

Table 4. Concentration, detection number, and detection frequency (%).

Range (%)	DBP	BBP	DEHP	DNOP	DINP-1	DINP-2	DIDP	TMPDIB	DEHA
<0.1	0	0	3	0	0	1	0	0	2
0.1 ~ 1<	1	0	1	1	1	0	1	0	2
1 ~ 10<	1	0	4	0	2	3	1	0	0
10 ~ 20 <	2	0	13	0	1	0	0	0	0
20 ~ 30 <	0	0	10	0	0	1	0	0	0
30 ≤	0	0	6	0	1	1	0	0	0
Detection number	4	0	37	1	5	6	2	0	4
Detection frequency	8.5	0	79	2.1	11	13	4.3	0	8.5

In addition, BBP and TMPDIB were not detected in the all samples in this study. Some samples, e.g., A6, contained several kinds of PAEs. Most samples contained DEHP and DINP in high concentrations (over 0.1 %). In some previous studies, detection frequencies of DINP in toy products in Japan^[22–24] and other countries^[33] were higher than the corresponding detection frequencies of DEHP. Furthermore, similar detection frequencies of DINP and DEHP in PVC toys and childcare articles were reported in the market survey carried out in Germany, Austria, and Switzerland.^[34] However, detection frequencies of DEHP in household products were higher than those of DINP,^[20] which is in agreement with the results of our study.

Two of the products studied, the float ring (C2) and the beach ball (C3), displayed the safety toy mark and safe use of plasticizer mark, indicating that their contents conform to the regulation of PAEs by the Japan Toy Association and the Japan Inflatable Vinyl Products Manufactures Association, respectively. However, the mouth-piece of the float ring (C2.1) contained 0.097 % of DEHP and 0.075 % of DINP-2. The presence of these PAEs might be due to impurities of other plasticizers or contamination during the manufacturing process.^[24,33] Four eraser samples were studied (A3, A7–9), and all of them contained high levels of DEHP and A7–9 also contained high levels of DBP. DEHP and DBP are often used together in household products;^[20,21] the total concentration of PAEs (DEHP + DBP) in the eraser samples was over 40 %. Erasers that do not contain PVC or phthalates have recently become available, but our results indicate that a large proportion of erasers currently sold still contain high levels of PAEs. DINP-1 and 2 showed similar detection frequencies in the PVC containing household products.

Since small amounts of DEHP can be present as an impurity in DINP,^[33] the DEHP detected in the emblem sample (B2) could be an impurity of DEHP. DINP and DEHP can be used as a mixture to improve resistance to low temperatures.^[1] Therefore, DINP might have been used intentionally with DEHP for the pipe chair (F2) that can be used outdoors. Even though DIDP production is greater than DBP production in Japan^[1] and it has been found in PVC toys in other countries,^[33,34] its levels were only recently investigated in toys from Japan.^[32] In our study, DIDP was detected in the cutting mat (A6) and mobile

phone strap (D7). Recently, Abe et al. investigated the concentration of DIDP in the PVC toys circulated in Japan.^[32] In this study as well, the presence of DIDP in household products was observed.

Exposure to PAEs from PVC household products

Our study has confirmed that high amounts of PAEs, particularly DEHP, are presented in PVC household products in Japan. In Japan, the tolerable daily intake (TDI) of DEHP was established as being 40–140 $\mu\text{g kgbw}^{-1} \text{day}^{-1}$ on the basis of its testicular and reproductive toxicity in rats^[2] and the TDI of DINP was established as being 150 $\mu\text{g kgbw}^{-1} \text{day}^{-1}$ on the basis of its hepatic toxicity in rats.^[5] Sugita et al. conducted an oral release study of DINP to estimate the amount of daily oral exposure of babies to PAEs derived from soft PVC baby toys.^[35]

For a migration test, they used PVC products containing 38–58 % of DINP, and concluded that there were no differences in migration amounts between PVC products containing different concentrations of DINP and that the migration tendency of DEHP was similar to that of DINP. In addition, they found the migration amounts of DINP and DEHP to be greater than those of TDI in certain cases. The erasers and the mobile phone straps (LCD cleaner type) investigated in this study are small; therefore, children could easily lick, chew, and ingest these products. As a result, it is important to reduce the probability of contact of children with such products containing high PAE concentrations.

In Japan, it has been reported that PVC products mounted at the edge of furniture to prevent children from injury (such as E1, E5, E6, and E7) can be chewed on and ingested by children.^[36] Large amounts of DEHP and DINP were contained in these products investigated in this study (maximum concentration of 47 %). Thus, it is advisable to replace plasticizers used in such household products with the suitable alternatives.

Hand-to-mouth contact is a important route of exposure to BFRs because of the dermal exposure of BFRs through house dust or direct contact with products containing BFRs.^[18] Bisphenol A (BPA) can be transferred from thermal printer paper to the finger skin.^[37] Since the PAE concentrations of the products considered in this study

