In vitro immunization can elicit the expansion of diverse repertoire of B cells from peripheral blood mononuclear cells

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Abstract We previously developed an in vitro immunization (IVI) protocol of human peripheral blood mononuclear cells (PBMC) for generating antigen-specific human antibodies. In order to clarify whether IVI protocol induces antigen-specific B cell responses in PBMC, we analyzed family gene usage and sequence of the variable region gene of immunoglobulin heavy chain (VH gene) of the antibody produced from the in vitro immunized PBMC. Sequence homology analyses of VH gene demonstrated that a larger repertoire of B cells can be sensitized with mite-extract than with cholera toxin B subunit and rice allergen. Further, antigen-specific B cells were efficiently expanded by using CpG oligodeoxynucleotide as adjuvant. These results suggest that appropriate combination of sensitizing antigen

and adjuvant is primarily important for expansion of antigen-specific B cells in IVI protocol.

Keywords CpG oligonucleotide · Human monoclonal antibody · In vitro immunization · Peripheral blood mononuclear cells · Variable region gene

Abbreviations

CTB	cholera toxin B subunit
FBS	fetal bovine serum
IL	interleukin
IVI	in vitro immunization
LLME	L-leucyl-L-leucine methyl ester
mAb	monoclonal antibodies
MDP	muramyl dipeptide
ME	mite extract
PBMC	peripheral mononuclear cells
RA	rice allergen

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Introduction

Monoclonal antibodies are suitable reagents for the diagnoses and treatments of cancer, allergy, infection, and other diseases. The monoclonal antibodies from laboratory animals are relatively easy to produce, but their therapeutic efficacy is restricted by their antigenicity (Co and Queen 1991). Although human monoclonal antibodies are the most ideal for clinical use,

methods for their generation have not been established (James and Bell 1987). Therefore, we developed an in vitro immunization (IVI) protocol for human peripheral blood mononuclear cells (PBMC) to generate antigen-specific human antibodies (Ichikawa et al. 1999). In this protocol, PBMC are primarily treated with L-leucyl-L-leucine methyl ester (LLME) (Borrebaeck et al. 1987; Thiele and Lipsky 1986) and then sensitized with antigen in eRDF medium supplemented with IL-2, IL-4, and muramyl dipeptide (MDP) (Bahr et al. 1984). Until now, we have succeeded in inducing antigen-specific B cells against cholera toxin B subunit (CTB) and rice allergen (RA) by using the IVI protocol (Shim et al. 2001; Xu et al. 2004). However, we need to clarify conditions that enable the efficient acquisition of antigen specific B cells with a high affinity to broaden the usefulness of IVI protocol. In this study, we revealed that a larger repertoire of antigen specific B cells can be expanded by the optimized combination of sensitizing antigen and adjuvant in IVI protocol.

Materials and methods

Antigen and reagents

RA was prepared according to the method described by Dr. T. Matsuda (Matsuda et al. 1988, 1991). CTB was purchased from List Biological Laboratories (Campbell, CA, USA). Mite-extract (ME) was purchased from LSL (Tokyo, Japan). Recombinant human Interleukin 2 (IL-2) was purchased from Genzyme (Cambridge, MA, USA). Recombinant human IL-4 was purchased from Pepro Tech (London, UK). MDP was purchased from Chemicon (Temecula, CA, USA). LLME was obtained from BACHEM (Torrance, CA, USA).

Isolation of human lymphocytes

PBMC from one healthy donor were separated by density-gradient centrifugation using lymphocyte separation medium (LSM; Organon Teknika, Durham, NC, USA), and were used throughout this experiment. Experimental protocol was described in elsewhere (Yamashita et al. 2002).

Experiments throughout this study were carried out in accordance with the principle of the Declaration of Helsinki.

In vitro immunization

PBMC were firstly treated with 0.25 mM LLME for 20 min at room temperature. After washed with eRDF medium (Invitrogen, Carlsbad, CA), the cells were sensitized with antigen ($10 \mu\text{g ml}^{-1}$) in the presence of IL-2 (1 unit ml^{-1}), IL-4 (1 ng ml^{-1}), MDP ($10 \mu\text{g ml}^{-1}$), and cultured in eRDF medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated fetal bovine serum.

Enzyme-linked immunosorbent assay (ELISA)

Total amounts of immunoglobulin (IgM and IgG) secreted into the culture supernatant were determined by ELISA using anti-immunoglobulin antibody (anti-human IgM antibody; anti-human IgG antibody, TAGO, Burlingame, CA, USA) as described previously (Yamashita et al. 2002).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared from the in vitro immunized PBMC by using Total RNA Extraction Kit (Sigma, St. Louis, MO, USA). Total RNA ($1 \mu\text{g}$) was used as a template for cDNA synthesis reaction using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and primers that hybridize to either the IgM or IgG constant regions of the heavy chain (CHm or CHg in Table 1). The variable region gene of immunoglobulin heavy chain (VH gene) was amplified by *Taq* DNA polymerase (Promega) using appropriate family-specific primer (TVH-1–TVH-6) and CH region primer (TCm or TCg) (Table 1) (Marks et al. 1991; Wang and Stollar 2000). Amplification was done through 28 PCR cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 1 min). A linear range of band intensity of amplified fragment vs. the number of PCR cycle was determined to estimate the appropriate number of PCR cycle to be used in the estimation of the usage of antibody gene family.

