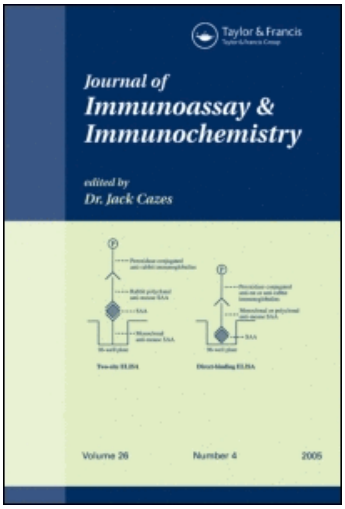

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Improved Sensitivity of Colorimetric Compared to Chemiluminescence ELISAs for Cytokine Assays

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ABSTRACT

Cytokines are often measured using ELISAs and chemiluminescence (CMIL) is reported to exhibit increased sensitivity compared to colorimetric (COL) assays. CMIL also has a wider dynamic detection range. We sought to directly compare ELISAs for measuring human TNF and IL-8 using CMIL or COL. CMIL substrates with glow fluorescence were obtained from 4 different commercial sources while the COL substrate was TMB. ELISAs for TNF and IL-8 were run under identical conditions and the

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standard curve extended from 0.5 to 4000 pg/ML. The COL substrate demonstrated a sigmoid shaped curve when plotted on a log-linear scale while the CMIL continued to increase up to the highest concentration. Both substrates were modeled most accurately by a 4 parameter equation with R values >0.99. The standard curves for both the IL-8 and TNF demonstrated a lower limit of detection (LLD) for the COL comparable to the CMIL detection system. To precisely define the LLD quadruplicate blanks were run and the mean plus 4 standard deviations were used. By these criteria, the COL assay routinely had a LLD of <1.5 pg/ML which was better than any of the CMIL substrates. Our data demonstrate the COL assays have the same or better sensitivity than CMIL and are significantly less expensive.

Key Words: Cytokines; ELISA; Immunoabsorbent assay; Chemiluminescence; TMB; Chemokines.

INTRODUCTION

Cytokines represent important components of the inflammatory response. These proteins have been implicated in the pathogenesis of several important disease states including asthma,[1] transplant rejection,[2] septic shock,[3] and ischemia reperfusion injury.[4] There is tremendous interest in accurate determination of the levels of cytokines in these disease states. In some situations the levels of the cytokines, or their endogenously occurring inhibitors, will predict disease progression or outcome.[5] Careful quantitation of the cytokines may be used to provide insight into the mechanism of disease, or determining an appropriate target for therapeutic intervention.

Cytokines may be measured by biological assays, but there are numerous problems associated with this technology.[6] In practice, most cytokines are measured by immunological assays such as a radioimmunoassay, or enzyme linked immunoabsorption assay (ELISAs). There has been recent interest in the chemiluminescent substrates for measuring cytokines since it was felt that they may exhibit better sensitivity compared to traditional colorimetric assays.

In this present submission, we directly compare the sensitivity of several chemiluminescent substrates to a colorimetric substrate using a standard ELISA format. When the ELISAs have been optimized for performance, the colorimetric substrates perform as well or better than the chemiluminescent substrates.

EXPERIMENTAL

Basic ELISA Protocol

ELISAs were performed using matched antibodies from R & D Systems (www.rndsystems.com) and our previously published protocols.[7] We tested 2 separate cytokines to ascertain if the results would be applicable to more than one assay. For the ELISAs, 96 well microtiter plates (Falcon Enhanced Binding, Becton Dickenson Labware Cat # 35–3941. www.bdbiosciences.com) were coated with capture antibody (TNF 2000 ng/ML, IL-8 500 ng/mL) in phosphate buffered saline overnight at 4°C. Both plates were blocked with Blotto (Pierce, www.piercenet.com) for 1 h. After washing with wash buffer (PBS with 0.05% v/v Tween 20, Pierce), standards were applied to the plate. The standard curves were prepared using recombinant standards from R & D systems in wash buffer supplemented with 10% Blotto. The standard curves ranged from 0.488 to 4000 pg/mL. For the IL-8 ELISA incubations were for 1 h while the TNF ELISA incubations were for 2 h. Following washing, the biotinylated detection antibodies were applied (IL-8 25 ng/mL, TNF 100 ng/mL). An IL-8 ELISA was also performed with higher concentrations of antibodies, coating at 5000 ng/mL and detection at 50 ng/mL. Different steptavidin coupled substrates and detection systems were utilized, as described in the individual assays.

