Synergistic Effects of Bovine Respiratory Syncytial Virus and Non-cytopathic Bovine Viral Diarrhea Virus Infection on Selected Bovine Alveolar Macrophage Functions

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ABSTRACT

The effect of bovine respiratory syncytial virus (BRSV) and non-cytopathic bovine viral diarrhea virus (ncpBVDV) infection on selected bovine alveolar macrophage (AM) functions was investigated. Alveolar macrophages were harvested from 2- to 6-month-old calves seronegative for BRSV and BVDV and inoculated with approximately 1 median cell culture infective dose of virus per AM. Control, BRSV infected, ncpBVDV-infected and BRSV-ncpBVDV coinfected AM cultures were evaluated for Fc receptor expression, phagosome-lysosome fusion, superoxide anion (O_2^-) production, and chemotactic activity on Days 1, 3, 5, and 7 post-infection. Both single and combined viral infections significantly depressed AM Fc receptor expression, phagosome-lysosome fusion, and secretion of chemotactic factors with a more significant synergistic depression seen in BRSV-ncpBVDV coinfection. Production of O_2^- by AM was not decreased by either BRSV or ncpBVDV infection, but was significantly decreased by coinfection with BRSV-ncpBVDV. The present study confirms previous reports of BRSV effects on AM functions and indicate that ncpBVDV affects AM functions in vitro. Coinfection with BRSV-ncpBVDV produced a synergistic depression on AM functions.

RÉSUMÉ

L'effet d'une infection par le virus respiratoire syncytial bovin (VRSB) et par le virus non-cytopathogène de la diarrhée virale bovine (VDVBncp) sur certaines fonctions des macrophages alvéolaires (MA) bovins a été étudié. Des MA récoltés chez des veaux âgés de 2 à 6 mois d'âge, séronégatifs pour le VRSB et le VDVB, ont été inoculés avec environ une dose infectante médiane de culture cellulaire de virus par MA. Des cultures de MA témoins, infectées par le VRSB, infectées par le VDVBncp et co-infectées avec le VRSB et le VDVBncp ont été examinées pour évaluer l'expression des récepteurs Fc, le degré de fusion phagosome-lysosome, la production d'anion superoxide (O_2^-) et l'activité chémotactique aux jours 1, 3, 5 et 7 post-infection. Les deux infections virales uniques de même que l'infection combinée ont amené une diminution significative de l'expression des récepteurs Fc, de la fusion phagosome-lysosome, et de la sécrétion de facteurs chémotactiques avec une diminution plus marquée lors de la co-infection VRSB-VDVBncp. La production d'anions O_2^- n'était pas affectée lors de l'infection avec le VRSB ou le VDVBncp seul, mais une diminution significative était observée lors de la co-infection VRSB-VDVBncp. Cette étude confirme les observations précédentes des effets du VRSB sur les fonctions des MA et démontre que in vitro le VDVBncp affecte les fonctions des MA. Une co-infection VRSB-VDVBncp a un effet dépresseur synergistique sur les fonctions des MA bovins.

(Traduit par le docteur Serge Messier)

INTRODUCTION

Respiratory tract disease of cattle has been known for years to have a multifactorial etiology involving a complex interaction between stressors, viruses, and bacteria. There is potential for various synergies between pathogens including virus-bacteria and virus-virus. Virus-bacteria synergies in respiratory tract disease are well documented (1). Less is known regarding synergism among viral respiratory pathogens. Serological evidence from cases of spontaneous respiratory disease in cattle indicates that multiple concurrent viral infections are common (2–4). The high prevalence of serum antibodies to bovine respiratory syncytial virus (BRSV) indicates that infection in cattle is common and often results in respiratory tract disease (5,6). Although bovine viral diarrhea virus (BVDV) primarily infects the gastrointestinal tract and lymphoid tissue, observations indicate that BVDV may be an important respiratory tract pathogen (3,7). The immunosuppressive effect of acute BVDV infection

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may enhance the clinical effects of other pathogens (3,8–10), and, therefore, may be an important part of the bovine respiratory tract disease complex. One study revealed that about 92% of multiple viral infections involved BVDV (3). Observations from a study where a BRSV inoculum was contaminated with non-cytopathic BVDV (ncpBVDV) indicate severe pulmonary lesions can be induced in sheep (11).

Alveolar macrophages (AM) play a critical role in the primary response of the lung to respiratory infections (12,13). Virus infection may predispose the respiratory tract to the development of secondary bacterial pneumonia by impairing AM functions (14). Previous studies indicate that BRSV replication is supported in AM in vitro, but prevalence of infection was low (15–19) and BRSV depresses AM functions (15,19,20), and that cytopathic BVDV (cpBVDV) can replicate in AM (18,21). No studies have been done with ncpBVDV to see what effect infection would have on AM functions. The objectives of this study were to determine the influence of ncpBVDV infection on bovine AM functional properties and to determine if co-infection of bovine AM with BRSV-ncpBVDV produced a synergistic depression on AM functional properties.

