



Immunopathologic evaluation of experimental transmission of mouse thymic virus  
by Doris Elaine Do

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in  
Veterinary Science  
Montana State University  
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Abstract:

Experimental congenital infection with a murine herpesvirus, mouse thymic virus (TA), in BALB/c mice was evaluated as a potential model to study the immunopathologic effects induced by herpesviruses in utero. The specific objectives of this investigation were to; a) evaluate the ability of TA to cross the placental barrier, b) determine the ability of TA to induce abortion, early fetal death, stillbirth or fetal infection following either intravenous or intra-peritoneal inoculation of pregnant mice, c) characterize the lesions present in the TA-infected offspring, and d) define the immunologic status of the offspring.

Immunopathologic studies were performed to determine the effects of TA on susceptible newborn mice less than 24-hours-old. Findings of a positive infection in virus inoculated newborn mice were utilized as a control for the comparative evaluation of the offspring from inoculated pregnant mice. Histopathologic evaluation of thymuses from TA-infected newborn mice indicated a necrosis of the thymus followed by an inflammatory response and eventual regeneration of new thymic tissue.

A total of five congenital experiments were conducted to characterize pathological changes and immunological alterations. Pregnant mice were inoculated in first, second, and third trimesters of gestation. The thymic structure of newborn offspring was evaluated before nursing, after nursing on the original inoculated dam, and after grafting to uninoculated lactating adult mice. In that mice allowed to nurse their natural dam demonstrated no histopathologic changes equivalent to those observed in mice infected as newborns, it was concluded that virus was not passed in colostrum, milk, or saliva. Moreover, since those mice born to dams infected during pregnancy and subsequently grafted to noninfected lactating dams shortly after birth also displayed no histopathologic changes, it can be additionally concluded that detectable viral infection was not passed transplacentally. An incidental finding was observed in the thymuses of two seven-day-old offspring from a pregnant mouse which had been inoculated on day 17 of gestation. In these animals, an absence of demarcation between cortex and medulla and accompanying lymphodepletion was found. Results of subsequent experiments conducted to determine consistency of these findings were negative.

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APPROVAL

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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## ABBREVIATIONS

TA	Mouse thymic virus
CHV	Canine herpesvirus
IBR	Infectious bovine rhinotracheitis
FVR	Feline viral rhinotracheitis
MCMV	Mouse cytomegalovirus
PRV	Pseudorabies virus
i.p.	Intraperitoneally
PHA	Phytohemagglutinin
Con A	Concanavalin A
FCS	Fetal calf serum
IMDM	Serum-free Iscove's medium
BSA	Bovine serum albumin
H&E	Hematoxylin and Eosin
IL2	Interleukin 2
CTLL2	Long term murine interleukin 2 dependent T-cells
MMT	Mouse mammary tumor cells
CPE	Cytopathogenic effect
[ <sup>3</sup> H]-Tdr	Tritiated thymidine
cpm	Counts per minute
SEM	Standard error of the mean
BN	Before nursing
i.v.	Intravenous

ABBREVIATIONS (continued)

dpi	Days post-inoculation
DO	Day old
LM	Light microscopy
EM	Electron microscopy
NK	Natural killer
HSV-1	Herpes Simplex Virus type 1

## ABSTRACT

Experimental congenital infection with a murine herpesvirus, mouse thymic virus (TA), in BALB/c mice was evaluated as a potential model to study the immunopathologic effects induced by herpesviruses in utero. The specific objectives of this investigation were to: a) evaluate the ability of TA to cross the placental barrier, b) determine the ability of TA to induce abortion, early fetal death, stillbirth or fetal infection following either intravenous or intraperitoneal inoculation of pregnant mice, c) characterize the lesions present in the TA-infected offspring, and d) define the immunologic status of the offspring.

Immunopathologic studies were performed to determine the effects of TA on susceptible newborn mice less than 24-hours-old. Findings of a positive infection in virus inoculated newborn mice were utilized as a control for the comparative evaluation of the offspring from inoculated pregnant mice. Histopathologic evaluation of thymuses from TA-infected newborn mice indicated a necrosis of the thymus followed by an inflammatory response and eventual regeneration of new thymic tissue.

A total of five congenital experiments were conducted to characterize pathological changes and immunological alterations. Pregnant mice were inoculated in first, second, and third trimesters of gestation. The thymic structure of newborn offspring was evaluated before nursing, after nursing on the original inoculated dam, and after grafting to uninoculated lactating adult mice. In that mice allowed to nurse their natural dam demonstrated no histopathologic changes equivalent to those observed in mice infected as newborns, it was concluded that virus was not passed in colostrum, milk, or saliva. Moreover, since those mice born to dams infected during pregnancy and subsequently grafted to noninfected lactating dams shortly after birth also displayed no histopathologic changes, it can be additionally concluded that detectable viral infection was not passed transplacentally. An incidental finding was observed in the thymuses of two seven-day-old offspring from a pregnant mouse which had been inoculated on day 17 of gestation. In these animals, an absence of demarcation between cortex and medulla and accompanying lymphodepletion was found. Results of subsequent experiments conducted to determine consistency of these findings were negative.

## CHAPTER 1

## INTRODUCTION

Congenital viral infections can have devastating consequences on a developing fetus. In the broadest sense, congenital infections can potentially produce three deleterious effects: a) malformations, b) abnormal function with or without tissue damage, and c) latent infection with subsequent induction of disease (11). By far the most striking observations concerning human congenital defects were those made on infants whose mothers were infected with rubella virus early in pregnancy (51,88). In addition to rubella, it has been established that human maternal infection with cytomegalovirus (25,44), vaccinia (2), or herpes simplex (28,79,93) can result in fetal damage.

Basically, three principles are involved in the production of congenital viral diseases: a) the ability of the virus to infect the pregnant animal, b) the timing of infection in relation to the stage of gestation, and c) the nature of the virus and its capacity to produce disease in the fetus (11). The sequence of events that may occur prior to and following viral infection of the fetus are illustrated in Appendix A.

Rowe and Capps (76) isolated a herpesvirus, "thymic agent" (TA, also termed mouse thymic virus) which produced acute necrosis in the medulla and cortex of the thymus of neonatal mice. Whereas thymic necrosis was characteristic of the infection in newborn animals, the salivary glands of adult mice became chronically infected and released virus into the saliva (19). Since it was highly tropic for the thymus of newborn animals, thymic lymphocytes were deficient in T-cell functions, i.e., the ability to produce antibody to thymus-dependent antigens and to proliferate in response to certain lectins or to allogeneic cells (13). Consequently, TA-infection appears to affect the immune system in a manner similar to rubella virus in humans, lymphocytic choriomeningitis in mice, avian leukosis, murine leukemia (25), and infectious bursal disease of birds (36,43,81,82,83).

Perhaps the most interesting aspect of TA-infection is the way it affects newborn mice as compared to adult animals. TA could be isolated from blood and viscera of young mice infected as newborns (i.e. less than 24 hours of age), but only from the salivary glands of infected adults (19). Most herpesvirus infections of humans and animals have been shown to have an affinity for epithelial tissue and produce latent infections (30). Infection during pregnancy, however, can induce abortion or

generalized fetal infection (28,78,93). The association of equine (15,20,59,90,91), bovine (48,61,64), swine (23,42,50), canine (10,33,86), feline (39), and murine herpesviruses (44) with abortion and congenital infection suggests common pathogenetic mechanisms by which indigenous herpesviruses affect the gravid uterus.

Relatively little information is available regarding the pathogenesis of abortion, fetal death, or fetal infection caused by herpesviruses. In order to study the pathogeneses of congenital infections with herpesviruses an appropriate animal model is needed.

The purpose of this study was to investigate experimental congenital infection with mouse thymic virus in BALB/c mice as a potential model for the study of the immunopathologic effects induced by herpesviruses in humans and other animals infected in utero.

## CHAPTER 2

## LITERATURE REVIEW

CANINE

Canine herpesvirus (CHV), apparently widespread in the canine population (as indicated by serologic studies), has been repeatedly isolated from normal dogs (9). Small foci of necrosis and ulceration in the urogenital tract of the bitch were undetected by many investigators while attention focused on viral associated mild respiratory infection in older dogs (4,16). In contrast, newborn pups which acquire the virus via transplacental infection or by exposure in passage through the vagina of the bitch develop a peracute, fatal systemic infection (16,86). The CHV is capable of passing the placental barrier and causing disease and death of some of the fetuses (86). In other apparently healthy animals the virus remains latent, becoming activated under certain conditions (86). Puppies which survive for several days exhibit a disseminated nonsuppurative meningoencephalomyelitis characterized by focal and segmental destruction of gray and white matter and diffuse and focal microgliosis (71). Histopathologically, the lesions, characterized by focal degeneration, necrosis, and presence of intranuclear inclusion bodies in the placental labyrinth (32), are

similar to those reported in cats experimentally infected with feline herpesvirus (39). Stuart and associates (86) suggest that there is a close correlation between the occurrence of pup runts, stillbirths, and CHV infection in the bitch.

#### BOVINE

Infectious bovine rhinotracheitis (IBR), recognized for decades as a febrile catarrhal upper respiratory disease, typically occurs where cattle are congregated. In adults, it has been found that virus infection by aerosol produces multiple foci of necrosis in the nasal passages, pharynx, larynx, trachea, and large bronchi (30). On occasions the virus is shown to produce meningoencephalitis in calves (27), keratoconjunctivitis (1), or abortions in pregnant cows (12,48,61,64,69). Owen and coworkers (69) isolated virus from aborted, dead in utero, and live fetuses during both the first and third trimesters of pregnancy. The IBR virus was isolated from most fetal tissues having microscopic lesions and rarely from fetal tissues without lesions. Therefore there appears to be a relationship between the presence of virus and microscopic lesions. The virus enters the fetus directly by the fetal circulation (hematogenous route) or directly by trans- versing the placenta and amniotic fluid (placento-amniotic route) (69). The fetal lesions which characterize

abortions caused by the IBR virus include focal necrotizing hepatitis, necrotizing placentitis, and irregularly distributed focal necrotic lesions in other organs (48).

#### EQUINE

Abortion in mares caused by equine rhinopneumonitis virus, originally described by Doll (20), has been recognized as a disease entity in many parts of the world. In weanlings, the disease is manifested by a mild febrile reaction accompanied by a rhinitis or nasal catarrh which appears in the fall months (30). The mortality in sucklings and weanlings is negligible. In aborting mares, clinical signs are not evident, but there are characteristic lesions in the fetuses (59). Histological changes include widely disseminated focal hepatocellular necrosis characterized by typical eosinophilic intranuclear inclusion bodies (15,59,90), interstitial pneumonia, and a bronchopneumonia (59). The interlobular septa of the lungs are both edematous and infiltrated with mononuclear inflammatory cells, and intranuclear inclusion bodies are observed in bronchial and alveolar cells (90). Studies conducted by Corner and associates (15) revealed the previously unrecorded presence of typical inclusion bodies in the pancreas, kidney, small intestine, and myocardium.

