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か）は3～4日で行う。

なお，1992年に採択された最初の方法では，用量は5，50，500，2000 mg/kg，動物数は各用量ごとに見当づけ試験に雌雄いずれか2匹ずつ，主試験では雌雄各5匹（すなわち計12匹）を用いることとされていたが，2001年の改定で上記のように改められた。3種の代替試験法間の相違を調整するためである。

2. TG423：急性毒性等級法（Acute Toxic Class Method）

TG401の別の代替法でドイツから提案されたTG423急性毒性等級法（Schlede *et al.*, 1995）は，やはりラットを用いて固定された用量の単回経口投与によって，結果を見ながら逐次試験する方法である。各段階で用いる動物は3匹，その結果によって上か下に用量を移すか，または同じ用量でもう一度，3匹での試験を繰り返し，その死亡率に応じて次の用量を選択する。従って，1用量段階で用いられる動物は最大6匹となる。固定用量は，5，50，300，2000 mg/kgの4段階（例外的に5000 mg/kgを加えて5段階）である。結果に基づいて化学物質危険度分類を行う。手順の概念をFig. 3に示す。開始用量によって進め方が多少変化する。

この試験法についても，最初の方法では用量は25，200，2000 mg/kgの3段階であったが，2001年の改定でTG420と合わせて変更された。また，最初の方法では，同じ用量での2回の試験は雄雌を順次試験して雌雄差を観察する方法であったが，改訂版では2回とも同じ性（通常雌）で行うことに変更された。

3. TG425：上げ下げ法（Up-and-Down Procedure）

米国は，固定用量法や毒性等級法の採用にあまり積極的でなかったが，それは農薬登録の資料として，LD_{50}とともに用量反応（死亡）関係の勾配のデータを要求していたからであった。米国は，従来法急性毒性試験の代替法として上げ下げ法を提案した。上げ下げ法の考え方は急性毒性試験の簡便法あるいは予備的な試験としてかなり古くからあったが，それをTG401の代替法として適当なように考案され提案されたものである。上げ下げ法の手順では，まず1匹に適当な用量を投与して観察し，その動物での結果によって，次の動物への用量を上か下かに調整する。動物が生存すれば次の動物への用量は上げ，死亡すれば〔次の用量は〕下げる。通常，直前の用量に対して係数（用量進行係数）を用いて（乗除して）次の用量を定めて行く。このように用量を上げ下げして試験を進め，最終的に死亡が起こった用量と動物数からLD_{50}を概算するのである。このTG425では，第1動物には，推定LD_{50}に近く，それより低い用量を投与し，その結果によって48時間以内ごとに1匹ずつに次の用量を投与していく。各動物の観察は死亡または14日まで続ける。最初からの連続した結果（生存か死亡か）が反転するとき，すなわち用量の上昇（または下降）の連続が逆転するとき，からさらに4匹の動物について同様の手順に従って試験した上で，最尤法に従ってLD_{50}を求める。通常雌動物のみを用いる。上げ下げ法の概要をFig. 4に示す。

用量進行係数は，用量反応曲線の勾配に従って最適値が異なるが，通例試験前にそのような情報を得られることはないので，勧告値を用いる。1998年に最初提案された方法では，用量進行係数は1.3とされていたが，2001年には係数3.2で用量を増減することに改められた。これは勾配が2の用量反応に相当する。また，提案された最初の方法では用量反応曲線の勾配も求めることとされていたが，改訂版ではなくなった。

急性毒性試験の目的：化学物質の分類・表示

これら3種の急性経口毒性試験代替法の主要な目的は，化学物質分類（および表示）にある。従来どの分類基準もLD_{50}の数値に基づいて作られていたから，すべての化学物質についてLD_{50}値は必要であった。これらの代替法はその目的を意識して作られており，その試験法としての有用性確認（validation）は，化学物質分類の一致率を評価の基準にしていた。しかし，それぞれ当時の自国の基準（たとえば欧州共同体分類）と比較したので，万国共通ではなかった。この問題については別途国際的に分類基準を統一する作業が国際連合を中心にして行われ，1998年に「万国共通分類法 Globally Harmonized Classification System for Chemical Substances and Mixtures (GHS)」(OECD, 1998) がまとめられた。そこで，これらの3種の代替

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法についてもいずれでもGHS.に従った化学物質分類が出来るように固定用量の修正等が行われたのである。動物操作の記載の統一も行われた。TG425に関して，LD_{50}のほかに用量反応関係の勾配の算定を求めていた米国環境保護庁の規制は変更され，試験法の簡略化もさらに進められた。その上で，OECD理事会は，TG401を廃棄し，急性経口毒性試験はTG420, 423, 425のいずれかによるべきことを決定した。

ちなみにGHSによる化学物質分類は，急性経口毒性に関してはTable 1のように定めている。各国は

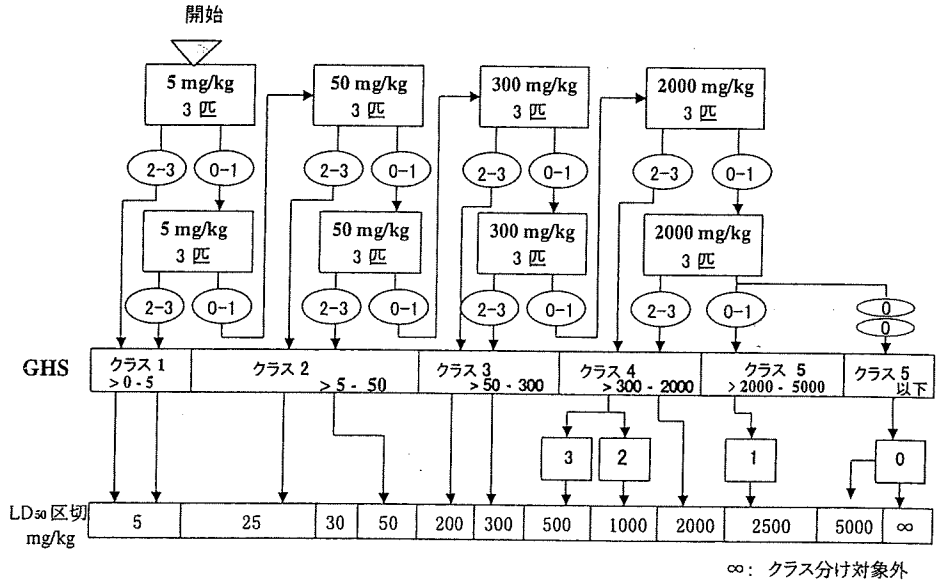

Fig. 3-1. Flowchart of Acute Toxic Class Method (TG423) : Starting at a dose of 5 mg/kg.

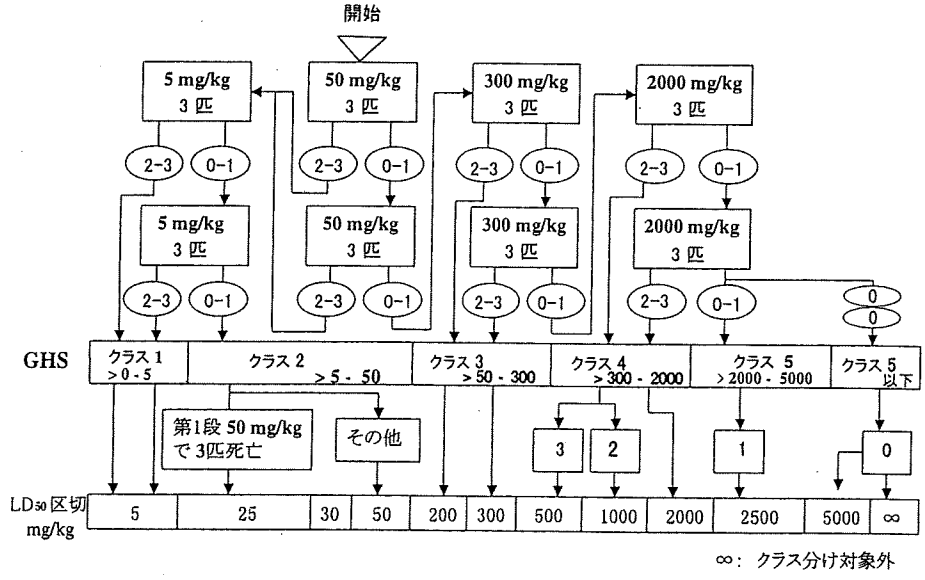

Fig. 3-2. Flowchart of Acute Toxic Class Method (TG423) : Starting at a dose of 50 mg/kg.

