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To cite this article: Stephanie Hom et al 2008 J. Breath Res. 2 041001

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COMMUNICATION

Matrix effect in exhaled breath condensate interferon-gamma immunoassay

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Received 27 December 2007
Accepted for publication 9 June 2008
Published 24 July 2008
Online at stacks.iop.org/JBR/2/041001

Abstract

Manuscripts presenting cytokine (and other protein) concentrations in exhaled breath condensate (EBC) are commonly published with insufficient control experiments performed to provide confidence that the assay was accurate. Our laboratory has identified false positives in EBC with several forms of immunoassay, including ELISA and multiplex bead arrays. In this current brief investigation, we studied EBC samples from ten subjects and examined for the effect of alteration of the matrix on the assay results reported by a commercially available ELISA assay for a commonly reported EBC cytokine (interferon-gamma (IFN-γ)). The commercial immunoassay reported measurable levels of the IFN-γ signal in all samples. However, when we added to the EBC samples the proteinaceous matrix of the zero-standard from the commercial kit, we could identify no IFN-γ signal. These simple findings are consistent with our earlier observations that the EBC matrix requires adjustment for there to be any validity to immunoassays.

Introduction

Exhaled breath condensate (EBC) is a non-invasive sampling procedure that has shown promise as a method to evaluate the chemical and inflammatory changes that occur in the lung during disease processes [1]. EBC consists of droplets of airway lining fluid (derived from unknown anatomical levels), condensed water vapor and trapped water-soluble gases [2]. Compounds measured in EBC range from the smallest molecule of all (the proton) to modest sized proteins. Several articles in the literature, and an increasing number of abstracts presented at international meetings, focus on cytokines in EBC [3–11]. However, there is substantial interlaboratory variation in the concentrations of these cytokines found. This variation is often attributed to variable dilution, although this has never been proven. Sometimes, the differences are attributed to different EBC collection equipment or condenser surfaces based on findings that certain other parameters are affected by these technical issues [12], although there appear to be few data in regard to cytokines.

Interferon-gamma (IFN-γ) is the defining cytokine characteristic of the Th1 immune paradigm. Studies have reported detectable levels of IFN-γ in EBC with variation in disease states [5, 6, 13, 14]. IFN-γ is approximately 20 kDa in size and is produced by multiple cells in the lung. It plays an antagonistic role with interleukin-4 (IL-4) to inhibit the production of immunoglobulin-E (IgE). It has therefore been of much interest to lung disease researchers. Invasive studies have reported increased IL-4 concentrations and decreased IFN-γ concentrations in asthmatic subjects in comparison to normal, healthy controls. It has been reported that EBC of steroid-naïve subjects with asthma had an average IFN-γ concentration less than that of controls [13].

In our experience reviewing manuscripts, reading abstracts, communicating with investigators and working on the exhaled breath condensate task force, rarely have we
been convinced that immunoassays for proteins in EBC have been performed with sufficient controls or with sufficiently validated assays. We therefore wished to perform a simple declarative experiment to show the potential of an assay artifact to alter profoundly the interpretation of immunoassay results from EBC. It is the purpose of this communication to report this issue clearly and simply, so that new investigators in the field recognize the importance of assay validation.

**Methods**

Informed consent was obtained from all subjects and the studies were approved by the Human Investigation Committee at the University of Virginia. Exhaled breath condensate collection was performed using the RTube™ EBC collection system (Respiratory Research, Inc., Charlottesville, Virginia). A convenience sample of ten human subjects was recruited from the laboratory staff to breathe tidally into an RTube for 10 min, without nose clips, with a starting condenser temperature of −20 °C. There were no relevant inclusion criteria, exclusion criteria nor efforts made at careful subject characterization.

IFN-γ immunoassays were performed using commercially available EASIA kits (IFN-γ EASIA, BioSource Europe, Nivelles, Belgium), designed and partially validated for use with several biological fluids (but with no mention of EBC in the accompanying literature). This is a sandwich ELISA that uses several monoclonal antibodies (attached to the ELISA plate by the manufacturer) to bind IFN-γ from the sample or standard. An additional monoclonal anti-IFN-γ antibody—conjugated with horse radish peroxidase—is then added, the purpose of which is to bind onto IFN-γ that has been captured by the plated antibodies. After 2 h, and then substantial rinsing to remove the unbound antibody, a chromogenic solution is added, upon which the horseradish peroxidase acts to cause an absorption change measured by spectrophotometry at 450 nm wavelength. The kits incorporate a set of standards consisting of lyophilized material (considered proprietary) which is solubilized prior to use with set volumes of distilled-deionized water. The standards range from 0 IU ml⁻¹ to 23.5 IU ml⁻¹. Standard curves were created for each microtiter plate, and unknown EBC IFN-γ sample concentrations were determined by interpolation from the standard curve. The level reasonably reported as a minimum lowest level of detection varies from standard curve to standard curve, but in our hands, intrasample concurrence commonly declined under 1 IU ml⁻¹. This level equates to 20 pg ml⁻¹.

