

***In vitro* immunization of naive human B cells yields high affinity immunoglobulin G antibodies as illustrated by phage display**

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SUMMARY

In vitro antibody responses to a synthetic immunogen, consisting of both a B cell [V3 loop of gp120 from human immunodeficiency virus type 1 (HIV-1)] and a T-helper epitope (15 amino acids of tetanus toxoid) was studied. The *in vitro* activation was performed by primary and secondary *in vitro* immunizations, using lymphocytes obtained from uninfected, seronegative donors. Analysis of the *in vitro* immune response demonstrated an antigen-specific isotype switch, which was dependent on the presence of antigen-specific T-helper cells, CD40 ligation and antigen. Antibody libraries were constructed from cells derived directly from the naive donors, or from primary or secondary *in vitro* immunized B cells. Five libraries were displayed on filamentous phage and selected for anti-V3-specific Fab fragments, using a selection approach that linked recognition and phage replication. A panel of 19 recombinant antigen-specific Fab, representing different phases of the humoral *in vitro* immune response, were sequenced, expressed and analysed using a biosensor. Recombinant Fab fragments derived from cultures on day 12 exhibited an increase in affinity of close to two orders of magnitude compared to those obtained from cells primary immunized for 7 days. This study provides the first evidence that an antigen-specific *in vitro* immune response can yield high-affinity immunoglobulinG antibodies.

INTRODUCTION

Stimulation of the B-cell surface molecule CD40 by its natural ligand (CD40L) found on activated CD4⁺ helper T (Th) cells has been demonstrated to play a critical role in the *in vivo* activation of antigen-specific B cells.¹⁻⁴ In lymph nodes, extrafollicularly activated B cells migrate into primary follicles where they undergo isotype switching, affinity maturation and antigenic selection. This is morphologically characterized as germinal centre formation,⁵ although the precise mechanism and molecules underlying this phenomenon are still not completely understood. However, CD40L, lymphokines and follicular dendritic cells have been shown to play a central role in the germinal centre reactions.⁶

Attempts to dissect this antigen-specific immune response *in vitro* have met with limited success due to difficulties in creating

an adequate micro-environment outside the human host. We recently demonstrated that a primary, antigen-specific immunoglobulin M (IgM) response against the V3 loop of human immunodeficiency virus type-1 (HIV-1) was enhanced by using a peptide antigen carrying a T helper cell epitope, thus recruiting specific T memory cells *in vitro*.⁷ Furthermore, using such a peptide immunogen an antigen-specific isotype-switching could be obtained in the presence of cloned Th0-type T helper cells.⁸ In the present investigation, we have rescued primary *in vitro* immunized human B cells by a secondary set of signals via CD40 and the antigen receptor as well as by non-clonal human CD4⁺ T helper cells. The results were evaluated with antibody library and phage display technologies,^{9,10} which allowed us for the first time to demonstrate that *in vitro* immunization of naive human B cells resulted in the selection of high affinity variants.

MATERIALS AND METHODS

Cell culture

Cultures of human cells were performed in RPMI-1640, supplemented with 4 mM L-glutamine, 100× non-essential amino acids (Seralab Ltd., Cramlington, UK), 50 µM 2-mercaptoethanol, 50 µg/ml gentamycin (Biological Industries, Haemek, Israel), and either 10% fetal calf serum (FCS) (Life Technologies Ltd., Paisley, UK) or 5% pooled human serum (University Hospital Blood Bank, Lund, Sweden).

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Abbreviations: PBMC, peripheral blood mononuclear cells; SAP, selection and amplification of phage; Th, CD4⁺ T helper cell; V, variable gene segment; V3, loop, neutralization determinant of gp120 from human immunodeficiency virus type 1.

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Phytohaemagglutinin (PHA) and staphylococcal enterotoxin A (SEA) were purchased from Pharmacia AB (Uppsala, Sweden) and Toxin Technology (Madison, WI), respectively.

Preparation of lymphocytes

Buffy coats from HIV-seronegative healthy donors were obtained from the University Hospital Blood Bank. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll–Paque (Pharmacia) and washed twice in phosphate-buffered saline. To prepare cell populations for primary *in vitro* immunization and T-cell stimulation, PBMC were treated with L-leucyl-L-leucine methylester hydrobromide (LeuLeuOMe) (Bachem Feinchemikalien AG, Budendorf, Switzerland), as previously described.¹¹ Briefly, the cells were incubated with freshly prepared 0.25 mM LeuLeuOMe in RPMI-1640, containing 2% FCS, for 15 min at room temperature. The cells were then washed three times with medium, containing 2% heat-inactivated human serum.

