INFECTION AND IMMUNITY, Jan. 1997, p. 261–266
0019-9567/97/$04.00+0

Vol. 65, No. 1

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

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Received 3 September 1996/Returned for modification 11 October 1996/Accepted 25 October 1996

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_κ 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_HIIIa and V_HIIIb families. V_L usage is more complex, with as many as 12 distinct Vκ and Vλ 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. Antigen-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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nated adult, can be cloned and isolated from bacteriophage libraries. The Fab fragments derived from the peripheral blood library bind Hib PS, express HibId-1, and use a V_H-V_L configuration characteristic of naturally derived Abs.

MATERIALS AND METHODS

Hybridomas and anti-Ids. The CA4 heterohybridoma secretes a human immunoglobulin G2 (IgG2), κ monoclonal Ab (MAb) specific for Hib PS. Maintenance of the hybridoma, the V region sequences, and the functional characterization of the CA4 MAb have been described previously (22). A MAb reactive with a CA4-associated Id was isolated by previously described immunization protocols and fusion methods (21). BALB/c mice received multiple immunizations with purified CA4 MAb. Three days after an intraperitoneal boost, spleen cells were taken and fused to the X63.Ag8-653 cell line. Hybridoma supernatants were screened for anti-Id activity by their ability to inhibit the binding of CA4 to Hib PS-coated microtiter wells (13, 21). A positive hybrid was identified and cloned by limiting dilution. This hybrid secreted an IgG1, κ MAb designated LuC11. LuC11 reacted with a CA4-associated Id, as shown by its ability to inhibit in a dose-dependent fashion the binding of CA4 to Hib PS in both a liquid-phase radioantigen binding assay (21) and a solid-phase enzyme-linked immunosorbent assay (ELISA) (13). LuC11 did not inhibit Hib PS binding of human MAbs ED.8 and LSF-2, which express HibId-1 and HibId-2, respectively (13). LuC11 did not react with Ig isotypic determinants, as determined in an ELISA with wells coated with either myeloma proteins representative of all the C_H isotypes and L chain types (provided by Hans Spiegelberg, University of California, San Diego), Hib PS-absorbed polyclonal IgG, or with well-defined IgM rheumatoid factors having κIII V regions (provided by Gregg Silverman, University of California, San Diego).

The isolation and characterization of murine MAb LuC9, which recognizes the $V_κ$II-A2-associated Id designated HibId-1, have been described in detail previously (21).

Isolation of human peripheral blood MNC and selection of HibId-1-positive cells. A 43-year-old white male received a subcutaneous injection of 40 μg of Hib PS vaccine (provided by Porter Anderson, University of Rochester, Rochester, N.Y.). Five days later, mononuclear cells (MNC) were isolated from 60 ml of peripheral blood with Lymphoprep (Organon Teknica, Durham, N.C.). Two cell populations were used for RNA isolation: unfractionated MNC and a HibId-1-enriched population, isolated with magnetic beads. Avidin-coated magnetic beads (Immunotech, Inc., Westbrook, Maine) were armed with biotin-conjugated LuC9 at a ratio of 50 μg of MAb per 1.0 mg of beads according to the manufacturer's instructions. Beads were washed three times with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (PBS-BSA). MNC (4×10^7) were mixed with 3 mg of LuC9-beads in a final volume of 3.0 ml of PBS containing 30% fetal calf serum. The mixture was incubated on ice for 10 min, after which time the beads were isolated by use of a magnet. The beads were washed two times with PBS-BSA by magnetic separation and then used directly for RNA isolation.

RNA isolation, cDNA synthesis, and PCR. RNA was extracted from either CA4 hybridoma cells, unfractionated MNC, or HibId-1-enriched MNC with the RNA STAT-60 (Tel-Test "B," Inc., Friendswood, Tex.) reagent according to a procedure based on the guanidium thiocyanate method (10). RNA was stored as an ethanol slurry at −70°C. cDNA was prepared from 5 μg of RNA with oligo$(dT)_{18}$ as a primer. Reverse transcriptase, nucleotides, and buffers were purchased from Pharmacia, Inc. (Piscataway, N.J.) and were used according to the instructions provided by the manufacturer.