#### FELINE

Feline viral rhinotracheitis (FVR) has been associated with acute febrile upper respiratory disease in the cat.

The characteristic histologic features of this disease include intranuclear inclusion bodies in the epithelial cells of the upper respiratory tract, tonsils and nictitating membranes (18). Clinically, the disease is characterized by fever, neutrophilic leukocytosis, paroxysmal sneezing and coughing, copious nasal exudate, dyspnea, anorexia, and pronounced weight loss (40). The virus is capable of causing several different ocular manifestations in affected cats, including ulcerative keratitis which closely resembles recurrent dendritic ulcerative keratitis in herpes simplex virus infection of humans (6).

Hoover and Griesemer (39) investigated experimental FVR infection in the pregnant cat and characterized the lesions produced in the uterus, placenta, and fetus. Intravenous inoculation of pregnant cats produces minimal illness in queens but results in abortion, intrauterine fetal death, and fetal infection. Placental lesions include multiple infarcts in the placental labyrinth, thrombosis of maternal vessels in the endometrium and placenta, and multifocal necrosis of the giant-cell trophoblast. Coagulative necrosis in the placental labyrinth has also been reported with congenital infection with herpes simplex virus (93) and IBR virus (64). Focal hepatic necrosis present in the fetus congenitally

infected with FRV is similar to the lesions associated with congenital infection by the equine (91), bovine (48,69), and human herpesviruses (34).

#### PORCINE

Pseudorabies virus (PRV, Aujeszky's disease) has long been recognized as a severe, highly fatal disease of newborn pigs in the United States (74). When virus is shed into the environment, overt disease is evidenced by abortion and stillbirths (50), tremors, and other signs of central nervous system disease in piglets (74); and is also a rapidly fatal disease of cattle (30). In most infections of adult pigs, PRV persists as a latent, sub-clinical infection of the nasopharynx. Small foci of necrosis and other evidence of herpesvirus infection are present in the nasal and tonsillar mucosa, and virus is shed in the nasal or oral secretions (30). In contrast to adult pigs, infection of the nasopharynx of neonatal piglets leads to disseminated encephalomyelitis which at times is rapidly fatal (14,74). Histopathologically, placental lesions are characterized by degeneration, necrosis, and presence of intranuclear inclusion bodies in the trophoblasts and mesenchymal cells of the chorionic fossae (42). These lesions are similar to those reported in CHV of dogs (32) and in cattle infected with IBR (48).

The isolation of PRV from the spleen and liver of aborted fetuses indicates the ability to cross the placental barrier, thus producing lesions in porcine fetuses and causing reproductive failure in sows (42).

#### CYTOMEGALOVIRUS

Cytomegalic inclusion disease has been reported in guinea pigs (63), mice (44,57), sheep (31), swine (23), and nonhuman primates (85). Nodules of hyperplastic epithelial cells with cytomegalic-type inclusions have also been observed in the lungs of elephants (60).

The highly species-specific cytomegaloviruses are capable of initiating prolonged active infections. Although they produce little, if any, clinically apparent disease in the adult, in some species they have an adverse effect on gestation (44). The human cytomegalovirus causes severe pathologic change in the fetus following placental transfer and invasion of fetal tissues (44). While studying the murine cytomegalovirus (MCMV) as a possible experimental model for the human infection, Mannini and Medearis (57) concluded that MCMV infection of pregnant mice causes fetal loss without evidence of fetal infection.

Inclusion body rhinitis of swine, caused by a cytomegalovirus type herpesvirus, has been shown to be principally a disease of the respiratory tract accompanied

by anemia in pigs less than four weeks of age (30). Cytomegalic inclusions are found in nasal mucosa, kidney, brain, liver, and adrenal cortex (47). Under experimental conditions, susceptible pregnant sows exhibit a mild disease accompanied by an increased number of mummified and stillborn fetuses. Virus can be isolated from various internal organs of some fetuses indicating transplacental transmission, and some survivors excrete virus and transmit the infection to other noninfected piglets (23).

In summary, the herpesvirus group includes a large number of cytopathogenic, epithelialtropic viruses in which there is a clear relationship between age and susceptibility. Neonatal animals succumb during a disease to which they would be relatively resistant a few weeks later, or become infected in utero. Such herpesviruses include: pseudorabies virus (14,74), canine herpesvirus (10,16,33,71,86), equine rhinopneumonitis (15,20,59,90), infectious bovine rhinotracheitis (12,48,61,69), feline rhinotracheitis (39), mouse cytomegalovirus (44,57), and inclusion body rhinitis (23). The evaluation of transplacental transmission with mouse thymic virus may contribute to a better understanding of the pathogenetic mechanisms involved in these disease processes.

## MOUSE THYMIC VIRUS

### Virus Isolation

The initial isolation of mouse thymic virus by Rowe and Capps (76), was an incidental finding during a blind passage series of a suspension of pooled lactating breast tissue, mammary tumor, and stomach contents of suckling mice. This suspension was inoculated into newborn out-bred mice and a blind passage series was initiated. No illness was observed until the fifth passage in which routine histologic sections indicated a small portion of the thymus was necrotic. These findings were consistent through 39 serial newborn mouse passages since the fifth passage of the initial series, with induction of thymic necrosis in 96% of mice examined at six to eight days, and 96% of the mice examined at 14 to 20 days. Twenty-one additional isolations of an apparently identical agent were made from tissues and mouth swabs of retired breeder mice of the same mouse colony. This isolation was indicative of a chronic phase.

### Ultrastructure

Electron microscopic studies conducted by Banfield (76) on thin sections of the thymus from infected mice six days post-inoculation indicated intranuclear and intracytoplasmic particles, the latter having an additional membrane, possibly of cellular origin. However, further

attempts were not made to identify or classify these particles due to a number of technical problems which made it difficult to obtain exact information on the virus.

Parker and associates (70) described some aspects of the morphology of the virus particle observed only in mice inoculated with TA. Intranuclear particles approximately 108 nm in diameter were limited by a single membrane and often had a complete nucleoid. The cytoplasmic and extracellular particles had a rough-surfaced outercoat measuring approximately 135 nm in diameter. The nucleoid of the particles in all locations was often a ribbon-shaped, oblong structure measuring approximately 74 by 45 nm. There was little evidence of margination of nuclear chromatin in any of the infected cells. There were accumulations of intranuclear filaments approximately 10 nm in diameter seen in cells with and without evidence of herpes-type particles. Based upon the morphology of TA particles described by Parker and associates (70), together with the physical properties of both heat and ether lability described by Rowe and Capps (76), this agent has been placed in the herpesvirus group.

#### Newborn Infection

When freshly prepared suspensions of infected tissue were inoculated into infant mice, (i.e. less than 24 hours

of age) they showed no evidence of overt disease or excess mortality (13). However, they exhibited a long lasting disability, associated with eye infections, a runting syndrome, hair loss, and enhanced susceptibility to infection.

Infection in newborn mice causes extensive necrosis of the thymic cortex and medulla from ten to 14 days after inoculation followed by recovery over several weeks. During the acute phase of infection, lasting about ten days, virus can be recovered from the thymus, salivary glands, blood, and viscera. For seven months after acute infection, virus can be demonstrated in the salivary glands but not elsewhere (19).

Cross and associates (19) conducted studies in which virus distribution in tissues of infected neonatal mice was examined. Litters of day old NIH Swiss mice were inoculated intraperitoneally (i.p.) with TA. At various points in time after infection, mice were bled and pools of thymuses, salivary glands, brains, and viscera (which included heart, lung, spleen, liver, and kidneys) were prepared. Samples from twenty mice were pooled between days one and 14 and on days 21 and 219; six mice comprised each sample. Results indicated that infectious virus was first detected in the thymus on day three, with a maximum titer of  $10^{3.5}$  ID<sub>50</sub> per 10. mg of tissue on day seven. Virus titers of the thymuses examined for TA were negative

from days 14 through 219. A viremia was present on day three with maximum titers on day seven, but by day ten viremia could not be detected. A trace amount of virus was detected on day 70 in a sample of undiluted blood (Refer to Appendix B).

#### Adult Infection

Cross and coworkers (19) evaluated adult NIH Swiss mice inoculated i.p. with TA. Extracts of pools of viscera and thymus were prepared from these animals at various points in time after inoculation. These pools were then inoculated into newborn mice, and the thymuses of these mice were passed once more into newborn mice. In contrast to the course of infection in newborn mice, a chronic infection of the salivary glands in adults became established without apparent infection of the thymus. No evidence of virus was detected in the thymus, brain, viscera, or blood. The histology of the thymus and lymph nodes harvested on days two through 14 was within normal limit (Refer to Appendix C).

Therefore, the analyses of data from both adult and neonatal infection with TA strongly suggests that this virus has a greater pathogenicity for newborn animals as manifested by thymic necrosis.

#### Pathogenesis in Newborn Mice

In a study reported by Cross and associates (19) on the pathogenesis of TA infection in newborn NIH Swiss mice,

both macroscopic and microscopic lesions of the thymus were evaluated. Histologic examination revealed nuclear inclusions on day five, but necrosis was not apparent macroscopically, even though infectious virus was detected in the thymus. Maximum thymic necrosis was observed on days ten and 14 but appeared first on day seven. Upon gross examination, visible lesions were first reported on day seven. Thymuses examined on days ten and 14 were very small and white, while focal necrosis was still visible as white areas on days 21 and 35. Scar tissue was the only visible lesion observed in thymuses 75 and 115 days post-inoculation.

Microscopic examination of tissues collected in the studies (19) revealed maximum inclusion bodies on day seven, which was directly correlated with virus titer. By day ten, nuclear inclusions decreased, virus titer dropped and maximum state of necrosis was observed. The medulla of the thymus had become a mass of necrotic material, and no areas rich in lymphocytes remained in the cortex. No nuclear inclusions were observed on day 14, but this was the first time giant cells were observed in the thymuses. The thymus appeared necrotic in some areas while in others, a granulomatous reaction was evident. On day 21 partial repopulation with thymocytes had occurred in the cortical areas of the thymuses and no

infectious virus was recovered. Giant cells were numerous with a granulomatous response. A histological granulomatous reaction was observed on day 35, with visible foci of necrosis. By day 77 the thymuses returned to normal except for some scar tissue. Cortical and medullary regions were clearly delineated.

