Obtention and Engineering of Non-Human Primate (NHP) Antibodies for Therapeutics

Thibaut Pelat[1], Michael Hust[2] and Philippe Thullier*[,1]

[1]*Groupe de biotechnologie des anticorps, Département de biologie des agents transmissibles, Centre de Recherche du Service de Santé des Armées, La Tronche, France;* [2]*Institut für Biochemie und Biotechnologie, Technische Universität Braunschweig, Germany*

Abstract: Recombinant antibodies are therapeutic molecules of choice regarding their efficacy, pharmacokinetics and tolerance - all the more if they are human. The efficacy of antibodies generally depends on their affinity for their antigens. The most straightforward approach to isolate human antibodies with high affinities is the construction and screening of human immune libraries, but Humans cannot be immunized with all antigens of interest, for ethical and practical reasons. We circumvented that difficulty by utilizing non-human primates (NHP) instead of Humans and, in the course of our different studies, we have obtained several antibody fragments with high or very high affinities (from 3 nM to 50 pM) and neutralizing properties. The framework regions (FR) - the regions most implicated in tolerance - of these NHP antibody fragments were shown to be very similar to FR encoded by human germline genes. In one case, in a process called "germline humanization" (or "super-humanization"), we have increased that level of similarity and managed to go even beyond the level observed for human antibodies, without any loss of affinity. Here, the methodological peculiarities of our approach, in comparison with immune libraries built from immunised Humans, and the rationales behind these peculiarities, will be reviewed. The isolation of NHP antibody fragments from immune libraries, followed by the "super-humanization" process, opens a new and highly efficient approach for the production of high-quality recombinant antibodies for therapeutic uses.

Keywords: Antibody, non human primate, immune library, humanization.

INTRODUCTION

Recombinant antibodies form a highly successful class of therapeutic molecules, with 21 products now licenced in Europe, generating between 20 and 30 billions of dollars of revenues globally in 2007. Thirteen of these antibodies, among the latest approved [1], have been isolated with the phage technology, which we use in a rather original but very efficient way. In effect, we have built phage-displayed libraries from immunised non-human primates (NHP), and all of these constructions have allowed the isolation of antibody fragments with high or very high affinities, ranging from the nano- to the picomolar range [2-6]. Furthermore, we have recently shown that NHP framework regions (FRs) might be mutated - in a process called "germline humanization" or "super-humanization" - into FRs encoded by human, germinal genes [7]. These FRs are encountered by every Human as part of IgMs, and are therefore perfectly tolerated, in particular they should be better tolerated than IgG FRs that bear mutations due to the natural process of affinity maturation. This germline humanization respected the parental affinity and neutralization capacity, so that the combined use of NHP immune libraries and germline humanization allows isolating of high-quality antibodies for therapeutic use, both in terms of efficiency and tolerance. This approach is an alternative to the construction of immune libraries from immunised Humans [8], which is indeed very constrained by ethical and practical aspects. It is also an alternative to the use of naïve or synthetic libraries, which is frequently constrained by commercial aspects because such libraries are often proprietary, and only yield antibodies with mediocre affinities that have to go through tedious *in vitro* affinity maturation. The use of NHP antibodies is patented [9] when the antigen is of human origin such as cancer markers or cytokines, and antibodies directed such targets form the bulk of the market. However, it is not patented for other antigens and, in Europe for instance, the patent will expire in 2012 so that scientists should prepare for this approach. Here, the peculiarities of the NHP combined strategy as compared with the use of human immune libraries, published elsewhere [10,11] will be reviewed according to our past experience and rationales for these peculiarities will be given.

1. CHOICE OF ANIMAL

The NHP utilized in our approach are macaques, of the *Macaca fascicularis* species. Chimpanzees, which are even more similar to Humans than macaques, are extremely difficult to access because of their close relation to Human and because they are endangered species. Nevertheless, they have been utilized sometimes for immunizations [12]. *Macaca rhesus* specimens will give poor results with the primers given below, and these animals are quite fragile so

*Address correspondence to this author Groupe de biotechnologie des anticorps, Département de biologie des agents transmissibles, Centre de Recherche du Service de Santé des Armées, 24 avenue des Maquis du Grésivaudan, 38702 La Tronche, France; E-mail: pthullier@yahoo.com

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that they are not recommended here. The *Macaca fascicularis* species is in no way endangered and the commerce of these animals is authorized under the Convention for International Trade of Endangered Species (CITES).