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この分類法に従って化学物質の有害度表示を行うことになるが，法的規定の常として対応にはある程度の時間を要するので，しばらくの間各国では従来の分類法が使われる見込みである。しかし，そのためにLD50を存続させることには強い抵抗があり，LD50の廃絶を先行させることとされた。3種の代替法は従来の分類法にも対応できるように「指針」(OECD, 2000) に解説されている。わが国における「毒物劇物取締法」では，従来LD50値を重視しており，他に問題がなければ，経口LD50 30 mg kg以下の物質を毒物，30～

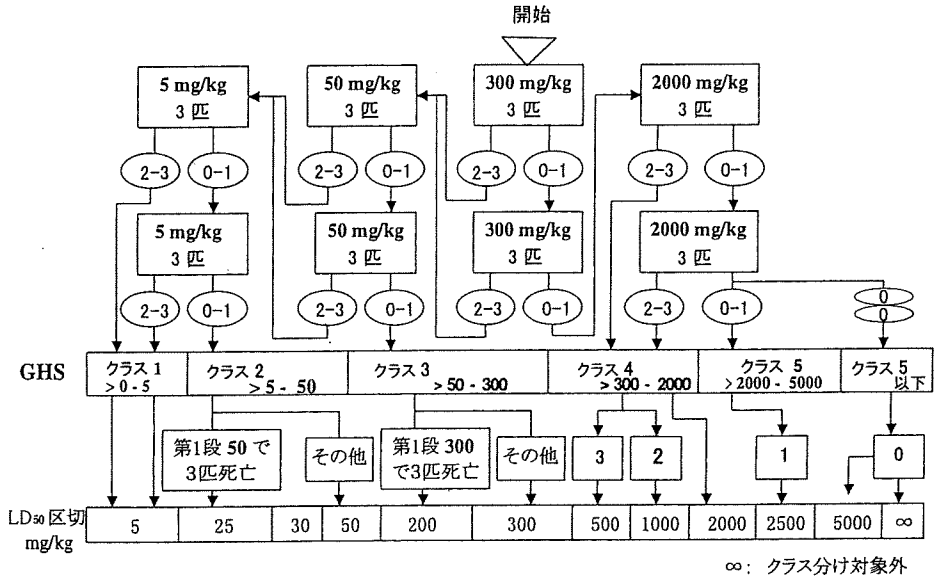

Fig. 3-3. Flowchart of Acute Toxic Class Method (TG423): Starting at a dose of 300 mg/kg.

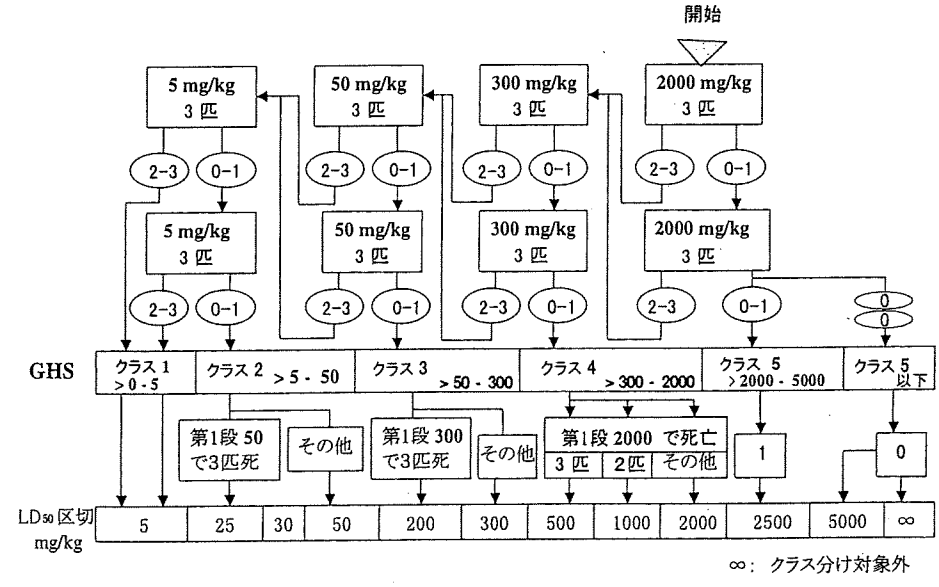

Fig. 3-4. Flowchart of Acute Toxic Class Method (TG423): Starting at a dose of 2000 mg/kg.

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300 mg/kg の物質を劇物と分類してきた。これを国際的な動向に合わせて，修正していく必要があり，現在，30 mg/kg の区切りを 50 mg/kg に変更する方針が打ち出されている。(毒物および劇物の数量的基準は法律で規定されたものではないので、法改正は必要ない。)

急性経口毒性試験代替法の問題点

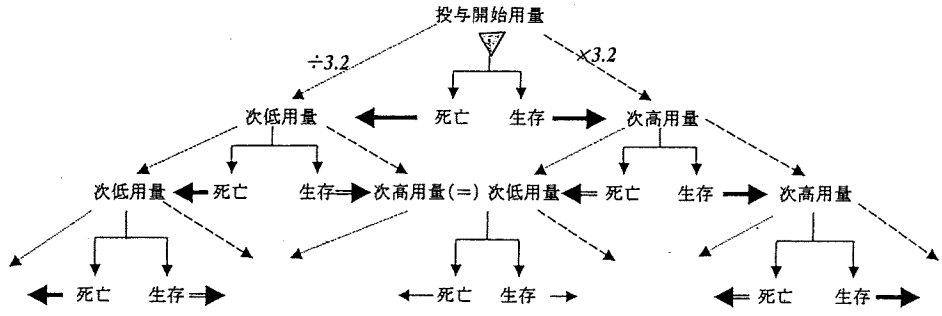
今回の毒性試験法の改定は相当に大きな変更であり，従来の毒性学の常識に挑戦するところも少なくない。今後これらの代替法に従って試験を行う必要も増えるであろうが，変更点を理解し，要求された手順に従うように注意して取り掛かるべきである。

1. 毒性の指標，試験の目標 (endpoint)

3つの代替法のうち TG423 と TG425 はまだ動物の死亡を毒性の指標にしているが，TG420 は動物が死亡しない用量での毒性徴候を指標にして急性毒性（単回投与毒性）を試験するものである。化学物質の毒性について致死量まで確認する必要があるかと問われれば，確かにその必要はないと言える。どのような化学物質でも大量の曝露を受ければ死亡する危険があると思っていればよいのであって，死亡することを確認する必要はない。TG420 に限らず，3 代替法はいずれも，重篤な毒性症状を示す動物は死亡を確認するまで待つことなく，人道的に屠殺することを求めている。しかし，従来急性毒性試験で致死量を求めていたのは，死亡が化学物質に共通の究極の毒性徴候であり，高度の技術的訓練を必要とせずに確認できる指標だからである。死亡以前に被験物質の「明らかな毒性」を的確に観察する能力を身に付けるには相当の熟達を要する。観察力と判断力つまり診断力が求められる。毒性徴候の標準化が望ましいが当分個々の毒性学者の判断に期待するしかない。LD_{50} 廃止の議論は LD_{50} の不確かさから始まったことであるが，その原因となる多数の変動要因（動物の個体差，飼育管理条件，被験物質調製法，安定性，手技の相違）の上に症状観察の個人差が加わると，急性毒性の定量的な確実さはますます失われることになろう。しかし，それでも固定用量の程度 (6〜10 倍) の誤差がなければ実用的に差し支えないということである。

2. 逐次試験法の問題

3 代替法はいずれも逐次試験法であり，各用量段階ごとに結果を確認して次の段階に進む手順となっている。毒性による死亡は必ずしも即死ではなく，非致死毒性の徴候にしても投与直後に発現するものばかりではないので，結果を出すまでには時間が掛かる。各用量段階の間に 2〜4 日の間隔が必要である。遅発毒性を有するいわば例外的な化学物質（一部の有機リン化



→ : 用量進行

⟹ : 用量逆転

3.2 : 用量進行係数

試験終了規定: (1) 2000 mg/kg で 3 連続生存

(2) 6 回の試行で 5 回の逆転

(3) 初回逆転から 4 試行以上で尤度条件を満足

Fig. 4. Concept of Up-and-Down Procedure (TG425).

