

Evaluation of antiviral activity and toxicity of recombinant human interferon alfa-2a in calves persistently infected with type 1 bovine viral diarrhea virus

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Objective—To evaluate antiviral activity and toxicity of recombinant human interferon alfa-2a in calves persistently infected with noncytopathic type 1 bovine viral diarrhea virus (BVDV).

Animals—5 Holstein heifers, 4 to 12 months of age.

Procedures—Calves persistently infected with noncytopathic type 1 BVDV were treated with recombinant human interferon alfa-2a every other day for 12 weeks. Viral loads were measured during the treatment period and compared with pre- and post-treatment values. Complete physical examinations were performed weekly, and calves were observed daily for signs of systemic illness. Complete blood counts and serum biochemical analyses were performed before, during, and after the treatment period. Because calves developed anemia during the treatment period, bone marrow biopsy specimens were collected. Anti-recombinant human interferon alfa-2a antibody concentrations in serum samples obtained before, during, and after the treatment period were measured by use of an ELISA.

Results—Recombinant human interferon alfa-2a had no antiviral activity against noncytopathic type 1 BVDV in persistently infected calves. All calves developed microcytic anemia during the treatment period that persisted for up to 13 weeks after cessation of treatment. Anti-interferon antibodies were detected during the treatment period and persisted for at least 2 weeks after cessation of treatment.

Conclusions and Clinical Relevance—Because of lack of in vivo antiviral activity against BVDV, recombinant human interferon alfa-2a has little promise as a therapeutic agent for the treatment of BVDV infection, at least in persistently infected cattle. Furthermore, treatment was associated with adverse immunologic and hematologic effects. (*Am J Vet Res* 2004;65:865–870)

Bovine viral diarrhea virus (BVDV) infection can cause economically important disease that may

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have enteric, reproductive, immunosuppressive, and hemorrhagic clinical manifestations.¹ Treatment of bovine viral diarrhea is purely supportive and nonspecific. Specific antiviral drug use for the treatment of retroviral, herpetic, and flavivirus infections in humans is rapidly increasing.² Humans infected with hepatitis C virus (a flavivirus closely related to BVDV) can be persistently infected; treatment with interferon alfa is frequently attempted, and response rates of up to 40% have been reported when interferon is administered with other antiviral agents.³ Persistent BVDV infection in cattle develops when the fetus is infected with a noncytopathic strain of BVDV in utero prior to development of fetal immunocompetence (approx days 100 to 120 of gestation) and the pregnancy goes to term.^{4,5} Persistently infected calves have detectable viremia for the rest of their lives; therefore, calves persistently infected with BVDV are excellent models in which to examine the in vivo efficacy of specific antiviral drugs. We have previously demonstrated lack of cytotoxicity and a profound in vitro antiviral activity against noncytopathic BVDV⁶ for recombinant human interferon alfa-2a. The purpose of the study reported here was to evaluate the toxicity and antiviral activity of recombinant human interferon alfa-2a in calves persistently infected with noncytopathic type 1 BVDV.

Materials and Methods

Cattle—Five Holstein heifers, 4 to 12 months of age and persistently infected with noncytopathic type 1 BVDV, were used in the study. Persistent infection was confirmed via repeated microplate ELISA tests^{7,8} that yielded positive results for BVDV over a 3-month period prior to the onset of the study. Use of a monoclonal antibody panel,^{9,10} both before and after the start of interferon treatment, confirmed that the persistent strain of BVDV was a type 1 virus in each calf. The calves were observed for signs of systemic illness daily for 3 months prior to treatment with interferon. The calves were kept in separate group housing at an accredited (Association for the Assessment and Accreditation of Laboratory Animal Care International) research facility, and all experimental procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Procedures—Calves were treated with recombinant human interferon alfa-2a^a (10,000 U/kg, IM) every other day for 12 weeks. Because only small volumes of interferon were required (0.15 to 0.4 mL), interferon was diluted in sterile physiologic saline (0.9% NaCl) solution to a final volume of 2 mL and injected into the semimembranosus-semitendinosus muscle mass. Calves were observed daily for signs of systemic illness, and complete physical examinations were

performed weekly. A weight tape was used to estimate body weight every other week; body weight was monitored as part of toxicity assessment of interferon treatment and to allow for adjustment of interferon dosage.

