5. 治療と予後

IRISを発症した場合も，有効な抗HIV治療をできるかぎり継続することが基本である．IRISへの対処方法には，その疾患自身に対する治療と過剰な炎症のコントロールがある．

疾患が感染症である場合には，病原体の増殖がなければ抗微生物薬の投与は不要とする考え方もある．しかし，臨床的に病原体の増殖がない証明は困難であり，抗微生物薬の開始・追加・変更が必要である．

炎症コントロールの方法には，NSAIDsや副腎皮質ステロイド薬の投与がある．副腎皮質ステロイド薬は，臓器の機能障害が重篤な場合，生命の危機がある場合，他の方法が無効な場合などに考慮する．プレドニゾロン1mg/kg/日で開始し，週から月単位で減量していく方法などが行われている．

IRISのために抗HIV治療を中止することもあるが，その基準も定まっていない．現時点では，抗HIV治療を継続することでIRISの病態が生命を脅かす場合や副腎皮質ホルモン薬が無効な場合などに中止する．

IRISの生命予後は良好なことが多い．しかし，非結核性抗酸菌症，CMV感染症，結核症は病状が安定するのに3カ月以上を要することがしばしばである．特に，非結核性抗酸菌症では副腎皮質ステロイド薬の併用や抗HIV治療の中止が必要となり，難渋することも多い[6]．一方，Parkら[10]は日和見感染症を発症し，かつIRISを発症した症例の長期予後がIRISを発症しなかった症例に比べ，良好であることを報告している．したがって，IRISを単純に抗HIV治療の副作用と位置づけてしまうとIRISの真の病態を見誤る可能性があり，今後の研究成果に注目する必要がある．

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症　例

Tenofovir 過量内服を含む HAART 開始後短期間に急性腎不全をきたした HIV 感染者の 1 例

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（平成 20 年 3 月 17 日受付）
（平成 20 年 7 月 14 日受理）

Key words: Tenofovir, human immunodeficiency virus (HIV), renal failure

序　文

新たな抗 HIV（human immunodeficiency virus）薬が開発され，HAART（highly active antiretroviral therapy）は進化を続けている．抗 HIV 作用が強く，服薬しやすい薬剤が登場し，HIV 感染者の予後はさらに改善している[1]．しかし抗 HIV 薬は副反応が発現しやすく，消化器症状のような各薬剤に共通するものと薬剤ごとに特徴的なものとが存在する．

今回，我々は tenofovir（TDF）を含む HAART 開始時短期間に急性腎不全を合併し，血液透析によって改善した症例を経験した．腎不全発症時の状況や腎生検の所見などが臨床医にとって示唆に富むと考えたので，若干の文献的考察を加えて報告する．

症　例

患者：58 歳，男性．

主訴：発熱・嘔気・嘔吐・無尿．

既往歴：48 歳時，急性 B 型肝炎．57 歳時，大腸ポリープ．

家族歴：特になし．

生活歴：喫煙歴　なし，飲酒歴　機会飲酒，同性間性的接触歴あり．

現病歴：2005 年 11 月に他院で大腸ポリープの経過観察目的で大腸内視鏡を受け，アメーバ腸炎と診断された．その際に HIV 感染を指摘されたため，2006 年 1 月 25 日に加療目的で当科を紹介された．初診時の CD4 陽性リンパ球数が 81/μL，HIV-RNA 量が 1.1×10^5 コピー/mL，血清クレアチニン値が 0.8mg/dL で尿蛋白は陰性あった．アメーバ腸炎に対して Metronidazole（2.25g/日）を 10 日間投与し，途中から血便は消失した．2 月に入り鼻水等の感冒様症状が，3 月初め頃にも微熱が出現したため市販の総合感冒薬を不定期に服用していた．2 月 27 日に薬剤師による抗 HIV 薬に関する服薬指導を受け，3 月 16 日から TDF＋lamivudine（3TC）＋lopinavir/ritonavir（LPV/r）による HAART を開始した．その後も発熱があると，消炎鎮痛薬などを併用していた．3 月 20 日に受診した際に血清クレアチニン値が 4.3mg/dL と上昇していたので，薬剤性の腎障害を疑い，全ての抗 HIV 薬を中断した．その後も水分を十分摂取しているにもかかわらず無尿となり嘔気や嘔吐もともなうようになったため，翌日救急外来を受診した．血清クレアチニン値が 8.0mg/dL とさらに上昇していたため，急性腎不全の診断で同日緊急入院となった．

入院時現症：身長 165.0cm，体重 62.0kg，体温 35.9℃，血圧 150/90mmHg，脈拍 72/分・整，呼吸数 16/分・胸腹式．眼瞼結膜に貧血・黄染はなかった．口腔内では舌に白苔を認めた．表在リンパ節は触知しなかった．心音は清で雑音なく，肺音も副雑音を聴取しなかった．腹部は平坦，軟であった．皮膚，四肢，神経系には異常所見を認めなかった．

入院時検査所見（Table 1）：末梢血検査では，ヘモグロビンが 10.9g/dL，白血球は 11,000/μL と増加し左方移動していた．CRP は 19.6mg/dL と上昇していた．生化学検査ではアルブミン 2.5g/dL と低値，血清学検査では IgA 1,236.8mg/mL，IgE 558.2U/mL と上昇，CD4 陽性リンパ球数が 127/μL と低下，β_2-MG 27.3 mg/L，HIV-RNA 量は 1.8×10^3 コピー/mL であった．ツベルクリン反応は陰性であった．発症直前に使用した薬剤（Loxoprofen Sodium, Salicylamide, Acetaminophen, Anhydrous caffeine, promethazine Meth-

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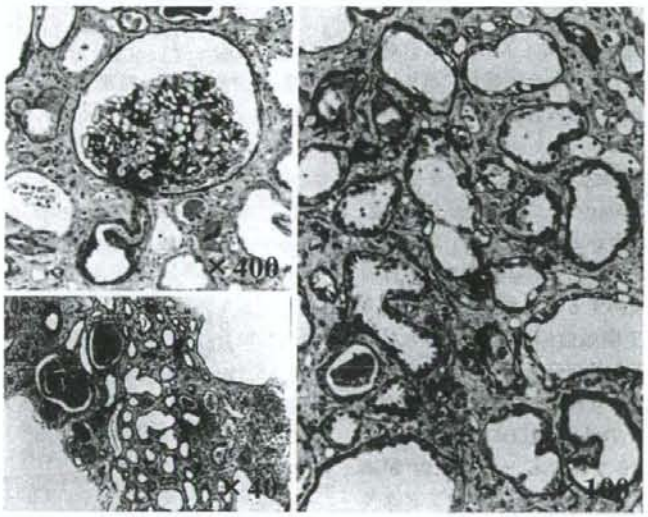
Table 1 Laboratory findings on admission

