性もある。また，部位によってはマクロファージの貪食作用による取り込みが積極的に進む可能性もある。一方，粒子の大きさによっては，消化管表層部に沈着する程度にも差が出て，体内への取り込みが変わってくることがありうる。このように，経口からの摂取とはいえ様々な条件の違いが生じ，その結果，体内への取り込みのされ方も異なってくることが予想される。経口摂取による吸収に関する検討は限られているが，これまで報告されている結果では，多くの場合，消化器管系では吸収されずに体内を通過し，排泄されている。放射性標識（^{14}C －）したフラーレンを環状付加反応させた極性トリメチレンメタンで水懸濁性にして，ラットに経口投与した結果では，48 時間内に糞便として 98％が排泄されている[1)]。しかし，残りは尿中に排泄されてはいることから，極一部は吸収されて血液循環系で全身に回っている可能性を示唆している。また，静脈注射した場合は，急速に各種の臓器に分布し，血液－脳関門も通過できることが明らかとなった。1 週間後にも約 90％が体内にとどまり，経時的に異なるが，73～80％が肝臓に貯留していた。この場合には急性毒性は認められなかったと報告されている。この報告の他に，種々の側鎖を付加して水懸濁性を高めたフラーレン類を経口投与や静脈注射した研究が報告されている。個々の物質により結果は大きく異なってはいるが，静脈注入したフラーレン類は吸収されて各種の臓器に分布することが示されている。これらの結果から，消化管から体内に取り込まれたフラーレンはその代謝物を含め，急速に全身の組織に分布し，長期に保持されることが推測される。取り込みによる影響は，急性毒性は低いようではあるが，長期間蓄積することから，慢性毒性の影響が懸念される。体内の保持期間は誘導体の種類により異なるが，半減期が 7 時間程度[2)]から 1 週間経過後もほとんど排泄されないものもあることが報告されている。体内に吸収されたフラーレン類の排泄は，腎臓を通して行われる例が報告されている[2,3)]。我々の検討においても，暫定的な結果ではあるが，コーン油に溶解させたフラーレンを単回強制経口投与した場合に，大部分（少なくとも 85％以上）が糞便中に排泄されたと算出され，消化管から体内に取り込まれにくい傾向が認められている（現在，詳細な検討中）。

2. ナノマテリアル取り込みの同定・定量方法の課題

次に，体内に取り込まれたかどうかを判定するためには，対象とするナノマテリアルを正確に同定・定量することが求められる。生体試料を扱う点から，物質単体としてではなく，多様な生体成分の夾雑物質の中から高感度に選択的に測定する方法が要求される。例えば，酸化チタン，白金，金や銀などの生体成分として存在量が少ない金属超微粒子は，誘導結合プラズマー質量分析装置による測定が有効である。この方法は，生体試料を灰化して，試料中に含まれる金属成分として同定・定量する方法であることより，精度や再現性が高く，単位重量あたりの濃度として

非常に低い濃度まで測定できる長所がある。しかし，生体試料に普遍的に存在量が多い金属には適用しにくく，生体内存在粒子径の大きさや細胞レベルの分布の状態までは明確にできないのが欠点である。この方法では，組織中の存在状態を同定するためには，電子顕微鏡を用いなければならない。カーボンナノチューブでは，透過型電子顕微鏡による同定方法が有効である。しかし，組織中の分布については多くの情報を得ることができるが，定量性に関しては課題が残っている。定量を行うためには，灰化した生体試料からカーボンナノチューブを効率よく分離・精製し，電子顕微鏡下で本数を目視で数える方法が可能と考え，現在検討中である。定量法については，開発・確立中であり，将来よい手法が提案されることもあるであろう。フラーレン類に関しては，特異波長における吸光度，および高速液体クロマトグラフィーの適用による定量が可能である。両方法は，単体の定性・定量には支障なく適用できるが，生体試料への適用については往々にして生体試料由来の物質が測定上の妨害となることが多く，前処理により夾雑物質の除去が必要なことが多い。我々は，生体試料からトルエンで抽出して，液体クロマトグラフィー－タンデム質量分析計で同定・定量する方法を確立して，高感度で選択性の高い測定法として提案している。この方法では，nグラムレベルの検出が可能となっており，極微量のフラーレンの体内分布が解析可能である。フラーレン誘導体についても同様の方法が適用できると考えているが，個々の物質で確認をしなければならない。また，各々の代謝産物についての同定も同時に解析できる可能性が示されている。カーボンナノチューブやフラーレン類では，先に記述した報告にあるように，炭素原子の放射性同位体や安定同位体を用いて標識し，放射性標識を検出することで同定・定量する方法もある。この方法は，これまでの生化学的な手法として広く使用されてきた方法であり，生体試料を対象として研究では高感度で測定できる技術が確立しており，極微量の対象物質の全身分布を解析できる長所がある。また，代謝産物を含めた体内挙動を追跡研究できる可能性があることが優れている。しかし，この手法は，製造が高価で，製造技術的にも解決しなければならない点がある上，使用に関して，取り扱いに注意を要するなどの欠点がある。短期的な体内挙動の研究には非常に有効な手段ではあるが，長期的な観察を必要とする研究には課題がある。さらに，これまで報告されている結果から推測すると，修飾基が異なるだけでも吸収の挙動が異なる可能性が予想され，対象とする物質の検討を進める際には個々の物質ごとに標識物質を作製しなくてはならないかもしれない点が課題として残っている。ナノマテリアルに，後から蛍光物質や分析が容易な修飾基を化学的に修飾する手法も考えられるが，先に述べたように，構造や表面状態により挙動や作用が異なる可能性が残されている現在，修飾基を導入することで体内への吸収・代謝・蓄積・排泄等の挙動に変化をきたさないとの情報が得られるまでは，修飾した物質の挙動は元の物質と全く異なった挙動をしていると考えるべきであり，修飾した物質での評価を安易に元の物質の評価としないことが重要である。したがって，修飾基の導入は目的により考慮すべき

