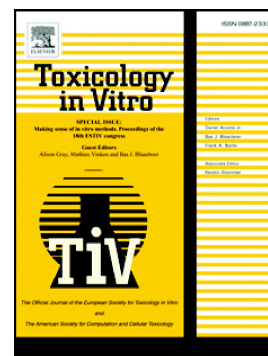


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ADAPTATION OF A SKIN SENSITIZATION ASSAY TO A CHEMICALLY DEFINED CULTURE

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ABSTRACT

There are currently three *in vitro* methods adopted by the Organization for the Economic Co-operation and Development for testing chemicals based on the third key event of the skin sensitization adverse outcome pathway, the activation of dendritic cells. All of them use culture medium supplemented with fetal bovine serum (FBS), which brings technical disadvantages and animal welfare concerns. The objective of this study was to analyze the possibility of eliminating the use of FBS in the human Cell Line Activation Test (h-CLAT). After successful implementation of the h-CLAT using THP-1 cells cultured in FBS-containing medium, several attempts to adapt THP-1 cells to four different serum-free media were made. The best results were obtained with gradual adaptation to RPMI-1640 medium with HL-1™ Supplement and to X-VIVO™ 10. Adapted cells were cryopreserved and submitted to the reactivity check. After being approved, they were used in dose finding and proficiency assays. Despite minor adjustments in the original protocol, it was possible to correctly predict the sensitizing potential of the ten proficiency substances using THP-1 cells adapted to X-VIVO™ 10, which indicates that it is possible to eliminate the use of FBS in the h-CLAT, using a chemically defined media.

Keywords: toxicity tests; animal use alternatives; culture media, serum-free; *in vitro* techniques; dermatitis, allergic contact.

Abbreviations used in the text:

UN GHS: United Nations Globally Harmonized System of Classification and Labelling of Chemicals; LLNA: Local Lymph Node Assay; AOP: adverse outcome pathway; OECD: Organization for Economic Co-operation and Development; h-CLAT: human-Cell Line Activation Test; FBS: fetal bovine serum; GLC: D-glucose; LAC: L-lactate; GLN: L-glutamine; GLU: L-glutamic acid; DT: doubling time; CV75: chemical concentration giving 75% cell viability; LA: lactic acid; DNCB: 2,4-dinitrochlorobenzene; NI: nickel sulfate.

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1. Introduction

Skin contact with xenobiotics — natural or synthetic chemicals that are foreign to our organism — can lead to allergic contact dermatitis, one of the most prevalent occupational skin diseases (Martin et al., 2011). According to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS), “a skin sensitizer is a substance that will lead to an allergic response following skin contact” (United Nations, 2013).

The prediction of skin sensitization has historically used animals, mainly guinea pigs, as in the Guinea Pig Maximization Test (Magnusson and Kligman, 1969) and the Buehler Test (Buehler, 1965). The Local Lymph Node Assay (LLNA) uses mice to analyze the proliferation of lymphocytes after chemical exposure, assessing the induction phase of skin sensitization (OECD, 2010a). LLNA and its non-radioactive variations (OECD, 2010b, 2010c) are considered alternative methods as they provide advantages in terms of reducing the number of animals used and improving animal welfare, thus applying two of the Three Rs, the concept of reduction, refinement and replacement of animals in scientific research (Russel and Burch, 1959). Ethical, social, and political concerns regarding the use of animals in the safety assessment of chemicals has fostered the development and validation of alternative methods that aim to replace them by *in vitro*, *in chemico* and/or *in silico* models. Due to the complex nature of skin sensitization, it is known that data from different non-animal methods should be combined to evaluate the safety of chemical compounds (Aeby et al., 2010).

Some mechanistically-based replacement methods have been recently proposed to identify the skin sensitization hazard potential, based on the key biological events occurring in the induction phase of sensitization, which can be summarized as an adverse outcome pathway (AOP). The skin sensitization AOP starts with the covalent binding of electrophilic substances to skin proteins (the first key event) as it penetrates the outer epidermis, forming hapten-protein complexes. The second key event occurs in keratinocytes and includes inflammatory responses and changes in gene expression in specific cell signaling pathways. The third key event is the activation of dendritic cells, which goes under a maturation process — including changes in specific cell surface markers, chemokines and cytokines — while migrating to proximal lymph nodes, where they present the hapten-protein complex to T-cells, leading to the fourth key event, the production of memory T-cells that will proliferate and act in the elicitation phase of allergic contact dermatitis (OECD, 2012a; Vocanson et al., 2009).

The Organization for Economic Co-operation and Development (OECD) applies the AOP for skin sensitization to develop integrated approaches for testing and assessment of chemicals, which incorporates different toxicological data into the decision-making process of hazard and risk assessment (OECD, 2012b). On October 2017, the OECD adopted the “Test guideline for the testing of chemicals based on key events – *in vitro* skin sensitization assays addressing the key event on activation of dendritic cells on the adverse outcome pathway for skin sensitization”(OECD, 2017), which comprises the following methods: U937 cell line activation Test (U-SENS™); interleukin-8 Reporter Gene Assay (IL-8 Luc Assay); and human Cell Line

Activation Test (h-CLAT). These methods, described in the OECD Test Guideline 442E, are different in terms of procedures and readouts, but can be used indiscriminately to support the *in vitro* identification of skin sensitizers, regarding the third key event of this AOP (OECD, 2017).

The target of the present study is the h-CLAT, an *in vitro* method designed for skin sensitization testing, based on the analysis of the biomarkers CD86 and CD54 following exposure to chemicals in THP-1 cells, a human monocytic cell line, (Ashikaga et al., 2006). According to the protocol, THP-1 cells are cultured in RPMI-1640 medium with 10% (v/v) fetal bovine serum (FBS) (Ashikaga et al., 2006).

