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Immunology

Influenza virus hemagglutinin-specific antibodies isolated from a combinatorial expression library are closely related to the immune response of the donor

(viral glycoprotein/variable region gene sequences/polymerase chain reaction/monoclonal antibodies/immunotherapy)

ANDREW J. CATON* AND HILARY KOPROWSKI

The Wistar Institute of Anatomy and Biology, 3601 Spruce Street, Philadelphia, PA 19104

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ABSTRACT **Antibodies specific for the influenza virus hemagglutinin have been isolated from a combinatorial expression library generated using mRNA obtained from an immunized donor mouse. Sequence analysis indicates that the antibody heavy chain variable regions were derived from members of an expanded hemagglutinin-specific B-cell clone, in conjunction with one of two light chain variable regions. Moreover, the most frequently identified heavy chain variable/light chain variable combination is extremely similar to a heavy chain variable/light chain variable combination that has previously been identified among hemagglutinin-specific hybridoma antibodies. The results, therefore, demonstrate that these antibodies bear a close relationship to the immune status of the donor mouse and suggest that simple adaptations of this procedure might allow evaluation of the immune responses of species, such as man, in which conventional hybridoma techniques have to date proven ineffective.**

The ability to generate human monoclonal antibodies (mAbs) would enormously facilitate the production of effective therapeutic reagents as well as allow genetic analysis of the human immune response. Attempts to produce human mAbs have been limited by the failure to develop effective procedures for immortalization of human peripheral blood lymphocytes. Most procedures have required the use of Epstein–Barr virus to first transform peripheral B cells prior to fusion (1). However, this process has not proven generally effective for the isolation of mAbs with high specificity for various antigens. An alternative approach that could potentially be used would rely on the use of the polymerase chain reaction (PCR; ref. 2) to amplify immunoglobulin variable (V) regions and facilitate their introduction into bacterial expression vectors. It has recently been found that some murine heavy (H) chain variable (VH) regions expressed in bacteria, in the absence of light (L) chain variable (VL) domains, are capable of binding to certain protein antigens (3). A second approach has been described that involves the generation of separate murine VH- and VL-containing libraries in bacteriophage λ. These libraries were then combined to produce a library of phage containing both H and L chains, termed a combinatorial library, from which mAbs (in the form of Fab fragments) that could recognize the hapten *p*-nitrophenyl phosphonamidate were isolated (4). Since these approaches rely on molecular biological techniques rather than cell fusion to immortalize particular antibody sequences, they have the potential to be readily adapted to generate human mAbs. However, because of the procedures that are used in their construction, how such antibodies might relate to the immune response of an individual is unclear. This is particularly apparent in the latter approach, which due to its capacity to produce a diverse array of VH/VL combinations might result in the formation of antibody structures that are not, or cannot usually be, found within a host.

In this report, we describe the construction of a combinatorial library from which mAbs that recognize a viral glycoprotein, the influenza virus hemagglutinin (HA), were isolated. HA is a well-characterized protein with regard to its antigenic structure and to its immune recognition (5, 6). In particular, an extensive panel of HA-specific hybridoma mAbs has been produced, and many of these antibodies have been analyzed for the sequences of their VH and VL regions (7–9). We wished to compare the antibodies that were generated from a combinatorial library with those produced by conventional hybridoma techniques, in terms of their diversity of V gene expression as well as their relationship to the immune status of the host. The structural diversity of HA-specific antibodies isolated from this library is limited. All of the HA-specific antibodies utilized a VH region derived from members of an expanded HA-specific B-cell clone. Strikingly, this VH region was in most cases found in association with the same L chain, and this VH/VL combination closely resembles a VH/VL combination that has previously been identified among HA-specific hybridoma mAbs. The results, therefore, demonstrate that despite the scrambling of VH and VL regions that was performed during construction of the library, the mAbs that were isolated bear a close relationship to the immune response of the donor mouse from which the library was generated.

MATERIALS AND METHODS

Viruses and Immunization. Influenza viruses A/PR/8/34 (PR8) and J1 were grown and purified, and their titer was determined as described (10). J1 is a reassortant virus that is genetically identical to PR8 but contains a serologically noncrossreactive (H3 subtype) HA (11). For immunization, a BALB/c mouse (Jackson Laboratories) was primed 24 days and given a booster injection 3 days prior to sacrifice and splenectomy with 1000 hemagglutinating units of PR8 by i.p. and i.v. routes, respectively. Hybridoma H36-6 was generated after similar immunization of a BALB/c mouse with PR8, followed by fusion with SP2/0-Ag14 cells as described (12).

