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The nature of the autoimmune antibody repertoire in human immunodeficiency virus type 1 infection

(human antibody repertoires/autoantibodies/phage surface expression)

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ABSTRACT **Human immunodeficiency virus type 1 (HIV-1) seropositive donors typically have high serum antibody titers to a range of autoantigens, and the corresponding autoantibodies have been suggested to be of importance in the pathogenesis of HIV-1 infection. We have prepared 38 IgG human monoclonal autoantibodies from asymptomatic HIV-1 seropositive donors with elevated serum titers to autoantigens by construction of Fab combinatorial libraries on the surface of phage and affinity selection using a range of autoantigens, including double-stranded DNA, major histocompatibility complex class II, CD14, epidermal growth factor receptor, and ganglioside GD2. The autoantibodies are shown to be of moderate affinity and exhibit marked cross-reactivity with a range of antigens. This contrasts with the specific high-affinity antibodies selected (*i*) against infectious agents using the same libraries and (*ii*) against one of the autoantigens using a library from a donor with established autoimmune disease. The results lend no support to the presence of specific autoantibodies in HIV-1 infection and instead suggest attention should be focused on the pathological significance of high serum levels of antibodies capable of interacting with multiple molecular species.**

Although most interest concerning the pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection has been focused on T cells, abnormal B-cell function is also a feature of the infection (1–6). Individuals infected with HIV-1 have serum antibody titers to a wide range of self- and exogenous antigens (7–11). The origin of the autoantibodies and their effects are unknown, although speculations as to a pathogenic role have been made (10–12). The autoantibodies in HIV-1 infection appear early postinfection and persist throughout the disease course (7). In general, HIV-1 seropositive patients, and in particular patients with AIDS, have elevated numbers of spontaneous immunoglobulin-secreting B cells in peripheral blood (1) giving rise to hypergammaglobulinemia (13).

Several hypotheses for the retroviral induction of autoantibodies have been suggested. One hypothesis is that the virus or viral proteins themselves, directly or indirectly, induce generalized polyclonal B-cell activation that leads to elevated levels of all antibodies, including those to self-antigens (14). Another hypothesis invokes molecular mimicry whereby a viral epitope elicits antibodies that cross-react with a self-antigen (15–17). Sequence similarities have been reported between major histocompatibility complex (MHC) class II, particularly HLA-DR and HLA-DQ, and gp120 (18, 19), HIV-1 Nef (20) and gp41 (21), and between the major retroviral capsid protein (CA) and regions in the Sm-BB′ autoantigen, the SSB/La antigen, the 70-kDa ribonucleoprotein, topoisomerase I, and acetylcholine receptor (22, 23). Finally, Hoffman and colleagues (24, 25) have proposed an autoimmune model of AIDS pathogenesis that involves an immune response both to HIV and to allogeneic stimuli. A synergistic effect of the two responses is postulated to cause collapse of the immune system.

Serum titers can only partially describe an antibody response. A more complete description requires access to monoclonal antibodies constituting the response. Until recently, investigation of the human B-cell repertoire was confined to Epstein–Barr virus-transformed B-cell clones and a relatively small number of hybridoma-produced monoclonal antibodies, many of which are IgMs (26). PCR amplification of heavy and light chain variable genes and the subsequent construction of combinatorial libraries on the surface of phage offer an efficient method to survey a large number (10^7–10^8) of clones for the human monoclonal antibodies of interest (27). We have previously described three combinatorial antibody IgG libraries from the bone marrow RNA of HIV-1 seropositive donors (28–30). Bone marrow was chosen because it has been shown, in humans, to be a major repository for differentiated B cells that spontaneously produce antibodies to maintain circulating antibody titers (31). From these libraries we have been able, by affinity selection, to retrieve specific, high-affinity human monoclonal antibodies to a wide selection of viruses, including HIV-1, respiratory syncytial virus, cytomegalovirus, varicella zoster virus, herpes simplex virus types 1 and 2, and rubella virus. To characterize the autoantibodies seen in association with HIV-1 infection, we decided to use these and new libraries to select a series of antibodies against a range of autoantigens. The molecular characteristics of these IgG human monoclonal autoantibodies are described in this report.¶