Table 1 Primers for amplification of antibody gene

Description	Name	Nucleotide sequence
cDNA synthesis	Constant regions	CHm CHg
		5'-TGGAAGAGGCACGTTCTTTTCTTT-3' 5'-TGCTGGGCTTGTGATT-3'
PCR	VH regions (Forward)	TVH-1 TVH-2 TVH-3 TVH-4 TVH-5 TVH-6
		5'-CAGGT(G/C)CAGCTGGT(G/A)CAGTC-3' 5'-CAG(G/A)TCACCTTGAAGGAGTC-3' 5'-(G/)AGGTGCAGCTGGTGGAGTC-3' 5'-CAGGTGCAGCTGCTGGAGTG-3' 5'-GA(G/A)GTGCAGCTGGTGCAGTC-3' 5'-CAGGTACAGCTGCAGCAGTC-3'
	CH regions (Reverse)	TCm TCg
		5'-GAAAGGGTTGGGGCGGATGC-3' 5'-GAC(G/C)GATGGGCCCTTGGTGA-3'

Sequence analysis

The amplified DNA fragments were sequenced with an automated DNA sequencer ABI Prism 3100 Avant (Applied Biosystems, Foster City, CA, USA) using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). The sequence homology of the VH region gene was analyzed by using Genetyx software (Genetyx Corp., Tokyo, Japan). The statistical difference was determined by two-sided student's *t*-test. The differences were considered to be significant if $P < 0.05$.

ELISPOT analysis

After the *in vitro* immunized PBMC were cultured in appropriate conditions for 6 days, cells were transferred to Multiscreen HA plate (Millipore, Bedford, MA, USA) coated with fish gelatin (FG) or ME. After the plates were washed with 2.24×10^{-2} M phosphate buffer containing 1.37×10^{-1} M NaCl and 0.05% Tween-20 (TPBS), the *in vitro* immunized cells (1×10^5 cells) were seeded and cultured in the eRDF medium supplemented with 10% FBS, IL-2 (1 unit m^{-1}), IL-4 (1 ng ml^{-1}) for 20 h. After washing the plates, we added diluted horseradish peroxidase-conjugated goat anti-human IgM antibody and subsequently incubated for 2 h at 37°C. After washing the plates, we added TrueBlue peroxidase substrate (KPL, Gaithersburg, MD, USA) and incubated for 20 min. After washing the plates, we measured the total spots area by using ImageJ image analyzing software.

Fig. 1 *In vitro* immunization augmented antibody production from PBMC. LLME-treated PBMC were cultured in eRDF medium supplemented with 10% FBS (Ctrl); eRDF medium supplemented with 10% FBS, IL-2, IL-4, and MDP (no Ag); eRDF medium supplemented with 10% FBS, IL-2, IL-4, MDP, and CTB (CTB) or ME (ME) or RA (RA). After 8 days of culture, the amount of IgM and IgG in the supernatant was determined by sandwich ELISA. The statistical difference was determined by two-sided student's *t*-test. The differences were considered to be significant, if $P < 0.05$

Results and discussion

ME is competent for augmenting antibody production by the IVI protocol

We tested for the abilities of three antigens such as CTB, ME and RA to induce antibody production in IVI protocol. After the LLME-treated PBMC were sensitized with these antigens, the total amounts of IgM and IgG

produced from the in vitro immunized PBMC were determined by ELISA. As shown in Fig. 1, antibody production was augmented even in the absence of antigen, thus suggesting that culture conditions, including the presence of IL-2, IL-4, and MDP, stimulate the proliferation of B cells in an antigen-non-specific manner. Further, ME among these antigens strongly stimulated the antibody production, thereby suggesting that ME can efficiently sensitize naïve B

Fig. 2 Usage of the VH family gene in the in vitro immunized PBMC. After eight days of culture, the expressions of the VH family gene in the in vitro immunized PBMC were evaluated by RT-PCR using family-specific primers. IgMc and IgGc indicate the constant region gene of IgM and IgG, respectively (the molecular weight marker (100 bp ladder marker from

Sigma-Aldrich, Tokyo, Japan) was loaded in the most right line) (a). The relative expression level of each family gene (VH1–VH6) was calculated by dividing the intensity of each band by that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (b, IgM variable region; c, IgG variable region). Experimental procedures are the same as Figure 1

cells and expand antigen-specific B cells by the IVI protocol.

