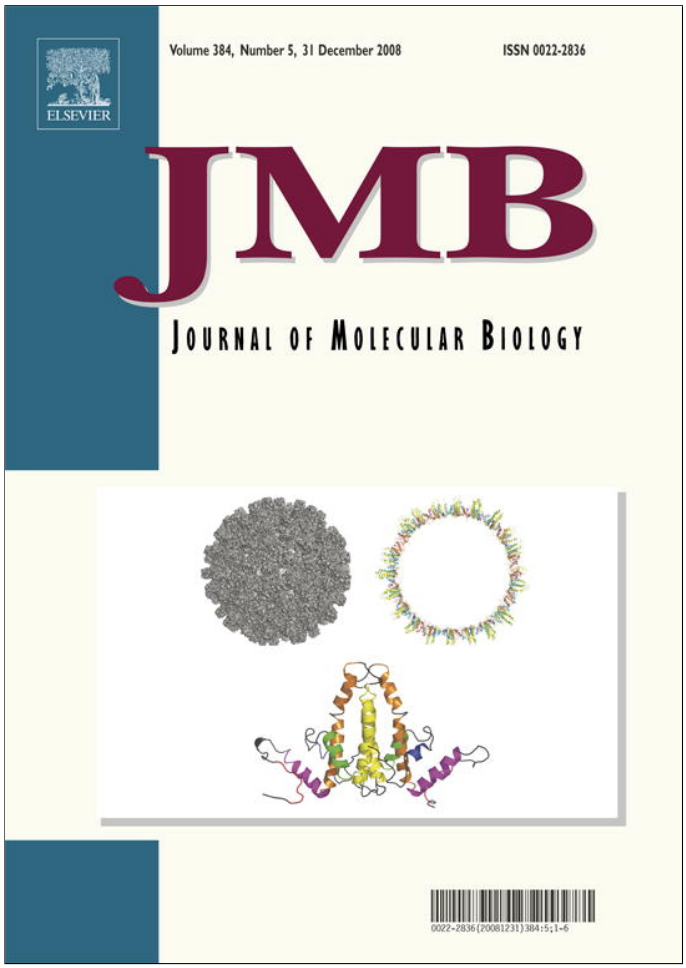
Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

doi:10.1016/j.jmb.2008.10.033 J. Mol. Biol. (2008) 384, 1400–1407

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

Germline Humanization of a Non-human Primate Antibody that Neutralizes the Anthrax Toxin, by *in Vitro* and *in Silico* Engineering

Thibaut Pelat[1], Hugues Bedouelle[2], Anthony R. Rees[3], Susan J. Crennell[3], Marie-Paule Lefranc[4] and Philippe Thullier[1]*

[1]*Groupe de Biotechnologie des Anticorps, Laboratoire d'Immunobiologie, Centre de Recherches du Service de Santé des Armées, 24 avenue du maquis du Grésivaudan, 38702 La Tronche, France*

[2]*Unit of Molecular Prevention and Therapy of Human Diseases (CNRS-URA 3012), Institut Pasteur, 28 rue Docteur Roux, 75724 Paris Cedex 15, France*

[3]*Department of Biology & Biochemistry, University of Bath, Claverton Down, Bath, BA2 7AY, UK*

[4]*IMGT, LIGM, UPR CNRS 1142, Institut de génétique humaine, 141 rue de la Cardonille, 34396 Montpellier Cedex 5, France*

Received 18 June 2008; received in revised form 7 October 2008; accepted 9 October 2008
Available online 19 October 2008

Edited by I. Wilson

Fab $35PA_{83}$ is an antibody fragment of non-human primate origin that neutralizes the anthrax lethal toxin. Human antibodies are usually preferred when clinical use is envisioned, even though their framework regions (FR) may carry mutations introduced during affinity maturation. These hypermutations can be immunogenic and therefore FR that are encoded by human germline genes, encountered in IgMs and thus part of the "self" proteins, are preferable. Accordingly, the proportion of FR residues in $35PA_{83}$ that were encoded by human V and J germline genes, i.e. the germinality index (GI) of $35PA_{83}$, was increased in a multistep cumulative approach. In a first step, the FR1 and FR4 residues of $35PA_{83}$ were changed simultaneously into their counterparts coded by $35PA_{83}$'s closest human germline genes, without prior modelling. The resulting derivative of $35PA_{83}$ had the same affinity as its parental Fab. In a second step, the 3D structures of this first $35PA_{83}$ derivative, carrying the same type of residue changes but in the FR2 and FR3 regions, were modelled *in silico* from sequences. Some of the changes in FR2 or FR3 modified the predicted peptide backbone. The changes that did not seem to alter the structure were introduced simultaneously in the Fab by an *in vitro* method and resulted in a loss of reactivity, which could however be fully restored by a single point mutation. The final $35PA_{83}$ derivative had a GI higher than that of a fully human Fab, which had neutralization properties similar to $35PA_{83}$ and which was used as a benchmark in this study.

© 2008 Elsevier Ltd. All rights reserved.

Keywords: antibody; humanization; germline genes; tri-dimensional modeling; non-human primate

Introduction

"Super-humanization" of a murine antibody has been defined as the modification of its framework regions (FRs) to increase the level of identity with FR encoded by human germline gene segments.[1,2] In effect, germline FR are expressed in humans as part of IgM immunoglobulins and should be well tolerated, like other self proteins.[1,3,4] In contrast, FRs that are expressed as part of IgGs carry somatic hypermutations, resulting from affinity maturation, and thereby potentially immunogenic sequences.[2] For medical use, human germline FR may thus be preferred to human expressed FR.

