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Human monoclonal antibodies against a plethora of viral pathogens from single combinatorial libraries

(human antibody repertoires/passive immunization/opportunistic infection/phage surface expression)

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ABSTRACT **Conventional antibody generation usually requires active immunization with antigen immediately prior to the preparation procedure. Combinatorial antibody library technology offers the possibility of cloning a range of antibody specificities at a single point in time and then accessing these specificities at will. Here we show that human monoclonal antibody Fab fragments against a plethora of infectious agents can be readily derived from a single library. Further examination of a number of libraries shows that whenever antibody against a pathogen can be detected in the serum of the donor, then specific antibodies can be derived from the corresponding library. We describe the generation of human Fab fragments against herpes simplex virus types 1 and 2, human cytomegalovirus, varicella zoster virus, rubella, human immunodeficiency virus type 1, and respiratory syncytial virus. The antibodies are shown to be highly specific and a number are effective in neutralizing virus *in vitro*.**

Viral infections, particularly in the fetus or newborn and in immunocompromised individuals, can produce severe, even fatal, disease. Indeed Herpesviridae infections are the major cause of morbidity and mortality in immunosuppressed individuals. Especially at risk are those patients seronegative for virus who have undergone organ or bone marrow transplants from seropositive donors. For example, cytomegalovirus (CMV) infection rates approach 75% in CMV-seronegative recipients of CMV-seropositive donor kidneys (1). AIDS patients frequently present with retinitis and gastroenteritis, among other problems with CMV as the causative agent. Chronic intrauterine infection is another major consequence of viral disease. Infection of the fetus may result from transplacental contact via the maternal circulation or from ascending pathogens from the cervix or from contact during passage through the birth canal. The infections most commonly acquired by these routes are CMV, toxoplasmosis, rubella, herpes simplex virus (HSV), hepatitis B, and human immunodeficiency virus type 1 (HIV-1). For CMV, there are >40,000 cases of congenital infection each year in the United States alone. Mental retardation and hearing loss will result from 8000–10,000 of these cases (2).

Current clinical management of viral infections is inadequate. At present, only a handful of antiviral drugs are licensed for systemic or topical administration in a restricted number of disease states—for example, acyclovir for herpesvirus infections and 3′-azido-3′-deoxythymidine (AZT) as applied to HIV infection. The greatest difficulty in development of classical pharmaceutical antiviral compounds is the inability to distinguish viral replicative pathways from those of the host.

An alternative approach to antiviral therapy is to boost the host immune defenses. It has been clearly established that prophylactic administration of human immune serum possessing high antiviral titers has been therapeutically beneficial, particularly in CMV (1, 3, 4), varicella zoster virus (VZV) (5, 6), and hepatitis A (7) and B (8) infections. These antisera preparations are shown to help prevent not only the incidence of serious chronic infection, typically in the context of mother to infant transmission (8), but also to reduce the clinical severity of ongoing infections such as those in immunocompromised individuals (6). In addition, the presence of maternal anti-viral antibodies has been observed to provide substantial protection against congenital infection (2, 9). Significantly, there is also an increasing body of evidence (10, 11) suggesting antibody efficacy in what has previously been thought to be the preserve of cellular immunity (in the form of cytotoxic T lymphocytes)—namely, the clearance of virus from infected tissues.

Human monoclonal antibody preparations for disease therapy and prophylaxis overcome many of the problems inherent in pooled sera (12, 13). In particular, the concentration of virus-specific antibodies is several orders of magnitude higher than in pooled sera, thereby decreasing the amount of immunoglobulin required for prophylaxis and therapy. This decreases the possibility of transmission of adventitious pathogenic agents. However, only a few human monoclonal antibodies suitable for passive immunotherapy have been produced to date. Conventional hybridoma technologies have been successfully applied in the generation of neutralizing murine anti-viral antibodies but have not been useful in generating human antibodies. The introduction of Epstein–Barr virus (EBV) transformation of human lymphocytes has also met with only limited success. Both technologies suffer from the paucity of sufficient virus-specific B cells in the peripheral circulation. Thus, not only will less-common specificities probably remain elusive, but a distorted picture of the immune response may be revealed. In addition, secretion of antibody by EBV-transformed cell lines is unstable and often of unsuitable isotype (14, 15).

