Flow Cytometric Detection of Bovine Viral Diarrhea Virus in Peripheral Blood Leukocytes of Persistently Infected Cattle

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ABSTRACT

Flow cytometry was investigated for detection of bovine viral diarrhea virus (BVDV) in peripheral blood mononuclear leukocytes of persistently infected cattle. The mononuclear leukocytes were purified by sedimentation in a gradient of Ficoll-Paque, fixed, permeabilized, and then labelled by indirect immunofluorescence using biotinylated immunoglobulins from a porcine antiserum to BVDV. Flow cytometric analysis of blood samples obtained from persistently infected cattle revealed virus in 3.0-21.0% (mean ± SD, 11.2% ± 6.4%) of the mononuclear leukocytes. Fluorescent cells were not observed in controls. Flow cytometric detection of BVDV in blood cells of persistently infected bovines is a rapid and objective technique which does not require cell culture facilities.

RÉSUMÉ

La cytométrie en flux a été éprouvée comme outil, afin de déceler la présence du virus de la diarrhée bovine virale (VDBV) dans les cellules mononucléées du sang périphérique chez des bovins immunotolérants. Les cellules mononucléées ont été isolées par sédimentation sur un gradient de Ficoll-Paque, fixées et leurs membranes fragilisées. Ces préparations ont par la suite été utilisées dans un test d'immunofluorescence indirecte, avec un antisérum de porc conjugué à la biotine et dirigé contre le VDBV. L'analyse par cytométrie en flux des échantillons sanguins provenant d'animaux infectés, révèle la présence du virus dans 3,0 à 21,0% (moyenne ± DS, 11,2 ± 6,4%) des leucocytes mononucléés. Aucune cellule fluorescente n'a pu être décelée chez les témoins. La cytométrie en flux, dans la détection de VDBV dans les cellules sanguines, se révèle être une méthode rapide et objective, qui ne nécessite pas de cultures cellulaires. ***(Traduit par Dr Pauline Brousseau).***

INTRODUCTION

Bovine viral diarrhea virus (BVDV) induces a wide variety of fetal and postnatal disorders in cattle. Prenatal infections can lead to the birth of persistently infected (PI) calves, and these are important transmitters of virus to other susceptible cattle (1). Therefore, identification of PI animals is an essential prerequisite for controlling the infection.

Demonstration of BVDV in blood samples is usually done by isolation of the virus in cell culture and subsequent identification by immunofluorescent or enzymatic techniques. The latter technique has been adapted to microtiter plates permitting samples to be tested in large numbers (2). Recently it was demonstrated that probe hybridization is a very sensitive technique for identification of BVDV in cell culture (3). An alternative method is detection of virus directly in blood cells from infected animals, either by immunocytochemical (4) or fluorescent techniques (5).

Flow cytometry has been used to quantitate viral antigens located in the cytoplasma of cells in culture. However, to our knowledge, this technique has only once been used for measuring viral antigens inside cells obtained directly from donors (6). The present study was undertaken to investigate whether BVDV antigens could be demonstrated by flow cytometry directly in peripheral blood mononuclear leukocytes (PBMLs) of PI cattle.

MATERIALS AND METHODS

VIRUSES AND CELL CULTURES

The NADL strain of BVDV was obtained from the American Type Culture Collection (Rockville, Maryland) and Madin-Darby bovine kidney (MDBK) cells were purchased from the National Veterinary Services Laboratories (Ames, Iowa). Virus was grown in MDBK cells shown to be free from contamination with adventitious BVDV. The cells were grown in Eagle's minimal essential medium (MEM) supplemented with 5% bovine serum (BS), derived from animals in the BVDV-free institute herd. Cells were maintained in Eagle's MEM with 2% BS.

