Immunofluorescence of Bovine Virus Diarrhea Viral Antigen in White Blood Cells from Experimentally Infected Immunocompetent Calves

David M. Bezek, John C. Baker and John B. Kaneene*

ABSTRACT

A study to evaluate the detection of bovine virus diarrhea viral antigen using immunofluorescence testing of white blood cells was conducted. Five colostrum-deprived calves were inoculated intravenously with a cytopathic strain of the virus. Lymphocyte and buffy coat smears were prepared daily for direct immunofluorescent staining for detection of antigen. Lymphocytes were separated from heparinized blood using a Ficoll density procedure. Buffy coat smears were prepared from centrifuged blood samples collected using ethylenediaminetetraacetic acid as an anticoagulant. Bovine viral diarrhea virus antigen was detected by immunofluorescence between 3 and 11 days postinfection in lymphocyte smears and 3 to 12 days postinfection in buffy coat smears. Isolation of virus from both lymphocytes and buffy coat preparations correlated with detection of immunofluorescence. Serum neutralizing antibody to bovine virus diarrhea virus was detected on day 10 postinfection. Buffy coat smears were as sensitive as lymphocyte smears for the detection of antigen by immunofluorescence. It appeared that immunofluorescent staining of white blood cells was an effective method of detecting bovine virus diarrhea viral antigen.

RÉSUMÉ

Cette expérience consistait à évaluer la technique d'immunofluorescence comme moyen de détecter le virus de la diarrhée à virus bovine dans les leucocytes circulants. Cinq veaux privés de colostrum reçurent à cette fin, par la voie intraveineuse, une souche cytopathogène du virus précité. On prépara quotidiennement des étalements des lymphocytes et de la couche leucocytaire qu'on colora par l'immunofluorescence directe, pour rechercher le virus. On sépara les lymphocytes, à partir de sang hépariné, à l'aide d'un procédé de densité Ficoll. On prépara les étalements de la couche leucocytaire, à partir d'échantillons centrifugés après leur prélèvement dans l'anticoagulant:acide éthylènediaminetétracétique. L'immunofluorescence permit de détecter l'antigène du virus expérimental, de trois à 11 jours après l'infection, dans les étalements des lymphocytes et, de trois à 12 jours après l'infection, dans ceux de la couche leucocytaire. L'isolement du virus, à partir de préparations de lymphocytes et de la couche leucocytaire, concordait avec sa détection par l'immunofluorescence. Le dixième jour après l'infection, on décela des anticorps sériques neutralisants, à l'endroit du virus. Les étalements de la couche leucocytaire se révélèrent aussi sensibles que ceux des lymphocytes, pour la détection de l'antigène par l'immunofluorescence. La coloration des leucocytes circulants, par l'immunofluorescence, sembla représenter une méthode efficace de détecter l'antigène du virus de la diarrhée à virus bovine.

INTRODUCTION

Cattle infected with bovine virus diarrhea virus (BVDV) display a broad spectrum of clinical manifestations, which have been recently reviewed (1-3).

Although immunofluorescence techniques using a variety of tissues are commonly used in diagnosis of BVDV infection (4), there is little published information on the use of such procedures for detection of BVDV antigen in bovine leukocytes. Direct immunofluorescence testing of white blood cells may have potential for diagnosis of BVDV infection. This study was undertaken to determine if direct immunofluorescent staining of white blood cells could be used to detect BVDV antigen in immunocompetent calves undergoing acute infection with a cytopathic strain of BVDV. A further objective of the study was to compare immunofluorescent staining of buffy coat cells and lymphocytes in detection of acute BVDV infection.

MATERIALS AND METHODS

VIRUS AND CELLS

Bovine turbinate cells (NADL, Ames, Iowa) determined free of noncytopathic strains of BVDV by immunofluorescence testing were used for virus isolation (VI) procedures and in a serum neutralization test (SNT) for BVDV. Both SNT and VI tests used minimal essential media with 10% horse serum (HyClone Laborato-

*Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan 48824-1314.
Reprint requests to Dr. J.C. Baker.
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ries, Logan, Utah) and antibiotics as previously described (5).

