

Antibody Evolution from the Centre to the Periphery: Applied to a Human Antibody Fragment Recognising the Tumour-associated Antigen Mucin-1

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Mucin-1 has proven to be a suitable target for antibody-based diagnosis and therapy of certain tumours, but no appropriate human antibody or antibody fragment displaying slow dissociation rate kinetics against this target is available. Since a rapid dissociation character prevents an antibody fragment from remaining at the site of the antigen, this fact may prevent the successful application of a human mucin-1 specific antibody in diagnosis and therapy. We have now used iterative antibody libraries to evolve a human antibody fragment originally obtained from a naïve antibody library. A strategy was devised whereby molecular variants displaying slow dissociation kinetics against the repetitive mucin-1 tumour-associated antigen can be selected *in vitro*. The evolved clones, that allowed for a reduced dissociation from the tumour antigen, carried substitutions in the outer parts of the binding site. This demonstrated the ability of this *in vitro* evolution technique to mimic the process whereby antibodies evolve *in vivo*. We have thus devised a strategy through which molecular variants displaying slow dissociation from a repetitive target like the mucin-1 tumour-associated antigen can be obtained *in vitro*. These or related molecules obtained by this approach will serve as a starting point for the development of fully human antibodies for use in mucin-1 specific tumour therapy or diagnosis.

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Keywords: antibody; molecular evolution; mucin-1; paratope; phage display

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Introduction

Mucin-1 is a highly glycosylated protein ubiquitously present in many human tissues. It is a protein that in tumour cells is often produced at an elevated level, shows an abnormal glycosylation pattern and a loss of polarisation. The post-translational modifications of mucin-1 produced by tumour cells make this molecule a possible target for therapeutic intervention and diagnostic applications. For these reasons, epitopes on mucin-1 recognised by the cellular and humoral immune responses have been extensively characterised. In particular, a large array of murine monoclonal antibodies have been raised and studied.¹ Indeed,

clinical trials employing the mouse mucin-1 specific monoclonal antibody HMFG-1 have been conducted successfully demonstrating the potential for using such specific antibodies in tumour therapy.² However, it is anticipated that antibodies of human origin would reduce problems associated with the administration of murine proteins to patients.³ Consequently, the development of human antibodies specific for the major epitopes on mucin-1 would provide the community with therapeutic agents of substantial value. Unfortunately, very few human antibodies specific for mucin-1 have ever been developed^{4–7} and several of these have been shown to be of low affinity and to display a rapid dissociation rate constant, probably making them less suitable for tumour therapy or diagnosis.

In order to develop effective tumour-specific human antibodies, we recently evolved antibody fragments specific for mucin-1 with a slower dissociation rate than those identified directly from a large naïve antibody library.⁸ These antibodies owed their specificity not only to the third

Abbreviations used: CDR, complementarity determining region; FR, framework region; V_H , variable domain of heavy chain; scFv, single chain antibody fragment.

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Table 1. Efficiency of mucin-1-specific selection approaches

Antigen	Selection frequency ^a		
	Round 1	Round 2	Round 3
Biotin-MUC-1 ₂	1/1700 ^b	1/900 ^b	1/3900 ^c 1/18500 ^d
Biotin-MUC-ID12	1/250000 ^b	1/590 ^b	1/2600 ^b

^a Number of output phage/number of input phage in the selection.

^b Elution with trypsin.

^c Elution with MUC-ID9.

^d Elution with trypsin after initial elution with MUC-ID9.

complementarity determining region (CDR) of the heavy chain,⁹ but also to other well recognised and defined features, in particular a set of core residues. These included in particular residues W34 and L55 as well as W107 and L116 of the heavy and light chain variable domains, respectively.⁸ Here, we employ combinatorial library and phage display technologies to define how to further reduce the dissociation rate constant of such human antibody fragments specific for the repetitive antigen mucin-1 by fine-tuning conditions for selection. We also demonstrate that human antibody fragments employ sequence variability in the periphery of the antigen binding site to enhance the interaction with the peptide

encompassing the major epitope of mucin-1. Thus, these data form a basis for generating a human mucin-1-specific antibody for clinical application in the treatment and diagnosis of e.g. breast carcinoma.

