Antigenic Analysis of *Bordetella pertussis* Filamentous Hemagglutinin with Phage Display Libraries and Rabbit Anti-Filamentous Hemagglutinin Polyclonal Antibodies

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Although substantial advancements have been made in the development of efficacious acellular vaccines against *Bordetella pertussis*, continued progress requires better understanding of the antigenic makeup of *B. pertussis* virulence factors, including filamentous hemagglutinin (FHA). To identify antigenic regions of FHA, phage display libraries constructed by using random fragments of the 10-kbp EcoRI fragment of *B. pertussis* fhaB were affinity selected with rabbit anti-FHA polyclonal antibodies. Characterization of antibody-reactive clones displaying FHA-derived peptides identified 14 antigenic regions, each containing one or more epitopes. A number of clones mapped within regions containing known or putative FHA adhesin domains and may be relevant for the generation of protective antibodies. The immunogenic potential of the phage-displayed peptides was assessed indirectly by comparing their recognition by antibodies elicited by sodium dodecyl sulfate (SDS)-denatured and native FHA and by measuring the inhibition of this recognition by purified FHA. FHA residues 1929 to 2019 may contain the most dominant linear epitope of FHA. Clones mapping to this region accounted for ca. 20% of clones recovered from the initial library selection and screening procedures. They are strongly recognized by sera against both SDS-denatured and native FHA, and this recognition is readily inhibited by purified FHA. Given also that this region includes a factor X homolog (J. Sandros and E. Tuomanen, Trends Microbiol. 1:192–196, 1993) and that the single FHA epitope (residues 2001 to 2015) was unequivocally defined in a comparable study by E. Leininger et al. (J. Infect. Dis. 175:1423–1431, 1997), peptides derived from residues 1929 to 2019 of FHA are strong candidates for future protection studies.

*Bordetella pertussis*, the agent of whooping cough, is responsible for more than 355,000 deaths annually, mostly of unvaccinated young children (7). Poor public acceptance (related to fears concerning vaccine safety) and the relatively severe reactivity of otherwise efficacious whole-cell vaccines led to the relatively recent development of efficacious acellular vaccines (ACVs) (4, 7, 8, 10, 11). Of the several *B. pertussis* virulence factors suggested for inclusion in ACVs (4, 5, 10, 27), the most commonly included are pertussis toxin and filamentous hemagglutinin (FHA), a multifunctional adhesin that is both cell associated and secreted into the external milieu. FHA improves vaccine efficacy when included in multicomponent ACVs, and in animal models, FHA alone elicits protective immunity (4, 5).

With the goal of improving long-term vaccine efficacy, ongoing research is directed toward exploring alternative approaches to vaccine delivery and improving our understanding of the immune response to *B. pertussis* antigens (4, 5, 10). Given this goal and the possibility that future vaccines may be recombinant proteins comprised of protective antigen subcomponents, an understanding of the antigenic makeup of components such as FHA is of fundamental importance.

*B. pertussis* pathogenesis (reviewed in references 5, 25, 27, and 42; see also reference 13) involves a diverse set of adhesins (FHA, pertactin, BrkA, fimbriae, and pertussis toxin) and toxins (pertussis toxin, adenylate cyclase-hemolysin, tracheal cytotoxin, and dermonecrotic toxin). The relative importance of FHA throughout infection can be illustrated by a simplified model (adapted from reference 20). After *B. pertussis* enters the upper airways, host factor-induced signalling (27, 34) leads to expression of the first of two temporally separated groups of virulence factors. The first group includes both FHA and fimbriae, and it is likely that an identified FHA lectin-like domain which mediates binding to ciliated cells (and to macrophages [26, 28]) is important at this stage. Once bacteria are attached, the second temporally expressed group of factors (includes pertussis toxin and adenylyl cyclase-hemolysin), as well as tracheal cytotoxin (constitutively expressed), mediate local and systemic damage associated with pertussis disease (4, 10, 27, 42). Toxin-mediated changes to the respiratory epithelium may now allow the FHA heparin-binding domain to mediate binding to targets other than ciliated cells, such as sulfated glycoconjugates of respiratory mucus and epithelial cell surfaces. The persistence of pertussis infection may also be partly due to FHA, for its RGD motif enables *B. pertussis* to bind to CR3 integrins and enter macrophages (16, 28, 33), conceivably allowing immune system evasion and establishment of an intracellular reservoir. FHA-mediated adherence to nonciliated epithelial cells and subsequent (pertactin-mediated) invasion may also play a role here (12, 19).