であろう。

繰り返しになるが，同定・定量法については，単純に重量を測定できるだけではなく，曝露溶液中の濃度，粒子もしくは凝集塊の粒度分布と数および表面積，生体内の状態，凝集塊の大きさ，粒子もしくは凝集塊の数，単位湿重量あたりの重さの評価，組織内の分布状態などの情報を可能な限り収集できる方法の組み合わせがとられるべきである。一方，スクリーニングやモニタリング手法としては，高感度ではあるが，迅速に結果を出すことができ，簡便で精度が高い方法を確立すべきである。

3. 実験動物による評価方法の課題

ナノマテリアルの吸収や生体内での挙動だけではなく，生体内物質との反応性についても不明な点が多い現在，より多くの物質に共通性の高い情報を得るために，個々の対象物質の特性に起因する性状や影響を調べることに加え，標準的な物質と手法による相互比較可能な情報の入手にも努めるべきである。そのためには，まず吸収の有無を正確に判定するために，最も吸収の可能性が高いと推測される手法の設定が必要となる。食品から吸収されることが予想される場合は食餌として，水から吸収されることが予想される場合は飲水で投与することが望ましい。この方法は長期的な曝露研究にとり最も優れた投与方法と考えられるが，短期的な曝露研究では投与量が限られ，たとえ吸収されても同定・定量できる濃度に達することができない危険性がある。したがって，短期的には強制的に胃に注入する方法で，吸収の有無や程度を評価する手法の選択も考慮しなければならない。次に，対象物質の分散剤の選択が課題となる。分散剤に関しても，標準化されておらず，現在検討が進められている。

例えば，実際の摂取状況を考慮してコーン油を代表とする食品中油脂，スクアラン，トルエン，キシレン，1-オクタノールなどの有機溶剤，1-メチル-2-ピロリドン，フォスファチヂルセリンやフォスファチジルコリンなどの膜構成脂質，ビタミンA，ビタミンD，ビタミンKなどの脂溶性生理活性物質等が候補として挙げられている。また，水に直接懸濁状態にさせる場合には，Tween20，Tween40，TritonX-100，NP-40などの生体に比較的毒性の低いと考えられる界面活性剤の使用による懸濁，リポタンパク質などの血液中成分と共存，膜構成成分によるリポソームの一部として構成させて懸濁させるなどの方法が検討されている。また，γ-シクロデキストリンやポリビニルピロリドン等を対象物質の周囲に含接させる方法で，水に分散しやすい状態にする方法が提案されている。ここで注意しなくてはならないのは，使用する分散剤が毒性や影響を及ぼさない物質であるかその濃度でなければいけない。例えば，刺激性の強い物質を分散剤に使用すると，胃壁を荒らして吸収を促進するか，逆に吸収を阻害する結果を招くからである。この

観点から，これまで多くの化学物質の溶剤として使用されて情報の蓄積があるコーン油が，最優先の分散剤として検討されるべき候補であろう。

4. ナノマテリアルの吸収評価の課題

次に，物理的な性状の課題として，分散剤の種類，対象物質の種類により粒子径が異なることが想定されることから，投与する分散溶液中の対象物質の粒子径の分布の測定が必要である。粒子径に従いマクロファージの貪食作用などによる取り込み率が異なるとの報告があるのに対し，超微粒子の形状では消化管表面の細胞間から体内に浸入する可能性が示唆される結果も得られており，粒子径が吸収に影響すると考えられるため，投与時の粒子径分布についての情報が重要である。Jani らは，ヨウ素（125）で放射化標識した球状のポリスチレンを SW 系雄ラット（平均体重 200g）に 1.25mg/kg/ 日で 10 日間強制経口投与した結果，50nm および 100nm の粒子が 34％と 26％吸収されたと報告している。この研究で，50nm の粒子は約 7％，100nm の粒子は約 4％が，血液，肝臓，脾臓および骨髄に達していた。1000nm の粒子は 0.8％が吸収されていたが，骨髄に達していなかった。3000nm の粒子は吸収されていなかった。300nm 以上の粒子は血液中から検出されず，心臓や肺からも検出されなかった。これらの結果は，粒子径が吸収の程度と体内分布に影響を及ぼすことを示唆している[4]。また，粒子径による吸収の相違に関して，150 ～ 500nm の酸化チタン粒子は吸収されて，血液から肝臓に達するとの報告がある[5]。

おわりに

これまで，経口からのナノマテリアルの摂取に関して概観したが，非常に限られた情報があるのみである。ナノマテリアルに関する環境動態，生物に対する影響評価の取り組みが始まったところで，多くの課題が今後の検討にゆだねられているのが現状であることを理解していただけたら幸いである。ここ数年後の検討の結果，新しい知見が集積し，これまでの化学物質で行われてきた同一の方法論で取り組みを進めてよいのか，別の方法論の導入を行わなくてはならないのかを含め，ナノに関する研究評価の方向性が明らかとなってくると思われる。

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[3] 皮膚接触

はじめに

ナノマテリアルの使用量の増加と使用範囲の拡大にともない，製造・使用工程の作業環境において作業従事者が非意図的に曝露する機会や，材料として含む製品から生活環境に放出されることにより多くの人々の曝露する機会が増加することが考えられる。しかし，これらの曝露の機会は，食品などを口から摂取や，皮膚から吸収される割合に比べ吸気からの曝露量が大きいと想像される。これに対して，皮膚からの吸収は，ナノマテリアルを含む製品に接触する機会や，意図的にナノマテリアルを添加した製品を塗布する場合を考慮しなければならない。

皮膚からの吸収や影響評価に関する知見は限られており，今後の検討課題となっている。これまでの検討では，すでに使用されている酸化チタンやカーボンナノチューブに関する報告例が多くなっている。本稿においても，これらの　物質を中心に得られている知見を中心に課題を明らかとしていく。

