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A Novel Microtiter Plate Based Method for Identification of B-cell Epitopes

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Abstract: A new type of microtiter plate capable of binding biomolecules covalently in a one step procedure was used to map linear B-cell epitopes in two different proteins using a peptide-based solid phase immunoassay. The method was compared with a conventional immobilization method using passive adsorption to microtiter plates. An array of 15-mer peptides, overlapping by five amino acids, representing the entire sequences of ubiquitin and murine tumor necrosis factor-α, respectively, was synthesized. The peptides were immobilized covalently using the new, specialized microtiter plates or non-covalently using conventional ELISA microtiter plates of the high binder type. Subsequently, specific antisera to ubiquitin or murine tumor necrosis factor-α were added to identify potential linear B-cell epitopes. All peptides, which were recognized on the conventional microtiter plates, were also recognized on the plates with the covalently bound peptides. In addition, the covalent immobilization method revealed epitopes that were not identified using the method for non-covalent binding although the peptides were in fact present on the non-covalent binding surface. The interaction with the hydrophobic surface of the conventional microtiter plate apparently interfered negatively with antibody recognition. The covalently binding microtiter plates described here could be useful for identification of new B-cell epitopes in protein antigens. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide ELISA; covalent binding; epitope scan; AquaBind; microtiter plate

INTRODUCTION

Information about the fine specificity of an antiserum towards a protein antigen is useful when establishing a peptide-based enzyme linked immunosorbent assay (ELISA) [1,2]. Cross-reactivity between related microorganisms can be avoided or minimized if specific peptide epitopes are identified and used in the solid phase assay instead of the entire protein antigen [3]. The epitope scan is furthermore a useful tool for identification of the specificity of a monoclonal antibody [4,5], e.g. to predict whether an antibody will be able to neutralize a biologically active protein [6,7].

Epitopes recognized by antibodies are either linear or non-linear, also known as discontinuous or conformational epitopes. Non-linear epitopes are composed of a combination of several parts of the protein. In practice, only linear epitopes are identified by the use of so-called epitope scans. One way to perform a linear epitope scan is to synthesize and immobilize overlapping peptides derived from the antigen and subsequently detect which peptides are recognized by the antibodies. This strategy requires a solid phase, e.g. a microtiter plate, to which peptides can be immobilized in a way that gives minimal interference with the subsequent antibody recognition.

Non-covalent immobilization of peptides involves multiple hydrophobic as well as hydrophilic interactions between the peptide molecule and the micro-

Abbreviations: mTNFα, murine tumor necrosis factor-α; PLL, poly L-lysine; aa, amino acid; BSA, bovine serum albumin; NHS, *N*-hydroxy succinimide; RT, room temperature.

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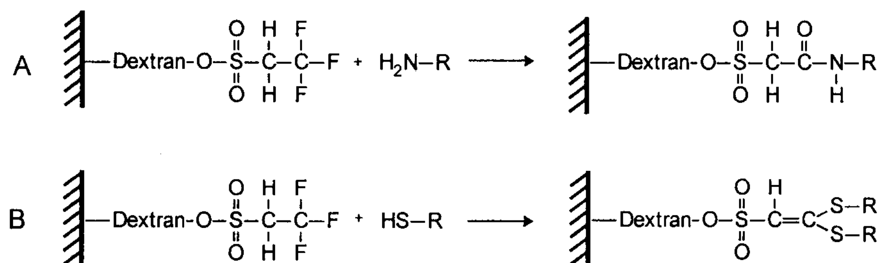

Figure 1 Reaction scheme showing the reaction between two different nucleophilic groups and the tresyl groups of the dextran tresylate on the surface of the AquaBind microtitre plate. If the nucleophile is an amino group (A), the result is an amide bond. The amino group can be the α-amino group from the N-terminus or it can be an ε-amino group in a lysine residue from the biomolecule R. When the nucleophilic group is a thiol group (B), e.g. from a cysteine residue in the biomolecule R, the major product is a dithioacetal [17].

titer plate surface, and such interactions could in theory negatively affect antibody recognition of the epitope [8]. In contrast, covalent immobilization involves in principle only one or few atoms of the peptide molecule. Hence, the ability of an antibody to recognize the epitope should be improved when the peptide is covalently immobilized.

