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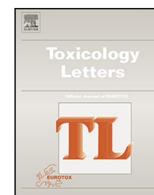

Characterization of human lymphoblastoid cell lines as a novel *in vitro* test system to predict the immunotoxicity of xenobiotics

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HIGHLIGHTS

- Human LCLs provide a novel *in vitro* method for evaluation of immunotoxicity.
- Immunomodulatory compounds exerted significant changes in IL-6 and IFNγ expression.
- IC_{50} values determined on LCLs and PBMCs were comparable.
- Human LCLs reflect inter-individual variability in response to xenobiotic.

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ABSTRACT

Evaluating immunomodulatory effects of xenobiotics is an important component of the toxicity studies. Herein we report on the establishment of a novel *in vitro* test system for the immunotoxicity screening of xenobiotics based on human lymphoblastoid cell lines (LCLs). Four immunotoxic compounds; tributyltin chloride, cyclosporine A, benzo(a)pyrene and verapamil hydrochloride, as well as three immune-inert compounds; urethane, furosemide and mannitol were selected for characterization. The treatment of LCLs with immunosuppressive compounds resulted in reduced viability. The IC_{50} values determined in human LCLs were in agreement with the data obtained for human peripheral mononuclear cells. Since cytokine production reflects lymphocytes responses to external stimuli, we evaluated the functional responses of LCLs by monitoring their pro-inflammatory and immunoregulatory cytokine production. Our findings prove that LCLs allowed for reliable differentiation between immunomodulatory and immune-inert compounds. Hence, pre-treatment with immunomodulatory compounds led to a decrease in the production of pro-inflammatory TNFα, IL-6 and immunoregulatory IL-2, IL-4, IL-10 and IFNγ cytokines, when compared to untreated ionomycin/PMA stimulated cells. Moreover, testing a panel of ten LCLs derived from unrelated healthy individuals reflects inter-individual variability in response to immunomodulatory xenobiotics. In conclusion, LCLs provide a novel alternative method for the testing of the immunotoxic effects of xenobiotics.

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1. Introduction

The immune system is an important and vulnerable target for xenobiotics. At present, the assessment of immunotoxicity depends on animal studies despite the substantial differences between species and species-specific effects in immune responses (Descotes, 2004; Haley, 2003; Hartung and Corsini, 2013). In line with European Union regulations, including REACH (Registration, evaluation, authorisation and restriction of chemical substances) (European Commission, 2006), the importance of immunotoxicological studies in determining the potential adverse effects of chemicals is increasing and the development and use of alternative methods is being increasingly endorsed (Galbiati et al., 2010). Until now, no *in vitro* method for the prediction of immunotoxicity has been fully validated or accepted by regulatory authorities and *in vitro* alternatives for the identification of immunotoxicity are only available for research (Fry et al., 2008; Galbiati et al., 2010;

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Hartung and Corsini, 2013). Moreover, little has changed in the sphere of immunotoxicity practices for regulatory purposes in recent years, especially with respect to immunosuppression (Hartung and Corsini, 2013). Therefore, alternative methods for the prediction of the immunotoxic properties of compounds are highly desirable.

Human LCLs are emerging as a novel tool for predicting drug responses, adverse drug reactions and for addressing inter-individual variability in drug responses (Lock et al., 2012; Morag et al., 2010, 2013). LCLs are generated from peripheral blood lymphocytes by Epstein–Barr virus (EBV) transformation. The cells retain most of the phenotypic properties of B-lymphocytes, including the expression of surface markers CD19 and CD20, as well as the production of antibodies (Hussain and Mulherkar, 2012; Morag et al., 2010). Their nuclear DNA remains intact after immortalization; therefore, LCLs are a preferred method of storing an individual's genetic material in biobanking (Sie et al., 2009).

The advantages of LCLs as an *in vitro* model have been proven by numerous studies. Firstly, controlled drug responses can be monitored without being confounded due to concomitant medication, as is often the case *in vivo* (Brown et al., 2014). Moreover, they constitute an excellent tool for the assessment of shared drug pathways and, thus, for the categorization of the responses of cells as a function of the mechanism of action (Morag et al., 2010). Furthermore, they can be implemented in a promising approach in pharmacogenomic research, wherein they are used to identify genes with clinical importance in drug responses, consequently facilitating the classification of new chemical entities into specific drug families (Brown et al., 2014; Morag et al., 2010).

