Mapping and structural analysis of B-cell epitopes on the morbillivirus nucleoprotein amino terminus

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INTRODUCTION

Rinderpest is a highly contagious viral disease of animals belonging to the order Artiodactyla, although cattle and buffaloes are the most susceptible hosts. The disease is characterized clinically by pyrexia, erosive then necrotic stomatitis, profuse diarrhoea and death (Plowright, 1968). The causal agent, Rinderpest virus (RPV), belongs to the genus Morbillivirus within the family Paramyxoviridae (Bratt & Hightower, 1977). Other members of this genus are Measles virus (MV), Peste-des-petits-ruminants virus (PPRV), Canine distemper virus (CDV), Phocine distemper virus (PDV) and cetacean morbilliviruses. With the success of the Global Rinderpest Eradication Programme (GREP), the FAO (Food and Agriculture Organization of the United Nations)-led international partnership programme, rinderpest is nearly eradicated from the world, with only an area in East Africa that needs further verification. To facilitate the implementation of that programme and serosurveillance of the disease (Anderson et al., 1990; Libeau et al., 1992). These assays were designed to differentiate between the two ruminant morbillivirus diseases: rinderpest and peste des petits ruminants. They were based on the use of monoclonal antibodies (mAbs) directed against either the viral haemagglutinin (H) or the nucleoprotein (N). H is the viral protein that binds to the cell receptor and against which most of the virus-neutralizing antibodies are directed (Choppin & Scheid, 1980; Merz et al., 1981; Giraudon & Wild, 1985). N is the most abundant and immunogenic viral protein (Barrett et al., 2006).

Sequence comparison of N within the genus Morbillivirus identified three main regions of differing similarity (Diallo et al., 1994): the amino-terminal region of medium similarity, the highly conserved central region and the poorly conserved carboxy-terminal domain covering the last 105 aa. Buckland et al. (1989) showed that mAbs specific to the amino and carboxy termini of the N protein of MV mapped to the least conserved amino acid sequences. Paradoxically, the carboxy-terminal tail of N may be involved in the immunosuppressive effects of morbilliviruses and the host immune response regulation (ten Oever et al., 2002; Zhang et al., 2002; Laine et al., 2003). The antigenic structure of N of both RPV and PPRV was studied by using the strategy of competition between mAbs. This approach has allowed the identification of six antigenic sites, of which four had epitopes that clearly distinguished between RPV and PPRV strains. Some of these mAbs have helped in the development of tests to differentiate between the two viruses (Libeau et al., 1992, 1994, 1995, 1997). Experience has shown, however, that the cELISA that was developed by using one of the specific RPV N mAbs (Libeau et al., 1992) for the serological diagnosis of rinderpest cross-reacts with PPRV antisera. Therefore, we...
decided to perform a more specific analysis of the antigenic and immunogenic properties of the RPV N protein.

The objective of this study was to use a combined strategy of truncated mutants and overlapping synthetic dodecapeptides, covering aa 115–150 (amino terminus) and 415–495 (carboxy terminus) corresponding to the RBOK strain (GenBank accession no. CAA83177), to map the epitope structure of sites I–IV and VI defined by mAbs on the N protein sequence more precisely.

**METHODS**

**Virus and cells.** The different RPV strains used in this study were the attenuated vaccine RBOK strain (Plowright, 1962), RPV Saudi, RPV Egypt, RPV Kuwait and RPV RBT1 (Taylor, 1986). They were propagated in Vero cells cultivated in Eagle’s minimal essential medium (MEM) supplemented with 5% fetal bovine serum and 1% antimycotics and antibiotics.

**mAbs and sera.** Five antigenic site-specific mAbs, IIH2 (1), 48-5 (II), 3-1 (III), IVB2 (IV) and 33-4 (VI) directed against the N protein of RPV RBOK strain, were used along with the anti-PPRV N mAb 38-4 (Libeau et al., 1997). The reactivity of positive RPV antisera [a rabbit rinderpest hyperimmune serum (RHS); a gift from J. Anderson, Institute for Animal Health, UK], a cattle serum against the Kudu strain (lineage II, gift from H. Wamwayi, Kenya Agricultural Research Institute, Kenya) and a naturally infected cattle serum from Chad were analysed. Additionally, a goat serum against the vaccine strain of PPRV, Nigeria 75-1, was used in this study.

