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Infectivity-Neutralizing and Hemagglutinin-Inhibiting Antibody Responses to Respiratory Coronavirus Infections of Cattle in Pathogenesis of Shipping Fever Pneumonia

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Respiratory bovine coronaviruses (RBCV) emerged as an infectious agent most frequently isolated from respiratory tract samples of cattle with acute respiratory tract diseases. Infectivity-neutralizing (IN) and hemagglutinin-inhibiting (HAI) antibodies induced by RBCV infections were monitored in sequential serum samples collected from cattle during a naturally evolving and experimentally monitored epizootic of shipping fever pneumonia (SFP). Cattle nasally shedding RBCV at the beginning of the epizootic started with low levels of serum IN and HAI antibodies. An increase in serum IN antibody after day 7 led to reduction of virus shedding in nasal secretions by the majority of the cattle between days 7 and 14. A substantial rise in the serum HAI antibody was observed during the initial phase among the sick but not the clinically normal cattle which were infected with RBCV. The RBCV isolation-positive cattle that developed fatal SFP had minimal serum IN and HAI antibodies during the course of disease development. Cattle that remained negative in RBCV isolation tests entered this epizootic with high levels of serum IN and HAI antibodies, which dramatically increased during the next two weeks. Protection against SFP was apparently associated with significantly higher levels of serum IN antibodies at the beginning of the epizootic. The RBCV-neutralizing activity is associated with serum immunoglobulin G (IgG), particularly the IgG2 subclass, while RBCV-specific HAI antibody is related to both serum IgG and IgM fractions.

Numerous wild-type coronavirus strains were recently isolated from nasal swab samples and lung tissues of cattle with signs of acute respiratory tract distress including a severe form of shipping fever pneumonia (SFP) (25–28). These virus isolates multiplied only in the G clone of human rectal tumor-18 cells, but not in Georgia bovine kidney and bovine turbinate cells, permissive for most of previously described respiratory viruses of cattle, and they were identified as respiratory bovine coronaviruses (RBCV).

The role of coronaviruses as bovine enteropathogens was first recognized in the 1970s when they were isolated from diarrheic samples of neonatal calves with severe gastroenteritis (16). Coronaviruses were also implicated in winter dysentery of adult cattle, principally dairy cattle, and occasionally in pneumoenteritis of young calves (2, 20). These coronaviruses are referred to as enteropathogenic bovine coronaviruses (EBCV).

The following phenotypic and genotypic properties differentiated RBCV from EBCV. (i) The RBCV were isolated in the first G clone cell passage without the use of trypsin enhancement (25–28). Trypsin activation was required for the isolation of wild-type EBCV (32). (ii) The RBCV have high cell-fusing activity for the G clone cells in the neutral pH ranges. (iii) The RBCV agglutinate only mouse and rat but not chicken red blood cells (RBC), while the prototype EBCV agglutinate both rodent and chicken RBC (29). (iv) The RBCV have high acetylesterase (AE) activity at 37°C, whereas the AE function of EBCV is much more active at 39°C (13). (v) Comparative analysis of wild-type RBCV and EBCV at the 3′ genomic region (9.5 kb) revealed that RBCV-specific nucleotide and amino acid changes are disproportionally concentrated within the hemagglutinin-esterase (HE) gene, the spike (S) gene, and the genomic region between the S and envelope (E) genes (1).

Bovine coronaviruses (BCV) belong to the *Coronaviridae* family of the order *Nidovirales* and are large, enveloped, positive-stranded RNA viruses with a genome of about 31 kb (6, 11). The viral RNA genome is associated with the nucleocapsid phosphoprotein (N) to form a helical nucleocapsid. Four structural proteins are part of the lipoprotein envelope: (i) membrane glycoprotein (M), (ii) S glycoprotein, (iii) HE glycoprotein, and (iv) the recently identified E protein. Specific monoclonal antibodies (MAbs) against EBCV glycoproteins S and HE inhibited virus infectivity, indicating that both glycoproteins elicit neutralizing antibodies in EBCV infections (4, 5, 9). The *N*-acetyl-9-*O*-acetylneuraminic acid was identified as the receptor determinant to which both S and HE bind in initiating infection and in agglutinating rodent erythrocytes. The S glycoprotein is considered to be the major viral structural protein to bind to the neuraminic acid-containing receptors (22, 23). Binding of HE glycoprotein to this determinant residue on the cell surface was suggested to function as a prereceptor interaction for EBCV (19, 24). The HE of EBCV also has receptor-destroying enzyme (RDE) function which is mediated by the AE potentially eluting adsorbed virions (24, 29). MAbs differentiated HE functions into hemagglutinin (HA) and RDE activities through relatively low activity inhibiting HA but high titers of activity inhibiting the RDE (30, 35).

