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Enhanced sensitivity of immunoblotting with peroxidase-conjugated antibodies using an adsorbed substrate method

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ABSTRACT

Immunoblots probed with immunoglobulin E (IgE)-containing sera from allergic patients are frequently used in allergy research. Current techniques for detection of specific IgE include radiolabeled and enzyme-linked methods. Although radiolabeled methods are very sensitive, many research groups prefer non-radioactive procedures with equal or greater sensitivity. Alkaline phosphatase (AP) and horse radish peroxidase (HRP) are the most frequently used conjugating enzymes for immunoblotting with the former generally recognized as more sensitive. We describe a method of immunoblot detection using HRP-conjugated immunochemicals with sensitivity equal to and for some systems greater than that of AP conjugates. An adsorbed substrate method for developing immunoblots probed with HRP immunochemical conjugates is compared with traditional AP and HRP methods. The adsorbed substrate system, when used to detect IgE binding to allergenic proteins, gives high resolution and delineates bands not otherwise seen. The system has advantages of high sensitivity, rapid development and conservation of immunochemicals. Problems of fading, sensitivity to heat and light, and high background can be solved with increased washing, prompt photography and computer scanning.

INTRODUCTION

Since Towbin *et al.* [1] first immobilized proteins on nitrocellulose, immunoblotting has become a frequently used tool for allergen research. Detection methods for blotted allergens ultimately must employ either radiolabeled or enzyme-conjugated proteins. Radiolabeling for detection, although exquisitely sensitive, is hampered by safety concerns and the production of radioactive waste products. Enzyme-linked methods have largely been adapted from histochemical staining techniques and, as a result, sensitivity is often compromised in favor of permanency and clarity of the end point. The most frequently used conjugating enzymes for immunoblotting are alkaline phosphatase (AP) and horse radish peroxidase (HRP) [2]. In both cases the final image is developed by producing an insoluble colored product either directly from the reaction of the enzyme conjugate with a suitable substrate or indirectly by reacting the proper chromagen with one of the products of the enzymatic reaction. For AP-conjugated systems there is general consensus that the best method utilizes the substrate 5-bromo-4-chloro-3-indole phosphate (BCIP) and derives the chromagenic material from Nitro Blue Tetrazolium (NBT) [3]. There is, however, no general consensus as to the best system for HRP conjugates. The leading candidates both utilize hydrogen peroxide as substrate and either 3,3′-diaminobenzidine (DAB) [4], 3,3′,5,5′-tetramethylbenzidine (TMB) [5] or 4-chloro-1-naphthol (CN) [6] as the chromagenic agent. DAB and TMB are reported to have the same sensitivity [7] and by far the most frequently used HRP chromogenic

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agents are DAB and CN [8]. The AP systems are considered to be more sensitive than the HRP methods [7] and the two benzidine methods for HRP, which are about equal in sensitivity, are superior to the CN methods [9].

Recently, two methods have evolved that do not have their roots in histochemistry but have resulted from the search for non-radioactive methods with sensitivity approaching the radiolabeled probes. The first of these methods is based on a chemiluminescence model [10]. Although the sensitivity of this method is reportedly better than AP, it does require ready access to dark room and film developing equipment. The second of these methods utilizes a commercially available enzyme substrate complex adsorbed on a plastic (WEB) matrix and requires only the ability to photograph the results in a timely manner. Additionally, the developed blot may be analyzed directly by computerized imaging if that is available to the researcher. To compare this adsorbed substrate method with the two most popular HRP methods and the traditional AP method the following studies were conducted.

EXPERIMENTAL

Chemicals and reagent

BCIP, NBT, DAB, TMB and CN chromagenic agents were obtained from Sigma (St. Louis, MO, USA). HRP- and AP-conjugated goat anti-human immunoglobulin E (IgE) were also obtained from Sigma. The prepared adsorbed substrate (Enzygraphic WEB) was obtained from International Biotechnologies (New Haven, CT, USA). All blotting solutions were made with deionized, glass-distilled water. Use of sodium azide as a preservative was strictly avoided.

