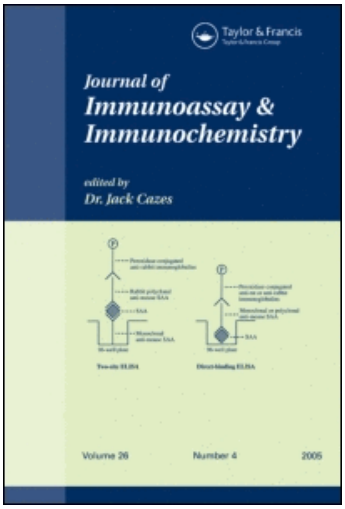

Journal of Immunoassay and Immunochemistry

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 Cleveland, Patrick H. , Wickham, M. Gary , Goldbaum, Michael H. , Ryan, Alan F. and Worthen, David M.(1981) 'Rapid and Efficient Immobilization of Soluble and Small Particulate Antigens for Solid Phase Radioimmunoassays', Journal of Immunoassay and Immunochemistry, 2: 2, 117 — 136

To link to this Article: DOI: 10.1080/15321818108056972

URL: http://dx.doi.org/10.1080/15321818108056972

JOURNAL OF IMMUNOASSAY, 2(2), 117-136 (1981)

RAPID AND EFFICIENT IMMOBILIZATION OF SOLUBLE AND SMALL PARTICULATE ANTIGENS FOR SOLID PHASE RADIOIMMUNOASSAYS

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(KEY WORDS: Antigen Immobilization, Staphylococcal Protein A)

ABSTRACT

A rapid method of antigen immobilization (10 min.) was developed using soluble antigens (bovine serum albumin, epidermal growth factor, and goat IgG) and small particulate antigens (Keyhole limpet hemocynanine and E. Coli) by drying them on filter paper discs. This technique results in a high % of the soluble antigen remaining firmly bound, goat IgG (89%), bovine serum albumin (73%). All the antigens we tested retained their antigencity after drying as detected by [^{125}I] labeled staphylococcal protein A radioimmunoassay. This method of antigen immobilization was compared to adsorption to plastic wells and was found to be much faster (10 min vs 18 hrs) and was 5 times more efficient than adsorption to plastic wells. Using this technique, we were able to detect as little as 40 ng of bovine serum albumin. These characteristics suggest that this technique of soluble antigen immobilization may be useful in rapid detection of antibodies to many different antigens, as well as detecting ng amounts of the antigens directly.

INTRODUCTION

The central feature of many immunoassays is how the antigen, or antibody or the antigen-antibody complex is immobilized. Presently immunoassays for antibodies to soluble antigens are usually done in one of two ways: 1) by labeling the soluble antigen with a radioactive isotope or enzyme, then reacting the test antibody with the labeled antigen. The labeled antigen-antibody complexes are separated from free labeled antigen by centrifugation after precipitating the complex with a) antiglobulin, b) polyethylene glycol, c) NH_4SO_4, d) charcoal or e) staphylococcus aureus bacteria; 2) by immobilizing the soluble antigen prior to assay (solid phase technique). This is done by covalently linking it to a solid substrate or adsorbing it to plastic wells, tubes or beads. Antibody is allowed to react with the immobilized antigen, the solid phase is washed and the amount of antibody bound determined by incubation with labeled antiglobulin or staphylococcal protein A (SPA).

The problems associated with these methods are as follows: centrifugation is cumbersome and slow especially if there are many samples involved; covalent binding procedures are complex and time consuming; adsorption to plastic requires at least three hours incubation and often overnight incubation is mandatory. The efficiency of the adsorption process is also highly dependent upon the plastic, edge effect, the time of incubation, pH and temperature, thus making well to well and plate to plate reproducibility difficult (1,2,3,4). Furthermore, the process is inefficient,

(3); this is an important consideration if the antigen is precious.

This paper describes a simple microfiltration procedure which immobilizes soluble or small particulate antigens by drying them on filter paper discs. This procedure employs a specially designed immunofiltration manifold (5) which greatly facilitates incubation and wash operations.

The purpose of the present study was to standardize the methodology of drying soluble antigens to filters, to apply the [^{125}I] SPA filter technique to the detection and quantitation of antibodies to soluble antigens, and to compare the sensitivity of this assay to assays where the antigen was adsorbed to plastic.