FBS is a widely used animal-derived culture medium supplement that brings important animal welfare concerns and serious technical disadvantages (van der Valk et al., 2018). It is obtained from bovine fetuses during the slaughter of pregnant cows. The blood is usually harvested by cardiac puncture, without anesthesia, under vacuum. After being allowed to clot and then centrifuged, the serum is obtained. As the fetus is to be alive during harvesting, it may be subjected to discomfort, pain and suffering, therefore, this practice is considered inhumane (Jochems et al., 2002).

Despite providing vitamins, hormones, trace elements and growth factors, this ill-defined mixture can also be a source of endotoxins, mycoplasma, virus and prions. The scientific disadvantages include undesirable interactions with test substances and batch-to-batch variability due to differences in components concentrations and biological activities, which can lead to intra and inter-laboratory discrepancies (Brunner et al., 2010; Hawkes, 2015; van der Valk et al., 2018; Wessman and Levings, 1999).

The limited availability of this renewable commodity and its high cost are also important drawbacks (Brindley et al., 2012).

On account of relevant moral concerns, several scientific and technical problems regarding the use of FBS in *in vitro* methods, particularly in those aimed at replacing animals in safety assessment, it is urgent that FBS be replaced by synthetic alternatives in existing methods and avoided in the alternative methods to animal testing that are under development and the ones to be developed in the future.

A series of workshops have been organized to bring awareness to this subject, to propose procedures regarding animal welfare and to discuss alternatives to reduce or replace the use of FBS, including strategies to develop serum-free media and the establishment of serum-free databases (van der Valk et al., 2018). In 2008, the Scientific Advisory Committee of the European Centre for the Validation of Alternative Methods (ECVAM) recommended the use of non-animal serum substitutes of FBS and other animal-derived supplements, whenever possible, and informed that where the replacement is not met, a justification must be provided (ESAC, 2008).

Recent pressures from legislation and consumers have driven the development of alternative methods to determine the skin sensitization of chemicals. More recently, cosmetic industries have shown preference for methods that are totally animal-free (Belot et al., 2017), which includes supplements used in cell culture, such as FBS.

Considering the skin sensitization AOP, at least one fully animal-free method for the first key event is available: the Direct Peptide Reactivity Assay (DPRA), an *in chemico* method that quantifies the reactivity of chemicals

towards synthetic peptides (OECD, 2015). For the second key event, it has recently been shown that the validated *in vitro* assay KeratinoSens™ can be modified to a fully human *in vitro* test by replacing bovine serum and porcine trypsin by human serum and a recombinant animal-free equivalent, respectively (Belot et al., 2017). For the third key event, the three methods included in the OECD Test Guideline 442E use at least one animal-derived product. In the U-SENS™, FBS is used in cell culturing and sample preparation for flow cytometry. The IL-8 Luc Assay uses a THP-1 derived cell line cultured in medium containing 10% FBS, although test chemicals are dissolved in a serum-free medium. As mentioned before, in the h-CLAT, THP-1 cells are cultured in RPMI-1640 medium containing 10% FBS; and PBS with 0.1% bovine serum albumin is used in cell staining for flow cytometry (OECD, 2017). Both U-SENS™ and h-CLAT use antibodies, whose production may or may not use animals or animal-derived products (Gruber and Hartung, 2004). Up to now, there is no OECD accepted method to assess the fourth key event of the skin sensitization AOP (van Vliet et al., 2018).

In order to have fully animal-free tests for the skin sensitization AOP, one strategy is to replace animal-derived products by synthetic ones in the OECD accepted methods for this endpoint. Therefore, the objective of this study was to analyze the possibility of eliminating the use of FBS in the culture of THP-1 cells for the h-CLAT.

2. Material and methods

2.1. Cells and culture conditions with fetal bovine serum

THP-1 cells (ATCC® TIB-202™) were obtained from the *Banco de Células do Rio de Janeiro* (BCRJ, Brazil) and cultured in RPMI-1640 medium

(BioWhittaker®, Lonza, USA; or Gibco®, Life Technologies™, Inc.) with 10% (v/v) of FBS (Gibco®, ThermoFisher Scientific, Inc.) and 0.05 mM 2-mercaptoethanol (Gibco™, ThermoFisher Scientific, Inc.), here called RPMI10%FBS. Cells were cultured in a humidified 5% CO₂ incubator at 37°C and sub-cultured every 2-3 days at the density of 0.1 or 0.2 x 10⁶ cells.mL⁻¹, not exceeding 1 x 10⁶ cells.mL⁻¹. They were propagated for no more than 2 months or 30 passages; and were routinely assessed for contamination with bacteria, fungi and mycoplasma.

2.2 Adaptation of cells to serum-free medium

One-step and gradual methods for the adaptation of cells to serum-free medium were performed in 25 cm² T-frasks (T25) or 6-well plates with a total of 4 different media, without adding antibiotics: HL-1™ with 2 mM L-glutamine (BioWhittaker®, Lonza, USA), here called HL1; RPMI-1640 with HL-1™ Supplement (BioWhittaker®, Lonza, USA), here called SHL1; X-VIVO™10 (Lonza, USA), here called XVIVO, which contains gentamicin; and DMEM/F12 (Lonza, USA), here called DMEMF12.

All the adaptation attempts were initiated with THP-1 cells in the exponential growth phase, with a cell density of 0.2 x 10⁶ cells.mL⁻¹ and at least 90% viability. Cells were passed every 2-4 days, when cell growth and viability were checked, using a hemocytometer and the trypan blue exclusion method, respectively.

For the one-step adaptation method, THP-1 cells cultivated in RPMI10%FBS were first transferred to medium containing 20% of conditioned medium (obtained from the previous THP-1 cell culture) and 80% (v/v) of the

chemically defined medium (HL1, SHL1, XVIVO or DMEMF12) and cultured for 2-4 days; then, cells were passaged using fresh chemically defined medium only. For the next passages, a 20% conditioned medium and 80% (v/v) of fresh chemically defined medium formulation was used.

