Cloning, sequencing and analyzing of the heavy chain V region genes of human polyreactive antibodies

ZHANG JINSONG, MING YEH[1].
Shanghai Institute of Cell Biology, Academia Sinica Shanghai 200031, China.

ABSTRACT

The heavy chain variable region genes of 5 human polyreactive mAbs generated in our laboratory have been cloned and sequenced using polymerase chain reaction (PCR) technique. We found that 2 and 3 mAbs utilized genes of the V_HIV and V_HIII families, respectively. The former 2 V_H segments were in germline configuration. A common V_H segment, with the best similarity of 90.1 % to the published V_HIII germline genes, was utilized by 2 different rearranged genes encoding the V regions of other 3 mAbs. This strongly suggests that the common V_H segment is a unmutated copy of an unidentified germline V_H III gene. All these polyreactive mAbs displayed a large NDN region (V_H-D-J_H junction). The entire H chain V regions of these polyreactive mAbs are unusually basic. The analysis of the charge properties of these mAbs as well as those of other poly-and mono- reactive mAbs from literatures prompts us to propose that the charged amino acids with a particular distribution along the H chain V region, especially the binding sites (CDRs), may be an important structural feature involved in antibody polyreactivity.

Key words: *human polyreactive antibody, heavy chain variable region gene, gene cloping and sequencing, polymerase chain reaction (PCR).*

1. Corresponding author

INTRODUCTION

In recent years, considerable interest has been aroused by reports dealing with the existence of antibodies with alternative binding characteristics: "polyreactive" or "multireactive" antibodies [1-3]. In humans, polyreactive antibodies are reported to be derived largely or exclusively from $CD5^+$ B cells[4-6]. The $CD5^+$ B cells constitute a distinct B cell subset committed to the production of natural antibodies or natural autoantibodies reactive with various self and foreign antigens. Whether they have a specialized function remains unclear. During our screening of the specific human hybridomas constructed from *in vftro* immunized human lymphocytes[7,8], many clones were of polyreactivity. However, when we studied the $CD5^+$ B cells in 14-days' *in vifro* induction cultures with FACS, they only accounted for a small percentage (2 % -3 %)[9]. Recent studies revealed that $CD5^-$ B cells may also participate in polyreactive antibody production. They are now designated as B-1b cell in mice, with $CD5^+$ B cells as B-1a cells[10]. In humans, a polyreactive antibody-secreting $CD5^-$ B cell subset has also been identified. They are speculated to represent the homologue of murine B-1b cells[11].

The precise mechanism by which polyreactive antibodies bind to difierent antigens is not known. Sequencing of V region genes have been made, but most results were obtained from mice. In this paper, the heavy chain V region genes of 5 human polyreactive mAbs estkblished in our laboratory have been cloned and sequenced using reverse transcriptase-polymerase chain reaction (RT-PCR) technique. The results show that the V_H segments of 2 polyreactive mAbs were encoded in the germline. An uncharacterized germline V_H gene of V_H III family was strongly suggested to encode the V_H segments of the other 3 polyreactive mAbs in unmutated configurations. All these polyreactive mAbs possessed a long NDN region (V_H- D-J_H junction). Analysis of the protein sequences of the H chain V regions suggest that the charged amino acids with a particular distribution along the V region, es-Pecially the CDRs, may represent an important structural feature for polyreactivity. Knowledge of the primary structures of the V regions of these antibodies may be informative in understanding the structural basis of po1yreactivity and possibly the role of the polyreactive antibody-secreting B cells as well.

MATERIALS AND METHODS

Human hybridoma clones

Clones B7H9, MD11, A8C8 and clones 104-A6, HB-1 used in this study were derived from fusions of mouse myeloma cells and human lymphocytes *in vitro* immunized with tetanus toxoid or HBV vaccine respectively, and all of these had been repeatedly cloned. Among them, A8C8 and MD11 came from the same fusion but from separate wells. HB-1 was a passaged line after 5th cloning, while 104-A6 was a clone after 8th cloning. All these clones produced IgM antibodies.

Elisa

Antigens used for coating ELISA plates included tetanus toxoid (TT, Shanghai Institute of Bio-

logical Products), ovalbumin (OVA, Dongfeng Reagents Factory), human transferrin (TF, Sigma), bovine insulin (IN, Sigma), trichosanthin (TCS, a plant protein, Wuhan Institute of Biological Products), fowl gamma globulin (FγG, prepared in our laboratory), HBsAg (Shanghai Medical Laboratory).

Assays were carried out as previously described[7,8]. Briefly, different antigens with same concentration (20 μg/ml) were coated on plates. Culture supernatants of hybridoma clones were added, followed by adding goat anti-human IgM-peroxidase conjugates (from Capel) after wash.

