Evaluation of cytotoxicity and antiviral activity of recombinant human interferon alfa-2a and recombinant human interferon alfa-B/D hybrid against bovine viral diarrhea virus, infectious bovine rhinotracheitis virus, and vesicular stomatitis virus in vitro

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Objective—To evaluate cytotoxicity and antiviral activity of recombinant human interferon alfa-2a and recombinant human interferon alfa-B/D hybrid against cytopathic and noncytopathic bovine viral diarrhea virus (BVDV), infectious bovine rhinotracheitis virus (IBRV), and vesicular stomatitis virus (VSV) in vitro.

Sample population—Primary bovine testicular cells and Mardin Darby bovine kidney cells.

Procedures—To evaluate cytotoxicity, cells were added to serial dilutions of each interferon. To evaluate antiviral activity of each interferon, interferons were serially diluted 1:10, and tissue culture cells were added; virus was then added at 3 time points. Prevention of viral infection by interferon was defined as failure to induce cytopathologic effect for VSV, IBRV, and cytopathic BVDV and failure to detect virus immunohistochemically for cytopathic and noncytopathic BVDV.

Results—No evidence of cytotoxicity in either cell line was detected after incubation with interferon alfa-2a or interferon alfa-B/D. However, reduced growth rates of tissue culture cells were detected for each interferon when undiluted interferon was tested. Comparable and profound antiviral activities against cytopathic and noncytopathic BVDV were evident for each interferon. Interferon alfa-2a and interferon a-B/D had comparable antiviral activities against VSV. Neither interferon had antiviral activity against IBRV.

Conclusions and Clinical Relevance—The safety and marked in vitro antiviral activity against noncytopathic BVDV, cytopathic BVDV, and VSV suggest that interferons alfa-2a and alfa-B/D may be useful for treatment of natural disease after infection with these viruses. (Am J Vet Res 2004;65:871–874)

Prevention and control of economically important viral diseases of cattle such as bovine viral diarrhea and infectious bovine rhinotracheitis have conventionally relied on vaccination to enhance passive and active immunity to these viruses and on preventive management practices designed to reduce exposure and susceptibility of cattle to these infectious agents. Increasingly specific antiviral drugs are being developed and used to treat retroviral, hepadnaviral, and herpetic infections in humans[1]; however, very few antiviral drugs have been evaluated for treatment of viral diseases of veterinary importance, particularly in large animals.[2] For economic and practical reasons, it is unlikely that specific antiviral drugs will be widely used to treat viral diseases in livestock in the near future. However, there is still a need to develop better treatments for many economically important viral diseases of calves and adult cattle. Numerous animal disease models exist in which the safety and efficacy of antiviral drugs can be investigated.[3]

The interferons are a family of cytokines with a broad range of biological activities, including inhibition of viral replication, antitumor activity, and induction of class I and II major histocompatibility complex antigens and Fc receptors.[4,5] In mammals, the alfa interferons are encoded by a closely related group of genes expressed predominantly in lymphoid and epithelial cells.[6,7] Quiescent lymphocytes do not produce detectable amounts of alfa interferon; however, after induction by a variety of agents, including viruses and double-stranded polyribonucleotides, detectable amounts are produced.[8-10] Previous investigations of potential therapeutic uses of interferons in cattle are limited to studies[11-14] that examined the ability of recombinant bovine interferon alfa-1 or recombinant human interferon alfa-1 to alleviate clinical disease caused by several common infectious agents. There is good reason to expect activity of human interferons in other species in vivo. Compared with cells of other species, bovine cells are the most sensitive to the antiviral effect of recombinant human interferons in vitro.[15,16]

Although human interferon alfa has a number of clinical indications and is federally licensed for treatment of hepatitis B and C virus infections as well as several types of leukemia, many patients develop dose-limiting adverse effects and respond incompletely to treatment.[17-19] These problems have stimulated considerable interest in the potentially improved clinical effi-

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cacy and safety of recombinant hybrid interferons. One of these interferons is recombinant human interferon alfa-B/D, a hybrid product of 2 human interferon alfa genes. Interferon alfa-B/D is comprised of amino acids 1 to 60 of interferon alfa-B and amino acids 61 to 166 of interferon alfa-D and is obtained by splicing the 2 parent genes and inserting part of the interferon alfa-D gene into the interferon alfa-B gene.[20] The cross-species activity of the hybrid molecule is substantially greater than that of either parent interferon; the hybrid molecule is biologically active in several experimental animal species and experimental virus infection models.[21,22]

The purposes of the study reported here were to evaluate the cytotoxicity and in vitro antiviral activity of recombinant human interferon alfa-2a and recombinant human interferon alfa-B/D hybrid against **infectious bovine rhinotracheitis virus (IBRV)**, cytopathic and noncytopathic **bovine viral diarrhea virus (BVDV)**, and **vesicular stomatitis virus (VSV)**.

