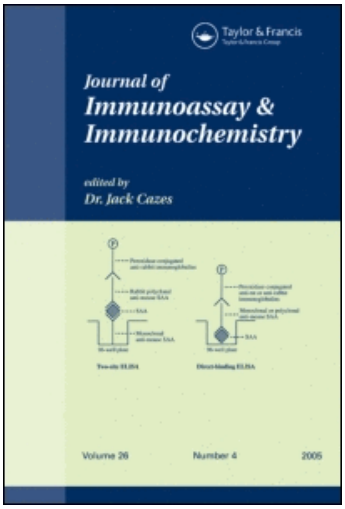

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Presentini, R. , Perin, F. , Ancilli, G. , Boraschi, D. , Volpini, G. and Antoni, G.(1989) 'Influence of the Peptide Insolubilization Method on Detection of Anti-Peptide Antibodies in Elisa. Evaluation of Nonspecific Interactions', Journal of Immunoassay and Immunochemistry, 10: 4, 395 — 412
To link to this Article: DOI: 10.1080/01971528908053249
URL: http://dx.doi.org/10.1080/01971528908053249

JOURNAL OF IMMUNOASSAY, 10(4), 395-412 (1989)

INFLUENCE OF THE PEPTIDE INSOLUBILIZATION METHOD ON DETECTION OF ANTI-PEPTIDE ANTIBODIES IN ELISA. EVALUATION OF NONSPECIFIC INTERACTIONS

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ABSTRACT

Different methods of peptide insolubilization in solid phase were compared in ELISA, to verify the influence of the peptide antigen presentation in the interaction with related antibodies.

Our studies were performed using as model the peptide fragment 163-171 of human Interleukin 1β, and polyclonal or monoclonal anti-peptide antibodies. It was found that the peptide, N-terminally linked to a protein carrier before the adsorption on microtiter wells, interacted with specific polyclonal and monoclonal antibodies with high sensitivity and specificity. In contrast the recognition of similar random conjugates, prepared using a bivalent cross-linking reagent or the peptide covalently linked to poly-L-Lysine-pretreated wells, was hampered generally by very high levels of nonspecific binding. On the other hand, the free peptide adsorbed directly to the solid phase interacted with antibodies with very low sensitivity and specificity.

Nonspecific interactions were found in particular between peptides and hyperimmune sera or nonrelated monoclonal antibodies. On the contrary pre-immune sera and normal mouse immunoglobulins never showed significant interactions with any of peptides. This nonspecificity was also overcome when N-terminally linked peptide-protein conjugates were used for the assay.

(KEY WORDS: Synthetic peptides - interleukin 1 - coating of microtiter plates - polyclonal and monoclonal anti-peptide antibodies - ELISA)

INTRODUCTION

Synthetic peptides have a wide range of immunochemical applications today, with particular reference to the study the antigenic structure of proteins (1-3), and for the production of antibodies with preselected specificity (4).

The evaluation of the interaction between synthetic peptides and specific antibodies is of great importance for these immunochemical studies, but this interaction generally involves weak binding forces, so that it is important to find suitable analytical conditions.

Several analytical methods have been proposed for the determination of peptide-antibody interactions involving the use of radiolabeled peptides (5), or peptides, prepared by solid phase method, left linked to the support after the synthesis (6). Methods for the analysis of the peptide-antibody interaction are based on the peptide insolubilization on microtiter plates, adsorbed directly on the solid phase (7), or on glutaraldehyde (8) or poly-L-lysine-pretreated wells (9). Moreover peptide-protein carrier conjugates have also been used as absorbents, either obtained by means of bivalent cross-linking reagents (10), or by the N-terminal-linkage of peptide to a protein carrier (11).

In this work we have tested in ELISA, different procedures for binding peptides to microtiter plate wells, to evaluate the influence of the peptide antigen presentation in the analytical environment on the sensitivity and specificity of the peptide recognition by related antibodies.

As a model for our studies we have used the peptide corresponding to the sequence 163-171 of human interleukin 1β (hu IL-1β), a fragment possessing immunostimulatory activity in vitro and in vivo, but devoid of the inflammatory properties of the entire molecule (12-14).

