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Research paper

Optimising the quantification of cytokines present at low concentrations in small human mucosal tissue samples using Luminex assays ☆

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ABSTRACT

Sensitive measurement of multiple cytokine profiles from small mucosal tissue biopsies, for example human gastric biopsies obtained through an endoscope, is technically challenging. Multiplex methods such as Luminex assays offer an attractive solution but standard protocols are not available for tissue samples. We assessed the utility of three commercial Luminex kits (VersaMAP, Bio-Plex and MILLIPLEX) to measure interleukin-17A (IL-17) and interferon-gamma (IFNγ) concentrations in human gastric biopsies and we optimised preparation of mucosal samples for this application. First, we assessed the technical performance, limits of sensitivity and linear dynamic ranges for each kit. Next we spiked human gastric biopsies with recombinant IL-17 and IFNγ at a range of concentrations (1.5 to 1000 pg/mL) and assessed kit accuracy for spiked cytokine recovery and intra-assay precision. We also evaluated the impact of different tissue processing methods and extraction buffers on our results. Finally we assessed recovery of endogenous cytokines in unspiked samples. In terms of sensitivity, all of the kits performed well within the manufacturers' recommended standard curve ranges but the MILLIPLEX kit provided most consistent sensitivity for low cytokine concentrations. In the spiking experiments, the MILLIPLEX kit performed most consistently over the widest range of concentrations. For tissue processing, manual disruption provided significantly improved cytokine recovery over automated methods. Our selected kit and optimised protocol were further validated by measurement of relative cytokine levels in inflamed and uninflamed gastric mucosa using Luminex and real-time polymerase chain reaction. In summary, with proper optimisation Luminex kits (and for IL-17 and IFNγ the MILLIPLEX kit in particular) can be used for the sensitive detection of cytokines in mucosal biopsies. Our results should help other researchers seeking to quantify multiple low concentration cytokines in small tissue samples.

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1. Introduction

Assessing cytokine profiles in small tissue biopsies presents a significant technical challenge, particularly the quantification of multiple cytokines when some are present at low concentrations. Multiplex methods using Luminex technology may offer an attractive solution. However these are often developed using soluble materials such as sera or cell culture supernatants spiked with recombinant cytokines and standard protocols are not available for tissue samples. Luminex assays use multiple sets of polystyrene or paramagnetic beads or 'microspheres' — see Vignali (2000) and Houser (2012). Each set is fluorescently colour-coded to be identifiable on a dedicated flow cytometer or other platform and pre-coated with antibody to capture a specific cytokine or other analyte, around which a sandwich immunoassay is built. Different bead sets can be

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combined to enable simultaneous measurement of multiple cytokine concentrations in a single sample against standard curve preparations. These assays require substantially less sample than traditional enzyme-linked immunosorbent assays (ELISAs) – typically 25–50 μL for multiple analytes compared with 200 μL for a single analyte – yet may offer similar sensitivity to Luminex (Vignali, 2000; Biagini et al., 2004; Elshal and McCoy, 2006).

Our research concerns the characterisation of immune responses to the pathogen *Helicobacter pylori* (*Hp*) which are linked to peptic ulceration and gastric cancer development (Atherton, 2006; Robinson et al., 2008). The challenges are broadly similar in other fields, particularly for gastrointestinal mucosal researchers: how to study immune responses using methodology that better reflects cytokine levels in the mucosa in vivo. Endoscopic mucosal biopsies are small (typically around 5–10 mg) and concentrations of many of the cytokines of interest are low, so assay sensitivity and sample volume requirements are critical. Other investigators have used semi-quantitative methods including immunohistochemistry (Lindholm et al., 1998; Lehmann et al., 2002; Holck et al., 2003) and western blotting (Luzza et al., 2000; Tomita et al., 2001), or PCR-based methods to quantify cytokine mRNA which are not always fully reflected at the protein level (Luzza et al., 2001; Robinson et al., 2008; Serelli-Lee et al., 2012). Cytokines have been measured in gastric biopsy homogenates using ELISA (Yamaoka et al., 2001; Shimizu et al., 2004; Caruso et al., 2008; Robinson et al., 2008; Serelli-Lee et al., 2012), but additional volume is needed for each analyte assayed which may require sample dilution. Therefore the number of cytokines, particularly those present at low concentrations, that can be assayed using this method is limited. Another common approach is to culture gastric biopsies in vitro, with or without stimulation, and measure cytokine concentrations in culture supernatants (Crabtree et al., 1991; Bodger et al., 1997; Mizuno et al., 2005). However, these methods may alter the cytokine profile (Veldhoen et al., 2009). The cytokine concentrations in homogenates of gastric biopsies should more closely reflect those found in the gastric mucosa in vivo.

