

Molecular mimicry of the unidentified antigen of myeloma antibody IgE-ND

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Antigen-specific human IgE is in short supply. Thus, we sought to determine the yet unknown specificity of a widely available human IgE, namely the myeloma cell line U266-derived IgE-ND. For this purpose highly specific peptides able to mimic the putative antigen recognized by IgE-ND were isolated from phage-display random peptide libraries. Interestingly, we found linear sequence homologies of the IgE-ND-binding peptides with self antigens and a xenoantigen from *Thiobacillus ferrooxidans*. However, none of these antigens was recognized by IgE-ND. Nevertheless, our approach may be applied to identify antigen specificities of myeloma antibodies. Importantly, the mimotopes were anaphylactogenic in a histamine release assay using human basophils sensitized with IgE-ND. Thus, our mimotopes represent functional albeit synthetic antigens and may be used to study human antigen-specific IgE responses.

Key words: Human IgE / Phage display / Mimotope / Random peptide

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1 Introduction

Since the first description of IgE [1, 2] its structure, function and *de novo* synthesis have been investigated in numerous studies and its central role in the pathogenesis of allergic diseases is undisputed [3]. In the past many experimental approaches were prevented by the fact that the only source of antigen-specific IgE was the limited amount available from human serum. In mice the synthesis and regulation of antigen-specific IgE are well documented [4–6], and murine systems for *in vitro* production of antigen-specific IgE are available [7]. In humans different approaches were undertaken to detect and isolate mature IgE-producing B cells [8] and to establish culture systems for the induction of antigen-specific human IgE *in vitro* [9]. The regulation of specific IgE synthesis *in vitro* by cytokines, allergens and Th2 cells has been investigated by numerous groups [10–12] and even a system for *in vitro* immunization of human B cells has been described [13]. Despite these intensive efforts, the supply of human IgE for use in basic research and for standardization purposes is still very limited, due to the difficulties in isolating and immortalizing mature B cells.

Different chimeric or myeloma-derived B cell lines are currently available for *in vitro* production of IgE, but only IgE-JW8 produced by a chimeric (mouse Fab fused to human Fc ϵ) cell line [14] has a known antigen specificity and, moreover, the “antigen” recognized by IgE-JW8 is a hapten (NIP) coupled to BSA. An alternative approach allowing definition of antigen specificity is that of random peptide phage display. Random peptide libraries expressed on filamentous phages have proven to be an efficient method for the determination of antibody epitopes and characterization of receptor ligands [15]. It has been shown that peptides displayed in a linear form or – flanked by two cysteine residues – as circular peptides are able to mimic the conformational structure of both continuous and discontinuous epitopes [16]. So far, however, it has never been attempted to use this technology for the identification of a functional peptide antigen for an antibody of unknown specificity. Additionally the isolation of a specifically binding peptide might allow the identification, via its primary amino acid sequence, of the “original” specificity of the myeloma protein. Different studies analyzing VH and VL genes of myelomas demonstrated frequent somatic hypermutation of variable genes of myeloma antibodies (reviewed in [17]), indicating that antigen selection of these B cell clones had occurred. Therefore, it would be interesting to investigate whether antigen specificity of myeloma antibodies is causatively related to the etiology and the development of disease. To date, however, no reports indicating the

[1 19563]

Abbreviations: HRP: Horseradish peroxidase ORF: Open reading frame

nature of the antigens recognized by myelomas are available.

In our study we intended to define the specificity of human IgE-ND, produced by the B cell line U266 derived from a myeloma patient [18–21]. For this purpose a circular random peptide library [22] and four linear libraries [23–25] were screened against IgE-ND. After four rounds of panning specific clones were isolated and sequenced. The binding specificity of the isolated peptides was shown in an inhibition assay using an anti-idiotypic Fab against IgE-ND. Binding of the ND-specific phage clones to receptor-bound IgE was demonstrated by FCM analysis. Importantly, the phage clones showed anaphylactogenic activity in a histamine release assay performed with IgE-ND-sensitized human basophils, demonstrating that our approach has led to the isolation of mimotopes capable of functionally mimicking the antigen recognized by IgE-ND.

