CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Jan. 2005, p. 114–121 Vol. 12, No. 1
1071-412X/05/$08.00+0 doi:10.1128/CDLI.12.1.114–121.2005

Antigenic and Immunogenic Investigation of B-Cell Epitopes in the Nucleocapsid Protein of Peste des Petits Ruminants Virus

Kang-Seuk Choi,[1]* Jin-Ju Nah,[1] Young-Joon Ko,[1] Shien-Young Kang,[2] Kyoung-Jin Yoon,[3] and Nam-In Jo[1]

Foreign Animal Disease Division, National Veterinary Research and Quarantine Service, Anyang, Kyoung-gi,[1] and College of Veterinary Medicine, Chungbuk National University, Heungduk-Ku, Cheongju, Chungbuk,[2] Korea, and Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa[3]

Received 11 July 2004/Returned for modification 28 September 2004/Accepted 22 October 2004

Attempts were made to identify and map epitopes on the nucleocapsid (N) protein of peste des petits ruminants virus (PPRV) (Nigeria75/1 strain) using seven monoclonal antibodies (MAbs) and deletion mutants. At least four antigenic domains (A-I, A-II, C-I, and C-II) were identified using the MAbs. Domains A-I (MAb 33–4) and A-II (MAbs 38–4, P-3H12, and P-13A9) were determined to be located on the amino-terminal half (amino acids [aa] 1 to 262), and domains C-I (P-14C6) and C-II (P-9H10 and P-11A6) were within the carboxy-terminal region (aa 448 to 521). Nonreciprocal competition between A-II MAbs and MAbs to C-I and C-II domains was observed, indicating that they may be exposed on the surface of the N protein and spatially overlap each other. Blocking or competitive enzyme-linked immunosorbent assay studies using PPRV serum antibodies revealed that epitopes on the domains A-II and C-II were immunodominant, whereas those on the domains A-I and C-I were not. The competition between MAb and rinderpest virus (RPV) serum antibodies raised against RPV strain LATC was found in two epitopes (P-3H12 and P-13A9) on the domain A-II, indicating that these epitopes may cause cross-reactivity between PPRV and RPV. Identification of immunodominant but PPRV-specific epitopes and domains will provide the foundation in designing an N-protein-based diagnostic immunoassay for PPRV.

Peste des petits ruminants (PPR) is an acute and highly contagious viral disease causing high morbidity and mortality in small ruminants, such as goats and sheep. The disease has accounted for significant economic losses to the livestock industry in many countries of Africa, the Middle East, the Near East, and South Asia where rinderpest has been present (34). There is a growing threat for the emergence of PPR in countries free of the disease, especially ones neighboring areas where PPR is endemic. PPR is caused by an enveloped RNA virus known as PPR virus (PPRV), which belongs to the *Morbillivirus* genus in the family *Paramyxoviridae* (2, 32). Other members of the genus *Morbillivirus* include rinderpest virus (RPV), measles virus (MV), canine distemper virus (CDV), phocine distemper virus (PDV), and dolphin morbillivirus (DMV) (2, 13). PPRV is genetically grouped into four distinct lineages (I, II, III, and IV) on the basis of partial sequence analysis of the fusion (F) protein gene (2, 11, 34), despite the fact that only a single serotype has been reported. Although PPRV mainly infects small ruminants whereas RPV mainly causes disease in large ruminants, PPR overlaps to some degree with rinderpest with respect to regions where outbreaks of these diseases occur, type of animals infected (hosts), and clinical manifestation.

Structural proteins of morbilliviruses consist of nucleocapsid (N) protein, fusion (F) protein, hemagglutinin (H) protein, matrix (M) protein, and polymerase (L) protein (13, 20). Among the structural proteins, N protein is antigenically the most conservative among morbilliviruses and is highly immunogenic in spite of its internal location (8, 28, 39). The N protein is expressed to a very high level in morbillivirus-infected cells (13, 17, 39). Hence, N protein can be used for serologic screening for naturally infected or vaccinated animals, although it may not be important for humoral immune protection (8, 10, 23, 27, 28). N protein also can be a good antigen candidate for the development of differential tests for differentiating infected animals from ones vaccinated with F- and/or H-recombinant marker vaccines (8, 24, 25, 28). Such recombinant maker vaccines have been used on an experimental basis to address concerns about the thermal stability of attenuated live PPRV vaccination, which has been practiced in countries where PPR is endemic (3, 12, 15, 16).

Despite a growing interest in diagnostic applications of N protein for PPRV as described above, epitopes on PPRV N protein and their immunological function have not been identified.

Previous studies on the N protein of RPV (525 amino acids [aa]) in our laboratory revealed that immunodominant epitopes are present at the amino-terminal half (aa 1 to 149) (7) and the carboxy terminus (aa 479 to 486) (9). For MV, another morbillivirus, antigenic determinants were also identified at both amino- and carboxy-terminal regions (aa 122 to 150, aa 457 to 476, and aa 519 to 523) of N protein, although it is not known whether these epitopes are immunodominant or not (5). Taken together, it is logical to assume that there should be immunodominant epitopes in both ends of the N protein of PPRV.

* Corresponding author. Mailing address: Foreign Animal Disease Division, National Veterinary Research and Quarantine Service, 480 Anyang-6 dong, Anyang, Kyoung-gi, 430-824, Korea. Phone: 82-31-467-1860. Fax: 82-31-449-5882. E-mail: choiks@nvrqs.go.kr.

In the following study, we attempted to topologically map epitopes on N protein of PPRV by using a series of gene deletion mutants and a panel of monoclonal antibodies (MAbs). In addition, relative immunogenicity of each of the identified epitopes was further analyzed in small ruminants. Such information may provide a better foundation for designing serological methods suitable for epidemiological surveillance, evaluation of immune response of vaccinated animals to PPRV, diagnosis of suspected animals in the early stage of infection, and differentiation from animals vaccinated with a marker vaccine.

MATERIALS AND METHODS

Virus. Nigeria 75/1 (Nig75/1) strain of PPRV (12), the seed virus for PPR vaccine production, was kindly supplied by G. Libeau (CIRAD-EMVT, Montpellier, France) and used for the study. The virus belongs to lineage I. The virus was propagated in Vero cells using a roller bottle culture system, concentrated, and semipurified as previously described (6). The amount of total protein in the purified viral antigen was determined using a GeneQuant II (Pharmacia Biotech), and then the antigen preparation (0.1 mg/ml) was stored at −70°C until it was used as an immunogen for MAb production or antigen in enzyme-linked immunosorbent assay (ELISA) to screen hybridomas.

Monoclonal antibodies. Production of MAb specific for PPRV was done as previously described (6). In brief, mice were injected via the footpad with viral antigens at a rate of 0.01 mg per mouse. Murine lymphocytes were collected from popliteal lymph node 10 to 14 days after the injection and were fused with murine myeloma SP2/O cells. PPRV antibody-secreting hybridomas were identified by an indirect ELISA (iELISA) using whole virus as described above and an immunofluorescence on PPRV-infected Vero cells. Hybridomas secreting MAb specific for PPRV N protein were then selected by testing against a recombinant PPRV N (rPPRV-N) protein in an indirect ELISA. Once N-protein-specific MAbs were produced, the isotype of each MAb was determined by a commercial isotyping ELISA kit (Boehringer, Manheim, Germany) according to the manufacturer's instructions. Two MAbs (33–4 and 38–4) raised against PPRV Nig75/3 strain were obtained from G. Libeau as reference (29). PPRV-neutralizing MAb P-13E5 directed against PPRV 75/1 produced in this study was used as a negative control for the N protein.

