Journal of Immunological Methods 427 (2015) 58–65

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

In vitro generation of rabbit anti-*Listeria monocytogenes* monoclonal antibody using single cell based RT-PCR linked cell-free expression systems

Teruyo Ojima-Kato [a,b,*], Dai Hashimura [b], Takaaki Kojima [b], Shiori Minabe [b], Hideo Nakano [b,**]

[a] Knowledge Hub Aichi, Aichi Science and Technology Foundation, 1267-1 Akiai, Yakusa cho, Toyota 470-0356, Japan
[b] Graduate School of Bioagricultural Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan

ARTICLE INFO

Article history:
Received 28 April 2015
Received in revised form 3 October 2015
Accepted 5 October 2015
Available online 8 October 2015

Keywords:
Rabbit monoclonal antibody
Cell-free protein synthesis
Listeria monocytogenes
Expression in *E. coli* cytoplasm
ScFv

ABSTRACT

Rabbit monoclonal antibodies (mAbs) have advantages over mouse antibodies in diagnostics and biotechnological applications owing to higher affinity and specificity. We developed a platform to generate rabbit mAbs by a novel monoclonal antibody generation method named "Single-Cell Reverse Transcription-PCR linked in vitro-Expression (SICREX)" system. In this method, we use single-cell based RT-PCR followed by sequential PCR steps of mAb genes and subsequent cell-free protein synthesis (CFPS) by using linear DNA fragments of mAbs. This platform enables the rapid generation and evaluation of mAbs derived from antigen-specific single B cells in the peripheral blood of immunized animals without mammalian cell cultivation. In this study, the antigen used was a food-borne gram-positive pathogen, *Listeria monocytogenes*, that is known to cause serious infection. Three active mAbs in CFPS were obtained by constructing the single chain of variable fragment (scFv) form. These scFvs were produced in the cytoplasm of *E. coli* Shuffle T7 Express strain as an active form and used for further investigation.

1. Introduction

Monoclonal antibodies (mAbs) are essential tools in the fields of therapeutic, diagnostic, and biological research. The mouse hybridoma method is a traditional and most widely used approach for obtaining mouse mAbs (Köhler and Milstein, 1975). This technology is based on cell fusion of spleen cells with immortal myeloma cell lines. In the past few decades, alternative selection methods for mAbs based on direct molecular cloning such as phage-display (Smith, 1985), yeast surface display (Murai et al., 1997; Rakestraw et al., 2011), bacterial display (Georgiou et al., 1996), ribosome display, (Mattheakis et al., 1994) and mRNA display systems (Roberts and Szostak, 1997; Nemoto et al., 1997) have been developed. Although these new antibody generation technologies are successfully employed for mAbs screening, these methods are complicated and require time-consuming operations such as cyclic panning and a series of selection steps. Therefore, it can take several months at the very least to obtain desired pairs of heavy chain (Hc) and light chain (Lc). In display systems, random combinations of antibody variable regions are used for the construction of libraries, which results in loss of natural cognate pairing of Hc and Lc and reduced specific diversity (Meijer et al., 2006; Smith et al., 2009). Recently, a single B cell-based method has been developed that allows direct sampling of the immune reactive single B cell repertoires followed by amplification of antibody genes, and transfer to mammalian expression cells (Tiller, 2011; Kurosawa et al., 2012; Clargo et al., 2014). After several days cultivation of the expression cells, the mAbs secreted from each cell are analyzed.

Alternatively, our group has developed a high-throughput screening method for mAbs called "Single-Cell RT-PCR linked in vitro-Expression (SICREX)" system. In this method, single-cell based mAb genes are amplified with specific DNA primers and subsequent cell-free production of fragment of antigen-binding (Fab) or single chain variable fragment (scFv) is combined. This enables the evaluation of affinity of mAbs derived from antigen-specific single B cells in peripheral blood of immunized animals within a few days (Jiang et al., 2006; Sabrina et al., 2010) (Fig. 1). Briefly, SICREX consists of the following short steps: (i) isolation of antigen-specific B cells from the peripheral blood, (ii) reverse transcription PCR (RT-PCR) with antibody-specific primers, (iii) amplification of variable and constant regions of Lc and Hc by PCR, (iv) nested PCR with primers carrying tails that are required to connect upstream and downstream dsDNA fragments in the following PCR, (v) connecting the three dsDNA fragments—T7 promoter, antibody gene amplified in step iv, and T7 terminator— by overlapping PCR, (vi) cell-free protein synthesis (CFPS) of the antibody genes, and (vii) enzyme-linked immunosorbent assay (ELISA) for their evaluation. In

