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Structural aspects of antibody–antigen interaction revealed through small random peptide libraries

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Summary

Two small random peptide libraries, one composed of 4550 dodecapeptides and one of 8000 tripeptides, were synthesized in newly developed credit-card format miniPEPSCAN cards (miniPEPSCAN libraries). Each peptide was synthesized in a discrete well (455 peptides/card). The two miniPEPSCAN libraries were screened with three different monoclonal antibodies (Mabs). Two other random peptide libraries, expressed on the wall of bacteria (recombinant libraries) and composed of 10^7 hexa- and octapeptides, were screened with the same three Mabs. The aim of this study was to compare the amino acid sequence of peptides selected from small and large pools of random peptides and, in this way, investigate the potential of small random peptide libraries. The screening of the two miniPEPSCAN libraries resulted in the identification of a surprisingly large number of antibody-binding peptides, while the screening of the large recombinant libraries, using the same Mabs, resulted in the identification of only a small number of peptides. The large number of peptides derived from the small random peptide libraries allowed the determination of consensus sequences. These consensus sequences could be related to small linear and nonlinear parts of the respective epitopes. The small number of peptides derived from the large random peptide libraries could only be related to linear epitopes that were previously mapped using small libraries of overlapping peptides covering the antigenic protein. Thus, with respect to the cost and speed of identifying peptides that resemble linear and nonlinear parts of epitopes, small diversity libraries based on synthetic peptides appear to be superior to large diversity libraries based on expression systems.

Introduction

Random peptide libraries are increasingly used to identify epitopes and investigate antigen–antibody interactions [1]. Successful strategies include screening of libraries in which the peptides are expressed on bacteria or phage [2], libraries in which the peptides are synthesized on beads [3], and libraries that contain soluble peptides [4,5].

Initially, epitopes were localized using small peptide libraries that contain overlapping synthetic peptides covering the complete linear amino acid sequence of a protein (PEPSCAN method). With this method, linear epitopes or linear parts of discontinuous epitopes are readily mapped [6–8]. However, the majority of epitopes are discontinuous to such an extent that they cannot be mapped using synthetic peptides that cover the linear sequence of the antigenic protein [9–11].

Using the PEPSCAN method, it has been found that, within a population of overlapping peptides covering the linear sequence of an antigenic protein, monoclonal antibodies (Mabs) can recognize peptides that are not part of the epitope. These peptides mimic a part of the actual epitope [12–14]. The presence of mimics within these relatively small populations of overlapping peptides suggests that it may be possible to identify mimics from relatively small synthetic peptide libraries. If this is the

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Abbreviations: ABTS, 2,2'-azino-di-3-ethylbenzthiazoline sulfonate; EGF, epidermal growth factor; Mab, monoclonal antibody; OD_{ccd}, optical density obtained using CCD camera; RAMPO, rabbit-antimouse peroxidase; SDS, sodium dodecylsulfate; TGEV, transmissible gastroenteritis virus.

