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Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens II. Further analysis of mammalian cell results, relative predictivity and tumour profiles

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Abstract

One of the consequences of the low specificity of the *in vitro* mammalian cell genotoxicity assays reported in our previous paper [D. Kirkland, M. Aardema, L. Henderson, L. Müller, Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. I. Sensitivity, specificity and relative predictivity, Mutat. Res. 584 (2005) 1–256] is industry and regulatory agencies dealing with a large number of false-positive results during the safety assessment of new chemicals and drugs. Addressing positive results from *in vitro* genotoxicity assays to determine which are "false" requires extensive resources, including the conduct of additional animal studies. In order to reduce animal usage, and to conserve industry and regulatory agency resources, we thought it was important to raise the question as to whether the protocol requirements for a valid *in vitro* assay or the criteria for a positive result could be changed in order to increase specificity without a significant loss in sensitivity of these tests. We therefore analysed some results of the mouse lymphoma assay (MLA) and the chromosomal aberration (CA) test obtained for rodent carcinogens and non-carcinogens in more detail. For a number of chemicals that are positive only in either of these mammalian cell tests (i.e. negative in the Ames test) there was no correlation between rodent carcinogenicity and level of toxicity (we could not analyse this for the CA test as insufficient data were available in publications), magnitude of response or lowest effective positive concentration. On the basis of very limited *in vitro* and *in vivo* data, we could also find no correlation between the above parameters and formation of DNA adducts. Therefore, a change to the current criteria for required level of toxicity in the MLA, to limit positive calls to certain magnitudes of response, or to certain concentration ranges would not improve the specificity of the tests without significantly reducing the sensitivity.

We also investigated a possible correlation between tumour profile (trans-species, trans-sex and multi-site *versus* single-species, single-sex and single-site) and pattern of genotoxicity results. Carcinogens showing the combination of trans-species, trans-sex and multi-site tumour profile were much more prevalent (70% more) in the group of chemicals giving positive results in all three *in vitro* assays than amongst those giving all negative results. However, single-species, single-sex, single-site carcinogens were not very prevalent even amongst those chemicals giving three negative results *in vitro*. Surprisingly, when mixed positive and negative results were compared, multi-site carcinogens were highly prevalent amongst chemicals giving only a single positive result in the battery of three *in vitro* tests.

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Finally we extended our relative predictivity (RP) calculations to combinations of positive and negative results in the genotoxicity battery. For two out of three tests positive, the RP for carcinogenicity was no higher than 1.0 and for 2/3 tests negative the RP for non-carcinogenicity was either zero (for Ames + MLA + MN) or 1.7 (for Ames + MLA + CA). Thus, all values were less than a meaningful RP of two, and indicate that it is not possible to predict outcome of the rodent carcinogenicity study when only 2/3 genotoxicity results are in agreement.
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1. Introduction

Current regulatory guidelines for genotoxicity testing require chemicals to be evaluated at high concentrations/molarity and/or cellular toxicity *in vitro* for a valid test. The low specificity in the mammalian cell assays reported in our previous paper [1] raises the question as to whether such protocol requirements for a valid assay or the criteria for a positive result (according to ICH [2] and OECD [3] guidelines) could be changed in order to increase specificity, thereby reducing the number of false-positive results obtained. Since the standard approach to determine the biological relevance of positive *in vitro* genotoxicity assays involves conduct of additional *in vivo* genotoxicity assays, efforts to reduce this high false-positive rate could lead to substantial reductions in animal testing, as well as the cost and time to run these tests. Therefore, we examined such questions as whether the sensitivity of a particular mammalian cell assay could be maintained, but the specificity improved, if lower levels of toxicity were required for a valid assay? Did the carcinogens induce genotoxic responses of greater magnitude than the non-carcinogens, such that by requiring a higher level of response for a positive result, specificity might be improved? Also, were the non-carcinogens positive at much higher concentrations than the carcinogens, such that by requiring a lower top concentration than the current 10 mM, specificity might be improved? In this paper we have analysed some of the mouse lymphoma assay (MLA) and chromosomal aberration (CA) test results with rodent carcinogens and non-carcinogens (as defined in our previous paper [1]) in light of these questions.

We also examined whether a consistent pattern of genotoxicity results e.g. positive in Ames, MLA and either *in vitro* micronucleus (MN) or CA tests correlated better with trans-species, trans-sex, multi-site carcinogens than with single-species, single-sex, single-site carcinogens.

Finally, the new concept of relative predictivity (RP) that we introduced in our previous paper [1] and that had been calculated and reported only for those chemicals giving all three results positive or all three results negative in the *in vitro* battery, was examined for other combinations of test results. Previously we reported that a reasonable correlation (RP of two or more) with rodent carcinogenicity was obtained when all three *in vitro* tests gave positive results (>3× more likely that a chemical would be a rodent carcinogen if all three tests were positive), and a reasonable correlation with non-carcinogenicity was obtained when all three tests were negative (>2× more likely that a chemical would be a non-carcinogen if all three tests were negative). As most compounds tested by industry give mixtures of positive and negative results across a battery of three tests (2 positive + 1 negative, or 1 positive + 2 negative), and not all regulatory guidelines request the conduct of an extensive *in vitro* battery, we calculated the RP for rodent carcinogenesis and non-carcinogenesis from these combinations of results.

2. Quantitative analysis of various aspects of the mammalian cell tests

An important source of poor specificity in our previous analysis [1] was for non-carcinogens where a single positive result was obtained in one of the mammalian cell tests in the battery. We therefore decided to examine whether changing the criteria for a valid assay or the criteria for a positive result would eliminate some of the isolated positives with non-carcinogens, whilst preserving the single positive results with carcinogens, thereby improving the specificity without decreasing the sensitivity. We decided to look at three parameters:

- Level of toxicity
- Magnitude of positive response
- Lowest clearly positive concentration

As indicated above, we focussed this analysis on those chemicals where the only positive response was in either the MLA or CA test, accompanied by negative results in the Ames test (we included one chemical with no Ames result), and either negative or no results in the other mammalian cell test.

Analysing these three parameters was relatively easy to do for the MLA because most published data (and many results came from NTP tests [4]) contained sufficient detail. However, very few CA publications, including the many NTP tests, provided detail on toxicity. We did have access to the laboratory notebooks of Dr. Ishidate, but were able to obtain toxicity information on too few clastogens to allow a meaningful analysis to be undertaken. For the MN test, where there are many fewer publications, there were so few situations where the MN was the only positive in the battery that this was not worthy of further analysis at this time.

