



VIEWPOINT

CHARACTERISTICS OF HUMAN ANTIBODY REPERTOIRES
FOLLOWING ACTIVE IMMUNE RESPONSES *IN VIVO*

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Abstract—Possibilities to develop human monoclonal antibody specificities have recently been much facilitated by improvements of human hybridoma technology but even more so by the emerging phage-display technique. However, until recently very little has been known about the characteristics at the molecular level of the induced, T cell-dependent human antibody response, frequently targeted by these techniques. Rather, the major part of available sequence information has been related to tumor-derived or autoreactive antibodies. We have now investigated high affinity, monospecific, human antibody repertoires as developed by hybridoma technology. The VH region gene usage among such *in vivo*-induced repertoires is in only some respects similar to that found in the total B cell population. A limited number of heavy-chain variable segment loci account for the majority of all induced antibodies. A particular VH gene locus (4-34) frequently employed by peripheral B cells and associated with autoreactive antibodies was rarely used by the induced repertoire. Furthermore, in particular antigen systems, V region usage differs from the total available repertoire, and heavy-chain CDR3 is generally longer among antibodies induced against foreign protein antigens than in the average B cell population. Light-chain gene usage is often restricted to just a few dominant genes frequently found among B cells in general. In comparison, variable regions derived by phage-display technology in some antigen systems display even longer heavy-chain CDR3 than hybridoma-derived antibodies. This technique also appears to select a different set of germline genes preferentially (both with respect to VH and JH) as compared to hybridoma technology. In summary, the T cell-dependent antibody response against foreign antigens appears to differ from the average circulating B cell in several ways, and thus does not seem to represent a random selection of the available repertoire. Copyright © 1996 Elsevier Science Ltd

Key words: human monoclonal antibodies, germline gene usage, V segment, J segment, somatic mutation, phage display, T cell-dependent antibody response, antibody variable domain.

INTRODUCTION

Specific antibodies induced as a consequence of infection or encounter with a foreign antigen have in many cases proved very effective in removing and controlling the invading organism or foreign molecule. Consequently, passive administration of antibodies and development of antibody responses by active immunization are frequently employed therapeutic modalities. However, antibodies may also perform detrimental activities *in vivo*. Certain antibodies induced against foreign antigens may directly interact with endogenous molecules inducing a destructive process. This is, for example, the case with antibodies against coxsackievirus which may be highly myocarditic (Gauntt *et al.*, 1995). Furthermore, antibodies developed against a foreign antigen or poly-

clonally induced by a foreign substance (e.g. an infectious agent) may present novel antigenic structures (their variable domains) to the immune system. Such epitopes may perturb the natural antibody network and consequently induce specificities against self structures, some of which may have pathological binding specificities. It has been proposed that such antibodies in some cases may participate in the development of autoimmune antibodies through idiotypic networks (Avrameas and Ternynck, 1993; Newkirk *et al.*, 1988; Rioux *et al.*, 1994a).

Despite the fact that antibodies specific for foreign antigens not only play an important role in the prevention of disease but also have the potential to participate in the development of autoimmune disease, little is known about such immune repertoires (e.g. with respect to V region gene usage) in humans. This can to a large extent be attributed to difficulties that have been encountered in the past to develop specific human monoclonal antibody-producing cell lines. The majority of data on human antibody gene utilization have actually focused on autoreactive specificities, e.g. displaying rheumatoid factor

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activities, although many of these antibodies represents natural as opposed to induced antibodies. Consequently, the knowledge of actual disease-associated induced antibodies is also rather limited.

When evaluating some small human repertoires, not displaying autoimmune specificities, a substantial degree of sequence similarity with the available repertoires of autoreactive antibodies has been observed (Newkirk *et al.*, 1988; Rioux *et al.*, 1994a, 1994b). These observations support the possible existence of an immune network connecting induced and autoreactive antibody populations. There has, however, not been a thorough evaluation of the characteristics of the immune, monospecific, non-autoreactive repertoire. Recently, a wider range of antibodies specific for foreign protein antigens has become available either through conventional hybridoma or phage display technology. This study focuses on an analysis of the variable regions used by the hybridoma-derived antibodies, because they provide a direct mirror of the *in vivo*-developed antibody repertoire. Furthermore, phage display-derived antibody fragments were studied in order to evaluate their characteristics in comparison to hybridoma-derived repertoires.