Several methods for epitope scanning using covalently immobilized peptides have been described. In one set-up, overlapping peptides were synthesized on polyethylene pins and used directly in an assay while still attached to the pins [9–11]. This method has two practical problems, (1) a new peptide synthesis usually has to be performed for each scanning experiment, and (2) proper analysis of the peptides is not possible. In another approach, purified overlapping peptides were coupled to a poly-L lysine (PLL) treated microtiter plate using glutaraldehyde as cross-linking agent [12]. The PLL–glutaraldehyde binds the peptides via amino groups, but the reaction is reversible and the resulting imine or Schiffs base must be reduced, e.g. by sodium borohydride, to a stable secondary amine to ensure that the peptide does not detach from the surface. Furthermore, the PLL coating generates a positively charged surface that may lead to problems with non-specific binding.

Other methods for covalent binding of biomolecules in general have been published, either based on a poly-phenylalanine–lysine copolymer coated surface [13] or by the introduction of amino groups by chemical modification of the polystyrene [14,15]. None of these methods, however, work as a one step procedure and they all require some kind of pre-activation using cross-linking agents.

In this paper, the authors present the results of peptide epitope scans on ubiquitin and murine tumor necrosis factor-α (mTNFα), respectively, using a method for direct covalent binding of peptides on microtiter plates modified with activated dextran molecules (AquaBind® microtiter plates). The AquaBind microtiter plate is capable of binding biomolecules, via for example an *N*-terminus α-amino group, an ε-amino group in a lysine residue or a thiol group in a cysteine residue. In contrast to what was believed for many years [16], the reaction between a tresylate and an amine leads to the formation of an amide [17]. Reaction with a thiol leads to the formation of a dithioacetal [17]. The chemical reactions are shown in Figure 1.

The authors compare this method with a conventional method in which the peptides were passively (non-covalently) adsorbed to the solid phase. The results of the epitope scan on mTNFα largely corresponded to a previously published epitope scan on human TNFα [10]. A full epitope scan of the mTNFα using overlapping peptides has not been published before. All the epitopes found using the conventional microtiter plates were also found using the AquaBind plates, but importantly, additional epitopes were identified using the AquaBind microtiter plates.

MATERIALS AND METHODS

If nothing else is noted, chemicals were of analytical quality from Ridel-de Häen, Seelze, Germany.

Peptide Synthesis

Overlapping peptides from ubiquitin and mTNFα were synthesized as 15-mer peptides with a five amino acid (aa) overlap on a Crystal Novasyn automatic peptide synthesizer (Novasyn, Nottingham, UK) using the Fmoc strategy. However, two ubiqui-

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tin peptides had a nine aa overlap and one mTNFα peptide was a 16-mer. The peptide sequences are shown in Table 1. The synthesis was performed on a cleavable Tentagel resin (Rapp Polymere, Tübingen, Germany). After cleavage from the resin and deprotection according to the protocol provided by the manufacturer (Novasyn), the peptides were freeze dried from 10% acetic acid and purified (>99% pure) by preparative reversed phase HPLC (Merck, Darmstadt, Germany) using a LiChrosorb RP18 (7 μm, 25 × 250 mm) column (Merck, Cat. No. 51494). The elution gradient of buffer B (90% acetonitrile, 9.9% H_2O and 0.1% trifluoroacetic acid) in buffer A (99.9% H_2O and 0.1% trifluoroacetic acid) was individual for the individual peptide in order to obtain the optimal separation of the actual peptide from impurities, such as truncated peptides. All peptides were subsequently control sequenced on an Applied Biosystems Sequencer 476A (Applied Biosystems, Foster City, CA). Furthermore, all peptides except ubiquitin 1–15, ubiquitin 62–76 and mTNFα 91–105 were characterized by analytical HPLC on a LiChrosorb RP 18 column (5 μm, 4 × 125 mm) using a linear elution gradient over 30 min ranging from 0 to 100% of buffer B (90% acetonitrile, 9.9% H_2O and 0.1% trifluoroacetic acid) in buffer A (99.9% H_2O and 0.1% trifluoroacetic acid). The retention time data is shown in Table 1. Biotinylation of the peptide MP9 (FAQKEPAFLKEYHLL) was performed by coupling biotin as the last step in the synthesis using biotin-*N*-hydroxy-succinimide (biotin-NHS, Sigma H-1759, Sigma, St. Louis, MO).