MATERIALS AND METHODS

VIRUS PREPARATION

Bovine respiratory syncytial virus, 375 strain (22), that had become contaminated with a ncpBVDV was used as the coinfecting inoculum (BRSV-ncpBVDV), because these 2 virus strains together had been shown to produce severe pulmonary lesions (11). BRSV free from the ncpBVDV was prepared from the coinfected inoculum by passage in Vero cells (23) in the presence of antiserum to BVDV. The BRSV was passaged 3 times in Vero cells and tested for ncpBVDV using an immunoperoxidase test (see below). Because the titer of the ncpBVDV was higher than that of the BRSV in the coinfected inoculum, the ncpBVDV was prepared through terminal dilution using bovine fetal turbinate (BT) cells. The lowest log dilution without BRSV-induced cytopathic effect (CPE) was subpassaged 3 times and monitored for the absence of BRSV-induced CPE. Both BRSV-ncpBVDV and purified BRSV stocks were prepared using ovine fetal turbinate (OFTu) cells. The virus titer determination for both the BRSV and BRSV-ncpBVDV inoculum was based on the characteristic CPE for BRSV and the titer for the ncpBVDV was based on the immunoperoxidase test. Aliquots of the virus pool were frozen at −85°C until they were used to inoculate AM cultures.

IMMUNOPEROXIDASE TEST

An immunoperoxidase test was conducted to determine ncpBVDV infection as described by Bolin et al (24), with some modifications. Cells were seeded into a flat-bottomed, 96-well microtiter plate to form a 90% confluent monolayer in 24 h. The wells were inoculated with culture fluid to be analyzed for the presence of ncpBVDV. After a 24-hour incubation period, the monolayers were fixed, dried, and BVDV antiserum was applied followed by peroxidase-conjugated recombinant protein G (Zymed Laboratories, Inc., San Francisco, CA, USA). Substrate solution (3-amino-9-ethylcarbazole; Sigma Chemical Co., St. Louis, MO, USA) was added to each well and the plate incubated for 10 to 15 min. Cells with reddish brown cytoplasm were considered positive for ncpBVDV.

CALVES

A group of six two- to six-month-old Jersey calves seronegative for both BVDV and BRSV was used as the source of alveolar macrophages. There was at least a 14-day interval between successive lung lavages. Calves were sedated with xylazine (0.05 mg/kg body weight (BW), intravenously (IV)) and placed in sternal recumbency for lavage. Following lung lavage, the calves were given tolazoline (0.5 mg/kg BW, IV) to reverse the effect of the xylazine. The experiment was performed with approval and under the guidelines of the Institutional Animal Care and Use Committee and the calves were housed in facilities accredited by the American Association for Accreditation of Laboratory Animal Care.

ALVEOLAR MACROPHAGE CULTURES

Alveolar macrophages were recovered by bronchoalveolar lavage as described by Trigo et al (19). Briefly, a 1.0 cm diameter polypropylene tube was passed intranasally and intratracheally until gently wedged into a bronchus. Fifty millilitres of lavage fluid (0.9% NaCl containing 200 μg/mL gentamicin, and 2.5 μg/mL amphotericin B) were infused into the lung and recovered using a 50-mL glass syringe connected to the end of the tube. The fluid recovered from the first 50-mL lavage was discarded. The lavage was repeated until 200 mL fluid was collected. The recovered lavage fluid was filtered through sterile gauze and centrifuged at 450 × *g* for 40 min at 4°C. The cell pellet was washed and resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 200 μg/mL gentamicin, and 2.5 μg/mL of amphotericin B at a concentration of 5×10^5 cells/mL. Viability of AM was determined by trypan-blue exclusion. Alveolar macrophages were cultured in 8-well tissue culture chamber slides, 100 μL/well, (Lab Tek Tissue Culture Chamber, Miller Scientific, Division of Miles Laboratories, Inc., Naperville, IL, USA), 8-well tissue culture plates, 1 mL/well, (LUX Scientific Corporation, Newbury Park, CA, USA), or 96-well flat-bottomed tissue culture plates, 50 μL/well (Becton Dickinson Labware, Lincoln Park, NJ, USA).

VIRUS INFECTION OF AM

The AM were inoculated with approximately 1 median cell culture infective dose ($CCID_{50}$) per cell for both BRSV and ncpBVDV. Inoculation of AM with BRSV-ncpBVDV was approximately 1 $CCID_{50}$/AM based on the titer of BRSV in the inoculum. Control AM cultures were sham-inoculated with virus-free cell culture medium. Following adsorption of virus for 2 h, the inoculum was removed, the cultures were gently washed 3 times and fresh RPMI-1640 supplemented with 10% BFS was added. Cultures from individual calves were evaluated for Fc receptor expression, phagosome-lysosome fusion, and superoxide anion (O_2^-) and chemotactic factor production on Days 1, 3, 5, and 7 post-inoculation.

