Transmission of Moloney murine leukemia virus (ts-1) by breast milk

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A murine model has been developed to study maternal transmission of the temperature-sensitive Moloney murine leukemia virus (ts-1). The goal of this study was to confirm early and late mother-to-offspring transmission of the virus and demonstrate transmission via breast milk. A series of six experiments was performed using six groups of BALB/c mice. Group 1 consisted of pups born to ts-1-infected mothers removed at birth to suckle from surrogate uninfected mothers. Groups 2 and 5 consisted of pups born to ts-1-infected mothers that suckled from ts-1-infected mothers (surrogate and biological). Group 3 consisted of non-infected pups removed at birth to suckle from ts-1-infected mothers. Groups 4 and 6 consisted of non-infected pups suckled from non-infected mothers. The combined in utero, intrapartum and breast-milk infection rate was 100 % to the offspring (groups 2 and 5). The in utero to early post-partum group (group 1) had an infection rate of 78 %. Breast milk alone (group 3) resulted in a 97 % infection rate. Control groups (groups 4 and 6) had a 0 % infection rate. The relative frequency of maternal CD4+ cells in peripheral blood mononuclear cells was consistently lower in infected mothers, whilst offspring did not show a significant decrease in CD4+ frequency. Pups infected via breast milk had a lower CD4+ frequency (group 3) than those infected by the uterine and/or intrapartum route (group 1). Breast milk from ts-1-infected mothers appears to be highly infectious for neonatal BALB/c mice.

INTRODUCTION

A temperature-sensitive mutant (ts-1) of the Moloney murine leukemia virus (MoMuLV) was first isolated in 1973 (Wong & McCarter, 1973, 1974). This retrovirus causes hindlimb paralysis and immunosuppression in susceptible strains of mice and has now been established as a small-animal model for retrovirus-induced neurodegenerative disease (Wong, 1990).

If susceptible strains of mice, such as BALB/c, are inoculated with ts-1 by intraperitoneal injection before 10 days of age, over 99 % develop a reproducible syndrome of progressive, bilateral hindlimb paralysis and immunodeficiency. This syndrome is also associated with the development of wasting, lymphoma, lymphopenia and thymic and splenic atrophy (Prasad et al., 1989). The initial viral target for infection is CD4+ cells, with continued virus replication in splenic and lymphoid tissues and associated continuous viraemia (Saha & Wong, 1992; Saha et al., 1994b). The virus is also neurocytopathic and replicates independently in the central nervous system (Wong et al., 1985, 1989). Among the murine retroviruses, ts-1 is similar to human immunodeficiency virus (HIV) in its use of CD4+ cells for infection, its secondary neurocytopathic effect, its continuous viraemia with free virus in circulation and depletion of CD4+ cells. These and other similarities have led to its use as a small-animal model for HIV (Clark et al., 2001).

Whilst extensive work has been done on development of this virus as a model for retrovirus-induced neurodegenerative disease (Wong, 1990), few studies were done until recently on the vertical transmission of this virus (Chakraborty et al., 2003; Duggan et al., 2004). Transfer of humoral immunity to ts-1 by breast milk was observed previously (Saha et al., 1994a). Saha et al. (1994a) reported that maternal antibody to ts-1 is primarily transferred to pups via breast milk. Some limited placental transfer of IgG was also reported. Vertical transmission of the ts-1 retrovirus has recently been demonstrated to occur in a dose-dependent manner (Chakraborty et al., 2003). When 72 h-old mice are inoculated intraperitoneally with 4 x 10⁶ focus-forming units (f.f.u.) ts-1 virus ml⁻¹, 99 % of mice develop neurodegenerative disease at > 6 weeks of age, but retain full reproductive capacity until the development of hindlimb paralysis and manifestations of immunosuppression. Vertical transmission occurs in utero in 39-6 % of mice and overall in 99 % of mice via combined in utero, intrapartum and post-partum routes (Duggan et al., 2004). The rate of transmission of ts-1 by the intrapartum and post-partum routes is unknown, and previous work has indicated a potentially protective role for..
breast milk in ts-1 infection (Saha et al., 1994a). In this paper, we examined post-partum transmission of ts-1 and also evaluated intrapartum-transmission rates. In addition, we postulate that ts-1 is an excellent small-animal model for the study of mother-to-fetus retroviral transmission.