Table 1 Sequences of Ubiquitin and mTNFα Peptides

aa Number	Sequence	Retention time[a] (min)
Ubiquitin peptides		
1–15	MQIFVKTLTGKTITL	nd[b]
7–21	TLTGKTITLEVEPSD	12.75
17–31	VEPSDTIENVKAKIQ	12.12
27–41	KAKIQDKEGIPPDQQ	10.10
37–51	PPDQQRLIFAGKQLE	13.36
47–61	GKQLEDGRTLSDYNI	12.25
57–71	SDYNIQKESTLHLVL	14.95
62–76	QKESTLHLVLRLRGG	nd
mTNFα peptides		
1–15	LRSSSQNSSDKPVAH	8.39
11–25	KPVAHVVANHQVEEQ	9.76
21–35	QVEEQLEWLSQRANA	13.93
31–45	QRANALLANGMDLKD	12.87
41–55	MDLKDNQLVVPADGL	13.93
51–65	PADGLYLVYSQVLFK	17.17
61–75	QVLFKGQGCPDYVLL	15.60
71–85	DYVLLTHTVSRFAIS	15.01
81–95	RFAISYQEKVNLLSA	14.24
91–105	NLLSAVKSPCPKDTP	nd
101–115	PKDTPEGAELKPWYE	12.89
111–125	KPWYEPIYLGGVFQL	17.71
121–135	GVFQLEKGDQLSAEV	13.68
131–145	LSAEVNLPKYLDFAE	15.31
141–156	LDFAESGQVYFGVIAL	17.53

[a] The retention time data is for analytical HPLC on a LiChrosorb RP18 column (5 μm, 4 × 125 mm) using a linear elution gradient over 30 min ranging from 0 to 100% of buffer B (90% acetonitrile, 9.9% H_2O and 0.1% trifluoroacetic acid) in buffer A (99.9% H_2O and 0.1% trifluoroacetic acid).
[b] Not determined.

Immobilization of Peptides and Performance of the Epitope Scan

AquaBind microtiter plates, containing tresyl-activated dextran molecules produced as previously described [18], were manufactured at M&E Biotech's production facilities. For non-covalent coupling experiments Maxisorp microtiter plates (NUNC, Roskilde, Denmark) were used. For immobilization on AquaBind as well as on Maxisorp plates, the peptides were dissolved in carbonate buffer (0.070 M $NaHCO_3$, 0.030 M Na_2CO_3, pH 9.6), diluted to 0.05 mg/ml and incubated for 2 h at room temperature (RT). After incubation, the wells were washed three times with washing buffer (0.001 M KH_2PO_4, 0.008 M Na_2HPO_4, 0.003 M KCl, 0.5 M NaCl, 1% (v/v) Triton X-100 (Sigma X-100), pH 7.2). The AquaBind microtiter plates were blocked at 37°C overnight with AquaBind blocking buffer (carbonate buffer including 1% (w/v) bovine serum albumin (BSA, Sigma A-2153), 0.01 M ethanolamine and 15% (w/v) polyethylene glycol 8000 (Sigma P-2139)). Maxisorp microtiter plates were blocked overnight at RT with 1% (w/v) BSA in carbonate buffer. After blocking, the wells were washed three times with washing buffer and serum was added in 1:500 dilution in AquaBind dilution buffer (washing buffer including 1% (w/v) BSA, 0.0005% (w/v) phenol red, pH 7.2), respectively. After 1 h of incubation at RT, the wells were washed with washing buffer and horseradish peroxidase (HRP) labeled rabbit-anti-mouse Ig (DAKO, Ejby, Denmark) was added diluted 1:1000 in dilution buffer. After 1 h of incubation at RT, the wells were washed with washing buffer and developed using *o*-phenylenediamine dihydrochloride

