

Simultaneous Determination of Total IgE and Allergen-Specific IgE in Serum by the MAST Chemiluminescent Assay System

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We have developed a chemiluminescent immunoassay system. The first commercial application of this chemiluminescent assay (CLA) is the measurement of total IgE and allergen-specific IgE in human serum. The CLA system is a second-generation adaptation of the MAST RIA allergy profiling system. The MAST CLA system assay protocol consists of three steps: overnight incubation of serum, a 4-h incubation with enzyme-labeled antibody, and a 30-min chemiluminescent reaction, which produces a visible image (immunograph) on high-speed Polaroid® instant film. The densities of the bands produced on the film are quantified with an inexpensive microprocessor-controlled infrared transmittance densitometer. The novel luminogenic substrates used yield a constant light output for over 2 h with an intensity at least 10-fold greater than that of commercial chemiluminescent reagents. The MAST CLA system exhibits sensitivity, specificity, and precision equal to that of the MAST RIA system ($r = 0.96$ for 40 serum samples analyzed with 25 allergens). As many as 35 different allergens per sample can be quantified in a single assay. The MAST CLA system requires no standard curve or volume-dependent pipetting steps, incorporates both positive and negative controls for each sample, and quantifies allergen-specific IgE at picomolar concentrations.

Additional Keyphrases: chemiluminescent immunoassay · allergy · autoimmunography · immunograph · densitometry

The diagnosis of allergy involves a patient history, physical examination, and confirmatory diagnostic testing to identify whether the patient's symptoms are of allergic or nonallergic origin. If allergy is responsible for the symptoms, then the allergens responsible must be identified. Possibly the most relevant type of diagnostic testing for allergy is provocation. However, this type of testing can be dangerous for the patient, is certainly unpleasant, and cannot be performed for multiple allergens at one sitting. Skin testing continues to be the most widely used diagnostic test for allergy, even though it has a high percentage of false positives, is subject to large differences in technique and interpretation, and cannot be used on patients taking certain drugs or patients with skin problems (1-4). In vitro techniques for allergy testing have improved since the specific immunoglobulin responsible for immediate allergic hypersensitivity was discovered and characterized as IgE in the mid-1960s by Ishazaka et al. (5) and Johansson and

Bennich (6). The first in vitro allergen-specific immunoglobulin assay was described by Millman et al. in 1964 (7). Later, Wide et al. (8) described the radioallergosorbent test (RAST), an immunometric "sandwich"-type immunoassay in which excess amounts of allergen are attached to a solid-phase. After the patient's serum is incubated with the solid-phase allergen, the amount of allergen-specific IgE in the serum is quantified by incubation with labeled anti-IgE. More recently, the radioactive label used in earlier RAST assays has been replaced with enzyme labels that generate color (9, 10) or fluorescence (11); these offer the convenience, stability, and nonhazardous features associated with nonisotopic assay methods.

We previously reported a unique RIA system for measuring allergen-specific (12) and total IgE (13) in which cellulose thread was the solid-phase matrix in a compact plastic test chamber. The MAST RIA Allergy Testing System allows up to 35 allergen-specific threads to be tested simultaneously along with a positive control, negative control, and a total IgE measurement in a single test with a very simple assay protocol. We have now extended this system to a nonisotopic version with a much shorter total assay time. In the MAST chemiluminescent assay system, enzyme-labeled anti-IgE interacts with substrates that generate luminescence upon enzymatic reaction. The light produced is photographed with Polaroid® high-speed film and quantified with an inexpensive infrared transmittance densitometer.

Materials and Methods

Apparatus

MASTpette test chamber. The MASTpette test chamber for the chemiluminescent assay system consists of a black silk-screened coverslip along with antigen-coated cellulose threads bonded in the plastic chamber shown schematically in Figure 1. Each chamber contains 38 cellulose threads for the simultaneous measurement of total IgE, as many as 35 clinically relevant allergen groups, and positive and negative controls. The total IgE test result provides an overall indication of the patient's allergic potential (manuscript in preparation) and is especially useful if all specific allergen results are negative. The positive control thread has human IgE covalently attached to assure the quality of the labeled antibody and film used to quantify the results. The negative control thread, devoid of allergen, measures the degree of nonspecific binding associated with each patient's sample and compensates for film variations. Concentrations of the patient's specific and total IgE minus the nonspecific binding are automatically calculated by the densitometer.