Results

Selection and analysis of mucin-1 specific single chain fragments

In previous studies^{6,8} antibody fragments recognising mucin-1 have been identified and characterised. These binders, which all recognized the immunodominant epitope on mucin-1, had very defined sequence characteristics within the proposed paratope. Evolution of initially selected clones had conferred an approximate tenfold improvement in the affinity constant of the specific, monovalent binders. In order to further evolve this type of antibody fragment, which may be a possible candidate for mucin-1-specific tumour therapy and diagnosis, we constructed a new medium-sized library by using CDR shuffling.^{8,10} CDRH3 and CDRL3, as well as features of CDRH1 and CDRH2 (in particular W34_H and L55_H) that were known to be associated with this specificity,⁸ were maintained at a high frequency in the library. Sequencing of unselected clones in the library confirmed a high frequency

		CDRH1						CDRH2										CDRL1										CDRL2								
		31	32	33	34	39	40	55	56	57	58	59	60	61	62	63	66	67	68	26	27	28	29	30	31	32	33	34	39	40	56	57	58	66		
germline sequences	IGHV3-07	S	S	Y	W	M	S																													
	IGHV3-30-3							V	I	S	Y	D	G	S	N	K	Y	Y	A																	
	IGLV1-44																			S	S	S	N	I	G	S	N	T	V	N						
	IGLV1-40																															G	N	S	N	
derived directly from n-CoDeR library	M23	.	.	.	.	.	.	L	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	N	S	N
	M67	A	N	T	.	.	.	L	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	N	N	Q	
	M201	.	.	.	.	.	.	L	.	.	G	S	.	G	S	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	
previously defined affinity matured clones ⁸	sMuc129	.	.	.	.	.	.	L	V	.	.	.	.	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	N	R	
	sMuc158	.	R	H	.	.	.	L	.	.	.	.	.	H	E	.	.	.	.	.	.	.	.	.	G	.	A	.	.	A	.	.	.	.		
	sMuc159	.	N	.	.	.	.	L	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	
randomly picked clones	sMuc1-ID9-1	.	.	.	.	.	T	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	H	G		
	sMuc1-ID9-2	.	.	.	.	.	.	L	.	.	N	.	.	D	T	.	.	.	.	.	.	.	.	.	.	.	S	.	.	A	.	N	.	.		
	sMuc1-ID9-3	.	N	.	.	.	.	L	.	.	S	G	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.		
	sMuc1-ID9-4	T	R	H	.	.	.	L	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	N	.		
	sMuc1-ID9-5	.	.	.	.	.	.	L	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.		
	sMuc1-ID9-6	G	.	H	.	.	.	T	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	sMuc1-ID9-7	N	N	.	.	.	.	T	L	.	.	G	S	.	G	S	I	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	N	Q		
	sMuc1-ID9-8	N	.	.	.	.	.	A	L	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	sMuc1-ID9-9	R	N	.	.	.	.	.	.	.	.	.	.	T	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	S	.	N	Q		
randomly picked clones	sMuc1-T-20	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	S	.	.	D	.	N	K		
	sMuc1-T-21	.	G	.	.	.	.	L	.	.	H	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.		
	sMuc1-T-22	.	R	.	.	.	.	T	L	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	T	Y		
	sMuc1-T-23	.	.	.	.	.	.	T	.	.	.	.	.	K	.	.	.	.	.	R	P	.	.	.	.	.	.	.	.	.	T	.	Y	.		
	sMuc1-T-24	R	N	.	.	.	.	L	.	.	G	S	.	G	S	I	.	.	T	.	.	S	.	.	.	.	.	.	.	.	.	.	N	.		
	sMuc1-T-25	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	D	.	N	.		
	sMuc1-T-27	.	.	.	.	.	.	L	.	.	.	.	.	K	Q	.	.	S	G	.	V	.	.	.	.	.	.	.	.	S	N	.	N	E		
clones picked for low k_{diss}	sMuc1-T-28	.	.	.	.	.	.	L	.	.	.	.	.	Q	.	.	.	.	N	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.		
	sMuc1-T-B1	R	N	.	.	.	.	T	L	.	.	.	.	N	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	T	.	
clones picked for low k_{diss}	sMuc1-T-C6	.	R	.	.	.	.	T	L	.	.	.	A	T	.	.	V	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd		
	sMucII-C1	.	R	.	.	.	.	L	.	.	.	.	.	Q	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	S	.	.	N	.		
	sMucII-E1	.	R	.	.	.	.	T	L	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	T	.	S	.	.	D	.	N	K		
	sMucII-F2	.	R	.	.	.	.	I	.	.	.	.	R	Q	.	.	.	.	.	N	.	.	.	.	A	.	.	.	.	.	.	.	N	.		

Figure 1. Sequences of residues residing within or in proximity to CDR targeted with modifications in this study. Residues belonging to the CDR as defined by the IMGT nomenclature are shown on a grey background. Critical R32_H and Q62_H residues are also highlighted in grey. Dots indicate identity with human germline sequences shown in the top part of each column. One clone carries three additional residues, not encoded by the germline gene at the position indicated by an asterisk. nd, not determined.

