lead project separate from the development of the DPRA TG, if found necessary by the working group.

Item 7 and 9: Issues specific to the TG for DPRA identified in the first WNT commenting round

16. The issue of applicability of the DPRA to mixtures and polymers was discussed. It was agreed that technically the assay is applicable to such substances but the set up and the interpretation of the results in these cases is complicated by the requirement to define the molar ratio of the test chemical and the peptide. It was agreed that for mixtures of known composition and for polymers an approach can be outlined to calculate apparent molecular weight and purity. The lead (ECVAM) undertook to elaborate the approach and include the changes in the second version of the TG.

17. The group also discussed how the reactivity classes were defined and agreed to retain them in the TG. However some changes were introduced (particularly in Tables 1 and 2 of the TG and others were agreed to be introduced along the same lines for the second revision of the TG) to more precisely specify: a) how to use the cut-off values of the DPRA prediction model for the purpose of supporting the discrimination between sensitisers and non-sensitisers (as the proposed use of the DPRA within the current TG) and b) the potential use of reactivity classes to inform potency assessment within an IATA. In addition a foot note was added to Tables 1 and 2, illustrating the DPRA prediction model, to avoid any misinterpretation on the potential use of a DPRA prediction for a final classification of the test chemical. In addition revisions were made to clarify that the cut-off values used in the DPRA prediction model refer to statistically generated values and are not related to the precision of the measurement. An additional sentence was introduced to clarify how to deal with test chemicals giving results close to the threshold to discriminate between sensitisers and non sensitisers in order to increase confidence in DPRA predictions for borderline chemicals.

18. All other individual comments for the DPRA were discussed with proposed changes by the developers and agreement was reached following discussion on: the order of choice of solvents, inclusion of the possibility of using a different positive controls and provision for this and a number of other details of the procedure description (all in track changes).

Item 8: Summary of outcomes of the day 1 meeting

19. The Secretariat outlined the outcomes of the discussions of day 1 and the agenda for day 2. All issues relating to DPRA discussed on day 1 were agreed upon and only minor changes were finalised in the TG based on the agreed conclusions (all described above in the joint summary of agenda items 7 and 9).

Item 10: Issues specific to the TG for KeratinoSens™ identified in the first WNT commenting round

20. The format of the TG for KeratinoSens™ contains performance standards because of the need to obtain a licence agreement when using the KeratinoSens™ cell lines within the ARE-Nrf2 luciferase test method. Furthermore, a licence agreement is needed regarding the use of the Luciferase gene inserted in the KeratinoSens™ cell lines, which is patented by Promega. The PS are intended to allow for me-too assays to be validated and included in the TG. Changes to the TG were incorporated into the TG to clarify that at this stage KeratinoSens™ is the only validated reference method (VRM) included by this TG.

21. Implications of the proprietary elements of the KeratinoSens™ assay were discussed and circumstances when licence agreement is needed were clarified. The Secretariat noted that further consideration may need to be given to this issue in the context of the OECD requirements for Material Transfer Agreements relevant to TG methods with proprietary elements. The secretariat also noted that this issue would need to be clarified before approval of the TG by the WNT.

22. Changes to the TG were discussed and agreed with regard to the limitations and applicability of the assay to hydrophobic substances and substances with particular value of logP. Applicability of the assay to mixtures was also discussed in the context of the limited data available on testing of mixtures using the KeratinoSens™ assay. It was agreed that although in principle the assay is applicable to mixtures having relevant physical properties making them technically testable, a cautionary statement needs to be included in the TG to trigger considerations by users as to whether the assay will provide acceptable results for the intended regulatory purpose. It was agreed to add such statement in the second draft of the TG.

23. The experts agreed to include an additional reference on the limited metabolic capacity of the test method. However, it was agreed that additional references regarding metabolic transformation of chemicals using liver S9 fractions to evaluate skin sensitisation potential is not needed within the test guideline since the metabolic competence of skin is very different than the one in liver. Relevant changes to the TG were agreed to be undertaken by the lead country for the second draft of the TG. In this respect it was indicated that considerations on potential inclusion of metabolic competence to the ARE-Nrf2 luciferase test method may be made in the context of the IATA Guidance Document and taking into account all relevant information sources.

24. A number of changes in the description of the procedure were also agreed by the expert group and incorporated in the TG (all in track changes).

25. The group discussed the performance standards as outlined in the first draft of the TG.

- Type of cells to be used in me-too assays - It was agreed that me-too assays need not necessarily be based on keratinocytes but because this is a test whose performance is evaluated relative to skin sensitisation, any other cell line used would need to be relevant to the skin sensitisation AOP.

- Number and identity of reference chemicals to be used for performance assessment - It was agreed that the number of reference chemicals for performance evaluation of me-too assays should be reduced from 30 to 20 and inclusion/exclusion of particular chemicals was discussed so as not to disadvantage prospective new assays. The developer undertook to propose the identity of substitute chemicals to obtain the best set of 20 reference chemicals for the second draft of the TG based on the discussion at the meeting.

- Performance assessment – The lead country reminded the group of the number and overlap of the chemicals used for within and between laboratory variability assessment (WLV and BLV, respectively) and outlined the issue this can pose in determining the best set of chemicals and the approach needed to assess these aspects for modified or new me-too assays. It was agreed that a weighed calculation should be used to take into account the fact that there will be a different number of experiments for the 20 Reference Chemicals that will have to be used for assessment

(some will have results from experiments for both BLR and WLR but others only for BLR). The expert group agreed that a weighed approach where positive and negative predictions for each chemical would be divided by the total number of predictions for that chemical is a good approach for assessment. However, the identity of the 20 chemicals was left to be agreed following proposal of substitute chemicals by the developer and review by the lead countries. In addition there was a suggestion for revisiting the performance target values based on the final set of reference chemicals.

Item 11: Conclusions, next steps, and closure of the meeting

26. The meeting was concluded with the agreement that the draft Test Guidelines were revised to a significant extent through the work of the lead countries in addressing the WNT comments before the meeting and the constructive contribution of the expert group during the meeting. The expert meeting effectively addressed all the WNT comments received on the two draft TGs and agreement within the expert group was achieved on all identified issues. As no major outstanding issues could be identified, there was a general feeling by the expert group that the TGs were ready to be proposed for discussion by the WNT at their 2014 April meeting and be considered for (provisional) adoption. The EC therefore asked the OECD Secretariat if it would be possible to send the revised TGs already to the WNT for consideration at their next meeting noting that we were still within the requested timeframe of six weeks for submission of the documentation. The Secretariat replied that this would not be possible because the agenda of the next WNT was already very full and the changes introduced in the draft TGs (or agreed by the experts to be introduced by the lead countries after the meeting) would first need to be submitted to the WNT for a second review and commenting round.

27. The Secretariat thanked the participants for their helpful contribution during the meeting and informed them that the Secretariat will update the WNT on the outcome of this expert meeting and the progress of the development of the TGs for DPRA and KeratinoSens™ at their 26th WNT meeting (WNT26) in April 2014.

28. The Chair closed the meeting at 16h00.

厚生労働科学研究費補助金（化学リスク研究事業）多色発光細胞を用いたhigh-throughput免疫毒性評価試験法の開発

分担研究報告書

化学物質のMulti-ImmunoTox assayによる解析，精度管理

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研究要旨

Multi-ImmunoTox assay（MITA）による免疫毒性評価法のラボ間バリデーション試験に参加、再現性を評価した。一方、MITA法の信頼性の確保(精度管理)のため、測定時に用いるルミノメーター機種間の測定結果変動の補正法を検討した。さらに毒性評価発光細胞の維持管理を行うため、レポータ遺伝子群を人工染色体に導入したコントロール発光細胞の樹立を目指しレポータ発光遺伝子群の人工染色体ベクター化を開始した。

キーワード：免疫毒性、動物実験代替法、*in vitro*

A．研究目的

我々はこれまでに多色発光タンパク質による新たな *in vitro* 免疫毒性評価試験法、いわゆるMulti-ImmunoTox assay（MITA）を確立し各種毒性評価発光細胞を樹立した[1)]。現在、これらの細胞群を用いた化学物質の免疫毒性評価法の確立を目指している。そこで本研究では、化学物質の免疫毒性評価のためのMITA法のOECDガイドライン化を視野に、ラボ間バリデーション試験の実施とMITA法の精度管理に必要な周辺技術の開発を目的とした。

より具体的には、東北大学病院で樹立されたJurkat細胞におけるINF-γ, IL-2, G3PDHプロモータ活性を測定する細胞株2H4及びTHP-1細胞におけるIL-8, IL-1β, G3PDHプロモータ活性を定量化できる細胞株6C12をモデル細胞としてバリデーション試験を実施、ガイドライン化するための手法の最適化を目指す。また、MITA法の精度管理として、ルミノメーターの機種間測定結果変動の補正法の開発及び人工染色体を用いた標準発光細胞の構築を目指した。

B．研究方法

B-1)プレバリデーション試験

東北大学で樹立した細胞株2H4（Jurkat細胞におけるINF-γ, IL-2, G3PDHプロモータ活性を測定）及び細胞株6C12（THP-1細胞におけるIL-8, IL-1β, G3PDHプロモータ活性を測定）を用いて数種類の化学物質を評価する。具体的な試験法を以下に示す。

1）細胞株2H4、6C12を用いた化学物質の免疫毒性試験法における細胞培養方法、被験物質調整及び添加方法、及びルシフェラーゼアッセイの方法についてはMulti-Immuno Tox Assay バリデーションプロトコール平成24年11月13日ver. 001.1準ずる。

