Humanization strategies for an anti-idiotypic antibody mimicking HIV-1 gp41

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Anti-idiotypic antibodies could represent an alternative vaccination approach in human therapy. The anti-idiotypic antibody Ab2/3H6 was generated in mouse and is directed against the human monoclonal antibody 2F5, which broadly and potently neutralizes primary HIV-1 isolates. Ab2/3H6 is able to mimic the antigen recognition site of 2F5 making it a putative candidate for HIV-1 vaccine purposes. In order to reduce immunogenicity of therapeutic proteins, humanization methods have been developed. The mouse variable regions of Ab2/3H6 were subjected to three different humanization approaches, namely resurfacing, complementarity determining region (CDR)-grafting and superhumanization. Four different humanized Ab2/3H6 variants were characterized for their binding affinity to 2F5 in comparison to the chimeric Ab2/3H6. The resurfaced and the ‘conservative’ CDR-grafted variants showed similar binding properties to 2F5 when compared to the chimeric version, while the ‘aggressive’ CDR-grafted antibody showed reduced affinity and the superhumanized type lost its binding ability. In this study, we developed humanized Ab2/3H6 variants that retained the same affinity as the parental antibody, and are therefore of potential interest for future clinical trials.

Keywords: 2F5/anti-idiotype/HIV-1/humanization/vaccine

Introduction

More than 30 years ago, Niels K. Jerne postulated ‘The Network Theory of the Immune System’, in which he presented the observation that antibodies (Abs) can elicit anti-idiotypic Abs (Ab2) directed against the paratope of the first Ab (Jerne, 1974). These Ab2 are expected to mimic the initial antigen (Jerne et al., 1982; Fields et al., 1995). After contact with the immune system, Ab2 induce anti-anti-idiotypic Abs (Ab3) (Shoenfeld, 1995) similar in the capability to the first Ab. Therefore, Ab2 are potent as vaccines, which has been shown in recent clinical trails (Hurvitz and Timmerman, 2005; Park et al., 2005; Inogés et al., 2006).

For most infectious diseases including smallpox, diphtheria, tetanus, hepatitis A, hepatitis B, influenza and others (Amanna et al., 2008; Plotkin, 2008), conventional vaccines are able to induce a humoral immune response. This, however, has not been achieved for AIDS. During 30 years of research, attempts to develop a vaccine eliciting broadly neutralizing HIV-1 Abs have only been of limited success (Burton et al., 2004; Flynn et al., 2005; Haynes and Montefiori, 2006; Rerks-Ngarm et al., 2009).

One of the most potent Ab identified so far is the monoclonal Ab (mAb) 2F5 (Trkola et al., 1995), which binds with high affinity to a conserved site of the membrane-proximal external region (MPER) of the virus envelope glycoprotein gp41 (Stoiber et al., 1997). It has been shown that mAb 2F5 broadly and potently neutralizes primary HIV-1 isolates (Purtscher et al., 1994). The mouse-derived Ab2/3H6 (ABP04229, ABP04230) was developed at the Department of Biotechnology (Kunert et al., 2002) and is directed against mAb 2F5. The chimeric version of Ab2/3H6 (chAb2/3H6) significantly inhibits the binding of mAb 2F5 to its synthetic epitope ELDKWA in an equimolar ratio and also decreases the in vitro neutralization potency of mAb 2F5 in a dose-related manner (Gach et al., 2007). Ab2/3H6 is therefore expected to mimic the epitope of mAb 2F5 and is of great therapeutic interest as an anti-idiotypic vaccine against HIV.