For use in competitive ELISA, MAbs were biotinylated with a biotin-labeling kit (Boehringer) after being purified through the ImmunoPure (A/G) immunoglobulin G (IgG) purification kit (Pierce, Rockford, Ill.) for the IgG antibody or a KappaLock Sepharose column (Zymed Labs, San Francisco, Calif.) for the IgM antibody according to the manufacturer's instructions. All MAbs were adjusted to 2 mg/ml by using a GeneQuant II before labeling.

Sera. Five PPRV-antibody-positive goat/sheep field sera, designated PPR 2–65, PPR 2–66, PPR 2–69, PPR 2–73, and PPR 2–81, from eastern Africa (PPR lineage III region) were kindly supplied by E. Couacy-Hymann (LANADA/LCPA, Bingerville, Ivory Coast). Two bovine anti-RPV sera (RP K9061 and RP K9062) were previously produced in our laboratory with the RPV LATC strain (8). All these sera had virus-neutralizing titers of >256 against homologous virus. Negative sera were obtained from 10 goats naive for both RPV and PPRV and were used as negative controls. Strong positive, weak positive, and negative controls included in an internationally available competitive ELISA kit for PPR serology were also used. In addition, a total of 22 sera periodically collected from three goats experimentally infected with PPRV (lineage I) were obtained from E. Couacy-Hymann.

Recombinant GST fusion proteins. A series of glutathione *S*-transferase (GST) fusion proteins containing either full-length N protein (aa 1 to 525) or partially overlapping fragments of N protein (aa 1 to 262, aa 1 to 447, and aa 405 to 521) of PPRV were expressed in *E. coli* BL21 cells using procedures previously described (6). Four primer sets, N-F1 (5′-GAGCTCATGGCGACTCTCCTCAAAG-3′) and N-R525 (5′-AACCATGGTCAGCTGAGGAGATCCTTGT-3′), N-F1 and N-R262 (5′-CGCCGGCGGAGTCCGGCTTCTACAATAT-3′), N-F1 and N-R447 (5′-CGCCGGCGCTTCGGACCCATTTGGGATC-3′), and N-F405 (5′-GAGCTCGAACGAACCGTTAGAGGGAC-3′) and N-R521 (5′-CCATGGCTATTCTCTGTTCTCAAACCAGT-3′) were used to amplify genes encoding aa 1 to 525, aa 1 to 262, aa 1 to 447, and aa 405 to 521, respectively (Fig. 1A), based on the published N amino acid sequence of PPRV strain Nig75/1 (GenBank accession number X74443). Restriction enzyme sites were incorporated at the 5′ ends of each primer to facilitate cloning and are indicated by the underlined nucleotides. cDNA of each N gene construct was reverse transcription-PCR amplified, cloned into pGEM-T Easy vector (Promega, Madison, Wis.), and then subcloned into pGEX vector (Amersham Pharmacia, Piscataway, N.J.). Recombinant pGEX plasmids pGEX-$N_{1\text{-}525}$ (aa 1 to 525), pGEX-$N_{1\text{-}262}$ (aa 1 to 262), pGEX-$N_{1\text{-}447}$ (aa 1 to 447), and pGEX-$N_{405\text{-}521}$ (aa 405 to 521) were transformed into *E. coli* BL21 cells (Amersham Pharmacia). Expression of GST fusion proteins (i.e., GST-$N_{1\text{-}525}$, GST-$N_{1\text{-}262}$, GST-$N_{1\text{-}447}$, and GST-$N_{405\text{-}521}$) was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to transformed cells and incubating them at ambient temperature for 4 to 6 h. The induced cells were harvested, washed three times with cold 0.01 M phosphate-buffered saline (PBS) by centrifugation at 10,000 × *g* for 1 min, and resuspended in a one-fifth volume of cold GST buffer (10 mM Tris–1 mM EDTA [pH 8.0], 0.1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 50 mM potassium glutamate, and 10% glycerol), and treated with lysozyme (final concentration, 0.2 mg/ml) and sodium sarkosyl (final concentration, 0.5%) for 10 min at ambient temperature. After a brief centrifugation (10,000 × *g* for 1 min), the supernatant was incubated with Triton X-100 (final concentration, 5%) for 30 min at ambient temperature to inactivate sarkosyl. Once extracted, the fusion proteins were then purified from Triton X-100-treated supernatants through a single-step glutathione Sepharose 4B affinity column (Amersham Pharmacia) according to procedures recommended by the manufacturer.

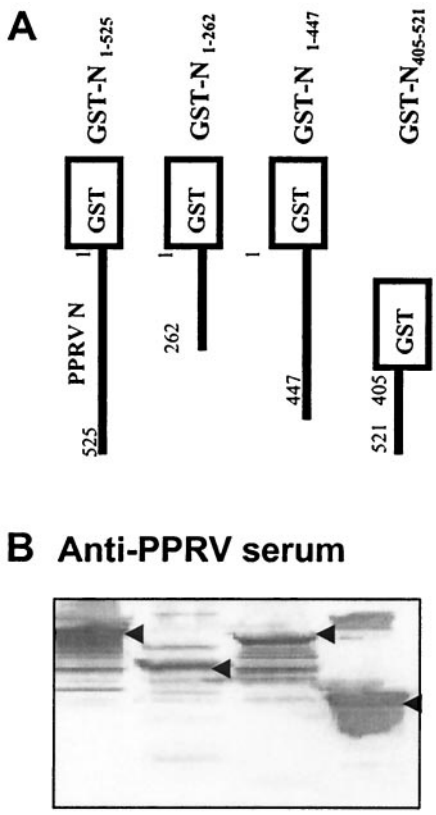

FIG. 1. Schematic diagram (A) of GST fusion protein constructs containing full-length or truncated nucleocapsid fragments of PPRV and Western immunoblot confirmation (B) of *E. coli*-expressed products using goat anti-PPRV serum.

Baculovirus-expressed recombinant N protein. cDNA of the full-length PPRV N gene was PCR amplified from recombinant pGEM-T Easy plasmid containing full-length N by PCR using two primers: PPR-N-NarI-F1 (5′-AAGGCGCCATGGCGACTCTCCTCAAAAG-3′) and PPR-N-R525-SacI (5′-AAGAGCTCTCAGCTGAGGAGATCCTTGT-3′). The resulting DNA product was inserted into the NarI and SacI restriction enzyme sites of pFastBac HT vector (Invitrogen, Carlsbad, Calif.). The resulting recombinant (pFastBac/PPRV-N) was transformed into *E. coli* DH10Bac cells (Invitrogen) to generate the recombinant (Bacmid/PPRV-N) by recombination with Bacmid plasmid in the cells. Recombinant N (rPPRV-N) protein was then expressed by infecting Bacmid/PPRV-N into Sf21 cells. The rPPRV-N protein was extracted from the infected cells by treating them with a lysis buffer containing protease inhibitors as described

previously (28). The cell lysate was kept at −70°C until it was used as an antigen for competitive ELISA. A recombinant RPV N (rRPV-N) protein previously expressed in baculovirus (8) was used as a control.