The need to develop a novel *in vitro* method for the evaluation of immunotoxicity led us to consider LCLs as a promising tool for assessing immunotoxicity in a population-related manner. Well-characterized immunomodulators and immune-inert substances were selected for the study. Cyclosporine A, a calcineurin inhibitor, is an immunosuppressant used in therapies for the prevention of organ graft rejection (Heidt et al., 2008; House, 1999); verapamil hydrochloride, a calcium channel blocker, is used in hypertension therapy and arrhythmias (Berrebi et al., 1994); benzo(a)pyrene, a polycyclic aromatic hydrocarbon, is found in exhaust fumes, cigarette smoke and environmental contaminants (Carfi et al., 2007; Lu et al., 2009), and organotin compound tributyltin chloride, which was widely used and still is in some parts of the world as a marine antifouling agent, agricultural pesticide and plastic stabilizer (Carfi et al., 2010; Kotake, 2012). All four compounds were confirmed as immunotoxic in several *in vivo* and *in vitro* studies and, moreover, were found to exert immunosuppressive effects on B-lymphocytes. The immune-inert compounds selected were urethane, furosemide and mannitol. Urethane is an intermediate for cosmetics and pharmaceuticals; mannitol is used in pharmaceutical formulations and food products as a tablet diluent and sweetener, respectively, while furosemide is a diuretic drug, prescribed for the treatment of oedema, with no impact on the immune system (Carfi et al., 2007).

In the search for a novel *in vitro* tool to assess immunotoxicity we characterized a panel of LCLs from ten unrelated donors based on their ability to predict the impact of xenobiotics on the viability and function of the immune cells. Herein, we report on human LCLs as a reliable *in vitro* test system for assessing immunotoxicity, capable of differentiating between immunotoxic and immune-inert substances. We also provide additional evidence that the use of a panel of ten LCLs derived from unrelated healthy individuals may offer an insight into variability in inter-individual response to a given compound of interest. An *in vitro* test system using LCLs could, therefore, be employed in future screening of chemical libraries and in the evaluation of the immunotoxicity of xenobiotics.

2. Materials and methods

2.1. Chemicals

Tributyltin chloride (96%) (Sigma–Aldrich), cyclosporine A (Sequoia Research Products Ltd., Pangbourne, United Kingdom) and furosemide (Sigma–Aldrich) were dissolved in ethanol. Verapamil (Fluka, Deisenhofen, Germany), urethane (Sigma–Aldrich) and mannitol (>98%) (Sigma–Aldrich) were dissolved in sterile water. Benzo(a)pyrene (Sigma–Aldrich) was dissolved in DMSO.

2.2. Cell culture

2.2.1. Lymphoblastoid cell lines

Human LCLs were obtained from the National Laboratory for the Genetics of Israeli Populations (NLGIP), a human diversity biobank at Tel-Aviv University, Tel Aviv, Israel. The cell lines in the NLGIP collection were generated from fresh lymphocytes isolated from blood samples donated for this purpose by consenting healthy adults. Age health status and ethnicity were self-reported by the donors. All cell lines were prepared with EBV generated from the same source of B-95-8 marmoset cell line as described (Morag et al., 2010, 2013). LCLs were cultured in an RPMI 1640 medium (Sigma–Aldrich) supplemented with 10% foetal bovine serum (Gibco, Grand Island/NY, USA), 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Sigma–Aldrich). Cells were maintained at a concentration of between 1×10^6 and 2×10^6 cells/ml and cultured in a humidified chamber at 37 °C and 5% CO_2.

2.2.2. Peripheral blood mononuclear cells

The experiments were conducted on freshly isolated primary PBMCs. Buffy coats from healthy volunteers were obtained by the Blood Transfusion Centre of Slovenia, in accordance with the institutional guidelines. The cells were isolated by density gradient centrifugation with Ficoll-Paque (Pharmacia, Sweden) and cultured in an RPMI 1640 medium (Sigma–Aldrich, St. Luis/MO, USA) supplemented with 10% foetal bovine serum (Gibco, Grand Island/NY, USA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol (all from Sigma–Aldrich). Cells were cultured in a humidified chamber at 37 °C and 5% CO_2.

2.3. Viability assay

Prior to each experiment, cells were counted and diluted to the respective concentrations of 1.0×10^6 cells/ml for PBMCs and 3.0×10^5 cells/ml for LCL cells. The viability of cells was assessed by means of tetrazolium MTS assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cells were treated with appropriate amounts of compounds of interest or corresponding vehicle (vehicle control). Assays were performed in triplicate in 96-well plates. Absorbance was measured at 492 nm on an automated microplate reader. Relative cell viability of treated cells was calculated by subtracting the negative control, which contained no cells, and normalizing to vehicle-treated controls.

2.4. Cytokine assay

Cells were plated (1.0×10^6 cells/ml) and pre-treated for 1 h with the compound of interest. Next, LCL cells were activated by 0.5 μM ionomycin and 3.33 ng/ml PMA and incubated for 24 h. The resting, untreated cells or the ionomycin/PMA stimulated cells were used as controls. The cell-free supernatants were obtained by centrifugation at 1200 rpm for 5 min and stored at −78 °C. Cytokine

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