ノクレイを0.1～1質量%分散させる。

歯磨剤：歯磨剤は、親水性粘土材、変性セルロースポリマー、カルボキシビニルポリマー及びアニオン性ポリマー由来の天然ゴム類を含む結合剤系（粘度 約10,000～450,000 Pa）、口腔ケア活性物質、極性溶媒キャリアから構成される。親水性粘土材には、天然及び合成層状ケイ酸塩鉱物類、ヒュームドシリカ類等があり、その使用目的は、結合剤系の増粘剤、柔らかい口感触、容易な分散、歯間部分への進入の改善である。ヒュームドシリカ類の場合、その粒径は約 5μm 未満、典型的には約 1 nm～1μm である。粘土材の添加量は全組成物の約0.01～4重量%が望ましいとされる。

ドラッグデリバリ（薬物送達用組成物）：薬物の有効成分を担持・送達する組成物（薬物送達用組成物）はナノコンポジットであり、そのフィラーにナノクレイが使用される。ナノクレイの使用目的は、ドラッグデリバリの機械的性質や生分解速度を、有効成分または治療用適用形態に合うように調整することである。適用されるナノクレイには、ベントナイト、モンモリロナイト等のスメクタイト系粘土であり、その添加量はナノコンポジットの1重量%～10重量%である。

有効成分を含むドラッグデリバリナノコンポジットの組成例として、ポリエチレングリコール95 %、クロイサイト 4%、有効成分パラセタモールはポリエチレングリコールに対して 5%がある。ポリエチレングリコールにクロイサイトを混錬して押出成形する。本ドラッグデリバリの剤形の範疇には、調節放出用の経口薬物送達系、インプラント系（生分解性または非生分解性）、経口送達、経鼻送達、医療デバイス、坐剤、皮膚用製剤等がある。

5) ナノクレイ・メーカーのヒアリング調査結果

＜質問項目＞と主な回答について以下にまとめた。

＜物質情報＞

モンモリロナイト：天然ベントナイトを粉砕した後、石英、長石、α-クリストバライト等の不純物を除去して、モンモリロナイト含有率を100%に精製製造したもの。基本構造は板状結晶（ケイ素四面体層-アルミナ八面体層-ケイ酸四面体層の３つの層が積み重なって一枚の結晶を構成）で、結晶の厚さ1nm, 層の長さ（幅）は300～500 nm程度である。モンモリロナイトの性質は、水中の膨潤性が高いほか、粘結性、チクソトロピー性、陽イオン交換性、吸着性等を持っている。製品モンモリロナイトの種類と特性の例は、表４の通りである。ベントナイトは産地により組成が異なるため、用途に応じて産地を選択している。

表 4. モンモリロナイトの製品特性例

	製品A	製品B
粘度	200～400mPa.s	200～400mPa.s
膨潤力	45 ml/2g 以上	50 ml/2g 以上
pH	10	10
水分	10 %以下	10 %以下
白色度	60以上	60以上
外観	微粉末	フレーク状
カチオン交換能	115 meq/100g	115 meq/100g

粘 度：4%分散液 BM型粘度計 60 rpm 25℃
白色度：ハンター白色度

＜含有製品の出荷量、使用量、配合率＞

ナノクレイの出荷量、使用量については、数値データは得られなかった。食品用フィルム・コーティング材のモンモリロナイトの添加量は、2～3 重量%である。

＜ナノクレイの具体的な使用形態＞

モンモリロナイトを樹脂中に分散させている状態。

＜食品分野におけるナノクレイ使用の役割期待＞

食品用包装容器材として、最近では、食品の味・鮮度等の質や、食品衛生が購買の選択要因になる傾向が強く、酸素（ガス）バリア性やフィルム・容器の透明性の保持について要求が高くなっているため。食品関連メーカーからの問い合わせは多い。

＜ナノクレイを用いた食品に対する安全の取組＞

食品添加物、健康食品のような分野では、ナノクレイのヒト健康影響（安全性）を懸念する

ことから、これらの用途開拓は現時点では積極的ではない。

化粧品でのナノクレイは、安全性が問われている。ナノ粒子であるために、皮膚に蓄積、目への混入、経口・吸入による体内影響などが懸念されている。

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D．考察

今回の調査では、①粘土鉱物とナノクレイの解釈とその取り扱い方の現状、②ナノクレイの使用分野の把握、③ヒトがナノクレイを経口する可能性のある用途（食品分野）の推測、④食品分野における使用実態例の情報収集、⑤食品分野におけるナノクレイ経口暴露量に関連した周辺データの収集について取り纏めることができた。 また、ナノクレイの用途として、包装容器以外に、直接経口摂取の可能性がある歯磨き等や、皮膚を接触する化粧品もあることが確認できた。 食品用容器包装は、鮮度保持や軽量（薄膜）化により、ガスバリア性に対する要求が高まり、ナノクレイの使用は増加すると思われる。

以上のことから、食品分野において、 ナノクレイを意図的に利用した用途について纏めてみると、ヒトがナノクレイを経口摂取する可能性があるものとしては、食品用包装容器材、液体農薬の沈降防止剤、洗浄剤（台所用洗剤）、歯磨き　等があり、粘土鉱物を利用した用途では、食品添加物、農薬造粒剤、動物用飼料添加物、医薬品　等があると推測された。

次年度以降は、食品分野のナノクレイの暴露評価の精度を上げるために、食品用包装容器材、農薬の沈降防止剤、歯磨き、洗剤など、ヒトがナノクレイを経口摂取する可能性のある用途における使用実態の詳細調査（ナノクレイの組成・配合率等）を継続することが必要であると考えられた。

E．結論

ナノクレイの食品・食品容器分野における使用実態の全体像を把握するために、ナノクレイの物質情報、使用形態、使用目的、開発または実用化の段階、出典等について調査した。その結果、ナノクレイの増粘性、分散性、吸着性（吸水性）、ガスバリア性、触感改良等の機能から期待されている用途として、クリーム・粉末化粧水・歯磨き等の化粧品、自動車部品やペットボトル等の部

品・容器包装材をはじめ、農薬プロアブル・接着剤・洗剤等の工業製品、塗料、難燃剤、触媒担体等があることが示された。実用化されている食品分野における主な用途は、包装容器材でガスバリア性の向上を主目的としてナノクレイが使われている他、液体農薬の沈降防止剤としても用いられていることが明らかとなった。

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G. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得
   (該当なし)
2. 実用新案登録
   (該当なし)
3. その他
   (該当なし)

表 1. ナノクレイの使用分野一覧

用途			ナノクレイの特性の利用	使用目的	使用段階	使用情報	2005年国内生産量(t)(%)
鋳物			△	鋳物砂を成形して鋳型を作る際の粘結剤	実用化	10～15 mm の球状ペレットにして使用。砂に対してベントナイトは数%混和。	194,708 (39.9 %)
土木・建築			△	場所打杭工法などの安定液材料、セメント混合材料、遮水材（漏水防止材）	実用化	粒状ベントナイト	163,008 (33.4 %)
ボーリング			△	ボーリング用泥水調整剤	実用化	水に対して粒状ベントナイト 20%の泥水を作製。	5,944 (1.2%)
猫砂			△	猫用トイレ砂（排泄物の固化材）	実用化	粒状ベントナイト	77,965 (16.0 %)
鉄・非ペレット			△	製鉄ダストの造粒助剤	実用化	粒状ベントナイト	7,666 (1.6 %)
土壌改良			△	土壌改良材、水田の漏水防止、園芸培土助剤、育苗培土助剤	実用化	粒状ベントナイト	1,528 (0.3 %)
農薬			△	農薬造粒助剤（粘結材）	実用化	粒状ベントナイト	14,434 (3.0 %)
練炭			△	成形助剤、可塑剤、粘結剤	実用化	粒状ベントナイト	897 (0.2 %)
肥料			△	肥料団結防止剤の団塊防止用バインダー	実用化	粒状ベントナイト	5,185 (1.1 %)
耐火物			●	難燃材、結合材、増粘性、安定性、成膜性	実用化／開発	ベントナイト配合は実用化、ナノクレイ配合は開発中	1,386 (0.3 %)
その他	塗料・顔料	塗料	●	成膜性、増粘性、分散性、触感改良	実用化	ナノクレイを使用	15,124 (3.1 %)
		顔料	△	沈降防止剤、垂れ防止剤	実用化	―	
	工業製品	製紙	△	填料（セルロース繊維による紙の凹凸を充填）、顔料（塗工を可能）	実用化	繊維に塗工	
		農薬プロアブル	●	液体農薬の沈降防止剤	実用化	2～3 重量%のベントナイトの添加から、ナノクレイの添加に移行	
		触媒担体	●	空気清浄機のフィルタ担体。イオン交換性を利用	開発	ナノクレイを使用	
		汚染物質	△	汚染物質の封じ込めバリア材	実用化	高分子フィルムに顆粒状のベントナイトを複合	
		エアロゾル	●	安定性、増粘性、分散性	実用化	ナノクレイを使用	
		水性ワックス	●	安定性、増粘性、分散性、吸着性、造膜性、触感改良	実用化	ナノクレイを使用	
		セラミックス	●	増粘性、分散性、チクソトロピー性	実用化	ナノクレイを使用	
		くれよん	●	安全性、安定性、増粘性	実用化	ナノクレイを使用	
		石油・油脂	●	接着剤の増粘剤、石油の脱色精製（吸着剤）	実用化	ナノクレイを使用	

