similar pattern. Virus titers were low during the first 2 weeks postinoculation, particularly on day 14, and increased to higher values on days 21 and 28 postinoculation despite the presence of significant levels of neutralizing CMV antibodies in the maternal circulation.

Time course of histological lesions in the placenta. To determine the development of CMV-induced histopathology in the placenta, placentas obtained from pregnant guinea pigs 7, 14, 21, and 28 days post-CMV inoculation were compared with placentas from control animals inoculated with uninfected salivary gland suspension, and with placentas from uninoculated controls. Forty-five placentas from 13 uninoculated mother guinea pigs were examined. Neither viral inclusions nor virus-specific lesions were observed, although one placenta showed hypotrophic villi and two other placentas had erythrocytic colonies in the fetal membranes. Forty-four placentas obtained from animals examined at 1 to 4 weeks after inoculation of uninfected salivary gland suspension also showed no virus-induced lesions. In contrast, placentas from CMV-inoculated animals showed specific changes at the various time points examined (Fig. 1).

FIG. 1. Time course of appearance of histological lesions in placentas from animals inoculated with guinea pig CMV.

A number of placentas examined demonstrated ischemic injury changes ranging from foci of coagulative necrosis to large areas of frank infarction. This coagulative necrosis was observed most frequently on days 14 and 21 post-CMV inoculation. Placentas demonstrating ischemic injury usually did not show areas of focal necrosis or viral inclusions. In addition, of a total of 16 placentas showing ischemic injury on days 14 and 21 postinoculation, 10 placentas were associated with fetal death. Typical CMV-specific histopathology, consisting of multiple areas of necrosis associated with acute and chronic inflammation, was only rarely observed during the first 2 weeks after inoculation, but was frequently seen on days 21 and 28 postinoculation. Some lesions were associated with acute inflammatory cell infiltrates and prominent necrosis. Other lesions included infiltrates rich in plasma cells, lymphocytes, and Kurloff cells, and were frequently associated with fibrosis and calcification. Typical intranuclear cytomegalic inclusions were only observed in placentas examined 4 weeks post-maternal inoculation (Fig. 2).

Localization of histopathology, antigens, and virus particles in placentas 4 weeks post-maternal CMV inoculation. To differentiate the infection into the various components that make up the placenta, placentas were examined by light microscopy for localization of CMV-specific histopathology and the presence of CMV antigens and by electron microscopy for the presence of viral particles. Placentas were all evaluated at the time when CMV-specific histopathology and intranuclear lesions were most frequently seen, i.e., 4 weeks postinoculation of the mothers with CMV. Areas of CMV-specific necrosis associated with inflammation and inclusions were all found to be strikingly localized at the transitional zone between the capillarized and noncapillarized syncytiotrophoblast. The mature guinea pig placenta is schematically represented in Fig. 3, showing the localization of CMV-induced histopathology. The hemomonochorial placenta of the guinea pig has a basic lobular arrangement. In these lobules, the maternal blood circulates from the center to the periphery through a complex of lacunae lined by syncytiotrophoblasts. The fetal vessels run centripetally. Different zones can be distinguished within this lobular array: (i) the capillarized labyrinth contains maternal arterial lacunae and fetal vessels which are venous in the center and arterial at the periphery; (ii) the noncapillarized syncytium or interlobium, which may be marginal or interlobar, contains only maternal venous lacunae. CMV-induced lesions

FIG. 2. Focal lesion demonstrating extensive necrosis in placenta from a Hartley guinea pig 28 days post-maternal inoculation. Typical CMV intranuclear inclusions are evident (hematoxylin and eosin, $\times 400$).

were found to be localized at the transitional zone between these two regions, in the marginal as well as the interlobar transitional zone (Fig. 4). This zone contains maternal venous lacunae and a reduced number of fetal arterioles.

To determine whether these lesions harbored CMV-specific antigens, immunohistochemical studies were performed using the ABC-immunoperoxidase method. A total of eight placentas were evaluated at 4 weeks postinoculation. In all placentas, the cytoplasm of cells within the lesions was found to stain positively when anti-guinea pig CMV antiserum was used but not when control sera were used (Fig. 5).

Under electron microscopic examination, intact virus-infected cells were not found easily in placental tissues because of extensive cellular degeneration. Typical intranuclear inclusions were seen within syncytiotrophoblastic cells (Fig. 6). The intranuclear inclusions consisted of electron-dense and electron-lucent areas. Viral nucleocapsids (100 nm in diameter) were associated primarily with the electron-dense areas. Virions and dense bodies were also

found in extracellular spaces surrounding the infected cells. Viral nucleocapsids, not associated with typical intranuclear inclusions, were also seen in the nuclei of other nontrophoblastic cells. The identity of these cells could not be determined with certainty because their morphology was altered significantly because of the cellular damage.

