J. Biochem. 125, 123-129 (1999)

Recombinant Human Monoclonal Fab Fragments against Rotavirus from Phage Display Combinatorial Libraries[1]

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Received July 17, 1998; accepted October 1, 1998

We prepared and characterized human monoclonal Fab fragments to rotavirus from IgG_1 κ combinatorial libraries (designated as N and O) constructed from peripheral blood lymphocytes (PBLs) from two healthy individuals. Approximately 30-fold enrichment in eluted phage was obtained in these libraries after five rounds of panning against rabbit polyclonal antibody-captured human rotavirus (HRV) Wa strain. Forty-eight clones from each library were tested for reactivity to HRV Wa in an enzyme-linked immunosorbent assay (ELISA), and the identities of positive clones were determined by *Bst*NI fingerprinting. As a result, eight individual clones (five from N library and three from O library) were isolated. In testing the cross-reactivity of Fabs against a panel of self- or non-self antigens, all Fab clones were found to be specific for HRV Wa. Fab clones from the two libraries showed distinct characteristics with respect to their reaction patterns with Wa and crossreactivities with rotavirus strains, and displayed variable heavy (V_H) chain gene usage, although they recognized the VP6 protein as determined by immunoblotting. The distinct epitope recognition by Fabs from two libraries suggests different courses of humoral immune response to rotavirus during infection in the two individuals.

Key words: combinatorial library, human antibody repertoire, phage display, recombinant Fab fragment, rotavirus infection.

Antibody phage display (*1, 2*) is a recently developed recombinant DNA technology for making human monoclonal antibodies that will replace generally used procedures, *e.g.*, production of heterohybridomas (*3*) and Epstein-Barr virus immortalization of human B cells (*4*). Human monoclonal antibody fragments can be obtained by cloning PCR-amplified heavy- and light-chain gene segments originating from immune cells, such as bone marrow cells, lymph node cells, or peripheral blood lymphocytes (PBLs), into a phage display vector, followed by panning of the phage-displayed antibody libraries against antigens of interest. The isolation of human monoclonal antibody fragments against viral pathogens or self-antigens by phage display has been reported (*5-17*). Antibody phage display technology would therefore provide more powerful diagnostic and therapeutic tools than the currently used polyclonal or monoclonal antibodies.

Rotaviruses are the leading cause of severe, life-threatening viral gastroenteritis and dehydrating diarrhea in young children and animals (*18*), and are associated with sporadic outbreaks of diarrhea in elderly (*19*) and immunocompromised patients (*20*). The rotavirus genome, which consists of 11 segments of double-stranded RNA encased within a triple-layered capsid, codes for six structural proteins (VP1, VP2, VP3, VP4, VP6, and VP7) and five non-structural proteins (NSP1-NSP5) (*21*). Among the structural proteins, VP4, VP6, and VP7 are considered to be important in defining the antigenic specificity of the virus. Two viral proteins in outermost protein coat, the viral hemagglutinin VP4 (*21*) and the viral glycoprotein VP7 (*21*), have been directly implicated as targets of serotype-specific neutralization *in vitro* and protection *in vivo* (*22*). The second protein layer component, VP6, makes up about 50% of the virion mass and is highly immunogenic. Because of its predominance in the virion, VP6 serves as the target of most diagnostic assays. In addition to the group A antigen, another antigenic specificity, termed subgroup, is present on VP6 (*23*). While antibodies to VP6 do not have neutralization activity *in vitro* (*22, 24*), VP6 appears to be the primary determinant of intracellular neutralization in infected epithelial cells *in vivo* (*25*). Although clinical studies have shown that

[1] This work was supported in part by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan. The nucleotide sequence data of mRNA for the anti-HRV Fabs' variable heavy and light chains reported in this paper are available from DDBJ/EMBL/GenBank under accession numbers, AB006834-49.