To conduct this study, nucleotide/protein sequences of human antibodies specific for foreign antigens were retrieved directly from the EMBL and genome sequence databases, the Kabat database of sequences of proteins of immunological interest, from data published in scientific journals or through personal communications. The sources of this information are summarized in Table 1.* The present investigation was focused only on specificities against non-human, protein antigens (thus eliminating autoantibodies). Because antibodies against polysaccharides, lipopolysaccharides etc. were not included, the studied antibodies are likely to have been developed following T cell-dependent *in vivo* responses. As some of the antigens are glycoproteins, some involvement of carbohydrate in the specificities cannot be excluded. Specificities developed following *in vitro*-immunization of non-immune lymphocytes or from recombinatorial libraries established from non-immunized individuals or semisynthetic libraries were not included in this investigation. Also, clones retrieved following repeated rounds of light-chain shuffling using phage display technology were not investigated. Furthermore, phage display-derived clones reported to carry identical sequences were only included once in the comparison. Analysis of the sequences were performed using the Wisconsin sequence analysis package (version 8; Genetics Computer Group Inc., Madison, WI, U.S.A.) using the FASTA algorithm.

For comparisons with known variable segment germline sequences, the V BASE sequence directory (release 1) kindly provided by Dr I. M. Tomlinson (MRC Centre

for Protein Engineering, Cambridge, U.K.) was used. In all but some of the phage display-derived clones, nucleotide sequences were used for germline gene assignments. When protein sequences only were available (which applies to some phage display-derived antibody fragments), only V region assignments were made. Heavy-, κ - and λ -chain germline genes were assigned to subgroups according to Cook and Tomlinson (1995), Cox *et al.* (1994), Tomlinson *et al.* (1992) and Williams and Winter (1993), respectively. Statistical comparisons of data sets were performed using the Student's *t*-test running on Microsoft Excel software (version 5.0).

GENERAL FEATURES OF THE REPERTOIRES

A large number of human antibody sequences have been determined to date. However, only relatively few fit the criteria put forward for inclusion in this study. Only human antibodies that specifically recognize non-human protein antigens were included. They would most probably be derived from T cell-dependent immune responses. Furthermore, the antibodies should not be reported to display multireactive properties. Much as would be expected, the vast majority of human antibodies that were included using these criteria were IgG, although some antibodies were of the IgA isotype. In addition, no exact homology to known heavy-chain germline sequences (83–98% homology) was detected. This finding differentiates these genes substantially from those retrieved from randomly chosen IgM-producing cells. In such populations, 67% of all sequences have been shown to be >99% homologous to known germline sequences (Demaison *et al.*, 1995). Consequently, many if not all of these antibodies had undergone affinity maturation processes. The locations of divergence from the known germline genes were investigated in the case of the frequently used DP-47 related genes and, as expected, they were found to occur mainly in the complementarity determining regions (CDR) regions (data not shown). In some cases (e.g. ITC33, 71-31, K14) the original germline gene/allele has most likely not yet been described, because the closest fits were obtained with sequences containing insertions/deletions not found in the antibody VH segment. However, with the rapid accumulation of knowledge of the gene segments found in the human variable region loci, germline gene assignments can now be made with increasing accuracy.

A variety of sources of immune lymphocytes have been used to establish the specificities described here although peripheral blood B cells predominate as the origin of antibodies produced by hybridomas. This fact may skew the observed in comparison to the total antibody repertoire. Furthermore, the mode of immortalization of the B cells and their genetic information (*in vitro* activation procedures, EBV infection and/or somatic cell hybridization) may target different B cell populations not necessarily being representative of the average antigen-specific lymphocyte. It can thus not be ruled out that some of the differences observed between different antibody repertoires may occur as a result of these factors.

*A complete compilation of the sequences (including GenBank accession numbers) used in this investigation as well as germline gene assignments are available upon request from the corresponding author or on the WWW as a word 5.1 document at URL:<http://www.kc.lth.se/immun/manuscripts/repertoires/rep.word>.

