Available online at www.sciencedirect.com
ScienceDirect
Veterinary Immunology and Immunopathology 117 (2007) 106–115
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Replication of infectious bursal disease virus in macrophages and altered tropism of progeny virus

Mahesh Khatri, Jagdev M. Sharma *

Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, USA

Received 14 November 2006; received in revised form 17 January 2007; accepted 7 February 2007

Abstract

We serially passaged classical infectious bursal disease virus (cIBDV) and antigenic variant IBDV (vIBDV) in an avian macrophage cell line, NCSU cells, referred as mcIBDV and mvIBDV respectively and examined the *in vitro* and *in vivo* characteristics of the macrophage-adapted viruses. NCSU adapted viruses caused earlier destruction of NCSU cells than the unadapted viruses. Nitric oxide (NO) was detected earlier in cultures infected with mcIBDV and mvIBDV than in cultures infected with cIBDV and vIBDV. cIBDV and vIBDV were able to infect DF-1 cells, a chicken embryo fibroblast cell line, only after one replication cycle in NCSU cells. The genetic basis of altered tropism of progeny virus from NCSU cells infected cultures was not identified. No aa substitutions were observed in hypervariable region of VP2 of cIBDV and vIBDV passaged 1 time in NCSU cells whereas both mcIBDV and mvIBDV had multiple aa substitutions. To assess protective efficacy of mcIBDV and mvIBDV, embryonated chicken eggs were inoculated with mcIBDV and mvIBDV at embryonation day 18 (ED 18) and challenged with a virulent cIBDV at 3 weeks of age. mcIBDV and mvIBDV were immunogenic and generated antibody responses and provided 100% protection against cIBDV.

Keywords: IBDV; Macrophages; Altered tropism

1. Introduction

Infectious bursal disease is an acute, highly contagious and immunosuppressive disease of chickens (Sharma et al., 2000). The causative agent, IBDV, belongs to the family *Birnaviridae*. The genome of IBDV consists of two segments of double-stranded RNA (dsRNA) (Dobos et al., 1979). The larger segment (the A segment, 3260 bp) contains two partly overlapping open reading frames (ORFs). The first, smaller ORF encodes the nonstructural viral protein 5 (VP5) (145 to 149 amino acids, 17 kDa). The second ORF encodes a polyprotein (1.012 amino acids, 110 kDa) that is autocatalytically cleaved to yield the viral proteins pVP2 (also known as VPX) (48 kDa), VP4 (29 kDa), and VP3 (33 kDa). During *in vivo* virus maturation, pVP2 is processed into VP2 (41 to 38 kDa), probably resulting from site-specific cleavage of pVP2 by a host cell-encoded protease (Kibenge et al., 1997). The smaller B segment (2827 bp) contains one large ORF, encoding VP1 (877 to 881 amino acids, 91 kDa) (Dobos, 1993).

Two serotypes of IBDV (serotypes 1 and 2) have been described. The serotype 1 IBDV isolates infect developing B-lymphoid cells in the bursa of Fabricius. Serotype 1 isolates are further divided into classical and antigenic variant isolates.

* Corresponding author at: 258 Veterinary Science Building, 1971 Commonwealth Ave., St. Paul, MN 55108, United States.
Tel.: +1 612 625 5276; fax: +1 612 625 5203.
E-mail address: sharm001@umn.edu (J.M. Sharma).

0165-2427/$ – see front matter
doi:10.1016/j.vetimm.2007.02.002

Pathogenic, bursa-derived IBDV is difficult to adapt to cell cultures. Adaptation has been possible only after extensive serial blind passages in cell cultures (Hassan et al., 1996) or the chorioallantoic membrane as well as in the yolk sac of embryonated chicken eggs (Yamaguchi et al., 1996a). A vIBDV isolate, E Del-IBDV, replicated in BMG-70 cells but failed to replicate in chicken embryo fibroblast (CEF) cells (Tsai and Saif, 1992).

Infection studies in chickens have revealed that replication of IBDV in the bursa is accompanied by an influx of T cells (Kim et al., 1999; Kim et al., 2000; Sharma et al., 2000; Tanimura and Sharma, 1997). Although T cells do not serve as targets for infection and replication of IBDV (Hirai and Calnek, 1979) cell-mediated immune responses of infected birds are compromised (Confer et al., 1981; Kim et al., 1998; Panigrahy et al., 1982). There are reports that macrophages and monocytes may be susceptible to infection with the virus (Burkhardt and Muller, 1987; Inoue et al., 1992; Kaufer and Weiss, 1976; Kaufer and Weiss, 1980; Khatri et al., 2005; Komine et al., 1989; Muller, 1986). Macrophages have been proposed to serve as virus carriers from the site of infection in the gut to the bursa and other peripheral tissues (Kaufer and Weiss, 1976; Kim et al., 1998; Lam, 1998; Sharma and Lee, 1983; van den Berg et al., 2000). We have shown previously that macrophages recovered from IBDV-exposed chickens had upregulated gene expression for several cytokines and produced elevated levels of nitric oxide (Khatri et al., 2005; Kim et al., 1998). Further, we have previously reported activation of the stress-activated protein kinase, p38 and NF-kB in chicken spleen macrophages and NCSU cells infected with IBDV (Khatri and Sharma, 2006). This apparent susceptibility of chicken macrophages to *in vitro* infection with IBDV contrasts with the observations of others (Abdel-Alim and Saif, 2001).

In the present study, we serially passaged cIBDV and vIBDV in NCSU cells and compared the molecular, *in vitro* and *in vivo* characteristics of macrophage-adapted and unadapted viruses.