Reagents

Moloney murine leukemia virus (MMLV) reverse transcriptase and deoxynucleotide triphosphates were from Boehringer Mannheim Biochemica. DNA polymerase from *Thermus aquaticus* (Taq polymerase) and TaqTrack sequencing system were from Promega Corp. Oligonucleotides, automatically synthesized on an Applied Biosystems 381 DNA Synthesizer and purified by electrophoresis on a denaturating polyacrylamide gel, were provided by Yingkui Wang (Shanghai Institute of Cell Biology).

Design of oligonucleotide primer

To test a general method for cloning the heavy chain variable region gene of any human antibody, we designed a degenerate 5' PCR primer referring to the method of Mark et al.[12]. The 3' primers were constructed from the constant region of human IgM μ chain. Primer Cμ1 was designed to synthesize the first chain cDNA. Primer Cμ2 was used in PCR. Restriction sites were incorporated into the PCR primers to facilitate the cloning of the amplified fragments.

Isolation of total RNA (modified from Chomczynski[13])

A modified AGPC method was used to isolate the total RNA from hybridoma cells[13]. Hybridoma cells (10^6) grown in monolayer were lysed in the tissue culture flask with 1 ml of lysis buffer (8 *M* guanidinium hydrochloride, 0.1 *M* Tris-HCl, pH 7.0). The lysate was transferred to a fresh tube. Sequentially, 0.1 ml of 2 *M* sodium acetate, pH 4, 1 m1 of water-saturated phenol, and 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were added with mixing after each addition. The final suspension was shaken vigorously for 10 s and cooled on ice for 15 min. After centrifugation, the water phase was transferred to another fresh tube. 0.1 vol of 3 M sodium acetate, pH 5.2, was added and the RNA was cold ethanol precipitated. The RNA pellet was washed twice with 70% ethanol, dried and dissolved in 20 μl of DEPC-treated water.

First strand cDNA synthesis and PCR amplification

First strand cDNA was synthesized at 37 ℃ for 1 h in a 50 μl reaction volume with Cμ1 priming. 50 μl of this reaction mixture contained: 5μl of the RNA solution, 1 μl of MMLV reverse transcriptase (200 U/μl), 1 μl of RNasin (50 U/μl), 10 μl of MMLV reverse transcriptase buffer (250 m*M* Tris-HCl, pH 7.5, 375 m*M* KCl, 15 m*M* $MgCl_2$), 5 μl of 100 m*M* dithiothreitol, 5 μl of 5 m*M* dNTP (dATP, dCTP, dTTP, dGTP) mix, and 50 pmol of primer Cμ1. After incubation at 37 ℃ for 1 h, the reaction mixture was heated to 100 ℃ for 5 min, and extracted once with phenol/chloroform (1:1), once with chloroform. The water phase fraction was frozen.

A 100 μl PCR mixture was prepared containing 1 μl of the cDNA solution, 10 μl of Taq DNA polymerase buffer (100 m*M* Tris-HCl, pH 9.0, 500 m*M* KCl, 15 m*M* $MgCl_2$, 1.0% Triton X-100), 4 μl of 5 m*M* dNTP mix, 50 pmol of 5' primer, 50 pmol of primer Cμ2 and 1 μl (5U) Taq DNA polymerase. The reaction mixture was overlaid with paraffin oil and subjected to 35 cycles of amplification. The cycle was 93 ℃ for 1 min (denaturation), 50 ℃ for 1.5 min (annealing) and 72 ℃ for 1 min (extension). The product was analyzed by running 5 μl on a 2% agarose gel. If necessary, the specific PCR product was isolated from agarose gel and subjected to another PCR amplification described above. The PCR product was digested with EcoR I and Pst I, and purified with 2% low

melting point agarose gel.

Cloning and sequencing of mAb heavy chain variable region genes

Digested and gel purified PCR products were ligated into pBluescript KS(+) or M13mp18/19 sequencing vectors. The ligation mixture was used to transform XL1-blue competent cells. The colonies were first screened for DNA inserts by minipreparation of dsDNA and digestion with EcoR I and Pst I. ssDNA was prepared from the positive colony containing the putative V region gene insert. Dideoxynucleotide chain termination sequencing was carried out using the sequencing grade Taq DNA polymerase according to the manufacturer's protocol. At least 3 independent colonies were sequenced.

Calculation of theoretical pI

The theoretical pI of the V regions was calculated using the "CHARGPRO" program of PC/GENE software (IntelliGenetics, Inc, Mountain View, CA, USA). Briefly, the fractional ionization of the N terminus and all ionizable side chains was first calculated, and then the net total charge at pH 7 was determined. When the pH at which the net total charge approached 0 was found, this value was taken as the pI. For calculation, it was assumed that the 2 invariant Cys residues in the V regions were involved in disulfide bonding, and their potential side chain contribution to the pI was therefore ignored.