MATERIAL AND METHODS

Library Construction. Eight HIV-1 seropositive donors were selected for this study whose designations and descriptions are as follows: Donors A and S demonstrated high serum neutralizing titers against laboratory isolates of HIV-1 virus; donors C, L, O, M, and P were long-term (>6 years) asymptomatic seropositives, and donor G was a seropositive individual immunized with recombinant gp160 (MicroGeneSys, West Haven, CT). Plasma and bone marrow samples were withdrawn concomitantly so that serology studies represented the immune status at the time at which bone marrow RNA was used to construct antibody libraries. Preparation of RNA from bone marrow lymphocytes and subsequent con-

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Abbreviations: HIV-1, human immunodeficiency virus type 1; MHC, major histocompatibility complex; BSA, bovine serum albumin; dsDNA, double-stranded DNA; EGFR, epidermal growth factor receptor; SLE, systemic lupus erythematosus; V_H, variable heavy chain.

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U07162 and U07194–U07196).

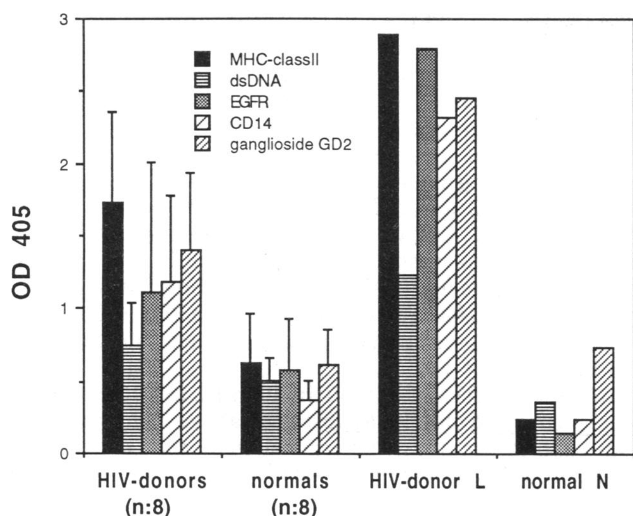

FIG. 1. Reactivity of sera (diluted 1:100) from eight asymptomatic HIV-1 seropositive male donors and eight healthy males against five selected autoantigens individually assessed by ELISA and then averaged as shown. Several HIV-1 positive donors (e.g., donor L) had strong serum responses to all of the autoantigens tested when compared to the healthy donors (e.g., donor N). Serum samples were taken concomitant with bone marrow aspiration for library construction.

struction of IgG1 κ/λ Fab libraries using the pComb3 M13 surface display system have been described (27, 28, 32).

Antigens. The autoantigens selected for biopanning were chosen primarily because of a reported immune response in HIV-1 patients. Several investigators have found homology between MHC class II and different parts of HIV-1, including gp120, gp41, and Nef, and it has been suggested that a human immune response directed against gp120 will also lead to antibodies against MHC class II (18–21). For the biopanning, purified human MHC class II antigen, type DR1, was kindly provided by L. Teyton (The Scripps Research Institute, La Jolla, CA). Elevated levels of anti-ganglioside antibodies have been reported associated with neuropathy (33) and AIDS (34) and increased levels of gangliosides have been found on lymphocytes from AIDS patients. The sialic acid-containing glycosphingolipid GD2 used for biopanning was obtained from Sigma. Purified human epidermal growth factor receptor (EGFR), a glycoprotein, was kindly provided by A. Komoriya (Federal Drug Administration, Rockville, MD). CD14, a phosphatidylinositol-linked protein, was kindly provided by A. Moriarty (R. W. Johnson Pharmaceutical Research Institute, La Jolla, CA). Double-stranded DNA (dsDNA) from human placenta (Sigma) was selected to compare the results with those obtained from systemic lupus erythematosus (SLE) patients and because of reported shared clinical features between HIV-1 infection and SLE (35). CD4 was not selected since it has been shown that antibodies obtained from HIV-1 seropositive donors to this antigen do not react with T lymphocytes (36–39). An HIV-1 library was biopanned against the Jurkat T-cell line. However, following several rounds of panning, none of the isolated Fab clones bound to the cell line.