Aliquots of the EBC sample were assayed in duplicate following the manufacturers’ instructions ‘neat’ (unaltered). Additionally, an aliquot of each EBC sample was processed as follows: lyophilized proteinaceous material from the kit’s zero standard was solubilized in 1 cm³ distilled-deionized water to form an eight-fold concentrated version of the zero standard. 5 µl of this concentrated standard matrix were added to 45 µl of EBC. This amount was determined so as to have the EBC samples contain close to the same concentration of this lyophilized material as the standards, with calculated addition of the proteinaceous lyophilized material being 3.9 mg/50 µl in the standard and 3.3 mg/50 µl in the samples. For comparison, EBC ‘neat’ (unaltered) has protein concentrations in the 1 µg ml⁻¹ range, several orders of magnitude lower [2]. Distilled-deionized water was used for additional controls. All samples were assayed in duplicate. Normality of the distributions was determined by Kolmogorov–Smirnov testing. Sample concentrations below the level of detection were assigned a value of 1 IU ml⁻¹. Comparisons were made using Wilcoxon signed rank testing using SigmaStat 3.0 software (SPSS, Inc.), and data are reported as median and interquartile range or mean and SD as appropriate.

**Results**

EBC was successfully collected from all subjects (five males, five females, mean age 30 ± 8 years). EBC samples run ‘neat’ (unaltered) were reported by the assay to have IFN-γ levels of 21.0 ± 13.8 IU ml⁻¹ (or a median of 22 (range 17–28.8) (n = 10)). These reported levels are firmly within the optimum range of the assay based on our standard curves. When aliquots of these same EBC samples were assayed after lyophilate from the zero standard was added to them, the levels of IFN-γ reported by the assay were all <1.0 IU ml⁻¹ (n = 10) (figure 1). The data from the unaltered samples were normally distributed (p > 0.20); however, the undetectable samples were assigned a numeric value of 1 IU ml⁻¹, and therefore comparison was performed with paired non-parametric testing, which confirmed these differences to be significant (p = 0.002). The EASIA reported IFN-γ levels for distilled-deionized water of 15.8 ± 3.6 IU ml⁻¹.

The experiment was repeated using three different kits (all by the same manufacturer), each resulting in the same artifact.
Discussion

Exhaled breath condensate is a dilute fluid, the volume of which is almost entirely water. However, it is clear that small amounts of airway lining fluid are also included in EBC samples, as is evidenced by the multiple non-volatile compounds that have been reported and confirmed with various assays. We have been in the position of reviewing numerous manuscripts reporting highly variable and often exceedingly high levels of cytokines in EBC, which most often seemed to be a methodologic failing. We therefore wished to show newer investigators in the field that there are problems with immunoassays in EBC that are addressable, but that indeed must be addressed for a manuscript to be considered acceptable.

In our hands, the EASIA technology employed is susceptible to a matrix-induced artifact when used for assaying EBC, and this artifact is overwhelming. Review of the literature does not clarify how most of the assays for cytokines have been performed in EBC samples. There is rarely a mention as to how the assay matrix effect was controlled. The matrix effect is a reasonably well-accepted phenomenon occurring in immunoassays in which the matrix of the standards is dissimilar to that of the samples—a situation akin to comparing apples to oranges. Several of the commercial companies (including the supplier of our kits) offer methods to address the matrix effect by providing proprietary reagents designed for addition to low-protein samples. Unfortunately, it seems likely that at least some of the reports in the literature, and certainly many of the manuscripts not acceptable for publication, relied on assaying EBC ‘neat’, without adding any matrix adjuster to the samples.

In our simple small experiment, we endeavored to compare EBC assayed neat with EBC assayed after the addition of lyophilized material from the zero standard of the kit. Although the assay reported a substantial IFN-γ signal for EBC samples assayed neat, this signal was abrogated by addition of the lyophilate from the zero standard. A strong and clearly artificial signal in the distilled-deionized water control—likewise eliminated by addition of the zero-standard lyophilate—assured us that the standard matrix material added to the samples did not eliminate the signal simply by binding true IFN-γ. It is reasonable to conclude that the EBC signals identified by the assay used in this study were primarily artifact. Caveats here need to be considered. First, our sample number was small, although sufficient statistically and scientifically. Second, we only report the effect for the IFN-γ assay kit from one manufacturer, and our findings are not necessarily applicable to other cytokines or ELISA techniques (although unreported experiments in our laboratory reveal such findings for a similar ELISA for IL-4).

We think it is likely that the EBC and distilled-deionized water act similarly to partially unblock the EASIA plate, allowing non-specific binding of the secondary antibodies to the plate, thus creating a false signal. Proprietary reagents formulated to address the matrix effect may work simply by means of preventing unblocking of the assay plate by having some extra proteins in the solution. One such material is Point-0 cytokine (BioSource Europe, Nivelles, Belgium), seemingly designed precisely for this purpose.

In this study, we did not endeavor to determine if EBC indeed has IFN-γ within it. We expect that it indeed does, in the range less than 1 IU ml⁻¹ (20 pg ml⁻¹) as has been reported for several cytokines. The assay we employed requires modification to obtain reliable data for low concentration samples. It is important to note that we found no major flaw with the assay itself, nor the manufacturer’s instruction, but rather the flaw lies in using this assay, unmodified, for EBC. To improve the sensitivity requires elimination of noise, as well as methods of concentrating the EBC sample before the assay. The first step in eliminating noise is elimination of the highly important matrix effect. We expect, but have not proven, that the issue of matrix effects is present whenever immunoassays are used in EBC.

We recommend that future studies of cytokines in EBC using any immunoassay technology be carefully validated and cautiously undertaken with effort to keep the matrix of the fluid as similar as possible to the standards, or to use some other methodology to assure that the signal identified is specific for the cytokine of interest. Validation of the assay is key to assuring appropriate conclusions. It is unfortunate if investigators perform the work of sample collection and assay and then attempt interpretations based on completely inaccurate assay information. However, EBC has much to offer investigators using a conscientious technique.

Disclaimer

Conflict of interest: John Hunt is a co-founder of Respiratory Research, Inc., which developed the RTube exhaled breath condensate collectors used in this study.

References