CATTLE

Six PI cattle, and an equal number of controls from the same herd of Jersey animals, served as blood

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Submitted January 11, 1990.

donors. Persistently infected animals were identified by two successive isolations of BVDV in cell culture (2). Samples from three PI heifers and three unmatched controls were used in initial experiments for optimization of the fixation and permeabilization procedure (see below).

SERA

Porcine antiserum to BVDV was prepared essentially as described by others (7). Briefly, pigs were intranasally inoculated with the Danish field strain UG 59 of BVDV and six weeks later challenged with the Alfort strain of hog cholera virus. Two weeks later the antisera to BVDV were collected and immunoglobulins were purified by affinity chromatography using protein-A immobilized on agarose (Kem-En-Tec, Copenhagen, Denmark). The immunoglobulins were biotinylated as described (8).

FIXATION AND IMMUNOSTAINING FOR FLOW CYTOMETRY

Approximately 10 mL heparinized blood were gently mixed with an equal volume of RPMI 1640 at room temperature. The PBMLs were obtained from this mixture by sedimentation in a gradient of Ficoll-Paque (Pharmacia, Uppsala, Sweden). The mononuclear leukocytes were washed three times in 0.01 M phosphate buffered saline, pH 7.2, with 0.5% normal rabbit serum (PBS-0.5 NRS), resuspended in the same buffer and counted. The cells were pelleted and fixed in 10 mL 1% paraformaldehyde in PBS for 5 min, except where otherwise noted. The mixture was centrifuged at 400 x *g* for 5 min, and the cells were washed in PBS-0.5 NRS with 0.1% saponin (Sigma) (PBS-0.5 NRS-SAP). Then approximately 10^6 cells in 50 μL of PBS-10.0 NRS-SAP were incubated for 45 min with 2 μL of biotinylated immunoglobulins from the antiserum to BVDV or normal porcine serum. The leukocytes were washed in PBS-0.5 NRS-SAP, incubated for 20 min with 2 μL fluorescein isothiocyanate (FITC)-conjugated streptavidin (Dakopatts, Gentofte, Denmark) diluted 1:25 in PBS-0.5 NRS-SAP and washed again in the same buffer. The fixed and stained cells were resuspended in 0.5 mL diluid (J.T. Baker, The Netherlands) with 0.04% ethidium bromide (EtBr) and analyzed by flow cytometry within 2 h.

Differential counts of granulocytes, monocytes and lymphocytes were performed in whole blood with lysed erythrocytes. Lysates were prepared by mixing 50 μL of heparinized blood with 2.5 mL lysis buffer containing 155 mM ammonium chloride, 130 mM potassium chloride and 0.1 mM ethylenediaminetetraacetate, pH 7.3. This mixture was incubated for 5 min. Then the cells were pelleted, washed three times in PBS-0.5 NRS and resuspended in diluid containing EtBr as described above.

FLOW CYTOMETRY

Samples were analyzed using a fluorescence-activated cell analyzer (FACScan, Becton Dickinson, Mountain View, California). The data were collected first into a two-parameter dot plot displaying forward scatter vs. fluorescence intensity and gated to exclude PBMLs binding normal porcine immunoglobulins. Cells passing through this gate were visualized in a histogram displaying fluorescence intensity vs. number of gated cells. The test-result of the specimen was calculated as the number of cells with fluorescence in a window of 148 channels located proximate to the peak of nonfluorescent cells. This number was expressed as a percentage of gated cells.

