PATHOGENESIS OF A HERPESVIRUS ISOLATED FROM AN OUTBREAK OF MUCOSAL DISEASE

by

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INTRODUCTION

During the past two decades, a number of similar disease conditions affecting cattle have been reported by various authors. These included virus diarrhea-New York (Olafson et al., 1946), mucosal disease (Ramsey and Chivers, 1953), virus diarrhea-Indiana (Pritchard, 1954), acute upper respiratory infection of cattle-California (Schroeder and Moys, 1954), an influenza-like entity-California (McKercher et al., 1954) and an infectious necrotic rhinotracheitis-Colorado (Miller, 1955).

The former three disease conditions are now grouped together under the name mucosal disease-virus diarrhea complex because of the close similarity in the clinical and pathological manifestations. Apart from the clinical and pathological similarities the viruses isolated from field cases of these syndromes are serologically related or identical. However, some workers believe that these syndromes have striking differences in the clinical signs and lesions.

Even though numerous antigenically related viral agents were isolated from field cases of mucosal disease and virus diarrhea, a typical disease syndrome terminating in death has not been reproduced in the laboratory with the possible exception of one or two cases.

The other disease conditions mentioned above are now described under infectious bovine rhinotracheitis (I.B.R.).

By use of cell culture methods, a cytopathogenic agent was isolated from this disease that has proven to be the etiologic agent (Madin et al., 1956).

It has been observed since 1950 that in some outbreaks of mucosal disease-virus diarrhea complex in which the mortality rate was high, the animals had a severe conjunctivitis and keratitis with marked lacrimation. In other herds these disease manifestations were absent. Lesions of the penis and prepuce of the male and vulva and vagina of the female were encountered in some outbreaks of this disease complex. These lesions in the female closely resembled those noted in cases of infectious pustular vulvovaginitis (I.P.V.).

I.B.R. virus has a wide range of pathogenicity in field and experimental cases. It has been found to produce conjunctivitis and keratitis (McKercher et al., 1958 and Abinanti and Plumer, 1961), encephalitis (French, 1962a), lesions in the reproductive tract often causing abortion (McKercher and Wada, 1964) and lesions in the gastrointestinal tract leading to a diarrhea (Baker et al., 1960).

The virus included in this study is one of two viral isolants from a field case diagnosed as mucosal disease. This agent was isolated from the Peyer's patch of the intestine.*

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*J. B. Gratzek, Associate Professor, Department of Veterinary Hygiene. Iowa State University of Science and Technology, Ames, Iowa. Isolation of a bovine herpesvirus. Private communication. 1963.

It is antigenically related to I.B.R. virus. The second isolant was recovered from the blood of the same animal and was found to be serologically related to virus diarrhea prototype Oregon C-24-V.* The above information indicates that dual infection does exist in outbreaks of mucosal disease-virus diarrhea complex. This has been confirmed by other workers who have also isolated viruses similar to I.B.R. virus from cases of mucosal disease-virus diarrhea complex (Noice and Schipper, 1959).

The present study was designed to determine the significance of the new viral isolant in the pathogenicity of the mucosal disease-virus diarrhea complex.

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*W. A. Malmquist, National Animal Disease Laboratory, Ames, Iowa. Data concerning the isolation and characterization of a virus associated with mucosal disease-virus diarrhea complex. Private communication. 1963.

REVIEW OF LITERATURE

Olafson et al. (1946) described a highly transmissible disease of dairy cattle which was named virus diarrhea-New York. This disease was characterized by severe diarrhea, rapid loss of condition and marked loss in milk production. Prominent gross lesions observed included ulcerations of the dental pad, palate and lateral surface of the tongue. Ulcerative and necrotic lesions were present on the pharyngeal and laryngeal mucosa. In most cases irregular, shallow, punched out linear ulcers were observed in the esophagus. Intestinal and abomasal mucosae were diffusely reddened with occasional ulcers present in the abomasum. Petechiae and small ulcerations were present in the cecum.

Mucosal disease was the name given to a disease syndrome in cattle described by Ramsey and Chivers (1953) and Ramsey (1956). This disease was characterized by gross erosions of the muzzle, lips, tongue, pharyngeal mucosa, buccal mucosa, esophagus, rumen, omasum and abomasum. Erosions were also consistently found in the small intestine, cecum and colon. Lymph nodes associated with the alimentary tract were swollen and hyperemic. Microscopically degenerative changes were present in the epithelium of the upper digestive tract. The necrosis extended from the surface to the basal layers. On microscopic examination Ramsey (1956) noted that many of the lesions in the abomasum which appeared to be ulcers on

gross examination were actually atrophic cystic glands with intact epithelium over the depressed area. The distended glands were filled with desquamated epithelial cells and leucocytes. Marked edema of the lamina propria and submucosa with leucocytic infiltration was usually noted. The inflammatory reaction of the small intestine was initially of an acute catarrhal type. The lesions were more severe in the jejunum and ileum. Histopathologic alteration of the small intestine included congestion, hemorrhage, defects in the epithelial lining and degenerative changes in the lymphoid tissue. Lesions varying from complete disappearance of lymphocytes to varying degrees of necrosis were present in the Peyer's patches.

Carlson et al. (1957) have reported on the experimental disease produced by the Indiana virus diarrhea agent. A biphasic pyrexia was observed. Throughout the course of both temperature reactions the calves were depressed, anorexic and developed a dry cough and nasal discharge. Mild diarrhea usually appeared during the second temperature elevation. Gross lesions of the gastrointestinal tract consisting of ulcerations and erosions were observed from the muzzle to the anus. These lesions were similar to those described by Ramsey (1956) varying only in the degree of involvement and severity. A catarrhal enteritis with congestion, edema and hemorrhage of the mucosa was observed in the intestine.

Necrosis of surface epithelium was observed in the cecum, colon and rectum. Lymphoid exhaustion and edema were very marked in the Peyer's patches.

Noice and Schipper (1959) isolated a cytopathogenic agent from a field case diagnosed as typical mucosal disease. This virus has been referred to as North Dakota bovine mucosal disease (B.M.D.) strain.

Barner et al. (1960) studied the experimental syndrome produced by the North Dakota (B.M.D.) agent in calves. The temperature was elevated at approximately 48 hours after inoculation. A concomitant leucopenia was observed at this time. Diarrhea did not occur. These workers observed a catarrhal to catarrhal-hemorrhagic enteritis in the posterior half of the small intestine. Petechial hemorrhages and/or erosions were present in the fundic portion of the abomasum. The Peyer's patches were edematous and hyperemic. On histologic examination there were hemorrhagico-necrotic alterations of the small intestine. Lymphoid depletion was frequently observed in the lymphatic tissues. The above lesions were similar to those produced by other isolants from the mucosal disease-virus diarrhea complex (Carlson et al., 1957).

Schipper (1961) later studied the serological characteristics of the North Dakota (B.M.D.) agent and concluded that it was similar to the virus of I.B.R.

From the study conducted by Tyler (1963) it was apparent

that combined infection with different viral isolants associated with mucosal disease-virus diarrhea complex resulted in a more severe clinical and pathological response. This was true especially when combinations of Sanders (Richter, 1962) and Merrell (Tyler, 1960) agents and of Sanders and North Dakota (B.M.D.) agents were administered.

Schroeder and Moys (1954) reported on an acute respiratory infection of cattle in California. This disease was characterized by high temperature, excessive salivation and extreme loss of condition. The nasal mucosa was inflamed and stringy mucus which later became mucopurulent was discharged from both nostrils. Gross lesions included hemorrhagic tracheitis and bronchitis. Necrotic lesions were scattered throughout the larynx and pharynx. Pneumonic changes were noted in the lungs. Severe inflammation of the small intestine was also noted. No lesions were reported in other organs.

The etiology of the above disease condition was undetermined at that time. However, it was possible to reproduce the disease by inoculation of homogenates of various tissues and exudates from naturally infected animals.

Miller (1955) described a disease condition in Colorado cattle similar to that reported by Schroeder. He called this disease condition "infectious rhinotracheitis of cattle".

After the etiologic agent of I.B.R. was isolated in cell

culture by Madin et al. (1956), the experimental disease was studied by Webster and Manktelow (1959) and Abinanti and Plumer (1961). The experimental disease was generally mild and complete recovery was the rule.

The most severe form of experimental disease of I.B.R. was seen in young calves as reported by Webster and Manktelow (1959). In these animals there was initial pyrexia, anorexia and an enlargement of superficial lymph nodes followed by development of a mucopurulent catarrhal rhinitis with small raised yellowish lesions on the nasal septum. Serous ocular discharge was consistently noted whereas purulent conjunctivitis was rarely observed. Postmortem lesions were almost entirely confined to the lymphatic and respiratory systems. Early in the course of the disease the lesions were more marked in the nasal cavity. In the animals killed later in the course of the disease lesions were more severe in the larynx, trachea and lungs. These included tracheitis with pseudomembrane formation and extensive areas of consolidation of the lungs. Microscopically the early acute inflammatory reactions were characterized by an outpouring of polymorphonuclear leucocytes and mononuclear cells in the subepithelial tissue with infiltration of overlying epithelium. This was followed by desquamation, cellular exudation and ulceration with much adherent necrotic cellular debris. Pulmonary lesions were those of an acute catarrhal bronchiolitis with

parenchymal collapse followed by necrotizing bronchiolitis. Later the inflammatory reaction spread to the adjacent parenchyma. The animals which received the inoculum by the intravenous route had focal hyaline necrotic lesions in the adrenal cortex.

The I.B.R. virus was found to have a predilection for the gastrointestinal tract in newborn calves as reported by Baker et al. (1960). This was in conflict with the earlier observation in older animals where there was a predilection for the respiratory tract (Schroeder and Moys, 1954). Extensive lesions were present in the gastrointestinal tract extending from the oral cavity through the forestomachs. Focal gray-white slightly raised areas were observed in the oral cavity. The above lesions were more numerous in the posterior part of the oral cavity, pharynx, larynx and cranial portion of the esophagus. Many epithelial nuclei on the edge of the ulcerative areas had eosinophilic inclusion bodies. In the forestomachs there were masses of gray soft material attached to the lining. Lymph nodes draining the area of epithelial necrosis were found to have massive areas of necrosis in the region of the peripheral sinuses. Usually the germinal centers were not affected until necrosis became so extensive as to involve the major portion of the node. Focal areas of necrosis and neutrophilic infiltration were observed in the liver, kidney and spleen.

I.B.R. virus was found to possess an unusually wide pathologic range. The virus causing I.P.V. was found to be the same as that causing I.B.R. (Gillespie et al., 1958; Bindrich, 1963). It has been demonstrated that the I.B.R. virus caused a conjunctivitis in experimental cattle following intranasal infection (McKercher et al., 1958). This was confirmed later in naturally occurring outbreaks of conjunctivitis in feeder cattle (Abinanti and Plumer, 1961). The initial isolation of I.B.R. virus in England was made from cattle affected with conjunctivitis unassociated with respiratory disease (Darbyshire et al., 1962).

In Australia a virus was isolated from the brains of cattle affected with an encephalitis (French, 1962a). The encephalitic syndrome was reproduced in calves following intranasal or intracerebral inoculation. Physical and serological studies of this agent revealed the relationship to that of I.B.R. virus (French, 1962b).

A virus similar to I.B.R. has been isolated consistently from cases of infectious infertility (epivag) of cattle in South Africa (Maré and van Rensburg, 1961).

Bindrich (1963) studied the viruses of I.B.R. and I.P.V. by means of the fluorescent antibody technique (Coons et al., 1942; Coons and Kaplan, 1950) in tissue culture. Fluorescence of the cell membrane was noted approximately 5 hours postinoculation. At about 10 hours postinoculation intense nuclear

fluorescence was observed. At 20-30 hours almost all nuclei were filled with fluorescing material.

In this study, the fluorescent antibody technique was used to detect virus in tissues and to determine its significance in the production of the lesions.

MATERIALS AND METHODS

Experimental Animals

A total of seven Hereford and one Holstein calves of four to six months of age were used in this study. All the Hereford calves were purchased from the disease free herd kept by the Veterinary Medical Research Institute, Iowa State University, Ames, Iowa. The Holstein calf was purchased from the Woodward State Hospital dairy herd at Woodward, Iowa. Preinoculation serum samples were assayed for antibody against the bovine herpesvirus strain used in this study, the National Animal Disease Laboratory strain of bovine virus diarrhea virus and the Colorado strain of I.B.R. virus. Only those calves without antibodies to the above agents were selected for the experiment.

Housing and Feeding

The calves were housed in the Veterinary Pathology research laboratory animal room. Wooden gates or metal fencing were used to form holding pens. No bedding was used. A rough rubber mat was used on the concrete floor to prevent slipping. Prior to each experiment the floor, panels, walls and utensils were thoroughly cleaned with lye solution and the room was fumigated with formaldehyde gas. Thorough precautions were taken to prevent contamination of the room after disinfection.

During the period of experiment, the calves were fed a diet consisting of cracked yellow corn - 43 per cent, crimped oats - 29 per cent, dehydrated alfalfa pellets - 15 per cent, soybean meal - 12 per cent, salt - 0.5 per cent and dairy mineral - 0.5 per cent. The ration was ground and mixed by a local feed mill. The calves were hand-fed twice daily and ample water was available in metal containers.

Virus

The virus was isolated by Gratzek* in bovine testicle cell cultures from the deep scrapings of Peyer's patch from a calf that died of mucosal disease. The virus was able to elicit cytopathic changes in cell monolayers which were characterized by rounding of the cells and detachment from the glass surface. This virus also formed plaques in 48 hours under agar overlay. The material used in this study was the virus at the fifth passage level in bovine testicle cell monolayers propagated with Hanks balanced salt solution containing 0.5 per cent lactalbumin hydrolysate.**

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*J. B. Gratzek, Associate Professor, Department of Veterinary Hygiene, Iowa State University of Science and Technology, Ames, Iowa. Isolation of a bovine herpesvirus. Private communication. 1963.

**Difco Laboratories, Detroit, Michigan.

Tissue Culture Media and Solutions

Medium used for cell propagation consisted of Hanks balanced salt solution (HBSS) with 0.5 per cent lactalbumin hydrolysate (LaH) (Melnik, 1955) and 10 per cent bovine or ovine serum.

HBSS-LaH (H-LaH)

NaCl	8.0 gm.
KCl	0.4 gm.
$MgSO_4 \cdot 7\ H_2O$	0.2 gm.
$Na_2HPO_4 \cdot 2\ H_2O$	0.06 gm.
KH_2PO_4	0.06 gm.
Glucose	1.0 gm.
$CaCl_2$	0.14 gm.
Phenol red	0.02 gm.
Lactalbumin hydrolysate	5.0 gm.
Double distilled water q. s.	1000 ml.

The medium was autoclaved at 120 C. and 20 pounds pressure for 20 minutes. After cooling to room temperature two and one half ml. of a 1.4 per cent sterile sodium bicarbonate solution and an antibiotic mixture containing 10,000 units of penicillin and 10,000 μg. of streptomycin were added per 100 ml. of the medium. The medium was stored at 4 C. Bovine or ovine serum was added prior to seeding of culture tubes and plates.

Saline used for washing and dilution of cells

Saline used for this purpose was the saline G of Ham and Puck (1962).

Glucose	1.1	gm.
Phenol red concentrate (0.002 gm./ml.)	0.6	ml.
NaCl	8.0	gm.
KCl	0.4	gm.
$Na_2HPO_4 \cdot 7\ H_2O$	0.29	gm.
KH_2PO_4	0.15	gm.
$MgSO_4 \cdot 7\ H_2O$	0.15	gm.
$CaCl_2 \cdot 2\ H_2O$	0.016	gm.
Lactalbumin hydrolysate	5.0	gm.