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(Sigma P-8287) as the chromogenic substrate, which was dissolved (1 mg/ml) in citrate–phosphate buffer (0.035 M citric acid, 0.075 M Na_2HPO_4, pH 5.0) and H_2O_2 (0.03%) was added. The reaction was stopped using H_2SO_4 (1 M, 100 μl/well) and the wells were read in a Dynatech MR5000 ELISA reader at 490 nm.

Detection of the Presence of Peptides on the Surface

Binding of the individual peptides to the surface was detected differently on the two types of microtiter plates. On the AquaBind microtiter plates, peptide binding was determined indirectly by measuring the number of residual binding sites after incubation with the individual peptides. From previous experiments, the authors knew that the peptide biotin-MP9 bound with high efficiency to the AquaBind microtiter plate (data not shown) and hence biotin-MP9 was used for detection of residual binding sites on the AquaBind surface. After 30 min pre-incubation at RT with the individual peptides (0.05 mg/ml in carbonate buffer), or with carbonate buffer alone, the wells were washed with water and biotin-MP9 was added (0.01 mg/ml in carbonate buffer). After 30 min incubation at RT, the wells were washed with washing buffer, and streptavidin-HRP (Amersham RPN1231, Amersham, Buckinghamshire, UK) in dilution buffer (1:1000) was added. After 1 h incubation at RT, the wells were washed with washing buffer and developed as described above. The binding % was calculated using the following formula:

$$\text{binding }\% = ((\text{OD-buffer} - \text{OD-peptide})/\text{OD-buffer}) * 100,$$

where OD-buffer is the OD_{490} value from wells with no peptide added in the pre-incubation step and OD-peptide is the OD_{490} value from wells with peptide added in the pre-incubation step.

On the Maxisorp microtiter plates a direct method for detection of the binding was used [8]: After incubation with the individual peptides (0.05 mg/ml in carbonate buffer, 2 h at RT), or with BSA (0.01 mg/ml in carbonate buffer) as a positive control (data not shown), the wells were washed with water and biotin-NHS (0.05 mg/ml in PBS, including 0.05% (v/v) Tween-20 (Sigma P-7949)), was added. After incubation (2 h at RT), the wells were washed with washing buffer, and streptavidin-HRP in dilution buffer, and developed as described above. In this assay, the OD 490 value is an indication of the number of available nucleophilic groups, primarily amino groups, on the surface and hence the amount of peptide.

Generation of Antisera

Antibodies were raised in mice by immunization with modified recombinant versions of mTNFα and ubiquitin, respectively. As described elsewhere [19], the recombinant molecules contained non-self T-cell epitopes, which upon immunization, induced cross-reactive autoantibodies against the non-modified self-proteins. In the recombinant ubiquitin, aa residues 21–32 of ubiquitin were exchanged with aa residues 325–336 of ovalbumin (QAVHAAHAEINE). In the recombinant mTNFα molecule, aa residues 124–138 were exchanged with aa residues 81–95 of hen egg lysozyme (SALLSSDITASVNCA). Consequently, antibodies directed against the sequences ubiquitin 21–32 and mTNFα 124–138 are not expected since these sequences are absent in the molecules used for immunization.

