Considerations regarding use of solvents in in vitro cell based assays

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Abstract Cell culture systems are widely used for the investigation of in vitro immunomodulatory effects of medicines and natural products. Since many pharmacological relevant compounds are water-insoluble, solvents are frequently used in cell based assays. Although many reports describe the cellular effects of solvents at high concentrations, only a few relate the effects of solvents used at low concentrations. In this report we investigate the interference of three commonly used solvents: Dimethyl sulfoxide (DMSO), ethanol and β -cyclodextrin with five different cell culture systems. The effects of the solvents are investigated in relation to the cellular production of interleukin (IL)-6 or reactive oxygen species

(ROS) after lipopolysaccharide (LPS) stimulation. We show that DMSO above 1 % reduces readout parameters in all cell types but more interestingly the 0.25 and 0.5 % solutions induce inhibitory effects in some cell types and stimulatory effects in others. We also found that LPS induced ROS production was more affected than the IL-6 production in the presence of ethanol. Finally we showed that β -cyclodextrin at the investigated concentrations did not have any effect on the LPS induced IL-6 production and only minor effects on the ROS production. We conclude that the effects induced by solvents even at low concentrations are highly relevant for the interpretation of immunomodulatory effects evaluated in cell assays. Furthermore, these results show the importance of keeping solvent concentrations constant in serial dilution of any compound investigated in cell based assays.

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Introduction

Cell culture systems incorporating cell lines or primary cells of human or murine origin are widely used for the investigation of in vitro immunomodulatory effects of medicines and natural products. These systems provide the possibility to investigate the influence of novel compounds on cellular functions [e.g. cytokine excretion, production of reactive

oxygen species (ROS), gene regulation and protein expression] with the complexity of functionally and structurally intact cells.

Predominantly, experiments with cell culture systems are performed in buffered saline or in growth medium. Therefore, water-soluble samples do not represent a problem with regards to solubility in the growth medium, while water-insoluble samples often require the inclusion of an extraneous solvent or additive to facilitate solubilization. Introduction of an extraneous solvent or additive to the growth medium will however alter the cellular environment and may therefore affect the outcome of the experiment.

Most papers that present results from cell culture experiments also provide data on the possible toxic effects of the used solvent. However, despite the fact that several authors report interactions of frequently used solvents with cellular based assays, it is seldom described how the applied concentrations of the solvents affect the actual readout.

In this report we investigate the interference of three commonly used solvents: Dimethyl sulfoxide (DMSO), β -cyclodextrin and ethanol with five different cell culture systems: peripheral blood mononuclear cells (PBMC), peripheral leukocytes, the human monocytic cell line Mono Mac 6 (MM6), the murine monocytic cell line RAW 264.7 and the HL-60 cell line differentiated to a neutrophil like cell type. The immunomodulatory effects induced by the solvents are investigated by stimulating the cells with lipopolysaccharide (LPS) and comparing the cellular responses from cells incubated in the absence of solvents to the response from cells incubated with increasing concentrations of solvents. The effects induced by the three solvents on the response were assessed using either production of interleukin (IL)-6 or ROS as readout parameters. The ROS production was measured by luminol enhanced chemiluminescence and the cytokine production was quantified using a time resolved dissociation-enhanced lanthanide fluoro immunoassay (DELFI A).

Materials and methods

Reagents

LPS from *Escherichia coli* 055:B5, 263 pg/EU (Lonza, Visp, Switzerland) was dissolved in pyrogen free water

and diluted with Hanks balanced salt solution with calcium and magnesium (HBSS; Gibco BRL—Life Technologies, Carlsbad, CA, USA). DMSO hybri-max (Sigma Chemical Co., St. Louis, MO, USA), ethanol (Kemetyl A/S, Koege, Denmark) and β -cyclodextrin (Sigma Chemical Co.) were likewise diluted with HBSS. Luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione) (Across Organics—Fisher Scientific, Pittsburgh, PA, USA) was dissolved in HBSS during constant stirring for 24 h and used at a final concentration of 283 μ M. Human plasma was isolated from venous blood of a healthy volunteer collected in heparinised anticoagulation vacutainers (BD, Franklin Lakes, NJ, USA). Plasma was obtained by centrifugation of whole blood (1,000 $\times$ g, 20 min) and stored at -80 °C until use. All buffers and reagents were purchased with a certificate of low endotoxin content or tested negative in our kinetic-turbidimetric LAL assay (detection limit < 0.01 EU/ml).

