Adverse reproductive outcomes in urban women with adeno-associated virus-2 infections in early pregnancy

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BACKGROUND: We demonstrated recently that adeno-associated virus-2 (AAV-2) DNA was detected significantly more frequently in placental trophoblast cells from cases of severe pre-eclampsia than from normal term deliveries. Here, we sought to determine if maternal AAV-2 infection early in pregnancy preceded adverse outcomes resulting from placental dysfunction. METHODS: We collected first trimester maternal serum samples and compared anti-AAV-2 IgM antibody levels (indicating primary infection or reactivation of latent AAV-2) between controls delivered at term (n = 106) and three groups of cases: spontaneous abortions (n = 34), spontaneous preterm deliveries (n = 24) and women with at least one outcome usually attributed to placental dysfunction, including pre-eclampsia, intrauterine growth restriction (IUGR) or stillbirth (n = 20). The seroprevalence of immunoglobulin G (IgG) antibodies against AAV-2 and IgM antibodies against viruses that promote AAV-2 replication [adenovirus and cytomegalovirus (CMV)] were also determined. RESULTS: First trimester maternal IgM seropositivity was 5.6 times more prevalent among pre-eclampsia/IUGR/stillbirth cases (P = 0.0004) and 7.6 times more prevalent among preterm deliveries (P < 0.0001) than among controls. CMV and adenovirus IgM antibodies and chronic AAV-2 infections (IgG seropositivity) were not associated with adverse pregnancy outcomes. CONCLUSIONS: Primary or reactivated AAV-2 infection (maternal IgM seropositivity) early in pregnancy was associated with adverse reproductive outcomes associated with placental dysfunction, including pre-eclampsia, stillbirth and spontaneous preterm delivery.

Keywords: adeno-associated virus; antibodies; placenta; pre-eclampsia; preterm birth

Introduction

Maternal infections with viruses such as human cytomegalovirus (CMV), herpes simplex virus and parvovirus have been associated with fetal anomalies, neonatal infection and intrauterine fetal demise (Anand et al., 1987; Fowler et al., 1992; Brown et al., 1997; Tolfvenstam et al., 2001). We hypothesized that maternal infection early in pregnancy with adeno-associated virus-2 (AAV-2) is a previously unrecognized cause of adverse reproductive outcomes that result, in part, from placental dysfunction.

Adeno-associated virus is a member of the parvovirus family, and six serotypes of AAV have been identified (Berns and Giraud, 1996; Berns and Linden, 1995). Of these, types 2 and 3 infect humans, although type 3 is rare and is probably a laboratory contaminant that developed during construction of recombinant AAV vectors (Berns and Linden, 1995; Berns and Giraud, 1996). AAV-2 is a common human isolate (40–80% of the adult population has been exposed), and infection involves hematogenous seeding (Erles et al., 1999; Grossman et al., 1992). Thus, the placenta may be exposed to AAV-2 during maternal infections. Although it has been shown that productive AAV-2 infections usually require co-infection with helper viruses (including adenovirus, CMV, human papillomavirus and herpes simplex virus), other investigators have demonstrated that AAV-2 can undergo full replicative cycles in keratinocytes, and we have demonstrated that placental trophoblast cells are uniquely susceptible to AAV-2 in the presence or absence of helper virus co-infection (Leonard and Berns, 1994; Fisher et al., 1996; Parry et al., 1998; Meyers et al., 2000; Arechaveleta-Velasco et al., 2006).

The pathogenicity of AAV-2 is largely unknown, but there are several reports that describe AAV-2 infection in association with adverse reproductive outcomes, including spontaneous abortion, gestational trophoblastic disease, spontaneous preterm birth and severe pre-eclampsia (Botquin et al., 1994; Tobiasch et al., 1994; Burguete et al., 1999; Kiehl et al., 2002; Arechaveleta-Velasco et al., 2006). In pregnant mice, infection with AAV induces early spontaneous abortion, and in humans, AAV-2 DNA has been detected in placentas from early spontaneous abortions (Botquin et al., 1994; Tobiasch...
et al., 1994; Malhomme et al., 1997; Burguete et al., 1999). Most recently, we demonstrated that AAV-2 infection reduces invasion of extravillous trophoblast cells through an extracellular matrix, and in a case–control study, we detected AAV-2 DNA significantly more frequently in trophoblast cells from cases of severe pre-eclampsia than from normal term deliveries (Arechavaleta-Velasco et al., 2006).