Luminex-based methods have been used to assess murine immune responses to *Hp* infection (Taylor et al., 2008) and vaccination (Taylor et al., 2007) in splenocyte culture supernatant and recently to quantify gastric cytokine concentrations in *Hp*-infected mice (Schumacher et al., 2012). A method to measure *Hp*-specific IgG in human saliva samples has also been developed, using Luminex beads conjugated with antigens including *Hp* whole cell sonicate and recombinant urease (Griffin et al., 2011). However, to our knowledge, Luminex assays have not been optimised for human gastrointestinal mucosal tissue samples, though were recently used to quantify interleukin-1β, interleukin-1 receptor antagonist, interleukin-6 and tumour necrosis factor-α in gastric tissue samples (Serelli-Lee et al., 2012). Careful kit selection and optimisation of tissue sample preparation in a limited volume of extraction buffer will theoretically facilitate cytokine detection in these samples.

Here we aim to systematically compare and contrast the accuracy and performance of several commercially available Luminex assays as well as different sample homogenisation protocols for quantification of cytokines in tissue biopsies. We purchased assays from three suppliers: Bio-Plex Pro (Bio-Rad Laboratories, CA, USA), MILLIPLEX MAP (Merck Millipore, Darmstadt, Germany) and VersaMAP (R&D Systems, MN, USA) with assays for interleukin-17A (IL-17) and interferon-gamma (IFNγ). This evaluation using cytokine spiked human gastric biopsies provides more widely relevant information on the technology's ability to quantify cytokines present at low concentrations in small tissue samples and optimisation of mucosal tissue preparation for this application. Finally we report on the suitability of our selected Luminex kit and optimised homogenisation protocol to detect endogenous cytokines in uninfected and *Hp*-infected clinical samples.

2. Materials and methods

2.1. Patients and samples

Patients attending for clinically-indicated routine upper gastrointestinal endoscopy at Queen's Medical Centre (Nottingham, UK) donated additional gastric mucosal biopsies for research. These were immediately snap frozen in liquid nitrogen and stored at −80 °C. Patients were ineligible for inclusion in the study if they had previous gastric surgery, were regularly taking non-steroidal anti-inflammatory drugs (those taking regular aspirin for cardiovascular prophylaxis were not excluded), regular steroids or other immunosuppressive therapy, or had taken antibiotics in the preceding four weeks or proton pump inhibitors in the preceding two weeks. Written informed consent was obtained from all participants after the nature and possible consequences of the studies had been fully explained. Ethical approval was granted by the National Research Ethics Service East Midlands — Nottingham 2 Committee (08/H0408/195).

For the kit and tissue processing comparisons, seven patients (mean age ± standard deviation (SD) [range]; 51 ± 19 years [21–69]; two male, five female) each donated nine antral biopsies which were stored for up to 10 weeks until sample preparation. For evaluation of uninfected and *Hp*-infected tissue by Luminex cytokine assays, antral biopsies from a further 24 patients were used (51 ± 15 years [17–75]; 13 male, 11 female) of whom 18 were *Hp*+ and none of the six *Hp*− patients had evidence of gastric inflammation by histology. To determine mRNA expression we used antral biopsies from a further 41 consecutive patients (51 ± 15 years [29–81]; 17 male, 24 female) such that each transcript was evaluated in 18 *Hp*+ and 6 *Hp*− patients as complete data were not available for every patient. *Hp* status was assessed by biopsy urease test, culture, histology and IgG serology, with patients classified as infected if supported by at least three parameters and non-infected if all four parameters were negative with no history of previous eradication therapy.

2.2. Sample preparation methods

Single biopsies from each patient were individually thawed on ice then immediately disrupted in extraction buffer, either: (1) manually with a mini pellet pestle (Kimble Kontes, NJ, USA) for 2 min, (2) a proportion of those disrupted by pestle were further homogenised by 5–10 repeated passes through a 23 G needle and 1 mL syringe, or (3) automatically with a bead-basher (TissueLyser LT, QIAGEN, Hilden, Germany) using

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