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Abbreviations: BSA, bovine serum albumin; BSA-PBS, 1% (w/v) BSA in PBS; CDR, complementarity determining region; ELISA, enzyme-linked immunosorbent assay; HRV, human rotavirus; IPTG, isopropyl β-D-thiogalactopyranoside; OD, optical density; PBLs, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RRV, rhesus rotavirus; SB, super broth; Tween-PBS, 0.05% (v/v) Tween 20 in PBS; V_H, variable heavy; V_L, variable light.

primary infection induces natural immunity to rotavirus and protects a child from subsequent episodes of severe disease (*26, 27*), the humoral immune response against rotavirus during infection is not yet fully understood.

In this study, we have attempted to optimize the phage display system for efficient human antibody production, and to clarify the humoral immune response to rotavirus during infection. In this effort, we isolated human monoclonal Fab fragments from PBLs of two healthy individuals with high serum titers to rotavirus. To achieve the above purposes, we designed a VP6-directed selection system based on the antibody capture procedure since purified VP6 protein was not available. The rabbit polyclonal antibody against human rotavirus (HRV) Wa and HRV Wa infected vero cell culture supernatant were used as a capture antibody and a captured antigen, respectively. Because polyclonal antibodies react with all viral proteins depending on their quantity, the major constituent was expected to be the VP6 protein. Here we report that human monoclonal Fab fragments specific for rotavirus VP6 protein can be efficiently isolated using optimized phage display and designed selection systems, and that the distinct immunological properties of the Fab clones from two libraries can mainly be attributed to molecular structural differences in their heavy chain variable regions.

MATERIALS AND METHODS

Virus Strains and Antibodies—HRV strain Wa, AU-1, AU64, and rhesus rotavirus (RRV) were maintained at the Department of Microbiology, Akita University School of Medicine. These strains were propagated in vero cells, and the cleared culture supernatants were used as antigens. The antigen content in the culture supernatants was predetermined and optimized before use for panning and enzyme-linked immunosorbent assay (ELISA). Rabbit polyclonal antibody against HRV Wa was purified by saturated ammonium sulfate precipitation and anion exchange chromatography from HRV Wa-hyperimmunized rabbit sera. The antibody concentration was determined by monitoring the optical density at 280 nm (OD_{280}), and the optimal concentration was determined by ELISA before use.

Selection of Donors—Serum was obtained from eleven healthy individuals (five males and six females), and the presence of antibodies against HRV Wa was tested by sandwich ELISA (see below). When the absorbance at 405 nm of a 1/500 dilution of serum sample was above 1.5, the serum was considered to show a high titer to HRV Wa.

Lymphocyte RNA Preparation and cDNA Synthesis—Fifty milliliters of heparinized venous blood was collected from a positive donor, and PBLs were separated by density gradient centrifugation using Histopaque 1077 (Sigma, St. Louis, MO). Total RNA from the purified PBLs (1×10^7) was extracted with an RNA isolation kit (Stratagene, La Jolla, CA) based on the guanidinium isothiocyanate extraction procedure (*28*). First-strand cDNA was synthesized from 5 μg of total RNA using an oligo $(dT)_{15}$ primer and an AMV reverse transcriptase first-strand cDNA synthesis kit (Life Sciences, St. Petersburg, FL).

PCR Amplification of Immunoglobulin Gene Segments—After reverse transcription, the γ1 Fd region and whole κ light chain were amplified by PCR with family-specific variable region and isotype-specific constant region primers (*29*). Reactions were carried out with the GeneAmp 9600 PCR system (Perkin-Elmer, Norwalk, CN) using 35 cycles of 15 s at 94°C, 1 min at 55°C, and 1 min at 72°C. Amplification was completed with a single cycle of 10 min at 72°C. PCR products of heavy- and light-chain gene segments were separately pooled and purified on 2% Seakem GTG agarose gels (FMC, Rockland, ME). Bands of approximately 660 bp were excised from the gel, and the DNA was extracted and purified with a QIAEX II gel extraction kit (QIAGEN, Santa Clarita, CA). To increase the efficiency of restriction enzyme digestion and subsequent library construction, purified PCR products were reamplified with extension primers containing a poly (GA) tail 5′ to the sequence of the original primers using the same reaction conditions as the original PCR (*9*). Amplified products were gel-purified and extracted as described above.

