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FOLLOWING VACCINATION AGAINST BOVINE VIRUS
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PATHOLOGIC AND IMMUNOLOGIC STUDIES ON FIELD AND
EXPERIMENTAL POSTVACCINAL REACTIONS FOLLOWING
VACCINATION AGAINST BOVINE VIRUS DIARRHEA

by

Chennekatu Paily Peter

A Dissertation Submitted to the
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Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa
1966
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INTRODUCTION

Since the recognition of virus diarrhea in 1946 (Olafson et al.,) and mucosal disease in 1953 (Ramsey and Chivers), considerable understanding of the relationship of these two syndromes has been established. Although they exist as two clinical entities, it has been shown that they are caused by the same etiologic agent and have the same basic pathologic response (Tyler and Ramsey, 1965). With the isolation of a cytopathic agent (Gillespie et al., 1960) a modified live-virus vaccine was developed (York et al., 1960) and eventually marketed in 1964. Although the vaccine has apparently been efficacious in preventing virus diarrhea, a condition resembling bovine virus diarrhea - mucosal disease (BVD-MD) complex has been observed in a few herds of cattle shortly after vaccination (Fuller, 1965). Confusion and concern about the safety of the vaccine have arisen among some practitioners and cattlemen. It was the purpose of this research to characterize the postvaccinal condition based on the gross, microscopic and ultrastructural changes and the immune status and to clarify its relationship to the BVD-MD complex, and more specifically, its association with vaccination. It is believed that the above information will add to the knowledge of the etiology and pathogenesis of BVD-MD complex.
Since the first published reports on bovine virus diarrhea (BVD) by Olafson et al., (1946) and mucosal disease (MD) by Ramsey and Chivers (1953) a large amount of work has been done toward the elucidation of the etiology and reproduction of the disease under laboratory conditions. Excellent descriptions of the clinical manifestation and gross and microscopic pathology of these two diseases have been made by several authors. A thorough review of the literature at the time concerning the BVD-MD complex was made by Trapp (1960).

Infectious bovine rhinotracheitis (IBR) was first reported as a respiratory disease of cattle by Schroeder and Moys (1954). Although the etiology of this syndrome was undetermined at that time, the report adequately describes the gross and microscopic lesions of the respiratory form of the disease. The experimental disease has been studied by several workers (Webster and Manktelow, 1959; Abinanti and Plumer, 1961; McKercher et al., 1963 and Peter, 1964) following the isolation of the etiologic agent in tissue culture by Madin et al., (1956).

There have been many reports concerning isolation of virus diarrhea and mucosal disease agents, some cytopathogenic and others noncytopathogenic, in cell cultures (Baker, 1954; Pritchard and Kniazeff, 1958; Tyler, 1960; Richter, 1962 and Malmquist, 1963) but their role in the etiology of these respective diseases has not been clearly determined. Using the

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cytopathogenic virus isolated by Gillespie (Oregon C-24-V) as the prototype, Kniazeff et al., (1961) have compared several viruses isolated from either virus diarrhea or mucosal disease-like syndromes from different parts of the world, and have shown that apparently most of these viruses are antigenically related.

There are also reports of the isolation of viral agents from either virus diarrhea or mucosal disease-like syndromes which are serologically identical to the virus of IBR (Noice and Schipper 1959; and Gratzek et al., 1966). In one of these reports (Gratzek et al., 1966) two antigenically different viral agents were isolated from the same animal that had died of BVD-MD complex. One of these viruses was isolated from the Peyer's patch and was found to be antigenically related to the virus of IBR (Gratzek et al., 1966). The second isolant was recovered from the blood by using buffy coat cultures and was found to be serologically related to the bovine viral diarrhea prototype Oregon C-24-V1.

The IBR virus was originally thought to cause primarily an upper respiratory disease. However, recently it has been found to possess an unusually wide pathologic range. It is of prime etiologic significance in infectious pustular vulvovaginitis (IPV) (Gillespie et al., 1958) a specific conjunctivitis (McKercher et al., 1958; and Darbyshire et al., 1962) and an encephalitis (French, 1962a; and French 1962b). A virus similar to IBR has been isolated consistently from cases of infectious infertility (epivag) of cattle in South Africa (Mare' and van Rensburg, 1961).

The gastrointestinal tract of newborn calves was reported to be susceptible experimentally to IBR virus by Baker et al., (1960). The lesions extended from the oral mucosa through the forestomachs. Focal gray-white slightly raised areas were observed in the oral cavity. These lesions were more numerous in the posterior part of the oral cavity, pharynx, larynx, and the cranial portion of the esophagus. Many nuclei of the epithelial cells on the edge of the ulcerative areas contained 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 had massive areas of necrosis in the region of the peripheral sinuses. Usually germinal centers were not affected until necrosis became so extensive as to involve the major portion of the lymph node. Focal areas of necrosis and neutrophilic infiltration were observed in the liver, kidney and spleen.

Using the fluorescent antibody technique, Peter (1964) studied the experimental syndrome produced by the IBR strain of virus isolated from an animal affected with BVD-MD complex (Gratzek et al., 1966). In this study three different routes of inoculation were utilized to determine the pattern of virus spread in the experimental animal. Gross lesions were not marked in any of the animals. Histopathologic lesions were usually confined to the lymphoid tissue and were characterized by lymphoid depletion, coagulative necrosis, and eosinophilic leukocyte infiltration. Involvement of the organs depended on the route of inoculation. Intravenous inoculation resulted in lesions in both the respiratory and digestive tracts whereas oral or intranasal administration produced lesions primarily confined to the digestive or respiratory tracts respectively. Mild to moderate
catarrhal enteritis and tracheitis were noted in these animals. In addition, cystic dilatation of intestinal glands with mucus, neutrophils and macrophages was frequently observed in the vicinity of the Peyer's patches.

Using the fluorescent antibody tracing method IBR viral antigen was usually detected in association with the microscopic lesions in the lymphoid tissues. In these areas viral antigen was consistently localized in the cytoplasm of large mononuclear cells. The viral antigen was found to persist in the adrenal gland, spleen and tonsils in these animals even after the appearance of circulating antibodies in the serum.

IBR virus was regularly reisolated from the nasal secretions up to the 7th day following inoculation.

Six different agents (IVD-46, Sanders, Merrell, NADL-MD, North Dakota (BMD), and Nebraska (C-1) isolated from animals affected with bovine virus diarrhea-mucosal disease complex were compared on the basis of clinical hematologic, immunologic, gross and histopathologic responses in experimental calves by Tyler and Ramsey (1965). The Colorado strain of IBR virus was also used in this study when it was learned that North Dakota (BMD) and Nebraska (C-1) agents were strains of IBR virus. Single inoculation of the Sanders, Merrell IVD-46 or NADL-MD agents resulted in a mild clinical syndrome but produced marked histologic lesions in the lymphoid tissues. These lesions consisted of lymphoid depletion, necrosis, and a fibrinoid residue in the germinal centers. These changes were most pronounced in Peyer's patches. Necrotic foci leading to pustules and eventual erosions were seen in esophageal and the rumen epithelium of those calves infected with IVD-46 and NADL-MD agents.

The lesions resulting from the intravenous inoculation with the North
Dakota, Nebraska or Colorado strain of IBR virus revealed a similarity to the lesions produced by the isolants of the BVD-MD complex. A lesion observed only in infections by the IBR virus was multiple focal, caseous necrosis of the adrenal cortex.

In general, combined inoculation of the Sanders agent with other isolants of BVD-MD complex resulted in a syndrome no more severe than had been recorded for single inoculations of the agents.

Simultaneous inoculation of Sanders and North Dakota agents resulted in a more severe disease syndrome than a single inoculation of either agents. A prolonged clinical course and erosions in the epithelium of the esophagus, rumen, and omasum characterized the syndrome.

Both field and modified strains of IBR virus has been incriminated as a cause of abortion in cattle. IBR virus was isolated from bovine fetuses aborted following vaccination against IBR (McKercher and Wada, 1964).

York and Schwarz (1956) have demonstrated that IBR virus modified by serial tissue culture passage can be used for a practical, safe, and effective vaccination for IBR. The safety as well as the potency of this live virus vaccine has been established by controlled field studies by Kendrick et al., (1956). All of the 20 vaccinated animals developed antibodies against IBR virus within 23 days. Serums from the controls reacted negatively to the serum neutralization test. Following challenge none of the vaccinated animals became ill while the controls experienced a temperature rise and were obviously sick. Two of the 11 unvaccinated controls died on the 10th day after challenge. Lesions in the upper respiratory tract typical of IBR were noted at necropsy.

York et al., (1960) described field experiments on the use of 2
vaccines for viral diarrhea, one consisting of rabbit adapted modified virus (New York-1), and the other, bovine kidney tissue culture modified live virus (Oregon C-24-V). Significant differences in serological titers were not found in 29 cattle recovered from virulent virus infection, 82 inoculated with rabbit adapted vaccine and 65 inoculated with tissue culture adapted vaccine. With the exception of 1 animal given tissue culture vaccine, all animals with negative serology at the time of vaccination developed antibody titers 2 - 4 weeks postinoculation. The titers ranged from 1:20 to as high as 1:10,000 per 0.1 ml. of serum. Experimentally inoculated cattle successfully withstood subsequent challenge with virulent viral diarrhea virus.

