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# Extended exposure duration of cultured intestinal epithelial cell monolayers in characterizing hazardous and non-hazardous proteins

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ABSTRACT

Recent studies suggest that human derived intestinal epithelial cells (IECs) cultured as polarized monolayers on Transwell® filters may respond differently when exposed to hazardous and non-hazardous proteins. This experimental platform was based on apical exposure of IEC monolayers to test proteins for 24 h followed by assessment of barrier integrity and cell viability. In this study, Caco-2 and T84 IEC polarized monolayers were evaluated for barrier integrity and cytotoxicity following exposure to hazardous and non-hazardous proteins for 24, 48 and 72 h. Hazardous proteins included *Clostridium difficile* toxin A (ToxA), Streptolysin O (SLO), Wheat Germ Agglutinin (WGA), and *Phaseolus vulgaris* haemagglutinin-E (PHA-E). Non-hazardous proteins included bovine serum albumin (BSA), porcine serum albumin (PSA), and fibronectin (Fbn). In general, evidence of diminished barrier integrity or cell viability observed following exposure to hazardous proteins for 24 h was more pronounced after 48 and 72 h for both IEC monolayers. Non-hazardous proteins exhibiting no impact following 24 h of exposure elicited minimal effects over longer exposure durations. These results support the utility of using cultured human IEC polarized monolayers to differentiate between hazardous and non-hazardous proteins and suggest that longer durations of exposure may further improve the ability to distinguish between them.

## 1. Introduction

Acute toxicology studies are routinely conducted with individual chemicals for the purposes of hazard characterization and package labeling requirements (Delaney, 2017). Relative to the industrial production scale of these chemicals, the quantity of test substance required to conduct these studies is trivial. Acute toxicology studies have also been conducted with proteins expressed in genetically modified (GM) crops because they are required by some regulatory agencies (Mendelsohn et al., 2003). However, in contrast to industrially produced chemicals, obtaining the quantities of protein to conduct acute toxicology studies can present a challenge. The concentration of newly expressed proteins in most GM crops is generally low, so it is not feasible to isolate large quantities of them from the crops in which they are expressed. Instead, acute toxicology studies have been conducted utilizing proteins purified from heterologous expression systems following extensive analytical characterization to establish that they were "sufficiently similar" to the protein expressed *in planta* (Raybould et al., 2013; Wang et al., 2016).

Prior to conducting acute toxicology studies, individual proteins are subjected to a multicomponent hazard identification process that has been called Tier I (Delaney et al., 2008a). Components in the hazard identification process for any individual protein include a bioinformatics analysis to determine if there are significant sequence or structural similarities between that protein and known hazardous proteins. Also included in Tier I analysis is information about the mechanism of action and/or specificity of the protein, and sensitivity to degradation in the presence of digestive enzymes (Delaney et al., 2008a). Conceptually, if no evidence of potential for hazard was identified following Tier I analysis, single and/or repeated dose toxicology studies in Tier II (hazard characterization) would not be necessary. These methods have proven to be robust and effective since all of the individual proteins to date that have been determined to be non-hazardous by this multicomponent analysis demonstrated no evidence of

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adverse effects with acute toxicology studies (Delaney et al., 2008b; Mathesius et al., 2009; Juberg et al., 2009; Xu et al., 2009; Cao et al., 2010, 2012; Stagg et al., 2012; Wang et al., 2016).

Membrane-bound proteins, transcription factors and other types of proteins are being investigated for use in future GM crop varieties to impart various phenotypic properties including drought tolerance, nutritional enhancement, and enhanced nitrogen utilization (Deikman et al., 2012; Rothstein et al., 2014; Hefferon, 2015). These proteins are expected to be difficult or virtually impossible to isolate in the quantity or quality necessary to conduct *in vivo* acute toxicology studies as they have been conducted historically. Collectively, and informally, they have been defined as "intractable" to indicate that, while it is difficult to isolate them, at least in some cases, it may be possible to isolate small quantities (Bushey et al., 2014). Since multiple regulatory agencies require acute toxicology studies for newly expressed proteins in GM crops, it is critical to develop and assess alternative approaches that will enable hazard characterization of intractable proteins that can be conducted using smaller quantities of protein. *In vitro* studies with polarized monolayers derived from human intestinal epithelial cell (IEC) lines have been investigated as a potential component of Tier II (hazard characterization) that could be considered *in lieu* of the acute toxicology study if there were uncertainty about potential hazard following the Tier I analysis. This model has demonstrated effectiveness at differentiating between hazardous and non-hazardous proteins with measurements of intestinal monolayer barrier integrity being the most sensitive indicator affected by hazardous proteins (Hurley et al., 2016a, 2016b; Eaton et al., 2017; Delaney, 2017). A major advantage to this particular *in vitro* method is that it provides a robust capacity to distinguish between hazardous and non-hazardous proteins and requires minimal amounts of test protein to conduct a thorough analysis.

Comparison of the effects of hazardous and non-hazardous dietary proteins on human IEC monolayers were investigated with results showing that most hazardous proteins damaged monolayer integrity while the non-hazardous dietary proteins did not (Hurley et al., 2016a, 2016b). This *in vitro* testing method for protein hazard characterization evaluated IEC responses to different types of proteins following exposure to a single concentration for approximately 24 h. The current studies were carried out to examine whether longer exposure durations with either hazardous or non-hazardous proteins would result in altered IEC responses compared with those observed at 24 h.

