Dermatology

戸倉新樹＜*Tokura Yoshiki*＞
産業医科大学皮膚科学教授

特集：光アレルギーはいま…

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光線過敏症における光アレルギーの位置

Photoallergy as an important disorder in photosensitivity

要　約

光アレルギーは光がトリガーとなり，免疫学的機序を介して起こる皮膚疾患である．光アレルギーの範疇に入りうる疾患は，①光接触皮膚炎，②薬剤性光線過敏症，③日光蕁麻疹，④慢性光線性皮膚炎(CAD)である．①と②は外因性光感受性物質による疾患であり，③と④は原因物質がはっきりしない疾患である．これら4疾患について解説していただき，光アレルギーという分野を浮き上がらせるのが本企画の目的である．

光アレルギー

アレルギーはCoombsとGellの分類として4タイプに分かれる．これを光アレルギーに踏襲すると，光接触皮膚炎と薬剤性光線過敏症はⅣ型に属することになる．日光蕁麻疹のかなりの部分はⅠ型になるであろう．慢性光線性皮膚炎(CAD)は何型に属するかは不明であるが，T細胞が起こす疾患であり，Ⅳ型かⅡ型になる．

KEY WORDS／光アレルギー／光接触皮膚炎／薬剤性光線過敏症／日光蕁麻疹／慢性光線性皮膚炎

Photoallergy as an important disorder in photosensitivity

1 光線過敏症はいろいろな原因で起こる

光線過敏症は，太陽光線に当たった皮膚が赤くなるなどの異常な反応を起こす疾患の総称である．ひどい場合は水疱形成など熱傷様になることすらあり，決して皮膚疾患として軽いものばかりではない．光に当たりやすい顔，項部，耳，手背，前腕伸側，上胸部などに皮疹が生じ，臨床現場ではまず皮疹の分布状態により光線過敏症を疑うことになる．

光線過敏症の原因は表1のように多種多様である．これらのうちで色素性乾皮症は先天性の光線過敏症の代表的なものである．後天性のものには，ペラグラ，光接触皮膚炎，薬剤性光線過敏症，種痘様水疱症，日光蕁麻疹，多形日光疹，慢性光線性皮膚炎(chronic actinic dermatitis；CAD)がある．ペラグラは先天性のHartnup病と同じようにニコチン酸欠乏による代謝性疾患である．ポルフィリン症には晩発性皮膚ポルフィリン症(PCT)と骨髄性皮膚ポルフィリン症(EPP)がある．ペラグラとPCTはどちらもアルコール多飲によることが多い．EPPは意外と軽症例は見逃されていることがあり，アトピー性皮膚炎(atopic dermatitis；AD)と誤診されている例すらある．種痘様水疱症は，発症以前に慢性のEBウイルス感染が存在することを土台とする疾患と考えられる．多形日光疹はわが国では小丘疹性日光疹という軽い光線過敏性疾患である．

これらのなかで光アレルギーの範疇に入りうる疾患は，外因性光感受性物質による疾患である，①光接触皮膚炎と②薬剤性光線過敏症，そのほか，③日光蕁麻疹，④CADということになる．各疾患についてエキスパートの先生に解説していただくのが今回の特集のねらいである．

2 外因性光感受性物質がはっきりしている光アレルギー

光線過敏症には明瞭な光線過敏性物質が存在する場合と，そうでない場合とがある．さらに明瞭な物質が存在する場合には，光毒性機序によって生ずるものと，光アレルギー性機序によって生ずるものとがある．臨床的には光アレルギー性による頻度のほうが高い．

通常のアレルギーには薬疹，接触皮膚炎を代表とするように抗原物質が明瞭なものと，AD，蕁麻疹などのように必ずしもアレルゲンを決定し得ないものとがある．この事情は光アレルギーについても同様であり，薬剤性光線過敏症，光接触皮膚炎は抗原となる光感受性物質が明らかであり，そのほかは明確でない疾患となる．

光アレルギーのひとつの特殊性として，光がアレルギー症状発現に必須であるため，光が当たる臓器すなわち皮膚だけが病変形成の場となることにある．すなわち，光アレルギーの症状は皮膚炎のみである．別の見方をすれば，光アレルギーはアレルギーのメカニズムを比較的

表1　光線過敏症の原因別分類

表1　光線過敏症の原因別分類
1．外因性物質によるもの：光毒性または光アレルギー性機序 経皮：光接触皮膚炎 経口：薬剤性光線過敏症(光線過敏型薬疹)
2．内因性物質によるもの：光毒性 ポルフィリン症(PCT，EPP)，ペラグラ，Hartnup病
3．DNA修復機構の異常 色素性乾皮症，Cockayne症候群
4．EBウイルス関連 種痘様水疱症
5．メラニン色素減少による閾値低下 白皮症，フェニルケトン尿症
6．日光により増悪ないし誘発される疾患 エリテマトーデス
7．原因不明のもの 日光蕁麻疹，多形日光疹，慢性光線性皮膚炎(CAD)

ピュアに調べることのできるシステムともいえる.

光接触皮膚炎は抗原が皮膚に塗られて，紫外線が当たって発症する．一方，薬剤性光線過敏症は抗原が薬剤という形で経口投与されて，紫外線が当たって発症する．現在，光接触皮膚炎の原因にはケトプロフェン，スプロフェンなどのNSAIDsや，サンスクリーン剤がある．薬剤性光線過敏症の原因には，ニューキノロンをはじめとして多くの薬剤がある.

通常の抗原とは異なり，光アレルギー性物質が抗原となるには紫外線照射が必要となる．この紫外線の作用による抗原性の獲得については，古くよりいくつかの考えが提唱されてきたが，大きく2つの説に集約される．ひとつはプロハプテンであり，もうひとつは光ハプテンという考えである．プロハプテン説は，光アレルギー性物質はUV照射により化学構造の変化が起き，通常のハプテンのようになり，蛋白との結合能力を獲得する，という単純明快な説である．一方，光ハプテン説は，UV照射がなされるとその化学構造の一部が光分解され，その分解と同時に近傍の蛋白と共有結合し完全抗原ができあがるという考えである．したがって，あらかじめUVAを照射した物質が蛋白と結合すればプロハプテン，一方，その物質と蛋白との共存下でUVAを照射し，両者が共有結合すれば光ハプテンということになる.

多くの光抗原は光ハプテンとしての性格をもっている．したがって，当該物質が光線過敏症の原因になっているかを検証するときは，まず物質を皮膚に塗っておいて，そこに紫外線を当てる方法，すなわち光パッチテストを行う．あらかじめ当該物質に紫外線を当てておいて，それを普通のパッチテストする方法は経験的に避けられてきたが，これはプロハプテンの証明方法であり，プロハプテンの性格をもつ薬剤が少ないことを知らず知らずのうちに実証してきたことになる.

ランゲルハンス細胞はプロフェッショナルな抗原提示細胞であり，通常の接触皮膚炎と同様に，光接触皮膚炎においても抗原提示細胞として働き，薬剤性光線過敏症においても光抗原を提示する細胞として機能する．ランゲルハンス細胞による光抗原の提示において，光ハプテンがランゲルハンス細胞上の主要組織適合抗原複合体(MHC)クラスII分子あるいはクラスII分子によって表出された自己ペプチドに直接光結合するのか，あるいは紫外線照射によってできた光ハプテンと蛋白の複合体がランゲルハンス細胞に一旦取り込まれ，クラスII分子とともに再表出されるのかは不明である．しかし，われわれは直接MHCクラスII分子と自己ペプチドとの複合体に光共有結合するとする実験結果を得ている．こうして光アレルギー性物質はT細胞を感作することになる.

