Monoclonal antibodies to the ORF5 product of porcine reproductive and respiratory syndrome virus define linear neutralizing determinants

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Complementary DNA encoding the ORF5 gene of a Quebec reference isolate (IAF-Klop) of porcine reproductive and respiratory syndrome virus (PRRSV) was cloned into the prokaryotic expression vectors pGEX-4T and pET21a to produce ORF5-glutathione S-transferase and ORF5–polyhistidine fusion proteins. Five hybridoma cell lines producing monoclonal antibodies (MAbs) to the 25 kDa viral envelope glycoprotein (GP5) were obtained from BALB/c mice immunized with the affinity chromatography-purified GST–ORF5 fusion protein. The polypeptide specificity of these anti-PRRSV MAbs, belonging to the IgG1 isotype, was confirmed by Western immunoblotting assays with recombinant and native viral proteins, and by radioimmunoprecipitation using [35S]methionine-labelled concentrated extracellular virus. All these MAbs showed virus-neutralizing (VN) activity, with VN titres ranging from 1:32 to 1:128. Two MAbs (IAF-1B8 and IAF-8A8) reacted with similar titres with the modified live attenuated vaccine strain ATCC VR-2332, but all five failed to react to the prototype European strain, the Lelystad virus, in VN and indirect immunofluorescence tests. The results obtained suggest that these five anti-PRRSV MAbs are directed to serotype-specific linear neutralizing epitopes which are not affected by the absence of carbohydrate residues.

Porcine reproductive and respiratory syndrome (PRRS) is an economically important viral disease first described in North America in 1987 (Goyal, 1993) and in Europe in 1990 (Wensvoort et al., 1991). The disease is characterized by inappetence and severe reproductive failure including late-term abortions, increased numbers of still-born, mummified and weak-born piglets, and respiratory problems affecting pigs of all ages (Goyal, 1993). Although the clinical syndromes associated with PRRS virus (PRRSV) infection are similar in North America and Europe, strains from the two continents represent two distinct genotypes (Mardassi et al., 1994; Meng et al., 1995), and significant antigenic differences have been observed (Dea et al., 1996; Nelson et al., 1993; Wensvoort et al., 1992).

PRRSV is a small enveloped RNA virus with a 25 to 30 nm isometric core, and is closely related to members of the genus Arterivirus in morphology, genome organization, transcription strategy and macrophage tropism (Plagemann et al., 1992). Other members of this group include lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV) and simian haemorrhagic fever virus. The PRRSV genome is a single-stranded polyadenylated RNA molecule of about 15 kb in length, being the largest amongst arteriviruses (Mardassi et al., 1995; Meulenberg et al., 1993). It contains two large open reading frames (ORFs), ORFs 1a and 1b, situated at the 5′ extremity of the genome and six smaller ORFs, ORFs 2 to 7, located at the 3′ part of the genome, which are expressed in the infected cells as a nested set of subgenomic RNAs (Meulenberg et al., 1995). ORF1a and ORF1b, which represent nearly 75% of the genome, code for proteins with apparent polymerase and replicase activity. ORFs 5, 6, and 7 code for the major structural proteins of the virion: a 25 kDa glycosylated envelope protein (GP5), an 18 to 19 kDa integral membrane (M) protein, and a predominant 14 to 15 kDa nucleocapsid (N) protein (Meulenberg et al., 1995; Mardassi et al., 1996). Recent studies indicate that ORFs 2, 3 and 4 of Lelystad virus (LV), the prototype European strain of PRRSV (Wensvoort et al., 1991), may also code for three other membrane-associated glycoproteins: a 29 to 30 kDa GP2, a 45 to 50 kDa GP3, and a 31 to 35 kDa GP4, respectively (van Nieuwstadt et al., 1996; Meulenberg et al., 1995; Meulenberg & den Besten, 1996). The role of PRRSV GP5 in relation to virus neutralization (VN) and cellular receptor binding is still to be determined, mainly due to the lack of specific immunological probes, while such functions...
have been clearly established for the \( G_L \) protein encoded by ORF5 of EAV (Balasuriya et al., 1995; Chirnside et al., 1995; Deregt et al., 1994).