As is the case in a retrospective analysis of this sort, it is important to recognise that the data from some studies may not meet current guideline requirements. Whilst it would be ideal to review each published result in terms of compliance with current guidelines, and focus our analysis only on those that complied, this goes beyond the scope of the project. As indicated previously [1], we did re-evaluate a significant number of the MLA studies from Mitchell et al. [6], and a number of published studies that we considered to be "technically compromised" were excluded from the analysis. However, there was insufficient information to do this for other study types, in particular the CA test. Nonetheless, we believe the trends observed here would largely still hold true if the analysis was limited to studies compliant with current guidelines.

2.1. Mouse lymphoma assay (MLA) results

The criteria for conclusions of positive, negative, equivocal or technically compromised have been described in our previous paper [1], as have the reasons why some previously reported MLA results were reassessed in order to fit our categorisation. The carcinogens and non-carcinogens where only the MLA gave a positive result (i.e. either both Ames and MN/CA were negative or results were not available) were identified from our Carcinogenicity Genotoxicity eXperience (CGX) database (as detailed in [1]; it can be viewed at http://www.lhasalimited.org/cgx). This amounted to only 45 chemicals, which is a small number, and analysis of other categories of chemicals in our database may provide more useful information. We re-examined the original reports and recorded the following:

- The highest relative total growth (RTG—i.e. lowest level of toxicity) at which a significant increase in mutant frequency occurred. Increases in MF above concurrent control levels of 90×10^{-6} for the agar method and 126×10^{-6} for the microwell method are considered significant according to the latest recommendations [5]. Most of the published studies in our database used the agar method.
- The maximum increase in MF at any concentration giving acceptable RTG (i.e. RTG >0.1).
- The lowest effective concentration at which a significant increase in MF (as defined above) was observed.

For the MLA-positive chemicals shown in Tables 1 and 2, all except one were negative in the Ames test—for pyrilamine maleate there was no Ames test result. Most were also negative for CA and/or MN but for a small number there was no result for either CA or MN, as noted in the Table. For pyrilamine maleate there was also no result in either CA or MN, so the MLA was the only result. For two carcinogens (*o*-benzyl-*p*-chlorophenol and C.I. Direct Blue 218) the MLA data were referred to by Mitchell et al. [6] in their revised EPA Gene-Tox review, and therefore included in our CGX database, but the original data were not available for review.

Tables 1 and 2 show the highest RTG levels (i.e. lowest levels of toxicity) at which significant positive responses were seen for carcinogens and non-carcinogens, respectively. As can be seen, there were no differences between carcinogens and non-carcinogens in the levels of toxicity at which the minimum positive response was observed. Carcinogens and non-carcinogens are distributed across the whole range of toxicity for the point at which the first positive response is seen. The lowest level of toxicity for a biologically relevant positive response ranged from 23 to 89% (RTG from 0.77 to 0.11) for carcinogens, and from 16 to 91% (RTG 0.84–0.09) for non-carcinogens.

Thus, if the upper limit of toxicity required for a valid assay were lowered, several carcinogens would be missed. Also, sufficient non-carcinogens were positive at low levels of toxicity that the specificity would not be improved. For instance, if the toxicity limit were lowered to 50% (consistent with the chromosomal aberration test), seven non-carcinogens would no longer be positive (see Table 2A) but 15 carcinogens would also no longer be positive (see Table 1A).

Tables 1 and 2 also show the maximum induced MF (IMF) at any concentration that produced acceptable (non-excessive) toxicity (i.e. RTG not lower than 0.1). It should be noted that, at the time most of the MLA studies were performed, there was limited appreciation of the importance of small-colony mutants, and therefore some tests may have achieved conditions that favoured growth of small-colony mutants, whereas others would not. Clearly this could have an impact on

Table 1
Toxicity, maximum mutagenic response and lowest mutagenic concentrations in the MLA for Ames-negative carcinogens

Chemical name	CAS number	RTG for minimum positive response[a]	Maximum induced MF ($\times 10^{-6}$) at any treatment with RTG of 0.1 or higher	Lowest effective concentration (μmol/l) for minimum positive or highest non-significant response[a]	Induction of DNA adducts [reference]
A: Carcinogens positive only in MLA (i.e. negative or equivocal in Ames and CA/MN)					
11-Aminoundecanoic acid	2432-99-7	0.51	149	99.4	No data
Benzofuran	271-89-6	0.22	172	1270	No data
o-Benzyl-*p*-chlorophenol	120-32-1	Data not publicly available			
Butylated hydroxytoluene	128-37-0	0.26	145	45	No data
Chlorobenzilate	510-15-6	0.30	244	277	No data
Chloroform	67-66-3	0.34	92	0.4	+ Calf thymus DNA[b] [9] and hepatocytes [10] *in vitro*
4-Chloro-*o*-toluidine	3165-93-3	0.17	71[c]	848	+ Liver *in vivo* [11]
C.I. Direct blue 218	28407-37-6	Data not publicly available			
Cinnamyl anthranilate	87-29-6	0.24	205	30.8	No data
Dicofol	115-32-2	0.30	36[c]	135	No data
N,N′-Diethyl-2-thiourea	105-55-5	0.42	2307	11344	No data
Ethylbenzene	100-41-4	0.34	529	754	No data
Kepone (AKA chlordecone)	143-50-0	0.38	62[c]	71.3	No data
Malonaldehyde sodium salt	24382-04-5	0.67	239	5553	+ Liver *in vivo* [12]
Methyl *tert*-butyl ether	1634-04-4	0.61	181	22.7	+ Hepatocytes *in vitro*[d] [13]
Piperonyl butoxide	51-03-6	0.17	443	148	No data
Piperonyl sulphoxide	120-62-7	0.70	85[c]	7.7	No data
Toluene	108-88-3	0.21	216	2442	+ HL-60 cells *in vitro* [14]
2,4,6-Trichlorophenol	88-06-2	0.11	144	608	No data
Trimethylthiourea	2489-77-2	0.21	377	35533	No data
4-Vinylcyclohexene	100-40-3	0.40	79[c]	740	+ Skin *in vivo* [15]
B: Carcinogens positive in MLA, negative Ames but no test (or TC) in CA/MN					
1,4-Benzoquinone	106-51-4	0.34	200	3.15	+ Plasmid DNA *in vitro* [16]
Dimethyl methylphosphonate	756-79-6	0.73	54[c]	40.3	No data
FD&C Red 1 (Ponceau 3R)	3564-09-8	0.36	100	3440	No data
Procarbazine HCl (Natulan)	366-70-1	0.77	356	49.7	+ Mammary tissue [17], liver and bone marrow [18] *in vivo*
Thioacetamide	62-55-5	0.69	177	10240	+ Liver *in vivo* [19]
Trichloroacetic acid	76-03-9	0.28	121	12241	+ Liver *in vivo*[b] [20]
Vinylidene chloride	75-35-4	0.70	212	64990	No data
C: Carcinogens positive in MLA but no test (or TC) in Ames and CA/MN					
Pyrilamine maleate	59-33-6	0.55	155	1619	No data

NB: There were no chemicals positive in MLA, negative in CA/MN but no test (or TC) in Ames.