MATERIALS AND METHODS

Peptide synthesis

Peptides 163-171 of hu IL-1β (VQGEESNDK, peptide 1), 199-208 of murine IL-1α (QGEDQPVLLK, peptide 2) and 166-174 of human chorionic somatomammotropin (FRKDMDKVE, peptide 3), were synthesized by solid phase (15) as previously described (12,16). All the peptides represented highly hydrophilic portions of the corresponding protein (17).

The addition of an N-terminal cysteine residue was performed using N-ter-butyloxycarbonyl-S-4-methyl-benzyl-L-cysteine (Vega Biotechnologies, Tucson, AR) as

the last residue. The protected cysteyl peptides were detached from the resin and deprotected by treatment with HF and then purified by gel permeation chromatography on Sephadex G-10 equilibrated in 0.1 M acetic acid.

Peptide-protein conjugates

Peptides used as immunogens were coupled to Keyhole Limpet Haemocyanin (KLH) by the reported method (18). Twenty-five mg of soluble KLH (Calbiochem Biochemical, La Jolla, CA) were dissolved in 2 ml of 0.05 M sodium-phosphate buffer (pH 7.5). Peptides (15-20 mg) were then added to the solution and the pH was brought to 7.5 with NaOH. Finally 1 ml of aqueous 20 mM glutaraldehyde was added with stirring and the product left for 1 h at room temperature. The conjugates were dialyzed against the same sodium-phosphate buffer PBS, then against water and lyophylized.

Two different soluble conjugates to be used as antigens in ELISA were also produced between peptides and BSA. The first conjugation method consisted in the use of adipic acid succinimidodiester (19) as cross-linking reagent. Ten mg of protein were dissolved in 1 ml of 0.1 M ammonium bicarbonate buffer and 5 mg of peptide were added; the solution was treated with 3 mg of diester dissolved in 0.1 ml of freshly distilled dioxane under stirring. After 2 h at room temperature,

the reaction mixture was dialyzed against the same buffer, then against water and finally filtered and lyophylized.

In the second method, N-terminal cysteyl peptides were coupled to maleimidated BSA (20). Ten mg of protein were dissolved in 0.15 ml of 0.05 M sodium--phosphate buffer pH 7, and treated with 25 μl of freshly distilled N,N-dimethylformamide containing 2 mg of m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce chemical Co., Rockford, IL), and stirred for 30 min at room temperature. After dialysis at 4°C against 0.1 M sodium-phosphate buffer pH 6 for 24 h, the solution was centrifuged at 3000 rpm for 10 min to discard the precipitate. The supernatant was adjusted to a final volume of 1 ml. This solution was mixed with 1.25 ml of a solution of 5 mg of cysteyl peptide in 10 mM sodium phosphate, 0.15 M sodium chloride buffer pH 7.4 (PBS) in the presence of 0.01 M EDTA, in a nitrogen environment; the pH was adjusted to 7 with NaOH and the mixture was left to react for 3 h at room temperature. A drop of mercaptoethanol was added to block non reacted maleimido residues. The conjugates were then extensively dialyzed against 0.1 M ammonium bicarbonate and then against water, filtered and lyophylized. The compositions of the peptide-protein conjugates were determined by aminoacid analysis (21).

Preparation of polyclonal anti-peptide antibodies

Two month-old New Zealand White rabbits were immunized with peptide-KLH conjugates. On day 0, 400 μg of conjugate in 0.1 ml PBS were mixed with an equal volume of complete Freund's adjuvant (CFA) and administered by intradermal injection at multiple sites. The animals were boosted on day 14, 21 and 42 with 400 μg of conjugate in incomplete Freund's adjuvant (IFA) and bled on day 48. Sera were collected and stored at -20°C.

