表-2　ニュージーランドの水質基準

（微生物）

項　目	最大許容値	項　目	最大許容値
大腸菌	100mL 中に 1 未満	ウイルス	設定なし
原虫	100L 中に 1 未満		

（無機化学物質）

項　目	最大許容値	項　目	最大許容値
アンチモン	0.02mg/L	フッ素	1.5mg/L
ヒ素	0.01mg/L	鉛	0.01mg/L
バリウム	0.7mg/L	マンガン	0.4mg/L
ホウ素	1.4mg/L	水銀	0.007mg/L
臭素酸	0.01mg/L	モリブデン	0.07mg/L
カドミウム	0.004mg/L	モノクロラミン	3 mg/L
塩素酸	0.8mg/L	ニッケル	0.08mg/L
塩素	5 mg/L	硝酸（短期）	50mg/L
亜塩素酸	0.8mg/L	亜硝酸（長期）	0.2mg/L
クロム	0.05mg/L	亜硝酸（短期）	3 mg/L
銅	2 mg/L	セレン	0.01mg/L
シアン	0.6mg/L	ウラン	0.02mg/L
塩化シアン	0.4mg/L		

（放射性物質）

項　目	最大許容値	項　目	最大許容値
アルファ線	0.10Bq/L	ベータ線	0.50Bq/L
ラドン	100Bq/L		

著者注記：水質基準項目は浄水場、配水区域ごとに定められた要件に該当する項目を水質検査する。詳細は 6 章の(1)の3) 水質検査と水質基準の適合基準を参照

（有機化学物質）

項　目	最大許容値	項　目	最大許容値
アクリルアミド	0.0005mg/L	フェノプロップ	0.01mg/L
アラクロール	0.02mg/L	ヘキサクロロブタジエン	0.0007mg/L
アルディカーブ	0.01mg/L	ヘキサジノン	0.4mg/L
アルドリン、ディエルドリン	0.00004mg/L	ホモアナトキシン-a	0.002mg/L
アナトキシン－a	0.006mg/L	イソプロツロン	0.01mg/L
アナトキシン－a (s)	0.001mg/L	リンデン	0.002mg/L
アトラジン	0.002mg/L	MCPA	0.002mg/L
グチオン	0.004mg/L	メコプロップ	0.01mg/L
ベンゼン	0.01mg/L	メタラキシル	0.1mg/L
ベンゾ（α）ピレン	0.0007mg/L	メトキシクロール	0.02mg/L
ブロマシル	0.4mg/L	メトラクロール	0.01mg/L
ブロモジクロロメタン	0.06mg/L	メトリブジン	0.07mg/L
ブロモホルム	0.1mg/L	ミクロシスティン	0.001mg/L
カルボフラン	0.008mg/L	モリネート	0.007mg/L
四塩化炭素	0.005mg/L	モノクロロ酢酸	0.02mg/L
クロルデン	0.0002mg/L	ニトリロ三酢酸	0.2mg/L
クロロホルム	0.4mg/L	ノデュラリン	0.001mg/L
クロロトルロン	0.04mg/L	オリザリン	0.4mg/L
クロルピリホス	0.04mg/L	オキサジアゾン	0.2mg/L
シアナジン	0.0007mg/L	ペンディメタリン	0.02mg/L
シリンドロスペルモプシン	0.001mg/L	ペンタクロロフェノール	0.009mg/L
2,4-D	0.04mg/L	ピクロラム	0.2mg/L
2,4-DB	0.1mg/L	ピリミホスメチル	0.1mg/L
DDT	0.001mg/L	プリミスルフォンメチル	0.9mg/L
フタル酸-ジ-2-エチルヘキシル	0.009mg/L	プロミシドン	0.7mg/L
1,2-ジブロモ-3-クロロプロパン	0.001mg/L	ピロパジン	0.07mg/L
ジブロモアセトニトリル	0.08mg/L	ピリプロキシフェン	0.4mg/L
ジブロモクロロメタン	0.15mg/L	サキシトキシン	0.003mg/L
1,2-ジブロモメタン	0.0004mg/L	シマジン	0.002mg/L
ジクロロ酢酸	0.05mg/L	スチレン	0.03mg/L
ジクロロアセトニトリル	0.02mg/L	2,4,5-T	0.01mg/L
1,2-ジクロロベンゼン	1.5mg/L	ターバシル	0.04mg/L
1,4-ジクロロベンゼン	0.4mg/L	テルブチラリン	0.008mg/L
1,2-ジクロロエタン	0.03mg/L	テトラクロロエチレン	0.05mg/L
1,2-ジクロロエチレン	0.06mg/L	チアベンザゾール	0.4mg/L
ジクロロメタン	0.02mg/L	トルエン	0.8mg/L
1,2-ジクロロプロパン	0.05mg/L	トリクロロ酢酸	0.2mg/L
1,3-ジクロロプロペン	0.02mg/L	トリクロロエチレン	0.02mg/L
ジクロロプロップ	0.1mg/L	2,4,6-トリクロロフェノール	0.2mg/L
ジメトエート	0.008mg/L	トリクロピル	0.1mg/L
1,4-ジオキサン	0.05mg/L	トリフルラリン	0.03mg/L
ジウロン	0.02mg/L	トリハロメタン	濃度比の総和が1以内
EDTA	0.7mg/L	塩化ビニル	0.0003mg/L
エンドリン	0.001mg/L	キシレン	0.6mg/L
エピクロロヒドリン	0.0005mg/L	1080	0.0035mg/L
エチルベンゼン	0.3mg/L		

れている。水質検査において、必要でない項目の測定をできるだけ減らすことを目的として検査項目、非検査項目の選択基準を定めている。

2)　基準項目の分類

基準項目の健康影響度及び各水道事業体の存在実態に応じて 4 つに分類し、水質検査を効率的に行う仕組みが導入されている。基本的に、大腸菌、原虫はプライオリティ 1 に分類され、それ以外の物質は水道水中の濃度に応じてプライオリティ 2 から 4 に分類される。プライオリティ 1 及び 2 に

分類された項目は水質検査を行う必要がある。

3)　水質検査と水質基準の適合基準

水質検査はプライオリティ1及び2に分類された水質項目の濃度がMAV未満であることを確認するために行われる。一方、水質基準の適合基準はそのような水道水が常に供給されていることを確実にするために設けられている。水質検査は当該物質を測定する検査と処理プロセスに関連した指標を測定する場合の二通りが設定されている。指標を測定する場合はMAVに代わって管理目標（Operational Requirement Limit）が適用される。

水道事業体の個別の浄水場、配水区域ごとに、下記の条件がすべて満たされている場合は水質基準に適合したと判定される。

・MAVまたは管理目標値を超過した回数が表-3に示されている許容回数以下であること。
・12ヶ月間にわたりプライオリティ1及び2に分類された項目と管理目標が、求められている頻度、条件を満たして測定されていること。
・水質検査の方法が定められた方法であること。
・MAVを超過した場合に、定められた対応が実施されていること。

表-3　水質基準を満足していると判定できる超過（陽性）数の上限

超過（陽性）数	全検査数
0	38－76
1	77－108
2	109－138
3	139－166
4	167－193
5	194－220
6	221－246
7	247－272
8	273－298
9	299－323
10	324－348
....	.....
....	.....
159	3,606－3,626

(2)　微生物及び原虫に関する水質基準の適合基準

大腸菌に関する適合基準は浄水場出口と配水区域それぞれに設定されており、連続測定しているかどうか、汚染のない井戸水と確認された原水かどうかなどの要因により異なる。詳細を7章に示した。

水質基準では原虫そのものの検査は求めておらず、処理による対応を求めている。処理方法は、原水の汚染リスク評価結果に基づいて選択することとされている。リスク評価は給水人口が1万人以上の場合は、原水中のクリプトスポリジウムを1年間に26回測定し、結果を表-4に当てはめ、処理によって達成すべき除去率を求める。水質基準には処理方法ごと、また処理方法の組み合わせによるオーシストの除去率が示されており、必要な除去率が保障できる処理方法を選択するか単独の処理を組み合わせて除去率を確保する。給水人口が1万人未満の水道では水源地域にある汚染源のリスク評価に基づいて必要な除去率を選択する。必要な除去率は、表流水ではlog 3（99.9%）～log 5（99.999%）とされている。汚染がないと確認された井戸水を原水とする場合は原虫の処理は求められておらず、それ以外の場合では井戸の状況に応じてlog 2（99%）～log 5（99.999%）の除去率を選択することが定められている。

表-4　原水中のオーシスト数と求められるlog除去率

原水10L中の平均オーシスト数	要求されるlog除去率
0.75未満	3（99.9%）
0.75～9.99	4（99.99%）
10以上	5（99.999%）

(3)　シアノトキシンに関する水質基準の適合条件

シアノトキシンは化学物質に分類されているが、一時的、または季節的に発生するなどの理由により、他の化学物質と異なるプライオリティの分類方法が提示されている。過去に水の華が発生したことがある水源、またはDWAが必要と判断した場合は、水道水中のシアノトキシン濃度を測定し、結果がMAVの50%を超える場合はプライオリティ2に分類した上で、定められた頻度で水質検査することが求められている。シアノトキシンがMAVを超過した場合は直ちに水質基準不適合と判断し、代替水源の水道水を供給することなどが

定められている。

(4) 化学物質に関する水質基準の適合基準

1) プライオリティの分類

水質基準では、シアノトキシン以外に109の化学物質をリストアップしている。各水道事業体ごとにこれらの物質について水源、浄水場、配水区域における汚染の可能性を評価し、その結果を基にDWAがプライオリティ2に分類する化学物質の候補を選定する。選定された物質をプライオリティ2化学物質特定計画（The Priority 2 Chemical Determinands Identification Programme）に基づいて測定し、MAVの50%を超過している物質がプライオリティ2に分類される。分類される物質は水道事業体ごとに異なることになる。水道事業体はこれらの物質の水質検査を行う。

