Analysis of the Antibody Response to Bovine Respiratory Syncytial Virus Proteins in Calves

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SUMMARY

The antibody response in calves to natural infections with bovine respiratory syncytial virus (BRSV) was analysed by radioimmunoprecipitation assays. Antibodies to virus proteins of Mr 200K (L), 87K (G), 46K (F), 41K (N), 35K (P), 28K and 24K (F2), 27K (M), 22K and < 14K could be identified. Recovery of 6- to 7-month-old calves from severe BRSV-associated disease was accompanied by the development of an antibody response to the virus, which was directed mainly to the F and N proteins. Calves of 2 to 3 weeks of age possessed moderate levels of maternal antibodies to BRSV particularly directed to the F and N proteins but became seriously ill after infection. The antibody response in these calves was severely suppressed. In the sera of 4- to 9-month-old calves that died in the course of infection, high antibody levels to the virus were found, which were directed at least to the F and N proteins. The presence or development of antibodies to the F and N proteins appears insufficient for protection against or recovery from BRSV infections.

INTRODUCTION

Human and bovine respiratory syncytial virus (HuRSV and BRSV) belong to the genus Pneumovirus of the family Paramyxoviridae (Kingsbury et al., 1978). They are important causes of lower respiratory tract disease in infants and calves (Stott & Taylor, 1985).

The genome of HuRSV codes for 10 unique virus proteins, eight of which are structural (for a review, see Collins & Wertz, 1986). Four proteins are associated with the envelope of the virus, i.e. the two glycosylated surface proteins, which are the large glycoprotein (G; 84K) and the fusion protein (F; 68K), and the matrix (M; 26K) and the 22K protein. The F protein consists of two disulphide-linked polypeptide fragments, F1 (48K) and F2 (20K), and causes fusion of infected cells (Walsh & Hruska, 1983). The G protein is the attachment protein (Levine et al., 1987). Three proteins, together with the viral RNA genome, constitute the nucleocapsid of the virus. These are the nucleocapsid protein (N; 42K), the phosphoprotein (P; 34K) and the large (L; 200K) protein with presumed RNA polymerase activity. In addition, a small 9.5K protein (IA) is found in association with purified virions.

The protein composition of HuRSV and BRSV strains is very similar, with only minor differences in M, between corresponding proteins (Cash et al., 1977). HuRSV and BRSV strains are also antigenically closely related. Two subgroups of HuRSV can be distinguished, the major antigenic differences of which are on the G protein (Anderson et al., 1985; Mufson et al., 1985). BRSV strains can be classified as a separate group, sharing epitopes with most proteins of the two human subgroups, but being distinct with respect to epitopes on the G protein (Taylor et al., 1984; Örvell et al., 1987).

A number of workers have analysed the antibody response to HuRSV in humans. Ward et al. (1983) showed that sera from infected infants reacted mainly with the F and N proteins, whereas sera from adults also recognized the G protein. Gimenez et al. (1987) found reactivity in infected infants and children mainly to the F and N proteins, whereas Vainionpää et al. (1985) detected
antibodies in children predominantly directed to the F and M proteins. Murphy et al. (1986a) and Levine et al. (1988) found maternal antibodies that reacted with the G and F proteins. Infected children, in which the antibody response was suppressed by maternal antibodies, responded to the G and F proteins (Murphy et al., 1986a) or mainly to the G and P proteins (Levine et al., 1988).

This is the first report on the protein specificity of the antibody response to BRSV in calves. We examined the response by radioimmunoprecipitation (RIP) assay in calves that recovered and calves that died during infections. We also analysed the specificity of maternal antibodies present in calves that, nevertheless, became infected.

METHODS

**Cells.** Bovine foetal trachea (BFT) cells were grown at 37 °C in tissue culture flasks in Eagle's MEM with Hanks' salts (EMEM) supplemented with 10% foetal calf serum and antibiotics.

**Growth of virus and radiolabelling.** Subconfluent monolayers of BFT cells were inoculated with a stock preparation of the Lelystad strain of BRSV at an m.o.i. of 0.1. Maintenance medium consisted of Eagle's MEM with Earle's salts (EMEM) supplemented with 2% chicken serum and antibiotics. When the first signs of c.p.e. were observed, usually 3 days after inoculation, medium was replaced with fresh maintenance medium, for labelling with [3H]glucosamine, or with medium containing 10% of the normal concentration of amino acids, for labelling with [3H]amino acids. After 1 h incubation at 37 °C, [3H]glucosamine (100 μCi/ml of medium) or [3H]amino acid mixture (100 μCi/ml of medium) (TRK 440; Amersham) was added. After about 48 h, when c.p.e. was extensive, cells were scrapped into the culture medium, collected by low speed centrifugation and washed in ice-cold buffer (10 mM-Tris-HCl pH 7.2, 1.5 mM-MgCl₂, 0.14 M-NaCl).

