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Biochemical and Biophysical Research Communications 417 (2012) 1164–1169

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Characterization of germline antibody libraries from human umbilical cord blood and selection of monoclonal antibodies to viral envelope glycoproteins: Implications for mechanisms of immune evasion and design of vaccine immunogens

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ARTICLE INFO

Article history:
Received 2 December 2011
Available online 27 December 2011

Keywords:
HIV-1
Human monoclonal antibody
IgM
gp120
Envelope glycoprotein
Immunogen

ABSTRACT

We have previously observed that all known HIV-1 broadly neutralizing antibodies (bnAbs) are highly divergent from germline antibodies in contrast to bnAbs against Hendra virus, Nipah virus and SARS coronavirus (SARS CoV). We have hypothesized that because the germline antibodies are so different from the mature HIV-1-specific bnAbs they may not bind the epitopes of the mature antibodies and provided the first evidence to support this hypothesis by using individual putative germline-like predecessor antibodies. To further validate the hypothesis and understand initial immune responses to different viruses, two phage-displayed human cord blood-derived IgM libraries were constructed which contained mostly germline antibodies or antibodies with very low level of somatic hypermutations. They were panned against different HIV-1 envelope glycoproteins (Envs), SARS CoV protein receptor-binding domain (RBD), and soluble Hendra virus G protein (sG). Despite a high sequence and combinatorial diversity observed in the cord blood-derived IgM antibody repertoire, no enrichment for binders of Envs was observed in contrast to considerable specific enrichments produced with panning against RBD and sG; one of the selected monoclonal antibodies (against the RBD) was of high (nM) affinity with only few somatic mutations. These results further support and expand our initial hypothesis for fundamental differences in immune responses leading to elicitation of bnAbs against HIV-1 compared to SARS CoV and Hendra virus. HIV-1 uses a strategy to minimize or eliminate strong binding of germline antibodies to its Env; in contrast, SARS CoV and Hendra virus, and perhaps other viruses causing acute infections, can bind germline antibody or minimally somatically mutated antibodies with relatively high affinity which could be one of the reasons for the success of sG and RBD as vaccine immunogens.

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1. Introduction

Elicitation of potent, broadly neutralizing antibodies (bnAbs) against HIV-1 by immunization remains a challenge. We had previously hypothesized that HIV-1 may use conserved structures that cannot initiate immune responses because of the existence of "holes" in the human germline B cell receptor (BCR) repertoire, i.e., lack of germline antibodies capable of binding those structures [1]. In support of this hypothesis, we showed that germline-like antibodies corresponding most closely to known HIV-1 bnAbs such as b12, 2G12 and 2F5 lack measurable binding to the HIV-1 envelope glycoprotein (Env) [1]. This observation led to investigation of the maturation pathways of two HIV-1 bnAbs b12 and X5 [2], whose structures and functions are well known [3,4], as well as to the identification and characterization of several human IgM-derived monoclonal antibodies (mAbs) selected from a large phage-displayed naïve human antibody library constructed from 59 healthy donors [5]. These studies demonstrated that germline intermediates corresponding to b12 also fail to bind the Env with high affinity, whereas the X5 putative germline-like predecessor antibody and other IgM-derived mAbs which diverged less from their corresponding germlines have high binding affinity for the Env; however, the latter enhanced or did not potently neutralize infection by HIV-1 primary isolates. Further studies on B cell lineages and maturation pathways of HIV-1 bnAbs may sidestep this impediment to HIV-1 vaccine developments [6].

As a part of our studies on the human antibodyome toward understanding initial responses to immunogens [7], we previously

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0006-291X/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.bbrc.2011.12.089

generated large IgM antibody libraries and developed and characterized IgM mAbs against SARS coronavirus (SARS CoV) protein receptor-binding domain (RBD), and soluble Hendra virus G protein (sG) [8,9]. Human umbilical cord blood B lymphocytes that presumably have not been exposed to exogenous antigens have been used as a source of naturally-occurring germline or minimally-mutated pre-immune antibodies [10,11]. For this reason, cord blood-derived IgM libraries might serve as a relevant source for selecting the closest germline antibodies corresponding to broadly neutralizing mAbs if they exhibit binding to target antigens. The current study addresses the hypothesis that the human cord blood does not contain high-affinity binders to HIV-1, although it has high-affinity antibodies against other human infectious agents such as SARS CoV and henipaviruses.