Peripheral blood		Blood chemistry		Serology/immunology	
RBC	363×10^4 /μL	TP	6.8 g/dL	CRP	19.6 mg/dL
Hb	10.9 g/dL	Alb	2.5 g/dL	β2-MG	27.3 mg/L
Ht	30.6 %	GOT	31 IU/L		
WBC	11,000 /μL	GPT	23 IU/L	IgA	1,236.8 mg/dL
		LDH	370 IU/L	IgG	1,649.1 mg/dL
stab	4 %	ALP	263 IU/L	IgM	54.6 mg/dL
seg	93 %	γ-GTP	62 IU/L	IgE	558.2 U/mL
eos	0 %	BUN	60 mg/dL		
bas	0 %	CRE	8.0 mg/dL	CD4 +	127 /μL
lym	1 %	Na	129 mEq/L	CD8 +	380 /μL
mon	2 %	K	3.8 mEq/L	CD4/CD8	0.32
		Cl	91 mEq/L	HIV-RNA	1.8×10^3 copies/mL
Plt	13.4×10^4 /μL	GLU	172 mg/dL		

Fig. 1 Renal biopsy specimen. (PAS staining)
Some glomeruli are globally sclerotic and the remainder show mild mesangial proliferation. Interstitia are widened and fibrosis exists together with mononuclear cell infiltration. Some tubes are lined by quite flattened epithelial cells and the lumina appear to be dialated. Small arteries show severe intimal thickening and hyalinosis.



ylenedisalicylate）のDLSTを行ったが，全て陰性であった．服薬中止2日後のTDF血中濃度は4,337ng/mLと高値であった．

腎臓超音波検査：のう胞を認めたが，両腎の萎縮はなかった．実質は高エコーだったが，血流信号は異常を認めなかった．

腎生検標本（Fig. 1）：メサンギウムの軽度増殖や細動脈の硬化，糸球体の硬化，間質の繊維化が見られた．さらに尿細管上皮の消失や空包化など尿細管壊死像も認めた．

入院後経過：薬剤性急性腎不全を疑い，全ての薬剤を中止した．翌日より4日連続して血液透析を施行し，以後週3回の透析を行った．徐々に尿量は回復し，入院2週間目頃からは尿量も確保できるようになった．腎機能も回復してきたため4月15日に血液透析を終了した．血清クレアチニン値が3.0mg/dL台で病状が安定したため，5月17日に退院した．入院後にTDFを誤って1日2回，つまり倍量服用していたことが判明した．

考　察

核酸系逆転写酵素阻害薬であるTDFは，その強力な抗HIV作用と1日1回服用という投与の簡便さから様々なガイドラインにおいて初回治療の推奨される組み合わせに含まれており[2]，処方の機会が増えている．HIV感染者における腎障害には，巣状分節性糸球体硬化症を伴う狭義のHIV関連腎症やHIV関連免

疫複合性増殖性腎炎・HIV 関連血栓性微小血管障害などが報告されている．一方で，抗 HIV 治療や日和見疾患治療のための薬剤による腎障害も報告され[3]，本邦でも TDF に関連した腎障害の報告例が散見されている[4)-7)]．Nelson ら[8]によると，市販後4年間の調査では TDF による重篤な腎障害の発現率は 0.5% と報告され，その危険因子として腎毒性を有する薬剤との併用・免疫不全が進行した時期・腎疾患の既往・敗血症の合併を挙げている．

本症例は，HIV 感染を家族に知られたくないために服薬指導の際に使用した説明用紙や処方したボトルの薬品名ラベルを捨てており，記憶に頼って服薬していた．その結果，TDF を誤って1日2回，倍量の 600 mg/日で服用していた．I/II 相試験[9]では，TDF の血中濃度は投与量が 75～600mg/日の範囲で用量に比例し，トラフ値は 300mg/日で 50ng/mL，600mg/日で 100ng/mL であったが有効性・安全性に差はないとされていた．本症例では投与中止2日後にもかかわらず血中濃度は 4,337ng/mL と異常高値を示していた．このことは，腎障害が高度になると，TDF の血中濃度は異常高値を長時間持続することを示唆しており，この血中濃度の高値がさらなる腎障害を惹起する可能性が推察された．

I/II 相試験において 600mg/日の TDF を投与された症例でのトラフ値は 100ng/mL で 300mg/日投与の症例と比し安全性に差はないとの結果から，誤って倍量の TDF を服用したことだけで本症例における腎障害の発症原因をすべて説明することができないと考える．本症例は，HAART 開始前の血清クレアチニン値が正常であったが，腎生検組織所見で糸球体の硬化像などが存在し，潜在的な腎障害があった可能性が考えられる．また，LPV/r の併用は TDF の体内動態に影響を与え AUC が 32% 上昇するという報告[9]がある．Whelton ら[10]によると，NSAIDs はプロスタグランジンの産生を抑制する薬理作用をもつ．しかしプロスタグランジンは腎血管拡張作用をもつため，その抑制は腎血流量を減少させ腎前性腎不全を誘発する可能性がある．さらに NSAIDs はアレルギー性の機序による尿細管の間質性腎炎などを引き起こし腎機能に影響の及ぼす可能性が指摘されている．

したがって，本症例では動脈硬化に伴う潜在的な腎障害がある上に TDF を過量に服用し，LPV/r や NSAIDs を併用したことが複合的に作用して腎不全に陥ったと推察する．

様々な事情を抱える HIV 感染患者は十分に服薬指導を行っても服薬方法や服薬量を間違えたり，さらに高齢者では潜在的な腎障害を合併している可能性もあり，その他様々な要因が絡み合って急性腎不全を発症する危険性のあるため，TDF 投与の際には十分な注意が必要であると考える．

なお，本論文要旨は，第20回日本エイズ学会（2006年12月，東京）にて発表した．

謝辞：稿を終えるにあたり，症例検討についてご指導頂きました当院 循環器・腎臓・代謝内科 吉本宗平先生，赤井靖宏先生，斎藤能彦教授に深謝致します．

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A Case of Acute Renal Failure Involving High Amounts of Tenofovir After HAART Start

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A 58-year-old man admitted for fever, nausea, vomiting, and anuria after the start of HAART, including tenofovir, had a viral load of 1.1×10^5copies/mL, a CD4-positive lymphocyte count of 81/μL, and serum creatinine of 0.8mg/dL before HAART. He underwent renal biopsy and temporary dialysis.

We concluded that the patient had acute tubular necrosis because of potentially impaired renal function and the high amount of medication, and judging from the renal biopsy specimen and clinical course.

When implementing HAART, physicians should be aware of and monitor potential patient misunderstanding of instructions on dosage and administration and for possible complications in medicinal combinations and potential side effects.

TDF taken together with lopinavir may increase the plasma concentration of TDF or other medications that could worsen renal function. It should also be noted that renal dysfunction is a potential complication in the elderly.

