Antibody responses to the *Bordetella pertussis* virulence factor P.69 Pertactin

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Chapter 6

Abstract

*Bordetella pertussis*, the causative agent of whooping cough, is re-emerging in several countries with a traditional high vaccine uptake. Analysis of clinical isolates revealed antigenic divergence between vaccine strains and circulating strains with respect to the *B. pertussis* virulence factor P.69 Pertactin. Polymorphisms in P. 69 Pertactin are mainly limited to regions comprised of amino acid repeats, designated region 1 and region 2. Region 1 flanks the RGD motif involved in adherence. Although antibodies against P. 69 Pertactin are implicated in protective immunity, and monoclonal antibodies against the variable regions have been shown to be protective, until recently, little was known about antibody specificity against Pertactin. Using P. 69 Pertactin variants and deletion derivatives, we investigated the immune response against the native protein, and against deletion derivatives. The N-terminus of P. 69 Pertactin was found to be immunodominant in both rabbits and humans. P. 69 Pertactin type specific antibodies were detected in rabbits, but not in humans. Furthermore, we found evidence for a role of the N- and C-termini and the variable region 1 in the evasion of antibody responses by conformational masking of epitopes. These results shed new light about the role of the variable regions and the N- and C-termini in the evasion of antibody responses.
Introduction

Despite high vaccine coverage, the causative agent of whooping cough (pertussis) has re-emerged in several countries. Several explanations have been suggested for the re-emergence of pertussis including antigenic divergence between vaccine and circulating strains. Antigenic divergence of P69 Pertactin (P69 Prn) has been suggested to be one of the causes for the return of pertussis in the Netherlands.

The role of P69 Prn in immunity to pertussis has been well documented. Antibody levels to P69 Prn have been shown to correlate with clinical protection. Passive and active immunization studies in mice and pigs have shown that antibodies (Abs) against P69 Prn confer protective immunity. Anti-P69 Prn Abs, but not anti-Ptx, anti-fimbriae, or anti-FHA Abs, were found to be crucial for *B. pertussis* phagocytosis. Recently it was described that P69 Prn induces type-specific Abs in humans. Furthermore, the efficacy of the Dutch whole cell vaccine was affected by variation of P69 Prn in a mouse model. Finally, acellular vaccines (ACV’s) containing Ptx, FHA and P69 Prn were more effective compared to ACV’s containing Ptx and FHA only.

P69 Prn, an autotransporter protein, is located at the cell surface of *B. pertussis*. P69 Prn is polymorphic but variation is mainly limited to two regions, designated region 1 and 2, which are comprised of Gly-Gly-X-X-Pro and Pro-Gln-Pro repeats, respectively. A total of 13 P69 Prn types have been isolated. In the Netherlands and Finland, the 3 most common *B. pertussis* P69 Prn types are P69 Prn1, P69 Prn2 and P69 Prn3. P69 Prn1 and P69 Prn3 only differ in 2 aminoacids (AA) in 1 of the repeats in region 1, whereas P69 Prn2 contains an additional GGFGP repeat (Fig. 1A). The variable regions 2 of P69 Prn1, 2 and 3 are identical (Fig. 1A). P69 Prn contains an N-terminally located Arg-Gly-Asp (RGD) motif, implicated in ligand-receptor interactions in eukaryotes, and most variation is found adjacent to this site. The second variable region, region 2, is located at the C-terminus of P69 Prn (Fig. 1A).

In a previous study, we showed that P69 Prn induces type-specific Abs in humans. However, since purified P69 Prn2 and P69 Prn3 were not available at that time, an indirect approach was used to identify P69 Prn-type-specific antibodies, involving synthetic peptides and a blocking ELISA. Recently we described the cloning, expression and purification of P69 Prn1, 2 and 3 and several P69 Prn mutants that lack parts of the protein. These proteins were used in this study where we investigated the presence or absence of P69 Prn type-specific Abs in humans and rabbits using a direct approach. In this direct approach we isolated the Abs of interest and tested their specificity. Based on the results described in this study, we propose an adjusted hypothesis about the effects of variation in Prn on the antibody response to Prn.
Materials and Methods

Recombinant Pertactin

P.69 Pertactin variants and Prn deletion mutants were expressed and refolded as described, except that 200 mM of L-Arginine was added to the first refolding buffer (1 mM EDTA, 100 mM NaCl, 50 mM Tris/HCl pH 8.8) resulting in higher refolding efficiencies 29.