VIRUS REPLICATION IN AM CULTURES

AM culture fluids harvested on Days 1, 3, 5, and 7 post-inoculation were assayed for the presence of virus by terminal dilution. For BRSV, OFTu cells in 25-cm^2 flasks were inoculated with 10-fold dilutions of AM culture fluids (3 replications per dilution) and observed for the presence of characteristic BRSV induced CPE. For ncpBVDV titer determinations, BT cells in flat-bottomed, 96-well microtiter plates were inoculated and examined for the presence of ncpBVDV using the immunoperoxidase test.

FC RECEPTOR ASSAY

The presence of Fc receptors was determined by the method of Bianco and Pytowski (25). Briefly, sheep red blood cells (SRBC), were collected, washed, and sensitized with an equal volume of a subagglutinating dilution (1/500) of rabbit anti-SRBC antiserum (IgG) (Organon Teknika Corporation, Durham, NC, USA) for 15 min at 37°C. Following sensitization, SRBCs were washed and resuspended with RPMI-1640 to a 0.5% (v/v) concentration. The AM cultures in chamber slides were washed with Hanks' Balanced Salt Solution (HBSS) and exposed to sensitized SRBC (200 μL/well) for 30 min at 4°C. The chambers were gently rinsed 4 times with HBSS to remove nonadherent SRBCs and wet-mounted with cover slips. Fc receptor expression was determined by the percentage of cells with 3 or more adherent SRBC (rosettes) in 10 microscope fields per replication. For each viral infection, the mean percentage of 6 replications on each test day was calculated.

PHAGOSOME-LYSOSOME FUSION ASSAY

The effect of virus on AM phagosome-lysosome fusion was assayed by a modification of the acridine orange (AO) method described by Jakab et al (26). *Candida albicans* (from Dr. G.B. Johnston, Iowa State University, Ames, IA, USA), grown overnight in 10% glycerol in 3.7% brain-heart infusion medium, was washed and resuspended in RPMI-1640 to a concentration of 1×10^7 cells/mL. On each test day, freshly prepared AO solution was added to virus-infected and noninfected AM cultures in chamber slides and incubated for 20 min at 37°C. Excess AO and nonadherent cells were removed by gently washing the slides. Cultures were then exposed to 30 μL of yeast suspension and incubated for 60 min at 37°C. Chambers and gaskets were removed and the slides dipped in phosphate-buffered saline (0.01 M, pH 7.2) to remove excess yeast. The slides were cover-slipped and observed with an epifluorescent microscope at 400× magnification. Phagosome-lysosome fusion occurred when yeast stained yellow, orange or red within phagosomes. For each viral infection on each test day, the mean percentage of 6 replications of phagosome-lysosome fusion in 200 AMs per replication was calculated.

TABLE I. Number of alveolar macrophage cultures positive for virus and virus titer on Days 1, 3, 5, and 7 post-infection from alveolar macrophage cultures inoculated with either bovine respiratory syncytial virus (BRSV), non-cytopathic bovine viral diarrhea virus (ncpBVDV), or both BRSV-ncpBVDV

					BRSV-ncpBVDV			
Days post-infection	BRSV		ncpBVDV		BRSV		ncpBVDV	
1	6/6[a]	1.75[b]	6/6	0.37	6/6	1.25	6/6	0.75
3	3/6	2.12	6/6	1.62	4/6	2.88	5/6	1.75
5	3/6	2.25	5/6	1.25	4/6	3.40	4/6	1.51
7	1/6	1.25	3/6	1.25	2/6	2.00	2/6	1.38

[a] Number of cultures positive/number sampled
[b] Virus titer $\log_{10}$ $TCID_{50}$ /mL

RESPIRATORY BURST ASSAY

Both virus-infected and noninfected AM were monitored for O_2^- production by the method described by Pick and Mizel (27). The first 4 columns of AM cultures in 96-well, flat-bottomed tissue culture plates contained no virus whereas subsequent columns were inoculated with BRSV, ncpBVDV, and BRSV-ncpBVDV respectively. During each test day, 300 U/mL of superoxide dismutase (Calbiochem Corporation, La Jolla, CA, USA) was added to columns 2 and 3 and incubated for 5 min at 37°C. Then Earle's balanced salt solution (EBSS) without phenol red (Life Technologies Inc., Grand Island, NY, USA) and HBSS was added to the first column and EBSS containing ferricytochrome C (Sigma Chemical Co.) plus opsonized zymosan (4 mg/mL) prepared as described by Nagahata et al (28) were added to the second and fourth through seventh columns and the plates incubated for 1 h at 37°C in a humidified atmosphere with 5% CO_2. Cytochrome C reduction was determined using microplate reader (Molecular Devices, Woodburry, MN, USA) at a wavelength of 550 nm. The amount of O_2^- produced per well was expressed by the formula: nanomoles O_2^- per well = (absorbance at 550 nm × 100)/6.3. For each viral infection on each test day, the mean of O_2^- production in 6 replications was calculated.

