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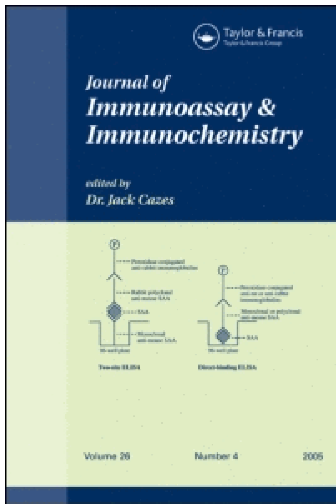

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Optimization, Comparison, and Application of Colorimetric vs. Chemiluminescence Based Indirect Sandwich ELISA for Measurement of Human IL-23

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Abstract: Currently, there is neither a published ELISA method nor it is clear whether chemiluminescence substrates would provide better sensitivity vs. colorimetric substrates for measuring human IL-23–a recently described Type-1 immunity associated cytokine. Initially, we optimized a colorimetric ELISA using *p*-nitro-phenyl phosphate substrate. Subsequently, we compared it with chemiluminescence substrates that provided ~5-fold enhanced sensitivity (mean sensitivity; 26.3 pg/mL vs. colorimetric assay, 131 pg/mL; $p < 0.01$). Both methods were reliable, with <10% inter- and intra-assay variations. We then found that the chemiluminescence method was useful in situations where human IL-23 was not readily measurable by a colorimetric method.

Keywords: Cytokine, Human IL-23, Colorimetry, Chemiluminescence, ELISA

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INTRODUCTION

Cytokines play a central role in inflammation, immune response, and health.[1,2] Accurate quantification of cytokines in human samples is a vital step in advancing basic and applied research in human health and disease. Human cytokines with key function in Type-1 and Type-2 immune responses have been classified broadly into at least three groups: Type-1 associated, Type-2 associated, and Type-3 or regulatory or suppressive cytokines.[1–3] Specific examples of cytokines belonging to these groups include: IFN-γ, IL-12, TNF-α (Type-1 associated); IL-4, IL-5, IL-13, IL-9 (Type-2 associated); and IL-10, TGF-β (Type-3 or regulatory or suppressive cytokines).[1–3] Whereas Type-1 cytokines are important in cell mediated immunity (e.g., delayed hypersensitivity reactions), Type-2 cytokines are implicated in allergic disorders. In contrast, Type-3 cytokines have been proposed to be critical in control of excessive immune and inflammatory responses that are thought to underlie allergy/asthma and autoimmune disorders.[3]

IL-23 is a heterodimeric cytokine containing IL-12 p40 and IL-23 specific p19 subunits.[4,5] This cytokine was recently identified as a member of the IL-12 related family of Type-1 immunity associated cytokines with IL-18 and IL-27 as other members.[5,6] Using IL-23 p19 knockout mice, it was shown that IL-23 is critical for cell mediated immunity as well as humoral immune responses.[7] It appears to participate in protection against intracellular infections and the pathogenesis of some autoimmune disorders.[6,8,9] One property that distinguishes IL-23 from IL-12 is that it targets memory but not naïve T helper cells.[8] Thus, measurement of human IL-23 in clinical samples will be very valuable for both basic and applied research in health and disease.

Here, we describe optimization and comparison of an indirect sandwich ELISA using colorimetric vs. chemiluminescence substrates for quantification of human IL-23. We also demonstrate that the optimized chemiluminescence method, as opposed to *p*-nitro-phenyl phosphate (PNPP) based colorimetric assay, provides ~5-fold enhanced sensitivity, but comparable reproducibility for human IL-23 quantification. Furthermore, we apply these two assays to measure IL-23 in human serum samples to demonstrate their utility.