Rabbit polyclonal anti-sera

Polyclonal rabbit anti-P.69 Prn1, 2 and 3 sera were raised at the Eurogentec facility (Eurogentec, Seraing, Belgium). New Zealand white Rabbits (n=3) were immunized 3 times with 50 µg of purified P.69 Prn per immunization using Freund’s complete adjuvant for the first immunization, followed by two boosts using Freund’s incomplete adjuvant.

Human sera

A set of paired sera from Finnish individuals were selected which included vaccine recipients and patients 22. Sera from 11 patients infected by Prn2 B. pertussis strains, 5 sera from patients infected with Prn3 B. pertussis strains, 10 sera from patients infected with B. parapertussis, and 10 sera from adolescents after booster vaccination with the GSK ACV (Boostrix) were selected. The antibody response of these sera to purified P.69 Prn1, 2, 3 and the Prn-R1-KO (lacking the variable region 1) was tested in ELISA as described previously 22, and a subset was used in the depletion assay.

Furthermore, a set of 21 sera from Dutch patients infected with B. pertussis was selected. Although, in the Netherlands, the P.69 Prn type of the strain causing an infection is not routinely characterized, surveillance studies have shown that over 90% of the strains are of the P.69 Prn2 type. The children were vaccinated at the age of 3, 4, 5, and 11 months with DTP-IPV which at that time contained the whole-cell pertussis vaccine from the Netherlands Vaccine Institute (NVI, Bilthoven, the Netherlands). The whole cell vaccine is derived from strains which produce P.69 Prn1 30. The children received a booster immunization with acellular vaccine containing 25µg FHA, 25µg PT and 8µg of P.69 Prn1 (Infanrix, Glaxosmithkline, Rixensart, Belgium) at the age of four years. The response of these sera to several P.69 Prn variants and deletion mutants was tested in ELISA and in a depletion assay.

Depletion of Sera

Human and rabbit sera were diluted 1:40 and 1:100 with PBS, respectively. Purified Histagged recombinant P.69 Prn or PBS was added to a final concentration of 25µg/ml. Sera were incubated overnight at 22°C under constant rocking. After incubation, possibly formed P.69 Prn-Ab complexes were removed by centrifugation for 15 min at 15,000 g. Additional purified recombinant His-tagged P.69 Prn was added to the supernatant to a concentration of 25 µg/ml, after which the sample was incubated for 1 hr at 22 °C while rocking. Complexes were removed by an additional centrifugation step at 15,000g for 15
min. Since not all of the Prn-Ab complexes were removed by centrifugation, a 1:20 volume of washed magnetic Ni\(^{2+}\) beads (His-Mag-Beads; Novagen) was added to the supernatant to remove the final Prn-Ab complexes via the His-tag of the recombinant Prn. After incubation at 22°C for 15 min, the beads were removed and the supernatant was used in ELISA.

ELISA
The binding of Abs to the recombinant proteins was tested in an ELISA as described previously. Polystyrene 96-well ELISA plates (Immulon II; Dynatech, Chantilly, Va.) were coated overnight at 22°C by adding 100 µl of 0.04 M carbonate buffer pH 9.6 containing 2 µg of protein/ml to each well. The plates were washed four times with 200 µl of PBS supplemented with 0.05% Tween 20 (PBST) per well by using a Titertek Plus M96V washer (ICN, Irvine, CA.). Sera were added to the wells and were incubated for 2 h at 37 °C followed by four washings as described above. Bound rabbit Abs were detected with alkaline phosphatase conjugated goat anti-rabbit total IgG (DakoCytomation, Glostrup, Denmark). Bound human Abs were detected with alkaline phosphatase conjugated goat anti-human total IgG (Sigma, Saint Louis, MO). The optical density at 405 nm (OD405) was measured with a plate reader (EL312e; BioTek Systems, Winooski, Vt.). To verify that the equal amounts of protein were coated in the ELISA assays, in all of the ELISA experiments, mAb PeM72 was used as a control. MAb PeM72 is directed against a linear epitope that is located in R1, and therefore does not react with mutants in which this region is deleted (Prn-R1-KO and Prn-R1+R2-KO).

