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Improvement of an Antibody Neutralizing the Anthrax Toxin by Simultaneous Mutagenesis of Its Six Hypervariable Loops

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The enhancement of antibody affinity by mutagenesis targeting only complementarity determining regions has the advantage of respecting the framework regions, which are important for tolerance if clinical use is envisaged. Here, starting from a Fab (antigen-binding fragment; $35PA_{83}$) capable of neutralizing the lethal toxin of anthrax and having an affinity of 3.4 nM for its antigen, a phage-displayed library of variants where all six complementarity determining regions (73 positions) were targeted for mutagenesis was built. This library contained 5×10^8 variants, and each variant carried four mutations on average. The library was first panned with adsorbed antigen and washes of increasing stringency. It was then screened in parallel with either small concentrations of soluble biotinylated antigen or adsorbed antigen and long elution times in the presence of soluble antigen. The stringencies of both selections were pushed as far as possible. Compared with $35PA_{83}$, the best selected clone had an affinity enhanced 19-fold, to 180 pM, and its 50% inhibitory concentration was decreased by 40%. The results of the two selection methods were compared, and the generality of these methods was considered.

Keywords: affinity; enhancement; phage display; antibody; *Bacillus anthracis*

Introduction

The pathogenicity of anthrax is mediated by lethal toxin (LT). Several studies have focused on the development of antibodies that are directed against one of the subunits of LT, the protective antigen (PA), with different approaches and the aim of neutralizing this toxin.[1–9] Previously, we isolated Fab (antigen-binding fragment) $35PA_{83}$ from a non-human primate. It has an affinity equal to 3.4 nM for PA and efficiently neutralizes LT.[10] Others found that the neutralization of LT by an antibody is correlated with the affinity between the antibody and the PA. Moreover, the antibody or antibody fragment needs to have an affinity for PA exceeding that of PA for its receptor (1 nM) for optimal LT neutralization.[11] Our goal was to improve the affinity of Fab $35PA_{83}$ for PA to subnanomolar values without generating immunogenic sequences, since we intend to use Fab $35PA_{83}$ as the basis for a therapeutic molecule. We therefore targeted only the complementarity determining regions (CDRs) for mutagenesis, in contrast to many other studies that used random mutagenesis, based on error-prone PCR.[12,13]

Selected mutagenesis of CDRs has been used previously to obtain affinities in the micromolar to

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Abbreviations used: Fab, antigen-binding fragment; LT, lethal toxin; PA, protective antigen; CDR, complementarity determining region; V_H, heavy chain variable domain; V_L, light chain variable domain; K_d, dissociation constant; IC_{50}, 50% inhibitory concentration; k_{off}, dissociation rate constant; k_{on}, association rate constant; LF, lethal factor; PBS, phosphate-buffered saline.

nanomolar range,[14–17] rather than the subnanomolar range.[18,19] In one of the studies that reached subnanomolar affinities, CDRs were sequentially mutated by iterative constructions and pannings of libraries, starting with CDR3, in a strategy named "CDR walking."[18] Several affinity-enhancing mutations were also combined to test the additivity of their effects, which was found to be "unpredictable and modest." This sequential approach allowed reaching an affinity of 15 pM, starting from an initial value of 6.3 nM. One recent study systematically searched for additive effects,[19] requiring the initial construction of six libraries, each with one mutated CDR, that were screened to identify individual mutations improving affinity. These individual mutations were then combined in heavy chain variable domain (V_H) or light chain variable domain (V_L) libraries to identify mutations presenting an additive effect in each variable domain. Finally, the beneficial mutations in V_H and V_L were combined and screened for further additive effects due to particular V_H/V_L combinations. This complex strategy made it possible to reach an affinity of 1.1 pM.

We developed a simpler and faster approach by constructing a single phage-displayed library of variants, which contained four mutations on average, located in any of the six CDRs. This mutagenesis method made it possible to search for additive effects right from the start of the study. This library was screened in two steps, adapted from a panning strategy described previously.[18] In the first step, we used adsorbed antigen and washes of increasing stringency to eliminate non-reactive and weakly reactive clones, while respecting the diversity of the library. In the second step, we selected the best clones under stringent conditions with two parallel methods, using either low concentrations of soluble antigen or long elution times from adsorbed antigen in the presence of soluble antigen. The stringencies of both selections were pushed as far as possible to select the best binders, avoid an arbitrary choice of the selection conditions, and thus be able to compare the two methods. The screening method utilizing long elution times from adsorbed antigen in the presence of soluble antigen was described earlier in a formal way[18,20] but apparently implemented here for the first time. This approach allowed us to reach the targeted affinity and to significantly improve the neutralization capacity of Fab $35PA_{83}$.

Results

Construction of the mutant Fab $35PA_{83}$ library

The final library was obtained by five rounds of Massive Mutagenesis® and contained 4×10^8 independent transformants. We sequenced 45 independent plasmid clones to determine their rate of mutation and diversity (Tables 1 and 2). The mean rate was 4 mutations per fragment (V_H+V_L), close to the anticipated 3.5 mutations per fragment. The sequences showed that 9 of the 45 targeted residues were not mutated for V_H (20%) and that 5 of the 28 targeted residues were not mutated for V_L (17.8%), giving a mean mutated/targeted position ratio of 80%. However, a ratio of 94% was measured for other sequences, obtained in the course of library construction (data not shown).