Library Construction. Total spleen RNA was prepared by the guanidinium isothiocyanate procedure followed by several rounds of ethanol precipitation (13). Half of this RNA was used in a 500-μl cDNA reaction mixture containing 0.1 M Tris·HCl (pH 8.3), 140 mM KCl, 10 mM $MgCl_2$, 20 mM

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Abbreviations: PCR, polymerase chain reaction; V, variable; H, heavy; L, light; C, constant; VH, heavy chain variable; VL, light chain variable; HA, hemagglutinin; BSA, bovine serum albumin; CDR, complementarity determining region; mAb, monoclonal antibody.

*To whom reprint requests should be addressed.

2-mercaptoethanol, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, 50 units of reserve transcriptase (Life Sciences, Saint Petersburg, FL), and 100 ng of oligonucleotide H12 and 100 ng of oligonucleotide L9 that hybridize to Cγ1- and Cκ-specific sequences, respectively (see ref. 4 for a full description of the oligonucleotide primers and their nomenclature; C stands for constant). After incubation for 1 hr at 42°C, the mixture was boiled and chilled on ice. A solution (900 μl) containing all four dNTPs, each at 0.33 mM, and 15 units of *Thermus aquaticus* polymerase (Perkin–Elmer/Cetus) was added, and 15 samples were removed and added either to 100 ng of oligonucleotide H12 and 100 ng of one of the oligonucleotides H1–H10 or to 100 ng of oligonucleotide L9 and 100 ng of one of the oligonucleotides L3–L7. The reaction mixtures (100 μl, final volume) were then subjected to 30 consecutive rounds of incubation at 50°C for 1 min, 72°C for 2 min, and 94°C for 30 sec, followed by a final incubation at 50°C for 1 min and 72°C for 10 min. Reaction mixtures were concentrated and applied to a 3% NuSieve GTG gel (FMC) containing ethidium bromide and DNA was visualized by brief exposure to ultraviolet light. Reaction products in the 700-base-pair (bp) size range were purified using GeneClean (Bio101) and digested with the appropriate restriction enzymes (4). The products were added in various amounts to restriction enzyme-digested and phosphatase-treated arms of the vectors λHC-2 and λLC-1 (4), which were prepared by standard methods (13), and packaged using Gigapack Gold (Stratagene). Libraries were titrated and assayed for the presence of Cγ1- or Cκ-specific sequences (see below), and the libraries containing the highest proportions of recombinants were plated at 5000–10,000 phage per 15-cm plate. Phage were eluted by soaking in SM (50 mM Tris·HCl, pH 7.5/0.1 M NaCl/10 mM $MgCl_2$) (13) and purified by banding to the interface of 3 M and 5 M CsCl in SM by ultracentrifugation in a SW 41 rotor (Beckman) for 2 hr at 22,000 rpm. Phage DNA was purified, and ≈10 μg of DNA was digested with appropriate restriction enzymes, treated with alkaline phosphatase, digested with *Eco*RI, religated, and packaged as described (4, 13). This library was stored at 4°C and analyzed directly in all screening procedures.

