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In order to establish a definitive test protocol for endocrine disruptors, a one-lifespan test was performed using rats and the aging process of reproductive function was observed. Neonates of Sprague-Dawley rats received forced oral administration of diethylstilbestrol (DES) at doses of 0 (vehicle), 0.05, 0.5 and 5 μg/kg for 5 days after birth. Sexual maturation (vaginal opening and preputial separation), estrous cycles (from 8 to 49 weeks of age), mating (at 12, 23, 34, 56 and 68 weeks) and litter size (of the 1st to 3rd parturitions) were observed. Each half of the males were examined for sperm counts and organ weights at 26 and 52 weeks of age. In half of the females, hCG induced ovulation and organ weights were examined at 54 weeks of age. Then the observation of remaining animals was terminated at 101 weeks and survival rate were determined.

Vaginal opening in the group received DES at 5 μg/kg was significantly earlier than the vehicle control group. Normal estrous cycles were observed in no animals of 5 μg/kg DES group throughout the study, and in less than 10% of 0.5 μg/kg DES group at 28 weeks and on. Fertility rate of 12 week-old females of the 5 μg/kg DES group was 0%, and that of 23 week-old females of the 0.5 μg/kg group was 33.3%. Mating rate of 0.05 μg/kg females of this age was reduced to 60%. Influence of neonatal DES exposure was not observed in the first delivery in any group, but in the second parturitions litter size was reduced significantly in the 0.5 μg/kg group. Organ weights of 54 week-old females showed dose-related significant increase of pituitary weight in the 0.05 to 5 μg/kg groups. Adrenal weight was increased in the 0.5 and 5 μg/kg groups. Weight of ovaries was lowered significantly in the 0.5 and 5 μg/kg groups. Testing of induced ovulation with hCG revealed lack of influence of DES on number of shed oocytes. No effects of neonatal DES exposure in males were observed on preputial separation, fertility, sperm counts and organ weights. The lower survival rate was observed in the 5 μg/kg group females.

These results showed that early life exposure of low doses of DES potentially cause precocious sexual maturation, and decreases in reproductive function such as estrous cyclicity, fertility or litter size in female rats. These effects were considered to cause through disruption of hypothalamo-pituitary system, not through direct disturbance on ovarian function. The effects of DES observed in this study indicate the usefulness of one-lifespan test as a definitive test protocol for endocrine disruptors.

緒言

現在，内分泌攪乱化学物質（環境ホルモン，EDC）研究の焦点は，化学物質の内分泌攪乱性を確定する試験法の開発にある．ホルモン活性を有する化学物質が環境中にも存在することは既知の事実で，化学物質のホルモン活性の有無を検討する方法はEDCのスクリーニング試験となり得る．しかし，ホルモン活性を有する化学物質が，生体に有害な影響すなわち内分泌攪乱性を示すか否かを判定する試験法は確立されていない．実

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際，これら外因性ホルモン活性物質よりはるかに強力な受容体結合性を持つ内因性ホルモンの影響が，従来の生殖発生毒性試験（多世代繁殖試験）では確認されないにもかかわらず，ジエチルスチルベストロール（DES）のような物質では内分泌系など高次調節系の遅発性の異常が臨床的に起っている．つまりEDCは実際に存在し，それを試験する方法が求められている．このような理由から，現在，EDCの確定試験として従来の多世代繁殖試験に代る「一生涯試験」が考案された．

本研究ではエストロゲン活性を有するDESをSprague-Dawley（SD）ラットの新生児期に投与し，児の発達，成熟および老化に至る各段階において生殖器系機能の変化を検索する「ラット一生涯試験」を試みた．本研究では，新生児期DES投与が引き起こす遅発性の生殖機能異常を検索するために，雌は8週齢から49週齢まで性周期を観察し，12，23および34週齢で交配実験を行った．雄については，26および52週齢で精子検査を行い，12，23，34，56および68週齢で交配実験を行った．

材料および方法

試験には，日本チャールス・リバーから8週齢で入手したCrl:CD（SD）雌雄ラットを使用した．SD系ラットは，毒性試験において一般的に用いられている系統であり，生殖毒性に関する背景データが豊富で，Wistar系ラットに比べて性周期の加齢性変化が早期に起り易いことが知られている[1]．11週齢時に交配し，交尾が確認された雌を1群12匹以上からなる4群に振分けた．動物は温度22～25℃，湿度50～65％，照明12時間（7時～19時点灯）に調節された飼育室で，固型飼料（CE-2，日本クレア）と水道水を自由摂取させて飼育した．妊娠雌は，紙パルプ製チップを入れた金属製ケージに1匹ずつ収容した．全ての実験操作は，「財団法人食品薬品安全センター秦野研究所 動物実験に関する指針」に基づいて実施した．

EDCには子宮内あるいは新生児期の曝露での影響が指摘されていることから，DESの投与経路は新生児への強制経口投与を選択した．投与量は，内分泌攪乱化学物質に対する厚生労働省の試験スキーム[2]を考慮し，子宮肥大試験の結果をもとに設定した．すなわち，0.05～15 μg/kg/dayのDESを卵巣摘出マウスに3日間反復経口投与し，最終投与の約24時間後に子宮重量を測定した結果，5 μg/kg/day以上を投与した群で子宮重量が有意に増加したことから，5 μg/kg/dayを確実影響量として一生涯試験の最高用量に設定し，無影響量と考えられる0.5 および0.05 μg/kg/dayをそれぞれ中用量および低用量に設定した．投与液は，DES（Sigma-Aldrich, St. Louis, MO）20 mgを1 mLのエタノールに溶解し，コーン油で段階希釈して調製した．

新生児は，生後1日（分娩日を生後0日とする）に性別および外表奇形の有無を検査し，異常のない雌雄各5匹を1腹毎に選抜し，四肢の皮下に墨汁を注入して個体識別した．投与は生後1日から生後5日まで1日1回，マイクロシリンジおよび新生児用カテーテル[3]を用いて行い，投与液量は10 mL/kgとした．投与終了後は同腹児数を雌雄各4匹に調整し，生後21日に離乳させた．離乳後は，金属製金網床ケージに2匹ずつ収容した．体重は，生後0～5日（毎日），7，14および21日に測定し，離乳後は週1回，10週齢以降は隔週1回，26週齢以降は4週間毎に測定した．

雌は生後25日から腟開口を，雄は生後35日から陰茎包皮分離[4]を性成熟の指標として毎日観察した．各腹の雌2匹は，8週齢から49週齢まで2週間間隔で連日2週間，腟垢を採取し，性周期を観察した．腟垢像は発情前期，発情期，発情休止期に分類し，渡辺らの報告[1]と同様に性周期の型を分類した．各腹の雌雄各2匹は，12，23および34週齢から2週間を限度に，兄妹交配を避けて1：1で群内交配させた．群内交配で交尾が確認されなかった場合，雄は無処置雌と，雌は交尾が確認された同群の雄と，いずれも2週間を限度に再交配させた．雄は，さらに56および68週齢から2週間を限度に無処置雌と交配させた．群内交配で交尾が確認された雌は自然分娩させ，妊娠日数および産児数を確認し，哺育0および4日の哺育児体重を測定した．無処置雌は妊娠13日以降に帝王切開し，妊娠の有無を確認した．

雄は26，52および101週齢時，雌は54および

101週齢時にペントバルビタールナトリウム麻酔下で採血し，剖検した．雌雄とも101週齢以外の剖検時には，脳，下垂体，甲状腺，肝臓，脾臓，腎臓，副腎，精巣，精巣上体，前立腺（腹葉），精嚢（凝固腺を含む），卵巣，子宮の重量を測定した．

26および52週齢の剖検時に雄から採取し，凍結保存した精巣上体尾部および精巣を用いて精子数および精子頭部数を測定した．精巣上体尾部および精巣は，解凍後，ホモジナイズした精子懸濁液をModified IDENT STAIN Kit（Hamilton-Thorne）により染色し，HTM-IVOSにより，精巣上体尾部および精巣重量当たりの精子数および精子頭部数を求めた[5]．

各腹とも雌1匹は，54週齢時に排卵可能な卵胞の有無を確認するため，剖検16～17時間前にヒト絨毛性性腺刺激ホルモン（hCG，Sigma）を10 IU尾静脈内投与し，剖検時に卵管内の誘起排卵数を数えた[1]．

離乳前の児に関するデータは腹単位，離乳以降のデータは個体を標本単位として解析した．体重，器官重量，産児数および精子数のデータは，一元配置型の分散分析を行い，群間に有意差が認められた場合はDunnett法による多重比較検定を行った．性成熟，妊娠日数および生存率のデータは，Kruskal-Wallisの順位検定を行い，群間に有意差が認められた場合には，順位化した値を用いてDunnett法による多重比較を行った．交尾率および受胎率の差は，Fisherの直接確率法による検定を行った．有意水準は5%および1%とした．

結果

体重：生後0日から離乳まで，および離乳後から26週齢までの体重は，各群とも順調に増加し，雌雄ともDES投与の影響は認められなかった．また，26週齢以降の体重推移についても，46週齢から50週齢にかけて対照群の雌の体重が低下した以外に異常は認められなかった．

性成熟：雌の腟開口時期（平均±S.D.，日）は，5 μg/kg投与群（29.8 ± 2.2）で対照群（32.9 ± 1.7）より有意に早まったが，雄の陰茎包皮分離時期にはDES投与の影響はみられなかった．腟開口時期が早まった5 μg/kg投与群では，雌の全例で尿道開口部の過剰開裂[6]が観察された．

性周期：正常な性周期を示した雌の割合を図1に示した．5 μg/kg投与群では，観察を開始した8週齢から正常な性周期を示す雌は認められなかった．0.5 μg/kg投与群では8週齢から13週齢にかけては80%以上の雌が正常な性周期を示したが，20週齢から25週齢時には約50%，28週齢以降は10%未満となった．0.05 μg/kg投与群は対照

