Development and Validation of a Fluorescent Microsphere Immunoassay for Soluble CD30 Testing

Igor Pavlov,1* Thomas B. Martins,1 and Julio C. Delgado1,2

ARUP Institute for Clinical and Experimental Pathology and Department of Pathology, University of Utah School of Medicine;2 Salt Lake City, Utah

Received 3 February 2009/Returned for modification 23 March 2009/Accepted 7 July 2009

Testing for soluble CD30 (sCD30), an indicator of Th2 immune response, is a useful prognostic marker in solid organ transplantation, lymphoproliferative disorders, autoimmunity, and various parasitic diseases. In this study we report the development and validation of a fluorescent microsphere immunoassay for the detection of sCD30 in serum, plasma, and culture supernatants. The dynamic range of this assay is 1 to 400 ng/ml, and the rate of recovery of various concentrations of recombinant sCD30 ranges from 97 to 116% (average recovery, 105%). The test showed a high degree of precision in both intra-assay and interassay studies (coefficients of variation, as high as 7% and 8%, respectively), with a sensitivity of 1 ng/ml. The normal reference range calculated for a cohort of 151 healthy individuals was 1 to 29 ng/ml. The clinical usefulness of the sCD30 fluorescent microsphere immunoassay was demonstrated by showing that levels of sCD30 have a positive correlation with specimens containing high titers of anti-double-stranded DNA antibodies and high titers of immunoglobulin G against Leishmania species. Given the multiplexing potential of the sCD30 fluorescent microsphere immunoassay reported in this study, it is expected that testing of sCD30 concentrations along with those of other cytokines will become an important diagnostic tool for selected immunological and inflammatory diseases where Th2-type cytokine responses have been reported.

CD30 (TNFRSF8) is a transmembrane protein, a member of the tumor necrosis factor (TNF) receptor superfamily. It was originally described as a marker for Reed-Sternberg cells (“Ki-1 antigen”) in Hodgkin’s disease (12, 18, 20). CD30 is expressed on CD4+ and CD8+ T cells that secrete Th-2 type cytokines (8, 17). Signaling through CD30 plays important roles in T- and B-cell growth, differentiation, and function. The soluble form of CD30 (sCD30) is produced after proteolytic cleavage of the membrane-bound CD30 ectodomain by the TNF-α-converting enzyme (9).

Numerous studies have reported that circulating levels of sCD30 may represent a biomarker for outcomes in solid-organ transplantation (16, 21). In addition, other studies have reported that levels of sCD30 have important prognostic value for various lymphoproliferative disorders (4, 15, 22), systemic lupus erythematosus (SLE) (5, 7), and leishmaniasis (1, 2). The current method for quantitation of sCD30 is the enzyme-linked immunosorbent assay (ELISA), which has good sensitivity and specificity. However, sCD30 production differs greatly between patients, and the dynamic range of ELISAs requires that many samples be diluted and retested. Moreover, ELISA measures only 1 analyte per well, which precludes the testing of multiple analytes in the same test. In this study, we report the development and validation of a fluorescent microsphere immunoassay suitable for multiplexed determination of sCD30 levels, along with those of other cytokines, in serum and plasma specimens and in tissue culture supernatants. We present data showing the positive correlation of sCD30 levels with titers of anti-double-stranded DNA (anti-dsDNA) antibodies in SLE and with immunoglobulin G (IgG) levels in patients with leishmaniasis.

MATERIALS AND METHODS

Antibodies and reagents. The following anti-sCD30 antibodies were used during the development of the sCD30 assay: mouse monoclonal antibodies (MAbs) Ki-2 (IgG1) and Ki-3 (IgG2b) from Bender MedSystems (Burlingame, CA), mouse MAb MEM-268 (IgG) from BioLegend (San Diego, CA), and a biotinylated goat polyclonal antibody from R&D Systems (Minneapolis, MN). Information on the antigen specificities of MAbs Ki-2 and Ki-3 (recognizing different, nonoverlapping regions of the CD30 molecule) has been reported elsewhere (10).

