Journal of Immunological Methods 413 (2014) 45–56

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Research paper

Comparative characterization of mAb producing hapten-specific hybridoma cells by flow cytometric analysis and ELISA

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ARTICLE INFO

Article history:
Received 20 June 2014
Received in revised form 13 July 2014
Accepted 14 July 2014
Available online 21 July 2014

Keywords:
Immunization
Hapten
Monoclonal antibodies
Hybridoma
Flow cytometry
ELISA

ABSTRACT

A novel method that optimizes the screening for antibody-secreting hapten-specific hybridoma cells by using flow cytometry is described. Cell clones specific for five different haptens were analyzed. We selectively double stained and analyzed fixed hybridoma cells with fluorophore-labeled haptens to demonstrate the target-selectivity, and with a fluorophore-labeled anti-mouse IgG antibody to characterize the level of surface expression of membrane-bound IgGs. ELISA measurements with the supernatants of the individual hybridoma clones revealed that antibodies from those cells, which showed the highest fluorescence intensities in the flow cytometric analysis, also displayed the highest affinities for the target antigens. The fluorescence intensity of antibody-producing cells corresponded well with the produced antibodies' affinities toward their respective antigens. Immunohistochemical staining verified the successful double labeling of the cells. Our method makes it possible to perform a high-throughput screening for hybridoma cells, which have both an adequate IgG production rate and a high target affinity.

1. Introduction

Their high affinity, selectivity and specificity make monoclonal antibodies (mAbs) a very important tool in research, diagnostics and therapy. Antibody production is routine. When the target is a protein or a microorganism, immunization can be done directly and an immune response is elicited that finally results in mAb-producing B cells in the spleen and antibodies in the serum. Low-molecular compounds with molecular weights below 1.000 Da do not cause an immune response and are called haptens. In order to initiate an immune response, haptens have to be linked to a carrier protein such as bovine serum albumin (BSA) (Dutton and Bulman, 1964; Walters et al., 1972; Fasciglione et al., 1996; Ramin and Weller, 2012). After triggering an immune reaction, the immune system produces B cells, which can be isolated from the spleen and fused with "immortal" myeloma cells in order to obtain so-called hybridoma cells (Köhler and Milstein, 1975). The hybridoma technique nowadays is applied almost in the same way as back then. The efficiency of polyethylene glycol (PEG)-stimulated fusion and electrofusion is very low. It ranges from 0.0001% to 0.01% successfully fused cells per initial cell number. Great care has to be given to dilution factors and cultivation conditions such as the use of feeder cells (De Blas et al., 1981). The indispensable identification and isolation of the desired antibody-producing

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http://dx.doi.org/10.1016/j.jim.2014.07.004
0022-1759/

hybridoma clone is still far from being trivial (Pasqualini and Arap, 2004; Chiarella and Fazio, 2008). The approaches are mostly empirical and guided by non-standardized protocols and individual experience. The immunoassay-based screening for single hybridoma cell clones that produce an antibody with the desired selectivity is mainly done by enzyme-linked immunosorbent assays (ELISAs). To isolate the cell clone of interest, multiple rounds of limiting dilution have to be carried out, which result in a high number of cells being lost during the process. This makes the entire procedure extremely time- and labor-intensive (Cervino et al., 2008; Zhang and Wang, 2009). Selective labeling of hybridoma cells with fluorophore-tagged antigens has been described, which allows for screening and isolation of such cells with the help of their cell-surface associated antibodies (Parks et al., 1979). Furthermore, it is also possible to use fluorescein-tagged antibodies directed against immunoglobulin G (IgG) antibodies to determine IgG on the surface of hybridoma cells and to screen for hybridoma cells, which secrete IgG variants (Liesegang et al., 1978; Kromenaker and Srienc, 1994). It was also reported that there is a relationship between the antibody production rate and the amount of IgG on the cell surface (Sen et al., 1990; Cherlet et al., 1995). However, no attempts have been made so far to correlate the amount of antibody production of the cell with the target-selectivity of the produced antibodies.

The aim of our work was to optimize the screening by flow cytometry for hybridoma cells producing specific antibodies of interest. The presented method is taking advantage of the fact that desired hybridoma clones carry antibodies on their cell surface. The membrane-based antibodies allow for double labeling of these cells by anti-mouse IgG antibodies, which are coupled to a fluorophore and should help to estimate the antibody expression of the cell. The second label is a fluorescein–hapten conjugate that characterizes the target-selectivity of the produced antibodies. In order to validate our newly developed flow cytometry method, ELISAs had to be performed using purified soluble antibodies from the supernatant of individual hybridoma clones. The presented studies were carried out with cell clones derived from immunizations against (i) the two mycotoxins aflatoxin B1 (Afla) and zearalenone (ZON), (ii) the steroid digoxigenin (DIG), and (iii) the natural estrogenic hormones, estrone (E1) and 17β-estradiol (E2).