Alternaria alternata growth and extraction

Several *Alternaria* extracts were obtained from Bob Esch (Greer Labs., Lenoir, NC, USA). The mold was grown for four weeks on a chemically defined sucrose medium (0.3% $NaNO_2$, 0.05% $MgSO_4$, 0.05% KCl, 0.01% K_2HPO_4, 3% sucrose, and trace elements consisting of 1.8 m*M* $Fe(NO_3)_3$, 0.097 m*M* $ZnSO_4$, 1.8 *M* $MnSO_4$) [11]. The cultures were exposed to 12-h periods of alternating light and dark during their growth. Fungal mats produced contained both spores and mycelia. The cultures were air-dried at 37°C and powdered with a mortar and pestle. Each preparation was extracted in 1:10 (w/v) distilled water at 4°C for 3 h and filtered with Whatman 4 filter paper followed by progressively finer pore size filters (Millipore, Bedford, MA, USA). After passing through a 0.45-μm filter, the clear extracts were dialyzed against 50 m*M* ammonium bicarbonate (pH 8.2) at 4°C for 48 h, and used or lyophilized. Lyophilized samples were stored in sealed bottles at −20°C. Reconstituted samples were kept at 4°C.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on vertical slab gels (Bio-Rad) was performed using the method of Laemmli [12]. Prestained molecular mass markers were obtained from Bio-Rad. Samples were reconstituted to 1 mg/ml from a lyophilized state using distilled water. Samples (10 μg) were diluted in 30 μl of sample buffer containing mercaptoethanol and denatured by boiling for 2 min. SDS-PAGE gels were blotted onto nitrocellulose using the method of Towbin *et al.* [1]. Typically blotting was carried out at 60 V for 1 h. Proteins were quantified by the use of a commercial Lowry kit [13] (Sigma).

Spot blots

For initial spot testing, serial dilutions of *Alternaria* were spotted directly onto nitrocellulose (Fig. 1). Spotted nitrocellulose was then blocked with 5% non-fat dry milk and probed with human IgG directed against *Alternaria*. The nitrocellulose sheet was then cut into strips each containing a series of dilutions and probed either with HRP- or AP-conjugated goat anti-human IgG. Probed strips were developed with either adsorbed substrate or BCIP–NBT for AP conjugates, or with DAB for HRP conjugates. For initial IgE testing, serial dilutions of human IgE-containing serum were spotted onto nitrocellulose (Fig. 2). Care was taken to insure that the

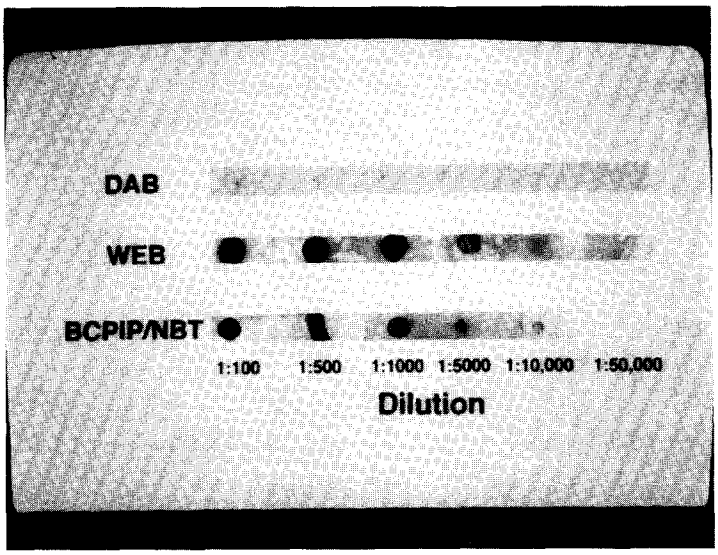

Fig. 1. Spot blot comparison of three staining methods: DAB, BCIP–NBT and WEB (adsorbed substrate). Five-fold dilutions of *Alternaria* are spotted onto nitrocellulose, probed with human IgG. The nitrocellulose is cut into strips and detected with the three different methods.

same volume was used on every spot. Spotted nitrocellulose was then blocked with 5% non-fat dry milk and probed with HRP-conjugated goat anti-human IgE. Probed strips were developed with adsorbed substrate and the diameter of the spot was correlated with dilution (Fig. 3).

