４．４　毒性試験結果のまとめ

４．１、４．２及び４．３の結果を表１にまとめる。

（表 1）平成１９～２１年度農薬吸入毒性評価手法確立調査における農薬の無毒性量のまとめ

薬剤名	試験条件*					エンドポイント	無毒性量（mg/m^3）
	試験系	使用動物数	ばく露濃度群（mg/m^3）	ばく露経路	ばく露期間		
フェニトロチオン（MEP）	ラット雌雄 (Crj:CD(SD))	雌雄各 5 匹/群	0, 2, 4, 8	鼻部ばく露による吸入	6 時間/日, 5 日間/週, 28 日間	脳及び赤血球コリンエステラーゼ活性阻害	雌雄：4
トリクロルホン（DEP）	ラット雌雄 (Crj:CD(SD))	雌雄各 10 匹/群	0, 10, 30, 100	全身ばく露による経気道投与	6 時間/日, 5 日間/週, 28 日間	赤血球アセチルコリンエステラーゼ活性阻害	雄：30 雌：100
イソキサチオン	ラット雌雄 (Crj:CD(SD))	雌雄各 10 匹/群	0, 1, 3, 10	全身ばく露による経気道投与	6 時間/日, 5 日間/週, 28 日間	赤血球アセチルコリンエステラーゼ活性阻害	雌雄：3

* OECD テストガイドライン 412 及びその改定案に準ずる

（参考）

経口と吸入のばく露経路の異なる試験における毒性の比較対照のため、28 日間の経口毒性試験の情報入手を試みたが、見出せなかった。従って、公表されている上記 3 剤の経口毒性試験結果（3 ヶ月、6 ヶ月、2 年間反復投与試験および繁殖毒性試験）と今回得られた吸入毒性試験結果をまとめた（表 2)。その結果を以下に要約する。

1. 28 日間吸入毒性が、3 ヶ月及び 2 年間経口毒性よりもやや強い場合（イソキサチオン）と、3 ヶ月経口毒性より強く 6 ヶ月あるいは 2 年間試験よりはやや弱い場合（フェニトロチオン、トリクロルホン）とが認められた。

2. ADI 設定の根拠となる各剤の経口毒性試験の中で最も感受性の高いデータ（最も低い NOAEL 値）と比較して 28 日間吸入試験での NOAEC は大きく乖離していなかった（フェニトロチオン；0.5 mg/kg/day(po)：0.74 mg/kg/day(inh)、トリクロルホン；2.5 mg/kg/day(po)：4.24 mg/kg/day(inh)、イソキサチオン；0.625 mg/kg/day(po)：0.405 mg/kg/day(inh)）。

3. 一方、最も感受性の低いデータ（最も高い NOAEL 値）との比較では 28 日間吸入試験での NOAEC は、フェニトロチオンで約 8.5 倍（6.3 mg/kg/day(po)：0.74 mg/kg/day(inh)）、トリクロルホンで約 6.6 倍（27.8 mg/kg/day(po)：4.24 mg/kg/day(inh)）、イソキサチオンでは約 3.1 倍（1.25 mg/kg/day(po)：0.405 mg/kg/day(inh)であった。

4. 繁殖毒性試験では NOAEL は親動物と児動物とで同じであり、繁殖毒性は認められなかった。また、繁殖毒性試験における NOAEL も吸入毒性の NOAEC（特に雌動物）と同等であった。

（表 2）

各　種　毒　性　試　験　結　果　一　覧

農薬名	ラット 28 日間吸入毒性試験 NOAEC （mg/kg/day 換算）	ラット 亜急性経口毒性試験 用量及び NOAEL（LOAEL）	ラット 慢性経口毒性試験 用量及び NOAEL（LOAEL）	ラット 繁殖毒性試験 用量及び NOAEL（LOAEL）
フェニトロチオン （ＭＥＰ）	雄：4 mg/m^3 (0.740 mg/kg/day) 雌：4 mg/m^3 (1.10 mg/kg/day)	・3 ヶ月間試験 [1)] 0, 32, 63, 125, 250, 500 ppm 63 ppm→6.3 mg/kg/day (125 ppm→12.5 mg/kg/day[2)]) ・6 ヶ月間試験 [1)] 0, 10, 30, 150 ppm 10 ppm→0.6 mg/kg/day (30 ppm→1.5 mg/kg/day[2)])	・2 年間試験 [1)] 0, 10, 30, 100 ppm 10 ppm→0.5 mg/kg/day (30 ppm→1.5 mg/kg/day[2)])	0, 10, 40, 120 ppm[1)] 10 ppm→0.65 mg/kg/day (40 ppm→3.1 mg/kg/day)
トリクロルホン （ＤＥＰ）	雄：30 mg/m^3 (4.24 mg/kg/day) 雌：100 mg/m^3 (20.7 mg/kg/day)	・3 ヶ月間試験 [3)] 0, 62, 185, 555, 1666, 5000 ppm 555 ppm→27.8 mg/kg/day[2)] *EP: 運動性低下、肝・腎・脾臓の重量増加 (1666 ppm→83.3 mg/kg/day[2)])	・2 年間試験 A[3)] 0, 50, 250, 500, 1000 ppm 50 ppm→2.5 mg/kg/day (250 ppm→12.5 mg/kg/day[2)]) ・2 年間試験 B[3)] 0, 100, 300, 1000⇒1750 ppm 100 ppm→4.4 mg/kg/day *EP: T.Chol 増加 (300 ppm→15.0 mg/kg/day[2)])	0, 100, 300, 1000, 3000 ppm[3)] 300 ppm→15 mg/kg/day[2)] (1000 ppm→50 mg/kg/day[2)])
イソキサチオン	雄：3 mg/m^3 (0.405 mg/kg/day) 雌：3 mg/m^3 (0.617 mg/kg/day)	・3 ヶ月間試験 A[4)] 0, 3.2, 6.3, 12.5, 25, 50, 100 ppm 25 ppm→1.25 mg/kg/day[2)] (50 ppm→2.5 mg/kg/day[2)]) ・3 ヶ月間試験 B[4)] 0, 12.5, 25, 50, 100, 200 ppm 12.5 ppm→0.625 mg/kg/day[2)] (25 ppm→1.25 mg/kg/day[2)])	2 年間試験 [4)] 0, 0.6, 1.2, 2.4, 35 mg/kg/day 1.2 mg/kg/day (2.4 mg/kg/day)	0, 2.5, 12.5 ppm[4)] 12.5 ppm→0.625 mg/kg/day[2)]

1) Pesticide residues in food 2000: FENITROTHION (http://www.inchem.org/documents/jmpr/jmpmono/v00pr06.htm)
2) PLINCIPLES FOR THE TOXICOLOGICAL ASSESSMENT OF PESTICIDE RESIDUES IN FOOD,WHO(1990) (http://www.inchem.org/documents/ehc/ehc/ehc104.htm) の換算係数を用いて体重あたり重量に換算
3) 厚生労働省 平成 15（2003）年 薬事・食品衛生審議会食品衛生分科会毒性・残留農薬合同部会報告
4) 三共研究所年報 vol.29, 51-54(1977)
* エンドポイント（EP）：繁殖毒性試験以外のすべての試験のエンドポイントは、当該試験を除きコリンエステラーゼ活性阻害である

４．５　気中濃度評価値の設定

３．４で述べた「気中濃度評価値の算出方法」に基づき、フェニトロチオン、トリクロルホン及びイソキサチオンについて、それぞれ気中濃度評価値を算出した。

なお、いずれの農薬においても、文献調査において妊娠動物や児動物への毒性影響は認められなかったことから、追加の安全係数を乗ずる必要はないと考えられた（平成 21 年度においては、児動物への毒性影響の評価の考え方について詳細な検討が行われたところ。その結果は別紙 4 のとおり。）。

