表 5. 誤嚥を生じる危険要因

1．寝たきり状態(原因疾患を問わない)	5．中毒・代謝障害
2．神経・筋疾患	薬物中毒
脳血管疾患(急性期、慢性期)	アルコール中毒
中枢性変性疾患	代謝性脳症(肝硬変、糖尿病、腎不全、電解質異常)
パーキンソン症候群	6．医原性
認知障害(脳血管性、アルツハイマー型)	全身麻酔
筋萎縮性側索硬化症	頭頸部食道手術
重症筋無力症	鎮静薬、睡眠薬
多発性筋炎	抗コリン薬など口腔内乾燥をきたす薬剤
3．口腔内の異常	テオフィリンなどの食道括約筋機能低下をきたす薬剤
歯の噛み合わせ障害(義歯不適合など)	経管栄養
口腔内乾燥	気管挿管
口腔・咽頭・喉頭癌	7．その他
4．胃・食道疾患	妊娠
食道憩室	腹水
食道運動異常(アカラシア、強皮症)	
胃・食道悪性腫瘍	
胃食道逆流(食道裂孔ヘルニアを含む)	
胃切除(全摘、亜全摘)	

(文献 9)より一部改変)

気道内に侵入することと定義されている。誤嚥には、急速に多量の胃内容物などを誤嚥する場合と気づかぬうちに少量の口腔・咽頭分泌物や胃内容物を繰り返して気道内に吸引する不顕性誤嚥とがある。

誤嚥性肺炎は、さまざまな程度の嚥下障害を背景にし、多くは高齢者に発症する。高齢者では咳反射や嚥下反射の低下を認めやすく、特に基底核領域の脳血管障害では昼間より就寝中に嚥下反射が低下し、誤嚥を起こしやすくなる。ほかにも、基礎疾患の影響や薬剤などの医原性要因などが複合的に関与して誤嚥性肺炎を発症すると考えられている(表 5)[9]。

誤嚥の存在を疑わないと誤嚥性肺炎の診断はできないので、誤嚥を起こしやすい病態があり、肺炎を繰り返す場合には積極的に誤嚥性肺炎を疑うようにする。誤嚥性肺炎の診断は、臨床経過と胸部 X 線写真や胸部 CT から行う。陰影は下葉、背側に多いが、繰り返している場合はびまん性に存在し、斑状影、スリガラス影、粒状影など多彩な性状を呈する。嚥下障害の診断には、嚥下造影検査(VF)や嚥下反射試験(2 段階簡易嚥下誘発テスト：東大法)などがある。

[2] 重要な病原体と治療の実際

誤嚥性肺炎の病原体は発症機序からみて、口腔内細菌が関与すると考えられている。しかし基本的には市中発症例では市中肺炎の原因菌、院内発症例では院内肺炎の原因菌と大差はなく、嫌気性

○メモ
Mendelson は 1946 年に全身麻酔による無痛分娩後に重篤な誤嚥性肺炎が高率に起こることを報告し、これは胃酸の誤嚥で生じる化学性肺炎(メンデルソン症候群)とされている。突然発症し数時間で重症化するが、60%以上が自然治癒する。しかし 12%は劇症化し死に至り、26%はいったん回復するも細菌による二次感染を引き起こす。

菌の重要性がより強調されている。また嘔吐に伴う誤嚥のときには細菌感染を生じていないこともあるが、鑑別は難しく、二次感染の可能性もあるので、抗菌薬を投与して経過をみる。

誤嚥性肺炎は高齢者や基礎疾患を有する症例に発症するので、抗菌薬の選択は肺炎の重症度を中等症以上として対処する必要がある。β-ラクタマーゼ阻害薬配合ペニシリン系薬、カルバペネム系薬、クリンダマイシン(CLDM：ダラシンS® 注、600～2,400 mg/日・1日2～4回)などを使用する(図3)。

[3] 誤嚥性肺炎の予防

誤嚥の危険があれば、食物形態の選択や適切な食事の体位を決定し、誤嚥を起こし難い食べ方や介助法を患者・家族に指導する。また口腔ケア(歯磨き、清拭、含嗽など)を実施し、口腔衛生を保つように努める。

咽頭から喉頭のサブスタンスPが枯渇すると嚥下反射が著しく低下することが動物実験で証明されている。降圧薬のアンギオテンシン変換酵素阻害薬(ACE阻害薬)にはサブスタンスPの分解を抑制する作用があるので、嚥下反射を改善させ、誤嚥性肺炎の予防が期待できる。

嚥下障害が強く自力での食事摂取が不可能な場合や誤嚥を繰り返す場合には、経管栄養が必要となる。この場合、経鼻経管栄養法よりも経皮内視鏡的胃瘻造設術(PEG)による栄養補給法の方が誤嚥性肺炎の予防に優れている。

(古西　満、三笠桂一)

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11 マクロライド系抗菌薬

1 化学構造—基本骨格と各製剤の構造

マクロライド系抗菌薬は巨大なラクトン環を有し，ラクトン環と糖とのグルコシド結合が化学構造の特徴である。市販中のマクロライド系薬は，ラクトン環内の炭素原子数で14員環，15員環，16員環に分類されている。14員環にはエリスロマイシン(EM；エリスロシン)，クラリスロマイシン(CAM；クラリシッド，クラリス)，ロキシスロマイシン（RXM；ルリッド）がある。15員環のアジスロマイシン(AZM；ジスロマック）はEMのラクトン部にメチル置換窒素が導入され，アザライド系抗菌薬ともよばれる。16員環にはキタサマイシン(LM；ロイコマイシン)，ロキタマイシン（RKM；リカマイシン)，アセチルスピラマイシン（AC-SPM；アセチルスピラマイシン)，ジョサマイシン(JM；ジョサマイシン)，ミデカマイシン(MDM；ミオカマイシン，メデマイシン）がある（**図1**)。

EMは胃酸で6位水酸基と9位ケト基の間にエノールエーテルが形成され，さらに9位と12位の間にスピロケタールが形成されると，抗菌活性を失うとともに，腸管蠕動作用を示し，胃腸障害をきたす欠点がある。CAMは6位水酸基をメチル化して酸に対する安定性を獲得している。9位カルボニル基を修飾した置換ヒドロキシアミン誘導体であるRXMは，経口吸収が速やかで，血中半減期が長くなる。AZMは9位カルボニル基がなくなったため，酸に極めて安定である[1]。

2 臨床的分類

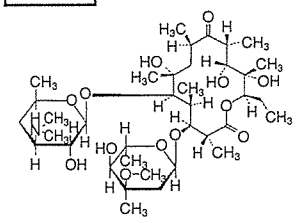
CAM，RXM，AZMは胃酸での不活化を受けにくく，経口吸収が優れ，血中濃度上昇，半減期延長，組織移行性増大などの特性をもち，ニューマクロライドとよばれている[2]。また16員環マクロライド薬では，RKMが吸収や組織移行性が改良された薬剤である（**図2**)。

EMとLMには経口薬以外に注射薬もある。LM，AC-SPM，RXMには小児適応がない（**図2**)。

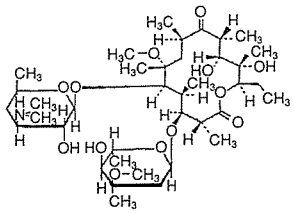
3 抗菌作用点

DNAからの遺伝情報はメッセンジャーRNA（mRNA）へ転写され，蛋白質が合

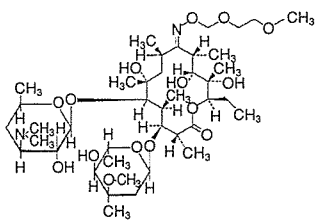
14員環



エリスロマイシン(EM)

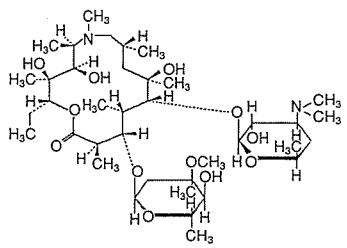


クラリスロマイシン(CAM)



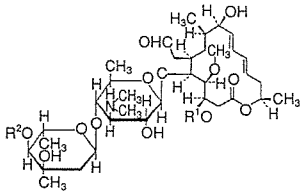
ロキシスロマイシン(RXM)

15員環

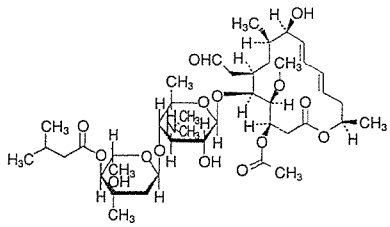


アジスロマイシン(AZM)

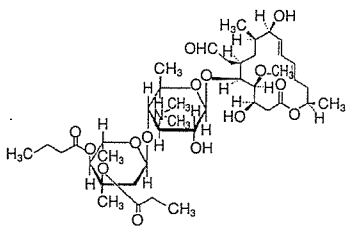
16員環



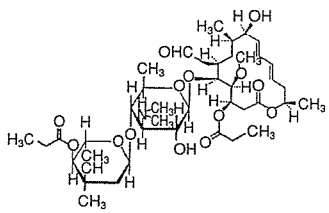
キタサマイシン(LM)