Immunochemical methods

Blot development methods using DAB without nickel chloride, CN and BCIP–NBT were taken from Harlow and Lane [3]. Human sera containing antibodies to *Alternaria* were drawn from volunteers immunized with *Alternaria* extracts or

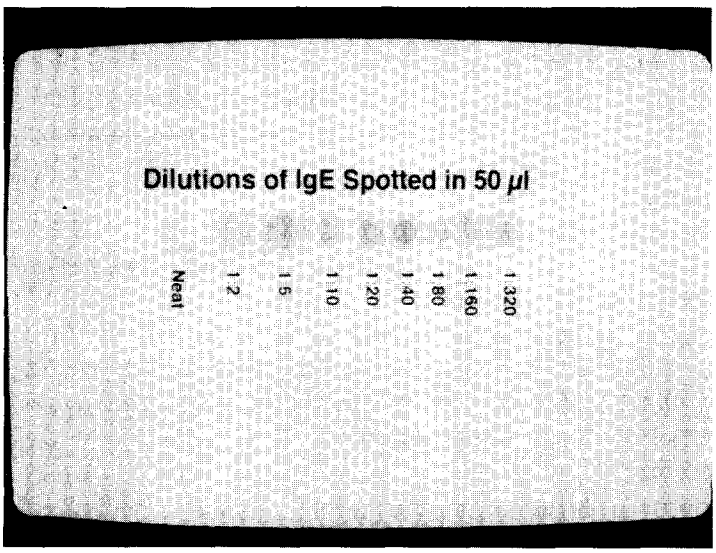

Fig. 2. Spot blot comparison of human IgE detected by the adsorbed substrate method. Serum containing IgE is diluted and identical volumes are spotted onto nitrocellulose, probed with HRP-conjugated anti-human IgE and detected with the adsorbed substrate method.

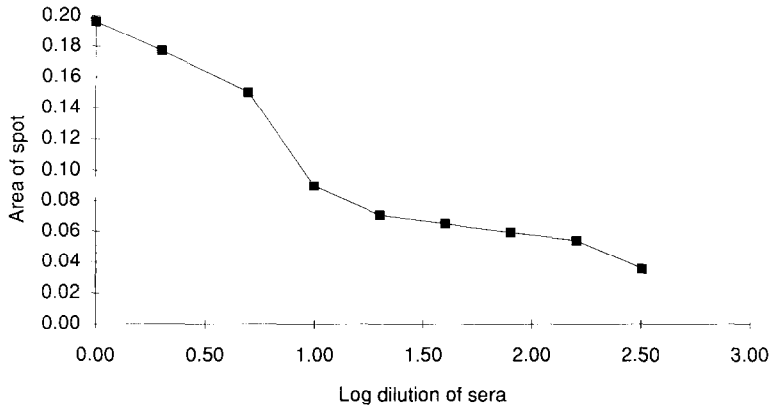

Fig. 3. Graph of IgE spot diameter *versus* dilution. The area of the spots in Fig. 2 is correlated with the log of the dilution of the sera.