RESULTS

Polyreactivity of antibodies secreted by human hybridoma clones

Results of 5 mAbs from 5 clones tested against various antigens were shown in Tab 1. They all reacted with various antigens to varying extent, thus displaying their polyreactivity.

Tab 1. Polyreactivity of human mAbs (ELISA, OD450)

	Antibody Titer to					
mAb	TT/HBsAg	OVA	TF	IN	TCS	FγG
104-A6	0.69 ± 0.00	0.72 ± 0.01	0.48 ± 0.10	0.42 ± 0.01	0.98 ± 0.13	0.55 ± 0.00
HB-1	0.89 ± 0.01	0.99 ± 0.04	0.99 ± 0.06	0.97 ± 0.02	1.04 ± 0.04	0.96 ± 0.03
B7H9	1.09 ± 0.06	1.32 ± 0.10	0.71 ± 0.00	0.69 ± 0.04	1.49 ± 0.04	0.76 ± 0.03
A8C8	0.46 ± 0.04	0.25 ± 0.01	0.16 ± 0.04	0.16 ± 0.04	0.39 ± 0.11	0.31 ± 0.02
MD11	0.28 ± 0.00	0.21 ± 0.02	0.12 ± 0.00	0.12 ± 0.01	0.26 ± 0.01	0.15 ± 0.00

In antigen coated wells, the OD value of adding conjugates alone varied within 0.08—0.12, but in TCS coated wells it may attain 0.17-0.18 in some cases.

Design of amplification primers

The nucleotide sequences encoding the immunoglobulin complementarity-determining regions (CDRs) are highly variable. However, there are several regions of conserved sequences (framework regions, FRs). The nucleotide sequence at the first 23 positions of the 5' end of human V genes is conserved when aligned by family[12]. Based on the conserved sequence, we have designed a degenerate 5' primer to be complementary to the first strand cDNA encoding the conserved N- terminal region of 6 human V_H families (antisense strand). The primer is a mixture of 32 primers with degeneracy at 5 positions (Tab 2). Restriction site Pst I, which is infrequently

present in the human V_H genes, was incorporated into the primer to facilitate the cloning of the amplified fragment.

The H chain 3' primer Cμ2 was constructed to be complementary to the constant region of human μ chain and contained a EcoR I restriction site (Tab 2).

Tab 2. Design of synthetic oligonucleotide primers corresponding to human immunoglobulin FR 1 and constant regions

Human heavy chain universal 5' primer[1]:

$P_{5'}$ (nucleotide positions 2-23, mixture of 32 primers):

5' AGGT(CG)(AC)A (AG) CTGCAG (CG) AGTC (AT) GG 3'

Pst I

Human IgM heavy chain 3' primer[2]:

Cμ1 (amino acid positions 164-172, used for synthesis of cDNA):

5' GAGGATCCGGGTGCTGCTGATG 3'

Cμ2 (amino acid positions 127-134, used for PCR):

5' CTGGATCCGACGGGGAATTCTCAC 3'

EcoR I

1. Bases in parentheses represent substitutions at a given position; i.e. (AT) means both A and T were present in equimolar amounts during the synthesis of a particular position.
2. Amino acid positions are numbered according to Kabat et al. (14).

cDNA synthesis and PCR amplification

cDNA was synthesized from total RNA isolated from hybridoma cells using a specific constant region primer Cμ1 (Tab 2) . The sequence encoding the variable region was enzymatically amplified from the cDNA , using a degenerate upstream Primer $P_{5'}$ and a nested downstream primer Cμ2. As seen in Fig 1, specially amplified DNA fragments were successfully obtained from 5 hybridomas. Each fragment should include the variable region and a small portion of the μ chain constant region flanked by 2 PCR primers. This resulted in an approximate size of the amplified fragment of 450 bp (Fig 1) which conformed to the expected fragment size of a variable region[14].

Cloning and sequencing of the V region genes

The amplified products were digested with EcoR I and Pst I and cloned into pBluescript KS (+) or M13mp l8/19 sequencing vectors. Positive colonies with inserts of approximate 450 bp were sequenced with ssDNA. The sequences were compared with published V_H gene sequences. A similarity reaching 80% was used as a criterion for assignment to a V_H gene family.

The nucleotide sequences and the deduced protein sequences of the V regions of these mAbs were reported in Fig 2, 3 and their gene usage was summarized in Tab 3.

As seen in Fig 2 and Fig 3, the H chain variable region sequences of HB-1 and A8C8 were completely identical with those of 104-A6 and MD11, respectively. The V_H segments of mAb 104-A6 and HB-1 shared an absolute nucleotide homology with that of the human germline gene V_H4.18, a member of the V_HIV family[15]. The V_H segments of mAb B7H9, MD11 and A8C8 were identical, and displayed 90.1% similarity to the V_H26 genomic V_HIII sequence[16]. It is interesting that a common V_H segment was utilized by 2 rearranged variable region genes of mAb B7H9 and mAbs MD11, A8C8, while the usages of D and J_H segments were different.

