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An on-bacterium flow cytometric immunoassay for protein quantification

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ABSTRACT

The polystyrene bead-based flow cytometric immunoassay has been widely reported. However, the preparation of functional polystyrene bead is still inconvenient. This study describes a simple and easy on-bacterium flow cytometric immunoassay for protein quantification, in which *Staphylococcus aureus* (SAC) is used as an antibody–antigen carrier to replace the polystyrene bead. The SAC beads were prepared by carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, paraformaldehyde fixation and antibody binding. Carcinoembryonic antigen (CEA) and cytokeratin-19 fragment (CYFRA 21-1) proteins were used as models in the test system. Using prepared SAC beads, biotinylated proteins, and streptavidin–phycoerythrin (SA–PE), the on-bacterium flow cytometric immunoassay was validated by quantifying CEA and CYFRA 21-1 in sample. Obtained data demonstrated a concordant result between the logarithm of the protein concentration and the logarithm of the PE mean fluorescence intensity (MFI). The limit of detection (LOD) in this immunoassay was at least 0.25 ng/ml. Precision and accuracy assessments appeared that either the relative standard deviation (R.S.D.) or the relative error (R.E.) was <10%. The comparison between this immunoassay and a polystyrene bead-based flow cytometric immunoassay showed a correlation coefficient of 0.998 for serum CEA or 0.996 for serum CYFRA 21-1. In conclusion, the on-bacterium flow cytometric immunoassay may be of use in the quantification of serum protein.

1. Introduction

Cytokine proteins can be secreted into surround microenvironment and peripheral blood to perform cellular communication by autocrine [1], paracrine and endocrine [2] pathway for maintaining organism homeostasis. The dysexpression of serum protein was evidenced to be associated with carcinoma [3], inflammation [4] and immunodeficiency [5]. Therefore, the serum protein analysis is frequently implemented for clinical diagnosis, treatment monitoring and prognosis evaluation.

Conventional methods for protein quantification, e.g. the sandwich enzyme-linked immunosorbent assay (sELISA), have important limitations. Both sELISA and Western blotting methods are not only time-consuming but also inefficient. The new generation of the polystyrene bead-based flow cytometric immunoassay economizes the time of traditional microtiter plate-based immunological assays by relying on the rapid binding of antibodies to antigens in solution [6,7]. It is administered by a flow cytometer with automatic sampler and analysis software, which also explains its high throughput and high sensitivity characteristics. Despite the widely reported application of the polystyrene bead-based flow cytometric immunoassay, the preparation of functional polystyrene bead is still inconvenient. And there are no studies on a bacterium-based flow cytometric immunoassay.

This study describes a simple and easy on-bacterium flow cytometric immunoassay, in which *Staphylococcus aureus* (SAC) is used as an antibody–antigen carrier to replace functional polystyrene bead. SAC expresses a 40–60 kDa surface protein, protein A, which can bind to the Fc region of antibody preferentially through interaction with the heavy chain [8]. Staphylococcal protein A appears unique binding properties for IgG from a variety of mammalian species and for some IgM and IgA as well. SAC and protein A coupled to solid supports have been applied in IgG purification from ascitic fluid, serum and culture supernatants. In addition, staphylococcal protein A functioning as a useful adapter molecule was reported to be conjugated with reporter reagents comprising fluorescence dye, horseradish peroxidase [9], biotin [10], colloidal gold and radioactive iodine [11] in immunological detection without affecting the antibody-binding site.

Carcinoembryonic antigen (CEA) and cytokeratin-19 fragment (CYFRA 21-1) proteins were used as models in the present test system. Some serum markers have been considered potentially prognostic and predictive in non-small cell lung cancer (NSCLC). Among these NSCLC markers, CEA and CYFRA 21-1 were proposed to be sensitive and valuable tumor markers for diagnosis, prognosis, and therapy monitoring. CEA is a 180–200 kDa glycoprotein

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member of the immunoglobulin supergene family, whose expression is limited in normal adult tissue whereas it is over-expressed in many types of primary and metastatic tumors, especially NSCLC [12,13]. Belonging to the human cytokeratins family, CYFRA 21-1 is an acidic (type I) subunit produced in all simple epithelia and in carcinomas such as NSCLC which arises from them [14,15].

This study is the first report addressing a bacterium-based flow cytometric immunoassay (Fig. 1).

2. Materials and methods

2.1. Bacterium, antibodies and reagents

S. aureus Cowan I was provided by Institute of Microbiology, Chinese Academy of Science (Beijing, China). Mouse monoclonal IgG_{2a} antibody against human CEA, mouse monoclonal IgG_2 antibody against human CFYRA 21-1, CEA protein and human IgG Fc fragment were purchased from Abcam Co. (Cambridge, UK). CYFRA 21-1 protein was from Shanghai Linc-Bio Science Co. Ltd. (Shanghai, China), mouse IgG_{2a} isotype control antibody was from BD Bioscience (NJ, USA), and an antibody clean-up kit was from Thermo Scientific (IL, USA). Streptavidin–phycoerythrin (SA–PE) reporter conjugate was obtained from Life Technologies Company (Grand Island, NY, USA). Polystyrene beads were from Spherotech Incorporation (Lake Forest, IL). Carboxyfluorescein diacetate succinimidyl ester (CFSE) and paraformaldehyde (PFA) were from Sigma Chemical Co. (St. Louis, MO, USA). Other biochemical reagents were from Sagon (Shanghai, China). All chemical reagents were analytic grade.