The preparation of random combinatorial libraries provides a route for selection of diverse high-affinity human monoclonal antibodies. The construction of such antibody libraries on the surface of M13 bacteriophage (16, 17) and their application to HIV-1, respiratory syncytial virus (RSV), and hepatitis B virus have been described (18–21). Here we report on the selection of human Fab molecules specific for a variety of pathogens from libraries derived from long-term asymptomatic HIV-1 patients who have experienced or who

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Abbreviations: HIV-1, type 1 human immunodeficiency virus; RSV, respiratory syncytial virus; CMV, cytomegalovirus; VZV, varicella zoster virus; HSV-1, type 1 herpes simplex virus; HSV-2, type 2 herpes simplex virus.

harbor other infections. Our observations clearly reveal the diversity of libraries generated by the combinatorial approach and the way in which existing antibody responses demonstrable at the serologic level may be directly cloned and characterized at the molecular level.

MATERIALS AND METHODS

Serological Evaluation of Donor Serum. Sera from two donors were evaluated for the presence of specific antibodies against the viruses studied by means of commercially available kits. Antibodies against CMV, HSV, and VZV were detected by ELISA (Behring). Sera were evaluated against both infected and noninfected cell lysates. Sera were considered positive when the OD (infected cell lysate minus noninfected cell lysate) at a dilution of 1:80 was higher than that of a negative control. This value is currently considered clinically protective. Anti-rubella virus antibodies were detected with the Abbott microparticle enzyme immunoassay (MEIA) system; antibody levels >10 international units, the accepted value for clinical protection during pregnancy, were considered positive. Anti-HIV-1 antibodies were assayed with commercially available Behring and Sorin Biomedica (Saluggia, Italy) ELISA kits following the manufacturers' directions. All the assays were repeated in double-blind tests, yielding reproducible results.

Lymphocyte RNA Preparation and Library Construction. Preparation of RNA from bone marrow lymphocytes and subsequent construction of IgG1κ Fab libraries using the pComb3 M13 surface display system have been described (16, 17). However, to increase the efficiency of restriction enzyme cutting of PCR-amplified material and subsequent library construction, a number of extension primers were designed. These oligonucleotides contain a poly(GA) tail 5′ to the sequence of the original primers, increasing the number of bases between the cutting site and the end of the molecule. Heavy-chain amplification was performed by using the 5′ primers VHaext [5′-$(GA)_{10}$S AGG TRC AGC TCG AGS AGT CWG G-3′] and VHfext [5′-$(GA)_{10}$S AGG TGC AGC TRC TCG AGT CKG G-3′], each paired with the 3′ primer CG1zext [5′-$(GA)_{10}$G GCA TGT ACT AGT TTT GTC AC-3′]. Similarly, for the light chain the 5′ primer VKext [5′-$(AG)_{10}$G AHA TYG AGC TCA CBC AGT CTC C-3′] was paired with the 3′ primer CK1zext [5′-$(AG)_{10}$C GCC GTC TAG AAC TAA CAC TCT C-3′] (S, G or C; R, A or G; W, A or T; K, G or T; H, A, T, or C; Y, T or C; B, G, C, or T). In later studies, the improved 3′ κ-chain primer CK1d (5′-GCG CCG TCT AGA ATT AAC ACT CTC CCC TGT TGA AGC TCT TTG TGA CGG GCG AAC TCA G-3′) with the extension primer CK1dext [3′-$(AG)_{10}$C GCC GTC TAG AAT TAA CAC TCT C-5′] was used. Subsequently, 10 ng of DNA pooled from reactions amplifying Fd, κ and λ, was subjected to a further 25 cycles of amplification by using the appropriate extension primer pairs. Extension PCR was performed on a Perkin Elmer 9600 with denaturation at 94°C for 30 sec, hybridization at 60°C for 20 sec, and extension at 72°C for 1 min.

