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Polarized monolayer cultures of human intestinal epithelial cell lines exposed to intractable proteins - *In vitro* hazard identification studies

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1. Introduction

Many genetically modified (GM) crops have been altered to express non-native proteins that endow the plant with agronomic traits including resistance to insects and tolerance to herbicides. Methods have been developed to evaluate the safety of proteins expressed in GM crops that focus on determining whether the individual proteins are likely to present a hazard if consumed orally. This approach includes consideration of the source of the protein and history of safe use, bioinformatics sequence comparison to other proteins, mechanism of action and specificity, and *in vitro* sensitivity to degradation in the presence of digestive enzymes (Delaney et al., 2008a). While no single component is necessarily more important than another, collectively this information has been applied to identify possible hazards from individual proteins. It is noteworthy that much of this information can be obtained without ever isolating any actual protein (Bushey et al., 2014).

In cases where the hazard identification data are uncertain, a second tier of hazard characterization studies can be considered that include acute and repeated dose toxicology studies. The general concept is to evaluate whether some type of adverse effect could occur upon *in vivo* exposure to a protein that was not identified using the hazard identification methodologies. To date, numerous single and repeated dose toxicology studies have been conducted with proteins expressed in GM crops though no evidence of adverse effects has been observed (Stagg et al., 2012; Juberg et al., 2009; Mathesius et al., 2009; Delaney et al., 2008b). The effectiveness of hazard identification tools in predicting the outcome of toxicology studies in laboratory animals has led some to question whether animal studies should be continued for this purpose (Hammond et al., 2013).

Very few proteins are known to cause adverse effects following oral exposure but those that do tend to encounter the intestinal epithelium (Ramadass et al., 2010; Lafont et al., 1988; Rossi et al., 1984). We recently published a paper that investigated the use of human intestinal epithelial cell lines (IECs) cultured as polarized monolayers for their ability to distinguish between known hazardous proteins and innocuous dietary proteins applied to the apical surface. The results of those studies indicated that hazardous proteins consistently produced changes in measurements of monolayer integrity or cytotoxicity whereas innocuous dietary proteins did not affect either (Hurley et al., 2016). Results of the studies cited above are important for a variety of reasons. First, they suggest that an *in vitro* approach to protein hazard identification is predictive of effects that could occur following oral exposure. Second, application of this method could decrease or eliminate the need to conduct studies in laboratory animals. Finally, an *in vitro* approach would require substantially smaller quantities of protein than required for studies in laboratory animals. This point is particularly important in the case of transmembrane, signaling, and transcription factor proteins that could be expressed in GM crops. These types of proteins are informally considered intractable because they are difficult or impossible to isolate in the quantities necessary to conduct *in vivo* toxicology studies (Bushey et al., 2014).

In the current study, four unrelated intractable proteins were evaluated for their impact on T84, Caco-2, and HCT-8 human polarized intestinal epithelial monolayers following apical surface exposure. These proteins included bacteriorhodopsin (transmembrane), human c-MET (signaling/transmembrane), follistatin (signaling glycoprotein) and activating transcription factor 2 (transcription factor).

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2. Materials and methods

2.1. Test substances

The following proteins were used in this study (Table 1): bacteriorhodopsin (Sigma, St. Louis, MO), human c-MET, follistatin and activating transcription factor 2 (all from Antibodies Online – Atlanta, GA). These proteins were added at the indicated concentrations to confluent monolayers of T84, Caco-2, or HCT-8 human intestinal epithelial cell lines as described previously (Hurley et al., 2016). Different positive control substances were used depending on the variable being measured. *Clostridium difficile* Toxin A (2 μg/ml; List Biological Laboratories, Inc., Campbell, California, USA) was used as a protein toxin positive control for barrier integrity/permeability effects; TritonX-100 (0.1%; Sigma) was the positive control for cytotoxicity and barrier integrity/permeability measurements; and TNFα/FliC (0.1 μg/ml each) were purchased from eBioscience, Inc. (San Diego, CA) and Enzo Life Sciences (Farmingdale, New York) respectively and this mixture served as the positive control for inducing the production of inflammatory cytokines.

2.2. Exposure

As described previously (Hurley et al., 2016), IECs were grown on 0.4 μm Transwell™ inserts (Corning Incorporated/Life Sciences, Tewksbury, Massachusetts, USA) coated with rat tail collagen (Invitrogen). IECs were seeded onto each collagen coated Transwell™ insert and cultured at 37 °C, 5% CO_2 for at least eight days prior to use. Integrity of the IEC monolayer was tested prior to administration of the indicated test substance for development of *trans*-epithelial electrical resistance (TEER) using a voltmeter (EVOM2, Epithelial Voltohmmeter, World Precision Instruments, Inc., Sarasota, Florida, USA). Test or control substances were added to the apical side of the IECs following establishment of a polarized monolayer after which they were incubated overnight (18–24 h) at 37 °C, 5% CO_2.

