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Review

"Fluorescent Cell Chip" for immunotoxicity testing: Development of the c-*fos* expression reporter cell lines

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Abstract

The Fluorescent Cell Chip for in vitro immunotoxicity testing employs cell lines derived from lymphocytes, mast cells, and monocytes–macrophages transfected with various EGFP cytokine reporter gene constructs. While cytokine expression is a valid endpoint for in vitro immunotoxicity screening, additional marker for the immediate-early response gene expression level could be of interest for further development and refinement of the Fluorescent Cell Chip. We have used BW.5147.3 murine thymoma transfected with c-*fos* reporter constructs to obtain reporter cell lines expressing ECFP under the control of murine c-*fos* promoter. These cells upon serum withdrawal and readdition and incubation with heavy metal compounds showed paralleled induction of c-Fos expression as evidenced by Real-Time PCR and ECFP fluorescence as evidenced by computer-supported fluorescence microscopy. In conclusion, we developed fluorescent reporter cell lines that could be employed in a simple and time-efficient screening assay for possible action of chemicals on c-Fos expression in lymphocytes. The evaluation of usefulness of these cells for the Fluorescent Cell Chip-based detection of immunotoxicity will require additional testing with a larger number of chemicals.
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Keywords: Immunotoxicity; Toxicity; c-Fos; EGFP; ECFP; In vitro; T cells; Cell chip; Heavy metal ions

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Introduction

There is a growing need to establish new in vitro alternatives for animal-based tests suitable for safety evaluation of industrial compounds (Hartung et al., 2003). This requirement could be exemplified by the fact that, in the near future, 30,000 compounds have to be tested for

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possible toxicity in Europe to meet the new standards of chemical safety (Hartung et al., 2003). New in vitro techniques that allow for high-throughput screening should increase the speed and reduce the cost per chemical entity for safety evaluation. At the same time, usage of such techniques in routine toxicity testing follows the three-R principle: to reduce, refine, and replace the use of laboratory animals.

While general toxicity could be assessed in vitro by determination of cell death observed upon exposure to tested substances, the more subtle forms of toxic activities of chemicals such as neurotoxicity or immunotoxicity would require different endpoints for in vitro assays. For example, in in vitro immunotoxicity testing, immunosuppressive activity is expected when proliferative response or physiological functions such as NK (natural killer) activity of immune cells are downregulated, and immunostimulation is expected when upregulation of these responses is detected (Exon et al., 1986). It might be expected that such complex changes in cell function are associated with changes in gene expression and the effects of tested compound on gene expression in proper cell lineage could be a good endpoint for toxicity testing in vitro. For example, certain types of immunotoxicity are associated with modulation of cytokine gene expression in immune or non-immune cells. Thus, heavy metal-mediated upregulation of interleukin-4 (IL-4) expression in lymphocytes and mast cells (Oliveira et al., 1995; Dastych et al., 1999) and amino acid derivative-mediated upregulation of IL-5 expression in lymphocytes (Yamaoka et al., 1994) are linked with immunotoxic effect of these compounds observed in vivo. It has also been reported that chemical allergens stimulate expression of chemokines in keratinocytes (Enk and Katz, 1992; Corsini et al., 1998). On the other hand, inhibition of cytokine expression is a mechanism of action of several immunosuppressants including cyclosporin A, FK 506, and azathioprine. (O'Keefe et al., 1992). Thus, assessment of cytokine expression could be employed to evaluate chemical exposure effects on the immune system in vitro (Corsini et al., 1998; Vandebriel et al., 1999). Stimulation and inhibition of gene expression could be efficiently measured in vitro by using a reporter construct, consisting of the regulatory region(s) such as promoter of a certain gene fused to a promoterless reporter gene. Usage of fluorescent proteins such as EGFP (enhanced green fluorescent protein) as reporter genes allows for very efficient measurements of the amount of translated reporter gene product in living cells. At the same time, instrumentation is already available for fluorescence-based high-throughput screening platform to screen for activities of a high number of tested compounds. Therefore, we have decided to explore the idea that the measurements of cytokine expression in cells exposed to tested substance in vitro could provide a good indication of possible immunotoxicity for development of a new system for in vitro immunotoxicity testing. The rationale and a proof-of-principle report for this technique have been laid down elsewhere (Ulleras et al., 2005). Briefly, a range of cell lines derived from lymphocytes, mast cells, and monocytes–macrophages were transfected with EGFP reporter DNA constructs, which contained 5′-upstream and 3′-downstream sequences controlling expression of IL-2, IFNγ (interferon-γ), IL-4, IL-10, TNF-α (tumor necrosis factor-α), or IL-1β. The critical feature expected from a resultant reporter cell line was EGFP expression closely mimicking expression of endogenous gene. Multiple cell lines obtained by stable transfection of immortalized cell lines with cytokine reporter gene constructs have met such criteria. In the next step of the development of the Fluorescent Cell Chip, we employed the panel of EL-4-derived reporter cell lines containing transgenes coding for four different cytokine reporter genes (IL-2, IL-4, IFN-γ, and IL-10) and reference reporter cell line containing transgene for β-*actin* reporter gene and the responses of this cell chip to selected chemicals were determined. Resultant data (Ringerike et al., 2005) showed statistically significant decrease in EGFP fluorescence in all activated cytokine reporter cell lines exposed to immunosuppressive compounds cyclosporin A and pentamidine. Another immunosuppressant rapamycine inhibited EGFP expression in IL-2, IL-4, and IL-10 reporters. Sensitizing compound benzocaine inhibited EGFP expression in activated IL-2, IFN-γ, and IL-10 but not IL-4 reporter cells while another sensitizer TDI (toluene diisocyanate) selectively upregulated EGFP expression in IL-4 reporter cells. Exposure to *Der p*-dust mite allergen has also resulted in increased EGFP fluorescence in IL-4 reporter cell line. Thus, immunosuppressive substances known to inhibit functions of immune cells in vitro and in vivo specifically and in a dose-dependent manner diminished fluorescence of cytokine reporter cells. Two of four tested allergens, namely, *Der p*-mite and TDI, generated specific pattern of changes in fluorescence level reflecting the immunomodulatory properties of these substances observed in vivo.

While cytokine expression seems to be a good endpoint for in vitro immunotoxicity testing, the expression of the immediate-early response genes is another indicator that could be employed for further development and refinement of the Fluorescent Cell Chip. These genes are known to be upregulated in eukaryotic cells exposed to different stressors and expression of these genes is important for cell survival or induction of apoptosis (Cochran, 1993). The c-*fos* proto-oncogene is a prototypic immediate-early response gene that encodes a DNA-binding protein (c-Fos) functioning as a component of the transcriptional factor AP-1. Its expression in mammalian cells is rapidly upregulated by a variety of stimuli such as heat shock (Andrews et al., 1987), UVB (Chen et al., 1998), serum, growth factors (Muller et al., 1984), cAMP (Tramontano et al., 1986), calcium ionophore or PMA (phorbol 12-myristate-13-acetate) (Moore et al., 1986), hypoxia, oxidative stress, or inflammation (Maki et al., 1992; Bernuau et al., 1993; Webster et al., 1993; Taniguchi et al., 1994; Bellavance and Beitz, 1996).

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