Entero virus infections during pregnancy have been associated with a variety of adverse fetal events. Gestational poliovirus infection causes spontaneous abortion and stillbirths, especially with severe maternal disease [1]. Infections with non-poliovirus enteroviruses have been linked to spontaneous abortions, stillbirths, and congenital anomalies, with enteroviruses detected in amniotic fluid, placenta, and fetal tissues by culture, immunofluorescence, polymerase chain reaction, and electron microscopy [2–10]. Axelsson et al. [11] reported higher rates of IgM antibody to coxsackieviruses in women with spontaneous abortions than in control women undergoing therapeutic abortions at the same gestational age. Brown and Karamus [12], in a serologic study of pregnancies resulting in congenital anomalies, showed a correlation between seropositivity to coxsackieviruses B2, B3, B4, and A9 and defects of the cardiovascular, urogenital, and digestive systems. Gauntt et al. [13] detected coxsackie B virus antibodies in ventricular fluid from 4 infants with severe anatomic defects of the central nervous system. Detection of enteroviruses in tissues from infants with congenital anomalies has not been widely reported. During a cluster of cases of hypoplastic right-sided hearts occurring during the spring and summer, possible enterovirus particles were seen by electron microscopy in 1 heart [9]. These latter studies suggest a link between maternal enterovirus infection and fetal anomalies, but a definitive association has not been proven despite high prevalence of adult enterovirus infections.

We previously described an animal model that uses a murine enterovirus, Théler’s murine encephalomyelitis virus (TMEV), to investigate the role of the placenta in the pathogenesis of gestational enterovirus infection and to explore the effects of enterovirus infection on the developing fetus. TMEV, like the human enteroviruses and human rhinoviruses, is a member of the picornavirus family. In the mouse, it behaves biologically similarly to human enteroviruses in humans and was used for many years as a murine model system for the study of poliomyelitis [14]. Intravenous inoculation of TMEV into splenectomized, pregnant mice in early, middle, and late gestation results in high rates of placental infection in each time period but infection of 78%, 7%, and 0 of fetuses, respectively. After early-gestation infection, in situ hybridization (ISH) detects TMEV RNA in fetal tissues and in all three layers of murine placenta, with most signal concentrated in the labyrinth layer (the layer closest to the fetus, where maternal and fetal blood exchange occurs). In contrast, middle- and late-gestation inoculation of TMEV results in viral RNA signal predominantly in the maternally derived decidua layer and the fetally derived spongiotrophoblast layer [15, 16]. A placental barrier to enterovirus transmission therefore appears to develop between early and middle gestation. The objective of this study was to specifically evaluate gestational outcome following murine enterovirus infection during early gestation.

Materials and Methods

Mice. Timed-pregnant outbred ICR mice seronegative for TMEV (Sasco Laboratories, Omaha) with a normal length of gestation of ~17–19 days were studied.

Viruses. Tissue culture–adapted DA virus, a group II strain of TMEV, was grown on BHK 21 cells and titered by TCID₅₀ assay [16]. Control inoculations used supernatant of freeze-thawed BHK 21 cells.

Adverse Effects of Maternal Enterovirus Infection on the Fetus and Placenta

April L. Palmer,* Harley A. Rotbart, R. Weslie Tyson, and Mark J. Abzug

Gestational outcome in a murine model of congenital enterovirus infection was evaluated. Pregnant mice were inoculated intravenously with Théler’s murine encephalomyelitis virus (TMEV), a murine enterovirus, or with BHK 21 cell lysate (control) at 6–7 days of gestation (early) and sacrificed 6 or 12 days later, and their placentas and fetuses were studied. High rates of gross and histologic abnormalities (50%–87%) were seen in placentas and fetuses from dams infected with TMEV and sacrificed 6 days later. TMEV-infected dams sacrificed 12 days after inoculation had lower rates of placental-fetal abnormalities (25%–57%) but an additional 42% rate of complete pregnancy loss. Pregnancy loss (9%) and placental-fetal abnormalities (4%–7%) were uncommon in control animals. Rates of fetal abnormalities and placental infection in infected dams exceeded fetal viral infection, suggesting that TMEV infection adversely affects pregnancy either directly by fetal damage or indirectly by placental compromise.

