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原　著

0，1，6カ月皮内接種方式による狂犬病抗体価の検討

Yanagisawa Naoki 柳澤　如樹[1)]　Takayama Naohide 髙山　直秀[2)]　Nakayama Eiichi 中山　栄一[2)]　Mannen Kazuaki 万年　和明[3)]
Suganuma Akihiko 菅沼　明彦[1)]

目　的

2006年11月に国内で相次いで輸入狂犬病患者が発生したのち[1,2)]，狂犬病ワクチンの需要が急増した．しかし，需要の増加が供給を上回っていたため，緊急避難的に狂犬病曝露前免疫が制限された．世界保健機関(WHO)は，狂犬病流行地において動物による咬傷を受けた場合，狂犬病免疫グロブリン(RIG)の投与と組織培養不活化ワクチン接種による曝露後発病予防を勧告しているが，曝露前免疫を受けていればRIGの投与が不要になる[3)]．RIGは入手が容易でないため，曝露後発病予防を確実なものにするためには，狂犬病曝露前免疫を普及させることが重要である．わが国における狂犬病ワクチンの生産量は少なく，急な増産ができないことに鑑みれば，ワクチンが不足する事態に備えて，少量のワクチンでも高い効果を上げることができる接種法の検討が必要である．

狂犬病常在地であるタイでは，1人当たりの狂犬病ワクチン接種量を減量した皮内接種法(タイ赤十字方式)が広く用いられ，曝露前免疫にも採用されている[4)]．われわれは，国産の狂犬病ワクチンを4週間隔で2回皮内接種する方式で，発症防御レベルまで狂犬病抗体価の上昇が認められたことを報告した[5)]．今回，日本の標準的な狂犬病ワクチンの接種時期である0，1，6カ月目にワクチンを皮内接種し，狂犬病曝露前免疫の効果と安全性を調査した．

1）がん・感染症センター都立駒込病院感染症科　2）同小児科　3）大分大学総合科学研究支援センター

対象と方法

1．対　象

本調査の目的，調査項目，接種ワクチンと予測される副反応について文書，および口頭で説明をして，同意が得られた医療関係者9例(男性7例，女性2例；平均年齢30.1±4.3歳)を対象とした．

2．接種ワクチン

化学及血清療法研究所製の組織培養不活化狂犬病ワクチンロットRB02およびRB03を，第1回目および第2回目接種時に用いた．第3回目接種時には，ロットRB04およびRB05を用いた．狂犬病ワクチンは溶解液1 mLに溶解した後，その0.1 mLずつを左右前腕に皮内注射した．

3．局所および全身反応

全例について，皮内接種15分後の接種局所における膨疹，発赤を視診で確認し，瘙痒感の有無を質問した．さらに，次回接種時および採血時に前回注射による局所の腫脹，発赤，疼痛，瘙痒感の自覚症状の有無について問診した．

4．抗体検査

狂犬病ワクチンは0，1，6カ月目に接種し，採血は2回目接種2～3週間後と3カ月後，また3回目接種直前および接種2～3週間後の計4回行った．酵素抗体法(ELISA)による抗体価はPLATELIA™ RABIES KIT Ⅱ(Bio-Rad Laboratories，France)を，中和抗体価は迅速蛍光フォーカス抑制試験法(RFFIT)を用いて測定した．

表1　ELISA法を用いた抗体価の推移

症　例	2回目接種 2～3週間後 (EU/mL)	2回目接種 3カ月後 (EU/mL)	3回目接種 直　前 (EU/mL)	3回目接種 2～3週間後 (EU/mL)
1	1.1	0.5	<0.5	1.9
2	5.5	1.5	0.5	2.1
3	1.6	0.7	<0.5	3.1
4	1.0	<0.5	<0.5	2.0
5	3.7	0.5	<0.5	0.9
6	3.6	0.8	<0.5	3.8
7	5.5	0.9	0.5	13.6
8	3.3	0.9	<0.5	13.4
9	2.5	<0.5	<0.5	6.4
幾何平均	2.6			3.6
範　囲	1.0～5.5			0.9～13.6

表2　RFFIT法を用いた中和抗体価の推移

症　例	2回目接種 2～3週間後 (IU/mL)	2回目接種 3カ月後 (IU/mL)	3回目接種 直　前 (IU/mL)	3回目接種 2～3週間後 (IU/mL)
1	3.5	1.5	<0.5	1.5
2	6.0	1.5	0.7	2.0
3	2.6	1.5	0.7	2.6
4	4.6	0.7	<0.5	2.0
5	4.6	<0.5	0.7	0.7
6	7.9	1.2	0.7	2.6
7	13.7	1.5	0.7	6.0
8	4.6	2.0	0.7	18.0
9	4.6	0.7	<0.5	6.0
幾何平均	5.2			3.0
範　囲	2.6～13.7			0.7～18.0

結　　果

1．血中狂犬病抗体価

ELISA法を用いた抗体価の測定結果を表1に示す．ワクチンを2回接種した2～3週間後の抗体価は全例で上昇しており，幾何平均は2.6 EU/mL(1.0～5.5 EU/mL)であった．2回目接種3カ月後の抗体価は全例で低下しており，3回目接種直前には7例で0.5 EU/mL未満であった．3回目接種2～3週間後の抗体価は全例で上昇しており，幾何平均は3.6 EU/mL(0.9～13.6 EU/mL)であった．

RFFIT法を用いた中和抗体価の測定結果を表2に示す．ワクチンを2回接種した2～3週間後の抗体価は全例で上昇しており，幾何平均は5.2 IU/mL(2.6～13.7 IU/mL)であった．2回目接種3カ月後の抗体価は全例で低下しており，3回目接種直前には3例で0.5 IU/mL未満であった．3回目接種2～3週間後の抗体価の幾何平均は3.0 IU/mL(0.7～18.0 IU/mL)と上昇を認め，全例発症防御レベルの0.5 IU/mLを超えていた．症例5は3回目接種前後で抗体価の変化が認められなかった．

2．接種後の局所反応および全身症状

ワクチン接種15分後，局所の発赤を呈した者は1例，腫脹を認めた者は4例，疼痛を認めた者は0例，瘙痒感を認めた者は1例であった．局所の発赤は，数日間残ったと報告した者があったが，発熱，頭痛，倦怠感などの全身症状を報告した例はなかった．

考　察

本邦での狂犬病曝露前免疫は，組織培養不活化ワクチン1回量1.0 mLを4週間隔で2回，その後6～12カ月後に1回皮下注射する方式が標準である[6]．しかし，ワクチン供給不足の状況において，1人に1回量1.0 mLを投与すれば，短期間にワクチンが枯渇して，実際，狂犬病ワクチン接種が不可能となるであろう．そのため，われわれは以前，接種量を減量しても効果が得られる方法として皮内接種法を考案し，4週間隔で2回皮内接種することで十分な抗体価の上昇が認められたことを報告した[5]．今回の小規模接種試験では，狂犬病ワクチン2回接種後，時間経過とともに抗体価の低下が認められた．この傾向は，国産の狂犬病ワクチンを用いて，高山らが考案した皮内・皮下併用法(狂犬病ワクチン0.1 mLを左右前腕に1カ所ずつ皮内注射し，残りを上腕1カ所に皮下注射する接種法)でも同様に認められている[7]．しかし，狂犬病抗体価が発症防御レベルを満たさなくなった場合でも，3回目接種後に十分な抗体価の上昇が認められ，追加免疫効果が確認された．また，全身症状を呈する重篤な副反応は認められなかった．

今回の検討では，狂犬病ワクチン3回目接種後，ELISA法で測定した抗体価の上昇は全例で認められた．しかし，3回目接種後，RFFIT法を用いた中和抗体価の上昇は1例(症例5)で認められなかった．また，中和抗体価の幾何平均が2回目接種後よりも3回目接種後が低値であった．狂犬病抗体価は，中和抗体価を用いるのが標準的で，WHOは0.5 IU/mL以上を発症防御レベルと定めている．本例において，中和抗体価の上昇が認められなかった原因は明らかでないが，中和抗体価と強い相関をもつELISA法[8]での抗体価は上昇していたため，実際には中和抗体価は上昇していたのではないかと推測される．加えて，2回目接種3カ月後で中和抗体価が0.5 IU/mL未満であったにもかかわらず，3回目接種直前には0.7 IU/mLと上昇していた点に鑑みると，測定時における手技的な問題も否定できない．

わが国における狂犬病ワクチンの生産量は，定期接種のワクチンに比べれば非常に少なく，急激な需要の増大が起これば，狂犬病ワクチンが不足する事態は避けられない．しかし，狂犬病はいったん発病すると有効な治療法はなく，現時点では狂犬病ワクチンの投与が唯一の発病予防策である．加えて，狂犬病ワクチンによる曝露後発病予防の効果を確実にするためには，RIGが入手困難である以上，曝露前免疫を提供することが極めて重要である．今回検討した皮内接種方式は，接種量が通常の5分の1と少ないにもかかわらず，効果が認められ，かつ副作用も軽微であった．小規模接種試験であるため，今後はより多くの被接種者を対象として，さらなる検討を行う価値がある．

追　記

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Pre-Exposure Intradermal Rabies Vaccination Using Japanese Rabies Vaccine Following the Japanese Recommended Schedule

Naoki Yanagisawa[1], Naohide Takayama[2], Eiichi Nakayama[2], Kazuaki Mannen[3] and Akihiko Suganuma[1]

1) Department of Infectious Diseases, Tokyo Metropolitan Cancer and Infectious diseases Center Komagome Hospital
2) Department of Pediatrics, Tokyo Metropolitan Cancer and Infectious diseases Center Komagome Hospital
3) Institute of Scientific Research, Oita University

In November 2006, two consecutive imported rabies cases were reported in Japan. The demand for rabies vaccine has grown rapidly, resulting in a shortage of the vaccine. In order to prepare for the vaccine shortage, it is necessary to consider a method that is effective yet uses less amount of the vaccine. We examined the efficacy and safety of the Japanese rabies vaccine when administered intradermally following the Japanese recommended schedule. The intradermal method we tested uses only 20% of the vaccine dose required under the standard method, but every subject tested had a sufficient rise in their anti-rabies antibody titer. Overall, the vaccine was well tolerated by all subjects. This was a small inoculation trial, but intradermal vaccination is an effective method, and may be used on a regular basis not only when vaccine is short.