Structure prediction
The published crystal structure of \(\text{P.69 Prn1}\) lacks the C-terminal AA residues 540-677. The tertiary structure of the passenger domain of \(\text{P.69 Prn1} \) (AA 1-677) (CAA09473) was determined using the Robetta server (http://robetta.bakerlab.org) with the published crystal structure (1DAB.pdb) as a reference.

Analysis
To calculate the remaining amount of antibody after depletion, a curve comparison was performed between the PBS depleted samples (un-depleted) and the \(\text{P.69 Prn}\) depleted samples. The OD405-reading of three subsequent data points in the linear range of a graph were multiplied with the corresponding dilution factor. The PBS-curve was set to a 100%. The statistical significance of the observed differences in binding of human serum Abs to \(\text{P.69 Prn}\) variants and its deletion derivatives was determined using a two-tailed T-test.
Chapter 6

Results

From here on, we will use the following nomenclature: P69 Prn1, 2 or 3 are termed Prn1, Prn2 or Prn3 respectively. The Prn variable regions 1 and 2 will be referred to as R1 and R2, respectively. The Prn region 1, 2 and 1+2 knockouts will be termed Prn-R1-KO, Prn-R2-KO and Prn-R1-R2-KO, respectively. The Prn mutants lacking parts of the C- or N-terminus will be referred to as C- and N-terminal Prn mutants, respectively. The rabbit anti-P69 Prn1, 2 or 3 sera will be referred to as P1, P2 or P3 serum, respectively. Furthermore, Abs that bind to all three Prn variants are referred to as cross-reactive Abs. Antibodies that are specific for only one of the P69 Prn types will be referred to as type-specific Abs. All of the data is summarized in Figure 1B.

Depletion of rabbit sera

In human sera, antibody levels against Prn are often low, and only small amounts of serum are available whereas in rabbits it is possible to generate Ab titers that are over a 1,000 times higher than those found in human serum. Therefore we decided to immunize rabbits with various Prn types and investigated their immune responses. After immunization, sera were tested for the presence of type specific Abs using a depletion ELISA. By adding purified Prn to the sera, we aimed to remove all of the cross reactive Abs while preserving the type specific Abs. Since His-tagged recombinant Prn was used, it was possible to remove Prn-Ab complexes by adding magnetic Ni²⁺ beads.

The 3 most common Prn types produced by current B. pertussis strains are Prn1, 2 and 3, we therefore focussed on the Ab responses against these 3 types. The presence of region- or type-specific Abs was investigated by depleting sera with Prn1, 2, 3 and with three deletion mutants that lack either R1, R2 or both variable regions. Furthermore, the Ab responses

Figure 1. (A) Structure of Prn variants and deletion derivatives. Amino acid sequence of the variable region R1 and R2 are indicated. Prn1 and Prn2 differ in 2 amino acids whereas Prn2 contains an additional repeat. The location and size of N-terminal, C-terminal and central deletions are indicated. R1, R2 and RGD indicate the variable regions 1, 2 and the RGD receptor binding motif, respectively. Abbreviations: del, deletion. AA, amino acid. Bp, base pair. (B) The residual activity of anti-Prn1, 2 and 3 rabbit Abs directed against the respective parts of P69 Prn are indicated in gray panels. The fraction of human sera that contain Abs directed against the respective parts of P69 Prn are also indicated in the gray panels. Only 2 of the 39 human sera had Abs whose binding was dependent on the presence of both R1 and R2.
to the N- and C-termini were characterized by depleting sera with N- and C-terminal Prn mutants (Fig. 1A). When the rabbit sera were depleted with Prn mutants, in many cases the reaction of the remaining Abs in a single sample was different to Prn1, 2 and 3 (Table 1 & Fig. 2). For a more straightforward presentation of the results, the range of the reaction to Prn1, 2 and 3 variants will be described in the first paragraphs. In the last paragraph, the difference in binding of depleted sera to Prn1, 2 and 3 will be discussed in more detail.

