In vitro immunization of Epstein–Barr virus-immortalized B cells augments antigen-specific antibody production

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Received: 12 March 2013 / Accepted: 3 June 2013 / Published online: 15 August 2013
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Abstract The current method for in vitro immunization (IVI) uses several antigens including toxins, food allergens, pathogenic bacteria, and self-antigen-derived peptides that induce an antigen-specific immune response in peripheral blood mononuclear cells (PBMCs). This protocol, however, requires donor blood collection and preparation of PBMCs before every IVI. In the present study, we aimed to design a more efficient system utilizing B cells immortalized with Epstein–Barr virus (EBV-B) as host cells for IVI to make antigen-specific antibodies. Results showed that previously antigen-sensitized, EBV-B cells exposed to the antigen along with IL-6, CpG oligonucleotides, and CD40 ligand signal produced antigen-specific antibodies. These results provide evidence for a novel and easy method to expand memory-type B cells and produce antigen-specific antibodies.

Keywords Epstein–Barr virus · B cells · Human antibody · In vitro immunization · CD40L · CpG oligonucleotide

Introduction

Monoclonal antibodies are suitable reagents for the diagnosis and treatment of diseases such as cancer, infection, and allergy. The monoclonal antibodies from laboratory animals are relatively easy to produce, but their therapeutic efficacy is restricted by their antigenicity (Co and Queen 1991). To overcome this challenge, an in vitro immunization (IVI) protocol that uses human peripheral blood mononuclear cells (PBMCs) to induce an antigen-specific immune reaction was developed (Ichikawa et al. 1999). We have previously induced an immune response in PBMCs against cholera toxin B subunit (CTB), rice allergen (RA), *Propionibacterium acnes* as well as peptides derived from RA, CTB, and tumor necrosis factor- α (TNF- α) by using this protocol (Fujiki et al. 2010; Ichikawa et al. 1999; Shim et al. 2001; Tamura et al. 2007). However, PBMCs must be collected and prepared before each IVI. We hypothesized that immortalized B cells may be a more efficient host for IVI and the production of antigen-specific antibodies, avoiding repetitive blood collection and preparation. B cells can be easily and stably immortalized with EBV, and furthermore, many EBV-immortalized B (EBV-B) cells have been deposited at cell banks available for research purposes. For example, EBV-B

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cells from Alzheimer's disease (AD) patients can be used for diagnosis of AD (Geylis and Steinitz 2006). In this study, we evaluated whether B cells immortalized with EBV can be sensitized with antigen and produce antibodies specific for that antigen, alleviating the need for collecting PBMCs during every IVI.

Materials and methods

EBV-B cells

Epstein–Barr virus cells (HEV0174) were purchased from RIKEN cell bank (Tsukuba, Japan) and cultured in eRDF media (Kyokuto, Tokyo, Japan) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS, USA).

Antigen and reagents

Bovine β -lactoglobulin (β -LG), keyhole-limpet hemocyanin (KLH), and cholera toxin B subunit (CTB) were purchased from Sigma (St. Louis, MO, USA). Fish gelatin (FG) was purchased from BioFX Laboratories (Owings Mills, MD, USA). D-type CpG oligodeoxynucleotide (ODN) (5'-ggTGCATCGATGCAGGGGggG-3') and K-type CpG ODN (5'-tegaggttctcC-3'; uppercase and lowercase letters indicate bases with phosphodiester and phosphorothioate-modified backbones, respectively) were purchased from Sigma Genosys (Hokkaido, Japan) (Verthelyi et al. 2001). Recombinant interleukin 6 (IL-6) was purchased from Pepro Tech (London, UK).

IVI

Epstein–Barr virus cells were sensitized with β -LG ($10 \mu\text{g mL}^{-1}$) in the presence of IL-6 (10 ng mL^{-1}), D-type CpG ODN ($1 \mu\text{M}$), and K-type CpG ODN ($1 \mu\text{M}$), in the wells containing the fixed and inactivated recombinant CHO cells (5×10^4 cells/mL) expressing CD40 ligand (CD40L), and were cultured in eRDF medium supplemented with 2-mercaptoethanol ($50 \mu\text{M}$, Sigma) and 10 % heat-inactivated FBS for 6 days.

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (Nunc, Naperville, IL, USA) were coated with anti-human IgM antibody (TAGO,

Burlingame, CA, USA) diluted in 0.1 M sodium carbonate buffer (pH 9.6) and incubated for 2 h at 37°C . The plates were washed three times with PBS containing 0.05 % Tween 20 (PBST). Aliquots of serially diluted supernatants from in vitro-immunized EBV-B cells were added, and the plates were then incubated at 4°C overnight. After washing three times with PBST, diluted horseradish peroxidase-conjugated goat anti-human IgM (TAGO) antibody was added, and the plates were subsequently incubated for 2 h at 37°C . The plates were again washed three times with PBST, and substrate solution [0.1 M citrate buffer (pH 4.0) containing 0.003 % H_2O_2 and 0.3 mg mL^{-1} 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] (ABTS; Wako, Osaka, Japan) was added followed by incubation for 20 min. The absorbance at 405 nm was measured using an ELISA plate reader.