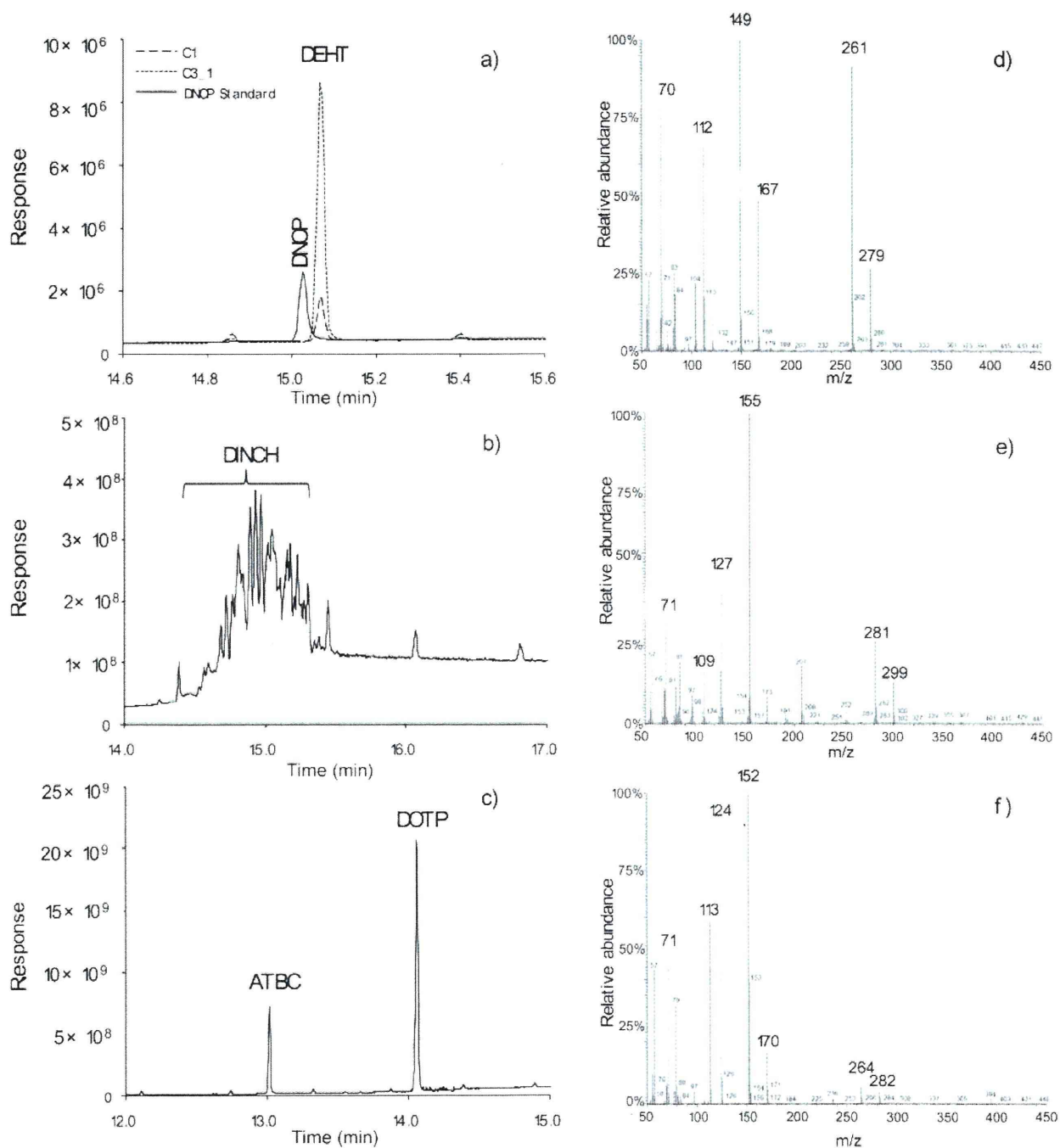


Fig. 1. Identification of unknown peaks in GC/MS chromatograms. a) Mass chromatogram of DNOP standard solution and samples C1 and C3.1 (selected ion monitoring mode: $m/z = 149$) and total ion chromatogram (scan mode: $m/z = 50-450$) of b) C2.2 and c) C5. Mass spectra of unknown peaks obtained by scan analysis (scan mode: $m/z = 50-450$) for d) DEHT (C1), e) DINCH (C2.2), and f) DOTP (C5).

are high, it is reasonable to assume that larger amounts of PAEs could be transferred to the skin surface from household products as compared to amounts of BFRs and BPA. Furthermore, PAEs may migrate easily from PVC products since they are not bound to plastic resin. Thus, a

large amount of PAEs might be transferred to the skin surface from the chair, sofa, or mat samples investigated in this study. Although the dermal absorption of PAEs is not immediate,^[7,8] determining of the level of dermal exposure is still important in order to estimate the amount of

PAEs ingested by hand-to-food or hand-to-mouth contact. Therefore, further study is necessary to estimate amounts of the PAEs transferred from household products to the skin surface.

Other plasticizers detected in PVC household products

Recently, Abe et al. detected and identified many kinds of non-phthalic plasticizers used in PVC toy products in Japan.^[32] In this study, other compounds were also detected in the household products samples that contained no or small amounts of the PAEs studied. We also carried out identification in these unknown peaks. The retention time of the unknown peak detected in samples C1, C3_1, and D4 was very close to DNOP and had a characteristic fragmentation ion of phthalate ($[m/z] = 149$) (Fig. 1a). This peak

was identified as based on EI-mass spectra reported in the previous study^[38] and estimated di(2-ethylhexyl) terephthalate (DEHT) (Figs. 1a and d). The unknown compound detected in samples C2_1, C2_2, C2_3, and C3_2 was identified as diisononyl 1,2-cyclohexanedicarboxylic acid (DINCH) on the basis of EI-mass spectra^[34,38] and the presence of isomers (Figs. 1b and e). Another unknown peak detected in samples C5 and D9 was identified as acetyl tributyl citrate (ATBC, Fig. 1c) using the NIST mass spectral library (NIST 05 ver2.0). ATBC is a non-phthalic plasticizer and has previously been detected in PVC toys and sports products^[21,24,32] similar to DEHA and TMPDIB. Sample C5 also contained an additional unknown peak identified using NIST as di(2-ethylhexyl) 4-cyclohexene-1,2-dicarboxylate (DOTP, Figs. 1c and f). DOTP is used as plasticizer for PVC and lubber products. It is a cyclic fatty