1. ナノマテリアルの皮膚からの浸透

単層カーボンナノチューブを高濃度に含むフラーレン煤の健康影響を，40 人のボランティアを対象としてパッチテストを実施した報告では，96 時間後の皮膚刺激性およびアレルギー反応は陰性の結果であった。同一の試料を，4 匹のウサギを用いて，改変ドレイズ法により，0.2ml の水に懸濁して眼に滴下し，24 時間後，48 時間後および 72 時間後の眼刺激性を観察した結果，異常は認められなかったと報告されている[1,2]。

日焼け止め製剤を連続塗布した後テープをはり，塗布部の角質層を剥ぎ取った後の皮膚の断面を組織学的観察し，日焼け止め製剤に含有している酸化チタンの浸透性を評価した結果，毛包脂腺開口部でのみ浸透が認められ，角質層の深部では認められなかったと報告している。しかし，毛包で検出された酸化チタンは塗布量の 1％未満であった[3]。これらの結果は，皮膚組織への酸化チタンの浸透は少ないと示唆している。

前述した 3 報に対して，豚の皮膚に，長さ 45 ～ 150nm，幅 17 ～ 35nm の酸化チタンを含む製品を塗布し 8 時間後，24 時間後，48 時間後の皮膚内部への浸透を，高エネルーギーイオンプローブを用いて測定した研究では，顆粒層では検出されたが，その下の有棘層や毛嚢では検出下限値以下であったと報告されている[4]。この結果からは，塗布した製品中の酸化チタンは細胞間を経て顆粒層に達したことが示唆される。

一方，微粒子化した3種の酸化チタン，I：20nm，立方晶，疎水性，II：100nm，針状，両親媒性，III：100nm，針状，親水性は，角質層の最も表面の部位にとどまり，深部まで達することはないとの報告もある[5]。

相反する結果が報告されているのは，皮膚への曝露条件が異なるのに加え，対象とする酸化チタンの性質や形状が異なっていることが原因の一つであると推測される。酸化チタンの超微粒子は結晶形と大きさ，表面の処理の方法により浸透の程度は大きく変わるとの結果もあり（私信），結論に至るには同一条件において系統立てた検討が必要である。

また，皮膚に塗布する酸化チタン微粒子の多くは，紫外線カットを使用目的とするものである。超微粒子としての挙動に加え，紫外線が照射された後の挙動や影響，製品中の表面処理剤の安定性，光エネルギーの吸収剤との化学反応生成物などに関して，総合的に評価しなければ添加されたナノマテリアルとしての酸化チタンを正しく理解できない点に留意すべきである。すなわち，超微粒子の酸化チタンの吸収の有無のみで健康影響を評価するのは十分とはいえない。また，皮膚表面は生理的に，順次，剥離していくものではあるので，沈着の程度，すなわち皮膚表層における生物的半減期に関しても今後情報の集積が必要である。

2. ナノ物質の皮膚組織への影響

ナノマテリアルが皮膚表面に付着した後，体内に浸透していくか，表層部にとどまるかは，物質により異なる可能性があることもあり，包括的な結論が出るには至っていないので，ここでは皮膚表層に曝露した研究結果に関して概論する。

皮膚表層から体内に浸入した場合には，循環系に入る可能性もあり，体内への取り込み経路にかかわらず類似の挙動をとる可能性がある。ただし，皮膚深部までの皮膚領域に留まる場合には，貯留領域の組織に特異的な影響を及ぼす可能性もあるが，現時点では知見は極限られている。細胞レベルの共通した生理的な機能に及ぼす影響が，皮膚系の培養細胞を用いて調べられているが，皮膚細胞特有の性質を反映した取り込みや影響について評価を示すには至っていない。これは，曝露するナノ物質の純度，物理的および化学的性状，曝露方法，曝露する細胞の種類，培養系の単層培養と三次元培養など，考慮すべき要件が多岐にわたっていることによる。

マウスの背部の皮膚に，ベンゼンに溶解したフラーレン（200 μg）を塗布した結果，72時間後のDNA合成とオルニチン脱炭酸酵素活性には変化が認められなかった。7,12-ジメチルベンゾアントラセンにより発がん誘導させた（イニシエーション）皮膚に24時間反復塗布曝露した結果，皮膚腫瘍の発生は認められなかった。しかし，TPA（12-O-テトラデカノイルホルボール-13-アセテート）によりイニシエーションさせた場合は良性の皮膚腫瘍が発生した[6]。この

結果は，フラーレンによる皮膚曝露の急性毒性は認められないながら，発がんプロモーション活性がある可能性を示唆している。

前述したように，皮膚に吸収された後，有害影響を及ぼす可能性が考えられるが体内に入った後は吸気を介して肺から取り込まれた影響と類似の作用を示すと考えられる。そこで，皮膚由来細胞を用いた in vitro 系による作用に関する報告例を示す。

ヒトの皮膚ケラチノサイト（HaCaT）および気管上皮細胞（BEAS-2B）を用い，単層カーボンナノチューブを曝露した研究において，超微細構造と形態学的変化，細胞の結合性の喪失，アポトーシス，酸化ストレスの影響増加，過酸化物の蓄積，抗酸化剤の枯渇を引き起こした結果を報告している。さらに，マイクロアレイ解析により，酸化ストレスに関連する遺伝子の転写発現パターンの変化を誘導することを明らかにしている[7]。これらの結果は，単層カーボンナノチューブが皮膚毒性を誘起し，酸化ストレスが細胞に傷害を及ぼす主要な原因であることを示唆している。しかし，カーボンナノチューブの有害影響は，含まれる不純物，幅や長さの形態，表面の状態に大きな影響を受けることを示唆する報告があり，製造方法，不純物の組成と含量，形態など，吸収と蓄積，体内移行に伴う分布，健康影響において，系統立てた評価をすることが重要である。

ヒト上皮ケラチノサイト（HEK）に 0.1，0.2，0.4mg/ml の多層カーボンナノチューブを 1，2，4，12，48 時間曝露させた研究では，透過型電子顕微鏡による観察で全ての曝露した細胞中に多層カーボンナノチューブが存在することを確認している。炎症性サイトカインの一種であるインターロイキン 8 が時間依存的に放出させていた結果を報告している[8]。この結果は，前述の単層カーボンナノチューブの研究と同様の課題を有しているが，曝露することにより短期刺激応答反応が誘起されることを示唆しており，特に高濃度の職業曝露を防止することが重要であることを示唆している。