Identification and Characterization of HA-Specific Antibodies. Recombinant phage were plated using the bacterial strain XL-1 blue (Stratagene) and the expression of antibodies was induced by overlay of isopropyl β-D-thiogalactoside-soaked nitrocellulose as described (4, 13). Filters were first blocked in phosphate-buffered saline (PBS)/3% (wt/vol) bovine serum albumin (BSA) and then incubated for 2–4 hr at 25°C with PR8 virus at 1000 hemagglutinating units/ml in PBS/3% BSA. Filters were washed extensively with PBS/0.1% BSA and then fixed by incubation with 3% (wt/vol) paraformaldehyde for 30 min at 25°C. The filters were washed, incubated with blocking solution, and then incubated at 4°C for 16 hr with HA-specific hybridoma mAb supernatant diluted 1:100 in PBS/3% BSA. The filters were washed as above and incubated for 2–4 hr at 25°C with a 1:1000 dilution of a goat anti-murine Fc-alkaline phosphatase conjugate (Sigma) in PBS/3% BSA, which had been preadsorbed with filters that were identical to those being screened except that neither virus nor HA-specific antibody had been added. Filters were washed as above and developed for the presence of alkaline phosphatase using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as described (13). Recombinant phage were plaque-purified and plasmid was excised into the Bluescript vector (Stratagene) as recommended by the manufacturer. Bacterial cultures (50 ml) were grown, antibody expression was induced as described (4), and the culture supernatants were concentrated 50- to 100-fold and dialyzed into PBS using colloidon dialysis/concentration bags (Schleicher & Schuell). Antibody binding to purified PR8 or J1 viruses was analyzed in the ELISA using serial dilutions of culture fluid followed by detection first with a murine Cκ-specific rat mAb/biotin reagent, then with extravadin-alkaline phosphatase (Sigma), and finally development with *p*-nitrophenyl phosphate essentially as described (14).

Nucleic Acid Sequence and Hybridization Analysis. Sequence analysis of plasmids was performed using Sequenase (United States Biochemical) under standard conditions for analyzing double-stranded DNA, using Cγ1- and Cκ-specific oligonucleotides (8) to direct analysis of the antibody V regions. Oligonucleotide hybridizations were performed using 5′-^{32}P-labeled oligonucleotides at 1 ng/ml in 5× SSPE (1× SSPE is 0.18 M NaCl/20 mM sodium phosphate, pH 7/1 mM EDTA)/5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% BSA)/0.5% SDS/denatured DNA (100 μg/ml). Filters were hybridized for 16 hr at their melting temperature −4°C, washed extensively at 25°C in 2× SSPE/0.2% SDS, and given a final wash in 5× SSPE/0.2% SDS at their melting temperature −2°C for 10 min (15). The λαPR8 chain and λαPR8-1 L chain junction-specific oligonucleotides had the sequence 5′-d[AGTCG(C/G)TACTATGGTAGTTT]-3′ and 5′-d(CGTCCGTAGAAGATTATCAT)-3′, respectively. The Cγ1- and Cκ-specific oligonucleotides have been described (8). Oligonucleotides were synthesized and purified by HPLC at the Wistar Institute DNA synthesis facility. Sequence analysis of the hybridoma H36-6 was performed as described (8).

RESULTS

Library Construction. A combinatorial library was constructed essentially as described (4), using mRNA from the spleen of a mouse immunized with influenza virus PR8. The 10 characterized VH-region specific and five Vκ-region specific oligonucleotide primers were used in conjunction with Cγ1- and Cκ-specific oligonucleotides to amplify sequences corresponding to the Fab portion of rearranged IgG1 H chain and Igκ L chain sequences. Independent amplification reactions were performed, and the reaction products were size-fractionated and purified by agarose gel electrophoresis. The amounts of the major 700-bp product obtained with the VH region-specific primers were variable; in particular, the H3 and H9 primers gave poor yields (Fig. 1). The Vκ region-specific primers all yielded roughly equal amounts of a major

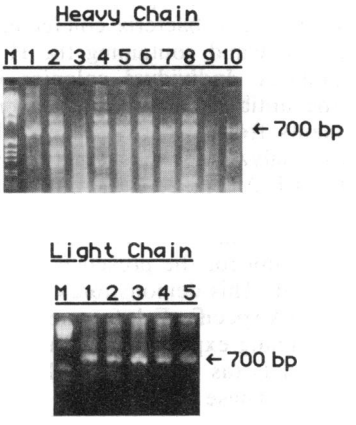

FIG. 1. Agarose gel electrophoresis of reaction products obtained from enzymatic amplification of rearranged V regions from immunized mouse spleen mRNA. For the H chains, VH-specific oligonucleotides H1–H10 (4) (lanes 1–10) were used in conjunction with the Cγ1-specific oligonucleotide H12. For the L chains, the Vκ-specific oligonucleotides L3–L7 (lanes 1–5) were used in conjunction with the Cκ-specific oligonucleotide L9. Lanes M contain a 1-kilobase ladder of molecular weight markers (Bethesda Research Laboratories). The location of the 700-bp reaction product is indicated.

