Induction of antigen-specific T-cell subset activation to bovine respiratory disease viruses by a modified-live virus vaccine

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**Objective**—To determine the efficacy of a modified-live virus vaccine containing bovine herpes virus 1 (BHV-1), bovine respiratory syncytial virus (BRSV), parainfluenza virus 3, and bovine viral diarrhea virus (BVDV) types 1 and 2 to induce neutralizing antibodies and cell-mediated immunity in naive cattle and protect against BHV-1 challenge.

**Animals**—17 calves.

**Procedures**—8 calves were mock-vaccinated with saline (0.9% NaCl solution) (control calves), and 9 calves were vaccinated at 15 to 16 weeks of age. All calves were challenged with BHV-1 25 weeks after vaccination. Neutralizing antibodies and T-cell responsiveness were tested on the day of vaccination and periodically after vaccination and BHV-1 challenge. Specific T-cell responses were evaluated by comparing CD25 upregulation and intracellular interferon-γ expression by 5-color flow cytometry. Titration of BHV-1 in nasal secretions was performed daily after challenge.

**Results**—Vaccinated calves seroconverted by week 4 after vaccination. Antigen-specific cell-mediated immune responses, by CD25 expression index, were significantly higher in vaccinated calves than control calves. Compared with control calves, antigen-specific interferon-γ expression was significantly higher in calves during weeks 4 to 8 after vaccination, declining by week 24. After BHV-1 challenge, both neutralizing antibodies and T-cell responses of vaccinated calves had anamnestic responses to BHV-1. Vaccinated calves shed virus in nasal secretions at significantly lower titers for a shorter period and had significantly lower rectal temperatures than control calves.

**Conclusion and Clinical Relevance**—A single dose of vaccine effectively induced humoral and cellular immune responses against BHV-1, BRSV, and BVDV types 1 and 2 and protected calves after BHV-1 challenge. (Am J Vet Res 2006;67:1179–1184)

**Abbreviations**

- IFN-γ: Interferon-γ
- BHV-1: Bovine herpes virus 1
- BRSV: Bovine respiratory syncytial virus
- PI-3: Parainfluenza virus 3
- BVDV: Bovine viral diarrhea virus
- IL-2: Interleukin-2
- TCR: T-cell receptor
- SVN: Serum virus neutralization
- CMI: Cell-mediated immunity
- PBMC: Peripheral blood mononuclear cell
- FBS: Fetal bovine serum
- PBSS++: PBS solution with 0.5% FBS and 0.1% sodium azide
- Δ%IFN-γ+: Net increases in the percentage of IFN-γ-positive cells
- EI: Expression indices
- NK: Natural killer

Resistance to infection by respiratory disease viruses involves several aspects of the immune response. Serum virus-neutralizing antibodies are important and are the component of immunity that is most often measured as an indication of resistance to infection by respiratory disease viruses. Other components of the immune response that may contribute to protection include IgA in mucus of the nasal passage, which can prevent virus attachment to epithelial cells; T-helper-1 cells, which can secrete IFN-γ and other cytokines; cytotoxic T cells, which can kill virus-infected cells; and γδ T cells, which are believed to help control viral infection of epithelial cells. Serum virus-neutralizing antibodies may be sufficient to protect healthy nonstressed animals from low-level challenge during experimental conditions. However, animals in field conditions exposed to various stressors and to varying amounts of multiple infectious agents may be better protected if all of the relevant aspects of acquired immunity have been effectively stimulated by vaccination.

Several modified-live and killed-virus vaccines containing the 5 major viruses that contribute to the bovine respiratory disease complex (BHV-1, BRSV, PI-3, and BVDV types 1 and 2) are licensed by the USDA and are commercially available. These vaccines may contain various strains and concentrations of viruses and various adjuvants, and they may induce various levels and types of immune responses.

