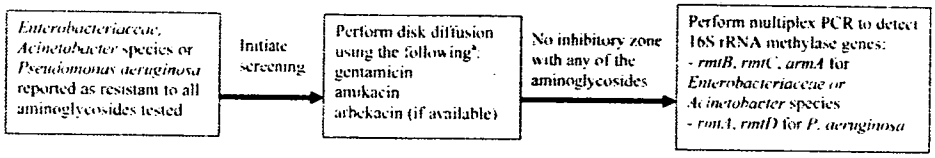

Figure 3. Suggested approach in identifying organisms producing 16S rRNA methylase. [a]Alternatively, MICs of 256 μg/mL to all aminoglycosides tested may be used.

CLINICAL IMPLICATION OF AMINOGLYCOSIDE RESISTANCE DUE TO 16S rRNA METHYLATION

Despite its currently low prevalence, the global spread of gram-negative bacilli producing 16S rRNA methylase is concerning for several reasons. First, these gram-negative bacilli show a very high level of resistance to most clinically useful aminoglycosides, including gentamicin, tobramycin, and amikacin, which cannot be overcome by dose adjustments. Second, all of the structural genes of known 16S rRNA methylases are associated with mobile genetic elements, such as transposon, some of which have been proven functional, providing them with the means to spread horizontally to other strains and species. Third, these organisms appear to possess a high potential for developing multidrug resistance, especially via acquisition of various β-lactamase genes. Of 35 ArmA- and RmtB-positive clinical isolates, 33 produced CTX-M- or SHV-type extended-spectrum β-lactamases (ESBLs) in a Taiwanese university hospital [22]. Similar observations have also been made in South Korea [20]. It has been reported that the structural gene for ArmA, the most prevalent methylase thus far, is located on a composite transposon Tn*1548* on a transferable plasmid and is frequently associated with CTX-M-3–type ESBL genes [14]. Production of CTX-M-9–type ESBLs is seen in many strains with RmtB [22] (K. Yamane, unpublished data). RmtD was initially reported in a *P. aeruginosa* strain that co-produced SPM-1 metallo-β-lactamase, which is endemic in Brazil. The latter combination would render ineffective a potent double-coverage regimen of carbapenem plus aminoglycoside. Currently, there are no data regarding clinical outcome in patients infected with these organisms. Nonetheless, it would be prudent to pay careful attention to the antibiogram and to maintain a low threshold to screen for ESBL production when these 16S rRNA methylase–producing gram-negative bacteria are encountered in clinical situations. Contact precautions should be used for patients when coproduction of 16S rRNA methylase and ESBL or metallo-β-lactamase is highly suspected or confirmed.

DETECTION OF AMINOGLYCOSIDE RESISTANCE DUE TO 16S rRNA METHYLATION

Screening for 16S rRNA methylase–producing organisms may be considered for epidemiologic purposes when nosocomial or foodborne spread of such bacteria is suspected. Detection of this resistance mechanism may pose a challenge in clinical laboratories. Gram-negative bacilli commonly produce aminoglycoside-modifying enzymes, such as acetyltransferases, nucleotidyltransferases, and phosphotransferases. When >1 of these enzymes are produced in single organisms, they could readily become resistant to multiple aminoglycosides. The hallmark of resistance mediated by 16S rRNA methylase that methylates residue G1405 is the very high level of resistance to all parenterally formulated aminoglycosides (MIC is typically ≥256 μg/mL), except streptomycin. This, however, may not be discernible in routine susceptibility testing conducted in the clinical laboratory, especially when automated susceptibility testing systems are used that only measure MICs close to the breakpoints of each aminoglycoside. One unique characteristic of these methylases is the high-level resistance to arbekacin that they confer. Arbekacin is a semisynthetic aminoglycoside derived from dibekacin [27]. It has activity against staphylococci,

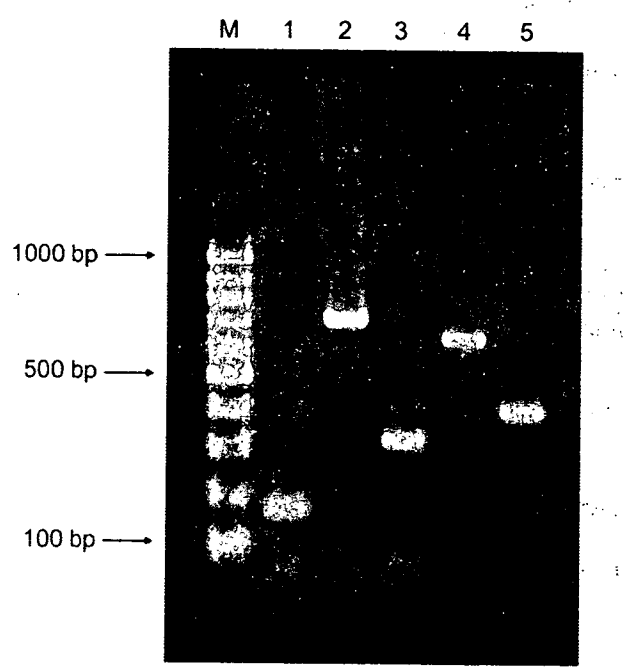

Figure 4. Electrophoresis profile of the PCR products. *Lane M*, 100–base pair (bp) marker; *lane 1, rmtB; lane 2, rmtC; lane 3, armA; lane 4, rmtA; lane 5, rmtD.*

Table 3. Primers for detection of 16S rRNA methylase genes.

Species, gene, primer	Sequence (5′→3′)	Amplicon size, base pairs
Enterobacteriaceae and *Acinetobacter* species		
rmtB		
rmtB-F	GCT TTC TGC GGG CGA TGT AA	173
rmtB-R	ATG CAA TGC CGC GCT CGT AT	
rmtC		
rmtC-F	CGA AGA AGT AAC AGC CAA AG	711
rmtC-R	ATC CCA ACA TCT CTC CCA CT	
armA		
armA-F	ATT CTG CCT ATC CTA ATT GG	315
armA-R	ACC TAT ACT TTA TCG TCG TC	
Pseudomonas aeruginosa		
rmtA		
rmtA-F	CTA GCG TCC ATC CTT TCC TC	635
rmtA-R	TTG CTT CCA TGC CCT TGC C	
rmtD		
rmtD-F	CGG CAC GCG ATT GGG AAG C	401
rmtD-R	CGG AAA CGA TGC GAC GAT	

NOTE. Both PCR for *rmtB, rmtC,* and *armA* and PCR for *rmtA* and *rmtD* may be performed in a mutiplex protocol. Thermal cycling condition is as follows: initial denaturation at 96°C for 5 min, followed by 30 cycles at 96°C for 30 s, at 55°C for 30 s, at 72°C for 1 min, with a final extension at 72°C for 5 min.

as well as against gram-negative bacteria, and it is currently approved only for treatment of multidrug-resistant *Staphylococcus aureus* infections in Japan. It is generally stable against the actions of aminoglycoside-modifying enzymes, with the exception of the bifunctional enzyme AAC(6′)/APH(2″), which is known to be produced by some multidrug-resistant *S. aureus* and enterococcal strains, and it may result in low-level arbekacin resistance. However, arbekacin is not readily available in many instances. Therefore, we propose the following approach in screening for 16S rRNA methylase production (figure 3). When a strain belonging to the family *Enterobacteriaceae* or glucose nonfermentative species, such as *P. aeruginosa* or *Acinetobacter* species, meets the criteria set forth by the Clinical and Laboratory Standards Institute for resistance to multiple aminoglycosides, disk diffusion test using gentamicin, amikacin, and arbekacin (if available) may be performed. Production of 16S rRNA methylase is suspected when no or little inhibitory zone is observed with any of the aminoglycoside disks. The addition of an arbekacin disk is desirable, because it raises the positive predictive value of this method to ≥90%, compared with ~60% when performed only with amikacin [20]. Alternatively, if the MICs of these aminoglycosides are to be used, a cutoff value of 256 μg/mL appears to provide excellent positive predictive value. All of the RmtA-, RmtB-, RmtC-, RmtD- and ArmA-producing isolates that we have tested to date have had MICs of these aminoglycosides ≥256 μg/mL—an observation confirmed elsewhere [20]. Currently, PCR is the only confirmatory method available. Multiplex PCR may be performed for *armA, rmtB,* and *rmtC* in strains belonging to the family *Enterobacteriaceae* and *Acinetobacter* species and for *rmtA* and *rmtD* in *P. aeruginosa* (figure 4). Recommended primers and thermal cycling conditions are listed in table 3. Of note, some other glucose nonfermentative organisms, such as *Stenotrophomonas maltophilia* and *Burkholderia cepacia,* may also present with panaminoglycoside-resistant phenotype, but the mechanism of this resistance has not been elucidated.

CONCLUSIONS

Methylation of the 16S rRNA in the 30S ribosomal subunit confers high-level resistance to most clinically useful aminoglycosides by inhibiting their access to the site of action. Gram-negative pathogens possessing this mechanism were first reported in 2003 and are increasingly reported worldwide. The organisms that produce 16S rRNA methylase are often multidrug resistant, especially against broad-spectrum β-lactams via production of ESBLs or metallo-β-lactamases, a process that is likely to be facilitated by the association of 16S rRNA methylase genes with genetic recombination systems. Although the clinical outcome for patients infected with these organisms is still unknown, early identification of the resistance mechanisms will be helpful in optimizing antimicrobial therapy and infection-control measures. A 2-tiered approach, consisting of disk diffusion tests followed by PCR confirmation, is recommended for detection of 16S rRNA methylase-mediated resistance.

Acknowledgments

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Potential conflicts of interest. All authors: no conflicts.

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16S rRNA Methylase–producing, Gram-negative Pathogens, Japan

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To investigate the exact isolation frequency of 16S rRNA methylase–producing, gram-negative pathogenic bacteria, we tested 87,626 clinical isolates from 169 hospitals. Twenty-six strains from 16 hospitals harbored 16S rRNA methylase genes, which suggests sparse but diffuse spread of pan-aminoglycoside–resistant microbes in Japan.

