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A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals

(AIDS/antibody repertoires/passive immunization/filamentous phage/phage surface expression)

DENNIS R. BURTON*†, CARLOS F. BARBAS III*, MATS A. A. PERSSON*‡, SCOTT KOENIG§, ROBERT M. CHANOCK¶, AND RICHARD A. LERNER*

*Departments of Molecular Biology and Chemistry, Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037; §Laboratory of Immunoregulation and ¶Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; †Krebs Institute, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2TN, United Kingdom; and ‡Department of Medicine, Karolinska Institute, Karolinska Hospital, Box 60500, S-104 01 Stockholm, Sweden

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ABSTRACT **A panel of human monoclonal antibody Fab fragments has been generated against the surface glycoprotein gp120 of type 1 human immunodeficiency virus (HIV) by antigen selection from a random combinatorial library expressed on the surface of filamentous phage. The library was prepared from 5 ml of bone marrow from an asymptomatic individual who has been HIV-positive for 6 years. The antibodies have high affinity for antigen (mostly with affinity constants of $>10^8$ M^{-1}) and notable sequence diversity. Given appropriate donor selection, the methods described should allow the generation of antibodies for the evaluation of passive immunization as a therapy for AIDS.**

Counter-AIDS strategies that are under intensive scrutiny include passive immunization to slow or halt the progression of human immunodeficiency virus (HIV) infection in seropositive individuals and vaccination to prevent infection. Both approaches could greatly benefit from the generation of large numbers of human HIV-specific monoclonal antibodies.

There is increasing evidence that passive immunization could be an effective strategy for the treatment and prophylaxis of the disease. Two groups have reported clinical benefit in patients with AIDS who were given plasma from healthy asymptomatic HIV-positive donors (1, 2). In the former case the plasma was known to contain high anti-HIV neutralizing titer. Two other groups have described a correlation between the presence of maternal antibodies against select epitopes of viral envelope glycoprotein gp120 and the failure of infected mothers to transmit the virus to their offspring (3–5). The appropriate passively transferred antibodies have also been shown effective in preventing HIV infection in chimpanzees (6) and in preventing HIV-2 and simian immunodeficiency virus (SIV) infection in cynomolgus monkeys (7).

Although many murine and a few human antibodies to HIV exist (for a recent review, see ref. 8), a broad-based program to evaluate the utility of antibodies in combatting HIV infection will likely require the facile generation of large panels of human anti-HIV antibodies. There are a number of reasons for this. First, the antibodies would need to be administered repeatedly over an extended period of time and so should not only be human to avoid anti-antibody (typically anti-rodent) responses but also be available in a number of idiotypic forms with retention of activity to avoid anti-idiotypic responses. Second, the broad spectrum of viral strains means that the generation of single or even small numbers of monoclonal antibodies against the virus is unlikely to suffice. Third, it may be necessary to examine many antibodies to find rare but highly effective molecules. Antibodies could be rare either because they are present as minor components of typical responses or because they are present in only a few individuals.

Similarly, the study of large numbers of human antibodies should accelerate vaccine design. Recent vaccination data in nonhuman primates have shown the development of protective immunity against HIV-1 in chimpanzees vaccinated with recombinant gp120 (9) and against HIV-2 in cynomolgus monkeys vaccinated with whole killed virus (10). These studies raise a number of important issues. For instance, the former report found that gp160, which includes the full gp120 molecule, was not protective. Given that antibodies are believed to be key in protective immunity (9), the molecular dissection of human antibody responses to HIV should allow assessment of the utility of protective epitopes in the context of the natural host.

Conventional technologies for antibody generation, such as hybridomas and Epstein–Barr virus transformation (11), cannot readily meet the challenge of assessing large numbers of human monoclonal antibodies from HIV-infected individuals at various stages of their clinical course. Our approach to the problem has been to prepare random combinatorial libraries. Initially, antigen binders were selected from libraries constructed using phage λ vectors (12). This method worked well for immunized mice (12, 13) and for "boosted" humans (14, 15), but we were unable to prepare specific antibodies from HIV-infected individuals. Recent papers have described the expression of random combinatorial antibody libraries on the surface of M13 phage and have shown how this allows much more rapid selection of specific antibodies from larger libraries (16, 17). Here we report that this method can be used to generate large panels of specific viral antibodies from the lymphocytes of HIV-positive individuals.‖

MATERIALS AND METHODS

Lymphocyte RNA Preparation. Five milliliters of bone marrow was removed by aspiration from HIV-1-positive donors. Immediately, 10 ml of 3 M guanidinium isothiocyanate containing 71 μl of 2-mercaptoethanol was added and then RNA was prepared by the standard method (18).