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Reprints or correspondence: Dr. Mark J. Abzug, B055, Infectious Diseases, Children’s Hospital, 1056 E 19th Ave., Denver, CO 80218.
*Present affiliation: Division of Pediatric Infectious Diseases, Department of Pediatrics, University of Mississippi Medical Center, Jackson.

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Figure 1. Histologic changes of placentas from dams inoculated with Theiler’s murine encephalomyelitis virus (TMEV) in early gestation and sacrificed 6 or 12 days later. A, Grade 0 placenta: Intact labyrinth layer (L) with thin cellular barriers (arrows) between maternal and fetal vascular beds. Parenchyma uniform throughout (hematoxylin-eosin, bar = 50 μm). B, Grade I placenta: Intact labyrinth layer (L) but some degree of thickening of cellular barriers between maternal and fetal vascular beds (arrows) (hematoxylin-eosin, bar = 50 μm). C, Grade II placenta: Replacement of normal labyrinth layer by ≤30%. Loss of integrity between maternal and fetal vascular beds in labyrinth (L) was accounted for by fibrin deposition (arrow) and dilated vascular lakes (open arrow); in other placentas (see E) by infarction (hematoxylin-eosin, bar = 50 μm). D, Grade III placenta: Replacement of normal labyrinth layer by 30%-70%. Specimen showed areas of infarction (I) and areas of dilated vascular lakes (arrow) within labyrinth (L). Other sections showed intact labyrinth (hematoxylin-eosin, bar = 100 μm). E, Grade IV placenta: Replacement of normal labyrinth layer by >70%. Specimen showed nearly 100% infarction (I) of the labyrinth (L) (hematoxylin-eosin, bar = 100 μm).
Inoculations. Pregnant mice were anesthetized by intraperitoneal instillation of Avertin (tribromoethanol/2-methyl-2-butanol/H₂O) and surgically splenectomized, as previously reported [15, 16]. They were then inoculated intravenously at 6–7 days of gestation (early gestation) with 8 × 10^5 TCID₅₀ of DA virus or an equal volume of BHK 21 cell lysate supernatant.

Specimen harvesting. Mice were phlebotomized and sacrificed 6 (range, 6–8) or 12 (range, 10–13) days after inoculation. Fetuses and placentas were removed and washed in PBS. One-half of the specimens from each mouse were frozen in PBS at −70°C for subsequent culturing. The other half of the specimens were either bisected, fixed in 4% paraformaldehyde, and paraffin-embedded for ISH and histologic evaluation or flash-frozen in liquid nitrogen for future studies.

Gross anatomic evaluation. Placentas were considered abnormal if markedly pale or small. Fetuses were considered abnormal if incompletely formed, degenerated, or absent in the presence of a placenta.

Histologic evaluation. Tissue sections were evaluated by an observer blinded to experimental group designation. Placentas were examined by light microscopy after hematoxylin-eosin staining and graded according to degree of damage seen in the labyrinth layer, the layer most proximal to the fetus, in which exchange of nutrients and wastes occurs. Placentas were considered abnormal when graded II–IV (figure 1). Fetal tissues were classified as histologically normal if organ morphology was appropriate for gestational age and abnormal if degenerate.

Specimen culturing. Frozen tissue specimens from virus- and control-inoculated mice were thawed, weighed, and homogenized in tissue dounces. After centrifugation, 150 μL of each supernatant (generally derived from 0.01–0.3 g of placenta or 0.01–1.6 g of fetus/1000 μL) was cultured on BHK 21 cells for 14 days. Maternal sera obtained immediately before sacrifice were similarly thawed and cultured (100 μL/specimen). Positive cultures were identified by the presence of cytopathic effect typical of TMEV.

