



A high-capacity manifold support for the detection of specific IgE antibodies in allergic individuals

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Abstract

A high-capacity manifold support with immobilized antigen was developed for the analysis of IgE-mediated immune reactivity in allergic subjects. Using this 96-pronged support, specific antibodies were trapped and detected from large sets of serum samples. We describe the binding of large amounts of antigen onto the expanded surface of the manifold support, permitting efficient identification of allergic individuals.

Key words: Solid support; Surface modification; Allergy; Immune assay; Manifold support; IgE

1. Introduction

Solid phase-based assay techniques permit the binding and isolation of specific ligands before a visualization step. A large number of solid phases are in use for immunoassays, including surfaces of reaction vessels such as microtiter wells, microparticles that may be isolated by centrifugation, filtration, or magnetism, and various three-dimensional matrices, offering large surface areas. A manifold support, with sets of 96 prongs projecting into individual microtiter wells, facilitates the convenient parallel handling of multiple

samples. Moreover, it permits the transfer of samples between consecutive reaction steps with minimal pipetting, eliminating risks of sample mix-up. Such supports have previously been used for peptide synthesis and in the subsequent screening for specific antibodies (Geysen et al., 1987), and similar devices have also been used for applications in molecular genetics (Lagerström et al., 1991; Rosenthal et al., 1987). In most cases the success of this strategy is critically dependent on the immobilization of large amounts of reagent on the limited area available on the manifold support.

For various reasons the detection of IgE antibodies specific for allergens immobilized on a multipronged support requires antigen preparations with a very high binding capacity. For example, antigens are frequently not available in a pure form. Secondly, different individuals may react to different components or groups of components in the antigen preparations, and these

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antigens sometimes bind poorly to solid phases. Furthermore, titers of specific IgE molecules are generally low and may be present together with excessive amounts of other classes of immunoglobulin having a similar specificity. In this study we have used a manifold support for the identification of individuals allergic to a specific group of antigens. We have achieved efficient coupling of antigen by greatly increasing the available surface through a layer of porous particles, attached to the polystyrene surface. In this manner, large numbers of samples from patients can be rapidly screened for evidence of specific allergy.

2. Materials and methods

2.1. Reagents

Specific reagents, including antigen preparations, antibody conjugates, and serum samples from normal and allergic subjects, were kindly provided by Dr. A. Kober, and Ms. M. Lönnberg, Kabi Pharmacia.

2.2. Preparation of antigen-coupled solid phases

The manifold support, used for the detection of specific IgE molecules, was configured as a microtiter plate lid with eight rows of 12 ball-and-pin extensions, projecting into individual wells of a microtiter plate located underneath (Falcon, Oxnard, CA). The surface of the prongs of this disposable polystyrene support was expanded by attaching Sepharose particles, modified with avidin, as previously described (Parik et al., 1993). A soy protein extract was biotinylated according to a standard procedure (Guesdon et al., 1979). Biotinylated antigen, 50 μ l at 0.2 mg/ml in 50 mM phosphate pH 7.4, 0.15 M NaCl, and 0.05% Tween 20, was subsequently bound to the avidin-coupled support in a 1 h incubation at room temperature. Alternatively, antigen was directly coupled to the particles as described below.

2.3. Coupling of antigen to Sepharose particles

Sedimented Sepharose particles (6 ml volume, HiTrap, NHS-activated HP Sepharose, Pharma-

cia) were washed with ice cold 1 mM HCl (3×10 ml) on a sintered funnel. The particles were transferred to 5 ml of 1.0 M NaCl and 0.4 M NaHCO_3 pH 8.3, containing 20 mg of soy protein extract. The suspension was incubated rotating end-over-end for 1 h, filtered, and the particles blocked in 0.1 M ethanolamine-HCl buffer pH 8.3 for 15 min. Antigen-conjugated Sepharose particles were washed with 0.1 M acetate buffer pH 4.0, followed by distilled water, and used immediately. Alternatively, the batches of conjugated particles were kept until use in a storage buffer of 50 mM Tris-HCl pH 7.3 with 0.02% (w/v) sodium azide.