Experimental setup

Experiments were conducted with cultured cells or cells isolated from healthy volunteers. For the monocytic cells, cell suspensions were incubated with solvents at various concentrations and with LPS (0.5 EU/ml) for 20 h. The produced cytokines were quantified by DELFIA and compared to the control stimulation where no solvents were added. For the ROS measurements we either differentiated HL-60 cells with all-trans retinoic-acid (ATRA) to obtain cells with neutrophilic characteristics or used the leukocyte fragment of freshly drawn whole blood. To measure the immunomodulatory effect of the solvents, cells were subjected to increasing concentrations of the solvents and stimulated with LPS. The production of ROS was measured by luminol enhanced chemiluminescence and the results quantified as the area under the curve (AUC) from 0 to 180 min.

Cells were stimulated with solvents at the following concentrations: DMSO: 0.25, 0.5, 1, 2 and 4 %, Ethanol: 0.01, 0.05, 0.1, 1 and 5 %, β -cyclodextrin: 0.1, 1, 10, 50 and 100 μ g/ml.

Cell cultivation and isolation

Mono Mac 6

The human monocytic cell line Mono Mac 6 was acquired from DSMZ (Braunschweig, Germany)

(ACC 124). The cells were cultured in pyrogen free RPMI 1640 (Gibco BRL) with 5 % heat inactivated fetal bovine serum (Biological industries, Kibbutz Beit Haemek, Israel), 200 U/ml penicillin, 200 µg/ml streptomycin (Sigma-Aldrich), 1 % glutamax, 1 % non essential amino acids (Gibco BRL, USA) and 1 % sodium pyruvate (Gibco BRL). The cells were incubated in humidified atmosphere containing 5 % CO₂ and 95 % air at 37 °C. The medium was replaced twice weekly and the cells were seeded at 2×10^5 cells/ml.

HL-60

The HL-60 cell line was acquired from ATCC (Manassas, VA, USA) (CCL-240) and cultured in growth medium consisting of RPMI 1640 (Biological industries) supplemented with 10 % heat inactivated fetal bovine serum (Biological industries), 1 % glutamax (Gibco BRL), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). The cells were incubated in humidified atmosphere containing 5 % CO₂, 95 % air at 37 °C and the culture medium was replaced twice a week. The cells were seeded at 1×10^5 cells/ml and the concentration was at all times kept below 1×10^6 cells/ml. The cells were used for a maximum of 25 passages after acquisition from ATCC. From the continuously growing cell culture, cells were collected for differentiation. To induce differentiation along the granulocytic pathway, cells were seeded at 3×10^5 cells/ml in RPMI 1640 supplemented with 1 µM all-trans retinoic-acid (ATRA; Fluka-Sigma-Aldrich). Cells were allowed to differentiate for 7 days without further renewal of culture medium or cell passage.

RAW 264.7

The RAW 264.7 cells ECACC (Salisbury, U.K.) (91062702) were cultivated in DMEM (D5546, Sigma) supplemented with 10 % heat inactivated fetal bovine serum (Biological industries), 1 % glutamax (Gibco BRL), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). The cells are semi-adherent and were subcultivated before they reached confluency. With a cell-scraper the cells were loosened from the culture flask and subcultivated at a concentration of 2×10^5 cells/ml.