700-bp reaction product corresponding to the rearranged L chain (Fig. 1). In a separate experiment, the same primers were used under identical reaction conditions to amplify rearranged IgG1- and Igκ-specific sequences using RNA isolated from the spleen of an unimmunized mouse. The Igκ-specific primers yielded equivalent amounts of the 700-bp reaction product as were obtained from the immunized spleen. However, little or none of the 700-bp product was detected using the IgG1-specific primers (data not shown). In view of the sensitivity of the PCR, the failure to obtain specific amplification of the IgG1-specific sequences was unexpected. This finding is nevertheless consistent with the previous reports describing the construction of libraries using VH-specific primers, which generally have involved prior immunization of the donor mouse (3, 4).

The individual reaction products were pooled and digested with appropriate enzymes to cleave restriction sites that were introduced by the primers used for amplification. The VH- and Vκ-specific reaction products were introduced separately into the bacteriophage λ expression vectors λHC2 and λLC1, respectively (4). A H chain library containing 125,000 phage and a L chain library containing 550,000 phage were generated. Approximately 95% of the phage from the H chain library and >95% of the phage from the L chain library were determined to contain Cγ1- and Cκ-specific sequences, respectively, based on their ability to hybridize to Cγ1- and Cκ-specific oligonucleotide probes. Each library was plated, phage particles were isolated, and DNA was purified and digested with restriction enzymes that destroyed either the right or left arm of phage DNA from each library. The remaining arms were then digested with *Eco*RI, mixed in equal weight, and ligated together. A combinatorial library containing 2.5×10^7 phage particles was generated, of which 75% were found to contain Cγ1-specific sequences and 100% contained Cκ-specific sequences.

Identification of HA-Specific Antibodies. Phage from the combinatorial library were plated and screened directly (without amplification) for their ability to bind intact PR8 virus particles (schematically represented in Fig. 2). In the first experiment, 125,000 phage per library from the VH-containing, the Vκ-containing, and the combinatorial libraries were screened. Ten strong positive phage were identified among phage from the combinatorial library; no strong candidates were identified in the library containing only VH- or Vκ-specific sequences. Positive phage were rescreened, and four phage were grown in bacteria coinfected with helper phage to excise a plasmid containing the H and L chain expression sequences. Individual colonies were grown, expression of the antibody sequences was induced, and culture supernatants from two of the recombinants were concentrated and analyzed for their ability to bind to influenza virus in the ELISA (Fig. 3). Supernatants from λαPR8-1- or λαPR8-9-infected bacteria showed clear binding to PR8 virus but not to the reassortant virus J1, which is genetically identical to PR8 except for the presence of a serologically noncrossreactive HA. This demonstrates that λαPR8-1 and λαPR8-9 produce HA-specific Fab fragments.

In a second screening experiment, several recombinants that gave weak signals (as well as one that gave a strong signal) were isolated. These recombinants continued to give weak signals in rescreening and yielded Fab fragments that did not bind either PR8 or J1 in the ELISA (data not shown). It appears then that the intensity of the signal obtained during screening may reliably distinguish between antibodies of high and low specificity or high and low affinity.

Sequence Analysis of HA-Specific Antibodies. Plasmids isolated from four of the recombinants from the first screening and from the strong positive identified in the second screening were subjected to partial sequence analysis of their VH and Vκ regions (Fig. 4). The antibody H chains were derived

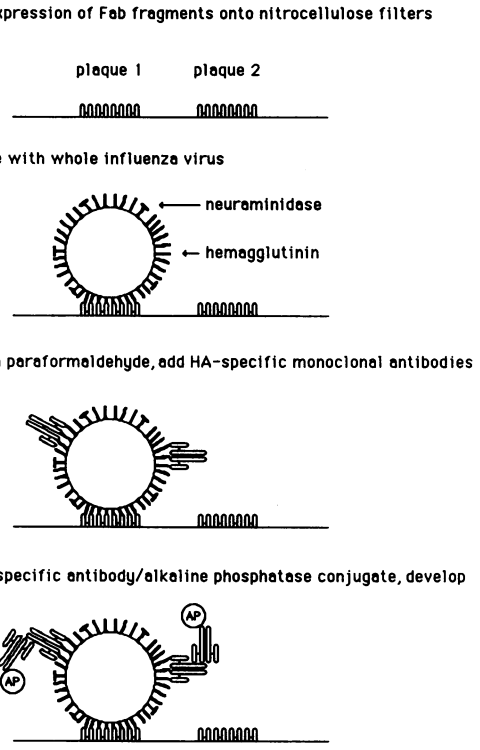

FIG. 2. Schematic representation of screening protocol used to identify recombinant phage secreting HA-specific antibodies. AP, alkaline phosphatase.