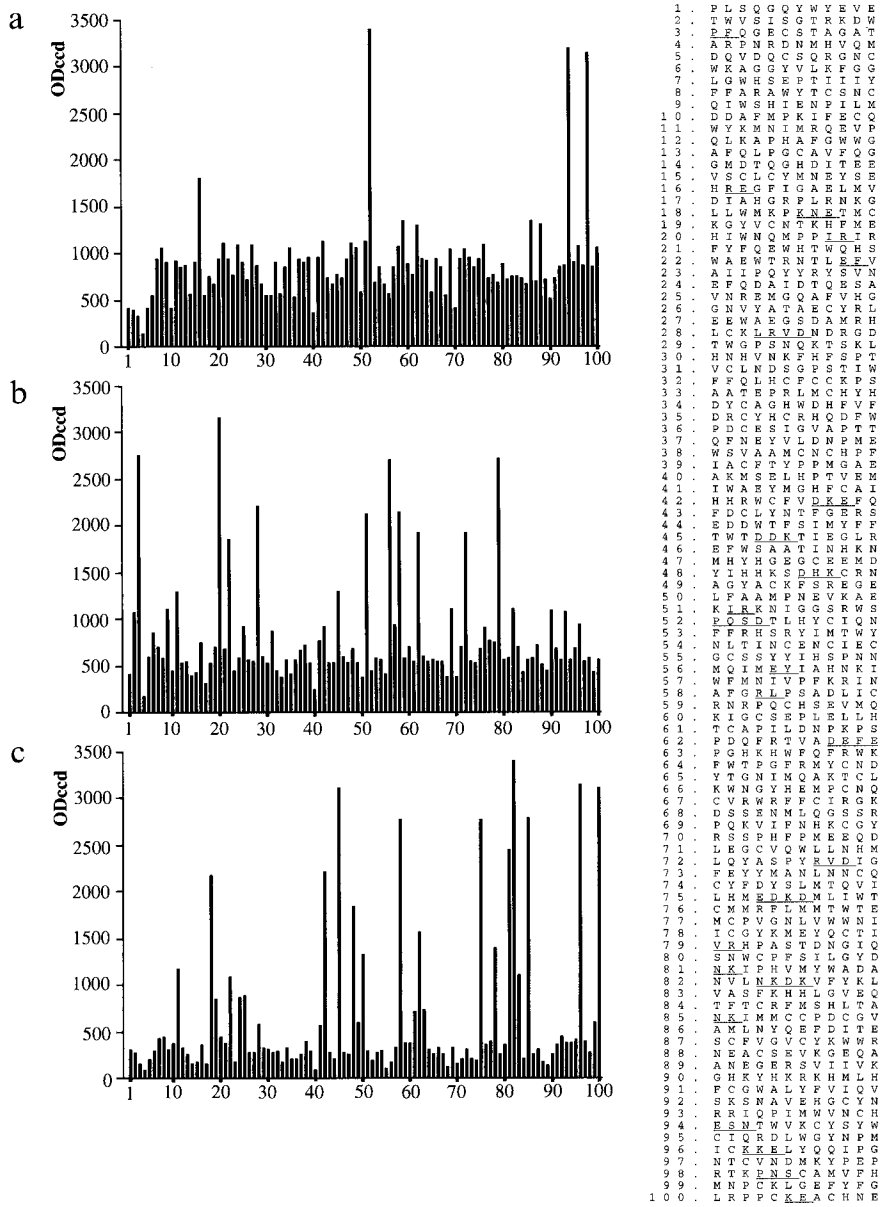

Fig. 1. PEPSCANs of the monoclonal antibodies (a) 6A.A6, (b) 57.9 and (c) 32F81 with the same 100 dodecapeptide sequences. The 100 peptide sequences correspond to 100 consecutive random dodecapeptides of miniPEPSCAN card 1. The x-axis represents the 100 random dodecapeptides; the y-axis depicts the OD_{ccd} values. Consensus sequences similar to the boxed sequences shown in Table 1 are underlined.

case, it would provide a more or less systematic and urgently needed way to identify peptides that mimic discontinuous epitopes. Therefore, we designed small random peptide libraries to map epitopes and we compared the potential of this new approach to that of large recombinant libraries composed of many millions of peptides.

Two relatively small and two relatively large random peptide libraries were screened with three different Mabs. The peptides in the libraries were tested for the ability to bind Mabs 6A.A6, 57.9 and 32F81. Mab 6A.A6 binds the transmissible gastroenteritis virus (TGEV) [14], Mab 57.9 binds and neutralizes TGEV [12,13] and Mab 32F81 binds and neutralizes the gametocyte of the malaria parasite *Plasmodium falciparum* [15].

Surprisingly, for each of the three Mabs the small random peptide libraries provided a very large number of antibody-binding peptides that appeared to mimic linear and nonlinear parts of the native epitopes. On the other hand, the large random peptide libraries, requiring much more effort to handle, provided a small number of antibody-binding peptides that only mimic linear parts of previously mapped linear epitopes.

To our knowledge, this is the first study that compares the potential of small and large random peptide libraries with respect to their ability to provide peptides that mimic epitopes. We found that, with respect to the costs, speed and amount of work involved, antigen–antibody interactions can be studied far more effectively using small ran-

dom peptide libraries than using large random peptide libraries.

Materials and Methods

Monoclonal antibodies

The monoclonal antibodies 6A.A6, 57.9 and 32F81 have been described previously [12–17]. Mabs 6A.A6 and 57.9 are directed against the peplomer protein-S of TGEV. Mab 32F81 is directed against an EGF-like domain of the surface protein pfs25 of *P. falciparum*.

Synthesis and screening of miniPEPSCAN libraries

Using the 20 L-natural amino acids, 4550 random dodecapeptides were generated with a random generator programmed in Quick BASIC, which runs on a 486 DX2 (66 MHz) computer system. In this library the frequency of each residue is approximately 5%. Furthermore, using the 20 L-natural amino acids all possible trimers, i.e. 8000 possibilities ($20 \times 20 \times 20$), were generated. The 4550 dodecapeptides and 8000 tripeptides were synthesized in credit-card format miniPEPSCAN cards (455 peptides per card) as described previously [6]. The dodecapeptide library was synthesized in miniPEPSCAN cards 1–10 and the tripeptide library in miniPEPSCAN cards 11–28.

The binding of Mabs 6A.A6, 57.9 and 32F81 to each peptide was tested in an ELISA based on pins. The 455-well cards, containing the covalently linked peptides, were incubated with Mab 6A.A6, Mab 57.9 or Mab 32F81 (antibody dilution 1/100). After washing, the cards were incubated with RAMPO (dilution 1/1000, 1 h, 25 °C) (Dako, Glostrup, Denmark). After washing, the peroxidase substrate ABTS and 2 µl/ml 3% H_2O_2 were added. After 1 h the color development was measured. The optical density of the color (green) was quantified using a CCD camera and an image processing system. The optical densities measured with the CCD camera (OD_{ccd}) are measured between 550–800 nm (orange filter). Using this filter, the green color becomes a grey value. Using the image processing software, this grey value is translated into an ELISA value between 0 and 3.5 (logarithmic scale). The setup consists of a CCD camera and a 55-mm lens (Sony CCD Video Camera XC-77RR, Nikon micro-nikkor 55 mm f/2.8 lens), a camera adaptor (Sony Camera adaptor DC-77RR) and the image processing software package TIM, v. 3.36 (Difa Measuring Systems, Breda, The Netherlands). TIM runs on a 486 DX2 (50 MHz) computer system. The volume of antibody solution that was used to screen both miniPEPSCAN libraries was 20 ml. After each assay the cards were cleaned in SDS and mercaptoethanol solutions, so that they could be re-used.

