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All is Not Butter that Comes from the Cow: The Bovine Viral Diarrhea. Understanding the Pathogenesis of Cytopathic and Non-Cytopathic Infection

Pinchuk LM[1*], **Ammari MG**[2] **and Pinchuk GV**[3]

[1]*Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, USA*

[2]*School of Animal and Comparative Biomedical Sciences, University of Arizona, Tucson AZ 85721, USA*

[3]*Department of Sciences and Mathematics, Mississippi University for Women, Columbus, MS 39701, USA*

[*]**Corresponding author:** Pinchuck LM, Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, USA, Tel: + (662)325-1461; Fax: + (662)325-1031; E-mail: Pinchuk@msstate.edu

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Abstract

Bovine viral diarrhea infections are seen in all ages and breeds of cattle worldwide and have significant economic impact due to the productive and reproductive losses. The infectious agents that cause this disease, Bovine viral diarrhea viruses (BVDVs) may occur as two biotypes, of which one is non-cytopathic (ncp) and the other cytopathic (cp), classified according to whether or not they produce visible changes in cell culture. Cytopathic biotypes of BVDV can be created through internal deletion or recombination of RNA of ncp biotypes. The mechanisms of this change are not sufficiently understood. In this review, we discuss the work that has been done to date in our laboratory using methods of proteomics, cellular and molecular biology, and immunology. We found that both biotypes cause numerous changes in the expression levels of the proteins in monocytes, including proteins related to professional antigen presentation, enzymes and receptors, and changes in the infected cell functions related to antigen uptake. However, the alterations caused by the cp and not by the ncp biotypes are consistent with the hypothesis that the virus cytotoxicity involves the mitochondrial dysfunction. Overall, our data show that, the three important signals, antigen-specific, co-stimulatory and cytokine, required to promote the effector activation of naïve T cells to be delivered by professional antigen presenting cells (APCs) are impaired by both types of BVDV. In addition, cp and ncp BVDVs differentially target mitochondrial proteins and antioxidant enzymes that control the fate of infected cells and determine whether BVDVs produce cytopathic effects or replicate noncytopathically to establish persistent infection. This research is aimed to discover foundational knowledge related to host-pathogen interactions and facilitate the development of innovative disease preventatives for pathogens causing significant animal losses.

Keywords Bovine viral diarrhea virus; Cytopathic; Non-cytopathic; Antigen presenting cells; Monocytes; Proteomics; Mitochondrial dysfunction

Introduction

Viruses defy all definitions. Are they alive? Are they organisms? If so, why are they not included into the taxonomical "tree of life" as the fourth domain, in addition to Bacteria, Archaea, and Eukarya [1]? If they are not a form of life, then why do they reproduce so fast, why do they possess all these sophisticated mechanisms of evading their host's defense [2]? Maybe the best definition of a virus is simply, "a virus is a virus?" [3]. Regardless of the definitions, we know all too well that viruses have been infecting cells for many millions of years. Studies show that, for example, baculoviruses infected ancient insects some 300 million years ago [4]. Viruses were isolated from hyperthermophilic Archaea of the genus *Sulfolobus*, which is thought to be one of the most ancient forms of life present on our planet today [5]. Infection of a cell by a virus does not necessarily lead to the cell's destruction and death. While many viruses are said to be cytopathic (cp) (able to destroy the infected cells using a variety of mechanisms), other are noncytopathic (ncp) (replicating in the infected cell in such a "benign" way that the cell remains viable) [6].

Some species of viruses include both cp and ncp variants, called biotypes [7]. It is not clear what makes a virus cp or ncp. Bovine viral diarrhea virus (BVDV) is a representative of the family Flaviviridae, which is thought to have emerged and dispersed during the early Ice Age [8], causes a very prevalent disease in cattle [9]. The viruses have both cp and ncp biotypes. The disease usually begins when an animal is infected by the ncp biotype, which then may or may not morph into a cp biotype. The cp biotypes cause fatal mucosal disease [10]. Within the recent 15 years, the work of our group focused on the attempts to understand molecular mechanisms of cytopathic and noncytopathic BVDV infection and how the cp biotype of the BVDV emerges from the ncp one. To this end, we analyzed differences between the two biotypes, using methods of cellular immunology, molecular biology biochemistry, and proteomics.