The purpose of the study reported here was to determine the efficacy of a new modified-live virus vaccine containing all 5 major bovine respiratory disease viruses to induce antigen-specific T-helper cells, cytotoxic T cells, and γδ T cells to heterologous strains of BHV-1, BRSV, and BVDV types 1 and 2 for as long as 6 months after vaccination. Flow cytometry was used to detect upregulation of CD25 (α chain of IL-2 receptor) and IFN-γ expression on CD4+, CD8+, and γδ TCR+ T-
strain via nebulization of 2 mL of virus suspension into (week 0), weeks 4, 5, 6, 8, 24, and 25 after vaccination, and collected from both groups of calves on the day of vaccination and at 14 days after challenge. 

Body weight was recorded immediately prior to challenge and at allocations and nasal secretions were collected daily. Body temperature and clinical observations in all cattle were recorded by a veterinarian who was unaware of treatment group. 

Virus vaccines of the same 5 viruses were included in the CMI assay as positive controls. The study was performed with a commercial facility. The secondary antibody conjugate mixture consisted of mouse anti-mouse IgG1-Alexa Fluor 488, goat anti-mouse IgG2a-R-phycocerythrin (R-PE), goat anti-mouse IgM-Alexa Fluor 647, and goat anti-mouse IgG2b-PE-Cy5. All antibodies were diluted in PBS++ to titrated optimum dilutions determined during preliminary titration studies. 

Vaccine—The vaccine used in the study was a pentavalent modified-live virus vaccine containing BVDV type 1 (cytopathic strain Singer, genotype 1a), BVDV type 2 (cytopathic strain 125A, genotype 2a), BHV-1 (strain Baker), BRSV (strain Lemkuhl 375), and PI-3 (strain Aboit). 

Seventeen colostrum-deprived calves (seronegative and CMI negative for the 4 viruses) were housed at the Veterinary Resources Inc facilities in Cambridge, Iowa, and randomly allocated to 2 groups. Calves in group A (n = 8) were used as negative controls and were mock-vaccinated with saline (0.9% NaCl) solution (control-group calves). Calves in the other group (n = 9) were vaccinated with a modified-live virus vaccine (2 mL, SC) at 15 to 16 weeks of age (vaccinated calves). After vaccination, the 2 groups were separated in the same facility. On week 25 after vaccination, all cattle received 4 mL of 10^7 TCID50/mL virulent BHV-1 Cooper strain via nebulization of 2 mL of virus suspension into each naris. For 14 days after BHV-1 challenge, rectal temperature and clinical observations in all cattle were recorded by a veterinarian who was unaware of treatment group allocations and nasal secretions were collected daily. Body weight was recorded immediately prior to challenge and at 14 days after challenge. 

Before blood samples for SVN testing and CMI assay were collected from both groups of calves on the day of vaccination (week 0), weeks 4, 5, 6, 8, 24, and 25 after vaccination, and weeks 1 and 2 after challenge (weeks 26 and 27). Specific T-cell-subset responses to BHV-1, BRSV, and BVDV types 1 and 2 were evaluated by measuring the upregulation of surface expression of CD25 and the expression of IFN-γ in cultured PBMCs by use of 5-color flow cytometry. Anticoagulated blood samples (approx 35 mL) for CMI assays were collected in 5 mL of acid citrate dextrose (0.15M sodium citrate, 0.076M citric acid monohydrate, and 0.287M dextrose). Blood samples from 3 cattle that received periodic administration of modified-live virus vaccines of the same 5 viruses were included in the CMI assay as positive controls. The study was performed with approval of the Iowa State University Committee on Animal Care (Assurance No. 8-04-5717-B). 

Serum virus neutralization test—Serum samples for the SVN tests were separated on the day of blood collection and frozen at -20°C until tested. Sera were submitted to the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University for standard SVN testing to BHV-1, BRSV, PI-3, and BVDV types 1 and 2. 