Chemiluminescent Reagents

All substrates were glow fluorescent type reagents. Alkaline phosphatase (AP) detection systems used streptavidin conjugated AP from Applied Biosystems (www.appliedbiosystems.com). CSPD and CDP-*Star* were obtained from Applied Biosystems, Bold APS 540 was obtained from Intergen (www.intergenco.com). Horseradish peroxidase (HRP) detection systems used streptavidin conjugated HRP from Jackson Immunoresearch (www.jacksonimmuno.com). Supersignal was obtained from Pierce (Supersignal ELISA Femto Maximum Sensitivity Substrate, catalog # 37075). PS-1 substrate was obtained from Lumigen (www.lumigen.com, catalog # PS-101).

Colorimetric Reagents

The following reagents were all purchased from Sigma (www.sigmaaldrich.com): TMB (3,3′,5′5′-tetramethyl-benzidine, catalog

T-2885), DMSO (catalog D-2650), sodium acetate, citric acid and 30% hydrogen peroxide. A stock of TMB was prepared in DMSO (0.1 g in 10 mL) aliquoted and frozen. A solution of 0.1 M sodium acetate was adjusted to a pH of 6.0 with 0.1 M citric acid and stored. On the day of use, reagents were brought to room temperature and the TMB was diluted 1:100 in the sodium acetate. Immediately prior to use, 4 μL of 30% hydrogen peroxide was added to 25 mL of the TMB solution. After color development, the reaction was stopped with sulfuric acid.

Polymeric Conjugate

As an alternative to the traditional monomeric streptavidin-HRP, we also tested different concentrations of Amdex™, a polymeric conjugate, obtained from Amersham (www.amershambioisciences.com, catalog RPN-4401).

Equipment

Chemiluminescent assays were read in a Packard Instrument Lumicount (www.packardbioscience.com), Model BS 10,000 with a read length of 1 s per well, 10 readings per well were averaged. For some chemiluminescent assays, plates were read in the Luminoskan from Themo Labsystem or the Lmax from Molecular Devices. All of the data presented were gathered from the Lumicount instrument. The colorimetric assay was read in a Biotek ELISA reader Model EL340 (www.biotek.com).

Calculations

Standard curves were prepared and modeled using a 4 parameter fit to the curve with the cytokine concentration on a log scale and the relative light units or optical density on a linear scale. Alternatively, standard linear regression was performed with both the relative light units and cytokine concentrations plotted on a log-log scale. To calculate the lower limit of sensitivity, blanks were performed in quadruplicates. The mean plus 4 standard deviations were used to determine the lower limit of detection based on the standard curve for the individual assays.

RESULTS

For the first experiment we directly compare the relative sensitivity of two different human ELISAs for cytokines using reagents coupled with the enzyme horseradish peroxidase (HRP). All of these ELISAs were performed multiple times and similar results were obtained each time. For these ELISAs, the antibody concentrations and all reagents were identical for both the chemiluminescent as well as the colorimetric procedures. The only difference was the substrate used for detection and the instrument used to measure the output. Figure 1 demonstrates the results

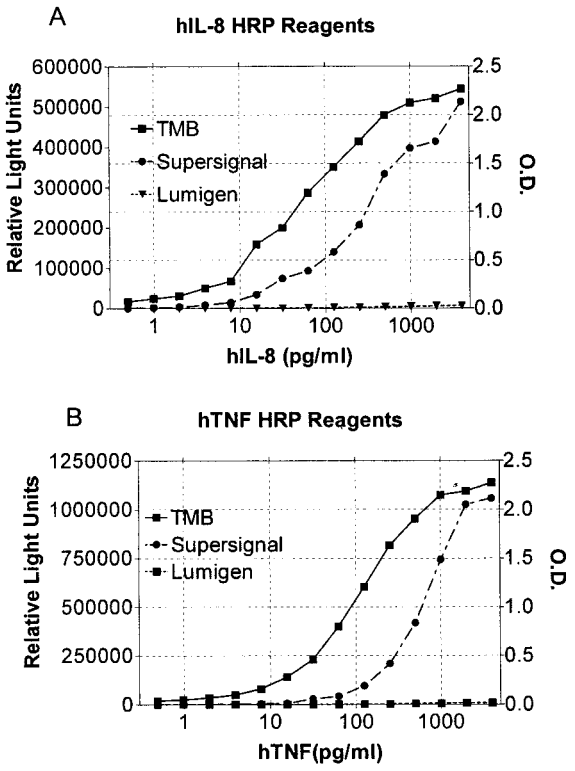

Figure 1. ELISAs with HRP conjugated streptavidin, chemiluminescent vs. colorimetric. An ELISA was performed by human IL-8 (A) or human TNF (B). The chemiluminescent substrates were Supersignal from Pierce and Lumigen PS-1 from Lumigen, which are plotted on the left axis as relative light units. The colorimetric was the standard TMB plotted on the right axis. The TMB showed better sensitivity.