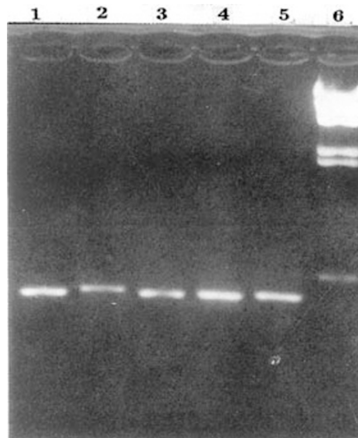

Fig 1. Agarose electrophoresis of H chain PCR products from the first-strand cDNA of human polyreactive antibodies.
Lane 1: A8C8,
Lane 2: HB-1,
Lane 3: MD11,
Lane 4: B7H9,
Lane 5: 104-A6,
Lane 6: Lambda DNA /Hind III markers.

Tab 3. Gene segment usage in IgM polyreactive antibodies

Antibody	V_H family	D	J_H
104-A6	V_HIV	DXP4	J_H6
HB-1	V_HIV	DXP4	J_H6
B7H9	V_HIII	DXP4+DLR5	J_H6
MD11	V_HIII	DLR2	J_H4
A8C8	V_HIII	DLR2	J_H4

The D gene segments

The D segments utilized by the 5 polyreactive mAbs were diverse (Fig 4) The D gene segments of 104-A6 and HB-1 shared complete homology with the genomic DXP4 D segment[17]. A stretch of 22 nucleotides of the genomic DLR2 D segment[18] was utilized in mAb MD11 and A8C8. A D-D fusion was observed in the D segment of mAb B7H9 resulted from nonconventional D gene recombination. The genomic DLR5 gene was originally reported by Zong et al.[19]. N addition regions were found at both V_H-D and D-J_H junctions. The NDN regions of mAbs 104-A6,

Fig 2. ▻ Nucleotide sequences of the H chains of polyreactive mAbs 104-A6, HB-1, B7H9, MD11 and A8C8. The CDRs and FRs are defined according to Kabat et al.[14]. The top sequence in each cluster is used for comparison. Identities are indicated by dashes. Asterisks denote the boundaries of the CDR. The V_H4.18 is a member of the V_HIV family[15]. The V_H26 gene is a member of the V_HIII family[16].

V GENE SEQUENCES

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                                                  FR 1
   VH4. l8    CAGCTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTC  60
mAb l04-A6              PCR 5’ pr imer -------------------------------------
mAb HB-1                PCR 5’ pr imer -------------------------------------
                                           *    CDR 1          *
   VH4. l8    ACCTGCAGTGTCTCTGGTGGCTCCATCAGCAGTAGTAGTTACTACTGGGGCTGGATCCGC  120
mAb l04-A6    ------------------------------------------------------------
mAb HB-1      ------------------------------------------------------------
                           FR 2                  *
   VH4. l8    CAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGAGTATCTATTATAGTGGGAGCACCTAC  180
mAb l04-A6    ------------------------------------------------------------
mAb HB-1      ------------------------------------------------------------
                CDR 2             *
   VH4. l8    TACAACCCGTCCCTGAAGAGTCGAGTGACCATATCCGTAGACACGTCCAAGAACCAGTTC  240
mAb l04-A6    ------------------------------------------------------------
mAb HB-1      ------------------------------------------------------------
                                      FR 3                         297*
   VH4. l8    TCCCTGAAGCTGAGCTCTGTGACCGCCGCAGACACGGCTGTGTATTACTGTGCGAGA
mAb l04-A6    ---------------------------------------------------------CAT  300
mAb HB-1      ------------------------------------------------------------
                                     CDR 3                        *
mAb l04-A6    CATTAGGATTTTTGGAGTGGTTATTATACGGCGCACTACTACGGTATGGACGTCTGGGGC  360
mAb HB-1      ------------------------------------------------------------
                         FR 4
mAb l04-A6    CAAGGGAGCACGGTCACCGTCTCCTCA 3'    387
mAb HB-1      ---------------------------