EXPERIMENTAL

Materials

The following materials were purchased from sources as indicated in parentheses. Capture antibody: Anti-human IL-23 p19 (R&D Systems, Minneapolis, MN), Biotin labeled developing antibody: anti-human IL-12 p40/p70 (Biolegend, San Diego, CA), recombinant human IL-23 (R&D Systems, Minneapolis, MN); *p*-nitro-phenyl phosphate (PNPP) (Sigma, St Louis, MO,

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USA); Streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA); CSPD Sapphire and Emerald (Applied Bio-Systems, Foster City, CA); ELISA plates: for both chemiluminescence assay as well as colorimetric assay (Costar, Corning Inc., Corning, NY).

Colorimetric Assay for Human IL-23

An indirect sandwich enzyme linked immunosorbent assay (ELISA) was optimized using similar principles as we have described previously.[10] All reagents were used at a final volume of 50 μL/well, except for blocking buffer that was used at 75 μL/well. Washing was done manually with 200 μL/well. Briefly, ELISA plates (96 well EIA/RIA plate, 96 well easy wash™, high binding, Corning Inc., NY) were coated with Anti-human IL-23 p19 antibody diluted in carbonate buffer (0.05 M, pH 9.6) and incubated at 4°C, overnight. Unbound antibody was discarded and the plates were blocked (0.17% BSA/PBS) at 37°C. After washing (0.05% Tween 20 in PBS), recombinant human IL-23 was added at various two-fold dilutions from 4,000 pg/mL to 7.8 pg/mL in dilution buffer (0.085% BSA, 0.05% Tween 20 in PBS). Following incubation, plates were washed and a biotin labeled anti-human IL-23 p19 antibody added at the indicated concentration. Plates were then washed and streptavidin alkaline phosphatase conjugate was added at 1/4,000 (in dilution buffer). Subsequently, plates were washed again and *p*-nitro phenyl phosphate (PNPP) substrate was added (1 tablet per 5 mL substrate buffer, according to manufacturer's instruction; Sigma). Reactions were allowed to develop at room temperature in the dark and absorbance was measured in a microplate reader with dual mode of wavelength at 405 nm (peak) minus 690 nm (background) using a KC4 software program (Synergy HT, Multifunction Reader, BioTek). According to the manufacturer's instructions, dual mode provides relatively better measurements, since it adjusts the reading for background interference (personal communication). Recombinant human IL-23 was used for optimizing these assays.

Chemiluminescence Assay for Human IL-23

The general method was similar to that described above, with the exception of using opaque ELISA plates for the assay. After the addition of SAAP, 50 μL of chemiluminescence substrate CSPD (Sapphire II or Emerald II) was used at 0.4 mM. The plates were read at the indicated times using a Synergy HT, Luminescence reader (BioTek). The ELISA plates were covered with aluminum foil at all times during the assay and in between readings. Plates were manually washed four times after each step (with 200 μL/well; washing buffer: 0.05% Tween 20 in PBS). Recombinant human IL-23 was used for optimizing these assays.

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Determination of Assay Sensitivity

The assay sensitivity was defined as the lowest amount of the analyte measurable using the assay.

Blood Collection

Blood was collected in glass tubes from subjects participating in the ongoing study (PEACH study). Serum was harvested, stored at -20°C, and shipped on dry ice to the laboratory where aliquots were made and stored at -70°C until they were used in the assay.

Statistical Analysis

Student's unpaired *t*-test and Mann-Whitney U-test were used to evaluate significance, using the Analyse-It^{TM} software program (Analyse-It Software Ltd, Leeds, UK). The statistical significance level was set at 0.05.

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RESULTS AND DISCUSSION

In order to determine the optimal capture (coating) antibody concentration for human IL-23 detection, an indirect sandwich ELISA was set up using two different amounts of capture antibody and developed with two different concentrations of biotin labeled antibody. As is evident from the results, a combination of coating antibody concentration at 1 μg/mL yielded relatively better profile. Since both biotin antibody concentrations provided comparable results, keeping the cost of the reagents in mind, we chose the lower antibody concentration (250 ng/mL) for further use. Incubation time of 2 hours after addition of substrate was found optimal for reading the plates. Representative data are shown in Figures 1A–C.