Fd and L chain cDNA were amplified by PCR. The light chain primers used included (5′→3′) VK3a (gaaattgagctcacgcagtctcca), VK2a (gatattgagctcactcagtc tcca), VK1s (gacatcgagctcacccagtctcc), VK2dr (gatattgagctcacccagactccactctc), and humkcl (gcgccgtctagaactaacactctcccctgttgaagctctttgtgacgggcgatctcag). The Fd primers included VH1a (caggtgcagctcgagcagtctggg), VH1f (caggtgcagctgctcg agtctggg), VH2f (caggtgcagctactcgagtcggg), VH3f (gaggtgcagctgctcgagtct), VH3a (gaggtgcagctcgaggagtctggg), VH4f (caggtgcagctgctcgagtcggg), VH6a (caggtacag ctcgagcagtcagg), VH6f (caggtacagctgctcgagtcaggtcca), G1hinge (gcatgtactagttttg tcacaagatttggg), G2hinge (ctcgacactagttttgcgctcaac), CMU1 (gctcacactagtaggcag ctcagcaatcac), and ALPHA (agttgaactagttgggcagggcacagtcac) and were derived from reference 18 or designed by us. The 5′ primers for both the H and L chain genes hybridize in framework 1 and introduce restriction sites (*Xho*I for the H chain and *Sac*I for the L chain) that facilitate their directional cloning into pComb 3. For the κ chain, the 3′ primer introduces an *Xba*I site and hybridizes at the end of the C region, resulting in a PCR product that includes the entire L chain gene. The 3′ H chain primers introduce *Spe*I sites and hybridize in the hinge region, thereby producing an Fd fragment consisting of V_H and C_H1. General conditions for PCR were *Taq* polymerase (Promega, Madison, Wis.) at 1 U/100-μl reaction mixtures, 200 μM deoxynucleoside triphosphates, 1 mM $MgCl_2$, 4 μl of cDNA per 100 μl of reaction mixture, 0.4 μM 5′ primer, and 0.4 μM 3′ primer in 1× buffer as supplied by the manufacturer (Promega). Reaction mixtures were cycled at 94°C for 1 min, 52°C for 1 min, and 70°C for 1 min for a total of 25 cycles. Total PCR volumes used for library construction were 200 μl. These conditions have generated products of the correct size (≈660 bp) on all samples tested to date. PCR was also used to screen bacterial colonies for a phagemid containing the appropriate insert. In this case, a bacterial colony was suspended in 10 μl of water, and this substrate was substituted for cDNA in the reaction described above.

Cloning of L chain and Fd in bacteriophage and preparation of Fab libraries. The M13 phage surface display vector pComb3, derived from pBluescript (Stratagene, La Jolla, Calif.) and developed by Barbas and colleagues (5), was kindly provided to us by The Scripps Research Institute, La Jolla, Calif. This vector permits display of Fab fragments on the phage surface fused to the carboxy-terminal domain of the gIII minor coat protein and thus allows phage to be selected by antigen binding. The insert encoding the gIII fragment can be removed by digestion with *Spe*I and *Nhe*I, producing a construct which results in the expression of the Fab in a soluble form that can be purified from the culture supernatant and periplasmic space (see below).

pComb3 phagemid was electroporated into XL1-Blue cells (Stratagene) and grown under ampicillin-tetracycline selection, and then the phagemid DNA was purified on Qiagen-100 columns (Chatsworth, Calif.). Five micrograms of the pComb3 vector was digested with *Sac*I and *Xba*I (Boehringer Mannheim, Indianapolis, Ind.) for 3 h. The restricted DNA was purified by electroelution from agarose gels after electrophoresis. L chain PCR fragments obtained by amplification of CA4 or HibId-1-selected MNC cDNAs were digested and purified in the same manner. Vector and L chain inserts were ligated at approximately 1:3 molar ratios with T4 DNA ligase (Promega) overnight at 4°C. After ligation, the reaction was diluted 1:1 with water and heated to 65°C for 10 min, and then 5 μl of the reaction mixture was electroporated into 300 μl of electrocompetent XL1-Blue cells. The L chain library was plated for isolation of clones and propagated in bulk as an overnight culture in super broth containing 50 μg of carbenicillin per ml and 10 μg of tetracycline per ml. Individual colonies were assayed by PCR to determine the fraction containing L chain inserts. Phagemid DNA, isolated from bacteria grown in bulk culture, was digested with *Xho*I and *Spe*I and gel purified. Purified Fd PCR fragments were digested with *Xho*I and *Spe*I, purified, and ligated into the restricted plasmid to produce a combinatorial library containing both Fd and L chain genes. After electroporation, cells were coinfected with the replication-deficient helper phage VCSM13 (10^{12} PFU) and propagated overnight in SB broth containing antibiotics (kanamycin at 70 μg/ml, carbenicillin at 50 μg/ml, tetracycline at 10 μg/ml). Phage were isolated from culture supernatants by precipitation with 4% (wt/vol) polyethylene glycol (PEG) 8000, and their titers were determined based on their ability to convert XL1-Blue cells to ampicillin resistance.