Western immunoblot. Recombinant GST fusion N proteins with various deletions (e.g., GST-N_{1-525}, GST-N_{1-262}, GST-N_{1-447}, and GST-$N_{405-521}$), rPPRV-N, and rRPV-N were electrophoretically separated through NuPAGE Novex Bis-Tris Gels using an Xcell SureLock Mini-Cell (Invitrogen) according to the manufacturer's instructions. Separated polypeptides were transferred from the gels onto nitrocellulose membranes by using the Xcell II Blot Module (Invitrogen) according to manufacturer's instructions. Immunoblotting was then performed by standard techniques using MAbs (1:1,000 dilution) or sera (1:100 dilution). Specific antigen-antibody reactions on each membrane were visualized by applying anti-species IgG (H+L) conjugated with alkaline phosphatase (1:1,000 dilution) followed by 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium substrate solution (Kirkegaard-Perry Laboratories Inc., Gaithersburg, Md.).

iELISA. ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 50 μl of optimally diluted native or recombinant N proteins for 1 h at 37°C. Plates were washed three times with 0.002 M PBS containing 0.05% Tween 20 (PBST) and then were incubated with 50 μl of serial dilutions of each MAb in a blocking buffer (0.01 M PBS, 3% skimmed milk, and 0.05% Tween 20) for 1 h at 37°C. MAb P-13E5 was used as a negative control. Plates were then washed three times with PBST. Antigen-antibody complexes were visualized by applying 50 μl of peroxidase-conjugated anti-mouse immunoglobulins (IgA+IgM+IgG) (Kirkegaard and Perry Laboratories, Inc.) diluted 1:1,500 in the blocking buffer and then *O*-phenylenediamine substrate (Sigma, St. Louis, Mo.). Colorimetric reactions were stopped with 1.25 M sulfuric acid solution, and the optical density (OD) of each well was measured at 492 nm. The net absorbance of each MAb was corrected by subtracting the OD for control MAb P-13E5 from that for recombinant fusion protein. MAbs with a net OD value of ≥0.2 were considered positive.

Blocking enzyme-linked immunosorbent assay (bELISA). To determine whether MAbs recognize the same or different epitopes, alternate rows of an ELISA plate coated with the purified whole-virus antigen in 0.01 M PBS at a predetermined optimal concentration was first incubated with a 1:100, 1:1,000, or 1:10,000 dilution of each unlabeled MAb (50 μl/well) in the blocking buffer for 45 min at 37°C. MAb P-13E5 was used as a negative control antibody. Without washing, 50 μl of each biotinylated MAb at 70 to 80% saturating concentration was added to paired wells (i.e., one with unlabeled MAb and the other without unlabeled MAb pretreatment) and incubated for an additional 1 h at 37°C. Plates were then washed three times with PBST, and binding of biotinylated MAbs was detected using 50 μl of peroxidase-labeled streptavidin diluted 1:1,500 in the blocking buffer. The rest of the procedure for the ELISA was identical to that of the iELISA described above. The optical densities (OD) were converted to percent inhibition (PI) values for each MAb pair by using the following formula: PI = [(100 − OD with MAb pretreatment) × 100]/(OD without MAb pretreatment). Two MAbs (i.e., unlabeled and labeled) were considered to share (overlap) an epitope when 50% or higher reduction occurred.

Another blocking ELISA (bELISA-II) was also performed to assess the relative immunogenicity of each of the epitopes identified on PPRV N protein as described above, with a few modifications. First, ELISA plates were coated with rPPRV-N instead of whole PPR viral antigens. Second, anti-PPRV field caprine or ovine sera were diluted 1:20 and 1:400 and applied first to wells coated with the antigen. Third, the plates were incubated with each unlabeled MAb at 70 to 80% saturating concentration. Fourth, peroxidase-labeled streptavidin was replaced with peroxidase-conjugated anti-mouse immunoglobulins (IgA+IgM+IgG) (Kirkegaard and Perry Laboratories, Inc.) diluted 1:2,000 in the blocking buffer in order to detect mouse antibody (i.e., MAbs). The net OD of each MAb was corrected by subtracting the OD of MAb P-13E5 from the OD of each MAb. Percent inhibition values (PI) by each serum were calculated by using the following formula: PI = [(100 − OD in the presence of test serum) × 100]/(Mean OD in the presence of negative sera). When 50% or higher inhibition was observed, the epitope recognized by the MAb of interest was considered to be blocked. The degree of immunogenicity of each MAb-reactive epitope was determined by comparing PI values of MAbs with 1:20 and 1:400 dilutions of PPR sera. When the binding of a MAb was blocked by 1:20 dilution of PPR-positive sera, the blocking of the MAb epitope (immunogenicity) was further tested by 1:400 dilution of the sera and was compared.

cELISA. A competitive enzyme-linked immunosorbent assay (cELISA) was performed for PPR serology according to the modified procedure of the commercial competitive ELISA kit for PPRV (28). Optimal running conditions of cELISA were predetermined by a checkerboard titration using PPR control sera, whole-virus antigen, and detecting MAb as previously described (36). For testing, alternate rows of ELISA plates coated with the antigen were incubated with an equal mixture of serum (final 1:20 dilution) and each MAb in the blocking buffer for 1 h at 37°C. Other alternate rows of ELISA plates were incubated only with each MAb. Plates were then washed three times with PBST, and binding of MAb was detected using peroxidase-labeled anti-mouse immunoglobulin and the substrate. The rest of the procedure for the ELISA was identical to that for the bELISAs described above. Percent inhibition by serum antibodies was calculated by using the following formula: PI = [1 − (OD of serum-MAb mixture/OD of MAb alone)] × 100. The sera with PI values of ≥50 were considered to be positive.

RESULTS

Characterization of MAbs. A total of five MAbs specific for N protein of PPRV Nig75/1 were produced, designated P-3H12, P-9H10, P-11A6, P-13A9, and P-14C6. MAbs P-9H10, P-3H12, P-11A6, and P-13A9 were IgG class, whereas MAb P-14C6 was IgM class. Among IgG MAbs, P-9H10 was IgG1, P-3H12 was IgG2a, and P-11A6 and P-13A9 were IgG2b. The two MAbs from G. Libeau (33–4 and 38–4) were both IgG1. All of these MAbs had kappa light chains.

Recombinant pGEX plasmids (pGEX-N_{1-525}, pGEX-N_{1-262}, pGEX-N_{1-447}, and pGEX-$N_{405-521}$) expressed high levels of GST fusion proteins (GST-N_{1-525}, GST-N_{1-262}, GST-N_{1-447}, and GST-$N_{405-521}$) with expected sizes, which were recognized by antibodies to PPRV (Fig. 1B). GST-$N_{405-521}$ was successfully extracted from the soluble fractions, and the other GST fusion proteins were harvested from insoluble fractions (data not shown). Degradation into smaller polypeptides was observed in all GST fusion proteins, although the majority of the fusion proteins remained intact (Fig. 1).