Synthetic peptides and antigen

Two synthetic peptides were used in this study: a 15-mer helper T-cell epitope of tetanus toxin,¹² covering amino acids 830–844 (ptt); and a 30-mer peptide containing the helper T-cell epitope (ptt) linked to the V3 region epitope, covering amino acids 309–323 of the HIV-1 LAI isolate (pV3) (kindly supplied by Dr B. Wahren, Stockholm), yielding the heterotope (ptt-V3). Amino acids were numbered according to the Database of Los Alamos Laboratory (Los Alamos, NM). Recombinant pB1,¹³ a fusion protein containing most of the C-terminal part of gp120, was a kind gift from Repligen Inc. (Cambridge, MA).

Primary in vitro immunization and T-cell stimulation

The LeuLeuOMe-treated PBMC were either subjected to a primary *in vitro* immunization by 7 nM ptt-V3 (20 ng/ml) to give pV3-specific IgM-secreting B cells, as recently described,⁷ or were stimulated by ptt to obtain ptt-specific T cells. For the generation of such antigen-stimulated T cells, 1×10^6 LeuLeuOMe-treated cells/ml were cultured in the presence of $10 \mu\text{M}$ ptt and 1×10^6 cells/ml of irradiated (4000 rads) autologous PBMC in 75 cm^2 culture flasks, using medium containing 5% human serum. The T cells were restimulated twice (day 3 and 5) by adding 1×10^6 PBMC/ml and $10 \mu\text{M}$ ptt. The T-cell stimulation cultures were devoid of any exogenously added lymphokines.

Secondary in vitro immunization

Cultures of primary immunized B cells or antigen-stimulated T cells were harvested on day 7 by centrifugation (400 g) through a cushion containing 40% Ficoll–Paque in phosphate-buffered saline (PBS). CD4^+ T cells were purified from the T-cell cultures using anti-CD4-coated M450 magnetic beads followed by DETACHaBEAD (Dynal International, Oslo, Norway), as described by the manufacturer. Secondary *in vitro* immunization was subsequently performed by a modification of a previously described method.⁸ Briefly, 10^4 ptt-stimulated autologous CD4^+ T cells or control T cells were co-cultured for 4 days with 10^5 primary immunized cells/ml on irradiated (10 000 rads) CD32-transfected fibroblasts in 75 cm^2 culture flasks, containing 500 ng/ml anti-CD40 monoclonal antibodies (mAb; generously provided by Dr S. Pauli, Stockholm) and

7 nM ptt-V3 (20 ng/ml) peptide. Control experiments included cultures where anti-CD40 mAb or CD32-transfected fibroblasts had been omitted, or where PHA was used as a polyclonal activator.

Primary or secondary *in vitro* immunized cells were infected with Epstein–Barr virus (EBV)⁸ and antigen-specific enzyme-linked immunosorbent assay (ELISA) for detecting HIV-specific human antibodies was performed, as previously described.⁷

Construction of phage displayed Fab libraries

Five different combinatorial Fab libraries were constructed. One library was constructed from a mixture of naive B cells derived from donors A and B. Libraries were also constructed after each immunization step, using antigen-specific EBV-infected cultures from each individual donor (A and B). These libraries were displayed on the surface of M13 phage, essentially using the primers described by Marks *et al.*,¹⁴ including restriction sites for cloning into the pEXmide3 phagemid vector.¹⁵ The cloning procedure was performed according to Burton *et al.*¹⁶ Briefly, mRNA was prepared from 10^5 EBV-infected cells and was reverse transcribed, using specific primers for γ , μ , κ and λ constant regions. The V region sequences were polymerase chain reaction (PCR)-amplified and cloned into the pEXmide3. Recombinant phagemids were electroporated into *Escherichia coli* SURE cells, as recently described.¹⁷ The heavy chains were cloned into the vector after the light chain had been introduced and library sizes of around 10^6 members over religated vector alone were obtained.