In this study, the concept of super-humanization was adapted to antibodies of non-human primate (NHP) origin. Because NHP and human antibodies

*Corresponding author. E-mail address: pthullier@yahoo.com.

Abbreviations used: PA, protective antigen; Fab, antigen binding fragment; Fv, variable fragment; FR, framework regions; IgG, immunoglobulin G; IgM, immunoglobulin M; CDR, complementary determining regions; NHP, non human primate.

0022-2836/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.

Author's personal copy

are very similar, computer programs such as IMGT/V-QUEST can find the human germline genes, V, (D) and J, which are the most similar to the sequences encoding any NHP variable region, i.e. the germline genes that would have been parental if the NHP antibody had been of human origin. We observed that such programs often fail when applied to murine antibodies because the sequences of the murine and human variable regions are too dissimilar. In previous studies, we have performed three analyses of NHP variable regions with the IMGT/V-QUEST program.[5–8] In particular, we have reported the proportion of FR residues that are identical between given NHP variable regions and the closest products of human germline gene segments. This proportion, which we call the germinality index (GI), can be considered as a predictor of tolerance, in line with the super-humanization concept. One of these analyses concerns a Fab fragment, $35PA_{83}$, that neutralizes the anthrax lethal toxin and was used as a starting point for the present study.[7]

Here, we used a combination of *in vitro* and *in silico* approaches to perform a super-humanization of $35PA_{83}$. In particular, we used two on-line programs, the IMGT/V-QUEST program for sequence analysis, and the WAM program to model the 3D structures of the antibody variable fragments (Fv) from their sequences.[9] This study was conducted in three steps: (i) super-humanization of FR1 and FR4 without any prior 3D modelling; (ii) super-humanization of FR2 and FR3 with such modelling; and (iii) restoration of activity. We propose to call this version of super-humanization applied to FR of NHP origin germline humanization, or "germlinization". In another parallel study, $35PA_{83}$ was submitted to an *in vitro* affinity maturation, whose mutations were strictly restricted to CDRs.[10]

Results

Comparison between $35PA_{83}$ and human germline genes

In a previous study, we reported the identification of the human germline gene segments most resembling the $35PA_{83}$ encoding genes.[7] For the H-chain, the V, D and J gene segments that we have identified are IGHV4-59*01, IGHD3-3*01 and IGHJ5*01, respectively. For the L-chain, the V and J gene segments are IGKV1-13*02 and IGKJ3*01, respectively. For this identification, we omitted the sequences that were brought by the oligonucleotides during the construction of the antibody library and located at the 5′-end of the Fab genes. The 3′-primers hybridized with the C_{H1} and C_L gene segments, which are outside the variable regions. Among the seven residues of H-FR1 that were encoded by the 5′ primer, only one, at position 5, differed from those encoded by IGHV4-59*01. Among the seven residues of L-FR1 that were encoded by the 5′ primer, only two, at positions 1 and 3, differed from those encoded by IGKV1-13*02. A total of 22 among the 178 residues of the eight FRs differed between $35PA_{83}$ and the products of the selected human germline gene segments (Table 1). The GI of $35PA_{83}$ was thus:

$$GI = (178-22)/178 = 0.876$$

Table 1. Residues in FR1 and FR4 differing between 35PA83 and those encoded by IGHV4-59*01 or IGHJ5*01 for the V_H domain, and IGKV1-13*02 or IGKJ3*01 for the V_L domain

Position	$35PA_{83}$	Hu_1	Position	$35PA_{83}$	Hu_1
H5	L	Q	L1	D	A
H12	L	V	L3	E	Q
H24	A	T	L14	Y	S
H122	A	T	L18	K	R
H123	V	L	L24	H	R
			L124	L	V

Table 2. Residues in H-FR2 and H-FR3 differing between the derivatives of $35PA_{83}$ and the product of IGHV4-59*01

Position	$35PA_{83}$	Hu_1	Hu_2	Hu_3	Hu_4
H45	S	S	P	P	P
H55	H	H	Y	H	H
H66	R	R	N	N	N
H80	K	K	V	V	K
H87	L	L	F	F	F
H90	Q	Q	K	Q	Q
H92	R	R	S	S	S

Germlinization of FR1 and FR4, resulting in $Hu_1 35PA_{83}$

Eight residues in both H-FR1 and L-FR1 differed between $35PA_{83}$ and the products of the closest human germline genes, IGHV4-59*01 and IGKV1-13*02, respectively. Likewise, three residues in both H-FR4 and L-FR4 differed between $35PA_{83}$ and the products of IGHJ5*01 and IGKJ3*01, respectively (Table 1). We derived a new sequence from the $35PA_{83}$ gene sequence, in which the 11 differences in FR1 and FR4 were changed into their human counterparts, and we named the Fab derived from $35PA_{83}$ in this way $Hu_1 35PA_{83}$. Its GI value was:

$$GI = [178-(22-11)/178] = 0.938$$

To obtain $Hu_1 35PA_{83}$, we also changed the C_{H1} and C_L domains of $35PA_{83}$ into their human counterparts IGHG1*01 and IGKC*01, respectively, in addition to the 11 changes in FR1 and FR2. These changes were not part of the germlinization process as such, but were made in the perspective of the expression of a full-size super-humanized IgG version of $35PA_{83}$ in prospect. Because the C_{H1} and C_L domains are not part of the variable region, these changes are not discussed further. The gene coding for the $Hu_1 35PA_{83}$ derivative of $35PA_{83}$ was synthesized and $Hu_1 35PA_{83}$ was expressed in *Escherichia coli*.

