Teratogenicity of Staphylococcus aureus L-forms using a mouse whole-embryo culture model

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INTRODUCTION

Staphylococcus aureus, a common and major human pathogen, is carried by 25–35 % of the population as resident bacterial flora, and can cause severe infection in pregnant women and their fetuses and in infants in the perinatal period (Dancer & Noble, 1991; Pinter et al., 2009). More recently, S. aureus has been reported as an emerging problem among pregnant women (Andrews et al., 2008; Méndez et al., 2011) due to its colonization of sites such as the genital tract, breast, buttocks, vulva and groin (Bourgeois-Nicolaos et al., 2010; Chen et al., 2007; Laibl et al., 2005). Such colonization among pregnant women facilitates the vertical transmission of S. aureus from a colonized mother to her infant. Such vertical transmission also has been reported among post-partum women (Saiman et al., 2003). Although several lines of study have reported that S. aureus colonization among third trimester pregnant women and post-partum women places the neonate or infant at increased risk for vertically transmitted infection, little is known about the influence of such S. aureus colonization on the fetus in the uterus during the first trimester of pregnancy.

It is known that wild-type S. aureus is not able to pass through the placental barrier. However, a S. aureus variant strain, a cell-wall-deficient bacterium named S. aureus L-form, that can pass through the placental barrier has been shown to emerge spontaneously in vivo (Michailova et al., 2000; Mattman, 2001) under certain conditions unfavourable to the organisms. Some clinical and experimental data have indicated that these bacterial L-forms frequently contribute to longevity and persistence in vivo (Acar & Sabath, 1978; Watanakunakorn, 1979) and are associated with atypical, chronic or latent infection (Domingue & Woody, 1997; Mattman, 2001). In contrast to the wild-type strain, S. aureus L-forms have a number of characteristics such as small size and a plastic shape (Mattman et al., 1961; Mattman, 2001) that contribute to their ability to pass through the placental barrier from the maternal side to the fetus and to cause vertical fetal infection similar to what occurs in viral infections. Our previous studies (Xia et al., 1999; Xu et al., 2007) have suggested that S. aureus L-forms are able to pass through the placental barrier in this way and affect fetal growth and development, but little is known about the direct influence of S. aureus L-forms on...
embryos during the critical period of organogenesis. To investigate this influence, a mouse whole-embryo culture system was chosen as the experimental approach because of the necessity of eliminating maternal influences (e.g. maternal toxicity or metabolism) in the study design, whilst retaining the structural and functional integrity of the embryo (New, 1978). This system allows whole embryos to grow and develop in vitro to approximately the same extent as they do in vivo (Tam, 1998).

**METHODS**

**S. aureus L-forms.** *S. aureus* Cowan I strain was purchased from the National Institutes for Food and Drug Control (Beijing, China) and induced into *S. aureus* L-forms as described previously (Mattman et al., 1961; Chatterjee et al., 1967). *S. aureus* L-forms were prepared at a concentration of 1×10^4 c.f.u. ml^-1.

**Animals.** BALB/c mice were purchased from the Anhui Provincial Center for Medical Experimental Animals (Anhui, China). Mice were housed under controlled conditions of temperature (25 °C), humidity (40–60 %) and light (12/12 h light/dark cycle). Rodent chow and deionized water were available to the animals. The maternal connective tissue and myometrium were removed, the decidua was removed along with the parietal yolk sac and the Reichert’s membrane was opened, leaving the conceptus intact. Up to three embryos were cultured in 30 ml serum bottles with a Teflon stopper containing 1 ml serum per embryo. Embryos from a given litter were cultured in the same serum bottle. The maternal yolk sac was removed and the conceptus was kept in the bottle for 48 h. After 48 h of culture, at the equivalent of GD 10.5, the embryos were examined for viability, growth and development by a researcher who was not aware of the study group assignment. Mean yolk sac diameter (YSD), crown–rump length (CRL) and head length (HL) were measured, and dry embryo weight (EW) was determined with an electronic balance after the mouse embryos had been dried for 24 h in an oven at 80 °C. Embryonic morphology was studied according to a standard morphological scoring system (Van Maele-Fabry et al., 1990), which gives a numerical score (0–5) to each of 17 morphological features based on their stage of development, with higher scores for more-developed fetuses. The score of each feature was added to get the total morphological score for the 17 features. Embryos with a total morphological score of <2 were most probably damaged as a result of explantation and were excluded from the analysis.