Our previous results and the results of other investigators indicate that AAV-2 infection is a previously unidentified cause of placental dysfunction and adverse obstetrical outcomes attributed to placental dysfunction. However, the detection of AAV-2 DNA in placental cells after delivery does not determine the timing of viral infection during pregnancy, which is critical for testing our hypothesis that AAV-2 infection during the first 20 weeks of gestation impairs placental invasion into the maternal uterine wall. Therefore, we sought to determine if maternal infection by AAV-2 early in pregnancy is associated with reproductive outcomes that may result from placental dysfunction, including spontaneous abortion, pre-eclampsia and spontaneous preterm delivery.

Materials and Methods

Case–control study design

Research nurses recruited study subjects and obtained blood samples from women presenting to the Emergency Department over a twelve-month period at the University of Pennsylvania Medical Center. All subjects underwent routine serum screening for human chorionic gonadotropin (hCG) and an obstetric ultrasound examination, confirming intrauterine pregnancies at less than 20 weeks’ gestation. Three groups of case subjects were compared separately with controls: (i) women who experienced spontaneous abortion before 20 weeks’ gestation; (ii) women who delivered between 20 and 37 weeks’ gestation; (iii) women who developed complications. Conceptuses that aborted spontaneously were not routinely karyotyped; therefore, we estimated that approximately one half of these fetuses were aneuploid (American College of Obstetricians and Gynecologists, 2002). Controls were defined as women who delivered at term without any medical or obstetric complications. Conceptuses that aborted spontaneously were not routinely karyotyped; therefore, we estimated that approximately one half of these fetuses were aneuploid (American College of Obstetricians and Gynecologists, 2002). Controls were defined as women who delivered at term without any medical or obstetric complications. Conceptuses that aborted spontaneously were not routinely karyotyped; therefore, we estimated that approximately one half of these fetuses were aneuploid (American College of Obstetricians and Gynecologists, 1995).

Immunofluorescence studies

In order to confirm that IgM antibodies detected by ELISA in maternal serum were directed against AAV-2, immunofluorescence studies were conducted. Briefly, human embryonic kidney (293) cells were seeded in 8-well multi-chamber slides (1 × 10⁵ cells/well) and transfected with an AAV-2 packaging plasmid p600 trans (containing genes that express VP1–3) complexed with FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN, USA) at a concentration of 3 μg plasmid DNA (Zhang et al., 2000; Auricchio et al., 2001). After transfections were complete, cells were washed and fixed with formaldehyde. Maternal serum samples, from which IgG antibodies had been removed by treatment with protein G agarose, were then incubated with fixed cells in each well for 1 h, after which the cells were washed and incubated with FITC-labeled goat anti-human IgM (Sigma). Transfected 293 cells treated with secondary antibody alone were used as negative controls. The cells were mounted with fluorescence medium under cover slips, and the number of fluorescent-labeled cells was visualized using fluorescent microscopy.

Enzyme-linked immunosorbent assay to detect IgM antibodies against AAV-2

Ten milliliter of blood was collected from each subject and centrifuged for 10 min at 3000 rpm. The supernatant (serum) was aspirated and stored at −70°C. Protein concentrations for serum samples were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). An aliquot of each serum sample containing 2 mg of total protein was mixed with 50 μl of protein G agarose (Invitrogen, Carlsbad, CA, USA) to remove immunoglobulin G (IgG) class antibodies that are present in serum samples at greater levels than IgM class antibodies, and can cause falsely elevated results when measuring IgM antibody levels (Martins et al., 1995; Erles et al., 1999). After 30 min, the serum was centrifuged at 1000 rpm for 10 min, and the supernatant was collected. Western blots were performed using an anti-human IgG antibody (Sigma, St Louis, MO, USA) to confirm that IgG antibodies were removed from the serum. In addition, proteins (including IgG and IgM antibodies) were removed from pooled serum samples using a thiophilic resin purification system (Thiophilic-uniflow resin, BD Biosciences, Franklin Lakes, NJ, USA), and these pooled samples were used as negative controls in enzyme-linked immunosorbent assay (ELISA) experiments. After centrifugation at 5000 rpm for 25 min, the pellet and supernatant (negative control) were collected, and the distribution of IgM and IgG antibodies was determined by western blotting.

