SESSION 4 : MOLECULAR BIOLOGY

Efficient utilization of available sequence space: generation of hAbs for medicine through consensus human Ab library and trinucleotide-based mutagenesis method

Liming Ge, Achim Knappik, Peter Pack, Vic Ilag, Bernhard Virnekäs and G. Wellnhofer
MorphoSys GmbH, Munich, GERMANY

Human antibodies (hAbs) against various disease-related antigens are highly desirable but not always available. Using the combinatorial approach, it is possible to generate human antibody fragments against those antigens with relatively high affinity. However, this approach requires enormous libraries which are not always feasible. It is reasoned that such large-sized libraries are necessary to represent the true functional repertoires. We have undertaken two approaches to address this problem. First, the human antibody repertoire is composed of sequences that are closely related and their diversity has little to do with the binding affinity. We have compared the available human antibody sequences in the database and generated a set of human consensus sequences which consists of 7 VH, 4 V(*k*) and 3 V (*lambda*) chains. With this set of consensus sequences, it is now possible to generate the entire human antibody repertoire with limited framework diversity. Due to their complete synthetic nature, these consensus sequences are designed to increase their expressibility in *E. coli* and to facilitate site-directed and random mutagenesis to imitate the affinity maturation process in nature. Secondly, even with the optimized 'rationally' designed random mutagenesis methods, it is not possible to exclude completely: (a) stop codons, (b) structure breaking residues, (c) residues that are known to lead to non-functional antibodies. Random mutagenesis using the NNK approach which is the most prevalently employed method, generates all possible combinations of sequences but at the expense of the quality of the available sequence space. We have developed the trinucleotide-based mutagenesis method with which we introduced only the residues that are known to give rise to functional antibodies, and thus were able to concentrate on the sequence space which is known to be entirely functional. The results from both of these approaches will be presented and their implications in the field of high affinity human antibody in medicine will be discussed.

Development and usage of semi-synthetic scFv repertoires displayed on phage

Ahuva Nissim†, Dario Neri & Greg Winter*
*MRC Centre for Protein Engineering and *MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, UK.* [†*Current address: Chaim Sheba Medical Center, Tel-Aviv University, Tel Hashomer, ISRAEL*]

The display of repertoires of antibody fragments on the surface of filamentous bacteriophage offers a new way of making antibodies, bypassing hybridoma technology and even immunization. To make human antibodies entirely *ex vivo*, a repertoire of rearranged V-genes were built using germline V-gene segments as building blocks. We used a bank of cloned VH gene segments to make a synthetic repertoire of VH-genes rearranged *in vitro* by incorporating a third hypervariable loop of between 4 and 12 residues of random sequence. The rearranged VH genes were then cloned with a single unmutated V(*lambda*)3 segment as single chain Fv fragment for phage display. This 'single pot' repertoire (5×10^8 clones) has been selected with a range of foreign and self antigens, obtaining specificities that have proven very difficult by the conventional hybridoma technology. Binding activities were isolated against haptens, secreted and cell surface proteins, viral coat proteins, and intracellular antigens from the lumen of the endoplasmic reticulum and the nucleus. Both phage and scFv fragments secreted from infected bacteria were used as monoclonal and polyclonal reagents in Western blots, epitope mapping and cell staining. The affinities of antibody fragments derived from this 'single pot' libraries fall in the range 10^6 - 10^7 M^{-1} which is typical of primary immune responses. To improve the affinities of single clones selected from this library, we built a repertoire of V(*lambda*)3 segment using the selected scFv as building blocks and rearranged it *in vitro* by incorporating random sequence of V(*lambda*)3-CDR3, while maintaining the original selected VH segment. This secondary repertoire was subjected for further rounds of selections improving the affinities of scFv anti-cytochrome C, maltose binding protein and Endoglin to nM ranges.