A human recombinant sCD30 standard was acquired from Bender MedSystems. Streptavidin conjugated with R-phycocerythrin and R-phycocerythrin conjugate diluent were obtained from Bioss, Inc. (Passadena, CA). Microspheres were acquired from Luminex Corporation (Austin, TX). N-Hydroxysulfosuccinimide (sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC) were obtained from Pierce (Rockford, IL). Polyoxethylene sorbitan monolaurate (Tween 20), phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA), morpholineethanesulfonic acid (MES), fetal bovine serum (FBS), bilirubin, and magnesium chloride were all obtained from Sigma (St. Louis, MO). Triglycerides (Intralipid 20%) were purchased from Baxter Healthcare Corp. (Deerfield, IL) and 96-well filter microplates from Millipore (Bedford, MA).

Coupling of the microspheres. For the development of the sCD30 fluorescent microsphere immunoassay, 50 μg of capture MAbs against CD30 was diluted in 400 μl of 50 mM MES, pH 5.0 (coupling buffer). Stock microspheres (1.25 × 107 beads/ml) were resuspended in 100 μl of 100 mM monobasic sodium phosphate, pH 6.2 (activation buffer). Microspheres were activated for 20 min at room temperature by the addition of 10 μl of 50-mg/ml sulfo-NHS (diluted in distilled H2O) and 10 μl of 50-mg/ml EDC. Activated microspheres were washed twice with coupling buffer and were incubated with capturing antibodies (50 μg MAb in a total of 500 μl of MES) for 2 h at room temperature on a rocker. The coupled microspheres were then washed twice with PBS (pH 7.2) plus 0.05% Tween 20 (PBS-T) and resuspended in 1 ml of PBS (pH 7.2) with 1% BSA and 0.05% sodium azide (PBBS). The microspheres were then incubated for 30 min on a rocker to permit the blocking of the unreacted sites and were stored at 4°C in PBBS.
sCD30 fluorescent microsphere assay. A standard curve for sCD30 was made by mixing a known concentration of recombinant sCD30 in assay buffer (PBSB plus 10% FBS). A six-point standard curve was generated by making 1:3 dilutions from 0 to 400 ng/ml. Each standard was run in duplicate. In addition, three controls containing high, medium, and low concentrations of sCD30, respectively, were run with each assay.

Ninety-six-well filter microtiter plates were washed with 150 μl of PBST well and were filled with 50 μl of assay buffer. Twenty microliters of sCD30 standards or testing specimens (undiluted serum, plasma, or culture supernatants) was added to each well. Fifty microliters per well of mixed biotinylated detector antibodies and microspheres containing 80,000 beads/ml was added, resulting in a final concentration of 4,000 microspheres per well. Foil-covered microtiter plates were incubated for 3 h at room temperature on a shaker. The microspheres were then washed three times by vacuum filtration with 150 μl of PBST, followed by the addition of 50 μl of streptavidin-conjugated R-phycoerythrin (5 μg/ml) to each well. After a 15-min incubation and a final wash, the microspheres were resuspended in 150 μl of PBST. The microplate containing the resuspended microspheres was placed in a Luminex 100 instrument with an XY platform (automated microtiter plate handler), where the microspheres were counted and analyzed. In each well of the microtiter plate, the fluorescence intensity of 100 beads is measured. The amount of sCD30 bound to the microspheres by this antibody sandwich technique is determined by the median fluorescence intensity of the reporter molecule phycoerythrin. When excited at 532 nm, phycoerythrin emits at 575 nm. The median fluorescence intensity of the unknown sample is then converted into a value (in nanograms per milliliter) based on the known sCD30 concentrations of the standard curve by using a four-parameter regression formula.

Clinical samples. Clinical samples were selected from the specimen repository of ARUP Laboratories. Samples included specimens received for testing of anti-dsDNA antibodies (titers determined by immunofluorescence) and for testing for leishmaniasis by specific IgG antibody determination. All patient samples included in this study were deidentified according to protocol 7275, approved by the University of Utah Institutional Review Board, in order to meet the patient confidentiality guidelines of the Health Information Portability and Accountability Act.

Also included in this study were culture supernatants from CD30+ lymphoma cell lines derived from a patient with cutaneous T-cell lymphoma (MAC-1 and MAC-2A) and a patient with anaplastic large cell lymphoma (LB-6), all kindly provided by Marshall Kadin, Roger Williams Medical Center, Providence, RI.

Statistical analysis. A reference range was created using the EP Evaluator software package (release 7; David G. Rhooids Associates, Inc.). A transformed parametric reference interval method utilizing Box-Cox transformation (3) was used. For statistical analysis of normality, the Shapiro-Wilk normality test (19) was used. To calculate the significance of the differences between cases and healthy controls, the Wilcoxon signed-rank test (23) was performed. The results of the Shapiro-Wilk normality test and the Wilcoxon signed-rank test were calculated using GraphPad Prism, version 5 for Windows (GraphPad Software, Inc.).