2. Materials and methods

2.1. Cells and viruses

NCSU, a chicken macrophage cell line (Qureshi et al., 1990) was obtained from the American Type Culture Collection (Rockville, Maryland, USA). The NCSU cells were maintained in L15 and McCoy 5A (1:1) medium (GibcoBRL, Gaitherburg, Maryland, USA) supplemented with 10% fetal bovine serum (FBS), 10% chicken serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin maintained at 41 °C and 5% CO_2. The classical strains IM (Winterfield et al., 1972) and the variant strain-E (Jackwood and Saif, 1987) were used for adaptation to NCSU cells. Macrophage adapted classical and variant isolates of IBDV were designated as mcIBDV and mvIBDV respectively. Virus stocks were made from the thirteenth serial passage of mvIBDV and the twelfth serial passage of mcIBDV. The virus stocks were examined by PCR for chicken anaemia virus (Sommer and Cardona, 2003) and reticuloendotheliosis virus (Rath et al., 2003) and were found to be negative. The NCSU cells were infected with virulent and NCSU cell adapted viruses at a multiplicity of infection (m.o.i.) of 1. The partial VP2 mRNA sequences of mcIBDV and mvIBDV have been deposited in the Gene Bank (Accession # AY819702 and AY819703 respectively). DF-1 cells (a chicken fibroblast cell line obtained from Dr. D. Foster, University of Minnesota) (Himly et al., 1998) were grown in Dulbecco minimal essential medium (DMEM) supplemented with 10% FBS, and were used for virus titration. Cell viability was determined by trypan-blue exclusion.

2.2. Immunofluorescence assay (IFA)

The expression of IBDV proteins was demonstrated in NCSU and DF-1 cells infected with cIBDV, vIBDV, mcIBDV and mvIBDV by immunofluorescence assay. Briefly, after 72 h of infection, the cells were washed with phosphate buffered saline (PBS) and fixed with acetone for 20 min at −20 °C. After washing with PBS, the cells were incubated with 1:2000 dilution of a rabbit anti-IBDV antiserum for 1 h (rabbit anti-IBDV serum was a gift from Dr. K. Tsukamoto, National Institute of Animal Health, Japan (Tsukamoto et al., 1995)). The cells were rinsed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 45 min. The cells were washed again with PBS and visualized using a Nikon Eclipse TE (Nikon, Tokyo, Japan) fluorescent microscope.

2.3. Detection of apoptosis

Cells were stained with acridine orange for determinations of nuclear morphology and with ethidium bromide to distinguish cell viability, at a final concentration of 1 μg/ml for each, as described (DeBiasi et al., 2001).

2.4. Measurement of NO production

NO production was determined by measurement of nitrite in the medium as described previously (Pertile et al., 1995). Briefly, an aliquot of the spent medium was mixed with an equal volume of 1:1 mixture of 1% sulfanilamide in water and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid. The absorbance was then read at 570 nm. Sodium nitrite dissolved in the culture medium was used to make the standard curve. NO concentration was measured in micromolars (μM).

2.5. Sequence analysis

The nucleic acid sequences of the viral genomes were determined by direct sequencing of the RT-PCR products. Viral RNA was extracted using Trizol (Invitrogen) and partial VP2 sequences were reverse transcribed, amplified by PCR using a one step RT-PCR (Qiagen, Carlsbad, CA, USA). The PCR products were sent for automated sequencing at the University of Minnesota Advanced Genetic Analysis Center.

2.6. Experiment design

To obtain mcIBDV and mvIBDV, cIBDV and vIBDV respectively were serially passaged in NCSU cells. NCSU cells were infected with these viruses and 72 h after infection, cells and supernatant were freeze thawed three times. Cellular debris was removed by centrifugation and supernatant was used to infect fresh NCSU cells for the next passage. The cIBDV and vIBDV were serially passaged 12 and 13 times respectively in NCSU cells.

In vivo characteristics of mcIBDV and mvIBDV were examined by conducting 3 experiments in specific-pathogen-free (SPF) chicken eggs purchased from HyVac Laboratories (Ames, IA). Groups of eggs at ED 18 were inoculated with 0.1 ml of virus suspension containing 2×10^3 tissue culture infectious $dose_{50}$ ($TCID_{50}$) of mcIBDV and mvIBDV and 2×10^3 egg infectious $dose_{50}$ (EID_{50}) of cIBDV and vIBDV as described earlier (Sharma and Witter, 1983). The hatched chickens were housed in separate isolation units. Handling of animals, including feeding and euthanasia, were in accordance with the University of Minnesota animal care guidelines. At 6 days post hatch (dph) bursal sections were subjected to histological examination. At 21 days of age, sera were tested for anti-IBDV antibody by enzyme linked immuno-sorbent assay (ELISA) (IDEXX, Portland, ME). The virus-exposed and unexposed chickens were examined for resistance to virulent cIBDV in a challenge test. In the challenge test, mcIBDV and mvIBDV inoculated chickens were challenged with the 10^4 EID_{50} of virulent cIBDV by the intraocular route. Five days post-challenge, chickens were euthanized by CO_2 inhalation. Bursae were examined for gross lesions. Bursal sections were fixed in 10% neutral buffered formalin for histological examination.

