Pathogenesis of in utero Infection in Porcine Fetuses with Porcine Reproductive and Respiratory Syndrome Virus

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ABSTRACT

Porcine fetuses were exposed in utero to porcine reproductive and respiratory syndrome virus (PRRSV) at stages of gestation ranging from 34 to 85 days and examined 17 to 31 days later to determine the effect of gestational age on fetal susceptibility. For each of the 8 litters tested during the study, all of the fetuses of 1 horn of the uterus were exposed to virus by intraamniotic injection; those of the other horn were exposed similarly to a sham inoculum that consisted of sterile cell culture medium. Viral infectivity titers associated with fetal tissues collected at necropsy indicated that, regardless of gestational age, the virus had replicated in fetuses exposed intraamniotically. In addition, virus had also spread and replicated in sham-inoculated littermates in 3 litters. On the basis of these findings it appears that there may be little or no temporal difference in fetal susceptibility to infection with PRRSV. If so, the lack of early fetal death as a commonly recognized feature of naturally occurring cases of PRRS may be due to a greater resistance of early gestational fetuses to the lethal effects of PRRSV, as suggested by this study, and/or a greater likelihood of transplacental infection during late gestation.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is a new disease of swine. The predominant clinical signs of a PRRS epizootic include a reproductive component characterized by an increase of late-term abortions and early farrowings with mummified fetuses, stillborn and weak piglets, late returns to estrus, and repeat breeders; the respiratory component has been described as an acute onset of a “flu-like” respiratory infection with a high morbidity for all ages of swine (1,2). The etiologic agent has been identified as a virus, known as the PRRS virus (PRRSV). It is classified as a member of the proposed virus family Arteriviridae, whose members include equine arteritis virus, lactate dehydrogenase elevating virus of mice, and simian hemorrhagic fever virus, all of which share similar morphology, genomic organization, and tissue tropism with the PRRSV (3,4).

Under experimental conditions, exposure of pregnant swine to PRRSV during the last trimester of gestation has typically resulted in transplacental infection followed by reproductive failure (5–7). These events appear to be much less common, however, when females are exposed to PRRSV earlier in gestation (8,9). As a result, it has been suggested that the consequences of infection of pregnant females with PRRSV may reflect a relative placental permeability to the virus during early versus late gestation, or an age difference in the ability of the fetus to support virus replication following transplacental exposure. In the study reported here we investigated the latter by exposing fetuses of different gestational ages to PRRSV by direct intraamniotic injection and then measuring the extent of virus replication at various times postexposure.

MATERIALS AND METHODS

VIRUS

The PRRSV isolate utilized in this study has been previously described as the swine infertility and respiratory syndrome virus (SIRSV) or ATCC-VR 2332 (10). It was propagated in CL2621 cells (Proprietary cell line CL2621, Boehringer Ingelheim Animal
Health Inc., St. Joseph, Missouri) maintained with minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 50 μg/mL of gentamicin. Viral inoculum was prepared by adsorption of virus to confluent monolayers of CL2621 cells for 1 h. Infected monolayers were incubated at 37°C in maintenance medium consisting of serum-free MEM and cell cultures were observed daily for cytopathic effect (CPE) until approximately 75% of cells degenerated. For harvesting, cell monolayers were submitted to 2 freeze–thaw cycles. Cell culture fluid was clarified at 1000 × g for 30 min and supernatant was filtered through a 0.45 micrometer pore-size membrane. Viral infectivity was determined in a 50% infective dose method as previously described (11). Infectivity titers were approximately 10^4 TCID50/mL. Virus (9th cell culture passage) was aliquoted and frozen at −80°C.

ANIMALS

Eight pregnant gilts with known breeding dates were purchased in groups of 2 or 3 from 2 commercial herds deemed free of PRRSV infection based upon herd history and serologic tests (Table 1). Fetuses were exposed by intraamniotic injection to either PRRSV (principals) or virus-free cell culture medium (controls) as previously described (12). The animal studies were conducted in accordance with the guidelines of the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

EXPERIMENTAL DESIGN

Each uterus was surgically exposed and fetuses were individually palpated and observed to distinguish live from dead. Principal fetuses were exposed by intraamniotic injection to a high or low dose of PRRSV in an alternating sequence. Each injection site was identified by a suture placed in the serosal surface of the uterus, thus marking the relative position of the fetus.