FHA is a large, complex molecule (20, 21) that is synthesized as a 367-kDa precursor (FhaB), translocated to the periplasm, and exported through the outer membrane (2, 17, 30). N-terminal processing (17) and cleavage of the C-terminal third of FhaB yield the 220-kDa mature FHA molecule (2, 30). Adhesin domains thus far identified within FHA include an RGD triplet (FHA<sub>1097–1099</sub> [29]), a heparin-binding domain...
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E. coli to reduce or eliminate recombinants from a library. Vectors used were fDRW836 (inserts followed by GAGTGA in one-letter amino acid code), fDRW863, fDRW867 (GAGSGA), and fDRW861 (GAGA).

An amber (TAG) codon within a stuffer fragment (B) can be removed with FspI and PvuII, creating a linear fragment for cloning blunt-end inserts (C) of length 3n + 2 (where n is an integer). Peptides are displayed near the N terminus of mature pIII and are flanked by Gly-Ala-rich linker sequences. The vector is propagated in an amber-suppressing host strain such as E. coli LE392 (SupE SupF), while libraries are constructed in a non-amber-suppressing host. Vector self-ligation yields a frameshift in gene III; since pIII is required for virion morphogenesis and infectivity, cells infected with self-ligated vector produce few, noninfectious virions. If the stuffer is not excised, the amber codon similarly prevents production of pIII. In principle, only recombinants possessing inserts of appropriate length contribute to the library.

Vectors and fDRW70 pseudorevertants. The type 3 pseudorevertants of fDRW70 (43) were constructed by replacing the SfiI restriction site (D) for receiving inserts of length 3n (E). Ligation products can be digested with SfII prior to transforming E. coli to reduce or eliminate recombinants from a library. Vectors used were fDRW866 (inserts followed by GVGTGA in one-letter amino acid code), fDRW863 (GAGSGA), fDRW864 (GAGTGA), fDRW887 (GAGSGA), and fDRW865 (GAGA).

The type 3 pseudorevertants of fDRW70 (43) were constructed by replacing the SfiI restriction site (D) for receiving inserts of length 3n (E). Ligation products can be digested with SfII prior to transforming E. coli to reduce or eliminate recombinants from a library. Vectors used were fDRW866 (inserts followed by GVGTGA in one-letter amino acid code), fDRW863 (GAGSGA), fDRW864 (GAGTGA), fDRW887 (GAGSGA), and fDRW865 (GAGA).

(continued)

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Materials and methods

Bacterial strains and bacteriophage f1. Escherichia coli K91-Kan (HfrC) and MC1061 (39) and bacteriophage f1 (47) were provided by G. P. Smith (University of Missouri).

Vectors and fDRW70 pseudorevertants. The type 3 (37) phage display vectors fDRW70 and the fDRW864 (43) were constructed by replacing the SfiI-excisable stuffer fragment of fDRW5 (45) with oligonucleotides appropriate to their design (Fig. 1). As derivatives of fd-tet and fUSE5 (35, 46), these vectors encode tetracycline resistance. fDRW70 pseudorevertants A and B are independently isolated variants in which a single base pair substitution altered the amber codon within the stuffer fragment to a codon encoding tyrosine (45).

FHA library construction. Four FHA-70 libraries (Table 1) were constructed with DNase I-generated (1, 36) fragments of the 10-kbp B. pertussis flaB EcoRI

FIG. 1. Phage display vectors. (A) Sequence of fd-tet, corresponding to the last residue of the pIII preprotein sequence and the first three residues of mature pIII, shown for comparison with fDRW70 and fDRW864 vectors. (B and C) Amber vector fDRW70 was designed to allow construction of libraries free of nonrecombinants. An amber (TAG) codon within a stuffer fragment (B) can be removed with FspI and PvuII, creating a linear fragment for cloning blunt-end inserts (C) of length 3n + 2 (where n is an integer). Peptides are displayed near the N terminus of mature pIII and are flanked by Gly-Ala-rich linker sequences. The vector is propagated in an amber-suppressing host strain such as E. coli LE392 (SupE SupF), while libraries are constructed in a non-amber-suppressing host. Vector self-ligation yields a frameshift in gene III; since pIII is required for virion morphogenesis and infectivity, cells infected with self-ligated vector produce few, noninfectious virions. If the stuffer is not excised, the amber codon similarly prevents production of pIII. In principle, only recombinants possessing inserts of appropriate length contribute to the library. (D and E) fDRW864 vectors were designed to display foreign peptides flanked by N-terminal Gly-Pro and variable C-terminal Gly-containing linker peptides. Each vector incorporates a rare SfII restriction site (D) for receiving inserts of length 3n (E). Ligation products can be digested with SfII prior to transforming E. coli to reduce or eliminate recombinants from a library. Vectors used were fDRW866 (inserts followed by GVGTGA in one-letter amino acid code), fDRW863 (GAGSGA), fDRW864 (GAGTGA), fDRW887 (GAGSGA), and fDRW865 (GAGA).
Table 1. Construction of B. pertussis fhaB DNase I fragment (FHA-70) libraries