Hematologic and serum biochemical analyses—Complete blood counts and serum biochemical analyses were performed immediately prior to the treatment period (week 0) and at weeks -10, -8, and -1. Blood samples were collected and analyzed weekly during the 12-week treatment period, then 1, 2, 4, 8, 12, 13, 18, 20, 24, and 32 weeks after cessation of treatment. Complete blood counts were performed by use of an electronic cell counting system,^b and leukocyte differential counts and blood smear evaluations were performed by technical staff in the Clinical Pathology Section, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin. An automated chemistry analyzer^c was used to measure serum alkaline phosphatase; γ -glutamyl transferase; aspartate aminotransferase; creatine kinase activities; and total bilirubin, sodium, potassium, chloride, calcium, inorganic phosphorus, glucose, BUN, creatinine, total protein, albumin, and cholesterol concentrations in serum.

Bone marrow examinations—Because all calves developed anemia during the treatment period, bone marrow aspirates and biopsy specimens were collected in week 7. Calves were sedated with xylazine (0.02 to 0.05 mg/kg, IV), local analgesia was administered, and bone marrow samples were collected from the midsternum by use of a standard technique. Slides of aspirated material were prepared, air-dried, stained with Wright-Giemsa, and examined via light microscopy. Biopsy specimens were fixed in neutral-buffered 10% formalin, stained with H&E, and examined via light microscopy. A second histologic section was stained with Gomori modified Prussian blue for evaluation of bone marrow iron stores.

Quantitation of BVDV load in plasma—The BVDV loads in plasma were measured 10 weeks, 1 week, and immediately prior to the treatment period; in weeks 1, 3, 6, 9, and 12 of the treatment period; and 2, 8, and 18 weeks after cessation of treatment. Blood samples were collected via jugular venipuncture into glass tubes containing sodium heparin. Blood samples were centrifuged at 1,200 \times g for 10 minutes within 2 hours of collection. Plasma was separated and stored at -80°C until analyses were performed. Thawed plasma samples were diluted 1:100 in Hank's medium.^d Serial dilutions (1:2) of the diluted plasma in tissue culture medium^e were made in duplicate across flat bottom tissue culture plates. Cells from a primary bovine testicular cell line^f were added to form a monolayer, and plates were incubated at 37°C in 5% CO₂ for 4 days. At the end of the incubation period, cells were fixed in stock fixative solution (20% acetone, 79.98% PBS solution 0.02% bovine serum albumin), stained via an immunoperoxidase technique, and examined for positive staining, as described.^{7,8} Viral load was expressed as the highest dilution resulting in 50% positive immunoperoxidase staining of the cell monolayer (TCID₅₀), as described.⁷

Detection of BVDV in plasma samples—To confirm the presence of noncytopathic BVDV and absence of cytopathic BVDV before and at the end of the treatment period, plasma samples were incubated with tissue culture cells. Presence of noncytopathic virus was confirmed via immunoperoxidase staining of tissue culture cells,⁷ and absence of cytopathic virus was confirmed via lack of detection of cytopathologic effect, as described.¹¹

Detection of anti-BVDV antibodies in serum—Neutralizing antibodies against BVDV were measured in

serum samples obtained for serum biochemical analyses, by use of a described technique.⁸

Evaluation of anti-recombinant human interferon alfa-2a antibodies—Because all calves developed anemia during the treatment period, and to investigate the possibility of an immune-mediated component to the anemia, a semiquantitative ELISA was developed to measure concentrations of anti-recombinant human interferon alfa-2a antibodies in serum. Serum samples that had been obtained for biochemical analyses and stored at -80°C were used.

