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Exploitation of human CD99 expressing mouse myeloma cells as immunogen for production of mouse specific polyclonal antibodies

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ABSTRACT

In this study, we describe the application of a molecular biology technique for the production of mouse polyclonal antibodies (pAbs) specific to human cell surface molecules. Production of the pAb specific to the human CD99 surface molecule was used as the study model. The retroviral expression system was employed to generate human CD99 expressing mouse myeloma cells. After cell sorting and single cell cloning, a myeloma clone which stably expressed high levels of human CD99 on its surface was established. The human CD99 expressing mouse myeloma cells were then used as the immunogen for immunization of BALB/c mice. As endogenous proteins of mouse myeloma cells possess self-non-immunogenicity for BALB/c mice, after immunization, only the expressed human CD99 molecules induce antibody response. After three immunizations, high titers of mouse anti-CD99 pAbs were successfully produced. The produced pAb specifically reacted to both recombinant human CD99 and native CD99 molecules expressed on human blood cells. The established technology is simple and valuable for the production of pAbs specific to human CD99 membrane proteins which can be used for characterization of the CD99 molecule.

1. Introduction

Currently, antibodies have been utilized as an important tool in biomedical research, clinical diagnosis, and therapeutics [1–6]. Polyclonal antibodies (pAbs) are antibodies that are produced from animals after antigen immunization. This type of antibody is produced by several B cell clones; therefore, they react to the multiple and distinct epitopes of their specific molecules. pAbs are employed for several purposes and, occasionally, have several advantages over monoclonal antibodies (mAbs) [4]. Production of specific pAbs, however, is still challenging, especially production of pAbs specific to mammalian cell membrane proteins [5].

In the production of pAbs appropriate to mammalian proteins, it is important to consider the properties of the immunogen, including purity, quality, and quantity [6,7]. Epitope alterations of antigens, such as changes in glycosylation, structure, or denaturation, can markedly affect the specificity of the generated antibodies [8,9]. Often, the produced pAbs can non-specifically react to other proteins due to the impurity of the immunogen used. To overcome this problem, using recombinant proteins as immunogens, instead of native proteins, has been recommended. Recombinant mammalian proteins produced in bacteria have been used as immunogens for production of antibodies [10,11]. Nevertheless, these types of immunogens often lack conformational epitopes and a proper glycosylation, because of which the generated pAbs can strongly react to the recombinant proteins but fail to react to the native proteins [12,13]. To overcome the disadvantage of using the prokaryotic expression system, an expression of the recombinant protein in the mammalian cells has been introduced. The proteins generated by the mammalian expression system have structures that are very similar to their native proteins.

Abbreviations: pAbs, polyclonal antibodies; mAbs, monoclonal antibodies; CD, cluster of differentiation; PE, Phycoerythrin; FITC, Fluorescein isothiocyanate; PBS, phosphate buffer saline; PBMCs, peripheral blood mononuclear cells; ELISA, enzyme-linked immunosorbent assay.

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In order to produce pAbs to human cell surface proteins, a mammalian expression system is necessary for the production of immunogen since it provides proper folding as well as post-translational modifications, for instance, glycosylation and phosphorylation [13–15]. With advanced biotechnologies, affinity tags have been introduced into the synthesized recombinant proteins for the purpose of purification. However, during the purification process, the conformational epitopes and the protein folding may be altered by any number of factors such as temperature, pH, and salt concentration, as well as mechanical forces [16]. Consequently, because of the use of such purified recombinant proteins, the produced pAbs may not bind to the native proteins.

In this study, we introduce a technique for the generation of recombinant proteins for use as immunogens to produce pAbs specific to human membrane proteins. The established method allows us to bypass immunogen purification processes which may induce conformational structural changes and also to decrease the time consumed during the preparation of the immunogen. The recombinant proteins were expressed on mouse cell line and then used as the immunogen for generating pAbs in mice. By this approach, only the expressed recombinant proteins become antigens in the immunized mice and induce specific antibody responses. In the present report, the human leukocyte surface molecule CD99, along with the production of pAb specific to CD99, was used as the study model.

2. Materials and methods

2.1. Preparation of mouse myeloma cells stably expressing human CD99 membrane protein

Mouse myeloma cells stably expressing human CD99 molecules were established using the retroviral expression system. In brief, 9×10^5 Phoenix-Eco packaging cell line (Origene, Rockville, MD, USA) were seeded in 2 ml of DMEM containing 10% fetal bovine serum. The cells were then transfected with 1.6 μg of pMSCV-CD99 (plasmid vector harboring human CD99 encoding gene) [17], using lipofectamine 2000 transfection reagent (Invitrogen, Grand Island, NY, USA), according to the manufacturer's protocol. After 2 days of cultivation, the culture supernatants containing virion were collected, and the cell debris were removed by centrifugation. One ml of the supernatant containing virion was used to spin-infect (1200 × g, 90 min at room temperature) the 5×10^5 P3-X63Ag8.653 mouse myeloma cells in the presence of 10 μg/ml of polybrene (Sigma-Aldrich, St. Louis, MO, USA). After overnight incubation at 37 °C in a 5% CO_2 incubator, the myeloma cells were re-infected using the same procedure. Three days after cultivation, the expression of human CD99 recombinant proteins on the myeloma cells was determined by immunofluorescence staining and flow cytometric analysis.