Following introduction of BVD vaccine on the market in 1964, a specific postvaccinal reaction has been frequently observed. Fuller (1965) reported on observations in 4 different herds of BVD vaccinated cattle, wherein approximately 1 per cent of the animals succumbed to a disease nearly identical to the field syndrome of bovine mucosal disease. No difference was observed in the numbers involved or the severity of the lesions between animals receiving BVD vaccine alone or a combination of BVD and IBR vaccines.

Shope (1964) has reported on the failure of antibody formation in natural fatal cases of the BVD-MD complex. The failure to produce antibodies was not due to complete immunological incompetence nor to the presence of the virus in a non-antigenic form. It has been hypothesized that this failure may be due to either the virus growing more rapidly than neutralizing antibody production or to the destruction of immunologically competent cells by viral infection, or to a combination of these two factors.
MATERIALS AND METHODS

Tissue Culture Media and Solutions

The 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 sheep or fetal calf serum.

\[
\text{HBSS-LaH (H-LaH)}
\]

\[
\begin{align*}
\text{NaCl} & \quad 8.0 \ \text{gm.} \\
\text{KCl} & \quad 0.4 \ \text{gm.} \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & \quad 0.2 \ \text{gm.} \\
\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} & \quad 0.6 \ \text{gm.} \\
\text{KH}_2\text{PO}_4 & \quad 0.6 \ \text{gm.} \\
\text{Glucose} & \quad 1.0 \ \text{gm.} \\
\text{CaCl}_2 & \quad 0.14 \ \text{gm.} \\
\text{Phenol red} & \quad 0.02 \ \text{gm.} \\
\text{Lactalbumin hydrolysate} & \quad 5.0 \ \text{gm.} \\
\text{Twice distilled water} & \quad \text{q.s.} \quad 1000 \ \text{ml.}
\end{align*}
\]

The medium was autoclaved at 120 C. and 20 pounds pressure for 20 minutes. After cooling to room temperature 2.5 ml. of a 1.4 per cent sterile sodium bicarbonate solution and an antibiotic mixture containing 10,000 units of penicillin and 10,000 ug. of streptomycin were added per 100 ml. of the medium. The medium was stored at 4 C. Serum was added just prior to seeding the culture tubes.
### Washing and dilutions

Saline G (Hamm and Puck, 1962) was used for this purpose.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>Glucose</td>
<td>1.1 gm.</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.0 gm.</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4 gm.</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.7H$_2$O</td>
<td>0.29 gm.</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.15 gm.</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.15 gm.</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>0.016 gm.</td>
</tr>
<tr>
<td>Phenol red concentrate</td>
<td>0.6 ml.</td>
</tr>
<tr>
<td>(0.002 gm./ml.)</td>
<td></td>
</tr>
<tr>
<td>Lactalbumin hydrolysate</td>
<td>5.0 gm.</td>
</tr>
<tr>
<td>Twice distilled water</td>
<td>q.s.</td>
</tr>
<tr>
<td></td>
<td>1000 ml.</td>
</tr>
</tbody>
</table>

The solution was sterilized by autoclaving. Antibiotic mixture was added to the saline after cooling to room temperature and stored at 4°C.

### Dispersion of cells

Saline containing EDTA was used for this purpose.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0 gm.</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.2 gm.</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>1.15 gm.</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 gm.</td>
</tr>
<tr>
<td>EDTA (disodium salt)</td>
<td>0.2 gm.</td>
</tr>
<tr>
<td>Twice distilled water</td>
<td>q.s.</td>
</tr>
<tr>
<td></td>
<td>1000 ml.</td>
</tr>
</tbody>
</table>

The solution was sterilized by autoclaving. Antibiotic mixture was added after cooling to room temperature and stored at 4°C.
Tissue Culture Cells

Tissue culture cells used in this investigation were an embryonic bovine kidney cell line developed in this laboratory.

Hyperimmune Serums

Hyperimmune serum against the IBR virus was produced in a calf according to the schedule described previously by Peter (1964). Hyperimmune serum against hog cholera virus was used in this study since high titered BVD antiserum was not available. It was shown that conjugate made from hog cholera antiserum was very adequate in detection of BVD antigen in tissue culture cells. This was obtained through the courtesy of Diamond Laboratories, Des Moines, Iowa.

Virus Reisolation Procedure

Procedures used for virus reisolation from nasal swabs were similar to a procedure previously described by Peter (1964). Virus reisolation from the blood was performed by using buffy coat cultures. Approximately 30 ml. of blood was drawn into 5 ml. of 10 per cent sodium citrate solution. After centrifugation at 1,000 r.p.m. for 15 minutes the plasma was carefully aspirated and the buffy coat transferred to another tube containing 10 ml. of phosphate buffered saline. The cell suspension was centrifuged at 1,000 r.p.m. for 15 minutes. The washing procedure was repeated at least 2 times after which the cells were resuspended in H-LaH medium with 10 per cent serum. The suspension was then seeded into tissue

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\(^1\)R. E. L. Taylor, Diamond Laboratories, Des Moines, Iowa. Data concerning the use of conjugate made from hog cholera antiserum in detection of BVD virus in tissue culture. Private communication. 1965.
culture tubes, incubated for 4 - 5 days and then inoculated on to bovine kidney cell monolayers. The tubes were incubated and observed for cytopathic effect. Two successive passages were made with each sample.

**Antibody Assay of Serum**

Serum samples were diluted twofold with saline G. Equal volumes of virus dilutions (NADL-MD and vaccine strain of IBR) in saline G containing approximately 2000 TCID$_{50}$ and serum dilutions were mixed and incubated at room temperature for 30 minutes. After incubation 0.1 ml. of the serum virus mixture was inoculated on to bovine kidney cell monolayers in tubes. The tubes were incubated at room temperature for 30 minutes prior to adding medium containing 2 per cent sheep serum. The cell monolayers were observed for cytopathic effect on the 5th day postinoculation and the 50 per cent end point was calculated by the method of Reed and Muench (1938).

**Fluorescent Antibody Procedure**

**Fractionation of serum**

The serums were fractionated by the procedure used by Kendall (1937). This was done by adding in a drop-wise-manner 40 ml. of a saturated solution of ammonium sulphate to 80 ml. of serum with constant stirring. The mixture was vigorously stirred and centrifuged at 3,000 r.p.m. for 15 minutes. The supernatant fluid was decanted and the sediment was redissolved in 40 ml. of 0.15 M NaCl. The globulin fraction was reprecipitated by a

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1 National Animal Disease Laboratory, Ames, Iowa.

2 Diamond Laboratories, Des Moines, Iowa.
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. The precipitate formed during dialysis was removed by centrifugation. All the above processes were carried out at 4 C. The globulin fraction was diluted with carbonate-bicarbonate buffer (pH 9) to a protein concentration of 1 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 fluorescent dye to the gammaglobulin was done according to the method of Clark and Shepard (1963). The gammaglobulin solution was placed in a dialysis bag in a graduated cylinder mounted on a magnetic stirrer at 4 C. Carbonate-bicarbonate buffer (pH 9) containing 0.1 mg. per ml. of fluorescein isothiocyanate \(^1\) (F.I.T.C.) (10 volumes of buffer per 1 volume of gammaglobulin solution) was placed in the cylinder. Labeling through the dialysis membrane was allowed to proceed for 24 hours. The labeled gammaglobulin was centrifuged to remove the precipitate formed during dialysis. The conjugate was filtered through a column of Sephadex G-25 \(^2\) prepared with 0.01 M phosphate buffer at pH 7.2 and eluted with the same buffer (Killander et al., 1961) and the rapidly passing labeled

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\(^1\) Nutritional Biochemicals Corporation, Cleveland, Ohio.

\(^2\) AB Pharmacia, Upsala, Sweden.
globulin fraction was collected.

Adsorption of the conjugate with tissue powder

Bovine liver powder was prepared as described by Coons and Kaplan (1950). The conjugated globulin was diluted with one-half of its volume of phosphate buffered saline prior to adsorption. Tissue powder was added to the diluted conjugate in a centrifuge tube at the rate of 100 mg. per ml. This mixture was constantly agitated for 2 hours at room temperature and was centrifuged at 12,000 r.p.m. for 15 minutes at 4 C. One additional adsorption was done prior to storage in small aliquots at -20 C.