2.7. Statistical analysis

Results are expressed as mean ± S.D. Statistical comparisons were made by Student's *t*-test. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Replication of IBDV in NCSU and DF-1 cells

NCSU cells were infected with cIBDV and vIBDV. At intervals, cell free and cell associated virus was titrated in DF-1 cells (Table 1). As expected, both cIBDV and vIBDV failed to replicate in DF-1 cells. Virus-exposed cells did not develop detectable cytopathic effect nor was viral antigen detected by IFA (Fig. 1a). However, IBDV recovered from NCSU cells after 1 or 12–13 passages readily infected DF-1 cells and propagated to appreciable titers (Fig. 1a and Table 1). Titers of cell-associated and cell-free viruses increased between 24 and 72 h following infection (Table 1). Approximately similar titers were detected for viruses after 1 or 12–13 serial passages in NCSU cells.

Viral proteins in DF-1 and NCSU cells were detected by IFA (Fig. 1a and 1b).

3.2. IBDV-induced cell lysis

To assess the relationship between virus replication and cell death, we examined cell viability in virus exposed and unexposed NCSU cells at 24, 48 and 72 h after virus exposure. Despite a similar pattern of virus replication among the viruses tested, at 24 h, there was a clear distinction between cell viability of the cultures infected with cIBDV and vIBDV versus those infected with mcIBDV and mvIBDV. In cultures infected with mcIBDV and mvIBDV, approximately 80% of the cells were destroyed compared to less than 30% destruction in cultures infected with cIBDV and vIBDV ($P < 0.05$) (Table 2). At 72 h after virus exposure, unadapted viruses caused more destruction of NCSU cells. This

Table 1
Susceptibility of DF-1 cells to cIBDV and vIBDV following replication in NCSU cells as determined by virus titration*

Virus	No. of serial Passages in NCSU cells	Titer of IBDV in DF-1 cells following replication in NCSU cells Time (h) post infection of NCSU cells†					
		24		48		72	
		CA	CF	CA	CF	CA	CF
cIBDV	1	5.40± 0.14‡	5.88± 1.2	6.66± 0.14	7.37± 0.18	6.58± 1.04	6.12± 0.53
	12	6.41± 0.15	7.32± 0.15	6.32± 0.13	6.87± 0.52	7.41± 0.29	7.5± 0.0
vIBDV	1	5.9± 0.28	6.83± 0.57	6.57± 0.15	7.37± 0.18	7.0± 0.4	7.37± 0.18
	13	6.16± 0.57	7.16± 0.38	6.07± 0.28	6.49± 0.01	6.5± 0.0	6.74± 0.7

*Virus titer in DF-1 cells (log $_{10}$ $TCID_{50}$/ml).
†NCSU cells (5×10^5) were infected with IBDV at a m.o.i. of 1. At indicated times, cell cultures were examined for cell associated and cell free virus by titration in DF-1 cells. Data presented are averaged from 2–3 different experiments. CA: cell associated virus; CF: cell free virus.
‡$P < 0.05$.

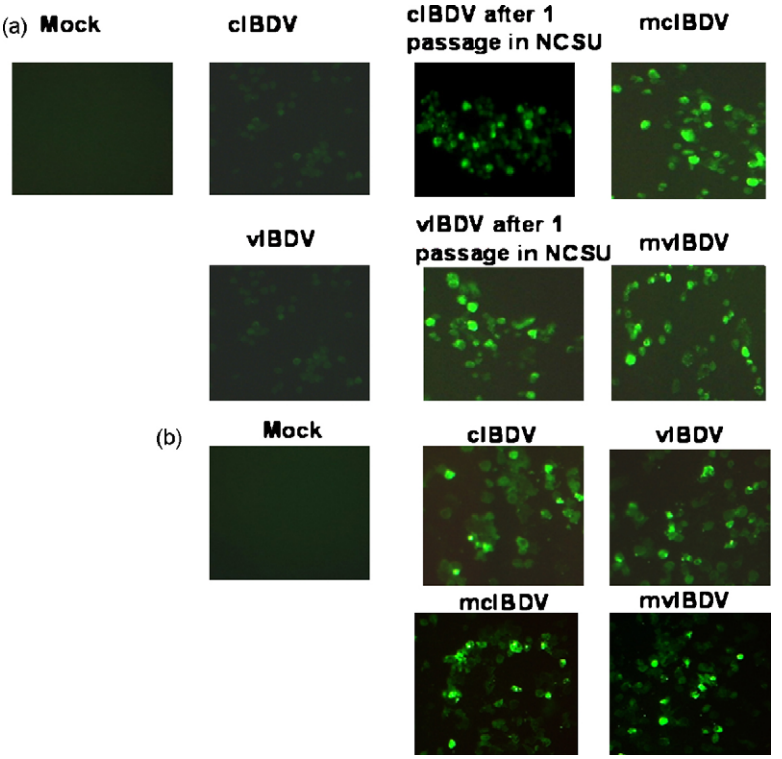

Fig. 1. Immunofluorescence detection of IBDV antigen in DF-1 cells (a) and NCSU cells (b). (a) DF-1 cells were infected with cIBDV, vIBDV, cIBDV after one passage in NCSU, vIBDV after one passage in NCSU, mcIBDV (12 serial passages in NCSU), and mvIBDV (13 serial passages in NCSU), at a m.o.i. of 1. (b) NCSU cells were infected with cIBDV, vIBDV, mcIBDV, and mvIBDV at a m.o.i. of 1. At 72 h postinfection, cells were stained with rabbit anti-IBDV serum, followed by detection with goat anti-rabbit FITC conjugate.