<table>
<thead>
<tr>
<th>Library</th>
<th>Insert size (bp)</th>
<th>Ligation products redigested with SrfI</th>
<th>Expected size of displayed peptide (amino acid residues)</th>
<th>Total yield Log_{10} transformants</th>
<th>Log_{10} virions recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHA-70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70-X</td>
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<td>5.5</td>
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<tr>
<td>70-A</td>
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<td>10–25</td>
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<td>9.6</td>
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<tr>
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<td>25–50</td>
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<td></td>
<td>50–100</td>
<td>5.2</td>
<td>9.1</td>
</tr>
<tr>
<td>70-D</td>
<td>300–600</td>
<td></td>
<td>100–200</td>
<td>5.2</td>
<td>8.7</td>
</tr>
<tr>
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<tr>
<td>80-A</td>
<td>30–75</td>
<td></td>
<td>10–25</td>
<td>4.5</td>
<td></td>
</tr>
</tbody>
</table>

* Each library was constructed with vector fDRW70 (FHA-70 libraries) or fDRW8nn vectors (FHA-80 libraries) and the indicated sizes of B. pertussis fhaB DNase I-digested fragments.

** Log_{10} yield values for these duplicates are as follows: library 80-Y, 3.4 and 3.7; and library 80-A, 4.2 and 4.2.

Restriction fragment (9) that has been subcloned from clone C1–5 of a Sau3A I cosmid library of B. pertussis 18-323 chromosomal DNA (M. J. Brennan, Food and Drug Administration, Bethesda, Md.) into pTZ18R. (Strain 18-323 is somewhat atypical among B. pertussis strains [see, for example, reference 40], and as noted by a reviewer, it is possible that the antigenic profile of 18-323 is not entirely typical of B. pertussis. Importantly, however, there is no evidence that FHA produced by 18-323 is atypical of B. pertussis. Rather, if the inversions and other rearrangements in the sequence which set 18-323 apart from other strains appear to have left fhaB unaffected [40], and [i] as described below, comparison of the sequences of the clones described in this report [derived from strain 18-323] with the sequences of GenBank entry M60351 [B. pertussis BP338] revealed only minor differences. For each library, ligations were performed with fDrp- and PsvII-digested fDRW70 (Fig. 1B) and B. pertussis fhaB fragments of a specific size range (Table 1). Control ligations were performed with fDRW70 alone. E. coli MC1061 was electroporated with portions of the ligation products, plated on LB containing 20 μg of tetracycline ml–1 (LB-Tet), and incubated overnight (37°C). Numbers of transformants recovered (Table 1) were estimated by counting ~1/200 of the total. After bacterial growth was washed from the surfaces of plates and the washes were centrifuged (80 min, 6,000 × g, 4°C), virions were precipitated from the supernatant with polystyrene glycol (PEG) (39) and quantitated (Table 1) by a plaque assay (43). Although vector fDRW70 was designed with the goal of eliminating nonrecombinants from these libraries (Fig. 1, legend) and the expected outcome of its use was that control library 70-X would produce few virions, this control library unexpectedly produced similar numbers of virions as did libraries 70-A to 70-D (Table 1); thus, the fraction of recombinants in these libraries could not be determined.

FHA library 80-A was similarly constructed, using fDRW8nn vectors and 30–75 bp SrfI fragments (Table 1) or, for control libraries 80-X and 80-Y, fDRW8nn DNA alone. The fDRW8nn vectors were designed (Fig. 1, legend) to allow redigestion of ligation products with SrfI (which recognizes a rare restriction site) to eliminate most nonrecombinants from library 80-A. Accordingly, ligation products for libraries 80-Y (one of the two control libraries) and 80-A were redigested with SrfI before electroporation of portions of the ligation products into E. coli MC1061. The numbers of transformants recovered from control libraries 80-X and 80-Y suggested that redigestion reduced nonrecombinants 5–10-fold (Table 1 and other data not shown).

Immunological materials. Rabbit polyclonal antibodies (Pabs) produced against wild-type phase 1b have been previously described (45). Three anti-FHA sera were raised in New Zealand White rabbits after four immunizations using FHA. Sera FN1/4 and FN2/4 were obtained from two rabbits immunized with native FHA, eluted from the hepB-Sepharose column by using a NaCl gradient (23). Serum FS1/4 was obtained from a rabbit immunized with sodium dodecyl sulfate (SDS)-denatured FHA, removed from a hepB-Sepharose column with 1% SDS.

Antibodies used in biopanning and plaque lifts (see below) were purified from sera FN2/4 and FS1/4 by ammonium sulfate precipitation, absorption with E. coli antigens by using an immobilized E. coli lysate (Pierce Immunochemicals), and protein A affinity chromatography (43). For biopanning, portions of the purified antibodies were biotinylated (Pierce Sulfo-NHS-biotinylation kit), and 4'-hydroxyazobenzene benzoic acid and avidin were used as recommended by the supplier (Pierce) to determine that the molar ratios of incorporated and noncovalently surface-exposed (accessible to streptavidin) biotin to antibody were ~2:1. For enzyme-linked immunosorbent assay (ELISA) and dot blotting, serum FN2/4 and FS1/4, but not FN1/4, were absorbed against E. coli antigens as described above.