To explore the diversity and specificity of cord blood-derived IgM antibodies, antibody libraries were characterized using large-scale Sanger sequencing to assess potential repertoire diversity, from which antibodies capable of binding to the Envs, RBD and sG could be identified. Although large-scale sequencing of a cord-blood derived IgM antibody repertoire revealed relatively high diversity, there was no enrichment observed by sequential panning against the Envs. However, considerable specific enrichments were seen when the libraries were panned against the RBD in which the antibodies produced were very close to their putative germline predecessors. These results suggest that HIV-1 could have evolved to elude strong binding to minimally somatically-diversified human antibodies as an escape mechanism from adaptive immune responses.

2. Materials and methods

2.1. Cord blood, viral proteins, human two-domain soluble CD4 (sCD4) and antibody

Cord blood was received from the National Disease Research Interchange (NDRI, Philadelphia, PA), and care was taken not to contaminate the cord blood samples with maternal blood. SARS CoV RBD [12] and human sCD4 were produced in our laboratory. HIV-1 Env $gp120_{Bal}$ [13] was provided by T. Fouts (Institute of Human Virology, Baltimore; currently at Profectus, Baltimore, MD). Soluble Hendra virus envelope glycoprotein G (sG) [14] and $gp140_{JRFL}$ were provided by C. Broder (Uniformed Services University of the Health Sciences, Bethesda, MD). The $gp140_{Con\text{-}s}$ [15] was provided by B. Haynes and H. Liao (Duke University, Durham, NC). Horseradish peroxidase (HRP)-conjugated mouse anti-M13 antibody was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Library construction

As a source for amplification of antibody gene fragments, cDNA was prepared from the cord blood of two babies [16] and libraries were constructed using phagemid pComb3x as previously described [17].

2.3. Analysis for sequence diversity of the libraries

The heavy (VH) and light (VL) chain variable domains of antibodies were sequenced using the standard Sanger sequencing method. Resulting sequences were sorted and trimmed to produce independent pools of heavy and light chain sequences, and the latter were further sorted into kappa (V_κ) and lambda (V_λ) light chains by searching their translated sequences for a set of mutually-exclusive motifs. These sequences were analyzed using the program JoinSolver [18], and those which it found to be the result of productive rearrangement and likely to yield functional products formed the data sets for further analysis. Using the outputs from JoinSolver, germline diversity, the heavy chain complementarity determining region 3 (HCDR3) lengths, and the number of mutations compared to the closest germline match from the V(D)J families were obtained for each sequence. Translated heavy and light chain variable segment library sequences were aligned using ClustalX2 [19] and trimmed to equivalent lengths within each group: V_H codons 3–91, V_κ codons 9–88, and V_λ codons 3–88. Functional V_H, V_κ, and V_λ translated germline sequences in the NCBI IgBLAST database (http://www.ncbi.nlm.nih.gov/projects/igblast) were similarly treated. Separate identity matrices for each stream were generated using BioEdit [20] and the number of amino acid (AA) changes between each query sequence and its closest germline match, as found by JoinSolver, were determined.

2.4. Selection of antibodies against HIV-1 Envs, SARS CoV RBD and Hendra virus sG

The combined cord blood libraries were used for selection of antibodies against antigens conjugated to magnetic beads (Dynabeads M-270Epoxy; DYNAL Inc., New Hyde Park, NY) as described previously [8]. Antigen was present in the amounts of 5, 5, 2.5 and 1 μg for the first, second, third, and fourth rounds of panning, respectively. Specific enrichment was determined by using polyclonal phage-based ELISA (ppELISA). Briefly, ppELISA was performed by using Corning high-binding 96-well plates coated with 1 μg/ml of antigen and blocked with 3% non-fat dry milk in PBS (MPBS). The microplate wells were inoculated with 50 μl per well of MPBS containing 10^{10} PFU of pooled phage purified from each round of panning for 2 h at room temperature. Following 4 washes with PBS containing 0.05% Tween 20 (PBST), bound phage was detected by adding 50 μl of 1:5000 diluted HRP-conjugated mouse anti-M13 antibody (Sigma, St. Louis, MO) to each well. Following incubation for 1 h at room temperature, the plates were washed 4 times with PBST and the assay was developed at 37 °C with ABST substrate (Roche, Indianapolis, IN) and absorbance monitored at 405 nm. Clones that specifically bound to antigens were identified from the fourth round by using monoclonal phage ELISA (mpELISA) as described [8].

