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Influence of the different genetic parameters and regulatory sequences on recombinant expression on a monoclonal antibody

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Recombinant human antibody production represents a major growing class of biopharmaceuticals based on the technological progress within the last decades especially in CHO cells. The HIV neutralizing human monoclonal antibody 2F5 was developed as hybridoma from human lymphocyte preparations. In order to estimate the potential of recombinant 2F5 expressing CHO cells, we generated five different recombinant CHO cell lines by varying regulatory sequences, the codon usage, the signal peptides and the transfection technique. These 2F5 expressing cell lines were developed by selection of the best producer, clone homogeneity and clone stability. The gene copy number of the clones differed significantly due to MTX amplification. In one cell line we identified only one copy of heavy chain and two copies of light chain. Neither the gene copy number nor the promoter was found to influence the amount of transcript exclusively emphasizing the positioning effect of the transgene. Codon optimization seemed to affect mRNA stability positively, but unexpectedly the amount of secreted product was not elevated in this configuration. Additionally, simple evaluation of intracellular product leaves the question if the product accumulates by stress response or if this phenomenon is positively linked with the secretion rate.

An optimized hybridoma method for cloning post-germinal center human IgG antibodies

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Hybridoma methods have the advantage that they can directly access these antibodies in their native configurations. We previously described a hybridoma method for cloning native human antibodies that uses a murine myeloma cell line that ectopically expresses the human telomerase catalytic subunit gene (hTERT) and the murine interleukin-6 gene (mIL-6). In the present study, we expressed hTERT and mIL-6 in a heterohybridoma (murine/human) cell lines to create a cell line (B5-6T) with improved stability of hTERT expression and improved cell fusion characteristics. Peripheral blood B-cells were selected for CD27 expression and cultured *in vitro* in the presence of IL-4, IL-10, and CD40 ligand activity and fused to the B5-6T cell line to create stable hybridomas secreting human antibodies. ELISA analysis and DNA sequencing of the cloned immunoglobulin genes showed that the antibody populations were highly enriched for post-germinal center IgG antibodies. The frequencies of hybridomas expressing IgG antibodies specific for botulinum neurotoxins and vaccinia virus antigens were increased by this method. Using this method, we have cloned human antibodies specific for botulinum neurotoxins using this method, some of which have nanomolar binding affinities and potent neutralizing activities.

A humanized bispecific antibody targeting EpCAM-expressing tumors and produced in B cell hybridoma

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Bispecific antibodies (bsAb) are excellent activators of the immune system, with clinical data showing that patients suffering from advanced ovarian cancer with ascites formation dramatically respond to treatment with an anti-EpCAMxanti-CD3 bsAb. Clinical use of bsAb, however, is hampered for technical reasons, namely lengthy purification, low yield, expensive production engendered by the conventional quadroma technology, mouse origin of the antibodies, and their large molecular weight that hinders diffusion into solid tumours.

To circumvent those bottlenecks we use B cell hybridomas trained by the immune system to produce high amounts of antibodies and genetic engineering to reduce immunogenicity (human constant domains) and to generate smaller fusion proteins for an improved tissue penetration (reduction of molecular weight from 150 kDa to 80 kDa). Here we describe a method to redesign the active antibody gene locus of an anti-EpCAM producing hybridoma line (HEA125) by homologous recombination. We cloned a gene cassette encoding a disulfide-stabilized anti-CD3 antibody (dsOKT3) fused to the human CH1 domain of the IgG1 heavy chain. The cassette was flanked by regions homologous to the endogenous antibody locus, where the sequences overlap with the 5'end of the CH1 domain and the 3' end of the CH3 domain. After homologous recombination, murine constant domains (CH1-3) are replaced by the fusion protein. The resulting small bsAb HEA125xdsOKT3 was shown to bind both EpCAM and CD3 antigens on target cells and is currently tested *in vitro* for its potency to prime T-cells for destruction of EpCAM-expressing tumor cells. Thereby the Fab fragment of the chimerized anti-EpCAM antibody functions as the tumour-targeting portion, while the second moiety of the bsAb is composed of a disulfide stabilized anti-CD3 single-chain antibody that enables activation of $CD3^+$ cytotoxic T-cells. The new method to produce humanized bsAb can easily be adapted to other specificities.

Hock Immunization: An alternative immunization route that streamlines the hybridoma development process

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Herein we report the results of a comparison study between two routes of immunization, footpad and hock, for the development of monoclonal antibodies via conventional hybridoma technology using standard rodent species. Immunization via the rear footpad, with adjuvant, is a commonly used route for antigen delivery. The footpad route has several advantages, including the low amounts ($\leqslant 2$ μg/animal) of antigen required and the rapid induction of high serum titers following hyperimmunization. However, in some instances the antigen, and/or adjuvant components, may induce pain and distress in the animals, which may ultimately impair the animal's immune response. In rats and mice, both the hock and footpad routes are equally easy to administer. Both routes also target lymphocyte recruitment to the popliteal lymph nodes. For the hock-based immunizations, the immune response observed was comparable to, or better than, the response observed following the footpad immunizations. Furthermore, FACS analysis confirmed that the populations of B and T cells in the lymph nodes were comparable for both the hock and footpad immunized animals, as were the number of positive clones identified from the comparative fusions. In summary, the hock route of immunization represents an easy, robust, and importantly, more humane technique that could be readily used for the development of mouse, rat and hamster hybridomas, including human IgG producing hybridomas derived from transgenic mice.