Peripheral blood mononuclear cell (PBMC)

Peripheral blood mononuclear cells were isolated by Ficoll separation. In short, whole blood was diluted 1:1 with 0.9 % pyrogen free saline. Ficoll-paque (3 ml) was transferred to a 11 ml centrifuge tube and 4 ml of whole blood was layered on top of the Ficoll-paque. After centrifugation ($400 \times g$, 40 min) the plasma layer was removed and the lymphocytes were isolated and washed twice with saline before they were resuspended in pyrogen free RPMI 1640 (Gibco BRL) supplemented with 5 % heat inactivated fetal bovine serum (Biological industries), 200 U/ml penicillin, 200 µg/ml streptomycin (Sigma-Aldrich), 1 % glutamax, 1 % non essential amino acids (Gibco BRL) and 1 % sodium pyruvate (Gibco BRL) and standardised.

Leukocytes

Leukocytes were isolated from human whole blood after lysis of red blood cells and centrifugation. The red blood cell lysis buffer comprised of 0.826 g NH₄Cl, 0.1 g KHCO₃, and 0.0037 g EDTA. The reagents were dissolved in 100 ml pyrogen free water and filtrated through an Ultrasart D20 ultrafilter (Sartorius, Göttingen, Germany). For the isolation procedure 2.5 ml whole blood and 7.5 ml red blood cell lysis buffer was inserted carefully in a 11 ml centrifuge tube for 2 min. The cell suspension was then centrifuged ($125 \times g$, 10 min) and the pellet resuspended in 5 ml red blood cell lysis buffer. The centrifugation was repeated and the pellet was washed twice in prewarmed (37 °C) HBSS before the stock solution was standardised to 1×10^7 cells/ml.

Stimulation of cells

Mono Mac 6 cells were seeded at 4×10^5 cells/ml and incubated overnight in supplemented RPMI 1640 medium. The cells were then centrifuged ($125 \times g$, 5 min), resuspended in supplemented RPMI 1640 medium and standardised to 2×10^6 cells/ml. Equal volumes of test solution (solvent diluted with HBSS and 50 pg/ml LPS) and suspension (250 µl) were gently mixed and incubated for 20 h. The culture was then centrifuged ($125 \times g$, 10 min), the supernatants were isolated and the content of IL-6 determined.

RAW 264.7 cells were seeded at 1×10^6 cells/ml and incubated overnight in supplemented RPMI 1640

medium. The supernatants were then discarded and the cells stimulated with 250 μ l test solution (solvent diluted with HBSS and 50 pg/ml LPS) and 250 μ l supplemented RPMI 1640 medium. After 20 h the supernatants were isolated and the content of IL-6 determined.

PBMCs were standardised to 2×10^6 cells/ml. Equal volumes of test solution (solvent diluted with HBSS and 50 pg/ml LPS) and cell suspension (250 μ l) were gently mixed and incubated for 20 h. The cell culture was then centrifuged ($125 \times g$, 10 min), the supernatants were isolated and the content of IL-6 determined.

Leukocytes and differentiated HL-60 cells were standardised to 1×10^7 cells/ml and used for ROS experiments as described below.

Quantification of interleukin from PBMC and monocytic cell lines

Interleukin-6 determination

The IL-6 concentration was determined using DELFIA. The assay was conducted as described previously (Moesby et al. 2008). Linearity between fluorescence and the IL-6 concentration was observed within the concentration range 5–4,000 pg/ml. Each standard and test was analyzed in triplicate. For the murine cell line RAW 264.7 the same procedure was applied for the determination of murine derived IL-6 using monoclonal anti-mouse IL-6 capture antibody (2 μ g/ml, 100 μ l/well; MAB406, R&D Systems, Abingdon, UK), standards prepared from recombinant murine IL-6 (406-ML-005, R&D Systems) and biotinylated polyclonal rat anti-murine IL-6 antibody (100 ng/ml; 100 μ l/well; BAF406, R&D Systems).