Selection by Biopanning. For biopanning, four wells of an ELISA plate (Costar) were coated overnight at 4°C with 50 μl of MHC class II, EGFR, and CD14 in 0.1 M bicarbonate buffer (pH 8.6). DNA in phosphate-buffered saline (PBS) and ganglioside GD2 in 96% ethanol were dried on the ELISA wells at 37°C. The wells were washed twice with water and blocked by completely filling the wells with 3% (wt/vol) bovine serum albumin (BSA) in PBS for 1 hr. The BSA

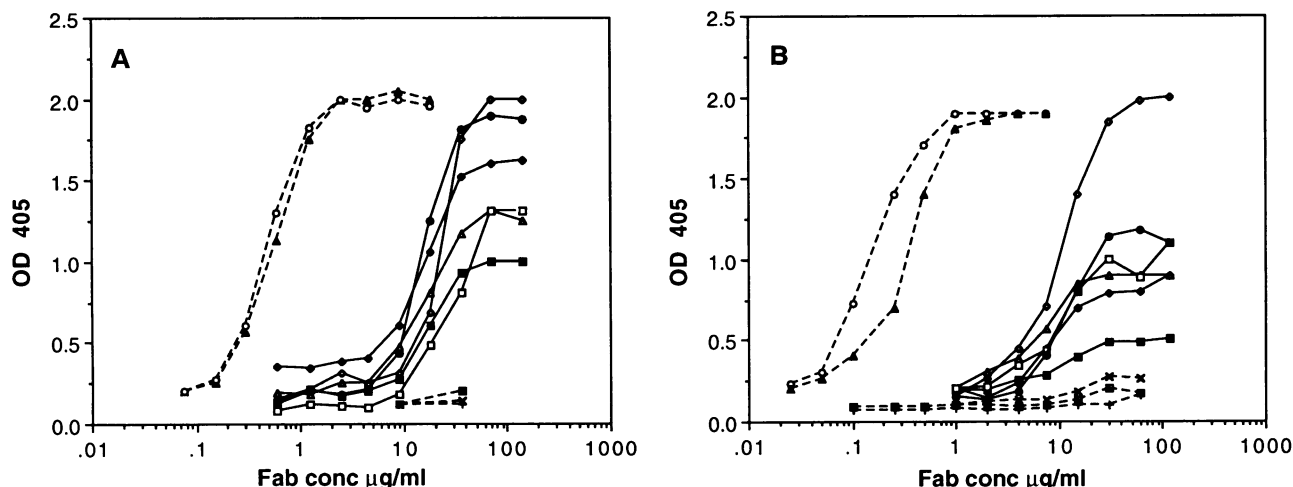

FIG. 2. (A) Comparison of monospecific anti-DNA antibodies from an SLE donor library (SI) and polyspecific anti-DNA antibodies from an HIV-1 seropositive donor library (donor L). The solid lines indicate the binding properties of a Fab [LNA3; donor L, antigen DNA (NA), clone 3] selected by biopanning the L library against human placental dsDNA. LNA3 was assayed for binding to solid-phase dsDNA (◇), ovalbumin (●), human transferrin (◆), BSA (■), human IgG Fc (△), and ganglioside GT1 (□). The dashed lines indicate the binding properties of two Fabs (ds3-40 and ds3-32) selected by biopanning the SI library against dsDNA. The steep dashed curves show the concentration dependence of ds3-40 (▲) and ds3-32 (○) binding to dsDNA. Also shown is the binding of ds3-32 to human IgG Fc (×), ovalbumin (+), and human transferrin (⊞). The binding of Fab ds3-40 to these latter antigens is essentially identical to that of Fab ds3-32. The much higher affinity (half-maximal binding at 10^{-8} M compared to 10^{-6} M) and lower cross-reactivity of the anti-DNA Fabs from the SLE library compared to the Fab from the HIV-1 seropositive donor are also illustrated. (B) Comparison of mono- and polyspecific antibodies isolated from the same HIV-1 library (L). The solid lines indicate the binding properties of a Fab (LNA12) selected by biopanning the library against dsDNA. LNA12 was assayed for binding to the same panel of antigens as in A. The dashed lines indicate the binding properties of two Fabs (L21 and L41) selected by biopanning the library against gp120. The steep dashed curves show the concentration dependence of Fab L21 (▲) and Fab L41 (○) binding to gp120. Also shown is the binding of Fab L21 to human dsDNA (×), ovalbumin (+), and human transferrin (⊞). The binding of Fab L41 to these latter antigens is essentially identical to that of Fab L21. The much higher affinity (≈100-fold, half-maximal binding at 10^{-8} M compared to 10^{-6} M) and lower cross-reactivity of the Fabs selected by panning against gp120 compared to the Fabs selected by panning against dsDNA are also illustrated. The IgG titers in the serum of the donor to gp120 and dsDNA were essentially equivalent (1:500) at the time of bone marrow aspiration for library construction. Comparison of A and B also shows that polyreactivity is more pronounced for Fab LNA3 than for LNA12.

