1985

Hemorrhagic enteritis virus infection of the spleen and bursa in turkey embryos and young pouls

Rebecca L. Wallskog Hyde
Iowa State University

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HEMORRHAGIC ENTERITIS VIRUS INFECTION OF THE SPLEEN AND BURSA IN TURKEY EMBRYOS AND YOUNG POULTS

Iowa State University

Ph.D. 1985

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Hemorrhagic enteritis virus infection of the spleen and bursa in turkey embryos and young poults

by

Rebecca L. Wallskog Hyde

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Veterinary Microbiology and Preventive Medicine
Major: Veterinary Microbiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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For the Major Department

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For the Graduate College

Iowa State University Ames, Iowa 1985
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### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>allantoic cavity</td>
</tr>
<tr>
<td>AGDP</td>
<td>agar gel diffusion precipitin</td>
</tr>
<tr>
<td>AI</td>
<td>avian Influenza</td>
</tr>
<tr>
<td>C</td>
<td>centigrade</td>
</tr>
<tr>
<td>CELO</td>
<td>chick embryo lethal orphan</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>DFAT</td>
<td>direct fluorescent antibody test</td>
</tr>
<tr>
<td>EID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>embryo infective dose, 50% end point</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>H+E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>HE</td>
<td>hemorrhagic enteritis</td>
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<td>HEV</td>
<td>hemorrhagic enteritis virus</td>
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<tr>
<td>HVT</td>
<td>herpesvirus of turkeys</td>
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<tr>
<td>IBD</td>
<td>infectious bursal disease</td>
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<tr>
<td>IC</td>
<td>intracardiac</td>
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<tr>
<td>IFA</td>
<td>indirect fluorescent antibody</td>
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<tr>
<td>IFAT</td>
<td>indirect fluorescent antibody test</td>
</tr>
<tr>
<td>IV</td>
<td>intravenously</td>
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<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>MSD</td>
<td>marble spleen disease</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PS</td>
<td>penicillin and streptomycin</td>
</tr>
<tr>
<td>SPF</td>
<td>specific pathogen free</td>
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<tr>
<td>TS</td>
<td>tenosynovitis</td>
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<tr>
<td>u</td>
<td>units</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
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<td>xg</td>
<td>times gravity</td>
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GENERAL INTRODUCTION

Hemorrhagic enteritis virus (HEV) causes disease in turkeys resulting in considerable financial loss to the turkey industry in parts of the United States. Presently, there is no federally licensed vaccine available to prevent this disease. One of the requirements of a federally licensed vaccine is that the vaccine agent be produced in a specific pathogen free (SPF) system which can be monitored and controlled to limit the presence of extraneous agents or antigens. Until recently, production of infectious HEV has been possible only in live, hatched avians. Production of a vaccine, live or inactivated, however, in hatched birds is not considered an acceptable procedure by the United States Department of Agriculture (USDA) due to the increased difficulty in maintaining vaccine standards.

Efforts have and are being made by different researchers, both governmental and private, to find a suitable SPF system in which infectious HEV can be produced. The experimental work presented in this dissertation has evolved out of such an effort. The initial goal was to examine the turkey embryo as a potential SPF system for HEV growth. Data collected indicated a possible relationship between the spleen and bursal infection of the turkey embryo with HEV, which was subsequently examined.
Hemorrhagic enteritis (HE) disease syndrome of turkeys was first described in the literature by Pomeroy and Fenstermacher in 1937. They reported a disease outbreak in turkey flocks in Minnesota resulting in 10% mortality. The birds were 7-12 weeks old, well-developed, well-nourished, and had few lesions other than profuse hemorrhage into the gut. Bacteriological examination, experimental animal inoculation, and serological examination did not point to a specific etiological agent.

Twenty years later, Gale and Wyne reported a similar outbreak in Ohio causing 1.5 to 3.6% mortality. They noted that the disease syndrome had been observed with some frequency in the field since Pomeroy's first description, but that little attention had been given to it in the literature. The etiological agent remained yet undetermined (Gale and Wyne, 1957).

It was not until 10 years later that Gross and Moore presented evidence that the etiological agent would pass a 0.22 micron filter (Gross and Moore, 1967), and Domermuth and Gross speculated that the agent might be a virus (Domermuth and Gross, 1971). In 1974, Carlson and Al-Sheikhly, using an electron microscope, observed adeno-like virus particles in tissues from infected birds and suggested this to be the causative agent (Carlson and Al-Sheikhly, 1974). Since then, substantial evidence has been presented to support the concept that the etiological agent of HE is an adenovirus (Itakura and Carlson, 1975a; Toin and Domermuth, 1975).

Outbreaks of HE have been reported from all turkey producing areas of the United States. It was estimated in 1978, that 30% of all turkey flocks had experienced the clinical disease. In addition, serological studies have revealed
that many flocks have antibody to HEV which have had no prior history of the clinical disease (Smith, 1981).

Outbreaks of HE have also been reported from foreign countries including Canada (Itakura et al., 1974), the United Kingdom (Arbuckle et al., 1979; McDougall, 1980), Germany (Winterol, 1977), Australia (Tham and Critchley, 1981), and Italy (Mandelli et al., 1977).

The viral agent of HE is classified as an adenovirus on the basis of its morphology and its nuclear site of replication. It is a non-enveloped virus, 70-80 nm in diameter, and has a bouyant density of approximately 1.34 g/cm³. The virions are of uniform size, have icosahedral symmetry, and are made up of 252 capsomers. Virions are observed primarily in the nucleus of the cell, both in diffuse array and in tight crystalline pattern (Carlson et al., 1974; Itakura and Carlson, 1975a; Ossa et al., 1983a; Toi In and Domermuth, 1975).

On the basis that HE antigen and antibody will not serologically cross-react with antigen or antibody from CELO or CELO-related viruses, it has been suggested that HEV be designated as a group II avian adenovirus and that CELO and related viruses be designated as Group I avian adenoviruses (Domermuth et al., 1979b).

The virus is resistant to quaternary ammonium compounds, chloroform, and ethyl ether (Smith, 1981), but can be rendered noninfectious by appropriate concentrations of sodium hypochlorite, chlorocide, phenocide, wescodyne, and lysol. Virus in serum and in infected feces can be inactivated by thorough drying for 1 week at 27 or 35°C (Domermuth and Gross, 1971; Domermuth and Gross, 1972). Virus has remained infectious, however, in spleen from infected turkeys
incubated at 37°C for 4 weeks (Domermuth et al., 1973) and in gut contents stored at 4°C for at least 6 months (Domermuth and Gross, 1971).

Attempts to propagate HEV in the laboratory in chicken and turkey embryos and fibroblast cell cultures have been unsuccessful (Carlson et al., 1974). Fasina and Fabricant have reported infection of dissociated spleen lymphocytes from HEV-free chickens, pheasants, and turkeys, but not from 18-day-old chick embryos. Viral antigens were detected in the cell nuclei as early as 24 hours post infection by the fluorescent antibody test. Demonstration of the production of infectious virus by the cultures during serial passage was unsuccessful (Fasina and Fabricant, 1982a).

Successful propagation of the virus in two lymphoblastoid cell lines—MDTC-RP15 and MDTC-RP19—has been reported by Nazerian and Fadly, (1982). Infected cultures displayed cell enlargement and lysis. These cell lines were established from B-cell tumors induced in turkeys by the GA strain of Marek’s disease (MD) virus, and a small percentage of the cells (less than 0.1%) produce MD viral antigens. HEV serially passaged in these cultures remained infective for turkey poulets. Unsuccessful propagation of the virus, however, was reported in this study in two lymphoblastoid cell lines from T-cell tumors.

There is considerable variance in the pathogenicity of isolates of HEV. Avirulent isolates exist which produce seroconversion but no clinical signs or death (Beasley and Clifton, 1979; Beasley and Wisdom, 1978; Harris and Domermuth, 1977), and virulent strains occur which vary from producing mild depression to severe intestinal hemorrhage and result in mortality rates of <1 to over 60% (Gale and Wyne, 1957; Gross and Moore, 1967; Pomeroy and Fenstermacher, 1937). In a laboratory situation, the severity of the clinical
lesions appeared directly related to the infecting dose size (Gross and Moore, 1967).

The natural host of HEV is the turkey, but it has been experimentally transmitted to chickens (Beasley and Clifton, 1979; Darbyshire, 1980; Fasina and Fabricant, 1982b; Gross and Moore, 1967; Slem et al., 1978) and to pheasants (Domermuth and Gross, 1978; Domermuth et al., 1979a) resulting in macro and microscopic lesions but no intestinal hemorrhage, depression, or death. Examination of 42 species of free ranging birds in Florida, Texas, and Virginia by Domermuth yielded no serologic evidence of past infection in those birds (Domermuth et al., 1977b).

