

**Identification of porcine alveolar macrophage glycoproteins  
involved in infection of porcine respiratory and reproductive  
syndrome virus**

Brief Report

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**Summary.** The aim of this study was to identify the receptor(s) for PRRSV on porcine alveolar macrophages (PAMs) by producing monoclonal antibodies (MAbs) against these cells. Hybridoma supernatants were selected for their ability to block PRRSV infection. Four MAbs, 1-8D2, 9.4C7, 9.9F2, and 3-3H2 inhibited infection and recognised cell surface, PAM-specific antigens as shown by immunofluorescence and immunoperoxidase monolayer assay. These MAbs were then used to identify cellular proteins involved in PRRSV infection by radioimmunoprecipitation assays (RIPAs). MAbs 1-8D2 and 9.9F2 each recognised a 150 kDa-polypeptide doublet, while MAbs 9.4C7 and 3-3H2 both recognised a 220 kDa-polypeptide. Glycosidase treatment demonstrated all these polypeptides to be N-glycosylated. Thus, multiple glycoproteins appear to be involved in infection of PAMs by PRRSV.

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*Porcine respiratory and reproductive syndrome virus* (PRRSV) is an enveloped, positive stranded RNA virus and a member of the *Arteriviridae* family. Other members of this family are *Equine arteritis virus* (EAV), *Lactate dehydrogenase-elevating virus* (LDV) and *Simian haemorrhagic fever virus* (SHFV) [1, 14]. PRRSV has a highly restricted tropism for macrophages, particularly for porcine alveolar macrophages (PAMs), both *in vivo* and *in vitro* [17]. Recently, PRRSV was also shown to replicate in testicular germ cells [19]. The only non-porcine cells known to support PRRSV replication are the African green monkey kidney

cells, MA-104 and its derivatives CL-2621 and MARC-145 [11, 23]. An important determinant of the restricted tropism of PRRSV is the presence of one or more specific receptors on the cellular membrane surface [6, 7, 10, 12, 16]. In order to identify the PRRSV receptor, Duan et al. [5] produced monoclonal antibodies (MAbs) against PAMs that inhibited PRRSV infection. These MAbs recognised a putative receptor protein of 210 kDa on the surface of PAMs that was absent on MARC-145 cells. However, the identity of this protein was not elucidated.

In the present study, we also aimed at identifying cell surface proteins involved in PRRSV entry. Likewise, we set out to generate infection-inhibiting MAbs that would allow us to characterise these proteins. To that end, two fusions were carried out with spleen cells from BALB/c mice that had been immunised with PAMs [9]. In addition, four fusions were performed with spleen cells from BALB/c mice that had been tolerised to porcine bone marrow cells prior to immunisation with PAMs [13]. Thus, we aimed at increasing the chances of obtaining MAbs inhibiting PRRSV infection since bone marrow cells are not susceptible to PRRSV [8]. A similar approach was used by Duan et al. [5], except that these authors used peripheral blood mononuclear cells (PBMCs) to tolerise their mice. Because a small percentage of the PBMCs consists of monocytes that are permissive for PRRSV [22], we initially selected peritoneal macrophages, a related cell type reported earlier to be non-permissive [7]. In our hands, however, these macrophages appeared to be permissive for PRRSV, unlike bone marrow cells (data not shown).

The resulting hybridomas were screened for the production of MAbs directed against PAMs in an immunoperoxidase monolayer assay (IPMA) [23]. Seventy stable hybridomas secreting anti-PAM antibodies were obtained that were sub-cloned twice by limiting dilution to ensure production of monospecific antibodies. Hybridoma supernatants were then assayed for their ability to block PRRSV infection of PAMs in a microtiter neutralisation assay on PAMs. PAMs were seeded into a 96-well-plate ( $10^5$  per well) and incubated at 37 °C. After 18 h, the cells were incubated with hybridoma supernatants for 1 h at 37 °C, washed once with culture medium, and subsequently inoculated with 100 or 300 TCID<sub>50</sub> of PRRSV (Lelystad isolate) per well for 1 h at 37 °C. After inoculation, cells were washed twice with culture medium, and incubated with fresh medium for another 24 h. Cells were finally fixed and an IPMA was performed with MAb 122.17, which is directed against the nucleocapsid protein [21].