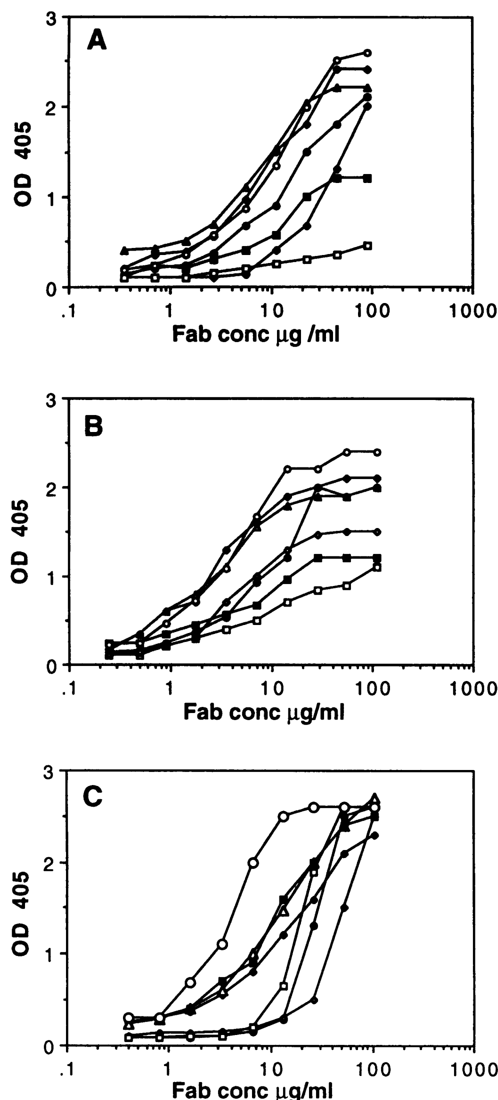

FIG. 3. Polyreactivity demonstrated by three representative Fab fragments, GII23 (*A*), SCD20 (*B*), and LEG36 (*C*), selected from HIV-1 libraries generated from three different donors (G, S, L) by biopanning against three different autoantigens [MHC class II (II), CD14 (CD), and EGFR (EG); numbers refer to clone designation]. The Fab fragments were assayed for binding to solid-phase human dsDNA (◇), ovalbumin (●), human transferrin (◆), BSA (■), human IgG Fc (△), ganglioside GT1 (□), and the autoantigen against which they were selected (○).

solution was discarded and 50 µl of phage library (typically 10^{11} colony-forming units) was added to each well and incubated for 2 hr. The soluble phage were then removed and the well was vigorously washed 10 times with PBS containing 0.05% Tween 20 (PBS/Tween). The ganglioside GD2-coated plates were washed instead with PBS containing 0.05% BSA. Bound phage were eluted with 0.1 M HCl (adjusted to pH 2.2 with solid glycine and containing 0.1% BSA) by pipetting up and down several times. Eluted phage were neutralized with 2 M Tris base and used to infect 2 ml of XL1-Blue for 15 min. Medium containing antibiotics and helper phage were then added sequentially as described (28) to amplify the diluted phage. The resulting phage library was then reapplied to the antigen to initiate a new round of biopanning. Generally four rounds of biopanning were carried out.