The saline solution was sterilized by autoclaving. After cooling, penicillin (100 units/ml.) and streptomycin (100 µg./ml.) were added and the saline was stored at 4 C.

Trypsin solution

Trypsin solution used for dispersion of cells consisted of the following:

Glucose	1.0	gm.
NaCl	8.0	gm.
KCl	0.4	gm.
$NaH_2PO_4 \cdot 7\ H_2O$	0.045	gm.
KH_2PO_4	0.03	gm.
$CaCl_2 \cdot 2\ H_2O$	0.016	gm.

Phenol red	0.0012 gm.
Penicillin	2×10^5 u
Streptomycin	0.2 gm.
Trypsin*	2.0 gm.
Double distilled water q. s.	1000 ml.

The solution was kept at 37 C. for one hour to dissolve the trypsin prior to filtration through a Selas 02 filter.** The solution was stored at 4 C.

0.01 M phosphate buffered saline (pH 7.2)

This solution was used for washing the tissue sections and in the preparation of the fluorescein conjugate.

KH_2PO_4	0.27 gm.
Na_2HPO_4	2.86 gm.
NaCl	8.18 gm.
Double distilled water q. s.	1000 ml.

0.5 M carbonate-bicarbonate buffer (pH 9)

This solution was used in the preparation of fluorescein conjugate.

Na_2CO_3	1.093 gm.
$NaHCO_3$	3.319 gm.

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*Difco Laboratories, Detroit, Michigan.

**Selas Corporation of America, Dresher, Pennsylvania.

Double distilled water q. s.	100 ml.

0.15 M NaCl

This solution was used to dilute the gamma globulin. It was prepared by dissolving 8.76 gm. of NaCl in one liter of distilled water.

Overlay medium for plaque assay

TC Medium Eagle HeLa 10 X*	200 ml.
5% L-glutamine	12 ml.
10% sodium bicarbonate	66 ml.
Penicillin	100,000 u.
Streptomycin	100,000 µg.
Double distilled water q. s.	1,000 ml.

The medium was sterilized by filtration and stored at 4 C. It was warmed to 45 C. prior to mixing with an equal volume of aqueous two per cent Special Noble Agar.*

Serum

Bovine and ovine sera were supplied by Microbiological Associates Inc., Bethesda, Maryland.

Cell Source

Bovine kidneys and testicles were obtained through the courtesy of the Rath Packing Company, Waterloo, Iowa. The

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*Difco Laboratories, Detroit, Michigan.

testicles and kidneys were removed from the slaughtered calves approximately six hours prior to arrival of tissues in Ames. During this time they were kept at approximately 4 C.

Sterilization

The glassware was sterilized by autoclaving at 120 C. and 20 pounds pressure for 20 minutes.

Cell Culture Procedure

Bovine kidney cells were prepared according to the method described by Youngner (1954).

Bovine testicles were processed in the same way as described by Gratzek (1961). The sedimented cells were resuspended in saline G and 30 per cent serum before cell counts were made. The cell suspension was added to the medium containing ten per cent serum at the rate of one to two million cells per ml. This suspension was then dispensed into culture tubes, bottles or disposable petri-dishes. The medium was changed each 24 hours until a monolayer of cells was formed.

Plaque Technique

The plaque technique used was similar to that described by Dulbecco and Vogt (1954). After inoculation of plates containing cell monolayers the virus was allowed to absorb for 30 minutes at room temperature before overlaying with medium.

After the medium had solidified the plates were inverted and incubated at 37 C. in a humidified atmosphere containing 5 per cent carbon dioxide for 48 hours. After this period the cells were stained by adding 1:10,000 neutral red in one per cent agar at 45 C. Plaque counts were made approximately 12 hours after staining.

Hyperimmunization

Hyperimmunization was carried out by using virus propagated in testicle cell cultures derived from the same calf to be hyperimmunized. The inoculum consisted of tissue culture fluid which titered to approximately $10^{7.5}$ 50 per cent tissue culture infective dose ($T.C.I.D_{50}$). The inoculation schedule followed in the hyperimmunization procedure was as follows:

Table 1. Hyperimmunization schedule

Day	Amount of inoculum	Route	Freund's adjuvant**
1	1 ml.	I/V	None
21	10 ml.	I/V	None
28	10 ml.	I/V	None
	10 ml.	S/C*	10 ml.
35	10 ml.	I/V	None
	10 ml.	S/C*	10 ml.
43	10 ml.	S/C*	10 ml.

*The inoculum for subcutaneous (S/C) injection was a mixture of equal parts of tissue culture fluid and Freund's adjuvant. The sites of subcutaneous injection were adjacent to the prefemoral and prescapular lymph nodes.

**Difco Laboratories, Detroit, Michigan.

Serum was collected one week after the last injection.

Preinoculation Procedures

The calves were given sufficient time to accommodate to the new environment before being used in experimentation. Observations were made at least twice daily to determine their state of health. After the calves had adjusted to the environmental changes blood samples were taken once a day and temperatures recorded twice a day. When a stable level was reached, the calves were judged ready to be inoculated.

Inoculation

The inocula consisted of tissue culture fluids which had been stored at -70 C. These fluids were thawed slowly prior to inoculation. Calves 2, 5, and 15 received five ml. of the inoculum by the intravenous route. Calves 11 and 12 were given two ml. of inocula intranasally using a nebulizer. The remaining two calves (7 and 14) received 60 ml. of inoculum orally.

Postinoculation Procedures

Following inoculation, the calves were observed for clinical signs of illness. During this period, blood samples were taken for cell counts once daily and rectal temperatures were taken and recorded twice daily. Blood samples and nasal swabs were also taken for virus reisolation. Serum samples

were collected daily for antibody assay. Rectal swabs were taken from calves 7 and 14 for reisolation attempts. All of the swabs were placed in sterile 25x125 mm. cotton-stoppered tubes containing one ml. of saline G. The swabs and blood samples for virus reisolation were stored at -70 C.

Virus Reisolation Procedures

After thawing, the swabs were washed in saline G. An antibiotic mixture containing penicillin (500 units/ml.) and streptomycin (500 µg./ml.) was added to the washings and incubation was carried on for 30 minutes at room temperature. The samples were centrifuged at 12,000 r.p.m. for 30 minutes at 4 C. The supernatant was used for inoculation of bovine kidney cell monolayers. The inoculated cell cultures were incubated at room temperature for one hour after which time one ml. of medium with two per cent ovine serum was added to each tube. The tubes were incubated at 37 C. and observed daily for cytopathic effects. After four days the medium from the tubes was pooled and reinoculated into cell cultures. Four such passages were made with each sample.

A similar procedure was used for the reisolation of virus from the blood.

Antibody Assay of Serum

A plaque reduction technique was used for measuring the antibody titer of the serum. Serum samples were diluted

fourfold with saline G. Equal volumes of virus dilution in saline G containing 120 plaque forming units (P.F.U.) and serum dilutions were mixed and allowed to incubate at room temperature for 30 minutes. After this period 0.5 ml. of the serum virus mixture was inoculated onto plates containing testicle cell monolayers. These plates were incubated at room temperature for 30 minutes prior to adding the overlay. The plates were incubated and stained in the same way as described before. The plaque counts were made and the 50 per cent end point was calculated by the method of Reed and Muench (1938).

Hematologic Procedures

Total leucocytic counts were made according to the method recommended by Benjamin (1958). The procedure described by the same author was used for absolute eosinophil count.

Necropsy Procedures

Calves were euthanatized by electrocution according to the schedule given in Table 4. Immediately after electrocution, necropsy was conducted. Gross observations were recorded and photographs were taken. Tissues were saved for both frozen and paraffin sectioning.

Tissues for paraffin sectioning were fixed in ten per cent buffered formalin for at least two days. The tissues

were trimmed, dehydrated, cleared and embedded in paraffin. Sections were cut at 6 microns with a rotary microtome.* Sectioning and staining were done according to the procedure given in the Manual of Histologic and Special Staining Technique by the Armed Forces Institute of Pathology (1957). All sections were stained with hematoxylin and eosin.

For frozen sectioning, small pieces of tissues (5 mm. thick) on tin foil were frozen in 2-methylbutane** kept cold by liquid nitrogen. Freezing was complete in about ten seconds. Frozen tissue blocks were stored at -20 C. prior to sectioning at 4 microns using a Cryostat*** set at -20 C. Tissue sections were placed on 0.8-1 mm. microscope slides and allowed to dry before they were fixed in fresh acetone. After fixation for ten minutes the slides were dried and either stored at 4 C. or immediately stained with fluorescein conjugate.

An alternative method used in the processing of tissues for staining with fluorescent conjugate was that of Sainte-Marie (1962). Tissue blocks were cut not more than five mm. thick and fixed in 95 per cent ethanol for 15-24 hours at 4

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*Scientific Products, 1210 Leon Place, Evanston, Illinois.

**Eastman Organic Chemicals, Rochester 3, New York.

***International Equipment Company, Boston, Massachusetts.

C. After fixation the tissues were dehydrated in four changes of precooled absolute ethanol for one to two hours each depending on the thickness of tissue. The tissues were cleared in three consecutive baths of precooled xylene for one to two hours each at 4 C. After placing the specimen in the last bath of xylene the container was removed from the refrigerator and allowed to equilibrate to room temperature (23-25 C.). Tissues were embedded in paraffin at 56 C. after passing through four consecutive baths of melted paraffin. The blocks were stored at 4 C. until sectioning. Sectioning was carried out as usual using a rotary microtome. The sections were dried at 37 C. for 30 minutes after which they were stored at 4 C.

Before staining the paraffin was removed by three changes of precooled xylene and the xylene in turn by three changes of precooled 95 per cent ethanol. Finally the alcohol was removed by three or four changes of cold phosphate buffer (pH 7.2). The sections were stained with the fluorescent conjugate.

Fluorescent Antibody Procedure

Fractionation of serum

The serum was fractionated by the procedure used by Kendall (1937). This was done by adding in a dropwise manner 40 ml. of a saturated solution of ammonium sulphate at 4 C.

to 80 ml. of serum with constant stirring. After the ammonium sulphate was added the suspension was vigorously stirred and centrifuged at 2500 r.p.m. for 15 minutes. The supernatant was decanted and the sediment was redissolved in 40 ml. of 0.15 M NaCl. The globulin fraction was reprecipitated by a further addition of 20 ml. of saturated ammonium sulphate solution. The resulting precipitate was centrifuged and redissolved in 40 ml. of 0.15 M NaCl. This fraction was dialyzed against 0.15 M NaCl with frequent changes of saline until the globulin fraction was completely free of any ammonium sulphate. Completion of dialysis was checked by the use of a saturated solution of barium chloride which will give a white precipitate if ammonium sulphate is present. After dialysis was complete, the precipitate formed during dialysis was removed by centrifugation. All of the above processes were carried out at 4 C. The globulin fraction was diluted with 0.15 M NaCl to a protein concentration of one per cent. Merthiolate was added to the diluted globulin to give a final concentration of 1:10,000 before storage at -20 C.

Conjugation

The conjugation of fluorescein to the globulin was done according to the method of Marshall *et al.* (1958). Five ml. of a 0.5 M carbonate-bicarbonate buffer at pH 9 were added to

45 ml. of one per cent gamma globulin solution in 0.15 M NaCl. Fluorescein isothiocyanate* (F.I.T.C.) was added to the above mixture at the rate of 0.05 mg. per mg. of protein. The conjugation was allowed to proceed overnight at 4 C. with constant stirring. After this period the conjugated gamma globulin was dialyzed against 0.1 M phosphate buffered saline (pH 7.2) until all of the unconjugated dye was removed. This was determined by the absence of fluorescence when exposed to a Wood's lamp. The fraction remaining in the dialysis tubing was centrifuged at 12,000 r.p.m. for 15 minutes to remove the precipitate formed during dialysis. The supernatant was distributed in small amounts (0.5-1 ml.) and stored at -20 C.

Gel filtration

An alternate method which was used for removing unconjugated dye was that of Killander et al. (1961). This consisted of passing the conjugated gamma globulin solution through a column of Sephadex G-25** packed to a volume of 2x5 cm. per ml. of conjugate. The Sephadex was previously washed with phosphate buffered saline (pH 7.4) to remove the fine particles. Elution was carried out with the same buffer. The rapidly moving zone containing the conjugated protein was collected. The unconjugated dye was retained at the top of

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*Nutritional Biochemicals Corporation, Cleveland, Ohio.

**AB Pharmacia, Uppsala, Sweden.

the column.

Absorption of the conjugate with tissue powder

Tissue powder used for absorption was prepared as described by Coons and Kaplan (1950). The conjugate was diluted with half of its volume of phosphate buffered saline prior to absorption. Tissue powder was then added to the conjugate in a centrifuge tube at the rate of 100 mg. per ml. This mixture was kept agitated for two hours at room temperature. The suspension was centrifuged at 12,000 r.p.m. for 15 minutes at 4 C. One additional absorption was done prior to collection and storage at -20 C.

Preparation of counterstain

The use of Eriochrome Black T was described by Hall and Hansen (1952) for counterstaining tissue sections without affecting the specific fluorescence. The counterstain was prepared by dissolving 1.7×10^{-5} mole of Eriochrome Black T* in one ml. of N,N-dimethylformamide and five ml. of chelating agent. The composition of the chelating agent was as follows:

N,N-dimethylformamide	50 ml.
Distilled water	20 ml.

*Fisher Scientific Company, 1458 N. Lamon Avenue, Chicago 51, Illinois.

0.1 M aluminum chloride	10 ml.
1 M acetic acid	10 ml.

The solution was adjusted to pH 5.2 with 1 M sodium hydroxide and made up to a volume of 100 ml. with distilled water. The chelated dye solution was allowed to develop maximum fluorescence before application on tissue sections. This required about 30 minutes.

Fluorescent staining procedure

Two sections from every block of tissue were used for staining in order to provide adequate controls. One section was pretreated with normal unlabelled gamma globulin prepared in an identical fashion as specific antiviral gamma globulin. The other section was pretreated with unlabelled specific gamma globulin. Both slides were incubated in a moist chamber at 37 C. for one to two hours. The slides were washed in phosphate buffered saline (pH 7.2) for five minutes with at least two changes of buffer. The excess buffer was drained from the sections and both were treated with labelled specific antiviral gamma globulin in a moist chamber for 30 minutes at 37 C. The sections were washed again in phosphate buffered saline for five to ten minutes after which they were counterstained by dipping in the chelated dye solution for five to ten seconds. Counterstaining was not used in all cases. The excess stain was washed off after which the

sections were mounted in buffered glycerine (equal parts of buffered saline and glycerine). The slides were examined by using a Leitz Ortholux microscope* with an Osram HBO 200 mercury vapour lamp, Type L 11, as light source and with 2 mm. UG1-UV filter. A dark field condenser was used to facilitate visualization of fluorescence.

Photography

A Leitz microcamera* attachment with a Leica M1 body was used for taking photographs of tissue sections. Photographs of gross lesions were taken with a Kodak Startech camera.

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*E. Leitz, Inc., 468 Park Avenue South, New York 16, New York.