Selection of Antigen Binding Phage Through Library Panning. Panning of the combinatorial libraries was carried out as described (17, 18) with viral antigens bound to ELISA wells. In the case of RSV, HIV-1, and CMV, a recombinant fusion glycoprotein (FG) (kindly supplied by M. Wathen, Upjohn), surface glycoprotein gp120, or viral extract (kindly provided by M. P. Landini, Italy), respectively, was coated to ELISA plates with 0.1 μg of antigen per well at 4°C overnight. Whole virus preparations of HSV-1, HSV-2, rubella, and VZV were reconstituted from ELISA-based diagnostic kits (Sigma) using phosphate-buffered saline (PBS)/1% bovine serum albumin for 1 hr at 37°C.

Preparation and ELISA Screening of Soluble Fab Fragments. Fabs were prepared as bacterial supernates through a freeze–thawing procedure as reported (20). To assess specificity, supernates were screened in an ELISA format (22) with equivalent amounts of the antigen against which they were initially panned and with bovine serum albumin (Sigma) used as a control preparation. Soluble Fab was purified from bacterial supernates by affinity chromatography over a protein A/G matrix (Schleicher & Schuell). The column was washed with a PBS solution and antibody was eluted in 0.2 M glycine·HCl buffer (pH 2.2) and immediately brought to neutral pH with 1 M Tris·HCl (pH 9.0).

Immunofluorescence of Virally Infected Cells. Antibody Fab found to be reactive with HSV-2 and VZV following ELISA screening was used in immunofluorescence studies of a virally infected monkey kidney epithelial cell line (Vero). The cells were infected at low multiplicity with HSV-2 strain G and fixed 6 hr postinfection in 4% paraformaldehyde in PBS. After washing in PBS, the cell layer was blocked for 30 min in PBS/0.3% Triton X-100/1 mg of bovine serum albumin per ml, before incubation for 1 hr at ambient temperature with the bacterial supernate containing the primary antibody Fab diluted 1:2 in blocking buffer. After washing in PBS, the cells were incubated for an additional hour with a 1:1000 dilution in blocking buffer of murine anti-HSV-2 protein 8 (ICP8) antibody (a gift of L. Pereira, University of California, San Francisco) and washed again in PBS. Subsequently, the cells were incubated for 1 hr with a 1:1000 dilution of secondary antibodies—i.e., conjugated goat anti-human Fab (BMBiochemicals, Indianapolis) and rhodamine-conjugated goat anti-mouse IgG (BMBiochemicals)—before being washed and mounted in *n*-propyl gallate (NPG) solution (80 ml of glycerol/20 ml of PBS/4% NPG). The preparations were observed under a Zeiss Axiophot microscope.

A similar procedure was performed for VZV. VZV (Webster strain)-infected cells were used as an innoculum for noninfected cells that were harvested 36 hr postinfection. The cells were then incubated as described earlier but with monoclonal antibody 8612 against VZV (Chemicon), exposed to labeled secondary antibodies, mounted, and examined.

Nucleic Acid Sequencing. Nucleic acid sequencing was carried out on a 373A automated DNA sequencer (Applied Biosystems) using a *Taq* fluorescent dideoxynucleotide terminator cycle sequencing kit (Applied Biosystems). Primers for elucidation of heavy-chain sequence were SEQGb (5′-GTCGTTGACCAGGCAGCCCAG-3′) hybridizing to the (+)-strand and the T3 primer (5′-ATTAACCCTCACTAAAG-3′) hybridizing to the (−)-strand. For the light-chain, SEQKb primer (5′-ATAGAAGTTGTTCAGCAGGCA-3′) and KEF primer (5′-GAATTCTAAACTAGCTAGTTCG-3′) were used, binding to the (+)- and (−)-strands, respectively.

