RESEARCH REPORT
SWINE HEALTH

Title: Immunity against PRRS virus – what is the role of 'auto-anti-idiotypic' antibody? - **NBP#02-015**

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Abstract: The antibodies against the envelope (Gp5) and matrix (M) proteins of porcine reproductive and respiratory syndrome virus (PRRS) neutralize the virus in vitro. Auto-anti-idiotype against the monoclonal anti-Gp5 and anti-M antibodies (Mab1s) was identified and purified from pigs experimentally infected with PRRSV. Auto-anti-idiotype was detected from serum samples collected at relatively early days post infection (DPI). Serological characterization indicates that auto-anti-idiotype recognized the idiotypes on Mab1s against the Gp5 and M proteins of PRRSV, but not on Mab1s against N protein, and blocked the Mab1s from binding to PRRSV-infected cells. We also found that if the auto-anti-idiotype developed at earlier DPI, the animals would clear the virus. In contrast, if it developed at later DPI, the animals would likely become persistently infected. These findings indicate that auto-anti-idiotype may recognize the idiotype located within or near the antigen-binding sites of Mab1s and should possess the virus neutralizing activity through binding potential viral receptor(s) on the cell. These results support further studies of developing anti-idiotype vaccine candidates against PRRSV.

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.

Results generated recently in our laboratories suggested a difference in the antibody responses between pigs that cleared the virus and pigs that remained persistently infected. Specifically, pigs experimentally infected with PRRSV produced not only anti-PRRSV antibodies, but also anti-anti-PRRSV antibodies. These anti-anti-PRRSV antibodies are referred to as auto-anti-idiotypic antibodies (auto-Ab2). In our research, the appearance of auto-Ab2s was associated with clearance of the virus. That is, pigs that produced auto-Ab2s earlier in the course of infection also cleared the virus faster.

The concepts behind auto-Ab2 require some explanation. An auto-Ab2 response occurs in individuals exposed to an antigen such that an anti-antigen (Ab1) response is generated that results in the induction of an auto-Ab2 produced against the individual's own Ab1. The concept of a series of Ab1-auto-Ab2 reactions was originally proposed by Jerne, which may serve as a feedback mechanism to control the immune responses. For example, an auto-Ab2 response has been detected when individuals were exposed to specific antigens, i.e., anti-thyroglobulin, trinitrophenylated Ficoll, insulin, and tetanus-toxoid. Perhaps one of the best-studied experimental systems for auto-Ab-2 is the nicotinic acetylcholine receptor (AcR). It has been demonstrated in rabbits that immunization with bis (a-trimethylammonio)-methy azobenzene (Bis Q), a potent agonist of the AcR, when coupled to BSA, can induce an auto-Ab2 response. In rabbits that produced the auto-Ab2 an experimental form of myasthenia gravis was induced. These findings directly implicated the induction of an anto-Ab2 with the development of an autoimmune disease. Auto-Ab2 response has also been shown in humans after infection with hepatitis B virus.

Objectives: The overall objective is to understand PRRSV immunity. Our goal is to examine auto-Ab2s in PRRSV- infected pigs and determine its role in eliminating the infection. In particular, our objectives are 1) to determine the relationship between the appearance of auto-Ab2s and clearance of virus in pigs; 2) serologically characterize auto-Ab2s purified from pigs; and 3) determine specific functions of auto-Ab2s by examining their ability to bind the cell surface and/or block the PRRSV infection of cells *in vitro*.

Materials and Methods:

1. Detection of Auto-Ab2 from Pigs Experimentally Infected with PRRSV.

To examine the appearance of auto-Ab2 in pigs following infection with PRRSV, sequential serum samples from a group of 60 PRRSV-inoculated pigs and 60 uninoculated controls collected at 0, 7, 14, 21, 35, 60, 90, and 120 DPI will be tested for the presence of auto-Ab2 using Mab1s against Gp5 and M antigens, along with normal mouse IgG. The swine serum samples are already available and were originally developed through NPPC Project #98-239. As described in the Final Report to NPPC (98-239: PRRS Eradication pilot study. I. Performance of diagnostic assays in identifying PRRS virus carrier animals), the carrier status of each animal is known and can be compared with the ontogeny of auto-Ab2 in the individual.