Cytopathic and Non-Cytopathic Bovine Viral Diarrhoea Viruses Interfere with Antigen Uptake, Cytokine and Toll-Like Receptor Gene Expression in Professional Antigen Presenting Cells (Apcs)

Earlier studies showed that the two biotypes differ in their influence on the functions of the infected immunocompetent cells, particularly monocytes/macrophages. For example, the cp biotypes induced infected macrophages to produce type 1 interferon (IFN), making uninfected cells die by apoptosis [11,12]. The cells infected with ncp biotypes did not produce type 1 IFN and acquire resistance to apoptosis [13]. On the other hand, no difference was found between

the cp and the ncp biotypes in their ability to compromise antigen presentation of the infected macrophages (but not dendritic cells) to antigen-specific T lymphocytes [14]. Our studies, which addressed the influence of BVDV on the functions of *in vitro*-infected bovine monocytes, showed that antigen uptake, an important APC function, was affected in bovine monocytes in the early stage of BVDV infection, and that both BVDV biotypes, cp and ncp, had similar effects on the monocyte antigen uptake. The evidence that 24 h BVDV infection augmented the mannose receptor-dependent active endocytosis and inhibited the fluid phase uptake and/or other mechanisms in bovine monocytes suggests that BVDV might use it in order to escape the appropriate presentation by professional APCs to the cytotoxic T cells which can efficiently destroy infected cells. [15].

However, we have found some major differences in the effect of cp and ncp biotypes in the expression of genes that play an important role in the innate and adaptive immunity that could be due to biological differences between cp and ncp BVDV strains [16] (Figure 1). Both biotypes suppressed the expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, Th1 type cytokine IL-15, Th2 type cytokine IL-10 and co-stimulatory molecules CD80 and CD86 genes. Neither biotype altered the expression of Th1 type cytokines genes IL-12 and IFN-γ, when measured 24 hours post-infection. Yet, only the ncp biotype up-regulated the expression of a Toll-like receptor (TLR), TLR3, as well as of type 1 IFN and IL-12 genes, when measured 1 hour post-infection. The gene expression of another TLR, TLR7, was up-regulated by both biotypes after 24 hours infection. We hypothesized that the difference between the two biotypes can partially be explained by their peculiar influence on the expression of TLR and on the work of the TLR-related signaling pathways [16]. After recognition of viral components, TLRs initiate the production of cytokines and stimulate inflammatory and adaptive immune responses through signal transduction [17,18]. Type I IFN is the most important cytokine in viral infection [19]. Unlike our study, previous *in vivo* and *in vitro* studies showed that cp BVDV induced type I IFN, such as IFN-α and IFN-β, whereas ncp BVDV failed to induce it in protein level in the bovine system [20-22]. Additionally, type I IFN protein expression was not detected in bovine monocytes infected with ncp BVDV [14]. Interestingly, recently identified plasmacytoid DC-like populations produced IFN type I *in vivo* in response to both ncp and cp BVDV [14,22].

In this study, as was mentioned above, type I IFN cytokine gene expression was significantly up-regulated in 1 h ncp, but not cp BVDV infection suggesting TLR3-mediated control of the type I IFN production in BVDV [16]. Signaling through TLR also induces pro-inflammatory cytokine gene expression, such as TNF-α, IL-1β, and IL-6 [17]. However, many viruses have mechanisms that inhibit inflammation and prevent apoptosis and are able to establish chronic infections [17,23,24]. The cytokine protein expression data supported our pro-inflammatory cytokine gene expression results. In particular, the decreases in the protein levels of TNF-a and IL-1β after cp BVDV infection and IL-1β after 24 h BVDV infection with both strains correlated with significant decreases in the corresponding cytokine gene expression levels [16]. We hypothesize that both cp and ncp BVDV could subvert, sabotage or escape innate immune responses, respectively by modulating TLR gene expression, followed by pro-inflammatory, type I IFN, Th1/Th2 type cytokine gene and protein expression, and by decreasing the levels of CD80/86 in professional APCs. Therefore, the two important signals, co-stimulatory and cytokine, required to promote the effector activation of naïve T cells to be delivered by professional APC [25] are impaired by both types of BVDV infection (Figure 1).

