“Self” and “Nonself” Manipulation of Interferon Defense during Persistent Infection: Bovine Viral Diarrhea Virus Resists Alpha/Beta Interferon without Blocking Antiviral Activity against Unrelated Viruses Replicating in Its Host Cells

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Bovine viral diarrhea virus (BVDV), together with Classical swine fever virus (CSFV) and Border disease virus (BDV) of sheep, belongs to the genus Pestivirus of the Flaviviridae. BVDV is either cytopathic (cp) or non-cytopathic (ncp), as defined by its effect on cultured cells. Infection of pregnant animals with the ncp biotype may lead to the birth of persistently infected calves that are immunotolerant to the infecting viral strain. In addition to evading the adaptive immune system, BVDV evades key mechanisms of innate immunity. Previously, we showed that ncp BVDV inhibits the induction of apoptosis and alpha/beta interferon (IFN-α/β) synthesis by double-stranded RNA (dsRNA). Here, we report that (i) both ncp and cp BVDV block the induction by dsRNA of the Mx protein (which can also be induced in the absence of IFN signaling); (ii) neither biotype blocks the activity of IFN; and (iii) once infection is established, BVDV is largely resistant to the activity of IFN-α/β but (iv) does not interfere with the establishment of an antiviral state induced by IFN-α/β against unrelated viruses. The results of our study suggest that, in persistent infection, BVDV is able to evade a central element of innate immunity directed against itself without generally compromising its activity against unrelated viruses (“nonself”) that may replicate in cells infected with ncp BVDV. This highly selective “self” and “nonself” model of evasion of the interferon defense system may be a key element in the success of persistent infection in addition to immunotolerance initiated by the early time point of fetal infection.

Viruses possess mechanisms ensuring their dissemination and persistence in a given population despite the existence of host defense mechanisms. If the host has a short generation time, if the population is large, or if the mobility of the individual host is high, a simple “hit-and-run” strategy suffices for the virus to sustain a chain of infection. The survival strategy of other viruses rather consists of causing persistent infection in their hosts to achieve persistence in the population. To persist in an individual, these viruses must overcome their host’s immune response. Virtually every aspect of host defense has been subverted by viruses. Most examples of viral immune evasion have been reported to target the adaptive immune response (5, 15, 59, 74). In recent years it has become increasingly clear that vertebrate (and probably also subvertebrate) hosts possess an innate immune system which represents a first line of defense. It is not surprising that a bewildering diversity of mechanisms has been adopted by viruses to evade innate immunity. Many studies have shown that evasion of innate immunity may be just as important for viral persistence as evasion of adaptive immunity (14, 32, 34).

Type 1 interferons (IFN-α/β) are an important defense mechanism against viral infections of eukaryotic cells. Once synthesized and secreted by an infected cell, IFN binds to its receptor on the surface of many infected as well as uninfected cells to induce an antiviral state.

More than 100 cellular proteins are induced by IFN-α/β (22). Some of the best-characterized antiviral proteins induced by IFN-α/β are protein kinase PKR and 2′,5′-oligoadenylate synthetase, both activated by double-stranded RNA (dsRNA), and Mx protein (references 32, 33, and 66 and references therein). During the long period of coevolution of host cells and pathogens, many viruses developed mechanisms to evade the establishment of an antiviral state by IFN-α/β (14, 29, 34, 41, 66, 70). Thus, some viruses prevent the induction of IFN synthesis; others prevent the action of IFN after it has been produced, e.g., they encode proteins that bind IFN; yet others interfere with the signal transduction pathway activated after IFN has bound to its receptor; and many viruses inhibit the activity of IFN-induced antiviral proteins.

Bovine viral diarrhea virus (BVDV), together with Classical swine fever virus and Border disease virus of sheep, belongs to the genus Pestivirus in the Flaviviridae family. BVDV exists in two biotypes, cytopathic (cp) and noncytopathic (ncp), depending on their effect on cultured cells. It is a close relative of hepatitis C virus (HCV), a pathogen causing persistent infection in at least 170 million humans worldwide (6). BVDV is a similarly successful pathogen that, however, persists in its host population by a combination of transient and persistent infections. The latter differs fundamentally from other persistent viral infections by its association with immunotolerance specific to the persisting viral strain. This unique type of persistent infection is initiated when an ncp biotype of BVDV invades the fetus in utero early in its development. Infected fetuses may develop normally, but the infection persists for life. Approximately 0.5 to 2% of newborn calves in dairy herds are persis-
tently infected (PI) (36, 64). Such animals play a crucial role in the epidemiology of BVDV, as they permit the virus to persist in the host population for months to years in the absence of susceptible animals. The early period of fetal infection is essential because fetuses infected in the later stages of development as well as animals infected postnatally mount an adaptive immune response to BVDV which terminates the infection. The latter type of infection is far more frequent than persistent infection, as indicated by antibody prevalences of 60% or higher in the cattle population (36, 64). The strategy of bypassing the adaptive immune system by establishing tolerance is highly unusual and permits the virus to be extremely successful without the more common evasion strategies seen in persistent viral infections of immunocompetent animals (e.g., antigenic drift, presentation of decay antigens, and viral latency).