2.4. Immobilization of particles on the polystyrene support

The surface of the 96-pronged support was modified by attaching antigen- or avidin-coupled particles as previously described (Parik et al., 1993). The particles were dried by washing with methanol (3×5 ml), followed by triethylamine (3×5 ml), and then suspended as a 50% (v/v) slurry in this solvent. The polystyrene support was washed with ethanol for 20 min in an ultrasonic bath and air dried. The prongs of the support were immersed in the slurry for 2 s, air dried, and the procedure repeated. After evaporation of triethylamine at room temperature and washing under running tap water, the non-specific binding capacity of the prongs was blocked by an overnight incubation in 0.5% dry milk, 50 mM Tris-HCl pH 7.5, 50 mM NaCl. (A blocking buffer containing 0.1 M glycine instead of dry milk may be used to avoid the risk of false positive reactions from milk allergic subjects.) The supports were kept in storage buffer at 4°C in a humidified container until they were used.

2.5. Enzyme-linked immunoassay

In the standard assay, manifold prongs with covalently bound antigen were washed in 50 mM phosphate pH 7.4 and 0.05% Tween 20, and then incubated in 50 μ l serum samples, dispensed into the wells of a polyvinyl microtiter plate (Falcon). The supports were incubated in microtiter wells on a shaking platform throughout the assay. After

30 min at room temperature, the supports were washed by three 5 min incubations in a washing buffer of 0.2 M phosphate pH 7.4 and 0.5% Tween 20. Next, the supports were incubated in antibody preparations, conjugated to β galactosidase. We used 50 μ l of a rabbit polyclonal anti-serum to human IgE (Kabi Pharmacia Diagnostics) at 0.5 μ g/ml in a 2 h incubation. As an alternative, Fab' fragments (1 μ g/ml) of a mouse monoclonal antibody to human IgE were used in a 1 h incubation (this conjugate is not commercially available). The supports were washed three times in washing buffer and incubated for 10 min at room temperature in 200 ml substrate solution, consisting of 4-methylumbelliferyl- β -D-galactopyranoside in 0.05 M phosphate, pH 6.5. After this incubation, the pH was raised by the addition of 200 μ l 5% glycerol in 0.4 M Na_2CO_3 pH 11.5. The fluorescence from 4-methylumbelliferone, formed by enzymatic hydrolysis of the substrate, was recorded in a Fluoroscan fluorescence reader (Pharmacia). Levels of specific IgE in samples from patients were also recorded using the Pharmacia CAP system (Pharmacia Diagnostics). In this test, serum samples and detection reagents were incubated within a cellulose matrix to which antigen had been coupled (Axén et al., 1988). All experiments were performed in triplicate.

2.6. Time-resolved fluorescence measurements

In some of the experiments another type of conjugate was used to reveal specifically bound IgE molecules. This was a conjugate between the same monoclonal antibody, specific for human IgE, and europium chelates (Hemmilä et al., 1984). The supports were incubated with the conjugate at 1 μ g/ml for 1 h at room temperature and then washed using a DELFIA assay buffer (Wallac). This buffer consisted of 50 mM Tris-HCl pH 7.75, 0.15 M NaCl, 0.5% (w/v) bovine serum albumin, 0.05% bovine globulin, 20 mM diethylenetriamine pentaacetic acid, 0.01% Tween 20, and 0.05% NaN_3 . Europium ions, bound to the solid supports in positive reactions, were visualized by immersing the prongs in an enhancement solution (0.1 M acetate-phthalate pH 3.2, 15 μ M 2-naphthoyl trifluoroacetone, 50 μ M tri-*n*-octylphosphine oxide, and 0.1% Triton X-100; Wal-

lac). After removing the support, the fluorescence from released europium ions was recorded in a time-resolved mode using a DELFIA research fluorometer (Wallac).

3. Results

3.1. Strategy for the analysis of specific IgE molecules in serum samples

A solid support with a set of prongs projecting into individual microtiter wells offers practical advantages in immuno assays although the limited surface available for coupling antigen may limit the applicability of the assay format. In order to circumvent this problem the surface was greatly expanded by affixing porous particles to the polystyrene support in a simple and efficient reaction. Fig. 1 illustrates schematically one of the prongs of the manifold, covered with antigen-modified particles and immersed in a microtiter well.