Broad-spectrum β-lactams and fluoroquinolones have been widely prescribed in the treatment of gram-negative bacterial infections; as a result, resistance to these antimicrobial agents has developed in some species. Although these agents are not immune to an increasing number of resistance mechanisms, they remain relatively potent and continue to be essential antimicrobial drugs for treating life-threatening bacterial infections.

Although the production of aminoglycoside-modifying enzymes is the most common mechanism of resistance in aminoglycosides, the emergence of pan-aminoglycoside–resistant, 16S rRNA methylase–producing, gram-negative bacteria has been increasingly reported in recent years. Five types of plasmid-mediated 16S rRNA methylases (ArmA, RmtA, RmtB, RmtC, and RmtD) have so far been identified in east Asia, Europe, and South America (*1–7*). RmtA was first identified in 2001 in Japan (*3*) and has so far been identified exclusively in *Pseudomonas aeruginosa* (*8*). RmtC was subsequently identified only in *Proteus mirabilis* (*4*). RmtB has been found among various gram-negative bacterial species, including *Serratia marcescens*, *Escherichia coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca*, isolated in Japan, South Korea, and Taiwan (*2,5,6,9*). Another new 16S rRNA methylase was initially identified in *C. freundii* in Poland, submitted to European Molecular Biology Laboratory (EMBL)/GenBank in 2002 (accession no. AF550415), and later characterized and assigned as ArmA in *K. pneumoniae*, *E. coli*, *Enterobacter cloacae*, *Salmonella enterica*, and *Shigella flexneri* in France, Bulgaria, and Spain (*10,11*). Moreover, ArmA was also identified in *E. coli*, *K. pneumoniae*, *E. cloacae*, *C. freundii* and *S. marcescens* in South Korea, Japan, and Taiwan (*5,8,9*). This enzyme has also been identified in a glucose nonfermentative *Acinetobacter* sp. in South Korea and Japan (*6,8*). Quite recently, RmtD was newly identified in the SPM-1–producing *P. aeruginosa* strain PA0905, which was isolated in Brazil (*7*). In Japan, arbekacin, a semisynthetic aminoglycoside, has been approved for treatment of methicillin-resistant *Staphylococcus aureus* infections, and this agent is also very efficacious for gram-negative bacteria. However, 16S rRNA methylase–producing microbes can adapt to this agent, and its prescription may well be a selective pressure on the kind of microbes in the clinical environment. Thus, this investigation was conducted to determine the exact isolation frequency of 16S rRNA methylase–producing, gram-negative pathogenic bacteria in Japanese medical facilities and assess the possibility of the future prevalence of these hazardous microbes.

The Study

From September 1 to October 31, 2004, 169 medical facilities with in-house microbiology laboratories participated in this investigation. Clinical specimens were collected from inpatients and outpatients with suspected infections. Bacterial isolates that belonged to the family *Enterobacteriaceae* or were nonfermentors of glucose, for example, *P. aeruginosa* and *Acinetobacter* spp., were included in this study. A total of 87,626 clinical isolates were analyzed. The results are shown in Table 1.

Twenty-nine strains (17 *P. aeruginosa*, 4 *A. baumannii*, 3 *E. coli*, 2 *P. mirabilis*, 1 *E. cloacae*, 1 *K. pneumoniae*, and 1 *Enterobacter aerogenes*) that grew on LB agar plates supplemented with 500 mg of arbekacin per liter were subjected to the typing of 16S rRNA methylase genes by a multiplex PCR. Primers used for the PCR amplification of bacterial 16S rRNA methylase genes were the following: RMTA-F 5′-CTA GCG TCC ATC CTT TCC TC-3′ and RMTA-R 5′-TTT GCT TCC ATG CCC TTG CC-3′, which amplify a 635-bp DNA fragment within *rmtA* gene (*3*); RMTB-F 5′-GCT TTC TGC GGG CGA TGT AA-3′ and RMTB-R 5′-ATG CAA TGC CGC GCT CGT AT-3′, which amplify a 173-bp DNA fragment within *rmtB* (*2*); RMTC-F 5′-CGA AGA AGT AAC AGC CAA AG-3′ and RMTC-R 5′-ATC CCA ACA TCT CTC CCA CT-3′, which amplify a 711-bp DNA fragment within *rmtC* (*4*); and ARMA-F 5′-ATT CTG CCT ATC CTA ATT GG-3′ and ARMA-R 5′-ACC TAT ACT TTA TCG TCG TC-3′, which amplify a 315-bp DNA fragment within *armA* (accession

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Table 1. Gram-negative strains investigated during September and October, 2004

Bacterial species	Strains, n			Rate of 16S rRNA methylase-producing strains, %
	Isolated	Resistant to all aminoglycosides tested	Harboring 16S rRNA methylase gene, n	
Pseudomonas aeruginosa	18,037	384	14	0.08
Escherichia coli	14,701	39	3	0.02
Klebsiella spp.	12,293	11	1	0.008
Enterobacter spp.	6,398	26	2	0.03
Acinetobacter spp.	3,116	33	4	0.13
Serratia marcescens	3,009	14	0	0
Citrobacter spp.	2,422	1	0	0
Proteus spp.	2,389	8	2	0.08
Alcaligenes spp.	443	0	0	0
Other	24,818	8	0	0
Total	87,626	527	26	0.03

nos. AY220558 and AB117519). PCR results and clinical data from these 29 strains are summarized in the Table 2. Genes for 16S rRNA methylases were absent in 3 arbekacin high-level-resistant strains of *P. aeruginosa* by PCR analyses that used 4 sets of 16S rRNA methylase-specific primers. In these strains, simultaneous production of multiple aminoglycoside-modifying enzymes was suggested as reported previously (*12*). Twenty-six strains harboring any of the four 16S rRNA methylase genes were identified in 16 hospitals, with no apparent geographic convergence in the locations of the hospitals (Figure 1). In hospital L, 3 different bacterial species (*E. coli*, *E. aerogenes*, and *K. pneumoniae*) harbored the *armA* gene, which suggests probable conjugal transfer of *armA*-carrying plasmids among different bacterial species.

Pulsed-field gel electrophoresis (PFGE) was performed on 9 strains of *P. aeruginosa* and 3 strains of *A. baumannii* isolated from 4 separate hospitals where 16S

Table 2. Bacterial species and type of 16S rRNA methylase gene detected*

Strain no.	Bacterial species	PCR type of 16S rRNA methylase gene	Hospital	Clinical specimen
40	*Proteus mirabilis*	*rmtC*	A	Sputum
64	*Pseudomonas aeruginosa*	*rmtA*	B	Sputum
101	*P. aeruginosa*	*rmtA*	C	Otorrhea
103	*P. aeruginosa*	*rmtA*	C	Otorrhea
109	*P. aeruginosa*	*rmtA*	C	Otorrhea
113	*P. aeruginosa*	*rmtA*	D	Bile
127	*P. aeruginosa*	*rmtA*	D	Pharynx
157	*P. aeruginosa*	*rmtA*	D	Pharynx
158	*P. aeruginosa*	*rmtA*	D	Stool
231	*Acinetobacter baumannii*	*armA*	E	Wound
249	*P. aeruginosa*	*rmtA*	F	Pus
252	*P. aeruginosa*	*rmtA*	F	Pleural fluid
328	*P. mirabilis*	*rmtC*	G	Sputum
353	*P. aeruginosa*	*rmtA*	H	Sputum
386	*Escherichia coli*	*rmtB*	I	Urine
422	*P. aeruginosa*	UD	J	Urine
463	*P. aeruginosa*	*rmtA*	K	Urine
469	*E. coli*	*armA*	L	Skin
470	*Enterobacter aerogenes*	*armA*	L	Stool
471	*Klebsiella pneumoniae*	*armA*	L	Stool
479	*P. aeruginosa*	*rmtA*	M	Unknown
499	*E. coli*	*armA*	N	Urine
509	*Enterobacter cloacae*	*armA*	O	Urine
525	*P. aeruginosa*	UD	P	Urine
527	*P. aeruginosa*	UD	Q	Blood
593	*P. aeruginosa*	*rmtA*	R	Vaginal secretion
615	*A. baumannii*	*armA*	S	Sputum
617	*A. baumannii*	*armA*	S	Sputum
619	*A. baumannii*	*armA*	S	Pus

*Strains for which MIC of arbekacin was ≥512 mg/L are listed; UD, undetected.

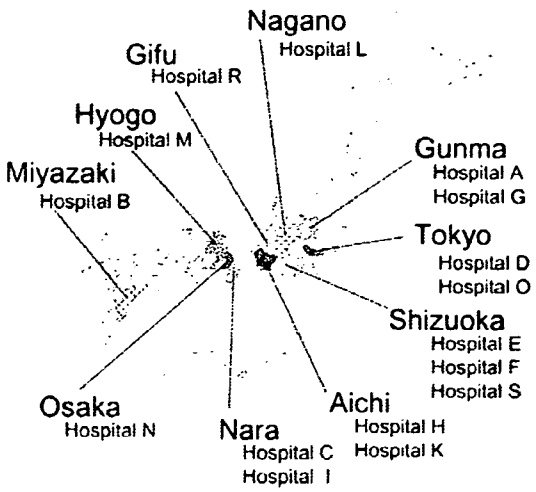

Figure 1. Geographic distribution of hospitals where 16S rRNA methylase gene-positive strains were isolated. Of 16 hospitals, 4 were located in the Kanto area (Gunma and Tokyo), 6 in the Chubu area (Aichi, Gifu, and Shizuoka), 1 in the Koushin-etsu area (Nagano), 4 in the Kinki area (Osaka, Nara, and Hyogo), and 1 in the Kyushu area (Miyazaki). This distribution suggests a sparse but diffuse spread of 16S rRNA methylase-producing, gram-negative pathogenic microbes in Japan. Bacterial species and type of 16S rRNA methylase identified in each hospital are shown in Table 2.

rRNA methylase genes were isolated (Figure 2). Genomic DNA preparations from *P. aeruginosa* and *A. baumannii* were digested with *Spe*I and *Sma*I, respectively. Clonality was inferred based on the criteria of Tenover et al. (*13*) Two of 3 *rmtA*-positive *P. aeruginosa* strains isolated in hospital C were estimated to be the same clone. Among 4 *rmtA*-positive *P. aeruginosa* isolates recovered in hospital D, 2 different clonal lineages were observed. This finding suggests possible conjugal transfers of *rmtA*-carrying plasmids among genetically different strains of *P. aeruginosa*. Three *armA* gene–harboring *A. baumannii* identified in hospital S were obviously the same clone. These findings imply probable nosocomial transmission of 16S rRNA methylase gene–harboring strains in hospitals C, D, and S, as well as frequent conjugal transfers of plasmids carrying 16S rRNA methylase genes among gram-negative pathogenic bacterial species.