[a] Minimum clear positive response considered where mutant frequency (MF) increased at least 90×10^{-6} (all studies used agar method) over concurrent control as recommended by Moore et al. [5]. In some cases, due to spacing of doses, the lowest significant increase in MF was much greater than the minimum, which would therefore have occurred at a higher RTG and lower concentration.

[b] Oxidative damage to DNA.

[c] Minimum increase in MF not achieved. These chemicals should be reclassified as equivocal rather than positive.

[d] DNA-protein crosslinks.

[e] Although induced MF was $<90 \times 10^{-6}$, the dose response was very steep and it is expected that a significant response would have been achieved at higher concentrations.

Table 2
Toxicity, maximum mutagenic response and lowest effective mutagenic concentrations in the MLA Ames-negative non-carcinogens

Chemical name	CAS number	RTG for minimum positive response[a]	Maximum induced MF ($\times 10^{-6}$) at any treatment with RTG of 0.1 or higher	Lowest effective concentration (μmol/l) for minimum positive or highest non-significant response[a]	Induction of DNA adducts [reference]
A: Non-carcinogens positive only in MLA (i.e. Ames and CA/MN negative or equivocal)					
Aldicarb	116-06-3	0.84	111	21024	No data
Anilazine	101-05-3	0.72	298	10.5	No data
Barium chloride dihydrate	10326-27-9	0.40	27[b]	4094	No data
1,2-Dichlorobenzene	95-50-1	0.81	357	68	+ Calf thymus DNA *in vitro* [21]
FD&C yellow number 6	2783-94-0	0.79	62[b]	8842	No data
4-Hexylresorcinol	136-77-6	0.29	110	154	No data
Hydrochlorothiazide	58-93-5	0.51	264	2519	No data
Isopropyl-*N*-(3-chlorophenyl)carbamate	101-21-3	0.13	68[b]	147	No data
Malaoxon	1634-78-2	0.22	75[b]	477	No data
Oxytetracycline HCl	2058-46-0	0.30	4329	616	No data
Phenyl-β-naphthylamine	135-88-6	0.36	140	59.3	No data
Sodium diethylcarbamate trihydrate	148-18-5	0.55	326	0.39	No data
Sulfisoxazole	127-69-5	0.39	96	3741	No data
2,3,5,6-Tetrachloro-4-nitroanisole	2438-88-2	0.55	151	24.1	No data
B: Non-carcinogens positive in MLA, negative in Ames but no test (or TC) in CA/MN					
Alpha-methyldopa sesquihydrate	41372-08-1	0.27	164	168	No data
Rhodamine 6G	989-38-8	0.09	94	16.7	No data

NB: There were no chemicals positive in MLA, negative in CA/MN but no test (or TC) in Ames.
[a] Minimum clear positive response considered where mutant frequency (MF) increased at least 90×10^{-6} (all studies used agar method) over concurrent control as recommended by Moore et al. [5]. In some cases, due to spacing of doses, the lowest significant increase in MF was much greater than the minimum, which would therefore have occurred at a higher RTG and lower concentration.
[b] Minimum increase in MF not achieved. Chemical should be reclassified as equivocal rather than positive.

the IMF, and highlights some of the difficulties of trying to apply current standards to old studies. Non-carcinogens (Table 2) were just as likely to produce very high IMF as were carcinogens (Table 1), and some carcinogens produced very low IMF. In fact, several carcinogens (as well as four non-carcinogens) failed to achieve the minimum IMF (90×10^{-6} for the agar method) currently recommended for a biologically relevant response [5] and should be re-classified as equivocal. These changes will be made in the web version of the CGX database (http://www.lhasalimited.org/cgx). Thus, if we were to consider increasing the required IMF for a positive response, several carcinogens would be missed.

The lowest concentrations of chemicals at which the minimum positive responses were seen (or in the case of those where minimum IMF was not reached, the highest non-significant concentration) are also shown in Tables 1 and 2. The lowest-effective concentrations clearly vary widely from <1 to >10,000 μM for both carcinogens (Table 1) and non-carcinogens (Table 2). It will be noted that some chemicals are listed where the concentration for a minimum positive response was >10 mM. If tested according to current standards such high concentrations would not be used. Thus five carcinogens and two non-carcinogens would drop out of the analysis if positive results were only included when obtained below 10 mM. However, the range of lowest effective concentrations would still be large, both for carcinogens and non-carcinogens. Thus, changing the maximum concentration requirement for a valid test might improve the specificity of the MLA but it would lower the sensitivity.

We investigated whether formation (or not) of DNA adducts might discriminate between MLA positives of different potency. The *in vitro* and *in vivo* DNA-adduct data that could be found in the literature are also included in Tables 1 and 2. The following comments can be made on these limited data:

- There were nine carcinogens positive only in the MLA that had adduct data, and all formed DNA adducts. Within this sub-group, the level of toxicity varied widely (from 23 to 83% toxicity for a minimum pos-

itive response), the maximum IMF varied from 71 to 356×10^{-6}, and the lowest effective concentration varied over six orders of magnitude from 0.4 to 64,990 μM.

- There was only one non-carcinogen giving an isolated positive result in the MLA for which adduct data could be found, and this compound formed DNA adducts. This was positive at low toxicity (19% toxicity for minimum positive response).

Since all the MLA-positive carcinogens and the one MLA-positive non-carcinogen induced DNA adducts, it is not possible to use these data to draw conclusions regarding DNA reactivity and the other parameters we studied (level of toxicity required, minimum effective concentration or magnitude of response). However, it does not appear that any changes to currently recommended toxicity levels or maximum concentration of test chemical, or requiring a higher induced mutant frequency for a positive "call" would improve the specificity of the MLA without causing serious deterioration in the sensitivity.

