Teratogenicity of the Semliki Forest Virus Mutant ts22 for the Foetal Mouse: Induction of Skeletal and Skin Defects

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SUMMARY

The maximum proportion of skeletal and/or skin defects induced by the Semliki Forest virus (SFV) mutant ts22 in the 17-day-old foetal mouse occurred following infection of the mother at day 10 of pregnancy. The skeletal defects were detected using a combination of Alcian blue staining for cartilage and Alizarin red staining for bone. Using immunogold–silver staining with anti-SFV IgG and in situ hybridization with a cDNA probe to SFV non-structural sequences, we have shown that mesenchymal cells in the dermis and surrounding developing cartilaginous plates were heavily infected in most foetuses at day 17 of pregnancy, following infection of the mother at day 10. Other infected foetal tissues contained less viral antigen and nucleic acid; they included the liver, muscle (including myocardium), lung and kidney. The central nervous system contained only small amounts of viral antigen and nucleic acid. It is proposed that the skeletal and skin defects induced in mouse foetuses by ts22 infection result from the tropism of the virus for mesenchymal cells involved in the development of such tissue.

INTRODUCTION

Many animal viruses are known to infect the developing embryo and/or foetus following infection of the mother. Such viruses may induce abortion or reabsorption, or teratogenic effects in the offspring. Several togaviruses are known to induce these effects; they include human rubella virus, bovine viral diarrhoea virus and border disease virus of sheep. Analysis of the mechanisms of foetal infection and teratogenesis has, however, been hampered by the lack of suitable animal model systems.

We have utilized Semliki Forest virus (SFV) infection of mice as a model to analyse foetal infection and teratogenesis (Atkins et al., 1982, 1985; Hearne et al., 1986, 1987). SFV is a togavirus of the genus Alphavirus. The avirulent A7 strain of this virus (A7-SFV) has been shown to infect lethally the developing foetus, following intraperitoneal (i.p.) infection of the mother. The course of infection is production of viraemia, followed by infection of the placenta from the maternal blood and hence infection of the foetus. Death of all foetuses occurs rapidly, within 2 to 4 days of maternal infection (Milner & Marshall, 1984; Hearne et al., 1987).

We have isolated four mutants of A7-SFV which differ from the wild-type in their effects on developing mouse foetuses. One mutant, ts22, was teratogenic for a proportion of foetuses, rather than rapidly lethal. Also, the process of foetal infection was slower for ts22 than for the
wild-type, and thus more amenable to detailed analysis (Hearne et al., 1987). Here we have begun an analysis of the mechanism of foetal infection by ts22, with particular reference to effects on the skeletal system and the skin.

METHODS

**Virus.** A plaque-purified stock of ts22 was grown at 30 °C and stored in aliquots at −70 °C. Plaque assays were carried out at 30 °C using BHK-21 cells, as described by Atkins et al. (1974).

**Mice.** As in our previous studies on embryonic and foetal infection (Hearne et al., 1986, 1987) the randomly bred Q/Fa mouse strain was used, since this strain has been selected for high body weight and produces large numbers of foetuses per mother. To ensure that the gestation period could be established, females and males were placed together in a ratio of 2:1 and the day of finding a vaginal plug was taken to be day 1 of pregnancy.

**Infection experiments.** Pregnant mice were injected i.p. with either 10⁴ or 10⁵ p.f.u. of ts22 in 0.5 ml of phosphate-buffered saline (PBS). At intervals up to day 17 of pregnancy (2 or 3 days before birth) the mothers were killed and the foetuses were examined. For immunocytochemistry and in situ hybridization, foetuses were fixed by immersion in a solution of 1% glutaraldehyde in PBS for 30 min, then quenched by immersion in solutions of 0.15 M ethanolamine hydrochloride, first at pH 7.4 for 30 min, and then at pH 8.6 for 30 min (Morris & Barber, 1983). The foetuses were then embedded in paraffin wax and sagittal sections of 4 μm were cut.

**Staining of foetuses for cartilage and bone.** This was based on the Alcian blue stain for cartilage described by Watson (1977) and the Alizarin red stain for bone described by Burdi (1965). Foetuses were placed in Alcian blue stain (80% v/v ethanol, 20%, glacial acetic acid, 0.015% w/v Alcian blue) for 3 days, washed in three changes of ethanol over 1 week, placed in warm (30 °C) 0.2% KOH until clear (<10 min), then transferred to Alizarin red stain (0.75% w/v Alizarin red in 0.2% w/v KOH) for 5 min. The foetuses were then washed with 50% (v/v) glycerol in 0.2% (w/v) KOH, with several changes over several days, until all the Alizarin red was removed from soft tissue. They were then placed successively for periods of 1 week in 75% (v/v) glycerol in 25% (w/v) KOH and in 90% (v/v) glycerol in 10% (w/v) KOH. Finally, the foetuses were transferred to glycerol for storage. This method specifically stains cartilage blue and bone red in a single specimen. Externally visible morphological abnormalities in the foetuses were generally photographed before Alizarin red staining, since this procedure tends to destroy soft tissue.