Apparently, PRRSV GP\(_5\) has low immunogenic potential since immunizing mice with the entire viral particle has so far resulted in isolation of MAbs directed against other viral structural proteins (Dea et al., 1996; Drew et al., 1995; Nelson et al., 1993; van Nieuwstadt et al., 1996). In this study we describe the characterization of neutralizing MAbs which were obtained to the Escherichia coli-expressed ORF5 gene product of a reference tissue culture-adapted Quebec strain of PRRSV, and the finding that targeted epitopes are linear and not affected by the absence of glycosylation. We further demonstrated that the resulting neutralizing MAbs selectively reacted with the homologous PRRSV isolate and the United States (US) vaccine strain ATCC VR-2332 but failed to react with the European prototype LV strain.

The Quebec reference cytopathic strain IAF-Klop of PRRSV (Mardassi et al., 1995) was plaque-purified twice and propagated in MARC-145 cells, a clone of MA-104 cells highly permissive to PRRSV (Kim et al., 1993), graciously provided to us by J. Kwang (US Meat Animal Research Center, USDA, Agricultural Research Service, Clay Center, Nebraska). Genomic RNA was extracted from concentrated extracellular virions by the one-step guanidinium isothiocyanate–acid phenol method (Chomczynski & Sacchi, 1987). Then, the entire ORF5 coding sequence of PRRSV was amplified by RT–PCR using the oligonucleotide primers 1005PS (sense) and 1005PR (antisense), corresponding to the sequence adjacent to the first ATG or the stop codon of the ORF5 coding regions of the Quebec strain IAF-exp91 of PRRSV (EMBL/GenBank accession number L40898) (Mardassi et al., 1995). The resulting cDNA was inserted into prokaryotic expression vectors pGEX-4T1 (Pharmacia) and pET21a (Qiagen), as previously described (Mardassi et al., 1996), to obtain pGEX-5 and pET21a-5 recombinant plasmids, respectively. Sequence analysis of these recombinant plasmids demonstrated that no alteration or mutation had occurred as a result of PCR amplification.

Fusion recombinant proteins were produced in BL21(DE3) (Novagen) competent Escherichia coli cells, as already described (Mardassi et al., 1996). Exponential cultures of transformed bacteria were induced at an \( \text{OD}_{600} \) of 1.2–1.5 by adding 0.5 mM IPTG to the culture media. pGEX-5 encoded a fusion protein (GST–ORF5) consisting of glutathione S-transferase (GST; about 26 kDa) fused to the N terminus of the cloned ORF5-encoded protein. pET21a-5 encoded a fusion protein (ORF5-pH) consisting of ORF5-encoded protein fused by its C terminus to a short polyhistidine tag providing a zinc-finger motive. The GST–ORF5 and ORF5–pH fusion recombinant proteins, which accumulated within the cells in the form of inclusion bodies, were solubilized in the presence of lysozyme, Triton X-100 and 8 M urea, then finally purified by affinity chromatography on glutathione–Sepharose 4B (Pharmacia) or electrophoresis following separation by SDS–PAGE, as described elsewhere (Chirnside et al., 1995; Mardassi et al., 1996). The protein concentration in purified antigen preparations was determined by spectrophotometry.

The recombinant fusion proteins were then analysed by SDS–PAGE and their molecular masses were estimated to be 45 kDa for GST–ORF5 and 23 kDa for ORF5–pH, in agreement with the values determined previously from the amino acid sequences of the IAF-Klop strain ORF5 product (Mardassi et al., 1995). Serological identification was confirmed by Western immunoblotting, using porcine anti-PRRSV (IAF-Klop strain) serum from experimentally infected pigs (Loemba et al., 1996) and porcine anti-GST–ORF5 monospecific hyperimmune serum (Mardassi et al., 1996). No reactivity was observed with the recombinant fusion proteins by SPF pig sera (data not shown).