2.2. Chromosomal aberration (CA) test results

As mentioned earlier, there were very few published examples where CA results were accompanied by toxicity data. Although one of us (MH) was able to look at the laboratory notebooks of Dr. Ishidate, who published many of the CA results in our previous analysis [1], we

Table 3
Analysis of CA test responses for Ames-negative carcinogens

Chemical name	CAS number	Survival (cell count, confluence or mitotic index as a % of control) for minimum positive response[a]	Maximum % of aberrant cells (excluding gaps) at any treatment with survival 50% or higher	Lowest effective concentration (μmol/l) for minimum positive or highest non-significant response[a]	Induction of DNA adducts [reference]
A: Carcinogens positive only in CA[b] (i.e. negative in Ames and MLA)[c]					
Pentachloronitrobenzene	82-68-8	Not given	19	8.1	No data
Naphthalene	91-20-3	Not given	16	234	No data
Zearalenone	17924-92-4	Not given	13	47.1	+ Kidneys, liver and ovaries *in vivo* [22]
B: Carcinogens positive in CA[b], negative in Ames but no test (or TC) in MLA[d]					
Atrazine	1912-24-9	64	28.7	5.0	− Liver *in vivo* [23]
Heptachlor	76-44-8	Not given	14	67.0	+ Hop plants *in vivo* [24]
Hexanamide	628-02-4	Not given	10	39071	No data
4-Methoxyphenol	150-76-5	Not given	6.8	250	No data
N-Methyloacrylamide	924-42-5	Not given	26	2473	No data
Methylphenidate HCl	298-59-9	Not given	9	3707	No data
Nafenopin	3771-19-5	100	Cannot be established	30.0	No data
Nitrobenzene	98-95-3	Not given	33.2	61.3	+ Liver *in vivo* [25]
C: Carcinogens positive in CA[b] but no test (or TC) in Ames and MLA[e]					
Haloperidol	52-86-8	Not given	Only one concentration used	Only one concentration used	No data
Retinol acetate[f]	127-47-9	Not given	13	200	No data

NB: There were no chemicals positive in CA, negative in MLA but no test (or TC) in Ames.

[a] Minimum positive response was considered as >5% cells with aberrations (excluding gaps) as this would usually be considered a positive response for CHO, CHL cells and human lymphocytes.

[b] Chemicals that were also positive in MN were excluded from these tables.

[c] Another chemical in this category, but for which there were insufficient data in the publications to complete any of the columns, was sodium saccharin.

[d] Other chemicals in this category, but for which there were insufficient data in the publications to complete any of the columns, were aldrin, aniline HCl, asbestos, carboxymethylnitrosourea, clofibrate, lead acetate, methimazole, styrene and 12-*O*-tetradecanoylphorbol 13-acetate.

[e] Other chemicals in this category, but for which there were insufficient data in the publications to complete any of the columns, were 1-amyl-1-nitrosourea, sodium barbital, manganese ethylenebisthiocarbamate and petasitenine.

[f] Equivocal in MN.

were only able to find toxicity data on very few additional clastogens. Therefore, it was not possible to analyse (as we did above for the MLA) whether any change to the required level of toxicity (currently at least 50% according to OECD [3]) would provide improved specificity whilst retaining sensitivity.

For a small number of chemicals that were positive only in this test, we could identify the maximum induced CA response (although we could not know whether this occurred at levels of toxicity much higher than 50%) and the lowest effective concentration required for a minimum positive response. For the latter, since most published data were from Chinese hamster cells, we accepted that CA frequencies (excluding gaps) of >5% would probably be statistically significant and exceed historical control ranges. These data are tabulated in Tables 3 and 4 for carcinogens and non-carcinogens, respectively. Unfortunately there were a number of chemicals for which the published reports contained insufficient details to establish the maximum frequency of aberrant cells or the minimum concentration for a positive effect.

From Tables 3 and 4 it can be seen that the maximum percentage of aberrant cells ranged from 9 to 33% for carcinogens and from 7 to 23% for non-carcinogens. The minimum positive concentration ranged from 5 to 39,000 μM for carcinogens and from 24 to 5000 μM for non-carcinogens. In other words, for this small sample of Ames-negative clastogens there were no clear differences in maximum response or minimum positive concentration between carcinogens and non-carcinogens. It will be noted that one chemical (hexanamide, Table 3) is listed where the concentration for a minimum positive response was >10 mM. If tested according to current standards such high concentrations would not be used. Thus this one carcinogen would drop out of the analysis if positive results were only included when obtained below 10 mM. However, the range of lowest effective concentrations would still be large, both for carcinogens and non-carcinogens. Thus it does not appear that any change to the maximum concentration, or the introduction of a requirement for the induced frequency of aberrant cells to reach a certain level would improve the specificity of the CA test without causing serious deterioration in the sensitivity.

As for the analysis of MLA data, we investigated whether DNA reactivity may correlate better with potency. Any *in vitro* or *in vivo* DNA-adduct data that could be found in the literature for these chemicals positive only in the CA test are shown in Tables 3 and 4.

Table 4
Analysis of CA test responses for Ames-negative non-carcinogens

Chemical name	CAS number	Survival (cell count, confluence or mitotic index as a % of control) for minimum positive response[a]	Maximum % of aberrant cells (excluding gaps) at any treatment with survival 50% or higher	Lowest effective concentration (μmol/l) for minimum positive or highest non-significant response[a]	Induction of DNA adducts [reference]
A: Non-carcinogens positive only in CA[b] (i.e. Ames and MLA negative)[c]					
Diphenhydramine HCl	147-24-0	Not given	17	343	No data
4,4′-Isopropylidenediphenol	80-05-7	45	23	399	+ Liver *in vivo* [26] and Syrian hamster cells *in vitro* [27]
Tin (II) chloride	7772-99-8	Not given	22	132	No data
B: Non-carcinogens positive in CA, negative in Ames but no test (or TC) in MLA[d]					
Carbromal	77-65-6	Not given	7	5061	No data
Chlorpheniramine maleate	113-92-8	Not given	18	1279	No data
Fenvalerate	51630-58-1	Not given	20	23.8	No data
Hexachlorocyclopentadiene	77-47-4	Not given	9.5	27.5	No data

NB: There were no chemicals positive in CA, negative in MLA but no test (or TC) in Ames.

[a] Minimum positive response was considered as >5% cells with aberrations (excluding gaps) as this would usually be considered a positive response for CHO, CHL cells and human lymphocytes.

[b] Chemicals that were also positive in MN were excluded from these tables.

[c] Other chemicals in this category, but for which there were insufficient data in the publications to complete any of the columns, were acetohexamide, benzoin, FD&C Red number 3 and tetracycline HCl.

[d] Other chemicals in this category, but for which there were insufficient data in the publications to complete any of the columns, were sodium benzoate, caffeine, chlorpropamide, FD&C Yellow number 5 and vinyl toluene.

Adduct data were found for only a small number of chemicals. However, the following comments can be made:

- Of the carcinogens that were positive only for CA, there were only four with adduct data, and one of these (atrazine) did not form DNA adducts. Whereas we might have expected DNA-reactive chemicals to be positive at lower concentrations, the non-DNA-reactive atrazine was positive at the lowest concentration of all the 13 carcinogens studied in this set.
- Of the carcinogens with adduct data the maximum CA response was quite variable (13–33%) as was the lowest effective concentration (5–67 μM).
- Only one non-carcinogen had adduct data (which was positive).