Processing of tissues for fluorescent antibody staining

Most of the tissues used for fluorescent antibody staining were prepared by the paraffin embedding technique described by Sainte-Marie (1962). Tissues were cut not more than 5 mm. thick and fixed in precooled 95 per cent ethyl alcohol for 24 hours at 4 C. The tissues were dehydrated in 3 changes of precooled absolute alcohol for 2 hours and cleared in 3 consecutive baths of precooled xylene for 2 hours. After placing the specimen in the last bath of xylene the container was removed from the refrigerator and allowed to equilibrate to the room temperature. The tissues were embedded in paraffin at 56 C. Sectioning was done by routine methods making certain that the temperature of water bath and drying plates did not exceed 40 C. Deparaffinization was accomplished by 2 changes of cold xylene, 3 changes of cold 95 per cent alcohol and 3 changes of cold 0.01 M phosphate buffered saline at pH 7.2.
**Staining procedure**

Two sections from every block of tissue were stained, one with IBR conjugate and the other with hog cholera conjugate. The sections were treated with the respective labeled antiviral gammaglobulin in a moist chamber for 30 minutes at 37 °C. The sections were washed with phosphate buffered saline (pH 7.2) for 5 minutes and mounted in buffered glycerine (equal parts of glycerine and phosphate buffered saline at pH 7.2). The slides were examined by using a Leitz Ortholux microscope with an Osram HBO-200 mercury vapour lamp, Type L 11, with a BG 38 heat absorbing filter and UG1 UV filter. When tissues revealed fluorescence, 2 additional slides were prepared in the same manner except for an additional treatment with respective nonlabeled specific antiviral gammaglobulin prior to treatment with labeled globulin. These slides served as controls.

**Electron Microscopy Procedures**

**Solutions**

**Acetate-Veronal buffer**

| Sodium diethyl barbiturate | 2.89 gm. |
| Sodium acetate             | 1.15 gm. |
| Distilled water            | q.s. 100.00 ml |

**2.5 per cent glutaraldehyde solution**

| 25 per cent glutaraldehyde | 1.0 ml. |
| Acetate-Veronal buffer     | 2.0 ml. |
| Distilled water            | 6.0 ml. |

---

Adjust to pH 7.5 with 0.1 N HCl.
Make to 10 ml. with distilled water.

1 per cent osmium tetroxide solution

2 per cent osmium tetroxide 10.0 ml.
Acetate-Veronal buffer 4.0 ml.
Adjust to pH 7.5 with 0.1 N HCl.
Make to 20 ml. with distilled water.

3:2 n-butyl:ethyl methacrylate solution

N-butyl methacrylate 3 parts
Ethyl methacrylate 2 parts
Add benzoyl peroxide to make 1 per cent by weight/volume.
Add divinylbenzene to make 1 per cent.
Filter through anhydrous powdered sodium sulphate.

**Fixation, dehydration and embedding**

Very thin pieces of tissues from spleen, lymph nodes and Peyer's patches were removed and immediately immersed in a 2.5 per cent glutaraldehyde solution at pH 7.5 (Sabatini et al., 1963). These tissues were cut into 1 mm. cubes and fixed for 2 hours. The cubes were transferred to a 1 per cent osmium tetroxide solution and fixed for 1 hour (Palade, 1952). Dehydration was accomplished in ascending grades of ethanol 2 changes each of 50 per cent, 75 per cent, 95 per cent, and absolute ethanol for 5 - 10 minutes. The tissues were infiltrated with 2 changes of 3:2 n-butyl:ethyl methacrylate for 30 minutes each. They were placed in number 0 gelatin capsules containing fresh methacrylate solution and polymeryzed overnight at 60 C.
Sectioning

The capsules were trimmed to a suitable block face after examining a thin section by a phase microscope to determine the desired area of the tissue. The sections were cut with an LKB ultramicrotome using glass knives. Sections showing silver or grey interference colors after flattening with chloroform vapours were placed on 200 mesh copper grids coated with a thin film of 0.5 per cent parlodion in amyl acetate.

Staining

Sections were stained with either 1 per cent potassium permanganate for 30 minutes (Lawn, 1960) or 1 per cent uranyl acetate for 1 hour (Watson, 1958).

Electron microscope

An Hitachi HU 11 A² operated at 50 kv was used for examination of sections.

Animals

The cattle used in this study were divided into 2 groups, one experimentally vaccinated and the other field cases of post-vaccinal condition. Two cases of natural BVD-MD complex were also included.

Experimental animals

Seven Holstein calves 3 - 4 months old were used. These animals were purchased from the dairy herd at the Woodward State Hospital at Woodward,

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2 Hitachi, Ltd., Tokyo, Japan.
Iowa, and a private herd at Kelly, Iowa. Preinoculation serum samples were negative for antibodies against National Animal Disease Laboratory strain of BVD virus and the vaccine strain of IBR virus.

Herd history of field cases

Calf 37 was from a group of 250 heads assembled from several herds in Kansas. They had been brought to a farm in Nebraska about the first week of September, 1964, and vaccinated on September 24 with a mixed single dose of IBR and BVD vaccines. Three weeks after vaccination several calves were observed to have clinical signs resembling that of BVD-MD complex. Morbidity for all animals on the farm was reported as 15 out of 4000 animals. Calf 37 was brought to the Veterinary Clinic at Iowa State University on October 7, and was killed by electrocution on October 10.

Calves 48 and 49, from a herd of 500, were brought to the clinic November 19, 1964, with a history of persistent diarrhea, thick mucous nasal discharge, and erosions on the muzzle. Seven animals had been involved similarly and 2 had died. The remainder of the herd was apparently healthy and gaining weight normally. Calf 48 was an Angus steer of approximately 10 months old from Nebraska. This animal had been brought to the present owner's farm on October 15, 1964, and vaccinated simultaneously with IBR, BVD and Leptospirosis vaccines on October 30. Calf 49 was a Hereford steer of approximately 8 months old. This animal arrived at the owner's farm September 28, 1964, from Colorado and was vaccinated with IBR, BVD and Leptospirosis vaccines on October 22, 1964. These animals were killed by electrocution on November 21, 1964, and complete necropsy examination was conducted.
Calf 57 was originated from a herd of 65 Angus steers from South Dakota approximately 6 months old. They were shipped on January 25, 1965, and vaccinated with IBR, BVD and Leptospirosis vaccines soon after they arrived at the farm. Mucopurulent discharge from the eyes, coughing, and erosions in the mouth appeared approximately 3 weeks after the animals had been vaccinated. Calf 57 was brought to the clinic February 25, 1965, and killed by electrocution on the same day.

Calf 65 was a Charolaise Holstein cross. Diagnosis of BVD-MD complex was made on the basis of clinical and necropsy observations. There was no history of vaccination against IBR or BVD. Information concerning morbidity in the remainder of the herd is not available.

Calf 72 was originated from a herd at Muscatine, Iowa, and was vaccinated on December 1, 1965, with IBR and BVD vaccines. Three animals were noted sick on January 20, 1966. This calf was brought to the clinic on January 30, 1966, and was killed by electrocution on February 4, 1966, and a complete postmortem was conducted. Two other animals had died previous to this date.

Calf 74 was from a group of 94 calves which were brought to a farm at Lake Park, Iowa, from a ranch in South Dakota. Two weeks after arrival clinical signs of IBR were observed in several of these animals. Considerable improvement occurred following treatment with Sulfonamides. On January 20, 1966, they were given BVD and IBR vaccines. Approximately 10 days after vaccination several calves became ill with BVD-MD complex. On February 2, 1966, one of the calves was dead. In an attempt at therapy calf 74 received 5 times the normal dose of IBR vaccine on February 14. This animal was killed by electrocution on February 18. Two other animals
recovered following a period of 10 - 15 days of severe illness.

Calf 76 was a 7 month old Angus steer from a herd of 30 animals. A diagnosis of BVD-MD complex was made on the basis of clinical signs and necropsy findings. These animals had not been vaccinated with IBR or BVD vaccines. Four animals had died during the course of the disease in the herd.

Calf 80 was from a herd consisting of 34 Hereford calves of 4 - 6 months old. They were kept in 2 groups, 12 of the younger calves in one group and the other ones in the other. During the middle part of October, 1965, 2 calves died of a condition which was diagnosed as BVD-MD complex. During the latter part of November the remaining calves, which were apparently healthy at this time, were vaccinated with a combined BVD and IBR vaccine. Two weeks later 14 calves of the older age group were observed to have erosions on the muzzle, dry cough, and a persistent watery diarrhea. Ten of these calves died and the remaining 4 recovered after a long period of severe illness.

Serum Samples

Serum samples were obtained from apparently normal animals during recovery following variable period of illness and from animals having clinical signs of BVD-MD complex. These animals originated from the same herds discussed previously. Serum samples were also drawn from the experimentally vaccinated calves.

Housing and Feeding

Experimental calves were housed in the isolation units at the Veterinary Medical Research Institute at Iowa State University, Ames, Iowa.
During the period of experiment, the calves were fed dehydrated alfalfa pellets and grains. The calves were fed twice daily and ample water was available in metal containers.

**Inoculum**

The inoculum consisted of 2 ml. of reconstituted BVD and IBR vaccines supplied by the Diamond Laboratories, Des Moines, Iowa.

**Preinoculation Procedure**

The experimental animals were given approximately 1 week to accommodate to the new environment. Observations were made at least twice daily to determine their state of health. Daily blood samples were taken for total leukocyte count. Temperatures were recorded twice daily. When a stable level was reached, the animals were considered to be ready for inoculation.

**Inoculation**

Seven calves were divided into 3 different groups before inoculation. The first group (calves 1, 2, and 3) received 2 ml. of reconstituted BVD vaccine by the intramuscular route. The second group (calves 4 and 5) were inoculated with 2 ml. each of BVD and IBR vaccines as a single dose by the intramuscular route. The third group of calves (6 and 7) received 2 ml. of IBR vaccine by the intramuscular route.