We developed an ELISA to detect IgM antibodies directed against AAV-2 using 96-well plates (Nunc, Denmark) coated overnight with recombinant AAV-2 particles containing the structural proteins VP1, VP2 and VP3 (Parry et al., 1998). The AAV-2 vectors were purchased from the Vector Core in the Institute for Human Gene Therapy at the University of Pennsylvania. The recombinant viral particles contained intact capsids and the same viral proteins (VP1, VP2 and VP3) as wild-type AAV-2 (Berns and Linden, 1995; Fisher et al., 1996). After washing the plates and blocking for 1 h, the wells were incubated with maternal serum diluted 1:16 with phosphate-buffered saline (PBS)/Tween-20 for another hour. The wells were washed after incubation with maternal serum samples and incubated with biotin-labeled anti-human IgM (Sigma), to which was bound streptavidin conjugated with alkaline phosphatase. The substrate to alkaline phosphatase, p-nitrophenyl phosphate (Sigma) was added to the wells, and the reaction of alkaline phosphatase with its substrate was stopped after 30 min. Optical density (OD) values were determined at a wavelength of 405 nm using an ELISA photometer (VWR, Bridgeport, NJ, USA) (Erles et al., 1999). Each sample was tested in duplicate, and the average OD value was calculated. To standardize our results, we divided the OD value of each sample by the background signal (mean OD value for negative controls) for each ELISA plate.
**Detection of IgG antibodies against AAV-2 in maternal serum samples**

To determine the prevalence of AAV-2 IgG seropositivity in our population, we measured levels of IgG antibodies against AAV-2 by ELISA in a representative number of maternal serum samples. Recombinant AAV-2 particles were used to coat 96-well ELISA plates as previously described, and maternal serum samples were diluted 1:16 with PBS/Tween-20 and added to each well for 1 h. The wells were washed after incubation with maternal serum samples and incubated with biotin-labeled anti-human IgG (Sigma), to which was bound streptavidin conjugated with alkaline phosphatase. p-Nitrophenyl phosphate was added to the wells, and the reaction of alkaline phosphatase with its substrate was stopped after 30 min. OD values were determined at a wavelength of 405 nm (Erles et al., 1999). Each sample was tested in duplicate, and the average OD value was calculated. The OD value of each sample was divided by the background signal (mean OD value for negative controls) for each ELISA plate.

In order to confirm that IgG antibodies detected by ELISA were directed against AAV-2, maternal serum samples were diluted 1:16 in Dulbecco’s modified Eagle’s medium (Gibco BRL, Grand Island, NY, USA) and incubated with a recombinant AAV-2 vector expressing green fluorescent protein for 3 h at 37°C. The AAV-2 vectors were purchased from the Vector Core in the Institute for Human Gene Therapy at the University of Pennsylvania. Human cervical carcinoma (HeLa) cells were seeded on 8-well multi-chamber slides (1 x 10^4 cells/well) and infected with wild-type adenovirus type 2 (ATCC number 216725, 100 viral particles per cell) as a helper virus 3 h before transduction with recombinant AAV-2 (100 particles/cell). Cells were then incubated with the serum/recombinant AAV-2 mixture for 16 h at 37°C, washed and fixed with formaldehyde. The percentage of green fluorescent-labeled (transduced) cells in six high-power fields was determined by direct visualization using fluorescent microscopy.

**Detection of IGM antibodies against CMV and adenovirus in maternal serum samples**

Although we demonstrated previously that trophoblast cells are susceptible to AAV-2 infection in the presence or absence of helper virus co-infection, we sought to determine if acute maternal infection with AAV-2 helper viruses (adenovirus, CMV) early in pregnancy were associated with adverse pregnancy outcomes (Malhomme et al., 1997; Parry et al., 1998; Arechaveleta-Velasco et al., 2006). We did not study the seroprevalence of antibodies against human papillomavirus and herpes simplex virus because those viruses do not spread hematogenously and serum antibody responses are less consistent. Thus, we screened maternal serum samples for IgM antibodies directed against CMV and adenovirus. Briefly, 1 ml of serum was diluted and added to wells coated with purified CMV antigen (CMV IgM ELISA, Calbiotech Inc., Spring Valley, CA, USA) or adenovirus antigen (Adenovirus IgM ELISA, IBL Immuno-Biological Laboratories, Hamburg, Germany). Serum diluent for IgM ELISAs contained absorbent to remove IgG antibody interference. After the wells were washed, enzyme conjugate and substrate were added to each well to bind antibody-antigen complexes according to the manufacturer’s protocol, and hydrolysis of the substrate by enzyme was detected at 450 nm using a microwell reader. For the CMV IgM ELISAs, optical densities (OD) corresponded to IgM concentrations, and antibody indices were calculated by dividing the OD of each sample by a calibration factor (see manufacturer’s instructions). CMV IgM antibody indices greater than 1.10 were considered positive. For the adenovirus IgM ELISAs, the cut-off for positive samples was the OD of the cut-off standard (as per manufacturer’s instructions).

**Statistical analyses**

Mean and median OD values and standard errors were calculated. Analysis of variance (ANOVA) was conducted to determine if overall differences occurred among control and case groups, and t-tests for independent samples were performed to compare controls with each group of cases. Rank order sum non-parametric tests (Kruskal–Wallis) that do not assume normal distributions also were conducted. Logistic regression models were developed to assess the ability of the OD values to differentiate between each case group and controls adjusting for gestational age and the presence of vaginal bleeding at enrollment.