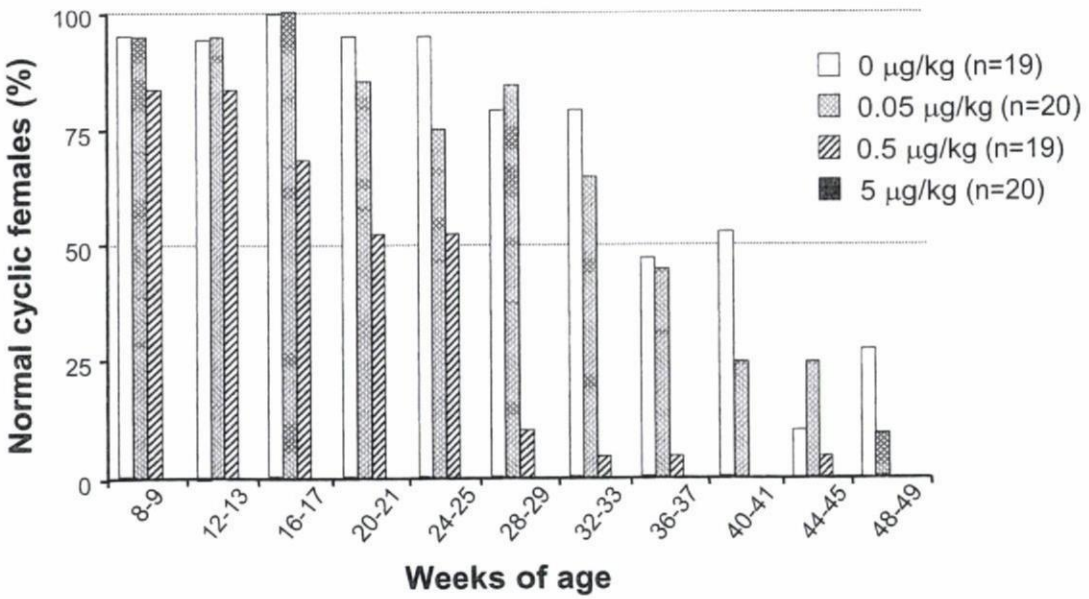

図1　新生児期にDESを投与したSD系雌ラットの性周期（正常性周期の割合の推移）

群とほぼ同様に推移し，正常な性周期を示す雌が29週齢までは80％以上，36週齢以降は50％未満となった．性周期を型別（図2）に見ると，5 μg/kg投与群で早期にみられた異常周期の型は連続発情であったのに対し，0.5 μg/kg以下の用量で加齢に伴って増加した異常周期の型は不規則周期や無発情であった．

交尾率・受胎率：交配結果を表1に示した．雄は12，23および34週齢のいずれの交配時期においても，交尾率および受胎率にDES投与の影響は認められなかった．また，56および68週齢の無処置雌との交配においても，DES投与の影響を示唆する変化は認められなかった．雌は12週齢の交配では，5 μg/kg投与群の交尾率は90％であったが，受胎率は0％となったため同群雌の23週齢以降の交配は中止した．23週齢の交配では，0.05 μg/kg投与群の交尾率が60％に低下し，0.5 μg/kg投与群の受胎率が33.3％に低下した．34週齢の交配では，対照群を含む各投与群の交尾率および受胎率が低下した．

分娩・哺育：分娩した雌の哺育成績を表2に示した．初回分娩では5 μg/kg投与群で産児が得られなかった以外にDES投与の影響は認められなかった．2産目では，0.5 μg/kg投与群の産児数が対照群より有意に減少し，0.05 μg/kg投与群の妊娠日数が対照群より延長する傾向にあった．3産目については，対照群を含む各投与群で受胎率が低下したことから，産児数の評価はできなかった．

雄の精子数および器官重量：26週齢および52週齢で精子数と器官重量を調べたが，いずれの時期においても，精巣上体尾部の精子数，精巣重量当りの精子頭部数，ならびに生殖器を含むいずれの器官重量にもDES投与の影響を示唆する変化は認められなかった．

雌の器官重量：54週齢の雌の器官重量を図3に示した．雌では，全てのDES投与群で下垂体重量が対照群より有意に増加し，5および0.5 μg/kg投与群で副腎重量が，5 μg/kg投与群で甲状腺重量が有意に増加した．また，5および0.5 μg/kg投与群で卵巣重量が対照群より有意に低下した．剖検時には，皮下に乳汁が貯留している例が0.05 μg/kg投与群で20例中2例，0.5 μg/kg投与群で18例中3例，5 μg/kg投与群で19例中9例みられた．その他，血中ホルモン濃度の測定では，0.05 μg/kg以上の投与群でプロラクチン濃度の上昇が，0.5 μg/kg以上の投与群でLH濃度の上昇

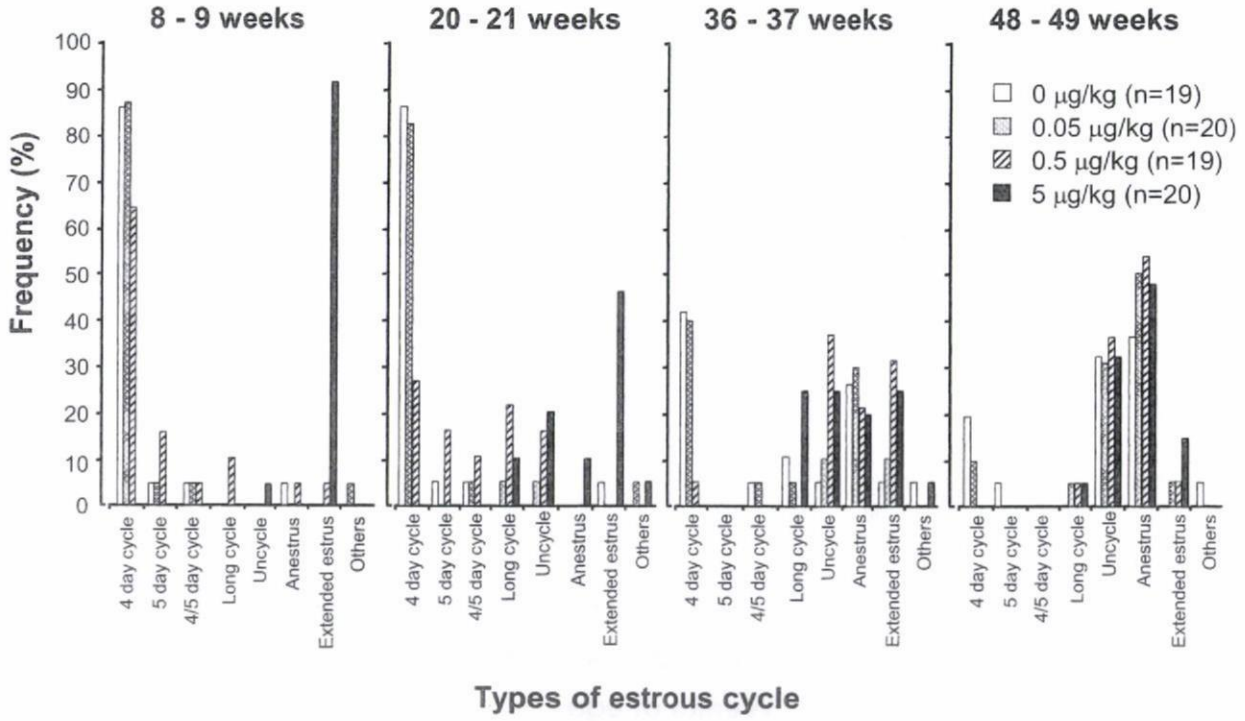

図2　新生児期にDESを投与したSD系雌ラットの性周期（性周期の型別の推移）

表1　新生児期にDESを投与したSD系ラットの交配成績

DES (μg/kg)	Males 0	Males 0.05	Males 0.5	Males 5	Females 0	Females 0.05	Females 0.5	Females 5
At 12 weeks of age								
Copulation index (%) (No. copulated/no. mated)	100.0 (20/20)	95.0 (19/20)	100.0 (20/20)	90.0 (18/20)	100.0 (20/20)	95.0 (19/20)	100.0 (20/20)	90.0 (18/20)
Fertility index (%) (No. pregnant/no. copulated)	100.0 (20/20)	84.2 (16/19)	95.0 (19/20)	100.0 (18/18)	90.0 (18/20)	81.3 (13/16)	80.0 (16/20)	0.0 ** (0/18)
At 23 weeks of age								
Copulation index (%) (No. copulated/no. mated)	100.0 (20/20)	95.0 (17/20)	100.0 (20/20)	90.0 (18/20)	100.0 (20/20)	60.0 ** (12/20)	90.0 (18/20)	
Fertility index (%) (No. pregnant/no. copulated)	89.5 (17/19)	100.0 (17/17)	100.0 (20/20)	83.3 (15/18)	80.0 (16/20)	58.3 (7/12)	33.3 ** (6/18)	
At 34 weeks of age								
Copulation index (%) (No. copulated/no. mated)	100.0 (20/20)	90.0 (18/20)	100.0 (20/20)	95.0 (19/20)	55.0 (11/20)	25.0 (5/20)	20.0 * (4/20)	
Fertility index (%) (No. pregnant/no. copulated)	95.0 (19/20)	72.2 (13/18)	90.0 (18/20)	84.2 (16/19)	54.5 (6/11)	60.0 (3/5)	25.0 (1/4)	
At 56 weeks of age								
Copulation index (%) (No. copulated/no. mated)	90.0 (18/20)	60.0 (12/20)	60.0 (12/20)	79.8 (15/20)				
Fertility index (%) (No. pregnant/no. copulated)	72.2 (13/18)	66.7 (8/12)	83.3 (10/12)	66.7 (10/15)				
At 68 weeks of age								
Copulation index (%) (No. copulated/no. mated)	57.9 (11/19)	47.4 (9/19)	55.6 (10/18)	55.6 (10/18)				
Fertility index (%) (No. pregnant/no. copulated)	72.7 (8/11)	66.7 (6/ 9)	80.0 (8/10)	50.0 (5/10)				