また，細胞内物質との反応性に関して，日焼け止め剤に含まれている表面処理をしているアナターゼ / ルチル型のチタン微粒子の存在下で，UVA および UVB を日光照射の想定で照射した研究において，DNA および RNA が部分的に切断を受け，ピリミジン塩基やプリン塩基は分解されて二酸化炭素とアンモニア，硝酸イオンに変化することが報告されている[9]。

おわりに

皮膚については，経口や経気道の曝露経路と異なり，ナノマテリアルを含む製品を意図して使用することによる曝露があり，吸収および貯留としての生物的半減期を明確に評価することが重要である。しかし，皮膚に対する評価についても，他の曝露経路と同様，検討の途についたところといえ，限られた情報が提供されているに過ぎない。

ナノ物質の形状や性状により得られる結果が異なる可能性があるため，系統立てた研究が進め

られることが望まれる。今後の取り組みに，大いに期待したい。

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Fourth International Workgroup on Genotoxicity testing: Results of the in vivo Comet assay workgroup

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Abstract

As part of the Fourth International Workshop on Genotoxicity Testing (IWGT), held 9–10 September 2005 in San Francisco, California, an expert working group on the Comet assay was convened to review and discuss some of the procedures and methods recommended in previous documents. Particular attention was directed at the in vivo rodent, alkaline (pH >13) version of the assay. The aim was to review those protocol areas which were unclear or which required more detail in order to produce a standardized protocol with maximum acceptability by international regulatory agencies. The areas covered were: number of dose levels required, cell isolation techniques, measures of cytotoxicity, scoring of comets (i.e., manually or by image analysis), and the need for historical negative/positive control data. It was decided that a single limit dose was not sufficient although the required number of dose levels was not stipulated. The method of isolating cells was thought not to have a qualitative effect on the assay but more data were needed before a conclusion could be drawn. Concurrent measures of cytotoxicity were required with histopathological examination of tissues for necrosis or apoptosis as the "Gold Standard". As for analysing the comets, the consensus was that image analysis was preferred

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but not required. Finally, the minimal number of studies required to generate a historical positive or negative control database was not defined; rather the emphasis was placed on demonstrating the stability of the negative/positive control data. It was also agreed that a minimum reporting standard would be developed which would be consistent with OECD in vivo genotoxicity test method guidelines.

Keywords: Single cell gel assay; Comet assay; DNA damage; Genotoxicity; Alkaline electrophoresis

1. Introduction

The Comet assay, also referred to as the single cell gel electrophoresis (SCG or SCGE) assay, is a rapid, visual, and quantitative technique for measuring DNA damage in eukaryote cells [1–7]. Under alkaline (pH >13) conditions, the assay can detect single and double-stranded breaks, incomplete repair sites, alkali labile sites, and also possibly both DNA–protein and DNA–DNA cross-links, in virtually any eukaryotic cell population that can be obtained as a single cell suspension.

As the Comet assay has gained in popularity as a standard laboratory technique for evaluating DNA damage and/or repair, the question of how it can be applied within the current regulatory strategy of genotoxicity testing has become a matter of debate [8]. The primary focus of interest has been on the alkaline (pH >13) version, as it is applied to *in vivo* genotoxicity testing strategies [6,8–11]. This is especially important now that acceptance of the *in vivo* Comet assay by regulatory agencies in a number of countries is growing, with some already citing it as an acceptable second test [12,13]. Part of the reason for this acceptance has been the development of a standard protocol and acceptance criteria for the assay through the IWGT working parties [6] and international Comet assay workshops [10]. The purpose of this meeting was to review the procedures and methods recommended in previous guidance documents [6,10], with particular attention being given to the *in vivo* rodent alkaline (pH >13) assay.

Prior to the actual IWGT session, the members of the working group were assigned to different subgroups with each subgroup responsible for reviewing a particular topic. At the IWGT meeting, the subgroups presented their conclusions and recommendations to the complete working group for consideration and discussion, with input from the audience. This report provides an overview of the topics discussed and the consensus reached by the working group with regard to the *in vivo* rodent alkaline (pH >13) Comet assay (hereafter designated as the *in vivo* Comet assay).

2. Discussion topics and recommendations

2.1. Multiple dose levels versus limit dose

For this topic, the discussions focused on the number of dose levels to be used in the *in vivo* Comet assay, especially for cases where there is no evidence of animal toxicity. For example, as stated in the Organisation for Economic Development and Co-operation (OECD) test guideline 474 (rodent bone marrow micronucleus test), a chemical which shows no sign of toxicity up to the limit dose of 2 g/kg need only be tested at that dose [14].

The consensus of the working group was that a single dose level was not sufficient even for substances that could be tested at the limit dose of 2 g/kg. The reasoning behind this consensus was that there were not yet sufficient data to conclude that downturns in the dose response curve (i.e., a bell shaped dose response curve) would not occur for some substances due, for example, to altered bioavailability at higher dose levels. The 'downturn phenomenon', was also a matter of discussion among the members of the IWGT *in vivo* micronucleus (MN) group [15], where this phenomenon has been shown to occur in some MN studies although the underlying mechanism(s) have yet not been identified. In such cases, positive responses occurred at the second highest dose level. Therefore, it was concluded that the use of a single dose level could lead to problems in data interpretation. There was also the feeling that positive responses at multiple dose levels could reinforce the biological relevance of the result.

2.2. Cell isolation process

The background behind this discussion point was the disparate *in vivo* rodent Comet assay data sets published about *ortho*-phenyl phenol. When tested by Sasaki et al. [16], *ortho*-phenyl phenol was positive in the mouse using stomach, liver, kidneys, lung, urinary bladder as target organs. However, when tested by Bomhard et al. [17] in the same species, *ortho*-phenyl phenol was negative in the tissues investigated. One possible explanation for the difference in results was how the tissues were pro-

cessed. In Sasaki et al. [16], isolated nuclei were used, whereas in Bomhard et al. [17], isolated whole cells were used. Although there was much discussion on this subject along with data from two groups which showed that the method of tissue processing (i.e., isolated cells versus isolated nuclei) did not have a qualitative effect on the comet response, it was decided that more data were needed before a conclusion could be made and that any international validation study should consider both processing methods.

