Human Fab Fragments Specific for the *Haemophilus influenzae* b Polysaccharide Isolated from a Bacteriophage Combinatorial Library Use Variable Region Gene Combinations and Express an Idiotypic That Mirrors In Vivo Expression

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To determine whether the human antibody (Ab) repertoire to the *Haemophilus influenzae* type b capsular polysaccharide (Hib PS) could be studied at the molecular level with phage display technology, we constructed a phage Fab library by using peripheral blood from a vaccinated adult. Phage were selected based on Hib PS binding. Two distinct Hib PS-specific phage clones were identified whose Fab fragments used the same V_H region paired with two different V_L regions. The V_L regions were derived from two independent rearrangements of the A2c gene with $J_{\kappa 1}$, and both contained a nontemplated arginine codon at the $V-J_{\kappa}$ junction. The two A2 V gene segments differed from the A2c germ line sequence in 0 and 5 bases. The V_H region consisted of the $V_H 26$ gene segment having 98% identity to the germline nucleotide sequence, a D region of 9 bases, and $J_H 4b1$. Usage of $V_H 26$ in combination with A2 V regions containing a junctional arginine is a predominant configuration of naturally occurring Hib PS-specific Abs. Liquid- and solid-phase assays showed that phage-derived Fab reacted with Hib PS and expressed HibId-1, an idiotype associated with the κII -A2 V region. These findings extend the database of V region polymorphisms that can contribute to the Hib PS repertoire and demonstrate that Hib PS-specific Fab fragments isolated from combinatorial phage libraries use V gene combinations which mirror the natural repertoire.

The protective antibody (Ab) response to the human pathogen *Haemophilus influenzae* type b is directed primarily against the *H. influenzae* type b capsular polysaccharide (Hib PS), which consists of repeating units of 3- β -D-ribose-(1-1)-ribitol-5-phosphate (25). The Ab repertoire to Hib PS has been intensively scrutinized and serves as one of the best characterized human Ab responses to foreign antigen (reviewed in references 8 and 24). V_H regions derive from four closely related members of the $V_H IIIa$ and $V_H IIIb$ families. V_L usage is more complex, with as many as 12 distinct V_{κ} and V_{λ} gene segments contributing to the repertoire. Diversity in the heavy (H) chain third complementarity determining region (CDR) is evident from the usage of D regions which vary in length from 1 to 34 bases in combination with five J_H regions. The use of multiple V_L genes and J segments indicates that V_L CDR-3 may also be diverse, although the recurrence of an arginine residue at the V-J joint in most κ and λ Abs suggests a crucial role for this residue in mediating Hib PS binding. Somatic hypermutation occurs to a variable extent in both V_H and V_L regions. Thus, the presumed random assortment of H and light (L) chains, the usage of multiple V gene elements, and hypermutation are processes which generate at the population level a repertoire with substantial potential for complexity.

Despite this potential, however, individual serum repertoires are usually oligoclonal (14, 15), and Abs using the A2 V_{κ} gene segment and expressing the HibId-1 idiotype (Id) dominate the serum population (21, 26). The mechanisms that account for this disparity between estimates of the size of the potential repertoire and the remarkably restricted heterogeneity of individually expressed repertoires are not well understood. An-

tigen-driven selection could play a role, since Id analyses have shown that V_L expression varies depending upon age at vaccination (19), and both Ab function and V_L usage in the infant are affected by the molecular form of the Hib PS immunogen (20). Skewing of the repertoire could also occur during early ontogeny if V region expression was not equiprobable or if particular V_H - V_L pairs were preferentially expressed and/or selected in early ontogeny. The possibility that H and L chains do not randomly assort and that particular combinations are preferred would further serve to limit diversity.

Efforts to address these issues experimentally are hampered by the technical difficulties in performing structure-function analyses and chain recombination experiments by using conventional Abs and hybridomas. Furthermore, the ontogeny of the repertoire has been intractable to molecular analysis, since the experimental strategies that have been successful in analyzing the adult Hib PS repertoire, such as purification of serum Abs and hybridoma technology, are not easily applied to infants.