Since all except one CA-positive carcinogen and the one CA-positive non-carcinogen induced DNA adducts, it is not possible to draw conclusions regarding DNA reactivity and the other parameters we studied (magnitude of response, or lowest effective concentration). Thus it does not appear that the specificity of the CA test can be improved, without serious deterioration in sensitivity, by changing the maximum required test concentration or setting a limit for magnitude of response.

3. Tumour profiles

We reviewed the tumour profiles as detailed in the Carcinogenic Potency DataBase (CPDB) of Gold [7] for those carcinogens giving positive results across all three *in vitro* tests (Table 5) or giving negative results across all three *in vitro* tests (Table 6). The prevalence of the trans-species, trans-sex and multi-site carcinogens amongst the two categories of results we examined (positive or negative across all three *in vitro* tests) is shown in Table 7A. The prevalence of chemicals showing "all positive" or "all negative" amongst the single-species, single-sex, single-site carcinogens is shown in Table 7B. The following conclusions can be drawn from these analyses:

- There are no differences between "all positive for genotoxicity" and "all negative for genotoxicity" amongst the carcinogens as far as inducing tumours in both males and females. In other words, trans-sex carcinogens are equally likely to be genotoxic in all *in vitro* tests as to be non-genotoxic in all *in vitro* tests.
- Trans-species carcinogens are 39% more prevalent in the "all positive for genotoxicity" group than in the "all negative for genotoxicity" group.
- Multi-site carcinogens are 31% more prevalent in the "all positive for genotoxicity" group than in the "all negative for genotoxicity" group.
- Carcinogens showing the combination of trans-species, trans-sex and multi-site tumour profile are much more prevalent (70% more) in the "all positive for genotoxicity" group than in the "all negative for genotoxicity" group.
- Single-species, single-sex, single-site carcinogens are not very prevalent even amongst those chemicals giving three negative results *in vitro*.

We also reviewed the tumour profiles for carcinogens giving positive results in only one of the *in vitro* genotoxicity tests whilst being negative in the other two tests in the battery. The results are shown in Table 8A for Ames-positive only, Table 8B for MLA-positive only, and Table 8C for MN- or CA-positive only. The prevalence of trans-species, trans-sex or multi-site carcinogens amongst these compounds positive in a single genotoxicity test is shown in Table 9. The following conclusions can be drawn:

- Multi-site carcinogens are surprisingly prevalent amongst those chemicals inducing only single positive genotoxicity results.
- Multi-site carcinogens are more prevalent amongst Ames-positive genotoxins, but the database is very small.
- The prevalence of trans-species or trans-sex or multi-site carcinogens amongst single genotoxicity test positive compounds is intermediate between those in "all positive" and "all negative" groups.

4. Relative predictivity for batteries with mixed positive and negative results

Because we did not observe any obvious changes to protocols for the mammalian cell tests that would improve specificity without impairing sensitivity, we revisited the topic of relative predictivity described in our previous paper [1] and we calculated relative predictivity (RP) for rodent carcinogenicity from different combinations of test results. As described previously, positive RP for predicting carcinogenicity was acceptable (>2) only for the Ames test of the single tests, but was most informative when all three tests were positive. We also calculated the RP for non-carcinogenicity from one, two or three *in vitro* tests all giving negative results. Only a few combinations of all negative results gave acceptable RP for non-carcinogenicity.

Table 5
Tumour profiles for carcinogens (tested in rats and mice, males and females) positive in Ames plus MLA plus either MN or CA

Carcinogen	CAS number	Tumour profile		
		Rats and mice	Males and females	Multi-site
N-Acetoxy-2-acetylaminofluorene	6098-44-8	Yes	Yes	Yes
2-Acetylaminofluorene	53-96-3	Yes	Yes	Yes
2-Aminoanthracene[a]	613-13-8	Yes	Yes	Yes
2-Aminofluorene[a]	153-78-6	Yes	Yes	Yes
2-Amino-4-nitrophenol	99-57-0	No	No	No
4-Amino-2-nitrophenol	119-34-6	No	No	No
2-Amino-5-nitrothiazole	121-66-4	No	No	Yes
5-Azacytidine	320-67-2	Yes	Yes	Yes
Benzo[a]pyrene	50-32-8	Yes	Yes	No[b]
Benzyl chloride	100-44-7	Yes	Yes	Yes
bis(2-Chloro-1-methylethyl)ether, technical grade	108-60-1	No	Yes	Yes
Calcium chromate	13765-19-0	Yes	Yes	Yes
Captan	133-06-2	Yes	Yes	Yes
Chlorodibromomethane	124-48-1	No	Yes	No
3-(Chloromethyl)pyridine HCl	6959-48-4	Yes	Yes	No
C.I. Disperse blue 1	2475-45-8	No	Yes	No
C.I. Disperse orange 2 (1-amino-2-methyl-anthrquinone)	82-28-0	Yes	Yes	Yes
Cyclophosphamide monohydrate	6055-19-2	Yes	Yes	Yes
Cytembena	21739-91-3	No	Yes	Yes
2,4-Diaminoanisole sulphate	39156-41-7	Yes	Yes	Yes
2,4-Diaminotoluene	95-80-7	Yes	Yes	Yes
1,2-Dibromo-3-chloropropane	96-12-8	Yes	Yes	Yes
1,2-Dibromoethane	106-93-4	Yes	Yes	Yes
Dichloroacetic acid	79-43-6	Yes	Yes	No
Dichloromethane	75-09-2	Yes	Yes	Yes
2,6-Dichloro-*p*-phenylenediamine	609-20-1	No	Yes	No
1,2-Dichloropropane	78-87-5	No	Yes	No
Dichlorvos	62-73-7	Yes	Yes	Yes
Diglycidyl resorcinol ether, technical grade	101-90-6	Yes	Yes	No
3,3′-Dimethylbenzidine	119-93-7	Yes	Yes	Yes
Dimethyl hydrogen phosphite	868-85-9	No	No	Yes
Epichlorohydrin	106-89-8	No	Yes	No
1,2-Epoxybutane	106-88-7	No	No	Yes
Ethyl methanesulphonate	62-50-0	Yes	Yes	Yes
Formaldehyde	50-00-0	Yes	Yes	Yes
Furylfuramide (AF-2)	3688-53-7	Yes	Yes	No[b]
Glycidol	556-52-5	Yes	Yes	Yes
Hydrazine sulphate	10034-93-2	Yes	Yes	Yes
Methylazoxymethanol acetate	592-62-1	No	Yes	Yes
4,4′-Methylenedianiline 2HCl	13552-44-8	Yes	Yes	Yes
N-Methyl-*N*′-nitro-*N*-nitrosoguanidine	70-25-7	Yes	Yes	Yes
2-Naphthylamine	91-59-8	Yes	Yes	No[b]
o-Nitroanisole	91-23-6	Yes	Yes	Yes
5-Nitro-2-furaldehyde semicarbazone (AKA Nitrofurazone)	59-87-0	Yes	No	Yes
1-[(5-Nitrofurfurylidene)amino]hydantoin (AKA Nitrofurantoin)	67-20-9	Yes	No	No[b]
2-Nitro-*p*-phenylenediamine	5307-14-2	No	No	No
4-Nitroquinoline-*N*-oxide	56-57-5	Yes	Yes	Yes
N-Nitrosodimethylamine (dimethylnitrosamine)	62-75-9	Yes	Yes	Yes
p-Nitrosodiphenylamine	156-10-5	Yes	No	No
4,4′-Oxydianiline	101-80-4	Yes	Yes	Yes
Phenobarbital	50-06-6	No	Yes	No
Beta-Propiolactone	57-57-8	Yes	Yes	No
1,2-Propylene oxide	75-56-9	Yes	Yes	Yes
Quercetin	117-39-5	No	Yes	Yes
p-Quinone dioxime	105-11-3	No	No	No
Selenium sulphide	7446-34-6	Yes	Yes	Yes
Styrene oxide	96-09-3	Yes	Yes	No
1,2,3-Trichloropropane	96-18-4	Yes	Yes	Yes
Zinc dimethyldithiocarbamate (Ziram)	137-30-4	No	Yes	Yes