〔J.J.A. Inf. D. 82 : 650～653, 2008〕

平成20年11月20日

研究成果の刊行に関する一覧表
平成 20 年度　信州大学医学部附属病院　内視鏡診療部　山崎喜隆

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
Tadashi Okabe, Yoshitaka Yamazaki, Miho Shiotani, Takefumi Suzuki, Mayumi Shiohara, Eriko Kasuga, Shigeyuki Notake, Hideji Yanagisawa	An amino acid substitution in PBP-3 in Haemophilus influenzae associate with the invasion to bronchial epithelial cells	Microbiological Research	***	***-***	2008

Microbiological Research ▮ (▮▮▮▮) ▮▮▮–▮▮▮

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An amino acid substitution in PBP-3 in *Haemophilus influenzae* associate with the invasion to bronchial epithelial cells

Q1

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Received 19 July 2007; received in revised form 15 February 2008; accepted 1 March 2008

KEYWORDS
Non-typeable *Haemophilus influenzae*;
fts-I;
Clarithromycin

Summary

Haemophilus influenzae is a common pathogen of respiratory infections. We examined whether β-lactamase-negative ampicillin-resistant (BLNAR) strains that are known to have ampicillin resistance due to a substitution of amino acid of penicillin binding protein (PBP)-3, differ from β-lactamase-negative ampicillin-susceptible strains with regard to invasion of bronchial epithelium. After 3 h incubation of each of 32 β-lactamase-negative ampicillin-susceptible and 59 BLNAR strains in the presence of BEAS-2B cells, a human bronchial epithelium cell line, extracellular bacteria were killed using gentamicin and intracellular bacteria numbered. All nine strains in which the efficiency of invasion was 1% or higher were BLNAR strains. The rate of invasion was significantly greater in strains with PBP-3 amino acid substitution (Met377 to Ile, Ser385 to Thr, Leu389 to Phe, and Asn526 to Lys) ($n = 34$) than in those with no amino acid substitution. Electron microscopy showed that high invasive BLNAR strains were observed in cytoplasm of BEAS-2B cell layer. The injured cells were 9.44 ± 1.76% among attaching cells examined by trypan blue staining after 6 h. These data may suggest that the amino acid substitution of the PBP in BLNAR strains may at least partly play roles in macropinocytosis, leading to the invasion and injury to epithelial cells.

Abbreviation: PBP: penicillin binding protein, BLNAR: β-lactamase-negative ampicillin-resistant.

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0944-5013/$ - see front matter © 2008 Published by Elsevier GmbH.
doi:10.1016/j.micres.2008.03.003

Please cite this article as: Okabe T, et al. An amino acid substitution in PBP-3 in *Haemophilus influenzae* associate with the invasion to bronchial epithelial.... Microbiol Res 2006; (2008), doi:10.1016/j.micres.2008.03.003

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Introduction

Haemophilus influenzae is a pathogenic bacteria responsible for diseases including otitis media, sinusitis, bronchitis, and pneumonia (Bartlett et al. 2000; Pfaller et al. 2001; Ehrlich et al. 2002). It is also known to repeatedly cause airway infection in patients with underlying diseases such as chronic obstructive pulmonary diseases (COPD) and cystic fibrosis (Murphy 2006). Most of the *H. influenzae* strains isolated and identified as pathogenic bacteria for these respiratory infections are non-typeable *H. influenzae* without a capsule in their outer layer (Murphy and Apicella 1987; Sethi et al. 2002; Bouchet et al. 2003).

Recently, the appearance of strains of *H. influenzae* resistant to β-lactam antimicrobial agents has emerged as a clinical problem. *H. influenzae* acquires resistance to β-lactam antimicrobial agents by either of two mechanisms. One is ampicillin resistance caused by the production of β-lactamases such as TEM-1 and Rob-1 and is called β-lactamase-positive ampicillin-resistant, the other is resistance acquired with a decrease in affinity to β-lactam antimicrobial agents due to amino acid substitution of penicillin binding protein (PBP)-3.

Strains are described here as β-lactamase-negative ampicillin-resistant (BLNAR) (Parr and Bryan 1984; Mendelman et al. 1990; Ubukata et al. 2001; Dabernat et al. 2002). Although BLNAR is defined by the Clinical and Laboratory Standards Institute/the National Committee for Clinical Laboratory Standards (CLSI/NCCLS) as *H. influenzae* showing with a minimum inhibitory concentration (MIC) of ampicillin of 4 μg/mL or higher, most strains with an ampicillin-MIC of 1 or 2 μg/mL were recently found by *ftsI* sequencing of the PBP-3 gene to have the same PBP-3 amino acid substitution as genetically BLNAR strains (Ubukata et al. 2001). Surveillance in Japan and Spain reported that BLNAR accounted for a high percentage of ampicillin-resistant *H. influenzae*: the genetic ratio of BLNAR was 52.0% (Sanbongi et al. 2006) and 65.1% (Hotomi et al. 2007) in Japan and 56% in Spain (García-Cobos et al. 2007). This trend differs from that in North America, where BLNAR accounts for less than 5%, while β-lactamase-positive ampicillin-resistant strains are dominant (Karlowsky et al. 2002, Fluit et al. 2005).

Recently, clarification about the mechanism of airway epithelium invasion by *H. influenzae* has been found to internalize in adenoid cells of children (Forsgren et al. 1994). Ketterer et al. (1999) observed that *H. influenzae* invasion of bronchial epithelial cells begins with extension of host cell microvilli and the formation of lamellipodia. Microvilli appear as the cytoskeleton is rearranged by polymerization of 20–30 actin filaments (Holmes and Bakaletz 1997; Ketterer et al. 1999). During macropinocytosis membrane folds extending from the surface fuse back with the plasma membrane.

Pathogenic bacteria of respiratory infections are mostly non-typeable *H. influenzae* (Murphy and Apicella 1987). Capsular polysaccharide, lipopolysaccharide (LPS), and various other polysaccharides are present in the surface layer of *H. influenzae* (Gotschlich et al. 1981; Kuo et al. 1985). Capsular polysaccharide is classified into six serotypes, a–f, based on the antigenicity. The surface structure of non-typeable non-capsulated strains is composed of outer membrane proteins, such as lipooligosaccharide (LOS), pili, outer membrane proteins (Flesher and Insel 1978; Weiser et al. 1990), and these are considered to be an important etiological factor related to the uptake of non-typeable *H. influenzae* by bronchial epithelial cells. Swords and colleagues showed glycoform containing phosphorylcholine to be of special importance for the invasion of bronchial epithelial cells among LOS of non-typeable *H. influenzae* (Swords et al. 2000). Moreover, its phosphorylcholine activates pertussis toxin-sensitive heterometric G protein complex by binding with platelet activating factor (PAF) receptors of bronchial epithelial cells and induces actin polymerization by putting the cell signal cascade into action (Swords et al. 2000; Wang et al. 2003).

The objective of this study was to investigate whether clinical isolates and the BLNAR strain of *H. influenzae* invade cultured bronchial epithelial cells, and clarify characteristics involved in the invasiveness. In addition, the influence of the BLNAR strain on the invaded bronchial epithelial cell injury was investigated.

Methods and materials

Clinical isolates

Ninety-one clinical isolates of *H. influenzae* were obtained at Shinshu University Hospital and Miroku Medical Laboratory Co. between January 2001 and March 2005 and stored by freezing at −78 °C in a MicroBank (Sanko Junyaku Co., Tokyo). All strains were cultured using chocolate II agar (Japan Becton Dickinson Co., Tokyo) at 35 °C in 5% CO_2 for 15–18 h.