The degree of reactivity of the MAbs to recombinant N proteins (GST-N_{1-525} and rPPRV-N) in comparison to that of native N protein is illustrated in Fig. 2. Based on endpoint titer and OD values, the reactivity pattern of each MAb to the baculovirus-expressed recombinant N protein (rPPRV-N) was determined to be similar to that of the native N protein. On both proteins, the ELISA reactivity of MAbs 38–4 and P-11A6 for all N proteins was the highest, while MAbs P-14C6 and 33–4 showed the lowest ELISA reactivity. In contrast, a wide-ranging difference in the reactivity to *E. coli*-expressed GST-N_{1-525} was found among some MAbs (i.e., 33–4, P-3H12, P-9H10, and P-13A9). MAbs P-3H12, P-9H10, and P-13A9 were barely bound to GST-N_{1-525}, whereas MAb 33–4 showed a relatively high degree of ELISA reactivity with GST-N protein.

Predicted location of epitopes. All MAbs were reactive to both native N proteins as well as to two recombinant (GST-N_{1-525} and rPPRV-N) N proteins on Western immunoblotting, demonstrating that antigenic structure of epitopes in recombinant proteins remained intact (Fig. 3). Besides being specific for PPRV N protein, MAbs 33–4 and P-13A9 were also reactive to rRPV-N, the recombinant N protein derived from RPV LATC strain, indicating that these two MAbs represent epitopes common between PPRV and RPV while the other MAbs are specific for epitopes present only in PPRV.

All GST fusion proteins expressed were probed with each MAb in Western blotting to determine the location of MAb epitopes. As illustrated in Fig. 3, four MAbs, 33–4, 38–4, P-3H12, and P-13A9, recognized GST fusion proteins containing the amino-terminal half (GST-N_{1-525}, GST-N_{1-262}, and GST-N_{1-447}) on Western blotting, indicating that these

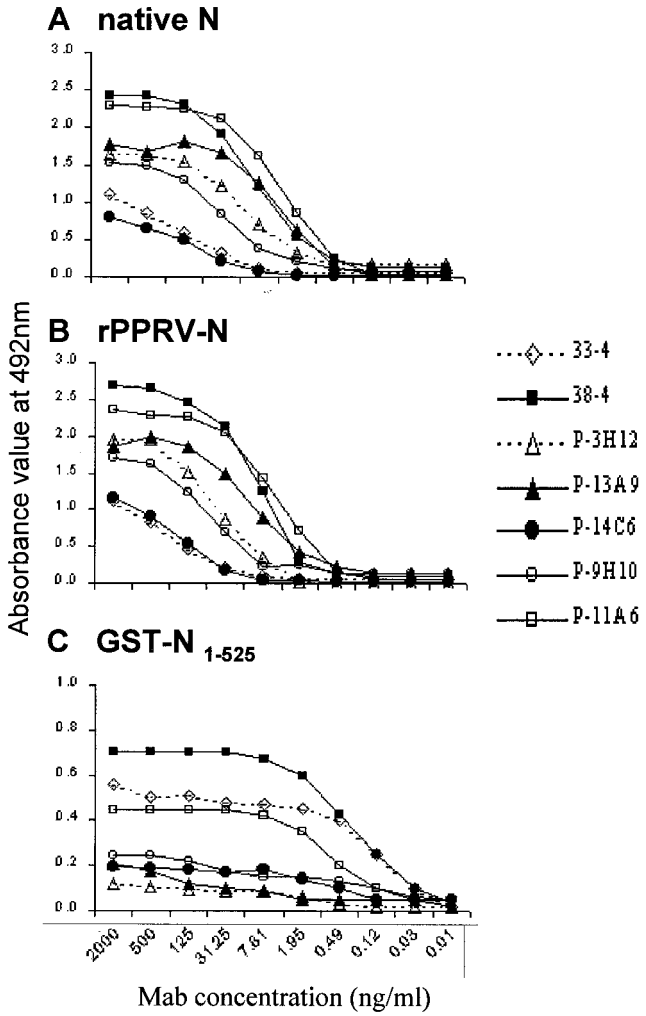

FIG. 2. Reactivity of anti-PPRV nucleocapsid MAbs with native (A), baculovirus-expressed recombinant (B), or *E. coli*-expressed recombinant GST fusion nucleocapsid proteins (C) in an indirect ELISA.

epitopes may be present between aa 1 and 262. In comparison, three MAbs, P-9H10, P-11A6, and P-14C6, were reactive to GST fusion proteins containing carboxy-terminal regions (GST-$N_{1\text{-}525}$ and GST-$N_{405\text{-}521}$), indicating that these epitopes may be located between aa 448 and 521 (Fig. 3).

Spatial relationship of epitopes. Blocking of each MAb by homologous and heterologous MAbs is summarized in Table 1. As expected, complete blocking was observed between homologous MAbs. MAb 33–4 binding was blocked only by homologous antibody. The other MAbs showed one-way or two-way competition with two or more heterologous MAbs (except for MAb 33–4) as well as homologous MAb. Based on the competition between the two MAbs, epitopes were categorized into at least four antigenic domains: A-I (33–4), A-II (P-3H12, P-13A9, and 38–4), C-I (P-14C6), and C-II (P-9H10 and P-11A6). Within the A-II and C-II domains, the MAb defining the epitopes differed slightly. Of the domain A-II MAbs which recognize epitopes in the amino-terminal half, MAbs P-3H12 and P-13A9 competed nonreciprocally with three MAbs (P-14C6, P-9H10, and P-11A6) which recognize epitopes in the carboxy-terminal region, while MAb 38–4 did not. MAbs P-3H12 and P-13A9 had identical relations with each other and with the other MAbs, except for MAb 38–4. Domain C-II MAbs P-9H10 and P-11A6 competed nonreciprocally with each other.

Immunogenicity of MAb epitopes in small ruminants. Binding of MAbs P-3H12, P-13A9, 38–4, P-9H10, and P-11A6 to rPPRV-N was significantly blocked (i.e., PI $\geq$ 50) by all anti-PPRV caprine and ovine sera that were applied after dilution to 1:20 (Fig. 4A). In contrast, MAb P-14C6 competed with some sera (PPR 2–65, PPR 2–81, and PPR positive control) while MAb 33–4 showed no competition with any of the PPR sera (Fig. 4A). When the same sera were applied after dilution to 1:400, binding of MAbs P-3H12, P-13A9, 38–4, P-9H10, and P-11A6 to rPPRV-N was blocked on average by 92.4, 90.7, 64.1, 49.1, and 25.7%, respectively (Fig. 4B). Taken together, of four domains identified in this study, three epitopes (P-3H12, P-13A9, and 38–4 epitopes) in domain A-II are more immunogenic than others, while the 33–4 epitope in domain A-I might play a small role in the induction of antibody response. Although P-3H12 and P-13A9 epitopes were considered to be immunodominant, it is worth noting that binding of P-3H12 and P-13A9 to the amino-terminal half was blocked by 71 to 95% and 66 to 95%, respectively, by low dilution of RPV bovine antisera, whereas binding of the other MAbs was not significantly affected (Fig. 4A).