NT: not tested in that species or sex.

[a] Carcinogens positive in Ames + MLA + MN but not tested or equivocal in CA. All others positive in Ames + MLA + CA.

[b] Single-site tumours only within a species. However, different tumours may have arisen in rats and mice.

Table 6
Tumour profiles for carcinogens negative in Ames plus MLA plus either MN or CA

Carcinogen	CAS number	Tumour profile Rats and mice	Males and females	Multi-site
3-Amino-1,2,4-triazole (Amitrole)	61-82-5	Yes	Yes	Yes
tert-Butyl alcohol	75-65-0	Yes	No[a]	No[b]
5-Chloro-*o*-toluidine	95-79-4	No	Yes	Yes
Decabromodiphenyl oxide	1163-19-5	No	Yes	No
Diethanolamine	111-42-2	No	Yes	Yes
Di(2-ethylhexyl)phthalate	117-81-7	Yes	Yes	No
1,4-Dioxane	123-91-1	Yes	Yes	Yes
DL-Ethionine	67-21-0	Yes	Yes	No
Melamine	108-78-1	No	No	No
Methyl carbamate	598-55-0	No	Yes	No
Nitrilotriacetic acid, trisodium salt, monohydrate	18662-53-8	No	Yes	Yes
N-Nitrosodiphenylamine	86-30-6	No	Yes	No
Progesterone	57-83-0	Yes	Yes	Yes
Pyridine	110-86-1	Yes	Yes	No[b]
Reserpine	50-55-5	Yes	Yes	Yes
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	1746-01-6	Yes	Yes	Yes
1,1,2,2-Tetrachloroethane	79-34-5	No	Yes	No
Tris(2-ethylhexyl)phosphate	78-42-2	No	No	No

By inhalation.
[a] Tumours in male rats but female mice.
[b] Single-site tumours only within a species. However, different tumours may have arisen in rats and mice.

Table 7A
Prevalence of trans-species, trans-sex or multi-site carcinogens amongst consistent positive or negative genotoxicity responses

	Trans-species carcinogens (%)	Trans-sex carcinogens (%)	Multi-site carcinogens (%)[a]	Trans-species, multiple site and trans-sex carcinogens (%)
Positive in Ames plus MLA plus either MN or CA	69.5 (41/59)	83.1 (49/59)	72.9 (43/59)	56 (33/59)
Negative in Ames plus MLA plus either MN or CA	50.0 (9/18)	83.3 (15/18)	55.6 (10/18)	33 (6/18)

[a] Including those carcinogens producing single-site tumours within one species but tumours at different sites in different species.

Table 7B
Prevalence of single-species, single-sex and single-site carcinogens amongst consistent positive or negative genotoxicity responses

	Single-species, single-sex, single-site carcinogens (%)
Positive in Ames plus MLA plus either MN or CA	6.8 (4/59)
Negative in Ames plus MLA plus either MN or CA	11.1 (2/18)

Table 8A
Tumour profile for carcinogens positive in Ames but negative in MLA, MN or CA

Carcinogen	CAS number	Tumour profile		
		Rats and mice	Males and females	Multi-site
D&C red 9	5160-02-1	No	No	Yes
Trifluralin, technical grade	1582-09-8	No	No	Yes
Urethane[a]	51-79-6	Yes	Yes	Yes

[a] There are a number of reports that urethane is negative in the Ames test. As noted in our previous paper [1] we decided to accept the deliberations of Zeiger [28] for this test with urethane.

Table 8B
Tumour profile for carcinogens (with results in rats and mice, males and females) positive in MLA but negative in Ames, MN or CA

Carcinogen	CAS number	Tumour profile		
		Rats and mice	Males and females	Multi-site
11-Aminoundecanoic acid	2432-99-7	No	No	Yes
Benzofuran	271-89-6	Yes	Yes	Yes
o-Benzyl-*p*-chlorophenol	120-32-1	No	No	No
Butylated hydroxytoluene	128-37-0	No	No	Yes
Chlorobenzilate	510-15-6	No	Yes	No
Chloroform	67-66-3	Yes	Yes	Yes
C.I. Direct blue 218	28407-37-6	Yes	Yes	No[a]
Cinnamyl anthranilate	87-29-6	Yes	Yes	Yes
Dicofol	115-32-2	No	No	No
N,N'-Diethyl-2-thiourea	105-55-5	No	Yes	No
Ethylbenzene	100-41-4	Yes	Yes	Yes
Kepone (AKA Chlordecone)	143-50-0	Yes	Yes	No
Malonaldehyde sodium salt	24382-04-5	Yes	Yes	Yes
Methyl *tert*-butyl ether	1634-04-4	Yes	Yes	Yes
Piperonyl butoxide	51-03-6	Yes	Yes	No
Piperonyl sulphoxide	120-62-7	No	No	No
Toluene	108-88-3	No	Yes	Yes
Trichloroethylene (with and without epichlorhydrin)	79-01-6	Yes	Yes	Yes
2,4,6-Trichlorophenol;	88-06-2	Yes	Yes	No[a]
Trimethylthiourea	2489-77-2	No	No	No

[a] Single-site tumours only within a species. However, different tumours may have arisen in rats and mice.