Quantification of ROS from isolated leukocytes and HL-60 cells

Production of ROS from isolated leukocytes and HL-60 cells was determined by luminol enhanced chemiluminescence (Timm et al. 2009). In short, the differentiated HL-60 cells were washed once in prewarmed (37 °C) HBSS, resuspended and standardised to 1×10^7 cells/ml and cells were used immediately hereafter. Isolated leukocytes were used within 30 min after the isolation procedure described above. Prior to measurements a reaction mixture was prepared

in a white polystyrene LumiNunc™96-Well Plate (Nunc, Roskilde, Denmark), each well containing a final concentration of 5×10^5 differentiated HL-60 cells or leukocytes, 283 μ M luminol and 2.5 % human plasma. The wells were supplemented with HBSS to a volume of 100 μ l and allowed to equilibrate for 15 min at 37 °C prior to addition of 50 μ l of LPS (100 pg/ml) and 50 μ l of solvent or control solution (HBSS). The microplate was read as follows: 1 s/well (every second minute) for 180 min in an ORION II Microplate luminometer (Berthold Detection Systems, Pforzheim, Germany) at 37 °C. The HL-60 cells and isolated leukocytes react to stimulation by releasing reactive oxygen species (ROS) quantified by luminol enhanced chemiluminescence. The light emitted from the reaction was assessed as relative luminescence units per second (RLU/s). The results were quantified as the area under the curve (AUC) of continuous measurements for 180 min.

Statistics

For evaluation of the effects of the solvents compared to the control without addition of solvent we used ANOVA unless otherwise stated. We considered $p \leq 0.05$ as significant.

Results

In the present study we examined the effects of solvents on stimulated cells in order to test the hypothesis whether solvents would either abrogate or enhance exogenous responses. To stimulate the cells we used LPS at a concentration that induces sub-maximal responses according to our standard curves for the five cell types (data not shown). We have displayed the results according to the solvent and normalised responses from all cell types to the activation induced by the LPS stimulation without any solvent present. Therefore the percentage displayed in Figs. 1, 2 and 3 corresponds to the activation induced or abrogated by the solvents for the various cell types.

Effects of DMSO

The effect of DMSO is recognisable for all cell types (Fig. 1). At 4 % DMSO the responses were reduced by

more than 90 % in all cellular systems tested. The cells most sensitive to DMSO were the isolated leukocytes and the RAW 264.7 cells. The ROS production of leukocyte was significantly affected by all concentrations of DMSO. The RAW 264.7 cells were also affected by DMSO concentrations above 0.5 % as evaluated by a ratio paired *t* test. However, only concentrations above 1 % resulted in significant differences in the IL-6 production based on the ANOVA test. For the PBMCs, HL-60 and the Mono Mac 6 cells the release of ROS or IL-6 was significantly reduced at concentrations above 1 %. For the Mono Mac 6 cells DMSO concentrations of 0.5 and 0.25 % significantly increased the IL-6 release. The same tendency was observed for the HL-60 cells where an increase in the ROS production could be recognised (*p* values of 0.0507 and 0.0533 for 0.5 and 0.25 %, respectively) when compared to control.

Effects of ethanol

Cellular sensitivity towards ethanol is more pronounced in the LPS induced ROS production of leukocytes and HL-60 cells than in the IL-6 production of the mononuclear cells (Fig. 2). At concentrations of up to 5 % ethanol the IL-6 release from isolated PBMC and Mono Mac 6 cells was not significantly

Fig. 1 Effects of DMSO. Influence of DMSO concentration (0.25–4 %) on the LPS induced ROS production in HL-60 cells (closed circles) and isolated leukocytes (closed triangles) and on the IL-6 production in isolated PBMCs (open squares), Mono Mac 6 cells (open triangles) and RAW 264.7 cells (open circles). All responses were normalized to the LPS induced responses of the respective cells incubated in HBSS in the absence of DMSO (=100 %). Results are shown as mean $\pm$ SEM (*n* = 3)