METHODS

Virus and assay. The MoMuLV-ts-1 (temperature-sensitive) virus was used for this study. The virus was cultured in TB (thymus–bone marrow) cells and assayed in 15F cells. The viral stock was a gift from Dr P. K. Y. Wong, University of Texas M. D. Anderson Cancer Center, Smithville, TX, USA. For further description of culture and assay methods, please see our previous paper (Chakraborty et al., 2003). Briefly, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 6% fetal calf serum, 4% newborn calf serum and 1% penicillin/streptomycin at 37°C. After brief polybrene treatment, 1 × 106 f.f.u. ts-1 virus was added to TB cells. The cells were washed and cultured, medium containing the virus particles was removed, filtered through a 0.45 μm filter, aliquotted and stored at −80°C. For viral assay, 15F cells were grown in culture medium as described for TB cells. Then, 9–6 × 105 cells ml−1 were plated onto 60 mm culture plates in polybrene. Assays were performed by serial dilutions of 0.2 ml viral stock in 24-well plates. Then, virus-infected cells were cultured in 60 mm plates for 5 days and the number of f.f.u. per plate was counted. The final viral titre was 4.0 × 106 f.f.u. ml−1.

Mice and experimental protocol. Eighteen timed pregnant female and 11 male mice were purchased from Charles River Laboratories (CRL), Wilmington, ME, USA. All experiments were performed after obtaining Institutional Animal Care and Use Committee approval and under direct supervision of the trained personnel of the Department of Laboratory Animal Medicine at the Medical University of Ohio. Eighteen females produced 80 pups in total. These 80 pups were divided into experimental and control groups. Experimental mice were injected intraperitoneally with virus (0.1 ml of 4 × 106 f.f.u. ts-1 virus ml−1) at 72 h after birth. At the same time, control animals were injected with 0.1 ml DMEM only. Six of the pups from the experimental group and two from the control group died, leaving 56 pups for the experimental group and 16 pups for the control group available for study. At day 25, the animals were weaned, sexed and ear-tagged. Of 56 experimental animals, 21 were male and were discarded. Three control males, along with 11 males purchased from CRL, were kept for mating. When the animals reached adulthood, they were allowed to mate. The day of pregnancy was determined by checking the vaginal plug and the date of delivery was calculated based on the day that the vaginal plug was identified. During the anticipated time of delivery, each of the females was checked every 4–12 h. Our initial plan was to remove the term fetuses by Caesarean section (C-section) at day 19 to avoid intrapartum infection. For that purpose, two females from each experimental and control groups were used for C-section. Unfortunately, none of the fetuses survived. Therefore, C-section delivery was not continued. However, as a result, we had 33 experimental and 11 control females available for the study. As soon as the pups were born, they were either transferred to the surrogate mothers or left with their own mothers. For surrogate control mothers, seven original CRL females were used. The rest of the surrogate came from our colony. Please see the flow diagram (Fig. 1) for the experimental design using 109 experimental and 59 control pups. Four to 12 hours after delivery, these pups were divided into the six following groups: group 1, ts-1-infected pups suckled from the surrogate control mothers; group 2, ts-1-infected pups suckled from the surrogate ts-1-infected mothers; group 3, control pups suckled from ts-1 surrogate mothers; group 4, control pups suckled from the surrogate control mothers; group 5, ts-1 pups left with their own mothers; group 6, control pups left with their own mothers.

Tissue collection. After weaning, all mothers were euthanized by a lethal injection of sodium pentobarbitol and thymus, spleen, lymph nodes, adrenals and blood samples were collected. The offspring were observed for 6 months for development of clinical symptoms and evidence of symptomatic infection. Animals were weighed on a biweekly basis. At the end of this period, the pups were euthanized and their tissue and blood samples were collected. Tissue samples were stored at −80°C for DNA extraction for PCR analyses. All procedures were performed under strict aseptic conditions.

