doi:10.1016/j.jmb.2005.03.072 J. Mol. Biol. (2005) 349, 814–824

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Llama Single Domain Antibodies as a Tool for Molecular Mimicry

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In camelids, a subset of the immunoglobulins consists of heavy-chain homodimers devoid of light chains, and are thus called heavy-chain IgGs (hcIgGs). Their variable region (VHH) is the smallest antigen-binding fragment possible, and being just one polypeptide chain it is especially suitable for engineering. In particular, camelid single domain antibodies might be very useful for molecular mimicry and anti-idiotypic vaccination. In the present work, we show that llamas immunized with an anti-DNA mouse mAb develop an important anti-Id response. Selection of VHHs by phage display, with specific elution of bound phages with the external antigenic DNA, shows that selected private anti-Id VHHs compete for binding to the external antigen and bear a functional mimicry of the DNA. These results indicate that llama anti-Id single domain antibodies would be an excellent tool for molecular mimicry studies.

Keywords: anti-idiotypic antibodies; camelid IgGs; phage display; DNA mimicry; anti-DNA immunity

Introduction

The complementarity-determining regions (CDR) of antibodies constitute its antigen combining sites. The combining sites of antibodies are large exposed areas on the surface of the variable domains and display great structural diversity. As a consequence, under appropriate experimental conditions, these highly variable determinants give rise to humoral immune responses in singeneic or xenogeneic systems. The antibodies elicited against the combining site are called anti-idiotypic (anti-Id) and react with antigenic determinants, the idiotopes.[1,2] The sum of idiotopes of an antibody constitutes its idiotype (Id).

Given the fact that external antigens and anti-Id antibodies compete for binding to their specific antibody, idiotopes are thought to share partially or completely its reactive surface with the combining site.[3] Also, as the external antigen and the anti-Id both bind to the same surface, they are thought to share structural similarity. This idea has led to proposals that anti-Id antibodies may resemble the external antigen, thus mimicking its structure.[2]

This "popular" idea has been the base of an enormous amount of work from different groups.[4] For example, functional mimicry of ligands of biological receptors by anti-Id has been described in several systems.[5] Besides, functional mimicry of external antigens has led to proposals to use anti-Id as surrogate antigens.[6]

Anti-idiotypic antibodies have been characterized structurally.[7–15] As any conventional antibody, their recognition surfaces are large flat areas composed of all six CDRs from heavy and light-chain variable domains. This fact seriously limits their use for mimicry of small ligands, carbohydrates and nucleic acids that interact with cavities or crevices of their target proteins.[7] In camelids, a subset of the immunoglobulins consists of heavy-chain homodimers devoid of light chains, and are thus called heavy-chain IgGs (hcIgGs). Their variable region (VHH) is the smallest antigen-binding fragment possible, and being just one polypeptide chain it is especially suitable for engineering. In particular, their different strategy of binding, longer CDR3 loops protruding from the binding site and the deviation of CDR conformations from the equivalent human or mouse loop structures, suggest that camelid single domain

Abbreviations used: CDR, complementarity-determining region; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; Fab, antigen-binding fragment; VHH, hcIgG variable region; NMS, normal mouse serum; Id, idiotype or idiotypic; SPR, surface plasmon resonance.

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antibodies might be very useful for molecular mimicry and anti-idiotypic vaccination.[16]

We have recently described two monoclonal antibodies with unusual high affinity and specificity against a double-stranded oligonucleotide of 18 bp, Site35.[17,18] This novel protein–DNA interaction constitutes a challenging system for molecular mimicry using anti-Id strategy. In the present work we show that llamas immunized with mouse IgG_1 ED84, one of the anti-DNA monoclonal antibodies (mAbs), develop an important anti-Id response using both conventional and hcIgGs. Selection of VHHs by phage display using specific elution strategies[19] rendered two types of anti-Id single domain antibodies, directed against public or private idiotopes. Furthermore, a selected anti-Id VHH competes for binding to the external antigenic oligonucleotide and should bear a functional mimicry of the DNA. These results indicate that llama anti-Id single domain antibodies would be an excellent tool for molecular mimicry studies.

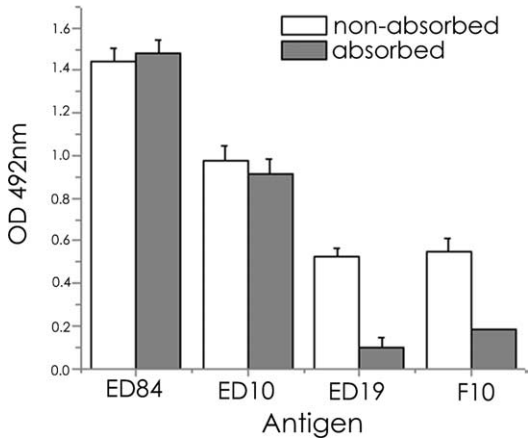

Figure 2. Sera reactivity is specific for ED84 idiotopes. Reactivity of llama serum, with or without absorption to normal mouse IgG, was determined by ELISA against a panel of Fab fragments. A 1:100 (v/v) dilution of serum after second immunization was used.

Results

Lama glama anti-Id response is present in conventional and hcIgG antibodies

In order to prove the efficacy of the immunization protocol, sera from two llamas prior to immunization and after two and three boosts with ED84[17,18] were analyzed by ELISA. When assayed against the whole ED84 molecule, sera from immunized animals showed a strong IgG response, with titers reaching 1/30,000 after three immunizations, as shown in Figure 1 for llama #8338.