The immunogenicity of epitopes recognized by MAbs 38–4, P-3H12, P-13A9, and P-11A6 was further investigated by assessing the antibody response of three goats to these epitopes after experimental PPRV infection using cELISA (Fig. 5). Antibody response (PI $\geq$ 50) in the infected goats was detected approximately 8 days after infection for 38–4, P-3H12, and P-13A9 and approximately 10 days after infection for the P-11A6 epitope. This suggests that the epitopes at the amino-terminal half of the PPRV N protein stimulate humoral response earlier than does the epitope in the carboxy-terminal region.

DISCUSSION

Epitopic profile of the N protein of PPRV was determined and characterized using full-length recombinant N and deletion mutants, MAbs, and polyclonal antisera. Two kinds of recombinant N proteins were used in this study. One is expressed in the baculovirus without any other fusion protein (rPPRV-N), and the other is expressed in *E. coli* with GST fusions (GST-$N_{1\text{-}525}$, GST-$N_{1\text{-}262}$, GST-$N_{1\text{-}447}$, and GST-$N_{405\text{-}521}$). GST-$N_{1\text{-}525}$ reacted with MAbs 33–4, P-3H12, P-9H10, and P-13A9 in Western immunoblotting but barely did so in indirect ELISA. Unlike GST-$N_{1\text{-}525}$, rPPRV-N showed an ELISA reactivity pattern similar to that of the native PPRV N protein, suggesting that the antigenic structure of rPPRV-N may be closer to the native form of PPRV N protein than GST-$N_{1\text{-}525}$. Previous studies have reported that expression of full-length MV N in prokaryotic, eukaryotic, or mammalian expression systems resulted in assembly of a nucleocapsid-like structure, which is morphologically and antigenically similar to the native form of N protein despite the absence of genomic RNA (17, 37, 41). In contrast, the expres-

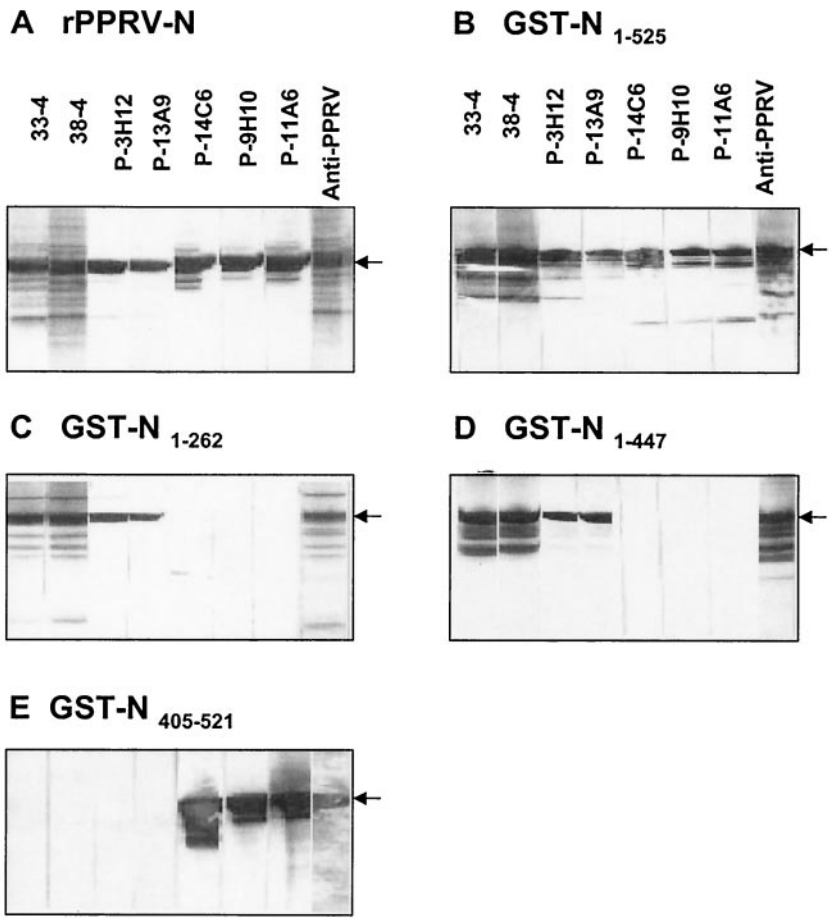

FIG. 3. Mapping of MAb epitopes using GST fusion proteins containing partially overlapped fragments of PPRV nucleocapsid protein. GST-N_{1-525} (B), GST-N_{1-262} (C), GST-N_{1-447} (D), and GST-$N_{405-521}$ (E) were separated electrophoretically and then transferred to nitrocellulose membranes. Baculovirus-expressed N protein rPPRV-N (A) was used as control. The binding of each MAb to GST fusion proteins on the membranes was detected using Western immunoblotting.

sion of MV N protein as a fusion protein (e.g., beta-galactosidase or maltose-binding protein) could not be assembled into a nucleocapsid-like structure (41). Therefore, the discrepancy in the reactivity with MAbs between GST-N_{1-525} and rPPRV-N in ELISA might be related to the inability of the recombinant N protein to form nucleocapsid structure by GST fusion, perhaps misfolding of N protein rather than conformational change by the glycosylation, because the N protein is not glycosylated in the native- and baculovirus-expressed N proteins the host cells. Such an inability would not be a problem in Western immunoblotting, as the protein is denatured. Diagnostically, our observations imply that rPPRV-N has much stronger merit than GST-N_{1-525} in place of the native PPRV N protein as an ELISA antigen for PPR serology.

TABLE 1. Mapping of epitopes on the N protein of PPRV Nig75/1 strain[a]

Competing MAb (isotype)	Domain	Labeled MAb						
		33-4	P-3H12	P-13A9	38-4	P-14C6	P-9H10	P-11A6
33-4 (IgG1)	A-I	++	−	−	−	−	−	−
P-3H12 (IgG2a)	A-II	−	+++	++	−	−	−	−
P-13A9 (IgG2b)	A-II	−	+++	+++	+	−	−	−
38-4 (IgG1)	A-II	−	+++	+++	+++	−	−	−
P-14C6 (IgM)	C-I	−	++	++	−	++	−	−
P-9H10 (IgG1)	C-II	−	+	+	−	−	+++	−
P-11A6 (IgG2b)	C-II	−	+++	+++	−	−	+++	+++

[a] −, <50% competition by unlabeled MAb at 1:100; +, >50% competition by unlabeled MAb at 1:100; ++, >50% competition by unlabeled MAb at 1:1,000; +++, >50% competition by unlabeled MAb at 1:10,000.