Antibodies against EBCV infections of cattle were analyzed

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in bovine sera by immunodiffusion, immunoblotting, and enzyme-linked immunosorbent assay (7, 8, 14, 33). Recent studies of sequential humoral immune responses to RBCV infections of cattle suggested that antibodies against S and HE glycoproteins play significant roles in clearing the infectious virus and in inducing protection against the virus infections (14). The purpose of this investigation was to assess the kinetics of infectivity-neutralizing (IN) and HA-inhibiting (HAI) antibodies against RBCV in sera of immunologically mature cattle during a naturally evolving epizootic of SFP, to define the correlation of IN and HAI activities with previously reported immunoisotype responses, and to relate the findings with isolation of RBCV from respiratory tracts during disease development and clearance of the virus during the recovery phase.

MATERIALS AND METHODS

Experimental design. As reported 105 6- to 8-month-old cattle were included in the epizootic occurring in 1997 (25, 26) and subjected to nasal and blood sampling and testing at the time of assembly at an order-buyer barn (day 0), after transport (day 7), and weekly throughout the pathogenesis of SFP (days 14, 21, 28, and 35). Nasal swab samples were taken for virological and bacteriological studies, while blood samples for serum harvest were collected for immunological investigation. These cattle were classified into five response groups based on clinical signs of respiratory tract diseases and results of RBCV isolation (14, 25, 26). Response group 1 included 72 cattle that exhibited clinical signs of respiratory tract disease and were nasally shedding RBCV on day 0, day 7, or both. Seven cattle shedding RBCV on day 7 were randomly chosen from this response group for testing in this study. Response group 2 contained five test cattle that secreted RBCV in nasal discharges without adverse respiratory signs. Ten cattle of response group 3 developed severe pneumonia and died on days 7 to 11, and nine that nasally shed RBCV were selected. Eighteen cattle remained RBCV isolation negative. Eleven of them were included in response group 4 because they had fever and other respiratory signs, while the remaining seven calves (response group 5) remained clinically healthy during the 5-week investigation. Samples of seven representative cattle from response groups 4 and 5 were serologically analyzed. Sequential serum samples from the selected 35 cattle were analyzed for their IN and HAI antibodies.

Cell line, virus isolate, and virus purification. The G clone of HRT-18 cells was used at the 24th passage level for RBCV propagation. A wild-type strain, RBCV-97TXSF-Lu15-2, was used at its second passage for antigen preparation after initial isolation from the lung tissue of a calf that died on day 8 (25). A stock of RBCV-97TXSF-Lu15-2 was prepared after partial purification was performed according to the method of Zhang et al. (35).

Infectivity neutralization assay. Serum samples were diluted 1:4 in Dulbecco's modified minimal essential medium (Sigma Chemical Co., St. Louis, Mo.) with 4.5 g of glucose per liter buffered with 44 mM $NaHCO^3$ and supplemented with penicillin (100 U/ml)-streptomycin (100 μg/ml) (Sigma Chemical Co.), heat inactivated at 56°C for 30 min, and then prepared as quadruplicates in serial twofold dilutions at 50 μl/well on 96-well flat-bottom cell culture plates (Costar, Corning, N.Y.). Serum 1745, with an IN antibody titer of 128, and BCV-antibody free serum from a normal calf with an IN titer of <8 were included as positive and negative controls, respectively (31, 33). The RBCV-97TXSF-Lu15-2 stock was diluted in Dulbecco's modified minimal essential medium to 100 50% cell culture infective doses and added at 50 μl/well. After the serum-virus mixture was reacted at 25°C for 30 min, 100 μl of G clone cell suspension containing 1,000 cells was dropped into each well. The plates were incubated at 37°C in a 5% CO_2 incubator and examined daily with an inverted microscope for RBCV-characteristic cytopathic changes, extensive cell fusion, for 4 to 5 days. The IN titers in serum were expressed as the reciprocal of the serum dilution that completely inhibited cytopathic changes in 50% of the quadruplicates.