We assayed anti-idiotypic specificity for ED84 by immunodepletion of antibodies against constant region and other shared IgG epitopes. An ELISA assay was performed to compare the reactivity against different mouse Fab fragments of the absorbed and non-absorbed sera. As shown in Figure 2 (ED84 bars), recognition of ED84-specific epitopes is as efficient as recognition of the whole Fab molecule, suggesting that there is an important response against ED84 idiotopes (i.e. anti-Id84 antibodies).

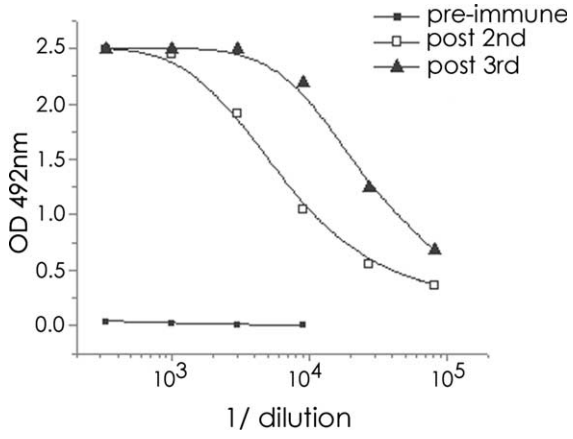

Figure 1. *Lama glama* IgG response against ED84. Serial dilutions of preimmune and post-immunization serum were analyzed by ELISA against 1 μg of ED84 IgG.

Recognition of ED10,[17,18] an IgG_1 identical with ED84 in specificity and very similar in heavy chain sequence (see Materials and Methods), is almost as efficient and specific as that of ED84. This result could imply that both mouse IgGs share idiotypic determinants (or idiotopes) regardless of their light chain sequence differences, which agrees with their identical specificity. An immunodominant heavy chain variable region, a scenario seen sometimes in anti-idiotypic responses,[20] would explain these results. In any case, the difference in reactivity between ED84 and ED10 is significant, indicating the presence of anti-Id84 antibodies, recognizing private ED84 idiotopes exclusively.

Recognition of control non-related IgG_1 Fabs ED19 and F10 is about 50% of that with ED84 in intact serum and almost completely abolished when absorbed to normal mouse IgG, indicating that a high proportion of anti-idiotypic ED84-specific IgGs were elicited during the immunization protocol. These results highlight the possibility of obtaining anti-Id antibodies that recognize conformational epitopes for molecular mimicry generation.

Phage display selection of antibodies is facilitated by the use of llama hcIgG variable regions (VHH) amplified with specific primers from total lymphocyte cDNA. This approach avoids the need of heavy and light chain polypeptides to be combined in conventional IgG libraries. To asses the presence of a-Id84 VHHs prior to library construction, we performed a similar ELISA as above with purified

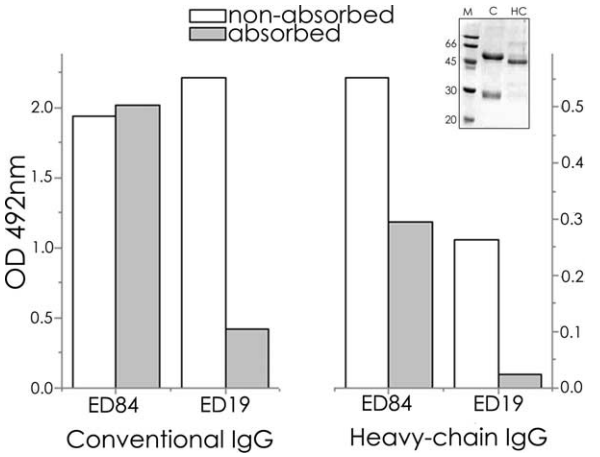

Figure 3. Specific reactivity against ED84 is present in conventional and heavy chain IgGs. IgGs were purified from sera after third immunization and assayed by ELISA against Fab ED84 and control Fab (ED19), adding 100 ng of llama IgG per well previously coated with 0.5 μg of the corresponding Fab. Absorption to normal mouse IgG was performed as described above. The different reactivity scales in both graphs cannot be ascribed to higher affinity of the conventional fraction, since it could be the result of a less efficient recognition of the heavy chain only IgG by the anti-llama IgG rabbit serum. Inset: PAGE showing purified conventional (C) and heavy chain (HC) IgGs; M, molecular mass marker.

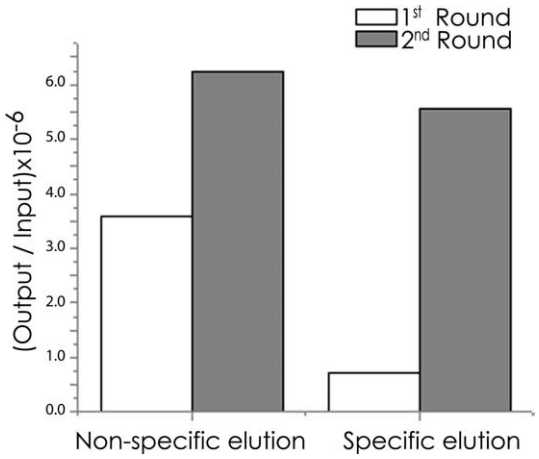

Figure 4. Enrichment in binders after two rounds of panning is achieved with both specific and non-specific elution. The size of the output relative to the input is shown. An equal input of approximately 3×10^{13} phage particles was used in every case. The output/input ratio from non-coated control wells is negligible ($\leq10^{-10}$).

heavy chain only IgG_2 and the conventional IgG_1 isotypes.