The human CD99-positive myeloma cells were isolated by magnetic cell sorting using MS column (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the MACS cell separation protocol. Briefly, 5×10^5 of the transduced myeloma cells were stained with anti-human CD99 mAb (MT99/1, IgM isotype) [18] followed by phycoerythrin (PE) conjugated anti-mouse IgM Abs (μ-chain specific) (Beckman Coulter, Marseille, France) and anti-PE microbeads (Miltenyi Biotec). The stained cells were then sorted according to the MACS cell separation protocol. The magnetically labeled cells were collected and single cell cloning was performed by the limiting dilution method. The human CD99 expression of the myeloma clones was then analyzed by flow cytometry. The clone with high CD99 expression was selected, grown, and used as the immunogen for the production of pAbs in mice.

2.2. Mouse immunization

Three BALB/c mice were immunized with human CD99 expressing mouse myeloma cells. CD99 expressing myeloma cells (2×10^7 cells) were suspended in 500 μl of phosphate buffered saline (PBS). Each of the mice was intraperitoneally immunized three times with the cells at 2-week intervals. Blood was collected before and after each immunization by tail bleeding. The sera were separated and stored at −20 °C.

2.3. Immunofluorescence staining and flow cytometric analysis

Cell lines or peripheral blood mononuclear cells (PBMCs) were used to block the Fc receptor with 10% fetal bovine serum for 30 min on ice. The cells were then stained with either 10 μg/ml of purified mAbs or mouse sera for 30 min on ice. After washing, the bound antibodies were detected by staining with PE-conjugated anti-mouse IgM Abs (μ-chain specific) (Beckman Coulter) or PE-conjugated anti-mouse IgG Abs (γ-chain specific) (Beckman Coulter) or FITC-conjugated anti-mouse Igs Abs (Chemicon, Melbourne, Australia) or Alexa fluor 488-anti-mouse IgG Abs (H andL chains specific) (Invitrogen) for 30 min on ice. The stained cells were analyzed by a FACSort flow cytometer (BD Biosciences).

2.4. Preparation of human CD99-IgGFc fusion protein and human CD147-IgGFc fusion protein

The human CD99-IgGFc fusion protein (CD99Rg) was generated in our laboratory. Briefly, the human CD99 encoding gene was amplified from the pCDM8-CD99 plasmid vector [19]. Subsequently, the CD99 genes were inserted by replacing the sequence of

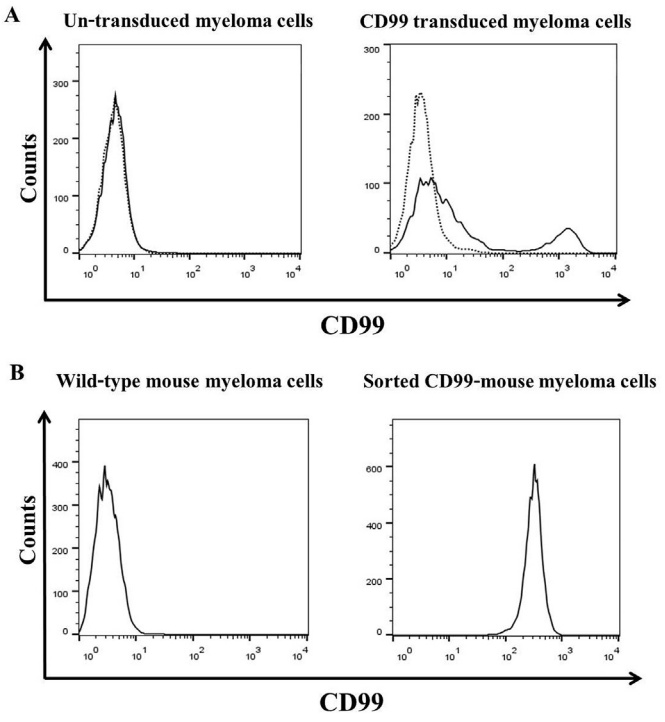

Fig. 1. The flow cytometric analysis of human CD99 expressing mouse myeloma cells. (A) Un-transduced and CD99-transduced myeloma cells were stained with anti-human CD99 mAb (solid line) or isotype matched control mAb (dotted line). (B) The wild-type mouse myeloma cells and the CD99-expressing mouse myeloma cells obtained after cell sorting and single cell cloning were stained with anti-human CD99 mAb. The stained cells were analyzed by flow cytometry.