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Isolation of a Mutant MDBK Cell Line Resistant to Bovine Viral Diarrhea Virus Infection Due to a Block in Viral Entry[1]

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A cell line, termed CRIB, resistant to infection with bovine viral diarrhea virus (BVDV) has been derived from the MDBK bovine kidney cell line. CRIB cells were obtained by selection and cloning of cells surviving infection with a highly cytolytic BVDV strain. CRIB cells contain no detectable infectious or defective BVDV as ascertained by cocultivation, animal inoculation, indirect immunofluorescence, Western immunoblot, Northern hybridization, and RNA PCR. Inoculation of CRIB cells with 24 cytopathic and noncytopathic BVDV strains does not result in expression of viral genes or amplification of input virus. Karyotype and isoenzyme analyses demonstrated that CRIB are genuine bovine cells. CRIB cells are as susceptible as the parental MDBK cells to 10 other bovine viruses, indicating that these cells do not have a broad defect blocking viral replication. Transfection of CRIB cells with BVDV RNA or virus inoculation in the presence of polyethylene–glycol results in productive infection, indicating that the defect of CRIB cells is at the level of virus entry. CRIB cells are the first bovine cells reported to be resistant to BVDV infection *in vitro* and may be a useful tool for studying the early interactions of pestiviruses with host cells. © 1995 Academic Press, Inc.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an enveloped virus of cattle with a single-stranded RNA genome 12.5 kb in length (Renard *et al.*, 1987; Collett *et al.*, 1988a,b). The naked viral RNA is infectious upon transfection into permissive cells (Diderholm and Dinter, 1966). BVDV is currently classified within the Flaviviridae family, in the genus *Pestivirus*, along with hog cholera virus and border disease virus (Francki *et al.*, 1991; Moennig and Plagemann, 1992).

Cattle are the natural hosts of BVDV but infection with BVDV has also been detected in sheep, swine, goats, buffalo, and a wide variety of wild ruminants, all members of the order Artiodactyla (Doyle and Heuschele, 1983; Dahle *et al.*, 1987; Nettleton, 1990). BVDV replicates in cultured cells from these species and often contaminates cells from other nonrelated species (Lee and Gillespie, 1957; Fernelius *et al.*, 1969; Onyekaba *et al.*, 1987; Wellemans and Opdenbosch, 1987; Potts *et al.*, 1989; Bolin *et al.*, 1994). All bovine cell lineages tested to date are susceptible to BVDV infection *in vitro* (Lee and Gillespie, 1957; Fernelius *et al.*, 1969; Truitt and Shechmeister, 1973; McClurkin *et al.*, 1974; Horzinek, 1981; Onyekaba *et al.*, 1987; Wellemans and Opdenbosch, 1987; Bolin *et al.*, 1994). In contrast, only selected cell populations become infected *in vivo* (Bielefeldt, 1988a,b; Bolin and Ridpath, 1990; Wilhelmsen *et al.*, 1990; Brownlie, 1990; Haines *et al.*, 1992; Marshall, 1993; Lopez *et al.*, 1991).

BVDV initiates infection by a mechanism of receptor-mediated endocytosis, followed by a low pH-dependent fusion of the virus envelope with the endosomal membrane (Hafez and Liess, 1972; Boulanger *et al.*, 1992; Donis, unpublished). The BVDV envelope glycoproteins E2 (gp53) and/or E0 (gp 48) are believed to mediate the initial interactions of virions with cells (Donis and Dubovi, 1987a,c; Donis *et al.*, 1988). A cell surface molecule(s) which interacts with viral envelope glycoproteins to bring about BVDV entry has not been identified and characterized. Monoclonal antibodies (MABs) directed against bovine cell surface proteins partially inhibit BVDV infection, but their molecular specificity remains unknown (Teyssedou *et al.*, 1987; Moennig *et al.*, 1988). In addition, anti-idiotypic antibodies mimicking the BVDV E2 (gp53) partially prevent infection with some strains of BVDV in MDBK cells (Xue and Minocha, 1993).

The evidence presented in this report suggests that a cell surface factor absent or dysfunctional in CRIB cells is required by most, if not all, BVDV strains to initiate infection in MDBK cells.

MATERIALS AND METHODS

Cells and viruses

BVDV-free Madin-Darby bovine kidney cells (MDBK, CCL-22) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) at passage 110 and routinely cultured for approximately 25 passages. Bovine

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testicle cells were prepared as described (Donis and Dubovi, 1987a). Cells were maintained in Eagle's minimum essential medium (MEM) (Gibco, Grand Island, NY) supplemented with 5% fetal horse serum (Sigma, St. Louis, MO). The source of the BVDV isolates is shown in Table 1. BVDV was propagated and quantitated in bovine testicle cells; viral stocks were stored at −70°. The following 10 viruses were obtained from the Veterinary Diagnostic Center at the University of Nebraska–Lincoln: bovine herpesvirus (BHV) types 1, 2, and 4; bovine respiratory syncytial virus (BRSV), bovine adenovirus (BAV), bovine enterovirus (BEV), vesicular stomatitis virus (VSV), parainfluenza-3 virus (PI-3), bovine parvovirus (BPV), and bovine leukemia virus (BLV).