                                                  FR 1
   VH26       GAGGTGCAGGTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTC  60
mAb B7H9                PCR 5  pr imer -------C----CA----------------------
mAb MD11                PCR 5  pr imer -------C----CA----------------------
mAb A8C8                PCR 5  pr imer -------C----CA----------------------
                                           *    CDR 1      *
   VH26       TCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCT  120
mAb B7H9      --------------------------C--T------AG-----A----------------
mAb MD11      --------------------------C--T------AG-----A----------------
mAb A8C8      --------------------------C--T------AG-----A----------------
                           FR 2          *
   VH26       CCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTAC  180
mAb B7H9      ---------------------------T C------A-----A--A--TA--T-------
mAb MD11      ---------------------------T-C------A-----A--A--TA--T-------
mAb A8C8      ---------------------------T-C------A-----A--A--TA--T-------
                 CDR 2          *
   VH26       GGAGACTCCGTGAAGGGCCGGTTCACCATCTGAAGAGACAATTCCAAGAACACGCTGTAT  240
mAb B7H9      -C------A-----------A-----------C---------CG-------T-A------
mAb MD11      -C------A-----------A-----------C---------CG-------T-A------
mAb A8C8      -C------A-----------A-----------C---------CG-------T-A------
                           FR 3                                   294*
   VH26       CTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAA
mAb B7H9      -----------------------------------T--G-------------G-GTCGAG  300
mAb MD11      -----------------------------------T--G-------------G-GATCGA
mAb A8C8      -----------------------------------T--G-------------G-GATCGA
                          CDR 3                               *
mAb B7H9      GGTTATTATAGGGAGGTTTTCTACTACTACTACTACATGGACGTCTGGGGCAAAGGGACC  360
mAb MD11      CCCGCTATTGTAGTGGTGGTAGCTGGAAACTTTGACTAC......TGGGGCCAGGGAACC
mAb A8C8      CCCGCTATTGTAGTGGTGGTAGCTGGAAACTTTGACTAC......TGGGGCCAGGGAACC
                 FR 4
mAb B7H9      ACGGTCACCGTCTCCTCA 3'    378
mAb MD11      CTGGTCACCGTCTCCTCA       372
mAb A8C8      CTGGTCACCGTCTCCTCA       372
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DEDUCED AMINO ACID SEQUENCES OF V REGIONS

                FR 1                 *CDR 1*      FR 2     *  CDR 2         *
  VH4. 18     PGLVKPSETLSLTCTVSGGSISSSSYYWGWIRQPPGKGLEWIGSIYYSGSTYYNPSLKSRVT
mAb 104-A6    ----------------------------------------------------------------
mAb HB-1      ----------------------------------------------------------------
                    FR 3              99*     CDR 3         *     FR 4
  VH4. 18     ISVDTSKNQFSLKLSSVTAADTAVYYCAR
mAb 104-A6    -----------------------------HHYDFWSGYYTAHYYGMDVWGQGTTVTVSS 129
mAb HB-1      ------------------------------------------------------------
                FR 1                 *CDR1*       FR 2     *  CDR 2         *
  VH26        GGLVQPGGSLRLSCAASGFTFSS YAMSWVRQAPGKGLEWVSAISGSGGSTYYGDSVKGRFTI
mAb B7H9      ----K------------------S-N-------------S--S-SSYI--A---------
mAb MD11      ----K------------------S-N-------------S--S-SSYI--A---------
mAb A8C8      ----K------------------S-N-------------S--S-SSYI--A---------
                    FR 3              99*     CDR 3      *      FR 4
  VH26        SRDNSKNTLYLQMNSLRAEDTAVYYCAK
mAb B7H9      ----A--S--------------------RVEGYYREVFYYYYYMDVWGKGTTVTVSS 126
mAb MD11      ----A--S--------------------RDRPAIVVVVAGNFDY..WGQGTLVTVSS 124
mAb A8C8      ----A--S--------------------RDRPAIVVVVAGNFDY..WGQGTLVTVSS 124
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Fig 3. Deduced amino acid sequences of the H chains of polyreactive mAb 104-A6, HB-1, B7H9, MD11 and A8C8. Identities are indicated by dashes. Asterisks denote the boundaries of the CDR.

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                                D SEGMENTS

                     Nucleotide Sequences                        Deduced Amino
                                                                 AcidSequences

                                DXP4
                    1         10        20        30
                  5' GTATTACGATTTTTGGAGTGGTTATTATACC3'
                      :: :::::::::: :::::::::::::
104-A6, HB-1       CATC ATTACGATTTTTGGAGTGGTTATTATAC GGCGC          HHYDFWSGYYTAH
                     N               D                N

                  DXP4
        1         10        20        30
      5' GTATTACGATTTTTGGAGTGGTTATTATACC3'
                  :: ::::::::       N       D
B7H9                 GTCGAG GGTTATTATA GGCAGGT TTTCTA               VEGYYREVFY
                       N        D                ::::::
                        5' AGAATATTGTAATAGTACTACTTTCTATGCC3'
               8          1         10        20        30
                                     DLR5