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Abbreviations: BSA, bovine serum albumin; CDR, complementarity-determining region; HIV, human immunodeficiency virus.

‖The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M80715–M80734).

Library Construction. Total RNA (typically 10 μg) was reverse-transcribed and γ1 (Fd region) and κ chains were amplified by PCR as described (14). The resulting γ1 heavy-chain DNA was cut with an excess of the restriction enzymes *Xho* I and *Spe* I and typically about 350 ng was ligated with 2 μg of *Xho* I/*Spe* I-linearized pComb3 vector (isolated by agarose gel electrophoresis) in a total volume of 150 μl with 10 units of ligase (BRL) at 16°C overnight. Following ligation, DNA was precipitated at −20°C for 2 hr by the addition of 2 μl of 2% (wt/vol) glycogen, 15 μl of 3 M sodium acetate (pH 5.2), and 330 μl of ethanol. DNA was pelleted by microcentrifugation at 4°C for 15 min. The DNA pellet was washed with cold 70% ethanol and dried under vacuum. The pellet was resuspended in 10 μl of water and transformed by electroporation into 300 μl of *Escherichia coli* XL1-Blue (16). After transformation, 3 ml of SOC medium (16) was added and the culture was shaken at 220 rpm for 1 hr at 37°C after which 10 ml of SB (super broth; 30 g of tryptone, 20 g of yeast extract, and 10 g of Mops per liter, pH 7) containing carbenicillin (20 μg/ml) and tetracycline (10 μg/ml) was added. At this point, samples (20, 1, and 0.1 μl) were withdrawn for plating to determine the library size. Typically the library had about 10^7 members. The culture was grown for an additional hour at 37°C while shaking at 300 rpm. This culture was added to 100 ml of SB containing carbenicillin (50 μg/ml) and tetracycline (10 μg/ml) and was grown overnight. Phagemid DNA containing the heavy-chain library was prepared from this overnight culture. To determine the insert frequency of this ligation, 10 colonies from the plates used to titer the library were picked and grown. DNA was prepared and then digested with *Xho* I and *Spe* I.

For the cloning of the light chain, phagemid DNA (10 μg) was digested as described above except that the restriction enzymes *Sac* I and *Xba* I were used. The resulting linearized vector was treated with phosphatase and purified by agarose gel electrophoresis. The desired fragment, 4.7 kilobases long, was excised from the gel. Ligation of this vector with prepared light-chain PCR DNA proceeded as described above for the heavy chain. After transformation, 3 ml of SOC medium was added and the culture was shaken at 220 rpm for 1 hr at 37°C. Then 10 ml of SB containing carbenicillin (20 μg/ml) and tetracycline (10 μg/ml) was added (samples were removed for titering as described above for the heavy-chain cloning) and the culture was shaken at 300 rpm for an additional hour. This culture was added to 100 ml of SB containing carbenicillin (50 μg/ml) and tetracycline (10 μg/ml) and then shaken for 1 hr. Helper phage VCS-M13 (10^{12} plaque-forming units) was added and the culture was shaken for an additional 2 hr. After this time, kanamycin (70 μg/ml) was added and the culture was incubated at 37°C overnight. The supernatant was cleared by centrifugation (4000 rpm for 15 min in a JA-10 rotor) at 4°C. Phage were precipitated by addition of 4% (wt/vol) polyethylene glycol 8000 and 3% (wt/vol) NaCl followed by incubation on ice for 30 min and centrifugation. Phage pellets were resuspended in 2 ml of phosphate-buffered saline (PBS: 50 mM phosphate, pH 7.2/150 mM NaCl) and microcentrifuged for 3 min to pellet debris. Supernatants were transferred to fresh tubes and stored at −20°C.

Titering of Colony-Forming Units. Phagemids that have been packaged into virions are capable of infecting male *E. coli* to form colonies on selective plates. Phage (packaged phagemid) was diluted in SB (dilutions: 10^{-3}, 10^{-6}, and 10^{-8}) and 1 μl was used to infect 50 μl of fresh *E. coli* XL1-Blue culture (OD_{600} = 1) grown in SB containing tetracycline (10 μg/ml). Phage and cells were incubated at room temperature for 15 min and then directly plated on LB/carbenicillin plates.