Indirect fluorescent antibody tests of infected thymuses revealed nuclear fluorescence in cells of the cortical and medullary areas of the thymus, with the strongest fluorescence seen in the cortex (19). The size and shape of the nuclear inclusions in the cells varied, and many cells contained more than one inclusion. The presence of nuclear inclusions corresponded in time to the detection of the infectious agent by virus isolation.

Although the histology of the thymus was almost normal (partial repopulation with thymocytes) by day 21, the weight of thymuses from TA-infected animals was significantly less than normal controls until day 42 (19). Maximum weight loss correlated with maximum necrosis (seven and 14 days).

A more recent study conducted by Wood and associates (94) was undertaken to determine if spleen and mesenteric lymph node necrosis occurred as a result of TA infection of newborn mice. Through histopathologic evaluation it was determined that necrosis was more severe in the

thymus, followed by mesenteric lymph nodes and spleen. Reconstitution of the damaged tissue occurred first in the thymus, followed by the spleen and finally the lymph nodes. It was concluded that all lymphoid tissues underwent necrosis following TA-infection, however with a lesser degree in the secondary lymphoid organs.

#### Immunologic Evaluation of Infected Newborn Mice

Cohen and associates (13) have shown that mice infected at birth with TA had a severe but temporary impairment of T-cell functions. Splenocytes from such animals failed to undergo stimulation by T-cell lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A). There was a small amount of proliferation in response to pokeweed mitogen, a T-cell lectin known to have some B-cell stimulating capacity. Reactivity to all three lectins returned around five to six weeks after birth and was equivalent to that of controls at eight weeks. Reactivity to bacterial lipopolysaccharide, a B-cell lectin, was unimpaired in virus-infected animals. It was also reported that despite the profound loss of spleen cell reactivity, thymocyte PHA reactivity was intact in virus-infected mice.

Cohen et al. (13) reported that spleens also failed to yield a primary in vitro antibody response to a T-dependent antigen, sheep red blood cells, despite normal

responses to a T-independent antigen, trinitrophenol-ficoll. In addition, both spleen and thymus cells from young virus-infected animals had diminished proliferative responses to allogeneic cells, as well as a decreased ability to generate cells which mediate lysis of allogeneic target cells (killer T-cells). It could be concluded from these studies that infection with TA results in depression of some parameters of T-cell function, while sparing others. Moreover, TA apparently has no effect on B-cell function, independent of helper T-cells. These in vitro studies suggest that TA might selectively affect particular subpopulations of T-cells (i.e., helper, cytotoxic, or suppressor T-cells).

Morse and associates (65) evaluated the in vivo effect of neonatal infection with TA on the ontogeny of cells regulating the antibody response to type III pneumococcal polysaccharide. Their results indicate that the virus does not affect B-cell function or the development of suppressor T-cells, but causes a transient delay in the development of helper T-cell function.

## CHAPTER 3

## MATERIALS AND METHODS

MICE

A breeding colony of BALB/c mice was purchased from Charles River Inc. (Wilmington, MA). Animals derived from that colony were used throughout this study. During the course of experimentation, animals were maintained in a controlled environment and housed in Nalgene polycarbonate cages (Cat. no. 10712-154, VWR Scientific Inc., Seattle, WA) which were fitted with filter tops (Lab Products, Rochelle Park, NJ). Bedding (SAN-I-CEL, Paxton Processing Co., Inc., Paxton, IL), diet (Wayne MRH 22/5 Lab Blox, Neff's Animal Specialties, Missoula, MT), and water were steam sterilized before use. A routine animal health program was implemented in which the stock colony was periodically tested for the following viruses from serum submitted to Microbiological Associates (Bethesda, MD): mouse hepatitis virus, minute virus of mice, lymphocytic choriomeningitis virus, Sendai virus, and pneumonia virus of mice. Serum samples were also assayed for the enzyme lactate dehydrogenase by Vetpath (Teterboro, NJ).

Mice termed newborn in experiments were less than 24-hours-old and adult mice were six to eight weeks of

age. In congenital experiments, conception was initiated by leaving one male with three females for 24 hours at which time the male was removed and the day of removal was termed day one.

#### MEDIUM

Powdered RPMI 1640 medium (Gibco, Gand Island, NY) was prepared to 10X concentration in distilled, deionized water including  $\text{NaHCO}_3$ , filtered through a 0.22  $\mu\text{m}$  filter, and stored at 4° C. Prior to use it was diluted to single strength concentration with sterile, distilled, deionized water and supplemented with 2-mercaptoethanol (Sigma, St. Louis, MO) 50  $\mu\text{M}$ ; glutamine (Gibco), 2 mM; penicillin (Pfizer, Groton, CT), 50 IU/ml; and gentamicin (Shering, Kenilworth, NJ), 50  $\mu\text{g/ml}$ . The pH was adjusted to 7.1 with 1 N HCl and osmolarity adjusted to 300 mOsm with 8.5% saline or water. In some experiments, this medium was further supplemented with 10% fetal calf serum (FCS, Hy-Clone, Sterile Systems, Logan, UT).

Serum-free Iscove's medium (IMDM) was prepared as previously described (5). Powdered Dulbecco's Modified Eagle Medium (Gibco) was dissolved to 1.25X concentration in distilled, deionized water. It was then supplemented with the following:  $\text{NaHCO}_3$ , 31 mM; HEPES (Research Organics, Cleveland, OH), 25 mM; cystine-HCL (Sigma), 120  $\mu\text{M}$ ;  $\text{Na}_2\text{SeO}_3$  (Sigma), 160  $\mu\text{M}$ ; 2-mercaptoethanol (Sigma),

50 uM; fatty acid-free bovine serum albumin (BSA, Sigma), 14.5 uM; human transferrin (Behring Diagnostics, Somerville, NJ), 1.13 mM, 1/3-saturated with  $\text{FeCl}_3$ ; alanine (Sigma), 222 uM; asparagine (Sigma), 131 uM; aspartic acid (Sigma), 180 uM; glutamic acid (Sigma), 410 uM; proline (Sigma), 8.7 mM; sodium pyruvate (Gibco), 8.7 mM; biotin (Sigma), 23 uM; Vitamin B<sub>12</sub> (Sigma), 4.0 uM; glutamine (Gibco), 2.0 uM; penicillin (Pfizer), 50 IU/ml; and gentamicin (Schering), 50 ug/ml. A suspension of cholesterol (Sigma); 19 uM; linoleic acid (Sigma), 10 uM; and 1-oleyl-2-palmitoyl phosphatidyl choline (Sigma), 1.0 mM, was prepared in 1X DMEM containing 290 uM fatty acid-free BSA (Sigma). This suspension was sonicated at 4° C for 10 to 12 minutes at 80 watts and added to the previous mixture at a ration of 1:400 (volume/volume). The medium was brought to working concentration with distilled, deionized water, the pH adjusted to 7.1, and osmolarity adjusted to 300 mOsm. It was then filtered through a 0.22 um filter and stored in 500 ml aliquots at -76° C until used.

#### STOCK VIRUS

TA virus seed (TA 9940/21, Microbiological Associates) was the fifth newborn mouse passage comprising a 10% extract of thymuses, livers, spleens, kidneys, and adrenals. The stock virus, used in subsequent experiments, was prepared by passing the TA virus seed a total of five times in newborn BALB/c mice. Newborn mice were inoculated

intraperitoneally (i.p.) with 0.05 ml (1 ID<sub>50</sub>) of virus with a 1.0 ml syringe fitted with a 23 gauge by 1 inch needle. For passage, thymic tissues were harvested seven days after inoculation and all tissues were homogenized on ice in 15 ml Ten Broeck tissue homogenizers (Cat. no. 62400-530, VWR Scientific Inc.) in 1640 RPMI medium. To prepare a 10% extract, 9 parts of medium (by volume) were added to 1 part of tissue (by weight), and both medium and tissues were held in an ice bath at 4° C. The tissue suspension was centrifuged at 4° C for 20 minutes at 800 xg in a refrigerated centrifuge with a swinging bucket rotor. The supernatant was removed, aliquoted into 1.0 ml vials, quick frozen on dry ice, and stored in both liquid nitrogen and at -64° C in a Kelvinator freezer. All experiments were carried out with frozen virus passages #3, 4, and 5.

The supernatant from the stock virus preparation was filtered through a 0.45 um Swiney filter. It was then inoculated i.p. (0.05 mls, 1 ID<sub>50</sub>, as before) into newborn mice. Thymuses were harvested seven days post-inoculation and evaluated histologically.

#### Electron Microscopy

To verify the presence of TA virus, the pellet from the stock virus preparation (passage #3) was negatively stained and observed with an electron microscope. The stock pool of virus was centrifuged at a low speed to pellet

the cells. The supernatant fluid was discarded and the pellet resuspended in 2.0 ml of sterile distilled water. Four to six drops of the suspension were added to a spot well containing two drops of 4% phosphotungstic acid, two drops of freshly prepared 0.3% suspension of BSA, and 15 to 30 drops of distilled water. The resulting suspension was mixed in a pipette and placed into an all-glass nebulizer (Ted Pella Inc., Tustin, CA). The preparation was sprayed onto a carbon coated formvar filmed grid and immediately examined using a JEOL 100CX transmission electron microscope operated at 80-100 KV.

#### Virus Titration

Since a tissue culture system had not been established for TA, it was necessary to titer the virus in newborn mice according to the method of Reed and Muench (75). Newborn mice were inoculated i.p. with 0.05 ml (1 ID<sub>50</sub>) of TA passage #3. The stock virus was diluted (serial log<sub>10</sub> dilutions) from 10<sup>-2</sup> to 10<sup>-8</sup> in RPMI 1640 medium, and control animals were inoculated with medium only since sham thymuses from seven-day-old normal BALB/c mice were not available at the time. Thymuses were harvested seven days post-inoculation and the following parameters evaluated: a) thymic weights, b) total body weights, c) imprints of the thymus, Carnoy's fixed (77) and stained with a rapid hematoxylin and eosin (H&E), and d) light microscopy samples of the thymus, fixed in 9 parts

mercuric chloride and 1 part concentrated formalin (76), paraffin embedded, and stained with a routine H&E.

Light microscopy sections of thymuses from individual mice were scored from 0 to +3 as follows: 0 normal thymus, +1 isolated foci of necrosis, +2 larger areas of necrosis with maintenance of thymic architecture, +3 the medullary area consisted of a mass of necrotic material and the cortex contained depleted areas of lymphocytes and scattered necrotic debris.