Table 2. Reaction rate constants of scFv specific for the major epitope on mucin-1 as determined on two antigen variants

Selecting antigen	Elution method	Clone	Reactivity to ala ₃ -MIC-1 ₃			Reactivity to MUC-ID12		
			$k_{\text{ass}} (\times 10^5 \text{ M}^{-1} \text{ s}^{-1})$	$k_{\text{diss}} (\times 10^{-3} \text{ s}^{-1})$	$K_A (\times 10^6 \text{ M}^{-1})$	$k_{\text{ass}} (\times 10^5 \text{ M}^{-1} \text{ s}^{-1})$	$k_{\text{diss}} (\times 10^{-3} \text{ s}^{-1})$	$K_A (\times 10^6 \text{ M}^{-1})$
Biotin-MUC-1 ₂	Trypsin	M67	4.8	130	3.6	nd	nd	nd
		sMuc159	5.2	14	38	8.3	13	64
		sMucI-T-B1	nd ^a	3.2	nd	nd	nd	nd
		sMucI-T-C6	nd	2.2	nd	nd	nd	nd
Biotin-MUC-ID12	Trypsin	sMUCII-C1	1.4	1.7	82	2.7	1.9	140
		sMUCII-E1	1.0	1.5	64	2.1	1.4	150
		sMUCII-F2	0.3	1.7	20	1.6	1.2	141

For comparison, two scFv selected directly from the n-CoDeR library (M67)⁶ and from a library designed to evolve this specificity (sMuc159)⁸ are also included.

^a nd, not determined.

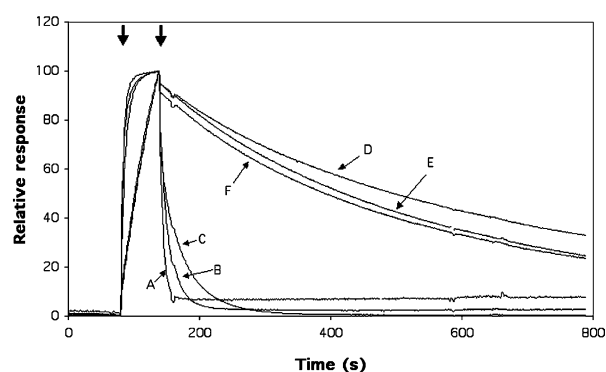


Figure 2. Biacore evaluation of kinetics of scFv obtained directly from the n-CoDeR library (M201 (A)), from a previous study⁸ allowing affinity maturation of mucin-1 specific clones (sMuc 158 (B) and sMuc 159 (C)), and from this study (sMucII-C1 (D), sMucII-E1 (E) and sMucII-F2 (F)). Arrows at the top of the graph indicate the beginning of the association and dissociation phases.

of the favoured residues. Such an analysis also demonstrated the presence of other sequence variants in CDRH1 and CDRH2, should new, emerging clones prefer other sequences within these critical, hypervariable loops (data not shown).

Selection of specific phages from the constructed library was efficient but dependent strongly on the number of repetitive units in the antigen, in particular in the first round of selection (Table 1). The much lower frequency of retrieved clones after selection with the monomeric antigen suggests that avidity effects caused by the physical linkage of two or more epitopes in antigens like biotin-MUC₂ (and probably also native, tumour-derived mucin-1), might compromise a selection based on affinity or dissociation rate constant. Consequently, the use of a monovalent antigen may be the key to finding slowly dissociating clones. When using the different selection protocols, we obtained different pools of single chain antibody fragment (scFv)-producing clones after three rounds of selection (Table 1). Members of these populations were assessed for apparent k_{diss} . It had previously been

observed that molecular variants of the herein-used scFv have a very low tendency to dimerise when produced as soluble fragments. We were thus able to initially determine k_{diss} of a large set of clones by using crude bacterial culture supernatants containing scFv with minimal interference from scFv dimers. In many cases where biotin-MUC-1₂ was used in the selections, no improvement in k_{diss} in comparison to the previously selected affinity-matured clone sMuc159 was observed among the selected ones. This was in particular true for clones eluted with soluble peptide (none out of 81 tested clones dissociated more than five times slower than sMuc159). However, a few clones (three out of 48 tested clones) showed evidence of a slow dissociation rate among the ones selected with biotin-MUC-1₂ and eluted with trypsin treatment after a first elution using a non-biotinylated peptide. In contrast, practically all clones (35 out of 38 tested) selected on the monomeric biotin-MUC-ID12 antigen showed evidence of a slow dissociation of the antigen–scFv complex. Among these, 13 were sequenced and they represented three different sequences (Figure 1) of which one (sMucII-E1) was present in multiple clones. ScFv were subsequently purified in monomeric form and their reaction rate constants were determined. As shown in Table 2, the dissociation rate constant had indeed been improved by approximately tenfold over the previously identified, affinity-matured clone sMuc159.⁸ However, with respect to affinity constant this gain was partly off-set by a reduction in k_{ass} , resulting in a twofold overall improvement in affinity constant as compared to the high affinity variant sMuc159. This was true both when investigating the reactivity towards the monovalent 12-mer peptide or a trimer repeat of a longer, 20-mer sequence. In relation to clones obtained directly from the original n-CoDeR library, a nearly 100-fold improvement of the dissociation rate constant was observed, resulting in a much prolonged persistence of the antigen–scFv complex (Figure 2). At this stage it was also confirmed that these evolved clones retained a specificity for the natural antigen on breast tumour cell lines, despite the fact that they had been selected using a very short synthetic peptide (Figure 3).