We measured the dissociation constants K_D between the PA_{83} antigen and either $35PA_{83}$ or

Author's personal copy

Table 3. Residues in L-FR2 and L-FR3 differing between the derivatives of $35PA_{83}$ and the product of IGKV1-13*02

Position	$35PA_{83}$	Hu_1	Hu_2	Hu_3	Hu_4
L68	Q	Q	E	Q	Q
L87	Y	Y	F	F	F
L96	S	S	P	P	P
L101	S	S	T	T	T

$Hu_1 35PA_{83}$ by Biacore, and found very similar values of 3.40 nM and 2.86 nM respectively. Thus, the changes in FR1 and FR4 did not affect the affinity of $35PA_{83}$ for its antigen substantially. A 3D structure for the variable fragment, Fv, of $Hu_1 35PA_{83}$ was modelled with the WAM program, and used as a reference structure for the remainder of the study.

Germlinization of FR2 and FR3, resulting in $Hu_2 35PA_{83}$

Eleven residues in the FR2 and FR3 regions differed between $Hu_1 35PA_{83}$ and the products of the closest human germline genes, IGHV4-59*01 and IGKV1-13*02 (Tables 2 and 3). We targeted these 11 residues in a second step of germlinization and derived a new sequence from the $Hu_1 35PA_{83}$ sequence where they were changed into their counterparts, coded by the selected human germline genes. We named $Hu_2 35PA_{83}$ the resulting $35PA_{83}$ derivative. The 3D structure of $Hu_2 35PA_{83}$ was modelled from its sequence with the WAM program and compared with that of $Hu_1 35PA_{83}$. We observed three differences in the two structural models: deviations in the conformations of H-CDR2 and H-CDR3, and a subtle but more extended deviation of the L chain (Fig. 1).

Structural modelling of $Hu_2 35PA_{83}$ revertants and formation of $Hu_3 35PA_{83}$

To identify the mutations that were responsible for the differences between the structural models of $Hu_2 35PA_{83}$ and $Hu_1 35PA_{83}$, we designed 11 reversion sequences from the $Hu_2 35PA_{83}$ sequence, in which the mutations in FR2 or FR3 were individually reverted. The structures of these 11 reverse derivatives of $Hu_2 35PA_{83}$ were modelled with the WAM program and compared with the structural model of $Hu_1 35PA_{83}$. The structures of three derivatives showed an improved fit with the parental structure. In V_H, reversion Y55H affected an anchor residue of H-CDR2 and restored the conformation of the corresponding CDR (Fig. 2). In V_H also, K90Q changed a positively charged residue into a polar one, in position 26 of H-FR3, i.e. in its middle, and restored the conformation of H-CDR3 (Fig. 3). In V_L, mutation E68Q changed a negatively charged residue into a polar one in position 2 of L-FR3 and restored the general light chain conformation. The eight other reversions had no significant effect. Thus, three point reversions were sufficient to restore the conformation of $Hu_2 35PA_{83}$, according to our *in silico* approach.

The three reversions Y55H and K90Q in V_H, and E68Q in V_L, were introduced simultaneously into

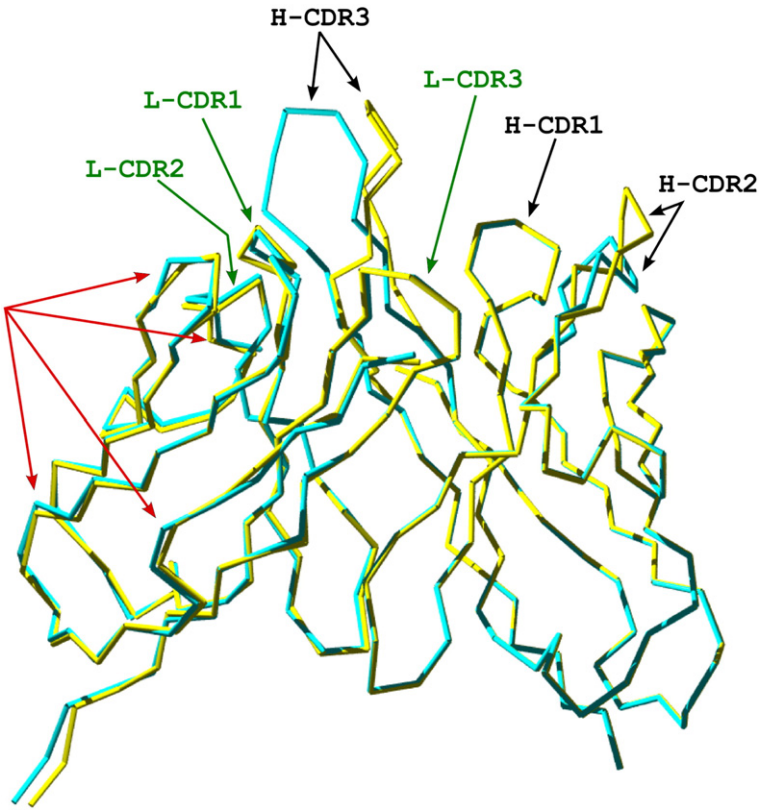


Fig. 1. Comparison between the C^{α} traces of the structural models for $Hu_1 35PA_{83}$ (yellow) and $Hu_2 35PA_{83}$ (blue). The light chain shows a general deviation, indicated by red arrows in the left-hand part of the figure, while H-CDR2 and H-CDR3 show two localized deviations. The figure was composed using Swiss-PDBviewer.