METHODS AND MATERIALS

Antigens

Bovine serum albumin (BSA) Cohn fraction V was purchased from Sigma Chemical Co., St. Louis, MO. Goat IgG was purchased from Antibodies, Inc. Davis, CA. epidermal growth factor (EGF) was purchased from Collaborative Research, Inc., Waltham, MASS. keyhole limpet hemocyanine (KLH) was purchased from Pacific Biomarine, Newport Beach, CA. *E. coli* was grown in trypticase soy broth.

Antisera

Antiserum to BSA or *E. coli* was prepared by repeated subcutaneous and footpad inoculation of young adult male Dutch belted rabbits with 100 ug of BSA or 10^8 heat killed *E. coli* in com-

plete Freunds adjuvant. Antiserum to KLH was prepared in guinea pigs by a single intradermal inoculation of 1mg of KLH. Preimmune serum was obtained from the same animals and used as the control serum. Rabbit anti-EGF serum was obtained from Collaborative Research, Inc.

Iodination of BSA, IgG, and SPA

BSA was labeled with [^{125}I] by the chloramine-T method of Hunter and Greenwood (6) as modified by Dorval, et al. (7). The [^{125}I] BSA had a specific activity (assuming 100% recovery) of 6 X 10^3 CPM/ng. Goat IgG was labeled with [^{125}I] by the same method. The [^{125}I] IgG had a specific activity (assuming 100% recovery) of 1.47 x10^4 CPM/ng. Purified freeze-dried SPA was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden and radioiodonated by the same method. The [^{125}I] SPA had a specific activity (assuming 100% recovery) of 3.87 x 10^4 CPM/ng.

The Design and Operation of the Diagnostic Immunofilter Manifold (DIFM)

The DIFM (V&P Enterprises, San Diego, CA.) consists of a filter manifold with 96 wells each with a small hole in the bottom (modified microtiter plate) and a vacuum manifold (5). The wells were loaded with pure cellulose filter paper discs VP102 or VP104. Because the hole in each well was small, surface tension forces retained the liquid in the well until vacuum was applied. Upon application of 380 mm Hg of vacuum, 0.3 ml of buffer wash can be drawn through each of the 96 filters within 5 sec, thus efficient-

ly removing soluble material. Rapid washing of all 96 filters in a 10 sec. period was achieved by adding 0.3 ml of buffer/well using a Cornwall repeating syringe with an 8 channel dispensing manifold.

[^{125}I] SPA Filter Assay

The [^{125}I] SPA filter assay was performed as illustrated in Figure 1. Two ul of various concentrations of the antigens in 0.01 M Physiologically buffered saline pH 7.4 (PBS), were added to each filter using a 100 ul Hamilton syringe with a repeating dispensor. The filter paper was dried by allowing the vacuum to draw air through the filters for 10 min. The filters were rehydrated with 0.1 ml of Ovalbumin (OA) buffer (PBS containing 1% OA, 0.3%

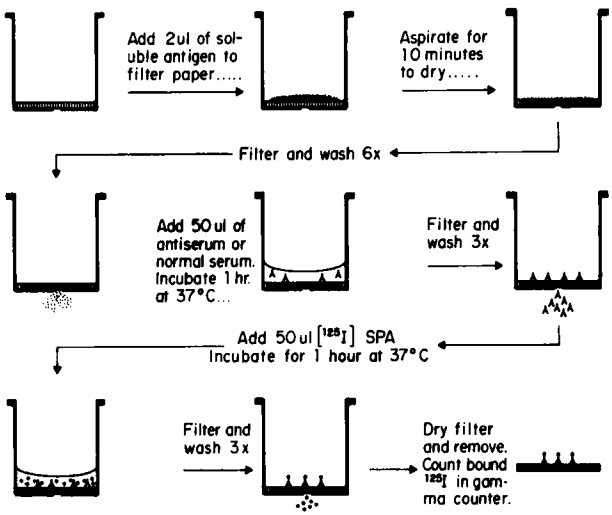

Figure 1. Flow diagram of the [^{125}I] SPA immunofiltration assay illustrating the various procedural steps.