MAbs were prepared as described previously (Dea & Tijssen, 1989), except that P3X63 Ag8–653 myeloma cells were used. Six to eight-week-old BALB/c mice were immunized intraperitoneally with 50–60 µg of GST–ORF5 fusion protein suspended in Freund’s complete (first injection) or incomplete adjuvant. After three injections given at 2 week intervals, titres of sera from mice providing the immune splenocytes ranged from 256 to 1024 by the indirect immunofluorescence (IIF) test using acetone-fixed PRRSV-infected MARC-145 cells (Nelson et al., 1993). Two weeks after the last inoculation, mice that also tested positive by Western immunoblotting against the density gradient-purified...
Table 1. Characterization and reactivity of MAbs directed against the IAF-Klop strain of PRRS virus

<table>
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<tr>
<th>MAb*</th>
<th>Titres of ascites determined by:</th>
<th>Protein specificity as defined by:</th>
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<tr>
<td></td>
<td>IIF†</td>
<td>ELISA‡</td>
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<td>MARC 145 cells</td>
<td>ORF5–pH fusion protein</td>
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<td>IAF-8A8</td>
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<td>IAF-1B8</td>
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<td>IAF-2A5</td>
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<tr>
<td>IAF-3A12</td>
<td>1 600</td>
<td>6250</td>
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<tr>
<td>IAF-1C10</td>
<td>1 600</td>
<td>1250</td>
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<td>Murine α-GST–ORF5 serum</td>
<td>250–1024</td>
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<tr>
<td>SDOW17</td>
<td>100 000</td>
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<tr>
<td>IAF-K8</td>
<td>100 000</td>
<td>–</td>
</tr>
<tr>
<td>IAF-K3</td>
<td>1 600</td>
<td>–</td>
</tr>
<tr>
<td>IAF-K6</td>
<td>1 600</td>
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* Ascitic fluids.
† Titres of PRRSV MAbs by indirect immunofluorescent test, expressed as the reciprocal of the highest dilution of ascitic fluid at which specific cytoplasmic fluorescence was observed.
‡ ELISA: endpoint dilution was defined as the reciprocal of the highest dilution of ascitic fluids producing an A₅₆₅ value > 2.5 × Aᵢ₅₆₅ value of an irrelevant murine ascitic fluid.
§ Virus neutralization: reciprocal of the highest dilution of ascitic fluids neutralizing cytopathic changes and expression of viral N protein in MARC 145 cells and primary culture, of porcine alveolar macrophages by 100 TCID₅₀ of virus.

PRRSV (Fig. 1, lane 7) received a final intravenous injection of 25 µg of the recombinant antigen in PBS.

Hybridoma culture media and mouse ascitic fluids were screened for the presence of anti-ORF5 protein antibodies by ELISA as described previously (Dea & Tijssen, 1989). Sucrose gradient-purified virus (0·5 µg of protein per well) (Mardassi et al., 1996) or gel-purified ORF5–pH fusion protein (0·1 µg of protein per well) in 0·05 M sodium carbonate buffer pH 9·6, was used to coat flat-bottomed microtitre plates. The ORF5–pH fusion protein was used to assess the humoral immune response of the above mice and screening the antibody-secreting hybridoma by ELISA and Western immunoblotting to avoid confusion resulting from reactivity of mice sera or hybridoma secretions with GST. After screening of 124 antibody-secreting hybridomas originating from three different fusions and subcloning by the limiting dilution method, only five subclones were found appropriate for production of ascitic fluids. These subclones were grown to high-density, and the subisotypes of MAbs secreted in the culture medium were determined to be IgG₁ by using a commercial enzyme immunoassay kit (Boehringer-Mannheim). Ascitic fluids were produced by intraperitoneal injection of 5 × 10⁸ to 5 × 10⁹ hybridoma cells into BALB/c mice that had been primed with Pristane (2,6,10,14-tetramethylpentadecane) (Sigma). Titres of MAbs secreted in the ascitic fluids ranged between 1600 and 12 800 by IIF with PRRSV-infected (IAF-Klop strain) MARC-145 cells, while no reactivity was observed to non-infected cells. By ELISA, titres of ascitic fluids ranged between 1250 and 6250 when tested against the recombinant ORF5–pH fusion protein (Table 1). In IIF tests, these MAbs reacted with variable titres to the attenuated vaccine strain ATCC VR-2332 (Collins et al., 1992) but failed to react to the European prototype LV strain of PRRSV (data not shown).

