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Grid-immunoblotting: a fast and simple technique to test multiple allergens with small amounts of antibody for cross-reactivity

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Abstract

Grid-immunoblotting is a procedure that allows the simultaneous testing of up to 20 different antibodies such as monoclonal antibody-containing hybridoma supernatants or human sera for specific antibodies to up to 20 different antigens or allergens on a single sheet of nitrocellulose membrane. Since only 150 to 200 μl of antibody-containing solution are required this technique is uniquely suited to test growing hybridomas and small amounts of sera (e.g. mouse and children's sera). Compared to a standard ELISA, approximately ten times less antibody is needed to obtain the same information.

Keywords: Food allergy; Grid immunoblotting; Antibodies

1. Introduction

In allergy diagnosis and research, allergen identification and characterization, a frequently encountered problem is limited amounts of sera available or other antibody-containing solutions. This is especially true for sera from allergic children since the quantities of blood that can be drawn are small and the reluctance to draw frequently blood for research purposes. Furthermore, the detection of sensitization to multiple allergens may require substantial amounts of sera since serum dilutions used to detect specific IgE antibodies in sera from food-allergic subjects are usually not very high. Another situation often encountered in food allergy research is the evaluation of food-allergic subjects who are not anymore available for additional blood donations. Another area where sample volumes are limited is the production of monoclonal antibodies. Mouse serum is often available in very small amounts. Furthermore, the selection of mAb-producing hybridomas in regard to the desired specificities should occur as early as possible during the production process. Such an early stage is the first screening of the growing hybridomas. However, approximately only 150 μl of supernatant are available at that stage and is usually

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not sufficient to test with more than four test antigens. All these problems can be solved using grid immunoblotting, and in this paper examples of this technique are presented.

2. Experimental

2.1. Grid blot

The grid blot technique was a modification of Alric [1] and Lane [2]. Grid immunoblotting is made up of three basic steps as most immunoblot procedures. First, the proteins (e.g. allergen extract) are immobilized on the a carrier membrane and unoccupied binding sites are blocked. Second, the blot is incubated with primary antibody (e.g. serum, monoclonal antibody) or other specific probes such as saccharide-specific lectins, and third, the specific binding is detected with a detection system. At each step several options are available and it is beyond the scope of this article to discuss all possible combinations and procedures. Instead, the experiments described below are a starting point for other experiments.

Since the membrane is exposed to significant stress, reenforced nitrocellulose membranes (Optitran BA-S 85, pore size 0.45 μm, Schleicher & Schuell) are used. To improve protein binding, the nitrocellulose membranes (12.5×12.5 cm) are activated with CNBr according to Demeulemester [3]. These membranes can be stored for extended periods of time at 4°C. The membranes are soaked for 1 min in Tris-buffered saline (TBS, 100 m*M* Tris–HCl, 100 m*M* NaCl, 2.5 m*M* $MgCl_2$, pH 7.4) before being placed onto the multi-channeled manifold of the Surf-blot apparatus (Idea Scientific, Minneapolis, MN) which is then assembled according to the manufacturer's instructions. Since the volumes of the channels are very small, it is important to aspirate any TBS before the membrane is coated with allergen.

Coating concentrations may vary, but protein concentrations of 100 μg to 1 ml/ml in standard ELISA carbonate/bicarbonate coating buffer (60 m*M* Na_2CO_3, 140 m*M* $NaHCO_3$, pH 9.6) have been successfully used. Two-hundred microlitres of allergen solution is pipetted into each channel. To mark the blot and avoid confusion later about the orientation of the membrane the last channel was filled with 200 μl of a 0.01% Pyronin Y solution instead of antigen solution resulting in a permanently stained pink lane on the blot. The grid blot apparatus is then incubated for 1 h at room temperature with end-over-end rotation. It is important that the antigen evenly coats the membrane and that no bubbles are trapped in the channels. After three washing steps with 2 ml per channel of washing buffer (TBS–T, TBS supplemented with 0.05% Tween 20) using a regular 1 ml pipette or an Eppendorf Multistepper with a 50 ml cartridge, the apparatus is disassembled and unoccupied binding sites on the membrane are blocked in 200 ml of TBS–T containing 1% (w/v) non-fat dry milk powder (Carnation, Nestle Food Company, Glendale, CA) for 1 h at room temperature. The membranes are rinsed twice for 10 min in 200 ml of TBS–T and air-dried until usage. For the detection of specific antibody reactivities, the membrane is soaked in TBS–T for 10 min and the membranes arranged such that the antigen-coated lanes on the membrane are perpendicular to the incubation channels of the manifold and thus allowing each antibody to react with each of the 20 antigens. Excess buffer is removed and 150 to 200 μl of diluted antibody-containing are pipetted into the lanes. The dilution may vary and depend on the allergens tested and the antibodies used; however, the dilution that is used for conventional western blotting is a good starting point. The blot is incubated for 1 h at room temperature with end-over-end rotation.