DNA extraction and PCR. Procedures for DNA extraction, PCR analyses and viral DNA sequencing were performed according to methods described previously (Duggan et al., 2004). As ts-1 is a retrovirus and contains RNA, the DNA that we used was from pro-viral DNA integrated in the host genome. Tissues from adrenals, spleens and thymus were used for PCR from each animal. All PCR analyses were performed by using a Perkin-Elmer Gene Amp PCR reagent kit with AmpliTaq DNA polymerase. Two sets of primers were used: one for the ts-1 env gene, which amplified a 264 bp region, and the second for a murine T-cell receptor gene (~200 bp; http://www.jax.org), which served as an internal control. Negative and positive-control samples were also used for each PCR run (Chakraborty et al., 2003). PCR bands were obtained by using agarose-gel electrophoresis. Gibco-BRL 100 bp DNA Ladder was used as a reference DNA marker.

Flow cytometry. Lymphocytes were gated on side scatter versus rat anti-mouse CD45 tricolour (TC)–phycoerythrin (PE Cy5) background. Fluorescence compensation was checked prior to analysis with single-labelled hamster IgG–fluorescein isothiocyanate (FITC), rat IgG2a–PE and rat anti-mouse CD45–TC. Rat anti-mouse CD4–PE and CD4–PE were used to analyse CD4 and CD8 T-cell subsets. All antibodies were obtained from Caltag Laboratories. Heparinized blood (50 μl) was incubated with appropriate antibodies for 15 min and 2 ml High Yield Lyse (Caltag) reagent was added and incubated for an additional 3 min and washed with PBS. The sample was centrifuged and supernatant was discarded before resuspending cells with 450 μl PBS. Fifty microlitres of 10% paraformaldehyde was added and frequency of the CD4+ and CD8+ T-cell subpopulations was obtained by counting approximately 5000 peripheral blood mononuclear cells (PBMCs) per sample using a flow cytometer (Coulter EPICS Elite).

RESULTS

Table 1 and Fig. 2 summarize the results of the PCR analysis of all offspring.

Group 1. This group contained offspring of ts-1-infected mothers nursed by control surrogate mothers. In this group, we had a total of 49 offspring. Of these, three died and another had enlargement of the spleen. Thirty-six of 46 surviving offspring (78%) tested positive for ts-1 viral DNA by PCR.

Group 2. This group contained offspring of ts-1-infected mothers nursed by ts-1-infected surrogate mothers. In this group, we had a total of 31 offspring. Of these, two died and two had enlarged spleens. All surviving 29 (100%) tested positive for ts-1 viral DNA by PCR.
Group 3. This group contained offspring of control mothers nursed by ts-1-infected surrogate mothers. In this group, we had a total of 40 offspring. Of these, two died, six developed splenomegaly and enlargement of lymph nodes and 37 (97%) of the surviving 38 tested positive for ts-1 DNA.

Group 4. This group contained offspring of control mothers nursed by control surrogate mothers. In this group, we had a total of nine offspring. None of these animals developed splenic enlargement. All (100%) tested negative for ts-1 DNA.