For the gradual adaptation method, cells were passaged 2-3 times a week and the percentage of FBS was gradually reduced, when cell growth and viability indicate they were adapted to the new condition. In each passage, a formulation containing 20% of conditioned medium and 80% (v/v) of chemically defined medium was used, except for the second passage, when only fresh chemically defined medium was used.

2.3 Cryopreservation of adapted cells

Three different protocols were tested for cryopreservation of cells adapted to XVIVO: the first one using 40% XVIVO, 50% FBS and 10% (v/v) dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA); the second one using 90% XVIVO and 10% (v/v) DMSO; and the third one with 15% DMSO, 35% XVIVO and 50% (v/v) ProFreeze™-CDM (Lonza, USA), following datasheet instructions. Cryopreserved cell banks of adapted cells were made using 15% DMSO, 50% ProFreeze™-CDM and 35% (v/v) SHL1 or XVIVO, respectively.

2.4 Kinetic assays

Three kinetic studies were done with THP-1 cells cultured with RPMI10%FBS and three others were done with cells adapted to XVIVO. Samples were collected at regular intervals of time from alternate T-flasks (25 cm²) to assess cell (x) growth and viability. The maximum specific growth

rates (μ_{Max}) were calculated for each experiment as the slope of the linear interval (exponential growth phase) observed at $\ln(x)$ curves *versus* time. In one assay with cells cultured in RPMI10%FBS and in two assays with cells adapted to XVIVO, cell culture supernatants were collected and stored at -20°C until the quantification of D-glucose (GLC), L-lactate (LAC), L-glutamine (GLN) and L-glutamic acid (GLU) using the YSI 2700 SELECT™ biochemistry analyzer (YSI, Ohio, USA); and of ammonia (NH_4) using Orion 4-Star pH/ISE Meter (Thermo Scientific, USA). Substrates to products yields (Y_x/GLC ; Y_x/GLN ; $Y_{\text{LAC}}/\text{GLC}$, $Y_{\text{NH}_4}/\text{GLN}$) were calculated as the slope of product *versus* substrate plots during exponential growth phase.

2.5 Human cell-line activation test

All the assays for the h-CLAT (doubling time monitoring, reactivity check, dose finding assay and proficiency test) were done following the DataBase on Alternative Methods (EURL ECVAM, 2014) and OECD (OECD, 2017) protocols, with minor adjustments regarding laboratory facilities: a digital flow cytometer (FACSAria III; BD Biosciences, USA) was used and, in the absence of a refrigerated plate centrifuge, sample preparation for flow cytometry was done using a non-refrigerated centrifuge with capacity for 24 microtubes, however, samples were maintained on ice during incubations and washes.

After checking the doubling time (DT) and passing the reactivity check, cells were submitted to the dose finding assay (to estimate the concentration giving 75% cell viability – CV75) and to proficiency tests with the ten proficiency chemicals (OECD, 2017), all purchased from Sigma-Aldrich. The

following monoclonal FITC-labelled mice antibodies were used: anti-CD86 (clone Fun-1, BD-Pharmingen, #555657), anti-CD54 (clone 6.5B5, DAKO, #F7143), and anti-IgG1 (BD-Pharmingen, #555748).

For THP-1 cells cultured in RPMI10%FBS, exposure to chemicals was performed using this medium plus 10 U.mL⁻¹ of penicillin and 100 µg.mL⁻¹ of streptomycin. For cells adapted to XVIVO and to SHL1, the respective chemically defined medium was used, without adding antibiotics (XVIVO contains gentamicin).

3. Results

3.1. *h-CLAT with cells cultured in FBS-containing medium*

The mean DT of four different lots of THP-1 cells cultured in RPMI10%FBS was 38.8 ± 5.7 hours, with cell viability above 95% during the 72 hours of the experiment, in accordance with h-CLAT protocols. Eight different lots of THP-1 cells cultured in RPMI10%FBS were submitted to the reactivity check and all were approved.

Using the ten proficiency chemicals, the dose finding assay resulted in all CV75 values within the expected range (Table 1). The measurements of CD86/CD54 expression, according to the h-CLAT prediction model (OECD, 2017), showed that the sensitization potential of all the ten proficiency chemicals were correctly predicted (Table 1).

Table 1. Proficiency chemicals, experimental data and reference values for the human-Cell Line Activation Test using THP-1 cells cultured in medium with 10% fetal bovine serum.

Proficiency chemicals	CAS RN	CV75 ($\mu\text{g.mL}^{-1}$)	CV75 reference range ^a ($\mu\text{g.mL}^{-1}$)	Sensitization prediction	Sensitization potential reference ^a
2,4-Dinitrochlorobenzene	97-00-7	6.5	2 – 12	S	S (extreme)
Nickel sulfate	10101-97-0	141.6	30 – 500	S	S (moderate)
Lactic acid	50-21-5	1,781.8	1,500 – 5,000	NS	NS
4-Phenylenediamine	106-50-3	13.4	5 – 95	S	S (strong)
2-Mercaptobenzothiazole	149-30-4	304.2	30 – 400	S	S (moderate)
R(+)-Limonene	5989-27-5	> 120	> 20	S	S (weak)
Imidazolidinyl urea	39236-46-9	36.4	25 – 100	S	S (weak)
Isopropanol	67-63-0	> 5,000	> 5,000	NS	NS
Glycerol	56-81-5	> 5,000	> 5,000	NS	NS
4-Aminobenzoic acid	150-13-0	> 1,000	> 1,000	NS	NS

CAS: Chemical Abstracts Service Registry Number; ^aAccording to the protocol (OECD, 2017); S: sensitizer; NS: non-sensitizer.

3.2 Adaptation of cells to serum-free medium

The process of adapting THP-1 cells to serum-free media was performed using two strategies: direct (one-step) and gradual reduction of FBS. For both, initial and final cell concentrations, as well as cell viability, were analyzed in all sub-cultures, as exemplified in Figure 1 (gradual adaptation to SHL1). The μ_{Max} calculations presented here are estimates done for each growing interval, disregard of the existence of a lag phase. This growth monitoring allowed evaluating the cell culture prevailing trend in each condition and enabled corrections in the strategy. At least two attempts were done for most of the formulations.