**Cloning of the RPV RBOK N gene and transient expression in Vero cells.** Vero cells were infected with the attenuated vaccine RBOK strain of RPV (Plowright, 1962). At 40–50% cytopathic effect, the cells were lysed and total RNA was extracted by using an RNaseasy RNA extraction kit (Qiagen) according to the manufacturer’s instructions. Following RNA extraction, cDNA was synthesized by reverse transcription using a First Strand cDNA synthesis kit and random primers (Amersham Biosciences). This cDNA was used to amplify the full open reading frame of the N gene by using the forward and reverse primers N-PR7 (5'-GATCCTATCGACTGGAGCAAGCTTA-3') and N-PR8 (5'-GGTAGGCTTGCTCCTCTGCCAT-3') with Tak polymerase. The PCR product was cloned into the pGEM-T plasmid (Promega) and the sequence of the insert was confirmed by sequencing. This insert was released by double digestion with restriction enzymes DraI and NotI and recloned into the eukaryotic expression vector pcDNA4/HisMax.B (Invitrogen) previously digested with EcoRV and NotI. In the resulting plasmid, pCN-RBOK, the insert encoding the full-length RPV N protein was placed under the control of the cytomegalovirus promoter. From this plasmid, two truncated mutants named pCN121–146 and pCN421–525 were generated by PCR mutagenesis (Allenberg & Silverman, 1997) using phosphorylated primers. Reverse and forward primers 120 R (5'-P-ACCCCTAGAGGCAAATGTC-3') and 146 N (5'-P-TGGTTTGGAGAAATCGGATA-3') led to the deletion of aa 121–145 in the amino-terminal half, whilst reverse and forward primers 420 R (5'-P-AAGGATTGAAAAACTCTGGGCTTTGTGG-3') and RTAG (5'-P-ACCTGATGATGAGGCCC-CA-3') led to the deletion of aa 421–525 in the carboxy-terminal half. *Escherichia coli* DH5α cells were used to propagate the various plasmids generated in this study. For downstream use, all plasmids were purified from bacteria by using a Qiagen EndoFree plasmid DNA preparation kit. For the transient expression of RPV N protein, Vero cells in 96-well plates (10 000 cells per well) were transfected with either pCN-RBOK plasmid or the truncated mutants by using Fugene 6 transfection reagent (Roche) according to the manufacturer’s instructions. After 72 h incubation at 37 °C, the transfected cells in the 96-well plate were fixed with 80% acetone for 30 min at -20 °C and subjected to indirect immunofluorescence assay (IFA) to assess RPV N protein expression using the following antibodies: RHS, anti-RPV N mAbs IIH2, 48-5, 3-1, 33-4 and IVB2 and anti-PPRV N protein mAb 38-4 (Libeau et al., 1997). mAbs and RHS were used at a dilution of 1/100. The cells were incubated with 50 µl test antibody for 30 min at 37 °C, then washed with PBS before incubation with 50 µl anti-mouse and anti-rabbit fluorescein isothiocyanate conjugates (Bio-Rad) diluted 1/80. After three washes with PBS, they were examined under a Canon inverted fluorescence microscope.

**Western immunoblot.** Cells transfected with the plasmid encoding the full-length RPV N protein, pCN-RBOK, or the truncated mutants pCN120–146 or pCN421–525 were lysed in RIPA buffer [100 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM PMSF] and analysed by SDS-PAGE. The proteins were transferred to a PVDF membrane 0.2 µm (Invitrogen) followed by immunostaining. The proteins were probed for 1 h with the primary antibody, RHS or mAb, at an appropriate dilution in PBS (pH 7.4) containing 0.5% Tween and 5% skimmed milk. Immune complexes were detected by using a secondary anti-rabbit antibody or anti-mouse immunoglobulin conjugated to horseradish peroxidase and subsequent detection using the ECL system (Amersham Biosciences) according to the manufacturer’s instructions.