Discovery and production of recombinant human oligoclonal antibodies

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Monoclonal antibodies have been successfully applied in multiple therapeutic indications but in many cases a ceiling in potency and efficiency has been reached: certain indications and/or patient populations cannot be treated with the current antibody products while rapidly mutating pathogens or cancer cells escape monoclonal antibody treatment by changing the target of the antibody. In this context, mixing of monoclonals has gained renewed interest. Both pre-clinical and clinical studies have shown that targeting of multiple mechanisms on cancer cells or pathogens can pro-

vide a synergistic enhancement in potency and reduce the generation of escape variants. However, serious drawbacks remain that hinder the widespread application of these approaches, such as the high cost associated with production and testing of the separate cocktail constituents as well as the reproducibility of production processes.

The Oligoclonic^{TM} platform combines the advantages of antibody cocktails with conventional monoclonal antibody production. In this approach, clonal cells are transfected with multiple IgG encoding genes. The cocktail constituents are carefully selected to maximize synergy and prevent escape generation. The stable Oligoclonal^{TM} cell lines thus generated produce a cocktail of antibodies in a manner as cost effective as a monoclonal antibody.

To generate an Oligoclonal^{TM} cell line, a single V_L region is required to pair with multiple V_H regions, each of which defines a unique specificity. To demonstrate proof-of-principle of the 'single V'_L concept, a phage display repertoire was constructed from a single human antibody light chain and the antibody heavy chains harvested from the blood of human donors immunized with tetanus toxoid (TT) vaccine. After selection, 129 unique anti-TT antibodies representing 53 V_H region rearrangements were isolated. Comparison of this repertoire with a published anti-TT repertoire that was obtained by a method that preserves cognate V_H and V_L pairing showed a very similar size, distribution of V_H, D_H and J_H gene segment utilization, epitope diversity and affinity. In addition, the antibodies are protective in-vivo and demonstrate synergy when used in cocktails. We conclude that a large fraction of human anti-TT-encoding V_H regions from an immunized repertoire readily combines with a single antibody light chain resulting in a large and diverse panel of high-affinity antibodies.

To validate the production of antibody cocktails in clonal cell lines, PerC6® cells were transfected with plasmids encoding three different antibodies. Clonal cells expressing these three specificities were identified, subcloned, adapted to growth in shake flasks and the resulting stable cell lines tested in batch production assays. The results show high IgG production levels and expression of the three specificities in stable ratios.

Thus the Oligoclonic^{TM} platform, based on the generation of defined single V_L antibody cocktails expressed in single clonal cell lines using conventional methods, provides a robust and economical route for the production of potent oligoclonal antibody preparations.

Low-similarity to the host proteome is a common denominator of B cell epitopes

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Meta-analysis of experimental data shows that the antigenic fragments targeted by antibodies are characterized by a low level of amino acid sequence similarity to the host proteome. Thus, the information about the antigen-antibody reaction appear to be packed into a single value: the similarity level of the epitopic peptide sequence to the host proteome. The similarity concept allows the cataloguing of the human peptide immunome and solves the immunological Self/Nonself problem in terms of Similar/Dissimilar sequences.

Comparing the outcome of the use of two (2) myeloma cells lines (NS0 & SP2/0) in the development of monoclonal antibodies against Hodgkin's Lymphoma and Human Endogenous Retrovirus cell lines, their reactivity profiles and growth characteristics

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SP2/0 and NS0 myeloma cell lines are characteristically different. SP2/0 myeloma cells are hybrid cells and NS0 are non-hybrid. It therefore was necessary to monitor their growth characteristics and if they make any difference at all in the Monoclonal Antibody development as well as the quality of hybridomas formed using these cells. The singular, independent monitor of the growth characteristic of these cell lines is a tendency to apoptosis and variable growth density and viability [1–3]. Reduced protein supplementations and introduction of cytokines including IL6 [3] made a difference to viability and quality of hybridoma cells formed when employed as fusing partners to splenocytes. The results showed monitor of growth, variably in Serum-Free (SFM) and Serum-Rich medium (SRM) without any particular stringency to the medium in which these cells were grown (if grown in SFM the cell lines were treated the same. This also applied to growth in SRM).

NS0 appears more robust. Seeded at 10^3 in 25 cm^3 on Day1. Well carpeted in a 25 cm^3 flask by Day 7, (Compared with SP2/0).

The subsequent programs carefully distributed the cell lines to various fusion programs and transfection with the HERV plasmids. The results did not show a definite advantage of NS0 cell line over SP2/0, although it could be argued out that there probably were more Hybridomas following fusion with NSO cell lines. This did not translate to qualitative advantage.