The distribution of mutants with zero to eight simultaneous mutations in this Massive Mutagenesis® library should follow a binomial law. The observed distribution was consistent with the theoretical distribution (Table 3). In particular, we expected 22% of the clones to have three mutations, and it was actually the case for 27% of them.

Table 1. Frequencies of mutations, before (f_{bp}) and after (f_{ap}) the first panning step, at any H-CDR position

	H-CDR1										
Residue	G	D	S	I	S	G	G	Y	Y	W	S
IMGT no.	27	28	29	30	31	31a	32	33	34	39	40
Kabat no.	26	27	28	29	30	31	32	33	34	35	35a
f_{bp}	8.5	4	11	6	2	2	17	2	6	2	8
f_{ap}	0	5.5	1.8	0	0	3.7	0	0	0	0	0

	H-CDR2																
Residue	H	I	Y	G	S	T	A	D	T	R	Y	N	P	S	L	K	S
IMGT no.	55	56	57	58	59	59a	60	61	62	66	67	68	69	70	71	72	74
Kabat no.	50	51	52	52a	53	54	55	56	57	58	59	60	61	62	63	64	65
f_{bp}	4	2	0	4	0	0	0	4	0	14	4	15	2	15	6	4	8
f_{ap}	1.8	0	0	0	0	0	0	0	0	4	0	3.7	3.7	1.8	0	1.8	0

	H-CDR3																
Residue	A	R	S	G	Y	N	F	W	S	G	E	Y	Y	G	L	D	S
IMGT no.	105	106	107	108	109	110	111	111.1	111.2	112.2	112.3	112.4	113	114	115	116	117
Kabat no.	93	94	95	96	97	98	99	100	100a	100b	100c	100d	100e	100f	100g	101	102
f_{bp}	2	0	2	0	2	2	0	0	13	8	8	8	8	12	14	4	6
f_{ap}	0	0	0	0	1.8	0	0	0	1.8	0	1.8	0	1.8	0	1.8	0	22

The numberings of residues according to the IMGT and Kabat nomenclatures are indicated. The residues belonging to the CDRs, according to each nomenclature, are underlined. Forty-five and 54 individual clones were sequenced before and after panning, respectively.

Table 2. Frequencies of mutations, before (f_{bp}) and after (f_{ap}) the first panning step, at any L-CDR position

	L-CDR1										
Residue	H	A	S	Q	G	I	N	S	W	L	A
IMGT no.	24	25	26	27	28	29	30	31	32	39	40
Kabat no.	24	25	26	27	28	29	30	31	32	33	34
f_{bp}	11	11	2	4	4	4	8	0	9	9	4
f_{ap}	3.7	0	0	11	0	0	1.8	0	3.7	3.7	0

	L-CDR2							
Residue	Y	K	A	S	S	L	Q	S
IMGT no.	55	56	57	58	66	67	68	69
Kabat no.	49	50	51	52	53	54	55	56
f_{bp}	0	6	0	8	13	6	12	10
f_{ap}	0	5.3	0	3.7	5.5	11	14	15

	L-CDR3								
Residue	L	Q	Y	D	S	A	P	L	A
IMGT no.	105	106	107	108	109	114	115	116	117
Kabat no.	85	90	91	92	93	94	95	96	97
f_{bp}	6	0	4	2	0	6	4	19	17
f_{ap}	0	0	0	1.8	0	7.4	3.7	1.8	3.7

See the legend to Table 1 for details.

The targeting of 73 positions, with the introduction of complete diversity at each position, gives $19 \times 73 = 1387$ single mutants. The number of possible double mutants is equal to $19^2C^2_{73} = 9.5 \times 10^5$, and the number of triple mutants is equal to $19^3C^3_{73} = 4.2 \times 10^8$. The library contained 4×10^8 independent clones. The binomial distribution of the library and its size, larger than the theoretical number of single and double mutants and equivalent to the theoretical number of triple mutants, suggested that this library should contain all possible single and double mutants in multiple copies, a significant portion of the triple mutants ($\sim 1/4$, as 27% of the 4×10^8 clones should present three mutations and the number of possible triple mutants is 4.2×10^8), and a smaller proportion of mutants with four or more mutations.

First step of selection based on adsorbed antigen and stringent washing

The number of eluted phages remained stable during the first two rounds of panning and then increased by a factor of 50 in the third and last rounds. Phage ELISA gave signals equivalent to background before panning. The signal doubled after the first round of panning, doubled again after the second round, and increased by a factor of 5 after the third round. The library thus became enriched in specific phages during the course of panning as expected, while these specific phages were initially undetectable. We sequenced 54 clones after this panning (Table 2). The most frequently mutated residue in V_H was Ser117 (22%). Other positions were mutated only half as frequently, possibly indicating a special role for this position. In V_L, it was less clear whether residues Leu67, Gln68, and Ser69 could be beneficially mutated, as mutation rates at these positions were already high before panning (from 6% before panning to 11% after panning for Leu67, from 12% to 14% for Gln68, and from 10% to 15% for Ser69). Conversely, mutations frequently observed before panning (e.g., Gly27 and Gly32 in V_H and Ala25 in V_L) were not recovered in the clones obtained after panning. The mutations selected after this panning therefore did not reflect possible bias in the library.