*, ** は対照群と比較して有意差（5%および1%）があることを示す．
データには無処置雌との交配結果も含まれる．

表2　新生児期にDESを投与したSD系母ラットの哺育成績

DES (μg/kg)	0	0.05	0.5	5
At the 1st parturition				
Number of dams	18	13	16	0
Gestation length in days	22.1 ± 0.3	22.2 ± 0.7	22.1 ± 0.5	
Number of newborns	13.9 ± 3.4	12.9 ± 3.9	14.1 ± 3.6	
Pup weight (g) Male	6.9 ± 0.3	6.8 ± 0.6	6.7 ± 0.7	
Female	6.5 ± 0.3	6.4 ± 0.6	6.3 ± 0.7	
Viability index on PND 4	99.7 ± 1.5	98.5 ± 3.0	99.0 ± 3.0	
At the 2nd parturition				
Number of dams	16	7	8	0
Gestation length in days	22.3 ± 0.5	22.9 ± 0.4 *	22.5 ± 0.5	
Number of newborns	12.8 ± 3.9	12.7 ± 3.7	7.6 ± 5.8 *	
Pup weight (g) Male	7.2 ± 0.8	6.9 ± 0.7	7.4 ± 0.9	
Female	6.7 ± 0.7	6.8 ± 0.7	6.6 ± 0.6	
Viability index on PND 4	93.3 ± 25.1	100.0 ± 0.0	100.0 ± 0.0	
At the 3rd parturition				
Number of dams	6	3	1	0
Gestation length in days	22.4 ± 0.5	22.7 ± 0.6	22.0	
Number of newborns	12.0 ± 4.3	11.0 ± 6.6	14.0	
Pup weight (g) Male	7.1 ± 0.5	7.2 ± 1.2	6.8	
Female	6.6 ± 0.5	6.8 ± 1.4	6.7	
Viability index on PND 4	96.5 ± 5.9	100.0 ± 0.0	100.0	

* は対照群と比較して有意差（5%）があることを示す．各値は平均±標準偏差を示す．

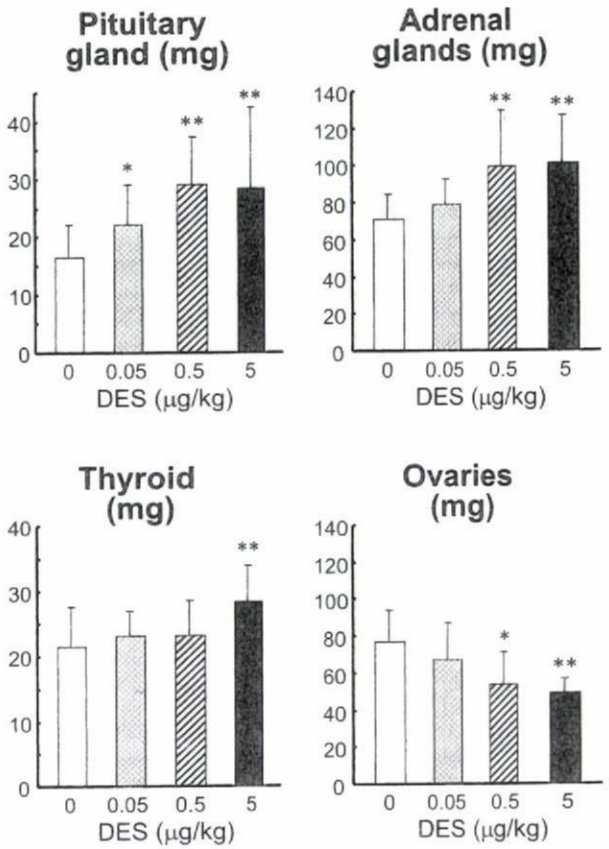

図3　新生児期にDESを投与したSD系雌ラットの54週齢における器官重量

*, ** は対照群と比較して有意差（5%および1%）があることを示す.

対照群（n=16），0.05 μg/kg群（n=20），0.5 μg/kg群（n=19），5 μg/kg群（n=19）

が確認されたが，T_3，T_4およびFSH濃度には，群間の差は認められなかった.

排卵検査：54週齢の排卵検査では，hCG投与により排卵した雌が対照群で8例中7例，0.05 μg/kg投与群で10例中6例，0.5 μg/kg投与群で10例中9例，5 μg/kg投与群で10例中8例みられ，誘起排卵数に群間の差は認められなかった.

生存曲線：雌雄の生存曲線を図4に示した．5 μg/kg投与群の雌では，生存日数が短縮したが，雄の生存日数にDES投与の影響はみられなかった.

考察

性成熟の観察では，5 μg/kg投与群で膣開口時期の早期化がみられ，同群では雌の尿道開口部の過剰開裂も認められた．性成熟の早期化[7]や尿道開口部の過剰開裂[6]を内分泌攪乱物質の生体に及ぼす有害影響と断定するには，さらに慎重に検討すべきであるが，一生涯試験の中では早期に検査できる項目であることから，後に得られる結果とあわせて内分泌攪乱性を判断する材料の一つになると考えられる．陰茎包皮分離時期に関しては，5 μg/kg投与群においても投与の影響はみられなかった．吉村ら[8]は，DESを出生後1～5日に投与したSD系ラットのうち，100 μg/kg以上の投与群で陰茎包皮分離時期の遅延を報告している．したがって，本研究で用いたDESの投与量では，雄の性成熟に影響を及ぼさないと判断される.

5 μg/kg投与群では8週齢から正常な性周期を示す雌動物は認められなかった．この結果から，同群ではDES投与により性成熟前の性腺刺激ホルモンが低下し，androgenizationを起し，膣開口後も排卵はなかったと推察される．一方，0.5 μg/kg投与群でも，16週齢以降に正常な性周期を示す動物の割合が減少した．TCDDの性成熟前投与[9]やビスフェノールAの胎生期投与[10]でも性周期の異常は対照群よりも早く起こることが示されている．これらのことは，内分泌攪乱化学物質の検索において，性周期を長期にわたって観察することの重要性を示している．本研究でみられた異常周期の型は，5 μg/kg投与群では主に連続発情であったのに対し，0.5 μg/kg投与群では不規則周期や無発情などが主であった．このことから，5 μg/kg投与群でみられた異常周期は無排卵に起因したのに対し，0.5 μg/kg投与群でみられた異常は，対照群にみられる加齢性変化が早期に誘発されたものと推察される.

12週齢の交配では，5 μg/kg投与群の雌で交尾が確認されたものの，受胎率は0%であった．これは，前述の排卵を伴わない連続発情を反映した結果と考えられる．一方，0.5 μg/kg投与群の雌では，23週齢の交配で受胎率が低下し，2産目の産児数が減少した．この変化も，同群で早期に加齢性変化，すなわち性周期の乱れを来したことと一致すると考えられ，2産目の産児数の低下は，エストロゲン分泌の低下に起因した排卵数の減少が原因と推定される[11]．なお，23週齢の交配で0.05 μg/kg投与群にみられた交尾率低下の原因は

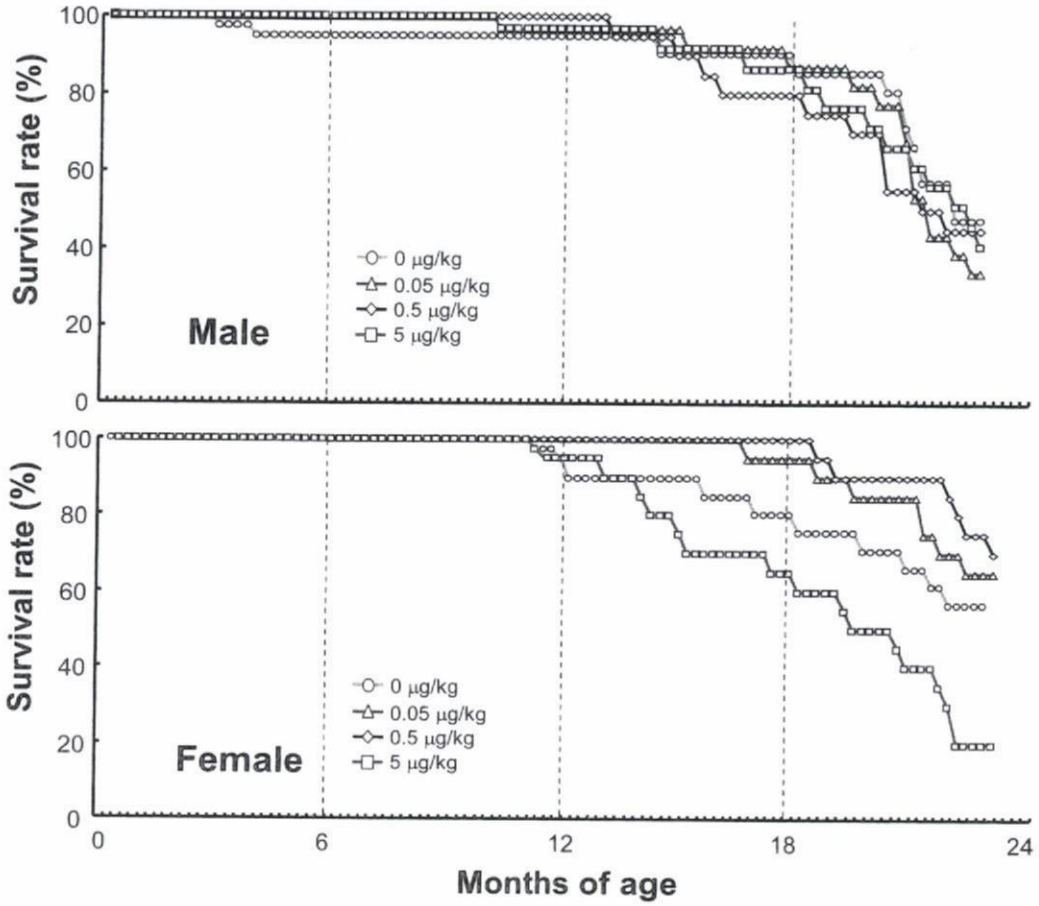

図4　新生児期にDESを投与したSD系ラットの生存曲線

不明である．

雄では，いずれの時期の交配においても，DES投与の影響は認められなかった．また，26週齢および52週齢では，ともに精子数および生殖器重量にDES投与の影響を示唆する変化も認められなかった．新生児期のDES投与では，雄動物の生殖能力への影響[12,13]が報告されているが，いずれも高用量（0.1 mg/pup/day以上）での報告であり，今回用いた5 μg/kgまでのDESは，新生児期投与で雄の生殖機能に影響を及ぼさないと考えられる．しかしながら，Vom Saalら[14]は，胎生期に0.02 μg/kgのDESを投与したマウスで前立腺重量の増加を報告していることから，同程度の投与量を用いて胎生期曝露を追試することが必要であろう．