**Lysis of radiolabelled cells.** After radiolabelling, cells were lysed by incubation for 30 min at 0 °C in 3 ml of RIP buffer and passing four times through a 26-gauge needle. Two different RIP buffers were used: [3H]glucosamine-labelled cells were lysed in 10 mM-Tris-HCl pH 7.2, 0.14 M-NaCl, 0.1% PMSF, containing 10% (v/v) Nonidet P40 (N buffer); [3H]amino acid-labelled cells were lysed in 10 mM-Tris-HCl pH 7.2, 0.14 M-NaCl, 0.1% PMSF, containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS (TDS buffer). Debris was removed by centrifugation at 2000 g for 15 min and at 10000 g for 30 min. Control lysates were prepared in the same way from mock-infected BFT cells. Lysates were stored at −20 °C.

**RIP assay.** Before use, radiolabelled lysates and test sera were centrifuged at 10000 g for 20 min. Lysate samples containing approximately 5 × 10⁶ c.p.m. were diluted to 250 μl with RIP buffer, and mixed with 4 μl of serum. Mixtures were incubated at 4 °C overnight. Then 50 μl of a 1:1 dilution of a rabbit anti-bovine immunoglobulin preparation (Dakopatts) in RIP buffer was added to each mixture and incubation was continued for 30 min at room temperature and 2 h at 4 °C. Mixtures were underlayed with 250 μl of 10% sucrose in Tris-buffered saline pH 7.2, and immune complexes were collected by centrifugation at 10000 g for 5 min. Pellets were washed once in the appropriate RIP buffer and once in demineralized water by suspending and re-pelleting. Finally, pellets were resuspended in 40 μl of sample buffer (Laemmli, 1970), heated for 4 min at 100 °C and analysed by SDS-PAGE.

**SDS-PAGE.** This was performed using the discontinuous buffer system described by Laemmli (1970). The resolving slab gel consisted of a 10% polyacrylamide gel for which bisacrylamide was used as a cross-linking agent. For stacking a 3.75% polyacrylamide gel was used. Electrophoresis was carried out for 2 h at 20 mA/gel and 2 h at 40 mA/gel. 14C-labelled Mr marker proteins (Amersham) were: myosin, 200K; phosphorylase b, 92.5K; bovine serum albumin, 69K; ovalbumin, 46K; carbonic anhydrase, 30K; lysozyme, 14-3K. After electrophoresis gels were processed for fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975). Dried gels were exposed to Kodak X-Omat-AR films at −70 °C.

**Tested sera.** Sera were collected from seven calves that recovered, and from eight calves that died during outbreaks of BRSV-associated disease. Of the calves that recovered, four (A₁, A₂, two from each of two herds) were 6 to 7 months old, and three (B₁ to B₃; from a third herd) were 3 weeks old at the onset of disease. The calves that died (C₁ to C₃) originated from different farms and varied in age from 4 to 9 months. Calves died 3 to 5 days after the onset of respiratory tract disease. From group A and B calves three series of sera were collected: in the acute stage of disease, and about 1 and 3 weeks later. From group C calves, blood was collected at necropsy from large veins and heart. In addition, serum was collected from a calf that had been inoculated intranasally with BRSV at 3 weeks of age and challenged intranasally and intratracheally at 3 months. The calf was involved in a study that will be published elsewhere (Kimman et al., 1989c).

**Diagnosis of BRSV infections.** For diagnosis of BRSV infections in group A and B calves, two previously reported ELISA procedures were used. Diagnosis by one ELISA (IgG-ELISA) was achieved on the basis of a fourfold or greater increase in BRSV IgG antibody titres between paired sera (Westenbrink et al., 1985). The other ELISA
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(IgM-ELISA) was used to detect a BRSV-specific IgM response. With the latter, BRSV infections in young calves with maternal antibodies can be diagnosed; these would usually be missed by other serological tests (Westenbrink & Kimman, 1987).

The involvement of BRSV in the outbreaks of respiratory tract disease during which group C calves died was reported previously (calves 539, 571, 573, 574, 590, 594, 593 and 600; Kimman et al., 1989a).

