RESEARCH REPORT
SWINE HEALTH

Title: Investigation into the ability of the anti-idiotype to block PRRS virus infection and characterization of a putative receptor on MARC-145 cells and porcine alveolar macrophages NBP #05-145

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Abstract:

The prevention and control of PRRS has been difficult, in part because our knowledge of the immunity against PRRS virus is limited. We know that antibodies generated during the early phase of infection cannot neutralize the virus, neutralizing antibodies and cellular immune responses appear much later in the course of infection, and animals can remain persistently infected despite an active immune response. Recently, a monoclonal anti-idiotypic antibody (designated Mab2-3H) specific for a monoclonal antibody to the PRRS virus envelope glycoprotein GP5 was produced in the investigator's laboratory. Serological characterization showed that Mab2-3H by functionally and/or structurally mimicking GP5 antigen of PRRS virus inhibited the binding of anti-GP5 antibodies to PRRS virus, directly bound MARC-145 cells and porcine alveolar macrophages (PAM), reacted with a soluble protein prepared from MARC-145 cells and PAM and blocked PRRS virus infection of these cells. This study may lead to the development of an immune therapeutic agent, the anti-idiotype-based receptor-blocker. The information on the characterized cellular receptor will lead to further elucidation of the mechanism of the receptor-virus interaction.

Introduction:

PRRSV is capable of producing catastrophic losses either upon introduction into naïve herds or as re-breaks in endemically infected herds. Once a herd is infected, virus continues to circulate within the herd and can be recovered years after the initial introduction. Currently, very little is known about the immunological events leading to the generation of protective immunity against PRRSV. However, it is recognized that neutralizing antibody and cellular immune responses are slow to develop against PRRSV. Vaccination against PRRSV infection is one of the strategies used to control this debilitating disease. However, the current vaccines are not consistently able to provide protective immunity at the herd level. Outbreaks of PRRS in Danish herds in causal association with the introduction of a modified-live virus vaccine illustrate this point. A better understanding of the mechanisms that regulate immunity to this virus will aid in the development of more effective vaccines. Our understanding of the immunobiology of the PRRSV and how this virus may be modulating the immune response is, at best, in its infancy. Given the long-term persistence of PRRSV in the presence of non-neutralizing antibodies, it is likely that the stimulation of a strong and early neutralizing antibody and cellular immune responses against this virus would be able to enhance protective immunity.

The development of "Immune Therapy" has been identified by the National Pork Board as one of the PRRS Initiative Objectives. This project is aimed to examine the ability of monoclonal anti-idiotype, by binding to a putative cellular receptor, to block PRRS virus infection of MARC-145 cells and porcine alveolar macrophages and to characterize this receptor. Anti-idiotype can in some cases bear a structural resemblance to the antigen. Obviously, the antigen and anti-idiotype have in common the ability to interact with the antigen receptor. This idea received its first experimental support in 1978 with the demonstration that rabbit anti-idiotype to anti-insulin antibodies reacted with the insulin receptors, and anti-idiotype to anti-thyrotropin (TSH) bound the TSH receptor and mimicked the activity of TSH. In a recent study, an anti-idiotype was used to identify the herpes simplex virus receptor, demonstrating that the identification of virus receptors using anti-idiotype is feasible. Results generated recently in this laboratory showed that a monoclonal anti-idiotype (designated Mab2-3H) directly bound MARC-145 cells and swine alveolar macrophages (PAM) and recognized a cellular protein prepared from these cells, which suggests that Mab2-3H recognizes a PRRS virus receptor on these cells. Based on these findings, we have examined the ability of Mab2-3H blocked PRRS virus infection of MARC-145 cell and PAM. The protection effects were observed when the cells interacted with F(ab')2 fragment of Mab2-3H and affinity purified the putative receptor protein. These results suggest that it is possible to develop an immune therapeutic agent, the anti-idiotype-based receptor-blocker.

Several separate studies have demonstrated the existence of putative PRRS virus receptors on MARC cells, macrophages, and other cells by direct or indirect binding of virus to the cells or by using antibodies generated against macrophages. Those putative receptors related to GP5 binding were only identified on PAM, not on MARC-145 cells, and with molecular weight of approximately 210 Kda, which is smaller than that identified in this laboratory. These findings suggest that the receptors identified by other researchers and that identified in this laboratory are not the same and more than one receptors are involved in PRRS virus binding and/or entry into the permissive cells.

Objectives:

a) **Examine the ability of Mab2-3H to block PRRS virus infection of MARC-145 cells and PAM.** Mab2-3H has been shown to bind MARC-145 cells and PAM via presumably antigen-binding site since the isotype matched mouse IgG did not bind these cells. Prevention of PRRS virus infection of cells by blocking the cellular receptor will potentially lead to design an effective therapeutic agent.

b) **Characterization of the soluble membrane protein from MARC-145 cells and PAM.** It is based upon the ability of Mab2-3H to mimic GP5 antigen and recognize a soluble protein prepared from those cells.