Table 1. Sources of sequence data used in this investigation^a

Antigen	Origin of sequences	Reference	GenBank access ^b
Cytomegalovirus	Cell line	Newkirk <i>et al.</i> (1988)	M20003, M20031
Cytomegalovirus	Cell line	Ohlin <i>et al.</i> (1994)	L26531-40
Cytomegalovirus	Cell line	Ohlin <i>et al.</i> (1996)	L37310-1
Cytomegalovirus	Cell line	Rioux <i>et al.</i> (1994a)	L26899-906
Cytomegalovirus	Cell line	Rioux <i>et al.</i> (1995)	L26890-6, L26898
<i>Haemophilus influenzae</i>	Cell line	Andris <i>et al.</i> (1993)	L21960-1
Hepatitis A virus	Cell line	Lewis <i>et al.</i> (1993)	M87789-90
Hepatitis B virus	Cell line	Andris <i>et al.</i> (1992)	L03677-84
Hepatitis B virus	Phage display	Zebedee <i>et al.</i> (1992)	M88309-19
Hepatitis C virus	Cell line	Esposito <i>et al.</i> (1995); Mondelli <i>et al.</i> (1994); de Lalla <i>et al.</i> (1993)	X77945-8, X82934-5
Herpes simplex virus	Cell line	Huang <i>et al.</i> (1992)	M97920-2, M97924
Herpes simplex virus	Phage display	Sanna <i>et al.</i> (1995)	–
Herpes simplex virus-1	Phage display	Burioni <i>et al.</i> (1994)	–
HIV-1	Cell line	Andris <i>et al.</i> (1991)	M67500-4
HIV-1	Cell line	Bagley <i>et al.</i> (1994)	–
HIV-1	Cell line	Felgenhauer <i>et al.</i> (1990)	X53612-3
HIV-1	Cell line	Moran <i>et al.</i> (1993)	S67981-8
HIV-1	Cell line	Marasco <i>et al.</i> (1992)	M88499-500
HIV-1	Cell line	Takeda <i>et al.</i> (1995)	L01410-3
HIV-1	Cell line	David and Zouali (1995)	Z34890, Z34909-15
HIV-1	Cell line	David <i>et al.</i> (1995)	Z35118-26
HIV-1	Cell line	van der Donk <i>et al.</i> (1994)	–
HIV-1	Phage display	Barbas <i>et al.</i> (1993)	L03139-75, L03177-80, L08898-901, L09078-87, L09089, L09092-103, L10139-40
HIV-1	Phage display	Ditzel <i>et al.</i> (1995)	–
Measles virus	Phage display	Bender <i>et al.</i> (1994)	–
<i>Neisseria meningitidis</i>	Cell line	Ayala Avila <i>et al.</i> (1993)	M97802-5
Rabies virus	Cell line	Ikematsu <i>et al.</i> (1993)	L08083, L08085-7, L08089-90
Rabies virus	Cell line	Cheung <i>et al.</i> (1992)	M98325
Respiratory syncytial virus	Phage display	Barbas <i>et al.</i> (1992)	–
Respiratory syncytial virus	Phage display	Moreno de Alboran <i>et al.</i> (1995)	–
STL-1	Cell line	Heveker <i>et al.</i> (1994)	–
Tetanus toxoid	Cell line	Larrick <i>et al.</i> (1992)	S55287, S55289, S55290, S55292
Tetanus toxoid	B cell	Lagerkvist <i>et al.</i> (1995)	L38015-8
Tetanus toxoid	Cell line	Gillies <i>et al.</i> (1989)	–
Tetanus toxoid	Cell line	Esposito <i>et al.</i> (1994)	X75387-8
Tetanus toxoid	Cell line	C. Furebring, unpublished	–
Tetanus toxoid	Phage display	Barbas <i>et al.</i> (1991)	L22156-7
Varicella-zoster virus	Cell line	Huang <i>et al.</i> (1992)	M97919, M97923

^aFull germline assignments and homologies are available over the WWW as a Word 5.1 document at URL: <http://www.kc.lth.se/immun/texter/manuscripts/repertoires/rep.word>.

^bSequences not available in GenBank were obtained directly from the author or from the relevant publications.

However, it has been shown that EBV-transformation used in many of these studies, as such, does not result in a selection of B cells representing a skewed VH-subgroup distribution (Victor *et al.*, 1991). Consequently, a comparison with repertoires of B cells was considered possible.