MIC determinations were performed according to the guideline of the CLSI (formerly National Committee on Clinical Laboratory Standards). All 16S rRNA methylase-positive strains were highly resistant (MICs >1,024 mg/L) of all 4,6-disubstituted deoxystreptamine group aminoglycosides (Table 3). In contrast, resistance to streptomycin and neomycin varied. Three16S rRNA methylase gene-negative *P. aeruginosa* strains were also highly resistant to arbekacin, but the MICs of some of the 4,6-disubstituted deoxystreptamine group aminoglycosides were relatively lower (256–512 mg/L) for these strains than those for 16S rRNA methylase gene–positive strains (>1,024 mg/L). Strains harboring 16S rRNA methylase genes tended to show resistance to oxyimino-cephalosporins such as cefotaxime and ceftazidime as well, but were susceptible to imipenem. As reported for the *armA*- or *rmtB*-bearing strains, the presence of β-lactamase genes was suggested in cefotaxime-resistant strains, and indeed the $bla_{CTX\text{-}M\text{-}14}$ gene was detected in several *rmtB*-positive strains tested in our study (data not shown). Some of these strains also demonstrated resistance to fluoroquinolones (Table 3).

Conclusions

The overall isolation frequency of 16S rRNA methylase-gene-positive gram-negative bacilli was very low (0.03%) in Japanese medical facilities in 2004, with the highest rates seen in *P. aeruginosa* and *Acinetobacter* spp. at 0.08% and 0.13%, respectively. Twenty-six bacterial isolates carrying 1 of the four 16S rRNA methylase genes were recovered from 16 (9.5%) of 169 hospitals that participated in this nationwide investigation. Of the 169 hospitals, 162 hospitals had ≥200 beds, accounting for 5.9% of all Japanese hospitals of similar scale. This implies that 16S rRNA methylase–producing strains might have been present in >250 Japanese hospitals during the investigation period, which in turn suggests sparse but diffuse spread of 16S rRNA methylase producers in Japan. Since several

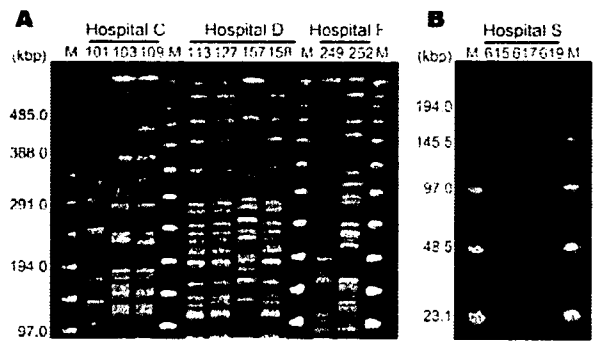

Figure 2. A) Pulsed-field gel electrophoresis (PFGE) fingerprinting patterns of *Spe*I-digested total DNA preparations from *Pseudomonas aeruginosa*. M, Lambda ladder PFGE molecular mass marker (Bio-Rad, Hercules, CA, USA). Strains 103 and 109 show similar patterns, which suggests probable nosocomial transmission of *rmtA*-positive strains in hospital C. Strains 113, 127, and 158 also demonstrate similar patterns, which implies possible nosocomial transmission in hospital D. However, 2 different PFGE patterns are observed in hospitals C, D, and F, which suggests transfer of plasmids carrying 16S rRNA-methylase genes among *P. aeruginosa* strains with different genetic backgrounds. B) *Sma*I-digested total DNA preparations from *Acinetobacter baumannii* isolated from hospital S. Three strains demonstrate the same PFGE pattern, which suggests probable nosocomial transmission of *armA*-positive *A. baumannii* in hospital S. M, lambda ladder low-range PFGE molecular mass marker (New England Biolabs, Ipswich, MA, USA).

Table 3. MICs of antimicrobial agents for arbekacin-resistant strains*†‡

Strain no.	MIC (mg/L)										
	ABK	AMK	TOB	ISP	GEN	SM	NEO	CTX	CAZ	IPM	CIP
40	>1,024	>1,024	>1,024	>1,024	>1,024	8	>1,024	<0.06	0.125	0.125	64
64	>1,024	>1,024	>1,024	>1,024	>1,024	8	>1,024	<0.06	0.5	0.125	64
101	>1,024	>1,024	>1,024	>1,024	>1,024	32	>1,024	8	2	0.5	32
103	>1,024	>1,024	>1,024	>1,024	>1,024	32	16	64	2	0.5	<0.06
109	>1,024	>1,024	>1,024	>1,024	>1,024	8	16	64	16	0.5	<0.06
113	>1,024	>1,024	>1,024	>1,024	>1,024	128	512	16	2	16	0.125
127	>1,024	>1,024	>1,024	>1,024	>1,024	128	128	16	2	16	<0.06
157	>1,024	>1,024	>1,024	>1,024	>1,024	32	32	64	4	2	0.5
158	>1,024	>1,024	>1,024	>1,024	>1,024	128	512	32	8	16	0.125
231	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	32	>128	128	4	16
249	>1,024	>1,024	>1,024	>1,024	>1,024	256	512	16	1	4	<0.06
252	>1,024	>1,024	>1,024	>1,024	>1,024	512	512	128	4	4	8
328	>1,024	>1,024	>1,024	>1,024	>1,024	8	512	>128	>128	2	32
353	>1,024	>1,024	>1,024	>1,024	>1,024	32	256	64	>128	4	32
386	>1,024	>1,024	>1,024	>1,024	>1,024	256	256	128	>128	0.5	>128
422	>1,024	>1,024	>1,024	256	>1,024	512	>1,024	>128	>128	8	128
463	>1,024	>1,024	>1,024	>1,024	>1,024	64	128	16	4	8	32
469	>1,024	>1,024	>1,024	>1,024	>1,024	64	32	>128	8	0.25	<0.06
470	>1,024	>1,024	>1,024	>1,024	>1,024	128	8	>128	>128	4	1
471	>1,024	>1,024	>1,024	>1,024	>1,024	64	8	128	4	0.25	<0.06
479	>1,024	>1,024	>1,024	>1,024	>1,024	256	1,024	64	4	0.25	0.25
499	>1,024	>1,024	>1,024	>1,024	>1,024	64	4	0.06	0.125	0.25	0.25
509	>1,024	>1,024	>1,024	>1,024	>1,024	64	1	>128	64	0.25	125
525	512	512	1,024	512	256	>1,024	>1,024	128	32	16	>128
527	1,024	512	1,024	>1,024	64	>1,024	>1,024	>128	>128	128	0.125
593	>1,024	>1,024	>1,024	>1,024	>1,024	128	64	>128	128	2	0.5
615	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	16	>128	>128	1	32
617	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	32	>128	>128	1	32
619	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	32	>128	>128	1	32

*ABK, arbekacin; AMK, amikacin; TOB, tobramycin; ISP, isepamicin; GEN, gentamicin; SM, streptomycin; NEO, neomycin; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; CIP, ciprofloxacin.
†MICs of kanamycin and sisomicin are not listed because values are >1,024 for all strain numbers.
‡See Table 2 for bacterial species and PCR type of 16S rRNA methylase gene of each strain number.

armA- or *rmtB*-positive strains have also been isolated in European and Asian countries, and given the potential for further dissemination, nationwide identification and ongoing surveillance of these isolates should be considered by all countries.

According to PFGE typing, nosocomial transmission of 16S rRNA methylase–producing *P. aeruginosa* and *A. baumannii* was suspected in 3 hospitals (hospitals C, D, and S). The banding patterns of *rmtA*-harboring *P. aeruginosa* isolated in hospitals C, D, and F were diverse, which excluded the possibility of an epidemic *P. aeruginosa* strain harboring the *rmtA* gene. Despite the observation of 2 different PFGE profiles among the 4 *P. aeruginosa* strains isolated in hospital D, they might share the same plasmids carrying the *rmtA* gene. For further characterization of genetic relations among *rmtA*-harboring *P. aeruginosa* strains, comparative analyses of plasmids and mobile elements that carry the *rmtA* gene (*14*) should also be pursued.

Nosocomial infections caused by multidrug-resistant, gram-negative bacteria have become a serious problem in clinical facilities. *P. aeruginosa* and *Acinetobacter* spp. have been especially efficient at developing multidrug resistance against broad-spectrum β-lactams, fluoroquinolones, and aminoglycosides (*3,6,7,9*). The identification of *armA* and *rmtB* genes in Europe and East Asia in both human (*1–11*) and livestock (*15*; EMBL/GenBank accession no. DQ345788) populations suggests that we must pay consistent attention to prevent further global proliferation. If 16S rRNA methylase–positive bacterial isolates disseminate widely and extensively, the high level of pan-aminoglycoside resistance will undoubtedly have an impact on illness, deaths, and costs of care in both clinical and livestock-breeding environments.

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REVIEW

Epidemiological approach to nosocomial infection surveillance data: the Japanese Nosocomial Infection Surveillance System

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Abstract Surveillance of nosocomial infection is the foundation of infection control. Nosocomial infection surveillance data ought to be summarized, reported, and fed back to health care personnel for corrective action. Using the Japanese Nosocomial Infection Surveillance (JANIS) data, we determined the incidence of nosocomial infections in intensive care units (ICUs) of Japanese hospitals and assessed the impact of nosocomial infections on mortality and length of stay. We also elucidated individual and environmental factors associated with nosocomial infections, examined the benchmarking of infection rates and developed a practical tool for comparing infection rates with case-mix adjustment. The studies carried out to date using the JANIS data have provided valuable information on the epidemiology of nosocomial infections in Japanese ICUs, and this information will contribute to the development of evidence-based infection control programs for Japanese ICUs. We conclude that current surveillance systems provide an inadequate feedback of nosocomial infection surveillance data and, based on our results, suggest a methodology for assessing nosocomial infection surveillance data that will allow infection control professionals to maintain their surveillance systems in good working order.

