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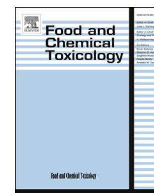

The development and validation of methods for evaluating the immune system in preweaning piglets

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ABSTRACT

The preweaning piglet has been found to be a valuable research model for testing ingredients used in infant formula. As part of the safety assessment, the neonates' immune system is an important component that has to be evaluated. In this study three concurrent strategies were developed to assess immune system status. The methods included (1) immunophenotying to assess circulating innate immune cell populations, (2) monitoring of circulating cytokines, particularly in response to a positive control agent, and (3) monitoring of localized gastrointestinal tissue cytokines using immunohistochemistry (IHC), particularly in response to a positive control agent. All assays were validated using white papers and regulatory guidance within a GLP environment. To validate the assays precision, accuracy and sample stability were evaluated as needed using a fit for purpose approach. In addition animals were treated with proinflammtory substances to detect a positive versus negative signal. In conclusion, these three methods were confirmed to be robust assays to evaluate the immune system and GIT-specific immune responses of preweaning piglets.

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1. Introduction

When evaluating the safety of new ingredients in infant formula it is important to select an appropriate research model that will replicate the conditions in infants during the nursing period. Some compounds in infant formula may have potential effects on the immune system; therefore testing ingredients within a formula for an inflammatory response is crucial to affirm safety (Nicklin and Miller, 1984; Tsuji et al., 2003). A comprehensive review of neonatal testing paradigms and their suitability for infant formula testing can be found in the literature (Flamm, 2013). Of the different animal models available, the neonatal piglet is considered to be the most appropriate for this type of an assessment (Guilloteau et al., 2010; Helm et al., 2007). In addition, swine are favored for toxicology testing due to the extensive historical data sets for toxicology endpoints and their anatomic, physiological and immunological similarity to humans (Barrow, 2012; Guilloteau et al., 2010; Helm et al., 2007; NIH, 1993; Odle et al., 2014; Penninks et al., 2012). For this study, an artificially reared preweaning Yorkshire cross-bred farm piglet was chosen because it is readily available; has a relatively short nursing period (3–4 weeks) and is similar in size to a human neonate. The Yorkshire piglet is a robust and relevant research model that has been used to study pediatric nutrition and safety, as related to growth and the development (Fedorova-Dahms et al., 2014; Hanlon and Thorsrud, 2014; Mahadevan et al., 2014; Weiner et al., 2015). Passive immunity is imparted by the sow through the colostrum during the first few hours of life and is necessary for the normal growth and development of the suckling pig and for its role in the maturation of the gastrointestinal tract (Dividich et al., 2005; Xu et al., 2002).

Abbreviations: CCE, circulating cytokine evaluation; CV, coefficient of variance; DDS, Dextran Sodium Sulfate; H&E, Hematoxylin & Eosin; IHC, Immunohistochemistry; IP, Immunophenotyping; GLP, Good Laboratory Practices; LLOQ, lower limit of quantification; LPS, lipopolysaccharide; NBF, Neutral Buffered Formalin; RT, Room Temperature; PBS, Phosphate Buffered Saline; QC, Quality Control.

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Using the piglet animal model, the development, assessment and validation of three different assays to monitor immune system function are described for use with infant formula ingredients. This work includes assessment of the innate immunologic subpopulations via immunophenotyping, and evaluation of systemic and local gastrointestinal competency after a pro-inflammatory challenge. The assays were chosen because they are recommended for use by current regulatory guidance for immunotoxic responses in adult laboratory animals (ICH, 1997). The FDA recommends that Good Laboratory Practice guidelines (GLP) be followed for all data to be submitted to the Agency (FDA, 2014). GLPs provides guidance regarding the organization, process and conditions under which laboratory studies are planned, performed, recorded, monitored and reported. Data collected following GLP guidelines are intended to promote the quality and validity of the test data. The GLPs mandate that all assays must be validated: proven reproducible and accurate for the specific purpose for which they will be generating data. Published GLP regulations do not specifically state validation implementation details, however, regulatory agencies, such as the FDA, have published guidance for analytical assay development and validation (FDA, 2015). There is still ongoing discussion and varying practices in the design of preclinical biomarker validations (Green et al., 2011; O'Hara et al., 2011; Owens et al., 2000). This ambiguity is due to a combination of challenges, such as heterogeneous cell populations, lack of reference material for accurate evaluation, and instrumentation complexity (Cunliffe et al., 2009; Green et al., 2011; Owens et al., 2000). Furthermore, due to the diverse nature of cytokine analysis via ELISA and Immunohistochemistry (IHC), neither FDA bioanalytical drug guidance, nor Clinical and Laboratory Science Institute (CLSI) guidance fully define a validation strategy ((CLSI), 2011).