用途		ナノクレイの特性の利用	使用目的	使用段階	使用情報	2005年 国内生産量(t) (%)
	水処理剤	●	安定性、増粘性、分散性、吸着性	実用化	ナノクレイを使用	
	断熱材・不燃材	●	増粘性、分散性、安定性	開発	ナノクレイを使用	
	ガスケット・パッキン	●	耐熱性・ガスバリア性	開発	ナノクレイを使用	
	太陽電池	●	バックシート材。水蒸気とガスバリア性	開発	ナノクレイ配合のPETコーティング	
	電子ペーパー	●	コーティング材、ガスバリア性	開発	ナノクレイ配合コーティング	
	フレキシブル基板	●	回路のコーティング材、ガスバリア性	開発	ナノクレイ配合コーティング	
	水素タンク	●	ガスバリア性、耐水性	開発	ナノクレイを使用	
化粧品・医薬品	化粧品	●	クレンジングクリーム、洗顔剤、粉末化粧水等のメーキャップ製品の賦形剤、増粘剤、触感改良	実用化	乳液ではナノクレイは0.5重量%、クリームでは2重量%配合	
化粧品・医薬品	医薬品	△	医薬部外品として湿布、クリーム、塗り薬（吸着剤、粉末製剤の滑沢剤）。 内服薬として制酸剤（吸着剤）	実用化	ナノクレイの適用ではない。カオリナイト、タルク、ベントナイトを使用。	
化粧品・医薬品	入浴剤	●	増粘性、分散性、触感改良	実用化	ナノクレイを使用	
化粧品・医薬品	洗剤（台所用を含む）	●	増粘性、分散性、触感改良	開発	界面活性剤とナノクレイを配合	
化粧品・医薬品	歯磨き	●	増粘性、分散性、触感改良	実用化	ナノクレイを使用	
食品	食品添加物	△	ワインや酢の清澄剤（吸着材）、健康食品（吸着性、膨満感剤）	実用化	天然ベントナイト	
食品	動物用飼料	△	食品添加物として動物の整腸剤、膨満感、便通を調整。	実用化	ベントナイト	
食品	包装容器材	●	自動車部品や食品・医薬品包装容器材のフィラー材（ガスバリア性、機械的特性、難燃性）	実用化	コーティング・フィルムの適用が多い。ナノクレイ1～2重量%を添加。	
家庭用品	クリーナー	●	安定性、吸着性、沈降防止、増粘性	実用化	ナノクレイを使用	

表 2. 食品分野におけるナノクレイの使用実態

ナノクレイの使用実態					書誌事項（日本特許）		
用途①	用途②	ナノクレイの状態	使用形態	使用目的	名称	出願人	出願番号
包装容器	食料製品、トレイ、パウチ	200nm以下の粒子、20Å以下の離隔距離。有機クレイを含む100m²/g より大きく、アスペクト比が10より大きい添加剤。	熱可塑ポリマーに1～20wt% (5～6wt%が適正)分散。	剥離可能な包装容器	包装用フィルムにおける可剥性複合材の熱可塑性シーラント	クラフトフーヅグローバルブランヅLTDライアビリテイ CO	2010-546866
包装容器	ボトル等の多層製品	無機ナノ粒子	芳香族ナイロン(5～85%)と芳香族/脂肪族ナイロン(5～95%)のブレンド物。芳香族ナイロンにナノ粒子を含む。芳香族ナイロンに対して、3.5 %のナノクレイ。	レトルト処理（オートクレーブ処理）可能な包装容器用。ガスバリア性、白濁化しない	結晶化が低減された芳香族ナイロン	アルカングローバルPHARMパツケージング INC	2010-523071
包装容器	カテーテル、ステントなどの医療用機器を被覆	モンモリロナイトをポリマー殺菌剤と接触させて抗菌性を有する	ポリマー抗菌剤(オニウム基含む)改質粘土を2～6 %。	抗菌性が必要な医療器具、包装容器、家庭用品、繊維	抗菌性ポリマーーナノ複合材料	ノツテインガム トレント UNIV	2010-511727
包装容器	食品容器	電荷密度0.25～0.6。層状ケイ酸塩	クレイに有機化剤を膨潤させ、樹脂に分散させた。	ガスバリア性を有し、白濁化しない樹脂	多層ポリエステル容器及びその製造方法	東洋製罐（株）	2009-509279
包装容器	食品容器	電荷密度0.25～0.6。層状ケイ酸塩	クレイに有機化剤を膨潤させ、樹脂に分散させた。	ガスバリア性を有し、白濁化しない樹脂	多層ポリエステル容器及びその製法	東洋製罐（株）	2009-506186
包装容器	食器	―	ポリオレフィンにナノクレイを含む層。ポレオレフィンは99%が望ましい。	レトルト用容器	レトルト処理可能な組成物	ソルベイINDフオイルズマネージメントアンドリサーチSA	2009-501963
包装容器	耐圧(炭酸飲料)容器	0.25～0.6の電荷密度を有する2-八面体型や3-八面体型の層状珪酸塩。	ポリエタレンテレフタレートとバリア性樹脂混合を中間にし、内・外層にポリエチレンテレフタレート。バリア性樹脂は樹脂100重量部、クレイ1～8重量部。	ガスバリア性、耐衝撃性を有する容器。炭酸飲料容器にも使用可能。	耐落下衝撃性に優れた多層プラスチック容器	東洋製罐（株）	2009- 49401
包装容器	容器	平均粒径10 nm～10 μm	芳香族系熱可塑性ポリウレタン樹脂100重量部に対して、脂肪族系ジ(ヒドロキシアルキル)アンモニウムイオンにより有機化処理した層状粘土鉱物0.1～10重量部	ガスバリア性ポリウレタン樹脂	熱可塑性ポリウレタン樹脂組成物	東ソー（株）	2009-287201
包装容器	ナノコンポジット	親水性粘土	ポリ塩化ビニリデンにナノケイ酸塩を1.5～4重量部	ブリスターパックの製造。低水蒸気透過・低酸素透過性を有する。	ポリ塩化ビニリデン層状ケイ酸塩ナノコンポジットおよびこれから製造されるフィルム	クライオバツク INC	2008-504344

ナノクレイの使用実態					書誌事項（日本特許）		
用途①	用途②	ナノクレイの状態	使用形態	使用目的	名称	出願人	出願番号
包装容器	容器（炭酸飲料）	スメクタイトクレイの粒子サイズ20～100nm、比重1.5～1.9	PET にナノクレイを500 ppm添加（樹脂に対し50 ～5000 ppmの割合で含まれるナノクレイ）	ガスバリア性	ポリエステル系ガスバリア樹脂およびそのプロセス	フツラポリエステルズLTD;クルカーニサンジヤイタムマジ;サンザナゴパラクリシユナンパラニアンデイヴアー;チユーラツブパンデユランガンモハン;マダナゴパルラマモーシイ;バラスンダラムデイリイラジ;モハンクマースンダラムマルラジヤ	2008- 99731
包装容器	包装容器	0.1～100nm、セピオライト型粘土	ポリエステル複合材の0.1～20重量 %	ガスバリア性	バリア用途用のポリエステル粘土ナノ複合材	イーアイデユポン デ ニモアスアンドCO	2007-548422
包装容器	チューブ容器	モンモリロナイトなど粘土化合物に45 %の有機化剤を含む。	ポリオレフィン60～96重量部	遮断性	遮断性チユーブ容器	エルジーーCHEM LTD	2007-538817
包装容器	フィルム、包装容器	スメクタイトナノクレイ（ケイ酸アルミニウム、ケイ酸マグネシウム）。径10～5000nm、層の厚み2nm未満。膨潤し1つの寸法は5nm未満。	ポリアミドに0.5～30重量 %ナノクレイを分散		水溶性ポリアミド組成物、その溶液、それからのフィルムおよびシート、ならびにそれからフィルムおよびシートを製造する方法	イーアイデユポン デ ニモアス アンド CO	2007-530470
包装容器	フィルム、容器	層状粘土化合物に45重量%の有機化剤を含む	エチレンビニルアルコール（EVOH）、ポリアミド、ポリビニルアルコールのいずれかの樹脂に、ナノクレイを4～30重量部	遮断性（ガス透過抑制及び液体透過抑制）	高遮断性物品	エル ジーーCHEM LTD	2007-523496
包装容器	容器	有機化モンモリロナイト	樹脂と粘土層、85.0：15.0～99.0：1.0 （実施例：EVOHに有機化モンモリロナイ3重量%）	ガスバリア性	遮断性ナノ複合体組成物及びそれを利用した物品	エルジーーCHEM LTD	2007-522414
包装容器	ボトル	粒径500 nm以下、望ましくは100 nm以下	ポリエステルにモンモリロナイトなどを0.1～3重量%添加。	ガスバリア性	ポリエステル樹脂	三菱化学（株）	2004-277776
包装容器	ポリプロピレン蓋容器	フィラー長径0.3～15 μm、アスペクト比15以上	ポリプロピレン系樹脂100重量部、フィラー20～180重量部	ガスバリア性、強度	板状フィラーを含む樹脂組成物及びフィルム	三菱樹脂（株）	2004-234284