However, the early period of initial infection is insufficient to explain why only ncp, and not cp, BVDV is capable of causing persistent infection. The key to recognizing the mechanisms underlying this type of infection may lie in understanding the differences in the interaction with its host cells. Previous work by this and other laboratories has revealed a number of significant differences in the outcome of the interaction of ncp and cp biotypes of BVDV with different cell types. Cells infected with ncp BVDV do not undergo apoptosis (2, 58, 68, 81), but this biotype does not generally interfere with cell death, e.g., it does not inhibit apoptosis induced by unrelated viruses (9, 69). Most significantly, ncp BVDV does not induce the synthesis of IFN in its host cells (2, 58, 68), which led us to propose that evasion of this defense mechanism of the innate immune system may be crucial for the establishment of persistent infection (2, 69). This in vitro observation was subsequently confirmed in vivo by B. Charlestan and colleagues, who showed that fetuses infected early in their development do not respond to ncp BVDV with IFN formation, whereas the cp biotype readily infected early in their development do not respond to ncp BVDV with IFN formation, whereas the cp biotype readily

**MATERIALS AND METHODS**

**Reagents.** Cell culture media were purchased from Seromed (Biochrom, Munich, Germany), and fetal calf serum (FCS) was from Sigma or Oxoid GmbH (Wessel, Germany). FCS was free of BVDV and antibody to BVDV as tested by virus isolation and serum neutralization assay, respectively. Synthetic poly(IC) and lipopolysaccharide (Escherichia coli O55:BS, L-2637) were from Sigma. Recombinant bovine (rbo) IFN-α1 was generously provided by Novartis (Basel, Switzerland), and a monoclonal antibody to MxA (M143 [27]) was kindly provided by O. Haller (Institute for Medical Microbiology and Hygiene, University of Freiburg, Germany). Peroxidase-labeled donkey anti-mouse antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA), and mouse anti-β-actin (clone AC-74) was from Sigma. All other chemicals were of the highest purity commercially available.

**Cells and viruses.** Primary bovine testicle (BT) cells and calf testicle cells were prepared from testes and from young calves, respectively, that were obtained from a local abattoir, and cells were maintained in Earle’s minimal essential medium supplemented with 15% FCS (2% FCS after viral infection if not otherwise stated), penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO₂ atmosphere. MDBK cells (American Type Culture Collection, Manassas, VA) were used at passages 118 to 135 and were maintained under the same conditions as BT cells. MDBK-V7 cells are MDBK cells transfected with the plasmid pEF-SVS-V-IR ES.neo and cultured in the presence of Geneticin (80). This plasmid coding for the V protein of the paramyxovirus simian virus 5 was kindly provided by R. Randall (University of St. Andrews, Scotland), and the MDBK-V7 cells were prepared and kindly provided by A. Metzler (Institute of Veterinary Virology, University of Zurich, Switzerland). Monocyte-derived macrophages (M4) were obtained from the blood of Red Holstein cows as described previously (40). All cells were found to be free of BVDV by immunoperoxidase staining.

The NADL, TGAC (62), R1935, P515-Cp, and SuwA Cp cytopathic BVDV strains and the TGAN (62), SD-1 (21), Suwa Ncp, P515-Ncp, Ncp7, and 890 noncytopathic viruses were used (all strains are of BVDV genotype I except for strain 890, which belongs to genotype II). The NADL virus strain was from the Animal Health Laboratory, R1935, TGAN, and TGAC strains were kindly provided by A. Moennig (University of Veterinary Medicine, Hannover, Germany); SD-1 was provided by K. Brock (Auburn University College of Veterinary Medicine, Auburn, AL); Pe515-Cp and Pe515-Ncp were provided by J. W. McCauley (Institute for Animal Health, Compton, United Kingdom); Ncp7 was provided by T. Rümennap (Justus Liebig University, Giessen, Germany); and strain 890 was provided by J. F. Ridpath (National Animal Disease Center, Ames, IA). SwaA Ncp and Suwa Ncp are a virus pair isolated at our institute from an animal with mucosal disease (58), Vesicular stomatitis virus (VSV, strain Indiana) and encephalomyocarditis virus (EMCV) were kindly provided by H. Hengartner (Institute of Experimental Immunology, Zurich, Switzerland) and J. W. McCauley, respectively. BVDVs were passaged and titrated on BT cells as described previously (3), and the titer of the virus stocks was calculated according to the method of Reed and Muench (61). Virus-containing cell culture supernatants were freed from cell debris by centrifugation at 3,000 × g for 4°C for 10 min prior to virus titration.

**Virus infection.** BT, MDBK, or calf testicle cells were seeded in microwell plates (96 wells) or six-well plates at a density of 10⁶ (microwell plate) or 1.2 × 10⁶ to 1.8 × 10⁶ (six-well plate) cells/well. Media were plated in 6- and 24-well plates at 2 × 10⁵ and 5 × 10⁴ cells/well, respectively. Cells were infected with the appropriate BVDV strain in a small volume of culture medium without FCS at a multiplicity of infection (MOI) as described in the figure legends for 1 hour at 37°C. After adsorption of the virus, the inoculum was removed by washing the cells in culture medium without FCS prior to the addition of complete medium with 2% FCS. Using an MOI of 1 or higher, expression of NS3/NS23 viral proteins was detectable at 6 h postinfection as analyzed by Western blotting or immunohistochemistry using an anti-NS3/NS23 antibody (3), and all cells expressed NS3/NS23 around 12 to 18 h postinfection. Using an MOI of 0.01, all cells expressed NS3/NS23 at 24 h postinfection.

