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Analysis of cellular phenotype during *in vitro* immunization of murine splenocytes for generating antigen-specific immunoglobulin

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Although various *in vitro* immunization methods to generate antigen-specific antibodies have been described, a highly effective method that can generate high-affinity immunoglobulins has not yet been reported. Herein, we analyzed a cellular phenotype during *in vitro* immunization of murine splenocytes for generating antigen-specific immunoglobulins. We identified a combination of T cell-dependent stimuli (IL-4, IL-5, anti-CD38 and anti-CD40 antibodies) plus lipopolysaccharides (LPS) that stimulates antigen-exposed splenocytes *in vitro*, followed by induction of the cells phenotypically equivalent to germinal center B cells. We also observed that LPS induced high expression levels of mRNA for activation-induced cytidine deaminase. We stimulated antigen-exposed splenocytes, followed by the accumulation of mutations in immunoglobulin genes. From the immunized splenocytes, hybridoma clones secreting antigen-specific immunoglobulins were obtained.

[**Key words:** *In vitro* immunization; Activation-induced cytidine deaminase; Germinal center; Somatic hypermutation; T cell dependent stimuli]

Monoclonal antibodies are now commonly used for the detection and quantification of antigens as well as for the detection and isolation of particular cell types, such as cancer cells. Recently, they have been used as therapeutics for various diseases such as rheumatoid arthritis (1) and cancer (2). The so-called antibody drugs are expected to have maximal therapeutic effect with minimal side effects.

Monoclonal antibodies are typically produced by the immunization of animals with a target antigen, followed by hybridoma cell culture. However, this method has various problems. It takes a long time (3–6 months) and is expensive because over 1 mg of antigen is usually required. It is difficult to prepare sufficient quantity of antigen, especially in the case of membrane proteins. Recent advances allow us to use *in vitro* selection methods such as phage display (3), ribosome display (4) and cell surface display (3,5–7) to select the desired antibody fragments. These display methods have the advantage that we could handle large libraries of antibody fragments. However, these methods still take a few weeks to carry out because it is necessary to repeat the selection step.

Therefore, a novel approach in immunotechnology for producing monoclonal antibodies, *in vitro* immunization, was proposed. B lymphocytes are exposed to antigens *in vitro* and are further stimulated by thymocyte-derived lymphokines. Usually, these thymocyte-derived lymphokines are produced by young thymocytes (8) or by mixed lymphocyte cultures (9) that have been used as cell culture supplements. Also a co-culture system using an antigen-activated T helper cell clone has been used (10). However, they are poorly reproducible methods due to lot-to-lot variation of thymocyte-derived lymphokines and clonal variation of antigen-activated T helper cells. Other supportive media including mitogens (11), adjuvant peptides (12) and a combination of cytokines and mitogens (13,14) have been used. However, these studies did not analyze whether such stimuli could trigger various immune responses such as somatic hypermutation (SHM).

The germinal center (GC) was recognized as an important region in the B-cell humoral immune response. The GC consists of activated B cells that exhibit rapid proliferation and mutation through SHM (15), and class switch recombination (CSR) (16). A key factor for SHM and CSR is activation-induced cytidine deaminase (AID), whose expression is induced by IL-4 and IL-5 in CD38-stimulated B lymphocytes (17). According to these findings, we thought that artificial induction of SHM and antibody affinity maturation would be possible by *in vitro* immunization. In this paper, we have carried out a systematic comparison of various stimuli to attain AID induction, induction of GC-like cells and accumulation of mutations in antibody genes, during *in vitro* immunization of murine splenocytes. As a result, we successfully obtained high-affinity immunoglobulins specific for hen egg lysozyme (HEL).

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MATERIALS AND METHODS

Preparation of murine splenocytes A cell suspension of splenocytes was prepared from 4–6-week-old female BALB/c mice. The splenocytes were dispersed through a sterilized nylon mesh (70 μm; Becton Dickinson & Co., Franklin Lakes, NJ, USA) into a single-cell suspension in phosphate buffered saline (PBS). Following lysis of the red blood cells with ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4), the remaining splenocytes were washed and re-suspended in warm (37°C) RPMI1640 containing 25 mM HEPES (Sigma–Aldrich, St Louis, MO, USA).