2.3. Concurrent measures of cytotoxicity

Cell death is a process that leads to DNA degradation. Thus, all test methods that evaluate primary DNA damage, including the Comet assay, have the potential to detect agents that are cytotoxic rather than genotoxic. However, since DNA damage in the Comet assay is assessed at the level of the individual cell, it is possible in some cases to identify dead or dying cells by their specific image. Under alkaline conditions, necrotic or apoptotic cells can result in comets with small or non-existent head and large diffuse tails [18] as observed in *in vitro* studies following treatment with cytotoxic, non-genotoxic compounds [19–21]. However, such images may not be uniquely diagnostic for apoptosis or necrosis since they may also be detected after treatment with high doses of radiation or high concentrations of strong mutagens [22]. For the *in vivo* Comet assay, only limited data are available to establish whether cytotoxicity results in increased DNA migration in tissues of experimental animals. It was discussed that migration levels detected at the time of sampling are dependent on the tissue and the slope of the dose response for a particular tested compound. For some chemicals, despite the presence of necrosis or apoptosis in target organs such as kidneys [23], testes [24], and liver or duodenum [11], an increase in DNA migration was not observed. In contrast, enhanced DNA migration was seen in cells isolated from the livers of mice dosed with carbon tetrachloride under conditions that also resulted in necrosis, as determined from a histopathological examination [25]. It is also possible that at cytotoxic doses, a decrease in DNA migration may be detected due to the loss of heavily damaged or dying cells during sample processing and/or electrophoresis.

There was consensus on the need to include measures of cytotoxicity and to address the possible effects of cytotoxicity in comet data interpretation. The suggested methods included: a dye exclusion test for membrane integrity and metabolic competency [26] and determining the frequency of cells with low molecular weight DNA using the neutral diffusion assay [6,27]. The "Gold Standard" for assessing levels of necrosis and apoptosis when an *in vivo* Comet assay gave positive results was concluded to be histopathology. It was pointed out that there was a need to standardize ways to present histopathological findings.

2.4. Image analysis (IA) or manual scoring

A variety of commercial and freeware IA systems are available for assessing DNA migration in individual cells. In addition, manual scoring can also be used to determine the length of DNA migration, the percentage of cells with and without migration, or the proportion of comets that can be "binned" into various migration categories (generally one of five, from undamaged to maximally damaged depending upon the tail length) [28]. However, a limitation of this categorization method may be a potential inability to take into account the density or shape of tails which can include short but dense tails and long but sparse tails depending on the effects of compounds tested. With IA systems, the most common parameters analyzed are the percentage DNA in the tail (% tail DNA), tail moment, and tail length and/or image length (referring to nucleus plus migrated DNA). The percentage DNA in the tail is generally defined as the fraction of DNA in the tail divided by the amount of DNA in the cell multiplied by 100, while the tail length is the distance from the middle or the estimated perimeter of the comet head to the last visible signal in the tail. There are several measures of tail moment. The one most commonly used, called the Olive tail moment, is the product of the amount of DNA in the tail and the mean distance of migration in the tail [29]. It is important to note that some parameters (e.g., tail moment) may be calculated differently among IA systems and this can lead to quantitative differences, which can be problematic when comparing inter-laboratory data.

The consensus was that IA is preferred but not required. Heavily damaged cells exhibiting a specific microscopic image (commonly referred to as hedgehogs) consisting of small or non-existent head and large and diffuse tails [18] potentially represent dead or dying cells and may be excluded from data collection. However, determining their frequency may be useful for data interpretation. If IA is used, then % tail DNA appeared to be the most linearly related to dose and the easiest to intuitively understand [30]. However, there was no consensus that this IA measure of DNA migration must be the only one used. If some measure of tail moment is used, than % tail DNA and tail length data must be provided also. Data on the distribution of migration among

cells should also be presented. This is accomplished by sorting cells within "bins" based on the metric used to evaluate DNA migration and presenting the data as the percentage of cells within each bin.

2.5. Historical negative/positive control data

The minimal number of studies needed was not defined but enough studies need to be conducted to demonstrate the stability of the negative/positive control data. Criteria for determining the acceptability of new studies, based on historical control data, should be developed for each tissue by each laboratory. There was discussion on the background responses for negative controls and there was a consensus that negative controls should exhibit measurable DNA migration. However, there was no consensus as to how much mean DNA migration was needed among the control cells. It was recognized that the ability to detect chemicals that predominantly induce DNA cross-linking, damage that reduces the ability of the DNA to migrate, depends on the extent of average DNA migration in the control cells. Investigators who are attempting to detect such chemicals will need to demonstrate the adequacy of their *in vivo* Comet assay protocol for this purpose.

2.6. Minimal reporting standards

It was agreed that to ensure that all studies can be independently evaluated, a minimum reporting standard for regulatory submissions and publications will be developed. This standard will be consistent with OECD *in vivo* genetic toxicology test method guidelines. Previous publications have covered some aspects of protocol design and reporting [10,31].

2.7. Conclusions

In recent years, the *in vivo* Comet assay has become increasingly used for regulatory purposes and acceptance of the test method by regulatory agencies is growing (reviewed in [8]). However, several issues on study design and on data analysis and assessment that required further investigation remain and it was these issues that were discussed by the IWGT working group. In addition to guidance provided in previous published guidelines [6,10], consensus among the participants of the working group was reached with regards to the selection of the number of dose levels, the need to include concurrent measures of cytotoxicity in the studies, the adequacy of manual scoring, and the need to develop historical control data. Consensus was also reached on the need for an international validation study to stringently evaluate the reliability and accuracy of the *in vivo* Comet assay (as well as *in vitro* versions). This validation study would compare, among other protocol issues, test results obtained using isolated nuclei versus isolated whole cells from various tissues.

