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Role of Primary and Secondary Maternal Viremia in Transplacental Guinea Pig Cytomegalovirus Transfer

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The modulation of the outcome of intrauterine guinea pig cytomegalovirus (GPCMV) infection by maternal viremia was investigated in the guinea pig model. Virus assay and in situ hybridization were used to study GPCMV infection of maternal blood, placentas, and fetuses following inoculation of pregnant guinea pigs by the subcutaneous, intracardiac, or intranasal route. Animals were inoculated in early gestation and were evaluated every 7 to 10 days throughout pregnancy. Although placental and fetal infections occurred in all groups examined, transfer of GPCMV to placentas and fetuses was most efficient in mothers inoculated subcutaneously. Primary viremia was followed by virus clearance from blood and by an episode of secondary viremia in the three groups of mothers examined. Placental and fetal infections in animals infected subcutaneously or intracardially were first detected at the time of primary viremia, persisted throughout gestation, and increased during secondary viremia. In contrast, placental and fetal infections in animals inoculated intranasally were demonstrated primarily during secondary viremia. Fetal infection was detected in all mothers with detectable primary and secondary viremia but in only 33% of mothers that experienced only primary viremia. These results suggest that secondary maternal viremia is associated with increased placental and fetal GPCMV infections.

Cytomegalovirus (CMV) is the most common cause of viral infection in the human fetus. Clinical manifestations in the newborn may range from asymptomatic infection to severe generalized illness, neurologic damage, and death (14). Clinically apparent infections in the newborn generally occur following primary maternal infection (25). These infections are believed to be acquired in utero through transplacental transmission. However, the determinants of CMV transfer from mother to placenta and fetus during primary maternal infection are not well understood. CMV viremia has been documented in mothers and their congenitally infected offspring (5, 20). Viremia is believed to be the hallmark of disseminated CMV infection, since the recovery of CMV from peripheral blood has been found to be increased in immunocompromised individuals with serious active disease as compared with patients with acute CMV mononucleosis (3, 4, 21–23, 27). However, the role of maternal viremia in transplacental transfer of human CMV has not been systematically investigated.

Guinea pigs infected with guinea pig CMV (GPCMV) represent a unique model for studying the mechanisms of transmission of CMV from mother to infant because transplacental transmission of GPCMV occurs in guinea pigs (1, 2, 10, 13, 15, 17, 19). Nonpregnant guinea pigs infected with GPCMV develop a mononucleosislike syndrome with viremia during acute infection (9). Enhancement of GPCMV infection has been demonstrated to occur in pregnant guinea pigs (11). In addition, delayed amplification of GPCMV infection in the placenta and maternal tissues has been described during late gestation, in association with episodes of secondary maternal viremia (7). Intrauterine infection occurs in animals that experience primary infection during gestation (2, 8, 13). However, the relationship between maternal viremia and the outcome of intrauterine GPCMV infection has not been studied in detail. To address this question, we hypothesized that the viremic spread of GPCMV in pregnant animals would differ depending upon the route of inoculation. In previous work, maternal guinea pigs were inoculated with GPCMV by the subcutaneous route (7, 8, 10, 13). In the present study, we determined whether the entry of GPCMV by the subcutaneous, intracardiac, or intranasal route resulted in different patterns of viremia in mothers and different efficiencies of transplacental transfer of GPCMV to fetuses.

MATERIALS AND METHODS

Animal inoculation. Hartley guinea pigs, 15 days pregnant, were purchased from Camm Research Institute (Wayne, N.J.). They were inoculated with the prototype strain of GPCMV (no. 22122; American Type Culture Collection, Rockville, Md.) serially passaged in guinea pigs. In the present study, salivary gland-passaged virus stocks prepared as described before (13) were used at passage levels 31 to 33 and were diluted in phosphate-buffered saline to contain 4.5 or 5.5 log PFU/ml. At the time of virus inoculation, serum was obtained from each animal by cardiac puncture. The serum was tested for the presence of neutralizing antibodies to GPCMV (9), and animals with preexisting antibodies were excluded from the study. Animals were injected with GPCMV subcutaneously (SC group) in the left axilla. Other animals were injected with GPCMV intracardially (IC group) or were given GPCMV intranasally (IN group). One milliliter of virus suspension containing 4.5 log PFU of virus was given to SC and IC group animals. One-tenth of 1 ml of virus suspension containing 4.5 log PFU was placed in the nasal cavity of each IN group animal.