Of seventy hybridoma supernatants, four MAbs consistently inhibited PRRSV infection, i.e. 1-8D2, 3-3H2, 9.4C7, and 9.9F2. The isotypes of these four MAbs were determined with the Mouse Immunoglobulin Isotyping kit (Roche). MAbs 1-8D2, 9.9F2 and 3-3H2 were found to belong to the IgG1 isotype, whereas MAb 9.4C7 was found to belong to the IgG2a isotype. MAbs 1-8D2, 9.4C7, 9.9F2 and 3-3H2 were subsequently tested for their cell specificity in an IPMA on several permissive and non-permissive cell types including PAMs. The only cells that could be immunostained in this assay were PAMs and Kupffer cells (Table 1). The absence of immunostaining of the other cell types is consistent with these cells not being permissive for PRRSV, except for the MARC-145

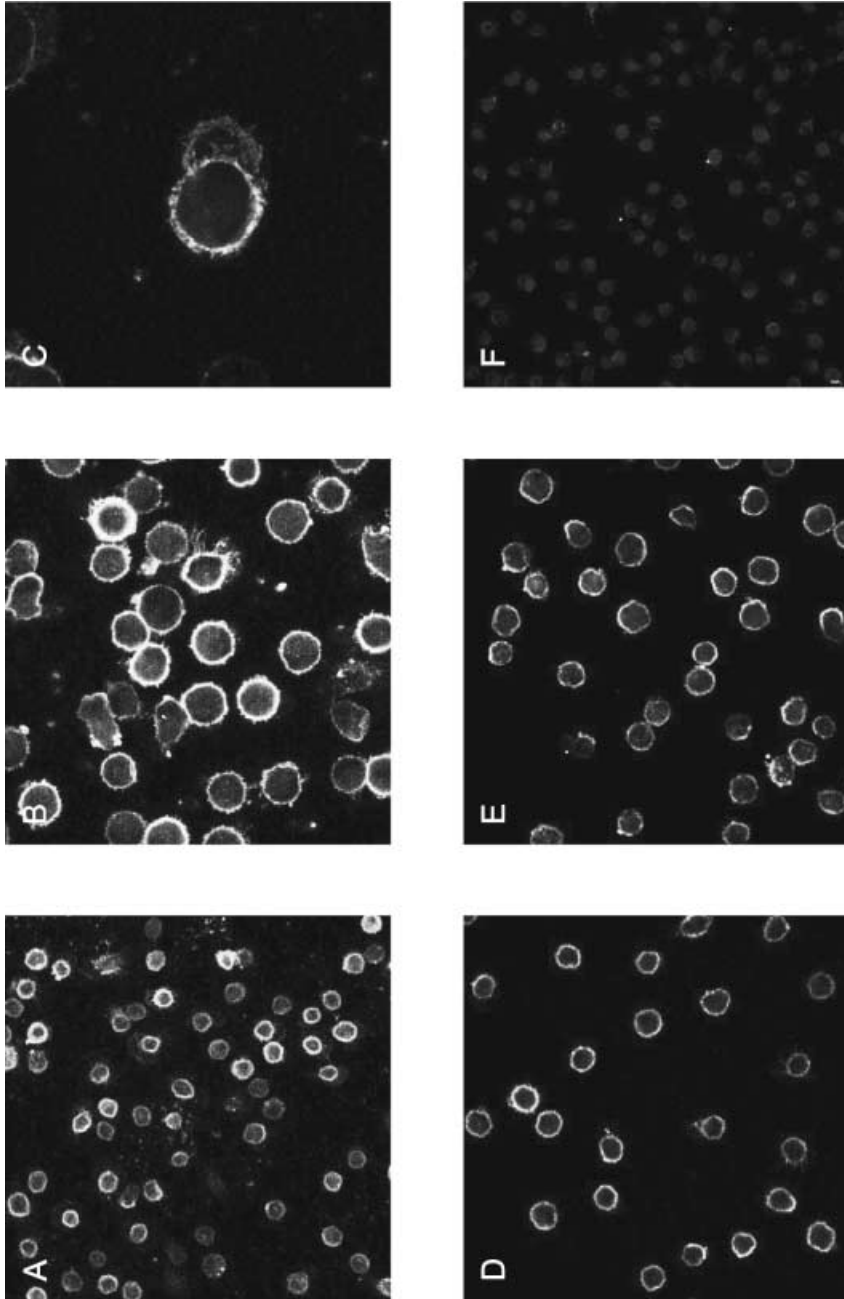
**Table 1.** Immunoperoxidase monolayer assay with MAbs 1-8D2, 9.4C7, 9.9F2 and 3-3H2 on different cell types, i.e. porcine alveolar macrophages (PAMs), porcine bone marrow cells, porcine Kupffer cells, African green monkey kidney cells (MARC-145), Baby Hamster Kidney (BHK)-21 cells, three porcine kidney cell lines (IB-RS-2, PK-15, and SK6 cells), and a bovine embryonic tracheal cell line (EBTr)