Serum samples collected 1 week and immediately prior to the treatment period; in weeks 2, 3, 6, and 8 of the treatment period; and 2, 12, and 24 weeks after cessation of treatment were used. Pure recombinant human interferon alfa-2a was diluted to a concentration of 1.0 μ g/mL in 0.1M carbonate (pH 9.6), and 100 μ L of the diluted interferon was added to each well of a 96-well plate and incubated for 12 hours at 4°C. The plate was washed 3 times with 0.15M sodium chloride solution followed by 0.1% Tween, blocked via addition of 1% bovine serum albumin (w/v) in PBS solution, incubated at room temperature (21°C) for 1 hour, and washed 3 more times. Fifty microliters of serum diluted 1:100 in sample diluent buffer (0.05% Tween in PBS solution) was added to each well, and the plate was incubated at room temperature for 1 hour. The plate was washed 3 times, 100 μ L of rabbit anti-bovine IgG conjugated with horseradish peroxidase and diluted 1:2,000 in 0.05% Tween in PBS solution was added to each well, and the plate was incubated at room temperature for 1 hour. The plate was washed 3 times, and 100 μ L of a substrate solution containing 98.7% 0.05M sodium citrate (pH, 4.0), 1% 0.4mM 2,2'-azinobis[3-ethylbenzthiazoline] sulfonic acid, and 0.3% hydrogen peroxide solution was added until a green color developed. The reaction was stopped with the addition of 200 μ L of 2N hydrochloric acid to each well. The optical density of each well was measured at 416 nm in a commercial microplate reader.⁸ Each ELISA was performed in duplicate.

Coombs' tests—To establish whether anti-recombinant human interferon alfa-2a antibodies detected via ELISA were associated with development of anemia, Coombs' tests were performed. Blood and serum samples collected during and after the treatment period were used. Direct Coombs' tests were performed on fresh blood samples collected from each calf 10 weeks after cessation of treatment. Indirect Coombs' tests were performed by use of packed RBCs from whole blood samples obtained 10 weeks after cessation of treatment and frozen serum collected in week 8 of the treatment period. This modification was necessary because only serum samples, and not RBCs, collected during the treatment period were available. Packed RBCs obtained from each calf 10 weeks after cessation of treatment were resuspended in 0.3 mL of PBSS that contained 10% recombinant human interferon alfa-2a and incubated at 37°C in 5% CO₂ for 1 hour. The sample was centrifuged, and the packed RBCs were resuspended in 200 μ L of serum obtained from each calf in week 8 of the treatment period (time point at which the highest antibody concentrations were measured via ELISA). Samples were incubated at 37°C in 5% CO₂ for 1 hour and centrifuged, and the serum was removed. Packed RBCs were resuspended in 0.4 mL of PBS solution, 100 μ L of bovine Coombs' (anti-bovine IgG and C3) reagent was added, and the samples were incubated at 37°C in 5% CO₂ for 30 minutes, after which they were examined for evidence of hemolysis and agglutination. Each Coombs' test was performed in duplicate.

Statistical analyses—Because of difficulties in obtaining age-matched control calves persistently infected with noncytopathic type 1 BVDV, calves acted as their own historical controls. Mean or median hematologic and serum biochemical values, viral titers, and concentrations of anti-recombi-

nant human interferon alfa-2a antibodies measured during the treatment period were compared with pretreatment values by use of the paired Student *t* test and Wilcoxon rank sum test for Gaussian and non-Gaussian data, respectively. A Bonferroni correction for multiple *t* tests was used. For all comparisons, $P \leq 0.05$ was considered significant.

Results

Treatment of calves persistently infected with noncytopathic type 1 BVDV with recombinant human interferon alfa-2a for 12 weeks was not associated with a significant decrease in plasma BVDV titers, compared with the median pretreatment titer (Fig 1). No significant differences in viral titers were found between the 18-week follow-up period values after cessation of treatment and pretreatment titers or titers measured during the treatment period. One calf died 15 weeks after cessation of treatment; therefore, no blood sample was available at the final time point for this calf. Calves were seronegative for antibodies against the persistently infecting noncytopathic strain of type 1 BVDV prior to the treatment period and remained seronegative during the treatment period and for 6 months after cessation of treatment. Only noncytopathic virus was detected in plasma samples obtained from each calf at the end of the treatment period.