RESULTS

Peptide Epitope Mapping and Detection of Immobilization Efficiency

Figure 2 shows the recognition of the overlapping ubiquitin peptides by an anti-ubiquitin antiserum on the AquaBind (Figure 2A) and Maxisorp (Figure 2B) microtiter plates, respectively, and the detection of immobilization efficiency of the ubiquitin peptides on the AquaBind (Figure 2C) and Maxisorp (Figure 2D) microtiter plates, respectively. Two peptides (ubiquitin 27–41 and ubiquitin 37–51) gave very strong signals on the AquaBind plate, and two others (ubiquitin 1–15 and ubiquitin 62–76) gave weak signals, although significantly above background. On the Maxisorp plate, only one peptide (ubiquitin 27–41) gave a signal only one third of the strongest signal on AquaBind.

All the ubiquitin peptides bound to the AquaBind surface with binding % values between 28 and 75% (Figure 2C). Five of the eight ubiquitin peptides gave binding % values above 50%. Doubling the coating concentration did not result in higher values (data not shown). Although the number of nucleophilic groups (ε-amino group in lysine residues) varies between the peptides, there was no direct correlation between the number of lysine residues in the individual peptides and the immobilization efficiency. The ubiquitin sequence does not contain cysteine residues. The presence of ubiquitin pep-

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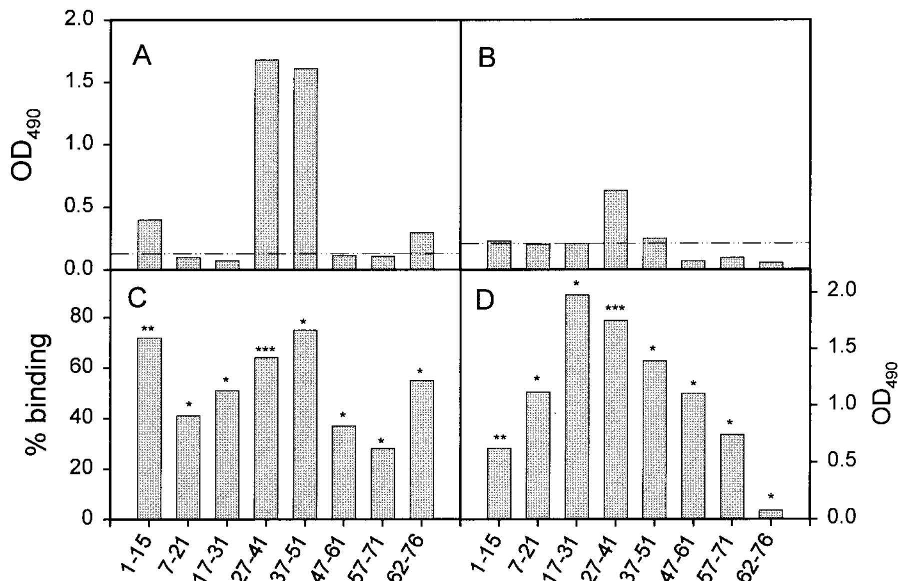

Figure 2 Epitope scan of ubiquitin performed on AquaBind (A) and Maxisorp (B) and the binding efficiency of the ubiquitin peptides on AquaBind (C) and Maxisorp (D). The numbers on the x-axis refer to the aa positions in ubiquitin from which the peptide is derived. The dashed/dotted line in A and B is the OD_{490} value obtained when serum was added to a peptide free well. An * in C and D indicates the presence of a lysine residue in the peptide.

tides, or more precisely, the presence of available amino groups on the Maxisorp plate after coating with the individual peptides (Figure 2D) varied significantly from peptide to peptide and did not correspond to the number of lysine residues in the peptides. One peptide (ubiquitin 62–76) gave no signal in the detection assay on the Maxisorp plate, although this peptide has two amino groups (ε-amine in one lysine as well as the N-terminus α-amino group) potentially available for reaction with the biotin-NHS. Apparently, this peptide did not bind to the Maxisorp plate. Increasing the peptide concentration did not change the result (data not shown).