Flow cytometry—Five-color flow cytometric analysis was performed by personnel of the Flow Cytometry Facility at Iowa State University, by use of a cytometer. Data were col-
lected from at least 10,000 live cells from each sample. Computer software was used to analyze flow data. Four major bovine T-cell subsets, CD4+CD8+γδ TCR– (CD4+ [28.3%]), CD4–CD8+γδ TCR– (CD8+ [19.1%]), CD4–CD8+γδ TCR+ (CD8+γδ TCR+ [2.5%]), and CD4–CD8–γδ TCR+ (γδ TCR+ [35.9%]) and cells with no T-cell marker (non–T cells [13.2%]) were identified. Percentages of each T-cell subset expressing CD25 and their geometric mean fluorescent intensities were determined. The CD25 EI were calculated by dividing the product (percentage CD25+ X mean fluorescent intensities) from antigen-stimulated cells by the product from non-antigen-stimulated cells of the same subset of the same calf. The Δ%IFN-γ were calculated by subtracting the percentage of IFN-γ+ of non-antigen-stimulated cells from the percentage of IFN-γ+ of antigen-stimulated cells of the same subset of the same calf. The T-cell subset CD4–CD8+γδ TCR+ (CD8+γδ TCR+) had low numbers of both CD25+ and IFN-γ+ cells and was not analyzed further.

Nasal secretion samples and virus titration—Samples of nasal secretions from all cattle were aseptically collected daily from the nares beginning on the day before BHV-1 challenge and continuing for 14 days after challenge. Samples were stored at –70°C until virus titration was performed. Samples of nasal secretions were thawed and centrifuged at 1,800 X g for 10 minutes. Clear supernatants were 10-fold serially diluted in CO2 independent medium up to a dilution of 10–7. Each dilution, including a sample of undiluted nasal secretions, was inoculated into 4 wells of confluent Madin-Darby bovine kidney cells in 96-well tissue culture plates (100 μL/well). An additional 100 μL of tissue culture medi-
um² with supplemental 15% FBS and 1.5% penicillin-streptomycin solution was added to wells inoculated with undiluted nasal secretions and dilutions of 10⁻¹ and 10⁻² to help dilute any potential cell toxicity from concentrated samples. Plates were incubated in a CO₂ incubator at 37°C for 3 days, and the typical round BHV-1 cytopathic effect was observed and recorded. The BHV-1 titers were calculated from the reciprocal of the highest nasal secretion dilutions that had cytopathic effects in 50% of the infected wells. Mean log virus titers for each experimental group for each day were obtained.

**Statistical analysis**—Statistical analysis was performed by use of computer software. The difference of the means of the vaccinated and control groups was compared by use of ANOVA. A t test or pooled t test was used accordingly to assess differences between the 2 groups for each period. Values of P < 0.05 were considered significant.

**Results**

All calves were seronegative to BHV-1, BRSV, PI-3, and BVDV types 1 and 2 prior to vaccination (Figure 1). Control-group calves remained seronegative throughout the study, except that they seroconverted to BHV-1 by week 2 after challenge. Vaccinated calves seroconverted to all 5 viruses by week 4 after vaccination, and neutralizing antibodies remained detectable throughout the study. The neutralizing antibody titer to BHV-1 in vaccinated calves increased significantly (P < 0.05) after challenge and was significantly (P < 0.05) higher than the titer in control-group calves, indicating an anamnestic response.

To simplify presentation of CMI assay results, the mean results from weeks 4, 5, 6, and 8 after vaccination as well as the mean results from weeks 24 and 25 are presented. Results from week 1 and 2 after challenge are depicted separately.