Group 1 consisted of 3 gilts (1, 2, and 3) that underwent surgery at 34, 65, or 45 d of gestation (DG), respectively (Table 1). All fetuses in 1 horn of each uterus were exposed to an intraamniotic injection of a 1 mL sham inoculum, i.e., the control fetuses. A 1 mL virus inoculum containing either 10^4 or 10^5 TCID50/mL was injected into the amniotic fluids of the principal fetuses in the contralateral uterine horns. The gilts were euthanized 21 d postsurgery (PS) and their uteri recovered and fetuses examined for the presence of virus.

Group 2 consisted of 3 gilts (4, 5, and 6) that underwent surgery at 49 DG. The virus inoculum for the 3 litters was prepared from the serum of P-4, a principal fetus from the litter of gilt 3, in the following manner. A 0.5 mL aliquot of the fetal serum containing about 10^8 TCID50/mL PRRSV was inoculated onto a monolayer of CL2621 and allowed to adsorb for 1 h. Following a media change, the cell culture was observed for CPE and virus was harvested as previously described. The virus inoculum had been passed 9 times in vitro, once through a porcine fetus and once again through CL2621. Its titer was 10^4 TCID50/mL. Principal fetuses were exposed to 1.0 mL of either the undiluted virus suspension or a 10-fold dilution of virus. Gilts were euthanized on 17, 19, and 21 d PS and fetuses examined for the presence of virus.

Group 3 consisted of 2 gilts (7 and 8) that underwent surgery at 34 DG and fetuses were inoculated in a similar fashion as in group 1. The virus inoculum consisted of a 10th passage of PRRSV diluted to concentrations of either 10^4 or 10^5 TCID50/mL. Principal fetuses received a 0.2 mL inoculum of either preparation. Control fetuses received a 0.2 mL sham inoculum of virus-free cell culture medium only. One gilt was euthanized 21 d PS and the 2nd gilt at 31 d PS.

NECROPSY PROCEDURES

Blood samples were collected from the gilts prior to surgery and at the time of euthanasia, which was effected by an intravenous overdose of pentobarbital. Each fetus was examined and crown–rump length (CR) determined for an estimation of fetal age (13). Fetuses were categorized as alive or dead based on detection of a heartbeat or obvious postmortem autolysis or mummification. Samples of lung, liver, heart, kidney, and spleen from each fetus were collected. Fetal fluids were collected from the umbilical cord of live fetuses or the thorax of dead fetuses.

VIRUS ISOLATION

Virus isolation was attempted on fetal fluids and tissues. A 0.1 mL aliquot of serum or thoracic fluid was added to 48- to 72-h-old monolayers of CL2621 cells on glass coverslips in Leighton tubes. The coverslips were fixed approximately 20 h later in 24:1 acetone/ethanol solution and observed for viral antigen by indirect fluorescent antibody (IFA) techniques. The primary seroconverting pig experimentally infected with ATCC-VR 2332 and the secondary serum was a goat serum containing an anti-swine IgG fluorescein isothiocyanate-labeled antibody (Dr. PS Paul, College of Veterinary Medicine, Iowa State University, Ames, Iowa). Pooled tissues of each fetus were ground with sterile sea sand in mortar with pestle and serum-free MEM added to produce an approximate 20% (w/v) solution. The suspension was clarified at 1000 × g for 30 min at 4°C. The supernatant was collected and 0.1 mL added to a 48- to 72-h-old monolayer of CL2621 cells in a 24-well tissue culture plate. The medium was changed 12 to 18 h later and the monolayers observed daily for CPE for 8 d. Fetuses were considered to be infected if virus was isolated from either their tissues or fluids or both.