Abbreviations: ELISA, enzyme-linked immunosorbent assay; SICREX, single-cell reverse transcription-PCR linked in vitro-expression; CFPS, cell-free protein synthesis; Hc, heavy chain; Lc, light chain; RT, room temperature; SA beads, DynaBeads M-280 Streptavidin; Fab, fragment of antigen-binding; ScFv, single chain of variable fragment.

* Correspondence to: T. Ojima-Kato, Knowledge Hub Aichi, Aichi Science and Technology Foundation, 1267-1 Akiai, Yakusa cho, Toyota 470-0356, Japan.
** Corresponding author.
E-mail addresses: teruyo.ojima@gmail.com (T. Ojima-Kato), hnakano@agr.nagoya-u.ac.jp (H. Nakano).

http://dx.doi.org/10.1016/j.jim.2015.10.001
0022-1759/

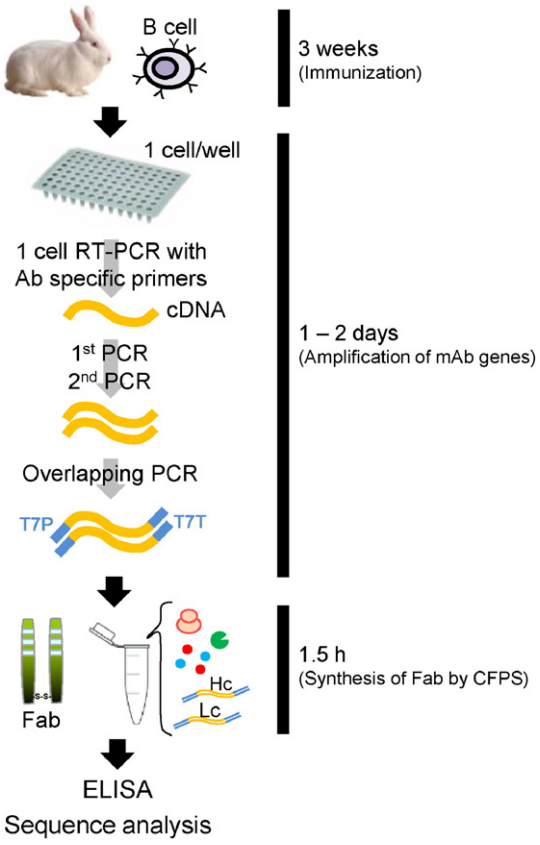

Fig. 1. Schematic workflow of SICREX. SICREX consists of the following short steps: (i) isolation of antigen-specific B cells from the peripheral blood in immunized animals, (ii) reverse transcription PCR (RT-PCR) with antibody-specific primers, (iii) amplification of variable and constant regions of Lc and Hc by PCR (1st PCR), (iv) nested PCR with primers carrying tails that are required to connect upstream and downstream dsDNA fragments in the following PCR (2nd PCR), (v) connecting the three dsDNA fragments—T7 promoter, antibody gene amplified in step iv, and T7 terminator—by overlapping PCR, (vi) cell-free protein synthesis (CFPS) of the antibody genes using PCR products, and (vii) enzyme-linked immunosorbent assay (ELISA) for their evaluation.

this approach, mAb genes of Hc and Lc from single B cells can be obtained rapidly because all the processes are undertaken in vitro. If necessary, the resultant mAb genes can be transferred to *E. coli* cells to prepare a large amount of mAbs for further analysis.