Keywords Epidemiology · Intensive care units · Japan · Nosocomial infections · Surveillance

Introduction

Infection control in the hospital setting is performed with the aim of improving the effectiveness of patient care and promoting patient safety. Infection control professionals need to recognize and explain nosocomial infections and design and implement interventions to reduce their incidence. These infection control activities should have their bases in a well-designed surveillance system of nosocomial infections (Pittet 2005).

Compared with the USA and other developed countries, Japan traditionally had limited sources of information on the epidemiology of nosocomial infections and, until recently, little was known about the incidence and outcome of nosocomial infections in Japanese hospitals. The Japanese Ministry of Health, Labour, and Welfare established the Japanese Nosocomial Infection Surveillance (JANIS) system in July 2000, when participating hospitals routinely started to collect and subsequently make their nosocomial infection surveillance data available for entry into a national database. The JANIS database has now become the most important source of information on the epidemiology of nosocomial infections in Japanese hospitals.

In the study reported here, we used the JANIS data to determine the incidence of nosocomial infections in intensive care units (ICUs) of Japanese hospitals and assess the impact of nosocomial infections on mortality and length of stay. We elucidated individual and environmental

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factors associated with nosocomial infections, which allowed us to identify high-risk patients and take appropriate measures to prevent infection. Infection rates are calculated as a fundamental measure for surveillance of nosocomial infections. The comparison of infection rates between hospitals and within a hospital over time contributes to the improved design, implementation and evaluation of infection control programs (NNIS 1991; Archibald and Gaynes 1997). We examined the benchmarking of infection rates and developed a practical tool for comparing infection rates with case-mix adjustment.

The JANIS system

The ICU component of the JANIS system consists of more than 30 ICUs of multidisciplinary hospitals throughout Japan that have more than 200 beds. All of the patients admitted to the participating ICUs are automatically enrolled in the survey. The following data are collected by trained physicians and nurses in each ICU and sent to the data management office by the Internet on a monthly basis: sex, age, underlying disease, severity-of-illness (APACHE II; Knaus et al. 1985), ICU admission and discharge (date, time and route), operation (elective and urgent), device use (ventilator, urinary catheter and central venous catheter), infection (pneumonia, urinary tract infection, catheter-related bloodstream infection, sepsis, wound infection and others) and hospital discharge (date and outcome). APACHE II uses a point score based on the initial values of 12 routine physiological measurements, age and previous health status to provide a general measure of severity of illness (Knaus et al. 1985). A higher point score indicates a greater mortality risk. Infections are diagnosed according to the JANIS criteria (RHLS 2000), which are based on and modified from those of the National Nosocomial Infections Surveillance (NNIS) system in the USA (Garner et al. 1988). Nosocomial (ICU-acquired) infection is defined as a newly developed infection at least 2 days after ICU admission (Garner et al. 1988). Pathogens are classified as drug resistant or drug susceptible according to the JANIS definitions that specify a drug-resistance pattern for each pathogen (RHLS 2000).

The JANIS system takes various measures to establish a standardized and formatted database: a specific database-oriented software and a written operating manual with uniform definitions and surveillance protocols are prepared in advance; workshops for data collectors are conducted on demand; reliability checks are routinely performed at the data management office. Although the participating ICUs may not represent all Japanese ICUs, the JANIS data is the only reliable source of information on the epidemiology of nosocomial infections in Japanese ICUs. Compared with the NNIS system, the JANIS system has the advantage of collecting data from each patient. The availability of the JANIS data enabled us to assess the incidence and outcome of nosocomial infections in the context of individual factors.

Epidemiology of nosocomial infections in Japanese ICUs

Incidence of nosocomial infections

Figure 1 shows the incidence of nosocomial infections in Japanese ICUs (Suka et al. 2004a). The study cohort

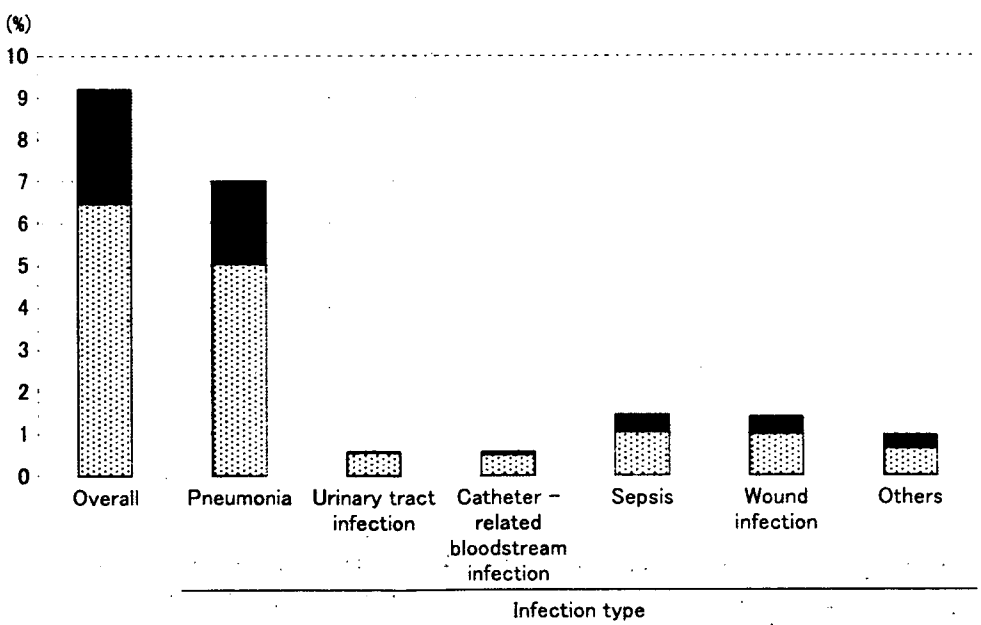

Fig. 1 Incidence of nosocomial infections (%) in Japanese intensive care units (ICUs) from July 2000 to May 2002. Pathogens were classified as drug resistant (*filled bar*) or drug susceptible (*shaded bar*) according to the JANIS definitions

consisted of 7374 eligible patients, aged ≥16 years, who had been hospitalized in 34 ICUs for ≥48 h. Of these 7374 patients, 678 patients (9.2%) had had at least one episode of nosocomial infection. The most common nosocomial infection was pneumonia (517 cases, 64%), followed by sepsis (106 cases, 13%), wound infection (102 cases, 13%), urinary tract infection (43 cases, 5%) and catheter-related bloodstream infection (42 cases, 5%). Drug-resistant pathogens were detected in 201 patients with nosocomial infection (29.6%). The majority of drug-resistant pathogens were methicillin-resistant *staphylococcus aureus* (MRSA).

A number of studies have been conducted to determine the incidence of nosocomial infections in ICUs. However, these studies differ considerably in terms of settings and protocols and, therefore, it is difficult to compare the reported rates accurately, although the incidence of urinary tract infection based on the NNIS data seems to be higher than that based on the JANIS data (annual reports of the JANIS system are available at: http://www.nih-janis.jp/report/list_index.html).

Outcome of nosocomial infections

The same cohort data were analyzed to assess the impact of nosocomial infections on hospital mortality (Suka et al. 2004a, b) and length of hospital stay for survivors (Suka et al. 2004b). The crude hospital mortality in the patients with nosocomial infection (45.8% for the cases of drug-resistant pathogens, 36.1% for the cases of drug-susceptible pathogens) was significantly higher than that in the patients without nosocomial infection (15.5%). Multivariate analysis adjusting for sex, age, APACHE II score, operation and device use showed that the patients with nosocomial infection caused by drug-resistant pathogens had a 1.4-fold higher risk of hospital mortality than those without nosocomial infection: the adjusted hazard ratio [95% confidence interval (CI)] of nosocomial infections for hospital mortality was 1.42 (1.15–1.77) for the cases of drug-resistant pathogens and 1.11 (0.94–1.31) for the cases of drug-susceptible pathogens. The patients with nosocomial infection had a significantly longer hospital stay than the patients without nosocomial infection. After adjusting for APACHE II score, we estimated the excess length of the hospital stay (95% CI) attributable to nosocomial infections to be 27.6 (18.6–36.5) days for the cases of drug-resistant pathogens and 12.8 (8.2–17.4) days for the cases of drug-susceptible pathogens.

Sepsis is a clinical syndrome describing infection and a subsequent systemic inflammatory response (Bone et al. 1992; Levy et al. 2001). A recent study of the JANIS data suggested that the development of sepsis leads to additional increases in mortality and length of stay among patients with nosocomial infection in Japanese ICUs (Suka et al. 2006a). In a study cohort of 20,909 eligible patients aged ≥16 years, hospitalized in 28 ICUs for ≥24 h, there were 928 episodes of nosocomial infection, including 168 episodes of sepsis (18.1%). The standardized mortality ratio (95% CI) was estimated at 2.43 (1.88–3.09) in those patients with nosocomial infection and subsequent sepsis and 1.18 (0.82–1.21) in those patients with nosocomial infection only. The mean length of stay (95% CI) following adjustment for the APACHE II score was estimated to be 15.0 (13.3–17.0) days in patients with nosocomial infection and subsequent sepsis and 11.8 (11.3–12.4) days in patients with nosocomial infection only compared with 3.8 (3.8–3.9) days in patients without nosocomial infection.

Factors associated with nosocomial infections

In order to implement interventions to reduce the incidence of nosocomial infections, infection control professionals need to understand factors associated with nosocomial infections. The identification of individual factors will allow them to identify high-risk patients, and the identification of environmental factors will allow them to take appropriate measures to prevent infection.