ナノクレイの使用実態					書誌事項（日本特許）		
用途①	用途②	ナノクレイの状態	使用形態	使用目的	名称	出願人	出願番号
包装容器	容器、包装資材	0.25～0.6電荷密度。2-八面体、3八面体。有機系化合物(C12以上のアルキル基またはアルケニル基を有する4級アンモニウム塩)に接触させてクレイ層間を広げる。	ポリアミド複合材料中0.3～10重量%。透明性が要求される場合は0.1～8重量%。有機系化合物に対してクレイは60重量%以下。	ガスバリア性	ポリアミド複合材料用組成物およびポリアミド複合材料	三菱瓦斯化学（株）	2004- 85266
包装容器	容器、包装用フィルム	ナノフィラー(モンモリロナイト、ベントナイトなど)	ナノフィラー比率0.1～50部、ポリアミド・ポリオレフイン	ガスバリア性、液体酸素バリアなど	ナノフィラーーを含むポリアミドをマトリツクスとしたポリアミドとポリオレフィンとの混合物	アルケマ　フランス	2004- 74560
包装容器	容器、透明性	膨潤性粘土鉱物	飽和ポリエステル樹脂表面に塗装。塗料中1～50重量%、水・溶液中5～70重量%	水蒸気、酸素バリア性、生分解性	バリアー性及び透明性を有する生分解性容器	東洋製罐（株）	2004- 66245
包装容器	容器、フィルム	層状粘土/ポリアミドまたはEVOH又はアイオノマー又はPVAのナノ複合体。層状粘土は、有機酸を含む。	実施例ではモンモリロナイト3.3重量%で遮断性ナノ複合体製造。遮断性ナノ複合体は基ポリマー100重量%に対し、1～30重量%	酸素遮断性、水遮断性	遮断性に優れたナノ複合体ブレンド組成物	エルジーー CHEM LTD	2003-556336
包装容器	―	―	ポリアミド	ガスバリア性	ポリアミド複合材料の製造方法	三菱瓦斯化学（株）	2003-337789
洗浄剤	台所用、皮膚用、毛髪用の洗浄剤組成物	水膨潤性粘土鉱物（モンモリロナイト等）「クニピア」（ナノクレイ）も適用可。	組成物全量に対し、二鎖二親水型界面活性剤は1～30%、水膨潤性粘土鉱物は0.1～1%を配合。粘土鉱物は組成物中にて分散。	増粘性、泡立ち、触感の改良	洗浄剤組成物	ライオン（株）	平11-363234
化粧品・医薬部外品	歯磨剤	親水性粘土材として、天然および合成層状ケイ酸塩鉱物類、ヒュームドシリカ類、増粘沈殿シリカなど。ヒュームドシリカ類の粒径は約1nm～1μm。好ましくは1μm～50 μm。	親水性粘土材(約0.01重量%～約4重量%）を水中に分散し均一混合したものと、変性セルロースポリマー、カルボキシビニルポリマーなどの結合剤系、口腔ケア活性物質、極性溶媒キャリアを混合。	増粘剤。研磨材レベルが低い／不要な歯磨材。（研磨材による）歯表面の摩耗減少、触感改良	親水性粘土材を含む結合剤系を備える歯磨剤組成物	ザプロクターアンドギャンブルカンパニー	2008-542918
医薬品（経口薬物）	薬物送達用組成物（ドラッグデリバリ）	ベントナイト、モンモリロナイト、フルオロヘクトライト、フルオロマイカなど。粒子径は1～1000 nm.	ポリエチレングリコール90～99重量%に対し、粘土ナノ粒子1重量～10重量%を分散。送達する薬物の有効成分は組成物の1～40重量%で使用することができる。	薬物送達用組成物の機械的特性、生分解性速度を治療適用形態に合わせる	ナノコンポジット薬物送達用組成物	ザクイーンズユニヴァーシティ オブ ベルファスト	2006-506219

ナノクレイの使用実態					書誌事項（日本特許）		
用途①	用途②	ナノクレイの状態	使用形態	使用目的	名称	出願人	出願番号
食品添加物*	食品製造助剤	—	タンパク質をモンモリロナイトに吸着させて、酵素を精製する。	—	粘土鉱物組成物を用いた濃縮・精製タンパク質の製造方法	日清オイリオグループ（株）	2003-544071
農薬*	昆虫忌避剤、皮膚用処理剤（ローションなど）	0.5～50 μm	昆虫忌避物質の0.1～50重量%の微粉を使用する。	—	徐放昆虫忌避性を有する組成物	イーアイデュポン デ ニモアス アンド CO	2008-501011
農薬*	農薬担体	—	本エステル化合物3.3部に、合成含水酸化珪素微粉末5部、ドデシルベンゼンスルホン酸ナトリウム 5部、ベントナイト 30部およびクレイ 56.7部を加え、よく攪拌混合造粒。	製剤担体	ワラジムシ類防除組成物	住友化学（株）	2009-295021
農薬*	農薬担体	—	本エステル化合物3.3部に、合成含水酸化珪素微粉末5部、ドデシルベンゼンスルホン酸ナトリウム 5部、ベントナイト 30部およびクレイ 56.7部を加え、よく攪拌混合造粒。	製剤担体	多足類防除組成物	住友化学（株）	2009-295020

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yamaguchi A., Fujitani T., Ohyama K., Nakae D., Hirose A., Nishimura T., Ogata A.	Effects of sustained stimulation with multi-wall carbon nanotubes on immune and inflammatory responses in mice.	J. Toxicol. Sci.	37	177-189	2012
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広瀬明彦、高木篤也、西村哲治、津田洋幸、坂本義光、小縣昭夫、中江大、樋野興夫、菅野純	ナノマテリアルの慢性影響研究の重要性	薬学雑誌	131	195-201	2011

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Original Article

Effects of sustained stimulation with multi-wall carbon nanotubes on immune and inflammatory responses in mice

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ABSTRACT — Possible effects of multi-wall carbon nanotubes (MWCNTs) on immune and inflammatory responses were examined in mice. Female ICR mice were given a single intraperitoneal administration (2 mg/kg body weight) of either MWCNTs, carbon black (CB), or crocidolite (blue asbestos) and controls received a vehicle of 2% sodium carboxymethyl cellulose (CMC Na). In the peritoneal cavity of MWCNT-administered mice, the liver had changed to a rounded shape and fibrous adhesions were seen on internal organs. Peritoneal cells overexpressed mRNA for genes of T helper (Th)2 cytokines (*interleukin [IL]-4*, *IL-5*, and *IL-13*), Th17 cytokine (*IL-17*), pro-inflammatory cytokines/chemokines (*IL-1β*, *IL-33*, *tumor necrosis factor α*, and *monocyte chemotactic protein-1*), and *myeloid differentiation factor 88* for at least 2 weeks after the administration of MWCNTs, while those of Th1 cytokine genes (*IL-2* and *interferon γ*) were overexpressed several weeks later and expression levels remained high up to 20 weeks. In MWCNT-treated mice, the numbers of leukocytes, monocytes, and granulocytes in the peripheral blood and the expression of the leukocyte adhesion molecules, cluster of differentiation (CD)49d and CD54, on granulocytes were increased 1 week after administration and remained high up to week 20. Production of ovalbumin-specific IgM and IgG_1 was enhanced by MWCNTs. These changes were not observed after CB or crocidolite administration. Thus, this study showed that MWCNTs exhibited sustained stimulating effects on immune and inflammatory responses, unlike the other mineral fibers with structural similarities.

Key words: Multi-wall carbon nanotube, Nanomaterial, Inflammation, Immunotoxicity, Hazard characterization

INTRODUCTION

Rapid progress in nanotechnology in recent years has made it possible to produce and apply numerous new and useful nanomaterials, such as nano-TiO_2, nano-SiO_2, nano-ZnO and nano-carbon materials. These are believed to be biologically inert, although inhalation of small-sized nanomaterials can cause pulmonary inflammation and fibrosis. (Mossman and Churg, 1998; Yazdi *et al.*, 2010). Carbon forms exist in many different shapes as both elementary substances and compounds, for example, diamond, charcoal, carbon black, graphite, fullerene, and carbon nanotubes are all carbon allotropes, while graphene is a single-wall product of graphite, whose structure consists of one-atom-thick planar sheets of hexagonal-bonded carbon atoms densely packed in honeycomb crystal lattices. Carbon nanotubes are seamless cylindrical structures comprising single or multiple graphene sheets. Both single-wall carbon nanotubes (SWCNTs) and multi-wall carbon nanotubes (MWCNTs) are several micrometers in length and approximately 1-20 nanometers in diameter. These needle-like structures resemble asbestos.