Biopanning. Biopanning (affinity selection) methods were adapted from reference 39. For each of eight FHA peptide and control libraries, a single round (39) of biopanning was carried out with each of the four indicated quantities (Fig. 2) of an equimolar pool of biotinylated Pabs FN2/4 and FS1/4 (pooled FN2/4–FS1/4). For each biopanning, antibodies were combined with the indicated quantities of FHA (50 μg) in a final volume of 100 μl of phosphate-buffered saline (PBS; 12 mM phosphate, 157 mM Na+, 4.4 mM K+, 140 mM Cl– [pH 7.4]) with 1% bovine serum albumin (BSA) and incubated overnight at 4°C. After incubation (2 h, 37°C, 1% BSA in PBS, 200 μl well–1), microtiter plate wells were coated with a covalently linked streptavidin (Pierce), blocking solution was discarded, and virion-antibody mixtures were added to wells, which were incubated for 20 min at room temperature. After wells were washed 10 times (PBS–0.5% Tween 20, 200 μl well–1), 150 μl of 0.1 N HCl (adjusted to pH 2.2) was added to each well to elute antibody-bound virions. After 20 min, 9 μl of 2 M Tris base (unadjusted pH) was added to each well, and samples were recovered immediately. The numbers of virions recovered (Fig. 2) were estimated by a transducing unit assay (43); assay adapted from that in reference 39) that derives from the ability of IDRW70 and fDRW8nn-derived virions to transduce tetracycline resistance into host cells.

Assessment of biopanning enrichment. Samples of unencrusted (viz., not bio-panned) library virions and biopanning eluates were plated on lawns of E. coli K91-Kan (43). Plaque lifts (described below) were probed with a 1/8,000 dilution of protein A-purified Pab FN2/4. Plaques visible (even faintly so) on nitrocellulose were counted as antibody reactive (Fig. 3A), while plaques on the correspondingly treated wells were counted as total plaques.

FHA-70 library antibody-reactive clones. Aliquots of library 70-A to -D eluates from biopanning with 3.6 μg as well as 360 ng of pooled FN2/4-FS1/4 (Fig. 2) were plated on E. coli K91-Kan. After plaque development, a nitrocellulose disc (Schleicher & Schuell) was applied to each lawn with light pressure. After ~40 min at 4°C, discs were removed and dried for 30 min at room temperature before application of 2-μl samples (100, 20, and 4 ng) of heparin-Sepharose affinity-purified FHA (a gift from F. D. Menozzi, Institut Pasteur de Lille) (23) as positive controls. Each disc (contained in a standard petri dish) was incubated for 1 h at room temperature in 10 ml of blocking buffer (1% skim milk powder–3% BSA in PBS) before being washed three times (per wash, 10 ml of PBS–0.05% Tween 20 for 20 min). After addition of (i) a 1/8,000 dilution (in blocking buffer, 10 μl per disc) of E. coli-absorbed protein A-purified Pab FN2/4 or (ii) a 1/8,000, 1/32,000, or 1/128,000 dilution of FN2/4 alone, each disc was incubated 1 h (room temperature) and washed three times. After addition of 10 ml of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G secondary antibodies (1:3,000 dilution in blocking buffer) (GIBCO/BRL), each disc was incubated for 1 h (room temperature) and washed three times with wash buffer and once with 10 ml of substrate buffer (100 mM Tris [pH 9.6], 40 mM MgCl2). Signal was developed by addition of 10 ml of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (50 μg ml–1)–nitroblue tetrazolium chloride (100 μg ml–1) in substrate buffer. Reactions were stopped by rinsing discs in water.

Antibody-reactive and total plaques (Fig. 3B) were counted as described above. Fifty-six antibody-reactive clones (Table 2) were chosen from those that reacted most strongly with antibody, as judged by relative color intensity (on plaque lifts) at a given antibody dilution or by any visible color at a high antibody dilution. For each, virions within an excised corresponding bacterial lawn were counted as total plaques.
weakly reactive clones were performed with a 1:4,000 dilution of FN2/4, and those for 10 other weakly reactive clones were performed with a 1:3,000 dilution of pooled FN2/4-FS1/4. During these rounds of plaque purification, antibody reactivity was lost for five clones; these were not included in subsequent analyses. As a final clonal isolation step, dilutions of each monoclonal virion eluate were used to infect *E. coli* K91-Kan. After overnight incubation (37°C) with shaking, virions harvested from these cultures by PEG precipitation of culture supernatant were used as a source of single-stranded template for sequencing flhB-derived inserts, in preliminary ELISAs, and as a source of virions to infect *E. coli* K91-Kan for later large-scale preparations of selected clones (43).