HEAVY CHAIN VARIABLE SEGMENT GENES IN HYBRIDOMA-DERIVED ANTIBODIES

A large array of human monoclonal antibodies specific for foreign protein antigens and most likely induced fol-

lowing a T cell-dependent *in vivo* immune response (induced by infection or vaccination) has recently been identified. In some cases, the genes encoding the variable regions have also been sequenced (Table 1), thus providing information on the primary sequence and germline origin of these specificities. Because these antibodies were identified from individual cells, the variable region gene combination is a good representation of the *in vivo*-developed variable gene combinations. In general, a wide spectrum of germline families (Table 2) has been used in the response towards these foreign antigens, and 73 investigated sequences were putatively assigned to 41

Table 2. Relative frequency of gene usage among human monoclonal antibodies and antibody fragments derived from phage display libraries specific for exogenous protein antigen and developed following an *in vivo* immune response (Table 1) and among peripheral blood mononuclear cells in general

Germline family	Representation among (%)		
	Hybridomas	Specific phage clones	Mononuclear cells ^a
	(n = 73)	(n = 86)	
VH1	26	35	11
VH2	4	0	3
VH3	40	47	52
VH4	22	13	19
VH5	8	2	10
VH6	0	3	5
	(n = 36)	(n = 57)	
Vκ1	50	42	43
Vκ2	8	5	9
Vκ3	36	53	29
Vκ4	6	0	19
	(n = 26)		
Vλ1	27	nd	nd
Vλ2	23	nd	nd
Vλ3	46	nd	nd
Vλ6	4	nd	nd

^aRelative frequency determined on adult peripheral blood B cells by *in situ* hybridization (Guigou *et al.*, 1990).

different VH segment germline sequences localized to 28 different loci (Cook and Tomlinson, 1995). However, a limited number of V segment loci (1-69 [and its close relative 1-e], 1-46, 3-21, 3-23, 4-59 and 5-51) accounted for 51% of all heavy-chain variable segments used by these antibodies. Among these, loci 1-69 and 3-23 have also been reported to be frequently utilized by the peripheral repertoire (Demaision *et al.*, 1995). The most frequently used germline sequence appeared to be DP-47 which alone accounted for 14% (10/73) of all VH sequences among the hybridoma-derived clones, a frequency similar to the one (12%) found among antibody sequences in general (Griffiths *et al.*, 1994). However, the various genes did not appear to be randomly used in the various repertoires. Three out of four clones using 5-51-related genes were specific for cytomegalovirus epitope AD-1 and four out of 10 clones representing the 3-23 locus (DP-47) were found among tetanus toxoid-specific antibodies, despite the fact that these specificities represented only 8% and 11% of the total number of investigated clones. Similarly, only 24% (7/29) of the HIV-specific clones used VH3-related genes whereas other specificities taken together used such genes in 50% (22/44) of the clones. The latter figure is more similar to VH3-gene usage reported elsewhere (Guigou *et al.*, 1990; Logtenberg *et al.*, 1989; Pascual *et al.*, 1990) (Table 2). Consequently the nature of the antigen (or even individual epitopes) will influence the V segment gene usage in human antibody responses. In some cases [as shown

for HIV (Berberian *et al.*, 1993, 1994)] such clonal selection/deletion may be influenced by a superantigen activity of the antigen/pathogen under study. Such superantigen activity could up- or down-regulate usage of particular genes (Zouali, 1995) depending on its ability to recruit suitable T cell-mediated co-stimulatory activity.

A high frequency of particular V segment genes has also been found among certain autoantibodies, e.g. the VH4.21 gene is frequently associated with autoimmune (anti-i/I cold agglutinins) (Silberstein *et al.*, 1991) antibodies. VH4.21 (loci 4-34) has also been found in a high frequency (approximately 10%) of peripheral blood B cells (Kraj *et al.*, 1995). Such lymphocytes are, however, rarely used for an induced immune response *in vivo* as only one out of 73 was found among the investigated clones. Although an involvement of the immortalization procedures in this observed skewedness of the induced repertoires can not be completely ruled out, it is highly probable that these T cell-dependent, induced repertoires *in vivo* are actually recruited from cells displaying characteristics different from the average B cell.