The viral protein specificity of the anti-PRRSV MAbs produced in ascitic fluids was determined by Western immunoblotting and radioimmunoprecipitation (RIPA), as described previously (Mardassi et al., 1996). Sucrose gradient-purified extracellular virus, as well as purified GST–ORF5 and ORF5–pH recombinant fusion proteins, were resuspended in Laemmli electrophoresis sample buffer containing 5% 2-mercaptoethanol, boiled for 4 min, and subjected to SDS–PAGE using a 12% polyacrylamide resolving gel. Proteins were electrophoretically transferred onto nitrocellulose membranes (pore size, 0·45 µm; Schleicher & Schuell). After saturation with 5% skim milk powder in 0·05 M Tris-buffered saline solution,
individual strips were incubated overnight at 4 °C in the presence of either a 1:50 dilution of homologous anti-PRRSV porcine serum or a 1:50 dilution of mouse ascitic fluid.

All five MAbs described above reacted with a 25 kDa band of viral protein (Fig. 1a, lanes 2 to 5; Fig. 1b, lanes 2 and 3) which was also revealed by the homologous murine and porcine anti-GST–ORF5 hyperimmune serum (Fig. 1a, lanes 6 and 7, respectively) and the porcine PRRS convalescent sera (Fig. 1b, lanes 1 and 6). Similarly to the porcine anti-GST–ORF5 and porcine PRRS convalescent serum, the antibodies contained in the ascitic fluids also reacted positively with the above mentioned recombinant ORF5 peptides (data not shown). For comparison, no reactivity to the 25 kDa virus protein was revealed following incubation with two previously characterized anti-PRRSV MAbs, MAbs IAF-K8 and IAF-K6 (Fig. 1b, lanes 4 and 5), directed against the N (15 kDa) and M (19 kDa) proteins of the IAF-Klop strain of PRRSV (Dea et al., 1996). As expected, these two MAbs also failed to react to purified GST–ORF5 and ORF5–pH recombinant fusion proteins (data not shown).

Immunoprecipitation assays were done with concentrated [35S]methionine-labelled extracellular virus, as previously described (Mardassi et al., 1996). Concentrated virions were disrupted in the lysis buffer (20 mM Tris–HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF) and aliquots (50 µl) were incubated overnight with 10 µl of ascitic fluid or rabbit monospecific antisera to the ORF5 (GP5), ORF6 (M) and ORF7 (N) products of PRRSV, available from previous experiments (Mardassi et al., 1996). The immune complexes were then adsorbed to protein A–Sepharose CL-4B beads (Pharmacia).

After washing, the immunoprecipitated proteins were dissolved in 50 µl of electrophoresis sample buffer in the presence of 2-mercaptoethanol, analysed by SDS–PAGE, and visualized by autoradiography.