CHEMOTACTIC FACTOR ASSAY

Chemotactic activity was assayed by using the procedure of Nelson et al (29) with modifications. On each test day, virus-infected and noninfected AM cultures on 8-well tissue culture plates were washed with RPMI-1640 and chased with 1 mL of either opsonized or nonopsonized zymosan (4 mg/mL). The fluids were harvested separately, centrifuged, and the supernatants stored at −85°C until tested. Neutrophils were recovered from freshly prepared heparinized normal calf blood by centrifugation on Ficol-Diatrizoate (Sigma Chemical Co.), and washed once with HBSS. The erythrocytes were lysed with two cycles of ice-cold 0.2% NaCl followed by the addition of 1.6% NaCl and centrifugation at 1000 × *g* for 10 min at 4°C. The leukocytes were washed 2 additional times with HBSS. Neutrophils were counted and resuspended at a concentration of 1×10^7 cells/mL in RPMI-1640 supplemented with 0.1% gelatin and 5 mM EDTA. Four series of 3 wells 3.0 mm in diameter and spaced 3.0 mm apart were cut in agarose plates (60 mm × 15 mm tissue culture dish; Becton Dickinson) containing 5 ml of 0.8% agarose in RPMI-1640 supplemented with 10% BFS. The center well of each 3-well series received a 10 μL volume of purified neutrophils. The outer well received 10 μL of culture fluid from nonopsonized zymosan stimulated cultures, while the inner

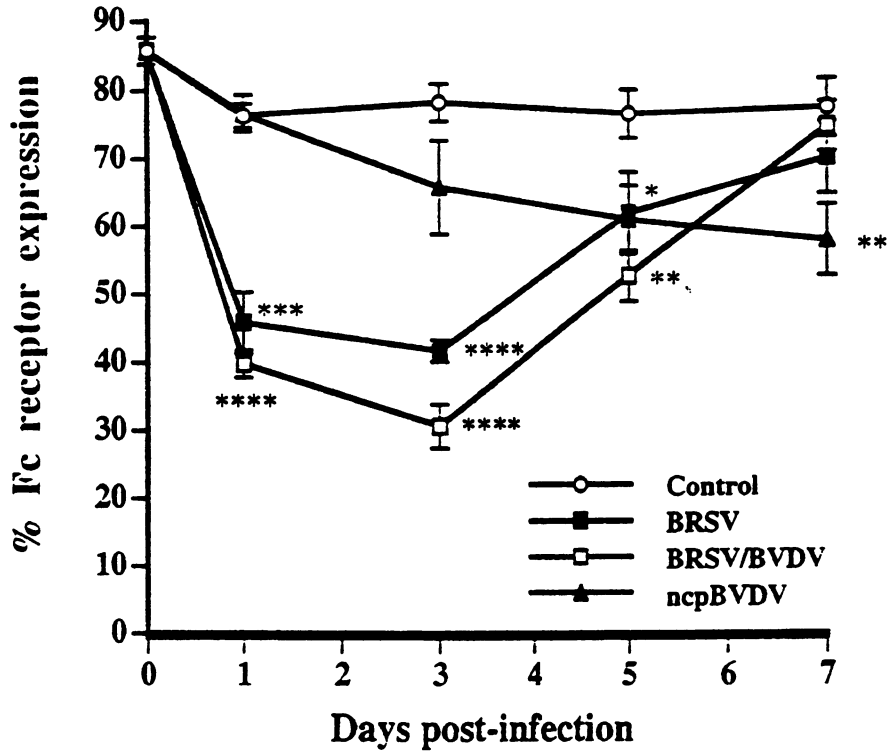

Figure 1. Percentage of alveolar macrophage in culture expressing Fc receptors on Days 1, 3, 5, and 7 post-infection from alveolar macrophage cultures inoculated with either bovine respiratory syncytial virus (BRSV), noncytopathic bovine viral diarrhea virus (ncpBVDV), BRSV-ncpBVDV, or sham-inoculum (control). Each point represents the mean ± SE of results obtained from alveolar macrophage cultures prepared from 6 calves. Values significantly different from control values are marked with asterisks. *$P < 0.05$, **$P < 0.02$, ***$P < 0.01$, ****$P < 0.001$.