Construction of fusion protein used for the SAP procedure

A fusion protein between the M13 protein 3 and pV3 was produced using the pEXmide3 vector, which was digested with *NcoI* and *EcoRI*.¹⁸ The fusion protein gene was assembled by PCR, using gene 3 as template and a primer encoding the pV3 peptide sequence plus 15 base-pair (bp) complementary to the 3'-residues of gene 3 (corresponding to residues 94–98 of protein 3). Thus, 5'-GGGAATTCTTA AAC GAA AGC ACG ACC CGG ACC ACG CTG GAT ACG GAT AGA TTT ACG GGATCC GCC ACC CTC AGA ACC-3' was used as the downstream primer and a primer complementary to the 5' end of gene 3 was used as the upstream primer: 5'-GGCCATGG CCG TGA AAA AAT TAT TAT TCGC-3'. These primers introduced restriction sites *NcoI* and *EcoRI* for cloning and one *BamHI* site between gene 3 and the gene encoding pV3. The PCR product was restriction digested, ligated into the vector and transformed into Y1089 competent cells by electroporation.¹⁷ Expression and function of the fusion protein was evaluated by the procedures described by Dueñas and Borrebaeck.¹⁸

Selection and amplification of specific phages

Antigen-specific phages were selected using the SAP system.¹⁸ Briefly, non-infectious antibody phage libraries were constructed, using a gene 3-deleted helper phage denoted M13MDΔ3. These non-infectious phages were then incubated overnight at 4° with 25% (v/v) of the culture supernatant containing the fusion protein (protein3–pV3). Subsequently, male bacteria (XL1 Blue strain) were infected and selected in ampicillin containing medium. After each round of selection, 30–100 colonies were grown for 16 h at room temperature in

96-well culture plates in 100 μ l of 2 $\times$ YT medium, containing 100 μ g/ml of ampicillin and 0.1 mM of IPTG. The plates were centrifuged at 1000 *g* and 50 μ l of the culture supernatant was analysed by ELISA, as recently described,¹⁸ against pB1 and unrelated antigens such as hen egg lysozyme and bovine serum albumin (BSA). The different Fab clones were coded as: donor (A or B); immunization status, i.e. U (= unimmunized) or P/S (= primary or secondary); number of selection rounds (1 or 2); and individual clone number.

DNA sequencing and analysis

Nucleic acid sequencing was carried out on double-stranded DNA, using *fmol*TM PCR sequencing system (Promega Corp., Madison, WI) and the primers 5'-TGTGCCCCAGAGG-TGC-3' annealing to the C γ 1 domain or 5'-CAGAGGCAGT-CCAGATTTC-3' annealing to the C κ domain.

Sequence alignment with germline sequences available in the July 1995 release of the V BASE Sequence Directory (Tomlinson *et al.*, MRC Centre for Protein Engineering, Cambridge, UK) was performed using the FASTA algorithm, available in the Wisconsin Sequence Analysis Package (UNIX version 8, Genetic Computer Group, Madison, WI).

Affinity constant analysis

The BIAcoreTM biosensor system (Pharmacia Biosensor AB, Uppsala, Sweden) was used to obtain affinity constants for the interaction between the Fab fragments and pB1. Briefly, pB1 was immobilized onto the sensorchip surface, using conventional amino coupling, at a concentration of 25 μ g protein/ml 10 mM sodium acetate buffer pH 4.5. This yielded an amount of 8000–10 000 RU of bound antigen. Fab fragments (0.75–16 μ g/ml) interacted with the antigen in HEPES complete buffer pH 7.4 (Pharmacia Biosensor AB), which also was used as flow buffer, at a flow rate of 3 μ l/min. The time for association was 11 min and the dissociation was studied for 30 min. The affinity constants were calculated for each Fab fragment, as described.¹⁹

RESULTS

Primary and secondary antigen-specific activation of human B cells

The experimental set-up, including the primary and secondary *in vitro* immunization of human peripheral blood lymphocytes, is outlined in Fig. 1. The result of the secondary *in vitro* immunization was analysed by EBV-infecting the cells and determining the isotype of individual lines. The result shown in Table 1 is given as the number of antigen-specific IgG-secreting EBV clones producing anti-pV3-specific antibodies. Only EBV-clones secreting antigen-specific IgM could be detected after the primary *in vitro* immunization, whereas no IgE or IgG response could be detected, not even after extensively pre-absorbing the primary culture supernatants on anti-human IgM-coated plates before analysis (data not shown). However, following the secondary stimulation a number of antigen-specific IgG-producing clones could be identified. The results, thus, demonstrated that specific IgG-producing B cells were generated during a secondary immunization period and that the isotype-switch was absolutely dependent on stimulation via CD40, antigen and autologous, specifically activated T cells. If any of these stimuli were omitted or if heterologous T cells were used, we could not detect any IgG-secreting B cells. Furthermore, if the autologous T cells were merely polyclonally activated, using SEA or PHA stimulation, only non-specific IgM-secreting B cells could be detected and in no case was antigen-specific IgG-secreting B cells found. The number of IgG secreting EBV clones was statistically evaluated by Linear-Rank exact test. The switch frequencies derived from experiments with cells from donor A and B (Table 1) was highly significant ($P < 0.001$), as compared to the control groups.