2.5. Expression, purification and binding of antibodies

Soluble antigen-binding fragments (Fabs) of antibodies were expressed, purified, and their binding activity was measured as previously described [21].

3. Results and discussion

3.1. Construction of two human antibody libraries by using phagemid vector pComb3x

Two relatively large (6.7×10^8 and 7.8×10^8 members) phage-displayed human IgM Fab libraries (designated ml32 and ml33, respectively) were constructed from 50 ml of cord blood from each of two babies as described in Section 2. To estimate the sequence

Table 1
Numbers of unique and duplicate sequences obtained from cord blood IgM libraries using the Sanger sequencing method.

	VH		V_κ		V_λ	
	Unique	Duplicate	Unique	Duplicate	Unique	Duplicate
ml32	747	6	316	41	265	113
ml33	792	4	359	64	261	106
Combined	1538	11	651	129	462	283

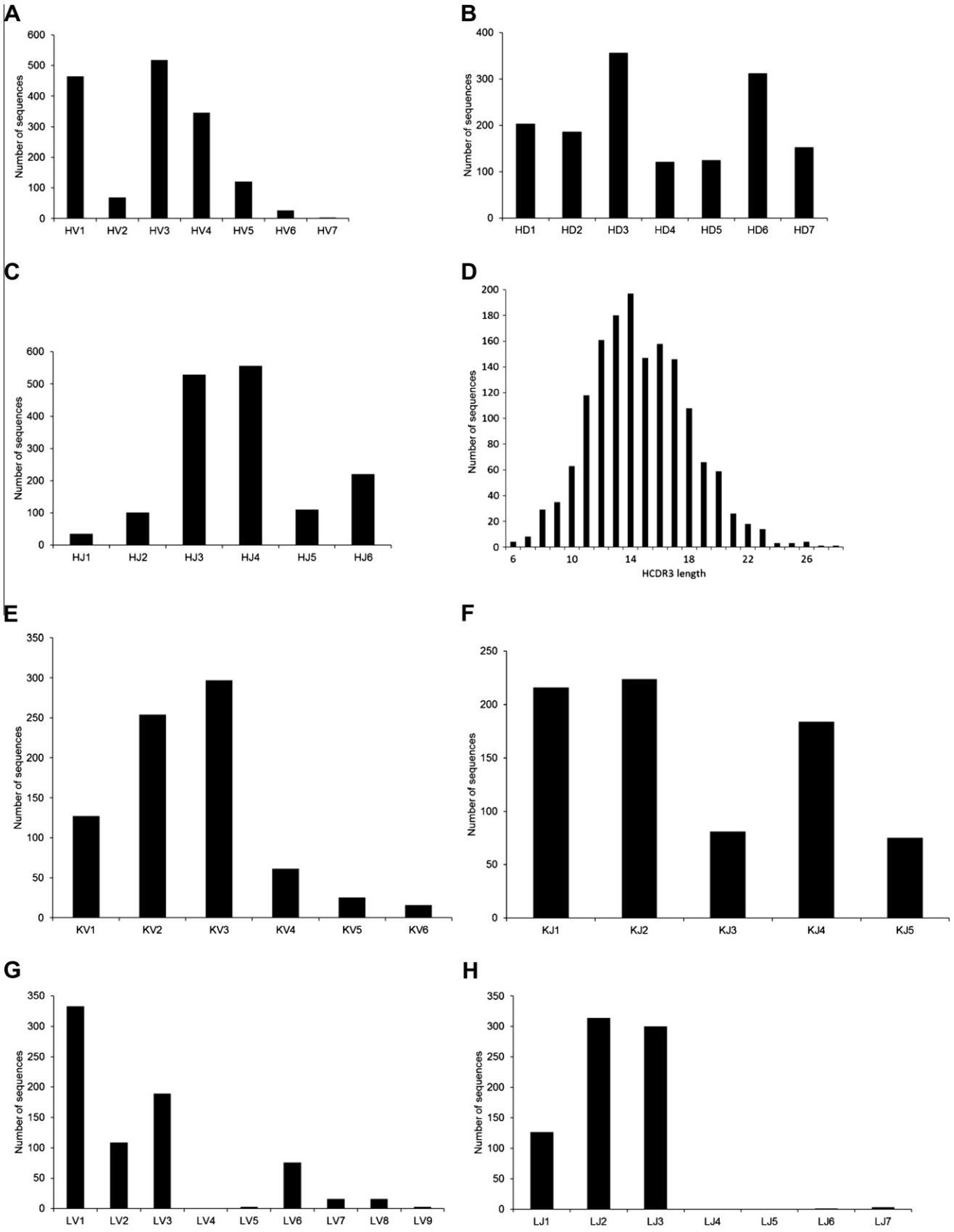