#### Test of Stock Virus for Murine Contaminants

The stock virus (passage #3) was tested for murine contaminants included in the routine animal health program. Sixteen female 35-day-old BALB/c mice were obtained from Charles River Inc.. Throughout the experiments they were maintained in a Trexler Flexible plastic isolator (Snyder Manufacturing Company, New Philadelphia, OH) with a blower, air filter, entry portal, and exhaust trap. Mice were divided into two groups consisting of eight mice each: TA-inoculated and sham-inoculated (thymus extract from normal seven-day-old BALB/c mice). Animals were inoculated i.p. with 0.5 ml ( $10^{5.0}$  ID<sub>50</sub>) of virus or normal thymus extract. The serum from each group was pooled at the end of the sixth week and tested for the presence of potential murine contaminants previously mentioned.

ADAPTION TO TISSUE CULTURE SYSTEM

Long term murine interleukin 2 (IL2) dependent T-cells (CTLL2, Dr. Paul Baker, Veterinary Research Lab, Bozeman, MT), mouse mammary tumor cells (MMT 060562, American Type Culture Collection, Rockville, MD), McCoy's cells (Flow Labs, Inglewood, CA), and cultured thymocytes from TA-inoculated newborn mice seven days post-inoculation were utilized as host cells. All cells were incubated at 37° C in a humidified atmosphere with 5% CO<sub>2</sub>.

Long term T-cell line

CTLL2 cells (5.0 ml) were seeded at  $5 \times 10^4$ /ml in a tissue culture flask (#3018, Falcon, Division Becton-Dickinson, Oxnard, CA) containing IMDM and 1.0 unit/ml of rat IL2. After two days in culture 1.0 ml (20 ID<sub>50</sub>) of passage #3 was added to the flask.

Mouse Mammary Tumor Cells

The use of mouse mammary tumor cells (MMT) as a host cell line for TA replication was based upon the initial isolation of the virus by Rowe and Capps (76). Cells (5.0 ml) were seeded at  $1 \times 10^5$ /ml in tissue culture flasks (#3018, Falcon) with IMDM supplemented with 5% FCS. Upon confluency, the cells were washed with phosphate buffered saline; and 3 to 4 ml of trypsin were added to the monolayer for one minute, removed and the flask incubated for ten minutes. Five ml of medium were added, cells resuspended and passed at a 1:10 dilution in IMDM containing 5% FCS.

Upon the removal of medium after two days in culture, cells were infected with 1.0 ml of TA passage #3 (20 ID<sub>50</sub>) and incubated for one hour. Four ml of IMDM supplemented with 5% FCS were added, incubated, and observed for cytopathogenic effect (CPE). Supernatant was removed, aliquoted into 1.0 ml vials, and stored at -64° C.

In order to possibly enhance viral attachment to MMT cells (7), DEAE-dextran (Sigma) prepared in sterile, distilled deionized water at a concentration of 10 mg/ml was added to the culture medium at a final concentration of 50 ug/ml. Cells (100 ul) were adjusted to  $1 \times 10^5$ /ml and plated in triplicate in sterile, 96-well, flat bottom microtiter plates (#IS-FB 96TC, Linbro) with varying concentrations of dextran: 200, 100, 50, 25, 12, 0 ug/ml. Both noninfected (medium only, IMDM supplemented with 5% FCS) and TA-infected (passage #3 or MMT infected) supernatants were cultured for various periods of time.

At times indicated, wells were pulsed with 50 ul of culture medium containing 10 uCi/ml [<sup>3</sup>H]-Tdr (sp. act., 1.9 Ci/mM, Schwartz/Mann, Spring Valley, NY). Plates were then incubated for an additional four hours prior to harvesting via a Bellco automated sample harvester. Glass fiber filter strips containing [<sup>3</sup>H]-Tdr-labeled well contents were air dried, placed

into a toluene-base cocktail (#NEF-903, New England Nuclear, Boston, MA), and counted for 0.5 minute on a Beckman LS100C liquid scintillation counter. Results were expressed as the mean cpm  $\pm$  1 SEM for triplicate wells.

MMT cells were cultured on sterile 12 mm cover glass discs (Rochester Scientific Co. Inc., Rochester, NY) placed in 24 well tissue cluster plates (Costar #3524, Linbro, Cambridge, MA). One drop (100  $\mu$ l) of cells at a concentration of  $1 \times 10^5$ /ml was plated per coverslip and incubated for one hour to ensure adherence. One ml of medium, IMDM supplemented with 5% FCS, was added and coverslips were incubated until confluent. At this time medium was aspirated off and cells inoculated with 100  $\mu$ l of virus passage #3 with the addition of 100  $\mu$ g/ml of dextran plus appropriate controls (TA + 0  $\mu$ g/ml dextran, medium + 100  $\mu$ g/ml dextran, medium + 0  $\mu$ g/ml dextran). After 45 minutes of incubation, coverslips were washed with 1.0 ml of medium and replaced with fresh medium. At various times, supernatants were removed and frozen and coverslips were fixed in Carnoy's fluid stained with a rapid H&E.

The supernatant from MMT-infected coverslips was inoculated (0.2 ml, 4 ID<sub>50</sub>) into newborn mice, harvested seven days post-inoculation, and thymuses were

evaluated histologically. Tissues were fixed in mercuric chloride concentrated formalin and stained with a routine H&E.

#### McCoy's Cells

Similar procedures utilized in the cultivation, infecting, and pulsing of MMT cells in microtiter plates and coverslips were followed for McCoy's cells, except that RPMI 1640 medium supplemented with 10% FCS was used to culture the cells.

#### Thymocytes from TA-Inoculated Newborn Mice

Thymuses from TA-inoculated newborn mice seven days post-inoculation were removed aseptically and placed in a petri dish containing sterile RPMI 1640 medium supplemented with glutamine (25 mM), penicillin (50 IU/ml), and gentamicin (50 ug/ml). Thymic pieces were repeatedly forced through a 10.0 ml syringe (without a needle) and transferred to a new petri dish containing medium. Thymic parenchyma was separated from supportive connective tissue by passing through a 20 gauge needle and placed into a 50 ml centrifuge tube to settle for ten minutes. The suspension was transferred with a pasteur pipette and centrifuged for ten minutes at 350 xg. The supernatant was then removed with a pasteur pipette and the pellet suspended in 200 ul (per thymus) of RPMI 1640 medium supplemented with 10% FCS and crude rat IL2, 1.0 unit/ml. Cells (100 ul) were plated on 12 mm glass coverslips in

a 24 well cluster plate (#3524, Linbro) with appropriate controls (i.e. thymocytes from sham-inoculated newborn mice seven days post-inoculation). Based upon observations of the cells in culture, coverslips were stained with an  $\alpha$ naphthyl acetate esterase stain (Sigma). The supernatant from cultured cells was inoculated (0.2 ml, 4 ID<sub>50</sub>) into newborn mice, and thymuses were harvested at seven days post-inoculation and evaluated histologically.

#### EVALUATION OF CONGENITAL TRANSMISSION

Since the exact time of onset of thymic necrosis in congenitally infected newborn animals was unknown, a pilot experiment was conducted to determine the specific time of infection. After this preliminary experiment, subsequent investigations would be conducted for this sequence and analysis performed in the areas of pathology and immunology.

#### Pathologic Evaluation

Both macroscopic and microscopic pathologic changes induced by the virus in TA-inoculated newborn animals were utilized as a positive control for the histologic evaluation of mice infected in utero. Newborn mice inoculated with 0.2 ml (4 ID<sub>50</sub>) of virus were sacrificed at various time intervals and thymuses were fixed in a mercuric chloride concentrated formalin fixative, paraffin embedded, and stained with a routine H&E.

The following five congenital experiments were conducted to evaluate both pathologic and immunologic investigations. Evaluation in all experiments included histopathologic analysis of individual thymuses including light microscopy samples (mercuric chloride concentrated formalin fixed, stained with a routine H&E), thymic imprints (Carnoy's fixed, rapid H&E stained), and thymic weights.

#### Experiment #1

The purpose of this pilot experiment was to examine the ability of TA to pass the placental barrier and induce an infection with pathologic changes in the offspring. The ability of the virus to induce such changes may be governed by the time in gestation in which the pregnant females were inoculated, therefore the inoculation schedule was varied. Pregnant BALB/c mice (four animals per day) were inoculated i.p. with 0.5 ml ( $10 \text{ ID}_{50}$ ) of virus passage #3 on days 1, 5, 10, 15, and 20 of gestation. Sham-inoculated controls were not included. Offspring were examined 1, 7, and 11 (one animal) days after birth to determine if an in utero infection had been established.

#### Experiment #2

The experimental design was based upon the results of the pilot experiment, specifically those obtained from the day 17 of gestation inoculation with 1.0 ml of TA

(10 ID<sub>50</sub>), in which the virus was present in the dam four days before delivery. The ability of the virus to pass through the placenta may be dose dependent (10 ID<sub>50</sub> vs 20 ID<sub>50</sub>) and the possibility of virus transfer in the milk was considered. Three pregnant females and one sham control were inoculated per day (days 10, 12, 14, and 16 of gestation) with 1.0 ml (20 ID<sub>50</sub>) of passage #4A. Offspring were examined before nursing (BN) and at seven days of age. Thymic extracts of day old mice (BN) from a female inoculated on day 16 of gestation were passed (0.2 ml, 4 ID<sub>50</sub>) into newborn BALB/c mice. The thymuses from these animals were harvested and examined at seven days post-inoculation.

#### Experiment #3

This experiment expanded upon the observations from the day 17 inoculation and additionally examined the possibilities of virus transfer by way of the milk and direct contact with the inoculated dam through the grafting of offspring (BN) from infected dams to uninoculated lactating animals. Four pregnant females plus one sham control were inoculated i.p. with 1.0 ml (20 ID<sub>50</sub>) of passage #4A on day 17 of gestation. Thymuses from offspring were evaluated on days 1, 5, and 7 after birth.

#### Experiment #4

The experiment was designed to repeat a day 17 of gestation inoculation (virus administered four days before

parturition) and took into consideration variables in earlier experiments. A variation in gestation period was compensated through the consideration of both a 20 and 21 day period. Previous experiments were conducted with two virus passages, passage #3 (used in experiment 1) and passage #4A (in experiments 2,3). Both passages were incorporated in this experiment. The route of inoculation may have a bearing on the ability of the virus to induce changes. In addition to i.p. 1.0 ml (20 ID<sub>50</sub>) inoculations used in earlier experiments, virus was administered via an intravenous (i.v.) route (0.2 ml, 4 ID<sub>50</sub>) into the retro-orbital sinus. Offspring were grafted to uninoculated animals (BN) and examined at one and seven days of age. Therefore the experimental design included the usage of two virus passages, two routes of inoculation and the grafting of offspring (BN). One to two animals were inoculated per group; sham inoculated animals were not included but normal BALB/c mice were used as controls for light samples. Salivary gland samples were evaluated histologically from two dams to observe viral inclusions which had been previously reported by Cross (19) in TA-inoculated adult mice.