LIGHT CHAIN VARIABLE SEGMENT GENES

With respect to κ light-chain variable segments it is also evident that the T cell-dependent immune response appears to restrict κ light-chain germline usage to a few members of the potentially available repertoire (Table 3). This agrees with previous reports (Cox *et al.*, 1994; Klein *et al.*, 1993) dealing with the characteristics of the entire light chain repertoire that has been reported. The κ-chain subgroup distribution and gene usage is consequently similar to previous reported frequencies found among lymphocytes in general (Cox *et al.*, 1994) (Table 3). A27 was the single most frequently used κ light-chain germline gene (7/36; 19%) among hybridomas. This, or closely related genes, has previously been implicated in various antibodies connected to autoimmune diseases (Mageed *et al.*, 1986; Newkirk *et al.*, 1993; Thompson *et al.*, 1994) but A27 is obviously also frequently found among high affinity antibodies directed against a diverse set of foreign antigens (cytomegalovirus glycoprotein B, HIV-1 gp41 and gp120, *Neisseria meningitidis* opc and tetanus toxoid). However, these induced antibodies differ from many autoimmune antibodies in that they carry numerous putative mutations (2–8% of all nucleotides) in contrast to many autoantibodies which use the A27 gene in an unmutated form. These putative mutations were, as expected and as observed with the DP-47 VH segment, mainly located within the CDR regions (data not shown). Furthermore, many A27-carrying antibodies have greatly reduced or absent expression of Vκ3-associated idiotypes (Ohlin *et al.*, 1994) which had previously been shown to correlate to autoreactivity (Newkirk *et al.*, 1993). The role of A27-derived light chains, if any, in autoimmunity is thus not clear. Over-representation of some sequences (such as those derived from A27) in some diseases might occur as a consequence of B cell superantigen activity of the pathogen directed against particular V segment subgroups (Berberian *et al.*, 1993, 1994; Goodglick and

Table 3. Relative frequency of $V\kappa$ -gene usage among human monoclonal antibodies and antibody fragments derived from phage display libraries specific for exogenous protein antigen and developed following an *in vivo* immune response (Table 1) and among reported sequences in general

Germline loci	Representation among (%)		
	Hybridomas	Specific phage clones	Rearranged sequences in general ^a
$V\kappa 1$	(<i>n</i> = 36)	(<i>n</i> = 57)	(<i>n</i> = 236)
O12/O2	11	21	13
O18/O8	8	5	9
L12	14	0	6
A20	6	9	4
L19/L5	6	5	2
L11	3	0	—
L8	3	2	4
$V\kappa 2$			
A19/A3	6	5	5
A17	3	0	5
$V\kappa 3$			
L2	0	4	8
L6	11	5	8
L16	3	4	1
A11	3	0	<1
A27	19	40	20
$V\kappa 4$			
B3	6	0	9

^aRelative frequency determined from known rearranged sequences (Cox *et al.*, 1994).

Braun, 1994; Goodglick *et al.*, 1995; Knight *et al.*, 1993; Silverman, 1994; Vasquez Kristiansen *et al.*, 1994). Multi- and/or autoreactive antibodies developing during an infectious disease process may thus be associated with a particular gene or gene family as a consequence of polyclonal activation by the pathogen, rather than by a direct association of the multi-/auto-reactive antibodies and the pathogen-specific antibodies through an antibody network.

Among the hybridomas, λ -bearing antibodies are approximately as common compared to the κ/λ ratio found among polyclonal antibodies in serum. The immortalized clones represent a set of germline genes belonging to four λ light-chain subgroups ($V\lambda 1$ -3, 6) (Table 2). However, a majority (64%) of the clones used one of only four [DPL8 ($V\lambda 1$), DPL12 ($V\lambda 2$), HSI-GLL150 and LV318 (both $V\lambda 3$)] out of a total of 48 germline genes used in this comparison. Consequently, the λ light-chain germline usage of the induced antibody repertoire appears, just as the κ light chains, to be dominated by a limited set of genes, although these genes do not match those previously suggested to be most frequently used [DPL5 ($V\lambda 1$), DPL11 ($V\lambda 2$), DPL16 and DPL23 (both $V\lambda 3$)] by the total B cell population *in vivo* (Griffiths *et al.*, 1994). As the total $V\lambda$ repertoire is only now being fully explored (Fripiat *et al.*, 1995), future investigations will have to determine whether this appar-

ent restriction is real or is a result of the as yet limited number of known germline genes.