RESULTS

Clinical Observations

Calves 2, 5 and 15 were inoculated by the intravenous route. The clinical response in calves 2 and 5 was characterized by temperature elevation within 24 hours following inoculation. However, in calf 15 the temperature rose slowly reaching a peak in approximately 72 hours. In all the animals peak temperatures were three to four degrees F. above normal and the temperature remained at this level for about 24 hours after which it started to decline slowly. By the fifth day postinoculation the temperatures were within the normal range (Fig. 1). During the febrile response the animals became depressed and developed a mucous nasal discharge, hyperpnea and exaggerated bronchial sounds. Slight coughing was noted in calf 15 but not in the others. Mild to complete anorexia was present in all calves during the febrile period. Diarrhea was observed on the day of the peak temperature and in calf 15 the diarrhea persisted for three days.

Calves 11 and 12 were inoculated by the intranasal route. In these calves temperatures rose slowly reaching a peak in approximately 72 hours postinoculation. The temperatures at this point were two to three degrees F. above normal and lasted for about two days (Fig. 2). The animals

developed a mucous nasal discharge and hyperpnea. On close examination the nasal passages were seen to be reddened. The calves retained a normal appetite throughout the experiment. Diarrhea was not observed.

Calves 7 and 14 received the inoculum by the oral route. These animals developed a febrile response at two days post-inoculation at which time the temperatures were elevated approximately three degrees F. (Fig. 3). The calves developed a nasal discharge, hyperpnea and excessive salivation accompanied by accumulation of froth at the angles of the mouth. During the febrile period the calves were anorexic and developed a marked diarrhea which persisted for approximately three days.

Hematologic Observations

In the calves inoculated intravenously (2, 5 and 15) the total leucocyte counts fell to approximately 50-60 per cent of the preinoculation value. This drop occurred at the time when the febrile response was at the peak. At this time the absolute eosinophil counts were found to be the same or slightly higher than the preinoculation value. At later stages when the total leucocyte counts were within the normal range the absolute eosinophil count was lower than the preinoculation level. In all the calves the highest eosinophil counts were observed at the peak of the febrile response (Fig. 1).

Fig. 1. Average clinical data on calves 2, 5, and 15 infected intravenously

□—Temperature (morning)

■--Temperature (evening)

O--Leucocytes x 10^3/cmm.

▽--Absolute eosinophils/cmm.

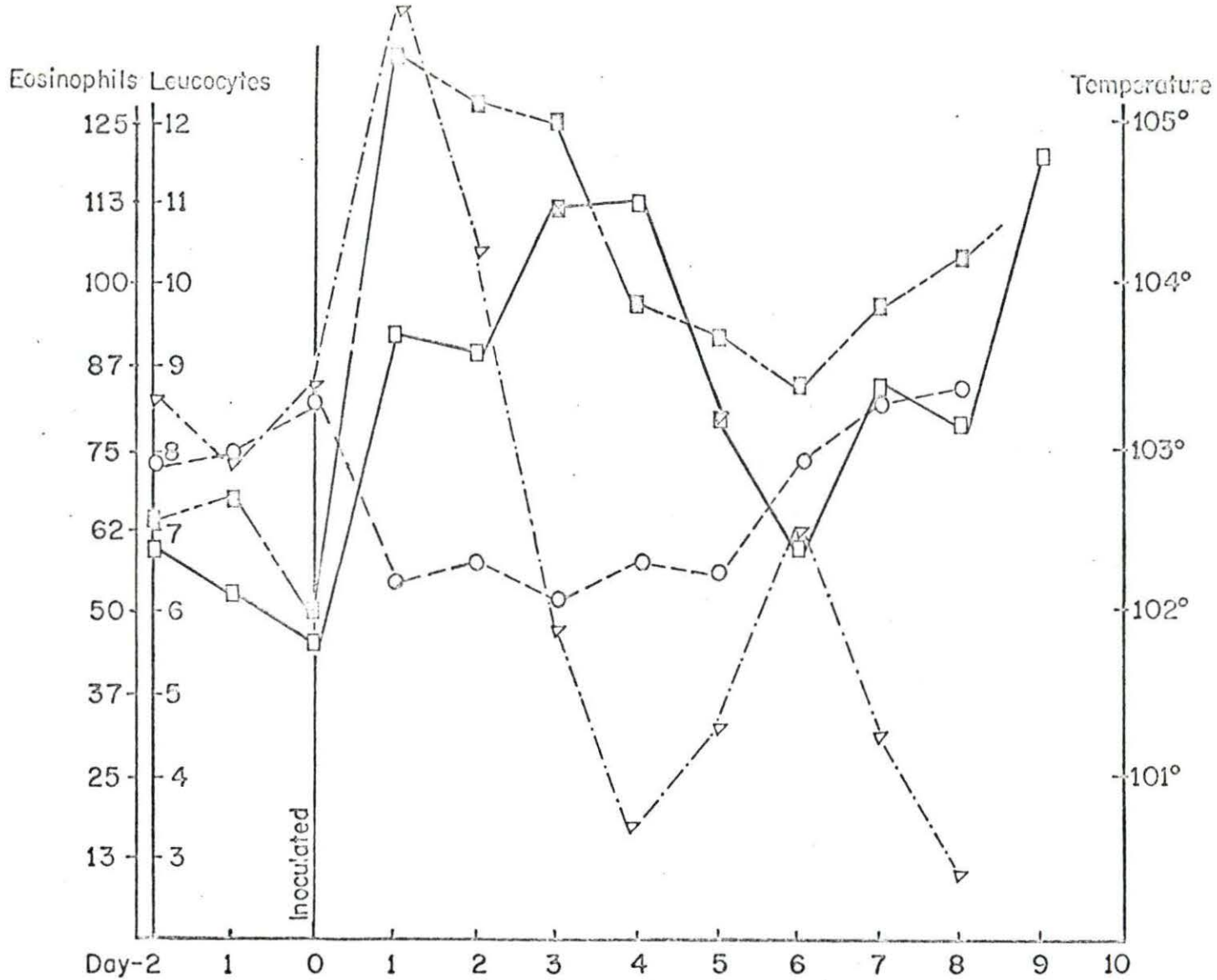
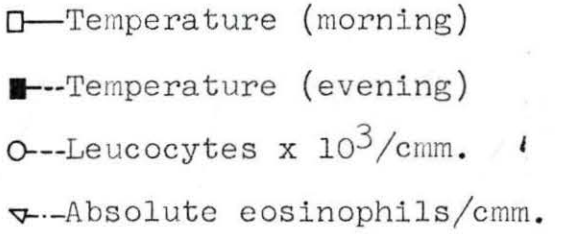


Fig. 1—Average Clinical Data on Calves (numbers 2,5,&15) Infected Intravenously

Fig. 2. Average clinical data on calves 11 and 12 infected intranasally



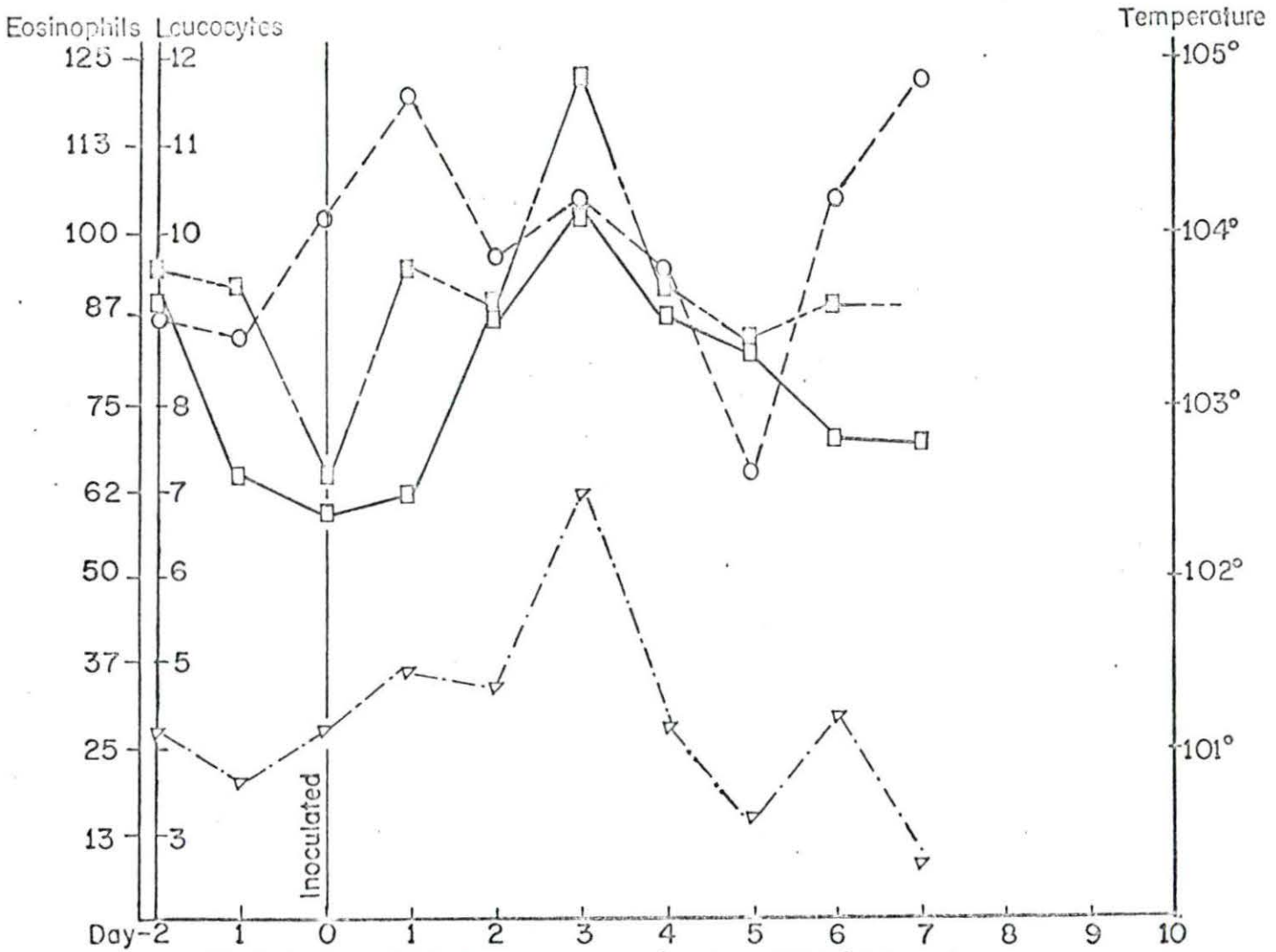


Fig.2-Average Clinical Data on Calves (numbers 11 & 12) Infected Intranasally

Fig. 3. Average clinical data on calves 7 and 14 infected orally

□—Temperature (morning)

■--Temperature (evening)

O---Leucocytes x 10^3/cmm.

▽--Absolute eosinophils/cmm.

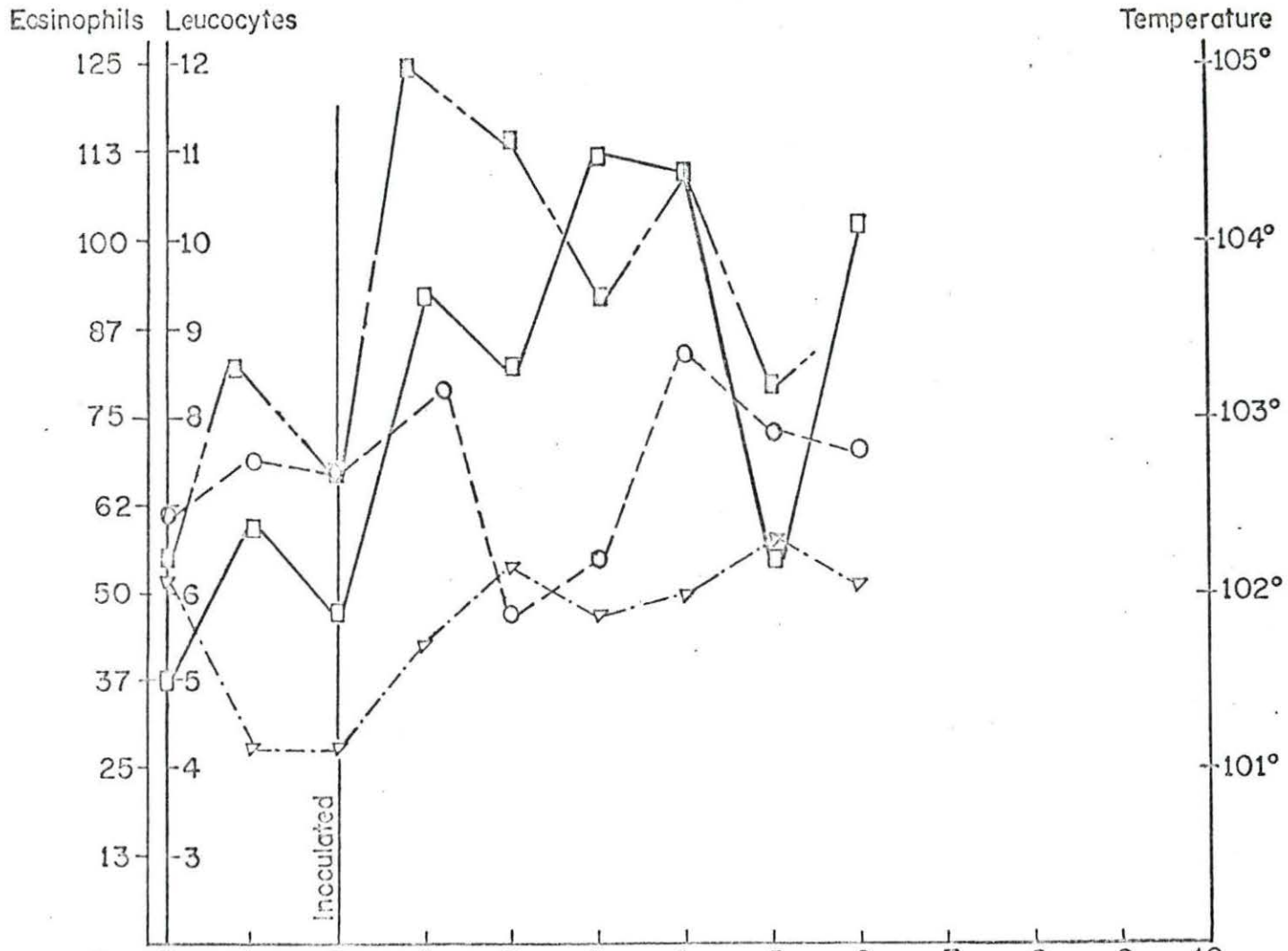


Fig. 3–Average Clinical Data on Calves (numbers 7 & 14) Infected Orally

In the calves infected intranasally the leucopenia was not marked. However, in calf number 11 the total white cell count was in the range of 60-70 per cent of the preinoculation level on one day. A great variation in the daily total white cell counts was noted in these calves during the experiment. The absolute eosinophil counts of the animals were twice that of the preinoculation level during the febrile period after which there was a decrease in the absolute counts (Fig. 2).

Calves infected by the oral route (7 and 14) developed a marked leucopenia during the febrile period. The total count returned to the normal range by five days postinoculation. Eosinophil counts in these calves were consistent with those inoculated by the intravenous and intranasal routes (Fig. 3).

Gross Pathologic Observations

Calves 2, 5 and 15 were inoculated by the intravenous route and were sacrificed on the third, fifth and ninth day postinoculation respectively. Calves 11 and 12 infected intranasally were sacrificed on tne fifth and seventh day postinoculation respectively. Calves 7 and 14 infected orally were also sacrificed on the fifth and seventh day postinoculation respectively.