#### Experiment #5

Offspring from animals inoculated in the first (days 1-7), second (8-14) and third (15-21) trimester of pregnancy were evaluated. Pregnant BALB/c mice were

inoculated with 1.0 ml (20 ID<sub>50</sub>) of passage #5B: first trimester: four mice, second trimester: five mice, third trimester: two mice. Control mice were inoculated with thymic extract from newborn mice seven days of age.

#### Immunologic Evaluation

In order to determine a more sensitive method of evaluation, the immunologic response of offspring infected on day 17 in utero was evaluated seven days after birth. Before congenitally infected newborn animals were analyzed, a known positive infection in such animals was established through the inoculation of newborn mice. These animals were then evaluated immunologically. TA-inoculated newborn mice seven days post-inoculation were the control animals used for the comparison of seven day old newborn mice infected on day 17 in utero. Therefore, this was an age matched control.

Thymuses and spleens from seven day old mice previously inoculated with TA at birth (0.5 ml, 10 ID<sub>50</sub>, passage #4B) were collected in sterile RPMI 1640 medium supplemented with glutamine (25 mM), penicillin (50 IU/ml), and gentamicin (50 ug/ml). The thymocyte suspension was prepared as previously described. Spleens placed in a petri dish containing medium were injected with a 1.0 ml tuberculin syringe containing additional medium and teased apart with forceps. The suspension was repeatedly forced

through a 10.0 ml syringe (without a needle) and transferred to a new petri dish containing medium. Splenic parenchyma was separated from supportive connective tissue by passing through a 20 gauge needle and placed into a centrifuge tube to settle for 20 minutes. The suspension was transferred to a second tube and centrifuged at 350 xg for ten minutes. The pelleted splenocytes were adjusted to  $1 \times 10^6$ /ml in RPMI 1640 medium supplemented with 10% FCS, and thymocytes were adjusted to  $2 \times 10^6$ /ml. Cells were cultured with Con A (two fold serially diluted with medium beginning with 10 ug/ml), crude rat IL2 containing a mitogenic dose of Con A, (1.0 unit/ml), and medium. Cells, lectin, IL2, and medium (100 ul) were plated in triplicate in sterile, 96-well, flat bottom microtiter plates (#IS-FB 96TC, Linbro) and incubated at 37° C in a humidified atmosphere at 5% CO<sub>2</sub>.

At times indicated, wells were pulsed with 50 ul of culture medium containing 10 uCi [<sup>3</sup>H]-Tdr/ml. Plates were then reincubated for an additional four hours prior to harvesting via a Bellco automated sample harvester and counted via liquid scintillation as described earlier. Results were expressed as the mean cpm  $\pm$  SEM for triplicate wells.

Thymocytes and splenocytes from congenitally-infected and sham-infected seven-day-old mice were prepared and

cultured as above except that Con A dilutions began with 5 ug/ml.

Offspring from IA-Inoculated Dam Bred to Littermates

Offspring from a dam inoculated on day 15 of gestation (1.0 ml, 10 ID<sub>50</sub>, passage #4B) were bred to littermates (six-weeks-old) and the thymuses of the offspring (second generation) were examined macroscopically at one and seven days of age.

## CHAPTER 4

## RESULTS

STOCK VIRUS

Histologic evaluation of thymuses from animals inoculated with a filtered preparation of passage #3 (1 ID<sub>50</sub>) and harvested seven days post-inoculation revealed extensive necrosis and scattered accumulations of nuclear debris as the result of karyorrhexis (Fig. 1).

Electron Microscopy

The negatively stained virion, constructed of capsomeres (Fig. 2) was very pleomorphic, ranging in size from 120 nm (Fig. 2) to 170 nm (Fig. 3).

Virus Titration

The virus titer calculated according to the method of Reed and Muench (75) in newborn mice inoculated with virus and harvested seven days post-inoculation was  $10^{2.6}$  (Refer to table 1). Light microscopy samples of infected thymuses indicated 100% necrosis for virus titered at  $10^{-2}$  and 16.6% necrosis for  $10^{-3}$  (Fig. 4). Thymic weights (Fig. 5) of TA-infected and control animals evaluated seven days post-inoculation revealed maximum weight loss in virus dilutions  $10^{-2}$  ( $9.45 \pm 5.16$  mg) and  $10^{-3}$  ( $17.04 \pm 6.60$  mg). Similarly, total body weights (Fig. 6) remained low at these

Table 1. Titration<sup>a</sup> of mouse thymic virus in newborn mice<sup>b</sup> according to the methods of Reed and Muench

DILUTION <sup>c</sup>	#ANIMALS IN LITTER	THYMIC <sup>e</sup> NECROSIS	% THYMIC NECROSIS	THYMIC WEIGHTS(mg)	TOTAL BODY WEIGHTS (g)	SCORE <sup>f</sup>
-2	4	4	100	9.45 ( <u>±</u> 5.16)	4.41 ( <u>±</u> .28)	3,3,3,2
-3	6	1	16.6	17.04 ( <u>±</u> 6.60)	4.37 ( <u>±</u> .29)	2,0.....
-4	6	0	0	23.62 ( <u>±</u> 10.47)	5.37 ( <u>±</u> .37)	0.....
-5	7	0	0	17.46 ( <u>±</u> 7.99)	4.72 ( <u>±</u> .35)	0.....
-6	6	0	0	26.42 ( <u>±</u> 2.53)	5.47 ( <u>±</u> .21)	0.....
-7	9	0	0	24.33 ( <u>±</u> 5.25)	4.66 ( <u>±</u> .33)	0.....
-8	5	0	0	29.62 ( <u>±</u> 3.99)	5.80 ( <u>±</u> .32)	0.....
control <sup>d</sup>	4	0	0	19.57 ( <u>±</u> 5.38)	4.96 ( <u>±</u> .29)	0.....

<sup>a</sup>Calculation of virus titer:  $10^{2.6}$

<sup>b</sup>BALB/c mice less than 24-hours-old were inoculated i.p. with 0.05 ml (1 ID<sub>50</sub>) of TA passage #3.

<sup>c</sup>Stock virus (TA passage #3) was diluted in RPMI 1640 medium.

<sup>d</sup>Mice were inoculated i.p. with 0.05 ml of RPMI 1640 medium.

<sup>e, f</sup>Based upon histologic evaluation.

dilutions,  $4.41 \pm .28$  g and  $4.37 \pm .29$  g respectively. The decrease in both thymic and total body weights correlated with the histologic finding of thymic necrosis. A fluctuation in the remaining weights was evident and may be attributed to individual variation.

Histopathologic evaluation of thymuses indicated the darkly stained cortical area of a 0 scored thymus appeared densely packed with thymocytes in comparison to the medulla, in which they were less abundant but replaced by reticular cells and supporting stroma (Fig. 7). A reduction in cortical area was apparent in the +2 scored thymus with subsequent maintenance of thymic architecture (Fig. 8). Massive necrosis was observed in a +3 thymus and individual cell necrosis was evident by the accumulation of nuclear debris (Fig. 9).

#### Test of Stock Virus for Murine Contaminants

Serum samples tested for contamination of murine viruses indicated the stock virus to be free of the following: mouse hepatitis virus, minute virus of mice, lymphocytic choriomeningitis virus, Sendai virus, and pneumonia virus of mice (Refer to Table 2). Samples were also examined for the enzyme lactate dehydrogenase which was found to be high in serum from TA-infected animals (4,521 IU/L) and low in the serum from sham-infected animals (1,496 IU/L), when compared to normal

Table 2. Laboratory results of serum samples tested for murine contaminants from TA- and sham-inoculated mice<sup>a</sup>

	<u>TA Serum</u>	<u>Sham Serum</u>
VIRUSES: <sup>b</sup>		
Pneumonia Virus of Mice (PVM)	----- <sup>c</sup>	-----
Sendai Virus	-----	-----
Minute Virus of Mice (MVM)	-----	-----
Mouse Hepatitis Virus (MHV)	-----	-----
Lymphocytic Choriomeningitis Virus (LCM)	-----	-----
ENZYME: <sup>d</sup>		
Lactate Dehydrogenase (LDH)	4,521 IU/L	1,496 IU/L

<sup>a</sup>Two groups consisting of eight female BALB/c mice (35-days-old) inoculated i.p. with 10 ID<sub>50</sub> of virus (passage #3) or normal thymic extract.

<sup>b</sup>Tested by Microbiological Associates, Bethesda, MD.

<sup>c</sup>Negative results

<sup>d</sup>Tested by Vetpath, Teterboro, NJ.

values ranging from 1,500 to 3,000 IU/L (67). The high values in the TA animals correlated with the presence of thymic necrosis.

#### ADAPTION TO TISSUE CULTURE

All cell lines utilized as host cells for TA replication were negative. However, when thymocytes from TA- and sham-inoculated ( $4 ID_{50}$ ) newborn animals harvested seven days post-inoculation were cultured with IL2, large cells were first observed on day six in culture. These cells were stained with  $\alpha$  naphthyl acetate esterase stain, which is an enzyme specific for cells of monocytic lineage and stains cytoplasmic granules black. RAW 264 cells, a known monocytic tumor cell line from BALB/c mice (73), were used as a positive stain control (Fig. 10). Monocytic cells were observed in cultures of normal thymocytes (Fig. 11), but a significant difference in size was noted as compared to cultured thymocytes from TA-inoculated animals (Fig. 12). The intensity of the granules almost obscured the nucleus.

Pathologic changes in the thymus of newborn animals inoculated ( $4 ID_{50}$ ) with supernatants from MMT-infected coverslips and cultured thymocytes from TA-inoculated newborn animals were not observed at seven days post-inoculation.

## EVALUATION OF CONGENITAL TRANSMISSION

Conditions encountered during in vivo congenital experiments included: a) cannibalism by adult mice towards newborn animals, b) the inability to determine pregnancy predominately in the first trimester, and c) the variation in gestation periods which interfered with inoculation schedules.

### Pathologic Evaluation

Both macroscopic and microscopic pathologic changes induced by the virus in the thymus of inoculated (4 ID<sub>50</sub>) newborn animals harvested seven days post-inoculation were utilized as a positive control for the evaluation of mice infected in utero.