3.2. Comparison of different preparations of antigen-modified manifold support

Allergy to specific allergens can be diagnosed by detecting IgE antibodies directed to an appropriate antigen preparation (Wide et al., 1967). We have analyzed serum from patients with known IgE reactivity for uncharacterized protein components in soybean extracts. We took advan-

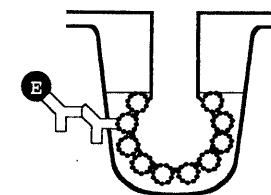


Fig. 1. A schematic illustration of an individual prong of the manifold support, incubated in a microtiter well. The polystyrene device is modified with antigen-conjugated particles (drawn out of scale). An antibody from a patient is shown, bound to the immobilized antigen and detected by an antibody conjugated to an enzyme molecule (E).

tage of the high binding capacity of the surface-modified manifold support by binding a biotinylated preparation of soybean protein to the avidin-conjugated support. This method of constructing an antigen-coated support is simple, the biotinylation procedure is gentle and the antigenic determinants in the protein preparation are preserved. For an assay that will be performed on large numbers of samples, it may be advantageous to prepare a large batch of particles to which antigen has been directly coupled. This simplifies the procedures and ensures consistent results. IgE antibodies, bound to the support, are then revealed using monoclonal or polyclonal β galactosidase-conjugated antibodies, specific for human IgE.

Fig. 2 illustrates that both means of preparing the antigen-coated supports resulted in the efficient identification of serum from an allergic subject. However, the direct immobilization of antigen onto the particles before affixing these to the manifold provided an even more sensitive assay, compared to that in which the antigen preparation was immobilized via an avidin-biotin interaction. Using directly coupled antigen, serum from an allergic individual gave a detectable reaction after more than 100-fold dilution in normal serum. This corresponds to less than 0.5 μ l of positive serum. The signal increased in a linear fashion until it reached a plateau at a volume of 100 μ l of patient serum (results not shown). In all subsequent experiments we used supports with directly coupled antigen.

3.3. Kinetics of binding of serum IgE and conjugate to the support

In order to provide a rapid and efficient assay the time required for the different incubation steps was investigated. The same set of serum samples employed in Fig. 2 were incubated with the manifold supports for different times before being washed, incubated with an enzyme-conjugated monoclonal antibody specific for human IgE, washed, and analyzed with respect to the release of a fluorescent product (Fig. 3). Prominent signals were obtained after a 10 min incubation of the support in serum. The signal did not, however, approach maximum until about 1 h. In

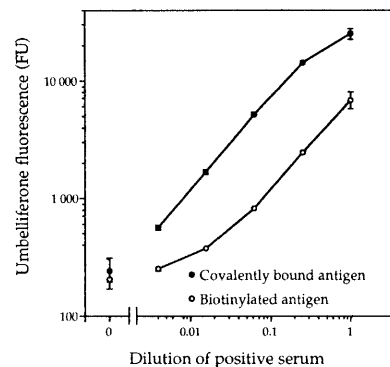


Fig. 2. Comparison between immune detection reactions for antigens bound covalently to the particle-coated support (filled circles) and for antigens immobilized via a biotin-avidin interaction (open circles). Serum from an allergic subject was diluted in normal serum and assayed for the presence of antigen-specific IgE antibodies. Results are given as means and standard deviations of triplicate determinations in this and subsequent figures.

contrast, a serum sample from a normal individual, supplemented with high concentrations of total human IgE, resulted in base-line signals.

Supports that had been incubated in serum for 30 min were then immersed in either of two conjugates for differing times. The incubation with enzyme-conjugated monoclonal or polyclonal anti-IgE antibodies resulted in a strong signal after 10 min, which was still rising after 60 min of incubation (Fig. 4). The monoclonal antibody-conjugate resulted in a stronger signal but the same level of background as the polyclonal conjugate.