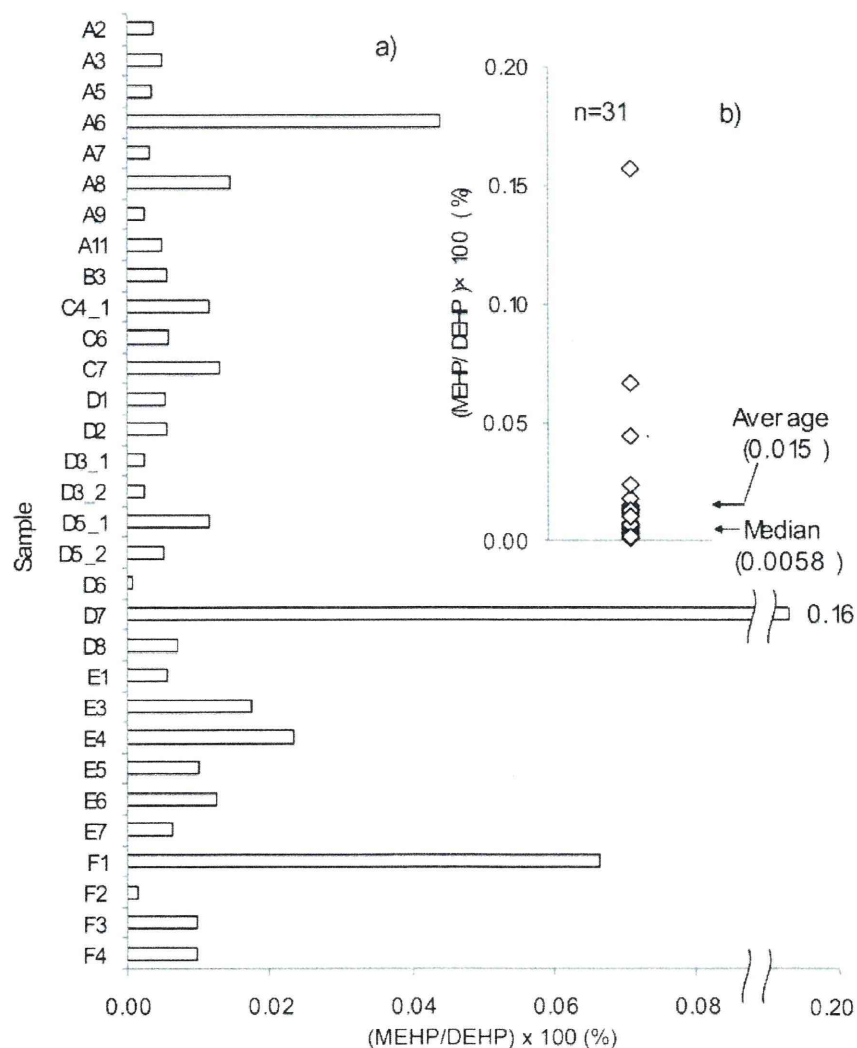


Fig. 2. MEHP in PVC household products. a) Ratios of MEHP against DEHP in the samples that contained MEHP at levels above the MQL, and b) their distribution.

acid ester highly suitable for sol preparing with less viscosity changes and is more resistant to cold temperatures than DEHP and other PAEs.^[39] To the best of our knowledge, this is the first case of DOTP detection in PVC household products. Identification of DEHT, DINCH, ATBC, and DOTP was confirmed by comparing retention times and mass fragment ion patterns from GC/MS to commercial standards.

During the last decade, new plasticizers have been used in PVC products as replacement for PAEs; some examples of such plasticizers include DINCH and DEHT.^[38] DINCH was produced by BASF (commercial name: Hexamoll) and initially used in medical devices and toys. DEHT is an isometric compound of DEHP and is also used in medical devices. Recently, DINCH and DEHT have been finding widespread use in products such as children's toys, childcare products, coatings, and sports goods.^[38] In the market survey carried out in European countries, many kinds of plasticizers in PVC toys and childcare articles were analyzed and it was reported that nearly half of the samples contained DINCH as principal plasticizers.^[34] Concentrations of DINCH and DEHT in house dust samples have increased significantly during the last decade in Germany.^[38] DINCH and DEHT have also been detected in PVC toy products in Japan.^[32] In our study, these compounds were detected mainly in sports goods for children (for example, C1, C2, and C3).

Concentrations of MEHP in PVC household products

MEHP was detected in 31 (70%) of the household products studied at concentration ranging from trace amounts to 40 $\mu\text{g g}^{-1}$ (Table 3). MEHP was only detected in samples that contained DEHP. The ratio (%) of MEHP against DEHP was calculated in samples containing both compounds above the MQLs (Fig. 2). The ratio ranged from 6.2×10^{-4} to $1.6 \times 10^{-10}\%$ with a median of $5.8 \times 10^{-3}\%$ and average of $1.5 \times 10^{-2}\%$. In particular, the ratio of the LCD cleaner of mobile phone (D7, $1.6 \times 10^{-10}\%$), the children's sofa (F1, $6.6 \times 10^{-20}\%$) and the cutting mat (A6, $4.4 \times 10^{-20}\%$) were relatively higher than the other samples. Our results are similar to a those of a previous study of MEHP in three PVC balls and two soft toys^[26] that revealed concentrations ranging from 0.04 to 41.81 $\mu\text{g g}^{-1}$ and ratio of MEHP against DEHP from 1.9×10^{-2} to 2.0×10^{-2} . The ratio of MEHP against DEHP in the house dust collected from 21 Japanese houses ranged from 0 to 81% (median 9.7%).^[25] Because the ratios obtained in this study were much lower than those of the house dust samples, MEHP in the household products investigated in this study might be presented as an impurity in DEHP. It is thought that DEHP in PVC household product is more important than MEHP to understand the exposure risk because large amount of DEHP is contained in PVC household products. Furthermore, DEHP easily migrate into mouth via saliva^[35] and then it is hydrolyzed to MEHP by endogenous esterase of many tissues.^[3,4]

However, migration rate of MEHP from PVC household product is unknown. Thus, it is necessary to investigate the migration tendency of MEHP from PVC household product for more accurate exposure risk analysis.

Conclusion

Household PVC products in Japan were analyzed for six kinds of PAEs (DEHP, DBP, BBP, DINP, DNOP, and DIDP), DEHA, TMPDIB, and MEHP. The products were selected on the basis of the probability of children placing them in their mouths and/or contacting with their skin (41 products; 47 samples). The detection frequencies of the compounds studied were as follows: DEHP (79%), DINP-2 (13%), DINP-1 (11%), DBP (8.5%), DEHA (8.5%), DIDP (4.3%), and DNOP (2.1%). Concentrations of these compounds ranged from 0.021% to 48%. BBP and TMPDIB were not detected in the all samples. Most samples contained DEHP and DINP at concentrations that were some orders of magnitude over the regulatory limit of 0.1%. The high concentrations of PAEs detected in products that children often place in their mouths reveal the importance of replacing plasticizers in common household products, and not just in children's toys, with safer alternatives. DEHT, DINCH, ATBC, and DOTP used as substitute plasticizers were observed in some samples. MEHP was presented in 70% of the samples, ranging from trace amounts to 140 $\mu\text{g/g}$, and was most probability an impurity in DEHP.