**Determination of cell viability.** Cell viability was determined either by DNA fragmentation analysis or by crystal violet staining. The fragmentation of cellular DNA was analyzed quantitatively by flow cytometry according to the method of Cossarizza et al. (19). Briefly, adherent and detached cells were collected by centrifugation at 250 × g and washed in phosphate-buffered saline (PBS), and the cell pellet (10⁶ cells) was lysed in 250 μl 0.1 M sodium citrate, pH 6.5, 1% Triton X-100, and 10 μg/ml propidium iodide. Nuclei were analyzed after a 30-min incubation at 4°C in the dark with a FACScan flow cytometer (Becton Dickinson, San Jose, CA), and a minimum of 10⁶ nuclei were analyzed. To determine the cell viability with crystal violet, adherent, viable cells in microwell plates were fixed and stained with 0.75% crystal violet in 50% ethanol, 0.25% NaCl, and 1.7% formaldehyde. Stained cells were rinsed with water, lysed in 0.6% sodium dodecyl sulfate (SDS), 40 mM HCl diluted in isopropanol, and the crystal violet intensity was measured at 590 nm with an enzyme-linked immunosorbent assay reader (1, 68).

**IFN-α/β activity.** Procedures for measuring biological activity of IFN-α/β in supernatants of virus-infected cells have been described previously. The as-
says used are based on the reduction of either Sendai virus or vesicular stomatitis virus growth by IFN-α/β contained in β-propiolactone-inactivated cell culture supernatants as determined by immunocytochemistry and crystal violet staining, respectively (57). To analyze the influence of BVDV infection on the activity of IFN-α/β, cells were mock infected or infected with ncp BVDV for 12 or 24 h prior to addition of IFN. Thereafter, the IFN-α/β activity was analyzed as described above and compared between uninfected and infected cells.

Passaging of BVDV-infected cells in the presence of IFN. To analyze whether BVDV can be eliminated from infected cells by prolonged treatment with IFN-α, MDBK cells were passaged 10 times in the absence or presence of IFN-α. Thus, MDBK cells were seeded in 25-cm² cell culture flasks at a density of 10⁶ cells/flask. Cells were infected with the BVDV strain SuwaNcp or 890 at an MOI of 1 as described above and incubated in complete medium with 7% FCS for 24 h (P1). Thereafter, the medium was replaced by medium without or with rbo IFN-α/1.1 at 1, 10, or 100 ng/ml. Cells were passaged twice weekly by transferring 10⁶ cells into new flasks and incubating them again in the absence or presence of IFN (P1 to P10). After 10 passages, the cells were passaged once more in the absence of IFN for all samples (P11). After each passage, the supernatant was used for virus titration, and total RNA was isolated from the remaining cells. Viral RNA was analyzed by real-time reverse transcription-PCR (RT-PCR) as described below.

In order to exclude the selection of IFN-resistant cells, parallel cultures of MDBK cells at P1, P7, and P10 treated with or without IFN were infected with VSV at an MOI of 0.01, and the amount of VSV in the supernatant was titrated 24 h postinfection.

Western blotting. Adherent BT, MDBK, or calf testicle cells were detached by trypsinization and collected by centrifugation at 250 × g, and the resulting cell pellet was washed in PBS. Adherent Mø were washed with PBS directly in the tissue culture plate. Cytosolic extracts were made using 50 to 100 μl per sample of M-PER mammalian protein extraction reagent (Pierce, Socochim SA, Lauzanne, Switzerland) according to the manufacturer's instructions. The protein content of the cytosolic extracts was determined using Coomassie Plus protein assay reagent (Pierce) and bovine immunoglobulin G as standard prior to lysis in 10% SDS-polyacrylamide gel electrophoresis sample buffer and boiling for 5 min. The proteins (20 to 50 μg total protein per lane) were separated on 10% SDS-polyacrylamide gels (Bio-Rad, Reinach BL, Switzerland) and electroblotted for 1.5 h at 110 V at 4°C in a Mini Trans-Blot cell (Bio-Rad) onto nitrocellulose membranes (Amersham Biosciences, Dübendorf, Switzerland). Unspecific binding sites on the membrane were blocked by 5% low-fat dry milk for 1 h at room temperature. To analyze for viral proteins, the blot was incubated with a monoclonal mouse anti-N3/N323 antibody (49DE9, generated at our institute) at a dilution of 1:50. To stain for cellular Mx protein (note that mouse Mx proteins can be found in the nucleus, whereas human or bovine Mx is exclusively located in the cytosol [4, 35, 49]), a mouse monoclonal antibody against human Mxα was used as the primary antibody (dilution, 1:10,000). To control the protein load per lane, the blot was stained with mouse anti-β-actin (dilution, 1:10,000) simultaneously with the anti-N3/N323 or the anti-Mx antibody. Peroxidase-labeled donkey anti-mouse antibody was used as secondary antibody (dilution 1:5,000) prior to ECL detection on Hyperfilm ECL (Amersham Biosciences) according to the manufacturer's protocol.