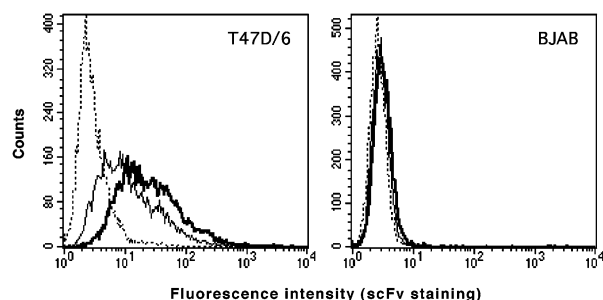


Figure 3. FACS analysis of the binding of sMuc 159 (thin line) sMucII-E1 (thick line) and a negative control (cytomegalovirus-specific) scFv (broken line) to the breast carcinoma cell line T47D/6, which carries mucin-1 and the negative control B cell lymphoma BJAB.

Sequence and structure analysis of selected clones

Sequencing of plasmids encoding scFv derived at different stages of the evolution process was performed in order to determine any specific features of the selected clones (Figure 1). We observed that selected clones preferentially used CDRH1 and CDRH2 sequences similar to those found in V_H genes IGHV3-07 and IGHV3-30/IGHV3-30-3, respectively. Such a divergent origin of CDR in these molecules is made possible by the modularity of the evolution system^{10,11} that brings together sequences, normally encoded by different

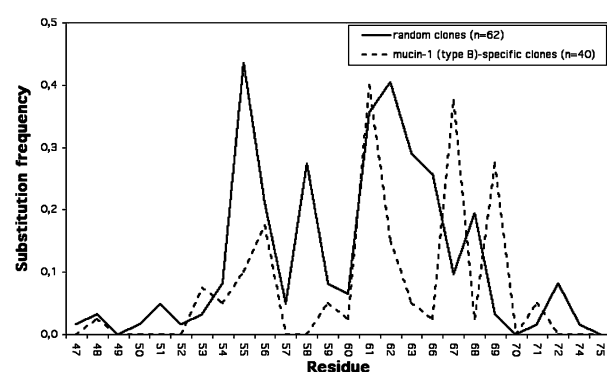


Figure 4. Frequency of substitutions encoded within CDRH2 and its vicinity, and derived from germline genes IGHV3-30 and IGHV3-30-3 as found in human antibodies in natural B cell populations (continuous line) and in human antibody fragments specific for mucin-1 and employing the MVGAHALDI CDRH3 sequence.

V genes, into one sequence. This agreed with previous observations that the CDRH3 employed here developed the MUC-1 specific paratope in conjunction with features found in CDRH1 and CDRH2 found in IGHV3-07 and IGHV3-30/IGHV3-30-3 genes, respectively.⁸ Among clones selected using the biotin-MUC-1₂ antigen, no accumulation of any given clone was observed, suggesting that no clone type has a clear selection advantage under these conditions. This is probably a consequence of the divalent nature of the target antigen. However, in the case of clones selected using the monovalent antigen biotin-MUC-ID12, one specific clone came to dominate the population. Furthermore, all the selected clones carried specific modifications targeting residues 32_H and 62_H. These specific modifications required substitutions in the genes that may not be common in the population. The S32_H → R and N62_H → Q substitutions require one and two single base-pair mutations, respectively, in comparison to the codons used in the unmutated germline genes. In fact, among 62 CDRH2 sequences[†] found in a set of human antibody genes derived from normal human B cells or hybridomas originating from the IGHV3-30/IGHV3-30-3 germline genes, Q62_H was not observed. Since the CDRH2 found in the present library originated from normal human B cells, it is expected that only the application of a strong selection pressure forced a selection for this rare mutation. To further investigate this matter, we used a larger set of similar clones recognising the same epitope and using a similar CDRH3, which we previously described.⁸ The peptide inhibition pattern as well as many sequence features, including critical parts of CDRH1 and

[†] Details on these sequences are available directly from the corresponding author and on the world-wide web at URL: http://www.immun.lth.se/manuscripts/mut_3-30-x.pdf

