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Endogenous peroxidases in sputum interfere with horse-radish peroxidase-based ELISAs

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Abstract

Peroxidase-based immunoassays are commonly used for detecting inflammatory mediators in biological samples. We suggest caution while interpreting assays particularly in sputum samples that have endogenous peroxidases like eosinophil peroxidase (EPX), which may interact with a horseradish peroxidase (HRP)-based ELISA. Using IL-8 as an example, we demonstrate that values generated with an HRP-ELISA (n=47) show significant positive correlation with sputum EPX content (r = 0.6, p=0.0004), which can be misconstrued to be affiliated to an eosinophilic event. This result can be misleading since the data-set generated with the same samples (n=47) using alkaline phosphatase (AP)-based ELISA and a non-enzymatic Milliplex system do not show any correlation with sputum EPX, confirming minimal to nil interaction of endogenous peroxidases in these two protocols (Milliplex r = -0.24, p = 0.13; AP r = 0.26, p = 0.09). We, therefore, recommend the use of alkaline phosphatase-based ELISA or Multiplex system rather than peroxidase-based ELISA for detecting soluble mediators in sputum samples with increased eosinophil activity.

Keywords

Eosinophil peroxidase; ELISA; Sputum Eosinophils; horseradish peroxidase; alkaline phosphatase; sputum immunoassay

Taxonomy

Immunology; Enzyme Immunoassays; Eosinophilia; Peroxidases; Sputum Analysis

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Author Contributions: MM designed the study. LK, KP, SRT, KR, MM, conducted all reported experiments with cytokines and autoantibodies. CJ and MPG made observations with SOCS3 data. LK, MM, PN analyzed data and prepared the manuscript. All authors have read and agreed. MM takes overall guarantee of the article.

Introduction

The assessment of airway inflammation has improved our understanding of the underlying pathophysiology of chronic airway diseases, such as asthma and COPD and has paved the way for optimal treatment options (Nair and O'Byrne, 2016). Measuring sputum biomarkers, in particular, cytokines, have emerged as promising tools for defining airway disease phenotypes (Westerhof et al., 2015; Robinson et al., 2017), development of targeted monoclonal antibody therapies (Hambly and Nair, 2014), evaluating and optimising treatment response (Chakir et al., 2010; Nair et al., 2013; Mukherjee and Nair, 2015). For assessing expression levels of 'target' antigen in the airways, enzyme-linked immunosorbent assays (ELISA) with a peroxidase-based colour development using either horseradish peroxidase (HRP)-conjugated or biotin-conjugated secondary antibodies remain the method of choice in sputum research (Pizzichini et al., 1996b; Keatings et al., 2002; Jee et al., 2010; Ntontsi et al., 2017). Indeed, adapting biotinylated secondary antibodies into an HRP-based system, otherwise conventionally followed by an alkaline phosphatase (AP) based colour development (Figure 1), has stemmed from the fact that the peroxidase-based protocol is often preferred over AP substrates. This is mainly due to better sensitivity of HRP substrates, the fewer number of steps involved in colour development, and the increased shelf-lives of the HRP-antibodies and peroxidase-based reagents. With this report, we would like to address our concern of using peroxidase-based immunoassays for assessing fluid-phase inflammatory markers and other soluble mediators in biological fluids like sputum with increased prevalence of endogenous peroxidases.

The disadvantage of an HRP-based system is its decreased sensitivity in a peroxidase-based micro-environment. Substrates commonly used in HRP-based ELISAs are readily converted by the endogenous EPX into the same chromogenic product. This concept is not new. There has been previous research indicating the interference of horseradish peroxidase reagents with high endogenous eosinophil peroxidase (EPX) levels in the gastrointestinal system (Hunyady B et al., 1996), and spleen (Strauss, 1979). None of the previously reported methods for blocking and neutralizing EPX could be applied satisfactorily to reduce the interference (Hunyady B et al., 1996). This is disconcerting since sputum in various airway diseases has high endogenous peroxidase levels. In fact, endogenous levels of EPX was recently demonstrated to be a biomarker for disease severity in asthma (Nair et al., 2013). It is particularly difficult to eliminate this interference even with multiple washings during the ELISA protocol, as EPX is an exceptionally adherent molecule to most surfaces owing to its strong cationic characteristic. This "stickiness" of EPX and the subsequent challenges in eliminating or neutralizing EPX to prevent its interference with detection systems have been previously established (Adamko et al., 2004). It can be argued that the presence of EPX in the sputum supernatants will interfere in a similar manner and to our knowledge, there has been no study investigating the validity of peroxidase-based ELISA protocols using sputum as the sample source.

