

Title: Investigation into the Ability of Anti-Idiotypic Antibodies to Protect Pigs from Porcine Reproductive and Respiratory Syndrome Virus Infection – **NPB #03-164**

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Abstract: Auto-anti-idiotypic (Aab-2) against the monoclonal anti-GP5 antibody (Mab-1) was identified and purified from pigs experimentally infected with PRRSV. Serological characterization demonstrated that Aab-2s represented the internal image of anti-idiotypic and mimicked GP5 antigen of PRRSV and bound MARC-145 cells and porcine alveolar macrophages (PAM) by recognizing a putative viral receptor(s) with approximate molecular weight of 210 KDa. Aab-2s neutralized PRRSV infection of MARC-145 cell presumably by blocking the viral receptor since the neutralization ability required a minimum 30 min and up to 60 hours direct contact between Aab-2s and cells. Further study has shown that Aab-2s significantly reduced PRRSV infection in pigs. These findings indicate that Aab-2s mimicking GP5 antigen recognized a viral receptor on PRRSV permissive cells and in turn blocked PRRSV infection. These results support further studies of developing anti-idiotypic vaccine candidates against PRRSV.

Introduction: Currently, very little is known about the immunological events leading to the generation of protective immunity against PRRSV. Vaccination against PRRSV is one of the strategies used to control this debilitating disease. However, the currently available vaccines are not able to provide protective immunity at the herd level. The occurrence, in 1996, of a severe PRRS epidemic among vaccinated pigs on southeastern Iowa swine farms illustrates this problem. Deficiencies in the pigs' ability to mount both humoral and cellular immune responses to PRRSV may have promoted the evolution of more virulent strains, in addition to predisposing the animals to infection. Indeed, the available data support the postulate that ability of the PRRSV to manipulate the early innate viral immune response may explain the pig's unique inability to mount good immune responses to this pathogen (1). Inasmuch as objective and subjective evaluations have shown that the protection provided by the current vaccines is inadequate, an alternative approach, like an anti-idiotypic vaccine, would be worth a trial. **Because anti-idiotypic antibodies are expected to bind the putative viral receptor(s) and block PRRSV infection, anti-idiotypic vaccines could be effective for this disease.**

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Anti-idiotypic antibodies have come a long way in a relatively short time. The concept that a series of idiotype (Id)-anti-idiotypic (anti-Id) reactions may be involved in regulating the immune response was originally proposed by Jerne (2). His proposal that the immune response to a given antigen can be regulated by a series of Ids and their serologic counterparts, anti-Ids or Ab-2s, has been found useful for the production of diagnostic reagents (3), vaccines (4) and specific immune response regulators (5). The demonstration that an auto-anti-Id response can be detected in individuals immunized with the antigen alone is the further evidence of idiotype networks function *in vivo* (6).

When exposed to antigens, animals produce not only anti-antigen (Ab-1) responses, but also the Aab-2 response proposed by Jerne (2). Aab-2s have been found in numerous experimental systems including, recently, PRRSV-infected pigs (7, 8). In the PRRS study, 45 of 52 infected pigs developed not only an anti-PRRSV antibody response, but also Aab-2 responses against anti-GP5 and anti-M monoclonal antibodies. Aab-2s detected in other systems include Aab-2s directed against antibodies to thyroglobulin (9), trinitrophenylated Ficoll (10), insulin (11), tetanus-toxoid (12) and acetylcholine receptor (reviewed in 13).

Objectives: The studies described below in 'Previous Work Related to The Proposed Study' showed the presence of Aab-2s in PRRSV infected pigs. The results raised important questions regarding the immunology of PRRS, i.e., the functional roles of the Aab-2 in PRRSV infection. **The objective of this study is to evaluate the direct binding of previously generated Aab-2s (NPB #02-015) to the putative viral receptor(s) and to test if this binding will protect cells and animals from PRRSV infection.**

Materials and Methods:

1. Direct binding of Aab-2s to swine macrophages: A standard immunohistochemistry (IHC) was performed to detect the binding of Aab-2s at various concentrations to macrophages from swine lung, tonsil, spleen and lymph nodes. The specific binding was detected using fluorescence labeled goat-anti-swine IgG and visualized under a light microscope in comparison with the binding of swine negative control IgG.

2. Western blot assay: Cell lysates from MARC-145 cells and macrophages were separated by SDS-PAGE in a 7.5 % gel under reducing conditions based on the procedure described in detail elsewhere (14). The separated proteins were transferred onto nitrocellulose membranes. After blocking the unbound sites on the membrane, the binding of Aab-2s were detected with a goat-anti-swine IgG conjugate.