Selection of antigen-specific phages from antibody libraries

To evaluate further the primary and secondary *in vitro* immunizations, we constructed five different libraries. One library from unimmunized cells, derived from a mixture of

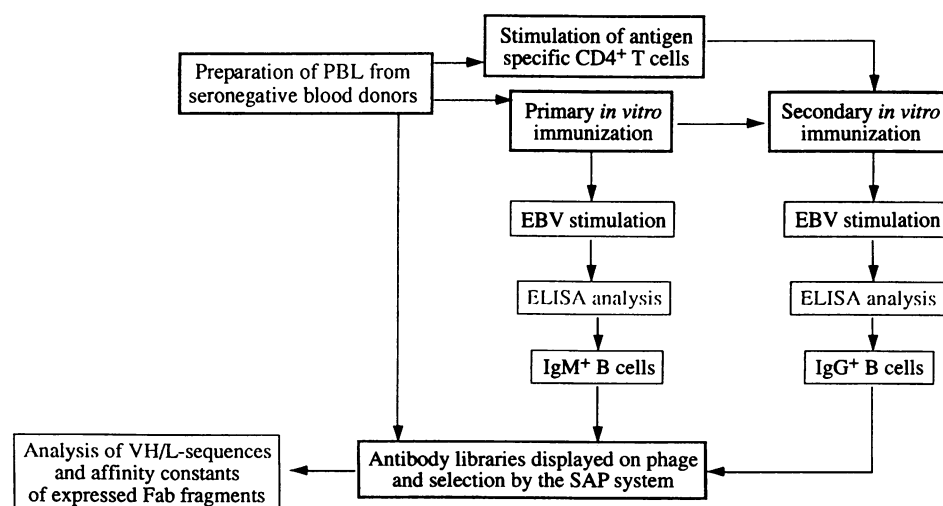


Figure 1. Schematic presentation of the experimental set-up, with antibody library construction using primary and secondary *in vitro* immunized PBL from seronegative blood donors.

Table 1. Effect of different signals during secondary *in vitro* immunization

Donor	α -CD40 mAb	FC γ RII ⁺ L cells	T _{aut} (ag+)*	T _{aut} (ag-)	T _{het} (ag+)	T _{het} (ag-)	SEA	PHA	No. of antigen-specific IgG ⁺ (specific/total)
A	+	+	+	-	-	-	-	-	12/384
	+	-	+	-	-	-	-	-	0/96
	-	+	+	-	-	-	-	-	0/96
	+	+	-	-	+	-	-	-	0/96
	+	+	-	+	-	-	-	-	ND†
	+	+	-	-	-	+	-	-	0/96
	+	+	-	-	-	-	+	-	0/96
	+	+	-	-	-	-	-	+	0/96
B	+	+	+	-	-	-	-	-	18/384
	+	-	+	-	-	-	-	-	0/96
	-	+	+	-	-	-	-	-	0/96
	+	+	-	-	+	-	-	-	0/96
	+	+	-	+	-	-	-	-	0/96
	+	+	-	-	-	+	-	-	0/96
	+	+	-	-	-	-	+	-	0/96
	+	+	-	-	-	-	-	+	0/96

*T_{aut}, autologous T cells; T_{het}, heterologous T cells; ag+, cultures stimulated with ptt; SEA, cultures stimulated with 1 ng/ml staphylococcal enterotoxin A; and PHA, cultures stimulated with 5 μ g/ml PHA.

†Analysis of antigen-specific IgG was performed on EBV-clones. An antigen-specific reactivity was scored as positive if: the ELISA OD obtained against pB1 was five times higher than the OD value obtained for a negative control; and the reactivity index (RI) was > 3, when defined as:

$$RI = \frac{OD(pB1) - OD(\text{medium control against pB1})}{OD(BSA) - OD(\text{medium control against BSA})}$$

‡ND, not determined.

donor A and B, and one after each immunization step, using cells from each individual donor. Antigen-specific phages were selected by the SAP approach,¹⁸ linking recognition and phage replication. Between 30 and 100 colonies from each library and round of selection were screened by antigen-specific ELISA (Table 2). The data show that the frequency of phages displaying antibodies specific for pV3 increased significantly during the *in vitro* immunization process and two rounds of selection had initially to be performed to obtain the V3-specific antibodies from the unimmunized donors. In comparison, we found a number of positive clones directly after the first

selection when libraries were constructed from primary or secondary immunized B cells. We observed an increase in antigen-specific phages from 1 to 40% in donor A and from 2 to 60% in donor B (Table 2) between the primary and the secondary immunizations. Nineteen clones, each with different VH/VL combinations, representing the different stages of immunization were then selected for sequence analysis, protein expression and biosensor analysis.