2）試験化学物質としてはデキサメサゾン（DEX）及び再クロスポリンA(CyA)とし、濃度はDEXの場合100ng/mL-1mg/mL、

CyAの場合、30pg/mL-1μg/mLとして、測定装置としてアトー社製Pheriosを用いた。

B-2）ルミノメーターの機種間測定結果変動の補正法

ルミノメーター用多色標準発光プレートの試作を検討した。

B-3)人工染色体を用いたコントロール発光細胞の作成

IL-8転写調節領域（約5.0kb）の橙色発光ルシフェラーゼ遺伝子を加えたものを人工染色体ベクターに搭載する。

1）pYM626-4からSalIとNotIでhIL-8 SLOカセットを切り出し、クローニングベクターに導入してphIL-8 SLOを構築する。

2）人工染色体ベクターを保持したCHO細胞に1）で構築したpMGP4 Neo hIL-8 SLO #20をトランスフェクション（invitrogen, Lipofectamine2000）する。

2）ランスフェクション6時間後に培地を交換し、トランスフェクション24時間後に細胞を継代。トランスフェクション48時間後に薬剤選択（Geneticin, 500μg/mL）を開始する。

3）薬剤選択耐性クローンを取得し、ゲノムPCRで人工染色体ベクター上の目的部位にモニターカセットが導入できていることを確認する。

（倫理面への配慮）

倫理的な問題が生じる実験を実施しておらず、特に配慮すべき問題はない。

C.結果

C-1）プレバリデーション試験

細胞株2H4、6C12を用いた化学物質の免疫毒性試験法は初めに東北大学病院にて手技の確認を行ってから産総研内で実験を行った。

細胞株2H4の結果を図1、2で示す。当初、東北大学のデータと同様な結果は得られず、特に緑色ルシフェラーゼの発光量は十分ではなかったが、抑制率を見る限りはほぼ同様の結果が得られている。さらに数回実験を繰り返した結果、東北大病院と発光量を含めて同様な結果が得られた。

細胞株6C12の結果を図3、4で示す。当初ばらつきがみられたが、回数を経るごとにばらつきは減少、東北大病院と同様な結果が得られた。2つの細胞株の応答性を確認、また、再現性の高い手法である点も確認した。

C-2）ルミノメーターの機種間測定結果変動の補正法

これまでLEDを用いた発光プレートは単色光であり、ルミノメーターの受光部分の性能確認、精度管理は可能であったが、多色発光では色分離を行うフィルターの活用が必須であるため、単色光のLEDでは十分な精度管理は難しい。そこで、3色の発光ルシフェラーゼ標品を作り、これらを用いることで精度管理が可能である。しかしながら、ルシフェラーゼ標品では供給が難しく、日々装置の校正をすることは容易ではない。そこで、図5にあるような3色のLEDを用いた発光プレートの試作を、アトー社と検討した。現在、試作中であり、次年度以降は多色発光標準プレートを用いて計測の精度管理を行う予定である。

C-3）人工染色体を用いたコントロール発光細胞の作成

発光細胞自体の精度管理を行うためレポータ遺伝子を安定に発現させることが可能な人工染色体に発光遺伝子を挿入したコントロール発光細胞の作製を目指す。まずはIL-8プロモータ領域5kbpと橙色発光ルシフェラーゼ遺伝子が挿入されているpYM626-4からSalIとNotIでhIL-8 SLOカセットを切り出し、クローニングベクターに導入してphIL-8 SLOを構築した(図6)。phIL-8 SLOは、ScaI、KpnI/NdeI、SfiI、SnaBIでそれぞれ消化し、電気泳動によりバンドパターンを確認(図7)し、phIL-8 SLO #1を得た。hIL-8 SLO #1を人工染色体ベクター導入用ベクターpMGP4 Neoに導入し、pMGP4 Neo hIL-8 SLOを構築した。pMGP4 Neo hIL-8 SLOは、EcoRI、NcoIでそれぞれ消化し、電気泳動パターンを確認(図7)し、pMGP4 Neo hIL-8 SLO #20を得た。

人工染色体ベクターを保持したCHO細胞に構築したpMGP4 Neo hIL-8 SLO #20をトランスフェクションした。トランスフェクション6時間後に培地を交換し、トランスフェクション24時間後に細胞を継代した。

トランスフェクション48時間後に薬剤選択（Geneticin, 500μg/mL）を開始した。薬剤選択耐性クローンを取得し、ゲノムPCRで人工染色体ベクター上の目的部位にモニターカセットが導入できていることを確認した。さらに2ヵ所のPCRを行い、すべて陽性のクローンのFISH解析を行った結果（図8）、hIL-8モニターカセット搭載人工染色体ベクターを保持するCHO細胞（クローン番号：hIL-8 SLO/MGP CHO #6）を取得した。来年度以降、THP-1細胞に導入し標準発光細胞として機能するか検証する。

