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Research paper

Evaluation of the effect of storage condition on cell extraction and flow cytometric analysis from intestinal biopsies

Manon E. Wildenberg[a,b], Marjolijn Duijvestein[a,b], Liset Westera[a], Tanja van Viegen[c], Christianne J. Buskens[d], Jarmila D.W. van der Bilt[d], Larry Stitt[c], Vipul Jairath[c,e,f], Brian G. Feagan[c,e,f], Niels Vande Casteele[c,g,*,1]

[a] Tytgat Institute for Liver and Intestinal Research, Amsterdam, The Netherlands
[b] Academic Medical Center, Department of Gastroenterology and Hepatology, Amsterdam, The Netherlands
[c] Robarts Clinical Trials, Inc., London, Canada
[d] Academic Medical Center, Department of Surgery, Amsterdam, The Netherlands
[e] University of Western Ontario, Department of Medicine, London, Canada
[f] University of Western Ontario, Department of Epidemiology and Biostatistics, London, Canada
[g] Univeristy of California San Diego, Department of Medicine, La Jolla, CA, United States

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ABSTRACT

Background: Flow cytometric (FC) analysis of intestinal tissue biopsies requires prompt cell isolation and processing to prevent cell death and generate valid data. We examined the effect of storage conditions prior to cell isolation and FC on viable cell yield and the proportions of immune cell phenotypes from intestinal biopsies.

Methods: Biopsies (N = 224) from inflamed or non-inflamed ileal and/or colonic tissue from three patients with Crohn's disease were processed and analyzed immediately in duplicate, or stored under different conditions. Cells were isolated and stained for specific markers, followed by FC.

Results: Decreased mean live CD45+ cell counts were observed after storage of biopsies at −80 °C dimethyl sulfoxide (DMSO)/citrate buffer compared with immediate processing (1794.3 vs. 19,672.7; p = 0.006]). A non-significant decrease in CD45+ live cell count occurred after storage at −20 °C in DMSO/citrate buffer and cell yield was adequate for subsequent analysis. CD3+ cell proportions were significantly lower after storage at 4 °C in complete medium for 48 h compared with immediate analysis. Mean CD14+ cell proportions were significantly higher after storage of biopsies at −80 °C in DMSO/citrate buffer compared with immediate analysis (2.61% vs. 1.31%, p = 0.007). CD4+, CD8+ and CD4+/CD8+ cell proportions were unaffected by storage condition.

Conclusion: Storage of intestinal tissue biopsies at −20 °C in DMSO/citrate buffer for up to 48 h resulted in sufficient viable cell yield for FC analysis without affecting subsequent marker-positive cell proportions. These findings support the potential shipping and storage of intestinal biopsies for centralized FC analysis in multicenter clinical trials.

1. Introduction

Flow cytometry (FC) is a powerful tool for elucidation of the mechanism of action of anti-inflammatory drugs in immune-mediated gastrointestinal diseases such as inflammatory bowel disease (IBD), celiac disease, and eosinophilic esophagitis. Flow cytometric measurement of changes in immune cell populations and relevant cellular targets in the intestinal compartment following therapy may accelerate drug development by providing insights into pharmacokinetic/pharmacodynamic relationships, target engagement, and identifying responder populations.(van Leeuwen, du Pre, van Wanrooij, et al., 2013; West, Hegazy, Owens, et al., 2017) Notwithstanding the potential

Abbreviations: ANOVA, two-way analysis of variance; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; FC, flow cytometry; IBD, inflammatory bowel disease; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; RPMI, Roswell Park Memorial Institute; RT, room temperature; SAS, Statistical Analysis System; SD, standard deviation

* Corresponding author at: University of California San Diego, School of Medicine, Division of Gastroenterology, IBD Center, 9500 Gilman Drive #0956, La Jolla, CA 92093, United States.
E-mail address: nvandecasteele@ucsd.edu (N. Vande Casteele).
[1] Guarantor of the article.

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value of FC, its application to clinical trials is constrained by rapid rates of cell death in biopsy specimens. This phenomenon makes the collection, storage and shipment of tissue samples from the clinic to an off-site laboratory challenging. Furthermore, methods for these three processes have not been standardized and consequently vary substantially from laboratory to laboratory. Given these circumstances, it is critical to develop standardized methods that optimize the yield of viable cells and reduce inter-laboratory variability. However, progress has been made in addressing these challenges. Recently, an international team of researchers published consensus guidelines for the use of FC and cell sorting in immunological studies.(Cossarizza, Chang, Radbruch, et al., 2017) Additionally, the variation in quantitation of immune cell phenotypes in peripheral blood mononuclear cells (PBMC) among international laboratories has also been studied by two separate groups using common protocols for permeabilization and staining.(Maecker, Rinfret, D'Souza, et al., 2005; Westera, van Viegen, Jeyarajah, et al., 2017) Although these studies showed acceptable inter-laboratory variation using central FC gating, the sample processing required for analysis of tissue biopsies, in distinction to blood samples, raises additional challenges due to the problem of decreased cell viability inherent to storage and processing of biopsy samples. Central processing and analysis of biopsies might reduce measurement variability, however storage and transport conditions must be optimized to ensure adequate and analyzable samples reach the central FC laboratory. Currently, the effects of storage temperature, buffer and shipping condition on the yield of viable cells and biomarker expression from intestinal tissue biopsies is unknown. The aim of the current study was to examine the effect of storage conditions prior to cell extraction and FC analysis on viable cell yield and marker-positive cell proportions from intestinal tissue biopsies. We hypothesized that an optimal cell preservation protocol could be identified that would allow transport of biopsies for valid FC central analysis.