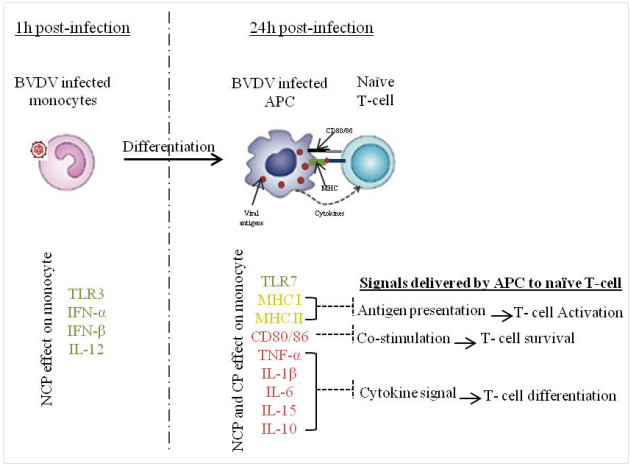

Figure 1: BVDV infection interferes with three major signals, antigen resentation, co-stimulation and cytokine signal delivered by professional APC required to promote the effector differentiation of naïve T cells. Only the ncp biotype up-regulated the expression of TLR3, as well as of type 1 IFN and IL-12 genes, when measured 1 hour post-infection in bovine monocytes (up-regulated genes shown in green) [16]. Both biotypes suppressed the expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 genes and proteins, Th1/Th2 type cytokines IL15 and IL-10 genes, respectively, and co-stimulatory molecules CD80/ CD86 genes when measured 24 hours post-infection in bovine APC (down-regulated genes and proteins are shown in red) [16]. The gene expression of TLR7, was up-regulated by both biotypes in 24 hours post-infection [16]. The MHC-related proteins have been altered in a similar or alternative way by both BVDV biotypes when evaluated in 24 hours post-infection (variable selectively regulated proteins shown in yellow) [16,27,29].

Pathogenesis of Bovine Viral Diarrhea Virus Infection through Proteomics

In the recent years, rapid advances in high-output nucleotide and amino acid sequencing as well as in bioinformatics allowed researchers to analyze very wide arrays of genes and their products. Using a proteomics approach, we evaluated the effect of cp and ncp BVDV biotypes on the expression of, approximately, 10,000 proteins extracted from bovine monocytes [26-28]. Of these, 378 proteins had high degree of amino acid homology with known protein kinases and related proteins. Because protein kinases play a major role in cellular signaling, we decided to concentrate our analysis on the difference in the expression of these molecules in bovine monocytes infected with either cp or ncp BVDV. We found that the expression of eighteen protein kinases was significantly altered by the BVDV infection regardless of the virus biotype. However, a number of protein kinases were altered differently by the two biotypes. Particularly, the expression of receptor-activated C kinase (RACK), pyridoxal kinase (PK), diacyglycerol kinase (DGK), and Bruton's tyrosine kinase (BTK) was significantly decreased in the bovine monocytes infected with cp

BVDV compared to the ncp biotype. We hypothesized that a loss of these protein kinases could contribute to the viral cytopathic effect, although it can also be a consequence of the cp biotype infection with other factors being responsible for its cytopathic effect [26].

