Simultaneous Quantitation of Specific IgE Against 20 Purified Allergens in Allergic Patients Sera by Checkerboard Immunoblotting (CBIB)

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A simple technique, checkerboard immunoblotting (CBIB), is described for simultaneous quantitation of specific IgE antibodies against several allergens in human sera. Using as little as 50 µl of each of the 20 sera examined against 20 different allergens, it was possible in a single run to achieve 400 tests. To guarantee high specificity and sensitivity of the assay, this new application of CBIB employs purified allergens, cyanogen bromide-activated nitrocellulose membrane, and Phosphorimager technology. Results are expressed both qualitatively (five classes)

and quantitatively in kilo units per liter equilibrated against the World Health Organization (WHO) standard for IgE. There was excellent agreement between the results of CBIB and the results of Pharmacia Cap System, an alternative method widely used for measuring serum-specific IgE. The CBIB method certainly could be useful in any laboratory interested in allergy clinical research for easy screening and relative quantitation of allergen-specific human IgE. *J. Clin. Lab. Anal.* 11:357–362, 1997. © 1997 Wiley-Liss, Inc.

Key words: screening; antibody; allergy; immunoblotting; phosphorimager

INTRODUCTION: TESTING FOR ALLERGEN-SPECIFIC IGE ANTIBODY

In vitro tests for IgE constitute one of the methods of choice for detection of allergen-specific IgE antibody in serum (1). In patients with suspected allergies, they are often used together with skin testing to confirm or negate clinical suspicions. The most commonly used in vitro assays include the radioallergosorbent test (RAST) (2) and various modifications of this test (3–5), such as the Pharmacia CAP system (6). They provide new solid phases with higher binding capacity to increase the limit of detection. However, these commercial tests designed for IgE-mediated hypersensitivity diagnosis have limited applications in clinical research because of the quality of allergen preparations. Since crude extracts are generally used instead of purified allergens, the amount of antigen coupled to the sorbent varies, making it difficult in the same sera to compare the amounts of IgE specific for each different allergen. Furthermore, allergen preparations not being standardized between alternative tests, results obtained by those different tests cannot be compared directly. Despite the variety of systems used to measure IgE antibody, very few systems use recombinant or purified allergens, although a large number of them have been prepared in recent years (7).

Moreover, in most of the techniques described so far, each

allergen is separately tested and a new amount of serum is therefore needed every time a new allergen is examined. Here, we describe a method adapted from checkerboard immunoblotting (CBIB) simultaneously to measure specific IgE antibodies against 20 purified allergens with as little as 50 µl of the serum tested. The CBIB, an uncomplicated method for assaying multiple antigen-antibody reactions, was originally described by Kazemi and Finkelstein (8) for the evaluation of monoclonal antibodies to cholera enterotoxins.

It was subsequently used for qualitative and semi-quantitative clinical applications in human sera. Ritchie et al. (9) have applied the method to specific antinuclear antibodies determination and Craig et al. (10) to antiviral IgG measurements (10). As a complement to these interesting possibilities, we present a rather original application of CBIB to assess specific IgE in allergic patients sera. The validity of the method is demonstrated by comparison with an alternative accepted assay, and its potential uses in clinical research are also discussed.

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MATERIALS AND METHODS

Sera

Serum samples from allergic patients were obtained from the allergy consultation, Pasteur Hospital. The diagnosis of allergy was based on case history, skin testing to one or more common allergens, and presence of specific IgE in serum. The allergen extracts employed for skin prick test (Stallergenes, France) were from house dust mite, *Dermatophagoides pteronyssinus* (100 IR/ml); graminaceae pollen mix (100 IR/ml); cat, *Felis domesticus* (100 IR/ml); and horse, *Equus caballus* (1:10 wt/vol). The 104 serum samples used were from subjects sensitized to mite (n = 32), grass pollen (n = 31), cat (n = 29), and horse (n = 12). Twelve serum samples from asymptomatic skin prick test negative subjects were also considered. Each serum sample was stored at -20°C until separate specific IgE antibody measurement by CBIB and Pharmacia CAP system were performed.