2 Results

2.1 Selection of IgE-specific mimotopes from random epitope libraries

In order to define the specificity of a human (myeloma) IgE termed IgE-ND [18] we screened various random epitope libraries displaying peptides between 6 and 15 amino acids in length as summarized in Table 1. The circular and the pool of the four linear libraries (Table 1) were enriched for phage-displayed peptides recognized by antibody IgE-ND in four rounds of panning. An enrichment of specific phages by a factor of 420 (IgE-ND, linear), or 700 (IgE-ND, circular), respectively, was

Table 1. Libraries used for panning

Library type	Name	Size of insert (aa) ^{a)}	Reference
Circular ^{b)}	Cx7C	7	[22]
Linear ^{c)}	6mer Amp EP Lib	6	[23]
Linear ^{c)}	fAFF 6mer Lib	6	[25]
Linear ^{c)}	am3-4	10	[24]
Linear ^{c)}	15mer Amp EP Lib	15	[23]

a) aa = number of amino acids in the random peptides.

b) This library is further denominated “circular”.

c) The indicated four linear libraries were pooled at equal ratios before use for panning and termed “linear” for further description.

Table 2. Sequence analysis of IgE-specific peptide phage clones

Libraries ^{a)}	Panning Ab	Sequence ^{b)}	Fre-quency	Name ^{c)}
Circular	IgE-ND	C C W L R M P C C	3/3	PhND-C
Linear	IgE-ND	L S P H L L W D L F R V G L P	3/3	PhND-L

a) Libraries as defined in Table 1.

b) Amino acid peptide sequence in single letter code.

c) Designation of phage clones for further use.

observed. After the fourth panning, 96 randomly picked clones were grown in a micro-culture system and the phages analyzed by ELISA for their ability to bind IgE-ND. The percentage of positive phage clones binding to IgE-ND was 79.1 % and 69.7 % for the circular and linear libraries, respectively. As shown in Table 2, sequence analysis was performed on three arbitrarily selected clones after the fourth round of panning for each of the panning procedures. Each selection procedure resulted in only one consensus sequence.

2.2 Inhibition/competition assays for ND-specific peptide phage clones

To verify that the two IgE-ND specific phage clones, PhND-C and PhND-L, bound specifically to the antigen binding site of IgE-ND, an inhibition assay using anti-idiotypic Fab against IgE-ND (anti-ND-Fab; [26]) was performed. As shown in Fig. 1, binding of IgE-ND to anti-ND-Fab was inhibited by PhND-C (Fig. 1A) or PhND-L (Fig. 1B) in a dose-dependent manner to background levels. In contrast, no inhibition of this interaction was observed by addition of a control phage displaying a peptide of irrelevant specificity (DPWATRIVLR). No binding of IgE-ND-specific phages to IgE-JW8 was observed (data not shown). These data show that the peptide phage clones reacted specifically with their selecting antibody and that this binding occurred via the variable part of IgE-ND as demonstrated by inhibition with an anti-idiotypic Fab.

2.3 Functional activity of circular and linear ND-specific peptide phages

Next we analyzed whether ND-specific phages might recognize IgE-ND under physiological conditions, *i.e.* bound to the high-affinity IgE receptor (FcεRI). For this purpose CHO cells transfected with the FcεRI α chain were sensitized with IgE-ND and binding of the phages

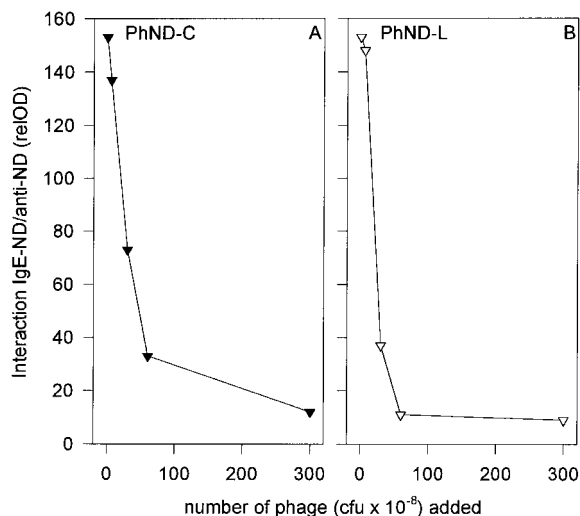


Figure 1. Inhibition of IgE-ND binding to anti-ND idiotype Fab (anti-ND Fab) with ND-specific peptide phage. Nitrocellulose strips were coated with anti-ND Fab, incubated with IgE-ND and bound IgE-ND detected with HRP-labeled anti-IgE Ab. (A) Inhibition of anti-ND Fab/IgE-ND interaction with circular ND-specific peptide phage (PhND-C). (B) Inhibition of anti-ND Fab/IgE-ND interaction with linear ND-specific peptide phage (PhND-L). Relative OD (relOD) as determined with a handheld densitometer [26] is shown.