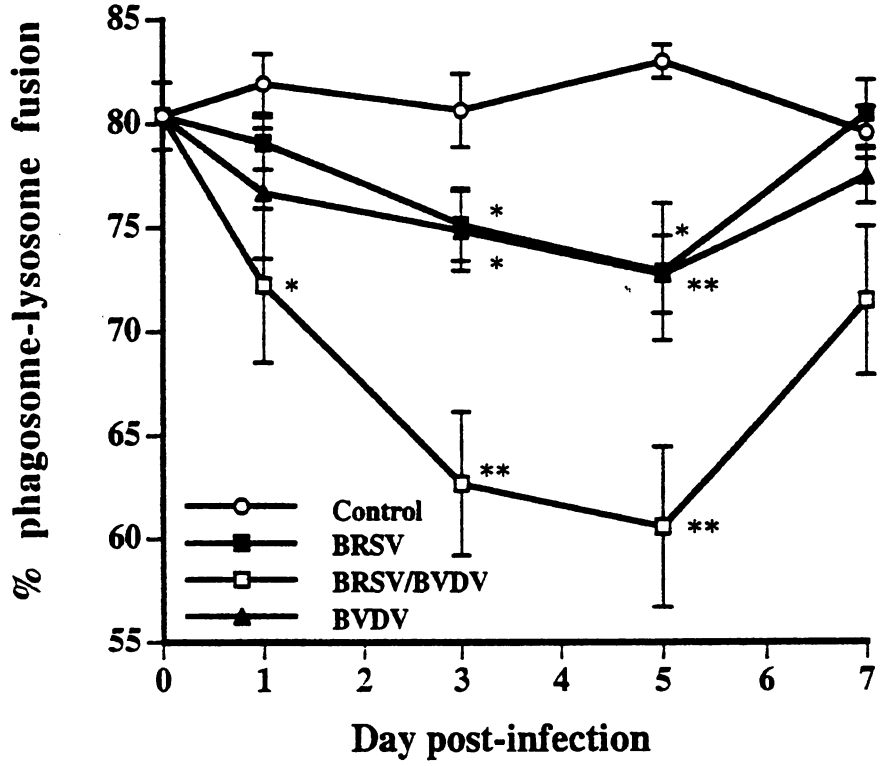

Figure 2. Percentage of alveolar macrophage in culture demonstrating phagosome-lysosome fusion on Days 1, 3, 5, and 7 post-infection from alveolar macrophage cultures inoculated with either bovine respiratory syncytial virus (BRSV), noncytopathic bovine viral diarrhea virus (ncpBVDV), BRSV-ncpBVDV, or sham-inoculum (control). Each point represents the mean ± SE of results obtained from alveolar macrophage cultures prepared from 6 calves. Values significantly different from control values are marked with asterisks. *$P < 0.05$, **$P < 0.01$.

well received 10 μL from opsonized-zymosan stimulated cultures. After a 2-hour incubation period, the cells were fixed, the gel removed and the plates were stained with Giemsa solution and air dried. The chemotactic differential was calculated for each replication by measuring the linear distance the cells had migrated from the margin of the well toward the opsonized medium (chemotactic factor) minus the linear distance the cells had migrated from the margin of the well toward the nonopsonized medium (spontaneous migration). The mean value and standard deviation of six replications for each viral-infection on each test day were determined.

STATISTICAL ANALYSIS

Student's *t*-test was utilized to test the differences between the means of the virus-infected and noninfected AM cultures and the differences were considered significant when $P < 0.05$. Six replications for each assayed group were conducted.

RESULTS

ALVEOLAR MACROPHAGE CULTURE AND INFECTION

The viability of AM was 87.27 ± 1.41% (mean ± SEM) on the day the cultures were established. After 7 d, the viability of remaining AM was greater than 95%, but the total number of AM in culture over 7 d decreased between 30 and 40%. Culture fluid positive for either BRSV, ncpBVDV, or both BRSV-ncpBVDV declined over the 7-day observation period (Table I). In BRSV inoculated cultures, BRSV isolation decreased from 6 on Day 1 post-infection to 1 on Day 7 post-infection. For BRSV-ncpBVDV inoculated cultures, 6 samples were positive on Day 1 post-infection, whereas 2 were positive for both viruses on Day 7 post-infection. Recovery of ncpBVDV from cultures inoculated with ncpBVDV decreased from 6 to 3 over the 7-day observation period. Data on virus titer are also presented in Table I. The BVDV titers were similar in the single and coinfected cultures. The BRSV titer in the coinfected cultures was 14 times greater on Day 5 post-inoculation than the AM cultures infected with BRSV alone.

FC RECEPTOR

Test results for Fc receptors are presented in Figure 1. Compared with sham-inoculated control cultures, BRSV infection significantly depressed Fc receptor expression starting on Day 1 post-inoculation (45.85 ± 4.46% vs 76.42%, $P < 0.01$), and reached the lowest point on Day 3 post-inoculation (41.73 ± 1.62% vs 78.35%, $P < 0.001$). On Day 5 post-inoculation (61.96 ± 6.04 vs 76.65%, $P < 0.05$), Fc receptor expression began to recover and returned to normal on Day 7 post-inoculation. Noncytopathic BVDV infection induced a significant decrease in Fc receptor expression starting on Day 5 post-inoculation (61.04 ± 4.97% vs 76.65%, $P < 0.05$) and persisted to Day 7 (57.99 ± 5.27% vs 77.49%, $P < 0.01$). The Fc receptor expression in coinfected cultures was like that produced by BRSV alone but was more severely depressed with 39.82 ± 1.98 vs 76.42% ($P < 0.001$) on Day 1 post-inoculation, reached the lowest point on Day 3 post- inoculation (30.72 ± 3.27% vs 78.35%, $P < 0.001$), and returned to normal by Day 7 post-inoculation.