In general gross pathological changes in all animals

were similar with only minor differences between the different groups receiving the inoculum by different routes.

Gross lesions were not found in the oral cavity or in the esophagus in any of the animals.

The tonsils of calves 7 and 14 were markedly enlarged and upon incision, small greyish necrotic foci and petechial hemorrhages were observed.

Erosions were visible on the pillars of the rumen of calf 15 (Fig. 4). However, similar lesions were absent in other calves. Edema and hyperemia of the abomasal ulcers varying from three to five mm. in diameter were present in the abomasal folds of the animals which received the inoculum by the intravenous route. Often the ulcers were surrounded by a hemorrhagic border.

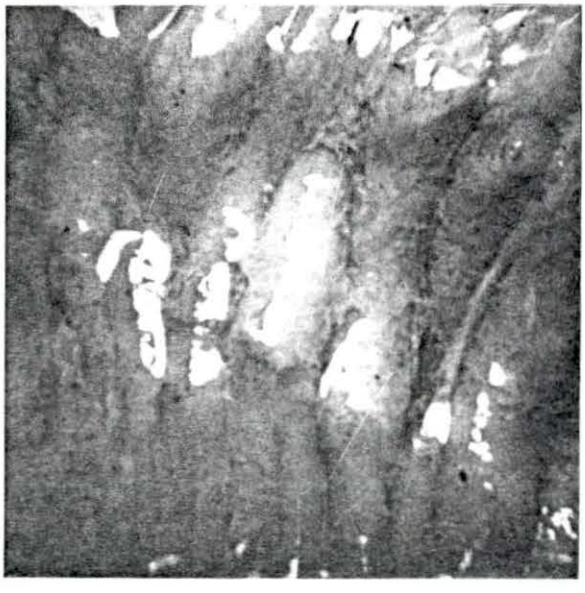
The lesions in the small intestine were characterized by a catarrhal enteritis of varying degrees in all animals except those infected by the intranasal route. The above lesion was most marked in calves 7, 14 and 15, and was characterized by the presence of an adherent catarrhal exudate on the mucosa (Fig. 5). The duodenum and ileum were most severely affected. When the exudate was removed, the mucosal surface was found to be hyperemic. In animals 11 and 12 the only gross lesions in the small intestine were mild hyperemia and edema. Peyer's patches in all animals were edematous and circumscribed with a hemorrhagic border. Incision of the Peyer's patches revealed

Fig. 4. Rumen pillar showing surface erosion.

(Calf 15 infected intravenously)

Fig. 5. Small intestine showing catarrhal exudate attached on to the mucosa. Note also the hyperemic border of the Peyer's patch.

(Calf 15 infected intravenously)



small yellowish white necrotic foci.

Infrequently, gross lesions were observed in the large intestine. In calves 5 and 15 there were ecchymotic hemorrhages measuring approximately three to five mm. in diameter on the cecal mucosa. In calf 7 there was a distinct fibrinous cast attached to the mucosa of the colon.

Gross lesions of the respiratory tract were usually mild and confined to the nasal cavity and trachea. Pulmonary lesions were rare. In the nasal cavity the macroscopic alterations were characterized by a mild to moderate catarrhal rhinitis and ecchymotic hemorrhages on the nasal septum. Diffuse redness of the turbinates was usually observed. A catarrhal exudate was present on the surface and in between the folds of the turbinates in calves 11, 12, 7 and 14. A marked catarrhal tracheitis with a layer of exudate adhering to the mucosa was observed in calves 2 and 15. No tracheal lesions were noted in other calves. A stringy yellowish exudate was present in the bronchioles of calves 2, 5 and 15.

Marked ecchymotic hemorrhages were present on the wall of the frontal sinuses of calves which received the virus by the intravenous and intranasal routes.

All the lymph nodes associated with the alimentary and respiratory systems were enlarged with petechiations usually being noted on incision. A marked gross finding in calf 14 was the hemorrhagic foci in the hepatic lymph nodes.

Distinct hemorrhagic lesions measuring approximately one to two mm. in diameter were present in the adrenal cortex of calves 5 and 15 whereas similar lesions were not visible in the adrenals of other animals.

Histologic Observations

No microscopic tissue changes were noted in the tongue, oral mucosa, pharynx or esophagus.

Marked tonsillar changes were observed in all animals. These consisted of lymphoid depletion and coagulative necrosis of the germinal centers. Excessive accumulation of necrotic cellular debris was noted in the tonsillar crypts.

Typical epithelial surface necrosis was present on the pillars of the rumen in calf 15 (Fig. 7). The lesion was that of an acute focal necrotic and ulcerative rumenitis. The base of the ulcer contained coagulated edema, cellular debris and abundant numbers of eosinophils. Eosinophilic infiltration was extensive in the lamina propria on either side of the necrotic lesion. In calf 12 histopathologic changes in the rumen folds were characterized by slight hypertrophy and hyperplasia of the stratified squamous epithelium accompanied by hydropic degeneration and cell death suggesting early stages of ulcerative rumenitis (Fig. 6).

Microscopic lesions in the abomasum consisted of focal linear accumulations of plasma cells, eosinophils and very

few neutrophils (Fig. 8). Apart from these lesions, there were epithelial necrosis and desquamation in animals infected by the intranasal and oral routes. Many of the crypts near the mucosal surface were filled with desquamated epithelium and leucocytes.

Histopathologic changes in the small intestine were similar in all animals. Extensive infiltration of lamina propria with leucocytes was consistently observed throughout the length of the intestine, the majority of the cells being eosinophils (Fig. 16). Lesions of varying degrees were often present in the Peyer's patches. These were characterized by mild to moderate depletion of lymphoid elements leaving behind a pink staining sponge-like stroma. Many of the lymph follicles contained pink staining hyaline droplets. Focal cystic necrosis was consistent in the Peyer's patches, the necrosis being more extensive in the two groups which received the virus by the intravenous and oral routes. The necrotic lesions contained numerous degenerating neutrophils and macrophages, the latter containing lipid droplets and necrotic cellular debris in their cytoplasm (Fig. 10). A few suppurative foci were also observed in the Peyer's patches of calf 15 (Fig. 11).

Microscopic changes in the large intestine consisted of mild lymphoid depletion, focal necrosis of lymphoid tissue and extensive leucocytic infiltration of the lamina propria,

the prominent cells being eosinophils.

Liver lesions were similar in all groups of animals and were characterized by minute focal infiltrations consisting of eosinophils, lymphocytes and a very few neutrophils (Fig. 13). Usually the total number of cells in each of these foci varied from ten to fifteen. Hepatic cells adjacent to these lesions revealed early degenerative changes.

Selective involvement of the lymph nodes was observed in all calves. The most prominent lesions were confined to the mesenteric, bronchial and retropharyngeal lymph nodes. These lesions were characterized generally by mild to moderate lymphoid depletion (Fig. 12) and a diffuse infiltration with eosinophils. In calf 15 there was an acute focal hemorrhagic lymphadenitis of the mesenteric lymph nodes with marked neutrophilic infiltration. The most prominent and consistent finding in the bronchial lymph nodes was the accumulation of eosinophils in the distended blood vessels.

A mild lymphoid depletion and evidence of necrosis of the Malpighian corpuscles of the spleen were frequently observed. Accumulation of eosinophils was consistent in splenic tissue from all animals. These cells often formed a cuff at the periphery of the Malpighian corpuscles.

Marked focal necrosis of the zona fasciculata of the adrenal cortex was noted in calves 5 and 15. The area of necrosis contained an abundance of macrophages with

eosinophilic droplets in the cytoplasm (Fig. 9). In calf 7 the adrenal lesions were of a very mild nature and were characterized by foci of cloudy swelling with mild leucocytic infiltration. The sinusoids in these areas were obliterated markedly by the swollen adrenal cells.

Microscopic lesions of the kidney were observed only in calf 7. They consisted of desquamated epithelial cells and hyaline casts in the lumen of the collecting ducts.

Varying degrees of histopathologic changes were observed in the respiratory tracts on microscopic examination. In the turbinate the changes were generally characterized by mild to moderate catarrhal rhinitis with catarrhal exudate between the folds. The exudate consisted of eosinophils, neutrophils and a few macrophages with cytoplasmic vacuoles. Mild eosinophilic infiltration was noted in the epithelial layer. Marked infiltration of subepithelial tissue with plasma cells was noted in calves 2 and 7 (Fig. 15). These cells had a large amount of bright pink staining cytoplasm. Tracheal lesions in calves 2 and 15 consisted of catarrhal tracheitis with exudate containing necrotic cellular debris adhering to the mucosa. Epithelial changes in the trachea were characterized by cystic foci, leucocytic infiltration and ballooning of the cells. In the lungs microscopic lesions varying from mild interstitial thickening, in calves 2, 5, 7 and 15, to mild interstitial pneumonia with fibrinous

exudate in the alveoli, in calves 11, 12 and 14, were noted. There was an increase in the number of macrophages in the interalveolar tissue (Fig. 14).

Virus Reisolation

Detailed results of virus reisolation attempts are given in Table 2. The results indicate that the nasal samples were usually positive for virus except in calves 7 and 14.

Antibody Assay

The antibody titers of different animals at successive days postinoculation are given in Table 3. Detectable antibody titers were found at seven days postinoculation in calf 15 which was inoculated intravenously. Calf 5 showed no antibody response at five days postinoculation. In the calves which received the virus by the intranasal or oral route, antibodies were not detected even by the seventh day postinoculation.

Fluorescent Microscopic Observations

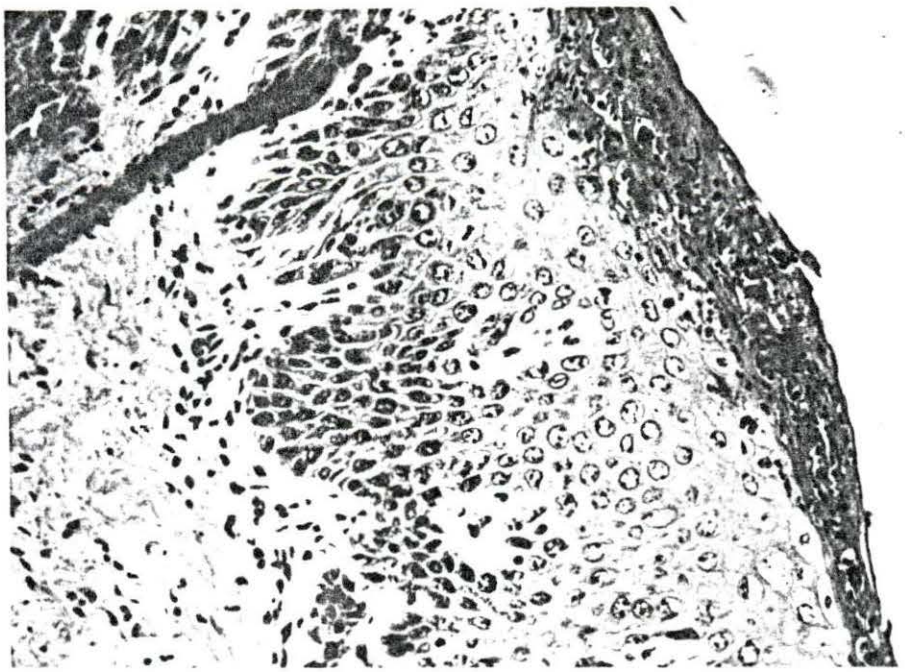
A number of different tissues and organs from each animal were examined for the presence of viral antigen. A detailed account of the distribution of the antigen in tissues as determined by the fluorescent antibody method is given in Table 4. The exact location and type of cells which contain the fluorescence varied from tissue to tissue. A description

Fig. 6. Note the surface necrosis of the stratified squamous epithelium of the rumen pillar which later sloughed off leading to erosion. X 130. Hematoxylin and eosin stain.

(Calf 12 inoculated intranasally)

Fig. 7. Note complete necrosis of the stratified squamous epithelium leading to an ulcer. Also note the extensive cellular infiltration in the lamina propria. X 50. Hematoxylin and eosin stain.

(Calf 15 inoculated intravenously)



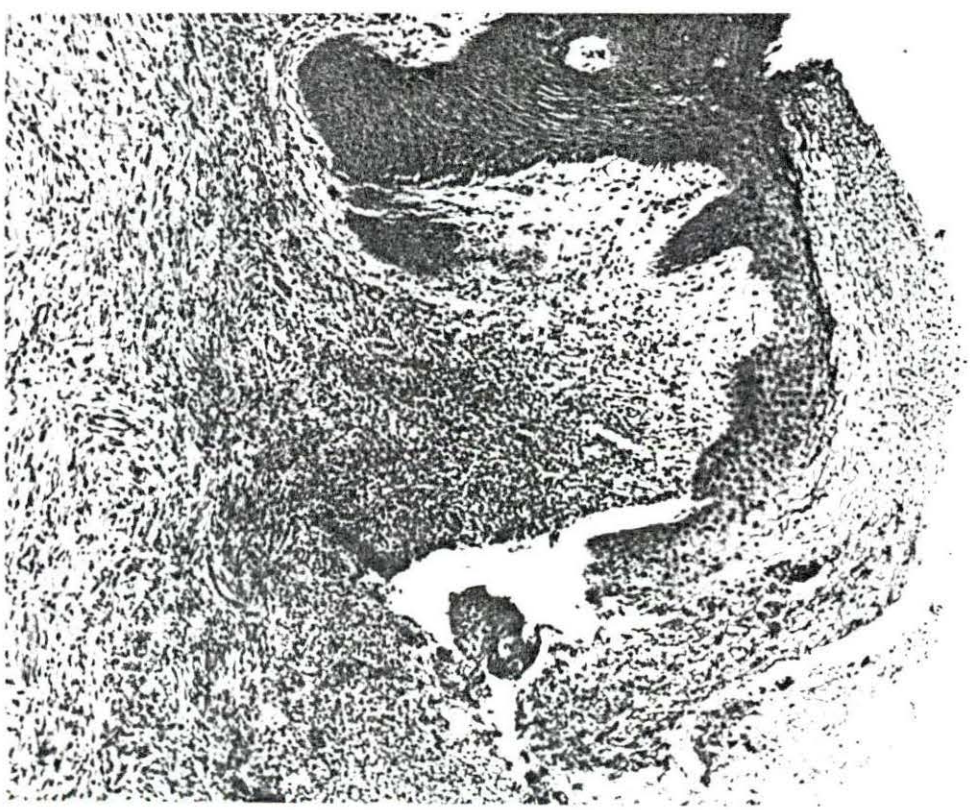


Fig. 8. Area of cellular infiltration in the lamina propria of the abomasum. Note extensive numbers of plasma cells. X 300. Hematoxylin and eosin stain.

(Calf 11 inoculated intranasally)

Fig. 9. Focal necrosis in the zona fasciculata of the adrenal cortex. X 70. Hematoxylin and eosin stain.