Photocassette. Because the MAST CLA system produces luminescence directly, we modified the photocassette design of the MAST RIA system by removing the phosphor screen. A permanent photographic print (immunograph) is produced by exposing Polaroid Type 57 (high-speed) film to the test chambers (the photocassette will accommodate up to five MASTpette test chambers per exposure). Light emitted

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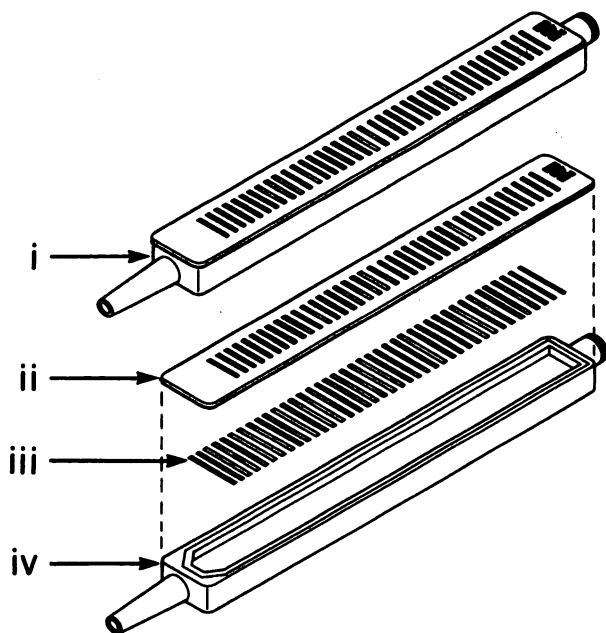


Fig. 1. The MASTpette test chamber: (i) the intact test chamber, (ii) black silk-screened polystyrene coverslip, (iii) individual cellulose threads, (iv) polystyrene test chamber body

from threads in the test chamber exposes the film, and a white band on the resulting print corresponds to each positive reaction (Figure 2).

Densitometer and reader card. Individual immunographs from the Polaroid prints are aligned in reader cards for proper presentation to the densitometer. The densitometer automatically scans the film strip to determine the amount of transmittance (voltage) produced by each thread. The use of the densitometer has been previously described (12). The degree of transmittance is directly proportional to the amount of bound IgE on each thread. If the transmittance of the positive control is too low, the densitometer will automatically indicate this with a double asterisk on the print-out. The negative control thread indicates the amount of nonspecific binding from each sample. The densitometer subtracts the transmittance of the negative control thread from the total IgE and the allergen-specific threads to

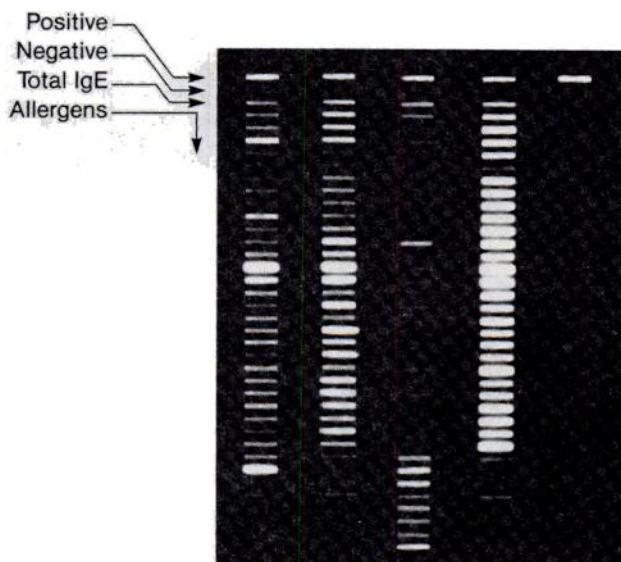


Fig. 2. MAST CLA immunograph showing four patients with positive (atopic) responses to specific allergens or groups of allergens and one nonatopic patient

Each MASTpette test chamber contains positive and negative control threads, a total-IgE thread, and 35 specific-allergen threads

provide net voltage results. Samples with an abnormally high background are indicated with a single asterisk. After the net voltage is determined, the densitometer converts the reading to a MAST class.