Animal evaluation and virus assay. Viremia was evaluated weekly throughout gestation. Two milliliters of blood was

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collected by cardiac puncture from each animal anesthetized with methoxyflurane (Metofane; Pitman-Moore, Inc., Washington Crossing, N.J.). The blood was collected in 0.5 ml of Alsever solution and centrifuged at 1,000 rpm for 10 min. The plasma was removed, and packed blood cells serially diluted were used for virus isolation studies.

Within each group, a number of pregnant guinea pigs were euthanatized at 10-day intervals throughout pregnancy (the duration of gestation in guinea pigs is 65 to 70 days), and maternal spleen, lung, salivary glands, and blood and placental and fetal tissues (brain, salivary glands, and pooled organs, including spleen, liver, kidney, and lung) were aseptically collected for virus studies. Portions of tissues were placed in 4% paraformaldehyde for in situ hybridization studies, and serial weight/volume suspensions were prepared from another portion of the same tissue for virus isolation. The presence of infectious virus was assessed by cocultivation with guinea pig embryo cells as described before (7). Cells were observed for 4 weeks for the presence of typical GPCMV-induced cytopathic effects. All isolates were identified by neutralization with specific antibodies as described before (9).

In situ hybridization. Sections of tissues were placed in a Unicassette (Miles Laboratories, Inc., Elkhart, Ind.) and fixed in freshly prepared 4% paraformaldehyde for 1 h at 4°C. These fixation conditions have been shown to result in optimum detection of GPCMV nucleic acids in guinea pig tissues (B. P. Griffith, H. C. Isom, and J. T. Lavallee, J. Virol. Methods, in press). Tissues were kept at 4°C in 70% ethanol until processed further. Following embedding in paraffin, 4-μm sections were prepared and placed on polylysine-coated slides. Slides were baked overnight at 56°C before in situ hybridization was performed. Biotinylated GPCMV DNA probes and a control pBR322 probe were prepared as described previously (6, 16, 24). The map location of each GPCMV probe on the GPCMV genome has been published (24). Two GPCMV probes were used. Probe 1 is composed of a mixture of cloned DNA fragments representing approximately 97% of the GPCMV genome. Probe 3 consists of eight GPCMV DNA fragments containing sequences that are transcribed at the immediate early, early, and late times. In situ hybridization was performed as described previously (24; Griffith et al., in press). Each tissue was hybridized with GPCMV probes 1 and 3. Controls for each experiment included (i) experimental tissues probed with the control probe pBR322 and (ii) salivary gland tissue from a GPCMV-positive animal containing intranuclear inclusions probed with GPCMV probe 1 and 3. GPCMV probes 1 and 3 have been shown to react with GPCMV-infected cells (24; Griffith et al., in press). The vector alone does not hybridize the GPCMV-infected cells (24; Griffith et al., in press). The presence of GPCMV nucleic acids in placental and fetal tissues was indicated by a purple precipitate.

RESULTS

Time course of maternal viremia. To determine whether inoculation of pregnant guinea pigs via different routes resulted in different kinetics of viremia, we assessed maternal blood from the three groups of animals weekly throughout pregnancy for the presence of infectious virus (Table 1). All animals in the IC and SC groups were found to be viremic at least once during gestation. Virus was not recovered from the blood of two IN group animals; however, these two guinea pigs seroconverted during gestation. Episodes of primary and secondary viremia were noted in each experimental group examined. The majority of animals experienced primary viremia. Secondary viremia was detected in a smaller percentage of animals. Similar kinetics of viremia were observed in SC and IC group animals, with peak rates of viremia detected at week 1, virus clearance from the blood of all animals detected at week 3, and secondary viremia detected during weeks 4 to 6 postinoculation. There was a slight delay in secondary viremia in the IC group in that a higher percentage of positive animals was seen at 5 to 6 weeks than at 4 weeks. In contrast, in IN group animals, the rates of viremia were lower during primary viremia. In addition, the episode of primary viremia tended to last longer so that virus clearance from blood occurred only during week 4 postinoculation in many animals and secondary viremia was detected during weeks 5 to 6 postinoculation.