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Reagents

Ceftriaxone (CTRX) provided by Chugai Pharmaceutical Co., Japan was dissolved in saline and adjusted to the intended concentration. Meropenem (MEPM) and trypan blue solution purchased from Sigma-Aldrich, US, was dissolved in distilled water and adjusted to the required concentrations. Clarithromycin (CAM), provided by Taisho Toyama Pharmaceutical Co., Japan, was dissolved with dimethyl sulfoxide (DMSO) and adjusted to the intended concentrations.

Antimicrobial susceptibility test and identification of β-lactamase

Susceptibility tests for 19 antimicrobial agents was performed for all 96 *H. influenzae* strains according to CLSI/NCCLS broth dilution method in *Haemophilus* Test Medium (HTM) (M100-A15:M7-MIC. The strains were evaluated with MICroFAST 4J (Date Behring Co., USA). Strains with a MIC value for ampicillin $>8\,\mu g/mL$, and gentamicin, were evaluated with the broth dilution method for CLSI/NCCLS.

The test was carried out in *Haemophilus* Test Medium broth (Japan Becton Dickinson), by 20–24 of incubation at 35 °C. The production of β-lactamase was confirmed by a nitrocefin test (Cefinase: Japan Becton and Dickinson Co.). The reaction time for *H. influenzae* took 1 min. The strains which showed ampicillin resistance and negative nitrocefin test results were classified as BLNAR strains. The serotype was classified into capsular types a to f by the slide-glass aggregation method (Denka Co., Tokyo, Japan).

Sequencing of *fts I* gene

The 1.3-kb DNA fragment encoding the PBP3 transpeptidase domain was amplified from the chromosomal DNA of *H. influenzae* by PCR using the primers 5′-GTTGCACATATCTCCGATGAG-3′ and 5′-CAGCTGCTTCAGCATCTTGC-3′. The PCR-products were cloned with pCR2.0 TOPO cloning kit (Invitrogen, USA). Double strand sequence was generated using an ABI automatic DNA sequencer (Applied Biosystems). BLNAR was defined as having amino acid substitution of PBP-3, which was reported previously (Ubukata et al. 2001): Met377 to Ile, Ser385 to Thr, Leu389 to Phe, Arg517 to His, and/or Asn526 to Lys.

Three hours invasion assay using BEAS-2B cells

BEAS-2B cell was a transfected human bronchial epithelial cell line (Reddel et al. 1993; Yamazaki et al. 2006) purchased from ATCC (Rochville, MD, USA), and a standard cell line for bronchial epithelial cell experiments. BEAS-2B cell were cultured in bronchial epithelium growth media (BEGM, Cambrex Bio Science, Walkerville, MD) at 37 °C in an atmosphere of 5% CO_2. For the invasion assay, BEAS-2B cells were seeded (1×10^5 cells/well) in a 24-well tissue culture plate (Coster, Corning NY) and incubated until greater than 90% confluence was obtained (approximately for 4 days).

Bacterial suspensions at about 6×10^6 CFU/mL were inoculated at 10 μL/well. The invasion assay was performed as reported previously (Ketterer et al. 1999; Swords et al. 2000). Briefly, monolayers were infected and incubated at 37 °C in a 5% CO_2 atmosphere for 3 h, washed 3 times with Hank's buffered salt solution (HBSS), and treated with gentamicin at a concentration of 200 μg/mL for 2 h. Gentamicin kills extracellular bacteria within 2 h, while intracellular bacteria remain viable. The monolayers were washed 3 additional times with HBSS, and the viable intracellular bacteria were released by incubation with 0.5 mL of 1% Triton X-100 (Sigma-Aldrich) in HBSS for 15 min. The samples were harvested and vortex agitated for 1 min to lyse cells. The numbers of viable bacteria were serially diluted and plated onto chocolate agar for colony counting. The experiment was repeated 3 times with each of the 91 strains. The invasion rate was calculated by the equation: {Bacterial amount recovered from BEAS-2B cells (CFU/mL)/inoculated bacterial amount (CFU/mL)} × 100 (%). Isolates with an invasion rate of 1% or higher and lower than 1% were defined as high- and low-invasive strains, respectively.

Electron microscopy (EM)

The polarized cell layer of BEAS-2B cells was grown on transwell membrane as reported previously (Yamazaki et al. 2006). Briefly, the BEAS-2B cells in BEGM were seeded at 0.2×10^5 cells/well on a transwell insert (Costar, USA) containing 0.33 cm^2 porous filter membrane (3.0 μm pore). Polarized monolayers achieved confluence after 5–7 days at 37 °C in 5% CO_2. Monolayers were incubated for two to three additional days until the transwell electrical resistance reached to 250 Ω, as measured with a Millicell-ESR apparatus (Millipore,

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USA). To facilitate electron microscopic examination, cells were treated for 3 and 6 h with bacterial suspensions at concentrations about 10 times higher than those used in the invasion assay.

Polarized monolayers on transwell inserts infected with bacteria were washed 6 times with HBSS and fixed in cold (4 °C) with 2.5% glutaraldehyde (Merk, USA) in 0.1 M phosphate buffer, pH 7.4, overnight. After washing with phosphate buffer, samples were dehydrated in series of ethanol and embedded in epoxy resin, and ultra-thin sections (80 nm) were cut out of blocks and mounted on grids and stained with uranyl acetate and lead citrate before examination in a JEOL JEM-1230 transmission electron microscopy (TEM). For scanning electron microscopy (SEM), the transwell membranes were fixed as described above, dehydrated in a critical point apparatus and examined with a JEOL JSM 6360 scanning electron microscope, after a Pt sputter coating.

Statistical analysis

Results are expressed as mean ± S.E. or mean ± S.D. Analysis of variance (ANOVA) was used for the comparison among three or more groups, and between-group comparisons were tested using the Mann–Whitney *U*-test. $p < 0.05$ was considered to be significant.

Results

H. influenzae strains

Sequencing of the *ftsI* gene of PBP-3 was performed in the 91 *H. influenzae* strains. All 91 strains were non-typeable *H. influenzae*. Gentamicin-MIC were below 4 μg/mL in all β-lactamase-negative ampicillin-susceptible and BLNAR strains. β-Lactamase-positive ampicillin-resistant strains were excluded by the nitrocefin method ($n = 5$).

H. influenzae, in the present study, was classified into seven types based on amino acid substitution of PBP-3, as cited from previous reports (Ubukata et al. 2001): type 0 (no amino acid substitution), type I (Met377 to Ile, Ser385 to Thr, and Arg517 to His), type II (Asn526 to Lys), type IIIa (Met377 to Ile, Leu389 to Phe, and Asn526 to Lys), type IIIb (Met377 to Ile, Ser385 to Thr, and Asn526 to Lys), type IIIc (Met377 to Ile, Ser385 to Thr, and Leu389 to Phe, and Asn526 to Lys), and type IV (Met377 to Ile, Ser385 to Thr, and Leu389 to Phe).