Construction of Immunoglobulin Combinatorial Library—Five micrograms of the purified extension PCR products were double-digested with restriction enzymes corresponding to the cloning site of the heavy chain (17 U/μg of *Spe*I and 70 U/μg of *Xho*I, Boehringer Mannheim, Indianapolis, IN) and light chain (35 U/μg of *Sac*I and 70 U/μg of *Xba*I, Boehringer Mannheim) for 3 h at 37°C. Digested products were purified on CHROMA SPIN 100 columns (CLONTECH, Palo Alto, CA). Light chain fragments (450 ng) were ligated into phage display vector pComb3 (*30*) (1 μg) using 10 U of T4 DNA ligase (Life Technologies, Bethesda, MA). The light chain libraries obtained were then electroporated into XL1-Blue cells (Stratagene, La Jolla, CA) by a Gene Pulser (Bio-Rad, Richmond, CA), and cultured overnight at 37°C in Super Broth (SB) with 50 μg/ml of carbenicillin and 20 mM glucose. The light chain library DNA was extracted from packed cells by the Wizard Minipreps DNA purification system (Promega, Madison, WI), and double-digested for 3 h at 37°C with 5 U/μg of *Spe*I and 10 U/μg of *Xho*I. The combinatorial libraries were finally constructed by ligating 450 ng of heavy chain fragments with 1 μg of gel-purified light chain libraries using 10 U of T4 DNA ligase. The constructed libraries were electroporated into XL1-Blue cells, and cultured in SB with 10 μg/ml of tetracycline and 50 μg/ml of carbenicillin. The phage libraries displaying recombinant Fabs were then rescued by infection with VCSM13 helper phage (Stratagene).

Library Panning for HRV Wa Binding Phage—The wells of an ELISA plate (Costar, Cambridge, MA) were coated overnight at 4°C with 50 μl of 4 μg/ml rabbit polyclonal antibody. The wells were washed once with phosphate-buffered saline (PBS) and blocked by completely filling the wells with 1% (w/v) BSA in PBS (BSA-PBS) for 1 h at 37°C. The BSA-PBS solution was discarded and 50 μl of a 1/5 dilution of HRV Wa was added to each well and incubated for 1 h at 37°C. The wells were washed five times with 0.05% (v/v) Tween 20 in PBS (Tween-PBS), and 50 μl of phage library (typically 10^{11} colony forming units) was added to each well and incubated for 2 h at 37°C. The unbound phage was discarded and the wells were washed vigorously 10 times with Tween-PBS. The bound phage was eluted with 0.1 M glycine-HCl (pH 2.2) by pipetting up and down several times. The eluted phage was neutralized with 2 M Tris base and used to infect 2 ml of XL1-Blue culture for 15 min at room temperature. SB

containing antibiotics and helper phage was added sequentially as described (*30*) to amplify the eluted phage. The resulting phage library was then reapplied to rabbit polyclonal antibody-captured HRV Wa to initiate a new round of panning. Five rounds of panning were carried out in this study.

Preparation of Soluble Fab Fragments—The selected library was reengineered for soluble Fab expression (*6, 30*). Briefly, 5 μg of plasmid DNA isolated after the final round of panning was double-digested with 10 U/μg each of *Spe*I and *Nhe*I (Boehringer Mannheim) to remove the coat protein III gene segment, self-ligated, and electroporated into XL1-Blue cells. Single colonies were picked and cultured at 37°C in 10 ml SB with 50 μg/ml carbenicillin and 20 mM $MgCl_2$ until an OD_{600} of 0.2 was achieved. Isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM was then added and culture was continued overnight at 30°C. The bacterial cells were pelleted by centrifugation, resuspended in 0.5 ml of PBS containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and lysed by four cycles of freezing and thawing. Cell debris was pelleted by centrifugation and the supernatants containing the soluble Fab were tested by sandwich ELISA as described below.