The virus has been transmitted in the laboratory to susceptible turkeys and chickens with spleen, serum, and feces from infected birds and soil from infected premises via oral, cloacal, intravenous, intraperitoneal, and intramuscular routes (Beasley and Clifton, 1979; Beasley and Wisdom, 1978; Domermuth et al., 1973; Domermuth and Gross, 1971; Gross and Moore, 1967; Itakura et al., 1974; Slem et al., 1978; Slem and Thorsen, 1981; Tolin and Domermuth, 1975).

In the laboratory, HEV which has been passaged serially in chickens has been shown to be still capable of infecting and causing clinical disease in turkeys (Slem et al., 1978).

The incubation period for the disease appears not to be greatly influenced by the quantity of inoculum in laboratory tests, and the clinical signs of depression, abnormal or bloody droppings, and/or death generally occur at 5 to 6 days post infection but may occur as early as 3 days or as late as 7 days. Birds which survive recover clinically in 5 to 6 days (Gross and Moore, 1967; Itakura et al., 1974). All of the birds in a flock may not be infected at the onset of the
outbreak, and as a result of contact spread of HEV, the disease may persist in the flock for up to 24 days, with mortality occurring over 10 days to 2 weeks (Domermuth and Gross, 1975a; Gale and Wyne, 1957).

The disease is acute and often the first indications of it are the finding of one or more turkeys dead. There may be evidence of a blood stained vent or of bloody droppings just prior to death. Short term depression, drowsiness, dark discoloration of the head, and ruffled feathers are less consistent signs, and a temporary drop in feed and water consumption may be noted (Gale and Wyne, 1957; Gross and Moore, 1967; Itakura and Carlson, 1975a; Itakura et al., 1974; Pomeroy and Fenstermacher, 1937). Birds may exhibit none of the clinical signs and infection is determined only after the fact by serology (Beasley and Clifton, 1979; Beasley and Wisdom, 1978; Harris and Domermuth, 1977).

The disease occurs in susceptible flocks of turkeys generally at 6 to 12 weeks of age (Carlson et al., 1974; Domermuth and Gross, 1975a; Gale and Wyne, 1957; Itakura et al., 1974; Pomeroy and Fenstermacher, 1937). Seroconversion indicates that nearly 100% of the birds in a flock are infected (Domermuth and Gross, 1978), but the severity of clinical signs and mortality will vary greatly within a flock. Survivors generally recover completely and show no adverse effects other than a temporary setback (Gross and Moore, 1967). In a laboratory study, turkeys inoculated with HEV showed a decreased capacity to produce antibodies to sheep red blood cells with the greatest inhibition occurring at day 19 post inoculation (Nagaraja et al., 1982b). Also, a suppression of the in vitro mitogenic response of lymphocytes has been reported to occur for up to 5 weeks after turkeys have been inoculated with HEV (Nagaraja et al., 1982a). In Canada
there have been reports of downgrading of carcasses attributed to HE disease (Itakura et al., 1974).

Due to the acute nature of HE disease, birds presented for post-mortem examination are generally in a good state of health and nutrition (Gale and Wyne, 1957; Itakura and Carlson, 1975b; Pomeroy and Fenstermacher, 1937). Death itself is caused by hemorrhage into the gut of an estimated 60 to 70% of the total blood volume of the bird (Gross and Moore, 1967). The most striking gross lesions in birds that have died of HE are sanguineous intestinal contents and congestion and petechial hemorrhage in the lining of the gut, particularly the duodenum. As a result of the hemorrhage, carcasses may appear anemic. The spleens in these dead birds are often small, possibly due to the blood loss, and may be either pale or darkly discolored (Carlson et al., 1974; Domermuth and Gross, 1975b; Gale and Wyne, 1957; Gross and Domermuth, 1976; Gross and Moore, 1967; Itakura and Carlson, 1975b; Pomeroy and Fenstermacher, 1937). The spleens of affected birds which do not die of the disease, however, are often large and may be mottled. Such spleens may range from 1.5 to 4 times normal size (Carlson et al., 1974; Domermuth et al., 1972; Gross and Domermuth, 1976; Itakura and Carlson, 1975b).

Histologically, the intestinal lesions begin as congestion near the tips of the villi, which is followed by the infiltration of the lamina propria with red blood cells, separation of the epithelium from the villus tips, and hemorrhage into the lumen of the gut (Gross, 1967; Itakura and Carlson, 1975b; Pomeroy and Fenstermacher, 1937; Pomeroy, 1972).

The spleen, which is the other organ to show prominent lesions during infection, is characterized by the proliferation of the cells around the sheathed
arteries and by the presence of intranuclear inclusion bodies in many of the cells. The intranuclear inclusions in the cells surrounding the sheathed arteries stain lighter than the intranuclear inclusions in cells of the red pulp of the spleen. The darker staining nuclear inclusions are also found in lesser numbers in reticular and mononuclear cells in the lamina propria of the intestine, in the medulla and cortex of the bursa of Fabricius, in the alveolar walls of the lung, and in the liver, thymus, bone marrow, kidney, pancreas, and testes (Carlson et al., 1974; Itakura and Carlson, 1975b; Itakura et al., 1974). HE viral antigen can be detected in peripheral blood lymphocytes by the fluorescent antibody test days 4-8 post inoculation, coinciding with the appearance of cells with intranuclear inclusion bodies in these tissues (Fasina and Fabricant, 1982b).

There is also a generalized lymphocytic hyperplasia in almost all of the tissues, except for the central nervous system, which may be a general response to viral infection (Itakura and Carlson, 1975b).

Turkeys under 4 weeks of age have been refractory to the clinical disease associated with HEV infection although there has been one report of HEV disease in the field in 2 1/2 week old poults (Harris and Domermuth, 1977). Poult's which are inoculated at 3 days of age with virulent HEV will develop intranuclear inclusions in the spleen but no intestinal hemorrhage (Fadly and Nazarian, 1982). Poult's that are chemically bursectomized shortly after hatching will develop spleen inclusions but no intestinal hemorrhage when inoculated at 2 or 5 weeks of age with virulent HEV (Fadly and Nazarian, 1982). Adult birds which have had their spleens removed will produce antibody to HEV but will not have intestinal hemorrhage when inoculated with virulent virus (Ossa et al., 1983b).
Virus and/or viral antigen can be detected in tissues, particularly spleen, from infected birds using agar gel diffusion precipitin (AGDP) tests (Domermuth et al., 1972; Domermuth et al., 1973), direct and indirect fluorescent antibody tests (DFAT and IFAT) (Smith, 1981), or enzyme-linked immunosorbent assay (ELISA) (Ilanconescu et al., 1984; Silim and Thorsen, 1981).

Cross reactions between the viral antigens and the antibodies of HEV and marble spleen disease (MSD) virus of pheasants occur (Domermuth and Gross, 1975a; Iltis et al., 1975b), and at this time the exact relationship of HEV and MSD virus has not been determined. They may be different strains of virus in the same group of viruses, or just similar viruses which share a common antigen (Iltis et al., 1975b). In addition, a third virus causing splenomegaly in chickens has been reported which also cross reacts on AGDP with HEV and MSD virus (Domermuth et al., 1979b). No cross reactions have been reported in AGDP tests of HEV with herpes virus of turkeys (HVT) (Domermuth et al., 1972) or with chick embryo lethal orphan (CELO) virus, bovine adenovirus, or porcine adenovirus (Silim et al., 1978).

Diagnosis of HE disease can be based on a combination of the following clinical signs; gross and histologic lesions; demonstration of viral antigen by AGDP tests, DFAT, IFAT, or ELISA; seroconversion of paired serum samples; and/or the ability to infect susceptible birds with tissues from dead or moribund turkeys (Domermuth and Gross, 1978; Domermuth et al., 1972; Domermuth et al., 1973; Ilanconescu et al., 1984; Silim and Thorsen, 1981; Smith, 1981).

Antibody to HEV can be detected as early as 3 days post infection with the ELISA test and 5 days post infection by the AGDP test (Silim and Thorsen, 1981).
The antibody is protective against challenge with virus and titers have been reported to persist at least up to 40 months (Domermuth and Gross, 1975a).

The disease should be differentiated from other intestinal disorders - protozoan, bacterial, dietary, sulfonamide toxicity, and mycotoxicosis, and also from reticuloendotheliosis, all of which share similar lesions or clinical signs with HE.