	Cell types						
	PAMs	Bone marrow cells	Kupffer cells	MARC-145	BHK-21	IB-RS-2, PK(15), SK6	EBTr
1-8D2	+	–	ND	–	–	–	–
9.4C7	+	–	+	–	–	–	–
9.9F2	+	–	+	–	–	–	–
3-3H2	+	ND	ND	–	–	–	–

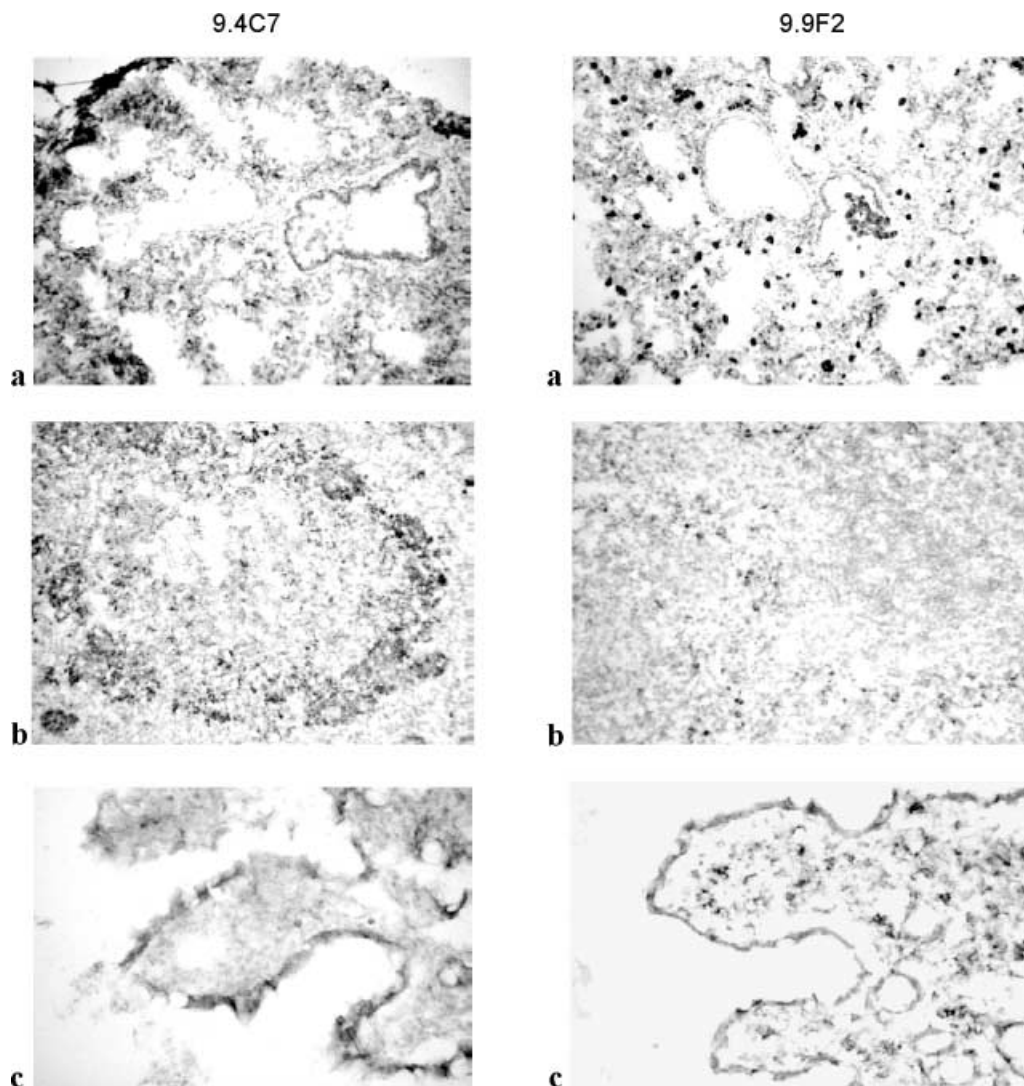
cells. It is of note that a putative receptor identified by Duan et al. [6] on PAMs was also not detected on MARC-145 cells. Apparently, PRRSV uses a different receptor or another member of the same receptor family to infect MARC-145 cells. Binding of PRRSV has also been reported to occur to non-permissive cells, such as BHK-21 and PK-15 cells [12, 20]. This suggests that the molecules recognised by the MAbs are involved in virus entry rather than in cell attachment. To confirm the plasma membrane localisation of proteins recognised by our MAbs 1-8D2, 3-3H2, 9.4C7 and 9.9F2, an indirect immunofluorescence assay (IFA) was performed on PAMs. The IFA was performed essentially as described previously [3] but without membrane permeabilisation to allow a cell surface staining. A MAb directed against feline coronavirus (23F4.5) was used as a negative control [2]. All four MAbs recognised antigen localised on the PAM membrane surface (Fig. 1).

To assess the recognition pattern of the MAbs, immunohistochemical staining was performed on thin sections of formalin-fixed, paraffin-embedded tissues from the lungs, lymph nodes, thymus, spleen, liver, bone marrow, small bowel, and central nervous system, collected from an outbred pig [18]. MAbs directed against specific subpopulations of PBMCs were used as controls. MAbs 1-8D2 and 9.9F2 displayed a similar recognition pattern that was clearly distinguishable from that of the two other MAbs, 9.4C7 and 3-3H2, the recognition patterns of which were also similar. Both MAb-pairs appeared to recognise – based on the morphology of the stained cells – macrophages and macrophage-like cells in lung, liver and spleen, and endothelial cells of small capillary vessels (Fig. 2). In addition, MAbs 1-8D2 and 9.9F2 stained distinct cells in bone marrow, thymus, and gut wall, contrary to MAbs 9.4C7 and 3-3H2.