ELISA Procedures—To test the reactivity of Fabs with HRV Wa, the wells of an ELISA plate (Costar) were coated overnight at 4°C with 50 μl of 0.67 μg /ml rabbit polyclonal antibody. The wells were washed once with PBS and blocked with BSA-PBS for 1 h at 37°C. The solution was discarded and 50 μl of a 1/10 dilution of HRV Wa was added to each well and incubated for 1 h at 37°C. The wells were then washed five times with Tween-PBS, and 50 μl of soluble Fab was added to each well and incubated for 2 h at 37°C. The wells were washed 10 times with Tween-PBS, and treated with 50 μl of a 1/500 dilution of alkaline phosphatase-labeled rabbit anti-human IgG $F(ab)'_2$ (Jackson ImmunoResearch, West Grove, PA). After incubation for 1 h at 37°C, the wells were washed as above, and treated with 50 μl of *p*-nitrophenyl phosphate (1 mg/ml, Sigma 104 phosphatase substrate, Sigma) in 1 M diethanol amine buffer (pH 9.8). Color was developed at 37°C, and the absorbance of the resultant *p*-nitrophenol was measured at 405 nm using a microplate reader (Model 550, Bio-Rad).

To estimate the Fab content in crude lysates, the wells of an ELISA plate (Costar) were coated overnight at 4°C with 50 μl of 1 μg/ml goat anti-human IgG $F(ab')_2$ (Jackson ImmunoResearch). The wells were washed once with PBS and blocked with BSA-PBS for 1 h at 37°C. The solution was discarded and 50 μl of serially diluted bacterial lysate was added to each well and incubated for 1 h at 37°C. The wells were washed 10 times with Tween-PBS, and treated with 50 μl of a 1/3,000 dilution of alkaline phosphatase-labeled goat anti-human IgG $F(ab)'_2$ (PIERCE, Rockford, IL). The following procedures were the same as described above. The Fab contents of the lysates were then calculated from the standard curve using authentic human IgG Fab fragment (Jackson ImmunoResearch).

BstNI Fingerprinting—Two micrograms of plasmid DNA from positive clones was digested with 10 U/μg of *Bst*NI (New England BioLabs) for 2 h at 60°C. Digestion products were then analyzed by electrophoresis in 3% agarose gels.

Nucleic Acid Sequencing—Nucleic acid sequencing was carried out on a 373A automated DNA sequencer (Applied Biosystems) using a *Taq* fluorescence dideoxyterminator cycle sequencing kit (Applied Biosystems). The primers for SeqT3 (5′-ATTAACCCTCACTAAAG-3′) and KEF (5′-GAATTCTAAACTAGCTAGTTCG-3′), both hybridizing to the (−)-strand, were used to sequence the heavy- and light-chain variable regions, respectively.

Immunoblotting—Partially purified HRV Wa particles were incubated for 15 min at 60°C with SDS-PAGE sample buffer under reducing conditions with 5% (v/v) β-mercaptoethanol. The incubation mixture was then subjected to 12% SDS-PAGE (Bio-Rad) and transferred to a nitrocellulose membrane (Immobilon NC Pure, Millipore, Bedford MA) by Trans-Blot SD Cell (Bio-Rad). The protein blotted membrane was immunostained with anti-HRV Fabs preincubated with rabbit anti-human IgG $F(ab')_2$, and the Fab-bound antigen was detected with biotinylated goat anti-rabbit IgG using an HRP-ABC system (Vectastain ABC Kit, Vector Lab, Burlingame, CA), and visualized with DAB/Ni (DAB Substrate Kit, Vector Lab).