**Postinoculation Procedure**

Following inoculation the calves were observed for clinical signs of illness until termination of experiment. During this period blood samples were drawn once a day for total leukocyte count and rectal temperatures recorded twice a day. Blood samples and nasal swabs were taken for virus
reisolation. Serum samples were collected at the termination of the experiment for antibody assay.

Necropsy Procedure

All animals were killed by electrocution and a complete necropsy was conducted. The schedule of termination of the experiment is given in Table 1. Tissues were collected for hematoxylin and eosin staining, fluorescent antibody staining, and electronmicroscopy.

Histologic Procedure

Tissues for hematoxylin and eosin staining were fixed in 10 per cent neutral formaldehyde. The remaining procedures were carried out as described in the Manual of Histologic and Special Staining Techniques (1960).
RESULTS

Clinical Signs

Field cases

Clinical signs of illness appeared between the 10th and 20th day after receiving the vaccines. The first evidence of the disease was depression, anorexia, and oculonasal discharge. These discharges were at first serous but rapidly progressed to mucous and mucopurulent in character and were accompanied by encrustation around the eye and nose and on the muzzle. Severe watery diarrhea accompanied or followed soon after these signs and persisted until the death or recovery of the animal. In advanced cases the feces contained mucus and often small flecks of blood. In severe cases violent tenesmus was noted. Lameness was a common observation.

During the earlier stages of the disease there was a marked temperature elevation ranging between 104 and 106°F and leukopenia of approximately 50 per cent. Differential counts revealed no significant selectivity in the cells involved in the leukopenia.

Death usually occurred in about 10 - 14 days after first being noticed sick. Those animals that survived longer than this generally recovered. The recovery time, however, was very prolonged.

Morbidity data were obtained from 17 herds containing a total of 9,891 cattle. Six of the herds had a morbidity of less than 1 per cent with an average of 0.39 per cent; 6 had a morbidity of more than 1 per cent but less than 5 per cent with an average of 2.9 per cent; and 5 herds had an incidence of greater than 5 per cent with an average of 13.8 per cent. The 6 herds with the lowest incidence each contained more than 500 head of
cattle. The highest morbidity levels were observed in the smaller herds. The range of morbidity for the 17 herds was from 0.2 - 40 per cent with an average incidence of 5.21 per cent. Mortality information has been difficult to obtain, but it is estimated that 75 per cent of the cattle that developed clinical signs died.

**Experimental animals**

There was a temperature elevation ranging between 102.5 and 103.0° F. appearing at approximately 3 - 5 days after experimental vaccination. In some calves there was also a leukopenia accompanying the febrile response. No other clinical signs were observed in any of the experimental calves.

**Gross Pathology**

**Field cases**

Encrustation of the muzzle resulting from accumulation of necrotic epithelium and nasal exudate was very marked (Fig. 1). Sloughing of the dried epithelium resulted in erosions and ulcers. Diffuse erosive to ulcerative stomatitis and glossitis involving the entire mucosa were consistent findings (Fig. 2). As the disease progressed, healing became evident at the base of the ulcers and erosions. This was most prominent on the dorsum of the tongue giving it a bluish-white, corrugated appearance.

Lesions in the esophagus were characterized by multiple foci of linear necrosis followed by erosion or ulceration. These lesions were more numerous and more severe in the posterior third of the esophagus.

The rumen, reticulum, and omasum were inconsistently involved. When present, the lesions in the rumen and the reticulum were seen as a diffuse
superficial necrosis of the epithelium (Fig. 3). The lesions in the rumen were more severe on the pillars where erosions and ulcers were often seen. The most common lesions in the abomasum were a diffuse superficial epithelial necrosis with some sloughing (Fig. 4). In a few animals there were discrete ulcers surrounded by a ring of petechial hemorrhages.

Considerable variation was observed in the lesions in the small and large intestines. A mild to moderate diffuse catarrhal enteritis was present in all animals. Lesions in the Peyer's patches were observed in approximately 50 per cent of the animals. When present, these lesions were characterized by a copious tenacious mucus adhering to the surface of the Peyer's patches (Fig. 5). Occasionally hemorrhagic to fibrinonecrotic exudation accompanied the excessive catarrh. In animals that had been ill for 2 weeks or more the Peyer's patches were seen as depleted and depressed areas in the intestinal mucosa. Severe lesions in the large intestine were observed only in the older cattle and were characterized by diffuse hemorrhage to fibrinonecrosis of the epithelium of the cecum, colon, and rectum (Fig. 6).

Gross changes were consistently found in the lymphatic tissues. The most frequent finding was a serous lymphadenitis with occasional foci of necrosis and hemorrhage. The nodes of the neck and the mesentery were most commonly involved.

A moist, seborrheic dermatitis especially prominent in the axillary, sternal, and inguinal regions was noted in the majority of the affected animals (Fig. 7). In nearly all animals there was a necrotic pododermatitis involving the interdigital skin of all 4 feet (Fig. 8).

Lesions were not observed in any other tissues except for occasional
Figure 1. Diffuse erosion and ulceration of the gingiva and encrustation of muzzle of a calf affected with the postvaccinal condition following vaccination against bovine virus diarrhea. Note the hemorrhagic appearance of the gingiva.

Figure 2. Erosion and ulceration of the dorsal surface of the tongue of a calf affected with the postvaccinal condition following vaccination against bovine virus diarrhea and infectious bovine rhinotracheitis.
Figure 3. Diffuse superficial necrosis of the epithelium of the omasum of a calf following vaccination with bovine virus diarrhea and infectious bovine rhinotracheitis vaccines. Note the chalky white appearance of the mucosa.

Figure 4. Diffuse superficial necrosis of the epithelium of the abomasal mucosa of a calf following vaccination against bovine virus diarrhea and infectious bovine rhinotracheitis.
Figure 5. Peyer's patch of a calf vaccinated with bovine viral diarrhea and infectious bovine rhinotracheitis vaccines. Notice the mucous exudate adhered to the mucosa above the lymphoid tissue (arrow).

Figure 6. Fibrinonecrosis of the mucosa of cecum of a calf following vaccination against bovine virus diarrhea and infectious bovine rhinotracheitis.
Figure 7. Matting of the hair by exudate in the axillary region of a calf vaccinated with bovine virus diarrhea vaccine and infectious bovine rhinotracheitis vaccine.

Figure 8. Necrosis and erosion of the epithelium in the interdigital space of a calf vaccinated with bovine virus diarrhea and infectious bovine rhinotracheitis vaccines.
pneumonic areas in the lungs which were considered either as residual foci from previous pneumonia or bacterial pneumonia secondary to the post-vaccinal condition.

**Experimental animals**

Significant gross lesions were not found in any of the experimentally vaccinated calves.

**Microscopic Lesions**

**Field cases**

Lesions in the oral mucosa were primarily fibrinonecrosis of the epithelium leading to erosion and sometimes ulceration. The lesions were often confined to stratum spinosum without affecting stratum germinativum (Figs. 9 and 10). The earliest lesion was extracellular edema which formed microvesicles. There was stretching of intercellular bridges and reduction in the volume of cell cytoplasm. The degenerating epithelial cell often seemed to be suspended in a clear space. Hyalinization of cytoplasm, pyknosis of the nuclei, and rounding up of cells preceded their eventual disintegration.

Infiltrations with neutrophils and some mononuclear cells leading to the formation of micropustules accompanied the necrotic changes. Coalescence produced larger pustules which extended to the surface resulting in an erosion. Occasionally these lesions also extended through the stratum germinativum forming ulcers. Subepithelial tissue was hyperemic and diffusely infiltrated with a mixture of inflammatory cells.

The lesions in the esophagus and rumen were similar to those in the
oral cavity.

Histopathological examination of the abomasum revealed a diffuse necrosis of the surface epithelium and small ulcers. Many of the glands were cystic and filled with mucus, necrotic desquamated epithelium, and small numbers of leukocytes. The distension of the glands stretched the normal columnar epithelial lining cells to a low cuboidal or even to a squamous type cells. Various stages of degeneration were observed in these flattened cells.

The lesions in the small intestine were usually confined to the Peyer's patches and immediately adjacent tissues. The intestinal glands embedded in the lymphoid tissue were markedly cystic and filled with mucus, necrotic desquamated cells and numerous leukocytes (Fig. 11). The marked distension of the glands resulted in pressure atrophy of glandular epithelium. Inflammatory cells predominantly neutrophils were observed in intact glandular epithelium and lumen of the distended glands. Although cystic many of these glands seemed to have patent openings into the lumen of the intestine.

The lymphoid tissue around the distended glands was extremely depleted of lymphocytes which had been replaced by large cells resembling reticular cells. In some of the lymphoid follicles, there was a complete disappearance of lymphocytes leaving a spongelike stroma. Epithelial necrosis followed severe involvement of the lymphoid follicles and was often accompanied by fibrinous exudation and hemorrhage. Submucosal edema was particularly prominent at the base of the Peyer's patches with the lymph vessels being distended with a fibrin rich fluid.