                                     DLR2
                          1         10        20        30
                        5' AGGATATTGTAGTGGTGGTAGCTGCTACTCC3'
                           : ::::::::::::::::::::
MD11, A8C8         CATCGACCC GCTATTGTAGTGGTGGTAGCTG GAA             DRPAIVVVVAGN
                     N                D               N
```

Fig 4. Nucleotide and deduced amino acid sequences of the D segments utilized by the polyreactive mAbs. The top and bottom nucleotide sequences (DXP4, DLR5 and DLR2) are presented for germline comparison. Sequences of NDN regions of the polyreactive mAbs are underlined. The homology to published germline D sequences is shown.

HB-1, mAb B7H9 and mAbs MD11, A8C8 were 37, 29 and 34 bp, respectively, in length. The deduced protein sequences of the D genes of the mAbs. were reported in Fig 4.

The J_H segments

The J_H segments in these polyreactive antibodies were virtually identical with the germline sequences (Fig 5). mAbs 104-A6, HB-1 and B7H9 utilized the truncated forms of germline J_H6 segments. Assuming that the difference of a G with a C displayed by the 3 mAbs was due to the expression of a J_H6 polymorphic allele, then mAbs 104-A6 and HB-1 displayed 100 % similarity to a truncated J_H6 segment, and mAb B7H9 displayed 3 nucleotides deletion and only one nucleotide difference, an A instead of a C (resulting in the deletion of Gly and the variation of a Gln with a Lys), when compared with the J_H6 germline sequence (Fig 5). The mAbs MD11 and A8C8 utilized a virtually complete form of germline J_H4 segment (Fig 5). The only variation, a G instead of an A was silent at the protein level and has recently been found in several expressed J_H4 genes[20].

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                              JH SEGMENTS

                          Nucleotide Sequences

JH6       5' TACTACTACTACTACGGTATGGACGTCTGGGGGCAAGGGACCACGGTCACCGTCTCCTCA3'
104-A6            -------------------------C---------------------------
HB-1              -------------------------C---------------------------
B7H9          -------------    --------------CA--------------------------

JH4                    5' TACTTTGACTACTGGGGCCAAGGAACCCTGGTCACCGTCTCCTCA3'
MD11                      --------------------G-------------------------
A8C8                      --------------------G-------------------------

                     Deduced Amino Acid Sequence6

JH6                  YYYYYGMDVWGQGTTVTVSS
104-A6                  -----------------
HB-1                    -----------------
B7H9                 ----  -----K--------

JH4                      YFDYWGQGTLVTVSS
MD11                        --------------
A8C8                        --------------
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Fig 5. Nucleotide and deduced amino acid sequences of the J_H segments utilized by the polyreactive mAbs. The top sequence (J_H6 or J_H4) in each cluster is used for germline comparison. Identities are indicated by dashes. Blank spaces represent deletions.

Charge properties of the H chain V regions of the polyreactive antibodies

In view of the interactions of charged groups may be important for a polyreactive phenotype, we computed the theoretical isoelectric points from the primary protein

sequences of the H chain V regions of the polyreactive mAbs. Two sets of poly- and mono-reactive mAbs from literatures were also calculated (Tab 4). The antibodies in each set were selected on the basis of the same "primary" antigen specificity [21, 22].

Tab 4. Charge characteristics of antibody H chain V regions

		mAb [1]	"Primary" specificity	Isoelectric point [2]	Net charge [3] CDR1	CDR2	CDR3	CDRs
This work	Polyreactive	104-A6	HBsAg	8.83	0	+2	-1	+1
		HB-1	HBsAg	8.83	0	+2	-1	+1
		B7H9	TT	9.23	0	+1	-1	0
		MD11	TT	9.46	0	+1	0	+1
		A8C8	TT	9.46	0	+1	0	+1
From literature	Polyreactive	BrM1	BrMRBC	8.89	0	+2	-1	+1
		BrM8	BrMRBC	9.60	-1	+2	0	+1
		CH12	BrMRBC	9.23	0	+2	-1	+1
	Monorecective	CH32	BrMRBC	4.57	0	-1	-3	-4
		BrM11	BrMRBC	5.57	0	+1	-2	-1
	Polyreactive	G8Ad3.8	GAT	9.16	0	+3	+1	+4
		G8Ca1.7	GAT	8.06	0	+2	+1	+3
		G5Bb2.2	GAT	8.06	0	+2	+1	+3
	Monoreactive	17C1	GAT	4.17	0	-1	-3	-4
		14C3	GAT	4.29	0	0	-3	-3

1. Including mAbs to BrMRBC (bromelain-treated mouse red blood cells) [21], and mAbs to GAT (synthetic copolymer poly (Ghu^{60} Ala^{30} Tyr^{10})) [22].
2. Calculations are based only on the amino acid sequence using the "CHARGPRO" Program of PC/GENE. The entire V region, comprising J, was all included for calculations (equivalent to amino acid residues 1-113, according to the nomenclature of Kabat et al.[14]).
3. Two flanking residues by each side of CDRs were included in calculations. Calculations are based on the charged amino acids.

As seen in Tab 4, the pI data of V regions of polyreactive mAbs were remarkably higher than those of monoreactive counterparts of the same “primary” specificity. The V regions of all of the polyreactive antibodies had basic pI, which would be expected to result in a net positive charge at neutral pH. In contrast, the V regions of the monoreactive antibodies were acidic and should have a net negative charge at neutral pH.