Macaca fascicularis specimen intended for laboratory use must be accompanied by a CITES certificate, be quarantined and tested for tuberculosis and herpes. Animals should be acclimated and observed for one month after arrival in the accredited animal house of the laboratory, and the evolution of their weight and their blood counts should be further checked. We have dismissed animals presenting hyperlymphocytis (possibly revealing a viral infection) or clinical signs (diarrhea for instance) during that observation. At the end of this period, bone marrow should be sampled and utilized for PCR as for library construction (see paragraph 3, below): no, or very little, PCR products should be amplified from a healthy macaque at this stage.

2. IMMUNIZATION

After pre-immune serum has been drawn, animals are usually immunized with 100 µg of non-toxic protein via subcutaneous injections, with Freund's complete (priming) and incomplete (boosting) adjuvant. The titres that were reached before library construction ranged from 1:100 000 to 1:250 000 but a human vaccine, thus utilizing alun as adjuvant, has been successfully utilized once, even though the titre of the response was a tenth of the titres obtained with Freund's adjuvant [2]. Freund's adjuvant was chosen according to its use in mice, for hybridoma production, and to best stimulate the immune system as this stimulation is an essential determinant of affinities and of the diversity of epitopes targeted. However, superficial granuloma generally appear within a few weeks of Freund's complete adjuvant injection (and, to a lesser extend, after incomplete adjuvant use) and have to be operated to prevent their ulceration. Once the hyperimmune status has been reached, the animal is put at rest for about three months, before the last boost and library construction. At the end of this period of time, the animal does no longer strongly respond to the previous injection and testing the bone marrow by PCR should yield a gel image comparable to the image observed after the acclimation.

3. PRE-CLONING

We have chosen the bone marrow as the source of activated lymphocytes (plasmocytes), secreting IgGs. Blood may be also utilized but, in our experience, only a short and unpredictable period of time will allow successful amplification. Bone marrow (BM) is the site where lymphocytes accumulate to secrete on long term [13] and it best allows harvesting the diversity of the immune response. We have thus utilized bone marrow for the construction of our libraries, in opposition to many human immune libraries built from blood [14]. Sampling of the bone marrow is to be performed no later than the 3^{th} day, then on the 7^{th} or 10^{th} days. Further sampling of BM is to be adapted to the pace of the macaque response, and a repetitive weekly sampling of 5 ml of BM is not deleterious to the animal. Sampling twice a week, but for two weeks maximum, is even possible. We have obtained the best RT-PCR amplifications on the 3^{th}, 7^{th} or 10^{th} days after the last boost (Fig. **1**).

To first extract RNA, bone marrow is homogenized in phenol and guanidine thiocyanate (TRI Reagent, Molecular research center Inc, USA), and the homogenate is separated into aqueous and organic phases by bromochloropropane (BCP, Molecular research center Inc, USA), according to the manufacturer's recommendations. Whole RNA was preferred to mRNA extraction as it allows the quantification of the nucleic acid, contrary to mRNA which is extracted in too minute quantities. A quantity of 200 µg RNA per 5ml of BM confirms the quality of the sample and of the first methodological step, and this RNA is retro-amplified using oligo(dT) priming (SuperScript II Reverse Transcriptase, Invitrogen,

