ARTICLES

薬の名前

ステムを知れば薬がわかる

Stems used in drug names : For the better understanding of pharmacological actions of drugs

第6回

国立医薬品食品衛生研究所
内田恵理子，川崎ナナ
ERIKO UCHIDA, NANA KAWASAKI
National Institute of Health Sciences

名古屋市立大学大学院薬学研究科
宮田直樹
NAOKI MIYATA
Faculty of Pharmaceutical Sciences, Nagoya City University

はじめに

本連載では，これまで化学薬品のステムについて紹介してきたが，今回から数回に分けて，生物薬品のステムを紹介する。生物薬品の第1回目は，生物薬品の一般名の命名に関する基本的ルールを紹介するとともに，サイトカイン類のステムについて紹介する。

生物薬品の国際一般名（INN）は，化学薬品と同様にWHOのINN委員会で決定される。生物薬品でも多くの場合，医薬品の分類ごとにステムが与えられ，ステムを用いて一般名が命名される。例えば，「som-」は成長ホルモンに関連する医薬品，「-stim」はコロニー刺激因子類，「-mab」はモノクローナル抗体などである。表1に，生物薬品の主な分類とステムの例を示した。一方，表1に示したインスリン類やインターフェロン類などにはステムがなく，学術用語と同じ「insulin」，「interferon」が命名に用いられている。命名ルールには統一されていない部分もあるが，本連載では，「insulin」や「interferon」もステムとして扱うことにする。

医薬品の分類をさらに小分類に分ける必要がある場合は，ステムから派生したサブステム（sub-stem）を用いる。表2に，インターロイキン類のサブステムの例を示

表1　生物薬品の主な分類とステムの例

ステムのある分類（Groups with International Stems）	ステム
成長ホルモン類（growth hormones）	som-
ホルモン放出促進／抑制ペプチド（hormone-release stimulating/inhibiting peptides）	-relin/relix
サイトカイン／インターロイキン類（cytokines/interleukins）	-kin
コロニー刺激因子類（colony stimulating factors）	-stim
エリスロポエチン類（erythropoietin type blood factors）	-poetin
モノクローナル抗体類（monoclonal antibodies）	-mab
成長因子類（growth factors）	-ermin
酵素類（enzymes）	-ase
血液凝固因子類（blood coagulation factors）	-cog
血液凝固カスケード阻害剤（blood coagulation cascade inhibitors）	-cogin
ペプチド，糖ペプチド類（peptides and glycopeptides）	-tide
受容体分子類（receptor molecules, native or modified）	-cept (pre-stem)*1
ヒルジン誘導体類（hirudin derivatives）	-irudin
ヘパリン誘導体類（heparin derivatives）	-parin
ステムのない分類（Groups without International Stems）	**ステム**
インスリン類（insulins）	insulin
インターフェロン類（interferons）	interferon

＊1：暫定ステム

した。

同一のステムに属するペプチドあるいはタンパク質性医薬品でアミノ酸配列が異なることを示す場合には，ステムに接頭語あるいは接尾語を付加してアミノ酸配列の違いを区別している。例えば，インターロイキン-2の場合，ステムは「-leukin」であるが，Celmoleukin(セルモロイキン)とTeceleukin(テセロイキン)は，N末端のメチオニン残基の有無が異なる。また，インスリン類の場合は，アミノ酸配列の違いを2語式(two-word name)の命名をして区別している。例えば，Insulin Aspart(インスリン アスパルト)は，Insulin(インスリン)のアミノ酸残基1カ所がアスパラギンに置換した誘導体である。

糖タンパク質や糖ペプチド医薬品で，アミノ酸配列は同一であるが糖鎖部分の構造が違うことを示す場合には，ギリシャ文字を略さずに記載したアルファ，ベータ，ガンマ(alfa, beta, gamma)等を用いた2語式の命名で糖鎖構造の違いを区別している。例えば，「-poetin」はエリスロポエチン類のステムであるが，糖鎖の異なるものは，Epoetin Alfa, Epoetin Beta, Epoetin Gamma等，命名されている。

しかし，例外的な命名ルールとして，インターフェロン類では糖鎖の違いではなく，インターフェロンの小分類を区別するためにギリシャ文字が用いられている。インターフェロンの名称については，ステム31「インターフェロン」の項で詳しく説明する。

なお，JANでは，遺伝子組換え技術を用いて製造された生物薬品の正名にはINNの後に括弧書きで(遺伝子組換え)，英名では(Genetical Recombination)と記載し，遺伝子組換えであることを明示するが，本連載では本文中では記載を省略した。

「-stim」:コロニー刺激因子類

「-stim」は，コロニー刺激因子(colony stimulating factor, CSF)類に共通のステムである。コロニー刺激因子とは，骨髄細胞に作用して，半固形培地で血液細胞のコロニー形成を促進する造血因子の総称であり，サイトカインの1種である。形成されるコロニーの種類によってさらにサブステムに分類される。

表2　インターロイキン類のサブステム

インターロイキン-1(IL-1)	-nakin	
インターロイキン-1α(IL-1α)	-onakin	Pifonakin(ピフナキン)
インターロイキン-1β(IL-1β)	-benakin	Mobenakin (モベナキン)
インターロイキン-2(IL-2)	-leukin	Adargileukin Alfa Aldesleukin Celmoleukin (セルモロイキン) Denileukin Diftitox Pegaldesleukin Teceleukin (テセロイキン) Tucotuzumab Celmoleukin
インターロイキン-3(IL-3)	-plestim	Daniplestim Muplestim
インターロイキン-4(IL-4)	-trakin	Binetrakin
インターロイキン-6(IL-6)	-exakin	Atexakin Alfa
インターロイキン-8(IL-8)	-octakin	Emoctakin
インターロイキン-10(IL-10)	-decakin	Ilodecakin
インターロイキン-11(IL-11)	-elvekin	Oprelvekin (オプレルベキン)
インターロイキン-12(IL-12)	-dodekin	Edodekin Alfa
インターロイキン-13(IL-13)	-tredekin	Cintredekin Besudotox
ニューロトロピン(インターロイキン-78, Brain derived neurotropic factor)	-neurin	Abrineurin
インターロイキン-1受容体アンタゴニスト	-nakinra	Anakinra
インターロイキン-4受容体アンタゴニスト	-kinra	Pitrakinra

(1)「-grastim」：顆粒球コロニー刺激因子類

「-grastim」は，顆粒球コロニー刺激因子(granurocyte-colony stimulating factor, G-CSF)類を示すサブステムである。G-CSFは顆粒球(好中球)の前駆細胞に特異的に作用してその増殖，分化を促進してコロニー形成を誘導する作用を有する。天然のヒトG-CSFは174個のアミノ酸残基からなり，Thr133に*O*-結合型糖鎖を有する分子量約20,000の糖タンパク質である。

ステム「-grastim」を持ち，現在，日本で承認されている医薬品には，Lenograstim(レノグラスチム)，Filgrastim(フィルグラスチム)，Nartograstim(ナルトグラスチム)の3品目がある(図1)。これらの医薬品は主にがん化学療法後の好中球減少症治療薬として用いられているほか，造血幹細胞の末梢血中への動員や造血幹細胞移植時の好中球数の増加促進にも用いられる。今後，日局への収載が予定されている医薬品である。

Lenograstim(レノグラスチム)はCHO細胞で製造された遺伝子組換えヒトG-CSFで，天然のものと同様に174個のアミノ酸残基からなり，*O*-結合型糖鎖を有する糖タンパク質である。Filgrastim(フィルグラスチム)は大

Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-Cys-Leu-Glu-Gln-Val-Arg-Lys-Ile-Gln-Gly-Asp-Gly-Ala-Ala-Leu-Gln-Glu-Lys-Leu-Cys-Ala-Thr-Tyr-Lys-Leu-Cys-His-Pro-Glu-Glu-Leu-Val-Leu-Leu-Gly-His-Ser-Leu-Gly-Ile-Pro-Trp-Ala-Pro-Leu-Ser-Ser-Cys-Pro-Ser-Gln-Ala-Leu-Gln-Leu-Ala-Gly-Cys-Leu-Ser-Gln-Leu-His-Ser-Gly-Leu-Phe-Leu-Tyr-Gln-Gly-Leu-Leu-Gln-Ala-Leu-Glu-Gly-Ile-Ser-Pro-Glu-Leu-Gly-Pro-Thr-Leu-Asp-Thr-Leu-Gln-Leu-Asp-Val-Ala-Asp-Phe-Ala-Thr-Thr-Ile-Trp-Gln-Gln-Met-Glu-Glu-Leu-Gly-Met-Ala-Pro-Ala-Leu-Gln-Pro-Thr-Gln-Gly-Ala-Met-Pro-Ala-Phe-Ala-Ser-Ala-Phe-Gln-Arg-Arg-Ala-Gly-Gly-Val-Leu-Val-Ala-Ser-His-Leu-Gln-Ser-Phe-Leu-Glu-Val-Ser-Tyr-Arg-Val-Leu-Arg-His-Leu-Ala-Gln-Pro

*O-結合型糖鎖結合位置

Lenograstim (Genetical Recombination)
レノグラスチム(遺伝子組換え)

Met-Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-Cys-Leu-Glu-Gln-Val-Arg-Lys-Ile-Gln-Gly-Asp-Gly-Ala-Ala-Leu-Gln-Glu-Lys-Leu-Cys-Ala-Thr-Tyr-Lys-Leu-Cys-His-Pro-Glu-Glu-Leu-Val-Leu-Leu-Gly-His-Ser-Leu-Gly-Ile-Pro-Trp-Ala-Pro-Leu-Ser-Ser-Cys-Pro-Ser-Gln-Ala-Leu-Gln-Leu-Ala-Gly-Cys-Leu-Ser-Gln-Leu-His-Ser-Gly-Leu-Phe-Leu-Tyr-Gln-Gly-Leu-Leu-Gln-Ala-Leu-Glu-Gly-Ile-Ser-Pro-Glu-Leu-Gly-Pro-Thr-Leu-Asp-Thr-Leu-Gln-Leu-Asp-Val-Ala-Asp-Phe-Ala-Thr-Thr-Ile-Trp-Gln-Gln-Met-Glu-Glu-Leu-Gly-Met-Ala-Pro-Ala-Leu-Gln-Pro-Thr-Gln-Gly-Ala-Met-Pro-Ala-Phe-Ala-Ser-Ala-Phe-Gln-Arg-Arg-Ala-Gly-Gly-Val-Leu-Val-Ala-Ser-His-Leu-Gln-Ser-Phe-Leu-Glu-Val-Ser-Tyr-Arg-Val-Leu-Arg-His-Leu-Ala-Gln-Pro

Filgrastim (Genetical Recombination)
フィルグラスチム(遺伝子組換え)

Met-Ala-Pro-Thr-Tyr-Arg-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-Ser-Leu-Glu-Gln-Val-Arg-Lys-Ile-Gln-Gly-Asp-Gly-Ala-Ala-Leu-Gln-Glu-Lys-Leu-Cys-Ala-Thr-Tyr-Lys-Leu-Cys-His-Pro-Glu-Glu-Leu-Val-Leu-Leu-Gly-His-Ser-Leu-Gly-Ile-Pro-Trp-Ala-Pro-Leu-Ser-Ser-Cys-Pro-Ser-Gln-Ala-Leu-Gln-Leu-Ala-Gly-Cys-Leu-Ser-Gln-Leu-His-Ser-Gly-Leu-Phe-Leu-Tyr-Gln-Gly-Leu-Leu-Gln-Ala-Leu-Glu-Gly-Ile-Ser-Pro-Glu-Leu-Gly-Pro-Thr-Leu-Asp-Thr-Leu-Gln-Leu-Asp-Val-Ala-Asp-Phe-Ala-Thr-Thr-Ile-Trp-Gln-Gln-Met-Glu-Glu-Leu-Gly-Met-Ala-Pro-Ala-Leu-Gln-Pro-Thr-Gln-Gly-Ala-Met-Pro-Ala-Phe-Ala-Ser-Ala-Phe-Gln-Arg-Arg-Ala-Gly-Gly-Val-Leu-Val-Ala-Ser-His-Leu-Gln-Ser-Phe-Leu-Glu-Val-Ser-Tyr-Arg-Val-Leu-Arg-His-Leu-Ala-Gln-Pro

Nartograstim (Genetical Recombination)
ナルトグラスチム(遺伝子組換え)

図1　顆粒球コロニー刺激因子類を示すステム「-grastim」を持つ医薬品

腸菌で製造された遺伝子組換えヒトG-CSFで，N末端にメチオニンが1残基付加したアミノ酸175個からなるタンパク質である。また，Nartograstim(ナルトグラスチム)は大腸菌で製造されたヒトG-CSF誘導体で，N末端にメチオニンが1残基付加しているほか，アミノ酸残基5カ所が置換されているアミノ酸175個からなるタンパク質である。天然型G-CSFと比べて高い比活性を示す。なお，図1には天然型と異なるアミノ酸残基を赤文字で示した。