Construction and screening of recombinant libraries

The construction of the two random recombinant libraries, one for hexa- and one for octapeptides, has been described previously [16]. An oligonucleotide with a completely degenerate sequence of 17 or 23 nucleotides was inserted into the bacterial expression vector pCL627. The libraries contain more than 10^7 clones with random peptides attached to a β-galactosidase hybrid protein. The colonies were grown on plates of 15×25 cm (approximately 10 000 clones/plate). The colonies were transferred onto nitrocellulose paper (Schleicher and Schüll BA85, Dassel, Germany) after applying a temperature shock of 42 °C (2 h). The nitrocellulose was incubated with one of the three Mabs (dilution 1/1000). Positive clones were purified by replating and were again incubated with the three Mabs (dilution 1/1000). Induction of gene expression, controlling of specificity of expression products (Western blotting) and sequencing of inserts were performed as described previously [16].

Modelling of the EGF-like domain of pfs25 protein of P. falciparum

A three-dimensional model of the EGF-like domain of the pfs25 protein that contains the linear epitope recognized by Mab 32F81 was constructed using the molecular modelling software SYBYL (v. 6.0, Tripos Associates, St. Louis, MO). SYBYL runs on a Silicon Graphics computer system (IRIS Indigo Elan, 3D graphics system). The data bank entry 1EGF.PDB [18], containing the 3D coordinates of mEGF, was extracted from the Protein Data Bank at Brookhaven Laboratories [19] and loaded into SYBYL. Using SYBYL residues of mEGF were replaced with residues of the EGF-like domain of the pfs25 surface protein of the malaria parasite *P. falciparum*.

Results

Peptides identified using the synthetic miniPEPSCAN dodecapeptide library

Screening of the synthetic random dodecapeptide library with Mabs 6A.A6, 57.9 and 32F81 resulted in three different sets of data of 4550 OD_{ccd} values linked to 4550 different dodecapeptide sequences. To illustrate the result, for each Mab, OD_{ccd} values of 100 consecutive random dodecapeptides (miniPEPSCAN card 1) are shown in Fig. 1. In addition, for each Mab and for the 50 dodecapeptides with the highest OD_{ccd} values, the sequences of these dodecapeptides and their OD_{ccd} values are shown in Table 1. Also listed are the linear epitopes previously identified using the PEPSCAN method [12–15] (Table 1).

Peptides identified using the synthetic miniPEPSCAN tripeptide library

Screening of the synthetic tripeptide library with Mabs 6A.A6, 57.9 and 32F81 resulted in three different sets of data, each consisting of 8000 OD_{ccd} values linked to 8000 different tripeptide sequences. These are all shown in Figs. 2–4.