3 原因物質のはっきりしていない光アレルギー

CADは，外因性光抗原を原因としない自己免疫性光線過敏症と呼ぶべき疾患である．このなかには，ある物質に光貼布試験陽性を示す患者がおり，光線過敏症は以前その物質に対する光接触皮膚炎であったものが，光アレルゲンなしに紫外線に感受性をもつようになってしまった状態と解される．同様に，ある薬剤による光線過敏症を示していた患者が，薬剤を中止しても光線過敏症が治癒することなく存続することもある．つまり引き金は光接触皮膚炎であったり，薬剤性光線過敏症であったものが光抗原が除去されても存続することがあることになる.

こうした光抗原なくして光アレルギーが起こるようになる機序はいまだ明瞭ではない．古典的には光感受性物質が皮膚に微量に残っている可能性がいわれていた．しかし，むしろ現在では紫外線が表皮細胞の表面に何らかの物質を誘導し，それを自己反応性T細胞が認識して皮膚炎を起こす可能性が考えられている．あるいは紫外線照射が自己蛋白の修飾を行い，それがアジュバント効果を発揮するのかもしれない．しかし，そもそもの過敏症を引き起こした光抗原反応性T細胞と自己反応性T細胞にはどんな関係があるのかは，まだまだ不明である.

もうひとつ重要な臨床的観察がある．それはCADがHIV陽性患者に多く報告されていることである．CADの病変組織にはCD8陽性T細胞が浸潤し，苔癬型組織反応を形成していることがしばしばある．一般にCD4陽性細胞のなかにはTh2やregulatory T細胞といったCD8陽性細胞傷害性T細胞の機能を抑制する細胞がある．HIV陽性者ではCD4陽性T細胞の数が減少するが，これが結果的にCD8陽性細胞傷害性T細胞を活性化させてしまい，CADを誘導してしまう可能性がある．最近，われわれは成人T細胞性白血病に伴ったCADを経験した[1]．この場合でもCD4陽性T細胞の機能障害を下地としてCD8陽性細胞傷害性T細胞を活性化させてしまい，CADを生じたと思われる.

以上のように，CADの発症には，自己反応性T細胞の抑制の解除が重要な因子となっているのかもしれない.

Photoallergy as an important disorder in photosensitivity

もうひとつの原因物質のはっきりしていない疾患，日光蕁麻疹については I 型アレルギーという観点から物質を探らなければならない．以上，光アレルギーの個々の疾患については各論での詳述に委ねたい．

Reference

1) Sugita K, Shimauchi T, Tokura Y : *J Am Acad Dermatol* 52 : 38-40, 2005

後天性の光線過敏症はなぜ起こる？

戸倉　新樹

日本皮膚科学会雑誌　第114巻　第13号　第2027-2029頁（平成16年12月臨時増刊）別刷
Reprinted from the Japanese Journal of Dermatology
Vol.114, No.13, pp.2027-2029, December 2004

日皮会誌：114 (13), 2027−2029, 2004（平16）

2004年私の仮説7

後天性の光線過敏症はなぜ起こる？

戸倉　新樹

1. はじめに

光線過敏症は，太陽光線に当たった皮膚が赤くなるなどの異常な反応を起こす疾患の総称である．光線過敏症の原因は多種多様であり，1）内因性の光感受性物質生成によるもの（ポルフィリン症，ペラグラ，Hartnup病），2）外因性光感受性物質投与によるもの（光接触皮膚炎，薬剤性光線過敏症），3）DNA修復機序の異常によるもの（色素性乾皮症），4）EBウイルスの関与により起こるもの（種痘様水疱症），5）その他原因不明のもの（日光蕁麻疹，多形日光疹，慢性光線性皮膚炎［chronic actinic dermatitis, CAD］）などに分けられる．

これらのうちで後天性のものは，ペラグラ，光接触皮膚炎，薬剤性光線過敏症，種痘様水疱症，日光蕁麻疹，多形日光疹，慢性光線性皮膚炎がある．ペラグラは先天性のHartnup病と同じようにニコチン酸欠乏による代謝性疾患である．種痘様水疱症は，発症以前に慢性のEBウイルス感染が存在することを土台とする疾患と考えられている．多形日光疹は我国では小丘疹性日光疹という軽い光線過敏性疾患である．従ってここで論じようとする"後天性の光線過敏"を起こす疾患は，外因性光感受性物質による疾患（光接触皮膚炎，薬剤性光線過敏症），慢性光線性皮膚炎，そして日光蕁麻疹ということになる．ここでは外因性光感受性物質による疾患のメカニズムをまず中心に述べ，慢性光線性皮膚炎のメカニズムを推論することにより「なぜ起こる？」の問いに答えたい．

2. 外因性光感受性物質による光線過敏症

光線過敏を獲得する際に，明瞭な光線過敏性物質が存在する場合と，そうでない場合とがある．明瞭な物質が存在する場合には，光毒性機序によって生ずるものと，光アレルギー性機序によって生ずるものとがある．臨床的には光アレルギー性による頻度の方が高いと考えられる．

通常のアレルギーには，薬疹，接触皮膚炎を代表とするように抗原物質が明瞭なものと，アトピー性皮膚炎，蕁麻疹などのように必ずしもアレルゲンを決定しえないものとがある．この事情はまさに光アレルギーについても同様であり，薬剤性光線過敏症，光接触皮膚炎は抗原となる光感受性物質が明らかであり，その他は明確でない疾患となる[1)2)]．

光アレルギーのひとつの特殊性として，光がアレルギー症状発現に必須であるため，光が当たる臓器すなわち皮膚だけが病変形成の場となることがある．すなわち，光アレルギーの症状は皮膚炎のみである．別の見方をすれば光アレルギーはアレルギー獲得のメカニズムを比較的ピュアに調べることのできるシステムということもできる．以後，外因性物質による光アレルギー，すなわち光接触皮膚炎，薬剤性光線過敏症のメカニズムをまず述べ，次いで慢性光線性皮膚炎の想定される機序を述べたい．

3. 光接触皮膚炎，薬剤性光線過敏症の機序

光接触皮膚炎は抗原が皮膚に塗られて，紫外線が当たって発症する．薬剤性光線過敏症は抗原が薬剤という形で経口投与されて，紫外線が当たって発症する．現在，光接触皮膚炎の原因には，ケトプロフェン，スプロフェンなどのNSAIDや，サンスクリーン剤がある．一方，薬剤性光線過敏症の原因には，ニューキノロンをはじめとして多くの薬剤がある．

通常の抗原とは異なり，光アレルギー性物質が抗原となるには紫外線照射が必要となる．この紫外線の作用による抗原性の獲得については古くよりいくつかの考えが提唱されてきたが，大きく2つの説に集約される[3)]．ひとつはプロハプテンであり，もうひとつは光ハプテンという概念である．プロハプテン説は，光アレルギー性物質は紫外線照射により化学構造の変化が起き，通常のハプテンのごとくなり，蛋白との結合能力を獲得する，という単純明快な説である．一方，光ハプテン説は，紫外線照射がなされるとその化学構造の一部が光分解され，その分解と同時に近傍の蛋白と

産業医科大学皮膚科学
別刷請求先：（〒807-8555）北九州市八幡西区医生ヶ丘1-1　産業医科大学皮膚科学　戸倉　新樹

共有結合し完全抗原ができあがるという考えである．したがって予めUVAを照射した物質が蛋白と結合すればプロハプテン，一方，その物質と蛋白との共存下でUVAを照射し両者が共有結合すれば光ハプテンということになる．