As shown in Figure 3, both fractions present antibodies against constant region and ED84-specific idiotopes. There is not a clear bias against these two specificities in these two populations of antibodies, allowing for the further use of the heavy chain only antibodies for the generation of an anti-idiotypic library.

Phage display selection of anti-Id llama antibodies is facilitated by antigen-specific elution procedures

Once established that anti-Id antibodies are present in the hcIgG fraction, we cloned their variable regions (VHH) in a phage display system. Out of ten sequenced clones, nine were clearly different (Table 2A), showing a good variability in the initial pool. No clone without insert or with non-VHH sequences was among those randomly selected clones, indicating a good quality of the initial library. We panned the phages displaying individual VHHs by its binding to ED84 IgG immobilized to plastic wells. The elution of the binders was performed by specific elution of phages bound to the combining site of ED84, as proposed by Goletz[19] for the isolation of anti-idiotypic antibodies, competing their binding with an excess of the cognate antigen for ED84, the oligonucleotide Site35. In a duplicate experiment, bound phages were eluted by classical, acidic pH, non-specific elution of all the bound phages, to compare the yield of anti-Id VHHs of both methods.

Two rounds of panning were performed for each elution protocol. The number of clones eluted in relation to the size of the input with each protocol after the first and second round of panning is shown in Figure 4. A similar number is achieved with both methods after two rounds. This indicates that, at this point, specific elution is as effective as non-specific elution and, to avoid loss of variability, we decided to analyze individual clones for their reactivity before proceeding to further rounds of selection.

To compare the number of a-Id84 VHHs eluted, we randomly picked 96 colonies from the second round of panning of both methods of elution and produced phages from each of them. Phage ELISA against ED84 IgG and the non-related IgG_1 ED5 were performed for every clone. The difference between the reading from both plates for each clone (ΔA) was calculated and clones showing a $\Delta A > 0.1$ were considered as anti-Id clones. This criterion is rather permissive, probably overestimating the number of real anti-Id clones present, but even with this low cut-off there is significant difference between both methods; with a more stringent one, the significance is even greater. When we compared the media of the ΔA obtained for all clones in each group, there were also highly significant differences, showing that ED84 specific recognition is achieved more effectively by phages eluted with Site35. Taken together, these results indicate that, with specific elution, we were able to effectively select clones recognizing ED84 by its variable region. The comparative results are shown in Table 1.

Table 1. Proportion of anti-Id clones and median ΔA of clones with site35 (specific) or 0.1 M glycine pH 2.2 (non-specific) after two rounds of panning

Elution method	Anti-idiotypic clones (%)*	Median ΔA**
Specific	61.5	0.620
Non-specific	37.5	0.096

ΔA is estimated as the reactivity for ED84 minus the reactivity for ED5 in phage ELISA. Every clone showing a $\Delta A > 0.1$ is considered as anti-Id for this analysis. *, $P < 0.001$; **, $P < 10^{-6}$.

Anti-Id reactivities against public and private idiotopes are selected from a llama single domain antibody library

We selected ten of the ED84 specific clones, showing the higher specific reactivity for ED84, from the specific elution protocol. We confirmed the anti-idiotypic nature of these clones by phage ELISA, against: (a) ED84, (b) non-related IgG_1 ED19, (c) IgG_1 390, highly homologous to ED84 in the heavy chain variable region sequence, but with significant differences in the CDR3, which render it non-reactive against DNA, and (d) GF6, an anti-DNA mAb with no similarities in sequence to ED84 (see Materials and Methods).

As shown in Figure 5, nine out of ten clones are specific for ED84, recognizing the private epitopes of the idiotype. The remaining clone, VHH28, does not recognize ED19 or GF6, but recognizes 390, even more strongly than ED84. We speculate that this is a public anti-Id antibody, the recognition of ED84 by this clone being restricted to VH epitopes, since this region is 88% identical in ED84 and 390, but there is only a 47.3% of identity in their VL sequences.

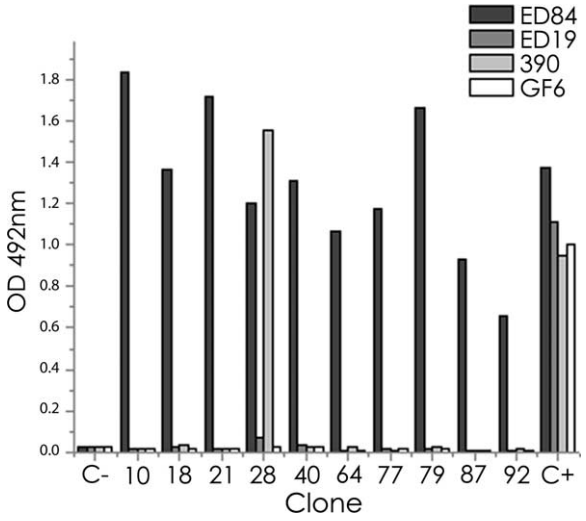

Figure 5. Selected clones from specific elution protocol are mainly private anti-Id VHH. Phage ELISA against ED84 and control IgGs (ED19, 390 and GF6, see Materials and Methods) was performed in duplicate wells sensitized with 1 μg of antibody and incubated with 10^6 phage particles, and bound phages were detected with anti-fd-HRP. C−, helper phage. C+, anti-isotypic clone (isolated from the glycine-eluted phages).

Sequence analysis was performed for these ten clones. We found that the nine private a-Id clones were very similar in sequence, all of them showing the same rearrangement of V, D and J genes. This presumably reflects a clonal relation of these VHHs, and denotes an effective selection of these consensus private anti-ID VHH sequences by the specific elution method. The VHH28 sequence shows no homology to the other nine, and its CDR3 is considerably shorter (11 *versus* 15 amino acid residues), providing a structural basis for its differential reactivity.