**Histology.** To evaluate whether *S. aureus* L-forms could go through the barrier of the visceral yolk sac from the medium to the embryo, Gram-staining and immunohistochemistry were used to determine the presence of *S. aureus* L-forms in embryonic tissues. For culture for 48 h in the presence or absence of 400 c.f.u. *S. aureus* L-forms ml^-1, at least five conceptuses were removed from the assay bottles, rinsed three times with 1 mM PBS at room temperature to eliminate the effect of the remnant *S. aureus* L-forms from the medium and transferred individually into 8 cm culture dishes. The visceral yolk sac surrounding the embryo was cut slightly and the embryo exteriorized. These mouse embryos were then fixed in 3% paraformaldehyde, dehydrated in increasing concentrations of alcohol, cleared in xylene, embedded in paraffin and serially sectioned (8 μm sections). For the bacteriological determination of *S. aureus* L-forms, the embryonic tissue sections were stained using the method of Brown & Hoppes (1973), a technique for Gram-staining in paraffin-embedded tissues. For the antigenic detection of *S. aureus* L-forms, immunohistochemistry was carried out using a Vectastain Elite ABC kit (Vector Laboratories) according to the instructions provided by the manufacturer. Briefly, the embryonic tissue sections were dehydrated and hydrated through successive dilutions of ethanol into PBS. Following this procedure, endogenous peroxidases were quenched using 0.3% H2O2 in methanol solution (30 min) and the sections were blocked with normal goat serum for 20 min to prevent nonspecific binding. The sections were incubated with a primary rabbit anti-L-form antibody (prepared by our institute) for 1 h at room temperature, followed by 30 min each with secondary goat anti-rabbit antibody conjugated with biotin and avidin–biotin complex reagent. After incubation with each antibody, a single PBS rinse was performed for 5 min. The chromogen Nova Red was used to localize the antigen of *S. aureus* L-forms. The sections were counterstained with haematoxylin, dehydrated with ethanol, cleared in xylene and mounted in mounting medium.

**Statistical analysis.** All results, except for the percentages of normality, are expressed as means±SD. To assess the significance of differences within experiments, Turkey’s b test in a one-way analysis of variance was used. Statistical significance was defined as *P*<0.05.

**RESULTS**

**Teratogenicity of S. aureus L-forms**

At 48 h after culture of the whole mouse embryos with *S. aureus* L-forms at different concentrations, the level of teratogenicity was determined. With increasing concentrations of *S. aureus* L-forms, fewer embryos developed normally and more embryos were shown to have abnormalities or...
Influence of *S. aureus* L-forms on the morphological development of cultured whole embryos

The difference in development of the embryos was measured at 48 h after explantation. Table 1 shows that both total morphological score and the number of somites of mouse embryos exposed to 100, 200 or 400 c.f.u. *S. aureus* L-forms ml\(^{-1}\) were significantly lower than those of the control group, but this was not true of the embryos exposed to 50 c.f.u. *S. aureus* L-forms ml\(^{-1}\). Fig. 3 shows that HL and CRL in the embryos infected with 50 c.f.u. *S. aureus* L-forms ml\(^{-1}\) were shorter than those of the control group \((P<0.01)\). In the groups exposed to 100, 200 or 400 c.f.u. *S. aureus* L-forms ml\(^{-1}\), YSD, HL and CRL (Fig. 3) and dry EW (Fig. 4) differed significantly from those of the control group \((P<0.01)\).

Fig. 1. Normality rate of in vitro-cultured mouse embryos exposed to different concentration of *S. aureus* L-forms. The percentage of normality was calculated using the ratio of the number of normal and total embryos in each group, based on numerical scores according to a standard morphological scoring system (Van Maele-Fabry et al., 1990). Values were calculated with data from three experiments carried out in triplicate \((n=18\) in each group).

![Graph showing normality rate](image1.png)

Fig. 2. Examples of abnormalities of in vitro-cultured mouse embryos exposed to different concentrations of *S. aureus* L-forms for 48 h. (a) Control mouse embryo showing a ‘G’-shaped body position. (b) Embryo exposed to 50 c.f.u. *S. aureus* L-forms ml\(^{-1}\) displaying a ‘C’-shaped body position. (c) Embryo exposed to 100 c.f.u. *S. aureus* L-forms ml\(^{-1}\) displaying abnormalities of the mid/hindbrain (thin arrows) and heart (thick arrow). (d) Embryo exposed to 200 c.f.u. *S. aureus* L-forms ml\(^{-1}\) displaying abnormalities of the fore/mid/hindbrain (thin arrows), heart (thick arrow) and hindlimb (dotted arrow). (e) Embryo exposed to 400 c.f.u. *S. aureus* L-forms ml\(^{-1}\) displaying an open fore/mid/hind neural tube (open arrow), pericardial effusion (arrowhead), a reversed ‘C’-shape body position and abnormality of the hindlimb (dotted arrow) and branchial bars.
Determination of *S. aureus* L-forms in embryonic tissues

To investigate whether *S. aureus* L-forms could pass through the barrier of the yolk sac from the medium to the embryo, cultured conceptuses were removed from assay bottles and rinsed three times with PBS to eliminate the remnant *S. aureus* L-forms from the medium. The visceral yolk sac surrounding the embryo was then removed and the embryo exteriorized. Using the method of Brown and Hopps (1973), we found spherical Gram-positive bacteria in the tissues of mouse embryos incubated with 400 c.f.u. *S. aureus* L-forms ml⁻¹ (Fig. 5), indicating that *S. aureus* L-forms passed through the barrier of the yolk sac from the cultured medium to the embryos.

