Selection of human anti-hapten antibodies from semisynthetic libraries

(Phage display; combinatorial libraries; CDR; surface plasmon resonance; panning)

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SUMMARY

Semisynthetic human Fab libraries were constructed, displayed on the surface of filamentous phage and selected for binding to three hapten conjugates. A number of Fabs were isolated and characterized with respect to affinity and specificity. Fabs exhibited affinities of between 80 and 29 nM, as determined by surface plasmon resonance, for the conjugate on which they were selected. Conservation of Asp in the third heavy-chain complementarity determining region (HCDR3) appears to be important in the construction of synthetically diverse repertoires.

INTRODUCTION

The in vitro generation of antibody (Ab) diversity offers an alternative to immunization for the production of mAb (Lerner et al., 1992). Molecular diversity in Ab combing sites may be accessed by the combinatorial association of heavy and light chain variable regions to produce combinatorial Ab libraries (Huse et al., 1989). To date combinatorial Ab libraries prepared from immune individuals have been applied to produce high affinity human antibodies with regular success (Barbas et al., 1991; 1992b,c,d; Burton et al., 1991; Zebede et al., 1992; Sungchoon et al., 1993; Williamson et al., 1993). Alternatively, libraries may be prepared from non-immune sources to generate 'naive' libraries which generally yield low affinity Ab (Marks et al., 1991; Gram et al., 1992). Low affinity Ab may, however, be improved for example by iterative chain-shuffling (Marks et al., 1992) or error-prone PCR (Gram et al., 1992) and reselction.

We are interested in exploring synthetic approaches to the generation of Ab wherein molecular diversity can be controlled at the level of oligo synthesis. Previously we had demonstrated that by randomizing, HCDR3 of a human anti-tetanus toxoid antibody and selecting the resulting library for binding to a different antigen, fluorescein, semisynthetic Ab could be isolated which possess combining sites which have been 'remodeled' to bind the new antigen with affinities in the 0.01–0.1 μm range (Barbas et al., 1992a). Furthermore, semisynthetic libraries have been utilized to directly select for Ab which coordinate metals and metal oxides (Barbas et al., 1993). Herein we report further investigations...
into the construction and selection of semisynthetic Ab libraries in an attempt to define rules for their generation.

RESULTS AND DISCUSSION

(a) Library construction

Semisynthetic Ab libraries were constructed by PCR as previously described (Barbas et al., 1992). The oligos used are shown in Fig. 1. Heavy-chain libraries were prepared with CDR3 lengths of 5, 10, and 16 aa. HCDR3 length of 10 is approximately the average length utilized in human Ab (Sanz, 1991). Lengths 5 and 16 were chosen to be representative of short and long CDRs, respectively, based on a previous report on the genetic diversity in this region. Complete randomization using an NNK or NNS doping strategy yielded libraries designated 5, 10, and 16. Alternatively, the penultimate position of the HCDR3 was fixed as Asp yielding libraries G, F, and E. The penultimate Asp, Kabat position 101, is conserved in 75% of human Ab (Kabat et al., 1991), and is thought to be structurally significant in stabilizing the loop structure (Chothia et al., 1987). Furthermore, its importance was further demonstrated by its selection from the first semisynthetic Ab library. In libraries F and E which are 10 and 16 aa in length, respectively, the first position was fixed as Gly as all clones selected previously to bind free fluorescein contained Gly at this position (Barbas et al., 1992a). Light-chain (LC) CDR3 (LCDR3) libraries were prepared in lengths 8, 9, and 10 aa, which cover the range of naturally occurring loop lengths in humans and are designated k8, k9, and k10. Diversity was limited to Kabat positions 92-96 as the remaining four positions are highly conserved in nature. Libraries in which both CDR3 regions were randomized were prepared by digestion of the LC library DNA with XhoI and SpeI to remove the natural HC and replace it with the library of synthetic variants. Nine crossed libraries were prepared by combination of k8, k9, and k10 with E, F, and G. To examine the role of LCDR3 in the previously selected fluorescein clone F22, crossed libraries were prepared by combining the HC of F22 with k9 and k10 libraries. All libraries were prepared in the monovalent Fab phage display vector pComb3 (Barbas et al., 1991). For the display of another protein in this vector system, see Pannekoek et al. (1993). All libraries consisted of at least 10⁸ independent transformants, except for F22/k9 and F22/k8 which contained 10⁷. Note that the crossed library k10/E consists of Fab fragments where 20 aa positions have been randomized. In order for this library to be 'complete', i.e., all members represented, more than 10⁸ transformants would be necessary. Randomly selected clones from each unselected library were sequenced to verify the targeted mutagenesis and the doping scheme.