The location of MAb epitopes was predicted by using deletion mutants. Of seven epitopes identified in this study, four epitopes (recognized by 33-4, P-3H12, P13A9, and 38-4) were located in the amino-terminal half (aa 1 to 262), while the others (recognized by P-14C6, P-9H10, and P-11A6) were localized in the carboxy-terminal region (aa 448 to 521). The presence of epitopes in both ends of N protein has been previously reported for other morbilliviruses, including measles virus (5) and rinderpest virus (7, 9). Topologically, MAbs-reactive epitopes were located in both N-terminal and C-terminal regions. Two antigenic domains were identified in each region (A-I and A-II or C-I and C-II). MAb 33-4 (recognizing domain A-I) did not display any competition with the other MAbs, suggesting that the domain was topologically separated from the other domains. MAbs reactive to epitopes on other

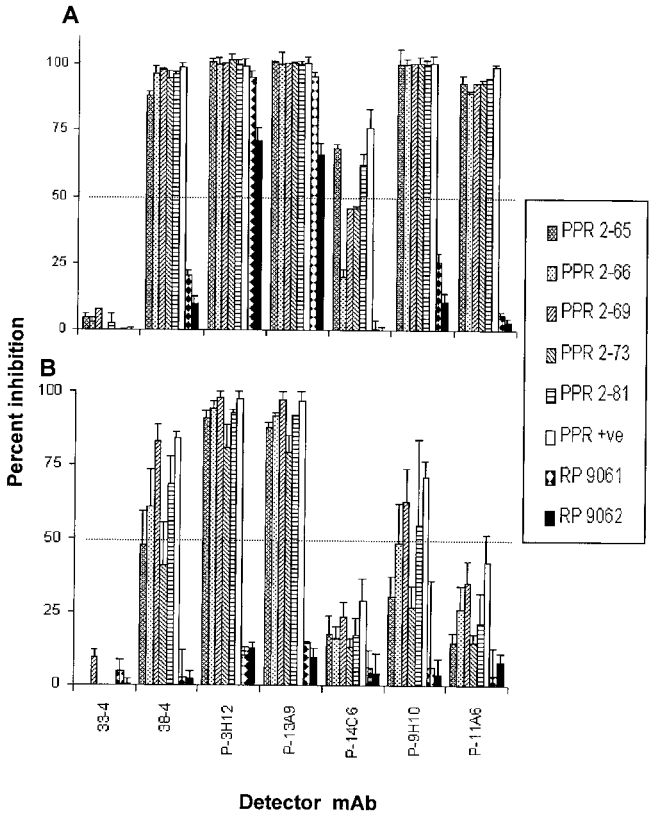

FIG. 4. Immunogenicity of seven epitopes on the nucleocapsid of PPRV as determined by the degree of blocking of their binding to the baculovirus-expressed recombinant PPRV N protein by anti-PPRV caprine sera (PPR 2–65, PPR 2–66, PPR 2–69, PPR 2–73, and PPR 2–81), PPRV hyperimmune serum (RPV positive), or anti-PRV bovine sera (RP K9061 and K9062) at dilutions of 1:20 (A) and 1:400 (B). Each epitope is designated by the name of each detector MAb.

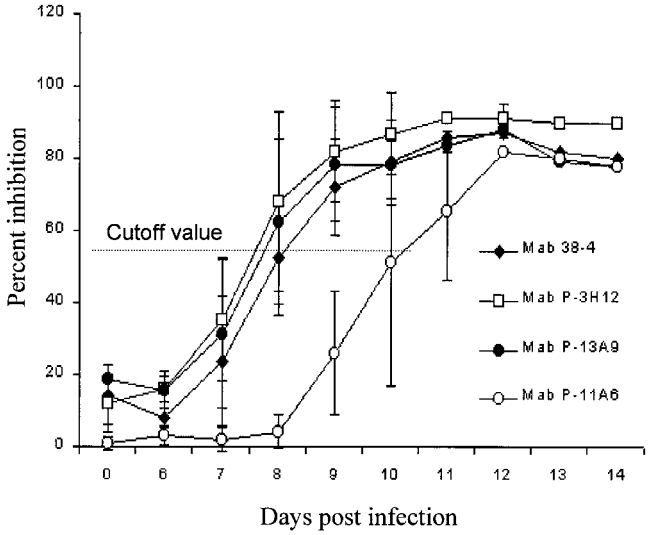

FIG. 5. Average humoral antibody response to four immunodominant epitopes (MAbs 38–4, P-3H12, P-13A9, and P-11A6) of PPRV N protein in three goats experimentally infected with PPRV of lineage I as measured by a competitive ELISA.

domains (i.e., A-II, C-I, and C-II) displayed competition nonreciprocally with each other. One-way competition (i.e., nonreciprocal competition) has been commonly observed among epitopes of other viruses because of their conformational structure (6, 22, 29, 33, 38, 42). However, it should be noted that epitopes (P-3H12 and P-13A9) on domain A-II were blocked by MAbs against epitopes (P-14C6, P-9H10, and P-11A6) in domains C-I and C-II but not vice versa. Such broad blocking effects allow us to draw some preliminary assumptions. First, the folding of PPRV N protein may bring distal epitopes at both ends close together and expose them on the surface of the N protein, as with Sendai virus (21). Second, domain A-II may be located close to but behind domains C-I and C-II so that MAb binding of domain C-I or C-II epitope sterically hinders binding of MAbs P-3H12 and P-13A9 to domain A-II. Third, the 38-4 epitope on domain A-II may be more topologically separated from domains C-I and C-II than P-3H12 and P-13A9 epitopes, because 38-4 reactivity was not affected by the binding of domain C-I and C-II antibodies.

From an immunogenicity point of view, epitopes in domain A-II (P-3H12, P-13A9, and 38-4) and domain C-II (P-9H10 and P-11A6) appeared to be immunodominant (see Fig. 4). Of them, P-3H12 and P-13A9 epitopes were more immunodominant. However, spatial overlap of epitopes should be taken into consideration for immunogenicity, because a higher PI value of MAb in bELISA-II could be achieved by not only direct competition but also indirect competition (i.e., steric hindrance). For example, P-3H12 epitope could be blocked by indirect competition with other antibodies against P-13A9, P-14C6, P-9H10, and P-11A6 epitopes in serum as well as direct competition by antibody to P-3H12 epitope in serum. Diagnostically, the use of antigen possessing an epitope with such broad blocking could improve the sensitivity of MAb-based blocking (or competitive) ELISA for PPR serology. This concept was supported in part by the results of cELISA on sera from goats experimentally infected with PPRV showing that antibodies against P-3H12, P-13A9, and 38-4 epitopes were detected earlier than those of P-11A6 epitope (Fig. 5).