○フェニトロチオン

①28 日間反復吸入毒性試験における無毒性量（NOAEC）

NOAEC：4 mg/m^3（雌雄）

平均体重：0.278 kg（雄）、0.187 kg（雌）

（雌雄ともに 4 mg/m^3 ばく露群の平均体重）

②NOAEC を基に雄体重から計算したヒト許容一日経気道ばく露量（ADAEL）

＝ 4 mg/m^3×1/0.278 kg 体重×1/1944

＝ 0.0074（mg/kg 体重/day）

NOAEC を基に雌体重から計算したヒト許容一日経気道ばく露量（ADAEL）

＝ 4 mg/m^3×1/0.187 kg 体重×1/1944

＝ 0.011（mg/kg 体重/day）

雌雄の体重に基づいて計算した ADAEL のうち、より小さい値である 0.0074（mg/kg 体重/day）を気中濃度評価値の算出に使用した。

③ADAEL から計算した気中濃度評価値（mg/m^3）

＝ 0.0074（mg/kg 体重/day）/｛0.403*（L/min/kg 体重）×1/1000（m^3/L）×60 min× 24 h/day｝

＝ 0.01 mg/m^3**

*小児の呼吸量は 0.403 L/min/kg 体重であり、成人の呼吸量 0.200 L/min/kg 体重に比べて大きいことから、小児の呼吸量を用いて計算した。トリクロルホン及びイソキサチオンについても同様とした。

**評価値設定の根拠試験である吸入毒性試験の無毒性量の有効数字は 1 桁であることから、評価値の有効数字は 1 桁とし、また、端数処理については、リスクを過小評価することのないよう、2 桁目を切り捨てて算出した。トリクロルホン及びイソキサチオンについても同様とした。

○トリクロルホン

①28 日間反復吸入毒性試験における無毒性量（NOAEC）

NOAEC：30 mg/m³（雄）、100 mg/m³（雌）

平均体重：0.364 kg（雄）、0.249 kg（雌）

（雄は 30 mg/m³ ばく露群、雌は 100 mg/m³ ばく露群の平均体重）

②NOAEC を基に雄体重から計算したヒト許容一日経気道ばく露量（ADAEL）

＝ 30 mg/m³×1/0.364 kg 体重×1/1944

＝ 0.042（mg/kg 体重/day）

NOAEC を基に雌体重から計算したヒト許容一日経気道ばく露量（ADAEL）

＝ 100 mg/m³×1/0.249 kg 体重×1/1944

＝ 0.21（mg/kg 体重/day）

雌雄の体重に基づいて計算した ADAEL のうち、より小さい値である 0.042（mg/kg 体重/day）を気中濃度評価値の算出に使用した。

③ADAEL から計算した気中濃度評価値（mg/m^3）

＝ 0.042（mg/kg 体重/day）/｛0.403（L/min/kg 体重）×1/1000（m^3/L）×60 min× 24 h/day｝

＝ 0.07 mg/m³

○イソキサチオン

①28 日間反復吸入毒性試験における無毒性量（NOAEC）

NOAEC：3 mg/m³（雌雄）

平均体重：0.381kg（雄）、0.250 kg（雌）

（雌雄ともに 3 mg/m³ ばく露群の平均体重）

②NOAEC を基に雄体重から計算したヒト許容一日経気道ばく露量（ADAEL）

＝ 3 mg/m³×1/0.381kg 体重×1/1944

＝ 0.0041（mg/kg 体重/day）

NOAEC を基に雌体重から計算したヒト許容一日経気道ばく露量（ADAEL）

＝ 3 mg/m³×1/0.250 kg 体重×1/1944

＝ 0.0062（mg/kg 体重/day）

雌雄の体重に基づいて計算した ADAEL のうち、より小さい値である 0.0041（mg/kg 体重/day）を気中濃度評価値の算出に使用した。

③ADAEL から計算した気中濃度評価値（mg/m^3）

＝ 0.0041（mg/kg 体重/day）/｛0.403（L/min/kg 体重）×1/1000（m^3/L）×60 min× 24 h/day｝

＝ 0.007 mg/m^3

(別紙 4)

有機リン剤の小児の感受性について

１．第 3 回吸入毒性部会（3 月 1 日）における議論

（１）これまでの農薬吸入毒性評価手法確立調査部会（以下、「毒性部会」という。）における委員指摘に基づき、毒性試験結果において児動物に対する明らかな影響が認められる場合、さらに追加安全係数を乗じることを検討する必要性があるとされていたところ。

（２）「農薬の登録申請に係る試験成績について」（平成 12 年 11 月 24 日付け 12 農産第 8147 号農林水産省農産園芸局長通知）に規定されている各種毒性試験のうち、小児への影響について確認する試験としては、繁殖毒性試験及び催奇形性試験があり、小児への影響を配慮した試験系が組まれている。

○繁殖毒性試験とは、被験物質を二世代にわたって投与し、発情周期、交尾、受胎、分娩、哺育等の生殖機能及び出生児の生育に及ぼす影響に関する科学的知見を得ることを目的とした試験であり、児動物に対する観察・検査項目としては、生存児数、一般状態の変化、体重、剖検観察、臓器重量、病理組織学的検査等を実施する。

○催奇形性試験とは、発生毒性試験とも呼ばれ、妊娠中の母動物が被験物質にばく露された場合の胎児の発生、発育に及ぼす影響、特に催奇形性に関する科学的知見を得ることを目的とした試験であり、胎児に対する観察・検査項目としては、生存胎児数、体重、外表異常検査、内臓異常検査、骨格異常検査等を実施する。

（３）第 3 回毒性部会においては、イソキサチオンの毒性試験結果について検討が行われ、児動物への影響について、事務局より、

・3 世代繁殖毒性試験において、生後の生育、摂餌量、一般症状、血液及び血液生化学的検査並びに病理組織学的検査においても特に異常は観察されていない。
・このため、イソキサチオンについては児動物への毒性影響は認められないと考えられることから、追加安全係数を乗じる必要はない。

との説明があった。

（４）これに対し、委員より、

①通常、毒性影響は全て成獣に対するばく露量で評価されており、胎児・新生児への直接のばく露量については評価されていないが、3 世代繁殖毒性試験において児動物への毒性影響は認められないことから、さらに安全係数を乗ずる必要はない、との意見があった一方、

②3 世代繁殖毒性試験は児動物への感受性を直接評価するものではないため、何らかの安全係数を乗ずることを検討すべき

③アメリカの EPA では、児動物への影響についての追加安全係数は、

・根拠がない場合：1

・児動物の発達神経毒性を予測させる等根拠がある場合：√10

・客観的に危惧されるデータがある場合：10

との意見が出された。

④その他、以下の点について総合的に勘案する必要があるとの意見もあった。

・繁殖毒性試験では、体重当たりに換算すると、児動物の被験物質摂餌量が親動物に比べ多いにもかかわらず、児動物への毒性影響は認められていない。

・イソキサチオンの今年度実施した吸入毒性試験の無毒性量設定のエンドポイントは、赤血球アセチルコリンエステラーゼ活性阻害という比較的軽いパラメーターであり、無毒性量より 3 倍以上高い 10mg/m^3 投与群においても、神経毒性の主要な指標である動物行動、脳アセチルコリンエステラーゼ活性に影響を与えていない。

・市街地等での農薬散布によるばく露が短期間であることに対し、今年度実施したラットの吸入毒性試験の試験期間は 28 日間であり、ヒトに換算すると十分に長い。

・今年度実施した吸入毒性試験は成獣を用いていることから、脳アセチルコリンエステラーゼ活性への無影響は、血液脳関門により薬剤が脳内に移行しないものであるという可能性が考えられ、血液脳関門が未成熟な児動物では、脳アセチルコリンエステラーゼ活性を阻害する可能性が考えられる。（２．（５）参照）

２．イソキサチオンの毒性評価における追加安全係数を乗じる必要性

（１）イソキサチオンの繁殖毒性試験及び催奇形性試験については下記のとおり。

〇繁殖毒性試験

親動物と比べ児動物に特異的な毒性影響は認められておらず、生後の生育、摂餌量、一般症状、血液及び血液生化学的検査並びに病理組織学的検査においても被験物質投与の影響を示唆する異常所見は特に観察されていない。