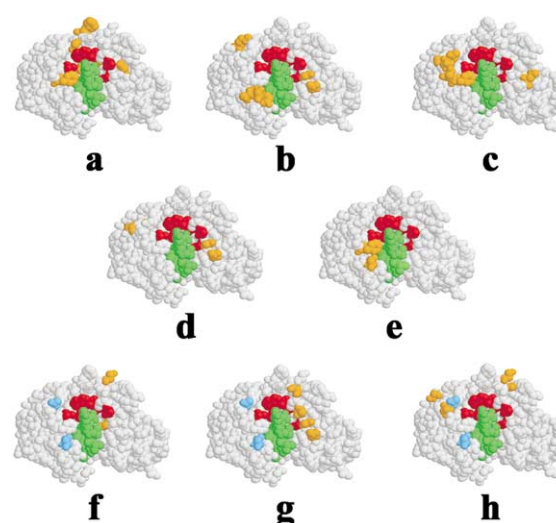


Figure 5. Localisation of substitutions that occurred as a consequence of the evolution of the mucin-1 specific scFv and which eventually resulted in a reduction of the dissociation rate constant. These sequence modifications are shown here on a model of the sMuc 159 scFv.⁸ Differences between a consensus sequence and (a) M23, (b) M67 and (c) M201 derived from the n-CoDeR library are shown. Similar differences are given for (d) sMuc 129 and (e) sMuc 159,⁸ as well as for the most slowly dissociating clones (f) sMucII-C1, (g) sMucII-E1 and (h) sMucII-F2 established here. CDRH3 residues 107–115 (VLAQQR) are highlighted in green. The core residues W34_H, L55_H, T34_L, N40_L, T106_L, W107_L and L116_L⁸ are shown in red. The occurrences of residues R32_H and Q62_H are highlighted in blue, while other differences from a consensus sequence (carrying germline IGHV3-07 CDRH1, germline IGHV3-30-3 CDRH2, germline IGLV1-44 CDRL1 and G₅₆NNNRPS₆₉ CDRL2) are shown in orange.

CDRH2, were common between this second set of sequences⁸ and the clones otherwise studied here, suggesting that their interaction with the target antigen was similar. By comparing substitution frequencies within or close to CDRH2 between these mucin-1-specific and naturally occurring antibody genes originating from the V_H genes IGHV3-30 and IGHV3-30-3, it was found that the sequence variation found in these populations differed (Figure 4). Selection pressure was put on a whole range of residues with CDRH2, either decreasing or increasing their ability to harbour sequence variation in the context of a mucin-1 specificity. To maintain this mucin-1 specificity, several residues including the critical 62_H residue were thus found to be unable to efficiently harbour the random sequence variants that are otherwise present among normal B cell-derived CDRH2 originating from these genes and introduced into the library in this study. Altogether this suggests that residue 62_H is important for this type of paratope and that the rare N62_H → Q substitution is particularly beneficial for the slow dissociation behaviour of the selected clones. The observed key mutations were mapped onto a previously

established structure model of the sMuc159 scFv.^{6,8} As shown in Figure 5, these mutations are located in the outer part of what could be proposed to be an antigen-binding site.

Discussion

The demonstration that mucin-1 specific antibodies provide beneficial effects in tumour therapy, such as in the treatment of ovarian cancer,² emphasises the importance of the availability of appropriate reagents for such applications. The development of a fully human antibody displaying appropriate binding specificity and reaction rate kinetics may provide an important reagent circumventing many of the disadvantages with currently existing murine antibodies. Combinatorial library and display technologies have proven to be very powerful methods to improve specificity and affinity. Not only do these and related technologies allow us to obtain antibodies of human origin, but they also permit the development of reagents with designed characteristics, like affinity constants. For instance, in certain cases an approximately 1000-fold improvement in affinity constants over that found in an initially available antibody fragments have been reported^{12,13} reaching affinity constants of $2 \times 10^{13} \text{ M}^{-1}$.¹² However, in many cases much less favourable results are obtained in evolution schemes, often depending on the nature of the target antigen. We have previously used combinatorial libraries and phage display to identify and evolve two types of mucin-1 specific human scFv.^{6,8} This approach had allowed us to define specific characteristics of the core of the binding site that was associated with the interaction with the immunodominant epitope of mucin-1.⁸ The antigen under investigation, though, poses specific problems with respect to affinity maturation of specific binders that at least partly are linked to the repetitive nature of the epitope. In fact, the antigen has a capacity to provide an avidity effect that negatively influences the efficiency of a dissociation rate constant, or affinity constant-based selection. In many applications, dissociation rate constants are of paramount importance for the successful use of an antibody fragment,^{13–15} and we set out to test how we could achieve selection favouring a slow dissociation rate with this biologically important antigen. A new antibody library was constructed to evolve a human antibody-specificity employing the modularity of the system and the CDR-shuffling technology.^{8,16} To improve the functionality of the iterative library, we took advantage of the features that were known to define the specific antigen binding site under investigation⁸ in order to suggest a new strategy upon which the binding site may be evolved. Specifically, attempts were made to incorporate a high frequency of key residues in the library making up the central part of the previously defined paratope. As we allowed

for some variability even in these critical residues we believe that we permitted the paratope not to be too rigid and to explore new areas of sequence space, whenever necessary. This allowed us to evolve the sequence beyond any limitations that would otherwise have been put upon the molecular evolution pathway if we rigidly had maintained an initially defined beneficial residue. If we would not have taken this approach we might have locked the paratope into a local affinity maximum from which it could not escape easily.¹⁷ In fact, we observed some clones that used such approaches to create a binding site by introducing other residues than those normally used (e.g. substituting L55_H for valine). However, in this particular case these clones were not among those that in the end proved to display the slowest dissociation rate kinetics.