Real-time RT-PCR. Total RNA from cultured cells was isolated using the QIAamp RNA blood minikit (QIAGEN, Basel, Switzerland) according to the manufacturer's protocol. Real-time TaqMan RT-PCR was performed using the TaqMan One-Step RT-PCR Master Mix reagent kit (Applied Biosystems, Rotkreuz, Switzerland) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) according to the manufacturer's protocol. Primers and probes were designed using Perkin-Elmer Primer Express 2.0 software for the SuwaNcp BVDV strain (GenBank accession number AF117700) and the 890 strain (GenBank accession number U18059). Probes were labeled with fluorescence reporter 6-carboxyfluorescein for analysis of viral RNA and with VIC for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, respectively, at the 3′ end and with the fluorescence quencher 6-carboxytetramethylrhodamine at the 5′ end of the probe (Applied Biosystems). The reactions for BVDV and GAPDH were always run in separate tubes on the same plate with the appropriate positive and negative controls. Primers were obtained from Microsynth GmbH (Balgach, Switzerland). The relative amount of viral RNA compared to a control sample was calculated using the ΔΔCt method with GAPDH as internal control as described by Applied Biosystems (Applied Biosystems User Bulletin #2: ABI PRISM 7700 Sequence Detection System).

RESULTS

ncp as well as cp BVDV inhibits Mx induction by dsRNA but not that by IFN-α/β. We previously showed that ncp BVDV inhibits poly(IC)-induced apoptosis in BT cells (69). However, taking cell death as a readout, it was not possible to investigate whether a cytopathic BVDV strain would also block poly(IC)-induced apoptosis. Since expression of Mx protein was detected by Western blotting already at 2 to 6 h after addition of poly(IC) (not shown), i.e., well before the development of cell death induced by cp BVDV, we used this assay to analyze whether cp BVDV influences the effects of dsRNA. Poly(IC) dose dependently induced the expression of Mx in MDBK cells (Fig. 1), which was blocked by 2-aminopurine (not shown), a known inhibitor of PKR and related serine-threonine kinases (37, 38). Infection of the cells by the ncp BVDV strain SuwaNcp prior to the addition of up to 100 μg/ml poly(IC) completely prevented the expression of Mx (Fig. 1A). Cells had to be infected for at least 6 to 12 h prior to the addition of poly(IC) in order to obtain complete protection (not shown). Similarly, preinfection of MDBK cells with the R1935 cp BVDV strain completely blocked the synthesis of Mx protein (Fig. 1B). This effect was not restricted to these two viruses, since all cp and ncp strains tested (see Materials and Methods) blocked the expression of Mx protein induced by dsRNA (not shown). It is worth mentioning that most commercial prepa-
strains tested were equally unable to block Mx synthesis, even when the cells were preinfected for up to 24 h prior to addition of IFN-α. At the same time as Mx was analyzed (Fig. 2B), NS23 and NS3 were detected by Western blotting, and the amount of these proteins was only slightly reduced in the presence of high concentrations of IFN (Fig. 2C), even when analyzed up to 3 days posttreatment (not shown). Despite the problem that the half-life of NS(2)3 is not precisely known—but might be on the order of 12 to 24 h (42)—this indicates that the expression of viral proteins was not markedly reduced by the subsequent addition of IFN.

In order to test whether Mx synthesis is indeed dependent on the presence of interferon, we tested whether this protein can be induced in cells that do not respond to IFN-α/β. For this purpose, we used MDBK-V7 cells expressing the V protein of the paramyxovirus simian virus 5, which targets the transcription factor Stat-1 for proteasome-mediated degradation, thus blocking IFN-α/β-dependent signaling (80). Surprisingly, poly(IC) was still able to induce the expression of the Mx protein in MDBK-V7 cells, albeit to a small extent, whereas the cells were completely resistant to IFN-α/β up to 30 ng/ml (Fig. 3A, lower panel). In contrast, wild-type MDBK cells strongly produced Mx protein in response to both poly(IC) and rbo IFN-α/β (Fig. 3A, upper panel). Especially with 30 and 100 μg/ml poly(IC), the expression of Mx was strongly enhanced in MDBK cells, which suggests that Mx expression induced by dsRNA occurs mainly through a positive feedback loop mediated by IFN-α/β and only to a minor extent by the IFN-independent mechanism.