〇催奇形性試験

胎児への影響は特に認められていない。

（２）追加安全係数については、食品安全委員会及び非食用農作物専用農薬安全性評価検討会で ADI 設定済みの全 158 農薬のうち、100 以外の安全係数を乗じた農薬は 6 農薬あり、必要な試験が実施されていない等、追加安全係数を乗じる場合は具体的な根拠が明示されている。

（３）従って、イソキサチオンの気中濃度評価値の設定においては、

・繁殖毒性試験及び催奇形性試験の結果、児動物への影響が認められないこと

・いずれの試験系においても無毒性量 NOAEL が得られていること

・追加安全係数を乗じることを説明できるだけの具体的な根拠がないことから、安全係数は種差及び個体差を考慮した 100 とし、追加安全係数を乗じる必要はないとすることが適当と考える。

（４）フェニトロチオン及びトリクロルホンについても、イソキサチオンと同様に、繁殖毒性試験及び催奇形性試験の結果､児動物への影響が認められないことから、当該 2 農薬についても、追加安全係数を乗じる必要はないとすることが適当と考える。なお、トリクロルホンについては、平成 15 年の厚生労働省薬事・食品衛生審議会食品衛生分科会残留農薬調査会において、安全係数を 100 として ADI が設定されている。

（５）なお、吸入毒性試験において脳コリンエステラーゼ活性阻害が見られなかったことについて、血液・脳関門により薬剤が脳内に移行しない可能性の指摘（１．（４）④）については、有機リン剤は成獣でも血液・脳関門を通過することが知られていることから（**別添**）、今回の吸入毒性試験で脳にコリンエステラーゼ阻害がみられなかった理由は用量に起因するものと考えられる。

また、小児への感受性を直接評価するためには、胎児、児動物等を評価対象とした試験系を構築する必要があるが、現行のガイドラインに基づく試験系は、すべて成獣に対するばく露量で評価されており、胎児・新生児へのばく露量は評価されていない。すなわち、胎児・新生児へのばく露量を算定するためには代謝物を含む被験物質の経胎盤移行率あるいは母乳中の被験物質濃度や新生児の母乳摂取量を求める必要があり、現時点では技術的手法を含め未解決な部分が多いことから、正確に児動物と成獣の感受性を比較・評価することは難しく、将来の毒性学（安全性試験評価）における検討課題である。

（別添）

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IgG-Paraoxonase-1 Fusion Protein for Targeted Drug Delivery Across the Human Blood-Brain Barrier

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Abstract

Paraoxonase (PON)-1 is the most potent human protein with organophosphatase activity against organophosphate (OP) toxins. OP compounds readily cross the blood-brain barrier (BBB), and have lethal mechanisms of action within the brain. The production of a brain penetrating form of human PON1, which crosses the BBB, is possible with the re-engineering of the enzyme as a fusion protein with a monoclonal antibody (MAb) against the human insulin receptor (HIR). The HIRMAb crosses the BBB via the endogenous insulin receptor, and acts as a molecular Trojan horse to ferry the PON1 into brain. The human PON1 was fused to the carboxyl terminus of the heavy chain of the chimeric HIRMAb. COS cells were dual transfected with the heavy chain gene and the light chain gene, and the HIRMAb-PON1 fusion protein was affinity purified with protein A chromatography. Western blotting with antibodies to human IgG or human PON1 showed the heavy chain of the HIRMAb-PON1 fusion protein was 40 kDa larger than the heavy chain of the chimeric HIRMAb. The ED50 of binding to the HIR extracellular domain was 0.55 ± 0.07 nM and 1.1 ±0.1 nM, respectively, for the chimeric HIRMAb and the HIRMAb-PON1 fusion protein. The PON1 enzyme activity of the fusion protein was approximately 25% of the enzyme activity in human plasma, based on a fluorometric enzymatic assay. In conclusion, human PON1 has been re-engineered as an IgG-organophosphatase fusion protein that penetrates the human BBB.

Keywords

blood-brain barrier; drug targeting; paraoxonase-1; chemical nerve gas

Introduction

Humans are subjected to organophosphate intoxication both chronically, in the form of pesticide exposure,[1] and acutely, in the form of chemical nerve gas agents.[2,3] Serum esterases, such as butyrylcholinesterase (BCE) or paraoxonase (PON)-1, inactivate organophosphates (OP).[4] PON1 organophosphatase activity is over 100-fold greater than BCE activity.[5] Recombinant PON1 is a potential new treatment for OP intoxication.[6–8] However, PON1 normally circulates bound to high density lipoprotein (HDL),[9] and, in the absence of extracellular lipoprotein, PON1 is not secreted by cells.[10] PON1 secretion by cells could be facilitated by the engineering and expression of IgG-PON1 fusion proteins, wherein the PON1 secretion is linked to the IgG secretion.

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New PON1 therapeutics for OP toxicity should be engineered to penetrate the central nervous system (CNS), following transport across the blood-brain barrier (BBB). Most OP molecules are low molecular weight, lipid soluble compounds that rapidly cross the BBB to enter the CNS. Moreover, the CNS is the principal site of action of the lethal effects of acute OP intoxication. The role of the CNS is implicated in several studies. Apnea and hypotension secondary to central muscarinic cholinergic stimulation by OPs occurs while diaphragm contractions can still be elicited by stimulation of the phrenic nerve.[11] Acetylcholinesterase (ACE) inhibitors, e.g., pyridostigmine, that are quaternary ammonium compounds, which do not cross the BBB, are less effective antidotes against OP toxicity as compared to tertiary amine ACE inhibitors, e.g., physostigmine, which do cross the BBB.[12] Muscarinic cholinergic receptor inhibitors, e.g., glycopyrrolate, that are quaternary ammonium compounds, which do not cross the BBB, are less effective antidotes against OP toxicity as compared to tertiary amine receptor blockers, e.g., atropine, which do cross the BBB.[13]

A large molecule such as PON1 does not cross the BBB. However, PON1 can be re-engineered to cross the BBB using molecular Trojan horse technology.[14] A molecular Trojan horse is an endogenous peptide, or peptidomimetic monoclonal antibody (MAb), that crosses the BBB via endogenous receptor-mediated transport. A MAb against the human insulin receptor (HIR) has been genetically engineered and shown to rapidly cross the primate BBB in vivo.[15] Similarly, genetically engineered fusion proteins of the HIRMAb also cross the BBB in vivo.[16–18] Therefore, the purpose of the present investigation was to examine the feasibility of engineering, expressing, and validating a fusion protein of the chimeric HIRMAb and human PON1. The amino terminus of human PON1 is fused to the carboxyl terminus of the heavy chain of the HIRMAb, as shown in Figure 1. This configuration places the PON1 in a dimeric conformation, which replicates the native state of PON1, which forms a homo-dimer.[19]

Methods and Materials

Cloning of human PON1 cDNA

The human PON1 cDNA (GenBank accession # NM_000446) corresponding to amino acids Met^{1}-Leu^{355} was cloned by the polymerase chain reaction (PCR) using the oligodexoynucleotides (ODNs) described in Table 1 and cDNA derived from reverse transcription of human liver PolyA+ RNA (Takeda/Clontech, cat# 6510–1). The forward (FWD) ODN primer introduces a Kozak sequence (i.e. CCCGACC) prior to the ATG initiation codon. The reverse ODN primer is complementary to the end of the open reading frame (orf) of PON1 plus 7 nucleotides of the 3'-untranslated region. The PON1 cDNA was cloned by PCR using 25 ng polyA+RNA-derived cDNA, 0.2 μM forward and reverse ODN primers (Table 1), 0.2 mM deoxynucleosidetriphosphates, and 2.5 U PfuUltra DNA polymerase (Stratagene, San Diego, CA) in a 50 μl Pfu buffer (Stratagene) containing 7% dimethylsulfoxide. The amplification was performed in a Mastercycler temperature cycler (Eppendorf, Hamburg, Germany) with an initial denaturing step of 95°C for 2 min followed by 30 cycles of denaturing at 95°C for 30 sec, annealing at 55°C for 30 sec and amplification at 72°C for 1 min; followed by a final incubation at 72°C for 10 min. PCR products were resolved in 0.8% agarose gel electrophoresis, and the expected major single band of ~1.1 kb corresponding to the human PON1 cDNA was produced (Figure 2A). The human PON1 cDNA was subcloned into the EcoRV site of the pcDNA3.1 eukaryotic expression plasmid, which was treated with alkaline phosphatase to prevent self ligation, and this PON1 expression plasmid is designated pCD-PON1. The engineering of this plasmid was validated by DNA sequencing in both directions, and by expression of PON1 in COS cells transfected with pCD-PON1.