Materials and methods

Sputum ELISAs

Using IL-8 as an example, we conducted a head-to-head comparison between HRP and AP-based detection, for 47 sputum supernatants collected between Jan 2017-May 2017 by the method described earlier (Pizzichini et al., 1996a). Sputum IL-8 was measured by IL-8 duo-set ELISA, (R&D Systems, MN, USA). For HRP-based detection, the manufacturer's instructions were followed. Since the detection antibodies supplied in this kit is biotinylated, we were able to adapt it for AP detection using BluePhos substrate kit (KPL Inc., MA, USA). The standards provided in the duo-set kit were used to generate the standard curve for both colorimetric methods and a 4PL method was used for interpolation of unknowns.

Non-enzymatic Multiplex immunoassay

For addressing the validity of the generated values from the two ELISA platforms, we used a non-enzymatic detection system for comparison. IL-8 levels were hence analysed with a Millipore Milliplex kit (Human High Sensitivity T-Cell Discovery Array 14-plex) on BioPlex 200 system by Eve Technologies Inc., (Calgary, Alberta, Canada). Care was taken to keep the freeze-thaw cycles to a minimum.

Sputum eosinophil peroxidase measurement

Endogenous EPX was measured using an in-house ELISA developed and validated for sputum samples (Nair et al., 2013).

Statistical analysis

GraphPad Prism version 7.00 (La Jolla, CA, USA) was used for all statistical analysis. Differences in the absolute values generated by the three methods were assessed by nonparametric Kruskal-Wallis test. After testing for normal distribution of data, Spearman's rank correlation was used to test the association between the data-sets. A P value of < 0.05 was considered to be significant.

Results and Discussion

To our knowledge, for the first time we report a validation study where protein levels of a commonly investigated cytokine, IL-8, is examined in 47 sputum supernatants using an ELISA platform (with same capture and detection antibodies), comparing two separate enzymatic reactions that employ HRP and AP, run in parallel. The validity of the two methods is compared against values generated by a Milliplex bead-based system (a non-enzymatic assay that utilises Streptavidin-conjugated phycoerythrin, PE, emission at 578 nm, refer to Figure 1C).

For the same sub-set of patients (n = 47 sputum supernatants), IL-8 levels were comparable between those generated using Milliplex beads and AP-ELISA (Figure 1D, $p > 0.05$), but not with HRP-ELISA (Figure 1D, $p = 0.03$). In accordance, the IL-8 values generated from the HRP-ELISA showed a weaker correlation with the Milliplex data-set (Figure 2B, $r=0.33$, $p=0.03$) compared to sputum IL-8 levels detected by AP-based ELISA (Figure 2A, $r = 0.57$,

$p < 0.0001$). Next, we compared the measured IL-8 values by each detection system with the concentrations of EPX in the sputum. The IL-8 levels detected by HRP system showed significant positive correlations ($r = 0.51$, $p < 0.0001$) to the increasing amount of EPX in the sputum (Figure 2D). In contrast, there was no statistically significant association with either Milliplex or AP-based system with the varying concentration of EPX (Milliplex $r = -0.24$, $p = 0.13$; AP $r = 0.26$, $p = 0.09$) (Figure 2C). The latter result is more likely since IL-8 is typically a neutrophil-mediated molecule and improbable to be increased in a micro-environment indicative of high eosinophil activity (Baines et al., 2010). Thus, the positive correlation coefficient values obtained from the HRP-generated IL-8 data-set is likely due to a consequence of endogenous EPX levels in the sputum rather than a meaningful association with airway eosinophilia. Post sub-group analysis, 22 patients were confirmed to have eosinophilic inflammation (sputum eosinophils $>3\%$), which was further corroborated by a significant increase in detected EPX levels compared to non-eosinophilic group (median [IQR]: 48.09 [86.3] $\mu\text{g/ml}$, vs. 1.91 [4.7] $\mu\text{g/mL}$, $P < 0.0001$). Within this group, the median concentrations of IL-8 detected by the three methods were comparable (Milliplex: 1675 vs. AP: 1670 vs. HRP: 1607) but not the variance (Milliplex: IQR - 1675, SD - 1218; AP: IQR - 2609, SD- 1983; HRP: IQR - 3514, SD- 3432). The comparable interquartile range and standard deviation values for the data sets generated by AP and Milliplex system in contrast to HRP-generated values further supports our current hypothesis.

We acknowledge our study is limited in terms of a small sample size and comparison with only one mediator, i.e. IL-8. Examining multiple mediators in a head-to-head comparison on three separate immunoassay platforms was not feasible with limited sample (sputum) volume. We considered IL-8 as an ideal candidate to test the current hypothesis since (i) the measurement on immunoassay is unaffected by dithiothreitol (DTT) used during sputum processing (Efthimiadis et al., 1997), (ii) is known to have an affinity for negatively charged molecules such as heparin (and not cations like EPX) (Miller and Krangel, 1992), and (iii) is not a typical marker of eosinophilic inflammation and therefore, unlikely to correlate with EPX levels.