Fig. 2 Effects of ethanol. Influence of ethanol concentration (0.01–5 %) on the LPS induced ROS production in HL-60 cells (closed circles) and isolated leukocytes (closed triangles) and on the IL-6 production in isolated PBMCs (open squares), Mono Mac 6 cells (open triangles) and RAW 264.7 cells (open circles). All responses were normalized to the LPS induced responses of the respective cells incubated in HBSS in the absence of DMSO (=100 %). Results are shown as mean $\pm$ SEM (*n* = 3)

different from the control. In the murine monocytic cell line RAW 264.7 a tolerance was also observed up to 1 % ethanol but at 5 % the response was reduced by more than 95 % compared to the control. All concentrations of ethanol significantly reduced the ROS

Fig. 3 Effects of β -cyclodextrin. Influence of β -cyclodextrin concentration (0.1–100 μ g/ml) on the LPS induced ROS production in HL-60 cells (closed circles) and isolated leukocytes (closed triangles) and on the IL-6 production in isolated PBMCs (open squares), Mono Mac 6 cells (open triangles) and RAW 264.7 cells (open circles). All responses were normalized to the LPS induced responses of the respective cells incubated in HBSS in the absence of DMSO (=100 %). Results are shown as mean $\pm$ SEM (*n* = 3)

production of isolated Leukocytes and likewise the ROS production of HL-60 cells was significantly reduced at concentrations of 1 and 5 % of ethanol.

Effects of β -cyclodextrin

β -cyclodextrin at the investigated concentrations did not have any effect on the LPS induced IL-6 production from monocytoid cells (Fig. 3). For the HL-60 cells a significant activation by cyclodextrin was observed at concentrations of 1 and 10 $\mu\text{g/ml}$. In the isolated leukocytes a slight but significant reduction of the ROS production was observed at all concentrations of cyclodextrin.

Discussion

A common concern when introducing extraneous solvents into cellular systems is the general cytotoxic effect of the solvent. It is therefore common practice to test the viability of the treated cells in a MTT assay (Mosmann 1983) or by using the trypan blue dye exclusion test (Strober 2001). However, usually only the highest concentration of the solvent is tested and conclusions on cell toxicity are drawn on this basis. However, as the results presented here display, that this is not always sufficient since the solvents may influence the outcome of the experiment even though used at concentrations far below those that would induce cytotoxicity.

DMSO is commonly used as solvent for water-insoluble samples to be tested in bioassays including cellular based assays. In a study by Forman et al. DMSO was found to exhibit a cytotoxic effect on HeLa cells at concentrations above 2 % and an inhibitory effect on cell growth at concentrations below 1 % (Forman et al. 1999). In another study DMSO was found to reduce the amount of measured superoxide production from primary neutrophils, eosinophils lymphocytes and monocytes at concentrations above 1 % (Kahler 2000). Our findings partly support this since the cellular functions in regards to IL-6 and ROS production in Mono Mac 6 cells, HL-60 cells and PBMC was not significantly reduced by 1 % DMSO. Isolated leukocytes and RAW 264.7 cells were however both negatively affected at these concentrations. An interesting finding is that concentrations of 0.25 and 0.5 % DMSO exerted a

stimulatory effect on the responses from Mono Mac 6 cells. This tendency could also be observed using HL-60 cells. However the same concentrations of DMSO reduced the response from RAW 264.7 and isolated leukocytes (Fig. 1). Only the response from isolated PBMC's seemed to be unaffected at these concentrations of DMSO. This may be important since dilutions of samples containing a solvent are seldom considered problematic. Therefore, serial dilutions of a sample (without equimolar concentrations of DMSO in the diluents) may in fact elicit an immunomodulatory response which could falsely be concluded to originate from the sample tested and not due to alterations of the solvent concentration. Thus, 1 % DMSO seems to be a critical concentration since lower concentrations will result in increased cellular activation while higher concentrations will exert inhibitory effects. Our results seem to indicate that different cell types respond very differently to DMSO at various concentrations in connection to various stimuli; this also seems to be the case in other studies (Xing and Remick 2005; Hollebeeck et al. 2011).