Regulatory white papers suggest constructing a validation plan based upon assay type and the endpoints of the assay (NCCLS, 1997). A “fit-for-purpose” approach has been recommended for biomarker validation due to the complexity of assay design and the necessity for biomarker assay customization (Lee et al., 2006). In a fit for purpose model, the validation plan should meet the predefined needs of the study, reflect the nature of the assay methodology, and take into consideration biomarker variability within and between study populations. In the assays validated here each parameter had a different validation plans and acceptance criteria based upon the objects of each assay.

ELISA assays are considered in the relative quantitative category of methods by the biomarker community, in other words, a calibration standard can be utilized to estimate absolute quantitative concentration for an unknown analyte sample (Lee et al., 2006). Since reference standard (calibrators) can be spiked into the sample or matrix, a calibration curve is established and utilized for accuracy estimation. Thus relative quantitative assays can be assessed for precision and accuracy values. In fit for purpose analytical assay, accuracy describe the closeness of the mean interpolated (actual) test results to the theoretical (expected) value of the sample (Lee et al., 2006). For the ELISA validations performed on this study inter- and intra-accuracy and precision were assessed. The matrix was spiked with a known concentration of the cytokines and these values observed in the assay were compared with expected value in terms of relative error (RE) between the values. Accuracy within ±20% RE of nominal concentration was used as a component of the acceptance criteria. Precision in the case of this assay describes the closeness or variance of individual measurements performed in triplicate on the same sample with the same conditions (Davis et al., 2013). Precision is expressed as coefficient of variance (% CV) which is the standard deviation of the replicates divided by the mean of the replicates. The establishment of CVs parameters for the acceptance criteria in ELISA-based assays was determined by a number of factors, such as how the quality of reagents is utilized in the assay and method of signal detection (Lee et al., 2006). Given the dynamic nature of cytokines in neonatal development, these analytes were designated to have a CV less than 30% in order to meet the validation acceptance criteria. In order to confirm the assay's ability to detect a positive inflammatory signal, a portion of the animals were LPS treated. LPS is routinely used as an inducer systemic inflammation for animal models making it an appropriate proinflammatory agent for testing assay development (Carroll et al., 2005).

Immunophenotyping (IP) assays generally fall into the quasi-quantitative assay category (Lee et al., 2006). This category is defined by the assay not having reference standards to establish a quantitative reference curve, as in the ELISA assay. In these types of assays, there is a numerical effect of the response and the measurement, however without a biological reference standard, the accuracy cannot be assessed. This study validated the IP panel by the assessment of numerous precision variables, in addition to whole and fixed cell stability assessment. Data sets for each precision parameter must have a CV less than 30% in order to meet acceptance criteria. Precision values describes the closeness or variance of individual measurements performed in triplicate on the same sample in identical conditions (Davis et al., 2013). The establishment of CV parameters for acceptance criteria in cytometry-based assays are determined by a number of variables such as the rarity of a cell population, the quality of reagents utilized in the assay, fluorochrome conjugate and the instrumentation used (Wood et al., 2013). Given the dynamic nature of these cell populations in neonatal development, these subpopulations were designated to have a CV less than 30% in order to meet the validation acceptance criteria.

Immunohistochemistry (IHC) assays commonly fall into the qualitative assay category (Lee et al., 2006). Qualitative assays do not generate results with direct numerical values. Assessment of histological and immunohistochemical targeted tissues with assessment by a board certified pathologist is an accepted method for evaluation of overt tissue changes in toxicology studies (Haschek and Rousseaux, 2013). Histological observation of microscopic cellular changes is a standard endpoint for toxicology and safety studies and is well characterized and cited within the literature (Crissman et al., 2004). The four step grading system used to define lesions in this study consisted of minimal, mild, moderate and severe gradations of severity. The severity gradings were determined based on the amount of tissue affected, the degree of tissue perturbation, and the pathologist's knowledge of potential systemic deleterious effects on the animal (Gibson-Corley et al., 2013). For the IHC staining, the acceptance criterion was a positive signal confirmation versus negative control assessed by a clinical pathologist. In order to confirm the assay's ability to detect a positive inflammatory signal, a portion of the animals were DSS treated. DSS is routinely used as an inducer of intestinal inflammation for animal models of inflammatory bowel disease and other enteric inflammatory disease, making it an appropriate proinflammatory agent for testing assay efficacy (Bassaganya-Riera and Hontecillas, 2006; Kim et al., 2012).

Multiple methods of evaluation focusing on a particular assessment are often necessary to reduce the overall uncertainty of scientific findings. By utilizing a weight of evidence approach using multiple assays the authors of this manuscript confirmed assay robustness and created a panel of tests which monitored immunotoxic effects of a potential proinflammatory compound with greater certainty (Weed, 2005). For instance, the physiological assessment of a positive control sample showed gastrointestinal damage in parallel with increased cytokine up regulation, thus

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