However, since Ab2/3H6 originates from mouse, a human anti-mouse immune response (HAMA) (Sgro, 1995) or, in case of the chimeric version, a human anti-chimeric response (HACA) (Hwang and Foote, 2005) might be triggered when Ab2/3H6 is applied in human therapy; an effect that is required to be circumvented. Induced Ab3 should theoretically be directed against the paratope of the Ab2 which in case of Ab2/3H6 is composed of the complementarity determining regions (CDRs). In contrast, Ab3 immune responses against the framework regions (FR) do not contribute to the anticipated effect and should be prevented. Therefore, the aim of humanization approaches is to reduce immunogenicity of FR regions and thus drive the Ab3 immune response towards the paratope of the Ab2, which is structurally similar to the mAb 2F5 HIV-1 epitope. In the past 25 years, several humanization methods have been developed and applied to various murine Abs before entering clinical trials (reviewed in Almagro and Fransson, 2008). In the present study, we applied three different humanization approaches to the mouse-derived Ab2/3H6 (muAb2/3H6) and analysed the newly generated proteins in comparison to the chAb2/3H6. The results of our studies are discussed according to the existing literature.

Materials and methods

Humanization of Ab2/3H6

Resurfacing. The idea of resurfacing is that the immunogenicity of a protein is determined by the surface accessibility of amino acids (aa) and protruding residues (Novotný et al.,
Therefore, a molecular model of muAb2/3H6 was generated by web-based Ab modelling software WAM (http://antibody.bath.ac.uk/index.html) (Whitelegg and Rees, 2000). This program predicts the side chains of the loops using ‘CONGEN iterative algorithm’ (Brucoleri and Karplus, 1987). The CDR-H3 of Ab2/3H6 is relatively long with 13 aa. Therefore, we identified the structure of the loop with ‘VFF side-chain energy screen’ (Dauber-Osguthorpe et al., 1988). The generated model of the murine Ab was used to identify surface accessible residues (Staelens et al., 2006) with a threshold that was set to 30% (Pedersen et al., 1994) using the Swiss Pdb Viewer Software (Gueix and Peitsch, 1997). In a second step, the sequence of the muAb2/3H6 variable heavy (V_{H}) and light (V_{L}) chain was searched using IGBLAST against the human IgG germline database (NCBI; http://www.ncbi.nlm.nih.gov/igblast/). The stated surface residues were exchanged manually to those found on the selected human germline sequence and then subjected to energy minimization [GROMOS 43B1 force field; (van Gunsteren et al., 1996)] using the software Swiss PDB Viewer program.

The resulting models were viewed and analysed with SwissPdb Viewer and PyMOL (http://pymol.sourceforge.net/). Based on this information, we superimposed the models of muAb2/3H6 and RS3H6 to determine changes in the loop structure.

**CDR graft.** During CDR grafting, the CDRs of the murine Ab are transferred to a human FR (Jones et al., 1986). For CDR grafting, the fixed FR approach was chosen in which the closest related existing human Ab structure is used as acceptor FR. Therefore, the muAb2/3H6 V_{H} and V_{L} chain was searched against the UniProt database (www.uniprot.org). This procedure might lead to a reduction or complete loss of binding affinity because certain FR residues contribute significantly to the conformation of the CDRs (Chothia et al., 1989; Foote and Winter, 1992) or are even directly involved in antigen binding (Mian et al., 1991). This problem is solved by reintroducing the murine amino acids into the human FR at positions that are critical for CDR loop conformation (Riechmann et al., 1988; Queen et al., 1989; Co et al., 1991; Foote and Winter, 1992). With this method, we generated two different CDR-graft variants, one ‘conservative’ graft (GC3H6) with various backmutations according to the mouse FR and one ‘aggressive’ graft (GA3H6) with less backmutations.

**Superhumanization.** The superhumanization approach is based on structural homologies between mouse and human CDRs. It is based on the axiom that if a mouse and human Ab have similar-structured CDRs, the human FR will also support the mouse CDRs, with retention of affinity. FR homology is not a factor and critical FR residues are not carried over from the mouse into the humanized Ab (Tan et al., 2002; Hwang et al., 2005). The comparison of CDRs which are structurally clustered into canonical structure classes was defined by Chothia and Lesk (1987). An automated determination of the Chothia canonical structure class from immunoglobulin sequences has been provided by Dr Andrew C.R. Martin’s laboratory and is available online at http://www.bioinf.org.uk/abs/chothia.html.