Fig. 1. Germline usage frequencies and HCDR3 length diversity observed from human cord blood IgM libraries are shown. (A–C) HV, HD and HJ gene families utilization in 1538 heavy chain sequences. (D) HCDR3 length diversity. (E–H) kV/kJ and LV/LJ gene families utilization in 651 and 462 sequences of V_K and V_λ, respectively.

diversity of the libraries, 25 and 22 clones were randomly selected from ml32 and ml33, respectively, and sequenced. No identical sequences of heavy and light chains were found; more than 80% of the clones showed productive V(D)J rearrangements. Of the 25

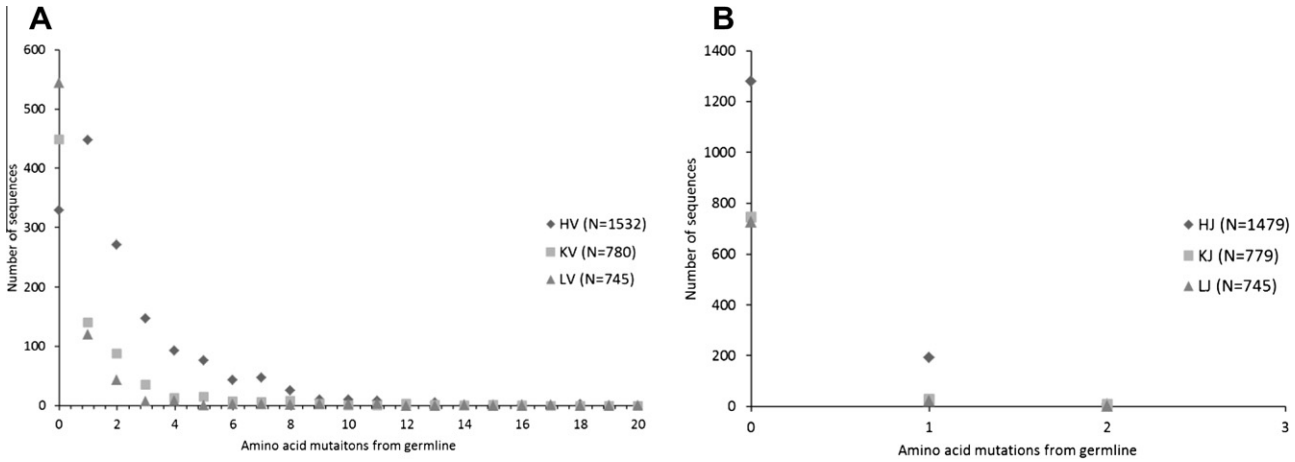

Fig. 2. Number of amino acid changes existing in antibodies found in the combined cord blood repertoire as compared to its closest germlines. (A) Number of mutations in the HVs, KVs, and LVs. (B) Number of mutations in the HJs, KJs, and LJs.