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TRANSFER OF PHTHALIC ACID DIESTERS FROM MODEL PVC SHEET TO SKIN SURFACE

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Introduction

Chemicals used in household products may diffuse to indoor environments, and humans may inhale these chemicals. Some of these chemicals are adsorbed on house dust, and humans may ingest the house dust¹. In addition, some chemicals may be transferred to the skin surface, and human exposure to these chemicals by skin absorption or hand-to-mouth contact has been indicated². Thus, studies investigating the transfer of chemicals, for example, parabens³, bisphenol-A⁴ and fragrances⁵, to human skin surfaces have been carried out. Phthalic acid diesters (PAEs) are widely used as plasticizers in various products, particularly those made from polyvinyl chloride (PVC). Since PAEs migrate easily from plastic products, and their content in plastic materials is high, these chemicals are of great concern globally. Although oral exposure of children to PAEs by chewing or licking toys and childcare products has been studied⁶, the transfer of PAEs from household products to skin surfaces have not been investigated. Thus, we examined the transfer of PAEs from PVC household products to forearm skin surfaces using model PVC sheets. We have already surveyed the PAE contents of PVC household products in Japan⁷, and reported that high detection frequencies and large amounts of di(2-ethylhexyl) phthalate (DEHP). In addition, we reported that diisononyl phthalate (DINP) and di-*n*-butyl phthalate (DBP) were also detected in PVC household products. Thus, we selected these PAEs as the target chemicals for this study. It was thought that sebum was an important factor in PAEs transfer to skin surface, and it has been reported that triglyceride (TG) is the dominant component in human sebum⁸. Therefore, we examined the relationships between the amounts of PAEs transferred to the skin surface and of TG collected from the skin surface.

Materials and methods

Eleven healthy adult subjects, seven men and four women, aged 31-57 years, participated in this study. The ethical review boards of the National Institute of Health Sciences approved this study (No.175). Written informed consent was obtained from all subjects.

Environmental analysis grade DBP, DEHP, and the deuterated PAEs [DBP, DEHP, di-*n*-octyl phthalate] used as internal standards were obtained from Kanto Chemical Co., Inc. DINP (CAS. 28553-12-0) was obtained from Wako Pure Chemical Ind., Ltd. Pesticide residue grade ethanol, acetone, and hexane were obtained from Kanto Chemical Co., Inc., Wako Pure Chemical Ind., Ltd. and Sigma-Aldrich, respectively.

PAEs analysis grade anhydrous sodium sulfate was obtained from Wako Pure Chemical Ind., Ltd. TG analysis was performed using a TG analytical kit purchased from Bio Vision. Triton-X 100 used for TG analysis was obtained from Sigma-Aldrich. Deionized water was produced by Milli-Q Synthesis A10 (Millipore). All utensils made of glass, metal, or Teflon were heated at 250°C for more than 12 h to prevent contamination. DEHP and DBP used for the production of model PVC sheets were obtained from Tokyo Chemical Ind., Co. DINP (Cas.28553-12-0) used for the PVC sheets was purchased from Wako Pure Chemical Ind., Ltd. The PVC polymer (S-1003) and stabilizer (AC-255) used for the PVC sheets were industrial materials. The concentrations of PAEs in the PVC sheet were determined by GC/MS⁷ and these are listed in Table 1.

A square model PVC sheet (1.5 cm x 1.5 cm = 2.25 cm², weight and thickness approximately 83 mg and 0.3 mm, respectively) was used for this study. The PVC sheet was contacted with inside of the forearm, and fixed by a rectangular silicone mat and surgical tape. After 30 min, the sheet was removed and the part of the skin surface contacted with the sheet was wiped with a clean applicator wetted with ethanol. The applicator was then placed in a test tube and PAE was extracted with acetone. The sample solution was dehydrated with anhydrous sodium sulfate, and the volume was adjusted to 10 ml. Finally, an internal standard solution was added to 1 ml of the sample solution and the sample was analyzed by GC/MS. The transfer experiment for each dose level was carried out in triplicate (n=3). Control samples (using a Teflon sheet of the same size) and blank samples were also analyzed to measure the PAEs background levels and to confirm contamination during the experimental process. The PAE amounts transferred to the skin surface were calculated as the average of three sheets. These experiments were carried out twice per subject [1st: DBP 28%, DEHP 15%, 28%, 37%, DINP 28%; 2nd: DBP 15%, DINP 15%, two-mixed sheets (DEHP and DBP, DEHP and DINP)] on different days. A control sample was used to measure the TG amounts. The control sample solution (9 ml) was concentrated and dried under a gentle N₂ stream. The residue was dissolved in 5% Triton-X aqueous solution and the TG was analyzed using a TG measurement kit according to the Bio Vision protocol.

Table 1. Concentrations of PAEs in the model PVC sheet (n=3).

PAEs	Blending ratio of PAE (%)	Average (%)	SD	CV (%)
DBP	15	11.9	0.47	3.9
	28	24.5	0.58	2.4
	14 ^a	11.4	0.18	1.6
DEHP	15	12.3	0.22	1.8
	28	23.5	0.14	0.6
	37	33.6	2.0	5.8
	14 ^a	12.5	0.13	1.0
DINP	14 ^b	12.3	0.54	4.4
	15	13.1	0.16	1.2
	28	26.1	0.72	2.8
	14 ^b	13.4	0.24	1.8

^amixture of DEHP and DBP. ^bmixture of DEHP and DINP.

Results and discussion:

DEHP was detected in the control samples collected from all subjects (n=22, 0.013-0.11 µg/cm²). DBP and DINP were not detected in the control samples collected from all subjects, excepting subject D. DBP was detected in the control samples collected from subject D (0.14 and 0.48 µg/cm²). In a previous study, benzyl butyl phthalate and DBP were detected at levels of 0.79 and 0.90 ng/cm² (as median) in the wiped

samples collected from children's skin surfaces⁹. The background amounts of DEHP detected on skin surfaces in this study were higher than those of other PAEs because DEHP is used with high frequency and in large amounts in PVC household products⁷. Stapleton et al. determined brominated flame retardants on skin surfaces (average 251 pg/cm²)². Methyl paraben has been detected on forefinger skin surfaces (approximately 0.2 µg/cm², maximum value)³. These differences in detection levels among chemicals used in household products probably reflect their physico-chemical properties and usage.