Other apparatus. Other useful apparatus, peripheral to the equipment necessary for the chemiluminescent test system, are a workstation, a wash buffer dispenser, a film splitter, and an allergy test record, described elsewhere (12). The MAST allergy test record is used to present results to the patient in an easily understood fashion: it lists the allergens present on the test chamber threads, has a guide for interpreting MAST classes, and provides a convenient way to store results in the patient's chart or file.

Materials

Allergens. Allergens were obtained from Hollister-Stier, Inc., Spokane, WA; Sharp and Sharp, Inc., Everett, WA; and Greer Laboratories, Inc., Lenoir, NC.

Sera. Blood samples were usually collected in Vacutainer Serum Separator Tubes (Becton Dickinson, Rutherford, NJ) and allowed to clot for 1 to 2 h at room temperature. Serum was removed by centrifugation and either used fresh or stored at -70°C until needed.

Enzyme-labeled antibody. Affinity-purified goat anti-human myeloma IgE was labeled with horseradish peroxidase (EC 1.11.1.7) at an enzyme/antibody protein molar ratio of 4; we used the two-step method of Nakane and Kawaoi (14). The antibody concentrate consists of 20 mg of enzyme-labeled antibody in, per liter, 10 mmol of phosphate (pH 7.2), 150 mmol of sodium chloride, and 0.1 g of thimerosal, plus a protein stabilizer.

Antibody diluent buffer. Antibody was diluted to its working concentration of 400 $\mu\text{g/L}$ in the antibody diluent buffer of, per liter, 10 mmol of phosphate (pH 7.2), 150 mmol of sodium chloride, and 0.1 g of thimerosal, plus additional protein stabilizers.

Enzyme substrate reagents (photoreagents). Photoreagent A: a cyclic diacylhydrazide compound described elsewhere (15) in 45.5 mmol/L NaOH. Photoreagent B: 50 mmol/L borate buffer, pH 9.4. Photoreagent C: 4 mmol/L hydrogen peroxide in phosphate-buffered saline (10 mmol/L phosphate, 150 mmol/L NaCl, pH 7.0). Photoreagent D: A mixture of erythrosine, Allura Red AC, and tartrazine dyes to absorb strongly ($A = 1.05$) from 418 to 520 nm in distilled, de-ionized water (15). Just before use, equal volumes of all photoreagents are mixed together.

Wash buffer. Per liter, 10 mmol phosphate buffer (pH 7.2), containing 150 mmol of sodium chloride, 1.0 mL of Tween 20 surfactant, and 0.1 g of thimerosal.

Other materials. For the chemiluminescent assays and RIA correlation studies we used the MAST RIA Allergy Profiling reagents (MAST Immunosystems, Inc., Mountain View, CA), sealing plugs, 3-mL syringes, and Polaroid Type 57 film. Luminol was obtained from Sigma Chemical Co., St. Louis, MO.

Procedures

MAST CLA assay. Attach a 3-mL syringe to the top port of the MASTpette test chamber and draw about 1.3 mL of the patient's serum up into the test chamber. Seal the bottom and top ports with rubber plugs, and incubate the chamber overnight (16–24 h) at room temperature; then remove the plugs and allow the serum to drain from the test chamber. Wash the chamber three times with 10 mL of wash buffer, voiding the test chamber of wash buffer between successive washes. Then draw enzyme-labeled antibody (1.3 mL per test chamber) up into the test chamber, seal the bottom and top ports again, and after a 4-h

incubation drain and wash the test chamber as above. Mix the four photoreagents in equal proportions (0.5 mL of each reagent per test chamber), and draw the photoreagent mixture (1.3 mL) into the test chambers. Seal the test chambers with plugs, and load them into the lid of the photocassette. Insert the film into the lower member of the photocassette and fasten both parts of the photocassette together. To initiate the film exposure, withdraw the film envelope from the photocassette and turn the control knob down fully to position the test chambers in contact with the film negative. Let the exposure proceed for 30 min at room temperature, and then turn the control knob up to raise the test chambers away from the negative. Immediately reinsert the film envelope into the photocassette and set the control level on the photocassette to the process position. Pull out the film packet, let the film process for 30 s, then print-coat and dry the positive print.