**Nucleotide sequencing of the N gene from wild-type strains of RPV.** RNA was extracted from Vero cells infected with different RPV strains (Saudi, Egypt, Kuwait or RBT1). The extraction was done as indicated above. The first-strand cDNAs were synthesized using random primers. For each virus, the coding sequence of the N gene was amplified by RT-PCR using forward and reverse N-PR7 and N-PR8 primers and cloned into the pGEM-T vector. The sequence of the entire N gene was obtained by sequencing internal primers. Forward primers were N-PR9, 5’-CAGCATTAAATTGTTG-GAGGTA-3’; N-PR11, 5’-AACAAATGGTCAAAGGGTC-3’; and N-PR13, 5’-ACAGGCGGAGGTTCTACTGACG-3’; and reverse primers were N-PR6, 5’-GTAGGCTGCTCCTGCACT-3’; N-PR14, 5’-ACCTCCACATTATGAGGCA-3’; and Nad2, 5’-GATT-GAGTCTCCTAAAGTCACT-3’. Cycle sequencing was performed by using dye-labelled terminators and Taq DNA polymerase followed by analysis on an ABI Prism 377 automatic sequencer (Applied Biosystems). By using the Vector NTI 9 package (Informax), amino acid changes between aa 115 and 150 of the N protein were evaluated by alignment of deduced amino acid sequences obtained for the above RPV strains with those of RPV RBOK, RPV lapinized strain, RPV Kuwait, PPRV 75-1, CDV Onderstepoort and MV Edmonston (GenBank accession numbers CAA83177, P37708, Z34262, CAA52454, P04685 and AAF85675, respectively). GenBank accession numbers for sequences obtained in this study are EF186057–EF186059 for RPV Egypt, RBT1 and Saudi, respectively.

**Computer-aided analysis.** The sequence–structure relationship of RPV strain RBOK N protein was built automatically by different programs that allowed the prediction of protein secondary structure according to amino acid sequence: PROFSEC (Rost, 2000), SUBSEC (Rost, 2001), gOBA (Garnier et al., 1996), PREDATOR (Frishman & Argos, 1996), PSIPRED (Jones, 1999), INEFT (Cuff & Barton, 2000) and SSPro (Baldi et al., 1999). The consensus secondary structure from all of these programs was used for further analysis.

**Immunosassay with cellulosose-bound peptides (Pepsan).** Cellulose-bound peptides were obtained from Fmoc amino acid chemistry (Synt: em). The Pepsan method used to locate the antibody-reactive peptides has been described previously (Mahé et al., 2000). Synthetic dodecapeptides overlapping by one residue and covering the sequence of N of the RBOK vaccine strain of RPV at positions 115–150 (amino terminus) and 415–495 (carboxy terminus) were examined. Antibody reactivity with Pepsan peptide was measured by indirect ELISA.
according to the manufacturer's instructions (SYNT: em) using mAb ascitic fluids (1:50–1:100) and sera (1:10–1:50) in blocking buffer (Genosys). Bound antibodies were detected on the membrane with an anti-mouse, anti-goat or anti-cattle alkaline phosphatase conjugate (Sigma) according to the origin of the primary antibody involved. The binding of the conjugate was further revealed by a precipitating phosphatase substrate (BCIP sodium salt, MMT and MgCl₂) that produced blue-coloured spots. A known peptide and its antiserum were used as a positive control and were subjected systematically to the different steps of the immunoassay. Reuse of the membrane was made possible by treatment with dimethylformamide, 6 M urea and 10% acetic acid in ethanol.