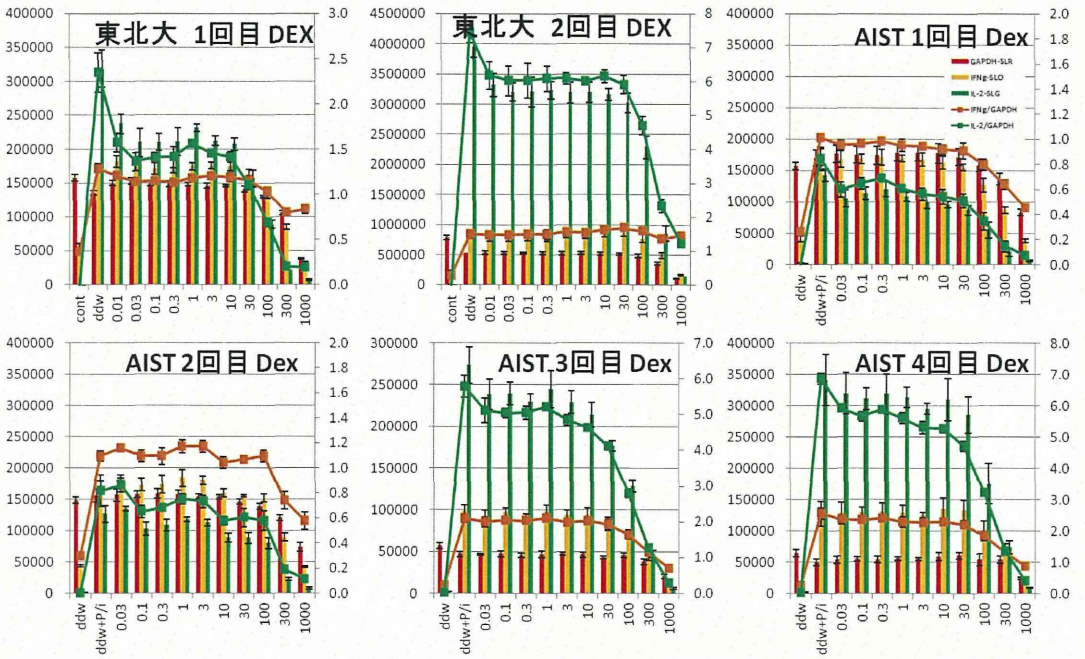

図1　細胞株 2H4 における DXE 刺激に対する細胞応答性

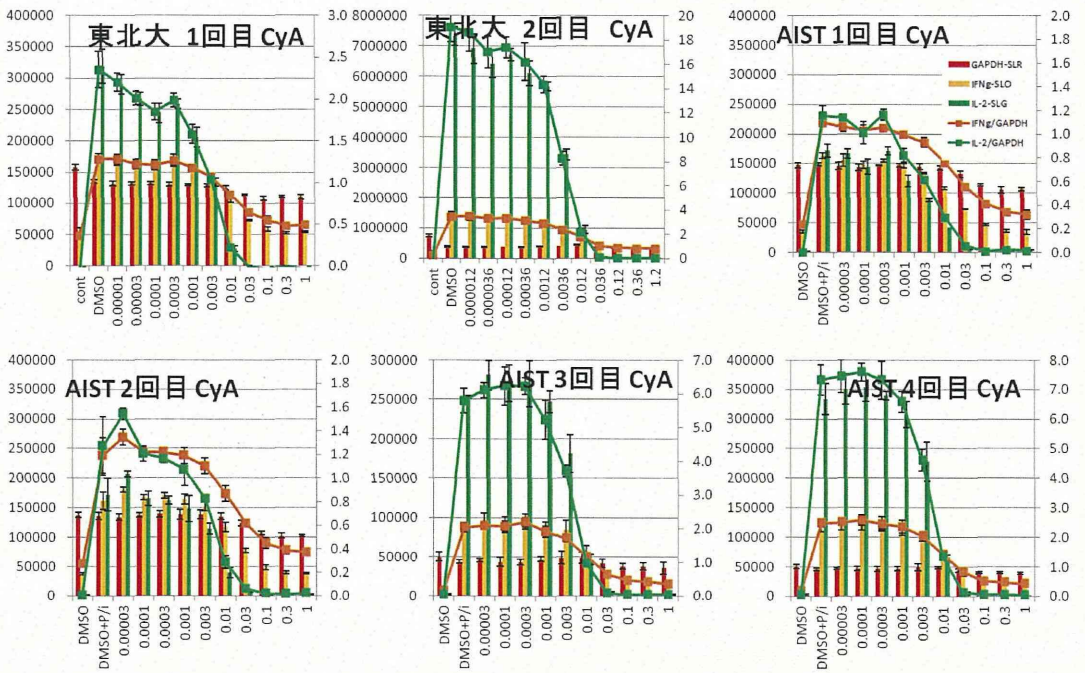

図2　細胞株 2H4 における CyA 刺激に対する細胞応答性

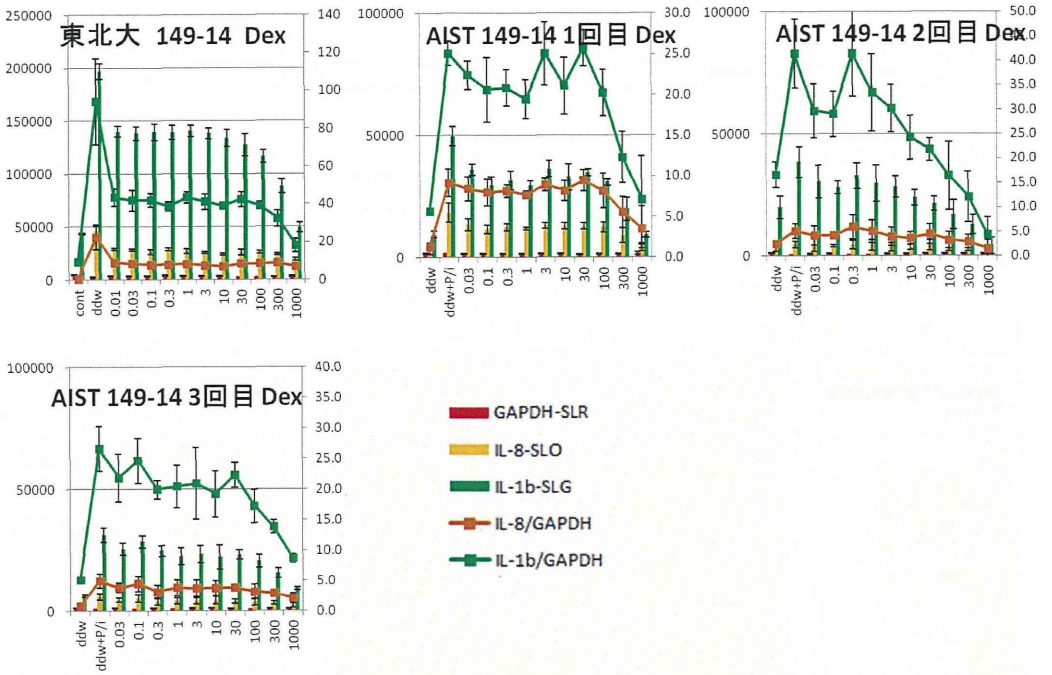

図３　細胞株 6C12 における DXE 刺激に対する細胞応答性

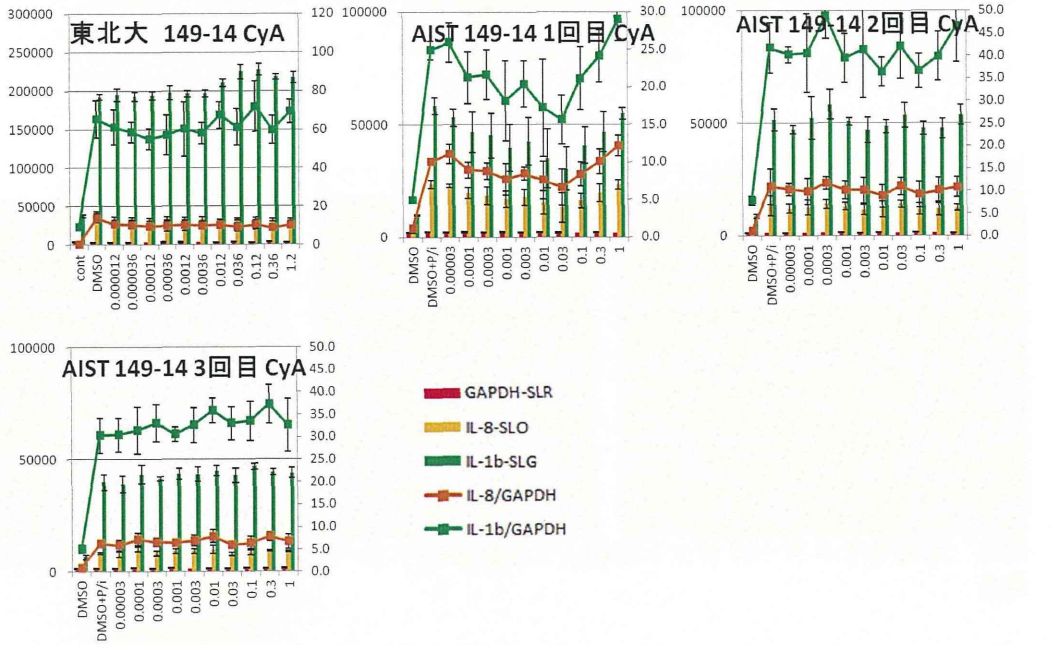

図４　細胞株 6C12 における CyA 刺激に対する細胞応答性

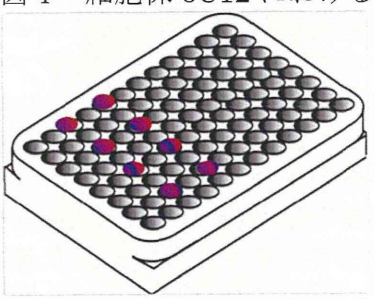

図５　3 色の LED を用いた発光プレートの概要

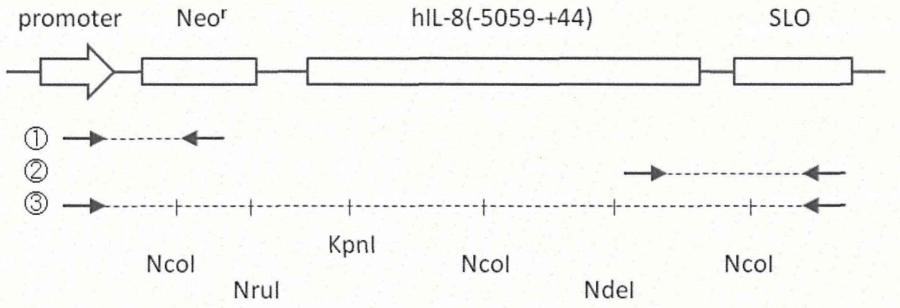

図６　phIL-8 SLO の遺伝子マップ

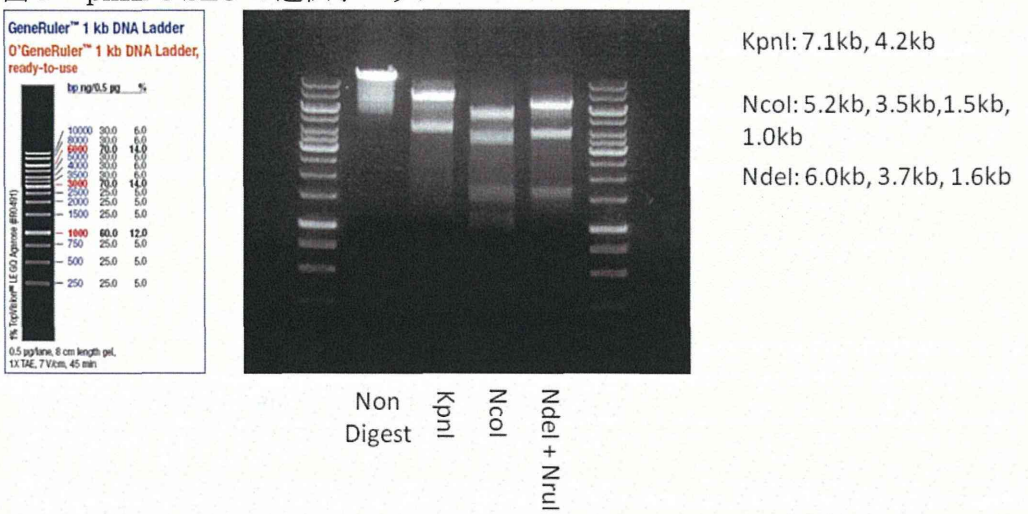

図７　phIL-8 SLO の遺伝子の制限酵素切断によるフラグメント解析

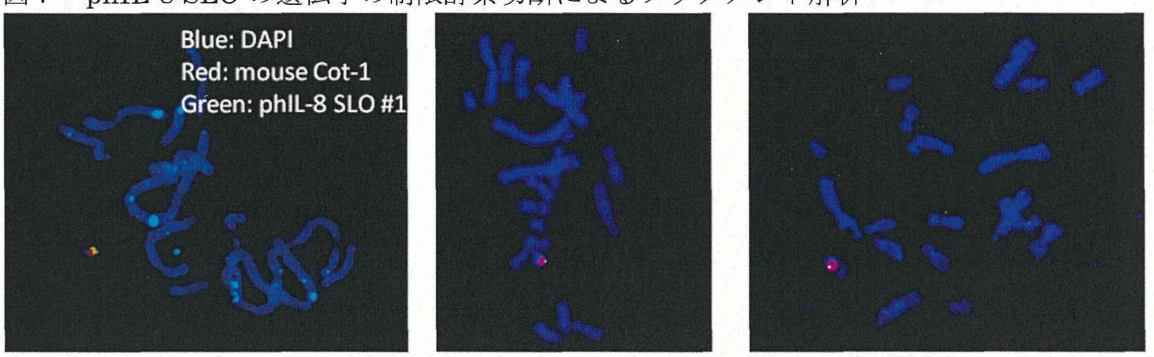

図８　hIL-8 モニターカセット搭載人工染色体ベクターを保持する CHO 細胞の FISH 解析

D. 考察

新たな*in vitro*免疫毒性評価試験法開発に当たり、ラボ間プレベリデーション試験の実施と精度管理法の開発を行った。東北大学病院で樹立された立した細胞株2H4 Jurkat細胞及び細胞株6C12　THP-１細胞を元に確立された実験手法を産業技術総合研究所に技術移転、プレバリデーション試験を行った結果、再現性の高い手法であることを確認した。今後、各種化学物質に対する毒性試験を実施する予定である。