It is well known that asbestos inhalation causes pulmonary inflammation and fibrosis, lung cancer, and malignant mesothelioma after relatively long latency periods (Mossman *et al.*, 1990; Hei *et al.*, 1992). However, the signaling pathways that lead to the development of these asbestos-associated diseases remain largely unknown. If

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carbon nanotubes can create the hazards and risks similar to those associated with asbestos, they must be appropriately assessed and managed to protect human health.

In vivo effects of MWCNTs have been studied using animal models with a variety of exposure methods, such as inhalation, intratracheal instillation, pharyngeal aspiration, and intraperitoneal injection, and their effects on inflammatory responses have been described. Mice exposed to MWCNTs by inhalation caused platelet-derived growth factor (PDGF) overexpression, inflammatory cell aggregation, and recruitment of macrophages that phagocytosed MWCNTs in the lung within 1 day, followed by the subsequent development of subpleural fibrosis during weeks 2-6 (Ryman-Rasmussen *et al.*, 2009a, 2009b). Pharyngeal aspiration of MWCNTs in mice caused the rapid development of fibrosis within 7 days and a persistent granulomatous inflammation throughout a 56-day post-exposure period (Porter *et al.*, 2010). Intratracheal instillation of MWCNTs in mice caused an increase in the number of neutrophils and the levels of cytokines in bronchoalveolar lavage (BAL) fluid within 1 day, and granulomatous lesions developed and persisted until day 14 of these experiments (Park *et al.*, 2009). Intraperitoneal injection of MWCNTs given to rats (Sakamoto *et al.*, 2009) or *p53* gene heterozygously deficient mice (Takagi *et al.*, 2008) induced a long-lasting inflammation and resulted in fibrous thickening and granuloma formation in the peritoneum in association with the induction of mesothelioma.

However, despite evidences from these studies, the potential immunotoxicity of MWCNTs has not been sufficiently established till date. Thus, the present study was conducted to assess a possible involvement of MWCNTs in immune and inflammatory responses of ICR mice. Intraperitoneal administration was chosen as the exposure route for MWCNTs. Although it may not be directly relevant to humans, intraperitoneal administration in a rodent model is sensitive enough to detect weak effects of MWCNTs, which was why this strategy was adopted to identify a possible carcinogenic hazard of MWCNTs (Sakamoto *et al.*, 2009; Takagi *et al.*, 2008). In addition, intraperitoneal administration can control and ensure the relationship between administration doses and agent exposure. Furthermore, some reports have clearly indicated the detection of inhaled MWCNTs in the subpleura (Ryman-Rasmussen *et al.*, 2009a), pharyngeally-aspirated MWCNTs in the pleura (Porter *et al.*, 2010), and intraperitoneally-administered MWCNTs in the liver and mesenteric lymph nodes (Sakamoto *et al.*, 2009). These results suggest that MWCNTs are distributed to a certain extent in the entire body, regardless of the exposure route used.

MATERIALS AND METHODS

Ethical approval

Our experimental protocols were approved by the Experiments Regulation Committee and the Animal Experiment Committee of the Tokyo Metropolitan Institute of Public Health prior to beginning of these experiments and were monitored at each step of experimentation for scientific and ethical appropriateness, including concerns for animal welfare, with strict adherence to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals, and other similar laws, guidelines, and rules provided domestically and internationally.

Animals

Specific pathogen-free female Crlj:CD1(ICR) mice, 6-7 weeks old, were purchased from Charles River Japan, Inc. (Kanagawa, Japan) and acclimatized for 1 week. Mice were housed individually in plastic cages (22 × 15 × 12 cm) with cedar chip bedding and had free access to a standard diet CE2 (Nihon Clea, Inc., Tokyo, Japan) and water. The animal room was maintained at 24°C-26°C with a relative humidity of 50%-60%, with 10 ventilations per hour (drawing fresh air through a high-efficiency particulate air filter, 0.3 µm, 99.9% efficiency), and on a 12 hr light/dark cycle.

Chemicals, reagents, and kits

MWCNTs (MITSUI MWCNT-7; lot number 060125-01k) were provided by National Institute of Health Science, Tokyo, Japan. These were exactly identical to those used in carcinogenicity studies with male Fisher 344 rats (Sakamoto *et al.*, 2009) and male C57BL/6-originated mice that were heterozygously deficient in the *p53* gene (Takagi *et al.*, 2008); these reports describe their physicochemical properties. Carbon black (CB; 22 nm in diameter) was purchased from Showa Chemical Industry Co., Ltd. (Tokyo, Japan). UICC-grade crocidolite was provided by the Tokyo Metropolitan Institute of Public Health.

Ovalbumin (OVA) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse IgM and IgG_1 antibodies were purchased from Zymed Laboratories (South San Francisco, CA, USA). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) tablets, a substrate for HPR-conjugated antibodies, were purchased from Roche

Diagnostics Division (Basel, Switzerland). Monoclonal anti-OVA-IgG_1 antibody (clone OVA-14) was purchased from Sigma-Aldrich. Phycoerythrin-conjugated (PE-) anti-CD3 (derived from T cell clone 145-2C11), fluorescein isothiocyanate-conjugated (FITC-) anti-CD45R (B220) (derived from B cell clone RA3-6B2), PE- anti-CD8 (clone 53-6.7), and FITC- anti-CD4 (clone GK1.5) were purchased from Beckman Coulter, Inc. (Fullerton, CA, USA). PE-Cy5.5--anti-CD3 (clone 145-2C11), PE-Cy5.5--anti-CD45 (derived from leukocyte clone 30-F11), PE--anti-CD14 (derived from monocyte clone: Sa2-8), PE--anti-Ly-6G (derived from granulocyte clone RB6-8C5), FITC--anti-CD54 (intercellular adhesion molecule [ICAM]-1; clone YN1/1.7.4), FITC--anti-CD49d (integrin α4; clone R1-2), FITC-anti-CD11b (integrin αM; clone 1/70), and anti-CD16/CD32 (Fcγ receptor III/II; clone 93) antibodies were purchased from eBioscience, Inc. (San Diego, CA, USA). RNAprotect Cell Reagent, RNeasy Mini kit, High Capacity RNA-to-cDNA kit, TaqMan Gene Expression Master Mix, and TaqMan Gene Expression Assays Inventoried were purchased from Life Technologies Co. (Carlsbad, CA, USA).

Animal experiments

Three independent animal experiments were conducted; protocols for handling test chemicals were identical in each. MWCNTs, CB, or crocidolite was suspended in 2% sodium carboxymethyl cellulose (CMC Na) to a concentration of 0.2 mg/ml. A single intraperitoneal dose (2 mg/kg body weight) of each of these was administered to mice. In a vehicle control group, CMC Na was administered with a single intraperitoneal volume of 10 ml/kg body weight.

The first animal experiment included histopathological examination and real-time polymerase chain reaction (PCR) assays for mRNA expression of cytokine/chemokine genes. Within 32 weeks, 2 of 6 mice that were administered MWCNT died; hence, the last experiment was conducted at the end of 34 weeks after administration. Mice were maintained up to 34 weeks after their exposure to test chemicals or vehicle. From each treatment or vehicle group, 3-6 animals were chosen for assays at the end of 2, 4, 10, 20, and 34 weeks. Under light ether anesthesia, cells were collected from the abdominal cavity and suspended in 5 ml of phosphate-buffered saline (PBS), centrifuged at 1,200 rpm for 10 min, and stored in RNAprotect Cell Reagent until RNA extraction for the real-time PCR assay. Tissues and organs were harvested for histopathological examinations. Samples were fixed in neutrally buffered formalin, embedded in paraffin, and stained with sirius red for collagen or hematoxylin-eosin.

The second animal experiment included flow cytometry analysis of the peripheral blood cells. Mice were maintained up to 20 weeks after their exposure to test chemicals or vehicle. From each treatment or vehicle group, 4 animals were chosen for the assays on day 2 and at the end of 1, 2, 4, and 20 weeks. Under light ether anesthesia, approximately 1 ml of blood was collected through cardiac puncture into a syringe with 20 µl of an anticoagulant, ethylenediaminetetraacetic acid, and used for flow cytometry.

The third animal experiment included determinations of OVA-specific immunoglobulins. After their exposure to test chemicals or vehicle, mice were immunized with OVA/alum intraperitoneally administered at a dose of 100 µg/mouse on days 2 and 10 as previously described (Ito *et al.*, 2002). Under light ether anesthesia, blood samples of approximately 0.1 ml were collected from a tail vein. Samples were taken from 10 to19 animals from each treatment or vehicle group from the tail vein 8 days after the last immunization for IgM and from 15 animals from each group 20 days after the last immunization for IgG_1. Serum was stored at -80°C until assayed.