BVDV infection and cloning of surviving MDBK cells

MDBK cells (5×10^6) were infected with a cytopathic BVDV strain (Singer-cp) at a multiplicity of infection (m.o.i.) of 10 $TCID_{50}$/cell (Reed and Muench, 1938). After 4 days, the medium was replaced and the very few survivor cells were cultured until they formed monolayers (approximately 6 weeks). The survivor cells, designated CRIB (Cells Resistant to Infection with BVDV), were cloned by limiting dilution, and clones derived from a single cell were subsequently propagated. Twenty clones, named CRIB-1 to -20, were selected, propagated, and stored in liquid nitrogen. The initial experiments performed to detect BVDV antigens by immunofluorescence and cocultivation, and the BVDV susceptibility assays using Singer-cp, were carried out with the mixed population of resistant cells (CRIB) and with clone 1 (CRIB-1). Isoenzyme analyses were performed in cells from clones 1 and 2 (CRIB-1 and CRIB-2) and the subsequent experiments utilized only cells from clone 1 (CRIB-1).

Search for infectious BVDV or viral subproducts in CRIB cells

Production of infectious virus by CRIB cells was assessed by cocultivation with indicator bovine testicle cells and animal inoculation.

(a) Cocultivation. CRIB and bovine testicle cells were dispersed with trypsin, mixed in a ratio of 1:1, and cultivated for up to 4 weeks. Cells were subcultured twice a week and production of BVDV proteins was monitored by indirect immunofluorescence (IFA).

(b) Animal inoculation. 2.5×10^7 viable CRIB-1 cells were injected intravenously into an 8-month-old, BVDV-seronegative calf. Blood samples were taken before virus inoculation and 2 and 4 weeks postinoculation and subjected to serum-neutralization assays as described elsewhere (Rossi and Kiesel, 1971).

BVDV proteins in cultured cells were detected by IFA as described previously (Donis *et al.,* 1988). Briefly, cells were fixed in acetone and probed with polyclonal or monoclonal antibodies of wide pestivirus-specific reactivity followed by a secondary anti-species fluorescein-conjugated antibody (Corapi *et al.,* 1990).

The presence of residual BVDV genetic material or proteins in CRIB cells was investigated by means of Northern blot hybridization, Western immunoblot, and RNA PCR.

(a) Northern blot. Total cellular RNA extracted from mock-infected or BVDV-infected (Singer-cp, m.o.i. of 10) CRIB-1 and MDBK cells by the acid guanidinium–phenol–chloroform method (Chomczynski and Sacchi, 1987) was processed for Northern blot hybridization according to standard protocols (Ausubel *et al.,* 1989). The nitrocellulose membranes were hybridized with a ^{32}P-labeled DNA probe (α-dATP; Amersham, Inc.). Radiolabeled probes were synthesized by random priming (Feinberg and Vogelstein, 1983) a template DNA fragment derived from the NS5 (p75) region of the BVDV NADL strain (Collett *et al.,* 1988b).

(b) RNA PCR. A set of two oligonucleotide primers (Reverse, 5'-CTTGTGTCTGGCTG, and forward, 5'-AACTGGGTGAAACAG) was used to amplify a fragment of 323 bases from the NS5 (p75) region of the BVDV genome (NADL nucleotide 11,102–11,424). Total cellular RNA from approximately 5×10^5 BVDV-infected or mock-infected MDBK or CRIB-1 cells, contained in 2 μl, was used as template. Reverse transcription and PCR were performed as previously described (Lopez *et al.,* 1991), for 35 cycles (1 min at 95°, 1 min at 34°, and 3 min at 56° for each cycle). PCR products were analyzed by agarose gel electrophoresis and by Southern blot hybridization, using the same probe described above.

(c) Western immunoblot. BVDV-infected or mock-infected CRIB or MDBK cells were lysed in sample buffer (Laemmli, 1970). Cell lysates at appropriate dilutions were separated by SDS–PAGE and proteins were electroblotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). To detect viral proteins with MABs, membranes were processed as described (Burnette, 1981; Donis *et al.,* 1988). HRPO signal was developed with a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL) following the manufacturer's protocol.

Assays for BVDV susceptibility

CRIB-1 and MDBK cells were inoculated with BVDV Singer-cp and VS115-ncp at m.o.i. ranging from 0.1 to 100; duplicate cultures were incubated at 34.5 or 37°. Infected cells were harvested and fixed in cold acetone at 24, 48, and 96 hr p.i. Production of viral proteins was monitored by MAB and polyclonal antibody staining by IFA. Production of BVDV RNA and proteins in CRIB cells following virus inoculation was also examined by Northern blot and Western immunoblot analyses, respectively, as described above. Release of low levels of infectious virus by CRIB-1 cells following inoculation with BVDV Singer-cp and VS115-ncp at m.o.i. of 1 was further investi-

gated by cocultivation with susceptible cells as described above, after neutralization of input virus with a mixture of neutralizing MABs for 48 hr. To ascertain the range of resistance of CRIB cells to different BVDV isolates, CRIB-1 and MDBK cells were inoculated with each of the 24 BVDV isolates at m.o.i. of 1 (see Table 1). To determine the spectrum of viral resistance, CRIB and MDBK cells were infected with 10 bovine viruses (BHV-1, 2, and 4, BRSV, BAV, BEV, VSV, PI-3, BPV, and BLV) from divergent families: Herpesviridae, Paramyxoviridae, Adenoviridae, Picornaviridae, Rhabdovbiridae, Parvoviridae, and Retroviridae. The relative yields of infectious progeny were determined for each of these viruses by limiting dilution (Mahy, 1985).

Karyotype and isoenzyme analyses of CRIB cells

The species derivation of CRIB cells was ascertained by karyotype and isoenzyme analyses. Karyotyping of CRIB-1 cells was performed by Dr. Warren Sanger (Genetics Dept., UNMC, Omaha, NE). Isoenzyme analyses of CRIB-1 and CRIB-2 cells were performed at the American Type Culture Collection (Rockville, MD), using the AuthentiKit System (Innovative Chemistry).