2) 水質検査の頻度

プライオリティ2物質は浄水処理によって付加される物質、それ以外の物質で浄水場以降で濃度が変化しない物質及び浄水場以降で濃度が変化する物質に分類される。この分類に基づいて、各物質の検査場所が決定される。これらの物質は、フッ化物イオン（フッ素添加を行っている場合）、塩素（MAVを超過していないことの確認）、シアノトキシンを除き、少なくとも1ヶ月に1回以上測定しなければならない。フッ化物イオン、塩素は1週間に1回以上、シアノトキシンは藻類の増殖期に1週間に2回以上測定することが求められる。12ヶ月間の測定でMAVの50%を超過しないことが確認できればDWAに当該物質のプライオリティ3への格下げと水質検査の終了を要請することができる。

(5) その他の項目の適合基準

「放射性物質に関する適合基準」及び「規模が小さい水道及び給水タンク車により給水する水道に関する適合基準」が定められている。

(6) プライオリティ2に分類された項目数と事業体数

プライオリティ2に分類され、定期的に水質検査しなければならないとされた水質項目と事業体数を表-5に示した。前述したようにニュージーランド国内の水道事業体数は2,000以上と想定されているが、MAVの50%を超過している項目について水質検査を行うことになっているため、水質検査が行われている浄水場及び配水区域の総数は300程度である。

7. ニュージーランドにおける水道の消毒に関する規則[9]

(1) 概要

水質基準では塩素、二酸化塩素、オゾン、紫外線を消毒剤として使用することを想定した規定が設けられているが、水道水の消毒は義務付けられていない。また、浄水場で消毒を行わない場合及び浄水場での消毒後に配水区域で消毒剤が残留しない場合を想定した規定も設けられている。いずれの場合においても浄水場の出口における大腸菌の基準に適合することを基本としており、消毒の

表-5 プライオリティ2に分類された項目と事業体数（2008年12月）

（浄水場出口）

水質項目	ヒ素	塩素酸	フッ素	硝酸態窒素
浄水場数	1	4	50	1

（配水区域）

水質項目	アンチモン	ヒ素	ホウ素	ブロモジクロロメタン	カドミウム	銅
区域数	5	16	2	4	15	16

水質項目	ジクロロ酢酸	フッ素	鉛	マンガン	総ハロ酢酸	総トリハロメタン
区域数	16	1	75	2	30	17

水質項目	ニッケル	硝酸態窒素	トリクロロアセトアルデヒド	トリクロロ酢酸
区域数	31	4	32	10

有無、消毒方法及び給水人口に応じて検査頻度がきめ細かく定められている。

消毒を行わずに給水する場合、安全であると認定された井戸水（Secure Bore Water）を水源とする浄水場の大腸菌検査は、それ以外を水源とする浄水場に比べて検査頻度が大きく緩和されており、同国の南島に位置するクライストチャーチ市ではこの規定を準用して消毒していない水道水を給水している。配水区域では大腸菌の基準が一律に設定されており、給水人口に応じて四半期毎の最低検査数が定められている。

(2)　浄水場出口の適合基準

浄水場での消毒処理について下記の５つの場合を想定し、それぞれについて満足すべき大腸菌検査及び他の技術的な要件を設定している。

①消毒なしまたは消毒されているが消毒剤が残留していない場合及び結合塩素消毒（クライテリア１）
②塩素消毒しており塩素濃度を連続監視している場合（クライテリア２A)、給水人口が5,000人以下で塩素消毒しており塩素濃度を連続監視していない場合（クライテリア２B）
③二酸化塩素消毒（クライテリア３）
④オゾン消毒（クライテリア４）
⑤紫外線消毒（クライテリア５）

それぞれのクライテリアに定められている大腸菌の最低検査頻度を表-6に示した。クライテリア１のうち、汚染されていないと認定された井戸水を水源とする場合は、他の場合と比べて検査頻度が大幅に緩和されている。一方、認定されていない井戸水などを水源とする水道において消毒されていない水道もしくは消毒剤が残留していない水道水は、表-6の頻度で行う検査をすべて満足すれば供給することが可能である。クライテリア２Aでは、連続的な残留塩素濃度の監視を大腸菌の検査に置き換えることができるとされており、大腸菌の検査は省略できる。残留塩素濃度の下限は0.2mg/L とされており、この濃度を下回った場合はDWA に報告することとされている。また、0.1mg/L 未満になった場合は大腸菌が陽性となった時と同じ対応を行うことが求められている。１日のうちで0.2mg/L 以上の測定結果が98％以上の場合は水質基準に適合していると判定される。

クライテリア２B では大腸菌の検査に加え、残留塩素濃度、pH、濁度の測定が求められており、給水人口に応じて１週間に１～２回の測定頻度が定められている。塩素濃度が0.2mg/L を下回った場合はクライテリア２A の場合と同じ対応が求められている。

クライテリア３ではクライテリア２A とほぼ同じ規定が設けられており、二酸化塩素の連続測定が求められている。クライテリア４ではオゾン消毒において一定のC･t値を確保することなどが規定されている。クライテリア５では紫外線消毒に

表-6　大腸菌の検査頻度

水道のタイプ クライテリア	給水人口	最低検査頻度	検査と検査の最大間隔日数	検査を行う曜日の数[1]
安全認定された井戸水 （クライテリア１）	すべて	１カ月に１回[2]	45(135)[2]	3（1）[2]
消毒なし 消毒剤が残留していない場合 結合塩素消毒 （クライテリア１）	500人以下 501人～10,000人 10,000人以上	１週間に１回 １週間に２回 毎日	13 5 1	5 6 7
塩素消毒しており塩素濃度を 連続監視していない場合 （クライテリア２B）	500人以下 501人～5,000人	２週間に１回 １週間に１回	22 13	3 5
オゾン（クライテリア４） 紫外線（クライテリア５）	すべて	２週間に１回	22	3

1：例えば「3」は月曜日、水曜日、金曜日など最低３つの曜日に分散させて検査する。
2：要件を満たせば最大３ヶ月に１回まで検査を省略できる。

おける線量などが定められている。

大腸菌検査が陽性になった場合、残留塩素濃度が0.1mg/L を下回った場合、オゾンのC・t 値及び紫外線線量が一定値を下回った場合、濁度が定められた濃度を超過した場合は、DWA への報告、配水区域での大腸菌検査、原因の究明と是正、水源調査（浄水場に問題がない場合）、応急給水などを緊急に行うよう規定されている。

(3) 配水区域の適合基準

配水区域の給水人口に応じた大腸菌の検査頻度が設定されている。配水区域の給水人口が500人以上で適切な残留塩素濃度が維持されている場合、大腸菌検査数の75％を配水区域で行う残留塩素検査で置き換えることが認められている。残留塩素濃度の下限は滞留部を除き0.2mg/L で、これを下回った場合は大腸菌検査を行うこととされている。滞留部では0.1mg/L を下回った場合に大腸菌検査を行うこととされている。いずれも、検査結果の陽性数が検査数に対して定められた数以下の場合、水質基準に適合していると判定される。大腸菌検査で陽性が確認された場合、浄水場で陽性が確認された場合とほぼ同様の対応を配水区域について行わなければならない。

(4) 汚染されていない井戸水（Secure Bore Water）の要件

1) 地表または気候の影響を受けていない井戸水

確認は次の３方法のいずれかによるとされている。滞留時間を要件とする場合、帯水層での滞留時間が１年未満の水の割合が0.005％未満であることが条件とされており、トリチウム、クロロフルオロカーボンもしくは六フッ化硫黄のいずれかを測定して判定する。水質項目の濃度変動による場合は、一定期間内（１年～３年）の測定における３つの水質項目の変動係数がすべて一定値を上回らないこととされている。対象水質項目は電気伝導率、塩化物イオン、硝酸態窒素で、変動係数の上限はそれぞれ３％、４％、2.5％である。確認に用いる化学物質が含まれていないなど、上記の方法で確認できない場合、根拠のある水理モデルを用いた方法の利用が認められる。

2) 防護された井戸からくみ上げた井戸水

井戸の防護に関して、頭頂部の保護と周辺部５m の範囲内での動物侵入防止措置及び井戸の建設資材に関する規定がある。また、井戸の防護に関して定期確認するよう規定されている。深さが10m 未満の井戸水、10m～30m の井戸水で大腸菌検査において陰性の結果が得られていない場合は「汚染されていない井戸水」とは認定されない。

8. 水道事業体の格付け制度[10,16,17]

(1) 格付け制度の目的

格付け制度の目的は水道事業体の安全で良質な水道水の安定供給能力を公表することであり、格付けの評価に当たっては公平で正確に行われていることが第三者機関により確認されるシステムが確立されている。同制度により、水道システムの改善・改良の取り組みの促進、利用者からの水道に対する改善要求内容の具体化の支援、水道の設置者からの水道の運営担当者に対する運営状況の提示、水道事業者間での最高格付けの取得に向けた取り組みが期待されている。現在は給水人口500人以上の水道事業体を対象としており、将来的には25人以上の事業体に拡大する予定にしている。格付けの結果は公表されており、概ね５年ごとに見直されている。

(2) 格付けの評価と活用

格付けの評価では、水道水が水質基準に適合していることの確認に加え、安全な水道水を送り続けるためのマルチバリアが適切に構築されていることが確認される。評価結果は①水源及び浄水処理、②配水システムを個別に、最高の評価（A1、a1）から最低の評価（E、e）まで６段階で評価し、結果は大文字と小文字の２文字を組み合わせて公表される。大文字は水源及び浄水処理を、小文字は配水システムの評価を示している。給水人口が１万人以上の水道事業体ではB、a、5,001～１万人の水道事業体ではB、b、５千人以下の水道事業体ではC、c 以上の格付け結果が求められている。

(3) 評価の手順

評価は定められた資格を有するDWA が行う。水源・浄水処理の格付けは33の要素を審査する。大腸菌、原虫、プライオリティ２物質などの基準への適合、記録の保存、水質モニタリング体制、内部監査の実施、水安全計画の策定と運用状況な

どが評価される。浄水に残留塩素が保持されない場合は最高でBの評価とされる。最も格付けが高いA1の取得には水道水の美観的（Aesthetic）項目が指針値に適合していること及びISOなどの品質管理システムを導入していることが必要である。

配水区域は配水管の経年度、水圧の監視、漏水防止計画、配水管洗浄計画など22の要素について審査を行い、減点制で評価される。不適合な事象に応じて減点ポイントが定められており、大腸菌基準への不適合は23ポイント、残留塩素が維持されていない場合は12ポイント、配水圧に関する記録の不備は2ポイントなどである。a、a1の評価を取得するためには減点ポイントが10以下、bの場合は20以下、cの場合は30以下でなければならないとされている。