TABLE 1. Incidence of viremia in guinea pigs inoculated with GPCMV via three different routes

Wk after GPCMV inoculation	% with viremia (no. tested) in the following group:		
	SC	IC	IN
1	94 (16)	100 (17)	53 (15)
2	50 (14)	79 (14)	62 (13)
3	0 (12)	0 (13)	8 (12)
4	18 (11)	9 (11)	0 (11)
5 to 6	14 (7)	17 (6)	33 (9)

Virus infectivity titers in maternal blood during primary and secondary viremia. Virus infectivity titers were determined throughout pregnancy in five animals from each of the three experimental groups to assess whether virus replication differed in the three experimental groups during primary and secondary viremia. Data for one representative animal in each group that showed both primary and secondary viremia are shown in Fig. 1. Similar trends were seen in the other animals. During primary viremia, virus infectivity titers reached similar levels in the blood of the SC, IC, and IN group animals (1.9, 1.7, and 1.5 $\log_{10}$ 50% tissue culture infective doses per 0.1 ml, respectively). Virus infectivity titers during secondary viremia were not significantly different in the three groups examined. However, as compared with virus titers during primary viremia, virus titers were lower during secondary viremia.

Incidence of primary and secondary maternal viremia. A separate experiment was carried out to determine whether each route of inoculation was associated with primary viremia only, secondary viremia only, or primary and secondary viremia. Viremia was assessed in 12 animals in the SC group, 13 animals in the IC group, and 12 animals in the IN group. Blood was obtained weekly throughout gestation (i.e., weeks 1 to 7 postinoculation) and examined for the presence of infectious virus by cocultivation. The majority of animals (9, 11, and 9 animals in the SC, IC, and IN groups, respectively) had only primary viremia, which was detectable during the first 2 weeks postinoculation. In a fraction of animals (three, two, and two animals in the SC, IC, and IN groups, respectively), an episode of secondary viremia was also detected later during the course of the infection (days 28 through 47 postinoculation). No animal was found to have evidence of secondary viremia only.

Incidence of placental and fetal infection. Maternal guinea pigs used for the viremia studies shown in Table 1 were also evaluated for placental and fetal infection at 10-day intervals throughout gestation. The presence of GPCMV in tissues

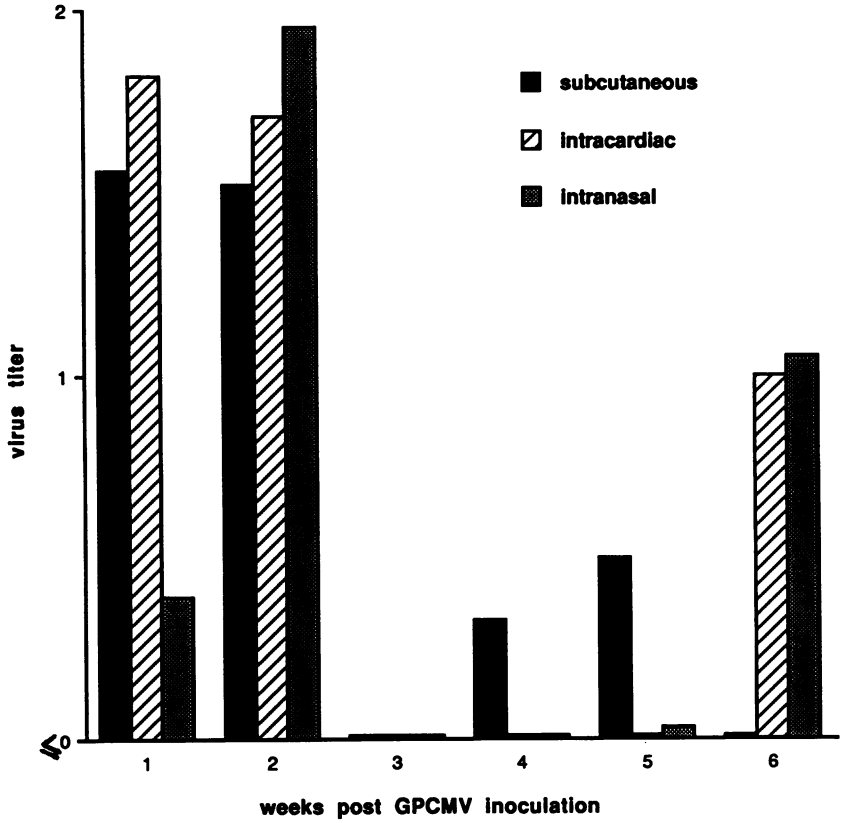

FIG. 1. Comparison of virus infectivity titers in blood from pregnant guinea pigs inoculated with GPCMV subcutaneously, intracardially, or intranasally. Data are shown for one representative animal in each group monitored throughout pregnancy. Virus titers are expressed as $\log_{10}$ 50% tissue culture infective doses per 0.1 ml of packed blood cells.