54週齢の雌では，下垂体および副腎重量が用量に依存して増加し，血中プロラクチンとLH濃度の上昇が確認された．剖検時に乳汁貯留も観察されていることから，高プロラクチン血症が疑われる．一方，5 μg/kg投与群では甲状腺重量の増加が認められたが，血中T_3およびT_4濃度に変化はなかった．卵巣重量は，0.5 μg/kg以上の投与群で低下したが，hCG投与による誘起排卵がすべての投与群で確認されたことから，新生時期のDES投与は，卵巣機能には直接影響を及ぼさず，下垂体からの刺激の低下，すなわち視床下部－下垂体系の内分泌攪乱作用に起因したものと推定される．

結論

低用量DESは雌の性成熟を早めるだけでなく，雌の老化過程における性周期，受胎率，交尾率あるいは産児数に影響を与える可能性が示唆された．また，それらの変化は視床下部－下垂体系の内分泌攪乱作用に起因したものと推定され，卵巣機能への直接的な影響ではないと考えられた．本研究から，内分泌攪乱性を確定する上で一生涯試

験は有効であることが示されたが，雄の生殖機能異常を検出することは出来なかった．

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OECD validation of the Hershberger assay in Japan: Phase 3. Blind study using coded chemicals

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Abstract

The Organization for Economic Co-operation and Development (OECD) has initiated the development of new guidelines for the screening and testing of potential endocrine disrupters. The Hershberger assay is one of the assays selected for validation based on the need for in vivo screening to detect androgen agonists or antagonists by measuring the response of five sex accessory organs and tissues of castrated juvenile male rats: the ventral prostate, the seminal vesicles with coagulating glands, the levator ani and bulbocavernosus muscle complex (LABC), Cowper's glands, and the glans penis. The Phase 1 feasibility demonstration stage of the Hershberger validation program has been successfully completed with a single androgen agonist and a single antagonist as reference substances. The Phase 2 validation study was performed, employing a range of additional androgen agonists and antagonists. Recently, the Phase 3 validation study was conducted and performed in several International laboratories. Three Japanese laboratories have contributed to the blind study using coded materials of Phase 3 validation. Four coded test substances in the agonistic version and seven substances in the antagonistic version were orally administered by gavage for 10 consecutive days, respectively. In the antagonist version of the assay, 0.2 mg/kg/day of testosterone propionate (TP) was coadministered by subcutaneous injection. All five accessory sex reproductive organs and tissues consistently responded with statistically significant changes in weight within a narrow window in both versions. Therefore, the Japanese studies support the Hershberger assay as a reliable and reproducible screening assay for the detection of androgen agonistic and antagonistic effects.

Keywords: Blind study; Endocrine; Hershberger assay; OECD validation

1. Introduction

Certain reproductive and developmental toxicants may have the potential to interfere with normal sexual differentiation and development in animals and humans by modulating or interfering with the endocrine system (McLachlan, 1993; McLachlan and Korach, 1995). The Organization for Economic Co-operation and Development (OECD) has initiated an activity to revise existing guidelines and develop new screening and testing guidelines to aid in the identification and assessment of such toxicants (OECD, 1998, 2000, 2002).

One proposed assay, referred to as the Hershberger assay, uses the androgen sensitivity of several accessory sex organs and tissues of the male reproductive tract. The assay was originally developed in the 1930s by Korenchevsky and coworkers, and a number of accessory sex organs and tissues were shown to be use-

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ful by these and other investigators including the ventral prostate (Deanesly and Parkes, 1936; Dingemanse et al., 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b), the seminal vesicles and coagulating glands (Deanesly and Parkes, 1936; Dingemanse et al., 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b), the preputial glands (Bülbring and Burn, 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b), Cowper's glands (Wainman and Shipounoff, 1941), and the glans penis (Bülbring and Burn, 1935; Dingemanse et al., 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b). In the 1940s, it was discovered that the levator ani and bulbocavernosus muscles also responded to androgens, but in a differential way from the other tissues (Wainman and Shipounoff, 1941; Eisenberg et al., 1949; Eisenberg and Gordan, 1950). The basis for this differential sensitivity is the presence of 5α-reductase in most accessory tissues of the male reproductive tract, but its absence in the muscle complex (Di Salle et al., 1994). The capabilities of the assay were demonstrated in 1953 by Hershberger et al. when they analyzed the response of the ventral prostate, seminal vesicles and coagulating glands, and the levator ani without the bulbocavernosus muscle to a number of active chemicals, including estrogens and progesterones (Hershberger et al., 1953).

In the 1970s and 1980s, with the discovery of the androgen receptor and the first compounds such as cyprotone acetate that were antagonists of the receptor, the assay was modified to address antagonistic activity. Briefly, a set dose of a reference agonist was coadministered to several groups of animals to whom a set of doses of the purported antagonist was also administered. This modified system was successfully used by several investigators for assaying androgen antagonists (Peets et al., 1973; Raynaud et al., 1980, 1984; Wakeling et al., 1981).

Therefore, based upon the recommendation of scientific workshops, both the US Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) (USEPA, 1998) and the OECD Endocrine Disrupter Testing and Assessment Group (EDTA) of the OECD (OECD, 2000) have proposed this assay as a Tier-1 screen to identify possible reproductive and developmental toxicants acting through androgen agonist and antagonist mechanisms.

The OECD Phase 1 validation program for the Hershberger assay was completed in 2001. In this phase, a standardized protocol using the ventral prostate, the seminal vesicles with coagulating glands, the levator ani and bulbocavernosus muscle complex (LABC), Cowper's glands, and the glans penis was successfully tested against a reference androgen compound, testosterone propionate (TP), and a reference antagonist, flutamide (OECD, 2002). The OECD proposed a Phase 2 validation program using additional androgen agonistic and antagonists as the next step to validate the assay, but the final results of Phase 2 studies were not opened by the OECD.

Recently, the OECD conducted a Phase 3 validation program as a final blind study using coded agonistic and antagonistic chemicals (OECD, 2003). In Phase 3, the coded test substances were to be used to investigate the reliability of the assay, including a demonstration of the protocol's transferability among laboratories and the reproducibility of the protocol's results. Three Japanese laboratories participated in the Phase 3 validation study using four coded agonistic test substances and seven antagonistic substances. The participation of the laboratories in the OECD Phase 3 validation study was performed as part of a national validation program in Japan.

2. Materials and methods

2.1. Laboratories

The three participating Japanese laboratories were: the Chemicals Evaluation and Research Institute (CERI); the Food Drug Safety Center; and the Japan Bioassay Research Center. Each laboratory performed the study in compliance with the principles of Good Laboratory Practice guidelines.

2.2. Test substance

All coded test substances except for TP were sent to each laboratory from a centralized chemical repository at TNO, Zeist, the Netherlands. TP and corn oil as vehicles were prepared in each laboratory. The coded substances A, B, L and E were used in the agonistic version, and F, G, I, C, K, D and H were used in the antagonistic version. We did not receive any information regarding the coded substances before all tests were started.

2.3. Animals

Laboratory details regarding rat strain, age of castration, age at start of dosing, day of autopsy, animal diet, and the number of animals housed per cage are summarized in Table 1. Two laboratories used Crj:CD (SD) rats castrated at 6-weeks old, and the test substances were administered 1 week after castration. One laboratory used Brl Han: WIST Jcl (GALAS) rats castrated at 6-weeks old, and the test substances were administered 2 weeks after castration. In all the laboratories, the rats were weighed, weight-ranked, and assigned randomly to each of the experimental and control groups after they had recovered from their operation. Body weight and clinical signs were recorded daily throughout the study. Rats were provided with water and

Table 1
Laboratory detail for rat strain, age of castration, age at start of dosing, day of autopsy, animal diet, and the number of animals housed per cage

Lab	Rat strain	Age of castration	Age at start of dosing	Day of autopsy	Diet	Number of rats per cage
1	Brl Han: WIST Jcl (GALAS)[a]	6-weeks old	8-weeks old	10-weeks old	MF[b]	3
2	Crj:CD (SD)[c]	6-weeks old	7-weeks old	9-weeks old	CE-2[d]	1
3	Crj:CD (SD)[c]	6-weeks old	7-weeks old	9-weeks old	CRF-1[d]	1

[a] Clear Japan Inc., Tokyo, Japan.
[b] Oriental Yeast Co., Ltd., Tokyo, Japan.
[c] Charles River Japan, Kanagawa, Japan.
[d] Clear Japan Inc.

a commercial diet ad libitum. The animals were kept under SPF conditions. All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by The Japanese Association for Laboratory Animal Science.

2.4. Administration

We performed each test according to the protocol proposed by the OECD (OECD, 2000, 2002, 2003). Each test substance was orally administered via a stomach tube for 10 consecutive days at approximately the same time each day. A vehicle control group receiving only corn oil was used in both versions. For the antagonistic version, 0.2 mg/kg/day of TP was coadministered each day by subcutaneous injection in the dorsal region after the oral administration of each chemical. The volume of the corn oil solution containing the TP was 0.5 ml/kg. In the agonistic version, a positive control group of animals received TP injections alone. The group size in all cases was six rats. The volume of the corn oil solutions containing each of the test chemicals was 5 ml/kg. The animals were killed by bleeding from the abdominal vein under deep ether anesthesia approximately 24 h after receiving their final dosage. The five mandatory tissues, the ventral prostate and fluid, seminal vesicle and fluid, LABC, glans penis, and Cowper's gland, were carefully dissected free of adhering fat and weighed to the nearest 0.1 mg. We also weighed the liver in three laboratories, and paired kidney and adrenal weights were measured in one laboratory.