Purified Allergens

Major mite allergens from *Dermatophagoides pteronyssinus* (Der p1) and from *D. farinae* (Der f1) were prepared according to Dandeu et al. (11). Proteinases from *Carica papaya*, papain, chymopapain, and caricain were purified by S-pegylation (12) as well as ananain and bromelain, the proteinases from *Ananas comosus*, the pineapple plant (13). Albumins from different animal species were purchased from Sigma Chemicals (St. Louis, MO): cat (CSA), dog (DSA), horse (*Equus caballus*) (ESA), and chicken (CkSA). Fel d1, the major allergen from the cat (*Felis domesticus*) was obtained as described by Dandeu et al. (14). Native horse allergen (nEqu c1) and its recombinant counterpart (rEqu c1) were, respectively, obtained from horse danders (15) and from cDNA cloning of horse salivary glands (16). Bet v1, the major allergen from birch tree pollen (*Betula verrucosa*) was a generous gift from Dr. Weyer (Institut Pasteur, Paris). Lol p1, the major allergen from rye grass pollen (*Lolium perenne*), was a kind gift from Dr. Mécheri (Institut Pasteur, Paris). Allergens from orchard grass pollen (*Dactylis glomerata*) were kindly provided by Dr. Peltre (Dac g1), Dr Guérin-Marchand (rDac g3), and Dr Leduc-Brodard (Dac g4). They were, respectively, prepared as described in Demeulemester et al. (17), Guérin-Marchand et al. (18), and Leduc-Brodard et al. (19).

Membranes

Nitro-cellulose membranes were obtained from Schleicher & Schuell (Keene, NH). Cyanogen bromide activation was performed according to Demeulemester et al. (20).

Checkerboard Immunoblotting (CBIB)

The checkerboard immunoblotting (CBIB) procedure of Kazemi and Finkelstein (8) was adapted as follows. A sheet

of cyanogen bromide-activated nitrocellulose membrane was wet for 5 min with phosphate-buffered saline (PBS), pH 7.0 and assembled into a Miniblotter 20SL (Immunetics, Cambridge, MA). Each individual lane was then filled with 50 µl diluted allergen in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6). The coating concentration of all the allergens tested was fixed at 50 µg/ml (2.5 µg for one lane) according to preliminar measurements. Five outer lanes were also coated with different concentrations of human IgE (Pharmacia, Gaithersburg, MD) to create a standard curve. After 60 min incubation at room temperature, the excess protein was aspirated and the lanes were dried. The membrane was then moved from the apparatus to a tray for blocking with 0.1% HSA in PBS-Tween, 2 hr at room temperature, followed by washing three times with PBS-Tween. The use of blocking buffer containing HSA, rather than other solutions such as bovine serum albumin (or gelatin), significantly reduced the amount of nonspecific background (21). Back to the Miniblotter, the membrane was turned 90° for patient serum incubation. Each channel was filled with 50 µL undiluted serum and incubated 1 hr at 37°C. All lanes were then washed simultaneously with the supplied manifolds using 100 ml PBS-Tween. The membrane was then transferred to a tray containing 10 ml of diluted ¹²⁵I radioactive labelled monoclonal antibodies antihuman IgE (Pharmacia) in blocking buffer (1,500 CPM/50 µl) and incubated for 1 hr with gentle shaking. It was then washed three times with PBS-Tween and dried before exposure to the Phosphorimager screen.

Quantitative Analysis by Phosphorimager

The Phosphorimager (400 A) was supplied by Molecular Dynamics (Sunnyvale, CA). All the membranes were exposed onto a reusable Phosphor screen for 16 hr. This screen provides a linear dynamic range of five orders of magnitude. The sensitivity is 10 times higher than films exposed at -80°C with an intensifying screen. It provides images of (¹²⁵I, ³⁵S, ¹⁴C, ³²P, ³³P)-labeled samples with a spatial resolution of 88 or 176 µm.

In this device, the sensible surface is a plate composed of fine crystals of BaFBr:Eu+2 in an organic binder (22). In close contact with a labelled source, the Phosphor screen stores the cartography of radioactivity by excitation of the crystals and returns the latent image by emission of luminescence at 365 nm by scanning with a helium-neon laser at 635 nm. Digitalisation of the signals were performed on the computer screen (480 × 640 pixels) with a 256 color card.