was assessed by cocultivation in 13, 12, and 15 mothers from the SC, IC, and IN groups, respectively. Numbers of placentas assessed were 41, 39, and 51 and numbers of fetuses assessed were 43, 44, and 62 in the SC, IC, and IN groups, respectively. GPCMV was detected in 51, 41, and 29% of placentas and in 17, 14, and 6% of offspring from SC, IC, and IN group animals, respectively. Figure 2 shows the kinetics of virus detection in placental and fetal tissues throughout gestation in SC and IN group animals. Eight to 17 placentas or fetuses were examined at each time point. Placental and fetal infections were first detected in SC group animals at the time of primary viremia and persisted during virus clearance from blood (day 21). The frequency of placental and fetal infections was highest at the time when viremia reappeared in maternal blood (4 weeks postinoculation). A similar pattern was observed in IC group animals (data not shown). In contrast, placental and fetal infections in IN group animals were demonstrated primarily during late gestation, at the time of secondary viremia. At the time of virus clearance from maternal blood, 30 days postinoculation, a small proportion of placentas and fetuses were found to be infected. The frequency of placental and fetal infections increased during secondary viremia. By day 50 postinoculation, six of nine placentas tested were found to be infected, but no fetuses had evidence of GPCMV infection.

Outcome of intrauterine infection in relation to the occurrence of primary viremia as compared with primary and secondary viremia. Pregnant guinea pigs evaluated for viremia throughout gestation were also assessed for placental and fetal infection by cocultivation to determine if the occurrence of primary or secondary viremia in the mother influenced fetal outcome. Rates of fetal infection in the mothers with primary viremia only as compared with those that had primary and secondary viremia are shown in Table 2. In the three experimental groups, all mothers in which two episodes of viremia were demonstrated had at least one infected fetus. In contrast, less than half of the mothers in which only one episode of viremia during early gestation was documented infected their fetuses. Four SC and four IC group mothers miscarried after week 4 postinoculation. In all eight animals, primary viremia but not secondary viremia was detected.

Detection of GPCMV nucleic acid sequences by in situ hybridization. To determine whether the type of cells infected by GPCMV differed in the three experimental groups, we studied, by in situ hybridization, placentas, fetal brains, and fetal salivary glands that were found to be virus positive by cocultivation. The detection of GPCMV nucleic acid sequences paralleled the recovery of infectious virus from placental and fetal tissues in all groups examined. In both placental and fetal tissues, similar types of cells were found to contain GPCMV nucleic acid sequences regardless of the route of virus inoculation used. Positive cells were seen within focal necrotic lesions in the placentas at the transitional zone between the capillarized labyrinth and the noncapillarized syncytium. The majority of positive placental cells appeared to be syncytiotrophoblastic cells. An example of cells containing hybridization signals in the placenta obtained 35 days after intracardiac inoculation of a pregnant guinea pig is shown in Fig. 3. Salivary glands obtained from

