3.2 毒性学のパラダイムシフト

低用量問題に取り組む中で，たくさんの事柄が明らかになってきた。ここで課題となった「性の可塑性」なども，多分に生命存続のための生物に備わった知恵であった。それが裏目に出た形でこの問題は発生している。その中には，様々の生命におしなべて影響の及んでいる事象が見いだされたが，他方，充分な生物学的蓋然性を持ちながらも齧歯類－霊長目－ヒトへの外挿性の明らかに否定されている事象もある。本稿で取り上げた齧歯類の性的二型核の変化などもこれに属し，霊長目での方法論は，また異なったものとなることを意味している。けだし蓋然性の蓋然性たる所以である。そうした中にあってもホメオステーシスの範囲内でのリスク，薬理と“毒理”のcontinuumに重なった生体障害の可能性は，毒性学の方法論の新たな段階を期するパラダイムシフトといえよう。低用量問題に関するノースカロライナ会議が従来の試験法そのものに疑問を投げかけることになったのは，こうした背景があってのことと考えている。

3.3 トキシコジノミクス[3)]

先にホメオステーシスの陰に隠れていて表面的には“見えない”現象を見いだすことが新しい課題となることにふれた。これに役割を果たすものと期待される手法として，マイクロアレイやDNAチップによる遺伝子の大量発現技術の試行的普及が進んでいる。それらの“ゲノム発現情報とリンクして包括的に把握される”比較的大容量の分子生物学的情報は，-omicsの接尾語を付してジノミクス，プロテオミクス，メタボノミクスなどと呼称される新しい生物学領域を形成しつつある。これらのトキシコロジーへの適用がトキシコジノミクスである[3),4)]。例えばDNA障害を伴った発がん物質の低用量域での閾値現象が，p53欠失動物では，無閾値性に観察され，通常動物でのそれは，修復に関与することが明らかになりつつある。このような通常観察されない静止変化や閾値現象は，トキシコジノミクスによって，“見えてくる”，可視化される。多次元的でネットワーク状の相互作用・拮抗関係にある様々のモメントの堪えざる平衡調節も可視化される，そういったことが期待される。これを通じていま明らかにされようとしている低用量問題は，今少し論理的な構成をもった現象として理解されるようになるものと思われる。

低用量問題は，内分泌撹乱物質問題を契機として，ヒトと外界との相互関係を探る本質的な生物学の課題の一つになろうとしている。

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Introduction:

Toxicogenomics - a new paradigm of toxicology

Tohru Inoue

Center for Biological Safety & Research, National Institute of Health Sciences
1-18-1 Kamiyohga, Setagayaku, Tokyo 158-8501, Japan

Summary. Molecular biology has enabled the elucidation of biological subjects with bilateral strategies, namely, an inductive approach and a deductive approach. Along with the development of the mouse whole-genome sequencing project, it has enabled elucidation of the science bilateral interrelationships between the toxicological phenotypes related to particular toxicants and expression profiles of pertinent genes induced by exposure to toxicants. While a conventional inductive approach permits exploration of the toxicological mechanism by cloning genes and analyzing gene and protein expression during the course of chemical exposure, the newly developed deductive approach potentially permits the elucidation of the toxicological phenotype(s) through gene expression.
Microarray technology has dramatically changed the time course of drug discovery in new drug development. Potential therapeutics can be screened for thousands of endpoints indicative of efficacy and adverse toxicity at one time using the microarray technology. Simultaneously, the same technology can be used to explore unique genomic "expression fingerprints", which can be used to group the biological effects of chemical actions at a various doses, time intervals, or target tissues, in a variety of animal species, into profiles as the bases of gene expression. Accumulation of the expression profiles (here and elsewhere) of whole genomes for reference chemicals for a variety of treatment conditions permits the establishment of an informatics profile (here and elsewhere) for reverse toxicology, which is conversely supposed to predict the toxicological phenotypes solely by analyzing gene expression. This translational introductory oversees the future prospects of how microarrays can be used in applied toxicology.

Key words. Toxicogenomics, DNA microarray, reverse science, reverse genetics, reverse toxicogenomics

DNA microarrays

As an introductory keynote to "Toxicogenomics", a discussion on what toxicogenomics can offer to conventional toxicology is given here in this

paragraph. Toxicogenomics is based on DNA microarray and DNA chip technologies that are similar to those in other genome science fields (Lovett, 2000; Hamadeh et al., 2001; Storck et al., 2002) i.e., the DNA microarray fixed with cDNA by a DNA spotter, and hybridized with fluorescence-labeled cDNAs from tissue samples (Schena et al., 1995, 1996), and the DNA chip, on which a number of oligonucleotide probes are photolithographically synthesized, followed by hybridization of biotiny lated cDNAs from samples (Fodor et al., 1993). Originally, DNA microarray and DNA chip technologies have been used to analyze a large number of gene expressions, and thus, have been applied to such functional genomics fields as transcriptomics (Storck, et al., 2002) pharmacogenomics (Lloyed A, 2000), mutagenomics (Aardema and MacGregor, 2002), oncogenomics (Herrmann, et al., 2001), pathogenomics (Liefers, et al., 2001), and predictive diagnostic medicine based on clinical prognosis (Nakamura, 2001), and specifically, the latter DNA chip technology is a potentially powerful tool for identifying DNA sequences, thus, such inductive information has been applied widely in the research for single nucleotide polymorphism, SNP, in a variety of drug-metabolizing enzymes, etc., to establish an individualized "tailor-made pharmacology".

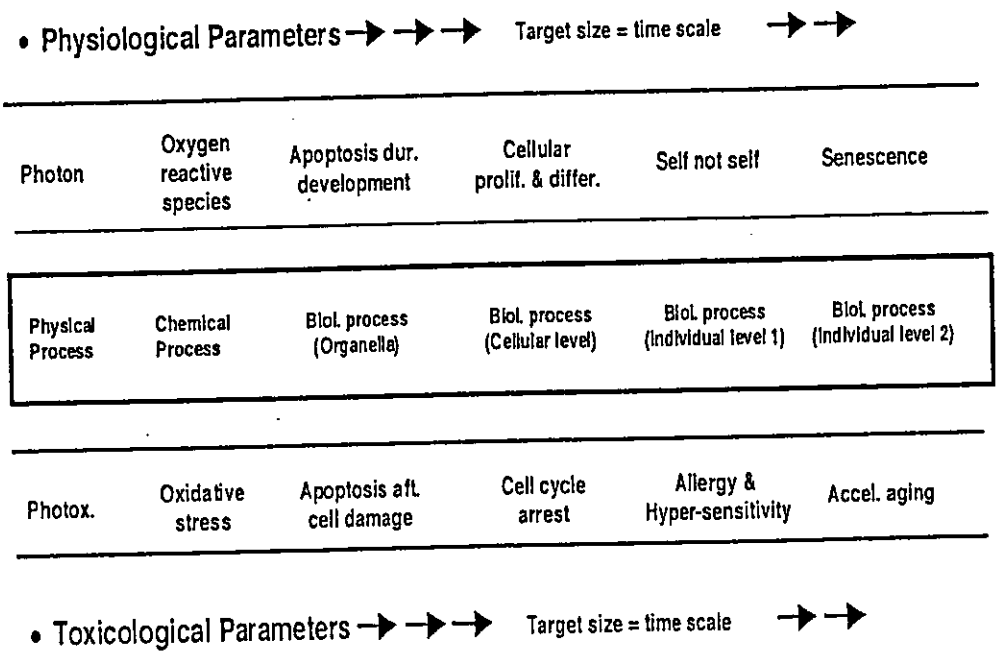


Fig. 1. Physiological parameters shown in the upper row vs. toxicological ones shown in the lower row along with the increase of participating target masses or time scales.