RESULTS

Combinatorial Library Construction—Serum samples from eleven healthy individuals were evaluated in a sandwich ELISA for the presence of anti-HRV Wa antibodies. Three persons were found to have high antibody titers to HRV Wa. We selected two of the three positives, ON and AO. The IgG_1 κ Fab phage libraries designated as N and O constructed from PBLs contained 2.5×10^7 and 4.0×10^6 members, respectively.

Library Panning and Selection of Anti-HRV Fabs—The Fab-displayed phage libraries were panned against rabbit polyclonal antibody-captured HRV Wa. Five rounds of panning produced up to an approximately 30-fold amplification (N; from 2.9×10^5 to 8.9×10^6 cfu, and O; from 3.8×10^5 to 1.1×10^7 cfu, respectively) in eluted phage, indicating enrichment of antigen binding clones.

Plasmid DNA obtained from the final round of panning was reconstructed to produce soluble Fab. Forty-eight individual colonies from each library were grown in 10 ml cultures, and the supernatants were tested for reactivity to HRV Wa in a sandwich ELISA. Fab clones reactive with HRV Wa with no crossreactivity to the capture antibody were selected for *Bst*NI fingerprinting analysis. Analysis of 21 positive clones from N library identified five individual clones and analysis of 10 positive clones from O library revealed three different clones (Table I).

Analysis of the Immunological Properties of Anti-HRV Fabs—Five clones (N6, N8, N27, N28, N31) and three clones (O25, O26, O27) were selected from the N and O libraries, respectively, and their immunological properties were analyzed. We first tested crossreactivity to a panel of antigens, including human serum albumin, the Fc part of human IgG, human transferrin, BSA, ovalbumin, tetanus toxoid, and virus-free vero cell culture supernatants. Rabbit polyclonal antibody-captured HRV Wa and the capture antibody were included as positive and negative controls, respectively. None of the anti-HRV Fabs bound to antigens other than HRV Wa (data not shown). They were revealed to be specific for HRV Wa. Next, we compared the anti-HRV Fabs binding to HRV Wa in a sandwich ELISA. All Fab clones reacted with HRV Wa in a concentration-

dependent manner. The major difference between the two libraries was that O clone Fabs reached a plateau at a higher optical density than N clone Fabs (data not shown).

The reactivities of the anti-HRV Fabs against several rotavirus strains were investigated to estimate viral antigenic specificity. We chose Wa (G1P1A[8], subgroup II), AU-1 (G3P3[9], subgroup I), AU64 (G1P1B[4], subgroup I), and RRV (G3P5[3], subgroup I). As shown in Fig. 1, all N clone Fabs reacted almost equally with all strains, whereas O clone Fabs showed dominant reactivity to the Wa strain with a variety of reaction patterns. O25 and O27 showed similar reaction patterns, but O27 was less cross-reactive than O25. Although O26 retains dominant reactivity to the Wa strain, the reaction pattern was similar to that of N clone Fabs. These results suggest that N clone Fabs may recognize an antigen commonly present in all tested strains (it may be a group antigen), while O clone Fabs may react with an antigen present dominantly in Wa strain (it may be a subgroup II antigen). It is known that group and subgroup antigens are presented in the VP6 protein. To confirm that they recognize the VP6 protein, an immunoblot analysis was performed using partially purified HRV Wa particles. We chose N28 and O27 for this experiment for two reasons; they are the most frequently represented clones in each library (Table I), and their reactivities to rotavirus strains differed markedly from each other. Since Fab has a single antigen binding site, the Fabs were preincubated with rabbit anti-human IgG $F(ab')_2$ to increase their avidity. As shown in Fig. 2, a single band of approximately 44 kDa was detected in the lanes of both Fabs. The target antigen was assigned to the VP6 protein because its migration through the gel was almost identical to the main band stained with rabbit polyclonal antibody. No band was seen in the rabbit anti-human IgG $F(ab')_2$ lane without human Fabs.

