

Multiplexed Particle-Based Anti-Granulocyte Macrophage Colony Stimulating Factor Assay Used as Pulmonary Diagnostic Test

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Pulmonary alveolar proteinosis (PAP) is characterized by the accumulation of lipoproteinaceous material within the lung alveoli. Recent studies indicate that PAP is an autoimmune disease characterized by a neutralizing anti-granulocyte macrophage colony stimulating factor (GM-CSF) antibody. At present the only definitive diagnostic test for PAP is open lung biopsy. We have previously published that anti-GM-CSF is diagnostic for PAP and correlates with disease pathogenesis using a traditional serial anti-GM-CSF antibody titer format (T. L. Bonfield, M. S. Kavuru, and M. J. Thomassen, *Clin. Immunol.* 105:342–350, 2002). Titer analysis is a semiquantitative method, and often subtle changes in antibody titer are not detectable. In this report we present data to support anti-GM-CSF detection by a quantitative highly sensitive multiplexed particle-based assay which has the potential to be a clinical diagnostic test.

Pulmonary alveolar proteinosis (PAP) is an anti-granulocyte macrophage colony stimulating factor (GM-CSF) autoimmune disease, which results in the accumulation of phospholipid surfactant material within the alveoli (14, 16, 19). All patients with PAP have systemic and localized levels of neutralizing anti-GM-CSF as determined by traditional serial antibody titer evaluation (2, 3, 9). We have shown that systemic antibody titers correlate with disease activity (2). A traditional serial dilution enzyme-linked immunosorbent assay (ELISA) titer assay is time-consuming and cumbersome. These assays have only a very limited capacity for evaluating multiple or sequential samples.

Autoantibody assays for evaluation of patients with lupus traditionally use indirect immunofluorescence to determine the presence of autoantibodies followed by more specific assays such as ELISA or immunodiffusion to specifically define the antigen-antibody recognition (12, 15). Recently, a U.S. Food and Drug Administration (FDA)-approved anti-nuclear antibody (ANA) multiplexed particle-based panel has been developed for clinical diagnosis resulting in high-sensitivity and high-volume specific antibody analysis (5). The assay takes advantage of the multiplexing ability of microparticles coupled with an analyte allowing the evaluation of multiple analytes within a single sample with one assay. Thus utilizing this technology, sample volume is conserved while augmenting sensitivity.

Multiplexed particle-based assay is a flow cytometric methodology which depends upon the recognition of fluorescent beads within the context of a biotin-labeled detection antibody using a streptavidin phycoerythrin substrate (8, 11). The advantage of this technology is that it is highly sensitive and quantitative (1, 4). In addition, the microparticle flow cytomet-

ric technology is fluid phase as opposed to traditional solid-phase assays employed with ELISA. Fluid-phase assays allow greater availability for antibody binding due to the three dimensional nature of the solid matrix (microparticle) (4, 20).

We propose that a multiplex microparticle-based assay using the Luminex format could be used to quantitate the amount of anti-GM-CSF in the patient sera. We hypothesize that the particle-based assay will be more quantitative. In addition, quantification of anti-GM-CSF could facilitate the understanding of pathogenesis by correlating antibody with PAP disease activity. Ultimately, we believe that this particle based anti-GM-CSF assay will become a screening pulmonary diagnostic tool for PAP.

MATERIALS AND METHODS

Study population. This protocol was approved by the Institutional Review Board, and written informed consent was obtained from all subjects. Healthy control (HC) individuals ($n = 23$) had no history of lung disease and were not on medication. The diagnosis of idiopathic PAP was established by histopathological examination of material from open lung or transbronchial biopsies showing the characteristic filling of the alveoli with eosinophilic amorphous material with preserved lung architecture and absence of inflammation and exclusion of secondary etiologies by negative lung cultures or occupational history (6, 7, 13, 14). All PAP ($n = 27$) patients were symptomatic with dyspnea, were hypoxemic on room air, and had typical alveolar infiltrates on radiographs. Disease controls (DC) consisted of patients with asthma ($n = 2$) and sarcoidosis ($n = 9$).

Serum. Serum samples were obtained from all patients with PAP and control subjects as previously described (7, 18). Blood was collected in serum separator tubes, aliquoted, and stored at -80°C until tested. PAP sera were evaluated over several serial dilutions and compared with healthy and disease control samples.