RESULTS

Transient expression of deletion mutants of the N protein

We speculated that the different antigenic sites defined on the N proteins of RPV and PPRV (Libeau et al., 1997) might be located within one of the two non-conserved regions of the N protein: aa 121–145 or 421–490. In order to map these antigenic sites, two plasmids able to express truncated proteins in eukaryotic cells were constructed. Vero cells were transfected with these plasmids and another expressing the full-length RPV N. Control of protein expression was assessed by indirect immunofluorescence using RHS. Each of the three transfected plasmids gave positive signals. No staining was observed with the negative serum or mAb 38-4, which is specific to the PPRV N protein. All of the other five mAbs specific to RPV N exhibited immunofluorescence staining on cells transfected with the full-length N protein plasmid pCN-RBOK and with one of the truncated mutant plasmids. Four of these mAbs (IIH2, 48-5, 33-4 and IVB2) reacted with the carboxy-terminally truncated mutant pCNA421–525, whilst mAb 3-1 reacted with the amino-terminally truncated mutant pCNA120–146 (Fig. 1). We confirmed the epitope localization of mAbs 33-4, 3-1 and IVB2 by Western blotting analysis of the different RPV N proteins expressed in Vero cells (Fig. 2). The antigenic characteristics of the full-length and truncated N proteins were examined on the basis of their reactivity with RHS (Fig. 2e), showing a strong cross-reaction. The reactivity pattern of the mixed mAbs was comparable to that of RHS (Fig. 2d). Neither mAb IIH2 nor mAb 48-5 bound to the N proteins in this test under denaturing conditions, an indication that they may be directed against a conformational epitope. We concluded that mAbs delineating sites I, II, IV and VI were located in the region comprising aa 121–145, whereas the mAb 3-1 epitope, defining site III, was located in aa 421–525 (see Table 1). To confirm these results, a peptide-mapping study in the amino-terminal part of the N protein was conducted.

Epitope mapping of the amino-terminal region using synthetic peptides

In order to further pinpoint the mAb-binding sites in the variable amino-terminal region, we analysed the capacity of short peptides derived from this region to bind to the four mAbs, IVB2, IIH2, 48-5 and 33-4, known to react with the carboxy-terminally truncated N proteins. The sequence corresponding to aa 115–150 was presented as a set of 25 overlapping dodecamer peptides (one residue frameshift). As a control, overlapping peptides corresponding to the variable sequence of the carboxy-terminal region (positions 415–495) were also analysed. The binding ability of mAb IVB2 (site IV) to these peptides was analysed by indirect ELISA on the cellulose membrane, along with mAbs classified as binding to the RPV N protein antigenic sites I (mAb IIH2) and II (mAb 48-5). The broadly reactive mAb 33-4, recognizing site VI, was also analysed. ELISA successfully identified epitopes for the four mAbs. As an example, Fig. 3 shows the reactivity pattern of peptides contributing to the interaction of two of these mAbs (IIH2 and 33-4) with the sequence derived from the amino and carboxy termini of RPV N. The most prominent peptide reactivity was found in the amino-terminal region on a stretch of 25 aa (from aa 115 to 150), whilst the carboxy-terminal region was found to be completely non-reactive. They had multiple recognition profiles, suggesting that the recognized epitopes were discontinuous, requiring two to three framework peptides displaying binding activity. Although shown to belong to different competition groups (Libeau et al., 1997), these results also indicated that the four mAbs had an epitope location within the same 30 aa, constituting the amino-terminal variable region of the N protein. mAb IVB2 reacted with this region; the most pronounced reactivity was against peptides 8–11, 13–17 and 22–23.