2. Serological Characterization of Auto-Ab2.

Once we have identified the presence of swine serum auto-Ab2, each type of auto-Ab2 against Mab1 anti-Gp5 and anti-M antibody will be purified using an affinity chromatography technique in which normal mouse IgG and the individual Mab1 IgG will be coupled to cyanogens bromide activated Sepharose 4B gel by a method described previously. By passing through the normal mouse IgG column, all swine anti-mouse IgG antibodies will removed. Then, by passing through the individual Mab1 column, the individual specific auto-Ab2 to each Mab1 will be purified and characterized along with the unbound portion of swine antiserum using various immunoassays as described below. Characterization will be based on (i) direct interaction between the auto-Ab2 and Mab1s using an ELISA; (ii) inhibition of interaction between Mab1 and auto-Ab2 by various swine anti-PRRSV antibodies using an ELISA; and (iii) ability of various auto-Ab2 to inhibit Mab1-PRRSV interaction using indirect immunofluorescence assay (IFA).

ELISA: An indirect ELISA will be used to detect auto-Ab2. The procedure will involve coating an ELISA plate with anti-Gp5 and anti-M, along with normal mouse IgG. Individual purified auto-Ab2 and unpurified swine antisera will be added to the Mab1-coated plate and goat anti-swine IgG conjugated to horseradish peroxidase (HRP) will be used to detect the presence of auto-Ab2.

To determine if auto-Ab2 recognizes a predominant idiotype on swine anti-PRRSV antibodies, affinity purified individual auto-Ab2 will be coated on an ELISA plate. Individual Mab1 will be mixed with individual swine anti-PRRSV antiserum collected from pigs that either cleared the virus or became persistently infected after the experimental infection with PRRSV. The mixture will then be added into a well of the ELISA plate. A goat anti-mouse IgG conjugated to HRP will then be used to detect the binding of Mab1 to the auto-Ab2.

IFA: This assay will be performed using the procedure routinely conducted in our laboratory. To detect the ability of auto-Ab2 to inhibit the binding of Mab1s to the viral antigen, individual Mab1 will be premixed with individual auto-Ab2 before interacting with the MARC-145 cell line infected with PRRSV. The binding of Mab1s to the viral antigens on the cell will be visualized using fluorescence labeled goat-anti-mouse IgG. We propose here to use IFA, rather than ELISA, simply because the amount of the M and Gp5 antigens expressed in cell culture is not sufficient to be used in an ELISA.

3. Examine the Binding of Auto-Ab2 to the Cell Surface by IFA:

The IFA procedure will be the same as that described above except that the cell line will not be infected with PRRSV and the fluorescence labeled goat-anti-swine IgG (H+L) will be used to detect the binding of auto-Ab2. To eliminate the potential cell surface biding of the auto-Ab2 IgG through its Fc portion, the auto-Ab2 antibody molecules will be cleaved using pepsin and papain to generate $F(ab')_2$ and Fab, respectively. These will be used in the assay to determine if the auto-Ab2 binds the cell surface, presumably by mimicking the viral antigen. Swine IgG molecules, $F(ab')_2$, and Fab fragments generated from normal swine serum and anti-PRRS antibodies will be used as controls.

4. Determine if the Auto-Ab2 Blocks PRRSV Infection.

The standard SN assay will be performed to examine if the auto-Ab2 blocks the PRRSV infection of MARC-145 cell line. The intact auto-Ab2 molecules, $F(ab')_2$ and Fab fragments and control IgG and its fragments will be reacted with the cell line which will then be infected with PRRSV strain ISU-P. The results will be shown by the cytopathologic effect of the cells induced by PRRSV infection. If the auto-Ab2 can block

the virus infection, there will be a marked reduction in the cytopathic effect in the cells pretreated with the auto-Ab2 as compared to the untreated controls.

Results:

1. Detection of Auto-Ab2s:

A group of 529 sequential serum samples collected from 5 groups of 59 pigs experimentally infected with PRRSV and 540 samples from 60 age-matched control pigs were used to evaluate the ELISA for detection of auto-Ab2s against $F(ab')_2$ fragments of Mab1s. To generate a background binding value of swine serum samples, normal mouse IgG $F(ab')_2$ fragments were used to coat ELISA plates and reacted with the 1069 swine sera. As measured by OD_{450} nm, the average OD value was 0.152 with STDEV of 0.975 and a confidence interval of 99.4%. Therefore, OD_{450} of 0.4 was used as the cut off value. With this cut off values, no auto-Ab2s against Mab1 anti-N were detected from these swine serum samples. In contrast, from 529 serum samples collected from 5 groups of 59 pigs, 199 were considered positive and 330 samples were negative for auto-Ab2s against Mab1 anti-Gp5. The 199 Aab-2 positive serum samples were from 5 groups of 47 pigs at DPI between 21 and 105 whereas most of negative samples were collected from DPI between 0 and 14. As shown in Table 1, the number of pigs with auto-Ab2s identified from Group 1 to Group 5 pigs was summarized as follows: 17 samples from 8 pigs, 24 samples from 8 pigs, 50 samples from 12 pigs, 46 samples from 9 pigs and 61 samples from 10 pigs, respectively. Two Aab-2 binding peaks were observed at relatively early (35 DPI) and later (77 DPI). The early auto-Ab2s were from 8 pigs between Group 2 and Group 5 pigs that were free of PRRSV and the late auto-Ab2s were from pigs that were virus carriers. The additional early auto-Ab2s were detected from two pigs of Group 1, which were determined as virus carrier when euthanized at 63 DPI.