Sequence identification of this soluble protein will lead to further understanding of PRRS virus pathogenesis and identify the region(s) of the protein responsible for binding to GP5.

V. Materials and Methods:

1. Examine the ability of Mab2-3H to block PRRS virus infection of MARC- 145 cells and PAM.

Preparation of cell monolayer. MARC-145 cell line was grown in minimum essential medium containing 10% fetal calf serum on a 96-well plate, according to the standard procedure used at ISU-VDL, and incubated at 37°C in a CO_2 incubator for 72 hours. PAM were collected from pigs negative for PRRS virus by serology and PCR. PAM were cultured in Eagle medium supplemented with 10% fetal bovine serum in a humidified 5% CO_2 incubator at 37°C as previously described (13).

Interaction of Mab2-3H with cells. The interaction between Mab2-3H and monolayered MARC-145 cells and PAM was conducted under the following conditions:

a. Mab2-3H was used at various concentrations: 1, 10, 100 and 1,000 ng/well,
b. The interaction was evaluated at various times: 15, 30, 45 and 60 minutes.

After washing the cells with PBS to remove any unbound Mab2-3H, the pre-titrated PRRS virus (ISU-P strain) was added to each well. After 48-hour incubation, the cells were fixed with acetone and the presence of PRRS virus was detected with a FITC-conjugated monoclonal antibody to PRRS virus N protein. The protection level was determined by measuring the 90% reduction of fluorescent foci unit (FFU) of Mab2-3H treated cells in comparison with the isotype matched control mouse IgG.

2. Characterization of the soluble membrane protein from MARC-145 cells and PAM.

Preparation of cell lysates. Monolayered MARC-145 cells and PAM prepared as described above was harvested into a centrifuge tube and centrifuged at 500 x g for 15 min at 4°C. The cell pellet was be treated with a lysing buffer [0.25M Tris, 0.11 M Tricine, pH 8.9, containing 0.14 M mM Calcium Lactate and 1% (v/v) Triton X-100] for three freeze-thaw cycles. After the final thawing, the cell suspension was incubated overnight at 4°C, with gentle agitation, centrifuged at 2,000 x g for 20 min at 4°C. The supernatant, which contains the soluble proteins, was collected and stored at -80°C until used.

Preparation of a Mab2-3H affinity column. Mab2-3H IgG was covalently coupled to CNBr-activated Sepharose 6MB using a procedure modified from a commercial protocol (Pharmacia). CNBr-activated Sepharose 6MB beads (1.5 g) was soaked in 1 mM HCl for 15 min, and the excess liquid removed through a 0.45 μm membrane. The beads were washed with 5 ml coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and immediately transferred to a solution of Mab2-3H IgG (2 mg/ml, 10 ml) dissolved in coupling buffer. After the suspension was mixed for 2 hours at room temperature and overnight at 4^oC, the Sepharose matrix was incubated with 0.1 M Tris-HCl (pH 8.0) for 2 hr at room temperature. The matrix was washed with 0.1 M glycine-HCl (pH 2.5) followed by phosphate buffered saline (PBS) before use.

Affinity purification of soluble protein using the Mab2-3H affinity column. MARC-145 and PAM cell lysate prepared as described above was filtered and passed through the Mab2-3H affinity column according to a standard affinity purification procedure described previously (14). Bound and unbound fractions will be collected. The protein concentration was determined by the Lowry method (15).

Sequencing of the purified membrane protein. The membrane protein purified by affinity chromatography were sequenced at the ISU Protein Facility.

Identify region(s) of the protein responsible for binding to PRRS virus. Once the protein's sequence is known, the region that bound to GP5 antigen was identified. Peptides that overlap the region were synthesized at the ISU Protein Facility. The peptides were tested for their ability to (a) bind to PRRS virus and Mab2-3H in an ELISA, in comparison with the binding of the whole protein, (b) block the binding of PRRS virus to MARC-145 cells and PAM in an IFA, and (c) prevent PRRS virus infection of MARC-145 cells and PAM in a fluorescent focus neutralization assay (described above).

Results:

Mab2-3H blocked PRRS virus infection of MARC- 145 cells and PAM

Preparation of cell monolayer. MARC-145 cell line and PAM were prepared and grown in designated cell culture media and incubated at 37°C in a CO_2 incubator. After 72 hours incubation, all cells were monolayered.

Interaction of F(ab')2 fragment of Mab2-3H with cells. F(ab')2 fragment of Mab2-3H was used at the concentrations of 1, 10, 100 and 1,000 ng/well. The interaction was allowed for 15, 30, 45 and 60 minutes.