Assays for HA and RDE. The assays for HA and RDE were performed as reported (29, 30) with washed rat RBC prepared as suspensions of 0.5% in phosphate-buffered saline at pH 7.4, containing 0.05% bovine serum albumin.

Test for HAI. Serum samples were diluted 1:4 in phosphate-buffered saline (pH 7.4; containing 0.05% bovine serum albumin), heat inactivated at 56°C for 30 min, and then prepared as serial twofold dilutions in 50-μl aliquots on 96-well V-bottom microtiter plates (Costar, Cambridge, Mass.). Again, serum 1745, with an HAI antibody titer of 128, was included as a positive control, while BCV antibody-free serum from a normal calf with an HAI antibody titer of <8 was used as negative control (31, 33). The partially purified stock of RBCV-97TXSF-Lu15-2 was diluted to contain 8 to 16 U of both HA and RDE and was used as an antigen. Fifty microliters of the antigen was added to each serum dilution. Serum-antigen mixtures reacted at 25°C for 30 min, and then the 0.5% (vol/vol) rat RBC suspension was added in a volume of 50 μl. The plates were shaken to disperse the RBC suspensions and incubated at 6°C for 2 h. The serum HAI antibody titers were expressed as the reciprocal of the highest dilution of serum sample that completely inhibited the aggregation of rat RBC.

Data analysis and statistical methods. The serum IN and HAI antibody titers were transformed to base 2 logarithms for statistical analysis. All data were presented as means ± standard errors of the mean (SEM). The IN and HAI activities of response groups were compared by an analysis of variance of repeated measures designed with a split-plot arrangement of treatments. Pairwise comparisons of treatment and day differences were conducted with Scheffe's test. Interaction effects were examined with pairwise *t* tests of least-square means for preplanned comparisons of treatments at specific days. All tests were considered significant at a probability of $P < 0.05$.

A total of 171 serum samples were collected from these 35 test cattle and analyzed for their IN antibody, HAI antibody, and immunoisotype levels (14). The IN or HAI activities were paired with HAI antibody, IN antibody, or immunoisotype levels for each serum sample. Serum samples with identical IN or HAI antibody levels were transformed to base 2 logarithms and grouped together. There were 33, 12, 11, 28, 30, 29, 14, 8, and 6 as well as 6, 26, 18, 24, 16, 24, 29, 26, and 2 serum samples for the nine specific IN and HAI antibody levels, respectively. To evaluate the sensitivity and specificity of the IN and HAI antibody assays, these groups of HAI antibody, IN antibody, or immunoisotype levels were compared with specific IN or HAI activities by linear regression analysis with the SAS system. They were presented as means ± SEM, and *P* values of <0.05 were considered statistically significant.

RESULTS

The IN and HAI activities of serial serum samples against RBCV-97TXSF-Lu15-2 strain. Isolation results for RBCV and overt signs of respiratory tract disease divided the 105 cattle of this experimentally assessed epizootic of SFP into five response groups (14, 25, 26). Overall kinetics of serum IN and HAI antibody levels between the seven sick cattle of response group 1 and the five clinically normal cattle of response group 2 did not show significant differences (Fig. 1A, B, F, and G). Levels of serum IN and HAI antibodies against RBCV were initially as low as 2.29 ± 0.18 and 3.71 ± 0.52 for cattle in response group 1 and 2.80 ± 0.80 and 3.60 ± 0.51 for cattle in response group 2, respectively. A significant increase in the level of IN antibody in serum was observed for all these cattle after day 7, reaching 4.86 ± 0.80 ($P < 0.0001$) and 4.60 ± 0.51 ($P = 0.0152$) on day 14 for cattle in response group 1 and 2 and then remaining high (Fig. 1A and B). An increase in the level of serum HAI antibody was statistically significant for the cattle in response group 1 between days 0 and 21 ($P = 0.007$), while a substantial rise in the level of serum HAI antibody was not detected for the cattle in response group 2 during this experiment ($P = 0.1072$) (Fig. 1F and G).