Treatment of calves persistently infected with noncytopathic type 1 BVDV with recombinant human interferon alfa-2a was associated with decreases in Hct (Fig 2) and mean corpuscular volume (Fig 3). Blood smear evaluation revealed marked anisocytosis and microcytosis and absence of polychromasia and basophilic stippling, consistent with a nonregenerative microcytic anemia. Median Hct and median mean corpuscular volume were significantly ($P \leq 0.05$) lower than the median value of the 3 pretreatment values from week 6 of the treatment period to 13 weeks after cessation of treatment for Hct and from week 2 of the treatment period to 8 weeks after the cessation of treatment for mean corpuscular volume. Examination of Prussian blue-stained bone marrow sections revealed subjectively low iron reserves in each calf, compared with iron

reserves in bone marrow of healthy adult cattle. Bone marrow examination revealed no abnormalities of myeloid and erythroid cell populations. Myeloid to erythroid ratios ranged from 0.7:1 to 1.4:1. Results of direct and indirect Coombs' tests for each calf were negative. No significant differences in other hematologic and serum biochemical values were found among pretreatment, treatment, and post-treatment periods. Calves retained their appetites, remained afebrile, and gained weight during the course of treatment. No signs of pain or swelling at the sites of injection, lameness, or high muscle enzyme activities were evident. One calf died as a result of diffuse acute enteritis 15 weeks after cessation of treatment; however, the remaining calves

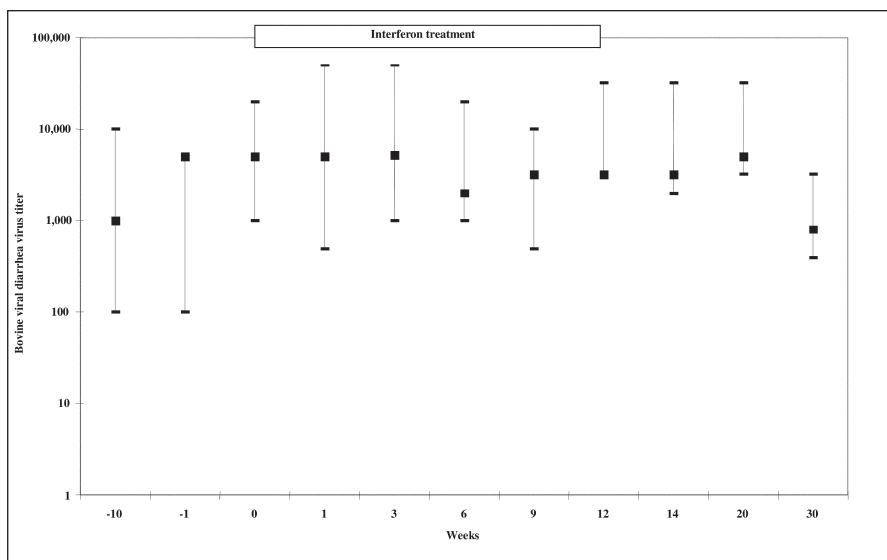


Figure 1—Serum bovine viral diarrhoea virus (BVDV) titers (median, range) before, during, and after treatment of 5 calves persistently infected with noncytopathic type 1 BVDV with recombinant human interferon α -2a. Bovine viral diarrhoea virus titer is expressed as the reciprocal of the highest dilution that caused infection of 50% of tissue culture cells (TCID₅₀). No significant differences were detected among values.

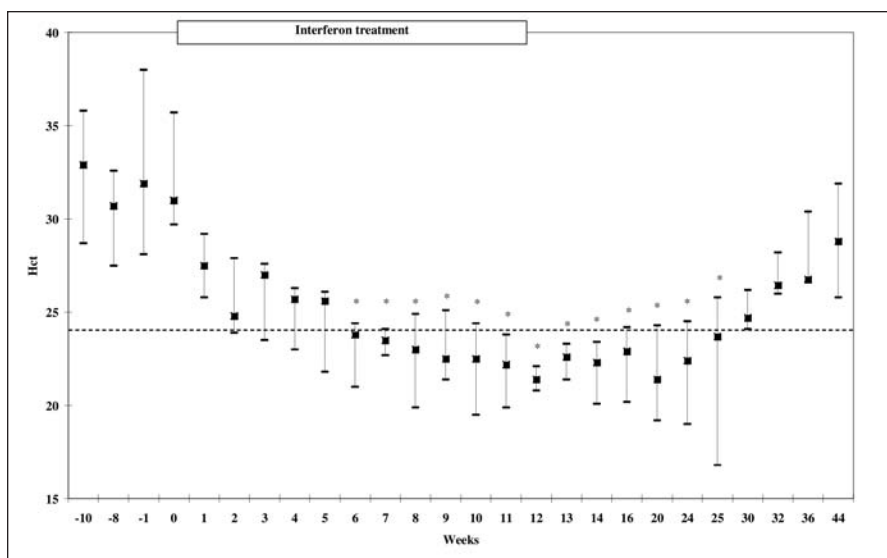


Figure 2—Hct (%; median, range) before, during, and after treatment of 5 calves persistently infected with noncytopathic type 1 BVDV with recombinant human interferon alfa-2a. The dotted line indicates the lower limit of the reference range (24% to 45%). *Significantly ($P \leq 0.05$) lower than median value of 3 pretreatment values.

survived for a minimum of 9 months after cessation of treatment.