2.5. Statistical analysis

We received the information from the coordinator of this Phase 3 validation after all tests were finished that the participating laboratories received pairs of the test chemicals (i.e. L and E, F and G, I and C, or K and D), so we analyzed the data using the following analytical methods between the vehicle control group and the same chemical groups in the agonistic version, and the TP group and the same chemical groups in the antagonistic version. In addition, coded A and F were nonylphenol, B and G were dinitrophenol, E and L were trenbolone, C and I were *p,p'*-DDE, and D and K were linurone. Body weight and organ weight data were analyzed by Bartlett's test for homogeneity of variance. When the variance was homogeneous at a significance level of 5%, one-way analysis of variance was performed. If a significant difference was found, the difference between the control group/TP group and each of the dosage groups was analyzed with Dunnett's test. If the variance was not homogeneous, the Kruskal–Wallis test was used. If a significant difference was found, the difference between the control group/TP group and each of the dosage groups was analyzed by the non-parametric Dunnett's test. On the other hand, differences in body weight and organ weight between the control group and the TP group, coded A or B in the agonistic version and between TP group and the group using coded H, G or F in the antagonistic version were assessed for statistical significance by the two-tailed Student's *t*-test. For graphical presentation, the sex accessory organ data were normalized to visually compare the shapes of the responses produced by each laboratory. For this normalization, the control value was set to 100% in the agonistic study, and 100% in the TP without coded compound in the antagonistic study. Analyses of variance were performed on the data from each laboratory and for the pooled laboratory data; these normalized values were not analyzed statistically.

3. Results

3.1. Agonistic version

3.1.1. Body weights, clinical observations, and optional organ weights

The body weights and the optional organ weight changes are shown in Table 2. Terminal body weights in rats given L were significantly lower than in rats given vehicle alone in Labs 2 and 3, and tendency towards lowering of the terminal body weights was observed in Lab 1. No abnormal clinical signs were observed in any of the rats that were treated with each substance. The paired kidney weights in rats given substance A and TP were significantly higher than in rats given only the vehicle in Lab 3, and the liver weights in rats given A and TP were also higher than in rats given the vehicle only in Lab 2.

Table 2
Optional organ weights including the liver, adrenal, and kidney in agonistic version

Lab	Body weights/organ weights	Substances					
		V.C.	A	B	L	E	TP
1	Starting body wt. (g)	214.8 ± 10.6	214.4 ± 11.0	213.1 ± 10.1	219.1 ± 10.5	215.0 ± 8.0	214.9 ± 9.3
	Terminal body wt. (g)	262.9 ± 17.0	250.6 ± 12.0	252.4 ± 12.8	243.7 ± 9.2	249.4 ± 12.9	266.0 ± 12.7
	Liver (g)	10.0 ± 1.1	10.2 ± 1.3	9.6 ± 0.5	11.1 ± 0.7	9.7 ± 0.7	10.1 ± 0.8
2	Starting body wt. (g)	231.0 ± 5.3	227.5 ± 5.0	229.6 ± 6.9	229.1 ± 3.9	226.8 ± 7.9	230.9 ± 6.9
	Terminal body wt. (g)	280.8 ± 7.9	275.9 ± 6.3	285.2 ± 12.4	261.5 ± 7.1*	281.8 ± 15.0	305.6 ± 15.0*
	Liver (g)	11.1 ± 0.6	12.1 ± 0.8*	10.7 ± 1.1	11.5 ± 0.6	11.5 ± 0.9	12.8 ± 1.2*
3	Starting body wt. (g)	257.1 ± 8.9	256.7 ± 8.9	256.0 ± 8.3	257.4 ± 7.9	256.8 ± 10.1	255.0 ± 12.2
	Terminal body wt. (g)	303.2 ± 15.5	297.3 ± 17.6	308.3 ± 14.9	264.6 ± 26.4*	300.8 ± 14.9	320.0 ± 22.7
	Liver (g)	12.9 ± 0.9	14.0 ± 1.9	13.0 ± 1.3	12.2 ± 1.6	13.0 ± 1.3	13.6 ± 2.0
	Adrenals (mg)	58.8 ± 9.3	57.0 ± 9.2	61.5 ± 10.6	49.9 ± 7.8	50.2 ± 5.1	51.0 ± 11.7
	Kidneys (mg)	2110 ± 72	2344 ± 138*	2290 ± 162	2229 ± 226	2189 ± 192	2435 ± 244*

V.C., vehicle control; TP, testosterone propionate. $n = 6$ rats/group/Lab.
* Significantly different from control group at $P < 0.05$.

3.1.2. Accessory sex organ weights

Five accessory sex organ and total five organ weight changes are shown in Table 3, and normalized organ weight changes are shown in Fig. 1. The accessory sex organ weights of rats given TP only in all laboratories were higher than these of rats given the vehicle alone, confirming the reliability of this study. Almost all accessory sex organ weights and total five organs in rats given L were higher than in rats given the vehicle in all laboratories. The LABC weights in rats given E was significantly higher than in rats given the vehicle in Lab 2, but the normalized change in this organ was

Table 3
Mean body weights and mean organ weights in agonistic version

Lab	Body weights/organ weights	Substances					
		V.C.	A	B	L	E	TP
1	Terminal body wt. (g)	262.9 ± 17.0	250.6 ± 12.0	252.4 ± 12.8	243.7 ± 9.2	249.4 ± 12.9	266.0 ± 12.7
	Ventral prostate (mg)	16.8 ± 1.0	17.0 ± 3.2	15.6 ± 3.4	34.1 ± 8.0	14.9 ± 1.2	93.6 ± 11.0*
	Seminal vesicles (mg)	27.9 ± 5.6	26.5 ± 1.9	25.7 ± 4.9	61.2 ± 9.6*	29.0 ± 3.5	190.4 ± 19.1*
	LABC (mg)	136.8 ± 22.2	128.2 ± 19.2	128.3 ± 11.0	298.5 ± 28.1*	141.5 ± 15.2	312.3 ± 26.9*
	Glans penis (mg)	29.5 ± 5.6	29.9 ± 2.8	28.3 ± 7.2	49.8 ± 6.5*	32.6 ± 5.1	64.4 ± 6.0*
	Cowper's glands (mg)	4.1 ± 1.3	4.4 ± 1.4	3.9 ± 1.5	10.3 ± 2.5*	4.5 ± 1.1	20.4 ± 3.5*
	Total of five organs (mg)	215.0 ± 21.3	206.0 ± 23.5	201.9 ± 21.3	453.8 ± 48.3*	222.4 ± 19.0	681.0 ± 42.3*
2	Terminal body wt. (g)	280.8 ± 7.9	275.9 ± 6.3	285.2 ± 12.4	261.5 ± 7.1*	281.8 ± 15.0	305.6 ± 15.0*
	Ventral prostate (mg)	16.0 ± 5.2	19.8 ± 5.1	15.8 ± 6.5	33.4 ± 6.3*	18.4 ± 3.2	121.8 ± 25.6*
	Seminal vesicles (mg)	42.0 ± 14.5	40.9 ± 11.2	38.8 ± 12.2	178.7 ± 60.4*	41.6 ± 11.5	420.4 ± 32.1*
	LABC (mg)	163.6 ± 38.3	178.6 ± 23.5	189.9 ± 30.4	426.8 ± 46.2*	216.3 ± 17.3*	527.5 ± 23.5*
	Glans penis (mg)	44.1 ± 4.3	42.9 ± 2.2	41.8 ± 2.3	58.5 ± 3.7*	45.5 ± 2.5	73.9 ± 3.9*
	Cowper's glands (mg)	5.8 ± 1.3	5.6 ± 1.1	4.4 ± 1.7	10.1 ± 2.6*	5.8 ± 1.8	34.4 ± 8.1*
	Total of five organs (mg)	271.6 ± 51.3	287.8 ± 29.7	290.7 ± 35.3	707.6 ± 104.7*	327.7 ± 14.6	1177.9 ± 35.1*
3	Terminal body wt. (g)	303.2 ± 15.5	297.3 ± 17.6	308.3 ± 14.9	264.6 ± 26.4*	300.8 ± 14.9	320.0 ± 22.7
	Ventral prostate (mg)	22.0 ± 3.1	20.6 ± 1.4	24.0 ± 1.7	43.7 ± 11.5*	26.2 ± 3.8	186.5 ± 48.4*
	Seminal vesicles (mg)	61.2 ± 5.9	58.1 ± 7.0	58.4 ± 8.2	165.5 ± 37.1*	61.2 ± 10.9	431.3 ± 55.1*
	LABC (mg)	191.3 ± 16.0	178.6 ± 25.2	190.7 ± 6.6	452.3 ± 34.5*	221.1 ± 35.8	543.5 ± 83.5*
	Glans penis (mg)	53.0 ± 8.0	54.6 ± 5.4	54.8 ± 5.6	72.9 ± 3.2*	52.0 ± 2.6	95.1 ± 8.0*
	Cowper's glands (mg)	8.5 ± 2.2	7.4 ± 1.8	8.0 ± 1.3	18.2 ± 5.2*	8.8 ± 2.4	37.1 ± 6.6*
	Total of five organs (mg)	336.0 ± 19.9	319.3 ± 29.6	336.0 ± 13.7	752.5 ± 66.4*	369.4 ± 45.8	1293.6 ± 112.7*

V.C., vehicle control; TP, testosterone propionate. $n = 6$ rats/group/Lab.
* Significantly different from control group at $P < 0.05$.