Microarray and /or DNA chip technologies applied in toxicology are called "toxicogenomics". Toxicogenomics can be applied bi-laterally, either inductively or deductively. Deductive approach of toxicogenomics shows a great, unexpected paradigm shift from conventional toxicology.

Toxicology and toxicogenomics

Before describing an over- view of toxicogenomics, what toxicology is, namely, its definition, entity, and scientific bases, should be reviewed first. Toxicology is an interdisciplinary area between biology/medicine and chemistry/physics. Key molecules participating in physiological responses and toxicological responses are presumably comparable (**Figure 1**), implying that physiological responses and toxicological responses may be a continuum. Pharmacology involves the identification of something available; on the other hand, toxicology involves the identification of not only the mechanism of toxicity but also clarifying a border of "nothing", i.e., NOEL, "no observed effect level", and/or NOAEL, "no observed adverse effect level". The goals of toxicology are to predict the effect of potential hazards on human health effects, and to identify the mechanism of toxicity, NOEL and/or NOAEL. In this regard, toxicogenomics is supposed to clarify comprehensively the border of "nothing". Although a prototype of "toxicogenomics" was developed in 1997 (Heller et al., 1997) to identify specific toxicological phenotypes, such as oxidative stress inducers, drug- metabolizing chemicals, and cell-cycle-specific modulators, comprehensive toxicogenomics became possible after the whole-genome sequencing project was accomplished in 2001. Because of the completion of the whole genome sequence, finally, the toxicology to predict "nothing" became possible.

Birth of reverse science & toxicology

In 1988, a new era of mouse genetics, reverse genetics, was started by generating the first knockout gene for mammalian species, murine int-2, by the group of Mario Capecchi's (Capecchi et al., 1988) and then Elizabeth Robertson's (Schwartzberg, et al, 1989). Thereafter, molecular biology has enabled the elucidation of biological subjects by bi-directional strategies, forward and reverse ones, i.e., the inductive and the deductive approach, respectively, where not only genes that possess a particular expression phenotype have been cloned by forward genetics, but also a number of genes of which functions were not known have been uncovered their function by reverse genetics, i.e., knockout technologies. The history of genetics teaches such bilaterally alternating strategies to strengthen scientific power. Thus, it is speculated that the inductive toxicology and deductive toxicology may complement each other.

Along with the development of the mouse whole-genome sequencing project, such bi-directional strategies for analysis became possible also in toxicology; the toxicologic phenotypes of particular toxicants and the expression profiles of pertinent genes reacting with the toxicants. While the inductive approach permits exploration of the toxicological mechanism by analyzing gene and protein expression during the course of toxicological testing, the deductive approach

permits prediction of the toxicological phenotype(s) solely by analyzing the gene expression. Microarray and/or DNA chip technologies have enabled the survey of a large number of gene expressions after exposure to a toxicant. Both inductive and deductive approaches have enabled application of DNA chip and/or the microarray in toxicological analysis, i.e., "toxicogenomics". Toxicogenomics enables exploration of the toxicological mechanism by analyzing a large number of gene chips inductively, and opens a new era of reverse toxicology, which is supposed to predict possible toxicologic phenotypes by distinguishing the expression patterns of particular genes from accumulated expression profiles. The DNA chip and the microarray technologies for the identification of specific toxicity groups are commercially available already, e.g., metabolic enzyme inducers, growth factor & receptor-mediated transducers, xenobiotic ligands for nuclear receptors, stress-response-gene modifiers, and cell-cycle regulator modifiers.

Reverse toxicology

Similar to reverse genetics, reverse toxicology is supposed to identify toxicological phenotypes solely by examining their expression profiles. Such deductive use of microarray technology for toxicology is called "Reverse Toxicogenomics", where it is expected to predict toxicological phenotypes solely by analyzing whole gene expression (**Figure 2**). This technique is requires a

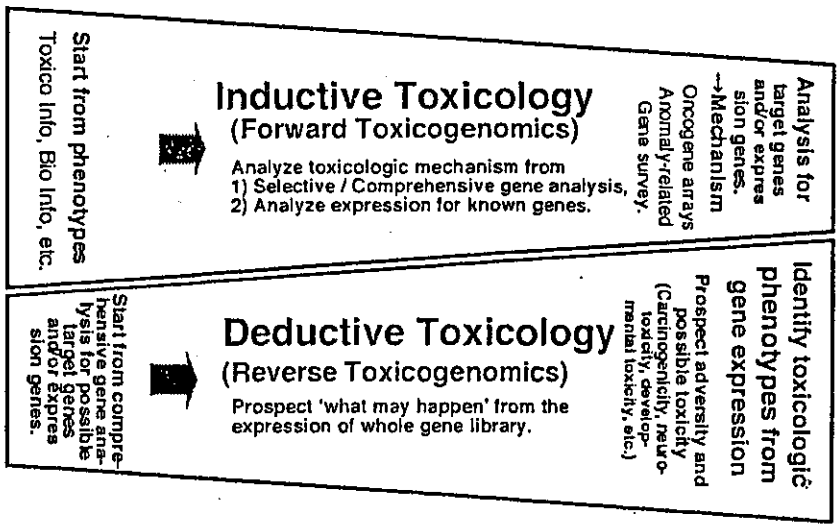


Fig 2. Structures of inductive toxicology vs. deductive toxicology. Former starts its analyses from toxicologic phenotypes toward the mechanism, whereas, the latter focuses in identigying toxicologic phenotypes solely from the gene expression profiling.

minimum number of animals, or even samples of in vitro-cultured cells after a relatively short period of exposure to a potential hazardous testing materials. The predictability by reverse toxicology depends upon the number of gene expression profiles accumulated, the number of phenotypes differentially linked to the gene expression profiles, and informatics linking such gene expressions and the phenotypes.