Next, we looked at the effect of the cp BVDV biotype on the expression of proteins that are directly involved in the professional antigen presentation in bovine monocytes [27,28]. Using the same proteomics approach, we found that of 445 proteins altered by the viral infection, 47 were identified as the ones involved in cell adhesion, apoptosis, antigen uptake, antigen processing and presentation. In our study, 18 MHC proteins (4%) out of 445 were significantly altered in cp BVDV-infected bovine monocytes. Firstly, 9 of MHC class I proteins were significantly down-regulated in cp BVDV-infected bovine monocytes. However, two proteins, were only detected in cp BVDV-infected group [27]. Secondly, 6 of MHC class II proteins, including the DQ isotype, were significantly decreased in the cp BVDV-infected monocytes, and one MHC class II DR-beta chain protein was increased in cp BVDV-infected monocytes only [27].

To confirm the significant changes in protein expression between the control and BVDV-infected monocytes by using DDF–MudPIT analysis and relative quantitation approach, Western blotting was performed to evaluate the protein expression of the MHC class I, class II and HLA-DQ isotype [27]. In our preliminary observation by DDF–MudPIT, ncp BVDV strain New-York decreased the protein expression levels of the MHC class I, class II and MHC-DQ isotypes to virtually undetectable levels (unpublished observation). The ncp BVDV had the strongest inhibitory effect on the MHC class I, MHC class II and MHC-DQ isotype protein expression levels and did not affect β-actin expression levels in bovine monocytes. As expected, cp BVDV infection significantly decreased the levels of the MHC proteins and did not affect β-actin expression levels in monocytes supporting our findings by DDF–MudPIT in cp BVDV-infected cells [27,28].

In our subsequent work [29], we used a proteomics approach to select and characterize only those bovine monocyte proteins that had been differentially altered by the cp and ncp BVDV biotypes. We have identified 2489 proteins from uninfected cells, 2356 proteins from ncp BVDV-infected cells, and 2028 proteins from cp BVDV-infected cells. Compared to uninfected bovine monocytes, 137 host proteins were altered in the ncp-infected monocytes, and 228 in the cp-infected monocytes. Some of these proteins were altered in the opposite fashion. For example, integrin α-2b and integrin β3 (molecules involved in caveolae-mediated endocytosis) were up-regulated in cp BVDV-infected monocytes and down-regulated in ncp BVDV-infected cells. Some proteins were altered in the monocytes infected with one but not the other biotype. For example, thrombin, subunit 2 of the actin-related complex, myosin, an interferon-inducible protein p78, and the product of a proto-oncogene v-yes-1 were up-regulated in monocytes infected with cp BVDV, but not by the ncp biotype.

Still other proteins (notably, HLA-DQ, an MHC Class II molecules) were down-regulated in the cells infected with cp BVDV, but not ncp BVDV. To summarize, both biotypes differentially affected multiple MHC molecules. Some of these proteins have been altered in the same way, another in the opposite way, and several MHC class I and II proteins were virtually absent either in cp or ncp BVDV-infected monocytes. Based on our data we hypothesize that both BVDV biotypes will affect the functional binding of the viral -peptide: self-MHC complex by the T cell receptor and both CD4 and CD8 co-receptors thus impairing or modulating the transmission to the naïve T cell that antigen has been encountered [25] (Figure 1). Future studies will determine how, exactly, do these differentially altered MHC proteins contribute to the impaired antigen presentation by professional APCs and viral cytopathic effect.

Pathway and network analysis of bovine proteins differentially altered by BVDV also identified significant biotype-related differences [29]. It is known that ncp BVD viruses can establish persistent infection (PI) as a result of infection of the embryo early in its development by interfering with a key mechanism of the innate immune system through the IFN type I production [30]. Since IFN is also important in the activation of the adaptive immune response, suppression of this signal may be essential for the establishment of PI [30]. The early stages of the host response to infectious agents include a number of physiological changes, collectively known as the acute phase response. Our previous report identified multiple acute phase response proteins altered by cp BVDV [27]. In this study, acute phase pathway was demonstrated to be the first significant pathway in both ncp and cp BVDV infection. Although, ncp and cp viruses altered different numbers of host proteins in general, they had the same effects on the monocyte protein expression levels [29].