原　著

Q熱コクシエラのマヨネーズおよびその構成成分中における生残性

福士秀人[1)†]　井上和幸[2)]　西藤　琳[1)]　大屋賢司[1)]
指原信廣[2)]　山口剛士[3)]　平井克哉[4)]

1）岐阜大学応用生物科学部（〒501-1193　岐阜市柳戸1-1）
2）キユーピー㈱研究所基盤技術センター（〒183-0034　府中市住吉町5-13-1）
3）鳥取大学農学部附属鳥由来人獣共通感染症疫学研究センター（〒680-8553　鳥取市湖山町南4-101）
4）天使大学看護栄養学部（〒065-0013　札幌市東区北13条東3-1-30）

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要　約

マヨネーズおよびその原材料中におけるコクシエラ菌の生残性を検討した．コクシエラの感染性はマヨネーズ中で時間経過とともに減少し，室温1週間では100分の1以下になった．マヨネーズの構成成分のうち酢酸では0.5％から2％，1週間では感染性に変化はなかった．卵白では感染性が減少する傾向がみられた．また，64℃7分間の加熱ではリン酸緩衝生理食塩水中で10分の1，卵黄中では100分の1に感染性が減少した．これらの結果からマヨネーズないし構成成分にコクシエラが混入したとしても，予想される汚染菌量や通常の流通過程を考慮すると，製造後7日以内に感染性が消失すると考えられる．
――キーワード：コクシエラ，マヨネーズ，生残性．
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Q熱はコクシエラ菌（*Coxiella burnetii*）による熱性の人と動物の共通感染症である．欧米では家畜の流産および非加熱乳製品を感染源とした発生がみられている．コクシエラ菌の感染は多くの動物種でみられ，牛，ヤギ，羊，犬，猫，霊長類，野生げっ歯類，は虫類，両生類，鳥類をはじめ多くのダニが上げられる．動物における慢性感染では子宮および乳腺が感染部位となる．感染動物の出産では胎盤に多量のコクシエラ菌が含まれ，感染源となる．また，乳汁中にもコクシエラ菌が排泄されることがわかっている．しかしながらわが国の動物におけるコクシエラ菌感染症の実態は明確ではない．

人のコクシエラ菌感染症であるQ熱はわが国では感染症法において四類に指定され，全数届け出となっている．感染症法が施行された1999年から数年は20から30例の届け出がなされたが，最近数年の届け出数は一桁台と非常に少ない．また，各症例における感染源は不明である．

わが国にQ熱の感染源の一つとして卵が示唆された[1]，しかし，この報告における実験結果は追試によって否定された．すなわち，市販の鶏卵の調査においてコクシエラ汚染は検出されなかった[2, 3]．さらに汚染食品としてあげられたマヨネーズについても，当初の報告よりも多数の検体を調査したにも関わらず汚染はまったく検出されなかった[4]．このようにわが国における鶏卵のコクシエラ汚染は否定された．スイスにおいてもわが国からの報告をうけ調査したが，鶏卵からはまったくコクシエラ菌は検出されなかった[5]．しかしながら，鶏卵がコクシエラに汚染された場合に，卵成分を含む食品においてコクシエラがどの程度生残するかを明らかにすることは食品公衆衛生上の意義があると考えられる．

卵を原材料とする代表的な食品としてマヨネーズがある．マヨネーズにおける微生物の生残性に関する報告は少ない．鳥インフルエンザウイルスはマヨネーズ中で速やかに不活化されるが[6]，サルモネラについては自家製マヨネーズにおいて殺菌されるまでに数時間を要するとされている[7]．コクシエラ菌の汚染についてはPCR法によりまったく検出されなかったことが報告されている[3]．しかしながら，これまでにマヨネーズにコクシ

† 連絡責任者：福士秀人（岐阜大学応用生物科学部獣医微生物学研究室）
〒501-1193　岐阜市柳戸1-1　☎・FAX 058-293-2946　E-mail : hfukushi@gifu-u.ac.jp

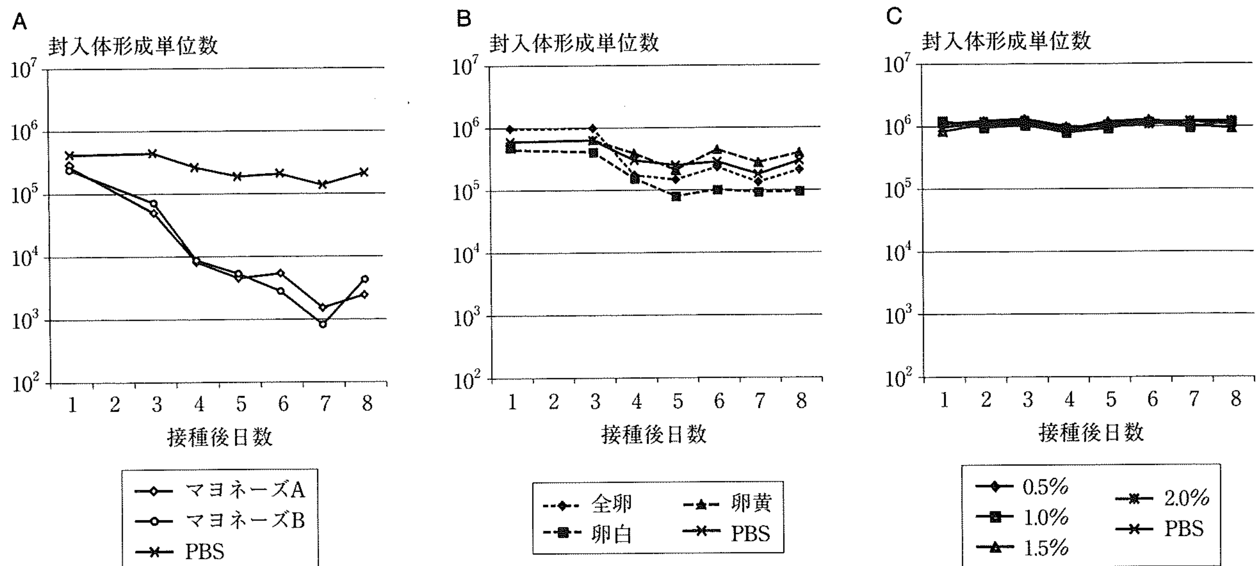

図1　マヨネーズ，卵成分および酢酸溶液におけるコクシエラ菌の生残性
マヨネーズ（A），全卵・卵白・卵黄（B）および0.5から2.0％酢酸（C）におけるコクシエラ菌の生残性を測定した．生残性は各溶液中の封入体形成単位数として示した．いずれもコクシエラ菌と卵成分を混合後，室温で7日間調べた．対象としてPBSを用いた．

エラが混入した場合のコクシエラの感染性についての検討はない．そこで，本研究ではマヨネーズおよびその原材料中におけるコクシエラ菌の生残性を検討した．その結果，マヨネーズ中ではコクシエラの生残性は著しく低下することがわかった．

材料および方法

用いたコクシエラ菌：コクシエラ菌Nine Mile II相菌を実験対象とした．培養細胞としてサル腎臓由来BGM細胞およびVero細胞を用いた．両細胞にコクシエラ菌の感受性に差はなかったが，接種材料に対する耐性に応じ，いずれかの細胞を用いた．コクシエラ菌の培養はBGM細胞を用い，既報［8］に従った．

マヨネーズ，全卵，卵黄，卵白および酢酸水溶液における生残性の定量：市販のマヨネーズを2種類および市販の鶏卵を用いた．鶏卵は手で割り，液卵とした．全卵はビーカー中で泡立て器を用い，均一になるまで攪拌混和した．卵白および卵黄はセパレーターでそれぞれを分取した後，泡立て器で均一にした．各液卵材料に抗菌剤としてストレプトマイシンを1mg/mlとなるように加えた．

次にマヨネーズ9gに菌液1.0mlの添加，もしくは各液卵材料（全卵，卵黄，卵白）4.5mlに菌液0.5mlを加えることにより，最終菌濃度5×10^5 IFU（封入体形成単位）/mlとなるように調整した．また，酢酸含有菌液は酢酸濃度0.5％，1.0％，1.5％，2.0％，菌濃度10^6 IFU/mlとなるように調整した．

各混合液は室温（約23℃）におき，混合直後，ならびに2日目から7日目まで毎日，50μlを採取した．酢酸含有菌はTris水溶液を最終濃度70mMになるように加え，中和した後に定量に用いた．

コクシエラ菌含有卵黄液は，56℃3.5分，60℃3.5分，61℃3.5分，54℃10分，58℃10分，59℃10分の法定殺菌条件および64℃7分間の割合で加熱した．加温にあたっては，実際の液卵がそれぞれの温度に達してから一定時間加熱し，終了後は氷水で急冷した．