Panning of the Combinatorial Library to Select Antigen Binders. The panning procedure is a modification of that originally described by Parmley and Smith (19). Four wells of a microtiter plate (Costar 3690) were coated overnight at 4°C with 25 μl of antigen (40 μg/ml in 0.1 M bicarbonate buffer, pH 8.6). The wells were washed twice with water and blocked by completely filling the well with 1% (wt/vol) bovine serum albumin (BSA) in PBS and incubating the plate at 37°C for 1 hr. Blocking solution was shaken out, 50 μl of the phage library (typically 10^{11} colony-forming units) was added to each well, and the plate was incubated at 37°C for 2 hr. Phage were removed and the plate was washed once with water. Each well was then washed 10 times with 50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.5% Tween 20 over a period of 1 hr at room temperature. The plate was washed once more with distilled water and adherent phage were eluted by the addition of 50 μl of elution buffer (0.1 M HCl, adjusted to pH 2.2 with solid glycine and containing 0.1% BSA) to each well and incubation at room temperature for 10 min. The elution buffer was pipetted up and down several times, removed, and neutralized with 3 μl of 2 M Tris base per 50 μl of elution buffer used. Eluted phage were used to infect 2 ml of fresh *E. coli* XL1-Blue cells (OD_{600} = 1) for 15 min at room temperature after which 10 ml of SB containing carbenicillin (20 μg/ml) and tetracycline (10 μg/ml) was added. Samples (20, 1, and 0.1 μl) were removed for plating to determine the number of phage (packaged phagemids) that were eluted from the plate. The culture was shaken for 1 hr at 37°C and then added to 100 ml of SB containing carbenicillin (50 μg/ml) and tetracycline (10 μg/ml) and shaken for 1 hr. Helper phage VCS-M13 (10^{12} plaque-forming units) were added and the culture shaken for an additional 2 hr. Then kanamycin (70 μg/ml) was added and the culture was incubated at 37°C overnight. Phage preparation and further panning were repeated as described above.

Following each round of panning, the percent yield of phage was determined as (no. of phage eluted/no. of phage applied) × 100.

Preparation of Soluble Fab Fragments. Phagemid DNA from positive clones was isolated and digested with *Spe* I and *Nhe* I. Digestion with these enzymes produces compatible cohesive ends. The 4.7-kilobase DNA fragment lacking the gene III portion was gel-purified (0.6% agarose) and self-ligated.

Transformation of *E. coli* XL1-Blue afforded the isolation of recombinants lacking the gene III fragment. Clones were examined for removal of the gene III fragment by *Xho* I/*Xba* I digestion, which yielded a 1.6-kilobase fragment. Clones were grown in 15 ml of SB containing carbenicillin (50 μg/ml) and 20 mM $MgCl_2$ at 37°C until OD_{600} of 0.2 was achieved.

Isopropyl β-D-thiogalactopyranoside (1 mM) was added and the culture was incubated overnight at 37°C. Cells were pelleted by centrifugation at 4000 rpm for 15 min in a JA-10 rotor (Beckman J2-21) at 4°C. Cells were resuspended in 3 ml of PBS containing 0.2 mM phenylmethylsulfonyl fluoride and lysed by sonication on ice (2–4 min, 50% duty). The debris was pelleted by centrifugation at 14,000 rpm in a JA-20 rotor at 4°C for 15 min. The supernatant was used directly for ELISA analysis and was stored at −20°C.

ELISA Analysis of Fab Supernatants. ELISA wells were coated with gp120 exactly as above, washed five times with water, blocked in 100 μl of 1% BSA/PBS for 1 hr at 37°C, and then incubated with 25 μl Fab supernatants for 1 hr at 37°C. After 10 washes with water, 25 μl of a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-human IgG $F(ab')_2$ (Pierce) was added and incubated for 1 hr at 37°C. Following 10 washes with water, 50 μl of *p*-nitrophenyl phosphate substrate was added and color development was monitored at 405 nm. Positive clones gave A_{405} values of >1 (mostly >1.5) after 10 min, whereas negative clones gave values of 0.1–0.2.

Inhibition ELISAs. Affinity measurement was carried out by inhibition ELISAs (20). Microtiter wells were coated with

gp120 (5 μg/ml in 0.1 M bicarbonate buffer, pH 8.6) overnight at 4°C. The wells were blocked with 1% BSA/PBS for 1 hr at 37°C. Dilutions of the samples previously determined in titration experiments to result in substantial reduction of OD values after 2-fold dilution (i.e., arranged on the "slope" of the titration curve) were mixed with free gp120 in the wells at a final concentration of 10^{-7} to 10^{-11} M in 0.5% BSA/0.025% Tween 20/PBS. The plates were incubated for 90–120 min at 37°C and carefully washed 10 times with 0.05% Tween 20/PBS before addition of goat anti-human IgG $F(ab')_2$ at a dilution of 1:500. After 1 hr of incubation and washes as above, *p*-nitrophenyl phosphate was added as substrate (Sigma). Plates were read at 405 nm after 15 min.