Preparation of GM-CSF coupled microspheres. Microspheres with a carboxylated surface (2.5×10^6 ; Luminex Corp., Austin, Tex.) were processed as recommended by Luminex Corporation. Briefly, microspheres were activated with $80 \mu\text{l}$ of $0.1 \text{ M NaH}_2\text{PO}_4$, pH 6.2, then pelleted ($5,000 \times g$ for 2 min) in 1.5-ml centrifuge tubes. The microspheres were then resuspended by sonication (mini sonicator; Cole Parmer, Vernon Hills, IL) followed by vortexing (VWR International, West Chester, PA). Microspheres were then processed in $80 \mu\text{l}$ of the activation buffer, to which an additional $10 \mu\text{l}$ of activation buffer containing 50 mg/ml of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Pierce Chemical Co., Rockford, IL) and $10 \mu\text{l}$ of activation buffer containing 50 mg/ml of *N*-hydroxysulfosuccinimide, sodium salt (sulfo-NHS; Pierce

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Chemical Co.) were added to make a total volume of 100 μ l. The mixture was allowed to gently rock for 20 min at room temperature. The microspheres were then washed twice in 500 μ l of coupling buffer [0.05 M 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma Chemical Co.), pH 5.0], followed by a 2-h incubation with 500 μ l of a solution containing 50, 25, 10, 5, or 1 μ g/ml GM-CSF coupling buffer (Berlex, Seattle, WA), again rocking gently. The coupled microspheres were then washed twice in 1 ml of wash buffer (phosphate-buffered saline [PBS] containing 0.05% Tween 20 [Sigma no. 3563]) and stored in 0.5 ml PBS containing 1% bovine serum albumin (BSA) (Sigma no. 3688), 0.05% sodium azide (pH 7.4; EM Sciences, Cherry Hill, NJ). Microsphere concentrations were determined using a hemacytometer. Microsphere preparations were stable for up to 1 year at 2°C as measured by no change in reactivity with a positive PAP serum or rabbit anti-human GM-CSF standard control.

Anti-GM-CSF titer ELISA. The titer ELISA procedure has been described in detail previously (3). Serum standards and sera for testing were prepared at the appropriate dilutions in phosphate-buffered saline (PBS) containing 5% skim milk and 0.5% Tween 20 (pH 7.4). A standard reference serum was run with every assay (a known positive PAP serum). Healthy and disease control sera and test sera were serially diluted twofold in PBS plus 5% skim milk. The minimum dilution of test serum was 1:100, with maximum at 1:12,800. The final volume in all wells was 100 μ l. Bound anti-GM-CSF immunoglobulin G (IgG) was detected by using horseradish peroxidase-conjugated mouse anti-human IgG Fc (Jackson Immunobiologicals, San Diego, CA). Color development was carried out over 30 min ($\pm$ 5) and was stopped by addition of 100 μ l of peroxidase stop solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD) to all wells of the test plates. Optical density values were read within 30 min of addition of the stop solution with Softmax Microtiter Plate Reader at a wavelength of 450 nm. Sera were evaluated for end titer by comparing test samples with healthy control sera and positive control sera. End titer was defined as the titer which yielded results within two standard deviations above the healthy control optical density at 450 nm. PBS controls for primary and secondary nonspecific binding were also included. The internal control of a known anti-GM-CSF titer was included in all of the assays.

Luminex anti-GM-CSF assay. Healthy control and PAP sera were diluted in PBS to 1:1,000, 1:5,000, 1:10,000, 1:50,000, and 1:100,000. GM-CSF coupled beads (5×10^3) were incubated with diluted sera on a rocker panel at room temperature for 1 h with the diluted sera. The beads were washed 3 times with PBS plus 0.1% BSA. Mouse anti-human IgG labeled with biotin (4 μ g/ml; Southern Biotech, Birmingham, AL) was added to detect the bound serum anti-GM-CSF and again incubated for 1 h on a rocker panel at room temperature. After washing the microparticles, the chromogenic substrate of streptavidin-phycoerythrin (2 μ g/ml; Molecular Probes, Eugene, Oregon) was added and incubated for an additional 30 min again with continued agitation on a rocker panel at room temperature. After the 30 min, the beads were washed, brought up into 100 μ l of PBS, and evaluated on the Luminex 100 platform. A commercial source of purified human anti-human GM-CSF is currently unavailable. Therefore, a standard curve was run in each assay consisting of rabbit anti-human GM-CSF followed by a goat anti-rabbit IgG biotin-labeled antibody (4 μ g/ml; Southern Biotech). To control for assay-to-assay variability an internal standard (a known positive PAP serum sample) was included in every assay. Samples were compared to the standard curve for quantitative analysis.