ISH. Paraffin sections (4–7 μm) of placentas and fetuses from infected and control animals were affixed to slides, deparaffinized, treated with 0.2 N HCl at room temperature for 20 min, and incubated in a solution of 2 mM CaCl₂, 5 μg/mL proteinase K (Sigma, St. Louis), and 10 mM TRIS-HCl buffer (pH 7.5) at 37°C for 20 min. Slides were then incubated in a prehybridization solution of 50% deionized formamide, Denhardt’s solution, dextran sulfate, yeast RNA, and standard saline citrate (SSC) for 1 h at room temperature. Hybridization was done with a single-stranded, digoxigenin-labeled RNA TMEV probe prepared by transcribing a PGEM-2 plasmid vector containing a cDNA fragment of TMEV strain BeAn 8386 in the presence of digoxigenin-labeled UTP (Boehringer Mannheim, Mannheim, Germany) and alkaline hydrolysis in a carbonate buffer, pH 10.2, at 60°C for 20 min [17]. Specificity of the TMEV probe was verified in previous experiments [15]. Slides were incubated overnight at 42°C with ~0.5 μg of this probe/slide in hybridization solution identical to the prehybridization solution. After hybridization, slides were washed in SSC and exposed to 20 μg/mL RNase A (Boehringer Mannheim). To detect hybridized product, slides were treated with a 2% normal sheep serum solution and then incubated overnight at room temperature in a 1:750 dilution of sheep anti-digoxigenin antibody conjugated to alkaline phosphatase. Color development was done overnight at room temperature with nitroblue tetrazolium and X-phosphate (Boehringer Mannheim). Slides were counterstained with eosin. Infected and uninfected placental tissues from previous experiments served as controls [15].

Statistical analysis. Data were evaluated with Fisher’s exact test or χ² analysis with Yates’s correction where appropriate.

Results
EIGHTEEN mice were inoculated in early gestation (6–7 days) with TMEV; 5 were sacrificed 6 days later, 1 delivered before sacrifice, and 12 were sacrificed 12 days after inoculation. All mice were confirmed to be pregnant by examination of uterine horns at time of splenectomy. All 5 dams inoculated with TMEV and sacrificed 6 days later had some placental or fetal material present at sacrifice. In contrast, 5 (42%) of the 12 TMEV-infected dams sacrificed 12 days after inoculation had empty uterine horns at sacrifice (complete pregnancy loss). Among the BHK-inoculated control animals, 1 was sacrificed 6 days after inoculation, 3 delivered before sacrifice, and 7 were sacrificed 12 days after inoculation. Only 1 control animal, sacrificed 12 days after inoculation, had complete pregnancy loss (P = .4, vs. complete pregnancy loss in TMEV-inoculated dams).

### Table 1. Placental examination, culture, and in situ hybridization (ISH) following early-gestation inoculation with enterovirus.

<table>
<thead>
<tr>
<th>No. of dams*</th>
<th>Abnormal gross</th>
<th>Abnormal histology</th>
<th>Culture-positive</th>
<th>ISH-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-day sacrifice</td>
<td>5</td>
<td>44/60 (73)%</td>
<td>15/19 (79)%</td>
<td>29/29 (100)%</td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>54/94 (57)%</td>
<td>16/37 (43)%</td>
<td>13/47 (28)%</td>
</tr>
</tbody>
</table>

NOTE. Data are no./total (%).
* Excludes dams with complete pregnancy loss or delivery before sacrifice.
1 P < .001 vs. controls (χ² with Yates’s correction).
² P < .001 6-day vs. 12-day sacrifice (χ² with Yates’s correction).
Figure 2. In situ hybridization of placentas from dams inoculated with Theiler’s murine encephalomyelitis virus (TMEV) (A) or control BHK 21 cell lysate (B) in early gestation. Dark hybridization staining indicating the presence of TMEV RNA (arrows) was present in spongiotrophoblast (S) and labyrinth (L) layers of placenta from infected mice but was not detected in placentas from control mice (eosin counterstain, bar = 50 μm).