PHAGOSOME-LYSOSOME FUSION

The percentage of control and virus-infected AM with phagosome-lysosome fusion are shown in Figure 2. A smaller decrease in phagosome-lysosome fusion was noted with both BRSV and ncpBVDV infection on Day 3 post-inoculation (75.16 ± 1.73% and 74.88 ± 1.92%, respectively, vs 80.67%, $P < 0.05$) and Day 5 post-inoculation (72.90 ± 3.31%, and 72.78 ± 1.87%, respectively, vs 83.03%, $P < 0.01$). Phagosome-lysosome fusion in coinfected cultures was depressed starting on Day 1 post-inoculation (72.25 ± 3.70% vs 81.94, $P < 0.05$) with the greatest reduction on Day 5 post-inoculation (60.58 ± 3.88% vs 83.03%, $P < 0.01$). By Day 7 post-inoculation, phagosome-lysosome fusion was recovering from the effect of both single and combined viral infections.

RESPIRATORY BURST

After stimulation with opsonized zymosan, neither BRSV nor ncpBVDV infected AM cultures produced significant changes in O_2^- production, compared with the sham-inoculated

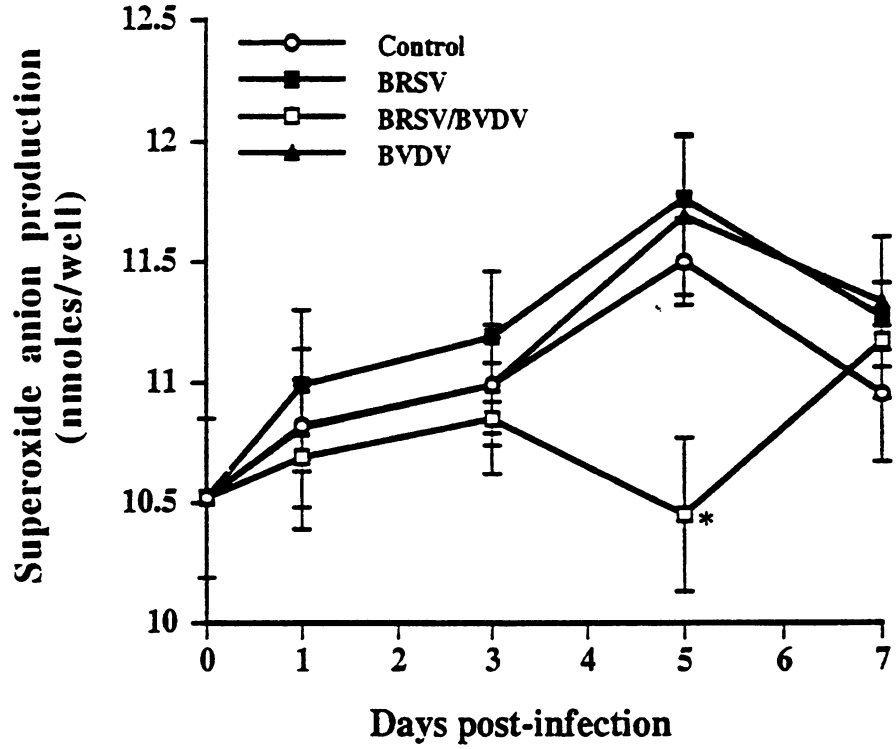

Figure 3. Superoxide anion production by alveolar macrophage cultures on Days 1, 3, 5, and 7 post-infection from alveolar macrophage cultures inoculated with either bovine respiratory syncytial virus (BRSV), noncytopathic bovine viral diarrhea virus (ncpBVDV), BRSV-ncp BVDV, or sham-inoculum (control). Each point represents the mean ± SE of results obtained from alveolar macrophage cultures prepared from 6 calves. Values significantly different from control values are marked with asterisk. *$P < 0.05$.