RESULTS AND DISCUSSION

It was previously demonstrated that a combinatorial library of heavy and light chains, derived from an asymptomatic HIV-1-positive donor and displayed on the surface of fila-

Table 1. Specific antibodies cloned from the first donor library

Infectious agent	Serology	Panning antigen	Characteristics of cloned Fabs		
			ELISA	Immunofluorescence	Neutralization
HIV	+	gp120	+	ND	+
RSV	+	FG protein	+	ND	ND
CMV	+	Viral lysate	+	ND	ND
HSV-2	+	Viral lysate	+	+	+
HSV-1	+	Viral lysate	+	+	+
VZV	+	Viral lysate	+	+	ND
Rubella	+	Viral lysate	+	ND	ND

ND, not done.

Table 2. Specific antibodies cloned from the second donor library

Infectious agent	Serology	Panning antigen	Characteristics of cloned Fabs		
			ELISA	Immuno-fluorescence	Neutrali-zation
HIV	+	gp120	+	ND	+
RSV	+	FG protein	+	ND	ND
CMV	+	Viral lysate	+	+	+
HSV-2	+	Viral lysate	+	ND	ND
HSV-1	+	Viral lysate	+	ND	ND
VZV	+	Viral lysate	+	+	ND
Rubella	+	Viral lysate	+	ND	ND

ND, not done.

mentous bacteriophage, can yield antibodies with specificities for HIV-1 (18, 21). Further reports described isolation of antibodies specific for RSV and hepatitis B (19, 20). We report here on the preparation of additional libraries from other long-term asymptomatic carriers of HIV-1 and characterization of antibodies to a group of clinically important viral pathogens.

IgG1κ/λ Fab libraries were prepared from the bone marrow of two donors, yielding libraries of 3×10^6 members in the case of one and 2×10^6 members in the case of the other.

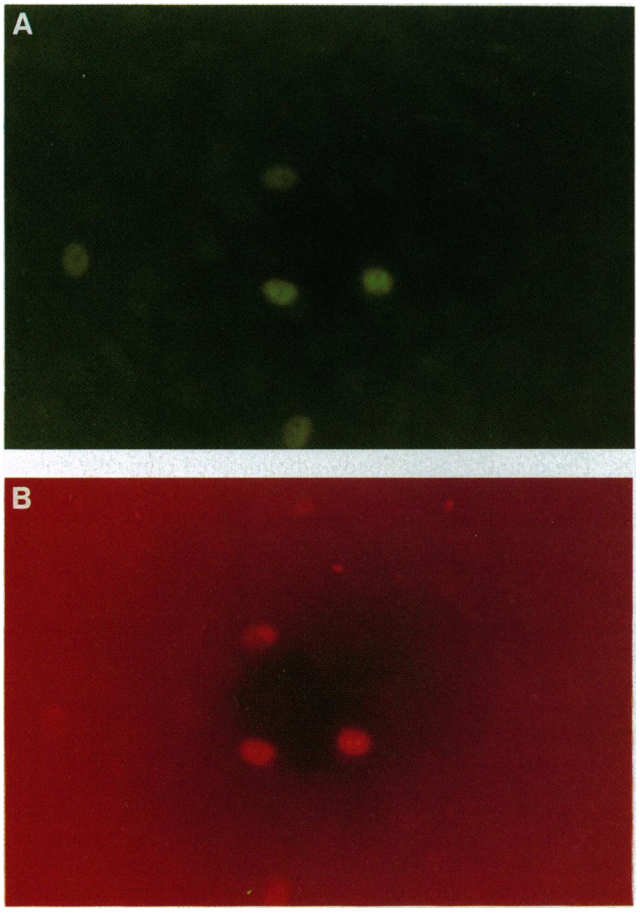

FIG. 1. Immunofluorescence of HSV-2-infected cells. Monkey kidney epithelial cells were infected at low multiplicity with HSV-2 strain G and fixed 6 hr postinfection. The cells were then incubated with recombinant human Fab and a virus-specific mouse monoclonal antibody. Subsequently, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-human Fab (*A*), and rhodamine-conjugated goat anti-mouse IgG (*B*). Infected cells, carrying fluorescent marker, may be seen against a darkened background of uninfected cells.