The selection system that was developed was aimed at evolving the affinity constant, with a specific pressure being applied on selection of clones displaying a reduced dissociation rate. The use of different antigens greatly affected the outcome of the selection system and dimeric antigen proved ineffective in achieving a reduction in k_{diss} . This may very likely be due to the occurrence of phages displaying more than one antibody fragment, giving them an avidity advantage in the selection process and ruling out a selection based on k_{diss} . We herein showed that a smaller fragment of the mucin-1 major epitope was more effective in selecting molecular variants displaying a slower dissociation rate constant. The reduced frequency of selected scFv in the first round of selection using the biotin-MUC-ID12 antigen clearly demonstrated that the selection pressure put on the system at this stage was much higher, most likely as a consequence of the monovalent nature of the selecting peptide. The clones that eventually emerged from selection on the monovalent antigen retain specificity for the epitope as defined by peptide inhibition and FACS analysis. This suggests that the choice of antigen as such and the observed mutations found in the evolved clones did not substantially change the antigen specificity of the emerging antibody fragments, an effect that has been observed in certain other cases.^{18,19}

Importantly, we were able to identify clones carrying novel combinations of rare mutations that together conferred the high-affinity character after employing the CDR-shuffling approach. The iterative process and re-organisation of initially selected fragments in new, dedicated libraries ensures that rare mutations will, if so required, be allowed to occur in new environments in which they will be able to create optimal binding sites. Similar results have previously been defined using evolution approaches involving error-prone PCR,²⁰ in particular in combination with iterative rearrangement (DNA shuffling) of selected variability.¹² These latter studies did not focus the variability to the antigen-contacting residues but allowed mutations to occur throughout the

Table 3. Sequences of synthetic peptides used here

Name of peptide	Sequence in the standard one-letter code
MUC-1 ₂	(PAHGVTSAPDTRPAPGSTAP) ₂
Ala ₃ -MUC-1 ₃	Ala ₃ -(PAHGVTSAPDTRPAPGSTAP) ₃
MUC-ID9	SAPDTRPAP
MUC-ID12	GVTSAPDTRPAP

molecule. It was observed that many beneficial mutations occurred in sites that were not located in the CDR, but which rather affected the binding site topography indirectly. Such findings corroborate with earlier studies demonstrating affinity-maturation effects as a consequence of mutations at distant sites.²¹ The herein-used approach does not specifically target framework residues with sequence modifications. Rather, a maturation of k_{diss} was achieved by introduction of substitutions at sites that frequently carry contact residues,²² but which could not be easily predicted to be the targets that should be explored in a mutagenesis scheme. For instance, residue 62_H is not among those targeted in one of the proposed schemes for affinity maturation,²³ and employing that approach would not have allowed us to reach this particular solution in structure space. However, observed beneficial mutations tended to occur at the edges of the proposed binding site, resulting in an evolution mimicking the process whereby an antibody population evolves *in vivo*.^{21,24,25}

Here, we achieved from the construction of a single library a tenfold improvement in k_{diss} , which is the factor that the selection process mainly selects for. Overall, the approach improved the dissociation rate constant approximately 100-fold over those clones that were retrieved directly from the original library by using an iterative process. The reduction in k_{ass} may be related to the limited amount of selective pressure exercised on this parameter, as well as on the fact that the primary clones obtained directly from the n-CoDeR library displayed a rapid k_{ass} , close to its proposed theoretical limit.^{26,27} Consequently, any introduced modifications may at best retain the original fast association rate. In fact, other selection approaches that dramatically improve the affinity constant have also achieved this at a cost of a reduced k_{ass} .¹²

In conclusion, by using a stepwise *in vitro* evolution process and an optimised selection procedure involving a monovalent core epitope we have managed to evolve the ability of an antibody fragment specific for the major tumour-specific epitope on mucin-1 to remain bound to its target by reducing its dissociation rate from the complex. Sequence analysis indicated that the number of substitutions leading to this improvement were small, resembling the *in vivo* situation. It also appeared that they tended to target the periphery of the binding site, into which molecular variability also disseminates during the *in vivo* affinity matu-

ration process. The herein-described approach has thus been used to functionally evolve antibody fragments recognising a tumour-associated epitope considered to be of great importance for the development of diagnosis and therapy. It is suggested that these or other similarly selected antibody specificities will provide us with antibodies of human origin that will be suitable for further clinical evaluation.