Examining sputum mediators with HRP-based ELISA kits may misrepresent experimental results and lead to a false assumption/association with an eosinophil-driven event/pathogenesis. Indeed, only the data-set generated from the HRP-based system implied a significant association between typically neutrophil-mediated IL-8 concentration and eosinophilic activity (i.e. EPX), and not those from AP-based ELISA or the non-enzymatic Milliplex values. That being said, eosinophil-driven mediators like IL-5, IL-13, eotaxins, etc., are likely to be co-present along with EPX (released/resultant of the same eosinophil-driven pathomechanism) and may not generate misleading correlation coefficients to the same degree when evaluated with an HRP-based kit. Therefore, we analysed an eosinophilic cytokine (IL-5) in 20 sputum samples that had adequate volume (out of 47). As expected, we did not see any disparity between HRP-ELISA generated values and those from AP-ELISA or Milliplex bead system (IL-5 HRP vs Bead: $r = 0.7$, $P = 0.003$; IL-5-AP vs Beads: $r = 0.8$, $P < 0.0001$). However, the values obtained from the HRP-ELISA might not be truly representative of the exact expression levels in the airways as suggested by the stronger correlation coefficients and P values of the AP-ELISA data-set with that of bead-based Milliplex for both IL-5 and IL-8. Hence, it is particularly pertinent to consider the impact of

endogenous peroxidase ‘interference’ in interpreting the clinical studies that investigate roles of soluble mediators in explaining eosinophil-mediated pathogenesis.

In addition, our observations using HRP-labelled secondary antibodies for detection of other soluble factors such as immunoglobulins (autoantibodies to EPX) and signalling molecules (suppressor of cytokine signalling 3, SOCS3) have been similar. During protocol optimisation of SOCS3 ELISA kit (Cloud-Clone Corp, TX, USA), optical densities (OD) generated for diluent control vs. diluent spiked with 1 ng/mL EPX (Lee Biosciences) were 0.0 and 0.158. When the detection antibody was switched to a biotinylated secondary antibody (AP-ELISA), the OD values generated for the EPX-spiked diluent control was considerably lowered (0.092). Similar observations were seen during protocol optimisation of an anti-EPX IgG ELISA (Mukherjee et al., 2017). The mean OD value ($\pm$ standard deviation) generated for the same set of samples (paucigranulocytic vs. eosinophilic sputa) with an HRP-labelled secondary antibody (Abcam) was 1.7 ± 0.9 vs. 2.2 ± 0.4 ($n = 4$, $p = 0.1$, paired t-test). In contrast, the difference between paucigranulocytic vs. eosinophilic sputa using a biotinylated secondary antibody (BD, Biosciences) was observed to be significant [0.6175 ± 0.08 vs. 1.9 ± 0.5 , $p = 0.007$, paired t-test]. This disparity could again be explained by the interference of endogenous EPX which we confirmed by comparing OD values between EPX-spiked (1 ng/mL) and unspiked sputum from healthy subjects. The former consistently showed higher OD values compared to its unspiked control. Care was taken that the healthy sputum samples used had undetectable EPX levels (measured as described previously).

In conclusion, our study demonstrates that endogenous peroxidases such as EPX in the sputum supernatants interfere with peroxidase-dependent detection system. We also raise concern that sputum samples from patients with infective bronchitis and increased myeloperoxidase levels (secreted by neutrophils) may pose a similar technical issue. This was beyond the scope of our current analysis. In conclusion, our study encourages cautious interpretation of data generated by HRP-based ELISAs.

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Abbreviations

ELISA	enzyme linked immunosorbent assay
HRP	horse radish peroxidase
AP	alkaline phosphatase
IL	interleukin
EPX	eosinophil peroxidase

IQR	interquartile range
SD	standard deviation
OD	optical density
SOCS3	suppressor of cytokine signalling 3

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Figure 1.

Schematic representation of commonly used immunoassays and colour-development protocols in sputum research: (A) AP-based ELISA, (B) HRP-based ELISA, It is to be noted that some ELISA protocols like the R&D system duo-sets, follows a peroxidase based colour development for biotinylated secondary antibodies. An intermediate streptavidin-HRP complex reagent is used where the streptavidin binds covalently to the biotin while the HRP catalyzes the substrate to generate the chromogenic product. (C) Milliplex or bead-based multiplex system employs polystyrene beads surface-conjugated with capture antibodies. Essentially, the sandwich structure (capture antibody-antigen-detection antibody) of an ELISA is maintained but without the requirement of an enzymatic reaction. Instead,

the detection is based on Streptavidin-conjugated phycoerythrin (PE, emission at 578 nm) which covalently binds to the biotinylated detection antibody. (D) IL-8 absolute values obtained from n=47 samples analysed by the three methods, Data represented as median (Interquartile range). Kruskal-Wallis test, Dunn multiple comparison test. $P < 0.05$ considered as significant difference.

Figure 2. Correlation of IL-8 data set generated by (A) AP-based ELISA (B) HRP-based ELISA against Bead-based Milliplex system ($n = 47$); Values of IL-8 sputum levels measured by (C) AP-ELISA and (D) HRP-ELISA plotted against presence of sputum EPX, Pearson. $P < 0.05$ is considered to be significant.