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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A thesis submitted in partial fulfillment
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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
[^{3}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 transversing 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, subclinical 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 outbred 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_{50} 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 macroscopicaly, 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