**Preparation of anti-SFV IgG.** Six confluent monolayers of BHK-21 cells in 500 ml Medical flat bottles were inoculated with A7-SFV at an m.o.i. of 1 p.f.u./cell. Following incubation of the monolayers at 37 °C for 18 h and the development of a c.p.e., the supernatant fluid (120 ml containing 10⁹ p.f.u./ml) was centrifuged at 3000 g for 10 min to remove cellular debris, then at 10⁵ g for 2 h to pellet the virus. The pellet was resuspended and layered onto a 20% to 65% (w/v) sucrose gradient in 50 mM-Tris-HCl, 0.1 M- NaCl and 1 mM-EDTA, pH 7.4 (TNE) and centrifuged at 105 g for 18 h. The virus-containing band was dispersed in 15 ml of TNE, then pelleted and resuspended in 5 ml of TNE. This purified virus preparation, which contained 10⁹ p.f.u./ml of infectious virus, was stored in 0.5 ml aliquots at −70 °C. Preimmune serum was taken from a 6-month-old New Zealand White rabbit, and the animal was injected intravenously with 1 ml and subcutaneously with 0.5 ml of the purified virus, and then subcutaneously with 0.5 ml of virus on days 7, 21, 35 and 49 after the first injections. The rabbit was exsanguinated 14 days after the final injection. The serum was precipitated with 45% (w/v) ammonium sulphate and resuspended to give a 10-fold concentration, dialysed extensively against 70 mM-phosphate buffer pH 6.3, and applied to a DEAE-cellulose column equilibrated in 70 mM-phosphate buffer pH 6.3. The presence of protein in the eluate was verified by reading the absorbance of fractions at 280 nm. Protein-containing fractions were pooled, and the purity of the IgG verified by SDS-PAGE. The specificity of the IgG for SFV was verified by Western blotting against protein extracts from BHK cells infected with A7-SFV, and extracts from uninfected cells.

**Immunogold–silver staining (IGSS).** Sections of infected and control foetuses were prepared as described above and mounted on glass slides. Three sagittal sections from each foetus were used for IGSS, and parallel sections were stained with haematoxylin and eosin for routine histological examination. A modification of the methods described by Holgate et al. (1983) and Springall et al. (1984) was used. Rabbit anti-SFV IgG was applied to the sections at a dilution of 1:80 (this was found in preliminary experiments to be the optimum concentration). This was followed by the secondary antibody, goat anti-rabbit IgG labelled with colloidal gold (Janssen Life Sciences Products, Olen, Belgium), diluted and used according to the manufacturer's instructions. The signal was amplified by silver enhancement (Intense II silver enhancement kit; Janssen) and the tissue sections were counterstained with methyl green.

**In situ hybridization.** Sections of infected and control foetuses were bound covalently to glass slides which had been pre-activated by Denhardt coating and organosilanation by the method of Tourtellotte et al. (1986). The cDNA probe was derived from the plasmid pKTH301, described by Lehtovaara et al. (1981), which was a gift of H. Söderlund, Department of Virology, University of Helsinki, Finland. This consists of a triplicated repeat of a non-structural sequence of SFV, cloned into the PstI site of the plasmid pBR322, and originally obtained from a
defective interfering particle. DNA derived from pKTH301 was restricted with PstI, and fragments of 484 kb and 489 kb, corresponding to viral sequences (Lehtovaara et al., 1981), were separated by agarose gel electrophoresis and labelled with $^{35}$SATP (New England Nuclear) by nick translation (Maniatis et al., 1982). Hybridization of the labelled probe to the tissue sections was performed according to the procedure of Brahic & Haase (1978), with the modifications of Moench et al. (1985). The hybridization mixture contained 0.2 $\mu$g/ml of $^{35}$S-labelled DNA and hybridization was carried out for 18 h at 25 °C. After washing, the slides were dehydrated through a graded alcohol series and air-dried. Autoradiography was carried out by coating slides with K-5 nuclear track emulsion (Ilford) which had been melted at 43 °C and diluted 1:1 with 0.6 M-ammonium acetate solution. After drying vertically, the slides were exposed in dessicant-containing boxes at 4 °C for 7 to 10 days, developed for 5 min with Kodak 19 developer at 16 °C, rinsed in water, fixed for 5 min with 30% (w/v) sodium thiosulphate, stained lightly with methyl green, dehydrated and mounted.