Introduction of cytokines/anti-apoptotic agents would have made a significant difference in the fusion yield at the very outset. Some studies in this regard of introducing appropriate anti-apoptotic stimuli [4–6] had been addressed by many researchers.

Here we also present the reactivity profiles and how they influence the validation techniques of the various monoclonal antibodies developed.

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Comparative account of 4 protein purification methods used as part of methodology in Development of novel Monoclonal antibodies

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Protein purification is pivotally an important step in development of Monoclonal antibody (MAb). The concentrations of the various immunoglobulins (proteins) in many ways reflect the reactivities and therefore the outcome in subsequent investigative works. Efficient protein purification could indeed therefore make a significant difference in terms of time, cost, quality of the results, reproducibility of results and convenience.

We here present the outcomes of four (4) *methodologies* in protein purification and the impact on the results of various investigations. The various techniques used include, manual purification, ammonium sulphation method and the use of 2 automation methods (Biological HR from Bio-rad, UK; and the AKTA FPLC multi-purpose equipment form GE Healthcare, UK).

In terms of quality of the purified proteins-expressed in reactivities – there is very little difference in the outcome of the various immuno-assays carried out. It is obvious that in terms of ease, convenience and purification of large quantities of immunoglobulins, the AKTA machine comes out the best. The manual technique is laborious, but the yield is appreciable and of equally high quality.

The various purified protein results were matched against using the fresh supernatant, unpurified. In our hands there probably is not a great deal of difference; if anything at all there seems to be a better quality to the Immuno-histochemistry (IHC) and immunoblot assays. The chemiluminescence and ELISA results are in this case very subjective (especially of the latter), with the use of non-purified, supernatant immunoglobulin.

Overall the use of automation is convenient, time saving and reproducible in terms of results; and large volume of supernatant can be reduced to small concentrations for more quality outcomes. The other noteworthy lesson is that the parent supernatant can always be consulted for verification of the outcome on immuno-assays, in cases of doubt.

TAKE HOME MESSAGE

1. Automation techniques are by far the most superior way of purification
2. In terms of doubtful results parental supernatant can be tested for confirmation of outcome, although in many cases the volume of protein use will be higher.
3. Unpurified supernatant can be unreliable at times in interpreting ELISA and Chemiluminescence results.
4. For higher concentration of supernatant and more elaborate work, automation techniques can be useful.
5. Ammonium sulphation can be further used to concentrate an already, automated purified protein.

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Improvement of EBV transformation in a mitogenic microenvironment by platelet activation

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Epstein – Bar virus (EBV) transformation of human B cells used as a source of permanent genetic materials and human antibody production. However, efficiency of different EBV transformation protocols is a live subject of research especially for production of antibody. We improved EBV transformation in a mitogenic microenvironment using activation of platelets by HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in vitro. All transformations of previously sensitized peoples with antigens of Rh blood group system were successful and clones of lymphoblastoid cell lines (LCLs) appeared on third day of transformation. Supernatant of 96 well plates screened for the presence of specific antibodies at 7-10 days of transformation. The time for raising LCLs is critical for a successful antibody production.

Cephalin as an efficient fusogen in hybridoma technology, can it replace Poly Ethylene Glycol?

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In this study we set up a simple, fast and highly efficient protocol to fuse cells and to produce human hybridoma using non-toxic cephalin as a fusogenic lipid and compared our proposed method with PEG mediated fusion as a known conventional method. Human lymphoblastoid cells were fused with F3B6 heteromyeloma cell line using cephalin or PEG as fusogenic compound. The viability of the cells and their fusion rate was determined microscopically and hybridoma (antigen specific and non- specific) production yield was calculated following HAT selection and screening. The fusion rates of cells in cephalin and PEG mediated methods was comparable (25.9 $\pm$ 5.73% versus 27.3 $\pm$ 6.07%) while the viability of the cells immediately and after overnight incubation was obviously more in cephalin method compared to PEG ($p < 0.001$). Our proposed cephalin mediated cell fusion method is about 5 times more efficient than PEG in production of hybridoma clones thus it may dismiss PEG as the most generalized fusogen of scene.

An extreme strategy to production of hybridoma

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Ethical issues of human immunization hamper human monoclonal antibody technology. Thus, it is reasonable to improve techniques to obtain maximum efficiency of hybridoma production. We used a new strategy to bypass toxic effects of poly ethylene glycol (PEG) as fusogen and hypoxanthine aminoptrin thymidine (HAT) as selective medium on newly fused cells. Cephalin used as a fusogen and the heteromyeloma pretreatment with emetine and actinomycin D as selection methods. The Epstein bar virus (EBV) transformed peripheral blood mononuclear cells (PBMC) of previously sensitized persons with antigens of Rh blood group system fused with pretreated heteromyeloma. The hybridoma production rate was about 19–34% of EBV

transformed human cells introduced in fusion process. This improvement may make hybridoma production as a plug and play technology and an ending to fusion efficiency problem; thus leads to new insight to basic concepts as the nature of antibody producing cells and definition of a good fusion partner.