MITA法の精度管理は2つのアプローチで達成することとした。第一のアプローチは発光測定装置ルミノメーターの精度管理であり、このための多色発光標準プレートの試作を実施中である。一方、発光細胞自身の発光の減弱等の細胞自体の管理を達成するため、挿入された遺伝子の発現が一定となるコントロール発光細胞を構築することを計画、人工染色体にIL-8プロモータでドライブされる発光遺

伝子ベクターを挿入、CHO細胞への導入に成功した。今後、THP細胞に導入しコントロール発光細胞とする予定である。

E. 結論

新たな*in vitro*免疫毒性評価試験法開発に当たり、MITA法のバリデーション試験の準備段階を終了した。一方、MITA法の精度管理のための多色発光標準プレートの試作、及びコントロール発光細胞の開発に着手した。

F. 参考文献

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G. 研究発表

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厚生労働科学研究費補助金（化学リスク研究事業）多色発光細胞を用いたhigh-throughput免疫毒性評価試験法の開発

分担研究報告書

化学物質のMulti-ImmunoTox assayによる解析

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研究要旨

Multi-ImmunoTox assay（MITA）による免疫毒性評価法のバリデーション試験を実施するにあたり、代表的4物質の反応性を確認し、良好な技術移転性が確認された。そこで、本法の施設内再現性および施設間再現性を検討するために、さらに新たな10物質について解析を行った。その結果、3回の反復実験でほぼ同様の反応性が確認され、本法は良好な施設内再現性を示すと考えられた。施設間再現性についても良好であることが示唆された。

キーワード：免疫毒性、動物実験代替法、*in　vitro*

A．研究目的

化学物質の免疫毒性の評価法として開発された Multi-ImmunoTox assay（MITA)は、Jurkat 細胞における IL-2, IFN-γ, G3PDH の各プロモーター活性および THP-1 細胞における IL-1β, IL-8, G3PDH の各プロモーター活性を定量化できる安定細胞株を用いた評価系である。平成 25 年度は、本法の技術移転確認、および代表的な免疫抑制剤を用いて本法が免疫毒性を正確に評価できるか否かを検討することを目的として研究を行った。

B．研究方法

B-1）MITAに用いた細胞

緑、橙、赤色の発光色の異なるルシフェラーゼ遺伝子をIL-2, IFN-γ, G3PDHの各プロモーター領域に繋いだベクター（それぞれ緑、橙、赤色）をJurkat細胞に導入した安定細胞株#2H4、および発光色の異なるルシフェラーゼ遺伝子をIL-1β, IL-8, G3PDHの各プロモーター領域に繋いだベクター（それぞれ緑、橙、赤色）をTHP-1細胞に導入した安定細胞株#149-14を使用した。このうち、#149-14の内標であるG3PDHが機能していないことから、IL-8, G3PDHの各プロモーター領域にそれぞれ橙、赤色のルシフェラーゼ遺伝子を繋いだベクターをTHP-1細胞に導入した安定細胞株#THP-G8を同条件で処理して、内標の代用とした。いずれの細胞株も、東北大学皮膚科より供与されたものを用いた。

B-2）使用した化学物質

主に技術移転性を確認する目的で4物質（$CoCl_2$、$NiCl_2$、Isophorone diisocyanate、2-MBT）を用いた。また、施設内再現性および施設間再現性を検討するために、以下の10物質を用いた。

① Benzethonium chloride（蒸留水を用いて100 mg/mlの原液調製）
② Dapsone（DMSO を用いて250 mg/mlの原液調製）
③ Acetaminophen （DMSOを用いて300

mg/mlの原液調製）

④ Ethanol（蒸留水を用いて100 mg/mlの原液調製）

⑤ Dibutyl phtahlate （DMSOを用いて500 mg/mlの原液調製）

⑥ Sodium bromate（蒸留水を用いて100 mg/mlの原液調製）

⑦ 5-Nitro-2-furaldehyde semicarbazone（DMSO を用いて125 mg/mlの原液調製）

⑧ Aluminium(III) chloride hexahydrate（蒸留水を用いて100 mg/mlの原液調製）

⑨ Chlorpromazine hydrochloride（蒸留水を用いて100 mg/mlの原液調製）

⑩ 4-Nitroanilin （DMSO を用いて500 mg/mlの原液調製）

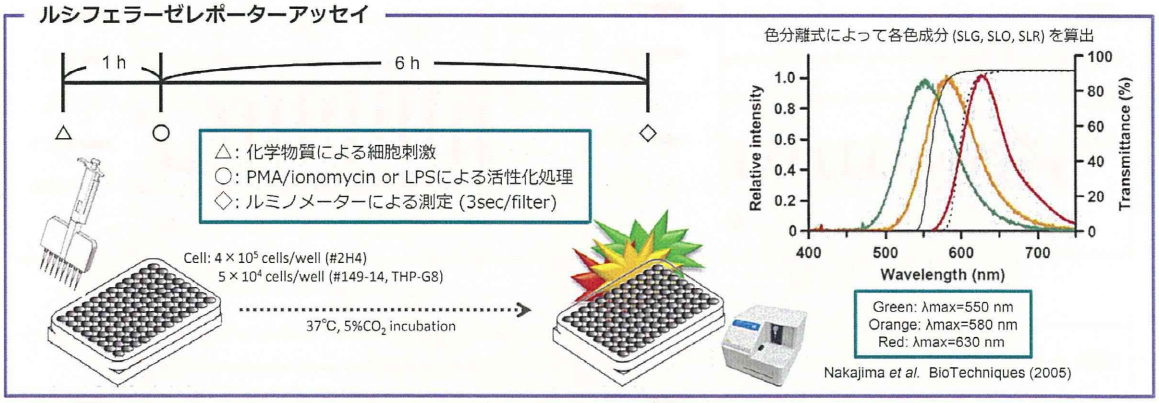
B-3）実験方法

基本的には、Multi-Immuno Tox Assay バリデーションプロトコール平成24年11月13日ver. 001.1準じて実験を行った。概要としては、各細胞を96wellプレートに播種し、各種濃度で化学物質を添加した。添加濃度は、最高血中濃度（Cmax）の100倍濃度から3倍希釈による10段階希釈で行った。1時間後にPMA/ionomycin（#2H4細胞）もしくはLPS（#149-14細胞、#THP-G8細胞）による活性化処理を行い、6時間後に各色ルシフェラーゼ活性を測定し、色分離式により各プロモーター活性を算出した（図1参照）。

C. 結果

技術移転性を主目的として実施した4物質の結果は、他の施設（東北大皮膚科、産業総合研究所）とほぼ同様であり、良好な結果が得られた。この結果を受け、さらに施設内および施設間再現性を検討するために10物質のMITAを実施した。なお、1物質につき3回の繰り返し実験を同日以外で実施した。その一部の結果を図2、3に示した。

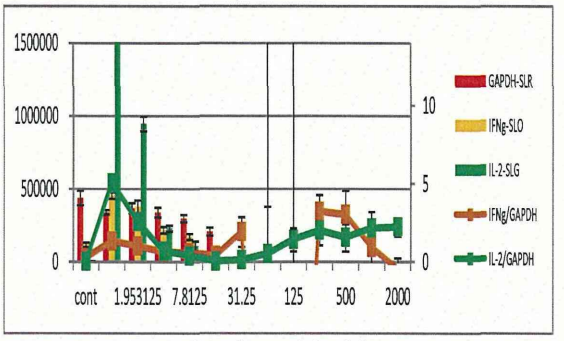
図1　MITAの試験法概要



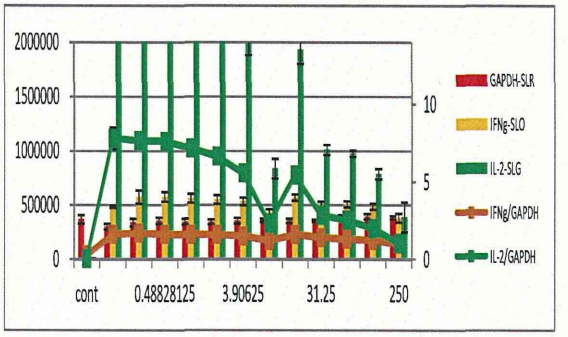
プレートデザイン (96-well)

cont	被験物質Aの溶媒のみ	0.003x Cmax	0.01x Cmax	0.03x Cmax	0.1x Cmax	0.3x Cmax	1x Cmax	3x Cmax	10x Cmax	30x Cmax	100x Cmax
	被験物質A（1/3希釈、10段階、n=4）										
cont	被験物質Bの溶媒のみ	0.003x Cmax	0.01x Cmax	0.03x Cmax	0.1x Cmax	0.3x Cmax	1x Cmax	3x Cmax	10x Cmax	30x Cmax	100x Cmax
	被験物質B（1/3希釈、10段階、n=4）										