FHA-80 library antibody-reactive clones. Samples of eluates from biopanning library 80-A with 3.6 µg as well as 360 ng of pooled FN2/4-FS1/4 (Fig. 2) were plated on *E. coli* K91-Kan, plaque lifts were probed with protein A-purified PAb FN2/4, and positive and total plaques were counted as described above for FHA-70 libraries. For each of 58 clones chosen from those identified with a 1:32,000 dilution of FN2/4 (Table 2), virions within a plaque-containing agar plug were eluted into 0.5 ml of LB overnight (4°C). For each clone, 10-µl samples of 10-fold serial dilutions of virion eluates were transferred to 90 µl of *E. coli* K91-Kan (optical density at 600 nm of 0.2) in microtiter plate wells. After 1 h of incubation (37°C, in LB with 0.2 µg of tetracycline ml−1), 10 µl of each well was transferred to 90 µl of LB-Tet in a microtiter plate well; after overnight incubation (37°C, shaking), the optical density at 595 nm of each culture was used to identify the highest dilution of each clone showing growth. For each clone, after a sample of each such dilution was incubated overnight on an LB-Tet plate, an isolated colony was used as a source of inocula for an overnight broth culture (37°C, shaking). Virions harvested from these cultures were used as described for FHA-70 library clones.

Propagation of selected FHA-70 and -80 clones. Forty-four recombinant clones selected after analysis by sequencing data (Table 3), together with (as controls) fDRW70 pseudorevertants A and B, were propagated and harvested by methods (43) that included (i) infecting *E. coli* K91-Kan with aliquots of virion preparations that had been used for sequencing, (ii) PEG precipitating virions from supernatants of overnight cultures of infected cells, and (iii) purifying virions by two additional PEG precipitations and by filtration through a 0.2-µm-pore-size syringe filter.

**Dot blots.** Triplicate 2-µl samples (800 ng of phage protein) of PEG-precipitated and filtered virions were applied to nitrocellulose discs. After samples had dried, discs were blocked and washed as for plaque lifts. After addition of 10 µl of primary antibody (per disc; a 1:5,000, 1:32,000, or 1:128,000 dilution in blocking buffer of [i] protein A-purified anti-FHA PAb or [ii] protein A-purified anti-FHA PAb [iii] FN2/4 or [iii] FN3/4 or [iv] crude anti-FHA serum FN1/4), each disc was incubated for 1 h at room temperature before washing, probing with secondary antibodies, and development of signal as for the above-described plaque lifts.

**Competition ELISA.** A competition ELISA was performed with anti-FHA PAbs prepared by preincubating 1:5,000 dilutions of *E. coli*-absorbed FN2/4 as well as FS1/4 (in blocking buffer, with NaCl adjusted to 233 mM) with heparin-Sepharose affinity-purified FHA (F. D. Menozzi) at final concentrations of 30 µg, 5 µg, 333 ng, and 0 ng ml−1 for 2.75 h (37°C) before dilution of the antibody preparations twofold immediately prior to their use in ELISA. Duplicate wells of Immulon-2 plates (NUNC) were coated with 1 µg of PEG-precipitated and filtered virions (100 µl well−1 in PBS). After overnight incubation at 4°C, plates were washed three times (PBS–0.05% Tween 20) before blocking (1% BSA–1% skim milk powder in PBS, 200 µl well−1) for 1 h at 37°C. After plates were washed three times, anti-FHA PAbs prepared as described above were added (100 µl well−1), and plates were incubated for 0.75 h at 37°C. After plates were washed three times, peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibodies (1:3,000 dilution in blocking buffer, 100 µl well−1; GIBCO/BRL) were added, plates were incubated for 1 h at 37°C and then washed six times; the reaction products were developed with o-phenylenediamine (1 mg ml−1 in 0.1 M citrate [pH 4.5] with 0.012% H2O2, Sigma), and the Amax of reaction products was read. Concurrently, additional Immulon-2 plates were coated with virions and washed as for ELISA before the relative quantities of virions remaining bound to wells were determined by a bicinchoninic acid (BCA) protein assay (45).

**RESULTS**

**Affinity selection of antibody-reactive clones.** Target clones reactive with rabbit anti-FHA PAbs were affinity selected from FHA-70 and -80 libraries (Table 1) by a single round of biopanning (37, 43) with four 10-fold serially different quantities of anti-FHA antibodies (Fig. 2). The recovery of a greater number of virions from libraries panned with 3.6 µg and 360 ng of PAb than from control libraries or libraries panned with smaller quantities of antibodies (36 and 3.6 ng) indicated (Fig. 2) that biopanning had been successful. This was confirmed in an assay showing, for eluates of libraries 70-A and 70-B panned with 3.6 µg and 360 ng of PAbs, increases in the fractions of...
antibody-reactive clones recovered after biopanning (Fig. 3A). Subsequent plaque lifts to identify antibody-reactive clones used eluates from biopanning with 3.6 μg and 360 ng of PAbs.