To verify each depletion, the reaction of the depleted serum was tested on a homologous protein used as coating antigen in ELISA (e.g. depletion of serum with Prn1 and ELISA plates were coated with Prn1). These controls were always negative, indicating that the sera were fully depleted (indicated with “0” in Table 1). Furthermore, as a second control, the reaction of every depleted serum was tested on all of the Prn variants and mutants. All data is summarized in Table 1. No differences were observed in the anti Prn titers of the rabbit sera (n=3), excluding the possibility of artefacts due to non-responder rabbits.

Table 1. Percentage of binding activity after the depletion of rabbit sera

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aRabbit sera that were depleted. bAntigens that were used for depletion of rabbit sera. PBS was used as the undepleted control and the response was set to a 100%. cResponse of depleted sera to the 8 different coating antigens.
Figure 2. Percentage of antibody binding to Prn variants after depletion. Antibodies raised against Prn variants were depleted with Prn variants and Prn3. % of Ab binding (A) Prn1 depletion, (B) Prn-R1-KO depletion, (C) (N-terminal mutant depletion) and (D) (C-terminal mutant depletion). The y-axis indicates the % of antibody binding to Prn variants after depletion and the x-axis indicates the % of antibody binding to Prn variants after depletion to Prn1, Prn2 and Prn3, respectively.
Only Prn3 induces type specific Abs in rabbits

To identify type specific Abs, rabbit P1, P2 and P3 sera were depleted with Prn1, Prn2 and Prn3. After depleting rabbit P1 and P2 sera with either Prn1, Prn2 or Prn3, no Ab response was detected against the three variants, indicating that the sera were fully depleted (Fig. 1A & Table 1). This indicates that the Abs directed to Prn1 or Prn2 are able to bind to all 3 Prn types. However when rabbit P3 serum was depleted with Prn1, a relative large Ab reactivity against Prn2 and Prn3 (>2%) was still detected (Table 1 & Fig. 2A). These are Prn3 type-specific Abs. When the rabbit P3 serum was depleted with Prn2, a much smaller Abs response (0.2%) was detected (not shown in Table 1 due to round off to integers). As expected, depletion of the rabbit P3 serum with Prn3 fully depleted the serum of anti Prn Abs. None of the Prn3-type specific Abs bound to the Prn-R2-KO, R1-R2-KO, N- or C-terminal mutant. Surprisingly, a response of 0.3% of the Prn3-type specific Abs was detected with the Prn-R1-KO (not shown in Table 1 due to round off to integers). This latter result was unexpected since Prn1 and 3 vary only in 2 AA in 1 repeat in region 1. Variation in region 1 apparently also affects the binding of Abs directed to other regions of Prn. Although the percentages seem very low, due to the high Ab titers of the rabbits, the presence of the Prn-type and region specific Abs could accurately be determined.

The immunogenicity of region 1 varies between the 3 main Prn types

Rabbit sera were depleted with the Prn-R1-KO to determine the amount of Abs directed against the variable region 1 of Prn. When rabbit antiserum, raised against Prn1, was depleted with the Prn-R1-KO, only a response of 2-3% was detected against the Prn variants (Table 1 & Fig. 2B). Rabbit serum raised against Prn3 also contained only a small amount of Abs specific for region 1. After depletion, only a response of 3-7% was observed against the Prn variants (Table 1 & Fig. 2B). In contrast, when serum raised against Prn2 was depleted with the Prn-R1-KO a response of 8-18% was detected, indicating that Prn2 induces more Abs, or Abs with a higher affinity against the variable region 1 compared to Prn1 and Prn3 (Table 1 & Fig. 2B).

Region 2 has a low immunogenicity in rabbits

To investigate the amount of Abs directed against R2, rabbit P1, P2 and P3 sera were depleted with the Prn-R2-KO. Subsequently, the ability of remaining Abs to bind to purified Prn and the Prn-deletion mutants was tested. Depletion of rabbit P1 sera revealed that only 0-1% of the Abs response was directed against R2. In P2 rabbit sera, only 1-2% of the Abs response was directed against R2. In the P3 rabbit sera, 1-3% of the Abs response was directed against R2 (Table 1).