Table 1. Summary of results of PCR analysis of six groups of animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Pup→surrogate mother</th>
<th>Sample size</th>
<th>Male (n)</th>
<th>Female (n)</th>
<th>Positive ts-1</th>
<th>Negative ts-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>1 (n=46)</td>
<td>Exp→Ctrl</td>
<td>46</td>
<td>21</td>
<td>25</td>
<td>36</td>
<td>78·3</td>
</tr>
<tr>
<td>2 (n=29)</td>
<td>Exp→Exp</td>
<td>29</td>
<td>14</td>
<td>15</td>
<td>29</td>
<td>100·0</td>
</tr>
<tr>
<td>3 (n=38)</td>
<td>Ctrl→Exp</td>
<td>38</td>
<td>15</td>
<td>23</td>
<td>37</td>
<td>97·4</td>
</tr>
<tr>
<td>4 (n=9)</td>
<td>Ctrl→Ctrl</td>
<td>9</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>0·0</td>
</tr>
<tr>
<td>5 (n=24)</td>
<td>Exp→Biological mother</td>
<td>24</td>
<td>8</td>
<td>16</td>
<td>24</td>
<td>100·0</td>
</tr>
<tr>
<td>6 (n=9)</td>
<td>Ctrl→Biological mother</td>
<td>9</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>0·0</td>
</tr>
</tbody>
</table>
Group 5. This group contained offspring of ts-1-infected mothers left with their own mothers. In this group, we had a total of 29 offspring. Five of these animals died, five developed splenic enlargement and two developed thymic enlargement. All surviving 24 pups (100%) tested positive for ts-1 viral DNA.

Group 6. This group contained offspring of control mothers left with their own mothers. In this group, we had a total of 10 offspring, one of which died. None of them developed splenic enlargement and all of the surviving nine pups (100%) tested negative for ts-1 viral DNA.

Analysis of tissues from offspring of groups 1, 2, 3 and 5 showed distinct PCR bands consistent with ts-1 env, whereas tissues of groups 4 and 6 were negative by PCR (Fig. 2). All ts-1-infected mothers showed distinct PCR bands (Fig. 2). These bands have been sequenced previously and were shown to be ts-1 env (Duggan et al., 2004).

Fig. 3 represents the flow-cytometric analysis of CD4+ and CD8+ cells, showing the relative percentage frequency of CD4+ or CD8+ cells in PBMCs of the offspring of control mothers nursed by surrogate infected mothers (group 3). Table 2 shows that the relative percentage frequencies of CD4+ and CD8+ PBMCs for offspring of control mothers nursed by surrogate infected mothers (group 3) were lower than those of the offspring of control mothers that remained with their biological mothers (group 6) or offspring of infected mothers nursed by surrogate control mothers (group 1).

Table 3 indicates that both the CD4+ and CD8+ frequencies were lowest in infected parents.

DISCUSSION
Although mother-to-offspring transmission of retroviruses has been reported in many species, including humans, the
mechanism by which this transmission occurs still remains unclear. During our previous studies of mother-to-offspring transmission of the murine retrovirus MoMuLV-ts-1, we have shown that about 39 % of transmission occurs in utero (up to day 20 gestation) and nearly 100 % transmission occurs if the offspring are allowed to be born and suckle breast milk from the infected mothers. Breast milk was considered as a major source of infection of this retrovirus to the offspring. However, previous work (Saha et al., 1994a) indicated that humoral immunity to ts-1 might also be transferred by breast milk. To further delineate the extent of infectivity of the breast milk, we designed the current experiment by using the surrogate infected and uninfected mothers, who were allowed to nurse the uninfected and infected pups, respectively.

Breast milk is a major source of viral transmission in both mice and humans and many parallels exist between this murine retrovirus model and human retrovirus transmission. According to a meta-analysis (Coutsoudis et al., 2004) of 4085 children from nine trials, 993 were infected with HIV and 225 (23 %) had late post-natal transmission through breastfeeding. This study showed that late post-natal (breast-milk) transmission contributed significantly to the mother-to-child transmission (MCT) of HIV and was associated with lower maternal CD4$^+$ cells and sex (male) of the child. Breast-milk transmission of HIV remains a major challenge in the developing countries. In an African study with 996 infants born to HIV-positive mothers, three routes of transmission were determined: intrauterine, intra- and early post-partum and late post-partum. HIV transmission occurred in offspring of 249 (25 %) mothers. Of these, 89 (36 %), 104 (42 %) and 21 (1 %) infants were infected by the intrauterine, intra- and early post-partum and late post-partum routes, respectively. This study shows that intrauterine and early post-partum transmission of HIV are major routes of infection among infants (Zijenah et al., 2004).