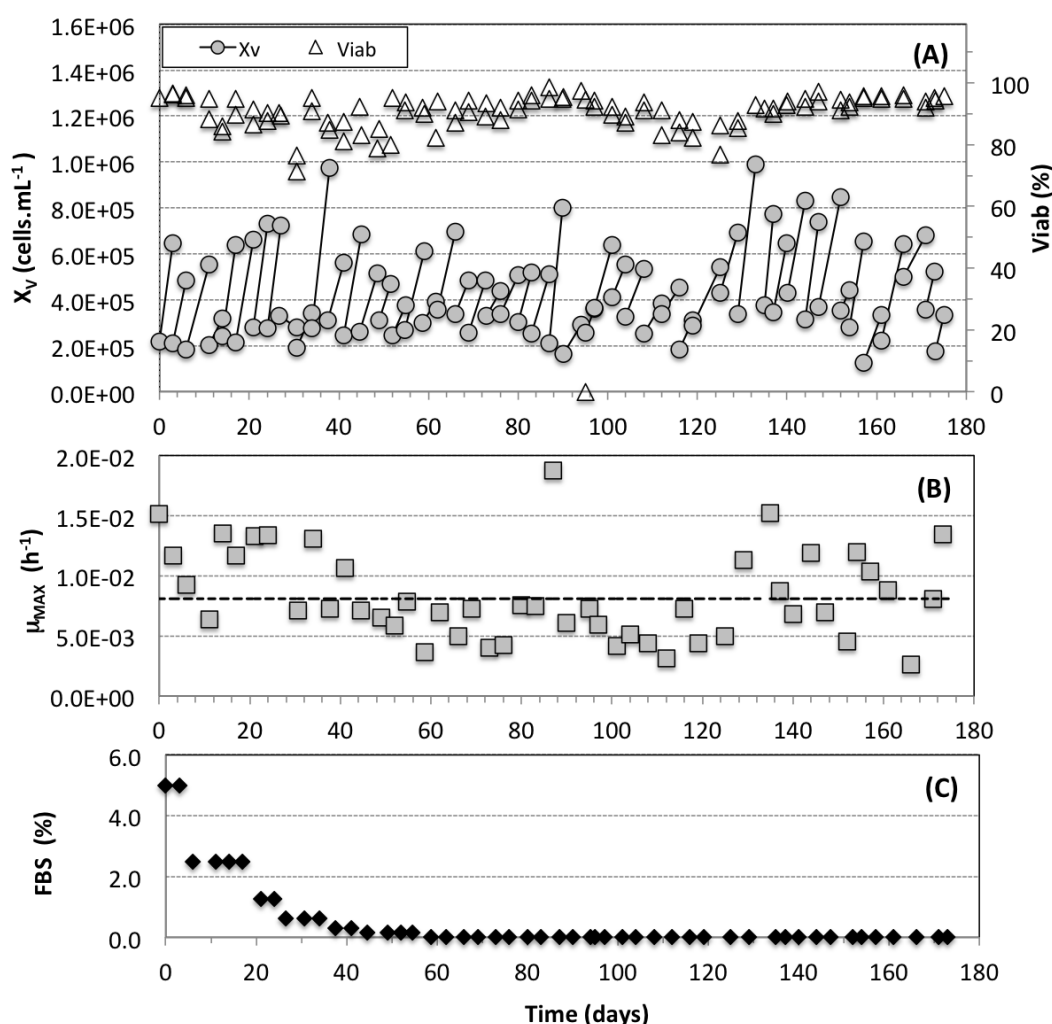


Figure 1. Gradual adaptation to RPMI-1640 medium supplemented with HL-1™ (SHL1). (A) Cell concentration and viability; (B) maximum specific growth rates (dashed line corresponds to the mean); (C) percentage of fetal bovine serum (v/v) in culture medium.

X_v : viable cells concentration; viab: cell viability; μ_{Max} : maximum specific growth rate; FBS: fetal bovine serum.

Table 2 summarizes the results of all attempts, indicating the successful cases, as well as the characteristic values for the better conditions of FBS reduction (analyzing the whole adaptation process for the successful cases; and for the unsuccessful ones, only the period before a sharp decline in cell viability hindered further culturing of cells). Here, adaptation success means having high cell viability and enough growth to continue the FBS-

reduction process until total removal of FBS (gradual reduction) or for at least two months in serum-free medium (one-step adaptation).

Table 2. Adaptation attempts and main characteristics of the cell cultures.

SFM	Method	Number of trials	Successful?	Values for the better conditions of FBS reduction during adaptation				
				Minimum FBS (%)	Days in SFM (mean)	μ_{MAX} (10^{-2} h^{-1}) (mean $\pm$ SD)	DT (h) (mean)	Viab (%) (mean $\pm$ SD)
XVIVO	D	1	No	0	42	1.37 ± 0.69	50.6	78.1 ± 8.5
	G	1	YES	0	11	0.56 ± 0.24	122.5	92.1 ± 3.7
SHL1	D	2	No	0	22	1.34 ± 0.55	56.3	86.2 ± 1.3
	G	2	YES	0	58	1.22 ± 0.66	66.2	92.1 ± 2.4
HL1	D	2	No	0	48	1.26 ± 0.20	55.7	91.7 ± 2.5
	G	2	No	0.25	7	1.38 ± 0.25	50.2	87.3 ± 5.5
DMEMF12	D	2	No	0	15	0.79 ± 0.19	89.9	89.0 ± 6.8
	G	3	No	0.38 – 1.25	8	1.14 ± 0.43	67.3	94.6 ± 0.6

D: direct (one-step); G: gradual; FBS: fetal bovine serum; SFM: serum-free medium; SD: standard deviation; DT: doubling time; viab: viability.