from individuals who had positive skin tests to *Alternaria* extract and whose sera also gave a strong enzyme-linked immunosorbent assay (ELISA) test for *Alternaria*-sensitive IgE. Washing and antibody incubation methods for the Enzygraphic WEB were taken from IBI Technical Bulletin 65-1. Blots were blocked for a minimum of 30 min with 5% non-fat dry milk (Carnation). After blocking, blots were washed by vigorous agitation for at least 1 min with three changes of Tris-buffered saline (TBS) containing 0.3% Tween 20 (TTBS). Primary antibody or human serum containing IgE was dissolved in TTBS and applied to blots for at least 2 h. The typical primary antibody application would contain 5–50 μg of antibody or 1–2 μg of IgE in 15 ml of TTBS for a 6 cm × 10 cm blot. After washing as before, secondary antibody was dissolved in TTBS about two times more dilute than the recommendation of the supplier (if the manufacturer recommended 1 μg/ml we would use the antibody at 0.5 μg/ml). The final wash was with three changes 15–20 min each in TTBS with agitation at room temperature and an additional three changes 10 min each in TBS alone with agitation. The wet blot was then placed onto filter paper, and excess moisture was removed until the surface of the blot was uniformly damp. The WEB substrate was then removed from the freezer and the blot was overlayed with the emulsion side of the plastic matrix. The matrix was pressed firmly onto the blot forceing all trapped air out. The blot was allowed to develop at room temperature until the background began to turn from white to light blue.

Computer and photographic methods

All blots were photographed several times during color development using a standard 35-mm SLR camera and copystand set-up. Photographs were taken under tungsten light using a tungsten-balanced Ektachrome film (Kodak Ektachrome T 160). Blots were then stored in the dark at −20°C until they were scanned into a Macintosh computer using a Microtek flat bed scanner and Photoshop software. Scanned images were analyzed with the NCSA GelReader for Macintosh (National Center for Supercomputing Applications, University of Illinois, Urbana-Champaign, IL, USA).

Human volunteers

All clinical samples were collected after obtaining approval from the pediatrics review board of the University of Missiouri at Kansas City Medical School (Kansas City, MO, USA). Written informed consent was obtained before any experiment was performed.

RESULTS

The dot blot comparison of DAB, BCIP and WEB (Fig. 1) clearly demonstrates that the adsorbed substrate system is 50–100 times more

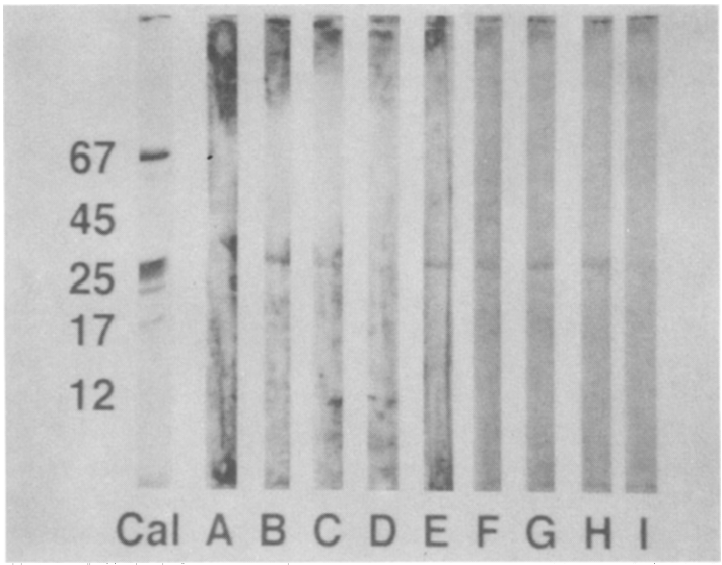

Fig. 4. SDS-PAGE comparison of AP and WEB methods. A sample of *Alternaria* is applied across the top of a SDS-PAGE gel, electrophoresed and blotted onto nitrocellulose. The blot is cut into strips and each strip is probed with a different dilution of human sera: lanes A and E with 1:10, lanes B and F with 1:100, lanes C and G with 1:1000, lanes D and H with 1:10 000 and lane I with 1:100 000. Lanes A, B, C and D are probed with AP-conjugated anti-human IgG and developed with BCIP–NBT. Lanes E, F, G, H and I are probed with HRP-conjugated anti-human IgG and developed with adsorbed substrate.