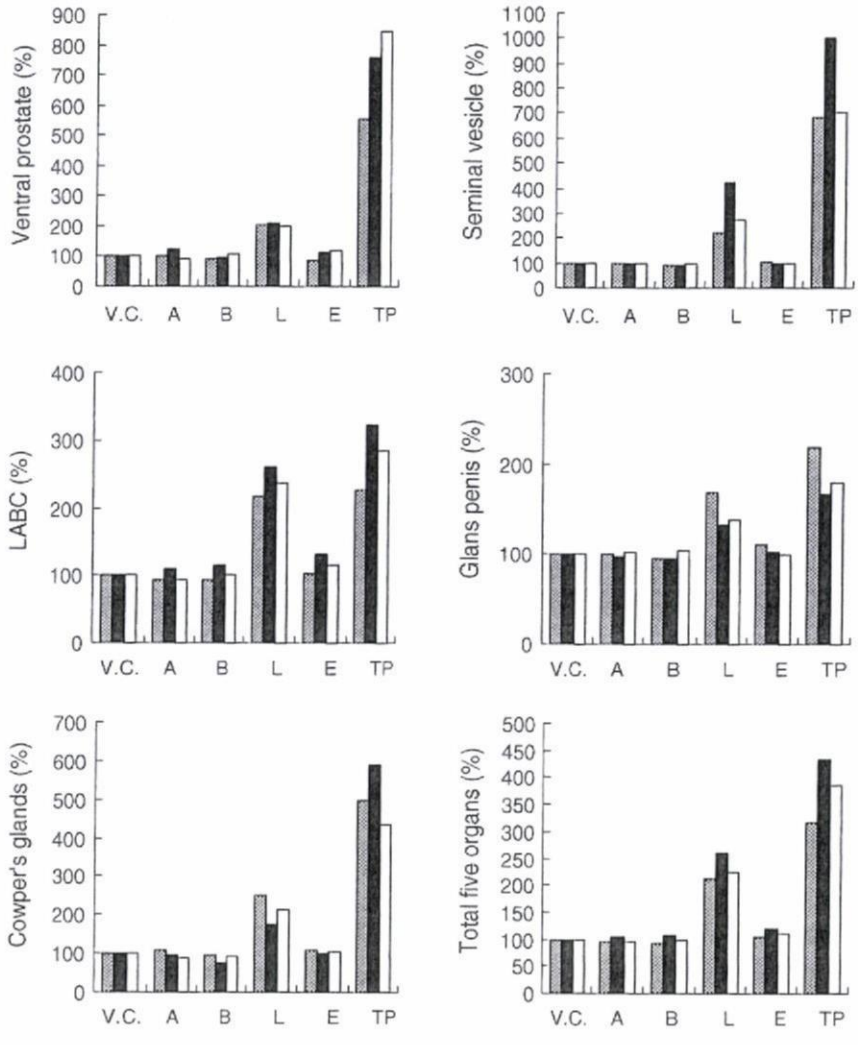

Fig. 1. Organ weights of accessory sex organs in agonistic version. Values from each laboratory were normalized to the control value set equal to 100%. LABC: levator ani and bulbocavernosus muscle; V.C.: vehicle control; A, B, L, and E: coded chemicals; TP: testosterone propionate. $n = 6$ rats/group/Lab (▩, Lab 1; ■, Lab 2; □, Lab 3).

not apparent. Normalized weight changes of the glans penis in rats given coded L showed the weakest response among five organs (Fig. 1).

3.2. Antagonistic version

3.2.1. Body weights, clinical general observations, and optional organ weights

The body weight changes and the optional organ weight changes are shown in Table 4. Two rats given I plus TP died with toxic signs such as decreasing body weight, soft feces, reddish urine, and weakness at 7–10 days after the administration in Labs 2 and 3, respectively. The terminal body weights in rats given I plus TP or K plus TP were significantly lower than in rats given TP only in two laboratories. The paired adrenals in rats given K plus TP were significantly higher than in rats given TP in Lab 3. The liver weights in rats given I plus TP were higher than in rats given TP in all laboratories, and increased liver weights were also observed in rats given C in Lab 1.

3.2.2. Accessory sex organ weights

Five accessory sex organ and total organ weight changes are shown in Table 5, and normalized organ weight changes are shown in Fig. 2. All accessory sex organ weights of rats given H, which is a positive compound, flutamide, plus TP were lower than those of rats given TP, confirming the reliability of this version. Almost all the accessory sex organ weights in rats given I plus TP and K plus TP were significantly lower than in rats given TP in all laboratories. Some accessory sex organ weights in rats given C plus TP and D plus TP were also lower than in the rats given TP. Although the

Table 4
Optional organ weights including the liver, adrenal, and kidney in antagonistic version

Lab	Body weights/organ weights	Substances								
		V.C.	TP	F	G	I	C	K	D	H
1	Starting body wt. (g)	216.2 ± 10.9	216.1 ± 8.8	214.9 ± 10.6	216.6 ± 10.8	218.1 ± 12.5	216.5 ± 8.1	216.0 ± 8.2	219.4 ± 11.3	215.2 ± 9.7
	Terminal body wt. (g)	260.0 ± 17.4	267.4 ± 16.5	263.3 ± 17.8	272.2 ± 9.5	248.7 ± 17.1	266.3 ± 11.7	245.7 ± 11.1*	263.6 ± 11.8	261.5 ± 12.5
	Liver (g)	10.0 ± 1.0	10.4 ± 0.9	11.3 ± 1.3	10.9 ± 0.9	17.3 ± 1.2*	12.5 ± 0.8*	9.6 ± 0.8	10.4 ± 0.5	10.0 ± 0.8
2	Starting body wt. (g)	256.8 ± 11.0	258.9 ± 9.2	259.3 ± 11.6	257.6 ± 10.9	256.8 ± 9.6	258.9 ± 10.8	258.0 ± 10.6	258.9 ± 10.7	255.4 ± 12.7
	Terminal body wt. (g)	313.2 ± 14.7*	331.9 ± 14.3	326.1 ± 16.4	332.2 ± 20.7	270.8 ± 44.6*	329.8 ± 18.7	309.5 ± 13.9*	335.3 ± 15.6	327.4 ± 23.1
	Liver (g)	13.8 ± 0.7	15.6 ± 2.1	15.8 ± 2.3	15.1 ± 0.9	22.8 ± 3.6*	17.7 ± 1.7	14.1 ± 0.8	15.6 ± 1.4	15.1 ± 2.2
3	Starting body wt. (g)	254.1 ± 13.8	254.4 ± 13.6	255.1 ± 12.3	255.1 ± 12.4	255.6 ± 13.1	255.3 ± 14.7	255.2 ± 14.9	254.3 ± 15.9	254.6 ± 16.6
	Terminal body wt. (g)	303.3 ± 16.4	314.7 ± 20.9	307.0 ± 24.0	317.5 ± 10.9	248.1 ± 62.4*	322.7 ± 23.5	292.7 ± 18.1	310.6 ± 23.1	313.2 ± 24.2
	Liver (g)	12.9 ± 1.2	13.7 ± 1.7	14.2 ± 1.7	13.7 ± 0.5	18.6 ± 2.5*	16.0 ± 1.3	13.0 ± 0.9	12.8 ± 1.4	13.5 ± 2.5
	Adrenals (mg)	57.0 ± 10.8	49.5 ± 8.3	54.3 ± 12.5	58.0 ± 5.9	55.5 ± 3.6	56.6 ± 3.5	63.2 ± 7.0*	56.5 ± 7.8	55.5 ± 7.7
	Kidneys (mg)	2106 ± 212	2207 ± 228	2316 ± 273	2342 ± 34	2152 ± 216	2329 ± 197	2236 ± 132	2184 ± 198	2195 ± 201

V.C., vehicle control; TP, testosterone propionate. Each substance was coadministered with 0.2 mg/kg TP. $n = 6$ rats/group/Lab.
* Significantly different from TP group at $P < 0.05$.

LABC weight in rats given G plus TP was significantly lower than that in the TP group in Lab 2, the normalized change of this organ was not so apparent. The total of the five accessory sex organ weights in rats given I plus TP and K plus TP was lower than in rats given TP in all laboratories. The seminal vesicle weight changes in rats given I plus TP and K plus TP were most sensitive among the five organs (Fig. 2).

4. Discussion

Japanese laboratories performed the validation studies of Phase 2 using methyltestosterone, vinclozolin, and *p,p'*-DDE as a part of the national validation program with the result that the Hershberger assay proposed by the OECD was suggested to be a good screening assay to detect androgen agonistic and antagonistic effect (Yamasaki et al., 2003a).

We also performed the Hershberger assay using coded chemicals as part of a national validation Phase 3 as the next step for the OECD guideline process of this assay. The weights of all the accessory sex organs from the experimental animals in all the laboratories exhibited significantly the same changes in the agonistic version; almost all organ weights increased in the rats given coded substance L, and no organ showed any response in rats given coded substances A and B. We received the information from the coordinator of this validation study after all tests were finished that a group of L and E was the same compound and a dose of L was higher than that of E, and that A and B were reported to have no agonistic properties and L and E were a weak agonistic compound. In addition, the normalized weights of all the tissues treated with coded substances in each assay fell within narrow ranges. Therefore, we think that the Hershberger assay is a good screening assay for detecting the androgen agonistic effects of chemicals. The findings that the terminal body weights in rats given coded L were depressed in all laboratories and no body weight changes were detected in rats given coded substance E in all laboratories means that a dose of L was a toxic level and a dose of E had no observed effect. The androgen agonistic effects were detected by the administration of toxic level in this study, but weak agonistic and antagonistic properties of some weak chemicals were detected when non-toxic level doses were administered (Yamasaki et al., 2003a,b).

In the antagonistic version, almost all the sex accessory organs decreased in rats given coded substances I plus TP and K plus TP in all laboratories compared with each organ weight in the rats given TP only, and some organ weights also decreased in the coded substance C