Screening of *H. influenzae* strains by 3 h invasion assays

Invasion assays were performed to screen 34 β-lactamase-negative ampicillin-susceptible and 57 BLNAR strains. As shown in Table 1, high-invasive strains were observed more often among the BLNAR strains as the MIC value for ampicillin increased. The invasion rates, ampicillin-MIC (μg/mL), and PBP-3 amino acid substitution types of these high invasion BLNAR strains were defined in (Table 2). The invasion rate was 1% or higher in nine strains, which were all BLNAR strains (Table 3). The invasion rate was studied 1 and 2 h after inoculation using high invasion BLNAR strains (BR49, BR56, and BR66) (Figure 1).

Relationships of the invasion rate with the MIC values for ampicillin and *ftsI* amino acid substitution

As shown in Table 1, the mean invasion rate increased with the ampicillin-MIC, but it did not differ significantly according to the ampicillin-MIC. The mean invasion rate was significantly higher in type IIIc than in type 0 ($p = 0.0075$)(Table 2).

Intracellular bactericidal effects of various antimicrobial agents (Figure 1)

Pharmacokinetics and pharmacodynamics are not reflected in the effects of antimicrobial effects against BLNAR that have invaded BEAS-2B cells. We investigated the intracellular bactericidal effects of MEPM, CTRX, and CAM, to which BLNAR is susceptible based on the US CLSI judgment criteria.

Three high-invasive BLNAR strains (BR49, BR56, and BR66) were selected. The MIC values of CTRX, MEPM, and CAM were 0.25, 0.5, and 8.0 μg/mL against BR49, 0.25, 0.5, and 8.0 μg/mL against BR56, and 0.5, 1.0, and 8.0 μg/mL against BR66, respectively. These antimicrobials were mixed with gentamicin to kill extracellular bacteria and cul-

Table 1. Correlation between percentage invasion and ampicillin-MIC in BLNAR

Ampicillin-MIC (μg/mL)	No.	Percentage invasion (%)
0.25	27	0.014 ± 0.033
0.5	5	0.011 ± 0.013
1	14	0.036 ± 0.013
2	13	0.497 ± 0.829
4	21	0.84 ± 2.251
8	9	0.967 ± 2.448
16	2	4.500

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Table 2. Correlation between invasion and amino acid substitution in PBP3 in BLNAR

Type	Amino acid substitution in PBP-3					(*n*)	
	377Met	385Ser	389Leu	517Arg	526Asn		
0	–	–	–	–	–	34	0.01±0.03%
I	Ile	Thr	–	His	–	5	0.65±1.27%
II	–	–	–	–	–	12	0.58±2.06%
IIIa	Ile	–	Phe	–	Lys	1	(0.05%)
IIIb	Ile	Thr		–	Lys	2	(0.38%), (1.8%)
IIIc	Ile	Thr	Phe	–	Lys	34	0.95±2.12%*
IV	Ile	Thr	Phe	–	–	4	0.01±0.02%

M±S.D.
*$p = 0.0075$.

Table 3. The high invasive BLNAR strainof over 1% or higher

Strain	Invasion (%)	MIC of ampicillin (μg/mL)	Type of amino acid substitution
BR17	5.75±2.33	16	IIIC
BR27	2.92±0.41	2	I
BR28	2.34±1.09	4	IIIC
BR42	1.37±0.33	4	IIIC
BR49	10.14±2.12	4	IIIC
BR53	7.33±2.46	8	II
BR56	4.67±2.91	4	IIIC
BR66	3.25±0.87	16	IIIC
BR99	1.80±0.89	2	IIIB

tured for 2 h. CAM at the concentration of MIC showed an intracellular bactericidal effect in all three strains (Figure 1).

SEM findings (Figure 2)

High-invasive BLNAR strain (BR49) showed microvilli extending from BEAS-2B cells and covering bacteria attached to the cell surface (Figure 2a and b), and showed bacteria entering cells and the formation of lamellipodia (Figure 2c). Bacteria entered cells individually without aggregating. However, low invasive β-lactamase-negative ampicillin-susceptible strain (H30) did not attach to the cell surface (Figure 2d).

TEM findings (Figures 3 and 4)

After 3 h, the surface of BEAS-2B cells was smooth (Figure 3a). Invasion of high-invasive BLNAR strain (BR49) was observed in cells close to the surface among layered cells. Most bacteria independently entered into cells by macropinocytosis, but a few bacteria were surrounded by vacuoles (Figure 3b and c).

After 6 h, the cell arrangement on the surface was irregular compared with the TEM images after 3 h (Figure 4a). A considerably larger number of bacteria had entered cells than after 3 h (Figure 4a). Moreover, bacteria were also observed in cells in areas deep from the surface (Figure 4a and b). The cytoplasm was expanded due to the presence of many bacteria, intercellular adhesion was physically detached, and some cells were detaching from the surface, in which bacteria clustered in some vacuoles. It was not clear whether the vacuoles were formed by the fusion of bacteria-containing linings or bacterial proliferation in the vacuoles. However, low invasion β-lactamase-negative ampicillin-susceptible strain (H30) was not observed in BEAS-2B cells (Figure 4d). Trypan blue dye staining among attaching BEAS-2B cells were significantly high at 6 h (9.44±1.76%, $p<0.001$) and at 3 h (1.78±0.63%, $p<0.01$) compared with those of control (0.78±0.34%, 0.54±0.24%, respectively). These data demonstrate that BEAS-2B cell injury was induced by high-invasive BLNAR strain.

Discussion

The rate of invading BEAS-2B cells was high in some BLNAR strains of *H. influenzae* that had acquired resistance to ampicillin. On screening by the 3 h invasion assay, this invasion rate exceeded 1% in 9 of the 91 *H. influenzae* strains. They were all BLNAR strains with a serotype of non-typeable *H. influenzae*. The invasion rate did not differ significantly according to the ampicillin-MIC but was significantly higher in type IIIc with PBP-3 amino acid substitution at BLNAR resistant sites (Met377 to Ile, Ser385 to Thr, Leu389 to Phe, and