Sequence analysis of antigen-specific heavy and light chains

The heavy chain sequences from the 19 different clones were analysed and Fig. 2 shows their sequences. From these clones we have 12 different VH sequences, one from the non-immunized group (U) and 11 from clones obtained from primary or secondary immunizations. The VH sequences derived from the naive, non-immunized group were both identical, although they were derived from different bacterial clones. All the heavy-chain sequences showed closest homology with germ line genes belonging to the VH4 family.

Analysis of the VL sequences showed an extensive chain promiscuity. Thus, different light chains paired with the same or similar heavy chains, without affecting the antibody affinity, as demonstrated by the light chains derived from the non-immunized group which were identical to light chains derived from the secondary immune response of donor A (U.2.1 and U.2.2 were identical to AS.1.28 and AS.1.29, respectively). All sequences are deposited in GenBank (accession numbers: L33418–L33447).

Table 2. Number of positive phage clones obtained after SAP selection

Donor	<i>In vitro</i> immunization	Clonal ratio after SAP selection*	
		1st	2nd
A + B	none	0/102	2/100 (2)†
A	primary	1/101 (1)	32/19 (2)
	secondary	12/18 (5)	ND‡
B	primary	2/100 (2)	50/1 (2)
	secondary	20/10 (5)	n.d.

*Positive wells/ negative wells by specific ELISA.

†Fab producing colonies selected for further characterizations.

‡ND, not determined.

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
4.36 (germline)	10.....20.....30	AB	...40.....50.....60.....	...70.....80..ABC.....90...	...100ABCDEFHIJK...	...A..110...	
U.2.1/2	QVQLQESGPGLVKPSQTLSTCAVYGGSF	GYWS--	WIRQPPGKLEWIG	EINISGSTYNPSLKS	RVTISVDTSKNQFSKLSSVTAADTAVYYCAR	GGYKWLAAQS----	WGO--GTLVTVSS
	T.....	----	C.....				
VH4.22 (germline)	QVQLQESGPGLVKPSQTLSTCTVSGYSIS	SCYYWG--	WIRQPPGKLEWIG	SIYHSGSTYNPSLKS	RVTISVDTSKNQFSKLSSVTAADTAVYYCAR		
3d24d (germline)	Q.....A.....	..SNW..-		Y..Y..I.....	..M.....V.....		
BP.1.3/4	Q.....G.....	..S..S-	..A.....	R..T..N.....	..MA..A.N.....R.....	LAAPIANN-----WGSTQY	WGLAGTTVTVSS
BP.2.5	Q.....G.....	..S..S-	..A.....M.	..S.....		R....YDYI-----	
AP.1.6/7	Q.....G.....	..S..S-	..R.A.....	RLFI.....S.....Q.	..MA..A.N.....R.....	GG...--YDYIAA-----Y.	
AP.2.8	Q.....G.....	..SSS.S-		RL.Y.....S.....Q.	..MA.....R.....	GVT...--YPMIAD-----Y.	
BS.1.21/24	Q.....G.....	..S..S-	..A.....	RFIY.....S.K..HQ.	..MA.G..N.....R.....	R....--YAYDYI-----	
BS.1.22/23/25	Q.....G.....	..S.F.S-	..A.....	R..Y.....N.....N	..MA..A.N.....R.....		
AS.1.28	Q.....G.....	..R..S-	..A.....S.	G..IT..N.N.P.....	..MA.....G.....	VPGYSW.....--TTKN.	
3d279d (germline)	QVQLQESGPGLVKPSQTLSTCTVSGGIS	SGSYWS	WIRQPPGKLEWIG	RIYTSGSTYNPSLKS	RVTISVDTSKNQFSKLSSVTAADTAVYYCAR	LAAPIAYN-----WGSTQY	WGLAGTTVTVSS
BP.2.9	N	..S..			..MA.A..S.....R..A.....	VPGYSWN.....--TTKN.	
AS.1.14/15	N	T.....	..S.....	G.H.....P.....	G.....	RGDYESTVGQHNYYYLDV	..Q-----
AS.1.29	N	..S..		VFH.....H.....	..I.M.....T.....	..S.APIANN-----KN	
AS.1.30	N	..N.....	..N.....S.	G.....P.....	G.....		

