Egyptian J. Virol. 1, 507-515 (2004)

Alterations of Immunologic Responses of Calves Experimentally Infected With Virulent Either Type 1 or 2 Non-Cytopathogenic Strains of *Bovine Viral Diarrhea Virus*

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Bovine viral diarrhea virus (BVDV) is world wide pathogen affecting cattle. Eleven Holstein calves between 6-8 months of age that were seronegative to BVDV were divided into three groups (n=4 for NY-1, n=4 for 890 and n=3 control. Fibroblast cells were prepared from calves' skin biopsies by trypsin digestion. Using immunofluorescence, the percentages of infected cells and MHC antigens expression were evaluated. The fibroblast and peripheral blood mononuclear (PBMC) cells were in vitro infected and stained by direct double staining with both human and bovine anti-MHC class I and II antibodies labeled with phycoerythrin (pink) 1:20 and anti-BVDV polyclonal antibodies labeled with fluorescence isothiocyanate, FITC (green) 1:60. There was a reduction in the percentages of infected fibroblasts and PBMC expressing both MHC-I and II, while, mild reduction was observed in the percentages of PBMC expressing MHC-I molecules. Eight calves of the two groups were infected intranasally with a virulent NCP BVDV type1 (NY-1) and 2 (890) biotypes and kinetic evaluation of its effect of *in vivo* infection on clinical and immunological parameters. All virus-inoculated calves developed a fever on day's 3-7-post infection. BVDV antigens were detected in the Buffy coat cells on days 3, 5, and 7 post infections. Most calves developed low neutralizing antibody titers as type-1 (3, 12, 18 and 44) and type-2 (6, 16, 20 and 48) on days 5, 7, 10 and 15) post infection respectively. There was a significant and minor diminution in the expression of the MHC-II and MHC-I molecules respectively in virus infected calves on days 3, 5, 7 and 10 post infection, when compared to control calves. There was close correlation between both in vitro and in vivo of virus immunodown regulatory effects associated with blocking the transport of MHC complexes and prevention of antigen presentation to the immune cells associated with eventual dysregulation and/or impairment of immunological functions accompanied with immunsuppression.

INTRODUCTION

BVDV is a worldwide pathogen affects cattle of all ages; causing variety of infections includes enteric form, abortions, congenital abnormalities, immunosuppression and mucosal disease (Baker, 1995).

BVDV is small, enveloped that belongs to the *Genus Pestiviruses* of the *Family Flaviviridae* along with the flavivirus and human hepatitis C virus (Francki *et al.*, 1991). The virus genome is a (+) ssRNA of 12.3 kb in length, encodes one polyprotein of 4000 amino acids. This polyprotein is post-translationally processed by cell and virus derived proteases to give rise the mature viral proteins. Based on the untranslated region, 5'UTR of the viral genome, BVDV exist as two genotypes type I and II. According to their effects on cell culture, each genotype includes

cytopathogenic (CP) and non cytopathogenic (NCP). (Ridpath *et al*., 1994). In addition, both NCP and CP BVDV can be isolated from infected animals (McClurkin *et al*., 1985). The CP biotype develops within animals persistently infected with a NCP BVDV, and results in the mucosal disease (Thiel *et al*., 1996).

Acute outbreaks of BVDV associated disease in young and adult calves characterized by thrombocytopenia, leukopenia, fever, hemorrhages, diarrhea and death was attributed to NCP strains (Corapi *et al*., 1989; Pellern *et al*., 1994). BVDV infection establishes a persistence infection in calves by immune evasion and suppression, which increases patterns of the host's susceptibility to secondary bacterial or viral infections (Potgieter, 1995). MHC molecules present the intracellularly processed antigens and result into stimulation and activation of the MHC-restricted immune cells and responses that play a major role in the control of infections. However, there is no information regarding the BVDV infection and its effect on MHC molecules. Therefore, this study investigates the relationship between BVDV infection and the expression of MHC antigen complex. Furthermore,, it provides wide footnotes on the virus virulence, clinical scores and antibody response of such infection.

MATERIALS AND METHODS

Virus

Two representative strains of NCP BVDV type I (NY-1) and type II (890) were used for in vitro and in vivo infections. Each strain has been propagated separately in BVDV free MDBK cells cultured in medium supplemented with equine serum (Fulton *et al*., 1997). The viruses were then harvested by two freezing-thawing, allocated and stored at -20°C until used. The proper infectious virus doses for each strain that were enough for induce infection were figured through titration of $9x10^6$ (NY-1) and $16x10^6$ $TCID_{50}$/ml (890).