TABLE 1
AMINO ACID SEQUENCES OF PEPTIDES IDENTIFIED USING THE SMALL DIVERSITY LIBRARIES BASED ON SYNTHETIC PEPTIDES

	No.	Mab 6A.A6		Mab 57.9		Mab 32F81	
linear epitopes	[1]	SRLPPNSDVVLG		SFFSYGEI		LDTSNPVKT	
	[2]	PICPSNSEANCG				FDDTDPIKK	
antibody binding dodeca-peptides	[1]	RSDSMGILLLPL	[3418]	HTEFFSSCSSQV	[3821]	FKCTADQYVTFA	[3546]
	[2]	PQSDTLHYCIQN	[3402]	YRTTMDFVVWSL	[3534]	LPWHEILKQNVN	[3458]
	[3]	CSDVVETRCQID	[3364]	NFIWAPFPMMNI	[3496]	QGSWDKFGMNEH	[3458]
	[4]	ESNTWVKCYSYW	[3364]	VQRQCVMASTEY	[3477]	EKCDHQCAQCVY	[3420]
	[5]	QSEFVLHDGCDF	[3168]	DDRLELPDHRWL	[3458]	DKIENYIQWPHD	[3420]
	[6]	EFMSDIALRGTV	[3168]	VEAIKFEQSIWP	[3458]	YWEKHSFSGIIT	[3420]
	[7]	RTKPNSCAMVFH	[3150]	AYRGTHVNVRDM	[3420]	HDKDRTEHTQDA	[3402]
	[8]	MYIFGDCAENDT	[3150]	IREPPRDGIKLT	[3420]	FWPQPKEEYTWD	[3402]
	[9]	LMSDQYNNTFVS	[3096]	PWQANFNFPKIV	[3420]	NVLNKDKVFYKL	[3402]
	[10]	DRIFTNSDYVHR	[3078]	IGRMQLMIIPFW	[3402]	WKHAPLFPADVT	[3402]
	[11]	ANSENRFDYVDW	[3072]	ANMGVREETSQF	[3402]	MWFKCEDRCSTQ	[3383]
	[12]	ADHREPDPNLVW	[3060]	QIVERIVPDGAS	[3402]	FKCNMMEQNHVI	[3383]
	[13]	GMQAKDNSDISS	[3060]	TFWRIDDPKHTC	[3383]	DWKTHMAVHSYC	[3383]
	[14]	ECDACKEETRVI	[3060]	VICSAATNIRKM	[3383]	WLPKFDPPATLN	[3383]
	[15]	ESWATWDMDTFW	[3042]	TDRALQMFFWQI	[3383]	VEKTAFNCNVMI	[3383]
	[16]	RQAPQWKPNSDQ	[3042]	PFVRPPNIVMHV	[3383]	KKECLEHGWLTH	[3383]
	[17]	AMSDKPFFHPGW	[3042]	SIWPIPMGALHV	[3364]	WSKIDDSHQPVI	[3383]
	[18]	MWIMIRFPINSD	[3036]	ARPIMPWRTTEM	[3364]	HKCNHFKMSEVR	[3383]
	[19]	WDMCNGVNSDIM	[3024]	PFPAMVNRICFM	[3364]	FWKNPVCEHIYL	[3383]
	[20]	ESEWSVMEIGAS	[3024]	VDVKERTPRTLY	[3364]	VEAIKFEQSIWP	[3364]
	[21]	QSELPSTINPLH	[3024]	VIDQFYSTAGGI	[3186]	QKEEFCVQFNSW	[3364]
	[22]	YDQPNGPNNGDW	[3024]	VPIRSDSAFTRH	[3168]	IVVDKCNMWTCV	[3364]
	[23]	PNSKDAPIHQAI	[3024]	KWAIEDMCSKIM	[3168]	DAVCGDFHKTNV	[3364]
	[24]	CSDDRHPSSHFI	[3006]	QKVDEKNCIATL	[3168]	PTVFKDHGLKHV	[3364]
	[25]	VSDFDRIKVYKC	[2850]	HIWNQMPPIRIR	[3168]	AWFNKHIGGAVL	[3364]
	[26]	TINTETCARAAV	[2821]	HSFDERARRFLW	[3150]	YRPRNPVPGKEY	[3364]
	[27]	DSDVSKILCLFK	[2821]	NKRPKIRHGAIV	[3132]	IVKGVMHFIDPI	[3364]
	[28]	PNGDPTKLILSN	[2821]	LRVCHAPVSFMC	[3132]	VSAFKHEIDWKI	[3186]
	[29]	ESDYLPFRDIMS	[2821]	TERSFWVMACHR	[3132]	VGKMFDTCVQVS	[3186]
	[30]	EQSEFFEFYRNC	[2821]	IRQGTDRRADWR	[3132]	KDDKPRIQLATL	[3186]
	[31]	ALHSDVLQRNEE	[2821]	FDKERKRVRIHE	[3132]	EKDDNSNNTAVL	[3186]
	[32]	GHEWNTPSEIAA	[2804]	VDGNFNQLILCG	[3132]	TNNMKDYHETIR	[3186]
	[33]	WMSEEPTWGGPW	[2804]	VQNIWQIRCMAR	[3114]	CFDYKTQNEIRI	[3186]
	[34]	EQQSDDTWSRPD	[2787]	RWVRVWMANNTK	[3114]	NKEKTGHKWVYF	[3186]
	[35]	ARCSDVPSWMWL	[2787]	QVTSATAWPNQS	[3114]	AWPPVMKSFDTY	[3186]
	[36]	NINNESDTDEIC	[2787]	RSVHNSQTIRQI	[3114]	AGIVPKDILLQV	[3186]
	[37]	SSDMQYCFCKSA	[2787]	VGKMFDTCVQVS	[3114]	IKHWTDKKDNTY	[3174]
	[38]	LSDTDCDRIWTW	[2787]	AVGSSIRLQIRD	[3114]	GYDKVETPLKIL	[3168]
	[39]	PRNSSDEEGMIG	[2770]	MIETLSMWLMHM	[3114]	HWHCIVNKMDYV	[3168]
	[40]	CWLENSEMMFLV	[2753]	YFDDRVNQMHRL	[3096]	KAIKNDSDCAYL	[3168]
	[41]	YKSDHPFISDTS	[2753]	GNRLNDCTRKGI	[3096]	HEKWLKDVSIPE	[3168]
	[42]	KSDKWEKDKIYI	[2736]	PALNRDNNVFRS	[3096]	FQWSECANPIKY	[3168]
	[43]	TPSEERLMTMTG	[2684]	HHTMIRGGDVVK	[3096]	RNKGEATQCCNF	[3168]
	[44]	QMSEFCVYPCQG	[2667]	NPINGLDPRLGI	[3096]	DPKLQCCTVYAI	[3168]
	[45]	YEVECAGMLESH	[2511]	ECSSISWPVEKN	[3078]	QLSKHWNQTTVC	[3168]
	[46]	RETDVSFTTHHE	[2511]	EIKSFGAIAETV	[3078]	FDKERKRVRIHE	[3168]
	[47]	GSHTLGREAQLL	[2511]	AFGSVMAMTNFF	[3078]	PWKAPLDTIVKC	[3156]
	[48]	PSEQRCQHMNSL	[2494]	RTGDKATTIYNC	[3078]	WFKCESERRYME	[3150]
	[49]	WSETVMDYGWNG	[2494]	PWKAPLDTIVKC	[3072]	TIVEKIPDLTPY	[3150]
	[50]	EFGEASNNVEAL	[2478]	CRRIEGIHPNGR	[3060]	PNKHTVIGYLKR	[3150]

For each Mab the 50 dodecapeptides with the highest OD_{ccd} are shown. Also shown are the linear epitopes previously identified using the PEPSCAN method [12–15]. Short linear sequences that are repeatedly present in the dodecapeptides are boxed and shown in bold face. Also boxed are sequences within the linear epitopes resembling the sequences that are repeatedly present in the dodecapeptides.