The family gene usage of the antibody produced from the in vitro immunized PBMC

Next, we analyzed the *VH* gene family usage of the antibody produced from the in vitro immunized PBMC. After amplifying the *VH* gene by using family-specific primer and CH region primer (Table 1) at the semi-quantitative condition, we investigated the relative expression level of each *VH* family member through calculating the expression level relative to the DNA size marker by using Image J (Fig. 2). The results revealed that the culture conditions, including IL-2, IL-4, and MDP, stimulated *VH* gene expression, and the addition of sensitizing antigens, including CTB, ME, and RA, further augmented *VH* gene expression by 1.4–1.8-fold. Further, we observed a similar usage

of each *VH* family gene in PBMC sensitized with different antigens. These results indicate that sensitizing antigens stimulated B cells to produce immunoglobulins by the IVI protocol through the biased usage of *VH* family gene such as *VH1*, *VH3*, *VH4*, and *VH5*. Next, we determined the sequence of randomly selected 7–10 genes from each *VH* gene corresponding to *VH1*, *VH3*, *VH4*, and *VH5* family that is derived from PBMC immunized with respective antigens.

Homology analysis of *VH* region gene of antibody produced from the in vitro immunized PBMC

Seven to ten amplified fragments against each *VH* family were sequenced in each sensitized antigen, and their homologies were analyzed in the round robin format. Although we performed homology analysis of *VH* gene for *VH1*, *VH3*, *VH4*, and *VH5*,

Fig. 3 Homology analysis of *VH* genes amplified from the in vitro immunized-PBMC. Seven to ten *VH3* (a), CDR1 (b), CDR2 (c), and CDR3 (d) genes amplified from the in vitro immunized PBMC were sequenced and their homologies were analyzed in the round-robin format by using the

Genetyx software. The dots indicate their homology (%) and the horizontal bar indicates the average values. The statistical difference was determined by two-sided student's *t*-test. The differences were considered to be significant (*), if $P < 0.05$

Fig. 4 ELISPOT analysis. LLME-treated PBMC were cultured in eRDF medium supplemented with 10% FBS (Ctrl); eRDF medium supplemented with 10% FBS, IL-2, IL-4, and MDP (no Ag); eRDF medium supplemented with 10% FBS, IL-2, IL-4, ME and MDP (ME + MDP) or CpG ODN 2006 (ME + CpG). After 6 days of culture, PBMC were seeded into Multiscreen HA plates coated with fish gelatin (FG) or ME, and antigen specific B cells were detected by TrueBlue staining (a). The total spot area was measured by ImageJ software (b). The statistical difference was determined by two-sided student's *t*-test. The differences were considered to be significant (*), if $P < 0.05$

significant differences in sequence homology could not be detected in VH1, VH4, and VH5 in all sensitized antigens. Thus, we focused on the changes in sequence homology observed in VH3 family depending upon the culture conditions and sensitizing antigens. The results revealed that although the sequence homologies of the VH3 gene that was amplified from PBMC sensitized with CTB or RA were low as compared to those from PBMC cultured in a medium supplemented with IL-2, IL-4, and MDP in the absence of an antigen (no Ag group), no significant differences existed between them (Fig. 3a). Further, we detected a significant difference in sequence homologies in the VH3 gene

between PBMC sensitized with ME and the no Ag group. Next, we focused on the complementarity determining region (CDR) of the VH3 gene and analyzed their homologies. The results revealed that no significant differences existed in the sequence homologies in CDR1 and CDR2 between PBMC sensitized with antigens and the no Ag group (Fig. 3b, c). However, we could detect a significant difference in the sequence homology in CDR3 between PBMC sensitized with ME and the no Ag group, but not between PBMC sensitized with CTB or RA and the no Ag group (Fig. 3d). All these results suggest that ME can stimulate a larger repertoire of B cells among these sensitizing antigens, and that antigen-specific B cells with varied specificities might be effectively established by using ME as sensitizing antigen in the IVI protocol.

Antigen-specific B cells were effectively expanded through the combined use of ME as sensitizing antigen and CpG oligodeoxynucleotide as adjuvant in IVI protocol

Finally, we performed an ELISPOT analysis to confirm whether ME-specific B cells can be established by using ME as sensitizing antigen in IVI protocol. However, we could not detect significant differences in the total spot area between PBMC sensitized with ME (ME + MDP) and the no Ag group (Fig. 4). These results suggest that although ME can stimulate a larger repertoire of B cells as mentioned above, ME-specific B cells can not be expanded and maintained by this IVI protocol. Thus, we used another strong adjuvant, CpG phosphorothioate oligodeoxynucleotide (ODN) 2006 (Hartmann and Krieg 2000), instead of MDP to induce the expansion of a larger repertoire and/or antigen-specific B cells. As shown in Fig. 4, expansion of ME-specific B cells was strongly augmented by using this CpG ODN 2006 in the IVI protocol. All these results demonstrate that antigen-specific B cells can be effectively expanded by the combined use of ME as sensitizing antigen and CpG ODN 2006 as adjuvant in the IVI protocol. By optimizing the IVI protocol using a suitable combination of individual PBMC and antigen (Yamashita et al. 2002), and by improving the IVI protocol using stronger adjuvant, antigen-specific B cells with a high affinity are expected to be induced and expanded. Further investigation to clarify the

relationship between repertoire of B cells and expansion of antigen-specific B cells is desirable.

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