The cell responses to gradual adaptation were variable. Although cell viability had remained high (mean viability above 87%) in the lower FBS percentage for all media, only with XVIVO and SHL1 it was possible to totally eliminate this component. For these, the FBS reduction steps were: 5; 2.5; 1.25; 0.625; 0.312 and 0% (v/v) FBS for XVIVO. For SHL1, the same FBS reduction scheme was done, with an additional step (reduction to 0.156 before 0% v/v FBS). In those cases in which it was not possible to totally eliminate FBS, cell viability remained above 85% until the reduction caused a

sharp fall in this parameter, without recovery, and the cell culture was discarded. The other evaluated parameter (μ_{Max}) presented a greater variability: the coefficients of variation (CV) were 54, 43, 38 and 18% for SHL1, XVIVO, DMEMF12 and HL1, respectively. Besides the greater dispersion, the adaptation processes also resulted in lower μ_{Max} values, compared to the reference condition (RPMI10%FBS, discussed further below). On average, these values were from 32 to 62% lower (for HL1 and XVIVO, respectively), but it was not possible to establish a direct correlation between the reduction of this parameter and the higher difficulty to adapt the cells to the respective serum-free medium. Indeed, the lower μ_{Max} value — $(0.56 \pm 0.24) \times 10^{-2} \text{ h}^{-1}$ — was observed with XVIVO, which, however, presented a substantial increase in this parameter (results showed further below) after the cryopreservation and additional subcultures of the adapted cells. At the same time, the higher μ_{Max} value was obtained using HL1, for which it was not possible to reduce the percentage of FBS below 0,25% (v/v).

As indicated in Table 2, the direct adaptation strategy was not successful for any of the four media evaluated. For most of the formulations, at least two attempts were made and, even though it was possible to maintain these cell cultures for up to 48 days) with mean cell viability above 78%, at some point during cell culture it was observed a sharp fall in cell viability, without recovery, and the culture was discarded. The mean μ_{Max} values observed were lower than those for cells cultured in medium with FBS (discussed further below). In this analysis, data variability was taken into account, in which the CV values were relatively low for HL1 and DMEMF12

(16 and 24%, respectively) and higher for XVIVO and SHL1 (51,1 and 41,0%, respectively).

Using the three different cryopreservation protocols, THP-1 cells adapted to XVIVO showed cell viability from 84.9 to 90.1% at thawing. There was a reduction in cell viability at the first passage, regardless of the protocol used, followed by a recovery at the next passages, but only using ProFreeze™-CDM the cell viability was fully recovered and maintained above 80% for at least 12 passages. It was noticed that THP-1 cells adapted to XVIVO tend to form cell aggregates at thawing, which were not easy to disrupt at the first passages. THP-1 cells adapted to SHL1 were only cryopreserved using ProFreeze™-CDM, and also showed above 90% cell viability after thawing and during the following passages.

3.3 Kinetic studies

In order to characterize the cell populations cultured in serum-containing medium and those adapted to serum-free medium, kinetic studies were performed with cells that were approved in the reactivity test (see next section): THP-1 cultured in RPMI10%FBS and those adapted to XVIVO. Typical profiles for both culture conditions are presented in Figure 2, where maximum viable cells concentrations (X_v) of approximately 2×10^6 cells.mL⁻¹ were equally achieved (Figure 2A), even though media composition on GLC and GLN was twice higher in XVIVO (Figures 2B and 2D). Table 3 summarizes the kinetic and stoichiometric data for those runs. All exponential phases were defined at $\ln(X)$ plots, and μ_{Max} values were determined from linear regression with good CV (superior to 0.99). Stoichiometric parameters

for GLC ($Y_{X/GLC}$ and $Y_{LAC/GLC}$) showed similar values for both media, despite of the differences in GLC availability.

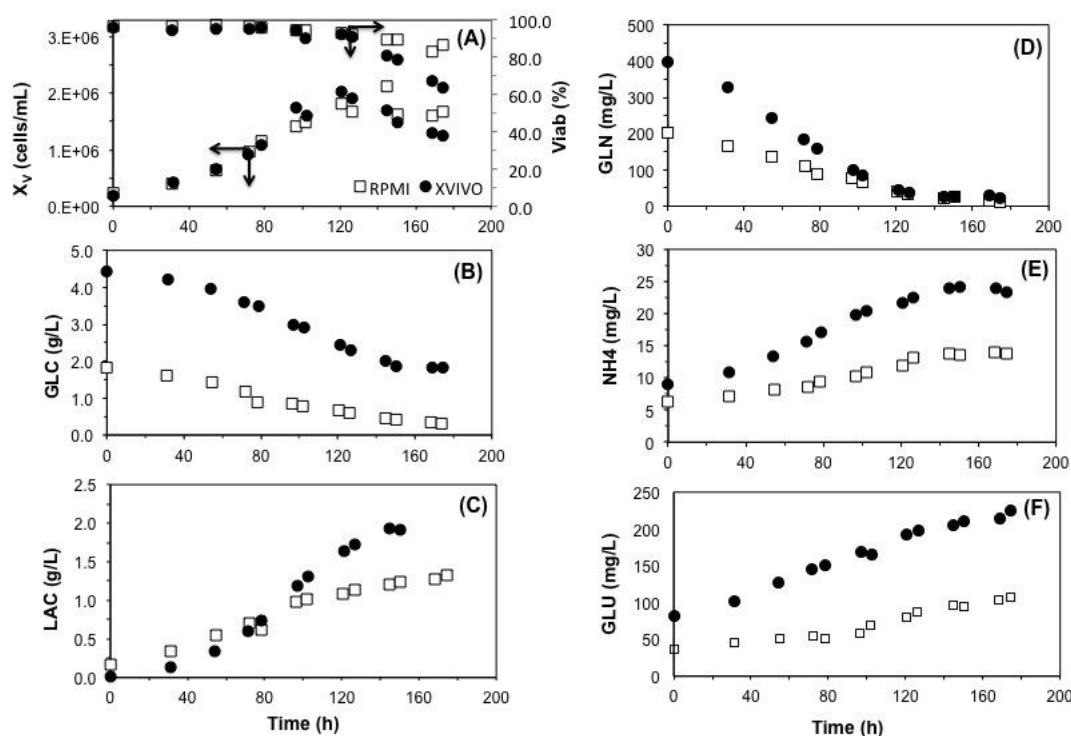


Figure 2. Kinetic and stoichiometric parameters for THP-1 cells cultured with FBS (RPMI10%FBS) and adapted to XVIVO.