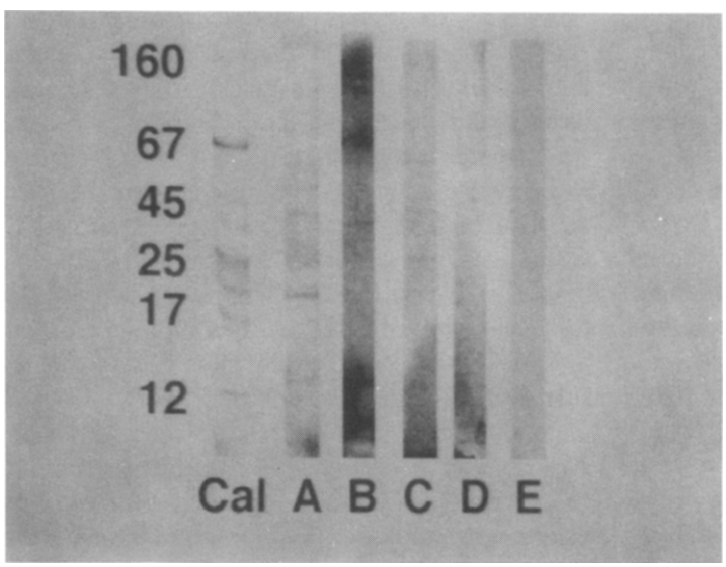

Fig. 5. SDS-PAGE comparison of WEB, HRP and AP methods. A sample of *Alternaria* is applied across the top of a SDS-PAGE gel, electrophoresed and blotted onto nitrocellulose. The blot is cut into strips. Strip A is protein-stained using colloidal gold. Strip B is probed first with human sera, second with HRP-conjugated anti-human IgE and developed with the adsorbed substrate. Strip C is probed first with human sera, second with AP-conjugated anti-human IgE and developed with BCIP–NBT. Strip D is probed first with human sera, second with HRP-conjugated anti-human IgE and developed with DAB. Strip E is probed first with human sera, second with HRP-conjugated anti-human IgE and developed with CN.

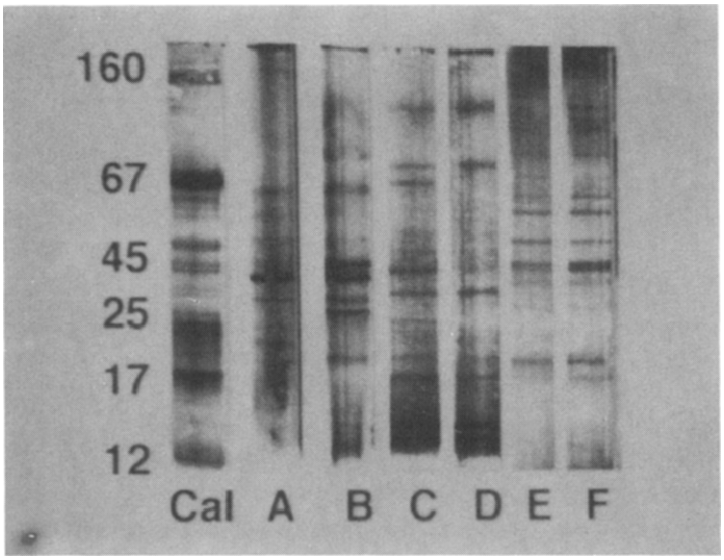

Fig. 6. IgE-probed SDS-PAGE blot of *Alternaria*. Extracted material from several strains of *Alternaria* is applied to a SDS-PAGE gel, electrophoresed, blotted onto nitrocellulose, probed first with sera from an *Alternaria*-sensitive individual, second with HRP-conjugated anti-human IgE and developed with adsorbed substrate. The calibration lane contains molecular mass markers. Lanes A, B, C, D, E and F contain extract obtained from six different strains of *Alternaria*.

sensitive than the other HRP substrate system and at least as sensitive as the AP substrate system. The *Alternaria* spot is delineated at a dilution of 100 μg/ml by the HRP-conjugated antibody using the DAB system and at a dilution of 1.0 μg/ml by the HRP antibody using the WEB system. The AP antibody delineates the spot clearly at 2 μg/ml and moderately at 1.0 μg/ml.