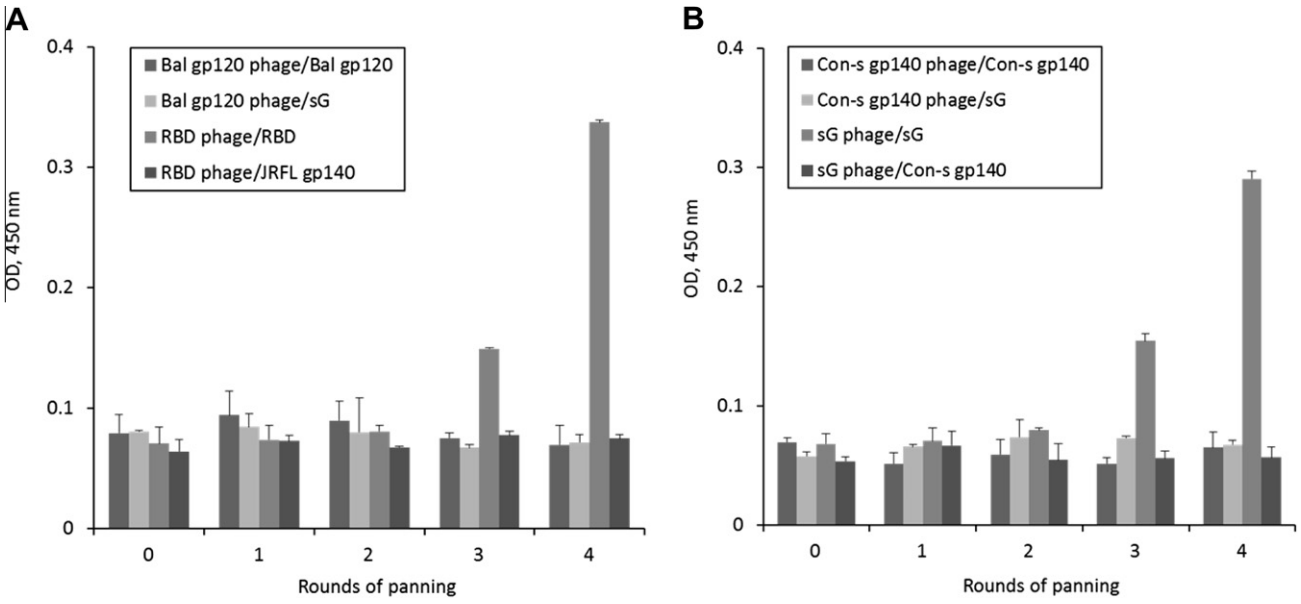

Fig. 3. Results of library panning experiments showing enrichments against (A) SARS CoV protein RBD and (B) Hendra virus protein sG.

m132 clones, 14 had V_κ light chains (families KV1–4) and 11 had V_λ light chains (families LV1–3, LV6–8 and LV10). Their heavy chains were also widely distributed (families HV1–5). Similar gene usage and family distribution were observed with m133. These results indicate that the cord blood libraries could have a high level of sequence diversity and contain antibody gene fragments from most of the families of both heavy and light chains.

3.2. High level of sequence diversity and low level of somatic diversification of antibodies randomly selected from the libraries

To more accurately estimate the diversity of the cord blood libraries, an additional 2112 clones from ml32 and ml33 were sequenced. Productive, full-length rearranged VH and VL sequences from each library were identified. Similarity between the sequences from the VH and VL repertoires was calculated, and 747 (99.2%) and 792 (99.5%) V_H sequences from m132and m133, respectively, were found to be unique (Table 1). The diversity of VL was significantly lower than that of VH; 70.1% and 88.5% for V_λ and V_κ of VL domains from m132, and 71.1% and 84.9% for V_λ and V_κ of VL domains from m133 were identified as unique. When the sequences from both libraries were combined, 1549 (99.3%), 780 (83.5%) and 745 (62.0%) sequences of V_H, V_κ, and V_λ, respectively, were unique. The combined cord blood antibody repertoire was then analyzed for gene usage, HCDR3 length, and somatic mutations compared to the closest germline counterparts. The heavy chain V (HV) regions were found to derive from all 7 HV germline families (Fig. 1A), and contained representatives from 45 subfamilies found in the IMGT database [22]. All heavy chain D (HD) and J (HJ) gene families were found but in different proportions (Fig. 1B and C). The lengths of HCDR3 ranged from 6 to 26 amino acid residues and the mode was a HCDR3 length of 14 (Fig. 1D). For V_κ, the V regions, KVs, were identified from gene families KV1–6 but KV7 was absent (Fig. 1E); the J regions, KJs, were distributed among all five gene families, KJ1–5 (Fig. 1F). For V_λ, all the V regions, LVs, were present except for three LV gene families, LV4, LV10 and LV11 (Fig. 1G); the J regions, LJs, were mostly distributed in LJ1–3 gene families while LJ4 and LJ5 were absent (Fig. 1H).