多くの光抗原は光ハプテンとしての性格を持っている．したがって，当該物質が光線過敏症の原因になっているかを検証する時は，まず物質を皮膚に塗っておいて，そこに紫外線を当てる方法，すなわち光パッチテストを行う．予め当該物質に紫外線を当てておいて，それを普通のパッチテストする方法，これはプロハプテンの証明方法であるが，この方法は通常とらない．

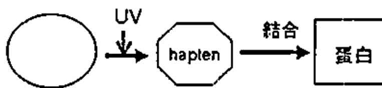
ランゲルハンス細胞はプロフェッショナルな抗原提示細胞であり，通常の接触皮膚炎と同様に，光接触皮膚炎においても抗原提示細胞として働き，薬剤性光線過敏症においても光抗原を提示する細胞として機能している[4)5)]．ランゲルハンス細胞による光抗原の提示において，光ハプテンがランゲルハンス細胞上の主要組織適合抗原複合体（MHC）クラスII分子あるいはクラスII分子によって表出された自己ペプチドに直接光結合するのか，あるいは紫外線照射によってできた光ハプテンと蛋白の複合体がランゲルハンス細胞に一旦取り込まれ，クラスII分子とともに再表出されるのかは不明である．しかし我々は，直接，MHCクラスII分子と自己ペプチドとの複合体に光共有結合するとする実験結果を得ている[6)]．こうして光アレルギー性物質はT細胞を感作することになる．

4. 慢性光線性皮膚炎の機序

慢性光線性皮膚炎は，外因性光抗原を原因としない自己免疫性光線過敏症と呼ぶべき疾患である．この中にはある物質に光貼布試験陽性を示す患者がおり，光線過敏症は以前その物質に対する光接触皮膚炎であったものが，光アレルゲン無しに紫外線に感受性を持つようになってしまった状態と解される．同様に，ある薬剤による光線過敏症を示していた患者が，薬剤を中止しても光線過敏症が治癒することなく存続することもある．つまり引き金は光接触皮膚炎であったり，薬剤性光線過敏症であったものが，光抗原が除去されても存続してしまうことがある．

こうした光抗原無くして光線過敏が起こるようになる機序はいまだ明瞭ではない．古典的には光感受性物質が微量に皮膚に残っている可能性が言われてきた．しかしむしろ現在では紫外線が表皮細胞の表面に何らかの物質を誘導し，それを自己反応性T細胞が認識して皮膚炎を起こす可能性が考えられている．あるいは紫外線照射が自己蛋白の修飾を行い，それがアジュバント効果を発揮することも考えられる．しかし，そもそもの過敏症を引き起こした光抗原反応性T細胞と自己反応性T細胞にはどんな関係があるのかはいまだ不明である．

もうひとつ重要な臨床的観察がある．それは慢性光線性皮膚炎が，HIV陽性患者に多く報告されていることである．慢性光線性皮膚炎の病変組織にはCD8陽性T細胞が浸潤し，苔癬型組織反応を形成していることがしばしばある．一般にCD4陽性細胞の中には，Th2やregulatory T細胞といったCD8陽性細胞傷害性T細胞の機能を抑制する細胞が備わっている．HIV陽性者ではCD4陽性T細胞の数が減少するが，これが結果的にCD8陽性細胞傷害性T細胞を活性化させてしまい，慢性光線性皮膚炎を誘導してしまうのかもしれない．最近我々は，成人T細胞性白血病に



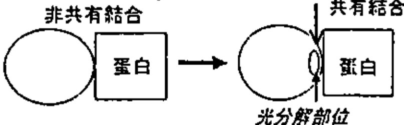


図1 光抗原の生成機序

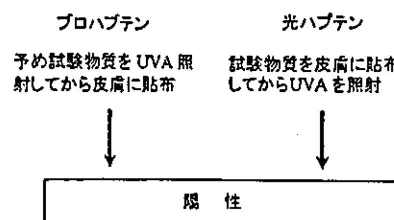


図2 貼付試験，光貼付試験でのプロハプテンと光ハプテンの違い

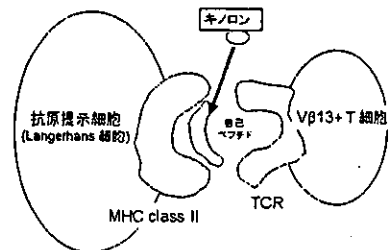


図3 キノロン光線過敏症での光抗原提示機構

表1 CADは外因性光感作物質による光線過敏症（光接触皮膚炎，薬剤性光線過敏症）から誘導されることがあることを示す論文

論文
Tribromsalan：Horio T. *Arch Dermatol* 1982
Musk ambrette：Lan LR *et al*, *Cutis* など多数
Benoxaprofen：Frain-Bell W, *BJD* 1989
Quinine：Guzzo C, Kaidbey K, *PPP* 1990
Olaquindox：Schauder S *et al*, *Contact Dermatitis* 1996
Dioxopromethazine：Schuder S, *Am J Contact Dermat* 1998

表2 光接触皮膚炎からCADへの移行を示す動物実験．これらの実験はcarrier proteinがphotomodificationを受け，自己抗原になっていくことを目指している

論文
Katsumura Y et al：guinea pigs, benzocaine. *JID* FCAとUVA量が重要.
Miyachi Y, Takigawa M：mice, TCSA. J Clin Lab immunol cell transferできず.
Ichikawa H et al：guinea pigs, hapten adjuvantとしてmycobacteria, muramyl dipeptide. JDS peritoneal exudate cellsによってtransfer可能.

表3 CD8＋T細胞とCAD（actinic reticuloid）の関係を示す論文

論文
Bakels V *et al*：*J Clin Pathol.* 皮疹部でCD8優位．末梢血でも4/7例がCD8増加.
Norris PG *et al*：*JAAD.* 皮疹部でCD8優位．末梢血でも1/6例がCD8増加.
Heller P *et al*：*Am J Dermatopathol.* 皮疹部でCD8優位.
Fujita M *et al*：*JDS.* 皮疹部でCD8優位.

表4 CD4＋T細胞の減少または機能不全とCADの関係を示す論文

論文
HIV陽性者にCAD発症. 症例報告多数. Berger TG, Dhar A：*Arch Dermatol.* Lichenoid photoeruptions.
HIV陰性だがCD4が少ない患者にCADが発症. Wolf P *et al*：*JAAD*
ATL患者にCADが発症. Sugita K *et al*：*JAAD*

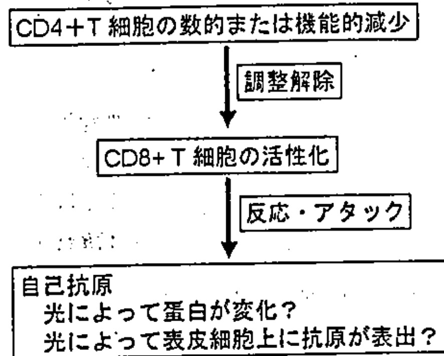


図4 CADの発症機序：仮説

伴った慢性光線性皮膚炎を経験した．この場合でもCD4陽性T細胞の機能障害を下地として，CD8陽性細胞傷害性T細胞を活性化させてしまい，慢性光線性皮膚炎を生じたと考えられる．

以上のように，慢性光線性皮膚炎の発症には，自己反応性T細胞の抑制の解除が重要な因子となっているのであろう．

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An assay method for the prediction of tumor promoting potential of chemicals by the use of Bhas 42 cells