Materials and Methods

Reagents

The n-CoDeR library was kindly provided by Bio-Invent Therapeutic AB (Lund, Sweden). Synthetic peptides, shown in Table 3, which had been prepared by automated peptide synthesis as described elsewhere,²⁸ were obtained from Professor Elias Krambovitis (IMBB, Heraklion, Greece). These peptides had been selected so as to encode the major immunogenic epitope on mucin-1. In cases where biotin was attached to the peptide this moiety was introduced at the N terminus. In the case of the peptide MUC-1₃, an amino acid spacer was introduced between biotin and the N-terminal proline residue to improve the binding of the peptide to streptavidin. sMuc159 and M67 were previously existing scFv specific for mucin-1 isolated from the n-CoDeR library,^{6,8} and an existing structure model⁶ of sMuc159 (PDB accession code 1KU4) was also used in this investigation.

Library construction

Antibody fragments specific for mucin-1 had previously been selected directly from the large naïve n-CoDeR library.⁶ All members in this library make use of the IGHV3-23 and IGLV1-47 germline gene framework regions (FR) into which sequences obtained from cDNA synthesised from genes expressed in human B cells and encoding hypervariable loops had been inserted. Subsequently, we evolved a clonotype derived from this library so as to achieve a higher affinity for the target antigen.⁸ Furthermore, characteristic features of the paratope were defined. A new antibody library was now constructed using CDR-shuffling.¹⁰ The approach taken was to try to maintain the paratope core of the antibody fragment sMuc159⁸ while allowing molecular diversity to spread into other parts of the binding site. The fact that all members of the library use the same FR allows us to easily substitute CDR in a clone with sequences found in other clones. For our current purpose CDRH3 and CDRL3 were left unchanged. To achieve this, sequences encoding CDR3 and adjacent FR were amplified from a plasmid carrying the sMuc159-encoding gene.⁸ It had also been defined that residues W34_H in CDRH1, L55_H in CDRH₂, and T34_L and/or N40_L in CDRL1 were all important for maintaining a functional binding site.⁸ Therefore, sequences encoding CDRH1 and adjacent FR were amplified from phagemids derived from the n-CoDeR library that had been selected on the mucin-1 peptide antigen. This gene population was enriched for two similar types of clones specific for the epitope studied that showed some sequence variation in CDRH3. Thus, a substantial fraction of the clones in this population was known to retain W34_H.⁸ Sequences encoding CDRH2 and adjacent FR were amplified from the unselected n-CoDeR library population. This ampli-

fied gene fragment population was, however, enriched for sequences carrying L55_H by using an upstream primer (AAGGGGCTGGAGTGGGTBKCWCT) that specifically incorporated the first two bases of residue 55_H encoding leucine. The use of the proof-reading capable *Pfu* polymerase (Stratagene, La Jolla, CA, USA) in this PCR amplification, however, also ensured that a part of the CDRH2-population would include other residues at this site following 3'-editing of primers annealing to a template not encoding leucine in position 55_H. Other PCR amplifications were carried out using either *Pfu* or TaqPlus (Stratagene). Sequences encoding CDRL1 and CDRL2 and adjacent FR were amplified directly from the n-CoDeR library as this population contained a high frequency of the residues within the light chain variable gene segment that were known to be critical⁸ for establishment of this paratope. In this way, five PCR products were obtained and these were assembled into an intact gene library by overlap-extension PCR, as described.^{8,10,11,29} The amplified genes were purified, cleaved with *Sfi*I and *Not*I, and ligated into the pFAB5-C.His phagemid vector.³⁰ After electroporation of ligated DNA into *Escherichia coli* Top10F' a library of 2×10^7 members was obtained. Phage stocks were prepared using R408 helper phage, and phages were concentrated by using a conventional polyethylene glycol-precipitation protocol.

Selection procedure

Selection of phages displaying antigen-specific scFv was carried out by using different approaches. In the first approach a biotinylated MUC-1₂ peptide was used as target antigen and scFv-phage/peptide complexes were captured on streptavidin-coated paramagnetic beads (Dynal A/S, Oslo, Norway). Three rounds of selections were performed. In the first round 10^{-8} M peptide was used, in the second and third round of selection, 10^{-9} M and 5×10^{-10} M were used. In rounds 1 and 2 the bound phages were eluted after incubation with trypsin, as this enzyme cleaves the scFv-protein 3 fusion protein at a site between the phage and the scFv molecule. This will dissociate the phage from the immobilized antigen but it will retain the infective properties of the phage. In the last round of selection the bound phages were eluted in two steps. Incubation of the bound phages with an excess of non-biotinylated MUC-ID9 for ten minutes was used as a first elution step, and remaining bound phages were released after treatment with trypsin as above.