9. ニュージーランドにおける水安全計画[12,18,19]

(1) 制度の概要

水安全計画としてPHRMPが制度化されており、保健（飲料水）改正法2007では給水人口が500人以上の水道事業体に作成と導入を義務付けている。また規模の小さい水道事業体にも策定を推奨している。健康省は、「水道におけるPHRMP策定のための指針（A Framework on How to Prepare and Develop Public Health Risk Management Plans for Drinking-water Supplies）」、「PHRMP策定のための各処理過程に関する情報（Public Health Risk Management Plan Guides）」など、水道事業体におけるPHRMPの策定を支援するプログラムを公開している。

(2) 策定のための支援

「水道におけるPHRMP策定のための指針」は水道事業体が水安全計画を策定する場合の詳細な手順を示したもので、PHRMP（水安全計画）の意義と策定、策定アプローチ方法の多様性の明示（策定方法は限定しない。）、個々の水道における独自の検討の必要性などを記述している。また、「PHRMP策定のための各処理過程に関する情報」は水道事業体がPHRMPの策定を容易に行うことを目的としたもので、水道の水源、浄水処理、配水施設において検討すべき事項が水源の種類、処理の種類毎に整理された総数40以上のガイドとして公開されている。個々の水道事業体の処理フローにしたがってガイドを選択することにより、水安全計画の体系とその概要がほぼ定まるように工夫されている。

10. サーベイランスと情報の公開[11]

(1) サーベイランスの制度

DWAによる水道事業体の格付け審査が概ね5年ごとに行われ、水質検査の実施状況、水質基準の適合状況、水質管理の記録、浄水場の運転記録などを監査するシステムが確立されている。

(2) 情報の公開

WINZは様々な水道水質に関する情報を収集するネットワークシステムであり、水道事業の特徴、各水道の格付け結果、水質基準の達成状況など水質管理に必要な情報が収集されている。一般に公開されている情報は、給水人口などの一般情報及び浄水場、配水区域ごとの水質基準適合状況、適合していない場合の不適合要件、最新の格付けの結果、プライオリティ2に分類された事業体ごとの水質基準項目などである。また、格付けの評価時に収集された情報、水安全計画策定時のリスク評価情報、水質検査計画に関する詳細情報なども収集されているとされているが、閲覧は関係者のみが可能で、一般には公開されていない。

化学物質及び微生物の水質検査結果は水質統計として毎年作成されており、保健省のホームページで全文が入手可能になっている。

11. まとめ

(1) ニュージーランド国政府保健省は保健（飲料水）法を2008年に改正、施行し、水道事業体に対して水質基準の遵守と基準に適合、水道事業者に対する水安全計画の導入、法律の遵守に関する記録の保管と公表などの制度を義務化した。

(2) ニュージーランドの水質基準には、微生物3項目、化学物質116項目、放射性物質3項目の最大許容値及び水質基準の適合基準が示されている。水質基準の検査項目は事業体によって異なり、原水中の最大濃度が最大許容値の50％を超える項目など、一定の基準に基づいて水道水評価官（DWA）が最終的に決定する。

(3) 消毒の強制的な規則は設けられておらず、水質基準に適合する限り、消毒を行わずに給水で

きる。安全であると認定された井戸水を原水として消毒を行わずに給水する場合、大腸菌検査頻度を大幅に減ずることができる。消毒剤は塩素、二酸化塩素、オゾン、紫外線を用いることができる。

(4) 水道事業体の安全で良質な水道水の安定供給能力を公表することを目的として水道事業体の格付け制度を制度化している。水道システムの改善・改良の取り組みの促進、利用者からの水道に対する改善要求内容の具体化の支援、水道の設置者からの水道の運営担当者に対する運営状況の提示、水道事業者間での最高格付けの取得に向けた取り組みが期待されている。

(5) 水安全計画の作成と導入が給水人口500人以上の水道事業体に義務付けられている。また規模の小さい水道事業体にも策定を推奨している。健康省は、「水道における PHRMP 策定のための指針」などの策定支援プログラムを公開している。

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本文中で使用した略号一覧

略号	英 語	和 訳
CAP	Capital Assistance Programme	財政支援プログラム
DHB	District Health Board	地区健康局
DWA	Drinking-Water Assessor	水道水評価官
MAV	Maximum Acceptable Value	最大許容値
NES	National Environmental Standard for Sources of Human Drinking Water	水道水源のための環境基準
PHRMP	Public Health Risk Management Plans	水安全計画
TAP	Technical Assistance Programme	技術支援プログラム
WINZ	National Drinking-Water Information System	水道情報提供サービス

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RESEARCH ARTICLE

Developmental toxicity of dibutyltin dichloride given on three consecutive days during organogenesis in cynomolgus monkeys

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Abstract

We previously reported that the administration of dibutyltin dichloride (DBTCl) by nasogastric intubation during the entire period of organogenesis, days 20–50 of pregnancy, was embryolethal, but not teratogenic, in cynomolgus monkeys. The present study was conducted to further evaluate the developmental toxicity of DBTCl given to pregnant monkeys on 3 consecutive days during organogenesis. Cynomolgus monkeys were given DBTCl at 7.5 mg/kg body weight/day by nasogastric intubation on days 19–21, 21–23, 24–26, 26–28, 29–31, 31–33, or 34–36 of pregnancy, and the pregnancy outcome was determined on day 100 of pregnancy. Embryonic/fetal loss was observed in 1 female given DBTCl on days 19–21, 2 females given DBTCl on days 24–26, and 1 female given DBTCl on days 34–36. There were no effects of DBTCl on developmental parameters in surviving fetuses, including fetal body weight, crown-rump length, tail length, or placental weight. No external, internal, or skeletal malformations were detected in fetuses in any group. DBTCl did not affect the incidence of fetuses with skeletal variation or skeletal ossification of fetuses. These data confirm our previous findings that DBTCl was embryolethal, but not teratogenic, in cynomolgus monkeys.

Keywords: *Developmental toxicity; embryolethality; dibutyltin; monkey*

Introduction

Organotin compounds are widely used in agriculture and industry (Quevauviller et al., 1991). Disubstituted organotin compounds are commercially the most important derivatives and are mainly used in the plastics industry, particularly as heat and light stabilizers for polyvinyl chloride (PVC) plastics to prevent degradation of the polymer during melting and forming of the resin into its final products, as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers (Piver, 1973; WHO, 1980). The most important nonpesticidal routes of entry for organotin compounds into the environment are through their use as PVC stabilizers (Quevauviller et al., 1991) and their use as antifouling agents, which introduces them to the aquatic environment (Maguire, 1991). Tributyltin (TBT) and dibutyltin (DBT) have been found in aquatic marine organisms (Lau, 1991; Sasaki et al., 1988) and marine products (Suzuki et al., 1992). TBT is degraded spontaneously and biochemically to DBT in the environment via a debutylation pathway (Seligman et al., 1988; Stewart and de Mora, 1990). These findings suggest that organotin compounds could be introduced into food products and subsequently consumed by humans.

We previously showed that dibutyltin dichloride (DBTCl) was embryolethal when orally administered during early pregnancy in rats (Ema and Harazono, 2000a, 2000b; Ema et al., 2003) and mice (Ema et al., 2007a). DBTCl was teratogenic when orally administered during organogenesis in rats (Ema et al., 1991); rat embryos were highly susceptible to the teratogenic effects of DBTCl when orally administered on days

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7 and 8 of pregnancy (Ema et al., 1992; Noda et al., 1993). Dibutyltin diacetate (DBTA) (Noda et al., 1992, 1993, 1994), dibutyltin maleate, dibutyltin oxide, and dibutyltin dilaurate were also teratogenic when orally administered during organogenesis in rats (Noda et al., 1993). Developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from that of tetrabutyltin (TeBT), TBT, and monobutyltin (MBT) in its mode of action because the period of susceptibility and the types of induced malformations are different (Ema et al., 1995a, 1996a). DBTCl showed dysmorphogenic potential in a rat whole-embryo culture system (Ema et al., 1995b, 1996b). DBT was detected in maternal blood at 100 ng/g and embryos at 720 ng/g at 24 hours after gavage of DBTA at 22 mg/kg on day 8 of pregnancy in rats (Noda et al., 1994). The dysmorphogenic concentrations of DBTCl in cultured embryos were within the range of levels detected in maternal blood after the administration of a teratogenic dose of DBT at 20–40 mg/kg. These findings suggest that DBT itself causes DBT teratogenesis, possibly via direct interference with embryos.

The developmental toxicity of organotin compounds has been extensively investigated in rodents (Ema and Hirose, 2006). We previously assessed the prenatal developmental toxicity of DBT in cynomolgus monkeys and reported that nasogastric intubation of DBTCl at 2.5 or 3.8 mg/kg body weight/day during the entire period of organogenesis (days 20–50 of pregnancy) was embryolethal but is unlikely to be teratogenic (Ema et al, 2007b). However, the treatment regimen in our previous study, which was designed to screen for embryofetal lethality/teratogenicity and included a longer duration of treatment, might have masked or diminished some effects. A shorter administration period can provide more information about developmental toxicity because it permits increased doses and reduces maternal toxicity. However, there have been no studies on developmental toxicity in monkeys after shorter durations of treatment with organotin compounds. Therefore, the present study was conducted to further evaluate the developmental toxicity of DBTCl given to pregnant monkeys on 3 consecutive days during organogenesis and to determine if phase specificity could be observed with the shorter duration of administration.

Materials and methods

Animal experiments were performed at Shin Nippon Biomedical Laboratories, Ltd. (SNBL; Kagoshima, Japan) during 2004–2007 in compliance with the Guideline for Animal Experimentation (1987) and in accordance with the Law Concerning the Protection and Control of Animals (1973) and the Standards Relating to the Care and Management of Experimental Animals (1980). This study was approved by the Institutional Animal Care and Use Committee of SNBL and performed in accordance with the ethics criteria contained in the bylaws of the SNBL committee.