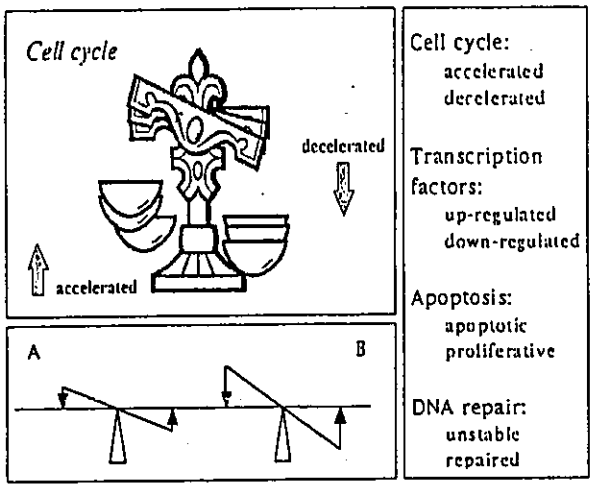
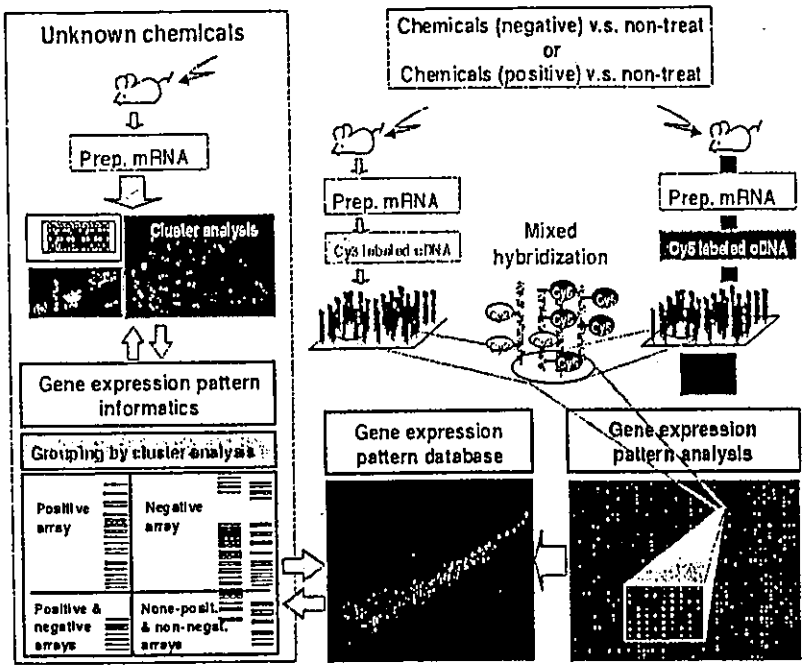


Fig. 3. Counter balancing gene expressions behind the homeostasis Visualization of different oscillatory balances A and B (left bottom).

At this moment, reverse toxicogenomics is still a theory. However, a variety of testing methods in toxicology will be replaced by reverse toxicogenomics, eventually. This strategy has the following advantages: it reduces the number of test animals, and the test period, and adopts simpler techniques by using established expression profile as new biomarkers rather than sophisticated methodologies requiring skill and experience. Furthermore, a specific proteome chip, expressing a series of specific genes, is supposed to function as "reverse proteomics" through a series of processes such as sample preparation, 2D gel electrophoresis, and mass-spectrometry for image analysis (Zhu et al., 2001). To set up an endpoint where NOEL or NOAEL exists, a traditional toxicology has been applied to incorporate something " invisible borders". Invisible borders are, in conventional toxicology, based on at least two major limitations: one in an endogenous factor(s) and the other in an exogenous factor. The former, for example, is hidden behind homeostasis, and the latter, for example, is behinbd a technological limitation. As shown in **Figure 3**, most living animals exhibit homeostasis between two (or more) counter- balancing vectors such as oxidation & reduction, apoptosis & anti-apoptosis, and acceleration & deceleration of cell cycle regulation. Since the counter-balancing counter-directional homeostasis, it appears static and one may not recognize the differences between one homeostatic stage, balanced at a low energy stage, to the other stage, balanced at a high energy stage (A and B in Figure 3). It is far more important to note that stage B is generally more risky. Toxicogenomics is expected to disclose such hidden homeostatic balances which are undetectable by conventional testing systems. The latter, an exogenous factor in a technological limitation, may be based on such resolution limit of light-microscopes, spectrophotometers, etc., and all



technologies exhibit their resolution point, i.e., "invisible barrier". Toxicogenomics also has a limit in terms of technical sensitivity; however, it may overcome presently available resolution limits in many ways, and hopefully identify a possible specific toxicological profiling.

Fig. 4. Practical toxicity-predicting system based on the gene expression microarray.

Practical approach

A sample of a chemical toxicity predicting system is shown in **Figure 4**. Mice are treated with, or without, a known toxic reference chemical (TRC), or treated with, or without, a known non-toxic reference chemical (NTRC). Then, the

Table 1: Possible toxicologic endpoints tested by in vitro or in vivo resources

Possibility of in vitro test	Toxicity Endpoint(s)
No [yes]*	*Morphogenesis, developmental anomaly*
No	*Identifying tissue-specific toxicity*
No/yes	*Epigenetic carcinogenesis (as modification of gene expression)*
	Metabolic activation
No	Hepatic activation leading to multi-tissue damage
Yes	Tissue-specific activation
	Receptor-mediated events
Yes/no	Neuronal tissue
Yes/no	Steroid hormonal tissue
Yes/no	Ah-receptors
Yes	*Cytotoxicity*
	Membrane activities
Yes	ion channels, ion pumps
	Inhibition of biochemical process
Yes	Uncoupling oxidative phosphorylation, inhibition of ATP production by redox cycling initiation to produce ROS
Yes	Oxidative phosphorylation to inhibit ATP production
Yes/no	Alteration of calcium homeostasis

*[yes] Limited possibility at this moment, e.g. whole embryo culture

messenger RNAs are extracted, and visualized with red color marker, cy3, for overexpression or with green color marker, cy5, for down-modulation. These color-labeled mRNAs will be processed into a competitive mixed-hybridization in a high-density hybridization array. Expression patterns are informatized in many ways. Along with accumulation of data to establish informatic profiles, specific gene clusters for TRC, NTRC, and those positive for both TRC + NTRC, andnegative for both TRC + NTRC, can be established. These databases can be compared with an expression profile that will be obtained from unknown chemicals (left box in Figure 4).

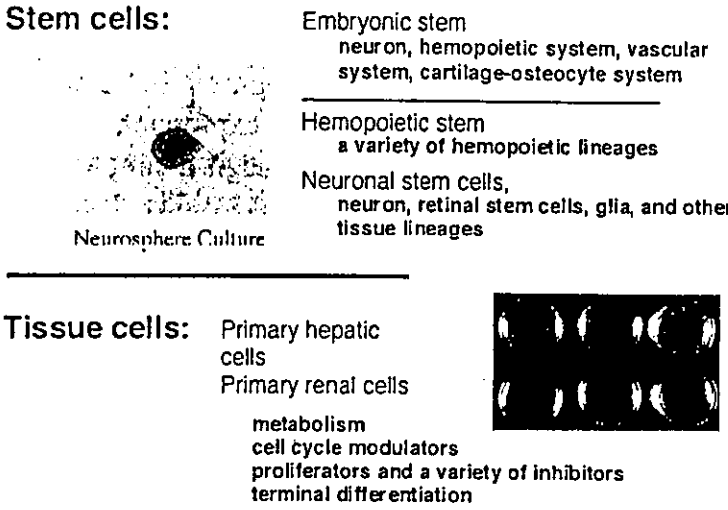


Fig. 5. A variety of in vitro resources for toxicologic gene expression array. Different cellular function for microarray analyses between stem cells and tissue cells. See text.