Receiver–operator characteristic (ROC) curves were constructed to determine the optimal threshold level for positive anti-AAV-2 IgM ELISA results. Sensitivity and specificity were calculated for adverse reproductive outcomes at each threshold level. In order to express a level of association using the optimal ROC curve cut point, we constructed 2 x 2 tables and calculated odds ratios (OR) and 95% confidence intervals (CI) to compare the incidence of IgM seropositivity between cases and controls. Stratified analyses were performed to determine if the relationship between anti-AAV-2 IgM antibodies and adverse obstetric outcomes was influenced by AAV-2 IgG seropositivity or CMV and adenovirus IgM seropositivity.

**Results**

Serum samples were obtained from 328 women who presented to the Emergency Department and were enrolled in this study. Among these 328 women, 144 were excluded from further analysis because they did not deliver at our institution (n = 139) or because they met other exclusion criteria (n = 5), including the development of chorioamnionitis during labor at term and the presence of multiple gestations or fetal anomalies not recognized at entry. Thus, our cohort consisted of 184 women, among whom 106 delivered at term without any obstetrical or medical complications (controls), 34 aborted spontaneously, 24 delivered preterm due to idiopathic preterm labor or PPROM and 20 experienced the composite outcome of pre-eclampsia (n = 5), intrauterine fetal demise (n = 2) or IUGR (n = 13). The most common reasons these women presented to the Emergency Department included vaginal bleeding (n = 78), suspected fever (n = 57) and vaginal discharge (n = 22). Among the 34 women who aborted spontaneously, 18 aborted spontaneously during the initial Emergency Department visit, whereas 16 aborted spontaneously at a later date (but before 20 weeks’ gestation). There were 29 women who were primigravid, 55 were nulliparous and $>$95% were African American; no differences in gravidity, parity and race were observed among cases and controls. A comparison of demographic characteristics between cases and controls is summarized in Table I.

The mean anti-AAV-2 IgM antibody level [OD value (±SE)] divided by the background signal] in samples from control subjects was 3.80 ± 0.35, which was similar to the OD value of cases who aborted spontaneously (3.95 ± 0.34) but lower than the OD value of women who delivered preterm (8.35 ± 0.92) and women whose pregnancy was complicated by pre-eclampsia, IUGR or stillbirth (8.96 ± 1.96) (Table II). ANOVA of mean OD values demonstrated significant differences among controls and case groups (P < 0.001). Thus,
individual comparisons were made between cases and controls using t-tests and this demonstrated that OD values were significantly higher among pre-eclampsia/IUGR/stillbirth cases and spontaneous preterm delivery cases than controls (P < 0.0001 for both comparisons). Because the OD/background values for the control group were not normally distributed, comparisons also were made between cases and controls using nonparametric tests (Table II). In a comparison between spontaneous abortion cases and controls, the mean rank score for spontaneous abortion cases was significantly greater than the mean rank score of controls (P = 0.04). The mean rank score of the two other case groups was also significantly greater than the mean rank scores of controls (P < 0.001 for both comparisons). After controlling for the presence of vaginal bleeding and gestational age at screening, significant differences persisted in comparisons between pre-eclampsia/IUGR/stillbirth and controls and between spontaneous preterm delivery cases and controls (P-values < 0.0001), but IgM levels in spontaneous abortion cases were not significantly different to controls (P = 0.10).

The area under ROC curves was calculated for each case–control comparison: (i) controls plus women who aborted spontaneously, area = 0.6178; (ii) controls plus women who experienced pre-eclampsia, IUGR or stillbirth, area = 0.7417 and (iii) controls plus women who delivered preterm, area = 0.7869. We utilized the ROC curve for controls plus pre-eclampsia/IUGR/stillbirth cases to determine that the optimal cut-off for a positive test (OD value of sample divided by background signal) was 6.00 (Fig. 1). At this cut-off level, 17.9% of controls and 55.0% of pre-eclampsia/IUGR/stillbirth cases were IgM seropositive, the sensitivity of a positive test was 0.50 and the specificity was 0.84 (Fig. 1). On the basis of this threshold, the incidence of seropositivity for IgM antibodies among cases of spontaneous preterm delivery and pre-eclampsia/IUGR/stillbirth was significantly greater than the incidence of IgM seropositivity in controls (Table III).