Figure 2. Amino acid sequences of heavy-chain variable regions of antigen-specific Fab fragments, derived from the different libraries. The Fab clones were coded as: Donor A or B; immunization status: U (unimmunized) or P/S (primary or secondary *in vitro* immunization); number of selection rounds (1 or 2); clone number. The sequences are aligned to their closest germ-line sequence. VH4.22 and 3d24d showed very similar homology to most sequences in the second group. The FR/CDR assignments were according to Kabat *et al.*²⁹

Biosensor analysis of Fab fragments showed selection of high-affinity variants

The antigen-specific Fab fragments were subsequently expressed and analyzed using the BIAcoreTM. Figure 3 shows the distribution of affinity constants derived from the five different libraries. The data clearly established that the secondary immunization period, between days 7 and 12, resulted in an increase in Fab affinity of close to two orders of magnitude with the highest constant of $1.8 \times 10^{10} \text{ M}^{-1}$. The Fab fragments derived from the primary immunization had very similar affinities as compared to fragments derived from the naive library of donor A/B. Furthermore, the observed increase in affinity constants was mainly due to a decrease in dissociation rate constants during the secondary immunization periods. The k_{diss} for antibodies (clones 1–9) derived between day 0 and 7 was in the range $(1.2–3.9) \times 10^{-4} \text{ s}^{-1}$, while the high-affinity clones (no. 28, 29, 30) all had k_{diss} around $8.5 \times 10^{-5} \text{ s}^{-1}$. In addition, it was confirmed by light-chain shuffling experiments that the increase in affinity was not

dependent on the particular light chain sequences used in the different clones.

DISCUSSION

Primary *in vitro* immunization of naive human B cells has been shown to result in the induction of antigen-specific IgM responses.^{7,11} An antigen-specific isotype-switch of naive B cells was also recently shown to be strictly dependent on the presence of antigen-specific autologous T-cell clones.⁸ The present results demonstrated that antigen-stimulated, non-clonal CD4⁺ T cells could replace specific autologous T-cell clones during the secondary stimulation period and still support an isotype switch of antigen-specific B cells. Evidence that ligation of CD40 was involved in isotype switching was recently reported when stimulation with antibodies to CD40, in the absence of IL-4, was sufficient to induce germline transcripts of most downstream isotypes.²⁰ The present data show that both CD40 ligation and the presence of antigen-stimulated, non-clonal CD4⁺ T cells were necessary to generate IgG expressing B cells *in vitro*. Furthermore, the effect of different T-cell sources showed that autologous CD4⁺ T cells stimulated by polyclonal activators, such as PHA or SEA, could not support the induction of antigen-specific IgG. Moreover, heterologous T cells did not support isotype switch events even if they were stimulated with antigen. Thus, these results support that isotype-switching of specific B cells *in vitro* depends on antigen-specific, MHC-restricted helper T cells.⁸ Another explanation is that antigen-specific, isotype-switched B cells were present already from the initiation of the primary cultures. This is, however, highly unlikely since in no case has antigen-specific IgG antibodies been isolated from primary immunizations of naive B cells, despite the presence of T helper cells.

The development of the combinatorial approach to antibody cloning has allowed unrestricted access to immunological repertoires. Indeed, that technology has been applied to immortalize the natural B-cell repertoire from most lymphoid compartments and has been focused on obtaining affinity matured antibodies from immunized^{9,21} or infected individuals.²² Most studies have generally failed to identify high-affinity binders in antibody libraries derived from

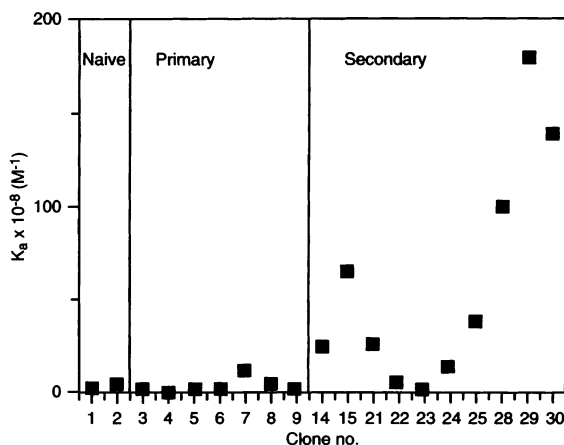


Figure 3. The affinity constants of all the antigen-specific Fab clones as a function of immunization status of the PBL used for library construction. First panel: unimmunized PBL; second panel: primary *in vitro* immunized PBL; third panel: secondary *in vitro* immunized PBL. The Fab clones were coded as in Fig. 2.