Infection of Mab2-3H contacted cells with PRRS virus. After washing the cells with PBS to remove any unbound F(ab')2 fragment of Mab2-3H, the pre-titrated PRRS virus (ISU-P strain) were added into each well. After 48-hour incubation, the presence of PRRS virus was detected with a FITC-conjugated monoclonal antibody to PRRS virus N protein. The protection level was determined by measuring the 90% reduction of fluorescent foci unit (FFU) of the cells. As shown in Table 1, treatment of Marc-145 cells and PAM with F(ab')2 fragment of Mab2-3H as low as 100 ng/well resulted in the significant reduction of PRRS virus infection level. The effect was observed as short as 15 minutes and as long as 60 minutes of the interaction between F(ab')2 fragment of Mab2-3H and the cells.(Table 1).

Characterization of the soluble membrane protein from MARC-145 cells and PAM

Preparation of cell lysates. Monolayered MARC-145 cells and PAM were prepared as described above and harvested into a centrifuge tube and centrifuged at 500 x g for 15 min at 4°C. The cell pellet was treated with lysing buffer for three freeze-thaw cycles. After the final thawing, the cell suspension was incubated overnight at 4°C, with gentle agitation, centrifuged at 2,000 x g for 20 min at 4°C. The supernatant, which contains the soluble proteins, was collected and stored at -80°C.

Affinity purification of soluble protein. Mab2-3H IgG was covalently coupled to CNBr-activated Sepharose 6MB using a procedure modified from a commercial protocol (Pharmacia). Cell lysate from MARC-145 and PAM was filtered and passed through the Mab2-3H affinity column. Proteins that bound to the column were collected and examined by Western blot immunoassay.

Analysis of purified soluble protein. The Mab2-3H affinity column purified soluble proteins prepared from MARC-145 cells and PAM were analyzed by Western blot immunoassay. The results showed that the affinity purified protein had the molecular weight of approximately 250Kd, the same sized as pre-purified soluble protein. They were specifically recognized by Mab2-3H, but not reacted with a control antibody preparation.

Sequencing of the purified soluble protein. The affinity purified protein was sent to the ISU Protein Facility for protein sequencing. After three trials, the protein could not be sequenced due to its size and potential N-

terminal blocked by some special amino acids. An alternative approach is to cut the protein into smaller fragments and sequence at a commercial facility. For this reason, the identification of region(s) of the protein responsible for binding to PRRS virus will be done in the near future.

Discussion:

The results generated from this study demonstrated that monoclonal anti-idiotypic antibody can bind the putative PRRS virus receptor on MARC-145 cells and PAM and blocked the virus infection of these cells. The ability of blocking PRRS virus infection was observed when monoclonal anti-idiotypic antibody was used at 100ng and contacted with the cells after 15 min, indicating this antibody is efficient to prevent PRRS virus in vitro infection of the cells.

The membrane protein prepared from MARC-145 cells and PAM that permit PRRS virus infection was affinity purified by the monoclonal anti-idiotypic antibody. The affinity purified membrane protein remained the immunological function as it was recognized by the monoclonal anti-idiotypic antibody. The initial trials of sequencing this protein were failed due to its size and potential N-terminal blockage; however, the alternative approach has been made to cut the protein into smaller pieces and sequenced. The fine characterization of this potential receptor protein will be conducted in the near future to elucidate the function of this protein in the involvement of PRRS virus binding and entry of MARC-145 cells and PAM.

Lay Interpretation:

PRRS is the most costly disease U.S. swine producers confront and little progress has been made in recent years to improve the situation. Prevention and control of PRRS has been difficult, in part because our knowledge of the immunity against PRRSV is limited. Recently, we produced a monoclonal anti-idiotypic antibody specific for a monoclonal antibody to the PRRS virus envelope glycoprotein GP5. This antibody functionally and/or structurally mimicked GP5 antigen of PRRS virus, inhibited the binding of anti-GP5 antibodies to PRRS virus, directly bound MARC-145 cells and porcine alveolar macrophages and reacted with a soluble protein prepared from MARC-145 cells and PAM. It blocked PRRS virus infection of MARC-145 cell and PAM and after affinity purified remained the immunological function by recognition with the monoclonal anti-idiotypic antibody. The information on the future characterization of the cellular receptor protein will lead to the development of an immune therapeutic agent, the anti-idiotype-based receptor-blocker and further elucidation of the mechanism of the receptor-virus interaction.

Table 1. Reduction of PRRS Virus Infection of Cells after Interaction with Mab2-3H

Incubation Time (minutes)	Percent (%) Reduction of PRRS Virus Infected Cells			
	Concentration (ng/well) of F(ab')2 fragment of Mab2-3H			
	1	10	100	1000
15	0	0	**65**	85
30	0	0	**68**	83
45	0	0	**63**	88
60	0	0	**66**	86