RESULTS

Development and validation of the sCD30 assay. The initial development of the sCD30 assay required the testing of different anti-sCD30 MAb combinations. The specificities of the different pair combinations were evaluated in samples spiked with different concentrations of recombinant sCD30. To ensure that the capture and detector antibodies used in the assay also recognized native human sCD30, similar studies were done using human serum samples. We established that antibody pairs utilizing the capture MAb Ki-2, Ki-3, or MEM-265 and a polyclonal secondary antibody did not perform optimally in the Luminex platform. No signal was detected when MAb MEM-268 was used as the detector antibody in combination with MAb Ki-2 or Ki-3 as the capture antibody. The combination of MAb MEM-268 as the capture antibody and Ki-3 as the secondary antibody was highly effective for detecting recombinant sCD30, but the signal was minimal in human serum samples. Good specific signal/background ratios of recombinant and native sCD30 were finally achieved by using the Ki-2 capture antibody and an in-house-biotinylated Ki-3 secondary antibody.

During the assay optimization, we observed that the detergent Tween 20, normally used in assay buffers, strongly inhibited the sCD30 signal in a dose-dependent manner (data not shown). As a consequence, we used an assay buffer containing PBS with 1% BSA and 10% FBS.

The linearity and recovery of the sCD30 fluorescent microsphere assay were assessed by the following methods. First, recombinant sCD30 was spiked and serially diluted in normal human serum. The percentage of recovery was calculated by dividing the mean observed value by the expected value. The percentages of recovery of the different spiked concentrations by the sCD30 fluorescent microsphere assay ranged from 97% to 116%. Then a human serum sample containing a high level of endogenous sCD30 was mixed either with assay buffer or with another human serum sample with a low level of the marker. In both cases, data were linear, with an allowable systematic error of 15% in the range between 1 and 442 ng/ml. Serial dilutions of recombinant sCD30 in assay buffer showed linearity from 4 to 348 ng/ml and accuracy within 15% from 11 to 348 ng/ml (Fig. 1).

To test whether plasma specimens were suitable for sCD30 testing using the fluorescent microsphere assay described in this study, 31 serum-plasma sample pairs from healthy donors were accessed. For two matrices to be statistically identical, the 95% confidence interval (95% CI) for the slope must include 1.00 and the 95% CI for the intercept must include 0.0. Analysis of serum-plasma comparisons (conducted with EP Evaluator software) yielded a Deming regression slope equal to 1.09 (95% CI, 0.95 to 1.23) and an intercept of −13.2 (95% CI, −27.3 to 0.6) with a correlation coefficient of 0.94. Thus, we conclude that plasma and serum specimens are suitable for sCD30 testing using this assay.

Analytical sensitivity was determined as the limit of blank, defined as a concentration corresponding to the average measured fluorescence intensity (MFI) of a zero-analyte sample plus 3 standard deviations (SDs). Six replicas of assay buffer samples (“zero analyte”) were measured; average MFIIs and SDs were calculated. Corresponding limit-of-blank concentra-
tions, determined using the calibration curve, were found to be 1 ng/ml.

Precision was determined by intra-assay and interassay studies of five serum samples containing different sCD30 concentrations ranging from 18 to 237 ng/ml. For intra-assay precision, the mean, SD, and coefficient of variation (CV) were calculated from seven replicates for each concentration. Data from intra-assay runs showed CVs of 8% or less. For interassay precision, the mean, SD, and CV (expressed as a percentage) were calculated from four runs each on five concentrations. Interassay CVs ranged from 4% to 7%.

To test analyte stability, four specimens (serum specimens containing low, medium, and high levels of endogenous sCD30 and a serum specimen containing recombinant sCD30 in assay buffer) were freeze-thawed once or three times; sCD30 measurements were compared to corresponding data for nonfrozen samples. The percentages of recovery after one or three cycles of freeze-thawing ranged from 90 to 110%. We concluded that serum samples can be freeze-thawed as many as three times with no effect on sCD30 assay results.