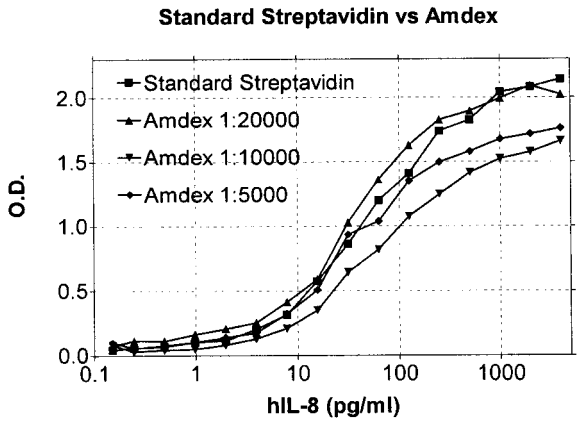

Figure 2. Polymeric Streptavidin vs. Monomeric Streptavidin. In place of the standard HRP-streptavidin, the polymeric HRP conjugated streptavidin Amdex™ from Amersham was tested at different concentrations using the colorimetric TMB. Amdex™ did not increase the sensitivity.

obtained with HRP reagents in either the ELISAs for tumor necrosis factor (TNF) or interleukin 8 (IL-8). These results demonstrated that TMB as the colorimetric substrate provides a robust standard curve with an excellent lower limit of sensitivity.

Polymeric streptavidin also has the possibility of producing a stronger signal than the monomeric streptavidin used in our system. We tested different dilutions of the polymeric streptavidin in order to investigate this possibility. Figure 2 demonstrates that the polymeric streptavidin offered no advantage over the monomeric streptavidin.

The ELISAs utilized in this paper have been optimized for colorimetric substrates. This optimization included using antibody concentrations which give a high signal-to-noise ratio. These concentrations are typically lower than those recommended by the manufacturers. Therefore, we tested higher concentrations of the coating and detection antibodies to ascertain if this would result in enhanced sensitivity. Figure 3 demonstrates the using greater concentrations of the antibodies and did not result in improved sensitivity.

Other chemiluminescent substrates are coupled to alkaline phosphatase and cytokine ELISAs were performed with these reagents also. An ELISA for tumor necrosis factor and interleukin 8 were again performed. Figure 4 demonstrates that none of the chemiluminescent substrates offered improve sensitivity over the colorimetric reagents.

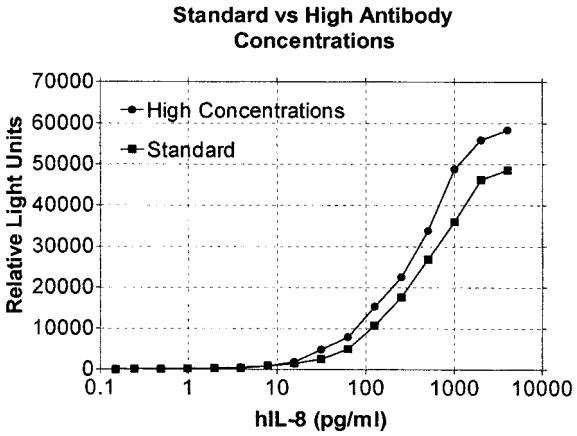

Figure 3. Higher antibody concentrations. Higher concentrations of the coating and detection antibodies were used in the IL-8 ELISA. There was no difference in the lower limit of detection with the higher antibody concentrations. For this ELISA, the chemiluminescent substrate Supersignal from Pierce was used.

The lower limit of detection may be calculated in several ways. A standard method is to take the detection value of 4 quadruplicates blank wells and determine the absorbance of these wells. The mean of the blank values plus 4 standard deviations is used to calculate the lower limit of detection. Using this method, we directly compare the sensitivity of the colorimetric and the chemiluminescent substrates. Table 1 indicates that the colorimetric substrate for either the IL-8 or the TNF ELISAs yields a lower limit of detection equal to or better than the chemiluminescent substrates.

DISCUSSION

There are several important characteristics for measuring cytokines. A significant concern for many of the cytokine measurements is the need to have a low limit of detection. While some cytokines such as interleukin-6 may be found in the plasma of patients at extremely high levels,[8] other cytokine concentrations are in the pg/ML range.[9,10] Therefore, a primary concern of any cytokine immunoassay is to have an extremely low-level of detection. This issue takes on further importance since frequently there are small sample volumes available, and multiple cytokines need to be measured. Additional problems may arise when