The lesions in the large intestine were similar to those described for
abomasum. In older animals there was also a fibrinonecrotic exudate on the surface of the mucosa and severe necrosis of the lamina propria with replacement by fibrous connective tissue which in some instances resulted in complete obliteration of the glands. The necrosis also extended to the submucosa in many places and in some cases there were hyalination and focal infiltration of inflammatory cells in the muscle layers.

Retropharyngeal, cervical, mesenteric lymph nodes were moderately to severely depleted of follicular lymphocytes. In extreme cases coagulation necrosis was evident throughout the lymph node and many of the lymph follicles were replaced by a fibrinoid material. Reticuloendothelial hyperplasia was very prominent in the medulla. The sinuses contained many macrophages with blood pigments in their cytoplasm. Multiple focal accumulations of plasma cells were occasionally observed in the cortex. Focal reactive areas were occasionally seen and were characterized by the presence of many mitotic figures. When present, lesions in the spleen were similar to those seen in the lymph node.

Microscopic examination of the skin revealed a surface accumulation of keratinized material mixed with neutrophils and cellular debris (Fig. 12). Necrosis often extended to stratum spinosum but generally left the stratum germinativum intact. Shallow erosion resulted from sloughing of the necrotic epithelium. The dermis was hyperemic and infiltrated with many inflammatory cells predominantly neutrophils. Folliculitis and follicular plugging accompanied the epidermal lesion.

Microscopic lesions in tonsils were confined primarily to squamous epithelium lining the crypts and were identical to those in the oral epithelium (Fig. 13).
Figure 9. Surface epithelial necrosis of esophageal mucosa of a calf following vaccination against bovine rhinotracheitis. Notice disorganization of cells and formation of microvesicles in the stratum spinosum. Hematoxylin and eosin stain. X 125.

Figure 10. Higher magnification of disorganized area in the stratum spinosum seen in Figure 9. Hematoxylin and eosin stain. X 800.
Figure 11. Cystic dilatation of intestinal glands embedded in the lymphoid tissue of the intestine of a calf vaccinated with bovine virus diarrhea and infectious bovine rhinotracheitis vaccines. The dilated glands are filled with mucus and cellular debris. Hematoxylin and eosin stain. X 125.

Figure 12. Surface accumulation of keratinized epithelium and neutrophils on the skin of a calf vaccinated with bovine virus diarrhea and infectious bovine rhinotracheitis vaccines. Also visible are folliculitis and cellular infiltration of the dermis. Hematoxylin and eosin stain. X 125.
Figure 13. Disorganization of the cells and microvesicle formation in the stratified squamous epithelium lining the tonsilar crypts of a calf vaccinated with bovine virus diarrhea and infectious bovine rhinotracheitis vaccines. Hematoxylin and eosin stain. X 250.
Experimental animals

Significant microscopic lesions were not observed in any of the experimentally vaccinated calves.

Fluorescent Microscopic Observation

Tissues from each animal were treated with both IBR and hog cholera conjugates.

In those animals receiving BVD vaccine (calves 1, 2 and 3) the specific antigen was detected in the spleen, mesenteric lymph node, tonsil, and suprapharyngeal lymph node. The widest distribution of the antigen was noted in calf 3, which was killed on the 9th day postvaccination. In the calf 1 that was killed on the 14th day following vaccination, only the spleen contained specific fluorescence.

Infectious bovine rhinotracheitis viral antigen was detected only in the spleen of one animal (calf 7) following vaccination with IBR vaccine.

Both BVD and IBR antigens were detected in lymphatic tissues of both animals (calves 4 and 5) following vaccination with combined BVD and IBR vaccines.

Tissues from 9 field cases were used for fluorescent antibody staining. Seven of the above 9 were postvaccinal reactions and the remaining 2 were natural cases diagnosed as BVD-MD complex on the basis of clinical signs and gross and microscopic lesions. BVD viral antigen was detected in the spleens of all these animals except calves 48, 57 and 72. Other tissues positive for specific fluorescence were mesenteric lymph nodes and suprapharyngeal lymph nodes. Peyer's patches and prefemoral lymph nodes were positive for BVD viral antigen in calf 37 and calf 76 respectively.
Sections from the spleens of all 9 animals were positive for IBR antigen. The other tissues most frequently positive for specific fluorescence were the tonsils and mesenteric lymph nodes. Suprpharyngeal lymph nodes, prefemoral lymph nodes and Peyer's patches contained specific fluorescence in calves 37 and 57, calf 76 and calf 37 respectively.

In general, lymphatic tissues contained BVD and IBR antigens most frequently.

A detailed account of the distribution of BVD and IBR antigens in tissues as determined by the fluorescent antibody method is given in Tables 1, 2 and 3.

Specific staining was observed in histiocytes of the medullary sinus as well as occasional scattered cells in the cortex of the lymph node. In the lymph node, tonsil, and Peyer's patch, the specific fluorescence was associated with cytoplasm and fine cytoplasmic processes of reticular cells at the periphery of the germinal centers (Figs. 14, 15 and 16).

In the spleen, the cells containing specific fluorescence were reticuloendothelial cells lining the sinuses (Figs. 17 and 18).

Serology

Serum antibody titers varying from 1:8 - 1:32 against the vaccine strain of IBR virus were observed in calves receiving IBR vaccine (calves 6 and 7) or IBR vaccine combined with BVD vaccine (calves 4 and 5). The calves vaccinated with BVD vaccine (calves 1, 2 and 3) or BVD vaccine combined with IBR vaccine (calves 4 and 5) developed significant titers of BVD virus-neutralizing antibodies. Table 5 summarizes the serologic observations made on the experimentally vaccinated calves.
Figure 14. Lymph node of a calf vaccinated with bovine virus diarrhea and infectious bovine rhinotracheitis (IBR) vaccines treated with conjugate made from IBR antiserum. Note the cytoplasmic fluorescence in the large mononuclear cells. X 540.

Figure 15. Tonsil of a calf vaccinated with bovine virus diarrhea (BVD) and infectious bovine rhinotracheitis (IBR) vaccines stained with conjugate made from IBR antiserum. Note two large mononuclear cells at the center of the field which contain specific cytoplasmic fluorescence. X 540.
Figure 16. Peyer's patch of a calf vaccinated with bovine virus diarrhea (BVD) and infectious bovine rhinotracheitis (IBR) vaccines stained with conjugate made from IBR antiserum. Note the cytoplasmic fluorescence in large mononuclear cells. X 540.

Figure 17. Spleen of a calf vaccinated with bovine virus diarrhea (BVD) and infectious bovine rhinotracheitis (IBR) vaccines stained with conjugate made from IBR antiserum. Note the specific cytoplasmic fluorescence. X 540.
Figure 18. Spleen of a calf vaccinated with bovine virus diarrhea (BVD) and infectious bovine rhinotracheitis (IBR) vaccines stained with conjugate made from hog cholera antiserum. Note two cells with specific cytoplasmic fluorescence. X 400.
With the exception of two calves (43 and 54), there was no significant antibody titer against the BVD virus in calves that had clinical signs of the postvaccinal condition at the time of sampling. In calves that were apparently recovering from the postvaccinal condition or had shown no evidence of illness, titers in excess of 1:64 were present in all but one calf (71).

Variable but significant antibody titers against IBR virus were present in all calves. Table 4 summarizes the serologic observations made in the 3 groups of cattle.

Virus Isolation

Attempts of virus isolation were unsuccessful in all the field and experimental animals except from calf 80. A virus antigenically related to NADL-MD virus was isolated from buffy coat cultures of this animal.

Ultrastructural Changes in Lymphatic Tissues

Field cases

Structural alterations were observed both in cytoplasm and nucleus. The cell membrane appeared to be infolded and was traced with difficulty. The infolding resulted in numerous cytoplasmic processes some encircling bundles of extracellular collagen (Fig. 19). Vacuolization of cytoplasm an vacuolization and swelling of mitochondria were often seen in the reticulum cell and the macrophage. Many of the large cytoplasmic vacuoles contained laminated structures (Figs. 20 and 21). These laminated structures were recognized previously (Adams and Prince, 1959) and referred to as "cytoplasmic inclusions". They are usually elliptical and may measure
Table 1. Distribution of viral antigens in tissues of calves following vaccination with bovine virus diarrhea (BVD) and/or infectious bovine rhinotracheitis (IBR) vaccines

<table>
<thead>
<tr>
<th>Vaccine used</th>
<th>BVD Days Post Inoculation</th>
<th>IBR Days Post Inoculation</th>
<th>BVD and IBR Days Post Inoculation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
<td>9</td>
<td>14</td>
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<tr>
<td>Calf 2</td>
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<td>Calf 3</td>
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<td>Calf 1</td>
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<td>Calf 6</td>
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<td>Calf 7</td>
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<td>Calf 4</td>
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<td>Calf 5</td>
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</tbody>
</table>

- Adrenal
- Spleen
- MLN - Mesenteric lymph node
- Tonsil
- Thymus
- SPLN - Suprathyroidal lymph node
- BLN - Bronchial lymph node
- S. Intestine (P.P.)