gelatin and 0.01% sodium azide) when BSA was the antigen. When EGF, goat IgG, E. coli or KLH were the antigens, BSA was used in the buffer rather than OA. After 15 min., vacuum was applied and the fluid removed by filtration and the filters were washed 6 times with 0.3 ml of gelatin buffer (PBS containing 0.3% gelatin and sodium azide). Foam was removed from the outside of the well bottoms by brief aspiration with a Pasteur pipet. Fifty ul of control or immune serum diluted in OA buffer were added to quadruplicate wells that contained antigen and also to wells that did not, and the filter manifold was incubated at 37°C for 1 hr. The unbound serum was removed by filtration and the filter washed 3 times with 0.3 ml of gelatin buffer, and the foam removed from the outside of the well bottoms. Next, 50 ul of [^{125}I] SPA in OA buffer (50,000 CPM) was added to each well. The filter manifold was then incubated at 37°C for 1 hr. The unbound [^{125}I] SPA was removed by filtration, the filter washed 3 times with 0.3 ml of gelatin buffer, and dried by allowing the vacuum to draw air through it for 3 to 4 min. The filters were removed from the manifold using jewelers forceps, placed in tubes, and the amount of bound [^{125}I] SPA determined with a Searle model 1195 automatic gamma counter.

[^{125}I] SPA Adsorption Assay

This assay was conducted in U bottom microtiter plates (#1-220-24, Dynatech Labs, Alexandria, VA). Two ul of various concentrations of BSA in 0.1 M carbonate buffer with 0.02% sodium azide pH 9.6 (8) were added to the wells. The BSA was allowed to

adsorb to the plates for 3 hrs at 37° and then 18 hrs at 4° C. The wells were aspirated and washed once with 0.3 ml of gelatin buffer and conditioned for 15 min with OA buffer. Next the wells were washed 6 times with gelatin buffer. Fifty ul of control or immune serum diluted in OA buffer were added to quadruplicate wells containing the adsorbed BSA and also to wells with no BSA and incubated at 37°C for 1 hr. The wells were washed 3 times with 0.3 ml of gelatin buffer and 50 ul of [^{125}I] SPA in OA buffer (50,000 CPM) was added to each well. After incubating for 1 hr at 37°C the plates were washed 3 times with gelatin buffer and dried. The wells were cut from the plate and the amount of bound [^{125}I] SPA determined.

Expression of Results and Statistical Analysis

The results were expressed as mean CPM $\pm$ (SD) of [^{125}I] SPA bound. The values were corrected for nonspecific binding to the filter paper or well by subtracting the mean CPM of [^{125}I] SPA bound to filters or wells which did not have antigen present but were incubated with the various serum dilutions (i.e., background corrected). The significance of the differences in [^{125}I] SPA binding between control and immune sera was determined using Student's t test. The titer of a serum was determined as the last dilution where the background corrected binding of the test serum was 500 CPM greater than the control serum, and the $P < 0.01$.

Elution of Immobilized [^{125}I] BSA

[^{125}I] BSA was immobilized by either drying on filters or adsorbing to wells as described previously for unlabeled BSA.

The following solvents were then added to the filters or wells and incubated for 15 min in an attempt to resolubilize the [^{125}I] BSA; 0.2M glycine-HCL buffer, pH 2.2, 1.0 N NaOH, pH 14.0, OA buffer, pH 7.4, 3.5 M $MgCl_2$, pH 7.1, 0.4 M KPO_4, pH 4.3, 0.1 M lithium 3,5-diiodosalicylate, pH 5.2, 0.1 M EDTA, pH 6.6, 0.1% Tween 20 in OA buffer, pH 7.4, 0.1% sodium dodecyl sulfate in OA buffer, pH 7.4, 0.1% triton X-100 in OA buffer, pH 7.4, Freon T.F., acetone, and ethanol.

After the incubation period the filters or wells were washed six times with the respective solvents, and the percentage of [^{125}I] BSA which remained on the filters or wells was determined.