SEROLOGY

All serum samples and fetal fluids collected from fetuses older than 60 d were heat inactivated at 56°C for 30 min prior to use. Virus neutralization (VN) tests were conducted as previously described (8). Glass coverslips containing PRRSV-infected monolayers of CL2621 cells were used for testing serum and fetal fluids for IFA antibody as described above.

STATISTICAL ANALYSIS

Differences in the incidence of abnormal fetuses and mortality rate between fetuses exposed to virus during the 1st or 2nd half of gestation were analyzed by Fisher’s exact test (14). Differences in the CR between live infected fetuses and live noninfected fetuses were analyzed with t-tests (15).

RESULTS

All gilts seroconverted to PRRSV following inoculation of their fetuses.
Table I. Virus neutralizing (VN) and indirect fluorescent antibody (IFA) tests on sera collected at the time of gilt surgery and euthanasia

<table>
<thead>
<tr>
<th>Gilt</th>
<th>Challenge*</th>
<th>Terminationb</th>
<th>VNc</th>
<th>IFAd</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Presurgery</td>
<td>Termination</td>
</tr>
<tr>
<td>1</td>
<td>85</td>
<td>21</td>
<td>&lt;4</td>
<td>56</td>
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<tr>
<td>2</td>
<td>65</td>
<td>21</td>
<td>&lt;4</td>
<td>111</td>
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<tr>
<td>3</td>
<td>45</td>
<td>21</td>
<td>&lt;4</td>
<td>28</td>
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<td>4</td>
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<td>&lt;4</td>
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<td>8</td>
<td>34</td>
<td>31</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

* Day of gestation when fetuses were exposed to virus by surgical methods
b Days postsurgery when gilt was euthanized
c VN titer expressed as the reciprocal of highest serum dilution neutralizing CPE induced by 100 TCID<sub>50</sub> of virus (mean titer from 5 tests)
d Reciprocal of the highest serum dilution given that permitted visualization of fluorescent foci in infected cell-monolayers

Table II. Condition* of porcine fetuses in gilts (litters) 1 to 8 at the time of necropsy

<table>
<thead>
<tr>
<th>Litter</th>
<th>Infected uterine horn</th>
<th>Control uterine horn</th>
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<tbody>
<tr>
<td></td>
<td>C-1</td>
<td>C-2</td>
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<tr>
<td>1</td>
<td></td>
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<tr>
<td>2</td>
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<td>8</td>
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</table>

* Fetuses were alive and normal at the time of necropsy of their dam unless noted otherwise
P = Principal fetus exposed to virus by experimental methods
C = Control fetus exposed to virus-free inoculum by experimental methods
+ = Positive virus isolation
- = Negative virus isolation
D = Fetus that had recently died
M = Fetus that had died and was mummifying
A = Fetus that was alive but abnormal in appearance
... = No fetus

Antibody was not detected by IFA in the fetal fluids examined. At the time of surgery, no obvious fetal pathology was noted in any of the litters. All gilts recovered uneventfully from surgery and were normal throughout the study. Fetal position was determined in relationship to the suture placed at the transuterine injection site during surgery. Fetuses were identified as control or principal and by their position in the uterine horn in relationship to the uterine body, e.g., the fetus located next to the body in the control uterine horn would be identified as C-1.

Group 1 consisted of gilts 1, 2, and 3 euthanized 21 d PS (Table II). In litter 1, 3 of the 5 principal fetuses were alive. Of the 2 dead fetuses, 1 had died recently (P-4) and 1 was undergoing mummification. Virus was recovered from all 3 live fetuses and from only 1 of the dead fetuses (P-4). All control fetuses were alive and no virus was detected. In litter 2, all of the principal fetuses had died and were mummified; no virus was detected in the principal fetuses. Four of 6 control fetuses were alive, virus was recovered from 1 live fetus (C-3) but not the 2 dead fetuses. In litter 3, all fetuses were alive, although principal fetuses were edematous and hemorrhagic with friable internal organs. Virus was recovered only from 1 of 3 principal fetuses. Group 2 gilts 4, 5, and 6 were euthanized 17, 19, and 21 d PS, respectively. In litter 4, 5 of 7 principal fetuses and 5 of 8 control fetuses were alive. Virus was recovered from all live principal fetuses but not the dead principal fetuses. Virus was recovered from 2 live control fetuses, C-1 and C-2, but not from the remaining live or dead control fetuses. In litter 5, 5 of 6 principal fetuses were alive.
Virus was recovered from the live principal fetuses, but not the dead one. Virus was not detected in the 3 live fetuses or 1 small (CR = 7.0 cm), mummified fetus in the control uterine horn. Litter 6 resembled litter 3 in that all principal fetuses were alive even though they were grossly affected and virus was recovered from each one. No virus was detected in the control fetuses that were alive and normal in appearance.