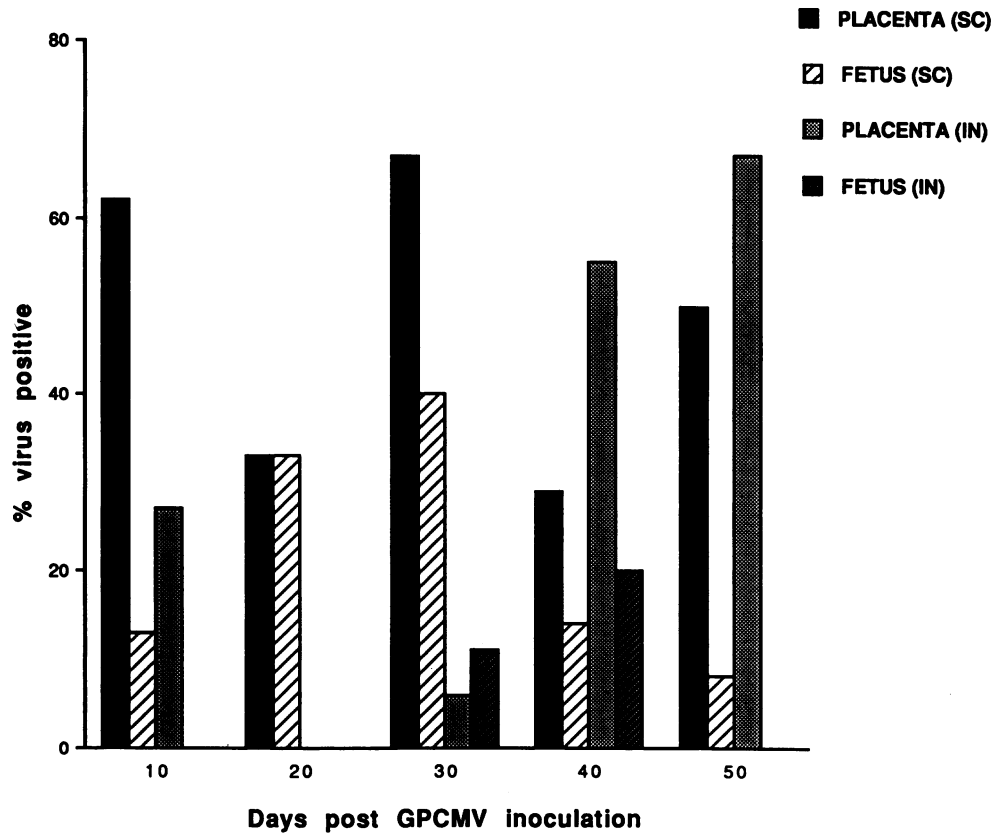

FIG. 2. Time course of GPCMV infection in placental and fetal tissues from mothers inoculated subcutaneously (SC) or intranasally (IN). No bar is shown when the percentage of virus-positive placentas or fetuses was zero.

infected fetuses were all found to have duct cells containing GPCMV nucleic acids. The salivary gland tissue obtained from a fetus 45 days after intranasal inoculation and containing many positive duct cells is shown in Fig. 3C. In contrast, fetal brains contained only isolated positively stained cells (Fig. 3D).

DISCUSSION

The need for continued studies of congenital CMV infections has been recently emphasized (26). Although it is known that primary maternal infection with human CMV results in maternal viremia and placental infection (5), infection of blood cells by human CMV during pregnancy has not been studied. The ways in which this maternal viremia influences the outcome of intrauterine infection are unclear. Studies of human congenital CMV infections are very difficult, and it is only in experimental models such as guinea pigs that mechanisms of cross-placental transfer can be dissected. The present study suggested that the outcome of intrauterine GPCMV infection is influenced by the occurrence of primary and secondary maternal viremia. The results showed that (i) the most efficient transfer of GPCMV to placentas and fetuses occurred in SC group animals; (ii) placental and fetal GPCMV infections were first detected during primary viremia in SC and IC group animals but were amplified in late gestation during secondary viremia; (iii) placental and fetal GPCMV infections occurred primarily during secondary viremia in IN group animals; and (iv) intrauterine GPCMV infections occurred in all animals with primary and secondary viremia but in only a fraction of animals with only primary viremia.

TABLE 2. Fetal outcome in mothers that experienced either primary viremia only or primary and secondary viremia

Viremia	Group	No. of mothers:		
		With viremia	With at least one infected fetus	That miscarried
Primary only	SC	5	2	4
	IC	7	3	4
	IN	9	2	0
Primary and secondary	SC	3	3	0
	IC	2	2	0
	IN	2	2	0

The majority of primary maternal CMV infections in humans are clinically silent. In addition, only approximately half of the mothers who seroconvert during pregnancy transmit CMV to their infants (25). Prediction of mothers who will transmit virus to their offspring has not been possible. Recovery of CMV from maternal urine or cervical secretions cannot distinguish women with from women without intrauterine infections. The present study showed an association of maternal GPCMV viremia during early and late pregnancy with intrauterine infections. Animals with primary and secondary viremia all transferred virus to their offspring, whereas only one-third of animals in which only