合物，グルココルチコイド）の場合はきわめて長期間を要することになる。TG425には，そのような物質はこの試験には向かない，と記述されている。また，TG425の用量進行係数の選び方，各試験法の初回開始用量の選び方によって多数の段階を経過することが必要になり，試験期間はきわめて長期化する。試験期間が長期化した際の技術的問題は，動物の均一性が保たれなくなることである。動物の体重変化を予想して，これらの代替法では，ラットならば8～10週齢の動物を用いることと規定している。この週齢であれば，通常毒性試験に用いられている4～6週齢のラットに比べ，体重の増加は日を追って急増することはなく，全投与作業が2週以内に終われば用量段階ごとの動物の体重の差は無視できると期待されている。

逐次試験の長期化のもう一つの問題は，被験物質の溶液中での安定性と均一性である。試験が長期に及ぶ時，試験の初めに調製した被験物質溶液を最後まで使い続けるには，溶液中での安定性が保証されなければならない。試験の開始時に試験の終了時期を予想することは（再試験でない限り）不可能であるから，あらかじめその期間の安定性を確認しておくことなど不可能である。従って，投与の都度溶液を調製する必要がある。すると，各回の調製溶液の同一性を含量試験によって確認しなければならないことになる。つまり，長期投与試験と同じような配慮が必要になるのである。この点は技術的に不可能という問題ではないが，非常に煩瑣な手間のかかる作業であり，得られる情報の程度と見比べると，過剰な作業であると思われる。

3. 雌雄差の観察を放棄したこと

3つの代替法はいずれも一方の性，主として雌を用いて試験することを規定している。その理由は使用動物数の削減にあるが，変更の根拠として，元来毒性の雌雄差というものはほとんどなく，いずれの性を用いてもほぼ同じ結果が得られるが，差があるとすれば，雌の方が感受性が高いことが多い，という調査(Lipnick *et al.*, 1995）があることによっている。雌より雄の方が感受性が高い場合も少なくないと予想されるが，その場合は雄を選択して片性で試験することとされている。どのようにしてそうした判断を事前に下せるのか，については一定の方策はない。これは建前なのである。

毒性試験の常識では，毒性の雌雄差の有無を調べることは不可欠の手順であった。従来のLD_{50}も雌雄両性について求めるのが常であったが，それが実際活用されていたとはいえない。LD_{50}による化学物質分類が雌雄別々になされるということはなかった。雄では毒物，雌では劇物というような扱いは（あり得たにも拘らず）存在しなかった。RTECSなど化学物質の毒性資料の記載でも，通例雌雄を分けてLD_{50}を記載することはなく，毒性の強い方のデータをその物質のLD_{50}と記載していた。毒性の雌雄差は重要な毒性情報であるという毒性学者の意識がある一方で，雌雄のLD_{50}の差は規制の上では無視されていたのである。

Table 1. Globally Harmonized Classification System.

急性毒性	Class 1	Class 2	Class 3	Class 4	Class 5
経口 (mg/kg)	5	50	300	2000	5000
経皮 (mg/kg)	50	200	1000	2000	試験実施の条件： ・ヒトにおける有意な影響の情報あり ・Class 4で死亡あり ・Class 4で相当の毒性徴候あり ・他の試験の情報から
吸入*					
ガス (ppm)	100	500	2500	5000	
蒸気 (mg/L)	0.5	2.0	10	20	
粉塵/煙霧 (mg/L·4hr)	0.05	0.5	1.0	5	

*4hr曝露の条件

つまり雌雄両性について急性毒性試験を行うことは実際的には無駄であったのだ。この認識はすでに1987年のTG401改定のときに取り入れられていた。しかし多くの試験機関でその変更は無視され，雌雄両性について試験を行い，LD_{50}を算定することが続けられていた。一部の行政が雌雄の動物についてのLD_{50}を要求していた事情もあるが，出来るだけ詳しい情報を提供することが毒性学者の使命であると信じてしたことでもあったに違いない。

ここで心配されるのが，雌の優先的使用による雄の余剰動物の発生である。生殖毒性試験で雌を多用することも合わせ，雛鳥生産のような状況が懸念される。しかし，この点に関して，動物繁殖業界から(なぜか)そのようなことはないという回答が得られている(OECD, 2000)。

おわりに

これまで，LD_{50}は毒性学の基本概念であり続け，認定トキシコロジストの認定試験でも必ず出題されてきた。それはわが国のみの事情でもなく，Casarett & Doull's Toxicology (6th ed., 2001) やHayesのPrinciples and Methods of Toxicology (4th ed., 2001) など著名な毒性学教科書のどれもが，依然としてLD_{50}を軸にした毒性学総論を繰り広げており，LD_{50}なしには毒性学は語れない状態は続いていくのではあるまいか。一方，急性毒性試験の代替法については，従来ほとんど記載されておらず，Hayesの新版には記載されたが，固定用量は（改訂の時期からして無理もないが）旧ガイドラインのままである。代替法は，実際の毒性試験の現場では重要な問題であり，学問の立場からもこれを理論的に援護していく必要がある。

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Relevance of chemical structure and cytotoxicity to the induction of chromosome aberrations based on the testing results of 98 high production volume industrial chemicals

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Abstract

Over a 6-year period (1991–1996), the chromosomal aberration testing of high production volume (HPV) industrial chemicals had been conducted using Chinese hamster lung (CHL/IU) cells according to OECD HPV testing program and the national program in Japan. A total of 98 chemicals were tested for the induction of chromosome aberration (CA), consisting of structural CA and polyploidy. Of the 98 chemicals, structural CA and/or polyploidy were induced by 39 chemicals (40%). Anilines and phenols tended to induce only structural CA. *p-tert*-Butylphenol had a peculiar feature in inducing not only structural CA but also polyploidy at considerably high frequency (93.2%) after continuous treatment for 48 h, posing an aneugenic potential. Not all, but six of 11 carboxylic acids or esters also showed the simultaneous induction of structural CA and polyploidy. The majority of organic phosphates, alcohols or ethers, alkyl benzenes and non-cyclic alkanes had no CA induction activity. For chemicals which were negative in the bacterial reverse mutation assay (Ames test), the proportion of the chemicals that induced CA at a severely cytotoxic dose (doses manifesting more than 50% cytotoxicity) was similar to that of the CA-negative chemicals manifesting severe cytotoxicity, suggesting that severely cytotoxic chemicals do not always induce CA.

Keywords: CHL/IU cells; Chromosome aberrations; Cytotoxicity

1. Introduction

It is estimated that 70,000–80,000 industrial chemicals may be present in our daily lives. However, the majority have not been evaluated for human health safety. Under the OECD high production volume (HPV) Testing Program, 467 out of about 1600 chemicals with HPV have insufficient safety data and were prioritized for safety testing. Chromosomal aberration tests of the HPV industrial chemicals have been conducted using Chinese hamster lung (CHL/IU) cells to examine the induction of chromosome aberration (CA) including structural CA and polyploidy.

Study of the relationship between chemical structure and the induction of CA is an important aspect for predicting the safety of HPV industrial chemicals.

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In this paper, the chemicals tested and published from 1992 to 1998 [1–6] through the Japanese Chemicals Investigation Promoting Committee, were classified based on their chemical structures to determine whether a particular structure correlated with CA induction. Furthermore, it was determined if the CA-inducing chemicals could be structurally categorized as "clastogen" (attack on DNA) or "polyploid inducer" (attack on mitotic apparatus).

In the chromosomal aberration test using CHL/IU cells, maximum doses are usually set at doses manifesting more than 50% cytotoxicity according to OECD guideline [7]. However, since severe cytotoxicity would be accompanied by the induction of cytotoxicity-dependent CA [8], appropriate levels of cytotoxicity have been previously discussed [9]. Some CA-positive Ames-negative chemicals are suspected to manifest severe cytotoxicity of more than 50% at doses which induce CA [10]. Also, so-called "high toxicity clastogens" (HTC), which only induce CA at severely cytotoxic doses, are regarded as less biologically relevant [11]. To discuss the relevancy of severe cytotoxicity to the induction of CA, we especially dealt with the chemicals known to be negative in bacterial reverse mutation assay (Ames test). Then, the numerical proportions of weakly cytotoxic (50% or less) and severely cytotoxic (more than 50%) chemicals were compared between CA-positive and CA-negative groups of the Ames-negative chemicals.

2. Materials and methods

2.1. Cells

CHL/IU cells used in all CA tests were obtained from Japanese Cancer Research Resources Bank (JCRB). The cells were cultured with Eagle's minimum essential medium (MEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum or calf serum. The 2×10^4 cells were plated in 5 ml medium on 60 mm plate and cultured for 72 h at 37 °C in a humidified incubator (5% CO_2) before the treatment with chemicals.

