TABLE 3. Position of $(CGG)_5$, $(CGG)_6$, and $(CGG)_7$ within the genome in three mycobacterial strains

Strain and position (bp)	No. of repeats	Gene no.	Product	Domain	Translation
M. tuberculosis					
55532	7	Rv0050	Probable penicillin-binding protein, PonA	ORF	poly(Pro)
261808	5	Rv0218	Hypothetical protein	ORF	poly(Ala)
340616	5	Rv0280	PPE family protein	ORF	poly(Ala)
362891	5	Rv0297	PE_PGRS family protein	ORF	poly(Gly)
672720	5	Rv0578c	PE_PGRS family protein	ORF	poly(Gly)
968964	5	Rv0872c	PE_PGRS family protein	ORF	poly(Gly)
1091589	5	Rv0977	PE_PGRS family protein	ORF	poly(Gly)
1189183	5	Rv1067c	PE_PGRS family protein	ORF	poly(Gly)
1189430	5	Rv1068c	PE_PGRS family protein	ORF	poly(Gly)
1191358	5	Rv1068c	PE_PGRS family protein	ORF	poly(Gly)
1213387	5	Rv1087	PE_PGRS family protein	ORF	poly(Gly)
1631645	5	Rv1450c	PE_PGRS family protein	ORF	poly(Gly)
2357161	5	Rv2098c	PE_PGRS family protein	ORF	poly(Gly)
2357267	6	Rv2098c	PE_PGRS family protein	ORF	poly(Gly)
2387312	5	Rv2126c	PE_PGRS family protein	ORF	poly(Gly)
2423539	5	Rv2126c	PE_PGRS family protein	ORF	poly(Gly)
2639030	5	Rv2356c	PPE family protein	ORF	poly(Ala)
2639330	5	Rv2356c	PPE family protein	ORF	poly(Ala)
2639442	5	Rv2356	PPE family protein	ORF	poly(Ala)
2802267	5	Rv2490c	PE_PGRS family protein	ORF	poly(Gly)
2922778	5	Rv2591	PE_PGRS family protein	ORF	poly(Gly)
3528969	5	Rv3159c	PPE family protein	ORF	poly(Ala)
3752989	5	Rv3347c	PPE family protein	ORF	poly(Ala)
3766907	5	Rv3350c	PPE family protein	ORF	poly(Ala)
3802146	5	Rv3388	PE_PGRS family protein	ORF	poly(Gly)
3803514	5	Rv3388	PE_PGRS family protein	ORF	poly(Gly)
3969420	5	Rv3532	PPE family protein	ORF	poly(Ala)
3972241	5	Rv3533c	PPE family protein	ORF	poly(Ala)
4029032	5	Rv3587c	Hypothetical protein	ORF	poly(Pro)
M. tuberculosis CDC1551					
55478	6	MT0056	Penicillin-binding protein	ORF	poly(Pro)
261924	5	MT0228	Hypothetical protein	ORF	poly(Ala)
340680	5	MT0292	PPE family protein	ORF	poly(Ala)
362955	5	MT0311	PE_PGRS family protein	ORF	poly(Gly)
674173	5	MT0607	PE_PGRS family protein	ORF	poly(Gly)
927976	5	MT0855	PE_PGRS family protein	ORF	poly(Gly)
968979	5	MT0894	PE_PGRS family protein	ORF	poly(Gly)
1091604	5	MT1004	Putative; PE_PGRS family protein, authentic frame shift	ORF	poly(Gly)
1189231	5	MT1096.1	PE_PGRS family protein	ORF	poly(Gly)
1189478	5	MT1096.1	PE_PGRS family protein	ORF	poly(Gly)
1191406	5	MT1097	PE_PGRS family protein	ORF	poly(Gly)
1213545	5	MT1118.1		UTR	poly(Gly)
1631528	5	MT1497.1	PE_PGRS family protein	ORF	poly(Gly)
2359430	5	MT2159	PE family-related protein	ORF	poly(Gly)
2359536	5	MT2159	PE family-related protein	ORF	poly(Gly)
2385890	5	MT2184 ?	Conserved hypothetical protein ?	Terminator ?	poly(Gly)
2422232	5	MT2220	PE_PGRS family protein	ORF	poly(Gly)
2633780	5	MT2423	PPE family protein	ORF	poly(Ala)
2634080	5	MT2423	PPE family protein	ORF	poly(Ala)
2636362	5	MT2425	PPE family protein	ORF	poly(Ala)
2636662	5	MT2425	PPE family protein	ORF	poly(Ala)
2636774	5	MT2425	PPE family protein	ORF	poly(Ala)
2797756	5	MT2564	PE_PGRS family protein	ORF	poly(Gly)
2918923	5	MT2668.1	PE_PGRS family protein	ORF	poly(Gly)
3524456	5	MT3247	PPE family protein	ORF	poly(Ala)
3526605	5	MT3248	PPE family protein	ORF	poly(Ala)
3745224	5	MT3453	Putative; PPE family protein, authentic frame shift	ORF	poly(Ala)
3759134	5	MT3458	Putative; PPE family protein, authentic frame shift	ORF	poly(Ala)
3793024	5	MT3495	PE_PGRS family protein	ORF	poly(Gly)
3794392	5	MT3495	PE_PGRS family protein	ORF	poly(Gly)
3961566	5	MT3636	Putative; PPE family protein, authentic point mutation	ORF	poly(Gly)
3964387	5	MT3637	PPE family protein	ORF	poly(Ala)
4021174	5	MT3693	Hypothetical protein	ORF	poly(Pro)
M. bovis					
262035	5	Mb0224	Probable conserved transmembrane protein	ORF	poly(Ala)
341620	5	Mb0288	PPE family protein	ORF	poly(Ala)
363940	5	Mb0305	PE_PGRS family protein	ORF	poly(Gly)
673964	5	Mb0593c	PE_PGRS family protein	ORF	poly(Gly)
1092029	5	Mb1002	PE_PGRS family protein	ORF	poly(Gly)
1189891	5	Mb1096c	PE_PGRS family protein	ORF	poly(Gly)
1192527	5	Mb1097c	PE_PGRS family protein	ORF	poly(Gly)

Continued on following page

TABLE 3—*Continued*

Strain and position (bp)	No. of repeats	Gene no.	Product	Domain	Translation
1214721	5	Mb1116	PE_PGRS family protein	ORF	Poly(Gly)
1627966	5	Mb1485c	PE_PGRS family protein	ORF	Poly(Gly)
2339003	5	Mb2125c	Conserved hypothetical protein PE_PGRS family protein	ORF	Poly(Gly)
2339109	5	Mb2125c	Conserved hypothetical protein PE_PGRS family protein	ORF	Poly(Gly)
2367710	5	Mb2150c	Conserved hypothetical protein PE_PGRS family protein	ORF	Poly(Gly)
2604931	5	Mb2376c	PPE family protein	ORF	Poly(Ala)
2607401	5	Mb2377c	PPE family protein	ORF	Poly(Ala)
2607513	5	Mb2377c	PPE family protein	ORF	Poly(Ala)
2769065	5	Mb2517c	PE_PGRS family protein	ORF	Poly(Gly)
3706526	5	Mb3380c	PPE family protein	ORF	Poly(Ala)
3720437	5	Mb3385c	PPE family protein	ORF	Poly(Ala)
3755777	5	Mb3420	PE_PGRS family protein	ORF	Poly(Gly)
3912639	5	Mb3562	PPE family protein	ORF	Poly(Ala)
3915460	5	Mb3563c	PPE family protein	ORF	Poly(Ala)
3972250	5	Mb3618c	Probable conserved membrane protein	ORF	Poly(Pro)

containing $(CGG)_7$, PonA, encoded a penicillin-binding protein (Table 3). In strain CDC1551, the genes containing $(CGG)_5$ encoded the PPE, PE_PGRS, and PE families of proteins. A gene containing $(CGG)_6$ encoded a penicillin-binding protein (Table 3). In *M. bovis*, all genes containing $(CGG)_5$ encoded PPE and PE_PGRS family proteins, with the exception of two genes that encoded probable conserved membrane proteins (Table 3). In all three strains, the $(CGG)_5$ in the PPE genes translated to poly(Ala), and the $(CGG)_5$ and $(CGG)_6$ in the PE_PGRS and PE genes translated to poly(Gly). In both *M. tuberculosis* strains, the $(CGG)_6$ and $(CGG)_7$ in genes encoding penicillin-binding proteins translated to poly(Pro) (Table 3). In *M. bovis*, the two $(CGG)_5$ repeats in genes encoding probable conserved membrane proteins translated to poly(Ala) and poly(Pro) (Table 3). Most of the $(CGG)_5$ repeats within the PPE genes were located in the N-terminal PPE domain of the genes (data not shown). All $(CGG)_5$ and $(CGG)_6$ repeats within the PE_PGRS genes consisting of PE and PGRS domains were located in the PGRS domain (data not shown). Two $(CGG)_5$ repeats within the PE family-related gene (MT2159) in strain CDC1551 were located in the C-terminal domain of the genes (data not shown).

Genomic stability. To examine whether $(CGG)_5$ repeats in the genome are stable, two *M. tuberculosis* strains (H37Rv and IMCJ 541) were analyzed for $(CGG)_5$- and IS*6110*-probed fingerprints. The fingerprint patterns among culture periods were identical for strain H37Rv (Fig. 1A). These findings were confirmed with strain IMCJ 541 (Fig. 1B). The data indicate that $(CGG)_5$ repeats are stable in the genome for at least a few months. In the IS*6110*-probed fingerprints, the patterns did not change during the 9 weeks of culture of strain H37Rv or strain IMCJ 541 (data not shown), indicating that IS*6110* inserts are also stable over a few months.

Comparison of fingerprints between *M. tuberculosis* strains H37Rv and H37Ra. The virulent *M. tuberculosis* strain H37Rv and its avirulent derivative strain H37Ra were originally derived from the same strain, H37 (22, 23). It was reported that there are distinct differences between these strains with respect to IS*6110*-probed fingerprint patterns (3, 11). We investigated whether differences exist between these strains with respect to $(CGG)_5$-probed fingerprint patterns. DNA derived from the H37Rv and H37Ra strains were digested with 16 restriction enzymes as described in Materials and Methods. Unexpectedly, the patterns of $(CGG)_5$-based hybridization showed no differences between the H37Rv and H37Ra strains (Fig. 2A). For example, the $(CGG)_5$-based RFLP patterns of PvuII-digested fragments of H37Rv were identical to those of H37Ra (Fig. 2A, PvuII). However, the IS*6110*-based RFLP patterns of H37Rv were markedly different from those of H37Ra, which were analyzed with the use of the same blot of PvuII-digested fragments used in the $(CGG)_5$-based RFLP analysis (Fig. 2B). In the IS*6110*-based RFLP patterns, H37Rv showed 9 bands, and H37Ra showed 11 bands. Strain H37Rv but not H37Ra showed one band of 5.1 kb. Strain H37Ra but not H37Rv showed three bands of 1.1, 2.3, and 3.0 kb.