Author's personal copy

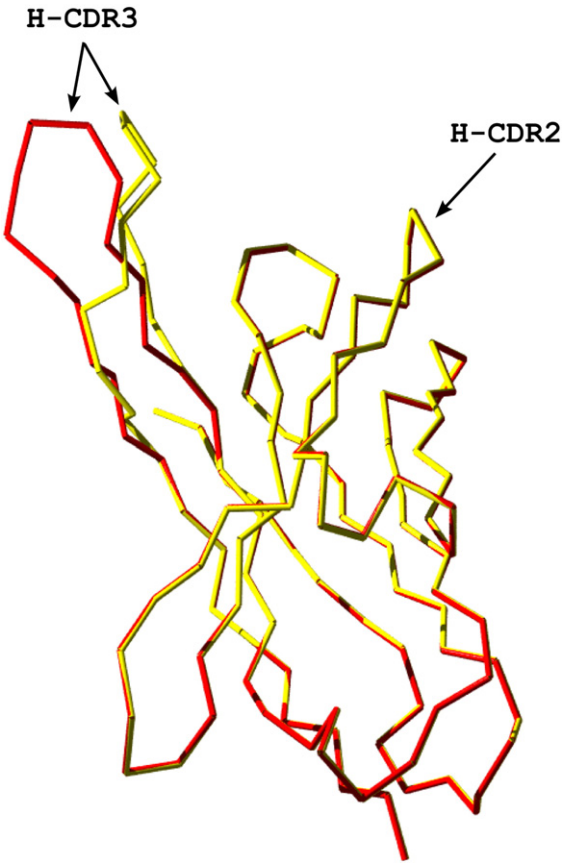


Fig. 2. Comparison between the C^{α} traces of the structural models for Hu_135PA_{83} (in yellow) and a derivative of Hu_235PA_{83} in which Tyr55 has been reverted into His55 in the V_H domain (in red). This reversion restores the conformation of H-CDR2 but not that of H-CDR3 as seen in Fig. 1. Only the V_H domain is represented.

Fig. 3. Comparison between the C^{α} traces of the structural models for Hu_135PA_{83} (in yellow) and a derivative of Hu_235PA_{83} in which residue Lys90 has been reverted into Gln90 in the V_H domain (in green). This reversion restores the conformation of H-CDR3 but not that of H-CDR2 as seen in Fig. 1. Only the V_H domain is represented.

the sequence of Hu_235PA_{83}, to give Hu_335PA_{83}. The latter thus corresponded to a Hu_135PA_{83} derivative with eight mutations (Tables 3 and 4). The Fv structural models of Hu_335PA_{83} and Hu_135PA_{83}, predicted with the WAM program, did not show any difference at the level of their peptide backbones. A gene encoding Hu_335PA_{83} was obtained by chemical synthesis and its product was expressed in *E. coli*. We found that Hu_335PA_{83} had lost all reactivity towards the PA_{83} antigen in an ELISA.

Restoration of the affinity between Hu_335PA_{83} and PA_{83} by point mutations

We analyzed the structural properties of the eight residues that differed between Hu_335PA_{83} and Hu_135PA_{83} in the corresponding structural models and observed that four mutations in V_H, S45P, R66N, K80V and L87F, could be responsible for the loss of affinity. Mutation S45P introduced a second Pro residue, adjacent to Pro46, within a β-turn of H-FR2 and could modify its conformation. R66N replaced an Arg residue that occupied an anchor position of H-CDR2 to the framework and contacted the side-chains of Tyr34 in H-CDR1 and Tyr112.4 in H-CDR3, by an Asn residue that contacted Asp61 in H-CDR2. K80V replaced a Lys residue , whose long side-chain made a hydrogen bond with the backbone oxygen atom of Ile30 in H-CDR1, by a Val residue that contacted Gly58 in H-CDR2. Finally, L87F introduced an aromatic residue in proximity of Trp39 (d = 3.5 Å), which is an anchor residue of H-CDR1.

These four mutations were reverted individually in Hu_335PA_{83}, at the genetic level, and the four

Table 4. Scheme of derivation and properties of Hu_1 to Hu_4

Derivative	Parent	Mutations	Level	GI	K_D (nM)	3D model
Hu_1	$35PA_{83}$	11 S	IV	0.910	3.4	Parent
Hu_2	Hu_1	11 S	IS	1.000	nd	≠
Hu_3	Hu_1	8 S	IS, IV	0.983	∞	=
Hu_4	Hu_3	1 R	IV	0.978	3.7	=

The method of derivation is indicated for each derivative: S, substitution; A, addition; IV, *in vitro*; IS, *in silico*. For comparison, $35PA_{83}$ has GI = 0.876 and K_D = 3.4 nM, whereas 83K7C had GI = 0.919 and K_D = 3.6 nM.

Author's personal copy

$Hu_3 35PA_{83}$ derivatives were produced in *E. coli.* The reverse mutation V80K in V_H restored the interaction between $Hu_3 35PA_{83}$ and the PA_{83} antigen in an ELISA. This reverse derivative was named $Hu_4 35PA_{83}$, and we observed that its structural model superimposed with that of $Hu_1 35PA_{83}$. The GI value of $Hu_4 35PA_{83}$ was:

$$GI = [(178-4)/178] = 0.977$$

An alignment of the sequence for $35PA_{83}$, $Hu_2 35PA_{83}$ and $Hu_4 35PA_{83}$ is presented in Fig. 4, with the positions of the FRs, CDRs, V, N, (D) and J regions.