Kiyomi Ohmori[a,*], Kiyoshi Sasaki[b], Shin Asada[b], Noriho Tanaka[b], Makoto Umeda[b]

[a] *Chemistry Division, Kanagawa Prefectural Institute of Public Health, 1-3-1 Shimomachiya, Chigasaki, Kanagawa 253-0087, Japan*
[b] *Department of Cell Biology, Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 257-8523, Japan*

Received 15 April 2003; received in revised form 27 October 2003; accepted 27 October 2003

Abstract

It has become an important task to develop a simple in vitro method for the detection of non-genotoxic carcinogens, among which tumor promoters are included. Bhas 42 cells are v-Ha-ras-transfected BALB/c 3T3 cells and are regarded as initiated cells in the 2-stage transformation paradigm. We designed a method for detecting tumor promoters by the use of Bhas 42 cells at advanced passage generation. In this method, the cells are cultured in six-well plates for 17 days during which test chemicals are added in the medium for 11 days from days 3 to 14. The end-point of the assay is the induction of transformed foci. When the tumor promoter TPA was used, a significant number of transformed foci were induced concentration-dependently, whereas only a few foci were observed in control cultures. When various chemicals were examined by the method, a reasonable correlation was observed with the reported tumor-promoting ability in animal experiments. We propose that the Bhas 42 cell transformation method is practical and useful for the detection of tumor promoters.
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Keywords: Tumor promoter; Transformation; Bhas 42 cell; BALB/c 3T3 cell; v-Ha-ras

1. Introduction

Non-genotoxic carcinogens have posed a major problem in the toxicity screening of chemicals [1]; that is, a considerable number of non-genotoxic chemicals have been shown to be carcinogenic in long-term animal experiments. Therefore, adoption of some screening tests related to carcinogenicity other than the genotoxicity screening is necessary before contemplating long-term animal cancer bioassays.

Carcinogenesis is known to be a multi-step process, involving at least initiation, promotion and progression [2]. Initiators induce changes in DNA and can be detected by various genotoxicity screening tests. Meanwhile, promoters, by the repeated application on initiated cells, can cause development of tumors. These chemicals can be considered to be one kind of non-genotoxic carcinogens, and several screening methods for the detection of promoters have been proposed; for example, in vitro cell transformation [3–6], inhibition of metabolic cooperation

* Corresponding author. Tel.: +81-467-83-4400; fax: +81-467-83-4457.
E-mail address: ohmori.n4yf@pref.kanagawa.jp (K. Ohmori).

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doi:10.1016/j.mrgentox.2003.10.014

through gap-junctional intercellular communication [7–9], promotion or inhibition of cell differentiation [10], expression of Epstein–Barr virus early antigen [11,12], and in vivo cell-proliferation (in vivo RDS test) [13]. However, none of these methods is yet included in the battery of regular safety screening tests for chemicals. One reason why they are not adopted for the regulatory screening tests is that some of these methods are not simple enough for routine screening.

In vitro cell transformation tests using BALB/c 3T3 cells or C3H10T1/2 cells can simulate the process of animal two-stage carcinogenesis [14]. For the detection of promoting chemicals in the in vitro cell transformation test, the cells treated with an appropriate concentration of an initiating agent are subsequently treated with test chemicals. In this method, treatment with an initiating agent and subsequent expression period are required before administration of test chemicals. Typically, these assays require 4–8 weeks to complete.

In order to improve experimental conditions for the examination of chemicals with tumor-promoting potential, Sasaki et al. [15,16] worked with a cell line, named Bhas 42, which was established from BALB/c 3T3 cells transfected with v-Ha-ras oncogene. According to their original procedure, Bhas 42 cells, co-cultivated with BALB/c 3T3 cells, could develop into transformation foci after treatment with chemicals having promoting potential. Here, treatment with an initiating agent and subsequent cultivation for expression period could be omitted. However, it takes a period of 6 weeks for the formation of transformed foci.

Recently, we found that using Bhas 42 cells after advanced sub-culturing and using an enriched basal medium, transformed foci can be efficiently induced in a single culture of the cells by treatment with promoting agents and without the need for co-cultivated BALB/c 3T3 cells. Furthermore, the period of focus formation can be shortened to 2.5–3 weeks. From these findings, we worked to establish a short-term screening method for the detection of promoting potential of chemicals.

The aim of this report is to describe a screening method for tumor promoters using Bhas 42 cells and to present test results for a range of chemicals.

2. Materials and methods

2.1. Media, cells and culture conditions

MEM, DMEM, RPMI 1640 and F12 media were obtained from Nissui Pharmaceutical Co., Tokyo, Japan. BME and DMEM/F12 were the products of GIBCO Laboratories, Grand Island, NY, USA. ITES, a mixture of insulin, transferrin, ethanolamine and sodium selenite, was obtained from Wako Pure Chemical Industries, Osaka, Japan. Fetal bovine serum (FBS) was purchased from Moregate, Australia.

Bhas 42 cells and BALB/c 3T3 A31-1-1 cells were routinely cultured in a medium consisting of MEM supplemented with 10% FBS (M10F), at 37 °C in an atmosphere of 5% CO_2 and 95% air. The cells were sub-cultured before confluence by the use of trypsin (Wako Pure Chemical Industries). Bhas 42 cells at passage generations between 12 and 20 were used in the present experiments.

Plastic culture dishes and plates were either products of Sumitomo Bakelite, Tokyo, Japan, or those of Costar, Corning Incorporated, Corning, NY, USA.

2.2. Chemicals

12-*O*-Tetradecanoylphorbol-13-acetate (TPA), okadaic acid, lithocholic acid, sodium phenobarbital, arsenic trioxide, sodium saccharin, catechol, acetone and ethanol were purchased from Wako Pure Chemical Industries. Phorbol 12,13-didecanoate (PDD), mezerein, phorbol, anthralin, progesterone, 17β-estradiol, dexamethasone, insulin and dimethyl sulfoxide (DMSO) were obtained from Sigma, St. Louis, MO, USA. *o,p'*-DDT and *p,p'*-DDT were obtained from GL Science, Tokyo, Japan. Diethylstilbestrol was the product of Tokyo Kasei Kogyo, Tokyo, Japan.

TPA was dissolved in DMSO at 1 mg/ml; stock aliquots were stored in a deep freezer. An aliquot was used in each experiment. Arsenic trioxide was dissolved in 0.1 mol/l sodium hydroxide solution and insulin was dissolved in 0.1 mol/l hydrochloric acid. The solutions had no effect on pH of the culture medium when diluted more than 1000 times. Other chemicals were dissolved in DMSO or directly in the culture medium.

2.3. *Cytotoxicity test*

A cell suspension of Bhas 42 cells in DMEM/F12+ 5% FBS (DF5F) at 2×10^4 cells/ml was distributed into each well of 24-well plates at 0.5 ml amounts (1×10^4 cells per well) and cultured. This cell number is almost equal to the 2 ml used per well of six-well plates. On day 3, medium in each well was changed with the medium containing test chemical. Three wells were used for one concentration. On day 7, the cells were fixed with 3.7% formaldehyde for 30 min, washed with water, and stained with 1% crystal violet (CV) in water for 30 min. After thorough rinsing with water, the plates were dried. Crystal violet from stained cells in each well was extracted with 0.5 ml of a solution consisting of 0.9% trisodium citrate dihydrate, 0.02 mol/l hydrochloric acid, and 50% ethanol. The optical density of the extracts was measured at 540–570 nm.