これらの他にINNに登録されている医薬品には以下のものがある。

Pegfilgrastim

Pegnartograstim

これらは，それぞれFilgrastim(フィルグラスチム)，Nartograstim(ナルトグラスチム)にポリエチレングリコールを結合した修飾タンパク質である。「Peg-」はポリエチレングリコール(PEG)が結合していることを意味する接頭語である。PEGによる修飾(PEG化)はDDS(Drug delivery system)の手法のひとつで，タンパク質性医薬品の体内での安定性の向上，血中消失半減期の延長や抗原性の低下を目的として行われる。欧米ではすでに持続性を高めたPegfilgrastimが承認されているが，日本ではまだ実用化されていない。

(2)「-gramostim」：顆粒球マクロファージコロニー刺激因子類

「-gramostim」は，顆粒球マクロファージコロニー刺激因子(granurocyte macrophage colony stimulating factor, GM-CSF)類を示すサブステムである。GM-CSFは，顆粒球(好中球)，マクロファージ，好酸球またはこれらの混合コロニー形成を誘導する作用を持つ。ヒトGM-CSFは127個のアミノ酸残基からなる分子量約18,000～24,000の糖タンパク質である。

ステム「-gramostim」を持つINNは以下のものがある。

Molgramostim

Ecogramostim

Regramostim

Sargramostim(サルグラモスチム)

Molgramostimは大腸菌で製造した遺伝子組換えヒトGM-CSF，Ecogramostimは大腸菌で製造したヒトGM-CSFでN末端にメチオニン残基が付加したもの，RegramostimはCHO細胞で製造したヒトGM-CSFで糖鎖が結合しているものである。Sargramostim(サルグラモスチム)はヒトGM-CSFの23番目のアルギニンをロイシンに置換したGM-CSF誘導体で，遺伝子組換えにより酵母で製造した糖タンパク質である。米国では化学療法後の白血球増加薬として承認されている。JANに登録され，クローン病患者の治療薬として臨床開発中である。

(3)「-mostim」：マクロファージコロニー刺激因子類

「-mostim」は，マクロファージコロニー刺激因子

(macrophage colony stimulating factor, M-CSF)類を示すサブステムである。M-CSFは，単球，マクロファージの前駆細胞に特異的に作用し，その分化，増殖を促進してコロニー形成を誘導する作用を持つ。ヒトM-CSFは149個または214個のアミノ酸残基からなる同一のサブユニット2分子で構成される，分子量約45,000と約84,000の2種類の糖タンパク質が知られている。

ステム「-mostim」を持つINNは以下のものがある。

Cilmostim

Lanimostim

Mirimostim(ミリモスチム)

これらのうち，Mirimostim(ミリモスチム)は，ヒト尿より精製したM-CSFで，214個のアミノ酸残基からなるタンパク質のホモ2量体で構成される糖タンパク質(分子量：約84,000)であり，日本で承認され顆粒球減少症治療薬として使用されている。

(4)「-plestim」：インターロイキン-3類

「-plestim」は，インターロイキン-3 (interleukin-3, IL-3)類を示すサブステムである。IL-3は多能性コロニー刺激因子(multi-CSF)とも呼ばれていたもので，顆粒球，マクロファージ，マスト細胞，赤血球，好酸球，巨核球系と多様な造血系細胞の分化，増殖を促進する作用を有する。IL-3はインターロイキンに分類されているが，ステムはインターロイキンのステム「-kin」ではなく，コロニー刺激因子のステム「-stim」が用いられている。ヒトIL-3は133個のアミノ酸残基からなり，4個の*N*-結合型糖鎖を有する糖タンパク質である。

ステム「-plestim」を持つINNには以下のものがある。

Muplestim(ムプレスチム)

Daniplestim

Muplestim(ムプレスチム)は，遺伝子組換えヒトIL-3で，JANに登録されているが，未承認である。Daniplestimは，IL-3の14番目から125番目のアミノ酸残基のうち，27個のアミノ酸残基を改変したIL-3誘導体で，IL-3よりも強力なIL-3受容体アゴニストとして開発中の医薬品である。

(5)「-distim」：2種類のコロニー刺激因子の融合タンパク質

「-distim」は，2種類の異なるコロニー刺激因子の融合タンパク質を示すサブステムである。INNでは以下の2種類が登録されている。

Leridistim

Milodistim

Leridistimは，IL-3誘導体とG-CSF誘導体との融合タンパク質，Milodistimは，GM-CSF誘導体とIL-3誘導体との融合タンパク質である。

(6)その他の「-stim」類

INNにはその他の「-stim」として，以下のものが登録されている。

Ancestim(アンセスチム)

Garnocestim

Pegacaristim

Ancestim(アンセスチム)は，造血幹細胞の増殖に重要な分子であるヒト幹細胞因子(stem cell factor, hSCF)の可溶性(分泌型)タンパク質を遺伝子組換えで製造したもので，hCSFの1-165番目のアミノ酸残基のN末端にメチオニン残基が付加したタンパク質の2量体からなる。JANに登録され，再生不良性貧血治療薬として開発が進められていたが，臨床開発は中止されている。

Garnocestimは，白血球遊走活性を有するCXCケモカインのひとつであるGROβ／マクロファージ炎症性タンパク質(macrophage inflammatory protein, MIP)2αの5-73番目のアミノ酸残基に相当するペプチドである。

Pegacaristimは，血小板産生を促進するヒトトロンボポエチン(thrombopoetin, TPO)の活性領域(recombinant human megakaryocyte growth and development factor, rhMGDF)にPEGを結合した修飾タンパク質で，血小板減少症治療薬として開発中である。

「-kin」：サイトカイン／インターロイキン類

「-kin」は，サイトカインの中の一群の分子種であるインターロイキン(interleukin)類に共通するステムである。インターロイキンはリンパ球や単球，マクロファージなどの免疫担当細胞が産生放出する(糖)タンパク質性の生物活性物質の総称で，細胞表面に存在する受容体を介して細胞の活性化，分化，増殖，細胞間相互作用などに関与する。インターロイキンはタンパク質として同定された順にインターロイキン(IL)の後に番号を付けて呼ばれている。インターロイキンのステムの「-kin」もイ

ンターロイキンの種類ごとにサブステムが与えられている。インターロイキンおよびインターロイキンに関連する医薬品のステムは表2に示した。

(1)「-leukin」：インターロイキン-2類

「-leukin」はインターロイキン-2(interleukin-2, IL-2)類を示すサブステムである。インターロイキン類の中で，日本で医薬品として実用化されているのはIL-2のみである。IL-2はT細胞増殖因子と呼ばれていたもので，T細胞より産生され，T細胞の増殖と分化を促進するほか，ナチュラルキラー細胞の活性化，B細胞の増殖など多様な作用を示す。ヒトIL-2はアミノ酸133個からなる糖タンパク質である。

ステム「-leukin」を持つINNは7品目が登録されている(表2)。これらのうち，Celmoleukin(セルモロイキン)，Teceleukin(テセロイキン)は新たに日局に収載された医薬品である(図2)。これらはいずれもヒトIL-2のcDNAを導入した大腸菌で製造されるタンパク質で，Celmoleukin(セルモロイキン)は天然のIL-2と同じ133個のアミノ酸残基から，また，Teceleukin(テセロイキン)はN末端にメチオニン1残基が付加した134個のアミノ酸残基からなるタンパク質である。いずれも天然のIL-2とは異なり糖鎖は付加していない。腎がん，血管肉腫の治療薬として使用されている。

Ala-Pro-Thr-Ser-Ser-Ser-Thr-Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu-His-Leu-Leu-Leu-Asp-
Leu-Gln-Met-Ile-Leu-Asn-Gly-Ile-Asn-Asn-Tyr-Lys-Asn-Pro-Lys-Leu-Thr-Arg-Met-Leu-
Thr-Phe-Lys-Phe-Tyr-Met-Pro-Lys-Lys-Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys-Leu-Glu-
Glu-Glu-Leu-Lys-Pro-Leu-Glu-Glu-Val-Leu-Asn-Leu-Ala-Gln-Ser-Lys-Asn-Phe-His-Leu-
Arg-Pro-Arg-Asp-Leu-Ile-Ser-Asn-Ile-Asn-Val-Ile-Val-Leu-Glu-Leu-Lys-Gly-Ser-Glu-
Thr-Thr-Phe-Met-Cys-Glu-Tyr-Ala-Asp-Glu-Thr-Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-
Trp-Ile-Thr-Phe-Cys-Gln-Ser-Ile-Ile-Ser-Thr-Leu-Thr

Celmoleukin(Genetical Recombination)
セルモロイキン(遺伝子組換え)

Met-Ala-Pro-Thr-Ser-Ser-Ser-Thr-Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu-His-Leu-Leu-Leu-
Asp-Leu-Gln-Met-Ile-Leu-Asn-Gly-Ile-Asn-Asn-Tyr-Lys-Asn-Pro-Lys-Leu-Thr-Arg-Met-
Leu-Thr-Phe-Lys-Phe-Tyr-Met-Pro-Lys-Lys-Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys-Leu-
Glu-Glu-Glu-Leu-Lys-Pro-Leu-Glu-Glu-Val-Leu-Asn-Leu-Ala-Gln-Ser-Lys-Asn-Phe-His-
Leu-Arg-Pro-Arg-Asp-Leu-Ile-Ser-Asn-Ile-Asn-Val-Ile-Val-Leu-Glu-Leu-Lys-Gly-Ser-
Glu-Thr-Thr-Phe-Met-Cys-Glu-Tyr-Ala-Asp-Glu-Thr-Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-
Arg-Trp-Ile-Thr-Phe-Cys-Gln-Ser-Ile-Ile-Ser-Thr-Leu-Thr

Teceleukin(Genetical Recombination)
テセロイキン(遺伝子組換え)

図2　インターロイキン-2を示すステム「-leukin」を持つ医薬品

また，Aldesleukin，Denileukin Diftitoxは海外で承認されている医薬品である。AldesleukinはIL-2の2-133番目のアミノ酸残基のうち，125番目のシステインをセリンに置換したIL-2誘導体で，適応症は腎がん，悪性黒色腫である。Denileukin DiftitoxはIL-2とジフテリア毒素との融合タンパク質で，IL-2受容体を介して標的細胞に取り込まれ，ジフテリア毒素により細胞死を誘導する。IL-2受容体α鎖(CD25)を発現している皮膚T細胞リンパ腫の治療薬として使用されている。

(2)その他の「-kin」類

IL-2以外のインターロイキン類はまだほとんど実用化されていない。しかし，インターロイキンの機能解明が進み，インターロイキンを利用したり，インターロイキンの機能を阻害する医薬品の開発が進められており，海外ではすでに承認されている医薬品もある。

①「-elvekin」：インターロイキン-11

「-elvekin」は，インターロイキン-11(IL-11)を示すサブステムである。IL-11は骨髄間質細胞や繊維芽細胞から産生される178個のアミノ酸残基からなる分子量23,000のタンパク質で，造血前駆細胞や間質細胞に作用し，巨核球の増殖と成熟，脂肪細胞分化の抑制などの作用を持つ。Oprelvekin(オプレルベキン)は遺伝子組換えで製造されたIL-11の2-178番目のアミノ酸残基に相当するタンパク質である。血小板増殖因子として開発が進められ，米国では血小板減少症治療薬として承認されているが，日本では承認申請が取り下げられている。

②「-nakinra」：インターロイキン-1受容体アンタゴニスト

「-nakinra」はインターロイキン-1受容体アンタゴニスト(interleukin-1 receptor antagonist, IL-1RA)を示すサブステムで，IL-1のステム「-nakin」と受容体アンタゴニスト(receptor antagonist)に由来する。IL-1RAは単球系細胞で産生分泌される分子量23,000～25,000の糖タンパク質で，IL-1受容体に結合し，IL-1がIL-1受容体に結合するのを競合阻害する生理的アンタゴニストである。IL-1は炎症性サイトカインで，慢性関節リウマチなどの炎症性疾患にも深く関与している。Anakinraは遺伝子組換えで製造されたN末端にメチオニン1残基が結合したIL-1受容体アンタゴニストで，欧米では関節リウマチ治療薬として承認されている医薬品である。しか

し日本での臨床開発は進んでいない。

「interferon」：インターフェロン類

インターフェロン(interferon, IFN)はウイルス感染などの刺激を受けた細胞が産生，分泌する分子量約20,000の一群の生理活性糖タンパク質で，サイトカインの1種である。ウイルス増殖抑制作用のほかに細胞増殖抑制作用，抗腫瘍作用，免疫調節作用等の生物活性を持つ。主な分子種として産生細胞や構造の異なるIFN-α，IFN-β，IFN-γの3種類がある。