After another washing procedure, the membrane is incubated at room temperature for 1 h and in 100 ml of TBS–T containing 1% non fat dry milk powder and detection antibody. For the detection of mouse antibodies, 1:20 000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG+IgM antibody (Jackson ImmunoResearch, West Grove, PA) is used. For the detection of IgE antibody reactivities, the blot is incubated with 1:1000 diluted alkaline phosphatase-conjugated monoclonal anti-human-IgE (Southern Biotechnology Associates, Birmingham AL, USA). The antibody binding is made visible by colorimetric detection [4]. After washing with TBS–Tween and TBS–AP (100 m*M* Tris–HCl; 100 m*M* NaCl; 5 m*M* $MgCl_2$; pH 9.5), the blots are incubated

in substrate/chromogen mixture for alkaline phosphatase at 37°C containing 450 μ*M* 5-bromo-4-chloro-indolyl-phosphate disodium salt (BCIP; Sigma, St. Louis, MO) and 400 μ*M* nitroblue tetrazolium chloride (NBT; Sigma) solubilized in TBS–AP. Alternatively, a chemiluminescence substrate for alkaline phosphatase can be used. Blots are washed with freshly prepared assay buffer (100 m*M* diethanolamine/HCl, 1.0 m*M* $MgCl_2$, pH 10.0), incubated in 1:20 diluted Nitroblock® chemiluminescence enhancer (Tropix, Bedford, MA) for 5 min and incubated in 250 μ*M* CSPD (disodium 3-(4-methoxy-spiro{dioxetane-3,2′-(5′ chloro) tricyclo-[3.3.1.1.^{3,7}]decan}-4-yl)phenyl phosphate; Tropix) for 5 min. Excessive liquid is drained, the blots are sealed between transparencies and exposed to autoradiography film for 15, 30, 60 and 120 s.

3. Results

3.1. Human IgE reactivities to meat extracts and tropomyosins

To evaluate the in vitro IgE antibody response to different vertebrate meats in meat-sensitized subjects, and the role of tropomyosin in meat allergy the cross-reactivities between vertebrate meats were analyzed [5] by grid immunoblotting (Fig. 1). Fifty-seven sera from suspected meat-allergic subjects were tested by grid blot. Approximately two-thirds of the sera had volumes of 1 ml or less. Extracts of beef, lamb, pork, venison, chicken and turkey and to four mammalian tropomyosins of different origins were used as test antigens. Most (91.5%) meat-allergic subjects have IgE antibodies to proteins in different mammalian meat extracts with some cross-reactivity (44.2%) to chicken. Weak IgE reactivity to tropomyosin was detected in only 2/57 sera tested.

3.2. Screening mouse sera for cross-reactivities to Penicillium and Aspergillus extracts

Aspergillus and *Penicillium* are two of the most diverse and commonly found fungal genera in indoor environments and have been associated with impaired indoor air quality and fungal allergy. The extreme diversity of *Aspergillus* and *Penicillium* species that may vary from environment to environment and the growth stage-specific expression of certain allergens present significant barriers for the application of species-specific mAbs for monitoring studies aiming at the establishment of overall exposure values to species of both genera. The goal was to produce monoclonal antibodies (mAbs) that react with a wide variety of *Aspergillus* or *Penicillium* species as the basis for a quantitative assay to monitor their combined fungal biomass in indoor environments. *Aspergillus* and *Penicillium* species were grown on malt agar plates containing doubled amount of agar. After sporulation the plates were washed carefully with approximately 10 ml ether and the spore ether suspension was collected in 15 ml cell culture tubes. The ether was allowed to evaporate over night and the spores were stored at −20°C. Glass beads (0.5 ml) were added to the harvested spores and the spores were vortexed vigorously for 10 min. Extraction buffer (1 ml; 0.125 *M* NH_4HCO_3, pH 8.1) was added and the suspension was again vortexed vigorously for 10 min. After removal of the extract, the glass beads were washed with 1 ml extraction buffer. The combined extract fractions were lyophilized and stored at −20°C until use. Mice were immunized with spore extracts of 20 *Aspergillus* or *Penicillium* species using Freund's complete and incomplete adjuvants. Out of several hundreds of isolates ten *Penicillium* species (*P. brevicompactum* Dierck, *P. chrysogenum* Thom, *P. citrinum* Pers., *P. expansum* Link, *P. herquei* Bain & Sart, *P. islandicum* Sopp, *P. purpurogenum* Stoll, *P. roquefortii* Thom, *P. sclerotiorum* van Beyma, *P. variabile* Sopp) and ten *Aspergillus* species (*A. candidus* Link, *A. clavatus* Dezm., *A. flavus* Link, *A. fumigatus* Fres., *A. japonicus* Saito, *A. niger* van Tieghem, *A. ochraceus* Wilhelm, *A. terreus* group, *A. ustus* (Bain.) Thom & Church, *A. versicolor* (Vuill.) Tiraboschi) were chosen since these species were most frequently found indoors. A few microliters of tail vein blood were obtained and tested for reactivity by grid immunoblotting; all immunizations resulted in reactivities to *Aspergillus* and *Penicillium* spore extracts (Fig. 2). In general, no preferred reactivity of a particular serum to the extract used for immunization was observed supporting our hypothesis of shared antigens among fungi. The overall reactivity to *A. fumigatus*, *P. islandicum*, and *P.*