If the selection of the consensus private a-Id VHHs was facilitated by specific elution, the initial set of a-Id clones in the library should be more diverse. To test this hypothesis, the non-specifically eluted anti-Id clones were analyzed. The sequence of ten ED84 specific clones from glycine-eluted phages shows that three out of ten clones correspond to the consensus. Compared to the 90% obtained with specific elution, we can ascribe the differences in enrichment to the elution method. The remaining seven clones are much more variable in sequence. Interestingly, one of the eluted clones (VHH75G) is identical with VHH28, an indication that this VHH would recognize an immunodominant public idiotope on ED84. The remaining six clones show some similarity to VHH28 but none to the private anti-Id consensus sequence. This finding shows that there is no strong selection of private anti-Id with acidic pH and that in this case, seven out of ten of the anti-idiotypic antibodies selected are probably of the public type. All the sequences, including ten clones picked randomly from the pre-panning library, are shown in Table 2.

Private anti-Id llama single domain antibodies inhibit binding to the external antigen

Our results indicate the facilitated selection of anti-Id VHHs by elution with the external antigen. In order to prove that the VHHs are capable of effectively binding the combining site of ED84, we performed inhibition assays of ED84 binding to Site35.

We expressed the VHH clones 28, 79 and 87 in a non-suppressor *Escherichia coli* strain and purified the His-tagged VHH fragments from periplasmic space with a nickel affinity chromatography column. The purified VHHs were incubated at different concentrations with ED84 until equilibrium and the mixture was added to ELISA plates sensitized with Site35. The results are shown in Figure 6. Both VHH79 and VHH87 effectively inhibit the binding of ED84 to Site35, with an I_{50} concentration close to two VHH per IgG molecule. They therefore bind the combining site of ED84 and are thus good candidates to elicit functional mimicry of DNA. On the other hand, VHH28 failed to inhibit the binding to Site35, even at a 100-fold higher concentration. This result is in agreement

Table 2. Alignment of sequences of ten randomly chosen clones from the library before panning, and from ten anti-idiotypic clones eluted with Site35 or glycine after two rounds of panning

A

Clone	FR1	CDR1	FR2	CDR2
6	QVKLQQSGGGLVQPGGSLRLTCSAS	GSIFRITEMG	WYRQAPGSQREMVA	FVSRD-HSTQYADSVTG
8	E			
4	HS S T			E
1	Q E S V	VS LS	F T K L	GIVDE PQ R F K
7	D Q A S S V	A VSLDD	K L	SITA GIAN EN MKD
2	E A A S VD	GA STYDL	KE F	AII GG A H E
10	Q E M L K S V	AT S NR	KE	RITSR DD D K
5	D Q A A S A	FV DDYQI	F	KE G S ISSKDG Y T K
9	D Q A E S	FN NDSNI	F R	KE GIS VIDKRDGREF R
3	D Q A A I S V	HTGDIRA	F	KE IF G GINWSGS L A K

Clone	FR3	CDR3	FR4
6	RFTISRDVAKNTVYLEMNSLKPEDTAVYFC	HAMH--LTAVYARQP-----Y	WGQG
8			
4	N D T		
1	I S A Q N TS	Y N HV	RARADSIAPTR
7	N K Q	I N DI	YGSS FGAGFREES
2	N M Q D S I	Y N VG	---PPL GWNLAYH
10	W D N Q VNV A I	Y N-VG	---AFI LSRKTYD
5	Y I N AH Q	Y AVEEAF	FPMTTMRPDMFE
9	M N S Q N R	Y A DTPETGGPHPSLH-AEYD	
3	A H R Q R E	Y A DD	--RP PTYHDNEYD

(*continued on next page*)

with its recognition of IgG 390, with similar VH and no DNA reactivity. It further indicates that this public anti-idiotypic VHH is not able to block the combining site.

To rule out that the differential inhibitory properties of VHHs were related to differences in binding affinities for ED84, the same preparations of purified VHHs were used to study their affinity to ED84 by biosensor analysis in an IAsys cuvette derivatized with IgG ED84. An example of the curves obtained for VHH79 is shown in Figure 7.

As shown in Table 3, the affinities of these VHH for ED84 are similar, all of them with K_D values in the sub-micromolar range. This affinity is ~100-fold lower than that of ED84 for Site35,[18] which is in agreement with the fact that they were displaced by the oligonucleotide during panning. These similar affinities further indicate that the lack of inhibition by VHH28 of Site35–ED84 interaction is due to

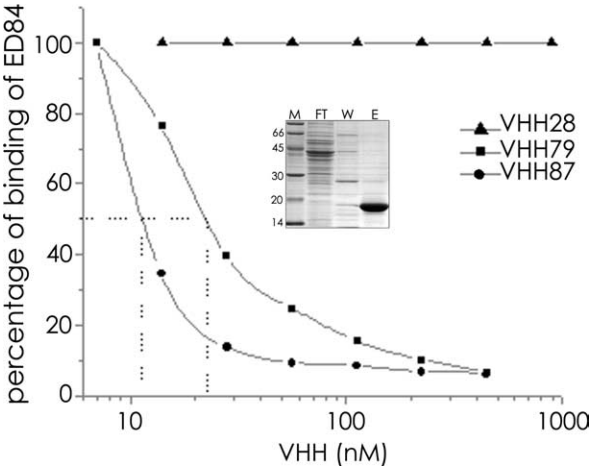

Figure 6. Inhibition of binding of ED84 to Site35 by purified anti-Id VHHs. ED84 IgG at 7 nM (giving a 14 nM binding site concentration) was incubated with increasing amounts of each VHH. After two hours, the mixture was added to Site35-coated wells and bound ED84 was determined by incubation with anti-Fc-HRP conjugate. Inset: purification of VHH79 by Ni-HiTrap column. F, flow-through; W, wash; E, eluted fractions; M, molecular mass standards.