RESULTS

Effect of BSA Concentration on the Efficiency of Immobilizing Soluble Antigens

We compared the relative efficiency of immobilizing [^{125}I] BSA by drying it on filter paper versus adsorption to plastic wells. In these experiments we prepared BSA mixtures in PBS or 0.1 M carbonate buffer which contained a constant amount of [^{125}I] BSA with varying concentrations of cold BSA. After the mixtures were immobilized on the filters or wells, they were washed 6 times and the amount of [^{125}I] BSA bound was determined. The results of these experiments are illustrated in Figure 2. We found that the efficiency of [^{125}I] BSA binding to the filter paper remained relatively constant from 5 to 300 ug/ml, with an average efficiency of 75%, but at concentrations greater

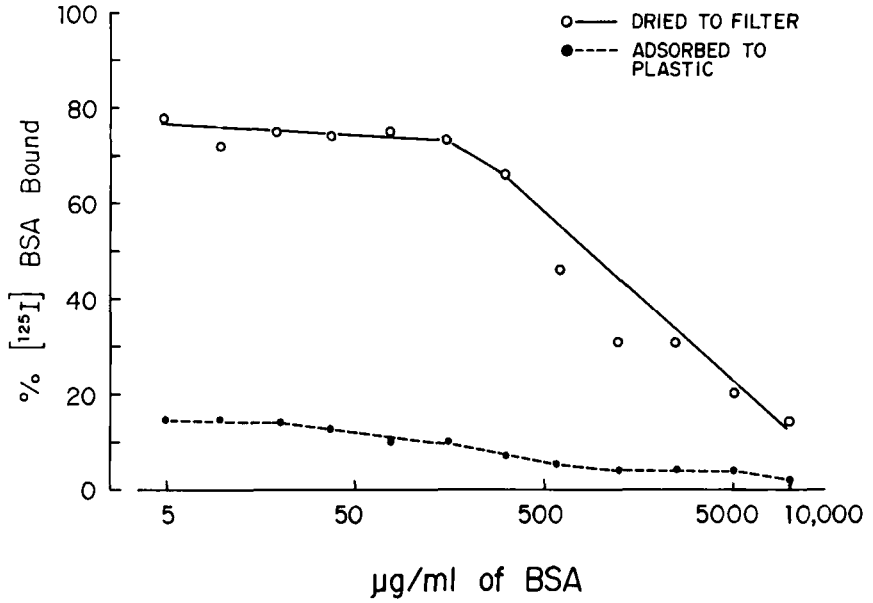

Figure 2. Effect of BSA concentration on the amount of [^{125}I] BSA immobilized by drying to the filter or adsorbing to the plastic wells. Mixtures of [^{125}I] BSA with cold BSA in either PBS (filters) or carbonate buffer (plastic wells) were prepared so they contained a constant concentration of [^{125}I] BSA with varying concentrations of cold BSA, and then were immobilized as described in the materials and methods.

than 600 ug/ml the efficiency steadily decreased, until it was only 14% at 10,000 ug/ml. The efficiency of [^{125}I] BSA immobilized by adsorption to plastic wells was only 15% over the range of 5 to 20 ug/ml and steadily decreased with increasing concentration of BSA until it was only 2% at 10,000 ug/ml.

Ability of Various Solvents to Elute Bound [^{125}I] BSA

We attempted to determine how the [^{125}I] BSA was bound to the filter paper by eluting with a variety of solvents which

contained either detergents, high salt concentration, high ionic strength, acid, alkali, disassociating agents, chelating agents and organic solvents described in the materials and methods. The results of these experiments demonstrated that only the detergents removed more [^{125}I] BSA than that normally removed by OA buffer (Tween 20 - 50%, SDS - 59% and Triton X100 - 72%).

Kinetic Studies on Elution of Antigens

The effect of conditioning time on the elution of BSA from filters was tested in time sequence experiments where OA buffer was incubated for 15 min to 2 hr on VP#102 filters to which [^{125}I] BSA was dried. A similar time sequence experiment was performed where BSA buffer was incubated for 15 min to 18 hr on VP#104 filters to which [^{125}I] IgG was dried. The results of these experiments indicate that longer conditioning times only slightly decreased the percentage of [^{125}I] BSA or [^{125}I] IgG bound to the filters (Table 1). Moreover, the coefficient of variance for the [^{125}I] IgG experiment was never larger than 3%.