Individual factors

The study cohort data, consisting of 8587 eligible patients aged ≥16 years who had been hospitalized in 34 ICUs for ≥48 h, were analyzed to elucidate individual factors associated with nosocomial infections (Suka et al. 2004c). Table 1 shows the adjusted odds ratios for nosocomial infections. A significantly high odds ratio was found for APACHE II, urgent operation, ventilator and central venous catheter, while a significantly low odds ratio was found for women and elective operation. Although the impact of APACHE II on the incidence of nosocomial infections became weaker with longer ICU stay (Suka et al. 2005a), APACHE II was recognized to be a good predictor of nosocomial infections. Patients requiring particular attention from health care workers to guard against infection are male, have a high APACHE II score, have an urgent operation and use ventilator and central venous catheter. Device use is the major changeable risk factor for nosocomial infections (Fridkin et al. 1997). As mentioned above, pneumonia is the most common nosocomial infection in Japanese ICUs, and about 90% of episodes of pneumonia are associated with mechanical ventilation (Suka et al. 2007). Improvements in the management of ventilators should be given the highest priority as a preventive measures against nosocomial infections in Japanese ICUs.

Table 1 Adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for nosocomial infection

Individual factors		OR (95% CI)
Sex	Men	1.00 (Reference)
	Women	0.74 (0.62–0.88)
Age (years)	16–44	1.00 (Reference)
	45–54	0.83 (0.60–1.15)
	55–64	0.83 (0.62–1.12)
	65–74	0.89 (0.68–1.18)
	75+	0.75 (0.56–1.00)
APACHE II	0–5	1.00 (Reference)
	6–10	1.57 (1.03–2.40)
	11–15	2.55 (1.70–3.85)
	16–20	3.62 (2.39–5.49)
	21–25	5.38 (3.50–8.27)
	26–30	5.14 (3.23–8.16)
	31+	7.09 (4.34–11.59)
Operation	None	1.00 (Reference)
	Elective	0.78 (0.63–0.98)
	Urgent	1.22 (1.00–1.49)
Ventilator	Nonuser	1.00 (Reference)
	User	2.11 (1.62–2.76)
Central venous catheter	Nonuser	1.00 (Reference)
	User	1.48 (1.14–1.93)

Environmental factors

In 2001, 25 ICUs participating in the JANIS system took part in a questionnaire survey on ICU characteristics. The relationship between ICU characteristics and the incidence of nosocomial infections was assessed to elucidate environmental factors associated with nosocomial infections (Suka et al. 2005b). The survey identified a number of ICU characteristics associated with an increased incidence of nosocomial infections, namely, the location of the ICU close by a critical care center, resident physicians present for night duty, ICU rounds conducted by attending physicians less than once a day, case conferences held fewer than four times a month, no local guidelines for antibiotic use, common use of instruments and personnel not always using gloves for patient care. Infection control professionals should recognize the potential impacts of these factors on the incidence of nosocomial infections and consider implementing interventions to reduce the risk involved.

Assessment of nosocomial infection surveillance data

Infection rates are calculated as a fundamental measure for surveillance of nosocomial infections. They must be meaningful for comparison, either from one hospital to another or within a hospital over time (NNIS 1991; Archibald and Gaynes 1997). A relatively high or increased infection rate may suggest a potential problem in the infection control program of the hospital, while a relatively low or decreased infection rate may suggest that the infection control program of the hospital is successful in preventing infection. However, the distribution of risk factors for nosocomial infections varies widely according to hospital and time. Failure to adjust adequately for case-mix will lead to erroneous conclusions (NNIS 1991). Benchmarking of infection rates is important when assessing nosocomial infection surveillance data. Infection control professionals fervently hope for a practical tool that allows them to compare observed infection rates with external standards (benchmark infection rates) in the proper way.

Benchmarking of infection rates

Infection rates are calculated as the total number of episodes of nosocomial infection divided by a measure of the population at risk. The choice of the denominator is crucial for interhospital comparison (NNIS 1991; Archibald and Gaynes 1997). The JANIS data has been used to examine the distributions of several infection rates with different denominators with the aim of identifying the optimum denominator for comparing infection rates between different Japanese ICUs (Suka et al. 2005c). Figure 2 shows the distributions of infection rates (per admissions vs. per patient-days) for pneumonia of 18 Japanese ICUs. These varied widely according to the ICU, but all 18 ICUs were assessed at the same order using either infection rate. The infection rates per admissions and per patient-days were significantly correlated to each other ($r = 0.99$). Generally speaking, infection rates per patient-days are a better tool for carrying out interhospital comparison than infection rates per admissions because the former can be adjusted for length of stay (NNIS 1991). It would be advisable to use infection rates per patient-days for benchmarking in order to reduce the risk of misleading.

A practical tool for comparing infection rates with case-mix adjustment

A spreadsheet was developed to calculate the standardized infection ratio (SIR) on the basis of the Japanese benchmark infection rates that were derived from the JANIS data (Suka et al. 2006b). The SIR is a well-known risk-adjusted indicator that is calculated by the indirect standardization method by dividing the total number of observed

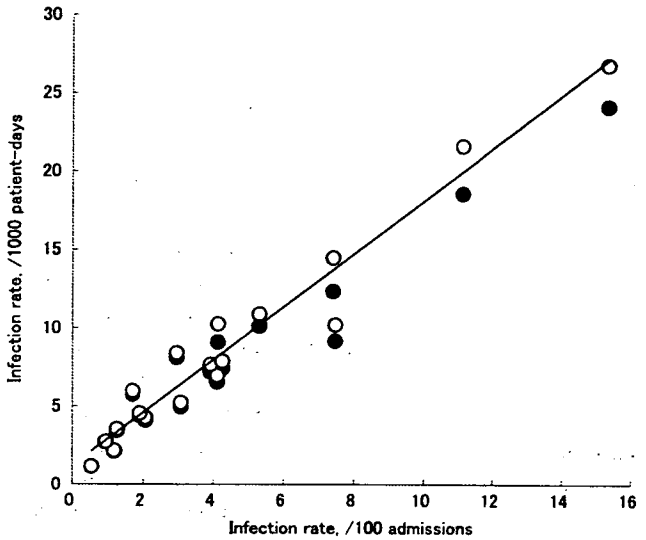

Fig. 2 Distributions of infection rates (per admissions vs. per patient-days) for pneumonia of 18 Japanese ICUs (June 2002–December 2003). Patient-days were counted as either total ICU stay (*filled circle*) or infection-free ICU stay (*open circle*)

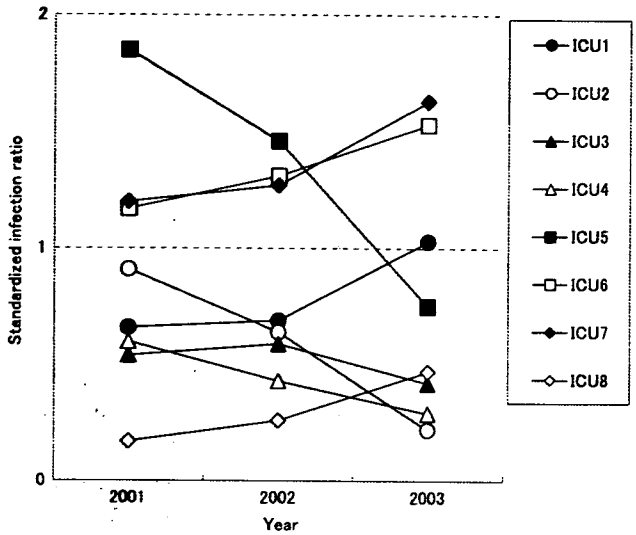

Fig. 3 Standardized infection ratios of eight Japanese ICUs. Standardized infection ratios were calculated by the indirect standardization method, which consists of dividing the total number of observed nosocomial infections by the total number of expected nosocomial infections based on the Japanese benchmark infection rates that were derived from the JANIS data

nosocomial infections by the total number of expected nosocomial infections (Gustafson 2000). The user of the spreadsheet inputs the number of observed nosocomial infections and patient-days by APACHE II, operation and ventilator, and then obtains a SIR adjusted for these three factors. A SIR value of more than one indicates that the incidence of nosocomial infections in the ICU is higher than the benchmark. Figure 3 shows SIRs of eight Japanese ICUs. For example, ICU5, ICU6 and ICU7 had a SIR of more than one in 2001. The SIR of ICU5 was gradually decreasing in 2002 and 2003, while, in contrast, those of ICU6 and ICU7 were increasing. One interpretation of these results is that ICU5 was successful in preventing infection while ICU6 and ICU7 had a potential problem in the infection control program and, therefore, should at the very least investigate the cause of the increasing incidence of nosocomial infection. The spreadsheet is simple and easy enough to be used by all infection control professionals, and it can reveal relative merits and secular changes in the incidence of nosocomial infections in the ICU. The use of the spreadsheet is expected to promote timely feedback of nosocomial infection surveillance data, which would allow infection control professionals to take prompt and efficient measures to prevent infection.

Conclusion

The studies of the JANIS data provide valuable information on the epidemiology of nosocomial infections in Japanese ICUs, and this information will contribute to the development of evidence-based infection control programs for Japanese ICUs. The primary aim of the surveillance of nosocomial infections is to introduce interventions aimed at reducing the incidence of nosocomial infections. Nosocomial infection surveillance data ought to be summarized, reported and fed back to health care personnel for corrective action (Pittet 2005). However, the current surveillance systems provide an inadequate feedback of nosocomial infection surveillance data. The studies of the JANIS data suggest a methodology of assessment of nosocomial infection surveillance data that will allow infection control professionals to keep the surveillance systems in good working order. For further developments of surveillance of nosocomial infections in Japanese hospitals, we may need to assess the effect of current surveillance systems on the incidence and outcome of nosocomial infections in future studies.

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Original article

Contribution of GM-CSF on the enhancement of the T cell-stimulating activity of macrophages

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Abstract

Mycobacterium leprae is an intracellular parasitic organism that multiplies in macrophages (MØ). It inhibits the fusion of mycobacterial phagosome with lysosome and induces interleukin (IL)-10 production from macrophages. However, macrophages are heterogenous in various aspects. We examined macrophages that differentiated from monocytes using either recombinant (r) granulocyte-MØ colony-stimulating factor (GM-CSF) (these MØ are named as GM-MØ) or rMØ colony-stimulating factor (M-CSF) (cells named as M-MØ) in terms of the T cell-stimulating activity. Although both macrophages phagocytosed the mycobacteria equally, GM-MØ infected with *M. leprae* and subsequently treated with IFN-γ- and CD40 ligand (L) stimulated T cells to produce interferon-gamma (IFN-γ), but M-MØ lacked the ability to stimulate T cells. While M-MØ mounted a massive IL-10 production, GM-MØ did not produce the cytokine on infection with *M. leprae*. *M. leprae*-infected, IFN-γ- and CD40L-treated GM-MØ expressed a higher level of HLA-DR and CD86 Ags than those of M-MØ, and expressed one of the dominant antigenic molecules of *M. leprae*, Major Membrane Protein-II on their surface. These results indicate that GM-CSF, but not M-CSF, contributes to the up-regulation of the T cell-stimulating activity of *M. leprae*-infected macrophages.