Table 2
Cell viability and apoptosis in NCSU cells after IBDV infection

	Cell viability (% control)			% Apoptotic cells	
	Time (h) post infection				
Virus	24	48	72	48	72
cIBDV	71 ± 6 *	41.1 ± 22	19.6 ± 18	25 ± 7	47 ± 8 *
mcIBDV	20.5 ± 9	20.1 ± 8	57 ± 9	20±4	24 ± 6
vIBDV	84 ± 24 *	36.3 ± 14	19.1 ± 6	34 ± 6	54 ±11 *
mvIBDV	20.4 ± 10	21 ± 5	53 ± 18	22 ± 8	29 ± 9

*$P < 0.05$.
NCSU cells (5×10^5) were infected with different IBDV at a m.o.i. of 1. At indicated times, cells were harvested for determination of cell viability by the tryban blue exclusion method and were subjected to acridine orange staining for apoptosis.
Data presented are calculated from 2–3 different experiments.

result indicated that macrophage-adapted viruses caused earlier destruction of NCSU cells whereas unadapted viruses caused more destruction of NCSU cells in later part of infection although both types of viruses replicated at about an equal rate.

3.3. Induction of apoptosis

NCSU cells were infected with cIBDV, vIBDV, mcIBDV and mvIBDV. At hours post infection apoptosis induced by viruses was determined by acridine orange-ethidium bromide staining. In apoptotic cell, nuclei were highly condensed or fragmented. At 72 h after infection, apoptosis induced by cIBDV and vIBDV was higher than mcIBDV and mvIBDV ($P < 0.05$) (Table 2). The higher apoptotic rate of cIBDV and vIBDV at 72 h correlated with the higher cell death rate induced by these viruses. The fact that IBDV-induced NCSU cell death was mediated by programmed cell death suggested that apoptosis plays an important part in the pathogenesis of IBDV (Tanimura and Sharma, 1998).

3.4. NO production

To examine the NO inducing ability of cIBDV, vIBDV, mcIBDV and mvIBDV, we infected NCSU cells with these viruses. At intervals following infection, spent media were quantified for NO. As shown in Fig. 2, in NCSU cultures infected with mcIBDV and mvIBDV, NO was detected earlier than in cultures infected with cIBDV and vIBDV. However, at 48 and 72 h after infection, higher levels of NO were detected in cultures infected with vIBDV than in those infected with mvIBDV ($P < 0.05$).

3.5. Sequence analysis of mcIBDV and mvIBDV

Analysis of the hypervariable region of VP2 protein was performed to identify possible aa substitutions associated with replication of the viruses in NCSU cells. No aa substitutions were observed in cIBDV and vIBDV passaged 1 time in NCSU cells. Sequence analysis of mcIBDV revealed multiple aa substitution including at aa 279 and 284 (Table 3). Previous data have shown that tissue culture adaptation in CEF was associated with substitution at aa 279 and 284 (Lim et al., 1999; van Loon et al., 2002). Similarly sequence analysis of

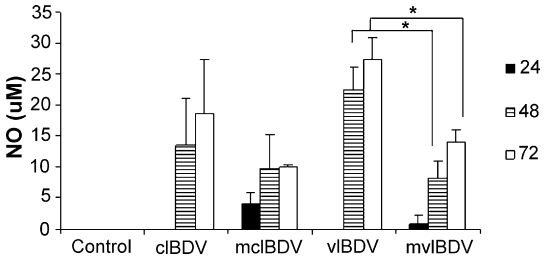

Fig. 2. Nitric oxide production by IBDV in NCSU cells. NCSU cells (5×10^5) were infected with different IBDV at a m.o.i. of 1 for 60 min at 37 °C. After washing out extracellular virus, cells were cultured in 24 well plates. At indicated times, the supernatant was harvested and examined for NO production by Griess assay. *$P < 0.05$.

Table 3
Sequence differences of the VP2 hypervariable region between the cIBDV, vIBDV and attenuated viruses mcIBDV and mvIBDV

Position	cIBDV	mcIBDV	Position	vIBDV	mvIBDV
213	D	N	247	V	M
222	P	T	253	Q	H
242	I	V	256	V	E
247	V	M	278	A	V
249	Q	K	284	A	T
253	Q	H	316	K	R
254	G	S	318	D	G
256	V	E	323	E	D
278	A	V			
279	D	N			
284	A	T			
286	T	I			

mvIBDV also showed multiple aa substitutions including at aa 253 and 284 (Table 3). Substitutions at aa 253 and 284 or 284 alone have previously been shown to be associated with tissue culture adaptation of variant viruses (Mundt, 1999). The significance of additional aa changes observed in NCSU adapted viruses and their contribution in tissue culture adaptation and attenuation will be the subject of future research.

3.6. *In vivo responses to mcIBDV and mvIBDV*

SPF chicken eggs were inoculated with mcIBDV and mvIBDV on ED18 and hatched chickens were examined for lesions, antibodies against IBDV and protection against challenge with virulent cIBDV.

3.6.1. *Effect on hatchability and lesions induction*

In ovo inoculation of mcIBDV and mvIBDV did not affect hatchability of eggs ($P > 0.05$) (Table 4). We examined the bursal lesions induced by cIBDV, vIBDV, mcIBDV and mvIBDV. At 6 dph, both cIBDV and vIBDV induced extensive bursal follicular damage as compared to minimal damage induced by mcIBDV and mvIBDV ($P < 0.05$) (Table 4 and Fig. 3a). cIBDV and vIBDV passaged 1 time in NCSU cells induced bursal lesions similar to the lesions induced by unpassaged virulent viruses (data not shown). These results indicate that *in ovo* inoculation of mcIBDV and mvIBDV did not affect the hatachability of eggs and induced minimum bursal damage.

3.6.2. *Antibody production and protection against challenge with virulent IBDV*

mcIBDV and mvIBDV were highly immunogenic and induced anti-IBDV antibody following *in ovo* administration. At 21 days of age, similar levels of antibodies were detected with both viruses ($P > 0.05$) (Table 4).