Noticeable differences in the amounts of PAEs transferred to the forearm skin surface were not observed among all subjects, except in the case of the 28% and 37% sheets for subjects D and I (Fig. 1, for example DEHP). Subjects D and I were women, and they did not use cosmetic items before these experiments. The reason for this high transferability is still unknown. The amounts of DEHP transferred from the 28% and 37% sheets were relatively high compared to those transferred from the 15% sheet. However, differences between the 28% and 37% sheets were not observed (Fig. 1). The amounts of each PAE transferred from the mixed sheets were higher than those from single component sheet (15% sheet). The plasticity of the mixed sheet was higher than that of the 15% sheet because of the large total amount of plasticizer. Thus, the PAEs included in the mixed sheet bled easily on contact with the skin. To examine the transferability of PAEs from the PVC sheet to the skin surface, the transferred amounts of DBP or DINP were compared with that of DEHP for the mixed sheets (Fig. 2). The relationships between the transferred amounts of DBP or DINP and that of DEHP were observed; the slopes of their regression lines were 0.503 or 1.16, respectively. The ratios of DBP/DEHP and DINP/DEHP in the mixed sheets were 0.912 and 1.09 (w/w), respectively. Thus, the order of the transferability of these PAEs to the skin surface might be DINP, DEHP, DBP. In addition, the average amount of PAEs transferred to the skin surface from the 15% sheet was correlated with their octanol-water partition coefficient (K_{ow}). Thus, the hydrophobic properties of the PAEs may influence their transferability to the skin surface from PVC sheet.

The average amount of TG collected from the skin surfaces of all subjects was 9.3 nmol/cm² (n=22, 3.4-18.3 nmol/cm²). This value was relatively high compared to those in a previous study that measured forearm sebum using a sebumeter¹⁰, and slightly lower than in another previous study which used standardized adhesive patches designed to collect sebum¹¹. Generally, men have higher amounts of sebum than woman¹². However, differences in sebum amounts, based on age and sex, were not observed among

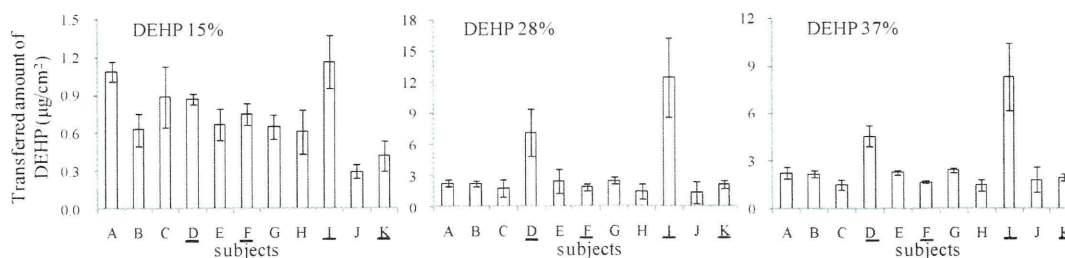


Fig. 1. Amounts of DEHP transferred from model PVC sheet to the skin surface (average \pm SD).
(Alphabetical order means in order of age and underline means female subject)

the subjects in this study. No obvious relationship between the amounts of PAEs transferred to the skin surface and the amounts of TG collected from subject's skin was observed. It was thought that the PAEs were transferred to a very thin layer of skin surface, although the TG collected in this study might be collected not only from this very thin layer but also from under the thin layer of surface skin. Therefore, the method of sampling sebum on the skin surface might affect examination of the relationship between PAEs transferred and TG. In addition, TG is composed of several kinds of fatty acids and other compounds such as wax-esters and squalene, which are also components of sebum on the skin surface. Thus, it is necessary to examine the relationships between PAEs transferred to the skin surface and sebum components to evaluate the transferability of PAEs to the skin surface.

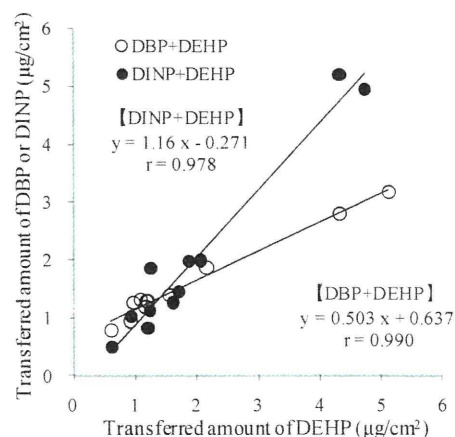


Fig. 2. Relationships between amount of DEHP and DBP or DINP transferred from mixture sheet.

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ヒトハザード評価における新しい流れと課題^{*1}

New Trends and Issues in the Evaluations of Hazard to Humans

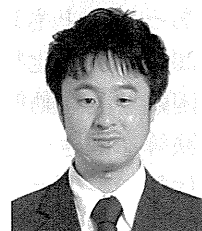
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1. はじめに

化粧品・食品・医薬品・その他一般化学物質等の人体でのハザード評価は、従来は専ら動物試験での情報を基礎としてきている。すなわち、試験で決定される用量作用関係に基づき、試験動物とヒトとの種差、ヒト集団内での感受性の差異等や現実的なシナリオでの曝露評価を考慮してリスク評価が行われ、その結果に基づいて実際の製品中での用量や社会での管理レベルが決定される。しかしながら近年、動物愛護という社会的要請や、科学的根拠に基づいたより正確な応答予測を求めるといった科学的要請から、非動物試験手法の開発と行政的導入が世界的に強く求められている。

本稿では、まず、化学物質のヒト影響評価と管理について昨今の新しい社会的・科学的背景を概説し、次に非動物試験に基づくヒト個体影響の試みとして、培養肺胞モデルと数理モデルを用いた経肺曝露時の体内取り込み量予測を紹介する。最後に、理想的なヒトハザード評価手法体系の将来像について考察する。

2. 動物実験代替という社会的要請

2.1 動物実験代替の3Rs原則

上述の社会的潮流を象徴する言葉が「動物実験代替」であり、それは3つのR原則(3Rs)からなる。すなわち、動物数の削減(Reduction)、苦痛の軽減(Refinement)および非動物試験法への置き換え(Replacement)である。動物の使用が科学的に必須である場合でも、使用数の削減と動物の苦痛の

軽減に最大限の配慮を払うべきであるし、究極の目的である非動物試験法への置き換えの実現のためには、本稿で述べるように実にさまざまな学問の成果を融合的に利用する必要がある。

この3Rs原則は、1998年8月にイタリアのボローニャで開催された第3回動物実験代替法世界会議で採択されたことから、ボローニャ宣言とも呼ばれる¹⁾。この3Rs原則は、今やわが国の改正動物愛護法(2005年)にも明記されている²⁾。