Keywords: Macrophage; *M. leprae*; GM-CSF; IFN-γ

1. Introduction

Mycobacterium leprae (*M. leprae*), a causative agent of human leprosy, is a representative parasitic pathogen that induces skin lesions and chronic progressive peripheral nerve injury, leading to systemic deformity [1,2]. Leprosy represents a clinical spectrum, in which clinical manifestations are associated with different levels of immune responses to *M. leprae* infection [3]. One representative type is a tuberculoid leprosy, in which patients exhibit innate and adaptive immunities to *M. leprae* and manifest a localized form of the disease with granuloma formation in infected tissues [4–6]. For the activation of an adaptive immunity, dendritic cells (DC) derived from inflammatory monocytes, play a central role [7,8]; and, in *in vitro* experiments, both $CD4^+$ and $CD8^+$ T cells are activated by DC infected with *M. leprae*, and these DC expressed Major Membrane Protein-II (MMP-II) as a dominant antigenic molecule [9,10]. Another representative manifestation is lepromatous leprosy, in which patients show reduced levels of host defense associated immunities and manifest a disseminated form of the disease with a broad spread of foamy MØ, in which an abundance of bacilli are usually involved [11,12]. *M. leprae* resides in the phagosome in MØ and replicates there without being digested by lysosomal enzymes [13]. Furthermore, *M. leprae* stimulates MØ to produce IL-10 [5,6] and suppresses the DC-mediated Ag-specific

Abbreviations: Ag, antigen; APC, Ag-presenting cells; BCG, *Mycobacterium bovis* BCG; DC, dendritic cells; GFP-BCG, BCG expressing GFP; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-gamma; IL, interleukin; L, ligand; mAb, monoclonal antibody; MØ, macrophages; M-CSF, MØ colony-stimulating factor; *M. leprae*, *Mycobacterium leprae*; MMP-II, Major Membrane Protein-II; PBMC, peripheral blood mononuclear cells; r, recombinant.

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adaptive immunity [14,15]. These observations may indicate that the induction of intracellular processing of *M. leprae* and that of expression of molecules, such as MMP-II could lead to the activation of IFN-γ producing type 1 $CD4^+$ T cells. Another important element that should be considered for the full activation of T cells is the suppression of IL-10 production from *M. leprae*-infected MØ.

So far, a variety of methods and tools, including cytokines, have been used for the differentiation of MØ from human peripheral monocytes *in vitro* [16–18]. One representative MØ can be differentiated by using M-CSF, termed M-MØ, and another by using GM-CSF, termed GM-MØ. Both MØ represent different functions on infection with mycobacteria. However, much remains not fully understood with regard to *M. leprae* infection and the T cell-stimulating activity of these MØ.

In this report, we analyzed the characteristics of *M. leprae*-infected GM-MØ and M-MØ, and tried to develop immunological methods to enhance the MØ-mediated host defense activities against the bacteria.

2. Materials and methods

2.1. Preparation of cells and bacteria

Peripheral blood was obtained from healthy PPD-positive individuals under informed consent. We are aware that PPD-negative individuals would help to provide more information for our study; however, in Japan, most healthy individuals are PPD-positive, because *Mycobacterium bovis* BCG vaccination is compulsory for children (0–4 years old). Moreover, PPD-negative individuals in the Japanese population are those who do not respond to BCG vaccination, and therefore, it is likely that they suffer from some immune insufficiency. Therefore, these individuals cannot be used as controls for our experiments. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [19]. For preparation of peripheral monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMC using immunomagnetic beads coated with anti-CD3 monoclonal antibody (mAb) (Dynabeads 450, Dynal, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on collagen-coated plates and the non-plastic adherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes [19]. MØ were differentiated by culturing monocytes in the presence of 20% fetal calf serum and either rM-CSF (R and D Systems, Abingdon, UK) (M-MØ) or rGM-CSF (Pepro Tech EC LTD, London, UK) (GM-MØ) [20]. Both GM-MØ and M-MØ were pulsed with *M. leprae*, treated with an optimal dose of IFN-γ on day 3 of culture, further treated with CD40L on day 4, and were used as a stimulator of T cells on day 5 [21]. *M. leprae* (Thai-53) was isolated from the footpads of BALB/c-*nu/nu* mice [22]. The isolated bacteria were counted by Shepard's method [22]. Killed *M. leprae* was prepared by heating the bacteria at 60 °C for 18 h. BCG (Pasteur strain) was cultured *in vitro* using Middlebrook 7H9 broth supplemented with 0.05% Tween 80 and albumin-dextrose-catalase. BCG expressing GFP was constructed as follows. The GFP sequence was amplified from pEGFP-1 vector (CLONTECH, Palo Alto, CA), and cloned into pMV261 [23]. Transformants were selected on a 7H10 plate containing 25 μg/ml kanamycin. The phagocytosis of BCG by GM-MØ and M-MØ after culture was determined using FACScalibur (Becton Dickinson Immunocytometry System, San Jose, CA). The multiplicity of infection (MOI) was determined based on the assumption that MØ were equally susceptible to infection with *M. leprae* [24].

2.2. Analysis of cell surface antigen (Ag)

The expression of cell surface Ag on MØ was analyzed using FACScalibur. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical Co., St. Louis, MO) and 1×10^4 live cells were analyzed. For analysis of cell surface Ag, the following mAbs were used: FITC-conjugated mAb against HLA-ABC (G46-2.6, PharMingen, San Diego, CA), HLA-DR (L243, PharMingen), CD14 (M5E2, BD Biosciences, San Jose, CA), TLR2 (TL2.3, Serotec, Oxford, UK), TLR4 (HTA125, Santa Cruz Biotech, Santa Cruz, CA), CD209 (DCN46, PharMingen), CD86 (FUN-1, PharMingen), and CD40 (5C3, PharMingen).

The expression of MMP-II, which is one of the dominant antigenic molecules of *M. leprae* [9] on *M. leprae*-infected MØ was determined using the mAb (IgM, kappa) against MMP-II, followed by FITC-conjugated anti-mouse Igs Ab (Tago-immunologicals, Camarillo, CA).

2.3. APC function of M. leprae-infected MØ

The ability of *M. leprae*-infected MØ to stimulate T cells was assessed using an autologous MØ-T cell co-culture as previously described [24,25]. Freshly thawed PBMC were depleted of $CD56^+$, MHC class II^+ and $CD8^+$ cells by using magnetic beads coated with mAb to CD56, MHC class II and CD8 Ags (Dynabeads 450; Dynal) [25]. The purity of $CD4^+$ T cells was more than 98% as assessed by FACS analyses. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates and MØ were added to give the indicated MØ:$CD4^+$ T cell ratio. Supernatants of MØ-T cell co-cultures were collected on day 4 and the concentration of cytokines was determined.

2.4. Cytokine production

Levels of the following cytokines were measured; IFN-γ produced by $CD4^+$ T cells, IL-10, IL-1β, TNFα and IL-12p40 produced by MØ stimulated for 24 h with *M. leprae*. The concentrations of these cytokines were quantified using the enzyme assay kits, Opt EIA Human ELISA Set (BD PharMingen International).

2.5. Statistical analysis

Student's *t*-test was applied to determine the statistical differences.

3. Results

3.1. Characteristics of MØ differentiated from monocytes using GM-CSF

MØ were differentiated from monocytes using either GM-CSF (GM-MØ) or M-CSF (M-MØ). To characterize these two types of MØ, surface markers expressed on GM-MØ and M-MØ were analyzed using the monocytes obtained from the same donor by flow cytometry (Fig. 1). MHC class I (HLA-ABC) and class II (HLA-DR) Ags were similarly expressed on GM-MØ and M-MØ, but the expression of CD14 Ag was significantly reduced in GM-MØ. While the expression level of TLR2, CD209, CD40 and TLR4 Ags was not different between GM-MØ and M-MØ, the expression of CD86 was significantly higher on GM-MØ than M-MØ. Then, we examined the phagocytic capacity of GM-MØ and M-MØ by using BCG expressing GFP (GFP-BCG), since *M. leprae* cannot be cultured *in vitro* or express GFP. The percentage of MØ expressing GFP after co-culture of MØ with GFP-BCG was similar between GM-MØ and M-MØ (Fig. 2). These results indicate that GM-MØ and M-MØ differed in the expression of some surface markers, but they similarly phagocytosed the mycobacteria.