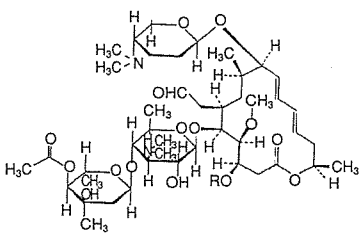
ジョサマイシン(JM)



ロキタマイシン(RKM)



ミデカマイシン(MDM)



アセチルスピラマイシン(AC-SPM)

図 1　マクロライド系抗菌薬の化学構造

(日本薬局方から抜粋)

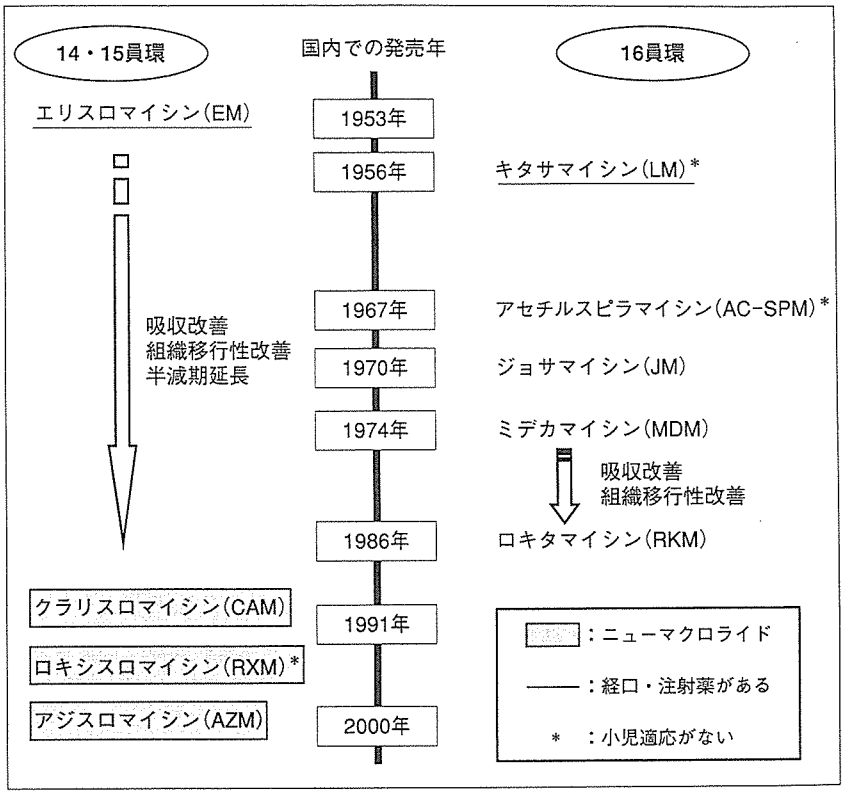


図 2 マクロライド系抗菌薬の開発経緯と臨床特性

成される。リボゾームはアミノ酸を運搬するトランスファーRNA（アミノアシルtRNA)がmRNAの対応するコドンに結合する場を提供する。リボゾームの小さなサブユニットはmRNAと結合し，大きいサブユニットは合成中のポリペプチドを運んでいる。リボゾームは5'末端から3'末端まで動き，コドンに対応したアミノアシルtRNAから1つのアミノ酸が結合し，ポリペプチド鎖が伸長して，蛋白質が合成される[3]（**図3**)。

マクロライド系薬は，細菌の70Sリボゾームの50Sサブユニットでペプチジル部位とアミノアシル部位の間に結合，ペプチド転移反応を阻害し，細菌の増殖を抑制する[3]（**図3**)。その作用は主に静菌的であるが，高濃度では殺菌的にも作用する。

4 実際に有効な疾患・菌種

マクロライド系抗菌薬，特にEMは，皮膚科領域感染症や呼吸器感染症を中心に幅広い適応疾患の承認を受けている。さらに，EMは破傷風，LMはつつが虫病，CAMはエイズの播種性マイコバクテリウム・アビウム・コンプレックス（*Mycobacterium avium* complex；MAC）症，胃潰瘍・十二指腸潰瘍におけるヘリコバクター・ピロリ（*Helicobacter pylori*）感染症に適応をもっている（**表1**)。

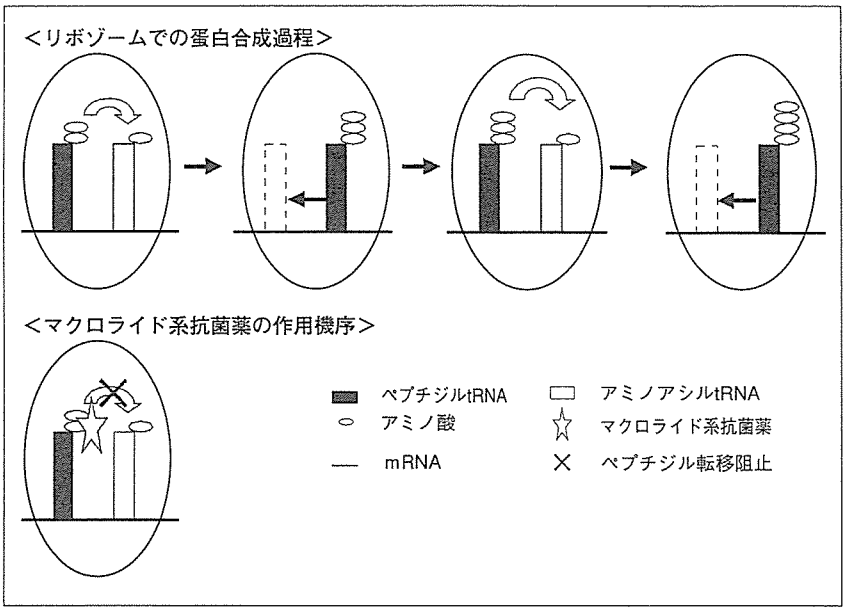


図 3　リボゾームでの蛋白合成過程の模式図とマクロライド系抗菌薬の作用機序

（文献 3 より）

表 1　マクロライド系抗菌薬（経口・注射薬）の適応症概要

薬剤＼適応症	敗血症	感染性心内膜炎	皮膚科領域感染症	外科・整形外科領域感染症	呼吸器感染症	尿路感染症	性感染症	胆嚢炎	感染性腸炎	産婦人科領域感染症	眼科領域感染症	耳鼻科領域感染症	歯科・口腔外科領域感染症	猩紅熱	ジフテリア	百日咳	破傷風	つつが虫病	エイズの播種性MAC症	ヘリコバクター・ピロリ感染症
EM			○	○	○	○	○		○	○	○	○	○	○	○	○	○			
CAM			○	○	○		○		○			○	○	○		○			○	○
RXM			○		○							○	○							
AZM			○		○		○					○	○							
LM	●	●	○		○		○	●				○		○	○	○		○		
RKM			○		○				○			○	○							
AC-SPM			○	○	○		○			○	○	○		○						
JM			○	○	○	○			○		○	○	○	○						
MDM			○	○	○	○					○	○	○							

●：注射薬のみの適応

（インタビューフォームより抜粋）

表 2　マクロライド系抗菌薬の臨床分離菌に対する抗菌力；MIC_{90}

	EM	CAM	RXM	AZM	JM
Staphylococcus aureus (MSSA)	>100	1.56	1.56	3.13	>100
Staphylococcus aureus (MRSA)	>100	>100	>100	>100	>100
Streptococcus pyogenes	0.1	0.05	0.39	0.1	0.39
Streptococcus pneumoniae	>100	12.5	>100	>100	3.13
Enterococcus faecalis	>100	>100	>100	>100	>100
Haemophilus influenzae	6.25	6.25	25	1.56	50
Moraxella catarrhalis	0.39	0.2	0.78	0.1	1.56
Escherichia coli	—	>128	—	16	—
Klebsiella pneumoniae	—	>128	—	64	—
Neisseria gonorrhoeae	1	1	—	0.125	—
Peptostreptococcus anaerobius	6.25	3.13	25	>100	3.13
Legionella pneumophila	2	0.125	—	2	—
Mycoplasma pneumoniae	0.0156	0.0078	—	0.00024	0.03125
Chlamydia pneumoniae	0.125	—	—	0.125	0.015
Helicobacter pylori	128	2	—	>128	—

(文献 4—6 より)

マクロライド系薬は β-ラクタム系抗菌薬が無効であるマイコプラズマ，クラミジア，レジオネラなどに有効である。一般細菌では黄色ブドウ球菌（*Staphylococcus aureus*），A 群溶血性レンサ球菌（*Streptococcus pyogenes*），肺炎球菌（*Streptococcus pneumoniae*），ジフテリア菌（*Corynebacterium diphtheriae*）などのグラム陽性菌，百日咳菌（*Bordetella pertussis*），淋菌（*Neisseria gonorrhoeae*）などのグラム陰性菌，ペプトストレプトコッカスなどの嫌気性菌が適応菌種とされている[4-6]（表 2）。