3. Immunization of pigs with Aab-2s and challenge with PRRSV: Fifteen 4-week-old pigs known to be free of PRRSV were divided into 3 groups with 5pigs/group. Group 1 to 3 pigs were immunized i.p. with 100mg/pig of affinity purified Aab-2s, normal pig IgG, and PBS buffer, respectively. All pigs were challenged intranasally 3 days later with 2 mls of inoculum containing PRRSV (ISU-P strain) at a concentration of $10^3 - 10^4$ TCID₅₀/ml. PRRS virus ISU-P were used in this study because it was the virus used to generate the monoclonal antibodies against GP5 and M antigens in the previous study (15) and the Aab-2s recognized these monoclonal antibodies (7, 8). Serum samples for virus isolation and serological assays were collected from all pigs at days post immunization (DPI) of -5, 0, 2, 3, 4, 6, 8, 15, 22, 29 and 32. All pigs were euthanized on 32 DPI or 29 days post challenge and tissue samples (lung, lung lavage, tonsil,

tracheobronchial lymph nodes) were collected post mortem for virus isolation according to the standard procedure routinely performed at VDL.

4. Detection of Aab-2s: The presence of passively transferred Aab-2s in the serum samples collected at between 2 DPI and 32 DIP were detected by coating an ELISA plate with the monoclonal antibodies along with control IgG. The level of binding of Aab-2s to immobilized monoclonal antibodies was quantified with an ELISA by coating an ELISA plate with anti-GP5 and normal mouse IgG. Swine antisera were added to the plate and goat anti-swine IgG conjugated to horseradish peroxidase (HRP) was used to detect the presence of serum Aab-2s.

Results:

1. **Aab-2 direct bound porcine alveolar macrophages (PAM):** Lung, tonsil, spleen and lymph nodes tissue samples were collected from PRRSV infected and uninfected pigs and immunohistochemistry (IHC) assays were used to detect the binding of Aab-2s to PAM. As shown in Table 1, the Aab-2 IgG prepared from 35 DPI and 77 DPI sera bound PAMs. However, the Aab-2 from 35 DPI serum had higher binding affinity than that from 77 DPI serum indicating that these early Aab-2 may have strong ability of binding to PAMs. Since it possesses the characteristics of internal image of anti-Id, i.e., mimicking PRRSV GP5 antigen and binding the viral receptor(s), the Aab-2 may have the ability of blocking PRRSV infection of PAMs.

2. **Aab-2 blocked PRRSV infection of Marc-145 cells:** The ability of Aab-2 to neutralize PRRSV infection of Marc-145 cells was determined by a standard fluorescent Foci Neutralization assay (FFN) routinely performed in the investigator's laboratory. In brief, 80% monolayered Marc-145 cells were incubated with the Aab-2. After a certain incubating time, the Aab-2 was removed and PRRSV at predefined fluorescent foci unit was added and allowed to infect cells for 1 hour. After 48 hours' culture, Marc-145 cells were fixed and probed with FITC-monoclonal anti-PRRSV antibody as routinely performed in the laboratory. The Aab-2 prepared from sera collected at early (35 DPI) and late (77 DPI) DPIs neutralized PRRSV infection of Marc-145 cells at dose dependent manner (Table 1). The neutralization ability was due to the blockage of viral receptor by the Aab-2 and required a minimum 30 mins and up to 6 hours contact between the Aab-2 and Marc-145 cells. These results showed that the Aab-2 (i) bind a putative viral receptor by mimicking PRRSV antigen (GP5) and (ii) neutralize PRRSV infection of Marc-145 cells, which suggest that the Aab-2 may bind the viral receptor on PAMs and neutralize PRRSV infection of pigs.

3. **Aab-2 detected cellular proteins of Marc-145 cells and PAMs:** The ability of Aab-2 mimic PRRSV antigen and bind Marc-145 and macrophage cellular proteins was examined using a Western blot immunoassay. Cell lysates from Marc-145 cells and macrophages were separated by SDS-PAGE in a 7.5% gel under the reducing conditions. The separated cellular proteins were transferred onto nitrocellulose membranes. As shown in Figure 1, a cellular protein with a molecular weight of approximately 210 KDa reacted with the Aab-2 but not with the control swine IgG. This result indicated that this 210 KDa protein may be the cellular receptor for PRRSV. Interestingly, other researchers have previously identified a putative PRRSV receptor with the same molecular weight (210 KDa) from PAMs (16).