The only treatment for HE in a flock is passive immunization of birds in the face of an outbreak with convalescent antiserum from recovered birds. Since HE spreads through a flock by contact, not all of the birds will be infected when the first birds start showing signs, and passive immunization will generally stop death losses immediately except in birds which are already moribund. In laboratory studies, Domermuth and Gross demonstrated that 0.5 to 1.0 ml of antiserum per poult, administered intramuscularly or subcutaneously, prevented all lesions and as little as 0.1 to 0.25 ml of antiserum per poult prevented intestinal lesions when given within 48 hours of cloacal inoculation with virulent virus (Domermuth and Gross, 1975a).

Turkeys from healthy growing flocks are no less susceptible to HE disease than turkeys from poorly managed flocks. Strict isolation of flocks may prevent spread of the disease from one farm to another and from one building to another via boots and equipment contaminated with feces. Thorough cleaning and drying, followed by application of disinfectants may help eliminate the virus from infected premises (Domermuth and Gross, 1971).

Several methods for immunizing turkeys against HEV have been developed with varying success. An avirulent strain of MSD virus propagated in turkeys, harvested as splenic material, and then administered as a live vaccine via the
drinking water has been reported to be fairly successful in protecting poultis
against HE disease (Domermuth et al., 1977a; Thorsen et al., 1982). Also, success
in a very limited laboratory study with a formalinized spleen vaccine of HEV
administered intramuscularly was reported (Beasley and Wisdom, 1978). More
recently, a live, oral vaccine of avirulent MSD virus propagated in the MDTC-RP19
cell line has proved efficacious in laboratory studies (Fadly and Nazarian, 1984).
Presently (1985), there are no USDA licensed vaccines for HEV on the market.

There is speculation that HEV of turkeys and MSD virus of pheasants belong
to the same viral group. A review of the literature shows that MSD virus: 1) has
a similar, if not identical, morphology and resistance to chemicals as HEV;
2) causes intranuclear inclusion bodies in infected cells as does HEV; 3) causes
similar gross and histologic lesions in the spleen, as does HEV, with the
exception of the presence of amyloid in MSD; 4) causes cross reactions with HEV
and antibody in A6DP tests; and 5) can experimentally infect turkeys (Domermuth
et al., 1975; Ilitis and Daniels, 1977; Ilitis and Wyland, 1974; Ilitis et al., 1975a,
1975b; Ilitis et al., 1977; Wyland et al., 1972). Also, MSD in pheasants can be
prevented with convalescent antiserum from turkeys recovered from HE disease
or by vaccination of pheasants with live HEV. Likewise, MSD virus from
pheasants can be successfully used as a live vaccine in the prevention of HE
disease in turkeys in the field (Domermuth et al., 1977a; Domermuth et al.,
1979a).

The MSD virus differs from HEV in that it does not cause a hemorrhagic
enteritis. Death in pheasants instead results from severe pulmonary edema (Ilitis
and Wyland, 1974; Jakowski and Wyland, 1972; Wyland et al., 1972).
MATERIALS AND METHODS

Turkey Embryos and Poults

Turkey embryos and poults from an HEV and CELO-free Beltsville white turkey flock were provided by the National Animal Disease Laboratories located in Ames, Iowa. The flock was monitored monthly for antibody to HEV and to CELO by the AGDP test.

Hemorrhagic Enteritis Virus

Source

Virulent HEV was obtained as a spleen homogenate from Dr. M. Hofstad at the Veterinary Medical Research Institute in Ames, Iowa. Dr. Hofstad had received the virus as a spleen homogenate from Dr. C. Domermuth at the Virginia Polytechnic Institute in Blacksburg, Virginia and had passaged it one time in turkey poults. The spleen homogenate was tested for HEV antigen by the AGDP test. It formed a line of identity with turkey HEV spleen antigen against turkey anti-HEV supplied by Dr. B. L. Patel at the University of Minnesota.

Virus suspension 076

One ml of the HEV spleen homogenate from Dr. M. Hofstad was given orally to a 6-week-old poult. The poult died 3 days post inoculation with severe hemorrhagic diarrhea. The spleen was removed and ground in 5.0 ml of physiological saline with 1000u of penicillin and 0.4 mg of streptomycin per ml (saline + PS), and 1.0 ml of this suspension was given to each of two 8-week-old poults. The poults died on day 3 and day 4 post inoculation. Their spleens were
removed, pooled, and ground in 10.0 ml of saline +PS. This pooled spleen suspension was designated 076, aliquoted into 1.0 ml amounts, and stored at -70C.

**Virus suspension 081**

One vial of virus suspension 076 was diluted 1 to 5 in saline +PS and 0.75 ml of this dilution was given orally to each of three 6-week-old pouls. On day 5 post inoculation the pouls were killed and the spleens were removed, pooled, and ground in 15.0 ml of saline +PS. This pooled spleen suspension, designated 081, was aliquoted into 1.0 ml amounts and stored at -70C.

**Virus suspension 089**

One vial of virus suspension 076 was diluted 1 to 5 in saline +PS and 0.7 ml of this dilution was given orally to each of six 8-week-old pouls. On day 4 post inoculation the pouls were killed and the spleens were removed, pooled, and ground in 40.0 ml of saline +PS. Fifteen ml of this suspension were further diluted with 285.0 ml of saline +PS, frozen and thawed 3 times, and then centrifuged at 600 xg for 15 minutes. The supernatant was designated 089, aliquoted into 3.5 and 6.0 ml amounts, and stored at -70C.

Virus suspension 089 was checked for extraneous agents by various means. By the AGDP test it was negative for antigen of avian influenza (Al) virus, tenosynovitis (TS) virus, HVT, infectious bursal disease (IBD) virus, and CELO virus. An aliquot was subcultured 3 times on chick embryo fibroblasts and produced no cytopathic effect, and cells and supernatant from each subculture were negative for avian lymphoid leukemia antigen by the complement fixation test. The virus suspension showed no mycoplasmal growth when examined by the
heart infusion media test as outlined in Volume 9 of the Code of Federal Regulations, Part 113.28. Twenty 10-day-old SPF chick embryos were inoculated with 0.2 ml of the virus suspension onto the chorioallantoic membrane and into the allantoic cavity (AC) by the Gorham technique (Gorham, 1957), incubated for 7 days, and then opened and examined for gross lesions. There were no embryo deaths and all embryos appeared normal.

Agar Gel Diffusion Precipitation

**Agar**

One percent Noble Agar plus 8% NaCl was prepared in 0.01 M phosphate buffer, pH 7.3. Fifteen ml of agar, autoclaved and cooled to 47°C, was poured into each 100 mm plastic petri dish, cooled, and stored at 4°C. Disc wells were cut into the agar.

**Positive control antigens and antisera**

HEV antigen and turkey anti-HEV serum were supplied by Dr. B. L. Patel from the University of Minnesota. Antigens and corresponding positive antisera for AI virus, TS virus, HVT, IBD virus, and CELO virus were supplied by Veterinary Services, USDA, Ames, Iowa.

**Indirect Fluorescent Antibody Test**

**Antisera**

**Chicken anti-HEV** A 1.0 ml portion of the HEV spleen homogenate obtained from Dr. M. Hofstad was passaged 3 times in 6 to 10-week-old SPF leghorn chickens, each time by oral inoculation of 1.0 ml of a pooled spleen
suspension diluted 1 to 5 in saline +PS. Spleens were harvested at 5 days post inoculation and at each passage were enlarged and marbled. Two weeks after a fourth chicken inoculation, the chickens were bled out and the sera separated from the blood cells and pooled. The pooled sera formed a line of identity in an AGDP test with turkey anti-HEV serum against turkey HEV spleen antigen. No lines of identity were formed with antisera to AI, TS, HVT, IBD, or CELO against the corresponding antigen. The pooled sera was then adsorbed with turkey liver powder for 24 hours at 4C, aliquoted into 1.0 ml amounts, and stored at -70C. The antisera was diluted 1 to 10 with saline for use.

Rabbit anti-chicken IgG  Fluorescein isothiocyanate conjugated rabbit anti-chicken IgG (heavy and light chains) was obtained from Cappel Laboratories, Cochranville, Pennsylvania. The lyophilized conjugate was restored with 2.0 ml purified water, centrifuged at 600 xg for 10 minutes, aliquoted into 0.05 ml amounts, and stored at -70C. The antiserum was diluted 1 to 40 with saline for use.

Impression smears

An impression smear of a tissue was made by lightly pressing the freshly cut surface of the tissue onto a clean glass slide. The smear was allowed to air-dry and then fixed in 100% acetone at 4C for 45 minutes to overnight. The smear was then air-dried again and stored at -20C in a tightly sealed container until stained.

Cryosections

Tissues for cryosectioning were placed in plastic cups containing Tissue Tek II compound marketed by Miles Laboratory, Naperville, Illinois, and frozen at
-70C. Three micron thick sections were then cut on an International Equipment Company, Needham Heights, Mass., CTF Mycotome-cryostat, placed on clean glass slides, air-dried, fixed for 10 minutes in 100% acetone at 4C, air-dried, and stored at -20C in a tightly sealed container until stained.