In order to confirm that anti-AAV2 antibodies detected by ELISA were IgM antibodies, western blots were performed after removal of all IgG antibodies from representative serum samples using protein G agarose (Fig. 2). IgG and IgM antibodies were detected in all samples before treatment with protein G agarose, but IgG antibodies were not detected in samples (supernatant) after treatment with protein G agarose and centrifugation. Conversely, IgG antibodies, but not IgM antibodies, were detected in resuspended pellets. Western blots also were performed to demonstrate that IgM and IgG antibodies were not present in background samples after purification using thiophilic resin.

The specificity of IgM antibodies against AAV-2 was determined by immunofluorescence studies (Fig. 3). The number of fluorescent-labeled 293 cells (transfected with AAV plasmid p600) per high-power field was greater after incubation with serum samples containing high levels of anti-AAV-2 IgM antibodies (OD/background ≥ 6.00) than after incubation with serum samples containing lower levels of anti-AAV-2 IgM antibodies (OD/background < 6.00). No fluorescence was detected in wells containing negative controls (cells treated with secondary antibody alone).

We calculated the seroprevalence of IgG antibodies against AAV-2 among 34 spontaneous abortion cases, 3 cases of pre-eclampsia/IUGR/stillbirth, 8 cases of spontaneous preterm delivery and 19 controls to determine if anti-AAV-2 IgG seroprevalence in our cohort was similar to that of previously

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**Table I.** Demographic characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Controls, n = 106</th>
<th>Spontaneous abortion, n = 34</th>
<th>PE/IUGR/SB, n = 20</th>
<th>SPTD, n = 24</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year) ± SE</td>
<td>23.15 ± 0.53</td>
<td>23.86 ± 0.86</td>
<td>24.30 ± 1.64</td>
<td>21.46 ± 0.59</td>
<td>0.27</td>
</tr>
<tr>
<td>Gravity ± SE</td>
<td>2.42 ± 0.19</td>
<td>2.65 ± 0.41</td>
<td>2.80 ± 0.58</td>
<td>2.25 ± 0.30</td>
<td>0.78</td>
</tr>
<tr>
<td>Parity ± SE</td>
<td>1.24 ± 0.13</td>
<td>1.41 ± 0.23</td>
<td>1.55 ± 0.44</td>
<td>1.25 ± 0.17</td>
<td>0.76</td>
</tr>
<tr>
<td>Race (% African American)</td>
<td>95.2</td>
<td>96.4</td>
<td>100</td>
<td>90.9</td>
<td>0.86</td>
</tr>
<tr>
<td>Gestational age at screen (week) ± SE</td>
<td>10.37 ± 0.43</td>
<td>9.66 ± 0.66</td>
<td>10.50 ± 0.78</td>
<td>10.62 ± 0.94</td>
<td>0.83</td>
</tr>
<tr>
<td>Chief complaint at enrollment (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal bleed</td>
<td>31.43</td>
<td>76.47</td>
<td>35.00</td>
<td>54.17</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fever</td>
<td>31.73</td>
<td>35.29</td>
<td>30.00</td>
<td>21.74</td>
<td>0.75</td>
</tr>
<tr>
<td>Vaginal discharge</td>
<td>12.62</td>
<td>8.82</td>
<td>5.00</td>
<td>20.83</td>
<td>0.75</td>
</tr>
</tbody>
</table>

**Table II.** Results of ELISA to detect IgM antibodies against AAV-2 in cases and controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>OD/background mean ± SE</th>
<th>OD/background median (range)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 106)</td>
<td>3.80 ± 0.35</td>
<td>2.09 (0.88–15.97)</td>
<td>NA</td>
</tr>
<tr>
<td>Spontaneous abortion (n = 34)</td>
<td>3.95 ± 0.34</td>
<td>3.86 (0.47–7.91)</td>
<td>0.04</td>
</tr>
<tr>
<td>PE/IUGR/IUFD (n = 20)</td>
<td>8.96 ± 1.92</td>
<td>6.28 (0.97–32.58)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SPTD (n = 24)</td>
<td>8.35 ± 0.92</td>
<td>9.03 (1.08–19.92)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

PE/IUGR/SB, composite group of women with pregnancies complicated by pre-eclampsia, intrauterine growth restriction and/or stillbirth; SPTD, spontaneous preterm delivery.