Phage bearing Fab fragments on their surface were selected by panning on antigen-coated wells. Microtiter wells were coated with 25 μg of Hib PS oligomers conjugated to human serum albumin per ml. Phage (≈10^{11} PFU) were incubated in wells for 2 h at 37°C. Wells were then washed 10 times with Tris-buffered saline–5% Tween. Phage adhering to the wells were recovered by elution with 0.1 M glycine HCl (pH 2.1) for 10 min, neutralized with 2 M Tris (pH 8.0), and used to infect XL1-Blue cells. The titers of newly infected cells were obtained to determine the number of phage eluted, infected with helper phage, and grown overnight. The phage were isolated and subjected to two further rounds of panning. To identify clones producing Hib PS-specific Fab fragments, 5-ml cultures were inoculated with single colonies, induced with IPTG (isopropyl-β-D-thiogalactopyranoside), grown overnight, lysed by repeated cycles of freezing and thawing, and cleared by centrifugation, and then the lysate was screened with a radioantigen binding assay (described below). The phage binding Hib PS were expanded, and DNA was prepared for sequencing.

Sequencing of V regions. Purified phagemid DNA was sequenced directly with Sequenase 2.0 (U.S. Biochemicals Corp., Cleveland, Ohio). To sequence the Fd region, M13 reverse (−48) (agcggataacaatttcacacagga), Hgamma (ccccaagcttttag gagcagggcgccaggggggaagac), and VH3seq (cggttcaccatctccagagacaa) primers were used. L chain inserts were sequenced with the VK2dr, A2CDR2, VK3a, and HK3′ (cccccaagcttttaatcagatggcgggaagat) primers.

Purification of Fab fragments. To prepare Fab in soluble form, pComb3 phagemid DNA containing L chain and Fd genes was restricted with *Spe*I and *Nhe*I to remove the M13 gene 3 coding sequence. The digested plasmid was gel purified, self-ligated, and electroporated into XL1-Blue cells. Small-scale overnight cultures of bacterial clones containing pComb 3 phagemids were used to inoculate 1 liter of SB broth containing antibiotics (50 μg of carbenicillin per ml, 10 μg of tetracycline per ml). Cultures were grown for 6 h at 37°C, induced with IPTG (1 mM), and then cultured overnight at 30°C with shaking. Bacteria were harvested by centrifugation, and soluble Fab was extracted from the periplasmic space by incubation for 30 min on ice in 20 ml of extraction buffer (20 mM Tris [pH 7.0], 1 mM EDTA, 20% [wt/vol] sucrose). Bacteria were removed by centrifugation at 12,000 × *g*. SOL10 and SOL17 Fab fragments were isolated from the supernatant and periplasmic extract by using agarose coupled with anti-Id MAbs LuC9 and LuC11, respectively. Supernatants and extracts were mixed with ~5 ml of the respective immunoabsorbent (having 5 mg of anti-Id Ab/ml) and rotated at room temperature for 2 h. After extensive washing with PBS, the Fab fragments were eluted with 3.5 M NaSCN and dialyzed extensively against PBS at 4°C. The Fab fragments were concentrated with a Centricon-10 centrifugal concentrator (Amicon Corp., Beverly, Mass.) and then passed through a column (1.5 by 100 cm) of Sephacryl S-300 (Pharmacia), equilibrated in PBS, and run at a flow rate of 4.0 ml/h. Fractions were monitored for A_{280}, and the fractions corresponding to a molecular mass of ~50 kDa were pooled and concentrated. Results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of

