Neonatal Infection with Mouse Thymic Virus: Spleen and Lymph Node Necrosis

By BEVERLY A. WOOD, WERNER DUTZ AND SUE S. CROSS*

Departments of Pathology and Microbiology, Box 662, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298, U.S.A.

(Accepted 12 June 1981)

SUMMARY

Mouse thymic virus (TA) is a naturally occurring herpesvirus of laboratory and wild mice, which produces massive thymic necrosis when inoculated into newborn mice. Our histopathological study showed necrosis not only of the thymus but also of the spleen and lymph nodes which was noticeable by day 7 and complete by day 14. Both spleen and lymph nodes regenerated to an almost normal histological pattern by day 70. The results show that TA infects multiple lymphoid tissues causing massive necrosis in all, and is not limited to a single site, the thymus. TA infection was found to be a persistent herpesvirus infection in both the lymph nodes and spleen. During the period of acute infection, as necrosis increased, the response of cell suspensions of lymph nodes to the T cell mitogens concanavalin A and phytohaemagglutinin was virtually non-existent. Activity returned to normal as the histological repair progressed.

INTRODUCTION

Mouse thymic virus (TA) was previously described by Rowe & Capps (1961) as an enzootic virus in certain colonies of laboratory and wild mice. It is so-called because of the marked tropism of the virus for the thymus and the distinctive biological property of producing massive necrosis of the thymus in newborn mice. Electron microscopic studies of particles in the thymus before massive necrosis showed that the morphology of the particle was similar to that of herpesviruses (Parker et al., 1973). TA has been compared to mouse cytomegalovirus (MCMV) and it has been established that TA and MCMV are two different, naturally occurring herpesviruses in mice (Cross et al., 1979).

The current study was undertaken to determine if spleen and lymph node necrosis occurred as a result of TA infection of newborn mice, and to determine the T cell responses of lymph node suspensions. We found that TA infection caused massive lymph node and splenic necrosis, and reduced responsiveness of lymph node-derived cells to concanavalin A (Con A) and phytohaemagglutinin (PHA). Tests of viable cells from TA-infected mice demonstrated virus persistence in the lymphatic tissues for a minimum of 3 months.

METHODS

Animals. Outbred pregnant Swiss mice and mice retired from breeding were obtained from M. A. Bioproducts (MA), Walkersville, Md., U.S.A. Mice referred to as neonates were less than 48 h old.

Virus. The mouse thymic virus seed pool (1281) was the fifth newborn passage provided by W. P. Rowe [National Institutes of Health (NIH), Bethesda, Md., U.S.A.] and was a 10%
extract of thymuses, livers, spleens, kidneys and adrenal glands. Seed virus was passaged five times in specific-pathogen-free (SPF) NIH Swiss mice as previously described (Cross et al., 1979). For passage, thereafter, MA newborn mice were injected with 0.05 ml extract intraperitoneally (i.p.). Livers, kidneys, thymuses and spleens were harvested 5 or 6 days after inoculation. Tissues were homogenized as 10% (w/v) suspensions in glass tissue grinders with Eagle's minimum essential medium (MEM) and frozen at -70°C. The 10% extracts for injections were prepared by centrifuging the tissue suspensions at 800 g for 10 min. For high-titred virus pools, only thymuses were collected.

Virus isolation. Salivary glands or lymphoid organs were harvested and frozen at -70 °C. After thawing, 10% suspensions were prepared in Eagle's MEM, clarified by centrifugation at 800 g for 20 min and injected i.p. in litters of newborn mice. The animals were examined 8 to 10 days later for macroscopic thymic necrosis.

Infectivity titration. TA was detected and titred by injecting 0.05 ml tissue extracts or viable cell preparations i.p. into newborn mice using 0.25 ml glass syringes and disposable 30-gauge needles. To determine virus titres, 8 to 10 newborn per 1 or 2 litters of mice were inoculated at each dilution of virus. Thymuses were examined 9 to 12 days post-infection for macroscopic necrosis. Necrosis was scored from 0 (no visible necrosis) to 4+ (entire thymus necrotic). Infectious dose (ID$_{50}$) was determined by the Reed-Muench method (Lennette, 1969).

Antisera. Six-week-old SPF NIH Swiss mice were inoculated i.p. five times with 50 to 100 ID$_{50}$s over a 3 month period. Serum was heated at 56 °C for 30 min before use. Control antisera were also prepared in a similar manner, using pooled extracts of normal mouse tissues including thymuses, livers, spleens and kidneys.