2.4. *Bhas 42 transformation test*

In the present experiments, several variables were examined, and the finally adopted protocol was as follows; a cell suspension of Bhas 42 cells was prepared in DF5F medium at 2×10^4 cells/ml, and distributed into each well of six-well plates at 2 ml amounts (4×10^4 cells per well). After cultivation for 3 days, medium was replaced with fresh medium containing test chemical (for the preparation of test chemicals, attention should be paid to ensuring the final concentrations of solvents is less than 0.1% in the case of DMSO and ethanol, and 0.5% in the case of acetone). The culture received medium containing test chemical on days 7 and 10, and then fresh DF5F medium on day 14. On day 17, the cells were fixed with methanol for 30 min and stained with 2.5% Giemsa solution for 30 min.

Transformed foci were characterized by the following morphological criteria: deep basophilic staining and dense multi-layering of cells; random orientation of cells at the edge of foci; more than 20 cells within a focus.

2.5. *Statistical analysis and criteria of judgment*

For evaluating the results, *t*-test analysis was performed. Chemicals showing significant increase ($P <$ 0.05) of focus number at more than two consecutive concentrations were considered to be positive (+). Chemicals which showed statistically significant effect at only one concentration, even after repeat tests, were judged to be equivocal (±). Negative chemicals were those which induced no statistically significant increase of transformed foci.

3. Results

3.1. *Fundamental conditions for efficient induction of transformed foci*

When the protocol of the original transformation procedure using Bhas 42 cells [15,16] at the 13th passage generation was followed, no transformed foci appeared after treatment with TPA. However, Bhas 42 cells at the 13th passage generation alone easily grew into a monolayer which contained only several spontaneously transformed foci. In addition, the number of transformed foci significantly increased by treatment with TPA. From these observations, the possibility of developing a short-term cell transformation assay for

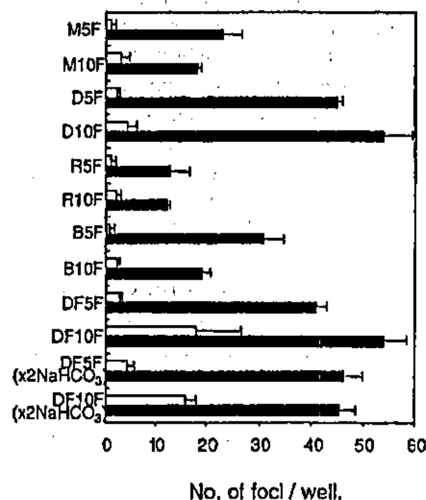

Fig. 1. Effects of various basal media and FBS concentrations on the transformation of Bhas 42 cells: (□), control; (■), TPA (20 ng/ml); M5F, MEM + 5% FBS; M10F, MEM + 10% FBS; D5F, DMEM + 5% FBS; D10F, DMEM + 10% FBS; R5F, RPMI1640 + 5% FBS; R10F, RPMI1640 + 10% FBS; B5F, BME+5% FBS; B10F, BME+10% FBS; DF5F, DMEM/F12+5% FBS; DF10F, DMEM/F12 + 10% FBS.

the detection of promotion potential of chemicals using a single culture of Bhas 42 cells at advanced passage generations was explored.

In order to establish a new transformation protocol using Bhas 42 cells, several variables which would influence the formation of transformed foci were examined. Essentially, the second stage of the two-stage transformation protocol using BALB/c 3T3 cells [6] was emulated. Bhas 42 cells near confluence were treated with TPA for 11 days and further cultured 3 or 7 days in fresh medium. The endpoint was the formation of transformed foci.

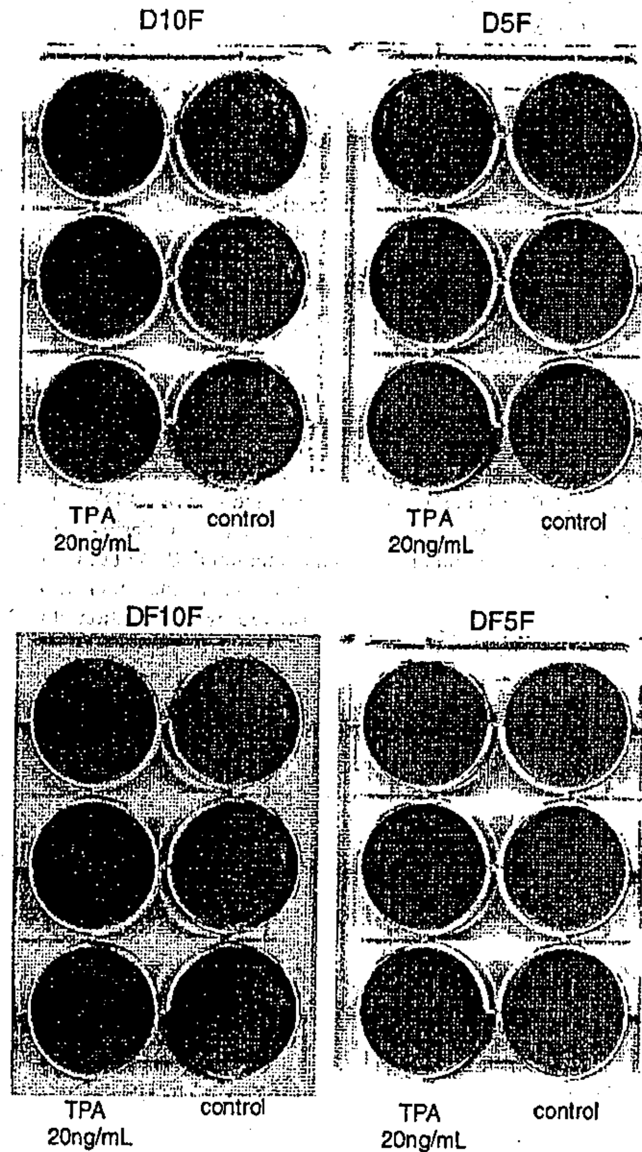

Fig. 2. Representative plates showing formed foci of Bhas 42 cells cultured in different media with or without TPA.

The induction of transformed foci with or without TPA was examined using various basal media added with 5 or 10% FBS (Fig. 1). The use of 10% FBS produced monolayers a little greater in cell density and induced more spontaneously arising foci than the use of 5% FBS. Among basal media examined, DMEM and DMEM/F12 gave great numbers of transformed foci on treatment with TPA. The foci formed were clearer and larger in DMEM/F12 than in DMEM (Fig. 2). From these results, DMEM/F12 supplemented with 5% FBS (DF5F) was selected as the medium for the subsequent experiments.

The effect of co-cultivation of Bhas 42 cells with BALB/c 3T3 cells was examined. The number of transformed foci of Bhas 42 cells was reduced by the presence of BALB/c 3T3 cells: the greater the number of BALB/c 3T3, the less the number of the foci. The culture of Bhas 42 cells alone resulted in the highest number of transformed foci (data not shown). Therefore, it was concluded that co-cultivation with BALB/c 3T3 cells was unnecessary in this protocol.

In order to determine the optimal time frame of treatment with test chemicals, various treatment periods were tested for TPA (Fig. 3). In this experiment, 2 ml of 2×10^4 Bhas 42 cells/ml (4×10^4 cells per well) were inoculated into each well of six-well plates, and TPA treatment was started from day 3. The number of foci was highest when the medium containing TPA was changed three times, i.e., on days 3, 7 and 10, and with treatment ceasing on day 14.

