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Molecular characterization of a human monoclonal antibody to B antigen in ABO blood type

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Abstract

A human anti-B antibody of clone BT97 was obtained from a healthy individual of type A of the ABO blood group without immunization. Cloning was performed by means of heterohybridoma formation of cell fusion between human peripheral lymphocytes and mouse myeloma cells. The antibody selectively reacted with B-antigen in flow cytometry using red blood cells and enzyme-linked immunosorbent assay. The VH and VL genes of BT97 were derived from the germline genes of DP-47 and 3p.81A4, respectively, with a couple of somatic mutational events. Comparative analysis with other reported anti-A, B and H antibodies revealed that the amino acid sequence of the VH region was more homologous than that of the VL region. The sequence of BT97 showed complete identity with one anti-H natural antibody reported by Marks et al., with the exception of the CDR3 region. It is not known whether the homologies include the common properties of the natural antibodies; however, a particular germline gene potentially changes to anti-ABH antibodies. We think that this method is suitable for cDNA preparation of human monoclonal antibodies to blood group antigens and for sequence analysis.

Keywords: Human monoclonal antibody; Heterohybridoma; Natural antibody; ABO histo-blood type

1. Introduction

Carbohydrate complexes combined with proteins and lipids decorate the plasma membrane of erythrocytes; moreover, these species are often polymorphic, which is known as blood types such as ABO, Ii and P type. In the ABO blood group, ABH antigens, which are carbohydrate structures present in many tissues, are synthesized under the control of highly related genes coding for glycosyltransferases [1]. The blood type A molecule is formed by an α1,3GalNAcT corresponding to the A allele of the ABO locus. The blood type B allele encodes an α1,3galactosyltransferase that forms the B determinate. O alleles encode functionally inert polypeptides that do not further modify H-active precursors and therefore represent null alleles at this locus. Four amino acid substitutions were identified between A transferase and B transferase. Most O alleles were found to contain a single base deletion near the N-terminus of the coding sequence. The phenotypes A, B, O and AB have been clinically examined by hemagglutination for transfusion. The antisera for laboratory examinations were, at one time, derived from immunized individual donors; however, these antisera are no longer available as a result of infectious hazard. To date, monoclonal antibodies (mAbs) to A and B-antigens raised in mice have replaced antisera. mAbs are sufficiently effective for regular laboratory examinations; however, the mAbs are less valuable than the former human antisera in cases involving the adsorption-elution test.

Antibodies to ABO antigen-structures are among the most predominant naturally occurring antibodies. Type A individuals display anti-B antibodies in their plasma. Type B individuals exhibit anti-A antibodies. Type O individuals possess both antibodies. The antibodies are oligoclonal; additionally, they consist mainly of IgM [2].

Abbreviations: mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; RBC, red blood cell; PBL, peripheral blood lymphocyte; RT-PCR, reverse transcription-polymerase chain reaction; CDR, complimentarity-determining region.

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Their high titer is maintained throughout the lifespan following birth despite no apparent specific stimuli. The physiological importance of natural antibodies remains unclear; however, roles for natural antibodies including nonspecific anti-bacterial defense and self-regulation of the immune system have been proposed [3,4]. Despite the classical theme, investigations concerning the production of specific antibodies by cloned cells display a number of shortcomings, which may be attributable to the difficulty associated with the production of human mAbs.

In order to obtain a human immunoglobulin gene, two major technical approaches are widely applied. The first technique involves the phage-display method, often combined with lymphocyte-transformation by Epstein–Barr virus (EBV) [5,6]. The second approach consists of heterohybridoma formation between EBV-transformed human lymphocytes and mouse myeloma cells [7–9]. However, both methods possess critical limitations. In the former technique, the combination of H and L-chains may or may not involve the original species expressed in the specific antibody producing cells. In contrast, production of human antibody is quite low and rapidly diminished under mouse genomic control in the latter case. However it is certain that the original combination of H and L-chains is obtained by the fusion protocol.