5　耐性動向

マクロライド系薬の抗菌力は，本来一般細菌では主としてグラム陽性球菌で良好であった。しかし黄色ブドウ球菌の耐性率はメチシリン感受性株（MSSA）で 10% 前後，メチシリン耐性株（MRSA）では 90%以上に達している。肺炎球菌は，重大な耐性の問題をもつ菌種の一つである。肺炎球菌のマクロライド耐性機序には，リボゾーム変異（*ermB* 遺伝子由来）と薬剤排出促進（*mefA* 遺伝子由来）とがある。前者は全てのマクロライド系薬に高度耐性を示し，後者は 14, 15 員環マクロライド系薬に軽・中等度耐性を示す。わが国の肺炎球菌のマクロライド耐性率は 70—80%

に及び，その半数は高度耐性である。A群溶血性レンサ球菌もマクロライド耐性率は増加傾向にある[7]。また，近年臨床的に耐性を疑う *Mycoplasma pneumoniae* による肺炎症例が経験され，その動向が注目されている。

6 常用量と極量

マクロライド系薬はβ-ラクタム系薬と同様に，時間依存性に抗菌活性を発揮する薬剤である。すなわち効果は抗菌薬の血中（組織内）濃度が有効濃度で保たれる時間が影響するため，1回投与量を増やすよりも投与回数を増やすことが有用である。そのため，EMなどは1日4—6回の投与回数が必要である。しかしニューマクロライド（CAM，RXM，AZM）は消化管吸収が優れ，血中半減期や組織移行性も改善されたので，投与量，投与回数とも減らすことが可能となっている（**表3**）。

7 体内動態の特徴

1．血中濃度

EM，CAM，RXM，AZM，MDMの薬動力学的パラメーターを**表4**に示す。最高血中濃度（Cmax）はRXMが最も高く，AZMが最も低い値である。曲線下面積（AUC）もRXMが最も大きく，EMとCAM，AZMとMDMがほぼ同等である[8]。

2．組織移行性

マクロライド系薬，特にニューマクロライドは，臓器移行性や好中球移行性が良好で，主な組織内濃度は血漿中濃度を上回る。EM，RXM，AZMは同様の臓器分布パターンを示し，肝＞腎＝脾＞肺＞心の順に高い濃度を示す。一方，CAMは肺＞脾＞肝＞腎＞心の順に高濃度であり，良好な肺組織への移行が特徴である[9]。ニューマクロライドは好中球内への移行も良好であり，特にAZMはEMの約10倍の移行性を示す（**表4**）。

3．代謝と排泄

マクロライド系薬は，肝臓のチトクロームP-450による脱メチル化を中心とした代謝を受け，主に胆汁中に排泄される。しかしCAMは，尿中回収率が36.9%と高い。また，血中半減期（$T_{1/2}$）はEMの1.78時間に対して，ニューマクロライドは各薬剤とも延長している（**表4**）。特に，AZMの$T_{1/2}$は投与後9時間までが2.8時間，48時間までが17.2時間，168時間までが68.1時間と時間経過とともに延長する[8]ため，1日1回，3日間投与に使用方法が限定されている。

8 臨床で使える領域

現在臨床で使うマクロライド系薬は，吸収や組織分布などを考慮すると，ニューマクロライドを選択することが多い。

マクロライド系薬が第一選択薬となるのは，マイコプラズマ，クラミジア，レジオネラによる呼吸器感染症，百日咳，カンピロバクター腸炎，ヘリコバクター・ピロリ感染症，クラミジアによる性感染症などである。また，エイズの播種性MAC症

表 3　わが国で認められているマクロライド系抗菌薬の用量・用法

薬　剤	成人投与量 (mg/日)	用　法 (回/日)	その他	小児投与量 (mg/kg/日)	用　法 (回/日)	注射薬投与量 (mg/日)	用　法 (回/日)
EM	800—1200	4—6	マクロライド長期療法 400 mg 分 2/600 mg 分 3	25—50	4—6	600—1500	2—3 (2 時間以上かけて点滴)
CAM	400	2	エイズ播種性 MAC 症 800 mg 分 2 *H.pylori* 感染症 400—800 mg 分 2×7 日	10—15	2—3	—	—
RXM	300	2		—	—	—	—
AZM	500	1 (3 日間)	尿道炎・子宮頸管炎 1000 mg×1 回	10	1 (3 日間)	—	—
LM	600—1600	3—4		—	—	400	2
AS-SPM	800—1200	4—6		—	—	—	—
JM	800—1200	3—4		30	3—4	—	—
MDM	800—1200	3—4		30	3—4	—	—
(酢酸ミデカマイシン)	600	3		20—40	3—4	—	—
RKM	600	3		20—30	3	—	—

表 4　マクロライド系抗菌薬の薬動力学的パラメーター

薬　剤	投与量 (mg)	Tmax (h)	Cmax (μg/ml)	AUC (μg・h/ml)	$T_{1/2}$ (h)	好中球への移行比 (細胞内/外)	尿中回収率 (%)
EM	400	2.2	1.88	6.05 (0-12 h)	1.78 (0-12 h)	6.5	3.6 (0-12 h)
CAM	200	1.7	1.11	6.87 (0-12 h)	3.53 (0-12 h)	12.6	36.9 (0-12 h)
RXM	150	1.34	5.35	58.8 (0-8 h)	6.83 (0-8 h)	21.9	5.1 (0-10 h)
AZM	500	2.5	0.58	3.32 (0-48 h)	2.8 (Peak-9 h)	65.0	9.0 (0-168 h)
MDM	600	0.59	1.64	2.45 (0-8 h)	0.69 (0-8 h)	—	2.1 (0-10 h)

(文献 8 より一部改変)

表 5　マクロライド系抗菌薬による治療を考慮すべき感染症

呼吸器感染症	1）マイコプラズマ，クラミジア，レジオネラ感染症 2）百日咳 3）難治性慢性下気道感染症（長期療法） 4）A 群溶血性レンサ球菌感染症（β-ラクタム薬が使用できない場合）
腸管感染症	1）カンピロバクター腸炎 2）ヘリコバクター・ピロリ感染症
性感染症	1）クラミジア感染症 2）軟性下疳
耳鼻科領域感染症	1）ジフテリア 2）慢性副鼻腔炎（長期療法） 3）滲出性中耳炎（長期療法）
その他	1）エイズの播種性 MAC 症 2）Q 熱

や Q 熱にも使用する。さらに，マクロライドの新作用を期待した使い方として，難治性慢性下気道感染症，慢性副鼻腔炎，滲出性中耳炎には長期療法で用いる[4]（**表 5**）。

A 群溶血性レンサ球菌，黄色ブドウ球菌，肺炎球菌，インフルエンザ菌などの一般細菌に対しては，β-ラクタム系薬が無効あるいは使用できない場合の二次選択薬とはなるが，黄色ブドウ球菌，肺炎球菌では耐性菌が多い点，インフルエンザ菌では AZM 以外は抗菌力が弱い点に注意する必要がある[4]。

9　小児・高齢者・妊婦への投与の注意点

RXM，LM，AC-SPM には小児適応がない。EM では新生児・乳児で肥厚性幽門狭窄の報告があり，他の薬剤では低出生体重児・新生児に対する安全性が確立していない。

高齢者では一般に生理機能が低下しているので，血中・組織内濃度が高くなることがある。使用する際には，用量に留意するなど慎重に投与する必要がある。

動物実験で CAM，RXM の高用量投与時に胎児毒性を認め，RKM では生児数の減少を認めている。他の薬剤でも妊娠中の投与に関する安全性は確立していない。そのため，治療上の有益性が危険性を上回ると判断される場合にのみ使用する。また，ヒト母乳中へ移行することが全ての薬剤で報告されており，薬剤投与中は授乳を避ける必要がある。

10　副作用・相互作用

マクロライド系薬の副作用では下痢や胃部不快などの消化器症状が最も頻度が高い。ほかには薬疹，肝機能障害，好酸球増多が比較的多くみられる副作用である。

表 6　クラリスロマイシン（CAM）での薬物相互作用の概要

	薬剤名	臨床症状など	機　序
併用禁忌	ピモジド	QT 延長，心室性不整脈	CYP 3 A 4 阻害により当該薬の血中濃度上昇
	エルゴタミン含有製剤	血管攣縮	同上
	シサプリド（国内承認整理済）	QT 延長，心室性不整脈	同上
併用注意	ジゴキシン	嘔気，嘔吐，不整脈等	腸内細菌叢への影響で当該薬の不活化抑制 P-糖蛋白質を介した当該薬の輸送阻害
	テオフィリン等	中毒症状（痙攣，横紋筋融解症等）	CYP 阻害により当該薬の血中濃度上昇
	ジソピラミド	QT 延長，低血糖等	CYP 3 A 阻害により当該薬の血中濃度上昇
	トリアゾラム	傾眠等	同上
	カルバマゼピン	嗜眠，眩暈，眼振，運動失調等	同上
	シクロスポリン	腎障害等	同上
	タクロリムス	クレアチニン上昇等	同上
	クマリン系抗凝固薬	プロトロンビン時間延長等	同上
	ミダゾラム	中枢神経系抑制作用の増強	同上
	カルシウム拮抗薬	血圧低下，頻脈，徐脈	同上
	シンバスタチン等	横紋筋融解症	同上
	スルホニル尿素系血糖降下薬	低血糖	機序不明だが，当該薬の血中濃度上昇
	コルヒチン	中毒症状（汎血球減少，肝障害，筋痛等）	肝臓での代謝阻害で当該薬の血中濃度上昇
	リトナビル	当該薬の血中濃度上昇	CYP 3 A 阻害により当該薬の血中濃度上昇
	イトラコナゾール	当該薬の血中濃度上昇	同上
	リファンピシン	CAM の血中濃度低下	当該薬の CYP 3 A 誘導で CAM 血中濃度低下