The technique requires only a limited number of animals, or even with cultured cells, in vitro, after relatively a short period of exposure. Depending upon the endpoints of toxicity aimed to focus on, even materials from in vitro culture may work efficiently (**Table 1**). As shown in Table 1, activities of membrane such as ion channels and ion pumps, the inhibitory effect of uncoupling oxidative phosphorylation, and the inhibition of ATP turnover by redox cycling, may be identified by the microarray. Possible toxicity related to developmental anomaly and morphogenesis (top of Table 1) may not be predictable by the use of in vitro cell culture; however, as seen in **Figure 5**, an in vitro system, for example, an embryonic stem (ES) cell, may predict some possible adverse effects of toxicity on the morphogenesis. Consequently, ES cells as well as hemopoietic and neuronal stem cells are particularly powerful tools for identifying the effect of toxicity on not only proliferation but also differentiation. Actually, one ES cell potentially corresponds to one individual; therefore, observing a microarray of ES cells may correspond to observing several millions of mice at the early developmental stage. Hepatic, renal and other types of primary cultured cells are limited but useful for observing such a variety of metabolic modulators, cell cycle regulators, and cell proliferation inhibitors and/or stimulators, in primary hepatic cells.

New paradigm of toxicology

Toxicogenomics, specifically reverse toxicogenomics, is about to open a new paradigm of toxicology from, at least, five aspects: first, the merging of such scientific borders as physiology and toxicology (also pharmacology and toxicology); second, a paradigm shift from "analog science" to "digital science"; third, visualization of hitherto unknown oscillatory changes behind homeostatic balances; fourth, comprehensive inter-species extrapolation; and lastly, a paradigm shift from inductive toxicology to deductive toxicology. As discussed previously in the first paragraph of this chapter, when we compare the aims of physiology, and toxicology, we find that they face opposite directions; however, participating molecules, seen in Figure 1, are presumably shared each other, physiologically as well as toxicologically, thereby implying that physiologic responses and toxicologic responses may be a continuum. In contrast, gene expression may not be continuum along with the dose response relationship, although a simple linear dose-response curve is generally accepted in the traditional toxicology. It appears to be clear that a different dose gives a different gene expression profile, in other words, the expression is expected to show not an analog change but a digital one, and a different dose behaves as a different chemical in the microarray. Although simple linear dose-response curves seem to apply in many cases, toxicological parameters may change discontinuously based on the genomic expression. This may be an advantage on one hand, because an appropriate array profiling of toxicologic responses can be eventually identified. On the other hand, there may still be a long way to go before reaching the final goal of defining the specific toxicologic array profiling for appropriate toxicologic phenotypes. The concept of safety borders, such as NOEL and NOAEL, may likely be re-established likely by means of such specific safety profiles. Visualization of invisible homeostatic balances is shown in the Figure 3. As was mentioned in the paragraph on oscillation strength, an additional new concept of "risk" may be re-established. Comprehensive 'interspecies'-extrapolation may be improved by informatics over different species such as mouse, rat, frog (Xenopus), and yeast. Interspecies extrapolation will be dealt with on a theoretical basis using a relatively small number of genes that are known to confer important allelic variations. This would result in better interspecies extrapolation, higher confidence of animal models, reduction in the number of animals needed for testing, shorter testing period, and most importantly, insights into pathways of toxicity and their mechanisms (US-EPA). Reverse toxicogenomics may be supported by the other four paradigm shifts mentioned above, and be used to predict toxicity and establish new concepts in risk assessment methodologies.

By means of toxicogenomics, we will be able to see a new toxicological world behind homeostasis and/or gene expression balance.

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3. ヒト全*MHC*遺伝子導入マウス
―染色体導入法によるヒト型モデル―

高木篤也・北嶋 聡・相賀裕美子

3. 1. はじめに

ヒトの腫瘍・組織をマウスに生着させるために，免疫機能の欠損したヌードマウス[1]やSCIDマウス[2]がよく用いられているが，これらのマウスでは免疫機能の一部が欠けているため，免疫療法などの研究への利用は限界がある．この問題を解決するため，近年，異種移植の拒絶の原因となるヒトタンパク質をコードする遺伝子をあらかじめマウスに導入することにより，ヒトタンパク質をマウスが拒絶しないようにする方法が試みられている[3]．異種移植の拒絶の原因として，糖鎖，自然抗体，補体など種々の因子があるが，それらのなかで重要なもののひとつに，主要組織適合遺伝子複合体(major histocompatibility complex，*MHC*)がコードするMHC分子がある[4]．なお，ヒトのMHC分子はヒト白血球抗原(human leukocyte antigen，HLA)とよばれる．MHC分子は同種移植や異種移植における細胞性免疫反応に関与しており[5]，その機能として，抗原ペプチドを結合し，T細胞に認識させることが知られている[6]．

そこで，この免疫反応に重要な役割をはたすヒトHLAをマウスに導入することにより，少なくともマウスとヒトのMHC分子の相違に起因する異種移植の細胞性免疫による拒絶反応を軽減させることが期待される．しかし，*HLA*遺伝子はその数がきわめて多いことが知られている．*HLA*遺伝子はクラスI遺伝子とクラスII遺伝子に大別され，さらに，両遺伝子の中間に位置する補体系遺伝子であるクラスIII遺伝子が存在する．クラスI遺伝子として，*HLA-A*遺伝子，*HLA-B*遺伝子，*HLA-C*遺伝子，*HLA-E*遺伝子，*HLA-F*遺伝子，および，*HLA-G*遺伝子が，クラスII遺伝子として，*HLA-DR*遺伝子，*HLA-DP*遺伝子，*HLA-DQ*遺伝子などが存在する[7](図3・1)．

これまで個々の*HLA*遺伝子を導入したトランスジェニックマウスはすでに作成されているが[8,9]，多数の*HLA*遺伝子を，その遺伝子制御領域までを含めてすべてマウスに導入するのは，多大の時間と労力を要するためこれまで作成されていない．そこで，ここでは，新しく開発されたミクロセル法を用いて[10]，全*HLA*遺伝子が存在する*HLA*遺伝子座を導入したマウスを作成したので紹介する．

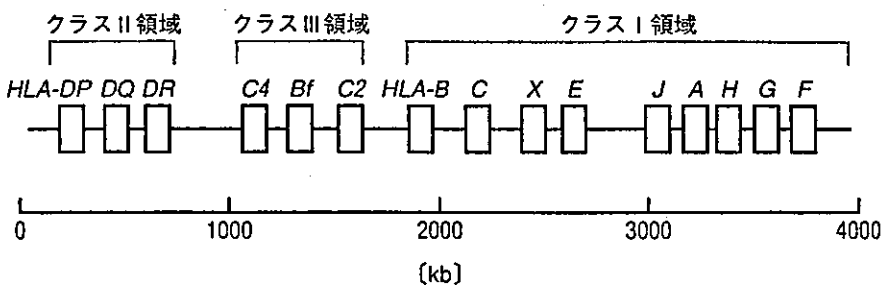

図3・1　ヒト*HLA*遺伝子領域に存在する遺伝子群．*Bf*：補体B因子遺伝子，*C2*：補体第2因子遺伝子，*C4*：補体第4因子遺伝子．

3. 2. 方法および結果

目的の*HLA*遺伝子座はヒト6番染色体短腕(6p21.3)に存在する．まず，ヒト6番染色体を1本だけもつようなマウスA9細胞の単離を行った．これには，正常ヒト細胞とマウスA9細胞をポリエチレングリコール法により細胞融合させ，ヒト-マウス融合細胞を作成し，さらに，コルセミドおよびサイトカラシンB処理により染色体を含むミクロセルを単離した．このミクロセルを再びマウスA9細胞と融合することにより，ヒト染色体を保持する多数の細胞クローンが得られた．ついで，この細胞よりDNAを抽出し，*HLA*遺伝子座をもつヒト6番染色体が存在する細胞をPCR法にてスクリーニングした．対象となる*HLA*遺伝子座(6p21.3)を含むヒト6番染色体の同定のために使用したマーカー遺伝子としては，ヒト6番染色体短腕に存在するエンドセリン-1遺伝子，プロラクチン遺伝子，TNF-α遺伝子，および，グアニル酸シクラーゼアクチベーター-1遺伝子と，長腕に存在する5′-ヌクレオチダーゼ遺伝子，インスリン様成長因子2受容体遺伝子を用いた(図3・2)．その結果，ヒト6番染色体短腕のみを有するクローンを得ることができた．さらに，染色体の状態を蛍光*in situ*ハイブリダイゼーションにより確認した結果，1クローンがヒト6番染色体短腕のみを独立に保持する良好なクローンであった(図3・3)．