The cytopathic Singer strain (NADL, Ames, Iowa) of BVDV was used to infect calves. Cell culture fluid was removed approximately 72 h postinoculation when cytopathic effects (CPE) involved 75% of the cells in the monolayer. Culture fluid was centrifuged and aliquots of the supernatant were prepared and frozen at -150° C to be used for inoculation of calves and in the SNT. All calves were infected with a single 10 mL intravenous injection containing $4 \times 10^{5.5}$ median cell culture infectious doses ($CCID_{50}$) of BVDV/mL.

EXPERIMENTAL ANIMALS

Five colostrum-deprived male Holstein calves ranging in age from one to four days at the time of inoculation were used. Housing and care of calves was in accordance with guidelines established by the National Institute of Health (6). Both SNT and VI techniques were used to confirm these animals as being seronegative and nonviremic for BVDV prior to inoculation. Animals were dosed orally with a monoclonal antibody to *Escherichia coli* (Molecular Genetics, Minnetonka, Minnesota) and vaccinated with a rotavirus/coronavirus vaccine (Norden Labs, Inc., Lincoln, Nebraska) upon arrival at isolation facilities. Procaine penicillin G (Pfizer, Inc., New York, New York) at 10,000 IU/1b was given subcutaneously twice daily for the duration of the study. Calves were fed milk replacer (Vita Plus Corp., Madison, Wisconsin) at 10% of body weight, divided between two daily feedings. Physical examination was performed daily throughout the study. Blood samples were collected daily in heparin, ethylenediaminetetraacetic acid (EDTA) and clot tubes.

SEPARATION OF LYMPHOCYTES

Heparinized blood was further processed with a Ficoll (Sigma Chemical Co., St. Louis, Missouri) density procedure (7) modified at the initial separation step by centrifuging 60 min at 500 x *g*. The lymphocyte-enriched fraction thus obtained was washed twice in an excess of phosphate buffered saline (PBS) and a portion of this fraction was used to make smears on glass slides for direct fluorescent antibody (DFA) staining while the remainder was used to inoculate cell cultures for VI procedures.

Blood collected in EDTA tubes was centrifuged at 100 x *g* for 10 min. The buffy coat layer formed was removed with a Pasteur pipette, a portion smeared on a glass slide, and the remainder used to inoculate cell culture as previously described for lymphocytes.

IMMUNOFLUORESCENT STAINING OF SMEARS

Lymphocytes and buffy coat smears were fixed in acetone, then immersed in 3% Tween 80 (Sigma Chemical Co., St. Louis, Missouri) in 0.01 M PBS (pH 7.6) for 10 min to enhance the antibody-antigen reaction. Air dried slides were then stained with a DFA specific for BVDV antigen (NADL, Ames, Iowa) for 30 min in a humidified chamber (37° C). Smears were then washed in 0.85% saline for 10 min and counterstained with 0.01% Evans Blue (Sigma Chemical Co., St. Louis, Missouri). Smears were then rinsed in distilled water and air dried prior to examining under fluorescence microscopy.

VIRUS ISOLATION

Bovine turbinate cells grown in 24-well cluster plates (CoStar, Cambridge, Massachusetts) on glass coverslips (Bellco Glass, Inc., Vineland, New Jersey) were inoculated with lymphocytes or buffy coats processed by rapidly freezing (dry ice and acetone) and thawing in three cycles. Plates were incubated 1 h at room temperature to allow time for viral adsorption and then rinsed three times with a solution containing 1% glucose, 8% NaCl, 0.2% KCl, 0.17% $NaHCO_3$ and phenol red. Cultures were observed daily for characteristic CPE and then subpassed at six days. The presence of BVDV was confirmed by DFA staining of coverslips.

SERUM NEUTRALIZATION TEST

Antibody titers to BVDV antigen were determined by a SNT following standard procedures (8). A total of 300 $CCID_{50}$ of test virus was added to each well (excluding control wells) and results were read six days later.