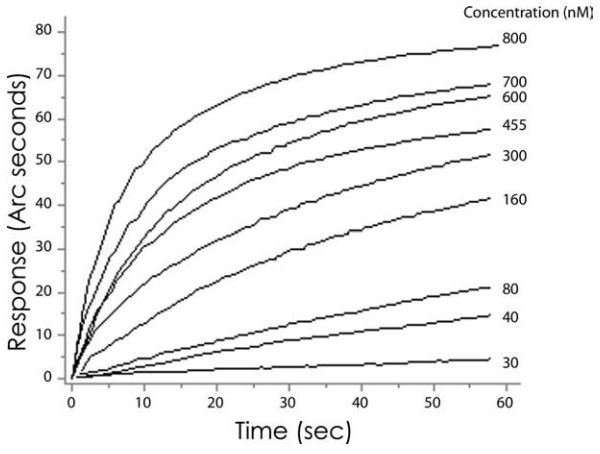

Figure 7. SPR binding curves of VHH79 to ED84. VHH79 at different concentrations was incubated for one minute on the ED84-derivatized biosensor chip. The response is shown for the 30–800 nM range, used to calculate the k_{on}.

Table 2 (*continued*)

(*continued on next page*)

B

Clone	FR1	CDR1	FR2	CDR2
79S	DVQLQASGGGLVQPGGSLRLSCLAS	GGIGTIDAMG	WYRQPPGKQRELVA	MSTSLG-TSYADTVKG
77S			A	T I
92S			A	T I
40S			A	T AI N
64S			A	T
10S	R		AS	L
18S	Q K E		A	T AV
21S	Q K E		A	T
87S	Q K E		A	L
39G	Q K E		A	T L
90G	Q Q Q		AA	T V T
12G	Q Q Q		A	QT R D T S
28S	DVQLQASGGGLVQAGGSLRLTCAAS	GRTFSTYDMG	WYRQPPGKEREPVA	VISWNGGSTYYPDSVKG
75G				
54G	Q Q S	L W	A G	D T L A
63G	Q E S		A V	SS S A
73G	Q K Q T S	N F	A FLG	G N S N S E
91G	Q K E T S	N F	A FLG	G N S N S E
36G	P S S	SI IGL R	A Q WIA	T DSG NIN LN-
44G	P S S	SI IGL R	A Q WIA	T DSG NIN LN-

	FR3	CDR3	FR4
79S	RFTISRSNALNTVSLQMNSLKPEDTAVYYC	AAGTTWSVAPGDMEY	WGKG
77S	N		D
92S			D
40S	N		D
64S			
10S	D	S A	D
18S	D		D
21S			
87S		S	D
39G	E	G	G
90G		S R	D
12G	A T L		N
28S	RFTISRDNAKNTVYLQMNSLKPEDTAIYYC	NAPLRI-HDLGS----	WGQG
75G			
54G		IAT QNPA	
63G		E QR LNP	
73G	YG G I V	A KVPTSREYNY	
91G	YG G I V	A KVPTSREYNY	
36G	V M	V V EV T TAV	AFAF
44G	V M	V	T

A, Pre-panning clones. B, Anti-idiotypic clones from specific elution are labeled with an S; non-specific elution clones are labeled with a G+ gray background.

differences in specificity, and not in affinity, with the private a-Id84 VHHs.

A llama anti-Id antibody elicits functional mimicry of DNA

The specific elution protocol followed by a simple ELISA assay for a-Id identification, allowed us to isolate private and public a-Id llama antibodies. We evaluated the ability of these antibodies to functionally mimic the DNA antigen by immunizing mice with the soluble forms of VHH79 and VHH28. We hypothesized that the private a-Id VHH79 could bear mimicry of DNA. The results from the ELISA assays performed with sera from mice after four immunizations, against salmon sperm double-stranded DNA, Site35 double-stranded DNA oligonucleotide, and the immunizing VHH, are shown in Figure 8.

The group immunized with VHH79 developed an important reactivity against double-stranded DNA and a smaller but significant reactivity against double-stranded Site35. This is a very interesting result, showing that this private a-Id VHH should be capable of bearing a mimic of DNA for presentation to the immune system. The reactivity

Table 3. Affinities measured for the different VHHs by biosensor are in the sub-micromolar range

Clone	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (nm)[a]	K_D (nm)[b]
VHH79	$1.04\times10^5\pm6.75\times10^3$	$1.60\times10^{-2}\pm6.2\times10^{-4}$	114 ± 16	123 ± 14
VHH87	$1.63\times10^5\pm1.09\times10^4$	$2.31\times10^{-2}\pm3.7\times10^{-3}$	142 ± 25	ND
VHH28	$1.58\times10^5\pm9.33\times10^2$	$1.82\times10^{-2}\pm1.1\times10^{-3}$	152 ± 9	155 ± 34

[a] K_D was calculated as k_{off}/k_{on}.
[b] K_D was calculated from the slope of Scatchard plots of response/concentration *versus* response measured at equilibrium.

to both DNA molecules cannot be compared directly, as the immobilization method for each antigen is different. Nevertheless, the difference is important when considering the ratio to the positive and negative controls of each case. This seems to imply that, somehow, the specificity for the oligonucleotide is lost during the passage through the anti-idiotypic network, as if the mimic of DNA could not be achieved to a sequence-specific level.