このクローンからマウスES細胞(TT2F細胞)への染色体導入は，前述のミクロセル法を用いて同様に行った[10]．なお，得られたES細胞の段階で，*HLA-A*遺伝子，*HLA-E*遺伝子，*HLA-F*遺伝子，および，*HLA-G*遺伝子がmRNAレベルで発現していることを，RT-PCR法を用いて確認している．

キメラマウスの作成は，ES細胞と8細胞期の卵との凝集法で行った[11]．すなわち，MCHマウスから得た8細胞期の卵を酸性タイロード処理して透明帯を除去し，ES細胞と一晩培養することにより得られた胚盤胞を，偽妊娠MCHマウスの子宮に戻した．のべ800個の胚盤胞を移植した結果，比較的ES細胞の寄与率の高い(30～50％)，1匹の雌キメラマウスを得ることができた．このキメラマウスは，外見上は正常であり，さらに，このキメラマウスを正常な雄と交配して仔マウスを正常に出産させることができた．しかし，残念ながら，導入した遺伝子が伝達した仔マウスを得るには至らなかった．なお，得られたキメラマウスにおける*HLA*遺伝子mRNAの発現については，末梢血白血

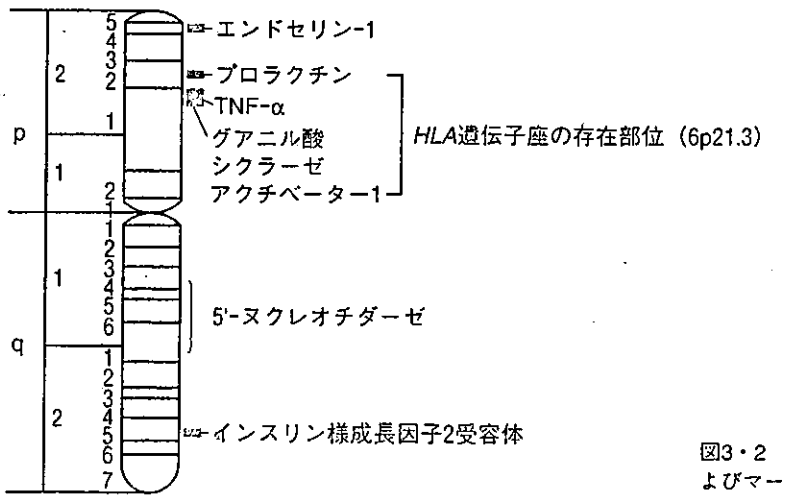

図3・2 ヒト6番染色体における*HLA*遺伝子およびマーカーとして用いた遺伝子の位置．

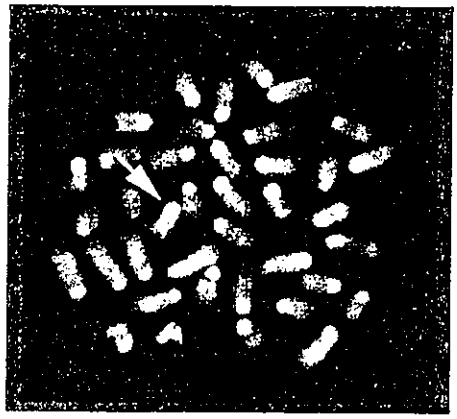

図3・3　ヒト6番染色体断片が導入されたES細胞の蛍光*in situ*ハイブリダイゼーション解析.

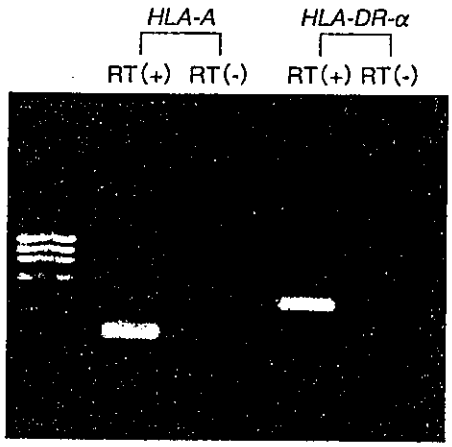

図3・4　ヒト6番染色体断片が導入されたキメラマウスの末梢血白血球における*HLA*遺伝子発現のRT-PCR法による解析．RT：逆転写酵素.

球のRNAを対象に，クラスI遺伝子に属する*HLA-A*遺伝子，および，クラスII遺伝子に属する*HLA-DR*遺伝子をRT-PCR法により検出した結果，それぞれ発現が確認された(図3・4)．さらに，HLAのタンパク質レベルの発現については，脾臓細胞を対象に，抗HLA-ABC抗体を用いたFACSにより解析した結果，発現が確認された．以上，導入した*HLA*遺伝子がキメラマウスの生体内で発現していることを確認することができた.

ヒト6番染色体をもつキメラマウスの骨髄細胞の機能解析，ならびに，次世代伝達によらない*HLA*遺伝子導入マウスの維持・拡大を目的に，ヒト全*HLA*遺伝子導入キメラマウスの骨髄移植を行った．種々の条件を検討した結果，ES細胞(TT2F細胞)と同じ系統のC57BL/CBA F1マウスをレシピエントとして用い，X線を全身に照射後，キメラマウス大腿骨から得た骨髄細胞を尾静脈より移植することにより良好な結果が得られた．すなわち，キメラマウスの骨髄移植の約4週間後に，骨髄移植されたC57BL/CBA F1マウスの脾臓細胞におけるHLAクラスI分子およびHLAクラスII分子の発現を，FACS解析により確認することができた.

これらの結果，マウスに導入された*HLA*遺伝子は正常な発現を示し，また，その骨髄をほかのマウスに移植しても，一定期間は*HLA*遺伝子が保持されることが示唆された．このことから，次世代伝達以外の方法で*HLA*遺伝子導入マウスを維持・拡張できる道筋が示され，今後，種々の免疫実験への利用が期待される.