Nucleic Acid Sequencing. Nucleic acid sequencing was carried out on double-stranded DNA with Sequenase 1.0 (United States Biochemical).

RESULTS AND DISCUSSION

Previously work has shown that the antibody phage surface expression system can be successfully used to isolate specific antibodies occurring in a combinatorial library at a frequency of about 1 in 5000 (16). Two rounds of antigen panning of a library prepared from an individual boosted with tetanus toxoid were sufficient to enrich the library to the extent that 27 out of 38 clones expressed tetanus toxoid-specific antibody following panning. In other studies a library was prepared that included known anti-tetanus toxoid antibody clones at a frequency of about 1 in 170,000. Three rounds of panning against toxoid were found to give enrichment such that 20/20 clones were antigen-specific, indicating that the method could access clones of low abundance from combinatorial libraries. This improved methodology for screening combinatorial libraries encouraged us to return to the HIV problem.

Libraries from a number of asymptomatic HIV positive donors have been prepared. We report now on the first donor studied, a 31-year-old homosexual male who has been HIV-positive for 6 years but has no symptoms of disease. Serological studies showed the presence of a significant ELISA titer (1:3000) against the HIV-1 surface glycoprotein gp120. After informed consent was given by the donor, bone marrow cells were obtained by aspiration. RNA was isolated from the lymphocytes and heavy (γ1, Fd region)- and light (κ)-chain genes amplified from the corresponding cDNAs by the PCR. The antibody genes were then cloned into the M13 phage surface expression vector pComb3 to give a library of 10^7 members.

The phage surface expression library was panned against recombinant gp120 (strain IIIB) coated on ELISA wells. Four rounds of panning produced an ≈100-fold amplification in eluted phage, indicating enrichment for specific antigen-binding clones. In the first panning, 4.6×10^{11} phage were applied to four wells and 7.7×10^5 phage were eluted. After the fourth panning 1.0×10^8 phage were eluted. Eluted phage were used to infect *E. coli* XL1-Blue cells. DNA was prepared from these cells, cut with *Nhe* I and *Spe* I to remove the gene III fragment, and religated. The reconstructed phagemid was used to transform XL1-Blue cells to produce clones secreting soluble Fab fragments. Forty such clones were grown up and the supernatants, containing Fab fragments, were screened in an ELISA for reactivity with recombinant gp120. The supernatants from 33 clones showed clear reactivity. The supernatants did not react with BSA-coated wells, and anti-tetanus toxoid Fab supernatants did not react with gp120-coated wells.

DNA samples from the reactive 33 clones were used as templates for sequencing of thymidine nucleotides of the variable regions of the heavy and light chains to reveal that at least 10 clones had unique heavy chains and 20 clones unique light chains. A representative number of chains were then sequenced. Fig. 1 compares the CDR3 sequences of these heavy and light chains and indicates the diversity of the panel of antibodies cloned. The details of the response to gp120 will be discussed elsewhere. Here we briefly note that clones identical with respect to heavy-chain CDR3 but differing in light-chain CDR3 sequences (e.g., nos. 14 and 31) were observed, as well as clones that were identical in both chains (e.g., nos. 11 and 29). The former is an example of chain promiscuity, which may be useful in altering idiotypy while retaining specificity (21).

To measure the affinities of the Fab fragments for gp120, inhibition ELISAs using soluble gp120 were performed. As shown in Fig. 2, most inhibition constants were $<10^{-8}$ M, implying monomer Fab–gp120 binding constants of the order of or greater than 10^8 M^{-1}.