1+ Viral antigen detected
2- Viral antigen not detected
Table 2. Distribution of bovine virus diarrhea viral antigen in tissues of calves affected with the post vaccinal condition following vaccination with bovine virus diarrhea and infectious bovine rhinotracheitis vaccines

<table>
<thead>
<tr>
<th>Tissues Examined</th>
<th>Calf</th>
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<th>49</th>
<th>57</th>
<th>65</th>
<th>72</th>
<th>74</th>
<th>76</th>
<th>80</th>
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<td>Small intestine (Peyer's patches)</td>
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<td>BLN - Bronchial lymph node</td>
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<td>MLN - Mesenteric lymph node</td>
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<td>PFLN - Prefemoral lymph node</td>
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</table>

1. + Viral antigen detected
2. - Viral antigen not detected
Table 3. Distribution of infectious bovine rhinotracheitis viral antigen in tissues of calves affected with the post vaccinal condition following vaccination with bovine virus diarrhea and infectious bovine rhinotracheitis vaccines

<table>
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<th>Tissues</th>
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<th>49</th>
<th>57</th>
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<td>BLN - Bronchial lymph</td>
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<td>PFLN - Prefemoral lymph</td>
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<td>node</td>
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<td>SPLN - Suprpharyngeal</td>
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<td>+</td>
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<td>lymph node</td>
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<td>Tonsil</td>
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<td>Adrenal</td>
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<td>-</td>
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</tbody>
</table>

1+ Viral antigen detected
2- Viral antigen not detected
Table 4. Antibody levels of serum from calves following vaccination against bovine viral diarrhea (BVD) and infectious bovine rhinotracheitis (IBR)

<table>
<thead>
<tr>
<th>Clinical signs of mucosal disease at the time of sampling</th>
<th>Stage of recovery after having clinical signs of mucosal disease</th>
<th>No clinical evidence of disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf Serum titer against BVD virus</td>
<td>Calf Serum titer against IBR virus</td>
<td>Calf Serum titer against BVD virus</td>
</tr>
<tr>
<td>37</td>
<td>&lt;4</td>
<td>16</td>
</tr>
<tr>
<td>39</td>
<td>&lt;4</td>
<td>32</td>
</tr>
<tr>
<td>42</td>
<td>&lt;4</td>
<td>16</td>
</tr>
<tr>
<td>43</td>
<td>16</td>
<td>&gt;64</td>
</tr>
<tr>
<td>52</td>
<td>&lt;4</td>
<td>&gt;64</td>
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<tr>
<td>54</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>68</td>
<td>&lt;4</td>
<td>32</td>
</tr>
<tr>
<td>75</td>
<td>&lt;4</td>
<td>32</td>
</tr>
</tbody>
</table>

1 All serum samples were obtained at least 3 weeks after vaccination.

2 Reciprocal of serum dilution. Neutralizing 100 TCID\textsubscript{50} of the BVD or IBR virus.
Table 5. Antibody levels of serum from calves following experimental vaccination with bovine viral diarrhea (BVD) and/or infectious bovine rhinotracheitis (IBR) vaccines

<table>
<thead>
<tr>
<th>Vaccine used</th>
<th>Days post-vaccination</th>
<th>Calf Serum titer against BVD virus[^1]</th>
<th>Calf Serum titer against IBR virus[^1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BVD</td>
<td>7</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>IBR</td>
<td>7</td>
<td>6</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>BVD and IBR</td>
<td>7</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5</td>
<td>24</td>
</tr>
</tbody>
</table>

[^1]: Reciprocal of serum dilution neutralizing 100 TCID_{50} of the BVD or IBR virus.

up to several microns in diameter. They have a limiting shell composed of a variable number of laminae.

The cytoplasm of the reticular cells also contained numerous small vesicles (alveolate vesicles) with characteristic projections at their surface (Figs. 24 and 27). Some of these vesicles contained dense material. In favorable sections these vesicles were seen to be connected to plasma membrane.

Numerous mitochondria under various stages of disintegration were observed. Many of the degenerating mitochondria contained granules presumed to develop from fragmentation of the cristae (Fig. 20).

In macrophages and plasma cells dilated rough surfaced vesicles were encountered (Fig. 23). Many of them were filled with floccular material probably protein in nature. The limiting membrane of these vesicles was irregularly covered by ribonucleoprotein (RNP) particles.
Surrounding the nucleus of blast cells and macrophages, there was often an area of cytoplasm which contained numerous filaments arranged in an irregular manner (Figs. 24 and 27). Occasionally they were observed in apparent contact with certain cellular structures (nuclear membrane and mitochondria). However, its relationship to these structures was not clear. These filaments were straight or slightly wavy and their apparent diameter was approximately 40 - 60 angstrom units.

The cytoplasm of some cells contained vesicles containing dense osmiophilic bodies probably lipid in nature (Figs. 21 and 26). These vesicles were limited by a double membrane.

The most frequent nuclear change was pyknosis. These shrunken nuclei still possessed an intact limiting membrane and were filled with dense granular material probably chromatin. Some nuclei exhibited an irregular outline with numerous invaginations. Margination and clumping of chromatin at the nuclear membrane was sometimes found (Figs. 22 and 27). These clumps sometimes consisted of numerous granules. The remainder of the nucleus consisted of a fine network of threads and small granules of low density instead of the normal granular component. It is possible that these changes denoted a stage of karyolysis. Karyorrhexis of the nucleus and release of homogeneous nuclear chromatin into the cytoplasm were seen at times (Fig. 25).

**Experimental animals**

Ultrastructure of lymphatic tissues from experimentally vaccinated calves was similar to those of the control animals.
Figure 19. Electron micrograph of an area in the germinal center of a mesenteric lymph node from an animal affected with the post-vaccinal condition. Notice cytoplasmic processes (CP) adjacent to extracellular collagen fibrils (F). Also evident at the bottom of the micrograph is a reticular cell which contains nucleus (N), mitochondria (M), and small vacuoles (arrow). Potassium permanganate stain. X 23,300.
Figure 20. A reticular cell from same area as Figure 19 with numerous cytoplasmic vacuoles containing laminated inclusions (arrow). Also evident are structures resembling mitochondria (W) which contain dense, granular material. Note the absence of uniformly dense cytoplasm. Uranyl acetate. X 105,000.
Figure 21. Electronmicrograph of a reticular cell from a mesenteric lymph node of an animal affected with the postvaccinal condition. Numerous vacuoles (V) are visible in the cytoplasm. Structures limited by double membrane are filled with a dense homogenous material (arrow). Also visible are laminated inclusions (L), mitochondria (M), and nucleus (N). Potassium permanganate. X 41,000.
Figure 22. Reticular cell from the mesenteric lymph node of an animal affected with the postvaccinal condition. Evident is the nucleus (N) with clumped chromatin (C) aggregated along the nuclear membrane (NM). Note the low density of the remainder of the nucleus. Also visible are mitochondria (M), some of which contain dense material, rough endoplasmic reticulum (ER) and small vacuoles (arrow) Uranyl acetate. X 56,000.
Figure 23. Electronmicrograph of a plasma cell from the mesenteric lymph node of an animal affected with the postvaccinal condition. Numerous vesicles are covered on their outer surfaces by ribosomes (R). Note the floccular material (F) inside the dilated vesicles. Also visible is the nucleus (N), which is pyknotic. Uranyl acetate stain. X 24,000.
Figure 24. Electronmicrograph of two reticular cells from the germinal center of the spleen of an animal affected with postvaccinal reaction. Note the irregular network of filamentous structures in cytoplasmic matrix. Also visible are the nuclei (N) and alveolate vesicles (A) connected to the plasma membrane. Uranyl acetate stain. X 51,000.
Figure 25. Reticular cell in the germinal center of the mesenteric lymph node of an animal affected with the postvaccinal condition. The cell contains dense fragments (NF) derived from the nucleus. Also visible are numerous vacuoles (V) and small laminated inclusions (I). The adjacent cell at the top of the field also has numerous vacuoles. Potassium permanganate stain. X 33,500.
Figure 26. Germinal center of the mesenteric lymph node of an animal affected with the postvaccinal condition. Extensive cytoplasmic vacuolation is visible (V). Also evident are numerous dense bodies limited by a double membrane (arrow), nuclei (N), and numerous cytoplasmic processes (CP). Potassium permanganate stain. X 35,000.
Figure 27. Cytoplasm of two adjacent reticular cells in the germinal center of the spleen of an animal affected with the post-faccinal condition. Present in the cytoplasm are alveolate vesicles (arrow), containing a dense material, and irregular network of filamentous material (F). Cytoplasm is empty except for the presence of the filaments and alveolate vesicles. Also visible are the plasma membrane (PM) and the nucleus. Note the clumped chromatin (C), aggregated along the nuclear membrane (NM). Uranyl acetate stain. X 105,000.
Figure 28. Reticular cell in the germinal center of the mesenteric lymph node from a control animal. Extracellular reticulum (R), nucleus (N), mitochondria (M), rough endoplasmic reticulum (ER), vacuoles (arrow), plasma membrane (PM), and Golgi zone (G). Uranyl acetate stain. X 46,000.
DISCUSSION

The above information indicates that a specific syndrome resembling BVD-MD complex occurs in a small number of cattle within 10 - 20 days after being vaccinated with BVD or BVD and IBR vaccines. Although it appears to be involved, the exact role the vaccine plays in the pathogenesis of the condition is not known.