Our finding indicates that ncp BVDV unlike the cp counterpart, inhibited the level of communication of the ECM and cell differentiation thus promoting the establishment of PI. The differences in the expression of integrins can also mean that cp BVDV infection induces monocytes to differentiate into macrophages, or, alternatively, that monocytes that have already embarked on the differentiation into macrophages, are more susceptible to cp BVDV infection [29]. In particular, up-regulation of proteins related to the acute phase response and cell adhesion while decreasing the expression of proteins involved in antigen uptake, processing and presentation suggests that cp BVDV infection is promoting monocyte migration, differentiation and activation while inhibiting their BVDV antigen presentation to immunocompetent lymphocytes, in particular, Th1 type and regulatory T cells, thus resulting in the uncontrolled inflammation mediated by activated macrophages and enhanced viral spread in the host. Taken together, the combined use of gene ontology (GO) information and systems biology network modelling extended our knowledge of the roles of ncp and cp BVDV biotypes in the production of PI and cytopathic effects respectively [27-29].

Mitochondrial Dysfunction Associated with the Cytopathogenicity of Bovine Viral Diarrhea Virus

Cell death can occur by necrosis ("opening up" and "spilling the cell's guts," as a result of hypoxia, chemical poisoning, burns etc.), or by apoptosis [31]. The latter, also known as programmed cell death, manifests mostly by shrinking of the dying cell without loss of the integrity of its membranes [32]. Many viruses evolved to destroy the cells they infect by inducing apoptosis [32-35] by causing mitochondrial dysfunction [36,37]. Studies show that while the BVDV ncp biotype fails to induce apoptosis [11], the cp biotype induces apoptosis of the infected cell [38,39]. In our studies, this effect of the cp biotype was, most likely, achieved by causing mitochondrial dysfunction [39]. Our results show that cp BVDV differentially affected proteins in multiple mitochondrial-related pathways by significantly decreasing their expression levels compared to the ncp BVDV biotype that affected a fewer number of proteins, by mostly enhancing their expression [39].

In our experiments [39], we asked whether the infection of a bovine turbinate (BT) cell line [40] with either of the two of the BVDV biotypes would alter some parameters of mitochondrial dysfunction assessed by flow cytometry. First, we looked at the ability of the two biotypes to trigger apoptosis in BT cells as judged from the pattern of staining with Annexin V and propidium iodine (PI). It turned out that the cp BVDV biotype significantly increased the number of cells stained with Annexin V (early apoptotic cells) as well as the number of cells stained with PI (late apoptotic cells. The ncp BVDV biotype clearly infected the BT cells, but failed to increase the numbers of the early and late apoptotic cells after 48 hours post infection. In kinetics studies, a tendency to cause apoptosis appeared in BT cells infected with the cp BVDV biotype on the 24^{th} hour post infection. The percentage of apoptotic cells was significantly bigger than in uninfected BT cells on the 36^{th} and 48^{th} hour post infection.

BT cells infected with the ncp BVDV biotype remained free of apoptosis [39]. Interestingly, in the same study [39] we observed that infection with the cp, but not the ncp BVDV biotype resulted in a significant disruption of the mitochondrial membrane potential ($\Delta\Psi m$), as judged from the shift in DePsipher staining. This was in line with our previous observations that the mitochondrial dysfunction pathways, as revealed by proteomic analysis, were the most affected pathways following cp BVDV infection [29]. Of the five known mitochondrial oxidative phosphorylation pathway-related protein complexes [41,42], the expression of proteins representing all these complexes except complex III, were altered by the cp biotype infection [39]. Proteomic profiling done by Diamond et al. [43] showed that in hepatitis C infection, the expression of proteins associated with the mitochondrial pathways was altered in human patients' biopsies showing signs of profound viral-induced tissue destruction. They also confirmed, by functional analysis, that the virus infection at this stage caused significant impairment of oxidative phosphorylation.