さらに，マヨネーズおよび液卵から50μlを採取し，450μlのPBS（0.1mg/mlストレプトマイシン含有）と混和後，10倍段階希釈した．

Vero細胞ないしBGM細胞を96穴プレートに3.5×10^4/穴となるようにまき，一晩培養後，検体を接種した．検体は0.1ml/穴，希釈あたり2穴，接種し，1時間吸着後，細胞を3回洗浄し液卵成分を除去した．5％牛胎児血清含有MEMを150μl/穴加え，5％CO_2存在下で5日間培養した．

その後，培養上清を除去し乾燥させた．メタノール固定を30分間行い，除去，乾燥させ，コクシエラ菌に対するモノクローナル抗体H106（1μg/ml）［9］を50μl/穴加え，1時間反応させた．またPBSで3回洗浄後，0.2％ Evans-blue含有FITC標識抗マウス抗体液（200倍希釈）を25μl/穴加え，1時間反応させた．PBSで2回，蒸留水で1回洗浄し，乾燥させ，蛍光顕微鏡で観察し，1穴あたりの特異的な蛍光を発する封入体をすべて数えた．その際，各穴の平均封入体数により検体における単位体積あたりのIFU数を算出した．

それぞれの実験は2回繰り返し，平均値として示し

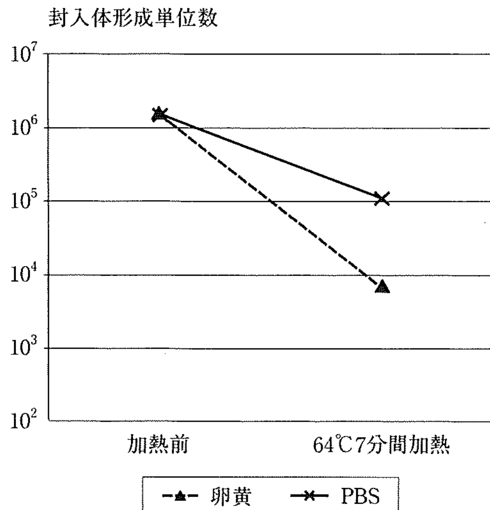

図2　卵黄の加熱によるコクシエラ菌の生残性
コクシエラ菌を卵黄と混合し64℃で7分間加熱した．対象としてPBSを用いた．

た．今回は実験回数が2回であり，統計検定は行わなかった．

成　　績

マヨネーズおよびその構成成分におけるコクシエラ菌の生残菌数をIFU測定により定量した．マヨネーズおよび卵成分は粘性が高く，IFUの実測値にばらつきがあったが，経時的な変動には再現性がみられた．マヨネーズ中ではコクシエラの生残菌数は日をおって減少し，7日目では1/100以下となった（図1A）．2種類のマヨネーズでコクシエラ菌の生残性に差はみられなかった．

マヨネーズの構成成分のうち，どの成分がコクシエラ菌の生残性に影響を与えているかをみるため，液卵および酢酸中における菌の生残について検討した．食用油については食用油そのものとコクシエラ菌を混入すると，細胞への感染がみられずIFUの定量ができなかったため，今回は検討できなかった．

全卵，卵白および生卵黄におけるコクシエラ菌の生残性をみたところ，全卵および卵白では5日後に1/5から1/10程度に生残菌数が減少したが，その後は生残菌数は維持された（図1B）．卵黄では生残菌数の減少はみられなかった．酢酸水溶液中でのコクシエラ生残菌数は濃度に関わらず一定で，ほとんど減少しなかった（図1C）．

マヨネーズの製造においては液卵の加熱殺菌が行われる．そこで，加熱による影響をみた．その結果，64℃7分間の加熱により生残菌数が1/100以下に減少した（図2）．いっぽう，56℃3.5分，60℃3.5分，61℃3.5分，54℃10分，58℃10分，59℃10分の法定殺菌条件では生残菌数は変化しなかった（成績は示していない）．

考　　察

今回の実験によりマヨネーズ中ではコクシエラ菌は生残しにくいことがわかった．マヨネーズ中でのコクシエラ生残性が著しく低下したことから，マヨネーズの構成成分のうちどれが有効かを調べた．基本的なマヨネーズの配合についてみると卵黄17％，食用油65から79％，食用酢9.5から13％，調味香辛料3.2から5％とされている［10］．食酢の酢酸濃度は一般に約4％であることから，マヨネーズ中の酢酸濃度は0.38から0.52％となる．

マヨネーズの成分のうち，卵黄中では生残菌数は5日目までで1/5になったがその後は変化がなかった．この生残菌数の減少は対象としたPBSでも同様であったことから卵黄の効果ではないと考えられる．卵白においては卵黄よりも生残菌数は少なかったが，マヨネーズ中より生残菌数は多かった．コクシエラ菌の培養に卵黄嚢内接種法が用いられることを考えると，卵黄や卵白がコクシエラの増殖や生残性に影響があるとは考えにくく，妥当な結果であると考えられた．

もう一つの成分である酢酸についてみると，今回の実験から少なくとも2％まではコクシエラ菌の生残に影響はないことがわかった．これはコクシエラ菌が細胞に感染後，酸性条件下のリソゾーム中で増殖可能であることと矛盾しない［11］．一般に食品中の酢酸は殺菌的ないし静菌的な効果をもつとされるが［12, 13］，マヨネーズ中の酢酸濃度はコクシエラ菌には無効と考えられた．

マヨネーズ体積の大半をしめる食用油について今回は検索できなかった．これは油成分中のコクシエラ生残菌数を定量する実験系の構築が困難であったためである．

マヨネーズの製造工程で液卵の加熱処理が行われている．今回の実験から液卵の法定殺菌条件ではコクシエラ菌は死滅しないことがわかったが，64℃7分間の条件であれば，生残菌数を減ずることがわかった．今後は加熱殺菌条件をさらに検討する必要がある．

今回の実験では検体に5×10^5IFU/mlとなるようにコクシエラ菌を添加した．これは通常の実験室におけるコクシエラの培養条件とほぼ同等である．野外における食品や食材への混入があるとしても混入菌数はもっと少ないと考えられる．市販の鶏卵のコクシエラ汚染量は検出限界以下であることから1個あたり10^2IFU/ml以下であると考えられる．また10^3～10^4IFU/個の鶏卵が混入しても他の汚染されていない鶏卵との混和により希釈され10^2IFU/ml以下となる．マヨネーズ中では数日で生残菌数が1/100以下になることを考慮すると，鶏卵にコクシエラ菌が混入したとしてもマヨネーズ製品中では製造後数日以内にほぼ死滅してしまうであろう．

さらに検討の余地があるものの，今回の実験結果から

はマヨネーズにコクシエラが混入しても人への感染源となる可能性はほとんどないと考えられた．

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Survival rates of *Coxiella burnetii* in mayonnaise and its constituents

Hideto FUKUSHI*†, Kazuyuki INOUE, Lin Saito, KENJI Ohya, Nobuhiro SASHIHARA, Tsuyoshi YAMAGUCHI and Katsuya HIRAI

** Faculty of Applied Biological Sciences, Gifu University, Yanagido 1-1, Gifu, 501-1193, Japan*

SUMMARY

The survivability of Coxiella burnetii in mayonnaise and its ingredients, including whole egg, yolk, albumen, and vinegar as an acetic acid was investigated. The infectivity of *C. burnetii* in mayonnaise decreased over time to be 1/100 after seven days at room temperature. No change in the infectivity of *C. burnetii* was observed for 0.5% to 2.0% acetic acid for seven days at room temperature. The incubation of *C. burnetii* in the albumen lowered the infectivity to 1/10. Heating the coxiella suspension at 64℃ for seven minutes decreased the infectivity to 1/100 in yolk compared to 1/10 in PBS. These results indicated that contaminated *C. burnetii* in mayonnaise may be able to be eliminated with seven days. ―― Key words : *Coxiella burnetii*, mayonnaise, survivability.

† *Correspondence to : Hideto FUKUSHI (Faculty of Applied Biological Sciences, Gifu University)*
Yanagido 1-1, Gifu, 501-1193, Japan
TEL・FAX 058-293-2946 E-mail : hfukushi@gifu-u.ac.jp

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ORIGINAL ARTICLE

Pre-exposure immunization against rabies using Japanese rabies vaccine following the WHO recommended schedule

Naoki Yanagisawa · Naohide Takayama ·
Eiichi Nakayama · Kazuaki Mannen ·
Akihiko Suganuma

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Abstract We examined the efficacy and safety of the Japanese purified chick embryo cell rabies vaccine (PCEC-K) when administered on days 0, 7, and 28, as recommended by the WHO. Post-vaccination serum samples were obtained from 53 human subjects, and rabies antibody titers were determined by a combination of enzyme-linked immunosorbent assay (ELISA) and neutralizing antibody (NA) assay. By day 42 of the experiment, which was 2 weeks after the third dose, all subjects had developed NA titers of 0.5 IU/ml or higher. The geometric mean titers of ELISA antibody and NA were 3.8 EU/ml and 5.7 IU/ml, respectively. Overall, the vaccine was well tolerated by all subjects. These results suggest that PCEC-K used for pre-exposure immunization according to the WHO schedule is as immunogenic and effective as the current pre-exposure immunization regimen in Japan, which consists of vaccines administered on days 0, 28, and 180. An accelerated schedule would be of great advantage to Japanese travelers, who could complete the required three doses for primary immunization in 1 month.