*P-values determined by ANOVA for continuous variables and chi square tests for categorical data.
Three cases and no controls were IgM positive against adenovirus in eight serum samples (five controls, two spontaneous abortion cases). We detected IgM antibodies (serum samples containing higher levels of IgG antibodies when recombinant AAV-2 vectors were incubated with greater levels of IgG antibodies against AAV-2 more effectively inhibited AAV-2 transduction of HeLa cells. Finally, neutralization studies demonstrated that maternal serum samples containing higher levels of anti-AAV-2 IgM antibodies bound with greater affinity to transfected 293 cells expressing AAV-2 capsid proteins. After neutralization studies confirmed that samples with greater levels of IgG antibodies against AAV-2 more effectively inhibited AAV-2 transduction of HeLa cells. When recombinant AAV-2 vectors were incubated with serum samples containing higher levels of IgG antibodies (n = 16, OD/background ≥3.00), 2.28 ± 1.93% of HeLa cells were transduced, but when AAV-2 vectors were incubated with serum samples containing lower levels of IgG antibodies (n = 11, OD/background ≤3.00), 63.55 ± 12.10% of HeLa cells were infected (P = 0.0001).

We also determined the seroprevalence of IgM antibodies against CMV and adenovirus in the entire cohort. We detected IgM antibodies against CMV in only nine serum samples (seven controls, one pre-eclampsia/IUGR/stillbirth and one spontaneous abortion case). We detected IgM antibodies against adenovirus in eight serum samples (five controls, two pre-eclampsia/IUGR/stillbirths and one spontaneous preterm delivery case). The overall seroprevalence of IgM antibodies against CMV and/or adenovirus was 8.2% (15/184 women), and there was no difference in the rate of CMV and/or adenovirus IgM seropositivity when comparing each of the case groups to controls (P-values >0.05, Fisher exact tests). The relationship between IgM antibodies against AAV-2 and adverse obstetric outcomes remained significant after excluding women who had IgM antibodies against CMV and/or adenovirus: pre-eclampsia/IUGR/stillbirth cases (10/18 were anti-AAV-2 IgM seropositive) versus controls (18/95, P = 0.0009) and spontaneous preterm deliveries (14/23 were anti-AAV-2 IgM seropositive) versus controls (P < 0.0001, chi square tests).

### Discussion

In this study, we demonstrated that elevated maternal serum levels of anti-AAV-2 IgM antibodies detected early in pregnancy were associated with adverse pregnancy outcomes, including spontaneous abortion, spontaneous preterm delivery, and a composite of pre-eclampsia/IUGR/stillbirth. From our data, we found that the odds of having elevated IgM antibody levels were between 5- and 7-fold greater among cases of spontaneous preterm delivery and pre-eclampsia/IUGR/stillbirth than among controls. We also showed that IgM antibody levels were significantly higher even among spontaneous abortion cases than among controls (P = 0.04).

One strong point of our study was that all samples were collected prospectively, indicating that maternal AAV-2 infections preceded the adverse obstetric outcomes. Our findings also were bolstered by the numerous experiments we conducted to demonstrate the specificity of the ELISA results. Because IgG antibodies generally are present in serum samples at levels several-fold greater than IgM antibodies, IgG antibodies may yield false positive results when attempting to detect IgM antibodies by ELISA (Martins et al., 1995; Erles et al., 1999). Therefore, we removed IgG antibodies from maternal serum samples before screening for IgM antibodies against AAV-2, and we demonstrated by western blotting that IgG, but not IgM, antibodies were removed by treatment with protein G agarose. In addition, immunofluorescence studies demonstrated that serum samples containing higher levels of anti-AAV-2 IgM antibodies bound with greater affinity to transfected 293 cells expressing AAV-2 capsid proteins. Finally, neutralization studies demonstrated that maternal serum levels of neutralizing (IgG) antibodies were associated with the ability of the samples to neutralize recombinant AAV-2 transduction of permissive cells.