The N-terminus is highly immunogenic in rabbits

The rabbit P1, P2, and P3 sera were depleted with a Prn mutant that lacks a large part of the N-terminus. A total of 18-25% and 14-25% of the Abs raised against respectively, Prn1
and Prn3 were directed against epitopes that are dependent on the presence of the first 110 AA (Table 1 & Fig. 1C). Prn2 induced less Abs directed against the N-terminus, 10-17% of the Abs were directed against this region (Table 1 & Fig. 1C).

The C-terminus of Prn3 is more immunogenic in rabbits than that of Prn1 and Prn2
When rabbit P3 serum was depleted with the C-terminal Prn mutant, a total of 3-11% of Abs remained, indicating they are directed against the C-terminus (Fig. 2D). In the rabbit P1 and P2 sera, 3-5% and 2-4% of the Abs were directed against the C-terminus, respectively (Fig. 2D). This could indicate that the C-terminus of Prn3 is more immunogenic.

Differences in binding of serum Abs to Prn variants
After depletion of the rabbit sera with the different Prn variants, the binding of the remaining Abs to Prn1, Prn2 and Prn3 was tested. One would expect the binding response of Abs to be the highest for the antigen they were raised against. In 4 out of 5 cases, the response of rabbit P1 serum Abs to Prn1, 2 or 3 was either equal to all 3 proteins, or higher to Prn1. However, the reaction of rabbit P2 serum Abs was always higher to Prn1 and Prn3 than to Prn2. Furthermore, in 3 out of 5 cases the reaction of rabbit P3 serum Abs was higher to Prn2 than to Prn3. A good example is the result of the depletion of rabbit P2 serum with the Prn-R1-KO. When the reaction of the remaining Abs was tested to Prn1, Prn2 and Prn3, the highest binding response was observed with Prn1 and 3, rather than with Prn2 (Table 1 & Fig. 2B). Another example is the depletion of rabbit P3 serum with the C-terminal Prn mutant. After depletion, the reaction of the remaining Abs to Prn1 was 3-4 times lower than to Prn2 and Prn3 (Table 1 & Fig. 2D). This result was rather unexpected since the C-termini of Prn1, Prn2, and Prn3 are identical (Fig. 1A).

ELISA with human sera and Prn variants
Anti-Prn Abs in the serum from Dutch children
We determined the titers of 21 sera from Dutch children to Prn variants and its deletion derivatives. When the response of a human serum sample to a Prn variants was at least 1.5 times higher or lower compared to the response to Prn1, it was statistically significant (P<0.05).

No significant differences in the response of any of the 21 sera to Prn1, Prn2 or Prn3 were observed. Interestingly, the response of 16 out of 21 sera was significantly higher to the C-terminal Prn mutant compared to the response with Prn1 (P=0.004) (Fig. 3A). Furthermore, the response of 10 out of 21 sera was significantly higher to the N-terminal Prn mutant compared to the response to Prn1 (P=0.0001) (Fig. 3A). From these 10 sera, the reaction of 9 sera was also significantly higher to the C-terminal mutant. Although the response of 3 out of 21 sera showed a decrease in the reaction with the Prn-R1-KO compared to Prn1, none of these decreases was significant (P=0.419). Although the response of 6 out of 21 of the sera to the Prn-R2-KO was lower than to Prn1, none of these responses was significant.
Antibody responses to Pertactin

(P=0.143). No differences were observed in binding of mAb PeM72 to Prn1, Prn2, Prn3, Prn-R2-KO, and the N- and C-terminal mutants. This indicated that the amount of protein coated in ELISA was similar for all proteins (Fig. 3B).

Since the amount of information about the sera was limited, no correlations between Ab responses and e.g. the type of strain causing the infection could be established.