To confirm that the MAbs specifically inhibited PRRS virus infection, an additional experiment was performed with MAbs 1-8D2, 3-3H2 and 9.9F2. For this purpose, the MAbs were purified using protein G sepharose (Amersham Pharmacia Biotech). The large-scale purification of MAb 9.4C7 was omitted for



**Fig. 1.** Cell surface localisation of proteins recognised by MAbs on PAMs as determined by immunofluorescence. **A** MAb 9.9F2; **B** MAb 9.9F2, detail; **C** MAb 1-8D2, detail; **D** MAb 3-3H2; **E** MAb 9.4C7; **F** MAb 23F4.5, directed against feline coronavirus [2]

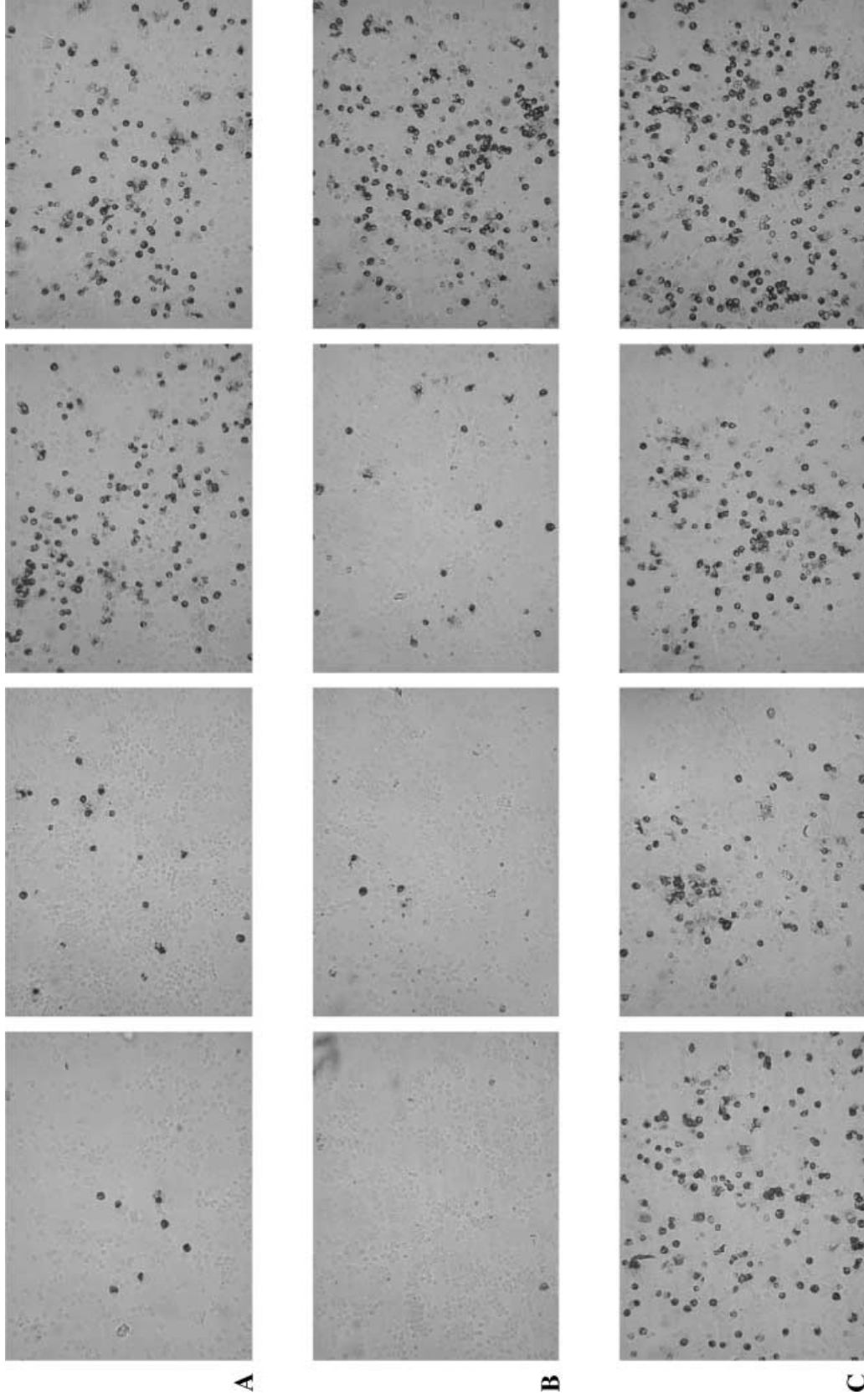


**Fig. 2.** Differences in immunohistochemical staining patterns in thin sections of formalin-fixed, paraffin-embedded tissues of lung (a), spleen (b), and gut wall (c) by MAbs 3-3H2 and 9.4C7 (on the left) versus MAbs 1-8D2 and 9.9F2 (on the right)

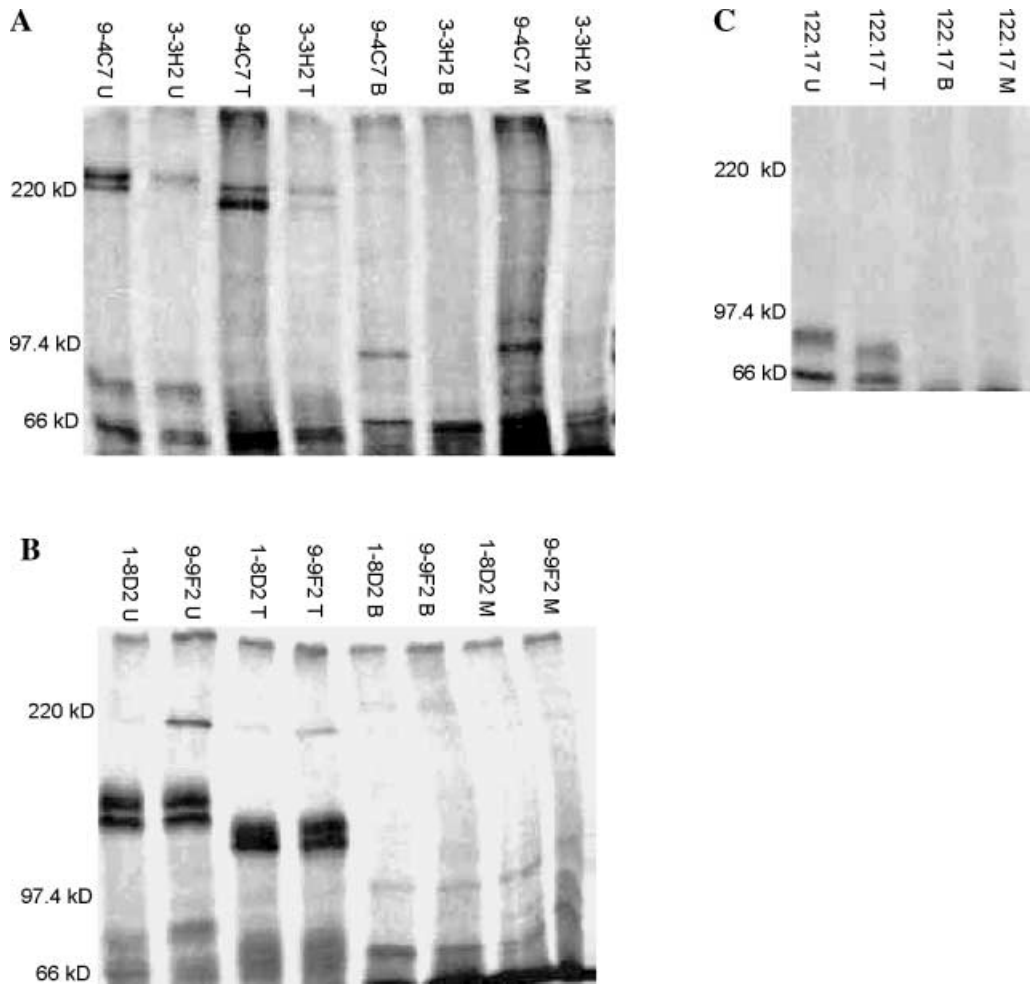
technical reasons. Two other porcine RNA viruses known to infect PAMs were used as control viruses, i.e. transmissible gastro-enteritis virus (TGEV) and classical swine fever virus (CSFV). PAMs were incubated with MAbs 1-8D2, 3-3H2, or 9.9F2 at concentrations ranging from 0.01 to 10  $\mu\text{g/ml}$ , washed, and subsequently infected with 100 or 300 TCID<sub>50</sub> of TGEV, CSFV, and PRRSV, respectively. MAb 3-3H2 appeared to block PRRSV infection completely at 10  $\mu\text{g/ml}$  and almost completely at 0.1  $\mu\text{g/ml}$ , whereas only partial inhibition was observed with MAb 1-8D2 at 10 and 1  $\mu\text{g/ml}$ . MAb 9.9F2 partly inhibited virus infection at the highest concentration of 10  $\mu\text{g/ml}$ , whereas at the lower concentrations

