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Epitope Structure of the *Bordetella pertussis* Protein P.69 Pertactin, a Major Vaccine Component and Protective Antigen

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***Bordetella pertussis* is reemerging in several countries with a traditionally high vaccine uptake. An analysis of clinical isolates revealed antigenic divergence between vaccine strains and circulating strains with respect to 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, which is involved in adherence. Although antibodies against P.69 pertactin are implicated in protective immunity, little is known about the structure and location of its epitopes. Here we describe the identification by pepscan analysis of the locations of mainly linear epitopes recognized by human sera and mouse monoclonal antibodies (MAbs). A total of 24 epitopes were identified, and of these only 2 were recognized by both MAbs and human antibodies in serum. A number of immunodominant epitopes were identified which were recognized by 78 to 93% of the human sera tested. Blocking experiments indicated the presence of high-avidity human antibodies against conformational epitopes. Human antibodies against linear epitopes had much lower avidities, as they were unable to block MAbs. Pepscan analyses revealed several MAbs which bound to both region 1 and region 2. The two regions are separated by 289 amino acids in the primary structure, and we discuss the possibility that they form a single conformational epitope. Thus, both repeat regions may serve to deflect the immune response targeted to the functional domain of P.69 pertactin. This may explain why the variation in P.69 pertactin is so effective, despite the fact that it is limited to only two small segments of the molecule.**

In the prevaccination era, pertussis was a major cause of infant death throughout the world. The introduction of effective pertussis vaccines 50 years ago led to dramatic reductions in morbidity and mortality. However, there has been a resurgence in the incidence of pertussis in the last 10 years in several countries, despite a high vaccine uptake (16, 25). Waning immunity in adolescents and adults, increased reporting, improved diagnosis of the disease, and the emergence of *Bordetella pertussis* escape variants are all proposed explanations for the reemergence of pertussis (3, 26). In The Netherlands, the emergence of escape variants has probably played an important role in the resurgence of pertussis (25, 26). An analysis of clinical isolates collected in the last 50 years revealed antigenic divergence between vaccine strains and circulating strains. Escape variants showed polymorphisms in at least two proteins implicated in protective immunity, namely P.69 pertactin (P.69 Prn) and pertussis toxin (Ptx) (25, 32). The level of antibodies to both P.69 Prn and Ptx have been shown to correlate with clinical protection (6, 30). Furthermore, acellular vaccines (ACVs) containing Ptx, filamentous hemagglutinin (FHA), and P.69 Prn were more effective than ACVs containing Ptx and FHA only, also implicating an important role for P.69 Prn in immunity (12, 24, 28). Finally, variations in P.69 Prn were shown to affect the efficacy of the Dutch whole-cell vaccine in a mouse model (18).

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P.69 Prn, the focus of this study, belongs to a class of so-called autotransporter proteins that undergo autoproteolytic processing (15). P.69 Prn is processed from a 93-kDa large precursor to 69- and 22-kDa proteins which are located at the cell surface and in the outer membrane, respectively (5). The 69-kDa product (referred to as P.69 Prn) is used in ACVs. P.69 Prn contains an Arg-Gly-Asp (RGD) motif, which is implicated in ligand-receptor interactions in eukaryotes. It has been shown that this motif is involved in the P.69 Prn-mediated attachment of *B. pertussis* to mammalian cells (19, 20). P.69 Prn is polymorphic, and 13 variants (P.69 Prn1 to P.69 Prn13) have been identified so far. Variation is mainly limited to two regions, designated region 1 and region 2, which are comprised of Gly-Gly-X-X-Pro (r1 repeat) and Pro-Gln-Pro (r2 repeat) repeats, respectively. Most variations are found in region 1, which is located proximal to the N terminus and flanking the RGD sequence. Region 2 is located more towards the C terminus. The results presented by He et al. suggest that the r1 repeat induces type-specific antibodies which show little cross-reactivity between P.69 Prn1 and P.69 Prn2 (14).

Several studies of both animals and humans have indicated that P.69 Prn can elicit protective antibodies (6, 17, 18, 30). However, information about the locations of epitopes to which these antibodies are directed is limited. The aim of this study was to define epitopes on P.69 Prn and to obtain insight into the role that the variable regions play in immunity and immune escape.

MATERIALS AND METHODS

Production of MAbs. Monoclonal antibodies (MAbs) were generated by the injection of BALB/c mice subcutaneously three times, either with purified P.69

TABLE 1. Binding of MAbs to peptides derived from region 2

Region and peptide	Amino acid sequence[a]	Binding of MAb[b]			
		PeM1	PeM2	PeM19	E4D7
Region 2	WSLVGAKAPPAPKPAPQPGPQPPQPPQPQPEAPAPQPPAGRELSA				
Pep11	WSLVGAKAPPAPKPA	−	−	−	−
Pep12	AKAPPAPKPAPQPGP	+	+	−	−
Pep13	APKPAPQPGPQPPQP	+	+	−	−
Pep14	PQPGPQPPQPPQPQP	−	+	−	−
Pep15	QPPQPPQPQPEAPAP	−	−	−	−
Pep16	PQPQPEAPAPQPPAG	−	−	−	−
Pep17	EAPAPQPPAGRELSA	−	−	+	+

[a] The amino acid sequence (aa 530 to 571) for the entire 2 region is given.
[b] +, binding; −, no binding.