Results in all calves were negative for CD25 EI to BHV-1, BRSV, and BVDV types 1 and 2 on the day of vaccination. In vaccinated calves, the mean CD25 EI of total PBMCs for all 4 viruses 4 to 8 weeks after vaccination were significantly higher than that in control-group calves. When analyzed by cell subset at weeks 4 to 8 after vaccination, all subsets, except CD8+ for BHV-1 and BRSV stimulation, had significant increases in mean CD25 EI, compared with control-group calves. By 24 to 25 weeks after vaccination, all cell subsets responded significantly to all 4 viruses tested. After BHV-1 challenge on week 25, vaccinated calves had markedly increased responses to BHV-1 within 1 week after challenge, especially in CD4+ and γδ TCR+ subsets. Control-group calves started to respond to BHV-1 stimulation by week 2 after challenge. Mean CD25 EI of vaccinated calves to both types of BVDV had signifi-

![Graphical representation of CMI assay results](image-url)
significantly higher responses to recall antigens throughout the study. The increased responses to BRSV in vaccinated calves were not significantly higher than background responses to BRSV in control-group calves at week 2 after challenge (Figure 2). This may have been because of increased variability in background expression of CD25 on PBMCs after challenge with BHV-1.

Results in all calves were negative for Δ%IFN-γ+ to BHV-1, BRSV, and BVDV types 1 and 2 on the day of vaccination. The mean Δ%IFN-γ+ of total PBMCs for all viruses at 4 to 8 weeks after vaccination were significantly higher in vaccinated calves than in control-group calves. When analyzed by cell subset for this same period in vaccinated calves all subsets, except CD8+ for BHV-1 and γδ TCR+ for BRSV stimulation, had significant (P < 0.05) increases in Δ%IFN-γ+, compared with that in control-group calves. By 24 to 25 weeks after vaccination, the mean Δ%IFN-γ+ for all tested viruses in vaccinated calves were not significantly (P > 0.05) higher than that in control-group calves, except for the CD8+ subset response to BVDV type 2. One week after BHV-1 challenge, all cell subsets in vaccinated calves had strong increases (P < 0.05) in mean Δ%IFN-γ+, compared with responses in control-group calves. These differences remained significant at week 2 after challenge, except for the CD8+ and γδ TCR+ subsets. This was apparently because control-group calves started to respond to BHV-1 by 2 weeks after challenge (Figure 3).

Results of BHV-1 titration in nasal secretion samples are depicted (Figure 4). The initial challenge inoculum replicated equally in both vaccinated and control-group calves. However, vaccinated calves had markedly (P ≤ 0.05) lower virus shedding in nasal secretions from 3 to 8 days after challenge, compared with control-group calves. Vaccinated calves stopped shedding BHV-1 on day 8 after challenge, 2 days earlier than control-group calves.

Mean rectal temperature in control-group calves was significantly higher from 3 to 7 days after challenge than that in vaccinated calves (Figure 5). Only mild clinical signs were observed after challenge with no obvious difference between groups. The mean body weight gain from day 0 to 14 after BHV-1 challenge in vaccinated calves (5.34 kg) was also higher than that in control-group calves (2.27 kg); however, this difference was not significant (P > 0.05).

**Discussion**

The modified-live virus vaccine evaluated in the study reported here induced antigen-specific SVN antibodies, T-helper cells, cytotoxic T cells, and γδ T cells from 1 to 6 months after vaccination as measured by upregulation of surface expression of the CD25 molecule. The observation that the T-helper cells synthesized IFN-γ 4 to 8 weeks after vaccination indicates that these were acting as T-helper-1 cells. Results of our study indicated that this vaccine induced a full spectrum immune response to BHV-1, BRSV, and BVDV types 1 and 2.

The CD25 response was evident in all T-cell subsets to all viruses 6 months after vaccination; however, the IFN-γ response was largely undetectable 6 months after vaccination. The fact that the IFN-γ response of CD4+ T cells to BHV-1 increased markedly 1 week after challenge, compared with control-group calves, indicated that vaccinated calves continued to have strong antigen-specific T-helper-1 memory 6 months after vaccination. In fact, the CD8+ and γδ TCR+ T cells also had strong anamnestic IFN-γ responses to BHV-1 after challenge.