RESULTS

Time course of teratogenesis

Groups of six pregnant mice were injected with 10^4 or 10^8 p.f.u. of ts22 at days 4, 6, 8, 10 or 12 of pregnancy, and the foetuses were examined at day 17 (2 or 3 days before birth). Foetuses were scored as defective by external appearance, and/or the presence of skeletal defects as revealed by staining. Fig. 1 shows the proportion of defective foetuses induced following injection at each of these times. For both doses, most defects were induced following injection at day 10 of pregnancy. Injection at day 4 produced no defects. As shown in Table 1, cartilage and bone defects were observed in the majority of defective foetuses examined; skin defects (fragility and/or haemorrhage) were also common. Many foetuses showed more than one defect. We have previously described the induction of skin haemorrhage by ts22 (Hearne et al., 1987). Examples of the skeletal defects induced by ts22 are shown in Fig. 2. They included phocomelia (reduction deformity in the limb), mandibular regression and scoliosis (spinal deviation). Many foetuses showing skeletal defects and/or defective ossification were of a size at which normal ossification could be detected in uninfected control foetuses, indicating a specific defect in skeletal development. In other foetuses, incomplete ossification and/or cartilage development was probably due to developmental retardation or arrest. In a few cases, bone was present but not cartilage; this was assumed to be due to tissue necrosis (Table 1 and Fig. 2; Hearne et al., 1987).

Immunogold-silver staining

The above results indicated that the optimum time for induction of skeletal and skin defects with a dose of 10^4 p.f.u. of ts22 was at day 10 of pregnancy. Therefore to study the mechanism of induction of such defects, pregnant mice were injected with ts22 at day 10 and foetuses from groups of three mothers were examined by IGSS at daily intervals from day 12 to day 17 of

Fig. 1. Time course of induction of skeletal and skin defects by ts22. A total of six pregnant mice were injected i.p. at different times post-conception. Doses were (□) 10^8 and (■) 10^4 p.f.u.
Table 1. Number of foetuses showing skeletal and skin defects after infection of the mother with ts22

<table>
<thead>
<tr>
<th>Dose (p.f.u.)</th>
<th>Time infected (days post-conception)</th>
<th>Total number of defective foetuses*</th>
<th>Location of defects</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Cartilage†</td>
<td>Mandible</td>
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<tr>
<td>$10^4$</td>
<td>8</td>
<td>4</td>
<td>3</td>
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<td>12</td>
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<tr>
<td>$10^8$</td>
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* Many foetuses showed more than one defect.
† Incomplete cartilage development and/or ossification, due to developmental arrest or retardation (Hearne et al., 1987).
‡ Haemorrhagic foetuses and/or fragile skin.

Table 2. Screening of foetuses from infected mothers for viral antigen and nucleic acid*

<table>
<thead>
<tr>
<th>Day of pregnancy†</th>
<th>Number positive by IGSS‡</th>
<th>Number positive by in situ hybridization§</th>
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<tr>
<td>12</td>
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<td>0/2</td>
</tr>
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<td>1/17</td>
<td>0/2‡</td>
</tr>
<tr>
<td>16</td>
<td>0/19</td>
<td>1/2</td>
</tr>
<tr>
<td>17</td>
<td>12/14</td>
<td>2/3¶</td>
</tr>
</tbody>
</table>

* Foetuses were taken from three infected mothers for each time point.
† Following infection on day 10 of pregnancy, mothers were killed and foetuses were examined at the times of pregnancy indicated.
‡ Out of the total number examined.
§ Out of the total number examined; foetuses were among those examined by IGSS.
¶ Both foetuses were negative by IGSS.
¶ Two foetuses were positive and one was negative by IGSS.

pregnancy. Table 2 shows the numbers of foetuses examined and the number positive for viral antigen. Foetuses from mothers injected with PBS alone were negative. Also, sections from foetuses showing a positive reaction to anti-SFV serum showed a negative reaction when treated with preimmune serum. As shown in Table 2, the majority of foetuses examined at day 17 were positive for viral antigen, but all younger foetuses, with the exception of one foetus at day 15, were negative. Viral antigen-positive foetuses showed varying degrees of degeneration and necrosis as shown by haematoxylin and eosin staining. For two foetuses, this degeneration was too extensive to permit accurate determination of the location of viral antigen. In all other positive foetuses the most intense immunostaining occurred in mesenchymal cells of the dermal stroma and surrounding developing cartilaginous plates (Fig. 3). Less intense staining occurred in skeletal muscle and myocardium, and in the liver, lung and kidney parenchyma. The central nervous system was unstained in the majority of positive foetuses examined.