(Calf 15 inoculated intravenously)

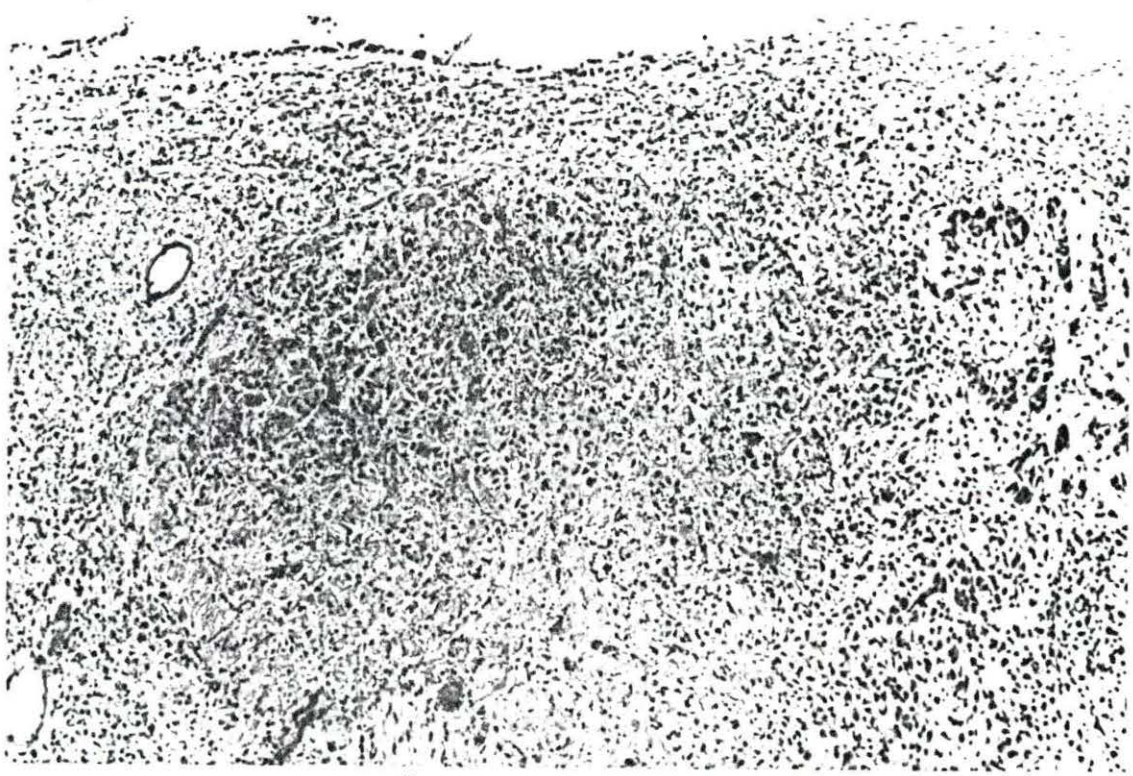
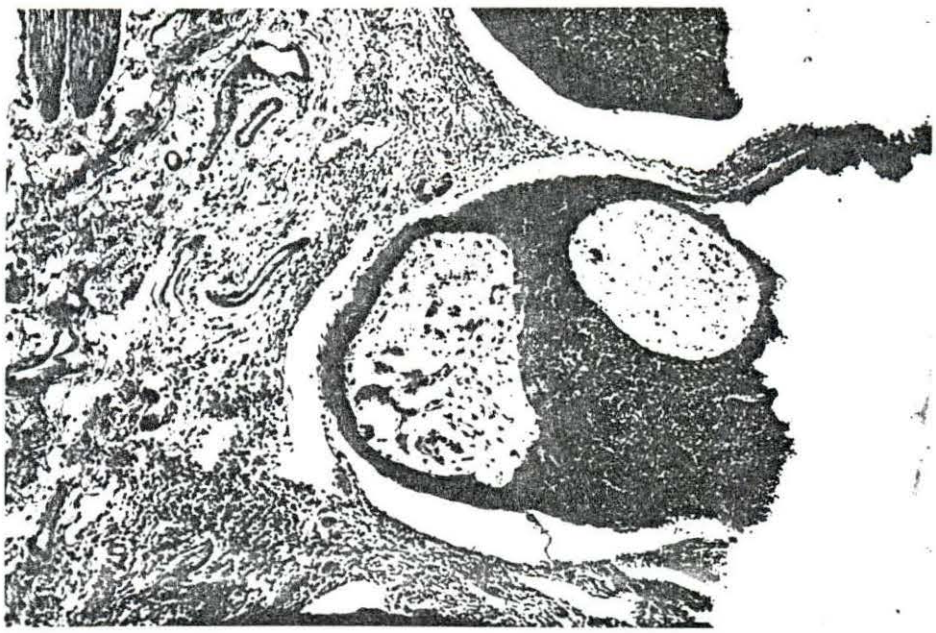


Fig. 10. Note the marked necrosis of the Peyer's patch of ileum with liquefactive changes leading to cyst formation. X 60. Hematoxylin and eosin stain.

(Calf 15 inoculated intravenously)

Fig. 11. Note the suppurative focus in the Peyer's patch of jejunum with neutrophils in the center. X 70. Hematoxylin and eosin stain.

(Calf 15 inoculated intravenously)



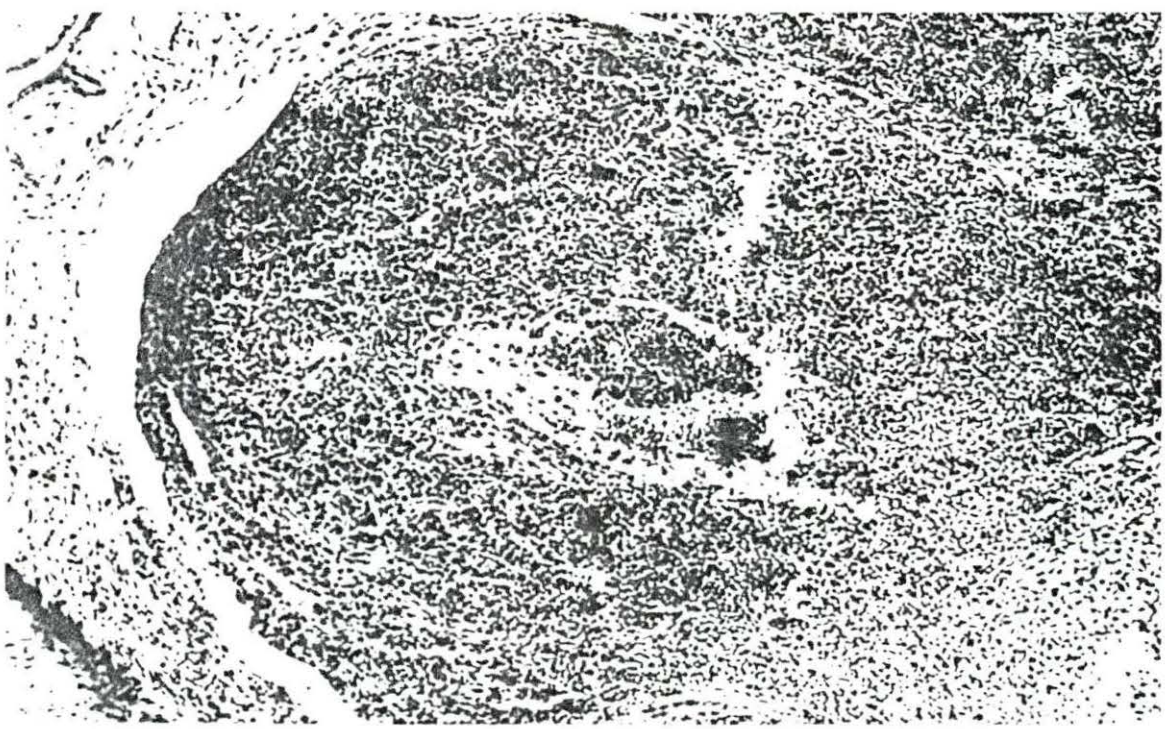


Fig. 12. Marked lymphoid depletion in one of the germinal centers of the mesenteric lymph node. Note a germinal center at the left top corner which appears normal. X 120. Hematoxylin and eosin stain.

(Calf 7 infected orally)

Fig. 13. Microfocus of cellular infiltration in the liver. X 400. Hematoxylin and eosin stain.

(Calf 12 inoculated intranasally)

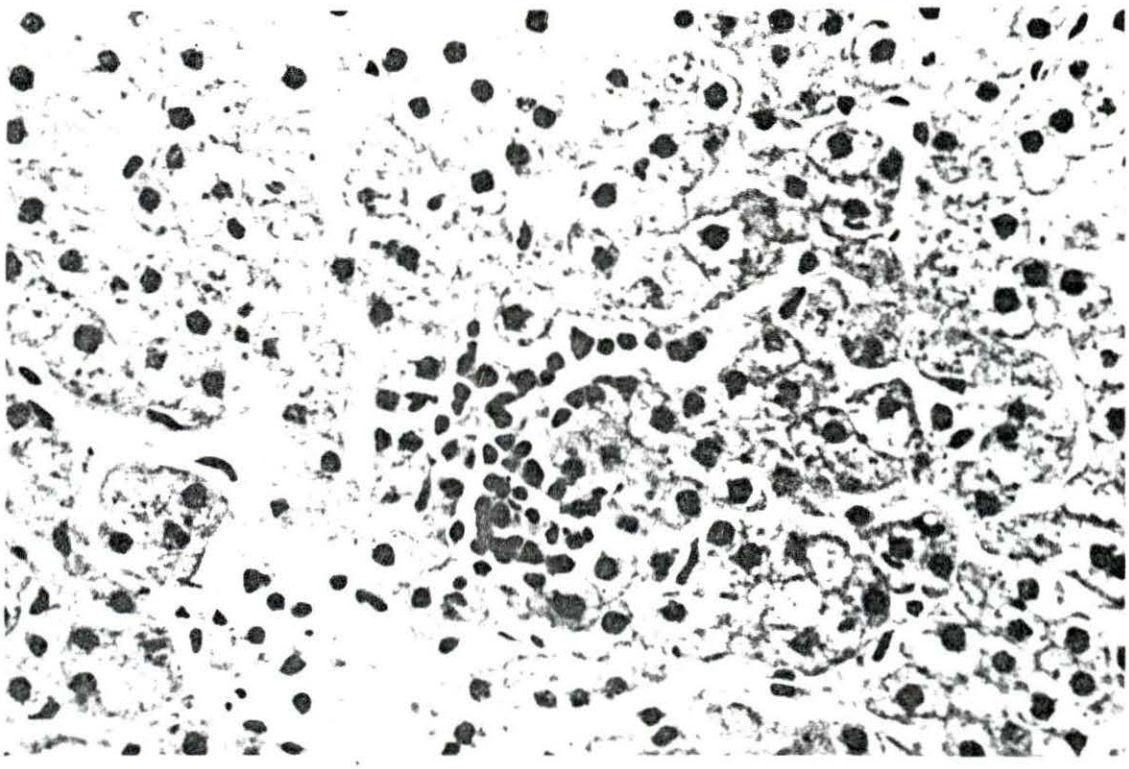
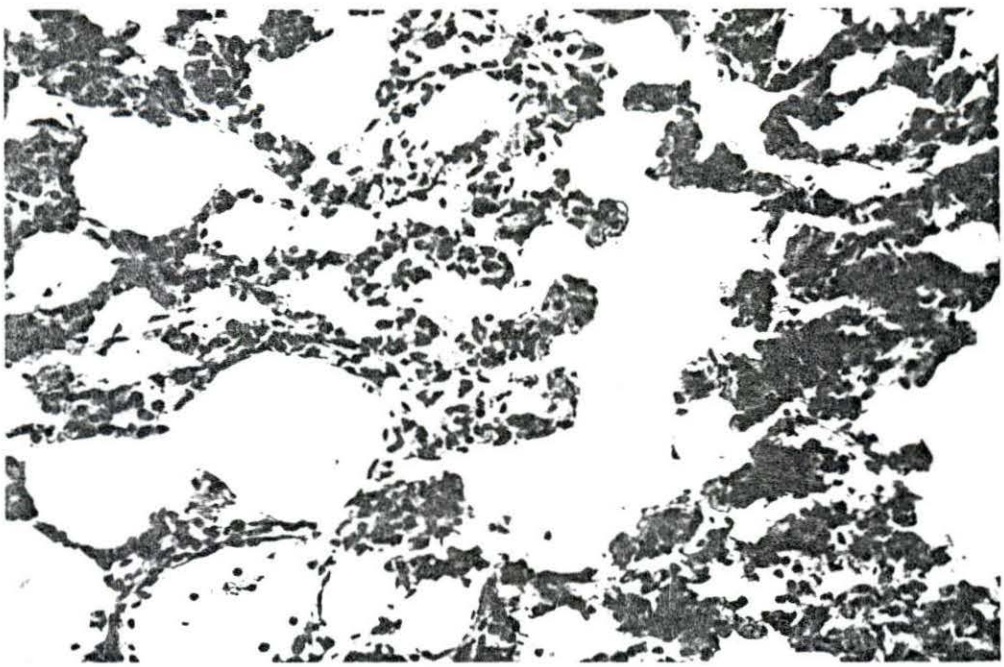


Fig. 14. Area of interalveolar thickening in the lung. Note the increase in number of cells in the interalveolar tissue. X 200. Hematoxylin and eosin stain.

(Calf 7 inoculated orally)

Fig. 15. Accumulation of highly cellular exudate between the folds of the turbinate. Note the cellular infiltration of subepithelial tissue. X 80. Hematoxylin and eosin stain.

(Calf 2 inoculated intravenously)



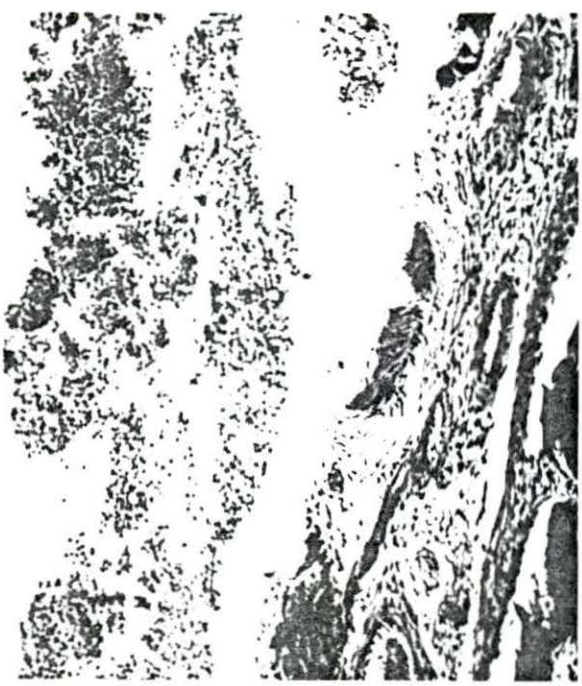


Table 2. Results of virus reisolation attempts from calves inoculated with a bovine herpesvirus by various routes

Calf Number	Route of Inoculation	Sample	Days postinoculation 2	3	4	5	7	9
2	Intravenous	Nasal	x*	x				
		Blood	-**	-				
5	Intravenous	Nasal	x	-	-	x		
		Blood	-	-	-	-		
15	Intravenous	Nasal	-	-	-	x	x	-
		Blood	-	-	-	-	-	-
11	Intranasal	Nasal	x	x	x	x		
		Blood	-	-	-	-		
12	Intranasal	Nasal	x	-	x	x	x	
		Blood	-	-	-	-	-	-
7	Oral	Nasal	-	-	-	-		
		Blood	-	-	-	-		
		Rectal	-	-	-	-		
14	Oral	Nasal	-	-	-	-	-	
		Blood	-	-	-	-	-	
		Rectal	-	-	-	-	-	

*Virus isolated.