Ethanol is often used as a solvent for plant extracts and hereof derived components for testing in bioassays (Zhijun 2008). There have been many studies indicating a general immunosuppressive effect of ethanol and other alcohols (Nair et al. 1990; Szabo 1999; Desy et al. 2010, 2008). Investigating the effects of ethanol on HeLa cells it was found that concentrations of 5 % and above compromised the cell viability (Forman et al. 1999). The influence of ethanol on cell viability of RAW 264.7 cells was evaluated by the trypan blue staining method and it was found that the cell viability was not affected at concentrations up to 600 mM (~ 2.8 %) (Wakabayashi and Negoro 2002). In the present study RAW 264.7 cells were found to tolerate concentrations of ethanol of 1 % while the response was greatly attenuated at 5 %. Interestingly, concentrations of ethanol up to 5 % did not affect the response of Mono Mac 6 cells. In contrast to this, all tested concentrations of ethanol (0.01–5 %) significantly reduced the response from isolated leukocytes (Fig. 2).

Cyclodextrins are cyclic oligosaccharides (mainly made up of six, seven or eight dextrose units; α , β and γ) which can form inclusion complexes with hydrophobic drug molecules and hereby enhance the aqueous solubility of these compounds (Stella and He 2008). Irie and Uekama have reviewed the cellular

interactions of cyclodextrins and found that they were able to induce shape changes of membrane invagination on human erythrocytes and at higher concentrations induce lysis (Irie and Uekama 1997). Furthermore, the hemolytic activity of cyclodextrins correlates with their ability to form inclusion complexes with cell membrane lipids which hereby are removed causing the cell membrane to become unstable and in turn cause cell lysis. The cytolytic effect of cyclodextrins have also been observed in other cells lines such as human skin fibroblasts, intestinal cells, and *E. coli*, suggesting that cyclodextrin-induced cell lysis is not cell type specific (Irie and Uekama 1997). Testing β -cyclodextrin at concentrations of 0.1–100 $\mu\text{g/ml}$ we observed that only the response from isolated leukocytes was significantly reduced. Contrary to this β -cyclodextrin at concentrations of 1–10 $\mu\text{g/ml}$ significantly augmented the response from HL-60 cells while no effect was observed for the response from isolated PBMC's, Mono Mac 6 and RAW 264.7 cells for the tested concentration range (Fig. 3).

In this study we observed a tolerance of the monocytic cells and cell lines towards ethanol. This tolerance is contradictory to our own and others' previous findings that reported great sensitivity of monocytic cells towards ethanol. One explanation is that the matrix affects the cellular tolerance. In our previous findings we have used pyrogen free water as matrix for studies of solvent interactions. However, in the experiments of the present study we have used a buffered salt solution (HBSS), and therefore suspect the matrix to be partly responsible for this increased tolerance in regards to previous findings. Control experiments showed that when using pyrogen free water as matrix the 5 % ethanol solution reduced the IL-6 production of the Mono Mac 6 cells (by approximately 90 %). The 1 % ethanol solution reduced the production by 25 % (data not shown) this pronounced reduction was however not observed in the HBSS matrix emphasising the relevance of the solvents used but also of the matrix.

Several compounds are known to scavenge reactive oxygen species. Consequently the observed effects of ethanol and DMSO on the isolated leukocytes and HL-60 cells may be due to enhanced scavenging of the solvents at high concentrations. However control experiments in HL-60 cells showed that the concentrations of DMSO and ethanol that inhibited the ROS

production also inhibited IL-8 production of HL-60 cells, suggesting a cellular effect of the solvents rather than a scavenging effect (data not shown).

Concluding remarks

The results reported here emphasize the importance of keeping equimolar solvent concentrations between test and control experiments even at very low concentrations. Furthermore we conclude that not only should cell based assays be validated with regards to cytotoxic effects of solvents used but also validated in relation to the effects of solvents on measurements of cellular responses as well as the matrix they are tested in. Therefore, when testing serial dilutions of novel compounds in cellular assays solvent concentrations should always remain the same even though the concentrations may not be cytotoxic. Furthermore it should be noted that results obtained with solvent interactions in one cell type cannot be transferred to other cell types.

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