3. 3. おわりに

以上，ヒトの細胞・組織のマウスへの移植に対する移植片拒絶免疫反応の軽減および解析のためのモデルマウスとして，全*HLA*遺伝子導入マウスの作成を行った．ミクロセル法によりヒト6番染色体断片を導入したマウスES細胞を用いることにより，種々のHLAを発現するキメラマウスを，効率は低いものの作成することができ，当初の目的はいちおう達成することができた．一方，このキメラマウスでは*HLA*遺伝子座をもつヒト染色体を次世代に伝達することができなかった．この原因として，用いたES細胞が操作の過程で次世代伝達の能力を失ったことが考えられるが，ほかの可能性として，ヒト6番染色体上に次世代伝達を阻害する遺伝子が存在することが考えられる．ヒト6番染色体は約180 Mbあり，今回，用いた短腕のみのものでも90 Mbもある．そこで，次の目標として，*HLA*遺伝子座(約4 Mb)に限定された領域の染色体断片のみをCre-*lox*P系を利用した新規染色体転座法によりES細胞へ導入し，これから作成したキメラマウスで染色体の次世代伝達をさせるべく研究を進めている．ひとたび，この次世代伝達が可能なマウスが樹立されれば，繁殖はもとより，*HLA*遺伝子以外の種々の拒絶に関与するヒト遺伝子を導入したトランスジェニックマウスやノックインマウス，あるいは，マウス本来の*MHC*遺伝子を欠失したノックアウトマウスなどと交配することにより，簡便により異種移植性にすぐれたマウスを作成できることが期待される．

なお，この研究は，医薬品機構井上プロジェクト「ヒト型モデル」(主任研究者 井上達)に基づく鳥取大学医学部の押村光雄教授との共同研究である．

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Evaluation of developmental toxicity of 1-butanol given to rats in drinking water throughout pregnancy

M. Ema [a,*], H. Hara [b], M. Matsumoto [a], A. Hirose [a], E. Kamata [a]

[a] *Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Ina Research, Inc., 2148 Nishininowa, Ina, Nagano 399-4501, Japan*

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Abstract

The objective of this study was to evaluate the developmental toxicity of 1-butanol in rats. Pregnant rats were given drinking water containing 1-butanol at 0.2%, 1.0% or 5.0% (316, 1454 or 5654 mg/kg/day) on days 0–20 of pregnancy. A significant decrease in maternal body weight gain accompanied by reduced food and water consumption was found at 5.0%. No significant increase in the incidence of pre- and postimplantation embryonic loss was observed in any groups treated with 1-butanol. Fetal weight was significantly lowered at 5.0%. Although a significant increase in the incidence of fetuses with skeletal variations and decreased degree of ossification was found at 5.0%, no increase in the incidence of fetuses with external, skeletal and internal abnormalities was detected in any groups treated with 1-butanol. The data demonstrate that 1-butanol is developmental toxic only at maternal toxic doses. No evidence for teratogenicity of 1-butanol was noted in rats. Based on the significant decreases in maternal body weight gain and fetal weight, it is concluded that the no observed adverse effect levels (NOAELs) of 1-butanol for both dams and fetuses are 1.0% (1454 mg/kg/day) in rats.

Keywords: 1-Butanol; Developmental toxicity; Teratogenicity; Fetal abnormality; Rat

1. Introduction

1-Butanol (CAS no. 71-36-3, *n*-butanol; *n*-butyl alcohol), a flammable colorless liquid with a rancid sweet odor, is widely used as an organic solvent and intermediate in the manufacture of other organic chemicals (IPCS/WHO, 1987). Exposure of the general population is mainly through its natural occurrence in food and beverages and its use as a flavoring agent (IPCS/WHO, 1987).

Several reports on the developmental toxicity of 1-butanol are available. Nelson et al. (1989a) reported the results of a developmental toxicity study in which SD rats were exposed to 1-butanol by inhalation for 7 hr/day on days 1–19 of pregnancy at 3500, 6000 and 8000 ppm (equivalent to estimated daily absorbed doses of 350, 600 and 800 mg/kg). They observed maternal deaths at 8000 ppm, decreases in maternal food consumption and fetal weight at 6000 and 8000 ppm, and an increased incidence of rudimentary cervical ribs at 8000 ppm, and concluded that 1-butanol was not a selective developmental toxicant in rats. Nelson et al. (1989b) conducted a behavioral teratology study in which female SD rats were given 1-butanol by inhalation at 3000 or 6000 ppm for 7 hr/day throughout pregnancy (the maternal exposure group); male rats were

Abbreviations: NOAEL, no observed adverse effect level

* Corresponding author. Tel.: +81 3 3700 9878; fax: +81 3 3700 1408.
E-mail address: ema@nihs.go.jp (M. Ema).

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similarly exposed for 6 weeks and mated to unexposed females (the paternal exposure group), and offspring were behaviorally and neurochemically examined. The data from all tests in their study were within the range of control data in other research conducted by their laboratory. Sitarek et al. (1994) reported a significant increase in the incidence of fetuses with abnormalities after administration of 1-butanol at 0.24–4.0% (300–5000 mg/kg/day) in drinking water during the pre-mating period for 8 weeks and throughout the mating and pregnant period. No maternal toxicity was found at any dose of 1-butanol. The no observed adverse effect level (NOAEL) was not derived from the results of their study, because significant increases in the incidence of fetuses with dilation of the subarachnoid space and dilation of the lateral ventricle and/or third ventricle of the brain were found even at the lowest dose (0.24%). They have concluded that 1-butanol is a developmental toxicant and produces anomalies in the skeleton and central nervous system.

The present study was conducted to determine whether or not morphological abnormalities could be produced in fetuses of rats given 1-butanol prenatally and designed to replicate the observations of the study by Sitarek et al. (1994).

2. Materials and methods

This study was performed in compliance with regulatory guidelines (MHW, 1997a) and accordance with the principles for Good Laboratory Practice (MHW, 1997b) and "Guidance for Animal Care and Use" of Ina Research, Inc.

2.1. Animals

International Genetic Standard (Crj: CD (SD) IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in reproductive and developmental toxicity studies and historical control data are available. Males at 10 weeks of age and females at 9 weeks of age were purchased from Tsukuba Breeding Center, Charles River Japan, Inc., (Yokohama, Japan). The rats were acclimated to the laboratory for 7 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Animals were reared on a basal diet (NMF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum and maintained in an air-conditioned room at 21–25 °C, with a relative humidity of 40–70%, a 12-h light/dark cycle, and ventilation with 16 air charges/hour. Virgin female rats were mated overnight with male rats. The day when sperm were detected in the vaginal smear was considered to be day 0 of pregnancy. The pregnant rats, weighing 217–273 g and 10–11 weeks of age, were distributed using a computerized randomization procedure (TOXstaff 21 system) into 4 groups of 20 rats each and housed individually.

2.2. Chemicals and dosing

1-Butanol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 1-butanol used in this study was 99.9% pure and a special grade reagent (Lot no. CER5688), and it was kept in a dark place at room temperature under airtight conditions. The purity and stability of the chemical were verified by analysis before and after the study. Rats were given 1-butanol in their drinking water at a concentration of 0 (control), 0.2%, 1.0% or 5.0% on day 0 through day 20 of pregnancy. The dosage levels were determined based on the results of our range-finding study in which administration of 1-butanol in the drinking water on days 0–20 of pregnancy caused decreases in maternal body weight gain and food and water consumption and tended to reduce in fetal weight at 4% and 7% in rats. 1-Butanol was dissolved in distilled water (Otsuka Pharmaceutical Factory, Inc., Naruto, Japan). The control rats were given only water. The stability of formulations in a dark and cool place under airtight conditions has been confirmed for up to 3 days. During use, the formulations were maintained under such conditions for no more than 3 days and were 95.7–103.5% of the target concentration.