Affinity and neutralization potency of $Hu_4 35PA_{83}$

The K_D value of 3.72 nM between $Hu_4 35PA_{83}$ and the PA_{83} antigen was measured by Biacore. Its 50% inhibitory concentration was measured in an *in vitro* neutralization test as 5.8±0.10 nM. Both K_D and 50 % inhibitory concentration of $Hu_4 35PA_{83}$ were equal to those for $35PA_{83}$, 3.4 nM and 5.6±0.13 nM, respectively, within experimental error.[7]

Discussion

Earlier, the simian Fab $35PA_{83}$ was isolated and shown to neutralize the anthrax lethal toxin, and a search with the IMGT/V-QUEST program has shown that its sequence has a high degree of identity with human germline sequences.[7] However, that study did not evaluate whether the Fv fragment of $35PA_{83}$, which could form the basis for a therapeutic full-length IgG, could be considered as human, and a benchmark was clearly needed for such an evaluation. Another Fab fragment, 83K7C, which also neutralizes the anthrax lethal toxin, has been isolated by others from a human immune library.[11] 83K7C and $35PA_{83}$ have very similar properties in terms of K_D for PA, 3.6 nM and 3.4 nM, respectively, and IC_{50} for the neutralization of the anthrax toxin, 4.8 nM and 5.6 nM, respectively. Therefore, 83K7C was chosen as a benchmark for the present study. The GI value of 0.919 for 83K7C is higher than the value of 0.876 for $35PA_{83}$. The GI value of 83K7C is not equal to 1.00 because somatic hypermutations have been introduced into its FR during the *in vivo* process of affinity maturation. The GI value of 0.919 for $35PA_{83}$ corresponds to 14 differences between its FR and those encoded by the closest human germline genes, when the actual number of differences was 22.

These 22 differences could either correspond to somatic hypermutations or to differences between the macaque and human germline genes. The two types of differences are indistinguishable and both could be immunogenic. We therefore decided to engineer

```
VH
                                 FR1                CDR1          FR2          CDR2
35PA83       QVQLLESGPGLLKPSETLSLTCAVS(GDSIS[GGYY)WS]WIRQSPGKGLEWIG[H(IYGSTADT)RYNP
Hu435PA83    QVQLQESGPGLVKPSETLSLTCTVS(GDSIS[GGYY)WS]WIRQPPGKGLEWIG[H(IYGSTADT)NYNP
Hu235PA83    QVQLQESGPGLVKPSETLSLTCTVS(GDSIS[GGYY)WS]WIRQPPGKGLEWIG[Y(IYGSTADT)NYNP
             |__________________________V__________________________________

                            FR3                           CDR3            FR4
35PA83       SLKS]RVTISKDTSKNQLSLQLRSVTAADTAVYYC(AR[SGYNFWSGEYYGL)DS]WGQGAVVTVSS
Hu435PA83    SLKS]RVTISKDTSKNQFSLQLSSVTAADTAVYYC(AR[SGYNFWSGEYYGL)DS]WGQGTLVTVSS
Hu235PA83    SLKS]RVTISVDTSKNQFSLKLSSVTAADTAVYYC(AR[SGYNFWSGEYYGL)DS]WGQGTLVTVSS
             _________________________________||_D_||___N____| |______J_____|

VL
                          FR1              CDR1        FR2           CDR2
35PA83       DIELTQSPSSLSAYVGDKVTITC[HAS(QGINSW)LA]WYQQKPGKAPKLLI[Y(KASS)LQS]GVPS
Hu435PA83    AIQLTQSPSSLSASVGDRVTITC[RAS(QGINSW)LA]WYQQKPGKAPKLLI[Y(KASS)LQS]GVPS
Hu235PA83    AIQLTQSPSSLSASVGDRVTITC[RAS(QGINSW)LA]WYQQKPGKAPKLLI[Y(KASS)LES]GVPS
             |___________________________V______________________________

                           FR3                       CDR3         FR4
35PA83       RFSGSGSGTDYTLTISSLQSEDFASYYC[(LQYDSAPLAF)]GPGTKLDIK
Hu435PA83    RFSGSGSGTDFTLTISSLQPEDFATYYC[(LQYDSAPLAF)]GPGTKVDIK
Hu235PA83    RFSGSGSGTDFTLTISSLQPEDFATYYC[(LQYDSAPLAF)]GPGTKVDIK
             ____________________________||_N_||_____J____|
```

Fig. 4. Alignment of the sequences (V_H and V_L) of the parental Fab ($35PA_{83}$), its fully germlinized version ($Hu_2 35PA_{83}$), and the most germlinized version that retained the parental affinity ($Hu_4 35PA_{83}$). The residues differing between these versions are written in bold. The CDRs according to the IMGT nomenclature are written in italics and between parentheses, the CDRs according to Kabat's nomenclature are written in italics and between square brackets. FRs (located between CDRs) and CDRs are numbered above the alignment. The V, N, (D) and J regions (corresponding to the human germline genes most resembling $35PA_{83}$; the N region is added without a template) are defined according to IMGT and they are indicated below the alignment.

Author's personal copy

$35PA_{83}$ and increase its GI value by a systematic approach. Other approaches, such as erasing potential T cell epitopes or resurfacing the exposed residues of the Fab, have been described but seemed less robust.[12–14] A systematic approach has been described under the name "super-humanization" as an evolution of the chimerization of murine antibodies.[1,2] In super-humanization, FRs encoded by human germline genes are preferred over FRs of expressed human IgGs because germline-encoded FRs are expressed unmutated in the IgM immunoglobulins of any human, except for allelic variations, and therefore should be as well tolerated as any other human self protein, in contrast to the mutated FR of expressed IgGs.[3] In the present study, we developed a method that we call germlinization, to engineer the FRs from non-human primate variable regions and increase their level of identity with FRs encoded by human germline genes. This method is similar to super-humanization, except for the origin of the antibody fragment, and its precise implementation and results.