To define the epitope location precisely and identify the shared areas corresponding to peptides showing significant reactivity, the binding patterns of the different mAbs were studied (Fig. 4) by scanning the sequence of interest (aa 115–150). mAb IIH2 showed high peptide reactivity, with prominent recognition of peptides 6, 8–11, 13–17 and 23. A generally low recognition of the peptides was displayed by mAb 48-5 on peptides 8–23, but only peptides 8–11, 13–17 and 22–23 had significant reactivity. Although it recognized shared antigenic sites of the morbilliviruses, unexpectedly, mAb 33-4 (antigenic site VI) showed a strong homogeneous reactivity in this variable region with peptides 8–11, 13–17 and 22–23. From these results, we concluded that the epitopes recognized by mAbs 48-5, IVB2 and 33-4 were all located between aa 122 and 148, with the exception of mAb IIH2, whose epitope extended from aa 120 to 148. The area corresponding to epitope reactivity was delimited within peptides 6–24, corresponding to the stretch in the amino terminus that is less well conserved between members of the genus Morbillivirus. This sequence is GTSMDEADRYTYEENPNDGEERQSYWFEN (Fig. 5a). The conserved regions flanking this variable sequence (peptides 1–5, 18–21 and 25), as well as the most variable region of the carboxy terminus, displayed no binding activity. The previously defined epitopes could be localized more precisely by defining the reactive framework residues displaying binding activity in overlapping peptides. The
analysis of the sequences with binding properties indicated that the possible delimitation of the binding epitope could be based on the appearance or disappearance of residues contributing to the binding activity. The study of mAb IIH2 in the recognition of its epitope is given as an example (Fig. 4). The sequence TSMDEADRYFT (peptide 7) had no activity, but the following overlapping sequence SMDEADRYFTY (peptide 8) gained activity with the adjunction of Y. In contrast, the decrease in the binding activity occurred with the disappearance of a residue from

Fig. 1. Indirect immunofluorescence microscopy of Vero cells analysed for intracellular transient expression of the full-length N protein of the RBOK strain of RPV (pCN-RBOK), the amino-terminally truncated protein pCNΔ120–146 or the carboxy-terminally truncated protein pCNΔ421–525. Seventy-two hours after transfection, cells were permeabilized and stained with the panel of anti-RPV mAbs (IIH2, 48-5, 3-1, IVB2 and 33-4). Non-transfected Vero cells were used as a negative control.

Fig. 2. Location of mAbs by Western blotting on the full-length N (lane 1) and on the deleted mutant proteins, NΔ120–146 (lane 2) and NΔ421–525 (lane 3), in transfected Vero cells. RBOK N proteins were separated by gel electrophoresis, blotted onto a membrane and detected by various antibodies: (a) mAb 33-4, (b) mAb 3-1, (c) mAb IVB2, (d) a mix of mAbs 3-1, 33-4 and IVB2, (e) RHS.
the peptide sequence: the loss of activity of peptide 12 compared with peptide 11 is attributed to the loss of the D residue. The whole motif contributing to the binding of the mAb was located in the so-defined epitope. When a unique peptide was recognized, only important residues were identified. The residues contributing to the epitope recognition were: 125D, 131F, 133Y, 138D and 148E for mAb IIH2; 125D, 131F, 133Y, 138D for mAb 48-5; and 125D, 131F, 133Y, 137N, 138D and 147F for mAbs IVB2 and 33-4.

The delimitation of the mAb interfaces and the residues identified above as important for the recognition of RBOK N are represented schematically in Fig. 5(b). Important residues were also located on peptide 6 (120G) and on peptide 23 (137N) for mAb IIH2. Therefore, two antigenic motifs were defined. The major one was delimited on peptide DEADRYFTYEEPND from 125D to 138D, defined as a common antigenic peptide recognized by all mAbs. In the case of mAb IIH2, it was extended to 120G. Close to this main sequence, two residues (147F and 148E) contributed to the epitope recognition defined below.

Identification by sequence analysis of residues required for efficient binding of the N protein of different RPV strains

mAbs IIH2, 48-5, IVB2 and 33-4 were mapped onto the aa 120–149 sequence of N deduced from the RBOK strain. The pattern of reactivity of different strains of RPV and representatives of PPRV, CDV and MV to mAbs in the IFA was described in a previous publication (Libeau et al., 1997) and is summarized in Fig. 5(b). The four mAbs reacted with the N protein of all of the RPV strains, although mAb 48-5 failed to recognize that of RBT1. Except for mAb 33-4, none of these mAbs reacted with PPRV. mAbs IIH2 and 33-4 reacted with CDV and MV. Consequently, to demonstrate whether the sequence variation among the strains was helpful to determine framework residues contributing to the difference in reactivity, the cDNA corresponding to the targeted area for the different RPV strains was amplified and sequenced. The following RPV strains were used: Saudi, Egypt, Kuwait and RBT1. The deduced amino acid sequences were aligned