IS*6110*- and $(CGG)_5$-probed DNA fingerprinting of *M. tuberculosis* clinical isolates. To assess the potential usefulness of $(CGG)_5$ as an epidemiologic marker for *M. tuberculosis*, 109 clinical isolates obtained from Tokyo (76 isolates) and Warsaw (33 isolates) and the H37Rv and H37Ra strains were analyzed by the IS*6110*- and $(CGG)_5$-probed fingerprint methods. For IS*6110*-probed hybridization, DNA of these isolates was digested with PvuII according to a standardized protocol (26). For $(CGG)_5$-probed hybridization, DNA of the isolates was digested with AluI. When DNA of the H37Rv and H37Ra strains was digested with AatII, EcoRI, MluI, NruI, NsbI, PstI, PvuII, SacI, SalI, or XhoI, relatively higher-molecular-weight DNA fragments were visualized by the probe with a minimum size of 1 to 3.5 kb and a maximum size of more than 10 kb (Fig. 2A). When digested with AfaI, AluI, HinfI, Sau3AI, SmaI, or XspI, DNA fragments of sizes of 0.5 to 8 kb were visualized. When DNA of five clinical isolates selected at random were digested with AluI, clear $(CGG)_5$ fingerprint patterns with 10 to 14 copies of DNA fragments of 0.75 to 8 kb were detected (data not shown). Although we used AluI for this fingerprinting method, other enzymes may also be used.

IS*6110* fingerprint patterns obtained from clinical isolates and the corresponding dendrogram are shown in Fig. 3A. IS*6110* copies were detected in 110 of 111 isolates. One isolate from Japan had no copy. As indicated in Fig. 3A, 10 of 111 isolates (9.0% of tested isolates), including 8 isolates from Japan and 2 from Poland, possessed fewer than 6 copies of IS*6110*, which was insufficient to distinguish polymorphisms. Except for these 10 isolates with fewer than 6 copies of IS*6110*, the IS*6110* fingerprint patterns of 101 isolates showed ≥28% similarity; 98 patterns were found (Fig. 3A). Five clusters with

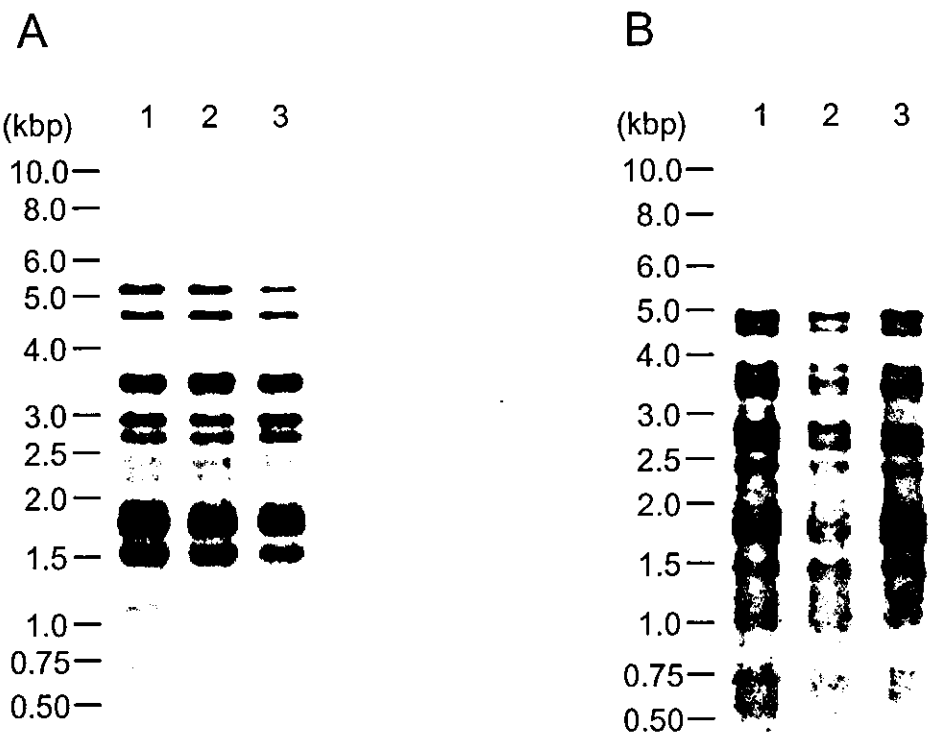

FIG. 1. (CGG)$_5$ fingerprinting of *M. tuberculosis* H37Rv (A) and clinical isolate IMCJ 541 (B), which were cultured and serially passaged weekly. The bacteria were harvested at 0 (lane 1), 3 (lane 2), and 9 (lane 3) weeks after culture.

≥44% similarity, including clusters Ia, IIa, IIIa, IVa, and Va, were detected (Fig. 3A). Cluster Ia was composed of seven Poland-derived isolates. Cluster IIa was composed of two H37 variants and 11 Japan- and 6 Poland-derived isolates. Cluster IIIa was composed of three Japan- and seven Poland-derived isolates. Cluster IVa was composed of four Japan- and five Poland-derived isolates. Cluster Va was composed predominantly of Japan-derived isolates (46 isolates from Japan and 2 from Poland). The majority of Japan-derived isolates (61%) and Poland-derived isolates (76%) belonged to cluster Va and to clusters Ia to IVa, respectively.

(CGG)$_5$ fingerprint patterns and the corresponding dendrogram are shown in Fig. 3B. (CGG)$_5$ copies were detected in all clinical isolates tested. The copy number ranged from 8 to 16, with a mean of 13.0 ± 1.5 per isolate. The number of (CGG)$_5$ copies of Japan- and Poland-derived isolates ranged from 8 to 16, with a mean of 12.9 ± 1.5 per isolate and from 11 to 15, with a mean of 13.2 ± 1.3 per isolate, respectively. A total of

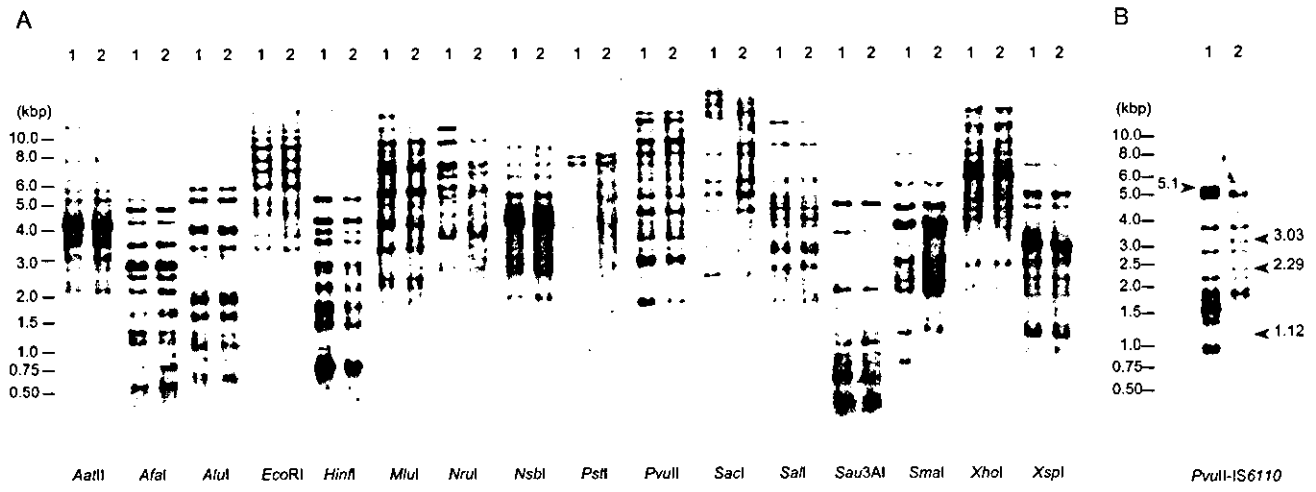

FIG. 2. (CGG)$_5$ (A) and IS*6110* (B) fingerprinting of *M. tuberculosis* strains H37Rv (lane 1) and H37Ra (lane 2). Genomic DNA was digested with 16 restriction enzymes. The digested fragments were separated by electrophoresis.

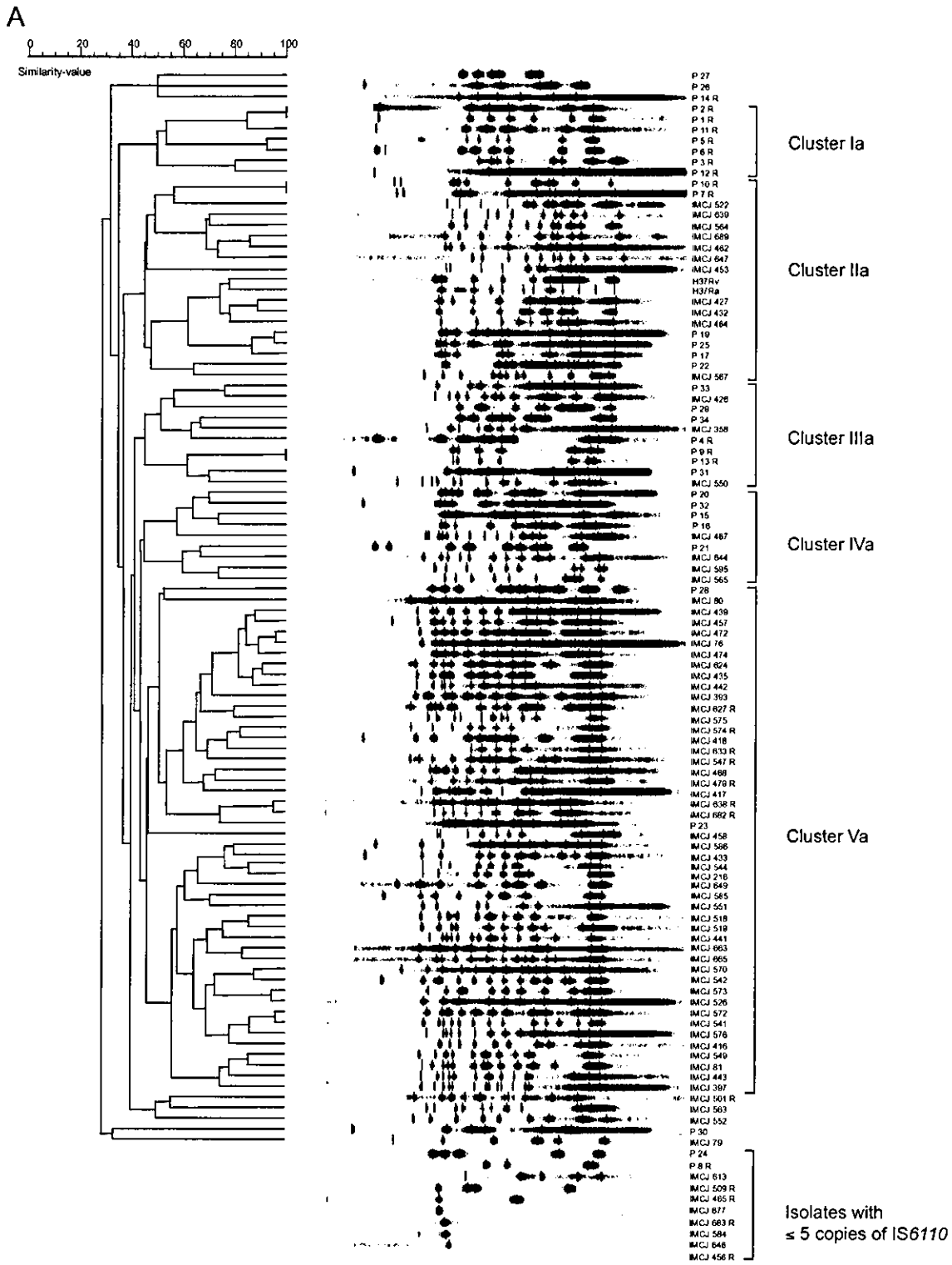

FIG. 3. IS*6110*- and $(CGG)_5$-probed DNA fingerprinting patterns of *M. tuberculosis* clinical isolates from Japan and Poland and the respective corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS*6110* (A) or $(CGG)_5$ (B) band is normalized so that the patterns for all strains are comparable. The scale depicts the similarity of patterns calculated as described in Materials and Methods. In IS*6110*-probed DNA fingerprint patterns, five clusters showing a similarity of more than 44% were designated clusters Ia, IIa, IIIa, IVa, and Va. Isolates with five or less than five copies are indicated in panel A. In $(CGG)_5$-probed DNA fingerprint patterns, four clusters showing a similarity of more than 70% were designated clusters Ib, IIb, IIIb, and IVb. The isolates are named according to their origin as IMCJ (Japan) or P (Poland); the suffix R indicates drug resistance For example, IMCJ 627 R is a Japan-derived drug-resistant isolate.