from the J558 gene family (19, 21) and display a high degree of homology including sequences of the third complementarity determining region (CDR3). Since this junctionally encoded region includes nucleotides that were generated by the random addition of *N* nucleotides during H chain assembly (22), this homology indicates that these H chains were derived from members of a single B-cell clone (23). There exist several individual differences between the H chain sequences and, at three positions, substitutions are shared by two or more of the antibodies. This overall pattern of mutation resembles that observed among clonally related antibodies that have been isolated by hybridoma techniques (7, 8, 23). Analysis of the L chain sequences indicates that four of the antibodies use L chains from the Vκ9 gene group

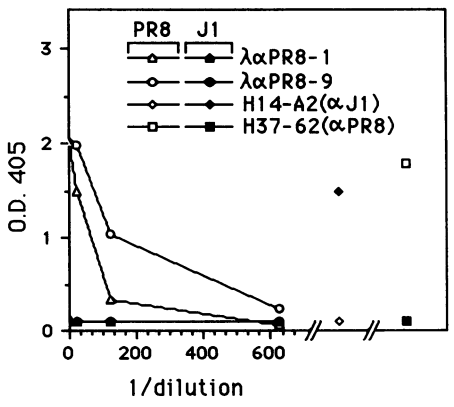

FIG. 3. Binding activity of antibodies to influenza viruses bearing serologically noncrossreactive HAs. Concentrated culture supernatants were incubated at various dilutions with influenza viruses PR8 (open symbols) and J1 (closed symbols), which had been adsorbed to plastic plates. Antibody binding was detected using a biotin-labeled anti-murine Cκ reagent followed by development in the ELISA. Also shown is the binding obtained with a 1:500 dilution of an anti-PR8 hybridoma mAb supernatant (H37-62; ref. 12) or an anti-J1 HA hybridoma mAb supernatant (H14-A2; ref. 16).