no inhibition was seen (Fig. 3). The difference in inhibition efficiency between MAbs 3-3H2 and 1-8D2 may be a result of a difference in affinity. The low level of inhibition obtained with MAb 9.9F2 might also be explained by a low affinity for the antigen. CSFV was not inhibited by any of the MAbs at the concentrations used (data not shown). Surprisingly, TGEV infection of PAMs was partially inhibited by MAb 3-3H2, although the inhibition was not as prominent as with PRRSV (data not shown). To investigate the possibility that MAb 3-3H2 recognises the porcine aminopeptidase N (pAPN) protein, the established TGEV receptor [4], experiments were performed using a cDNA clone encoding the porcine APN (jAP1) and the anti-pAPN antibody G43 (both generously provided by Dr. B. Delmas). It was found that BHK-21 cells transfected with jAP1 could not be immunostained with MAb 3-3H2, indicating that this MAb does not recognise pAPN, unlike MAb G43 that did stain these cells (data not shown). From these data we conclude that MAb 3-3H2 does not recognise pAPN. The inhibition of TGEV infection by MAb 3-3H2 might just be the result of sterical hindrance. Alternatively, TGEV might use a coreceptor for entry into PAMs that is identical to the protein recognised by MAb 3-3H2.

Since MAbs 1-8D2, 9.4C7, 9.9F2 and 3-3H2 inhibited PRRSV infection, it may be postulated that these MAbs recognise cell surface proteins that are involved in virus attachment and/or entry. Therefore, RIPAs were performed with <sup>35</sup>S-methionine labelled lysates of PAMs to define the polypeptide specificities of the MAbs. Similarly labelled lysates of MARC-145 and BHK-21 cells were used as negative controls. Metabolic labelling and immunoprecipitation of proteins were performed essentially as described previously [15]. Immunoprecipitates were analysed by SDS-PAGE using a 5% polyacrylamide gel. As illustrated in Fig. 4, MAbs 3-3H2 and 9.4C7 both recognised a protein with a relative molecular mass of approximately 220 kDa (Fig. 4A). In addition, a 210 kDa protein was observed using these MAbs. However, this protein is most likely aspecific, since it was also observed in RIPAs with MAbs 1-8D2 and 9.9F2, and in RIPAs with cell lysates of MARC-145 or BHK-21 cells. Interestingly, MAbs 1-8D2 and 9.9F2 both specifically recognised a protein doublet with a relative molecular mass of approximately 150 kDa (Fig. 4B). SDS-PAGE analysis under non-reducing conditions also resulted in a doublet of approximately 150 kDa, indicating that the two polypeptides of the 150 kDa doublet are not covalently linked by disulphide bridges (data not shown). Both the 220 kDa protein and the 150 kDa protein doublet are specific for macrophages, as these proteins were not precipitated from cell lysates of MARC-145 or BHK-21 cells. To analyse whether the antigens detected by the MAbs were glycoproteins, and whether the 150 kDa doublet was composed of two identical polypeptide chains that are differently glycosylated, the immunoprecipitates were treated with N-glycosidaseF, which removes N-linked oligosaccharides [15]. As can be seen from Fig. 4, this treatment indeed caused a shift in mobility of the proteins. After deglycosylation, the apparent molecular mass of the 220-kDa protein was reduced to approximately 200 kDa (Fig. 4A), whereas that of the 150-kDa protein doublets was reduced to approximately 130 kDa. The 150-kDa protein doublets appeared to be composed of different