Prn or with a fusion protein comprised of region 1 of P.69 Prn and region 1 of the maltose binding protein (18). Specol was used as an adjuvant (ID-DLO, Lelystad, The Netherlands). Three days before the fusion injection, mice were boosted intravenously. Spleen cells were fused with mouse SP2/0 myeloma cells by using 50% polyethylene glycol 1500 (Boehringer, Mannheim, Germany). Hybridomas secreting antibodies to P.69 Prn were selected by an enzyme-linked immunosorbent assay (ELISA) and cloned twice by limiting dilution (18). MAbs were purified by protein G affinity chromatography (Pharmacia, Uppsala, Sweden) (1).

Human sera. We selected sera from nine children (median age of 4 years) who had high immunoglobulin A (IgA) titers (median of 192 U/ml) against *B. pertussis*, which is indicative of a recent infection (27). In addition, we selected sera from five children who at 4 years of age received a booster immunization with an ACV containing 25 μg of FHA, 25 μg of pertussis toxin, and 8 μg of P.69 Prn1 (Glaxo Smith Kline, Rixensart, Belgium) in one arm and a regular diphtheria-tetanus-inactivated polio vaccine booster in the other arm. The children were previously vaccinated with four doses of diphtheria-tetanus-pertussis-inactivated polio vaccine (containing a whole-cell pertussis vaccine; Netherlands Vaccine Institute, Bilthoven, The Netherlands) at the ages of 3, 4, 5, and 11 months. Prebooster sera ($n = 3$) were used as negative controls.

Synthesis of peptides derived from region 2. Synthetic penta-decapeptides spanning the r1 and r2 repeats and overlapping by 10 amino acids were synthesized. Synthesis of the r2 repeat peptides was performed as described previously for the r1 peptides (18). The amino acid sequences of the r2 peptides are listed in Table 1.

R1 and R2 peptide ELISA. Polystyrene 96-well ELISA plates (Immulon II; Dynatech, Chantilly, Va.) were coated overnight at 22°C by adding 100 μl of PBS containing 1 μg of peptide/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, Calif.) and were subsequently blocked by incubation with PBST supplemented with 0.5% (wt/vol) bovine serum albumin (BSA; Sigma, St. Louis, Mo.) for 1.5 to 2 h at room temperature. Murine MAbs diluted in 0.5% BSA in PBST to a concentration of 1 μg/ml were added to the wells and were incubated for 2 h at 37°C followed by four washings as described above. Bound antibodies were detected by using horseradish peroxidase-conjugated anti-mouse total IgG (Cappel, Organon Technica, Boxtel, The Netherlands). Extinction (the optical density at 450 nm [OD_{450}]) was measured with a plate reader (EL312e; BioTek Systems, Winooski, Vt.). To increase the sensitivity of the assay, we used higher concentrations of the coating peptide (5 μg/ml) and MAb (10 μg/ml).

Synthesis of peptides for pepscan analysis. For pepscan analysis, 15-mer linear, 15-mer loop, and 30-mer linear peptides spanning the entire amino acid sequence of P.69 Prn were synthesized. There was a shift of one amino acid residue between subsequent 15- and 30-mer peptides. The synthesis of the 15-mer linear and 15-mer loop peptides was performed on credit-card-format mini-Pepscan cards (455 peptides/card) (Pepscan Systems, Lelystad, The Netherlands) as described previously (29). The 30-mer peptides were synthesized by linking two 15-mer peptides as follows. The sequence 1 to 14 was synthesized with an additional cysteine at position 15, whereas the sequence 17 to 30 was bromoacetylated at its N terminus. The peptides [1-14]-Cys and BrAc-[17-30] were coupled by the formation of a thioether bond. The thioether-containing spacer replaced two amino acid residues (i.e., 15 and 16) in the 30-mer sequence.

Pepscan ELISA. The binding of antibodies to peptides was tested in a pepscan-based ELISA (29) (Pepscan Systems). The 455-well credit-card-format polyethylene cards containing the covalently linked peptides were incubated (at 4°C overnight) with a MAb (10 μg/ml) diluted in blocking solution, which contained 5% (vol/vol) horse serum and 5% (wt/vol) ovalbumin or human serum (diluted 1:100). After being washed, the peptides were incubated for 1 h at 25°C with rabbit anti-mouse IgG–peroxidase conjugate (diluted 1:1,000) (Dako, Glostrup, Denmark), and subsequently, after being washed, with the peroxidase substrate 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) and 2 μl of 3% H_2O_2/ml. After 1 h, the color development was measured by determining the OD_{450}. The color development of the ELISA was quantified with a charge-coupled device camera and an image processing system.