医薬品としてのインターフェロン類にはステムはなく，学術用語と同じ「interferon」がINNとしても用いられている。Interferon(インターフェロン)がINNになったのは非常に古く1962年のことで，「動物細胞とウイルスの相互作用により産生される(糖)タンパク質で，動物細胞にウイルス感染に対する抵抗力を持つようにする物質」と定義された。1980年代になり，IFN-α，IFN-β，IFN-γやこれらのバリアント(アミノ酸変異体)が遺伝子組換えで製造されるようになり，名称や定義の変更が検討された。INN委員会は「Alfaferon」，「Betaferon」，「Gammaferon」等の名称を検討したが，これらはすでに製品名として登録されており変更はできなかった。そこでINNでは，

Interferon Alfa(インターフェロン アルファ)

Interferon Beta(インターフェロン ベータ)

Interferon Gamma(インターフェロン ガンマ)

とα，β，γのアルファベット綴りを略さずに記載し，2語式で表す方法が採用された。前にも説明したように，INNには糖タンパク質の糖鎖の異なるものに対して名称の後にアルファ，ベータとつけて2語式に表して区別するというルールがある。しかし，インターフェロンでは，アミノ酸配列の異なるインターフェロンの分類を示すために生化学名に使われているアルファ，ベータ，ガンマをINNでもそのまま例外的に使用している。さらに必要に応じて数字やアルファベットを付加したり，混合物の場合にはコードを付加することにより，遺伝子の違いやアミノ酸の違いを区別するというルールが採用されている。

(1)「Interferon Alfa」：インターフェロン アルファ類

ヒトIFN-αは，白血球インターフェロンとして知られていたもので，ウイルス感染した白血球で産生分泌される糖タンパク質ファミリーである。塩基配列の異なる14種類以上のIFN-α遺伝子群から発現されるサブタイプが存在する。アミノ酸残基165-172個からなり，*N*-結合型糖鎖を持つものが多い。

INNではヒトIFN-α遺伝子のサブタイプはハイフンの後に数字を付けて，Interferon Alfa-2(インターフェロン アルファ-2)のように表す。Interferon Alfa-2には23番目と34番目のアミノ酸残基の異なるバリアントがあり，これらは数字の後にアルファベットをつけて区別する(Alfa-2a, Alfa-2b, Alfa-2c)(図3)。また，混合物の場合はInterferon Alfa-n1, Interferon Alfa-n2などと表す。

現在，日本では以下の7品目が承認されている。

Interferon Alfa(NAMALWA)(インターフェロン アルファ(NAMALWA))

Interferon Alfa(BALL-1)(インターフェロン アルファ(BALL-1))

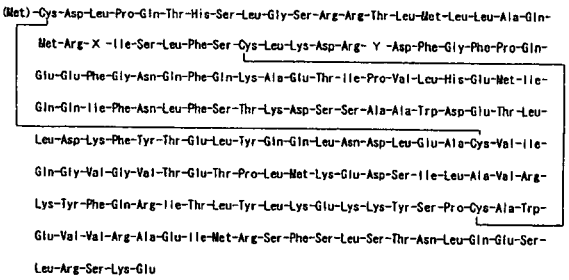
Interferon Alfa-2a(Genetical Recombination)(インターフェロン アルファ-2a(遺伝子組換え))

Interferon Alfa-2b(Genetical Recombination)(インターフェロン アルファ-2b(遺伝子組換え))

Interferon Alfacon-1(Genetical Recombination)(インターフェロン アルファコン-1(遺伝子組換え))

Peginterferon Alfa-2a(Genetical Recombination)(ペグインターフェロン アルファ-2a(遺伝子組換え))

Peginterferon Alfa-2b(Genetical Recombination)(ペグインターフェロン アルファ-2b(遺伝子組換え))



	各位置のアミノ酸残基	
	23(X)	34(Y)
Alfa-2a	Lys	His
Alfa-2b	Arg	His
Alfa-2c	Arg	Arg

図3 Interferon Alfa-2(インターフェロン アルファ-2)のアミノ酸配列

これらの医薬品のうち，Interferon Alfa(インターフェロン アルファ)は，INNでは1種類であるが，細胞培養技術を用いて製造したIFN-αは用いる細胞によりサブタイプの組成が異なるため，JANでは，INNの後に用いた細胞の名称を括弧書きで記載して区別しているため2品目となる。これらの医薬品は慢性C型肝炎の治療薬として用いられているほか，慢性B型肝炎，腎がん，慢性骨髄性白血病，多発性骨髄腫の治療薬としても用いられる。

Interferon Alfa(NAMALWA)(インターフェロン アルファ(NAMALWA))は，ヒトリンパ芽球NAMALWA細胞をセンダイウイルスで誘発することにより産生される分子量17,000～30,000の糖タンパク質で，サブタイプの混合物であり，日局収載候補品目となっている。

Interferon Alfa(BALL-1)(インターフェロン アルファ(BALL-1))は，ヒトリンパ芽球BALL-1細胞をセンダイウイルスで誘発することにより産生される分子量13,000～21,000の糖タンパク質で，IFN-α2，α7およびα8のサブタイプから構成される。

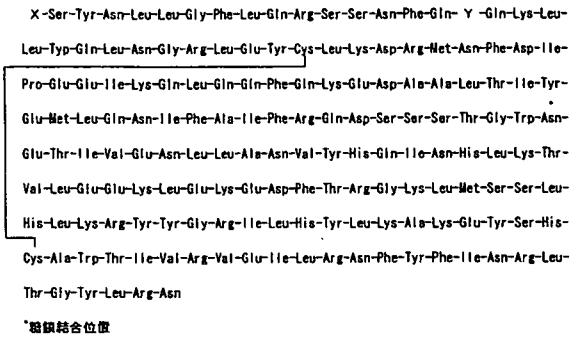
Interferon Alfa-2a(インターフェロン アルファ-2a)，Interferon Alfa-2b(インターフェロン アルファ-2b)は，それぞれ対応する遺伝子を導入した組換え体で産生される165個のアミノ酸残基からなるタンパク質である。

Interferon Alfacon-1(インターフェロン アルファコン-1)は，ヒトIFN-αの12種類のサブタイプのアミノ酸配列において，各位置の出現頻度が最も高いアミノ酸残基をコードするように人工的に設計した遺伝子の発現により組換え体で産生される，一部N末端にメチオニン残基が付加している166個のアミノ酸残基からなるタンパク質で，インターフェロン アルファよりも高い生物活性を示す。

Peginterferon Alfa-2a(ペグインターフェロン アルファ-2a)，Peginterferon Alfa-2b(ペグインターフェロン アルファ-2b)は，それぞれInterferon Alfa-2a(インターフェロン アルファ-2a)，Interferon Alfa-2b (インターフェロン アルファ-2b)をPEG化したもので，血中半減期が延長され，投与回数を減らすことが可能な医薬品である。

(2)「Interferon Beta」：インターフェロン ベータ類

ヒトIFN-βは，繊維芽細胞インターフェロンとして知られていたもので，ウイルスや2本鎖RNAの刺激により繊維芽細胞で産生される166個のアミノ酸残基からなるN-結合型糖鎖を持つ糖タンパク質である。IFN-β遺伝子はIFN-αと異なり1種類である。



	各位置のアミノ酸残基 1(X)	17(Y)	糖鎖結合
Beta-1a	Met	Cys	Asn80
Beta-1b	-	Ser	-

図4　Interferon Beta(インターフェロン ベータ)のアミノ酸配列

INNではIFN-βのサブタイプはハイフンの後に数字を付けて，Interferon Beta-1(インターフェロン ベータ-1)と表す。Interferon Beta-1では，1番目と17番目のアミノ酸残基および糖鎖結合の有無が異なるものがあり，これらは数字の後のアルファベットで区別する(Beta-1a, Beta-1b)(図4)。また，混合物の場合はInterferon Beta-n1, Interferon Beta-n2などと表す。

現在，日本で承認されているのは以下の3品目である。

Interferon Beta(インターフェロン ベータ)

Interferon Beta-1a(Genetical Recombination)(インターフェロン ベータ-1a(遺伝子組換え))

Interferon Beta-1b(Genetical Recombination)(インターフェロン ベータ-1b(遺伝子組換え))

Interferon Beta(インターフェロン ベータ)は，ヒト繊維芽細胞に誘発剤を作用させて産生した166個のアミノ酸残基からなる糖タンパク質であり，悪性黒色腫，膠芽腫，髄芽腫，星細胞腫，慢性B型肝炎，慢性C型肝炎などの治療薬として使われている。

Interferon Beta-1a(インターフェロン ベータ-1a)は，CHO細胞を用いて遺伝子組換えにより製造した166個のアミノ酸残基からなる糖タンパク質で，天然型IFN-βと同じアミノ酸配列でN-結合型糖鎖を持つ。一方，Interferon Beta-1b(インターフェロン ベータ-1b)は，17番目のシステインをセリンに置換し，分子内ジスルフィド結合が正しく架橋されるようにしたもので，大腸菌

で製造した165個のアミノ酸からなるタンパク質である。これらはともに，多発性硬化症の治療薬として使用されている。

(3)「Interferon Gamma」：インターフェロン ガンマ類

ヒトIFN-γは，免疫インターフェロンとして知られていたもので，マイトジェンや特異抗原刺激によりT細胞で産生される，146個のアミノ酸残基からなるN-結合型糖鎖を持つ糖タンパク質で，IFN-γ遺伝子は1種類である。IFN-αとIFN-βは構造上の類似性が高く受容体も共通しているが，IFN-γとIFN-α，IFN-βに類似性はなく，αとβはⅠ型，γはⅡ型インターフェロンに分類される。

INNではIFN-γのサブタイプはハイフンの後に数字を付けて，Interferon Gamma-1(インターフェロン ガンマ-1)と表す。Interferon Gamma-1ではN末端，C末端のアミノ酸配列の異なるものを数字の後のアルファベットで区別し，Gamma-1a，Gamma-1b，Gamma-1cが定義されている(図5)。また，混合物の場合はInterferon Gamma-n1，Interferon Gamma-n2などと表す。

現在，日本では以下の2品目が承認されており，腎がん，菌状息肉症，慢性肉芽腫，成人T細胞白血病の治療薬として用いられている。

Interferon Gamma-1a(Genetical Recombination)(インターフェロン ガンマ-1a(遺伝子組換え))

Interferon Gamma-n1(インターフェロン ガンマ-n1)

Interferon Gamma-1a(インターフェロン ガンマ-1a)は，対応する遺伝子を導入した組換え体で産生されるアミノ酸146個からなるタンパク質である。また，Interferon Gamma-n1(インターフェロン ガンマ-n1)は，ヒトミエロモノサイト細胞株HBL-38をリポポリサッカライドで刺激して産生される，126，127，128，129および138個のアミノ酸残基からなる分子量約15,000～26,000の糖タンパク質の混合物である。

X-Gln-Asp-Pro-Tyr-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-Asn-Ala-Gly-His-Ser-
Asp-Val-Ala-Asp-Asn-Gly-Thr-Leu-Phe-Leu-Gly-Ile-Leu-Lys-Asn-Trp-Lys-Glu-Glu-Ser-
Asp-Arg-Lys-Ile-Met-Gln-Ser-Gln-Ile-Val-Ser-Phe-Tyr-Phe-Lys-Leu-Phe-Lys-Asn-Phe-
Lys-Asp-Asp-Gln-Ser-Ile-Gln-Lys-Ser-Val-Glu-Thr-Ile-Lys-Glu-Asp-Met-Asn-Val-Lys-
Phe-Phe-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Glu-Lys-Leu-Thr-Asn-Tyr-Ser-Val-
Thr-Asp-Leu-Asn-Val-Gln-Arg-Lys-Ala-Ile-His-Glu-Leu-Ile-Gln-Val-Met-Ala-Glu-Leu-
Ser-Pro-Ala-Ala-Lys-Thr-Gly-Lys-Arg-Lys-Arg-Ser-Gln-Met-Leu-Phe-Arg-Gly-Arg-Y

	末端アミノ酸配列		糖鎖結合
	X	Y	
Gamma-1a	Cys-Tyr-Cys	Arg-Ala-Ser-Gln	-
Gamma-1b	Met	-	-
Gamma-1c	Met	Arg-Ala-Ser-Gln	-