The inhibition by BVDV of Mx synthesis was not restricted to the MDBK cell line. Thus, similar results were obtained using BT cells and monocyte-derived Mφ, as well as monocyte-derived dendritic cells (not shown). This clearly shows that the inhibition of Mx induced by dsRNA, but not by IFN-α/β, is inherent to BVDV, independent of its biotype, of the virus strain, or the host cell type. Notably, avirulent as well as virulent strains (13) and viruses of genotypes I and II (56) were similarly effective. Recently, it was reported that cp BVDV induces the synthesis of Mx in calf testicle cells in the absence of any stimulation, e.g., by IFN or poly(IC) (9). None of our cp BVDV strains tested induced Mx expression in either BT or MDBK cells and only occasionally induced expression in monocyte-derived Mφ (Fig. 1B and data not shown). To clarify whether this difference is based on the cell types used, we infected calf testicle cells with various ncp and cp BVDV strains and tested for Mx expression by Western blotting at 1 or 2 days postinfection (Fig. 3B). All cp BVDV strains tested except the NADL strain strongly induced the synthesis of the Mx protein. Interestingly, also many of the ncp BVDV strains, and especially the 890 strain, were able to induce the expression of Mx at 2 days postinfection. Nevertheless, ncp BVDV strains (cp strains could not be tested due to strong Mx expression induced by the virus on its own) also inhibited the expression of Mx induced by dsRNA but not that induced by IFN-α in calf testicle cells, as described for the other cell types (not shown).

Although Mx is expressed upon IFN treatment in BVDV-infected cells, the replication of BVDV is not inhibited by subsequent addition of IFN-α/β. As described above, the expression of viral proteins was only marginally reduced by addi-
whether BVDV is resistant to type I IFN once infection has begun. IFN-β/H9251 was added to cultures of MDBK cells (upper panel) or MDBK-V7 cells (lower panel) were treated with calf testicle cells and nonresponsive (MDBK-V7) cells. (A) MDBK cells were either mock infected or infected with the ncp BVDV strains SuwaNcp (S-Ncp), Pe515-Ncp (515Ncp), TGAN, R1935, and NADL, respectively, at an MOI of 5 and incubated for 1 or 2 days at 37°C. Twenty micrograms of poly(IC) (pIC) per milliliter or 20 ng/ml rbo IFN-β/H9251 were added to the cultures. p.i., postinfection.

FIG. 3. Expression of Mx protein in IFN-responsive (MDBK and calf testicle cells) and nonresponsive (MDBK-V7) cells. (A) MDBK cells (upper panel) or MDBK-V7 cells (lower panel) were treated with poly(IC) at concentrations of 0 to 100 μg/ml and with rbo IFN-α/H9251 at concentrations of 0.01 to 30 ng/ml, respectively, as indicated. Cytosolic extracts were prepared 24 h later, and the expression of Mx protein was analyzed by Western blotting as described in Materials and Methods. Simultaneous staining of β-actin on the same membrane was used as a control for the protein loading of the individual lanes. The film was exposed for 10 seconds or 4 min as indicated. (B) Calf testicle cells were either mock infected or infected with the ncp BVDV strains SuwaNcp (S-Ncp), Pe515-Ncp (515Ncp), TGAN, and 890 and the cp BVDV strains SuwaCp (S-Cp), Pe515-Cp (515Cp), TGAC, R1935, and NADL, respectively, at an MOI of 5 and incubated for 1 or 2 days at 37°C. Twenty micrograms of poly(IC) (pIC) per milliliter or 20 ng/ml rbo IFN-α/H9251 (IFN) was used as a positive control for the expression of Mx protein. Cytosolic extracts were prepared at the time points indicated, and the expression of Mx protein and β-actin was analyzed by Western blotting as described in Materials and Methods. p.i., postinfection.

A dose-dependent manner, reducing the virus titer more than 200 times at 100 ng/ml IFN. Similarly, all cells remained infected by BVDV after treatment with IFN as judged by immunoperoxidase staining, whereas the percentage of infected cells was strongly decreased by treating the cells with IFN prior to infection (not shown). Nevertheless, even the highest concentration of IFN tested (100 ng/ml) could not completely prevent the infection of the cell monolayer with either biotype of BVDV (not shown). The reduction in virus particle release was mirrored by the reduced replication of viral RNA in the host cells. Thus, treatment of cells with IFN after infection reduced the level of viral RNA less than threefold compared to non-IFN-treated cells, whereas addition of IFN prior to infection reduced the amount of viral RNA up to 75-fold compared to non-IFN-treated cells (Fig. 4B). Similar results were obtained with monocyte-derived Mφ (Fig. 4C and 4D), except that the effects were more pronounced. Thus, the virus titer in the supernatant of Mφ (Fig. 4C) and the amount of intracellular viral RNA (Fig. 4D) were only slightly reduced by adding interferon after virus infection, whereas IFN treatment prior to infection led to a reduction of several orders of magnitude.