The results for the MAbs obtained with the three sets of peptides were essentially the same, and only in a few cases was the intensity of binding of the MAbs to the peptides somewhat different. For the human sera, a background correction was performed by subtracting the average extinction of the three negative sera from the positive sera data points. The binding of antibodies was considered significant when three successive data points reached values which were 1.5 times higher than the average value of 10 flanking data points. Most of the responses found with the human sera were observed for all three sets of peptides. When a reaction was observed with only one of the three peptide series or with two of the three series, it was also included in the data set, but this was observed only occasionally.

Blocking ELISA. A blocking ELISA was performed as described previously (1). Briefly, plates were coated with P.69 Prn1 (2 μg/ml) as described above. After being washed and blocked, the wells were incubated with 100-μl aliquots of serial twofold dilutions of a pool of human sera with IgG titers of ≥500 IU/ml, diluted in 0.5% BSA in PBST for 2 h at 22°C. The pool comprised a mix of sera from several recently infected pertussis patients who were fully vaccinated. Subsequently, the plates were washed and incubated with a P.69 Prn-specific murine MAb (2 to 3 μg/ml) for 2 h at 22°C. After a final washing step, the amount of MAb was quantified as described above. A MAb was considered to compete with human sera when at least 50% of its binding capacity was blocked by the binding of human antibodies.

RESULTS

Binding of MAbs to peptides derived from regions 1 and 2. In view of the fact that polymorphisms in P.69 Prn are mainly restricted to the two regions comprised of repeats (regions 1 and 2), we initially focused on these regions by using a limited set of overlapping peptides. To distinguish these two limited sets of peptides from the more extensive sets used in the pepscan analysis (see below), we will refer to the two sets of peptides as r1 and r2 peptides, respectively. Previously, we tested 14 MAbs for the ability to bind to synthetic peptides derived from region 1 of P.69 Prn (18). Of these 14 MAbs, six (PeM3, PeM4, PeM68, PeM71, PeM72, and F6E5) were found to bind r1 repeats, whereas one MAb (PeM70) bound to the sequence ATIRR, which forms the N-terminal border of the r1 repeats (Table 2). Due to some discrepancies with the pepscan analysis (see below), these binding experiments were repeated

TABLE 2. Characterization of P. 69 Prn MAbs

MAb	Antigen[a]	Blocking ELISA result[b]	Region 1 peptide	Region 2 peptide	Pepscan analysis peptide(s)[a]
PeM1	P.69 Prn1	+	–[f]	APKPAPQPGP[d]	TWDDD, GGFGPVLDGW, PAPQPP
PeM2	P.69 Prn1	–[c]	–[c]	PQPGP	PQP, GGFGPGGFGP
PeM3	P.69 Prn1	–[c]	GGFGPGGFGP[c]	–	ND
PeM4	P.69 Prn1	–[c]	GGFGPGGFGP[c]	–	GGFGPGGFGP
PeM5	P.69 Prn1	+[c]	–[c]	–	–
PeM6	P.69 Prn1	+	–	–	–
PeM7	P.69 Prn1	+[c]	GGFGPGGFGP[d]	–	GGFGPGGFGP
PeM19	P.69 Prn2	–	–	RELSA	RELSA
PeM21	P.69 Prn2	+	–	–	–
PeM29	P.69 Prn2	+	GGFGPGGFGP	–	GERQH+GDTWDDD+GGFGP
PeM38	P.69 Prn2	+	–	–	–
PeM64	P.69 Prn2	+	–	–	–
PeM68	FP	–	GDAPAGGAVP[c]	–	ND
PeM70	FP	–	ATIRR[c]	–	ND
PeM71	FP	–	GGFGP[c]	–	GGFGPGGFGP
PeM72	FP	–	GGFGP[c]	–	GGFGPGGFGP
PeM80	P.69 Prn5	+	–	–	RFAPQ
PeM84	P.69 Prn5	+	–	–	GGFGPGGFGP
PeM85	P.69 Prn5	+	GGFGPGGFGP[d]	–	GGFGPGGFGP
E4D7	P.69 Prn1	–	–	RELSA	ND
F6E5	P.69 Prn1	–[c]	GGFGPVLDGW[c]	–	ND

[a] FP, fusion protein containing region 1.
[b] A pool of sera from pertussis patients was tested for the ability to block at least 50% of binding of the MAb. +, blocking; –, no blocking.
[c] Determined previously by King et al. (18).
[d] Binding was observed with high concentrations of coating peptide and MAb.
[e] ND, not done.
[f] –, experiment performed but no binding observed.

with higher concentrations of coating peptide and MAbs. Under these conditions, two additional MAbs (PeM7 and PeM85) were identified that bound to r1 peptides. We extended this previous work further by testing the ability of seven additional MAbs, raised against P.69 Prn, to bind to synthetic peptides derived from region 1. Binding was also determined at high concentrations of coating peptide and MAbs. Of these seven MAbs (PeM6, PeM19, PeM21, PeM29, PeM38, and PeM64), only PeM29 showed binding to the r1 peptides (Table 2).