### Gross Evaluation:

During the acute phase of the disease, TA-inoculated newborn mice exhibited a runted condition as compared to age matched control animals. A slight difference in total body size was first noted five days post-inoculation (Fig. 13) and the thymus appeared normal, still maintaining opacity (Fig. 14). However, at six days the thymus took on a more whitish color in comparison to that of control mice (Fig. 15, 16). The striking difference in total body size at seven days (Fig. 17) correlated with previous observations of maximum virus titer (19) and a reduction in thymic dimensions attributed to the

prominence of the lobes (Fig. 18). At ten days, normal thymic architecture was obscured and the organ appeared small and block-like (Fig. 19, 20). Histopathologic evaluation revealed that the thymus was at a stage of maximal necrosis at ten days. At day 23 a repair process was evident as the thymus regained opacity (Fig. 21), and the difference in total body size was less dramatic (Fig. 22). The left thymic lobe exhibited a mottled surface due to foci of necrosis (Fig. 23). The thymic structure was comparable to that of the normal control at both 29 (Fig. 24) and 35 days post-inoculation (Fig. 25).

#### Histopathologic Evaluation:

Through histopathologic evaluation it was evident that TA induced a necrosis of the thymus followed by a characteristic reaction referred to as granulomatous inflammation. Therefore, this inflammatory response set into motion a series of events which healed and aided in reconstitution of the damaged tissue. At six days post-inoculation, the cortical area was thinner in comparison to the medulla, a ratio of 1:3 as compared to a normal of 2:1 (Fig. 26). The cortex exhibited a paucity of cells with no clear demarcation from the medulla on day seven. The loss of normal thymic architecture was evident in the lymphodepleted cortical

and medullary areas (Fig. 27, 28). On days eight to ten, the thymus was observed at its height of necrosis in which diffuse necrosis (90%) was evident by the extension from the medulla to the capsule of degenerative cellular debris (Fig. 29). At this stage, intranuclear inclusion bodies were observed in the thymocytes with margination of nuclear chromatin (Fig. 30). On days 12 to 14 there was evidence of an inflammatory response, as indicated by the appearance of multinucleated giant cells (Fig. 31). Additionally, partial repopulation of thymocytes was apparent around blood vessels (Fig. 32) and the outer margin of the cortical area (Fig. 33). At 16 days, repopulation occurred locally as evidenced by an increase in mitotic figures (Fig. 34). Discrete foci of mineralized necrotic material were first observed on day 21 (Fig. 35) and progressed to day 24 at which time mineralization was prominent with multinucleated giant cells in close proximity (Fig. 36) and the majority of the medullary area was mineralized (Fig. 37). However, on days 29 to 35 isolated foci of mineralization were observed without evidence of giant cells (Fig. 38).

In summary, the pathologic changes induced by TA in the thymus of inoculated newborn mice included: a) thymic necrosis, initially beginning with the mature T-cells of the medulla and consequently spreading to the cortex, b) an inflammatory response in which multinucleated giant

cells phagocytized necrotic debris, c) mineralization of necrotic tissue (dystrophic calcification), and d) ultimately a partial repopulation of the thymus with new thymocytes perhaps from remaining viable cells of the thymus and/or from a nonthymic source (i.e. bone marrow).

#### Experiment #1

Histologic evaluation of all thymic samples of offspring from dams inoculated with  $10 \text{ ID}_{50}$  of passage #3 on days 5, 10, 15, and 20 of gestation did not reveal pathologic changes. During the course of the experiment as these thymuses were harvested and macroscopic changes were not evident (block-like thymus), one pregnant female (day 17 of gestation) was inoculated with  $20 \text{ ID}_{50}$  of passage #3. Thymic evaluation of two seven-day-old runt offspring from this female revealed no demarcation between cortical and medullary areas (Fig. 39) accompanied by lymphodepletion and evidence of pyknotic nuclei (Fig. 40). It appeared that either the virus concentration ( $20 \text{ ID}_{50}$ ) or the day in gestation of inoculation, day 17, attributed to the experimental results.

#### Experiment #2

The offspring from adult mice inoculated with  $20 \text{ ID}_{50}$  of passage #4 on days 10, 12, 14, and 16 of gestation, examined before nursing and at seven-days-old were not runt and thymuses were histologically comparable to sham-inoculated controls. Additionally, histologic evaluation

of thymuses from newborn mice (seven days post-inoculation) which had been inoculated with thymic extracts ( $4 ID_{50}$ ) from day old mice (BN) infected on day 16 of gestation did not reveal alteration of thymic structure. Therefore the experiment indicated the virus concentration  $20 ID_{50}$  had no bearing on the ability of the virus to induce pathologic changes of the thymus of animals inoculated in utero.

#### Experiment #3

This experiment was designed to repeat results of a day 17 inoculation with  $20 ID_{50}$  of virus administered four days before parturition (from Expt. #1). However due to variation in gestation period, animals delivered one to three days post-inoculation. Two offspring (nursed by an uninoculated female) from a dam administered virus one day before delivery exhibited a runt condition as compared to littermates. Thymuses from these five-day-old animals displayed a reduction of the cortical area by  $\frac{1}{2}$  (Fig. 41) compared to a five-day-old normal control (Fig. 42). Medullary thymocytes remained well defined (Fig. 43).

#### Experiment #4

Two virus passages and routes of inoculation were incorporated into the experiment. Three pregnant mice were inoculated i.p. with  $20 ID_{50}$  of virus four days before parturition. Two of these animals were inoculated with

passage #4, of which the offspring of one dam were switched to an uninoculated female. Offspring of the third animal (inoculated with passage #3) were nursed by the original dam. Histopathologic evaluation of thymuses from one- and seven-day-old offspring switched to uninoculated animals and those nursed by the same dam did not reveal alteration of thymic structure. Additionally, histologic evaluation of salivary glands from these inoculated adults seven days following parturition did not reveal viral inclusions.

#### Experiment #5

Thymuses from seven-day-old offspring of dams inoculated in first (day 1-7), second (8-14), and third (15-21) trimester of pregnancy did not exhibit pathologic changes when compared to thymuses from seven-day-old mice of sham-inoculated females.

#### Immunologic Evaluation

Incorporated [<sup>3</sup>H]-Tdr Response of Inoculated Newborn Mice:

The proliferative response of spleen and thymus cells obtained from sham- and TA-infected newborn mice (harvested seven days post-inoculation) to IL2 and various concentrations of Con A was measured as cpm of incorporated [<sup>3</sup>H]-Tdr.

Thymocytes: Thymocytes from sham-infected newborn mice cultured with Con A remained unstimulated on days one through five compared to those of TA-infected newborn mice (Fig. 44). One day after initiation of culture of

thymocytes from TA-infected newborns, [ $^3\text{H}$ ]-Tdr incorporation peaked at 5.0 ug/ml Con A with a maximum of 19,810  $\pm$  1,208 cpm compared to sham-infected newborn thymocytes of 1,980  $\pm$  146 cpm. On succeeding days 2, 3, 4, and 5 stimulation was not observed.

Optimum T-cell blast transformation in IL2 of thymocytes from TA-infected newborns resulted on days 1, 2, and 3 after initiation of culture as evidenced by cpm of incorporated [ $^3\text{H}$ ]-Tdr of 14,430  $\pm$  160 cpm, 14,819  $\pm$  1,099 cpm, and 8,534  $\pm$  619 cpm respectively, in comparison to thymocytes from sham-infected newborn animals which did not incorporate radionucleotide label in IL2 significantly greater than medium alone (Fig. 45).

Splenocytes: The proliferative response of splenocytes from sham- and TA-infected newborns cultured with various concentrations of Con A was assayed (Fig. 46). Splenocytes from TA-infected newborn mice were unstimulated on days 2, 3, 4, and 5 at which time counts remained below 5,000 cpm of incorporated [ $^3\text{H}$ ]-Tdr. Maximal activity was found to be that of splenocytes from sham-inoculated newborn mice stimulated with 10.0 ug/ml Con A, harvested two days after initial culture and consequently exhibiting counts of 34,359  $\pm$  964 cpm compared to splenocytes from TA-infected animals of 3,600  $\pm$  355 cpm which gradually decreased to 2,441  $\pm$  307 cpm on day five.

Splenocytes from TA-inoculated animals cultured in both medium and IL2 did not exhibit significant blastogenesis on day 2 ( $2,327 \pm 205$  cpm,  $5,075 \pm 388$  cpm respectively), day 3 ( $2,130 \pm 300$  cpm,  $2,262 \pm 246$  cpm respectively), day 4 ( $1,815 \pm 234$  cpm,  $2,007 \pm 153$  cpm respectively), and day 5 ( $1,233 \pm 196$  cpm,  $1,437 \pm 299$  cpm respectively) (Fig. 47). Blast transformation in medium and IL2 was observed in cultured splenocytes from sham-inoculated mice from day one to day five with maximum counts of  $3,140 \pm 203$  cpm (medium) and  $22,447 \pm 1,203$  cpm (IL2) on day three.

Incorporated [ $^3$ H]-Tdr Response of Offspring from an Inoculated Adult Mouse:

The proliferative response of spleen and thymus cells obtained from offspring (seven-days-old) of adults inoculated on day 17 of gestation was assayed with IL2 and various concentrations of Con A.

Thymocytes: Thymocytes harvested from offspring of sham- and TA-inoculated females cultured with Con A did not exhibit lymphocyte proliferation on days one through six. The cpm of incorporated [ $^3$ H]-Tdr for both offspring from TA- and sham-inoculated animals remained below 2,000 cpm (Fig. 48). This was compared to thymocytes from TA-inoculated newborn mice cultured with Con A which exhibited optimal blastogenesis on day one with a maximum of  $19,810 \pm 1,208$  cpm in 5.0 ug/ml Con A (Fig. 44).

Thymocytes from offspring of TA- and sham-inoculated females remained unstimulated in both medium and IL2 compared to thymocytes from TA-inoculated newborn animals cultured with IL2 in which lymphocyte blast transformation was observed on days 1, 2, and 3 with optimum blastogenesis on day 2 and a maximum count in IL2 of  $14,819 \pm 1,099$  cpm (Fig. 49).

Splenocytes: Splenocytes from offspring of both TA- and sham-inoculated females cultured with Con A did not exhibit lymphocyte blastogenesis on days 1, 2, 3, 4, and 6 (Fig. 50). However, splenocytes from offspring of sham-inoculated adults displayed a slight proliferation in 2.5 ug/ml Con A on day five with a maximum count of  $2,902 \pm 432$  cpm in contrast to splenocytes from offspring of TA-inoculated females with  $471 \pm 86$  cpm. This was not significant when compared to splenocytes from sham-inoculated newborns in which maximum counts were  $34,359 \pm 964$  cpm of incorporated [ $^3$ H]-Tdr (Fig. 46).

Splenocytes from offspring of TA- and sham-inoculated females cultured in both medium and IL2 exhibited a proliferative response on days one through six with optimal blastogenesis on day three (Fig. 51). Offspring of sham-inoculated females displayed maximum counts of  $230 \pm 36$  cpm,  $11,833 \pm 600$  cpm. Additionally, offspring from TA-inoculated females exhibited counts of  $975 \pm 368$  cpm,  $15,398 \pm 1,352$  cpm. However, the degree of stimulation

between the offspring of TA- and sham-inoculated females was not significantly different. This was compared to splenocytes from TA-inoculated newborn mice which did not exhibit blastogenesis in IL2 (Fig. 47).