Selection of clones for characterization. The anti-FHA antibodies used in biopanning had been purified from three rabbit sera elicited with two preparations of FHA: (i) native FHA, used to raise sera FN1/4 and FN2/4; and (ii) SDS-denatured FHA, used to raise FS1/4. To recover maximal numbers of target clones, biopannings used a pool of E. coli-absorbed, protein A-purified FN2/4 and FS1/4 (pooled FN2/4-FS1/4) and subsequent preliminary plaque lifts of the biopanning output used these same pooled sera. Because these plaque lifts yielded an unexpectedly large number of antibody-reactive clones, clones to be characterized were chosen from later plaque lifts that used FN2/4 alone and were selected from plaques that yielded the greatest color intensity at a given antibody dilution or any visible reactivity at a high antibody dilution. In this manner, 56 antibody-reactive clones were selected (Table 2) from plaque lifts of biopanning output from FHA-70 libraries (Fig. 3B). During the several rounds of plaque purification that followed, 5 of the 56 clones were lost. Whether this was due to insert instability or mishap such as incorrect excision of a nonreactive plaque was not determined. An additional 58 clones were selected (Table 2) from plaque lifts of biopanning output from library 80-A (Fig. 3C).

Sequencing. Sequences of most fhaB-derived inserts (Table 3) agreed with the published sequence of fhaB (GenBank entry M60351). Base substitutions were found in three clones (I-a, XI-c, and XI-d; see footnotes to Table 3). No base insertions or deletions were identified. Two clones contained inverted sequences. The first of these (I-b) contained an additional noninverted fragment encoding an FHA peptide, while the second (clone 30) encoded a peptide unrelated to FHA. The latter clone was used as a control in later assays.
The chosen clones encoded 31 combinations of vector-FHA sequences that mapped to 14 regions of FHA (Table 3; Fig. 4A). More than half mapped to region I; here, a 126-residue peptide (clone I-a) was represented by 11 siblings, and an 11-residue peptide (I-d) was represented by 22 siblings. Relatively high numbers of clones were also found for regions III (seven siblings for III-a and for III-b) and XI (eight siblings for XI-a, and nine for XI-d). These findings suggested that regions I, III, and XI contained immunodominant antigenic determinants of FHA, an idea confirmed by subsequent dot blots and ELISA.

**Dot blots and ELISA.** Dot blots of purified virions applied to nitrocellulose and probed with FS1/4, FN2/4, and FN1/4 (a serum not used in biopanning or screening) were used to compare immunoreactivities of the clones. A composite blot, constructed from individual blots by using computer graphics software, is shown in Fig. 5. Importantly, variability among triplicate samples and among siblings was minimal, and all but three (nonreactive) clones showed titerable reactivity with antibodies elicited by SDS-denatured FHA (FS1/4 versus native FHA) (FN2/4 and FN1/4) and (ii) the way in which preincubation with FHA (Fig. 6A) was readily titrated to near zero with increasing concentrations of FHA, while recognition of less reactive region III, IV, and VI clones was also inhibited but not to quite the same low levels. Recognition of region VIII, IX, and X clones was only marginally inhibited.

**DISCUSSION**

**Candidate immunogens for eliciting protective antibodies.** Four groupings of clones (Table 4) were suggested by considering dot blot and ELISA data in terms of (i) recognition by antibodies elicited by SDS-denatured FHA (FS1/4 versus native FHA) (FN2/4 and FN1/4) and (ii) the way in which pre-
incubation of antibodies with FHA influenced this recognition. Although the analysis is (i) limited by the small number of sera used and correspondingly conjectural and (ii) simplistic in that it ignores the polyclonal nature of the antibodies used and variability in the immune response among animals, it may nevertheless provide insight.

(i) **Group A.** Region VIII, IX, and X clones were recognized by FS1/4 but not FN2/4 or FN1/4 (Fig. 5 and 6); preincubating FS1/4 with FHA had little effect on recognition. From this it may follow that the phage-displayed peptides and corresponding sequences of SDS-denatured FHA adopt similar nonnative conformations, with native conformation being required for generation of antibodies capable of recognizing FHA. The sequences may thus have little immunogenic value.

(ii) **Group B.** Region IV and VI clones were recognized strongly by FS1/4 but weakly by FN2/4 and FN1/4 (Fig. 5 and 6); preincubating FS1/4 and FN2/4 with FHA diminished recognition of these clones only moderately. For reasons similar to those suggested for group A clones, sequences encoded by group B clones appear to have little ability to generate cognate anti-FHA antibodies. However, because of their possible importance in pathogenesis, the immunogenicity of these sequences may warrant further experimental study.