BVDV RNA isolation and transfection

Viral RNA was isolated from concentrated BVDV virions by extraction with acid guanidinium–phenol–chloroform as previously described (Dubovi *et al.*, 1987; Chomczynski and Sacchi, 1987). MDBK and CRIB-1 cells (10^6) were transfected with approximately 2 μg of viral RNA by electroporation. Electroporation was carried out in a 0.4-cm cuvette at 400 Ω, 25 μF, 400 V, with a Gene Pulser device as previously described (Bio-Rad, Hercules, CA) (Liang *et al.*, 1991; Vassilev and Donis, unpublished). Transfected cells were plated on glass coverslips and cultured for 16 hr, then culture supernatants were harvested for quantitation of progeny virus and cells were fixed for IFA.

Virus binding assays

Virus binding assays were performed at 37° on MDBK and CRIB cell monolayers previously treated with 1 mM of phenylarsine oxide (Sigma, St. Louis, MO) to inhibit endocytosis (Milton and Knutson, 1993). Treated cells were incubated with BVDV Singer-cp at m.o.i. of 5 for 1 hr at 37°. After virus adsorption, the cell monolayers were washed five times with MEM to remove unbound virus and were lysed in 2 ml of culture medium by three rounds of freezing at −70° and thawing for 15 min at 4°. Cell debris was sedimented by centrifugation (400 *g* for 5 min at 4°) and virus infectivity in the supernatant fluid was quantitated. Results are presented as means of three independent experiments with four repetitions each.

PEG-mediated BVDV infection of CRIB cells

A suspension of 10^6 CRIB-1 cells in 0.5 ml of culture medium was mixed with an equal volume of cell culture fluid containing 10^7 $TCID_{50}$ of BVDV (Singer-cp or VS115-ncp) and 5% polyethylene–glycol (PEG) (MW 6000; Fisher, Pittsburgh, PA). The mixture was incubated 1 hr at 37°. Cells and attached virus were sedimented by centrifugation (400 *g* for 5 min at 25°), the medium was removed, and the cell pellet was treated with warm MEM containing 37.5% PEG (MW 1450; ATCC, Rockville, MD) for 1 min. After treatment, PEG concentration was slowly reduced by addition of serum-free MEM, and the cells were plated on glass coverslips in culture dishes and incubated at 37°. The frequency of cells producing BVDV proteins was assessed by IFA performed in cells fixed at 24 hr p.i. For quantitation of progeny virus yield, cells were extensively washed after attachment, culture medium was replaced, and culture supernatants were harvested at 24 hr p.i. for virus quantitation. CRIB and MDBK cells inoculated with BVDV in the absence of PEG were used as controls. An identical procedure was used to determine the effect of PEG on VSV and BRSV infectivity in CRIB and parental cells.

RESULTS

Selection of a bovine cell line resistant to bovine viral diarrhea virus infection

A bovine cell line (CRIB) that displays resistance to infection with BVDV was derived from MDBK cells that survived infection with a highly cytolytic BVDV strain (Singer-cp). Infection of bovine cells with cytopathic BVDV at high m.o.i. usually results in lysis of virtually 100% of the cells. Surviving cells develop into cultures which are normally persistently infected. To select BVDV-resistant cells, MDBK cells were inoculated with BVDV Singer-cp at high input multiplicity and the few cells surviving the initial infection were cultured until they formed monolayers. During this time, the cells alternated between periods of cell growth and periods of crisis in which many cells showed characteristic BVDV-induced vacuolation and died. Six weeks after infection, a sample of cells from the culture was fixed and stained by IFA with MABs to BVDV proteins. A large proportion of cells surviving this process did not express BVDV antigens. Attempts to infect this surviving cell population with BVDV Singer-cp at m.o.i. of 10 were unsuccessful; BVDV-inoculated cells did not show cytopathic effect and did not produce viral proteins as judged by IFA. These candidate BVDV-resistant cells were cloned by limiting dilution and 20 independent clones resistant to infection were obtained.

CRIB cells contain no detectable infectious BVDV or viral subproducts

Cells surviving lytic infection with BVDV *in vitro* normally develop into persistently infected cultures. Often,