Using the film splitter, cut the film print into five individual immunographs and use the reader card to align the bands on each immunograph for presentation to the densitometer, placing the top band (positive control thread) within the top window, marked "POS." When the reader card is fed into the densitometer, the instrument automatically records the gross voltage readings for the positive and negative bands and net voltage and class readings for the total IgE and allergen-specific bands. Concentration of IgE is expressed in MAST Class units or net voltages, as defined in the MAST product inserts.

Comparison luminescence measurements. For comparison we monitored luminescence with the photomultiplier portion and circuitry of an Ames fluorocolorimeter (Miles Labs Inc., Elkhart, IN).

Results

Luminescence Measurements

Our first experimentation with commercially available luminol showed the chemiluminescent assay to be less sensitive than the MAST RIA. Subsequent modification and experimentation led to the discovery of an improved cyclic diacylhydrazide compound with greater luminescence yield in the MAST CLA system (15). Maximum emission for both luminol and the MAST luminescence reagent was at 450 nm. The luminescence yield for equimolar concentrations of luminol and MAST CLA luminescence reagent in solution

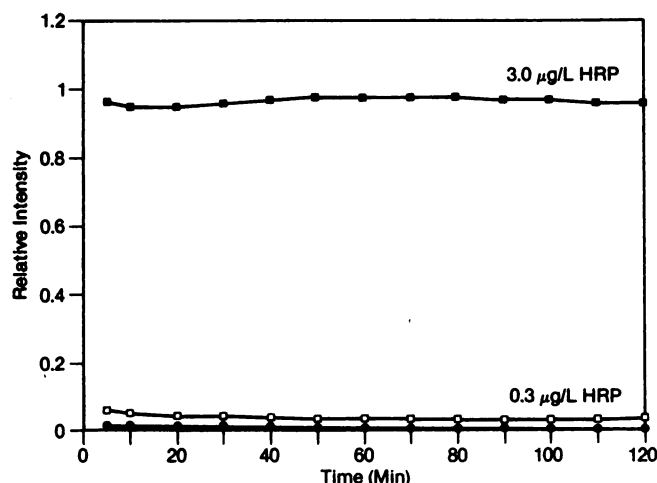


Fig. 3. Light emission profiles of MAST luminescent reagent in solution. Measurements taken in an Ames fluorocolorimeter demonstrate fairly constant light emission of MAST luminescent reagent added to 2.0 mL of 1 mmol/L hydrogen peroxide in 50 mmol/L borate buffer, pH 9.4, containing either 0.3 or 3.0 µg of horseradish peroxidase (HRP) per liter. The light emission intensity of the MAST luminescent reagent is more than 10-fold greater than that of an equimolar concentration of commercially available luminol (●) mixed with the same concentration of HRP (3.0 µg/L).

are shown in Figure 3. The MAST luminescence reagent yielded over 10-fold the emission of luminol, and its luminescent output was constant for up to 2 h. Results under assay conditions with Polaroid film were similar: when new film packets were placed in the photocassette at sequential 30-min intervals, the response from the threads was constant for up to 2 h (Figure 4).

Assay Optimization

Serum incubation time. A patient's serum was incubated at room temperature for various times in MASTpette test chambers. After completing the subsequent assay steps as described above, we found that, although sufficient intensity for determining the final class of allergen-specific IgE is achieved after 6–8 h, a longer incubation results in slightly greater sensitivity (Figure 5, top). We recommend overnight incubation for both convenience and maximum sensitivity.

Antibody incubation time. Figure 5 (middle) shows typical results for selected MAST classes when antibody incubation times were varied from 1 to 5 h. Optimal sensitivity was achieved with a 4-h incubation.

Exposure time. Light emitted from the reaction of the enzyme-labeled antibody with the photoreagent mixture exposes the film; thus a white band on the resulting photograph corresponds to each positive reaction. The transmittance of each band is measured with the MAST densitometer. The degree of transmittance (voltage) is directly proportional to the allergen-specific IgE and the total IgE concentration in the patient's serum. Figure 5 (bottom) illustrates the effect of varying the time of photographic exposure. Threads with high amounts of total IgE or allergen-specific IgE reach their maximum transmittance relatively quickly, and the transmittance plateaus with continued exposure; threads with low amounts of antigen have a lower rate of increase in transmittance. We chose an exposure time of 30 min to correlate the MAST CLA test response with that of the current MAST RIA system, given its demonstrated clinical utility and assay sensitivity (12, 16–18).