We used a multistep approach to germlinize $35PA_{83}$ (Table 4). In the first step, FR1 and FR4 were modified by *in vitro* methods without special precaution because the planned changes, 11 mutations, were located at the ends of the variable domains, and thus expected to cause few constraints on their cores and on the antigen-binding site. As expected, the resulting Hu_135PA_{83} retained the parental affinity and its GI value of 0.938 was already higher than the benchmark value of 0.919.

A 3D structure of Hu_135PA_{83}, restricted to its Fv fragment, was modelled from a sequence with the WAM program. The structure of the parental $35PA_{83}$ could not be modelled, because the WAM program requires certain key residues that were absent from its sequence, i.e. the sequence of $35PA_{83}$ contained the disallowed residues Ala122 and Val123 in positions 5 and 6 of H-FR4. In contrast, Hu_135PA_{83} had the allowed residues Thr122 and Leu123, and was submitted successfully to the WAM program. The structural model of Hu_135PA_{83} was used as a reference for the remainder of the study, since the affinities of $35PA_{83}$ and Hu_135PA_{83} for their common antigen were similar.

The second step of the germlinization process concerned FR2 and FR3 at the core of the Fv fragment, which was studied *in silico* before implementation. We designed the sequence of Hu_235PA_{83}, a fully germlinized version of $35PA_{83}$, where the 11 differences between the FR2 and FR3 regions of Hu_135PA_{83} and the products of its closest human germline genes were removed. We modelled the 3D structure of Hu_235PA_{83}, compared it with that of Hu_135PA_{83}, and observed that they did not superimpose exactly. Therefore, we undertook to revert each mutation, individually and *in silico*, to unveil potentially additive or compensating effects of the other mutations. Three reversions restored the parental model: in V_H, Y55H and K90Q restored the conformations of H-CDR2 and H-CDR3, respectively, whereas in V_L, E68Q restored the general light chain conformation. The Fab fragment Hu_335PA_{83}, derived from Hu_235PA_{83} and carried these three reversions, was produced from a synthetic gene but no longer recognized the PA_{83} antigen.

The third step of the germlinization process consisted of restoring the activity of Hu_335PA_{83} through the rational design of point mutations. Some of the eight residue changes between Hu_135PA_{83} and Hu_335PA_{83} had abolished antigen binding, likely by modifying the conformation of the antigen binding site. An *in silico* analysis of these changes revealed that four of them, in V_H, could induce such a conformational change; S45P, R66N, K80V and L87F. We found that reversion V80K, in V_H, when introduced in Hu_335PA_{83} by *in vitro* methods to give the Hu_435PA_{83} derivative, was sufficient to restore the full parental affinity and neutralization potency of the parental $35PA_{83}$ Fab. An analysis of the V_H structural models suggested that either Val80 prevented a productive interaction between H-CDR2 and the antigen or Lys80 stabilized the active conformation of H-CDR1 by making a hydrogen bond with the oxygen atom of Ile30 (see Results). Hu_435PA_{83} had a GI value of 0.978, which was well above the benchmark value of 0.919.

To evaluate the quality of the germlinization process, we asked whether the four differences between the FR of Hu_435PA_{83}, and the FR that are encoded by the selected human germline genes, could form an epitope and be recognised by the human immune system. In V_H, His55 was not exposed to the solvent in the structural model of Hu_435PA_{83}, and Lys80 was moderately exposed (28%) but only through its backbone atoms. In V_L, Gln68 belonged to a string of 10 amino acid residues, ASSL**Q**SGVPS, which is also encoded by several human IGKV germline

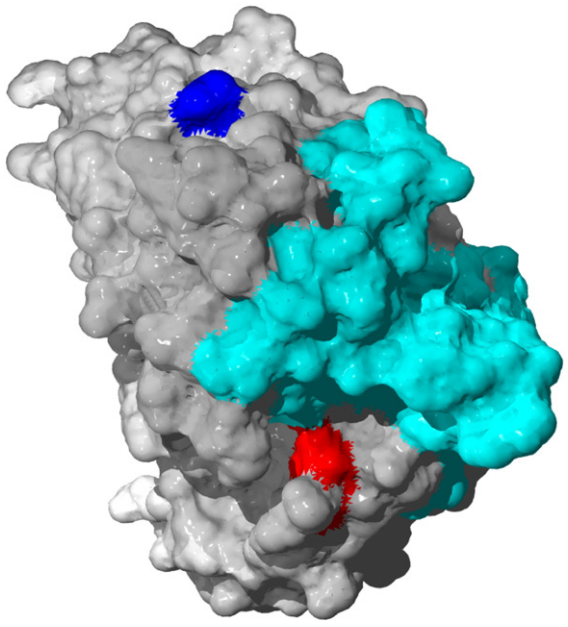


Fig. 5. Structural model of Hu_435PA_{83}, showing Gln68 in the V_L domain (in red) and Gln90 in the V_H domain (in blue) located on opposite sides of the paratope (in cyan).

Author's personal copy

genes (1-6*01, 1-12*01, 1D-16*01, 1D-16*02, 1D-17*01, 1D-39*01, 1D-43*01) and should be well tolerated. In V_H, Gln90 belonged to a string of 10 residues, QLSLQLRSVT, whose closest human equivalent, Q*F*SLQL*N*SVT, is encoded by IGH6-01*01 and differs by only two residues (in italics). Moreover, Gln68 in V_L and Gln90 in V_H were located on opposite sides of the Fab in the structural model, at a distance of 30 Å and should not form an epitope (Fig. 5).