![Image](http://vir.sgmjournals.org)
Fig. 4. Determination of the fine specificity of mAbs by indirect ELISA. Synthetic dodecapeptides (overlapping by one residue) spanning aa 115–150, corresponding to the variable amino-terminal sequence in the N protein of RPV, were tested on a derivative membrane to identify reactive peptides. Shaded areas correspond to significant binding. Residues in red delineate the recognized epitope.
with published data of the N protein of RPV (RBOK and lapinized strain), PPRV, CDV and MV (GenBank accession numbers CAA83177, Z34262, CAA52454, P04865 and AAF85675, respectively) (Fig. 5b). Reactive amino acids that were shared between all morbilliviruses were not considered as effective when the mAb had a differential reactivity. The remaining residues were then compared to define the minimum amino acid sequence involved in the difference in reactivity among strains.

mAb IIH2 reacts with RPV, CDV and MV. It gave a negative result with PPRV 75-1, but reacted with some other PPRV strains (G. Libeau, unpublished data). Therefore, a conserved sequence could be involved in the delineated epitope with the contribution of a variable residue. 124D and/or 125D, which are conserved among RPV, PPRV, CDV and MV strains, could be involved. Taken together with the previously identified residues, the contributing motif for IIH2 was found to be 120G---125D---131F---133Y---137ND138 and 148E.

mAb 48-5 reacted with all RPV strains except RBT1. In the area of peptides recognized by this mAb, there are three main amino acid changes between RBT1 and other RPV strains: 131L, 136S and 144F in the protein sequence alignment. In the same region, four other amino acids are not conserved between different RPV strains: 135F for RPV Saudi, 138G for RPV lapinized and 143R for RPV Egypt. In total, within the sequence between aa 131 and 144, there are seven changes. In addition to this region, the cooperation of the motif 147FE148 was found to be necessary for an efficient reaction with the RPV N protein. The motif recognized by mAb 48-5 was therefore determined to be 125D---RYFTY---PN138 144S-FE148. As this does not react with RPV RBT1 strains, the three amino acid changes noted for this strain, 131L, 136S and 144F, might be critical for the formation of the epitope.

By using the same deductive method, the contributing motifs for mAb IVB2 were found to be 125D---RYFTY---ND138 145Y-F147. mAb 33-4 is different from the preceding mAbs in that it recognizes different morbilliviruses, including MV, CDV and PPRV (DMV was not tested). Alignment analysis between the four viruses suggests that the amino acid sequence implicated in the interaction of antigen with this mAb was 125DEAD--F-Y---ND138 146WFEN149.

Antibody-binding sequence comparison between mouse mAbs and sera from immunized or convalescent goats and cattle: correlation with the secondary structure of the epitopes

To determine whether the epitopes that we identified within the amino-terminal region of RBOK N are...
peptides located in the region of aa 129–133, strong (Saudi) overall reactivity; they also reacted with the region encompassing residues 126–133, whereas residues 145–147 did not appear to be involved in the binding. The other sera had weak (RBOK), moderate (lineage II) or generally strong reaction, with maximum reactivity in the carboxy terminus of the N protein of the RPV RBOK strain, analysed with the ruminant sera also indicated that this region induced a strong immune response (data not shown). The efficiency of a mAb in cELISA to detect the peptide-reactivity pattern obtained with the different mAbs studied with those with sera from immunized or convalescent goats and cattle. To this end, five different sera were analysed. The history of the sampled animals is as follows: a bovine that recovered from rinderpest during an outbreak in Chad in 1987, an RPV RBOK-vaccinated bovine, a bovine infected experimentally with an RPV strain isolated from an antelope (RPV Kudu strain; H. Wamwayi, personal communication), a goat inoculated against a different and non-competitive epitope should demonstrate that these amino acids were strongly immunodominant for the humoral response to RPV infection. The similar reactivity observed for the four anti-RPV animal sera suggests that the immunogenicity of the region encompassing aa 129–133 is conserved among the virus strains and corresponds to the recognition sites of the mAbs. On the other hand, the anti-PPRV goat serum had weak or no reaction with the region encompassing these residues, confirming the difference in the amino acid sequence. The slight reactivity observed with peptides covering the variable sequence 137–143 could be explained by a similar amino acid conformation or a cross-reactivity with an unknown RBOK sequence. Overlapping synthetic peptides covering aa 415–495, corresponding to the variable sequence at the carboxy terminus of the N protein of the RPV RBOK strain, analysed with the ruminant sera also indicated that this region induced a strong immune response (data not shown). The efficiency of a mAb in cELISA to detect the humoral response to RPV is primarily due to the sharing of the same recognition epitope between mAbs and immune sera. Hypothetically, the immune response against a different and non-competitive epitope should not be taken into account.