Effect of BSA Concentration on Immune Binding

To optimize the concentration of BSA or EGF dried on the filters, we added varying concentrations of BSA or EGF to replicate wells. After drying, the unbound BSA or EGF was eluted and the filters incubated with either a 1/1000 dilution of anti-BSA serum, or anti-EGF serum or a 1/1000 dilution of preimmune serum or OA buffer for 1 hr. and then with [^{125}I] SPA for 1 hr. We also performed a parallel experiment where 2 ul aliquots of vari-

TABLE 1

STABILITY OF ANTIGEN BINDING TO FILTER PAPER

Elution time[c]	% of [^{125}I] IgG bound to filter[b]	% of [^{125}I] BSA Bound to filter[a]
15 min	89	72
30 min	90	73
2 hr	91	69
4 hr	85	NT[d]
18 hr	84	NT

[a] Eight ng of [^{125}I] BSA (2,944 CPM) were dried on VP#102 filters and then OA buffer was added. [b] Seven ng of [^{125}I] IgG (16,602 CPM) were dried on VP#104 filters and then BSA buffer was added. [c] The filters were conditioned for 15 min to 18 hrs then washed 6 times with gelatin buffer and the % of [^{125}I] BSA or [^{125}I] IgG still remaining bound was determined. [d] NT= not tested.

ous concentrations of BSA were adsorbed to plastic wells. The filters or wells treated with the antigens plus preimmune serum or those left untreated plus immune serum, bound less than 300 cpm [^{125}I] SPA in both the filter and adsorption experiments, regardless of antigen concentration: The [^{125}I] SPA bound to filter or wells treated with antigen plus immune serum increased steadily with increasing antigen concentration (Figure 3). The

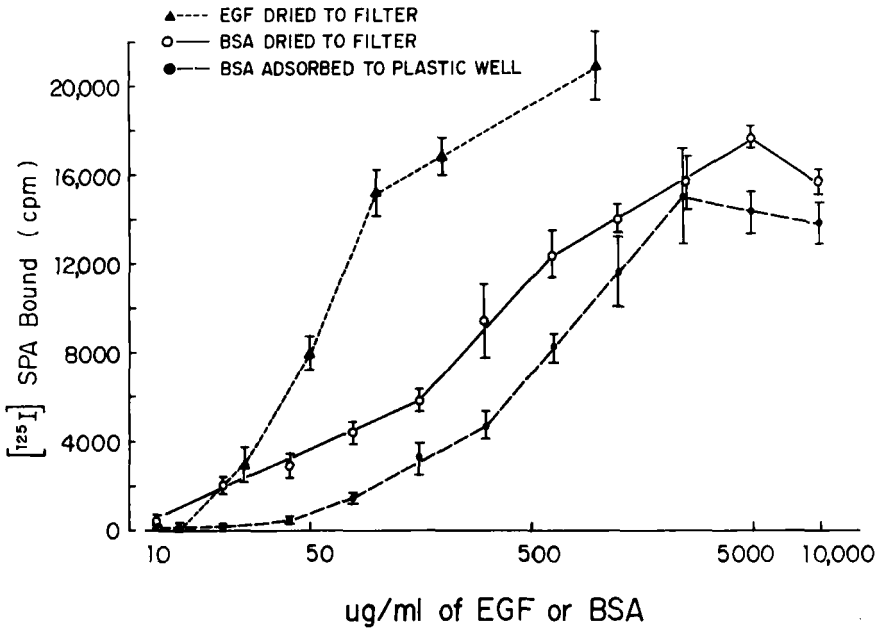

Figure 3. Effect of BSA or EGF concentration on the amount of immune binding with anti-BSA or anti-EGF as detected with [^{125}I] SPA, using the immunofiltration method to immobilize the BSA (o—o) and EGF (▲---▲). A parallel experiment was also performed where BSA was adsorbed to plastic wells (●--●). After various concentrations of BSA were immobilized and the filters or wells washed to remove the unbound BSA or EGF, a 1/100 dilution of anti-BSA or anti-EGF was added to all the wells and [^{125}I] SPA assays were conducted as described in the materials and methods.

filter method was able to detect as little as 40 ng of BSA and 50 ng of EGF, while the smallest amount of BSA detected by the adsorption method was 160 ng. Using extrapolation techniques it was shown that for most of the BSA concentration range the adsorption technique required 4 to 5 times as much BSA to bind an equivalent amount of [^{125}I] SPA as the filter method.