Generation of monoclonal anti-peptide antibodies

Five eight week-old BALB/c female mice were immunized with 50 μg of each peptide-KLH conjugate in CFA in the footpads. After 13 days, the same amount of conjugate was again administered in IFA in the same site. Popliteal lymphnodes were removed 3 days later and dissociated to obtain a single cell suspension. Lymphnode cells were fused with myeloma cells P_3X63-Ag8.653 at a ratio of 4:1 with 50% PEG 4000 (Merck, Darmstadt, West Germany). Cells were resuspended in HAT medium and plated at $5\text{-}10x10^4$ myeloma cells/well of Cluster^{96} (Costar, Cambridge, MA) microtiter wells on a feeder of mitomycin C-treated peritoneal macrophages of C3H/HeN female mice. Supernatants from wells containing growing hybridomas were screened by RIA, one week after seeding, for

reactivity to the antigen adsorbed on the wells, with an anti-mouse immunoglobulin radiolabeled antibody. Cells from positive wells were first fractionated and then cloned twice by limiting dilution. Anti-peptide monoclonal immunoglobulins were purified from ascitic fluid by protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) affinity chromatography. Immunoglobulin classes were determined by immunodiffusion and immunoelectrophoresis.

Coating of peptide antigens to plates for ELISA

ELISA measurements were carried out using flat-bottomed microtiter plates (Immulon II Dynathec, Denkendorf, FRG).

Free peptides were adsorbed directly by incubation of 10 μg/well of peptide in sodium-bicarbonate buffer pH 9.6 (7). After overnight incubation at room temperature plates were washed three times with PBS.

Plates coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO) were prepared adding 0.1 ml of polymer, 1 mg/ml in PBS pH 8. After stirring overnight at room temperature, microtiter wells were washed three times with PBS and 10 μg of peptides dissolved in 0.1 ml of PBS were added. Wells were then treated with 10 μl of a solution of 10 mg/ml of adipic acid hydroxysuccinimido diester in dioxane, as cross-linking reagent in substitution for glutaraldehyde (9). The plates were

then left overnight at room temperature and finally washed three times with PBS. BSA-peptide conjugates were adsorbed to microtiter wells using 0.1 ml/well of 0.1 mg/ml solution of the conjugate in PBS pH 8. After overnight incubation at room temperature plates were washed three times with PBS.

ELISA procedure

All antigen-coated plates were treated with 200 μl/well of 3% BSA in PBS to prevent nonspecific adsorptions.

After 6 h at room temperature plates were washed three times with PBS and 100 μl of two-fold dilutions of sera or mAbs (1 mg/ml) in PBS containing 1% BSA were added. Plates were left 4 h at 37°C, then washed ten times with water.

Wells containing rabbit antibodies were treated with 100 μl of protein A-peroxidase conjugate (Sigma) diluted 1:500 in PBS containing 1% BSA. Wells containing mAbs were treated with 100 μl of anti mouse Ig-peroxidase conjugate (Cappel, West Chester, PA) diluted 1:500 in PBS with 1% BSA for 1 h at 37°C.