RESULTS

Identification of BRSV-specific proteins

When we used procedures that had previously been successfully applied to purification of HuRSV (Lambert & Pons, 1983; Wechsler et al., 1985), we obtained virus preparations that were heavily contaminated with cellular proteins. For identification of BRSV proteins, we were therefore restricted to RIP assays. For identification of glycosylated viral proteins we used infected cells labelled with \[^{3}H\]glucosamine and extracted with N buffer, and for the remaining virus proteins we used \[^{3}H\]amino acid-labelled cells extracted with TDS buffer. Fig. 1 shows results obtained with serum from a naturally infected calf (A) collected 1 month after the onset of disease (a, lanes 4 and 5; b, lanes 5, 6 and 7) and with serum from an experimentally infected calf (E) (a, lanes 6 and 7; b, lanes 8, 9 and 10). Both sera precipitated proteins of Mr 46K, 41K, 35K, 27K, 24K, 22K and < 14K from the \[^{3}H\]amino acid-labelled lysate (Fig. 1 a), and of Mr 46K and 24K from the \[^{3}H\]glucosamine-labelled lysate. A glycosylated protein of Mr 87K was precipitated only by serum E (Fig. 1 b). This protein was not precipitated from the \[^{3}H\]amino acid-labelled lysate. Although in this experiment similar results were obtained with \[^{3}H\]glucosamine-labelled cellular lysates prepared with either one of the two RIP buffers (Fig. 1 b), in other experiments slightly better results were obtained with respect to precipitation of the 87K glycoprotein when the lysate prepared with N-buffer was used. We therefore used this lysate to study antibody activity against the glycosylated virus proteins.

With the different sera a total of eight BRSV proteins were identified, with Mr values corresponding to the equivalent HuRSV proteins (see Fig. 1 to 4). Three of the proteins, of Mr 87K (G), 46K (F1) and 24K (F2), were glycosylated. The F2 protein was usually resolved into two bands of Mr 28K and 24K, as was also reported for the F2 protein of HuRSV (Lambert & Pons, 1983). Five non-glycosylated proteins were identified, with Mr values of 200K (L) (see Fig. 2), 41K (N), 35K (P), 27K (M) and 22K (corresponding to the 22K envelope protein of HuRSV), and a polypeptide band of Mr < 14K was detected near the front during electrophoresis.

Analysis of sera from recovered calves (group A)

Sera were collected from four calves during outbreaks of BRSV-associated disease and infections were diagnosed in all of them. With the IgG-ELISA a significant increase in antibody titre between paired sera (samples 1 and 3) was measured; with the IgM-ELISA samples 2 and 3 of each calf were scored positive (Table 1). The results obtained in the RIP assays are shown in Fig. 2. All first serum samples were negative, all second samples reacted predominantly with the 46K (F1) and, to a lesser extent, with the 41K (N) protein. The second and third samples reacted to various degrees with additional proteins, i.e. the 35K (P), 27K (M), 22K and < 14K proteins and the 28K and 24K F2 bands. Only the third sample of calf A4 reacted with the 200K (L) protein. The virus specificity of the precipitated proteins was demonstrated by their absence in precipitates obtained with the first serum samples and with the control lysate.

Analysis of sera from recovered calves with maternal antibodies (group B)