Placental CMV infection and outcome of CMV infection in the fetus. To evaluate the role of placental CMV infection on the outcome of CMV infection in the fetus, the frequency of fetal CMV infection was determined in relation to the infection status of the associated placentas. Pregnant animals were evaluated on days 8 to 28 postinoculation. A total of 63 placentas were found to contain CMV; 17 of the 63 fetuses (27%) showed evidence of CMV infection. In contrast, CMV was not isolated from the fetuses from 11 virus-negative placentas.

The development of fetal CMV infection during the course of maternal infection was also determined, to assess whether the placenta played a role in delaying the establishment of the infection in the fetus. The frequency of fetal CMV infection was highest on day 21 post-maternal inoculation, with 9 of 12 (75%) fetuses showing virus. On days 8, 14, and 28 post-maternal inoculation, respectively, 0 of 20, 3 of 17 (18%), and 5 of 14 (36%) of the fetuses tested harbored virus. Uninfected fetuses were obtained at all times after maternal infection. In all instances, each pregnant animal tested had at least one uninfected fetus.

To determine whether, within each fetal-placental unit, there was an association between high concentrations of virus in the placenta and fetal CMV infection, virus infectivity titers were compared in placentas from CMV-infected and uninfected fetuses (Fig. 7). Placental virus titers were assessed on days 14 ($n = 8$), 21 ($n = 13$), and 28 ($n = 15$) post-maternal inoculation. There was no significant difference between placentas from CMV-infected and uninfected fetuses at each of the time points examined. The mean $\pm$ standard deviation virus infectivity titers ($\log_{10}$ PFU per milliliter) in placentas from CMV-infected guinea pigs were 1.37 ± 1.50 , 2.58 ± 2.99 , and 2.63 ± 2.55 on days 14, 21, and 28 post-maternal inoculation. These mean values are not

FIG. 3. Schematic representation of the guinea pig placenta showing localization of CMV-specific histopathology, 4 weeks post-maternal inoculation.

FIG. 4. Necrosis and inflammation localized at the interlobar noncapillarized syncytium with extension into adjacent capillarized labyrinth in the placenta, 4 weeks post-maternal inoculation with CMV (hematoxylin and eosin, $\times 100$).

significantly different from those in placentas from uninfected fetuses: 0.76 ± 0.38 , 2.63 ± 2.66 , and 1.99 ± 2.15 .

DISCUSSION

The present study has demonstrated that the guinea pig placenta has the ability to support CMV replication during primary acute infection of pregnant outbred guinea pigs. As has been reported before (5, 12), initial placental infection occurred at the time of maternal viremia, i.e., 1 and 2 weeks

post-maternal inoculation. The virus remained detectable in placental tissue later during the course of maternal CMV infection, at the time when the maternal viremic stage was terminated and when CMV-specific antibodies were present. The findings that highest placental virus infectivity titers and typical intranuclear inclusions containing viral nucleocapsids were observed only at 3 to 4 weeks post-maternal inoculation indicate that replication of CMV occurred in placental tissue with a delayed time course as compared to primary maternal viremia. CMV has been isolated from the human placenta (8, 11, 14), but replication of human CMV has only been demonstrated after infection *in vitro* of passaged human placental cells (29). The placenta has also been reported to be a site of murine CMV replication, although the infection is not readily transmitted to the fetus in this animal model (18).

Histological evidence of human CMV placental infection has been described by several investigators (1, 2, 4, 6, 22–26, 28). Placental changes associated with human CMV infection range from no apparent change to plasmacytic villitis with inclusion bodies. In the present study, histological changes similar to those described in human placentas were observed to appear in guinea pig placentas in a defined chronological order. The finding of severe ischemic injury in several placentas early in the postinoculation period, and in association with abortion, suggests the maternal CMV infection may be accompanied, even before the appearance of classical viral lesions, by placental vascular compromise that may result in fetal death. It is interesting that CMV-induced lesions containing intranuclear inclusions, CMV-specific antigens, and mature virions were only observed late during the course of maternal CMV infection. This suggests that CMV-specific histological findings may depend on the duration of maternal CMV infection, although the stage of gestation at the time of sampling may also have played a role. Indeed, all animals were inoculated at midgestation, and therefore when they were tested at 4 weeks postinoculation, most animals were close to the time of delivery. Investigations in several species, including guinea pig, have shown that major changes in placental structure and function

FIG. 5. Localization of CMV antigen in a lesion situated within the transitional zone of the placenta, 4 weeks post-maternal inoculation with CMV (ABC-immunoperoxidase method with hematoxylin counterstain, $\times 100$).

FIG. 6. Electron micrograph of the placenta from a pregnant guinea pig, 28 days post-CMV inoculation, showing syncytiotrophoblast with intranuclear viral inclusion (VI) containing viral nucleocapsids. Virions are also present surrounding the infected cell (bar, 1 μ m). Insert, Details of viral nucleocapsids within the intranuclear inclusion (bar, 200 nm).

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