Indirect fluorescent antibody (IFA) test. Impression smears were made by gently pressing a clean 22 x 10 mm coverslip against the cut surface of the tissue being examined. Coverslips were fixed in cold acetone for 10 min, air-dried and stored at -70 °C. Thawed coverslips were flooded with anti-TA antiserum and incubated in a moist chamber at 37 °C for 30 min. The coverslips were then washed three times in phosphate-buffered saline (PBS) and flooded with fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Downingtown, Pa., U.S.A.). Roger Wilsnack, Huntington Research Center, Brooklandville, Md., U.S.A. kindly supplied the lissamine rhodamine counterstain. Following another 30 min incubation at 37 °C, the coverslips were again washed three times in PBS, rinsed once in distilled water and mounted on glass slides with phosphate-buffered glycerin (9:1, glycerin:PBS). The slides were examined for fluorescein-stained nuclear inclusions.

Mitogen stimulation. Single-cell suspensions of TA-infected and non-infected mesenteric lymph nodes or spleens were washed and prepared in RPMI 1640 medium (Gibco) supplemented with 10% foetal calf serum. In 0.2 ml medium, 4 x 10$^5$ cells per well were cultured in microtitre plates (Costar, Cambridge, Ma., U.S.A.). Cells were stimulated with 3 µg/ml PHA (Wellcome) or 2.5 µg/ml Con A (Sigma). Earlier experiments showed that these concentrations of PHA and Con A provided maximum response. Control wells contained only cells and media. Cultures were incubated for 48 h in a humidified 5% CO$_2$ atmosphere at 37 °C. One µCi of methyl [³H]thymidine with a specific activity of 6.7 Ci/mmol (New England Nuclear) was added to each well. Plates were incubated an additional 18 to 20 h and cells were harvested in a Bellco microharvester (Vineland, N.J., U.S.A.). [³H]thymidine incorporation was measured in a scintillation counter. Cell viability was determined before and after incubation by staining with trypan blue and was found to be comparable for virus-infected and non-infected cells.

Skin grafting. A 2 x 3 cm piece of skin was removed from the abdominal area of donor TRF$_1$ mice (F$_1$ generation of T$_6$T$_6$ females mated to RFM males). RFM mice, acquired from Oak Ridge National Laboratory through the courtesy of Dr Robert Allen, were the
descendants of outbred RFM (Un) mice which had been derived from RF mice. The T6T6 (T6) Harwell-derived mice homozygous for the characteristic chromosome markers were originally provided by Dr John Trentin of Baylor University, Houston, Tx., U.S.A. Both strains have been inbred since their receipt. The TRF donor skin grafts were age- and sex-matched with recipient mice and grafted on to the backs of five normal and nine neonatally TA-infected MA Swiss mice 4 to 6 weeks old. The grafts were sutured with wound clips and considered rejected, when the donor skin became hard and necrotic.

**RESULTS**

**Examination of MA Swiss mice for natural infection and susceptibility to TA**

Previous reports show that TA infection may occur in some commercial mouse colonies as a natural infection and contribute to the production of variable results due to differences in susceptibility of individual mice (Rowe & Capps, 1961; Cross et al., 1979). Therefore, for our experiments MA Swiss mice were tested for the absence of TA to ensure that the virus did not occur as a natural infection. From 20 MA mice (retired from breeding), salivary glands were obtained, prepared as 10% extracts and inoculated into litters of newborn mice. Absence of thymic necrosis on macroscopic inspection 9 to 12 days after inoculation indicated that the exbreeders were free of TA.

Infectious virus titres and patterns of necrosis for MA Swiss mice were determined. The highest virus titres developed on day 7, and the maximum necrosis of the thymus was observed on day 8. The results were consistent with previous data on MA Swiss mice (Cross et al., 1979). Since these mice showed the same susceptibility patterns of the thymus to TA, they were selected for additional studies on the lymph nodes and spleen.

**Evidence for persistence of mouse thymic virus in spleen and lymph node cells**

To demonstrate virus persistence, newborn mice were injected i.p. with approx. 40 ID508 TA. The thymus, spleen and lymph nodes from 3 to 15 mice were harvested and frozen at −70 °C until virus assays were performed. The tissues were harvested 2, 5, 7, 10, 14, 30 and 42 days after injection. Lymph nodes and spleens were examined every month thereafter; thymuses were examined every 2 months until the seventh month. To determine the sensitivity of tissue extracts for detection of TA compared to injections of viable cells prepared from tissues of virus-infected mice, approx. 10⁶ viable cells in 0.05 ml Eagle’s MEM were injected into neonates.