Full circles: XVIVO; empty squares: RPMI10%FBS; X_v : concentration of viable cells; Viab: cell viability; GLC: D-glucose; LAC: L-lactate; GLN: L-glutamine; NH_4 : ammonia; GLU: L-glutamic acid.

Table 3. Kinetic studies with THP-1 cells cultured in medium with fetal bovine serum and adapted to XVIVO.

Cultured medium	Assay	Exponential phase								Stationary phase	
		t_{End}	μ_{max} ($10^{-2} h^{-1}$)		DT	$Y_{X/GLC}$	$Y_{LAC/GLC}$	$Y_{X/GLN}$	$Y_{NH_4/GLN}$	t_s	X_v
		(h)	Values	Mean $\pm$ SD	(h)	($10^9 cells.g^{-1}$)	($g.g^{-1}$)	($10^7 cells.g^{-1}$)	($mg.g^{-1}$)	(h)	($10^6 cells.mL^{-1}$)
RPMI with 10% FBS	1	95.0	2.16	2.03 $\pm$ 0.118	32.1	ND	ND	ND	ND	97.5	1.85
	2	90.0	1.93		35.9	ND	ND	ND	ND	140	1.31
	3	96.5	2.00		34.6	1.27	0.84	1.15	30.0	120	1.82
XVIVO	1	72.0	1.52	2.09 $\pm$ 0.519	45.7	ND	ND	ND	ND	102	2.14
	2	97.0	2.23		31.1	1.04	0.82	0.36	35.7	120	2.03
	3	94.0	2.53		27.4	1.02	0.90	0.81	88.4	104	2.22

t_{End} : time for the end of the exponential growth phase; μ_{Max} : maximum specific growth rate; SD: standard deviation; DT: doubling time; $Y_{\text{X/GLC}}$: D-glucose to cell yield; $Y_{\text{LAC/GLC}}$: D-glucose to L-lactate yield; $Y_{\text{X/GLN}}$: L-glutamine to cell yield; $Y_{\text{NH}_4/\text{GLN}}$: L-glutamine to ammonia yield; t_{S} : time for the beginning of the stationary phase; X_{V} : maximum concentration of viable cells; FBS: fetal bovine serum; ND: not determined.

3.4 *h-CLAT with cells adapted to serum-free media*

Reactivity tests were done with cells adapted to SHL1 and XVIVO. For cells adapted to SHL1, two different lots of cells were used, and both have not passed. Despite correctly identifying LA as a non-sensitizer, they were not able to correctly identify the sensitizers 2,4-dinitrochlorobenzene (DNCB) and nickel sulfate (NI).

In reactivity tests with THP-1 cells adapted to XVIVO, cell viability of non-treated cells was below 90%. However, these cells were able to correctly identify: LA as a non-sensitizer (negative response for both CD86 and CD54); DNCB as a sensitizer (positive response for both CD86 and CD54); and NI as a sensitizer (positive response only for CD54), despite cell viability below 50% for this chemical.

Analyzing four different lots of THP-1 cells adapted to XVIVO, DT ranged from 30.5 to 48.7 h. In all four experiments, cell density exceeded 1×10^6 cells.mL⁻¹ after culturing for 72 hours, with cell viability remaining above 92% during all the experiment.

In dose finding assays with the proficiency substances, 9 out of 10 CV75 values were within the expected range. Only R-limonene resulted in a CV75 below the reference value (Table 4).

Table 4. Proficiency chemicals, experimental data and reference values for the human Cell Line Activation Test using THP-1 cells adapted to XVIVO.

Proficiency chemicals	CV75 ($\mu\text{g/mL}$)	CV75 reference range ^a ($\mu\text{g/mL}$)	Sensitization prediction	Sensitization potential reference ^a
2,4-Dinitrochlorobenzene	4.7	2 – 12	S	S (extreme)
Nickel sulfate	136.7	30 – 500	S	S (moderate)
Lactic acid	2,837.0	1,500 – 5,000	NS	NS
4-Phenylenediamine	21.3	5 – 95	S	S (strong)
2-Mercaptobenzothiazole	137.8	30 – 400	S	S (moderate)
R(+)-Limonene	16.9	> 20	S	S (weak)
Imidazolidinyl urea	34.3	25 – 100	S	S (weak)
Isopropanol	> 5,000	> 5,000	NS	NS
Glycerol	> 5,000	> 5,000	NS	NS
4-Aminobenzoic acid	> 1,000	> 1,000	NS	NS

^aAccording to the protocol (OECD, 2017); S: sensitizer; NS: non-sensitizer.

In the proficiency assay with THP-1 cells adapted to XVIVO, 16 out of the 20 experiments (10 chemicals in duplicate) have met all the acceptance criteria (considering the acceptance criterion of cell viability above 50% for medium and vehicle controls, when using THP-1 cells adapted to XVIVO, discussed afterwards). However, according to the h-CLAT prediction model, the sensitization potential of all the ten proficiency substances were correctly predicted (Table 4).

4. Discussion

THP-1 cells cultured in RPMI10%FBS presented a mean DT (38.8 h) below the mean (43 h) and within the expected range (30 to 55 h) for these cells (EURL ECVAM, 2014). They were approved in reactivity checks and all CV75 values and measurements of CD86/CD54 expression were in

accordance to the expected for all the ten chemicals (OECD, 2017). Therefore, the h-CLAT was correctly implemented in this laboratory (proficiency proven).