Animals

Cynomolgus monkeys (*Macaca fascicularis*) were used in this study. The monkeys were obtained from Guangxi Primate Center of China (Guangxi, China) through Guangdong Scientific Instruments and Materials Import/Export Co. (Guangzhou, China). The monkeys were quarantined for 4 weeks and confirmed to be free from tuberculosis, *Salmonella*, and *Shigella*. The animals were maintained in an air-conditioned room at 23.0–29.0°C, with a relative humidity of 35–75%, a controlled 12–12-light and dark cycle, and a ventilation rate of 15 air changes/hour. Monkeys were housed individually, except during the mating period and fed 108 g/day of diet (Teklad global 25% protein primate diet; Harlan Sprague-Dawley Inc., Madison, Wisconsin, USA) and *ad libitum* tap water from an automatic supply (Edstrom Industries Inc., Waterfold, WI, USA). Healthy male and female monkeys were selected for use. Only females showing 25–32-day menstrual cycles were used in the experiment. Each female monkey was paired with a male of proven fertility for 3 consecutive days between days 11–15 of the menstrual cycle. Visual confirmation of copulation and/or the presence of sperm in the vagina were considered evidence of successful mating. When copulation was confirmed, the median day of the mating period was regarded as day 0 of pregnancy. Pregnancy was confirmed 18–23 days after copulation by ultrasound (SSD-4000; Aloka Co., Mitaka, Japan) under anesthesia induced by an intramuscular injection of 5% ketamine hydrochloride (Sigma Chemical Co., St Louis, Missouri, USA). Pregnant females, weighing 2.51–4.50 kg on day 0 of pregnancy, were allocated randomly to seven groups, each with 5 monkeys, and housed individually.

Dosing

Monkeys were dosed once-daily with DBTCl (Lot no. GG01, 98% pure; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) at 7.5 mg/kg by nasogastric intubation on either days 19–21, 21–23, 24–26, 26–28, 29–31, 31–33, or 34–36 of pregnancy. The dosage levels were determined in previous studies where the administration of DBTCl at 2.5 or 3.8 mg/kg body weight/day

by nasogastric intubation during the entire period of organogenesis caused embryolethality (Ema et al., 2007b). DBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The dose volume was adjusted to 0.5 mL/kg body weight, based on the most recent body weight. The present study was performed almost at the same time as the previous study, in which the control monkeys were given olive oil on days 20–50 of pregnancy (Ema et al, 2007b), and the administration period in the previous study covered the administration period in the present study. Therefore, cynomolgus monkeys that received only olive oil in our previous study were used as the control group for this study and compared with the DBTCl-treated groups.

Observations

The pregnant monkeys were observed for clinical signs of toxicity twice a day during the administration period and once a day during the nonadministration period. Body weight was recorded on days 0, 20, 27, 34, 41, 51, 60, 70, 80, 90, and 100 of pregnancy. Food consumption was recorded on days 20, 23, 27, 30, 34, 37, 41, 44, 48, 51, 55, 58, 62, 80, 90, and 99 of pregnancy. Embryonic/fetal heartbeat and growth were monitored by using ultrasound under anesthesia on days 18, 19, 22, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, and 99 of pregnancy. For dams in which embryonic/fetal cardiac arrest was confirmed by ultrasound, the uterus, including the embryo/fetus and placenta and ovaries, were removed from the maternal body and stored in 10% neutral buffered formalin. The dead embryos/fetuses and placentae were morphologically examined.

Terminal caesarean sectioning was performed on day 100 of pregnancy, under anesthesia induced by an intramuscular injection of 5% ketamine hydrochloride (0.1–0.2 ml/kg) and inhaled isoflurane (0.5–2.0%; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Salivation was inhibited by atropine (0.01 mg/kg; Tanabe Seiyaku Co., Ltd., Osaka, Japan). The fetus and placenta were removed from the dams. The placenta was morphologically examined, weighed, and stored in neutral buffered 10% formalin. Dams that underwent caesarean sectioning were not necropsied.

After fetal viability was recorded, fetuses were anesthetized by an intraperitoneal injection of pentobarbital sodium and euthanized by submersion in saline for 30–40 minutes at room temperature. Fetuses were weighed, sexed, and examined for external anomalies after confirmation of the arrested heartbeat. The anogenital distance (AGD), crown-rump length (CRL), head width, tail length, chest circumference, paw and foot length, distance between the eyes, umbilical cord length, volume of amniotic fluid, and diameters of the primary and secondary placentae were measured. After completion of the external examinations, the fetuses were examined for internal anomalies. The peritoneal cavity was opened, and the organs were grossly examined. The brain, thymus, heart, lungs, spleen, liver, kidneys, adrenal glands, and testes/uterus and ovaries were weighed and stored in 10% neutral buffered formalin. The eyeballs, stomach, small and large intestine, head skin, and auricles were stored in neutral buffered 10% formalin. Fetal carcasses were fixed in alcohol, stained with alizarin red S (Dawson, 1926), and examined for skeletal anomalies. The number of ossification centers in the vertebral column and the lengths of each humerus, radius, ulna, femur, tibia, and fibula were recorded.

Data analysis

The data were analyzed by using MUSCOT statistical analysis software (Yukums Co., Ltd., Tokyo, Japan). Data were analyzed by using Bartlett's test (Snedecor and Cochran, 1980) for the homogeneity of variance. When the variance was homogeneous, Dunnett's test (Dunnett, 1996) was performed to compare the mean value of the control group with that of each DBTCl group. When the variance was heterogeneous, the data were rank-converted and a Dunnett-type test (Miller, 1981) was performed to compare the mean value of the control group with that of each DBTCl group. The incidence of females showing toxicological signs was analyzed by Fisher's exact test. The fetal parameters were not statistically analyzed because the size of the groups was limited to a small number.

Results

Table 1 shows maternal findings for monkeys given DBTCl on 3 consecutive days during organogenesis. No maternal death occurred in any group. Soft stool and/or diarrhea in all groups, including the control group and vomiting in all DBTCl-treated groups, were observed. Significant increases in the incidence of females showing soft stool and/or diarrhea after the administration of DBTCl on days 19–21, 21–23, 24–26, or 26–28 of pregnancy, and females showing vomiting after the administration of DBTCl on days 19–21 of pregnancy were noted.

Figure 1 presents maternal body weight gain during pregnancy in monkeys given DBTCl on 3 consecutive days during organogenesis. Body weight gain on days 0–20 (during the preadministration

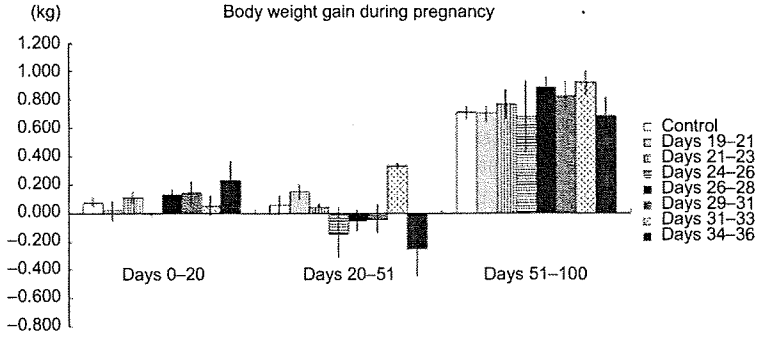

Figure 1. Maternal body weight gain during pregnancy in cynomolgus monkeys given DBTCl on three consecutive days during organogenesis.

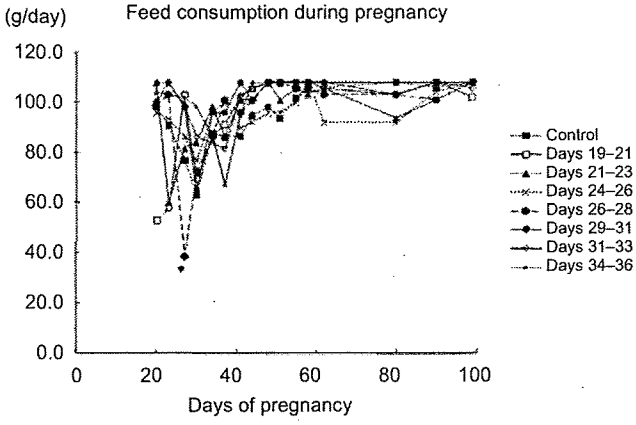

Figure 2. Maternal feed consumption during pregnancy in cynomolgus monkeys given DBTCl on three consecutive days during organogenesis. *Significantly different from the control group; $P < 0.05$.

Table 1. Maternal findings in cynomolgus monkeys given DBTCl on three consecutive days during organogenesis.

Dose (mg/kg)	0 (Control)	7.5						
Dosing days of pregnancy	20–50	19–21	21–23	24–26	26–28	29–31	31–33	34–36
No. of pregnant females	12	5	5	5	5	5	5	5
No. of females showing toxicological sign								
Death	0	0	0	0	0	0	0	0
Soft stool/diarrhea	1	4*	4*	5*	4*	3	3	3
Yellowish white stool	0	2	0	2	0	1	0	0
External genital bleeding	0	1	0	0	1	0	0	0
Vomiting	0	3*	2	2	1	1	1	2
Initial body weight (kg)[a]	3.53±0.59	3.42±0.60	3.20±0.48	3.71±0.63	3.71±0.63	3.38±0.41	3.26±0.17	4.06±0.61

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

period) did not significantly differ between the control and DBTCl-treated groups. Although body weight gain on days 20–51 was reduced in groups given DBTCl on days 24–26, 26–28, 29–31, and 34–36 of pregnancy, there were no statistically significant differences between the control and DBTCl-treated groups. No significant decreases in body weight gain on days 51–100 were found in the DBTCl-treated groups.

Figure 2 illustrates maternal feed consumption during pregnancy in monkeys given DBTCl on 3 consecutive days during organogenesis. Significantly

Table 2. Reproductive and developmental findings for cynomolgus monkeys given DBTCl on threee consecutive days during organogenesis.