Engineering of HIRMAb-PON1 expression vector

For the engineering of the pHIRMAb-PON1 heavy chain (HC) expression plasmid, the mature human PON1 cDNA corresponding to amino acids Met^{1}-Leu^{355} was cloned by PCR using the pCD-PON1 as template. The ODNs used for PCR are 5'-phosphorylated for direct insertion into the HpaI site of the pHIRMAb-HC expression plasmid (Figure 2B), as described previously.[16] The pHIRMAb-HC plasmid encodes the HC of the chimeric HIRMAb, and dual transfection of COS cells with this plasmid and a light chain (LC) expression plasmid, pHIRMAb-LC, allows for transient expression of either the chimeric HIRMAb, or a fusion protein, in COS cells. The mature human PON1 forward PCR primer introduces "CA" nucleotides to maintain the open reading frame and to introduce a Ser-Ser linker between the carboxyl terminus of the CH3 region of the HIRMAb HC and the amino terminus of the PON1. The PON1 reverse PCR primer (Table 1) introduces a stop codon, "TGA," immediately after the terminal leucine of the mature PON1 protein. The engineered pHIRMAb-PON1 expression vector was validated by DNA sequencing.

The HIRMAb HC and LC cDNA expression cassettes are driven by the cytomegalovirus (CMV) promoter and contain the bovine growth hormone (BGH) polyadenylation (pA) sequence (Figure 2B). The engineering of the universal pHIRMAb-HC vector was performed by insertion of a single HpaI site at the end of the HIRMAb HC CH3 open reading frame (orf) by site directed mutagenesis (SDM), as described previously.[16]

Site-Directed Mutagenesis

The cloned human PON1 expressed Met-55 and Arg-192. The more active PON1 allozyme was produced with site-directed mutagenesis, as described previously.[16] The SDM produced the following PON1 allozymes: Leu-55/Arg-192 and Leu-55/Gln-192. The SDM was verified by bi-directional DNA sequencing.

Transient expression of HIRMAb-PON1 fusion protein in COS cells

COS cells were dual transfected with pHIRMAb-LC and pHIRMAb-HC-PON1 using Lipofectamine 2000, with a ratio of 1:2.5, ug DNA:uL Lipofectamine 2000. Following transfection, the cells were cultured in serum free VP-SFM (Invitrogen, Carlsbad, CA), with or without 1% Ex-Cyte (Cellianca, Kankakee, IL), a lipoprotein supplement. The conditioned serum free medium was collected at 3 and 7 days. For screening assays, the COS cells were plated in 6-well cluster dishes. For production assays, the COS cells were plated in 10xT500 flasks with a proportionate scale-up of the Lipofectamine 2000 and the plasmid DNA.

Protein A affinity chromatography

Approximately 2 liters of serum free conditioned medium was collected over 7 days from 10xT500 flasks of COS cells dual lipofected with the pHIRMAb-HC-PON1 and pHIRMAb-LC plasmids. This medium was reduced to a 400 mL volume with tangential flow filtration, and the HIRMAb-PON1 fusion protein was purified by affinity chromatography with a 5 mL column of protein A Sepharose 4 Fast Flow (Amersham, Chicago, IL). The protein A column was equilibrated with buffer containing 1 mM CaCl2, and the fusion protein was eluted with 0.1 M sodium acetate/pH=3.7/1 mM CaCl2, followed by neutralization with 1 M Tris base. The neutralized acid eluate was concentrated and buffer exchanged with 0.01 M Tris/0.15 M NaCl/pH=7.4/1 mM CaCl2 with an Ultra-15 concentrator (Millipore, Bedford, MA), and stored at −20C. The protein content was measured with the bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL).

Human IgG ELISA

Human IgG ELISA was performed in Immulon-2 high binding plates (Dynex Tech., Chantilly, VA) with COS cell conditioned medium. A goat anti-human IgG primary antibody (Zymed-Invitrogen, Carlsbad, CA) was plated in 0.1 M NaHCO3 (100 μl, 2 μg/ml) and incubated for overnight at 4C. Plates were washed 0.01 M Na2HPO4/0.15 M NaCl/pH=7.4/0.05% Tween-20 (PBST), and blocked with 1% gelatin in PBST for 30 min at 22°C. Plates were incubated with 100 uL/well of either human IgG1 standard or the fusion protein for 60 minutes at room temperature (RT). After washing with PBST, a goat anti-human kappa LC antibody conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was plated for 60 min at 37°C. Color development was performed with p-nitrophenyl phosphate (Sigma) at pH=10.4 in the dark. The reaction was stopped with NaOH, and absorbance at 405 nm was measured in a BioRad ELISA plate reader.

Western blotting

The immunoreactivity of the HIRMAb-PON1 fusion protein was measured for both the human IgG part and the human PON1 part of the molecule. For human IgG Western blotting, the primary antibody was a goat anti-human IgG (H+L) antiserum from Vector Labs (Burlingame, CA), and binding was detected with a biotinylated horse anti-goat IgG (Vector Labs). For human PON1 Western blotting, the primary antibody was a mouse monoclonal antibody against human PON1 from Abcam (Cambridge, MA), and binding was detected with a biotinylated horse anti-mouse IgG (Vector Labs). Human IgG1 standard was from the Sigma Chemical Co. (St. Louis, MO), and the human PON1 standard was from American Research Products, Inc. (Belmont, MA).

HIR receptor assay

The affinity of the fusion protein for the HIR extracellular domain (ECD) was determined with an ELISA using the lectin affinity purified HIR ECD. CHO cells permanently transfected with the HIR ECD were grown in serum free media (SFM), and the HIR ECD was purified with a wheat germ agglutinin affinity column, as previously described.[20] The HIR ECD (0.2 ug/well) was plated on Immulon-2 high binding 96-well plates, and the binding of the chimeric HIRMAb, or the HIRMAb-PON1 fusion protein to the HIR ECD was detected with a biotinylated goat anti-human IgG (H+L) antibody (0.3 ug/well), and the ABC Elite detection system (Vector Labs). The concentration that caused 50% binding to the HIR ECD, the ED50, was determined by non-linear regression analysis using the WinNonlin software.

PON1 enzyme assay

PON1 enzyme activity was measured with a fluorometric assay using diethylphospho-6,8-difluoro-4-methyl-umbelliferone (DEPFMU) as the substrate,[21] which was custom synthesized by Molecular Probes-Invitrogen (Carlsbad, CA). The assay buffer was 0.02 M Tris/pH=8.0/0.15 M NaCl/2 mM CaCl2. The standard curve (3 to 300 pmol/tube) was generated with the reaction product, 6,8-difluoro-4-methylumbelliferone (Invitrogen). Human plasma was used as a positive control. Fluorometric readings were obtained with a Farrand filter flurometer using a filter with an emission wavelength of 460 nm and a filter with an excitation wavelength of 355 nm. Enzyme activity is reported as nmol/hour/mL. Enzyme activity was measured after both 20 and 40 min incubations, and was linear with respect to time of incubation.

Results

DNA sequencing of the expression cassette of the pCD-PON1 encompassed 2,196 nucleotides (nt), including a 714 nt CMV promoter, a 6 nt Kozak sequence, a 1,068 nt PON1 open reading

frame, and a 408 nt BGH sequence, which produced a 355 amino acid human PON1 protein, including the amino terminal Met, with 100% identity with the known sequence for human PON1 (NM_000446, AAB25717). The predicted molecular weight, minus glycosylation, of the PON1 was 39,773 Da with an isoelectric point (pI) of 5.15. The sequence analysis indicated the PCR cloned human PON1 was the Met-55/Arg-192 allozyme. SDM was used to convert the Met-55 residue to the more active Leu-55 (Methods).