Table 5
Mean body weights and mean organ weights in antagonistic version

Lab	Body weights/organ weights	Chemicals								
		V.C.	TP	F	G	I	C	K	D	H
1	Terminal body wt. (g)	260.0 ± 17.4	267.4 ± 16.5	263.3 ± 17.8	272.2 ± 9.5	248.7 ± 17.1	266.3 ± 11.7	245.7 ± 11.1*	263.6 ± 11.8	261.5 ± 12.5
	Ventral prostate (mg)	14.4 ± 1.5*	102.4 ± 17.6	82.0 ± 7.7	85.9 ± 19.3	28.3 ± 5.9*	78.9 ± 14.6*	41.4 ± 5.9*	80.6 ± 10.7*	24.9 ± 3.6*
	Seminal vesicles (mg)	23.8 ± 3.1*	194.8 ± 38.9	191.4 ± 40.0	185.7 ± 33.5	35.5 ± 6.2*	144.5 ± 29.1	60.6 ± 8.2*	154.7 ± 32.5	28.2 ± 3.8*
	LABC (mg)	119.0 ± 7.0*	306.2 ± 39.1	307.8 ± 40.0	302.3 ± 29.4	125.7 ± 13.4*	280.7 ± 29.7	155.8 ± 21.8*	302.9 ± 40.4	148.3 ± 18.6*
	Glans penis (mg)	29.4 ± 2.9*	60.9 ± 5.3	61.9 ± 5.5	61.2 ± 2.1	32.0 ± 3.9*	53.6 ± 3.3*	41.7 ± 6.4*	64.5 ± 6.1	30.3 ± 4.2*
	Cowper's glands (mg)	3.2 ± 1.3*	18.4 ± 4.6	20.1 ± 1.7	16.2 ± 3.5	4.9 ± 0.8*	16.4 ± 1.9	8.3 ± 1.8*	18.1 ± 3.3	4.7 ± 1.3*
	Total of five organs (mg)	189.9 ± 10.6*	682.7 ± 85.8	663.2 ± 81.3	651.2 ± 65.6	226.5 ± 20.6*	573.9 ± 62.2	307.7 ± 25.4*	620.8 ± 74.6	236.3 ± 24.2*
2	Terminal body wt. (g)	313.2 ± 14.7*	331.9 ± 14.3	326.1 ± 16.4	332.2 ± 20.7	270.8 ± 44.6*	329.8 ± 18.7	309.5 ± 13.9*	335.3 ± 15.6	327.4 ± 23.1
	Ventral prostate (mg)	17.7 ± 2.7*	144.9 ± 19.4	135.0 ± 12.9	129.0 ± 31.5	28.0 ± 8.9*	126.5 ± 40.0	61.6 ± 25.7*	115.5 ± 26.3	28.2 ± 8.9*
	Seminal vesicles (mg)	47.5 ± 8.4*	463.2 ± 70.1	405.9 ± 69.8	439.7 ± 68.7	77.8 ± 26.4*	352.5 ± 80.3*	177.7 ± 43.8*	357.1 ± 40.3*	65.9 ± 11.2*
	LABC (mg)	217.7 ± 25.2*	575.3 ± 31.8	539.6 ± 53.0	529.9 ± 32.2*	218.1 ± 39.6*	496.4 ± 71.6*	336.0 ± 46.3*	532.4 ± 56.5	235.9 ± 29.0*
	Glans penis (mg)	49.9 ± 2.9*	73.0 ± 3.7	75.6 ± 5.9	75.2 ± 2.1	49.8 ± 1.7*	71.7 ± 2.5	63.9 ± 6.2*	73.0 ± 3.3	49.7 ± 2.0*
	Cowper's glands (mg)	5.7 ± 0.9*	33.0 ± 5.4	28.5 ± 5.6	31.0 ± 2.6	10.5 ± 3.8*	30.9 ± 6.5	22.9 ± 9.1	25.7 ± 6.2	10.4 ± 3.4*
	Total of five organs (mg)	338.4 ± 29.0*	1290.2 ± 111.4	1184.6 ± 97.1	1204.6 ± 115.7	384.2 ± 71.6*	1078.0 ± 174.3*	662.0 ± 111.1*	1103.7 ± 89.4*	390.0 ± 29.6*
3	Terminal body wt. (g)	303.3 ± 16.4	314.7 ± 20.9	307.0 ± 24.0	317.5 ± 10.9	248.1 ± 62.4	322.7 ± 23.5	292.7 ± 18.1	310.6 ± 23.1	313.2 ± 24.2
	Ventral prostate (mg)	20.7 ± 3.5*	159.1 ± 31.2	142.0 ± 34.5	132.8 ± 20.1	36.2 ± 9.4*	107.2 ± 17.8*	67.8 ± 24.5*	133.6 ± 24.2	39.8 ± 5.2*
	Seminal vesicles (mg)	53.9 ± 5.2*	459.7 ± 97.2	437.1 ± 120.5	411.6 ± 59.7	73.9 ± 7.9*	323.7 ± 59.0	159.6 ± 45.0*	317.1 ± 62.3*	76.3 ± 10.8*
	LABC (mg)	193.1 ± 11.3*	518.1 ± 74.2	460.2 ± 84.7	494.4 ± 50.1	165.4 ± 35.5*	396.4 ± 50.9*	262.6 ± 47.8*	452.4 ± 64.2	221.8 ± 51.5*
	Glans penis (mg)	50.9 ± 3.8*	93.5 ± 4.6	86.3 ± 7.9	89.6 ± 7.6	60.2 ± 7.3*	83.9 ± 4.3	73.2 ± 10.0*	90.2 ± 6.1	60.8 ± 3.4*
	Cowper's glands (mg)	8.3 ± 2.8*	35.6 ± 6.5	37.5 ± 11.1	33.4 ± 5.3	9.3 ± 2.5*	23.5 ± 5.9*	20.9 ± 4.6*	34.8 ± 6.7	12.0 ± 3.8*
	Total of five organs (mg)	326.9 ± 21.3*	1266.0 ± 151.6	1163.1 ± 217.1	1161.6 ± 84.1	345.0 ± 57.2*	934.7 ± 130.1*	584.2 ± 90.7*	1028.0 ± 110.7*	410.7 ± 61.4*

V.C., vehicle control; TP, testosterone propionate. Each substance was coadministered with 0.2 mg/kg TP. $n = 6$ rats/group/Lab.
* Significantly different from TP group at $P < 0.05$.

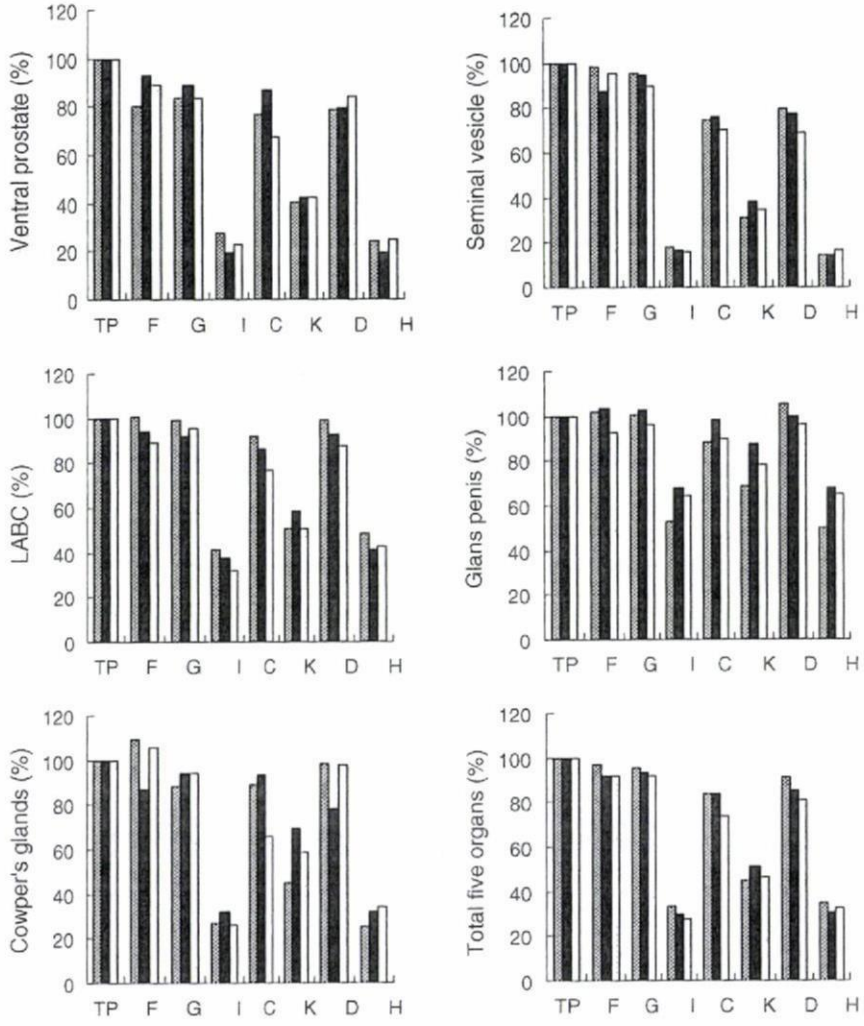

Fig. 2. Organ weights in antagonistic version. Values from each laboratory were normalized to the value of TP group set equal to 100%. LABC: levator ani and bulbocavernosus muscle; F, G, I, C, K, D, and H: coded chemicals; TP: testosterone propionate. $n = 6$ rats/group/Lab (▩, Lab 1; ■, Lab 2; □, Lab 3).

plus TP and D plus TP groups. No changes were detected in rats given coded substances F and G. These findings demonstrate that coded substances I, C, K, and D had antagonistic properties and coded substances F and G had no antagonistic properties. We accepted the information; substances F and G were negative compounds, I, C, K, and D were weak antagonistic compounds, and H was a positive control compound, flutamide; the groups of substances I and C, or K and D were the same compound, and dose levels of I and K were higher than those of C and D. We also received the information that C and I were *p,p'*-DDE and D and K were linurone. The ventral prostate and glans penis in Lab 1, the seminal vesicle and LABC in Lab 2, and the ventral prostate, LABC and Cowper's glans in Lab 3 were significantly affected in the rats given coded substance C plus TP. In addition, the ventral prostate in Lab 1 and seminal vesicle in Labs 2 and 3 were significantly affected in rats given coded substance D plus TP. The differential effects across laboratories were observed in rats given coded C plus TP and D plus TP. We found that the most sensitive organ among the five accessory sex organs was the prostate and/or seminal vesicle in our previous validation Phase 2 study, and in the Hershberger assays using various chemicals (Yamasaki et al., 2003a,b). The ventral prostate and/or seminal vesicle were responded with or without significant differences in rats given coded substances C plus TP and D plus TP, so we determined that coded C and D have androgen antagonistic properties. On the other hand, the LABC weight in rats given coded substance G plus TP was significantly lower than that in the TP group in Lab 2, but the normalized change of this organ was not so apparent. Therefore, the Japanese data in this study demonstrated that the Hershberger assay

is considered to be a good screening assay for detecting the androgen antagonistic effects of chemicals. The findings that some animals died in rats given coded substance I and decreased body weights were detected in rats given K and I, and the liver weights increased in rats given I means the coded substances I and K were at a toxic dose level. In addition, the liver weights increased in rats given C in one laboratory, so a dose of C may be at a toxic level. The general toxicity is considered to be important for this assay, because a 10% change in terminal weight is suggested to affect some Hershberger assay endpoints (Marty et al., 2003).

In conclusion, we performed the OECD validation study Phase 3 using coded chemicals. All five accessory sex organs responded with statistically significant changes in weight within a narrow window in the agonistic and antagonistic versions, and no false positive or false negative results were observed in this study. Therefore, the Japanese studies support the Hershberger assay as a reliable and reproducible screening assay for the detection of androgen agonistic and antagonistic effects.