Nine RBCV isolation-positive cattle of response group 3 which developed fatal SFP had low IN and HAI levels on day 0, which were 2.00 ± 0.00 and 3.56 ± 0.18, respectively (Fig. 1C and H). No increases in the serum IN antibody level were detected, but the HAI antibody level rose minimally during the 7-day course of disease development.

Significant differences were not observed in the kinetics of serum IN and HAI antibody levels between response groups 4 and 5 (Fig. 1D, E, I, and J), but they were all remarkably higher than those in response groups 1 and 2 (Fig. 1A, B, F, and G). The level of IN antibody in serum significantly increased dur-

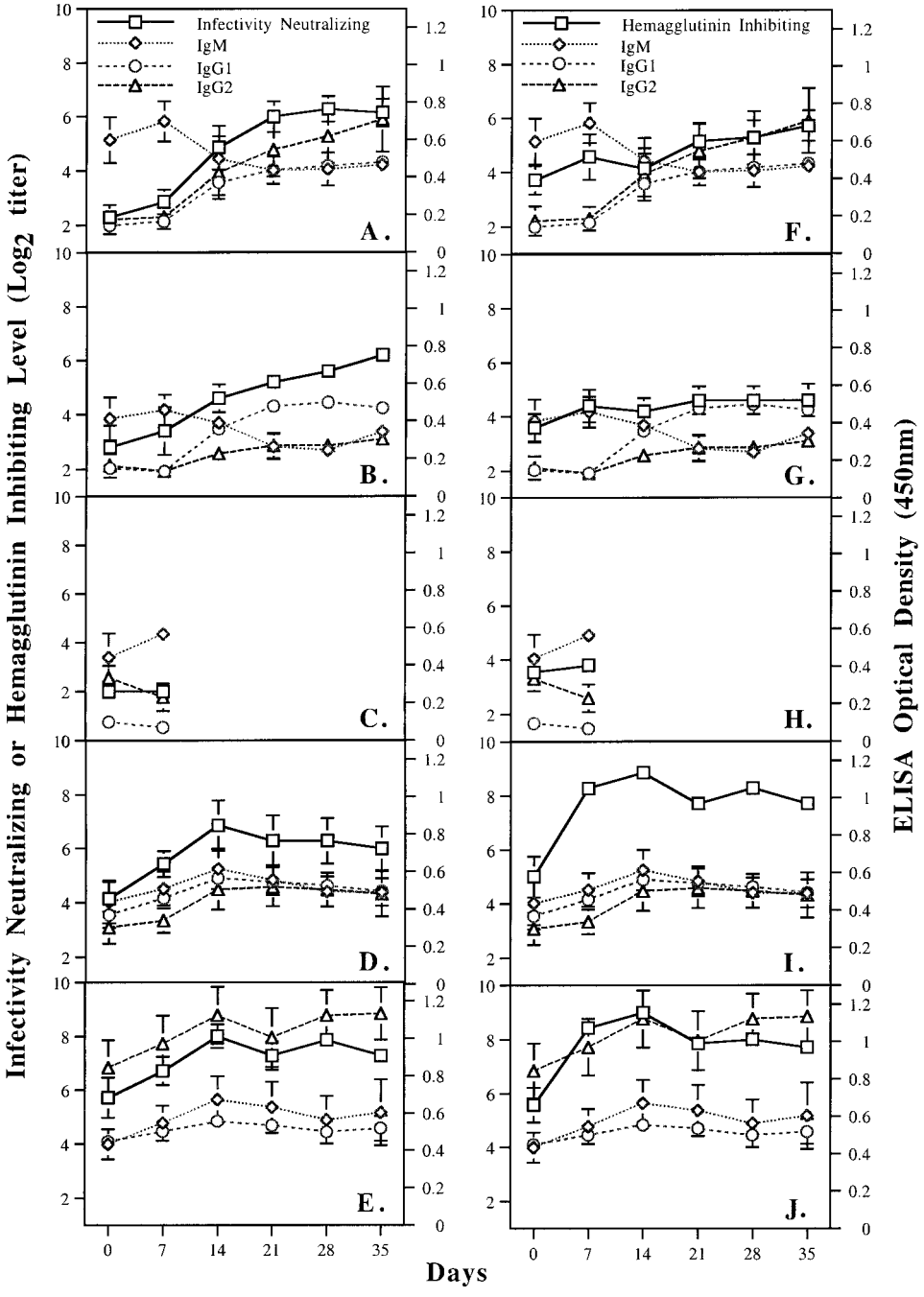

FIG. 1. Levels of IN and HAI activities against RBCV-97TXSF-LU15-2 strain in serum samples of cattle that were nasally shedding RBCV on day 7 and were clinically sick (A and F), that secreted RBCV in nasal discharges without adverse respiratory signs (B and G), that nasally shed RBCV and developed fatal pneumonia (C and H), and that remained RBCV isolation negative with mild respiratory signs (D and I) or without any adverse clinical signs (E and J). Data are means ± SEM (error bars) (n = 7, 5, 9, 7, and 7 for A + F, B + G, C + H, D + I, and E + J, respectively).

ing the first 2 weeks; reached 6.86 ± 0.94 ($P < 0.0001$) and 8.00 ± 0.44 ($P = 0.0003$) on day 14 for cattle in response groups 4 and 5, respectively; and then remained at high levels (Fig. 1D and E). The serum IN antibody levels for cattle in response group 5 were 5.71 ± 0.75 on day 0 and 6.71 ± 0.52 on day 7, which were significantly higher than those for cattle in response group 4, which were 4.14 ± 0.63 on day 0 ($P = 0.0122$) and 5.43 ± 0.48 on day 7 ($P = 0.0395$). The level of HAI antibody in serum was initially at 5.00 ± 0.76 and 5.57 ± 0.65; dramatically increased to 8.29 ± 0.36 ($P < 0.0001$) and 8.43 ± 0.20 ($P < 0.0001$) by day 7 for cattle in response group 4 and 5, respectively; and remained high (Fig. 1I and J).

Correlation of serum IN and HAI activities with immunoglobulin M (IgM), IgG1, and IgG2 levels. The results for