（インタビューフォームより抜粋）

重篤な副作用には，ショック，アナフィラキシー様症状，QT 延長，心室性頻拍，皮膚粘膜眼症候群，中毒性表皮壊死症，剝脱性皮膚炎，劇症肝炎，横紋筋融解症，急性間質性腎炎などがある（いずれも頻度不明）。

薬物相互作用は薬物吸収，分布，代謝，排泄の過程で生じる。マクロライド系抗菌薬が関係する薬物相互作用の多くは，代謝酵素阻害を介するものである。マクロライド系薬は主に肝臓で代謝される。ラクトン環に結合したアミノ糖の 3 級アミン部位での N-脱メチル化反応で生じたニトロソ体マクロライドは，チトクローム P-450（CYP）のヘム鉄と共有結合するため，CYP 分子種は不活化される。マクロライド系薬の代謝に関わる CYP 分子種は，CYP 3 A 4 分子種であるので，これによって代謝される多くの薬物と相互作用を生じる可能性がある[10]。**表 6** には，マクロライド系薬の中でも CYP 阻害活性の高い CAM の薬物相互作用を示す。

（古西　満）

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研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Dilip Patel, Lia Danelishvili, Yoshitaka Yamazaki, Marta Alonso, Michael L. Paustian, John P. Bannantine, Lisbeth Meunier-Goddik, and Luiz E. Bermudez	The Ability of Mycobacterium avium subsp. paratuberculosis To Enter Bovine Epithelial Cells Is Influenced by Preexposure to a Hyperosmolar Environment and Intracellular Passage in Bovine Mammary Epithelial Cells	INFECTION AND IMMUNITY	74	2849-2855	2006
Yoshitaka Yamazaki, Lia Danelishvili, Martin Wu, Eiko Hidaka, Tsutomu Katsuyama, Bernadette Stang, Mary Petrofsky, Robert Bildfell and Luiz E. Bermudez	The ability to form biofilm influences Mycobacterium avium invasion and translocation of bronchial epithelial cells	Cellular Microbiology	8	806-814	2006
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The Ability of *Mycobacterium avium* subsp. *paratuberculosis* To Enter Bovine Epithelial Cells Is Influenced by Preexposure to a Hyperosmolar Environment and Intracellular Passage in Bovine Mammary Epithelial Cells

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***Mycobacterium avium* subsp. *paratuberculosis* is the cause of Johne's disease in cattle and other ruminants. *M. avium* subsp. *paratuberculosis* infection of the bovine host is not well understood; however, it is assumed that crossing the bovine intestinal mucosa is important in order for *M. avium* subsp. *paratuberculosis* to establish infection. To examine the ability of *M. avium* subsp. *paratuberculosis* to infect bovine epithelial cells in vitro, Madin-Darby bovine kidney (MDBK) epithelial cells were exposed to *M. avium* subsp. *paratuberculosis*. It was observed that bacteria can establish infection and replicate within MDBK cells. *M. avium* subsp. *paratuberculosis* also has been reported to infect mammary tissue and milk, and we showed that *M. avium* subsp. *paratuberculosis* infects bovine mammary epithelial cells (MAC-T cell line). Using polarized MAC-T cell monolayers, it was also determined that *M. avium* subsp. *paratuberculosis* crosses apical and basolateral surfaces with approximately the same degree of efficiency. Because *M. avium* subsp. *paratuberculosis* can be delivered to the naïve host by milk, it was investigated whether incubation of *M. avium* subsp. *paratuberculosis* with milk has an effect on invasion of MDBK cells. *M. avium* subsp. *paratuberculosis* exposed to milk entered epithelial cells with greater efficiency than *M. avium* subsp. *paratuberculosis* exposed to broth medium or water ($P < 0.01$). Growth of *M. avium* subsp. *paratuberculosis* within MAC-T cells also resulted in augmented ability to subsequently infect bovine MDBK cells ($P < 0.001$). Microarray analysis of intracellular *M. avium* subsp. *paratuberculosis* RNA indicates the increased transcription of genes which might be associated with an invasive phenotype.**

Mycobacterium avium subsp. *paratuberculosis* is the etiologic agent of Johne's disease in cattle and other ruminants. It is assumed that *M. avium* subsp. *paratuberculosis* infects the young calf by crossing the intestinal barrier. Previous work (3, 26) has indicated that the interaction of *M. avium* subsp. *paratuberculosis* with bovine epithelial cells is a complex process which might involve participation of several bacterial and host factors. For example, it has been reported that both in calves and in mice, challenge by the gastrointestinal route results in *M. avium* subsp. *paratuberculosis* infecting M cells in the Peyer's patches (23, 26). Recently, Secott and colleagues have suggested that the invasion of the intestinal mucosa by *M. avium* subsp. *paratuberculosis* is secondary to the binding to fibronectin (26). In addition, Bannantine and colleagues demonstrated a role for a 35-kDa *M. avium* subsp. *paratuberculosis* protein in the invasion of cultured bovine epithelial cells (5). The 35-kDa protein is exposed in the outer layer of *M. avium* subsp. *paratuberculosis* and has also been associated with *Mycobacterium avium* invasion of human intestinal cells (22).

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After *M. avium* subsp. *paratuberculosis* crosses the intestinal mucosa, the infection spreads to other organs, leading to the advanced stages of disease. Several studies have reported the presence of *M. avium* subsp. *paratuberculosis* in the mammary glands and in milk of symptomatic or asymptomatic animals (6, 28–30). This observation raises the possibility that the mammary gland could be a reservoir for *M. avium* subsp. *paratuberculosis*. The environment inside the mammary gland is believed to be hyperosmolar (milk) and hypoxic (28). In addition, *M. avium* subsp. *paratuberculosis* organisms infecting mammary epithelial cells might suffer the influence of the intracellular environment. In fact, *M. avium* subsp. *paratuberculosis* may remain in contact with either the intracellular or milk environment for periods of up to 24 h before being excreted. Previous studies using *Mycobacterium avium* subsp. *avium* have shown that the environment to which the bacterium is exposed in the host can influence the expression of genes associated with its ability to enter epithelial cells. Incubation of *M. avium* subsp. *avium* in low oxygen tension or increased osmolarity conditions significantly enhances its ability to enter intestinal epithelial cells (8). A previous study on interactions between *M. avium* subsp. *avium* and environmental amoebae has shown that infection of amoebae results in enhanced virulence (14). Similar findings were reported regarding the *M. avium* subsp.

paratuberculosis 35-kDa protein (5), which was shown to be expressed under anaerobic and hyperosmolar conditions (5).

It is reasonable to hypothesize that bacterial exposure to the complex conditions in the mammary gland or mammary gland epithelial cells may have an effect on virulence gene expression and, therefore, on the efficacy of *M. avium* subsp. *paratuberculosis* invasion of the intestinal mucosa.

The objectives of this work were to determine whether *M. avium* subsp. *paratuberculosis* has the ability to enter cultured bovine epithelial cells in vitro and whether this characteristic can be influenced by exposure to milk or the intracellular environment of mammary epithelial cells.

MATERIALS AND METHODS

Cell lines. A bovine epithelial cell line (Madin-Darby bovine kidney [MDBK]) was purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained on Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. A bovine mammary epithelial cell line (MAC-T) was kindly provided by Lewis Sheffield (Department of Dairy Science, University of Wisconsin). MAC-T cells were maintained on Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, 5 μg/ml insulin, and 1 μg/ml hydrocortisone.

Bacteria. *M. avium* subsp. *paratuberculosis* ATCC 19698, a bovine clinical isolate from an animal with Johne's disease, was purchased from ATCC. It was grown at 37°C on modified Middlebrook 7H11 agar supplemented with Mycobactin J (2 mg/liter) and oleic acid-albumin-dextrose-catalase (OADC) (10%, vol/vol) or in 7H9 broth with Mycobactin J (2 mg/liter) and OADC (10%, vol/vol). For the invasion assay, individual colonies were selected, and a bacterial suspension was prepared using Hanks' balanced salt solution (HBSS) to give turbidity equivalent to a 0.5 McFarland standard. The *M. avium* subsp. *paratuberculosis* suspension (5 ml) was then passed through a 30-gauge needle 10 times, and clumps in the final suspension were allowed to settle. The top 1 ml out of the 5-ml suspension containing dispersed bacteria was used for the invasion assay.