Molecular Structural Analysis of Anti-HRV Fabs—To elucidate the contributions of the variable heavy (V_H)- and variable light (V_L)-chains and to define their antigenic specificities, the V_H and V_L domains of anti-HRV Fabs were sequenced and compared. Gene usage and the deduced amino acid sequences of the Fab clones are shown in Table

TABLE I. **Selection and identification of Fab clones from enriched libraries.**[a]

Library	ELISA (Positive/Total)	*Bst*NI fingerprinting (Identical/Total)	Selected Fab
N	21/48	14/21 (66%)	N28
		4/21 (19%)	N31
		1/21 (5%)	N6
		1/21 (5%)	N8
		1/21 (5%)	N27
O	10/48	6/10 (60%)	O27
		3/10 (30%)	O25
		1/10 (10%)	O26

[a]The reactivity of Fabs to HRV Wa was determined in a sandwich ELISA. Plasmid DNA from the positive clones was then subjected to *Bst*NI fingerprinting. The numbers in parentheses indicate the frequency of appearance.

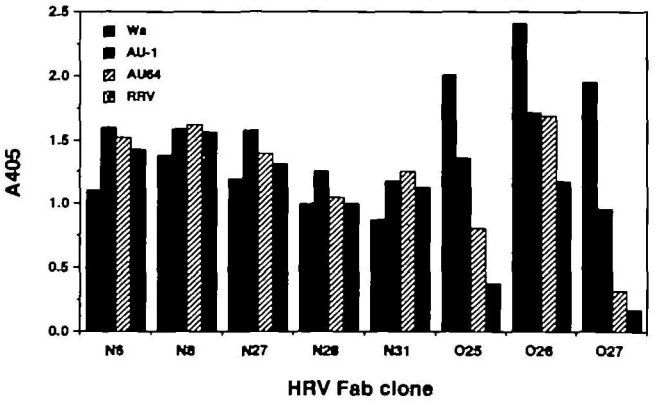

Fig. 1. **Comparison of the reactivity of anti-HRV Fabs to HRV Wa, AU-1, AU64, and RRV by sandwich ELISA.**

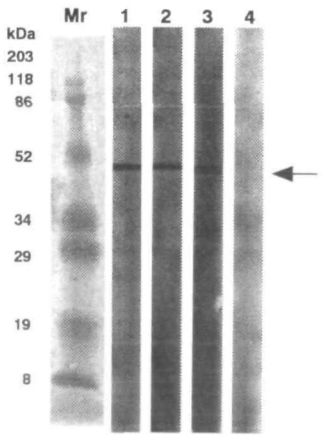

Fig. 2. **Identification of proteins recognized by anti-HRV Fabs.** Viral proteins of HRV Wa blotted on the membrane were stained with N28-anti-Fab complex (lane 1), O27-anti-Fab complex (lane 2), rabbit polyclonal antibody against HRV Wa (lane 3), or anti-Fab without human Fab (lane 4).

TABLE II. **Comparison of gene usage and structural homologies of the heavy- and light-chain variable regions of anti-HRV Fabs.**

Fab	V_H gene usage					V_L gene usage				
			% homology with germline					% homology with germline		
	V_H family	Nearest V_H	V_H DNA	V_H protein	J_H	V_L family	Nearest V_L	V_L DNA	V_L protein	J_L
N6	4	DP-65	86.8	78.1	J_H4b	κI	DPK9	97.2	94.7	Jκ5
N8	4	DP-65	86.5	76.0	J_H4b	κII	DPK15	96.0	92.0	Jκ2
N27	4	DP-65	86.8	76.0	J_H4b	κIII	DPK22	96.5	95.8	Jκ2
N28	4	DP-65	86.5	76.0	J_H4b	κIII	DPK22	96.5	96.9	Jκ1
N31	4	DP-65	86.5	76.0	J_H4b	κI	DPK9	96.5	93.7	Jκ4
O25	3	V3-21	91.2	86.3	J_H4b	κI	L12a	95.8	92.6	Jκ2
O26	3	V3-21	91.2	86.3	J_H4b	κIII	DPK22	96.9	94.8	Jκ1
O27	3	V3-21	91.2	86.3	J_H4b	κI	DPK9	95.8	91.6	Jκ2