To determine the level of somatic mutations of the antibodies, each sequence from the combined cord blood repertoire was compared to its closest germline and the number of amino acid (AA)

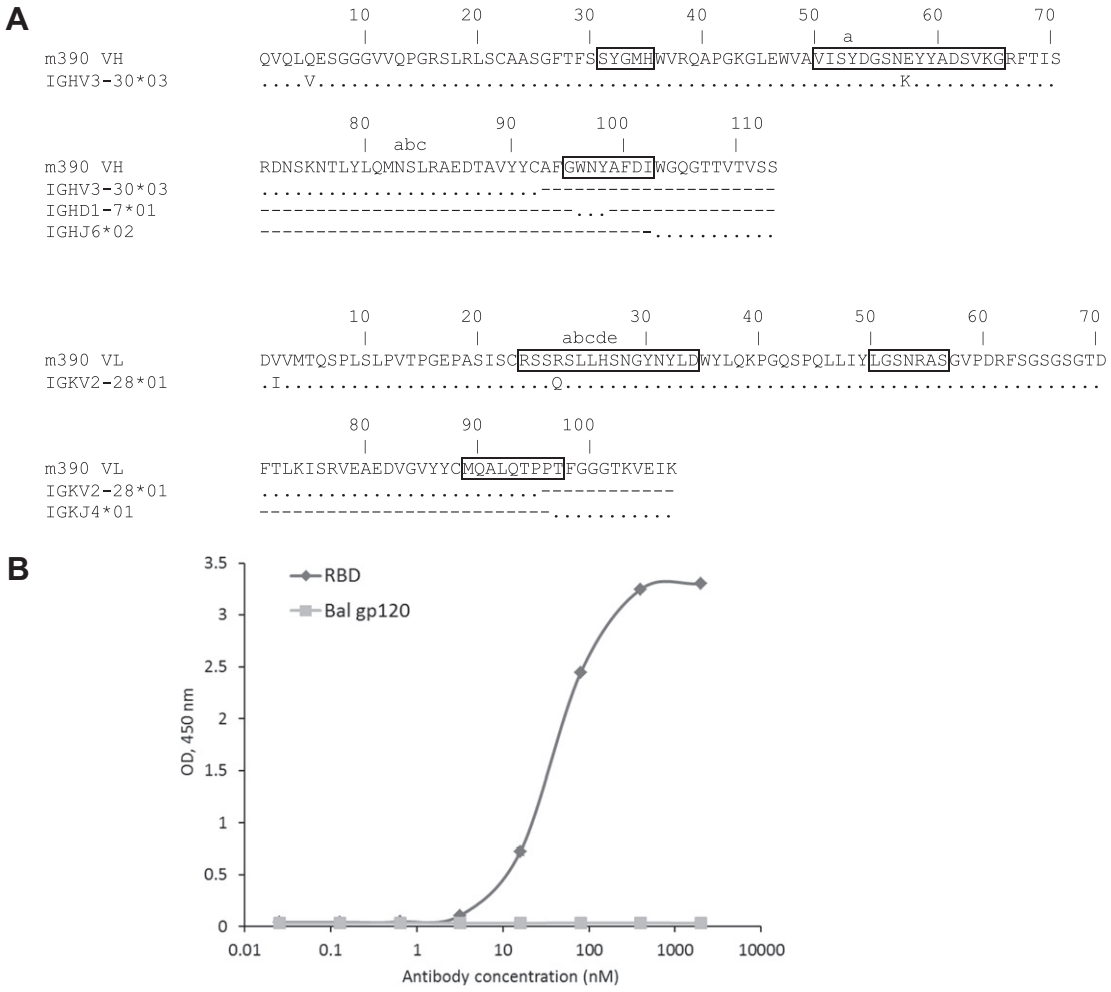

Fig. 4. Characterization of a representative antibody against SARS CoV. (A) The VH and VL sequences of m390 are shown along with potential germline precursors. The six CDRs are depicted in bold face following the Kabat numbering scheme. Only mutated residues are shown for the germline sequences. (B) ELISA binding of Fab m390 to SARS CoV protein RBD and an irrelevant antigen HIV-1 $gp120_{Bal}$ showing the specificity of the binding.

mutations was calculated. Of the HVs, 21.5% were the same as germline sequences; 39.2% of the sequences contained only one AA mutation; the remaining 39.3% had two or more AA mutations, but very few sequences with more than 8 mutations were found (Fig. 2A). In contrast, the light chains were significantly less mutated, with 57.6% and 73.0% of the KVs and the LVs, respectively, giving translations identical to germlines. The numbers of mutations occurring in the J regions of heavy and light chains were also calculated. Similarly to what was observed with the variable regions, relatively more mutations were observed in the HJs than in the KJs and LJs (Fig. 2B). The mutations in D regions were not estimated because of the difficulties in determining the D gene germlines for many antibodies due to the events of recombination involving insertion and deletion. These results suggest that the sequence diversity of the libraries is likely to be high although the light chains appear to be less diverse than the heavy chains, and the libraries exhibit low levels of somatic hypermutations.