Normal reference ranges. One hundred fifty-one serum samples (66 males, 85 females) from an ARUP collection from healthy individuals was found to be positively skewed and did not pass the Shapiro-Wilk normality test calculated with GraphPad Prism (Table 1). The median sCD30 concentration of healthy individuals was found to be 29 ng/ml (90% CI, 26 to 32 ng/ml) of sCD30 were units. A low value of 1 ng/ml (90% CI, 0 to 1 ng/ml) and a high mean value of 10 ng/ml; the interquartile range was 7 to 15 ng/ml. For the high-positive-titer (1:160) (Fig. 2).

We found significant levels of sCD30 in the supernatants from three different CD30+/H9251 cell lines (described in Materials and Methods) that had been incubated for 24 or 48 h. We found significant levels of sCD30 in the supernatants, determined using the calibration curve, were found to be 1 ng/ml.

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Normal reference ranges. One hundred fifty-one serum samples (66 males, 85 females) from an ARUP collection from healthy individuals was used to investigate the distribution of sCD30 levels in the healthy population (median age, 29 years; interquartile range, 26 to 40 years; range, 18 to 65 years). The distribution of sCD30 concentrations determined for the group of healthy individuals was found to be positively skewed and did not pass the Shapiro-Wilk normality test calculated with GraphPad Prism (Table 1). The median sCD30 concentration was 10 ng/ml; the interquartile range was 7 to 15 ng/ml. For estimation of the reference range, the transformed parametric reference interval method was used. EP Evaluator software utilizes Box-Cox transformation to change the scale of the data so that it has a Gaussian distribution. Then it computes the mean ± 2 SDs and converts the answer back to the original units. A low value of 1 ng/ml (90% CI, 0 to 1 ng/ml) and a high value of 29 ng/ml (90% CI, 26 to 32 ng/ml) of sCD30 were adopted.

Specificity of the sCD30 fluorescent microsphere assay. To study the specificity of the sCD30 fluorescent microsphere assay, 41 ng/ml of each of the following human recombinant cytokines/markers was spiked into assay buffer: interleukin-1b (IL-1b), IL-2, IL-2 receptor, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, gamma interferon, TNF-α, and soluble CD40 ligand. Normal concentrations for these cytokines/markers in serum are generally less than 30 pg/ml. The level of sCD30 in this spiked sample was equal to 0 ng/ml, indicating the high specificity of the sCD30 assay.

Utility of sCD30 testing in clinical laboratory testing. To validate the clinical usefulness of the sCD30 fluorescent microsphere assay, sCD30 concentrations in two panels of clinical samples were assessed. First, levels of sCD30 in 125 randomly selected clinical specimens that tested positive (titers, 1:10 or greater) for anti-dsDNA IgG antibodies were measured. Considering non-Gaussian distributions of the data, the Wilcoxon signed-rank test was used to estimate differences in median concentrations of sCD30 between groups of samples. The median concentration of sCD30 was significantly higher in anti-dsDNA antibody-positive specimens than in healthy-control samples (Table 1). Given that high titers of antibodies to dsDNA are specific for SLE, we divided the anti-dsDNA antibody panel into a low-positive-titer (1:10 to 1:160) (n = 48) and a high-positive-titer (>1:160) (n = 77) group. Both the low- and the high-positive anti-dsDNA group were statistically different from healthy controls; P values by the Wilcoxon signed-rank test were all less than 0.01 (Table 1). We found that the difference in the distribution of sCD30 concentrations between anti-dsDNA-positive groups and controls was higher for the high-positive group than for the low-positive group (Fig. 2).

We then measured the levels of sCD30 in a panel of 26 specimens containing IgG against Leishmania species. We observed a positive correlation between sCD30 levels and anti-Leishmania IgG titers (coefficient of correlation [R2], 0.616). The difference in median sCD30 concentrations between anti-Leishmania IgG-positive specimens and healthy-control samples was statistically significant (P < 0.01 by the Wilcoxon signed-rank test [Table 1]). Figure 3 shows that the difference from healthy controls was higher for the high-anti-Leishmania IgG (titers, ≥1:128) group (15 samples) than for the low-anti-Leishmania IgG (titers between 1:32 and 1:64) group (11 specimens).

Finally, sCD30 levels were measured in tissue culture supernatants from three different CD30+ lymphoma cell lines (described in Materials and Methods) that had been incubated for 24 or 48 h. We found significant levels of sCD30 in the super-
natants of all three cell lines tested; the concentration of sCD30 correlated positively with the duration of the culture (Table 2).