In *vitro* immunization of murine splenocytes For *in vitro* immunization, splenocytes were re-suspended in RPMI1640 medium without FBS (1×10^7 cells in 1 mL). The splenocytes were placed in a 15 mL tube and exposed to 1 μM HEL (Sigma–Aldrich) and 50 μg/mL *N*-acetylmuramyl-L-alanyl-D-isoglutamine hydrate (also known as muramyl dipeptide; Sigma–Aldrich) for 15 min at room temperature (RT). The following stimuli were added to antigen-activated splenocytes in various combinations: 50 ng/mL of IL-4 (Sigma–Aldrich), 50 ng/mL of IL-5 (Sigma–Aldrich), 200 μg/mL of LPS (*Escherichia coli* 0111:B4; Sigma–Aldrich), 5 μg/mL of anti-CD38 antibody (αCD38, NIMR-5; Southern Biotechnology Associates, Birmingham, AL, USA), 5 μg/mL of anti-CD40 antibody (αCD40, 1C10; R&D Systems, Minneapolis, MN, USA).

RPMI1640 (4 mL) supplemented with 25 mM HEPES, 2 mM L-glutamine, non-essential amino acids (Invitrogen, Carlsbad, CA, USA), 1 mM sodium pyruvate, 50 U/mL penicillin, 50 μg/mL streptomycin, 55 μM 2-mercaptoethanol and 40% FCS was added to stimulated splenocytes immediately without any washing step, and the cells were seeded into a 60-mm dish. Thus, the final concentration of stimuli is 10 ng/mL IL-4, 10 ng/mL IL-5, 40 μg/mL LPS, 1 μg/mL anti-CD38 antibody and 1 μg/mL anti-CD40 antibody. The splenocytes (1×10^7 cells/5 mL) were cultured for several days without refreshing the culture medium.

Real-time PCR analysis Total RNA was isolated from immunized cells using an Isogen kit (Nippon Gene, Tokyo, Japan) and solubilized in sterile water. Complementary DNA was synthesized from 0.5 to 2 μg of total RNA using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan) and an oligo-dT primer in a total volume of 20 μl, of which 1 μl cDNA was used for each sample in a real-time PCR assay. The primers used to amplify AID, Bcl-6 and β-actin were: AID (F), 5′-GGA GCC CGT GCT ATG ACT GT-3′; AID (R), 5′-GGC TGA GGT TAG GGT TCC ATC T-3′; Bcl-6 (F), 5′-TCA TTT GCG CCA GAA GCA-3′; Bcl-6 (R), 5′-GAC ACG CGG TATT GCA CCT T-3′; β-actin (F), 5′-CCA GTT CGC CAT GGA TGA-3′; and β-actin (R), 5′-ATG CCG GAG CCG TTG TC-3′, respectively. The reference gene, β-actin, was used to control sample variation in RNA isolation and integrity, RNA input, and reverse transcription.

Reactions using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were set up according to the manufacturer's instructions, and carried out using a 7500 Fast Real-Time PCR System (Applied Biosystems). Amplification conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 30 s. The expression level of mRNA was analyzed by ΔΔCt method. For each sample, the differences in threshold cycles between AID or Bcl-6 and β-actin genes (ΔCt) were detected, and a calibrated ΔCt value (ΔΔCt, $\Delta Ct_{AID \text{ or } Bcl\text{-}6}$, $-\Delta Ct_{\beta-actin}$) was analyzed. The expression level of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized against that of unimmunized splenocytes on day 0 to give the relative expression level value of mRNA. The mRNA expression level was calculated from at least three assays for each immunization treatment. The mean ± SEM is shown in the figures.

Flow cytometric analysis Cells were harvested at various time points and labeled using combinations of PE anti-CD45R (RA3-6B2; Miltenyi Biotec, Bergisch Gladbach, Germany), FITC anti-CD38 (NIMR-5; Beckman Coulter, Miami, FL, USA), FITC anti-CD45R (RA3-6B2; Beckman Coulter), anti-mouse Syndecan-1 (CD138; R&D Systems), PE anti-goat IgG (R&D Systems) and polyclonal FITC goat IgG (Beckman Coulter) antibodies. The cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson and Company), and the data were analyzed with FlowJo (Treestar Inc., Palo Alto, CA, USA). Total cell numbers were obtained by counting live lymphocytes after PI staining. The percentages and numbers of each cell population were calculated from at least triplicate assays for each immunization. Mean ± SEM is shown in the figures.

Cloning of V_H and V_L genes The scFv library was constructed using PCR as described previously (Pluckthun et al, 1996). Briefly, V_H and V_L were amplified from a cDNA library that was constructed from total RNA of immunized splenocytes and were assembled by PCR. The amplified fragment was digested with *Eco*RI and *Hin*dIII restriction enzymes and ligated into an *Eco*RI- and *Hin*dIII-digested plasmid (pMal-c2E; New England Biolabs Inc., Beverly, MA).

Sequence analysis of V_H and V_L The DNA sequences of V_H and V_L were determined by MegaBACE500 (GE Healthcare, Waukesha, WI). The closest germ line V_L and V_H for each clone were assigned using IgBLAST (http://www.ncbi.nlm.nih.gov/igblast/). CDRs and FRs were defined by the rules of Kabat et al (18). Because there was no sequence list corresponding to a CDR3 in the IgBLAST database, we analyzed the sequences except for CDR3.