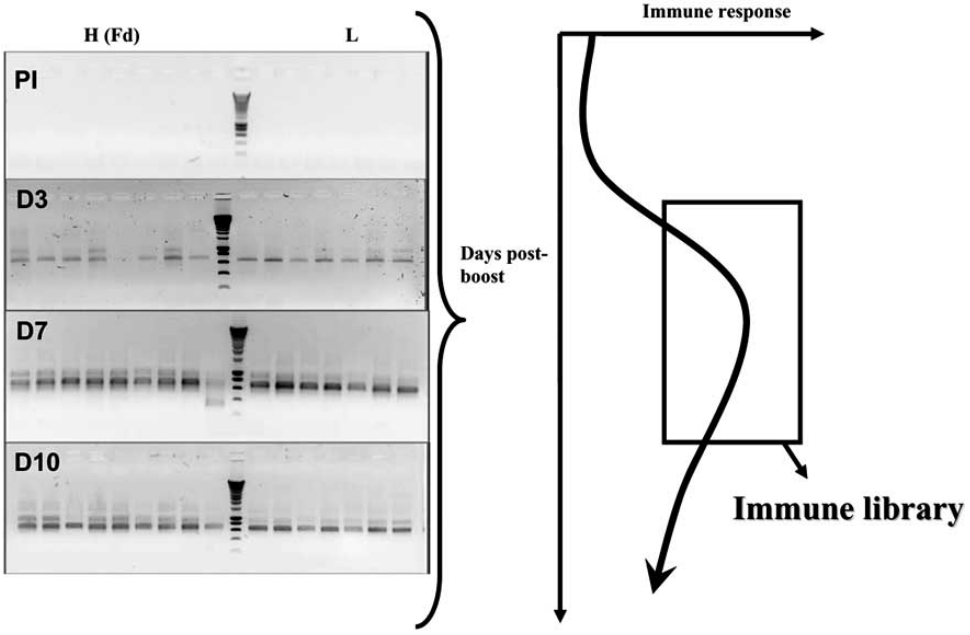

Fig. (1). Follow-up of the immune response by iterative BM sampling and RT-PCR. PI (PreImmune) corresponds to sampling prior to the last immunisation. D3 is to the third day after the last immunisation, and correspondingly for D7 and D10.

USA). Resulting cDNAs is PCR amplified using mixes of 7 κ sense primers (MacVK-SacI primers, Table **I**), 8 λ sense primers (MHMacVL primers) and 2 CL antisense primers (MacIgGCKappaFab-Xba1_r or MHMacLambdaCL_r, respectively κ and λ) to obtain DNA coding the light κ and λ chains. Nine VH (MacVH-XhoI) sense primers and 1 CHγ1 antisense primer (MacIgGCH1Fab-SpeI_r) are utilized for PCR amplification of DNA coding the Fd fragment. Of note, the κ and γ primers were published early [15] but the λ primers have been recently developed [16].

Table I. Oligonucleotide Primers Utilised for the First Amplification, Starting from cDNA (See Text).

MacVH- *Xho*I _f1 : 5'-CAGGTGCAGCTCGAGCAGTCTGGG-3'

MacVH- *Xho*I _f2 : 5'-CAGGTGCAGCTGCTCGAGTCTGGG-3'

MacVH- *Xho*I _f3 : 5'-CAGGTGCAGCTACTCGAGTCGGG-3'

MacVH- *Xho*I _f4 : 5'-GAGGTGCAGCTCGAGGAGTCTGGG-3'

MacVH- *Xho*I _f5 : 5'-GAGGTGCAGCTGCTCGAGTCTGGG-3'

MacVH- *Xho*I _f6 : 5'-CAGGTACAGCTCGAGCAGTCAGG-3'

MacVH- *Xho*I _f7 : 5'-AGGTGCAGCTGCTCGAGTCTGG-3'

MacVH- *Xho*I _f8 : 5'-CAGGTGCAGCTGCTCGAGTCGGG-3'

MacVH- *Xho*I _f9 : 5'-CAGGTGCAGCTACTCGAGTGGGG-3'

MacIgGCH1Fab- *Spe*I _r :5'-
AGGTTTACTAGTACCACCACATGTTTTGATCTC'-3'

MacVK- *Sac*I _f1 :5'-GACATCGAGCTCACCCAGTCTCCA-3'

MacVK- *Sac*I _f2 :5'-GACATCGAGCTCACCCAGTCTCC-3'

MacVK- *Sac*I _f3 :5'-GATATTGAGCTCACTCAGTCTCCA-3'

MacVK- *Sac*I _f4 :5'-GAAATTGAGCTCACGCAGTCTCCA-3'

MacVK- *Sac*I _f5 :5'-GAAATTGAGCTCACACAGTCTCCA-3'

MacVK- *Sac*I _f6 :5'-
GAGCCGCACGAGCCCGAGCTCCAGATGACCCAGTCTCC-3'