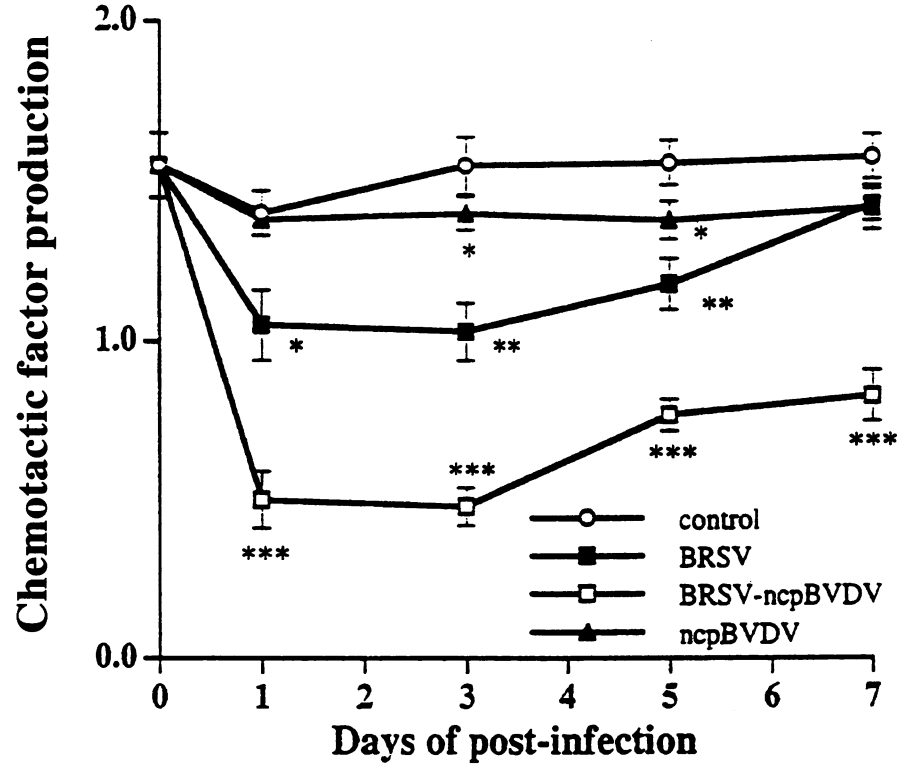

Figure 4. Chemotactic factor production by alveolar macrophage cultures on Days 1, 3, 5, and 7 post-infection from alveolar macrophage cultures inoculated with either bovine respiratory syncytial virus (BRSV), noncytopathic bovine viral diarrhea virus (ncpBVDV), BRSV-ncp BVDV, or sham-inoculum (control). Each point represents the mean ± SE of results obtained from alveolar macrophage cultures prepared from 6 calves. Values significantly different from control values are marked with asterisks. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

control (Figure 3). However, BRSV-ncpBVDV coinfection significantly depressed O_2^- release by AM on day 5 post-inoculation (10.45 ± 0.32 nmol/well vs 11.50 nmol/well, $P < 0.05$). In cultures stimulated in the presence of superoxide dismutase, almost all O_2^- release was eliminated indicating that O_2^- contributed to the specific reduction of cytochrome C (data not shown).

CHEMOTACTIC FACTOR

Production of chemotactic factor(s) by virus-infected and noninfected AM cultures was determined by measuring chemotactic differentials. The results are presented in Figure 4. Chemotactic factor production by AM was depressed by ncpBVDV infection on Day 3 post-inoculation (0.1400 ± 0.0052 cm vs 0.1550 cm, $P < 0.05$) and on Day 5 post-inoculation (0.1383 ± 0.0060 cm vs 0.1567 cm, $P < 0.05$). By Day 7 post-inoculation, chemotactic factor production was back to normal. Chemotactic factor production was significantly depressed by BRSV infection over the 7-day observation period with the greatest effect on Day 1 post-inoculation (0.1050 ± 0.115 cm vs 0.1400 cm, $P < 0.05$) and Day 3 post-inoculation (0.1033 ± 0.0092 cm vs 0.1550 cm, $P < 0.01$) By Day 7 post-inoculation, chemotactic factor production was returning to normal. For BRSV-ncpBVDV coinfected cultures, chemotactic factor production was severely depressed for at least 7 d with the greatest depression on Day 3 post-inoculation (0.0483 ± 0.0060 cm vs 0.1550 cm, $P < 0.001$).

DISCUSSION

Synergistic depression of Fc receptor, phagosome-lysosome fusion, O_2^- production and chemotactic factor production was observed following in vitro coinfection with BRSV-ncpBVDV, while the effect was more limited in AM cultures infected by either BRSV or ncpBVDV alone.

Bovine respiratory syncytial virus and BVDV recovery from AM culture fluid decreased over the 7-day period in our study (Table I). Residual virus should have been removed following adsorption when the cultures were washed. Thus, we feel the virus detected in our study represents newly replicated virus. The decline in virus titer most likely reflects the restriction of virus replication over time and correlates with recovery of AM function. Our observations were based on qualitative tests (cytopathic effect and immunoperoxidase test) as well as the titration of virus in the culture fluids. Previous reports (18,19) suggested that BRSV infection in AM culture is abortive, but more recently, Adair and McNulty (15) demonstrated increased numbers of BRSV infected AM in cultures prepared from 2 of 10 calves over the 7- to 10-day observation period of their study. Schrijver et al (17) also presented evidence that BRSV replicates to a low level in AM. Both abortive and low level permissive replication of human RSV have been reported to occur in human AM cultures (30,31). The present results also show that ncpBVDV can replicate in AM cultures and confirm previous results using cpBVDV (18,21), where low level replication of virus was observed. Coinfection dramatically increased the titer of the BRSV Day 5 post-inoculation. The enhanced BRSV replication may provide the basis for the synergism between BRSV and ncpBVDV infection.