IFN-α/β cannot cure BVDV-infected cells. To determine whether BVDV can be eliminated from infected cells by prolonged IFN treatment, we passaged ncp BVDV-infected MDBK cells several times in the presence of various amounts of rbo IFN-α. Even 10 cell culture passages of MDBK cells infected with the SuwaNcp BVDV strain in the presence of up to 100 ng/ml rbo IFN-α were not able to eliminate the virus (Fig. 5), even though the virus titer in the cell culture supernatant was reduced in the presence of rbo IFN-α/H9251 (Fig. 5A). Similarly, intracellular viral RNA was only marginally reduced in the presence of 1 and 10 ng/ml IFN-α/H9251, whereas it was reduced 5- to 50-fold in the presence of 100 ng/ml IFN-α/H9251 compared to untreated cells (Fig. 5B). All cells remained infected by BVDV after treatment with IFN as judged by immunoperoxidase staining of parallel cultures of the last passage (P10, not shown), thus excluding the possibility that the reduction in viral replication observed in the presence of high concentrations of IFN is attributable to the elimination of the virus from a subset of cells. In addition, the MDBK cells remained fully sensitive to the activity of IFN-α, since the replication of the challenge virus VSV was inhibited by IFN to a similar extent at the 7th and 10th passages compared to the 1st passage (Fig. 5C). This excludes the possibility that the failure to eliminate BVDV is due to selection of IFN-resistant cells, which was reported for an HCV replicon-harboring cell line (52). Interestingly, the inhibition of viral replication by rbo IFN-α/H9251 was more prominent during the first five to six passages and then decreased. Additionally, after IFN was omitted during the last passage, the virus titer in the supernatant of the sample which was passaged 10 times in the presence of 100 ng/ml IFN was more than 1 magnitude higher (Fig. 5A) and the amount of viral RNA was three times higher (Fig. 5B) than in the control sample.

IFN treatment of cells persistently infected with ncp BVDV efficiently protects against infection with unrelated viruses. Recently, we showed that the induction of IFN-α/β synthesis stimulated by poly(IC) is completely inhibited in ncp BVDV-infected bovine Mφ (69). Here, we studied the effect of IFN on BVDV-infected cells. Addition of rbo IFN-α/H9251 to uninfected cells (Fig. 5)}
BT cells led to the induction of an antiviral state as judged by the dose-dependent prevention of VSV-induced apoptosis (Fig. 6A) and inhibition of VSV replication (Fig. 6B). Infection of BT cells with ncp BVDV 24 h before addition of IFN only marginally inhibited the activity of IFN-α/β against VSV (Fig. 6). Only the inhibition of VSV-induced apoptosis by 0.1 ng/ml IFN-α/β was significantly different between infected and uninfected cells (Fig. 6A). In addition to VSV, EMCV is often used as a challenge virus, and it was reported that the expression of the HCV proteins allowed significantly higher yields of EMCV, but not of VSV (28). However, infection of BT cells with BVDV 24 h before addition of IFN neither interfered with inhibition by IFN-α/β of EMCV replication (not shown). Thus, VSV- as well as EMCV-induced apoptosis and replication of these viruses were inhibited to a similar extent in BVDV-infected and noninfected cells.

**DISCUSSION**

Here, we report that, while being resistant to induction of IFN, cells infected with ncp BVDV are capable of responding to exogenous IFN with a robust antiviral state that protects from infection with unrelated viruses without, however, eliminating the ncp BVDV residing in the same cells. The experiments showing this unique evasion of the host’s IFN system by BVDV were initiated by comparing the effects of infection with ncp and cp biotypes on Mx synthesis induced by dsRNA. The expression of this antiviral protein was chosen as a readout because it precedes cell death by several hours. The Mx protein is believed to be induced exclusively via signaling through the type I IFN receptor (79), at least in human and murine cells (G. Kochs [University of Freiburg, Germany], personal communication). Surprisingly, Mx protein was induced by dsRNA also in cells in which the type I IFN signaling pathway had been reliably inactivated by expressing the V protein of simian virus 5, a paramyxovirus (80). This suggests that dsRNA may activate Mx expression in these cells also through a pathway independent of Stat-1, the protein targeted by V (Fig. 3A). The results of these experiments showed that, like ncp BVDV, the cp biotype blocks Mx induction by dsRNA. In addition, the fact that BVDV completely inhibits the synthesis of Mx induced by dsRNA indicates that BVDV inhibits the IFN production (thus avoiding IFN-induced Mx synthesis) as well as the IFN-independent, dsRNA-induced Mx expression.

The inhibition of Mx induction in response to dsRNA and the lack of interference with Mx expression in response to IFN are in accordance with the results of Baigent et al. (9), who showed that ncp BVDV inhibits Mx induction by poly(IC) or Semliki Forest virus, but not that by recombinant IFN-α. These authors also found that infection by the Pe515-Cp BVDV strain itself induced the synthesis of Mx in calf testicle cells.
Confirming and extending these observations, all cp BVDV strains tested—except the NADL strain—strongly induced the synthesis of Mx in calf testicle cells (Fig. 3B). The lack of Mx induction by NADL suggests that, at least with regard to Mx induction in bovine testicle cells, this widely used BVDV reference strain may in fact not be representative of the cp biotype. Remarkably, in calf testicle cells, several ncp BVDV strains were also able to induce the expression of Mx. These observations suggest that the difference between the cp and ncp biotypes might be gradual and depend on the strain as well as the biological context.