Keywords Rabies vaccine · Pre-exposure immunization · Post-exposure prophylaxis

N. Yanagisawa (✉) · A. Suganuma
Department of Infectious Diseases, Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan
e-mail: naokiy@cick.jp

N. Takayama · E. Nakayama
Department of Pediatrics, Tokyo Metropolitan Komagome Hospital, Tokyo, Japan

K. Mannen
Institute of Scientific Research, Oita University, Oita, Japan

Introduction

Infection with the rabies virus, a Lyssavirus, causes acute, progressive, and fatal viral encephalitis. Mortality is almost 100% once clinical symptoms of rabies appear, because we lack effective therapy [1]. Rabies is a serious cause of human mortality in many developing countries. Post-exposure prophylaxis (PEP) using the rabies vaccine is the only means of preventing clinical rabies. In cases of severe exposure, WHO recommends the administration of rabies immunoglobulin (RIG) in addition to vaccination. However, the supply of RIG is scarce worldwide. Moreover, RIG is not marketed in Japan, which makes it impossible to follow the WHO recommendation regarding PEP for severe cases [2].

The problems associated with the unavailability of RIG could be mitigated by routine pre-exposure immunization (PEI). With regard to PEI, the WHO recommends rabies vaccines to be administered intramuscularly on days 0, 7, and 21 or 28 [2]. In contrast, the approved PEI regimen in Japan is performed by administering Japanese purified chick embryo cell rabies vaccine (PCEC-K) subcutaneously on days 0, 28, and 180. Therefore, completing PEI by administering the recommended three doses is inconvenient because of the long intervals between doses [3]. Following the WHO recommended schedule would present great advantages for potential travelers, who could complete the three-dose rabies vaccine needed for primary immunization in 1 month.

The aim of this study was to determine the efficacy and safety of PCEC-K when following the WHO recommended schedule. We have previously reported that this accelerated schedule could result in protective titers, as measured by enzyme-linked immunosorbent assay (ELISA), but neutralizing antibody (NA) assay was not performed in that

study [4]. NA assays are considered by the WHO to be the gold standard for measuring successful seroconversion against rabies [2]. However, ELISAs present key advantages over other serological techniques [5], including speed, ease, and lower biosafety requirements, and we were interested in measuring how results obtained with an ELISA correlated with those obtained with the NA assay.

Subjects, materials, and methods

Ethics approval and informed consent

This study was conducted in accordance with the Declaration of Helsinki. The study protocol was submitted to and approved by the institutional review board. All participants were informed of the study procedures, and written consent was obtained from all subjects.

Subjects

A total of 53 subjects (40 men and 13 women; ages ranging from 24 to 59 years, with a mean of 37.0 ± 10.5 years) were enrolled. Of these, 26 and 27 subjects were enrolled in 2007 and 2008, respectively. The subjects were interviewed and examined by a physician and confirmed to have had no previous animal bites; no previous rabies vaccines; no acute or chronic infectious diseases; and no concomitant use of corticosteroid therapy or antimalarial or immunosuppressive drugs. The subjects were all volunteers who considered receiving the rabies vaccine for their travel abroad in the near future.

Administration of vaccines

The PCEC-K vaccine used in this study was manufactured by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). The 26 subjects enrolled in 2007 were vaccinated with lot RB02 for the first and second doses. For the third dose, 18 subjects were vaccinated with lot RB02 and 8 subjects with lot RB03. The 27 subjects enrolled in 2008 were vaccinated with lot RB06 for the first and second doses, and lot RB07 was used for the third dose. Vaccination was performed by subcutaneous injection in the anterolateral aspect of the upper arm, using 1.0 ml of PCEC-K, on days 0, 7, and 28.

Serological testing

Blood samples were collected from all vaccinees on days 7, 28, and 42. ELISA antibody titers were measured with the PLATELIA™ RABIES KIT II (Bio-Rad Laboratories, Marnes-la-Coquette, France) according to the manufacturer's protocol. NA titers were measured by using the rapid fluorescent focus inhibition test (RFFIT), following the international standard procedure [6]. ELISA antibody titers of 0.5 EU/ml or higher, or NA titers of 0.5 IU/ml or higher were considered positive.

Adverse effects

The subjects were told to notify the investigators of any adverse effects after the vaccine injection on their next visit.

Statistical analysis

The correlation of $\log_{10}$ ELISA antibody titers and $\log_{10}$ NA titers was calculated using Pearson's correlation test. Statistical analysis was conducted using StatView 5.0 (SAS Institute Japan, Tokyo, Japan). $P < 0.05$ was considered to be statistically significant.

Results

ELISA antibody titers measured on days 7, 28, and 42 are shown in Table 1. On day 7, 1 week after the first dose, none of the subjects had a detectable ELISA antibody titer. On day 28, 3 weeks after the second dose, 84.9% (45/53) were seroconverted, as defined as 0.5 EU/ml or higher. Among the seroconverted subjects, the geometric mean titer (GMT) was 1.7 EU/ml (range <0.5–3.5 EU/ml). On day 42, 2 weeks after the third dose, all subjects (53/53) were seroconverted, with a GMT of 3.8 EU/ml (range 0.8–17.3 EU/ml).

The NA titers measured on days 7, 28, and 42 are shown in Table 2. Again, on day 7, no subject had a detectable NA titer. On day 28, 94.3% (50/53) were seroconverted, defined as 0.5 IU/ml or higher. Among the seroconverted subjects, GMT was 1.2 IU/ml (range <0.5–6.0 IU/ml). On

Table 1 ELISA antibody titers after administration of Japanese rabies vaccine on days 0, 7, and 28

Day	7	28	42
GMT (EU/ml)	–	1.7	3.8
Range	All <0.5	<0.5–3.5	0.8–17.3
$n = \geq 0.5$ EU/ml	0/53	45/53	53/53
Seroconversion (%)	0	84.9	100

Blood samples were collected on days 7, 28, and 42. Subjects were considered to have seroconverted when antibody titers were 0.5 EU/ml or higher

ELISA enzyme-linked immunosorbent assay, *GMT* geometric mean titer, *range* lowest–highest value

Table 2 Neutralizing antibody titers after administration of Japanese rabies vaccine on days 0, 7, and 28

Day	7	28	42
GMT (IU/ml)	–	1.2	5.7
Range	All <0.5	<0.5–6.0	0.7–23.7
$n = \geq 0.5$ IU/ml	0/53	50/53	53/53
Seroconversion (%)	0	94.3	100

Blood samples were taken on days 7, 28, and 42. Subjects were considered to have seroconverted when antibody titers were 0.5 IU/ml or higher

GMT geometric mean titer, *range* lowest–highest value

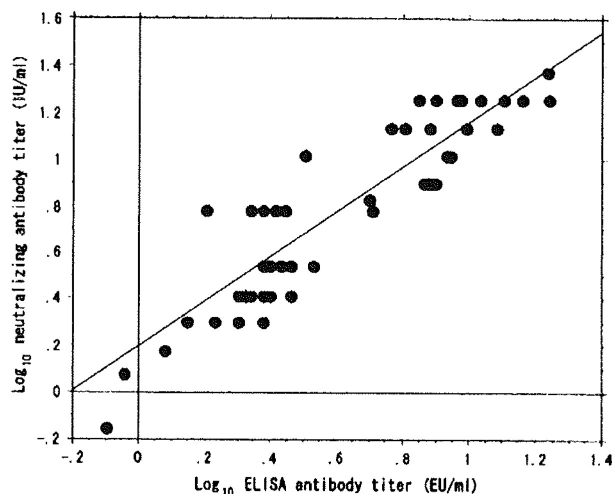

Fig. 1 Correlation of $\log_{10}$ enzyme-linked immunosorbent assay (*ELISA*) antibody titers with $\log_{10}$ neutralizing antibody titers in 53 subjects. The correlation analysis was performed using Pearson's correlation test ($r = 0.891$, $P < 0.0001$, $n = 53$)

day 42, all subjects (53/53) were seroconverted, with a GMT of 5.7 IU/ml (range 0.7–23.7 IU/ml).

Figure 1 shows the comparison between the $\log_{10}$ ELISA antibody titers and $\log_{10}$ NA titers. There was a clear correlation between the two assays ($r = 0.891$, $P < 0.0001$, $n = 53$).

Overall, the vaccines were well tolerated by all subjects. One subject complained of a headache, which resolved on its own the following day. Other systemic reactions such as fever or malaise were not reported. Mild local reactions were observed, erythema being the most frequent, recorded in 18.8% (10/53). This was followed by pruritus in 17.0% (9/53), swelling in 13.2% (7/53), and local pain in 7.5% (4/53).

Discussion

We studied the efficacy of PCEC-K following the schedule recommended by the WHO. After three doses of PCEC-K, all subjects developed protective ELISA and NA titers. There were no significant adverse effects.

Previous studies have reported on the efficacy of the officially approved PEI in Japan, with NA GMT values ranging between 2.7 and 4.0 IU/ml [7, 8]. In our study, all subjects had seroconverted after the third dose of the vaccine, with an NA GMT of 5.7 IU/ml. Our results cannot be directly compared to those in these previous studies, due to differences in the age, gender proportions, sample sizes, and timing of the samples taken. However, the fact that all subjects in the present study seroconverted suggests that administering PCEC-K on days 0, 7, and 28 is as effective as administering it on days 0, 28, and 180.

There was a strong correlation between the ELISA antibody and NA titers in the present study, suggesting that the ELISA test could be as reliable as the neutralizing test in this context. Feyssaguet et al. [5] likewise reported that the ELISA test was as sensitive and specific as the current standardized reference method, with sensitivity of 98.6% and specificity of 99.4%. Moreover, the ELISA kit is simple, safe, and quick to use compared to the neutralizing test, which is time-consuming and expensive, and requires extensively trained technicians and laboratory facilities equipped to handle the live rabies virus.

A difference in the seroconversion rate between the two assays used in the present study was observed on day 28. The seroconversion rate was significantly lower when titers were measured by ELISA as compared to measurement by the neutralizing test (84.9 vs 94.3%, respectively). Moreover, none of the subjects with elevated ELISA antibody titers had negative NA titers. Taken together, these data suggest that the ELISA test could be more stringent in determining seroconversion. All subjects had seroconverted after the third dose, indicating that the completion of the recommend schedule was the most important factor in achieving protection.