(b) Selection for hapten binding

Three haptons were chosen for this investigation and are shown in Fig. 2. Conjugate 1 was used in the previous investigation (Barbas et al., 1992). Conjugate 2 was chosen as an analog for the selection of catalytic antibodies which catalyze a decarboxylation reactions, a reaction which has been demonstrated with mouse mAb (Lewis et al., 1991). Conjugate 3 was chosen as it is similar to the other two haptons in that it contains a flat aromatic ring system but lacks their anionic character.

Phabs were selected for binding to a microtitrering dish coated with the BSA conjugates as described previously (Barbas et al., 1991). In the first round of selection, Phab libraries were added to separate microtiter wells. Following washing and acid elution, the phage eluted from the individual libraries were combined for application. Panning was performed in this competitive fashion.

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**Fig. 1.** Oligos for the construction of semisynthetic Fab libraries. For the construction of libraries 5 and 10, primers 5 and 10 were used in place of the previously described primer 7ECDR3, which was used to construct library 16. PCR assembly and library construction were as described (Barbas et al., 1992a). The construction of libraries E, F, and G have been reported (Barbas et al., 1993). Light-chain libraries were constructed using the same template DNA as above. Primers k8, k9, and k10 were paired with T7B to produce a fragment consisting of the 3' side of the gene. Primers kEF and kV12B were paired to produce the 5' side of the gene. The gene was assembled as described above with primers kEF and T7B. PCR conditions were as described for the heavy-chain libraries. In general, 10-20 independent PCR reactions were performed in each step. Selection of Fab from the libraries was as described (Barbas et al., 1991; 1992a). N, A or C or O or T; S, C or G; K, G or T.

**Fig. 2.** Structures of hapten conjugates used for selection. Conjugate 1 was reported previously (Barbas et al., 1992a). Conjugates 2 and 3 were prepared as described (Lewis et al., 1991).
to identify the highest affinity clones from the collection of libraries. Following sequencing, the clones' source could be identified. Since the hapteners were conjugated to BSA, selective pressure was applied to select for hapten binding and against BSA binding. This was accomplished by resuspending phage in TBS containing 1% BSA prior to selection and by alternating 3% BSA and 2% Blotto blocking of the microtiter dish at each round of selection. Following five rounds of selection and conversion of the phagemid from surface display form to soluble antibody producing form, 20/20 clones selected for binding the fluorescein conjugate 1, 18/20 selected for binding conjugate 2 and 1/20 selected for binding conjugate 3 were positive in enzyme-linked immunosorbent assay (ELISA) analysis. All clones from F22-derived libraries were also positive following selection for binding conjugate 1.

(c) Sequence analysis of selected clones

The sequences of the selected Ab are shown in Table I. A number of features are immediately obvious. No clones derived from libraries containing HCDR3 length of five have survived the competitive selection. Furthermore, no clones derived from libraries with LC variation only were selected. All clones are derived from HIC libraries where the first and penultimate residues have been fixed as Gly and Asp, respectively. Clone FL18 contains a Ser at the first position that is likely an artifact of the synthesis and assembly and is the result of a single nt change (GGT-AGT). This has been noted in previous examinations of libraries E and F. These results suggest that completeness of a semisynthetic Fab library does not necessarily correlate with the quality of antibodies which can be derived from it. Libraries k8, k9, 5, and G all contained sufficient members to be judged as 99% complete and yet no clones from these libraries survived the competitive selection. Indeed most clones are derived from the crossovers libraries which are the most incomplete but probably most structurally diverse. These results may highlight the fact that we are remodeling an evolved combining site which may be best achieved with more extensive mutation rather than less. This argument may explain the low affinity clones isolated by the randomization of five aa reported previously (Hoogenboom et al., 1992). For an example of an approach which utilizes CDRs of biased sequence see Garrard et al. (1993).