In the present study, in order to characterize the variable region of anti-ABH antibody, the simplest protocol of the fusion methodology between human peripheral blood lymphocytes (PBL) and mouse myeloma cells was selected [10]. A clone producing anti-B antibody was obtained. Following determination of the sequence coding the variable region of the specific antibody, a comparative analysis was conducted with those anti-ABH antibodies previously reported.

2. Materials and methods

2.1. Cell fusion

Peripheral blood (100 ml) was extracted from a healthy volunteer of type A. Mononuclear cells were isolated by centrifugation on Ficoll-Paque Plus (Amersham Pharmacia Biotech); isolated cells were washed twice with phosphate-buffered saline (PBS). Mouse myeloma cells, P3U1, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Morgate) at 37 °C in a saturated atmosphere of 5% CO_2. Following harvest, myeloma cells were fused with human mononuclear cells of $1.2–3.8 \times 10^8$ at a ratio of 1:10 in a warmed mixture of 1 ml of PEG-1000 (Wako), 1 ml of FBS- free RPMI 1640 and 50 μl of dimethylsulfoxide. After a 2-min incubation and centrifugation, the pellet was resuspended in 100 ml of HAT selective medium (Gibco BRL) supplemented with 10% FBS in RPMI 1640 medium. To exclude fibroblast cells, the cell suspension was placed on 75-cm^2 culture dishes overnight. The fused cells were gently collected from the dishes, and subsequently transferred into 96-well culture plates in a volume of 200 μl (2×10^5 human cells per well). Following a 1-week incubation, half of the supernatant was discarded from each well and an identical volume of HT medium was added. After 4 weeks, the supernatants were screened by flow cytometry analysis with red blood cells (RBCs) and enzyme-linked immunosorbent assay (ELISA).

2.2. Flow cytometry analysis

RBCs of types A, B and O were prepared using peripheral blood extracted from healthy volunteers. The culture supernatant was incubated with 5 μl of the 3% RBC suspensions for 1 h. After washing twice with PBS, the RBCs were incubated with a 100-fold dilution of FITC-conjugated goat anti-human IgM (μ) (Kirkegaard & Perry Laboratories) for 1 h. The reactions were performed at room temperature. Following additional washing and fixation with 0.25% para-formaldehyde in PBS, cells were analyzed with a FACS Calibur (Becton Dickinson) flow cytometry device.

2.3. ELISA

A-and B-tri-oligosaccharides conjugated to human serum albumin (IsoSep AB) were independently coated onto microtiter plates (Sumitomo Bakelite) at 1 μg/ml for 1 h. After blocking with 0.3% gelatin, the plates were incubated with the culture supernatant for 1 h. Following washing with 0.05% Tween 20 in PBS, the plates were incubated with rabbit antibodies to human IgM, IgG, λ light chain and κ light chain (Dako) for 1 h, followed by peroxidase-conjugated goat $F(ab')_2$ to rabbit IgG (Medical & Biological Laboratories) for 1 h. Following subsequent washing, the enzymatic reaction was conducted with a substrate solution consisting of 0.55 mg/ml 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 0.03% H_2O_2. The optical density was measured at 405 nm with an auto-plate reader. These reactions were performed with a volume of 50 μl at room temperature.

2.4. Isolation of the variable regions of immunoglobulin cDNA

Total RNA was prepared from the heterohybridoma cells employing a RNeasy mini kit (Qiagen). The initial strand cDNA was synthesized by AVM-reverse transcriptase (Takara) via priming nine random oligomers and oligo-dT. In order to amplify Fab portions, PCR amplification was effected with oligonucleotide mixtures

of V_L+C_L for κ and λ chains and of V_H+C_{H1} for μ and γ chains. Primers were designed according to those reported by Takekoshi et al. with minor modifications [11]. The reaction progressed with HotStar *Taq* polymerase (Qiagen). Reaction conditions were as follows: the initial denaturation step occurred at 95 °C for 15 min, followed by 35 rounds of a temperature cycle at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. Following electrophoresis on 1% agarose gels, PCR products were purified from the excised pieces utilizing a spin column (Gibco BRL). DNA fragments were subcloned into a plasmid vector, p123T (Mo Bi Tec); subsequently, DNA fragments were labeled with the BigDye terminator cycle sequencing kit and analyzed with the ABI prism 310 auto-sequencer (Perkin–Elmer).