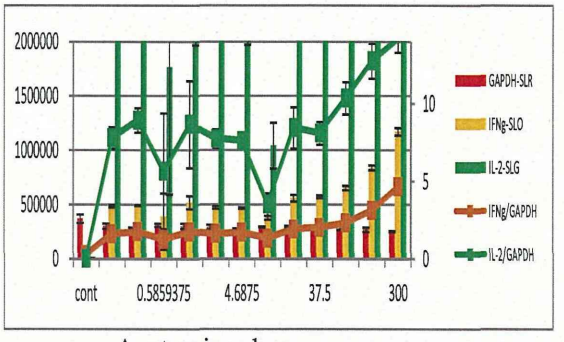
図２　#2H4 細胞を用いた 10 物質の結果



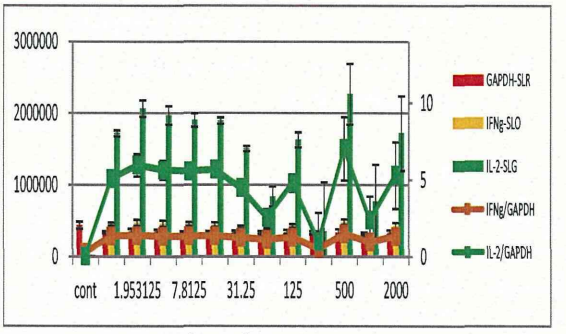
Benzethonium chloride



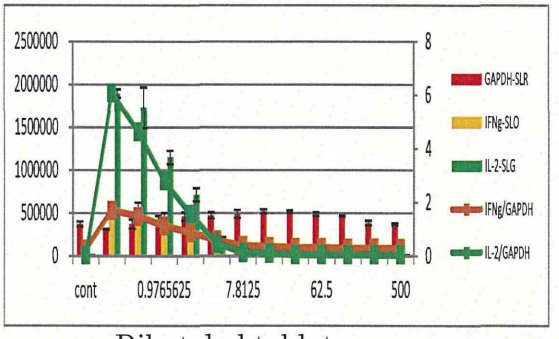
Dapsone



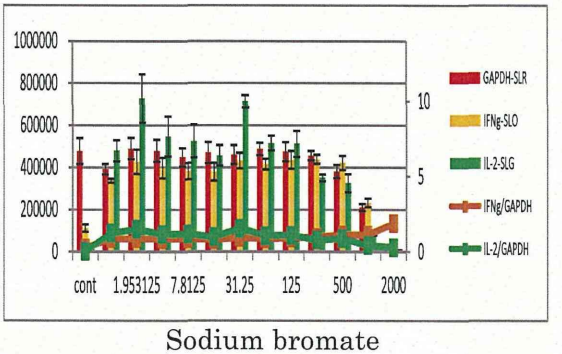
Acetaminophen



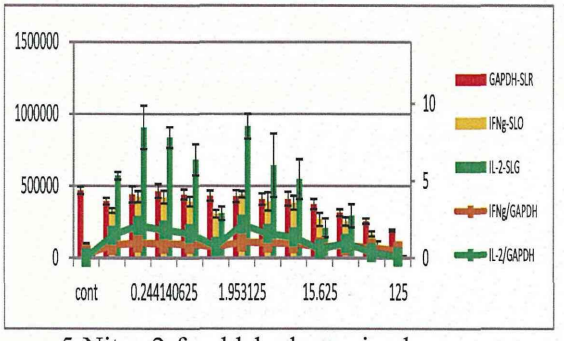
Ethanol



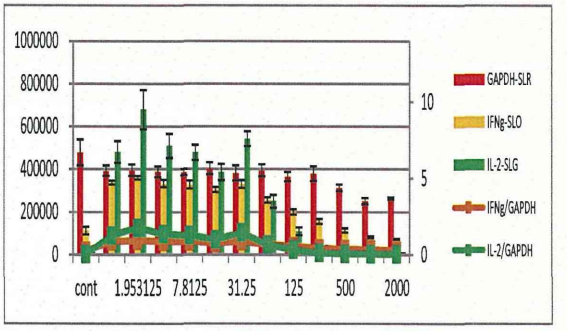
Dibutyl phtahlate



Sodium bromate

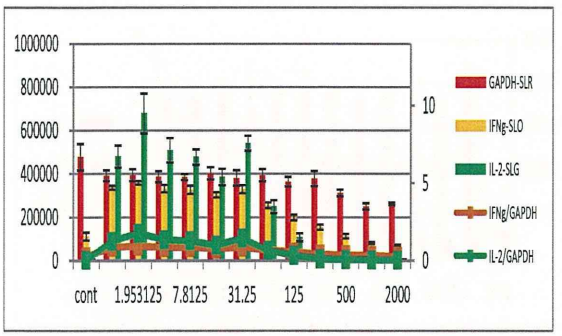


5-Nitro-2-furaldehyde semicarbazone

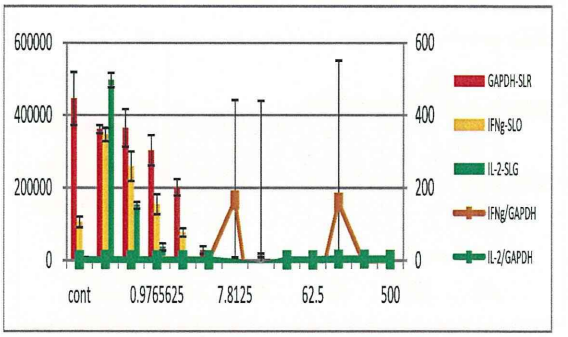


Aluminium(III) chloride hexahydrate

図２　#2H4 細胞を用いた 10 物質の結果（続き）

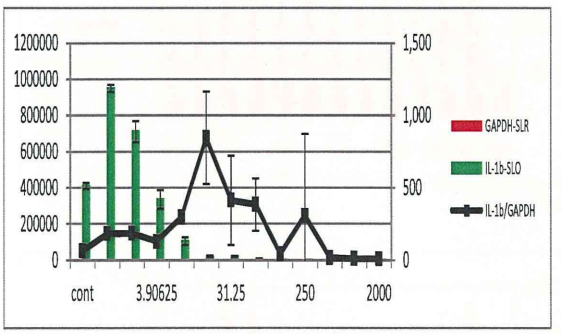


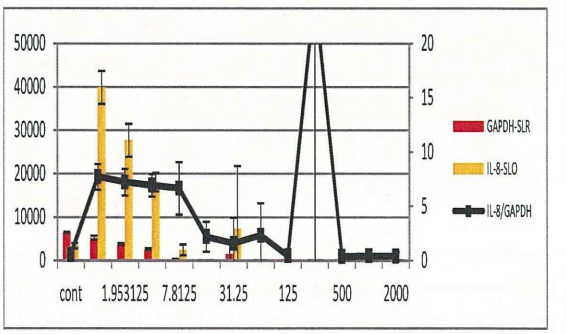
Chlorpromazine hydrochloride



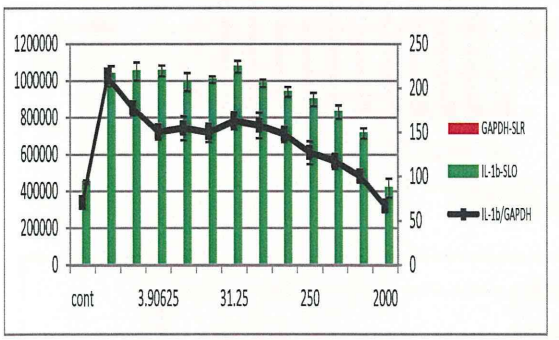
4-Nitroanilin

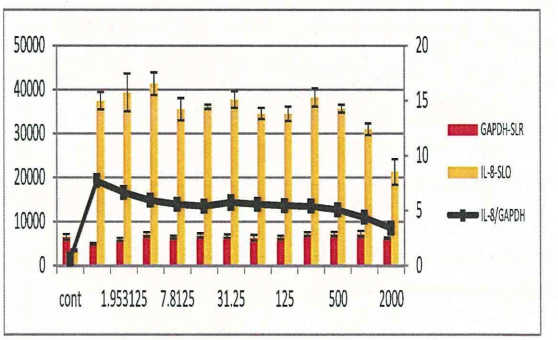
図３　#149-14 細胞（左）および#THP-G8 細胞（右）を用いた 10 物質の結果