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During the preparation of this report, the Japanese Center for the Validation of Alternative Methods (JaCVAM) announced that they were forming a study management team including participants from the European Centre for the Validation of Alternative Methods (ECVAM), the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), US National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the Mammalian Mutagenicity Study Group/Japanese Environmental Mutagen Society (MMS/JEMS) to conduct an international Comet assay validation study. This validation study is scheduled to start in late 2006 and will focus initially on the *in vivo* Comet assay, to be followed by the validation of various *in vitro* Comet assays. ECVAM has also implemented an initiative to evaluate the validity of the *in vitro* Comet assay.

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Mouse lymphoma thymidine kinase gene mutation assay: Meeting of the International Workshop on Genotoxicity Testing, San Francisco, 2005, recommendations for 24-h treatment

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Abstract

The Mouse Lymphoma Assay (MLA) Workgroup of the International Workshop on Genotoxicity Testing (IWGT), comprised of experts from Japan, Europe and the United States, met on September 9, 2005, in San Francisco, CA, USA. This meeting of the MLA Workgroup was devoted to reaching a consensus on issues involved with 24-h treatment. Recommendations were made concerning the acceptable values for the negative/solvent control (mutant frequency, cloning efficiency and suspension growth) and the criteria

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to define an acceptable positive control response. Consensus was also reached concerning the use of the global evaluation factor (GEF) and appropriate statistical trend analysis to define positive and negative responses for the 24-h treatment. The Workgroup agreed to continue their support of the International Committee on Harmonization (ICH) recommendation that the MLA assay should include a 24-h treatment (without S-9) in those situations where the short treatment (3–4 h) gives negative results.

Keywords: Mouse lymphoma assay; In vitro mutation; Thymidine kinase

1. Introduction

The Mouse Lymphoma Assay (MLA) Workgroup of the International Workshop on Genotoxicity Testing (IWGT), comprised of experts from Japan, Europe and the United States, met on September 9, 2005, in San Francisco, CA, USA. This meeting of the MLA Workgroup was devoted to reaching a consensus on issues related to the use of 24-h treatment.

The first meeting of the MLA Workgroup was held as a part of the International Workshop on Genotoxicity Testing Procedures in Washington, DC, in the spring of 1999. Since that time, the Workgroup has been working to address three main issues of importance to the assay. These include: (1) the conduct of a data-based analysis and a final recommendation for using the relative total growth (RTG) as the appropriate measure for cytotoxicity; (2) the criteria for data acceptance (based on the negative/vehicle and positive controls) and a new method [the global evaluation factor (GEF)] for data evaluation; (3) the issues related to the International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) recommended use of a 24-h treatment time (including the ability of the assay to detect aneugens). This is the 5th meeting of the Workgroup in which consensus has been reached and reported. The previous four meetings are reported in Moore et al. [1–4].

2. 24-h treatment

Following the ICH recommendation requiring that the MLA be conducted using a 24-h treatment (without S-9) in situations where the short treatment (3–4 h) was negative, laboratories have conducted such experiments. With the goal of determining the approximate frequency at which chemicals require a 24-h treatment to express their mutagenic potential, and to make recommendations concerning the conduct of the 24-h treatment, the Workgroup solicited data from laboratories conducting both the agar and microwell versions of the assay.

Laboratories were asked to evaluate data obtained since 2002 (some earlier data were submitted) and to base a positive result on the assay evaluation criteria in force for each participating laboratory at the time of the performance of the assay. They were asked to identify: (i) compounds uniquely positive following 24-h treatment in the absence of S-9, (ii) compounds positive following short (3- or 4-h) treatment times in the absence of S-9, but negative following 24-h treatment in the absence of S-9, and (iii) compounds either known or suspected to be aneugens.

An estimated 990 data sets (compounds) were reviewed by the individual laboratories to identify compounds fitting into one of these three categories. The majority of assays (approximately 900) were performed using the microwell method; approximately 90 assays used the agar method. Of these approximately 990 tests, 71 (7%) were positive, as assessed by the individual laboratory. It should be noted that the nine laboratories that submitted data only provided the actual data for these 71 test agents. These data were compiled, analyzed and summarized by three members of the Workgroup and the summary information was used in the deliberations of the entire Workgroup. It should be noted that only five data sets were submitted for the third category (known or suspected aneugens). This small number was considered insufficient to permit meaningful analysis and although the data was compiled, there was no discussion of this category by the Workgroup.

2.1. Category 1 responses

A total of 56 data sets (54 in the microwell assay and 2 in the agar assay) were uniquely positive following 24-h treatment in the absence of S-9. The negative controls for each of the 56 data sets were evaluated to determine whether they met all of the revised assay acceptance criteria agreed in the 3rd Workgroup meeting, held in Plymouth in 2002 [3] and the acceptable range for solvent control mutant frequencies (MF) agreed in the 4th Workgroup meeting held in Aberdeen in 2003 [4]. The application of these acceptance criteria eliminated 19 data sets. See Fig. 1 for a breakdown as to the causes of the unacceptable experiments.