Preparation and ELISA Screening of Soluble Fab Fragments. Clones were grown in superbroth 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 then added and the culture was incubated overnight at 30°C. Cells were pelleted by centrifugation, resuspended in 2 ml of PBS containing 0.2 mM phenylmethylsulfonyl fluoride, and lysed by freeze-thawing three times. Debris was pelleted by centrifugation and the supernate containing the soluble Fab was tested in an ELISA format for binding to the antigens. Coating of antigens on the ELISA wells was carried out as described above. The Fab was incubated with antigen for 2 hr at 37°C followed by washing 10 times with PBS/Tween (GD2 was washed with PBS/BSA). Bound Fab fragments were detected with an alkaline phosphatase-labeled goat anti-human IgG Fab antibody (1:500 in PBS) (Pierce) and visualized with *p*-nitrophenyl phosphate substrate (Sigma) as monitored at 405 nm.

Inhibition ELISA. For the inhibition ELISA, the antigens were coated and blocked as described above. Dilutions of the Fab samples, previously determined in titration experiments to result in 75% of maximum OD, were mixed with soluble antigen at a final concentration of 0.3–200 µg/ml in PBS/Tween for 1 hr before transferring the mixture to antigen-coated ELISA wells. The mixture was incubated for 2 hr and bound Fab was detected as described above.

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). Sequencing primers for the heavy chain were SEQGz (5'-GAAGTAGTCCTTGACCAG-3') hybridizing to the (+)-strand and the T3 primer (5'-ATTAACCCTCACTAAAG-3') hybridizing to the (−)-strand. For the light chain, primer SEQKb (5'-ATAGAAGTTGTTCAGCAGGCA-3') hybridizing to the (+)-strand and primer KEF (5'-GAATTCTAAACTAGCTAGTTCG-3') were used.

RESULTS AND DISCUSSION

The sera from eight HIV-1 seropositive male donors were first tested in ELISA for binding to the purified autoantigens: MHC class II, dsDNA, CD14, EGFR, and the ganglioside GD2. As shown in Fig. 1, higher average serum IgG titers against all five antigens were found in the eight HIV-1 seropositive donors compared to eight healthy seronegative control male donors. Serum IgG responses to the autoantigens varied significantly between HIV-1 donors, and some had serum levels comparable to healthy controls. Bone marrow IgG combinatorial libraries were constructed from the eight HIV-1 seropositive donors and selected libraries were biopanned against antigens from the set above to retrieve a panel of monoclonal autoantibodies. The library from donor L, with the highest serum titer, was panned against four of the five antigens (Fig. 1). Other libraries were panned against antigens, where deemed appropriate by a high corresponding serum titer. As a control, an IgG combinatorial library constructed from bone marrow of a healthy individual (donor N) was also panned against the panel of antigens. The serum IgG response of this donor against the five autoantigens is also depicted in Fig. 1.

Four rounds of biopanning of the HIV-1 libraries produced up to a 30-fold amplification in eluted phage, indicating enrichment for antigen-binding clones. Plasmid DNA was prepared from the fourth round of panning and gene III was excised. The reconstructed phagemid was used to transform XL1-Blue cells to produce clones secreting soluble Fab fragments. Forty clones from each panning were grown in 10-ml culture and the supernates were examined for binding to the antigen in ELISA. Positive clones were obtained from all panning experiments using the libraries from the HIV-1 donors, whereas positive clones were obtained only from panning of the normal library against dsDNA. A total of 38 clones was obtained from the HIV-1 libraries. These clones