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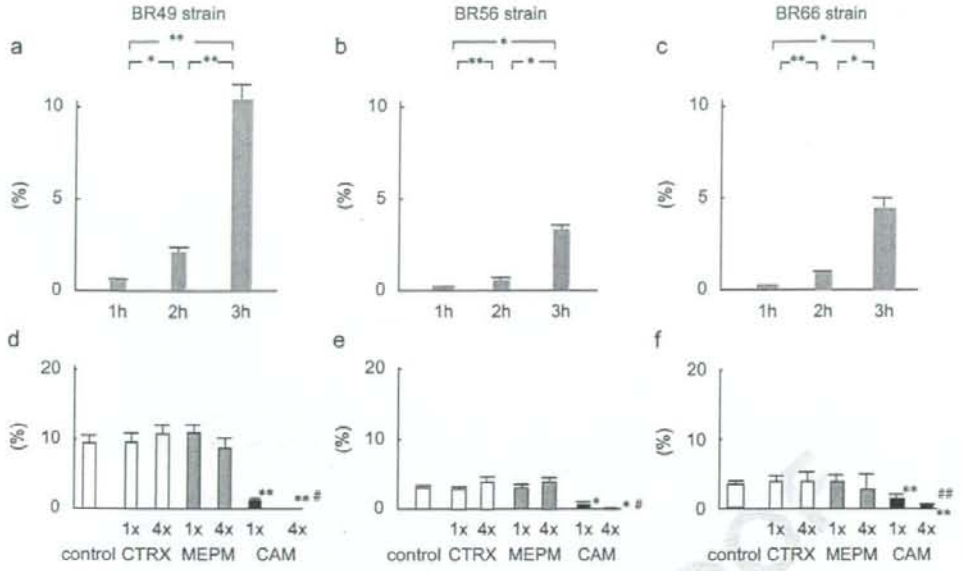

Figure 1. Time-course changes of three high-invasive BLNAR strains invasion rates and intracellular bactericidal effects of three antimicrobial agents to BLNAR strains. The invasion rates of BR49, BR56, and BR66 strains (a, b and c) increased significantly after 2 and 3 h. The invasion rates after 3 h were significantly higher than those after 2 h ($n = 3$, mean ± S.E., $^{*}p < 0.05$, $^{**}p < 0.01$). The invasion rates of BR49, BR56, and BR66 strains (d, e, and f) were significantly reduced when they were treated with clarithromycin (CAM) at the CAM-minimum inhibitory concentration (1 × MIC) and 4 × MIC compared with the control invasion rate ($n = 3$, mean+S.E., $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with control, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with 1 × MIC). No significant difference was noted in the invasion rate after treatment with ceftriaxone (CTRX) or meropenem (MEPM) at 1 × ABPC-MIC or 4 × MIC.

Asn526 to Lys) than in the types with no substitution.

Ubukata et al. (2001) showed that, in BLNAR, 5 amino acid substitution of PBP-3, i.e., Met377 to Ile, Ser385 to Thr, Leu389 to Phe, Arg517 to His, and/or Asn526 to Lys, were related to ampicillin resistance. Moreover, PBP-3 with multiple substitutions (Met377 to Ile, Ser385 to Thr, and/or Leu389 to Phe) together with Asn526 to Lys resulted in increased resistance compared to that for PBP-3 with the Asn526 to Lys substitution alone (Sanbongi et al. 2006). Some 93.2% of the BLNAR strains had amino acid substitutions at the Lys-Thr-Gly (KTG) motif, the two most common being Asn526 to Lys (83.9%) and Arg517 to His (9.3%). Amino acid substitutions at positions 377, 385, and 389, which conferred cefotaxime and cefixime MICs 10–60 times higher than those of susceptible strains, were found for the first time in Europe (García-Cobos et al. 2007).

Macropinocytosis defines a series of events to internalized bacteria initiated by extensive plasma membrane reconstitution or ruffling (Jones 2007). On the other hand, micropinocytosis defined to uptake of extracellular molecules by cells, which can be either nonspecific endocytosis to form small vesicles at the plasma membrane, or cell surface receptor-mediated endocytosis of substances (Marino and McCluskey 2000). In this study, SEM images Q5 are considered to correspond to "micropinocytosis". Rearrangement of the cytoskeleton occurs in microvilli. The mechanism of this phenomenon is signal transduction induced by contact of bacteria with cell surface receptors and subsequent actin rearrangement (Ketterer et al. 1999; Kunishima et al. 2000), i.e., 20–30 actin filaments inside microvilli form bundles and are arranged in the same direction. Swords et al. (2000) reported that PAF receptors are involved in the invasion mechanism of *H. influenzae*, speculating that binding of the outer membrane of *H. influenzae* with PAF receptors activates small GTP, which induces actin rearrangement. Based on our study, it may be suggested that the amino acid substitution of high-invasive BLNAR strain possesses a bacterial factor inducing host macropinocytosis.

According to electron microscopic findings, the non-invasive β-lactamase-negative ampicillin-susceptible *H. influenzae* strain did not adhere to the epithelial cell surface, suggesting that adhesion to

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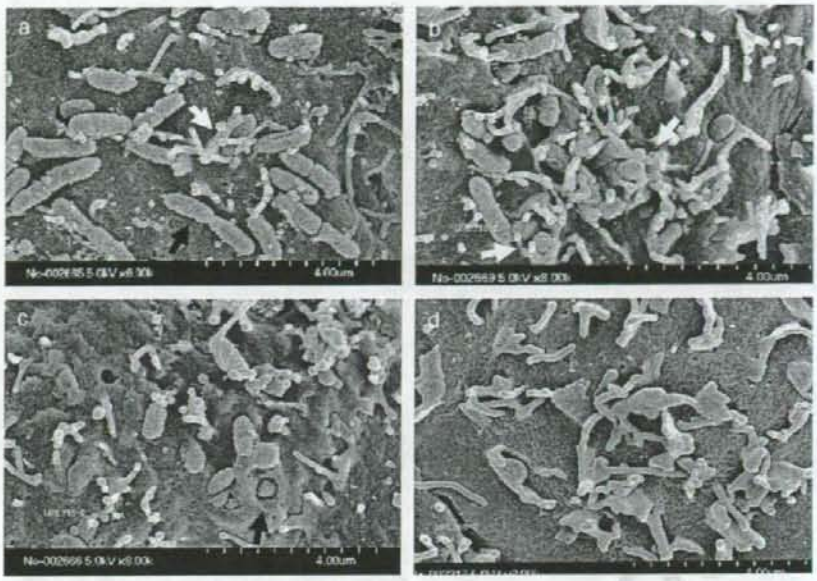

Figure 2. Scanning electron microscopy (SEM) of the polarized cell layer of BEAS-2B cells allowed to develop over the transwell membrane. Three hours after inoculation of a high invasion BLNAR strain (BR49) to the culture supernatant, the cells were washed gently 3 times with HBSS using a pipette and fixed (a) (× 8000), (b) (× 8000). Bacteria attached to the surface of BEAS-2B cells (black arrow) were covered by microvilli extending from cells (white arrow). Some bacteria have entered cells or formed lamellipodia (two white arrows). Bacteria entered cells individually without aggregating. In (c) (× 8000), some bacteria have entered cells nearly completely, with only the tail being visible, or be completely covered by the cell surface (black arrow). In (d) (× 8000), low invasive β-lactamase-negative ampicillin-susceptible strain (H30) did not attach to the cell surface ((d) × 8000).

the cell surface is necessary for *H. influenzae* to enter epithelial cells. Analysis of the BLNAR strain, which entered epithelial cells, suggested that PBP-3 with several amino acid substitutions acts as an adhesion molecule. According to reports on bacterial PBP and epithelial cell invasion, it is possible that BPB-2b also is an adhesion molecule, as well as a cell surface antigen and lipoprotein, in adhesion to and invasion to cultured cervical epithelial cells by Group B *Streptococcus* (GBS) (Johri et al. 2007). It has also been reported that expression of ampH, which is homologous to PBP of *Escherichia coli*, in addition to the *salmonella* pathogenicity island 1 (SPI1) gene regulatory genes (hilD and invF), is involved in invasion of intestinal epithelial cells by *Salmonella enterica* (Gantois et al. 2006). These findings suggested that PBP-3 with amino acid substitutions is an adhesion molecule. To clarify adhesion to the epithelial cell surface or bind to receptors, it is necessary to investigate the structure of amino-acid substituted PBP-3 including hydrophilic or hydrophobic characteristics.