Dose (mg/kg)	0 (Control)	7.5						
Dosing days of pregnancy	20–50	19–21	21–23	24–26	26–28	29–31	31–33	34–36
No. of pregnant females	12	5	5	5	5	5	5	5
No. of females with embryonic/fetal loss	1	1	0	2	0	0	0	1
No. of females with live fetuses	11	4	5	3	5	5	5	4
No. of live fetuses	11	4	5	3	5	5	5	4
Sex ratio of live fetuses (male/female)	6/5	3/1	4/1	2/1	3/2	2/3	1/4	3/1
Body weight of live fetuses (g)[a]	126±14	122±12	124±16	100±12	110±7.5	117±21	111±16	124±13
Crown-rump length (cm)[a]	12.7±0.5	12.3±0.3	12.6±0.2	11.9±0.7	12.5±0.3	12.3±0.9	12.1±0.5	12.4±0.4
Tail length (cm)[a]	11.8±1.0	11.8±0.6	11.2±0.2	10.5±0.2	11.5±0.6	11.6±0.9	10.6±0.9	12.1±0.8
Anogenital distance (cm)[a]								
Male	4.2±0.5	4.3±0.1	4.2±0.2	3.8	4.0±0.3	4.2	3.4	4.5±0.3
Female	1.0±0.1	0.8	1.0	0.9	1.0	0.9±0.2	0.9±0.1	0.9
Placental weight (g)[a]	42.1±7.0	41.3±9.4	38.9±4.2	39.8±15.2	37.1±4.08	42.3±6.7	44.7±7.2	50.0±14.3
No. of single placentae	1	0	1	1	0	0	0	0
No. of fused placentae	0	0	1	0	0	0	1	1

[a]Values are given as the mean±SD.

Table 3. Summary of morphological examinations for fetuses of cynomolgus monkeys given DBTCl on three consecutive days during organogenesis.

Dose (mg/kg)	0 (Control)	7.5						
Dosing days of pregnancy	20–50	19–21	21–23	24–26	26–28	29–31	31–33	34–36
No. of fetuses examined	11	4	5	3	5	5	5	4
External examinations								
No. of fetuses with malformations	0	0	0	0	0	0	0	0
Internal examinations								
No. of fetuses with malformations	0	0	0	0	0	0	0	0
No. of fetuses with variations	0	0	0	0	0	0	0	0
Skeletal examination								
No. of fetuses with malformations	0	0	0	0	0	0	0	0
No. of fetuses with variations	0	1	2	2	0	0	1	0
Full supernumerary ribs	0	1	2	2	0	0	0	0
Shortening of 12th ribs	0	0	0	0	0	0	1	0
Cervical ribs	0	0	0	0	0	0	1	0
Ossification								
No. of ossified centers of vertebral column[a]	53.6±0.8	53.0±1.4	53.4±1.3	53.7±1.5	53.4±1.1	53.2±1.6	52.8±1.3	53.8±0.5
Skeletal length (mm)[a]								
Humerus	23.6±0.8	23.1±1.3	23.3±1.3	21.2±0.4	22.8±0.5	23.2±1.5	22.2±1.3	23.3±0.8
Radius	23.0±1.0	22.4±1.7	22.9±1.6	20.7±0.4	22.2±1.0	22.2±1.6	21.6±1.2	22.3±1.1
Ulna	24.6±1.0	24.0±1.1	24.3±1.1	22.4±0.5	23.9±0.8	23.5±1.5	23.0±1.1	22.8±2.2
Femur	22.3±1.2	22.0±1.4	21.7±1.5	20.2±0.6	21.3±0.3	22.2±1.6	20.9±1.7	22.5±1.3
Tibia	21.5±1.3	21.2±1.9	21.2±1.6	19.6±0.5	20.3±0.6	21.1±1.1	19.9±1.6	21.4±1.4
Fibula	19.8±1.0	20.0±1.8	19.6±1.4	18.1±0.1	18.7±0.4	19.6±1.0	18.5±1.5	19.6±1.0

[a]Values are given as the mean±SD

reduced feed consumption was only found on days 27–28 in the group given DBTCl on days 26–28 of pregnancy.

Table 2 shows the reproductive and developmental findings for monkeys given DBTCl on 3 consecutive days during organogenesis. There was an abortion on day 90 for 1 female given DBTCl on days 19–21, an abortion on day 35, and an embryonic loss on day 35 for females given DBTCl on days 24–26, and fetal death on day 90 for 1 female given DBTCl on days 34–36. No embryonic/fetal loss or abortions were found for females given DBTCl on days 21–23, 26–28, 29–31, or 31–33. No difference was observed in the incidence of embryonic/fetal loss between the control and DBTCl-treated groups. A shortened tail length was detected in fetuses of dams given DBTCl on days 31–33 of pregnancy. There were no changes in sex ratio, body weight, AGD or CRL of live fetuses, or placental weight. A single placenta was observed for one dam each from the control group and groups given DBTCl on days 21–23 and 24–26, and a fused placenta was found in one dam each in the groups given DTBCl on days 21–23, 31–33, and 34–36. There were no differences in head width, chest circumference, paw and foot length, or distance between the eyes of fetuses. There were also no differences in umbilical cord length, volume of amniotic fluid, or diameters of the primary and secondary placentae between the control and DBTCl-treated groups (data not shown).

Table 3 summarizes the results of morphological examinations of monkey fetuses given DBTCl on 3 consecutive days during organogenesis. No external, internal, or skeletal malformations were found in fetuses in any group. No internal variations were observed of any group. Skeletal examinations revealed full supernumerary ribs in 1 fetus in the groups given DBTCl on days 19–21 and 2 fetuses each in the groups given DBTCl on days 21–23 and 24–26, as well as shortening of the 12th and cervical ribs in 1 fetus of the group given DBTCl on days 31–33. There were no differences in the number of ossified centers of the vertebral column or the length of the radius, femur, tibia, or fibula between the control and DBTCl-treated groups. However, the humerus, radius and ulna all were shortened in the group given DBTCl on days 24–26. Although a decrease was observed in the absolute brain weight of monkey fetuses given DBTCl on days 24–26, 26–28, and 31–33, and a decrease was also observed in the weights of the hearts of fetuses given DBTCl on days 24–26, there was no difference between the control and DBTCl-treated groups in the relative weight of any organ (data not shown).

Discussion

Many studies on the developmental toxicity of DBT have been performed using rodents, primarily rats (Ema and Hirose, 2006). No single species has yet clearly emerged as a superior model for the testing of developmental toxicity (Schardein, 2000). Nonhuman primates appear to provide an especially appropriate model for the testing of teratogenicity because of their high ranking on the evolutionary scale (Hendrickx and Binkerd, 1979). The close phylogenetic relationship of old-world monkeys to humans may render them the most desirable models for teratology studies (Schardein, 2000). The similarities in placentation and embryonic development between monkeys and humans are of considerable value for investigating the developmental toxicity of chemicals (Poggel and Günzel, 1988). Therefore, we previously determined prenatal developmental toxicity in monkeys given DBTCl during the entire period of organogenesis (Ema et al., 2007b). In the present study, relatively high doses of DBTCl were administered to monkeys during the early and middle periods of organogenesis, because teratogenic effects have been noted following the administration of DBTCl to rats during early organogenesis (Ema et al., 1992; Noda et al., 1993).

The doses of DBTCl used in the present study were expected to induce maternal toxicity, thereby allowing the characterization of the effects of DBTCl on embryonic/fetal development. Maternal toxicity, as evidenced by the increased incidence of pregnant females showing soft stool/diarrhea and vomiting, was found in all groups given DBTCl and was observed after the administration of DBTCl on days 19–28 of pregnancy. These findings indicate that more severe general toxicity is induced by DBTCl administration at earlier time points during pregnancy in cynomolgus monkeys.

In our previous study in which DBTCl was given to cynomolgus monkeys during the entire period of organogenesis, DBTCl at 2.5 mg/kg was sufficient to induce embryonic/fetal loss around days 35–60 of pregnancy (Ema et al., 2007b). In the present study, embryonic/fetal loss was found in females given DBTCl on days 19–21 and 34–36 and in 2 females given DBTCl on days 24–26 of pregnancy. It is, therefore, likely that days 24–26 of pregnancy may be more susceptible to the lethal effect of DBTCl on embryos/fetuses.

Decreased absolute weights of the brain and/or heart observed in fetuses of monkeys given DBTCl on days 24–26, 26–28, and 31–33 were not thought to be due to toxic effects on embryonic/fetal development because the changes were small and the

relative weights were not decreased. Short tail length was observed in fetuses of dams given DBTCl on days 24–26 and 31–33 of pregnancy. The tail lengths in the background control data during 1994–2006 in the laboratory performing the current study were 8.6–15.1 mm (mean ± SD = 12.3 ± 0.6) in 239 fetuses. The short tails observed in the present study are unlikely to have toxicological significance, because the change was small and within the range of the control background data. However, the embryonic/fetal changes observed in the 24–26-day group may be associated with the adverse maternal effects observed at these dosage levels. Collectively, these findings suggest that DBTCl is not toxic to embryonic/fetal growth *per se* at 7.5 mg/kg when administered on 3 consecutive days during organogenesis, but that the delays in development may be associated with maternal toxicity.

Although a single placenta was found in 1 female in the control group and 1 female each in groups given DBTCl on days 21–23 and 24–26, and a fused placenta was found in 1 female each in groups given DBTCl on days 21–23, 31–33, and 34–36, the appearance of a single placenta or fused placenta is not uncommon in developmental toxicity studies in cynomolgus monkeys. The incidences in our historical control data during 1994–2006 were 0–66.7% (mean = 12.6% of 255 pregnancies) for a single placenta and 0–11.1% (mean = 4.2% of 255 pregnancies) for a fused placenta. The incidences of females with a single or fused placenta in the present study were within the range of or slightly higher value than that of the background control data, respectively. We are unaware of any studies of the relationship between these types of placenta and the development of monkey embryos/fetuses, and we do not have any evidence suggesting that these types of placenta adversely affect the normal development of embryos/fetuses in cynomolgus monkeys.

