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High-Yield Production of Recombinant Antibody Fragments in HEK-293 Cells Using Sodium Butyrate

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ABSTRACT

To develop new recombinant monoclonal antibody fragments for therapy and imaging, it is indispensable to have a simple and easy procedure to handle the eukaryotic expression system for production of proteins in high amounts. Gene amplification techniques such as the dehydrofolate reductase (DHFR) system in Chinese hamster ovary cells or the glutamine synthase system in myeloma cells have a couple of disadvantages. The selection procedure is complex, time-consuming, and not fruitful in all cases. The toxic drug methotrexate

(for the DHFR system) can increase the production rate but decreases the specific growth rate of the cells. The production rate is not always stable over a long-term cultivation period. To overcome these problems, we are using stably transfected human embryonic kidney (HEK-293) cells in combination with an efficient screening method. Sodium butyrate can increase the expression of recombinant antibody fragments in the transfectomas up to $500 \mu\text{g}/4.2 \times 10^7$ cells/24 h corresponding to $175 \mu\text{g}/\text{mL}$ culture medium. This strategy allows a rapid development of new recombinant monoclonal antibody fragments and allows one to proceed rapidly to in vivo testing.

INTRODUCTION

Mammalian cells are the best available expression system for the production of biologically active functional monoclonal antibodies (mAbs). High expression levels can be achieved by gene amplification systems, for instance in Chinese hamster ovary (CHO) cells with dehydrofolate reductase (DHFR) marker in combination with methotrexate or in myeloma cells with glutamine synthase marker in combination with L-methionine sulfoximine. Increasing concentrations of the drugs will lead to cells with a higher copy number of the marker genes. The copy numbers of the co-transfected objective genes are increasing simultaneously (1–4). Multiple rounds of selection by increasing concentrations of the drugs make this approach very time consuming (1–3). We (unpublished results) and others (5) observed that the specific growth rate of methotrexate-tolerant cells often decreased with increasing concentrations of the drug and that the production rate of the selected clones is not always stable for a long cultivation period. To solve these problems, we recombined the standard calcium phosphate transfection procedure (6) in human embryonic kidney (HEK-293) cells with a simple and efficient screening procedure (Reference 7, modified) and optimized the expression rate of the selected clones with sodium butyrate (8,9). We could not observe any significant decrease of the production rate of our selected cell clones without G418 in

the growth medium. However, we have not tested the expression stability for a long time period in particular (over 12 cell passages).

MATERIALS AND METHODS

Cell Culture

HEK-293 cells (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were grown in DMEM containing 4.5 g/L glucose, supplemented with 10% FCS, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 0.25 $\mu\text{g}/\text{mL}$ fungizone, and 4 mM glutamine in a 7.5% CO_2 incubator at 37°C . All tissue culture reagents were purchased from BioConcept (Basel, Switzerland).

Plasmid Construction

The cDNAs for chCE7 light and heavy chain (a gift from J. Jean-Mairet, ETH, Zurich) were cloned into the *HindIII/BamHI* site of pcDNA3.1+ (Invitrogen, Basel, Switzerland). All mutations were introduced in the cDNA of chCE7 heavy chain by high-fidelity PCR and standard molecular biology techniques (6). All mAb fragments have a 6xHis tag at the C-terminal end of the heavy chain for affinity purification via Ni-NTA agarose (Qiagen, Basel, Switzerland). All mutations were confirmed by DNA sequencing (Mycrosynth, Balgach, Switzerland). The sequences of oligonucleotides used for mutagenesis in this study are available on request.

Transfections

Cells were transfected using the calcium phosphate method (6). Briefly, 1×10^6 cells per transfection were seeded one day before transfection. Fifteen micrograms of pcDNA3chCE7L (light chain) and 15 μg pcDNA3chCE7H (heavy chain, respectively mutants of the heavy chain) were dissolved in 440 μL water, and 61 μL 2 M CaCl_2 were added. The DNA mixture was dropped into 500 μL 2x HBS (HEPES-buffered saline: 50 mM HEPES, pH 7.10, 280 mM NaCl, 1.5 mM Na_2HPO_4), and the calcium phosphate DNA precipitate was allowed to stand for 20 min at

room temperature. Fresh complete medium (10 mL) was added to the cells before the transfection mixture was added. After 24 h, the cells were washed twice with medium and grown for 2–3 days in complete medium. The cells were trypsinized, and a quarter of the cells were replated onto 10 new 10-mm cell culture dishes in complete medium supplemented with 1 mg/mL G418, which was changed every 5 days. After about 2 weeks, resistant colonies were isolated by overlaying the culture plates with agarose and lifting the agar along with the colony beneath with a 1 mL syringe (Reference 7, modified). For the lifting procedure, sterilized 1.8% agarose was melted and kept at 55°C. Cell culture plates were washed twice with complete medium warmed up to 37°C before a mixture of 6 mL complete medium warmed up to 37°C and 2 mL of the 1.8% agarose were poured on the plates. After the agarose cooled down to room temperature, the plates can be stored for several days in the cell incubator. Before lifting the agar, the cell culture dishes were cooled in the refrigerator for 5 min.

