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Research paper

Toxin–antigen conjugates as selection tools for antibody producing cells

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ABSTRACT

The generation of antibodies with designated specificity requires cost-intensive and time-consuming screening procedures. Here we present a new method by which hybridoma cells can be selected based on the specificity of the produced antibody by the use of antigen–toxin-conjugates thus eliminating the need of a screening procedure. Initial experiments were done with methotrexate as low molecular weight toxin and fluorescein as model antigen. Methotrexate and a methotrexate–fluorescein conjugate were characterized regarding their toxicity. Afterwards the effect of the fluorescein-specific antibody B13-DE1 on the toxicity of the methotrexate–fluorescein conjugate was determined. Finally, first results showed that hybridoma cells that produce fluorescein specific antibodies are able to grow in the presence of fluorescein–toxin-conjugates.

1. Introduction

Monoclonal antibodies (mAbs) are the most important binding molecules in biomedical research as well as in clinical diagnostics and therapy. Although mAbs are such invaluable tools the conventional technology used for the generation and screening of antibody-producing hybridoma cells has not much changed since its invention in the 1970s (Köhler and Milstein, 1975). For the production of monoclonal antibodies by hybridoma technology B lymphocytes of an immunized laboratory animal – in most cases of a mouse (*Mus musculus*) – are fused with myeloma cells. B cells provide the information for antibody production, and myeloma cells the information for unlimited cell division. After HAT selection of hybrids all generated hybridoma cells have to be screened for the production of the antibody of interest. Furthermore, the hybridoma cells have to be cloned using limiting dilution techniques before mass production can be established. This process is up to now a very expensive, time-consuming and low-throughput process. Although several selection strategies for high producing cell lines are described the conventional screening procedures are still state of the art. This might be due to complex sorting processes with specific equipment as described in several publications (Lee et al., 2008; Caron et al., 2008; Hanania et al., 2005). To find a general, simple and well-working selection principle is difficult because the combination of multiple chromosome sets often leads to instability which could affect the antibody related genes of the cell resulting in a loss of antibody production. The present study was aimed to find a procedure by which the generation of antigen-specific monoclonal antibodies by the hybridoma technique will turn out to become easier, faster and more cost effective. The new technique used a selection process which allows the survival of only those hybridoma cells that produce the antibody of interest. This process is based on toxin–antigen conjugates which induce cell death when hybridoma cells secrete unspecific antibodies. When an antigen-specific antibody is bound to the antigen the toxin will be neutralized i.e. it is no longer toxic for the cell. That means the addition of the toxin–antigen conjugate to a mixture of antigen-specific and non-specific hybridoma cells should enable only those cells to survive that produce the antigen-specific antibody (Fig. 1). Therefore screening and cloning procedures could be avoided to find and isolate those hybridoma cells that

Abbreviations: ATCC, American Type Culture Collection; mAbs, monoclonal antibodies; OVA, ovalbumin; BSA, bovine serum albumine; FITC, fluoresceinisothiocyanate; OVA-FITC, fluoresceinisothiocyanate linked to ovalbumin; BSA-FITC, fluoresceinisothiocyanate linked to bovine serum albumin; PBS, phosphate-buffered saline; HAT, hypoxanthine-azaserine-thymidine; FCS, fetal calf serum; NCS, neonatal calf serum; MTT, methylthiazolyldiphenyltetrazolium bromide; OD, optical density.

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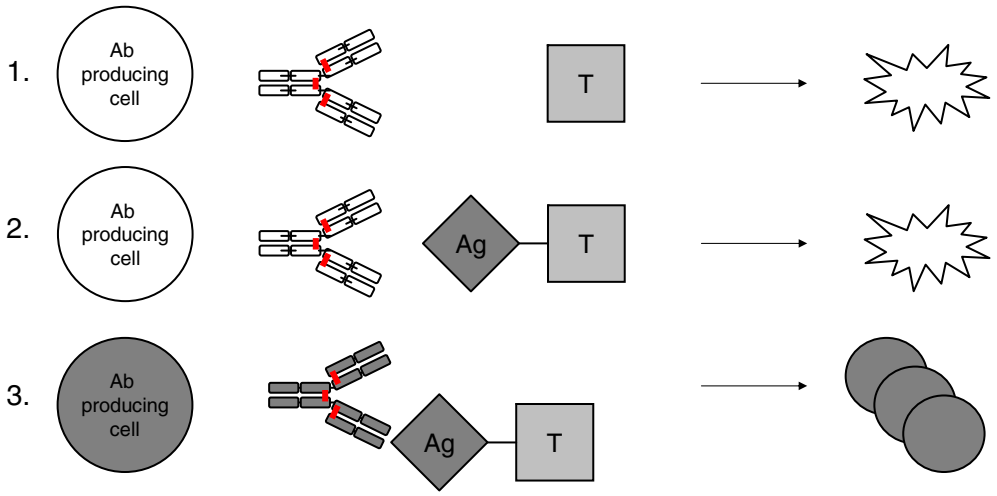