2.2. Biomaterials preparation

2.2.1. CFSE labeling and PFA fixation

SAC was propagated in BPY medium (pH 7.0) containing 5% beef extract, 10% peptone, 5% NaCl, 5% yeast extract, 5% glucose, and 1% agar. A 2.5 μl SAC was incubated with 10 μl of CFSE (5 mM in dimethyl sulfoxide) and 2.5 ml of 0.01 M phosphate-buffered saline (PBS), pH 7.2, at 37 °C for 20 min. The SAC was washed twice in PBS and then fixed with PFA (0.5% in PBS) at room temperature for 30 min. After washing twice in PBS, the SAC beads were stored in PBS with 0.5% PFA at 4 °C in the dark.

2.2.2. Protein biotinylation

CEA and CYFRA 21-1 proteins contained in PBS were condensed using freeze-dried method and resuspended in 0.1 M Na_2CO_3 buffer (pH 8.8) to a final concentration of 1 mg/ml, respectively. NHS–biotin was dissolved in dimethyl sulfoxide to a final concentration of 10 mg/ml. One microlitre of NHS–biotin solution was mixed with 100 μl of CEA or CYFRA 21-1 solution. And this was incubated at room temperature for 4 h. Next, 1 μl NH_4Cl solution (1 M) was added to terminate the reaction.

2.3. Protein quantification

2.3.1. On-bacterium flow cytometric immunoassay

The stored SAC beads were washed and resuspended in binding buffer comprising 150 mM NaCl, 1.0% NP-40, 50 mM Tris, pH 8.0. A 0.5 ml SAC solution (2×10^6 SAC beads/ml in binding buffer) was incubated in a Falcon tube with 50 μl of the monoclonal antibody against human CEA or CYFRA 21-1 at 4 °C for 1 h. The antibody-bound SAC beads were washed twice, collected by centrifugation at $1699 \times g$ for 10 min, and resuspended in binding buffer.

In the on-bacterium flow cytometric immunoassay, the biotinylated protein and the corresponding protein in sample competitively bind to SAC-coated antibody. Briefly, a 50 μl antibody-bound SAC beads solution containing 6000 cells was incubated with 50 μl of biotinylated CEA or CYFRA 21-1 (7.5 ng/ml in binding buffer) and 50 μl of sample at 4 °C for 1 h in the dark with gently mixing. Then a 50 μl SA-PE was added in an amount equivalent to the total amount of biotinylated proteins for a 30 min incubation in the dark. After washing twice and resuspending in 100 μl binding buffer containing 1% bovine serum albumin (BSA), the SAC beads were examined using a FACSCalibur flow cytometer with Cell Quest Pro software (BD Bioscience, NJ, USA).

2.3.2. Polystyrene bead-based flow cytometric immunoassay

The monoclonal antibodies against human CEA and CYFRA 21-1 proteins were respectively purified using a Pierce antibody clean-up kit (Thermo Scientific, IL, USA) according to the manufacturer's instructions. Using a Falcon tube, carboxylated polystyrene beads (1×10^5) were co-incubated with 10 μg of the purified monoclonal antibody against CEA or CYFRA 21-1 protein in 500 μl of 0.1 M 2-(N-morpholino)ethanesulfonic acid buffer (pH 5.0) containing 0.5% sulfo-NHS and 0.5% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride at 4 °C for 2 h in the dark with occasional shaking. The antibody-bound beads were washed twice in PBS using a filter plate (MSBVS1210, Millipore, US), a vacuum bracket (Multiscreen Vacuum Manifold, Millipore, US), and a vacuum (wp6122050, Millipore, US), and then resuspended in 100 μl of PBS with 0.1% BSA and 0.02% NaN_3.

In the polystyrene bead-based flow cytometric immunoassay, the biotinylated protein and the corresponding protein in sample also competitively bind to bead-coated antibody. A 50 μl antibody-bound beads solution containing 6000 beads was co-incubated with 50 μl of biotinylated CEA or CYFRA 21-1 (7.5 ng/ml in PBS) and 50 μl of sample at 4 °C for 1 h in the dark with gently mixing. The beads were washed twice in PBS containing 0.1% BSA and incubated in 100 μl SA-PE (5 μg/ml in PBS with 0.1% BSA) at room temperature for 30 min in the dark. After washing twice and resuspending in 100 μl of PBS containing 1% BSA, the beads were measured using a flow cytometer.

2.4. Instrument settings

Samples were detected using a FACSCalibur flow cytometer with Cell Quest Pro software and a count of 6000 gated events. Photomultiplier tube (PMT) voltages were adjusted by using SAC beads or polystyrene beads in either side scatter (SSC) versus FL1 (CFSE) dot plot or the FL2 (PE) histogram with logarithmic amplification. A fluorescent compensation for the FL1 versus FL2 dot plot was implemented prior to data acquisition using Quantibrite PE beads (Becton-Dickinson, Franklin Lakes, NJ, USA) and CFSE-labeled SAC.

2.5. Statistics

Quantitative data were expressed as the mean ± SD. The data were analyzed using OriginPro 8.0 statistical software. A Mann–Whitney *U*-test was conducted to compare mean values between different samples. A value of $p < 0.01$ was considered statistically significant in all cases.

3. Results

3.1. Conjugation of SAC bead with antibody

In the present study, the conjugation of SAC bead with antibody may be affected by CFSE labeling and PFA fixation. In order to validate whether the CFSE-labeled and PFA-fixed SAC beads efficiently bind antibody by the protein A-Fc region recognition, the labeled and fixed SAC beads were preincubated with Fc fragment for 30 min and then incubated with mouse PE-labeled IgG_{2a} isotype control antibody at 4 °C for 1 h in the dark. Electron microscopy demonstrated the monodisperse form of the SAC beads (Fig. 2). And flow