The gp120 used in these experiments was derived from the IIIB strain for reasons of availability. However, this strain is thought to be very rare in the United States (22) and therefore it is likely that most of the antibodies we have selected are strain-crossreactive. Indeed, initial studies indicate that the binding of a number of the Fab fragments to gp120 is inhibited

LIGHT CHAIN				HEAVY CHAIN			
Clone	FR3	CDR3	FR4	Clone	FR3	CDR3	FR4
3	YYC	QQYGDSPLYS	FGQG	3	YYCAT	KYPRYSDMVTGVRNHFYMDV	WGKG
7	YYC	QQYGSSRYT	FGQG	7	YYCAR	VGPYTWDDSPQDNYYMVV	WGKG
11	YYC	QQFGDAQYT	FGQG	11	YYCAT	RYPRYSEMMGGVRKHFYMDV	WGKG
14	YYC	QKYQSAPRT	FGQG	14	YYCAR	ERRERGWNPRALRGALDF	WGQG
20	YYC	QNYDSAPWT	FGQG	20	YYCAT	QKPRYFDLLSGQYRRVAGAFDV	WGHG
21	YYC	QVYGASSYT	FGQG	21	YYCAR	VGPYTWDDSPQDNYYMDV	WGKG
22	YYC	QQSYSTPYT	FGQG	22	YYCAR	DIGLKGEHYDILTAYGPDY	WGQG
24	YSC	QQYGTSPWT	FGQG	24	YFCAR	ERRERGWNPRALRGALEV	WGQG
29	YYC	QQFGDAQYT	FGQG	29	YYCAT	RYPRYSEMMGGVRKHFYMDV	WGKG
31	YYC	QKYNSAPRT	FGQG	31	YYCAR	ERRERGWNPRALRGALDF	WGQG

FIG. 1. Amino acid sequences of heavy- and light-chain complementarity-determining region 3 (CDR3) from HIV gp120-binding clones. The flanking sequences of the framework regions FR3 and FR4 are also shown.

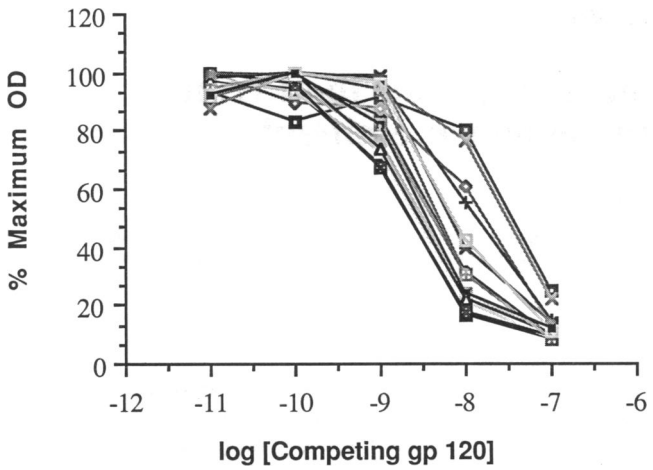

FIG. 2. Affinities of Fab fragments for gp120, estimated by inhibition ELISA for 15 different clones.

by soluble CD4, suggesting that they may recognize the CD4 interaction site.

The phage surface library was also panned against the HIV glycoprotein gp160. After four rounds of panning a 200-fold enrichment was observed. Supernatants containing soluble Fab fragments were then screened in an ELISA against gp160 and gp120. Of 35 clones reacting with gp160, 8 reacted also with gp120. The remainder are likely to be against gp41 or epitopes on gp120 that are sensitive to the presence of gp41. Screening against gp41 peptides may be useful in distinguishing these possibilities.

For generation of the present library, we have focused only on antibodies from the major heavy-chain variable-region subgroup families and κ light chains. Yet, from only 5 ml of bone marrow we have been able to obtain a large panel of high-affinity antibodies against HIV. This study, along with several others (12–16, 23), stands in contrast to theoretical arguments that the chances of isolating high-affinity antibodies from random combinatorial libraries were "remote" (24). As previously pointed out (15), the fact that libraries are constructed from immunologically amplified mRNA and not DNA is a crucial fact in any calculations concerning the probability of obtaining the original pairings of heavy and light chains. Immunized animals give libraries rich in functional chains that are recombined with one another relatively frequently to generate high-affinity antibodies, many of which probably represent the original *in vivo* pairings. In a real sense, by its sensitivity to mRNA, the combinatorial approach is reporting on the current antibody response of the donor.

The sensitivity of the method in combination with cell enrichment techniques should also allow us to draw upon the "fossil record" of the antibody response of an individual. This may be important in situations where immunological competence has deteriorated such as in AIDS or aging. For example, in AIDS we may wish to resurrect antibodies from different times in the host–virus encounter, for either analytical or therapeutic purposes (25).

At any rate, after addition of the Fc region, human antibodies from combinatorial libraries could form the basis of clinical trials of the efficacy of passive immunization in AIDS. While the present studies constitute a proof of principle, for trials of passive immunization in patients it will be necessary to decide what donor(s), how many antibodies, and which specificities should be used.

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