Alternative approaches are therefore required in order to analyze the ontogeny of the Hib PS repertoire, the pairing potential of V regions, and the relationship between V gene polymorphisms and Ab function. The expression of Ab Fab fragments in bacteriophage combinatorial libraries may provide the means to address these issues. This methodology has been used successfully to isolate a number of pathogen-specific human Fab fragments (3–7, 28, 29), although human Fab fragments specific for carbohydrate determinants have not been isolated with this technology. Anticarbohydrate specificities are typically considered to be of low intrinsic affinity, and therefore it is essential to determine whether Hib PS paratopes could be isolated by this methodology. In this report, we show that Hib PS-specific Fab fragments, derived from either a Hib PS-specific hybridoma or from peripheral blood of a vacci-

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the purified Fab fragments under both nonreducing and reducing conditions were consistent with the presence of disulfide-linked L and Fd chains. The protein concentration of the Fab fragments was determined by A_{280} with extinction coefficients calculated from the amino acid composition by the formula of Gill and von Hippel (12). The A_{280} s for a 1.0-mg/ml solution of SOL17 and SOL10 were 1.19 and 1.37, respectively.

Evaluation of Hib PS binding and Id expression. Hib PS binding by Fab fragments was analyzed with an ELISA as described previously (13). Briefly, dilutions of phage-derived Fab fragments or control Abs were incubated for 3 h at 37°C on wells coated with Hib PS oligomers coupled to human serum albumin. The wells were washed, and anti-human κ Abs conjugated with alkaline phosphatase (Biosource International, Camarillo, Calif.) were added. After 3 h at 37°C, the wells were washed, and *p*-nitrophenyl phosphate in diethanolamine buffer (pH 9.8) was added. A_{410} was determined after ~60 min.

Fab fragments were also analyzed by a previously described radioantigen binding assay, except that PEG 8000 rather than ammonium sulfate was used as the precipitant (22). The diluent consisted of PBS with 10% fetal calf serum. Fifty microliters containing ~250,000 cpm of ^{125} I-Hib PS (specific activity, ~45 μ Ci/ μ g) was mixed with 50 μ l of diluted Fab or control Abs. The mixtures were incubated for 2 h at 37°C and overnight at 4°C. One hundred microliters of 25% (wt/vol) PEG 8000 in 2% fetal calf serum-PBS was added, and the mixtures were incubated for 8 h at 4°C. The precipitates were harvested by centrifugation (16,000 $\times$ g), washed once with 12.5% PEG 8000, and counted in a dry-well gamma counter.

Id expression of Fab fragments was evaluated by measuring anti-Id inhibition of ^{125} I-Hib PS binding with a previously described assay, except PEG 8000 was used as the precipitant rather than ammonium sulfate (21).

Nucleotide sequence accession number. The V_L sequences of SOL10 and SOL1 have been assigned GenBank accession numbers U41569 and U41570, respectively. The SOL10 V_H sequence has been assigned GenBank accession number U41571.

RESULTS AND DISCUSSION

We adopted two approaches for determining the utility of the phage system for displaying Hib PS-specific human Fab fragments. In the first approach, we cloned and expressed a Fab fragment derived from the CA4 hybridoma, which secretes a functionally characterized and sequence-defined anti-Hib PS MAb (22). This strategy was chosen to determine whether a known Hib PS-binding Fab could be selected by panning and recovered in active form with the appropriate V regions and Id. In the second approach, we used MNC from a Hib PS-vaccinated adult as a source of polyclonal Ig mRNA for library construction. Successful isolation of Hib PS-specific Fab fragments from a combinatorial library consisting of randomly assorted V_H - V_L pairs would demonstrate the value of this methodology for repertoire analysis, particularly if Fab fragments could be isolated which recapitulate V_H - V_L pairs that are known to occur *in vivo*.