Group 3 consisted of gilts 7 and 8, which were euthanized 21 and 31 d PS, respectively. At the time of surgery, 7 principal and 5 control fetuses were thought to be present in the uterus of gilt 7. At necropsy, 8 live fetuses were in the principal uterine horn and 2 live fetuses (C-1 and C-4) and remnants of what were thought to be fetuses C-2 and C-3 were identified in the control horn. A principal fetus was located in the body of the uterus and was not identified by suture. It was misidentified at the time of surgery as a control, and is now identified as P-1, a sham inoculated fetus in the principal horn. Virus was recovered from principal fetuses P-1 through P-7. Virus was not recovered from fetuses P-8 or the control fetuses. At the time of surgery, 6 principal and 8 control fetuses were thought to be present in the uterus of gilt 8. When the gilt was euthanized, 4 live fetuses, 1 mummified fetus, and the remnants of a 6th fetus were identified in the principal uterine horn. Virus was detected only in the 4 live principal fetuses. Seven control fetuses were identified in the control horn, 2 of which had recently died (C-3 and C-7) and were undergoing mummification. No virus was detected in these fetuses and no physical evidence was found for the 8th control fetus thought to be present at the time of surgery.

Of the 39 fetuses positive for virus, virus was isolated from the serum of all 39, whereas virus was isolated from tissue homogenates of only 35. The serum-virus titers ranged from $10^{2.3}$ to $10^{7.0}$ TCID$_{50}$/mL in a fetus (P-1, litter 7) suspected of being recently infected by intrauterine spread of virus to $10^{7.0}$ TCID$_{50}$/mL in a fetus (P-4, litter 7) that had been exposed to virus for 21 d. Principal fetuses positive for virus were considered infected by their original intraamniotic inoculation, titers of which ranged from $10^{2.3}$ to $10^{7.0}$ TCID$_{50}$/mL. No discernable differences in fetal susceptibility or mortality were noted between low and high titered inoculums. A significant difference ($P < 0.05$) was observed in mortality rates between principal fetuses exposed to virus in the 1st (litters 3 to 8) and 2nd (litters 1 and 2) half of gestation; however, no difference ($P = 0.08$) was found in the incidence of abnormal fetuses (hemorrhagic and edematous live fetuses or dead fetuses) between the 1st and 2nd half of gestation. Although the mean CR of live infected fetuses was less than that of live noninfected littermates in litters 1 to 7, no statistical difference was found. In litter 8, the CR mean was the same for live infected and live noninfected fetuses.

**DISCUSSION**

In this study porcine fetuses from early through late gestation were susceptible to a direct inoculation of PRRSV; however, an age-related difference in the onset of mortality following inoculation may exist between the 1st and 2nd half of gestation. Fetuses inoculated during the 1st half of gestation could replicate virus up to 31 d without severe, gross pathologic consequences. However, inoculation during the 2nd half of gestation may result in death within days of exposure. In this method of fetal inoculation, we speculate that fetuses ingest the virus along with amniotic fluids and a viral infection is established. This route of infection is unnatural; nevertheless, it demonstrates that fetuses can support virus replication from 34 through 106 DG and that fetus-fetus and fetus-dam-fetus transmission may occur.