Group B calves, 3 weeks of age, originated from a herd that has been described previously (herd G; Kimman et al., 1986). The calves had maternal antibodies to BRSV and showed no increase in IgG antibody titre during infection. Two of the three calves showed a BRSV-specific IgM response. The third calf had a relatively high IgG antibody titre to BRSV, the suppressive effect of which probably explains the absence of an IgM response in this particular calf (Table 1). In the RIP assay (see Fig. 3), all first samples reacted with the 46K (F1) glycoprotein and with the 41K (N) protein. The first sample from calf B1 reacted also with the 24K (F2) glycoprotein.
Fig. 1. SDS-PAGE analysis of [3H]amino acid-labelled (a) or [3H]glucosamine-labelled (b) virus-infected (subscript V) or uninfected (subscript C) cellular lysates and of immunoprecipitates. (a) Mr markers (lane 1), virus-infected (lane 2) and uninfected (lane 3) cellular lysates, and immunoprecipitates obtained with a serum of a naturally infected calf (A) (lanes 4 and 5) and with serum from an experimentally infected calf (E) (lanes 6 and 7). (b) Mr markers (lane 1), virus-infected cells lysed in TDS buffer (lane 2) or N buffer (lane 3), uninfected cells lysed in N buffer (lane 4), and immunoprecipitates obtained with serum A (lanes 5, 6 and 7) and E (lanes 8, 9 and 10) using virus-infected lysates prepared with TDS buffer (lanes 5 and 8) or N buffer (lanes 6 and 9) and uninfected lysates prepared in N buffer (lanes 7 and 10).
Fig. 2. SDS–PAGE analysis of immunoprecipitates obtained with sera of group A calves, using [3H]amino acid-labelled (a) or [3H]glucosamine-labelled (b) virus-infected (lanes 1 to 3) or uninfected (lanes 4) lysates. Serum samples were collected from four calves (A1 to A4) at the acute stage of disease (lanes 1) and about 1 week (lanes 2) and 3 weeks later (lanes 3 and 4). Each third sample was tested on virus-infected and control lysates.
Fig. 3. SDS–PAGE analysis of immunoprecipitates obtained with sera of group B calves, using [3H]amino acid-labelled (a) or [3H]glucosamine-labelled (b) virus-infected (lanes 1 to 3) or uninfected (lanes 4) lysates. Serum samples were collected from three calves (B1 to B3) at the acute stage of disease (lanes 1) and about 1 week (lanes 2) and 3 weeks later (lanes 3 and 4). Each third sample was tested on virus-infected and control lysates.
Fig. 4. SDS-PAGE analysis of immunoprecipitates obtained with sera of calves that died during BRSV-associated outbreaks of disease group C calves. Sera were tested on virus-infected (lanes 1) and uninfected control lysates (lanes 2), radiolabelled with [3H]amino acids (a) or [3H]glucosamine (b).
Table 1. Diagnosis of BRSV infections by IgG- and IgM-ELISAs

<table>
<thead>
<tr>
<th>Calf</th>
<th>Age</th>
<th>Sample no.</th>
<th>IgG titre*</th>
<th>IgM score*</th>
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<tr>
<td>A1</td>
<td>7 months</td>
<td>1</td>
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<td>3</td>
<td>2560</td>
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<tr>
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<td>&lt;80</td>
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<td>2</td>
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<td>4 months</td>
<td></td>
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</table>

* Diagnosis by the IgG-ELISA was based on measurement of a fourfold or higher increase in BRSV antibody titre between paired sera collected on approx. a 3-week interval (samples 1 and 3) (Westenbrink et al., 1985); diagnosis by the IgM-ELISA was based on the detection of BRSV-specific IgM in a single serum (Westenbrink & Kimman, 1987).

† For group C calves, only one serum sample was available, collected at necropsy.

and with the 27K (M) protein. Reactivity with a 30K protein was also observed with the control lysate and was thus considered to be non-virus-specific. Of the three calves, only B2 showed a clear response to the infection with BRSV in the RIP assay, with additional reactivity to the 35K (P) and 27K (M) proteins in the second and, to a lesser extent, the third sample. The other two calves showed a decrease in reactivity to the 46K (F1) glycoprotein in the successive sera. All sera were weakly reactive to the 87K (G) glycoprotein. Similar results were obtained with sera from four calves of comparable age in another herd suffering an outbreak of BRSV-associated disease.

Analysis of sera from calves that died during disease (group C)

From group C calves, only single serum samples were available, of which all but one reacted positively in the IgM-ELISA. Six sera had moderate to high IgG antibody levels to BRSV (Table 1). Results obtained by RIP assay with sera from these calves are shown in Fig. 4. The sera of calves C1, C3, C6 and C8 reacted clearly with the 46K (F1) and 24K (F2) glycoproteins and the 41K (N) protein. The sera of calves C3 and C8 also reacted strongly with the 35K (P), 27K (M) and 22K proteins, but weakly to the 87K (G) glycoprotein, whereas the sera of calves C1 and C6 showed a weak reaction to the first three of these. The serum of calf C7 was an exception, and reacted most strongly with the 41K (N) protein.
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DISCUSSION

In outbreaks of BRSV-associated disease, calves of 1 to 3 months of age are usually most severely affected (Kimman et al., 1988). Even calves of 2 to 3 weeks of age can become seriously ill (Kimman et al., 1988), indicating that moderate to high levels of maternal antibodies to BRSV provide insufficient protection. The incidence of disease is lower in calves younger than 2 weeks; different factors may be responsible for this, such as a high level of maternal antibodies and/or herd management (Kimman et al., 1989b). Reinfections of cattle have been observed, but normally these are not accompanied by clear clinical symptoms (Holzhauer, 1978; Martin, 1983), suggesting that natural infection offers good protection to BRSV-induced disease.