The negative control compensates for imprecision in the timing of the exposure and eliminates false positives if the film is exposed for longer than 30 min.

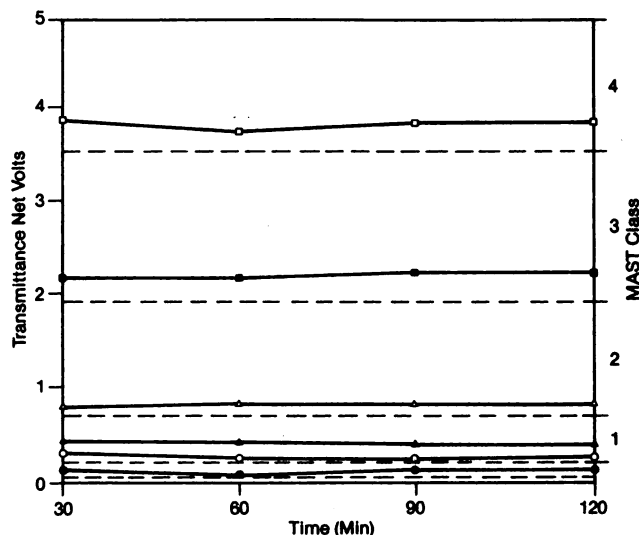


Fig. 4. Light emission profile of luminescent reagent under MAST CLA assay conditions.

Serum from an atopic individual was assayed in triplicate. After addition of the luminescent substrate, successive 30-min exposures were taken, with new film for each exposure, for 120 min. Net voltage readings indicate a steady light emission at each of the various classes of allergen-specific IgE (demarcated by broken lines). □, grass mix; ■, total IgE; △, oak mix; ▲, walnut/hickory/pecan; ○, mite; ●, dog.

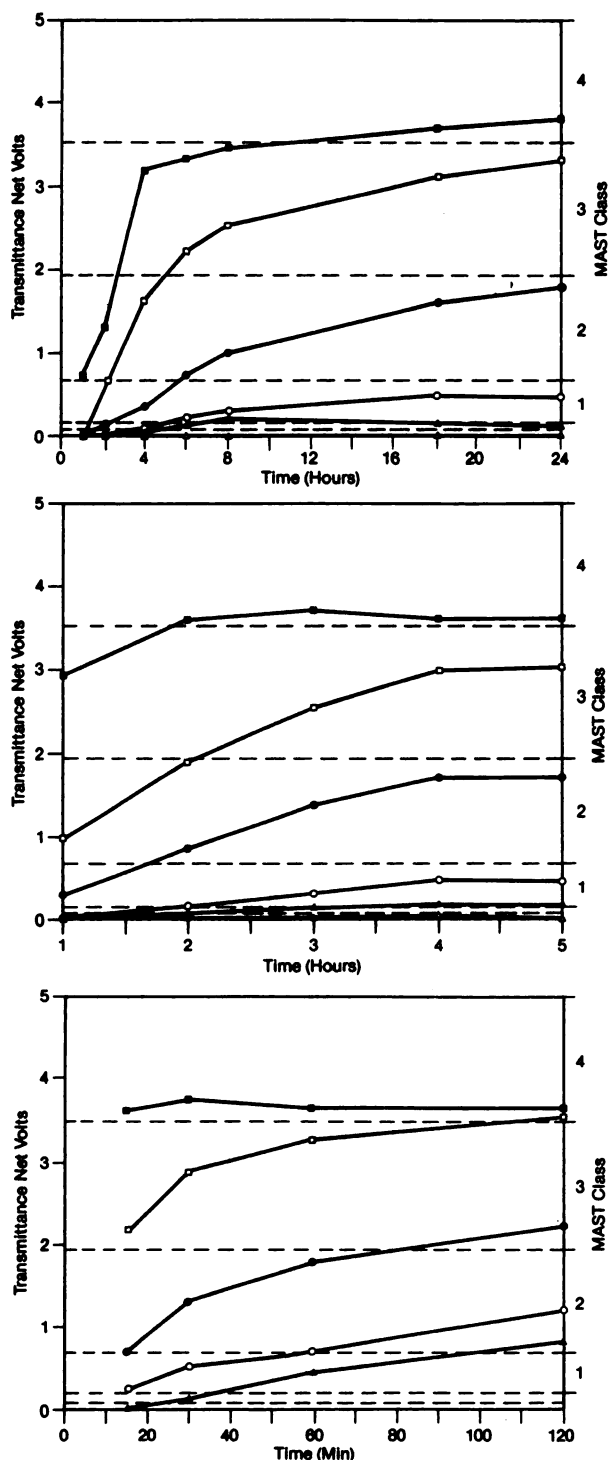


Fig. 5. Assay optimization for times of serum incubation (*top*), antibody incubation (*middle*), and film exposure (*bottom*)