**Fig. 3.** Inhibition of PRRSV infection by PAM-specific MAbs. **A:** MAb 1-8D2, **B:** MAb 3-3H2, **C:** MAb 9.9F2, at concentrations of 10, 1, 0.1, and 0.01  $\mu\text{g/ml}$  from the left to the right, respectively. PRRSV-infected PAMs were detected in an IPMA with MAb 122.17 directed against the PRRSV nucleocapsid [21]



**Fig. 4A–C.** Sodium dodecyl sulfate-polyacrylamide (5%) gel electrophoretic analysis of proteins immunoprecipitated from  $^{35}\text{S}$ -methionine labelled lysates of alveolar macrophages, BHK-21-cells and MARC-145 cells using the PRRSV-inhibiting MAbs. Labelling of the cells, preparation of the cell lysates, immunoprecipitation and deglycosylation of proteins was performed as described earlier [15]. Relative molecular masses (in kDa) and positions of marker proteins analysed in the same gel are indicated at the left. *U* = untreated, *T* = N-glycosidase F-treated, *B* = BHK-21 cells, *M* = MARC-145 cells

polypeptide chains, as they remained doublets after deglycosylation (Fig. 4B). Two major species of 56 kDa, and of 80 kDa were co-immunoprecipitated by MAbs 1-8D2, 3-3H2, 9-4C7, and 9-9F2. To investigate their specificity, RIPAs were carried out with an unrelated antibody directed against the PRRSV nucleocapsid (Fig. 4C), and with various other PAM-specific antibodies (data not shown). The 56 kDa and the 80 kDa proteins were also observed using these MAbs in RIPAs with PAM lysates, but not with cell lysates of MARC-145 or BHK-21 cells. These results suggest that the 56 kDa and 80 kDa macrophage proteins nonspecifically coprecipitate with various MAbs. In conclusion, two sets of MAbs



were obtained that specifically inhibit PRRSV infection. These two sets have clearly distinguishable antigen recognition patterns as shown by immunohistochemistry. One set, comprising MAbs 3-3H2 and 9.4C7, recognises an approximately 220 kDa N-glycosylated polypeptide, whereas another set, consisting of MAbs 1-8D2 and 9.9F2, recognises a doublet of N-glycosylated polypeptides of approximately 150 kDa. Endoglycosidase treatment showed that the protein moieties of these polypeptides are approximately 200 kDa and 130 kDa, respectively. Since MAbs 1-8D2 and 9.9F2 recognise an epitope that is present in two distinct polypeptides with almost identical relative molecular masses, the two proteins within this doublet are probably related and possibly represent protein isoforms.

The putative PRRSV receptor reported by Duan et al. [6] was a protein estimated to be 210 kDa. Though its glycosylation was not investigated, this protein most likely corresponds with the 220-kDa N-glycosylated proteins identified in our study. These authors did not detect receptor candidates in the 150-kDa-size class. Our results leave us with several intriguing questions to be answered in the future. One obvious issue is the identity of the two sets of glycoproteins that we detected, and their possible relationship. Another issue is their role in PRRSV infection: are they alternative receptors or co-receptors, or do they serve different functions during viral entry. To address these issues, we will clone and sequence the gene(s) for the N-glycosylated proteins in the near future.

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