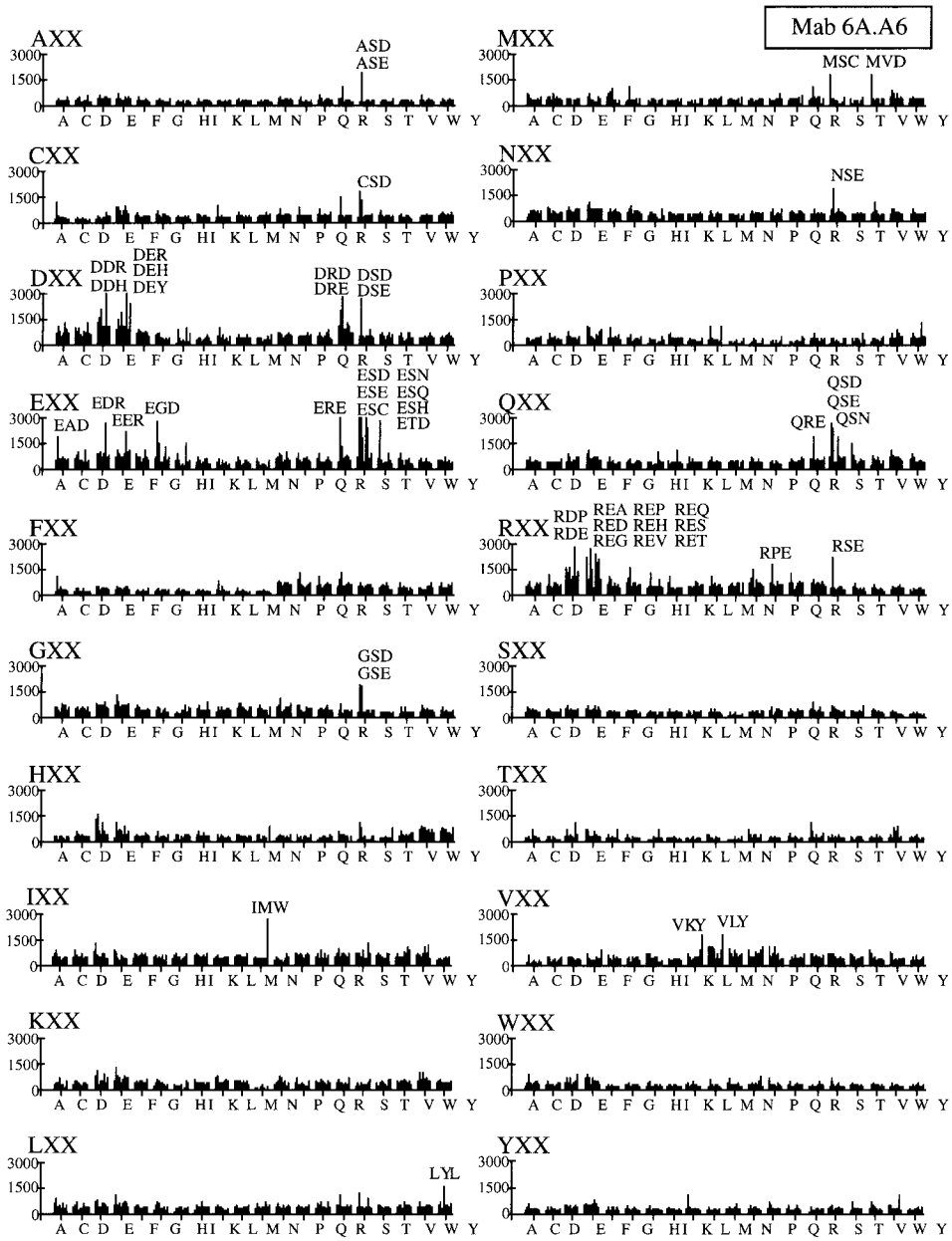

Fig. 2. PEPSCAN of Mab 6A.A6 with all 8000 tripeptide sequences. The x-axis shows 20 groups of 20 tripeptides with consensus sequence AAX (AA = one of the 400 possible dipeptide pairs; X = one of the 20 natural L-amino acids). Along the y-axis, OD_{ccd} values are given. The amino acid sequences of the 50 tripeptides with the highest OD_{ccd} values are printed.

Peptides identified using the two recombinant libraries

Screening of the two recombinant libraries with Mabs 6A.A6 and 57.9 has been described previously [16]. With Mab 6A.A6, six peptides were identified using the hexapeptide library and two identical peptides were identified using the octapeptide library. With Mab 57.9, one peptide was identified using the hexapeptide library. With Mab 32F81, three peptides were identified using the octapeptide library. The results for the three Mabs are collected in Table 2. The hexapeptide library was not screened with Mab 32F81, and the octapeptide library was not screened with Mab 57.9.

Discussion

To investigate whether small random peptide libraries can be used to identify peptides that mimic a part of the native epitope, two miniPEPSCAN libraries were screened with three different Mabs. We found that a large number of peptides could be easily identified.

The potential of small random peptide libraries was investigated further through comparison of the amino acid sequences of these peptides with those of the peptides derived from two large recombinant libraries. We found that peptides derived from the small random peptide li-