図5 Interferon Gamma(インターフェロン ガンマ)のアミノ酸配列

「-poetin」：エリスロポエチン類

「-poetin」は，エリスロポエチン(erythropoetin，EPO)型の血液因子に共通のステムである。EPOは，赤血球前駆細胞に作用して赤血球への分化と増殖を促す造血因子で，主として腎臓から分泌される。天然のヒトEPOは，165個のアミノ酸残基からなる分子量約30,000の糖タンパク質で，Asn24，38，および83にN-結合型糖鎖，またSer126にO-結合型糖鎖が結合している。糖鎖の非還元末端に結合しているシアル酸数が多いものほど血中半減期が長く，高い生物活性を示す。

ステム「-poetin」を持ち，現在，日本で承認されている医薬品には以下の2品目がある(図6)。

Epoetin Alfa(Genetical Recombination)(エポエチン アルファ(遺伝子組換え))

Epoetin Beta(Genetical Recombination)(エポエチン ベータ(遺伝子組換え))

これらの医薬品は主に透析施行中の腎性貧血治療薬として用いられているほか，未熟児貧血にも用いられる。今後，日局への収載が予定されている医薬品である。

Epoetin Alfa(エポエチン アルファ)は，ヒトEPOをコードするゲノムDNAを導入したCHO細胞で産生され

Ala-Pro-Pro-Arg-Leu-Ile-Cys-Asp-Ser-Arg-Val-Leu-Glu-Arg-Tyr-Leu-Leu-Glu-Ala-Lys-
Glu-Ala-Glu-Asn-Ile-Thr-Thr-Gly-Cys-Ala-Glu-His-Cys-Ser-Leu-Asn-Glu-Asn-Ile-Thr-
Val-Pro-Asp-Thr-Lys-Val-Asn-Phe-Tyr-Ala-Trp-Lys-Arg-Met-Glu-Val-Gly-Gln-Gln-Ala-
Val-Glu-Val-Trp-Gln-Gly-Leu-Ala-Leu-Leu-Ser-Glu-Ala-Val-Leu-Arg-Gly-Gln-Ala-Leu-
Leu-Val-Asn-Ser-Ser-Gln-Pro-Trp-Glu-Pro-Leu-Gln-Leu-His-Val-Asp-Lys-Ala-Val-Ser-
Gly-Leu-Arg-Ser-Leu-Thr-Thr-Leu-Leu-Arg-Ala-Leu-Gly-Ala-Gln-Lys-Glu-Ala-Ile-Ser-
Pro-Pro-Asp-Ala-Ala-Ser-Ala-Ala-Pro-Leu-Arg-Thr-Ile-Thr-Ala-Asp-Thr-Phe-Arg-Lys-
Leu-Phe-Arg-Val-Tyr-Ser-Asn-Phe-Leu-Arg-Gly-Lys-Leu-Lys-Leu-Tyr-Thr-Gly-Glu-Ala-
Cys-Arg-Thr-Gly-Asp

Epoetin(Genetical Recombination)
エポエチン(遺伝子組換え)

図6 Epoetin(エポエチン)のアミノ酸配列
*N-結合型糖鎖結合位置，**O-結合型糖鎖結合位置

る165個のアミノ酸残基からなる糖タンパク質で，グリコフォームαからなる。一方，Epoetin Beta(エポエチン ベータ)は，ヒトEPOをコードするcDNAを導入したCHO細胞で産生される165個のアミノ酸残基からなる糖タンパク質で，グリコフォームβからなる。すなわち，この2つの医薬品のアミノ酸配列は同一であるが，結合している糖鎖の分布は異なっている。なお，グリコフォームとは，タンパク質部分の一次構造が同一で，糖鎖部分のみが異なるサブユニットのことである。

その他，ステム「-poetin」を持ちJANに登録されている医薬品として以下のものがある。

Epoetin Epsilon(Genetical Recombination)(エポエチン イプシロン(遺伝子組換え))

Darbepoetin Alfa(Genetical Recombination)(ダルベポエチン アルファ(遺伝子組換え))

Epoetin Epsilon(エポエチン イプシロン)は，ヒトEPOをコードする遺伝子を導入したBHK細胞で産生される165個のアミノ酸残基からなる糖タンパク質で，グリコフォームεからなる。Darbepoetin Alfa(ダルベポエチン アルファ)は，ヒトEPOの5カ所のアミノ酸残基を置換することによって新たに2本の*N*-結合型糖鎖を導入し，分子あたりのシアル酸数を最大14から22へと増加させた改変型糖タンパク質で(図7)，ヒトEPOよりも血中半減期が長い。欧米ではすでに貧血治療薬として承認されている。図7には天然型と異なるアミノ酸残基を赤文字で示した。

その他，INNにはステム「-poetin」を持つ以下のものが登録されている。いずれもヒトEPOと同じアミノ酸配列を有し，異なるグリコフォームからなる糖タンパク質である。

Epoetin Gamma

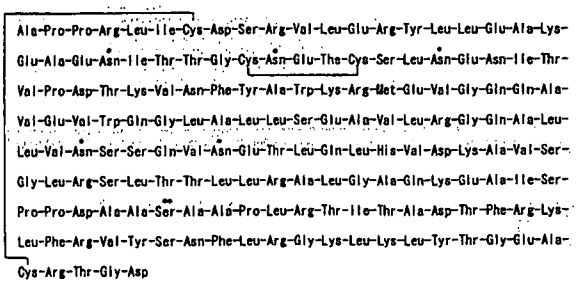
Epoetin Delta

Epoetin Zeta

Epoetin Theta

Epoetin Iota

Epoetin Omega



Darbepoetin(Genetical Recombination)
ダルベポエチン(遺伝子組換え)

図7　Darbpoetin(ダルベポエチン)のアミノ酸配列
*N-結合型糖鎖結合位置，**O-結合型糖鎖結合位置

おわりに

今回は，生物薬品のステムとして，サイトカイン類に属するコロニー刺激因子類のステム「-stim」，インターロイキン類のステム「-kin」，インターフェロン類「interferon」，およびエリスロポエチン類のステム「-poetin」を紹介した。次回以降も順次，生物薬品のステムを紹介していきたい。

筆者紹介：
内田恵理子：
厚生労働省国立医薬品食品衛生研究所遺伝子細胞医薬部第一室長。医薬品の名称に関して，医薬品医療機器総合機構のJAN専門委員およびJP名称委員を務める。
川崎ナナ：
厚生労働省国立医薬品食品衛生研究所生物薬品部第一室長。医薬品の名称に関して，医薬品医療機器総合機構のJAN専門委員およびJP名称委員を務める。
宮田直樹：
本連載第1回(本誌2006年8月号)を参照。

■参考文献

1) INTERNATIONAL NONPROPRIETARY NAMES(INN) FOR BIOLOGICAL AND BIOTECHNOLOGICAL SUBSTANCES(A REVIEW), INN Working Document 05.179, 15/06/2006
http://www.who.int/medicines/services/inn/BioRevforweb.pdf
2) pINNおよびrINNのリストhttp://www.who.int/druginformation/general/innlists.shtml
3) 日本医薬品一般名称データベースhttp://moldb.nihs.go.jp/jan/Default.htm
4) 医療用医薬品の添付文書情報(医薬品医療機器総合機構)
http://www.info.pmda.go.jp/info/pi_index.html
5) 医薬品一般名称辞典 JAN1996，(財)日本公定書協会(1996)
6) FDA Drug information-Product approval information-http://www.fda.gov/cder/drug/default.htm
7) 生化学辞典，第2版，東京化学同人(1991)
8) 免疫学辞典，第1版，大沢利昭・小山次郎・奥田研爾・矢田純一【編】，東京化学同人(1993)
9) 分子細胞生物学辞典，第1版，村松正實編集代表，東京化学同人(1997)
10) 日経バイオ年鑑2006，バイオセンター編集，日経バイオテク・日経バイオビジネス(2006)
11) 薬学用語解説，日本薬学会http://www.pharm.or.jp/dictionary/
12) 宮田直樹，中野達也，川崎ナナ，内田恵理子，滝明子，長谷川式子，山本美智子：平成15年度「日本薬局方の試験法に関する研究」研究報告－日本薬局方収載医薬品などの名称，構造式，化学名の国際調和に関する研究(第3報)－，医薬品研究，35(12)，627-637(2004)

Genes and Environment, Vol. 28, No. 2 pp. 68–73 (2006)

Regular article

Microcystin-LR is not Mutagenic *in vivo* in the λ/*lacZ* Transgenic Mouse (Muta™Mouse)

Li Zhan[1,2], Masamitsu Honma[2], Li Wang[1], Makoto Hayashi[2], De-Sheng Wu[4], Li-Shi Zhang[4], Palanichamy Rajaguru[3,5] and Takayoshi Suzuki[2,3,6]

[1]National Chengdu Center for Safety Evaluation of Traditional Chinese Medicine, West China Hospital, Sichuan University, Chengdu, China
[2]Division of Genetics and Mutagenesis, [3]Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, Tokyo, Japan
[4]West China School of Public Health, Sichuan University, Chengdu, China
[5]Department of Biotechnology, School of Engineering and Technology, Bharathidasan University, Tiruchirappalli, India

(Received April 2, 2006; Accepted April 18, 2006)

The water pollution of toxic cyanobacteria (blue-green algae) is causing a serious public health problem in many parts of the world. Microcystin-LR (MCLR) is a potent cyclic heptapeptidic hepatotoxin produced by the cyanobacterium *Microcystis aeruginosa*. MCLR presents acute and chronic hazards to human health and has been linked to primary liver cancer in humans chronically exposed to this peptide toxin through drinking water. To assess the *in vivo* mutagenecity of MCLR, the λ/*lacZ* transgenic mice (Muta™Mouse) were treated with MCLR (1 mg/kg per week x 4) and examined for mutant frequencies (MFs) in the *lacZ* and *cII* genes of liver and lungs. Micronucleus induction in peripheral blood cells was also assessed. Co-mutagenic effect of MCLR was studied in combination with *N*-nitrosodiethylamine (DEN). MCLR did not increase either MFs of the target genes in liver and lungs or micronucleus frequency in the peripheral blood cells of the λ/*lacZ* transgenic mouse. While DEN treatment increased MFs significantly, the co-administration of MCLR did not potentiate its mutagenicity. We conclude that pure MCLR has no *in vivo* mutagenicity as it failed to induce gene mutation and micronucleus in transgenic mouse. Its tumor promoting effect is independent of its interaction to DNA.

Key words: Microcystin-LR, *N*-nitrosodiethylamine, *lacZ*, *cII*, Muta™Mouse

Introduction

The water pollution of toxic cyanobacterial bloom (blue-green algae) is an increasing problem worldwide and worsens with eutrophication of drinking- and recreational- water reservoirs due to industrialization (1,2). Cyanobacteria produce lethal toxins, and often associated to death of livestock and cases of human illness caused by drinking water contaminated by these toxins, which have drawn the attention of the World Health Organization (WHO) (3). Microcystins are the most common group of cyanobacterial toxins comprised of over 60 structurally related cyclic heptapeptides (4) with potent hepatotoxicity and tumor promotion ability (5,6). Among them, Microcystin-LR (MCLR) is the most frequent secondary metabolite produced by *Microcystis aeruginosa* (2,7). MCLR presents acute and chronic hazards to human health (8,9). Although human illnesses attributed to microcystins include gastroenteritis and allergic/irritation reactions, the primary target of the toxin is the liver (10–14). It has been suspected to be involved with promotion of primary liver cancer in humans chronically exposed to doses of these peptide toxins through drinking water (15,16).

Algal toxins were reported to cause chromosomal breakages in human lymphocytes *in vitro* (17). Genotoxicity of cynobacterial extract has been demonstrated by SOS chromotest with *Escherichia coli* PQ37 and the comet assay with human lymphocytes (18) and in four strains of *Salmonella typhimurium* (TA97, TA98, TA100 and TA102) in Ames test with or without S9 mix (19). In the same study, however, pure MCLR did not show any mutagenicity in all these strains. MCLR was reported to damage the mitotic spindle apparatus and thus induces polyploidy and apoptosis and necrosis in Chinese hamster ovary (CHO-K1) cells (20), and to induce gene mutation with base substitution in human RSa cells (21). Recently we have demonstrated mutagenic and clastogenic activities of MCLR in human lymphoblastoid cells (TK6) after 24 h treatment *in vitro*

[6]Correspondence to: Takayoshi Suzuki, Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel: +81-3-3700-1926, Fax: +81-3-3700-1926, E-mail suzuki@nihs.go.jp

(22). Because MCLR induced mainly LOH type mutations rather than point mutations, mutagenicity of MCLR might be exerted by a clastogenic mechanism. Nevertheless, *in vivo* genotoxicity of this cyanotoxin is less convincing and relatively undescribed. Therefore, the present study was conducted to evaluate the *in vivo* mutagenicity of MCLR using transgenic (TG) mouse mutation assay. TG system has been shown to be useful for studying chemical mutagenesis and clastogenesis *in vivo* (23–25). This is largely attributed to its ability to detect tissue-specific gene mutations. TG assay also permits analysis of mutation at the molecular level and allows examination of the relation between mutagenesis and carcinogeneis *in vivo* in detail (25). Since MCLR has been found to promote tumor initiated with *N*-nitrosodiethylamine (DEN) in rats (5), the co-mutagenic (potentiation) effects of MCLR in combination with DEN was also studied.