Hybridoma formation and selection of HEL-specific immunoglobulin HEL-immunized cells were harvested after 5-day culture and then fused with SP2/0–Ag14 myeloma cells. Fusion, single step selection and cloning of hybridomas were performed using the ClonaCell-HY™ system (StemCell Technologies Inc., Vancouver, BC, Canada) following the instructions of the manufacturer. To select antigen-specific hybridomas, we performed antigen ELISA. Separated hybridomas were cultured in RPMI1640 medium containing 10% FBS and HT supplement (Invitrogen), and immunoglobulin was purified by using IgG Purification kit-G (Dojindo Molecular technologies, inc., Gaithersburg, MD, USA).

Antigen ELISA ELISAs were performed using 96-well Maxisorb™ MTPs (Thermo Scientific Inc., Bremen, Germany). Each well was coated with 1 μg HEL in 50 μl PBS overnight at 4°C. The antigen-coated wells were then blocked with Protein Free Blocking solution (Thermo Scientific Inc.) for 1 h at RT followed by three washes with PBS. The antibody-containing supernatant (150 μl) was added to the wells for 1 h at 37°C followed by three washes with PBS. The amount of antigen-bound antibody was detected using HRP-labeled anti-mouse IgG (R&D Systems, 1:2000 dilution in Protein Free Blocking solution). HRP activity was measured using 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate and the reaction was stopped by adding 100 μl of 1 M HCl. Absorbance at 450 nm was measured using a microtiter plate reader (Bio-Rad, Richmond, CA, USA).

Surface plasmon resonance (SPR) analysis Binding kinetics of immunoglobulin was determined by SPR analysis using a BIAcore™ X-100 system (GE Healthcare). Immunoglobulins were immobilized on research grade CM5 sensor chips (GE Healthcare) in 10 mM sodium acetate (pH 4.5), using the amine coupling kit supplied by the manufacturer. Unreacted moieties on the surface were blocked with ethanolamine. All measurements were carried out in HBS-EP buffer that contained 10 mM HEPES pH 7.4, 150 mM NaCl, 3.3 mM EDTA and 0.005% Surfactant P-20 (GE Healthcare). Analyses were performed at 25°C and at a flow rate of 3 μl/min. Interaction between antibody and HEL was monitored in real-time and analyzed with BIAevaluation software (GE Healthcare) to determine the kinetic parameters of interaction.

RESULTS

Real-time PCR analysis of stimulated splenocytes by T cell-dependent stimuli and LPS To achieve high-affinity antibody generation, we first planned to induce AID, a key factor for SHM, using a combination of various cytokines and agonist antibodies known as T cell-dependent stimuli. We modified previously described protocols to develop our *in vitro* immunization methods (Fig. 1A). We used various stimulants at commonly used concentrations to stimulate splenocytes or B cells (19,20). Splenocytes were immunized with HEL (1 μM) as an antigen and cultured for 4 days. The stimulated cells were harvested and their total RNAs were extracted to measure AID mRNA expression level. The expression level of AID is shown in Fig. 1B. The expression level was decreased by antigen stimulation in most conditions. However, the combination of IL-4 + anti-CD38 antibody, IL-5 + anti-CD38 antibody, IL-4 + anti-CD38 antibody + anti-CD40 antibody and IL-4 + IL-5 + anti-CD38 antibody + anti-CD40 antibody exhibited over 3-fold higher expression level than unstimulated control (Fig. 1B, closed bar). Among these conditions, the expression level was elevated by co-stimulation with antigen and IL-4 + IL-5 + anti-CD38 antibody + anti-CD40 antibody compared to the absence of antigen stimulation. Therefore, we decided to use the mixture of all components of T cell-dependent stimuli (IL-4, IL-5, anti-CD38 and anti-CD40 antibodies), named as TDS, for further study. Furthermore, B lymphocytes are also activated by T cell-independent stimuli, like LPS, through Toll-like receptors, but the effect of LPS remained to be determined. Therefore, we measured the expression level of AID after stimulation with LPS and TDS. Although LPS strongly induced AID mRNA (17-fold increase compared with unstimulated control, Fig. 1C), these induction was independent of antigen stimulation The elevated expression of AID mRNA dependent on antigen stimulation and higher expression level of AID mRNA was observed by LPS + TDS stimulation. To maximize the AID mRNA induction dependent on antigen stimulation, we chose LPS + TDS stimulation for further studies.

Time-course analysis of AID mRNA expression levels To examine the optimal period for cell culture, we measured mRNA expression levels at various time points. AID mRNA expression levels gradually increased after 2 days in culture and reached a peak