No virus was detected in 10% extracts of mesenteric lymph nodes throughout the 7 months of testing. TA was found in low titres in spleen extracts 5 and 7 days after inoculation (Table 1). Virus titres were high in the thymus on days 5 and 7, and low by day 10. The presence of virus antigens in the thymus was confirmed by IFA tests for TA nuclear inclusions. After the acute infection subsided, virus was not detected in extracts of lymphatic tissues.

However, both the cyclic patterns observed in the responses of lymph node cells to Con A and PHA mitogens, and the extended period of abnormal pathology in the lymph nodes suggested probable persistence of virus in the lymphatic tissues. To avoid the loss of infectious virus that occurred in the freeze-and-thaw cycle of extract preparations, viable cells from virus-infected mice were inoculated into newborn mice. During the acute period of infection, TA was detected from lymph node, spleen and thymus cells from 1-week-old virus-infected mice (Table 2). At the time of peak necrosis, 2 and 3 weeks after inoculation, no virus was found in thymus, lymph nodes or spleens. As necrotic tissue was replaced with normal tissue, virus was detected in both the lymph nodes and thymus 8 weeks after inoculation and in the spleen after 12 weeks (Table 2).
Table 1. Comparison of the growth of mouse thymic virus in the thymus and spleen of inoculated newborn mice

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>Infectious virus in tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thymic necrosis†</td>
</tr>
<tr>
<td>2</td>
<td>0/8§</td>
</tr>
<tr>
<td>5</td>
<td>1/8</td>
</tr>
<tr>
<td>7</td>
<td>8/8</td>
</tr>
<tr>
<td>10</td>
<td>8/8</td>
</tr>
<tr>
<td>14</td>
<td>6/8</td>
</tr>
<tr>
<td>21</td>
<td>0/8</td>
</tr>
<tr>
<td>30</td>
<td>0/8</td>
</tr>
</tbody>
</table>

* MA Swiss mice were injected i.p. within 48 h of birth with 40 ID₉₀ TA in 0.05 ml. Spleens, thymuses and lymph nodes were harvested from 3 to 15 mice at each time point, prepared as 10% extracts and titrated in litters of newborn mice. Thymic necrosis was scored in these mice 9 to 12 days after inoculation.
† Correlation of the occurrence of macroscopic thymic necrosis with virus isolation.
‡ No virus was isolated from lymph node extracts. Thymus and spleen were negative for all times tested from day 30 to 210.
§ No. positive/no. observed.
‖ Expressed as ID₉₀ s/5 mg tissue.

Table 2. Evidence for persistent mouse thymic virus in lymphoid cells by inoculation of newborn mice with viable cells from virus-infected mice

<table>
<thead>
<tr>
<th>Time after infection (weeks)</th>
<th>Virus isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mesenteric lymph node</td>
</tr>
<tr>
<td>1</td>
<td>4/7†</td>
</tr>
<tr>
<td>2</td>
<td>0/12</td>
</tr>
<tr>
<td>3</td>
<td>0/10</td>
</tr>
<tr>
<td>5</td>
<td>8/8‡</td>
</tr>
<tr>
<td>6</td>
<td>9/9</td>
</tr>
<tr>
<td>8</td>
<td>9/16</td>
</tr>
<tr>
<td>12</td>
<td>0/6</td>
</tr>
<tr>
<td>27</td>
<td>0/16‡</td>
</tr>
<tr>
<td>36</td>
<td>0/12</td>
</tr>
</tbody>
</table>

* MA mice were injected i.p. within 48 h of birth with approx. 10⁶ viable cells from virus-infected mice. Macroscopic thymic necrosis was scored 9 to 12 days later.
† No. of mice with necrosis/no. observed.
‡ Brachial and axillary lymph nodes also tested.
§ ND, Not done.

Effects of mouse thymic virus infection on reactivity of lymph node cells to PHA and Con A

The PHA and Con A responses of mesenteric lymph node cells from neonatally infected mice was compared to that of normal cells 1, 2, 3, 4, 5, 6, 9, 10, 13, 15, 19, 28, 31, 5 and 39 weeks post-infection. Spleen cells from normal and virus-infected mice were tested at these time points for comparison. The data for each time assayed represented from two to five experiments with four to six replicate wells in each experiment. About 3 to 20 mesenteric nodes were pooled for each assay.