The non–T cells are those that did not have CD4, CD8, or γδ TCR on their surfaces. These are presumably B cells and NK cells. The B cells are known to upregulate CD25 in response to specific antigen, and NK cells are known to produce IFN-γ in response to IL-2. Therefore, the CD25 response in non–T cells is likely caused by B-cell response to a specific antigen. The IFN-γ response in non–T cells is likely because of a bystander effect of IL-2, secreted from antigen-specific T cells, on NK cells.

The SVN antibody responses to BHV-1, BRSV, PI-3, and BVDV types 1 and 2 were detected when first tested 4 weeks after vaccination and remained detectable throughout the study. The SVN antibody titers in vaccinated calves increased more quickly after challenge than did titers in control-group calves, indicating B-cell memory to BHV-1 at least through 6 months after vaccination.

In our laboratory, flow cytometry has been used to...
detect the upregulation of activation marker CD25 and the expression of intracellular IFN-γ in PBMCs after stimulation with recall antigens. Two-color flow cytometry has been widely used by many laboratories because of the simplicity in fluorochrome combination and analysis. On the other hand, 2-color flow cytometry requires several identical antigen-stimulated wells to stain for all studied markers. This generates well-to-well variability and is time consuming and costly. We developed a 4-color flow cytometry assay to simultaneously detect 3 major T-cell markers (CD4, CD8, and TCRγδ) and an activation marker (CD25) from the same activated PBMCs at the same time. This diminishes any well-to-well variation, and the percentage of each T-cell subset can be studied more accurately. The T-cell subsets with double positive T-cell markers can be identified with no overlapping results with single T-cell marker cells. In our study, CMI assay via 3-color flow cytometry was developed by addition of a fifth color for detection of intracellular IFN-γ expression from the same cells. The technique was applied to prove the negative status of all calves prior to vaccination and the T-cell-subset responses to BHV-1, BRSV, and BVDV types 1 and 2 after vaccination.

The virus shedding patterns of calves in vaccinated and control groups were similar through day 4 after BHV-1 challenge. On day 5 after challenge, virus shedding increased in control-group calves, but decreased in vaccinated calves. This indicates that the original virus inoculum replicated in the nasal epithelium similarly in vaccinated and control-group calves, but that the anamnestic immune response limited subsequent virus replication in vaccinated calves. Virus shedding in nasal secretions was similar between the 2 groups on days 3 and 4 after challenge; however, compared with vaccinated calves, control-group calves had higher rectal temperatures on those 2 days. This suggests that, in control-group calves, BHV-1 had increased replication in tissues other than the nasal epithelium on days 3 and 4, compared with vaccinated calves. Vaccinated calves shed significantly less virus than control-group calves beginning 5 days after challenge and stopped shedding virus sooner than control-group calves. The immunity induced by the vaccine effectively decreased virus replication and febrile responses to BHV-1 challenge 6 months after a single dose of vaccine was administered.

Results of the study reported here indicated that administration of a single dose of modified-live virus vaccine induced significant SVN antibody, T-helper-1-cell responses, cytotoxic T-cell responses, and T-cell responses to BHV-1, BRSV, and BVDV types 1 and 2 as measured by both upregulation of CD25 and induction of IFN-γ. In vaccinated calves, the mean CD25 EI increased early and remained significantly higher than in control-group calves throughout the study, whereas the mean ΔΔIFN-γ increased significantly then decreased by weeks 24 to 25 after vaccination. After BHV-1 challenge at week 25, vaccinated calves had anamnestic antibody and T-cell-subset responses to BHV-1 as early as 1 week after challenge. Vaccinated calves shed significantly less BHV-1 in nasal secretions for a shorter duration and had significantly lower mean rectal temperatures, compared with control-group calves. All viruses induced neutralizing antibodies beginning at week 4 after vaccination through the end of the study.

References