Sera of the two donors, taken concomitantly with bone marrow samples, were assessed for serological activity with CMV, HSV-1 and -2, VZV, rubella, RSV, and gp120 derived from HIV strain IIIB. Antibody titers for all the antigens were found in both donors. The libraries were then independently panned to enrich for specific antigen binding clones. In each case, detection of serum antibodies for a virus was associated with subsequent recovery of antigen-specific clones from the library. Tables 1 and 2 summarize the results obtained from the library pannings.

Various degrees of amplification as determined by the numbers of phage eluted from the coated ELISA wells were observed after four rounds of panning against different antigens. Specific antibody was recovered from only an 8-fold amplification in the case of VZV, whereas there was an amplification of >130-fold in the CMV panning. Soluble Fab was subsequently prepared through removal of the gene encoding the phage coat protein 3 and induction with isopropyl β-D-thiogalactopyranoside (17). The soluble antibody was then assayed by ELISA and immunofluorescence studies of virus-infected cells. A large proportion of phage clones (16/20) eluted from the fourth round of CMV panning were antigen specific; only 25% (5/20) were specific in the case of VZV. Examples of the immunofluorescence assays shown in Figs. 1 and 2 clearly illustrate the specificity of the recombinant Fab for virus-infected cells against a background of uninfected cells.

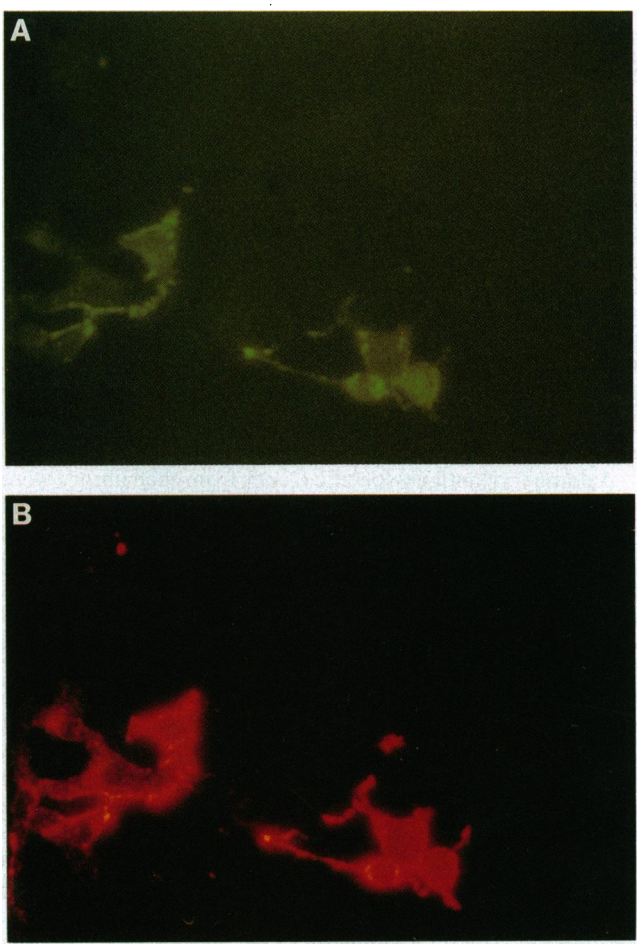

FIG. 2. Immunofluorescence of VZV-infected cells. In a procedure similar to that used for HSV-2, virally infected cells 36 hr postinfection were specifically labeled by using recombinant human (*A*) and mouse monoclonal (*B*) antibody against a background of uninfected cells.