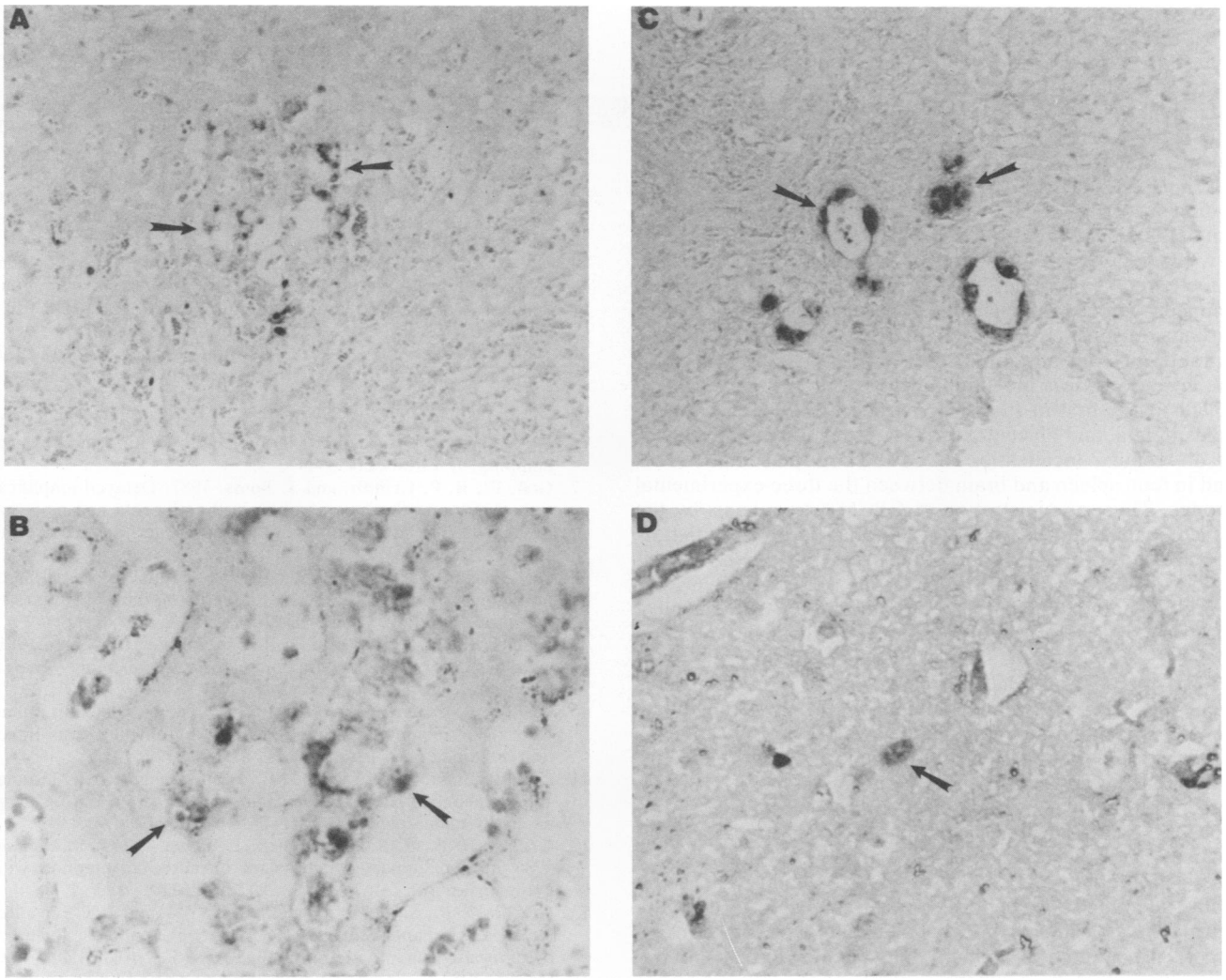

FIG. 3. Detection of GPCMV DNA by in situ hybridization in placental and fetal tissues. (A and B) Placenta obtained 35 days after intracardiac inoculation of a pregnant guinea pig. Tissue was hybridized with GPCMV DNA probe 3. Arrows point to positive cells. Magnifications: A, ca. ×19; B, ×83. (C and D) Fetal tissues obtained 45 days after intranasal inoculation of a pregnant guinea pig. Tissues were hybridized with GPCMV DNA probe 1. Arrows point to positive cells. Magnifications: C (salivary gland), ×83; D (brain), ×83.

primary viremia was detected transmitted GPCMV to their offspring. These results suggest that intrauterine GPCMV transfer may be enhanced by episodes of secondary viremia in the latter part of gestation and that analysis of GPCMV viremia in pregnant mothers may be used to predict transmission of GPCMV to the fetus.