The proportion of positions remaining unmutated after the first step of panning increased to 66.6% (30 positions) in V_H and to 35.7% (10 positions) in V_L, from 20% and 17.8%, respectively (see above). These data suggest that mutations were detrimental to affinity at a large proportion of positions. Note that some of the peptide sequences that we identified as parental were actually encoded by variants of the parental nucleotide sequence that contained silent mutations.

Table 3. Frequencies of clones after construction of the library as a function of the number of mutations

Mutation(s)	f_{th} (%)	f_{obs} (%)
0	3	9
1	10	0
2	18	12
3	22	27
4	19	15
5	13	9
6	8	12
7	4	9
8	1	6

Column 1, number of mutation(s) per clone. Column 2, theoretical frequency of the clones that carry the number of mutations indicated in column 1, according to the binomial law. Column 3, observed frequencies.

Second step of selection using low concentrations of soluble antigen (second step of selection, method 1)

After the first step of panning, the best binders were searched in parallel by two methods, where stringencies of elution were stretched to their limits. One of these two methods was screening with various concentrations of biotinylated PA_{83}, ranging from 100 nM to 0.01 pM and corresponding to conditions 1 to 8 in Table 4. The lowest concentration of antigen, which resulted in the elution of more clones than the negative control, was equal to 1 pM. This condition (no. 6) was regarded as the most stringent attainable. Fabs isolated under condition 5, to retain diversity, and condition 6 were studied

Table 4. Number of eluted phages as a function of the concentration of soluble biotinylated antigen (Bt-PA_{83})

Condition	Bt-PA_{83} (nM)	Phage(s) (10^3)
1	10^2	250
2	10	192
3	1	55
4	10^{-1}	10
5	10^{-2}	8.8
6	10^{-3}	3.4
7	10^{-4}	1
8	10^{-5}	0.7
9	0	0.9

further. Ninety individual clones, eluted with 10 and 1 pM PA_{83} (conditions 5 and 6, respectively), were sequenced and induced for expression.

We isolated 47 clones in condition 5 and found that 17 of them (36%) corresponded to the parental Fab $35PA_{83}$. Only 6 of the 30 variants were expressed sufficiently for affinity measurement by surface plasmon resonance. Two Fabs with enhanced affinities were isolated (Table 5). Clone 5.39 displayed a 2-fold improvement in affinity (dissociation constant $K_d = 1.71$ nM) and clone 5.25 displayed a 1.7-fold improvement ($K_d = 2$ nM) relative to the parental Fab $35PA_{83}$ (3.4 nM).

We isolated 43 clones in condition 6 and found that 15 of them (35%) corresponded to the parental Fab. Only 8 of the 28 variants were strongly expressed. Clone 6.20 displayed a 19-fold improvement in affinity ($K_d = 0.18$ nM) (Fig. 1), mainly due to a decrease in the dissociation rate (Table 5). The improvement for clone 6.20 was higher than improvements for the other clones isolated under the same condition, displaying only a 2-fold improvement in affinity.

Second step of selection using long elution times from adsorbed antigen in the presence of soluble antigen (second step of selection, method 2)

The second method utilized for the search of the best binders was elution with long elution times in the presence of the antigen, and the stringency of this method was also stretched to its limit. The number of clones eluted remained steady for almost 3 weeks and then fell 200-fold, to 18 clones, on day 24, with no clone eluted on day 25 (Table 6). Elution on the 24th day was thus the most stringent attainable condition of this selection, and the 18 corresponding Fabs were further studied. Only 14 of these 18 clones could be completely sequenced, the others being partially recombined. Three of these 14 clones corresponded to the parental Fab, and 6 of the 11 variants were strongly expressed. Fabs J24.7 and J24.15 displayed an almost 5-fold enhancement of affinity (0.78 and 0.88 nM, respectively) (Table 7), mainly due to improvements in the association rates. The two clones with the next best affinity enhancement displayed a 2-fold improvement, and the two last clones displayed almost no affinity improvement.

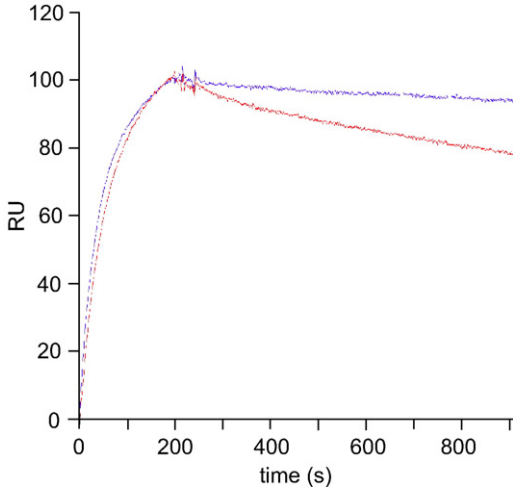

Fig. 1. Sensorgram of the parental Fab, $35PA_{83}$ (shown in red), and its best derivative, 6.20 (shown in blue).