Similar results were seen for Auto-anti-idiotypes against Mab1 anti-M except that additional 11 positive samples (total of 210) were detected from DPI between 21 and 91.

2. Characterization of Auto-Ab2s:

The auto-Ab2s recognized idiotype shared by mouse and swine anti-PRRSV: one of the characteristics of Ab-2 that possess internal image of antigen is the idiotype they recognize is shared by the same antibodies from different species. In an inhibition ELISA, we demonstrated that the interaction was inhibited between Aab-2 and Mab1 anti-Gp5 (85-89%) or Mab1 anti-M (85-87%) by four swine polyclonal anti-PRRSV antisera known of lacking of auto-Ab2s from four individual pigs. The specificity of the inhibition was demonstrated by the observation that the control swine serum sample had no effect on the interaction (inhibition <14%). The binding between swine auto-Ab2s and Mab1 anti-Gp5 or Mab2 anti-M was also blocked by the mouse Ab-2s as the idiotype-specific manner, i.e., mouse Ab-2s against Mab1anti-Gp5 inhibited the interaction between auto-Ab2s and Mab2 anti-Gp5 but not between Aab-2 and Mab1 anti-M or vice versa. The mouse presera did not inhibit the interaction (inhibition <17%). These results further demonstrated that the idiotype on anti-PRRSV antibodies was predominant that induced auto-Ab2s and mouse Ab-2s.

The idotypes were located within or near the antigen-combining sites: another characteristic of internal image Ab-2 is that they mimic the antigen. In the inhibition ELISAs, the interaction between Aab-2 and monoclonal anti-PRRSV antibodies were inhibited by PRRSV antigen (75-78% inhibition). The specificity of the inhibition was confirmed since the cell control antigen did not inhibit the interaction (inhibition <14%).

Discussion: Antibody responses to PRRSV result in the formation of verity of antibodies against N, GP5, M and other structural and non-structural proteins. The anti-N, anti-GP5 and anti-M antibodies were detected in the serum samples collected from 59 pigs experimentally with PRRSV as early as 7 or 14 DPI and in the serum samples from the same pigs, the auto-Ab2s were also detected to anti-GP5 and anti-M antibodies starting at DPI 21 and up to DPI 105. Auto-Ab2s antibody responses have been identified in numerous systems suggesting the existence of idiotype-anti-iditoype complex in a given antibody response even though Aab-2 against a single monoclonal anti-N antibody was not identified in this study.

The results from this study demonstrated different time frames of the onset of the auto-Ab2s against anti-GP5 and anti-M antibodies between pigs that were free of the virus and that became virus carriers. Following the anti-GP5 and anti-M antibodies were identified in pigs free of the virus (at 14 DPI), the auto-Ab2s were identified starting at DPI 21, peaked at DPI 35 and diminished at DPI 49 or 63. In contrast, the auto-Ab2s from virus carrier pigs were not identified until DPI 49, peaked at DPI 77 and declined thereafter. The early auto-Ab2s were identified from two pigs that were considered the virus carriers since PRRSV was isolated from these pigs when they were euthanized at 63 DPI. This contradictory could be explained that it is possible that these pigs might be free of the virus at later DPI. In fact, all 11 pigs in this group were identified as virus carriers and all pigs free of the virus were identified from other 4 groups of pigs. This fact indirectly suggests that pigs may not clear the virus until 77 days post infection. Therefore, it is reasonable to conclude that pigs developed the auto-Ab2s at relatively early stage of the infection with PRRSV may become free of the virus.

The idiotype were shared by mouse and swine anti-PRRSV antibodies since the interaction between auto-Ab2s and the murine Ab-1s was inhibited by the swine polyclonal Ab-1s and swine auto-Ab2s and murine Ab-2s recognized the same or similar idiotype since their interaction with murine Ab-1s was inhibited by each other. Together, these findings along with the fact that PRRSV antigens inhibited the Ab-1-Aab-2 interaction indicated that the idiotype is located within or near the antigen-combining sites and that at least part of the swine auto-Ab2s and murine Ab-2s possessed the characteristics of internal image anti-idiotype.