VHH28, on the other hand, is not a private a-Id antibody, and therefore is not likely to provide a structural mimicry of DNA. The results arising from the immunization experiment confirm that that is the case. No anti-DNA reactivity was observed in the mice after four boosts with VHH28. Even when the overall reactivity against VHH28 is somehow weaker than that achieved against VHH79, the absence of anti-DNA reactivity in the VHH28-immunized group can be attributed to the lack of mimicry borne in the structure of this public anti-idiotypic single domain fragment.

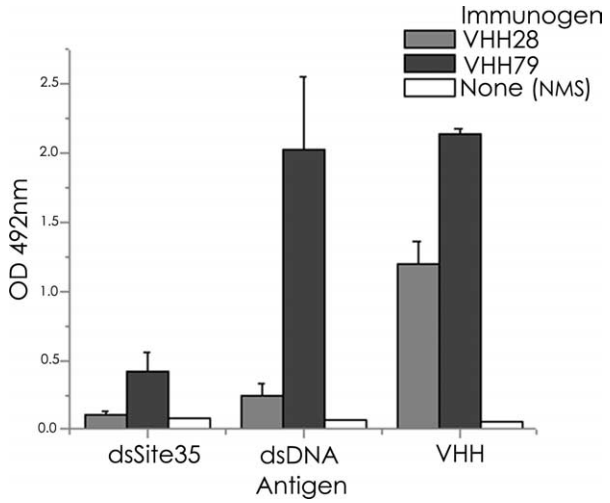

Figure 8. Anti-DNA reactivity is generated by immunization with a private anti-idiotypic VHH. Mean group reactivity of mice ($n=5$) after four immunizations with VHH79 or VHH28, against salmon sperm double-stranded DNA, double-stranded oligonucleotide Site35 and against the respective immunogen is shown. The reactivity of individual sera at 1:100 dilution against each antigen was determined by ELISA as described in Materials and Methods. Mean and SD was calculated from the five measures of each group. The positive control of this assay, 50 ng of purified ED84, reacts with an $A\geq2.0$ against the three antigens (not shown). A 1:100 dilution of NMS was included as negative control.

Besides, as both a-Id llama antibodies react with similar affinity with ED84, we can conclude that the anti-DNA antibodies observed upon immunization of mice with VHH79 are not produced solely by back-elicited ED84+ clones.

Discussion

In the present work, we describe a simple and straightforward strategy to generate molecular mimicry taking advantage of camelid single domain antibodies. The idea of using anti-idiotypic antibodies for molecular mimicry has attracted the attention of many researchers, but the methodology has several drawbacks: (a) it is difficult to generate ant-Id mAbs in syngeneic systems; (b) it is difficult to generate anti-Ids that mimic small ligands; (c) once the anti-Id are obtained and characterized, it is difficult to use their structure to design peptide mimetics for drug design, given the discontinuous nature of the anti-Id combining site.

The strategy outlined in our work tends to overcome most of the limitations for the use of anti-idiotypic antibodies for molecular mimicry. As we have shown here, llama antibodies can be easily generated against a murine monoclonal antibody by immunization and further selection by phage display. More importantly, the anti-Id response is based in both conventional and hcIgG response. Single domain antibody libraries are by far more easily generated than heterodimeric single chain antibodies. The selection method shown here, applying specific elution with the antigen, allows for an important enrichment of private anti-Id binders, increasing the possibility of selecting mimetic antibodies. This specific selection appears as a method of choice for small ligands, but also for macromolecules such as nucleic acids (as the case presented here) or proteins. The smaller size of llama single domain antibodies facilitates the steps of recombinant production and structural studies. Besides, their particular strategy of binding could allow for selecting mimetic anti-Id carrying this capacity in their long CDR3s, allowing the use of synthetic peptides or peptide mimetics for the design of binders. In this sense, we are currently undertaking the structural studies that should identify the molecular basis of mimicry of DNA by a single domain anti-Id antibody.

Notably, the private anti-Ids elicited against the mAb specific against double-stranded oligonucleotide show an oligoclonal profile, since nine out of

the ten clones selected share almost the same sequence. These results suggest that there are not many different structural solutions to create a specific binder against a given idiotope, as shown previously in other systems.[21,22] Thus, the fact that we have been able to select a family of anti-Ids single domain antibodies showing functional mimicry strongly suggests that the combination of the generation of single domain binders plus specific selection would be a straightforward strategy for the generation of molecular mimicry.

Materials and Methods

Immunization of llamas

Two llamas (*Lama glama*) were immunized every 21 days with 1 mg of purified mouse IgG ED84[17,18] in Stimune (Cedi Diagnostics) at a 0.8:1 protein to adjuvant ratio, for a total of three immunizations. Prior to each boost, blood samples were collected and the sera were used to ascertain the immune response from the last immunization. Twenty-one days after the third immunization, 150 ml of heparinized blood was obtained from the better responsive animal and used for mononuclear cell isolation.

Polyclonal anti-Id response

Sera from llamas after each immunization were assayed by standard ELISA procedures. For titer determination, microtiter plates (Maxisorp, NUNC) were sensitized with 50 μl of 20 ng/μl of ED84 in PBS for one hour at room temperature. After blocking with 200 μl of blocking buffer (3% (w/v) skim milk, 0.05% (v/v) Tween-20 in PBS) for one hour and washing each well three times with PBST, the sera were serially diluted in blocking buffer, added to the plate in duplicate wells (50 μl/well) and incubated for one hour at room temperature. Following washing, the assay was revealed with polyclonal rabbit anti-llama IgGs (1:800 dilution in blocking buffer, 50 μl, one hour at room temperature), washed, incubated for one hour at room temperature with goat anti-rabbit IgGs conjugated to HRP (Sigma) at 1:500 dilution, and washed again. Substrate (OPD, Sigma) was added, reaction was stopped with 2M H_2SO_4 and $A_{492\ nm}$ was measured on an ELISA reader (Σ960 Metertech Inc.). The titer was considered as the dilution at which the reactivity is 50% of the 1:100 dilution reactivity.