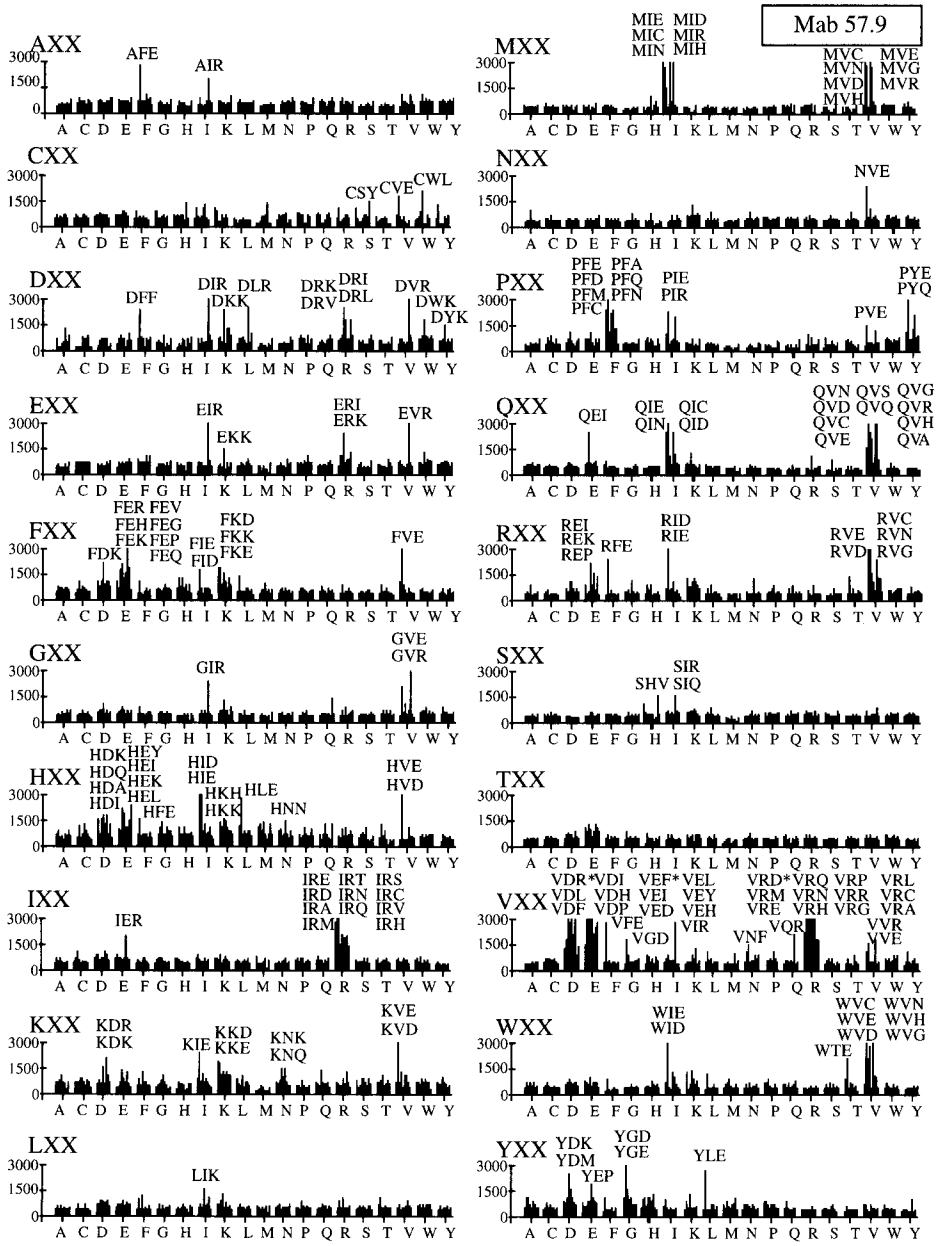

Fig. 3. PEPSCAN of Mab 57.9 with all 8000 tripeptide sequences. The x-axis shows 20 groups of 20 tripeptides with consensus sequence AAX (AA = one of the 400 possible dipeptide pairs; X = one of the 20 natural L-amino acids). Along the y-axis, OD_{ccd} values are given. The amino acid sequences of the 200 tripeptides with the highest OD_{ccd} values are printed. *: also included VDD, VDK, VDM, VDN, VEA, VEC, VEE, VEK, VEN, VEQ, VER, VEV, VEW, VRF, VRK, VRI, VRS, VRT, VRV and VRY.

braries mimic linear or nonlinear parts of the native epitope (Table 1), whereas the amino acid sequences of the peptides derived from the large random peptide libraries only mimic linear parts of the native epitope (Table 2). The results obtained with each Mab will be discussed in detail.

Mab 6A.A6

The many peptides derived from the small random peptide libraries often contain sequences that are identical to parts of the amino acid sequence PNSD, a major part of the native epitope [14]. For instance, 72% of the 50 peptides with the highest OD_{ccd} values contain the amino acid sequences SD or SE (Table 1). This suggests a prominent role for the amino acid sequence SD in the native epitope.

Interestingly, some of the peptides recognized by Mab 6A.A6 do not resemble the linear sequence of the native epitope. Examples include peptides that often contain a Glu and an Arg (Fig. 2). Two possibilities exist. Either these peptides mimic a part of the epitope or they are 'nonspecific' binders, i.e., they bind outside the antibody-combining site. For the peptides containing glutamates, aspartates and/or arginines (Fig. 2), the latter possibility seems not to be the case since these peptides, although