Staining procedure for HEV antigen.

Impression smears on slides were incubated for 30 minutes in a humidified chamber at 37C. They were washed in 2 baths of phosphate buffered saline pH 7.3 (PBS) for 3 minutes and for 8 minutes, next in 2 baths of distilled water for 30 seconds and for 3 minutes, and then dried. They were then incubated for 30 minutes with rabbit anti-chicken IgG for 30 minutes in a humidified chamber at 37C, and washed and dried as before.

Microscope.

The stained impression smears were read on a Leitz Orthoplan microscope equipped with a 200 watt mercury light source and an incident-light illuminator.

Immunofluorescent score.

An immunofluorescent score was assigned to each tissue on the basis of the number of fluorescing cells or nuclei observed in the entire smear. No allowances were made for the varying sizes of the smears, and the scores were meant only to give an indication of the degree of infection of the tissue.

Score of 0 A score of 0 was assigned to a tissue when 0 or 1 fluorescing cell or nucleus was observed.

Score of 1 A score of 1 was assigned to a tissue when 2-5 total fluorescing cells or nuclei were observed. Generally, the pattern of
immunofluorescence in these tissues was that of scattered, individual fluorescing cells or nuclei.

**Score of 2** A score of 2 was assigned to a tissue when 6-19 total fluorescing cells or nuclei were observed. The pattern of immunofluorescence in these tissues was generally that of scattered, small groups of fluorescing cells or nuclei.

**Score of 3** A score of 3 was assigned to a tissue when 20 or more fluorescing cells or nuclei were observed. In this instance, there was often a fairly uniform speckling of fluorescing cells or nuclei in the tissue.

**Average immunofluorescent score of the positive tissues**

The average immunofluorescent score of 2 or more positive tissues in any group of tissues was calculated by adding together the individual scores of the tissues and then dividing by the number of positive tissues. This average was intended only to give an indication of the degree of infection in several tissues in any given group.

**Poul and Embryo Inoculation**

**Allantoic cavity inoculation of 17 and 22-day-old turkey embryos**

Seventeen or 22-day-old turkey embryos were candled and only eggs having normally positioned air cells were retained for inoculation. Eggs were then placed in egg cartons with their air cells positioned upward and swabbed with 2% tincture of iodine in 70% alcohol. A small hole was drilled over the center of the air cell, and the inoculum was placed in the allantoic cavity via a 1" 20 gauge needle inserted vertically with the hub of the needle resting on the shell. Of 74, 22-day-old embryos inoculated with dye by this method and then opened, the
inoculum was found in the allantoic cavity of 67 (90% accuracy), in the amniotic cavity of 5, and in the yolk sac of 2. The dye was found in the allantoic cavity of 6 of 6 (100% accuracy) 17-day-old embryos inoculated by this method.

Embryonic death during the first 24 hours post inoculation and during the hatching process was disregarded.

**Intravenous inoculation of embryos**

Embryos were candled and a 0.5 x 1.5 cm box was drawn in pencil on the shell over a prominent blood vessel lying under the shell membrane. The shell was then removed from this area and mineral oil was applied to clear the shell membrane and thus expose the blood vessel. A 3/4" 30 gauge hypodermic needle was used to place the inoculum in the vein. Proper placement of the inoculum was assumed only if actual blanching of the vessel was seen. The hole in the shell was covered with tape.

Embryonic death during the first 24 hours post inoculation and during the hatching process was disregarded.

**Intracardiac inoculation of newly hatched poults**

Poults were placed in dorsal recumbency and inoculated via the heart with a 3/8" 26 gauge needle inserted at the apex of the furcula. Proper placement of the inoculum was assumed only if blood could be withdrawn into the syringe both pre- and post-inoculation.

**Oral inoculation of poults**

Poults were inoculated with a ball-tipped needle placed well into the esophagus.
**Cloacal inoculation of poults.**

Poults were inoculated with a ball-tipped needle placed 0.5 cm into the cloaca.
A PRELIMINARY STUDY OF INFECTION OF THE TURKEY EMBRYO BURSA AND SPLEEN WITH HEMORRHAGIC ENTERITIS VIRUS

The Turkey Embryo in the Last One-third of the Embryonation Period

Introduction

In a search for a suitable SPF system in which to grow HEV for the vaccination of turkeys, the turkey embryo in the last one-third of the embryonation period was selected as a possible system. The turkey embryo, particularly at this age, offered several distinct advantages for the virus growth: 1) the turkey is the natural host of HEV; 2) the turkey embryo and its associated tissues offer a vast array of differentiated cell types from which the virus may select for self-propagation; and 3) the microenvironment of the cells is far more sophisticated than a cell culture environment if the virus is fastidious. Although there is previously published work (Carlson et al., 1974) and a general consensus among researchers that have worked with the virus that HEV will not grow in turkey embryos, the possibility remained that virus growth was simply not detected by the usual means of embryo death or deformities.

Adenoviruses are known for producing large amounts of excess antigen. Consequently, the indirect fluorescent antibody (IFA) and the AGDP tests were selected as the tools to detect possible virus infection.

The spleen and the bursa were picked as the initial tissues to be evaluated for HEV infection. The spleen was selected because it appears to be a target organ of infection in the adult turkey. The bursa was selected due to a prior observation of what appeared to be numerous, atypical, intranuclear inclusion bodies in cells of the bursa of a 6-week-old poult 24 hours post inoculation with virulent HEV.
Experimental design

This preliminary study was actually a composite of 8 individual experiments which consisted of embryo inoculation with 0.5 ml of a 1 to 20 dilution in saline +PS of a crude ground spleen suspension from HEV infected turkeys, designated as virus suspension 081. The embryos ranged in age from 15 to 23 days. The route of inoculation was undefined – the inoculum was simply placed anywhere from 1 to 1 1/2" straight down into the egg from the center of the egg shell over the air cell. Inoculated and control embryos were opened at varying days post inoculation and examined for gross lesions, and spleen and bursa were collected for IFA (impression smears and cryosections) and AGDP testing.

Results

Gross lesions All embryos, both HEV-inoculated and controls, appeared normal in shape, size, and activity. Likewise no difference was observed in the size or coloring of the internal organs, in particular the bursa and spleen, between the two groups.

IFA The results of the experiment are presented in Table 1. Positive immunofluorescence was observed in the bursae of 32 of the 81 inoculated embryos and in 0 of the 27 control embryos. The fluorescence in the bursal cells was of 3 types: 1) well-defined, brilliant fluorescence of a portion of the nucleus, often oval in shape; 2) well-defined, evenly distributed fluorescence throughout the entire nucleus; or 3) general fluorescence of what appeared to be the entire cell. See Figures 1, 3, and 4.
Impression smears of spleen tissue were made from 63 of the inoculated embryos and 21 of the control embryos. Of these, positive immunofluorescence was observed in 6 of the 63 spleens from the inoculated embryos and 0 of the 21 spleens from the controls. A general fluorescence of what appeared to be the entire cell was most frequently observed. See Figure 2.

**AGDP** The AGDP test was conducted on a total of 66 bursae. Of 23 of these bursae that were positive for HEV antigen by the IFA test, only 11 were positive for HEV antigen by the AGDP test. Of 43 bursae that were negative for HEV antigen by the IFA test, all were negative by the AGDP test.

**Incubation time for infection** The time that elapsed between inoculation of the embryo and the first signs of positive immunofluorescence in the tissues indicating infection, varied from 2 days for the 23-day-old embryo inoculates to 6 days for the 18-day-old embryo inoculates. See Table 1.

**Discussion**

Despite the fact that this study was conducted with a crude spleen suspension of HEV, that the data are a summary of 8 separate experiments, and that the route of inoculation in the embryos was not specifically known, some preliminary observations were still made and some tentative conclusions drawn. First, the positive immunofluorescence in the tissues with the IFA test and the positive lines of identity with the AGDP test did indicate that the virus was able to infect and at least produce antigen in the turkey embryo. A lack of gross lesions in the infected embryos was not surprising considering that 3-day-old poults which are infected also show no gross lesions with the possible exception of some spleen enlargement (Fadly and Nazarian, 1982). Second, infection of the embryo did not seem to occur at a specific time interval after inoculation, but
rather infection became apparent on or after day 24 of embryo incubation. This suggested a possible age dependence of the embryo to support virus infection. Third, the bursa seemed more susceptible to infection by HEV than the spleen of the embryo.

The above observations had not been reported by other workers. Further studies were planned to prove replication of the virus in the turkey embryo and to determine the possible relationship between the spleen and bursa.