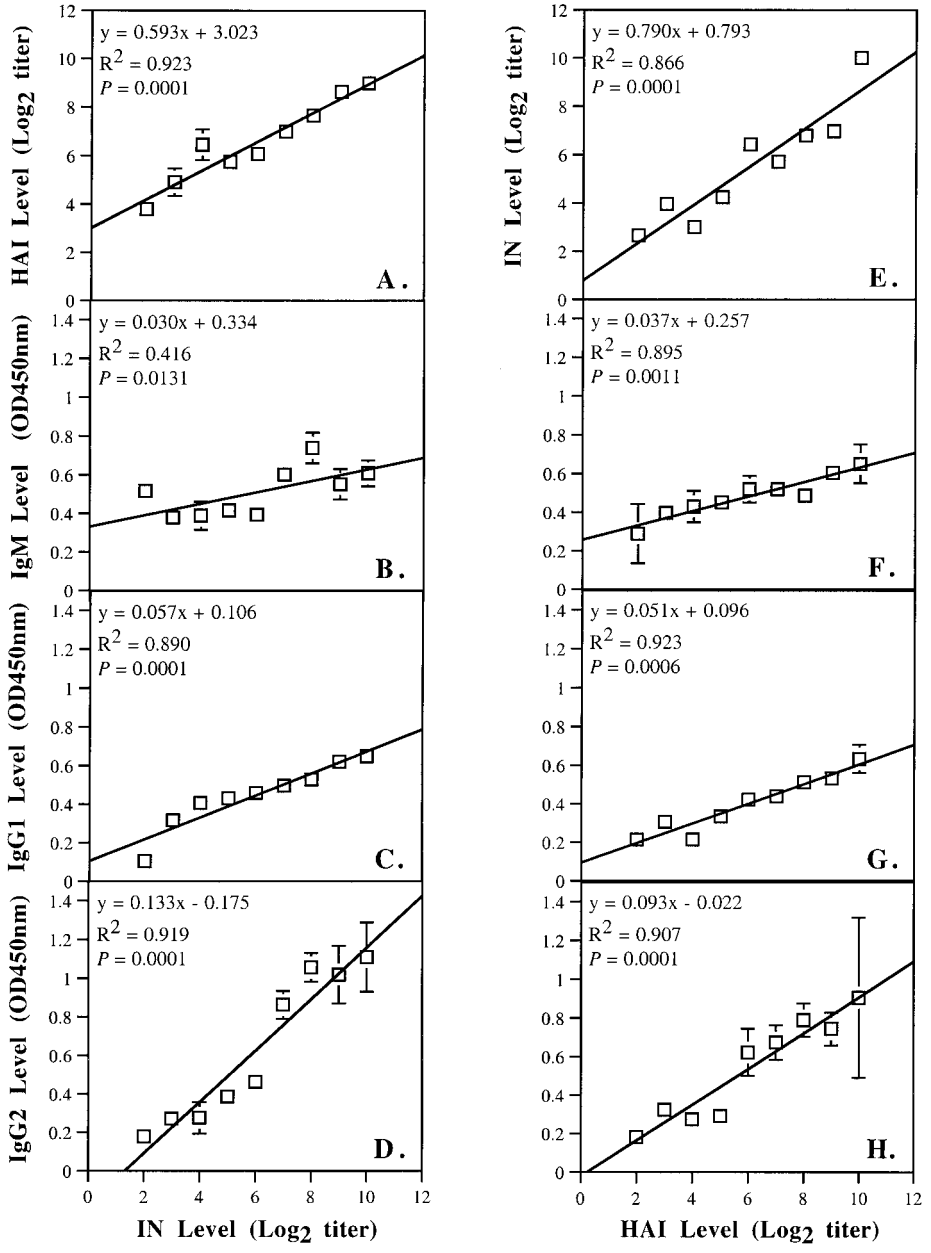

FIG. 2. Correlation of serum IN antibody level versus levels of HAI antibody (A), IgM (B), IgG1 (C), and IgG2 (D) in serum and serum HAI antibody level versus levels of serum IN antibody (E), IgM (F), IgG1 (G), and IgG2 (H) in serum. The mean ± SEM (error bars) for 33, 12, 11, 28, 30, 29, 14, 8, and 6 serum samples are shown for base 2 logarithms of IN antibody titers of 2, 3, 4, 5, 6, 7, 8, 9, and 10, respectively in panels A, B, C, and D. The means ± SEM (error bars) for 6, 26, 18, 24, 16, 24, 29, 26, and 2 serum samples are shown for base 2 logarithms of HAI antibody titers of 2, 3, 4, 5, 6, 7, 8, 9, and 10, respectively in panels E, F, G, and H.

comparisons between serum IN activity and HAI level are shown in Fig. 2A and E. The correlation between serum IN activity and corresponding HAI level was especially significant ($P = 0.0001$) and was excellent, with a coefficient of determination (R^2) of 0.923 for IN level versus HAI level (Fig. 2A) and 0.866 for HAI level versus IN level (Fig. 2E).

The correlation between the serum IN activity with IgM, IgG1, and IgG2 levels is presented in Fig. 2B, C, and D, respectively. The best correlation existed with the IgG2 level, which had a P value of 0.0001 and an R^2 of 0.919 (Fig. 2D). The correlation of the serum IN activity with IgG1 level was slightly lower ($R^2 = 0.890$), but the results were still in excellent agreement and especially significant ($P = 0.0001$) (Fig. 2C). The correlation between the IN activity and IgM level was the lowest ($R^2 = 0.416$) and not remarkable ($P = 0.0131$) (Fig. 2B).

The serum HAI activity was also compared with IgM, IgG1, and IgG2 levels (Fig. 2F, G, and H). Serum IgG1 and IgG2 levels were highly correlated with serum HAI activity, with an R^2 value of 0.923 and 0.907 and a P value of 0.0006 and 0.0001, respectively (Fig. 2G and H). The correlation of IgM with the

serum HAI activity was much higher ($R^2 = 0.895$) and more significant ($P = 0.0011$) than that with the serum IN activity (Fig. 2B and F).