Real-time PCR assays for mRNA expression of cytokine/chemokine gene

Total RNA was isolated from 5 x 10^4 peritoneal cells obtained in the first animal experiment as described above, using RNeasy Mini kit. RNA from untreated 8-16-week-old female ICR mice were prepared separately, pooled, and used as a basal expression control. First-strand cDNA was prepared from 0.9 µg of RNA using a High Capacity RNA-to-cDNA kit. PCR used TaqMan Gene Expression Master Mix for genes (*IL-1β*, Mm01336189_m1; *IL-2*, Mm00434256_m1; *IL-4*, Mm99999154_m1; *IL-5*, Mm99999063_m1; *IL-6*, Mm99999064_m1; *IL-8*, Mm00436450_m1; *IL-10*, Mm99999062_m1; *IL-13*, Mm00434204_m1; *IL-17*, Mm00439619_m1; *IL-18*, Mm00434225_m1; *IL-33*, Mm00505403_m1; *IFNγ*, Mm99999071_m1; *MCP-1*, Mm00441242_m1; *MyD88*, Mm00440338_m1; *TGFβ1*, Mm03024053_m1; *TNFα*, Mm99999068_m1; *TATA box binding protein [TBP]*, Mm00446973_m1; *hypoxanthine phosphoribosyltransferase [HPRT]*, Mm00446968_m1), cDNA-specific TaqMan Gene Expression Assays, and an ABI 7500 Real-Time PCR System (Life Technologies). All PCR reactions were performed in duplicates. The quantity of PCR product was determined by the Comparative Ct Method as described by the manufacturer, in which each sample was normalized against the value of a housekeeping gene, *HPRT*. Fold-changes were expressed as either an increase or decrease compared with the basal expression control level.

Flow cytometry analysis of the peripheral blood cells

After 15 minute pre-incubation with an anti-CD16/32 monoclonal antibody to prevent non-specific binding, a peripheral blood sample (100 μl) obtained in the second animal experiment described above was reacted with various combinations of antibodies. After a 30-min incubation in the dark, erythrocytes were lysed with 4 ml of Tris (1 g/500 ml)plus NH_4Cl (2.8 g /500 ml) for 10 min, suspended in 4 ml of PBS, and centrifuged at 1,200 rpm for 10 min. The cell pellet was washed in PBS with 0.5% BSA. Fluorescence intensity and cell numbers were determined using a Cell Lab Quanta SC (Beckman Coulter). The number of leucocytes was counted as positive cells of PE-Cy5.5- anti-CD45 antibody. The number of lymphocytes was distinguished based on CD45 fluorescence and side scatter. T and B cells were distinguished based on PE and FITC fluorescence from PE-Cy5.5- CD45 positive cells. CD4 and CD8 cells were distinguished based on PE and FITC fluorescence from PE-Cy5.5- CD3 positive cells. Percent of CD11b, CD49d, and CD54 positive cells was measured based on FITC fluorescence from CD45 and CD14 or CD45 and Ly6G positive cells.

Serum OVA-specific immunoglobulin concentrations

Concentrations of OVA-specific IgM and IgG_1 in serum were determined using ELISA. We added 100 μl of 100 μg/ml of OVA to wells of a microtest plate and incubated the plates overnight at 4°C. Wells were washed 6 times with 0.05% Tween20/PBS (0.05T/PBS) and blocked with 5% BSA in PBS (5B/PBS) for 2 hr at room temperature. Diluted serum (IgM: 1/150, IgG_1: 1/5 x 10^6) was then added to each well and incubated for 2 hr. After 6 washes with 0.05T/PBS, the wells were blocked with 5B/PBS for 1 hr at room temperature. HRP-labeled anti-mouse IgM and IgG_1 antibodies were added to each well, and the plates were incubated for 2 hr at room temperature. After 6 washes with 0.05T/PBS, a substrate solution prepared using ABTS Tablets according to the manufacturer's instructions was added and the color reaction was allowed to develop in the dark at room temperature for 30 min. Optical density (OD) at 405 against 492 nm was determined using a microplate reader (SUNRISE REMOTE; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Values for anti-OVA antibody were used as basal expression control.

Statistical analysis

Intergroup comparisons were made using Student's *t*-test. Significance level was set at p value < 0.05 .

RESULTS

General findings

During the course of the experiments, the body weights of mice increased within the same range after intraperitoneal administration of CMC Na, MWCNTs, CB, or crocidolite. In the first animal experiment, morphological assessments were conducted for mice that were given a single intraperitoneal administration of MWCNT, CB, or crocidolite. In the abdominal cavities of MWCNT-treated mice as compared with CMC Na-treated mice, liver edges had lost their sharpness, fibrous adhesions were seen on internal organs, and deposits were observed on the surfaces of the liver and diaphragm (Figs. 1a and 1b). In CB-treated mice, deposits were scattered in the abdominal cavity, especially on intestinal surfaces (Fig. 1c). No noteworthy changes were observed in the abdominal cavities of the CMC Na- or crocidolite-treated mice (Figs. 1a and 1d), or anywhere outside of the abdominal cavity in any of the groups.

Peritoneal cells obtained from MWCNT-treated mice contained small amounts of erythrocytes as compared with CMC Na-treated mice (Figs. 2a and 2b), whereas numerous erythrocytes were found for the crocidolite-treated mice (Fig. 2d). The peritoneal cells obtained from the CB-treated mice looked black, presumably because of the engulfment of the test chemical (Fig. 2c).

Fig. 3 showed the micrograms of liver, and Fig. 3a and 3b were the liver of CMC Na treated-mice that had thin layered mesothelium. Histopathological examinations revealed that the hepatic visceral peritoneum had fibrous thickening along with mesothelial cell hypertrophy in the MWCNT-treated mice (Figs. 3c and 3d). Inflammatory cells had infiltrated into this fibrously thickened visceral peritoneum. The majority of these infiltrating cells were macrophages containing MWCNTs, along with eosinophils, plasma cells, and immature myeloid cells (Fig. 3e) that occasionally formed a granulation (Fig. 3c). These changes were not observed in the CB- or crocidolite-treated mice.

No tumorigenic changes were observed either macroscopically or histopathologically in any of the mice treated with any of the test chemicals within the 34-week experimental period.

Expression of cytokine mRNA in peritoneal cells

mRNA expression levels of certain cytokine genes were substantially increased in the peritoneal cells obtained from MWCNT-treated mice, and these high levels were maintained up to the ends of 20 and 34 weeks. Th2 cytokine gene mRNA levels for *IL-4*, *IL-5,* and *IL-13*

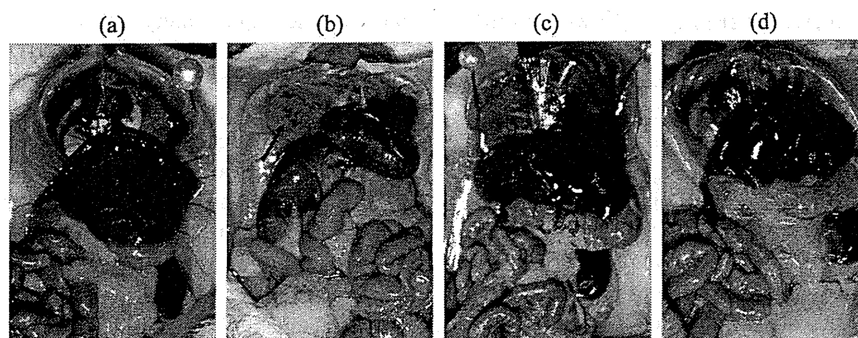

Fig. 1. Representative macroscopic appearances of the mouse abdominal cavity in the first animal experiment. Observations were made at 10 weeks after exposure to (a) CMC Na, (b) MWCNTs, (c) CB, or (d) crocidolite. Arrows indicate deposits of test chemicals.

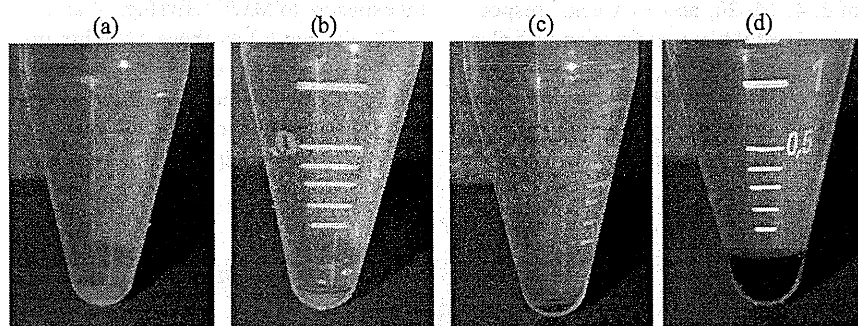

Fig. 2. Representative macroscopic appearances of peritoneal cells obtained from mice in the first animal experiment. Observations were made at 10 weeks after exposure to (a) CMC Na, (b) MWCNTs, (c) CB, or (d) crocidolite.