2.3. Observations

The maternal body weight and water consumption were recorded daily, and food consumption was recorded every 3 or 4 days. The pregnant rats were euthanized by exsanguinations under ether anesthesia on day 20 of pregnancy. The peritoneal cavity was opened, and the numbers of corpora lutea, implantation sites and live and dead fetuses and resorptions were counted. The live fetuses removed from the uterus were sexed, weighed, measured among their crown-rump length, and inspected for external malformations and malformations within the oral cavity. Approximately one-half of the live fetuses in each litter were randomly selected and fixed in alcohol, stained with alizarin red S (Dawson, 1926) and examined for skeletal anomalies. The remaining live fetuses in each litter were fixed in Bouin's solution. Their heads were subjected to a free-hand razor-blade sectioning (Wilson, 1973) and the thoracic areas were subjected to microdissecting (Nishimura, 1974) to reveal internal abnormalities. The placental weight was also measured.

2.4. Data analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. The initial body

weight, body weight gain and food and water consumption of pregnant rats, numbers of corpora lutea, implantations and live fetuses per litter, fetal weight and crown-rump length and placental weight were analyzed with Bartlett's test (Snedecor and Cochran, 1980) for homogeneity of variance at the 5% level of significance. If it was homogeneous, the data were analyzed using Dunnett's multiple comparison test (Dunnett, 1955) to compare the mean of the control group with that of each dosage group, and if it was not homogeneous, the mean rank of the 1-butanol-treated groups was compared with that of the control group with the Dunnett type test. The Dunnett type test was used for the incidences of pre- and postimplantation embryonic loss and fetal anomalies and sex ratio of fetuses to compare the mean rank of groups treated with 1-butanol and that of the control group. The incidence of dams with anomalous fetuses was analyzed by Chi-square test or Fisher's exact test. The significance of differences from the control group was estimated at probability levels of 1% and 5%.

3. Results

Table 1 shows the maternal findings in rats given 1-butanol during pregnancy. No death was found in female rats of any group. All females in all groups became pregnant. The body weight gains on days 0–7 of pregnancy were significantly reduced at 5.0%. The body weight gain during the whole period of pregnancy was also significantly decreased at 5.0%. No significant decrease in the body weight gain was noted at 0.2 or 1.0, except for a transient decrease on days 0–2 of pregnancy at 1.0%. The food consumption on days 0–7, days 7–14, days 14–20 and days 0–20 of pregnancy was significantly lower in the 1.0% and 5.0% groups than the control group. The water consumption on days 0–7 at 1.0 and 5.0% and on days 7–14, days 14–20 and days 0–20 at 5.0% was significantly decreased. The mean daily intakes of 1-butanol were 316 mg/kg for the 0.2% group, 1454 mg/kg for the 1.0% group and 5654 mg/kg for the 5.0% group.

Reproductive findings in rats given 1-butanol during pregnancy are presented in Table 2. No litters totally resorbed were found in any group. No effects of the administration of 1-butanol were observed on the numbers of corpora lutea, implantations, pre- or postimplantation loss, resorptions or dead or live fetuses or sex ratio of live fetuses. The body weights of male and female fetuses were significantly lower in the 5.0% group than in the control group. There was no significant difference in the crown-rump length of male and female fetuses or placental weight between the control and groups treated with 1-butanol.

A summary of morphological findings in live fetuses of rats given 1-butanol during pregnancy is shown in Table 3. One fetus with spina bifida in the control group and one fetus with thread-like tail and anal atresia in the 0.2% group were observed. Skeletal examination

Table 1
Maternal findings in rats given 1-butanol on days 0–20 of pregnancy

Dose (%)	0 (Control)	0.2	1.0	5.0
No. of rats	20	20	20	20
No. of pregnant rats	20	20	20	20
No. of dead rats	0	0	0	0
Initial body weight	245 ± 14	247 ± 13	245 ± 11	244 ± 12
Body weight gain during pregnancy (g)[a]				
Days 0–7	44 ± 7	45 ± 7	40 ± 6	20 ± 28**
Days 7–14	40 ± 6	41 ± 5	41 ± 7	42 ± 10
Days 14–20	78 ± 14	82 ± 8	84 ± 7	75 ± 11
Days 0–20	162 ± 19	168 ± 16	165 ± 15	146 ± 16**
Food consumption during pregnancy (g)[a]				
Days 0–7	179 ± 12	180 ± 16	164 ± 12*	138 ± 21**
Days 7–14	193 ± 14	194 ± 17	177 ± 14**	160 ± 11**
Days 14–20	176 ± 14	175 ± 15	161 ± 12**	143 ± 11**
Days 0–20	548 ± 38	548 ± 46	503 ± 34**	441 ± 34**
Water consumption during pregnancy (ml)[a]				
Days 0–7	284 ± 28	305 ± 37	258 ± 29*	175 ± 34**
Days 7–14	318 ± 35	337 ± 48	299 ± 40	239 ± 80**
Days 14–20	328 ± 47	342 ± 47	334 ± 46	256 ± 85**
Days 0–20	930 ± 105	983 ± 126	890 ± 106	669 ± 182**
Mean daily intakes of 1-butanol (mg/kg)[a]	0	316 ± 30	1454 ± 186	5654 ± 1402

*,** Significantly different from the control, *$P < 0.05$ and **$P < 0.01$.
[a] Values are given as the mean ± SD.

Table 2
Reproductive findings in rats given 1-butanol on days 0–20 of pregnancy

Dose (%)	0 (Control)	0.2	1.0	5.0
No. of litters	20	20	20	20
No. of litters totally resorbed	0	0	0	0
No. of corpora lutea per litter[a]	16.4 ± 3.6	16.7 ± 3.0[d]	16.1 ± 2.1	16.3 ± 2.6
No. of implantations per litter[a]	14.3 ± 2.8	15.1 ± 1.7	15.2 ± 1.2	14.7 ± 2.5
% Preimplantation loss per litter[b]	9.0	9.0[d]	4.4	9.2
% Postimplantation loss per litter[c]	6.0	5.4	3.7	8.0
No. of live fetuses per litter[a]	13.4 ± 2.6	14.3 ± 1.4	14.7 ± 1.5	13.5 ± 2.5
Sex ratio of live fetuses (male/female)	128/139	145/140	149/144	131/139
Body weight of live fetuses (g)[a]				
Male	4.18 ± 0.27	4.00 ± 0.24	4.04 ± 0.25	3.83 ± 0.18**
Female	3.97 ± 0.25	3.86 ± 0.20	3.83 ± 0.16	3.59 ± 0.17**
Fetal crown-rump length (mm)[a]				
Male	40.5 ± 1.2	40.3 ± 1.4	40.2 ± 1.2	39.7 ± 1.3
Female	39.4 ± 1.2	39.4 ± 1.2	39.3 ± 1.1	38.5 ± 1.4
Placental weight (g)				
Male	0.50 ± 0.05	0.49 ± 0.05	0.48 ± 0.06	0.50 ± 0.06
Female	0.49 ± 0.05	0.48 ± 0.05	0.47 ± 0.05	0.49 ± 0.06

** Significantly different from the control, $P < 0.01$.
[a] Values are given as the mean ± SD.
[b] (No. of preimplantation embryonic loss/no. of corpora lutea) × 100.
[c] (No. of resorptions and dead fetuses/no. implantations) × 100.
[d] Value was obtained from 19 pregnant rats.

revealed one fetus with supernumerary thoracic vertebral bodies and malpositioned thoracic vertebrae at 1.0%. Although the total number of fetuses with skeletal variations was significantly increased at 5.0%, the number of fetuses with individual skeletal variations was not significantly increased, except for fetuses with short supernumerary ribs at 5.0%. A significantly lower number of forepaw proximal phalanges was observed at 5.0%. Membranous ventricular septum defect occurred in one fetus of the control and 0.2% groups and 3 fetuses in 3 dams of the 5.0% group. One fetus with a double aorta in the control group and one fetus with a left umbilical artery in the control and 2.0% groups were observed. Thymic remnants in the neck were found in 4–11 fetuses of the control and groups treated with 1-butanol. However, there was no significant difference in the incidence of fetuses with internal abnormalities between the control and groups treated with 1-butanol.