Figure 3 shows the results from similar experiments with peptides derived from mTNFα peptides and anti-mTNFα antiserum. The recognition of the overlapping mTNFα peptides by anti-mTNFα antiserum on AquaBind (Figure 3A) and Maxisorp (Figure 3B) microtiter plates, respectively, and the mTNFα peptide immobilization pattern on the AquaBind (Figure 3C) and Maxisorp (Figure 3D) microtiter plates, respectively. A number of peptides in the N-terminus part, and especially the peptides mTNFα 21–35 and mTNFα 41–55, gave strong signals on the AquaBind plate (Figure 3A), while peptides derived form this part of the mTNFα molecule were not recognized on the Maxisorp plate at all (Figure 3B). Four peptides from mTNFα (91–105, 101–115, 111–125 and 131–145) were recognized both on AquaBind and Maxisorp plates. On both types of plates mTNFα 101–115 gave the strongest signal. All peptide epitopes identified on the Maxisorp microtiter plates were also identified on the AquaBind microtiter plates but generally with a stronger signal.

All the mTNFα peptides bound to the AquaBind microtiter plate. Four peptides (mTNFα 21–35, 41–55, 71–85 and 81–95) gave low ($<40\%$) binding % (Figure 3C) but mTNFα 21–35 anyway gave a very good response in the epitope mapping assay (Figure 3A). Doubling the peptide concentration did not give higher binding % (data not shown). All the mTNFα peptides also bound to the Maxisorp plate, although mTNFα 51–65 and mTNFα 71–85 gave weak signals (Figure 3D).

In both sets of peptide binding detection experiments on the Maxisorp microtiter plates, the BSA control gave an OD_{490} value of 0.13 (data not shown).

DISCUSSION

Identification of B-cell epitopes in protein antigens generates valuable information, e.g. about the fine

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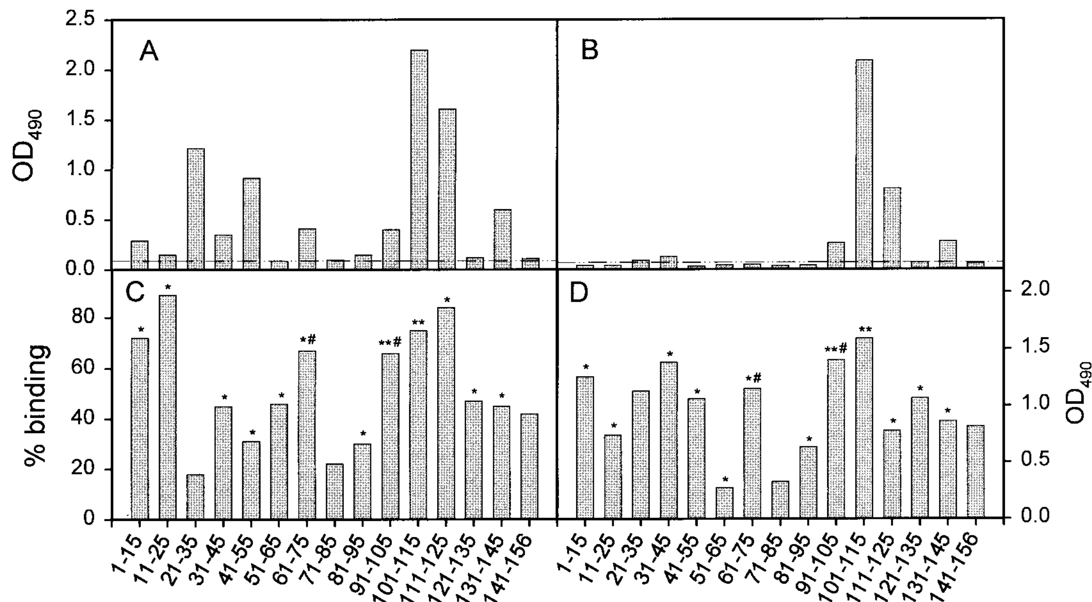

Figure 3 Epitope scan of mTNFα performed on AquaBind (A) and Maxisorp (B) and the binding efficiency of the mTNFα peptides on AquaBind (C) and Maxisorp (D). The numbers on the x-axis refer to the aa positions in mTNFα from which the peptide is derived. The dashed/dotted line in A and B is the OD_{490} value obtained when serum was added to a peptide free well. An * in C and D indicates the presence of a lysine residue(s) in the peptide. A # indicates the presence of a cysteine residue in the peptide.