was analyzed by FCM. As shown in Fig. 2 both PhND-C and PhND-L recognized IgE-ND when bound to α chain, whereas the control phage (DPWATRIVLR) did not bind. No binding of ND-specific peptide phages to IgE-JW8-

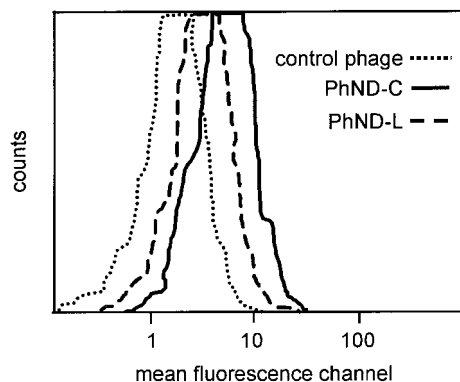


Figure 2. FCM analysis of circular and linear ND-specific peptide phage for binding to human Fc ϵ RI sensitized with IgE-ND. CHO cells transfected with human Fc ϵ RI α -chain were sensitized with IgE-ND. Binding of unspecific control phage DPWATRIVLR (dotted line), linear ND-specific peptide phage (dashed line) and circular ND-specific peptide phage (straight line) is shown. Bound phages were revealed with FITC-labeled anti-phage antibody [45].

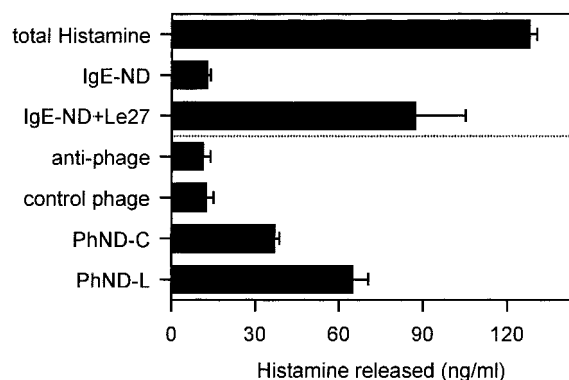


Figure 3. Histamine release induced by circular and linear ND-specific peptide phage. Freshly isolated and enriched basophils (4×10^5 cells containing 30 % basophils per sample) isolated from peripheral blood were sensitized with IgE-ND and primed with IL-3. Histamine release induced by control phage DPWATRIVLR, PhND-C and PhND-L, all cross-linked with a polyclonal anti-phage antibody, is shown. The anaphylactogenic anti-IgE mAb Le27 [47] was used as positive control. Mean from duplicate cultures is shown.

sensitized CHO cells or to non-transfected wild-type CHO cells was observed (data not shown).

Subsequently we tested the isolated phage clones in a histamine release assay using enriched, IL-3-primed human basophils, sensitized with an excess amount of IgE-ND. Circular and linear ND-specific peptide phages induced a release of 29 % for PhND-C or 51 % for PhND-L of the total cellular histamine (Fig. 3). Thus, the ND-specific peptide phage clones isolated in this study showed not only specificity for IgE-ND but upon cross-linking were capable of mimicking the anaphylactogenic activity of the putative antigen recognized by IgE-ND.

3 Discussion

In this study we have demonstrated the isolation and characterization of synthetic peptides, recognized by human myeloma IgE-ND. Specificity of the isolated peptides was demonstrated in an inhibition assay. Importantly, using the IgE-ND-specific peptide phages in a histamine releasing assay, anaphylactogenic activity was demonstrated.