**FIG. 5.** Passaging of ncp BVDV-infected cells in the presence of rbo IFN-α is unable to eliminate the virus. MDBK cells in 25-cm² tissue culture flasks were infected with the SuwaNcp BVDV strain as described in Materials and Methods. At 24 h postinfection, the virus titer in the supernatant was analyzed (P1), and the medium was replaced with fresh medium without (A) or with 1 (●), 10 (○), or 100 (△) ng/ml rbo IFN-αL1. Thereafter, cells were passaged twice a week for 10 times as indicated (P1 to P10) with or without IFN as described for the first passage. For the last passage (P11), all samples were incubated in medium without IFN. After every passage, the BVDV titer in the supernatant (A) and the amount of intracellular viral RNA (B) were analyzed as described for Fig. 4 (representative experiments are shown). In order to exclude the selection of IFN-resistant cells, parallel cultures of MDBK cells at passages 1, 7, and 10 were infected with VSV at an MOI of 0.01, and the amount of VSV in the supernatant was titrated 24 h postinfection (C). TCID₅₀, 50% tissue culture infective dose.

**FIG. 6.** ncp BVDV does not inhibit or only slightly inhibits the activity of rbo IFN-α against unrelated viruses in BT cells. BT cells were mock infected (●) or infected with the TGAN (A), 890 (B), or Ncp7 (C) ncp BVDV strains (■), respectively, at an MOI of 3 at 24 h prior to the addition of rbo IFN-αL1 at various concentrations as indicated (w/o, without IFN). Six hours (A and B) or 24 h (C) after the addition of IFN, the cells were infected with VSV (A and B) or EMCV (C) at an MOI of 0.01 and incubated for another 20 to 24 h. (A) DNA fragmentation was quantified by propidium iodide staining of nuclei followed by fluorescence-activated cell sorting analysis as described in Materials and Methods. The percentages on the y axis indicate the relative number of nuclei with subgenomic DNA compared to the corresponding sample in the absence of IFN, which was set to 100%. For clarity, the mean ± 95% confidence interval (n = 3 to 8) is shown only for cells treated with more than 0.01 ng/ml IFN. (B) The titer of the challenge virus VSV was analyzed as described in Materials and Methods. For clarity, the mean ± 95% confidence interval (n = 2 to 6) is shown only for cells treated with at least 0.01 ng/ml IFN. TCID₅₀, 50% tissue culture infective dose. (C) Viable, adherent cells were stained with crystal violet (CV) and the intensity measured at 590 nm as described in Materials and Methods (average of triplicates ± standard deviation). OD₅₉₀, optical density at 590 nm.
parameters investigated. Interestingly, the fact that ncp and cp BVDV strains are more similar than anticipated is corroborated by the work of N. Tautz and associates, showing that also during the replication cycle of ncp, and not only cp, BVDV strains, NS23 cleavage and thus the expression of NS3 occur transiently (42). However, in bovine turbinate or MDBK cells we did not observe Mx induction with any of the cp and ncp BVDV strains tested, and Mx induction was only rarely detected with cp strains of BVDV in monocyte-derived Mo. The unusually high sensitivity to IFN induction observed in cultured testicle cells might be related to the immunologically privileged status of the testicles which is possibly reflected in the cell types present in cell cultures of low passage numbers used in our experiments. Interestingly, ncp BVDV has been demonstrated to persist in testicles and even to be excreted with sperm for an extended time after virus has been cleared from the bloodstream in transiently infected bulls (31, 78), suggesting that the action of the innate immune system alone might be insufficient for a rapid elimination of BVDV from this organ.

The induction of Mx protein by recombinant IFN-α in BVDV-infected cells suggested that signaling through the type I IFN receptor was possible despite the complete block of synthesis of bioactive IFN in response to dsRNA, thus indicating a complete separation of IFN induction from IFN action in these cells. The inhibition of replication of VSV and EMCV in BVDV-infected cells treated with recombinant IFN-α prior to infection with the challenge viruses provided direct evidence that BVDV-infected cells were still capable of mounting an antiviral state similar to that achieved in BVDV-uninfected cells. The two different viruses were used because IFN was demonstrated to inhibit the replication of VSV via the activity of Mx and possibly also PKR (7, 10, 73, 75) and the replication of EMCV via the 2’,5’-oligoadenylate synthase/RNase L pathway (43, 45). This suggests that the most important mechanisms mediating the antiviral activity of IFN remained intact in ncp BVDV-infected cells although these cells were refractory to IFN induction in response to dsRNA. The apparent contradiction of earlier reports that had demonstrated IFN induction in response to dsRNA may be explained by the presence of infected as well as uninfected cells in those studies (24, 63). Specifically, in cultures containing uninfected as well as infected cells the net response to dsRNA may be IFN production by uninfected cells, which obfuscates the interpretation. To overcome this problem, we carefully checked if all cells were infected (or uninfected) when comparing BVDV effects on IFN induction and IFN action. The observation of interference with IFN induction has now been demonstrated in various cell types with various strains of BVDV and also with classical swine fever virus (65, 69, 76).