Since the antibody - binding site (CDR s) should be mainly responsible for antibody specificity, the charge properties of the H chain CDRs of poly - and mono -reactive antibodies were further investigated. In view of less than 10 % of histidine residues (with a pK of 6. 0) carry a positive charge at pH 7. 0, their contribution to the charge properties described below was therefore ignored. The net charges of each CDR and CDRs were calculated and the results are also shown in Tab 4. There were obvious differences in the net charges of the CDRs between poly - and mono - reactive antibodies. The net charges of the CDRs of monoreactive antibodies were negative (-1 ～ -4), while those of polyreactive antibodies usually positive (0 ～ +4) (Tab 4). Furthermore, regarding the charge distribution in the CDRs, the negative net charges of the CDRs of monoreactive antibodies were mainly offered by the CDR3, which carried high negative net charges, whereas the CDR2 were less charged (Tab 4). In contrast, the net charges of the CDRs of polyreactive antibodies were mainly determined by the CDR2, which carried relatively high positive charges, while the CDR3 were less charged (Tab 4).

DISCUSSION

Methodology

Design of upstream PCR primers is a crucial factor for efficient amplification of special variable region genes of immunoglobulins due to variability of the V gene sequences. Rearranged human V genes have been amplified from several hybridomas using PCR primers based on the leader exon and the C region exon[23]. However, the variability in the H chain leader sequence necessitates the use of 3 groups of 5’ primers with high degeneracy. Furthermore, the location of the PCR primers compromises the direct cloning of the amplified V genes for bacterial expression. Recently, Marks et al reported a new set of upstream PCR primers based on the 5’ end of human V_H gene exons [12], which consisted of 5 different family - based primers without degeneracy. Results of Marks et al showed that the family - based primers lacked the specificity in 6 human V_H gene families. Our results suggested that the design of different family - based primers was unnecessary.

Germline configuration of the V_H segments of human polyreactive antibodies

Previous studies have shown that the V_H segments of human polyreactive antibodies were encoded by germline genes in unmutated configuration[24-26]. Our results reported in this paper were consistent with this viewpoint. The V_H seg-

ments of mAbs 104-A6 and HB-1 shared a complete homology with genomic $V_H4.18$ sequence. A common V_H segment was utilized by 2 different rearranged $V_H DJ_H$ genes of mAb B7H9 and mAbs MD11, A8C8. Considering they were from 2 different fusions and the higher number of yet undetermined members of the V_H III family [14,20,27,28], these results strongly suggested that this common V_H segment is also encoded by an unmutated germline gene, which has not been identified so far. This genomic V_H gene is a member of V_HIII family and its coding sequence should be identical with the common V_H sequence of mAb B7H9, MD11 or A8C8.

It is noticeable that apart from the V_H segment, the D and J_H segments of mAbs 104-A6 and HB-1 also exhibited perfect identity with the genomic donors. To the best of our knowledge, human antibodies having this property, i.e. V_H, D, J_H segments all in germline configuration, have not been reported.

Origin of human polyreactive antibody-secreting B cells

Recently, Kantor et al demonstrated the existence of a third B-cell lineage, CD5— Ly-1 B cells, or B-1b cells, in mice[10]. The morphologic and functional feature of B-1a and B-1b cells are essentially identical, distinguished only by the presence or absence of CD5 cell-surface antigen. In humans, Kasaian et al. have recently identified a $CD5^-$ B-cell subset, $CD5^-$ $CD45RA^{low}$ B cells, which were found to share a distinctive functional feature of B-1a cells in their ability to produce polyreactive antibodies [11]. They speculated that these $CD5^-$ B lymphocytes may represent the homologue of the murine B-1b cells. Sequence data of this type of B cells has not yet been reported in both humans and mice so far.

In our study[9], FACS analysis data showed that $CD5^+$ B cells maintained only at a few percent level assayed on different culture days, indicating no significant proliferation of $CD5^+$ B cells throughout the 14 days' *in vitro* immunization period. However, the frequency of the polyreactive antibody-secreting hybridoma clones was so high that a non-immunizing antigen, usually OVA, should be routinely included along with the specific antigen during each screening in our lab. This raises the possibility that the polyreactive antibody-secreting B cells in the culture were mainly, but not exclusively, $CD5^-$. This may support Kasaian et al's view that in humans there was also presence of a counterpart of mouse B-1b cell population.

The high frequency of the occurrence of polyreactive antibody-secreting hybridoma clones in cell fusions possibly suggested that polyreactive antibody-secreting B cells had expanded through antigen stimulation during *in vitro* immunization, which implied that the polyreactive antibody-secreting B cells might play an important role in primary antibody responses. It is possible that the *in vitro* immunization of human lymphocytes we adopted was liable to induce primary antibody responses, since we never detected high affinity TT/HBsAg-specific clones in almost all the 50 fusions. Polyreactive antibody-secreting B cells may represent a reservoir for further antigen-driven mutation and selection, and may play a role in dual antigen recogni-