Comparison of Assay Sensitivity on Filters Versus Wells

The titer of a single anti-BSA serum and its corresponding preimmune serum (normal) were determined using [^{125}I] SPA assay employing both the filter and well adsorption methods of immobilizing the BSA. By the well adsorption method the titer was 1/25,000 and by immunofiltration 1/130,000 or 5.2 times greater (Figure 4). The activity of the normal serum was insignificant in

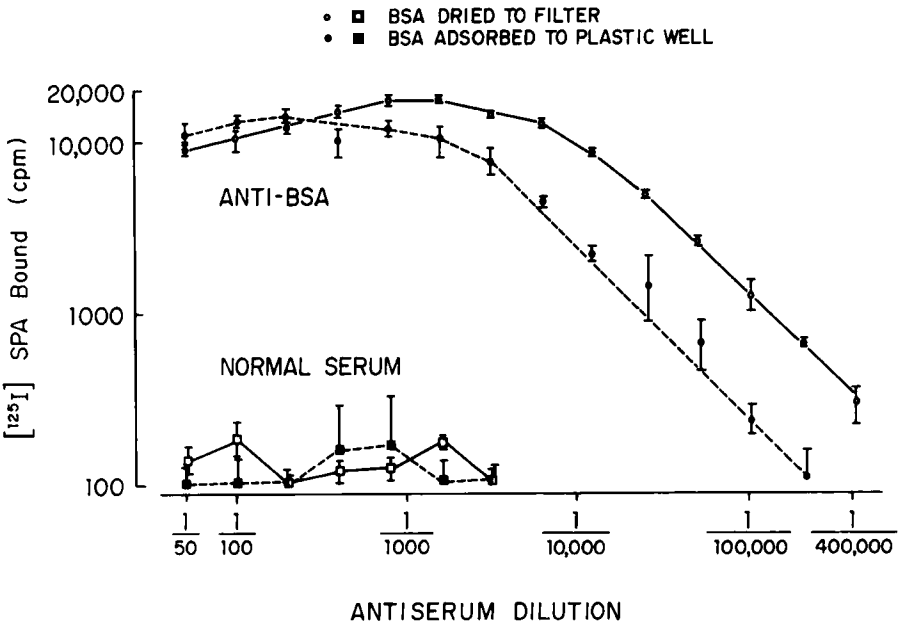

Figure 4. Sensitivity comparison of immunofiltration and plastic adsorption assays. Dilutions of an anti-BSA serum (o—o) and its corresponding preimmune serum (□—□) were tested in a [^{125}I] SPA assay where BSA (2,500 ug/ml) was immobilized by drying to filters. In a parallel experiment conducted concurrently the same anti-BSA serum (●---●) and corresponding preimmune serum (■---■) were tested in a [^{125}I] SPA assay where BSA (2,500 ug/ml) was immobilized by adsorption to microtiter wells.

both the assays even at low dilution (less than 200 cpm of [^{125}I] SPA bound).

Application of the Drying Technique to Small Particulate Antigens

We also demonstrated that small particulate antigens such as KLH and E. coli bacteria could be immobilized by drying on the filters. In these experiments as few as 25,000 E. coli cells could be detected using the [^{125}I] SPA immunofiltration technique.

DISCUSSION

We have described a new assay methodology for performing solid phase radioimmunoasays, which significantly reduces the time required to immobilize soluble or small particulate antigens and the assay processing time. The assay employed a commercially available microfiltration device (5) which efficiently immobilized these antigens by drying them on to filter discs.

Antigens or antibodies have been immobilized for immunoassay by attaching to solid substrates using either adsorption or covalent coupling agents (9,10,11,12,13). Covalent coupling agents can be used to reliably attach antigens or antibodies to agarose or polyacrylamide beads, however the involved coupling protocols and long preparation times have compromised their usefulness in solid phase immunoassays. Because adsorption techniques do not have complex protocols they have been widely used in solid phase immunoassays, however, they also require long incubation periods in order to complete the adsorption process (2,3) and recently the reproductibility of this technique has been challenged (4).