**Virus not isolated.

Table 3. Antibody levels of calves inoculated with a bovine herpesvirus by various routes

Calf Number	Route of Inoculation	Log. 50% neutralization end point: Days after inoculation 2	3	4	5	6	7	8	9
2	Intravenous	0	0						
5	Intravenous	0	0	0	0				
15	Intravenous	0	0	0	0	0	$10^{-1.9}$	$10^{-2.9}$	$10^{-2.97}$
11	Intranasal	0	0	0	0				
12	Intranasal	0	0	0	0	0	0		
7	Oral	0	0	0	0				
14	Oral	0	0	0	0	0			

Table 4. Distribution of bovine herpesvirus antigen in tissues of calves following inoculation

Tissue Examined	Route of inoculation: Intravenous, Days postinoculation 3, Calf 2	Intravenous, Days postinoculation 5, Calf 5	Intravenous, Days postinoculation 9, Calf 15	Oral, Days postinoculation 5, Calf 7	Oral, Days postinoculation 7, Calf 14	Intranasal, Days postinoculation 5, Calf 11	Intranasal, Days postinoculation 7, Calf 12
Tongue	-*	-	-	-	-	-	-
Esophagus	-	-	-	-	-	-	-
Rumen	-	-	x**	-	-	-	-
Abomasum	x	-	-	x	-	-	-
Duodenum	-	-	-	-	-	-	-
Jejunum	-	x	-	-	x	-	-
Ileum	-	x	-	-	-	-	-
Cecum	x	x	-	-	-	-	-
Ileocecocolic valve	-	x	-	-	x	-	-

*Antigen not detected.

**Antigen detected.

Table 4. (Continued)

Tissue Examined	Route of inoculation: Intravenous, Days postinoculation 3, Calf 2	Intravenous, Days postinoculation 5, Calf 5	Intravenous, Days postinoculation 9, Calf 15	Oral, Days postinoculation 5, Calf 7	Oral, Days postinoculation 7, Calf 14	Intranasal, Days postinoculation 5, Calf 11	Intranasal, Days postinoculation 7, Calf 12
Colon	-	-	-	-	-	-	-
Rectum	-	x	-	-	-	-	-
Turbinate	x	-	-	-	x	-	x
Trachea	-	-	-	-	-	x	-
Lung	-	-	-	-	x	x	-
Bronchial lymph node	-	-	x	-	-	-	-
Mesenteric lymph node	-	x	-	-	-	x	-
Prescapular lymph node	-	-	-	-	-	-	-
Retropharyngeal lymph node	-	-	-	-	-	-	-
Tonsil	-	-	x	x	x	-	-
Epiglottis	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-

Table 4. (Continued)

Tissue Examined	Route of inoculation: Intravenous, Days postinoculation 3, Calf 2	Intravenous, Days postinoculation 5, Calf 5	Intravenous, Days postinoculation 9, Calf 15	Oral, Days postinoculation 5, Calf 7	Oral, Days postinoculation 7, Calf 14	Intranasal, Days postinoculation 5, Calf 11	Intranasal, Days postinoculation 7, Calf 12
Adrenal	-	x	x	-	-	-	-
Spleen	x	x	x	x	x	x	-
Myocardium	-	-	-	-	-	-	-

of the location and type of cells which contain the fluorescence is included in the following paragraphs.

Digestive system

In the jejunum, ileum, ileocecocolic valve, cecum and rectum the viral antigen was detectable in the lymphoid tissue. The location of the fluorescent material in these tissues corresponded with necrotic or depleted germinal centers. The fluorescence was chiefly cytoplasmic but some was seen in the nucleus. Brighter fluorescence was detectable at the cell membrane. The cells resembled those of the macrophage series (Fig. 17).

In the abomasum viral antigen was present in cells which resemble macrophages. These cells were situated in the lamina propria proximal to the mucosal surface (Fig. 18). This type of fluorescence was found only in those animals which were infected by the oral route.

In the rumen the base of the ulcerative lesions contained cells with cytoplasmic fluorescence. On staining with hematoxylin and eosin, it appeared that the cells which contained the viral antigen were in the areas of eosinophilic infiltration.

Respiratory system

Most of the fluorescence associated with the turbinate was in the cells contained in the exudate adherent to the

mucosa (Fig. 20). Very few cells with antigenic material were present in the subepithelial tissue and inside of the blood vessels. Both nuclear and cytoplasmic fluorescence were detectable. In the trachea, cells containing viral antigen were located in the mucosa. Only a very few cells contained the antigenic material.

In the lungs, many of the macrophages in the interalveolar tissue contained viral antigen. The antigen was present both in the nucleus and cytoplasm (Fig. 24). Pretreatment with specific gamma globulin was found to block the staining reaction (Fig. 25).

Reticuloendothelial system

Viral antigen in the mesenteric lymph nodes was detectable in the necrotic or depleted germinal centers. The fluorescent pattern resembled that seen in the Peyer's patches. In the bronchial lymph node, the area of fluorescence correlated with the cells present inside of the distended blood vessels. On staining with hematoxylin and eosin, these cells were found to be eosinophils.

In the tonsils, the fluorescing material was detected in cells of the mononuclear series associated with the necrotic lesion (Fig. 22). The fluorescence was also found in the necrotic material contained in the crypts. Specificity can be proved by blocking the staining reaction by unlabelled

specific gamma globulin (Fig. 23).

In the spleen, the cells with fluorescent antigenic material were distributed throughout the tissue with very little localization. In areas where there was some localization, the cells were situated at the periphery of the splenic corpuscles. These cells with cytoplasmic fluorescence resembled those of the mononuclear series (Fig. 21).

Endocrine system

Among the organs of the endocrine system, only the adrenals showed the presence of antigenic material. The specific fluorescence observed in the necrotic lesion was clearly distinct from the surrounding normal tissue. The fluorescence was chiefly cytoplasmic with deeper staining of the cell membrane (Fig. 19).

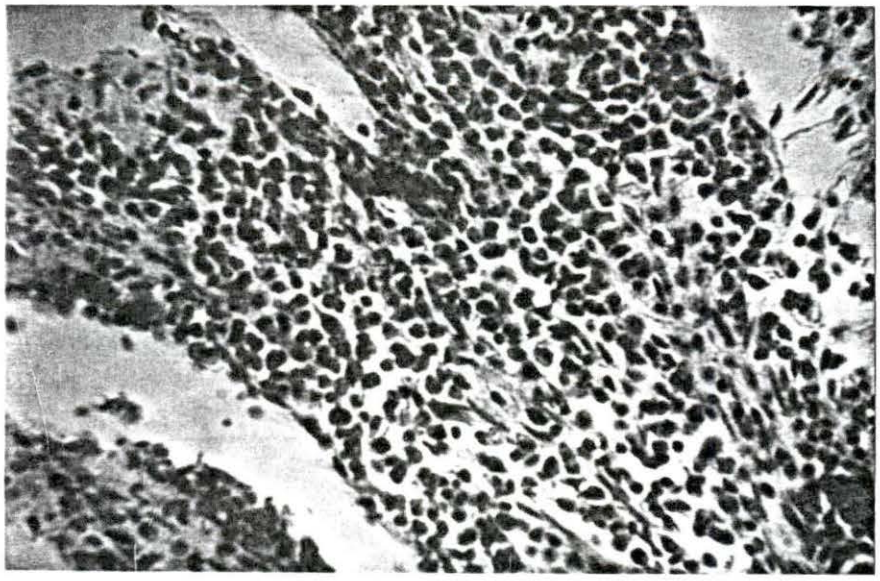
Fig. 16. Excessive eosinophilic infiltration of the lamina propria of the small intestine. X 250. Hematoxylin and eosin stain.

(Calf 7 infected orally)

Fig. 17. Small intestine. The area of fluorescence is represented by the yellowish color. The red-brown fluorescence is that of the counterstain. X 450.

(Calf 5 infected intravenously)

(The yellowish fluorescence is caused by the overlapping of the fluorescence of the counterstain in the process of photography)



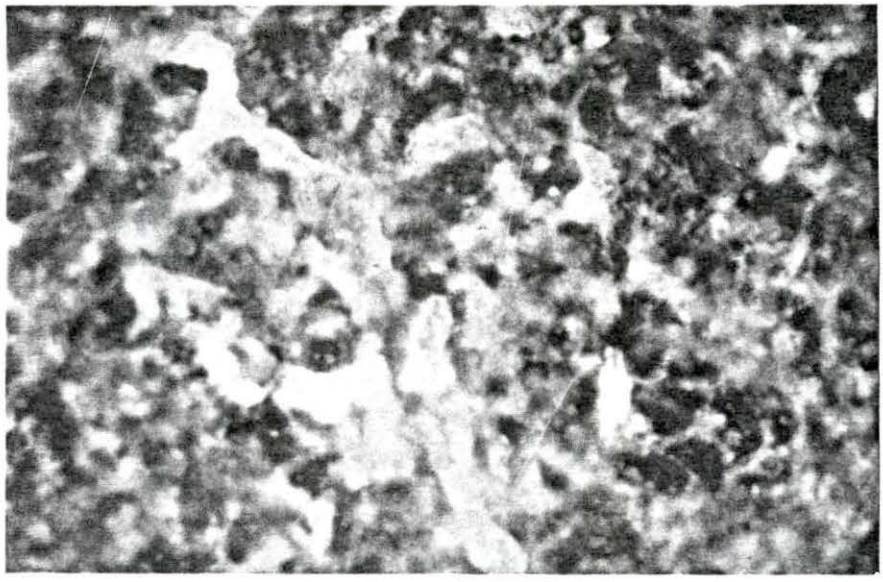
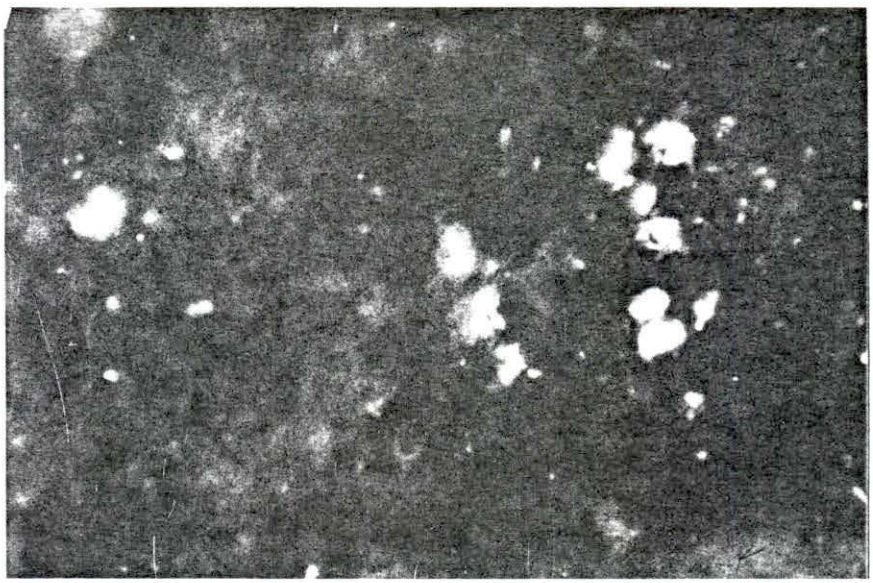


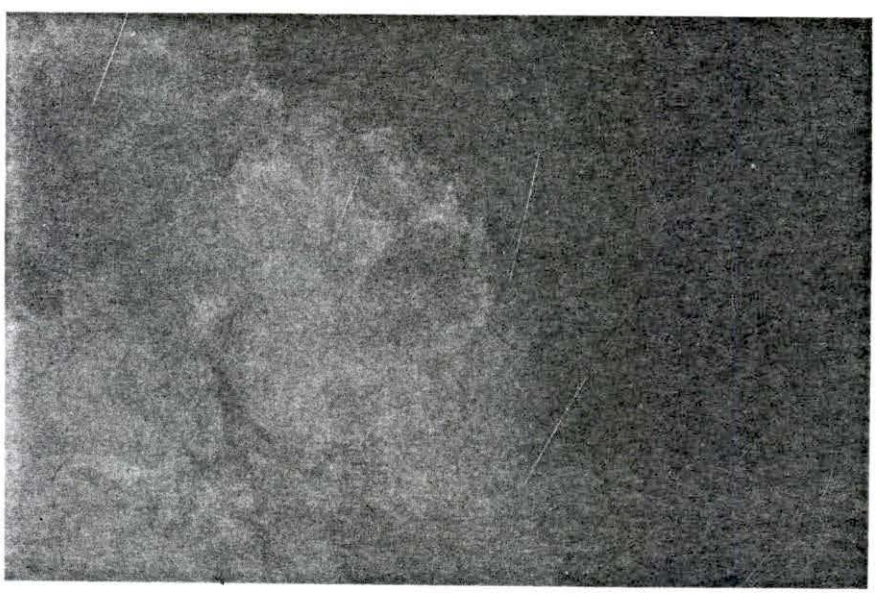
Fig. 18. Abomasum. The cells containing the fluorescing antigen are located in the lamina propria. X 540.

(Calf 2 infected intravenously)

Fig. 19. Adrenal. Note the fluorescence of the lesion on the left half of the picture. X 450.

(Calf 15 infected intravenously)





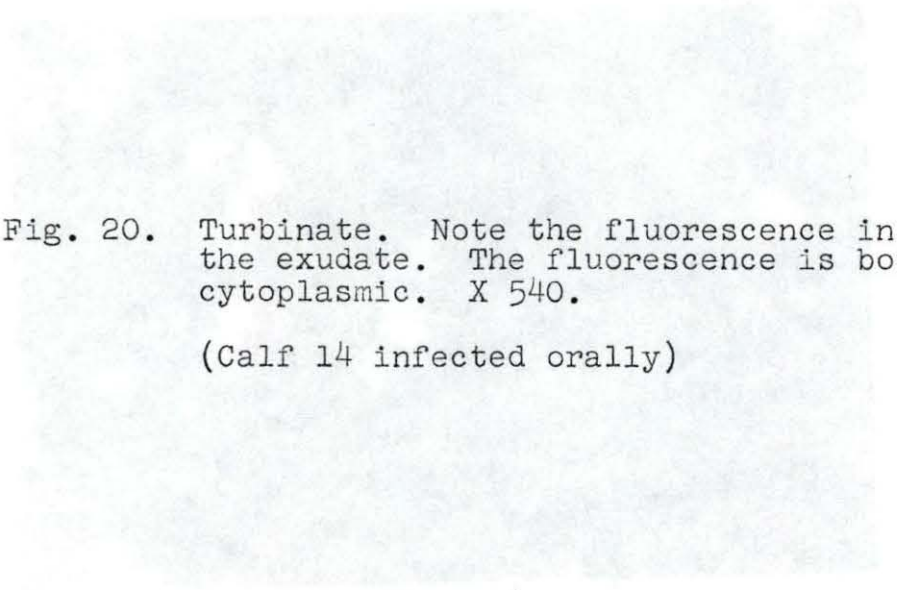


Fig. 20. Turbinate. Note the fluorescence in the cells of the exudate. The fluorescence is both nuclear and cytoplasmic. X 540.