Table 5. K_d, k_{on}, and k_{off} values for the derivatives of Fab $35PA_{83}$ that were isolated by panning against soluble antigen

Variant	V_H mutations	V_L mutations	K_d (nM)	k_{off} (10^{-4} s^{-1})	k_{on} (10^4 M^{-1} s^{-1})
Wild type	None	None	3.4	3.2	9.3
6.20	H55L (2), S74G (2)	Q68L (2)	0.18	0.51	28.6
6.49	P69G (2), S111.2T (3)	None	1.55	1.5	9.6
5.39	G31aE (1), D28G (1)	None	1.71	1.08	6.3
6.46	S70A (2), E112.3Q (3), S117R (3)	S69R (2)	1.82	1.26	6.9
6.5	None	Q27R (1), A114P (3)	1.9	1.5	8
6.2	S74G (2)	A114G (3)	2	3.2	16
6.36	Y113T (3)	P115S (3)	2	0.126	0.62
5.25	None	Q68R (2)	2	0.9	4.5
5.47	Y112.4T (3)	P115S (3)	2.11	1.86	8.81
5.7	S29R (1), S117R (3)	W32D (1)	2.57	1.4	5.5
5.15	S117A (3)	None	3.5	3.11	8.85
6.7	None	Q27V (1), S66L (2)	8.11	1.5	1.9
5.20	None	S69R (2)	12.2	2.91	2.4

The rate constants were measured by BIAcore; $K_d = k_{off}/k_{on}$. The K_d values are listed in increasing order (decreasing affinities). The positions of the mutations are numbered according to the IMGT nomenclature, and the corresponding CDR is indicated in parentheses.

Combinations of mutations

Selected light chains and Fd fragments present in different clones, isolated after panning, were combined to determine whether these combinations had an additive effect on affinity enhancement. In particular, Fab J24.7 had an affinity of 0.78 nM, with two mutations located in V_L only. Fabs 6.49 and 5.39 had affinities of 1.55 and 1.71 nM, respectively, with mutations in V_H only. These mutated chains were combined. The combination of the V_L of J24.7 with the V_H of 6.49 gave no improvement over J24.7

Table 6. Number of phages eluted at various times after adsorption on immobilized antigen and incubation in the presence of soluble antigen

Day	Phage(s)
3	4000
13	5700
18	4000
24	18
25	0

Table 7. K_d, k_{on}, and k_{off} values for the derivatives of Fab $35PA_{83}$ that were isolated 24 days after adsorption on immobilized antigen and incubation in the presence of soluble antigen

Variant	V_H mutations	V_L mutations	K_d (nM)	k_{off} (10^{-4} s^{-1})	k_{on} (10^4 M^{-1} s^{-1})
Wild type	None	None	3.4	3.2	9.3
J24.7	None	H24R (1), S69E (2)	0.78	3.4	43.5
J24.15	S117L (3)	S58R (2)	0.88	3	34.1
J24.3	P69G (2)	None	1.5	2.9	20
J24.13	None	D108E (3)	1.5	1.86	12
J24.14	D28G (1), G32E (1), S117L (3)	None	3.3	2.03	6.14
J24.12	None	A114D (3)	3.5	2.8	7.8

See Table 5 for details.

in terms of affinity (recorded value of 0.92 nM). The same was true of the combination of the V_L of J24.7 with the V_H of 5.39 (1.08 nM).

In vitro LT neutralization assay

The 50% inhibitory concentration (IC_{50}) of Fab 6.20 was 3.3±0.15 nM, 40% lower than that of the parental $35PA_{83}$ Fab (5.6±0.13 nM). At a concentration of 3.3 nM, Fab $35PA_{83}$ protected only 5%±3% of toxin-treated cells.

Discussion

Validity of the randomization strategy

The aim of this study was to reach a subnanomolar affinity, starting from a value of 3.4 nM, by mutagenesis targeting CDRs only, as the Fab concerned was developed and improved with possible clinical use in mind. Its other aim was to compare two screening methods when both were pushed to their limits. Targeted mutagenesis is more complicated than random PCR methods to carry out. Here, we resorted to Massive Mutagenesis® technology to induce an average of 3.5 mutations per Fab, targeting all six CDRs simultaneously. It is the first use of this technology for affinity maturation. We adopted this strategy to accelerate the process, because few additive effects of mutations improving affinity have been demonstrated,[18] and we wanted to avoid the complex strategies involving the construction and screening of several sublibraries that have been described to identify rare combinations of mutations with additive effects.[19]

A two-step screening process, based on phage technology, was used. In the first step, we aimed to eliminate weakly reactive clones without decreasing library diversity; in the second step, we aimed to select the clones with the highest affinities. Difficulties were encountered during the expression of several Fabs, possibly due to genetic instability or toxicity of the variants.

Positions of the mutations

Some positions were mutated more frequently after than before screening. These positions included H-Ser117, which was mutated into Leu (S117L) not only in clone J24.15, with subnanomolar affinity, but also in clones 5.7 and 5.15, displaying little affinity enhancements. Therefore, the impact of this mutation on affinity remains unclear.

We observed trends in the location of the mutations involved in affinity improvement. Five positions (111.2, 112.3, 112.4, 113, and 117) in H-CDR3 were mutated in eight variants with enhanced affinity. This observation is consistent with the suggestion that H-CDR3 optimization can be used for the rapid improvement of affinity.[21] However, only one (S117L) of the seven mutations in the variants with subnanomolar affinities was located in H-CDR3. Therefore, the mutations of H-CDR3 were not particularly associated with major improvements in affinity. Four positions (28, 29, 31A, and 33) in the center of H-CDR1 were mutated in five clones, but mutations at these positions improved affinity only slightly. Similar observations apply to positions 108, 114, and 115 in the center of L-CDR3, mutated in five clones. Thus, three preferential locations of mutations were identified in this study, but they represented no clear trend regarding the location of mutations strongly improving affinity. Overall, the results justify our initial decision to target all six CDRs. On a tridimensional model, we noted that the mutations that were present in the variants with subnanomolar affinities (red in Fig. 2) were generally located at the periphery of the CDR (light blue and green) with the exception of mutation H-H55L, present in the best variant 6.20 and located in H-CDR2, and at the center of the CDR.