tion by B cells[29] through the formation of antigen- antibody - complement complexes binding to both antigen receptors and complement receptor-2 on B cells for enhancing signal transduction during primary antibody responses.

Structural basis for antibody polyreactivity

Numerous studies have been conducted on naturally occurring polyreactive mAbs obtained from normal mice and humans[2-4]. However, the molecular basis of their ability to bind to multiple antigens is still elusive. The possibility that domains other than the variable region may influence the binding of polyreactive antibodies to antigens was excluded. The C_H region is not responsible for polyreactivity[30]. Harindranath et a1[25] found that the length of the NDN regions utilized by the polyreactive mAbs (36 and 45 bp) and those utilized by their monoreactive counterparts (15 and 24 bp) were remarkably difierent. This fact raised the issue of whether the configuration of the NDN segnmnt may contribute to antibody poly- or monoreactivity[25]. The possibility that a long NDN region may be a prerequisite for antibody polyreactivity was further supported by the length, 28-60 bp, of the NDN regions of other polyreactive natural antibodies of various Ig classes from $CD5^+$ B cells (24). In the present paper, the NDN regions of polyreactive mAbs 104-A6, HB-1, mAb B7H9 and mAbs MD11, A8C8 were 37, 29 and 34 bp, respectively, in length. The H chain CDR3 of these antibodies were 57, 51 and 45 bp, respectively, in length. These results provided further evidences for the above- mentioned viewpoint that a long NDN region may be a determinant factor involved in antibody polyreactivity.

The H chain V regions of the polyreactive antibodies were all predicted to have basic pI, which is significantly higher than the acidic pI predicted for the monoreactive antibodies (Tab 4). The presence of a positively charged V region in the polyreactive antibodies suggested that charge interactions might play a role in conferring the polyreactive phenotype. Indeed salt bridges are more energetic and act over longer distances than hydrogen bonds, hydrophobic and Van der Waals interactions. Furthermore, charged residues possess a certain flexibility because of their large side chains and their interactions are not limited by a given orientation in contrast to the highly linear hydrogen bonds.

Conger et al.[21] reported that polyreactive antibodies displayed a significant binding with both the acidic and basic panel of antigens, while monoreactive antibodies bind neither of the 2 antigen panels but only the specific antigen. Obviously, a H chain V region with net positive charge might facilitate antibody interaction with acidic molecules, but this does not explain the binding patterns using cationic antigens. Therefore, while the charge across the entire V region is likely to be an important contributor to polyreactivity, other factors must also be relevant.

It is interesting to note that the charge properties of the H chain CDRs of polyreactive antibodies are also different from those of monoreactive counterparts (Tab 4).

This is consistent with the fact that the CDRs are the binding sites involved in antibody-antigen interaction. Both the net charge and the charge distribution of the H chain CDRs show differences between these 2 types of antibodies. In polyreactive antibodies, the net charges of the CDRs are mainly determined by CDR2. It is of interest that despite the overall basic nature of the polyreactive antibodies, especially in amino acid composition of CDR2, the CDR3 is comparatively more acidic. Most of the polyreactive antibodies have a negatively charged CDR3 with a large, loose character. This long, loose CDR3 might enable it to bind a variety of structures with a relatively low affinity via extensive hydrophilic or hydrophobic interactions. Particularly, the acidic amino acid residues in this region may be important in binding of positively charged epitopes by the polyreactive antibodies. In contrast, the negative net charges of the CDRs of the monoreactive antibodies are mainly offered by the CDR3. A strong negative pocket may be formed by acidic residues in this short, highly negative region and may be partly responsible for the high affinity and specific binding phenotype of the monoreactive antibodies we discussed. These considerations suggested that charged residues and their distribution along the H chain V regions, especially the CDRs, may be important for the antibody binding pattern.

These specific characteristics of the H chain variable region of the polyreactive antibody may contribute to a particular microenvironment of the binding site which enables it to complement various antigens. Of course, a more direct answer to polyreactivity shall wait for the study of the conformational structure of Ag-Ab complex[31].

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