In the next step, a human germline sequence with the same canonical structure class as the mouse Ab with the best residue to residue match in the CDRs is defined as acceptor FR. Afterwards, mouse CDRs are grafted onto the chosen acceptor FR to create the superhumanized variant SH3H6.

**Antibody expression**

The genes of the V_{H} and V_{L} of the humanized Ab2/3H6 variants RS3H6, GC3H6, GA3H6 and SH3H6 were synthesized by Genearth (Regensburg, Germany).

The synthetic V_{H} genes were inserted into the plasmid vector phuIgG1 containing a human IgG1 HC leader/constant region under the control of the SV40 promoter and a dihydrofolate reductase (dhfr) cassette. In parallel, the synthetic V_{L} genes were inserted into the plasmid vector phuKappa containing a human kappa LC leader/constant region under the control of the CMV and a neomycin selection cassette. Similarly, the chAb2/3H6 was recombinantly expressed and used as reference material in all analyses (Gach et al., 2007).

Stable cell lines were generated by cotransfection of corresponding HC and LC plasmids into CHO dhfr negative cells [ATCC CRL-9096; (Urlaub and Chasin, 1980)]. Clones were selected with Geneticin G418 (Fisher Scientific) and methotrexate (MTX) (Sigma) in combination with limiting dilution subcloning. Collected supernatants were concentrated by Stirred cell 8200 with UF Discs Ultraceal RC 10 kD (Millipore) before purification.

**Purification via affinity chromatography**

Purification of all Ab2/3H6IgG1 variants was performed on a BioLogic Duo Flow chromatography system (Biorad). Ten millilitre concentrated animal cell culture supernatant was filtered through a 0.22 μm syringe filter (Millipore) and diluted 1:2 in buffer A (100 mM Glycin, 100 mM NaCl, pH 7.0). The UNOsphere SUPra Mini cartridge column (Biorad) was equilibrated with 5 column volumes (cv) buffer A. Twenty millilitre of the concentrated sample were loaded onto the column using direct injection via the DuoFlow F40 pump at a flow rate of 1 ml/min corresponding to a linear velocity of 243.6 cm/h. The column was washed with buffer A until the optical density returned to base line. IgGs were eluted from the Protein A column using a three-step gradient of 40, 60 and 100% of buffer B (100 mM Glycin pH 3.5) in buffer A in 5 cv at a flow rate of 1.5 ml/min (364.4 cm/h). Quantification of Ab2/3H6IgG1 was done by quantitative double sandwich ELISA.

**Antibody affinity**

Bio-Layer Interferometry (BLI), a label-free technology, was used for measuring the interactions of Ab2/3H6IgG1 with mAb 2F5. Affinity measurements were performed with an Octet QK equipped with streptavidin (SA) biosensor tips (forteBio, Menlo Park, CA, USA). The assay was performed at 30°C in 1× Kinetics assay buffer (forteBio). Samples were agitated at 1000 rpm. Prior to analysis, SA sensors were humidified for 15 min. Tips were loaded with 1 μg/ml of biotinylated mAb 2F5 (mAb2F5-B) for 80 s which typically resulted in capture levels of 0.7 to 0.8 nm within a row of eight tips. Ab2/3H6IgG1 variants were prepared in a 500 nM concentration. Association was monitored for 60 s. Data were processed and analysed using the Octet data analysis software 6.3 (forteBio).
Competition ELISA

Apparent affinity constants (aK) of Ab2/3H6 IgG1 variants were calculated by 505 residual binding of mAb2F5-B in a competition ELISA (Kunert et al., 2000). Briefly, the synthetic peptide GGGLDLDWASL was coated onto MaxiSorp 96-well plates (Nunc). Culture supernatant of the Ab2/3H6 IgG1 variants was serially diluted and mixed with a constant amount of mAb2F5-B (50 ng/ml). The mixture and the serially diluted standard (mAb2F5-B; 100 ng/ml) were incubated for 1 h and afterwards transferred onto the pre-coated plates. Unbound mAb2F5-B was detected with horseradish-peroxidase labelled SA conjugate (Roche).