Various suggestions have been considered as to what this involvement may be. The presence of an unrecognized contaminating virus in the vaccine seems unlikely in view of the very few numbers of animals that are affected. Likewise, limited cultural studies have failed to reveal any such agent. Another possibility would be that the calves were in the incubative stage of BVD-MD complex at the time of vaccination and the vaccine merely contributed to the stress which actually precipitated the disease. Since so little is known about the epidemiology of BVD-MD complex, it is difficult to discount this theory. It becomes less tenable, however, if one considers that BVD-MD complex has not been a problem following vaccination with the IBR and Leptospirosis vaccines which could act in a similar stressor capacity. The specificity of the incubation time and the unique lesions of this syndrome would also cast doubt on the vaccine acting merely as a stressor.

Many have felt that the Oregon C-24-V strain of virus diarrhea virus was not attenuated enough during vaccine production and that it was actually producing BVD-MD complex in some animals. This proposition is not very probable considering that the vast amount of field testing completed before the vaccine was released revealed no indication of pathogenicity. In
addition, calves experimentally inoculated with low tissue culture passages of this strain experienced little or no clinical response (York et al., 1960).

Early in the investigation most of the reported cases of the post-vaccinal condition had received IBR and BVD vaccines simultaneously. This suggested that the reaction may be due to the synergistic activity of the two viruses as had been shown earlier with experimental inoculations (Tyler and Ramsey, 1965). As more cases were studied, however, it became evident that the condition occurred as commonly in animals receiving the BVD vaccine alone as in those receiving both vaccines together.

Our observations suggest that there is a small number of cattle population that are uniquely susceptible to the virus diarrhea virus. It seems to make no difference as to whether the virus is of field or vaccine origin. What constitutes this unique susceptibility is not known, but it is believed to involve a failure of the immune mechanism. Support for this belief can be found in evaluating the serologic findings in Table 4. In calves with clinical signs of BVD-MD complex at the time serum was obtained, there was evidence of significant production of antibodies against the BVD virus in only two calves (43 and 54). The specificity of the immune failure to BVD virus is evident from the fact that significant antibody titers against the IBR virus were readily developed in the affected cattle. Similar observations have been previously reported in studies of natural cases of BVD-MD complex.

In the present study it has been observed that in a few of the affected animals significant levels of antibodies against the BVD virus developed late in the course of the disease (Table 4). These animals
generally recovered while those that failed to develop antibodies invariably died.

As further evidence of possible immune failure, this study has shown that one of the principal lesions in the postvaccinal condition is a necrosis of the germinal centers of lymphoid tissues. Similar lesions have been reported previously for field and experimental cases of BVD-MD complex, and in animals treated with actinomycin D or X-radiation (Hanna and Wust, 1965; DeBruyn, 1948). In each of these conditions, including the present study, failure to produce antibodies against a specific antigen has been reported.

It is possible that this immune failure or immune delay can be explained by the destruction of lymphoid tissue and inhibition of the proliferation of surviving immunologically competent cells of germinal center. Since some of the affected animals survived it would appear that this destruction and inhibition is neither complete nor permanent. It is possible that in some cases the surviving cells can proliferate and establish an immunologically active center. Such a mechanism has been proposed by Hanna and Wust (1965) for animals treated with actinomycin D. It was their observation that a few of the large, pyronin staining cells of the germinal centers survived the treatment. It was shown that subsequent proliferation of these surviving pyronin staining cells was responsible for the repopulation of the germinal center and the eventual production of specific antibody.

Although the lesions associated with the postvaccinal condition closely resemble those of natural cases of BVD-MD complex, there are some differences. The skin, foot, and eye lesions which were seen in almost
every postvaccinal case are observed less frequently in BVD-MD complex. The necrosis and subsequent erosion of the alimentary tract were more diffuse but generally more superficial than in natural cases. The superficiality of the necrosis was indicated by the evidence of early reepithelialization in many of the animals that had been ill for several days. Early healing was especially prominent on the dorsum of the tongue.

Although the lesions in the abomasum and the Peyer's patches were of the same pattern as those of BVD-MD complex, only rarely were they as severe or as extensive.

In attempting to determine the pathogenesis of the lesions associated with the postvaccinal condition, it was noted that the conditions in the following studies had similar lesions. Shope (1964) administered 6 mercaptopurine (6-MP), an inhibitor of protein synthesis, both singly and in combination with BVD virus to calves. In both instances the lesions produced were similar to those of BVD-MD complex. Jacobson (1964) observed that folic acid antagonists, aminopterin and amethopterin, inhibited mitotic activity in rapidly proliferating tissues. Lesions in the alimentary tract consisted of ulcerations of mucosa and cystic dilatation of glands. It was Jacobson's belief that the cystic distention of the glands was caused by failure of germinative cells to replace the glandular epithelium. Loss of the cells of the glandular neck by surface emigration allowed the distal portion to become isolated and eventually cystic.

As has already been pointed out, X-radiation and Actinomycin D which are inhibitors of cell proliferation produce lesions in lymphoid tissue almost identical to those of the BVD-MD complex.

In consideration of the close similarity of lesions produced by
chemical or physical agents which interfere with cell proliferation to those of BVD-MD complex, it is probable that the BVD virus may exert its influence upon the cell by way of similar mechanism.

IBR viral antigen was usually present in the lymphoid tissues of animals affected with the postvaccinal condition and in natural cases. The significance of this observation is unknown. It is possible that many of the field cases of BVD-MD complex is as a result of combined infection with BVD and IBR viruses. This concept is further strengthened by the fact that BVD and/or IBR viruses have been isolated from field outbreaks of mucosal disease (Gratzek, et al., 1966; Noice and Schipper, 1959). However, combined inoculation of these two viral agents did not produce a syndrome similar to the field case of mucosal disease, although it resulted in a more prolonged course and more severe lesions in some animals (Tyler and Ramsey, 1965).

It has been shown that IBR virus can localize in lymphoid tissue and cause lesions similar to those seen in BVD-MD complex following experimental inoculation (Peter 1964). IBR antigen was demonstrable in the lymphoid tissues even after the appearance of detectable virus neutralizing antibody in the serum.

Fine Structure of Lymphoid Tissue

This study revealed the fine structure of normal lymphoid tissue of cattle to be similar to lymphoid tissue of human, rabbit, and rat as described earlier (Weiss, 1957; 1964).

The presence of "cytoplasmic inclusions" was one of the common findings in the reticulum cell and macrophage of the lymphoid tissue of
animals affected with postvaccinal condition. Similar inclusions were not
detected in tissues from control animals or in tissues from animals ex­
perimentally vaccinated with bovine virus diarrhea vaccine or a combination
of bovine virus diarrhea and infectious bovine rhinotracheitis vaccines.

The significance of these inclusions remains speculative. It is very
unlikely that these are characteristic to this particular virus-cell system.
Although none of the control preparations in the present study contained
such inclusions, similar appearing laminated structures have been described
by other investigators in other virus-cell systems (Adams and Prince, 1959).
They have been seen on rare occasions, in uninfected Ehrlich cells grown
in vitro. These workers have also reported similar inclusions in Ehrlich
cells infected with Newcastle disease virus. Such observations suggest
that these inclusions are simply a particular example of a more general­
ized reactive phenomenon on the part of the cells.

It is possible that these inclusions are formed by compression of the
cristae by vacuoles developing within the mitochondria. Morgan et al.,
(1957) have observed similar laminations inside vacuolated structures be­
lieved to be mitochondria. Another possibility is that the laminar portion
of the inclusion shell is formed by a collapse and fusion of vesicles of
smooth surfaced endoplasmic reticulum. Adams and Prince (1959) have ob­
served these lamellae to be continuous with the wall of the adjacent
vesicles. In the present study, however, it is unlikely that the lamellae
are derived from smooth surfaced reticulum since many of the cells lack a
significant membranous component in the cytoplasm.

Localized dilatation of mitochondria which are frequently observed
in this study are considered to be a fixation artifact since similar
changes were also seen in the control preparations. Similar observations
have been made by others following aldehyde fixation.

The granular material seen in some mitochondria are presumed to have
developed from the fragmentation of cristae. These changes are probably
not unique since they have been described previously by Gansler and
Rouiller (1956) in cells from animals that had been subjected to starvation.

The dense osmiophilic bodies which were limited by double membrane are
most likely derived from mitochondria. Although these bodies were seen
only in tissues from animals affected with the postvaccinal condition,
recent studies of necrosis induced by several injurious agents in a variety
of cells indicate that the mitochondria of dying cells contain similar
characteristic dense aggregations (Ashworth et al., 1963; Hruban et al.,
1963; Svoboda and Higginson, 1963). Chemical nature of these dense bodies
are unknown. Their appearance in osmium fixed tissues suggests a lipid or
lipoprotein composition which may be derived from the mitochondrial mem­
branes. Studies by Ashworth et al., (1963) indicate that these bodies may
contain protein or other components which are capable of scattering elec­
trons. Similar bodies have been observed by others in degenerating cells
in both infected and uninfected cell cultures (Morgan et al., 1957; 1961).
However, in these cases a limiting double membrane was not distinct. It
is also plausible that these dense bodies may have originated from
cisternae of the smooth endoplasmic reticulum. Another possibility would

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1J. F. Munnell, Asst. Professor, Department of Veterinary Anatomy,
Iowa State University of Science and Technology, Ames, Iowa. Use of
gluaralydehyde as a fixative for electron microscopy. Private communica­
tion. 1966.
be that these structures were lysosomes.