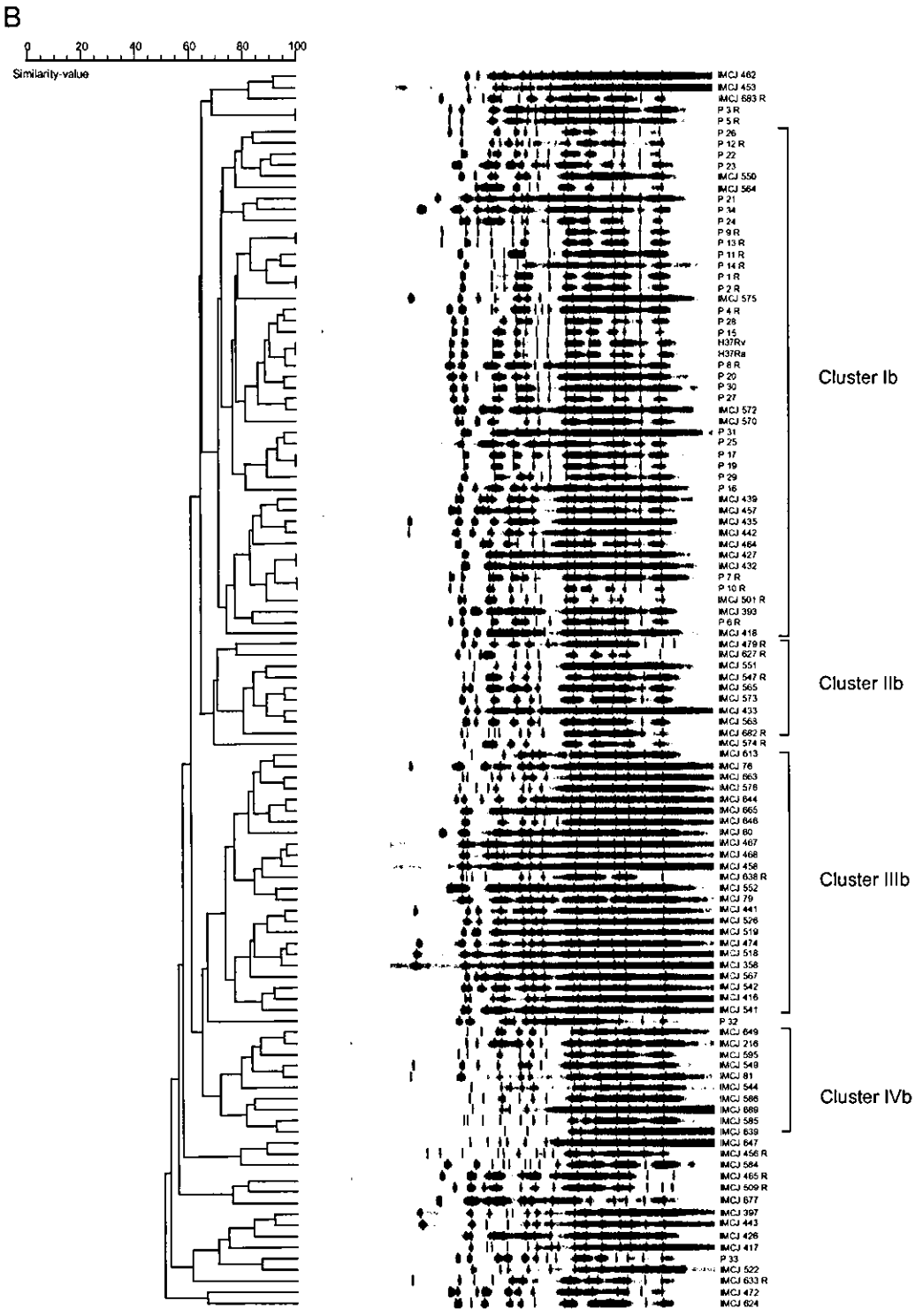

FIG. 3—*Continued.*

104 (CGG)$_5$ fingerprint patterns were found with ≥50% similarity (Fig. 3B). Four clusters with ≥70% similarity, including clusters Ib to IVb, were detected (Fig. 3B). Cluster Ib was composed of two H37 variants and 15 Japan- and 29 Poland-derived isolates. Clusters IIb, IIIb, and IVb were composed of 9, 24, and 10 Japan-derived isolates, respectively. Over half of the Japan-derived isolates (57%) and the majority of the Poland-derived isolates (88%) belonged to clusters IIb to IVb and to cluster Ib, respectively (Fig. 3B).

Both the IS*6110* and (CGG)$_5$ fingerprint analyses showed an association between fingerprint pattern and geographic origin, indicating a correlation between them. Ten isolates that were

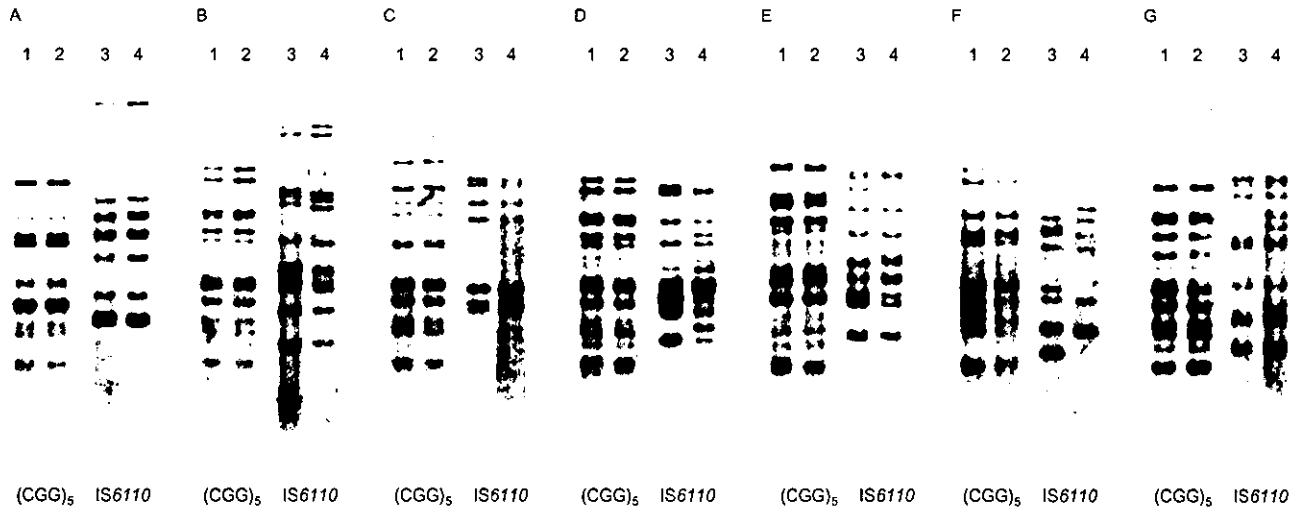

FIG. 4. $(CGG)_5$- and IS*6110*-probed DNA fingerprinting patterns of *M. tuberculosis* isolates that shared identical $(CGG)_5$ fingerprinting. (A) Lanes 1 and 3, P1; lanes 2 and 4, P2. (B) Lanes 1 and 3, P 7; lanes 2 and 4, P 10. (C) Lanes 1 and 3, P 9; lanes 2 and 4, P 13. (D) Lanes 1 and 3, H37Rv; lanes 2 and 4, H37Ra. (E) Lanes 1 and 3, IMCJ 427; lanes 2 and 4, IMCJ 432. (F) Lanes 1 and 3, P 3; lanes 2 and 4, P 5. (G) Lanes 1 and 3, P 17; lanes 2 and 4, P 19.

indistinguishable by IS*6110* RFLP because of the presence of few copies of the marker could be analyzed by $(CGG)_5$ marker. Three and seven pairs of isolates were identical to each other in the IS*6110* and $(CGG)_5$ fingerprint patterns, respectively (Fig. 4). The three pairs P 1 and P 2, P 7 and P 10, and P 9 and P 13 were identical to each other in the IS*6110* and $(CGG)_5$ fingerprint patterns (Fig. 4A to C, respectively). The four pairs H37Rv and H37Ra, IMCJ 427 and IMCJ 432, P 3 and P 5, and P 17 and P 19 were identical to each other in the $(CGG)_5$ fingerprint pattern but different in the IS*6110* fingerprint pattern (Fig. 4D, E, F, and G, respectively). The data suggest that the $(CGG)_5$ fingerprint patterns are more stable than the IS*6110* patterns.

Occurrence of $(CGG)_5$ among various mycobacterial strains. We investigated the presence of $(CGG)_5$ repeat sequences in mycobacterial species. $(CGG)_5$ hybridization patterns from various mycobacterial species are shown in Fig. 5. Bands ranging from 0 to 20 in number were seen. *Mycobacterium szulgai* possessed 20 bands. *M. bovis* BCG, *Mycobacterium marinum*, and *Mycobacterium kansasii* possessed 16 bands. *Mycobacterium nonchromogenicum, Mycobacterium terrae, Mycobacterium gastri, Mycobacterium simiae, Mycobacterium smegmatis*, and *Mycobacterium intracellulare* possessed 14, 12, 8, 5, 5, and 3 bands, respectively. *Mycobacterium peregrinum* possessed two bands. *Mycobacterium fortuitum* and *Mycobacterium chelonae* possessed one band. *Mycobacterium scrofulaceum, Mycobacterium avium, Mycobacterium xenopi*, and *Mycobacterium abscessus* showed no bands.

DISCUSSION

In this study, we found that various bacterial strains contain TRS in their genomes. In humans, TRS are associated with hereditary neurologic and neuromuscular disorders, including myotonic dystrophy, Huntington's disease, Fragile X syndrome, and Friedreich's ataxia (27). These diseases result from TRS expansion such as $(CTG)_n$, $(CGG)_n$, and $(GAA)_n$ (27). The TRS sizes associated with these diseases are usually quite large. For example, 80 to 3,000 repeats of CTG have been found in myotonic dystrophy, 230 to 2,000 repeats of CGG have been found in Fragile X syndrome, and 200 to 900 repeats of GAA have been found in Friedreich's ataxia (21). These expanded TRS can form hairpin structures or intramolecular triplex structures that result in genetic instability (21). The TRS sizes found in bacteria were relatively small. The largest size TRS identified was 21 repeats of GAA in *M. leprae*. The most frequently identified TRS was five repeats of CGG in *M. tuberculosis* and *M. bovis*. TRS found in bacteria are not likely to be linked to genetic instability because of the lower repeat number.