Data are either supplied qualitatively (five classes) and quantitatively in kilo units per liter (kU/l) equilibrated against the WHO standard for IgE.

Detection of IgE by Pharmacia CAP System

Cap, a recently developed immunoassay for the detection of allergen-specific IgE antibody, uses cyanogen bromide-

activated cellulose derivatives as the solid phase encased in a capsule (23). The radioimmunoassay version was used as described by the manufacturers. The standard curve is equilibrated against the WHO standard for total IgE antibody, and it measures specific IgE antibody in concentrations from 0.35 to 100 kilo units per liter (kU/l) and allergen classes from 0 to 6.

Allergens Coating Concentration for CBIB

A single coating concentration was selected for all the allergens tested. It was determined as follows. Each of the three most common allergens (Der p1, Dac g1, Fel d1) were applied to the membranes at various concentration between 1 and 250 µg/ml and tested for specific IgE by CBIB with a pool of sera highly positive to the respective allergen. Three dose/response related graphs were obtained. The coating concentration meeting the two following criteria was chosen: (1) the concentration has to be in the linear range of all the three curves, (2) the concentration has to give a signal higher than the inferior limits of the highest class (17,5 kU/l).

Concordance of Specific IgE Antibody Classes Between CBIB and CAP Systems

In the practice of clinical allergy, the results of specific IgE in vitro measurements are usually given as classes. For that reason, five classes were also used in the CBIB system. As indicated in Table 1, to compare properly both immunoassays, CAP classes 4, 5, and 6 were treated as a single class since the corresponding values obtained in CBIB system with the Pharmacia standards for IgE were out the linear range of the standard curve.

RESULTS

Allergens Coating Concentration

As can be seen in Figure 1, coating with increasing concentrations of allergen gave higher values in a linear fashion over a range from 1 µg/ml to 250 µg/ml. This was shown for three representative allergens (Der p1, Dac g1, Fel d1) using a pool of sera highly positive to the respective allergen. After linear regression analysis, the average of the linear correlation coefficient (r^2) following five independent experiments

TABLE 1. Absolute Values of Specific IgE Antibody Detected by CBIB and CAP Systems

CBIB		CAP	
kU/l	Class	kU/l	Class
0.35	0	<0.35	0
0.35–0.7	1	0.36–0.7	1
0.71–3.5	2	0.71–3.5	2
3.51–17.5	3	3.51–17.5	3
>17.50	4	17.51–50.0	4
		50.10–100.0	5
		>100.0	6

Fig. 1. The effect of coating a CNBr-activated nitro cellulose membrane with increasing concentrations of Der p1 (▲), Dac g1 (□) and Fel d1 (○). The respective specific IgE were detected with 125 I radioactive labelled monoclonal antibodies antihuman IgE and quantified by Phosphorimager analysis. Linear correlation coefficient (r^2): Der p1, $r^2 = 0.994$; Dac g1, $r^2 = 0.996$ and Fel d1, $r^2 = 0.991$.

was calculated for each of the allergens tested: Der p1, $r^2 = 0.994$; Dac g1, $r^2 = 0.996$ and Fel d1, $r^2 = 0.991$.

The concentration of 50 µg/ml was chosen for all the allergens tested in CBIB assays because of satisfying the two criteria defined in Materials and Methods.

Fully Developed CBIB System

Results from a full analysis of sera against the various allergens tested are shown in Figure 2. Different allergens were applied to vertical lanes A–T and tested with 20 sera from allergic patients (horizontal lanes 1–20) as described in Materials and Methods. In the case of lane F and lane 11, the membrane was incubated, respectively, with buffer and with serum from a nonallergic individual as controls. Each positive reaction appeared as small square at the intersection of allergen/antibody lanes, giving a checkerboard appearance to the image released from the Phosphor screen.

A serum from a patient suffering from mite allergy was incubated in lane 20 (Fig. 2). As expected, this serum was positive for the major mite allergens Der p1 (lane A) and Der f1 (lane B). No other allergen tested was recognized, suggesting that this patient was monosensitized to mite. However, a serum from a patient suspected of being polysensitized was analysed in lane 1 and strong reactions were effectively observed with several allergens (lanes A, B, P, R, S, T), confirming the clinical suspicion of severe atopy.