A viremic stage during early acute GPCMV infection of both pregnant and nonpregnant guinea pigs has been described (2, 9, 11). It usually peaks on days 5 to 10 postinoculation; virus clearance then follows 2 weeks after GPCMV inoculation at the time when neutralizing antibodies can first be detected. The present study showed that episodes of secondary viremia occurred in pregnant animals inoculated with virus by the subcutaneous, intracardiac, or intranasal route. In a previous report, viremia was found to reappear towards the end of pregnancy in 6 of 15 mothers inoculated subcutaneously in early gestation, whereas none of 12 nonpregnant animals had an episode of secondary viremia (7). The fact that episodes of secondary viremia are associated with pregnancy is also suggested in the present report. None of eight maternal guinea pigs that miscarried had demonstrable secondary viremia.

The cell type(s) infected with GPCMV during primary and secondary maternal viremia was not identified, although previous studies of primary viremia in nonpregnant guinea pigs showed that GPCMV can be recovered from the granulocyte-enriched fraction and the mononuclear cell-enriched fraction (9). Levels of GPCMV replication were similar during primary and secondary viremia, suggesting that the target blood cells during both episodes of viremia can efficiently support the growth of the virus. Episodes of secondary viremia might be due to reactivation of GPCMV that remains present in a nonreplicating form in blood cells after episodes of primary viremia. Alternatively, seeding from maternal or placental tissues with high GPCMV titers might also occur.

The differences between the kinetics of placental and fetal GPCMV infections in IN group animals as compared with SC and IC group animals are interesting to note. Although levels of virus infectivity were similar in the maternal blood

of all groups of animals, the frequency of GPCMV recovery from the blood of IN group animals was lower, suggesting that this route is less efficient in producing viremia. In addition, fetal infection was delayed and rates of fetal infection were lower in this group of animals than in the other groups. It is possible that the virus inoculum, when given intranasally, had lost some of its infectivity before reaching the maternal circulation because of partial inactivation or loss at the time of inoculation. In a previous study, animals inoculated with low GPCMV doses also showed a 2-week delay of fetal infection with respect to placental infection (7). Placental and fetal infections in IN group animals were evident primarily at the time of secondary viremia, suggesting that secondary viremia may determine intrauterine transmission of GPCMV, particularly in mothers given low virus inoculum doses.

This is the first in situ demonstration of GPCMV nucleic acids in fetal and placental tissues. We found no differences in the cellular localization of GPCMV in placental tissues and in fetal spleen and brain between the three experimental groups. Thus, administration of GPCMV to animals by the subcutaneous, intracardiac, or intranasal route ultimately appears to result in infection of the same target cells, at least in the tissues we examined. All tissues were examined 10 or more days after inoculation of the mothers. Assessment of the tissues earlier during the establishment of the infection might provide clues for subtle differences in the patterns of GPCMV spread. In the placenta, GPCMV nucleic acids were localized at the interlobium labyrinthine transitional zone. Using immunocytochemistry, we previously showed a similar localization of GPCMV antigens in the placenta (13). Duct cells were found to be the main target of GPCMV infection in salivary glands of offspring. A similar localization of GPCMV nucleic acids has been reported for adult guinea pigs inoculated subcutaneously (Griffith et al., in press). Cells harboring GPCMV genes in fetal brain could not be identified with certainty because cells in the developing brain were difficult to recognize. Histopathology consisting of glial nodule encephalitis has been described in congenitally infected newborn guinea pigs (10). Lesions consisted of mononuclear cell infiltrates containing mainly macrophages. It is not known if these infiltrating cells or brain cells themselves contained positive signals.

Human CMV and GPCMV are different viruses; therefore, results obtained with GPCMV may not be directly applicable to human CMV, despite the fact that the congenital infections produced by both viruses are similar. Nevertheless, studies of factors that influence the transmission of GPCMV from mothers to fetuses are important as they improve our understanding of congenital human CMV infections. We have shown in the present study that although intrauterine GPCMV infection can be detected at the time of primary viremia, maternal secondary viremia appears to be needed for efficient GPCMV transfer to the offspring. Further study is needed to establish the factors that modulate episodes of secondary viremia in the blood of maternal guinea pigs.

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