We studied changes in the invasion rate of high invasion BLNAR with the 1 h invasion rate in BR49, BR56, and BR66, but the 3 h invasion rates were 5 times higher that the 2 h invasion rates. Based on these findings, bacterial invasion of BEAS-2B cells increased with time. Moreover, findings on TEM and trypan blue staining suggested progressive cell injury. A comparison of TEM images after 3 and 6 h indicated bacterial invasion causing an increase in bacteria in vacuoles, the swelling of cells, and their detachment from other cells after 6 h. It was not clear whether the presence of bacteria in one vacuole was due to the fusion of bacteria-containing linings or bacterial proliferation in the vacuole. The mechanism of bronchial epithelial cell impairment by the BLANR strain may be clarified in the future experiment.

H. influenzae infection is known to repeatedly cause to exacerbation of COPD, and induces airway epithelial disorder and excess of secretion, aggravating symptoms (Sapey and Stockley 2006). This Q6 study suggested that *H. influenzae* invade and destroy airway epithelial cells and damage the airway. The prevalence of BLNAR suggested that antibacterial drug resistance may increase. Macrolides and quinolones are thought to transfer and accumulate intracellularly and have intracellular bactericidal effects. We should pay attention to the

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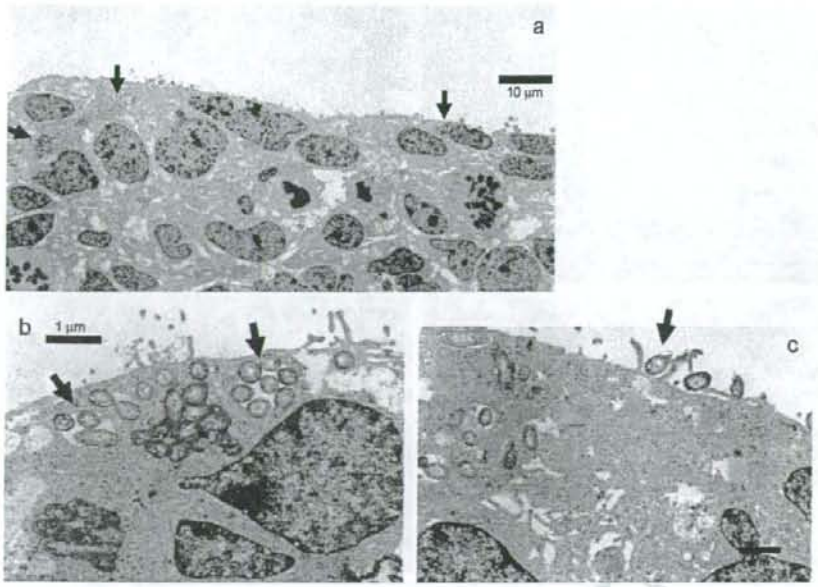

Figure 3. Transmission electron microscopy (TEM) of the polarized cell layer of BEAS-2B cells allowed to develop on the transwell membrane after 3 h. In this photograph obtained 3 h after inoculation of a high-invasive BLNAR strain (BR49) to the culture supernatant, the cell surface is smooth. Invasion of bacteria is observed in cells near the surface (three black arrows) (a) (× 1000). Bacteria are surrounded by vacuoles and are observed in multiple numbers (two black arrows) (b) (× 8000). In (c) (× 8000), microvilli adhering to bacteria, i.e., an image of macrospinosis, is observed (black arrow).

antimicrobial agents when the pathogenic bacteria are BLNAR.

It has been recognized that antimicrobial drug therapy based on pharmacodynamics and pharmakinetics is necessary for the treatment of infectious Q7 diseases (Craig 1998). As shown in the BLNAR strains, there is no treatment against bacteria that have invaded airway epithelial cells. The high-invasive BLNAR strains (BR49, BR56, and BR66) were susceptible to MEPM, CTRX, and CAM based on the US CLSI MIC criteria, but only CAM exhibited an intracellular bactericidal effect in BEAS-2B cells. Considering the pathology of airway infection by the high-invasive BLNAR strains, an antimicrobial agent that readily penetrates airway epithelium to which bacteria show susceptibility is necessary.

The concentrations of β-lactams, MEPM, and CTRX, in airway epithelial lining fluid are not easily elevated, but those of macrolides are high compared with serum concentrations. Furthermore, macrolides also penetrate cell membrane and exhibit a bactericidal effect, suggesting that these are appropriate for the treatment of BLNAR infection. Investigation of the clinical usefulness of macrolides is needed in the future.

The characteristics of macrolide antibiotics have the superiority in being taken up by phagocytes, lymphocytes, and epithelial cells (Kunishima et al. 2000; Bosnar et al. 2005). In a reported *in-vitro* experiment using an epithelial cell line (A549 and MDCK), when a macrolide antibiotic was added to the culture medium, the intra- and extracellular antibiotic concentrations became similar within 1 h (Bosnar et al. 2005).

In conclusion, the BLNAR strains isolated from clinical samples included isolates capable of invading BEAS-2B cells, and an amino acid substitution in PBP-3 was assumed to be a factor involved in invasion. It was also clarified that *H. influenzae* causes epithelial cell damages in invaded BEAS-2B cells.

Conflicts of interest

Tadashi Okabe, Yoshitaka Yamazaki, Miho Shiotani, Takefumi Suzuki, Mayumi Shiohara, Eriko Kasuga, Shigeyuki Notake, Hideji Yanagisawa have no conflicts of interest.