Invasion assay. The invasion assays were carried out using the methods described by Bermudez and Young and by Sangari et al. (10, 24). Briefly, 24-well tissue culture plates (Corning Costar, New York) were seeded with 10^4 MDBK or MAC-T cells, and monolayers were grown in an atmosphere of 5% CO_2 at 37°C until confluence. Before infection, the medium was replaced with fresh culture medium. Monolayers were then infected with approximately 2×10^6 bacteria (approximate ratio, 10 bacteria:1 cell) and incubated at 37°C in 5% CO_2 for up to 4 h, depending upon the experimental design. After incubation, monolayers were washed three times with HBSS and then treated with 200 μg/ml of amikacin for 2 h at 37°C to kill extracellular bacteria, as shown in the *M. avium* system (8, 10) and confirmed using *M. paratuberculosis* (data not shown). Following incubation, the supernatant was removed and cell monolayers were washed three times with HBSS. To lyse the cells, wells were treated with 0.5 ml Triton X-100 (0.1%) for 10 min. Subsequently, 0.5 ml 7H9 broth containing Mycobactin J (2 mg/liter) and OADC (10%, vol/vol) was added to each well, and cells were disrupted by vigorous pipetting. Lysates were collected, serially diluted, and plated onto 7H11 agar for colony count. The percentage of invasion was calculated as the fraction of the inoculated bacteria that was recovered from the cell lysate.

***M. avium* subsp. *paratuberculosis* survival in MDBK and MAC-T cells.** Confluent monolayers of bovine epithelial cells were incubated with approximately 10^6 *M. avium* subsp. *paratuberculosis* organisms for 2 h. After the incubation period, extracellular bacteria were killed by amikacin treatment (as described above), the supernatant was removed, and fresh culture medium was added to the monolayers. Infected cells were incubated for up to 4 days. At the end of the incubation period, monolayers were lysed, the lysate was diluted, and the number of intracellular bacteria was determined by plating cell lysates onto 7H11 agar.

Translocation assay. The translocation assay was performed using the Transwell 2-chamber culture system (Corning Costar, New York). Monolayers were established either on the top or bottom sides of the membrane by seeding it with 1×10^5 MAC-T cells. Some monolayers had their apical surface exposed to the medium in the bottom chamber, whereas others had their basolateral side exposed to the medium in the top chamber. The culture medium was changed daily, and the integrity of the monolayers was determined by the following methods: (i) microscopic observation, (ii) measuring the transepithelial resistance, and (iii) the trypan blue (0.25%) permeability assay (optical density at 580 nm), as previously described (9, 24). Trypan blue (0.25%) was added to the monolayer, and 3 h later the supernatant of the lower chamber was obtained for spectrometer reading. Controls included medium only (baseline). The top chamber was infected with 3×10^7 bacteria as described above. After 1, 2, 3, and 4 days infection, 600 μl of filtrate was collected from the bottom chamber, and fresh culture medium (600 μl) was replenished. The ability to translocate was calculated as the cumulative percentage of the initial inoculum recovered in the bottom chamber at each time point.

Passage of *M. avium* subsp. *paratuberculosis* in MAC-T cells. Confluent monolayers of MAC-T cells in cell culture flasks (Corning Costar, New York) were inoculated with approximately 10^7 *M. avium* subsp. *paratuberculosis* bacteria. After 2 h of contact time, extracellular bacteria were removed, followed by amikacin treatment as described previously, and new medium was added to the monolayer. Internalized bacteria were allowed to grow at 37°C for a total of 1 or 4 days. At the end of the incubation period, MAC-T cells were lysed by adding 0.5 ml Triton X-100 (0.1%) for 10 min. Lysates were then submitted to differential centrifugation as reported previously (8). The *M. avium* subsp. *paratuberculosis* cells were collected into 7H9 broth containing Mycobactin J (2 mg/liter) and OADC (10%, vol/vol). The bacteria were recovered by centrifugation at $3,500 \times g$ for 30 min at 4°C to maintain their phenotype and then used in the invasion assays.

Preexposure of *M. avium* subsp. *paratuberculosis* to milk and milk components. Approximately 10^7 *M. avium* subsp. *paratuberculosis* organisms were inoculated into 5 ml of raw milk (from a pool of several cows) containing polymyxin B (5.5 mg/liter), amphotericin (11 mg/liter), carbenicillin (25 mg/liter), and trimethoprim (2.5 mg/liter). Incubation was carried out at 37°C. After 24 h, *M. avium* subsp. *paratuberculosis* was recovered by centrifugation at $3,500 \times g$ for 30 min at 5°C. Subsequently, bacteria were resuspended in HBSS, quantified by both McFarland turbidity standard and colony count, and used for infection of MDBK cells. Sterile water and 7H9 broth treated in the same manner as milk were used as controls. To determine the role of milk components, casein and serum protein fractions were separated by centrifuging milk at $90,000 \times g$ for 2 h at 5°C (16). The casein fraction formed the pellet, while serum protein and the lactose fraction remained in the supernatant. The volumes of the casein fraction and serum-protein-plus-lactose fractions were brought to the level of the original volume of milk by adding water. The casein fraction with 0.9% NaCl was prepared by addition of NaCl to the casein fraction. The 4.8% lactose sample was prepared by dissolving lactose in water.

RNA isolation and DNA microarray. An *M. avium* subsp. *paratuberculosis* whole genome microarray, containing 70-mer oligonucleotides representing 98% of *M. avium* subsp. *paratuberculosis* coding sequences identified in the *M. avium* subsp. *paratuberculosis* K-10 genome, was provided by the National Animal Disease Center, Ames, Iowa. For RNA isolation, *M. avium* subsp. *paratuberculosis* bacteria were kept in contact with confluent monolayers of MAC-T cells grown in T75 flasks for 2 h. Extracellular bacteria were removed by washing, followed by amikacin treatment for 2 h. Intracellular bacteria were recovered after 24 h by lysing MAC-T cells with sterile water. Cell debris and nuclear fractions were removed by low-speed centrifugation at 500 rpm for 5 min at 4°C (20). The bacterial pellet was recovered from the supernatant after centrifugation at $3,000 \times g$ for 10 min at 4°C. Pelleted bacteria were stored at −70°C. The RNA extraction procedure used a combination of guanidine-thiocyanate-based buffer (TRIzol; Invitrogen, Carlsbad, CA) and rapid mechanical cell lysis, as described previously (18). Briefly, 1 ml TRIzol reagent was added to the bacterial pellet and gently mixed. The mixture was added to a (2-ml) screw-cap tube containing 0.4 ml glass beads (0.1 mm). Cell disruption was carried out in a bead beater using three beatings for 30 s each. The TRIzol solution was transferred to a heavy phase lock gel (Eppendorf, Hamburg, Germany) after centrifugation at 3,000 rpm for 90 s at 4°C. Subsequently, 300 μl of chloroform:isoamyl alcohol (24:1) was added to the heavy phase lock gel tube. This was followed by rapid mixing for 15 s. Mixing was continued periodically for 2 additional min, followed by centrifugation at 6,000 rpm for 10 min at 4°C. The aqueous layers were precipitated with cold isopropanol. Isopropanol was then carefully removed without disturbing the RNA pellet, and the final washing was done in 80% ethanol. The RNA was stored at −70°C until further use. RNA quality was determined spectrophotometrically (optical density at 260/280 nm). A ratio of ≥2.0 was considered significant. RNA was treated with DNase as per the manufacturer's instructions (QIAGEN, Valencia, CA). The RNA-DNA hybridization and microarray analysis were carried out at the Central Service Laboratory, Oregon State University, Corvallis, Oregon. RNA prepared from *M. avium* subsp. *paratuberculosis* bacteria incubated in 7H9 broth served as a control, whereas *M. avium* subsp. *paratuberculosis* bacteria incubated in MAC-T cells served as the treatment. For the DNA microarray, the Array 900 MDX (Gemisphere, Hatfield, PA) labeling and detection system was used as per instructions.

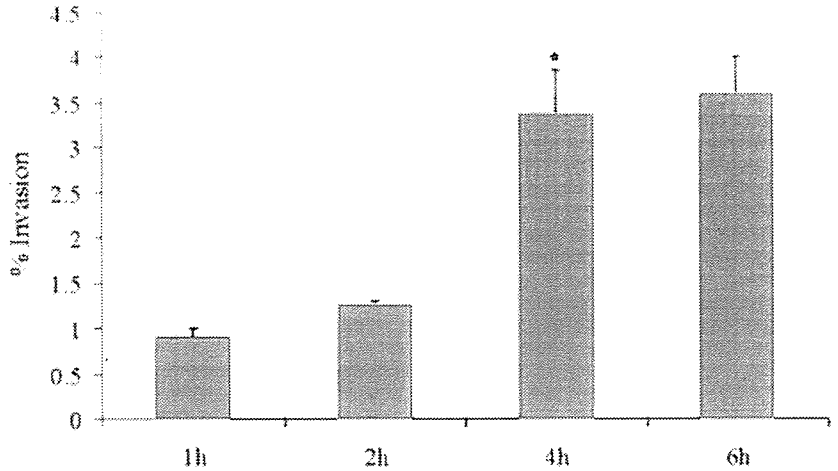

FIG. 1. Invasion of bovine epithelial cells (MDBK) by *M. avium* subsp. *paratuberculosis*. The percentage of invasion was defined as the fraction of inoculated bacteria that became internalized after the incubation period. Values represent the means of three experiments ± standard errors of the means (SEM). *, $P < 0.05$ compared with the percent invasion at 2 h.