Transfection of COS cells with pCD-PON1 resulted in an increase in PON1 enzyme activity in the medium, and the addition of 1% ExCyte lipid supplement resulted in a 19-fold increase in medium PON1 enzyme activity (Table 2). The medium PON1 enzyme activity was comparable to PON1 enzyme activity in 10% human plasma (Table 2).

The cDNA corresponding to the 355 amino acid PON1/Leu-55/Arg-192 was amplified by PCR using custom ODNs and the pCD-PON1 as template, and this cDNA was subcloned into the HpaI site of the pHIRMAb-HC plasmid, as outlined in Figure 2B. DNA sequencing of the expression cassette of the pHIRMAb-PON1 plasmid encompassed 3,560 nt, including a 714 nt CMV promoter, a 9 nt full Kozak site (GCCGCCACC), a 2,460 nt HIRMAb HC-PON1 fusion protein open reading frame, and a 303 nt BGH polyA sequence. The plasmid encoded for a 819 amino acid protein, comprised of a 19 amino acid IgG signal peptide, the 443 amino acid HIRMAb HC, a 2 amino acid linker (Ser-Ser), and the 355 amino acid human PON1. The predicted molecular weight of the heavy chain fusion protein, minus glycosylation, is 88,553 Da, with a predicted pI of 6.31.

Dual transfection of COS cells with the pHIRMAb-PON1 and the pHIRMAb-LC resulted in the secretion of detectable human IgG in the medium, as determined with a human Fc specific ELISA. The addition of 1% lipid supplement (ExCyte) to the medium did not result in an increase in secretion of the HIRMAb-PON1 fusion protein, based on the IgG ELISA. The HIRMAb-PON1 fusion protein was purified by protein A affinity chromatography from serum free medium conditioned by dual transfected COS cells. On Western blotting, the LC of either the HIRMAb or the HIRMAb-PON1 fusion protein react equally with a primary antibody directed against the human IgG (H+L), as shown in Figure 3A. The size of the HC of the fusion protein is about 40 kDa larger than the size of the HC of the HIRMAb on both Western blots using either the anti-human IgG primary antibody (Figure 3A) or the anti-human PON1 primary antibody (Figure 3B). The anti-PON1 primary antibody reacts with the HC of the fusion protein, and with recombinant PON1, but does not react with the HIRMAb (Figure 3B).

The affinity of the fusion protein for the HIR extracellular domain (ECD) was determined with a ligand binding assay using lectin affinity purified HIR ECD (Methods). There is comparable binding of either the chimeric HIRMAb or the HIRMAb-PON1 fusion protein for the HIR ECD with ED50 of 0.55±0.07 nM and 1.1±0.1 nM, respectively (Figure 4).

The PON1 enzyme activity of the protein A purified HIRMAb-PON1/Leu-55/Arg-192 fusion protein is shown in Table 3, in comparison with the PON1 enzyme activity in 20% human plasma. PON1 activity for the fusion protein is linear with respect to incubation time and concentration (Table 3). The PON1 enzyme activity of the HIRMAb-PON1/Leu-55/Arg-192 fusion protein, at a concentration of 68 μg/mL, is comparable to the PON1 enzyme activity in 20% human plasma (Table 3). The PON1 enzyme activity against substrates such as paraoxon is higher for the Arg-192 allozyme, as compared to the Gln-192 allozyme, although enzyme activity against chemical nerve gas agents is higher for the Gln-192 allozyme.[22] Therefore, SDM was used to convert the Arg-192 residue to the Gln-192 in the HIRMAb-PON1 fusion protein (Methods). Similar to paraoxon, the Arg-192 HIRMAb-PON1 allozyme has a higher enzyme activity against DEPFMU, the substrate used for the fluorometric assay (Table 4).

Discussion

The results of this study are consistent with the following conclusions. First, a bi-functional IgG-PON1 fusion protein has been genetically engineered, wherein human PON1 is fused to the carboxyl terminus of the heavy chain (HC) of a chimeric HIRMAb (Figure 1), and expressed and secreted in COS cells without lipid acceptor molecules (Results). Second, the HIRMAb-PON1 fusion protein is correctly processed in COS cells, resulting in the production of the expected size of the heavy chain of the fusion protein on IgG or PON1 Western blotting (Figure 3). Third, the HIRMAb-PON1 fusion protein is bi-functional and binds the HIR (Figure 4), and exhibits PON1 enzyme activity (Table 3). Fourth, the organophosphatase activity against DEPFMU of the Arg-192 fusion protein allozyme is higher, as compared to the Gln-192 fusion protein allozyme (Table 4).

The level of PON1 enzyme activity in the serum free medium conditioned by COS cells transfected with the pCD-PON1 plasmid was increased nearly 20-fold by the addition to the medium of 1% ExCyte, a lipoprotein supplement (Table 2). This observation parallels other reports that PON1 is secreted poorly by transfected eukaryotic cells, in the absence of a lipid acceptor.[10] The finding that PON1 secretion is enhanced by the addition of a lipoprotein supplement to the medium (Table 2) suggests that binding of PON1 to lipoproteins is an obligatory requirement for PON1 secretion by cells. In contrast, the addition of ExCyte to the medium has no effect on the secretion of the HIRMAb-PON1 fusion protein (Results). Therefore, fusion of PON1 to an IgG molecule, which is normally secreted by cells, provides an alternative pathway for secretion of PON1 by transfected cells. Similarly, the HIRMAb-PON1 fusion protein is secreted to the medium without the requirement for lipid acceptors in the medium by permanently transfected Chinese hamster ovary (CHO) cells (unpublished observation).

Following transfection of COS cells, the HIRMAb-PON1 fusion protein heavy and light chains are correctly assembled, as demonstrated by the Western blotting of protein A affinity purified fusion protein (Figure 3). An anti-human IgG (H+L) primary antibody reacts with the light chain, and heavy chain, of both the chimeric HIRMAb and the HIRMAb-PON1 fusion protein, but not with PON1 (Figure 3A). Conversely, an anti-human PON1 primary antibody reacts with recombinant PON1, and the heavy chain of the HIRMAb-PON1 fusion protein, but not with the heavy chain of the chimeric HIRMAb (Figure 3B). There is comparable binding to the HIR extracellular domain of the chimeric HIRMAb and the HIRMAb-PON1 fusion protein (Figure 4). The high affinity of the HIRMAb-PON1 fusion protein for the HIR is attributed to the fusion of the PON1 at the carboxyl termini of the HIRMAb heavy chains (Figure 1). In contrast, the HIR binding sites on the HIRMAb-PON1 fusion protein are formed by the complementarity determining regions, which are located at the amino terminal portion of the HIRMAb-PON1 fusion protein.

The PON1 enzyme activity of the HIRMAb-PON1 fusion protein was measured in these studies with the fluorometric assay, which uses DEPFMU as the organophosphate substrate.[21] PON1 enzyme activity is typically measured with paraoxon as the substrate using a spectrophotometric assay of human plasma samples.[23] However, given the limited production of the HIRMAb-PON1 fusion protein by transiently transfected COS cells, the more sensitive fluorometric assay was used in these studies. Human plasma was used as a positive control in the PON1 fluorometric assay. The PON1 enzyme activity of 20% human plasma was 63.5 ± 1.4 nmol/hr/mL (Table 3). Since the concentration of PON1 in human plasma is about 50 ug/mL,[24] the human plasma PON1 specific activity against the DEPFMU substrate is 6.3 nmol/hr/ug enzyme, as determined with the fluorometric assay. The PON1 specific activity against paraoxon in human plasma is 114 nmol/hr/ug enzyme, as determined with the spectrophotometric assay.[24] Therefore, the PON1 activity against DEPFMU is lower as

compared to paraoxon, and this observation parallels other reports showing a reduced catalytic rate constant for DEPFMU as compared to paraoxon for organophosphorus hydrolase.[21] The PON1 enzyme activity of the HIRMAb-PON1/Leu-55/Arg-192 fusion protein is 165 ± 4 nmol/hr/μg (Table 3); therefore, the PON1 enzyme specific activity of the HIRMAb-PON1 fusion protein is 0.6 nmol/hr/ug protein. Since the molecular weight of the HIRMAb-PON1 fusion protein is about 3-fold greater than the PON1 homo-dimer, the PON1 enzyme specific activity of the HIRMAb-PON1 fusion protein is about 25% of the enzyme activity of the native PON1 in human plasma.