CDR3 AND J SEGMENT USAGE AMONG HYBRIDOMA-DERIVED SEQUENCES

It is widely believed that the third complementarity determining region (CDR3) of the heavy chain is the major contributor to the antigen-specificity of a large part of antibodies. As little is known about the factors determining the conformation of this loop, its length distribution had to be used as an indicator of normality of distribution. When compared to previously reported sets of data (Wu *et al.*, 1993; Yamada *et al.*, 1991) regarding heavy chain CDR3 length, overlapping distributions were seen although the average length of CDR3 was significantly longer ($P < 0.01$) in the secondary repertoire ($\bar{x} = 15.1$ amino acids; SEM = 0.5 amino acids) when compared to the two random, B cell repertoires ($\bar{x} = 13.1$ amino acids and 13.3 amino acids, respectively) (Wu *et al.*, 1993; Yamada *et al.*, 1991) (Fig. 1). Whether this difference in CDR3 length is caused by partial differences in the origin of the different sets of clones is not known. It might be proposed that an unselected antibody repertoire may contain clones similar to fetal lymphocytes which do not add N nucleotides when rearranging their germline genes (reviewed by Hardy, 1992), thus accounting for a shorter CDR3. If these clones were not subsequently to participate in any secondary response involving class switch, affinity maturation and monospecificity, a collection of such selected clones would on average use longer CDR3 regions than an unselected repertoire. Alternatively, one might suggest that the antigens (protein epitopes) which the specific clones recognize in some way skew the heavy-chain variable region repertoire towards longer CDR3 loops by allowing more contact points for the antibody binding sites with their epitopes and thus possible higher affinity. It should, however, be kept in mind though that there is no strict correlation of the number of contact points or the size of the buried surface with the displayed affinity for an antigen (Searle *et al.*, 1995). Finally, if particular (in this case, longer) CDR3 lengths would be more flexible than the average CDR3 they might be more likely to display conformational isomerism (Foote and Milstein, 1994). It may be hypothesized that antibodies displaying an enhanced level of conformational isomerism and thus different antigen-binding surfaces may be more likely to be selected during a primary immune response. Such a phenomenon would result in a skewed CDR3 length population in induced repertoires. It is of interest in this context to note that, regardless of the underlying mechanism for determining the forces operating in the T cell dependent immune responses, the canonical structures of the other CDR also seem to be a factor when determining the likelihood that a particular antibody will be monospecific for a protein antigen as opposed to smaller haptens (Vargas-Madrado *et al.*, 1995).

In general, a diverse set of heavy- and light-chain J segments was used by the high affinity, monospecific anti-

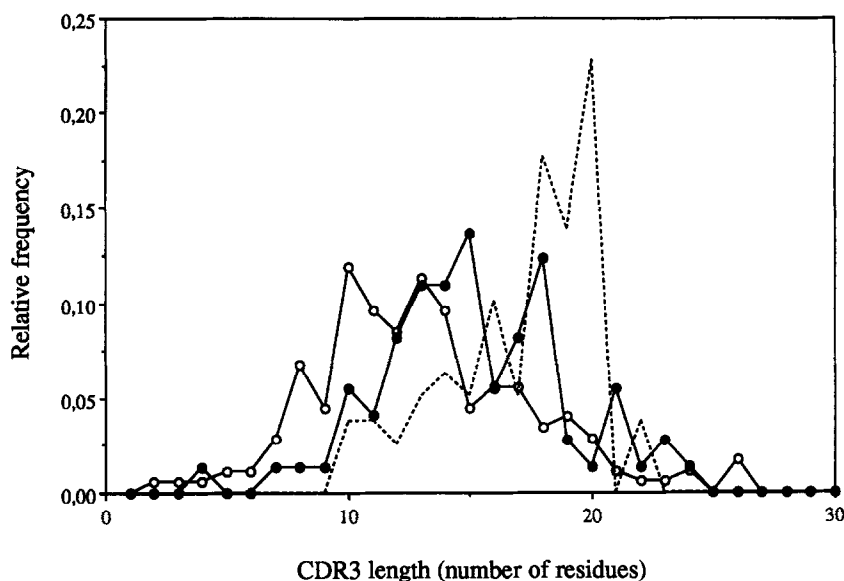


Fig. 1. Distribution of heavy chain variable region CDR3 length among antibodies derived from a random selection of antibodies (○) (Wu *et al.* (1993), hybridoma-derived antibodies (Table 1) induced *in vivo* and specific for T cell-dependent protein antigens (●) and recombinant antibody fragments obtained by phage display (Table 1) (dashed line). The distribution of CDR3 length in another investigation of a random selection of antibodies (Yamada *et al.*, 1991) essentially overlapped the repertoire described by Wu *et al.* (1993) (data not shown).

bodies studied in this investigation. Furthermore, the various JH segments were represented in frequencies normally found among randomly selected B lymphocytes (Table 4). There is thus no evidence suggesting that B cells participating in T cell-dependent immune responses would use other J segments as compared to peripheral blood lymphocytes in general.