2.2 代替法の分野ごとの導入可能性

このような動物実験代替の可能性を対象製品・物質ごとに示した(図1)。導入可能性はおおよそ4つの指標で決定されている。すなわち、愛護運動・潜在的ヒトリスク・代替法開発の困難性・対象物質数である。

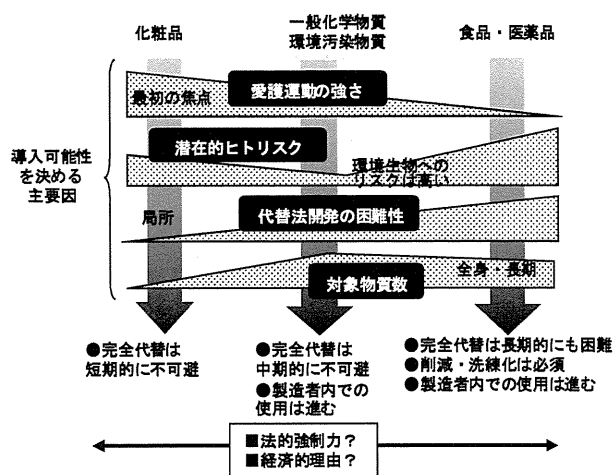


図1 分野ごとの代替法導入の可能性

*1 原稿受理 2011年4月18日

代替法の開発と導入が最も早くから進んだのは化粧品分野である。これは愛護運動がドレイズ試験のひとつであるウサギを用いる目刺激性試験を最初の焦点としたことが最大の理由である。化粧品は局所的に投与されることから、潜在的ヒトリスクは比較的小さく、代替法開発は比較的容易であったことも理由となる。現にEUでは、すでに2009年より化粧品指令第7次改正により、代替が困難な薬物動態試験・発生毒性試験・反復投与試験等を除き(これらは2013年が非動物試験による代替の目標とされている)、動物による安全性試験が化粧品分野においては禁止されており、動物試験で安全性が確認された製品はEU域内で販売ができない²⁾。

一方、対極に位置するのは、食品・医薬品であり、潜在的ヒトリスクは高く、個体の長期影響を評価する必要があることから、代替法開発の難易度は非常に高い。また、動物試験は高コストであるため、良好な結果が得られる適切な非動物試験が開発されれば、経済原則に従って、各企業内での導入が図られることもある。

2.3 一般化学物質・環境汚染物質

次の代替法導入の焦点となっているのが、図1で化粧品と食品・医薬品との中間に位置する一般化学物質である。環境汚染物質も同じ場所に位置づけられよう。EUでは、新たな化学物質管理制度REACH(Registration, Evaluation, Authorisation and Restriction of Chemicals)を施行しているが、もし代替法なしにこれに適用するとより多くの動物を使用してしまうという矛盾をはらんでいる³⁾。そこで、最終的にはOECDの化成品テストガイドライン化を目指した代替法開発に関するEUプロジェクトが、多数実施されている⁴⁾。

代替法をめぐる世界的な最新の状況とわが国の寄与については、本分野におけるわが国の世界への窓口となっている国立医薬品食品安全性研究所・安全性生物試験研究センター内の新規試験法評価室が事務局を務める日本動物実験代替法検証センター(Japanese Center for the Validation of Alternative Methods, 通称JaCVAM)のホームページを参照されたい⁵⁾。

3. メカニズムベース評価という科学的要請

3.1 従来の代替法開発と利用

上述の社会的要請とは別に、非動物試験の利用の拡大は科学的必然でもある。それが展望できるまで、少なくとも多様な生物ツール・解析手段が進歩してきている。

非動物試験には後述のようにさまざまカテゴリーがあるが、生物学的応答を得ることができる培養バクテリアや細胞を用いた試験法が中核であった。個別の試験法は、個体内で起こる重要な毒性発現メカニズムのみに着目し、飼育が容易な生物や培養細胞を用いて、そのメカニズムに基づいた生物学的応答を簡便に再現・可視化するものである。例えばエイムズ法やumu法といった変異原性試験はバクテリアを用いているが、発ガンの第一段階である化学物質の遺伝子損傷性を検出できる。

昨今の細胞培養技術・ステムセル分化誘導技術の進捗を考えれば、それらの試験法への適用が十分に考えられるが、少なくとも現状では試験法としてのガイドライン化(例えばOECDテストガイドラインへの収録)にはやや時間がかかる。これは、一般に代替法としての収録には国際的なバリデーションを経ることが必須であり、従来は長い時間を要してきたことが理由である。一方で、上記のREACHの施行に伴い、代替法バリデーション結果の国際的な相互認証体制の構築が急速に進んでおり、今後はより早く代替法のガイドライン化が進むと予想できる⁶⁾。

3.2 米国および欧州における新たな動き

このような個別の代替法開発というややボトムアップ的なアプローチに対して、“そもそも理想的な非動物試験とは何か”、との根源的な問いかけから出発したトップダウン的な思考に基づく方法論も提唱されている。ヒト全身を対象としたメカニズムベースでの定量的なハザード予測を目指す立場から、人体システムの階層構造に着目した手法体系を作り上げる必要がある(図2)。例えば、培養細胞で得られる用量作用関係を、物質の体内動態を記述する数理モデルで個体レベルに積み上げるという考え方は、以前から提案されている⁶⁾。

最近米国環境保護局は、基本的には同様の考え方に立ちながらも、欧州に比べて米国が優位に

ある網羅的解析や数理解析との統合利用を重視した新たな化学物質管理戦略を提唱した⁷⁾。一言で言えば、“毒性発現メカニズム(機序)=Toxicity pathwayに基づいた定量的予測”のために、培養細胞試験や数理解析に代表される非動物手法を統合的に利用することで、動物実験の削減を目指す、というものである。図2に示すように、曝露された化学物質はヒト個体内の各階層を行き来すると同時に種々の代謝を受け、最終的に標的組織臓器内で分子レベルでの反応を起こす。この過程をメカニズムベースで予測するためには、培養細胞試験、標的臓器内での分子間相互作用を記述するToxicodynamics (TD)モデル・体内各臓器での吸収・分配・代謝・排泄を記述するToxicokinetics (TK)モデルとを組み合わせることが重要である。すなわち、培養細胞試験およびそれを対象とした網羅的解析・インフォーマティクス解析によって毒性発現メカニズム=Pathwayを同定し、それをTDで定量的に記述、TKと組み合わせることで個体のハザードを記述予測するという流れである。

