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

Tracey L. Bonfield,¹ Nejimol John,¹ Barbara P. Barna,¹ Mani S. Kavuru,¹ Mary Jane Thomassen,¹,³ and Belinda Yen-Lieberman²

Departments of Pulmonary, Allergy and Critical Care Medicine,¹ Clinical Pathology,² and Cell Biology,³
The Cleveland Clinic Foundation, Cleveland, Ohio 44195-5038

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Pulmonary alveolar proteinosis (PAP) is characterized by the accumulation of lipoproteinoicaceous 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.

Multiplexed Particle-Based Anti-Granulocyte Macrophage Colony Stimulating Factor Assay

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 cytometric 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 cosinophilic 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 × 10⁶; Luminex Corp., Austin, Tex.) were processed as recommended by Luminex Corporation. Briefly, microspheres were activated with 80 µl of 0.1 M NaH₂PO₄, pH 6.2, then pelleted (5,000 × 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 µl of the activation buffer, to which an additional 10 µl of activation buffer containing 50 mg/ml of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Pierce Chemical Co., Rockford, IL) and 10 µl of activation buffer containing 50 mg/ml of N-hydroxysulfosuccinimide, sodium salt (sulfo-NHS; Pierce

* Corresponding author. Mailing address: Department of Pulmonary, Allergy and Critical Care Medicine, 9500 Euclid Avenue, Cleveland Clinic Foundation, Desk A90, Cleveland, Ohio 44195-5038. Phone: (216) 445-7298. Fax: (216) 444-5172. E-mail: bonfield@ccf.org.
Chemical Co.) were added to make a total volume of 100 μl. The mixture was allowed to gently rock for 20 min at room temperature. The microspheres were then washed twice in 500 μl 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 μl of a solution containing 50, 25, 10, 5, or 1 μg/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 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 μg/ml; each concentration was run in duplicate for three separate curves. Reactivity of anti-GM-CSF resulted in a linear

![FIG. 1. GM-CSF can be efficiently coupled to Luminex microbeads. Human recombinant GM-CSF at concentrations of 50 μg, 25 μg, 10 μg, 5 μg, and 1 μg 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 μg and 25 μg were efficient at generating a linear standard curve with the antibody control.](http://cvi.asm.org/)

![FIG. 2. The appropriate coupled GM-CSF concentration is 25 μg. Healthy control and PAP sera were compared when anti-GM-CSF was quantitated using microbeads coupled with both 25 μg and 50 μg recombinant human GM-CSF. The dynamic range for PAP sera was greater at the 25-μg 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.](http://cvi.asm.org/)
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 \( \mu \)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 ELISA titer assay. Mean titers were obtained and plotted against the quantified anti-GM-CSF determined in the Luminex assay. Titters 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 \((n = 27)\), 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 \((299 \pm 34 \mu \text{g/ml})\) whereas all of the healthy control and disease control values fell below 34 \( \mu \text{g/ml} \) \((P = 0.0001)\). The line represents the mean for each group.

**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 \((n = 12, 11 \pm 2.2 \mu \text{g/ml})\), 1:10 \((n = 2, 21.5 \pm 5)\); PAP is 1:1,600 \((n = 2, 57 \pm 5)\), 1:3,200 \((n = 2, 131 \pm 3)\), 1:6,400 \((n = 6, 201 \pm 54.4)\), 1:12,800 \((n = 9, 433 \pm 65)\). Titters 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 \((299 \pm 34 \mu \text{g/ml})\) whereas all of the healthy control and disease control values fell below 34 \( \mu \text{g/ml} \) \((P = 0.0001)\). The line represents the mean for each group.

**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 Luminox assay based upon the high correlation ($r^2 = 0.99$) for the detection of the antibody in PAP patients using Luminox 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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