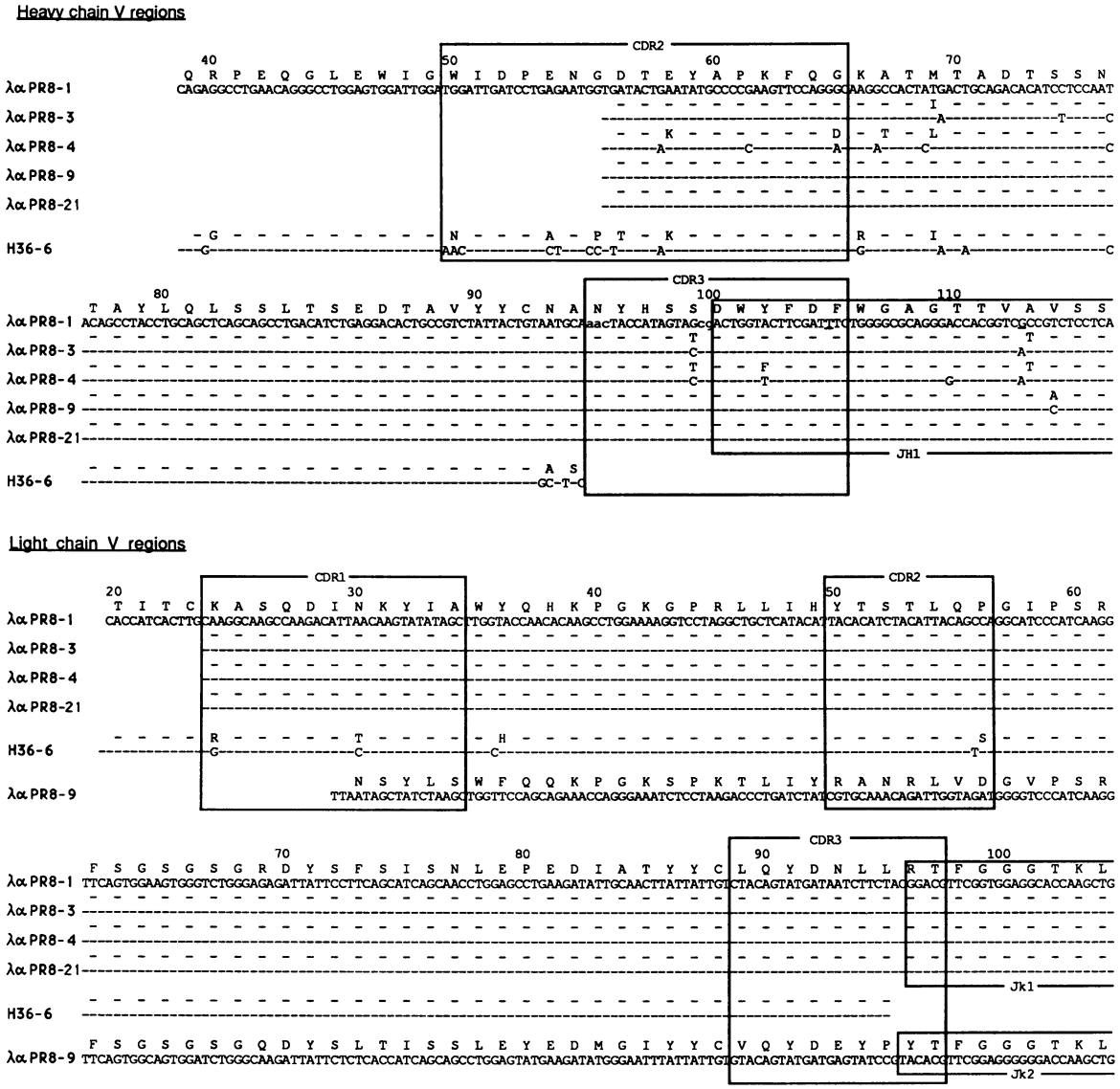

FIG. 4. Partial sequence analysis of antibody H and L chain V regions. The nucleotide and deduced amino acid sequences are shown relative to those of λαPR8-1, except for the L chain sequence of λαPR8-9, which is shown in full. Dashes indicate identity with the λαPR8-1 sequence. The VH and Vκ region sequences of hybridoma mAb H36-6 are shown for comparison. The amino acid sequences are numbered according to Kabat (19), and the locations of sequences encoding CDR regions and J regions are indicated. In the λαPR8-1 CDR3 sequence, lowercase letters indicate possible N region nucleotides; these N sequences bound an inverted DSP2.5 germ-line gene element (20), which is in capital letters. The underlined nucleotides at amino acids 105 and 112 in the JH1 sequence indicate mutations relative to the JH1 germ-line gene sequence (19).

(19) that have identical sequences over the region examined, whereas the fifth (λαPR8-9) uses a different member of the Vκ9 gene group. Thus, although the majority of the antibodies express a common VH/Vκ combination, unique VH/Vκ combinations can be generated by this approach. When the λαPR8-1 VH and Vκ sequences were used separately to search a data base of VH and Vκ sequences identified among >50 PR8 HA-specific hybridoma mAbs, greatest similarity was found with the VH and Vκ regions of the same hybridoma, H36-6 (Fig. 4). Accordingly, this specific VH/Vκ gene combination is closely related to that of an identified HA-specific hybridoma mAb.

In view of the repetitive isolation of identical or closely related antibody sequences, we determined the relative representation of these sequences and the overall degree of structural diversity of the library. Oligonucleotide probes that correspond to the junctionally encoded sequences of the H chain CDR3 regions and of the L chain CDR3 region of λαPR8-1 were used to screen duplicate filters containing 20,000 phage from the combinatorial library. The H chain CDR3 probe hybridized to 97 plaques, indicating that this H chain sequence is present at a frequency of ≈1 in 200. The L chain probe hybridized to 18 plaques, indicating a frequency of ≈1 in 1100. The combined frequency (1 in 22,000) is roughly comparable to the frequency with which antigen-specific phage were identified (1 in 12,500). To assess the overall diversity of the library, 12 plaques were isolated at random, and plasmids were excised and analyzed for their VH- and Vκ-specific sequences. Eight of the plasmids contained VH-specific sequences, consistent with the frequency estimated by hybridization with a Cγ1-specific probe; these sequences were derived from either the J558 or the X24 gene families (refs. 21 and 24; data not shown). The plasmids yielded Vκ-specific sequences, which were derived from the Vκ1, the Vκ4/5, and the Vκ9 gene groups (ref. 19; data not shown). Based on this random sampling, the overall diversity of gene family representation in this library is fairly limited, although it remains possible that other VH and Vκ families