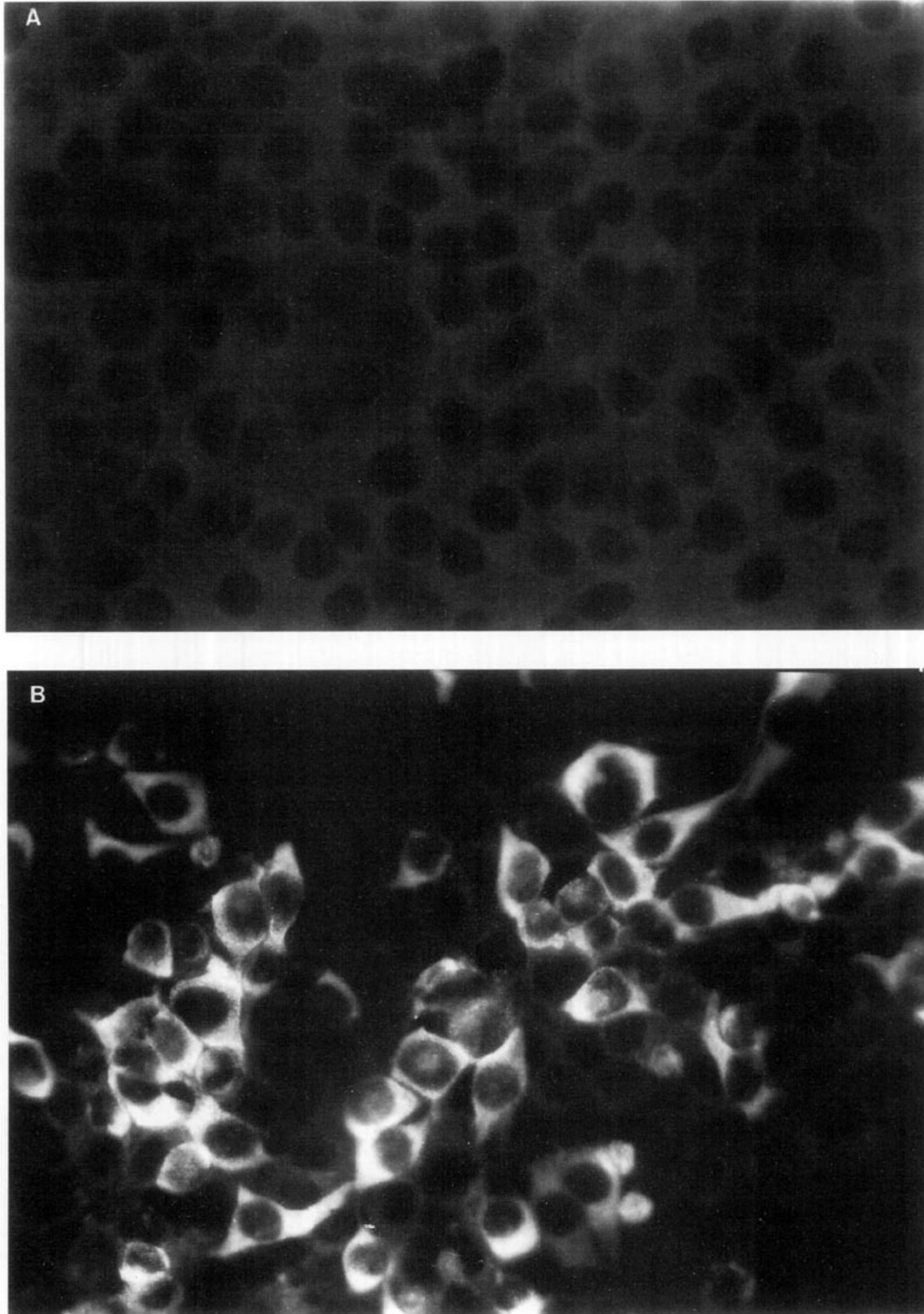

FIG. 1. Immunofluorescence of MDBK (A, B) and CRIB (C, D) cells, mock-infected (A, C) or inoculated with BVDV (B, D). Cells were inoculated with BVDV Singer-cp at m.o.i. of 1 and viral proteins were detected by IFA in cells fixed 24 hr p.i. (magnification = 320×).

the predominant viruses in these cultures are temperature-sensitive mutants which can strongly interfere with the growth of standard virus (Youngner *et al.*, 1986). As CRIB cells were derived from cells that survived lytic BVDV infection, it was conceivable that a residual level of standard or mutant virus or subgenomic RNA replication was inducing resistance by interference with replication of the homologous virus (Gillespie *et al.*, 1962; Shirai *et al.*, 1984). Different approaches were taken to investigate if CRIB cells express viral proteins or contain infec-