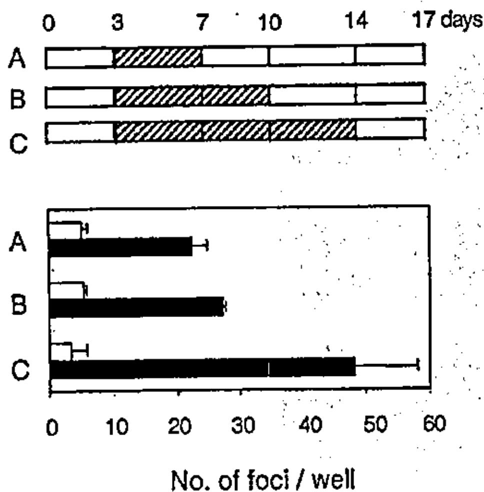

Fig. 3. Effects of period of TPA treatment on the transformation of Bhas 42 cells: (▨) period of TPA treatment; (□) control; (■) TPA (20 ng/ml).

The transformation assay was carried out with Bhas 42 cells inoculated at various cell numbers (Fig. 4). The cells were treated with TPA on days 3, 7 and 10, and fixed on day 17 or 21. When seeded at 4×10^4 cells per well, the cells reached near confluence on day 3. In this condition, the number of transformed foci was highest. The foci obtained from cultures fixed on day 21 were clearer and larger than those fixed on day 17. In addition, the number of foci fixed on day 21 was slightly increased at every inoculum size, but this was not statistically significant in comparison with those fixed on day 17. From these results, an inoculum size of 4×10^4 cells per well of six-well plates and 17 days of culture as the experimental period were adopted. Due to this shorter culture period, it was designed to score foci consisting of 20 or more cells.

3.2. *Variables influencing the formation of transformed foci*

During the course of experiments it was found that the concentration of DMSO used as a solvent affected focus formation. Various solvents were tested. DMSO and ethanol at 0.5% but not 0.1% decreased the number of transformed foci. In contrast, little effect was observed by the addition of 0.5% acetone.

In order to assure the stable supply of Bhas 42 cells, the effect of further passages on transformation was examined using 2 kinds of media, M10F and DF5F. Cells at the 13th passage generation were further cultured for 1 month. The cells before confluence were sub-cultured at a 3–4-day interval. At appropriate intervals, the cells were stocked frozen at −80 °C. Each stock of the cells was thawed and examined for the formation of transformed foci with or without TPA. Fig. 5 reveals that even after a month of culture the number of transformed foci induced by TPA did not change significantly when cultured in M10F. Also, spontaneous formation of foci did not increase after the passages. In contrast, the cells cultured in DF5F formed a gradually increasing number of transformed foci irrespective of the treatment with TPA, suggesting that cultivation in M10F was most appropriate for the proper maintenance of the cells for the assay.

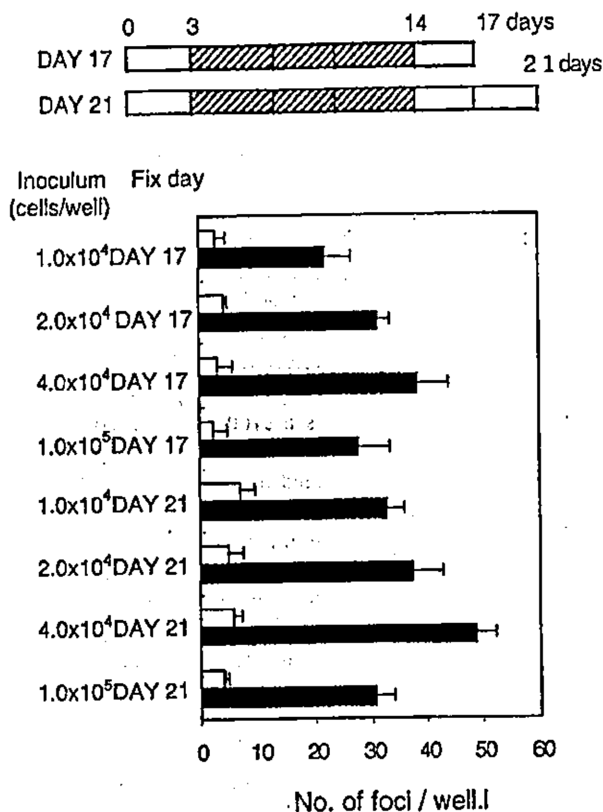

Fig. 4. Effects of inoculum sizes and fixation days on the transformation of Bhas 42 cells: (▨) period of TPA treatment; (□) control; (■) TPA (20 ng/ml).

3.3. Determination of test concentrations for each chemical

From the cytotoxicity tests as described in Materials and Methods, test concentrations of each chemical were determined, covering from 60 to 80% of growth inhibition to no inhibition. Fig. 6a shows the result of the toxicity experiment with TPA. Cell number did not decrease over a large concentration range up to 1 μg/ml, but concentrations inducing transformed foci were far less, even as low as 5–10 ng/ml (Fig. 7a). TPA was an extreme case, and in such a case it was necessary to examine cytotoxicity over a wide range of concentrations. Fig. 6b and c show cytotoxicity with okadaic acid and lithocholic acid at 50 ng/ml and 50 μg/ml, respectively.

3.4. Examination of various chemicals in the transformation protocol

In order to evaluate this cell transformation assay for a variety of chemicals, known tumor promoters together with related and other chemicals were examined (Fig. 7). Dose-response of TPA is shown in Fig. 7a. The number of transformed foci began to increase from 5 ng/ml and reached a plateau at 20–50 ng/ml. PDD and mezerein induced transformed foci from 0.05 ng/ml and reached a maximum level at 0.5 ng/ml (Fig. 7b and c). These chemicals induced transformed foci at concentrations showing overgrowth of the cells. Phorbol, with the basal structure of TPA and PDD but weak tumor promoting activity [17], did not induce transformed foci up to 100 ng/ml (Fig. 7d).

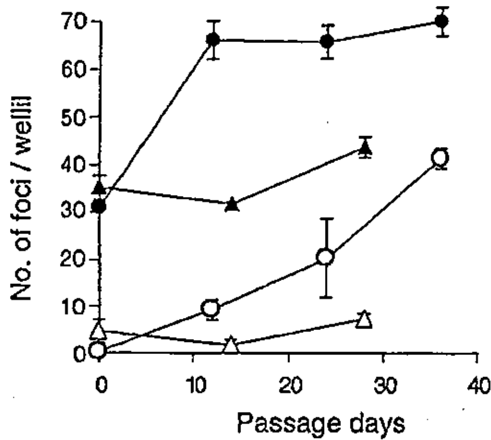

Fig. 5. Effects on transformation frequency of passage generations of Bhas 42 cells cultured in M10F or DF5F: (○) DMEM/F12+5% FBS (DF5F) in the absence of TPA; (△) MEM+10% FBS (M10F) in the absence of TPA; (●) DF5F in the presence of TPA; (▲) M10F in the presence of TPA.

Representatives of other class tumor promoters were examined. Okadaic acid showed growth inhibition at 10 ng/ml and above (Fig. 6b), and induced transformed foci at a narrow concentration range from 5 to 10 ng/ml (Fig. 7e). The highest number of induced foci was relatively low, about 25 foci per well. In the case of anthralin, the highest transformed foci density was 15 foci per well at 1 μg/ml (Fig. 7f). Lithocholic acid induced transformed foci from 1 to 20 μg/ml (about 30 foci per well maximum) (Fig. 7g) and at doses that were marginally cytotoxic (Fig. 6c). Both o,p'-DDT and p,p'-DDT showed significant increase of foci at 5 and 10 μg/ml (Fig. 7h and i). Sodium phenobarbital induced statistically significant effect only at one concentration, and was judged equivocal (Fig. 7j).