Table 8C
Tumour profile for carcinogens (with results in rats and mice, males and females) positive in MN or CA but negative in Ames and MLA

Carcinogen	CAS number	Tumour profile		
		Rats and mice	Males and females	Multi-site
DDT	50-29-3	Yes	Yes	Yes
17-β-Estradiol	50-28-2	Yes	Yes	Yes
Nitrilotriacetic acid	139-13-9	Yes	Yes	Yes
Oxazepam	604-75-1	No	Yes	Yes
Pentachloronitrobenzene	82-68-8	No	No	No
Saccharin, sodium[a]	128-44-9	No	No	No
Zeralenone	17924-92-4	No	Yes	Yes

[a] Sodium saccharin induces bladder tumours via chronic irritation from the formation of precipitates at high doses, and the CA positive response was only obtained at concentrations >10 mM, so it is unlikely these effects are connected.

Table 9
Prevalence of trans-species, trans-sex or multi-site carcinogens amongst single-positive genotoxicity responses

	Trans-species carcinogens (%)	Trans-sex carcinogens (%)	Multi-site carcinogens (%)[a]	Trans-species, multiple site and trans-sex carcinogens (%)
Positive Ames only	33.3 (1/3)	33.3 (1/3)	100 (3/3)	33.3 (1/3)
Positive MLA only	55.0 (11/20)	70.0 (14/20)	60.0 (12/20)	45.0 (9/20)
Positive MN or CA only	42.9 (3/7)	71.4 (5/7)	71.4 (5/7)	42.9 (3/7)
Positive in any single test but Negative in the other two	50.0 (15/30)	66.7 (20/30)	66.7 (20/30)	43.3 (13/30)

[a] Including those carcinogens inducing single-site tumours within one species, but inducing tumours at different sites in different species.

Table 10A
Numbers of carcinogens and non-carcinogens with 2/3 results positive or negative[a]

	Ames plus MLA plus MN (%)	Ames plus MLA plus CA (%)
Carcinogens		
2/3 Tests positive	16/50 (32.0)	51/176 (29.0)
2/3 Tests negative	6/50 (12.0)	30/176 (17.0)
Non-carcinogens		
2/3 Tests positive	8/12 (66.7)	22/76 (28.9)
2/3 Tests negative	0/12 (0)	22/76 (28.9)

[a] E and TC results excluded from analysis (counted as blank = no result).

Table 10B
Relative predictivity of carcinogen/non-carcinogen status when 2/3 results indicative (data from Table 10A)

Combination of three tests	Chemicals positive in 2/3 tests (%)		Relative predictivity that 2/3 positive results indicate a carcinogen A/B	Chemicals negative in 2/3 tests (%)		Relative predictivity that 2/3 negative results indicate a non-carcinogen C/D
	Carcinogens (A)	Non-carcinogens (B)		Non-carcinogens (C)	Carcinogens (D)	
Ames plus MLA plus MN	32.0	66.7	0.48	0	12.0	0
Ames plus MLA plus CA	29.0	28.9	1.00	28.9	17.0	1.70

As a battery of three *in vitro* tests most often gives a mixture of positive and negative results, we decided to calculate RP for predicting carcinogenicity when two tests were positive but one negative, and to calculate RP for predicting non-carcinogenicity when two tests were negative but one positive. The numbers of results falling into each category are shown in Table 10A and the RP values in Table 10B. It can be seen that in all cases the RP values are less than the meaningful value of two, and in some cases are <1, which means there would be a better chance of predicting the outcome of the carcinogenicity studies by flipping a coin.

As all of the RP values were worse for 2/3 tests than for 3/3 tests, we decided not to analyse further the RP from 1/3 tests.

5. Discussion and conclusions

Although limited data were available, our analyses of chemicals giving isolated positive genotoxicity results in one of the mammalian cell tests, suggest that revising the requirements for toxicity limits or highest test concentration, or to apply some "threshold" response that must be exceeded, would improve the poor specificity of the MLA and CA tests, but would at the same time significantly impair sensitivity. An examination of DNA reactivity within these small groups did not suggest any associations between DNA reactivity and level of toxicity or concentration needed for a minimum positive response, or the magnitude of the genotoxic response. It is possible that if we examine all MLA and CA positives, and view separately those carcinogens known to interact with DNA, and/or known to have structural alerts, we may find that the "profile" of the genotoxicity results (concentration, level of toxicity, magnitude of response) would be more useful in predicting carcinogenic outcome. This is currently being explored and will be the subject of a future manuscript.

An analysis of tumour profiles indicated that trans-species, trans-sex and multi-site carcinogens are much more prevalent amongst those chemicals giving positive results in all three *in vitro* genotoxicity tests than amongst those giving negative results in all three tests. This is consistent with the relative predictivity (RP) analysis we reported previously [1] where chemicals giving positive results in all three *in vitro* tests would be at least 3× more likely to be rodent carcinogens than non-carcinogens. However, unexpectedly, single-species, single-sex and single-site carcinogens were not prevalent amongst those chemicals giving negative results in all three *in vitro* tests. This suggests that non-genotoxic carcinogens are just as likely to produce single-site tumours in one sex of one species as to produce multi-site tumours in both sexes of rats and mice. It was also surprising that multi-site carcinogens were highly prevalent amongst those chemicals positive in one but negative in the other two genotoxicity tests. Multi-site carcinogens were particular prevalent amongst Ames-positive chemicals, but the

database was small and further examples are needed for a rigorous evaluation. Thus, it appears that the profile of genotoxicity results cannot be used with any confidence to predict the tumour profile in subsequent carcinogenicity studies. This is consistent with a more modern view that mutational events and chromosomal rearrangements are, although required for tumourigenesis, not a major driving factor for prevalence and incidence of tumours in lifetime rodent carcinogenicity tests with chemical compounds [8]. In such long-term experiments, hormonal disturbances, genetic strain background, general tumour promotional effects, etc. are very important. Such effects may equally be exerted by many of the investigated chemicals that also possess genotoxic properties.

Although our previous RP analysis [1] indicated that chemicals giving positive results in all three *in vitro* tests were >3× more likely to be rodent carcinogens than non-carcinogens, and that chemicals giving all negative results were >2× more likely to be non-carcinogens than carcinogens, in industry mixed positive and negative results are most common. As might be expected, RP analysis of "mixed" genotoxicity results revealed that these cannot be used to predict carcinogenic outcome. It should be noted that predictivity analyses are dependent on the choice of compounds analysed and influenced by the prevalence of carcinogens and non-carcinogens in the data set. Different predictivities from those presented here could be obtained with different numbers of carcinogens and non-carcinogens in the data set. Also, only some of the mechanisms (e.g. genotoxic) giving rise to cancer are the same as those leading to mutations and chromosomal aberrations, and non-genotoxic mechanisms would not be expected to be predicted by genotoxicity assays. Therefore, the predictivity of genotoxicity tests for cancer will depend on the mechanism.