Animals and experimental design

Eleven BVDV seronegative Holstein calves, 6-8 month old, were randomly selected with history of no BVDV infection and conventionally housed in separate groups. Animals were divided into three groups where 4 calves got infected with type-I (NY-1), 4 calves got infected type 2 (890) and 3 animals were lift without infection.

The calves were infected with BVDV strains, as a single dose of 6 ml infected culture (3ml/nostril) in the form of intranasal spray. The control group was given the same dose intranasally of sterile culture medium.

Clinical Scores

The clinical scores such as temperature, nasal discharges and diarrhea for the experimentally infected calves were also evaluated.

Collection and preparation of fibroblasts cells

Prior to any treatment, fibroblasts were aseptically collected

from each calf in the form of dermal biopsies and prepared using trypsin enzymes. Fibroblasts then were cultured in DMEM (Gibco, BRL, USA), supplemented with 10% equine serum until monolayer confluency was reached (Freshney, 1994).

Normal distribution of MHC molecules on fibroblast and PBMC cells using immunofluorescence

Fibroblast cells were trypsinized and adjusted $2x10^5$ cells /ml DMEM. PBMC were also collected, separated using Ficoll-histopaque and adjusted $5x10^6$cell /ml RPMI 1640 containing 10% fetal bovine serum (Xue and Minocha, 1996). The cells were dispensed, adhered on slides, fixed with 80% acetone for 10 minutes at room temperature then directly stained with either anti-Human MHC-I or MHC-II Mabs (Dako & Sigma, USA) labeled with Phycoerythrin (1:20). The numbers and percentages of stained and unstained cells in 3-6 fields were counted using fluorescent microscope. PBMC were indirectly stained with unlabeled bovine anti-MHC-1 (H58A) Mabs or MHC-2 (TH14b) (VMRD-USA), and then countered by goat anti-mouse IgG with FITC. Similarly the numbers and percentages of PBMC that expressed MHC molecules were evaluated using fluorescent microscope (Lalor *et al.*, 1986)

In vitro effect of BVDV infection and expression of MHC antigens

As previously mentioned fibroblasts and PBMC cells were plated into into 6-well plates and incubated at 37°C in 5% Co2. Expression of both MHC-I and II was also assessed after in vitro infection with BVDV biotypes at (moi-1). Dual staining using anti-MHC-I or MHC-II monoclonal antibodies (VMRD-USA) along with anti-BVDV polyclonal antibody labeled with FITC-conjugate (green) diluted 1:60 (NVSL, USA) then examined as above (Lopez *et al.*, 1993; Steinmassi and Hamprecht, 1994).

Further more, kinetic evaluation of in vivo effect of BVDV on MHC expression on PBMC was examined on days 1, 3, 5, 7, 10, and 15 post infections.

Detection of the BVDV antigen and immune response post experimental infection

Heparinized (for PBMC and buffy coat) and clotted (sera) blood samples were collected on days 0 (before), 1, 3, 5, 7, 10, and 15 post infection (PI). These samples were used for detection of both BVDV antigens in Buffy coat on days 3, 5, 7, and 10 PI using ELISA Kit (LSI BVD/BD antigen capture, Lab Service Internation, Lissieu, France) and antibodies using serum neutralization on days 5, 7, 10 and 15 PI, using 100 $TCID_{50}$ of cytopathic BVDV type 1 (NADL) and 2 (biotype-125), Fulton *et al.*, (1997).

RESULTS AND DISCUSSION

Normal distribution of MHC-molecules on fibroblast and PBMC cells

The expression of MHC molecules was present on both

fibroblasts and PBMC cells isolated from calves. The distribution of MHC-I was similar in both PBMC and fibroblasts while the expression of MHC-II was noted to be less on fibroblasts compared to PBMC.

In vitro effect of BVDV infection on expression of MHC molecules

MHC-I or II stained with PE were visualized in the form of a peripheral yellow to pink coloration while BVDV-FITC bound antigen was seen intra-cytoplasmically as green coloration. There was a reduction in the infected fibroblasts expressing MHC-I molecules while PBMC showed a reduction in MHC-II expression after in vitro infection of these cells. No significant reduction was observed in the PBMC expressing MHC-I at their surfaces.

Kinetic evaluation of in vivo effect of BVDV infection and MHC antigens expression on PBMC

For the calves infected with NY-1, the percentages of PBMC expressed MHC-I were 90, 89, 78, 78, 80 and 82, while class II were 75, 68, 47, 49, 51 and 56 on days 1, 3, 5, 7, 10 and 15 PI. With regard to the 890 infected calves, the mean percentages of cells expressed class I were 88, 84, 79, 82, 78 and 83 while class-II were 81 55, 42, 40, 33, and 57 at the same sampling intervals. The obtained data showed down regulation of both MHC-class I and II expression after BVDV infection. A significant down regulation and diminution was reported in the PBMC expressed MHC II on days 5, 7, and 10 PI, in both groups of NY-1 and 890 virus-infected calves when compared to control group. The down regulation of class-II was significantly in 890 infected calves than that of NY-1. On the other hand, no significant variation in the PBMC expressing either MHC-I or MHC-II was observed among the control animals (Figure 1a and b).