There is evidence for selection of consensus sequence in the clones. For example, in the eighth position of HCDR3 of clones S4, S10, and S12 is an aromatic one. Their corresponding light chains contain the basic doublets KK, RR, and KR respectively. Furthermore, sequence similarity is noted in clones S4 and S2 which

<table>
<thead>
<tr>
<th>Clone/conjugate used for selection</th>
<th>HCDR3&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LCDR3&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Library designation&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL1/1</td>
<td>GWSRWGSLDQW</td>
<td>QQYLPGGRYT</td>
<td>k10/F</td>
</tr>
<tr>
<td>FL1/8/1</td>
<td>STKIMKLDST</td>
<td>QQVRVEGQG</td>
<td>k9/F</td>
</tr>
<tr>
<td>FL1/9/1</td>
<td>GMFBRGFDVR</td>
<td>QQVGGSPW</td>
<td>E</td>
</tr>
<tr>
<td>FL1/12/1</td>
<td>GVRFNFRWHVWVDS</td>
<td>QQVGGSPW</td>
<td>E</td>
</tr>
<tr>
<td>FL1/13/1</td>
<td>GAVRGSKRLVGLYDR</td>
<td>QQVGGSPW</td>
<td>E</td>
</tr>
<tr>
<td>FL1/15/1</td>
<td>GRGVRVRRRIAPRMDI</td>
<td>QYVYRIPLW</td>
<td>k10/E</td>
</tr>
<tr>
<td>FL1/17/1</td>
<td>GPKGVFRPGGMAFDR</td>
<td>QQVGSSSW</td>
<td>16</td>
</tr>
<tr>
<td>F22/1</td>
<td>GVNLFRVNSRPDLM</td>
<td>QYTPRGV</td>
<td>F22/fk</td>
</tr>
<tr>
<td>P2/1</td>
<td>GVNLFRVNSRPDLM</td>
<td>QQYSGK</td>
<td>F22/k9</td>
</tr>
<tr>
<td>P4/1</td>
<td>GVNLFRVNSRPDLM</td>
<td>QQYGKRK</td>
<td>F22/fk</td>
</tr>
<tr>
<td>P5/1</td>
<td>GVNLFRVNSRPDLM</td>
<td>QQYTPRGAT</td>
<td>F22/fk</td>
</tr>
<tr>
<td>P6/1</td>
<td>GVNLFRVNSRPDLM</td>
<td>QQYTPRGV</td>
<td>F22/fk</td>
</tr>
<tr>
<td>P7/1</td>
<td>GVNLFRVNSRPDLM</td>
<td>QQYKGYRGMT</td>
<td>F22/fk</td>
</tr>
<tr>
<td>S4/2</td>
<td>GLRGSRSGFDR</td>
<td>QQYGKQKVQV</td>
<td>k10/F</td>
</tr>
<tr>
<td>S10/2</td>
<td>GSWLGRPGYD</td>
<td>QYVFRSGT</td>
<td>k9/F</td>
</tr>
<tr>
<td>S12/2</td>
<td>GTVSGEGGYDR</td>
<td>QQYGRKSPV</td>
<td>k10/F</td>
</tr>
<tr>
<td>S2/2</td>
<td>GWRSSGRGVWVFSGDA</td>
<td>QQYAWRATG</td>
<td>k10/E</td>
</tr>
<tr>
<td>C13/3</td>
<td>DGDWGFTVRATWRPDP</td>
<td>QYVFRSFRSRT</td>
<td>k10/E</td>
</tr>
</tbody>
</table>

<sup>a</sup>The QYQ triplet and the last residue in LCDR3, T, were fixed and internal aa residues randomized.

<sup>b</sup>Clones isolated by selection for binding to conjugate 1, 2 or 3 (see /1, 2, 3).

<sup>c</sup>HCDR3 aa sequence following selection.

<sup>d</sup>LCDR3 aa sequence following selection, the unmutated sequence is QQYGGSPW.

<sup>e</sup>The clone was derived from the designated library based on sequence composition; clone F22/1 (library designation 16) was previously reported (Barbas et al., 1992a).
Fig. 3. Examination of the specificity of selected Fabs by ELISA. The antigens are (from left to right) tetanus toxoid (Barbas et al., 1991), conjugate 1, BSA as a background control, conjugate 2, and conjugate 3. Antigens were coated on Costar No. 3600 ELISA plates. Standard ELISA was performed with PBS 0.05% Tween as a washing buffer. F22 and P2 are clones derived from selection with conjugate 1, S2, S4, and S10 are clones derived from selection with conjugate 2, and C15 is a clone derived from selection with conjugate 3.