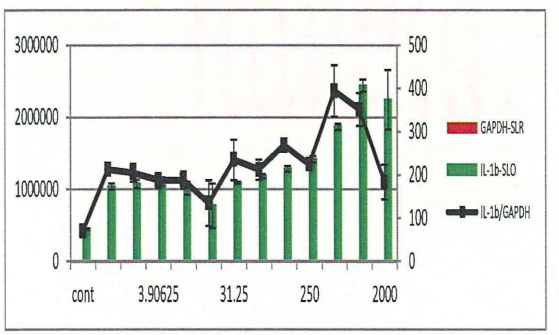


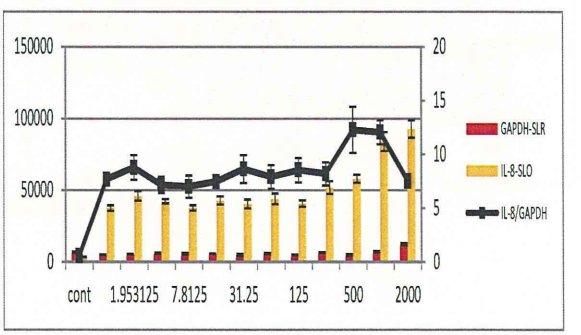
Benzethonium chloride





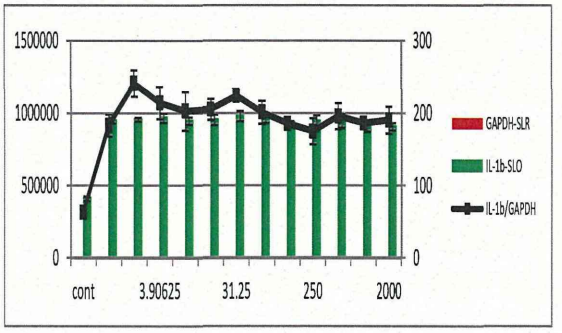
Dapsone

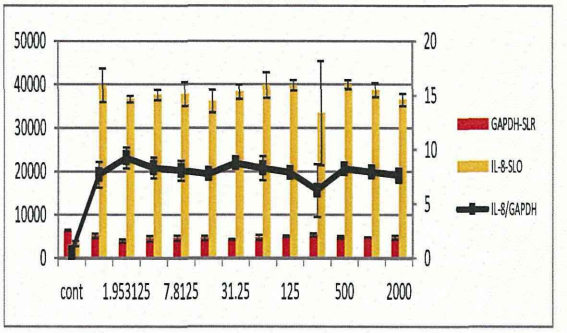




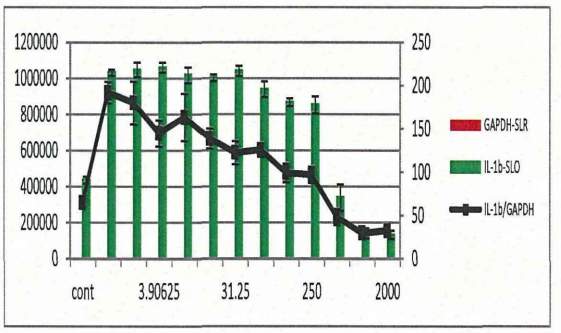
Acetaminophen

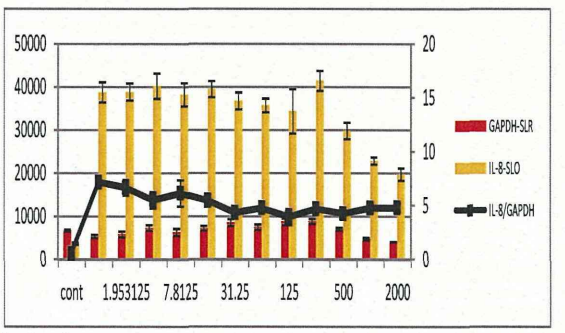
図３　#149-14 細胞（左）および#THP-G8 細胞（右）を用いた 10 物質の結果（続き）



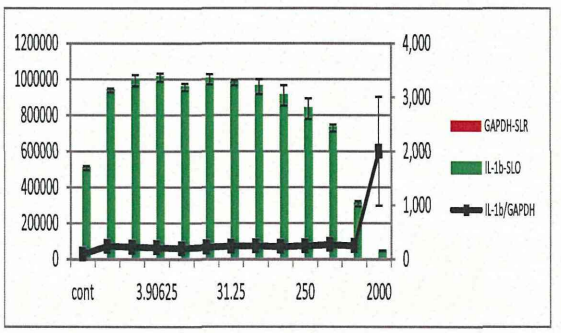


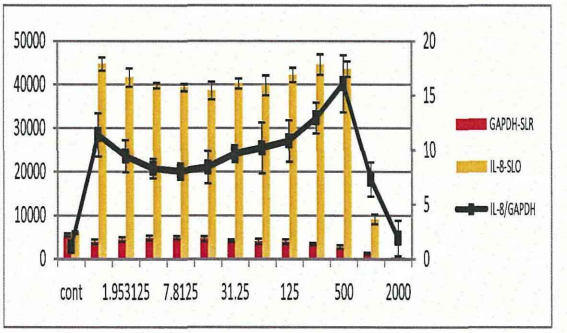
Ethanol



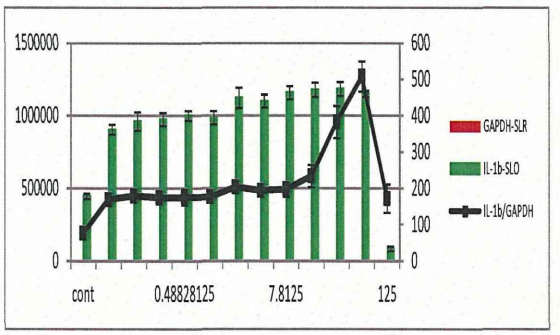


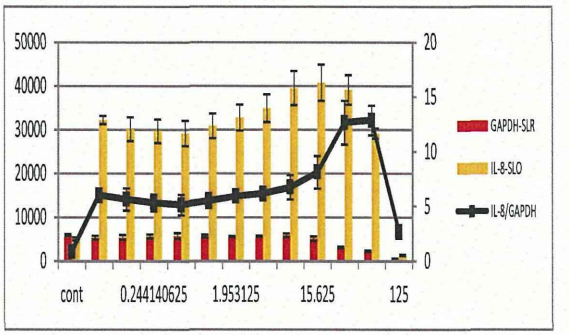
Dibutyl phthalate





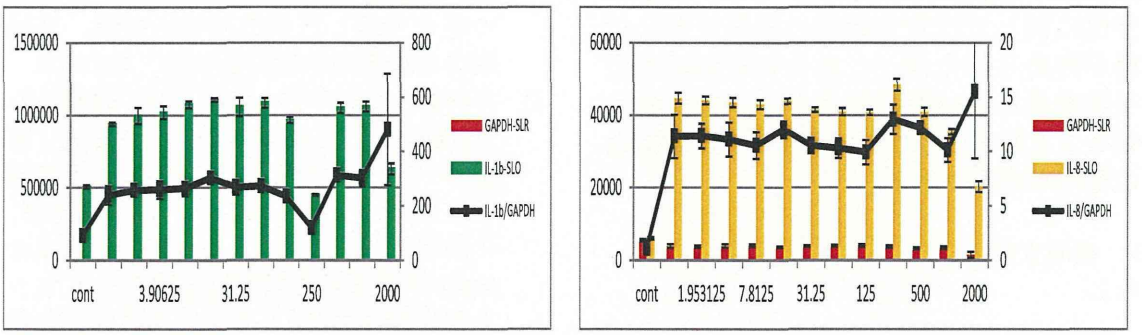
Sodium bromate



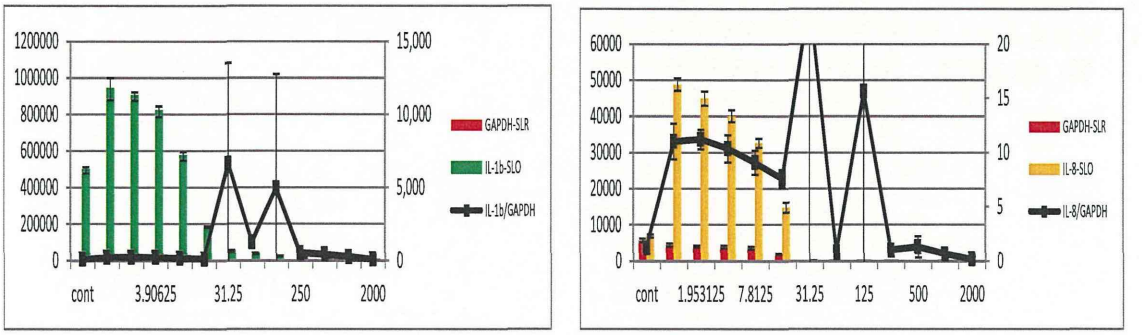


5-Nitro-2-furaldehyde semicarbazone

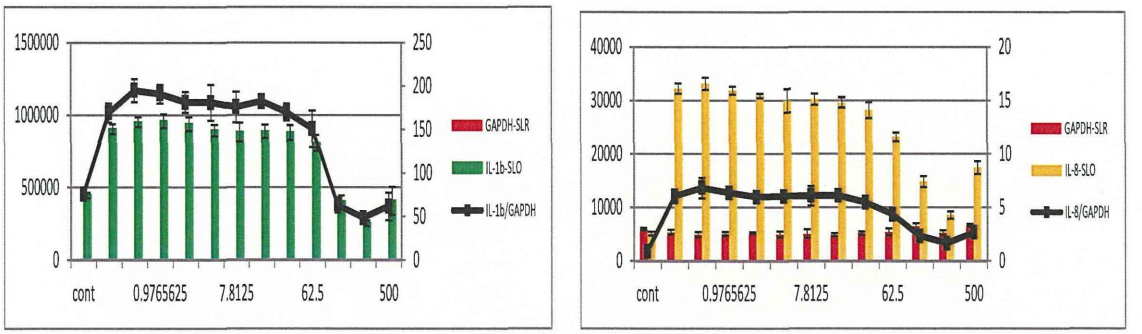
図３　#149-14 細胞（左）および#THP-G8 細胞（右）を用いた 10 物質の結果（続き）



Aluminium(III) chloride hexahydrate



Chlorpromazine hydrochloride



4-Nitroanilin

D. 考察

新たな*in vitro*毒性評価試験法開発に際しては、技術移転性、施設内再現性および施設間再現性の高い試験法開発は重要な課題である。今回我々は、*in vitro*免疫毒性評価を目的に東北大学病院で開発されたMITAの有用性を確認するために、14物質について3回の反復実験を実施した。その結果、最終的判定法については、未確立の状況ではあるが、各物質の全体的反応性を見る限り、良好な施設内再現性が得られる試験系であると考えられた。また、施設間比較においてもほぼ同様の反応性が認められていることから、施設間再現性も高い試験系であると考えられる。しかしながら、今回の我々結果の詳細に検討した場合、強い毒性（G3PDHの低発現）を示す濃度範囲以外で濃度依存性を逸脱するような結果が散見されることから、その原因を検討しさらに精度の高い試験系の確立が必要かもしれない。

今後は、さらに多数の物質を試験し、免疫抑制物質の予測性も加味した新たな試験系としての有用性を検討する。

E. 結論

技術移転性、施設内再現性および施設間

再現性を確認するために、合計14物質について、3回の繰り返し実験を実施した。全体的に良好な結果が得られた。なお、濃度依存性に関しては若干確認の必要な懸念材料が認められたが、これらの検討結果を手技改良あるいはプロトコールに反映させることにより、さらに精度の高いMITAの確率が期待される。

F. 参考文献

なし

G. 研究発表

1. 学会発表

1) 丸谷あおい，相場節也，木村裕，渡辺美香，鈴木紀之，岩城知子，山影康次，斎藤幸一，中島芳浩，近江谷克裕，山崎晶次郎，小島肇，田中憲穂，小林眞弓，森梓，大森崇：IL-8 Luc assayにおけるばらつきを考慮した判定基準の提案，第26回日本動物実験代替法学会，2013.12.