3.2. Effect of M. leprae infection to GM-MØ on the T cell-stimulating activity

Since *M. leprae* is an intracellular parasitic bacterium and is hardly digested with lysosomal enzyme in MØ unless MØ are activated [26], we analyzed the T cell-stimulating activity of *M. leprae*-infected GM-MØ and M-MØ (Table 1). When M-MØ were infected with up to MOI 80 of *M. leprae* and treated with IFN-γ and CD40L, they did not stimulate $CD4^+$ T cells to secrete a significant dose of IFN-γ. In contrast to M-MØ, when *M. leprae*-infected, IFN-γ- and CD40L-treated GM-MØ were used as Ag-presenting cells (APC), T cells produced significant levels of IFN-γ in a manner dependent on the dose of *M. leprae*. Since GM-MØ express CD40, and are activated by IFN-γ, we examined the effect of treatment with IFN-γ and CD40L on the T cell-stimulating activity of *M. leprae*-infected GM-MØ (Table 2). While IFN-γ production from $CD4^+$ T cells was not significantly induced by GM-MØ untreated or treated with either IFN-γ or CD40L, the cytokine production was significantly enhanced by the treatment of GM-MØ with

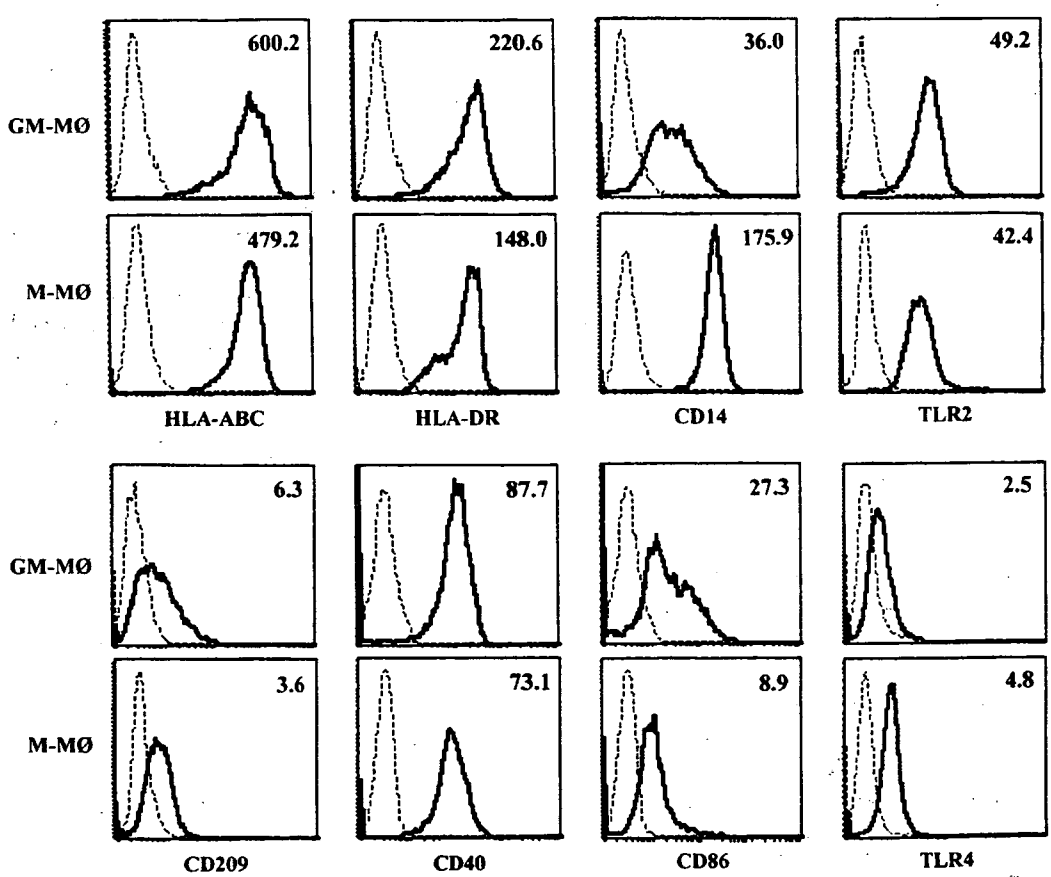

Fig. 1. Phenotype of GM-MØ and M-MØ differentiated from monocytes. Plastic adherent monocytes were differentiated into MØ by 3 days culture with either rGM-CSF or rM-CSF. Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dashed and solid lines. Representatives of three independent experiments are shown.

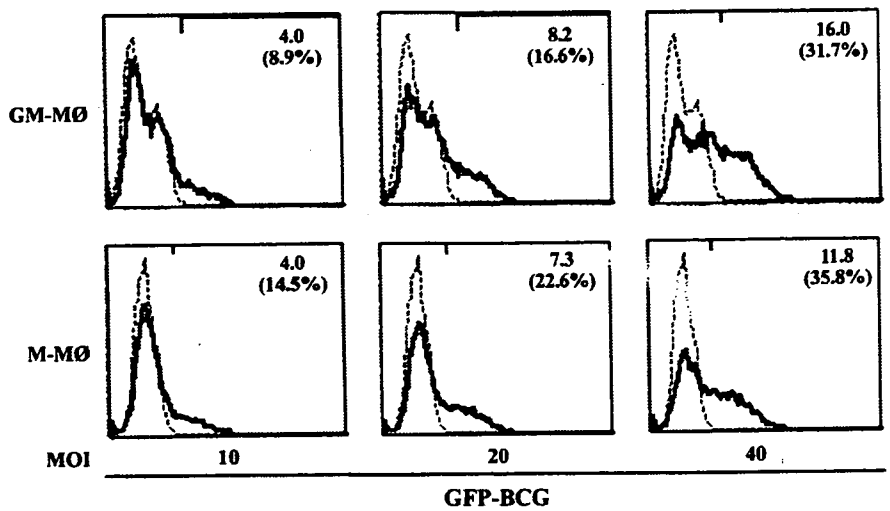

Fig. 2. Phagocytic activity of GM-MØ and M-MØ. Plastic adherent monocytes were differentiated into MØ by 3 days culture with either rGM-CSF or rM-CSF. For analysis of the phagocytic activity of MØ, GM-MØ and M-MØ were pulsed with BCG expressing GFP and assessed on day 4 of culture. Dashed lines, unpulsed cells; solid lines, GFP-BCG pulsed cells. The number represents the difference in mean fluorescence intensity between the dashed and solid lines. The number in parenthesis indicates the percent GFP-positive cell number. Representatives of three independent experiments are shown.

both IFN-γ and CD40L. Then, we compared the T cell-stimulating activity of live and heat-killed *M. leprae* (Table 3). Both forms of *M. leprae* stimulated $CD4^+$ T cells when pulsed to GM-MØ, but the heat-killed *M. leprae* more efficiently induced T cell activation than live bacteria. When we examined the effect of heat-killed *M. leprae* on M-MØ, they did not stimulate $CD4^+$ T cells significantly, even when IFN-γ and CD40L were administered (data not shown). Also note that, when GM-MØ and monocyte-derived DC were compared in terms of their T cell-stimulating activity, GM-MØ were less efficient in this respect (data not shown).

3.3. Factors associated with the enhancement of the T cell-stimulating activity of GM-MØ

Various factors may be responsible for enhancing the T cell-stimulating activity of APC. When we examined the expression of APC associated molecules on *M. leprae*-infected MØ (Fig. 3), the expression of HLA-DR and CD86 on GM-MØ was higher than on M-MØ, although there was no significant difference in the expression of HLA-ABC between GM-MØ and M-MØ. The cytokines produced from APC should also be considered to be another important factor that should be monitored and MØ produce a variety of cytokines, including IL-10, IL-1β, TNFα and IL-12 [6,11,27]. IL-10 was efficiently produced from M-MØ by stimulation with *M. leprae*, but it was hardly produced from GM-MØ (Fig. 4a). When macrophages were differentiated by using both GM-CSF and M-CSF, the function of GM-CSF was dominant and, the production of IL-10 was suppressed (Fig. 4a). Similarly to the production of IL-10, IL-1β was more efficiently produced from M-MØ than GM-MØ (Fig. 4b). In contrast, TNFα, which is important for granuloma formation, was more efficiently produced from GM-MØ (Fig. 4c). However, there was no significant difference in the production of IL-12p40 between GM-MØ and M-MØ (Fig. 4d). Finally, we assessed whether *M. leprae*-infected GM-MØ expressed dominant antigenic molecules of *M. leprae* on the surface (Fig. 5). To this end, we examined the expression of MMP-II on GM-MØ and M-MØ. No apparent expression of

Table 1
T cell-stimulating activity of *M. leprae*-infected GM-MØ and M-MØ[a]

Stimulator of $CD4^+$ T cells	*M. leprae* infection of macrophages (MOI)	IFN-γ (pg/ml) production by $CD4^+$ T cells after stimulation with macrophages at ratio (T:MØ)		
		2:1	4:1	8:1
GM-MØ	0	$0.6 \pm 0.2^{*,\dagger}$	$0.5 \pm 0.1^{\ddagger,\S}$	$1.4 \pm 0.2^{\P,\|}$
	40	$38.1 \pm 3.8^{*}$	$34.2 \pm 2.3^{\ddagger}$	$23.4 \pm 3.8^{\P}$
	80	$230.7 \pm 21.4^{\dagger}$	$120.5 \pm 16.9^{\S}$	$74.7 \pm 6.8^{\|}$
M-MØ	0	0.9 ± 0.1	3.1 ± 1.2	13.9 ± 2.2
	40	0.9 ± 0.1	2.6 ± 1.3	12.2 ± 3.1
	80	11.8 ± 0.3	17.5 ± 2.1	12.2 ± 2.9

$^{*}p < 0.005$; $^{\dagger}p < 0.005$; $^{\ddagger}p < 0.01$; $^{\S}p < 0.01$; $^{\P}p < 0.01$; $^{\|}p < 0.005$.

[a] $CD4^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous GM-MØ or M-MØ at the indicated dose of macrophage. GM-MØ or M-MØ were pulsed with *M. leprae* and IFN-γ (100 IU/ml) on day 3, treated with CD40L (1 μg/ml) on day 4, and were used as APC on day 5. Representatives of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean ± SD. Groups with identical symbols were compared using Student's *t*-test.

Table 2
Contribution of IFN-γ and CD40L on T cell-stimulating activity of GM-MØ[a]

M. Leprae infection of GM-MØ (MOI: 80)	Treatment of *M. leprae*-infected GM-MØ with		IFN-γ (pg/ml) production by $CD4^+$ T cells after stimulation with GM-MØ at ratio (T:MØ)		
	IFN-γ (100 IU/ml)	CD40L (1.0 μg/ml)	2:1	4:1	8:1
−	+	+	2.3 ± 0.3	0.1 ± 0.2	0.8 ± 0.5
+	−	−	4.0 ± 1.1*	5.5 ± 1.9§	6.0 ± 2.1#
+	−	+	21.4 ± 3.1†	22.7 ± 4.0¶	14.8 ± 2.2**
+	+	−	20.3 ± 1.7‡	15.9 ± 1.3‖	10.7 ± 2.3††
+	+	+	226.1 ± 20.9*,†,‡	107.8 ± 13.7§,¶,‖	94.8 ± 9.7#,**,††

*$p < 0.005$; †$p < 0.005$; ‡$p < 0.005$; §$p < 0.005$; ¶$p < 0.005$; ‖$p < 0.01$; #$p < 0.005$; **$p < 0.005$; ††$p < 0.005$.
[a] $CD4^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous GM-MØ at the indicated dose of macrophage. GM-MØ were pulsed with *M. leprae* and IFN-γ (100 IU/ml) on day 3, treated with CD40L (1 μg/ml) on day 4, and were used as APC on day 5. Representatives of three separate experiments are shown. Assays were done in triplicate, and the results are expressed as the mean ± SD. Groups with identical symbols were compared using Student's *t*-test.