2.2. Chemicals tested

All chemicals tested are listed in Table 1 . These were supplied from several industrial companies in Japan under management by Ministry of Health and Welfare in Japan.

2.3. Chemical treatment

Chemicals were dissolved in water when appropriate. For chemicals insoluble in water at 100 mM or 50 mg/ml, dimethyl sulfoxide (Wako Pure Chemical Industries, Osaka, Japan) or acetone (Wako Pure Chemical Industries, Osaka, Japan) was used to dissolve them to at least 1000 mM or 500 mg/ml. When the chemicals could not be dissolved in these solvents, a 0.5% solution of carboxymethyl cellulose sodium was used as a vehicle to suspend them.

In order to examine the metabolic activation of the chemicals, the proliferating cells were treated with the chemicals for 6 h in serum-free MEM with S9 mix (S9(+)), or without S9 mix (S9(−)), then cultured a further 18 h in the fresh MEM with serum. S9 (Kikkoman, Chiba, Japan) and co-factors were mixed immediately before use, then applied to cultures to expose the cells to the S9 at 5% (v/v) [12]. Moreover, the cells were also treated with the chemicals for 24 and 48 h continuously in the absence of S9 mix. Duplicate cultures were used for each dose.

2.4. Growth inhibition test for cytotoxicity evaluation

Preliminary growth inhibition test was conducted to determine the cytotoxicity of the chemicals. Relative cell confluence or cell number to solvent control was measured with a Monocellater™ (Olympus Optics, Tokyo, Japan) or a Coulter Counter™ (Coulter Electronics, Bedfordshire, UK), respectively. Relative metaphase frequency (relative mitotic index) to the solvent control was also used as an indicator of cytotoxicity when appropriate. A maximum dose was set for the chromosomal aberration test at 50% or more of the cytotoxic dose determined by the growth inhibition test. When cytotoxicity was 50% or less, 5 mg/ml or 10 mM was set as the maximum dose. The other doses were sequential half dilutions.

2.5. Chromosome preparation

Before harvesting, the cells were treated with 0.1 μg/ml of colcemid for 2 h, then chromosome

Table 1
List of 98 HPV industrial chemicals tested

Classification	No.	Chemical name [CAS No.]
Aniline	1	2,3-Dimethylaniline [87-59-2]
	2	3,4-Dimethylaniline [95-64-7]
	3	*N*-Ethylaniline [103-69-5]
	4	*N*-Methylaniline [100-61-8]
	5	4-Nitro-*o*-anisidine [97-52-9]
	6	*m*-Toluidine [108-44-1]
Sulfonic acid	7	4-Methylbenzenesulfonamide [70-55-3]
	8	3-Aminobenzenesulfonic acid [121-47-1]
	9	2-Amino-5-methylbenzenesulfonic acid [88-44-8]
	10	2-Amino-5-chloro-4-methylbenzenesulfonic acid [88-53-9]
logenated benzene	11	1,4-Dibromobenzene [106-37-6]
	12	1,4-Dichloro-2-nitrobenzene [89-61-2]
	13	1,2-Dichloro-3-nitrobenzene [3209-22-1]
	14	2,4-Dichloronitrobenzene [611-06-3]
	15	2,4-Dichloro-1-methylbenzene [95-73-8]
	16	Trifluoromethylbenzene [98-08-8]
Phenol	17	4-Aminophenol [123-30-8]
	18	3-Methyl-4-nitrophenol [2581-34-2]
	19	4-(1-Methylpropyl)phenol [99-71-8]
	20	Thymol [89-83-8]
	21	*p-tert*-Butylphenol [98-54-4]
	22	6-*tert*-Butyl-2,4-xylenol [1879-09-0]
	23	*p-tert*-Octylphenol [140-66-9]
Bisphenol	24	2,2′-Methylenebis(6-*tert*-butyl-*p*-cresol) [119-47-1]
	25	4,4′-Thiobis(6-*tert*-butyl-*m*-cresol) [96-69-5]
Organic phosphate	26	Dibutyl phosphate [107-66-4]
	27	Diphenyl cresyl phosphate [26444-49-5]
	28	Tris(2-ethylhexyl)phosphate [1806-54-8]
	29	Tris(*p*-cymenyl)phosphate [26967-76-0]
	30	Tris(2-butoxyethyl)phosphate [78-51-3]
	31	Trimethyl phosphate [512-56-1]
olycyclic aromatic hydrocarbon	32	Acenaphthene [83-32-9]
	33	2-Amino-1-naphthalenesulfonic acid [81-16-3]
	34	Dimethyl 2,6-naphthalenedicarboxylate [840-65-3]
	35	1-Methoxynaphthalene [2216-69-5]
	36	1-Naphthylacetic acid [86-87-3]
	37	Potassium 7-hydroxy-1,3-naphthalenedisulfonate [842-18-2]
	38	Monosodium 4-amino-5-hydroxy-2,7-naphthalenedisulfonate [5460-09-3]
	39	1-Aminoanthraquinone [82-45-1]
Pigment	40	D & C Red No. 7 [5281-04-9]
	41	Pigment green No. 7 [14832-14-5]
Heterocyclic compound	42	*N*-Cyclohexyl-2-benzothiazolesulfenamide [95-33-0]
	43	*N,N*-Dicyclohexyl-2-benzothiazolesulfenamide [4979-32-2]
	44	*N-tert*-butyl-2-benzothiazolesulfenamide [95-31-8]
	45	Thiophene [110-02-1]
	46	Tetrahydrothiophene-1,1-dioxide [126-33-0]
	47	2-Mercaptobenzimidazole [583-39-1]
Aldehyde	48	2-Hydroxybenzaldehyde [90-02-8]

Table 1 (*Continued*)

Classification	No.	Chemical name [CAS No.]
Alkyl benzene	49	1,3-Bis(aminomethyl)benzene [1477-55-0]
	50	1,2,4-Trimethylbenzene [95-63-6]
	51	1,4-Diethylbenzene [105-05-5]
	52	1-Methylethenylbenzene [98-83-9]
	53	Diisopropylbenzene [25321-09-9]
	54	Divinylbenzene [1321-74-0]
Alcohol or ether	55	Methoxymethanol [4461-52-3]
	56	*N*-(Aminoethyl)ethanolamine [111-41-1]
	57	Diacetone alcohol [123-42-2]
	58	1,4-Butanediol [110-63-4]
	59	3-Methyl-1,5-pentanediol [4457-71-0]
	60	1,2-Butanediol [584-03-2]
	61	2,2-Dimethyl-1,3-propanediol [126-30-7]
	62	2-Ethyl-2-hydroxymethyl-1,3-propanediol [77-99-6]
	63	Propylene glycol monomethyl ether acetate [108-65-6]
	64	Pentaerythritol [115-77-5]
	65	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate [6846-50-0]
	66	Tripropylene glycol [24800-44-0]
	67	3,5,5-Trimethylhexan-1-ol [3452-97-9]
	68	Glycerol triacetate [102-76-1]
	69	Methyl acetoacetate [105-45-3]
Carboxylic acid or ester	70	2,3-Dibromosuccinic acid [526-78-3]
	71	4-Hydroxybenzoic acid [99-96-7]
	72	Dibutyl adipate [105-99-7]
	73	Diethyl fumarate [623-91-6]
	74	2-(Dimethylamino)ethyl acrylate [2439-35-2]
	75	2-Hydroxyethyl methacrylate [868-77-9]
	76	2,3-Epoxypropyl methacrylate [106-91-2]
	77	2-(Dimethylamino)ethyl methacrylate [105-16-8]
	78	2-Hydroxypropyl methacrylate [923-26-2]
	79	Methyl dodecanoate [111-82-0]
	80	1,2,4-Tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate [3319-31-1]
Cyanide	81	2-Hydroxypropanenitrile [78-97-7]
	82	2,2-Azobis(2-methylpropionitrile) [78-67-1]
	83	1,3-Dicyanobenzene [626-17-5]
	84	1,4-Dicyanobenzene [623-26-7]
	85	Isocyanuric acid [108-80-5]
Non-cyclic alkenes	86	Triisobutylene [7756-94-7]
	87	3,4-Dichloro-1-butene [760-23-6]
Non-cyclic alkanes	88	*n*-Pentadecane [629-62-9]
	89	*n*-Hexadecane [544-76-3]
	90	1-Chlorobutane [109-69-3]
	91	Undecane [1120-21-4]
	92	2,2,4,4,6,8,8-Heptamethylnonane [4390-04-9]
Others	93	Dicyclohexylcarbodiimide [538-75-0]
	94	Tetrahydromethyl-1,3-isobenzofuranedione [11070-44-3]
	95	3a,4,7,7a-Tetrahydro-1*H*-indene [3048-65-5]
	96	Dicyclopentadiene [77-73-6]
	97	5-Ethylidene-2-norbornene [16219-75-3]
	98	Ethyl methyl ketoxime [96-29-7]

specimens were made by the usual air-dry method.

