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In vitro cytokine release from human peripheral blood mononuclear cells in the assessment of the immunotoxic potential of chemicals

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ABSTRACT

Alternative methods to the use of animals in testing of chemicals are needed. We investigated if the immunotoxic potential of 12 dietary toxicants could be predicted from effects on cytokine release from human peripheral blood mononuclear cells (PBMC) after *in vitro* exposure. Nine cytokines were selected to reflect different types of immune responses. The toxicants were classified as immunotoxic or non-immunotoxic substances according to the published *in vivo* data.

Isolated human PBMC were exposed for 20 h to three concentrations of each of the 12 substances in the presence of human liver S9 fraction. After further incubation of PBMC in fresh medium containing the mitogen phytohemagglutinin (PHA, 10 μg/ml) for 48 h, release of the nine selected cytokines into the supernatant as well as cell proliferation were measured by Luminex technology™ and the BrdU incorporation assay, respectively.

All 12 substances investigated affected the release of one or more cytokines, and each of the substances showed different cytokine release patterns. Within the limitations of the study design, the present study suggests that the effect of the substances on mitogen-induced cytokine release from PBMC cannot predict their immunotoxic potential, but may be useful in mechanistic studies.

1. Introduction

Scientists are in search of alternative methods to the use of animals in testing of chemicals, because of animal welfare considerations and relevance to risk assessment for humans, as well as for economical reasons (Balls et al., 1995). This aspect is important, e.g. with regard to the new European Community regulation REACH (registration, evaluation, authorisation and restriction of chemical substances), which will require reassessment of thousands of existing substances and the use of millions of animals (European Commission, 2010).

Many toxic substances exert adverse effects on the immune system *in vivo*, resulting in immunosuppression, hypersensitivity or autoimmunity, due to direct or indirect mechanisms of action (Galbiati et al., 2010; Gennari et al., 2005; Lankveld et al., 2010). A substance is considered to exert direct immunotoxicity when effects on organs or cells of the immune system and immune function are observed at doses without overt general toxicity (De Jong and van Loveren, 2007). *In vivo*, a substance may exert indirect immunotoxicity, other than general toxicity, if the immune system is influenced by other organs affected by the substance. In contrast, indirect effects other than general toxicity will not play a role in the *in vitro* situation.

Studies have been performed to establish *in vitro* assays able to predict *in vivo* effects of substances on the immune system (Carfi' et al., 2007; Galbiati et al., 2010; Hymery et al., 2006; Koeper and Vohr, 2009; Langezaal et al., 2001; Lankveld et al., 2010; Lebrec et al., 1995; Ringerike et al., 2005; Wagner et al., 2006). Assessment of cytokine release from leukocytes *in vitro* has been reported to be promising and is a frequently applied method to evaluate immunotoxic effects of substances (Gennari et al., 2005; House, 1999). However, a panel of cytokines may have to be analyzed to detect immunotoxicity since different substances act via different mechanisms (Ringerike et al., 2005).

The present study is part of the EU-funded project NewGeneris with the main aim to investigate if maternal exposure to dietary

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toxicants results in *in utero* exposure and in molecular events in the unborn child, leading to increased risks of cancer and immune disorders in childhood (Merlo et al., 2009). An intermediate aim of the workpackage is to investigate immunotoxic effects of dietary exposure and to develop biomarkers of immunotoxic risk based on both *in vitro* and *in vivo* studies. The purpose of the present *in vitro* study was to examine if the direct immunotoxic potential of 12 dietary toxicants, selected within the NewGeneris project, could be predicted from effects on mitogen-induced cytokine release after *in vitro* exposure of PBMC. Dietary toxicants from different chemical classes were chosen to include substances with different mechanisms of toxicity. Nine cytokines were selected to reflect different types of immune responses. Principal component analysis (PCA) was performed to examine the multivariate cytokine release data and is to our knowledge a novel approach to investigate cytokine release data. The *in vitro* results were compared with *in vivo*-based classification of the dietary toxicants as immunotoxic or non-immunotoxic substances.

2. Materials and methods

2.1. Blood donors and collection of blood samples

Venous blood was drawn from healthy non-smoking Caucasian males ($n = 25$) and females ($n = 35$) (20–35 years of age) into sodium heparinised vacutainers (Becton Dickinson, Plymouth, England) using butterfly blood collection sets (Becton Dickinson, Franklin Lakes, NJ, USA). "Healthy" was defined as absence of self-reported infections, chronic diseases like autoimmune disorders, or use of medication at the time of blood sampling. The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics, and all donors gave their written consent.