(Calf 14 infected orally)

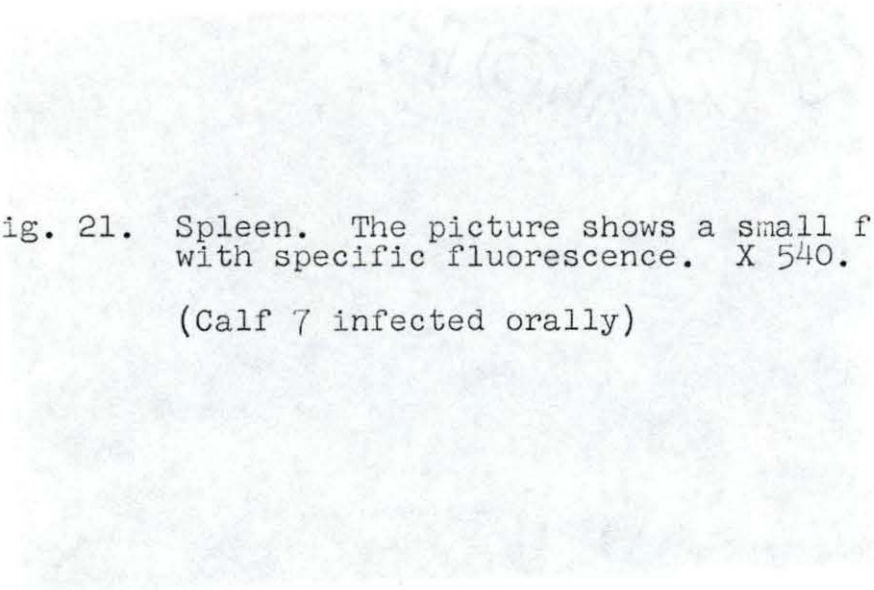
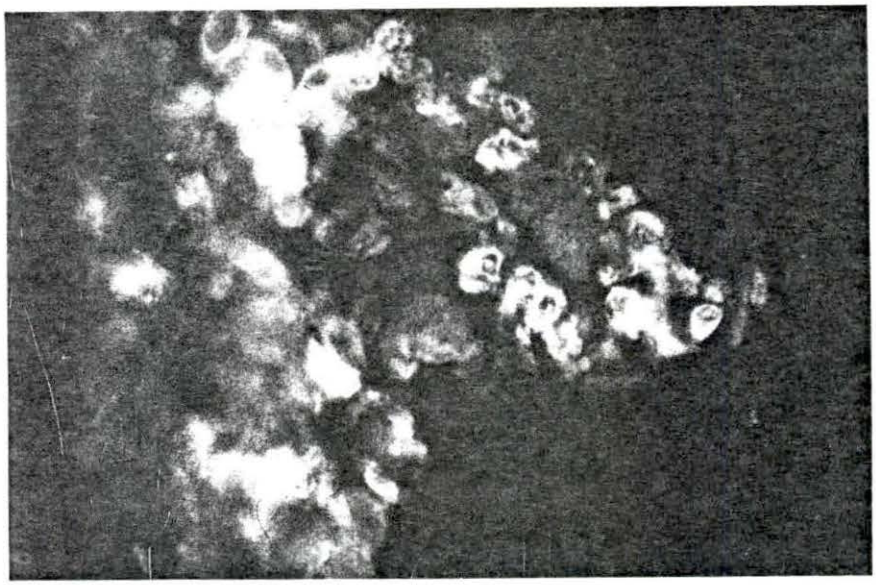


Fig. 21. Spleen. The picture shows a small focus of cells with specific fluorescence. X 540.

(Calf 7 infected orally)



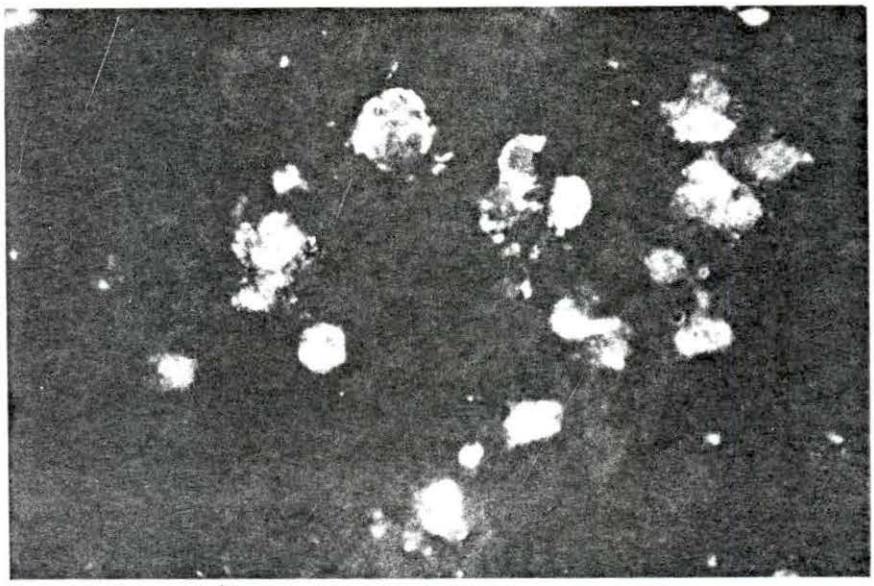
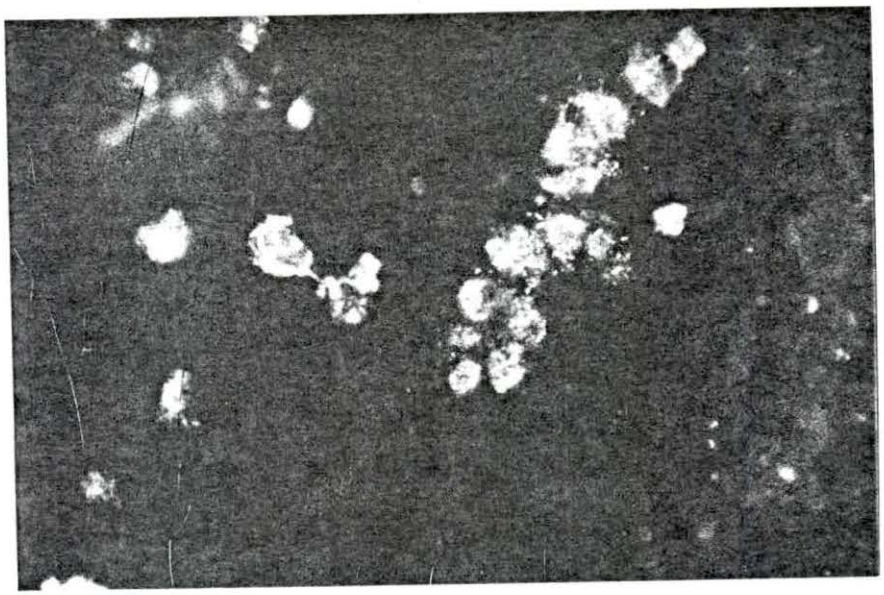


Fig. 22. Tonsil. Section through a necrotic focus showing the specific cellular fluorescence. X 540.

(Calf 7 infected orally)

Fig. 23. Tonsil. Note the absence of fluorescence in this section. This was the next serial section to that shown in Fig. 22. The reaction has been blocked by pretreatment with specific unlabelled antiviral antibody. X 540.

(Calf 7 infected orally)



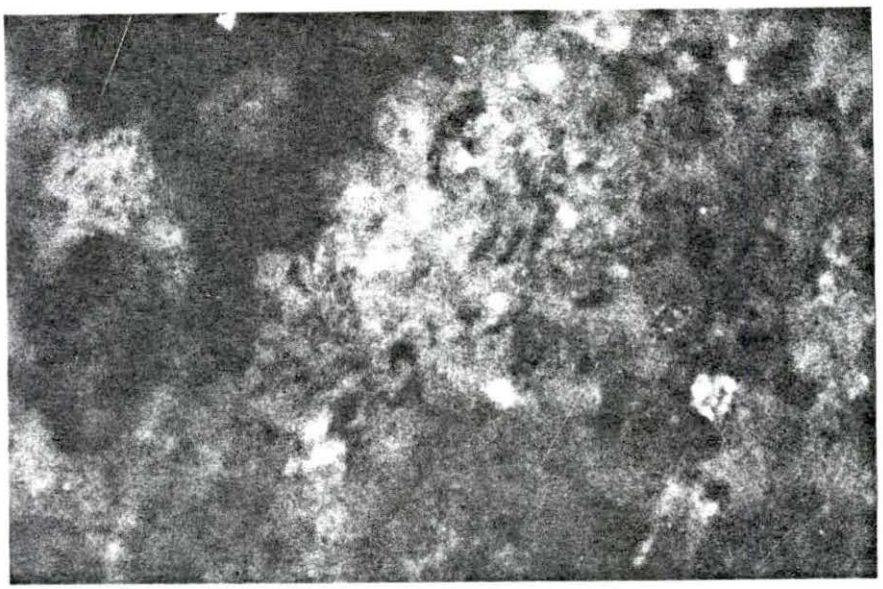
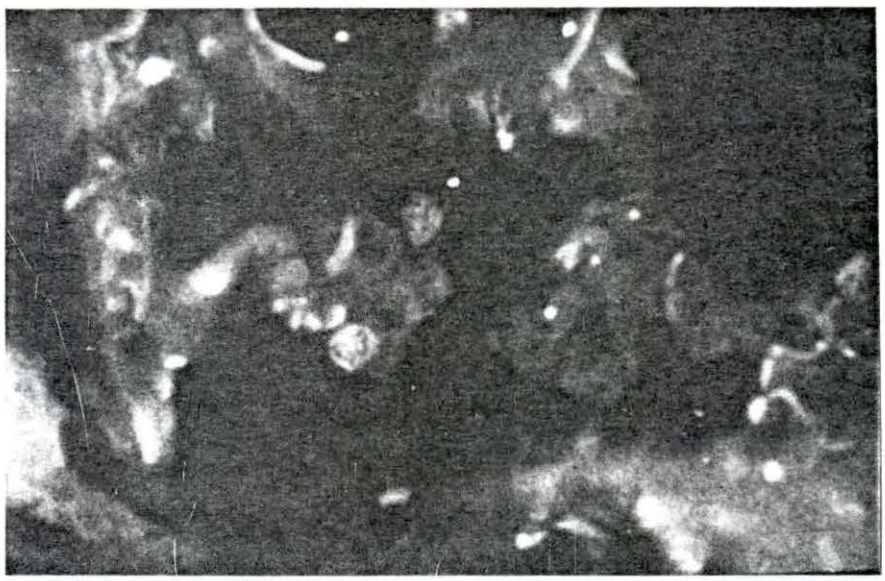


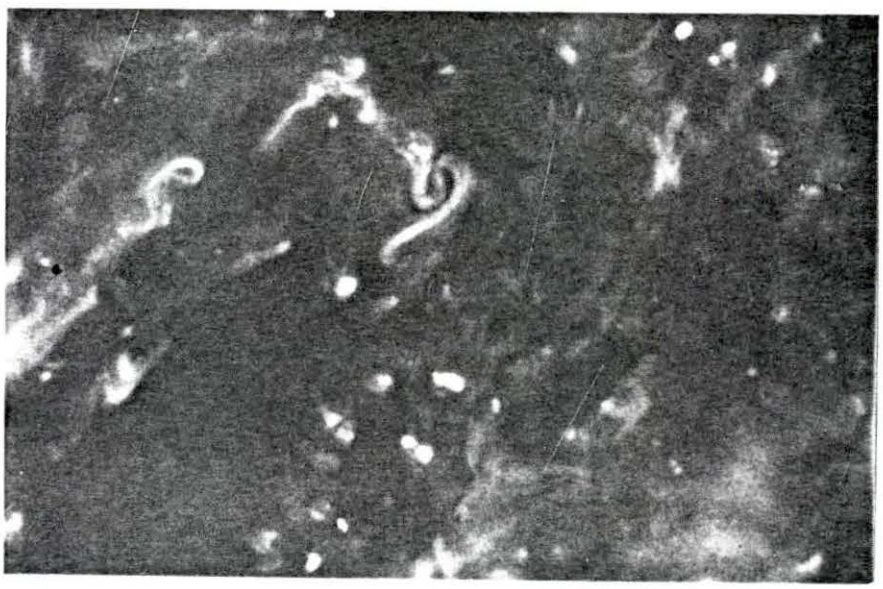
Fig. 24. Lung. The two macrophages at the center of the field contain the fluorescing antigen. X 540.

(Calf 14 infected orally)

Fig. 25. Lung. Cells similar to those in Fig. 24 are absent in this section. X 540.

(Calf 14 infected orally. The tissue section was treated with specific unlabelled antiviral gamma globulin prior to treatment with specific conjugate)





DISCUSSION

The virus used in this study was one of the agents isolated from an animal that died of mucosal disease. From preliminary transmission studies in calves, it was found to cause a clinical syndrome similar to that produced by the viruses of mucosal disease-virus diarrhea complex as reported by Carlson et al. (1957), Barner (1960), Richter (1962) and Tyler (1963). Even though this agent was shown to be antigenically related to the I.B.R. virus, it was thought that this agent might have some significant role in the production of lesions similar to those seen in mucosal disease. Various routes of inoculations were employed in order to determine any differences in the tissue localization of the virus. The experimental animals were killed at successive intervals following inoculation in order to study the pathogenesis of this disease.

Clinical and Hematologic Response

Clinical and hematologic findings were generally similar in all groups of animals, the only difference being in the time at which these signs appear. The time of onset of clinical signs was dependent on the route of administration.

Clinical and hematologic findings were characterized by a pyrexia of approximately 105 degrees F. with a concomitant leucopenia at 24, 48 or 72 hours postinoculation in animals

inoculated intravenously, orally or intranasally respectively. This was in agreement with the findings of Richter (1962) and Tyler (1963) in cases of experimental mucosal disease. These authors have reported a second febrile response approximately on the seventh or eighth day postinoculation with a concomitant leucocytosis. In this study all but one calf were killed by the seventh day postinoculation. In calf 15 which was killed on the ninth day postinoculation, a second febrile response was noted on the eighth day postinoculation. From the preliminary studies in calves, a second febrile reaction was apparent on the eleventh or twelfth day after inoculation with an accompanying leucocytosis.

Marked leucopenia was noted during the initial febrile period in all calves except those inoculated intranasally. Similar findings were reported in experimental mucosal disease-virus diarrhea complex (Carlson et al., 1957; Richter, 1962 and Tyler, 1963) and experimental I.B.R. (Tyler, 1963). However, in experimental I.B.R. when calves were inoculated by the intranasal route, the white blood cell count did not fluctuate beyond normal limits at any time (McKercher et al., 1955).

Previous studies with viral agents of the mucosal disease-virus diarrhea complex have shown an extensive infiltration of various tissues by eosinophils (Tyler, 1963). Also there were reports of adrenal necrosis following intravenous

inoculation of I.B.R. virus (Webster and Manktelow, 1959 and Tyler, 1963). Because of the above reports, it was decided to take absolute eosinophil counts of every animal. In general, there was a slight eosinophilia during the febrile period. In the later stages of the disease there was an eosinopenia. Even though the absolute eosinophil counts were twice that of the preinoculation level, as was the case in some animals, it cannot be considered as significant because of the very low preinoculation levels. Speirs (1958) has reported eosinophilia in animals as a response to injections of any antigen. He found both local and systemic eosinophilic responses which were maximal when repeated doses of the antigen were given. According to Speirs, these eosinophils will eventually be trapped within the tissues of the reticuloendothelial system. Based on these findings, the eosinophilia could be explained as a result of viral antigenic stimulation and the eosinopenia which was noted in the later stages of this disease could be explained by a reticuloendothelial clearing mechanism.

Signs of mucous nasal discharge, anorexia and diarrhea usually paralleled or followed the febrile phase. These observations correspond with what has been described in cases of experimental mucosal disease-virus diarrhea complex and I.B.R.