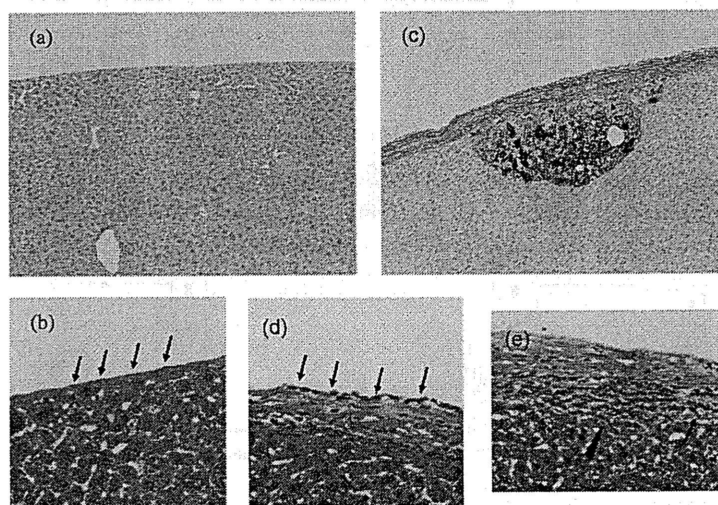

Fig. 3. Representative histology of the mouse liver in the first animal experiment. Examinations were made at 20 weeks after exposure to (a, b) CMC Na or (c-e) MWCNTs. Arrows indicate mesothelial cells and arrowheads indicate the infiltrations of eosinophils and immature myeloid cells. (a) and (c) were stained with sirius red staining, x 100, and (b), (d), and (e) were stained with hematoxylin-eosin, x 400.

were significantly increased from 2 to 20 weeks after administering MWCNTs. When compared with the basal expression control value for untreated animals, mRNA levels of *IL-4* were 34, 43, 24, 60, and 3 times higher, those of *IL-5* were 110, 127, 63, 226, and 69 times higher, and those of *IL-13* were 55, 38, 11, 28, and 3 times higher at 2, 4, 10, 20, and 34 weeks, respectively, after administering MWCNTs (Fig. 4).

Overexpression of mRNA of Th1 cytokine genes, *IL-2* and *IFNγ*, were delayed compared with mRNA of Th2 cytokine genes, but were also sustained; however, these were not significantly higher than basal expression levels except for *IL-2* at the end of 34 weeks. mRNA expression levels of *IL-2* were 0.3, 0.6, 1.5, 5.4, and 4.8 times higher, and those of *IFNγ* were 0.5, 0.3, 0.6, 1.6, and 4.3 times higher at the end of 2, 4, 10, 20, and 34 weeks, respectively (Fig. 4). Sustained mRNA overexpression was also found for a Th17 cytokine gene, *IL-17*, and these increases were significant at the end of 10 to 20 weeks.

mRNA for genes of proinflammatory cytokines, *IL-1β*, *IL-33*, and *TNFα*, and an inflammatory chemokine, *MCP-1*, were increased significantly at the end of 2 to 20 weeks (*IL-1β* and *TNFα*) and at 2 to 34 weeks (*IL-33* and *MCP-1*). mRNA level of an adapter protein of Toll-like receptors (TLR), *MyD88*, was also increased significantly at the end of week 2 to 20. mRNA levels of *IL-17* were 9, 13, 9, 26, and 25 times higher, those of *IL-1β* were 29, 23, 32, 28, and 19 times higher, those of *IL-33* were 13, 20, 5, 13, and 20 times higher, those of *TNFα* were 3, 2, 2, 3, and 2 times higher, those of *MCP-1* were 17, 28, 41, 49, and 42 times higher, and those of *MyD88* were 3, 3, 2, 2, and 1 time higher at 2, 4, 10, 20, and 34 weeks, respectively, after MWCNT administration (Figs. 4 and 5). mRNA levels of other inflammatory cytokine genes (*IL-6*, *IL-8*, and *IL-18*), anti-inflammatory cytokines (*IL-10* and *TGFβ*), and a housekeeping gene (*TBP*) were not affected by exposure to MWCNTs (Figs. 4 and 5).

CB did not affect these cytokine mRNA expressions in peritoneal cells. For crocidolite-treated mice, sustained mRNA overexpression was observed only for an inflammatory cytokine gene, *IL-6* (4, 5, 6, and 8 times higher at the end of 2, 4, 10, and 20 weeks, respectively), which

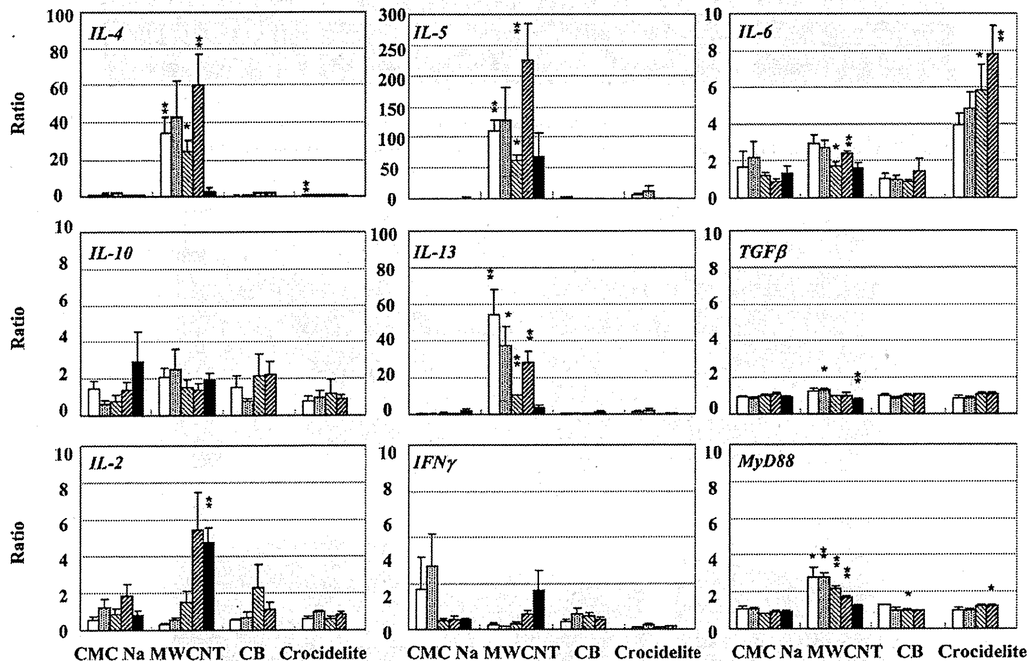

Fig. 4. mRNA expression of Th2 cytokine genes (*IL-4, IL-5, IL-10, IL-13,* and *TGFβ*), Th1 cytokine genes (*IL-2* and *IFNγ*), and a TLR adaptor protein (*MyD88*) in peritoneal cells obtained from mice in the first animal experiment. For each group of mice exposed to a test chemical or vehicle, columns from the left to the right are average values (n = 3-6) at 2, 4, 10, 20, and 34 weeks after exposure. These determinations were not made at the end of week 34 for the CB- and crocidolite-treated groups. (*$p < 0.05$, **$p < 0.01$).

was a more pronounced change than that with MWCNTs (Fig. 4). In addition, mRNA levels of *IL-5* were 5, 11, 1, and 1 times higher (Fig. 4), and those of *MCP-1* were 14, 9, 2, and 4 times higher (Fig. 5) at the end of 2, 4, 10, and 20 weeks, respectively; however, these changes were faint and transient.

Effects on the peripheral blood cells

MWCNT treatment increased the total number of leukocytes, granulocytes, and monocytes in the peripheral blood as early as 1 week after its administration, and these high levels were maintained up to the end of week 20 (Figs. 6a, 6b and 6c). The number of total lymphocytes was also increased, but only at the end of week 20. B and T cells were increased, although not significantly, within the 20-week experimental period in the MWCNT-treated mice (Figs. 6d, 6e and 6f). In the crocidolite treatment mice, the numbers of leukocytes, granulocytes, and monocytes exhibited a statistically significant, although minimal, transient increase at the end of week 1 (Figs. 6a, 6b and 6c). CB and crocidolite treatment increased the numbers of lymphocytes, B, and T cells at the end of day 2 and 1 week, but not significantly, and then decreased (Figs. 6d, 6e and 6f).

Expression of leukocyte adhesion molecules on the peripheral blood cells

MWCNT treatment induced overexpression of CD49d and CD54, but not CD11b, on granulocytes as early as 2 and 1 weeks, respectively, after its administration, and these high levels were maintained up to the end of week 20 (Fig. 7a). The expression of adhesion molecules was not altered on monocytes, with the exception that a statistically significant, although minimal, transient overexpression was observed for CD49d at the end of week 4 (Fig. 7b). CB and crocidolite did not induce overexpression of any of the leukocyte adhesion molecules on the peripheral blood cells, and in fact their expression was transiently decreased in some cases (Fig. 7).