Materials and Methods

Chemicals: MCLR and DEN were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MCLR was dissolved in saline immediately before use. Phenyl-β-D-galactoside (P-gal) was purchased from Sigma.

Treatment of Muta™Mice: Male Muta™Mice (5-6-week old, ca. 25 g body weight) supplied by Covance Research Products (PA, USA) were acclimatized for 1 week before use. The animals were divided into 4 groups of 5 mice each and administered with weekly doses of either vehicle (saline), MCLR (1 mg/kg, 1/10 of LD_{50} in mice), DEN (25 mg/kg, 1/4 of LD_{50} in mice) or DEN + MCLR (25 mg/kg and 1 mg/kg, respectively) for 4 weeks. Saline and MCLR were administered intragastrically while DEN was intraperitonealy injected. No apparent sign of toxicity was observed in any mice.

Micronucleus assay in peripheral blood cells: Forty eight hours after the first treatment, 5 μL of peripheral blood was collected from the tail vein without anti-coagulant. The blood thus collected from each animal was placed on an acridine orange-coated glass slide, covered with a cover slip, and supravitally stained (26). Type I, II, and III reticulocytes (RETs) with red fluorescent reticulum in the cytoplasm were scored under a fluorescent microscope. One thousand RETs were examined per animal within a few days after the slide preparation. The number of RETs with micronucleus (MNRETs) was recorded.

Mutation assay: 1) Tissue collection: Mice were killed by cervical dislocation 7 days after the last treatment. Liver and lungs were removed, immediately frozen in liquid nitrogen, and stored at −80°C until DNA extraction. MFs of *lacZ/cII* transgenes derived from liver and lungs were determined as described previously (27–29). DNA sequencing of mutants isolated from the control and MCLR treated animals were carried out as described below.

2) Sequence analysis of *cII* gene: The *cII* mutant plaques were transferred into a microtube containing 50 μL SM buffer and 5 μL chloroform. The λ phage *cII* region was amplified directly from mutant plaque solution by Taq DNA polymerase (Takara Shuzo, Tokyo, Japan) with primers P1, 5′-AAAAAGGGCAT-CAAATTAAACC-3′; and P2, 5′-CCGAAGTTGAG-TATTTTTG- CTGT-3′. Amplification was done by the Minicycler PTC-150-25 (MJ Research, Inc., MA, USA) under the following thermal cycling: 95°C 5 min→ (95°C 20 s, 53°C 30 s, 72°C 40 s)×30 cycles→72°C 10 min. Amplification of 446 bp PCR product was checked by 2100 Bioanalyzer using lab DNA chips (Agilent Technologies, USA) and purified with a microspin column (Amersham Pharmacia, Tokyo, Japan) before being used for a sequencing reaction with the Ampli Taq cycle sequencing kit (PE Biosystems, Tokyo, Japan). The sequencing reaction was performed by Minicycler PTC-150-25 with 25 cycles of denaturing at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min, with the primer P1. The reaction product was purified by ethanol precipitation and analyzed by the ABI PRISM® 310 Genetics Analyzer (PE Biosystems, Tokyo, Japan).

3) Statistical analysis: The results of the different treatment groups were compared using Students' *t*-test. Significance was indicated by P values <0.05.

Results

Micronucleus induction in peripheral blood: Results of the micronucleus test 48 h after the first administration of chemicals in the Muta™Mice is shown in Fig. 1. The mean frequency of MNRETs did not increase significantly ($P>0.05$) in any of the treatment group in comparison with that of the control group.

Mutant frequency of *lacZ* and *cII* genes: The mutant frequencies (MFs) observed in the DNA preparations extracted from the liver and lung tissues 7 days after the last treatment are shown in Table 1. In MCLR-treated mice, the MFs of *lacZ* and *cII* genes in liver were not different significantly ($P>0.05$) from that of the background levels. Although a slight increase was observed in the lungs, it was not statistically significant ($P>0.05$). DEN treatment significantly ($P<0.05$) increased MFs of both the target genes in both liver and lungs (for *lacZ* gene 6.1 fold and 3.7 fold respectively and for *cII* gene 11.0 fold and 4.6 fold, respectively). We did not observe significant difference in MFs between DEN-treated and DEN+MCLR co-treated animals.

***cII* mutation spectrum:** Thirty four MCLR-in-

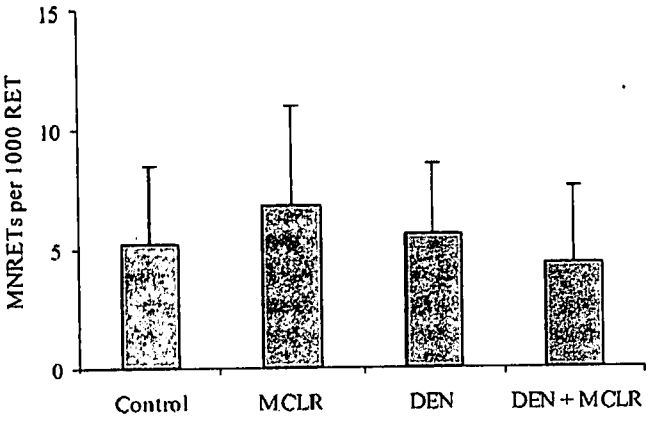


Fig. 1. Incidence of MNRET in the peripheral blood of Muta™Mouse 48 h following treatment with MCLR (1 mg/kg), DEN (25 mg/kg) and DEN (25 mg/kg)+MCLR (1 mg/kg).

duced and 46 DEN-induced mutants together with 42 spontaneous mutants from the liver were subjected to sequence analysis. The mutation spectra are summarized in Table 2. Spontaneous mutations consisted mainly of base substitutions (37/42). Among them, G:C to A:T transitions (21/26) predominated and most of them (17/21) occurred at CpG sites. DEN-induced mutations also consisted mainly of base substitutions (42/46). Compared to the control, G:C to A:T transitions were decreased in DEN treated group (50% versus 24% respectively) while A:T to T:A transversions were increased (2% versus 28%, respectively). However no obvious change was observed for incidences of mutations induced by MCLR including transitions (62% versus 62%) and transversions (27% versus 21%).

Table 1. MFs in the *lacZ* and *cII* gene from liver and lung of Muta™Mouse treated with MCLR (1 mg/kg), DEN (25 mg/kg) and DEN (25 mg/kg)+MCLR (1 mg/kg)

Organ	Treatment	*lacZ*				*cII*			
		Total plaques	Mutants	MF ($\times 10^{-6}$)	Mean ± SD	Total plaques	Mutants	MF ($\times 10^{-6}$)	Mean ± SD
Liver	Control	3311250	138	41.7	43.8 ± 11.7	3486000	69	19.8	20.5 ± 8.2
	MCLR	4053750	173	42.7	40.3 ± 13.7	4282500	94	21.9	21.1 ± 3.5
	DEN	3175000	963	261.7	268.4 ± 62.4*	3,495,000	788	225.5	226.6 ± 54.2*
	DEN+MCLR	2122500	472	222.4	206.9 ± 83.4†	2,149,500	391	181.9	176.4 ± 77†
Lung	Control	3823750	144	28.8	32.1 ± 13.9	4305000	110	25.6	25.7 ± 4.3
	MCLR	3823750	134	35.0	35.7 ± 4.89	2468250	93	37.7	36.9 ± 21.3
	DEN	3622500	416	114.8	117.5 ± 17.2*	2874000	332	115.5	118.1 ± 10.1*
	DEN+MCLR	2576250	264	102.5	109.9 ± 44.7†	1,136,250	141	124.1	132.2 ± 20.6†

*Compared to the control group $P < 0.05$
†Compared to the DEN-treated group $P > 0.05$

Table 2. Summary of *cII* mutations in the liver of control, MCLR- and DEN-treated Muta™Mice

Mutation class	Liver					
	Control	CpG	MCLR (%)	CpG	DEN (%)	CpG
Base	37 (89)		28 (82)		42 (91)	
Transitions	*26 (62)*		*21 (62)*		*20 (43)*	
G:C to A:T	21 (50)	17 (40)	20 (59)	17 (52)	11 (24)	6 (13)
A:T to G:C	5 (12)		1 (3)		9 (20)	
Transversions	*11 (27)*		*7 (21)*		*22 (48)*	
A:T to T:A	1 (2)		1 (3)		13 (28)	
A:T to C:G	4 (10)		2 (6)		2 (4)	
G:C to T:A	4 (10)		4 (12)		7 (15)	
G:C to C:G	2 (5)		0 (0)		0 (0)	
−1 Frameshift	1 (2)		2 (6)		1 (2)	
+1 Frameshift	3 (7)		4 (12)		0 (0)	
Deletion	0 (0)		0 (0)		1 (2)	
Insertion	0 (0)		0 (0)		0 (0)	
Complex	1 (2)		0 (0)		2 (4)	
Total	42 (100)		34 (100)		46 (100)	
MF ($\times 10^{-6}$)	43.8		40.3		268.4	

Discussion

The occurrence of toxic cyanobacterial blooms found in eutrophic, municipal, and residential water supplies is an increasing public health problem. Frequent deaths of domestic and wild animals are caused by drinking water contaminated by lethal toxins produced by cynobacteria. MCLR is the most commonly encountered and among the most toxic algal cyclic peptide hepatotoxins. Epidemiological studies have indicated a close relationship between primary liver cancer in human and cynobacteria contaminated drinking water (15,16). While there are several reports showing the *in vitro* genotoxicity of MCLR (21,22) or cyanobacterial extract (18,19), the evidence for the *in vivo* genotoxicity of this toxin is less convincing. Therefore, the main objectives of this study were to assess the *in vivo* genotoxicity of MCLR (if any) and its role in potentiation of DEN induced mutations for its suggested tumor promoting effects. To meet out these objectives male Muta™Mouse were administered with MCLR alone or in combination with DEN and examined for two end points- point mutation in transgenes, and micronucleus induction in peripheral blood cells. Considering the strong correlation between organ specific genotoxicty and organ specific carcinogenicity, the assessment of genotoxicity in multiple organs *in vivo* may indicate its target organ in humans and provide useful information for the evaluation of chemical safety. In the present research, hence, two target organs -liver and lungs- were examined for the evidence for mutagenicity.

Intraperitoneal injection of the raw cyanobacterial extracts containing several other microcystins besides MCLR induced micronucleus in the mouse bone marrow cells (19) and degradation and fragmentation of DNA in the liver cells (30). In the present study a pure MCLR (1 mg/kg = 1/10 of LD_{50}) was used, but no mutagenicity was observed. In another study, neoplastic nodule formation has been observed in the livers of mice received 100 intraperitoneal injections of sublethal doses of MCLR (20 μg/kg) over a period of 28 weeks (31). In the same study, oral administration of relatively higher doses of MCLR (80 μg/kg) under similar experimental conditions did not induce characteristic chronic injuries. Similarly, as suggested by the authors, fragmentation of DNA observed in hepatocytes of mice treated with the extract or MCLR (0.5-2.0 folds of LD_{50} doses) might be a consequence of endonucleolytic DNA degradation associated with cytotoxicity, rather than by a direct toxin-DNA interaction (30). In support to this, recently Zegura *et al.* (32) have reported that the genotoxicity of MCLR could be mediated by reactive oxygen species. So it may be inferred that some other mutagenic toxins present in the extracts or different routes of administration might be responsible for the positive results observed in those studies. However, MCLR treatment caused enhanced formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in a time- and dose-dependent manner *in vitro* in primary cultured hepatocytes and *in vivo* in rat liver cells that could involve in the formation of hepatic tumors during long-term exposure to this cyanobacterial hepatotoxin (33). In contrast, in our study, under present experimental conditions, MCLR failed to induce mutation in both target genes (*lacZ* and *cII*) in liver and lungs of TG mouse. The *in vivo* micronucleus test in peripheral blood cells also yielded negative results. These results indicate that MLCR is capable of inducing neither point mutation nor chromosomal breakage *in vivo* in mouse organs.