Offspring from TA-Inoculated Dam Bred to Littermates

Macroscopic evaluation of thymuses from seven-day-old newborns of offspring originally born to a female inoculated on day 15 of gestation (20 ID<sub>50</sub>, passage #4B) did not reveal gross pathologic changes.

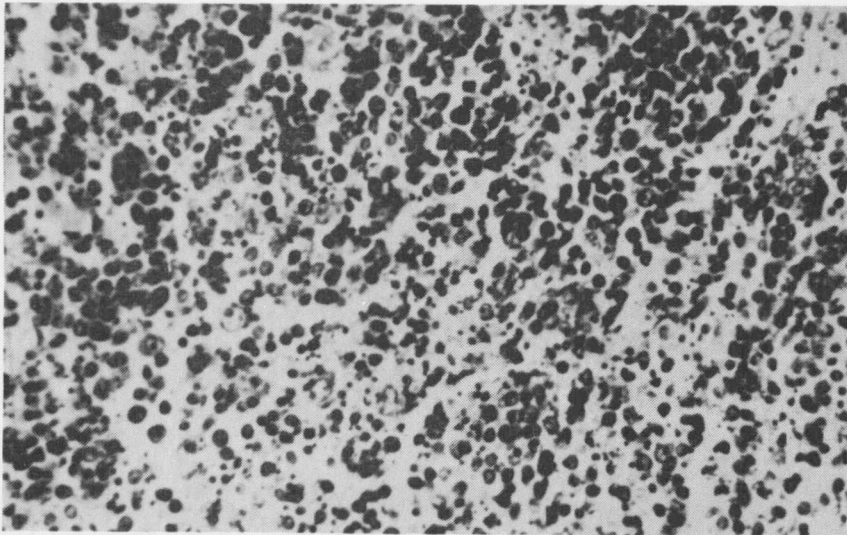


Fig. 1. LM. H&E of thymus from a newborn BALB/c mouse inoculated with filtered TA suspension (harvested seven days post-inoculation) exhibiting extensive necrosis in the medulla. X400.

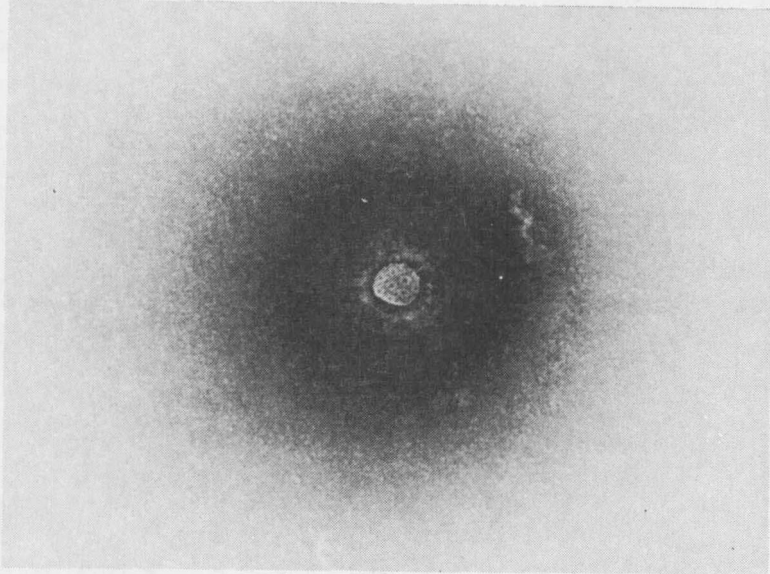


Fig. 2. EM. Negatively stained virion (120 nm) displaying capsomeres. X42,900.

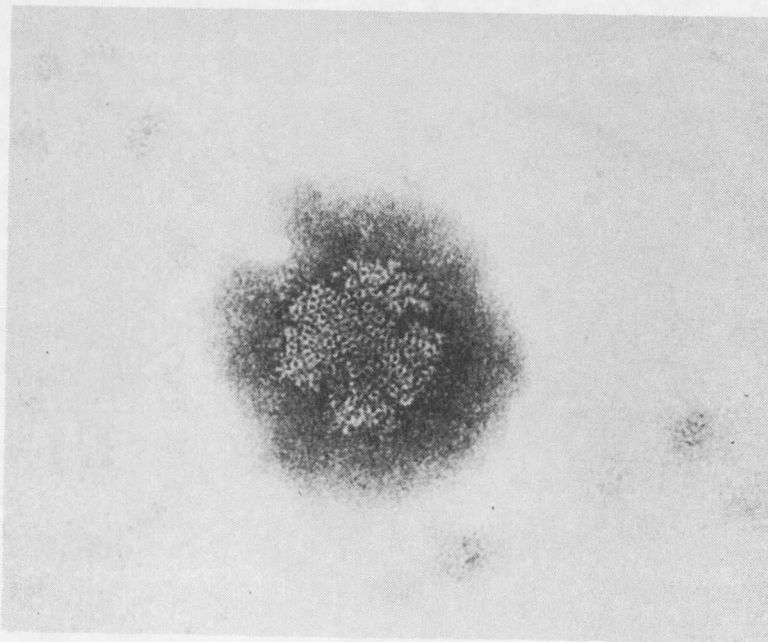


Fig. 3. EM. Negatively stained pleomorphic virion (170 nm). X65,000.

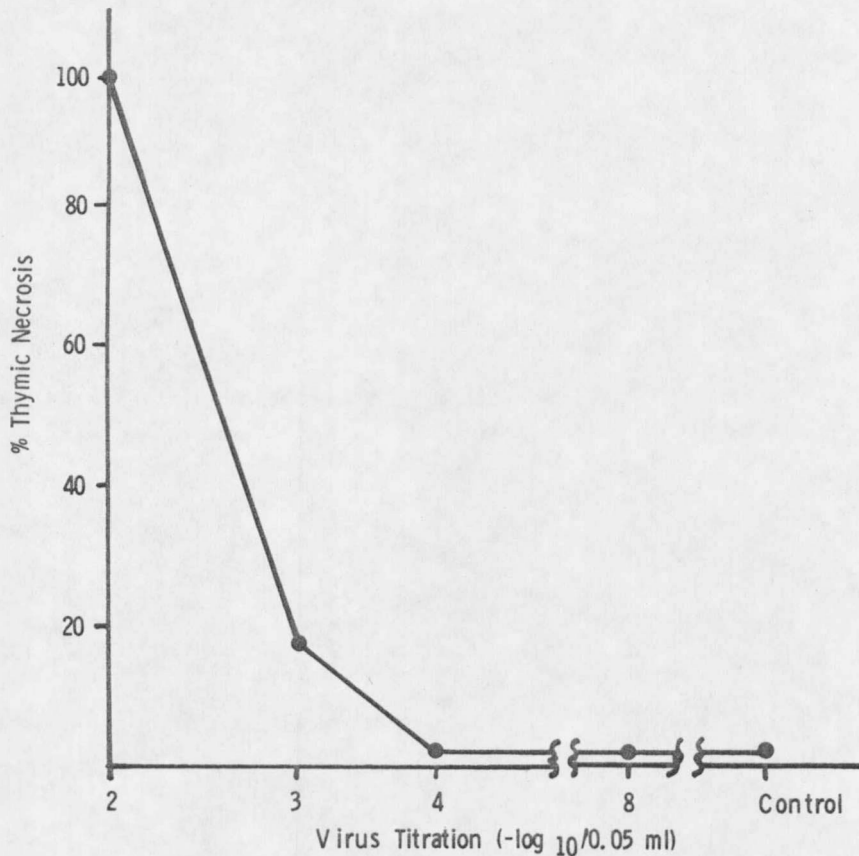


Fig. 4. Percent thymic necrosis based upon histologic evaluation of newborn mice (7 dpi) inoculated with TA. The virus titer as calculated according to the method of Reed and Muench was  $10^{2.6}$ . Newborn mice were inoculated i.p. with 0.05 ml (1 ID<sub>50</sub>) of virus. The stock virus was diluted (serial log dilutions) in RPMI 1640 medium and control animals were inoculated with medium only. One litter was inoculated per dilution ( $10^2$  through  $10^8$  plus controls) which included 4, 5, 5, 7, 6, 8, 5, 3 animals respectively. Thymic necrosis was evaluated according to: imprints of the thymus, Carnoy's fixed and stained with a rapid H&E, and light microscopy samples of the thymus, fixed in a mercuric chloride concentrated formalin fixative, paraffin embedded, and stained with a routine H&E.

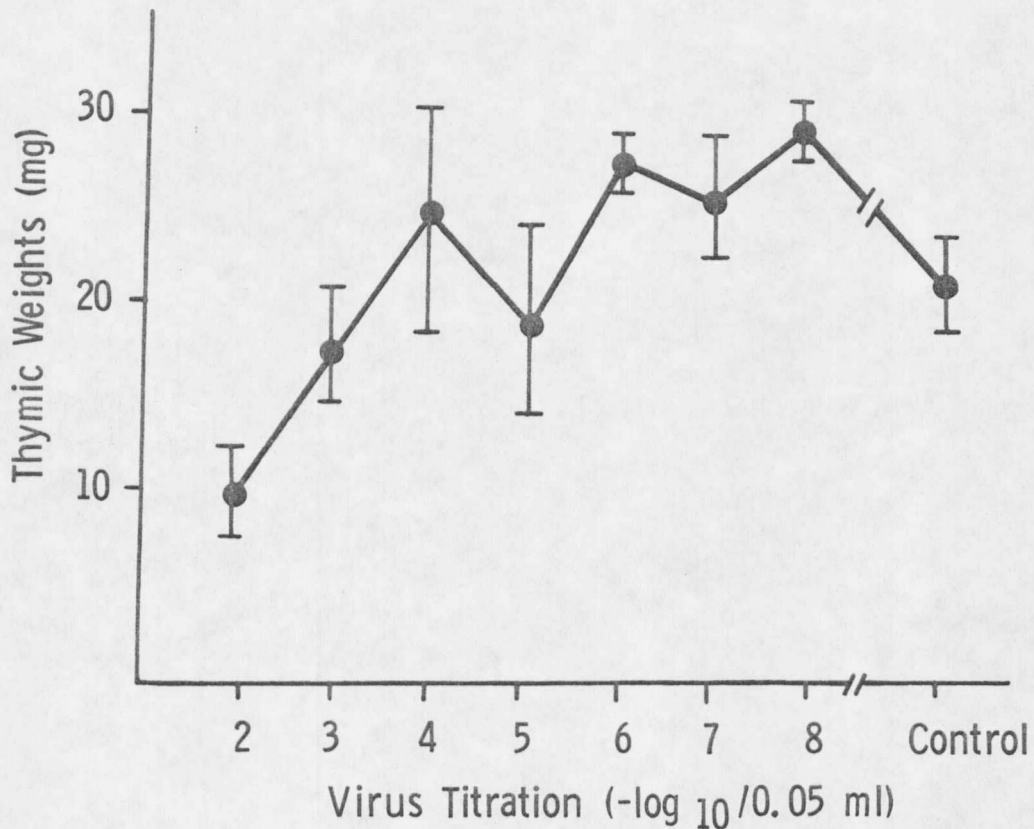


Fig. 5. Thymic weights of newborn mice (7 dpi) inoculated with TA with the standard error of litter samples at each point. The virus titer as calculated according to the method of Reed and Muench was  $10^{2.6}$ . Newborn mice were inoculated i.p. with 0.05 ml (1 ID<sub>50</sub>) of virus. The stock virus was diluted (serial log<sub>10</sub> dilutions) in RPMI 1640 medium and control animals were inoculated with medium only. One litter was inoculated per dilution (10<sup>2</sup> through 10<sup>8</sup> plus controls) which included 4, 5, 5, 7, 6, 8, 5, 3 animals respectively. Thymuses were harvested and individual weights were calculated.