RESULTS

Results of immunofluorescence testing of lymphocyte and buffy coat preparations, and of virus isolation, are presented in Fig. 1. Nonspecific immunofluorescence was not observed with either buffy coat or

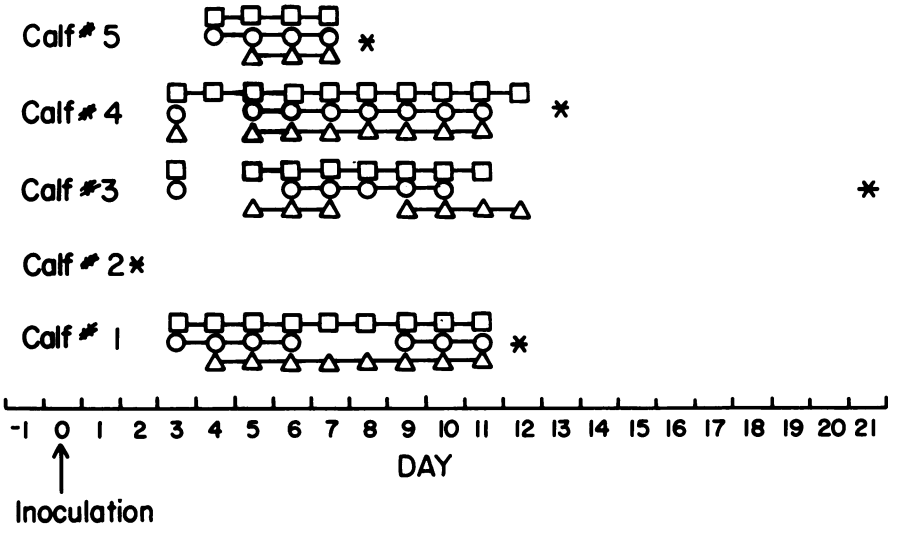

Fig. 1. Comparison of immunofluorescence (IF) testing of blood buffy coat and lymphocyte preparations for bovine viral diarrhea virus (BVDV) antigen and isolation of BVDV from five experimentally infected calves.
□, blood buffy coat preparation IF;
○, lymphocyte preparation IF;
△, isolation of BVDV;
***, death.**

lymphocyte preparations. Immunofluorescence for BVDV antigen was not detected prior to three days postinfection (PI) including the preinfection period. Buffy coat and lymphocyte smears began to show immunofluorescence for BVDV antigen at three days (three of four calves) and four days (one of four calves) PI and persisted until 11 to 12 days PI. Buffy coat smears displayed immunofluorescence comparable to or in excess of the lymphocyte smears prepared on the same day. Bovine viral diarrhea virus was first isolated from lymphocytes and buffy coats at day 4 (one of four calves) and day 5 (three of four calves) PI. After 12 days PI, BVDV was no longer isolated in the calves remaining alive.

Calves Nos. 1, 2 and 5 died or were euthanized prior to the detection of antibody to BVDV antigen. Antibody to BVDV antigen was first detected in Calf No. 3 on day 13 PI and in Calf No. 4 on day 10 PI.

DISCUSSION

Lymphocyte and buffy coat smears obtained from calves infected with cytopathic BVDV demonstrated specific immunofluorescence for BVDV antigen. Both cell preparations demonstrated BVDV antigen during the same time period and at a comparable amount and intensity of immunofluorescence. Since the lymphocyte smear required up to 2 h longer to process, the buffy coat smear appeared to be a more practical technique. Results of immunofluorescence testing of white blood cells correlated with results of VI on buffy coat cells and lymphocytes, and appeared to be a reliable diagnostic procedure in immunocompetent cattle undergoing acute infection with a cytopathic strain of BVDV.

Although specific immunofluorescent staining of noncytopathic BVDV infected lymphocytes has been described (9), as well as direct immunofluorescent staining of BVDV infected macrophages (10), studies utilizing similar techniques for buffy coat preparations are lacking. Immunofluorescent staining of blood buffy coats may have application in detection of persistently infected cattle, but this remains to be tested.

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