The CA4 hybridoma L chain and H chain Fd were cloned in the pComb 3 vector. Phage displaying the CA4 Fab were added at a frequency of 10^{-3} to a control Fab library known not to contain Hib PS-specific Fab fragments. The resulting mixture of phage particles was subjected to repetitive rounds of panning with microtiter wells coated with Hib PS. The percentage of clones binding Hib PS was determined after each round of panning with the radioantigen binding assay as described in Materials and Methods. Hib PS-binding phage were present at frequencies of <5, 8, 18, and 30% after one, two, three, and four rounds of panning, respectively. Sequence analysis of clonal isolates confirmed that these phage expressed V_L and V_H regions identical to those of the donor hybridoma (22). These findings demonstrate that the CA4 anti-Hib PS paratope can be expressed on the surface of M13 phage and can be enriched by specific panning. We also found that panning the starting phage library against the LuC11 MAb, which recognizes an Id determinant of CA4, efficiently enriched for the relevant phage such that after three rounds, >90% of the recovered phage expressed the CA4 Fab.

Our second objective was to determine if phage technology could be used to isolate Hib PS-specific Fab fragments from a

combinatorial library constructed from MNC mRNA. MNC obtained from a Hib PS-vaccinated adult were separated into two groups. One group, enriched for HibId-1-positive cells, was used to construct a phage L chain library. This tactic was chosen so that we could increase the probability of obtaining Fab fragments having the A2 V region, which comprises the predominant V_L gene used in the adult *in vivo* repertoire. The second group consisted of unfractionated cells, which were used to derive H chain Fd DNA. The H-L combinatorial library was prepared by directly cloning the Fd PCR products into the appropriately restricted bulk L chain phagemid DNA. After electroporation and productive phage growth, the library was subjected to repetitive rounds of panning against Hib PS-coated wells. Lysates of randomly picked colonies were evaluated for Hib PS binding at each step of the procedure. Hib PS-binding phage comprised <10, 10, and 93% of the isolates after one, two, and three rounds of panning, respectively. Eight Fab clones having Hib PS-binding activity were chosen for sequence analysis. As might be expected of a population enriched for HibId-1, all eight V_L regions were products of the A2c V gene segment. A2c is a newly described allele of the A2 gene whose coding sequence differs from the original A2 gene (6) at a single base in framework 2 (C to T) that results in replacement of a proline with a serine (2). Two independent V_L rearrangements were identified, as shown by nucleotide differences in the A2 segment and by the usage of different codons at the junction between the A2 V segment and the $J_{\kappa 1}$ region (Fig. 1). Interestingly, these junctional codons encode a nontemplated arginine residue, a feature characteristic of most naturally derived Hib PS Abs, irrespective of V gene segment and J usage (1, 8, 26). As has been shown for Abs binding other negatively charged antigenic determinants (17), the recurrence of this junctional arginine suggests an essential role in binding Hib PS. The A2 gene used by the SOL10 Fab was identical to A2c, and the A2 gene used by the SOL1 Fab differed from the A2c germ line sequence at 5 bases. Three of the 5 base changes in SOL1 result in amino acid replacements.

Sequence analysis of the V_H region of the eight clones identified a single rearrangement in all clones (Fig. 2). This rearrangement consisted of the V_H 26 germ line gene segment, a D region encoding three amino acids, and the J_H 4b1 region. Sequencing of the C_H 1 region identified the H chain as IgG1 (not shown). Of the 8 bases in the V gene segment which differed from the reported sequence for V_H 26 (GenBank accession no. J00236), 5 resulted in amino acid differences, 3 of which were in the CDRs. Naturally occurring anti-Hib PS Abs frequently utilize the V_H 26 gene, and three of four anti-Hib PS hybridomas that express the A2 V region use it in combination with V_H 26 (1, 8, 23). Thus, these phage-derived Fab fragments recapitulate a common *in vivo* H-L pairing configuration. Sequence diversity in naturally derived anti-Hib PS V_H CDR3 can be considerable; D regions vary in length from 1 to 39 bp (8). The 9-bp D region used in these Fab fragments appeared to derive from DN1 or DN4, but no definitive assignment could be made.