Serological cross-reactivity has been observed among morbilliviruses because of common antigens present among the viruses (35, 38). Considering that the N protein is highly immunogenic and conserved among morbilliviruses (8, 14), it is apparent that the N protein would play an important role in such an antigenic cross-reactivity. In our earlier work (8), anti-RPV sera displayed strong reactivity with the PPRV N protein (rPPRV-N) as well as PPRV whole-virus antigen in an indirect ELISA. In this study, MAbs P-13A9 and 33-4 reacted with the N protein of strains PPRV Nig75/1 and RPV LATC (8, 29), suggesting that these two epitopes can serve as common antigens. While the P-13A9 epitope likely plays a major role in cross-reactivity due to its immunogenicity, the 33-4 epitope would not play an important role in cross-reactivity between the two viruses because it appeared to be the least immunogenic. However, it should be noted that P-3H12 antibody strongly blocked P-13A9 epitope, suggesting that P-3H12 MAb may be in competition with RPV antiserum (i.e., cross-reaction) in a blocking ELISA for PPR serology, although the MAb was not directly bound to the RPV. This was evident in bELISA-II, where MAb P-3H12 competed with RPV sera (1:20 dilution) while the others did not (see Fig. 4A). Such a

competition was not, however, observed between MAb P-11A6 (domain C-II) and PRV antibody cross-reacting with P-13A9 epitope of PPRV, even though MAb P-11A6 could block P-13A9 epitope nonreciprocally. Two-way competition appears to be important for cross-reactivity due to steric hindrance.

In conclusion, our observations on the immunogenicity and spatial relationships of epitopes on the four domains of the PPRV N protein would be useful in designing sensitive and specific immunoassays based on the N protein. For example, MAb P-3H12 (recognizing the immunodominant domain A-II) would be applicable for the detection of antibodies in competitive ELISA for serosurveillance and the diagnosis of suspected animals under certain circumstances (i.e., poor sample quality and low laboratory capacity). This test would be useful in PPR- and RP-free countries for the screening of animals either naturally infected or vaccinated with attenuated PPRV vaccine. It could also be used to differentiate infected animals from those vaccinated with F- and/or H-recombinant marker vaccines in areas where the virus is endemic, although weak cross-reactivity with RPV antibody was found in the competition immunoassay in this study. PPR-specific MAb P-9H10 (recognizing domain C-II) would be suitable for the detection of PPRV-specific antibody and/or antigen, although domains C-I and C-II are less immunodominant than domain A-II. Nonetheless, the exact location of MAb epitopes remains to be further investigated by immunoassays (i.e., immunospot) using synthetic peptide fragments (18), phage display libraries (40), or site-directed mutagenesis with an infectious clone (1).

In addition, anti-N MAbs produced in this study may be applicable to further studies of structural biology and replication of PPRV nucleocapsid (4, 19, 21, 26, 30, 31).

ACKNOWLEDGMENTS

We thank G. Libeau (CIRAD-EMVT) and E. Couacy-Hymann (LANADA/LCPA) for supplying us with diagnostic reagents and RPV and PPRV sera, respectively. Hoe-Wol Lee and Sang-Mi Kang are gratefully acknowledged for their excellent technical assistance.

This work was supported by funding from the National Veterinary Research and Quarantine Service, Korean Ministry of Agriculture and Fishery.

REFERENCES

1. **Balasuriya, U. B., J. C. Dobbe, H. W. Heidner, V. L. Smalley, A. Navarrette, E. J. Snijder, and N. J. MacLachlan.** 2004. Characterization of the neutralization determinants of equine arteritis virus using recombinant chimeric viruses and site-specific mutagenesis of an infectious cDNA clone. Virology **321:**235–246.
2. **Barrett, T., I. K. Visser, L. Mamaev, L. Goatley, M. F. van Bressem, and A. D. Osterhaust.** 1993. Dolphin and porpoise morbilliviruses are genetically distinct from phocine distemper virus. Virology **193:**1010–1012.
3. **Berhe, G., C. Minet, C. Le Goff, T. Barrett, A. Ngangnou, C. Grillet, G. Libeau, M. Fleming, D. N. Black, and A. Diallo.** 2003. Development of a dual recombinant vaccine to protect small ruminants against peste-des-petits-ruminants virus and capripoxvirus infections. J. Virol. **77:**1571–1577.
4. **Bourhis, J. M., K. Johansson, V. Receveur-Brechot, C. J. Oldfield, K. A. Dunker, B. Canard, and S. Longhi.** 2003. The carboxy-terminal domain of measles virus nucleocapids belongs to the class of intrinsically disordered proteins that fold upon binding to their physiological partner. Virus Res. **99:**157–167.
5. **Buckland, R., P. Giraudon, and F. Wild.** 1989. Expression of measles virus nucleoprotein in *Escherichia coli*: use of deletion mutants to locate the antigenic sites. J. Gen. Virol. **70:**435–441.
6. **Choi, K. S., J. J. Nah, Y. J. Ko, C. U. Choi, J. H. Kim, S. Y. Kang, and Y. S. Joo.** 2003. Characterization of antigenic sites on the rinderpest virus N protein using monoclonal antibodies. J. Vet. Sci. **4:**57–65.
7. **Choi, K. S., J. J. Nah, Y. J. Ko, S. Y. Kang, and Y. S. Joo.** 2003. Localization of antigenic sites at the amino-terminus of rinderpest virus N protein using deleted N mutants and monoclonal antibody. J. Vet. Sci. **4:**167–173.
8. **Choi, K. S., J. J. Nah, C. U. Choi, Y. J. Ko, H. J. Sohn, G. Libeau, S. Y. Kang, and Y. S. Joo.** 2003. Monoclonal antibody-based competitive ELISA for simultaneous detection of rinderpest virus and peste des petits ruminants virus antibodies. Vet. Microbiol. **96:**1–16.
9. **Choi, K. S., J. J. Nah, Y. J. Ko, S. Y. Kang, K.-J. Yoon, and Y. S. Joo.** 2004. Characterization of immunodominant linear B-cell epitopes on the carboxy-terminus of the rinderpest virus nucleocapsid protein. Clin. Diagn. Lab. Immunol. **11:**658–664.
10. **Couacy-Hymann, E., F. Roger, C. Hurard, J. P. Guillou, G. Libeau, and A. Diallo.** 2002. Rapid and sensitive detection of peste des petits ruminants virus by a polymerase chain reaction assay. J. Virol. Methods **100:**17–25.
11. **Dhar, P., B. P. Sreenivasa, T. Barrett, M. Corteyn, R. P. Singh, and S. K. Bandyopadhyay.** 2002. Recent epidemiology of peste des petits ruminants virus (PPRV). Vet. Microbiol. **88:**153–159.
12. **Diallo, A., W. P. Taylor, P. C. Lefevre, and A. Provost.** 1989. Atténuation d'une souche de virus de la peste des petits ruminants: candidat pour un vaccin homologue vivant. Rev. Elev. Méd. Vét. Pays Trop. **42:**311–319.
13. **Diallo, A.** 1990. Morbillivirus group: genome organization and proteins. Vet. Microbiol. **23:**155–163.
14. **Diallo, A., T. Barrett, M. Barbron, G. Meyer, and P. C. Lefevre.** 1994. Cloning of the nucleocapsid protein gene of peste-des-petits-ruminants virus: relationship to other morbilliviruses. J. Gen. Virol. **75:**233–237.
15. **Diallo, A., C. Minet, G. Berhe, C. Le Goff, D. N. Black, M. Fleming, T. Barrett, C. Grillet, and G. Libeau.** 2002. Goat immune response to capripox vaccine expressing the hemagglutinin protein of peste des petits ruminant. Ann. N. Y. Acad. Sci. **969:**88–91.
16. **Diallo, A.** 2003. Control of peste des petits ruminants: classical and new generation vaccines. Dev. Biol. **114:**113–119.
17. **Fooks, A. R., J. R. Stephenson, A. Warnes, B. A. Dowsett, B. K. Rima, and G. W. G. Wilkinson.** 1993. Measles virus nucleocapsid protein expressed in insect cells assembles into nucleocapsid-like structures. J. Gen. Virol. **74:**1439–1444.
18. **Frank, R.** 2002. The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports–principles and applications. J. Immunol. Methods **267:**13–26.
19. **Gombart, A. F., A. Hirano, and T. C. Wong.** 1993. Conformational maturation of measles virus nucleocapsid protein. J. Virol. **67:**4133–4141.
20. **Grubman, M. J., C. Mebus, B. Dale, M. Yamanaka, and T. Yilma.** 1988. Analysis of the polypeptides synthesized in rinderpest virus-infected cells. Virology **163:**261–267.
21. **Heggeness, M. H., A. Scheid, and P. W. Choppin.** 1981. The relationship of conformational changes in the Sendai virus nucleocapsid to proteolytic cleavage of the NP polypeptide. Virology **114:**555–562.
22. **Heinz, F. X., C. Mandl, R. Berger, W. Tuma, and C. Kunz.** 1984. Antibody-induced conformational changes result in enhanced avidity of antibodies to different antigenic sites on the tick-borne encephalitis virus glycoprotein. Virology **133:**25–34.
23. **Hummel, K. B., D. D. Erdman, J. Heath, and W. J. Bellini.** 1992. Baculovirus expression of the nucleoprotein gene of measles virus and utility of the recombinant protein in diagnostic enzyme immunoassays. J. Clin. Microbiol. **30:**2874–2880.
24. **Ismail, T., S. Ahmad, M. d'Souza-Ault, M. Bassiri, J. Saliki, C. Mebus, and T. Yilma.** 1994. Cloning and expression of the nucleocapsid gene of virulent Kabete O strain of rinderpest virus in baculovirus: use in differential diagnosis between vaccinated and infected animals. Virology **198:**138–147.
25. **Kamata, H., S. Ohkubo, Y. Matsuura, Y. Kamata, K. Tsukiyama-Kohara, K. Imaoka, C. Kai, Y. Yoshikawa, and K. Yamanouchi.** 1993. Expression in baculovirus vector system of the nucleocapsid protein gene of rinderpest. J. Virol. Methods **43:**159–166.
26. **Karlin, D., S. Longhi, and B. Canard.** 2002. Substitution of two residues in the measles virus nucleoprotein results in an impaired self-association. Virology **302:**420–432.
27. **Libeau, G., A. Diallo, F. Colas, and L. Guerre.** 1994. Rapid differential diagnosis of rinderpest and peste des petits ruminants using an immunocapture ELISA. Vet. Rec. **134:**300–304.
28. **Libeau, G., C. Préhaud, R. Lancelot, F. Colas, L. Guerre, D. H. L. Bishop, and A. Diallo.** 1995. Development of a competitive ELISA for detecting antibodies to the peste des petits ruminants virus using a recombinant nucleoprotein. Res. Vet. Sci. **58:**50–55.
29. **Libeau, G., J. T. Saliki, and A. Diallo.** 1997. Caractérisation d'anticorps monoclonaux dirigés contre les virus de la peste bovine et de la peste des petits ruminants: identification d'épitopes conservés ou de spécificité stricte sur la nucléoproteine. Rev. Élev. Méd. Vét. Pays. Trop. **50:**181–190.
30. **Liston, P., R. Batal, C. DiFlumeri, and D. J. Briedis.** 1997. Protein interaction domains of the measles virus nucleocapsid protein (NP). Arch. Virol. **142:**305–321.
31. **Longhi, S., V. Receveur-Brechot, D. Karlin, K. Johansson, H. Darbon, D. Bhella, R. Yeo, S. Finet, and B. Canard.** 2003. The C-terminal domain of the measles virus nucleoprotein is intrinsically disordered and folds upon bind-