Japan is one of the few rabies-free countries in the world. Yet two human imported rabies cases were reported in Japan, in November 2006 [9, 10]. The victims in question did not receive rabies PEP after being bitten by a rabid dog. To prevent further tragedies, travelers must be encouraged to receive PEP immediately after being bitten by an animal suspected of being rabid. Furthermore, it is important to encourage PEI to insure the efficacy of PEP and economize on the short supply of RIG worldwide.

In conclusion, PCEC-K used for PEI and administered according to the schedule recommended by the WHO is as immunogenic and effective as the currently approved PEI in Japan. The use of the schedule recommended by the WHO would also foster traveler compliance with vaccination recommendations by providing protective immunity with a shorter, more manageable time course.

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Coxiella burnetii Isolates Cause Genogroup-Specific Virulence in Mouse and Guinea Pig Models of Acute Q Fever[∇]†

K. E. Russell-Lodrigue,[1,3]‡ M. Andoh,[1]‡ M. W. J. Poels,[1,2] H. R. Shive,[3] B. R. Weeks,[3] G. Q. Zhang,[1] C. Tersteeg,[1,2] T. Masegi,[4] A. Hotta,[5] T. Yamaguchi,[5] H. Fukushi,[5] K. Hirai,[5] D. N. McMurray,[1] and J. E. Samuel[1]*

Department of Microbial and Molecular Pathogenesis, Texas A&M Health Science Center, College Station, Texas[1]; Institute of Life Sciences and Chemistry, Utrecht, The Netherlands[2]; Department of Veterinary Pathobiology, Texas A&M University, College Station, Texas[3]; and Department of Pathogenetic Veterinary Science[4] and Department of Applied Veterinary Science,[5] United Graduate School of Veterinary Sciences, Gifu University, Gifu, Japan

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Q fever is a zoonotic disease of worldwide significance caused by the obligate intracellular bacterium *Coxiella burnetii*. Humans with Q fever may experience an acute flu-like illness and pneumonia and/or chronic hepatitis or endocarditis. Various markers demonstrate significant phylogenetic separation between and clustering among isolates from acute and chronic human disease. The clinical and pathological responses to infection with phase I *C. burnetii* isolates from the following four genomic groups were evaluated in immunocompetent and immunocompromised mice and in guinea pig infection models: group I (Nine Mile, African, and Ohio), group IV (Priscilla and P), group V (G and S), and group VI (Dugway). Isolates from all of the groups produced disease in the SCID mouse model, and genogroup-consistent trends were noted in cytokine production in response to infection in the immunocompetent-mouse model. Guinea pigs developed severe acute disease when aerosol challenged with group I isolates, mild to moderate acute disease in response to group V isolates, and no acute disease when infected with group IV and VI isolates. *C. burnetii* isolates have a range of disease potentials; isolates within the same genomic group cause similar pathological responses, and there is a clear distinction in strain virulence between these genomic groups.

Coxiella burnetii, the etiologic agent of acute and chronic Q fever, is an obligate intracellular bacterium with worldwide distribution and a diverse host range. Livestock serve as the organism's primary reservoir and may be asymptomatic carriers or exhibit reproductive disorders. Ticks are important in the maintenance of the disease in nature and have been shown to transmit the infection transovarially (37). Humans are most often infected through inhalation of the bacterium in fine-particle aerosols, though transmission may also occur through ingestion of the organism from contaminated, unpasteurized dairy products (22, 27). Although a high percentage of infections may result in subclinical or asymptomatic infection, humans can become ill from exposure to as few as 10 organisms (6) and may display signs of (i) an acute flu-like illness with or without pneumonia and/or hepatitis (30, 31) or (ii) a chronic disease manifesting most frequently as endocarditis and/or hepatitis (40, 41).

C. burnetii isolates have been obtained from natural Q fever infections in humans and other animals. Several theories have been proposed to explain the dichotomy in development of acute and chronic Q fever. Unique sequence differences between genomic groups are correlated with the clinical expression of Q fever (44). Biochemical markers have grouped *C. burnetii* isolates from chronic-disease patients separately from acute-disease/arthropod/domestic animal isolates, but whether these groupings predict virulence potential and acute/chronic-disease outcomes has not yet been fully resolved (20). Samuel et al. were the first to separate these isolates and their resulting diseases based on plasmid patterns (44). Hackstadt used variations in lipopolysaccharide (LPS) banding patterns to divide isolates of *C. burnetii* into three groups, and group distinction was noted in correlation with acute or chronic disease (16). Hendrix et al. separated *C. burnetii* isolates into six genomic groups (20). Group I to III isolates have a QpH1 plasmid and have been isolated from ticks, acute human Q fever cases, cow's milk, and livestock abortions. Groups IV and V have a QpRS plasmid or no plasmid (with plasmid-related sequences integrated into the chromosome), respectively, and have been associated with livestock abortions and human chronic endocarditis or hepatitis. Group VI isolates were collected from wild rodents in Dugway, UT, and were infectious but avirulent in rodent models of disease (47, 48). Jager et al. used restriction fragment length polymorphism (RFLP) to differentiate 80 *C. burnetii* isolates and reproduced distinguishable patterns for reference isolates in groups I, IV, V, and VI (23). More recently, multiple-locus variable nucleotide tandem repeat analyses (49) have validated these groupings. Infrequent-restriction-site PCR of 14 livestock and tick isolates resulted in six groups; subsequent multiple-locus variable-number tandem repeat analysis typing of 42 isolates revealed 36 genotypes (2). Glazunova et al. used multispacer sequence typing to analyze 173 isolates, a majority of which were acquired from chronic-

* Corresponding author. Mailing address: Department of Microbial and Molecular Pathogenesis, Texas A&M Health Science Center, College Station, TX 77843-1114. Phone: (979) 862-1684. Fax: (979) 845-3479. E-mail: jsamuel@medicine.tamhsc.edu.

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‡ K.E.R.-L. and M.A. contributed equally to this work.

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TABLE 1. Isolates evaluated for virulence

Genomic group	Isolate	Notation in this study	Original source			
			Sample	Yr	Location	Disease
I	Nine Mile RSA493	NM	Tick	1935	Montana, US	NA[a] (acute; flu-like in humans)
	African RSA334	African	Human blood	1949	Central Africa	Acute; Congolese Red Fever
	Ohio 314 RSA270	Ohio	Cow's milk	1956	Ohio, US	Persistent
IV	MSU Goat Q177	Priscilla	Goat Cotyledon	1980	Montana, US	Abortion
	P Q173	P	Human heart valve	1979	California, US	Endocarditis
V	G Q212	G	Human heart valve	1981	Nova Scotia, Canada	Endocarditis
	S Q217	S	Human liver biopsy specimen	1981	Montana, US	Hepatitis
VI	Dugway 5J108-111	Dugway	Rodents	1958	Utah, US	NA

[a] NA, not applicable.

disease patients, and identified 30 genotypes in three monophyletic groups; an association between the plasmid type, some genotypes, and the nature of disease was observed (15). These monophyletic groups supported the early RFLP groups and placed groups I, II, and III in one monophyletic group; group IV in the second monophyletic group; and group V in the third monophyletic group. A comprehensive microarray-based whole-genome comparison by Beare et al. confirmed the relatedness of RFLP-grouped isolates and added two more genomic groups, VII and VIII (4). Differences in novel gene contents and pseudogenes may be factors in the variations in virulence seen among group I, IV, V, and VI isolates (5). It has been shown in an intraperitoneal (i.p.)-challenge guinea pig model that 10^1 organisms of the acute-disease-associated group I isolate Nine Mile RSA493 (NM) caused fever, but 10^6 chronic-disease-associated group IV isolate MSU Goat Q177 (Priscilla) organisms were required to induce fever (36).

In opposition to the theory of genotype/pathotype correlation, Stein and Raoult evaluated 28 human isolates and found that isolates bearing the QpH1 plasmid were present in both acute and chronic Q fever patients in France and that isolates without the QpH1 plasmid were able to cause acute disease (46). QpH1 plasmid-containing isolates have also been isolated from chronic-endocarditis patients (50). Several groups have speculated that host factors are primarily responsible for the outcome of infection with *C. burnetii*. Individual differences in immune function lead to varying sensitivity to infection and disease development. In this model, acute and chronic disease could be caused by organisms from the same isolate group, and chronic disease could develop because of compromised resistance of the host rather than as a consequence of a specific property of the pathogen. For example, human immunodeficiency virus infection is a risk factor for the development of chronic Q fever endocarditis (9, 29). Deficiencies in the host-specific cell-mediated immune response in Q fever patients have been associated with the suppression of monocyte and macrophage activities (25), and monocytes from chronic-Q fever patients have been shown to be defective in phagosome maturation and to have impaired *C. burnetii*-killing potential, regulated in part by elevated interleukin-10 (IL-10) expression (14). There is strong clinical evidence to support the role of increased host production of IL-10 in the development of both Q fever endocarditis and chronic fatigue syndrome (11, 12, 21, 39). A recent study suggested that chronic Q fever endocarditis may be associated with atypical M2 polarization and stimulation of bacterial replication (7), but the pathogenic process that mediates this polarization was undefined.

The route of infection may also be an important determining factor in the manifestation of acute and chronic Q fever. La Scola et al. and Marrie et al. demonstrated that the route of infection and the size of the inoculum affected clinical illness and pathology associated with infection in mouse and guinea pig models (26, 33). Differences in the geographic distributions of the diseases have also been noted (32); in Nova Scotia, for example, the primary manifestation of acute Q fever is pneumonia (34), but in France it is hepatitis, possibly due to ingestion of raw milk and unpasteurized cheeses (51).