MacVK- *Sac*I _f7 :5'-
GAGCCGCACGAGCCCGAGCTCGTGTTGACACAGTCTCC-3'

MacIgGCLkappaFab- *Xba*I _r :

CTAGAATTAACACTCTCCCCTGTTGAAGCTCTTTGTGACGGG-CGAACTCAG-3'

MHMacVL-f1 : 5' cagtctgtgctgactcagccrcc 3'

MHMacVL-f2 : 5' cagtctgccctgactcagcct 3'

MHMacVL-f3 : 5' tcctatgagctgacwcagccacc 3'

MHMacVL-f4 : 5' tcttctgagctgactcaggaccc 3'

MHMacVL-f5 : 5' cwgcctgtgctgactcagcc 3'

MHMacVL-f6 : 5' cagccggcctccctctcagcatct 3'

MHMacVL-f7 : 5' cagrctgtggtgacycaggagcc 3'

MHMacVL-f8 : 5' cagcctgtgctgactcagcca 3'

MHMacLambdaCL_r: 5' AGAGGAGGGCGGRAAWAGAGTGAC 3'

Final PCR products are pooled and cloned into pGemT vector to obtain 3 sub-libraries (κ, λ, γ), each of more than $5x10^3$ clones. The PCR products obtained from the various BM samples, corresponding to the different days of sampling, are to be compared and the most diverse products, according to the number of pairs of primers allowing amplification, will be utilized for library construction. Because no amplification was possible before the boost (see PI on Fig. **1**), these PCR products should code for antibody fragments with the desired specificity.

4. CONSTRUCTION OF THE PHAGE-DISPLAYED LIBRARY

Phage-displayed scFv libraries are built after re-amplification of the precloned PCR products. A first amplification allows to retrieve the DNA coding the VH or VL regions utilizing primers indicated on (Table **I**), and a second amplification (primers indicated on Table **II**) adds the restriction sites.

Table II. Primers used for the second PCR of scFv-coding DNA for the heavy (VH), κ light (VK) or γ light chain (VL) chain (see text)

MHMacVH-NcoI_f1 : 5′ gtcctcgca-
CATGGCCSAGGTGCAGCTGGTGSAGTCTGGG 3′

MHMacVH-NcoI_f2 : 5′ gtcctcgca-
CATGGCCCAGGTGCAGCTRCTCGAGTCKGG 3′

MHMacVH-NcoI_f3 : 5′ gtcctcgca-
CATGGCCSAGGTGCAGCTGCTCGAGTCKGG 3′

MHMacVH-NcoI_f4 : 5′ gtcctcgcaCATGGCCCAGGTACAGCTC GAG-CAGTCAGG 3′

MHMacVH-NcoI_f5 : 5′ gtcctcgca-
CATGGCCGAGGTGAGCTGCTCGAGTCTGG 3′

MHMacVH-NcoI_f6 : 5′ gtcctcgca-
CATGGCCCAGGTGCAGCTRCTCGAGTSGGG 3′

MHMacIgGCH1scFv-HindIII_r : 5′ GTCCTCGCAAAGCTT
TGGGCCCTTGGTGGA 3′

MHMacVK-MluI_f1 : 5′ accgcctccACGCGTA-
GAHATCGAGCTCACNCAGTCT CC 3′

MHMacVK-MluI_f6 : 5′ accgcctccACGCGTAGAGCTCCWGATGACM-CAGTCT CC 3′

MHMacKappaCL-NotI_r : 5′ ACCGCCTCCGCGGCCGCGACA-GATGGTGSAGCCAC

MHMacVL-MluI_f1:5' ACCGCCTCCACGCGTA-
CAGTCTGTGCTGACTCAGCCRCC 3'

MHMacVL-MluI_f2:5' ACCGCCTCCACGCGTA-
CAGTCTGCCCTGACTCAGCCT 3'

MHMacVL-MluI_f3:5'
ACCGCCTCCACGCGTATCCTATGAGCTGACWCAGCCACC 3'

MHMacVL-MluI_f4:5'
ACCGCCTCCACGCGTATCTTCTGAGCTGACTCAGGACCC 3'

MHMacVL-MluI_f5:5'
ACCGCCTCCACGCGTACWGCCTGTGCTGACTCAGCC 3'