IMMUNOFLUORESCENCE MICROSCOPY

Monolayers of MDBK cells were infected with the NADL strain of BVDV or mock infected. At 48 h postinfection the cells were incubated with PBS containing 0.25% trypsin and 0.1% titriplex (Merck, Germany) for 20 min. After trypsinization, the cells were washed in Eagle's MEM supplemented with 5% BS and resuspended in the same medium. Fifty μL of the cell suspension were applied to a glass slide and left at 37° C to let the medium evaporate. The surplus of the cells were fixed and immunostained for analysis by flow cytometry as described above. The cells were fixed on the glass slide with cold acetone, washed in PBS with 0.05% Tween 20 (PBS-T) and incubated for 1 h with 0.1% bovine serum albumin (BSA) in PBS-T (PBS-T-BSA). Biotinylated primary antibody was added and after 1 h the slides were washed 5 x 10 min. Then the cell preparations were incubated for 1 h with FITC-conjugated streptavidin (Dakopatts, Gentofte, Denmark) and after five washings in PBS-T and soaking in water the slides were dried at 37° C. A drop of glycerol with 10% 0.05 M 5,5-diethylbarbituric acid sodium salt, pH 8.5, was applied to each slide, which finally was mounted with a cover slip and examined under fluorescence microscopy.

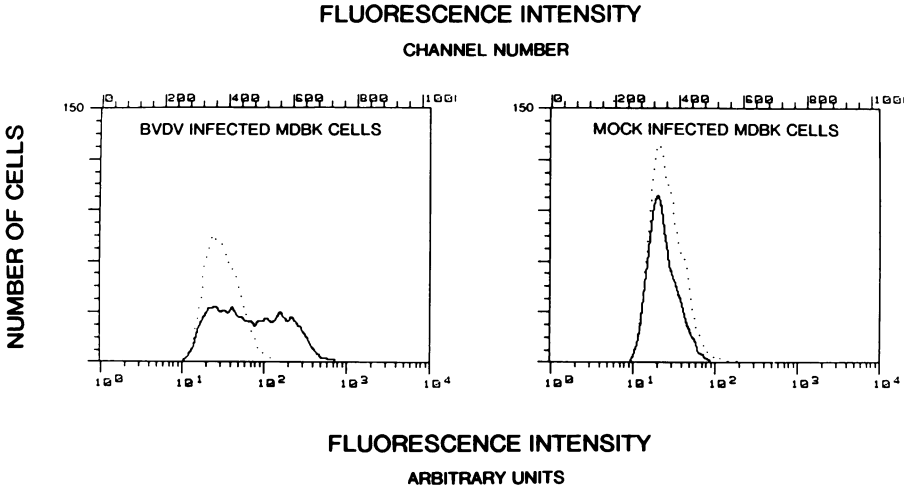

Fig. 1. Flow cytometric analysis of BVDV-infected and mock infected MDBK cells. Monolayers of MDBK cells were infected with the NADL strain of BVDV or mock infected. At 48 h postinfection cells were trypsinized, fixed, permeabilized, and then labelled by indirect immunofluorescence using biotinylated immunoglobulins from the antiserum to BVDV (——) or normal porcine serum (·····). Fluorescence of individual cells was analyzed by flow cytometry and results presented in a histogram.