Anti-Prn Abs response of Finnish sera

The response of 36 Finnish sera from patients that were vaccinated with Prn1 or infected with B. pertussis Prn2, Prn3 or B. parapertussis strains was tested to Prn1, Prn2, Prn3 and the Prn-R1-KO. No significant differences in the Ab responses to these variants were found. From 8 of these sera the response was also tested to the Prn-R2-KO, Prn-R1-R2-KO, and N- and C-terminal Prn mutants. Similar as described for the response of the Dutch sera, the response of 7 out of 8 sera to the N-terminal Prn mutant was significantly higher then the response to Prn1. All of the 8 sera showed a significant increase to the C-terminal Prn mutant. Interestingly, 4 of the 8 sera showed a significant decrease in binding to the Prn-R1-R2-KO mutant, but not to the Prn-R1-KO, indicating that Abs directed to region 2, but not region 1 were present. From these 4 sera, 3 were from children that had just been vaccinated (data not shown) indicating that vaccination with Prn1 induces a relatively large amount of Abs directed against region 2. No further correlations were found between the Prn type of the strain causing the infection and the direction of the Ab response.

Depletion of human sera

A set of 39 samples, comprising Dutch and Finnish human sera, were depleted in a similar manner as the rabbit sera. The titers of the human sera varied between 15 and 3,798 EU/ml. Unless stated otherwise, no differences in the reaction of the depleted sera were observed with the different Prn-coating antigens. In 38 out of 39 depletions, the response of the human sera depleted with the N-terminal Prn mutant was similar as the response of the

![Figure 3. Response of antibodies to Prn variants and deletion derivatives. Reaction of (A) Randomly selected human serum and (B) mAb Pem72 to Prn variants and deletion derivatives. The OD405 is indicated on the Y-axes, and the Ab dilution on the X-axes.](image-url)
undepleted (PBS) serum (Fig. 4A+B). This could indicate that a very large amount of Abs is directed against the N-terminus of Prn. Only when the sera that were depleted with the N-terminal Prn mutant were tested with the N-terminal Prn mutant as coating antigen in an ELISA, the response was abolished, indicating that the depletions were successful. The only serum sample that after depletion with the N-terminal mutant had no remaining Abs, had the lowest anti-Prn IgG titer (15 EU) of all the sera tested.

In 6 out of 39 sera, a residual activity of approximately 15% was observed after depletion with mutants Prn-R1+R2-KO and the C-terminal Prn mutant. This response was abolished when either the Prn-R2-KO or the C-terminal mutant was used as coating antigen, indicating that the Abs were directed against R2. Of these 6 sera, 3 were from children that had just been vaccinated. Furthermore, the IgG titers of these 3 sera were among the highest. In 2 of the 39 sera, a response was observed after depletion with the Prn-R1-KO or the Prn-R1-R2-KO (Fig. 4B). When the Prn-R1-KO or the Prn-R2-KO was used as coat in an ELISA, the response was halved. Only when the Prn-R1-R2-KO was used as a coating antigen, the response was abolished. This indicated that this serum contained Abs directed against both variable regions. In contrast to the results obtained with the rabbit sera, depletions of human sera with Prn1, Prn2 or Prn3, fully depleted the sera from Abs (Fig. 4A+B). Besides the observation that vaccination induces a large amount of Abs (15%) against region 2, no correlations were found between the Prn type of the strain causing the infection and the specificity of the Ab response.
Antibody responses to Pertactin

Discussion

There has been a resurgence of pertussis in several countries, including the Netherlands. One of the factors which may have contributed to this re-emergence is the variation in Prn, a protein that has been shown to elicit protective immunity in both animals and humans. Most variation in Prn is found in R1 which is comprised of GGXXP repeats and is located proximal to the N-terminus. Of the 3 most prevalent Prn types, Prn2 is the most distinct since it contains 1 additional repeat. Variation between Prn1 and 3 is limited to 2 AA. In this study we investigated the antibody response of rabbits and humans, immunized or infected with different Prn variants. The results are summarized in figure 1A.