Results

Humanization of 3H6

Resurfacing. The human germline VH sequence IGHV1-3*01 (64.9% identity to muAb2/3H6 VH) and VL kappa sequence IGKV5-2*01 (63.2% identity to muAb2/3H6 VL) were defined as corresponding human germlines.

The model of the muAb2/3H6 was used to identify surface accessible residues. Single aa were substituted manually with human germline residues. The side chains were rotated adapting to the human version (GlnH5Val, ThrH14Pro and HisH41Pro). Additionally, the missing residue GlnH1 was inserted and the buried SerH40 was changed to the corresponding human Ala since the model revealed that SerH40 and HisH41Pro). Moreover, the missing residue GlnH1 was inserted and the buried SerH40 was changed to the corresponding human Ala since the model revealed that SerH40 might clash with ProH41. In case of muAb2/3H6 VH, 7 out of 16 surface accessible aa differed from the human germline sequence and were subjected to energy minimization using the Swiss PDB Viewer program. Sixteen aa in the VH of muAb2/3H6 were identified as surface accessible residues. Only three of them differed from the human germline sequence and were adapted to the human version (GlnH5Val, ThrH14Pro and HisH41Pro). Additionally, the missing residue GlnH1 was inserted and the buried SerH40 was changed to the corresponding human Ala since the model revealed that SerH40 might clash with ProH41. In case of muAb2/3H6 VH, 7 out of 16 surface accessible aa differed from the human germline sequence and were substituted to their corresponding aa (SerH40Thr, LeuH11Met, IleH1.5Pro, GlnH1.7Asp, ArgL41Ile, SerL60Pro and GlnL100Gln). The molecular model showed a possible clash between the introduced AspL1.7 and the SerL1.4 therefore, the buried SerL1.4 was changed to Thr. Further, the model revealed that additional H-bonds were formed between the buried MetL13GlnL105 as well as LysL107. Thus MetL13 was changed to Ala.

Afterwards, the models of the mouse variable region and the resurfaced Ab2/3H6, named RS3H6, were superimposed to determine conformational changes in the loop regions. As depicted in Fig. 1, the RS3H6 CDRs coloured in red showed only minor differences in the loop structures in comparison to the muAb2/3H6 CDRs coloured in green. Additionally, we analysed the root-mean-square deviation (RMSD), which can be used as a measure for the similarity in three-dimensional structures (Maiorov and Crippen, 1994). High RMSD values mean dissimilar and zero means identical in conformation. RMSD cut-off values have been published for different applications proposing RMSD value <1.5 Å is suitable for defining antibody epitopes (Baker and Sali, 2001). The RMSD values of the CDR loop structures model of RS3H6 in comparison to Ab2/3H6 ranged from 0.50 to 1.34 Å indicating a similar conformation. The only difference between RS3H6 and muAb2/3H6 was observed in the loop of CDR-H2. Figure 2 shows conformational changes at position YH53 resulting in an RMSD value of 2.213 Å. The Y at position 53 in the RS3H6 variant has a different side-chain conformation in comparison to the muAb2/3H6 resulting in the higher RMSD value. The model of the CDR-H2 showed that different angles of the YH53 side chain can be present in the loop. The formation seen in RS3H6 CDR-H2 has a slightly better energy minimum in comparison to the conformation seen in muAb2/3H6 CDR-H2 (data not shown). Due to this minor energy differences and the fact that surface exposed side chains are flexible in solution, we suppose that YH53 will exist in both conformations and might not influence the correct CDR-H2 conformation.

CDR graft. The human Ab EU VH (P01742.1) and VL (P01598.1) sequences were defined as acceptor FR. The sequence of muAb2/3H6 VH showed 53% identity to human Ab EU VH and muAb2/3H6 VL has 47% identity to EU VL. We identified 36 aa in the FR of muAb2/3H6 VH and 34 in the FR of muAb2/3H6 VL that differed from the acceptor FR sequence. The alignment of muAb2/3H6 VH and VL sequences with their corresponding EU sequence is shown in Fig. 2.