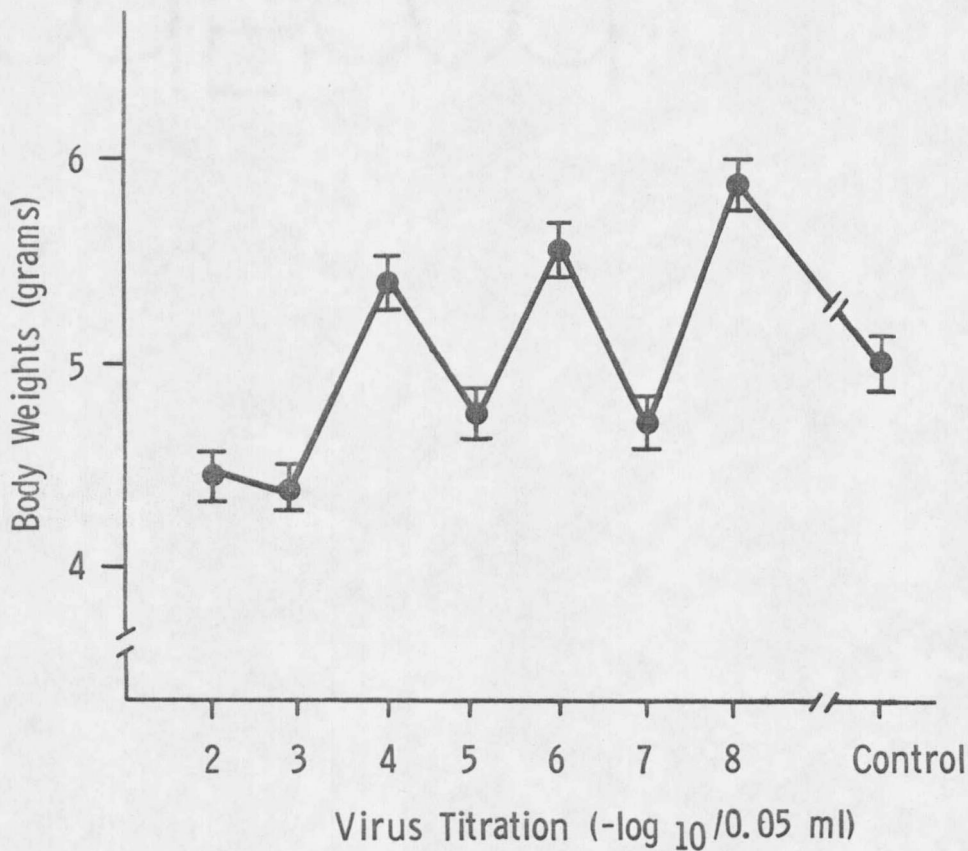


Fig. 6. Total body weights of newborn mice (7 dpi) inoculated with TA with the standard error of litter samples at each point. The virus titer as calculated according to the method of Reed and Muench was  $10^{2.6}$ . Newborn mice were inoculated i.p. with 0.05 ml (1 ID<sub>50</sub>) of virus. The stock virus was diluted (serial log<sub>10</sub> dilutions) in RPMI 1640 medium and control animals were inoculated with medium only. One litter was inoculated per dilution ( $10^2$  through  $10^8$  plus controls) which included 4, 5, 5, 7, 6, 8, 5, 3 animals respectively. Animals were euthanized and total body weights were calculated.

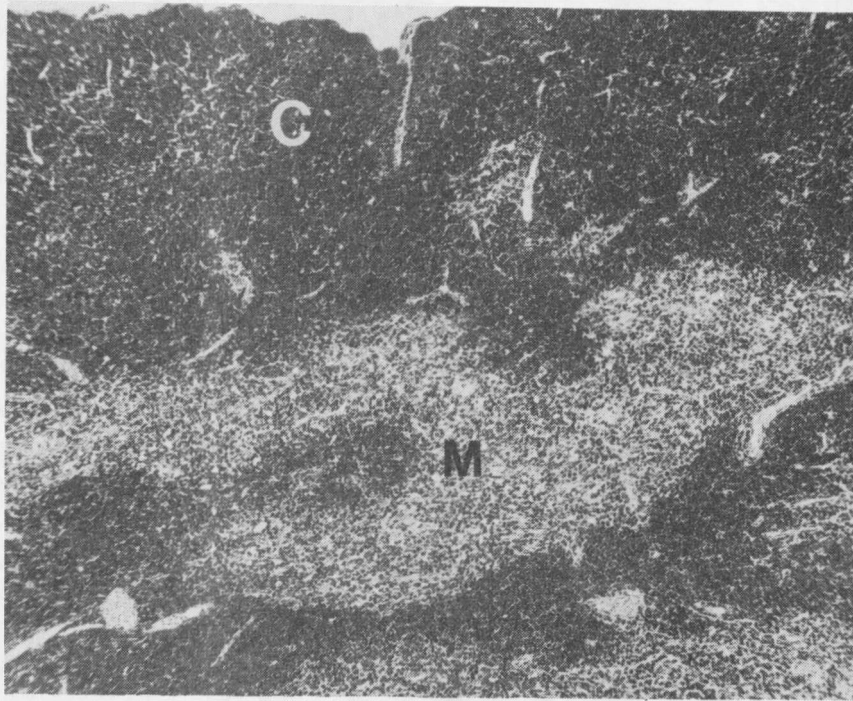


Fig. 7. LM. H&E of 0 scored thymus from a control newborn mouse. Densely packed thymocytes in the cortical area (C), medulla (M) composed of reticular cells and supporting stroma. X100.

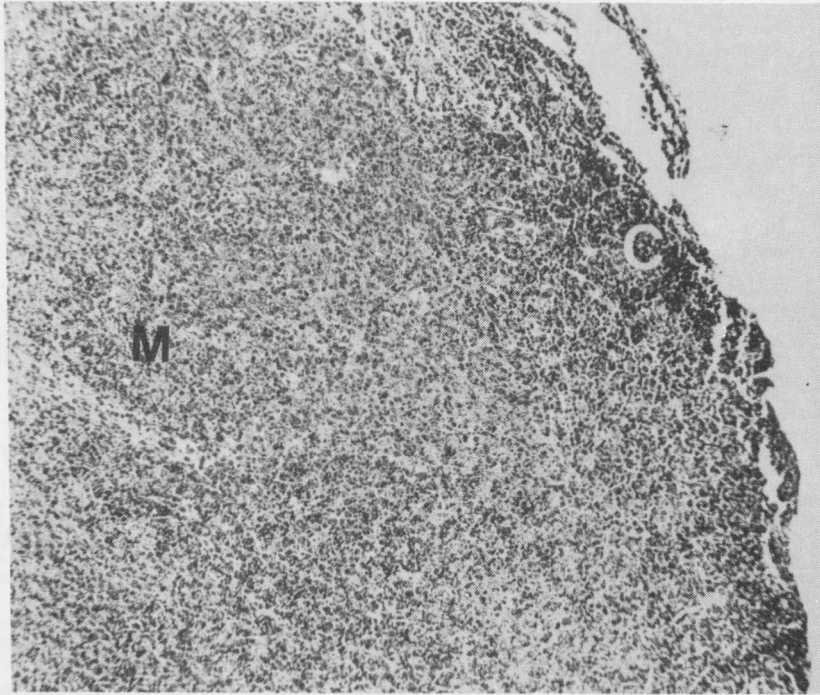


Fig. 8. LM. H&E of +2 scored thymus from a virus infected newborn mouse harvested seven days post-inoculation. Thinner cortical area (C) with maintenance of thymic architecture. X100.

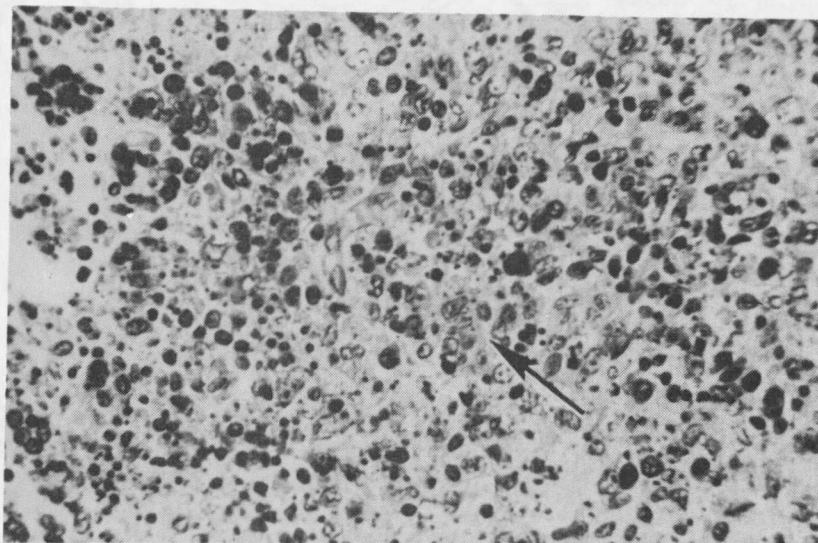


Fig. 9. LM. H&E of +3 scored thymus from a virus infected newborn mouse harvested seven days post-inoculation. Massive necrosis of the entire thymus, individual cell necrosis as evident by the accumulation of nuclear debris (arrow). X400.





















































































































