In a first attempt to identify Prn specific Abs, we tested the response of rabbits vaccinated with Prn1, 2 or 3. Using a depletion assay we were able to detect the Abs of interest. Depletion of rabbit P1, P2 and P3 sera, revealed that P2 serum contained 3-4 times more R1 specific Abs compared to P1 and P3 serum (Fig.2B). It is possible that the larger number of repeats in R1 of Prn2 caused this region to become more exposed and therefore more immunogenic compared to the R1 of Prn1 and Prn3. In contrast, rabbit P1 and P3 sera contained almost 2 times more Abs directed against the N-terminus of Prn than anti Prn2 sera (Fig. 2C). We previously hypothesized that the variable R1 acts as a shield that hides important epitopes located in the N-terminus from the immune system. Region 1 appears as a loop that protrudes from the backbone of Prn, and folds towards the N-terminus. The R1 of Prn1 covers at least parts of 4 loops in the N-terminus (Fig. 5). It is likely that the increased size of the Prn2-R1 covers an even larger part of the N-terminus than the R1 of Prn1 and 3 (Fig. 5). The observation that Prn2 induces more Abs directed against R1, but less to the N-terminus (compared to Prn1 and Prn3), supports this line of reasoning. When rabbit P2 serum was depleted with the Prn-R1-KO, and subsequently the reaction of the remaining Abs was tested to Prn1, Prn2, and Prn3, the lowest response was observed with Prn2 (Fig. 2B). Apparently Prn2 evolved a way to circumvent the increased immunogenicity of its R1. The amount of binding energy needed for an interaction between a highly flexible loop and an Ab is likely to be larger than the amount of energy needed for an interaction between a conformationally stable defined epitope and a mAb. It is possible...
that R1, due to its increased size is more flexible and that therefore Abs pay a significant entropy penalty to bind to this flexible region. This could explain the observation that the reaction of P2-R1 Abs is higher to the R1 of Prn1 and Prn3 then that of Prn2, since the amount of energy needed for this interaction is possibly lower.

Pertactin type specific Abs were only observed when rabbit P3 serum was depleted with Prn1. Interestingly, these Abs were unable to bind to any of the Prn deletion mutants. This was rather unexpected since Prn1, Prn2 and Prn3 only vary in R1. This observation however, is in line with our previously described hypothesis that the two variable regions interact \(^\text{31}(\text{Hijnen et al. Submitted})\). However, due to structural limitations this cannot be a direct interaction. It is more likely that the N- and C-termini are interacting and the variation in R1 and R2 indirectly affects this interaction.

Due to the close proximity of R1 (located in the N-terminus) and the C-terminus (Fig. 6), it is possible that Abs are induced that recognize a conformational, type-specific, epitope that is comprised of both R1 and the C-terminus. It is conceivable that when either R1, R2, the N- or C-terminus are deleted, the conformational epitope is destroyed, explaining that Prn3-specific Abs are not able to bind to either one of these deletion mutants.

To test whether similar antibody responses were induced in humans after infection or vaccination, identical experiments were performed with human sera. When the binding of Abs from human sera were tested to Prn1, Prn2 and Prn3 in a regular ELISA, no differences were measured. In contrast to the rabbit sera, depletion of human serum with Prn1, Prn2 or Prn3 did not reveal Prn type specific Abs. Possibly, Prn specific Abs are not induced in humans, or the amount of these Abs is so small that they are not detectable. Even if Prn-type-specific Abs are induced in humans, the role of the Abs is questionable. We were only able to detect Prn-specific Abs in Prn3 immunized rabbits whereas humans are vaccinated with Prn1, which did not induce Prn-specific Abs in rabbits. Furthermore, infections with Prn3 strains are very rare.

When we tested the response of human sera to N- and C-terminal mutants, an unexpected result was found. From the 30 sera tested, 24 sera reacted significantly better to the C-terminal mutant, and 17 of the 30 sera reacted significantly better to the N-terminal mutant compared to their reaction to Prn1 (Fig. 2A). This result seems to contrast with the results obtained from the human serum depletion assay. In 38 out of the 39 cases when a human serum was depleted with the N-terminal mutant, almost all of the Abs remained present. In contrast, only in 6 of the 39 depletions with the C-terminal mutant, Abs remained present. These results may indicate that deletion of the N-terminus results in the exposition of additional epitopes allowing an increased Ab binding. Apparently, some regions of Prn are immunogenic, but not very well accessible to antibodies in native Prn. The second result however, suggests that a large amount of Abs is directed against the N-terminus since depletion with this mutant did not result in a decrease of Ab binding. The latter result is consistent with one of our previous studies where 6 of the 17 mapped epitopes recognized by human sera were located in the N-terminus of Prn \(^\text{31}\). Although these results
Antibody responses to Pertactin