non-immunized individuals,¹⁴ unless very large libraries were constructed,²³ e.g. it was not possible to obtain gp120 binders by panning of a bone marrow immunoglobulin library from an HIV seronegative donor.²⁴ In this study we could, however, detect the presence of anti-V3-specific B cells in naive donors and during the development of an *in vitro* immune response due to the powerful homogeneous selection procedure of phage-displayed antibody fragments.^{18,25}

A restricted usage of VH gene families has previously not been reported among Fab fragments specific for gp120 in antibody libraries from infected donors.^{22,26} Our observation of VH4 gene usage in all of the five libraries might, thus, reflect a distinct *in vitro* response against the V3 loop, in particular since we and also Marks *et al.*¹⁴ seem to amplified all the VH families equally well, using similar sets of primers. For a recent overview on antibody repertoires against HIV-1 see ref. 27.

When a comparison was performed between the heavy-chain V-region sequences and germ line sequences several VH4.22-related sequences (clone 3/4, 6/7, 8, 21/24, 22/23/25, 28) showed only around 91% homology, thus, preventing an analysis of somatic mutations. A comparison between sequences derived from the primary and secondary *in vitro* immunizations revealed, however, some cases of high homology. A complete homology in the VDJ recombination of the CDR3/FR4 regions could even be observed in sequences derived from primary (clone 3 & 4) and secondary immunizations (clone 22, 23, 25) from donor B, which indicate a common clonal origin²⁸ (Fig. 2). The sequences derived from clones 22/23/25 contained three amino acid replacements, which might indicate somatic mutations. However, due to our polyclonal culture system such a common ancestry cannot at present be confirmed. Since affinity maturation involves both antigenic selection and somatic mutation, could the observed increase in affinity merely be a result of new heavy- and light-chain recombinations during construction of the phage libraries? This is highly unlikely, since we showed by light chain shuffling that the affinity constants were dependent only on the heavy chain variable domain (data not shown). This agrees with the light chain promiscuity described above, again suggesting that the light chain variable domains contributed marginally to the antigen binding. Furthermore, we never observed an increased affinity in clones with novel recombinations derived from libraries constructed from primary immunized B cells. The major component of the observed increase in affinity of antibody fragments derived from prolonged cultures was most probably antigenic selection. However, this antigenic selection of *in vitro* immunized B cells became only evident in libraries derived from secondary *in vitro* immunized B cells and the exact mechanisms need to be further evaluated. Possibly, an increase in frequency of relevant V regions during the culture period became evident in the subsequent antibody selection process.

In conclusion, we have shown by phage display technology that secondary *in vitro* immunized human B cells yielded antibody fragments with affinities of almost two orders of magnitude higher than those derived from primary immunized cells. This combination of technologies will most likely prove to be a valuable tool in the search for binders of human origin when *in vivo*-induced repertoires are not easily accessible.

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REFERENCES

1. ARMITAGE R.J., FANLOW W.C., STROCKBINE L. *et al.* (1992) Molecular and biological characterization of a murine ligand for CD40. *Nature* **357**, 80.
2. SPRIGGS M.K., ARMITAGE R.J., STROCKBINE L. *et al.* (1992) Recombinant human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion. *J Exp Med* **176**, 1543.
3. HOLLENBAUGH D., GROSMARE L.S., KULLAS C.D. *et al.* (1992) The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. *EMBO J* **11**, 4313.
4. GRAF D., KORTHAUER U., MAGE H.W., SINGER G. & KROCEK R.A. (1992) Cloning of TRAP, a ligand for CD40 on human T cells. *Eur J Immunol* **22**, 3191.
5. JACOB J. & KESOE G. (1992) In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periaarteriolar lymphoid sheath-associated foci and germinal centers. *J Exp Med* **176**, 679.
6. LIU Y.J., MASON D.Y., JOHNSON G.D. *et al.* (1991) Germinal center cells express bcl-2 protein after activation by signals which prevent their entry into apoptosis. *Eur J Immunol* **21**, 1905.
7. CHIN L.-T., HINKULA J., LEVI M., OHLIN M., WAHREN B. & BORREBAECK C.A.K. (1994) Site-directed primary *in vitro* immunization: production of HIV-1 neutralizing human monoclonal antibodies from lymphocytes obtained from seronegative donors. *Immunology* **81**, 428.
8. CHIN L.-T., MALMBORG A.-C., KRISTENSSON K., HINKULA J., WAHREN B. & BORREBAECK C.A.K. (1995) Mimicking the humoral immune response *in vitro* results in antigen-specific isotype switching supported by specific autologous T helper cells: generation of human HIV-1 neutralizing IgG monoclonal antibodies from naive donors. *Eur J Immunol* **25**, 657.
9. HUSE W.D., SASTRY L., IVERSON S. *et al.* (1989) Generation of a large combinatorial library of immunoglobulin repertoire in phage lambda. *Science* **246**, 1275.
10. MCCAFFERTY J., GRIFFITHS A.D., WINTER G. & CHISWELL D.J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**, 552.
11. OHLIN M. & BORREBAECK C.A.K. (1992) Production of human monoclonal antibodies. In: *Methods of Immunological Analysis* (eds R.F. Masseyeff, W.H.W. Albert & N.A. Staines), p. 298. VCH Verlagsgesellschaft GmbH, Weinheim.
12. DEMOTZ S., LANZAVECCHIA A., EISEL U., NIEMANN H., WIDMANN C. & CORRADIN G. (1989) Delineation of several DR-restricted tetanus toxin T cell epitopes. *J Immunol* **142**, 394.
13. PUTNEY S.D., MATTHEWS T.J., ROBEY W.G. *et al.* (1986) HTLV-III/LAV-neutralizing antibodies to an E. coli produced fragment of the virus envelope. *Science* **234**, 1392.
14. MARKS J.D., HOOGENBOOM H.R., BOUNERT T.P., MCCAFFERTY J., GRIFFITHS A.D. & WINTER G. (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J Mol Biol* **222**, 581.
15. SÖDERLIND E., SIMONSSON-LAGERKVIST A.C., DUEÑAS M. *et al.* (1992) Chaperonin assisted phage display of antibody fragments on filamentous bacteriophages. *Bio/Technology* **11**, 503.
16. BURTON D.R., BARBAS C.F., PERSSON M.A., KOENIG S., CHANOCK R.M. & LERNER R.A. (1991) A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from