As indicated previously [1], the disappointing findings from these analyses suggest it may be time for a complete rethink with regard to *in vitro* genotoxicity testing. It does not appear that a redefinition of the conditions (cytotoxicity, solubility, etc.) for *in vitro* testing will improve the low specificity for prediction of rodent tumourigenesis, and therefore new, more robust assays may be needed. We should also consider, however, that the rodent bioassay is not relevant for predicting human carcinogenicity in many cases, and therefore new, more robust carcinogenicity assays may also be needed.

Regarding the availability of complete data sets, or of critical information (e.g. cytotoxicity) related to single-assay results, the authors are astonished that only small subsets of chemicals have full data sets. Hence, despite more than 30 years of genotoxicity testing, the published literature still contains large data gaps. These gaps certainly do not facilitate far-reaching and important conclusions on correlations between important parameters. Thus in many cases our only recourse is to some kind of patchwork analysis when working with published data. As expected, most adduct data have been reported for the carcinogens and there are very few studies of adducts formed by non-carcinogens.

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

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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.

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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

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 Micro-Flow^{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

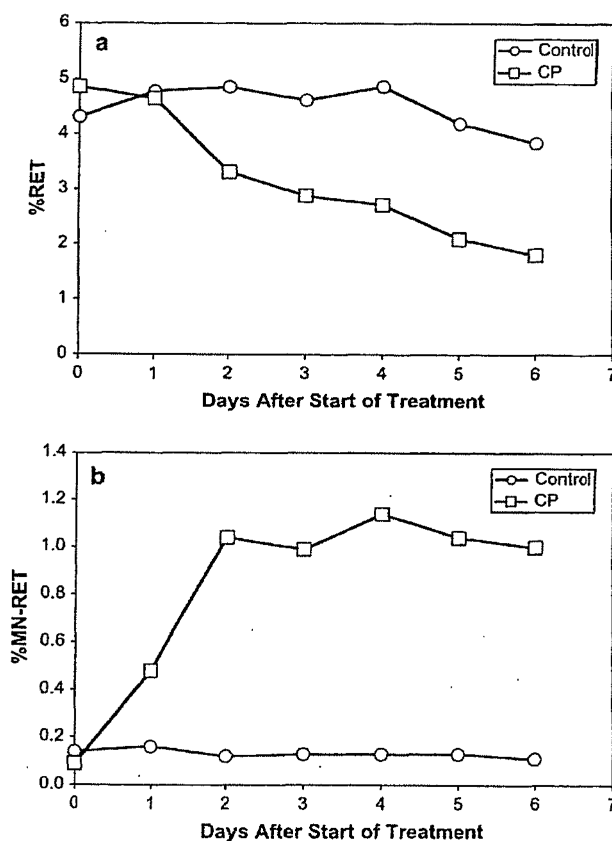

FIG. 1. The frequency of peripheral blood RETs (%RET, panel a) and peripheral blood micronucleated RETs (%MN-RET, panel b) as a function of time in the individual rats used to generate reference samples for analytical comparison. These data were acquired by flow cytometric analysis (laboratory L2) and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

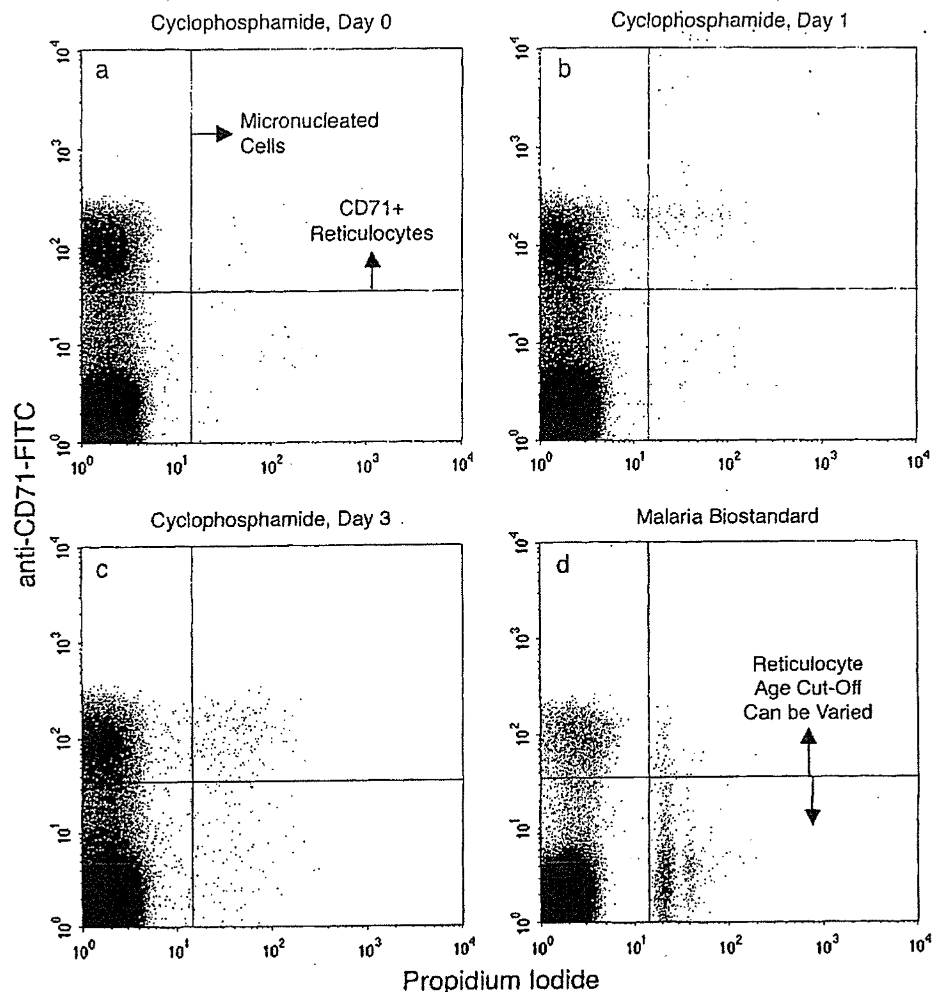

FIG. 2. Panels (a–c): Bivariate graphs illustrate the staining characteristics of rat blood specimens over the course of several days of CP treatment. Note the appearance of micronuclei at Day 1 in the very youngest (highest anti-CD71-FITC fluorescence) RETs (Panel b) and the more uniform distribution 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.