It is widely believed that MCLR has tumor promoting effect (5,6). To test the possible potentiating effect of MCLR on mutagenicity of DEN, in our study, mice were simultaneously treated with DEN (25 mg/kg) and MCLR (1 mg/kg) once a week for four weeks. Relative to control mice, no significant increase in micronucleus frequency was observed either in DEN- or DEN+ MCLR-treated mice. This is in consistent with the negative results observed with DEN as previously reported (24). Further, simultaneous administration of MCLR with DEN did not increase MF caused by DEN in either of the target genes, although DEN treatment resulted in a significant increase in MFs in both *lacZ* and *cII* genes from liver and lungs. This indicates that the tumor promoting effects of MCLR is independent of mutagenicity of DEN. Because MCLR is known as an inhibitor of protein phosphatase 1 and 2A (5,34), the tumor promoting activity might be exerted by a disturbance of protein phosphorylation. Okadaic acid, which is known as a tumor promoter and a strong inhibitor of protein phosphatases (35), has similar mutagenic properties as MCLR (non-mutagenic in Salmonella and mutagenic in mammalian cells (36,37)). It is possible that tumor promoting activity of both compounds has a common mechanism through the inhibition of protein phosphatases.

In conclusion, pure MCLR has no *in vivo* genotoxicity as it is failed to induce gene mutation and micronucleus in transgenic mouse. Also lack of potentiation of DEN induced mutations in transgenes, as observed in the present study, indicates that the tumor promoting effects of MCLR is independent of its interaction to DNA.

Acknowledgement: This work was supported by a grant from the Japan-China Sasagawa Medical Fellowship and a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan.

References

1 Carmichael WW. Toxins of freshwater algae. In: Tu AT,

editor. Handbook of natural toxins. New York: Marcel Dekker; 1988. p. 121-47.
2 Codd GA, Bell SG, Kaya K, Ward CJ, Beattie KA, Metcalf JS. Cyanobacterial toxins, exposure routs and human health. Eur J Phycol. 1999; 34: 405-15.
3 Chorus I. Introduction: cyanotoxins-research for environment safety and human health. In: Chorus I, editor. Cyanotoxins. Germany: Springer; 2001. p. 1-4.
4 Carmichael WW. Health effects of toxin-producing cyanobacteria: the CyanoHABs. Hum. Ecol Risk Assess. 2001; 7: 1393-407.
5 Nishiwaki-Matsushima R, Ohta T, Nishiwaki S, Suganuma M, Kohyama K, Ishikawa T, Carmichael WW, Fujiki H. Liver tumor promotion by the cyanobacteria cyclic peptide toxin microcystin-LR. J Cancer Res Clin Oncol. 1992; 118: 420-4.
6 Carmichael WW. The toxins of cyanobacteria. Sci Am. 1994; 270: 78-86.
7 Carmichael WW. Cyanobacteria secondary metabolites: the cyanotoxins. J Appl Bacteriol. 1992; 72: 445-59.
8 Carmichael WW. The cyanotoxins. In: Callow JA editor. Advances in botanical research. London: Academic Press; 1997. p. 211-56.
9 Hernandez M, Macia M, Padilla C, Del Campo F. Modulation of human polymorphonuclear leukocyte adherence by cyanopeptide toxin. Environ Res Sci. 2000; 84: 64-8.
10 Codd GA, Ward CJ, Bell SG. Cyanobacterial toxins: occurrence, modes of action, health effects and exposure routes. Arch Toxicol Suppl. 1997; 19: 399-410.
11 Miura GA, Robinson NA, Geisbert TW, Bostian KA, White JD, Pace JG. Comparison of *in vivo* and *in vitro* toxic effects of microcystin-LR in fasted rats. Toxicon. 1989; 27: 1229-40.
12 Rao PVL, Bhattacharya R, Parida MM, Jana AM, Bhaskar AS. Freshwater cyanobacterium *Microcystis aeruginosa* (UTEX 2385) induced DNA damage *in vivo* and *in vitro*. Environ Toxicol Pharmacol. 1998; 5: 1-6.
13 Bhattacharya R, Rao PV, Bhaskar AS, Pant SC, Dube SN. Liver slice culture for assessing hepatotoxicity of freshwater cyanobacteria. Hum Exp Toxicol. 1996; 15: 105-10.
14 Ding WX, Shen HM, Zhu HG, Ong CN. Studies on oxidative damage induced by cyanobacteria extract in primary cultured rat hepatocytes. Environ Res. 1998; 78: 12-8.
15 Yu SZ. Primary prevention of hepatocellular carcinoma. J Gastroenterol Hepatol. 1995; 10: 674-82.
16 Ueno Y, Nagata S, Tsutsumi T, Hasegawa A, Watanabe MF, Park HD, Chen GC, Chen G, Yu SZ. Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. Carcinogenesis 1996; 17: 1317-21.
17 Repavich WM, Sonzongni WC, Standridge JH, Wedephohl RE, Meisner LF. Cyanobacteria (blue-green algae) in Wisconsin waters: acute and chronic toxicity. Water Res. 1990; 24: 225-31.
18 Mankiewicz J, Walter Z, Tarczynska M, Palyvoda O, Wojtysiak-Staniaszczyk M, Zalewski M. Genotoxicity of cyanobacterial extracts containing microcystins from Polish water reservoirs as determined by SOS chromotest and comet assay. Environ Toxicol. 2002; 17: 341-50.
19 Ding WX, Shen HM, Zhu HG, Lee BL, Ong CN. Genotoxicity of microcystic cyanobacteria extract of a water source in China. Mutat Res. 1999; 442: 69-77.
20 Lankoff A, Banasik A, Obe G, Deperas M, Kuzminski K, Tarczynska M, Jurczak T, Wojcik A. Effect of microcystin-LR and cyanobacterial extract from Polish reservoir of drinking water on cell cycle progression, mitotic spindle, and apoptosis in CHO-K1 cells. Toxicol Appl Pharmacol. 2003; 189: 204-13.
21 Suzuki H, Watanabe MF, Wu Y, Sugita T, Kita K, Sato T, Wang X, Tanzawa H, Sekiya S, Suzuki N. Mutagenicity of microcystin-LR in human RSa cells. Int J Mol Med. 1998; 2: 109-12.
22 Zhan L, Sakamoto H, Sakuraba M, Wu de S, Zhang LS, Suzuki T, Hayashi M, Honma M. Genotoxicity of microcystin-LR in human lymphoblastoid TK6 cells. Mutat Res. 2004; 557: 1-6.
23 Douglas GR, Gingerich JD, Gossen JA, Bartlett SA. Sequence spectra of spontaneous *lacZ* gene mutations in transgenic mouse somatic and germline tissues, Mutagenesis 1994; 9: 451-8.
24 Suzuki T, Hayashi M, Sofuni T. Initial experiences and future directions for transgenic mouse mutation assays. Mutat Res. 1994; 307: 489-94.
25 Nohmi T, Suzuki T, Masumura K. Recent advances in the protocols of transgenic mouse mutation assays. Mutat Res. 2000; 455: 191-215.
26 Hayashi M, Morita T, Kodama Y, Sofuni T, Ishidate Jr. M. The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. Mutat Res. 1990; 245: 245-9.
27 Suzuki T, Wang X, Miyata Y, Saeki K, Kohara A, Kawazoe Y, Hayashi M, Sofuni T. Hepatocarcinogen quinoline induces G:C to C:G transversions in the *cII* gene in the liver of lambda/lacZ transgenic mice (Muta™ Mouse). Mutat Res. 2000; 456: 73-81.
28 Kohara A, Suzuki T, Honma M, Ohwada T, Hayashi M. Mutagenicity of aristolochic acid in the *lambda/lacZ* transgenic mouse (Muta™Mouse). Mutat Res. 2002; 515: 63-72.
29 Yamada K, Suzuki T, Kohara A, Hayashi M, Hakura A, Mizutani T, Saeki K. Effect of 10-aza-substitution on benzo[*a*]pyrene mutagenicity *in vivo* and *in vitro*. Mutat Res. 2002; 521: 187-200.
30 Rao PV, Bhattacharya R. The cyanobacterial toxin microcystin-LR induced DNA damage in mouse liver *in vivo*. Toxicology 1996; 114: 29-36.
31 Ito E, Kondo F, Terao K, Harada I. Neoplastic nodular formation in mouse liver induced by repeated intraperitoneal injections of microcystin-LR. Toxicon. 1997; 35: 1453-7.
32 Zegura B, Lah TT, Filipic M. The role of reactive oxygen species in microcystin-LR-induced DNA damage. Toxicology 2004; 200: 59-68.
33 Bouaicha N, Maatouk I, Plessis MJ, Perin F. Genotoxic potential of Microcystin-LR and nodularin *in vitro* in primary cultured rat hepatocytes and *in vivo* in rat liver. Environ Toxicol. 2005; 20: 341-7.

34 Guzman RE, Solter PF, Runnegar MT. Inhibition of nuclear protein phosphatase activity in mouse hepatocytes by the cyanobacterial toxin microcystin-LR. Toxicon. 2003; 41: 773–81.
35 Gehringer MM. Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response. FEBS Lett. 2004; 557: 1–8.
36 Aonuma S, Ushijima T, Nakayasu M, Shima H, Sugimura T, Nagao M. Mutation induction by okadaic acid, a protein phosphatase inhibitor, in CHL cells, but not in S. typhimurium. Mutat Res. 1991; 250: 375–81.
37 Nakagama H, Kaneko S, Shima H, Inamori H, Fukuda H, Kominami R, Sugimura T, Nagao M. Induction of minisatellite mutation in NIH 3T3 cells by treatment with the tumor promoter okadaic acid. Proc Natl Acad Sci U S A. 1997; 94: 10813–6.

TOXICOLOGICAL SCIENCES 94(1), 83–91 (2006)
doi:10.1093/toxsci/kfl075
Advance Access publication August 3, 2006

Flow Cytometric Analysis of Micronuclei in Peripheral Blood Reticulocytes: I. Intra- and Interlaboratory Comparison with Microscopic Scoring

Stephen D. Dertinger,* Michelle E. Bishop,† James P. McNamee,‡ Makoto Hayashi,§ Takayoshi Suzuki,§ Norihide Asano,¶ Madoka Nakajima,‖ Junichiro Saito,‖‖ Martha Moore,† Dorothea K. Torous,* and James T. MacGregor‖‖‖,[1]

*Litron Laboratories, Rochester, New York 14623; †U.S. Food and Drug Administration, National Center for Toxicological Research, Jefferson, Arkansas 72079; ‡Health Canada, Ottawa, Ontario, Canada K1A 0L2; §National Institute of Health Sciences, Tokyo 158-8501, Japan; ¶Nitto Denko Corporation, Osaka 567-8680, Japan; ‖An-Pyo Center, Shizuoka 437-1213, Japan; ‖‖Astellas Pharma Inc., Tokyo 174-8511, Japan; ‖‖‖U.S. Food and Drug Administration, National Center for Toxicological Research, Rockville, Maryland 21012

Received February 2, 2006; accepted July 31, 2006

Accumulating evidence suggests that reticulocytes (RETs) in the peripheral blood of rats may represent a suitable cell population for use in the micronucleus assay, despite the ability of the rat spleen to selectively remove micronucleated erythrocytes from the peripheral circulation. To evaluate the analytical performance of a previously described flow cytometric method (Torous *et al.*, 2003, *Toxicol. Sci.* 74, 309–314) that may allow this assay to be conducted using peripheral blood *in lieu* of bone marrow sampling, we compared the sensitivity and performance characteristics of the flow cytometric technique with two established microscopy-based scoring methods. Peripheral blood samples from single Sprague-Dawley rats treated for 6 days with either vehicle or cyclophosphamide were prepared in replicate for scoring by the three methods at different laboratories. These blood-based measurements were compared to those derived from bone marrow specimens from the same animals, stained with acridine orange, and scored by microscopy. Through the analysis of replicate specimens, inter- and intralaboratory variability were evaluated for each method. Scoring reproducibility over time was also evaluated. These data support the premise that rat RETs harvested from peripheral blood are a suitable cell population to assess genotoxicant-induced micronucleus formation. The interlaboratory comparison provides evidence of the general robustness of the micronucleus endpoint using different analytical approaches. Furthermore, data presented herein demonstrate a clear advantage of flow cytometry–based scoring over microscopy—significantly lower inter- and intralaboratory variation and higher statistical sensitivity.

Key Words: flow cytometric analysis; reticulocytes; micronucleus test; CD71.