The $(CGG)_5$ TRS found in two strains of *M. tuberculosis* (H37Rv and CDC1551) and in one strain of *M. bovis* existed in genes encoding PE protein families, including a PE_PGRS subfamily and PPE protein families comprising 88 to 101 and 61 to 69 kinds of proteins, respectively, which occupy approximately 8% of the genome (4, 7, 8). The functional properties of $(CGG)_5$ in these genes are unknown, but $(CGG)_5$ should not play an important role in the development of the variations among different strains. $(CGG)_5$ in the PPE genes was located in the conserved N-terminal domain PPE but not in the C-terminal variable domain containing the major polymorphic tandem repeats with the consensus sequence of GCCGGT GTTG (10, 18). $(CGG)_5$ in the PE_PGRS genes was within the C-terminal variable domain containing the PGRS with the consensus sequence of CGGCGGCAA (18, 19). $(CGG)_5$ in the PE_PGRS genes did not comprise part of the consensus sequence of PGRS. $(CGG)_5$ was contained in 13 and 12 PE_PGRS genes in H37Rv and CDC1551, respectively. Among these genes, deletion or insertion was detected at one site of Rv1068c, two sites of Rv1087, and two sites of Rv1450c compared with their orthologs, MT1097, MT1118.1, and MT1497.1, respectively (data not shown). However, $(CGG)_5$ was not near these sites, indicating that it did not directly affect the deletion and insertion of PE_PGRS genes. $(CGG)_5$ in

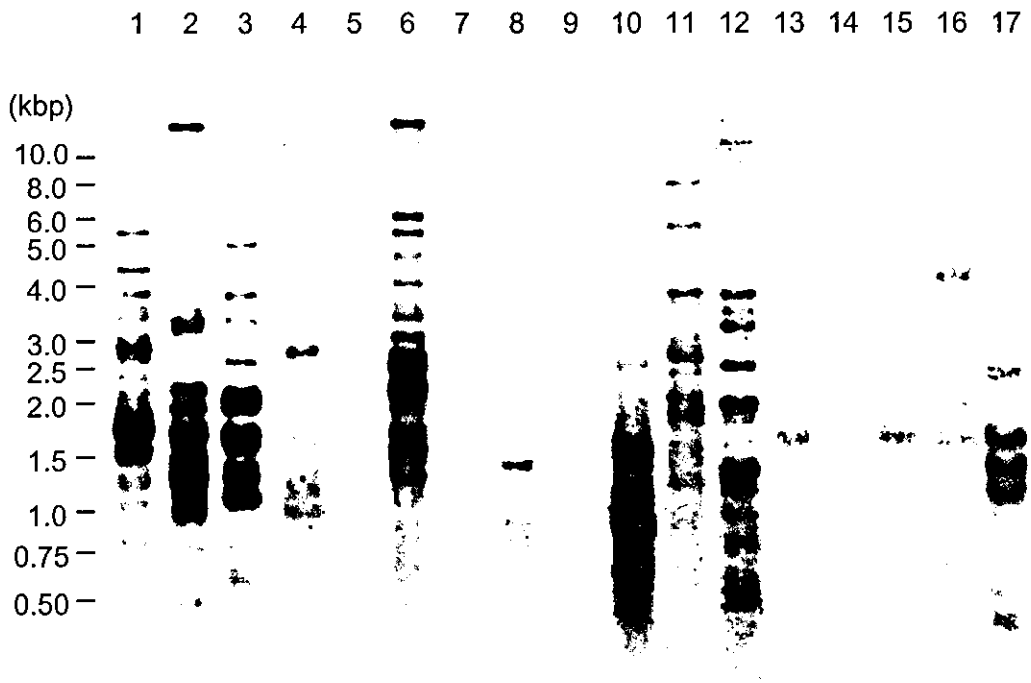

FIG. 5. (CGG)$_5$-probed fingerprinting of AluI-digested DNA from various mycobacterial species. Lane 1, *M. bovis* BCG; lane 2, *M. marinum*; lane 3, *M. kansasii*; lane 4, *M. simiae*; lane 5, *M. scrofulaceum*; lane 6, *M. szulgai*; lane 7, *M. avium*; lane 8, *M. intracellulare*; lane 9, *M. xenopi*; lane 10, *M. gastri*; lane 11, *M. terrae*; lane 12, *M. nonchromogenicum*; lane 13, *M. fortuitum*; lane 14, *M. abscessus*; lane 15, *M. chelonae*; lane 16, *M. peregrinum*; lane 17, *M. smegmatis*.

PPE, PE, and PE_PGRS genes translated to neutral-charged amino acids of poly(Ala) and poly(Gly), respectively, with no special substitution, indicating that these regions do not participate in the formation of unique structures within these proteins. Thus, the (CGG)$_5$ sequences in these genes will likely not have characteristic properties regarding function.

It is unclear whether TRS in bacteria, particularly (CGG)$_5$ in *M. tuberculosis* and *M. bovis*, participate in their pathogenesis. There was no difference between virulent strain H37Rv and the derived avirulent strain H37Ra in (CGG)$_5$-probed fingerprinting (Fig. 2). No correlation was found between the virulency of mycobacterial species and the numbers of bands in (CGG)$_5$-probed fingerprinting or copies of (CGG)$_5$ (Table 2 and Fig. 5). For example, *M. leprae* had no (CGG)$_5$ repeats (Table 2). Some rare etiologic agents of nontuberculous mycobacteria, such as *M. smegmatis* and *M. szulgai* (20), did possess several copies of (CGG)$_5$ in their genomes (Fig. 5), whereas some common etiologic agents, such as *M. avium*, *M. xenopi*, and *M. abscessus* (20), possessed no (CGG)$_5$ repeats (Fig. 5). These results indicate that (CGG)$_5$ repeats do not participate directly in the virulency of mycobacterial species.

Whereas fingerprinting analysis showed that both (CGG)$_5$ and IS*6110* were sufficiently stable epidemiologic markers, (CGG)$_5$ appeared to be more stable than IS*6110* (Fig. 1). We were unable to find any differences between strains H37Rv and H37Ra in (CGG)$_5$-probed fingerprinting by extensive studies with various restriction enzymes. However, four different bands were detected between these strains with PvuII-IS*6110* fingerprinting (Fig. 2B). Lari et al. (11) compared H37Rv and H37Ra strains maintained at their institution by IS*6110* fingerprinting with EcoNI, PstI, and PvuII and found different patterns between these strains. Bifani et al. (3) compared the PvuII-IS*6110* fingerprints of 15 and 3 different catalogued variants of H37Rv and H37Ra, respectively. Ten distinct fingerprint patterns, making up nine H37Rv variants and one H37Ra variant, were identified. A discrepancy between IS*6110*- and (CGG)$_5$-probed fingerprints of laboratory strains was observed in three pairs of clinical isolates (Fig. 4). In these cases, each isolate was identical in (CGG)$_5$ fingerprinting pattern but differed in its IS*6110* fingerprinting pattern. Our recent epidemiological case report of intrafamilial tuberculosis transmission showed that two clinical isolates from a father and son were identical in (CGG)$_5$-probed fingerprinting patterns, whereas one different band was detected between them by IS*6110*-probed fingerprinting (25). Collectively, IS*6110*-probed fingerprint patterns changed more rapidly than did (CGG)$_5$-probed patterns, suggesting that there are different mechanisms by which these patterns change. In other terms, although (CGG)$_5$-probed fingerprinting will hardly detect a few mutations in a clone of *M. tuberculosis*, it will easily detect an origin among the clones. The (CGG)$_5$-probed fingerprinting combined with IS*6110*-probed fingerprinting will provide more powerful information about tuberculosis epidemiology.

We collected and analyzed the isolates in this study in Japan and Poland. If isolates could be collected worldwide, it would provide more exact epidemiological data. In conclusion, the (CGG)$_5$ repeat is a useful probe for DNA fingerprinting of *M. tuberculosis*, because all strains tested here possessed more than eight copies. In addition, (CGG)$_5$-probed fingerprinting will be a useful tool for the investigation of *M. bovis*, *M. marinum*, *M. kansasii*, and *M. szulgai*.

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Laboratory and Epidemiology Communications

Detection of *Mycobacterium bovis* Bacillus Calmette-Guerin Using Quantum Dot Immuno-Conjugates

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Luminescent quantum dots (QDs) are a novel and promising class of fluorophores for cellular imaging (1,2). The benefits of QDs include their photostability, high brightness, multi-target labeling with several colors, and single-source excitation for QDs of all colors. We have developed procedures for using QDs to detect mycobacteria in a species-specific manner.

Mycobacterium bovis BCG strain 172 was obtained from Japan BCG Laboratory, Tokyo, Japan. A green fluorescent protein (GFP) expressing *M. bovis* BCG, containing plasmid pGFM-11, was supplied by C. Locht, Institut Pasteur de Lille, France. The BCG strains were grown in liquid Middlebrook 7H9 medium (Difco Laboratories, Detroit, Mich., USA) supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC, Difco) and incubated at 37°C. Ten microliters of liquid medium was mounted on a glass coverslip beneath a hole in a plastic petri dish bottom (Matsunami Glass Industry., Ltd., Tokyo, Japan; code. D110100) and were subsequently air dried. Two percent glutaraldehyde in PBS was applied for 1 h at room temperature. After several rinses with PBS, the 1% bovine serum albumin (BSA) in PBS (BSA/PBS) was applied for 20 min at room temperature to block nonspecific binding. Antiserum obtained from rabbits immunized with heat-killed BCG was applied at a dilution of 1:4000 with BSA/PBS, and the dishes were incubated for 1 h at room temperature. After several rinses with 0.02% Tween 20 in PBS (PBS/Tween 20), Qdot™ 655 goat $F(ab')_2$ anti-rabbit IgG conjugate (H+L) highly cross-absorbed (antibodies QD-conjugate: Quantum Dot Corp., Hayward, Calf., USA) was applied at a dilution of 1:1000 with 1% BSA for 1 h at room temperature. The dishes were then rinsed three times with PBS/Tween 20, and microscopic examinations were conducted with a confocal laser scanning microscope (LSM 510, Carl Zeiss, Oberkochen, Germany) equipped with a × 100/1.40 oil immersion objective, an HBO 50 illuminator, and an FITC/Rhodamine dual-band filter set.

The results of immunofluorescent staining (A, B), conventional mycobacterial staining (C, D), and Ziehl-Neelsen staining (E, F) are shown in Fig. 1. BCG strains were labeled in red when treated with anti-BCG antibodies (Fig. 1A), whereas *Mycobacterium smegmatis* (Fig. 1B) was not labeled when treated with anti-BCG antibodies, indicating that these antibodies was specific to *M. bovis* BCG.

