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Detection and isolation of anti-hapten antibody-secreting cells by cellular affinity matrix technology

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ABSTRACT

We developed a method to detect and isolate plasma cells that produce antigen-specific antibodies. An affinity matrix of hapten was constructed on a cell surface, and subsequent incubation allowed cells to secrete antibodies. Anti-hapten antibodies preferentially bound to the affinity matrix on the cells from which they were secreted. We showed that the combination of surface biotinylation and streptavidin which was conjugated with a high valence of hapten was suitable for sensitive detection of antibody binding. Using this protocol, anti-hapten plasma cells from immunized mouse spleen were detected and enriched by flow cytometry. This method allows for isolation of intact plasma cells according to the antibody specificity and may be useful for highly efficient and precise analysis of an antibody repertoire.

1. Introduction

The dynamics of an antibody repertoire in an immune response to T cell-dependent antigens is dependent on isotype switching, somatic hypermutation, and clonal selection in germinal centers. During these processes, B cells which produce antibodies with increased suitability for host defense are selected and differentiate into memory B cells or antibody-secreting plasma cells (PCs). Analysis of selected antibody repertoires should provide useful information about the nature of these evolved antibodies. It was commonly believed that affinity-matured and isotype-switched cells are the B cells which are usually selected (Eisen, 2014). However, heterogeneity in the memory B cell population has been suggested (Weill et al., 2013). Memory B cells with low affinity B cell receptors (BCRs) can be generated without a germinal center reaction (Takemori et al., 2014), and we found very low affinity IgM antibodies produced in secondary immune responses (Murakami et al., 2014). The precise properties and functions of these novel subsets of memory B cells and antibodies are not clear, and it is important to re-evaluate the dynamics of an antibody repertoire in the course of an immune response.

Haptens are small chemicals which induce a T cell-dependent immune response when they are conjugated to proteins. Anti-hapten antibody production has been commonly used in studies of the immune response in animal experiments. Studies examining the immune response to hapten are also important for understanding the response to allergens in humans (Erkes and Selvan, 2014). Because of the chemical simplicity of haptens, affinity measurement and repertoire analysis of anti-hapten antibodies are much easier to carry out than those examining anti-protein antibodies. Therefore, hapten-conjugated proteins have been commonly used for studying the dynamics of the T cell-dependent antibody response (Eisen, 2014).

Hapten-specific memory B cells or germinal center B cells can be detected and isolated by flow cytometry using hapten-conjugated fluorescence proteins (Nishimura et al., 2011; Kaji et al., 2013). Results of these studies may not correctly reflect the antibody repertoire because memory B cells and germinal center B cells do not secrete antibodies. In order to analyze the repertoire of antibodies produced in vivo, it is important to isolate and analyze PCs because these terminally differentiated B cells secrete large amounts of antibodies. However, it is difficult to detect antigen-specific PCs using the antigen-binding capacity of their BCRs because BCR expression on the cell surface is down-regulated (Oracki et al., 2010) during differentiation into PCs.

For detection of PCs secreting antigen-specific antibodies, the ELISPOT assay has been used (Crotty et al., 2004; Tuaillon et al., 2006). However, it is not possible to recover the cells detected with this assay. In the fluorescent foci method, antigen-specific PCs can be isolated with a micromanipulator (Clargo et al., 2014), although more convenient and scalable methods such as flow cytometry are preferred. Cells producing antigen-specific antibodies can be detected by intracellular staining (Manz et al., 1998), but the staining procedure requires cell fixation and permeabilization, and this method cannot be employed