As an additional test of the sensitivity of the WEB detection system, serial dilutions of human IgE-containing sera were spotted onto nitrocellulose (Fig. 2). The same volume of sample was used on every spot. The diameter of the spot developed by the WEB substrate can be correlated with the log of the dilution in a linear fashion (Fig. 3).

SDS-PAGE blots of whole *Alternaria* probed with human IgG are compared using the HRP-conjugated antibody-adsorbed substrate system and the AP-conjugated antibody–BCIP substrate system (Fig. 4). The WEB substrate system identifies the major allergen at a 1:1000 dilution of human serum (1.0 ng/ml total IgE) as well as several minor allergens at higher serum concentrations. The AP–BCIP system is also able to delineate the major allergen at 1:1000 dilution of human serum (1.0 ng/ml total IgE) but minor allergen bands are not seen.

When IgE-probed SDS-PAGE blots of whole *Alternaria* extract are compared using the four substrate systems (Fig. 5) only the AP detection system and the adsorbed substrate system successfully exposed the allergens. The 31-kDa range allergen [14] and the 70-kDa range allergen [15] are both seen on each blot. The adsorbed substrate gave a stronger signal in the 70-kDa region and showed several minor bands that were not clearly visible on the AP substrate system.

When blots of extracts from several *Alternaria* strains are compared using the adsorbed substrate system (Fig. 6) the typical 70- and 31-kDa bands are seen as well as a larger number of secondary bands. These complex patterns are noted to vary from strain to strain both in the intensity of the major bands and in the weight of the stained protein.

DISCUSSION

One of the first observations we made when using the adsorbed substrate system was the rapidity of the color development. The signal-to-background ratio is most often greatest in 16 min. Care must be taken to prevent this contrast from deteriorating due to the increase in non-specific background. Elevated temperature and exposure to strong light or direct sunlight must be carefully avoided to obtain good results.

The sensitivity of the adsorbed substrate is clearly superior to that of any of the other HRP substrate systems and at least as good as the AP substrate system. This increased sensitivity can be especially valuable when immunochemicals are only available as HRP conjugates or when HRP is necessary due to significant inherent levels of the phosphatase enzyme in the material to be stained. An additional advantage of the adsorbed substrate system is that in many instances conjugated immunochemicals are required at levels up to five times more dilute than other methods. This can be a great advantage when immunochemicals are either expensive or available in limited quantities. Additionally many of the commonly used substrates are regulated carcinogens (DAB) and all are shipped with material safety data sheets and thus are regulated substances in the US and require proper handling and disposal.

The one potential disadvantage of the adsorbed substrate system is the rapid development of non-specific background upon exposure to high temperature or bright light. If left unattended, a very clear staining pattern can be obscured in a matter of minutes. In practical experience this has not been a problem in our laboratory. We routinely set up to photograph the blot before the WEB substrate is applied. When the blot is completely developed it is photographed and then stored at −20°C. We have stored blots for several weeks without deterioration of blotting patterns. At convenient intervals, blots are removed from the freezer and transported in a cool dark container to an adjacent building where they are scanned into digitized images for subsequent labeling and publication. Scanned images are routinely printed on a laser printer and these prints are pasted into laboratory notebooks.

The results of the IgE-probed blot of *Alternaria* allergic material (Fig. 6) revealed more bands and greater delineation of banding patterns than blots developed utilizing AP methods [16]. This sensitivity is very desirable when dealing with human IgE, for serum levels can be up to 100-fold less than equivalent IgG levels.

CONCLUSIONS

The adsorbed substrate system has the advantages of high sensitivity, rapid speed of development and conservation of immunochemicals. Problems of fading with time, sensitivity to heat and light and high background can be solved with increased washing, prompt photography and computerized scanning.

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