In situ hybridization

Sections from two foetuses examined by IGSS at each of the time points (three at day 17) were also examined by in situ hybridization. Foetuses from mothers injected with PBS alone were negative for viral nucleic acid. Two foetuses examined at day 17 of pregnancy were positive by in situ hybridization and by IGSS, and one was negative by both techniques. The positive foetuses showed similar patterns of staining by both techniques, except that epidermal cells showed only occasional small stain deposits with IGSS, but were markedly positive by in situ hybridization.
SFV teratogenicity

Fig. 2. Mouse foetuses at day 17 of pregnancy, stained with Alcian blue and Alizarin red, from uninfected mothers and mothers infected with ts22. (a) Foetus from uninfected mother showing staining of spine and (b) foetus from infected mother showing scoliosis. (c) Foetus from uninfected mother showing staining of mandible and (d) foetus from infected mother showing mandibular regression. (e) Foetus from uninfected mother and (f) foetus from infected mother showing general inhibition of ossification, particularly in the head region. Bar markers represent 4 mm (a, b, e and f) or 2 mm (c and d).
Fig. 3. IGSS using anti-SFV IgG and in situ hybridization using 35S-labelled SFV cDNA to sections of foetuses from mothers infected at day 10 of pregnancy with ts22 and controls injected with PBS alone. All sections were counterstained with methyl green. (a) Positive IGSS reaction in the skin and (b) surrounding a cartilaginous plate in a 17-day-old foetus. (c) In situ hybridization to skin and (d) cartiligenous plate in an uninfected 17-day-old foetus. (e) In situ hybridization to skin and (f) cartiligenous plate in an infected 17-day-old foetus. (g) In situ hybridization to skin and (h) cartiligenous plate in an infected 16-day-old foetus; this foetus was negative by IGSS. All photographs were taken at the same magnification; the bar marker represents 10 μm.
Another foetus was negative by both techniques. Examples of the staining reaction in skin and surrounding cartilaginous plates by in situ hybridization are shown in Fig. 3. One foetus from day 16 of pregnancy, which was negative by IGSS, was positive by in situ hybridization. In this foetus, focal areas of staining in the dermis and surrounding cartilage could be detected by in situ hybridization (Fig. 3g, h). This may reflect the greater sensitivity of in situ hybridization, compared to IGSS, in these experiments.

**DISCUSSION**

Our results indicate that developmental defects in the skin and skeleton of the foetal mouse may be optimally induced by infection of the mother with the ts22 mutant of SFV at day 10 of pregnancy. However, the defects in many of the foetuses were extreme and resulted in foetal death. Also, many foetuses escaped detectable damage from virus infection. Teratogenesis is probably therefore an intermediate effect of infection between foetal death at one extreme and no damage at the other. Postnatal survival of foetuses showing developmental defects is difficult to achieve in the mouse, due to cannibalism by the mother. Also, there is great variation in the infectious virus content of individual foetuses from infected mothers (from undetectable to $10^9$ p.f.u./g at day 17 of pregnancy following infection at day 8 or 10), although infectious virus content does not correlate with severity of foetal damage (Hearne et al., 1987; M. Mabruk, unpublished results). We have previously shown that immunity to SFV in the mouse may be transferred across the placenta to the foetus (Atkins et al., 1982). Since ts22 is lethal for non-immune neonatal mice (Hearne et al., 1987), the spectrum of effects on the foetus following ts22 infection of the mother may be related to the rapidity of induction of antiviral immunity in the mother and its transfer to the foetus. The wild-type A7 strain of SFV, from which ts22 is derived, is rapidly lethal for the foetus (Atkins et al., 1982) and therefore foetal infection probably always precedes the development of foetal immunity. The time course of multiplication of ts22 in the pregnant mouse is slower than A7 (Hearne et al., 1987), and may allow the transfer of immunity to some foetuses before a lethal threshold of virus-induced damage occurs.

We have shown in this study that ts22 shows a tropism for mesenchymal cells surrounding developing cartilaginous plates and in the dermis of infected foetuses, following infection of the mother at day 10 of pregnancy, although cells in other organs may also be affected to a lesser extent. This probably explains the large number of skin and skeletal defects induced by day 17 of pregnancy. A surprising aspect of the present study is the poor multiplication of ts22 in the central nervous system of the foetus, following infection of the mother at day 10 of pregnancy. A7-SFV, from which ts22 is derived, is neurotropic for adult mice, and we have shown that ts22 retains its neurotropism for adult mice (E. King & G. Atkins, unpublished results). One explanation for this may be that the tissue tropism of ts22 varies with the state of differentiation of the host cells.

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**REFERENCES**


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