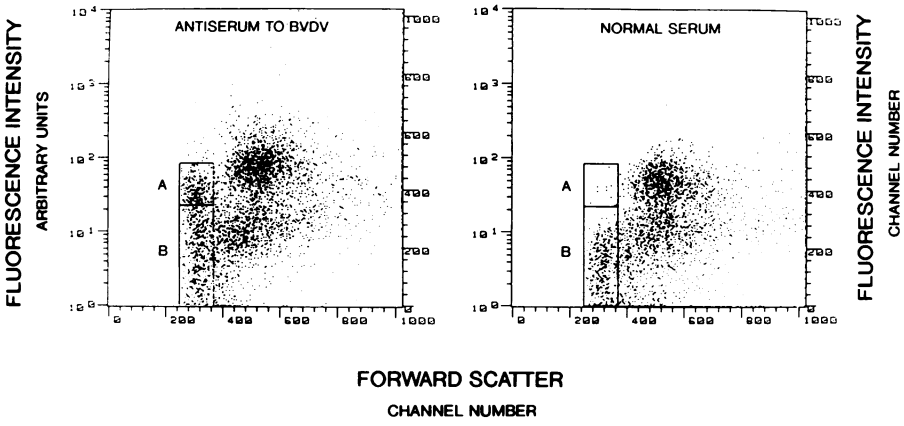

Fig. 2. Flow cytometric detection of BVDV in PBMLs obtained from a PI heifer. The PBMLs were purified from blood of a PI heifer (No. 554) by sedimentation in a gradient of Ficoll-Paque. The PBMLs were fixed, permeabilized, and then labelled and analyzed as described in the legend to Fig. 1. Results were collected in a two-parameter dot plot displaying forward scatter vs. fluorescence intensity. Gates were set to exclude cells binding normal immunoglobulins. Finally the test result, F, of the specimen was calculated according to the formula (F=a/(a+b), where a and b denotes the number of cells in region A and B, respectively.

ELECTRON MICROSCOPY

Mononuclear leukocytes, suspended in PBS-0.5 NRS-SAP after fixation and permeabilization, were collected in a cytospin centrifuge. The cells were applied to a glass slide and dehydrated in acetone. Then the cells were embedded in Vestopal W (Serva) by placing a BEEM-capsule, filled with the embedding medium, upside down on top of the cell spot. Sections of the embedded mononuclear leukocytes were stained with uranyl acetate and lead citrate and examined in a JEM 100B electron microscope.

RESULTS

Initially the use of flow cytometry for detection of BVDV antigens on the surface of viable cells was investigated. This was not possible (data not shown). To measure intracellularly located antigens a number of different procedures for fixation and permeabilization of PBMLs were compared. Fixation in any of the following solutions did not produce cells with fluorescence above background (data not shown): (a) cold acetone, (b) 0.1 M lysine in 0.05 M Na_2PO_4 containing 0.5% paraformaldehyde, 0.5% glutaraldehyde and 0.2% sodium p-periodate, pH 6.0, (c) methanol followed by 0.5% paraformaldehyde in PBS. However, fixation with 1% paraformaldehyde for 5 min (or less) generated cells positive for BVDV antigens when analyzed by flow cytometry. The effects on the cells of the different methods of fixation and permeabilization were examined by electron microscopy, and 1% paraformaldehyde for 5 min preserved most of the cytoplasma (not shown).

The PBMLs analyzed by flow cytometry were inadequately stained when examined by fluorescence microscopy (not shown). Instead, cells from virus-infected MDBK monolayers were analyzed by both techniques. The cytometer distinguished two cell populations from virus-infected monolayers (Fig. 1), and this was confirmed by fluorescent microscopic examination of the cells (not shown).

Differential counts of granulocytes, lymphocytes and monocytes in lysed blood samples from six PI cattle and six controls were performed (data not shown). Differences in the geometric mean of the fraction of the three leukocyte subpopulations in the PI animals and controls were not statistically significant (*t*-test). The means for all 12 animals were 28% ± 14%, 64% ± 15% and 8% ± 2% for granulocytes, lymphocytes and monocytes, respectively.

When purified PBMLs from both PI bovines and controls were incubated with biotinylated immunoglobulins from normal porcine serum numerous monocytes had high fluorescence. Therefore, gates were set to include only a subpopulation of small lymphocytes in calculation of percentage of cells with viral antigens (Fig. 2). These cells constituted 23-55% of all PBMLs purified from the blood (data not shown). Fluorescence was detected in 6.0-21.8% (geometric mean, 12.4%) of gated PBMLs from PI cattle, when immunoglobulins from the BVDV antiserum were used (Table I). Less than 0.2% (mean, 0.2%) of gated PBMLs from controls had fluorescence of the same intensity, and this difference in means was statistically significant (P = 0.0003, *t*-test). The corresponding difference between PI cattle and controls, obtained with immunoglobulins from normal porcine serum, was not statistically significant (*t*-test). The BVDV specific antigens were demonstrated in 3.0-21.0% (mean, 11.2%) of gated PBMLs from PI bovines. The virus specific response was defined as difference in test result obtained with antiserum to BVDV and normal serum.