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 μCi/μg) was mixed with 50 μl 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 × *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 $J_κ1$ 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_H26 germ line gene segment, a D region encoding three amino acids, and the J_H4b1 region. Sequencing of the C_H1 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_H26 (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_H26 gene, and three of four anti-Hib PS hybridomas that express the A2 V region use it in combination with V_H26 (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

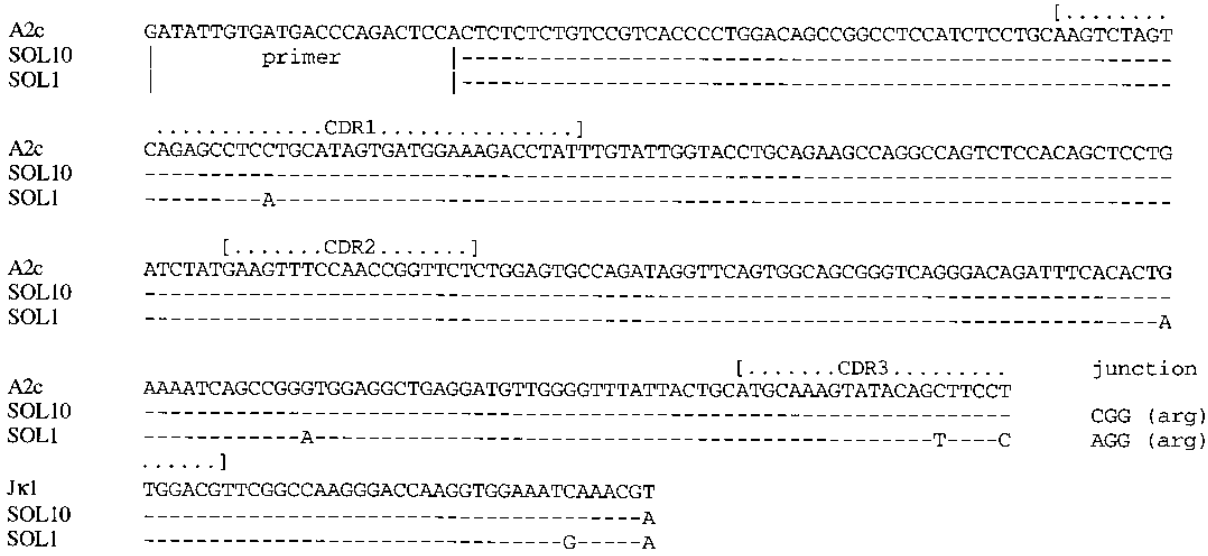

FIG. 1. Nucleotide sequences of V_L regions used by two Hib PS-specific Fab clones (SOL10 and SOL1) isolated from an MNC combinatorial library. The germ line sequences of the A2c V gene (GenBank accession no. U41644) and the $J_\kappa 1$ gene (GenBank accession no. J00242 and J00243) are shown for comparison.

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 paring 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-