To further elucidate the epitopes recognized by all 21 MAbs, we synthesized a set of seven overlapping peptides corresponding to region 2 (Pep11 to -17 in Table 1). These r2 peptides were comprised of 15 amino acids with an overlap of 10 residues. Of the 21 MAbs tested, two (PeM1 and PeM2) bound to peptides harboring the PQP repeat, whereas two MAbs (PeM19 and E4D7) bound to the sequence RELSA, which forms the C-terminal border of the PQP repeat region (Table 1). The binding of PeM1 to the r2 peptides was found only when high peptide concentrations were used in the assay.

Pepscan analysis of binding of MAbs to peptides derived from P.69 Prn. A more extensive mapping of MAb epitopes was performed with pepscan analyses in which the complete primary structure of P.69 Prn was represented. Three sets of peptides were synthesized that spanned the entire amino acid sequence of P.69 Prn, namely the 15-mer linear, 15-mer loop, and 30-mer linear sets (670 peptides in each). In the case of the 15-mer loop peptides, both ends were connected to the plastic card, as this may mimic loops in the protein and hence possibly result in an improved representation of the natural epitope (4, 23).

Of the 16 MAbs tested in the pepscan analyses, 11 showed binding to the peptides (Table 2). Five MAbs (PeM4, PeM7, PeM19, PeM71, and PeM72) recognized a single peptide, as illustrated for PeM72 in Fig. 1A. In contrast, PeM80, PeM84, and PeM85 recognized multiple peptides (between 5 and 10) distributed along the whole length of P.69 Prn. However, one peptide was preferentially bound and is indicated in Table 2. Five MAbs (PeM5, PeM6, PeM21, PeM38, and PeM64) failed to show any reactivity with peptides, suggesting that they recognize discontinuous or conformational epitopes. Interestingly, three MAbs (PeM1, PeM2, and PeM29) bound to two or three nonoverlapping peptides in the pepscan analysis, as shown for PeM29 in Fig. 1B. The binding specificity of these MAbs confirmed the reaction with the r1 and r2 peptides, but the results were extended to additional peptides which were found by pepscan analysis. There was no obvious similarity in the primary structures of the peptides recognized by any one of the three MAbs.

Some minor discrepancies were observed between the pepscan analysis and the analyses with the r1 and r2 peptides (Table 2). Several MAbs (PeM1, PeM2, PeM7, PeM29, PeM84, and PeM85) showed binding to the r1 or r2 repeat in the pepscan analysis, whereas no, or very weak, binding was observed with the r1 or r2 peptides. This suggested that the pepscan analysis has a higher sensitivity, an assumption that was confirmed by the fact that four MAbs (PeM1, PeM7, PeM29, and PeM85) bound to the r1 or r2 peptides when higher concentrations of MAbs and coating peptides were used.

Of the 17 MAbs raised against native P.69 Prn, 11 were found to bind to r1 or r2 peptides, suggesting that these regions are immunodominant in mice (Table 2). The remaining four