Microarray analysis was carried out as follows. Briefly, RNA samples were reverse transcribed using deoxynucleoside triphosphates and a random primer. The cDNA was labeled at the 3′ end with a terminal deoxynucleotidyltransferase tailing reaction followed by ligation to the 3DNA capture sequence. The tagged cDNA was purified using a PCR purification kit (QIAGEN, Valencia, CA), and then successive hybridization of cDNA and 3DNA was carried out using the *M. avium* subsp. *paratuberculosis* genome microarray.

RT-PCR. cDNA amplification for genes that have been shown to be upregulated by DNA array was carried out as previously described (22). We used specific primers for five selected genes: MAP0482, MAP0706, MAP2450c, MAP2751, and MAP3305c. PCR amplification was carried out at 95°C for 3 min (1 cycle); 95°C for 3 s, 62°C for 30 s, and 72°C for 2 min in a linear range (35 cycles); and then 72°C for 10 min (1 cycle). Equivalent amounts of cDNA were used for the reverse transcriptase (RT)-PCR. The products obtained by PCR amplification were quantified using the Kodak EDEAS 290 system and the Kodak ID image analysis software (Eastman Kodak Company, Rochester, NY). The 16S cDNA was used as a control.

Transmission electron microscopy. Approximately 5×10^4 MAC-T cells were grown in T25 tissue culture flasks (Corning Costar, New York) until confluence was established and then inoculated with approximately 10^7 *M. avium* subsp. *paratuberculosis* bacteria. Incubation was carried out for 24 h under 5% CO_2 at 37°C. After the incubation period, MAC-T cells were treated with trypsin (0.5%) for 10 min and subsequently fixed in 0.1 M cacodylate buffer (pH 7.3) containing 3% glutaraldehyde and 2% paraformaldehyde. After fixing, cells were pelleted and surrounded by 1% agarose, cut into quarters, and processed as per the following schedule: 0.1 M cacodylate buffer (2×), 30 min at room temperature (RT); 1% osmium tetroxide in 0.1 M cacodylate buffer, 1 h at RT; 0.1 M cacodylate buffer (2×), 30 min at RT; acetone (10%, 30%, 50%, 70%, 80%, 95%, and 100%), all 10 min at RT; 3:1 acetone:resin, 30 min at RT; 1:1 acetone:resin, 30 min at RT; 1:3 acetone:resin, 30 min at RT; 100% resin, 1 h at 30°C; and 100% resin, 20 h at RT. Fresh resin was placed in polymerization capsules for 24 h at 60°C. The blocks were sectioned on an MT-5000 ultramicrotome (Sorvall, Newton, CO) using a diamond knife, and sections were placed on 300-mesh copper grids. Grids were stained with saturated uranyl acetate and lead citrate and viewed using a Zeiss 10A transmission electron microscope (Carl Zeiss, Thornwood, NY).

Statistical analysis. Each experiment was repeated at least three times, and significant differences were determined using Student's *t* test (unpaired) and analysis of variance. A *P* value of <0.05 was considered significant.

RESULTS

***M. avium* subsp. *paratuberculosis* invasion and survival in MDBK cells.** Invasion assays were carried out to examine the ability of *M. avium* subsp. *paratuberculosis* to enter bovine epithelial cells using MDBK cells as a model of intestinal mucosa. The results shown in Fig. 1 indicate that *M. avium* subsp. *paratuberculosis* can invade MDBK cells. The efficiencies of invasion at 1-h and 2-h time points were comparable; however, a significant increase ($P < 0.05$) in invasion was observed after 6 h of contact time.

The ability of *M. avium* subsp. *paratuberculosis* to survive inside MDBK cells was evaluated by incubating intracellular bacteria for 24 h or 96 h after invasion. The number of *M. avium* subsp. *paratuberculosis* bacteria increased at 96 h of infection compared with the inoculum taken at 2 h (Table 1), suggesting that *M. avium* subsp. *paratuberculosis* is able to survive inside MDBK cells.

Mammary gland as a reservoir for *M. avium* subsp. *paratuberculosis*. The results of *M. avium* subsp. *paratuberculosis* invasion of the MAC-T cell line are shown in Fig. 2A. *M. avium* subsp. *paratuberculosis* entered MAC-T cells, although the efficiency of invasion did not change during the first 2 h of incubation. However, invasion was greater at 4 h ($P < 0.05$) than at 2 h. *M. avium* subsp. *paratuberculosis* could be observed within vacuoles by using transmission electron microscopy (Fig. 2B). The survival experiments whose results are shown in Table 2 indicate that *M. avium* subsp. *paratuberculosis* is able to persist over time within MAC-T cells.

In the mammary gland, the apical surface of epithelial cells faces the alveolar lumen, whereas the basolateral side faces the

TABLE 1. Intracellular survival of *M. avium* subsp. *paratuberculosis* in bovine epithelial cells (MDBK)

Time (h)[b]	Mean no. of intracellular bacteria ± SEM (CFU/ml)[a]
2	$2.36 \times 10^3 \pm 0.44 \times 10^3$
24	$2.81 \times 10^3 \pm 0.39 \times 10^3$
96	$3.26 \times 10^3 \pm 0.43 \times 10^3$

[a] Values represent means of three experiments ± SEM. A *P* value of >0.05 resulted for the comparison between the values for 24 h and 96 h and the value for 2 h.

[b] MDBK cells were exposed to *M. avium* subsp. *paratuberculosis* for 2 h. Extracellular bacteria were removed in all experiments after 2 h. Internalized bacteria were allowed to grow for 24 h or 96 h.

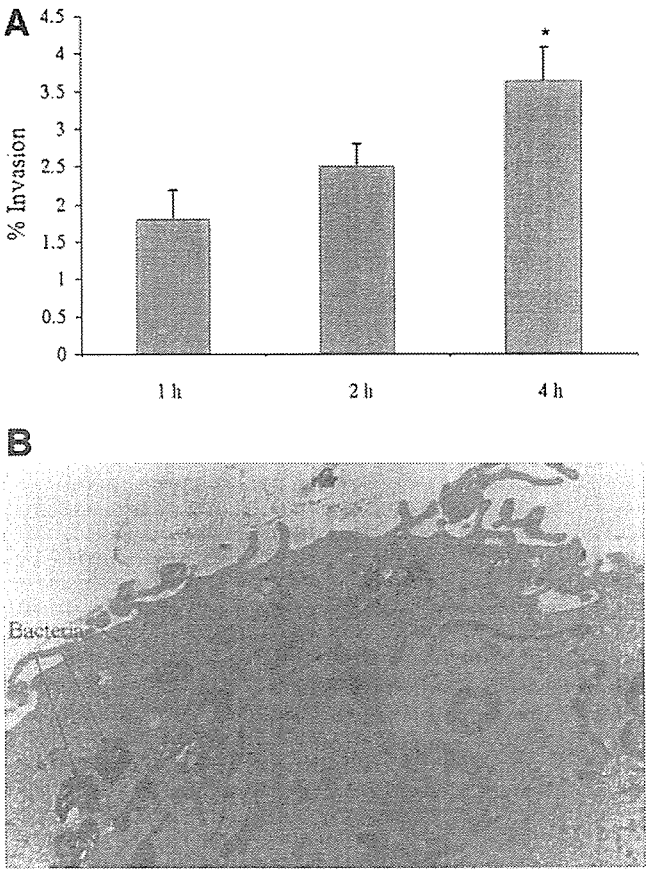

FIG. 2. (A) Invasion of bovine mammary epithelial cells (MAC-T) by *M. avium* subsp. *paratuberculosis*. The percent invasion was defined as the fraction of inoculated bacteria that became internalized after the incubation period. Values represent the means of three experiments ± SEM. *, $P \leq 0.05$ compared with the percent invasion at 2 h. (B) Representative transmission electron micrograph of mammary epithelial cells (MAC-T) after 24 h of infection by *M. avium* subsp. *paratuberculosis* bacteria (arrows). Bacterial cells can be seen within vacuoles. Magnification, ×10,000.

bloodstream. To investigate whether there was a preferential route for entry, invasion assays were performed using polarized cell monolayers. *M. avium* subsp. *paratuberculosis* crossed the MAC-T cells' polarized monolayers equally well from the apical and basolateral surfaces (Table 3). These results in vitro indicate that infection of mammary epithelial cells could potentially occur across both membrane surfaces.