Statistical analysis. Statistical analysis was performed by student's *t* tests and linear regressions using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

GM-CSF can be efficiently coupled to Luminex microbeads.

Luminex microbeads were coupled with several different concentrations of recombinant human GM-CSF. Dose response curves were generated with rabbit anti-human GM-CSF. Both 25 μ g and 50 μ g were linear at 25 ng/ml anti-GM-CSF (Fig. 1). Each dose was run twice in duplicate. The coupling dose of 50 μ g had a greater dynamic range of detection. To determine the most effective coupling dose at detecting anti-GM-CSF in a complex mixture such as serum, dilutional analysis of a healthy control ($n = 2$) and PAP ($n = 2$) sera were done with both 50 μ g and 25 μ g coupled beads (Fig. 2). The 50 μ g coupling dose had a slightly higher level of nonspecific binding with healthy control sera. Both the 50 and 25 μ g were efficient at detecting

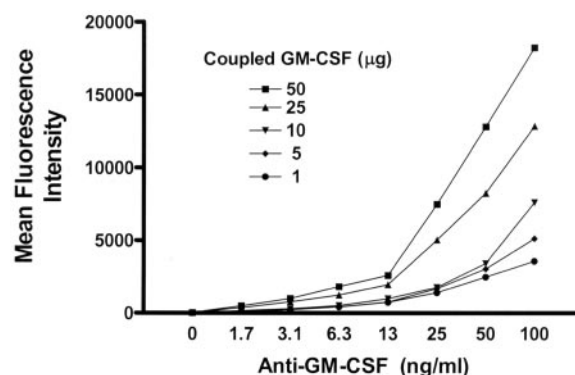


FIG. 1. GM-CSF can be efficiently coupled to Luminex microbeads. Human recombinant GM-CSF at concentrations of 50 μ g, 25 μ g, 10 μ g, 5 μ g, and 1 μ g were coupled to Luminex microbeads. Coupling efficiency was determined by evaluating each set of prepared beads with a rabbit anti-human GM-CSF standard curve in duplicate. Each experiment was repeated twice. Both the 50 μ g and 25 μ g were efficient at generating a linear standard curve with the antibody control.

the anti-GM-CSF in our patient samples with almost superimposable curves. The upper dynamic range for PAP sera was better at the 25- μ g coupling dose with more linearity at the upper end of the curve and with a low level of nonspecific binding in our healthy controls compared to the PAP sera. Therefore, we chose 25 μ g as our final coupling dose for the development of our anti-GM-CSF multiplex particle-based assay. We obtained similar results with an additional patient and healthy control samples (data not shown).

Anti-GM-CSF Luminex assay is sensitive. Purified rabbit anti-human GM-CSF was incubated with GM-CSF coupled Luminex beads at concentrations ranging from 0.7 ng/ml to 100 ng/ml. Each concentration was run in duplicate for three separate curves. Reactivity of anti-GM-CSF resulted in a linear

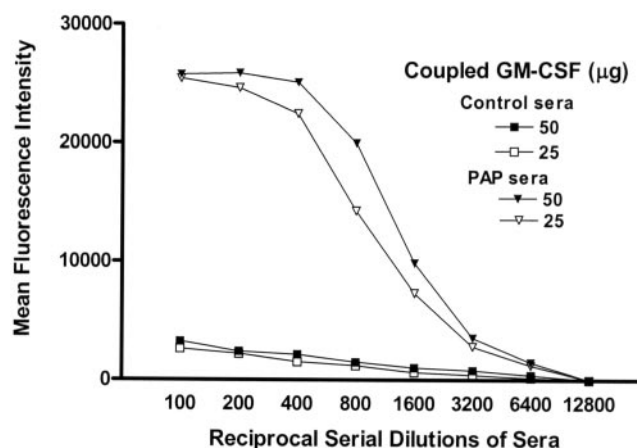


FIG. 2. The appropriate coupled GM-CSF concentration is 25 μ g. Healthy control and PAP sera were compared when anti-GM-CSF was quantitated using microbeads coupled with both 25 μ g and 50 μ g recombinant human GM-CSF. The dynamic range for PAP sera was greater at the 25- μ g coupling dose with more sensitivity at the upper end of the curve and less antigen saturation. This also gave low levels of nonspecific binding in our healthy controls compared to PAP sera. These data are representative of two experiments run in duplicate.