**DISCUSSION**

In this study we report the development and validation of a fluorescent microsphere immunoassay suitable for the determination of sCD30 concentrations in serum, plasma, and culture supernatants. This assay has a large dynamic range (1 to 400 ng/ml), which is convenient for measuring the varied and widely ranging concentrations of sCD30 under clinical conditions and in cell culture experiments. Percentages of recovery for the sCD30 assay ranged from 97 to 116%, and results from the validation showed high degrees of precision in both the intra-assay and interassay studies (CVs, as high as 7% and 8%, respectively). These findings are better than those of previous studies reporting percentages of recovery ranging from 80 to 120% and intra- and interassay variabilities as high as 20% for multiplexed fluorescent microsphere assays (6, 13). The analytical sensitivity or detection limit for the sCD30 fluorescent microsphere immunoassay was 1 ng/ml. This sensitivity is similar to the sensitivity reported for the standard ELISAs available.

The clinical usefulness of the sCD30 fluorescent microsphere immunoassay was demonstrated by measuring the concentrations of sCD30 in serum samples from patients with SLE (5, 7). Anti-dsDNA antibody production is one of the main features of SLE, and the determination of anti-dsDNA antibody titers in serum samples from patients with SLE is important for the prognosis of the disease (reviewed in references 11 and 14). Our results showed that sCD30 concentrations have a positive correlation with the presence of anti-dsDNA antibodies. Similar results were found when levels of sCD30 were measured in samples with positive anti-\textit{Leishmania} IgG titers. These results suggest a role for sCD30 testing in the clinical laboratory as a diagnostic tool for selected diseases where increased Th2-type cytokine responses have been reported.

The sCD30 fluorescent microsphere immunoassay described in this study has the potential to be used in a multiplexed format, in which multiple cytokines can be measured in the same reaction mixture. Preliminary studies using the sCD30 fluorescent microsphere immunoassay in a multiplexed format have revealed accurate and reliable results for the simultaneous testing of 14 cytokines, including sCD30, from only 25 µl of patient serum or plasma (data not shown). Studies are currently being carried out in our laboratory to measure sCD30 levels along with those of multiple cytokines under

**TABLE 2. Concentrations of sCD30 in tissue culture supernatants of CD30$^+$ lymphoma cell lines**

| Cell line | 24 h sCD30 concn (ng/ml) | 48 h sCD30 concn (ng/ml) |
|-----------|--------------------------|--------------------------|
| JB-6      | 419                      | 800                      |
| MAC-1     | 135                      | 551                      |
| MAC-2A    | 738                      | >1,800                   |

FIG. 2. Comparison of the concentrations of sCD30 in samples from a healthy population (n = 151) with those in samples positive for anti-dsDNA IgG. Levels of sCD30 in 125 randomly selected clinical specimens with anti-dsDNA IgG antibody titers of 1:10 or greater were measured. Anti-dsDNA antibody-positive specimens were divided into a low-intermediate-positive-titer (1:10 to 1:160) group (n = 48) and a high-positive-titer (>1:160) group (n = 77) for comparison with healthy-control specimens. The results are presented in “box-and-whisker” plots. Boxes represent the 25th to 75th percentiles; whiskers represent the 5th to 95th percentiles. The median is represented by a solid horizontal line and the mean by a plus sign.

FIG. 3. Comparison of the concentrations of sCD30 in samples from a healthy population (n = 151) with those in samples positive for anti-\textit{Leishmania} IgG. Levels of sCD30 in 26 specimens containing anti-\textit{Leishmania} IgG were measured. Anti-\textit{Leishmania} IgG-positive specimens were divided into a low-positive-titer (1:32 to 1:64) group (n = 11) and a high-positive-titer (≥1:128) group (n = 15) for comparison with healthy-control specimens. The results are presented in “box-and-whisker” plots. Boxes represent the 25th to 75th percentiles; whiskers represent the 5th to 95th percentiles. The median is represented by a solid horizontal line and the average by a plus sign.
different clinical conditions. It is expected that these studies will be able to show correlations between production of different cytokines that can be used for the diagnosis and monitoring of several immunological and inflammatory disorders.

ACKNOWLEDGMENTS

This work was supported by the ARUP Institute for Clinical and Experimental Pathology.

We thank Marshall Kadin of the Roger Williams Medical Center, Providence, RI, for providing human lymphoma cell culture supernatants and Anne Tebo and Christine Litwin of ARUP Laboratories, Salt Lake City, UT, for providing clinical samples.

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