Treatment of calves persistently infected with noncytopathic type 1 BVDV with recombinant human interferon alfa-2a was associated with the induction of anti-recombinant human interferon alfa-2a antibodies (Fig 4). Significant ($P \leq 0.05$) increases in median ELISA optical density above pretreatment values were detected as early as week 3 of the treatment period and persisted for at least 2 weeks after cessation of treatment.

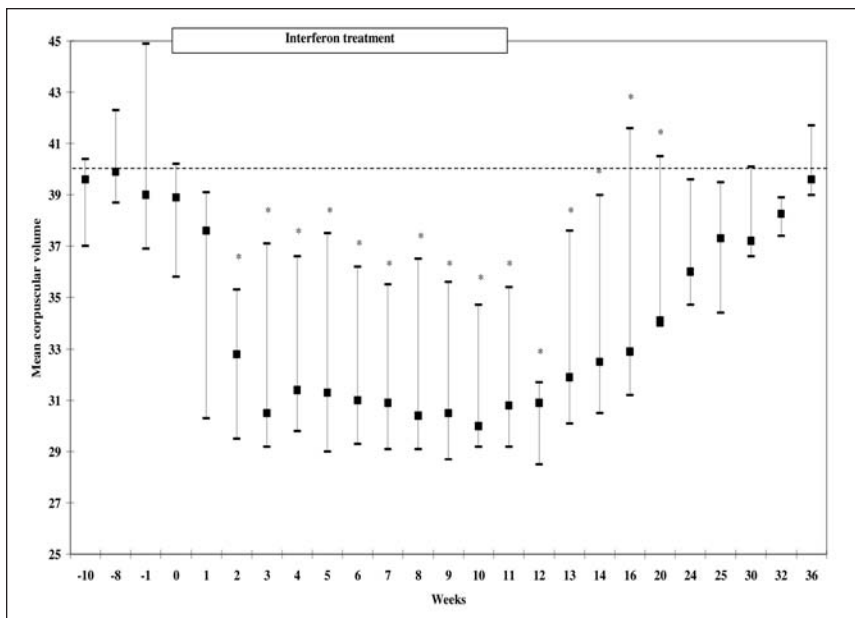


Figure 3—Mean corpuscular volume (fL; median, range) before, during, and after treatment of 5 calves persistently infected with noncytopathic type 1 BVDV with recombinant human interferon alfa-2a. The dotted line indicates the lower limit of the reference range (40 to 60 fL). *Significantly ($P \leq 0.05$) lower than median value of 3 pretreatment values.