Conclusions

This study started with a simian immune library because we had no access to humans immunized with the antigen of interest,[7] a situation that is widely encountered. Having obtained a Fab with an affinity and a neutralizing potency equivalent to those of a Fab of human origin, we engineered our Fab to increase the degree of identity of its FR with FR encoded by human germline genes. A high degree of identity is arguably a factor of tolerance from a therapeutic perspective, and the degree that was reached with $Hu_4 35PA_{83}$ was higher than that of its human counterpart. This process, which we call germline humanization or germlinization, was made possible by on-line internet tools (a study is underway to systematically evaluate WAM predictions in another germlinization study) and facilitated by affordable molecular biology services. It did not cause any loss of affinity or neutralization potency. Therefore, our results contrast with those that have been reported in two occurrences of the application of the same process to murine antibodies (super-humanization), and which have shown 6-fold and 30-fold reductions of affinity.[1,2]

In conclusion, our study allowed us to obtain a "better than human" Fab — according to the GI parameter — starting from a simian Fab. In addition, it led us to question and quantify the "human nature" of antibodies, a concept that is central in the design and use of recombinant antibodies as therapeutic molecules, and has been the object of some quantification through the calculation of *Z*-scores in a recent study.[15] Following the above rationale, the next step in the development of recombinant antibodies for therapy might be the germlinization of the human antibodies themselves.

Materials and Methods

Nomenclature

The variable domains were denoted V_H for the heavy (H) chain and V_L for the light (L) chain. The first constant domains were denoted C_{H1} for the H-chain and C_L for the L-chain. The framework regions (FR) were denoted H-FR1 to H-FR4 for the H-chain, and L-FR1 to L-FR4 for the L-chain. They were delimited according to the IMGT® unique numbering.[16] The complementary determining regions (CDRs) were denoted correspondingly. The residue positions of $35PA_{83}$ were numbered according to the IMGT® unique numbering.[7]

Identification of human parental genes and calculation of an index of germinality

The on-line analysis of the $35PA_{83}$ nucleotide sequence using IMGT®, the International ImMunoGeneTics information system®†,[17] and in particular the IMGT/V-QUEST,[5] and IMGT/JunctionAnalysis tools,[18] was as described.[7] This analysis identified the human germline genes most similar to the $35PA_{83}$ encoding genes. These human germline genes were translated and the percentage of residues identical between the FR of these translated genes and those of $35PA_{83}$ (four FR in V_L and four FR in V_H) was calculated and called the germinality index (GI). This calculation represented a change over the former identity evaluation, where only six FR, three encoded by the germline V_L gene segment and three encoded by the germline V_H gene segment, were taken into account.[7]

Gene synthesis

The synthetic genes for $Hu_1 35PA_{83}$ and $Hu_3 35PA_{83}$, which are derivatives of $35PA_{83}$, and the four point mutations in the $Hu_3 35PA_{83}$ gene were obtained by Entelechon (Regensburg, Germany).

Gene expression, ELISA and affinity measurements using surface plasmon resonance

The synthetic genes were inserted into the plasmid vector pComb3X,[19] and expressed in the *E. coli* strain HB2151.[20] Cultures were grown until they reached an $A_{600\ nm}$ of 1.5, and then induced with 1 mM IPTG for 12 h at 22 °C. The content of the periplasmic space, including the recombinant Fabs, was extracted with polymyxin as described.[21] The recombinant Fabs were purified by affinity chromatography on Ni-NTA columns, according to the manufacturer's instructions (Qiagen, Valencia, CA). They were tested by ELISA as described.[7] Affinities were measured by surface plasmon resonance with a Biacore X instrument and related consumables and programs (Biacore, Upsala, Sweden). PA was immobilized at ≤200 resonance units on a CM5 chip via amine coupling, according to the manufacturer's instructions. The binding experiments were performed in HBS-EP buffer at a flow rate of 30 μL/min. At least six concentrations of Fab (0.1–10 μg/mL) were tested for 900 s each. The chip was regenerated between runs with Glycine 1.5 reagent at a flow rate of 10 μL/min for 30 s. The binding constants were calculated as described and verified by the internal consistency tests of the BiaEvaluation program.[22]

Structural predictions and comparisons

The structures of $35PA_{83}$ derivatives, restricted to their variable fragment, were modelled from their sequences by the WAM program‡.[9,23] The comparisons of the modelled structures were performed with the Swiss-PDBviewer program.[24]

† http://imgt.cines.fr
‡ http://antibody.bath.ac.uk

Author's personal copy

Neutralization test

The mouse macrophage cell line J774A.1 was plated overnight at 14,000 cells/well in 96-well microtitre plates. Lethal toxin components (400 ng ml^{-1} of PA and 40 ng ml^{-1} of LF), purchased from List Biological Laboratories (Campbell, CA), were added simultaneously to Fab or to medium alone (positive control), incubated for 1 h at 37 °C, then added to macrophages and incubated at 37 °C for 4 h.[25] The Cytotox® assay96 kit (Promega, Madison, WI) was used according to the manufacturer's instructions to assess cell viability and evaluate IC_{50} for the neutralization of the lethal toxin by the Fab.[11]