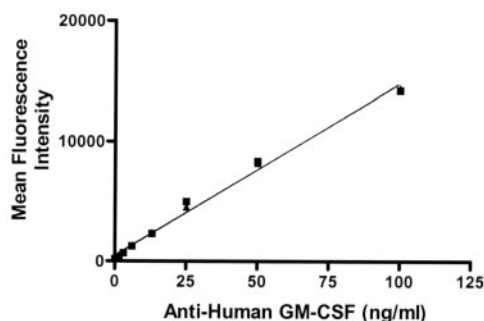


FIG. 3. The anti-GM-CSF Luminex assay is sensitive. Purified rabbit anti-human GM-CSF was incubated with 25 μ g GM-CSF coupled Luminex beads at concentrations ranging from 0.7 to 100 ng/ml. Data are expressed as mean fluorescence intensity of the sample versus the concentration (in nanograms/milliliter) of rabbit anti-human GM-CSF. Reactivity of the anti-GM-CSF resulted in linear curve ($n = 3$, $R^2 = 0.98$, $P < 0.05$).

concentration curve versus mean fluorescence intensity (Fig. 3, $n = 3$, $R^2 = 0.98$, $P < 0.05$). Further, we used a nonspecific antibody control (goat anti-human M-CSF; 2 μ g/ml; R&D Systems, Minneapolis, MN) to define the specificity of the coupled beads. There was no binding of anti-M-CSF with the microbeads (data not shown).

Anti-GM-CSF Luminex assay correlates with ELISA titer assay. Patient samples were run concurrently in both the Luminex assay and the titer assay. Mean titers were obtained and plotted against the quantified anti-GM-CSF determined in the Luminex assay. Titers in the ELISA assay correlated with the quantity of anti-GM-CSF determined by the Luminex assay (Fig. 4, $n = 19$, $R^2 = 0.99$, $P < 0.05$).

Anti-GM-CSF Luminex assay is specific for PAP anti-GM-CSF autoimmune disease. PAP patients ($n = 27$, previous patients from Fig. 4 as well as an additional 8) with verified anti-GM-CSF end titers using the ELISA were evaluated in the Luminex assay (Fig. 5). Healthy control sera ($n = 23$,

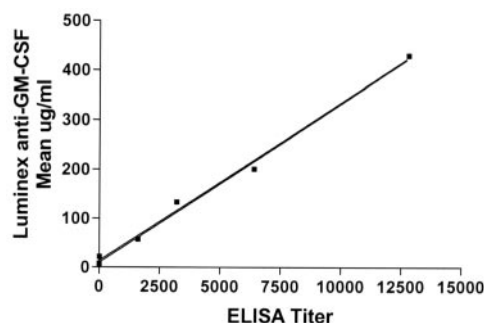


FIG. 4. The anti-GM-CSF Luminex assay correlates with the ELISA titer assay. Samples from 19 PAP patients and 18 healthy controls were run concurrently in both the multiplex particle-based assay and the ELISA titer assay. Mean concentrations of PAP and healthy control samples were plotted against their respective end titers. The distributions of the samples with titer values are as follows: healthy control is 0 titer ($n = 12$, 11 ± 2.2 μ g/ml), 1:10 ($n = 2$, 21.5 ± 5); PAP is 1:1,600 ($n = 2$, 57 ± 5), 1:3,200 ($n = 2$, 131 ± 3), 1:6,400 ($n = 6$, 201 ± 54.4), 1:12,800 ($n = 9$, 433 ± 65). Titers in the ELISA correlated with the quantity of the anti-GM-CSF determined by the developed Luminex assay ($R^2 = 0.99$, $P < 0.05$).