Detection of *S. aureus* L-form antigen in embryonic tissues by immunohistochemistry

Embryonic tissues treated with 400 c.f.u. *S. aureus* L-forms ml⁻¹ as described above were subjected to immunohistochemistry using anti-L-form antibody and *S. aureus* L-form antigens were detected in the mouse embryo tissues (Fig. 6).

DISCUSSION

In the present study, a whole-embryo culture model was chosen to study the teratogenicity of *S. aureus* L-forms. Mice embryos in this model were cultured in vitro from GD 8.5 to 10.5, which is the critical period of organogenesis in the mouse, equivalent to 3–6 weeks after fertilization in human embryos (Tanaka *et al.*, 1991). This model has been used extensively in studies in the field of teratogenesis and related mechanisms (Longo *et al.*, 2010; Guo *et al.*, 2011). Unlike other *in vivo* animal models, this model enables the direct assessment of external factors on embryogenesis and is not affected by any metabolic and kinetic differences between humans and other animals (Webster *et al.*, 1997). Of course, fundamental biological differences exist between humans and other animals;

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total morphological score</td>
<td>49.7 ± 0.5</td>
<td>48.4 ± 0.7</td>
<td>44.4 ± 1.3*</td>
<td>39.9 ± 2.2*</td>
<td>30.3 ± 3.3*</td>
</tr>
<tr>
<td>Number of somites</td>
<td>28.5 ± 0.3</td>
<td>28.0 ± 0.8</td>
<td>25.7 ± 1.1†</td>
<td>23.2 ± 0.9*</td>
<td>19.4 ± 1.6*</td>
</tr>
</tbody>
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*P<0.01 compared with the control group.
†P<0.05 compared with the control group.

Table 1. Total morphological score and number of somites of mouse embryos (*n*=18 per group) exposed to different concentrations of *S. aureus* L-forms

For each group, *n*=18.

Fig. 3. Development characteristics of *in vitro*-cultured mouse embryos exposed to different concentrations of *S. aureus* L-forms. Values were calculated with data from three experiments carried out in triplicate (*n*=18 in each group). Results are shown as means ± SD. *P<0.01 compared with the control group.

Fig. 4. Dry EW of *in vitro*-cultured mouse embryos exposed to different concentrations of *S. aureus* L-forms. The value was calculated with data from three experiments carried out in triplicate (*n*=18 in each group). Results are shown as means ± SD. *P<0.01 compared with the control group.
therefore, results generated from animal studies should be interpreted with caution.

We investigated the direct effect of *S. aureus* L-forms on mouse embryos during the critical period of organogenesis. Our results showed that *S. aureus* L-forms had a significant effect on the morphogenesis of the embryos. When mouse embryos were exposed to *S. aureus* L-forms at a concentration of 50 c.f.u. ml\(^{-1}\), both embryonic CRL and HL decreased but the total morphological score, number of somites, EW and YSD remained the same. However, a significant reduction in all of the above indices was found in mouse embryos exposed to *S. aureus* L-forms at concentrations of \(\geq 100\) c.f.u. ml\(^{-1}\), and appeared in a dose-dependent manner. We also found that there were fewer normally developed embryos, and more embryos were shown to have abnormalities or retardation in growth with the increase in concentration of *S. aureus* L-forms in infection. Furthermore, we found the presence of *S. aureus* L-forms and its antigen in the cultured embryos incubated with *S. aureus* L-forms. These data suggested that *S. aureus* L-forms can directly affect the growth and development of mouse embryos and are teratogenic, consistent with some results in an *in vivo* study using *S. aureus* (Xia *et al.*, 1999).

A series of studies has shown that apoptosis plays an important role in embryonic growth and development, and mediates the teratogenicity of some factors (Nakajima *et al.*, 2008; Tung & Winn, 2011). Therefore, further studies are required to elucidate whether *S. aureus* L-forms can induce the apoptosis of mouse embryos.

To our knowledge, this study is the first to investigate the teratogenicity of a microbe using a mouse whole-embryo model. *S. aureus* L-forms, compared with the original wild-type *S. aureus*, present the characteristics of slower growth and proliferation (Mattman *et al.*, 1961; Mattman, 2001), so that they fail to cause the serum medium to become too poor in terms of nutrition to meet the growth of mouse embryos in the *in vitro* culture system. Furthermore, *S. aureus* is a clinically common residential pathogen and causes increased risk of vertical infection in the perinatal period. Therefore, *S. aureus* L-forms were chosen to study their teratogenicity on mouse embryos. In this study, we found direct effects of *S. aureus* L-forms on the *in vitro*-cultured mouse embryos. These results showed that *S. aureus* L-forms could retard the growth of mouse embryos. Although the results from such animal teratogenicity studies may not reflect the circumstances in humans, our findings suggest that women during their first trimester of pregnancy should be cautious about infections caused by *S. aureus* and its L-forms.

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