Since part of the objective of this study was to evaluate effects of the evolution process on kinetic parameters, the selection procedure described above has a potential disadvantage. As the peptide used in the selections presents two epitopes in close proximity, any phages displaying more than one copy of the scFv will have a clear advantage during the selection due to avidity effects obscuring selection for improved kinetics. In order to avoid this problem, a monomeric peptide antigen, biotinylated MUC-ID12 peptide, was used in a second selection procedure. The first round of selection was performed essentially as above. In the second and third rounds of selection, 10^{-8} M and 10^{-9} M peptide was used. To minimise any avidity effects on multivalently displaying phages, the capture on the beads was made in the presence of 10^{-7} M non-biotinylated MUC-ID9 peptide. In all, this approach has the potential to provide a more stringent affinity and off-rate based selection.

Protein production and determination of kinetic parameters

To assess the outcome of the selections, plasmid was prepared from the pools retrieved after the third round of selection and converted into a format that allows production of soluble scFv. For this purpose, an *Eag*I restriction-fragment encoding the C-terminal part of filamentous phage fusion protein 3, was removed from the vector, using conditions recommended by the supplier of the enzyme (New England Biolabs, Beverly, MA, USA). Ligated vector was subsequently transformed into *E. coli* Top10F', and individual clones were picked for further analysis. Clones derived from the selection using the first approach with the MUC-1₂ peptide, eluted with the 9-mer peptide, are named sMucI-ID9 followed by a clone number. Similarly, the trypsin-eluted clones from this selection process are called sMucI-T and a clone number. The clones derived from the second selection approach using the monomeric antigen are identified by the prefix sMucII followed by a clone number. The different clones were grown and induced to produce soluble scFv. In initial screening approaches, individual crude culture supernatants were analysed for a slower dissociation from the antigen using Biacore analysis.⁸ Some clones that were identified to potentially have a slower k_{diss} as compared to the scFv sMucI59 that had been established previously⁸ were subjected to large-scale production. Antibody fragments were isolated either directly from the media or from the periplasmic space and purified by metal affinity chromatography over a Ni-NTA agarose Superflow column (Qiagen, Hilden, Germany). The monomeric form of the scFv was obtained after gel permeation chromatography on a Superdex 75 column equilibrated with Dulbecco's phosphate-buffered saline (PBS).

The reaction rate kinetics for the purified, monomeric clones was determined using Biacore analysis for two different versions of MUC-1 peptides. The biotinylated version of the MUC-1₂ or the MUC-ID12 peptide was immobilised *via* streptavidin at a low density (approximately 300 units of streptavidin) to a CM5 sensorchip, using the amine coupling kit (Biacore AB, Uppsala, Sweden). Biotinylated peptides were coated onto all available binding sites. The kinetics of both the association and the dissociation phase were determined with the BiaEvaluation 3.0 software (Biacore AB) at a flow rate of 20 μ l/minute, using different concentrations of the monomeric scFv.

Flow cytometry

The binding of monovalent scFv to the mucin-1 producing breast carcinoma cell line T47D/6 was investigated by flow cytometry. After incubation of cells with 5 μ g monovalent scFv/ml in PBS containing 1% (w/v) bovine serum albumin, bound scFv were detected with the anti-FLAG M2 antibody (Sigma Chem. Co., St. Louis, MO, USA) and finally with R-phycoerythrin-conjugated rabbit F(ab')₂-anti-mouse immunoglobulins (DAKO A/S, Glostrup, Denmark). The analysis was performed on a FACScan (Becton Dickinson, San Jose, CA, USA). The human B cell lymphoma BJAB was used as a control cell line.

Genetic analysis

To determine the sequences of clones derived from the different selection rounds, sequencing reactions were

performed using the Big Dye Terminator kit (PE Biosystems, Warrington, England). The reactions were analysed using an ABI377 automated sequencing equipment (Applied Biosystems, Foster City, CA, USA) and the sequences were evaluated using the MacVector 6.5.3 software (Oxford Molecular Group Ltd, Oxford, UK). The origin of each CDR was determined by comparison with known variable gene sequences available in the March 1997 release of the VBASE sequence directory.³¹ Gene names and the sequence numbering schemes follow the official IMGT nomenclature.^{32–33} We also compared the substitution pattern in CDRH2 of antibody populations of mucin-1 specific scFv derived either directly from the n-CoDeR library or from sublibraries that had been further evolved. Such antigen-specific scFv clones employed CDRH2 derived from the IGHV3-30 and IGHV3-30-3, which encode the same CDRH2 sequence, at a high frequency. For comparison, we used sequences of naturally occurring antibodies derived from these two germline genes.

Acknowledgments

We are grateful to BioInvent Therapeutic AB (Lund, Sweden) for providing the n-CoDeR antibody library and to Professor Elias Krambovitis (Institute of Molecular Biology and Biotechnology, Heraklion, Greece) for providing synthetic peptides. This study was supported by a grant from the National Research Council for Engineering Sciences.

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Edited by I. Wilson

(Received 2 October 2001; received in revised form 7 February 2002; accepted 8 February 2002)