In accordance with previous reports (30, 53, 71), we found that BVDV was sensitive to IFN in cells pretreated with IFN. A most remarkable and unexpected finding was that, in spite of inducing an antiviral state against unrelated superinfecting viruses, treatment with IFN did not eliminate BVDV from the cells even after 10 passages (Fig. 5) in the presence of a concentration of IFN that strongly protected the cells against BVDV when given prior to infection (Fig. 4). Different from previous reports that had also reported a failure of IFN to cure cell cultures containing infected as well as uninfected cells (12, 25), we showed that all cells remained infected during the passages in the presence of a high concentration of IFN. These experiments clearly show that cells infected with ncp BVDV are capable of mounting an antiviral state in response to IFN that protects against infection by unrelated viruses. It can also be inferred from these experiments that BVDV may have been passed on to daughter cells via cell division rather than via the extracellular space and that BVDV is largely resistant to IFN over several generations of persistently infected cells. Remarkably, the resistance to the action of IFN of BVDV residing in the cells was not correlated with the emergence of a general resistance of the cells to IFN, as has been reported in reference 52. Recently, prolonged treatment of persistently infected animals with large doses of recombinant human IFN-α2a, which is also active in bovine cells (54), failed to reduce viremia in these animals (55), which suggests that the resistance to elimination observed in vitro may apply also to the situation in vivo.

The resistance of BVDV towards IFN added postinfection is reminiscent of that of simian virus 40 or of respiratory syncytial virus (RSV). Thus, simian virus 40-specific RNA synthesis is inhibited by IFN added prior to infection but is not inhibited when IFN is added after infection (17, 20). Human RSV was inhibited to more than 90% by up to 10,000 U/ml IFN-α when pretreated for 20 h before infection, whereas treatment after infection had no effect. Moreover, RSV did not suppress the establishment of an antiviral state, since the block by IFN of parainfluenza virus type 3 replication was not affected (8). In contrast, the NS1 and NS2 proteins of bovine RSV (BRSV) were reported to cooperatively inhibit the activity of IFN-α/β (67), indicating that the NS proteins of BRSV interfere with the action of IFN rather than its induction. In contrast, a later report by the same group showed that BR SV does not inhibit Mx induction induced by IFN-α, indicating that the Jak-Stat pathway is not suppressed (16). Dengue virus, a flavivirus in the Flaviviridae family, is inhibited by IFN-α/β, provided that the cytokine is given at least 4 h prior to infection (23). However, it was recently shown that NS2A, NS4A, and NS4B of dengue virus inhibit the activity of IFN-α/β (39, 50, 51), in contrast to our study with BVDV. Although much has been learned of the way in which HCV, a close relative of BVDV, evades the IFN defense of its host, it is difficult to compare this directly to IFN evasion by BVDV, most prominently because data obtained with HCV replicons in cultured cells do not necessarily reflect the behavior of replication-competent parental virus in vivo.

The fact that both challenge viruses, VSV and EMCV, were susceptible to IFN in ncp BVDV-infected cells suggests that this virus may escape the Mx and PKR (7, 10, 73, 75) as well as the 2’,5’-oligoadenylate synthase/RNase L (43, 45) pathways which mediate the antiviral activity against VSV and EMCV, respectively. The selective evasion of the antiviral mechanisms by BVDV without interfering against their action against unrelated viruses suggests the existence of a “self” and “nonself” discrimination between a virus persistently infecting a cell and unrelated viruses capable of superinfecting the host cell of the virus residing in this cell. As a possible mechanism, the intracellular site of BVDV replication may be compartmentalized by lipid membranes and various proteins that coat the viral RNA, which both prevent and reduce the exposure of host defenses such as PKR or RNase L, as was suggested in elegant studies for HCV and flaviviruses (26, 46, 48, 77).

The unique evasion strategy of BVDV most likely has a
bearing on its interaction with individual cells and in fact also on its successful persistence in the cattle population. Approximately 0.5 to 2% of the cattle population are persistently infected (36, 64). Some PI animals show signs of ill thrift, but many others are clinically healthy (47) to the extent of giving birth to calves or being detected only when tested for BVDV prior to entry into artificial insemination stations. In PI animals, a variable percentage of diverse cells are infected, including monocytes, T and B cells, and cells of the stratum basale of the skin (11, 44, 60, 72). With regard to BVDV, these animals may be viewed as chimeric, with only some cells infected with a BVDV strain to which these animals are immuno-tolerant. Without discounting the complexities of extending observations made in vitro to the situation in vivo, we propose that the unique interaction of ncp BVDV with the IFN defense system may be a key to its success in maintaining persistent infection without globally compromising its host’s IFN defense system to fight viral infections. Thus, while BVDV-infected cells do not produce IFN in response to infection with unrelated viruses, IFN produced by BVDV-infected cells in response to these heterologous viruses might still protect many other cells, including those harboring BVDV, from infection with other viruses (“nonself”) while not terminating the persistence of BVDV virus in these cells (“self”). The largely intact IFN defense system against unrelated viruses (“nonself”) might explain why many PI animals remain healthy despite constantly producing large quantities of virus throughout their lives. Since shedding of virus by PI animals is not linked to disease, attenuated viruses would be favored over more virulent ones in their spread in the population. Hence, BVDV very elegantly manipulate the host’s IFN defense system without grossly compromising its host’s immune system: By not inducing IFN when invading the fetus early in its development, BVDV profits by the establishment of persistence and immuno-tolerance. Later in the life of the PI animal, BVDV is resistant to IFN in its host cells while not blocking its action against unrelated viruses. Finally, persistent infection may go undetected for years, and this increases the chance of transmission of BVDV to new susceptible animals due to the long life span of its host, and some persistently infected animals may even give birth to several PI calves.

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