Table 1 shows the results of placental examination, viral culture, and ISH. Seventy-three percent and 57% of placentas from dams inoculated with TMEV at early gestation and sacrificed 6 and 12 days later, respectively, were grossly abnormal, rates that were significantly higher than in control animals. Histologic abnormalities were also significantly higher than in controls (figure 1). Most of these histologically abnormal placentas had extensive damage in the labyrinth layer, with grade IV pathology in 73% and 50% of the abnormal placentas from dams sacrificed 6 and 12 days after TMEV inoculation, respectively. The apparent decline in gross and histologic abnormalities with longer time to sacrifice most likely reflects complete resorption of some abnormal placental and fetal tissue over time, resulting in a decreased percentage of abnormal tissue grossly identified at sacrifice.

One hundred percent of placentas from infected dams sacrificed 6 days after inoculation but only 28% of placentas from infected dams sacrificed 12 days after inoculation were viral
### Table 2. Fetal examination, culture, and in-situ hybridization (ISH) following early-gestation inoculation with enterovirus.

<table>
<thead>
<tr>
<th></th>
<th>No. of dams*</th>
<th>Abnormal gross</th>
<th>Abnormal histology</th>
<th>Culture-positive</th>
<th>ISH-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-day sacrifice</td>
<td>5</td>
<td>52/60 (87)²</td>
<td>4/8 (50)¹</td>
<td>7/25 (28)²</td>
<td>0/13</td>
</tr>
<tr>
<td>12-day sacrifice</td>
<td>7</td>
<td>54/94 (57)²</td>
<td>8/32 (28)³</td>
<td>1/45 (2)</td>
<td>1/36 (3)</td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>6/95 (6)</td>
<td>2/48 (4)</td>
<td>0/42</td>
<td>0/17</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no./total (%).

* Excludes dams with complete pregnancy loss or delivery before sacrifice.

¹ *P* < .001 vs. controls (χ² with Yates’s correction).

² *P* < .01, 6-day vs. 12-day sacrifice (Fisher exact 2-tailed or χ² with Yates’s correction).

³ *P* < .02 vs. controls (Fisher exact 2-tailed).

culture–positive. Despite a decline in culture positivity with longer time to sacrifice, ISH signal remained common in placentas from infected dams. Ninety-five percent and 88% of placentas from dams sacrificed 6 and 12 days after virus inoculation, respectively, contained hybridization signal. TMEV RNA was frequently detected in all three layers of the placenta (decidua, spongiotrophoblast, and labyrinth). None of the placental tissues from control animals were viral culture– or ISH-positive (figure 2).

Table 2 shows the results of fetal examination, viral culture, and ISH. Eighty-seven percent and 57% of fetuses from dams inoculated with TMEV in early gestation and sacrificed 6 or 12 days later, respectively, were grossly abnormal, rates that were significantly higher than in control animals. Most of these gross abnormalities, in fact, reflected complete resorption of fetal tissue, despite presence of placental tissue. Fifty percent and 25% of any fetal tissue present in dams sacrificed 6 and 12 days after virus inoculation, respectively, were histologically abnormal, rates also significantly higher than in controls. These histologically abnormal fetuses had degenerative changes of some or all tissue, with replacement by a monotonous array of necrotic cellular debris lacking organization (figure 3). No specific organ defects could be detected in nondegenerative fetal tissue.

Twenty-eight percent of fetuses from dams sacrificed 6 days after virus inoculation were viral culture–positive, while only 2% of fetuses from dams sacrificed 12 days after TMEV inoculation were viral culture–positive. Another fetus, from a dam sacrificed 12 days after inoculation with TMEV, had ISH signal detected in the brain; no other case nor control fetus had detectable hybridization (figure 4).