Fig. 2. Results from fully developed CBIB method. Allergens were coated in vertical lanes. A = Der p1, B = Der f1, C = ananain, D = bromelain, E = CSA, F = buffer control, G = Fel d1, H = papain, I = chymopapain, J = caricain, K = CkSA, L = nEqu c1, M = ESA, N = rEqu c1, O = DSA, P = Lol p1, Q = Bet v1, R = Dac g1, S = rDac g3, T = Dac g4. Sera from allergic patients were incubated in horizontal lanes 1–20, except control lane 11, which was incubated with a nonallergic individual serum.

Several sera from patients sensitized to pollen were tested in lanes 1, 4, 5, 6, 8, 12, and 16. A great heterogeneity was observed in their reactivities against the five pollen allergens tested from three different sources: *Lolium perenne* (lane P), *Betula verucosa* (lane Q), and *Dactylis glomerata* (lanes R, S and T). Sera 1, 4, 5, 6, and 8 were positive for at least two out of three allergen sources, whereas sera 12 and 16 only shown specific IgE against allergens from *Dactylis glomerata*, respectively Dac g1 (lane R) and Dacg 4 (lane T). The four sera tested from patients with cat allergy (sera 10, 15, 17, and 18) were positive either for the major cat allergen Fel d1 (lane G) or for cat albumin (lane E). These observations were also reported by Goubran-Botros et al. (24). Two sources of the major horse allergen were tested. The native allergen nEqu c1 (lane L) was purified from horse dander, and its recombinant counterpart rEqu c1 (lane N) was obtained by cloning cDNA from horse sublingual salivary glands. Both molecules were recognized by IgE antibodies when tested with sera from patients sensitized to horse (lanes 1, 4, 5, and 14).

Potential fruit allergens purified from the papaya tree (lanes H, I and J) and from the pineapple plant (lanes C and D) were also examined. Like Der p1 and Der f1, they belong to the cysteine proteinase family of enzymes. One of these enzymes, ananain (lane C) was weakly recognized by specific IgE in sera 1, 4, 7, 9, 12, and 13. As can be seen in the interspaces between both allergens and sera lanes (Fig. 2), blocking with

HSA (21) was satisfying since the background obtained was low and the small squares representing positive reactions well defined. The CBIB method was found able to produce 400 tests within 24 hr.

Standard Curve of Human IgE

Each membrane tested was also coated with different concentrations of human IgE (ranging from 1 to 150 kU/l) to create a standard curve. The results of a typical standard curve are shown in Figure 3. The dilution of IgE gave an almost linear curve from 1 to 50 kU/l.

Concordance of Class Levels

The relative values of specific IgE antibody were expressed in classes that were used for the evaluation of concordance between the CBIB system and the Pharmacia Cap. We compared the data for the clinically most common allergens. Figure 4 shows the results. Very good concordance was found between both immunoassays for all the allergens tested. The comparison of overall data demonstrated a coefficient of correlation of 0.718.

DISCUSSION

CBIB as a Tool for Allergen-specific IgE Quantitation

Considerable progress has been made to improve the sensitivity of in vitro tests for allergen-specific IgE in serum. In

Fig. 3. Standard curve of human IgE. Human IgE (●) equilibrated against the WHO standard for IgE were coated at different concentrations. Quantitative analysis was made by Phosphorimager technology. Linear regression analysis (-----) in the range 0–50 kU/l, $r^2 = 0.969$.

Fig. 4. Comparison between class results for Pharmacia CAP system (x) and CBIB system (y). The class definition is based on absolute values in kU/l according to Table 1. Three common inhalent allergens are represented by following symbols: ▲ Der p1 (n = 40); ● Dac g1 (n = 40), ■ Fel d1 (n = 40). Spearman's coefficients of correlation (r): all allergens, $r = 0.718$; Der p1, $r = 0.938$; Dac g1, $r = 0.860$; Fel d1, $r = 0.773$.

particular, it has been shown that the Pharmacia CAP System, which uses a cyanogen bromide-activated cellulose derivative as the solid phase, is superior to RAST for measuring IgE antibodies (6). However, this method widely used for the diagnosis of allergy has restricted performances when used for research purposes. Since each test performed requires its own amount of allergen and serum to be examined, CAP is a time- and material-consuming method. In addition, the crudeness of allergen preparations can be considered as a source of artifacts.