Regarding direct adaptation, HL1 and XVIVO showed the best results, as they supported the culture of THP-1 cells without FBS for a longer time (average of 48 and 42 days, respectively) with cell viability above 80% for HL1 for almost 2 months in the first trial, followed by a sharp fall in this parameter, without recovery, which hindered further assays.

Although previously proven to be possible (Tsuchiya et al., 1986), gradual adaptation using DMEMF12 was not successful in this study, even using the same weekly reduction schedule described in the literature. As in two out of three attempts with DMEMF12 cell viability remained above 90% up to reducing FBS to less than 1%, some strategies may be used in a future attempt, as the supplementation with insulin, transferrin and selenium, which has been proven to be useful in the adaptation of other cell lines (Gstraunthaler, 2003).

The HL1 datasheet informs that this medium is suitable for THP-1 cells (Lonza, 2016), but gradual adaptation was not successful in the first attempt. The second one seemed to be more promising, as cell viability remained high (above 90%) during almost all the process, with quick cell viability recoveries after the last reduction steps. However, this recovery did not happen when cells were transferred to HL1 with 0% FBS.

For the four serum-free medium used in this research, gradual adaptation was only successful with SHL1 and XVIVO. In the case of SHL1, although the mean DT (66.2 h) was above the range (30 to 55 h) expected for

THP-1 cells cultured in FBS-containing medium, cell viability was high during all the experiment, including a long period of almost 4 months after reducing FBS to 0% in the first trial. Therefore, THP-1 cells were considered to be adapted to SHL1 and a cryopreserved cell bank was made. Gradual adaptation using XVIVO was successful in the first attempt and cell viability remained high during all the adaptation process. The mean DT during adaptation (122.5 h) was well above the DT using other media, which may be due to the low number of passages in serum-free medium. However, DT and μ_{Max} values were improved after cells being cryopreserved, thawed and subcultured (as shown in kinetic studies), which indicates that the cryopreservation may have been done before the complete selection of adapted cells.

As THP-1 cells adapted to XVIVO and cryopreserved using ProFreeze™-CDM showed the high cell viability after thawing, this protocol was adopted to make the cryopreserved cell banks of cells adapted to XVIVO and SHL1 and has proved to be successful.

DT assays with THP-1 cells adapted to XVIVO showed good cell viability in all experiments. After culturing cells for 72 hours, all lots of cells showed cell density exceeding 1.0×10^6 cells/mL (possibly indicating that cells were not anymore at the exponential growth phase), which is not recommended for THP-1 cells cultured in medium containing FBS. As it is important to passage and use cells in the experiments when they are at the exponential growth phase, it is recommended to analyze the growth kinetics of these cells in order to guarantee the time limit to use them after each passage. The good cell viability and DT within the reference range for THP-1

cells cultured in medium containing FBS indicate that THP-1 cells adapted to XVIVO are good candidates to be used in the h-CLAT.

In kinetic studies, the end of the exponential growth phase occurred from 90.0 to 96.5 h for cells cultured in medium with FBS and from 72.0 to 97.0 h for cells adapted to XVIVO. So, in order to guarantee that cells are always passaged during the exponential growth phase, as recommended for THP-1 cells cultured in medium with FBS, it is important to passage cells adapted to XVIVO each 2-3 days when cell culture is initiated 0.2×10^6 cells.mL⁻¹. Kinetic studies showed that cell growth is very similar between THP-1 cells cultured in medium with FBS and those adapted to XVIVO, indicating that the latter could be good surrogates in h-CLAT.

Two different lots of THP-1 cells adapted to SHL1 did not meet the acceptance criteria for the reactivity check. Despite showing adequate cell growth and DT, even after cryopreservation, they were not able to correctly identify the sensitizers DNCB and NI. The h-CLAT protocol (EURL ECVAM, 2014) suggests that if the acceptance criteria for the reactivity check are not met for the sensitizers, one possible cause is that CV75 values for these chemicals are not appropriate for the cell lot, so CV75 should be re-determined and the newly re-calculated CV75 value is to be used in a new reactivity assay. This was not done with cells adapted to SHL1 as the cell viability of the 3 test chemicals in both experiments was near 75%, indicating that CV75 values were not the problem in this case. Therefore, THP-1 cells adapted to SHL1 were not used in further assays to check their usefulness in the replacement of cells cultured in medium with FBS in the h-CLAT.

Kinetic and stoichiometric data of cells adapted to XVIVO are similar to those for cells cultured in FBS-containing medium. Therefore, the adaptation process does not seem to have caused metabolic changes in THP-1 cells.

Different lots of THP-1 cells adapted to XVIVO were submitted to the reactivity check, and cell viability of non-treated cells was below 90% in flow cytometry assay (discussed afterwards). While LA produced negative response for both CD86 and CD54, and DNCB produced positive response for both CD86 and CD54, NI only produced positive response to CD54, with very low cell viability. Following the h-CLAT protocol recommendations, CV75 was re-determined for NI and the newly re-calculated dose was used in another reactivity check, which also resulted in cell viability below 50% and positive response only for CD54. This led us suppose that plasma membrane of THP-1 cells adapted to XVIVO and exposed to NI are even more susceptible to shear stress. This assumption, as well as the benefit of using additives to improve cell resistance to shear stress may be investigated in further studies.

Regarding CV75, using THP-1 cells adapted to XVIVO exposed to the proficiency substances, only R-limonene resulted in CV75 narrowly under the expected range. One possibility is that the calculated CV75 is not accurate. This can have occurred due to the fact that R-limonene is a colorless liquid chemical (which makes the visual confirmation of solubility a difficult task) with low solubility (Log Kow higher than 3.5). In the h-CLAT validation study, different concentrations of R-limonene (ranging from 50 to 200 $\mu\text{g.mL}^{-1}$) were used by the laboratories as the highest soluble concentration (EURL ECVAM,

2013). Despite the R-limonene CV75 was out of the expected range, it was correctly identified as a sensitizer in the present study.