specificity of antibodies and it can be useful in the prediction of the neutralizing effects of, for example, a monoclonal antibody [6,7]. Furthermore, the identification of B-cell epitopes may give information about the three-dimensional structure of the native protein from which the peptides are derived, e.g. about those parts of the native protein antigen that are exposed on the surface of the molecule [20]. Finally, the identified peptide epitopes can be used diagnostically in peptide-based solid phase immunoassays [1].

It was shown previously that the covalent immobilization method is generally superior to the conventional, non-covalent adsorption method with regard to recognition of peptides by antibodies [18]. In this paper, the authors demonstrate that the use of the covalent immobilization method in a peptide-based epitope scan gives a more complete picture of the distribution of linear epitopes in a protein antigen. Furthermore, they show that a peptide, which is non-covalently adsorbed on a microtiter plate, can be completely shielded for antibody recognition.

Epitope mapping of ubiquitin and mTNFα using the covalently immobilized peptides (Figure 2A and Figure 3A) on the AquaBind microtiter plates was superior to the conventional non-covalent binding technology represented by the Maxisorp microtiter plates (Figure 2B and Figure 3B). Ubiquitin 27–41 was recognized both on AquaBind and Maxisorp (Figure 2A and B). However, on the Maxisorp microtiter plate, the signal was only about one third of the signal on the AquaBind microtiter plate. Ubiquitin 37–51 was only recognized on the AquaBind plate, although this peptide indeed was present on the Maxisorp microtiter plate as well (Figure 2D). Apparently, this peptide bound to the Maxisorp surface in a way that interfered with the subsequent recognition by the antibody. Ubiquitin 1–15 and ubiquitin 62–76 gave weak signals, but significantly above background on the AquaBind plate in the epitope scan (Figure 2A). However, these peptides gave strong and medium signals, respectively, in the binding detection assay (Figure 2C) and probably low affinity antibodies were present against epitopes included in these peptides. As expected, no antibodies were found against peptides containing the ubiquitin sequence 21–32 that was substituted by ovalbumin 325–336 in the recombinant ubiquitin molecule used for immunization. Recognition of the peptide ubiquitin 27–41 must be due to epitopes located within aa residues 33–41. These observations correspond to previous findings [21].

Four TNFα peptides (91–105, 101–115, 111–125 and 131–145) were recognized both on Maxisorp

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and on AquaBind microtiter plates (Figure 3A and B) but in addition several epitopes, e.g. in peptides such as mTNFα 21–35 and mTNFα 41–55, were identified only on the AquaBind microtiter plate and with strong signals. Furthermore, mTNFα 31–45 and mTNFα 61–75 were also recognized on the AquaBind microtiter plate but with weaker signals.

An epitope scan on human TNFα (hTNFα) using peptides synthesized with an uncleavable linker on polyethylene pins has been published previously [10]. Epitopes identified by Yone *et al.* [10] in the hTNFα are located in regions of the hTNFα molecule homologous to regions in the mTNFα, where epitopes were identified. Furthermore, by the use of AquaBind plates, the authors identified epitopes in mTNFα 61–75 and in mTNFα 111–125 that were not identified by Yone *et al.* Monoclonal antibodies that can neutralize the cytotoxicity of hTNFα have been mapped to aa residues 63–71 and 111–127 in hTNFα by enzyme digestion protection experiments [20]. Apparently, these regions must to some extent be exposed on the surface of the hTNFα molecule and hence potentially contain B-cell epitopes. Either the antiserum used here has a broader specificity than the antiserum used by Yone *et al.* or the peptides synthesized by Yone *et al.* derived from these regions (aa 63–71 and 111–127) could not be recognized. The technology used by Yone *et al.* does not allow proper analysis of the peptides in the epitope scan.