Table 4. Variable region sequences of mouse IgGs used to asses the specificity of anti-idiotypic VHHs

Heavy chain variable region

IgG	FR1	CDR1	FR2	CDR2
ED84	QVQLQQSGPELVKPGASVKISCKASGYTFT	DYYIN	WVKQKPGQGLEWIG	WIYP-GSDNIKYSEKFKG
ED10	E		LR	V -- N D
390	----			G T N
GF6	KE G A SQ LS T TV FSL	S GVH	GR P K L	V WAD TEYNSALMS--

	FR3	CDR3	FR4
ED84	KATLTVDSSSSTAYMQVSSLTSEDTAVYIC	TRWTYGSSFDY-----	WGQGTTVTVSS
ED10	A T V HL D N F		
390	T L F	A GG YVP	
GF6	RL ISK N KRQVFLKMN QTD M Y	A GGSRYGSS DSMDF	

Light chain variable region

IgG	FR1	CDR1	FR2	CDR2
ED84	DILMTQSPASLSASVGKTVTSTC	---EASENIYGALN--	WYQRKQVKSPQLLIY	GATNLAD
ED10	V T L PV L DQASIS	RSSQSIVHSN NTYLE	LQ PGQ K	KVS RFS
390	-V T L PV L DQASIS	RSSQSIVHSN NTYLE	LQ PGQ K	KVS RFS
GF6	V L PV F DQAFIS	RSSQNL SN NTYLN	LQ PGQ	RVSSRFS

	FR3	CDR3	FR4
ED84	GMSSRFSGSGSGRQYSLKISCLHPDDVATYYC	QNVLSTPYT	FGGGTKLEIKR
ED10	VPD TDFT L RVEAE LGV	FQGSHI L	A- DV
390	VPD TDFT RVEAE LGV	FQGSHV W	
GF6	VLD NS TDFT RVEAE LGV F	LQ THV W	

The sequence of ED84 is compared to the sequence of IgG_1Ed10[18,19] (with identical anti-DNA reactivity as ED84), to IgG1 390, with no anti-DNA reactivity (GenBank accession nos AY816717 (VH) and AY816716 (VL)] and to IgG_{2a} GF6, an anti-DNA MAb with no CDR sequence similarities to ED84 (GenBank accession nos AY816715 (VH) and AY816714 (VL)]. The sequences of ED5, ED19 and F10, used as negative controls, are not shown. They are all IgG_1 against protein antigens, with no variable region similarities with the IgGs presented here.

For analysis of the reactivity against different mouse IgGs, similar procedures were followed, in which sera at 1:100 dilution, with or without absorption to normal mouse IgG-agarose (Sigma), was added to plates sensitized with 0.5 μg/well of the different Fab fragments. (Relevant mouse IgG variable region sequences are listed in Table 4.) Absorption was performed by incubating with gentle agitation for three hours at room temperature, a 5:1 mixture of the diluted sera and IgG-agarose. Following centrifugation, supernatant was added directly to the plate wells.

For conventional and hcIgG fractionation, serum was passed through HiTrap Protein A and HiTrap Protein G columns (Pharmacia) following described procedures.[23] Purity of the different fractions was analyzed by SDS-PAGE. Reactivity against Fab ED84 and control mouse IgG (ED19) of both fractions at 100 ng of llama IgG/well, with or without absorption to normal mouse IgGs, was determined by ELISA as described above.

Construction of the library and phage display selection of anti-Id llama antibodies

Mononuclear cells were isolated from llama #8338 heparinized blood by Ficoll-Hypaque (Pharmacia) gradient centrifugation.

Total RNA from 10^7 cells was purified by TRIZOL reagent (Pharmacia) and subjected to first strand synthesis. VHH specific primers:[23]

Lam07f-NotI
(5′gatggtgatgatgatgtgcggccgcgctggggtcttcgctgtggtgcg3′)
VH1b-SfiI
(5′gctggattgttattactcgcggcccagccggccatggcccaggtsmarctgcagsagtcwgg3′)
VH6b-SfiI
(5′cgtggattgttattatctgcggcccagccggccatggccgatgtgcagctgcaggcgtctggrggagg3′)

were used in PCR to amplify VHH cDNAs. PCR products (~500 bp) were purified from agarose gels (GFX PCR DNA & Gel band purification kit, Pharmacia), digested with SfiI and NotI sequentially and repurified.

For phage-display library construction, 1 μg of SfiI-NotI-digested plasmid pHEN[24] and the digested fragments were ligated in a 1:3 ratio. The ligation reaction was purified with phenol/chloroform, precipitated and resuspended in sterile MilliQ water.

Electrocompetent XL1-Blue cells were transformed and library size was calculated by plating aliquots on LB Amp agar, yielding a total of 1.1×10^8 clones. Ten clones were used for plasmid DNA preparation (Quiaprep Spin Miniprep Kit, Quiagen) and sequenced for diversity analysis.