- combinatorial libraries of asymptomatic seropositive individuals. *Proc Natl Acad Sci USA* **88**, 10134.
17. POTTER H. (1993) Application of electroporation in recombinant DNA technology. *Meth Enzymol* **217**, 461.
18. DUEÑAS M. & BORREBAECK C.A.K. (1994) Clonal selection and amplification of phage displayed antibodies by linking antigenic recognition and phage replication. *Bio/Technology* **12**, 999.
19. BORREBAECK C.A.K., MALMBORG A.-C., FUREBRING C. *et al.* (1992) Kinetic analysis of recombinant antibody-antigen interactions: relation between structural domains and antigen binding. *Bio/Technology* **10**, 697.
20. JUMPER M.D., SPLAWSKI J.B., LIPSKY P.E. & MEEK K. (1994) Ligation of CD40 induces sterile transcripts of multiple Ig H chain isotypes in human B cells. *J Immunol* **152**, 438.
21. PERSSON M.A., CAO R.H. & BURTON D.R. (1991) Generation of diverse high-affinity human monoclonal antibodies by repertoire cloning. *Proc Natl Acad Sci USA* **88**, 2432.
22. BARBAS C.F., COLLET T.A., AMBERG W. *et al.* (1993) Molecular profile of an antibody response to HIV-1 as probed by combinatorial libraries. *J Mol Biol* **230**, 812.
23. GRIFFITHS A.D., WILLIAMS S.C., HARTLEY O. *et al.* (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J* **13**, 3245.
24. DUCHOSAL M.A., EMING S.A., FISCHER P. *et al.* (1992) Immunization of hu-PBL-SCID mice and the rescue of human monoclonal Fab fragments through combinatorial libraries. *Nature* **335**, 258.
25. DUEÑAS M., MALMBORG A.-C., CASALVILLA R., OHLIN M. & BORREBAECK C.A.K. (1996) Selection of phage displayed antibodies based on kinetic constants using the SAP system. *Mol Immunol* **33**, 279.
26. BARBAS C.F., BJÖRLING E., CHIOLDI F. *et al.* (1992) Recombinant human Fab fragments neutralize human type 1 immunodeficiency virus *in vitro*. *Proc Natl Acad Sci USA* **89**, 9339.
27. OHLIN M. & BORREBAECK C.A.K. (1996) Characteristics of human antibody repertoires following active immune responses *in vivo*. *Mol Immunol* **33**, 583.
28. SHLOMCHIK M., MASCELLI M., SHAN H. *et al.* (1990) Anti-DNA antibodies from autoimmune mice arise by clonal expansion and somatic mutation. *J Exp Med* **171**, 265.
29. KABAT E.A., WU T.T., PERRY H.M., GOTTESMAN K.S. & FOELLER C. (1991) *Sequences of Immunological Interest*, 5th edition. NIH Publication No. 91-3242. National Institutes of Public Health, Bethesda, MD.