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for the isolation of viable PCs. In addition, it is difficult to obtain cDNA of antibody genes because mRNAs are lost through permeabilization. Cellular affinity matrix technology is used to identify and sort cells by their secreting molecules without fixation and permeabilization (Manz et al., 1995). With this method, cells are first coated with the capture antibody which recognizes the molecule secreted from the cells. Then, cells are incubated to allow for protein secretion. During this period, the secreted target molecules preferentially bind to the capture antibodies on the secreting cells. Finally, these target molecules are detected by fluorescence-conjugated detection antibodies so that the secreting cells can be detected by flow cytometry. This method is used to analyze cytokine-producing cells (Brosterhus et al., 1999; Campbell, 2003). PCs have been detected and isolated using anti-IgG or IgE antibodies as capture and detection antibodies (Manz et al., 1995, 1998; Carroll and Al-Rubeai, 2005). However, antigen specificities were not considered in these studies. For a more precise analysis of antigen-specific antibodies produced by PCs, it is better to isolate antigen-specific PCs among total PCs. Recently, it was reported that anti-ovalbumin-specific PCs were detected on an affinity matrix using antigen-conjugated anti-CD138 monoclonal antibody (mAb) (Taddeo et al., 2015). We attempted to apply affinity matrix technology for the detection of anti-hapten antibody-producing PCs.

In order to isolate hapten-specific PCs, we generated a cellular affinity matrix by labeling cells with a hapten, (4-hydroxy-3-nitrophenyl)acetyl (NP). Using cultured cell lines, we showed that anti-NP antibodies can bind to NP on the cells, and that antibody-secreting cells can be detected and sorted by flow cytometry. We also showed that anti-NP antibody-producing PCs were detected and enriched from splenocytes of NP-chicken gammaglobulin (NP-CGG)-immunized mice. We concluded that this method may be useful for isolating hapten-specific PCs.

2. Materials and methods

2.1. Antibodies, cells, and reagents

Recombinant anti-NP antibodies were prepared by introducing heavy chain genes of anti-NP mAb into Igλ-producing J558L cells (Oi et al., 1983). These heavy chain genes were constructed by fusing cDNAs of the variable region from hybridoma clone B2 (Furukawa et al., 1999) and the constant regions of either mouse IgM or IgG1, and cloned into pEF1/*Myc-HisA* vector (Invitrogen). Stable transfectant clones were established by selection with G418 (Wako) and limiting dilutions. Established clones VBCM and VBCG1 produce IgM and IgG1, respectively. Recombinant antibodies, termed B2-IgM and B2-IgG1, were purified from the culture supernatants of VBCM or VBCG1 cells on a NP-bovine serum albumin (BSA) column. A mouse T cell lymphoma, EL4 (Gorer, 1950), was used as a source of cells which do not produce antibodies. Unlabeled, FITC, PE, or alkaline phosphatase-conjugated goat polyclonal antibodies to mouse Igλ, IgM, IgG, and IgG1 were purchased from SouthernBiotech. APC-conjugated anti-mouse CD138 mAb and anti-mouse B220 mAb were purchased from BioLegend. FITC-anti-mouse CD90.2 mAb was purchased from Biodesign. Magnetic beads conjugated with sheep anti-rat IgG antibody were purchased from Invitrogen Dynal. Succinimide esters of NP (NP-OSu) were prepared by reacting (4-hydroxy-3-nitrophenyl)acetic acid with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide in DMSO. NP conjugates were prepared by reacting SA (Wako), CGG (Rockland), or BSA (Sigma) with NP-OSu, and unreacted NP was removed by dialyzing against PBS. The concentration of NP was determined by measuring absorbance at 430 nm and was calculated using a molar absorption coefficient of $4.23 \times 10^3\ M^{-1}\ cm^{-1}$ at pH 8.4. The protein concentration of the NP conjugate was determined by measuring absorbance at 280 nm. After correction for the UV absorption of NP at 280 nm, concentrations were calculated using molar absorption coefficients of $1.65 \times 10^5\ M^{-1}\ cm^{-1}$ for SA.