Screening for Expression

The isolated colonies were propagated in 24-well cell culture plates to confluency. Cells were washed twice with serum-free medium, and 200 μ L serum-free medium were added to each well. The cells were incubated for 5 h at 37°C, 7.5% CO₂, and the supernatants were centrifuged to remove cell debris. The supernatants (150 μ L) were used to transfer the proteins on Immobilon™-P membranes (Millipore, Volketswil, Switzerland) with a Bio-Dot SF apparatus (Bio-Rad Laboratories, Glattburg, Switzerland). Complete medium was added to the cells to facilitate further cultivation of high producer cell lines.

The membranes were blocked with 10% dry milk/PBS for 1 h, incu-

bated with a 1:15 000 dilution of an anti-human IgG (H+L) HRP conjugate (Promega, Wallisellen, Switzerland) in 5% dry milk/PBS for 30 min, and were washed three times for 10 min each with TBST (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween® 20). The membranes were rinsed with water, and the antibody fragments were detected with SuperSignal® chemiluminescence substrate from Pierce Chemical (Socochim, Lausanne, Switzerland).

Cell Binding Assay

To quantify the amounts of immunoreactive chCE7 mAb fragments in the supernatant of the producer cell lines, we used a cell binding assay with SK-N-AS neuroblastoma cells (10). Briefly, antibody chCE7 (100 μ g) was labeled in 300 μ L PBS with 5 mCi (18.5 mBq) of ¹²⁵I using a glass tube coated with 20 μ g iodogen (Pierce Chemical). Labeled antibody was purified on a Sephadex® G10 column (PD10; Amersham Biosciences, Dübendorf, Switzerland).

SK-N-AS cells were used to coat 24-well microplates as described previously (10). Cells were presaturated with 500 μ L PBS containing 0.5% BSA for 60 min at 37°C. Binding assay were performed in duplicate by incubating 10⁵ cpm ¹²⁵I-chCE7 with in-

Figure 1. Slot-blot analysis of selected HEK-293 clones for chCE7 F(ab')₂ expression. Thirty selected clones were grown in 24-well cell culture dishes, and secreted chCE7 F(ab')₂ fragments were collected in 200 μ L serum-free medium over a time period of 5 h. Samples were centrifuged, and 150 μ L of the supernatant were transferred to PVDF membrane with a slot-blot apparatus. The blot was blocked with 10% dry milk/PBS for 1 h, incubated with an anti-human IgG (H+L) HRP conjugate in 5% dry milk/PBS for 30 min. The membrane was washed three times with TBST, and the antibody fragments were detected with chemiluminescence substrate. Arrows indicate clones with a high expression rate. As control, 150, 15, and 1.5 ng chCE7 mAb were transferred.

Figure 2. Stimulation of HEK-293 transfectomas with sodium butyrate. Transfectomas (4.2×10^7 cells) were grown in 25 mL DMEM/10% IgG-free FCS supplemented with different concentrations of sodium butyrate in 14-cm cell culture dishes for 8 days. An aliquot of the cell culture medium was used for quantification of secreted bioactive chCE7 mAb/F(ab')₂ using a cell binding assay with SK-N-AS cells. We did this experiment twice with the same result. Cell clones stimulated with the optimal concentration of sodium butyrate (6 mM) yield $\pm 20\%$ of the production rate.

creasing concentrations of unlabeled chCE7 (0.003–3 µg) as standard or with different amounts of the cell supernatants in PBS/0.5% BSA (total volume 500 µL). The probes were incubated for 16 h at 37°C. The plates were washed three times with ice-cold PBS/BSA, and the cells were dissolved

in 500 µL 1 M NaOH. Radioactivity was counted in a γ -counter. Nonspecific binding was determined in parallel in the presence of 10 µg unlabeled chCE7 and was subtracted. Analysis of the saturation curves was done according to the Scatchard method.