The PON1 enzyme exists as different allozymes with natural Met/Leu and Arg/Gln polymorphisms at amino acids 55 and 192, respectively.[22] Enzyme activity is higher for the Leu-55 allozyme, as compared to the Met-55 allozyme,[25] and the HIRMAb-PON1 fusion protein described in these studies was engineered as the Leu-55 allozyme. With respect to position 192, HIRMAb-PON1 fusion proteins were engineered with either the Arg-192 or the Gln-192 polymorphism. The PON1/Arg-192 allozyme has a higher activity toward substrates such as paraoxon as compared to the PON1 /Gln-192 allozyme.[22] Similarly, the HIRMAb-PON1/Arg-192 allozyme has a higher activity toward DEPFMU as compared to the HIRMAb-PON1/Gln-192 allozyme (Table 4). However, the Gln-192 allozyme may be the more potent therapeutic form of the fusion protein. The Gln-192 polymorphism confers on PON1 both a higher enzyme activity against chemical nerve gas agents,[22] and a higher anti-atherogenic effect of the enzyme.[26]

In summary, these studies describe a new IgG-PON1 fusion protein that is bi-functional, and both binds the HIR, to induce transport across the human BBB, and has PON1 organophosphatase enzyme activity (Figure 1). PON1 is the most potent human protein with organophosphatase activity.[5] Re-engineering of human PON1 as a fusion protein with the HIRMAb not only allows for CNS targeting of the enzyme, but also the secretion of the enzyme by transfected host cells without lipid acceptors.

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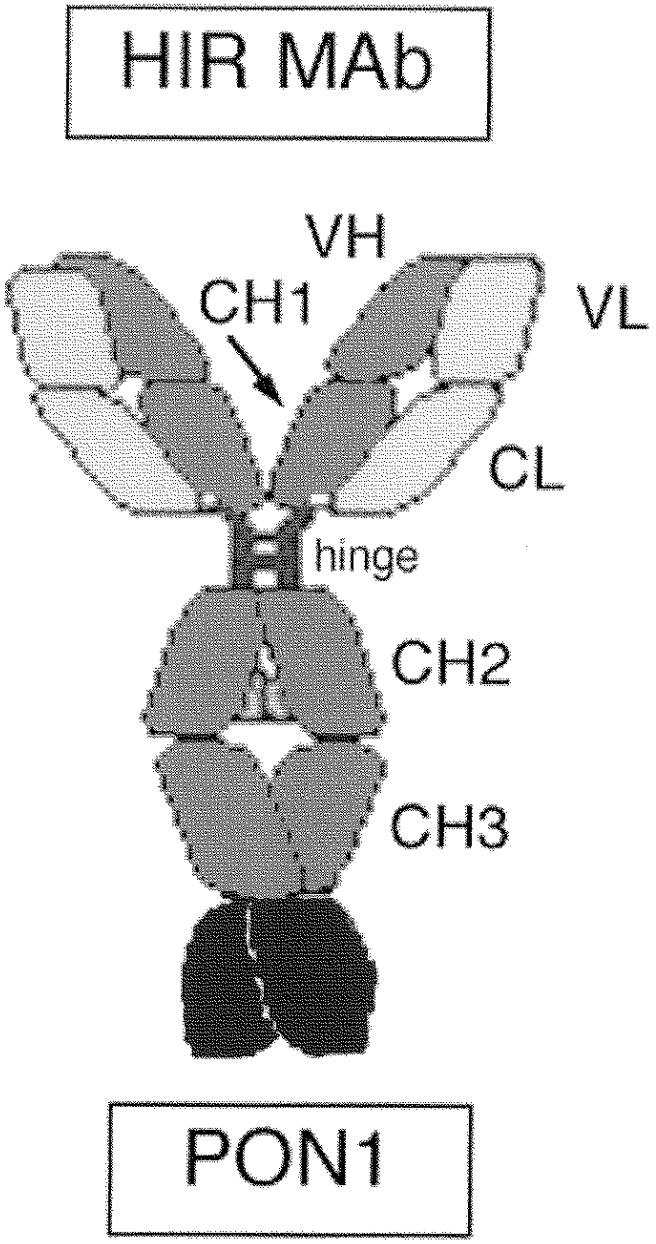

Figure 1.
The HIRMAb-PON1 fusion protein is formed by fusion of the amino terminus of human PON1 to the carboxyl terminus of the CH3 region of the heavy chain of the chimeric HIRMAb. The fusion protein is a bi-functional molecule: the fusion protein binds the HIR, at the BBB, to mediate transport into the brain, and has PON1 organophosphatase enzyme activity. VH=variable region of heavy chain; VL=variable region of light chain; CL=constant region of light chain; CH1, hinge, CH2, and CH3 are sub-domains of the constant region of the heavy chain of the HIRMAb.

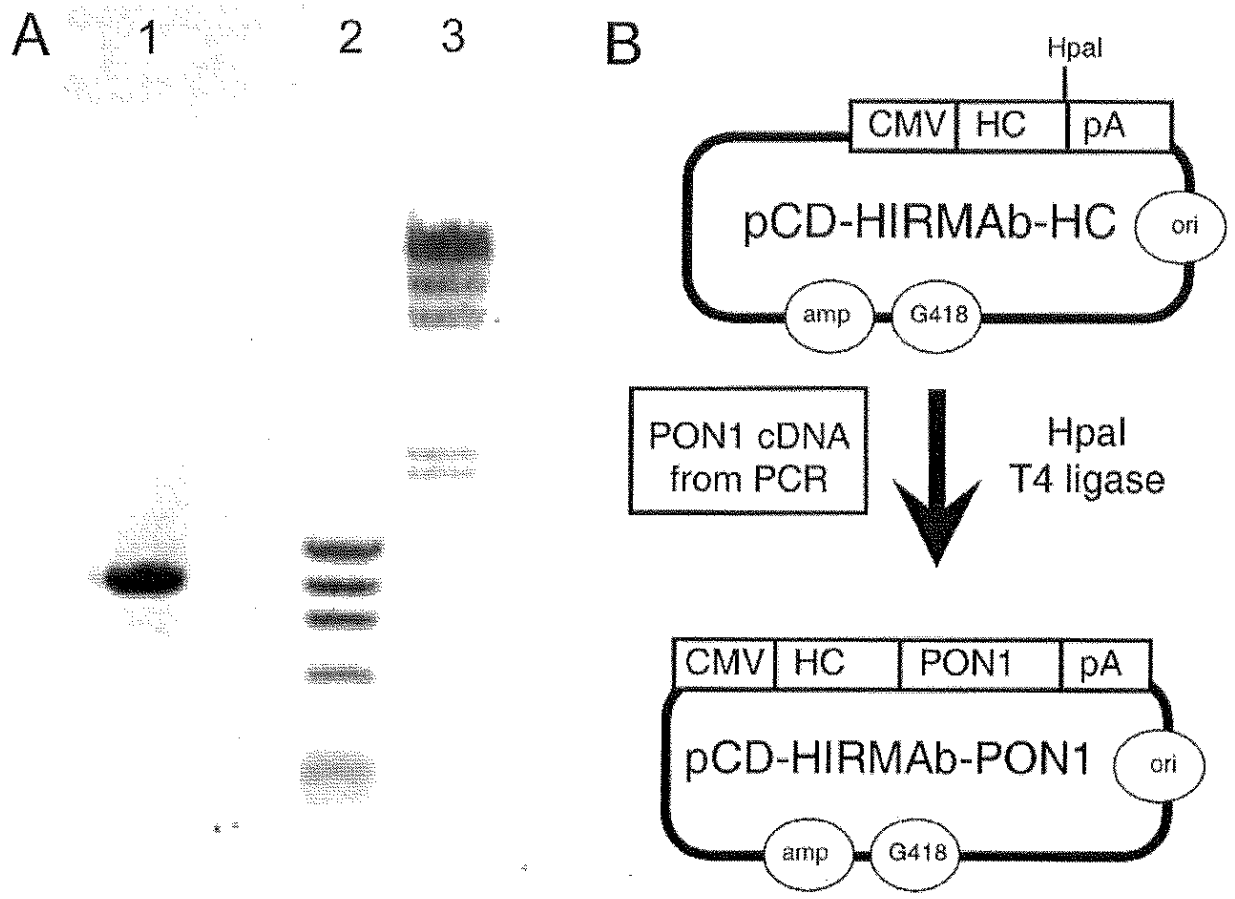

Figure 2.