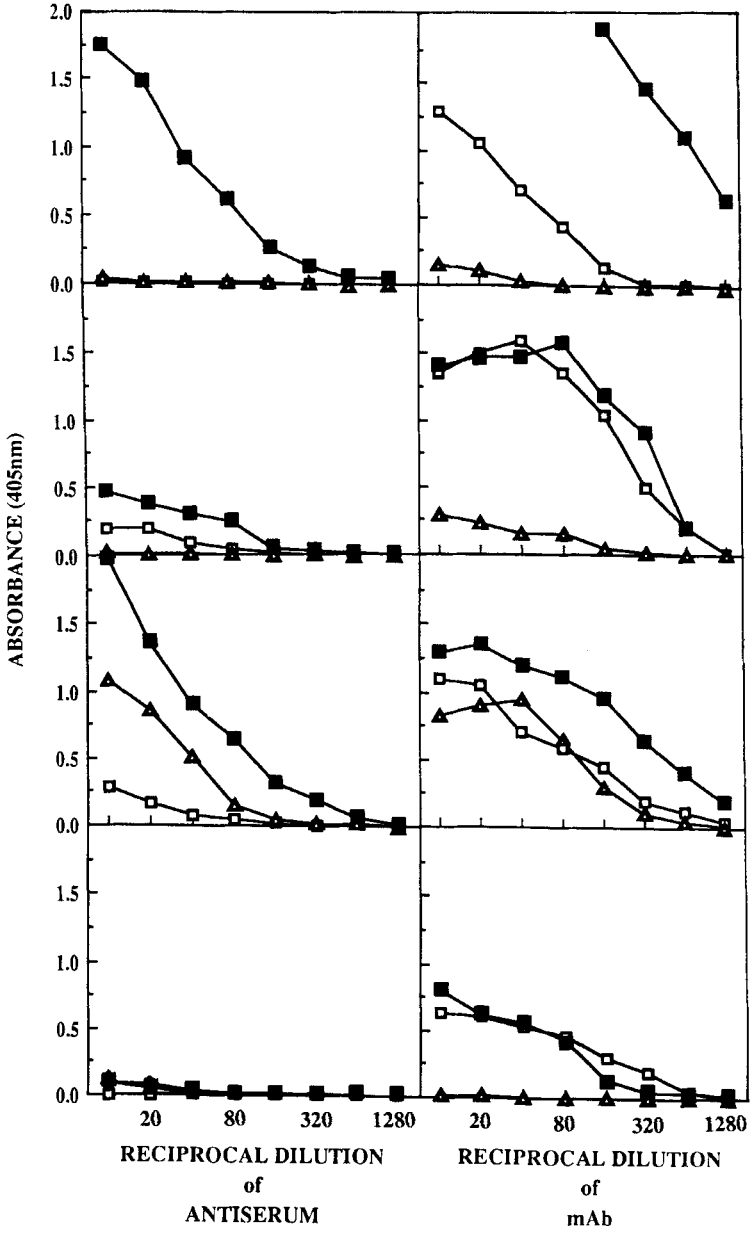
Plates were washed five times with water and the color was developed using 100 μl/well of ortho-phenylen-diamine, 3 mg/ml in 0.1 M phosphate-citrate buffer pH 5.5 containing 0.02% hydrogen peroxide (final pH 5). The reaction was stopped in all plates after 15 min by

adding 75 µl of 1 M sulfuric acid. Absorbances were read at 492 nm using a Titertek multiscanner (Flow Laboratories, McLean, VA).

RESULTS

Peptide 1 and peptide 2 were coupled to KLH and used as immunogens in rabbits to induce anti-peptide pAbs and in mice for the preparation of mAbs. The composition of the peptide-protein conjugates are reported in table 1. Anti-peptide 1 mAb was identified as IgG_{2a}, while the anti-peptide 2 mAb turned out to be of the IgG_1 subclass. The polyclonal and monoclonal antibodies anti-peptide 1, analyzed by RIA, could react with the entire hu IL-1β. On the other hand, the anti-peptide 2 antibodies did not recognize the same protein and therefore served as negative control. As a further control of specificity, the possible interaction of the antibodies with an unrelated peptide antigen was checked. For this purpose peptide 3 was submitted to the same insolubilization procedures as the analytical peptide 1 (table 1).

As reported in Figure 1A, the anti-peptide 1 pAb gave a strong positive reaction with the immunizing peptide when peptide 1 was specifically linked to the protein carrier via N-terminal. The anti-peptide 1 mAb



proved extremely reactive in the same assay at the concentrations used. The use of a more diluted mAb solution confirmed the strong specific interaction of the anti-peptide 1 mAb with the immunizing peptide (data not shown). Conversely, unsatisfactory results were obtained using a random conjugate (Figure 1B). In this case, in fact, a very high nonspecific interaction was obtained between anti-peptide 1 antibodies and peptide 3. This reactivity was particularly evident with the anti-peptide 1 mAb which could bind equally well both to the specific and to the unrelated peptide.

When peptides were coupled to poly-L-lysine-pretreated plates, positive binding with antibodies was obtained (Figure 1C), but with a very complex panel of

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Fig. 1. ELISA of polyclonal (left) and monoclonal (right) anti-peptide antibodies with peptides insolubilized on microtiter wells by different methods.

A) Spatially oriented peptides: peptide-BSA conjugates via N-terminal.

B) Random peptides: peptide-BSA conjugates prepared by means of the bifunctional cross-linker adipic acid succinimidodiester.

C) Peptides linked to poly-L-lysine pretreated plates by bifunctional cross-linker.

D) Free peptides directly adsorbed to the plates.