We used the optimized capture (coating) and biotin-labeled antibody with buffer conditions of the colorimetric assay for the initial experiment to optimize the chemiluminescence assay. As is evident, the conditions provided an assay with a sensitivity of 31.25 pg/mL with 30 minutes of developing time after addition of the substrate for reading the plate (Figure 2A). We then tested the impact of different blocking buffers (0.17% BSA vs. 0.5% BSA vs. 5% Gelatin) on chemiluminescence assay. As is evident, blocking with 0.17% (Figure 2B) provided better results. Finally, we tested whether different chemiluminescence substrates affect the assay sensitivity. We found that the use of the CSPD substrate with Sapphire yielded better sensitivity compared to the CSPD substrate with Emerald (Figure 2C).

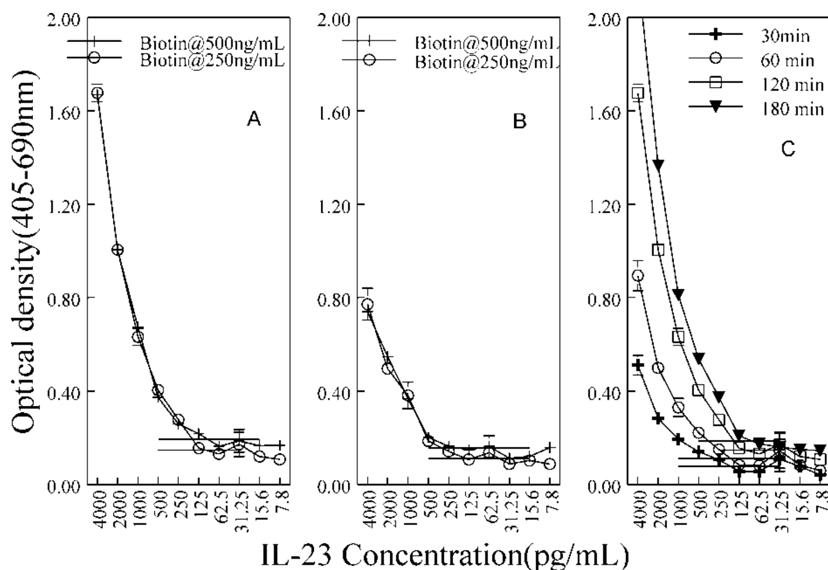

Figure 1. Optimization of an indirect sandwich ELISA based on colorimetric method for human IL-23. A) shows the assay that was performed using the capture antibody (anti-IL23 p19 antibody) at 1 μg/mL and biotin labeled anti-p40/p70 antibody at 250 and 500 ng/mL. B) shows the assay that was performed using the capture antibody at 0.5 μg/mL and biotin labeled anti-p40/p70 antibody at 250 and 500 ng/mL. Both figures show the data read at 2 hour time point after adding the substrate that was found optimal as evident in Figure 1C. C) shows the assay that was performed using capture antibody at 1 μg/mL and biotin labeled anti-p40/p70 antibody at 250 ng/mL, and read at different time periods (30, 60, 120 and 180 minutes) after addition of the substrate. In these assays, recombinant human IL-23 was used as the standard. Data is presented as mean ± SE. Horizontal line indicates background +3 SD values for each condition tested in duplicates.

We performed a series of experiments to compare the two types of substrates in terms of their impact on assay sensitivity, time of incubation after adding the substrate, and reproducibility of the assay (based on inter- and intra-assay variations). As is evident from the results, whereas colorimetric assay provided an average sensitivity of 131.25 pg/mL (range: 62.5–250 pg/mL), chemiluminescence assay yielded ~5-fold enhanced average sensitivity of 26.3 pg/mL (Table 1). We found that the chemiluminescence assay reads faster (30 minutes) relative to the colorimetric assay (2 hours) to obtain optimal sensitivity. Results from the analysis of inter- and intra-assay variation between the two assays is shown in Figures 3A, B, C, and D. As is evident, both assays yielded comparable data and the coefficients of variation were <10% in each case.