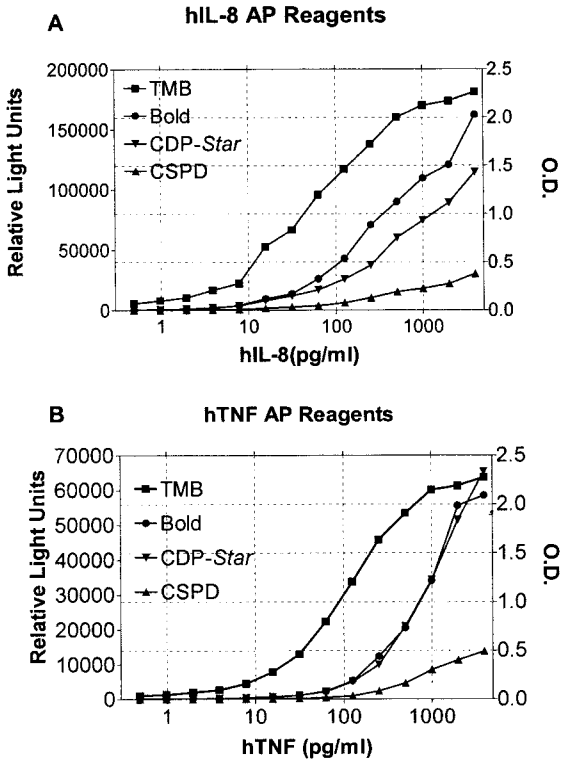

Figure 4. ELISAs with alkaline phosphatase conjugated streptavidin vs. colorimetric. An ELISA for human IL-8 (A) or human TNF (B) was run with the standard TMB colorimetric ELISA with the results graphed as optical density (O.D.) on the right axis of the graph. The chemiluminescent substrates Bold, CSPD, and CDP-*Star* were run and graphed as relative light units on the left axis. The TMB shows better sensitivity.

attempting to measure cytokines in protein rich fluids such as plasma or serum although simple modifications may be made to overcome these problems.[7,11]

Other groups have compared CMIL to COL ELISAs for measuring cytokines. When measuring interferon alfa-2b[12] or IL-5[13] there was an increase in sensitivity with the CMIL approach. Using a 3 day CMIL assay, Lewkowich et al. reported an increase in the lower limit of detection for mouse IL-12, human IL-4, and mouse IL-4.[14] Finally, an assay for tumor necrosis factor had a lower limit of detection of 1000 pg/ML using a chemiluminescent approach.[15] Our data indicates that the COL

Table 1. Lower limit of sensitivity. The lower limit of sensitivity was calculated for each of the ELISAs. A standard curve was modeled using a 4 parameter fit, the R^2 indicates the goodness of fit for the curve. The lower limit of detection was calculated by running blanks in quadruplicate, determining the standard error of the background and calculating the background plus 4 standard deviations. This value was used with the formula for the standard curve to determine the lower limit of detection.

Substrate	Enzyme	R^2	Sensitivity (pg/ML)
hIL-8 ELISA			
TMB	HRP	0.9918	1.2
Supersignal	HRP	0.9907	2.1
Lumigen	HRP	0.9971	0.9
Bold 540	AP	0.9925	3.6
CDP-*Star*	AP	0.9986	4.0
hTNF ELISA			
TMB	HRP	0.9959	< 0.31
Supersignal	HRP	0.9907	1.87
Lumigen	HRP	0.9971	0.57
Bold 540	AP	0.9977	< 0.31
CDP-*Star*	AP	0.9995	28.35

assay achieves a lower limit of detection equal or better than that of the CMIL. The reason for our improved sensitivity probably lies in the careful optimization of the ELISA conditions.

Another concern with cytokine immunoassays is the need to have an overall low-cost. Cytokine ELISAs are routinely performed by research laboratories and not clinical labs, and research labs frequently operate on an extremely low budget. One method to reduce the cost is to use commercially available matched antibody pairs rather than ELISA kits.[7] We extended our finding from this previous report to look carefully at different substrates. The issue of cost for the chemiluminescent substrates is significant. The cost of the chemiluminescent reagents to perform 10 ELISA plates ranges from $152 to $275, while the cost of the TMB is less than 1 dollar.

A consideration for designing cytokine ELISAs is the ease with which they may be performed. Additional, dedicated, specialized equipment must be purchased or leased in order to read the chemiluminescent assays. In contrast, many laboratories have traditional ELISA readers already in place. These ELISAs are readers can be used not only for measuring ELISAs, but for cell viability assays and other protocols.

This manuscript demonstrates that standard colorimetric ELISAs can be performed with the same sensitivity as the chemiluminescent assays. Close attention to detail when optimizing all the steps in the traditional colorimetric ELISA permits the development of sensitive, cost efficient measurement of cytokines.

ABBREVIATIONS

CMIL	Chemiluminescence
COL	Colorimetric
LLD	Lower limit of detection

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