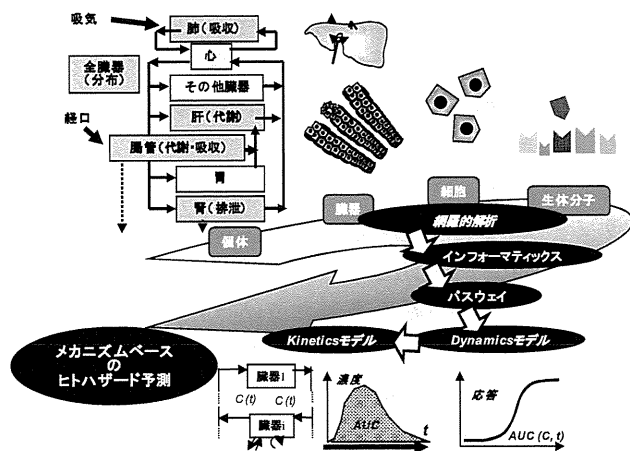


図2 階層に注意したメカニズムベースでの予測における生物学的情報と数理解析の融合

しかしながら、この手法には現状では多数の困難な点がある。個別の物質についてTK/TDモデルと構築して評価したことがまだまだ一つの学術論文となるような状況を見れば容易に理解できるし、しかもそのためには動物実験で得られる生物学的知見を使用することが普通である。後述のように、個体と培養細胞のシステム階層の違い・ヒトと動物との種差という2つの違いを乗り越えてヒト個体影響評価を行うためには、どこかで同種

の生物を用いた*in vitro/in vivo*の橋渡しを行う必要がある。現実的には、動物実験を含むさまざまな手法を総合的に用いていくしか解決の道はない。

このように米国は、“科学的根拠を重視すれば動物試験をやらざるを得ない”，という立場を暗にとる。これに対して欧州は、図1に示したように潜在的ヒトリスクがそもそも低い化学物質については、“代替ありき”，という社会的要請を重視し、今利用できるベストの技術とその組み合わせ利用を含めて考える。そして、“現状で最良と思われる手法を文書化・国際標準化する”，という戦略をとることとなる。

4. 培養細胞と数理モデルのリンクの例

4.1 培養肺胞モデル

ガス状物質の人体への摂取経路として肺胞は極めて重要である。ここでは、揮発性環境汚染物質や各種燃焼プロセス排ガス・浮遊粒子状物質 (Suspended Particulate Matter, SPM) を対象として行ってきた筆者らの研究を簡単に紹介すると共に、図2のようなフレームを想定し、ヒトハザード評価を真に目指した場合に抽出される様々な問題点を指摘する。

肺胞内腔を主に覆っているI型上皮細胞の初代培養については、単離したII型細胞を用いた分化誘導が可能になってきたが、依然として容易ではない⁸⁾。またstemセルからの分化誘導法も確立されていない。そこで多用されるのは、ヒト肺胞ガン由来のA549細胞株でありII型由来の特徴を持つ。これをマイクロプレートにセットできる半透膜型培養器(カルチャーインサート)上で気液界面培養する(図3)。これを基礎として、ガラス製閉鎖型曝露チャンバーを用いた揮発性化学物質評価⁹⁾や実SPM試料の評価^{10), 11)}を行ってきた。いずれも急性毒性レベルの極めて高い負荷量でA549細胞の生存率は低下することから、肺急性障害の代替法としては使用可能であると考えている。しかし実際には、そのような高濃度曝露が行われる実ケースはほぼ皆無で、培養肺胞モデルを肺吸収率の評価系として使用することが適切である。肺での吸収過程は、その極めて広い面積(30-50m²)と薄い構造(0.5-1.0μm)とから瞬時に平衡に達し、たとえばオクタノール・水間や空気・水間の分配係数を用いて推算するのが普通である¹²⁾。これ

に対して、培養肺胞モデルを用いることで、肺胞内腔に存在する界面活性剤様物質の液層(厚さ約10 μm)や細胞における代謝・極性輸送といった従来は考慮されてこなかった生物学的影響の考慮と定量化が期待できる。

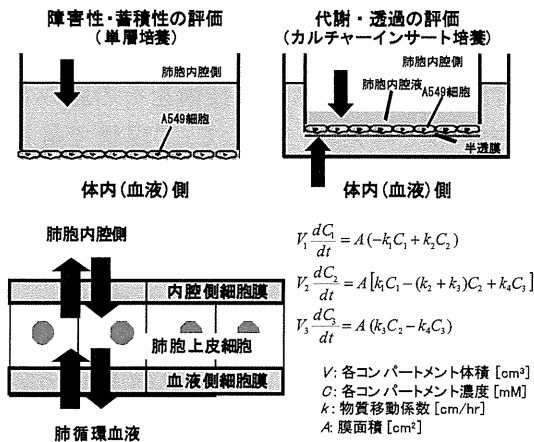


図3 培養肺胞モデルにおける肺障害・透過とその簡単な数理モデル化

4.2 簡単な数理モデルとのリンク

肺胞内腔液・上皮細胞内・肺循環血液の3つのコンパートメントを仮定、それぞれの物理学的・生物学的差異から異なった濃度にて平衡に達する(動的分配平衡関係にある)として、より汎用性を高めた。この場合には順および逆輸送のフラックスが存在することとなり、その大きさは4つの速度定数で示される。これを元に、各コンパートメントでの添加物質のマスバランスを記述する基礎式(連立常微分方程式)を立てた。

4つのパラメータ決定のために、図3に示す3つの曝露実験を行った。すなわち、細胞層への分配を見るための単層培養を用いる実験と、半透膜型培養器を用いて、肺胞→血液および血液→肺胞の輸送を見る実験である。培養液中の化学物質の濃度を経時的に測定する。時刻ゼロにおいては、細胞内または反対側の培養液中の濃度はほぼゼロとみなせること、十分時間がたち3つのコンパートメント間が平衡に達した状況では、順輸送と逆輸送の速度定数の比が分配平衡定数になること、などを利用してパラメータを決定することができる。このように構築した簡便な数理モデルで、SPMからの溶出物質や揮発性化学物質に対しては、培養系での2日間の挙動を概ね記述可能であっ