The IFA test was a better tool than the AGDP test for detecting HEV antigen for 3 reasons: 1) the cellular location of the antigen could be assessed; 2) the test could be conducted on impression smears of tissue and the bulk of the tissue could be fixed for sectioning or frozen and stored for later processing; and 3) it had greater sensitivity than the AGDP test.
Table 1. Results of the IFA test for viral antigen in the bursae of HEV-inoculated turkey embryos—a composite of 8 experiments

<table>
<thead>
<tr>
<th>Age of embryo at inoculation</th>
<th>Embryo group</th>
<th>Day post inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>15 day</td>
<td>Inoculates</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>-</td>
</tr>
<tr>
<td>18 day</td>
<td>Inoculates</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>-</td>
</tr>
<tr>
<td>21 day</td>
<td>Inoculates</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>-</td>
</tr>
<tr>
<td>22 day</td>
<td>Inoculates</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>-</td>
</tr>
<tr>
<td>23 day</td>
<td>Inoculates</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>0/1</td>
</tr>
</tbody>
</table>

*a* Not done.

*b* Number of positive tissues/total number of tissues.
Figure 1. Positive fluorescence in dural cells. Impression smear from a HEV-infected turkey embryo

Figure 2. Positive fluorescence in spleen cells. Impression smear from a HEV-infected turkey embryo
Figure 3. Positive fluorescence in bursal cells. Cryosection from a HEV-infected turkey embryo.

Figure 4. No fluorescence in bursal cells. Cryosection from an uninoculated turkey embryo.
EVALUATION OF THE AGE DEPENDENCE OF THE TURKEY EMBRYO FOR INFECTION BY HEMORRHAGIC ENTERITIS VIRUS

The 17 Versus the 22-day-old Turkey Embryo

Introduction

The purpose of the following experiment was to determine if infection by HEV was dependent on the age of the embryo, and hence the stage of maturation, rather than on a specific incubation time.

To conduct the following experiment, virus suspension 089 was prepared, evaluated for extraneous antigens, aliquoted, and stored, and the technique for embryo inoculation via the allantoic cavity (AC) was verified, as described in the Materials and Methods section.

Experimental design

Seventy 17-day-old and 50, 22-day-old turkey embryos were inoculated with 0.5 ml of virus suspension 089 via the AC. Fourteen turkey embryos were retained as uninoculated controls. Embryos were sacrificed and bursal and spleen impression smears were made daily from 10 embryos of each inoculation group starting 4 days post inoculation (at 21 days of embryonation) for the 17-day-old inoculates and 1 day post inoculation (at 23 days of embryonation) for the 22-day-old inoculates. Bursal and spleen impression smears were made daily from 2 embryos of the control group starting at 21 days of embryonation. Tissues were examined for immunofluorescence by the IFA test. An immunofluorescent score was recorded for each positive tissue, and the average score determined (see Materials and Methods).
Results

The results of this experiment are presented in Table 2. Positive immunofluorescence first appeared in the 17-day-old inoculated embryo on day 25 of embryonation, 8 days post inoculation. At this time 2 of the 10 bursal smears were positive with an average immunofluorescent score of 2.5. The number of bursal smears with positive immunofluorescence increased daily, with 8 of 10 positive day 26 of embryonation and 10 of 10 positive day 27 of embryonation. Positive immunofluorescence for viral antigen appeared in 1 of 10 bursal smears of the 22-day-old inoculated embryos on day 24 of embryonation, 2 days post inoculation. The number of bursal smears with positive immunofluorescence for this inoculated group likewise increased daily, with 3 of 10 positive day 25, 10 of 10 positive day 26, and 9 of 9 positive day 27.

Positive immunofluorescence in the spleen impression smears from the inoculated groups appeared in each case 2 days after the first appearance of positive immunofluorescence in the bursal impression smears. On day 27 of embryonation, only 1 of 10 spleens from the 17-day-old inoculated group and 3 of 10 spleens from the 22-day-old inoculated group showed positive immunofluorescence.

The number of fluorescing cells in the positive tissues, indicated by the average immunofluorescent scores, appeared to be somewhat greater in the bursal tissues than in the spleen tissues.

Discussion

The data support the hypothesis that turkey embryo infection with HEV is dependent on the embryo maturation state rather than on a specific incubation time, at least when the virus is inoculated via the allantoic cavity. Regardless of
whether an embryo was 17 or 22-days-old at the time of inoculation, the first indications of HEV infection occurred at the 24th-25th day of embryonation. The relationship of HEV infection in the bursa to HEV infection in the spleen is of interest. In both groups of embryos, HEV antigen was detected in the spleen 2 days after it was detected in the bursa. Considering that the allantoic cavity communicates directly with the hind gut, and therefore the bursa, one could argue that the bursa is the first to be exposed to the virus and the spleen is infected secondarily to the bursa. On the other hand, it is probable that by day 25 virus, which had been inoculated into the allantoic cavity of a 17-day-old embryo, is dispersed generally throughout all of the tissues and fluids of the egg, and thus would be in contact with both the bursa and the spleen. This suggests that the cells of the bursa and spleen, capable of supporting HEV antigen expression, mature at different times or that infected cells pass from the bursa to the spleen.
Table 2. Seventeen and 22-day-old turkey embryos inoculated with HEV by the AC route--IFA test results

<table>
<thead>
<tr>
<th>Embryo group</th>
<th>Tissue</th>
<th>21</th>
<th>22</th>
<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>27</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-day inoculates</td>
<td>Spleen</td>
<td>0/10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>1/10 (1.0)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Bursa</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>2/10 (2.5)</td>
<td>8/10 (2.0)</td>
<td>10/10 (2.9)</td>
</tr>
<tr>
<td>22-day inoculates</td>
<td>Spleen</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>1/10 (1.0)</td>
<td>3/9 (2.0)</td>
</tr>
<tr>
<td></td>
<td>Bursa</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0/10</td>
<td>1/10 (3.0)</td>
<td>3/10 (1.0)</td>
<td>10/10 (2.6)</td>
<td>9/9 (3.0)</td>
</tr>
<tr>
<td>controls</td>
<td>Spleen</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>Bursa</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of positive tissues/total number of tissues.

<sup>b</sup> Average fluorescent score of positive tissues.

<sup>c</sup> Not done.
THE RELATIONSHIP OF THE INFECTION IN THE BURSA AND IN THE SPLEEN OF THE TURKEY EMBRYO INOCULATED WITH HEMORRHAGIC ENTERITIS VIRUS

The 22-day-old Turkey Embryo, Experiment 1

Introduction

The following experiment was so designed as to expose the spleen and the bursa to the HEV inoculum at approximately the same time and therefore determine if both tissues are capable of becoming infected with HEV simultaneously at some stage of embryo development, or if spleen infection is dependent on prior bursal infection.

Experimental design

Twenty-six, 22-day-old turkey embryos were inoculated with 0.25 ml of virus suspension 089 intravenously (IV). Five turkey embryos were retained as uninoculated controls. Bursal and spleen impression smears were made daily from 5 embryos of the inoculated group and from 1 embryo of the control group starting 3 days post inoculation, at 25 days of embryonation. Tissues were examined for immunofluorescence by the IFA test. The immunofluorescent score was recorded for each positive tissue, and the average score determined.

Results

The results of this experiment are presented in Table 3. Positive immunofluorescence was already present in 5 of 5 bursae and in 3 of 5 spleens on the first day that tissues were examined. The rates of infection of the bursae and the average immunofluorescent scores of the bursae were greater each day than those of the spleens.
Discussion

Antigen was detected in the spleens and in the bursae earlier in these IV-inoculated embryos than had been expected drawing from the previous data obtained from AC-inoculated embryos. The design of this experiment therefore did not allow for the detection of the earliest time of infection for either tissue, and hence no observations could be made concerning the relationship of the initial bursal and spleen infections.

The 22-day-old Turkey Embryo, Experiment 2

Introduction

A second experiment was conducted to look at tissues from embryos at earlier times post inoculation in order to determine the initial occurrence of HEV infection in the bursa and the spleen.

Experimental design

The experimental design was identical to that of the previous experiment with the exception that the impression smears were made daily from day 1 to 3 post inoculation instead of day 3 to 7 post inoculation.

Results

The results of this experiment are presented in table 3. Four of the 5 bursal impression smears exhibited positive immunofluorescence by the IFA test on day 1 post inoculation whereas positive immunofluorescence was not observed in the spleen impression smears until day 3 post inoculation in 4 out of 5 spleens.
Discussion

The 2 day lag phase of HEV infection of the spleen following HEV infection of the bursa was observed in the IV-inoculated embryos as it had been observed in the AC-inoculated embryos. However, since HEV infection occurred earlier in both tissues of the IV-inoculated embryos than it had in the AC-inoculated embryos, the data still do not clarify the issue of whether or not spleen infection occurs sequentially only after bursal infection. It could be that day 24 of embryonation is just the earliest stage of development at which the spleen could be infected regardless of bursal infection, and that both tissues could be simultaneously infected at a latter stage of embryonation.