The *in vivo* rodent erythrocyte micronucleus (MN) test is widely used in research and regulatory safety assessment to evaluate the potential of chemical and physical agents to cause chromosomal damage. Historically, MN studies based on rat peripheral blood have been avoided as it has been assumed that the efficiency by which the rat spleen filters out erythrocytes with intracellular inclusions would reduce assay sensitivity (Hayashi *et al.*, 2000; Wakata *et al.*, 1998). However, accumulated data suggest that peripheral blood from intact rats can be used effectively to detect chemical-induced genotoxicity (Abramsson-Zetterberg *et al.*, 1999; Asanami *et al.*, 1995; Hamada *et al.*, 2001; Hayashi *et al.*, 1992; Hynes *et al.*, 2002; Romagna and Staniforth, 1989; Torous *et al.*, 2000, 2003; Wakata *et al.*, 1998). Thus, it appears that MN studies using peripheral blood sampling in the rat have the potential to substitute for labor-intensive, bone marrow–based tests. In addition, the ability to use low-volume blood samples will facilitate integration of the assay into routine toxicology and/or pharmacokinetic studies and may make it unnecessary to conduct separate assays for the evaluation of chromosomal damage (Asanami *et al.*, 1995; Hamada *et al.*, 2001; MacGregor *et al.*, 1995; Wakata *et al.*, 1998).

Before rat blood–based MN assays gain wider acceptance, especially in the context of regulatory testing requirements, additional information that allows direct comparisons between bone marrow and blood data is needed. Furthermore, the performance characteristics of the most widely utilized scoring techniques require further study. The experiments described herein were designed to address these issues of analytical performance by directly comparing values in blood and bone marrow obtained at different laboratories with three widely used methodologies, comparing values derived from two microscopy-based methods with a flow cytometry–based method that incorporates a calibration standard.

For each of the three scoring techniques, at least three proficient laboratories received replicate, coded samples for reticulocyte

[1] To whom correspondence should be sent at the present address: Toxicology Consulting Services, 201 Nomini Drive, Arnold, MD 21012. Fax: 410-975-0481. E-mail: jtmacgregor@earthlink.net.

TABLE 1
Participating Laboratories

Laboratory	Code	Specimens analyzed	Scoring method	Instrumentation (magnification)
U.S. FDA-NCTR, Rockville, MD and Jefferson, AR	L1	BM, PB	MeOH-AO	Zeiss Axioskop 50 (×630), Zeiss PlanApomat ×63 oil objective
			FCM	BD-FACSort, BD-FACScan
Litron Laboratories, Rochester, NY	L2	PB	FCM	BD-FACSCalibur
Health Canada, Ottawa, Ontario, Canada	L3	PB	FCM	BD-FACSCalibur
National Institute of Health Sciences, Tokyo, Japan	L4	Coordinated SV-AO laboratories		
Nitto Denko Corporation, Osaka, Japan	L5	PB	SV-AO	Olympus AHBT3-RFC (×600)
An-Pyo Center, Shizuoka, Japan	L6	PB	SV-AO	Olympus BX50-RFL (×800)
Astellas Pharma Inc., Tokyo, Japan	L7	PB	SV-AO	Olympus BH-RFL (×600)
N/A to this study	L8			
Contract testing laboratory 1*	L9	BM, PB	MeOH-AO	Leitz Laborlux 12 (×1000)
Contract testing laboratory 2*	L10	BM	MeOH-AO	Olympus BH2 (×1000)
Contract testing laboratory 3*	L11	BM, PB	MeOH-AO	Zeiss STD 14 (×1000)

Note. Abbreviations: FDA-NCTR = U.S. Food and Drug Administration, National Center of Toxicological Research; BM = bone marrow; PB = peripheral blood; MeOH-AO = acridine orange staining of methanol-fixed smears; FCM = flow cytometry; SV-AO = supravital staining using acridine orange–coated slides; N/A = not applicable. *The three contract testing laboratories are BioReliance, Covance, and SRI International, but their identities as L9, L10, or L11 is confidential.

(RET) and MN-RET scoring. Proficiency was assumed based on the high level of training that has occurred at these laboratories (L1, L2, L3, L5, L6, and L7) and/or the frequency with which they contribute *in vivo* rodent MN data for regulatory submission purposes (L9, L10, and L11). See Table 1 for more detailed information regarding collaborating laboratories.

Data presented herein describe the performance characteristics of the three scoring methods evaluated, address the sensitivity of the rat peripheral blood compartment for detecting genotoxicant-induced micronuclei, and support recommendations concerning the minimum number of rat blood RET that should be evaluated for micronuclei.

MATERIALS AND METHODS

Chemicals and other reagents. Cyclophosphamide (CP) (CAS No. 6055-19-2) was purchased from Sigma, St Louis, MO. Acridine orange (AO)–coated slides used for supravital staining, prepared according to the method of Hayashi *et al.* (1990), were provided by the National Institute of Health Sciences, Japan. Flow cytometry reagents, including fixed malaria-infected rat blood (malaria biostandard) were from Rat MicroFlow^{PLUS} Kits contributed by Litron Laboratories (available from Litron Laboratories, Rochester, NY and BD Biosciences PharMingen, San Diego, CA).

Animals and treatment regimens. Animal studies were conducted in compliance with guidelines of the National Research Council (1996) "Guide for the Care and Use of Laboratory Animals" and were approved by the appropriate Institutional Animal Care and Use Committees. Two female Sprague-Dawley rats, 4- to 5-weeks old, were purchased from Taconic, Germantown, NY. Animals were housed singly and were assigned randomly to treatment groups. The animals were acclimated for approximately 2 weeks before the experiment was initiated. Food and water were available *ad libitum* throughout the acclimation and experimentation periods. One rat was treated via oral gavage with distilled water, and the other rat was treated by the same route with 10 mg CP/kg/day for 6 consecutive days.

Blood/bone marrow sample collection and storage. Each day, before vehicle or CP treatment, low-volume blood samples (approximately 100 µl) were collected from the tail vein using a 26.5-gauge needle and syringe after a brief warming period under a heat lamp. These samples were fixed for flow cytometric analysis of RET and MN-RET frequencies according to procedures described in the Rat MicroFlow^{PLUS} manual (v020213). Fixed samples were stored at – 85°C until analysis. Approximately 24 h after the last administration of vehicle or CP, blood samples were collected into tubes containing heparin solution (500 USP units heparin per milliliter of phosphate buffered saline) as follows: into a small tube containing 75 µl heparin solution, blood was collected until a final volume of approximately 750 µl was obtained; into a second tube containing 5 ml heparin solution, approximately 1 ml blood was collected. To tubes with the 750 µl blood suspension, an equal volume of heat-inactivated fetal bovine serum (FBS) was added. These FBS-diluted suspensions were used to prepare replicate AO-supravital (SV) slides (8 µl per slide) according to the method of Hayashi *et al.* (1990, 1992). These slides were frozen, shipped to collaborating SV-AO laboratories on dry ice, and stored frozen until analysis. FBS-diluted blood suspensions were also used to prepare slides for conventional acridine orange staining of methanol-fixed smears (MeOH-AO) staining (5 µl per slide). These blood smears were prepared by drawing the cell suspensions behind a second slide with smoothed edges (a "spreader slide"). These smears were allowed to air dry and were then fixed with absolute methanol for 10 min. The slides were stored in a slide box until they were shipped to collaborating MeOH-AO laboratories for MN scoring according to their standard operating procedures. Replicate bone marrow slides were prepared as smears, air dried, methanol fixed, and shipped similarly. These bone marrow cells were harvested from two femurs per rat, whereby both ends of each femur were cut and its contents flushed with 1 ml FBS. The cells were centrifuged at approximately at 1100 rpm for 5 min and then resuspended with approximately 600 µl FBS. As with the peripheral blood, 5 µl of cell suspension was applied to each slide. The 6 ml heparinized peripheral blood suspensions were fixed with ultracold methanol according to procedures described in the Rat MicroFlow^{PLUS} manual (v020213) in order to preserve cells for flow cytometric analysis. These cell suspensions were stored at – 85°C until analysis or shipment on dry ice to collaborating flow cytometry laboratories.

The samples obtained were divided into three identical pools, and replicate samples of each pool were provided to participating laboratories with three separate codes. Thus, laboratories received triplicate samples of each condition, but were not aware that they were from an identical pool. Thus, the analyses

conducted allow assessment of both intralaboratory variability of replicate analysis of identical samples and interlaboratory variability of the same analysis. Each laboratory also conducted analysis of each of these pools on three separate occasions, allowing assessment of variability of analysis over time.

Standard acridine orange slide scoring (MeOH-AO). Blood and bone marrow smears were scored using the MeOH-AO scoring technique at the Food and Drug Administration-National Center for Toxicological Research laboratory (L1) and three contract testing laboratories (L9, L10, and L11). Methanol fixation leads to a diffuse distribution of RNA, and erythrocytes are classified as normochromatic or as RETs based on the presence or absence of RNA-associated fluorescence. This technique is not well suited for visually classifying subpopulations of RETs. RET frequencies were determined by inspecting 500 or 1000 total erythrocytes per bone marrow or blood sample, respectively. MN-RET incidence was determined by inspecting 2000 RETs per sample. At L1, micronuclei were defined by the criteria of Schmid (1976) with the added requirements that they exhibit the characteristic yellow to yellow-green fluorescence characteristic of AO staining and that they exhibit the smooth boundary expected from a membrane-bound body. Laboratories L9, L10, and L11 were instructed to follow the standard operating procedures they use for regulatory submissions to support new drug or food additive development. Thus, the acquisition of data by these facilities allows for comparisons with three highly experienced contract laboratories under conditions associated with regulatory testing.

Supravital acridine orange slide scoring (SV-AO). Laboratories L5, L6, and L7 scored peripheral blood samples using the SV-AO scoring technique. This staining procedure aggregates RNA, leading to punctate staining patterns. These staining characteristics allow RET to be classified into four age cohorts: Type I (youngest) through Type IV (oldest) RETs as described by Hayashi *et al.* (1990, 1992). The frequency of MN-RETs was determined by analyzing 2000 Type I and Type II RETs (L5 and L7) or 2000 Type I RETs (L6). An index of cytotoxicity was obtained by inspecting at least 400 RETs and calculating the percentage of Type I and Type II RET among total RETs (L5 and L7) or the percentage of Type I RETs among Type I and Type II RETs (L6). AO-coated slides were purchased from TOYOBO (Osaka, Japan). Supravitally stained triplicate slides were frozen and sent to the Japanese reference laboratory (Nitto Denko) with dry ice. Each set of slides was also sent to two other laboratories for replicate scoring by fluorescence microscopy.

Flow cytometry–based scoring. Methanol-fixed blood samples were washed and labeled for flow cytometric analysis by L1, L2, and L3 according to procedures described in the Rat MicroFlow^{PLUS} Kit (v020213). Samples were analyzed with 488-nm capable instruments (FACSCalibur, FACSort, and FACScan, all from Becton Dickinson, San Jose, CA). Anti-CD71-FITC and propidium iodide fluorescence signals were detected in the FL1 and FL3 channels, respectively (stock filter sets). Calibration of the flow cytometers for the MN scoring application, across laboratories and between experiments within each laboratory, was accomplished by staining *Plasmodium berghei*–infected rat blood (malaria biostandards) in parallel with test samples on each day of analysis (Dertinger *et al.*, 2000; Tometsko *et al.*, 1993; Torous *et al.*, 2001). By adjusting voltages applied to the photomultiplier tube, it was possible to standardize the FL3 fluorescence channel into which erythrocytes with single (MN like) parasites fell. In this manner, analysis regions were consistent across laboratories and between experiments. Flow cytometry–based MN-RET measurements reported herein are based on an immature fraction of peripheral blood RETs (approximately the youngest 30–50% of propidium iodide–positive erythrocytes, based on CD71 expression level; Torous *et al.*, 2001, 2003). This is thought to be analogous to scoring the youngest (Types I and II) RETs using the SV-AO method, which may be beneficial in view of reports which have suggested that the influence of rat spleen filtration function can be minimized by scoring the younger RETs (Abramsson-Zetterberg *et al.*, 1999; Hayashi *et al.*, 1992; Hynes *et al.*, 2002; Torous *et al.*, 2000, 2003). Data were acquired with CellQuest software (v3.3, BD-Immunocytometry Systems, San Jose, CA), with the stop mode set so that 20,000 high CD71-expressing RETs were analyzed per blood sample. The number of mature (CD71 negative) erythrocytes was determined concurrently, providing an index of cytotoxicity (%RETs).

Calculations. All calculations were performed with Excel (Office X for Mac or Microsoft Office Excel 2002 for XP Windows Professional, Microsoft Corp., Seattle, WA). The incidences of MN-RETs are expressed as frequency percent. The percentage of RETs among total erythrocytes was measured by the flow cytometric and MeOH-AO laboratories and served as an index of bone marrow cytotoxicity. The three SV-AO laboratories used percentage of RETs in different stages of maturity as an index of toxicity; therefore, these indices are not directly comparable to those obtained by the flow cytometric and MeOH-AO microscopy laboratories. Percent coefficient of variance values (%CV, i.e., standard deviation (SD) as percent of the mean) were used to describe intralaboratory variability associated with multiple readings of replicate samples and also interlaboratory variation of vehicle control and CP-induced MN-RET measurements that were pooled according to scoring method.