3.3. Lack of human germline antibodies binding to the exposed epitopes on HIV-1 Envs

Previously, it was noted that the known bnAbs against HIV-1 were highly divergent from their germline sequences in contrast to bnAbs against SARS CoV and henipaviruses which were less diversified, possessing a limited number of mutations. In addition, it was found that germline-like predecessors of several HIV-1 bnAbs lack measurable binding to HIV-1 Envs [1]. Based on these findings, we hypothesized that there is a scarcity or absence of high affinity binders to HIV-1 Envs in the minimally somatically-hypermutated human cord blood antibody repertoire, whereas high affinity antibodies against other human infectious agents including SARS CoV and henipaviruses can be easily found. To test this hypothesis, the combined cord blood library of ml32 and ml33was panned against $gp140_{Con\text{-}s}$, a synthetic Env designed by aligning the consensus Env sequences of group M [15], and the Hendra virus protein sG in parallel. After four rounds of panning, enrichment and binding specificity of pooled phage were determined by ppELISA. Interestingly, significant and specific enrichment was observed with selection against sG only (Fig. 3). In a separate experiment, the mixed library was panned against another Env, $gp120_{Bal}$, and simultaneously the RBD. In line with previous observations, no marked enrichment was achieved with panning against $gp120_{Bal}$ while considerable and specific enrichment was seen when the library was panned against the RBD (Fig. 3). These results support the hypothesis and suggest that HIV-1 Envs could have evolved strategies to avoid binding to the germline repertoire by presenting epitopes unsuitable for minimally somatically-diversified human antibodies to bind with, thereby denying them the chance to bind with high affinity. This strategy may represent a way exploited by the virus to escape from strong immune responses.

3.4. The selected antibodies against SARS CoV RBD are very close to germlines in sequence and have relatively high binding activity

To determine the extent to which antibodies against SARS CoV selected from the cord blood library are mutated, a number of individual clones with high affinity for the SARS CoV RBD were identified by mpELISA as described in Section 2. Sequencing of these clones revealed them to be identical. This unique clone, designated m390, contains gene products of HV3–30 and KV2–28 in the heavy and light chain V regions, respectively, (Fig. 4A). M390 has only 2 AA mutations each in the V regions of both heavy and light chains and no mutations in the D and J regions. The purified soluble m390 Fab bound to SARS CoV RBD with an EC_{50} of about 50 nM while no binding to an irrelevant antigen $gp120_{Bal}$ was observed suggesting the specificity of the binding (Fig. 4B). Previously, vesicular stomatitis virus (VSV) specific antibodies with $V_HQ52/V_\kappa19–28$ gene combination elicited in a mouse model were found devoid of somatic mutations but showed high binding avidities [23]. These results are in line with our previous findings and provide supporting evidence for our hypothesis that extensive somatic hypermutations could be a unique feature for bnAbs against HIV-1 and perhaps other viruses causing chronic infections.

In summary, panning of a large phage-displayed minimally somatically-diversified antibody library derived from human cord blood against the HIV-1 Envs did not yield any significant binders. However, considerable specific enrichments were seen after panning against the SARS CoV RBD and Hendra virus sG, yielding an antibody against the RBD whose sequence is close to its parent germline. As we used the whole Envs in this study, one would expect the binding activity due to highly immunogenic epitopes such as the V3 loop which actually could bind and initiate immune response though it may be capable of diverting immune responses from the conserved epitopes. Therefore, the fact that there is no relatively high affinity binding of Envs might indicate that immune responses may not be so fast or in general are different than in the case of the other viruses. These results provide further evidence supporting and expanding the hypothesis that HIV-1 uses a strategy based on absent or weak binding of its conserved epitopes to germline antibodies for escaping neutralizing immune responses.

Acknowledgments

We thank Drs. C. Broder, B. Haynes, H. Liao and T. Fouts for reagents. This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research, and the Gates Foundation to DSD, and by Federal funds from the NIH, National Cancer Institute, under Contract No. NO1-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the US Government.

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