(A) Variable heavy chain sequence

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
DP65	LQESGPGLVKPSQTLSLTCTVSGGSIS	SGGYYWS	WIRQHPGKGLEWIG	YIYYSGSTYYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR		
N6	.L.....E.....Y.V.	.TS....	.....R.....	F.SNN.DPF.....	.F...I.....T.R.Y...S....K.....	QSIDFWSGSKAAYFDS	WGQGALVTVSS
N8	.L.....V..S.....A..N	TAN....	.....R.....	F.SNN.DPF.....	.F...I.....T.R.Y...S....K.....	QSIDFWSGSKAAYFDS	WGQGALVTVSS
N27	.L.... .....V..S.....A..N	TAN....	.....R.....	F.SNN.DPF.....	.F...I.....T.R.Y...S....K.....	QSIDFWSGSKAAYFDS	WGQGALVTVSS
N28	.L.....V..S.....A..N	TAN....	.....R.....	F.SNN.DPF.....	.F...I.....T.R.Y...S....K.....	QSIDFWSGSKAAYFDS	WGQGTLVTVSS
N31	.L.....V..S.....A..N	TAN....	.....R.....	F.SNN.DPF.....	.F...I.....T.R.Y...S....K.....	QSIDFWSGSKAAYFDS	WGQGALVTVSS
V3-21	LVESGGGLVKPGGSLRLSCAASGFTFS	SYSMN	WVRQAPGKGLEWVS	SISSSSSYIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR		
O25	.L.....G.....	..GFS	.....	A.....TF.T.....	...V.K....S.....K	KGSYKGGRKGDWLGN	WGQGTLVTVSS
O26	.L.....G.....	..GFS	.....	A.....TF.T.....	...V.K....S.....K	KGSYKGGRKGDWLGN	WGQGTLVTVSS
O27	.L.....G.....	..GFS	.....	A.....TF.T.....	...V.K....S.....K	KGSYKGGRKGDWLGN	WGQGTLVIVSS

(B) Variable light chain sequence

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
DPK9	DIQMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP	
N6	ELV.....A.....F..	.....	.....	.....	.....	.....IT	FGQGTRLEIKRTVA
N31	EL.....	.....	.....	.....	.....	..AMRF.LT	FGQGTKVEIKRTVA
O27	EL.....P.....	....T.N....	.....	G.T....	.....R.....	.....RT	FGQGTKLEIKRTVA
DPK15	DIVMTQSPLSLPVTPGEPASISC	RSSQSLLHSNGYNYLD	WYLQKPGQSPQLLIY	LGSNRAS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQALQTP	
N8	EL.L.....	.....N.FM.	....R.....	.....	.....	..G....YT	FGQGTKLEIKRTVA
DPK22	EIVLTQSPGTLSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	QQYGSSP	
N27	.LT.....	.....	.....	.....	.....	.....RGYT	FGQGTKLEIKRTVA
N28	.LT.....D.....	.....	.....	.....	.....	.....TT	FGQGTKVEIKRTVA
O26	.L.M....A.....G.....	.....G._...	.....	.....	.....	.....GT	FGQGTKVEIKRTVA
L12a	DIQMTQSPSTLSASVGDRVTITC	RASQSISSWLA	WYQQKPGKAPKLLIY	KASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYNSYS	
O25	E.....	.....	.....	E.....	.....S..S...H.	...K..PYT	FGQGTKLEIKRTVA

Fig. 3. **Deduced amino acid sequences of anti-HRV Fab V_H and V_L domains from N and O libraries in comparison with the closest known germline.** (A) V_H sequences, (B) V_L sequences.