TABLE I. Frequency of BVDV in PBLs of cattle as demonstrated by flow cytometry

Percentage of gated PBLs with fluorescence[a]	Immunoglobulin	
	Anti-BVDV (F_s)	Normal (F_b)
Persistently infected cattle		
Calf 544	21.8%	0.8%
Heifer 613	14.3%	0.5%
Heifer 638	14.2%	0.5%
Heifer 554	9.2%	0.5%
Heifer 636	8.8%	2.1%
Cow 544	6.0%	3.0%
	12.4% ± 5.6%[b]	1.2% ± 1.1%
Controls		
Heifer 689	0.2%	0.2%
Heifer 602	0.2%	0.3%
Heifer 648	0.1%	0.2%
Heifer 649	0.1%	0.4%
Heifer 572	0.2%	1.2%
Heifer 566	0.2%	1.4%
	0.2% ± 0.1%[b]	0.6% ± 0.5%

[a]Gated cells comprised 23-55% of the 10,000 PBLs recorded for each specimen
[b]Geometric mean ± SD
BVDV = bovine viral diarrhea virus
PBLs = peripheral blood leukocytes

DISCUSSION

A limited number of studies on flow cytometric detection of intracellular located viral antigens has been published (6,9-13). The difficulties in obtaining a signal above the usually high background may in part be the

reason for the relatively few publications (11). Each study used different procedures for fixation and permeabilization and the present work was initiated by optimization of these two parameters. The intensity of the signals obtained with the cytometer were strongly influenced by the various treatments, and only low concentrations of paraformaldehyde for very short periods of time preserved the labile antigens of BVDV. It seems to be essential to carefully evaluate several procedures for fixation and permeabilization for each biological system under study.

The ability of the BVDV antiserum to separate virus- and mock-infected cells was clearly demonstrated by flow cytometry. This finding was confirmed by fluorescent microscopic examination of the cells, and it was therefore concluded that the positive signals obtained with the cytometer represented identification of BVDV antigens.

The observed percentage of each of the three circulating leukocyte subpopulations was in good agreement with an earlier study (14), demonstrating 65% lymphocytes, 32% granulocytes and 3% monocytes compared to 64% ± 15% (mean ± SD), 28% ± 14% and 8% ± 2%, respectively, obtained in this study. However, shifts in the frequencies of monocytes and T lymphocytes in PI calves compared to uninfected calves have been observed (4).

Even though the leukocytes were incubated in buffer containing 10% normal rabbit serum, numerous cells still bound the biotinylated antibodies prepared from normal porcine serum. This could be due to inadequate blocking of F_c-receptors on the monocytes. Therefore, only a subpopulation of small lymphocytes was used in the analysis, despite the fact that BVDV has been demonstrated in monocytes (4). In the same study BVDV antigens were detected in the cytoplasma of approximately 15% of T+B lymphocytes of PI cattle (4). This finding is in agreement with the results of the present study identifying specific BVDV antigens in approximately 11% of the gated cells. The gated cells, however, do not comprise all lymphocytes. Infectious noncytopathic BVDV has been demonstrated in 4.9% ± 3.9% (mean ± SD) of PBMLs from PI cattle (15). This difference could be caused by inability to isolate infectious virus from leukocytes containing BVDV antigens. More likely the presence of infectious virus and viral antigens is highly correlated, and the observed discrepancy could be due to differences in either virulence of the viruses (or host susceptibility) or in the cell population under study.

The present study demonstrated that flow cytometry can be used for the detection of BVDV antigens located in the cytoplasm of PBMLs obtained from PI cattle. The technique is rapid and does not require cell culture facilities. With minor modifications it has considerable potential for mass screening as it is easily automated. Therefore, flow cytometry could be a useful alternative for identification of PI cattle as part of a future program for controlling the infection.

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