Challenge data are shown in Table 5. Chickens immunized with mcIBDV or mvIBDV were protected against challenge with cIBDV. Protection was assessed by absence of mortality and gross and microscopic bursal lesions. The challenge virus was highly pathogenic and induced mortality and gross and microscopic bursal lesions in unimmunized chickens (Fig. 3b). Theses results indicated that macrophage-adaptation of cIBDV and vIBDV resulted in attenuation

Table 4
Response of chickens to *in ovo* exposure to mcIBDV and mvIBDV.

Treatment Group	Hatchability	Microscopic bursal lesions at 6 dph		ELISA antibody titer	
None	93 ± 2.3	0		4 ± 2	
cIBDV	85 ± 0	4 ± 0	*	19823 ± 524	*
mcIBDV	93 ± 6.2	0.75 ± 0.5		10455 ± 575	
vIBDV	60 ± 15	2.66 ± 1.15	*	8401 ±3407	
mvIBDV	87 ± 7.5	0.5 ± 0.57		11013±1503	

dph: days post hatch Groups of embryonated eggs were inoculated at ED18 with cIBDV, vIBDV, mcIBDV and mvIBDV. Percent hatchability, microscopic bursal lesions at 6 dph and ELISA antibody titers at 21 days of age were recorded. Hatchability data are mean of 3 experiments with 16–20 chickens in each group in each experiment. Microscopic bursal lesions values are mean of 2 experiments with 4–5 chickens in each group in each experiment. ELISA antibody titer values are mean of 3 experiments with 6–8 chickens in each group in each experiment.
*$P < 0.05$.

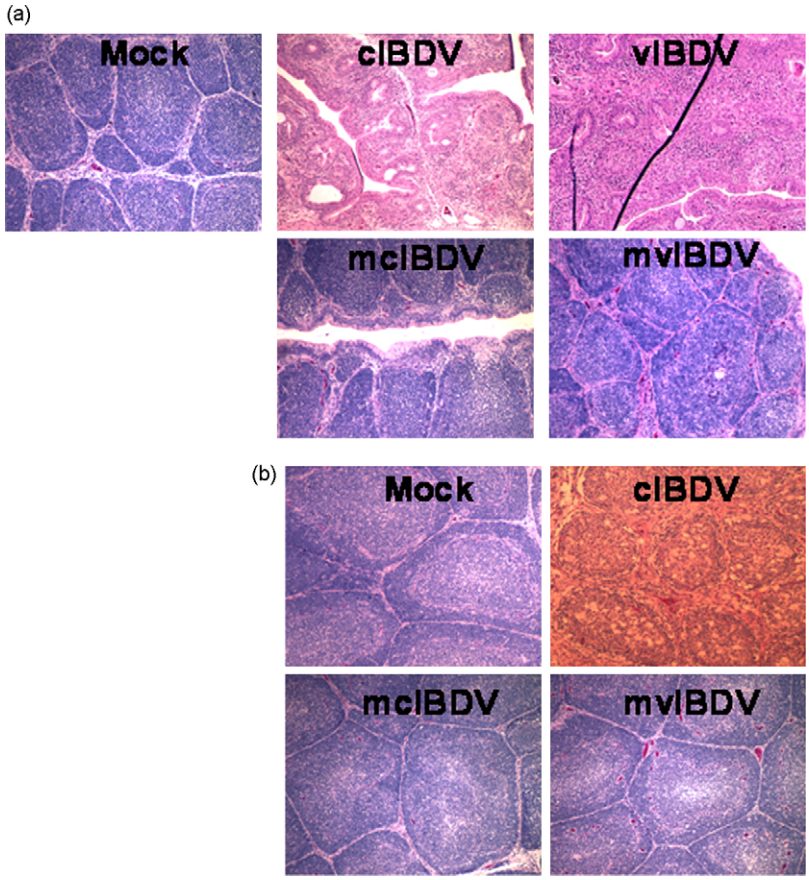

Fig. 3. Induction of bursal lesions (a) and protective ability of mcIBDV and mvIBDV (b). (a) Embryonated eggs were inoculated with cIBDV, vIBDV, mcIBDV and mvIBDV at ED 18. At 6 dph, bursal sections from chickens uninoculated or inoculated with cIBDV, vIBDV, mcIBDV and mvIBDV were examined. Haematoxylin and Eosin staining revealed extensive damage of bursal follicles in cIBDV and vIBDV exposed chickens whereas bursal follicles in mcIBDV and mvIBDV exposed chickens showed little damage. (b) Embryonated eggs were inoculated with mcIBDV and mvIBDV at ED 18 and challenged with virulent cIBDV at 21 dph. Five days post challenge, bursal sections were examined for histopathological lesions. Bursal section from unvaccinated chicken challenged with virulent cIBDV showed extensive follicular destruction. No lesions were detected in bursa sections from chickens exposed to mcIBDV and mvIBDV and challenged with virulent cIBDV.

Table 5
In ovo exposure to mcIBDV and mvIBDV induced protection against challenge with virulent cIBDV

Treatment at ED18	% Response to challenge with cIBDV at 5 days after challenge			
	Mortality	Bursal lesions		Protection
		Gross	Microscopic	
None	32 ± 10	100	100	0
mcIBDV	0*	0*	0*	100*
mvIBDV	0†	0†	0†	100†

(*) and (†) values significantly different from untreated group ($P < 0.05$).
Values are means of 3 different experiments with 6–8 chickens in each group in each experiment.

of these viruses but attenuated viruses retained immunogenicity.