Clinical response to BVDV type 1 and 2 infections

The clinical scores reported among the BVDV exposed calves were fever, depression, nasal discharge, transient soft diarrhea and dyspnea on days 3, 5, and 7 PI. No clinical signs were observed among the control calves throughout the experiment

Detection of BVDV antigen by ELISA

From the corrected positive absorbencies of ELISA results, abundant BVDV antigens were detected in Buffy coat samples collected from both type 1 and 2 virus inoculated calves on days 3, 5, 7 and 10 PI (Table 1).

BVDV serology post virus inoculation

BVDV specific antibodies were increased gradually to titers means of BVDV NY-1 (3, 12, 18 and 44) and 890 (6, 16, 20 and 48) on days 5, 7, 10, and 15 PI. Type-2 antibody response was significantly higher than type-1. No antibodies were detected in control calves (Table 1).

Both type 1 and 2 NCP BVDV strains induced in 6 to 8 month old calves infection characterized by common variable clinical signs that declined by the development of virus

specific antibody and are in a similarity with previously reported findings (Abd El-Samie, 1998; Cortese *et al.*, 1998) in different else where. Although hemorrhage has previously been associated with acute BVDV type 2 infections (Corapi *et al.*, 1989), it was not reported on any of the infected calves. This was might due to different virus isolates, virulence, conditions and calves breeds. BVDV antigens were consistently detected in Buffy coat by ELISA in virus-inoculated calves on days 3, 5, and 7 PI. This demonstrated the induction of infection and in agreement with (Ellis *et al.*, 1998). The inoculated calves with NCP BVDV types had low titers of neutralizing antibodies in their sera on days 5, 7, 10, and 15 PI. There was a correlation between the detection of the virus in Buffy coat and the slow development of circulating antibody that was eventually clearing the virus. Similar findings using immunoperoxidase assay were previously reported by (Fulton, *et al.*, 1997).

MHC-I and II antigens are primarily expressed at the surface of fibroblasts and antigen-presenting PBMC (B, MQ and dendritic) cells. They are the key-molecules associated with antigen presentation and the interactions between the immune cells in the induction of immune response. Reduction of MHC expression results in down regulation of the presentation of viral antigenic peptides, alters the host's defense and could result in inadequate protective immune responses and susceptibility to viral infections (Abbas *et al.*, 1997). A number of viruses have been shown to influence MHC expression on cells such as adenovirus type-2 and herpes simplex-I and reduced the susceptibility of the virus infected cells to cytotoxic cell mediated lysis (Burgert and Kvist, 1985; Jennings *et al.*, 1985).

A double immunofluorescence was established to demonstrate the effect of BVDV infection versus expression MHC antigens (Ahmed Abd El-Samie, 1998). The results here indicated that in vitro BVDV infection down regulated the class I MHC on the fibroblasts and in close similarity with (Steinmassi and Hamprecht, 1994), who's reported that with human cytomegalovirus. In virus-infected calves, there was minor variation in the percentages of PBMC expressing MHC-I. In contrast, a significant diminution in the percentages of PBMCs expressing MHC II molecules was reported on days 5, 7, and 10 PI when compared to control animals. As the infected cells expressing high levels of viral antigens bear low levels of MHC antigens particularly class-II. The results demonstrated that BVDV infection either type 1 or 2 induced marked negative and down regulation of trafficking of endoplasmic MHC molecules. The negative regulation might have an impact on the host immune system, have been considered as an explanation for the BVDV enzootic infection in cattle herds, were consistent with and supported by (Beliveau and Archambault, 1997; Archambault *et al.*, 2000) Other studies have indeed reported that BVDV in vitro and in vivo infection results in concomitant decrease in immune cell populations (monocyte / macrophage, lymphocytes) with impairment of their various functions

(Ketelsen *et al.*, 1979; Jensen and Schultz, 1991; Welsh *et al.*, 1995). The reduction in intensity of fluorescence and diminution in the MHC II- expressing cells might reflect a defect in the trafficking and expression of MHC molecules at the surface of these cells rather than a diminution in the absolute numbers of cells. However, type 1 and 2 NCP BVDV exerts dual immunoregulatory effects on the host different subsets of monocytic immune cells represented by negative down regulation in MHC expression (obtained here) and positive up regulation of cell phagocytic capability (Arachambault *et al.*, 2000) of immune cell In conclusion, BVDV infection is associated with immunological modulation and dysregulation of the host specific immune response by impairment and/or blocking the transport of peptide loaded MHC complexes into the medial Golgi compartments as a possible mean to evade antigen presentation with subsequent immunesuppression. A future work with advanced equipments should design for more quantitative demonstration and description of MHC antigens.