A phage clone having the Fab derived from the CA4 hybridoma (designated SOL17) and a single clone isolated from the peripheral blood library (designated SOL10) were chosen for antigen binding and Id analysis. Gene III was removed from the phagemid. The modified phagemid was reintroduced into XL1-Blue cells, and soluble Fab was produced in bulk. The Fab fragments were purified by affinity and molecular sieve chromatography and characterized immunochemically. The Fab fragments SOL10 and SOL17 were evaluated for Hib PS binding in a radioantigen binding assay and in an ELISA. The data shown in Fig. 3 demonstrate that both SOL17 and SOL10

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A2c      GATATTGTGATGACCCAGACTCCACTCTCTCTGTCCGTCACCCCTGGACAGCCGGCCTCCATCTCTGCAAGTCTAGT [.....]
SOL10    |      primer      |-----
SOL1     |-----

.....CDR1.....]
A2c      CAGAGCCTCCTGCATAGTGATGGAAAGACCTATTGTATTGGTACCTGCAGAAGCCAGGCCAGTCTCCACAGCTCCTG
SOL10    -----
SOL1     -----A-----

[.....CDR2.....]
A2c      ATCTATGAAGTTTCCAAACCGGTCTCTCTGGAGTGCCAGATAGGTTTCTAGTGGCAGCGGGTCAGGGACAGATTTACACTG
SOL10    -----
SOL1     -----A-----

[.....CDR3.....] junction
A2c      AAAATCAGCCGGGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCATGCAAAGTATACAGCTTCCT
SOL10    -----
SOL1     -----A-----T-----C      CGG (arg)
                                           AGG (arg)
.....]
Jk1      TGGACGTTTCGGCCAAGGGACCAAGGTGGAAATCAAACGT
SOL10    -----A-----
SOL1     -----G-----A-----

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FIG. 1. Nucleotide sequences of V_L regions used by two Hib PS-specific Fab clones (SOL10 and SOL1) isolated from an MNC combinatorial library. The germ line sequences of the A2c V gene (GenBank accession no. U41644) and the J_k1 gene (GenBank accession no. J00242 and J00243) are shown for comparison.

bound Hib PS in the liquid phase (Fig. 3A) and the solid phase (Fig. 3B). Interestingly, the relative binding to Hib PS as a function of Fab concentration was consistent between the Fab fragments and their respective control Abs. SOL17 appeared to have a higher binding affinity for Hib PS than did SOL10, since approximately 10-fold less SOL17 was required to achieve an equivalent level of Hib PS binding. Fab fragments bound to Hib PS with a lower affinity than the respective control Ab, a phenomenon presumably related to their monovalency.

The ability of Fab fragments to express Id was evaluated in the radioantigen binding assay, in which anti-Id was used to inhibit binding to ^{125} I-Hib PS. Each Fab expressed the appropriate Id (Fig. 4). SOL10 expressed HibId-1, a marker for the A2 V region, as shown by the ability of the HibId-1-specific MAb LuC9 to inhibit SOL10 Hib PS binding (Fig. 4A). Similarly, SOL17 expressed its respective Id, as shown by the ability of LuC11 to inhibit the Hib PS binding of SOL17. The specificity of the anti-Id Abs was demonstrated by the appropriate inhibition of the control Abs.

Several conclusions which bear on the technical suitability of phage display as a method for studying the human Hib PS-specific repertoire can be drawn from these experiments. First, surface-displayed or monomeric soluble Fab fragments that have disparate affinities for Hib PS can be isolated by panning or affinity chromatography. Thus, the concern that monovalent anticarbohydrate combining sites may be difficult to retrieve because of their low intrinsic affinities does not seem to be warranted. Our findings agree with the studies by Deng and colleagues which showed that murine single chain FVs having a broad range of affinities for the *Salmonella* serogroup B O-PS can be isolated by phage display (11). Second, the faithful retention of antigen binding and Id expression by the phage-derived Fab fragments indicates that experiments aimed at examining the structural determinants of Hib PS binding and Id expression likely can be performed with this technology. Third, despite the random pairing of L chain and H chain gene fragments during the construction of the library, anti-Hib PS Fab fragments can be isolated from an MNC library which recapitulate H-L combinations and junctional residues com-

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VH26     GAGGTGCAGCTGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGTCCCTGAGACTCTCTGTGCAGCCTCTGGA
SOL10    |      primer      |-----