2) 木村裕，藤村千鶴，渡辺美香，齋藤るみ子，鈴木紀之，岩城知子，山影康次，斎藤幸一，中島芳浩，近江谷克裕，酒井綾子，丸谷あおい，大森崇，山崎晶次郎，小島肇，田中憲穂，相場節也：IL-8 Luc assayの施設間差試験およびデータセットの作製，第26回日本動物実験代替法学会，2013.12.

III. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kimura Y, Fujimura C, Ito Y, Takahashi T, Aiba S.	Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs.	Toxicol in Vitro	28	759-768	2014
Onami K, Kimura Y, Ito Y, Yamauchi T, Yamasaki K, Aiba S.	Nonmetal Haptens Induce ATP Release from Keratinocytes through Opening of Pannexin Hemichannels by Reactive Oxygen Species.	J Invest Dermatol	in press		2014
Tsujita-Inoue K, Hirota M, Ashikaga T, Atobe T, Kouzuki H, Aiba S.	Skin sensitization risk assessment model using artificial neural network analysis of data from multiple in vitro assays.	Toxicol in Vitro	28	626-639	2014
Hirota M, Kouzuki H, Ashikaga T, Sono S, Tsujita K, Sasa H, Aiba S.	Artificial neural network analysis of data from multiple in vitro assays for prediction of skin sensitization potency of chemicals.	Toxicol in Vitro	27	1233-1246	2013

Toxicology in Vitro 28 (2014) 759–768

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journal homepage: www.elsevier.com/locate/toxinvit

Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs

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Alternatives to animals

ABSTRACT

We established a luciferase reporter assay system, the Multi-ImmunoTox Assay (MITA), to evaluate the effects on key predictive *in vitro* components of the human immune system. The system is composed of 3 stable reporter cell lines transfected with 3 luciferase genes, SLG, SLO, and SLR, under the control of 4 cytokine promoters, IL-2, IFN-γ, IL-1β, and IL-8, and the G3PDH promoter. We first compared the effects of dexamethasone, cyclosporine, and tacrolimus on these cell lines stimulated with phorbol 12-myristate 13-acetate and ionomycin, or lipopolysaccharides, with those on mRNA expression by the mother cell lines and human whole blood cells after stimulation. The results demonstrated that MITA correctly reflected the change of mRNA of the mother cell lines and whole blood cells. Next, we evaluated other immunosuppressive drugs, off-label immunosuppressive drugs, and non-immunomodulatory drugs. Although MITA did not detect immunosuppressive effects of either alkylating agents or antimetabolites, it could demonstrate those of the off-label immunosuppressive drugs, sulfasalazine, chloroquine, minocycline, and nicotinamide. Compared with the published immunological effects of the drugs, these data suggest that MITA can present a novel high-throughput approach to detect immunological effects of chemicals other than those that induce immunosuppressive effects through their inhibitory action on cell division.

1. Introduction

Environmental contaminants, food additives, and drugs can target the immune system, resulting in adverse health effects, such as the development of allergies, autoimmune disorders, cancers, and other diseases. Accordingly, immunotoxicity, which is defined as the toxicological effects of xenobiotics on the functioning of the immune system, has raised serious concerns from the public as well as regulatory agencies. Currently, the assessment of chemical immunotoxicity relies on animal models and assays that characterize immunosuppression and sensitization. However, animal studies have many drawbacks, such as expense, ethical concerns, and eventual relevance to risk assessment for humans. Therefore, European policy is promoting alternative testing methods and assessment strategies to reduce the use of laboratory animals and, if possible, replace animals employed for scientific studies (Balls et al., 1995).

A workshop hosted by the European Centre for the Validation of Alternative Methods (ECVAM) in 2003 focused on state-of-the-art *in vitro* systems for evaluating immunotoxicity (Galbiati et al., 2010; Gennari et al., 2005; Lankveld et al., 2010). In that workshop, a tiered approach was proposed, since useful information can be obtained from regular 28-day general toxicity tests. Namely, prescreening for direct immunotoxicity starts with the evaluation of myelotoxicity. Compounds that are capable of damaging or destroying the bone marrow will most likely have immunotoxic effects. If compounds are not potentially myelotoxic, they are tested for lymphotoxicity. Then, they are tested for immunotoxicity by approaches such as human whole-blood cytokine release assay (HWBCRA), lymphocyte proliferation assay, mixed lymphocyte reaction, natural killer cell assay, T-cell-dependent antibody response, dendritic cell maturation, and fluorescent cell chip. Among these assays, HWBCRA has undergone formal prevalidation, although other techniques are being examined or have been previously examined in a rigorous prevalidation effort by ECVAM and other groups.

The principle of HWBCRA, described by Langezaal et al. (2002), is based on the well-known human whole-blood method for pyrogen testing (Hartung, 2002). In brief, human blood is treated with lipopolysaccharide (LPS) or staphylococcal enterotoxin B (SEB),

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which causes monocytes and Th2 lymphocytes to produce IL-1β and IL-4, respectively. After incubation for 40 h in the presence or absence of immunotoxic and non-immunotoxic test compounds, the levels of IL-1β and IL-4 in the supernatant are quantified, and the 50% inhibitory concentration (IC50) and the fourfold stimulating concentration (SC4) are calculated to establish the immunotoxic potency (Langezaal et al., 2002). According to the ECVAM workshop, this method has several advantages, such as the avoidance of species differences between humans and animals, employment of human primary cells, simple culture techniques, and reduced expense and time requirements as compared to animal experiments. The interindividual variation in leukocyte numbers and their response to stimuli is a major concern when using HWBCRA. Although cryopreservation techniques for human whole blood can overcome these problems (Schindler and Hartung, 2002), this method is not suitable as a high-throughput assay to evaluate vast numbers of chemicals.

In the present study, to develop a high-throughput screening system to evaluate chemical immunotoxicity, we first established 3 stable reporter cell lines transfected with luciferase genes under the control of IL-2, IFN-γ, IL-8, and IL-1β promoters. We selected these 4 cytokines because IL-2 and IFN-γ are mainly produced by T cells and reflect T-cell function, while IL-8 and IL-1β are mostly produced by monocytes or dendritic cells and correspond with their activity. Next, we examined the effects of 3 well-characterized immunosuppressive drugs, dexamethasone (Dex), cyclosporine A (CyA), and tacrolimus (Tac), on luciferase activities of these three cell lines stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io) or lipopolysaccharide (LPS). Then, we compared the results with their effects on mRNA expression by the mother cell lines, Jurkat cells or THP-1 cells, under the relevant stimulation. Furthermore, we also compared their effects on luciferase activities with mRNA expression by human whole blood cells stimulated with PMA/Io or LPS in the presence of these immunosuppressive drugs. Finally, we treated these cell lines with immunosuppressive drugs, immunomodulatory drugs, or drugs without known immunomodulatory effects and estimated the performance of our screening system for immunotoxicity.

2. Materials and methods

2.1. Reagents

Water-soluble dexamethasone (Dex), cyclosporin A (CyA), tacrolimus (FK-506), rapamycin, cyclophosphamide (CP), azathioprine (AZ), mycophenolic acid (MPA), mizoribine (MZR), methotrexate (MTX), sulfasalazine (SASP), colchicine, chloroquine (CQ), minocycline (MC), nicotinamide (NA), acetaminophen (AA), digoxin, warfarin, phorbol 12-myristate 13-acetate (PMA), ionomycin (Io), and lipopolysaccharides from E. coli 026:B6 (LPS) were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Cell lines and reporter cell lines

The human acute T lymphoblastic leukemia cell line Jurkat and the human acute monocytic leukemia cell line THP-1 (ATCC, Manassas, VA) were cultured in RPMI-1640 (Gibco, Carlsbad, CA) with antibiotic–antimycotic (Invitrogen, Carlsbad, CA) and 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) (Growth medium) at 37 °C with 5% CO_2. We previously established 2 reporter cell lines, #2H4 derived from Jurkat cells containing stable luciferase green (SLG) regulated by IL-2 promoter, stable luciferase orange (SLO) regulated by IFN-γ promoter, and stable luciferase red (SLR) regulated by G3PDH promoter (Saito et al., 2011) and THP-G8 cells derived from THP-1 cells containing SLO regulated by IL-8 promoter and SLR regulated by G3PDH promoter (Takahashi et al., 2011).

In the present study, we further established THP-G1b cells derived from THP-1 cells containing SLG regulated by IL-1β promoter and SLR by G3PDH promoter. Full details are available in Supplementary Methods.