MHMacVL-MluI_f6:5' ACCGCCTCCACGCGTA-
CAGCCGGCCTCCCTCTCAGCATCT 3'

MHMacVL-MluI_f7:5' ACCGCCTCCACGCGTACAGRCTGTGGTGA-CYCAGGAGCC 3'

MHMacVL-MluI_f8:5' ACCGCCTCCACGCGTA-
CAGCCTGTGCTGACTCAGCCA 3'

MHMacLambdaCL-NotI_r:5' ACCGCCTCCGCGGCCGCAGAG-GAGGGCGGRAAWAGAGT GAC 3'

The libraries will be constructed in two subsequent steps: first the VL fragments are cloned into pHAL14 [3,17,18], in the second step the VH fragments are cloned into pHAL14 containing the VL repertoire. Typically, the size of the final library size is about 10^7-10^8 independent clones, as for immune libraries of human origin. Afterwards the library will be packaged using Hyperphage [1,19,20] to gain polyvalent display for the first panning round.

5. PANNING

For panning, the antigen to be utilized is preferably the molecule to be neutralized [16], in opposition to its subunits or its inactivated form, in order to target epitopes that are naturally present. The screening is realized as exposed previously [11] except that PBS-Tween 0.1% is utilized for stringent washing because high affinities are expected, and immobilized phages are eluted by trypsin cleavage to ensure the retrieval of even the very high-affinity binders [17]. According to our experience, the panning will include 3 to 4 rounds, with 5 washing for the first round, then 10 for the second, 15 or 20 for the third and possibly 30 or 40 washes for the fourth round. At a point in the panning, typically at the end of the third or fourth round, the number of eluted phages should increase, indicating in theory that the panning had successfully enriched the library in antigen-specific clones. At this point, the panning is stopped and either another panning strategy (long elution steps, see below) is utilised or individual clones are analysed. To verify the quality of the first part of the panning, a phage-ELISA utilising relevant and non-relevant antigens is recommended to best assess reactivity of the selected phages, as our (unpublished) experience has shown that the increasing number of eluted clones may also be observed after the sole selection of anti-polystyrene phages. To ensure the reliability of this phage-ELISA, it is important that the non-relevant antigen used as negative controls be different from the agent utilized for phage blocking and plate saturation (KLH and BSA respectively, for instance). The positive phage-ELISA signal read from phages eluted after the last round should be at least 5 times higher than on the negative control, after a progressive increase often seen in the previous steps of the panning. If the phage-ELISA shows such results, clones may be isolated on Petri dishes and induced by IPTG [11,17], in order to be tested in standard ELISA. The best reactive scFv will then be characterized by affinity measurements, typically in a search for nanomolar affinities (for instance, see [3,5]) or better [2]. Because very high-affinity scFvs may be isolated from NHP immune libraries [16], an additional, extremely stringent screening step based on long elution times may be performed to select these scFvs, as we exposed previously [21]. This additional screening step aims at isolating the very best binders, more particularly those with the best K_{off} rates. This step starts as exposed above until phages are incubated on plate. The incubation step is then performed in presence of the soluble antigen (from 0.1 to 1 mg/ml), at 4°C and for long very period of times (from one to 20 days). The scFvs with mid-level affinities will detach over time, and will bind to the soluble antigen present in high concentration thus not re-attach to the antigen adsorbed on plate. At the end, only the scFvs that did not detach from the adsorbed antigen over time, thus those with the best Koff - and consequently the best affinities - will be eluted from the plate by the usual trypsin cleavage. These eluted clones should then be characterized, and the scFvs with the best affinities may then be germline-humanized.