In different studies biological activity mediated by recombinantly expressed proteins, e.g. the thrombin inhibitor hirudin [27], alkaline phosphatase [28] or streptococcal nuclease [29] on the surface of M13 bacteriophages was demonstrated. However, to our knowledge this is the first description of biological activity mediated

by peptide-displaying phage particles, as opposed to chemically synthesized peptides isolated by phage display. Thus, phages displaying ND-specific mimotopes or, probably more practically, recombinantly expressed fusion proteins of ND-specific mimotopes with another protein of choice may be used for the study of antigen-specific IgE responses. To date in the majority of models to analyze such responses, a rat basophilic leukemia cell line [30–32], transfected with single or multiple chains of the human FcεRI [33–35] and hapten-specific rat [30–32] or mouse/human chimeric IgE [33, 36] is used. In addition, an alternative model using bone marrow-derived mast cells from mice transgenically expressing human FcεRI α chain sensitized with NIP-specific, chimeric IgE was described [36]. In contrast to these rather artificial systems our peptides would allow the use of human (myeloma) IgE and human basophils and thus may represent a valuable and practical alternative to these other reported models.

No established human lymphoma or myeloma cell lines expressing IgE or IgG of defined specificity are known to date. Nevertheless, an increasing body of evidence indicates that the VH and VL genes of most known myelomas show frequent somatic hypermutation indicative of a post-germinal center origin, and thus development of myeloma from antigen-selected B cells [37–40]. However, no indications were given in these reports on antigens recognized by these myelomas, even though it would be interesting to assess whether specificities of myelomas/lymphomas are restricted to certain types of antigens (e.g. self vs. exogenous antigens) and whether

antigen specificity is causatively involved in the development of disease. In only one report two cell lines isolated from an HIV patient suffering from Burkitt's lymphoma producing polyreactive IgM against actin, HIV gp160 or human IgG have been described [41]. Sequence analysis of the VH gene of these IgM revealed 95 % homology to VH germ-line genes, and it was suggested that B cells of HIV-positive individuals responding to chronically present antigens might be at increased risk for lymphoma development. For IgE-ND the level of somatic hypermutation of VH and VL genes is not known, even though IgE produced by U266 had been used to determine the human ε chain DNA sequence [42]. Thus, it remains unclear whether selection for binding to a naturally occurring antigen had occurred or whether the V genes of IgE-ND are in germ-line configuration. Given the reports mentioned above and the fact that IgE-ND is produced by a myeloma cell line that has switched isotype to IgE *in vivo*, we assume that U266 probably developed from a B cell clone that had undergone antigen selection.

It has been reported that mimotopes isolated from random peptide libraries may display structural relationships to the natural antigen recognized by the antibody used for selection [43]. Thus, such libraries might also be used to define natural antigens recognized by antibodies of yet unknown specificities. To eventually define a natural antigen for IgE-ND, we performed sequence homology analysis of the peptide sequences isolated in our study with a large array of previously published protein sequences using the EMBL Gene Bank. As summarized in Table 3, sequence homologies to different self anti-

Table 3. Proteins with homology to IgE-ND-specific peptides

Origin ^{a)}	Sequence ^{b)}																		
PhND-C		C	C	W	L	R	M	P	C	C									
keratin	22 ^{c)}	C	C	Y	-	R	D	P	C	C	30								
PhND-L		L	S	P	H	L	L	W	D	L	F	R	V	G	L	P	G	A	A
galactosyl-transferase	359	L	S	P	E	Y	C	W	D	-	Y	Q	/	G	L	P	373		
aldolase	153	/	S	P	Q	F	L	S	D	L	F	S	V	G	165				
T. ferrooxidans ORF	276	L	G	P	H	L	L	W	D	283	308	V	G	L	S	G	A	A	314

a) Origin of sequences: PhND-C, PhND-L are phage clones specific for IgE-ND; sequences of keratin, galactosyltransferase and aldolase were retrieved from EMBL Gene Bank. The sequence of *T. ferrooxidans* ORF is as described in [44].

b) Identical amino acids are shown in bold front, similar amino acids in italic font. The last three amino acids shown for PhND-L (GAA) are part of the pIII protein of M13.

c) Amino acid position.