The use of rabbit mAbs has advantages in diagnostics and biotechnology since they exhibit both high affinity and specificity toward antigens that are weakly immunogenic in mice (Steinberger et al., 2000; Rossi et al., 2005). In spite of these favorable features, rabbit mAbs are not commonly used in studies because the procedures to produce rabbit mAbs are more complex and difficult than the conventional hybridoma method used in mouse or rat (Spieker-Polet et al., 1995). Recently, new platforms have been developed for generating rabbit mAbs from B cells or plasma cells from peripheral blood by combining cultivation of single sorted B cells for screening, subsequent cloning of VH and VL region, and expression of recombinant mAbs in animal cells like HEK293 or CHO (Kurosawa et al., 2012; Ozawa et al., 2012; Seeber et al., 2014). Although they may be robust screening method for antigen-specific mAbs, the entire screening process including immunization takes several months as it involves the transfer and cultivation of animal cells.

Listeria monocytogenes is one of the important food-borne gram-positive bacteria, which can cause serious disease and large-scale outbreaks (Liu, 2006). It often contaminates various ready-to eat foods including milk, fish, coleslaw and strict regulatory standards are in place in Europe and the US to ensure food safety. Serovar 1/2ab type is known to be the causative pathogen in the majority of listeriosis infections and a mAb against *Listeria* would be valuable for the immunological detection of the pathogen for food safety and clinical purposes. mAbs from mouse hybridoma that bind *L. monocytogenes* have been reported (Lin et al., 2006; Portanti et al., 2011; Ronholm et al., 2013), but no genetic information is available.

This work describes for the first time the use of the SICREX system with rabbits for obtaining anti-*L. monocytogenes* mAbs. Furthermore, we attempted to express mAbs as scFv forms in *E. coli* cytoplasm for practical uses.

2. Materials and methods

2.1. Immunization

L. monocytogenes ATCC 15313 obtained from American Type Culture Collection (Manassas, VA) was aerobically cultured overnight in Brain Heart infusion medium (Becton Dickinson, Franklin Lakes, NZ) at 30 °C. Cell pellet was harvested by centrifugation, washed with phosphate-buffered saline (PBS), and then treated with 0.25% formalin in PBS at 80 °C. This suspension was used as an antigen for immunization and ELISA. A New Zealand White rabbit (4 weeks old) was immunized with 5×10^8 cells of *L. monocytogenes* in PBS supplemented with 2.5 mL of complete Freund's adjuvant by hypodermic injection. After an interval of 2 weeks the rabbit was immunized a second time and after another 10 days the third boost was given. Two days after the third boost, blood samples were collected to monitor titers and isolate B cells. This study was approved by the committee of animal experiments of the Graduate School of Bioagricultural Sciences, Nagoya University (permit number 2012031511) and performed according to the Regulations on Animal Experiments in Nagoya University.

2.2. Isolation of B cells

A fraction of lymphocytes was collected from the peripheral blood by density-gradient centrifugation with pancoll (PAN-Biotech, Aidenbach, Germany). The upper layer of serum was used for titer evaluation in ELISA. The lymphocytes including B cells in the middle layer were collected and washed with PBS thrice by repeated centrifugation (400 ×*g*, 10 min, RT) and resuspension. The cells were counted under a microscope (BX53, Olympus) with a Burker-Turk hemocytometer and immediately used for the experiment described in Section 2.3.

2.3. Screening of antigen-specific B cells

First, 1 mL of lymphocyte suspension (10^6 cells) was mixed with 5 μL of DynaBeads M-280 Streptavidin (SA beads, Life technologies, Foster City, CA) and incubated for 2 min at RT. Cells nonspecifically bound to the magnetic beads were discarded. Antigen-specific B cells were collected by antigen-coated magnetic beads that were prepared as follows: Fresh cells of *L. monocytogenes* (2×10^9 cells) were suspended in 100 μL of 50 mM MES buffer (pH 5.0) containing 1 mM EZ-link pentylamine-biotin (Thermo Fisher Scientific, Waltham, MA). After incubating on a rotary shaker at RT for 15 min, 3 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added, and the mixture was further incubated for 2 h. The pellet collected by centrifugation (3000 ×*g*, 5 min, RT) was conjugated with SA beads (6×10^7 beads) by incubation in 100 μL of PBS at RT for 1 h. The bead-cell complexes were collected using a magnetic stand and washed with PBS twice. They were subsequently incubated with the B cells prepared above for 1 h and then the complexes of beads, *L. monocytogenes*, and B cells were again collected using a magnetic stand. The construction of the complexes was confirmed under a microscope. Loeffler's methylene blue alkaline was used when staining the bacterial cells.