Effects on the IECs caused by the added test or control substances were measured using methods described previously (Hurley et al., 2016). Cytotoxicity was monitored by MTT conversion and release of intracellular lactate dehydrogenase (LDH). Monolayer integrity was monitored by measurement of TEER and flux of both a smaller ([^{3}H]-inulin) and larger (horseradish peroxidase) molecular weight substance from apical to basolateral side of the Transwell™ insert. Concentrations of the inflammatory cytokines IL-6 and IL-8 were determined in media collected from both the apical and basolateral sides using ELISA kits from R&D Systems, Inc. (Minneapolis, Minnesota, USA). All experiments were performed on at least three separate occasions. Each data point represents the mean ± standard deviation (SD) of triplicate wells assayed for each treatment or control group from a representative internally controlled experiment. Differences were considered significant at $p \leq 0.05$ when compared to negative control (monolayers treated with assay media alone) using an unpaired two–tailed student's T test within an internally controlled experiment.

3. Results

Culture conditions utilized to produce polarized monolayers for each of the three IECs were previously reported (Hurley et al., 2016). Treatment of the apical surface with assay media alone established the baseline for each assay as the negative control. TritonX-100 (TX100; 0.1%) or *Clostridium difficile* Toxin A (ToxA; 2 μg/ml) served as positive controls for disruption of barrier integrity and/or cytotoxicity. As expected, TX100 resulted in maximal cytotoxicity (>70% LDH release and minimal MTT conversion) as well as loss of monolayer integrity (increased flux of [^{3}H]-inulin/HRP and reduction in TEER to background levels) in monolayers of all three cell lines (Tables 2–4). ToxA produced reproducible adverse effects on monolayer integrity when administered to all three cell lines. With respect to cytotoxicity, ToxA elicited a statistically significant and reproducible decrease in MTT conversion within Caco-2 monolayers (Table 3), but did not impact Caco-2 LDH release, nor did ToxA exposure impact either of the cell viability assays for the other two IEC monolayers (Tables 2–4). A mixture of TNFα + FliC consistently triggered increased production of IL-6 and IL-8 in all IEC monolayers except HCT-8, which did not produce IL-6 under any circumstances tested. TNFα + FliC served as a positive control for evaluating inflammatory responses in these IEC monolayers. ToxA also stimulated a small but significant and reproducible increase in IL-8 release, but this was observed only in T84 monolayers (Table 2). The overall impact of ToxA as a representative known hazardous protein is depicted in Fig. 1.

Bacteriorhodopsin is a 26 kDa seven helix integral membrane protein found in Archaea such as Holobacteria. It has both extracellular and intracellular segments that utilize light as a source of energy to pump intracellular protons outside of the cell (Wickstrand et al., 2015). It is not implicated in any known adverse effects and is not being considered as a candidate protein for any known GM crops. It was included in these studies as an example of an integral membrane protein. Addition of bacteriorhodopsin at concentrations of up to 10 μg/ml did not cause changes in any of the

Table 1
Proteins and controls.

Protein/toxin	Abbreviation	Category	Vendor*	Range tested
Bacteriorhodopsin	BRh	Transmembrane	Sigma-Aldrich	0.01–10 μg/ml
Human c-MET	MET	Signaling	Antibodies-online.com	0.01–10 μg/ml
Follistatin	FST	Signaling glycoprotein	Antibodies-online.com	0.005–5 μg/ml
Activating transcription factor 2	ATF2	Transcription Factor	Antibodies-online.com	0.01–10 μg/ml
Control	**Abbreviation**	**Category**	**Vendor***	**Range tested**
Assay media	(–)	(–) control	Invitrogen	(–)
TritonX-100	TX-100	(+) control[a,b]	Sigma-Aldrich	0.1%
Clostridium difficile Toxin A	ToxA	Enterotoxin	List Laboratories	2 μg/ml
Flagellin + TNFα	FliC + TNFα	(+) control[c]	Enzo Life Sci. & eBioscience	0.1 μg/ml each

*Sigma-Aldrich (St. Louis, MO), Antibodies-online.com (Atlanta, GA),, List Biological Laboratories, Inc. (Campbell, California), Invitrogen Corporation (Carlsbad, CA), Enzo Life Sciences, Inc. (Farmingdale, NY), and eBioscience, Inc. (San Diego, CA).

[a] Treatment of IEC apical surface with 0.1% TX-100 as (+) control for cytotoxicity.

[b] Treatment of IEC apical surface with 0.1% TX-100 as (+) control for disruption of monolayer integrity.

[c] Treatment of IEC apical surface with 0.1 μg/ml FliC + TNFα as (+) control for induced IL-6 & IL-8 release.