Our study had some limitations. First, the cohort was comprised of inner city pregnant women who presented to our Emergency Department early in pregnancy and were almost exclusively African American, so our findings may not be reproducible in other populations seeking routine prenatal care early in pregnancy. However, the results of serum screening for IgG antibodies against AAV-2 demonstrated a seroprevalence rate in our cohort (82.8%) that was similar to previously published study groups, and seropositivity for IgG antibodies was not associated with adverse obstetric outcomes.
and IgG negative (e.g. primary infection), but women with reactivation of latent AAV-2. Three women were IgM positive whether our findings represent primary maternal infection or was relatively modest. Finally, we were not able to establish between anti-AAV-2 IgM antibodies and spontaneous abortions, which may explain why the association episodic, whereas placental samples are not available until after delivery, by which time AAV-2 may not be detected. Thirdly, we were not able to perform karyotyping after spontaneous abortions, which may explain why the association between anti-AAV-2 IgM antibodies and spontaneous abortions was relatively modest. Finally, we were not able to establish whether our findings represent primary maternal infection or reactivation of latent AAV-2. Three women were IgM positive and IgG negative (e.g. primary infection), but women with episodic, whereas placental samples are not available until after delivery, by which time AAV-2 may not be detected. Thirdly, we were not able to perform karyotyping after spontaneous abortions, which may explain why the association between anti-AAV-2 IgM antibodies and spontaneous abortions was relatively modest. Finally, we were not able to establish whether our findings represent primary maternal infection or reactivation of latent AAV-2. Three women were IgM positive and IgG negative (e.g. primary infection), but women with episodic, whereas placental samples are not available until after delivery, by which time AAV-2 may not be detected. Thirdly, we were not able to perform karyotyping after spontaneous abortions, which may explain why the association between anti-AAV-2 IgM antibodies and spontaneous abortions was relatively modest. Finally, we were not able to establish whether our findings represent primary maternal infection or reactivation of latent AAV-2. Three women were IgM positive and IgG negative (e.g. primary infection), but women with episodic, whereas placental samples are not available until after delivery, by which time AAV-2 may not be detected. Thirdly, we were not able to perform karyotyping after spontaneous abortions, which may explain why the association between anti-AAV-2 IgM antibodies and spontaneous abortions was relatively modest. Finally, we were not able to establish whether our findings represent primary maternal infection or reactivation of latent AAV-2. Three women were IgM positive and IgG negative (e.g. primary infection), but women with episodic, whereas placental samples are not available until after delivery, by which time AAV-2 may not be detected. Thirdly, we were not able to perform karyotyping after spontaneous abortions, which may explain why the association between anti-AAV-2 IgM antibodies and spontaneous abortions was relatively modest. Finally, we were not able to establish whether our findings represent primary maternal infection or reactivation of latent AAV-2. Three women were IgM positive and IgG negative (e.g. primary infection), but women with.

Table III. Rates of seropositivity of IgM antibodies against AAV-2 among cases and controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>Percent seropositive</th>
<th>P-Value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 106)</td>
<td>17.9</td>
<td>NA</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Spontaneous abortion (n = 34)</td>
<td>20.6</td>
<td>0.73</td>
<td>1.19 (0.45, 3.13)</td>
</tr>
<tr>
<td>PE/IUGR/SB (n = 20)</td>
<td>55.0</td>
<td>0.0004</td>
<td>5.60 (2.04, 15.38)</td>
</tr>
<tr>
<td>SPTD (n = 24)</td>
<td>62.5</td>
<td>&lt;0.0001</td>
<td>7.63 (2.91, 20.01)</td>
</tr>
</tbody>
</table>

PE/IUGR/SB, composite group of women with pregnancies complicated by pre-eclampsia, intrauterine growth restriction and/or stillbirth; SPTD, spontaneous preterm delivery.

Seropositivity defined by ROC curve analysis as OD value of each sample/OD value of background signal ≥6.00.

Chi square tests, comparing rates of seropositivity for each group of cases with controls.

Figure 2: Western blots demonstrating the distribution of IgM and IgG antibodies in maternal serum samples treated with protein G agarose (to remove IgG antibodies)

(A) IgM antibodies detected by band at 900 kD; Sr, serum from two patients (1 and 2) before extraction of IgG antibodies; Sp, supernatants after treatment with protein G agarose and centrifugation, demonstrating the absence of IgG antibodies in samples before screening for anti-AAV-2 antibodies; P, pellets after treatment with protein G agarose and centrifugation, demonstrating that IgG antibodies were not removed from samples before screening for anti-AAV-2 antibodies. (B) IgG antibodies detected by band at 150 kD; P, pellets from two patients (1 and 2) after treatment with protein G agarose and centrifugation, demonstrating that IgG antibodies were removed from samples; Sp, supernatants after treatment with protein G agarose and centrifugation, demonstrating the absence of IgG antibodies in samples before screening for anti-AAV-2 antibodies

(Figure 3: Immunofluorescence studies

Human embryonic kidney (293T) cells were transfected with a plasmid (p600) expressing the AAV-2 capsid proteins VP1, VP2 and VP3 and incubated with maternal serum samples for 12 h. Secondary fluorescent-labeled anti-human IgM antibodies were added and fluorescent-labeled cells were visualized microscopically. (A) Cells incubated with serum from a patient with borderline anti-AAV-2 IgM levels (OD/background between 3 and 6). (B) Cells incubated with serum from a patient with elevated anti-AAV-2 IgM levels (OD/background >6). (C) Negative control, cells incubated with secondary antibody only. (D) Cells incubated with serum from a patient with negative anti-AAV-2 IgM levels (OD/background ≤3)

positive IgM and IgG titers may have had primary infection or reactivation of AAV-2, both of which may be embryotoxic and cannot be distinguished by serial antibody titers (Erles et al., 1999).