In order to obtain a functional CDR-grafted Ab2/3H6 Ab, a so-called ‘conservative’ graft (GC3H6) harbouring backmutations at all identified positions that might influence the CDR conformation was developed. In GC3H6, 22 aa in the VH and 13 aa in the VL FR were identified for backmutation (highlighted in grey in Fig. 2) and mutated in the human acceptor FR to their murine counterpart (Table I). As summarized in Table I the reasons for switching single aa are (i) influences on the structure of the antibody, (ii) contribution to the canonical structure class, (iii) regions of venier-zones, (iv) contribution of aa to the VH/VL interface or (v) lack of single positions in the EU antibody. In order to reduce the amount of backmutations, making the grafted Ab more humanlike, a so-called ‘aggressive’ graft (GA3H6) harbouring only backmutations at probable critical positions like venier-zones or regions contributing to the canonical structure classes was designed. This variant has only 10 backmutations in the VH and 5 backmutations in the VL (Table I).

Superhumanization. Canonical structure classes of muAb2/3H6 were identified as class 1-2 for VH and class 2-1-1 for VL (Table II) according to http://www.bioinf.org/abs/chothia.html. Human germline VH/JH and VL/Jk gene sequences were extracted from the V-Base database (http://vbase.mrc-cpe.cam.ac.uk/). Table II describes the structure classes of the muAb2/3H6 and the nearest related human germline VH and VL gene sequences. The human germline VH sequence with the highest sequence identity in the CDRs representing the canonical structure class 1-2 is IGKV1-3*01 with 80% identity in CDR-H1, and 31% in CDR-H2. The JH segment contributes a variable number of residues of CDR-H3. Therefore, JH4 which has YFDY at position 100d-102 was chosen as the best match. The human germline VL sequence with the highest sequence identity in the CDRs representing the canonical structure class 2-1-1 is IGKV5-2*01 with 64% identity in CDR-L1 and 43% in CDR-L2. The Jk segment encodes two residues of CDR-L3. Jk2 has YT at position 96–97 and was chosen as the best match. Afterwards, the muAb2/3H6 CDRs were grafted onto the above-defined human acceptor FR.
The resulting Ab SH3H6 is composed of human germline FR regions and the original mouse CDRs, respectively. No murine FR residues were carried over to the humanized construct. Figure 3 shows sequence identities in the CDR regions between muAb2/3H6 and the nearest related human germline sequence, which was selected by identities with the same canonical structures to demonstrate similarity.

**Binding properties of 3H6 humanization variants**

The purified IgGs of all Ab2/3H6 variants were tested for their binding capacity with the Octet QK and in a competition ELISA. The binding of the Ab2/3H6IgG1 variants to mAb2F5 was determined in an affinity binding study using BLI. The binding curves (Fig. 4a) of RS3H6 and GC3H6 show a similar interaction with mAb2F5 as chAb2/3H6. The lower signal of GA3H6 indicates a lower affinity to its antigen, whereas SH3H6 shows no interaction. The binding rate ($k_{obs}$) calculated by the forteBio Octet QK software package is summarized in Fig. 4b. RS3H6, GC3H6 and chAb2/3H6 showed similar $k_{obs}$ values ranging from $6.2 \times 10^{-2}$ to $7.5 \times 10^{-2}$ s$^{-1}$ indicating the same affinity to mAb2F5, whereas GA3H6 ($3.8 \times 10^{-2}$ s$^{-1}$) has a two-fold reduced affinity and SH3H6 does not bind to mAb2F5.