(A) Ethidium bromide stain of agarose gel of human PON1 cDNA (lane 1), which was produced by PCR from cDNA produced by reverse transcription of RNA from human liver, and PON1-specific ODN primers (Table 1). The expected band of ~1.1 kb band corresponding to the human PON1 cDNA is shown in lane 1. DNA standards are PhiX174 HaeIII digested DNA, 1.4, 1.0, 0.8, 0.6, 0.3-0.1 (lane 2); and Lambda HindIII digested DNA, 23, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kb (lane 3). (B) Genetic engineering of pHIRMAb-PON1, the eukaryotic expression plasmid encoding the fusion protein of PON1 and the heavy chain (HC) of the chimeric HIRMAb. The fusion gene is 5'-flanked by the cytomegalovirus (CMV) promoter and 3'-flanked by the bovine growth hormone polyA (pA) sequence.

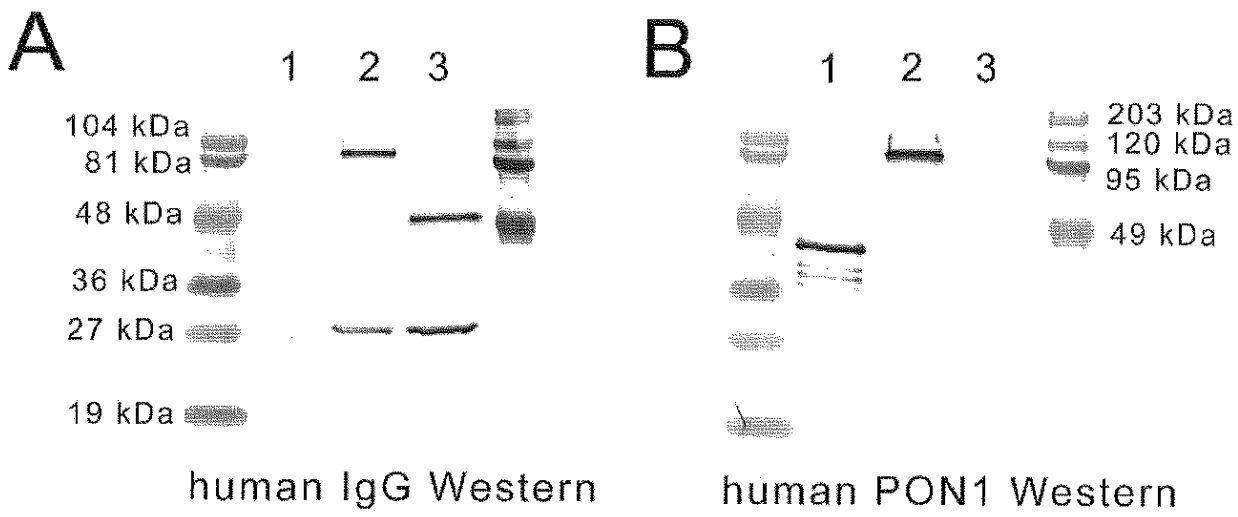

Figure 3.

Western blot with either anti-human IgG primary antibody (A) or an anti-human PON1 primary antiserum (B). The immunoreactivity of the HIRMAb-PON1 fusion protein (lane 2) is compared to the chimeric HIRMAb (lane 3) and to recombinant PON1 (lane 1). Both the HIRMAb-PON1 fusion protein and the HIRMAb have identical light chains on the anti-hIgG Western. The HIRMAb-PON1 fusion heavy chain reacts with both the anti-IgG and the anti-human PON1 antibody, whereas the HIRMAb heavy chain only reacts with the anti-IgG antibody. The size of the HIRMAb-PON1 fusion heavy chain, 95 kDa, is about 40 kDa larger than the size of the heavy chain of the HIRMAb, owing to the fusion of the 40 kDa PON1 to the 55 kDa HIRMAb heavy chain.

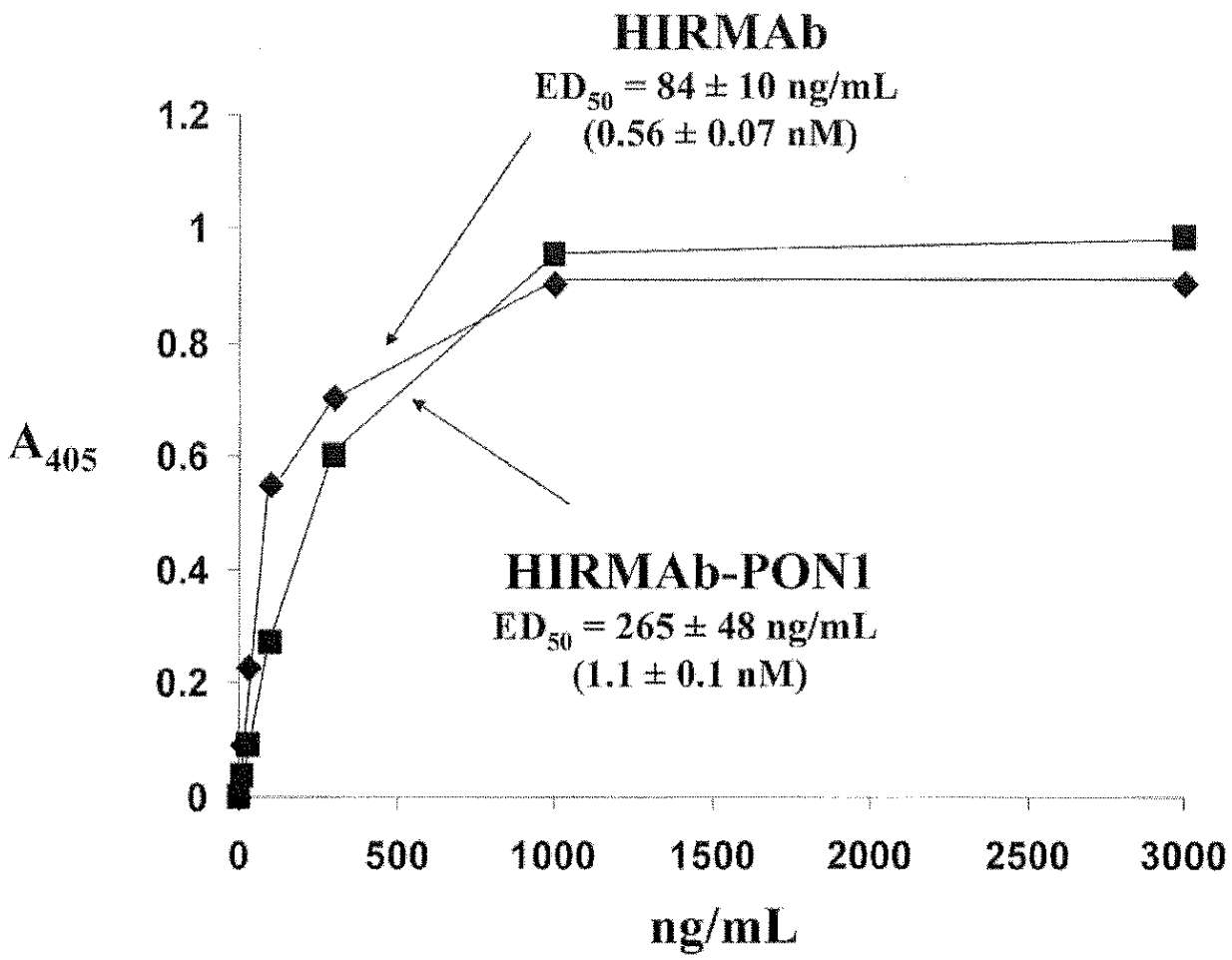

Figure 4.
Binding of either the chimeric HIRMAb or the HIRMAb-PON1 fusion protein to the HIR extracellular domain (ECD) is saturable. The ED50 of HIRMAb-PON1 binding to the HIR ECD is comparable to the ED50 of the binding of the chimeric HIRMAb.