### Table 2. Reactivity pattern of cattle and goat antibodies to the amino terminus of the RPV RBOK N protein

An indirect ELISA spot test was performed to identify the location of the B-cell epitopes. Sera were tested when the response reached the plateau. Results were expressed as 0, ±, + and ++ for low, medium and high reaction, respectively.

<table>
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<th>Peptide no.</th>
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The immunogenic properties of the N protein correlated with the secondary-structure prediction that was generated (Fig. 6). An α-helix structure from 124D to 133Y fitted inside the major antigenic motif delimited on peptide DEADRYFTYEEPND. Helical configuration may lead to better fitting with immune-system molecules, rendering them more immunogenic.

DISCUSSION

RPV, as all other morbilliviruses, induces an efficient immune response, but also, paradoxically, a transient immunosuppression, in its host (Heaney et al., 2002, 2005; Laine et al., 2003, Kerdiles et al., 2006). This latter characteristic is responsible for secondary bacterial infections, which are common to all morbillivirus infections. N is the most abundant viral protein and anti-N antibodies are produced rapidly after infection, supporting the view that, during antibody synthesis, there is a direct release of RPV N into the extracellular compartment, where it binds to B-cell receptors (Laine et al., 2003). Previously (Libeau et al., 1997), we identified six antigenic sites on the RPV N by a competitive-binding assay of mAbs to the protein. In the present study, we used a combined strategy of truncated mutants and peptide analysis to map these antigenic sites more precisely. Our results point to a more efficient immunogenicity of the amino-terminal than the

Fig. 6. Secondary-structure prediction of the N protein of the RBOK strain of RPV. A sequence consensus predicting probable secondary structures and folding classes along the polypeptide chain was deduced from the programs PROFSEC (Rost, 2001), SUBSEC, GOR4 (Garnier et al., 1996), PREDATOR (Frishman & Argos, 1996), PSIPRED (Jones, 1999), JNET (Cuff & Barton, 2000) and SSPRO (Baldi et al., 1999). Structure elements (β-sheets, α-helices and loops) are mapped onto the sequence.
carboxy-terminal region of RPV N in the mouse immune system. All but one mAb bound to the non-conserved amino acids at positions 115–150 within the amino-terminal domain of RPV. This short sequence of low similarity (40%) fits into a well-conserved region among members of the genus Morbillivirus and has already been described by Giraudon et al. (1988) and Buckland et al. (1989) on the MV N protein. Located in a low-hydrophobicity stretch of the protein, it may be exposed to the protein surface (Karlin et al., 2002). It was surprising that only one mAb, 33-4, bound to the carboxy-terminal sequence, which is known to be highly hydrophilic and also predicted to be exposed to the surface of morbillivirus N proteins (Liston et al., 1997; Karlin et al., 2002; Longhi et al., 2003). Our results are similar to those of Choi et al. (2003a, b, 2004), who mapped the binding sites of the anti-N mAbs that they produced against the RPV LATC strain to the amino terminus (Choi et al., 1998), but identified immunodominant linear B-cell epitopes at the carboxy terminus when using anti-RPV bovine sera. Buckland et al. (1989), in contrast, identified three antigenic sites on the MV N recognized by anti-N mAbs: one in the amino-terminal region and two in the carboxy terminus. If the amino as well as the carboxy termini of RPV N are exposed on the surface of the protein, the amino-terminal epitopes are certainly better recognized by the mouse immune system. The data presented here confirm that the region located between aa 115 and 150 is highly immunogenic in mice.