4. Discussion

The inability of cIBDV to infect CEF cells is well documented (Tsai and Saif, 1992). Adaptation of this virus to CEF often requires extensive serial blind passages in a variety of tissues and cells (Hassan et al., 1996; Yamaguchi et al., 1996a). We show here that cIBDV replicated extensively in CEF after one passage in an avian macrophage cell line. We serially passaged cIBDV and vIBDV in NCSU cells and the resultant viruses infected and replicated in CEF cells. DF-1 cells exposed to cIBDV and vIBDV showed no evidence of cytopathology or the presence of viral antigens detectable by IFA. Infection of macrophages by IBDV

is not unexpected as these cells are known to be permissive to wide range of viruses, including other double stranded viruses such as reoviruses (O'Hara et al., 2001). Previously we and others have shown that macrophages are susceptible to infection with IBDV (Burkhardt and Muller, 1987; Inoue et al., 1992; Kaufer and Weiss, 1976; Kaufer and Weiss, 1980; Khatri et al., 2005; Komine et al., 1989; Lam, 1998; Muller, 1986). In addition, we have shown that IBDV enhanced gene expression for cytokines and elevated levels of nitric oxide in macrophages (Khatri et al., 2005; Kim et al., 1998). To understand the cellular signaling pathways involved in IBDV induced cytokine and NO production, we examined the activation of p38 MAPK and NF-kB in chicken macrophages. Our observations indicated that p38MAPK and NF-kB regulate the cytokine and NO production by IBDV (Khatri and Sharma, 2006).

The mechanisms responsible for infectivity of CEF by IBDV after a single passage in NCSU cells are currently not known. Similar to our observation, herpes simplex virus HSV1716 grown once in BHK cells started to grow in non-permissive CHO cells. In that study authors concluded that mechanism responsible for this switch is most likely to be through the acquisition (or loss) of factor(s) during replication in BHK cells (Conner et al., 2005). During the replication cycle of virulent IBDV in MQ-NCSU cells, the virus might have attained some additional receptor binding sites or has attained higher binding affinity for the receptors present on CEF. In addition, virus might have acquired aa substitutions in proteins other than VP2 protein of virus which might play a role in receptor binding and or post-attachment viral entry processes.

The replication of cIBDV and vIBDV in non-permissive CEF cells following one passage in NCSU has practical implications. IBDV is a wide-spread naturally occurring, immunosuppressive virus of chickens. Anti-IBDV vaccines are extensively used to protect commercial flocks against the immunosuppressive effects of the virus. Because of the difficulty of replicating the virus in avian cell cultures, most commonly used live vaccines are produced by propagating IBDV in chickens or chicken eggs. Our observation that both classical and variant virulent, highly antigenic, viruses could be replicated in CEF should facilitate the production of live IBDV vaccines of avian cell culture origin.

Although the replication pattern of cIBDV, vIBDV, mcIBDV and mvIBDV was similar in NCSU cells, there were some differences between virulent and macrophage-adapted viruses. mcIBDV and mvIBDV caused earlier apoptosis and cellular lysis of NCSU cells than cIBDV and vIBDV. In addition, NO was detected earlier in NCSU cells infected with mcIBDV and mvIBDV than with cIBDV and vIBDV. However, as the infection progressed, cIBDV and vIBDV were better NO inducers than mcIBDV and mvIBDV.

Previous studies have shown that mutations at aa279 and aa284 in VP2 of very virulent IBDV resulted in adaptation to CEF and aa253, 279 and 284 were involved in the virulence of IBDV (Bayliss et al., 1990; Brandt et al., 2001; Lim et al., 1999; van Loon et al., 2002; Yamaguchi et al., 1996b). Similarly, substitutions at aa253 and aa284 or aa284 alone of the VP2 protein of the variant virus were necessary for tissue culture infectivity (Mundt, 1999). Adaptation of IBDV in tissue culture by serial passaging resulted in reduced virulence *in vivo* (Cursiefen et al., 1979; Hassan et al., 1996; Lange et al., 1987; Yamaguchi et al., 1996a). In the present study, no aa substitutions were observed in the hypervariable region of VP2 protein of cIBDV and vIBDV passaged 1 time in NCSU cells whereas mcIBDV and mvIBDV revealed aa substitutions at several positions including aa279 and 284 for cIBDV and at aa253 and 284 for vIBDV (Table 3). As observed by others, these mutations may be responsible for tissue culture adaptability and attenuation of virulent cIBDV and vIBDV. Additional studies are needed to identify the genetic mutations in the whole genome of cIBDV and vIBDV passaged 1 time in NCSU cells which might be responsible for infectivity of CEF.

In vivo studies revealed that both mcIBDV and mvIBDV did not cause clinical disease or extensive bursal damage in SPF chickens. Following *in ovo* inoculation, the lesion scores were 0.75 and 0.33 in mcIBDV and mvIBDV group respectively compared to the scores of 4.0 and 2.66 for cIBDV and vIBDV respectively. This result indicated that adaptation of cIBDV and vIBDV to macrophages resulted in attenuation of the virus. Attenuation was further confirmed by reduced mortality and morbidity in *in ovo* inoculated chickens. The adapted viruses were immunogenic and induced high levels of antibodies and protected chickens against challenge with virulent serotype 1 IBDV. It is possible that mcIBDV and mvIBDV because of their tropism for macrophages as well as B cells may induce an immune response that cross-protects against classical and variant strains of IBDV. Our preliminary results support this hypothesis (data not shown).