-■-■-■- : binding of anti-peptide 1 antibodies to peptide 1 (specific interaction).
-□-□-□- : binding of anti-peptide 1 antibodies to peptide 3 (negative control)
-△-△-△- : binding of anti-peptide 2 antibodies to peptide 1 (negative control).

TABLE 1

Composition of the peptide-protein conjugates

Peptide	Protein carrier (a)	Coupling reagent (b)	Conjugation ratio (mol peptide:mol carrier)
Peptide 1	KLH	GA	26
Peptide 2	KLH	GA	13
Peptide 1	BSA	AAS	4
Peptide 3	BSA	AAS	3
Peptide 1(c)	BSA	MBS	4
Peptide 3(c)	BSA	MBS	5

a) KLH, Keyhole Limped Haemocyanin (mol.wt 100,000); BSA, Bovine serum Albumin (mol.wt 68,000).

b) GA, glutaraldehyde; AAS, adipic acid succinimidodiester; MBS, m-maleimidobenzoyl-N-hydroxysuccimidoester.

c) An N-terminal cysteine residue was added to the peptide during solid phase synthesis, to be used in this conjugation method.

nonspecific interactions. The anti-peptide 1 mAb was found to interact also with peptide 3, whereas peptide 1 cross-reacted nonspecifically with both pAb and mAb anti-peptide 2.

The free peptide 1 directly absorbed onto the plates was not recognized by the specific pAb, while only nonspecific interactions were seen with the corresponding mAb. The latter reacted in the same fashion with both peptides 1 and 3 (Figure 1D).

Preimmune serum anti-peptide 1 and normal mouse Ig were also tested in all assays and showed no interaction with any of the peptides (data not shown).

DISCUSSION

During our studies on the preparation of polyclonal and monoclonal antibodies against synthetic peptides representing different portions of several proteins, we generally found it easier to detect the interaction of anti-peptide antibodies with the entire protein, rather than with the immunizing peptide, using solid phase immunoassays.

This evidence probably indicated the need for a correct conformation of the peptide in the analytical environment, to facilitate the interaction with specific antibodies. It was therefore felt to be

important to find an adequate insolubilization method which could stabilize the peptide in a more correct spatial conformation.

Different methods of peptide binding to the solid phase were tested in this study and the degree of interaction of the immobilized peptide with specific antibodies was evaluated. It was found that the use of a peptide directionally linked via N-terminal to a protein carrier could significantly increase the sensitivity of the ELISA assay with both polyclonal and monoclonal antibodies. The efficiency of the assay clearly decreased when random conjugates, or the peptide linked to poly-L-lysine-pretreated wells were employed.

On the contrary using free peptides as antigens adsorbed directly to the solid phase, only poor results were obtained both in terms of sensitivity and specificity. We supposed that this result might be caused by the high hydrophilicity index of the peptides employed in this work. In fact, the adsorption of these peptides to polystyrene wells can be hindered because the adsorption of proteins on solid surfaces occurs mainly through hydrophobic bonds (22). Consequently, the efficiency of all the assay is affected drastically.

Finally, neither preimmune rabbit serum nor normal mouse Ig gave any response in all assays tested. On the

contrary nonspecific interactions were displayed by the cross-reaction between the peptide antigens and nonspecific polyclonal or monoclonal anti-peptide antibodies.

Our experiments, although hitherto limited to a single antigenic peptide, clearly show that many problems arise when the antigenic reactivity of a small synthetic peptide has to be assessed. In fact, the interaction of the peptide with specific antibodies is often too weak and therefore difficult to evaluate. On the other hand, incorrect reactivities can be often observed, unless an accurate control of every possible source of nonspecific interaction is performed.

These results confirm the importance of a correct conformation of the antigen in the analytical enviroment to increase the reactivity with related antibodies. The use of ordered peptide-protein conjugates, to be adsorbed on solid phase in ELISA, appears an useful tool for the resolution of the problem. We tested successfully the use of conjugates prepared by reaction of maleimidated BSA with N-terminal cysteyl peptide. Obviously other methods of tidily binding peptides to carriers, besides the one proposed, may also give good results and should therefore be evaluated.

Acknowledgments

We wish to thank Fabrizio Zappalorto for treating the animals, and Giorgio Corsi for graphic work.

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