4. **Anti-Id induced reduction of PRRS virus infection:** Based on the ability of Aab-2

that neutralized PRRSV infection *in vitro* by recognizing the putative viral receptor, the *in vivo* experiment was conducted using Aab-2 to reduce PRRSV infection of pigs. Pigs were inoculated intraperitoneally (i.p) with 100mg of Aab-2 or control antibodies in PBS. Three days later, all pigs were challenged intranasally with 2 mls of inoculum containing PRRSV strain ISU-P at a concentration of 10^3 - 10^4 TCID₅₀/ml. This immunization and challenge regime was based on the results from a separate study (17) in which, two days after the passive transfer of IgG by i.p. immunization, IgG was detected in the peripheral blood of the recipients.

Peripheral blood and serum samples were collected from all pigs at days post immunization (DPI) -5, 0, 2, 3, 4, 6, 8, 15, 22, 29 and 32 for virus isolation and serum Aab-2 detection. All pigs were euthanized on 32 days post challenge and tissue samples (lung, tonsil, tracheobronchial lymph nodes) were collected post mortem for virus isolation according to the standard procedure routinely performed in our laboratories. **The results in Table 2 clearly demonstrated that pretreatment of pigs with Aab-2 (Group 1) significantly reduced PRRSV infection.**

5. Detection of passively transferred Aab-2: Passive transferred Aab-2 was detected in an ELISA by coating the solid-phase with the F(ab')₂ fragments of Mab-1 (Mab-25C against GP5 antigen) along with the normal mouse IgG F(ab')₂ fragments. As shown in Figure 2A, Aab-2 was detected starting from 2 days post immunization (DPI), reached the peak level at 3 DPI and decreased gradually from 4 DPI to 32 DIP. The Aab-2 binding to Mab-1 was specific since the serum samples did not react with normal mouse IgG (Figure 2B).

Discussion: Antibody responses to PRRSV result in the formation of variety of antibodies against N, GP5, M and other structural and non-structural proteins. Pigs infected with PRRSV produced not only antibodies (Ab-1s) to PRRSV antigens, but Aab-2s against anti-GP5 and anti-M antibodies as demonstrated previously (7, 8). Aab-2 antibody responses have been identified in numerous systems suggesting the existence of idiotype-anti-idiotypic complex in a given antibody response.

The results generated from this study clearly demonstrated that the Aab-2 (i) recognized a cellular receptor on MARC-145 cells and PAMs by mimicking GP5 antigen of PRRSV, (ii) identified a soluble cellular protein prepared from MARC-145 cells and PAM, (iii) blocked PRRSV infection of MARC-145 via the receptor binding and (iv) partially protected pigs from PRRSV infection.

Previous studies conducted in this laboratory have shown that the different time frames of the onset of the Aab-2s against anti-GP5 and anti-M antibodies between pigs that were free of the virus and that became virus carriers (7, 8), suggesting that pigs developed the Aab-2s at relatively early stage of the infection with PRRSV may clear the virus and therefore stop the potential transmission of the infection to other animals. The results generated from this study support this hypothesis that pigs immunized with Aab-2 were partially, but significantly, protected from subsequent PRRSV infection. Taking together, the results from these research studies provided the scientific evidence that using anti-idiotypic approach to prevent PRRSV infection is possible.

The limited supply of Aab-2 will prevent its use as the potential vaccine or therapeutic agent since Aab-2 was isolated from pigs infected with PRRSV. To overcome this limitation, a monoclonal anti-idiotypic antibody (designated Mab2-3H) has been produced in this laboratory against Mab-1 (Mab-25C against GP5 antigen). Since Mab2-3H was secreted by a hybridoma cell line, the production of Mab2-3H in needed quantity is unlimited. The study showed that Mab2-3H is an internal image anti-Id of GP5 antigen,

recognized the putative PRRSV receptor on MARC-145 cells and PAMs, identified the soluble cellular protein prepared from these cells and blocked PRRSV infection of MARC-145 cells. The *in vivo* study is in progress to examine the ability of Mab2-3H to protect pigs from PRRSV infection.

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. 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 remain persistently infected despite an active immune response.

In studying swine antibody responses, this laboratory has discovered the presence of serum auto-anti-idiotypic antibodies in pigs experimentally infected with PRRSV. These auto-anti-idiotypic antibodies are specific for anti-GP5 and anti-M antibodies. One set of the auto-anti-idiotypic antibodies mimics GP5 antigen, identifies a putative virus receptor on swine macrophages. Both *in vitro* and *in vivo* studies showed that this auto-anti-idiotypic antibody blocked PRRSV infection. The anti-idiotypic technique represents a new approach representing a major advance for further understanding the pigs' immunological response to PRRSV infection and providing a new tool to bring a potential alternative anti-idiotypic vaccine to the field.