gens like keratin, galactosyltransferase and aldolase were found, but binding studies showed no recognition of these proteins by IgE-ND. More strikingly, a strong correspondence to the amino acid sequence of an unidentified open reading frame (ORF) present in the genome of *Thiobacillus ferrooxidans* [44] (13 out of 15 amino acids identical) was determined (Table 3). After cloning and recombinant expression in *Escherichia coli* this protein was tested in an ELISA for binding to IgE-ND. However, no recognition by IgE-ND was observed (data not shown). These findings allow several interpretations: (1) As there are no antisera, mAb or functional tests available for the analysis of this newly cloned protein of *T. ferrooxidans*, we cannot assess whether recombinant expression of the protein in *E. coli* yielded a correctly folded form of the protein. (2) It is widely accepted that analysis and comparison of linear sequences do not necessarily allow identification of corresponding structures as not only the amino acid sequence but also the conformational and atomic/electronic structure of an antigen are critical for the recognition by antibodies, especially in the case of discontinuous [17] and conformational epitopes [45]. Consequently, our findings imply a conformational epitope for IgE-ND. Interestingly, comparison of the sequence of the ND-specific linear peptides to those of the circular peptides revealed no homologies, suggesting again the presence of a conformational epitope for IgE-ND or the existence of two different epitopes recognized by IgE-ND. Similarly, no homology between linear and circular IgE-ND-specific peptides and anti-ND-Fab was observed. (3) It can be speculated that there may exist a natural antigen recognized by IgE-ND which, however, is not cloned and sequenced and thus cannot be retrieved from a data bank. Nevertheless, the approach of using random peptide libraries to identify the antigen specificities of myeloma antibodies by sequence comparison of the isolated peptides with gene banks may contribute to the delineation of the etiological pathways leading to the development of myelomas.

4 Materials and methods

4.1 Reagents and phage libraries

E. coli strain K91Kan was purchased from Stratagene (Zürich, Switzerland) and grown in terrific broth [46] or in NZY (Gibco Life Technologies, Basel, Switzerland). Kanamycin (Boehringer Mannheim, Rotkreuz, Switzerland) was used at a concentration of 100 µg/ml, tetracycline (Boehringer Mannheim) at 20 µg/ml. Human keratin, galactosyltransferase and rabbit aldolase were purchased from Sigma (Fluka Chemie AG, Buchs, Switzerland). IgE-ND [18] and IgE-JW8 [14] were produced and purified in our laboratory. Anti-ND-idiotype Fab-phage clones [26] are described elsewhere.

Random epitope phage libraries were kindly provided by Drs. E. Rouslahti, La Jolla Cancer Research Foundation, CA (Cx7C) [22], George P. Smith, University of Columbia, Tucker Hall, MO (6mer Amp EP Lib and 15mer Amp EP Lib) [23], B. A. Malcolm, Chiron Corporation, Emeryville, CA, USA (am3-4) [24] and J. A. Stoute, Walter Reed Army Medical Center, Washington DC, USA (fAFF 6mer Lib) [25] and were used as described in Table 1. Polyclonal rabbit anti-phage Ab was produced and purified in our laboratory as described [26]. Le27 and BSW17 are anti-IgE mAb produced in our laboratory [47]. As control phage displaying a peptide of irrelevant specificity a phage clone isolated from am3-4 [24] displaying the peptide DPWATRIVLR was used.

4.2 Panning and amplification

Polystyrene tubes (Falcon 2063, Becton Dickinson, Temse, Belgium) were coated with 4 ml IgE (2.4×10^4 U/ml IgE in 0.05 M NaHCO₃ pH 9.6) at room temperature overnight, and thereafter blocked with PBS containing 0.15 % casein (Sigma) (PBS-C) for 1 h at 37 °C. Then the linear or circular phage pools (Table 1) were incubated for 2 h by shaking at 37 °C. The phage solution was removed and tubes washed once with H₂O followed by ten washing cycles with TBS containing 0.1 % Tween (TBS-T) and one final wash with H₂O. Bound phages were eluted with 1 ml 0.1 M HCl pH 1.2 for 10 min and neutralized with 100 µl 2 M Tris. Aliquots were removed for determination of the elution titer and the rest of the eluted phages amplified by propagation in K91Kan.

4.3 Sequence analysis

Plasmid DNA from 100 ml bacterial culture was purified using a commercial kit (Macherey-Nagel, Oensingen, Switzerland) according to the manufacturer's instructions. A fragment was amplified by PCR using the primers FK344n 5'-CTGTAGCATTCCACAGACAAC-3' and FK350n 5'-TCTCACTCGGCCGACGGGGCT-3'. The resulting 150-bp fragment was purified and sequenced.