OVA-specific immunoglobulins in serum

Figure 8 summarizes the results for the serum con-

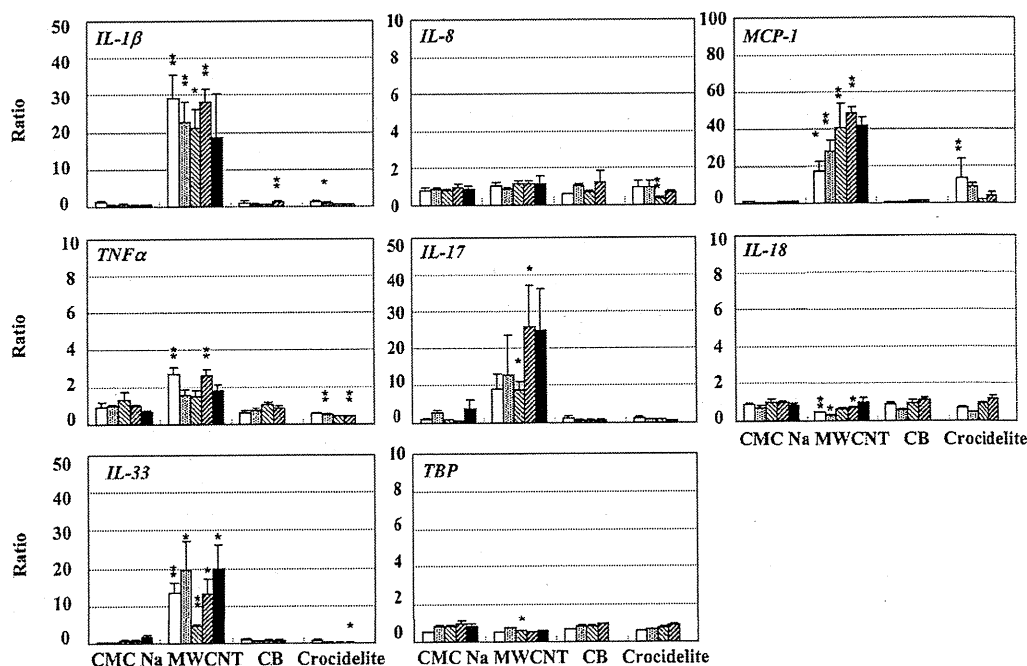

Fig. 5. mRNA expression of inflammatory cytokine genes in peritoneal cells obtained from mice in the first animal experiment. For each group of mice exposed to a test chemical or vehicle, columns from the left to the right are average values (n = 3-6) at 2, 4, 10, 20, and 34 weeks after exposure. These determinations were not made at the end of week 34 for the CB- and crocidolite-treated groups. (*$p < 0.05$, **$p < 0.01$).

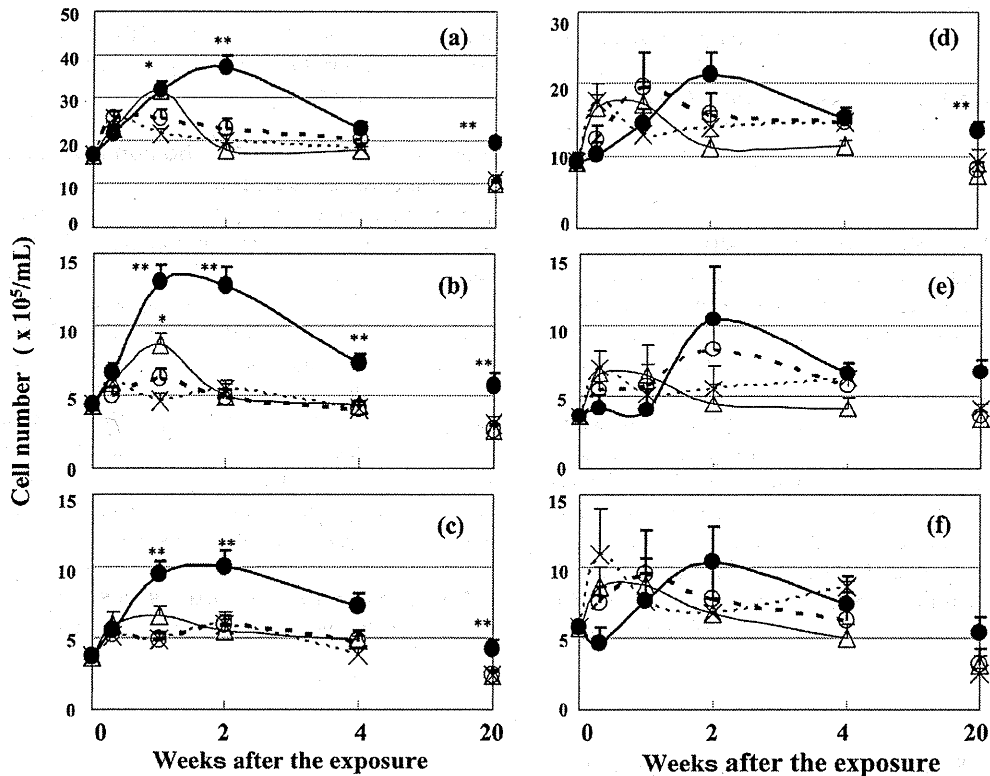

Fig. 6. Flow cytometry results for the peripheral blood cells obtained from mice in the second animal experiment. Changes in the numbers of (a) leukocytes, (b) granulocytes, (c) monocytes, (d) lymphocytes, (e) B cells, and (f) T cells after exposure to CMC Na (open circles), MWCNTs (closed circles), CB (crosses), and crocidolite (open triangles). Results are means ± standard deviations (n = 4). Asterisks indicate that results are significantly different from those of controls (*$p < 0.05$, **$p < 0.01$).

centrations of OVA-specific IgM (Fig. 8a) and IgG_1 (Fig. 8b). For mice treated with MWCNTs, CB, crocidolite, and CMC Na, the relative amounts (arbitrary units; AU) of OVA-specific IgM were, 1.33 ± 0.20, 1.07 ± 0.20, 1.07 ± 0.15, and 0.79 ± 0.12 AU, respectively, while those for OVA-specific IgG_1 were, 3.68 ± 0.57, 2.49 ± 0.29, 2.13 ± 0.32, and 2.28 ± 0.35 AU, respectively. Thus, MWCNT and not CB or crocidolite, significantly enhanced the production of OVA-specific immunoglobulins in mice.

DISCUSSION

The present study clearly shows that MWCNTs stimulated immune and inflammatory responses in mice and these effects sustained until the mice died. It has been previously shown in other animal models that a single intraperitoneal administration of MWCNT caused severe inflammation throughout the abdominal cavity and mesothelioma. Male Fisher 344 rats died at 37-52 weeks after administration (Sakamoto *et al.*, 2009) and and male C57BL/6-originated mice heterozygously deficient in the *p53* gene died within 25 weeks of administration (Takagi *et al.*, 2008).

The toxicity caused by MWCNTs in the present study did not involve tumor formation, but did induce severe inflammation, and 2 of 6 mice had died by the end of 32 weeks. The differences in the magnitudes of MWCNT toxicity between the present and previous studies was apparently because of differences in species, strains, and/or genders. To extrapolate the animal toxicity data to information important for human health concerns, further investigations are required. The most aggressive morphological change we observed was the infiltration of macrophages, eosinophils, plasma cells, and immature myeloid cells into the fibrously thickened visceral peritoneum of the liver with occasional granulation, and severe fibrous adhesions to the internal organs.