The seven mutations that were present in our three clones with subnanomolar affinities (6.20, J24.7, and J24.15; Tables 5 and 7) were compared with beneficial mutations in other antibodies. For antibody 14B7, which is also directed against the PA antigen, four of the five mutations that improve affinity are located in positions L-68 and L-69, at the C-terminal end of L-CDR2, and in the same positions as mutations L-Q68L and L-S69E in our clones 6.20 and J24.7 [ImMunoGeneTics (IMGT) numbering; see Tables 1 and 2 for the correspondence with the Kabat numbering].[11] For antibody D2E7, which is directed against tumor necrosis factor-α, most of its derivatives with improved affinities carry mutations in position L-24, thus at the same position compared with the mutation L-H24R seen in our clone J24.7 and located at the N-terminal end of L-CDR1, and in positions L-68 and L-69 as above. Moreover, several improved derivatives of D2E7 also carry a mutation in position H-117, at the C-terminal end of H-CDR3, and in the same position as mutation H-S117L in our clone J24.15.[19] This set of studies, including ours, suggests that the N-terminal

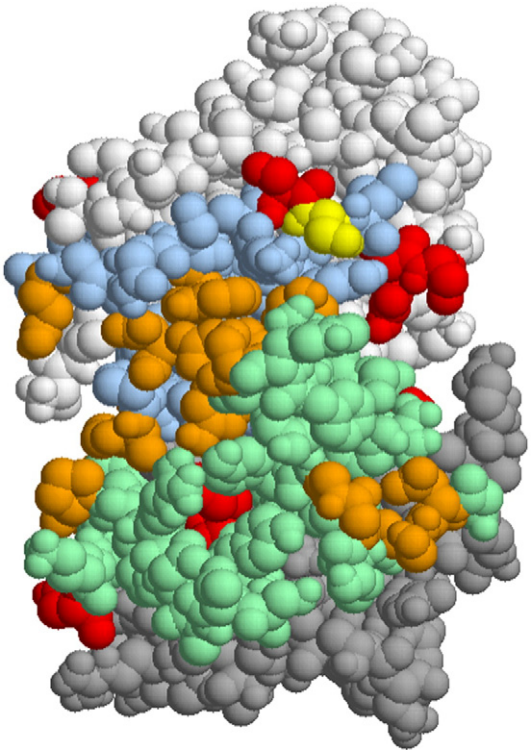

Fig. 2. Mutated residues in a three-dimensional model of antibody $35PA_{83}$. The model was obtained with the WAM Internet server. The V_L and V_H are shown in light and medium gray, respectively. The CDR loops of V_L and V_H are shown in light blue and green, respectively. The residues that were mutated in mutant antibodies with values of K_d that were <1 nM are shown in red. From left to right: His24 (half-hidden), Ser58, and Gln68–Ser69 in V_L and Ser74, His55, and Ser117 in V_H (half-hidden). The residues that were mutated in mutant antibodies with values of K_d that were decreased but >1 nM are shown in orange. Residue Ser66 in V_L, which was mutated only in a mutant antibody with an increased value of K_d, is shown in yellow.

end of L-CDR1 and C-terminal ends of L-CDR2 and H-CDR3, defined according to the Kabat nomenclature, could constitute preferential targets to improve the affinity of antibodies.

Nature of the mutations

We identified several trends concerning the type of mutations most frequently found. Seven of the 19 mutations in V_H and 7 of the 13 mutations in V_L involved in affinity enhancement led to the appearance of electrostatic charges, either positive (corresponding to mutations into Arg: S>R, H>R, and Q>R) or negative (G>E, S>E, and W>D). Some were observed repeatedly (5×S>R, 2×G>E, and 2×Q>R) and involved long side chains (S>R and G>E). For example, the Q68R mutation in V_H was the only mutation involved in Fab 5.25. The D108E mutation was the only mutation in clone J24.13 and involved no change in negative charge but an elongation of the side chain, thus a possible improvement in charge exposure. Overall, the introduction of electrostatic charges, or improvement in the exposure of such charges, was the mechanism most frequently involved in affinity enhancement. The second most frequent type of mutation in this study was a decrease in the size of the side chain (D>G, P>G, and S>G, all observed twice; S>A and A>G, each observed once), and it occurred more often in V_H (seven occurrences) than in V_L (one occurrence). Such mutations were observed twice at positions 28, 69, and 74 of V_H, where they could make room for two bulky residues (Y33 and Y34, close to position 28) or allow some conformational change of H-CDR2 (positions 69 and 74). This could be the case for mutation P69G, which was observed twice. Clone J24.3 showed that this mutation resulted in a twofold enhancement of affinity. Hydrophobic interactions, created by the introduction of hydrophobic residues (S>L, observed twice; H>L, S>A, and Q>L, each observed once), were observed less frequently and mostly affected H-CDR2. These mutations accounted for two of the three mutations in the best clone (6.20), thus confirming their efficiency.[11]