On morphological examination, fetuses with full supernumerary ribs as well as shortened 12th ribs and cervical ribs were found in the DBTCl-treated groups. A recent survey of international experts in the field of reproductive/developmental toxicology resulted in high agreement that full supernumerary ribs and cervical ribs should be considered as variations, and in poor agreement that shortened 12th ribs should be considered as malformations (Solecki et al., 2001). Therefore, our findings would be classified as skeletal variations, based on the above survey. Chahoud et al. (1999) noted that variations are unlikely to adversely affect survival or health and might result from delayed growth or morphogenesis; the fetuses otherwise follow a normal pattern of development. The incidences in our historical control data were 0–33.3% (mean = 9.5%, 24 of 239 fetuses) for full supernumerary ribs and 0–18.2% (mean = 2.0%, 5 of 239 fetuses) for cervical ribs. In the present study, a relatively higher incidence of full supernumerary ribs was observed after the administration of DBTCl on days 19–26 of pregnancy. We defined the ribs present in the lateral potion of the first lumbar vertebra and the distal cartilagious portion as full supernumerary ribs. Full supernumerary, but not rudimentary, ribs are thought to be an indicator of toxicity during the embryonic development of rats (Kimmel and Wilson, 1973) and mice (Rogers et al., 2004). Branch et al. (1996) noted that supernumerary ribs might be induced in embryos on gestation day 8, prior to any morphological differentiation of the axial skeleton, and cartilaginous supernumerary ribs were visible in fetuses on gestation day 14 prior to ossification in mice. These findings may be consistent with the present findings that full supernumerary ribs were found in cynomolgus monkeys given DBTCl during early organogenesis. In monkeys, however, the toxicological significance of supernumerary ribs is still unknown.

Shorter lengths of the humerus, radius, and ulna were observed in fetuses of dams given DBTCl on days 24–26. The lengths of the humerus and ulna in our background control data during 1994–2006 were 19.1–26.6 mm (mean ± SD = 23.4 ± 0.6) and 18.4–28.9 mm (mean ± SD = 24.7 ± 0.7), respectively, for 239 fetuses. The shortened lengths of these bones observed in the present study are probably associated with maternal toxic exposure. Morphological examinations of dead embryos/fetuses in the DBTCl-treated groups revealed no anomalies.

Collectively, these findings suggest that the morphological alterations observed in fetuses in the present study do not indicate a teratogenic response, and that DBTCl possesses no teratogenic potential in cynomolgus monkeys, although it does retard development and increase variations at maternally toxic doses.

Conclusion

In conclusion, the administration of DBTCl to pregnant cynomolgus monkeys on 3 consecutive days during organogenesis had an adverse effect on embryonic/fetal survival, retarded fetal growth, and produced a slight increase in skeletal variations, but no malformations.

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Fetal malformations and early embryonic gene expression response in cynomolgus monkeys maternally exposed to thalidomide☆

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ABSTRACT

The present study was performed to determine experimental conditions for thalidomide induction of fetal malformations and to understand the molecular mechanisms underlying thalidomide teratogenicity in cynomolgus monkeys. Cynomolgus monkeys were orally administered thalidomide at 15 or 20 mg/kg-d on days 26–28 of gestation, and fetuses were examined on day 100–102 of gestation. Limb defects such as micromelia/amelia, paw/foot hyperflexion, polydactyly, syndactyly, and brachydactyly were observed in seven of eight fetuses. Cynomolgus monkeys were orally administered thalidomide at 20 mg/kg on day 26 of gestation, and whole embryos were removed from the dams 6 h after administration. Three embryos each were obtained from the thalidomide-treated and control groups. Total RNA was isolated from individual embryos, amplified to biotinylated cRNA and hybridized to a custom Non-Human Primate (NHP) GeneChip® Array. Altered genes were clustered into genes that were up-regulated (1281 genes) and down-regulated (1081 genes) in thalidomide-exposed embryos. Functional annotation by Gene Ontology (GO) categories revealed up-regulation of actin cytoskeletal remodeling and insulin signaling, and down-regulation of pathways for vasculature development and the inflammatory response. These findings show that thalidomide exposure perturbs a general program of morphoregulatory processes in the monkey embryo. Bioinformatics analysis of the embryonic transcriptome following maternal thalidomide exposure has now identified many key pathways implicated in thalidomide embryopathy, and has also revealed some novel processes that can help unravel the mechanism of this important developmental phenotype.

1. Introduction

Thalidomide (α-phthalimidoglutarimide) was synthesized in West Germany in 1953 by the Chemie Grünenthal pharmaceutical firm, and was marketed from October 1957 into the early 1960s. It was used for treating nausea and vomiting late during pregnancy and was also said to be effective against influenza. The first case of the phocomelia defect, although not recognized at the time as drug-related, was presented by a German scientist in 1959; subsequently, malformed children were reported in 31 countries [1]. A pattern of defects of limbs as well as the ocular, respiratory, gastrointestinal, urogenital, cardiovascular and nervous systems caused by maternal thalidomide exposure during early pregnancy was observed. Limb defects such as phocomelia, amelia, micromelia, oligodactyly, and syndactyly were the most common malformations [2]. After removal from the global market in 1962, thalidomide was reintroduced in 1998 by the biotechnology firm Celgene as an immunomodulator for the treatment of erythema nodosum leprosum, a serious inflammatory condition of Hansen's disease, and in orphan status for treating Crohn's disease and several other diseases [1].

Animal species are not equally susceptible or sensitive to the teratogenicity of chemical agents, and some species respond more readily than others [3]. For thalidomide, a variety of developmental toxic effects were reported in 18 animal species, but the responses have been highly variable across species. Limb defects that mimic

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human thalidomide embryopathy have only been observed and replicated in a few strains of rabbits and in primates [1,3,4]. Eight of nine subhuman primates treated with thalidomide showed characteristic limb reduction malformations ranging from amelia to varying degrees of phocomelia at a dosage and timing comparable to those observed in human thalidomide embryopathy [3,5]. Since the first report of thalidomide embryopathy appeared 50 years ago, considerable information regarding the therapeutic applications of this drug has accumulated, but the mechanisms by which thalidomide produce congenital malformations are still not well understood [2,3,5].

The non-human primate *Macaca fascicularis* (cynomolgus monkey) is widely used in prenatal developmental studies because of year-round rather than seasonal breeding behavior [6]. Kalter [5] noted that non-human primates, especially macaques and baboons, are favorable for mechanistic studies; however, only two full reports of the teratogenicity of thalidomide in cynomolgus monkeys are available [7,8]. In those studies, cynomolgus monkeys were given thalidomide by gavage at doses of 5–30 mg/kg-d during gestation days 20–30, and fetuses were examined morphologically. The findings of these studies determined the critical period and doses of thalidomide required for the production of fetal malformations in this macaque species. Although amounts taken were not always accurately recorded in humans, available documents show that typical malformations resulted from the ingestion of as little as 25 mg three times a day or 100 mg/day for 3 days during the sensitive period, equivalent to an astonishingly small dosage of about 1 mg/kg-d [5]. In teratology studies using cynomolgus monkeys, the timing of dosing was comparable to the human one and the doses were estimated to be 5–30 times higher than those which produced typical malformations in humans [5,7,8].

Knowledge of the patterns of altered gene expression in embryonic target organs on a global scale is an important consideration for understanding the mechanisms of teratogenesis [9–13]. The application of cDNA microarray technology, a genome-wide analysis technique, to cynomolgus monkeys facilitates the rapid monitoring of a large number of gene alterations in this species [14]. In order to obtain information about the molecular mechanisms underlying the detrimental effects of thalidomide teratogenicity, the present study has determined the experimental conditions required to produce thalidomide-induced fetal defects that mimicked human abnormalities in cynomolgus monkeys and then profiled altered patterns of gene expression in these embryos during the critical period. The dosing used in the present study was 15 or 20 mg/kg-d thalidomide given by gavage to pregnant dams at days 26–28 of gestation for teratological evaluation, and 20 mg/kg given on day 26 for gene expression profiling 6 h post-treatment.

2. Materials and methods

2.1. Teratological evaluation

The teratology study was performed at SNBL USA, Ltd. (Everett, WA, USA) in compliance with the Animal Welfare Act and recommendations set forth in The Guide for the Care and Use of Laboratory Animals [15]. Only females showing 25–32-day menstrual cycles were used in these experiments. Each female monkey was paired with a male of proven fertility for 3 days between days 11 and 15 of the menstrual cycle. When copulation was confirmed, the median day of the mating period was regarded as day 0 of gestation. Pregnancy was confirmed on day 20 or day 25 by ultrasound (SSD-4000, Aloka Co., Mitaka, Japan) under sedation induced by intramuscular injection of 5% ketamine hydrochloride (Sigma Chemical Co., St Louis, MO, USA). The monkeys were given (±)-thalidomide (Lot no. SEH7050, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 15 or 20 mg/kg-d by oral administration using gelatin capsules (Japanese Pharmacopiae grade) on days 26–28 of gestation. The dosage was adjusted to the body weight on day 25 of gestation. Cesarean section was performed on day 100–102 of gestation under deep anesthesia induced by intramuscular injection of 5% ketamine hydrochloride (0.1–0.2 ml/kg) and inhalation of isoflurane (0.5–2.0%, Baxter, Liberty Corner, NJ, USA). Salivation was inhibited by atropine (0.01 mg/kg, Phenix Pharmaceutical, St. Joseph, MO, USA). Fetal viability was recorded, and the fetuses were euthanized by intraperitoneal injection of pentobarbital and phenytoin solution (Euthasol®, Virbac Corp., Fort Worth, TX, USA). Fetuses were sexed and examined for external anomalies after confirmation of the arrested heartbeat. After the completion of external examinations, fetuses were examined for internal abnormalities.

2.2. Microarray experiments

The animal experiments were performed at Shin Nippon Biomedical Laboratories (SNBL), Ltd. (Kagoshima, Japan) in compliance with the Guideline for Animal Experimentation (1987), and in accordance with the Law Concerning the Protection and Control of Animals (1973) and the Standards Relating to the Care and Management of Experimental Animals (1980). This study was approved by the Institutional Animal Care and Use Committee of SNBL and performed in accordance with the ethics criteria contained in the bylaws of the SNBL committee.

Each female monkey was paired with a male of proven fertility for 1 day between day 11 and day 15 of the menstrual cycle. Pregnant females, aged 5–8 years and weighing 2.84–3.76 kg on day 22 of gestation, were allocated randomly to two groups, each with three monkeys, and housed individually. The monkeys were orally dosed with (±)-thalidomide (Lot no. SDH7273/SDJ3347, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 0 or 20 mg/kg by oral administration of a gelatin capsule on day 26 of gestation, which was during the critical period for thalidomide-induced teratogenesis [7,8]. Dosage was adjusted to the body weight on day 22 of gestation. Control monkeys received the capsule only.