We believe that our findings provide the first evidence that maternal AAV-2 infections early in pregnancy are related to adverse reproductive outcomes, and these findings add validity to previous studies describing AAV-2 infections during pregnancy. For example, AAV-2 DNA was detected in placental cells in 12/30 cases of spontaneous abortion and 28/49 hydatidiform moles, but comparison groups (i.e. placental samples from elective abortions) were not presented in these studies (Tobiasch et al., 1994; Kiehl et al., 2002). In our laboratory, we found that AAV-2 DNA was found more frequently in trophoblast cells from cases of severe pre-eclampsia (22/40) than from normal term deliveries (5/27, P = 0.002) (Arechaveleta-Velasco et al., 2006). In another study with a limited sample size, IgM antibodies against AAV-2 were detected in 7/24 maternal serum samples from cases of spontaneous abortion
compared with 6/61 controls (P = 0.03), but many of the controls were not pregnant (Tobiasch et al., 1994). Conversely, Friedman-Einat et al. (1997) detected AAV-2 DNA in 11/61 genital swab specimens from women suspected of having herpes simplex virus infections, but they were unable to detect AAV-2 DNA sequences in 38 placental samples from spontaneous abortions. Finally, AAV-2 DNA was detected in 7/32 amniotic fluid samples following amniocentesis for various indications between 15 and 19 weeks’ gestation, and the presence of AAV-2 DNA was associated with an increased risk of PPROM and preterm labor (AAV-2 DNA in 5/7 samples) occurring later in pregnancy (Burguete et al., 1999).

Unlike pre-eclampsia and fetal growth restriction, spontaneous preterm delivery traditionally has not been related to placental dysfunction. However, evidence has accumulated in the past several years that associates placental dysfunction with spontaneous preterm delivery. One group of investigators found that women who delivered preterm after idiopathic preterm labor had higher rates of placental ischemia than controls and proposed that impaired placentation led to ischemia (Germain et al., 1999). Another group observed that abnormal placentation (defined as failure of physiological transformation of maternal spiral arteries resulting in reduced blood flow to the placental intervillous space) was more common in patients with spontaneous preterm labor and preterm premature rupture of the fetal membranes than in controls who delivered at term (Kim et al., 2002, 2003). Most recently, a large multicenter collaboration of investigators demonstrated that decreased first trimester maternal serum levels of pregnancy associated plasma protein A, which is a protease produced by trophoblast cells, were associated with a significantly increased risk of preterm premature rupture of membranes and preterm delivery (Dugoff et al., 2004). These findings support the hypothesis that spontaneous preterm delivery, at least in part, has its origins in abnormal placental function at the beginning of pregnancy. Finally, as stated above, AAV-2 DNA was detected more frequently in amniocytes from women who later experienced preterm premature rupture of membranes than in those from controls (Burguete et al., 1999). Thus, we believe that it is biologically plausible that viral infection and/or the immune response to viral infections early in pregnancy might be directly related to an increased incidence of spontaneous preterm delivery.

We hypothesize that AAV-2 infection of invasive trophoblast cells at the maternal–fetal interface induces pathologic changes that interfere with placental invasion into the uterine wall, resulting in reduced placental perfusion, placental dysfunctions, and adverse obstetric outcomes. Because productive AAV-2 infections usually require co-infection with helper viruses, we decided to determine if maternal co-infection with CMV or adenovirus influenced the association between AAV-2 infection and adverse outcomes. After excluding subjects who had IgM antibodies against CMV and/or adenovirus, we found that cases of pre-eclampsia/IUGR/stillbirth and spontaneous preterm delivery remained significantly more likely to be anti-AAV-2 IgM seropositive than were controls. These results are consistent with our previous observation that placental trophoblast cells are uniquely susceptible to AAV-2 in the presence or absence of helper virus co-infection (Arechavala-Velasco et al., 2006; Parry et al., 1998). Thus, the placenta may be exposed to AAV-2 during maternal infections early in gestation, and AAV-2 may induce pathologic changes in invasive trophoblast cells in the presence or absence of other viral co-infections.

In conclusion, we have shown that pre-eclampsia/IUGR/stillbirth and spontaneous preterm delivery are associated with a 5- to 7-fold increased rate of positive screening for IgM antibodies against AAV-2 in maternal serum early in pregnancy. Our findings should be corroborated in other populations, and the relative importance of primary and/or reactivation infection of placental cells should be determined. Future studies may provide additional insights into the role of AAV-2 and other viral infections in the pathogenesis of placental dysfunction and may inspire the development of novel strategies to reduce the frequency of associated complications.

Funding
This research was supported by the NIH grant HD4214100 (S.P.).

References