The clinical signs of late gestation reproductive failure have been reproduced with the North American prototype PRRSV isolate (SIRSV or ATCC-VR 2332). Transplacental infection did occur within days in all susceptible sows following oronasal exposure to virus at 93 DG (6). Transplacental infection did not occur by 7, 14, or 21 d in susceptible sows following oronasal exposure to PRRSV at or near 45 d of gestation; however, transplacental infection did occur in 1 litter within 65 d postexposure as evidenced by isolation of virus from 2 piglets at birth (9). In an analogous study, pregnant gilts were exposed to PRRSV by intravenous inoculation at or near 30, 50, 70, and 90 DG (8). Transplacental infection did not occur following inoculation of pregnant swine at 30 DG and only limited evidence was found for transmission following virus challenge at 50 and 70 DG while inoculation of gilts at 90 DG resulted in the typical signs of PRRS late-term reproductive failure.

In a fetal susceptibility study, fetuses were exposed to PRRSV by intramuscular or intraamniotic exposure at or near 45 DG and when examined 4 and 11 d postinoculation the fetuses were alive, replicating virus, and normal in appearance (9). The PRRSV studies described here suggest that this isolate does not readily cause transplacental infections early in gestation, although fetuses apparently are susceptible to the virus at this time.

We completed a pilot study to determine the effects of direct inoculation of amniotic fluid with PRRSV in fetuses at 45, 65, or 85 DG (16). The gilts were euthanized 7 d later and all fetuses inoculated with virus were alive, normal in appearance, and infected with virus. The in utero infection study was repeated with the fetuses of group 1 being examined 21 d postexposure in an attempt to cause fetal death with the virulent virus. Gross pathology was evident in each litter, and 1 of 10 principal and 2 of 12 control fetuses had died in the litters exposed to virus during the 2nd half of gestation, at 65 and 85 DG (Table II). All principal fetuses in litter 2 were dead and mummified when examined at 86 DG, and based on condition and CR length, they probably died 1 to 2 wk prior to examination. In this litter, intrauterine spread of virus from principal to control uterine horn was presumed because a live control fetus (C-3) was positive for virus and 2 control fetuses nearest the uterine body had recently died. The principal fetuses infected during the 1st half of gestation in litter 3 were alive when examined at 66 DG although severely affected. The results of these litters suggested that a fetus may undergo a developmental change around 65 to 70 DG, at which time it changes from being able to support virus replication without apparent
gross pathology to an increased susceptibility and subsequent fetal death.

The absence of spontaneous abortions in this study following direct inoculation of amniotic fluids could be due to a variety of mechanisms, one of which could be the attenuation of virus by repeated passages in cell culture. The inoculum for litters 4, 5, and 6 of group 2 was prepared from the serum of fetus P-4, litter 3, a fetus that had a viremia of $10^6$ TCID$_{50}$/mL. This inoculum was administered to fetuses in group 2 to test for an increase of virulence by in vivo passage. In addition, the fetuses in group 2 were exposed to virus at 49 DG and recovered 17 to 21 d later to test the observation that fetuses infected earlier in gestation could replicate the virus until about 65 to 70 DG, at which time they would begin to succumb to the viral infection. Results from group 2 indicated that fetuses could replicate the virus to a very high serum titer and maintain life until they reached the projected stage of susceptibility, at which time some of them had recently died and death appeared imminent for others. All principal fetuses were alive in litter 6 (necropsied at 70 DG) and replicating virus even though each one was very hemorrhagic and edematous with swollen, friable internal organs; this litter closely resembled litter 3 in size and appearance. We believe the virulence of the PRRSV isolate was unaffected by the single in vivo propagation and subsequent in vitro propagation of inoculum for group 2 and the additional in vitro propagation of virus for group 3 had no discernable effect.