Peptides 1–15, 21–35, 31–45, 41–55 and 61–75 from mTNFα all bound well to the Maxisorp microtiter plate (Figure 3D) but the epitopes represented by these peptides that were identified on the AquaBind microtiter plate, were not recognized on the Maxisorp surface. This result clearly shows that the covalent immobilization technology gives a more complete picture of the distribution of B-cell epitopes and it furthermore emphasizes the problems that arise when small molecules are passively adsorbed for use in ELISA. The recombinant mTNFα used for immunization had mTNFα aa residues 124–138 exchanged with hen egg lysozyme aa 81–95 and as expected, no reactivity against this region (peptide mTNFα 121–135, Figure 3A) was seen. Hence, the reactivity observed against mTNFα 131–145 must be directed against an epitope in the C-terminus part of this peptide.

The covalent immobilization of peptides to the AquaBind microtiter plate takes place via the α-amino group in the N-terminus, via an ε-amino group in a lysine residue or via a thiol group in a cysteine residue (Figure 1). The binding % of each peptide to the AquaBind microtiter plate shown in Figure 2C and Figure 3C does not reflect the number of reactive side chains (lysine and cysteine) in the individual peptides. For example, the peptides mTNFα 11–25 and mTNFα 81–95 in Figure 3C both have one lysine residue but the binding % of mTNFα 11–25 is about three times that of mTNFα 81–95. Hence, the amino acid composition, apart from lysine and cysteine residues, also seems to influence the binding ability of the peptide to the AquaBind microtiter plate. The ε-amino group from a lysine residue can probably be more or less available for reaction, depending on the structure of the peptide. The peptides ubiquitin 27–41, mTNFα 11–25 and mTNFα 111–125 all have a lysine residue in the N-terminus and these three peptides all show high binding % (Figure 2C and Figure 3C). If this is a general rule, all peptides should be synthesized with a lysine residue in the N-terminus to increase the immobilization efficiency. Peptides that lack both lysine and cysteine residues bind to the AquaBind microtiter plate via the N-terminus α-amino group and there is a tendency that such peptides (mTNFα 21–35, mTNFα 71–85 and mTNFα 141–155) do not react as readily to the reactive sites on AquaBind microtiter plate than peptides with, e.g. one lysine residue. However, peptide mTNFα 21–35 neither contains lysine nor cysteine residues and it showed low binding % ($<20\%$), but was still readily recognized by the antiserum (Figure 3A). The authors conclude that peptide mTNFα 21–35 contains a very strong epitope, maybe even stronger than the epitope in peptide mTNFα 101–115, although mTNFα 101–115 gave a stronger signal in the epitope scan. This strong epitope in mTNFα 21–35 would not have been identified if the epitope scan had been performed on the Maxisorp microtiter plate only.

When a peptide is immobilized only via the α-amino group in the N-terminus, the major part of the molecule will be available for recognition by an antibody. In contrast, if the peptide is immobilized via one or several internal side chains, it is likely that recognition is sterically hindered. On the AquaBind microtiter plate, the presence of internal lysine and cysteine residues in a peptide could therefore interfere with antibody recognition, especially if a lysine residue is part of the epitope. Synthesizing, purifying and immobilizing the peptide with intact side-chain protection on lysine and cysteine residues may solve this problem. The covalent bond between a peptide and the AquaBind surface is resistant to treatment with 95% trifluo-

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roacetic acid, 6 M HCl, 1 M NaOH, 50% dimethyl sulfoxid, 50% dimethylformamide, etc. (data not shown). Consequently, deprotection of lysine and cysteine residues could be performed in the well after the partially protected peptide has been immobilized via the N-terminus α-amino group. Studies are underway to demonstrate this.

Previously published results [18] and the results presented here document that when peptides are covalently immobilized, recognition of the peptides by antibodies is improved. On the AquaBind microtiter plate, peptides are covalently immobilized in a one step procedure and this makes the AquaBind microtiter plate very useful as a screening tool and in the development of peptide based diagnostics kits.

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