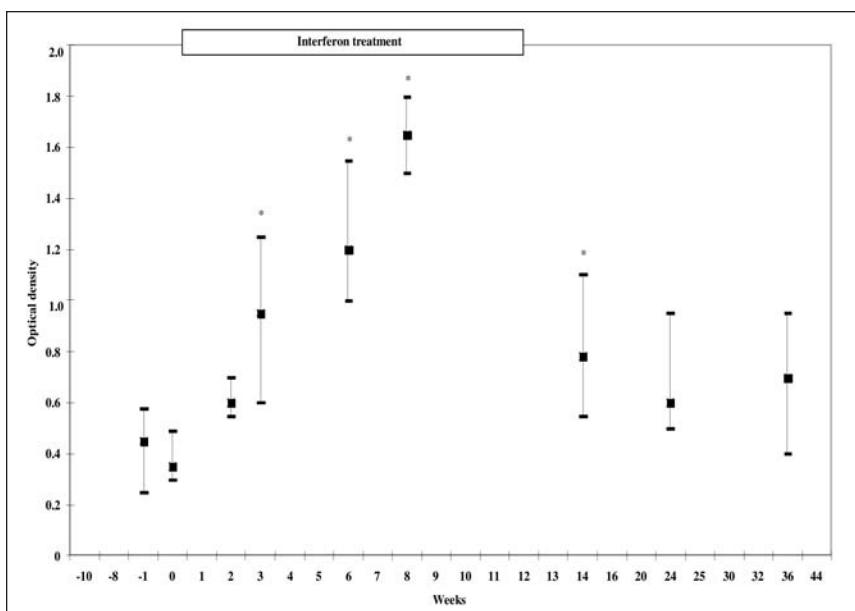


Figure 4—Concentration of anti-recombinant human interferon alfa-2a antibodies (optical density; median, range) before, during, and after treatment of 5 calves persistently infected with noncytopathic type 1 BVDV with recombinant human interferon alfa-2a. *Significantly ($P \leq 0.05$) higher than median value of 2 pretreatment values. Values at weeks -1 and 0 represent background readings.

Discussion

Recombinant human interferon alfa-2a had no antiviral activity against noncytopathic type 1 BVDV in persistently infected calves. The absence of in vivo antiviral activity was unexpected; recombinant human interferon alfa-2a had profound antiviral activity against noncytopathic (except when noncytopathic BVDV, interferon alfa-2a, and cell culture were combined at the same time [T0] and incubated) and cytopathic BVDV in vitro.⁶ The activities of interferons in vivo, however, particularly when administered to a nonhomologous species, are impossible to predict purely on the basis of in vitro activity.¹² Evaluation of antiviral activities of pure bovine lymphoblastoid or recombinant bovine alfa interferons against BVDV in vivo would have been ideal; however, these products were unavailable. Recombinant human interferon alfa-2a was used in our study because it had profound antiviral activity against BVDV in vitro⁶ and because bovine cells, compared with cells of other species, are the most sensitive to the antiviral effect of recombinant human interferons in vitro.^{13,14} Strong binding affinities between recombinant human alfa-interferons and the bovine alfa-interferon receptor have been reported. These receptors are responsible for triggering downstream cellular events that lead to the antiviral effects of the alfa interferons.¹⁵ Whether nonhomologous species ligand-receptor affinity in vitro translates to quantitatively substantial downstream induction of antiviral effector molecules (ie, bovine 2-5A synthetase, latent RNAases, and P1 kinase) in vivo is not known. Whether anti-interferon antibodies such as those detected in our study are capable of impairing or blocking the critical ligand-receptor interaction in vivo in a manner that cannot be predicted in vitro is also not known.

Bovine viral diarrhea virus-host-interferon interactions may be different in persistently infected cattle than in immunocompetent cattle. Establishment of persistent infection is the result of failure of alfa interferon response to the infecting noncytopathic BVDV in early gestational embryos.⁴ Noncytopathic BVDV can inhibit the induction of endogenous interferon production by double-

stranded viral RNA.⁵ Whether the inhibition of endogenous interferon induction can also explain the lack of response to exogenously administered alfa interferons is not known. The absence of *in vivo* antiviral activity of recombinant human interferon alfa-2a in our study may have been the result of inadequate dosage. The interferon dosage was selected on the basis of the *in vitro* activity of recombinant human interferon alfa-2a against a common challenge virus (vesicular stomatitis virus) in bovine and human cell cultures^{14,16} and extrapolation from the National Institutes of Health standard used to calculate interferon dose for human use.¹⁴

Use of a monoclonal antibody panel against various BVDV strains before and after the start of interferon treatment revealed that the persistent strain of noncytopathic BVDV was a type 1 strain in each calf. Genomic and strain analyses were not performed. All calves originated from the same farm that had had a history of BVDV-related disease over the preceding 12 months, suggesting, but not confirming, that all calves were infected with the same BVDV strain. Different strains of BVDV may have different susceptibilities to the antiviral activity of interferon; however, this issue was not addressed in our study. Mutations within hypervariable regions of the flavivirus genome may occur during specific antiviral treatment of human hepatitis C virus infections and are frequently associated with poor therapeutic responses.^{17,18} In our study, the persistently infecting noncytopathic strain of BVDV did not mutate to the cytopathic form during the treatment period; no cytopathologic effect was detected in tissue culture cells when plasma containing BVDV obtained at the end of the treatment period was added.