Initially, the uterus of each gilt was flaccid when first exposed surgically; but following physical examination and manipulation, it would become firm. This phenomena made the identification and exact location of fetuses difficult to determine in the gravid uterus of group 3. In litter 7 at necropsy, the apparent remnants of 2 fetuses were identified in the control horn. The cause of death for these fetuses is unknown; but due to the scant amount of tissues found, the fetuses are thought to have died around the time of surgery, implying that PRRSV probably was not involved. In this litter all principal fetuses were alive and normal in appearance including fetus P-1, the principal fetus that was mistakenly treated like a control. Virus was recovered from the serum of P-1 but not from its tissues, which was uncharacteristic for fetuses infected with virus for 21 d. The recovery of virus from the serum only and the low titer suggest this fetus may have been recently infected or had supported virus replication at a low rate. Virus was not recovered from 1 principal fetus, P-8, that was thought to have been inoculated at surgery. This fetus may not have been exposed to the inoculum or the concentration of virus inoculum ($10^{2.3}$ TCID$_{50}$) may have been near its threshold of infectivity. Since infected fetuses in litter 7 appeared healthy 21 d postexposure, gilt 8 was not euthanized until day 65 of gestation, or 31 d following exposure to virus.

In litter 8, 2 control fetuses had recently died. Virus was not recovered from them and their cause of death is unknown. It is possible that the fetuses were infected by a natural transuterine route since the dam was apparently exposed to virus, based on her seroconversion to PRRSV. In this study all gilts seroconverted to PRRSV following the in utero exposure of their fetuses and they were clinically normal throughout the study. The dams’ exposure to virus may have been by contamination of maternal tissues or blood at the time of transuterine injection and/or their fetuses replicated the PRRSV and served as the source of infection. Virus was isolated from control fetuses in litters 2 and 4, indicating the fetuses had probably become infected by intrauterine spread of virus (Table II). Recent control fetus deaths had also occurred in these litters as well as in litter 8. These deaths may be the result of a recent PRRSV infection. Based on uterine position, the control fetus deaths in litter 8 and possibly litter 4 could have occurred following a transuterine infection and not from intrauterine migration. This scenario suggests a low frequency of transuterine infections during the 1st half of gestation following infection of the dam.

The inability to recover virus from the 2 dead control fetuses in litter 8 is not surprising, since PRRSV is apparently labile in autolytic tissues based upon positive isolation results: 33/34 (97%) live principal fetuses and only 1/12 (8%) dead principal fetuses. All of these fetuses were thought to have been exposed to the virus at the time of surgery. The single virus isolation from a dead fetus was from the thoracic fluids of a fetus that had recently died with minimal autolysis. One principal fetus, P-1 from litter 7, was not included in the above comparison because it was not exposed to virus experimentally but became infected later in gestation.

Although the actual mechanism is unknown, pathogenic viruses are thought to cross the maternal–fetal junction as free virus and/or as virus associated with a maternal cell that migrates through the barrier. Once on the fetal side, the virus could infect placental tissues or enter the vasculature and/or the amnion and thus the fetus. The amniotic route may have a low probability, but we believe the fetus can become infected by this route as demonstrated in this study. It is not known if a difference exists between young and old fetuses in the mechanics of ingesting amniotic fluid and what influence it may have in the mortality reported in this study. We suggest that any potential difference would be inconsequential in establishing a lethal fetal infection.

Several possibilities may exist for the putative lack of clinical signs associated with PRRSV infection during the 1st half of gestation: 1) PRRSV-induced reproductive failure occurs at all stages of gestation and the signs observed during the 1st half of gestation are not equally reported; 2) differences in PRRSV virulence may exist between geographic regions; 3) transplacental infection of fetuses can occur at any time of gestation but fetuses do not succumb to viral infection until the 2nd half of gestation; or 4) transplacental infection of fetuses does not occur until the 2nd half of gestation.

Perhaps a unique pathogenesis exists for natural PRRSV infections in which susceptible fetuses are not readily infected during early gestation. Further investigation is warranted to test this hypothesis because of its potential impact on managing a breeding herd during a PRRS epizootic. Studies could be designed to evaluate the efficacy of immunizing susceptible sows with live virus or
feedback of contaminated tissues prior to breeding or during early gestation. This immunization could protect sows in the herd from the reproductive failure that frequently, if not always, occurs following late gestation exposure.

ACKNOWLEDGMENTS

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REFERENCES