ANTIBODY SEQUENCES DERIVED BY PHAGE DISPLAY TECHNOLOGY

For comparison of the variable regions found in hybridoma and phage display-derived repertoires a compilation of sequences derived from immunized individuals by the latter technique was also established. Both these sets of antibodies contain a large proportion of HIV-1 specific clones although the exact range of epitope specificities in the two repertoires might differ. It was evident that the heavy-chain variable region gene usage differed substantially between these two sets of repertoires, although a similar proportion of the various VH families were found (Table 2). In particular, the phage display-derived VH segments were mainly derived from germline genes at loci 1-e and 1-69 (two highly similar sequences) (19%), 3-15 (19%) and 3-30.3 (12%). Of these the latter two were found in a frequency of 0% and 5%, respectively, in the hybridoma-derived repertoires. In contrast, the loci 3-23-related gene (DP-47) often used by human B cells was found more frequently in hybridoma (14%) vs phage display-derived (3%) repertoires. Several explanations may be proposed for these differences. First of all, the characteristics of the genes derived by the phage display technique may be affected by particular characteristics of the limited number of libraries that were examined. Second, the main epitopes recognized in the

two systems are different, a fact that might have influenced the V segment gene usage. However, when limiting the investigation to epitopes expressed by the gp41/120 of HIV-1, substantial differences remained in particular with respect to the frequent usage of variable region genes originating from germline loci 3-15 in phage display-derived repertoires (Fig. 2). The observed discrepancies may, thus, very well suggest that the phage display technique biases the selection of V region sequences. Such effects may arise as a consequence of a bias in the PCR-based gene amplification process or differences in the ability of products of the various antibody-encoding genes to be expressed in *E. coli* (Dueñas *et al.*, 1995). Alternatively, the combinatorial approach used in phage display technology would preferentially select such V-region genes that produce a functional paratope with a variety of different light chains (high light-chain promiscuity), because such heavy chains are more likely to find a suitable partner in a limited library. Indeed, many (but not all) heavy chain variable segments obtained by phage display technology are promiscuous with respect to light-chain usage (Collet *et al.*, 1992), a fact that is not observed in a hybridoma-derived antibody (Ohlin *et al.*, 1996). Similarly, the somewhat higher frequency (40% vs 19% in hybridomas) of light chains derived from the A27 germline sequence among all κ -chain sequences (Table 3) might be related to an inherent ability of its protein product to expose a specificity encoded mainly by a promiscuous heavy-chain sequence. This is a plausible explanation because it was recently shown that an A27-derived light chain promoted the multireactive properties of a heavy-chain variable region (CDR3), without itself encoding such properties (Ichiyoshi and Casali, 1994). In addition to the skewed V segment gene usage in phage display-derived antibody fragments, unusually long

Table 4. Relative frequency of J segment usage among human monoclonal antibodies and antibody fragments derived from phage display libraries specific for exogenous protein antigen and developed following an *in vivo* immune response (Table 1) and among peripheral blood mononuclear cells in general

Germline family	Representation among (%)		
	Hybridomas	Specific phage clones	Mononuclear cell ^a
	(n = 73)	(n = 46)	
JH1	0	0	1
JH2	1	0	0
JH3 ^b	21	33	9
JH4 ^c	48	22	53
JH5 ^c	14	9	15
JH6 ^b	16	37	22
	(n = 36)	(n = 40)	
JK1	25	18	nd
JK2	25	52	nd
JK3	19	5	nd
JK4	25	25	nd
JK5	6	0	nd
	(n = 26)		
Jλ1	19	nd	7
Jλ2/3	81	nd	89
Jλ7	0	nd	4

^aRelative frequency of Jλ family usage were as reported by Combriato and Klobbeck (1991) and JH usage was determined on adult peripheral blood B cells by Yamada *et al.* (1991).

^bPolymorphic variants of these genes are grouped together but both JH3a, JH3b and JH6a, JH6b were identified among the investigated hybridoma clones. JH6c was frequently found only among clones obtained by phage display.

^cOnly the described polymorphic variants JH4b and JH5b were found among the investigated clones.