Although vesiculation of cisternae and tubules of the endoplasmic reticulum appears to give rise to some vacuoles others apparently develop from nonevident cytoplasmic components. Swelling and vacuolation of mitochondria may add to the number of vacuoles.

The extensive and elaborate cytoplasmic processes or protrusions of the reticular cells which greatly increase the cell surface area which are closely apposed to other cells membranes was very conspicuous in the germinal center of lymphoid tissue. As suggested by Miller and Nossal (1964) and Swartzendruber (1966) these processes may contain antigen which would be more readily accessible to contact with migrating lymphocytes. This concept is further strengthened by the fact that the present study has shown the presence of virus diarrhea viral antigen by immunofluorescence in the processes of reticular cells of germinal centers.

The significance of alveolate vesicles have been discussed by Roth and Porter (1964) who consider them to be protein uptake mechanism. The significance of these in the present study is not known since they were seen in both control and diseased tissues. Generally however, there were more of these vesicles in diseased tissues than in control tissues.

Filamentous formation similar to those described in this study have been reported in leukemic and hepatoma cells and in normal mononuclear phagocytes (Bairati, 1961; DePetris et al., 1962; Freeman and Samuels, 1958). In the leukemic cells these formations had either a barrel shaped structure or a whorl open on both ends with long axis of the fibrils oriented in a circular direction. A granular component was contained within these formations. In hepatoma cells, normal macrophage and in the
large blast type cells in the present study structural organization of the filamentous formation was not observed.

The significance of these filaments has created a great deal of controversy. It has been suggested by some investigators (Freeman and Samuels, 1958) to be a simple specific form of cellular organoid in pathologic states. On the other hand DePetris et al., (1962) considered these filaments as normal structures in macrophages and that modification by some pathologic process gives rise to such formation as the whorl or the barrel structure described by Freeman and Samuels.

Histochemical observation of Ackerman et al., (1960) suggests these filaments to be composed of protein. These same investigators, however, on the basis of electron microscopic observations consider these structures to be membranous rather than filamentous in character and to be derived through modification of the cisternae of endoplasmic reticulum. The present study along with observations of DePetris et al., (1962) do not confirm the electron microscopic observation of Ackerman et al.

With regard to the function of these filaments different theories have been postulated. Although morphologically distinct from tonofibrils, one cannot exclude the possibility that these filaments may constitute a type of supporting framework in normal cells. Another view is that these fibrils are contractile in nature and are related in some way to dynamic activity within the cells.

Since a limited number of sections were examined, the absence of similar filaments in tissues of control animals in the present study does not exclude the possibility that these filamentous structures are a normal component in blast cells or macrophages. In the tissues from
animals affected with postvaccinal condition, the irregular filamentous network seen throughout the cytoplasm may indicate a qualitative modification or degeneration of normal filaments.

The presence of dilated rough surfaced vesicles probably indicates an attempt to produce antibody. The floccular material in the vesicles is probably protein in nature and may be antibody globulin. Similar alterations and conclusions have been reported by Bessis (1961) in plasma cells during antibody formation. Only a very limited number of cells containing these rough surfaced vesicles were observed in the present study. This insufficiency may account for the lack of a significant antibody titer in the serum of affected animals.

The nuclear changes characterized by "clumping" of the chromatin and aggregation of these compact masses along the nuclear membrane can be considered as a nonspecific degenerative change since they have been seen in cells injured by anoxia (Caulfield and Kliosky, 1959), infectious agents (Svoboda, 1962) and radiation (Parsons, 1962). However, margination of chromatin has been suggested by some investigators to be a specific response to virus infection (Morgan et al., 1956; Reissig and Melnick, 1955). The fragmentation of the nuclei can be considered as an advanced stage of cellular degeneration.
CONCLUSION

Considering the clinical, pathologic, and hematologic findings it would appear that the condition appearing in calves following vaccination against BVD or BVD and IBR is identical to the BVD-MD complex. Most animals that do become severely ill do not produce detectable amount of virus neutralizing antibodies against the BVD virus and these invariably die. However, in a small percentage of severely ill animals a delayed antibody response was evident and these animals generally recovered.

Evidence from this study indicate that the failure to produce antibodies is not due to complete immunologic incompetence but due to partial damage to immunologically competent cells which are in contact with the specific antigen.

It has been hypothesized that the lesions in lymphoid tissue and gastrointestinal epithelium are as a result of inhibition of cell mitosis in these sites due to the direct effect of the virus or to the effect of the products of cell-virus interaction.

In considering the ultrastructural changes in lymphoid tissues, it appears that these are not specific to this particular cell-virus system and this is probably a nonspecific degenerative change.

It should be emphasized that although the vaccine is etiologically involved in the postvaccinal condition, the fault lies basically with the unique susceptibility of very limited number of cattle and not with the vaccine. Since the postvaccinal condition is highly sporadic and generally has a very low morbidity, it is not considered to be of sufficient significance to merit serious concern in using the vaccine. It is suggested,
however, that the vaccine be used as prophylactic measure and not as a treatment. Many of the herds with highest incidence of the postvaccinal condition had received the vaccine during the course of an outbreak of BVD-MD complex in the herd.
SUMMARY

Pathogenesis of a condition appearing in cattle following vaccination against bovine virus diarrhea-mucosal disease (BVD-MD) complex was studied. This condition usually appeared between 10th and 12th day postvaccination.

This study included 7 experimentally vaccinated calves and 9 calves affected with the postvaccinal condition. Also included were 23 serum samples obtained from animals from different herds vaccinated against bovine virus diarrhea and infectious bovine rhinotracheitis (BVD and IBR) and seven samples obtained from experimentally vaccinated calves. Virus neutralizing antibody titers against National Animal Disease Laboratory (NADL-MD) strain of bovine virus diarrhea virus and vaccine strain of infectious bovine rhinotracheitis in these samples were determined by using tissue culture.

The calves vaccinated with BVD vaccine were killed on the 7th, 9th and 14th day postvaccination. The calves which received IBR vaccine or a combination of IBR and BVD vaccines were killed on the 7th and 9th day postvaccination. Only clinical signs noted in these calves were a mild temperature elevation and leukopenia for about 2 days beginning the 3rd or 4th day following vaccination. Tissues from these calves did not reveal any gross or microscopic lesions. Varying, but significant amounts of virus neutralizing antibodies against IBR and/or BVD virus were detected in the serums of these calves.

The first evidence of the disease in calves affected with the postvaccinal condition was anorexia, depression and occlusal nasal discharge. Other signs included encrustation of muzzle and severe watery diarrhea.
During the early stages of the disease there was marked temperature elevation and a 50 per cent reduction in total leukocyte count. Death usually occurred in 10 - 14 days after the first signs of illness.

The range of morbidity in 17 herds studied was from 0.2 - 40 per cent with an average incidence of 5.21 per cent. The mortality rate was estimated to be about 75 per cent of those cattle that develop severe clinical signs.

Gross and histopathologic changes were confined to the gastrointestinal tract and associated lymphoid tissue. Erosion and ulceration were prominent in the mouth and esophagus. Diffuse superficial epithelial necrosis was the predominant lesion in the abomasum. In a few animals there were discrete ulcers surrounded by a ring of petechial hemorrhages.

Copious tenacious mucous exudate adhered to the surface of the Peyer's patches. Occasionally the lesions in the Peyer's patches were characterized by fibrinonecrosis and cystic dilatation of the glands embedded in the lymphoid tissue. Lesions in the large intestine were characterized by a diffuse hemorrhagic to fibrinonecrotic inflammation of the mucosa.

A moist seborrheic dermatitis especially prominent in the axillary, sternal and inguinal region was noted in majority of animals. There was also a pododermatitis involving the interdigital space.

With the exception of two animals, there was no significant antibody titer against the BVD virus in calves that had clinical signs of the post-vaccinal condition at the time of sampling. In calves that were apparently recovering from the post-vaccinal condition, or calves that have shown no evidence of illness, significant serum antibody titers were present. Variable but significant titers against IBR were present in all calves.
IBR viral antigen was consistently detected in lymphoid tissues of almost every animal by fluorescent antibody technique. BVD viral antigen was less frequently detected in lymphoid tissues.

The principle ultrastructural changes in the lymphoid tissue were in the reticular cells in the germinal center. Laminated "cytoplasmic inclusions" were observed in many cells. Cytoplasmic vacuoles of varying size and shape were frequently observed. Occasionally the cytoplasm of the reticular cells contained an irregular network of filamentous structures and varying number of alveolate vesicles. The most frequent nuclear change was pyknosis. The chromatin of many of the nuclei were clumped and aggregated along the nuclear membrane.
LITERATURE CITED


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