seem contradictory; they could be explained as follows. The N-terminus harbors important discontinuous conformational epitopes to which Abs with a high affinity are induced (Hijnen et al. Submitted). Abs binding to this region block the subsequent binding of other Abs. When in an ELISA the response of sera is tested to the N-terminal mutant, the binding of Abs to other parts of Prn is not blocked by Abs directed against the N-terminus, resulting in a higher response. When the N-terminal mutant is used in a depletion assay, the Abs directed against the discontinuous conformational epitopes in the N-terminus remain present in the sample. When subsequently the depleted sample is tested to Prn variants, still a large response is measured.

It is possible that a similar mechanism as just described for the N-terminus is applicable for the C-terminus. However, in contrast to the N-terminus, only a small amount of human sera contained Abs directed against the C-terminus. Although the tertiary structure of the C-terminus has not been determined, structure prediction programs predicted it to be a flexible linear coiled region. It is conceivable that the C-terminus folds into the direction of the N-terminus, thereby covering a large part of the molecule. This hypothesis was confirmed when the tertiary structure of P.69 Prn1 including the C-terminus was predicted by the Robetta server (Fig. 6). We previously showed that the center and the N-terminus of Prn harbor several conformational epitopes to which Abs with a high affinity are induced. It is possible that the C-terminus blocks the binding of these Abs. When the C-terminus is deleted, the binding of Abs directed against the remainder of the protein will not be blocked, resulting in a higher response then obtained with the entire protein.

A second explanation remains for the results obtained with the N-terminal Prn mutant. It is possible that the tertiary structure of the N-terminal mutant is affected by the deletion

Figure 6. The predicted tertiary structure of Prn including the C-terminus. The tertiary structure of the entire passenger domain of P.69 Prn1 was determined by the Robetta server with the published crystal structure as a reference.
of the 110 N-terminal AAs. This could result in the inability of Abs directed against conformational epitopes to bind. A depletion assay with this mutant could result in the depletion of Abs directed to linear epitopes. When subsequently the binding of this depleted serum was tested to Prn1, Abs directed against conformational epitopes would be able to bind. This would indicate that antibodies directed against linear epitopes are induced in large numbers, which block the binding of antibodies directed against conformational epitopes. The latter are often considered to be more important. However, it was possible to partly deplete rabbit sera with this mutant, which speaks against this hypothesis. Together, these and previously described results, explain the effects of variation in region 1 on the antibody response. The variation does not only affect the Ab response directed against region 1 as suggested by us previously, but rather affects Ab binding to the N-terminus of Prn. As previously described, Prn2 infected patients do not develop Abs that block the binding of mAbs PeM1 and PeM5 to Prn. Recently, PeM5 was found to recognize an N-terminal loop as its primary binding site and not a strictly R1 dependant epitope. The binding of PeM1 was found to be affected by deletion of parts of the N- and C-terminus, but not by deletion of region 1. However mutation of the RGD site led to an increase and decrease in binding of PeM1 and 5 respectively, suggesting the epitopes of these mAbs are located in the vicinity of region 1. Since the Prn2-region 1 is larger than that of Prn1 and 3, no, or less Abs are induced against the epitopes recognized by mAbs PeM1 and 5, which could explain that no blocking was observed due to improved conformational masking. Depletion of rabbit sera showed that, compared to Prn1 and 3, Prn2 induces more Abs against region 1, and less Abs directed to the N-terminus. These results support our hypothesis that variation in region 1 affects the Ab response directed against the N-terminus.

It is feasible that Prn1 induces antibodies to epitopes that are present in Prn2 but are not exposed due to the conformational masking by the larger region 1 of Prn2. Therefore, in theory, immunization with Prn2 would be more efficient, as this would not induce Abs to epitopes that are hidden in Prn1 or Prn2. These results could facilitate the development of new pertussis vaccines that direct the Ab response to epitopes that are exposed in all Prn variants.
Antibody responses to Pertactin

References


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