2.3. RNA sample collection

Hysterectomy was performed under terminal anesthesia at 6 h after the administration of thalidomide on day 26 of gestation. Whole embryos were rapidly removed from the uterus using a stereomicroscope and immersed in sterilized physiological saline. Three embryos each in the thalidomide-treated and control groups were obtained for RNA analysis and stored at −70 °C until further processing. General factors of maternal age, weight and date of processing these samples are shown in Table 1. Embryos were processed simultaneously, and aside from the blocking factors in Table 1, all six samples were handled concurrently through RNA isolation and hybridization.

2.4. RNA preparation and labeling

Total RNA was isolated from each day-26 embryo, amplified to cRNA, and biotin-labeled for analysis on the Affymetrix NHP GeneChip® Array at Gene Logic Inc. (Gaithersburg, MD, USA) using the TRIzol method and RNeasy columns according to protocols from Affymetrix (Santa Clara, CA, USA). The 28S/18S rRNA ratio of isolated RNA was assessed using a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and found to be of sufficiently high quality. Biotinylated cRNA was finally cleaned up and fragmented by limited hydrolysis to a distribution of cRNA fragment sizes below 200 bases.

2.5. Affymetrix NHP GeneChip® Array and hybridization

Biotinylated cRNA samples from control and exposed embryos ($n = 3$ each) were hybridized using Biogen Idec's (NASDAQ: BIIB) proprietary Affymetrix NHP GeneChip® Array platform. This microarray chip contains a comprehensive representation of the Cynomolgus genome derived from Biogen Idec's proprietary sequencing efforts, from which Gene Logic (www.genelogic.com/) subsequently obtained the exclusive rights to provide as a service (personal communication, Jun Mano, Gene Logic). The steps for hybridization followed a protocol described in the Gene Logic GeneChip® Analysis Manual (Gaithersburg, MD, USA). Probe-sets for this analysis consisted of cynomolgus expressed sequence tags (ESTs), published rhesus monkey ESTs, predictive coding sequences from the rhesus genome, and human genes not represented by monkey sequences. Because of the incomplete state of annotation for the cynomolgus genome at the time this study was undertaken, we used human, mouse and rat gene annotations to characterize monkey genes on the NHP GeneChip® Array. This reasonably assumes that most cynomolgus sequences are well-annotated by human ortholog information. After hybridization the GeneChip® Arrays were scanned and raw signal values were subjected to subsequent normalization and processing.

2.6. Microarray data processing and analysis

Probe-level data normalization from the six *.cel files used the robust multi-chip average (RMA) method with perfect-match (PM) but not mismatch (MM) data from the microarrays. RMA returns a single file containing the 51,886 probes in six columns of normalized data, representing the log 2-intensity of each probe. To query differential transcript abundance between sample groups, the log 2 ratio of treated (Q) to reference (R) was computed for all six samples, with R being the average of the three controls. The six columns were centered to MEDIAN = 0.00 and scaled to STDEV = 0.50 [10,12]. These data were loaded to GeneSpring GX7.3 software (Agilent Technologies, Redwood City, CA, USA) for one-way analysis of variance (ANOVA) by treatment group. Due to the small sample size ($n = 3$) and limited annotation of the cynomolgus genome for this preliminary analysis we relaxed the selection criterion

Table 1
Procurement of cynomolgus embryos at SNBL for microarray study.

Group	Embryo	Maternal age in years	Maternal bw in kg (day 22)	Date of embryo collection (day 26)	*.cel filename (NIHS)
Control	001	6	3.76	November 2, 2006	137255bpcyna11.cel
	002	7	2.84	December 2, 2006	137256bpcyna11.cel
	003	8	3.68	December 2, 2006	137257bpcyna11.cel
Thalidomide	101	5	2.97	October 30, 2006	137258bpcyna11.cel
	102	6	3.01	November 6, 2006	137259bpcyna11.cel
	103	8	3.14	November 24, 2006	137260bpcyna11.cel

by not applying a false-discovery rate filter. Genes or probes passing the statistical (ANOVA) filter at a P value of 0.05 were subjected to K-means clustering, with cluster Set 1 and Set 2 that were up-regulated and down-regulated, respectively, in the thalidomide-exposed versus control embryos. Entrez gene identifiers were used for bioinformatics evaluation (http://www.ncbi.nlm.nih.gov/).

3. Results

3.1. Teratological evaluation

To confirm thalidomide embryopathy in the cynomolgus colony under the conditions used for this study, pregnant dams were given thalidomide at 15 and 20 mg/kg on days 26–28 of gestation. Four fetuses were obtained at each dose for teratological evaluation (Table 2). Although we did not observe a clear dose–response in this limited number of fetuses, we did observe a number of cases with limb defects consistent with human thalidomide embryopathy. Fig. 1 shows external appearance of fetuses of dams exposed to thalidomide on days 26–28 of gestation. Bilateral amelia in the fore-/hindlimbs was noted in one female fetus at 20 mg/kg, and bilateral micromelia in the hindlimbs was observed in four fetuses at 15 mg/kg. Deformities of the paw and/or foot including hyperflexion, ectrodactyly, polydactyly, syndactyly, brachydactyly, and/or malpositioned digits, were observed in all fetuses at 15 mg/kg and in two fetuses at 20 mg/kg. Tail anomalies were found in one fetus at 15 mg/kg and three fetuses at 20 mg/kg. Small penis was noted in one fetus each in both thalidomide-treated groups. No internal abnormalities were noted in any of the thalidomide-treated fetuses examined here. This confirmed the relevant sensitivity of cynomolgus embryos to thalidomide, based on a maternally administered dose of 15–20 mg/kg during days 26–28 of gestation.

3.2. Genes altered by thalidomide

The embryonic transcriptome was evaluated at 6 h after 20 mg/kg maternal thalidomide exposure on day 26. For this analysis, we used a proprietary Non-Human Primate (NHP) microarray having representation of the cynomolgus genome (see Section 2 for details). The NHP array includes 18,293

Table 2
Morphological findings in fetuses of cynomolgus monkeys given thalidomide on days 26–28 of gestation.

Target	Dose	15 mg/kg				20 mg/kg			
Findings	Fetus no.	1	2	3	4	5	6	7	8
	Gender	Female	Male	Female	Female	Male	Male	Male	Female
Forelimb									
Amelia		–	–	–	–	–	–	–	B
Paw									
Hyperflexion		B	–	–	–	–	–	–	–
Ectrodactyly		L	–	–	–	–	–	–	–
	Accessory digit(s)[a]	L	–	–	–	–	–	–	–
Polydactyly[a]		–	R	–	–	–	–	–	–
	Brachydactyly	–	–	–	B	–	–	–	–
Hindlimb									
Micromelia		B	B	B	B	–	–	–	–
Amelia		–	–	–	–	–	–	–	B
Foot									
Hyperflexion		–	B	B	B	–	–	–	–
Ectrodactyly		–	B	R	R	–	–	–	–
Polydactyly		–	–	–	–	B	B	–	–
Syndactyly		R	–	B	–	–	–	–	–
	Brachydactyly	–	–	–	L	–	–	–	–
	Malpositioned digit(s)	–	–	L	–	–	–	–	–
Craniofacial		–	–	–	–	–	–	–	–
Trunk		–	–	–	–	–	–	–	–
Tail									
	Bent or curled tail	–	–	–	+	–	+	+	+
Short tail		–	–	–	–	–	–	+	–
External genital organs									
Small penis		–	+	–	–	+	–	–	–

–: No anomaly was observed.
+: Anomaly was observed.
B: Bilateral anomaly was observed.
R: Unilateral (right side) anomaly was observed.
L: Unilateral (left side) anomaly was observed.
[a] Polydactyly means (almost) complete extra digits existed, and accessory digit incomplete "digit like tissue" attached to a normal digit.

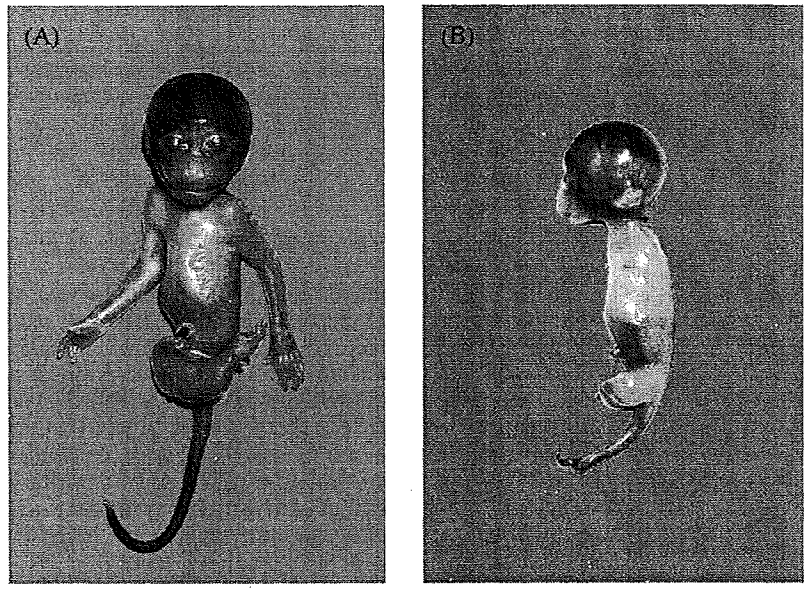

Fig. 1. Malformed fetuses of cynomolgus monkeys exposed to thalidomide on days 26–28 of gestation. (A) The fetus of maternal monkey given thalidomide at 15 mg/kg-d exhibiting brachydactyly in the paw, micromelia in the hindlimb, hyperflexion, ectrodactyly and brachydactyly in the foot and curled tail. (B) The fetus of maternal monkey given thalidomide at 20 mg/kg-d exhibiting amelia in the fore- and hindlimb and bent tail.

cynomolgus genes and 8411 Rhesus genes as well as genes from several other species. The six-array dataset conforming to MIAME standards resides in the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo/) under platform accession number GPL8393 (series GSM389350–GSM389355). A thalidomide-sensitive subset of genes in the embryonic transcriptome was reflected in the high-percentage of present calls for genes whose expression levels showed ≥1.5-fold difference between thalidomide-treated and control embryos.