2. Materials and methods

2.1. Materials

All solvents and chemicals were obtained from Merck KGaA (Darmstadt, Germany), Sigma-Aldrich (Taufkirchen, Germany), and Steraloids (Newport, RI, USA) and were of best available quality. 5-(Aminoacetamido)fluorescein (FITC) was obtained from Invitrogen (Carlsbad, California, USA). Horseradish peroxidase (HRP) was EIA-grade and obtained from Roche (Mannheim, Germany). Bovine serum albumin (BSA) was purchased from Protea Biosciences (Morgantown, WV, USA). The immunogens ZON coupled with keyhole limpet hemocyanin (KLH) and Afla-KLH and the conjugates ZON–FITC and Afla–FITC were obtained from aokin AG (Berlin, Germany). All buffers and solutions were prepared with ultrapure water from a Synthesis A10 Milli-Q® water purification system.

2.2. Derivate synthesis and conjugation

2.2.1. Immunogen

The immunogen estrone (E1) was synthesized in our laboratory by coupling of E1 to the carrier protein BSA. The coupling of E1-6-CMO (1,3,5(10)-estratrien-3-ol-6,17-dione-6-O-carboxymethyloxime) to BSA was performed via the carbodiimide method adapted from Schneider and Hammock (1992). First, the hapten is activated by NHS/DCC followed by the coupling to BSA. Ten micromole (3.6 mg) E1-6-CMO was dissolved in 430 µl anhydrous N,N-dimethylformamide, and 12 µmol N-hydroxysuccinimide (24 µl from a 0.5 M stock solution) was added, followed by 12 µmol dicyclo hexylcarbodiimide (24 µl from a 0.5 M stock solution) under nitrogen atmosphere. After stirring for 18 h at room temperature, the solution was centrifuged for 10 min at 14,000 rpm and 20 °C. The clear supernatant was collected. The molar ratio of BSA and the activated ester was 1 : 50. BSA (13.4 mg) was dissolved in 4.8 ml carbonate buffer (0.13 M $NaHCO_3$) and 480 µl of the supernatant containing the activated ester was added dropwise to the BSA solution and stirred for 4 h at room temperature. The solution was pipetted onto a G-25 Sephadex column (GE Healthcare, Munich, Germany) conditioned with 1/10 PBS buffer (10 mM sodium dihydrogen phosphate, 70 mM disodium hydrogen phosphate, 145 mM sodium chloride, pH 7.6). The same buffer was used for the elution of the conjugate. Fractions were collected in a microtiter plate (UV star, Greiner Bio-One, Frickenhausen, Germany) and their absorbance was measured photometrically at 280 nm. The degree of labeling (DOL) was determined by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS) (Autoflex III, Bruker Daltonics, Bremen, Germany). Sample preparation, measurement and data processing were performed according to Bahlmann et al. (2009). The mean coupling ratio of the E1–BSA conjugate was 20 (±6.6) molecules of E1 per BSA molecule. The concentration of the E1–BSA was determined photometrically at 280 nm and 620 nm using a calibration curve of BSA. The concentration was 7.3 mg/ml.

2.2.2. Enzyme tracer synthesis

For ELISA enzyme tracers are required. Several conjugates with HRP were synthesized.

The synthesis of E1–HRP was performed via coupling of E1-6-CMO to HRP. The method was adapted from Munro and Stabenfeldt (1984) for the conjugate synthesis of progesterone. First, the hapten is activated followed by the coupling to HRP. Five micromole (1.8 mg) E1-6-CMO, in the presence of 1 µl N-methylmorpholine, was dissolved in 100 µl anhydrous N,N-dimethylformamide (DMF) and activated by 1 µl iso butyl chloroformate at a temperature of −21 °C under nitrogen atmosphere. The molar ratio of HRP to the activated ester was 1 : 50. After stirring for 30 min at room temperature, the solution was added dropwise to 4 mg HRP dissolved in 50 µl H_2O and 30 µl DMF at a temperature of −21 °C. The mixture was stirred for 1 h at −21 °C and additional 2 h at 0 °C. Afterwards, the solution was pipetted onto a G-25 Sephadex column conditioned with 1/10 PBS buffer (10 mM sodium dihydrogen phosphate, 70 mM disodium hydrogen phosphate, 145 mM sodium chloride, pH 7.6). The same buffer was used for the elution of the