The pathogenicity of *C. burnetii* has been evaluated using guinea pigs, mice, and chicken embryos. Febrile response, splenomegaly, and mortality in guinea pigs; splenomegaly and mortality in mice; and mortality in chicken embryos are indicators of virulence for *C. burnetii*. The establishment of an aerosol model of *C. burnetii* infection in guinea pigs (43) provides a relevant model in which to test isolate virulence. Additionally, severe combined immunodeficient (SCID) mice are highly sensitive to the *C. burnetii* prototype (NM isolate) (1), and the 50% lethal dose (LD_{50}) of NM in SCID mice was at least 10^8 times less than in wild type mice. We speculated that with these highly sensitive rodent models it may be possible to observe intra- and intergroup pathogenicity differences of *C. burnetii* isolates. To confirm whether SCID mice could be used to model isolate-specific virulence, we gave multiple infectious doses of a group IV Q fever isolate to immune-competent CB-17 and SCID mice (on the same background) to compare them with previously reported group I isolate (NM) infections (1). Eight isolates from four genomic groups (Table 1) were then evaluated for the ability to cause acute disease in SCID mouse i.p.-challenge and guinea pig aerosol challenge models. We hypothesized that isolates within the same genotypic group would cause similar diseases and that there would be a distinct difference in disease manifestations between isolate groups. Finally, we evaluated the potential of a vaccine composed of one *C. burnetii* isolate to protect guinea pigs against infection with an isolate from another group, since cross-protection between disparate isolate groups is a further indication of antigenic relatedness.

MATERIALS AND METHODS

Animals. The female 6- to 7-week-old CB-17/Icr-scid/scid (SCID) and wild-type CB-17/Icr$^{+/+}$ (CB-17) mice used in Japan were purchased from Japan

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CLEA (Tokyo, Japan); A/J mice were purchased from Japan SLC (Shizuoka, Japan). A/J mice were used because they are considered more susceptible to *C. burnetii* than other inbred mouse strains (45). The female 6- to 8-week-old SCID and wild-type CB-17 mice used in the United States were purchased from Taconic (Hudson, NY). Female Hartley guinea pigs weighing approximately 350 to 450 g were purchased from Charles River Laboratories (Wilmington, MA).

All infected animals were housed in approved animal biosafety level 3 facilities, and immunodeficient mice were housed under sterile conditions. All animals used in this study were acclimated to the facility and assessment procedures during the week prior to infection to decrease stress-related abnormalities. Animal health was assessed daily by a veterinarian.

Mouse experiments performed in Japan adhered to the guidelines for animal experiments at Gifu University. The Texas A&M University Laboratory Animal Care Committee reviewed and approved the mouse and guinea pig research at Texas A&M University, and experiments were carried out in AAALAC-approved facilities in accordance with university and federal regulations.

C. burnetii. Eight *C. burnetii* isolates from four genomic groups (Table 1) were used. For the initial dose-effect experiment in Japan, *C. burnetii* MSU Goat Q177 (Priscilla), obtained from J. Kazar, Institute of Virology, Brastislava, Slovakia, was maintained in mice by passage in spleen homogenates at Gifu University. The spleen homogenates were stored at −80°C until they were used. The absence of contamination with other pathogens was confirmed by direct staining (Giménez and Gram staining), detection of *Mycoplasma* DNA using a PCR *Mycoplasma* detection set (Takara, Shiga, Japan), and inoculation of the spleen homogenate into cell culture and SCID mice (independent experimental infection from the study described here). The bacterial dose was evaluated as the 50% tissue culture infectious dose ($TCID_{50}$) in BGM cells (buffalo green monkey fibroblasts), the 50% infectious dose (ID_{50}) in CB-17 mice, and the LD_{50} in SCID mice. The $TCID_{50}$ was determined by detecting the bacteria 6 days after infection using immunofluorescence staining with anti-*C. burnetii* rabbit antiserum. The ID_{50} was determined by detecting seroconversion (immunoglobulin G [IgG], >1:16) using indirect microimmunofluorescence. The LD_{50} was determined as reported previously (1).

For all subsequent experiments, all of the *C. burnetii* isolates were maintained at the Texas A&M Health Science Center. The *C. burnetii* isolates were cultivated in embryonated chicken eggs, purified by gradient centrifugation as previously reported (19, 44, 53), and stored at −80°C until they were used. The absence of contamination by other pathogens was confirmed as described above. *C. burnetii* was quantified by optical density (OD) (53), direct viable-particle count using the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR), and quantitative real-time PCR (qPCR) using primers amplifying the *com1* gene (8) (see Table S1 in the supplemental material). The bacterial dose used for mouse infections was determined by qPCR; guinea pig doses were calculated using the OD.

Experimental infection in mice. (i) Dose/effect experiment with the Priscilla isolate. Six mice per group were used for the dose/effect experiment. SCID, CB-17, and A/J mice were inoculated i.p. with serial 10-fold dilutions of Priscilla (10^2 to 10^{-7} $TCID_{50}$ per animal) or sterile phosphate-buffered saline (PBS) (sham infection). SCID mice were observed for 112 days (16 weeks), and CB-17 and A/J mice were observed for 30 days.

(ii) Genomic group comparison. Four mice per group were used for the genomic group comparison. Each of eight *C. burnetii* isolates described in Table 1 (10^5 genome copies/animal) or PBS was administered i.p. to SCID and CB-17 mice. Two independent infections were performed, and the mice were observed for 28 days (for all of the *C. burnetii* isolates in SCID and CB-17 mice) or until death (for four representative *C. burnetii* isolates in SCID mice).

Clinical signs were evaluated every 2 days by visual observation (ruffled fur, hunched-back appearance, and lethargy) and body weight measurement. Body weight changes were evaluated using a body weight index (BWI) derived as follows: BWI = relative body weight/mean relative body weight of the control group; relative body weight = body weight on day "*x*" of infection/body weight on the day of infection. Cachexia was diagnosed when a mouse was lethargic and had a BWI of less than 0.85. At necropsy, the spleen weight was measured as an indicator of *C. burnetii* infection (54), and tissues were collected. To quantify the growth of *C. burnetii*, DNA was extracted from spleen tissue and *C. burnetii com1* gene copies were detected by qPCR as previously described (8). The heart, lung, liver, spleen, kidney, and femur were formalin fixed, embedded in paraffin, sliced, and then prepared by hematoxylin-eosin staining and immunocytochemistry, as described previously (1, 8), to evaluate histopathologic changes and bacterial distribution in tissues. The degree of inflammation present in each tissue sample was scored numerically by the following system: 0, none; 1, mild; 2, moderate; 3, marked; 4, severe. IgG titers for phase I and II *C. burnetii* in the sera of CB-17 mice were measured by microimmunofluorescence as described elsewhere (1).

For cytokine assays, blood was collected from the lateral saphenous vein at 3, 7, 10, 14, and 21 days postinfection (p.i.) and via cardiac puncture at 28 days p.i. after euthanasia, and the group pooled sera were stored at −80°C until they were used. Sixteen cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12p40, IL-12p70, IL-10, granulocyte-macrophage colony-stimulating factor, gamma interferon [IFN-γ], KC, macrophage inflammatory protein 1α [MIP-1α], RANTES, and tumor necrosis factor alpha [TNF-α]) were measured using the Bio-Plex cytokine assay system (Bio-Rad, Hercules, CA) following the manufacturer's protocol. The cytokine quantification assay was performed in duplicate for each sample. The cytokine levels of infected sera were evaluated as the induction values compared to the values of uninfected sera.

Experimental infection in guinea pigs. A chamber specially designed to deliver droplet nuclei directly to the alveolar spaces (College of Engineering Shops, University of Wisconsin, Madison), allowing the infection of multiple guinea pigs simultaneously and ensuring uniform infection within each challenge group (35, 43, 52), was used for all guinea pig infection studies. (i) Three guinea pigs per group were infected with low (10^2), mid-level (10^4), or high (10^6) doses of one of the phase I *C. burnetii* isolates described in Table 1. Four negative control animals were sham infected with sterile PBS. Body weight, rectal temperature, and behavioral attitude were recorded, along with any abnormalities noted on thoracic auscultation and abdominal palpation. A rectal temperature of ≥39.5°C was defined as fever. The guinea pigs were observed for 28 days p.i. The spleens and livers were weighed at necropsy. Tissues were collected and formalin fixed for histopathologic evaluation. Serum was obtained from each animal for serologic testing. (ii) In a separate experiment, three guinea pigs per group were exposed to PBS or 2×10^6 particle equivalents of NM, P, G, or Dugway. Daily assessment of these animals was performed as described above, and the organs were weighed at necropsy 14 days p.i. to detect splenomegaly and/or hepatomegaly. (iii) In the heterologous-protection study, guinea pigs were vaccinated twice with 40 μg of formalin-inactivated group I (NM) or group V (S) *C. burnetii* in Freund's incomplete adjuvant or with adjuvant alone, with 2-week intervals between the vaccinations and infection. The animals were then infected with high doses of either NM or S. Three animals per group were separated into the following six groups: (a) nonvaccinated, NM infected; (b) nonvaccinated, S infected; (c) NM vaccinated, NM infected; (d) S vaccinated, S infected; (e) NM vaccinated, S infected; and (f) S vaccinated, NM infected. The guinea pigs were monitored for 14 days p.i. for development of fever and other clinical signs of illness.