3.4. Comparison between enzyme-linked and europium-chelate fluorescence detection

Different visualization techniques were examined to ensure optimal sensitivity and linearity of the assay. The enzyme-linked visualization technique provides an efficient detection reaction. By attaching rare earth metal chelates to the anti-IgE antibodies, bound reagents may instead be detected through the long-lived fluorescence from

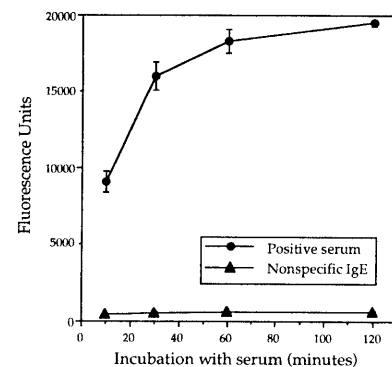


Fig. 3. Effect of time of incubation of the antigen-coated supports with serum on the signal in the assay. The results obtained using a serum, derived from an individual allergic to soybeans, are shown as filled circles. This serum contained 6 kU_A IgE/l of allergen-specific IgE as defined by the British National Institute for Medical Research. A serum from a healthy control, spiked with 1000 kU/l of total "nonspecific" human IgE (filled triangles), was also analyzed.

excited europium ions in the form of time-resolved fluorescence (Soini and Lövgren, 1987). This type of label is stable when stored and under a wide range of assay conditions, and instrumentation is available for detection. Moreover, this

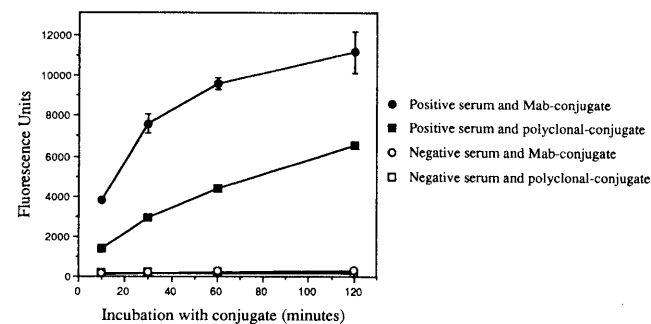


Fig. 4. Effect of time of incubation with conjugate on the signal in the assay. A serum sample from an allergic individual (filled symbols) or a healthy control (open symbols) were reacted with the antigen-coated support. Bound IgE-antibodies were revealed by incubating for differing times with β galactosidase conjugated to a monoclonal anti IgE-antibody (circles) or to a polyclonal anti IgE-antiserum (squares).

visualization technique offers the possibility of using two or more different lanthanide ions to permit the simultaneous analysis of different target molecules (Iitiä et al., 1992; Samiotaki et al., 1994).

In order to compare these detection methods, a serum sample from an individual allergic to soy protein was diluted stepwise in serum from a normal individual. As shown in Fig. 5 enzymes and chelates revealed the presence of immune IgE in a very similar fashion. Both the umbelliferone- and the europium-dependent fluorescent signals exhibited very low variability with, on average, less than 5% coefficient of variance in triplicate determinations. Both detection principles resulted in similar thresholds of detection, and both provided efficient visualization over an extended titration range.

3.5. Screening of serum samples using a manifold support or the CAP test

The antigen-coated high-capacity manifold was used in a practical test to analyse sera from soybean-allergic subjects using two types of solid support. The results obtained using the manifold support were compared with those in a commercial test, the Pharmacia CAP system (see Fig. 6). This latter support is a three-dimensional cellulose matrix, offering a very large available surface

area for the coupling of antigen. Results obtained using either support were in excellent agreement. Specifically, samples weakly positive in one assay were weakly positive in the other, and clearly resolved from the two negative serum samples. This suggests that the detection thresholds of the two supports were very similar. Furthermore, no obvious plateau-effect was observed among the investigated samples as would have been expected if the capacity of one of the supports had been substantially lower than that of the other. In conclusion, the manifold support offers similar binding characteristics to those of the cellulose matrix in a very convenient assay format.