Pathology

Gross and microscopic lesions similar to those seen in field cases of mucosal disease-virus diarrhea complex were not observed in the oral cavity, pharynx or esophagus. Ulceration of the esophagus may be absent in one third of the field cases of mucosal disease. They were frequently observed by Richter (1962) in experimental infection of calves with a virus of mucosal disease-virus diarrhea complex. Baker et al. (1960) in a study of I.B.R. virus in newborn calves noted lesions of the alimentary tract extending from the oral cavity through to the forestomach. However, in older animals similar lesions were absent both in experimental and natural cases of I.B.R.

Distinct ulcerative and necrotic rumenitis was noted in the pillars of the rumen in calf 15 which closely resembled those described by Ramsey (1956) in field cases of mucosal disease and by Baker et al. (1960) in experimental I.B.R. infection in newborn calves. However, inclusions in the epithelial cells as reported by Baker et al. were not found in this case. Fluorescence of the cells at the base of the lesion indicate that these lesions are the effect of virus itself or possibly the result of toxic substances released during virus-cell interaction.

The ulcers present in the abomasal mucosa in these animals did not have any resemblance to those described by Ramsey

(1956) in field cases of mucosal disease. However, the lesions in the abomasum consisting of ulceration, edema and hyperemia were quite similar to those found in experimental cases of mucosal disease-virus diarrhea complex. Similar lesions have not been reported in experimental or field cases of I.B.R.

Catarrhal enteritis present in those calves infected by the intravenous and oral routes was similar to that described by Richter (1962) and Tyler (1963) in calves infected with Sanders agent. Similar lesions were absent in experimental I.B.R. infection. The absence of the marked intestinal lesions in those calves inoculated intranasally may be due to a lesser number of infective virus particles reaching the intestine because of natural defense mechanism of the animal. The necrotic lesions present in the Peyer's patches were more severe than those reported in experimental mucosal disease-virus diarrhea complex. A consistent histological finding was the leucocytic infiltration of the lamina propria which was most severe in animals infected orally or intravenously. Eosinophils were the most prominent cell type of the leucocytic infiltrate. A similar finding was reported by Tyler (1963) in his studies with viruses of the mucosal disease-virus diarrhea complex. Speirs (1958) has observed that the presence of any antigen in tissues attracts eosinophils. He found that these eosinophils then form enzymatic templates with antigen which

in turn are phagocytized by the macrophages. This was postulated to be an earlier step in the immune mechanism of the animal. Based on this theory, the eosinophilic infiltration could be explained as a result of attraction by the virus present in the lymphoid tissue or in the lamina propria. An increase in the number of eosinophils was also found in the lymphoid tissue of the intestinal wall, especially around the necrotic lesion which was shown to contain antigenic material by fluorescent antibody method. In most cases excessive amounts of mucus were present in the intestinal crypts. This could have been a result of direct cellular stimulation by the virus or by-products of cell-virus interaction. The latter explanation appears to be more reasonable because viral antigen was not detected in the glandular epithelium.

Ecchymotic hemorrhages were present in the cecum. In calf 7 (infected orally) a thin fibrinous cast adhered to the mucosa of the colon. As of this writing there has been no report on cecal and colonic lesions in experimental cases of mucosal disease-virus diarrhea complex or I.B.R. However, cecitis, colitis and proctitis varying in degree from catarrhal to hemorrhagic, ulcerative or fibrinonecrotic were always found in field cases of mucosal disease (Ramsey, 1956).

In general the gastrointestinal lesions produced by the new viral isolant resemble more closely the lesions of mucosal

disease-virus diarrhea complex than those of I.B.R. In many cases the lesions were more severe than those of experimental cases of mucosal disease-virus diarrhea complex.

The gross and microscopic lesions of the respiratory tract were characteristic of an acute catarrhal rhinitis and tracheitis which resembled those reported by McKercher et al. (1955) and Webster and Manktelow (1959) in experimental cases of I.B.R. These lesions were much less severe than those described in natural cases of I.B.R. There was some variation in the severity of the lesions between the different groups, the most severe lesion being found in those groups infected by the intranasal or intravenous routes. The inflammatory cells (plasma cells) in the subepithelial tissue of the turbinates in calves 7, 11 and 12 appeared to be very active because of the greater amount and deeper staining character of the cytoplasm. Even though large amounts of viral antigen was detected in the cells of the exudate adhering to the epithelial surface, specific fluorescence was very rare in the cells in the epithelium or subepithelial tissue. The low concentration of viral antigen in the epithelium or subepithelial tissue of the turbinate could be explained on the basis of local antibody production by plasma cells in these areas. If this were so, the spread of the virus to other parts of the body would be limited. Interstitial thickening in the lung was usually associated with the presence of virus in the macrophages.

The ecchymotic hemorrhages present on the wall of the frontal sinuses of calves 5, 15, 11 and 12 are probably associated with the presence of virus. It is not difficult to speculate that the frontal sinuses are infected by a direct extension from the nasal cavity. McKercher et al. (1963) have isolated I.B.R. virus from the frontal sinuses after intranasal inoculation.

Gross and microscopic lesions in the lymph nodes resembled those seen by Trapp (1960) in natural cases of mucosal disease. Marked hemorrhages were found in two animals killed at the seventh and ninth day postinoculation (calves 14 and 15). At this stage most of the lymph nodes of these animals had large numbers of eosinophils in the distended blood vessels. In the bronchial lymph node of calf 15, these eosinophils showed intense fluorescence indicating the presence of antigenic material. However, no fluorescence was detected in the lymphoid elements in these lymph nodes. This could be explained by the fact that location of the antigenic material might have been missed when sections were taken for fluorescent staining or that the antibodies in the tissue might have neutralized the antigenic sites. Thus the changes in the lymph nodes of these animals could be attributed to the direct action of virus on the vascular endothelium or to an antigen-antibody reaction occurring at the surface of the cells resulting in an anaphylactic type of reaction. One function of

the eosinophil is to phagocytose antigen-antibody complexes (Sabesin, 1963). The fluorescence of these cells within the blood vessels may be due to the presence of phagocytosed viral antigen-antibody complexes. It is assumed that some reactive sites are still available for the attachment of the specific conjugate. Eosinophils were found to appear in large numbers in the bronchial lymph nodes as early as three days postinoculation. This is also an indication of the significance of the eosinophils in the immune mechanism of the animal. Lymphoid depletion and coagulative necrosis of the germinal centers are probably a result of direct killing effect of virus because antigenic material was detected in these areas. Generally the lymph nodes associated with the respiratory and gastrointestinal tracts were affected most severely.

Tonsillar changes were quite marked in those animals infected by oral route. They were distinctly enlarged and necrotic foci were visible on sectioning. On microscopic examination, these foci contained excessive amounts of necrotic epithelial tissue and eosinophilic hyaline-like droplets. These changes were probably caused by the direct effect of virus because of the fact that viral antigen was detected in these cells. The role of tonsils as portals of entry for viruses and pathogenic microorganisms is well established.

Necrosis of the zona fasciculata in the adrenal cortex

was found only following the intravenous inoculation. Similar lesions have been reported by Webster and Manktelow (1959) with experimental I.B.R. infection and Barner (1960) and Tyler (1963) with the North Dakota (B.M.D.) agent. The necrotic lesions in calves 5 and 15 contained a high concentration of antigenic material suggesting that these were caused by the virus.

Depletion of the lymphoid tissues of the splenic corpuscles was similar to that described by Ramsey (1956), Trapp (1960) and Tyler (1963) in their studies of the mucosal disease-virus diarrhea complex. A marked eosinophilic cuffing was found at the periphery of the corpuscles. As in other tissues these cells may be involved in the immune mechanism which is thought to include phagocytosis of antigen-antibody complexes or the formation of enzymatic template with antigen. Many of the cells in this area were found to contain fluorescent material. However, identification of cell types in this area was difficult. It is possible that both the eosinophils and mononuclear cells contained the viral antigen.

Liver lesions similar to but smaller than those described by Baker et al. (1960) were noted in all animals. This type of lesion was also noted in cases of experimental mucosal disease-virus diarrhea complex (Tyler, 1963). However, the pathogenesis of this lesion has not been described. In this study no antigenic material was detected in these minute hepatic

necrotic foci by the fluorescent antibody method. The significance of the virus or products of virus-cell interaction in the production of this lesion has not been assessed.

Pathogenesis

The presence of viral antigen in tissues detected by the fluorescent antibody method can be explained by primary localization and multiplication and by extension from the primary sites to secondary ones. The site of primary localization and multiplication appeared to vary with the route of administration. Spread of the virus to secondary foci may be by direct extension or through the lymphatics or blood stream.

When the virus was administered by the intravenous route it was rapidly distributed throughout the body. Fluorescent antibody analysis indicated that there were some specific locations where the virus localized and multiplied. These areas were the spleen, tonsils, Peyer's patches and other lymphoid areas of the body. Microscopic lesions in these areas varied greatly in severity. This could reflect a difference in the rate of viral multiplication which in turn could be attributed to difference in cellular metabolism and possible differences in local immune mechanism. Of all the lymphoid areas, the lesions were most severe in the Peyer's patches. Even though the virus was present in the abomasal mucosa of one animal

this does not seem to be an important locus of multiplication. Localization of virus in the turbinate was observed only in one animal. However, isolation attempts from nasal swabs were successful in most cases. This result could indicate a regular excretion of virus in the nasal discharge originating from the tonsils or from the frontal sinus. In calf 15 the absence of virus in the nasal cavity on the ninth day may be correlated with specific antiviral antibody (Table 3). Similarly the intestinal lesions in calf 15 also lacked the fluorescence. However, at this time viral antigen was present in the rumen, tonsil, adrenal, bronchial lymph nodes and spleen. These differences may be due to low concentration of antibody at these sites. It may be possible that the lack of fluorescence in the intestinal lesions was due to the fact that these areas represent primary foci of viral multiplication with early local antibody response and thus an early disappearance of viral antigen. Another possibility for the difference is that in the rumen, tonsil, adrenal, bronchial lymph nodes and spleen the antigenic sites associated with antigen-antibody complex within the eosinophils are responsible for the fluorescence. In this latter case the intracellular location of the antigen may protect antigenic sites from viral specific antibody.

At no stage of the experimental disease, could virus be isolated from the blood. This may be as a result of

phagocytosis of the virus by the cells of the reticuloendothelial system soon after inoculation. Later on, the virus probably multiplies in these cells of the reticuloendothelial system and disseminated to various parts of the animal body by the lymphatic route.

When the virus was administered by the intranasal route the primary localization was in the lungs and probably in the nasal cavity from where it is distributed to the lymphoid tissues in other parts of the body such as the spleen and mesenteric lymph nodes by way of the lymphatics. Viral antigen was detected in these organs in calf 11 but not in calf 12. The absence of fluorescence in the animal killed later may be explained on the basis of neutralization of antigen by tissue antibodies. The virus detected in the cells of the exudate may have originated from the lungs or frontal sinus. The absence of fluorescence in the nasal mucosa probably is due to the local antibodies produced by the infiltrated plasma cells in the subepithelial tissue. In tissues of the kidney and central nervous system viral antigen was not detected by the fluorescein antibody method. Ocular tissues were not examined in these animals. McKercher et al. (1963) has isolated I.B.R. virus from the tissues of the central nervous system, eye and kidney following intranasal inoculation. The extent of distribution and persistence of the virus was found to be less pronounced than when infected intravenously or orally

which may be explained by a more active defense mechanism along the respiratory tract of the animal.

When the virus was administered by the oral route the primary localization of the virus was most likely in the tonsil and probably in the Peyer's patches and abomasum because viral antigen was detected in these tissues as early as five days postinoculation. However, in these calves viral isolation attempts from the nasal cavity and fecal samples were unsuccessful even though viral antigen was detected in the nasal exudate by the fluorescent antibody method. The failure of viral reisolation attempts was probably the result of the small number of infective viral particles present or the presence of inhibitory substances in these samples. These findings concur closely with the classical work of Bodian (1957) where the primary sites of poliovirus replication were found to be the tonsils and Peyer's patches. He was able to reisolate the virus from the pharyngeal secretion and feces.

From the primary site or sites of infection the virus in all probability is disseminated to other parts of the body such as the respiratory or reticuloendothelial systems by way of lymphatics because the presence of virus was not detected in the blood at any stage of the disease. Extension to the respiratory system, as observed in these animals, could also be the result of extension of virus excreted into the nasopharynx from the tonsils by a combination of gravity and

coughing.

Conclusion

Considering the clinical, pathologic and hematologic findings, it is evident that experimental disease in calves resembles more closely that produced by the viruses of mucosal disease-virus diarrhea complex than I.B.R. Lesions of the gastrointestinal tract were quite marked while those of the respiratory tract were minimal even though the agent was shown to be serologically related to I.B.R. group.

In general, the distribution of the viral antigen following inoculation is primarily confined to the lymphoid tissue, the organs involved being dependent upon the route of inoculation. There are some exceptions, one being the involvement of the adrenal gland after intravenous inoculation. Lesions are quite generalized following intravenous inoculation affecting both the digestive and respiratory systems to a similar degree. However, oral and intranasal infection resulted in more severe involvement of the digestive and respiratory systems respectively.

The virus replicates in the cells of the lymphoid series as determined by the fluorescent antibody method. Most of the pathologic changes observed were associated with the presence of viral antigen and these changes must then be attributable to the direct effect of virus or to the effect of the products of cell-virus interaction in that area.

SUMMARY

Pathogenesis of a bovine herpesvirus isolated from the Peyer's patch of a calf that died of typical mucosal disease was studied. A total of eight calves of four to six months of age were used. Different routes of inoculation were employed. Three calves were inoculated intravenously, two intranasally and two orally. The experimental disease syndrome was very similar to that produced by the viruses of mucosal disease-virus diarrhea complex.

The clinical and hematological findings were characterized by a biphasic pyrexia with the peaks occurring at the second or third and eighth or ninth day postinoculation. Concomitant to the initial febrile response there were mucous nasal discharge, diarrhea, leucopenia and slight eosinophilia. At later stages of the disease there was an eosinopenia in most cases accompanied by normal leucocytic count.

The animals were killed at successive intervals following inoculation and tissues were saved both for histopathologic and fluorescent antibody studies.

Histopathological changes were usually confined to the lymphoid tissue, the organs involved depending on the route of inoculation. Intravenous inoculation resulted in the involvement of both the respiratory and digestive tract whereas when the virus was administered by the oral and intranasal routes

the lesions were primarily confined to the digestive and respiratory systems respectively. Mild to moderate catarrhal enteritis and tracheitis were noted in most cases. In the lymphoid tissue the lesions were characterized by lymphoid depletion, coagulative necrosis and eosinophilic infiltration. Marked eosinophilic infiltration of the lamina propria of the intestine was noted in most of the animals.

Pathogenesis of the disease was studied by using the fluorescent antibody method. Viral antigen was usually associated with macroscopic and microscopic lesions in the lymphoid tissue of the digestive tract even when different routes of inoculation were employed. In the respiratory tract the viral antigen was usually associated with the inflammatory cells in which it was mainly intracytoplasmic. These cells included primarily those of the mononuclear series and rarely eosinophils. The viral antigen was found to persist in the adrenal gland, spleen, rumen and tonsils in some animals even after the appearance of antibodies in the serum. This virus was usually associated with the lesion in these organs.

Detectable amounts of circulating antibody were present only in one calf at the seventh day following intravenous inoculation. Serum samples from all of the other animals were negative for antibodies.

Virus was consistently isolated from the nasal cavity starting from two days postinoculation except in those infected

by the oral route.

From this study it is evident that this bovine herpesvirus can definitely cause lesions, particularly in the gastrointestinal tract, which are very similar to those caused by the viruses of the mucosal disease-virus diarrhea complex.

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