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Table 1. Binding of Auto-Anti-Id to Macrophages

Auto-Anti-Ids prepared from	Concentration (ug/ml)	Binding Macrophages Collected from ¹				Neutralizing Marc-145 Cells ²
		Lung	Tonsil	Spleen	Lymph Nodes	
35 DPI serum	100	++++	++++	++	++	+
	10	+++	+++	++	++	+
	1	+	++	-	+	-
77 DPI serum	100	+++	++	+	++	+
	10	++	+	-	-	-
	1	-	-	-	-	-

- a. Auto-anti-Id binding to swine macrophages was detected by standard IHC. “++++”, “+++”, “++”, “+”, and “-” indicated 100, 75, 50, 25, and 0% binding of an examined tissue, respectively.
- b. The neutralization was observed when auto-anti-Id incubated with Marc-145 cells between 30 min and 6 hours.

Table 2. Effect of anti-Id treatment on PRRS virus infection

Groups (5 pigs/ group)	Immunized with ¹	Isolation of virus (Log ₁₀ TCID ₅₀ /ml) from serum and tissues at necroscopy ²											
		Serum Samples (DPI)										Tissues(L/T/LN) ³	
		-5	0	2	3	4	6	8	15	22	29		32
1	Aab-2	-	-	-	-	-	1	1	-	-	-	-	-/-/-
2	Pig IgG	-	-	1	2	4	5	5	4	2	-	-	+/+/-
3	PBS	-	-	1	2	5	5	5	4	2	-	-	+/+/-

1. Each pig was immunized i.p. with 100mg of Aab-2 or normal pig IgG in 10mls of PBS or PBS along.
2. The values were the mean \pm SDV of 5 pigs in each group.
3. L: lung; T: tonsil; LN: tracheobronchial lymph nodes.

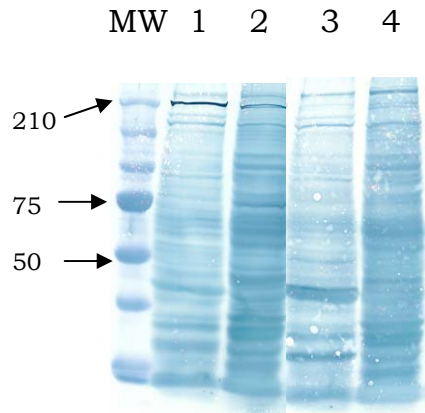


Figure 1. Western blot immunoassay of auto-anti-Id recognition of cellular proteins from Marc-145 cells (lane 2 and 4) and porcine alveolar macrophages (lane 1 and 3). Lanes 1 and 2 was reacted with auto-anti-Id and lanes 3 and 4 reacted with normal pig IgG. Molecular marker (MW) is indicated in KDa.

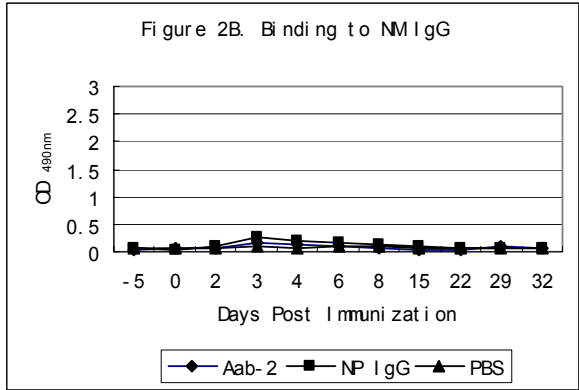
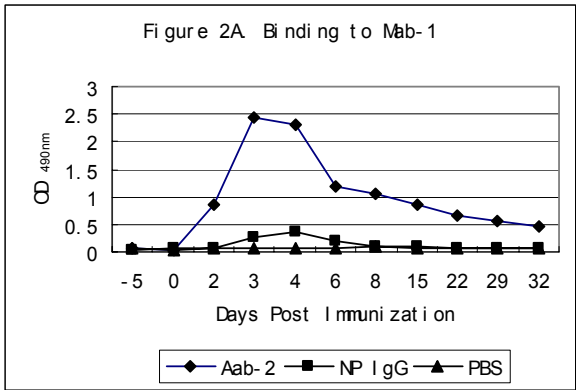


Figure 2. Detection of passive transferred Aab-2 from pigs immunized with Aab-2. The values represent the mean + 3 SDV from 5 pigs in each group. Group 1-3 pigs were immunized with Aab-2, normal mouse IgG and PBS, respectively.