2.5. Sequence analysis

Nucleotide sequences of anti-ABH antibodies, retrieved from the DDBJ/EMBL/GenBank database, include the following accession numbers: ABO.B1-VH = Z23026; ABO.B1-VL = Z23027, ABO.HI1-VH = Z23028; ABO.HI1-VL = Z23029; HuA-VH = L41175; HuA-VL = L41174. Nucleotide sequences of A-ϕAb and B-ϕAb were provided by Dr Curiel-Quesada [12]. The sequence of FB5.7 was provided by Dr Siegel [13]. To characterize the nucleotide sequence of the positive clones, a database search was conducted of the IMGT (http://imgt.cines.fr:8104/home.html) [14], V-BASE (http://www.mrc-cpe.cam.ac.uk/) and DDBJ/EMBL/GenBank (http://www.ddbj.nig.ac.jp/) databases. Multiple alignments were effected employing the CLUSTAL W program, ver. 1.60 [15]. Results were illustrated with MACBOXSHADE, ver. 2.11. The amino acid identity (%), designated to be the identical amino acid number by the number of total regions except for gaps, was calculated using the MACBOXSHADE program.

3. Results

3.1. Cloning of human anti-B mAb

In order to obtain human antibodies to ABH antigens mAb, heterohybridomas between human PBL and mouse myeloma cells, P3U1, were generated according to the common procedure. Pre-culture in the dishes was effective with respect to removal of excessive fibroblasts. Hybridoma colonies were observed in 16–38% of the culture plates 4 weeks later. This observation indicated that the lymphocytes were successfully fused at a frequency of approximately 4×10^{-7}. Flow cytometry analysis and ELISA were conducted to clone the hybridoma cells and to evaluate the antibodies to ABH antigens. Hemagglutination was not readily apparent even in the presence of the Coombs reagent. As displayed in Fig. 1, the presence of specific antibodies was evident based on flow cytometry using RBCs. When the supernatant from all wells containing colonies was examined by flow cytometry analysis, three positive clones were obtained, which secreted antibodies reactive with B-RBCs. After a single step of limiting dilution, one cloned hybridoma cell line was obtained, which was designated BT97. The BT97 cells maintained antibody production for approximately 10 weeks after fusion. To ensure specificity and to determine the subclass, ELISA was conducted employing soluble antigen coated plates (Fig. 2). BT97 mAb selectively reacted with the B-antigen as well. In addition, ELISA with subclass-specific second antibodies demonstrated that the BT97 mAb was comprised of Hμ and Lλ chains; moreover, the whole serum of the donor also contained anti-B antibodies composed of Hμ and Lλ chains.

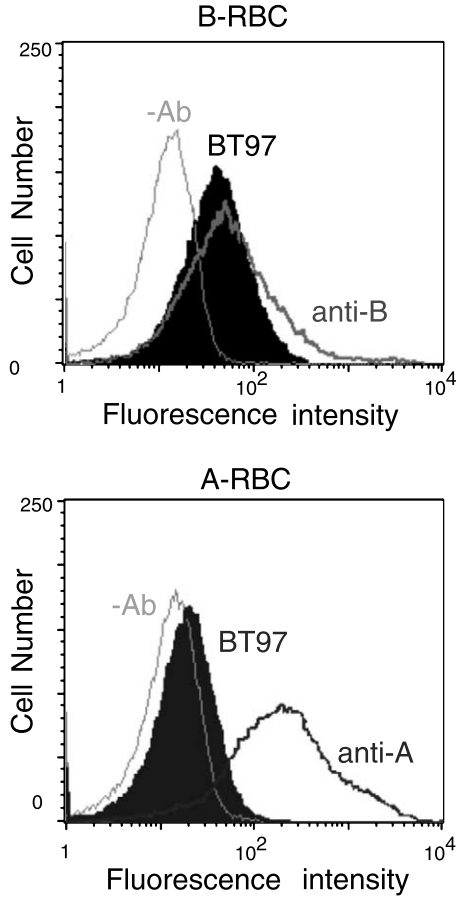


Fig. 1. Flow cytometry analysis using B-RBC (upper) and A-RBC (lower) to detect positive hybridoma clones. The shadowed area represents the peaks stained with the supernatant of BT97. Bold line represents the peaks stained with human anti-A antiserum to B-RBC and anti-B to A-RBC as a control. Thin line depicts the peaks stained in the absence of the first antibody as a negative control.