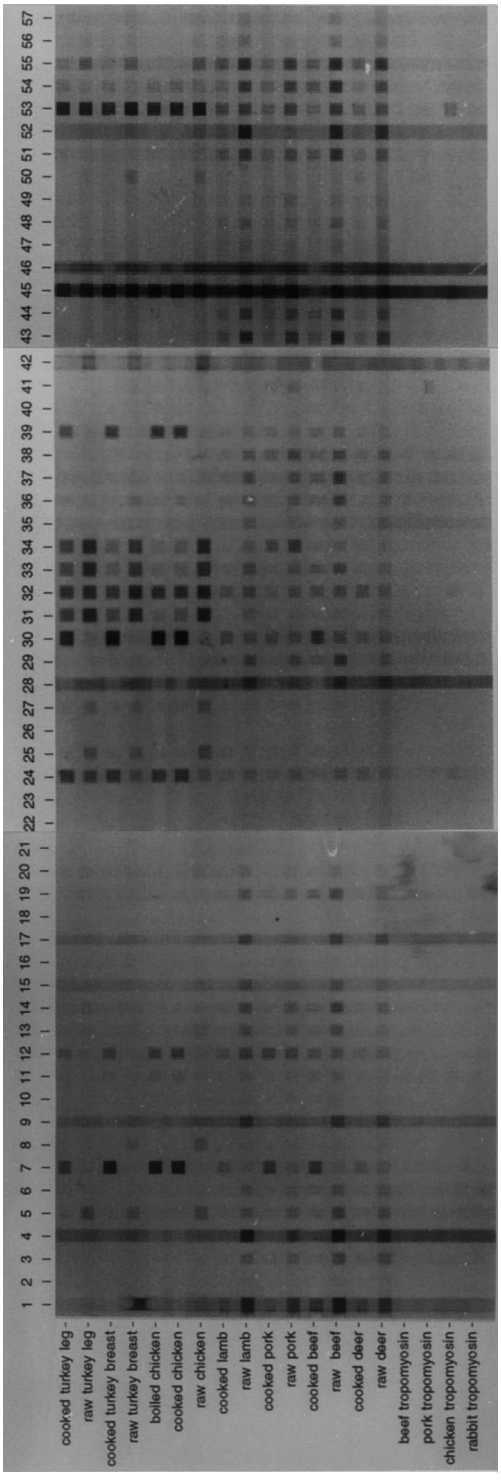

Fig. 1. IgE reactivities to the meat extracts and tropomyosins by grid blot. The lanes represent the different vertebrate meats and tropomyosins tested. Columns: patients' sera 1–57. A reactivity was considered positive when IgE antibody binding to the extract was stronger than that of the background.