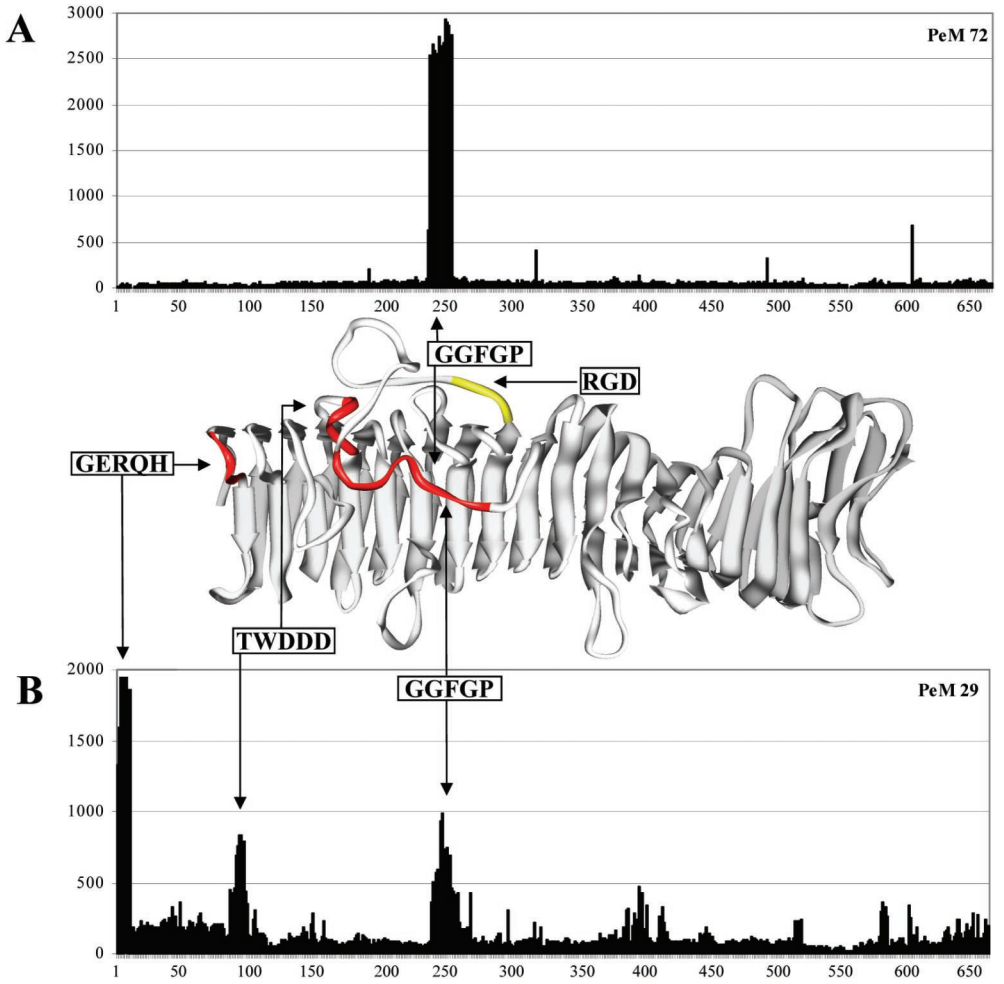


FIG. 1. P.69 Prn pepscan analysis of a MAb (PeM72) that binds to a single linear epitope (A) and a MAb (PeM29) that binds multiple linear epitopes (B). The peptides recognized by PeM72 and PeM29 are indicated in the P.69 Prn crystal structure. The *x* axis shows the residue numbers starting at the N terminus of the processed protein. The OD_{450} values are given on the *y* axis.

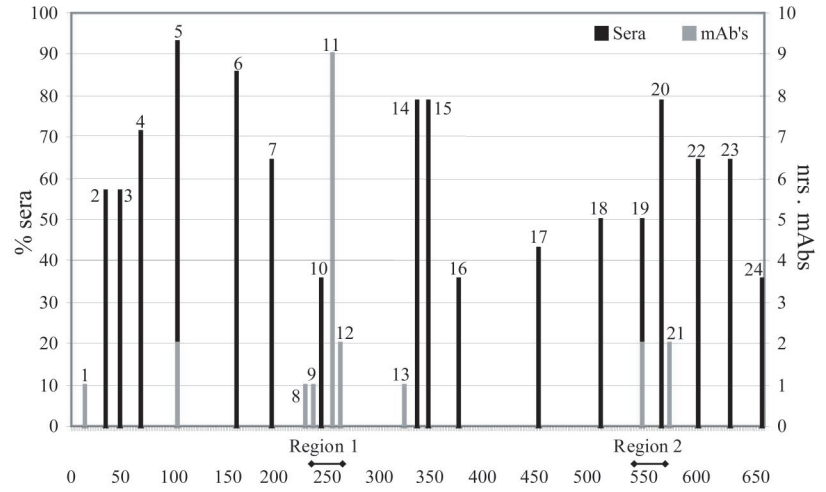
MAbs were raised against a fusion protein containing region 1, and as expected, only recognized region 1.

Pepscan analysis with human sera. Human sera were also subjected to pepscan analyses. The sera were obtained from 4-year-old children who received a booster vaccination with a three-component vaccine comprised of Ptx, FHA, and P.69 Prn ($n = 5$). In addition, sera from children with high titers against *B. pertussis*, which is indicative of a recent infection, were analyzed ($n = 9$). Epitopes that were unique for antibodies from vaccinated or infected individuals were not observed, and the results were combined (Fig. 2). Only epitopes that were recognized by at least 5 of 14 sera (36%) are shown in Fig. 2. Epitopes that showed a lower percentage of binding with the human sera were rare. A total of 17 different peptides were recognized by the sera. Three regions with immunodominant linear epitopes were identified in P.69 Prn. The first region was located in the N-terminal part of P.69 Prn and harbored the peptides TWDDD (amino acids [aa] 99 to 103) and SLQPED (aa 156 to 161). The two epitopes were recognized by 93 and 86% of the sera, respectively. The second region was located in the center of P.69 Prn and harbored the peptides SITLQA GAH (aa 327 to 335) and KALLYR (aa 339 to 344), which were both recognized by 78% of the sera. The third region was found in the C-terminal domain of P.69 Prn, with the peptide PAPQPP (aa 562 to 567), which was recognized by 78% of the sera and is located within the r2 repeat region, as the immunodominant epitope.