Efficiency of the selection and recombination methods

Two screening methods were used in parallel for the second part of the study, aiming to identify the Fabs with the best affinities. The most stringent conditions still allowing the elution of phages should have selected the best binders present in the library, whatever the method. We studied those phages in detail and were thus able to compare the two methods. Panning based on adsorbed antigen and long incubation times has been described before,[18] as a modification of another method.[20] However, the high cost of the antigen had led to include the parental Fab, and not the antigen, in the soluble phase during the dissociation reaction. To our knowledge, the method utilizing long elution times from adsorbed antigen in the presence of soluble antigen was used here for the first time. Fewer parental Fabs (3/14 or 21%) were obtained with this method compared with soluble antigen (17/47 or 36% and 15/43 or 35%). Furthermore, fewer Fabs with unchanged or decreased affinities (1/6 or 16.6%) were obtained with this method compared with soluble antigen (4/14 or 28.5%). Two of the three clones with subnanomolar affinities were isolated with long elution times, and some of the next best clones were also obtained by this method. Conversely, the high affinity of 6.20 seems to be abnormal for Fabs obtained with soluble antigen, as the next best affinity obtained with this method was 10 times less favorable. We analyzed twice as many Fabs eluted with soluble antigen (14 Fabs) as those eluted with long elution times (6 Fabs), so that this bias may account for this apparent abnormality. More broadly, the method based on adsorbed antigen and long elution times appeared more efficient at selecting high-affinity clones than the method based on soluble antigen, but very high-

affinity clones remained rare in both cases. It is generally assumed that the selection methods that are based on long elution times select clones with improved dissociation rates, because dissociated phages would bind to the antigen in solution and later be eliminated. Such methods have therefore been named "off rate."[18] The off rate method was pushed here to its limit, and clones were eluted after 24 days. The observed dissociation rate constant (k_{off}) values were not in a range that would account for stability of antigen–Fab complexes in this time frame, such that other mechanisms might have played a role. Unchanged or increased dissociation rates have been observed in at least another study, based on an off rate method (clones 3B3/L3.13, 3B3/L3.15, and h1.3B/h3.36 in Ref. 18). Together, these data suggest that the method based on long elution times from adsorbed antigen in the presence of soluble antigen may also select for an improved association rate constant (k_{on}), which would allow for rapid rebinding to the same or nearby adsorbed antigen molecules.

In the present study, two combinations of mutations were voluntarily generated and tested. One of the parental clones, J24.7, was used in both recombinations as it showed a strong improvement in affinity and had mutations in V_L only. The other parental clones, 6.49 and 5.39, showed a twofold improvement in affinity and had mutations in V_H only. This V_L mutation was combined with these V_H mutations, but these two combinations had poorer affinities compared with the best parental Fab, demonstrating that increases in affinity are not always additive, as previously reported.[18] This result supported our initial decision to combine mutations before screening.

Affinity *versus* neutralization

Earlier studies have shown the existence of a strong correlation between the affinities of antibodies directed against viral antigens and their neutralization potencies.[21] The relation between affinity and neutralization potency of antibodies directed against PA has been studied previously but with a neutralization assay that differed from the standard assay in several experimental conditions.[11] Here, we used the standard assay; therefore, the neutralization potencies in the two studies cannot be compared directly. However, the relations between affinity and neutralization may still be compared. The 19-fold improvement in the affinity of Fab 6.20 over that of the parental Fab led to a 40% decrease in IC_{50}. In the previous study, a Fab (14B7) with an affinity of 12 nM protected 10% of the cells at a concentration of 3 nM, whereas a variant with a 48-fold higher affinity (1H, 250 pM) was nine times more neutralizing (90% cells protected) at the same concentration. In the present study, the $35PA_{83}$ Fab (with an affinity of 3.4 nM) protected 32% of the cells at a similar concentration of 3.3 nM.[10] Its variant 6.20, corresponding to a 19-fold improvement in affinity, was 1.5-fold more neutralizing, protecting 50% of the cells at this concentration. The relationships between neutralization and affinity were thus of comparable magnitude in the two studies.

Conclusions

Our initial objective was to reach a subnanomolar affinity with limited means and in a limited time frame, starting from a nanomolar value. The approach presented here made it possible in one cycle of diversification and selection and gave the best reported improvement from a single cycle to date. Much greater improvements have been obtained by sequential or iterative approaches, and iteration of the strategy presented here could indeed be used to replicate the natural process of affinity maturation, in a search for further improvement. The 19-fold affinity enhancement obtained here, down to 180 pM, decreased IC_{50} by 40% relative to the parental Fab, thus significantly. This study also has wider implications, as the use of the screening method with long elution times and soluble antigen, utilized here apparently for the first time, appeared to be an effective method for identifying fragments with high affinities. It requires no more material than general panning, and its systematic use could be recommended, more particularly after standard panning of immune libraries, which may contain rare clones with very high affinities.

Materials and Methods

Strains and toxins

The DH10B and SURE strains (Stratagene, Amsterdam, The Netherlands) of *Escherichia coli* were used for library construction and panning, respectively. The bacterial strain HB2151, a non-suppressor strain of *E. coli*, was used for the expression of soluble Fabs.[22] LT components [PA_{83}; lethal factor (LF)] were purchased from List Laboratories (Campbell, CA, USA).