Enzyme-linked immunospot (ELISPOT) assay

Multiscreen HA filtration plates (Millipore, Bedford, MA, USA) were coated with $1 \mu\text{g}$ of β -LG per well in 0.5 M carbonate buffer (pH 9.6) and incubated overnight at 4°C . The plates were then blocked with 1 % FG in PBS for 2 h at 37°C . After washing the plates with PBS, in vitro-immunized EBV-B cells were added to plates in triplicates at 1×10^5 cells/well $^{-1}$ and cultured for 18 h in a humidified atmosphere at 37°C and 5 % CO_2 . The plates were again washed with PBST and incubated with diluted horseradish peroxidase-conjugated goat anti-human antibody (IgM-HRP; Biosource, Camarillo, CA, USA) for 2 h at 37°C . After washing the plates with PBST, TrueBlue substrate solution (KPL, Gaithersburg, MD, USA) was added, and the plates were incubated at 37°C for 10 min. The reaction was terminated by washing the plates with water, and the plates were then dried in the dark. The number of spots was counted using Image J software.

Flow cytometry

The antigen specificity of antibodies produced by EBV-B cells was evaluated by flow cytometry. EBV-B cells were harvested and resuspended in cold eRDF medium with 2 % BSA containing fluorescently labeled antigen (β -LG labeled with Alexa647 or FG labeled with Alexa488) (Brezinsky et al. 2003).

Fig. 1 EBV-B cells produced antigen-specific antibodies after IVI. **a** EBV-B cells were sensitized with β -LG ($10 \mu\text{g mL}^{-1}$) in the presence of IL-6 (10 ng mL^{-1}), D-type CpG ODN ($1 \mu\text{M}$), and K-type CpG ODN ($1 \mu\text{M}$) in the wells fixed with or without CHO cells producing CD40L. IgM production was determined by ELISA. **b** EBV-B cells were sensitized as above with CD40L+ CHO cells. Cells producing antigen-specific

antibodies were detected by ELISPOT assay. The number of spot was counted by using Image J software, and indicated at the *upper right corner* of the well. **c, d**. Antigen-specific antibodies were detected by flow cytometry. In vitro-immunized EBV-B cells were resuspended in eRDF medium with 2 % BSA containing fluorescently labeled antigen, and then analyzed by flow cytometer

Fluorescence was then measured by flow cytometry (BD FACSAria, Becton–Dickinson, Franklin Lakes, NJ, USA).

Results and discussion

β -LG-specific immune response was elicited in EBV-B cells by IVI

Until now, we have developed a method to induce antigen-specific immune response in human PBMCs by in vitro immunization using CTB, RA, and various peptides as sensitizing antigens (Fujiki et al. 2010;

Ichikawa et al. 1999; Shim et al. 2001; Tamura et al. 2007). This protocol, however, requires donor blood collection and preparation of PBMCs before every IVI. In the present study, we first tested whether an antigen-specific immune response could be elicited in EBV-B cells after IVI against the sensitizing antigen β -LG. To enhance the response, recombinant CHO cells expressing CD40L were used as stimulators (Katakura et al. 1999). CD40–CD40L interaction increases the antigen-specific immune response of B cells (Oxenius et al. 1996). Although the amounts of antibody did not change upon the addition of β -LG and adjuvant, IgM production was enhanced by sensitizing EBV-B cells in the wells fixed with recombinant CHO

Fig. 2 EBV-B cells were sensitized with KLH or CTB by in vitro immunization. EBV-B cells were stimulated with KLH or CTB. Cells producing antigen-specific antibody were detected by ELISPOT assay

cells expressing CD40L (Fig. 1a). These results suggest that CD40–CD40L interaction would enhance the immune reaction in EBV-B cells. Thus, we sensitized EBV-B cells with β -LG in the presence of IL-6 in the wells fixed with CD40L-expressing CHO cells. We next assessed the antigen specificity of IgM antibodies produced by EBV-B cells cultured in the presence of IL-6, CpG ODN, and CD40L signal via ELISPOT assay. The amount of EBV-B cells producing β -LG-specific antibodies increased after the addition CpG ODN (Fig. 1b). In addition, we tested the antigen specificity of IgM antibodies produced by EBV-B cells using flow cytometry. Referring to a method described by Brezinsky et al. (2003) secretory IgM proteins were captured on the cell surface by fluorescently labeled antibodies at a low temperature and then detected by flow cytometry. The amount of EBV-B cells producing antigen-specific antibodies, as detected by flow cytometry, also increased upon β -LG sensitization using CpG ODN as adjuvant (Fig. 1c and d). These results suggest that antigen sensitization in the presence of IL-6, CD40L, and CpG ODN gives the greatest expansion of antigen-specific EBV-B cells.

In vitro immunization of EBV-B cells with an unsensitized antigen

We next tested whether IVI expands the EBV-B cells specific for unsensitized antigens such as KLH and CTB. As shown in Fig. 2, the number of EBV-B cells producing antibodies specific for KLH or CTB was

small compared with the result against β -LG (Fig. 1). Furthermore, we could hardly detect spots specific for bovine serum albumin, which is present in the culture during IVI, but is unsensitized antigen. These results suggest that further optimization is needed for the efficient expansion of in vitro-immunized EBV-B cells producing antibodies specific for unsensitized antigens.

Summary

In conclusion, IVI is able to expand EBV-B cells producing antigen-specific antibodies against previously sensitized antigens. Therefore, EBV-B cells can be used as an alternative to human PBMCs for IVI. Improvements in the amount of antigen-specific antibodies generated from EBV-B cells will be the focus of future studies.

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