2.2. Isolation of peripheral blood mononuclear cells

Heparinised blood was diluted with an equal volume of physiological saline (B. Braun Melsungen AG, Melsungen, Germany). Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation, using Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in Leucosep filter tubes (Greiner Bio-One GmbH, Frickenhausen, Germany), at 1000*g* for 20 min at 18 °C. The interface layer consisting of mononuclear cells was collected. The mononuclear cells were washed once with physiological saline to remove remaining platelets, and twice with sterile phosphate buffered saline (PBS, 10 mM, pH 7.4, Norwegian Institute of Public Health, Oslo, Norway), being centrifuged at 350*g* for 10 min at 18 °C. The cells were resuspended in culture medium consisting of RPMI 1640 medium with L-glutamine, 10% heat-inactivated foetal calf serum (FCS) (Gibco, Paisley, Scotland), 100 U/ml penicillin G and 0.1 mg/ml streptomycin (PAA Laboratories GmbH, Pasching, Austria). The cells were counted under a microscope using a Bürker chamber and trypan blue staining of dead cells, and diluted to a final concentration of 2.4×10^6 viable cells/ml.

2.3. Chemical substances

The selected substances and abbreviations, their chemical class and immunotoxic potential according to published *in vivo* data, are shown in Table 1. The substances 2-amino-3-methyl-3H-imidazo(4,5-f)quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) were obtained from Toronto Research Chemicals Inc. (North York, Canada), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) from AccuStandard Inc. (New Haven, CT, USA), 4-hydroxy-2-nonenal (4-HNE) from Calbiochem (San Diego, CA, USA) and ethanol (EtOH) from Arcus (Oslo, Norway). The remaining substances were obtained from Sigma–Aldrich (St. Louis, MO, USA). Stock solutions of the different chemicals were prepared in dimethylsulfoxide (99.9% DMSO, Sigma–Aldrich) except for EtOH

Table 1
Overview of the 12 selected substances and their immunotoxic potential based on published *in vivo* data. (+) denotes immunotoxic substances and (−) denotes non-immunotoxic substances.

Substance	Chemical class	Immunotoxic potential	References
BaP – benzo[a]pyrene CAS no. 50-32-8	Polycyclic aromatic hydrocarbons	+	Ladics and White (1996) and De Jong et al. (1999)
EtOH – ethanol CAS no. 64-17-5	Alcohols	+	Szabo (1997) and Szabo and Mandrekar (2009)
4-HNE – 4-hydroxynonenal CAS no. 75899-68-2	DNA reactive aldehydes	+	Wang et al. (2008) and Kim et al. (2009)
MDA – tetramethoxypropane CAS no. 102-52-3	DNA reactive aldehydes	+	Tuma (2002) and Wang et al. (2008)
PCB – polychlorinated biphenyl 153 CAS no. 35065-27-1	Organochlorines	+	Smialowicz et al. (1997), Weisglas-Kuperus et al. (2000), Lyche et al. (2004), and Heilmann et al. (2006)
TCDD – 2,3,7,8-tetrachlorodibenzo-*p*-dioxin CAS no. 1746-01-6	Organochlorines	+	Holsapple et al. (1991), De Heer et al. (1994), Kerkvliet (2002), and Quintana et al. (2008)
AFB1 – aflatoxin B1 CAS no. 1162-65-8	Mycotoxins	+	Hinton et al. (2003) and Meissonnier et al. (2008)
DON – deoxynivalenol CAS no. 51481-10-8	Mycotoxins	+	Tryphonas et al. (1986), Robbana-Barnat et al. (1988), Rotter et al. (1996), and Pinton et al. (2008)
DMNA – dimethylnitrosamine CAS no. 62-75-9	Nitrosamines	−	
AA – monoacrylamide CAS no. 79-06-1	Acrylamides	−	
PhIP – 2-amino-1-methyl-6 phenylimidazo-[4,5-b]pyridine CAS no. 105650-23-5	Heterocyclic amines	−	
IQ – 2-amino-3-methylimidazo-[4,5-f]quinoline CAS no. 76180-96-6	Heterocyclic amines	−	