A number of the remaining 56 data sets showed only a very small induced MF (IMF). In fact, the maximum

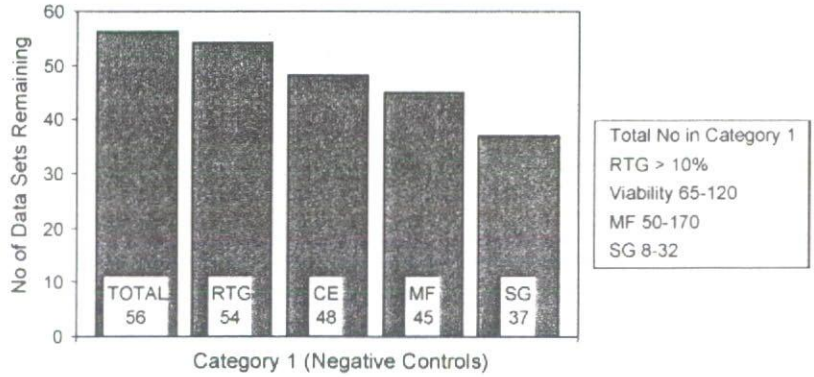

Fig. 1. Column graph demonstrating how many Category 1 (unique 24-h positive) data sets would be excluded as the acceptance criteria [4] are applied sequentially left to right. Note that SG as described in this instance were the values recommended for the short term treatment. They were applied to the 2 day expression period and excluded the 24-h treatment.

increase in MF observed at any data point (compared to concurrent controls) did not exceed the GEF in 26 out of 56 data sets (Fig. 2). Therefore, these data sets did not meet the new criteria required for a positive response, as agreed in the 4th Workgroup meeting in Aberdeen in 2003 [4]. Positive responses are defined as those that exceed the GEF and show statistically positive dose response trends (see discussion below concerning data evaluation).

After applying both the acceptance criteria for the negative controls and the GEF, the number of unique 24-h positive compounds was reduced to 18.

2.2. Category 2 responses

Only 10 data sets (9 in the microwell assay and 1 in the agar assay) were positive following the short (3- or 4-h) treatment in the absence of S-9, but apparently negative following 24-h treatment in the absence of S-9. Application of the acceptance criteria to the negative controls in these data sets reduced this number to 7 and the additional application of the GEF to the data further reduced the number to 4 (Fig. 3). The Workgroup made no additional recommendations concerning the 3–4 h treatment, based on this data.

2.3. Acceptance criteria for negative/vehicle controls (24-h treatment)

Previously, following an extensive evaluation of negative/vehicle control data from a number of laboratories using the short (3- or 4-h) treatment time, the Workgroup reached consensus on the acceptance criteria for individual experiments based upon several negative/vehicle control parameters [4]. With the exception of the suspension growth (SG) parameter, the Workgroup recommended that the same criteria be applied to the 24-h treatment experiments. Because the 24-h treatment includes 3 rather than 2 days of suspension growth, the acceptance criteria for the 24-h SG was revised to 32–180. The theoretical optimum suspension growth is about 5-fold per day, or 125 over the 3-day

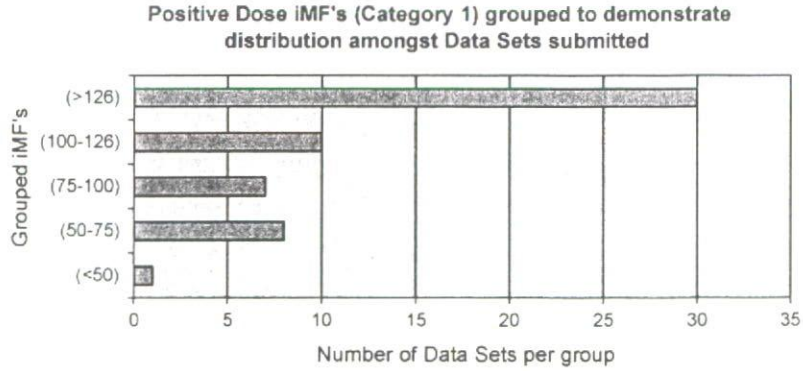

Fig. 2. Bar chart demonstrating breakdown of the 56 Category 1 (unique 24-h positive) studies in terms of the induced MF of highest positive data point.

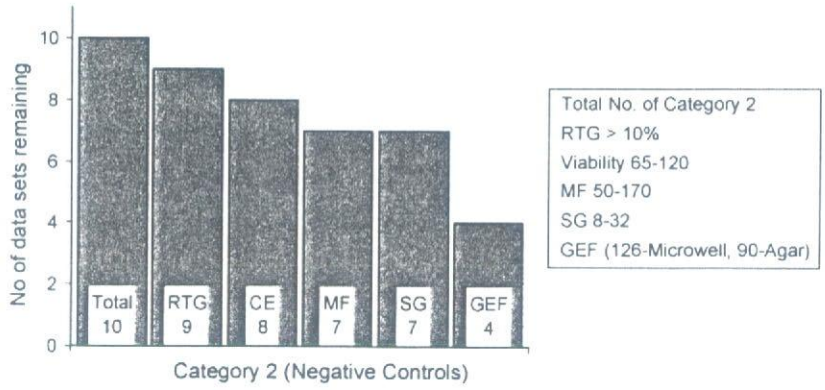

Fig. 3. Column graph demonstrating the number of Category 2 (unique 3- or 4-h positive) data sets that would be excluded as stated acceptance criteria [4] are applied in sequence of left to right.

period. However, there is variability in growth rates and the Workgroup would be very concerned with suspension growth less than 32. The high limit of 180 allows for reasonable errors in cell counting and dilution. As with the short treatment, the acceptance criteria for the background MF are specific to the agar or microwell version of the assay. For both methods the cloning efficiency (CE) referred to in the criteria is the absolute CE obtained at the time of mutant selection. The suspension growth of the negative/vehicle control refers to the growth during both the 24-h treatment and the 2-day expression period following treatment. It is defined as the fold-increase of the cell number during this 3-day period. The SG is calculated by the treatment period fold-increase multiplied by both the expression day 1 and expression day 2 fold-increases in cell number.

The acceptance criteria for the negative/vehicle control parameters for the soft agar and microwell methods of performing the MLA using 24-h treatment are now as follows:

Agar method	Microwell method
MF: 35–140 × 10^{-6}	MF: 50–170 × 10^{-6}
CE: 65–120%	CE: 65–120%
SG: 32–180	SG: 32–180

2.4. Acceptance criteria for positive controls (24-h treatment)

As with the short treatment time experiments, positive control cultures should be included in every 24-h treatment MLA experiment. To assess the adequacy of detection of both small and large colony mutants in the 24-h treatment, the Workgroup agreed that it is appropriate to apply the same acceptance criteria developed for the short treatment time. There are two equally acceptable approaches to assuring an adequate positive control response. (1) The laboratory should use a dose of a mutagenic chemical that yields an absolute increase in total MF that is an increase above the spontaneous background MF [an induced MF] of at least 300 × 10^{-6}. The small colony MF should account for at least 40% of that IMF. For instance, in a culture showing an IMF of 300 × 10^{-6}, the small colony IMF should be at least 120 × 10^{-6}. (2) The second approach requires the use of a dose of a chemical that increases the small colony MF at least 150 × 10^{-6} above that seen in the concurrent negative/vehicle control (a small colony IMF of 150 × 10^{-6}).