Differ in length but contain very similar N-terminal HCDR3 regions RGSRG and RSSRG, respectively. Note clones S10 and S2 were found three and two times, respectively, identical at the nt level following sequencing of seven clones.

Examination of the role of LCDR3 in the previously selected clone F22 (Barbas et al., 1992) reveals that considerably different sequence may be tolerated in this region as compared to the starting clone. The predominant clone was P2 which was found five times identical at aa level among the ten clones sequenced. This clone was found to be encoded by four unique nt sequences. Naturally occurring murine and human LCRD3 regions show a strong conservation of Pro at Kabat position 95 (Kabat et al., 1991). None of the clones derived from the semisynthetic libraries contain Pro at this position. This may indicate that Pro is conserved for something other than structural reasons or there is editing of this sequence at some level, perhaps in E. coli.

(d) Characterization of specificity and affinity

Cross-reactivities of purified clones were examined by ELISA and are shown in Fig. 3. Clones F22, P2, S4, and S10 are specific for the conjugate on which they were selected. Clone S4 retains some reactivity to the parent antigen tetanus toxoid. Clones S2 and C15 are more promiscuous in binding. Selection against binding to BSA was effective as indicated by the limited reactivity of the Fab to this antigen. The affinities of several purified Fab were examined by surface plasmon resonance. To date we have only observed monomeric Fab as judged by gel filtration in contrast to a recent report of single-chain Ab dimerization (Griffiths et al., 1993). Plots for the determination of $k_{on}$ and $k_{off}$ are shown in Fig. 4. A compilation of kinetic and equilibrium constants is given in Table II. All $K_d$ values approach the nanomolar range. Clone P2 which was strongly selected from F22 derived

\[ \text{[Fab]} + \text{[An]} = \text{[Fab-\text{An}]} \quad \text{and} \quad \frac{d[\text{Fab-\text{An}}]}{dt} = k_{on} \text{[Fab][An]} - k_{off} \text{[Fab-\text{An}]} \]

in surface plasmon resonance measurements, instrument response, $r$, is proportional to [Fab-\text{An}]. Since [An] = [An]_o - [Fab-\text{An}], then

\[ \frac{dr}{dt} = k_{on} \text{[Fab]} r_{max} - (k_{on} \text{[Fab]} + k_{off}) r_1, \]

plotting $\frac{dr}{dt}$ vs. $r_1$ allows for the determination of the slopes of a family of lines such that slope = $k_{on}$ [Fab] + $k_{off}$, so in A, a replot of slopes vs. [Fab] yields a new line where slope = $k_{on}$. For the determination of $k_{off}$, the decay of the [Fab-\text{An}] complex is observed under conditions where [Fab] = 0, such that $\frac{dr}{dt} = k_{off} r_1$ then $\ln (r_1/r_0) = k_{off} (t_0 - t_1)$, the plot of which is shown in B.
libraries had a slightly lower affinity than the parent clone. The affinity of F22 for conjugate 1 by surface plasmon resonance is in close agreement with affinity as determined by competitive analysis.

(e) Conclusions
A variety of anti-hapten Ab can be directly selected from semisynthetic Ab libraries derived from the randomization of one or two CDR regions. Like naturally occurring antibodies, semisynthetic Ab exhibit differing degrees of cross-reactivity. Libraries with greater structural diversity, i.e., with more residues randomized, were functionally superior over complete but structurally limited libraries. However, constraining diversity in HCDR3 to the extent of holding the penultimate position fixed as Asp improved the quality of the library and highlights the structural role of this residue. No such phenomenon has been observed in LCDR3 though four positions in this region have yet to be examined. These results support the idea that there are many solutions to a particular binding problem and likely many solutions for the improvement of semisynthetic mAb. Semisynthetic mAb may become important tools in drug design as they can be regarded as optimized conformationally constrained peptides which could serve as important leads in the design of nonpeptide drugs (Barbas et al., 1992a). The ability to produce human anti-hapten Ab may be significant in the development of catalytic Ab as pharmaceuticals (Lerner et al., 1992; Landry et al., 1993).

REFERENCES


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