Further we compared the four humanized Ab2/3H6 variants and the chAb2/3H6 in a competition ELISA (Fig. 5). The optical density resulting from binding of mAb2F5 to its peptide epitope GGGELDKWASL is plotted versus the logarithm of the concentration of Ab2/3H6 preparations in the liquid phase. RS3H6 and GC3H6 showed a nearly identical behaviour of concentration-dependent mAb2F5 inhibition. Binding capacity of mAb2F5 to its peptide epitope (GGGELDKWASL) was reduced by 50% after incubation of mAb2F5 with a 5-fold excess of RS3H6, GC3H6 or chAb2/3H6. Besides, GA3H6 inhibited 50% mAb2F5 only with a 17-fold excess. In contrast, SH3H6 did not inhibit mAb2F5 binding to the solid phase even in an 80-fold excess. Table III summarizes the $aK$ values calculated as the reciprocal value of the Ab2/3H6 concentration required to inhibit 50% of the maximal binding in a competitive ELISA. RS3H6 and GC3H6 showed similar $aK$ values ($2.0 \times 10^{9} \text{ M}^{-1}$) as the ch3H6 ($2.5 \times 10^{9} \text{ M}^{-1}$), whereas GA3H6 had a reduced affinity ($7.1 \times 10^{8} \text{ M}^{-1}$) compared to the
original Ab and SH3H6 lost the ability to inhibit mAb2F5 binding to its epitope. An equimolar amount of the negative control (unspecific human IgG) did not show interaction with mAb2F5.

Taken together, the results of the BLI and the competition ELISA assay show that RS3H6 and GC3H6 retained the same binding affinity and neutralizing potency as chAb2/3H6, while GA3H6 had a minor loss in its affinity and SH3H6 completely lost its binding ability to mAb2F5.

Discussion
In the past 20 years, rational and empirical humanization methods have been developed (reviewed in Almagro and Fransson, 2008), but there is still no generally applicable method available which can be used to eliminate potential immunogenic sites while keeping the specificity of the Ab. Each Ab has to be analysed separately and different methods have to be tested in each particular case to obtain a
humanized variant that features the same affinity and biological activity as the parental Ab.

Variable domain resurfacing emerged as an alternative to CDR grafting. The basic idea of this method is that only surface residues in the non-human Ab are able to induce HAMA and therefore only these few murine surface aa are changed to human residues (Pedersen et al., 1994; Roguska et al., 1994). The resurfacing method is quite promising in terms of retaining antigen affinity because no changes are introduced to the protein core. Since resurfacing only changes the residues at the surface of the molecule, the expectation is to eliminate potential B-cell epitopes while

Fig. 3. Alignment of amino acid sequences of the heavy chain CDRs (a) and light chain CDRs (b) of human germline sequences IGHV1-f*01/JH4, IGKV5-02*01/JK2 and mouse antibody Ab2/3H6.

Fig. 4. Binding curves of Ab2/3H6IgG1 variants to mAb 2F5 (a) and comparison of the binding rate ($k_{obs}$) of the Ab2/3H6IgG1 variants (b).
retaining the specificity resulting in an Ab that would show little or no immunogenicity when applied in human therapy. Unfortunately T-cell epitopes are not considered using this method. However, recent clinical evaluation of the resurfaced Ab conjugate huC242-DM1 (Cantuzumab mertansine) showed no anti-human globulin response in patients (Rodon et al., 2008), proving that the resurfacing approach results in Abs with reduced immunogenicity when applied in human therapy.

The conventional method for humanization of a murine Ab is CDR grafting. This approach has been used for the majority of humanized Abs approved by the Food and drug Administration (FDA) including Herceptin® (trastuzumab) (Carter et al., 1992), Campath® (alemtuzumab) (Riechmann et al., 1988) or Xolair® (omalizumab) (Presta et al., 1993). Commonly, affinity decreases after CDR grafting as a consequence of incompatibilities between non-human CDRs and human FRs. In order to circumvent this problem, backmutations have to be introduced to restore or prevent affinity losses. These backmutations have to be defined individually for each Ab and each aa positions even if Abs have a high sequence homology or similar antigen specificity (Rosok et al., 1996). In some cases, backmutations at known critical positions are counterproductive (Caldas et al., 2000; Gonzales et al., 2003). Thus, introducing backmutations is difficult and not always straight forward, which has been shown for the humanized Ab Xolair® (omalizumab) where 22 different variants were tested experimentally to determine the best version (Presta et al., 1993) or Omnitarg® (pertuzumab) where 10 variants have been developed to obtain an humanized variant with the same affinity as the parental Ab (Adams et al., 2006). More than 10 CDR grafted Abs for human therapies have been approved by the FDA to date. Clinical studies revealed that CDR grafted Abs have a reduced immunogenicity when applied in human therapy (Hwang and Foote, 2005).