For all mAbs studied except one, the epitopes that they bind to are within the same area, although they defined different binding sites by absence of mutual competition. mAbs IVB2 and 33-4 were directed against linear epitopes, whereas mAbs IIH2 and 48-5 demonstrated anti-conformational reactions. These two last mAbs may therefore recognize residues distant in sequence from the short, variable amino-terminal cluster, but near their three-dimensional position. The sequential epitope defined by peptide mapping is part of a more complex conformational epitope and explains the lack of competition between these two mAbs and the anti-sequential mAb as defined in the precedent work (Libeau et al., 1997). Nevertheless, we defined for these four mAbs a major antigenic motif, DEADRYFTYPEPND, from 125D to 138D. To confirm the prominent immunogenic role of this short sequence in the amino-terminal part of RPV N, in silico sequence-structure analysis was performed and this identified an α-helical configuration between 124D and 135Y, thus defining overlapping residues as essential for mAb recognition. The presence of structures such as short α-helices or β-turns seems to be critical for increased flexibility and enhanced immunogenic properties of epitopes, due to a better activation of the immune system. Indeed, Alba et al. (2003) demonstrated for Plasmodium falciparum that peptides presenting an α-helical fragment between residues 5 and 10 maintained greater flexibility in the rest of the molecule and were immunogenic and protective. Although the region comprising aa 115–150 of N has low similarity between morbilliviruses, we mapped the epitope recognized by mAb 33-4 (site VI) in that region. This antibody is the only mAb to recognize all morbilliviruses and its binding site is composed of residues that are, in fact, well conserved within the genus: 126EAD128----131F-------148EN149.

Among our mAbs, those determining sites II, III and IV were the most interesting for use in a cELISA for differential serodiagnosis between rinderpest and peste des petits ruminants. Indeed, mAb IVB2, belonging to the group directed against site IV, has already been used in such a test (Libeau et al., 1992). However, whilst unable to react with PPRV strains in an immunofluorescence test, mAb IVB2 showed a cross-reaction of approximately 10% with anti-PPRV sera in the cELISA format (data not shown). Cross-reactivity among morbilliviruses is known to be important and has hampered the development of highly sensitive and specific serological tests to differentiate between RPV and PPRV infections, two serologically related infectious agents of ruminants that also give rise to similar clinical symptoms. When we developed cELISA tests for the detection of the humoral response to RPV or PPRV, high sensitivity and specificity depended primarily on the correspondence of recognition sites of the specific mAb and antibody from natural immunity response to the virus. In this study, sharing of the recognition epitope between mAb IVB2 and anti-RPV sera from ruminants was demonstrated, whereas it was shown not to be the case with PPRV antisera. Indeed, looking at the 10 aa that are critical for the antigenic site recognized by mAb IVB2, only three residues, 130Y, 131F and 147F, are conserved between RPV and PPRV and are insufficient to create an antigenic epitope for cross-reactivity. Thus, in the absence of sequence identity between PPRV and RPV in the so-defined immunodominant region of the amino-terminal variable region of N, the serological cross-reactivity observed between RPV and PPRV with the IVB2 mAb-based cELISA could be explained by steric hindrance for the recognized epitope, due to the proximity of an epitope common to both RPV and PPRV in the conserved regions upstream of aa 120 and downstream of aa 145. In an attempt to alleviate the steric hindrance, three histidine residues were inserted between aa 120 and 121, and three others between aa 145 and 146. The N mutant that was obtained was not recognized by mAb IVB2 (S. C. Bodjo, unpublished data). The insertion of the six histidine residues has probably introduced a dramatic change in the conformation of the epitope. The major antigenic motif delimited on peptide DEADRYFTYPEPND bearing the α-helix structure is therefore a promising candidate for consideration as an antigen for peptide-based ELISA diagnostic tools. Practically, the diagnostic capacity of small polypeptides or synthetic peptides composed of this antigenic motif remains to be validated in the indirect ELISA format by using extended RPV and PPRV sera.
REFERENCES