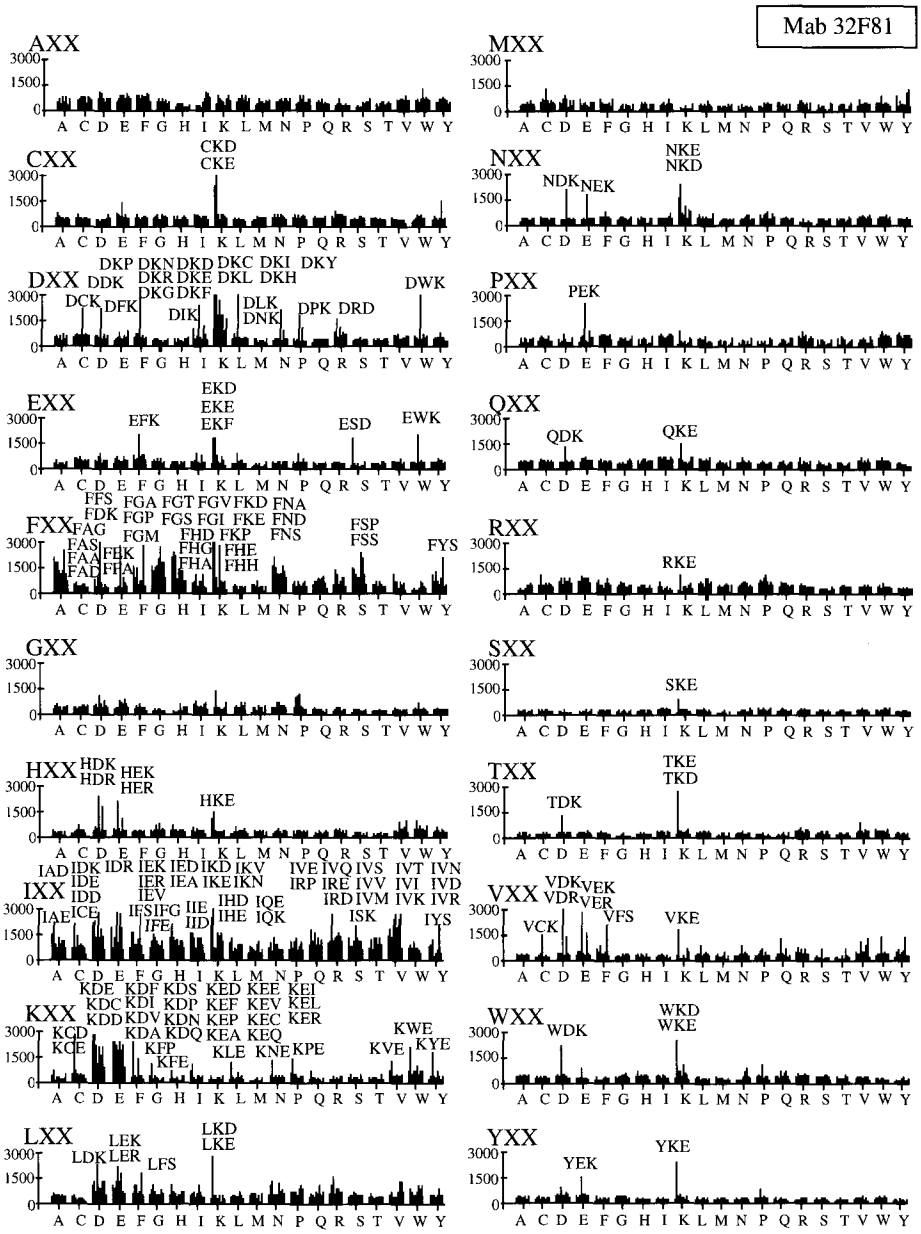

Fig. 4. PEPSCAN of Mab 32F81 with all 8000 tripeptide sequences. The x-axis shows 20 groups of 20 tripeptides with consensus sequence AAX (AA = one of the 400 possible dipeptide pairs; X = one of the 20 natural L-amino acids). Along the y-axis, OD_{ccd} values are given. The amino acid sequences of the 150 tripeptides with the highest OD_{ccd} values are printed.

this is not directly obvious, appear to mimic a nonlinear part of the epitope. The high turn propensity of PPNS in SRLPPNSDVVLG suggests that the Arg and the Asp are located spatially next to each other. Therefore, sequences such as EDR and RDP (Fig. 2) probably mimic a nonlinear region composed of the Arg, the Asp and one of the Arg residues in SRLPPNSDVVLG.

Mab 57.9

A small selection of the peptides that were derived from the small random peptide libraries contain sequences that mimic parts of SFFSYGEI, a major part of the native epitope [12,13]. Examples are the sequences PFS and PFP, which were previously shown to be allowed replacements of FFS in SFFSYGEI (Table 1) [12,13]. However, most of the peptides that bind Mab 57.9 and that were derived from the small random peptide libraries do not resemble the sequence SFFSYGEI; they contain IR and VR at a high frequency (28% of the 50 peptides with the highest OD_{ccd} values contain IR or VR; see Table 1 and Fig. 1). This suggests that IR or VR are part of the epitope.

Because previous studies have shown that the epitope recognized by Mab 57.9 is composed of SFFSYGEI and an until now unidentified region [12,13], we speculate that

TABLE 2
AMINO ACID SEQUENCES OF THE PEPTIDES IDENTIFIED USING THE LARGE DIVERSITY LIBRARIES BASED ON RECOMBINANT SYSTEMS

	No.	Mab 6A.A6	Mab 57.9	Mab 32F81
Linear epitopes	[1]	S R L P P N S D V V L G [a]	S F F S Y G E I	L D T S N P V K T
	[2]	P I C P S N S E A N C G [a]		F D D T D P I K K
Antibody-binding hexa- and octapeptides	[1]	S P N S E A [a]	P F S F G S [a]	D G S D P I R K
	[2]	P A H S E A [a]		D T S D P T L K
	[3]	P I N S E A [a]		N Y S D P V R K
	[4]	P S H S E A [a]		
	[5]	P S N S E A [a]		
	[6]	P S H S D H [a]		
	[7]	V G G V N S E A [a]		

[a] Taken from Ref. 16. Also shown are the linear epitopes previously identified using the PEPSCAN method [12–15].

antibody-binding peptides containing IR or VR mimic the 'missing' part of the native epitope [12,13].