As shown by the confocal image in Fig. 2A, the surface of

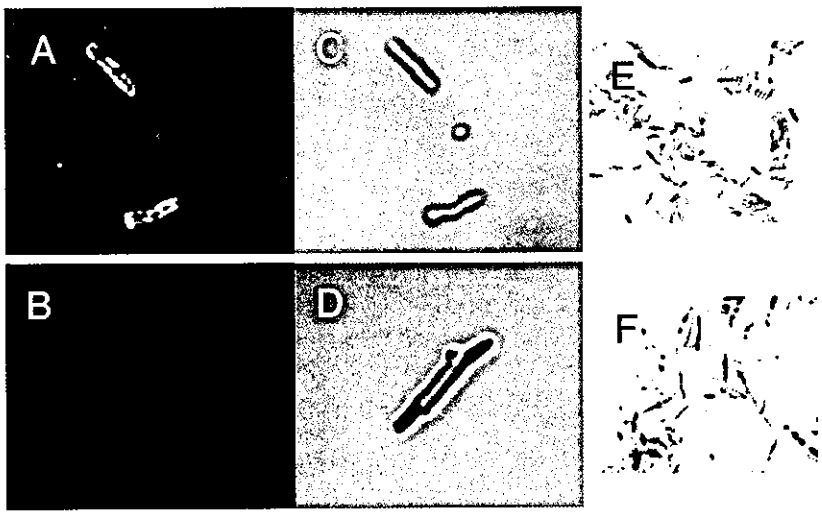

Fig. 1. Immunofluorescence staining of BCG (A, C, E) and *M. smegmatis* (B, D, F) strains (×1000).

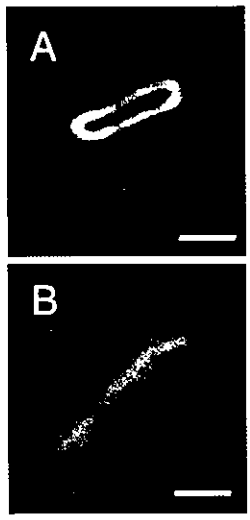

Fig. 2. Labeling of BCG (A) and GFP-expressed BCG (B) with anti-BCG antiserum and QD-conjugated anti-rabbit IgG. Scale bar, 1 μm.

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BCG strain 172 was labeled with red-colored QD-conjugated anti-rabbit IgG when treated with antiserum against BCG. The size of the labeled BCG was 3.5 (SD: 0.4) × 0.5 (SD: 0.1) μm (n = 4). The microorganisms were not labeled when treated with pre-immune serum. GFP-expressing BCG was stained using the same procedure (Fig. 2B). GFP was detected in the bacteria's intracellular region and was labeled only negligibly by QD-conjugate. The anti-BCG antibodies in combination with the QD-conjugated anti-IgG antibodies labeled the surface of BCG in a specific manner.

Acid-fast staining, such as Ziehl-Neelsen stain and auramine-rhodamine stain, are well-established procedures for detecting *Mycobacterium tuberculosis* and other mycobacterial spp. The immunostaining using QD-conjugates may be useful for identification of mycobacterial-specific antigen.

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Lipopolysaccharide Induces CD25-Positive, IL-10-Producing Lymphocytes Without Secretion of Proinflammatory Cytokines in the Human Colon: Low MD-2 mRNA Expression in Colonic Macrophages

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Despite the huge number of colonized Gram-negative bacteria in the colon, the normal colon maintains its homeostasis without any excessive immune response. To investigate the potential mechanisms involved, human colonic lamina propria mononuclear cells (LPMCs) obtained from uninflamed mucosa were cultured with lipopolysaccharide (LPS) prepared from *Bacteroides vulgatus* (BV-LPS) or *Bacteroides fragilis* (BF-LPS), as representatives of indigenous flora, or pathogenic *Salmonella minnesota* (SM-LPS). Colonic LPMCs failed to produce inflammatory cytokines in response to any type of LPS. Colonic macrophages barely expressed mRNA for MD-2, an essential association molecule for LPS signaling via Toll-like receptor 4. Further, BV-LPS induced CD25 and Foxp3 expression in lymphocytes and $CD4^+CD25^+$ cells expressed IL-10 mRNA. Thus, the low expression of functioning LPS receptor molecules and induction of IL-10-producing $CD4^+CD25^+$ lymphocytes by indigenous LPS may play a central role in the maintenance of colonic immunological homeostasis.

KEY WORDS: Human; lipopolysaccharide; colon; mucosa; cytokines.

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INTRODUCTION

There is accumulating evidence to support the notion that suppression of immunoreactions against resident flora is a major factor for the homeostasis of gastrointestinal immunity. For example, gene-manipulated immunodeficient mice, such as TCRα IL-2, and IL-10 gene knockout (−/−) mice, developed spontaneous colitis in a specific pathogen-free (SPF) or conventional environment; however, the severity of the inflammation was reduced under germ-free conditions (1). In humans, it is now clear that the bacterial flora play a role in the pathogenesis of inflammatory bowel diseases (IBD) including Crohn's disease and ulcerative colitis (UC). Tolerance toward autologous intestinal microflora was disturbed in patients with active IBD (2, 3). Studies in murine models also suggest that IBD likely represents a failure of the mucosal immune system to either attenuate its reaction or become tolerant to luminal bacterial proteins (4). These studies suggest that the failure of appropriate immune responses toward resident microflora causes critical intestinal disease.

In the large intestine, there are 10^{10}–10^{12} microorganisms per gram of feces. Intestinal bacterial flora comprise an estimated number of more than 400 bacterial species, most of which are anaerobic Gram-negative bacteria including *Bacteroides*, *Bifidobacteria*, and *Clostridia* species, as well as Gram-positive *Peptostreptococci* and *Peptococci* (5). Among these species of resident flora, *Bacteroides* species have been implicated in the pathogenesis of colitis. For example, colitis induced in guinea pigs by carrageenan was exacerbated after immunization of the animals with the outer membranes of *Bacteroides vulgatus* (BV) (6) or administration of a bacterial cocktail (7).

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Although the development of spontaneous colitis in HLA-B27/human β_2-microglobulin transgenic rats was not observed under germ-free conditions (8), colonization with a single strain of BV in these rats induced colitis of the same severity as seen under the SPF condition (9). BV was detected at a high incidence in TCR$\alpha^{-/-}$ mice in association with the development of colitis (10). In general, the inflammatory activity in IBD has been shown to be subdued by administration of antibiotics that modify the anaerobic flora in the intestine (11). Further, some patients with UC showed a high titer of antibody (Ab) against a protein in the outer membrane of BV (12). Some T cell clones established from IBD patients responded to the indigenous anaerobic bacteria including BV (3). Since *Bacteroides* species is a major component of the resident flora and is not pathogenic, these studies show that host immunity against indigenous flora plays a pivotal role in intestinal homeostasis.

Lipopolysaccharide (LPS) is an integral component of the outer membrane of Gram-negative bacteria and is present in large quantities in the human gut. Thus, the intestinal mucosal surface is constantly exposed to a high concentration of LPS. In contrast, when LPS happens to be in the bloodstream, even at a relatively low concentration, the systemic response is dramatic and life threatening. There, LPS induces the rapid appearance of circulating proinflammatory cytokines such as TNFα, IFN-γ, IL-1, and IL-12, which results in sepsis or septic shock. Thus, there must be a critical difference in the response to LPS between the systemic environment and the colonic mucosal surface. CD14 and LPS-binding protein (LBP) are known receptors for LPS (13). However, the lack of an intracellular domain in CD14 or in LBP suggests the presence of a coreceptor capable of inducing transmembrane signals in the cells. Recently members of the Toll-like receptor (TLR) family were found to recognize pathogen-associated molecular patterns, which are motifs conserved on pathogens but not found in higher eukaryotes (14). The combination of membrane-bound TLR4 and soluble associated MD-2 was identified as a signal transducer for LPS (15). The different expression of TLRs in a variety of cells including immune cells and epithelial cells (16, 17) may modulate innate immunity in a tissue-specific manner.

The responses of colonic lamina propria mononuclear cells (LPMCs) to LPS from indigenous bacteria are poorly understood. The aim of this study was to investigate the responses of human colonic LPMCs isolated from noninflammatory colonic mucosa to LPS isolated from indigenous bacteria. We used LPS purified from the pathogenic bacterium *Salmonella minnesota* (SM), and LPS derived from indigenous nonpathogenic flora, i.e., BV and *Bacteroides fragilis* (BF). Here we show the unique responses of LPMCs to LPS, which stand in contrast to those of the systemic immune system.

MATERIALS AND METHODS

LPS

Bacteroides vulgatus IMCJ1204 was isolated from the feces of a patient with Crohn's disease. *Bacteroides fragilis* NCTC10581 was obtained from ATCC. LPS samples from BV and BF were extracted by the phenol–water method. The extract was subjected to enzymatic digestion with DNase and RNase followed by proteinase K and then phenol–water extraction again to yield the crude LPS preparation. For use, LPS was further purified by hydrophobic-interaction chromatography (18). To assess the purity of prepared LPS, the portion of BV-LPS was hydrolyzed, and subsequently the protein content was estimated using the Automatic Amino Acid Analyzer (Model L-8500; Hitachi). The protein content in the BV-LPS fraction was 1.056%. LPS from SM was obtained from Sigma (L-2137; Tokyo).

Cell Preparation

This study was approved by the ethics committee of the International Medical Center of Japan, and informed consent was obtained for the taking of all samples. LPMCs were prepared from surgical specimens obtained from 80 patients (average age, 65.5 ± 10.8 years) treated for colorectal cancer at the International Medical Center of Japan. Patients had not received chemotherapy or radiation therapy prior to operation. The purpose of surgery was curative resection of the lesion in all cases. Cases that satisfied these conditions were subjected to our study in the order of the date of surgery without any selection for specific assays. Colonic mucosal samples from macroscopically and microscopically unaffected areas, which were at least 10 cm distant from the tumor, were used as the source of LPMCs. Autologous PBMCs were obtained from five patients, and PBMCs from healthy subjects were also prepared. LPMCs were isolated from freshly resected mucosa by a previously described method, with modification (19). In brief, a strip of mucosa (6- to 8-g total weight) was washed in Hank's balanced salt solution free of calcium and magnesium (HBSS-CMF), cut into pieces, and then incubated for 15 min at room temperature in HBSS-CMF containing 1 m*M* DTT (Wako, Tokyo), 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 1 μg/ml fungizone. After three washes in HBSS-CMF, the tissue was incubated for 45 min at 37°C in HBSS-CMF containing 0.75 m*M* EDTA, 10 m*M* HEPES

buffer (pH 7.2–7.4), 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 1 μg/ml fungizone and, again, washed twice to remove epithelial cells and intraepithelial cells. The residual tissue was minced and then incubated for 1 hr at 37°C in HBSS-CMF containing 1 mg/ml collagenase V (Sigma), 0.4 mg/ml Dispase II (Roche, Tokyo), 0.01% DNase (Roche), 10 m*M* HEPES buffer, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 1 μg/ml fungizone. The cells released into the supernatant were collected, washed twice in HBSS-CMF, and then subjected to centrifugation through a 30–40–60–100% Percoll gradient (Pharmacia Biotech AB, Uppsala, Sweden) to obtain LPMCs at the 40/60% interface. PBMCs were isolated from heparinized blood obtained from patients with colonic cancer and from healthy controls by density-gradient centrifugation using Ficoll–Paque (Amersham Pharmacia Biotech, Tokyo).

Cell Culture

LPMCs (1×10^6/ml) were cultured in RPMI 1640, supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 2 μg/ml fungizone, 2 m*M* glutamine, and 1 m*M* pyruvic acid in 96-well, U-bottom culture plates, with or without LPS at various concentrations for a specified period; and the culture supernatants were then collected and stored at −20°C until the cytokine assay could be done. All LPMC samples were examined for their response to stimulation with PHA, to confirm the viability of the cell populations.