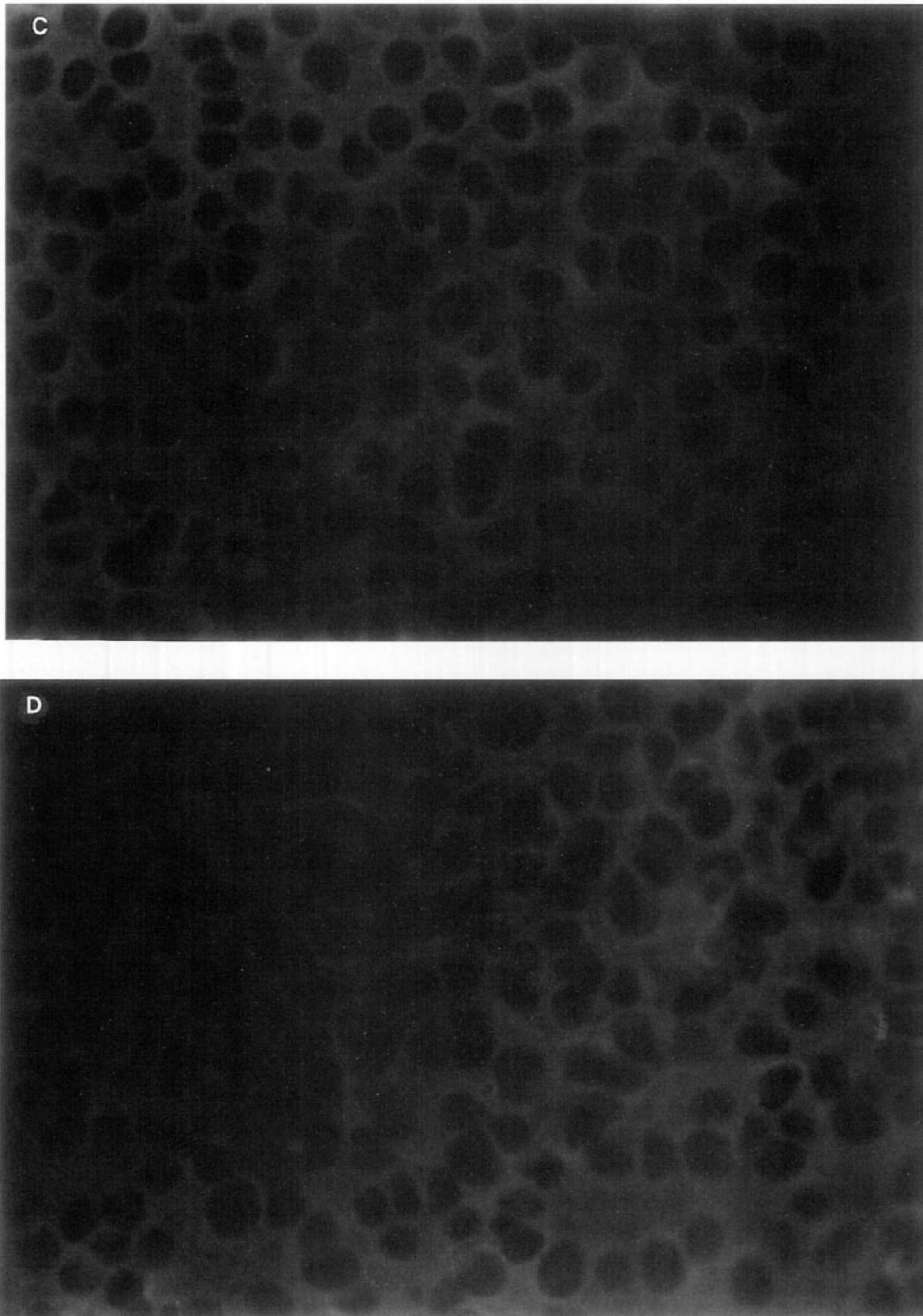

FIG. 1—*Continued*

tious virus or viral RNA. CRIB cells grown at 37 or 34.5° were stained with polyclonal antisera or a mixture of MABs by IFA (Fig. 1). No BVDV antigens were detected in CRIB cells at standard or reduced culture temperatures. Western immunoblots failed to detect BVDV proteins in CRIB cells (Fig. 2). Cocultivation of CRIB cells with bovine testicle cells for up to 4 weeks did not reveal production of infectious virus, as judged by IFA. Moreover, a suspension containing approximately 2.5×10^7 CRIB cells was inoculated intravenously into an 8-month-old BVDV-free, seronegative calf. Serum-neutralization assays performed on blood samples collected 4 weeks after inocu-

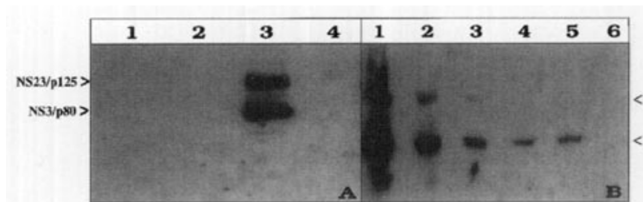

FIG. 2. Western immunoblot probed with MAB to NS3 (p80). (A) mock-infected MDBK (lane 1) and CRIB-1 (lane 2), MDBK cells infected with BVDV at m.o.i. of 10 (lane 3), and CRIB cells infected with BVDV as before (lane 4); cells were harvested by lysis at 24 hr p.i. (B) Serial 10-fold dilutions of BVDV-infected MDBK cells, lysed and tested by Western immunoblot to assess sensitivity of the assay. Neat lysate from ~500,000 infected cells (lane 1); 1:5 dilution (lane 2); 1:50 dilution (lane 3); 1:500 dilution (lane 4); 1:5,000 dilution (lane 5); 1:50,000 dilution (lane 6). Chemiluminescence was recorded on X-ray film, the image was captured with a video camera and Image v. 1.49 software (courtesy of Wayne Rasband, NIH). Figures were printed without filtration in a Tektronix printer. The positions of NS3 (p80) and its precursor NS23 (p125) are indicated.

lation showed absence of immune response to BVDV antigens. Northern blot and PCR analyses which could detect BVDV RNA from 200 and 30 infected cells, respectively, have also failed to detect residual BVDV genetic material in samples of 10^6 and 10^5 CRIB cells, respectively (not shown). These results taken together indicate that CRIB cells do not produce infectious virus or contain detectable levels of viral RNA or proteins.

CRIB cells are resistant to BVDV infection

BVDV inoculation of 10^5 CRIB cells with BVDV Singer-cp and VS115-ncp at m.o.i. ranging from 0.1 to 100 failed to initiate a BVDV infection of the monolayer (Figs. 1 and 2A, Tables 1 and 2). Progeny infectious BVDV was not detected in 10^7 CRIB cells inoculated with VS115-ncp at m.o.i. of 1 and subsequently cocultivated with susceptible bovine testicle cells for 5 days (data not shown). A Northern blot assay, capable of detecting BVDV RNA in total RNA extracted from 200 BVDV-infected MDBK cells, failed to detect BVDV RNA in cellular RNA extracted from 10^6 CRIB cells inoculated with BVDV Singer-cp at m.o.i. of 10 (data not shown). Western blot analysis failed to detect BVDV proteins in 5×10^5 CRIB cells after inoculation with BVDV Singer at m.o.i. of 10 (Fig. 2A). Under identical conditions, this assay detected 100 BVDV-infected cells (Fig. 2B).

However, inoculation of large numbers of CRIB cells with high input m.o.i. (>10) results in occasional infected cells. Examination of ~100 microscopic fields (at 400× magnification) of these cells stained by IFA reveals that the frequency of infection of CRIB cells nears 10^{-5}. The infection is productive (Table 2). These occasional infected cells may be a consequence of infection through aberrant pathways of virus entry or reversion of CRIB cells to the wild-type phenotype. Virus propagation to adjacent cells by cell-to-cell spread is inefficient, since no infected cell foci were found.