We then used these assays to measure human IL-23 in subjects. Serum samples collected from a group of adult humans were examined for the

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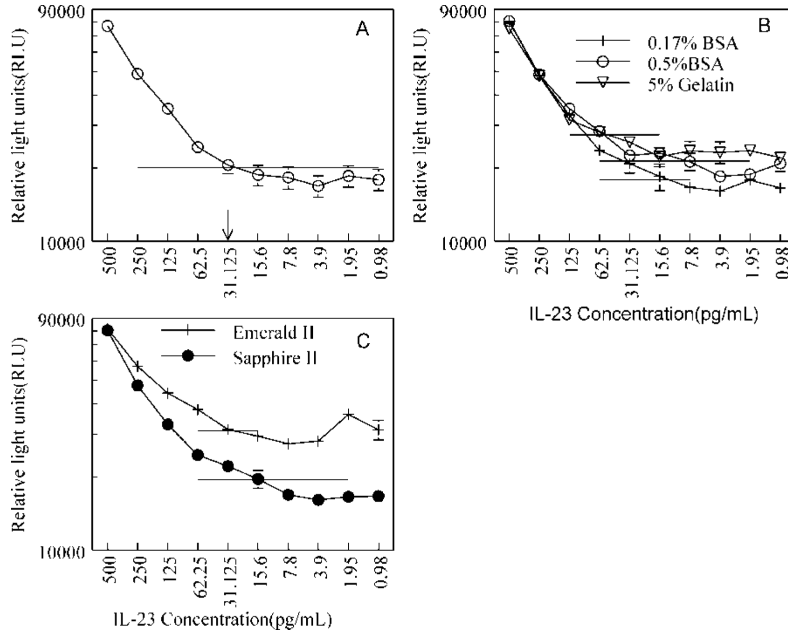

Figure 2. Optimization of an indirect sandwich ELISA for human IL-23 using chemiluminescence substrates. A) A chemiluminescence assay was optimized using capture anti-IL23 p19 antibody (1 μg/mL) and biotin labeled anti-p40/p70 antibody (250 ng/mL). Recombinant human IL-23 was used as the standard in these assays and plates were read at 30 min after addition of the substrate. Data is shown as mean ± SE (n = 4). B) This figure shows the chemiluminescence assay performed using three different blocking buffers (0. 17% BSA; 0.5% BSA, 5% gelatin). Data is shown as mean ± SE. Recombinant human IL-23 was used the standard in these assays and plates were read at 30 min after addition of the substrate. C) This figure shows the chemiluminescence assay profile using two different substrates (Sapphire II vs. Emerald). Data is shown as mean ± SE. Recombinant human IL-23 was used the standard in these assays and plates were read at 30 min after addition of the substrate.

presence of circulating IL-23 levels. As is evident, IL-23 was measurable in 6 out of 10 subjects by colorimetric assay (Figure 4A). We then examined whether the chemiluminescence assay with enhanced sensitivity would be useful to measure IL-23 in those subjects where the colorimetric assay was unsuccessful. As is evident, in 3 out of 4 subjects, IL-23 was measurable using the chemiluminescence assay (Figure 4B).

The use of the chemiluminescence substrate provides an exciting opportunity to improve current cytokine detection technology, since such substrates have been reported to yield enhanced assay sensitivities for certain cytokines.[11] However, one study reported that colorimetric assay provides better sensitivity than chemiluminescence assay for other

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Table 1. Comparison of colorimetric assay vs. chemiluminescence assays for measurement of human IL-23

Type of assay	Assay properties					
	x/n[a]	Average sensitivity (pg/mL)	Range of sensitivity (pg/mL)	ODT[b] (min)	Student's *t*-test (P value)	Mann-Whitney *U*-test (P value)
Colorimetric	12/5	131.25	62.25–250	120		
Chemiluminescence	12/5	26.3	7.8–62.5	30	<0.01	<0.01

[a]Total number of replicates/number of experiments.
[b]ODT- Optimal development time.

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cytokines.[12] For human IL-23 quantification, we are not aware of any previously published study comparing colorimetric vs. chemiluminescence methods. In an effort to fill this need, we established the present method for IL-23, a recently identified Type-1 immunity associated cytokine.