Light microscopic examination revealed that MWCNT

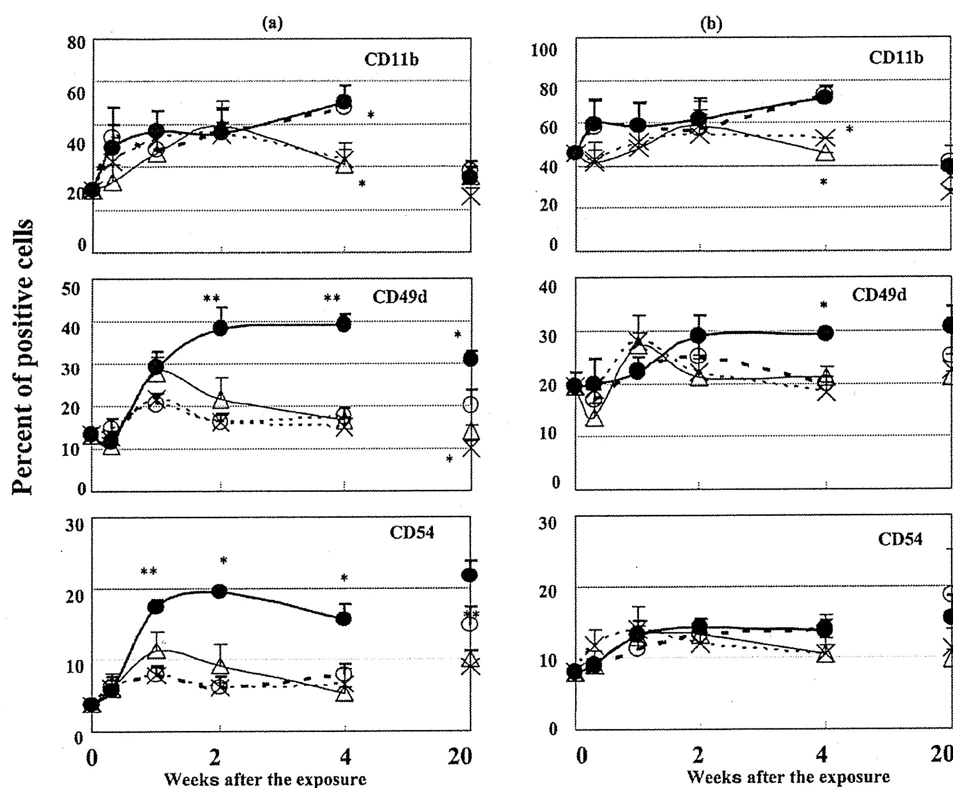

Fig. 7. Flow cytometry results for the peripheral blood cells obtained from mice in the second animal experiment. Changes in the expression of adhesion molecules on the surfaces of (a) granulocytes and (b) monocytes after exposure to CMC Na (open circles), MWCNT (closed circles), CB (crosses), and crocidolite (open triangles). Results are means ± S.D. (n = 4). Asterisks indicate that values are significantly different from those of controls (* $p < 0.05$).

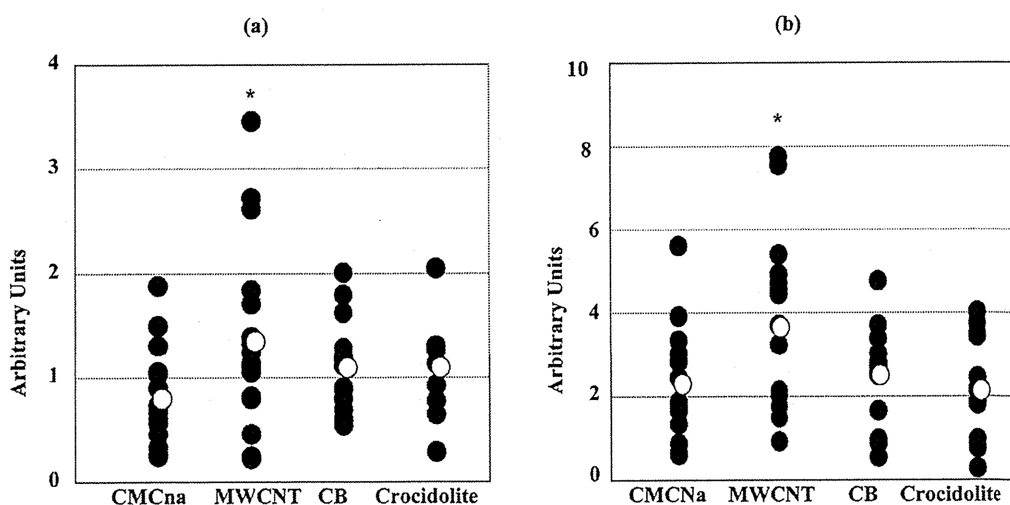

Fig. 8. Production of OVA-specific antibodies by mice in the third animal experiment. Serum concentrations of OVA-specific (a) IgM and (b) IgG_1 Open circles are average values, and closed circles are individual values (n = 10-19 for IgM, and n = 15 for IgG_1). Asterisks indicate that values are significantly different from those of controls (* $p < 0.05$).

were present in macrophages in these lesions. Frustrated phagocytosis has frequently been postulated to be involved in the mechanisms by which MWCNTs causes toxicity, including inflammation and carcinogenesis (Poland *et al.*, 2008). Thus, these lesions may have some fundamental biological importance. The sustained overexpression of cytokine mRNA in peritoneal cells may suggest one possibility, although no MWCNTs were observed in the peritoneal cells by light microscopy. An *in vitro* time lapse experiment revealed that dead cells with MWCNTs had been re-engulfed by other macrophages (data not shown), suggesting a cycle of sustained inflammation. MWCNTs either could not be limited by granuloma formation or subvisible MWCNTs may remain in the cavity. Thus, macrophages may continue their phagocytic activity, and as a result inflammatory cytokines/chemokines would be continuously produced.

Our results indicated that MWCNTs caused a systemic inflammation that was sustained for at least 20-34 weeks after a single intraperitoneal administration, because the numbers of leukocytes, granulocytes, and monocytes in the peripheral blood were increased from 1 to 20 weeks after MWCNT administration. During a similar period, CD49d and CD54 were overexpressed on granulocytes, which may have been involved in their infiltration into the inflammatory sites past vascular endothelial cells. Crocidolite increased the numbers of leukocytes, granulocytes, and monocytes at 1 week, but returned to the basal level after 2 weeks, and the effects were weaker than MWCNTs. The number of leukocytes was slightly increased at 2 days and 1 week after CMCNa exposure, and mRNA level of *IFNγ* was increased at 2 and 4 weeks after CMCNa exposure, but decreased thereafter. However the effect of CMCNa has not been known, there is a possibility CMCNa acts as a xenobiotic although the effect is little. Furthermore, the number of peripheral lymphocytes was also increased at 20 weeks after MWCNT administration, and this corresponded to the enhanced T cell-dependent production of OVA-specific antibodies, as indicated by their increased serum concentrations. The overexpressed mRNA of Th2 cytokine genes seen in peritoneal cells suggested that these cytokines have been involved in this enhanced antibody production. Although the underlying mechanisms need to be clarified, MWCNTs may promote these immune responses by acting as an adjuvant (Inoue *et al.*, 2009; Nygaard *et al.*, 2009).

The present study demonstrated the overexpression of mRNA for various cytokines/chemokines in peritoneal cells after a single intraperitoneal administration of MWCNTs. To the best of our knowledge, this is the first report of results obtained for peritoneal cells with regard to changes in cytokine/chemokine mRNA expression after MWCNT exposure *in vivo*. Previous reports focused primarily on short-term effects of MWCNT exposure. Mitchell *et al.* (2007) reported that *IL-10* levels increased in spleen homogenates after 14 consecutive days of whole body inhalation exposure for male C54BL/6 mice. Park *et al.* (2009) found that the protein levels of proinflammatory cytokines were increased both in the BAL fluid and in the peripheral blood, in which Th2 cytokines were increased to a greater extent than Th1 cytokines, in mice given intratracheal administrations of MWCNTs. In these reports, the levels of cytokines reached a peak at day 1 after the exposure and remained high at day 14.

In a study by Ryman-Rasmussen *et al.* (2009b) intratracheally administered MWCNTs potentiated the development of airway fibrosis in mice with allergic asthma induced by OVA sensitization, in which the levels of *IL-13* and *IL-5* increased at day 1, but returned to normal levels at day 14 when airway fibrosis became significant. Inoue *et al.* (2009) used MWCNT instillation for 6 weeks. At 24 hours after the final treatment, they observed significant exacerbation of murine allergic airway inflammation and high levels of Th1 and Th2 cytokine proteins. In the present study, the time-courses of changes in mRNA levels corresponded to functional groups of cytokines/chemokines.

mRNA overexpression of some pro-inflammatory cytokine genes, *IL-1β and IL-33*, occurred within 2 weeks and remained elevated up to the end of week 34. These are known to induce Th2 cytokines (Schmitz *et al.*, 2005; Amatucci *et al.*, 2007; Komai-Koma *et al.*, 2007; Kondo *et al.*, 2008). Therefore, mRNA of Th2 cytokine genes, *IL-4*, *IL-5* and *IL-13*, were also overexpressed within 2 weeks and remained elevated up to week 20. Among these, mRNA level for *IL-5* was still high, but levels for *IL-4* and *IL-13* decreased at the end of week 34.

mRNA level of a Th17 cytokine gene, *IL-17*, was also increased within 2 weeks, it was increased significantly after 10 to 20 weeks, and was still high, although not significantly, at the end of week 34. mRNA of Th1 cytokine genes, *IL-2* and *IFNγ*, were also overexpressed; however, this occurred at 20-34 weeks (*IL2*) and 34 weeks (*IFNγ*) after MWCNT exposure. While the details of the underlying mechanisms need to be clarified, the differential, time-dependent overexpression of Th2 and Th17 cytokine genes at first followed by Th1 cytokine genes may provide for some optimum balance between these inflammatory mediators for the sustained stimulating effects of MWCNTs on immune and inflammatory responses.

The present study indicated a rapid, drastic and sus-