2.6. Chromosome analysis

Chromosome specimens were stained with 3% Giemsa solution for 8 min. The number of cells with chromatid- and chromosome-type breaks and exchanges were scored per 200 cells at each dose. Polyploid cells were also scored per 800 cells at each dose. A polyploid cell was defined as a metaphase with more than 37 chromosomes, i.e. theoretical ypertriploid cell", containing approximately three imes the number of chromosomes than half the modal chromosome number (25) in CHL/IU cells.

3. Results and discussion

3.1. Chemical structure and CA-induction

The 98 chemicals tested were classified by their structural similarity (Table 2), and the numbers of chemicals in each category were summarized (Table 3). Of the 98 chemicals, 39 (40%) were judged as positive for the induction of CA (Table 3). These CA-positive chemicals seemed to be non-randomly distributed among the chemical classes and were over-represented in the anilines, sulfonic acids, phenols, polycyclic aromatic hydrocarbons and carboxylic acids or esters (24 of the 39 chemicals, Table 2). However, of the 24 chemicals, three sulfonic acids os. 8–10), two polycyclic aromatic hydrocarbons (Nos. 33 and 36) and one carboxylic acid (No. 71) may have induced CA indirectly by their acidification of the culture medium [13].

The majority of organic phosphates, alkyl benzenes, alcohols or ethers, non-cyclic alkanes tested were negative for the induction of CA (Table 2).

3.2. Anilines

Structural CA was induced by four of six anilines (Nos. 1 and 3–5, Tables 2 and 3). Recently, 2,4-dimethylaniline and 2,4,6-trimethylaniline were reported to induce DNA damage detected by single cell gel electrophoresis assay (Comet assay) in mouse liver cells [14]. 2,3-Dimethylaniline (No. 1) and 4-nitro-*o*-anisidine (No. 5) induced CA in CHL/IU cells with metabolic activation and were Ames-positive [5], representing a similar property to that of *o*-anisidine and its metabolites in the induction of DNA damage [15]. Anilines with a methyl residue located at position 2 in the benzene ring seem to be preferentially active in inducing CAs or gene mutations.

3.3. Phenols

Structural CA was induced by four of seven phenols (Nos. 17, 18, 20 and 21) being Ames-negative. The structural CA increased significantly in the CHL/IU cells treated with 4-(1-methylpropyl)phenol (No. 19), but the induction of CA was finally judged as negative from the result of an in vitro micronucleus test using CHL/IU cells. Two bisphenols 2,2′-methylenebis(6-*tert*-butyl-*p*-cresol) (No. 24) and 4,4′-thiobis(6-*tert*-butyl-*m*-cresol) (No. 25) were tested, but CA was not induced (Table 2). Some bisphenols, which are well-known as endocrine disrupting chemicals, were reported to induce aneuploidy detected as micronuclei revealing the centromere signals of anti-kinetochore antibody [16]. The bisphenols studied here showed no induction of polyploidy, suggesting that they may not affect the mitotic apparatus.

Structural CA and polyploid cells were induced by *p*-*tert*-butyl phenol (PTBP, No. 21), but neither structural CA nor polyploidy were induced by the similar chemicals, 6-*tert*-butyl-2,4-xylenol (No. 22) and *p*-*tert*-octylphenol (No. 23). Among the phenols, phenolic amines (Nos. 17 and 18) and PTBP induced CA in the CHL/IU cells at more than 10% (Table 2). The hydroxybenzoic structure is suspected to be the primary determinant for CA induction.

3.4. Carboxylic acid or ester

Seven of the 11 carboxylic acids or esters (Nos. 72–78), especially acrylate and methacrylate compounds (Nos. 74–78) induced structural CA, showing more than 20% increase over controls (Table 2). Also, six of these seven CA-inducers had a tendency to induce polyploidy. Of the six, five chemicals were assayed in the Ames test, with four (Nos. 73, 75, 77 and 78) of the five showing no mutagenic activity

Table 2
Results of chromosomal aberration test using CHL/IU cells treated with 98 HPV industrial chemicals

Classification	No.	[CAS No.]	CA and[a] cytotoxicity	Induction of structural CA in each system[b]				Polyploidy (%)[h]	Remarks	Ames test[i] positive: +; Negative:−	References (Volume No.)
				Short-term treatment		Continuous treatment					
				S9(+)[f]	S9(−)[g]	24 h	48 h				
Aniline	1	[87-59-2]	B	++	−	−	−	−		+	5
	2	[95-64-7]	C	−	−	−	−	−		No data	3
	3	[103-69-5]	B	−	+++	+	−	−		−	3
	4	[100-61-8]	A	++	−	+++	+++	−		−	3
	5	[97-52-9]	A	++	−	−	−	−		+	5
	6	[108-44-1]	C	−	−	−	−	−		No data	2
Sulfonic acid	7	[70-55-3]	D	−	−	−	−	−		−	1
	8	[121-47-1][c]	a	++	−	−	−	−	Low pH[e]	−	2
	9	[88-44-8][d]	a	+	−	−	−	−	Negative under neutral pH[d]	−	4
	10	[88-53-9][c]	a	++++	−	+	++	−	Low pH[e]	−	1
Halogenated benzene	11	[106-37-6]	A	+++	−	−	−	−		−	2
	12	[89-61-2]	B	−	−	−	++	−		+	3
	13	[3209-22-1]	A	−	+	−	+	48 h, 15.5%		No data	1
	14	[611-06-3]	C	−	−	−	−	−		No data	3
	15	[95-73-8]	D	−	−	−	−	−		−	1
	16	[98-08-8]	D	−	−	−	−	−		−	4
Phenol	17	[123-30-8]	A	+	+	++++	+++	−		−	5
	18	[2581-34-2]	A	++	+	−	−	−		−	2
	19	[99-71-8][c]	D	+	−	−	+	−	Negative in MN test[e]	−	2
	20	[89-83-8]	B	+	−	−	−	−		−	4
	21	[98-54-4]	A	++	−	−	−	48 h, 93.2%		−	4
	22	[1879-09-0]	C	−	−	−	−	−		−	3
	23	[140-66-9]	D	−	−	−	−	−		−	1
Bisphenol	24	[119-47-1]	C	−	−	−	−	−		−	4
	25	[96-69-5]	C	−	−	−	−	−		−	4
Organic phosphate	26	[107-66-4]	C	−	−	−	−	−		−	2
	27	[26444-49-5]	A	++	−	−	−	−		−	2
	28	[1806-54-8]	C	−	−	−	−	−		−	2
	29	[26967-76-0]	D	−	−	−	−	−		−	2
	30	[78-51-3]	C	−	−	−	−	−		−	5
	31	[512-56-1]	D	−	−	−	−	−		No data	3
Polycyclic aromatic hydrocarbon	32	[83-32-9]	B	++	−	−	−	−		−	5
	33	[81-16-3][c]	a	+++	−	−	−	−	Low pH[e]	+	3
	34	[840-65-3]	D	−	−	−	−	−		−	5
	35	[2216-69-5]	B	+++	−	−	−	−		−	4
	36	[86-87-3][c]	a	+	+	−	−	−	Low pH[e]	+	2
	37	[842-18-2]	D	−	−	−	−	−		−	6
	38	[5460-09-3]	D	−	−	−	−	−		−	1
	39	[82-45-1]	C	−	−	−	−	−		+	3