Calves persistently infected with noncytopathic type 1 BVDV and treated with recombinant human interferon alfa-2a for 12 weeks developed microcytic nonregenerative anemia associated with subjectively low bone marrow iron reserves. Hematocrit returned to reference range and mean corpuscular volume returned to pretreatment values after cessation of treatment, suggesting that the anemia was the direct result of interferon treatment; however, this took up to 20 weeks after cessation of treatment. Microcytic nonregenerative anemia with low bone marrow iron reserves suggests iron deficiency anemia that is typically the result of chronic hemorrhage; however, no macroscopic evidence of blood loss from the gastrointestinal or genitourinary tracts and no evidence of ectoparasitism were found. Chronic blood loss from the gastrointestinal tract is plausible in cattle persistently infected with BVDV; however, a specific drug effect is strongly suggested by the return of RBC parameters to pretreatment values after cessation of treatment. Significant increases in concentrations of anti-recombinant human interferon alfa-2a antibodies during the treatment period suggested the possibility of an immune-mediated mechanism of anemia; however, direct Coombs' tests were performed 10 weeks after cessation of treatment, at which time concentrations of anti-recombinant human interferon alfa-2a antibodies may have decreased to concentrations undetectable via direct Coombs' test. Indirect Coombs' tests were performed on samples obtained during the treatment period

because only sera obtained during the treatment period were available. Anemia of chronic or inflammatory disease was also considered, particularly given the immunocompromised state of persistently infected cattle; however, no hematologic or serum biochemical indicators of inflammatory disease and no physical examination findings consistent with chronic antigenic stimulation and inflammation (fever, chronic respiratory or enteric disease, or injection site reactions) were detected.

Dose- and treatment-limiting adverse effects may be associated with interferon alfa treatment of infectious and neoplastic diseases in humans. The recombinant human interferon alfa-2a used in our study is licensed in the United States for treatment of hairy cell leukemia, acquired immunodeficiency syndrome-related Kaposi's sarcoma, chronic myelogenous leukemia, and chronic hepatitis C virus infection. Bovine viral diarrhea virus and hepatitis C virus are closely related flaviviruses. This fact influenced our choice of interferon. Virologic response rates (defined as viral clearance to undetectable amounts) of up to 40% have been reported in patients chronically infected with hepatitis C virus who were treated with interferon alfa in combination with other antiviral agents (ie, ribavirin); however, a sustained response rate to recombinant human interferon alfa-2a treatment alone could be as low as 9%.³ However, the criterion used for virologic response rate was viral clearance to undetectable amounts; a lesser decrease in viral load was found in a higher proportion of patients who completed treatment.¹⁹ The effect of recombinant human interferon alfa-2a treatment on viral load in our calves was not significant, and it is unlikely that a different effect would have been found had a larger number of calves been treated.

The most commonly reported adverse effects in human patients treated with recombinant human interferon alfa include myalgia, fatigue, headache, nausea, vomiting, and neuropsychiatric effects including depression and insomnia.^{20,21} No physical abnormalities were detected in calves during our study, and calves continued to eat and gain weight throughout the study period. The absence of signs of pain or swelling at the sites of injection, lameness, or high muscle enzyme activities suggest that interferon treatment was well tolerated. Hematologic abnormalities detected in non-leukemic human patients treated with recombinant human interferon alfa-2a include leukopenia, neutropenia, and thrombocytopenia but not anemia.^{19,22} Neutralizing antibodies against recombinant human interferon alfa-2a have been detected in up to 9% of patients with chronic hepatitis C virus infection treated with this interferon 3 times/week over a 3-month period.²³ The clinical importance of anti-interferon antibodies in humans is not known; however, a higher rate of treatment failure was evident in patients that developed antibodies against interferon.^{19,23}

^aRoferon-A (Interferon alfa-2a recombinant), Hoffman-LaRoche Inc, Nutley, NJ.

^bAdvia 120, Bayer Corp, Pittsburgh, Pa.

^cVitros Chemistry System, Orthoclinical Diagnostics, Raritan, NJ.

^dGibco Life Technologies, Grand Island, NY.

^eEagle minimum essential medium, Sigma Chemical Co, St Louis, Mo.

[†]American Type Culture Collection, Manassas, Va.

[‡]Bio-Rad model 550, Bio-Rad Laboratories, Hercules, Calif.

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