Human antibodies isolated from a large non-immunized phage repertoire

A. J. Roberts, T. J. Vaughan, A. J. Williams, J. K. Osbourn, J. C. Earnshaw, J. Thompson and K. S. Johnson

Cambridge Antibody Technology Ltd, Melbourn, Cambs. SG8 6JJ, UK

We have recently described the design and construction of a large human antibody repertoire displayed on phage (Vaughan TJ, *et al. Nature Biotechnol* March 1996; pp. 1 - 7). This resource - comprising 1.3 x 10^{10} scFvs - has allowed us to isolate a number of antibody fragments to a diverse panel of antigens ranging from haptens to proteins and cell surface markers. Two of these clones have affinities of 0.3 nM (against fluorescein) and 0.8 nM (against DTPA) demonstrating that sub-nanomolar affinity clones can be isolated directly from this library without further manipulation. Routinely, we isolate antibodies with $k_a < 10^{-8}$ M, and k_{off} of 10^{-3} - 10^{-4} s^{-1}, characteristics normally associated with antibodies of a secondary immune response. A further advantage of this large single pot repertoire is that we can isolate antibodies to antigens that have proven difficult using traditional hybridoma techniques. An example of this is doxorubicin, which is both immunosuppressive and toxic. We are currently developing a number of high affinity antibodies isolated from this library for therapeutic use, for example antibodies reactive with carcinoembryonic antigen (CEA) and we describe our current work with antibodies that neutralize the potent biological activity of transforming growth factor ß.

A rationalized mutagenesis and selection strategy to generate subnanomolar affinity human scFvs specific for CEA

Anne Field, Jane Osbourn, Elaine Derbyshire, Jane Wilton, John Earnshaw, Deborah Allen and John McCafferty

Cambridge Antibody Technology Ltd, Melbourn, Cambs. SG8 6JJ, UK

A completely human single chain Fv (scFv) with high affinity and specificity for human carcinoembryonic antigen (CEA) has been isolated from a large (2.0 x 10^9) phage display library, which is a subset of of the 1.38 x 10^{10} library described by Vaughan TJ, *et al.* (*Nature Biotechnol* March 1996; pp. 1 - 7). The dissociation constant of the scFv is 7.7 x 10^{-9} M with an off rate constant (k_{off}) of 6.4 x 10^{-3} s^{-1}. In order to investigate directly whether increased affinity leads to improved targetting of CEA positive tumours, this scFv has been affinity matured. The initial clone was mutagenized both by targetting CDR3 of heavy and light chains and by chain shuffling, where a wider range of residues was diversified. Selection of mutagenized repertoires was carried out to bias for clones with an improved off rate. The range of improvements obtained was up to sevenfold as determined by surface plasmon resonance using human CEA coupled to the sensor chip. All clones have been analysed further to demonstrate CEA specificity by immunocytochemistry on human colon tumour sections. We have also investigated differences in fine specificity using epitope mapping on recombinant CEA and flow cytometry on CEA expressing cell lines. It is apparent that certain mutations associated with an improvement in off rate also give rise to subtle changes in fine specificity which may have influenced the performance of these antibodies in the targetting of human colon cancer.

Multicombinatorial libraries: new vectors and construction of a very large human naive antibody repertoire

R. Sodoyer, F. Geoffroy, I. Peubez, C. Pion, J. Dubayle, A. Jacquemot, A. Bouchardon and L. Aujame

Pasteur Mérieux, Marcy l'Etoile, FRANCE

We present easy and efficient techniques for the construction of large antibody repertoires (10^{10} different clones) through the recombination of two separate heavy (VH) and light (VL) chain gene libraries. The process, based on combinatorial infection, makes use of (*lambda*) phage att recombination sites and integras, and leads to the irreversible physical association between vectors carrying respectively VH and VL sequences. Selection of the recombinants and complexity calculation are made possible by the assembly, *in vivo*, of a new genetic marker. The functionality of the system has been tested through the display of anti-Ig gp160 Ab. In order to improve the efficiency or solve intrinsic problems of the initial system, different sets of new vectors have been designed and tested. The assembly of a large scale 'naive' human antibody repertoire is also presented. Requirements for the assembly and handling of such large scale libraries will be described.