Purification of 6×His-Tagged mAb Fragments

High producer transfectomas were stimulated with 6 mM sodium butyrate in the growth medium for eight days. The cell culture medium was clarified by filtration through a 0.2 µm Express Plus filter (Millipore). The concentration of immunoreactive mAb fragments was quantified using a cell binding assay as described previously (10). NaCl (150 mM), 15 mM imidazole, and sufficient quantity of Ni-NTA agarose were added to the media, and the samples were incubated for 16 h at 4°C. The resin was washed on a column with 10 volumes of PBS, 10 volumes of PBS/1 M NaCl, 20 mM imidazole, and 5 volumes of PBS.

Figure 3. SDS-PAGE analysis of secreted chCE7 mAb fragments of a chCE7 F(ab')₂ transfectoma cell line. Affinity-purified mAb fragments of a chCE7 F(ab')₂ stably transfected cell line were separated by SDS-PAGE under nonreducing conditions (A). Protein bands I and II were cut out of the gel and were analyzed under reducing conditions (B). The gels (7.5% and 12.5%, respectively) were stained with Coomassie Brilliant Blue®.

Bound proteins were eluted in 1 mL fractions with PBS/500 mM imidazole. Fractions were checked by SDS-PAGE, and the peak fractions were polished using HILoad™ 16/60 Superdex 200 gel filtration column and Äkta™prime system (Amersham Biosciences). The flow rate was 1 mL/min using PBS buffer.

RESULTS AND DISCUSSION

Our goal was to combine known simple techniques for establishing high producer cell lines for production of recombinant mAb fragments and to increase the expression rate with sodium butyrate. We are applying these techniques to a clinically relevant antibody. The chimeric chCE7, a IgG type 1 mAb that is used in a clinical trial for radioimmunodiagnosis of neuroblastoma patients, binds to a 200 kDa cell-surface protein known as L1-CAM (11,12). With our outlined expression strategy, we are able to test various different chCE7 mAb fragments for radioimmunotherapy in an in vivo xenograft model.

After selection of transfected HEK-293 cells with G418, we isolated single cell colonies from the cell culture dish by overlaying the plate with agarose and lifting the agarose with the colony beneath with a syringe (Reference 7, modified). This method allowed us to isolate a sufficient number of clones for each mAb construct to find clones with high expression rates. In general, we isolated 48 colonies. For the screening, monolayers of the transfectomas were grown in 24-well cell culture plates to confluency. Secreted mAb fragments were collected over a 5-h time period and were transferred to a PVDF membrane with a slot-blot apparatus. The membranes were analyzed with a peroxidase-coupled anti-human IgG and SuperSignal reagent. The transfection and selection techniques are applicable for almost all antibody constructs. The specificity of the peroxidase-coupled antibody for the screening procedure depends on the produced antibodies. Figure 1 shows an example [chCE7 F(ab')₂]. High-expression cell clones (Figure 1, arrows) were propagated, and the expression rates of immunoreactive chCE7 mAb fragments were analyzed using a cell binding assay (10). Clones with the highest expression

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rates were used for optimizing the sodium butyrate concentration (Figure 2). Cells were grown on 14 cm cell culture dishes until confluency. Twenty-five milliliters of complete medium (IgG-free) were added to the cells supplemented with the indicated concentrations of sodium butyrate. Cells were grown for eight days at 37°C, 7.5% CO₂. The quantification was done using a cell binding assay with SK-N-AS cells (10).

We analyzed affinity-purified recombinant chCE7 mAb (data not shown) and chCE7 F(ab')₂ fragments by SDS-PAGE under nonreducing conditions (Figure 3A). In contrast to the full covalently assembled chCE7 mAb, we observed two bands with at molecular mass of approximately 120 kDa and approximately 60 kDa for the chCE7 F(ab')₂ (Figure 3A). SDS-PAGE under reducing conditions shows (Figure 3B) that both bands comprise the truncated heavy chain and the light chain of chCE7. From this, we conclude that the upper band in Figure 3A presents the fully assembled chCE7 F(ab')₂ fragment and the lower band presents the monovalent chCE7 Fab fragment. We observed this phenomenon also for a chCE7 [CH2 fragment (data not shown)]. This partially incomplete assembly of recombinant mAb fragments in mammalian cells was also observed by others (13).

With our expression system, for instance, we are able to produce recombinant chCE7 mAb at about 400 µg/4.2 × 10⁷ cells/24 h corresponding to 130 µg/mL medium. Fully functional recombinant chCE7 F(ab')₂/Fab fragments (with a 40%–60% ratio) (Figures 2 and 3) can be produced at 500 µg/4.2 × 10⁷ cells/24 h corresponding to 175 µg/mL medium. The original chCE7 myeloma cell line produces 2.5 µg/mL medium in a continuous culture system (14). The use of amplifiable markers for mAb production resulted in production rates of 200 µg/mL (Campath-1H) (3), 560 µg/mL (cB72.3 IgG4) (1), and between 30 and 500 µg/mL culture fluid for five different mAbs (15), for example. This shows that the use of HEK-293 cells in combination with sodium butyrate is comparable with the production of mAbs with a gene amplification system.