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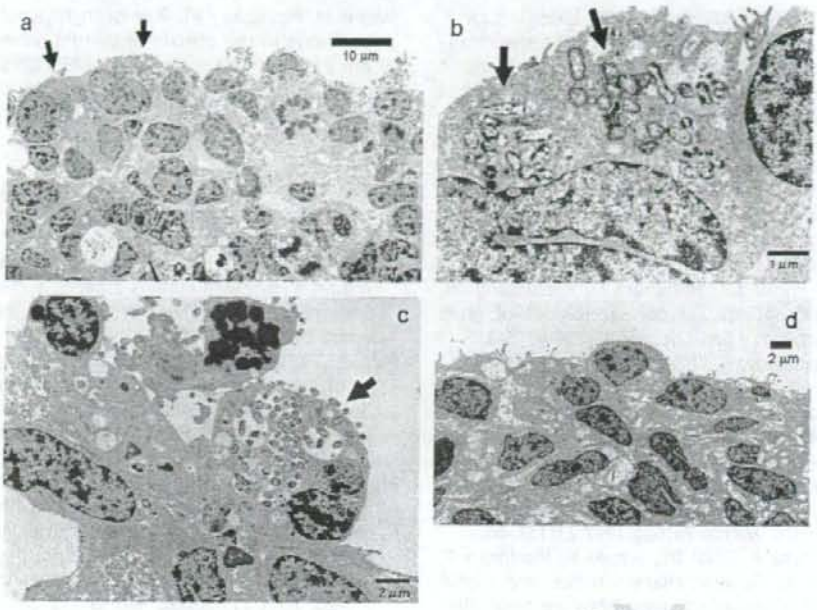

Figure 4. Transmission electron micrographs (TEM) of the polarized cell layer of BEAS-2B cells allowed to develop on the Transwell membrane after 6 h. This is a TEM image 6 h after inoculation of a high-invasive BLNAR strain (BR49). The cell surface is irregular, showing elevations and depressions compared with the image after 3 h. Considerably more bacteria are observed in cells than after 3 h (black arrows) (a) (× 1000), (b) (× 8000). Some cells on the surface were swollen and became spherical due to the presence a large number of bacteria in the cells (black arrow) (c) (× 3000). Low invasion β-lactamase-negative ampicillin-susceptible strain (H30) was not observed in BEAS-2B cells (d).

Q8 ## Uncited references

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Please cite this article as: Okabe T, et al. An amino acid substitution in PBP-3 in *Haemophilus influenzae* associate with the invasion to bronchial epithelial.... Microbiol Res 2006; (2008), doi:10.1016/j.micres.2008.03.003

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研究成果の刊行に関する一覧表
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雑誌

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肺結核診断の決め手と治療開始までに遭遇するジレンマ

永井 英明*

はじめに

結核の診断は，症状，臨床経過，検査所見などから結核症を疑わなければ難しい．日本は，結核罹患率が欧米先進国に比べ高く，結核の中蔓延国といわれており，日常臨床のなかでは，常に結核を念頭に置いて対応しなければならないと考えている．

どのような患者に，結核の検査を進めるかというと，「15日以上長びく咳を訴える患者」，「抗菌薬に反応の悪い不明熱」，「1年以内に塗抹陽性患者と接触した人」，「他疾患の治療中に咳，発熱が出現し治りがたい症例」，「他医で結核疑いとされた症例」などがあげられる[1]．また，初診時に胸部異常陰影がある症例については，原則として喀痰の抗酸菌検査を行うべきである．

結核の胸部X線写真所見

肺結核でみられる胸部X線写真所見を**表1**に示したが，画像所見だけで結核の確定診断をくだすことは不可能であり，ほかの呼吸器疾患の鑑別が必要である．典型的な胸部X線写真所見は，上葉(S^1，S^2，S^6)を中心とする空洞影とその周辺の散布影を伴う陰影であるが，胸水貯留，縦隔リンパ節腫大を認めることもある．

肺結核の進展は基本的には気道散布であり，それを端的に示す胸部X線所見は多発小粒状影であり，散布性粒状影といわれる．それは終末細気管支から肺胞道周辺に形成される結核性病変を反映している．散布性粒状影はほとんどの肺結核症でみられ，ほかの疾患との鑑別が問題となった場合，ほかの疾患を否定する重要な所見となる．この陰影は胸部CTでは小葉中心性の粒状影として認められ，ときに分岐状影を呈する．粒状影とそれを連結する細気管支の樹枝状陰影を，tree-in-bud(**図1**)といい特徴的であり，終末細気管支程度の太さの気道からその末梢気道を埋めるように充満する炎症性物質を反映する像を表している．

表1 肺結核の胸部X線写真所見と鑑別すべき疾患

肺結核の典型的所見	鑑別すべき疾患
空洞影	肺膿瘍，肺腫瘍，肺真菌症
散布性粒状影	比較的結核に特有
浸潤影	肺炎
結節影	肺腫瘍
粟粒影	過敏性肺臓炎，転移性腫瘍
胸水	心不全，悪性腫瘍
肺門・縦隔リンパ節腫大	悪性腫瘍，肺真菌症，サルコイドーシス

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図1 肺結核の胸部CT：散布性粒状影(tree-in-bud像)

血行性播種性結核症である粟粒結核の典型的な胸部X線写真所見は，全肺野に認められる均一な直径1～3 mmの粒状影であり，粒状影が密になると血管影が不明瞭になる．CTでは胸膜に接した粒状影も認められる．このような，典型例では診断は容易であるが，免疫不全症例，ステロイド薬・抗がん薬の投与を受けているような症例では，粒状影の大小不同，結節影，浸潤影などの非典型的所見を呈することがあり，生前診断が困難な場合もある．一般に肺の粟粒結核結節は，はじめは肺胞壁に形成されて円形であるが，その後，肺胞腔内に破れて肺胞腔内の変化を伴い病変がひろがる．したがって，粟粒結核症の診断が行われた時期により，胸部X線写真は典型的な粟粒影のみの所見から，粟粒大よりも大きい結節影，さらに浸潤影を伴うようになると考えられる．HRCT(High Resolution CT：高精度CT)を検討した報告[2]では，粟粒結核では粟粒影に次いで多い陰影はスリガラス陰影であったという．非典型的所見は，発病から診断までの期間が長いほど出現する確率が高くなる．病状が進行し成人呼吸促迫症候群(ARDS)を合併すると，両肺に広範な浸潤影を呈する．肺門および縦隔リンパ節腫大はHIV感染症に合併した粟粒結核では高頻度で認められるが[3]，合併症のない粟粒結核症での頻度は高くない．

結核感染の新しい診断法

従来，結核感染の診断はツベルクリン反応(ツ反)によって行われてきた．この方法はBCG未接種者においては感度，特異度ともに高く基本的には優れた方法であるが，BCG接種者においては，現れる反応が過去のBCG接種によるものか，最近受けた結核感染によるものかが区別できないという大きな問題がある．BCG接種に積極的に取り組んできたわが国では，結核感染の有無をツ反で判定するのはしばしば困難をきわめる．そこにBCG接種の影響を受けない新しい結核診断法が開発された．特異的抗原刺激に対するリンパ球のインターフェロンγ(IFN-γ)産生能を測定することによって結核感染の診断を行う方法(QuantiFERON-TB 第2世代；QFT-2G)である．

QFT-2Gは，結核菌由来の特異抗原early secreted antigenic target 6(ESAT-6)とculture filtrate protein 10(CFP-10)の刺激による末梢血リンパ球のIFN-γ産生能を測定する検査法で，結核感染の診断有用性は高い．Moriら[4]によればQFT-2Gの結核感染の診断における特異度は98.1%，感度は89%である．

QFT-2GはBCG接種の影響を受けないため，接触者検診，医療関係者の結核管理，結核の補助診断などにツ反に代わって用いられることになるだろう[5]．

結核菌の検出

結核の診断は臨床検体の塗抹・培養検査に