Group A calves, 6 to 7 months of age, were seronegative in the IgG-ELISA (titre < 80) and thus had no maternal antibodies at the onset of disease. The recovery of these calves was accompanied by the development of a strong antibody response to BRSV, predominantly directed to the F and N proteins. Reactivity to other viral proteins except the G protein was also seen. Previous observations identified the F protein as a major antigen inducing protection. Antisera and monoclonal antibodies to the F protein showed neutralizing activity and prevented cell-to-cell spread of the virus in vitro (Walsh & Hruska, 1983; Walsh et al., 1985). Passive transfer of monoclonal antibodies to the F or G protein offered protection against challenge with HuRSV, while monoclonal antibodies to internal proteins did not (Taylor et al., 1984; Walsh et al., 1984). Immunization with recombinant vaccinia viruses expressing the F or G protein offered protection to HuRSV, the F protein appearing to be the more effective (Olmsted et al., 1986).

Moderate levels of maternal antibodies to the F, N and, to a lesser extent, the G protein were measured in 2 to 3-week-old calves. Despite this, calves became infected with the virus and developed severe disease. Recovery of one calf was accompanied by an antibody response to the P and M proteins. Group C calves died during the course of infection with BRSV. High antibody levels to the virus were found in serum and/or lung washings of these calves (Kimman et al., 1989a; Table 1). Five of the eight sera examined had antibody activity in the RIP assay, and four of these reacted with at least the F and N proteins. The serum of calf C7 was an exception, recognizing predominantly the N protein. An incomplete antibody response may have contributed to the severity of the infection in this particular calf.

Previous analysis of the human antibody response to HuRSV infections by RIP assay (Ward et al., 1983) or by immunoblot analysis (Gimenez et al., 1987) demonstrated antibodies in convalescent sera of infants and adults to be directed mainly to the F and N proteins. Only a high level of maternal antibody to the N protein was associated with protection (Ward et al., 1983). Vainionpää et al. (1985) found the antibody response in infected children of 4 months and older to be directed mainly to the F and M proteins, with only a weak response towards the N protein. Murphy et al. (1986a) used ELISAs with immunoaffinity-purified F or G protein, and Levine et al. (1988) used immunoblot analysis to study the antibody response in HuRSV-infected infants and children with or without maternal antibodies. Maternal antibody reactivity to both the F and G proteins was found. Most infected children without maternal antibodies responded to both proteins (Murphy et al., 1986a). The response in children with maternal antibodies was suppressed, but the antibodies present were directed to the G and F proteins at least (Murphy et al., 1986a), or mainly to the G and P proteins (Levine et al., 1988). These results may indicate that the ELISA and immunoblot assay are more sensitive than the RIP assay in detecting antibody reactivity to the G protein, although with the latter, activity to the G protein was detected in sera of adults (Ward et al., 1983), calves after secondary infections (Kimman et al., 1989c) and calves containing maternal antibodies (this study). Alternatively, the failure to detect antibodies to the G protein may be due to the existence of distinct subgroups of BRSV, as in the case of HuRSV in which the two subgroups are distinct mainly in epitopes on the G protein (Taylor et al., 1984; Örvell et al., 1987).

We found no essential differences in antibody response between calves that recovered and calves that died in the course of severe infections with BRSV, both with respect to the individual virus proteins recognized, and to the level of virus-specific antibodies (see also Kimman et al.,
High levels of virus antibodies, mainly directed to the F and N proteins, appear unable to prevent a fatal course of BRSV-associated disease. In humans, high antibody levels to HuRSV protected against infection. Therefore, moderate levels of maternal antibodies to the G, F and N proteins are not necessarily protective. Additionally, the specificities of the immune response to BRSV (and HuRSV) infections, such as an efficient mucosal IgA response (Kimman et al., 1987a, b) and cytotoxic T cell activity (Bangham et al., 1986; Pemberton et al., 1987). Also, the functional activity of BRSV or HuRSV antibodies, i.e. neutralizing, complement activating or 'non-functional' activity, may play a role in the recovery from, protection against or even the immune-mediated pathogenesis of BRSV- and HuRSV-associated disease. Non-neutralizing antibodies to the F and G proteins possibly mediated enhancement of the disease which was observed in infected children previously vaccinated with formalin-inactivated HuRSV vaccine (Prince et al., 1986; Murphy et al., 1986b). We have distinguished epitopes on the F protein of BRSV that induced neutralizing and non-neutralizing monoclonal antibodies (Kimman et al., 1989c). Further examinations are needed to determine the role that antibodies to BRSV play in field infections.

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