II and Fig. 3. Distinct V_H gene usage was seen in the two libraries (Table II). All N clone Fabs were members of the V_H4 family and derived from the DP-65 germline gene, whereas all O clone Fabs were members of the V_H3 family and derived from the V3-21 germline gene. The heavy chain complementarity-determining region (HCDR) 3 sequences of the Fab clones in the two libraries were also different from each other (Fig. 3). On the other hand, shared V_L gene usage and similar light chain CDR3 sequences were seen in the two libraries (Table II and Fig. 3). These results suggest that the distinct immunological properties of Fab clones in the two libraries may be mainly attributed to differences in their V_H sequences.

DISCUSSION

In this report, we describe the isolation and characterization of human recombinant Fab fragments from PBLs of two healthy individuals with high serum titers to rotavirus. Eight different Fab clones specific for rotavirus were successfully isolated from the PBL-based phage display combinatorial libraries by panning and selection with HRV Wa captured on a rabbit polyclonal antibody. We demonstrated that PBLs from seropositive donors are a satisfactory source for library construction. Bone marrow cells or lymph node cells have generally been used for library construction (*6, 8-10, 14, 17*) because they are the major repositories of differentiated B cells that produce antibodies to maintain circulating antibody titers (*31*). Although PBLs contain a smaller population of differentiated B cells compared with bone marrow cells or lymph node cells, successful retrieval of human Fabs from PBLs has been reported (*5, 7, 11, 13, 15, 16*). PBLs can therefore be chosen as an alternative source when bone marrow cells or lymph node cells are not available from the donor.

Although purified antigens are essential for obtaining antigen-specific antibodies, the purification of target antigens is laborious and time consuming when they are presented as a minor population. If polyclonal or monoclonal antibodies with predetermined specificities are available, antibody captured crude antigens can be applied instead of purified antigens. Since purified viral antigens were not available in this study, we used a polyclonal antibody against HRV Wa to capture viral antigens, and antigen-specific Fab clones were successfully isolated from the two libraries. If monoclonal antibodies with different specificities are available, Fab clones recognizing different epitopes could be isolated from a single library using this system. The selection of human Fabs to herpes simplex virus glycoproteins using monoclonal antibodies with different specificities has been reported (*32*).

Since successful panning yields a large number of identical positive clones, the identity of those clones should be determined for efficient downstream analysis. Although nucleotide sequencing has been employed for identification, this procedure is expensive and time consuming. Alternatively, we employed *Bst*NI fingerprinting, which has been utilized for the identification of individual clones (*12*) or for monitoring the enrichment of a library by panning (*14*). Comparing the fingerprinting pattern of each clone, ELISA-positive clones from the N and O libraries, eight and three, respectively, were found (Table II). The usefulness of this procedure is supported by the nucleotide sequencing of selected clones because they have different sets of V_H and

V_L sequences even though they have an identical V_H sequence (Fig. 3).

Although the Fab clones from the two libraries reacted with an identical antigen, the VP6 protein (Fig. 2), they recognize different epitopes because of their distinct strain crossreactivities (Fig. 1) and different V_H sequences, especially their HCDR3 sequences, which are known to be major determinants of antigenic specificity (*13, 33*). These observations suggest different courses of humoral immune response to rotavirus during infection in the two individuals. VP6 is a hydrophobic protein that is highly antigenic and immunogenic (*34-36*), but it remains unclear whether VP6 plays a role in inducing protective immunity. Epitope analysis of N28 and O27 using a phage display random peptide library (*37*) is now in progress to identify their recognition sites and to investigate the role of VP6 protein in the induction of protective immunity.

We thank Dr. Dennis R. Burton (The Scripps Research Institute, La Jolla, CA, USA) for the generous gift of pComb3 and for critical reading of the manuscript.

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