4. Discussion

The present study was conducted to determine the developmental toxicity of 1-butanol and designed to replicate the observations of the study by Sitarek et al. (1994). The data showed that prenatal administration of 1-butanol did not produce morphological anomalies in fetuses of rats. Thus, we have been unable to confirm the results of Sitarek's study in which prenatal exposure to 1-butanol produced fetal anomalies.

The doses of 1-butanol used in the present study expected to induce maternal and/or developmental toxicity, such as a decrease in maternal body weight gain and fetal weight, were given to pregnant rats during the whole period of pregnancy to characterize the effects of 1-butanol on embryonic/fetal development. Maternal toxicity, a significant decrease in body weight gain, was found at 5.0%. Maternal food and water consumptions were also reduced in this dose group. Although the only significant decrease in maternal body weight gain was observed on days 0–2 of pregnancy at 1.0%, this decrease was occasional and discontinuous and seems unlikely to be of toxicological significance. In this dose group, decreases in the maternal food consumption during the whole period of pregnancy and water consumption during the early period of pregnancy, which were unaccompanied by the continuous changes in body weight gain, were observed. No significant changes in maternal parameters were noted in the 0.2% group. These findings in maternal rats indicate that 1-butanol exerts maternal toxicity at 5.0% (equivalent to 5654 mg/kg/day) when administered during the entire period of pregnancy in rats.

No significant increase in the incidence of postimplantation loss was found at any dose of 1-butanol, and significantly decreased weights of male and female fetuses were found at 5.0%. No significant adverse effects on reproductive parameters were detected at 0.2% and 1.0%. These findings indicate that 1-butanol is not toxic to embryonic/fetal survival up to 5.0% or fetal growth up to 1.0% when administered during the whole period of pregnancy.

As for morphological examinations in the fetuses of exposed mothers, a few fetuses with external, skeletal

Table 3
Morphological examinations in fetuses of rats given 1-butanol on days 0–20 of pregnancy

Dose (%)	0 (Control)	0.2	1.0	5.0
External examination				
Total no. of fetuses (litters) examined	267 (20)	285 (20)	293 (20)	270 (20)
Total no. of fetuses (litters) with abnormalities	1 (1)	1 (1)	0	0
Spina bifida	1 (1)	0	0	0
Thread-like tail and anal atresia	0	1 (1)	0	0
Skeletal examination				
Total no. of fetuses (litters) examined	139 (20)	147 (20)	152 (20)	140 (20)
Total no. of fetuses (litters) with abnormalities	0	0	1 (1)	0
Supernumerary of thoracic vertebral bodies and malpositioned thoracic vertebrae	0	0	1 (1)	0
Total no. of fetuses (litters) with variations	28 (11)	23 (12)	52 (17)	69 (20)**
Bipartite ossification of thoracic centra	1 (1)	1 (1)	1 (1)	7 (5)
Dumbbell ossification of thoracic centra	0	1 (1)	2 (2)	3 (3)
Bipartite ossification of lumbar centra	0	0	0	2 (2)
Supernumerary lumbar vertebrae	4 (1)	1 (1)	5 (3)	5 (2)
Lumbarization	0	0	1 (1)	1 (1)
Bipartite ossification of sternebrae	1 (1)	1 (1)	1 (1)	1 (1)
Misaligned sternebrae	0	0	0	1 (1)
Cervical ribs	2 (2)	3 (3)	3 (3)	7 (5)
Full supernumerary ribs	5 (2)	1 (1)	10 (5)	9 (5)
Short supernumerary ribs	20 (10)	18 (9)	43 (16)	55 (19)**
Wavy ribs	0	0	0	1 (1)
Degree of ossification[a]				
No. of sacral and caudal vertebrae	8.4 ± 0.5	8.4 ± 0.4	8.3 ± 0.5	8.1 ± 0.3
No. of sternebrae	5.9 ± 0.2	5.8 ± 0.2	5.8 ± 0.2	5.8 ± 0.2
No. of forepaw proximal phalanges	1.6 ± 1.3	1.6 ± 0.9	1.2 ± 1.1	0.3 ± 0.4**
Internal examination				
Total no. of fetuses (litters) examined	128 (20)	138 (20)	141 (20)	130 (20)
Total no. of fetuses (litters) with abnormalities	7 (6)	9 (6)	11 (8)	14 (9)
Membranous ventricular septum defect	1 (1)	1 (1)	0	3 (3)
Double aorta	1(1)	0	0	0
Left umbilical artery	1 (1)	0	1 (1)	0
Thymic remnant in neck	4 (4)	8 (5)	10 (8)	11 (8)

** Significantly different from the control, $P < 0.01$.
[a] Values are given as the mean ± SD.

and/or internal abnormalities were found in all groups. The abnormalities observed in the present study are not thought to be due to the administration of 1-butanol, because they have occurred at a very low incidence and are of types that occur sporadically among control rat fetuses (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000). Several types of skeletal variations were also found in the control and groups treated with 1-butanol. These skeletal variations are frequently observed in fetuses of rats at term (Kimmel and Wilson, 1973; Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000). In the 5.0% group, a significant increase in the incidence of fetuses with skeletal variations and fetuses with short supernumerary ribs, but not full supernumerary ribs, and a significant decrease in the degree of ossification were accompanied by a significant decrease in the fetal weight. These findings show a correlation between these morphological alterations and growth retardation in fetuses. Although a skeletal variation, i.e., full supernumerary ribs, is a warning sign of possible teratogenicity, short supernumerary ribs, sternebral variations, and bilobed centra of the vertebral column are normal variations (Kimmel and Wilson, 1973). Chahoud et al. (1999) noted that variations are unlikely to adversely affect survival or health and this might result from a delay in growth or morphogenesis that has otherwise followed a normal pattern of development. Consideration of these findings together suggests that the morphological changes in fetuses observed in the present study do not indicate a teratogenic response and that 1-butanol possesses no teratogenic potential in rats.

In Sitarek's study (1994), significant increases in the incidences of wavy ribs at 300 mg/kg/day, dilation of the subarachnoid space and dilation of the lateral ventricle and/or third ventricle of the brain at 300 mg/kg/day and higher, dilation of the renal pelvis and external hydrocephaly at 1000 mg/kg/day, internal hydrocephaly at 1000 mg/kg/day and higher, and supernumerary ribs and delayed ossification at 5000 mg/kg/day were found. A significant decrease in fetal crown-rump length was