CDR3-regions ($x = 16.8$; SEM = 0.3; $P < 0.01$) (Fig. 1) were preferentially selected in such studies (Table 1). This specific phenomenon was observed only among the HIV-specific, phage display-derived binders ($x = 18.5$; SEM = 0.3) whereas other specificities developed by this technique did not use longer CDR3 as compared to hybridoma/B cell-derived antibodies. However, these long CDR3 used by HIV-1-specific phage-derived clones were not a general feature of HIV-specific specificities because there was no statistical difference between the length of CDR3 in HIV-specific hybridomas ($x = 15.5$; SEM = 0.6) in comparison to CDR3 lengths of hybridoma-derived antibodies in general. In addition to using longer CDR3 regions, these clones showed a reduced frequency of JH4 usage (Table 4) and preferentially used JH6c (16/17; 94%) over JH6b usually (10/12; 83%) found in hybridoma-derived sequences of the JH6 type.

Together, these results suggest that individual VH/VL repertoires isolated by combinatorial approach may not exactly match those found *in vivo* and further experience with the phage display technology will be required firmly to establish the ability of this approach to mirror the *in vivo* repertoire, as previously discussed for autoantibody-related specificities (Rapoport *et al.*, 1995). In order to further investigate the general features of phage display-derived specific antibody fragments, it would be of value to investigate repertoires simultaneously developed from single individuals using phage display and conventional human hybridoma technology. In addition, an evaluation of the effect of a skewed antibody population to display enhanced conformational isomerism (Foote and Milstein, 1994) would be most valuable. Possibly such a capacity would make it easier for a particular VH segment to accommodate different light-chain sequences and thus display enhanced light-chain promiscuity. Regardless of these mechanisms, it should be pointed out that even if the phage display technology preferentially selects

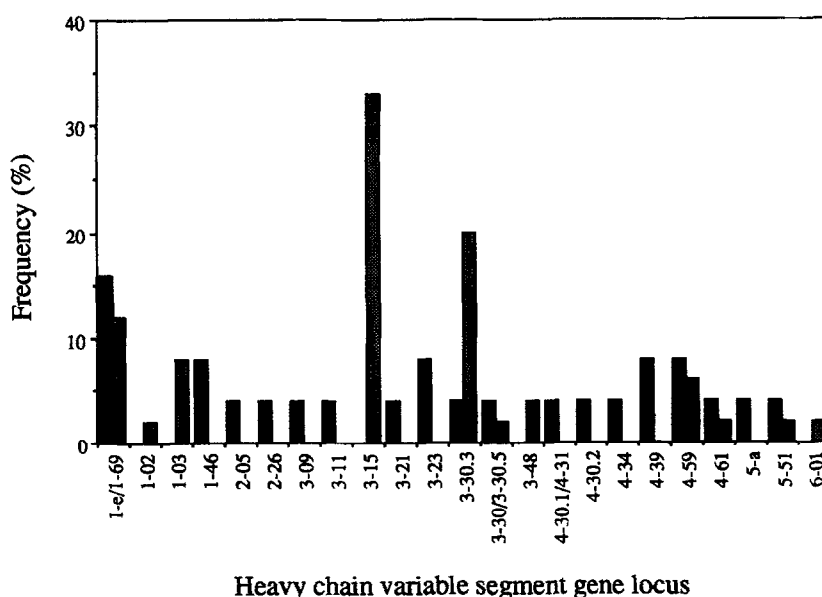


Fig. 2. VH segment loci used by hybridoma/B cell (filled columns; $n = 25$) and phage display (grey columns; $n = 49$)-derived HIV-1 gp41/120-specific binders (Table 1).

for a limited part of the available repertoire this fact will not as such disqualify specificities obtained by this technique for inclusion, e.g. in clinical studies.

CONCLUSIONS

From this investigation it is clear that the humoral immune response induced in humans as a consequence of interaction with a foreign, immunogenic protein regularly results in a diverse antibody repertoire. No general bias for particular variable segment subgroups appears to occur as a consequence of the cellular immortalization procedures used (in many cases initiated by a primary step of EBV immortalization) in agreement with previous observations (Victor *et al.*, 1991). However, the CDR3 of induced repertoires appear to be longer than those found in circulating B cells in general, suggesting that recruitment of B cells in a T cell-dependent immune response is not random in humans. Furthermore, at least one Vh locus frequently found in the periphery and also associated with autoreactivity is rarely used in induced repertoires against foreign antigens. Certain VH subgroups appear to be over- or under-represented in some immune responses, a fact that may involve B cell super-antigen activity of such infectious agents/antigens. Furthermore, repertoires derived by phage display technology seems to differ from regular *in vivo* repertoires in several respects (VH and JH segment usage and heavy-chain CDR3 length) some of which may relate to the combinatorial approach as such.

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