Quantification of Cytokines in the Culture Medium

TNFα and IL-6 were determined using Development Kits (Genzyme Techne, Minneapolis, MN). IL-10 was measured by a sandwich ELISA using anti-human IL-10 mAb JES3-9D7 (rat IgG1; PharMingen International, Tokyo) as the capture Ab and biotinylated anti-human IL-10 mAb JES3-12G8 (rat IgG2a; PharMingen International) as the detection Ab, as described previously (20). The sensitivity of the assay was 16 pg/ml.

Flow Cytometric Analysis and Sorting

Phenotypes of LPMCs were analyzed by laser flow cytometry on a FACS Calibur Flow Cytometer (Beckton Dickinson, Tokyo). FITC-, PE-, PC5-, or biotin-labeled Abs to CD3 (clone UCHT1), CD3 (UCHT1), CD4, (13B8.2), CD4 (T4: SFCI12T4D11), CD8, (B9.11), CD33 (MY9), CD14 (MY4), and CD25 (B1.49.9) were purchased from Beckman Coulter (Tokyo). FITC-anti-CD19 Ab (CLB-CD19) was purchased from Nichirei (Tokyo). Labeled cells were analyzed with a FACS Calibur Flow Cytometer. In some experiments, LPMCs stimulated with LPS for 24 hr were stained with FITC-labeled anti-CD25 Ab and PE-labeled anti-CD4 Ab. $CD4^+CD25^+$, $CD4^+CD25^-$, and $CD4^-CD25^+$ cells were then purified using a FACS Vantage (Beckton Dickinson). The purity of each cell population was >95% as assessed by flow cytometric analysis.

Isolation of CD33-Positive Cells 24 hr After LPS Stimulation

CD33-positive cells were isolated from the LPMC population by selection using an AutoMACS (Miltenyi Biotec GmbH, Germany) according to the manufacturer's instructions. In brief, cells were cultured with LPS for 24 hr, then stained with FITC-labeled anti-CD33 Ab (Beckman Coulter), and subsequently incubated with immunomagnetic beads conjugated to monoclonal mouse anti-FITC isomer-1 Ab (IgG1; Miltenyi Biotec). The resultant CD33-positive cell population contained >95% CD33-positive cells as assessed by flow cytometric analysis.

RT-PCR

Total RNA was prepared from cell pellets using an RNeasy Mini Kit (Qiagen, Tokyo). Complementary DNA was synthesized from RNA by reverse transcription (RT). PCR primers for human TLR4 were 5′-TGGATACGTTTCCTTATAAG and 5′-GAAATGGAGGCACCCCTTC, yielding a PCR product of 507 bp. The step-cycle program was set for denaturing at 95°C for 1 min, annealing at 55°C for 40 sec, and extension at 72°C for 1 min for a total of 35 cycles. PCR primers for human MD-2 were 5′-GAAGCTCAGAAGCAGTATTGGGTC and 5′-GGTTGGTGTAGGATGACAAACTCC, yielding a PCR product of 402 bp. The step-cycle program was set for denaturing at 94°C for 45 sec and annealing and extension at 72°C for 1 min for a total of 30 cycles. Expression of human IL-10 mRNA was assessed by quantitative PCR using a SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). PCR primers for human IL-10 were 5′-CTATCCCAGAGCCCCAGATCCGA and 5′-ATGCCCAAGCTGAGAACCAAGAC, yielding a PCR product of 333 bp. Primers for GAPDH were 5′-GGGAGCCAAAAGGGTCATCATCTC and 5′-CCATGCCCAGTGAGCTTCCCGTTC (352 bp). The step-cycle program was set for denaturing at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min for a total of 40 cycles. PCR primer for human Foxp3 were 5′-CCTTGAACCCCATGCCACCA and 5′-GCACTGGGATTTGGGAGGGT, yielding a PCR

product of 225 bp. The step-cycle program was set for denaturing at 95°C for 1 min, annealing at 60°C 1 min, and extension at 72°C for 1 min for a total of 40 cycles. Threshold cycle numbers (Ct) were determined with Sequence Detector software (version 1.7; Applied Biosystems) and transformed using the ΔCt or ΔΔCt method as described by the manufacturer, with GAPDH used as the calibrator gene where indicated.

Statistical Evaluation

Data are presented as the mean ± the standard error of assessment. The statistical significance of differences in the results was tested by the Mann–Whitney *U* test or paired Student *t*-test.

RESULTS

Cell Composition of LPMC Population

Since many of our experiments were performed using total LPMC cultures, we first analyzed the cell composition of the LPMC population by flow cytometry (Table I), in comparison with that of PBMCs. The percentage of $CD19^+$ B cells, including plasma cells, was higher in the LPMC population than in the PBMC one. The percentage of $CD33^+$ macrophages was twofold higher in the PBMC preparation than in the LPMC one. $CD14^+$ cells accounted for the majority of the $CD33^+$ macrophages in the PBMC population, whereas the percentage of $CD33^+$ macrophages that were $CD14^+$ in the LPMC preparation was only 50%. Thus, LPMCs were characterized by higher numbers of B cells than in the PBMC population and $CD14^-$ macrophages, which were a rare population in the PBMC population. The cell composition of the PBMC population in cases with colonic cancer used in this study was not different from that for the healthy controls.

Table I. Surface Phenotypes of LPMCs and PBMCs (Percentage)[a]

Cells	LPMCs (n = 12)	PBMCs (healthy controls; n = 7)
$CD3^+$	22.5 ± 2.6	48.6 ± 3.0
$CD4^+CD8^-$ in $CD3^+$	69.5 ± 3.0*	56.5 ± 4.5
$CD4^-CD8^+$ in $CD3^+$	20.0 ± 2.1	34.0 ± 6.3
$CD4^+CD8^+$ in $CD3^+$	5.2 ± 2.3	4.6 ± 3.3
$CD4^-CD8^-$ in $CD3^+$	5.3 ± 0.6	5.0 ± 1.2
$CD19^+$	37.4 ± 2.1*	10.8 ± 2.0
$CD33^+$	10.5 ± 2.8*	21.9 ± 4.4
$CD14^+$ in CD33	52.9 ± 3.2	94.8 ± 1.0
CD3/CD19 ratio	0.59 ± 0.07*	5.4 ± 1.14

[a]Results are expressed as the mean percentage ± SE.
*Statistically significant differences from PBMCs ($P < 0.05$) using the Mann–Whitney *U* test.

Secretion of TNFα and IL-6 in Response to LPS

It is known that LPS stimulation of human peripheral monocytes causes production of proinflammatory cytokines, including TNFα and IL-6. We first examined the time course and dose dependency of the secretion of TNFα and IL-6 from LPMCs after stimulation with LPS samples prepared from BV (BV-LPS), BF (BF-LPS), and SM (SM-LPS). LPMCs secreted minimal levels of TNFα (Fig. 1A) regardless of the source of the LPS. In contrast, PBMCs produced high levels of TNFα, which reached its peak within 12 hr after the addition of LPS (Fig. 1A). Subsequently, we measured the dose dependency of the level of TNFα 24 hr after adding various concentrations of LPS to the culture medium of LPMCs prepared from 12 patients. The concentration of TNFα did not show significant changes from the basal secretion level, response to the LPS at concentrations below 1 μg/ml, irrespective of their source (Fig. 1B). Even at a high concentration (10 μg/ml) of SM-LPS, no more than 100 pg/ml of TNFα was secreted persistently in preparations from all patients (Fig. 1B). In contrast, autologous PBMCs obtained from five cases showed high levels of TNFα production at a low concentration (1 ng/ml) of SM-LPS, and this cytokine release reached its plateau at the concentration of 100 ng/ml of SM-LPS (Fig. 1B) 24 hr after stimulation with LPS. TNFα responses to BV- or BF-LPS by PBMCs required higher concentrations of LPS than those to SM-LPS (Fig. 1B). Production of IL-6 was hardly detected in LPMCs in response to LPS either, while LPS induced secretion of high levels of IL-6 from autologous PBMCs. Thus, none of the types of LPS stimulated the LPMCs to produce proinflammatory cytokines, TNFα and IL-6. We also tested the LPS-induced TNFα secretion by LPMCs after a certain recovery time. After the digesting enzymes had been washed off the total LPMCs, the cells were cultured for 5 hr in complete medium and then stimulated with LPS for 19 hr. The levels of TNFα in the culture supernatant were similar to those in cultures stimulated immediately after cell separation. Thus, we conclude that the hyporesponsive state is not a transient one induced by enzyme digestion but rather reflects the true nature of colonic LPMC. Although pathological examination demonstrated no abnormality in the tissue we used for the source of LPMCs, we further confirmed that there was no significant difference in the level of TNFα production by LPMCs according to tumor stage, gross type, or location, as well as age and sex.

Low Expression of Messenger RNA for MD-2 in Colonic Macrophages

The hyporesponsiveness in terms of TNFα production by LPMCs suggests that macrophages in the colonic

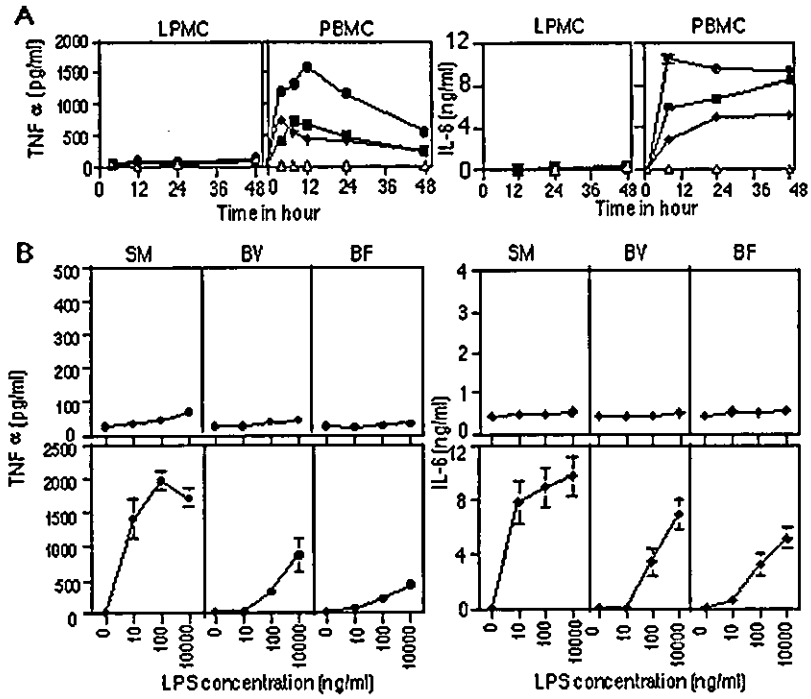

Fig. 1. Production of TNFα and IL-6 by LPMCs and PBMCs in response to LPS. (A) Kinetics of production of TNFα and IL-6. LPMCs isolated from normal colonic tissue from patients with colorectal cancer or PBMCs prepared from healthy controls (1×10^6/ml) were cultured with or without the indicated LPS (10 μg/ml). Culture supernatants were collected at the indicated time points, and concentrations of TNFα and IL-6 were then measured. Open triangles, without LPS; filled circles, SM-LPS; filled squares, BV-LPS; filled diamonds, BF-LPS. Data on LPMCs and PBMCs represent typical results of four cases of LPMCs and three cases of PBMCs. (B) Dose dependency of LPS in TNFα and IL-6 production. LPMCs (top panel) or PBMCs (bottom panel) (1×10^6/ml) were cultured with or without LPS (1 ng/ml–10 μg/ml) for 24 hr. Culture supernatants were collected and analyzed for cytokine levels. Data are shown as the mean ± SE of LPMCs from 12 cases and of PBMCs from 5 cases with colorectal cancer.