Superhumanization is based on structural comparisons of CDRs in which a FR homology is irrelevant. Using this strategy, three Abs have already been successfully humanized in the past (Tan et al., 2002; Hwang et al., 2005; Hu et al., 2007). This approach has the advantage over CDR grafting that only one version has to be created because it was postulated that the CDRs between Abs with the same canonical loop structure can be substituted without altering the conformation (Tan et al., 2002). This leads to a humanized Ab with a complete human germline FR and therefore immunogenicity is expected to be minimal because the remaining murine residues lie inside the CDRs. Using this strategy, the murine anti-human CD28 Ab 9.3 was humanized resulting in a variant with a 30-fold loss in affinity but the biological activity was retained (Tan et al., 2002). In an other case, the superhumanized form of the Ab D1.3 had a better affinity (6-fold loss in comparison to the murine variant) (Hwang et al., 2005) than the CDR-grafted version (70-fold loss in affinity) (Foote and Winter, 1992).

To conclude on this study, the induction of neutralizing 2F5-like Abs is challenging and has not been achieved so far with conventional epitope vaccination approaches (Burton et al., 2004). A possible reason might be that the mAb2F5 epitope is only weakly immunogenic. As part of the MPER, it is difficult to keep a stable and correct conformation when used as a conventional vaccine (Ofek et al., 2004; Cardoso et al., 2005). Therefore, an Ab2 vaccine approach would be a

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**Table III.** $\alpha K$ values determined for different Ab2/3H6 preparations

<table>
<thead>
<tr>
<th>3H6 variants</th>
<th>IC50 (nM)</th>
<th>$\alpha K$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ch3H6</td>
<td>0.5</td>
<td>2.5 x 10$^6$</td>
</tr>
<tr>
<td>RS3H6</td>
<td>0.4</td>
<td>2.0 x 10$^6$</td>
</tr>
<tr>
<td>GC3H6</td>
<td>0.4</td>
<td>2.0 x 10$^6$</td>
</tr>
<tr>
<td>GA3H6</td>
<td>1.4</td>
<td>7.1 x 10$^6$</td>
</tr>
<tr>
<td>SH3H6</td>
<td>No binding to 2F5</td>
<td>No binding to 2F5</td>
</tr>
<tr>
<td>Irrelevant IgG</td>
<td>No binding to 2F5</td>
<td>No binding to 2F5</td>
</tr>
</tbody>
</table>

IC50, 3H6 concentration required for 50% inhibition of maximal binding in competitive ELISA. $\alpha K = 1/IC50$.  

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*Fig. 5.* Experimental determination of the apparent affinity constant ($\alpha K$) of Ab2/3H6 variants.
promising alternative. In former studies, muAb2/3H6 was able to induce 2F5-like Abs in mice (Kunert et al., 2002; Gach et al., 2007). In order to use Ab2/3H6 in a clinical vaccination study, the reduction of its immunogenicity in the FRs is essential to reduce adverse effects.

We have applied three different state of the art humanization approaches to the muAb2/3H6. The humanized Ab2/3H6 variants RS3H6, GC3H6 showed the same affinity and GA3H6 had a 2-fold loss in affinity to muAb2F5 activity in comparison to chAb2/3H6. SH3H6 lost the ability to bind muAb2F5. This approach was not suitable for humanization of Ab2/3H6. We postulate that humanizing the FR of Ab2/3H6 will drive the immune response towards the paratope of the Ab2 and therefore enhance elicitation of Ab3 when administered during therapy. Affinity to mAb2F5 is therefore not the crucial factor in evaluating the best candidate for a clinical study. Our results show that the Ab2/3H6 variants RS3H6 and GA3H6 would be suitable candidates for further studies but due to regulatory reasons and the fact that the majority of humanized Abs approved by the FDA are CDR-grafted GA3H6 is the candidate of choice.

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