TABLE 1

Susceptibility of MDBK and CRIB Cells to Infection with Bovine Viral Diarrhea Virus (BVDV)[a]

Strain/isolate	Biotype	MDBK	CRIB	Isolate reference/origin
Singer	cp	+	−	McClurkin *et al.*, 1966, 1974
NADL	cp	+	−	Gutenkust and Malmquist, 1964
CP-1	cp	+	−	Meyers *et al.*, 1991
NCP-1	ncp	+	−	Meyers *et al.*, 1991
VS115	ncp	+	−	Donis, unpublished
2541	ncp	+	−	Bolin *et al.*, 1991
232176	ncp	+	−	Donis and Dubovi, 1987b
VS83	cp	+	−	Dubovi, E. J., unpublished
VS53a	cp	+	−	Dubovi, E. J., unpublished
C24v	cp	+	−	Gillespie *et al.*, 1960
VS186	cp	+	−	Dubovi, E. J., unpublished
VS60	cp	+	−	Osorio, F. A., unpublished
Burns	cp	+	−	Donis and Dubovi, 1987b
VS21	cp	+	−	Bolin *et al.*, 1991
VS15	ncp	+	−	Bolin *et al.*, 1991
VS84	cp	+	−	New York, unpublished
VS91	cp	+	−	New York, unpublished
VS46	cp	+	−	New York, unpublished
VS110	cp	+	−	Osorio, F. A., unpublished
VS182	ncp	+	−	New York, unpublished
VS183	ncp	+	−	New York, unpublished
VS184	ncp	+	−	New York, unpublished
VS191	ncp	+	−	Odeon *et al.*, 1994
15486	cp	+	−	Osorio, F. A., unpublished

[a] Cells were inoculated at a m.o.i. of 1. Infection status was assessed by IFA performed 24 hr postinfection for the cp and 36 hr p.i. for the ncp isolates.

To determine if there is a virus-strain dependence in the resistance of CRIB cells to infection, 24 different BVDV isolates were inoculated onto cells at m.o.i. of 1. When production of viral proteins was investigated by IFA, none of the cell cultures inoculated with each of 24 isolates had infected cells (Table 1).

The course of infection, cytopathology, and yield of

TABLE 2

Effect of Polyethylene-Glycol (PEG) in BVDV Infection of MDBK and CRIB-1 Cells[a]

	Yield ($Log_{10}TCID_{50}$/ml)		IFA-positive cells (%)	
Cell	Control	PEG	Control	PEG
MDBK	7.51	7.64	>95	>95
CRIB-1	2.11	5.74	<0.01	~1−3

[a] Cells were inoculated with BVDV Singer-cp at m.o.i. of 10 in the presence or absence of PEG. The percentage of BVDV-infected cells was estimated by counting 500 cells from cultures fixed for IFA 24 hr postinfection. Culture supernatants were harvested 24 hr p.i. and titer was determined.

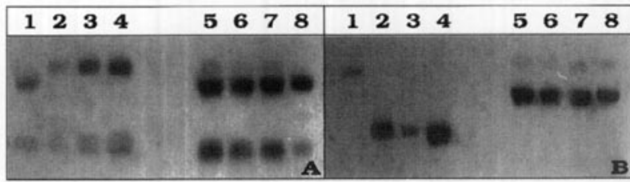

FIG. 3. (A) Glutamic oxaloacetate transaminase isoenzyme analysis. (B) Nucleoside phosphorylase isoenzyme analysis. Murine NCTC clone 929 (CCL1) (lane 1); human HeLa (CCL2) (lane 2); human breast medulla carcinoma (HTB24) (lane 3); and human colon adenocarcinoma (HTB38) (lane 4) were used as controls; MDBK (lane 5); CRIB-1 (lane 6); CRIB-2 (lane 7); and bovine endothelial (CRL8659) (lane 8). The stained zymograms were photographed using a video camera and Image software and printed without filtering.

infectious progeny of 10 bovine viruses, BHV-1, 2, and 4, BRSV, BAV, BEV, VSV, PI-3, BPV, and BLV, in CRIB cells did not differ significantly from those observed in MDBK cells.

These results taken together demonstrate that CRIB cells are refractory to BVDV infection since no RNA, protein, or progeny virus synthesis is detected upon virus inoculation.

CRIB are genuine bovine cells

CRIB cell growth, behavior, and microscopic appearance in culture is virtually identical to parental MDBK cells. Population doubling time of CRIB cells is slightly shorter than parental MDBK cells, near 30 hr. To ascertain the true derivation of the CRIB cells, we carried out karyotype and isoenzyme analyses. Analysis of 35 GW-banded metaphases of CRIB cells revealed a modal distribution of chromosomes of 50/51/52; with 5/5/5 metacentrics; 8/8/8 submetacentrics, and 37/38/39 acrocentrics or telocentrics in modal cell type. CRIB cells present a chromosome distribution very similar to that described for the constitutional chromosome complement of the domestic cow, *Bos taurus,* and almost identical to the parental MDBK cells (ATCC, CCL-22). Isoenzyme analysis showed that the gel mobility of a set of six enzymes of CRIB cells, including glutamate oxaloacetate transaminase and nucleoside phosphorylase, is identical to that of the parental MDBK and other bovine cells (Figs. 3A and 3B). Taken together, these results demonstrate that CRIB cells are genuine bovine cells and not a cell contaminant from another animal species.

CRIB cells are defective in BVDV entry

The relative ability of MDBK and CRIB cells to bind BVDV virions was assessed by quantifying cell-associated infectivity following virus adsorption. Attachment of BVD virions to cells at 4° is ~100 times less efficient than at 37° (P. Willson and R. Donis, unpublished). Virus binding assays were carried out at 37° on cell monolayers treated with an endocytosis inhibitor, phenylarsine oxide. Adsorption of BVD virions to the surface of CRIB cells was reduced approximately eightfold compared to the parental MDBK cells (Fig. 4). Reduced virus attachment may contribute to resistance and hints toward candidate areas for future research.