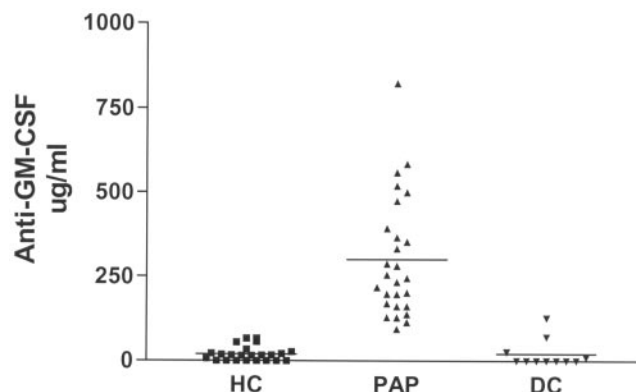


FIG. 5. The anti-GM-CSF Luminex assay is specific for PAP anti-GM-CSF autoimmune disease. PAP ($n = 27$), healthy control ($n = 23$), and disease control sera ($n = 11$) were evaluated in the multiplex particle-based assay. All of the patients had detectable levels of anti-GM-CSF (299 ± 34 μ g/ml) whereas all of the healthy control and disease control values fell below 34 μ g/ml ($P = 0.0001$). The line represents the mean for each group.

previous 18 patients from Fig. 4 plus an additional 6) with end titer values of 0 ($n = 13$) and 1:10 ($n = 5$) were assayed in the Luminex assay. In addition, we evaluated several disease controls ($n = 11$, 2 asthmatics and 9 sarcoidosis patients). All of the patients had detectable levels of anti-GM-CSF (299 ± 34 , range of 94 to 821 μ g/ml, $P = 0.0001$), whereas all of the healthy control and disease control values fell below 34 μ g/ml (20 ± 2.4 , range of 0 to 34 μ g/ml, and 21 ± 12 , range of 0 to 33 μ g/ml, respectively).

DISCUSSION

We have presented for the first time the development of an anti-GM-CSF multiplex particle-based assay for diagnostic utility in PAP. Microparticle assays are cytometry-based methods and use antigen-coupled microbeads containing a proprietary combination of fluorescent dyes (1, 8, 11). In our studies we have coupled human recombinant GM-CSF to the surface of these beads, which are then used to detect the presence of anti-GM-CSF (1). Purified rabbit anti-human GM-CSF was used to determine coupling efficiency and ultimately to define the upper and lower ranges of assay detection. We have used a Luminex based methodology for our multiplex particle-based assay. This Luminex anti-GM-CSF assay directly correlates with the traditional ELISA anti-GM-CSF titer assay ($R^2 = 0.99$).

PAP is an anti-GM-CSF autoimmune disorder which culminates in the accumulation of surfactant phospholipids in the alveoli (14, 16, 17, 19). All PAP patients have both systemic and localized levels of anti-GM-CSF (2, 3, 9, 10). In patients with appropriate history and consistent imaging studies, data from bronchoscopy (i.e., gross appearance of lavage fluid) and open biopsy are usually performed to confirm a diagnosis of PAP (6, 13, 14). This is an invasive procedure, which requires general anesthesia. We developed a traditional ELISA titer assay to detect the presence of anti-GM-CSF in the sera of these patients showing that anti-GM-CSF titer correlates with disease (2). The ELISA is cumbersome and requires a consid-

erable amount of time and reagents. Further, only four patient samples can be evaluated at once making longitudinal evaluations more imprecise.

The multiplex particle-based assay was developed by coupling the microbeads with a series of different concentrations of recombinant GM-CSF to define the appropriate coupling efficiency. We found that 25 µg/ml is the most efficient concentration for GM-CSF coupling of the microbeads. The anti-GM-CSF ELISA has a sensitivity of 100% and specificity of 91% (2). This is the minimum sensitivity and specificity of the Luminex assay based upon the high correlation ($r^2 = 0.99$) for the detection of the antibody in PAP patients using Luminex as compared to the ELISA titer assay.

Particle-based flow cytometric assays are offered by three different companies: Becton-Dickson, DiaSorin, and Luminex (reviewed by Vignali [20]). These assays have significant advantages over the ELISA titer assay. First, multiple samples from the same patient can be evaluated during the same run using less than 200 µl of sera, second, the assay format is more time efficient, and third, the fluid phase nature allows for greater dynamic range.

In the presence of anti-GM-CSF, open lung biopsy can be avoided and the potential diagnostic and therapeutic choices can be made earlier with potentially better outcomes. We believe that this newly developed multiplex particle based anti-GM-CSF assay will become an important diagnostic tool for pulmonary diseases and PAP specifically.

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