Construction of the $35PA_{83}$ mutant library

Obtention of $35PA_{83}$ Fab phage was described previously.[10] CDR definition has not been standardized, so we targeted any residue belonging to the CDRs, defined according to the nomenclature used by Wu and Kabat[23] and the IMGT[24] (Table 2). This gave 73 target positions in the six CDRs. Starting from the phagemid (pComb3X-$35PA_{83}$) coding $35PA_{83}$ and aiming at generating 3.5 mutations on average per clone, we generated a mutant phage antibody library by Massive Mutagenesis®[25] (Fig. 3), described in Ref. 26. All the oligonucleotides used for Massive Mutagenesis® were complementary of the same strand of the phagemid coding $35PA_{83}$. For each of the 73 targeted positions, a 33-mer oligonucleotide incorporating an NNS in its center coding for the complete diversity to be introduced was prepared, while the 30 remaining bases (the first 15 and the last 15) were complementary to the sequences flanking the positions to be substituted. The 73 oligonucleotides were mixed, and 1 nmol of the oligonucleotide mixture was phosphorylated in a final

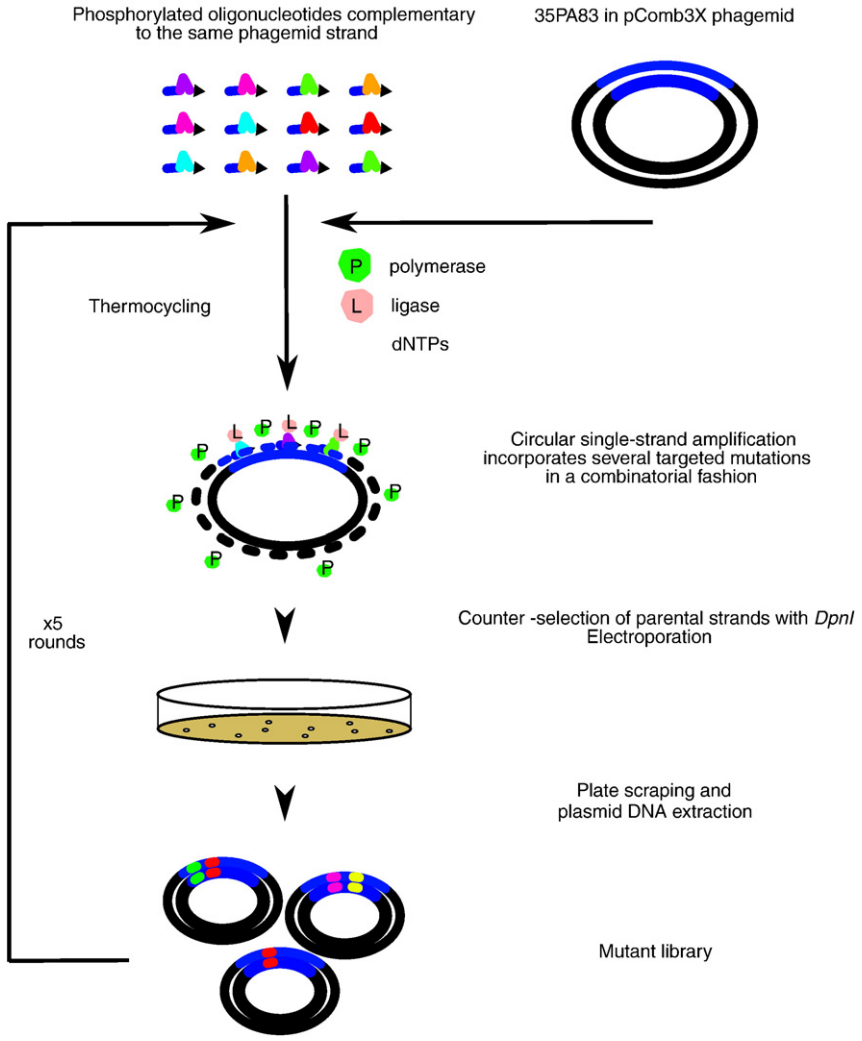

Fig. 3. Representation of mutant library generation using Massive Mutagenesis®. Phosphorylated oligonucleotides are used to introduce mutations in a combinatorial fashion by circular single-strand amplification. The product is selected by DpnI digestion and used to transform *E. coli* cells. The library is obtained by plate scraping and plasmid DNA preparation. Several successive rounds can be performed to increase the average number of mutations per molecule.