In the proficiency assay with THP-1 cells adapted to XVIVO, as previously observed in the reactivity tests, it was not possible to use the same acceptance criteria applied for cells cultured in medium containing FBS, mainly due to cell viability below 90% for medium and vehicle controls. It is important to mention that FBS not only provides nutrients and growth factors, it also provides biological and physico-chemical protection to cells (Papoutsakis, 1991). The absence of a cell wall makes mammalian cells more susceptible to hydrodynamic forces, including shear stress, which can lead to cell death due to damages in the plasma membrane (Hu et al., 2011). Although THP-1 cells are cultured in static liquid inside T-flasks, the protocol for preparing them for flow cytometry includes several sample centrifugation and pellet resuspension steps, exposing cells to shear stress, which can lead to low viability when, at the end, cells are analyzed by flow cytometry. This seems to be the case here, as THP-1 cells adapted to XVIVO show viability above 90% during cell culturing in T-flasks but low cell viability after being prepared for flow cytometry analysis. Also, in CV75 assays (a protocol with less centrifugations and resuspensions), cell viability of non-treated cells was from 84.5 to 92.4%, higher than non-treated cells in the experiments for CD86/CD54 analysis (from 65.8 to 80.2%, excluding the assays in which appropriate temperature control was not possible, discussed afterwards).

By analyzing the results of the proficiency assay (obtained with two experiments for each of the ten proficiency substances), it was noticed that even when cell viability was above 90% (but above 50%), the sensitization

potential of the test substances was correct. Therefore, we proposed to adapt the acceptance criteria regarding cell viability of control cells for both the reactivity check and proficiency assay when using cells adapted to XVIVO: cell viability of non-treated cells (medium and vehicle controls) should be above 50%. The decision of reducing this acceptance criteria from 90 to 50% was based on two facts: (1) in the proficiency assay, when non-treated cells showed viability above 50%, the sensitization potential was correctly predicted for all substances; and (2) in the h-CLAT protocol, one of the acceptance criteria is that cell viability should be more than 50% in at least 4 tested concentrations, indicating this is a reliable value for the evaluation of CD54 and CD86 expression.

In the last four experiments with THP-1 cells adapted to XVIVO, due to an equipment failure, it was not possible to adequately control the samples temperature during the processing for flow cytometry. This may be the cause of the failure to obtain cell viability above 50% for at least 4 tested concentrations in these experiments. It is important to notice that, even though cell viability was low, in these four experiments the sensitization potential of the test chemicals were correctly indicated. As these four experiments were the second run for chemicals that were also correctly identified as sensitizers (NI) and non-sensitizers (isopropanol, glycerol and 4-aminobenzoic acid) in the first run with THP-1 cells adapted to XVIVO, and as in all the other experiments with these cells the other acceptance criteria (including cell viability of non-treated cells higher than 50%) were met and test substances were correctly identified as sensitizers and non-sensitizers, we considered that THP-1 cells adapted to XVIVO have passed the proficiency

assay, with minor adjustments in the acceptance criteria. In future studies, it is recommended that a refrigerated plate centrifuge be used, in order to better control the temperature of the samples during processing for flow cytometry and to reduce time and labor of this assay. Strategies to increase cell viability of adapted cells are also recommended, such as the use of Pluronic F-68, polyethylene glycol (Papoutsakis, 1991), polaxamers or other media additives to protect cells from shear stress (Chang et al., 2017).

It has been recently shown that a modified method using THP-1 cells adapted to RPMI-1640 medium supplemented with human serum (pooled human AB plasma) can correctly classify all ten proficiency substances, with minor adaptations from the original h-CLAT protocol. However, it is known that fully defined cell culture conditions are the final goal, especially for enhanced reproducibility (Edwards et al., 2018). Besides this technical advantage of chemically defined media over media supplemented with human sera, the former is also less expensive and do not present the risk of contamination, especially by viruses. Therefore, the use of human sera in alternative *in vitro* methods is justified in order to address the urgent need for cosmetic testing without using animals or animal-derived products, but may not be seen as a final solution.

As the other two OECD-accepted *in vitro* methods for the third key event of the skin sensitization AOP use cells that are similar to the THP-1 and also cultured in RPMI-1640 medium supplemented with 10% FBS — U-SENS™ uses the U937 cell line and the IL-8 Luc Assay uses a THP-1 derived IL-8 reporter cell line (THP-G8) — it is reasonable to suppose that these cells

can also be adapted to XVIVO, making these methods also serum-free by using a chemically-defined medium.

It is important to mention that, in order to turn these methods fully free of animal-derived products, and consequently, really cruelty-free, it is necessary to eliminate the use of bovine serum albumin in h-CLAT, as well as to make sure that the antibodies used in these methods do not use animals or animal-derived products, if produced *in vitro*. At least for the h-CLAT, the use of human serum albumin and phage display antibodies do not seem to have negative impacts on the results (Edwards et al., 2018).

5. Conclusions

It is possible to adapt THP-1 cells to different serum-free media. THP-1 cells adapted to XVIVO show similar cell growth and viability as those cultured with FBS. THP-1 cells adapted to XVIVO were approved in the reactivity check and showed similar results in dose finding (CV75) and proficiency assays, with minor adjustments in the original h-CLAT protocol, which indicates that it is possible to eliminate the use of FBS in the h-CLAT, by adapting THP-1 cells to a chemically defined medium.

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ACCEPTED MANUSCRIPT

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Highlights

- THP-1 cells can be adapted to chemically defined media, with no animal serum;
- cells cultured in serum-free medium are able to identify skin sensitizers;
- THP-1 cells adapted to chemically defined medium may be used in the h-CLAT.