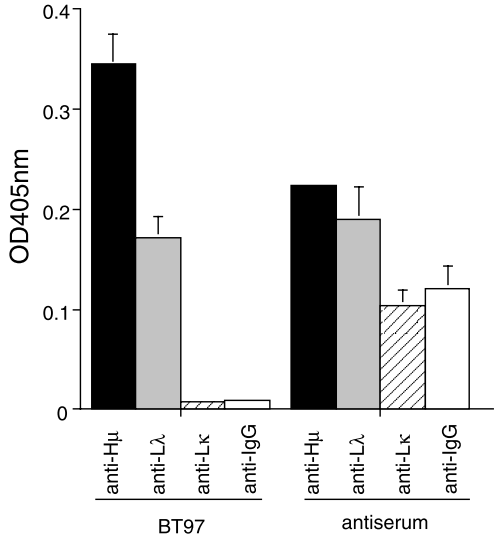


Fig. 2. Recognition of A and B antigens by the antibody of the BT97 clone supernatant and the donor antiserum. The microtiter plates were coated with B-tri-oligosaccharides. The O.D. value is expressed as the mean ± SD.

3.2. Sequence analysis of BT97 mAb

To deduce amino acid sequence of variable regions, reverse transcription-polymerase chain reaction (RT-PCR) was performed on the RNA extract from the BT97 cells during production of the antibody. PCR products were subcloned into a vector, followed by complete sequencing from both strands. The nucleotide sequence of H and L-chain variable regions of the BT97 mAb are deposited in DDBJ/EMBL/GenBank data bank (accession numbers AB087877 and AB087878, respectively). The sequence of BT97 mAb was comparatively analyzed against several human mAbs to ABH antigens that have been reported. B-ϕAb [12], ABO.B1 [16] and FB5.7 [13] are anti-B antibodies, A-ϕAb [12] and HuA [17] are anti-A antibodies, and ABO.HI1 [16] is an anti-HI antibody. Five of the six reported anti-ABH antibodies were obtained employing the phage-display method. The remaining HuA was produced by cloned EBV-transformed lymphocytes that were stabilized by fusion with a human–mouse hybridoma [18].

In a fashion similar to BT97 mAb, five of the six anti-ABH antibodies, B-ϕAb, ABO.B1, A-ϕAb, HuA and ABO.HI1, were comprised of Hμ chains; the remaining FB5.7 mAb demonstrated an Hγ chain. The VH genes of BT97, B-ϕAb, ABO.B1, HuA, and ABO.HI1, belong to IGHV3 in the IMGT classification, whereas the other two genes of FB5.7 and A-ϕAb belong to IGHV4. In contrast, the VL and JL genes of these species exhibited a variety of the IGLV and IGLJ groups.