Results examined for other Ames' negative chemicals with reports on carcinogenicity were the followings. Progesterone induced about 20 transformed foci per well at 2 μg/ml (Fig. 7k), but 17β-estradiol was not active in the assay (Fig. 7l). Diethylstilbestrol, a synthetic estrogen, induced statistically significant effect at two non-consecutive concentrations, being judged equivocal (Fig. 7m). Dexamethasone which is a synthetic glucocorticoid, showed significant increase of foci at 12.5 and 25 μg/ml, and was evaluated as positive (Fig. 7n). Arsenic trioxide induced transformed foci dose-dependently in the concentration range 0.02–0.2 μg/ml (Fig. 7o). Arsenic trioxide was not cytotoxic below 0.5 μg/ml (data not shown). Sodium saccharin induced maximum number of transformed foci at 3000 μg/ml (about 30 foci per well) (Fig. 7p). The number of induced transformed foci increased dose-dependently by treatment with catechol at 0.1–2 μg/ml, at up to 25 foci per well (Fig. 7q). Insulin induced about 10 transformed foci per well at 20 and 50 μg/ml, and was evaluated as positive (Fig. 7r).

The average number of foci per well in 18 experiments was 2.9 ± 0.9 in the controls and 37.4 ± 9.9 in cultures treated with 20 ng/ml TPA, demonstrating the stable sensitivity of Bhas 42 cells in the repeated assays.

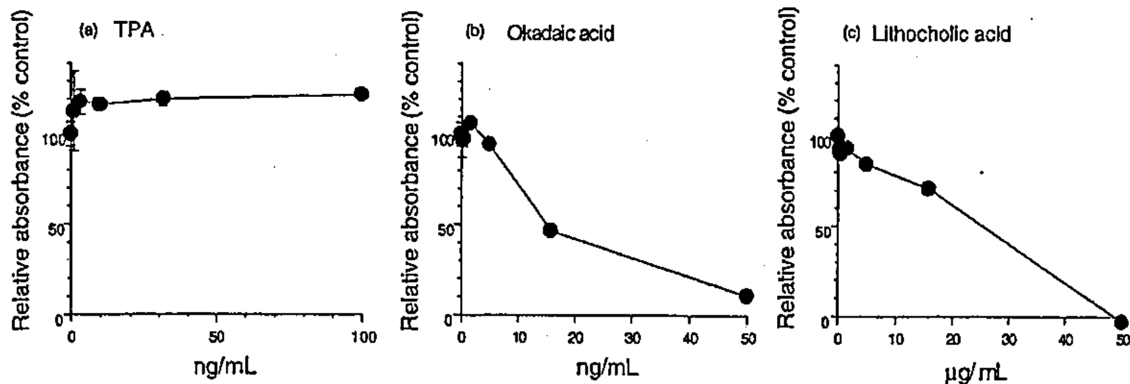

Fig. 6. Cytotoxicity experiments using TPA, okadaic acid and lithocholic acid.

4. Discussion

In most screening tests, chemicals insoluble in water were dissolved in some solvent and diluted with the medium. DMSO is one of the most widely-used solvents. Usually, the final concentration of DMSO was adjusted to be less than 0.5% in the medium. In the current experiments using TPA as a promoting agent, DMSO and ethanol were without effect at 0.1% or less, whereas with acetone up to 0.5% could be used. Kennedy and Symons [18] reported that DMSO at 0.1% or more is capable of suppressing TPA enhancement of radiation-induced transformation by OH free radical scavenging. They also reported that ethanol scavenges OH free radical.

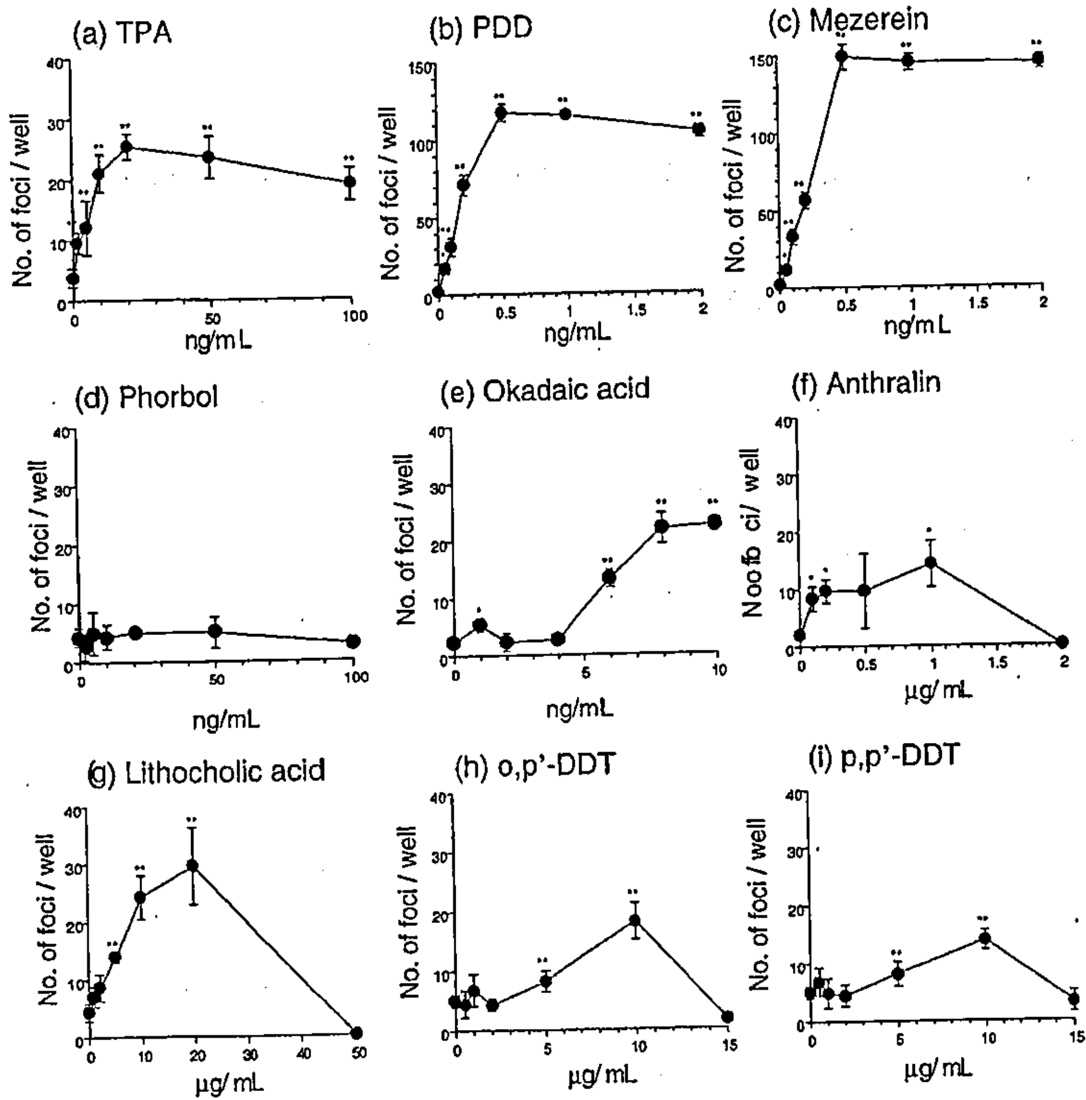

Fig. 7. Transformation experiments using various chemicals: * $P < 0.05$; ** $P < 0.01$.