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特集　環境化学物質の作用メカニズムを解き明かす

トキシコゲノミクスの新展開

Percellomeプロジェクトによる2,3,7,8-TCDD－2,3,7,8-TCDF比較

An Attempt for Adding a New Dimension to Toxicogenomics Research : 2,3,7,8-TCDD−2,3,7,8-TCDF Comparison Trial in The Percellome Project

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Percellomeトキシコゲノミクスはマイクロアレイという数万遺伝子の発現レベルを一気に測定するハイスループット技術を利用し，全遺伝子のカスケード解明を最終目標としつつ，従来に比べてより早く安くかつ正確な毒性評価系の確立を目指すものである．筆者らはこのような次世代の毒性評価・予測技術を開発するために，細胞1個当たりのmRNAコピー数を測定するPercellome法を開発した．今までに90以上の化学物質についての網羅的遺伝子発現情報を得て，なお追加中である．本稿では環境化学物質の一例としてダイオキシンの分子毒性に関わる知見を紹介する．

key words

Percellome Project，遺伝子発現カスケード，分子毒性学

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1985年東京医科歯科大学大学院医学研究科博士課程修了．人体病理学，実験病理学専攻．国立医薬品食品衛生研究所毒性部室長を経て，2002年より同部長．内分泌撹乱関連などの分子毒性学研究，Percellomeトキシコゲノミクスプロジェクトなどを厚生労働所掌業務の一環として有機的に推進．

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はじめに

毒性学は生体（Biosphere）と外来性物質（Chemosphere）との相互作用を研究する分野であり，目的は“ヒトの安全”である．日常遭遇する化学物質（生活化学物質，環境化学物質，医薬品や食品を含む）が摂取された際の安全性を担保するため（毒性評価）に，人体実験が困難な場合，身代わりとしての実験動物の毒性所見をヒトに外挿することが行われてきた．これは両者が基本的に同等の生体反応を示すという前提に基づいている．そして，酵素，膜，DNAなど比較的普遍的かつ基本的な標的が主な検討対象となってきた．現在の分子毒性学は，生体反応メカニズムに踏み込み，受容体，転写因子などとの選択的結合によるシグナル伝達障害などの標的特異性の高いものや，エピジェネティックな遅発影響なども直接的な対象とするようになり，基礎分子生物学と直結する時代に入っている．古い話ではあるもののいまだに分子機構の解明が完結していないサリドマイドの催奇形性問題，あるいは，最近の健常人ボランティアに対するバイオ医薬品（治験薬）の微量投与がその全員を集中治療室送りにした事件は，種差問題の解決を含む分子毒性評価法の確立の重要性と，その現状を示していると考えられる．

Ⅰ．トキシコゲノミクス

分子毒性メカニズム解析のためのツールの1つにmRNAを対象とするトキシコゲノミクスがあり，見落としのない網羅性が要求される毒性学では全遺伝子のカスケード解明がその最終目標となる．これにより従来に比べて早く安く正確な毒性評価を目指すことができる．そして，種差・個体差，一生涯の反応性を修飾する胎生期・周産期影響，あるいは複合作用などを包括的に扱う際には，生命科学の各分野との緊密な連携が必須となる．また，従来の毒性学に対してのトキシコゲノミクスは，例えとしては光学顕微鏡に対しての電子顕微鏡のような立場にあると考えられる．電子顕微鏡が広く用いられるようになるには，教科書や図譜が必要であったように，トキシコゲノミクスの実用化にはある程度の量のデータの蓄積と解析のための基礎研究（関連分野との連携を含む）が必要である．そこで，筆者らは，情報の互換性を確保するために細胞1個当たりのmRNA発現コピー数を得るPercellome手法を開発した．これを基盤としたプロジェクトを展開中であり，今までに90以上の化合物についてのデータを蓄積し，その解析ツールを開発している．

Ⅱ．Percellome法

原理は単純で，サンプルの細胞数を測る代わりにDNA濃度を精密に計測し，それをもとに外部標準mRNA（スパイクRNA）を細胞1個当たり決まった分子数だけそのサンプルに添加し，そしてRNA抽出・測定に移る．スパイクRNAの測定値を基準に，サンプルの各RNAの測定値を細胞1個当たりのコピー数に換算する[1)〜3)]．これにより，実験操作，試薬やマイクロアレイのロット差などによる系統誤差を相殺するという本来の目的が果されるほか，測定過程における各種の異常が高感度に検出されることから，品質管理精

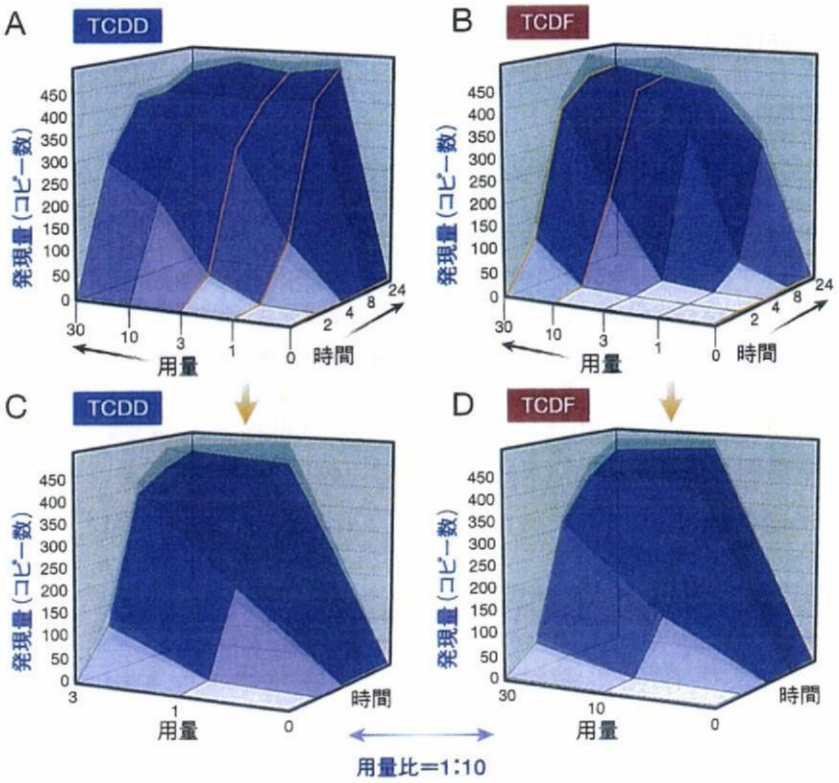

図1. TCDDとTCDFのPercellomeデータ：TEF依存遺伝子の抽出法（1）

TCDDおよびTCDFの単回経口投与をC57BL/6雄マウスに行った．用量は両実験とも0（溶媒対照），1，3，10，および30 μg/kgとし，投与後2，4，8，および24時間後に肝を採取しマイクロアレイ解析を行った（両動物実験は1カ月を隔てて，国立医薬品食品衛生研究所，環境保全型動物実験施設内にて厳重管理の下に実施された）．代表例としてcytochrome P450, family 1, subfamily a, polypeptide 1（Cyp1a1; Affymetrix probe ID 1422217_a_at）を示す．
A：TCDDによるCyp1a1の発現変動のSurface（反応曲面）表示．
B：TCDFによるCyp1a1の発現変動のSurface表示．丁度，用量について10倍ずれた反応を示している．
C：TCDDの3，1，0から作製したSurfaceとD：TCDFの30，10，0から作製したSurfaceが形状および発現値ともにほぼ完全に一致している．このような遺伝子をTEF依存性とした．

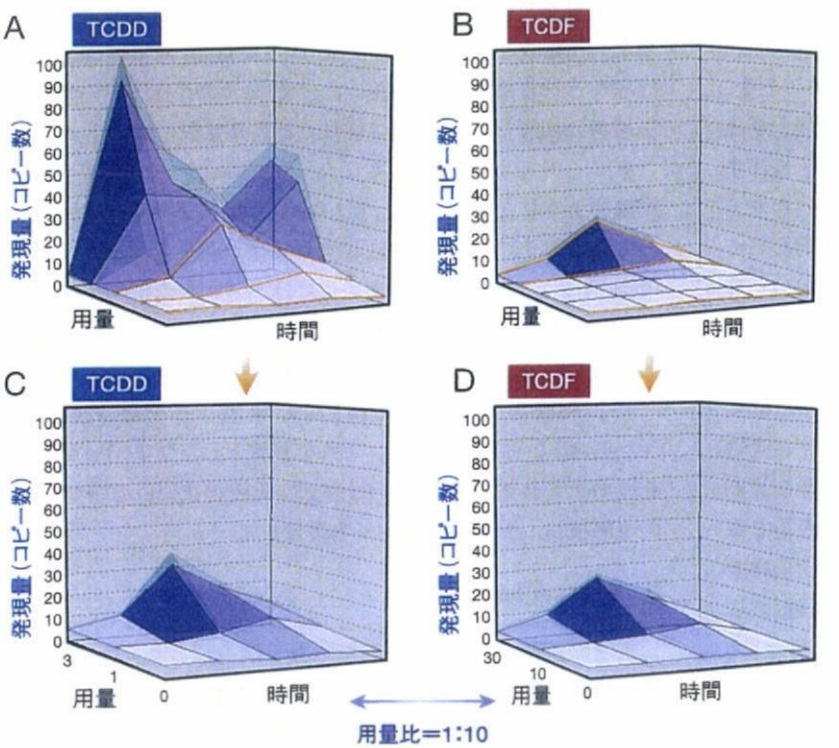

図2. TCDDとTCDFのPercellomeデータ：TEF依存遺伝子の抽出法（2）

TEFに従うもう1つの例としてTCDD-inducible poly (ADP-ribose) polymerase（Tiparp；1452160_at）を示す．A，B，C，Dは図1と同様の表示．AとBを比較すると一見違った反応をしているようだが，CとDを比較するとTEF依存性であることがわかる．

度の向上が図られている．例えば，高密度マイクロアレイで問題となるプローブの飽和によるダイナミックレンジの狭小化の検証・回避に役立っている．新世代Affymetrix GeneChipにおいて高発現遺伝子プローブが容易に飽和し高用量域で定量性を失う現象は，一般的なデータ標準化手法では検出困難であり，Percellome法を用いて初めて直接的に感知することができる．現在，筆者らはサンプルRNA量をメーカー推奨プロトコールの半量にすることなどにより効率的にこれを回避している．