The 24-day-old Turkey Embryo

Introduction

This experiment was designed to determine whether the spleen infection required an earlier bursal infection. Embryos were inoculated IV one day prior to the age at which it had been shown in previous studies that the spleen could support viral antigen expression. Data from this study would show if the spleen could be infected with HEV at the same time as the bursa, hence without a dependence on prior bursal infection.

Experimental design

Twelve 24-day-old turkeys were inoculated with 0.25 ml of viral suspension 089 IV. Three turkey embryos were retained as uninoculated controls. Bursal and spleen impression smears were made daily from 4 embryos of the inoculated group and from 1 embryo of the control group starting day 1 post
inoculation. Tissues were examined for immunofluorescence by the IFA test. The fluorescent score was recorded for each positive tissue and the average score determined.

Results

The results of this experiment are presented in table 4. Positive immunofluorescence indicating HEV infection was observed in 1 of 4 bursal impression smears day 1 post inoculation and in 4 of 4 tissues each days 2 and 3 post inoculation. Immunofluorescence was not observed in spleen tissue until day 3 post inoculation when 1 of 4 tissues was positive.

Discussion

In both this experiment involving 24-day-old inoculated embryos and in the previous experiment involving 22-day-old embryos, the spleen did not show positive fluorescence until 2 days after fluorescence was observed in the bursa. If the stage of spleen maturation were the only critical factor for the HEV infection, then simultaneous infection of the spleen and bursa should have occurred with the 24-day-old inoculated embryos. These data therefore support the hypothesis that infection of the spleen with HEV is somehow related to or dependent on prior infection of the bursa.

These data do not define the nature of this dependence. One could hypothesize a number of scenarios whereby spleen infection results subsequent to bursal infection. For a few examples: 1) Virus could have a tropism for the bursa. Bursal lymphocytes become infected and, in the natural course of the maturation of the immune system of the embryo, the infected bursal cells migrate out and home to the spleen as whole cell infectious units. 2) The bursal
cells could produce infectious HEV with a tropism for spleen cells. 3) The bursal lymphocytes could produce very early immunoglobulin in which forms a complex with virus and complement. As such, the virus could be more easily taken up by various spleen cells such as macrophages, B lymphocytes, or polymorphonuclear cells.

These data also are not contradictory to the possibility that other tissues of the embryo are involved in the infection of the spleen. Spleen infection may result from the infection of any one of several tissues, or it may follow the infection of a sequence of tissues which includes the bursa.

The 17-day-old Turkey Embryo

Introduction

In the previous experiments, it was shown that spleen infection with HEV is preceded by bursal infection by 2 days. The purpose of the following experiment was to determine if this time interval likewise occurs at earlier stages of embryonation.

Experimental design

Eighteen 17-day-old turkey embryos were inoculated with 0.25 ml of virus suspension 089 intravenously. Six turkey embryos were retained as uninoculated controls. Bursal and spleen impression smears were made daily from 3 embryos of the inoculated group and from 1 embryo of the control group days 3-8 post inoculation. Tissues were examined for immunofluorescence by the IFA test. The immunofluorescent score was recorded for each positive tissue, and the average score determined.
Results

The results of this experiment are presented in Table 5. Three of 3 bursae were positive for infection on the first day the tissues were collected (day 3 post inoculation) and the bursal tissues remained positive throughout the experimental period with the exception of 1 bursa on day 4 post inoculation. None of the spleens on any of the days were positive.

Discussion

Impression smears were not made on days 1 and 2 post inoculation so it is not possible to say when infection of the bursa first occurred, but it is obvious that bursal infection did occur considerably earlier by the IV route of inoculation (at least by day 20 of embryonation) than by the AC route (day 24 of embryonation). This delay in infection by the AC route could be attributed to an immature state of the bursal epithelium existing prior to its differentiation into follicle-associated epithelium (Glick, 1983), and hence a lack of transport of virus into the follicle by pinocytosis.

Spleen infection did not follow bursal infection at least through day 8 post inoculation (day 25 of embryonation). In the previous studies of IV-inoculated 22-day-old embryos, spleen infection was detected as early as day 25 of embryonation. Coupling the data from these three experiments and from the IV-inoculated 24-day-old embryo experiment, it appears that spleen infection does depend on prior bursal infection but that one or a combination of the following situations occurs: 1) the spleen infection is dependent on the production of virus by the bursa with a tropism for the spleen but that this virus production does not occur prior to day 24 or 25 of embryonation; 2) that spleen infection results from the migration of infected cells from the bursa to the spleen and that this
migration of cells does not occur prior to day 24 or 25 of embryonation; or 3) the spleen is not capable of antigen expression until day 25 of embryonation.
Table 3. Twenty-two-day-old turkey embryos inoculated with HEV by the IV route—IFA test results

<table>
<thead>
<tr>
<th>Embryo Group</th>
<th>Tissue</th>
<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>27</th>
<th>28</th>
<th>29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculates expt. 1</td>
<td>Spleen</td>
<td>___</td>
<td>___</td>
<td>3/5</td>
<td>3/5</td>
<td>4/5</td>
<td>3/5</td>
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<td></td>
<td></td>
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<td></td>
<td>(1.3)</td>
<td>(1.0)</td>
<td>(1.3)</td>
<td>(1.7)</td>
<td>(1.5)</td>
</tr>
<tr>
<td></td>
<td>Bursa</td>
<td>___</td>
<td>___</td>
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<td>(1.8)</td>
</tr>
<tr>
<td>Controls expt 1</td>
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<td>___</td>
<td>___</td>
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<td>0/1</td>
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</tr>
<tr>
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<td>0/5</td>
<td>0/5</td>
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<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
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<td></td>
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</tr>
<tr>
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<td>5/5</td>
<td>5/5</td>
<td>___</td>
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<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
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<td>0/1</td>
<td>0/1</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
</tbody>
</table>

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\(^a\) Not done.

\(^b\) Number of positive tissues/total number of tissues.

\(^c\) Average fluorescent score of positive tissues.
Table 4. Twenty-four-day-old turkey embryos inoculated with HEV by the IV route--IFA test results

<table>
<thead>
<tr>
<th>Embryo group</th>
<th>Tissue</th>
<th>25</th>
<th>26</th>
<th>27</th>
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</thead>
<tbody>
<tr>
<td>Inoculates</td>
<td>Spleen</td>
<td>0/4 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/4</td>
<td>1/4 &lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td>Bursa</td>
<td>1/4</td>
<td>4/4</td>
<td>4/4 &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.0)</td>
<td>(1.5)</td>
<td>(2.5)</td>
</tr>
<tr>
<td>Controls</td>
<td>Spleen</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>Bursa</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of positive tissues/total number of tissues.

<sup>b</sup> Average fluorescent score of positive tissues.
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<th>Embryo group</th>
<th>Tissue</th>
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<th>20</th>
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</thead>
<tbody>
<tr>
<td>Inoculates</td>
<td>Spleen</td>
<td>—</td>
<td></td>
<td>0/3 b</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
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<td>Bursa</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>(1.7) c</td>
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<td>(2.7)</td>
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<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
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<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
</tbody>
</table>

* a Not done.

* b Number of positive tissues/total number of tissues.

* c Average fluorescent score of positive tissues.
THE RELATIONSHIP OF THE INFECTION IN THE BURSA AND IN THE SPLEEN OF THE NEWLY HATCHED POULT INOCULATED WITH HEMORRHAGIC ENTERITIS VIRUS

The Newly Hatched Poult

Introduction
The results of the previous experiments in turkey embryos support an hypothesis that infection of the spleen with HEV is dependent on prior infection of the bursa. The data additionally lend support to the hypothesis that infection is dependent on the stage of maturation of the two tissues. The following experiment was conducted in newly hatched poults to evaluate how continued maturation of the tissues affects the bursal-spleen relationship.

Experimental design
Forty-eight newly-hatched poults (less than 24 hours old) were inoculated with 0.25 ml of virus suspension 089. Sixteen were inoculated cloacally, 16 orally, and 16 via the heart. Four newly hatched poults were retained as uninoculated controls. Bursal and spleen impression smears were made daily from 4 poults from each inoculated group and 1 poult of the control group, days 1-4 post inoculation. Tissues were examined for immunofluorescence by the IFA test. The immunofluorescent score was recorded for each positive tissue, and the average score determined.