In summary, virulent cIBDV and vIBDV replicated in NCSU cells and a single round of replication in NCSU cells resulted in replication of these viruses in non-permissive CEF cells. The ability to culture virus from infected bursal tissue is extremely valuable in that

it is now possible, for the first time, to produce high titer infectious stocks of wild type field strains of IBDV in avian cells by first propagating them in macrophages. Furthermore, this tissue culture model will help elucidate stages of IBDV life cycle and identify cellular factors essential for viral replication. This information may unravel strategies for improving management and control of a naturally occurring disease of commercial chickens. Further, serial passaging of cIBDV and vIBDV in macrophages resulted in attenuation of virus. The adapted viruses caused minimal bursal lesions, induced high levels of antibodies and protected chickens against challenge with virulent IBDV.

Acknowledgements

Variant E-IBDV was obtained from Dr. Y.M. Saif, Food Animal Health Research Program, The Ohio State University, OH, USA.

References

Abdel-Alim, G.A., Saif, Y.M., 2001. Pathogenicity of cell culture-derived and bursa-derived infectious bursal disease viruses in specific-pathogen-free chickens. Avian Dis. 45, 844–852.

Bayliss, C.D., Spies, U., Shaw, K., Peters, R.W., Papageorgiou, A., Muller, H., Boursnell, M.E., 1990. A comparison of the sequences of segment A of four infectious bursal disease virus strains and identification of a variable region in VP2. J. Gen. Virol. 71, 1303–1312.

Brandt, M., Yao, K., Liu, M., Heckert, R.A., Vakharia, V.N., 2001. Molecular determinants of virulence, cell tropism, and pathogenic phenotype of infectious bursal disease virus. J. Virol. 75, 11974–11982.

Burkhardt, E., Muller, H., 1987. Susceptibility of chicken blood lymphoblasts and monocytes to infectious bursal disease virus (IBDV). Arch. Virol. 94, 297–303.

Confer, A.W., Springer, W.T., Shane, S.M., Donovan, J.F., 1981. Sequential mitogen stimulation of peripheral blood lymphocytes from chickens inoculated with infectious bursal disease virus. Am. J. Vet. Res. 42, 2109–2113.

Conner, J., Rixon, F.J., Brown, S.M., 2005. Herpes simplex virus type 1 strain HSV1716 grown in baby hamster kidney cells has altered tropism for nonpermissive Chinese hamster ovary cells compared to HSV1716 grown in vero cells. J. Virol. 79, 9970–9981.

Cursiefen, D., Kaufer, I., Becht, H., 1979. Loss of virulence in a small plaque mutant of the infectious bursal disease virus. Arch. Virol. 59, 39–46.

DeBiasi, R.L., Edelstein, C.L., Sherry, B., Tyler, K.L., 2001. Calpain inhibition protects against virus-induced apoptotic myocardial injury. J. Virol. 75, 351–361.

Dobos, P., 1993. *In vitro* guanylylation of infectious pancreatic necrosis virus polypeptide VP1. Virology 193, 403–413.

Dobos, P., Hill, B.J., Hallett, R., Kells, D.T., Becht, H., Teninges, D., 1979. Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. J. Virol. 32, 593–605.

Hassan, M.K., Nielsen, C.K., Ward, L.A., Jackwood, D.J., Saif, Y.M., 1996. Antigenicity, pathogenicity, and immunogenicity of small and large plaque infectious bursal disease virus clones. Avian Dis. 40, 832–836.

Himly, M., Foster, D.N., Bottoli, I., Iacovoni, J.S., Vogt, P.K., 1998. The DF-1 chicken fibroblast cell line: transformation induced by diverse oncogenes and cell death resulting from infection by avian leukosis viruses. Virology 248, 295–304.

Hirai, K., Calnek, B.W., 1979. *In vitro* replication of infectious bursal disease virus in established lymphoid cell lines and chicken B lymphocytes. Infect. Immun. 25, 964–970.

Inoue, M., Yamamoto, H., Matuo, K., Hihara, H., 1992. Susceptibility of chicken monocytic cell lines to infectious bursal disease virus. J. Vet. Med. Sci. 54, 575–577.

Jackwood, D.H., Saif, Y.M., 1987. Antigenic diversity of infectious bursal disease viruses. Avian Dis. 31, 766–770.

Kaufer, I., Weiss, E., 1976. Electron-microscope studies on the pathogenesis of infectious bursal disease after intrabursal application of the causal virus. Avian Dis. 20, 483–495.

Kaufer, I., Weiss, E., 1980. Significance of bursa of Fabricius as target organ in infectious bursal disease of chickens. Infect. Immun. 27, 364–367.

Khatri, M., Palmquist, J.M., Cha, R.M., Sharma, J.M., 2005. Infection and activation of bursal macrophages by virulent infectious bursal disease virus. Virus Res. 113, 44–50.

Khatri, M., Sharma, J.M., 2006. Infectious bursal disease virus infection induces macrophage activation via p38 MAPK and NF-kappaB pathways. Virus Res. 118, 70–77.

Kibenge, F.S., Qian, B., Cleghorn, J.R., Martin, C.K., 1997. Infectious bursal disease virus polyprotein processing does not involve cellular proteases. Arch. Virol. 142, 2401–2419.

Kim, I.J., Gagic, M., Sharma, J.M., 1999. Recovery of antibody-producing ability and lymphocyte repopulation of bursal follicles in chickens exposed to infectious bursal disease virus. Avian Dis. 43, 401–413.

Kim, I.J., Karaca, K., Pertile, T.L., Erickson, S.A., Sharma, J.M., 1998. Enhanced expression of cytokine genes in spleen macrophages during acute infection with infectious bursal disease virus in chickens. Vet. Immunol. Immunopathol. 61, 331–341.