Histopathologic samples were prepared by hematoxylin and eosin staining or by immunohistochemistry using a Vectastain ABC kit and a Vector NovaRed substrate kit (Vector Laboratories, Burlingame, CA) and in-house-generated rabbit anti-*C. burnetii* NM (3) and by counterstaining them with hematoxylin. All slides were evaluated in a blinded fashion. Serum samples collected at necropsy were tested by enzyme-linked immunosorbent assay for IgG titers against phase I *C. burnetii* NM antigen as previously described (43). Sera from uninfected guinea pigs were used as negative controls.

Statistical analyses. The results were expressed as means for each group and were compared using one- and two-way analysis of variance or Student's *t* test, as appropriate. Differences were considered significant at a P value of <0.05.

RESULTS

***C. burnetii* Priscilla is infective and exhibits delayed virulence in SCID mice.** A detailed analysis of dose-effect in an immunocompromised-mouse model supported the previous study by Moos and Hackstadt that evaluated the ability of the Priscilla isolate to cause fever in i.p.-challenged guinea pigs (36). The infectious titer of the Priscilla isolate in the splenic homogenate used for the multiple-dose infection was 2×10^4 $TCID_{50}$/ml in BGM cells, $2 \times 10^{9.3}$ ID_{50}/ml in CB-17 mice, and 2×10^{10} LD_{50}/ml in SCID mice (1 $TCID_{50}$ corresponded to $10^{5.3}$ ID_{50} in CB-17 mice and to 10^6 LD_{50} in SCID mice). The LD_{50} in CB-17 mice could not be determined because no CB-17 mice died from any infectious dose used in this study, and the ID_{50} in SCID mice could not be determined due to lack of antibody production. The ID_{50} in CB-17 mice and the LD_{50} in SCID mice were similar, suggesting that SCID mice could be lethally infected with very few viable organisms.

Multiple-dose infection of SCID mice with the Priscilla iso-

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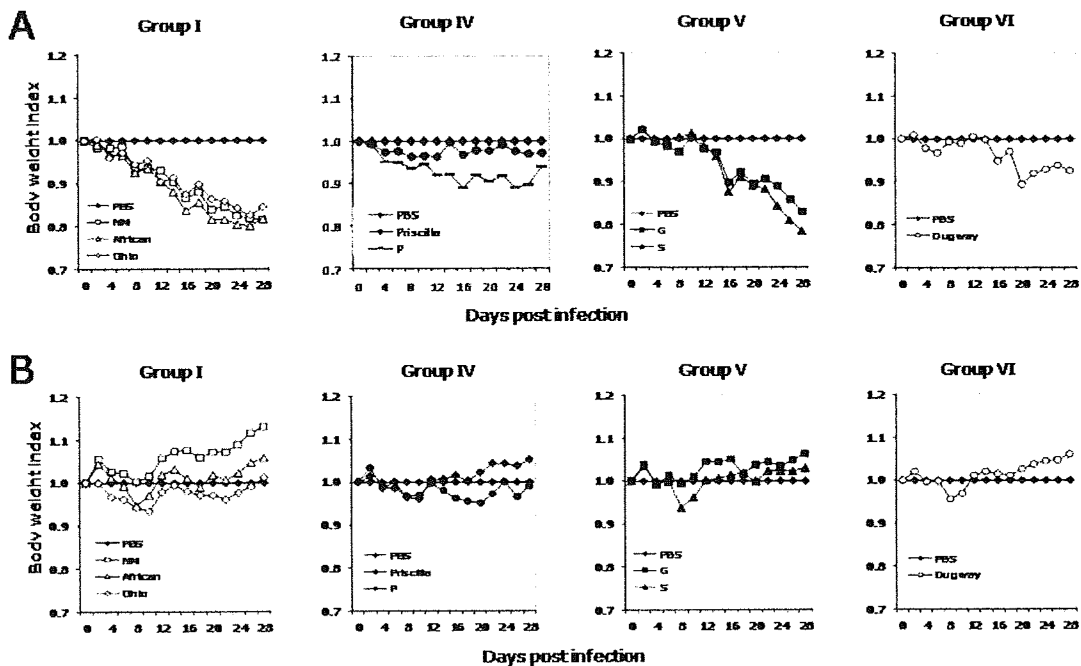

FIG. 1. Average body weight changes in SCID mice (A) and CB-17 mice (B) infected with *C. burnetii* isolates during 28 days of infection. Body weights were significantly lower in SCID mice throughout the infection period and transiently in CB-17 mice infected with all isolates except Priscilla compared to PBS-injected controls ($P < 0.05$).

late resulted in slow, progressive, and long-term-persistent disease. Clinical signs included ruffled fur, extremely distended abdomens, and death. Body weight loss, inactivity, and cachexia were not observed until a few days prior to death. Survival time ranged from 55 to 109 days p.i. Progression of clinical signs and survival times were dose dependent, with shorter times corresponding to higher infectious doses (see Table S2 in the supplemental material). Similar lesions were found in all of the SCID mice that died, most notably severe hepatosplenomegaly, and all organs had cellular infiltration, primarily macrophages containing bacteria. The severity of the lesions in infected SCID mice was not dependent on the *C. burnetii* challenge dose.

On the other hand, CB-17 and A/J mice displayed transitory clinical signs only after infection with the highest dose of Priscilla. Both mouse strains showed ruffled fur from 4 to 13 days p.i., but only A/J mice demonstrated transient body weight loss (data not shown). No other clinical signs were observed. At 28 days p.i., CB-17 and A/J mice had mild splenomegaly and seroconversion as evidence of infection (data not shown). Small granulomas were present in the spleen and liver, but bacterial antigen was not detectable by immunohistochemistry.

Genomic-group-specific virulence in mice. It was important to establish whether the results of infection seen with the Priscilla isolate and those previously noted with the NM isolate were genomic group specific (24). To determine this, the pathogenicities of multiple isolates were compared by delivering a single dose of eight *C. burnetii* isolates from four genomic groups (Table 1) to mice by i.p. injection. The infections were initially compared in SCID and CB-17 mice sacrificed at 28 days p.i.

All *C. burnetii* isolates caused disease in SCID mice, with various clinical courses. There was no mortality during the 28-day infection period. Clinical signs, including significant body weight loss ($P < 0.05$) and cachexia, summarized in Fig. 1A and in Fig. S1A in the supplemental material, were most apparent in mice infected with group I isolates, followed by those given group V, IV, and VI isolates. In CB-17 mice, only mild transient disease was noted, with minimal loss of body weight, in response to all isolates and noticeably ruffled fur with group I isolate infection (Fig. 1B).

Splenomegaly in response to infection was more severe in SCID than in CB-17 mice (Fig. 2A). The number of bacteria in the spleens was determined by qPCR (Fig. 2B), and consistently higher numbers of *com1* genes were detected in SCID than in CB-17 mice. SCID mice showed phylogenetic-group-characteristic spleen size and growth of bacteria. Splenomegaly was greatest in SCID mice with mild clinical disease infected with bacteria from groups IV and VI. However, the number of organisms in the spleen was greater in mice with severe clinical disease following infection with phylogenetic groups I and V. In CB-17 mice, splenic enlargement and numbers of bacteria increased with the severity of clinical disease. CB-17 mice displayed differences between infection with the *C. burnetii* isolates that caused acute disease (phylogenetic group I) and infection with the *C. burnetii* isolates that caused chronic disease (phylogenetic groups IV and V), but there was no difference between groups infected with isolates that caused chronic disease. All infected mice developed significant splenomegaly,

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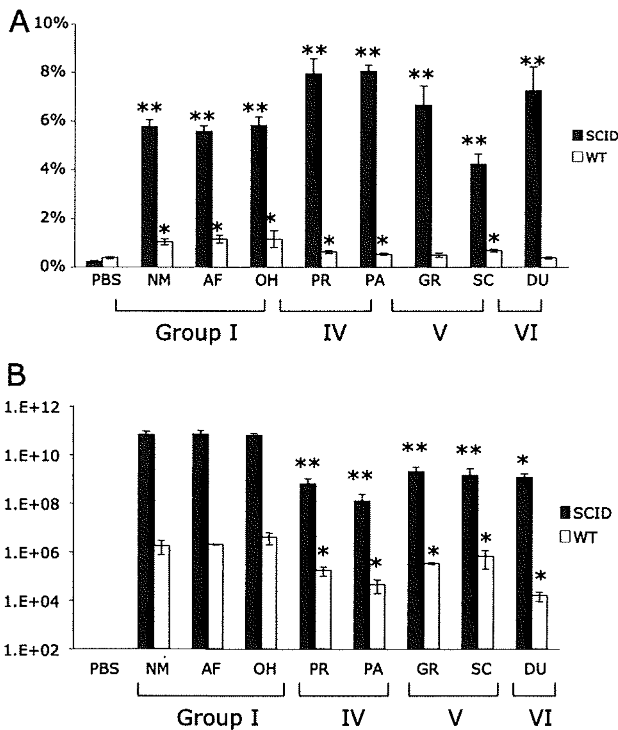

FIG. 2. Splenomegaly (A) and splenic bacterial loads (B) in mice at 28 days p.i. (A) All infected animals developed significant splenomegaly compared to controls, and infected SCID mice had significantly larger spleens than CB-17 mice ($P < 0.05$). (B) Mice infected with group IV, V, and VI isolates had significantly fewer bacteria than those infected with group I isolates ($P < 0.05$). *, $P < 0.05$. The error bars indicate standard deviations.

but mice infected with group IV, V, and VI isolates had significantly fewer splenic bacteria than mice infected with group I isolates ($P < 0.05$).

Evaluation of histopathology at 28 days p.i. revealed more lesions in SCID mice than in CB-17 mice (see Table S3 in the supplemental material). SCID mice showed histopathologic changes in all organs investigated. Group I isolates caused the most inflammation, followed by groups V, IV, and VI. The inflammatory-cell populations were similar in all groups and consisted of few neutrophils and numerous macrophages containing abundant intracytoplasmic bacteria. *C. burnetii* antigen was diffusely distributed in all organs examined. CB-17 mice had mild histopathologic changes in some organs, but even in the tissues with an inflammatory response, *C. burnetii* antigen was rarely detected.