2.2. Construction of an NP affinity matrix on the cell surface

Cells were washed twice with cold PBS before labeling. For direct labeling, cells were reacted with 0.3 mM NP-OSu on ice for 10 min, followed by washing twice with PBS containing 0.5% BSA. For biotinylation, cells were reacted with 0.2 mg/ml sulfo-NHS-LC-biotin (Thermo Scientific) on ice for 10 min. Cells were then washed two times with PBS containing 0.5% BSA, and reacted with 8 μg/ml NP-SA on ice for 10 min. In some experiments, concentrations of NP-SA were changed as indicated in the text. Binding of anti-NP antibodies to an NP affinity matrix was detected by PE-conjugated anti-Igλ antibody and analyzed with a FACScan cell analyzer and Cell Quest Pro software (BD Biosciences).

2.3. Detection of anti-NP antibody production in cell lines

After construction of the NP affinity matrix, cells were suspended with DMEM containing 10% FCS and 20 mM HEPES buffer (pH 7.2) in 1×10^6 cells/ml, and incubated at 37 °C for 30 min. During the incubation, cells were mixed every 3–5 min. Cells were then washed two times with cold PBS, and stained with PE-conjugated anti-IgM, or IgG1 antibodies. 10 μg/ml NP-BSA was added in order to block additional binding of anti-NP antibodies which were spontaneously released during the washing and staining. After washing, cells were suspended in buffer containing 7-amino-actinomycin D (7AAD) (Sigma), which was added to exclude dead cells. Stained cells were analyzed with a FACScan cell analyzer or a FACSVantage SE cell sorter (BD Biosciences). Sorted cells were further stained with anti-CD90.2 mAb and analyzed by flow cytometry.

2.4. RT-PCR

IgM^+ or $IgG1^+$ cells were sorted from a mixture of EL4 and antibody-expressing transfectant cells by the affinity matrix method or by intracellular staining. Intracellular staining was performed using fixation and permeabilization buffers (BioLegend) according to the manufacturer's instructions. 1×10^4 cells were directly collected into 500 μl of TRI REAGENT (Molecular Research Center) by the cell sorter, and total RNA was purified by chloroform extraction and isopropanol precipitation. cDNA was synthesized with a PrimeScript II 1st strand cDNA synthesis kit (TAKARA) using oligo dT primer. The genes of anti-NP antibodies as well as hypoxanthine guanine phosphoribosyl transferase (HGPRT) were amplified by PCR and analyzed by agarose gel electrophoresis. The primer sequences were GCTGGTGAAAAGGACCTCT and CACAGGAC TAGAACACCTGC for HGPRT, Vλ (ACWGCCTGCTGCTGACCAATATTG) and Cλ1 (ATGTTTCTGATCTCAGCCTCTGTG) for Igλ, V_H (CTGTATCATGCT CTTCTTGGC) and Cμ (AGCATGGTCAATAGCAGGTGCC) or Cγ1 (GATCAT TTACCAGGAGAGTGGG) for IgM or IgG1.

2.5. Detection of anti-NP antibody-producing PCs from mice

C57BL/6 mice were purchased from Japan SLC, and were bred and maintained in our animal facility under specific pathogen-free conditions. The animal experiments were approved by the Committee for Care and Use of Laboratory Animals at Kochi University. Mice were immunized with 100 μg of NP-CGG with alum. Then, 8 to 10 weeks after immunization, mice were boosted with 100 μg of NP-CGG in PBS. Seven days after the boost immunization, mice were sacrificed and spleens were taken. Single cell suspensions were prepared, and red blood cells were lyzed with Tris–ammonium chloride. After washing with 10 mM EDTA in PBS, cells were incubated with anti-B220 mAb, and $B220^+$ cells were depleted using magnetic beads conjugated with anti-rat IgG antibody. $B220^-$ cells were incubated with PE-conjugated anti-Igλ antibody to stain surface BCRs, followed by incubation with unlabeled anti-Igλ antibodies to block residual BCRs. A cellular affinity matrix was constructed by biotinylation and NP-SA, and labeled cells