Previously, chemiluminescence based ELISA methods have been described for other type-1 associated cytokines: TNF-α, IFN-γ (human study); IL-12 (murine study).[11–13] In one of the former studies (TNF-α), the chemiluminescence assay was found to have similar or reduced sensitivity compared to the colorimetric assay.[12] In the latter study, an opposite conclusion was reached.[11] We also found that chemiluminescence provides an enhanced sensitivity for IL-23, as well as for some type-2 cytokines, IL-5 and IL-13 (data not shown). Thus, the improved sensitivity offered by the chemiluminescence method may not be generalizable to any and every cytokine measured. Consequently, it is clear that one might need to optimize methodology for each target cytokine protein in question, to evaluate suitability of chemiluminescence vs. colorimetric substrates as readouts.

There are previous reports examining human IL-23 p19 gene expression by measuring either mRNA levels in macrophages or IL-23 p19 protein detection by Western blotting.[14] Notably, interpretation of mRNA data on cytokines, in general, becomes difficult due to the potential role of post-transcriptional regulation of protein synthesis. Consequently, it is difficult to accurately estimate the amount of protein secreted by cells based on mRNA data.[15] Furthermore, the sample volume required, lower assay sensitivity, and difficulty in accurate quantification are potential problems for analysis of cytokine protein levels by Western blotting analysis. We are not aware of previous published data on levels of human IL-23 proteins present in serum/plasma samples or cultured cell supernatants in health or disease. However, we have been able to measure

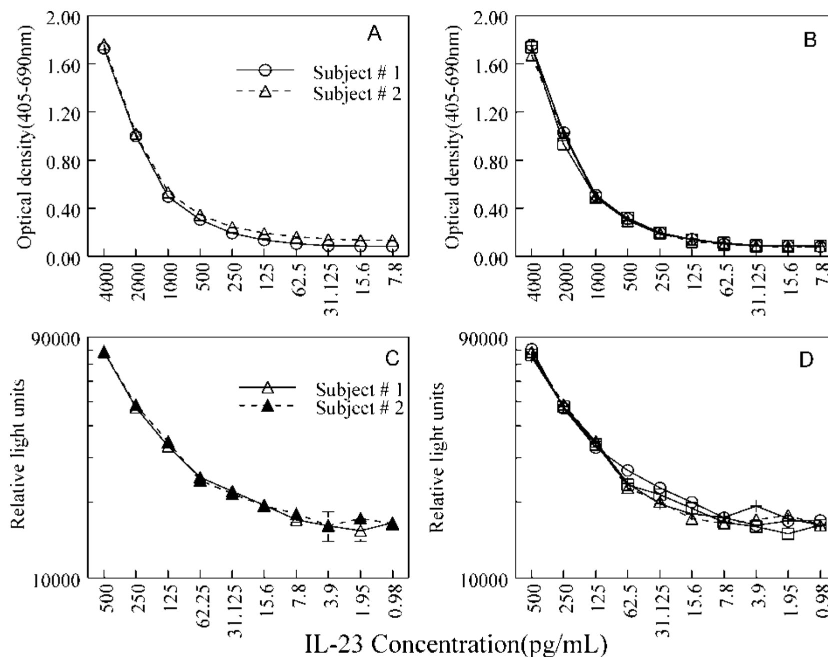

Figure 3. Inter- and intra-assay variations of colorimetric vs. chemiluminescence assays for human IL-23. A) This Figure illustrates two experiments performed by two different individuals using the optimized colorimetric method (inter-assay variation). Data is shown as mean $\pm$ SE (n = 4). B) This Figure illustrates four experiments performed by one individual using the optimized colorimetric method (intra-assay variation). C) This Figure illustrates two experiments performed by two different subjects using the optimized chemiluminescence method (inter-assay variation). Data is shown as mean $\pm$ SE (n = 4). (D). This Figure illustrates four experiments performed by one subject using the optimized chemiluminescence method (intra-assay variation). Recombinant human IL-23 was used as the standard in these assays.

IL-23 in human serum samples using the combination of presently described methods.