ACKNOWLEDGMENT

The author's thanks Dr. Bassam Sheidifat. post graduate student and the farmers allowed the use of their farms for collection of samples.

Table (1): Detection of BVDV antigen from Buffy coat and type-1 and 2 virus specific neutralizing antibody responses of infected calves.

Group/calf number	Days post virus infection / antigen detection in Buffy coat (+/-) / Serum Neutralizing antibodies (# *)					
	1	3	5	7	10	15
NY-1: 2040	-	-	+ / 2	+ / 16	- / 16	*NT / 64 **
2041	-	-	+ / 4	+ / 16	+ / 8	NT / 16
2043	-	-	+ / 2	+ / 8	+ / 16	NT / 32
2044	-	+	+ / 4	+ / 8	- / 32	NT / 64
NY-1 Mean titer			3	12	18	44
890: 2045	-	+	+ / 8	+ / 16	- / 16	NT / 32
2046	-	-	+ / 8	+ / 8	+ / 32	NT / 64
2047	-	-	+ / 4	+ / 8	+ / 16	NT / 32
2051	-	+	+ / 4	+ / 32	+/ 16	NT / 64
890 / Mean titer			6	16	20	48
Control	-	-	-	-	-	-

(-): No viral antigen detected by ELISA in Buffy coat. (+): Detectable viral antigen.
(NT): Not tested for virus antigen.
(# *): Reciprocal of virus specific serum neutralizing (SN) antibody titers.

Figure-1a: Percentages of PBMC Expressed MHC-I Molecules post Infection with BVDV Biotypes NY-1 and 890

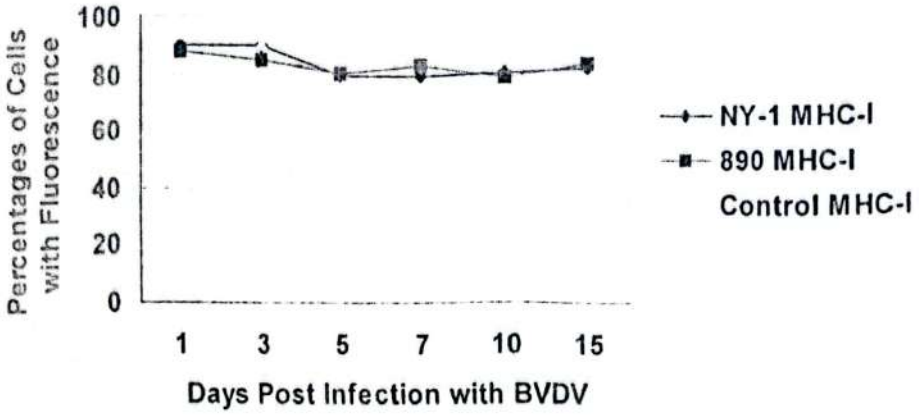

Figure-1b: Percentages of PBMC Expressed MHC-II molecules Post Infection with BVDV Biotypes NY-1and 890

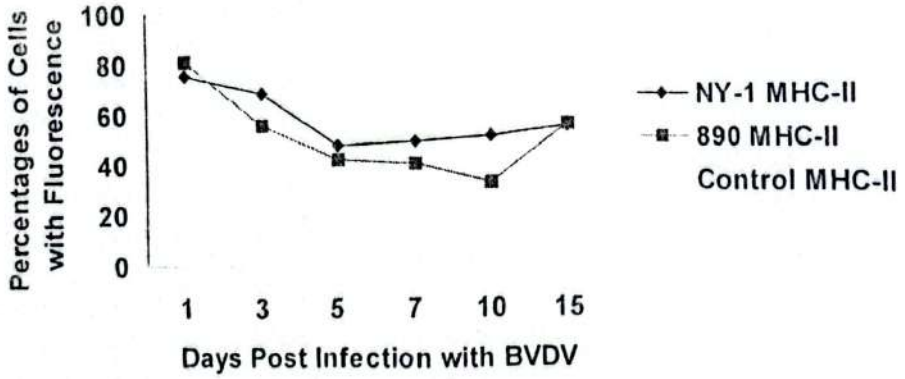

Figure (1a and b): Sequential percentages of PBMC expressed both MHC-I and MHC-II molecules post infection with BVDV NCP biotypes NY-1 and 890. NY-1: Calves infected with BVDV strain NY-1. 890: Calves infected with BVDV strain 890. Control: Non infected calves.

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