TABLE 2. Intracellular survival of *M. avium* subsp. *paratuberculosis* in bovine mammary epithelial cells (MAC-T)

Time of infection (h)[b]	Mean no. of intracellular bacteria ± SEM (CFU/ml)[a]
2	$0.91 \times 10^4 \pm 0.25 \times 10^4$
24	$0.96 \times 10^4 \pm 0.28 \times 10^4$
96	$1.29 \times 10^4 \pm 0.39 \times 10^4$

[a] Values represent means of three experiments ± SEM.
[b] MAC-T cells were exposed to *M. avium* subsp. *paratuberculosis* for 2 h. Extracellular bacteria were removed in all experiments after 2 h. Internalized bacteria were allowed to grow for 24 h or 96 h.

TABLE 3. Translocation of *M. avium* subsp. *paratuberculosis* across polarized monolayers[b] of bovine mammary epithelial cells[c] (MAC-T)

Invasion surface	*M. avium* subsp. *paratuberculosis* inoculum recovered after designated incubation period (%)[a]			
	1 day	2 days	3 days	4 days
Apical	0.11 ± 0.02	0.25[d] ± 0.03	0.35[d] ± 0.04	0.49[d] ± 0.05
Basolateral	0.15 ± 0.02	0.26[e] ± 0.01	0.34[d] ± 0.02	0.47[d] ± 0.03

[a] Translocation percentage was defined as the percentage of *M. avium* subsp. *paratuberculosis* inoculum that was recovered from the bottom chamber of the Transwell apparatus. The results represent the means of three experiments within rows ± SEM.
[b] Monolayer integrity was verified by a trypan blue dye exclusion assay and by measuring transepithelial resistance.
[c] MAC-T cells were exposed to *M. avium* subsp. *paratuberculosis* for 1, 2, 3, or 4 days from either the apical or the basolateral surface.
[d] $P < 0.05$, cumulative percent translocation after 2, 3, and 4 days compared with that after 1 day within individual rows.
[e] $P < 0.01$, cumulative percent translocation after 2 days compared with that after 1 day within individual row.

Incubation with milk and efficiency of invasion. Since *M. avium* subsp. *paratuberculosis* in the mammary gland may be exposed to milk, we attempted to evaluate the effect of incubation of *M. avium* subsp. *paratuberculosis* in milk on the ability to enter bovine epithelial cells. *M. avium* subsp. *paratuberculosis* bacteria were exposed to milk (increased osmolarity conditions), 7H9 broth (iso-osmolar medium), or water (hypo-osmolar medium) at 37°C for 24 h in the presence of antibiotics (polymyxin B [5.5 mg/liter], amphotericin [11 mg/liter], carbenicillin [25 mg/liter], and trimethoprim [2.5 mg/liter]). The antibiotics were used to prevent the growth of other microorganisms present in milk. After incubation, the ability to enter MDBK cells was evaluated. The efficiency of invasion was significantly greater when *M. avium* subsp. *paratuberculosis* was preincubated in milk than when *M. avium* subsp. *paratuberculosis* was exposed to other environments (Fig. 3A).

To determine whether a specific milk component was associated with increased invasion, *M. avium* subsp. *paratuberculosis* was exposed to different milk components prior to invasion of MDBK cells. Figure 3B shows that the efficiency of invasion of *M. avium* subsp. *paratuberculosis* after incubation in casein alone (low-osmolar medium) was lower than with milk ($P < 0.05$). However, differences were not significant when milk was compared to casein with 0.9% NaCl, lactose, or lactose plus serum protein (hyperosmolar conditions) (Fig. 3B).

Intracellular phenotype. Because *M. avium* subsp. *paratuberculosis* is encountered intracellularly during infection and is potentially eliminated from the mammary gland within detached epithelial cells, it is of paramount importance to determine whether *M. avium* subsp. *paratuberculosis* infection of mammary epithelial cells impacts its ability to enter bovine MDBK epithelial cells. MAC-T cells infected with *M. avium* subsp. *paratuberculosis* were maintained for 1 or 4 days in culture before they were lysed. Intracellular bacteria recovered after lysis were then used to infect MDBK cells. Results shown in Fig. 4 indicate that the efficiency of invasion by *M. avium* subsp. *paratuberculosis* collected from infected MAC-T cells was approximately 10-fold greater than that of *M. avium* subsp. *paratuberculosis* incubated in medium.

DNA microarray. To determine whether *M. avium* subsp. *paratuberculosis* genes are upregulated during the intracellular

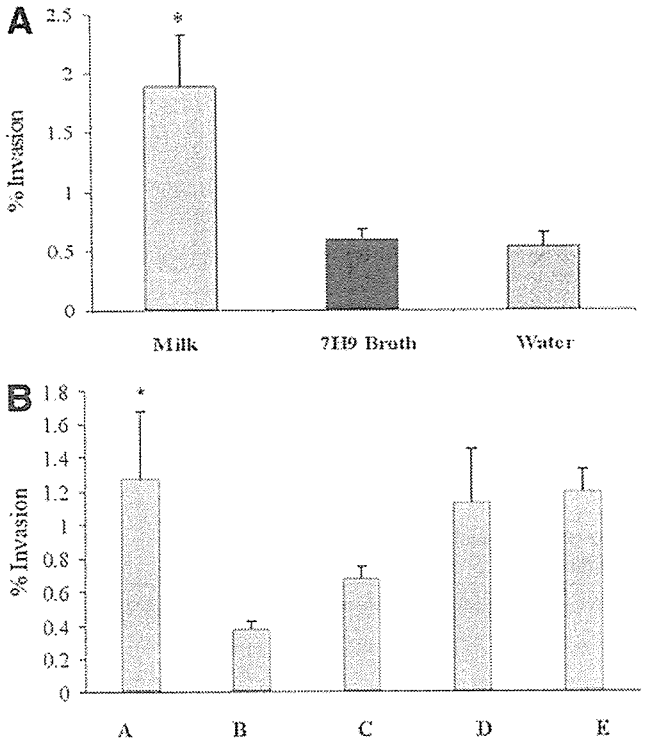

FIG. 3. (A) Ability of *M. avium* subsp. *paratuberculosis* to invade MDBK epithelial cells following exposure to three different environments: milk, 7H9 broth, or water for 24 h at 37°C. Values are the means of three experiments ± SEM. *, $P < 0.01$ compared with 7H9 broth or water. (B) Invasion of bovine epithelial cells (MDBK) by *M. avium* subsp. *paratuberculosis*. Prior to invasion, *M. avium* subsp. *paratuberculosis* bacteria were incubated in the presence of milk (column A), casein (column B), casein plus 0.9% NaCl (column C), serum protein plus lactose (column D), or lactose (4.8% solution) (column E) for 24 h at 37°C. Values represent the means of three experiments ± SEM. *, $P < 0.05$ for difference between the invasion percent for milk (column A) and casein (column B) casein plus NaCl (column C).

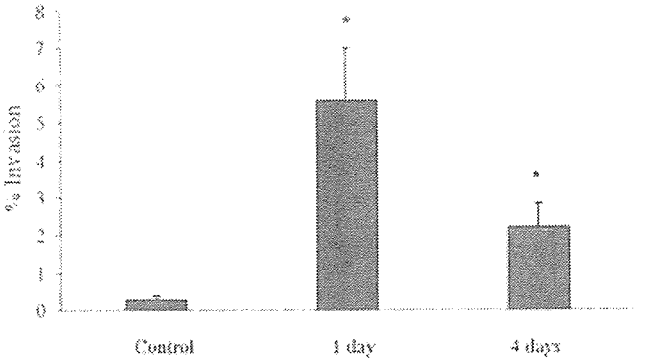

FIG. 4. Invasion of bovine epithelial cells (MDBK) by *M. avium* subsp. *paratuberculosis* after passage in MAC-T cells. *M. avium* subsp. *paratuberculosis* bacteria were incubated in MAC-T cells for 1 or 4 days and then used to infect MDBK epithelial cells. *M. avium* subsp. *paratuberculosis* incubated in medium was used as a control. Values represent the means of three experiments ± SEM. *, $P < 0.001$ compared with the invasion percent for the control.

stage in mammary epithelial cells, DNA microarray analysis was carried out. MAC-T cells were infected for 24 h and *M. avium* subsp. *paratuberculosis* RNA was obtained. The 24-h time point was chosen based on the greater invasion of MDBK cells. The results in Table 4 indicate that upregulation of a number of *M. avium* subsp. *paratuberculosis* genes occurs during infection of MAC-T cells.

M. avium subsp. *paratuberculosis* whole genome microarray analysis identified 20 genes that showed gene expression threefold or higher than control. The in silico analysis of these genes suggests a diverse array of regulatory, metabolic, and candidate virulence-associated factors. For example, genes MAP0482, MAP1695c, MAP3404, MAP1259, and MAP2652c encode transcription-regulatory proteins. Among other differentially expressed genes, MAP0392 belongs to an operon encoding an upstream transcription-regulatory protein. MAP0462, which encodes a urease alpha subunit, and MAP3404, encoding a biotin carboxyl bifunctional carrier protein, are essential genes for survival based on their homology with *Mycobacterium tuberculosis* (25). Among other differentially expressed genes are MAP2450c (encoding a probable ATP synthase), MAP0462 (encoding a tRNA synthetase), and MAP3224 (involved in secretion of β-lactamase). Finally, MAP2751, which has previously been shown to be present uniquely in *M. avium* subsp. *paratuberculosis* (4) and has no known function, also showed increased transcription within MAC-T cells. The expression of five of those genes was evaluated using RT-PCR and shown to correspond to the DNA array analysis (Table 4).