Discussion

In this report we have proposed a so-called matrix-matrix format for immune assays in which manifold solid phases are co-ordinately moved between sets of reaction wells in order to sequen-

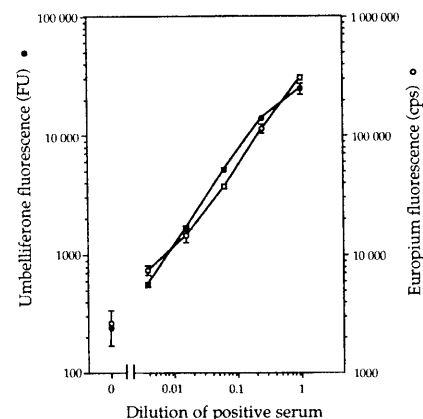


Fig. 5. Comparison between detection using a β galactosidase-linked procedure (filled circles) and time-resolved fluorescence of lanthanide chelates (open circles). In the first case, a fluorescent group, 4-methylumbelliferone, is released by the action of the enzyme. In the second, chelated europium ions are released and detected by time-resolved fluorometry. Serum from a soybean-allergic individual were diluted stepwise in serum from a normal individual.

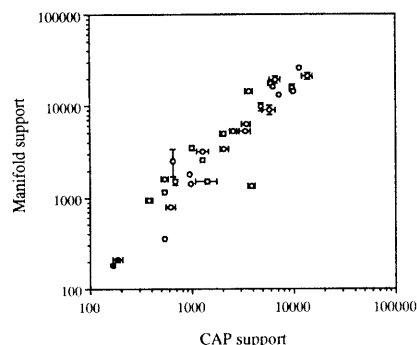


Fig. 6. Comparison of results obtained in assays for IgE reactivity to soy protein among 32 serum samples using a commercial test format, the Pharmacia CAP System, and the present manifold support. Results using serum samples from two non-allergic subjects are shown as filled circles in the lower left hand part of the figure.

tially trap specific antibodies from serum samples, expose the bound antibodies to a labelled reagent with specificity for the antibodies, and develop the signal. The strength of this strategy lies in the possibility of performing large numbers of reactions in parallel, moving between consecutive reaction steps with little or no pipetting, and with a concomitant reduction in sample mix-ups and/or contamination.

The problem of attaching sufficient quantities of reactant to the support has been solved by greatly expanding the surface available. This was effected by partially embedding porous particles on the surface of the plastic support by a simple procedure. We have previously established that this method of immobilizing proteins results in a greatly increased capacity over that achieved by non-specific adsorption to the same polystyrene support or to microtiter wells. Prongs with avidin-conjugated particles exhibited a 15-fold greater biotin-binding capacity compared to the best microtiter wells passively coated with avidin (Parik et al., 1993) and large batches of particles may be conjugated with antigen and stored for later immobilization to the manifold. The batch-wise modification of the support simplifies the

standardization of assays, resulting in consistent results both within and between different experiments. The suspension of protein-conjugated Sepharose particles in triethylamine did not appear to affect the function or antigenic determinants of any of the proteins used. In addition to the antigen preparations investigated here we have used the same immobilization technique for avidin (Parik et al., 1993), streptavidin, and for IgG and protein A (J.P. unpublished data) with excellent results. As demonstrated here, an avidin- or streptavidin-conjugated support can also be used to bind any molecule that has been modified with biotin.

The present assay takes approximately 3.5 h to perform but the time can be reduced to 1 h, whilst preserving good detection sensitivity. Using time-resolved fluorescence detection of rare-earth metal ions, at least three different colors are available, permitting the simultaneous analysis of different substances in the sample, e.g., different antibody classes with similar specificity. Depending on the configuration of the support, any number up to 96 or more samples can be analyzed in parallel, or several of the prongs can be incubated in the same serum sample, either sequentially or simultaneously. Different prongs of a manifold may be coated with the same or with different antigen preparations. In this manner a single serum sample may be investigated for evidence of antibodies to a large repertoire of antigens.

We have successfully used the same type of support for routine molecular genetic reactions, including the distinction of allelic nucleotide sequence variants using a ligase-mediated assay (Samiotaki et al., 1994), and the preparation of DNA sequencing templates, performing the sequencing reactions, and loading these on an automated sequencer (Lagerkvist et al., 1993). The possibility of immobilizing high concentrations of reactants on very limited surfaces will be of general value in attempts to miniaturize assay formats and perform large numbers of reactions in a

wide range of analytical and preparative situations.

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