Mab 32F81

Most of the many peptides derived from the small random peptide libraries contain the sequences DK and KD, which at first glance do not seem to resemble the sequence LDTSNPVKT, a major part of the native epitope [15] (Figs. 1 and 4). However, LDTSNPVKT probably forms a loop within an EGF-like domain. Figure 5 shows a model of this loop containing LDTSNPVKT or FDDTDPIKK (FDDTDPIKK is a mimic of LDTSNPVKT with a 2000-fold increased affinity for Mab 32F81 [17]). In the model of LDTSNPVKT, the Asp and the Lys are located spatially next to each other (Fig. 5). This suggests that the amino acids Asp and Lys in the peptides containing DK or KD mimic this spatial feature of LDTSNPVKT or FDDTDPIKK. This suggestion is supported by a replacement analysis of LDTSNPVKT, showing that Asp and Lys cannot be replaced by any other residue [17]. Furthermore, since the Leu, Asp, Thr and Ser in LDTS cannot be replaced by lysine, and Pro, Val, Lys and Thr in PVKT cannot be replaced by aspartate [15], the dipeptides DK or KD probably mimic a spatial arrangement of Asp near Lys.

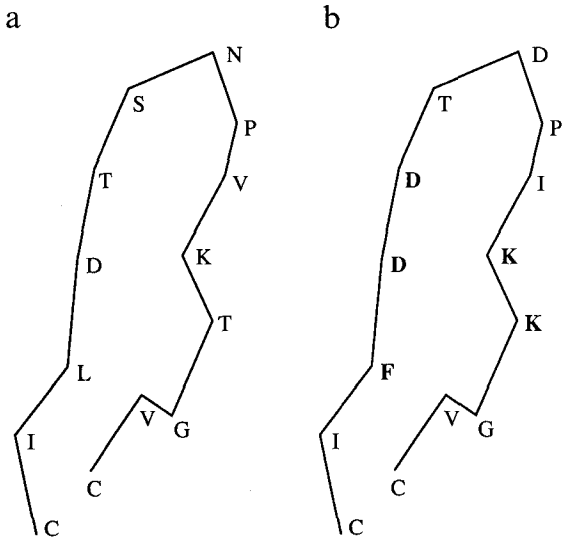

Fig. 5. Model of the three-dimensional structure of the loop containing LDTSNPVKT or FDDTDPIKK within the EGF-like domain of the surface protein pfs25 of *P. falciparum*. Residues in bold face are part of a nonlinear region that shows similarity to the peptides identified using the synthetic peptide libraries.

Finally, since many of the peptides that were found with Mab 32F81 and Mab 6A.A6 (see above) seem to mimic nonlinear regions, we speculate that small random peptide libraries can be used not only to obtain insight into the structure of linear epitopes, but in the structure of discontinuous epitopes as well.

Concluding remarks

We have compared small and large diversity libraries and we have shown that both types of libraries can be used to identify peptides that mimic parts of epitopes. However, the amino acid sequences and the number of identified peptides were clearly different. Using small libraries based on synthetic peptides, many peptides were identified that appear to mimic linear and nonlinear parts of the epitopes. Using the large libraries based on expression systems, a few peptides were identified that only appear to mimic linear parts of the epitopes.

An explanation for these differences could be the concentration of antibody that was used to screen the libraries. The recombinant libraries were screened with a 10-fold lower antibody concentration. However, when the small libraries were screened with this lower antibody concentration the overall patterns did not change, although fewer and lower peaks were obtained (not shown).

Alternatively, the difference between the small and large random peptide libraries could be explained by the difference in length of the peptides. The small libraries are composed of tri- and dodecapeptides, whereas the recombinant libraries are composed of hexa- and octapeptides. Screening of the tripeptide and dodecapeptide libraries gave similar results with respect to the large number of

identified antibody-binding peptides. This suggests that results obtained from screening synthetic miniPEPSCAN libraries composed of hexa- or octapeptide libraries would not be dramatically different from screening tri- or dodecapeptide libraries.

Finally, the difference between the miniPEPSCAN and recombinant libraries could be due to the different ways in which the peptides are presented to the Mabs. For instance, the density of peptides on the surface of the PEPSCAN support could be much higher than that of peptides on the surface of the bacteria. It has been shown that the concentration of peptide/surface area in a microtiter plate strongly affects antibody binding [20]. If the peptide concentration is low, antibodies bind only to sequences that represent a large part of the epitope. If the peptide concentration is very high, antibodies can bind to sequences that represent a relatively small part of the epitope. Thus, a relatively high concentration of peptides synthesized on PEPSCAN support and a relatively low concentration of peptides expressed on the wall of bacteria could also explain why much more peptides were derived from the miniPEPSCAN libraries in comparison to the recombinant libraries.

A question that remains is how useful are short consensus sequences such as SD, found with Mab 6A.A6, to understand antibody–antigen interactions. Do these sequences contribute significantly to the total binding energy? For various antigen–Mab interactions it has been shown that within an epitope a 'hot spot' of only two to five residues contributes significantly to the total binding energy [11,21]. Functional hot spots have also been found in hormone–receptor binding interfaces. Recently, it has been shown that a hot spot containing a relatively small set of approximately eight residues dominates binding between growth hormone and its receptor [22,23]. Thus, dipeptide sequences such as SD, found with Mab 6A.A6, IR, found with Mab 57.9 and DK, found with Mab 32F-81, may constitute such functional hot spots.

In conclusion, our approach using 455-well credit-card format mini-PEPSCAN cards, which contain small diversity libraries based on synthetic peptides and which can be re-used many times, appears to form a useful tool to study structural aspects of antibody–antigen interactions.

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