To ascertain the degree of permissiveness of the intracellular milieu of CRIB cells for BVDV replication, we assessed it after bypassing or enhancing the virus entry step by: (1) transfection of BVDV RNA and (2) addition of PEG to the virus inoculum during adsorption. MDBK and CRIB cells were transfected by electroporation with BVDV genomic RNA. Transfection efficiency was low in both cell lines and the percentages of BVDV IFA-positive cells at 16 hr p.i. were identical. Quantification of infectious virus in culture fluid from transfected monolayers indicated that MDBK cells produced $10^{3.31}$ $TCID_{50}$/ml and CRIB cells $10^{3.51}$ $TCID_{50}$/ml. Virus yield per infected cell in a single cycle of growth was estimated to be near 100 $TCID_{50}$ for both cell lines.

Addition of 5% PEG to the BVDV inoculum during adsorption, followed by treatment of cells with 37.5% PEG leads to infection of 1 to 3% of CRIB cells (Fig. 5, Table 2). This represents a 1000-fold increase in the frequency of infected CRIB cells compared to cells inoculated with virus in the absence of PEG (Table 2). In contrast, similar PEG treatment of vesicular stomatitis virus or respiratory syncytial virus infecting CRIB cells enhanced infectivity 10- and 5-fold, respectively (not shown).

Since BVDV infection after bypassing entry by transfection or enhancing it by PEG treatment is equally efficient and produces similar amounts of progeny virus on a per cell basis, we conclude that the intracellular permissiveness of CRIB cells is identical to parental cells. It follows that resistance to viral infection is associated with a block in viral entry.

DISCUSSION

Cultured bovine cells, either primary or continuous cell lines, are invariably very susceptible to BVDV infection *in vitro* (Lee and Gillespie, 1957; Fernelius *et al.*, 1969;

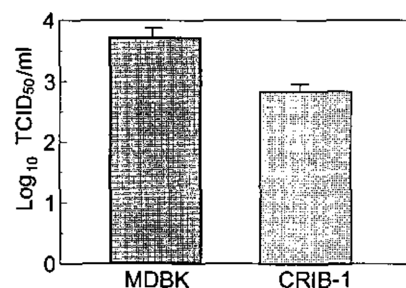

FIG. 4. Cell-associated infectivity after BVDV adsorption to MDBK and CRIB cell monolayers. Virions (Singer-cp, m.o.i. of 5) were allowed to attach for 1 hr at 37° to cells previously treated with the endocytosis inhibitor phenylarsine oxide. Cell-bound virus was quantitated and expressed as $TCID_{50}$/ml.