Materials and Methods

Cytotoxicity assays—A stock solution containing 11.1 µg of pure recombinant human interferon alfa-2a/mL[a] and a stock solution containing 11.1 µg of a novel recombinant human interferon alfa-B/D hybrid/mL were prepared. Recombinant human interferon alfa-B/D hybrid was produced as described.[20] The interferon preparations were stored carrier-free in phosphate-buffered saline solution at 4°C at a concentration of 0.2 mg/mL (3.5×10^7 U/mL).

A 10-µL aliquot of each interferon stock solution was serially diluted 1:10 in sterile endotoxin-free physiologic saline (0.9% NaCl) solution across a 96-well plate, yielding concentrations of 11.1 µg/mL to 1.11×10^{-12} µg/mL. Ninety microliters of growth media was added to each well. Tissue culture cells sufficient to create a viable monolayer were added, and plates were incubated at 37°C in 5% CO_2 for 72 hours. Mardin Darby bovine kidney cell line[b] and a primary bovine testicular cell line[b] were used; both cell lines are known to be BVDV-free. Each interferon was tested in triplicate. At the end of the incubation period, each well was examined for evidence of cytotoxicity via inverted light microscopy and compared with control wells without interferon.

In vitro antiviral activity of interferons—Stock solutions of each interferon and serial dilutions were prepared as described for the cytotoxicity assays. Growth media and tissue culture cells were added as for the cytotoxicity assays. Three 96-well plates were prepared simultaneously for each interferon to allow for addition of virus at 3 time points. Vesicular stomatitis virus,[c] IBRV (IBR-CJC) obtained from a clinically affected animal and maintained as a stock laboratory culture, a cytopathic strain of BVDV (BVDV-NADL) obtained from stock laboratory cultures, and a noncytopathic strain of BVDV (BVDV-63) obtained from a persistently infected animal and maintained as a stock laboratory culture were used. For each virus, 100 $TCID_{50}$ were added either immediately after addition of tissue culture cells to interferon (**time 0 [T0]**), after 16 hours of incubation of tissue culture cells with interferon (T16), or after 24 hours of incubation of tissue culture cells with interferon (T24). Each assay was performed in triplicate in Mardin Darby bovine kidney and primary bovine testicular cell lines. All plates were incubated at 37°C in 5% CO_2 for 5 days. Wells were examined via inverted light microscopy on each of the 5 days of incubation for evidence of cytopathologic effect for cytopathic BVDV, IBRV, and VSV. Cytopathologic effect was used to determine the end point.[23,24]

Noncytopathic and cytopathic BVDV infection of tissue culture cells was detected by use of an immunoperoxidase staining technique. After 5 days of incubation, cells were fixed in stock fixative solution (20% acetone, 79.98% PBS solution, 0.02% bovine serum albumin), processed, stained by use of an immunoperoxidase technique, and examined for positive staining as described.[25]

Statistical analyses—The highest dilution at which each interferon prevented either infection (detected via immunoperoxidase staining technique) or cytopathologic effect (detected via inverted light microscopy) when virus was added at T0, T16, and T24 was recorded. Experiments were performed in triplicate, and the median value of 3 results for each time point was compared between interferon types by use of the Wilcoxon signed rank test. For each virus-interferon treatment data set, comparisons between values obtained at T0, T16, and T24 were initially performed by use of Kruskal-Wallis 1-way ANOVA by ranks with significance set at $P \leq 0.05$. Subsequent correction for all pair-wise comparisons within each virus-interferon treatment data set was performed by use of a commercial software program.[d] Values of $P \leq 0.05$ were considered significant.

Results

Cytotoxicity assays—No evidence of cytotoxicity was detected for either interferon in all interferon dilutions tested. All plates revealed growth of tissue culture cells to confluence within 72 hours, comparable to that observed in control wells without interferon. However, wells containing undiluted interferon (11.1 µg/mL) revealed reduction of growth to 80% of confluence for each interferon after 72 hours of incubation, compared with control wells without interferon. This negative influence of each interferon was interpreted as growth retardation and not cytotoxic effect.