4.4 Inhibition assay for ND-specific phage clones

Anti-ND Id Fab-phages (10^9) [26] were dotted onto nitrocellulose (Millipore, Volketswil, Switzerland), dried and blocked with PBS-C. Aliquots (1 ml) of ND (200 IU) in PBS-C were complexed with different concentrations of linear or circular ND-specific peptide phage clone for 2 h at 37 °C. Strips were incubated by shaking overnight with the IgE-ND/phage complexes. After washing (3 × 10 min with PBS-T), strips were incubated with the horseradish peroxidase (HRP)-labeled anti-IgE Ab Le27 for 2 h and developed with enhanced chemiluminescence (Amersham, Zürich, Switzerland) following the manufacturer's instructions. Dots were quantified using a handheld Densitometer (Gretag Limited, Regensdorf, Switzerland) as described [26].

4.5 FCM analysis

CHO cells (3×10^5) transfected with human Fc ϵ RI α chain were sensitized with 400 IU IgE-ND for 20 min. After washing, 50 μ l of phage (ND-specific and unspecific phage as negative control) were added for 20 min at 4 °C and after washing developed with 1 μ g FITC-labeled anti-phage Ab. As a positive control IgE-sensitized cells were stained with 1 μ g FITC-labeled BSW17. Cells were analyzed by FCM (EPICS Profile II, Coulter Electronics, Hialeah, FL).

4.6 Histamine release assay

Histamine release was performed as described previously [48]. Briefly, basophils from peripheral blood were enriched by dextran sedimentation and percoll gradient centrifugation. Thereafter cells were incubated with 50 μ g IgE-ND for 60 min at 4 °C and then washed twice. For the assay 4×10^5 cells (4×10^6 /ml, 100 μ l per sample) containing 30 % basophils were used. The whole procedure was performed at 37 °C with duplicate samples. After priming with 100 ng/ml IL-3 for 15 min cells were stimulated with 200 μ l phage (2×10^{12} phages extensively dialyzed in PBS) preincubated with 8 μ g anti-phage Ab. The reaction was stopped after 20 min by transferring the samples into ice water. As a positive control 1 μ g anaphylactogenic anti-IgE antibody Le27 was used. Samples were analyzed using an automated fluorimetric method [49] and quantified according to a histamine standard.

4.7 Cloning of “unidentified ORF” of *T. ferrooxidans* into pComb3H and analysis

A 4.47-kb fragment was isolated from plasmid pT130 [44] by digestion with EcoRI (AGS, Axon Lab AG, Baden, Switzerland) and HindIII (AGS) and separation on and elution from a 0.8 % agarose gel. This fragment was used as a template to isolate and unidentified ORF from *T. ferrooxidans* [44] by PCR using the following primers: TFORF5: GCCGAGCTC-ATGCAAGGTGTAATGTTAC and TFORF3: GCCACTAGT-TTCCGCCTTGCGCCGACGCG. The underlined parts of the primers serve to introduce restriction sites for SacI and SpeI. The resulting 1010-bp fragment was digested with SacI (AGS) and SpeI (AGS) and ligated into pComb3H, previously digested with the same enzymes. The resulting plasmid was transformed into *E. coli* XL-1 using a CaCl₂ transfection method described by Sambrook et al. [46]. Clones were analyzed for the presence of the insert by restriction digest and sequence analysis. The protein was produced by induction of the lacZ promoter with isopropyl β -D-thiogalactoside and growth at 30 °C by shaking for 16 h. The pellet was lysed with lysozyme and by sonication and the supernatant purified over a TALON resin (Clontech, Stehlin AG, Basel, Switzerland). The purified protein was used to coat ELISA plates and nitrocellulose. After blocking, IgE-ND and differ-

ent control IgE antibodies were incubated overnight at room temperature. Thereafter, plates or strips were incubated with HRP-labeled anti-IgE and developed with 2.4 mg/ml 3,3',5,5'-tetramethylbenzidine (Fluka) dissolved in acetone:ethanol (1:9) containing 80 mM H₂O₂ and diluted 1:20 in 30 mM sodium citrate pH 4.1. The reaction was stopped with 1 M H₂SO₄ and absorbance measured at 450 nm. Immunodots were developed as described in Sect. 4.4 (immunodot assay).

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