Mitochondrial dysfunction manifests not only as a drop in the $\Delta\Psi m$, but also as an impairment of the production of antioxidant enzymes, and/or of their ability to neutralize the reactive oxygen species (ROS) produced by mitochondria, resulting in oxidative stress [44]. We asked whether the infection of BT cells by either of the BVDV biotypes resulted in the above impairment, as judged from an increase of ROS production evaluated by flow cytometry of the cells stained with the carboxyH2DCFDA reagent [45]. The results indicated that the infection of the BT cell line with the cp BVDV biotype resulted in an early (1 hour post infection) decrease in ROS production, followed by a strong (up to tenfold) increase in ROS production. On the other hand, the content of ROS in BT cells infected with the ncp biotype did not cause any significant change in ROS production at any time post infection [39]. We interpreted these data as meaning that in cp BVDV infection, a slight temporary reaction against oxidative stress early post infection is followed by a failure to protect the cells from oxidative stress. Yet another way of assessing mitochondrial dysfunction is to measure the concentration of antioxidant enzymes in permeabilized cells of interest by indirect flow cytometry with the specific antibodies [46].

Our studies revealed that the concentration one of these enzymes, catalase, tended to be decreased in BVDV-infected cells of both biotypes, but this tendency was small and statistically insignificant. However, another important antioxidant enzyme, peroxiredoxin PRDX1 [47], was altered depending on the BVDV biotype. In the BT cells infected with the cp biotype, its concentration during the first 24 hours post infection, compared to the cells infected with the ncp biotype, was significantly lower. Yet, 48 hours post infection it was significantly (more than threefold, $P<0.05$) higher in the cp BVDV-infected cells compared to the ncp BVDV-infected cells [39]. These results were confirmed by Western blotting. This result is different from the one we obtained by proteomics analysis of the bovine monocytes, where the expression of catalase was decreased in the cp BVDV-infected cells [29]. Moreover, we observed a significant increase of some isoforms of PRDX1 in the ncp BVDV-infected monocytes. Taken together, these results imply that in different cell types the coping with oxidative stress after BVDV infection may occur differently. Still, it is remarkable how profound was the observed oxidative stress (as judged from the increase in ROS), and an attempt of cells to cope with it (as judged from the increase in PRDX1) in BVDV-infected cells, and, in the case of the BT cell line, especially in the cp BVDV-infected cells.

Conclusion

To summarize, our studies, as well as studies by others [48], revealed that the cp and the ncp BVDV biotypes cause alterations in the proteomic profile and in the functions of the infected cells. These alterations may be found during the comparison between BVDV-infected cells (monocytes, bovine turbinate cell line) and the uninfected cells. Also, notably, some alterations seem to be peculiar for the cp BVDV biotype, but not for the ncp BVDV biotype. We suggest that by altering expression levels in multiple proteins related to immune responses such as cell adhesion, apoptosis, antigen uptake, processing and presentation, and other acute phase response proteins cp BVDV could significantly compromise immune defense mechanisms. Our data suggest that cp BVDV infection induces monocytes to differentiate into macrophages, or, alternatively, that monocytes that have already embarked on the differentiation into macrophages are more susceptible to cp BVDV infection.

Our finding indicates that ncp BVDV unlike the cp counterpart, by inhibiting the level of communication of the ECM and cell differentiation promotesthe establishment of PI. Our data indicate that three major signals, antigen recognition, co-stimulatory and cytokine signal, required to promote the effector activation of naïve T cells to be delivered by professional APC are impaired by both types of BVDV infection (Figure 1). We are especially intrigued by the observation that the cytopathic virus of cows causes infection characterized by mitochondrial dysfunction, making it similar to other (including human) viruses that kill cells, for example influenza virus. In our future work, we will attempt to shed some light on the question, how exactly the mitochondrial dysfunction develops and causes death of virus-infected cells of cattle and other organisms.

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