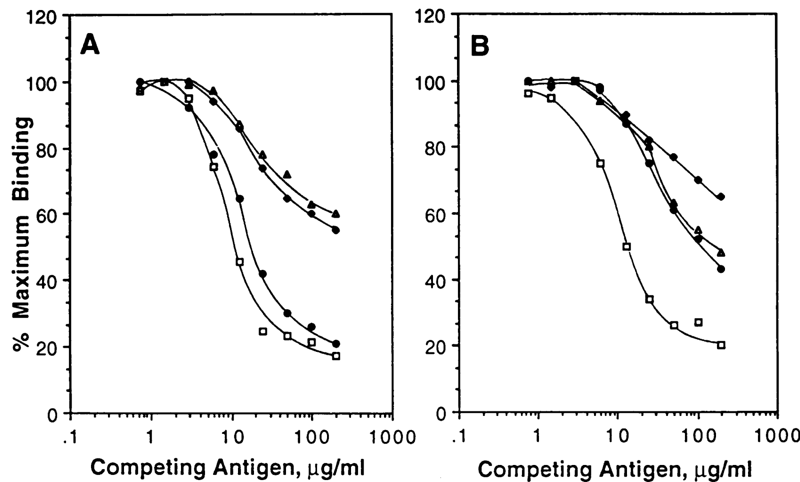

FIG. 4. Inhibition of the binding of LNA3 (*A*) and LNA12 (*B*) human Fab fragment (Fig. 2 *A* and *B*) to solid-phase human dsDNA by soluble homologous [human dsDNA (□)] and heterologous [human transferrin (◆), ovalbumin (●), and human IgG Fc (△)] antigens.

were regrown in 1-liter cultures and affinity-purified using an anti-human IgG $F(ab')_2$ column.

The specificities and affinities of the Fabs for a panel of autoantigens were determined using ELISA. As depicted in Figs. 2 and 3, all selected Fabs showed moderate affinity for antigen and cross-reactivity with several other autoantigens as well as exogenous antigens. The pattern of cross-reactivity was individual to a given Fab. These findings contrast with our recent observation of high-affinity anti-DNA antibodies from a library constructed from an SLE patient (Fig. 2*A*) (S.M.B., E. M. Salonen, H.J.D., C.F.B., G. J. Silverman, and D.R.B., unpublished data). These Fabs showed no cross-reactivity with the same panel of autoantigens.

The relevance of antibodies derived from libraries in reporting on antibody responses has been discussed elsewhere (40–42). The important point for this study is that the libraries used to select for antibodies to infectious agents are the same as those used to select for antibodies to self-antigens. In a number of cases, the serum titers to the exogenous antigens are very similar to those to self-antigens. However, the resulting cloned antibodies are, as described, of a very different character. For example, the serum from donor L has titers against dsDNA and gp120 of 1:500 and 1:400, respectively. However, as depicted in Fig. 2*B*, two human anti-gp120 Fabs retrieved from the L library are of high affinity and monospecific, in contrast to the autoantibodies selected by panning against DNA, which are polyspecific and of lower affinity.

The polyreactivity of the Fabs from the HIV-1 seropositive donors was confirmed in ELISA competitive inhibition assays in which the binding of a given Fab to a solid-phase antigen was tested in the presence of both homologous and heterologous soluble antigens (Fig. 4). The apparent affinities of these antibodies were of the order of 10^6–10^7 M^{-1}.

To establish the identity of the antigen-binding clones obtained from the different biopanning experiments, the variable heavy chain (V_H) and variable light chain domains were sequenced and compared. A detailed comparison of the sequences will be provided elsewhere (H.J.D. and D.R.B., unpublished data). This analysis established that several of the clones obtained by panning against a given antigen had identical, or very similar, sequences, which is further indication of specific selection of these antibodies. It was further found that a V_H sequence retrieved from panning the L library against DNA was also retrieved by panning the same library against MHC class II antigen. Another V_H sequence obtained from panning against DNA was also retrieved by panning against EGFR (Fig. 5). These findings strongly suggest that the repertoire of polyreactive antibodies is restricted. None of the clones showed any resemblance to the virus-specific antibodies previously retrieved from these libraries.