6. SEQUENCE ANALYSIS FOR GERMLINE HUMANIZATION

Germline humanization (or "super-humanization") aims at turning the NHP FRs into human FRs coded by germinal V(D)J genes, as these latter FRs are encountered by every Human as IgM and thus are perfectly tolerated. In particular, these are preferable as a basis for humanization to IgGs FRs, which are randomly mutated in the course of affinity maturation and these mutations may be immunogenic [22]. The ImMunoGenetics information system® (http://-www.imgt.org) proposes the "DomainGapAlign" tool (http://www.imgt.org/3Dstructure-DB/cgi/DomainGapAlign.-cgi), which is the best suited for germline humanization because it performs its analysis at the peptidic (and not nucleotidic) level - indeed the level relevant for assessment of immunogenicity. The goal of the analysis is to find the peptidic sequences encoded by human germinal V(D)J genes that are most similar to the NHP heavy or light variable domains. The "DomainGapAlign" tool clearly outlines (in bold, under the NHP and the human sequences) the residues differing between NHP FRs and the selected V(D)J-encoded FRs; these residues will later be mutated in the germline humanization process. Of note, such a peptidic analysis may effectively yield results slightly different from the results obtained with a nucleotidic analysis : for instance, a κ light chain numbered [FJ178347] in Genbank/EMBL is most similar to the nucleotidic sequence of gene IGKV1-5*1 but to the peptidic sequence encoded by gene IGKV1-17*01.

7. GERMLINE HUMANIZATION ("SUPER-HUMANIZATION")

The sequence analysis presented above pinpoints the differences existing between NHP FRs, and human FRs encoded by germinal genes. Whether these differences correspond to differences between the human and the NHP species, or to mutations introduced during the affinity maturation of the NHP antibody, is of no practical importance because both types of differences are possibly immunogenic and should be suppressed. We have called "germinality index" (GI) [7] the percentage of identity between the FRs of any immunoglobulin, and the most similar FRs encoded by human, germinal V(D)J genes. It was measured (unpublished data) that the GI of human IgGs is around 95% when it is approximately 90% in the case of NHP IgGs. The goal of germline humanization is thus to tend toward a GI of 100% (corresponding to human IgM FRs), and practically to reach at least a GI of 95% (corresponding to human IgG FRs), without altering the affinity of the antibody. It may then be analysed on structural models (such as obtained on-line from WAM: http://antibody.bath.ac.uk/) if the macaque residues still present in the final super-humanised variant are enough proximal to each other to risk forming a B epitope, to which Humans may react.

When erasing these differences, we have observed ([7] and unpublished data) that FR1s and FR4s might be readily super-humanized, as they are located at the extremities of the

variable domain and should not bring high constraints to its core. This is in contrast to the superhumanization of FR2s and FR3s, and we have utilized two approaches for the super-humanization of these FRs. One of these approaches was the three-dimensional modelling of FR2s and FR3s, and only mutations that did not alter the parental structure were realized [7]. This approach is as good as the modelling is, and for instance we encountered one error that was corrected by manual analysis of locations and physico-chemical properties of mutated residues. The super-humanization of another scFv was realized as all point mutations being physically performed in parallel, without modelling, and affinities of the corresponding variants measured. All mutations respecting the parental affinity were finally associated in a synthetic gene.

In a published example of germline humanization [7], we followed the first strategy, starting from a GI of 92 % to reach a GI of 97.8%, while respecting the parental affinity and neutralisation properties This is in opposition to the germline humanization applied to murine FRs, for whom super-humanization was twice published but with far less satisfactory results [23,24]. Remarkably, the GI of the germline-humanized variant was superior to the GI of a fully human antibody with similar affinity and neutralization properties [10]. Also remarkably, the germline humanized variant only retained four macaque residues, which were showed to be so scattered at the surface of the variable domain that they could not form a B epitope, at risk of being immunogenic in Humans. When the germline humanization of an scFv is performed with such good results, the germlinized variant may be expressed in the full- size IgG format with confidence regarding its clinical tolerance.

CONCLUSION

Immune libraries are preferable to laborious naïve libraries to isolate antibody fragments of high affinities. Construction of immune libraries of human origin is however very constrained because Human immunization is difficult to perform for ethical and practical reasons. Immunization of non human primates (NHP) is not limited in the same fashion and NHP antibody fragments of high affinities, directed against a variety of immunogens, may be isolated from NHP immune libraries. In addition, NHP antibody fragments may be germline-humanized to present FRs very similar to human IgM FRs - arguably the best tolerated FRs for therapeutic use - while retaining the parental affinity. If directed against non-human proteins, germlinized antibodies may already be obtained without limitations brought by patents as we did, and against all antigens starting in 2012, after patent expiry. The rarity of articles presenting NHP antibodies has been formerly noted [11] but this situation should change.

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