た。しかし、以下に示すようにフェーズの異なる多くの問題点が浮かび上がった。

4.3 数理モデルとのリンクにおける問題点

ハザード予測への利用を考えると、培養系での数理モデルを基に、ある曝露シナリオでの経肺曝露における血中取り込み量の長期の増大を時間軸に対して予測する必要がある。

ここで浮かび上がる最大の問題は、用いた培養系が閉鎖系であり、分配平衡に達した以後はそれ以上の吸収は起こりえないことで、絶えず血液が循環している*in vivo*肺内の状況と異なる。この点を考慮しない数理モデルでは、SPM吸着物質について、実際に試したほとんどの物質は肺胞内に分配されることとなってしまい、肺胞障害は起こさず他の臓器に障害を与える、という動物実験の結果¹³⁾と大きく異なってしまう。この解決のためには、数理モデルにおいて、肺循環血液コンパートメントの濃度を絶えずゼロにするという境界条件を置くことが必須であった。また、A549が形成する細胞層が10 μm 程度と*in vivo*に比べて10-20倍厚いことに関しては、物理化学的性質は変わらないとして、数理モデル上で肺胞膜を薄くすることで、添加物質はより迅速に体内に移行することが表現できた。

このように、培養モデルで得られた生物学的パラメータ(4つの速度定数など)はそのままとして、培養モデルと*in vivo*の状況の違いのうち、少なくともスケールや操作条件にかかわるパラメータを*in vivo*の状況に合わせて置き換えることが必須である点にまず注意を要する。さらに、現在詳細を検討中ではあるが、化学物質によっては、3つのコンパートメント内での器壁への吸着や代謝(血清中や細胞内の酵素による)による分解が顕著に起こる。代謝物が原体よりも毒性が高い場合には、図3の基礎式セットをその代謝物についても立て、必要な実験を実施することでパラメータを決定しなければならない。

4.4 経肺摂取ハザードに向けた広範な課題

このように、一旦メカニズムベースでのヒト外挿を目指そうとすれば、多岐に渡る現象に注意を払い、数理モデル化と培養実験とを真に融合的に利用することは必須といってもよい。しかしなが

らこれは、動物試験に比べてはるかに容易である点が培養モデルの利点である。

他の手法とのリンクも必須である。上記はあくまで肺胞と血液の輸送のみに着目した例であり、実際の予測には肺の幾何学的構造を表現する肺呼吸モデルとの統合利用が必須である。また、得られる情報は経肺摂取時の取り込み量の時間変化のみであり、しかも短期間のものである。現実的には経口投与の慢性毒性試験の結果を利用し、総体内取り込み量を基礎として最終的な影響予測を行うなどの手段を用いる必要がある。

さらには、ヒト由来であるとはいえ上皮細胞株のみからなる肺胞モデルの生物学的妥当性は大きな問題で、根本的解決のためには、ステムセルからの肺胞I型細胞の分化誘導・成熟化技術や正常肺胞マクロファージとのI型細胞膜上での安定共培養技術等の発展も強く望まれる¹⁴⁾。

5. まとめ—融合活用が期待される様々な技術—

さて、このような新しいハザード評価体系において、利用が期待される技術・手法を、動物・ヒトという種の違い、個体と細胞というシステムの違いに着目して配置したものが図4である。

生理学的応答取得が可能な培養組織モデルについては、吸収・代謝・排泄といった主要動態プロセスを評価できる臓器と標的になりやすい臓器についての両方が必要である。システム階層差に潜む未知のメカニズムが関与するため、細胞から個体までを一貫して評価できる動物の使用が少なくとも中期的には欠かせない。

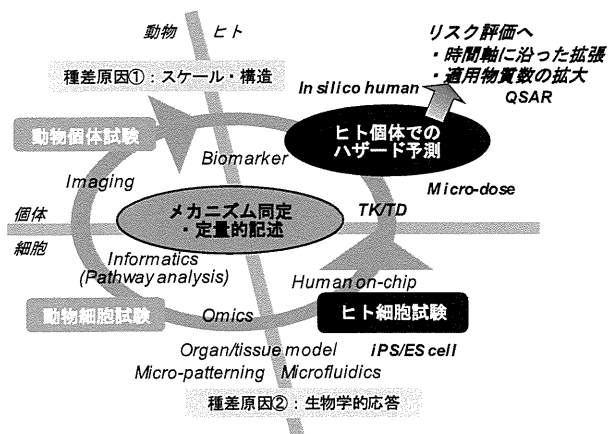


図4 メカニズムベースのヒト影響評価において融合利用が期待される種々の技術

様々な生物学的試験においては、iPS/ES細胞からの正常細胞の分化誘導と成熟化に加えて、ハイスループットまたはハイコンテントスクリーニングにも対応可能なように、マイクロパターニングやマイクロ流体デバイス技術といった微細化技術の利用が特に望まれる^{15,16)}。また、生物各階層を材料とする実験系を対象として、遺伝子発現からタンパク・代謝物までの網羅的解析とそのデータから主要メカニズムを抽出するためのパスウェイ解析・同定が主要な応答評価手法となろう。イメージングは情報量は少ないが、個体から細胞まで階層縦断的に用いられる点に有用性がある。主要物質の主要パスウェイについてTDモデルで記述し、リアルタイムの臓器間相互作用の関与に配慮しつつTKモデルで個体レベルに積み上げる。TK/TDモデルで、図2に示すような階層構造を跨ぐメカニズムベースの縦型評価を行い、ハイスループットな培養細胞試験と定量的構造活性相関(QSAR)を併せて用いることで、対象物質数を増やすという横型の拡張予測を行うこととなろう。バイオマーカーは、イメージング技術と結び付けられることで、個別の試験系での応答可視化に役立つばかりでなく、個体と細胞レベル・予測影響(事前評価)と実影響(疫学的調査等の事後的評価)の間をつなぐ重要な指標ともなる。

図4に掲げた様々な技術・方法論の重要性について異論はないと思われるが、それらは「理想的なヒトハザード評価手法体系」のための必要条件に過ぎず、実際にどの手法がどの程度役に立つのか(役に立たないのか)、については、現状では誰も予測が不可能である。各自が理想像を描いておき、最新の到達水準を適宜アップデートしつつ臨機応変かつ広い視野で柔軟に融合するしかないが、全体としては図4の流れに沿っていくものになるであろう。

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