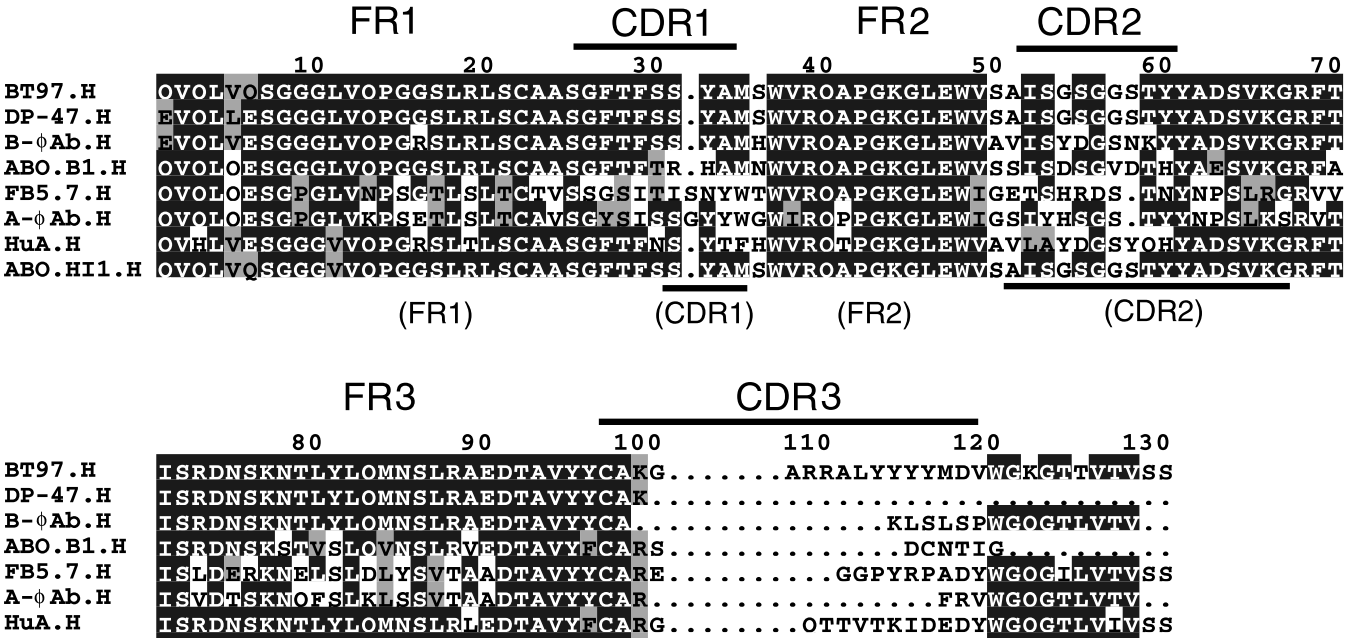
Based on their homology, it was evident that the VH and VL genes of BT97 were derived from the germline DP-47 gene [19] corresponding to the IGHV3 subgroup in the IMGT database, and the germline 3p.81A4 [20] corresponding to the IGLV3-10 subgroup, respectively. ABO.B1 and ABO.HI1 were also derived from the DP-47 gene. B-ϕAb and HuA were derived from the DP-46 gene. Additionally, FB5.7 and A-ϕAb were derived from the DP-70 and DP-67 genes, respectively. Fig. 3 shows the multiple alignment along the VH and VL regions among the eight anti-ABH mAbs including BT97 and its germlines. It was clear that the VH genes revealed high homology, with the exception of the third complimentarity-determining region (CDR3). In contrast, the VL genes displayed less homology than that of the VH genes. Employing this multiple alignment, the amino acid sequence identity (%) of the CDR and the framework (FR) regions in the VH and VL genes of BT97 to the germline and the six antibodies was calculated (Table 1). Germline sequences (DP-47 and 3p.81A4) did not completely include the CDR3 region and its downstream regions; thus, the estimation of the identity of the CDR3 region was relatively low. The VH gene of BT97 and DP-47 are identical, with the exception of a three-nucleotide difference in the region of the 5′ primer used in PCR. Thus, the differences between the VH gene of BT97 and DP-47 in the FR1 region had potentially been introduced by mismatches during the PCR priming cycles. The VL gene of BT97 differs from 3p.81A4 by seven nucleotides, which are accompanied by two amino acid substitutions in the CDR3 region. Except for these differences, the VH and VL genes of BT97 exhibited no other substitution in their nucleotide sequences. With respect to the FR regions of the VH gene of anti-ABH antibodies, high homology was evident with 53–100% residual identities. Particularly, BT97 and ABO.HI1 possessed identical sequences in the VH gene except for the CDR3 region downward. On the other hand, FR regions of the VL gene displayed identities of 35–78%.