Fc receptor expression is an important indicator of AM function because it reflects the phagocytic ability of AM. Trigo et al (19) and Adair and McNulty (15) showed that BRSV-infected bovine AM had a significantly impaired ability to phagocytize antibody-coated erythrocytes over the 3- to 11-day observation period. Significant depression of Fc receptor expression by BRSV occurred beginning on Day 1 post-inoculation and lasted through Day 5 post-inoculation in our study. A steady decrease in the percentage of AM expressing Fc receptors was observed in the ncpBVDV-infected cultures over the 7-day observation period. Welsch et al (21) also showed that BVDV infection both in vivo and in vitro can decrease AM Fc receptor expression. The effect on Fc receptor expression by infection with either BRSV alone or BRSV-ncpBVDV coinfection was alike but coinfection produced greater depression in Fc receptor expression, indicating that BRSV may play the dominant role in this combined virus infection.

After being ingested by AM, foreign pathogens are usually enclosed within phagosomes that move to the cell interior and fuse with lysosomes to form phagolysosomes in which the pathogens are acted upon by digestive enzymes. Viral infection can either impair phagocytic ability or prevent phagosome-lysosome fusion in AM (26,32,33). Thus, phagosome-lysosome fusion has become a valuable index in measuring virus-induced macrophage dysfunction. In the present study, AM infected with either BRSV or ncpBVDV produced a limited effect on phagosome-lysosome fusion on Days 3 and 5 post-inoculation, while BRSV-ncpBVDV coinfection resulted in severe depression on Days 1, 3 and 5 post-inoculation. In normal macrophages, fusion of lysosomes with phagocytic vacuoles is controlled by intracellular levels of cyclic AMP. Prostaglandin E_2 (PGE_2), one of the prostaglandins produced by AM, competes with cyclic AMP to down-regulate activated macrophages (34,35). Virus infection has been shown to enhance PGE_2 production by macrophages (34). The mild down regulation of phagosome-lysosome fusion in BRSV and ncpBVDV-infected AM may be due to the low levels of PGE_2 production, whereas severe depression of BRSV-ncpBVDV coinfected AM may result from 2 mechanisms, the high level of PGE_2 production or the inhibition of phagocytosis.

The present study shows that neither BRSV nor ncpBVDV infection in AM depressed O_2^- release from AM, but that BRSV-ncpBVDV coinfection significantly depressed O_2^- production on Day 5 post-inoculation. The normal production values for O_2^- by AM after exposure to either BRSV or ncpBVDV indicated that the virus infection had not depleted AM energy. The synergistic depression of O_2^- production on Day 5 post-inoculation could have been due to severe reduction of Fc receptor expression by BRSV-ncpBVDV coinfection. Kobzik et al (36) verified that both membrane-derived and mitochondrial components of the AM respiratory burst have important roles in the response to opsonized particles. Thus, O_2^- production by AM can be maintained at normal levels unless the numbers of Fc receptors expressed

during virus infection are reduced. The coinfected AMs probably responded to extremely low numbers of opsonized zymosan to decrease markedly the generation of both mitochondrial and membrane-derived oxygen metabolites.

Alveolar macrophages can secrete a series of chemotactic factors including LTB4 and NAP-1/IL-8 attracting neutrophils to the lung (37). Infection of human AM by human RSV was shown in a previous study to induce expression of IL-8 mRNA and the release of IL-8 protein (30). The low levels of IL-8 production induced by RSV infection were shown to have some degree of inhibition of AM function. Results of our study indicate that BRSV, ncpBVDV or BRSV-ncpBVDV infection also depressed production of chemotactic factor(s). Noncytopathic BVDV infection induced a mild but significant depression of chemotactic factor(s) on Days 3 and 5 post-inoculation, while a significant depression that lasted for at least 7 d was produced by BRSV. On Day 7 post-inoculation, production of chemotactic factor(s) was returning to normal. These results contrast with those reported by other researchers who demonstrated that RSV stimulated the increased production of neutrophil chemotactic factors in cultures of infected macrophages (15). The differences could be due to the variation in procedures. In our study, before challenging with opsonized zymosan, the virus-infected AM culture fluids were discarded, the adherent AMs washed and the culture fluid tested for chemotactic factor(s) following stimulation with opsonized zymosan. Virus may stimulate chemotactic factor production during the early stage of virus infection, followed by a decrease due to viral inhibition of chemotactic factor mRNA expression.

The synergistic effects of BRSV and ncpBVDV on AM functions were clearly demonstrated. The mechanisms involved in this process are probably different from that involved in viral-bacterial synergism. The interaction of viruses and the immune status of the host may be the key factors in determining virus-virus synergism. The synergistic decrease in AM function produced by BRSV-ncpBVDV coinfection may explain the pathogenesis leading to severe clinical respiratory disease.

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