Results
The results of the experiment are presented in Table 6. In the poults inoculated by the cloacal route, spleen infection followed bursal infection by 2 days. In the poults inoculated by the oral route, spleen and bursal infection
occurred later and almost simultaneously, with 1 of 4 bursae positive day 3 post inoculation and 2 of 4 spleens and 3 of 4 bursae positive day 4 post inoculation. The poult's which received the HEV via intracardiac inoculation however, displayed HEV antigen first in the spleen (3 of 4 spleens positive day 1 post inoculation) and then in the bursa (3 of 4 bursae positive day 2 post inoculation).

Discussion

Poult's inoculated via the cloacal route showed the same pattern of spleen infection occurring 2 days after bursal infection as was seen with the 22 and 24-day-old inoculated embryos. These data are consistent with the hypothesis that spleen infection can result from the systemic spread of virus following local infection of the bursa.

When poult's were inoculated via the intracardiac route, it was observed that the spleen could be infected without prior infection of the bursa. Thus, at this stage of maturation, the spleen possessed cells capable of viral infection, whose origin might be the bursa.

When poult's were inoculated by the oral route, which is considered by many to be the natural route, infection of the spleen and bursa was not only delayed, but also occurred almost simultaneously. Such a situation could result, for example, if a local infection occurred first in the lymphoid tissue along the gut with the spleen and bursa becoming infected subsequently due to systemic spread of the virus.
Table 6. Newly-hatched turkey poults inoculated with HEV--IFA test results

<table>
<thead>
<tr>
<th>Embryo group</th>
<th>Tissue</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<tbody>
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<td>4/4</td>
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<tr>
<td></td>
<td></td>
<td>(1.7)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(1.7)</td>
<td>(3.0)</td>
<td>(2.0)</td>
</tr>
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<td>3/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
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<td>(1.7)</td>
<td>(2.8)</td>
<td>(2.8)</td>
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</tr>
<tr>
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<td>0/4</td>
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</tr>
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<td>(2.0)</td>
</tr>
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<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>Bursa</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of positive tissues/total number of tissues.

<sup>b</sup> Average fluorescent score of positive tissues.
HEMORRHAGIC ENTERITIS VIRUS INFECTION IN THE TURKEY EMBRYO DURING THE LATTER STAGES OF EMBRYONATION AND AFTER HATCHING

The 22-day-old Turkey Embryo Followed Through Day 6 Post-hatching

Introduction

The purpose of this experiment was three-fold. One objective was to inoculate embryos in the latter stage of embryonation and observe the duration of HEV infection in the spleen and the bursa. A second objective was to collect tissues in formalin for light microscopic examination to determine the location of the HEV-infected cells. The third and primary objective was to determine if infectious virus is produced in the bursa or the spleen. No data were obtained from the previous experiments to prove that infectious virus is actually produced in the positive immunofluorescing cells. The IFA test confirms viral infection only, by detecting cellular viral antigen. For the following study, a titration method for HEV was set up using turkey embryos.

Experimental design

Fifty-six 22-day-old turkey embryos were inoculated with 0.5 ml of virus suspension 089 via the AC. Ten embryos were retained as uninoculated controls. Bursal and spleen impression smears were made daily from 6 embryos of the inoculated group and from 1 embryo of the control group daily, days 3-11 post inoculation. Tissues were examined for immunofluorescence by the IFA test. The fluorescent score was recorded for each positive tissue, and the average score determined.

One-half of the spleen and 1/2 of the bursae from each of 2 embryos or poultis randomly selected each day from the inoculated group were fixed in
formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H+E). One-half of the spleen and 1/2 of the bursa from the control embryo was similarly treated.

The pools of bursal tissues and of spleen tissues from days 21, 29, and 31 post setting were titrated. Each pool of tissues was processed for inoculation as follows. The tissue volume was estimated and the tissues were minced, mixed with 2 parts saline + PS, and ground in a ten Broeck grinder for 5 minutes. An additional 7 parts saline + PS was added to bring the total tissue dilution to $10^{-1}$. The mixture was then frozen to -70°C, thawed, and centrifuged at 600xg for 10 minutes. The supernatant was saved and $10^{-2}$ through $10^{-4}$ dilutions were made from a portion of it.

Viral suspension 089 was titrated to serve as a basis for comparison. Three aliquots were thawed and dilutions $10^{-1}$ through $10^{-4}$ were made in saline + PS from each aliquot.

Each titration was conducted by inoculating five 22-day-old embryos with 0.5 ml each of the appropriate dilution $10^{-1}$ through $10^{-4}$ via the AC. On day 27 post setting impression smears were made from the bursae of the embryos, or in some cases of the newly hatched poults, and the tissues were examined for immunofluorescence by the IFA test. A 50% endpoint of infection was calculated using the method of Reed and Meunch (1938) and virus titers reported as log $10$ EID$_{50}$'s per bursa.

Results

The IFA test results of this experiment are presented in Table 7. The first positive immunofluorescence in the spleen was observed on day 5 post inoculation when 2 of 6 spleens had an average fluorescent score of 1.5. On days
6-10 post inoculation, there were 4 to 6 spleens out of 6 positive daily with an average fluorescent score ranging from 1.8 to 2.8. On day 11 post inoculation, all 8 spleens were negative. Positive immunofluorescence was already present on day 3 post inoculation in the bursa with 2 of 6 impression smears having an average fluorescent score of 2.0. Days 5 through 9 post inoculation all bursae were positive with average fluorescent scores ranging from 2.0-3.0. Days 10 and 11 post inoculation showed a decline in incidence of infection with 3 of 6 and 2 of 8 bursae respectively positive, with average fluorescence scores of 2.3 and 1.0.

Cells with large, dark staining and irregularly shaped intranuclear inclusion bodies were observed in the medulla of the bursae on all days 3-11 post inoculation. See Figure 5. By day 9 post inoculation, there was an obvious lymphoid depletion of the bursa. See Figures 6 and 7. Cells with large, dark staining and irregularly shaped intranuclear inclusion bodies were also observed in the periellipsoid white pulp of the spleen, primarily days 6 and 7 post inoculation. See Figure 8. Cells with large, light staining, round to oval shaped intranuclear inclusion bodies were present in the ellipsoid areas of the spleen starting day 8 post inoculation. See Figure 9.

Hatched poults appeared healthy and alert with no clinical signs of HE disease.

The results of the titrations of virus in spleen and bursa are presented in Table 7. On day 5 post inoculation, no virus was detected in the spleen but a titer of $0.8 \log_{10} \text{EID}_{50}$'s was detected per bursa. On day 7 post inoculation, $0.9 \log_{10} \text{EID}_{50}$'s was titrated per spleen and $1.8 \log_{10} \text{EID}_{50}$'s was titrated per bursa. On day 9 post inoculation, both tissues had virus titers of $1.7 \log_{10} \text{EID}_{50}$'s.
The virus titers of the 3 aliquots of virus suspension 089 were 2.5, 2.5, and 2.6 log_{10} EID_{50}'s per 0.5 ml.

**Discussion**

Not surprisingly, bursal infection preceded spleen infection, but the data also showed that bursal infection persisted at least one day past the last day that HEV antigen was detected in the spleen. In addition, the daily average fluorescent scores were generally higher for the bursal tissues than for the spleen tissues. One could interpret this as a higher rate of infection in the bursa than in the spleen, and/or one could postulate that this difference is a function of the cell type infected. For example, if the hypothesis was made that the target cell of infection was the lymphocyte, the fact that the proportion of lymphocytes is greater in the bursa than in the spleen would account for the lower proportion of cells observed fluorescing in the spleen impression smears.

HEV infection of the 22-day-old inoculated embryo appears to run its course by day 11 post inoculation, which is approximately 6 days post hatching. The design of the experiment was not such that an actual end point of bursal infection was observed, but the trend of the infection days 10 and 11 post inoculation was one of decreasing bursal infection.

It is not possible to identify the cell type or types infected by HEV unequivocally by light microscopic examination of paraffin sections stained with H&E. They may be lymphocytes, macrophages, cells of the reticular framework, secretory cells, or some other cell type. An evaluation of the cell type by immunolabelling and/or by electron microscopy may help elucidate the nature of the bursal-spleen relationship in infection.
The poult's appeared physically unaffected by the HEV infection. They may not fare so well, however, in a field situation due to decreased immune capacity resulting from the lymphoid depletion of the bursa.