The CBIB assays, whose results are reported on, combine an optimized allergen-coated solid phase (the cyanogen-bromide activated nitrocellulose membrane) with a highly sensitive radioactive detection system (the Phosphorimager). Moreover, the technique allows 400 tests to be performed simultaneously. When CAP and CBIB results were compared, we found significant agreement both when results are expressed as classes and when we compare the absolute levels of allergen-specific IgE antibodies. In the last case, despite good correlation coefficients (r), we observed slopes significantly different from 1. Since the calibration systems of the two methods are similar as well as the principle of allergen binding to the solid phase, the discrepancy may be due to the difference in quality of allergen preparations. In CBIB assays, the allergens used were obtained as pure preparations, whereas most of the immunoassays described so far for specific IgE determination use either crude or partially purified extracts. When such extracts are used, rather than purified allergens, it becomes difficult to find out the contribution of

each of the components to the signal observed (results not shown) and thus to compare properly the specific IgE content for various allergens in the same patient's serum. Some observations are only made possible when pure material is exclusively used. For example, as shown in Figure 2, sera 4, 5, and 6 all appear to react strongly with Dac g1, the major allergen of pollen *Dactylis glomerata*. However, if serum 4 equally reacts well with Dac g3 and Dac g4, serum 5 shows reactivity against Dac g4, but not against Dac g3, whereas serum 6, on the contrary, possesses reactivity against Dac g3, but not against Dac g4. Another advantage of using purified allergens is to validate the comparison between different sera for their IgE titers against a particular allergen. To ensure that both intra and interserum comparisons were correctly made, a single coating concentration was chosen for all the allergens tested throughout the CBIB assays. The value of 50 $\mu\text{g/ml}$ was selected because of satisfying two criteria: first, to be in the linear range of the dose/response graphs obtained with the three most common allergens when tested with a pool of patients sera highly positive to the respective allergen, and second, to give a signal higher than the inferior limit of the highest class (17.5 kU/l).

Besides the multiple reactions (i.e., close to 400) that can be examined simultaneously using the checkerboard system employed herein, the method has several advantages over other in vitro assays to measure specific IgE. The volume and amount of reagent required is small: 50 μl of serum to test 20 different allergens, whereas in the CAP system, 50 μl is the volume of serum needed to test a single allergen. The results are highly specific and reproducible: the same amount of pure allergen is deposited on each channel, within each antigen lane. The same is true for the antibodies. Furthermore, the Phosphorimager system of detection has an excellent sensitivity and allows the results to be displayed in a permanent form (a single checkerboard includes 400 results), which is easily read and interpreted. Nevertheless, the method suffers from some drawbacks. The overall experiment including the various incubation periods, exposure to the Phosphorscreen, and data treatment takes a minimum of 24 hr since every step remains to be automated.

CBIB as a Clinical Research Tool

Despite the validity of the CBIB method for specific IgE quantitation demonstrated by comparison with the CAP system, the technique should be more appropriate for clinical research purposes than for the diagnosis of allergy in a routine laboratory. As the aim of the diagnosis is to allow the definition of the allergen source against which a patient is sensitized, the use of partially purified allergens is sufficient. On the contrary, fully purified natural or recombinant allergens are a prerequisite in research and the technique herein described could therefore be useful in any laboratories interested in allergy. CBIB has already proven practical for preliminary screening of IgE cross reactions between mite and

plant cysteine proteinases (25). Since more and more works are revealing cross-reactivity of IgE in allergic patients with related as well as unrelated allergens, the CBIB technique seems to be an interesting tool for this kind of study, and it may help to select a few model allergens that bear most IgE epitopes and that can be used for diagnosis and therapy.

One more application of CBIB could be the precise determination of the patients allergen profile (allergogram). Potential uses of this technique also include easy epitope mapping by comparison of IgE levels against different peptides derived from a pure allergen. Thus if the above in vitro test is to be used as a clinical research tool in allergy, the performance characteristics described are of importance.

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