DISCUSSION

The kinetics of serum IN and HAI activities with RBCV were defined for the first time during a naturally evolving and experimentally assessed epizootic of SFP and correlated with virus clearance from respiratory tracts. Such a characterization of the functional effector mechanism of the bovine humoral immune response is not possible through enzyme-linked immunosorbent assay, immunodiffusion test, or immunoblotting assay. Similar investigations of young calves with EBCV infections have not been reported except for in vivo studies after inoculating attenuated and virulent EBCV into the surgically isolated Thirty-Vella intestinal loops of 4-day-old, colostrum-deprived calves (15). Serum IN antibody was present 6 to 7 days postinoculation, and IgM and IgA but no virus were detected in the intestinal loop fluid 9 to 10 days postinoculation.

Strong primary IN antibody responses were detected among the cattle which nasally shed RBCV during the early stage of the epizootic. The serum IN antibody level was initially low and significantly increased after day 7, with a simultaneous decline of nasal RBCV shedding in most cattle within a week. Intranasal vaccination of calves with modified live virus of infectious bovine rhinotracheitis induced neutralizing activity in the serum by day 21 (12). This finding, along with our previous studies on the antigenicity of RBCV structural proteins by immunoblotting assays (14), highlights the importance of S and HE glycoproteins in inducting IN antibodies in RBCV infections, similar to reports on EBCV and human respiratory coronavirus infections (4, 5, 9, 21).

Serum HAI activity was higher than that of serum IN during the first week of this investigation. Our previous observation on the antigenicity of RBCV structural proteins in RBCV infections indicates that both HE and S are viral antigens recognized during the initial stages of the bovine immune response to RBCV infections (14). The HE glycoprotein induced antibodies 1 week earlier than S glycoprotein, whereas S glycoprotein induced a more persistent antibody response. The structure favoring exposed epitopes and the abundance of the HE glycoprotein might have facilitated the early strong HAI antibody response (11, 13, 19). Interestingly, a remarkable rise in the level of serum HAI activity was observed among the sick but not among clinically normal cattle between days 0 and 21. Immunologic injury, mediated largely through antibody to the S glycoprotein, plays an important role in the pathogenesis of feline infectious peritonitis (FIP) (3, 10, 17, 18). Immune complex formation and complement deposition incite pyogranulomatous lesions of FIP, and MAbs to the S glycoprotein of FIP virus can enhance this infection of macrophages in vitro. Furthermore, purified S glycoprotein of EBCV was documented to exhibit higher HA activities with rodent RBC than purified HE glycoprotein (23, 24). Therefore, we proposed that a rapid increase in the level of HAI antibody primarily against S glycoprotein could result in deposition of antibody-antigen complex and complement and aggravation of the RBCV infection as a possible pathologic mechanism of early disease enhancement.

Infectivity neutralizing and HAI antibodies could only be detected at minimal levels in the serum of cattle with fatal SFP. High titers of RBCV, reaching up to 5.0×10^6 PFU/g were detected in the pneumonic lung tissues of these cattle (25). Inability to develop neutralizing antibodies against S and HE glycoproteins could have resulted in severe RBCV infections of lungs, which evidently favored *Pasteurella haemolytica* infections, typical for SFP (25, 34). The interactive infection of the lung with RBCV and *P. haemolytica* in SFP requires additional study.

High levels of IN and HAI antibodies against RBCV infections in serum developed initially and further increased in a subgroup of cattle that remained completely RBCV isolation negative during this 5-week investigation. Importantly, a major difference between the cattle with and without signs of respiratory tract diseases was the serum IN antibody level during the first week. These clinically normal cattle had significantly higher IN levels than the cattle developing clinical signs, suggesting that a high level of IN antibody against RBCV enabled the cattle to resist RBCV infections more efficiently, thus preventing clinical signs of SFP.

A high correlation between serum IN and HAI activities was documented. However, results of the correlation of serum IN and HAI activities with immunoisotype levels, along with the detailed illustrations in Fig. 1, suggest that the IgG, especially IgG2, might contain the majority of the RBCV-specific IN activity in the bovine serum, whereas both IgG and IgM might play an important role in serum HAI activity. This result implies that detection of serum RBCV-specific HAI antibodies is more useful at the early stage of RBCV infection, whereas monitoring of serum RBCV-specific IN activity may be a better indicator of overall outcome of disease development. These results further indicate that measurement of RBCV-specific IgG, particularly IgG2, may provide a good estimate of protective IN antibody levels.

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