ing to the C-terminal moiety of the phosphoprotein. J. Biol. Chem. **278:** 18638–18648.
32. **Mitra-Kaushik, S., R. Nayak, and M. S. Shaila.** 2001. Identification of a cytotoxic T-cell epitope on the recombinant nucleocapsid proteins of rinderpest and peste des petits ruminants viruses presented as assembled nucleocapsids. Virology **279:**210–220.
33. **Riddell, M. A., F. Li, and D. A. Anderson.** 2000. Identification of immunodominant and conformational epitopes in the capsid protein of hepatitis E virus by using monoclonal antibodies. J. Virol. **74:**8011–8017.
34. **Shaila, M. S., D. Shamaki, M. A. Forsyth, D. Diallo, L. Goatley, R. P. Kitching, and T. Barrett.** 1996. Geographic distribution and epidemiology of peste des petits ruminants viruses. Virus Res. **43:**149–153.
35. **Sheshberadaran, H., E. Norrby, K. C. McCullough, W. C. Carpenter, and C. Orvell.** 1986. The antigenic relationship between measles, canine distemper and rinderpest viruses studied with monoclonal antibodies. J. Gen. Virol. **67:**1381–1392.
36. **Singh, R. P., B. P. Screenivasa, P. Dhar, R. N. Roy, and S. K. Brandyopadhyay.** 2000. Development and evaluation of a monoclonal antibody based competitive enzyme-linked immunosorbent assay for the detection of rinderpest virus antibodies. Rev. Sci. Tech. **19:**754–763.
37. **Spehner, D., A. Kirn, and R. Drillien.** 1991. Assembly of nucleocapsidlike structures in animal cells infected with a vaccinia virus recombinant encoding the measles virus nucleoprotein. J. Virol. **65:**6296–6300.
38. **Sugiyama, M., N. Minamoto, T. Kinjo, N. Hirayama, H. Sasaki, Y. Yoshikawa, and K. Yamanouchi.** 1989. Characterization of monoclonal antibodies against four structural proteins of rinderpest virus. J. Gen. Virol. **70:**2605–2613.
39. **von Messling, V., T. C. Harder, V. Moennig, P. Rautenberg, I. Nolte, and L. Haas.** 1999. Rapid and sensitive detection of immunoglobulin M (IgM) and IgG antibodies against canine distemper virus by a new recombinant nucleocapsid protein-based enzyme-linked immunosorbent assay. J. Clin. Microbiol. **37:**1049–1056.
40. **Wang, L. F., and M. Yu.** 2004. Epitope identification and discovery using phage display libraries: applications in vaccine development and diagnostics. Curr. Drug Targets **5:**1–15.
41. **Warnes, A., A. R. Fooks, A. B. Dowsett, G. W. Wilkinson, and J. R. Stephenson.** 1995. Expression of the measles virus nucleoprotein gene in Escherichia coli and assembly of nucleocapsid-like structures. Gene **160:**173–178.
42. **Wensvoort, G.** 1989. Topographical and functional mapping of epitopes on hog cholera virus with monoclonal antibodies. J. Gen. Virol. **70:**2865–2876.