In addition, the upper limit of cytotoxicity observed in the positive control culture should have a relative total growth (RTG) that is greater than 10% [2]. The Workgroup recognizes that some laboratories prefer to use more than one dose of their positive control and/or to use a dose that gives a small increase in MF. For these laboratories, it is sufficient if only a single dose of the positive control meets the acceptance criteria.

2.5. Data evaluation

Once the criteria for experimental acceptance have been satisfied, the data from each individual experiment can be evaluated to determine whether the response is positive, negative or equivocal. The Workgroup agreed that data generated using 24-h treatment should be evaluated using the same method previously developed by the Workgroup for use with the short treatment times. A brief summary of the previous analyses conducted by the Workgroup and the rationale for developing the new method using the GEF in conjunction with appropriate statistical analysis to ascertain the presence of a dose-related positive trend is included in the Plymouth and Aberdeen Meeting Reports [3,4]. It should

be noted that the GEF approach takes into account previous guidance documents (i.e. FDA Redbook [http://www.cfsan.fda.gov/~redbook/red-toca.html] and OECD [5], which states that biological relevance should be a major factor in data evaluation.

The GEF evaluation method requires that the IMF exceeds a value based on the global distribution of the background MF for each method (agar or microwell). This value, the GEF, was established by the Workgroup, based on short treatment experiments, to be 126 for the microwell version of the assay and 90 for the agar version. The GEF is applied as follows: if the negative/vehicle control MF in a microwell experiment is 100×10^{-6}, then one of the treatment groups must have a MF of at least $100 + 126$ (the microwell GEF) $= 226 \times 10^{-6}$ in order to meet the GEF criterion for a positive call. An appropriate statistical trend test should be applied to determine whether there was a positive dose-related increase.

A test agent response in an experiment is positive if both the IMF for any treatment meets or exceeds the GEF and a positive trend test is obtained. A test agent response is clearly negative if both the trend analysis and the GEF are negative. Situations where either (but not both) the GEF or statistical analysis is positive should be evaluated on a case-by-case basis. It should be noted that it is generally advisable to conduct one or more additional experiments to better define the assay response (particularly in the 30–10% RTG cytotoxicity range).

For more detail on the Workgroup recommendations on the steps for proper assay evaluation, the reader is referred to the summaries of the New Orleans, Plymouth and Aberdeen meetings [2–4]. All of these recommendations are equally applicable to the short treatment and the 24-h treatment.

3. Conclusions

From this analysis, it is clear that only a very small percentage of chemicals (less than 2%) are uniquely positive at 24 h, and an even smaller percentage appear to be uniquely positive at short (3- or 4-h) treatment times. The low numbers of unique 3- or 4-h positive results may be attributable to the regulatory guideline requirements that positive results observed following 3- or 4-h treatments do not need to be further evaluated at 24 h. In some of the unique 24-h treatment cases, the longer treatment time provides for the effective treatment of a higher dose of compound. This is particularly true when a chemical's insolubility prevents testing to adequate toxicity in the short treatment time. There are also some situations in which the maximum recommended concentration (5000 μg/ml) was not mutagenic and was insufficiently toxic in the short treatment, but was mutagenic following a 24-h treatment. There is also evidence that some (but not all) aneugens require longer treatment time [6].

Based upon all the available data, the Workgroup agreed to continue its support of the ICH recommendation that 24-h treatment be used when the short treatment time is negative or equivocal.

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Strategy for genotoxicity testing—Metabolic considerations

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Abstract

The report from the 2002 International Workshop on Genotoxicity Tests (IWGT) Strategy Expert Group emphasized metabolic considerations as an important area to address in developing a common strategy for genotoxicity testing. A working group convened at the 2005 4th IWGT to discuss this area further and propose practical strategy recommendations. To propose a strategy, the working group reviewed: (1) the current status and deficiencies, including examples of carcinogens "missed" in genotoxicity testing, established shortcomings of the standard *in vitro* induced S9 activation system and drug metabolite case examples; (2) the current status of possible remedies, including alternative S9 sources, other external metabolism systems or genetically engineered test systems; (3) any existing positions or guidance. The working group established consensus principles to guide strategy development. Thus, a human metabolite of interest should be represented in genotoxicity and carcinogenicity testing, including evaluation of alternative genotoxicity *in vitro* metabolic activation or test systems, and the selection of a carcinogenicity test species showing appropriate biotransformation. Appropriate action triggers need to be defined based on the extent of human exposure, considering any structural knowledge of the metabolite, and when genotoxicity is observed upon *in vitro* testing in the presence of metabolic activation. These triggers also need to be considered in defining the timing of human pharmaceutical ADME assessments. The working group proposed two strategies to consider; a more proactive approach, which emphasizes early metabolism predictions to drive appropriate hazard assessment; and a retroactive approach to manage safety risks of a unique or "major" metabolite once identified and quantitated from human clinical ADME studies. In both strategies, the assessment of the genotoxic potential of a metabolite could include the use of an alternative or optimized *in vitro* metabolic activation system, or direct testing of an isolated or synthesized metabolite. The working group also identified specific areas where more data or experiences need to be gained to reach consensus. These included defining a discrete exposure action trigger for safety assessment and when direct testing of a metabolite of interest is warranted versus the use of an alternative *in vitro* activation system, a universal recommendation for the timing of human ADME studies for drug candidates and the positioning of metabolite structural knowledge (through *in silico* systems, literature, expert analysis) in supporting metabolite safety qualification. Lastly, the working group outlined future considerations for refining the initially proposed

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