Pigment	40	[5281-04-9]	C	−	−	−	−	−		−	2
	41	[14832-14-5]	C	−	−	−	−	−		−	2
Heterocyclic compound	42	[95-33-0]	D	−	−	−	−	−		−	5
	43	[4979-32-2]	A	−	−	−	−	48 h, 6.0%		−	3
	44	[95-31-8]	A	+	−	−	−	−		−	5
	45	[110-02-1]	D	−	−	−	−	−		−	4
	46	[126-33-0]	D	−	−	−	−	−		−	4
	47	[583-39-1]	A	++	−	−	+	−		−	3
Aldehyde	48	[90-02-8]	B	++	+++	+	−	48 h, 18.1%		−	4
Alkyl benzene	49	[1477-55-0]	C	−	−	−	−	−		−	3
	50	[95-63-6]	C	−	−	−	−	−		−	4
	51	[105-05-5]	C	−	−	−	−	−		−	2
	52	[98-83-9]	D	−	−	−	−	−		−	3
	53	[25321-09-9]	C	−	−	−	−	−		−	6
	54	[1321-74-0]	D	−	−	−	−	−		−	6
Alcohol or ether	55	[4461-52-3]	B	++	+++	+++	+	24 h, 5.9%		+	2
	56	[111-41-1]	A	−	−	−	−	48 h, 4.0%		No data	4
	57	[123-42-2]	D	−	−	−	−	−		−	5
	58	[110-63-4]	D	−	−	−	−	−		−	5
	59	[4457-71-0]	D	−	−	−	−	−		−	5
	60	[584-03-2]	D	−	−	−	−	−		−	1
	61	[126-30-7]	D	−	−	−	−	−		−	1
	62	[77-99-6]	D	−	−	−	−	−		−	1
	63	[108-65-6]	D	−	−	−	−	−		−	6
	64	[115-77-5]	D	−	−	−	−	−		−	3
	65	[6846-50-0]	C	−	−	−	−	−		−	2
	66	[24800-44-0]	D	−	−	−	−	−		−	2
	67	[3452-97-9]	D	−	−	−	−	−		−	5
	68	[102-76-1][c]	b	+++	−	−	−	−	Low pH[c]	−	6
	69	[105-45-3][d]	a	++	−	−	−	S9(+), 2.8%	Negative under neutral pH[d]	−	6
Carboxylic acid or ester	70	[526-78-3]	D	−	−	−	−	−		−	2
	71	[99-96-7][d]	a	+++	−	+++	++	−	Negative under neutral pH[d]	−	5
	72	[105-99-7]	A	++	−	−	−	−		−	3
	73	[623-91-6]	A	−	++	+++	+	24 h, 50.5%		−	2
	74	[2439-35-2]	A	++	++	+++	+	S9(−), 10.9%		+	5
	75	[868-77-9]	A	++	−	++++	++++	S9(−), 6.1%		−	5
	76	[106-91-2]	A	+	+++	++++	+++	48 h, 9.4%		No data	5
	77	[105-16-8]	A	+++	−	−	−	24 h, 7.3%		−	6
	78	[923-26-2]	A	++++	−	++	++++	48 h, 4.2%		−	4
	79	[111-82-0]	C	−	−	−	−	−		−	4
	80	[3319-31-1]	D	−	−	−	−	−		−	4

Table 2 (*Continued*)

Classification	No.	[CAS No.]	CA and[a] cytotoxicity	Induction of structural CA in each system[b]				Polyploidy (%)[h]	Remarks	Ames test[i] positive: +; Negative:−	References (Volume No.)
				Short-term treatment		Continuous treatment					
				S9(+)[f]	S9(−)[g]	24 h	48 h				
Cyanide	81	[78-97-7]	A	++	++	+	+++	48 h, 9.0%		−	1
	82	[78-67-1]	D	−	−	−	−	−		−	5
	83	[626-17-5]	D	−	−	−	−	−		−	4
	84	[623-26-7]	D	−	−	−	−	−		−	3
	85	[108-80-5]	D	−	−	−	−	−		No data	5
Non-cyclic alkenes	86	[7756-94-7]	C	−	−	−	−	−		−	2
	87	[760-23-6]	A	+++	+	+++	++	S9(+), 1.8%		+	4
Non-cyclic alkenes	88	[629-62-9]	D	−	−	−	−	−		−	1
	89	[544-76-3]	D	−	−	−	−	−		−	1
	90	[109-69-3]	D	−	−	−	−	−		−	2
	91	[1120-21-4]	D	−	−	−	−	−		−	4
	92	[4390-04-9]	D	−	−	−	−	−		−	1
Others	93	[538-75-0]	D	−	−	−	−	−		−	1
	94	[11070-44-3]	A	−	−	−	−	S9(+), 1.9%		−	5
	95	[3048-65-5]	B	++	−	−	−	−		−	5
	96	[77-73-6]	C	−	−	−	−	−		No data	3
	97	[16219-75-3]	C	−	−	−	−	−		−	6
	98	[96-29-7]	D	−	−	−	−	−		−	4

[a] A: CA-positive with the minimum effective dose manifesting 50% or less than 50% cytotoxicity, B: CA-positive with the minimum effective dose manifesting over 50% cytotoxicity, C: CA negative at doses manifesting over 50% cytotoxicity, D: CA-negative at doses manifesting 50% or less than 50% cytotoxicity (or at 5 mg/ml or 10 mM), a and b: cytotoxic response same as the A and B, respectively, but under low pH acidic condition in culture medium.

[b] CA negative (−). Induction of CAs at 5% =<, <10% (+); 10% =<, <20% (++); 20% =<, <50% (+++); 50% =< (++++).

[c] Chromosomal aberrations might be induced by lowering of pH in culture medium.

[d] There was no increase of CAs when the pH of culture medium as changed from acidic to neutral conditions.

[e] In vitro micronucleus test was conducted, then finally judged as negative.

[f] Short-term (6 h) treatment with S9.

[g] Short-term (6 h) treatment without S9.

[h] Maximum frequency and treatment system were described.

[i] Results were cited from references.

Table 3
No. of HPV industrial chemicals judged on CA

Judgements or types of CA-induction	No. (%) of chemicals
Negative	59 (60)
Positive	39 (40)
Structural CA	16[a]
Structural CA + polyploidy	12[b]
Polyploidy	3[c]
CA induced under non-physiological culture condition (pH<6)	8[d]
Total	98 (100)

Chemical Nos. (denoted in Tables 1 and 2).
[a] Nos. 1, 3, 4, 5, 11, 12, 17, 18, 20, 27, 32, 35, 44, 47, 72 and 95.
[b] Nos. 13, 21, 48, 55, 73, 74, 75, 76, 77, 78, 81 and 87.
[c] Nos. 43, 56 and 94.
[d] Nos. 8, 9, 10, 33, 36, 68, 69 and 71.

(Table 2). The acrylate and methacrylate compounds are usually polymerized and produced as industrial materials in a polymer form. Though the monomer form of acrylates and methacrylates tested here had clastogenic activity and/or activity as polyploid inducer, their risks to humans should be further evaluated on the basis of the form to which humans are exposed.

3.5. Polyploidy induced by p-tert-butylphenol

Among phenols that induced structural CA, only *p-tert*-butylphenol (PTBP, No. 21) also induced polyploidy, showing a considerably high frequency (13.2%) in the 48 h treatment (Table 2). We confirmed the aneugenic potential of PTBP by using fluorescence in situ hybridization (FISH) technique on interphase human peripheral blood lymphocytes treated with PTBP for 48 h (unpublished data). If it is possible that all polyploid inducers can always induce aneuploid cells, the polyploidy induced in CHL/IU cells has significant meaning no less important than that of the heritable risk of structural CA for humans.

PTBP, 2(3)-*tert*-butyl-4-methoxyphenol and 2-*tert*-butyl-4-methylphenol are known to induce hyperplasia and tumorous lesion in forestomach of golden hamster [17]. This and the present results indicate that the molecular species of tertiary-butyl phenols possess genotoxicity.

3.6. Induction of polyploidy alone

N,N-Dicyclohexyl-2-benzothiazolesulfenamide (No. 43), *N*-(aminoethyl)ethanolamine (No. 56) and tetrahydromethyl-1,3-isobenzofuranedione (No. 94) did not induce structural CA but did induce polyploid cells at low frequencies (6.0, 4.0 and 1.9%, respectively, Table 2) with significant difference (Fisher's exact probability test, $P < 0.01$) from the number of polyploid cells scored in solvent/vehicle control (control data not shown). These frequencies may not appear to be clearly positive, but lend some difficulty in evaluating the long-term genetic hazard from exposure to these chemicals.

3.7. Simultaneous induction of structural CA and polyploidy

PTBP (No. 21) and carboxylic acids or esters (Nos. 73–78), i.e. simultaneous inducers of structural CA and polyploid cells, may widely act on intracellular proteins which include histones and the proteins associated with the mitotic apparatus (kinetochores, centrioles and microtubules) rather than act directly on DNA or mitotic apparatus. This is partly supported by the fact that PTBP and five of the seven CA-positive carboxylic acids or esters (Nos. 72, 73, 75, 77 and 78) were negative in the Ames test (Table 2). A similar case is seen with arsenite compounds. Sodium arsenite was known to induce both structural CA and aneuploidy simultaneously [18] though arsenite compounds showed no mutagenicity in the Ames test [19]; indeed, arsenite has a high affinity for sulfhydryl residues of protein molecules which can result in interference of polymerization of tubulin protein. Thus, PTBP and the five carboxylic acids and/or their metabolic derivatives possibly act by attacking intracellular protein, although testing data with a range of genotoxicity and non-genotoxicity endpoints are needed to elucidate this.