### Discussion

This study demonstrated that maternal infection during early gestation with TMEV resulted in high rates of complete pregnancy loss, as well as gross and histologic abnormalities of the placenta and fetus. After completion of these experiments, we retrospectively reviewed our previously published work [16] and identified an intermediate level of gross placental (33%) and fetal (33%) abnormalities in mice inoculated with TMEV in late gestation, when the placental barrier restricts transmission of TMEV to the fetus. With either early or late gestation inoculation, fetal abnormalities occurred at a rate greater than that of fetal infection as detected by culture or ISH, suggesting placental insufficiency as a significant pathophysiologic mechanism. The disruption of placental vasculature in the labyrinth, which we observed commonly after maternal TMEV infection, would be expected to disturb transfer between maternal and fetal blood supplies and impair fetal development.

Other murine models of congenital enterovirus infection show varying amounts of placental and fetal infection, often with adverse effects on fetal outcome exceeding evidence of fetal infection. Maternal inoculation with poliovirus results in high rates of fetal infection and spontaneous abortion, especially during early gestation and when maternal paralysis is present [18, 19]. Inoculation with the non-poliovirus enteroviruses coxsackievirus B3, B1, B4, and A13 also results in placental and fetal infection [20–24]. In these models, infection rates and virus titers are lower and of shorter duration in fetal than in placental tissue. Although fetal infection rates are relatively low, spontaneous abortion rates remain high, which underscores the important role of virus-induced placental insufficiency as a cause of fetal loss. Susceptibility to spontaneous abortion may vary with time of inoculation, but previous reports are conflicting. With coxsackievirus B1, Modlin and Crumpacker [21] found that spontaneous abortion rates are higher when maternal infection occurs during early gestation, but Dalldorf and Gifford [25] found higher spontaneous abortion rates in mice inoculated during late gestation.

In the current study, placental culture positivity declined with longer time to sacrifice, whereas ISH signal remained frequent. There are several potential explanations for this observation. Virus in placental tissue may be neutralized by maternal antibody, inhibiting our ability to cultivate the virus. Alternatively, virus (or its remnants) may persist in a nonreplicating form, rendering the tissue culture-negative but allowing TMEV RNA detection by ISH. In experiments by Klingel et al. [26],
in which murine heart tissue was chronically infected with coxsackievirus B3 (Nancy strain), viral RNA was detected long after tissue was culture-negative. The investigators hypothesized that persistent infection may involve restriction of viral replication at the level of RNA synthesis but still contribute to ongoing immunologic destruction of myocardial tissue. Enteroviruses may similarly persist in placental tissue, although the physiologic consequences of viral persistence for the placenta are unknown.

Direct correlation between our model and human infection should be made cautiously. TMEV is phenotypically closer to another murine enterovirus, encephalomyocarditis virus, than to the human enteroviruses [27]. However, similar to the extensive damage present in our infected murine placentas, human placentas infected with coxsackieviruses and echoviruses frequently have histologic evidence of villitis with necrosis, fibrin deposits, and inflammatory cell infiltrates; venous thrombosis is also commonly seen [4, 7]. Human placentas with other viral
infections, such as cytomegalovirus and rubella, show similar pathologic changes [28]. Postmortem examination of aborted and stillborn fetuses infected with coxsackieviruses and echoviruses typically show no external or internal abnormalities other than visceral congestion, hemorrhage, thrombosis, necrosis, or edema and minimal inflammatory infiltrates [4, 5, 7, 8, 10], although several stillbirths with histologic myocarditis and coxsackievirus B antigen in heart tissue have been described [6, 29]. In some reports of stillbirths associated with enterovirus infection, enteroviruses have been isolated from placental and fetal tissues [7, 8], while in others, enteroviruses were grown from placental but not fetal tissue [4]. The latter observation suggests that, in some cases, placental injury and insufficiency rather than transplacental infection of the fetus may be a pathophysiologic mechanism causing fetal demise after human enterovirus infections. Similarly, in our animal model, despite relatively low fetal infection rates, fetal abnormalities consisting of incomplete development or complete loss of tissue, rather than recognized specific organ defects, were frequent. The parallels between our murine model and human data suggest that enterovirus infection may adversely affect the outcome of pregnancy either by direct fetal damage or indirectly by placental compromise.

References