When studying the passive immunity against PRRSV, researchers have shown both in vitro and in vivo the antibody-dependent enhancement of PRRSV infection when using swine polyclonal antibody preparation at the sub-neutralizing concentration. It is also demonstrated the fully protection of pregnant gilts from PRRSV infection after received large amount (105 g/pig) of polyclonal anti-PRRSV antibodies, which had the neutralizing titre of 1:256, produced from pigs multiply infected and challenged with several PRRSV stains. These studies suggest that the possible involvement of the auto-Ab2s against anti-GP5 and anti-M antibodies in protection or enhancement of PRRSV infection. Further studies on anti-PRRSV antibody and anti-idiotypic antibody responses may be necessary for understanding the immune responses against PRRSV infection and development of strategy to control 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, we discovered the presence of serum auto-anti-idiotypic antibodies in pigs experimentally infected with PRRSV. More importantly, clearance of virus from these animals was associated with the earlier appearance of auto-anti-idiotypic antibodies post infection. Our goals were 1) to determine the relationship between the appearance of auto-anti-idiotypic antibodies and clearance of virus in pigs; 2) serologically characterize auto-anti-idiotypic antibodies purified from pigs; and 3) determine specific functions of auto-anti-idiotypic antibodies by examining their ability to bind the cell surface and/or block the PRRSV infection of cells *in vitro*. This proposal represents a new approach, which if successful, would represent a major advance both in terms of understanding the pigs' immunological response to PRRSV infection and providing new tools to bring an alternative anti-idiotype vaccine to the field.

Unfortunately, the proposal was only funded for one year (proposed for 2 years). We were only able to identify and characterize the auto-anti-idiotypic antibodies.

Table 1. Detection of PRRSV, anti-PRRSV antibodies and Aab-2s from swine serum samples

Groups of Pigs[a]	Samples collected at DPI	PRRSV[b]	Anti-N[c] / Aab-2	Anti-GP5[c] / Aab-2	Anti-M[c] / Aab-2
Group 1: euthanized at 63 DPI	0	0[d]	0/0	0/0	0/0
	7	11	3/0	0/0	0/0
	14	11	11/0	5/0	4/0
	21	11	11/0	10/0	10/2
	35	10	11/0	11/2	11/2
	49	0	11/0	11/7	11/7
	63	11[e]	11/0	11/8	11/8
Group 2: euthanized at 77 DPI	0	0	0/0	0/0	0/0
	7	12	5/0	0/0	0/0
	14	12	12/0	4/0	5/0
	21	12	12/0	9/1	8/1
	35	9	12/0	12/1	12/1
	49	2	12/0	12/8	12/8
	63	0	12/0	12/8	12/8
	77	11[5]	12/0	12/7	12/8
Group 3: euthanized at 91 DPI	0	0	0/0	0/0	0/0
	7	12	4/0	0/0	0/0
	14	12	12/0	6/0	5/0
	21	12	12/0	11/4	9/5
	35	7	12/0	12/4	12/6
	49	0	12/0	12/12	12/11
	63	0	12/0	12/12	12/12
	77	0	12/0	12/9	12/12
	91	11[5]	12/0	12/9	12/9
Group 4: euthanized at 98 DPI	0	0	0/0	0/0	0/0
	7	12	4/0	0/0	0/0
	14	12	12/0	6/0	5/0
	21	12	12/0	11/2	9/3
	35	2	12/0	12/2	12/8
	49	1	12/0	12/9	12/9
	63	0	12/0	12/9	12/9
	77	0	12/0	12/9	12/9
	91	0	12/0	12/8	12/9
	98	10[5]	12/0	12/7	12/6
Group 5: euthanized at 105 DPI	0	0	0/0	0/0	0/0
	7	12	6/0	0/0	0/0
	14	12	12/0	4/0	4/0
	21	12	12/0	10/1	11/1
	35	3	12/0	12/1	12/4
	49	1	12/0	12/9	12/8
	63	0	12/0	12/10	12/9
	77	0	12/0	12/10	12/10
	91	0	12/0	12/10	12/10
	98	0	12/0	12/10	12/8
	105	11[5]	12/0	12/10	12/7

a. Each group consisted of 12 pigs.
b. PRRSV was detected by the standard virus isolation method from serum samples collected from sequential samples.
c. Anti-N antibodies were detected by the HerdChek ELISA (IDEXX Laboratories Inc., Westbrook, MA). Anti-GP5 and anti-M antibodies were detected by an inhibition IFA in which swine serum samples were used to inhibit Mab-25C and Mab-19B, respectively, binding to PRRSV in the infected MARK-145 cells.
d. The numbers represented total positive number of pigs.
e. Virus isolation was performed from oropharyngeal scrapings and tonsil samples collected from euthanized pigs (Horter et al. 2002).