Responsiveness of lymph node cells to Con A was virtually non-existent 1 week following infection but gradually returned to normal by 4 weeks (Fig. 1). Reductions in Con A responsiveness occurred again at 13 and 28 weeks. Results of PHA stimulation were comparable to those of Con A (Fig. 2). Brachial and axillary nodes were also tested 9 and
Lymph node necrosis induced by TA

Fig. 1. Con A responses of mouse thymic virus-infected (——) and normal (-----) lymph node cells.

Fig. 2. PHA responses of mouse thymic virus-infected (——) and normal (-----) lymph node cells.

31.5 weeks following infection with similar results. Spleen cell responses to PHA and Con A from normal and virus-infected mice gave comparable results to previously published data (Cohen et al., 1975).

Comparison of total cell number in the lymph nodes of TA-infected and normal mice

Litters of newborn Swiss mice were injected i.p. with 13 ID$_{50}$s TA. Mesenteric lymph nodes were harvested from the infected mice and from age-matched normal controls. Two pools containing nodes from 5 to 20 mice were prepared at each time point for 5 consecutive weeks. The nodes were disrupted in glass tissue grinders and repetitively aspirated into pipettes to produce single-cell suspensions. Cell suspensions were counted and the average number of cells per mesenteric lymph node was computed. The number of cells in the mesenteric lymph nodes of TA-infected mice was significantly decreased ($P < 0.05$) at 1 and 2 weeks post-infection. By 3 weeks post-infection, the total number of cells in the mesenteric node of infected mice was comparable to that of normal mice.

Comparison of skin graft rejection time of normal mice to TA-infected mice

Normal mice were able to reject the skin grafts in an average of 13-6 days (range of 13 to 15 days). TA-infected mice required an average of 24 days to reject the graft (range of 17 to 30 days). Interestingly, one TA-infected mouse did not reject the TRF$_1$ skin graft, but died 30 days after grafting. Following autopsy, it was found that the thymus had not regenerated in this mouse. Histological sections of the skin showed no evidence of immunological reactions against the graft.

Histopathology studies of lymphoid tissue from TA-infected mice during the acute and chronic stages of infection

At various time points following neonatal infection with 40 ID$_{50}$s TA, at least 5 infected and 3 age-matched control mice were sacrificed and the thymuses, mesenteric lymph nodes
Fig. 3. Haematoxylin-eosin stain. Day 7, mesenteric lymph node from mouse infected within 24 h after birth with mouse thymic virus. Depletion of small lymphocytes except in subcortical area (between arrows) and reduction of overall cellularity are shown.

Fig. 4. Haematoxylin-eosin stain. Day 14, mesenteric lymph node from mouse infected within 24 h after birth with mouse thymic virus. Nuclear and cellular debris (short arrow), nuclear inclusion bodies (long arrow) and total destruction of small lymphocytes are shown. Only cells of reticular structure remain viable.
and spleens were removed for histological examination. Thymuses and spleens were examined on days 2 and 5 post-infection and all three tissues were examined on days 7, 10, 14, 21, 30, 35, 42, 70, 126 and 182. Tissues were fixed in neutral-buffered formalin and embedded in paraffin. Sections were cut and stained with haematoxylin and eosin. The histological pattern of thymic necrosis was found to conform to previously published reports (Rowe & Capps, 1961; Cross et al., 1979). Five days post-infection cellular breakdown occurred in the thymic medulla and nuclear inclusions were seen. By day 7, the thymus was markedly necrotic and nearly all the medullary cells contained viral inclusions. By day 10, giant cells were seen and 10% repopulation with lymphocytes had occurred. Thirty days after infection the thymus was 80% reconstituted. Six weeks post-infection, the thymus was histologically normal except for small areas of scarring and calcification of less than 5% total volume.

At 7 days post-infection, the mesenteric lymph nodes showed necrosis similar to that reported in the thymus (Fig. 3). By day 10 almost total lymph node necrosis with few viable cells had occurred and at day 14 necrosis was practically complete. The stroma was destroyed and nuclear inclusions were present (Fig. 4). The recovery process of the lymph node had begun by day 21 with an approx. 25% repopulation of the node with lymphocytes and the development of occasional giant cells (Fig. 5). The lymph nodes of mice examined on day 30 showed marked blast proliferation of the follicular centres and reticulum cell hyperplasia. Reticulum cell hyperplasia together with some residual necrosis was still found on day 35. Marked lymphocyte predominance occurred on day 42. By day 70 the lymph nodes appeared almost normal showing only focal residual fibrosis. Hyperplasia was still observed on day 126, but not on day 182.