Thus, the region IV sequence FHA<sub>1229-1244</sub> is almost entirely contained within a possible lectin-like binding domain (FHA<sub>1224-1242</sub> [32]), and antibodies to this domain may prove to be of protective value. Also, the overlapping VI-a and VI-b sequences encode all (VI-a) or most (VI-b) of a sequence with homology to C3bi (FHA<sub>1407-1417</sub> [32]) and may play a role in adherence (32). Antibodies to this region may thus be of protective value.

(iii) **Group C.** Clones of groups I and XIV were recognized by FN2/4, and in some cases by FN1/4, but not by FS1/4; preincubating FN2/4 with FHA strongly inhibited recognition (Fig. 5 and 6). Lack of recognition by FS1/4 may reflect use of SDS-denatured FHA as an immunogen; the sequences may thus have immunogenic value, provided they are presented in a suitable conformation.

Region I clones accounted for more than half of the 109 sequenced clones (Table 3), and some were strongly recognized by antibody. Although a common epitope may lie within the shared sequence S<sub>50</sub>G<sub>50</sub> (Fig. 7), clones (1-c to h) displaying only these or a few additional residues were not recognized by FN1/4, while a clone (1-b) displaying an N-terminally extended sequence, G<sub>579</sub>Gln. . .G<sub>595</sub> (Fig. 7), was both recognized by FN1/4 and more strongly recognized by FN2/4. These sequences map within a 422-residue region (FHA<sub>422-863</sub>) that contains an FHA heparin-binding domain (15) and is flanked by arginine-rich sequences. Given that heparin-binding domains are believed to be defined in part by patterns of clustered positively charged residues (6, 22), the sequence S<sub>579</sub>RVRGKGV DLHDLSAAGADISGEGRVNIGRARSDDSVK<sub>510</sub> (within clone I-a; charged residues in bold) may thus be part of a
heparin-binding domain and might be a candidate for eliciting protective antibodies.

The value of region XIV may be limited, for only a single region XIV clone (FHA 2226–2255) moderately reactive with FN2/4 but only weakly reactive with FN1/4) was identified.

(iv) Group D. Region III and XI to XIII clones were recognized by FS1/4, FN2/4, and (for regions XI to XIII) FN1/4 (Fig. 5 and 6). Preincubation of FS1/4 and FN2/4 with FHA strongly inhibited recognition. It might be argued that (i) since SDS-denatured group D sequences can elicit antibodies that recognize FHA and (ii) phage displaying group D peptides are recognized by antibodies against both SDS-denatured and native FHA, it follows that group D peptides used as immunogens may be able to elicit responses against native FHA.

Because the three region III clones were equally and strongly recognized by FS1/4 and FN2/4, the shared sequence Arg...Glu...Arg (Table 3) likely contains the residues critical to recognition. Although this sequence maps within the sequence (FHA 442–863) that contains the FHA heparin-binding domain (15), the sequence is relatively charge poor and may play no direct role in heparin binding.

The sequences of the five region XI clones overlap (Fig. 7) in a way that suggests the existence of at least two epitopes. Clones XI-a and XI-b include only the first of these; clone XI-a (1951Leu...Pro1964), noticeably better recognized than XI-b, is longer than XI-b by two residues. Clone XI-c includes only a portion (1957Glu...Tyr1963) of the sequence common to XI-a and XI-b but was better recognized, expectedly because it also includes the second and larger immunogenic region. The clones that include this region—XI-c, XI-d, and XI-e—are the most strongly recognized of all clones analyzed, the 71-residue XI-d sequence being recognized most strongly. Since XI-d lacks N-terminal residues (1952Asp...Tyr1956) found in the more weakly recognized XI-c and contains additional C-terminal residues (1951Lys...Ala1960), some of these C-terminal residues may account for the stronger recognition. The similarity of the additional C-terminal sequence2018KKLQGEYKA2027 to the preceding overlapping2011RKIFGEYK

FIG. 5. Dot blots of antibody-reactive clones. (A) Relative positions within FhaB of antibody-reactive clones assayed by means of dot blotting with anti-FHA antibodies and serum. (B) Thirty unique clones (I-a, I-b, etc.) were assayed. In many cases (e.g., clone ID I-a), more than one sibling (e.g., clones 43A, 46, and 52) were assayed to control for variability. Controls included clone 30 (see Table 3) and two variants (pseudorevertants) of vector fDRW70, prA and prB. Triplicate 2-μl samples (800 ng of protein) applied to nitrocellulose were probed with the indicated dilutions of E. coli-absorbed sera FS1/4 and FN2/4 and crude serum FN1/4; protein A-purified antiphage (α-f1) antibodies, as a control to assess virion quantities bound to nitrocellulose; and no primary antibody, as a control for recognition of virions by secondary antibody alone. Only the first of the triplicates are shown for these latter controls. After blot development, scanned computer images were imported into the format shown here. Two sets of nonconcurrent dot blots, (i) and (ii), are shown.
KL2020 (similar residues in bold) raises the possibility that both sequences are antibody reactive. Clones XI-c, XI-d, and XI-e include the factor X homolog 1979Leu...Lys1984 (32), and as reviewed earlier, antibodies to this sequence may not be beneficial.