lamina propria possess characteristics distinct from those of PBMCs with respect to LPS. Therefore, we examined the expression of the major molecules that transduce LPS signals, i.e., TLR4, and that of an associated molecule, MD-2, by RT-PCR. Messenger RNA for TLR4 is known to be expressed in human monocytes, dendritic cells, and B cells in PBMC populations (21). However, there has been no agreement on the expression of TLR4 in human LPMCs. One group reported the expression of TLR4 mRNA in small intestinal macrophages (22), whereas another showed that colonic macrophages expressed neither mRNA nor surface protein of TLR4 (23). Yet another group reported that a total LPMC population expressed low levels of mRNA for TLR4 and MD-2 (24). In our study, we purified colonic macrophages by utilizing a cell surface marker, $CD33^+$, and assessed the expression of mRNA by RT-PCR. TLR4 was equally detected in both macrophage and nonmacrophage $CD33^-$ cell fractions of the LPMC population in all cases tested (Fig. 2). On the other hand, the mRNA for MD-2 was barely detected in colonic macrophages, whereas colonic nonmacrophage cells did express this mRNA (Fig. 2). These results stand in contrast to those for PBMCs, in which case the macrophages expressed both TLR4 and

Table II. Examples of Cell Viability in the Culture

	Total cell viability (%)[a]	Percentage of total living cells[b]		
		$CD4^+$	$CD19^+$	$CD33^+$
Freshly isolated LPMCs	91.4	17.0	41.6	7.6
After 24-hr culture				
Without LPS	59.2	19.8	18.0	1.7
With SM-LPS	51.8	22.7	18.5	1.1
With BV-LPS	57.0	21.6	18.3	2.5
With BF-LPS	69.1	20.7	18.6	1.4

[a] Viability was determined by dye-exclusion test.
[b] Determined by flow cytometry after staining with antibody against the indicated antigen and propidium iodide.

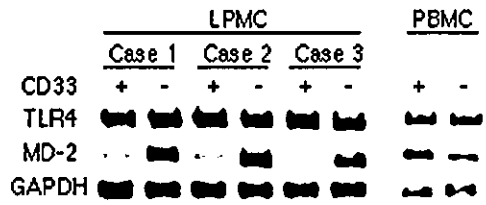

Fig. 2. RT-PCR analysis of mRNAs for TLR4 and MD-2 from freshly isolated $CD33^+$ or $CD33^-$ cells in LPMC and PBMC populations.

MD-2 mRNAs (Fig. 2). We tried to detect TLR4 on the cell surface by flow cytometry, but it was not detectable on LPMCs. Thus, in the colon, it is likely that TLR4, if expressed in any part of the cells including intracellular organella (16, 17), does not function to stimulate cytokine production by macrophages in response to LPS due to low expression of MD-2.

Production of IL-10 in Response to LPS

Since IL-10 is a key cytokine controlling inflammatory responses, and because it is well known that LPS induces secretion of IL-10 as well as TNFα by PBMCs, we next assessed the secretion of IL-10 by LPMCs after stimulation of the cells with SM, BV, or BF-LPS. In most of the 59 cases tested, the basal levels of IL-10 secretion were under the sensitivity of the assay or less than 100 pg/ml; however, in about 50% of the cases, an increase in the secretion of IL-10 by the LPMCs occurred in the presence of any type of LPS, while in the rest of the cases IL-10 was not detected even in the presence of LPS (Figs. 3A and B). Therefore, we considered that LPS stimulated secretion of IL-10 by LPMCs at low levels that are not detectable in total cell culture in half of cases but may be significant as an auto- or juxtacrine cytokine. To this point, due to the strictly limited availability of human healthy colonic tissue, we had to control our study using unaffected tissue from patients with colorectal cancer: the systemic effects of cancer may have impacted IL-10 production. However, IL-10 as well as TNFα secretion levels by PBMCs in response to any type of LPS were not statistically different between patients with colonic cancer and healthy controls (data not shown), which means that there was no obvious systemic effect of cancer regarding response to LPS. In addition, despite great individual diversity, we could not find any significant relation between the level of IL-10 production by LPMCs and age, sex, or gross type or location of the sampling in the colon. Therefore, we regard the enhancement of IL-10 secretion at a low level in the presence of LPS to be a feature of the normal colon. In 16 cases, we also measured the release of IFN-γ and IL-4 after stimulation with each of the three LPSs; however,

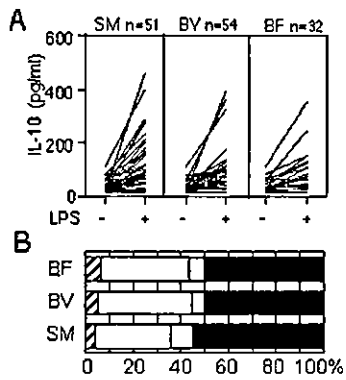

Fig. 3. Production of IL-10 by LPMCs in response to LPS. (A) LPMCs at a density of 1×10^6/ml were cultured with (+) or without (−) the indicated LPS (10 μg/ml) for 24 hr. Culture supernatants were then collected and analyzed for levels of IL-10. When the level was under the lower limit of detection (16 pg/ml), the value was plotted as 16 pg/ml. (B) Summarized IL-10 responses in the cases shown in A. Black bar shows the percentage of cases in which IL-10 levels in the supernatant of cultures stimulated with LPS were higher than those obtained without LPS, and the differences in the levels were more than 16 pg/ml; shaded bar, same as for black bar except that the differences in the levels were less than 16 pg/ml; white bar, percentage of cases in which the concentration of IL-10 was under the lower limit of detection with or without LPS; hatched bar, percentage of cases in which the IL-10 concentration in the supernatant of cultures stimulated with LPS was lower than that obtained without LPS (differences in the levels were up to 11 pg/ml).

none of these cytokines were detected in the supernatant of LPMC cultures. Thus, IL-10 release was not associated with the secretion of these Th1 or Th2 cytokines. Further, we assessed the production of TGFβ. The active form of TGFβ was not detected in the cultures, and the level of the latent form of TGFβ did not show a significant difference between the supernatant with LPS and that without it.

LPS Upregulated IL-10 mRNA in Both $CD33^+$ and $CD33^-$ Cells in LPMCs

Next we investigated the source of IL-10 especially in response to LPS derived from indigenous flora. After incubation for 24 hr with LPS, LPMCs were separated into macrophages ($CD33^+$) and nonmacrophage cells ($CD33^-$). Although four cases tested showed various levels of IL-10 secretion in the presence of BV-LPS, mRNA levels for IL-10 were increased in response to BV-LPS in macrophages in three of the four cases (Fig. 4). In nonmacrophage cells, enhancement of IL-10 mRNA expression was seen in two of four cases (Fig. 4). Considering the total IL-10 output of each case, both macrophages and nonmacrophage cells, including lymphocytes, were probable sources of IL-10 in the LPMC population.

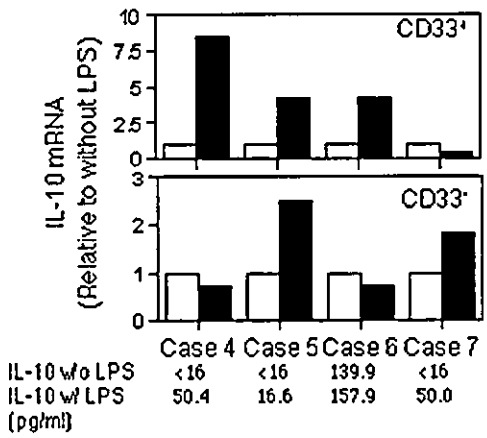

Fig. 4. Induction of mRNA for IL-10 in $CD33^+$ or $CD33^-$ cells in the LPMC population after stimulation with BV-LPS. LPMCs (1×10^6/ml) were cultured with (black column) or without (white column) BV-LPS (10 μg/ml) for 24 hr. Cultured LPMCs were separated into $CD33^+$ (top panel) and $CD33^-$ (bottom panel) cells, purified, and subjected to quantitative RT-PCR for IL-10 and GAPDH. The results are expressed relative to the expression level of each transcript in cells without LPS. Levels of IL-10 in the culture supernatant with or without LPS in each case are also shown.

BV-LPS Enhanced CD25 Expression on Colonic Lamina Propria Lymphocytes

The upregulated mRNA for IL-10 in nonmacrophage cells by LPS in some cases led us to more specific analysis of lymphocytes. The CD25 molecule is known to be expressed on immunosuppressive regulatory T cells as well as on activated T cells in humans. Therefore, we next assessed the alteration of the surface CD25 on colonic lamina propria lymphocytes (LPLs) in response to LPS by flow cytometry. There was individual diversity in the percentage of $CD25^+$ cells in lymphocytes among cases; however, the numbers of both $CD4^+$ and $CD4^-$ lymphocytes expressing $CD25^+$ in the presence of BV-LPS was increased in all six cases examined (Fig. 5A). The induction of $CD25^+$ cells by SM-LPS was observed in both $CD4^+$ and $CD4^-$ lymphocytes at levels similar to or somewhat lower than those seen in experiments using BV-LPS.

Messenger RNA for IL-10 Was Detected in $CD4^+$ $CD25^+$ Cells Induced by Stimulation with BV-LPS

Since response to indigenous bacteria is more important in terms of gut homeostasis, we further characterized $CD25^+$ lymphocytes induced by BV-LPS, by cell sorting we purified $CD4^+CD25^+$, $CD4^-CD25^+$, and $CD4^+CD25^-$ cell populations from LPMC cultures stimulated with BV-LPS and then quantitatively compared the levels of mRNA for IL-10 in each cell fraction. Compared with $CD4^+CD25^-$ cells, $CD4^+CD25^+$ cells expressed evidently higher levels of IL-10 mRNA in all four cases examined (Fig. 5B). Although increased IL-10 protein secretion was not necessarily detected in these cases, upregulation of IL-10 transcription in $CD4^+CD25^+$ cells was evident in all cases. $CD4^-CD25^+$ cells also showed enhancement in three of the four cases. Since the mRNA for IL-4 was barely detected in all cases even using a sensitive quantitative RT-PCR system, these results indicate that $CD4^+CD25^+$ cells induced by BV-LPS were not activated conventional T helper type 2 cells, but a unique IL-10-producing cell population. For further characterization of the LPS-induced changes in lymphocyte population, we analyzed expression of mRNA for the transcription factor Foxp3, which is a unique marker for T regulatory cells (25, 26). In two cases tested, we found that Foxp3 expression was enhanced in total LPMCs or in $CD33^-$ cells (which population includes total lymphocytes) stimulated with BV-LPS in the two cases tested.

DISCUSSION

In the current study we demonstrated the responses of LPMCs to LPS to be distinct from those of PBMCs, indicating a potential mechanism for the homeostasis of the colonic immune response. First, LPMCs were hyporesponsive in producing TNFα and IL-6 in response to both indigenous and pathogenic LPSs, possibly due to low expression of MD-2 association molecule with the LPS receptor TLR4 in macrophages. Second, LPMCs produced low levels of IL-10 without other cytokines in response to LPS. Third, $CD4^+CD25^+$ lymphocytes were induced by stimulation with BV-LPS, which was accompanied by the expression of mRNA for IL-10 in this cell fraction. The significance of these features of the colonic immune response is discussed below.