2. Methods

This prospective single center study was performed at the Tytgat Institute (Academic Medical Center, Amsterdam, the Netherlands) where tissue resected from patients undergoing surgery for IBD was used to sample biopsies. The Independent Ethics Committee of the Academic Medical Center approved the use of resected tissue for research purposes and all patients provided informed consent. Patient information collected complied with the requirements for the protection of privacy of individually identifiable health information.

2.1. Materials

Complete medium was prepared with RPMI 1640 (Invitrogen, Carlsbad, CA), 2 mM L-Glutamine (Lonza, Allendale, NJ), 100 U/ml penicillin/streptomycin (Lonza), 50 ng/ml gentamycin (Lonza), and 50 ng/ml Fungizone (Amphotericin B, Gibco, Breda, the Netherlands). Dimethyl sulfoxide (DMSO)/citrate buffer (pH 7.6) was prepared with 250 mM sucrose (Prolabo, Boxmeer, the Netherlands), 40 mM citrate (Merck, Schiphol-Rijk, the Netherlands), and 5% DMSO (Sigma, Zwijndrecht, the Netherlands). Digestion medium was prepared with complete medium and 1 mg/mL collagenase D (Roche, Basel, Switzerland; activity: > 0.15 U/mg according to Wünsch), 1 mg/mL soybean trypsin inhibitor (Invitrogen), and 50 µg/mL DNase I, grade II from bovine pancreas (Roche, activity approximately 2000 U/mg according to Kunitz [+25 °C; DNA as substrate]). Fluorescence-activated cell sorting (FACS) buffer consisted of phosphate buffered saline (PBS) and 1% bovine serum albumin (BSA, Sigma).

2.2. Biopsy collection and storage

In this exploratory study, biopsies were procured from surgical resection specimens due to the large number of biopsies required for evaluation of multiple temperature and buffer conditions. Resected tissue was collected directly from the operating room and biopsies were taken immediately or within 1 h of receipt of the resected specimen using standard-size (2.2 mm outer diameter) spiked double-bite biopsy forceps. Biopsies (n = 28) were collected from each of 8 ileal or colonic surgical specimens from macroscopically inflamed or non-inflamed tissue. Two biopsies were pooled and processed as a single sample, resulting in 14 samples per surgical specimen for analysis in duplicate for 7 different conditions. One set (2 sets of pooled biopsies, 4 total) of biopsies per specimen was analyzed immediately. The remaining 6 sets of biopsies were stored according to the conditions outlined in Table 1. For conditions 4 and 6, a CoolCell® (Corning, Amsterdam, the Netherlands) container was used to ensure a controlled freezing rate of −1 °C/min. The samples used for testing conditions 5 and 7 were soaked in 1.5 mL DMSO/citrate buffer for 10 min before freezing to allow the buffer to penetrate the tissue.

Table 1
Storage conditions.

Condition #	Temperature	Buffer	Time
1	Not applicable, immediate analysis	Complete medium	< 1 h after receipt
2	4 °C	Complete medium	24 h
3	4 °C	Complete medium	48 h
4	−20 °C with CoolCell®	DMSO/citrate buffer	48 h
5	−20 °C	DMSO/citrate buffer	48 h
6	−80 °C with CoolCell®	DMSO/citrate buffer	48 h
7	−80 °C	DMSO/citrate buffer	48 h

DMSO, dimethyl sulfoxide.

2.3. Cell isolation and antibody staining

After thawing at 37 °C (if necessary) and removal of storage buffer, biopsies were transferred to a 10 mL round-bottom tube pre-filled with 2.5 mL digestion medium and incubated for 1 h at 37 °C with shaking, followed by transfer to a 50 mL tube containing a 100 µm mesh cell strainer (BD Biosciences, San Jose, CA). The rubber end of a syringe plunger was used to gently mash the tissue through the strainer, while frequently rinsing with digestion medium. Digestion medium was added to each tube to a final volume of 20 mL followed by centrifugation for 5 min at 500 g at room temperature (all subsequent centrifugation steps were performed at room temperature unless otherwise noted). The supernatant was removed and cells were resuspended in FACS buffer containing fixable amine-reactive live/dead marker AQUA (Thermo Fisher Scientific/Life Technologies, Carlsbad, CA), transferred to an Eppendorf tube and incubated in the dark for 30 min at room temperature. Cells were then washed in PBS and resuspended in FACS buffer. Cells were subsequently stained using anti-CD45-APC-eFluor780 (clone HI30), anti-CD14-PE-Cy7 (clone 61D3), anti-CD4-PerCp-eFluor710 (clone SK3, all eBioscience, San Diego, CA), anti-CD3-AF488 (clone UCHT1), anti-TCRab-BV421 (clone IP26), and anti-CD8-AF700 (clone HIT8, all Biolegend, San Diego, CA). After incubation for 20 min in the dark at 4 °C, cells were washed, centrifuged, resuspended in FACS buffer, and stored at 4 °C until analysis on the same day.

2.4. Acquisition and analysis

Cell acquisition was performed on a cell analyzer (BD LSRFortessa) with FACSDiva software (both BD Biosciences, San Jose, CA). Single-stained beads were run as compensation controls. The complete sample was acquired and all samples were analyzed using FlowJo software