Statistical (ANOVA) analysis identified 2362 genes that differed significantly between control and thalidomide groups ($P \leq 0.05$). The heat map for these genes showed a clear pattern (Fig. 2). *K*-means clustering partitioned them into primary sets of up-regulated (1281) genes and down-regulated (1081) genes for thalidomide relative to control embryos.

3.3. Annotation systems

Ranking functional categories of genes in an expression cluster is an important step to unravel the cellular functions and pathways represented in the differentially expressed gene list. To derive the highest ranking biological themes across the up-/down-regulated gene lists, Entrez gene IDs were annotated by Gene Ontology (GO) category using the Database for Annotation, Visualization, and Integrated Discovery (http://apps1.niaid.nih.gov/david/). Table 3 lists the significantly over-represented themes when the 1281 up-regulated genes (Table 3A) and 1081 down-regulated genes (Table 3B) were mapped by GO category. We used level-4 annotation for Biological Processes, Cellular component and Molecular Function as well as curated pathways from the KEGG (Kyoto Encyclopedia of Genes and Genomes) open source pathway resource to obtain categories passing by Fisher exact test ($P \leq 0.05$). For clarity and greater specificity we limited the categories in Table 3 to those having at least 10 hits for sensitivity and no more than 50 hits to improve specificity.

Integrated biological processes evident across the up-regulated categories addressed the regulation of cellular growth, including cell cycle progression, DNA repair and nucleic acid transport. Other up-regulated biological processes addressed the regulation of metabolism, the cytoskeletal cycle, heart development and vesicle transport. Many of these processes were logically reflected in the ontologies for cellular components addressing the nucleo-ribosomal system, the microtubule network, and molecular functions for GTPase activity and actin binding. Up-regulated signaling pathways (KEGG) included several oncogenic growth pathways as well as the TGF-beta, GnRH and insulin signaling pathways.

Integrated biological processes evident across the down-regulated categories addressed ion homeostasis and cellular secretion. These processes were logically reflected in the ontologies for cellular components addressing the endoplasmic reticulum, GTPase activity and transferases. Other down-regulated biological processes addressed cell growth, muscle and vasculature development, and the inflammatory response—consistent with KEGG pathways for hematopoietic cells and antigen processing.

4. Discussion

The results from this study show that a teratogenic dose of thalidomide (20 mg/kg) significantly alters global gene expression profiles in the cynomolgus monkey embryo within 6 h of exposure on day 26 of gestation. Bioinformatics analysis of the embryonic transcriptome following maternal thalidomide exposure revealed up-regulation in several signaling pathways with roles in morphogenesis and oncogenesis (e.g., TGF-beta, insulin signaling), and down-regulation of the endoplasmic reticulum and inflammatory response. As might be anticipated, this implies a broad reaction of the embryo to the mechanism of thalidomide and a generalized reprogramming of pathways known to be important in development and teratogenesis.

The dosing scenario used in the present study was 15 or 20 mg/kg-d thalidomide given by gavage to pregnant dams on days 26–28 of gestation for teratological evaluation, and 20 mg/kg given on day 26 for gene expression profiling 6 h post-treatment. The teratological exposure induced limb malformations consistent with earlier studies with thalidomide in pregnant macaques. For example, it was previously reported that two fetuses with amelia were obtained from two of four cynomolgus monkeys given thalidomide by gavage at 10 mg/kg-d on days 32–42 after commencement of menses (approximately equivalent to days 20–30 of gestation)

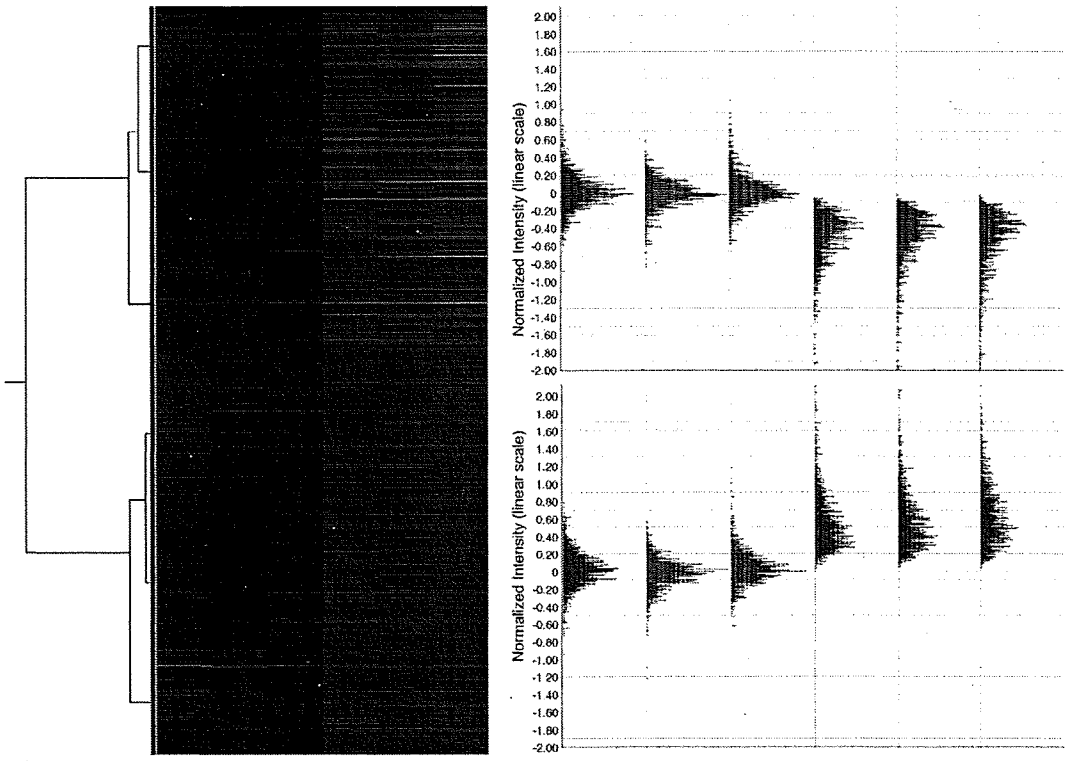

Fig. 2. Molecular abundance profiles of the thalidomide-sensitive genes in the cynomolgus embryonic transcriptome on day 26 of gestation. RNA was isolated from day 26 embryos 6 h after maternal exposure to 20 mg/kg thalidomide or vehicle control. Values represent log 2 ratios of treated/reference, where the reference is an average of all three controls for each gene. ANOVA returned 2362 genes that were significantly different between the groups ($n = 3$, $P \leq 0.05$). The heat map visualizes the genes in rows and the embryos in columns, and the histogram shows the distribution of genes in each cluster. Columns left to right: 1–3 from control embryos (#001, #002, #003) and 4–6 from thalidomide embryos (#101, #102, #103). Genes were partitioned by *K*-means clustering into two primary expression clusters with 1281 up-regulated genes (red) and 1081 down-regulated genes (green).

and that the fetal malformations were similar to malformations reported in children whose mothers had taken thalidomide during pregnancy [7]. Forelimb malformations in the cynomolgus fetus were noted following a single oral administration of thalidomide on days 25, 26 or 27 of gestation at 10 and 30 mg/kg and daily administration on days 25–27 of gestation at 5 mg/kg, and both fore- and hindlimb malformations were observed following a single oral administration on day 25 or 28 of gestation at 30 mg/kg [8]. The present study, taken together with the previous studies [7,8], indicate that orally administered thalidomide induces fetal malformations in cynomolgus monkeys similar to human pregnancies and furthermore localizes the vulnerable period to days 25–28 of gestation and the effective doses to 5–30 mg/kg-d.

Given the limitations of working with this species the preliminary application of a custom NHP microarray, the analysis at one dose and time point, and the incomplete state of annotation of the macaque genome, the current study design focused on RNA collected from individual embryos rather than the specific target organ system (forelimb, hindlimb). Ideally a follow-up study on focused gene expression analysis should be performed for specific embryonic limbs in which malformations have been induced with thalidomide; however, the present study is among the first to provide genomic information on the initial changes in gene expression occurring in macaque embryos during the critical events following a teratogenic dose of thalidomide. A total of 43 and 26 functional categories of redundant genes were up- and down-regulated, respectively, based on the GO annotation system for human Locus Link identifiers.

Statistically, the top-ranked 20 up-regulated genes included 4 hits to cell shape and polarity genes: KIAA0992 (twice), FNML2, FMNL3. Palladin, encoded by the KIAA0992 gene, plays a role in cytoskeletal organization, embryonic development, cell motility, and neurogenesis [16]. Formin-related proteins play a role in Rho GTPase-dependent regulation of the actin cytoskeletal cycle and have been implicated in morphogenesis, cell movement and cell polarity [17]. Several genes in the focal adhesion/actin cytoskeleton pathway were up-regulated. Guanine nucleotide exchange factors (GEFs) *DOCK1*, which forms a complex with RhoG, and *VAV2* and *ARHGEF7* that act on Rho family GTPases, play a fundamental role in small G-protein signaling pathways that regulate numerous cellular processes including actin cytoskeletal organization [18–22]. To further understand the mechanisms of thalidomide-induced teratogenicity the regional and developmental stage of expression for these genes and corresponding proteins should be determined; however, these preliminary findings suggest that thalidomide perturbs a general program involving the up-regulation of Rho family GTPases and their GEFs.

One candidate pathway for the control of cytoskeletal remodeling evident in studies of early induction of the Fetal Alcohol Syndrome (FAS) in mouse embryos is the receptor tyrosine kinase (RTK) signaling pathway, mediating insulin-like growth factors [12]. Genes in the RTK insulin signaling pathway were significantly up-regulated by thalidomide treatment as in FAS. AKT1 and GSK3β, which were up-regulated by thalidomide, are key genes in this pathway. AKT1, a serine–threonine protein kinase, is regulated by PDGF and insulin through PI-3 kinase signaling [23–25]. GSK3β, a substrate of AKT, is a proline-directed serine–threonine kinase that was initially identified as a phosphorylating and inactivating glycogen synthase [26]. IGF-I and IGF-II are expressed in the anterior and posterior mesodermal cells of the developing limbs [27–29]. IGF-I can influence chick limb outgrowth [29–31] and regulate mus-