Mono- and divalent recombinant mAb fragments can be separated using

HiLoad 16/60 Superdex 200 gel filtration column. This approach allows us to test various mAb fragments for in vivo evaluation within a reasonable time.

REFERENCES

1. Bebbington, C.R., G. Renner, S. Thomson, D. King, D. Abrams, and G.T. Yarranton. 1992. High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. *Biotechnology (NY)* 10:169-175.
2. Crouse, G.F., R.N. McEwan, and M.L. Pearson. 1983. Expression and amplification of engineered mouse dihydrofolate reductase minigenes. *Mol. Cell Biol.* 3:257-266.
3. Page, M.J. and M.A. Sydenham. 1991. High level expression of the humanized monoclonal antibody Campath-1H in Chinese hamster ovary cells. *Biotechnology (NY)* 9:64-68.
4. Wood, C.R., A.J. Dorner, G.E. Morris, E.M. Alderman, D. Wilson, R.M. O'Hara, Jr., and R.J. Kaufman. 1990. High level synthesis of immunoglobulins in Chinese hamster ovary cells. *J. Immunol.* 145:3011-3016.
5. Yoshikawa, T., F. Nakanishi, Y. Ogura, D. Oi, T. Omasa, Y. Katakura, M. Kishimoto, and K. Suga. 2000. Amplified gene location in chromosomal DNA affected recombinant protein production and stability of amplified genes. *Biotechnol. Prog.* 16:710-715.
6. Sambrook, J. and D. Russel. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd ed. CSH Laboratory Press, Cold Spring Harbor, NY.
7. Walls, J.D. and B.W. Grinnell. 1990. A rapid and versatile method for the detection and isolation of mammalian cell lines secreting recombinant proteins. *BioTechniques* 8:138-142.
8. Gorman, C.M., B.H. Howard, and R. Reeves. 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res.* 11:7631-7648.
9. Grünberg, J., U. Luginbühl, and E.E. Sterchi. 1992. Proteolytic processing of human intestinal lactase-phlorizin hydrolase precursor is not a prerequisite for correct sorting in Madin Darby canine kidney (MDCK) cells. *FEBS Lett.* 314:224-228.
10. Novak-Hofer, I., H.P. Amstutz, A. Haldermann, K. Blaser, J.J. Morgenthaler, P. Blauenstein, and P.A. Schubiger. 1992. Radioimmunolocalization of neuroblastoma xenografts with chimeric antibody chCE7. *J. Nucl. Med.* 33:231-236.
11. Hoefnagel, C.A., M. Rutgers, C.K. Buitenhuis, L.A. Smets, J. de Kraker, M. Meli, F. Carrel, H. Amstutz, et al. 2001. A comparison of targeting of neuroblastoma with mIBG and anti L1-CAM antibody mAb chCE7: therapeutic efficacy in a neuroblastoma xenograft model and imaging of neuroblastoma patients. *Eur. J. Nucl. Med.* 28:359-368.
12. Meli, M.L., F. Carrel, R. Waibel, H. Amstutz, N. Crompton, R. Jaussi, H. Moch, P.A. Schubiger, and I. Novak-Hofer. 1999. Anti-neuroblastoma antibody chCE7 binds to an isoform of L1-CAM present in renal carcinoma cells. *Int. J. Cancer* 29:401-408.
13. Lund, J., N. Takahashi, A. Popplewell, M. Goodall, J.D. Pound, R. Tyler, D.J. King, and R. Jefferis. 2000. Expression and characterization of truncated forms of humanized L243 IgG1. Architectural features can influence synthesis of its oligosaccharide chains and affect superoxide production triggered through human Fcγ receptor I. *Eur. J. Biochem.* 267:7246-7257.
14. Amstutz, H., Ch. Rytz, I. Novak-Hofer, M. Spycher, P.A. Schubiger, K. Blaser, and J.-J. Morgenthaler. 1993. Production and characterization of a mouse/human chimeric antibody directed against human neuroblastoma. *Int. J. Cancer* 53:147-152.
15. Trill, J.J., A.R. Shatzman, and S. Ganguly. 1995. Production of monoclonal antibodies in COS and CHO cells. *Curr. Opin. Biotechnol.* 6:553-560.

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