are represented but at frequencies that are too low to have been detected in this sample.

DISCUSSION

mAbs specific for the influenza virus HA have been isolated from a combinatorial library that was generated using mRNA derived from a mouse that had been immunized with influenza virus. Sequence analysis indicated that these antibodies used VH regions derived from members of a single B-cell clone. Most of these antibodies expressed this VH region in association with a specific L chain; however, in one case a different L chain was used, demonstrating the capacity of such libraries for combinatorial diversification of individual V gene sequences through different pairwise associations to produce novel VH/VL combinations. The high structural similarity of the antibodies reflects two major factors that influence the representation of individual sequences in the library. The first is that procedures used to produce this library clearly acted to limit its overall structural diversity. In particular, the VH library contained sequences that were predominantly derived from two gene families (J558 and X24). The presence of large numbers of J558 gene family members is consistent with it being the largest gene family (21). The X24 gene family, on the other hand, contains only two members (24) and is relatively infrequently observed either among B cells from BALB/c mice in general (17) or among HA-specific hybridoma antibodies (refs. 7–9; unpublished observations). Its frequent representation (and concordant absence of members of other families) is most likely a consequence of the particular primers that were used for library construction (or of the specific conditions under which they were used here) and suggests a need for refinement of the procedures for amplification and expression of heterogeneous populations of V gene sequences.

A second major factor that influenced the relative representation of individual VH and Vκ sequences was the use of mRNA from an immunized individual to construct the library, since this enriched the library for those sequences that were represented among B cells that proliferated in response to the immunizing agent. All antibodies isolated here used H chains derived from the same B-cell clone, as indicated by their sharing of junctionally encoded sequences that act as unique markers for common clonal origin (23). The pattern of individual differences between these sequences is extremely similar to that observed among clonally related HA-specific hybridoma antibodies (7, 8), suggesting that the high representation of these sequences is a consequence of antigen-specific B-cell clonal expansion in the immunized donor mouse. It is possible that some of these differences were introduced during the PCR, which has the capacity to introduce mutations (18). However, detailed examination of the sequences suggests that it is unlikely that all of these differences were introduced during amplification. The antibodies all share a nucleotide difference from the JH1 germ-line gene sequence that changes the germ-line encoded valine at position 105 to phenylalanine. For this difference to have been caused by PCR, it must have been introduced in one of the first rounds of amplification to give rise to the various H chains. The three shared differences (at amino acids 76, 99, and 113) by which $\lambda\alpha$PR8-3 and $\lambda\alpha$PR8-4 systematically differ from the other antibodies would then necessarily have had to have been introduced in the next few rounds of amplification. This model requires the introduction of mutations at a rate that is much greater than has been determined for PCR-induced mutation (18), and it is much more likely that most of these differences were introduced during expansion of an HA-specific B-cell clone.

The scrambling of VH and VL gene segments that occurs during library assembly has the capacity to generate unusual specificities and to remove from the library the combinatorial information that is represented by the specific VH/VL combinations that are induced during an immune response. It is noteworthy that the antibodies described here do not represent random combinations of VH and VL gene segments. Despite its frequent representation, this expanded H chain was identified in association with one of only two L chains. Moreover, the most frequently isolated L chain gave rise to a VH/Vκ combination that is extremely similar to a VH/Vκ combination that has previously been identified among HA-specific hybridoma mAbs. Thus the results indicate that the antibodies that were isolated bear a close relationship to the immune status of the donor mouse. Based on these findings, it seems likely that the adaptation of these procedures to the generation of libraries from human peripheral blood lymphocytes may provide an effective means of assessing the immune responses of humans to a variety of pathogenic agents.

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