volume of 20 µl for 1 h at 37 °C using 2 µl of polynucleotide kinase buffer (New England Biolabs, Beverly, MA, USA), 2 µl of 10 mM ATP, and 1 µl of 10-U/µl T4 polynucleotide kinase (New England Biolabs). The following mixture was then prepared: 400 ng of $35PA_{83}$ plasmid, 200 pmol of the phosphorylated oligonucleotide mixture, 1 µl of 25 mM deoxyribonucleotide triphosphate, 1 µl of 100 mM $MgSO_4$, 1 µl of 10 mM ATP, 0.2 µl of 100 mM NAD, 0.2 µl of 1 M DTT, 2.5 µl of 10× Pfu Pol buffer (Promega, Madison, WI, USA), 0.8 µl of Pfu DNA polymerase (10 U/µl; Promega), and 0.8 µl of Tth ligase (10 U/µl; ABgene, Epsom, Surrey, UK) in a final volume of 25 µl. The resulting mixture was subjected to 12 temperature cycles (at 94 °C for 1 min, 50 °C for 2 min, and 68 °C for 20 min). Four microliters of 10× buffer 4 (New England Biolabs), 0.5 µl of DpnI (20 U/µl; New England Biolabs), and 15.5 µl of deionized water were added to the 25-µl final volume of the previously amplified products and incubated for 30 min at 37 °C. This digestion leads to the linearization of the methylated parental phagemids and permits only the newly *in vitro* synthesized molecules containing the mutations to be transformed. For this, the product of the digestion was desalted by simple membrane dialysis and electroporated into bacteria DH10B, which were plated on an LB medium supplemented with 1% glucose and 100 µg/ml ampicillin, and grown overnight at 30 °C. Bacteria were then scraped and the plasmidic DNA was extracted, before being submitted to another round of Massive Mutagenesis® using the same oligonucleotide mixture.

First step of selection based on the use of adsorbed antigen and increasingly stringent washings

The library was subjected to three successive rounds of panning/reamplification, with 5, 10, and then 15 washes, as previously described.[27] The enrichment in specific phages resulting from this panning procedure was evaluated by phage ELISA with plates coated with 5 µg/ml of PA.[27] After these three rounds of panning and reamplification, the library was reamplified to obtain a titer of 5×10^{10} pfu/ml and used for the second step of the selection process, which was designed to identify the Fabs with the highest affinities for PA. Sequences of the light and heavy chains of selected clones were determined by Genome Express (Meylan, France) using the primers ompseq and newpelseq.[28] Residues were numbered according to the IMGT nomenclature.

Second step of selection using low concentrations of soluble antigen (second step of selection, method 1)

The PA_{83} antigen was biotinylated (Prot On Biotinylation kit, Vector Laboratories, Burlingame, CA, USA) using

a PA_{83}/biotin molar ratio of 1:25. μMACS streptavidin-coated magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) and glass tubes were saturated by incubation with 2% milk powder in phosphate-buffered saline (PBS) for 1 h at room temperature. A modified version of the protocol described by Schier *et al.*[29] was utilized. Ninety microliters of reamplified phage was incubated for 30 min at 37 °C in glass tubes with 10 μl of biotinylated PA_{83}, at eight concentrations generated by 10-fold dilutions of the original stock solution, ranging from 100 nM to 0.01 pM plus a negative control with no PA_{83}. Phages bound to biotinylated PA_{83} were captured by incubation with 100 μl of μMACS streptavidin-coated magnetic beads for 5 min at room temperature. The beads were then washed once in 0.1% Tween 20 in PBS and five times in PBS, according to the manufacturer's protocol. Bound phages were eluted by incubating the beads with trypsin (100 μl; 10 μg/ml in PBS; Sigma, St. Louis, MO, USA) at 37 °C for 15 min, and the eluate was used to infect *E. coli* (SURE strain) in the exponential growth phase. Phage plaques were then counted. The two lowest concentrations of PA_{83} permitting the elution of more clones than the negative control were selected for further study.

Second step of selection using long elution times from adsorbed antigen in the presence of soluble antigen (second step of selection, method 2)

ELISA wells (Nunc Maxisorp) were coated by incubation overnight at 4 °C with PA_{83} in PBS (0.06 mM) and blocked by incubation with 5% milk powder in PBS for 1 h at 37 °C. Reamplified phages (50 μl) were incubated in each well for 2 h at 37 °C, and the wells were washed five times with 0.1% Tween 20 in PBS and twice with PBS. The wells were then incubated for various periods at 4 °C with 0.6 mM soluble PA_{83} in PBS. Phages that remained bound after incubation and five additional washes were eluted with 50 μl of trypsin at 37 °C for 15 min, used to infect log-phase *E. coli* (SURE strain), and counted. The longest elution time permitting the elution of phages was selected for further study.

Soluble Fab production and affinity measurements

Soluble Fabs were produced as previously described.[30] Kinetic constants for interactions between PA_{83} and soluble Fabs were determined with a BIAcore X surface plasmon resonance system (BIAcore, Uppsala, Sweden). PA_{83} was immobilized on a CM5 sensor chip, using the amino coupling procedure, via the injection of 30 μl of 2-μg/ml PA_{83} in 10 mM sodium acetate, pH 4.5. Binding rates for different Fab concentrations, ranging from 5 to 400 nM in PBS, were determined at a flow rate of 30 μl/min, with minimal amounts of coupled antigen (<500 resonance units). We fitted the 1:1 Langmuir model of BIA evaluation software to the binding data. The on and off rate constants (k_{on} and k_{off}, respectively) for the binding of Fab to PA_{83} were determined at 25 °C.

In vitro LT neutralization assay

The mouse macrophage cell line J774A.1 was incubated overnight at a density of 14,000 cells/well in 96-well plates. LT components (400 ng/ml of PA and 40 ng/ml of LF) were added simultaneously to Fab or medium alone and incubated for 1 h at 37 °C. These mixtures were then added to macrophages and incubated for 4 h at 37 °C. A Promega CytoTox 96 assay kit was used, according to the manufacturer's instructions, to determine the IC_{50} values of Fab for LT neutralization.[30]

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