Kim, I.J., You, S.K., Kim, H., Yeh, H.Y., Sharma, J.M., 2000. Characteristics of bursal T lymphocytes induced by infectious bursal disease virus. J. Virol. 74, 8884–8892.

Komine, K., Ohta, H., Kamata, S., Uchida, K., Yoshikawa, Y., Yamanouchi, K., Okada, H., 1989. Complement activation by infectious bursal disease virus and its relevance to virus growth in the lymphoid cells. Nippon. Juigaku. Zasshi. 51, 1259–1262.

Lam, K.M., 1998. Alteration of chicken heterophil and macrophage functions by the infectious bursal disease virus. Microb. Pathog. 25, 147–155.

Lange, H., Muller, H., Kaufer, I., Becht, H., 1987. Pathogenic and structural properties of wild type infectious bursal disease virus (IBDV) and virus grown *in vitro*. Arch. Virol. 92, 187–196.

Lim, B.L., Cao, Y., Yu, T., Mo, C.W., 1999. Adaptation of very virulent infectious bursal disease virus to chicken embryonic fibroblasts by site-directed mutagenesis of residues 279 and 284 of viral coat protein VP2. J. Virol. 73, 2854–2862.

Muller, H., 1986. Replication of infectious bursal disease virus in lymphoid cells. Arch. Virol. 87, 191–203.

Mundt, E., 1999. Tissue culture infectivity of different strains of infectious bursal disease virus is determined by distinct amino acids in VP2. J. Gen. Virol. 80, 2067–2076.

O'Hara, D., Patrick, M., Cepica, D., Coombs, K.M., Duncan, R., 2001. Avian reovirus major mu-class outer capsid protein influences efficiency of productive macrophage infection in a virus strain-specific manner. J. Virol. 75, 5027–5035.

Panigrahy, B., Misra, L.K., Adams, L.G., 1982. Humoral and cell-mediated immune responses in chickens with infectious bursal disease. Vet. Microbiol. 7, 383–387.

Pertile, T.L., Sharma, J.M., Walser, M.M., 1995. Reovirus infection in chickens primes splenic adherent macrophages to produce nitric oxide in response to T cell-produced factors. Cell. Immunol. 164, 207–216.

Qureshi, M.A., Miller, L., Lillehoj, H.S., Ficken, M.D., 1990. Establishment and characterization of a chicken mononuclear cell line. Vet. Immunol. Immunopathol. 26, 237–250.

Rath, N.C., Parcells, M.S., Xie, H., Santin, E., 2003. Characterization of a spontaneously transformed chicken mononuclear cell line. Vet. Immunol. Immunopathol. 96, 93–104.

Sharma, J.M., Kim, I.J., Rautenschlein, S., Yeh, H.Y., 2000. Infectious bursal disease virus of chickens: pathogenesis and immunosuppression. Dev. Comp. Immunol. 24, 223–235.

Sharma, J.M., Lee, L.F., 1983. Effect of infectious bursal disease on natural killer cell activity and mitogenic response of chicken lymphoid cells: role of adherent cells in cellular immune suppression. Infect. Immun. 42, 747–754.

Sharma, J.M., Witter, R.L., 1983. Embryo vaccination against Marek's disease with serotypes 1, 2 and 3 vaccines administered singly or in combination. Avian Dis. 27, 453–463.

Sommer, F., Cardona, C., 2003. Chicken anemia virus in broilers: dynamics of the infection in two commercial broiler flocks. Avian Dis. 47, 1466–1473.

Tanimura, N., Sharma, J.M., 1997. Appearance of T cells in the bursa of Fabricius and cecal tonsils during the acute phase of infectious bursal disease virus infection in chickens. Avian Dis. 41, 638–645.

Tanimura, N., Sharma, J.M., 1998. In-situ apoptosis in chickens infected with infectious bursal disease virus. J. Comp. Pathol. 118, 15–27.

Tsai, H.J., Saif, Y.M., 1992. Effect of cell-culture passage on the pathogenicity and immunogenicity of two variant strains of infectious bursal disease virus. Avian Dis. 36, 415–422.

Tsukamoto, K., Tanimura, N., Mase, M., Imai, K., 1995. Comparison of virus replication efficiency in lymphoid tissues among three infectious bursal disease virus strains. Avian Dis. 39, 844–852.

van den Berg, T.P., Eterradossi, N., Toquin, D., Meulemans, G., 2000. Infectious bursal disease (Gumboro disease). Rev. Sci. Tech. 19, 509–543.

van Loon, A.A., de Haas, N., Zeyda, I., Mundt, E., 2002. Alteration of amino acids in VP2 of very virulent infectious bursal disease virus results in tissue culture adaptation and attenuation in chickens. J. Gen. Virol. 83, 121–129.

Winterfield, R.W., Fadly, A.M., Bickford, A., 1972. Infectivity and distribution of infectious bursal disease virus in the chicken. Persistence of the virus and lesions. Avian Dis. 16, 622–632.

Yamaguchi, T., Kondo, T., Inoshima, Y., Ogawa, M., Miyoshi, M., Yanai, T., Masegi, T., Fukushi, H., Hirai, K., 1996a. *In vitro* attenuation of highly virulent infectious bursal disease virus: some characteristics of attenuated strains. Avian Dis. 40, 501–509.

Yamaguchi, T., Ogawa, M., Inoshima, Y., Miyoshi, M., Fukushi, H., Hirai, K., 1996b. Identification of sequence changes responsible for the attenuation of highly virulent infectious bursal disease virus. Virology 223 (1), 219–223.