[.....CDR1.....] [.....]
VH26     TTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGTGGAGTGGGTCTCAGCTATTAGT
SOL10    -----C-----G-----

.....CDR2.....]
VH26     GGTAGTGGTGGTAGCACATACTACGGAGACTCCGTGAAGGGCCGGTTTACCATCTCAAGAGACAATTCCAAGAACACG
SOL10    -----T-----C-----C-----T-----A-----

CTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAA
SOL10    -----C-----

[.....CDR3.....]
D              J
JH4b1     TTTGACTACTGGGGCCAGGGAACCCCTGGTTCACCGTCTCTCTCA
SOL10     GGGTACGGC -----G-----

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FIG. 2. V_H nucleotide sequence used by the Hib PS-specific Fab SOL10 isolated from an MNC combinatorial library. The identical V region is used by the SOL1 Fab. The germ line sequences of the V_H26 gene (GenBank accession no. J00236) and the J_H4b1 gene (GenBank accession no. X86355) are shown for comparison.

FIG. 3. Binding of Hib PS by phage-derived Fab fragments SOL17 (▲) and SOL10 (●) as determined in a radioantigen binding assay (A) and ELISA (B). SOL17 was derived from the CA4 hybridoma, and SOL10 was derived from the MNC library. Fab binding is compared with that of the control Abs the CA4 MAb (△) and purified HibId-1-positive anti-Hib PS serum Abs (○). O.D., optical density.

monly found in the *in vivo* repertoire. Caton and Koprowski demonstrated that a murine influenza virus hemagglutinin-specific Fab derived from a combinatorial library was able to recreate an H-L combination found *in vivo* (9), but to our knowledge, the results reported here provide the first demonstration that this can be accomplished with a human library. The ability to retrieve functional Fab fragments from combinatorial libraries suggests that this tactic can be fruitfully applied to analyzing V gene usage in the infant and to unraveling the molecular basis for the functional disparities between Hib PS Abs induced by different vaccines (20).

Finally, the results presented here confirm and extend our knowledge of the A2 V region sequence polymorphisms that can contribute to the Hib PS repertoire. Previously, four complete A2 V gene sequences of anti-Hib PS hybridomas have been reported (1, 8, 23). One of these sequences was identical to the originally described A2 germ line gene, which is now known as the A2a allele (2). The other three A2 sequences differed from the A2a germ line sequence by 5, 7, and 9 bases. In this report, we show that the newly described c allele of the A2 gene can be expressed in a germ line configuration and can form a functional Hib PS binding site. In addition, we identified from the same individual what appears to represent a somatically generated mutant of the A2c gene, and this variant

FIG. 4. Id expression by Hib PS-specific Fab fragments as determined in a radioantigen binding assay. Various concentrations of LuC9 (A) and LuC11 (B) were tested for their ability to inhibit Hib PS binding of a fixed concentration of SOL17 Fab (▲), SOL10 Fab (●) and control anti-Hib PS Abs. The control Abs are the CA4 MAb (△) and HibId-1-positive anti-Hib PS serum Abs (○).

can also form a Hib PS site. Previous studies based upon partial protein sequences of V_L regions suggested that anti-Hib PS L chains may fall into two categories based upon somatic mutability: A2 V regions which mutate little if any and non-A2 V regions which do mutate (27). However, Insel and colleagues have shown that the majority of A2 V regions isolated from peripheral blood after vaccination were somatically mutated variants (16). Although not directly demonstrated, it is likely that these A2 V regions were contributors to the Hib PS repertoire, because they had the 10-amino-acid, 95a arginine-containing V_L CDR-3 that is the signature of A2 anti-Hib PS Abs. Thus, these findings when taken with the aforementioned hybridoma analyses and the results presented here, support the contention that somatically mutated variants of the A2 V region can contribute to the Hib PS repertoire. Whether these polymorphisms hold functional significance remains to be determined, but it is interesting to speculate that differences in the extent of A2 hypermutation may account for the disparities in avidity and bactericidal activity that are observed between HibId-1 Abs elicited in infants by different Hib PS conjugate vaccines (20).

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