Table 1

Oligodeoxynucleotide primers used in the RT-PCR cloning of human PON1

PON1 FWD: 5'-phosphate-CCCGACCATGGCGAAGCTGATTG
PON1 REV: 5'-phsphate-CGGTCTGTTAGAGCTCACAGTAAAG

Table 2

PON enzyme activity in the medium of COS cells following transfection with pCD-PON1

Sample	PON1 enzyme activity (nmol/hr/mL)	
	0% ExCyte	1% ExCyte
COS medium, pCD-PON1	1.24 ± 0.01	23.3 ± 0.1
COS medium, Lipofectamine 2000	0.26 ± 0.02	0.29 ± 0.01
Human plasma (10%)	41.3 ± 3.1	n.m.

Mean ± SE (n=3 dishes per point); n.m.=not measured. Clone pCD-PON1 produces the Met-55/Arg-192 allozyme; serum free medium was harvested 7 days after transfection of COS cells with pCD-PON1.

Table 3

PON1 enzyme activity of protein A purified HIRMAb-PON1 fusion protein

Enzyme source	Enzyme assay units		PON1 enzyme activity (nmol/hr/mL or nmol/hr/ug)
	20 min	40 min	
Human plasma			
20%	140 ± 2	273 ± 1	63.5 ± 1.0
HIRMAb-PON1/Leu-55/Arg-192			
270 μg/mL	492 ± 6	999 ± 10	165 ± 4
68 μg/mL	117 ± 2	233 ± 1	156 ± 4

Fluorometric assay units were converted into enzyme activity based on the standard curve (Methods). The units of human plasma PON1 enzyme activity are nmol/hr/mL; the units of HIRMAb-PON1 enzyme activity are nmol/hr/ug protein.

Table 4

PON1 enzyme activity of protein A purified HIRMAb-PON1/Arg-192 and HIRMAb-PON1/Gln-192 fusion protein allozymes

Enzyme source	Assay units		
	10 min	20 min	40 min
HIRMAb-PON1/Leu-55/Arg-192			
270 μg/mL	184 ± 1	362 ± 2	717 ± 4
135 μg/mL	92 ± 1	183 ± 2	363 ± 2
68 μg/mL	45 ± 2	92 ± 1	181 ± 2
HIRMAb-PON1/Leu-55/Gln-192			
500 μg/mL	100 ± 2	200 ± 1	404 ± 2
250 μg/mL	52 ± 1	101 ± 2	203 ± 2
125 μg/mL	26 ± 2	52 ± 1	101 ± 2

５．　農薬の吸入毒性等に係る情報の収集及び文献調査の結果

５．１　欧州食品安全機関（EFSA：European Food Safety Authority）ガイダンスドキュメント翻訳（要約部分）

圃場労働者，散布作業者，通行者および近隣住民の農薬ばく露評価に関するガイダンス文書の作成
農薬および残留物に関する委員会の科学的意見書
（Question No. EFSA-Q-2008-261）

委員会メンバー
Damia Barcelo Culleres, Jos Boesten, Claudia Bolognesi, Alan Boobis, Arne Büchert Ettore Capri, David Coggon, Anthony Hardy, Andy Hart, Hervert Köpp, Matthias Liess, Robert Luttik, Otto Meyer, Stella Michaelidou-Canna, Mark Montforts, Angelo Moretto, Markus Müller, Bernadette Ossendorp, Walter Steurbaut, Maria Tasheva and Christiane Vleminckx

要約
欧州食品安全機関（EFSA）は，通常のリスク評価に用いる圃場労働者，散布作業者，通行者および近隣住民に対する農薬ばく露量評価に関するガイダンス文書を作成するため，農薬および残留物に関する委員会に諮問した。

意見書は，英国病害虫安全局（PSD）および Ghent 大学（UG）が合同で実施した外部発注プロジェクトに端を発しており，このプロジェクトは関連する情報源を体系的に考察ならびに評価している。さらに，重要なデータが見落とされないよう意見書の草案が 2009 年 8 月にパブリックコメントに出された。［コンサルテーションの結果の説明］

EC 規制 91/414 では，適切な植物防疫方法に準拠した施用による農薬（PPPs; plant protection products）の残留物には“ヒトや動物の健康に悪影響”があってはならないことを求めている。現在，散布作業者，圃場労働者，通行者および近隣住民のリスク評価には，合理的な毎日の全身ばく露量の上限推定値が，関連する毒性参照値，許容作業者ばく露量（AOEL）未満であることを確認する決定論的方法を用いている。得られているデータにおいて，現在の散布作業者，圃場労働者，通行者および近隣住民に対するリスク評価方法に大きな不具合は示されなかった。

しかし，現在のリスク評価方法は完全に満足できるものではない。いくつかのばく露シナリオでは推定ばく露量を裏付ける実証データが少なく，推定値は統計学的信頼性に乏しい。その他には，どのばく露を評価するか，どのばく露場所について行うか

によってばく露評価に 1 以上のモデルが利用できることから，規制当局に承認されたアプローチ間で矛盾が生じることがある。さらに，実証データセットの 50 ないし 75 パーセンタイルのばく露値が 1 日のうちに合理的に起こり得る最大ばく露を実質的に過小評価している可能性があり，急性毒性を有する農薬（PPPs）の安全許容範囲を危うくしている。

そのため，ガイダンス文書の作成にあたり PPR（plant protection products and their residues）委員会により現行の規定に対しての変更が何度も提案された。個別農薬の通常のリスク評価は従来の決定論的方法を用いることおよびばく露評価の段階的アプローチが適していることが提案された。しかし，農薬が急性毒性を有する場合に散布作業者，圃場労働者および通行者の急性リスク評価をさらに導入することについて活発に議論された。これにより，急性毒性を有する農薬の経口摂取によるリスク評価に用いる急性参照用量に類似した別の毒性参照値，“急性 AOEL”（“AAOEL”）が要求されるであろう。長期リスク評価では 75 パーセンタイルが始点となるが，急性リスク評価では，関連するデータセットの 95 パーセンタイルに基づきばく露を推定するべきである。

さらに，データセットが少ないパーセンタイルの統計学的不確実性を考慮し，測定全体の分布が対数正規であると仮定した場合，リスク評価に用いるばく露値は初期値として a）関連するデータセットにおける適切なパーセンタイルまたは b）データセットが由来する理論的母集団の測定において対応するパーセンタイルのパラメータによる推定値の高いほうのいずれかを採用することを提案する。しかし，対数正規であるという仮定が不合理であることを確信することができるような証拠がある場合，ケースバイケースで代替的な方法を適用できるよう規制はオープンとすべきである。

推奨される方法を適用し，規制実施にあたり最もよく合う各ばく露シナリオについて第一段階のリスク評価における標準化されたばく露量推定法を提案している。リスク管理の要素は，この分類のいずれのスキームにおいても絶対的なものである。本提案を立案するにあたり，PPR 委員会は現在適用している予防措置と同様あるいは若干高いレベルを目指している。しかし，リスク管理者が希望する場合予防策のレベルを修正することができる（例えば，ばく露推定値の基にするパーセンタイルの変更）。

意見書ではばく露推定値が最低限満足されるようなシナリオを特定し，現在の不確実性を軽減させる更なる研究を推奨している。

最後に，付表に，委員会がガイダンス文書の草案を示した。ここにはリスク管理者によりガイダンス文書の最終版が同意されたら，要求される計算を容易にするワークシートを開発することを提案している。

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