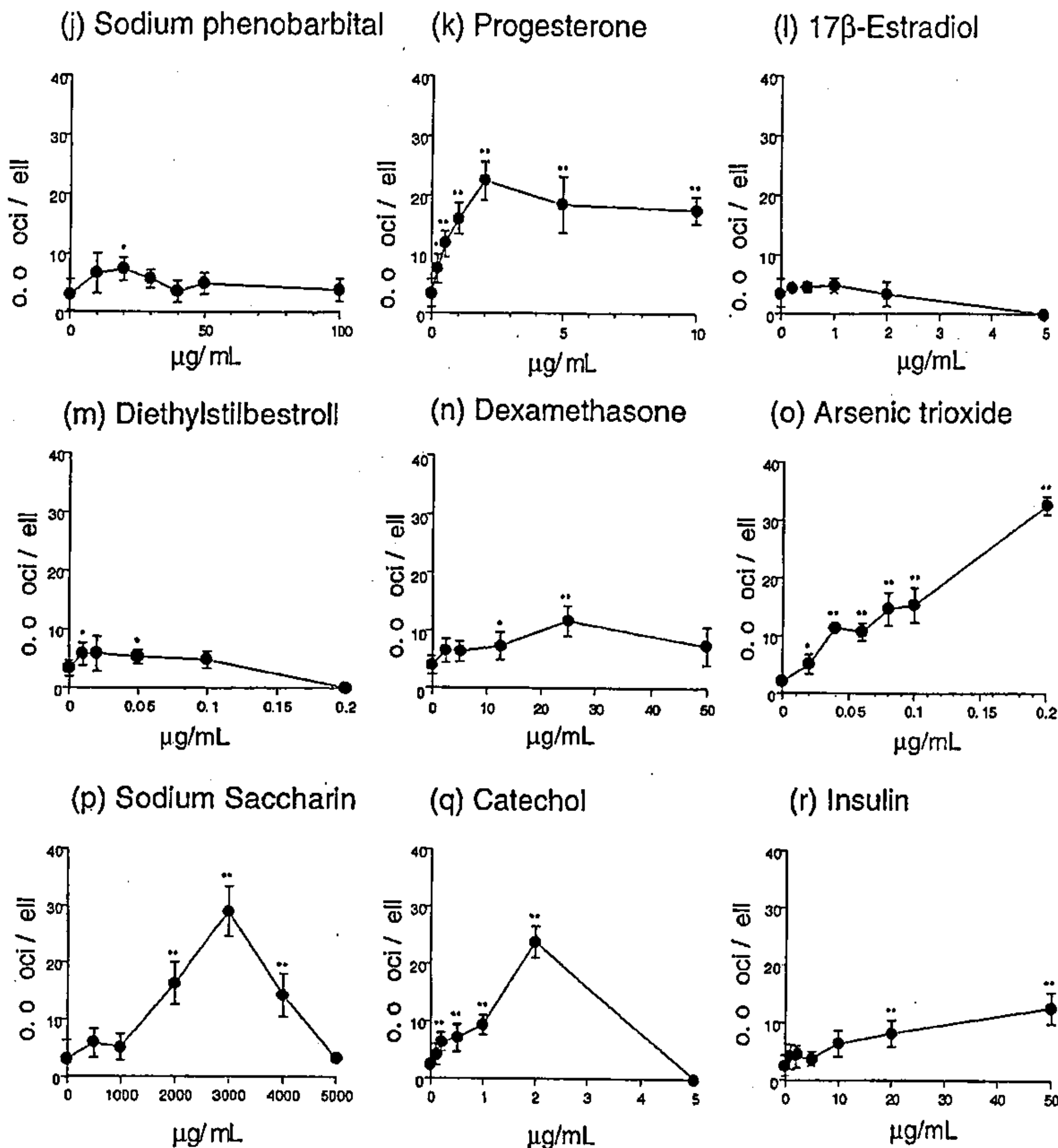

Fig. 7. (*Continued*).

Employing our newly developed promoter assay method, various chemicals were examined (Fig. 7), and the results are summarized together with other information on short-term tests and animal carcinogenicity (Table 1). Typical tumor promoters such as TPA, PDD, mezerein, okadaic acid, anthralina and lithocholic acid showed positive results. It is noteworthy that PDD and mezerein were extremely

Table 1
Summary of results in the Bhas 42 cell transformation assay together with those in other short-term assays [12,16,31] and carcinogenicity evaluation from IARC

Chemical	Bhas assay[a]	Bhas original	Balb 3T3	C3H10T1/2	SHE	JB6	V79	Raji	Ames	IARC
TPA	+	+	+	+	+	+	+	+	−	
PDD	+	+	+		+		+		−	
Mezerein	+	+	+	+	+	+	+		−	
Phorbol	−					−	−		−	
Okadaic acid	+		+	+	+	±	−	+	−	
Anthralin	+		+		−		±		−	3
Lithocholic acid	+		+	+			+	±	−	
o,p'-DDT	+	+	+		+		+	±	−	2B
p,p'-DDT	+	+	+		+		+		−	2B
Phenobarbital (sodium salt)	±	+	±	±			±	+	−	2B
Progesterone	+				±			+	−	
17β-Estradiol	−		−	+	+		−	−	−	1
Diethylstilbestrol	±		−	±	+		−	+	−	1
Dexamethasone	+			+			−		−	
Arsenic trioxide	+								−	1
Saccharin (sodium salt)	+	−	−	+	−	−	±	−	−	2B
Catechol	+		+				±	−	−	2B
Insulin	+	±	+			−	−		−	

[a] +: positive, +: equivocal, −: negative.

potent in inducing transformation foci in this assay. High sensitivity to mezerein was reported by Tsang et al. [19] using a bovine-papilloma DNA-carrying C3H10T1/2 cell line. Stability in the medium or other factors may influence the sensitivity difference of these chemicals among various assays.

Both DDT's [20] and phenobarbital [21,22] are typical tumor promoters of the rodent liver. The present results showed that DDT's were positive and phenobarbital was equivocal. This may suggest that these compounds have different mechanism of promotion action. Progesterone [23] showed a positive result. In contrast, 17β-estradiol [24] and diethylstilbestrol [23] showed negative or equivocal results. These female sex hormones show their activity through their hormone receptors and carcinogenicity to female organs [25]. These results suggest that this assay has difficulties in detecting some specific types of tumor promoters.

Arsenic trioxide [26], dexamethasone [27], saccharin [22], catechol [28] and insulin [29] are related to promotion effect without genotoxicity. In the present study these chemicals gave positive results. More investigations on many other chemicals are required in order to evaluate this method as a predictor of tumor-promoting potential for a wide variety of chemicals.

Here we have used v-Ha-ras-transfected BALB/c 3T3 cells. Similarly, Kowalski et al. [30] demonstrated that an assay employing a bovine-papillomavirus DNA-carrying C3H10T1/2 cell line could well predict carcinogens, promoters and non-carcinogens. Our present study was focused on promoters among non-genotoxic carcinogens, and the method is more simple and economical than the preceding transformation experiments using BALB/c 3T3 cells. Further studies with more chemicals are necessary to establish the reproducibility, reliability and relevancy of the method. In time, this test method will be evaluated for its utility in predicting the tumor promoting potential of chemicals.

Acknowledgements

This study was supported by Grants-in-Aid from Japan Chemical Industry Association, and from Kanagawa Prefectural Government for the Encouragement of Basic Science and Technology.

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