Top, MASTpette test chambers were filled with serum from an atopic individual and incubated at room temperature for various periods, up to 24 h, in duplicate samples for each period. Then antibody reagent was added to each test chamber and incubated at room temperature for 4 h. Substrate solution was added, and all test chambers were exposed to film for 30 min. The average net voltage of representative threads from each MAST class (demarcated by broken lines) are shown. Assay sensitivity is maximum for an incubation time of between 16 and 24 h. *Middle*, MASTpette test chambers filled with serum from an atopic individual were incubated for 18 h at room temperature. Antibody added to each test chamber was incubated at room temperature for periods from 1 to 5 h, in duplicate samples for each time, then exposed to film as above. The optimum antibody incubation time was 4 h. *Bottom*, duplicate samples of serum from an atopic individual were incubated in MASTpette test chambers for 18 h at room temperature, then with antibody for 4 h. After substrate was added to all test chambers, they were loaded into the photocassettes and exposed to film for various times from 15 to 120 min. Net voltage readings at 30 min correlated well with results for samples run with the current MAST-RIA procedure; increasing the exposure time, however, increases assay sensitivity

Suppression of background luminescence. A dye reagent that absorbs between 400 and 600 nm was incorporated in the luminescent reagent to suppress "cross talk" luminescence emitted between the threads in the MASTpette test chamber. The incorporation of the dye is essential to eliminating false-positive results on threads located next to strongly positive threads (15).

Precision. Within-run variation is illustrated in Figure 6 (*top*). Five replicates of four serum samples were assayed on the same day with the same reagents. The mean CV of the responses of all the allergen threads, calculated as net transmittance voltage, was 13.9%.

Between-run variation (Figure 6, *bottom*) was determined by running five replicates of four serum samples on four different days. The CV of the responses of all the allergen threads, calculated as net transmittance voltage, was 15.9%.

Correlation study. We analyzed a group of 40 patients' serum samples with various concentrations of allergen-specific IgE by both the MAST RIA and MAST CLA systems. The sera were tested against 25 different allergens, including materials from trees, grasses, weeds, molds and animal danders. The classes of allergen-specific IgE correlated identically in 77% (601/778) of the cases, and 97%