4. Discussion

We obtained a human mAb of BT97 that reacted with B-antigen of the ABO blood group. Heterohybridoma formation in this study is characterized by methodological limitations, including low fusion rate and unstable antibody production. Large volumes of the antibody-containing supernatant were not obtained; however, it was sufficiently effective to clone the cells and to prepare cDNA coding the specific antibody. Although this protocol is in need of improvement, the advantage that the combination of the obtained H and L-chains is original indicates that this approach is suitable for the analysis of the sequence of immunoglobulins.

As screening procedures, hemagglutination and ELISA are common in preparation of anti-RBC antibodies [12,13,16,17]. In this series of experiments, hemagglutination was rarely observed with the culture

Heavy chain



Light chain

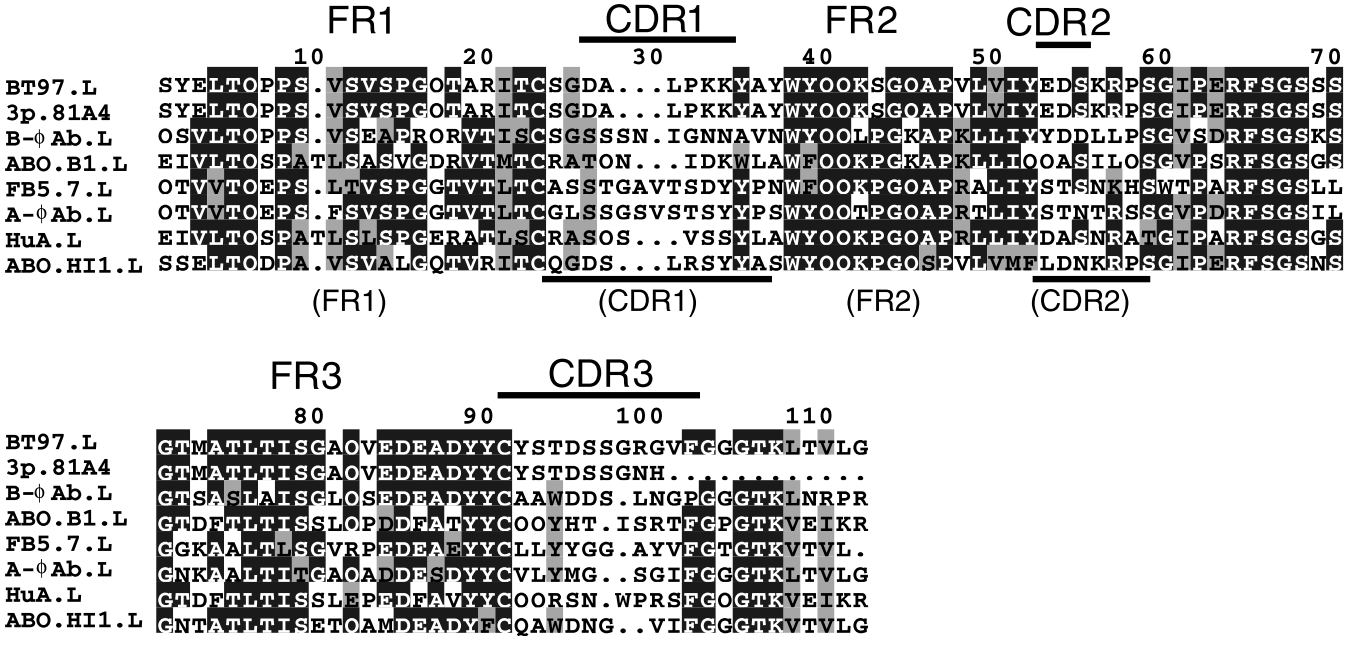


Fig. 3. The multiple alignment of the amino acid sequence of the H and L-chains. Amino acids are designated by single-letter code. Amino acids identical to BT97 are shown on a black background; amino acids similar to BT97 are highlighted with a gray background. The CDR and FR regions defined by the IMGT database are indicated over the top; those defined by the V-BASE are in parenthesis at the bottom.