The single region XII clone contains the factor X homolog 2062ETKEVDG2068 (32); as noted earlier for the region XI factor X homolog, the benefit of antibodies to this sequence are uncertain.

Comparison with other studies. Although the libraries used in this work were constructed with random, DNase I-generated fragments of fhaB, they were only partially characterized for completeness, diversity, and insert stability. Considering this as well as our concerns (44) that not all peptide sequences can be successfully displayed on phage surfaces, it became important to confirm that our results provided a comprehensive antigenic analysis of FHA.

In this context, it is thus noteworthy that antibody-reactive clones were found within each antigenic domain identified in two earlier studies as well as within regions not identified in this earlier work. One such study, carried out in our laboratory, used a Pseudomonas aeruginosa OprF expression system to identify 19 antibody-reactive OprF-FHA fusion proteins that mapped to four domains (Fig. 4B). The present study identified antibody-reactive clones within each of these domains (Fig. 4A and B) and additionally identified clones in regions I, X, XIII, and XIV. A more limited study by Delisse-Gathoye et al. (9) used FHA-derived recombinant proteins and anti-FHA MAbs to show that a 1,200-residue immunoreactive domain contained at least four epitopes (Fig. 4C). The present study identified 10 or more epitopes within the same domain (Fig. 4A and C) and additionally identified epitopes of regions I and III.

The present study also serves to extend the recently published work of Leininger et al. (18), who used 23 mouse MAbs to identify five antigenic domains (Fig. 4D) of FHA. In the C-terminal half of FHA (residues 1200 to 2300), we identified eight antibody-reactive clones in three regions (X, XI, and XII) that lie within two antigenic domains (Ia and IB [Fig. 4D]) identified by Leininger et al., as well as clones in six regions (IV, VI, VIII, IX, XIII, and XIV) that map outside domains identified by Leininger et al.

Notably, the single epitope unequivocally defined by Leininger et al. (18) by using Pepsan analysis (FHA_1929-2017 [Fig. 4E]) is included in our clones XI-c (FHA 1929–2017), XI-d (FHA1957–2027), and XI-e (FHA 1967–2019) (Fig. 4A; Table 3). Considering that region XI clones accounted for a relatively large fraction (20 of 109) of all clones sequenced (Table 3) and were strongly recognized by sera against both native and SDS-denatured FHA (Fig. 5), region XI sequences may contain the most dominant linear epitope of the entire FHA molecule and are thus strong candidates for future protection studies.

In the N-terminal half of FHA (residues 1 to 1100), our results varied in some ways from those of Leininger et al. (18). First, Leininger et al. identified two domains (IIA and IIC [Fig. 4D]) in which we identified no antibody-reactive clones (Fig. 4A). Second, we identified a number of clones in region I (Fig. 4A; Table 3) that do not map within a domain identified by Leininger et al. Finally, we identified three clones (region

<table>
<thead>
<tr>
<th>Group</th>
<th>Regions of FHA</th>
<th>Recognized by FS1/4?</th>
<th>Recognized by FN2/4?</th>
<th>Effect of preincubating antibodies with purified FHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>VIII, IX, X</td>
<td>Yes</td>
<td>No</td>
<td>Little or no effect</td>
</tr>
<tr>
<td>B</td>
<td>IV, VI</td>
<td>Yes</td>
<td>Poorly</td>
<td>Some inhibition of recognition</td>
</tr>
<tr>
<td>C</td>
<td>I, XIV</td>
<td>No</td>
<td>Yes</td>
<td>Inhibited recognition</td>
</tr>
<tr>
<td>D</td>
<td>III, XI, XII, XIII</td>
<td>Yes</td>
<td>Yes</td>
<td>Inhibited recognition</td>
</tr>
</tbody>
</table>
the numbers of residues omitted. Uppercase letters are FhaB sequences; lowercase letters are vector-derived sequences. Boxed regions of overlap are discussed in the text.

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The present study has provided an antigenic analysis of FHA that has both enhanced our understanding of previously identified antigenic domains and identified a number of additional antigenic regions. Taken in context of both this earlier work as well as published structural and functional analyses of FHA, the present findings serve to define sequences that merit investigation as candidates for inclusion in recombinant subcomponent vaccines.

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