Transformants were grown in SB medium + 1.5% (w/v) Glc + Amp + Tet for two hours at 37 °C, and infected with 20-fold excess of VCS helper phage (Promega) for one hour at 37 °C. Infected cells were harvested by centrifugation, resuspended in 700 ml of SB + Amp + Tet + Kan and incubated overnight at 30 °C with agitation. Phage particles were precipited form culture supernatant with 4% PEG 8000, 0.5 M NaCl, resuspended in sterile PBS and used for panning.

Panning was performed on 24-well culture plates. Four wells were sensitized with 2 μg of IgG ED84 and two wells were left uncoated. After blocking with 2% skim milk in PBS, 2.8×10^{13} phage particles in 1% skim milk/PBS were added to each well, incubated for two hours at room temperature, and washed ten times with PBST. Bound phages were eluted either with 0.1 M glycine (pH 2.2) (non-specific elution) or with a solution of 2.5 ng/μl of dsSite35 in PBS (specific elution) in duplicate wells. The two non-coated wells were subjected to the same procedures and were used as the negative control of panning for each elution method.

Eluted phages were titrated and subjected to a second round of panning, following the same procedure. Phage titers of input and output at all steps were estimated by infection of XL1-Blue cells and plating on LB Amp plates.

For estimation of anti-Id VHH presence, of XL1-Blue cells were infected with the phages eluted in each method. Ninety-six individual colonies from each method were grown in culture plates in 2×TY + Amp + Glc, three hours at 37 °C with agitation, and superinfected with 2×10^9 pfu of VCS helper phage for 30 minutes. Cells were pelleted, resupended in 2×TY + Amp + Kan and incubated for 16 hours at 30 °C. Culture supernatants containing the phages were diluted twofold in 2% skim milk in PBS and added to ELISA plates sensitized with 1 μg of ED84 or control IgG_1 (ED5) and blocked with 2% skim milk in PBS. After washing with PBST, bound phages were revealed with 1/2000 anti-fd rabbit IgG conjugated to HRP (Sigma). Readings from both ED84 and control wells were done and those clones with a $\Delta A > 0.1$ were considered as presumptive a-Id clones. Statistical comparison by χ^2 test of the proportion of anti-Id clones eluted with each method and one-factor ANOVA of ΔA values from both populations were performed with Origin 6.0 professional (Microcal Software, Inc.).

From the strongest anti-Id clones of both methods, showing only background recognition of ED5, ten clones were selected, their plasmidic DNA isolated and sequenced.

The selected phage clones from the specific elution method were expressed in higher scale and re-evaluated in their specificity for ED84, using 10^6 particles/well in duplicate wells, in a similar ELISA. Reactivity was measured against ED84, against IgG_1 3-90, highly homologous to ED84 but with no anti-DNA activity, and against GF6, an anti-DNA IgG_{2a} with no sequence similarity to ED84 (Table 4). This time IgG_1 ED19 was used as control.

Inhibition assays

The selected clones were expressed as soluble periplasmic proteins in *E. coli* non-suppressor strain Top10 (Invitrogen) for ED84 inhibition studies. Two of the VHH clones with specificity for ED84 (VHH79 and VHH87) and the clone with clearly different sequence and recognition pattern, VHH28, were expressed. Periplasmic space proteins were isolated following established techniques[25,26] and purified by Hi-Trap Ni column (Pharmacia). Their purity was assessed by SDS-15% PAGE electrophoresis and their concentration by $A_{280\ nm}$ reading (average yield, 2 mg/l of pure VHH). Their theoretical concentration factor was obtained from the amino acid sequence with ProtParam at ExPASy.[27]

ELISA plates were sensitized for one hour with 0.5 μg of streptavidine (Sigma), blocked and incubated with 25 ng of biotinilated Site35 for 30 minutes. ED84 (7 nM) was preincubated with the VHHs at different concentration ratios for two hours and added to the plate for one

hour. Bound ED84 was determined with aFC-HRP as described above.

Affinity measurements

The affinities of purified VHHs for ED84 were determined using an IAsys biosensor. A CMD cuvette was sensitized with 100 ng/μl of IgG ED84 following the manufacturer's instructions. VHHs were incubated at different concentrations on the cuvette, either for one minute (k_{on} determination) or until equilibrium (K_D determination). The dissociation was measured for the higher concentrations of VHH, after washing with PBS. All parameters were calculated using GraFit 5.0.3 (Erithacus Software Ltd.) and Origin 6.0 professional (Microcal Software, Inc).

Immunization of mice with VHH fragments and anti-DNA response

Groups of five female BALB/c mice of six to eight weeks of age were immunized every 15 days with 50 μg of protein per boost with either VHH79 or VHH28 in Stimune. After four immunizations, blood was taken from the retro-orbital sinus and the individual sera were assayed at ELISA at 1:100 dilution in 1% (w/v) BSA. The plate was sensitized in duplicate wells with: 50 ng of biotinilated Site35 (as described above), 0.5 μg of salmon sperm DNA (dried overnight at 37 °C) or 0.5 μg of VHH (one hour at room temperature; mice immunized with each VHH were assayed with the corresponding protein). As a negative control, normal mouse serum (NMS) was used and ED84 was used as a positive control at 50 ng of IgG per well. The ELISA was performed essentially as described above for ED84.

Data Bank accession codes

Sequences of the ten clones have been deposited with GenBank, with accession numbers from AY816718 to AY816744.

Acknowledgements

We are grateful to Vanina Alzogaray for help with the immunizations of llamas, to Gonzalo Pérez Zabala and Guillermo Vila Melo for providing the llamas, farm site and veterinarian care, and to Maria Laura Cerutti for her helpful comments about this work.

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Edited by I. Wilson

(Received 30 November 2004; received in revised form 19 February 2005; accepted 25 March 2005)