Looking at the titers of the tissues from the 22-day-old inoculated embryos, it would appear that the tissues do produce infectious virus and that the bursa produces virus prior to the spleen. Such data are consistent with the observation that the bursa is the first of the two tissues to show positive fluorescence, and supportive of the hypothesis that the spleen infection results somehow directly or indirectly from the bursal infection. However, the virus titers detected in the spleen and bursal tissues are lower than the titer of the original inoculum, the 089 virus suspension. Considering the stability of the virus, one could argue that perhaps the titers detected in the tissues merely represented residual virus from the original inoculum. The average bursa/embryo + associated tissues weight ratio of 3, 24-day-old embryos was calculated to be 0.0008 or 0.08%, and if the titers were entirely due to residual virus, then much of the inoculated virus would have to have been concentrated in these tissues. On the other hand, the titers from the tissues could be artificially low due to inadequate release of virus from the cells during the tissue preparation. Additional freeze-thaw steps and/or sonication may substantially increase the measurable titer. If the titers are indeed just low, then serial passage of the virus in embryos could supply proof of virus multiplication.
Table 7. Twenty-two-day-old turkey embryos inoculated with HEV by the AC route--IFA test and titration results

<table>
<thead>
<tr>
<th>Embryo group</th>
<th>Tissue</th>
<th>Method</th>
<th>Hatch day</th>
<th>Day post setting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>25 26 27 28 29 30 31 32 33</td>
<td></td>
</tr>
</tbody>
</table>

| Inoculates | Spleen | IFA | 0/6<sup>a</sup> 0/6 | 2/6<sup>b</sup> (1.5) | 5/6 (2.4) | 6/6 (2.0) | 5/6 (2.8) | 6/6 (1.8) | 4/6 (1.8) | 0/8 |
| Bursa       | IFA    | 2/6 (2.0) | 2/6 (2.5) | 6/6 (2.8) | 6/6 (3.0) | 6/6 (2.0) | 6/6 (2.3) | 3/6 (2.3) | 2/8 (1.0) |
| Controls   | Spleen | IFA | 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/2 |
| Bursa      | IFA    | 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/1 0/2 |

<sup>a</sup> Number of positive tissues/total number of tissues.

<sup>b</sup> Average fluorescent score of positive tissues.

<sup>c</sup> Not done.

<sup>d</sup> Average log<sub>10</sub> EID<sub>50</sub>′s per bursa.
Figure 5. Intranuclear inclusion bodies in cells of the bursal medulla 5 days post inoculation, H + E
Figure 6. Lymphoid depletion of bursa 9 days post inoculation, H+E

Figure 7. Control bursa, H+E
Figure 8. Intranuclear inclusion bodies in cells in the periellipsoid area of the spleen 7 days post inoculation, H + E

Figure 9. Intranuclear inclusion bodies in cells in the ellipsoid area of the spleen 9 days post inoculation, H + E
OVERALL SUMMARY AND DISCUSSION

HEV can infect turkey embryos and the infection can be detected by the IFA test and to a lesser degree by the AGDP test. The route of inoculation — allantoic cavity or intravenously — has a significant effect on the time of appearance of viral antigens in the tissues. When embryos were inoculated by the AC route, bursal infection was not evident until days 24-25 of embryonation regardless of whether the embryos were 17 or 22 days old at the time of inoculation. It appeared that infection of the bursa is dependent on the stage of maturation of the embryo. However, when 17-day-old embryos were inoculated by the IV route, bursal infection was already present on the first day that tissues were sampled, day 20 of embryonation. Therefore, the hypothesis that HEV infection in embryos is dependent on the stage of maturation rather than on an incubation time only holds true when embryos are inoculated by the AC route. The exact mechanism of this dependency is not known. The fluid of the allantoic cavity communicates directly with the hind gut and virus which is inoculated into this cavity would be expected to come into contact with the bursa considering the location of the bursa on the dorsal wall of the cloaca. Bursal infection may simply be delayed until the epithelial cells have differentiated into the specialized follicle-associated epithelium which is responsible for transporting material from the lumen of the bursa into the medulla of the bursal follicle by pinocytosis (Cooper et al., 1972; Glick, 1983). Inoculation of embryos by the IV route demonstrates that susceptible cells are either already present in the bursa at or before day 20 of embryonation, or that they enter as infected cells, possibly from the blood.

Spleen infection is preceded by bursal infection when turkey embryos are inoculated with HEV regardless of whether the route of inoculation is the AC or
IV. This phenomenon can form the basis for two hypotheses concerning the
pathogenesis of infection in the embryo. The first hypothesis would be that cells
capable of supporting viral replication are present initially in the bursa and that
these cells subsequently migrate to the spleen. Infectious virus would reach the
spleen through the blood vascular system as free virus or through the agency of
the movement of infected cells. Intracardiac inoculation of virus into newly
hatched poult results in approximately simultaneous infection of the spleen and
bursa supporting this hypothesis.

The second hypothesis would be that cells are initially infected in the bursa
and then migrate from the bursa to the spleen and precipitate infection or that
virus must first be produced in the bursa which then has a tropism for the spleen.
From the data collected in these experiments, it would appear that this
movement of cells or production of infectious virus does not occur in the 17-24
day period of embryonation, but occurs only during the last few days of
embryonation, with the earliest evidence of cells with positive
immunofluorescence appearing in the spleen day 25 of embryonation.

These two hypotheses are in no way exclusive of one another and the two
systems accounting for spleen infection may co-exist. However, the most likely
hypothesis is that cells capable of supporting viral replication first develop in
the bursa and occur only later in embryonic development in the spleen.

Migration of bursa-derived cells has been shown to occur in 2-week old
chickens by demonstration of the presence of a statistically significant amount
of H3-thymidine label in the spleen occurring at day 6 after injection of label into
the bursa (Woods and Linna, 1965). Much indirect evidence, derived from
experiments which involve the bursectomy of chick embryos or young chickens
and which result in decreased antibody production and/or a lack of development of the peri-ellipsoidal lymphoid tissues and germinal centers of the spleen, has been used in support of the migration theory of bursa-derived lymphoid cells, B-lymphocytes (Chang et al., 1957, 1958; Eerola et al., 1983; Glick et al., 1956; Granfors et al., 1982; Hoshi, 1972; Sugimura and Hashimoto, 1980). In addition, a cell has recently been described, the secretory cell, which is found in the bursa and it is suggested that it contributes to the microenvironment necessary for B-cell development. Similar appearing cells have also been found in the germinal centers of the spleen and the cecal tonsil. Furthermore, some evidence has been presented that these secretory cells are the same cells as the ellipsoid-associated cells of the spleen which bind a variety of substances and which are capable of tissue migration and of systemic circulation (Glick and Olah, 1984; Olah and Glick, 1978, 1979, 1982; Olah et al., 1984). On the other hand, a lymphocyte-differentiating hormone, termed bursopoietin, has also been extracted from the bursa of the chicken (Brand et al., 1976), and one might argue that it is a hormonal effect, rather than a migration of cells in the chicken, that contributes to the microenvironment necessary for B-cell development. However, it has been suggested that this may only act locally, within the bursa, so the hormone producing cell would have to migrate to the spleen (Glick and Olah, 1984).

Although cell identification was not possible in any of these experiments, infection either of lymphocytes or secretory cells could result in the lymphoid depletion observed in the paraffin sections of HEV-infected bursae and could account for the suppression of the in vitro mitogenic response of lymphocytes
and the decreased capacity to produce antibodies to sheep red blood cells of HEV-infected turkeys (Nagaraja et al., 1982a and b).

The tissue tropism of infection in the embryo may also include other tissues besides the spleen and bursa. The relationship of HEV-infection in the spleen and bursa presented in these studies does not preclude the possibility that the bursal infection triggers infection in a second tissue in the embryo and that this second tissue in turn precipitates the infection in the spleen. It also does not preclude the possibility that HEV initially infects a number of tissues, any one of which could transmit infection to the spleen. A study to detect HE viral antigen in other selected tissues—for example the thymus—would be of interest.

The low virus titers detected in the HEV-infected tissues were a disappointment from the practical standpoint of vaccine production. Much higher titers had been anticipated in light of the large number of fluorescing cells observed, and several possibilities exist to account for this discrepancy: 1) Embryonic cells may be capable of producing large quantities of early HEV proteins but limited quantities of late proteins or viral nucleic acid. 2) Accurate titers of virus were not obtained due to virus being inadequately released from cells during the tissue preparation. Additional freeze-thaw cycles or sonication of cells may improve this situation. 3) The low titers may simply represent residual virus. Serial passage of virus in embryos would supply proof of virus multiplication.

These studies developed from the need to find an in vitro system for HEV replication, and it appears that the turkey embryo may prove suitable. Further efforts should be made to adapt the virus to the embryo by serial passage. In addition, the organ tropism of the virus should be further examined to attempt to
Identify the cell or cells susceptible to infection, including cells of other organs of the embryo. This should then be compared with the tropism of the virus for cells and organs of turkey poults when they are at an age at which the HE disease syndrome can be experimentally produced. An understanding of the pathogenic mechanisms of HEV in inducing the HE disease syndrome may result from the elucidation of the virus cell tropism.


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