While chemiluminescence based substrates provide enhanced sensitivity, there are a few limitations in terms of the cost involved for ELISA plates and substrates. For development of 100 ELISA plates it costs ~US$ 274 for PNPP based substrate vs. ~$1573 for Sapphire II based chemiluminescence method. These estimates include plate plus substrate costs (excluding cost of antibodies, recombinant protein and other reagents). Thus, chemiluminescence assay are ~6 times as expensive, relative to the colorimetric assay. Another limitation is the cost of chemiluminescence readers, which tend to be expensive compared to the traditional colorimetric ELISA readers.

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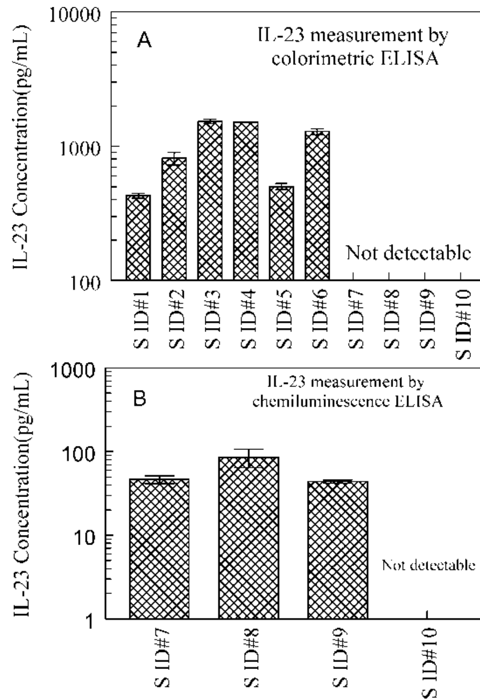

Figure 4. Application of colorimetric and chemiluminescence assays for measurement of human IL-23 in serum samples. A) This figure illustrates the utility of colorimetric method for measurement of IL-23 in human serum samples. Each bar represents one subject. Data is shown as mean $\pm$ SE of duplicate analysis. B) In this experiment, the serum samples from the experiment performed in Figure A where IL-23 was not detectable by colorimetric method (subject ID #7–10) were analyzed by the chemiluminescence method. This data illustrates the utility of the chemiluminescence assay for measuring IL-23 in serum where colorimetric assay was not useful. Each bar represents one subject. Data is shown as mean $\pm$ SE of duplicate analysis.

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Conventionally, human cytokines have been measured using ELISA based colorimetric assays. In many studies, the source of the human specimens are invasive in nature (e.g., serum, peripheral blood cell stimulated culture supernatants). A major attraction of the more recently developed technology of chemiluminescence is the potential for higher sensitivity of detection. The enhanced sensitivity suggests potential application of these assays for detection of disease relevant cytokines, especially in non-invasive human samples where: (i) sample volumes might be limited (e.g., tears, sexual fluids, etc.), so that samples can be diluted and used for quantification; and (ii) where samples are naturally diluted (e.g., saliva, urine) and cytokines are expected to be present in very low quantities.[16–20]

CONCLUSIONS

In summary, (i) we have optimized and compared indirect sandwich ELISA methods using colorimetric vs. chemiluminescence substrates for quantification of human IL-23; (ii) we have demonstrated that the optimized chemiluminescence method, as opposed to a PNPP based colorimetric assay, provides enhanced sensitivity, but comparable reproducibility; and (iii) we demonstrated that the chemiluminescence method is useful to measure serum IL-23 in situations where it is not readily measurable by the colorimetric method.

ABBREVIATIONS

OD, optical density; ELISA, enzyme linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate buffered saline; SD, standard deviation, SE, standard error; PNPP, para-nitro-phenyl phosphate; CSPD, chloro-5-substituted adamantyl-1,2-dioxetane phosphate; IL, Interleukin; TNF-α, tumor necrosis factor-alpha; TGF-β, transforming growth factor-Beta; IFN-γ, Interferon-gamma; EIA, Enzyme immunoassay; RIA, radioimmunoassay.

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