MMP-II was observed on M-MØ, but, on GM-MØ, significant expression of MMP-II was induced. The expression was dependent on the dose of *M. leprae* (Fig. 5). However, the MMP-II expression on *M. leprae*-infected GM-MØ required both IFN-γ and CD40L, and apparent expression was not induced by sole treatment of macrophages with either IFN-γ or CD40L (data not shown).

4. Discussion

In order to avoid the intracellular multiplication and intercellular spread of *M. leprae*, the activation of adaptive immunity, especially of IFN-γ-producing type 1 T cells, plays an important role [5,6]. In fact, paucibacillary (tuberculoid) leprosy patients activate $CD4^+$ T cells through DC, although the bacteria cannot be eliminated completely [8,28]. The *M. leprae*-infected DC digest the bacteria and express dominant antigenic molecules for the efficient IFN-γ production from T cells [9]. In contrast, multibacillary (lepromatous) leprosy patients retain a large number of *M. leprae* in their MØ, and concordantly induce reduced levels or completely lack the ability to effectively stimulate T cells [11,12]. Since tissue resident MØ are heterogenous with regard to functional aspects [17,29], we assessed two different types of MØ: GM-MØ and M-MØ, and found that GM-MØ, but not M-MØ, stimulated T cells. GM-MØ were generated from monocytes using cytokine GM-CSF whilst M-MØ were produced using M-CSF. Although there were some differences in the expression levels of MHC class I, II, CD14 and CD209 Ags on GM-MØ and M-MØ, both forms were equally susceptible to mycobacteria as far as phagocytosis of BCG-GFP was examined. However, there was a striking difference between *M. leprae*-infected GM-MØ and M-MØ in the expression of antigenic molecules; only GM-MØ expressed MMP-II, which is one of the dominant antigenic molecules capable of stimulating T cells in *M. leprae*-infected individuals. The induction of MMP-II expression on GM-MØ requires not only GM-CSF, but also the co-stimulation of MØ with IFN-γ and CD40L. In case of *M. leprae*-infected DC, the phagosomal bacteria could be processed by lysosomal enzymes, and MMP-II expression was observed on DC [9]. The MMP-II expression observed on GM-MØ may indicate that at least some intracellular *M. leprae* were processed. However, the processing of *M. leprae* by GM-MØ still seemed partial, since the heat-killed *M. leprae* induced T cell activation more vigorously than live bacteria, and *M. leprae*-infected DC stimulated T cells more efficiently than GM-MØ, although other factors, such as an induction of IL-12, cannot be ruled out completely. The cell wall architecture including surface-exposed molecules, of heat-killed mycobacteria is globally altered [29,30], resulting in the exudation of some soluble antigenic molecules which may be feasibly digested in macrophages (unpublished observation). Therefore, T cells are more efficiently activated by heat-killed bacteria than by live bacteria.

Table 3
Comparison of T cell-stimulating activity of live and heat-inactivated *M. leprae*[a]

M. leprae pulsed on GM-MØ (MOI)	IFN-γ (pg/ml) production by $CD4^+$ T cells after stimulation with GM-MØ at ratio (T:MØ)		
	2:1	4:1	8:1
None	2.3 ± 1.1	2.1 ± 1.2	2.4 ± 0.9
HK (40)	406.5 ± 49.3*	157.3 ± 20.1‡	75.4 ± 6.8¶
HK (80)	399.8 ± 33.2†	187.7 ± 17.8§	106.9 ± 11.2‖
Live (40)	101.5 ± 8.8*	30.2 ± 4.6‡	3.2 ± 1.9¶
Live (80)	152.0 ± 12.7†	82.9 ± 7.4§	32.7 ± 2.8‖

*$p < 0.01$; †$p < 0.005$; ‡$p < 0.005$; §$p < 0.005$; ¶$p < 0.005$; ‖$p < 0.005$.
[a] $CD4^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous GM-MØ at the indicated dose of macrophage. GM-MØ were pulsed with either heat-killed (HK) or live *M. leprae* and IFN-γ (100 IU/ml) on day 3, treated with CD40L (1 μg/ml) on day 4, and were used as APC on day 5. Representatives of three separate experiments are shown. Assays were done in triplicate, and the results are expressed as the mean ± SD. Groups with identical symbols were compared using Student's *t*-test.

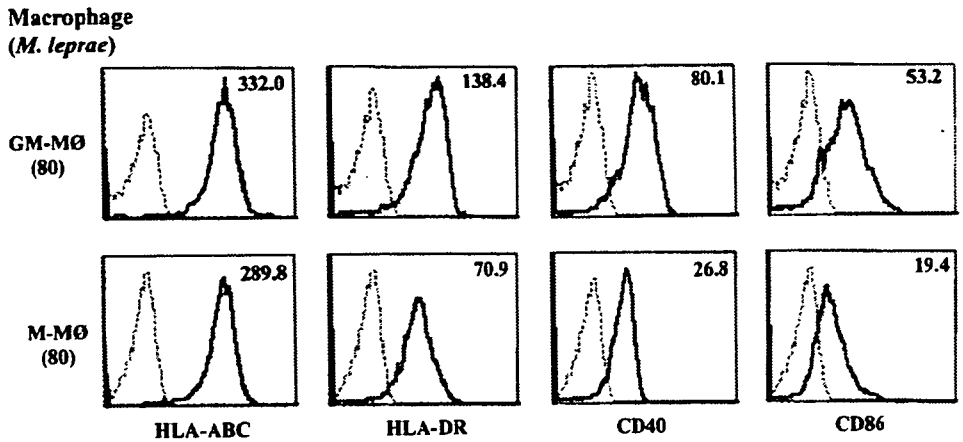

Fig. 3. Phenotype of *M. leprae*-infected GM-MØ and M-MØ. GM-MØ and M-MØ differentiated from monocytes by 3 days culture with rGM-CSF or rM-CSF were infected with *M. leprae*, treated with IFN-γ (100 IU/ml) on day 3, and further treated with CD40L (1 μg/ml) on day 4 of culture. On day 5, the phenotype of GM-MØ and M-MØ was analyzed. Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dashed and solid lines. Representatives of three independent experiments are shown.

Ottenhoff et al. have also reported that GM-CSF up-regulates the T cell-stimulating activity of MØ, but not M-CSF, and mycobacteria-infected GM-MØ promoted the type 1 cell-mediated immunity against pathogens [31]. Our observations are in line with their data and provide ways to enhance the cell-mediated immunity, especially in cases progressing towards lepromatous leprosy. To facilitate the T cell activation and MMP-II expression, it was required to use

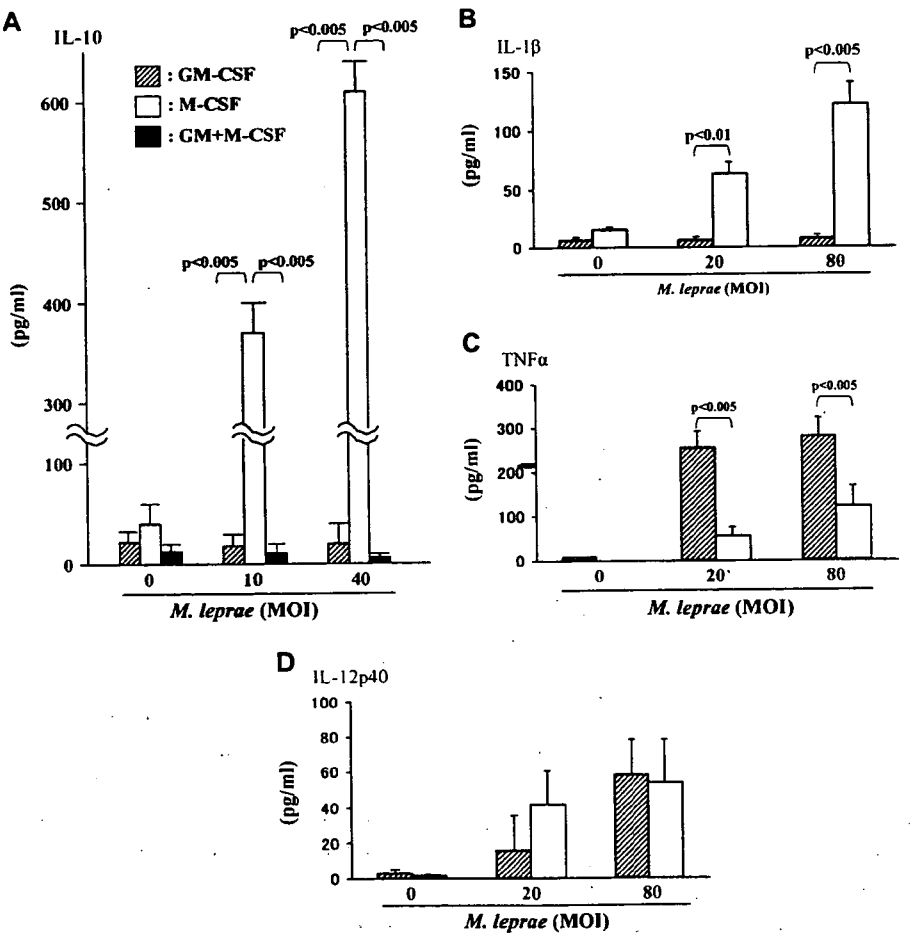

Fig. 4. Cytokine production from GM-MØ and M-MØ. MØ were differentiated by 3 days culture with rGM-CSF, rM-CSF or rGM-CSF + rM-CSF, and were stimulated with *M. leprae* for 24 h. The cytokines: (a) IL-10; (b) IL-1β; (c) TNFα; and (d) IL-12p40 were measured by ELISA. Representatives of three independent experiments are shown.