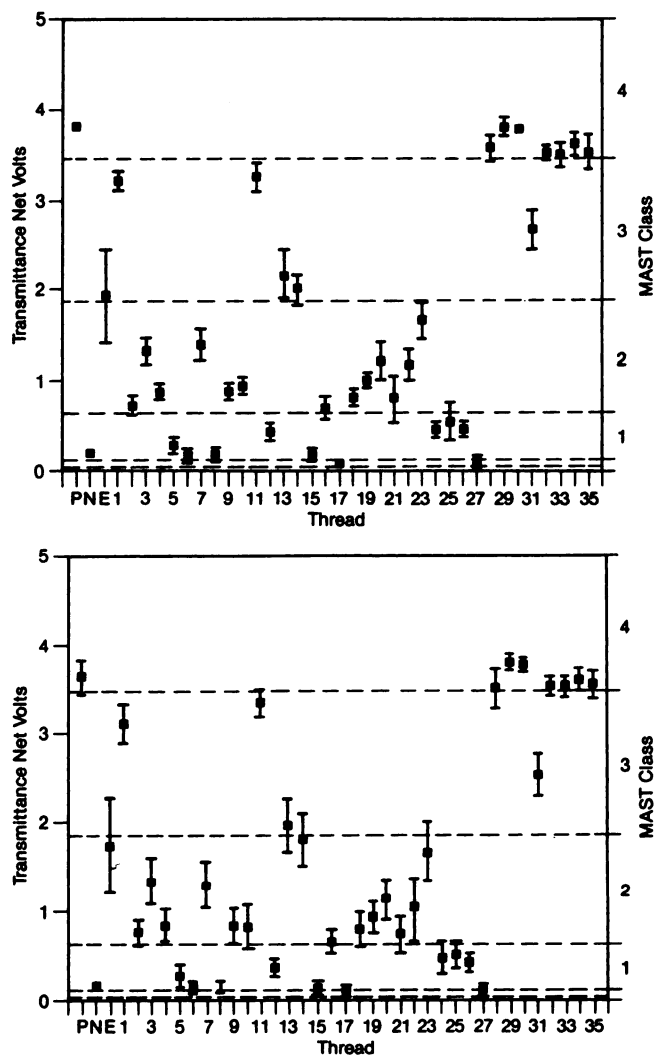


Fig. 6. Within-run (*top*) and between-run (*bottom*) precision of MAST-CLA system

Top: a positive serum pool was assayed in replicates of five on the same day with the same reagents. Results for a positive, negative, and total IgE thread and for 35 inhalant and food threads are presented as average net volts (± 1 SD). Class borders are indicated as broken lines. *Bottom*: a positive serum pool was assayed in replicates of five on four different days on MASTpette test chambers as described above

(754/778) of the results were within one class difference (Figure 7). The correlation coefficient was 0.96, the y-intercept was 0.09, and the slope was 0.96 ($n = 778$).

Discussion

The MAST RIA technology has several advantages over existing RAST tests as an in vitro method for determining IgE responses to individual allergens. The ability to miniaturize RAST-type testing for a panel of allergens plus total IgE offers a clear improvement in the determination of the allergic or nonallergic nature of a patient's symptoms. Furthermore, the MAST RIA has shown good correlation with other RAST assay results (16, 17) and also with skin-prick testing (18). The chemiluminescent assay system (MAST CLA) we have described produces results that compare well with those of the clinically proven MAST RIA but also offers several technical and procedural improvements.

The MAST CLA reagents provide a much greater intensity of light emission than do other commercially available luminescent reagents. Combined with a prolonged (2 h) constant light emission, this provides flexibility to the system; i.e., the chemiluminescent reaction may be initiated several minutes before exposure to film, and the sensitivity of the assay can be increased by increasing exposure times. Unlike the MAST RIA system, which requires an overnight film exposure at refrigerated temperatures, in the MAST CLA system this takes 30 min at room temperature. For intercomparability of results, the chemiluminescent assay response has been tempered to equal that of the MAST RIA system ($r = 0.96$). The assay time has been considerably reduced; results can be obtained within 24 h, with little "hands-on" time. And finally, the CLA system not only alleviates the precautions necessary for handling isotopic materials but also has the advantage of long-term reagent stability (at least six months). The chemiluminescent system retains the quality-control features of internal standards, to assess and compensate for nonspecific binding in a patient's serum sample, which minimizes the chance of

falsely increased results inherent in other RAST-type assays.

Like other nonisotopic immunoassays, chemiluminescent assays are becoming an increasingly attractive alternative to radiolabels in new immunoassay methods (19, 20). Current methods for measuring luminescence reactions typically record peak light emissions or integrate the total light output (21, 22). Immunoassays with luminescent tracers generally require instrumentation to facilitate the simultaneous initiation of the luminescent reaction and the measurement of the light emission in very short time frames (generally between 0 and 60 s, more commonly between 0 and 20 s). Luminescent immunoassays for analytes of clinical interest have been described (23–26); however, efforts toward a clinically feasible chemiluminescence reagent and instrument system package have not heretofore been proven clinically feasible.

The MAST CLA technology is a simple and practical approach for in vitro measurement in the diagnosis of allergy. The MAST CLA Allergy System is the first of several planned clinical diagnostic products that will involve the MAST chemiluminescent technology.

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4					10	85
3			1	25	74	22
2	1	2	28	36	7	
1	11	11	28	12		
1/0	13	14	10			
0	364	15	8	1		
	0	1/0	1	2	3	4

Fig. 7. MAST RIA vs MAST CLA correlation of 40 patients' serum samples assayed against 25 inhalant allergens

The MAST RIA assay was a double-overnight serum-antibody incubation followed by a 16-h film exposure (12). Inhalant allergens included those from trees, grass, weeds, animal danders, housedust, and mite

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