Circulating cytokines are altered in *C. burnetii*-infected CB-17 mice. The variations in pathology and inflammation associated with these isolate group infections suggest differences in the immune responses. To expand on this observation, the serum levels of 16 cytokines and chemokines were measured. In CB-17 mice, serum cytokine levels differed between mice infected with group I isolates and those given isolates from other groups. Group I isolates induced persistently high cytokine secretion throughout the 28-day experiment; group IV and V isolates caused moderate cytokine secretion at the peak of clinical disease (7 to 14 days p.i.) (Fig. 3). After 14 days p.i., group I isolates induced higher secretion of IL-3, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IFN-γ, TNF-α, MIP-1α, and RANTES than other groups. The KC and granulocyte-macrophage colony-stimulating factor levels of mice infected with group I isolates were higher than those in mice infected with other groups prior to 14 days p.i. Serum IL-1α, IL-1β, IL-2, and IL-5 levels and eotaxin secretion were not increased during the infection period (data not shown).

Lethal potentials of all genomic groups in SCID mice. The lethal potentials of representative isolates from each phylogenetic group were investigated in SCID mice, and it was determined that all of the isolates evaluated could eventually lead to clinical illness and death in the immunodeficient model (see Fig. S1B in the supplemental material). Isolates that caused a long period of cachexia led to severe body weight loss in infected mice (see Fig. S2 in the supplemental material). A group I isolate (NM) induced the earliest and longest period of cachexia and, correspondingly, the most severe body weight loss. Mice infected with isolates from groups V (G) and VI (Dugway) had similar survival times, but those given group V isolates had longer periods of cachexia and more severe body weight loss than group VI-infected mice. Infection with group IV isolates (Priscilla and P) resulted in the shortest period of cachexia, and body weight loss was not observed until the terminal stage of infection. The survival time was shortest in mice challenged with group I isolates (32.0 ± 0.8 days), followed by those infected with groups V (36.0 ± 0.0 days), VI (35.5 ± 1.0 days), and IV (47.5 ± 0.6 days for P and 77.3 ± 2.8 days for Priscilla). The probable cause of death was multiple-organ failure due to massive systemic infection.

The pathological changes in SCID mice at mortality were more advanced than those observed at 28 days p.i. (data not shown). The severity of inflammatory changes in the liver and spleen was similar in all groups of infected mice, but animals given group I isolates exhibited a greater degree of inflammation in the heart and lungs than those given group IV, V, and VI isolates. The extent of splenomegaly changed with survival time; however, the numbers of bacteria in the spleen were similar in all groups, suggesting that the number of bacteria (10^{10} genome copies/spleen) detected is the saturation point in SCID mice. *C. burnetii* antigen was diffusely distributed in all tissue sections.

Genomic-group-specific outcome of acute Q fever pneumonia in the guinea pig aerosol model. Aerosol challenge in the guinea pig provides a physiologically relevant model that simulates both the natural route of infection and common clinical presentations associated with human acute Q fever, making this a choice model for evaluating the comparative levels of virulence of different *C. burnetii* isolates, and thus, it was used in the logical progression of experiments after different levels of virulence were observed in mouse models of infection. Guinea pigs challenged with group I and V isolates developed significant fever in response to infection ($P < 0.01$), whereas those given isolates from groups IV and VI were afebrile even at the highest challenge dose (Fig. 4).

Fever response, weight loss, and other clinical signs displayed a dose-dependent relationship in guinea pigs infected with the group I *C. burnetii* isolates African and Ohio, as has been described for the reference isolate in this group, NM (43). All animals that received African or Ohio organisms at a high

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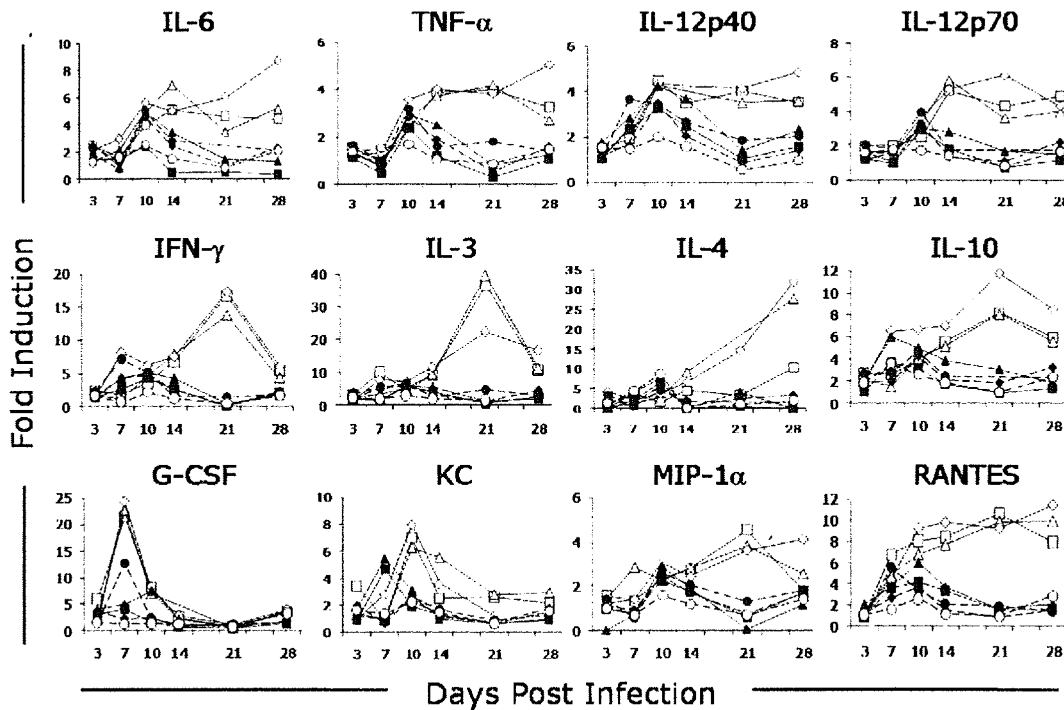

FIG. 3. Mean circulating cytokine levels in response to infection in CB-17 mice with different *C. burnetii* isolates. Isolates from genomic group I induced persistently high cytokine secretion with increased levels of IL-6, TNF-α, IL-12p40, IL-12p70, IFN-γ, IL-3, IL-4, IL-10, MIP-1α, and RANTES compared with other genogroups ($P < 0.05$). ◆, PBS; □, NM; △, African; ◇, Ohio; ●, Priscilla; ◆, P; ■, G; ▲, S; ○, Dugway.

dose died within 7 to 9 days p.i., as did two of three that received NM; lower infectious doses were not lethal. Gross lung consolidation and overall lack of normal body fat were noted on necropsy at 7 to 9 days p.i. in guinea pigs infected with the highest dose of organisms. Histologically, these animals had severe panleukocytic bronchointerstitial pneumonia with bronchial and alveolar exudates. Lung tissues from the surviving NM-infected guinea pig and those given the mid-level dose of group I organisms were evaluated at 28 days p.i. for comparison to animals infected with other isolates evaluated at this time, and they exhibited moderate multifocal lymphohistiocytic pneumonia with granuloma formation.

No significant fever or other overt clinical signs were noted in guinea pigs infected with group IV isolates. Mild lymphohistiocytic pneumonia was seen histologically at 28 days p.i. in animals given the highest dose of organisms.

Group V isolate-infected guinea pigs all developed fever when given the highest challenge dose, and dose-dependent temperature increases and other clinical signs were again noted, with no fever development, in those animals receiving the lowest dose of organism. Though auscultation confirmed respiratory compromise, none of the infections were lethal. At 28 days p.i., the lungs had mild to moderate lymphohistiocytic interstitial pneumonia and a few small granulomas.

No major clinical or pathological changes were noted in guinea pigs infected with the group VI isolate or in negative control animals. Table S4 in the supplemental material compares the severity of histopathologic changes in guinea pigs infected with high doses of *C. burnetii* isolates from each group at 28 days p.i. Immunohistochemistry confirmed the presence of *C. burnetii* organisms, primarily in macrophages, in the lungs, livers, and spleens of infected animals.

Experimental guinea pigs in all dose groups for each isolate seroconverted by the time of euthanasia, with the exception of animals infected with high doses of NM, African, and Ohio necropsied at 1 week p.i. and low-dose Dugway-infected guinea pigs. The degree of seroconversion was dose dependent and varied among isolates (data not shown). No PBS-injected control animals seroconverted.

Genomic-group-specific severity of hepatitis and splenomegaly in guinea pigs. The doughnut granulomas common in human acute Q fever hepatitis (31) had not been previously described in animals experimentally infected with *C. burnetii* and were also not seen in the guinea pigs in this study. Mild hepatitis and severe hepatic lipidosis were noted at death 7 days p.i. in guinea pigs challenged with high doses of group I isolates, as had been previously reported for NM aerosol-infected guinea pigs (43). Tissue sections from the remaining NM-infected guinea pig and those infected with mid-level doses of the group I organisms were evaluated for comparison with animals infected with other isolates at 28 days p.i. and revealed vacuolization and degeneration of centrilobular hepatocytes, lymphocyte infiltration in periportal regions, and multiple small granulomas.

Group IV-infected guinea pigs also had periportal lymphocytic infiltration, as well as multiple granulomas of various sizes. The granulomas in Priscilla- and P-infected guinea pigs were more defined, with more histiocytic involvement than was

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