In vitro antiviral activity of interferons—Identical results were obtained in both cell lines (**Table 1**). No

Table 1—In vitro antiviral activity of recombinant human interferon alfa-2a (IFN alfa-2a) and recombinant human interferon alfa-B/D hybrid (IFN alfa-B/D) against cytopathic bovine viral diarrhea virus (BVDV-NADL), noncytopathic bovine viral diarrhea virus (BVDV-63), vesicular stomatitis virus (VSV), and infectious bovine rhinotracheitis virus (IBRV) in Mardin Darby bovine kidney cell line and a primary bovine testicular cell line. Virus was added to cell culture containing interferon at time 0 (T0), after 16 hours of incubation of cell culture with interferon (T16), and after 24 hours of incubation of cell culture with interferon (T24). Identical results were obtained in both cell lines. Each interferon dilution is the median value of 3 determinations

		Interferon dilution (µg/mL)		
Virus	**Interferon type**	**T0**	**T16**	**T24**
BVDV-NADL*	IFN alfa-2a	1.11×10^{-5}	1.11×10^{-6}	1.11×10^{-6}
	IFN alfa-B/D	1.11×10^{-5}	1.11×10^{-5}	1.11×10^{-7}
BVDV-NADL†	IFN alfa-2a	1.11×10^{-1}	1.11×10^{-5a}	1.11×10^{-5a}
	IFN alfa-B/D	1.11×10^{-1}	1.11×10^{-5a}	1.11×10^{-5a}
BVDV-63†	IFN alfa-2a	$< 1.11 \times 10^{-1}$	1.11×10^{-6a}	1.11×10^{-7a}
	IFN alfa-B/D	$< 1.11 \times 10^{-1}$	1.11×10^{-6a}	1.11×10^{-7a}
VSV*	IFN alfa-2a	1.11×10^{-6}	1.11×10^{-12a}	1.11×10^{-10a}
	IFN alfa-B/D	1.11×10^{-6}	1.11×10^{-11a}	1.11×10^{-10a}
IBRV*	IFN alfa-2a	$< 1.11 \times 10^{-1}$	$< 1.11 \times 10^{-1}$	$< 1.11 \times 10^{-1}$
	IFN alfa-B/D	$< 1.11 \times 10^{-1}$	$< 1.11 \times 10^{-1}$	$< 1.11 \times 10^{-1}$

*Highest dilution at which interferon prevented virus from causing cytopathologic effect. †Highest dilution at which interferon prevented viral infection of cells.
[a]Significantly ($P \leq 0.05$) different than value at T0.

significant difference in antiviral activity against any of the viruses was detected between the 2 interferons.

Recombinant human interferon a-2a and interferon alfa-B/D hybrid had marked antiviral activity against noncytopathic and cytopathic BVDV (except for noncytopathic BVDV when virus was added at T0; Table 1). When cytopathic BVDV was added to cell culture and interferon at T0, prevention of cytopathologic effect (detected via inverted light microscopy) was detected at higher dilutions of interferon than was prevention of infection (detected via immunoperoxidase staining technique), suggesting that the immunoperoxidase staining technique is a more sensitive indicator of in vitro viral infection. Infection by cytopathic BVDV (detected via immunoperoxidase staining technique) was prevented at T0 by the 1.11×10^{-1} μg/mL dilution for each interferon, whereas for noncytopathic virus, even this dilution did not prevent infection. Interferon alfa-2a and interferon alfa-B/D had comparable and profound antiviral activity against VSV. Neither interferon was capable of preventing cytopathologic effect after IBRV infection.

For VSV, noncytopathic BVDV, and cytopathic BVDV (detected by the immunoperoxidase technique only), significant differences ($P = 0.018$) in the highest dilutions of both interferons that prevented cytopathologic effects or infection were detected among the 3 time points (T0, T16, and T24). A multiple pair-wise comparisons procedure was used to detect which of the values at the 3 time points were different from the others. In all instances, significance in the Kruskal-Wallis ANOVA was attributable to differences between T0 and T16 or T24; no differences were found between the T16 and T24 time points. However, when multiple comparison criteria were used, *P* values exceeded 0.05 but were < 0.10 for these comparisons.

There was no significant difference in the highest dilutions of either interferon when virus was added at T0 and T16 when cytopathic BVDV infection was detected via inverted light microscopy (cytopathologic effect). The highest dilutions of interferon at which either cytopathologic effect or infection were prevented were not significantly different for noncytopathic and cytopathic BVDV and VSV when virus was added at T24 compared with when it was added at T16.