Table 1
Amino acid identity (%) of VH and VL regions compared to BT97 mAb

VH region								VL region							
Antibody	Total length	FR1	CDR1	FR2	CDR2	FR3	CDR3	Total length	FR1	CDR1	FR2	CDR2	FR3	CDR3	Reference
Germ line															
DP-47	97	88	100	100	100	100	13								[19]
3p.81A4								98	100	100	100	100	100	64	[20]
Anti-B															
B-ϕAb	89	88	100	82	38	100	13	57	56	0	59	33	69	18	[12]
ABO.B1	79	92	63	88	63	76	7	46	35	14	53	33	61	18	[16]
FB5.7	52	60	0	71	25	53	19	50	44	11	59	33	56	18	[13]
Anti-A															
A-ϕAb	57	60	44	59	50	58	13	56	48	11	59	0	64	27	[12]
HuA	67	80	75	71	13	92	20	51	39	14	71	33	64	18	[17]
Anti-HI															
ABO.HI1	87	96	100	100	100	100	23	66	72	33	71	33	78	18	[16]

supernatant, which may be attributable to a low concentration of the antibody. In addition, ELISA, utilizing plates coated by the artificially synthesized soluble antigens, was not able to determine whether the obtained values precisely reflected the actual interaction of the antibody to RBCs. For these reasons, analysis by flow cytometry has been established in our trial. Flow cytometry employing RBCs was more sensitive and demonstrative than was hemagglutination.

The nucleotide sequence of the variable regions of the BT97 mAb was determined by RT-PCR; moreover, the H and L-chains were derived from the DP-47 and 3p.81A4 germline genes, respectively. Both the VH and VL genes shared high amino acid identities of 97 and 98%, respectively, with their corresponding germline genes. These genes appeared to contain a minimum number of somatic mutational events, suggesting that a germline gene such as DP-47 might originally have an affinity to ABH antigens. It has been repeatedly suggested that fetal antibodies and auto-antibodies are dominated by rarely mutated or unmutated germline VH genes and that these antibodies are often polyreactive [21–24]. We think that the antibodies to ABH-antigens appear to be naturally occurring in the repertoire of germline genes.

In an attempt to evaluate the amino acid sequence and its organization of the BT97 mAb, a comparative analysis was performed with the six reported anti-ABH antibodies. Among these anti-ABH antibodies, the amino acid sequence of the H-chains exhibited greater homology than that displayed by the L-chains. The human mAbs of B-ϕAb, FB5.7, A-ϕAb and HuA were obtained from immune disease patients. ABO.B1 and ABO.HI1 were acquired from a normal human; however, Siegel et al. notes that the donor for ABO.B1 was immunized [25]. Therefore, only two mAbs of BT97 and ABO.HI1 are naturally occurring mAbs. Of particular interest, the VH region of BT97 showed the highest homology with ABO.HI1, with the exception of the CDR3 region. As for natural anti-ABH antibodies, the reactive species are possibly derived from the same germline genes. Furthermore, since BT97 is anti-B and ABO.HI1 is anti-HI, the differences in the CDR3 of the VH region or the VL region may distinguish the antigen structures. Anti-B mAb of B-ϕAb also exhibited high homology to BT97 with the complete identities in the CDR1 and FR3 regions of the VH gene. In contrast, FB5.7 revealed the lowest homology in the VH region with BT97, particularly of the CDRs, which should be caused by the step of affinity maturation.

In conclusion, we obtained human anti-B mAb, BT97, via human–mouse heterohybridoma formation. Comparative sequence analysis of variable regions was performed with previously reported species. Strong homology, particularly in CDRs of the H chain, was revealed. The variety of antibody repertoire indicates the

general occurrence of natural antibodies in the ABO blood group, whereas the ternary structure of the specific antibodies should be constrained for the carbohydrates of antigen. In the future, further accumulation of data regarding the sequence of specific antibody repertoire will be necessary in order to draw a conclusion. Experiments involving targeted mutagenesis are also required to identify the antigen-reactive site on antibodies.

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