## 2. Materials and methods

### 2.1. Test substances

Proteins and chemical substances used in this study are listed in Table 1. Fibronectin (Fbn), Porcine Serum Albumin (PSA), Streptolysin O (SLO), and Triton X-100 (TX-100) were purchased from Sigma Chemical Company (St. Louis Missouri, USA). Wheat Germ Agluttinin (WGA) and Unconjugated *Phaseolus vulgaris* haemagglutinin (PHA-E) were purchased from Vector Laboratories (Burlingame, California, USA). *Clostridium difficile* Toxin A (ToxA) was purchased from List Laboratories, Inc. (Campbell, California, USA). Bovine serum albumin (BSA) was purchased from Fisher Scientific (Waltham, MA, USA). All proteins were diluted in assay media (phenol red free, serum free, antibiotic free DMEM/F12) purchased from Invitrogen Corporation (Carlsbad, CA) and examined at the concentrations indicated in Table 1.

### 2.2. Cell culture

Intestinal epithelial carcinoma adherent cell lines derived from colon (T84 and Caco-2) were purchased from American Type Culture Collection (ATCC; Manassas, Virginia, USA) and grown on the inner membrane of 0.4 μm Transwell™ inserts (Corning Incorporated/Life Sciences, Tewksbury, Massachusetts, USA) as previously described (Hurley et al., 2016a, 2016b).

**Table 1**
Protein and Controls Evaluated.

| Protein / toxin | Abbreviation | Category | [b]Vendor | Dose Tested |
|---|---|---|---|---|
| Bovine Serum Albumin | BSA | Non-hazardous protein | Sigma-Aldrich A2153 | 1 mg/ml |
| Streptolysin O | SLO | hemolysin | Sigma-Aldrich S0149 | 2500 units/ml |
| *Clostridium difficile* Toxin A | ToxA | enterotoxin | List Laboratories #152B | 2 μg/ml |
| Wheat Germ Agglutinin | WGA | food toxin | Vector Laboratories L1020 | 1 mg/ml |
| Porcine Serum Albumin | PSA | Non-hazardous protein | Sigma-Aldrich A4414 | 1 mg/ml |
| *Phaseolus Vulgaris* Erythroagglutinin | PHA-E | food toxin | Vector Laboratories L1120 | 1 mg/ml |
| Fibronectin bovine plasma | Fbn | Non-hazardous protein | Sigma-Aldrich F4759 | 100 μg/ml |
| **Control** | **Abbreviation** | **Category** | **Vendor[b]** | **Range Tested** |
| Assay media | (-) | (-) control | Invitrogen | (-) |
| [a]TritonX-100 | TX-100 | (+) control | Sigma-Aldrich | 0.1 % |

[a] Treatment of the apical surface of IECs with.1% TX-100 serves as a (+) control for induced cytotoxicity of IECs and for disruption of IEC monolayer barrier integrity.

[b] Sigma-Aldrich (St. Louis, MO), List Biological Laboratories, Inc. (Campbell, California), Vector Laboratories, Inc. (Burlingame, CA), and Invitrogen Corporation (Carlsbad, CA).

### 2.3. Exposure to proteins

IECs seeded on transwell inserts were grown for one or two weeks in medium (DMEM/F12, 10% FBS, 10% Pen/Strep) to achieve confluence and monolayer polarity. On the day of the experiment, IEC monolayers were washed, equilibrated in serum free assay media, and *Trans*-Epithelial Electrical Resistance (TEER) was evaluated using a voltohmmeter (EVOM2, Epithelial Voltohmmeter, World Precision Instruments, Inc., Sarasota, Florida, USA). A total of 72 individual IEC monolayers grown on transwells were separated into three plates containing 24 transwells/plate. Within each plate, 4 IEC monolayers were exposed to either a protein or control along with barrier integrity probes FITC-inulin 500 μg/ml and horseradish peroxidase (HRP) 450 ng/ml and incubated at 37 °C, 5% $CO_2$. Incubation times for each of three separate plates were approximately 24, 48, and 72 h respectively. Following the designated incubation period, IECs were evaluated for barrier integrity (TEER, FITC-inulin flux, and HRP flux) and cytotoxicity (LDH release and MTT conversion to formazan) as described previously (Hurley et al., 2016a, 2016b). FITC-inulin and HRP were purchased from Sigma-Aldrich (St. Louis, MO). The Pierce LDH Cytotoxicity Assay Kit and the Vybrant® MTT Cell Proliferation Assay Kit were purchased from Fisher Scientific (Waltham, MA).

### 2.4. Data analysis/statistics

All experiments were performed at least 3 times (≥3 biological replicates). Each data point within an internally controlled experiment represents the mean and standard deviation of quadruplicate IEC monolayers (4 technical replicates/internally controlled experiment). Differences from the negative control (no protein) were considered statistically significant when $p < 0.05$ using an unpaired two-tailed student's T test within an internally controlled experiment. Statistical