Under the electrophoresis conditions described above, all five MAbs obtained following immunization with GST–ORF5 recombinant fusion protein precipitated a 25 kDa protein corresponding to GP5 (Fig. 2, lanes 7 to 10), whereas monospecific rabbit antisera directed against the ORF5, 6, and 7 products precipitated proteins of 25, 18, and 15 kDa (Fig. 2, lanes 1 to 3), corresponding to the GP5, M and N proteins of PRRSV, respectively. As in the case of the monospecific rabbit antiserum directed against the recombinant GST–ORF5 fusion protein (Fig. 2, lane 3), the five anti-ORF5 protein MAbs coprecipitated both the GP5 and M proteins. Similarly, rabbit anti-M monospecific antiserum precipitated both M and GP5 proteins. This coprecipitation is explained by the association of the GP5 with M protein into disulfide-linked heterodimers in arteriviruses (de Vries et al., 1995; Mardassi et al., 1996). The N protein was also precipitated along with M or GP5 immune complexes, but the amount was substantially smaller than the amount precipitated by the monospecific anti-N rabbit serum (Fig. 2, lane 1). However, neither M nor GP5 proteins were precipitated by the anti-N hyperimmune serum. This phenomenon has already been reported and is believed to be due to a non-specific precipitation of N protein by protein A–Sepharose (de Vries et al., 1995) or an unidentified interaction between the GP5 and the N protein of PRRSV (Mardassi et al., 1996). As previously demonstrated for EAV structural proteins, incorporation of 0.5 mM DTT (dithiothreitol) in the RIPA lysis buffer resulted in the loss of non-specific coprecipitation of N.
protein and substantially decreased coprecipitation of M protein by the anti-GP\textsubscript{3} MAbs (Fig. 2, lanes 12 to 15). Serum samples collected from SPF pigs prior to experimental infection (negative control) failed to precipitate radiolabelled PRRSV structural proteins (Fig. 2, lane 4).

To determine if the anti-ORF5 protein MAbs could neutralize virus infectivity, ascitic fluids were heat-inactivated (56 °C for 60 min) and serial dilutions were incubated with 100 TCID\textsubscript{50} of PRRSV in a microneutralization assay (Dea & Tijssen, 1989). Cytopathic changes (CPE) or infected cells expressing the N protein were visualized by microscopic observation following staining by indirect immunoperoxidase (Wensvoort et al., 1991) using anti-N MAb IAF-K8 and a commercial peroxidase-conjugated anti-mouse IgG (Boehringer-Mannheim). The VN tests were done on both MARC-145 cells and a primary culture of porcine alveolar macrophages (PAM cells) (Loemba et al., 1996). The VN titres were expressed as the reciprocal the highest serum or ascitic fluid dilution leading to total inhibition of CPE or expression of viral N protein in the PRRSV-infected cultures.

The five anti-GP\textsubscript{3} MAbs obtained in the present study were found to be neutralizing, with VN titres ranging from 32 to 128 when tested against the homologous virus propagated in MARC-145 cells. These MAbs also neutralized, with approximately similar titres, the homologous virus propagated in PAM cells (Table 1). Furthermore, two of these five anti-ORF5 protein MAbs (MAbs IAF-8A8 and IAF-1B8) displayed neutralizing activity towards the heterologous modified live attenuated vaccine strain ATCC VR-2332, but none of these MAbs neutralized the European prototype LV strain (kindly provided by G. Wensvoort, NVLC, Lelystad, The Netherlands) when tested on MARC-145 cells (data not shown).

In this report we described for the first time the production and characterization of MAbs to PRRSV GP\textsubscript{3}, which specifically react with the native viral envelope glycoprotein or the recombinant ORF5 fusion protein in Western immunoblotting, IIF, ELISA and RIPA. Interestingly, the five MAbs described in this report showed neutralizing activity against the homologous Quebec reference IAF-Klop strain. This implies that PRRSV GP\textsubscript{3} has a role in virus infectivity and may function in attachment to cell receptors and/or in virus penetration into the cytoplasm of target cells. The data obtained also suggest that at least one neutralizing antigenic determinant is associated with PRRSV GP\textsubscript{3}. Since all MAbs reacted with both the glycosylated and unglycosylated forms of the viral envelope protein, it appears that glycosylation is not associated with the neutralizing epitope(s) recognized by these MAbs. This phenomenon also indicates that an important linear neutralizing epitope exists in the PRRSV GP\textsubscript{3}, although conformational epitopes in the protein cannot be excluded. Indeed, fusion proteins expressed in prokaryotic vectors may not effectively mimic the native viral proteins due to differences in polypeptide folding, disulfide bond formation or post-translational modifications, notably N-glycosylation.