Regarding the definitions of in utero and intrapartum transmission in humans, the paediatric Virology Committee of AIDS Clinical Trials group developed a working definition. According to their definition, if HIV genome is detected by PCR within 48 h of birth, then the infant is considered to have in utero infection. However, if the infant is negative for PCR during the first week of life and has not been breastfed, but becomes positive 7–90 days afterwards, then it is considered intrapartum infection (Bryson et al., 1992). Rate of MCT also depends on the genetic strain of the virus. For example, HIV-2 transmission occurs at a much lower rate than HIV-1 transmission (Renzifo et al., 2004). These investigators also reported significant differences in MCT according to HIV-1 subtype. Of 253 HIV-infected infants, 39 % had subtype A, 29 % subtype C and 21 % subtype D. They found that transmission of subtypes A and D occurred mostly in the intrapartum and post-natal periods, whereas subtype C transmission occurred mostly in utero. Moreover, no difference in CD4$^+$ cell count or viral load was associated with the subtypes of HIV-1. Magder et al. (2005) carried out a study with 1709 children. In this group, 166 showed HIV infection, with 34 % presumed to have in utero infection and 66 % with intrapartum infection. These authors concluded that, although perinatal HIV transmission has declined, the rate of in utero transmission is increasing. Other retroviruses, such as human T-lymphotropic virus 1, are transmitted through breast milk, with lower rates of intrauterine infection seen when compared with post-partum transmission (Kawase et al., 1992; Ichimaru et al., 1991).

During the current investigation, we obtained 78 % combined in utero and intrapartum infection, which is comparable to 66 % observed in humans (Magder et al., 2005). However, in mice, the overall rate of infection is higher than in humans. Overall, MCT occurs at a rate of 16–50 % in humans (Renzifo et al., 2004). It is possible that higher maternal viraemia and the virulence of the virus play a role in transmission to the offspring in this model. Also, a high maternal viral inoculum might result in higher viraemia, which may yield a higher rate of transmission. Although we removed the pups from the infected mothers within 4–12 h of birth, there is a possibility of a few infections from breast milk during this short period of exposure to the infected mothers. In our previous experiments, we observed a 39 % in utero infection rate when the fetuses were removed by C-section (Duggan et al., 2004). This transmission rate correlates very well in this murine model when compared with rates and results of transmission of HIV in humans.

In the current experiment, we also evaluated the mothers’ CD4$^+$ frequency in relation to transmission of the virus. In general, the mothers’ CD4$^+$ frequency was consistently low. Our preliminary results indicate that CD4$^+$ frequency was lowest in offspring of control mothers nursed by the infected mothers, probably due to the continuous transmission of the virus particles through breast milk. However, no further conclusion on this finding can be made at this time. The offspring of infected mothers nursed by control surrogate mothers showed a smaller drop in CD4$^+$ frequency. This could be due to a lower non-continuous viral inoculum. We are currently evaluating CD4$^+$ levels and viral loads in this model as a correlate of virus transmission, but a larger sample size observed over longer periods of time is needed for analysis. In conclusion, although the ts-1 mouse model is not homologous to HIV-induced AIDS in humans, it could serve as an excellent tool to investigate the mechanism of viral transfer from mother to fetus. Also, as transfer of HIV

<table>
<thead>
<tr>
<th>Parent</th>
<th>Mice sample size</th>
<th>CD8$^+$ (mean ± SD, %)</th>
<th>CD4$^+$ (mean ± SD, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>4</td>
<td>16.0 ± 11.5</td>
<td>42.8 ± 28.9</td>
</tr>
<tr>
<td>Exp</td>
<td>10</td>
<td>10.1 ± 8.5</td>
<td>23.8 ± 15.2</td>
</tr>
</tbody>
</table>

Table 3. Flow-cytometric analysis of mean relative percentage frequency of CD4$^+$ and CD8$^+$ cells in PBMCs of adult mice infected with ts-1 (Exp) and control (Ctrl)
from mother to child via breast milk is a common occurrence in humans, the ts-1 model in mice can be used effectively to examine how the virus is concentrated in breast milk and how it establishes the infection in the offspring.

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REFERENCES