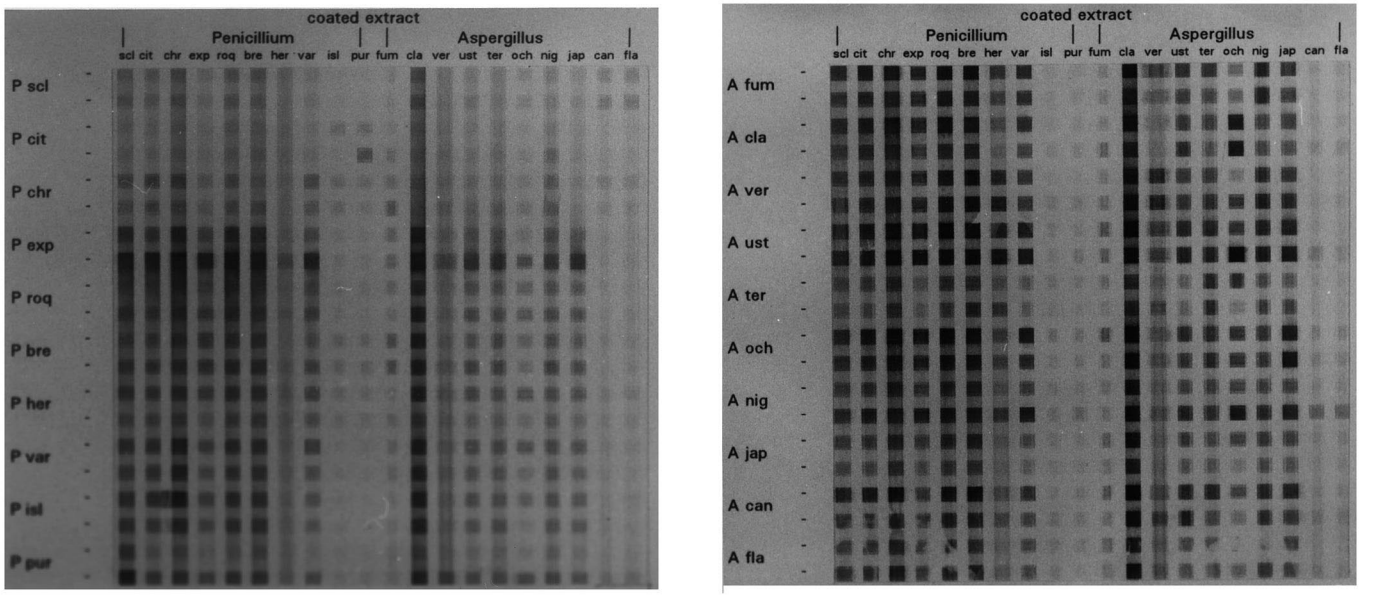

Fig. 2. a: Grid blot analysis of sera of mice immunized with *Penicillium* (P scl: *P. sclerotinium*, P cit: *P. citrinum*, P chr: *P. chrysogenum*, P exp: *P. expansum*, P roq: *P. roquefortii*, P bre: *P. brevicompactum*, P her: *P. herque*, P var: *P. variabile*, *P* isl: *P. islandicum*, P pur: *P. purpurgenum*) spore extracts (vertical lanes: coated extracts, horizontal lanes: mice sera). b: Grid blot analysis of sera of mice immunized with *Aspergillus* (A fum: *A. fumigatus*, A cla: *A. clavatus*, A ver: *A. versicolor*, A ust: *A. ustus*, A ter: *A. terrus*, A och: *A. ochraceus*, A nig: *A. niger*, A jap: *A. japonicus*, A cand: *A. candidus*, A fla: *A. flavus*) spore extracts (ventrical lanes: coated extracts, horizontal lanes: mice).

purpurogenum was weak, whereas most of the murine sera reacted strongly with *A. clavatus*. The immunization with *P. expansum* resulted in the strongest cross-reactive serum for both *Aspergillus* and *Penicillium* species and thus, the *P. expansum*-immunized mice were chosen for the production of monoclonal antibodies.

4. Discussion

In our laboratory, the grid immunoblotting has been successfully used to perform a number of experiments in which the amount of sera were extremely limited and could not been performed by conventional techniques such as ELISA. For example, the serum volume needed to test for IgE antibodies to up to 20 allergens using a dilution of 1:2 is 1 ml (20×50 μl) by ELISA whereas 80–100 μl are sufficient for grid immunoblotting; this technique allows to stretch limited serum resources. Semi-quantitative visual comparison between different reactivity patterns is much easier then the comparison of numeric ELISA readings and if quantitative results are required a chemiluminescence substrate such as CSPD (Tropix, Bedford, MA) or a radioactive anti-IgE can be used, and the resulting autoradiographs can be quantified by densitometry. Another advantage is the easy storage of antigen-coated membranes. Dried membranes were stored at room temperature for more than 6 months without losing their activity. Since the Surf Blot apparatus can also be used for Western blotting, it is a versatile tool that allows quick and easy characterization of allergen-specific antibody response with minimal amounts of sample.

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