3.8. Induction of CA under non-physiological culture condition

For eight of the CA-positive chemicals (Nos. 8, 9, 10, 33, 36, 68, 69 and 71) tested for 6 h with metabolic activation, the color of the culture medium shifted from orange or red (pH 7–8) to yellow (pH<6)

just after applying the treatment solution, suggesting that CA was generated by the non-physiological culture conditions. Culture medium with low pH (pH<6.4) was known to induce structural CA in CHL/IU cells [13], but it could not be assumed without confirmation that structural CA in the current tests were caused by lowering pH. Of the eight chemicals, 2-amino-5-methylbenzenesulfonic acid (No. 9), methyl acetoacetate (No. 69) and 4-hydroxybenzoic acid (No. 71) did not induce CA when the pH of the culture medium was adjusted to pH 7–8 by adding 1N NaOH into the medium containing the test substances. Although it is possible that intrinsic chemical features were altered after adjusting pH, the decrease of pH was relevant to the induction of CA by the three chemicals.

3.9. Results of chromosomal aberration test and Ames test

Of the 36 CA-positive chemicals that have been assayed in Ames test, only eight (Nos. 1, 5, 12, 33, 36, 55, 74, 87) were Ames-positive (Table 2) [1–5]. Thus, chromosomal aberration tests of HPV industrial chemicals detected a much larger number of positive chemicals than the Ames tests. Furthermore, the overall numerical proportions of the CA-positive and Ames-negative pharmaceutical compounds tested in another laboratory [9] were almost the same as the proportions demonstrated with the HPV industrial chemicals tested in our laboratory (Ames-positive versus CA-positive: 10 and 34% for HPV chemicals, 8 and 29% for pharmaceutical compounds, Table 4), suggesting good interlaboratory agreement for screening the clastogenic activity of chemicals or polyploid inducers.

Table 4
Numbers of HPV industrial chemicals and pharmaceutical compounds with positive results

	Ames positive (%)	CA positive (%)
HVP industrial chemical	9/89 (10)	31/90 (34)[a]
Pharmaceutical compound	23/298 (8)[b]	77/266 (29)[b]

[a] Results of eight chemicals that induced CAs under non-physiological culture condition (see Table 3) were excluded.
[b] Müller and Sofuni [9].

3.10. CA-induction and cytotoxicity

It has been previously suggested that non-DNA targeted chemicals (e.g., Ames-negative) would show high cytotoxicity (more than 50%) at doses inducing CA, based mainly on information from pharmaceutical drugs [10]. In that study, it was discussed whether the maximum dose (more than 50% cytotoxic dose) recommended by OECD Guideline [7] was an appropriate level to screen chemicals for clastogenic activity or polyploid induction.

Table 2 also indicates the cytotoxicity levels at doses where the induction of CA is first apparent (i.e. minimum effective dose) and the results of Ames test from the publications [1–6] cited. Typical examples of CA-positive responses with accompanying "weak" and "severe" cytotoxicity are given in Figs. 1 and 2, respectively. 4-Aminophenol (No. 17) induced structural CA quadratically with increasing doses, and showed "weak cytotoxicity" (50% or less than 50% cytotoxicity, Fig. 1). On the other hand, 2,3-dimethylaniline (No. 1) did not induce structural CA at low and middle doses but showed a marked increase in the number of cells with structural CA only at the maximum dose manifesting "severe cytotoxicity" (more than 50% cytotoxicity, Fig. 2), thus representing a typical high toxicity clastogen (HTC) [11].

In order to discuss the relevance of cytotoxicity to the induction of CA, the data on the eight chemicals

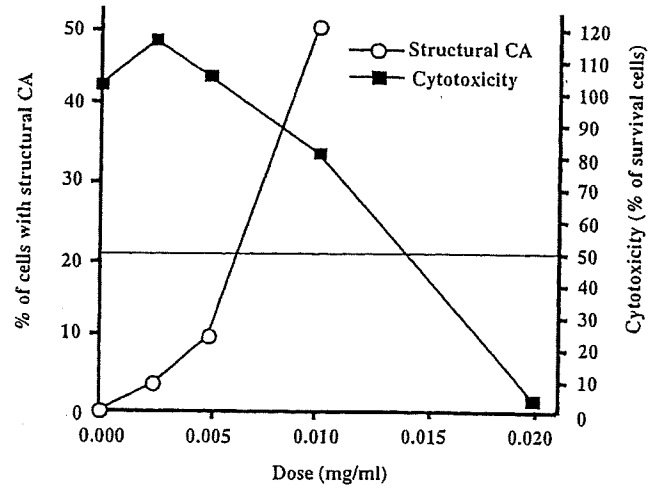

Fig. 1. Structural CA and cytotoxicity induced by 4-aminophenol (No. 17, Ames-negative chemical) after continuous treatment for 24 h. Note that the structural CA increased quadratically at the doses showing weak (50% or less) cytotoxicity.

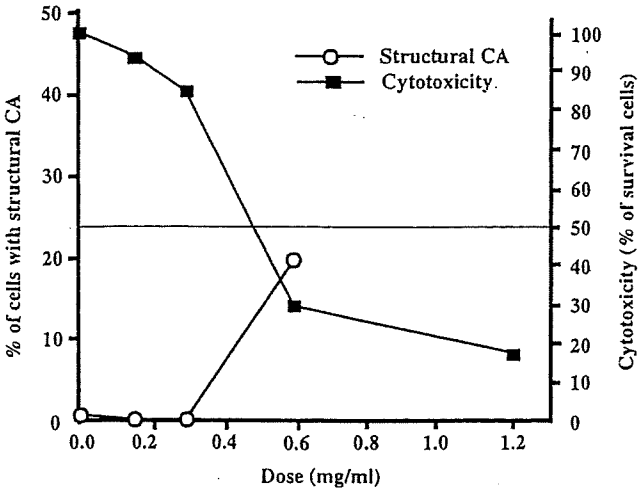

Fig. 2. Structural CA and cytotoxicity induced by 2,3-dimethylaniline (No. 1, Ames-positive chemical) after short-term treatment for 6 h under metabolic activation. Note that the structural CA increased steeply only at the maximum dose, which showed severe (more than 50%) cytotoxicity.

tested under non-physiological culture conditions (lowered pH) were excluded from the consideration in Table 4. This was done because five of the eight had not been tested repeatedly under physiological culture conditions adjusted to neutral pH, so, the actual clastogenicity of the test chemicals could not be evaluated.

We assumed firstly that the CA-positive chemicals with negative Ames test result did not tend to directly attack DNA molecules, but indirectly induced chromosome damage in CHL/IU cells via a cytotoxic effect. If the cytotoxic effects were more relevant to the induction of CA than the attack of chemical molecules or derivatives on DNA, then the numerical proportion of CA-positive chemicals manifesting severe cytotoxicity (i.e. HTCs or the chemicals with HTC-like response) would be clearly greater than that of the CA-negative chemicals also manifesting severe cytotoxicity. However, compared with the number of Ames-negative chemicals, the numerical proportion of the CA-positive chemicals manifesting severe cytotoxicity was 27% (six of 22 chemicals) being similar to that of the CA-negative chemicals (31%, 16 of 52 chemicals) (Fig. 3). Also, among the HPV industrial chemicals, unknown mutagenic responses unable to be screened by Ames test or the other epigenetic reactions proceeding within cells may take part in the induction of CA by the CA-positive chemicals

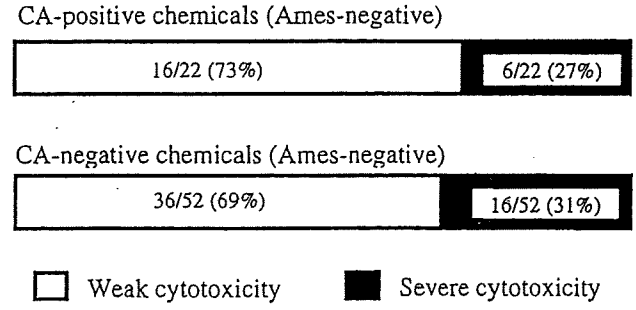

Fig. 3. Relevance of the cytotoxic action to the induction of CA by Ames-negative HPV industrial chemicals. There is little difference in the numerical proportions between CA-positive and CA-negative chemicals manifesting severe cytotoxicity.

judged as Ames-negative. Therefore, since most of the Ames-negative but CA-positive chemicals are possibly DNA-targeted clastogens, other mutation assays using cultured mammalian cells will be helpful to discriminate DNA-targeted from non-targeted clastogens.