References

1. Tan, P., Mitchell, D. A., Buss, T. N., Holmes, M. A., Anasetti, C. & Foote, J. (2002). "Superhumanized" antibodies: reduction of immunogenic potential by complementarity-determining region grafting with human germline sequences: application to an anti-CD28. *J. Immunol.* **169**, 1119–11125.
2. Hwang, W. Y., Almagro, J. C., Buss, T. N., Tan, P. & Foote, J. (2005). Use of human germline genes in a CDR homology-based approach to antibody humanization. *Methods*, **36**, 35–42.
3. Williams, G. T., Jolly, C. J., Kohler, J. & Neuberger, M. S. (2000). The contribution of somatic hypermutation to the diversity of serum immunoglobulin: dramatic increase with age. *Immunity*, **13**, 409–417.
4. Harris, W. J. & Cunningham, C. (1995). Humanized antibodies. In *Antibody Therapeutics*, chapt. 6, pp. 85–110, R. G. Landes Company, Austin, TX.
5. Giudicelli, V., Chaume, D. & Lefranc, M. P. (2004). IMGT/V-QUEST, an integrated software program for immunoglobulin and T cell receptor V-J and V-D-J rearrangement analysis. *Nucleic Acids Res.* **32**, W435–W440.
6. Chassagne, S., Laffly, E., Drouet, E., Herodin, F., Lefranc, M. P. & Thullier, P. (2004). A high-affinity macaque antibody Fab with human-like framework regions obtained from a small phage display immune library. *Mol. Immunol.* **41**, 539–546.
7. Laffly, E., Danjou, L., Condemine, F., Vidal, D., Drouet, E., Lefranc, M. P. *et al.* (2005). Selection of a macaque Fab with framework regions like those in humans, high affinity, and ability to neutralize the protective antigen (PA) of Bacillus anthracis by binding to the segment of PA between residues 686 and 694. *Antimicrob. Agents Chemother.* **49**, 3414–3420.
8. Pelat, T., Hust, M., Laffly, E., Condemine, F., Bottex, C., Vidal, D. *et al.* (2007). High-affinity, human antibody-like antibody fragment (single-chain variable fragment) neutralizing the lethal factor (LF) of Bacillus anthracis by inhibiting protective antigen-LF complex formation. *Antimicrob. Agents Chemother.* **51**, 2758–2764.
9. Whitelegg, N. R. & Rees, A. R. (2000). WAM: an improved algorithm for modelling antibodies on the WEB. *Protein Eng.* **13**, 819–824.
10. Laffly, E., Pelat, T., Cedrone, F., Blesa, S., Bedouelle, H. & Thullier, P. (2008). Improvement of an antibody neutralizing the anthrax toxin by simultaneous mutagenesis of its six hypervariable loops. *J. Mol. Biol.* **378**, 1094–1103.
11. Wild, M. A., Xin, H., Maruyama, T., Nolan, M. J., Calveley, P. M., Malone, J. D. *et al.* (2003). Human antibodies from immunized donors are protective against anthrax toxin in vivo. *Nature Biotechnol.* **21**, 1305–1306.
12. Baker, M. P. & Jones, T. D. (2007). Identification and removal of immunogenicity in therapeutic proteins. *Curr. Opin. Drug Discov. Dev.* **10**, 219–227.
13. Zhang, W., Feng, J., Li, Y., Guo, N. & Shen, B. (2005). Humanization of an anti-human TNF-alpha antibody by variable region resurfacing with the aid of molecular modeling. *Mol. Immunol.* **42**, 1445–1451.
14. Delagrave, S., Catalan, J., Sweet, C., Drabik, G., Henry, A., Rees, A. *et al.* (1999). Effects of humanization by variable domain resurfacing on the antiviral activity of a single-chain antibody against respiratory syncytial virus. *Protein Eng.* **12**, 357–362.
15. Abhinandan, K. R. & Martin, A. C. (2007). Analyzing the "degree of humanness" of antibody sequences. *J. Mol. Biol.* **369**, 852–862.
16. Lefranc, M. P., Pommie, C., Ruiz, M., Giudicelli, V., Foulquier, E., Truong, L. *et al.* (2003). IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains. *Dev. Comp. Immunol.* **27**, 55–77.
17. Lefranc, M. P. (2005). IMGT, the international ImMunoGeneTics information system: a standardized approach for immunogenetics and immunoinformatics. *Immunome Res.* **1**, 3.
18. Yousfi Monod, M., Giudicelli, V., Chaume, D. & Lefranc, M. P. (2004). IMGT/JunctionAnalysis: the first tool for the analysis of the immunoglobulin and T cell receptor complex V-J and V-D-J JUNCTIONs. *Bioinformatics*, **20**, i379–i385.
19. Andris-Widhopf, J., Rader, C., Steinberger, P., Fuller, R. & Barbas, C. F., 3rd (2000). Methods for the generation of chicken monoclonal antibody fragments by phage display. *J. Immunol. Methods*, **242**, 159–181.
20. Carter, P., Bedouelle, H. & Winter, G. (1985). Improved oligonucleotide site-directed mutagenesis using M13 vectors. *Nucleic Acids Res.* **13**, 4431–4443.
21. Renard, M., Belkadi, L., Hugo, N., England, P., Altschuh, D. & Bedouelle, H. (2002). Knowledge-based design of reagentless fluorescent biosensors from recombinant antibodies. *J. Mol. Biol.* **318**, 429–442.
22. Karlsson, R., Michaelsson, A. & Mattsson, L. (1991). Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. *J. Immunol. Methods*, **145**, 229–240.
23. Whitelegg, N. & Rees, A. R. (2004). Antibody variable regions: toward a unified modeling method. *Methods Mol. Biol.* **248**, 51–91.
24. Guex, N. & Peitsch, M. C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*, **18**, 2714–2723.
25. Little, S. F., Leppla, S. H. & Friedlander, A. M. (1990). Production and characterization of monoclonal antibodies against the lethal factor component of Bacillus anthracis lethal toxin. *Infect. Immun.* **58**, 1606–1613.