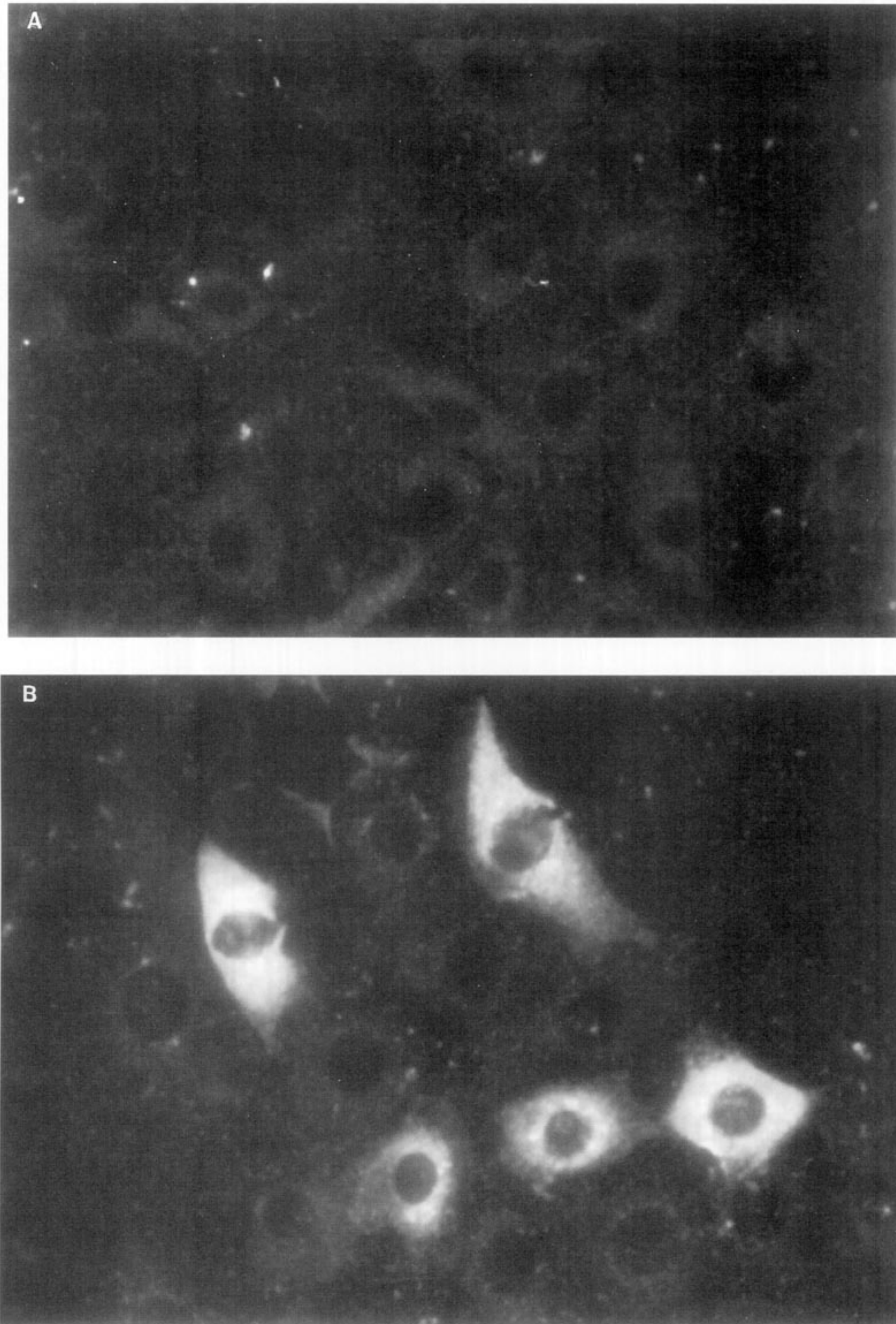

FIG. 5. BVDV infection of CRIB cells by virus inoculation in the presence of polyethylene–glycol (PEG). CRIB cells were inoculated with BVDV Singer-cp at m.o.i. of 10 in the absence (A) or in the presence of PEG (B), as described under Materials and Methods. Viral proteins were detected by IFA in cells fixed 24 hr p.i. as described in Fig. 1 (magnification = 320×).

Horzinek, 1981; Nettleton and Entrican, 1992; Bolin *et al.,* 1994). In contrast, a remarkable tropism for certain cell types is seen *in vivo* (Bielefeldt, 1988a,b; Bolin and Ridpath, 1990; Brownlie, 1990; Wilhelmsen *et al.,* 1990; Haines *et al.,* 1992; Marshall, 1993). The reasons for the differences between host cell ranges *in vivo* and *in vitro*

are not well understood but are likely derived from differential expression of host cell molecules controlling virus replication and spread. As the first step toward the identification of cellular molecules mediating BVD virus infection, we have isolated and characterized a mutant bovine cell line (CRIB) that lacks a function required for BVDV entry.

The genetic events that led to the development of the resistant phenotype remain to be determined. Possible scenarios include the presence of rare BVDV-resistant cells among the MDBK population selected by the initial infection and subsequent crises of the persistently infected culture. Alternatively, mutation took place after high multiplicity infection and was followed by a multiple-step selection process. Evolution of cells through the course of persistent infection resulting in reduced permissiveness to viral replication has been reported in other viral systems (Ahmed *et al.,* 1981; Ron and Tal, 1986; Kaplan *et al.,* 1989; Kaplan and Racaniello, 1991; De la Torre *et al.,* 1988).

Since CRIB cells originated from cells that survived lytic BVD virus infection, interference by temperature-sensitive mutants or residual replicative forms of the viral RNA would be an immediately attractive explanation for the resistance to infection with homologous virus. Homologous interference between noncytopathic (ncp) and cytopathic (cp) BVDV strains results in delayed replication, reduced progeny yield, and dampened cytopathology of the latter (Gillespie *et al.,* 1962; Shirai *et al.,* 1984). Several lines of evidence argue against this hypothesis. No BVDV nucleic acids or proteins were detected in CRIB cells at standard or reduced temperatures. CRIB cells display a degree of resistance to viral infection that greatly exceeds the levels of interference observed between BVDV strains. Evidence discussed above indicating that the intracellular milieu of CRIB cells is permissive to BVDV replication does not support a homologous interference mechanism as the basis for the resistance. The latter usually involves impairment of RNA replication (Polacino *et al.,* 1985).

PEG treatment enhances BVDV infectivity by 1000-fold compared to cells inoculated with virus in the absence of PEG. In contrast, PEG treatment of CRIB cells enhances infectivity of other viruses by less than 10-fold (not shown). The exact mechanism by which PEG is mediating BVDV infection of CRIB cells remains to be determined. In several viral systems, polyethylene–glycol treatment has been shown to enhance viral infection of tissue culture cells by enhancement of virus binding (Hoekstra *et al.,* 1989; Gripon *et al.,* 1993; Sarmanti *et al.,* 1994) and by inducing virion-cell membrane fusion (Rohde *et al.,* 1978; Sarmiento *et al.,* 1979; Hoekstra *et al.,* 1989; Fehler *et al.,* 1992; Asanaka and Lai, 1993; Herrmann *et al.,* 1993). PEG-mediated BVDV infection of CRIB cells is dramatically reduced by inhibitors of endocytosis (phenylarsine oxide) and by agents that prevent endosomal acidification (chloroquine, ammonium chloride) (results not shown). Thus, enhancement of BVDV infection of CRIB cells by PEG depends on normal endosome formation and acidification. These observations suggest that PEG enhances BVDV infection by favoring the physiological entry pathway and argue against a fusogenic activity at the plasma membrane.

Ten different bovine viruses did not discriminate between CRIB and MDBK cells for growth, indicating that the virus-resistant phenotype of CRIB cells appears to be BVDV-specific and not a consequence of expression of a nonspecific anti-viral activity or a broad defect in a metabolic pathway required for viral replication.

Previous observations have suggested that different BVDV strains may use alternative cell receptors to initiate infection in bovine cells (Moennig *et al.,* 1988; Xue and Minocha, 1993). CRIB cells were selected by infection with a single virus isolate (Singer) and display resistance to all BVDV isolates tested to date. These findings suggest that a common cell surface molecule is required for all these strains to initiate infection in MDBK cells. The identity of this molecule and its involvement in virus attachment or subsequent steps in internalization are currently under investigation.

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