An overlap between the 17 epitopes recognized by human antibodies and the 9 epitopes of the mouse MAbs was observed in two cases, for the epitopes TWDDD and PQP (epitope no. 5 and 19 in Fig. 2). In two other cases, the epitopes recognized by human antibodies were located in close proximity to epitopes recognized by mouse MAbs. In the r1 region, epitope 10 is flanked by two epitopes recognized by mouse MAbs. The sequence RELSA, recognized by mouse MAbs, forms the C-terminal border of the r2 repeat region and is just a few amino acids removed from epitope 20, which is recognized by human antibodies.

Competition between MAbs and human sera for P.69 Prn. A blocking ELISA was performed with all 21 MAbs to test their ability to compete with a pool of human sera (Table 2). Six MAbs (PeM2, PeM3, PeM4, PeM5, PeM7, and F5E6) were

A



B

Nr.	AA's	Epitope	Nr.	AA's	Epitope	Nr.	AA's	Epitope
1	11-15	GERQH	9	227-236	GDALAGGAVP	17	445-450	LAGSGL
2	31-36	SGTIKK	10	237-246	GGAVP	18	503-509	TFTLANK
3	44-49	GILLEN	11	247-251	GGFGP	19	545-547	PQP
4	71-77	GIRRFLL	12	252-261	GGFGPVLDGW	20	562-567	PAPQPP
5	99-103	TWDDD	13	317-321	RFAPQ	21	570-574	RELSA
6	156-161	SLQPED	14	327-335	SITLQAGAH	22	596-603	SNALSKRL
7	190-194	GASEL	15	339-344	KALLYR	23	627-631	DNRAG
8	222-226	ATIRR	16	367-374	TELPSIPG	24	657-664	HLGGLAGY

C

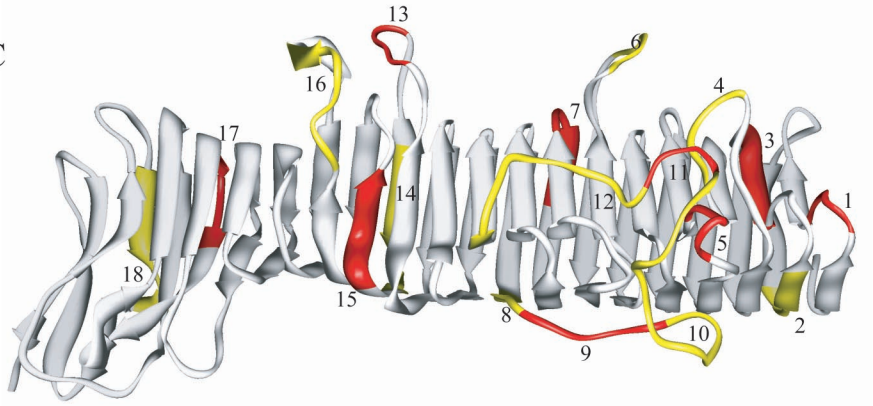


FIG. 2. P.69 Prn epitopes recognized by MAbs and human sera. (A) The numbers of MAbs binding to a particular peptide and the percentages of human sera that contain antibodies binding to a particular peptide are indicated on the right and left *y* axes, respectively. The numbers on the *x* axis refer to the P.69 Prn amino acid residues. Numbering starts with the N terminus of the processed protein. Epitopes recognized by <36% of the human sera are not shown. Epitopes are numbered to facilitate identification. (B) A minimal sequence with the corresponding amino acid residues of the epitopes. (C) Visualization of epitopes recognized by human and mouse antibodies. Epitopes are numbered to facilitate identification. Numbering starts with the N terminus of the processed protein. Epitopes recognized by <36% of the human sera are not shown. The crystal structure does not include the last 138 C-terminal amino acids of P.69 Prn, which harbor region 2. Successive epitopes are colored red and yellow to facilitate discrimination.

tested previously (18). Of the 21 MAbs tested, the binding of 10 was not inhibited by human antibodies. Interestingly, seven of these MAbs (PeM3, PeM4, PeM68, PeM70, PeM71, PeM72, and F5E6) were directed against linear epitopes that were localized in region 1. This indicated that human antibodies bound with a low avidity to this region relative to the MAbs. One of the 10 MAbs whose binding was not affected was PeM2, which recognized the PQP peptide in the r2 repeat. Even when the peptide was used as a coat in the assay instead of purified P.69 Prn and when many different human sera were tested (>50), no binding competition was observed. Since 50% of the human sera contained antibodies against this part of the r2 repeat, the lack of inhibition was probably due to a low avidity of the human antibodies relative to the MAbs. The observation that the remaining 2 of 10 MAbs (PeM19 and E4D7) that were not blocked by human sera were directed against the RELSA epitope, which is just a few amino acids away from the PAPQPP epitope in the r2 repeat (which is recognized by 78% of the human sera), can be explained in a similar manner.