RESULTS AND DISCUSSION

Confirmation of Steady State

RET and MN-RET measurements obtained from the daily low-volume blood specimens were analyzed to confirm that the

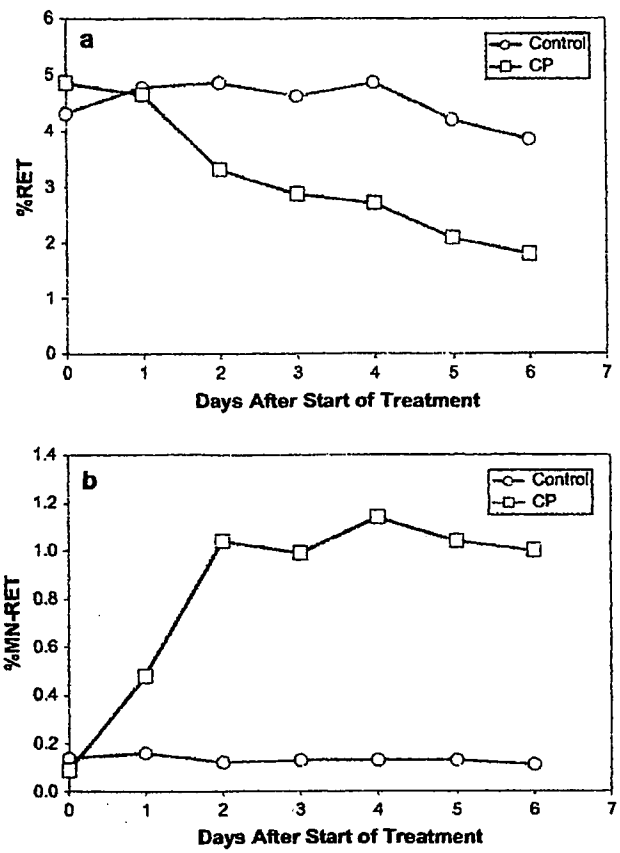


FIG. 1. The frequency of peripheral blood RETs (%RET, panel a) and peripheral blood micronucleated RETs (%MN-RET, panel b) as a function of time in the individual rats used to generate reference samples for analytical comparison. These data were acquired by flow cytometric analysis (laboratory L2) and demonstrate the attainment of a steady-state MN-RET frequency, facilitating subsequent comparisons between bone marrow and peripheral blood compartments.

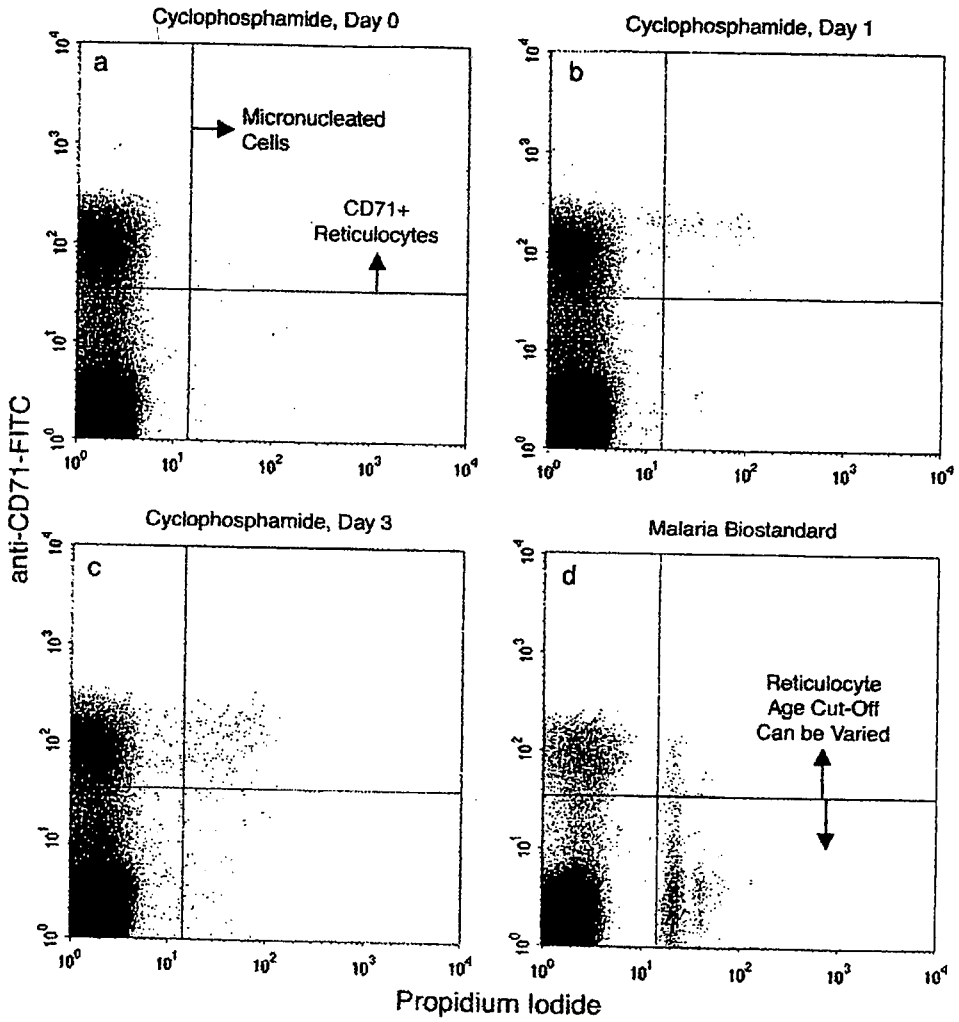


FIG. 2. Panels (a–c): Bivariate graphs illustrate the staining characteristics of rat blood specimens over the course of several days of CP treatment. Note the appearance of micronuclei at Day 1 in the very youngest (highest anti-CD71-FITC fluorescence) RETs (Panel b) and the more uniform distribution among RETs after a steady state has been reached on Day 3 of treatment (Panel c). Panel (d) illustrates the use of the malaria standard, with distinct fluorescence intensities corresponding to inclusion of one, two, or three parasites. This allows the instrument settings to be standardized to the DNA content of the parasite, which is controlled biologically to a quantity similar to that in an average MN.

MN-RET frequency of the vehicle-treated animal was stable over the duration of the experiment and that CP treatment caused the MN-RET frequency to increase to a steady-state level of approximately 10-fold the control frequency (Fig. 1). Since the frequency of MN-RETs was at steady state in both cases, the values in bone marrow and peripheral blood should be directly comparable—that is, expected to be equal in the absence of selective removal of MN-RETs from blood or methodological differences in measurement. Thus, the samples collected in this manner allow the direct comparisons between measurements in the bone marrow and blood compartments that follow. The use of large samples from a single treated and a single control rat allows differences in methodology and scoring laboratory to be assessed independently of sample variation.

The dose of CP (10 mg/kg/day) had a moderate effect on erythropoiesis, as indicated by the decline in RET frequency (terminal day specimen showed a greater than 50% decrease from pretreatment value; see Fig. 1, panel a). This level of bone marrow cytotoxicity is well within the range of target toxicity recommended by current regulatory guidances (i.e., $\leq$ 80%, see Organisation for Economic Cooperation and Development, 1997, Guideline 474; U.S. Food and Drug Administration, 2000.

To illustrate the nature and source of the flow cytometry–based data described above, bivariate fluorescence intensity plots are provided (Fig. 2). Note the appearance of micronuclei on Day 1 in the very youngest (highest anti-CD71-FITC fluorescence) RETs (Panel b) and the more uniform distribution

TABLE 2
Reticulocyte Data (cytotoxicity determinations)

Laboratory	Method	Compartment	Treatment	Cytotoxicity index[a]	Average %RET[b] ± SEM	%CV	%Change
L1	MeOH-AO	BM	Vehicle	%RET	81.0 ± 0.70	1.5	
			CP		67.8 ± 2.36	6.0	− 16
L9	MeOH-AO	BM	Vehicle	%RET	65.4 ± 1.03	2.7	
			CP		51.9 ± 1.83	6.1	− 21
L10	MeOH-AO	BM	Vehicle	%RET	58.2 ± 1.65	4.9	
			CP		60.0 ± 0.66	1.9	+ 3
L11	MeOH-AO	BM	Vehicle	%RET	63.1 ± 1.67	4.6	
			CP		57.1 ± 1.14	3.5	− 10
Pooled[b] L1, 9, 10, 11			*Vehicle*		*66.9 ± 2.63*	*13.6*	
			CP		*59.2 ± 1.87*	*10.9*	*− 12*
L1	MeOH-AO	PB	Vehicle	%RET	7.7 ± 0.19	4.2	
			CP		5.7 ± 0.27	8.2	− 26
L9	MeOH-AO	PB	Vehicle	%RET	6.2 ± 0.27	7.4	
			CP		5.6 ± 0.90	27.8	− 9
L11	MeOH-AO	PB	Vehicle	%RET	6.6 ± 0.52	13.5	
			CP		4.9 ± 0.33	11.7	− 26
Pooled L1, 9, 11			*Vehicle*		*6.9 ± 0.29*	*12.5*	
			CP		*5.4 ± 0.32*	*17.6*	*− 21.7*
L5	SV-AO	PB	Vehicle	%Type I + II/I–IV	55.2 ± 1.95	6.1	
			CP		42.2 ± 0.12	0.5	− 24
L6	SV-AO	PB	Vehicle	%Type I/I + II	42.4 ± 2.8	11.5	
			CP		29.1 ± 2.1	12.4	− 31
L7	SV-AO	PB	Vehicle	%Type I + II/I–IV	52.3 ± 2.1	6.8	
			CP		34.8 ± 3.1	15.6	− 34
Pooled[c] L5, 7			*Vehicle*		*53.7 ± 1.4*	*6.5*	
			CP		*38.5 ± 2.2*	*13.8*	*− 28*
L1	FCM	PB	Vehicle	$\%RET^{High\ CD71+}$	3.40 ± 0.02	1.18	
			CP		1.53 ± 0.01	0.75	− 55
L2	FCM	PB	Vehicle	$\%RET^{High\ CD71+}$	3.32 ± 0.02	1.26	
			CP		1.44 ± 0.01	1.44	− 57
L3	FCM	PB	Vehicle	$\%RET^{High\ CD71+}$	3.32 ± 0.05	2.42	
			CP		1.40 ± 0.08	9.88	− 58
Pooled L1, 2, 3			*Vehicle*		*3.34 ± 0.02*	*1.93*	
			CP		*1.46 ± 0.03*	*6.33*	*− 56*

Note. Abbreviations: RET = reticulocyte; MeOH-AO = acridine orange staining of methanol-fixed smears; SV-AO = supravital staining using acridine orange–coated slides; FCM = flow cytometry; BM = bone marrow; PB = peripheral blood; CP = cyclophosphamide; SEM = standard error of the mean.

[a]Each laboratory evaluated cytotoxicity based on immature erythrocyte parameters. This was accomplished in several different manners: %RET = percentage of RETs relative to total erythrocytes; %Type I + II/I–IV = percentage of Type I and Type II RETs relative to total RETs; %Type I/I + II = percentage of Type I RETs relative to Type I and Type II RETs; and $\%RET^{High\ CD71}$ = percentage of RETs that express high levels of CD71 relative to total erythrocytes.

[b]Values are the mean of three separately coded, but identical, samples. By "Pooled" it is meant that like-method data from two, three, or four laboratories were combined for these calculations.

[c]Only data from the two SV-AO laboratories that measured toxicity similarly (%Type I + II/I–IV) were combined for these calculations.

among RETs after a steady state has been reached on Day 3 of treatment (Panel c). Panel (d) illustrates the use of the malaria biostandard, with distinct fluorescence intensities corresponding to inclusion of one, two, or three parasites. This allows the instrument settings to be standardized to the DNA content of the parasite, which is controlled biologically to a quantity similar to that in an average MN. For research purposes, the regions may be adjusted to allow measurements in different age populations of RETs and/or micronuclei with different DNA contents. For analytical purposes, the standard can be used to achieve comparable instrument performance across time within a laboratory or across different instruments in different laboratories.

Intra- and Interlaboratory Variability

Replicate bone marrow and/or peripheral blood specimens obtained after 6 consecutive days of treatment were provided to each collaborating laboratory. As noted above, the frequencies of MN-RETs were at steady state and therefore not changing as a function of time. Each laboratory received three separately coded samples from each of the high and low MN-RET frequency pools but were not aware that the three separately coded samples were identical. Tabular values are the means of the values of the three separately coded samples.