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
GLCMV 11	LEESGAEMKKPGSSVRVSCKASGFTFH	NHAVS	WVRQAPGEGLEWMGG	LIPIVGLANLQPRFQG	RVTISADESTNTAYMEMRSLTSDDTAIYYCVR	HGDDSSGFPPFDL	WGQGALVIVSS
GLCMV 10,15, 20,18	LEQSGAEVKKPGSSVKVSCKASGGTFS	SYAIH	WVRQAPGQGLEWMG	RITPMFSPAIYAQKFDG	RVTITADESTTTAYMEMNSLRSDDTAVYYCAR	PSAYTGSLAY	WGQGTLVTVSS
GLCMV 17	LEQSGAEVKQPGSSMKVSCKVSGGIFR	TNAFS	WVRQAPGQGLEWMGI	SIPMFATVNYAGTFQG	RITISADESTSTVDMELSSLRPDDTAIYYCAR	GGRFLEFFEYGLDV	WGQGTTVIVSS
GLCMV 8,1	LEQSGAEVKKPGSSVKVSCKASGGSFS	SYAIN	WVRQAPGQGLEWMGG	LMPIFGTTNYAQKFQD	RLTITADVSTSTAYMQLSGLTYEDTAMYYCAR	VAYMLEPTVTAGGLDV	WGQGTTVTVAS
GLCMV 3,7,16	LESGPGLVKTSETLSLTCTVSGGSVSS	NSDYWA	WIRQTPGKGLEYFG	SILFGGTTYYNPSLKS	RVTMSVDTSTNQFSLDLSSVTAADTAVYYCAR	HTVTGFLEWSPPNWFFDL	WGRGTLVTVSS
GLCMV 2,4,5,13	LESGGGVVQPGRSLRLSCAASGFTFR	TYGMH	WVRQAPGKGLEWVA	VISYDGSKNYYADSVKG	RFTISRDNSKKTLYLQMNSLRAEDTAVYYCAK	DFWSGSTKNVFDL	WGQGTLVTVSS
GLCMV 14,	LEQSGGGVVQPGRSLRLSCAASGFTFR	TYGMH	WVRQAPGKGLEWVA	VISYDGSKNYYADSVKG	RFTISRDNSKKTLYLQMNSLRAEDTAVYYCAK	DFWSGSTKNVFDL	WGQGTLVTVSS
GLCMV 12	LEQSGAELKRPWSSVKVSCKASGGTLR	STAVN	WVRQPPGQGLEWMGG	LIPLFGTPNYAQKFQG	RVTFTADESTSTAYMELSSLRSDDTAVYYCAG	TSRGLNWFDP	WGQGALVTVSS
GLCMV 9	LEESGAEVKKPGESLRITCKAVGYSFT	NAWIS	WVRQVPGKGLEWLG	RINPIDSSRNYSPSFQG	HVTISADTSITSASLHWSSLEASDTAMYYCAR	HMSDSSGYSNRGAYDI	WGQGTMVIVPS

FIG. 3. Amino acid sequence of heavy chains binding specifically to CMV-infected cells and derived from the second library. The sequence group including clones 2, 4, 5, and 13 is identical to clone 14 in all but the N-terminal amino acids. This difference arises from independent amplification of this gene by two different primers, each designed to amplify sequences of the VH3 gene family.

It is worth noting the variability we encountered when assessing Fab in an ELISA format. Some clones appearing negative by ELISA were shown to be virus-specific by immunofluorescence. This probably arises from a difference in sensitivity of detection between phage panning and soluble Fab binding in ELISA. The percentage of antigen molecules available in native or recognizable conformation on the well surface may be extremely small, or the epitope may be rare yet still sufficient to support enrichment of specific antibody phage as monitored by amplification of eluted phage through successive rounds of panning ($<1 \times 10^7$). However, the number of molecules of Fab required to bind before a spectrophotometric signal is measurable is probably considerably higher.