The spleen showed moderate lymphocytic proliferation 5 days after infection. By day 10 atrophy of the peritrabecular lymphoid tissue as well as medullary hyperplasia were noted. A few inclusion bodies were also seen. By day 10 proliferation of perifollicular lymphoblasts as well as marked erythropoiesis and vacuolation of nuclei of individual lymphocytes of the
medulla was noticed. At 14 to 21 days there was a reduction in small lymphocytes, presence of giant cells and excessive haematopoiesis as well as nuclear inclusions and some cell necrosis. Lymphocyte reconstitution had begun 30 days post-infection and lymphoblastic proliferation persisted through day 42. By day 70 the spleen returned to a normal histological appearance.

All lymphoid tissues underwent necrosis following TA infection. It was more severe in the thymus, followed by lymph nodes and spleen. Recovery occurred first in the thymus, followed by the spleen and finally the lymph nodes.

DISCUSSION

Earlier reports had suggested that the lymph nodes and spleen were involved in TA infection (Cohen et al., 1975; Cross et al., 1976). This study demonstrates that histological changes and virus replication occur in both of these tissues. The mechanism by which the spleen and lymph nodes become infected is still unclear. It is possible that virus is transported to these tissues during the viraemia which occurs with the acute infection. Alternatively, infected thymocytes may leave the thymus and seed the spleen and lymph nodes. The first possibility is favoured at this time because necrosis is detected in the spleen and lymph nodes at approximately the same time as it is seen in the thymus.

Our virus isolation studies show that TA persists in lymphoid tissues for at least 8 weeks following neonatal infection. The inability to isolate virus from cell-free extracts suggests that the TA particles do not survive the freeze-thaw step in that procedure. Failure to demonstrate TA in the spleen and lymph nodes by IFA supports this. Additional tests are needed for quantifying the virus recovered from viable cell preparations. It would be interesting to see if immunosuppression prior to removal of organs for viable cell injection increases the incidence of TA isolation.

The histopathological study shows that the lymph node undergoes necrosis and recovery similar to that seen in the thymus. During the acute infection a significant reduction in the number of lymphocytes occurs in the mesenteric lymph nodes and spleens in TA-infected animals. The thymus recovers from the infection first, followed by the spleen and finally, the lymph nodes. At the time of maximum necrosis, little or no virus is isolated from the lymphoid organs. This would seem reasonable because few cells are left to support replication during the period of maximum necrosis.

Neonatal TA infection interferes with immunological function. Earlier work suggests a viral tropism for specific T lymphocyte subsets with subsequent immunological defects (Cohen et al., 1975; Cross et al., 1976). This study demonstrates that TA interferes with the ability to reject allogeneic skin grafts. TA-infected mice maintained skin grafts from histo-incompatible donors almost twice as long as normal controls.

The inability of cells from TA-infected lymph nodes to respond to PHA and Con A is similar to that reported earlier for spleen cells (Cohen et al., 1975). One week after infection virtually no response is detected. The responsiveness gradually returns to normal by week 4. Surprisingly, a cyclic pattern of response to these mitogens is demonstrated. Reduction in responsiveness occurs again 13 and 29 weeks post-infection. Further experimentation is needed to explain this cyclic response pattern. One possibility, which would agree with earlier data, is that the subset of lymphocytes responsible for mitogen responsiveness is also the subset susceptible to TA infection. The cycles may represent periods of infection and death of susceptible cells followed by regeneration.

It is possible that chronic infection is maintained in the lymph nodes and spleen by a specific subset of T cells or their precursors. Alternatively, the persistence of TA in high titres in the salivary glands may provide a source of virus for reinfection of lymphoid tissue (Cross
et al., 1979). Future studies may identify the specific subsets of T cells involved in TA infection and determine the role of these cells in the maintenance of infection.

Temporary thymic atrophy or necrosis due to nutritional or infectious stress in animals and man causes prolonged and profound changes in the immune status and host defence (Dutz et al., 1973; Smythe et al., 1971). Studies in premature human orphans with severe thymic atrophy due to infection and marasmus during the first 4 months of life showed persistent cell-mediated immune deficiency after 2 to 5 years of life with marked implications for the disease patterns in populations of the third world (Dutz et al., 1976). Recent studies in mice with intra-uterine malnutrition reveal a marked persistent immune deficiency, which manifests itself to a minor degree in the second generation offspring (Chandra, 1975). We suggest that this model of thymic necrosis is an excellent vehicle for examination of disease modulation in mice and to determine the type of T cell responsiveness and deficiency of the perinatal period.

This research was supported by American Cancer Society Grant IM-125 B.

REFERENCES


(Received 10 April 1981)