To shorten preparation time and to simplify the procedure we compared drying BSA on filter discs versus adsorption to plastic wells. We demonstrated that not only was the filter drying procedure faster (10 min versus 3 to 18 hrs) allowing assays to be initiated and completed within 3 hrs, but it was also 5 times more efficient (75% vs 15%), and required 4 to 5 times less BSA to achieve the same degree of immune binding (Figure 3). We also noted a 5 fold increase in antibody assay sensitivity between the filter and plastic well adsorption assays (Figure 4). These later two observations also indicate that the BSA did not lose its antigenicity after being immobilized on the filters. Thus in systems where precious antigens are used such as EGF, the filtration technique offers a significant advantage. One of the reasons for the higher binding efficiency is probably due to the greater surface area provided by the filter. However the chemical make-up of the filter may also play a role in determining just how the antigens are bound, as we have noted significant differences in the degree of binding by different filter materials (unpublished observations). The observation that binding efficiency decreases with increasing concentration of BSA agrees with reports on albumin and immunoglobulin adsorption to plastic (3,14).

Although a number of elution experiments were performed to determine the nature of the bond that holds the dried BSA to the filter paper, we were not able to resolve this question. Perhaps there were several different binding or adsorption mechanisms operating in this system. However, other experiments elucidated

the salient points that are most germaine to solid phase immunoassays, 1) a high % of BSA was firmly bound to the filter, 2) the BSA retained its antigenicity, 3) it could not be eluted or desorbed from the filter after 2 hrs. incubation with OA buffer and 4) several different antigens could be used in this system.

Previous attempts to use filtration techniques for radioimmunoassays employing IgG have been disappointing because of high backgrounds caused by nonspecific binding of both the antiserum and labeled antiglobulin to the filter material (15,16). Green, et al. (15), were able to reduce this nonspecific binding somewhat by using polycarbonate membranes in place of mixed cellulose acetate/cellulose nitrate filters. However, the nonspecific binding was still unacceptably high. We have been able to markedly reduce nonspecific binding in our assay by judicious choice of certain cellulose filter material, by using buffers that contain gelatin and high protein concentration (BSA, OA or FCS), by using [^{125}I] SPA and higher dilutions of antiserum. In addition, we have shown (5) that the addition of Tween 20 to antiserum dilutions increases the nonspecific binding of IgG to filter material. This observation may explain, at least in part, the high background binding to filter materials which have previously been reported, because Tween 20 has been widely used as a wetting agent in immunologic assays.

We have found [^{125}I] SPA to be a very useful reagent because it has a high (40 to 50%) specific binding efficiency and because it can bind to the immunoglobulins of many mammalian

species, whereas antiglobulins are species specific. Others have used it to measure the binding of IgG to many different antigens, and the sensitivity achieved has been equal to or greater than that achieved with [^{125}I] antiglobulin (1,17,18,19,20). Another important advantage of SPA is that its tendency to bind nonspecifically is lower than that of antiglobulin (17,18,21). Furthermore Kessler (17) has reported that the binding affinity of SPA is significantly greater for antibody which is complexed with antigen than uncomplexed antibody.

In terms of processing the assays, the immunofiltration procedure also has several advantages over adsorption in microtiter plates: 1) it is easier and faster to place the filters in tubes for gamma counting than to cut out the plastic wells and place them in tubes; 2) an incubation fluid can be removed and the filters washed three times within 45 sec, and if infectious reagents (such as human serum which may contain infectious hepatitis) or radioactive compounds are used, then they are safely confined to the vacuum trap. With the microtiter plates, the reagents and wash solutions may be merely "flipped" out into a sink. However, they must be laboriously aspirated if they contain a biohazard or radioactive compound.

In summary we demonstrated that drying soluble or small particulate antigens to filter discs was an efficient and rapid method of immobilizing these antigens. The [^{125}I] SPA filter assay was simple to perform, required only small volumes of antiserum and ng quantities of soluble antigen, and yielded results

which were readily and objectively quantitated. It can easily accomodate 768 specimens and is amenable to automation.

ACKNOWLEDGEMENTS

This work was supported in part by the Veterans Administration and by USPHS Research Grant no. EY-02434-02 and by a grant from the NIH-NINCDS, 5 K04 NS00176.

We thank Cathy Amundsen Vincent for her excellent technical assistance.

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