Monoclonal human anti-CD34 antibodies generated from combinatorial immunoglobulin libraries

D. R. Rubinstein, T. Guillaume, M. Symann, M. Sekhavat and P. Leblanc

Boston University School of Medicine, Boston, MA, USA, and Université Catholique de Louvain, Brussels, BELGIUM

Murine monoclonal antibodies (MAb) to CD34 have proven very useful for the identification and isolation of human hematopoietic stem cells. In order to circumvent the need for single cell cloning required in EBV and hybridoma techniques and produce a new class of higher affinity, human anti-CD34 MAb, human combinatorial immunoglobulin (Ig) phage display libraries were generated. Because autoimmune individuals often have immune cytopenias, we reasoned that autoAbs against the CD34 antigen on normal hematopoietic progenitors may be present in the repertoire of such individuals. For the combinatorial Ig libraries, all Ig heavy (H) and light (L) chains were amplified and transcribed *in vitro* and their gene products displayed monovalently as Fabs on the surface of filamentous phage. Libraries of > 10^6 Fabs were generated. To select specific binding of CD34 on normal hematopoietic cells, 10^{10} to 10^{12} Fab bearing phage were mixed with CD34+ cells following adsorption with autologous CD34- cells. Fab-bearing phage adherent to the CD34+ cells were then enriched by successive rounds of re-infection into fresh bacteria and repetition of exposure to CD34+ and CD34- cells for a total of five selections. The repeated adsorptions against the CD34- cells favored enrichment of Fabs that were directed specifically against epitopes present on the CD34+ cells. Following 5 rounds of enrichment, a 4-log increase was seen, from 4.8×10^3 to 6.5×10^7 Fab-bearing phage binding to CD34+ cells. Following cloning, induction and purification of Fab, ELISA with purified CD34 protein was used to confirm specific binding and high affinity of a group of selected Fabs to CD34, and nucleic acid sequencing confirmed each was encoded by different V genes. Efforts are in progress to identify autoAb from the library binding heretofore unidentified antigens on subsets of hematopoietic precursors. Human anti-CD34 MAbs generated by this approach may provide an alternative to murine MAbs for isolation of human hematopoietic stem cells for clinical applications and for use in stem cell targetting for gene therapy. In addition, the approach has applicability to a wide variety of disease-associated autoAbs.

An analysis of humanization methodologies

José Saldanha, Andrew Henry*, Tarran Jones, Olivier Léger, Catherine Kettleborough, David Ricketts* and Anthony Rees*

*MRC Collaborative Centre, Mill Hill, London NW7 1AD, UK, and *Oxford Molecular Ltd, The Medawar Centre, Oxford OX4 4GA, UK*

Humanization of rodent monoclonal antibodies (MAbs) has been used to circumvent the human anti-mouse antibody response and to improve the activation of the human immune system; indeed there are now more than 20 humanized MAbs in clinical trials. However, simple grafting of the rodent CDRs onto human frameworks does not always reconstitute the binding affinity and specificity of the original MAb. It has been found to be necessary to include back mutations where murine residues are substituted in the framework regions. The position of back mutations have been identified by analysis of canonical residues or by homology modeling. Similarly, many approaches have been used to choose which antibody to mimic. Some groups use variable regions with high amino acid sequence identity to the rodent variable regions ('homology matching'); others use consensus or germline sequences while a recently developed technique is to select fragments of the framework sequences within each VH or VL region from several human antibodies. In addition, some groups use a limited subset of human variable regions irrespective of the sequence identity to the rodent MAb ('fixed frameworks'). There are also approaches to humanization developed which replace the surface rodent amino acid residues with the most common residues found in human MAbs ('resurfacing' or 'veneering'). Here we present a comparison of the methods which have been used to humanize a range of MAbs. Furthermore, we discuss the predicted performance of these methods had they been used to humanize all the mouse MAbs in the Kabat database. This evaluation provides a wealth of information to be applied in future humanization studies.