For 11 MAbs, the binding to P.69 Prn could be blocked by

human antibodies. These MAbs included the five (PeM5, PeM6, PeM21, PeM38, and PeM64) that did not bind to peptides and therefore probably recognized conformational or discontinuous epitopes. In fact, the pepscan analysis indicated that 2 of these 11 MAbs recognized discontinuous, linear sequences (PeM1 and PeM29). It is conceivable that human antibodies have a stronger affinity for discontinuous epitopes than do mouse MAbs. The peptide TWDDD, which was recognized by 93% of the sera, forms a part of the epitope recognized by PeM1 and PeM29. The remaining four MAbs (PeM7, PeM80, PeM84, and PeM85) which were blocked by human sera bound to linear epitopes that were not recognized by human antibodies according to pepscan analyses. This suggested that human antibodies might recognize these epitopes as part of a larger discontinuous epitope that was not revealed by the pepscan analyses. The observation that three MAbs directed against the r1 repeat (PeM7, PeM84, and PeM85) were inhibited by human sera indicated the presence of human antibodies directed against this region of P.69 Prn. This was also suggested by the pepscan analyses.

DISCUSSION

Epitopes on P.69 Prn were mapped with human sera and MAbs by using synthetic peptides derived from the repeat regions 1 and 2 and with a more extensive set of peptides covering the complete P.69 Prn polypeptide. The interpretation of the results was facilitated by the availability of the crystal structure of part of P.69 Prn (8, 9).

Human sera. Pepscan analyses did not reveal any differences between epitopes recognized by sera from humans infected with *B. pertussis* and from humans vaccinated with a vaccine containing P.69 Prn. However, pepscan analysis mainly detects linear epitopes, and it is conceivable that infection and vaccination elicit antibodies that differ with respect to the conformational epitopes recognized. The pepscan analyses revealed 17 linear epitopes that were recognized by at least 36% of the human sera. The N-terminal region harbored a large cluster of epitopes (no. 2 to 7 in Fig. 2). Located within this cluster was the most immunodominant linear epitope of P.69 Prn (TWDDD), which was recognized by 93% of the sera. The central region of P.69 Prn harbors two main epitopes (no. 14 and 15 in Fig. 2), which were both recognized by 78% of the sera. A large number of epitopes were observed in the C-terminal region, with the r2 repeat region being immunodominant (epitopes 19 and 20 in Fig. 2). Most of the identified epitopes (13 of 18) that could be visualized in the crystal structure of P.69 Prn were located in loops (Fig. 2C).

Comparisons of pepscan analyses of MAbs and human sera. There was little overlap between the epitopes recognized by MAbs and those recognized by human antibodies (Fig. 2). Of the 24 epitopes identified, only 2 were recognized by both human and mouse antibodies. One of these epitopes was TWDDD, emphasizing the immunodominance of this linear epitope.

Only 35% of the human sera contained antibodies directed against linear r1 epitopes. In contrast, a high percentage of the human sera (86%) recognized linear r2 repeat epitopes. The percentages of mouse MAbs reacting with these two epitopes showed an opposite trend. Of the 17 MAbs raised against P.69 Prn, 59% were directed against the r1 repeat and only 12% were directed against the r2 repeat (or 24%, if the RELSA epitope was included in the r2 repeat).

By combining the results of the pepscan analyses and blocking ELISAs, we identified three different kinds of epitopes that are recognized by human and mouse antibodies: (i) strictly linear epitopes localized within regions 1 and 2 that are not recognized or are recognized only with a low avidity by human antibodies compared to that by mouse MAbs, as the binding of MAbs to these epitopes was not blocked by human sera; (ii) conformational epitopes which could not be localized with linear peptides but were recognized by human antibodies with a high avidity compared to that by mouse MAbs, as the MAbs were blocked by human sera; and (iii) discontinuous and possible discontinuous epitopes which could be (partly) localized with peptides and were also recognized by human antibodies with a high avidity compared to that by mouse MAbs, as the binding of MAbs was blocked by human sera. Thus, blocking experiments indicated the presence of high-avidity human antibodies against conformational epitopes. Interestingly, human antibodies against linear epitopes had much lower avidities, as they were unable to block MAbs. It should be realized that blocking of the binding of MAbs by human sera may be due to competition for identical or overlapping epitopes, thus identifying epitopes recognized by human sera. However, blocking may also result from steric hindrance due to the proximity of distinct epitopes that are recognized by human antibodies and the MAbs tested. At present, we cannot distinguish between these possibilities and therefore the identification of discontinuous and possible discontinuous epitopes defined by blocking experiments should be viewed as tentative.