2.3. Chemical treatment

Based on the previous reports (Saito et al., 2011; Takahashi et al., 2011), #2H4 cells (2×10^5 cells/50 μl/well), THP-G1b cells, or THP-G8 cells (5×10^4 cells/50 μl/well) in 96-well black plates (Greiner bio-one GmbH, Frickenhausen, Germany) were pretreated with different concentrations of chemicals for 1 h. The optimum cell numbers at seeding were based on the previous reports. Afterwards, #2H4 cells were stimulated with 25 nM of PMA and 1 μM of ionomycin (PMA/Io) for 6 h, while THP-G1b cells or THP-G8 cells were stimulated with 100 ng/ml of LPS for 6 h. In some experiments, we changed the stimulation time to determine the optimum incubation period for the luciferase assay. Three luciferase activities, SLG luciferase activity (SLG-LA), SLO luciferase activity (SLO-LA), and SLR luciferase activity (SLR-LA), were simultaneously determined by using a microplate-type luminometer with a multicolor detection system, Phelios (Atto Co., Tokyo, Japan), and the Tripluc luciferase assay reagent (TOYOBO) according to the manufacturer's instructions. To rule out the variation of cell number or cell viability after chemical treatment, we obtained normalized luciferase activity as follows:

Normalized SLG-LA (nSLG-LA) or normalized SLO-LA (nSLO-LA) = SLG-LA or SLO-LA/SLR-LA.

We also calculated percent suppression as follows:

% suppression = (1 − nSLG-LA or nSLO-LA of the reporter cells treated with drugs/nSLG-LA or nSLO-LA of non-treated reporter cells) × 100.

To eliminate the data affected by cytotoxic effects of drugs or cell death, we also defined the inhibition index of SLR-LA (II-SLR-LA) as follows:

II-SLR-LA = SLR-LA of reporter cells that were treated with chemicals/SLR-LA of untreated reporter cells.

Since our previous study has reported that, in the treatment showing more than 5% in II-SLR-LA, more than 75% of cells are PI-excluding living cells (Takahashi et al., 2011), we presented only the data that demonstrated more than 5% in II-SLR-LA in this study.

2.4. Human whole-blood cytokine mRNA expression test (HWBCMET)

The human whole-blood cytokine mRNA expression test (HWBCMET) was performed by modifying the HWBCRA protocol by Langezaal et al. (2002)) and Thurm and Halsey (2005). The following studies were approved by the ethics committee of Tohoku University Graduate School of Medicine, Sendai, Japan, and conducted according to the Declaration of Helsinki principles. Full details are available in Supplementary Methods.

2.5. mRNA expression by Jurkat and THP-1 cells

Jurkat or THP-1 cells (3×10^6 cells) in 6-well plates were pretreated with different concentrations of drugs for 1 h and then stimulated with PMA/Io or LPS for 6 h, respectively. Total RNA was extracted by using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The total RNA concentration was measured by using a NanoDrop spectrophotometer.

2.6. Quantitative RT-PCR

Complementary DNAs (cDNAs) were synthesized by using the TaKaRa RNA PCR Kit (AMV) (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed by using the Mx3000p QPCR System (Stratagene; Agilent Technologies Division, Santa Clara, CA). Sequences for each target gene were obtained from GenBank. Forward and reverse primers and TaqMan probes were selected by Primer Express 1.0 (Applied Biosystems) and synthesized by SIGMA GENOSYS (Ishikari, Japan). Each primer and TaqMan probe set used is described in our previous publication (Saito et al., 2011). qPCR reaction mixtures (25 μl) contained 10 ng of template cDNA, 400 nM of forward and reverse primers, 60 nM TaqMan probe, 30 nM ROX, and Brilliant II Fast QPCR Master Mix (Stratagene; Agilent Technologies Division). The thermal cycling conditions were 2 min for polymerase activation and cDNA denaturation at 95 °C and 45 cycles of 95 °C for 5 s and 60 °C for 20 s. Constitutively expressed G3PDH served as a normalization control by using the ΔΔCt method (Livak and Schmittgen, 2001). In the examination of mRNA from whole blood cells, percent suppression was calculated as follows:

% suppression = (1 − the normalized mRNA expression of WBC in the presence of drugs/the normalized mRNA expression of WBC in the absence of drugs) × 100.

2.7. Statistics

Representative data from at least three independent experiments for each analysis is shown. For each experiment, a one-way ANOVA test followed by Dunnett's post hoc test was used to evaluate statistical significance. For comparison of three independent experiments, the Student's *t*-test was used to evaluate statistical significance. *p* values <0.05 were considered statistically significant.

3. Results

3.1. Three reporter cell lines, #2H4, THP-G1b, and THP-G8, responded with relevant stimulations by augmenting their SLG-LA or SLO-LA

First, we stimulated #2H4 cells with PMA/Io and THP-G1b or THP-G8 cells with LPS and measured SLG-LA, SLO-LA, and SLR-LA after stimulation. PMA/Io significantly augmented SLG(IL2)-LA and SLO(IFN)-LA of #2H4 cells corresponding with IL-2 and IFN-γ promoter activities, respectively, from 6 h after stimulation, while it suppressed SLR(G3PDH)-LA corresponding with G3PDH promoter activity (Fig. 1A). Similarly, LPS significantly augmented SLG(IL1)-LA of THP-G1b cells corresponding with IL-1β promoter activity and SLO(IL8)-LA of THP-G8 cells corresponding with IL-8 promoter activity without affecting SLR(G3PDH)-LA of both cell lines from 3 h after stimulation (Fig. 1B and C). In Fig. 1A, B, and C, we also presented nSLG(IL2)-LA and nSLO(IFN)-LA of #2H4 cells, nSLG(IL1)-LA of THP-G1b cells, and nSLO-LA(IL8) of THP-G8 cells at various time periods after stimulation. PMA/Io significantly and time-dependently augmented both nSLG(IL2)-LA and nSLO(IFN)-LA of #2H4 cells from 6 to 10 h after stimulation. On the other hand, LPS significantly and time-dependently augmented nSLG(IL1)-LA of THP-G1b cells from 4 to 9 h after stimulation, while it significantly augmented nSLO(IL8)-LA of THP-G8 cells from 3 to 10 h after stimulation, with maximum induction at 5 h.

3.2. The effects of 3 immunosuppressive drugs on the reporter activity of the three reporter cells correlate with their effects on mRNA expression by Jurkat or THP-1 cells

Next, we examined whether the effects of 3 well-characterized immunosuppressive drugs, Dex, CyA, and Tac, on nSLG-LA or nSLO-LA of 3 reporter cells stimulated with PMA/Io or LPS correlate with their effects on the corresponding mRNA expression by Jurkat or THP-1 cells (Fig. 2). When we stimulated #2H4 cells with PMA/Io in the presence of Dex, CyA or Tac, Dex significantly suppressed nSLG(IL2)-LA at concentrations of 0.01 μg/ml and greater (≥0.01 μg/ml) and nSLO(IFN)-LA at ≥100 μg/ml, while CyA suppressed nSLG(IL2)-LA at ≥0.03 ng/ml and nSLO(IFN)-LA at ≥0.001 μg/ml, and Tac suppressed nSLG(IL2)-LA and nSLO(IFN)-LA at ≥0.016 ng/ml. Although Dex suppressed nSLO(IFN)-LA, the concentration to decrease nSLO(IFN)-LA was greater than 100 μg/ml, and the magnitude of the suppression was small. When we stimulated THP-G1b cells with LPS in the presence of Dex, CyA, or Tac, Dex significantly suppressed nSLG(IL1)-LA at ≥0.01 μg/ml, but CyA and Tac did not. Similarly, when THP-G8 cells were stimulated with LPS in the presence of Dex, CyA, or Tac, only Dex significantly suppressed nSLO(IL8)-LA at ≥0.01 μg/ml.

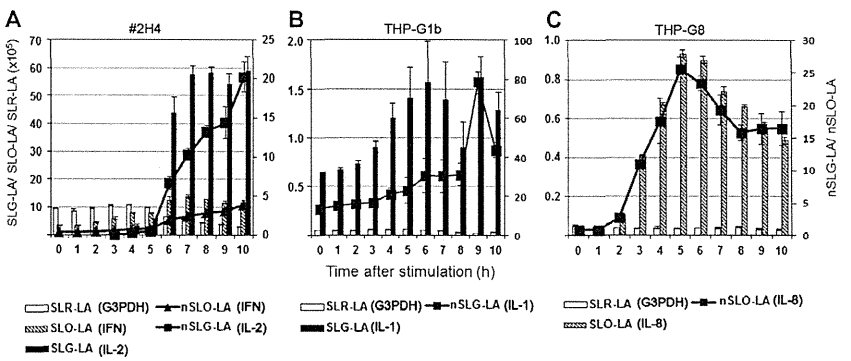

Fig. 1. Time course of IL-2, IFN-γ, IL-1β, and IL-8 reporter activities in PMA/Io-stimulated #2H4 cells and LPS-stimulated THP-G1b or THP-G8 cells. #2H4 cells (2×10^5 cells/100 μl/well) (A) in 96-well black plates were stimulated with PMA/Io, while THP-G1b (B) or THP-G8 cells (5×10^4 cells/100 μl/well) (C) were treated with LPS. Then, SLG-LA, SLO-LA, and SLR-LA were measured after stimulation by using a microplate-type luminometer with a multi-color detection system. To rule out the variation of cell number or cell viability after chemical treatment, normalized SLG luciferase activity (nSLG-LA) or SLO luciferase activity (nSLO-LA) was obtained by dividing SLG-LA or SLO-LA with SLR-LA. Data represent means +/− SD ($n = 4$). SLG-LA (IL-2), SLO-LA (IFN-γ), and SLR-LA (G3PDH) in A, SLG-LA (IL-1β) and SLR-LA (G3PDH) in B, and SLO-LA (IL-8) and SLR-LA (G3PDH) in C.