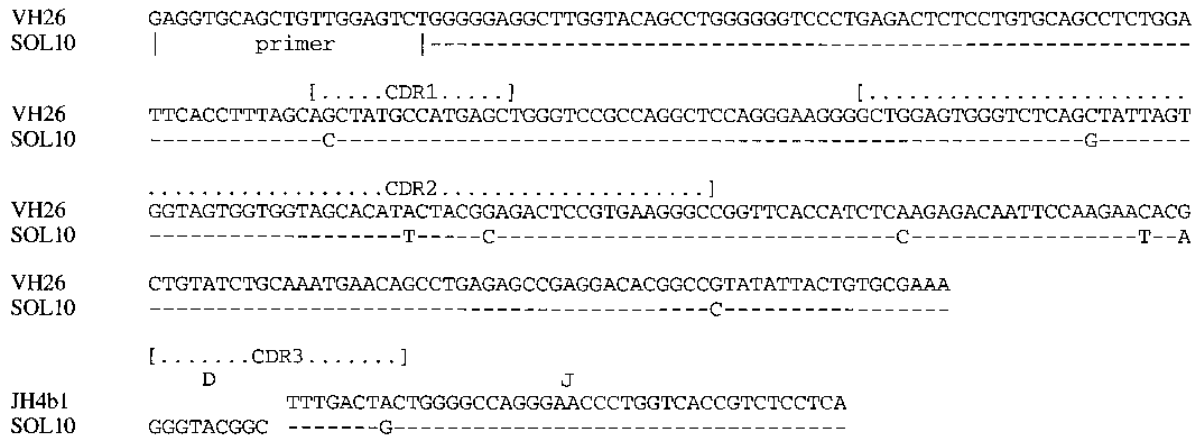

FIG. 2. V_H nucleotide sequence used by the Hib PS-specific Fab SOL10 isolated from an MNC combinatorial library. The identical V region is used by the SOL1 Fab. The germ line sequences of the V_H26 gene (GenBank accession no. J00236) and the J_H4b1 gene (GenBank accession no. X86355) are shown for comparison.

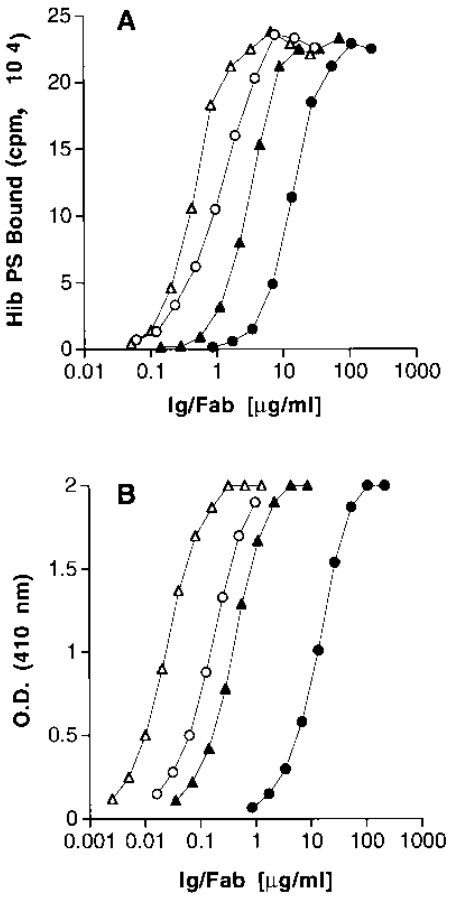

FIG. 3. Binding of Hib PS by phage-derived Fab fragments SOL17 (▲) and SOL10 (●) as determined in a radioantigen binding assay (A) and ELISA (B). SOL17 was derived from the CA4 hybridoma, and SOL10 was derived from the MNC library. Fab binding is compared with that of the control Abs the CA4 MAb (△)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

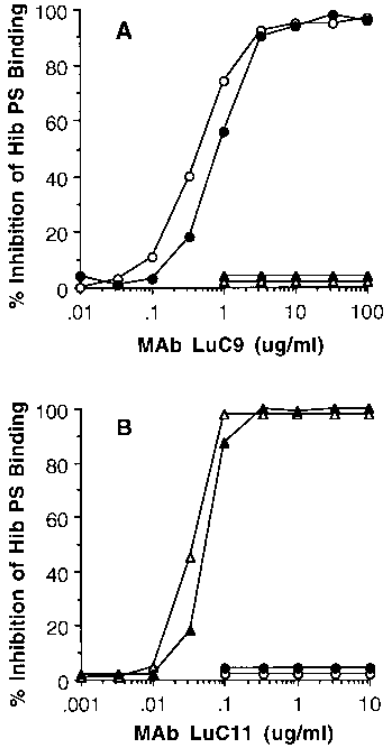

FIG. 4. Id expression by Hib PS-specific Fab fragments as determined in a radioantigen binding assay. Various concentrations of LuC9 (A) and LuC11 (B) were tested for their ability to inhibit Hib PS binding of a fixed concentration of SOL17 Fab (▲), SOL10 Fab (●) and control anti-Hib PS Abs. The control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

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 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).

ACKNOWLEDGMENTS

We thank volunteers for blood donations and Carlos Barbas and his colleagues at The Scripps Research Institute, La Jolla, Calif., for providing the pComb 3 vector and their protocols.

This work was supported by Public Health Service grant AI25008 from the National Institute of Allergy and Infectious Diseases.

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Editor: J. R. McGhee