Soluble Fab supernates specific for HSV-2 and CMV were further screened for their ability to neutralize virus infectivity. While a number of clones of each group exhibited weak neutralizing activity, 1 of 20 anti-HSV-2 (R.A.W., R.B., P.P.S., C.F.B., and D.R.B., unpublished data) and 1 of 20 anti-CMV (R.B., R.A.W., L. Pereira, C.F.B., and D.R.B., unpublished data) clones were highly efficient neutralizers. In particular, Fab generated against HSV-2, but also cross-reactive with HSV-1, was highly active. When affinity-purified to >95% homogeneity (through examination by SDS/PAGE; data not shown), the Fab was consistently able to totally neutralize HSV-2 at 0.5 μg/ml (10 nM) and HSV-1 at 5 μg/ml (100 nM); 50% inhibition was observed at 0.05 μg/ml (1 nM) and 0.25 μg/ml (5 nM), respectively.

A number of antigen-specific clones from each library were sequenced. The extent of heavy-chain diversity observed varied between antigens. Of eight antigen binding clones derived from panning the second library against the FG protein of RSV, only one sequence was seen, reflecting a pattern similar to that of another library constructed from a different donor (20). Similarly, in the first library, six of seven heavy-chain sequences taken from clones binding to a VZV library were identical. In contrast, 8 of 10 heavy-chain sequences of HSV-2 binding clones derived from the first library were greatly different from each other, and 8 of 16 heavy-chain sequences binding CMV derived from the second library were equally different (Fig. 3), illustrating the range of host response against these pathogens in the two individuals. Interestingly, 9 of 10 heavy-chain sequences binding HSV-1 from the donor of the first library were different but were mostly closely related somatic mutants. Despite the structural similarity of the herpes simplex 1 and 2 virions, only one of the eight heavy chains binding to HSV-2, derived from the first library, was similar to those found binding to HSV-1. However, all 10 first library anti-HSV-1 clones were found to be cross-reactive with HSV-2 when evaluated by ELISA.

The extent to which the combinatorial approach, by its random recombination of heavy- and light-chain components of the Fab molecule, can be used as a window through which to examine the immune response of an individual to a particular antigen or pathogen as a whole is uncertain. The demonstrable phenomenon of light- and heavy-chain promiscuity (23–26) within the libraries means that one cannot be certain of the original B-cell chain pairing. However, heavy-chain sequences identified by this method are likely to be those used *in vivo* (24, 25). It should thus be possible to show whether the host response is diverse or restricted to a given pathogen and to which epitopes antibodies (and, more importantly, protective antibodies) are directed.

The antibodies described here were selected from libraries prepared from HIV-1-seropositive individuals. It may be that such individuals are better potential bone marrow donors than seronegative donors because of generalized B-cell activation (27) and/or viral reactivation (28) occurring in HIV-1 infection. We have, however, isolated a Fab fragment specific for measles from a HIV-1-seronegative individual with a low titer (<1:20) to antigen (E. Bender, G. J. Pilkington, and D.R.B., unpublished data).

Human monoclonal anti-viral reagents capable of mediating neutralization may provide a valuable clinical agent for virus therapy and prophylaxis, overcoming the well known problems associated with clinical use of nonhuman antibodies. The quantity of antibody administered clinically should be reduced over current human pooled sera IgG preparations because of the marked increase in concentration of virus-specific antibody. Positive and negative synergy of particular monoclonal antibody combinations could also be investigated. In addition, the identification of genes encoding virus proteins shown by human antibodies to be important targets of therapy or prophylaxis may make an important contribution to subunit vaccine design. There may also be diagnostic applications, with the possibility of linking the antibody to a marker group in a single construct.

The development of large panels of highly specific human recombinant monoclonal Fabs against infectious agents, made possible by the combinatorial approach, and the ease with which these specificities may be linked to induce the effector system desired (29) allow us to access the full therapeutic potential of antibodies in this area of human disease.

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