DISCUSSION

Mycobacterial invasion of intestinal epithelial cells is a complex event, requiring participation of several bacterial and host factors. However, *M. avium* subsp. *paratuberculosis* infection is difficult to study in large animal model systems. Tissue culture cell models have been shown to be useful to obtain insights into the host-pathogen interactions. For example, Madin-Darby canine kidney cells have been used by several laboratories to study bacterial pathogenesis (17, 20). In addition, a number of studies employed cultured epithelial cells to examine the interaction between the bacteria and host cells (8, 9, 14). Earlier work has established MDBK epithelial cells as a model of bovine intestinal mucosa (5). When confluent monolayers of MDBK cells were exposed to *M. avium* subsp. *paratuberculosis*, it was observed that *M. avium* subsp. *paratuberculosis* was capable of invading epithelial cells. Since epithelial cells are not phagocytic in nature, *M. avium* subsp. *paratuberculosis* invasion of MDBK cells indicates that the bacteria might trigger their own uptake, probably by inducing cytoskeleton reorganization. We did not observe significant replication of *M. avium* subsp. *paratuberculosis* inside cells after 4 days, which should be due to the long replication time (15), which might be even longer when inside epithelial cells.

Previous work has demonstrated the recovery of *M. avium* subsp. *paratuberculosis* from various sites in the body during advanced stages of infection. For instance, *M. avium* subsp. *paratuberculosis* has been isolated from milk, colostrum, and mammary lymph nodes from both asymptomatic and symptomatic cows (28–30), making it plausible that mammary epithelial cells could be a site of *M. avium* subsp. *paratuberculosis* infection. Our results demonstrated that *M. avium* subsp. *para-*

TABLE 4. DNA microarray profile showing differential expression of *M. avium* subsp. *paratuberculosis* genes

Gene	Fold increase in DNA array[a]	Function(s) or characteristic(s)	Fold increase in RT-PCR
MAP2450c	6.35	*atpC*; probable ATP synthase, *M. leprae*	7.1
MAP3305c	6.35	Conserved hypothetical protein, *M. leprae*	6.8
MAP0482	5.76	Putative transcription regulator, *Nocardia farcinica*	6.3
MAP2751	5.47	Unique to *M. avium* subsp. *paratuberculosis*	5.5
MAP0706	5.47	Probable cytoplasmic peptidase, *Listeria monocytogenes*	5.8
MAP0741c	4.91	Possible oxidoreductase	ND[b]
MAP3404	4.78	Biotin carboxyl bifunctional carrier protein; essential gene in *M. tuberculosis*	ND
MAP1259	4.49	Probable transcription regulatory protein in *M. tuberculosis*	ND
MAP2708c	4.33	Probable glutamine amidotransferase	ND
MAP3224	4.33	Possibly involved in secretion of β-lactamase in *M. tuberculosis*	ND
MAP1695c	4.25	Transcription factor mediated by hypoxic conditions in *M. tuberculosis*; essential gene in *M. tuberculosis*	ND
MAP2652c	4.02	Probable phosphate acetyl transferase	ND
MAP2524c	3.89	Oxidoreductase	ND
MAP0462	3.79	*ureC*; urease alpha subunit; essential gene in *M. tuberculosis*	ND
MAP0369	3.72	Probable nitrate reductase	ND
MAP1758c	3.56	*nrtC*; possible acyl coenzyme A dehydrogenase	ND
MAP3374	3.43	Probable F 420 biosynthesis protein	ND
MAP0392c	3.23	Probable bifunctional membrane associated penicillin binding protein	ND
MAP4310c	3.08	Possible acyl coenzyme A dehydrogenase	ND

[a] *M. avium* subsp. *paratuberculosis* bacteria were incubated intracellularly in MAC-T cells for 1 day prior to RNA microarray analysis. The expression was compared to that for bacteria grown in 7H9 broth.
[b] ND, not done.

tuberculosis infects MAC-T cells in vitro and that the infection is possible from both the apical and basolateral surfaces with comparable efficiency. Therefore, the implication of the observation is that infection in mammary gland tissue may potentially occur by either the systemic or the ascending route. Similar findings have been reported for other pathogens such as *Streptococcus dysgalactiae* and *Staphylococcus aureus* (1, 2). The results of transmission electron microscopy (Fig. 2B) confirmed that *M. avium* subsp. *paratuberculosis*, once inside MAC-T cells, is encountered within cytoplasmic vacuoles, similar to what has been described for *M. avium* subsp. *paratuberculosis* in macrophages (15) and other mycobacteria in epithelial cells (8). Furthermore, it was observed that *M. avium* subsp. *paratuberculosis* survived within mammary epithelial cells for several days in vitro. Collectively, these findings support the idea that the infection of the mammary gland can occur through the systemic route and that mammary gland epithelial cells may serve as a reservoir for *M. avium* subsp. *paratuberculosis* and a potential source of infection for young calves.

It is assumed that the intracellular environment in the mammary gland has high osmolarity, while the mammary gland milk is also a hyperosmolar fluid in nature. *M. avium* subsp. *paratuberculosis* incubated in milk prior to infecting MDBK epithelial cells became significantly more invasive than *M. avium* subsp. *paratuberculosis* that had been previously incubated in broth or water. The augmented ability to invade cells was then attributed to the hyperosmolar conditions of milk, a hypothesis that was strengthened by the observation that *M. avium* subsp. *paratuberculosis* incubated in four different hyperosmolar milk fractions acquired a similar phenotype. It appears that the environment with high osmolarity may serve as a trigger for expression of invasion-related determinants. In fact, osmolarity has been shown to be associated with the expression of virulence determinants in a number of bacteria, e.g., the *toxR* gene in *Vibrio cholerae* and *ompR* genes in *Salmonella* and *Shigella* (11, 13, 21). Previously, it was also shown that when *M. avium* was preincubated under high osmolarity conditions, a change in phenotype was induced, resulting in enhanced efficiency in entering human intestinal epithelial cells (8). These studies also showed that the invasive phenotype was likely to be related to the upregulation of genes involved in invasion, since incubation under high-osmolarity conditions in the presence of subinhibitory concentrations of amikacin, which inhibits protein synthesis, failed to result in expression of the invasive phenotype (8).

Prior incubation of *M. avium* subsp. *paratuberculosis* in MAC-T cells enhanced the efficiency of invasion of MDBK cells. DNA microarray analysis of *M. avium* subsp. *paratuberculosis* genes regulated during MAC-T cell infection showed that several genes had their expression altered. The upregulated *M. avium* subsp. *paratuberculosis* genes, for example, MAP0482, MAP1695c, MAP3404, MAP1259, MAP2652c, and MAP0392, encode proteins with transcription-regulatory functions. MAP0482 encodes a putative transcriptional regulator in *Nocadia farcinica*. The upstream gene MAP0483 encodes a transcription-regulatory protein in *M. tuberculosis*. Another transcription protein, encoded by MAP1695c, acts as a cochaperone in *M. tuberculosis* (19, 27). The upstream gene is for Hsp18, a stress protein induced by anoxia. Homology with *M. tuberculosis* suggests that the MAP1695c operon encodes a response regulator having an important role. MAP3404 belongs to an operon having an upstream sigma factor. MAP0392 encodes a probable bifunctional membrane-associated penicillin binding protein (PonA2, murine polymerase) under the control of the transcription-regulatory protein encoded by MAP0393. Studies with *M. smegmatis* and *M. tuberculosis* have suggested that transposon disruption of *ponA* resulted in a penicillin-binding-deficient mutant that was sensitive to beta-lactam antibiotics and grew slowly in culture (7, 12). The genes

identified may be associated with other functions, such as intracellular survival. Efficiency of invasion among intracellular bacteria peaks at 24 h, probably reflecting the fact that the mammary gland is emptied at least once a day. The function of the majority of the identified genes is unknown, and further studies are necessary to understand the role of the identified genes in intracellular survival.

In summary, we have examined the different conditions of *M. avium* subsp. *paratuberculosis* invasion and survival. A working model can be identified from the present results. Infection of the mammary gland and milk are observed in the majority of the infected cows. Therefore, it is plausible to hypothesize that *M. avium* subsp. *paratuberculosis* (within or outside of cells) fed to calves in milk is an organism with the ability to cross the intestinal barrier with efficiency, compared with organisms present in the water. Fecal material may be another important source of *M. avium* subsp. *paratuberculosis* expressing an invasive phenotype. Future work will address the role of genes upregulated within milk and mammary epithelial cells and will attempt to put together the 35-kDa protein, identified previously (5), and the present model of infection.

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