Discussion

Our findings of profound in vitro antiviral activities of recombinant human interferon alfa-2a and recombinant human interferon alfa-B/D hybrid against bovine viruses in bovine cell lines complement the findings of previous studies that examined in vitro activity of pure recombinant human interferons against BVDV,[23] IBRV, parainfluenza 3 virus, and respiratory syncytial virus.[26] Fulton et al[26] found that human interferon alfa-1 and human interferon alfa-A/D could reduce BVDV and IBRV viral loads in bovine monolayer cultures. In our study, however, cytopathologic effect caused by IBRV was not prevented by either interferon in either cell line. Absence of in vitro antiviral activity against IBRV may be explained by the observation that DNA viruses are less affected by interferons than are RNA viruses. This difference is partly explained by the fact that 2 well-characterized interferon-induced compounds with antiviral effects, 2-5A synthetase and P1 kinase, require double-stranded RNA as a cofactor for activation.[8-10]

For all viruses tested, no significant differences between the highest dilutions at which each interferon prevented cytopathologic effect or infection at each time point were detected. This suggests that there is no difference between in vitro antiviral activity of recombinant human interferon alfa-2a and that of recombinant human interferon alfa-B/D hybrid against cytopathic BVDV, noncytopathic BVDV, and VSV. The highest dilutions recorded for each interferon were identical or within a 1:10 dilution. These results differed from the results of previous studies that compared in vitro antiviral activity of interferon alfa-B/D hybrid with that of the pure lymphoblastoid[21,27] or its parent recombinant interferons alfa-B[20,22] or alfa-D molecules. The recombinant human interferon alfa-B/D hybrid had enhanced antiviral activity compared with either of its purified parental molecules.[20,22] The interferon alfa-B/D hybrid had affinity for bovine cells; however, it is not known whether the mechanism of receptor binding affinity is the consequence of receptor recognition by a portion of either parental molecule or the result of a unique conformational change that occurs during hybridization and enhances binding affinity.[21] The profound in vitro activities of many human interferons in bovine cells suggests that substantial interferon receptor homology exists between bovine and human cells.[21,28,29]

The absence of cytotoxicity and the profound antiviral activities against cytopathic BVDV and noncytopathic BVDV (except at T0 for noncytopathic BVDV) found for recombinant human interferon alfa-2a and recombinant human interferon alfa-B/D hybrid suggest that recombinant human alfa interferons may be useful treatments for cattle infected with BVDV. Both interferons had the ability to prevent infection of cells with cytopathic BVDV when virus was added to cell culture and interferon at T0; however, even the lowest dilution of both interferons did not prevent infection of cells with noncytopathic BVDV at T0.

For noncytopathic BVDV, cytopathic BVDV (detected via immunoperoxidase staining technique only), and VSV, the time of virus addition to cell culture containing either interferon was important with respect to antiviral effect. Statistical analyses by use of Kruskal-Wallis ANOVA indicated that, compared with T0, significantly higher dilutions of interferons were able to abrogate infection or cytopathologic effect when interferon and cell culture were incubated for 16 or 24 hours prior to addition of these viruses. The differences between T0 and the latter time points (Table 1) suggest that multiple comparisons of pair-wise differences with the attendant requirement for a lower significant *P* value may be unnecessary and in fact may lead to a type 2 statistical error. However, there were no significant differences in the highest dilutions of each interferon that prevented cytopathologic effects caused by cytopathic BVDV at all time points. These findings may have negative clinical implications; exogenous interferon would most likely only be administered after virus infection has occurred. An antiviral effect was

detected at T0 for cytopathic BVDV and VSV but not noncytopathic BVDV, although it is also unlikely that exogenous interferon would be given coincident with the onset of natural infection.

The pathogenicity of persistent infection with BVDV may be attributable to the ability of noncytopathic BVDV to inhibit fetal interferon production during the first trimester of pregnancy by use of a process that depends on double-stranded RNA.[30,31] Whether or not exogenous interferon treatment of persistently infected cattle could result in reduction of viral load, or result in seroconversion and clearance of the virus, is uncertain. Seroconversion alone is unlikely to clear persistent infection; persistently infected cattle frequently have partial immune responses to the heterogeneous strains of BVDV encountered in nature or to vaccine strains.[32] However, persistently infected cattle may spontaneously clear the persistently infecting BVDV strain.[e] Although it is unlikely that persistently infected cattle will be treated with interferon in the future, investigations of therapeutic approaches for persistent flavivirus viremia are important from the standpoint of animal and human health.

[a]Roferon-A, Hoffman-LaRoche Inc, Nutley, NJ.
[b]American Type Culture Collection, Manassas, Va.
[c]Provided by Dr. L. E Carmichael, Baker Institute, Cornell University, Ithaca, NY.
[d]Statistix, Analytical Software, Tallahassee, Fla.
[e]Bonds MD, Larson LJ, Schultz RD. Three animals persistently infected with BVDV that became virus negative (abstr), in *Proceedings.* 81st Annu Conf Res Work Anim Dis 2000;76.

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