Fig. 1. Principles of the described selection process. 1. A toxin that kills hybridoma cells is identified. 2. Toxin is covalently linked to an antigen. Toxin–antigen conjugate is still toxic to hybridoma cells. 3. Toxin–antigen conjugate is no longer toxic if an antigen-specific antibody is bound to the conjugate.

produce antigen-specific antibodies in the vast multitude of antibody-producing cells after a hybridoma fusion. To show the feasibility of this new selection process a methotrexate–fluorescein conjugate was used as model conjugate. Using this conjugate a fluorescein-specific antibody-producing cell line could be selected and established after hybridomas were generated from a mouse immunized with a fluorescein carrier protein conjugate.

2. Methods

2.1. Toxicity assay

All assays to identify the concentration of optimum toxicity of methotrexate (Hexal, Holzkirchen, Germany) and the methotrexate–fluorescein conjugate (Invitrogen, Darmstadt, Germany) were performed in 96 well tissue culture plates. Hybridoma cells (clone E17-FH10, producing antibodies specifically binding horseradish peroxidase) were seeded (5000 cells/ml, 100 μl/well) in cell culture medium (RPMI 1640, PAN Biotech, Aidenbach, Germany) containing 10% fetal calf serum (FCS, Gibco Invitrogen, Darmstadt, Germany) and 2 mM glutamine. Methotrexate or methotrexate–fluorescein was added at different concentrations (100 μl/well). Cells without toxin or toxin–antigen-conjugate were cultivated in parallel as positive control. After incubation for seven days (under 7% CO_2 and 100% humidity) the amount of viable cells was determined using a cell proliferation assay. To evaluate the effect of the fluorescein-specific antibody B13-DE1 (Micheel et al., 1988) on the toxicity of methotrexate and methotrexate–fluorescein (both at a concentration of 6.25 μM) identical toxicity assays were performed with and without the addition of the purified fluorescein-specific antibody B13-DE1 (600 μg/ml = 4 μM).

2.2. Cell proliferation assay

Methylthiazolyldiphenyltetrazolium bromide (MTT) was used to determine the amount of viable cells. MTT (characterized by a yellow color) is cleaved to formazan (characterized by a purple color) by mitochondrial enzymes of living cells exclusively. Therefore the quantity of formazan produced correlates directly to the number of viable cells. At first, cell culture medium was removed completely and a fresh medium (100 μl/well) was added to the cells, followed by the addition of MTT (5 mg/ml in PBS, 10 μl/well). After incubation for 3 h (under 7% CO_2 and 100% humidity) the medium was removed completely and cells were lysed using dimethylsulfoxide (100 μl/well). The amount of produced formazan was determined by measuring the optical density (OD) at 550 nm.

2.3. Conjugation of fluoresceinisothiocyanate to proteins

Fluoresceinisothiocyanate (FITC) was linked to bovine serum albumin (BSA) or ovalbumin (OVA) as described in Gani et al. (1980). Briefly, a mixture of BSA or OVA (5 mg/ml), FITC (400 μg/ml) and NaH_3PO_4 (10 mM) in phosphate buffered saline (PBS) was incubated for 4 h at room temperature. The reaction products (designated BSA-FITC and OVA-FITC) were dialyzed against PBS at 4 °C overnight.

2.4. Purification of murine monoclonal antibody

Culture supernatants of antibody producing hybridoma cells were centrifuged (16,000 ×*g*, 5 min, 4 °C), filtered (0.45 μm) and mixed 4/1 with binding buffer (4 M NaCl, 2 M Glycin NaOH pH 8.9) before applying to a protein A column (HiTrap, Amersham GE Healthcare, Buckinghamshire, UK). The column was washed with diluted binding buffer (1/4) and antibodies were eluted using 0.1 M citrate pH 5.5 or 3.5. The eluted antibodies were neutralized with 500 μl 1 M Tris–HCl, pH 9.0 immediately. Purified antibodies were characterized using indirect and competitive enzyme-linked immunosorbent assays (ELISA).

2.5. Indirect enzyme-linked immunosorbent assay (ELISA)

Production of fluorescein-specific antibodies by hybridoma cells was shown using indirect ELISA as follows. Microtiter plates were coated with BSA-FITC (incubation overnight with 5 μg/ml protein in PBS, 50 μl per well), washed with tap water