When the V_H gene usage of the 26 unique clones was analyzed, it was found that only 2 of the clones used the V_H3 family. V_H3 is the largest family of immunoglobulin V_H regions, consisting of 30 or more related gene segments (43, 44). The primers used for library construction should generally lead to roughly equal representation of V_H1 and V_H3. Whether the above findings reflect the nature of these polyreactive antibodies or are caused by a general depletion of B-cell-expressing V_H3 gene products as recently described in HIV-1 (45) is difficult to determine. However, it is worth noting that many of the antibodies specific for different viruses retrieved from the same libraries used the V_H3 family and no V_H3 deficiency has been observed. The V_H6 family has been reported to be associated with certain autoantibodies in humans (46). The 2 clones retrieved from the normal library here were from the V_H6 family as were 2 polyreactive clones previously selected from this library against measles antigens (47). In contrast, only 2 of 36 polyreactive clones from the HIV libraries were from the V_H6 family.

Clearly it is not possible to examine all autoantigens and therefore the present study cannot unequivocally eliminate the presence of specific autoantibodies in HIV-1 infection. However no support for this hypothesis can be found, particularly in cases where sequence similarities between HIV-1 proteins and self-antigens have been reported. Instead, the results suggest that an increased level of polyreactive antibodies is probably responsible for the elevated serum titers to autoantigens in the serum of HIV-1 seropositive donors. Polyreactive antibodies have also been de-

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	VH family
LNA1	LESGAEVKNPGASLKVSCQATGQRLI	DSPLH	WLRLAPGRGLEWMG	RINLYNGDTNYARRLQG	RVTMTRDKTINAAYMELRRLKYDDTAVYFCTL	EVEDIAARTFWFAP	WGRGTQVTVSS	VH1
LEG32	....	.....	....	....	....	....	....	...
LNA11	LESGTGLLKTSETLSLTCAVYGGSLS	NHYWS	WIRQAPGKGLEWLG	EINQSANRHHNPSLKS	RLTTQVDTSKNQFSLKLTSMTAADTGMYFCAR	RRMSVGYGSMWYRGFNWFDT	WGQGTQVTVAP	VH4
LNA12	....	.....	....	....	....	...A................	....	...
LII3	....	.....	....	....	....	...A................	....	...
LNA3	LESGPGLVKPSQTLSLTCSVSGGSIS	SGSYYWS	WIRQPAGKGLEWIG	RIYSSGSTNYNPYLKS	RVAMSLGTSKNQFFLKLTSVTPTDTAEYLCAR	LEPGKYSYKPGPFDI	WGRGTMVTVSS	VH4

FIG. 5. Amino acid sequence analysis of the V_H domains of a selected number of the autoimmune human Fab fragments. The sequences are designated as previously: first letter, library donor identifier; second and third letters, autoantigen against which they were selected (NA, dsDNA; EG, EGFR; II, MHC class II). The sequences LNA1 (panning antigen DNA) and LEG32 (EGFR) are identical as are the sequences LNA12 (DNA) and LII3 (MHC class II). The V_H sequences of the polyreactive HIV-1 clones analyzed in Figs. 2 and 4 are shown: LNA3 and LNA12. Dots indicate identity with the preceding sequence. FR, framework region; CDR, complementarity-determining region.

scribed from healthy individuals where they occur at low levels and have been referred to as "natural" autoantibodies (48–50). The discrete $CD5^+$ B-lymphocyte subtype has been implicated in the production of polyreactive antibodies (51, 52), and recent data have suggested that many HIV patients have elevated levels of this cell type (53). The polyreactive antibodies observed may reflect this increase.

Any role of polyreactive antibodies in the pathogenesis of HIV-1 infection is unclear. However, the affinities of the monovalent Fab fragments described here are of the order of 10^6–10^7 M^{-1} for a range of antigens. As bivalent IgG molecules, the affinity for antigen (avidity) may well be an order of magnitude higher, which would be expected to permit *in vivo* antibody–antigen interactions. The polyreactivity of the combining site may introduce novel features to the *modus operandi* of the antibody molecule, e.g., self-association, cross-linking of unrelated antigen molecules, and focusing of diverse species to the surface of Fc receptor-bearing cells. Given considerations such as these, the effects of high serum levels of antibodies capable of interacting with diverse molecular species are difficult to predict and warrant further investigation.

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