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Low-dose bisphenol A does not affect reproductive organs in estrogen-sensitive C57BL/6N mice exposed at the sexually mature, juvenile, or embryonic stage

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Abstract

Bisphenol A (BPA) is used on a large scale in the manufacture of polycarbonate plastics. BPA has been shown to bind weakly to both estrogen receptor (ER) α and ER β. The objective of this study was to evaluate the effects of low-dose BPA on male sexual development after exposure at various stages of development. Mice of the estrogen-sensitive strain C57BL/6N were exposed to BPA orally at doses of 2, 20, or 200 μg/kg at various stages, i.e. adulthood, the immature stage just after weaning, or the embryonic/fetal stage, to evaluate the effects of low-dose BPA on male reproductive organs. Body weight changes, weights of reproductive organs (testes, epididymides, seminal vesicles), cauda epididymal sperm density, and histology of reproductive organs including the ventral prostate were not affected by exposure to BPA at any dose examined. The results of this study indicate that exposure of estrogen-sensitive C57BL/6N mice to low-dose BPA did not reduce sperm density or disrupt development of the male reproductive organs.

Keywords: Bisphenol A; Low-dose exposure; C57BL/6N mice; Reproduction; Testicular toxicity

1. Introduction

Bisphenol A (BPA) is an industrial compound that has generated a great deal of concern on the part of regulatory agencies and scientists due to its high level of production and widespread use. BPA is the monomer used in the manufacture of the resin used to line food and drink cans and from which polycarbonate plastic is made. BPA is also used to make dental sealants, which are often used to protect children's teeth [1]. BPA has been reported to be weakly estrogenic both in vitro and in vivo. Krishnan et al. [2] reported that BPA leached from polycarbonate flasks competed with [^{3}H]-estradiol for binding to estrogen receptors (ER) from rat uterus, induced progesterone receptor expression, and promoted cell proliferation in cultured human mammary cancer cells (MCF-7). BPA binds to both ER α and ER β with low affinity and transactivates reporter genes in vitro [3,4].

Recently, experiments by Nagel et al. [5] and vom Saal et al. [6] indicated that administration of low oral doses of BPA to pregnant mice on days 11 through 17 of gestation produced statistically significant increases in the weights of the prostate and preputial glands, a decrease in epididymis weight, and reduced efficiency of sperm production in male offspring. However, the low-dose effects of BPA have been controversial. Other researchers reported no treatment-related effects of BPA at the same and additional low-dose levels given at the same time of pregnancy to mice [7–12].

Large (more than 16-fold) differences in sensitivity to disruption of juvenile male reproductive development by 17β-estradiol (E_2) were found between strains of mice. Spermatid maturation was eliminated by low doses of E_2 in strains such as C57BL/6J and C17/Jls. In contrast, mice of the widely used CD-1 line showed little or no inhibition of spermatid maturation even in response to 16-fold higher doses of E_2 [13].

In the present study, we examined the effects on development of male reproductive organs in C57BL/6N mice, which were confirmed to be sensitive to estrogens similarly to C57BL/6J, as a result of embryonic or fetal exposure to environmentally relevant doses of BPA. In addition, the effects on reproductive organs in males exposed to low

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doses of BPA at the sexually mature or immature stages after weaning were also investigated in this strain.

2. Materials and methods

2.1. Animals and treatment

C57BL/6N and ICR mice were purchased from Charles River, Atsugi, Japan, at 3 or 8 weeks of age. The animals were acclimated to the laboratory for 3 days to 2 weeks prior to the start of the experiments. Animals were housed individually in polycarbonate cages in a room with controlled temperature (24 ± 1°C) and humidity (50 ± 5%), with lights on from 07:00 to 19:00 daily. Mice were given access to food (PLD, phytoestrogen-low diet, Oriental Japan) and tap water (distilled water) ad libitum. The contents of phytoestrogens in the diet, tap water, and wood bedding were determined, and genistein and daidzein levels were below 0.5 mg/100 g.

2.2. Experiment I (exposure of C57BL/6N and ICR juvenile male mice to E_2)

To compare the susceptibility to 17β-estradiol (E_2, Sigma Chemical Co., St. Louis, MO) between C57BL/6N and ICR males, 6 to 8 males from each strain were treated subcutaneously (s.c.) with E_2 at 10 μg/kg from postnatal day 27 to 48. The administration period was determined according to the protocol of the study by Spearow et al. [13]. Administration was performed at a defined time (12:00). Ten males of each strain were given corn oil (2 ml/kg) as controls. On postnatal day 43, male mice were weighed and subjected to necropsy. Subsequently, the testes, epididymides and seminal vesicles with coagulating glands were weighed. These reproductive organs were fixed in Bouin's solution for histologic observation.

2.3. Experiment II (exposure of C57BL/6N adult males to BPA)

Groups of twenty C57BL/6N male mice at 10 weeks of age were exposed to bisphenol A (BPA, Tokyo Kasei, purity, GC min. 99.0%) at 2, 20, or 200 μg/kg by oral gavage for 6 consecutive days. The dosages were determined on the basis of body weight on the day of the treatment. Administration was performed at a defined time (12:00). Twenty males were given 0.5% carboxymethyl cellulose (5 ml/kg) as controls. Six weeks after the final administration, male mice were weighed and 15 were subjected to necropsy. The administration period and the day of necropsy after the last administration were determined based on the results of the previous study by Ohsako et al. [14]. Subsequently, the testes, epididymides, and seminal vesicles with coagulating glands were weighed. The ventral prostate was not weighed in the present study since it was difficult to sample only the prostate in mice, and to determine the precise weight of this organ. The left cauda epididymis was homogenized in 1 ml distilled water. The homogenates were stained with an IDENT staining kit (Hamilton Thorne prepackaged DNA-specific dye based on Hoechst 33342). The stained samples were placed onto Cell-Vu slides (Fertility Technologie, MA, USA), and the numbers of sperm were counted using an HTM-IVOS analyzer (Hamilton Thorne Research, MA, USA) and the IDENT software supplied with the HTM-IVOS. Other reproductive organs were fixed in Bouin's solution for histologic evaluation. The remaining mice (5 mice per group) were anesthetized. Transcardiac perfusion was carried out with a mixture of 0.1 M phosphate-buffered 1.25% glutaraldehyde and 2% paraformaldehyde. Following fixation, the testes, epididymides, seminal vesicles, and prostates of these mice were rinsed three times in phosphate buffer, postfixed for 2 h at 4°C in 2% osmium tetroxide, and dehydrated in alcohol; these organs were embedded in epoxy resin. Tissue sections (1 μm thick) were stained with toluidine blue for light microscopy. Ultrathin sections stained with uranyl acetate and lead citrate were observed with an electron microscope (H-7100, Hitachi, Japan).

2.4. Experiment III (exposure of C57BL/6N juvenile males to BPA)

To obtain pregnant animals, 10-week-old virgin C57BL/6N females were cohabited overnight on a 1:1 basis with males of the same strain at 11 weeks of age or older. The next morning, females with vaginal plugs were regarded as pregnant, and the day of gestation was designated as day 0. All pregnant mice were allowed to give birth. On postnatal day 0, all female pups were discarded, and the number of males per litter was adjusted to 3. Male pups (30 males from 10 litters/group) were weaned on postnatal day 21, and exposed to BPA at 2, 20, or 200 μg/kg by oral gavage from postnatal day 21 to 43. The dosages were determined on the basis of body weight on the day of treatment. Administration was performed at a defined time (12:00). Thirty males from 10 litters were given 0.5% carboxymethyl cellulose (5 ml/kg) as controls. At 6 weeks old, all males were weighed. Five mice per group were anesthetized and transcardiac perfusion was carried out. The remaining males were subjected to necropsy. Subsequently, the testes, epididymides, and seminal vesicles with coagulating glands were weighed, and the left cauda epididymis of each male was homogenized to determine the sperm density. Finally, weighed organs were fixed in Bouin's solution for histologic evaluation.

2.5. Experiment IV (exposure of C57BL/6N embryos/fetuses to BPA)

Pregnant mice were obtained as described in Experiment III. Groups of 10 C57BL/6N mice were exposed to BPA at