Regions 1 and 2 possibly form a single epitope. Interestingly, pepscan analyses revealed several discontinuous epitopes, of which region 1 is a part. We found three MAbs (PeM1, PeM2, and PeM29) that bound to two or more nonoverlapping peptides. There was no obvious similarity in the primary structures of the peptides recognized by a single MAb, suggesting that these peptides are part of a discontinuous epitope. Apparently, the MAbs retained some affinity for the different peptides of which the discontinuous epitope is comprised (22). This assumption was corroborated for the epitopes of PeM29 and PeM1 by the crystal structure of P.69 Prn. Although the three peptides recognized by PeM29 are separated by 85 and 235 amino acids in the primary structure, they are proximate in the crystal structure (Table 2 and Fig. 1B). Similarly, two of the three peptides recognized by PeM1 (TWDDD and GGFGPV LDGW), although separated by 153 amino acids in the primary structure, are in close proximity in the crystal structure (Fig. 2C). Significantly, PeM1 and PeM2 bound peptides derived from both the r1 and r2 repeats. The r2 repeats are not part of the published crystal structure of P.69 Prn (8); however, our results suggest that they are in close proximity to the r1 repeats in P.69 Prn.

Immune evasion and repeats. Tandemly repeated sequences such as the r1 and r2 repeats are found in the surface antigens of many pathogens and have been implicated in immune escape. This occurs by insertion or deletion of the repeat unit (11). This type of antigenic variation is unlikely to affect linear epitopes, as the deletion or insertion of repeats does not create novel sequences. Thus, to be effective, repetitive sequences

involved in immune escape should not induce protective levels of antibodies directed against the repeat unit but should induce antibodies directed against conformational epitopes. There is evidence that this indeed occurs. The protective alpha C protein of group B streptococci harbors a variable number of tandem repeats. It has been shown that deletions in this region are selected for under specific antibody pressures, and they appear to decrease the organism's susceptibility to killing by an antibody specific to the alpha C protein. Importantly, it was shown that antibodies to a repeat harbored by the protective alpha C protein of group B streptococci are recognized predominantly as a conformational epitope (10, 11, 21). This is not to say that antibodies to the repeat are not induced, as 36 and 78% of the sera we tested harbored antibodies against regions 1 and 2, respectively. However, such antibodies may be less effective than conformational antibodies, e.g., due to a low avidity, the concentration, or the specific isotype. One reason why the repeats per se may be less protective than when an epitope is presented as a conformational epitope is that antigens with repeating epitopes may activate B cells without the assistance of T-helper cells. T-cell-independent antibody responses tend to be weaker and are not boosted with repeated immunization. We propose that protective P.69 Prn antibodies induced during infection or vaccination recognize a conformational epitope comprised of r1 and r2 repeats. Antibodies directed against the repeat unit are proposed to be much less effective. This hypothesis is confirmed by the observation that variations in region 1 affected vaccine efficacy in a mouse model (13, 18). Boursaux-Eude et al. (2) used a mouse model to compare the efficacies of ACVs against isolates that produced different pertactin and pertussis toxin variants. They concluded that a tricomponent vaccine was highly effective in promoting the lung clearance of all isolates expressing different pertussis toxin and pertactin variants. The data obtained with different isolates were not compared on a statistical basis, however. Thus, relative efficacies against different strains were not determined. The role of pertactin variation in pertussis epidemiology is still under debate (25). The extent to which polymorphisms affect vaccine efficacy is probably dependent on the potency of the vaccine used. In The Netherlands, in which a relative weak vaccine has been used, pertussis epidemics have been associated with the emergence of non-vaccine-type strains (7, 25, 31). Furthermore, the fitness advantage of strains carrying nonvaccine types of pertactin may be limited to older age categories in which vaccine-induced immunity has waned.

The assumption that region 1 is recognized mainly as a conformational epitope by the human immune system is suggested by the results of He et al. (14). These authors analyzed the type specificity of the P.69 Prn immune response in humans infected with a P.69 Prn2 or Prn3 strain or vaccinated with P.69 Prn1. P.69 Prn1 and P.69 Prn3 differ by two amino acid substitutions in region 1, while P.69 Prn2 differs from both by the deletion of a repeat unit comprising five amino acids. Thus, of the three P.69 Prn variants, P.69 Prn2 is the most structurally distinct, while P.69 Prn1 and P.69 Prn3 are structurally more similar. Consistent with this, individuals infected with P.69 Prn2 strains had significantly lower antibodies to P.69 Prn1 than those infected by P.69 Prn3 strains and those who were vaccinated with P.69 Prn1. Moreover, in contrast to vaccinees and subjects infected with P.69 Prn3 strains, individuals infected with P.69 Prn2 strains had hardly any antibodies specific to the variable region of P.69 Prn1.

In summary, our results suggest that the two most variable regions of P.69 Prn may form a single conformational epitope. Since the RGD motif, which is involved in adherence to host cells (20), is separated from the first r1 repeat by three amino acids, it seems likely that both repeat regions serve to deflect the immune response targeted to the functional domain of P.69 Prn.

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