In general, the results of the present study suggest that antigenically the PRRSV GP\textsubscript{3} is the counterpart of the major envelope glycoprotein of other arteriviruses (Plagemann & Moennig, 1992), notably EAV, the prototype of the genus (Chirnside et al., 1995). The ectodomain of the G\textsubscript{L} glycoprotein of EAV, which is located in its N-terminal half, contains a highly immunogenic region consisting of not more than 44 amino acid residues. An immunodominant epitope maps to this region which induces neutralizing antibody in horses. Immunizing horses with an E. coli-expressed linear protein encoded by this region of G\textsubscript{L} or a synthetic peptide representing close to 50% of this immunogenic region induces EAV-neutralizing antibody (Chirnside et al., 1995). Moreover, characterization of different neutralization-resistant escape mutant viruses with a panel of six anti-EAV neutralizing MAbs, as well as competitive binding assays, indicated that this linear immunodominant region of G\textsubscript{L} comprises at least three interactive neutralizing epitopes (Balasuriya et al., 1995; Deregt et al., 1994). Generation of such mutant viruses for PRRSV, as well as competition binding assays, should allow us to determine how many neutralizing epitopes are clustered on the N-terminal half of GP\textsubscript{3}.

PRRSV GP\textsubscript{3} is rather abundantly present in the virion and is partially exposed in association with the lipidic envelope (Mardassi et al., 1996; Meulenberg et al., 1995). Although it has been recently demonstrated that ORFs 2, 3 and 4 also code for envelope-associated glycoproteins of LV (Meulenberg et al., 1995; Meulenberg & den Besten, 1996), and that MAbs generated to GP\textsubscript{4} can also neutralize viral infectivity in vitro (van Nieuwstadt et al., 1996), these proteins are generally only weakly recognized by convalescent pig sera (Loemba et al., 1996; Nelson et al., 1994), and thus can only be considered as minor viral structural proteins whose role in the protection or recognition by immune cells is still to be determined. Several authors have demonstrated by Western immunoblotting and RIPA, using purified or concentrated virus preparations, that GP\textsubscript{3} is the major viral envelope glycoprotein, being recognized by most convalescent pig sera (Loemba et al., 1996; Nelson et al., 1993; Meulenberg et al., 1995).

Further studies are needed in order to establish if distinct neutralizing antigenic domains are present on PRRSV GP\textsubscript{3}. Nevertheless, the results obtained from this study of the comparative reactivity of the five anti-ORF5 protein MAbs to the modified live attenuated vaccine strain ATCC VR-2332 and the European prototype LV strain provide preliminary evidence for the existence of at least two neutralizing epitopes on GP\textsubscript{3}. This assumption is based on the fact that only two of the five MAbs tested neutralized the US strain with titres similar to the homologous strain, while all five failed to react to the LV strain. These results could be anticipated since previous genomic studies established that the amino acid sequence identity between the Quebec IAF-Klop strain and the reference US strain was 89% for the ORF5-encoded glycoprotein, whereas the predicted product of the Quebec and LV strains
display only 52% amino acid identity (Mardassi et al., 1995; Meng et al., 1995). Of particular interest is the recent identification within the N-terminal half of the ORF5 protein of North American field isolates of an hypervariable region with antigenic potential (Meng et al., 1995). Similar findings were also described for Canadian field isolates (Conference of Research Workers in Animal Diseases, Chicago, November 1996). Since three of the anti-ORF5 protein MAbs tested failed to neutralize the ATCC VR-2332 strain, we cannot exclude the possibility that they may be directed to this region. Competitive binding assays and the study of the reactivity of the MAbs with the truncated ORF5-encoded protein are in progress in order to establish the number of immunodominant epitopes associated with PRRSV GP5.

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References


MAbs to GP$_s$ of porcine arterivirus


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