One of the important features of LPMCs is their hyporesponsiveness to LPS, even to high concentrations of pathogenic SM-LPS, in terms of production of proinflammatory cytokines such as TNFα and IL-6. These results are in accord with previous reports that LPS-induced proinflammatory cytokine response was low for jejunal macrophages obtained from healthy subjects undergoing gastrojejunostomy for morbit obesity (22) or for colonic macrophages obtained at surgery for diverticulitis (23). It is known that both TNFα and IL-6 are produced by activation of TLR4 in response to LPS (27). In our study, we clearly showed that colonic macrophages expressed TLR4 mRNA, but not MD-2 mRNA, in contrast to peripheral blood monocytes, which are fully equipped with both TLR4 and MD-2. MD-2 is an essential association molecule for LPS recognition by TLR4 and regulates the redistribution of TLR4 from the Golgi apparatus to the

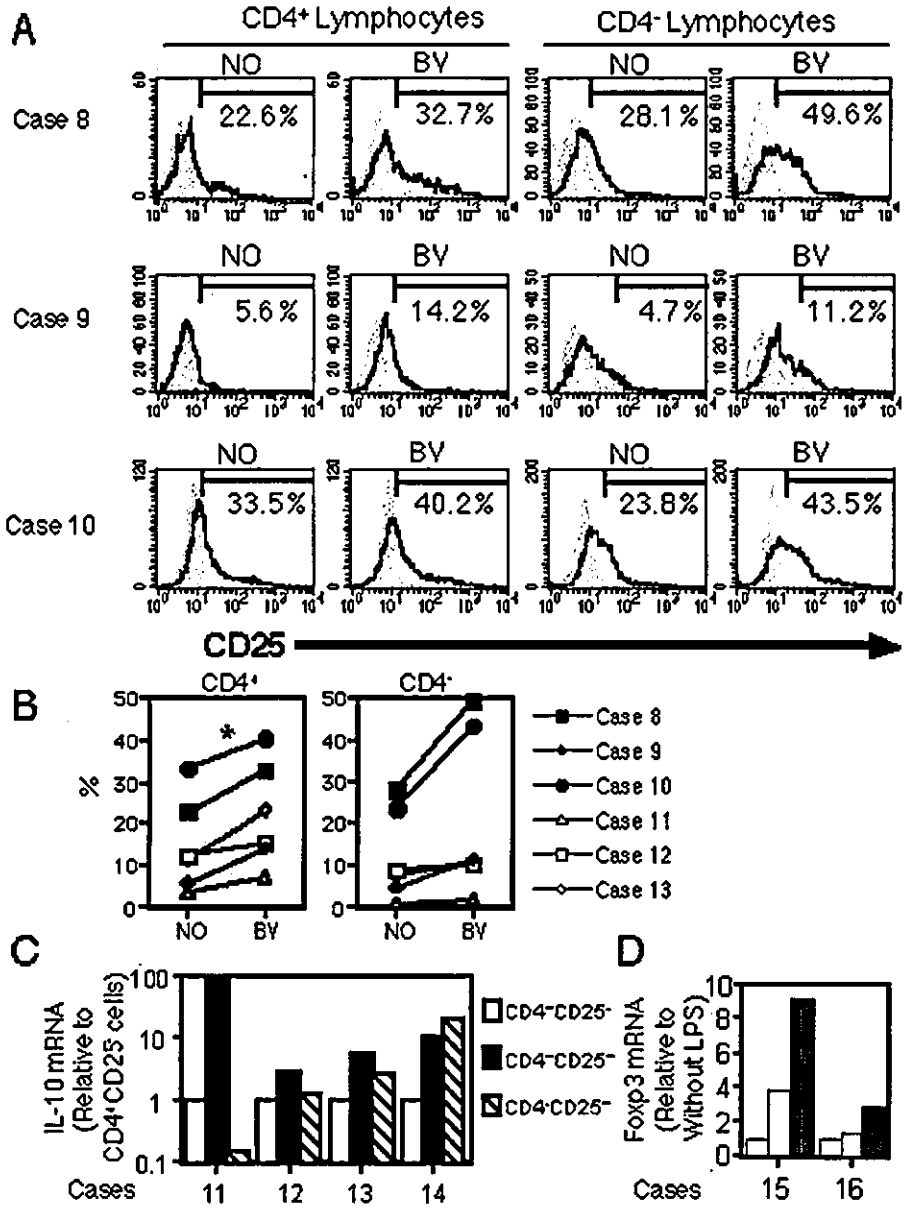

Fig. 5. Induction of CD25-expressing cells in lymphocytes of the LPMC population after stimulation with BV-LPS. (A) LPMCs (1×10^6/ml) were cultured with or without 10 μg/ml of BV-LPS. After 24 hr in culture, the cells were harvested and stained with antibodies against CD25 and CD4. Histograms show cells gated for $CD4^+$ or $CD4^-$ lymphocytes. Staining with anti-CD25 Ab is shown as open histograms. Shaded histograms indicate background staining of cells with a control (IgG_1) monoclonal Ab. Typical results from three of six cases tested are shown. All cases showed a similar pattern. (B) Percentage of $CD25^+$ cells obtained in six cases with or without BV-LPS was determined by flow cytometry as shown in A. A statistically significant difference was found in the $CD4^+$ cell population (*, paired Student *t*-test). (C) Expression of mRNA for IL-10 in lymphocytes in the LPMC population after stimulation with BV-LPS. LPMCs (1×10^6/ml) were cultured with BV-LPS (10 μg/ml) for 24 hr. Cells were harvested and sorted into $CD4^+CD25^+$, $CD4^-CD25^+$, and $CD4^+CD25^-$ lymphocyte fractions. Isolated lymphocytes were tested by quantitative RT-PCR for expression of mRNA's for IL-10 and GAPDH. The results are expressed relative to the expression in $CD4^+CD25^-$ cells. (D) Expression of Foxp3 mRNA in LPMCs. After stimulation of LPMCs with SM-LPS (shaded column) or BV-LPS (black column) or without LPS (open column) for 24 hr as above, RNA was extracted from total LPMC (case 15) or $CD33^-$ cells (case 16) and tested by quantitative RT-PCR for expression of mRNA's for Foxp3 and GAPDH.

plasma membrane (28). A functional TLR4–MD-2 complex is thus necessary for producing TNFα and IL-6 in response to LPS. Our result strongly suggests that the hyporesponsiveness of LPMC to SM-LPS was caused by the lack of MD-2 in colonic macrophages, which may prevent the expression of the TLR4 molecule on the cell surface. A recent study showed that human TLR4 discriminates differences in the lipid A structure of LPS from various bacterial strains (29), although it is not yet known whether LPS from *Bacteroides* species might also stimulate cells through the human TLR4. *Bacteroides* LPS was earlier reported to elicit a weaker biological reaction than *Salmonella* LPS (30), and such was the case in our experiment using PBMCs (Figs. 1B). On the other hand, LPMCs did not seem to distinguish SM-LPS from BV-LPS (Figs. 1 and 3). In our results, apparently LPMCs do not utilize TLRs to receive signals from LPS to produce TNFαs and that may be one of the reasons that LPMCs do not recognize any difference between pathogenic and indigenous LPSs.

The second important finding was that LPS stimulated IL-10 transcription in either $CD33^+$ or $CD33^-$ cells, even in cases with modest IL-10 protein secretion into the culture supernatant. IL-10 mRNA was also high in purified $CD4^+CD25^+$ cells, which was induced by BV-LPS. IL-10 is produced by peripheral blood monocytes, and probably by T cells, in response to LPS (31, 32). However, we confirmed that LPS-induced IL-10 production by LPMCs was not affected in the presence of blocking Ab against TLR4 (data not shown). Further, since we could not exclude that minor ingredients in our LPS preparation, such as lipoproteins, could have an effect on LPMC in our experiment, we also tested the effect of blocking Ab against TLR2; anti-TLR2 blocking Ab did not suppress LPS-induced IL-10 secretion by LPMCs (data not shown). These results suggest the presence of a unique pathway, which is independent of TLR4 and TLR2, to produce IL-10 in response to LPS. Although the TLR-independent pathway that induces IL-10 is not obvious at this point, the finding that LPS-induced TNFα responses in TLR4 mutant mice were not completely abolished (33) indicates the presence of an alternative minor pathway for LPS signaling. It is also well known that macrophages are equipped with a range of scavenger-type receptors including those directly recognizing carbohydrates and lipids. Little is known about the expression of these molecules and signaling systems triggered by them, especially in the case of macrophages in the gut. The properties of resident macrophages are determined by their local microenvironment, including surface and secretory products of neighboring cells. Since we stimulated total LPMCs reflecting the environment in the gut, T cells and B cells and their secretory products, such as Ab to LPS, may interact with macrophages and modify their responses. In fact, ligation of Fcγ receptors of macrophages reversed Th1 biasing (34) and induced IL-10 secretion (35). Thus, we speculate that the mechanism for secretion of low levels of IL-10 by colonic LPMCs may involve both innate and acquired immunity.

On the other hand, the hyporesponsiveness in proinflammatory cytokine production by LPMCs in response to LPS reminds us of a situation known as LPS tolerance. LPS tolerance was initially described as unresponsiveness and survival of animals to a lethal dose of LPS, if they had been pretreated with a sublethal LPS injection (36). LPS tolerance is demonstrable both *in vitro* and *in vivo*. Human monocytes pretreated with LPS produced low levels of TNFα in response to a subsequent challenge with LPS, with upregulation of TNF receptor type II (37) and IL-10 (38) and downregulation of surface-expressed MHC class II molecules and CD86 (39). Dramatically decreased production of TNFα with persistent or enhanced release of IL-10 was a feature of human monocytes under the condition of LPS tolerance both *in vivo* (40) and *in vitro* (38). Thus, LPS tolerance reflects a reciprocal response of downregulation of a proinflammatory cytokine and upregulation of an antiinflammatory one. The molecular mechanism of LPS tolerance is not fully clear yet, but what we know now is the following: the expression of CD14 on human monocytes was unchanged upon LPS tolerance *in vitro* (41). Although downregulation of surface TLR4 to some extent was seen in murine cells upon LPS tolerance (42), LPS tolerance was induced *in vitro* in Chinese hamster ovary cells with overexpressed TLR4 on their cell surface (43). These observations are consistent with what we observed in LPMCs, i.e., expression of mRNA for TLR4 and, secretion of IL-10 and a minimal amount of TNFα. Since the colonic mucosa is continuously exposed to Gram-negative bacteria, it is attractive to hypothesize that a mechanism similar to that governing LPS tolerance is responsible for the hyporesponsiveness of colonic mononuclear cells including macrophages. However, according to a recent study, LPS-tolerant human peripheral blood monocytes expressed enhanced levels of MD-2 mRNA (44), which does not coincide with our results on colonic macrophages, i.e., TLR expression without that of MD-2. Thus, our data suggest that colonic macrophages are not identical to peripheral blood monocytes rendered LPS tolerant.

Our final important finding was that the colonic lamina propria lymphocyte population also actively responded to LPS from indigenous flora by inducing CD25 expression on both $CD4^+$ and $CD4^-$ lymphocytes. Furthermore, LPS induced $CD4^+CD25^+$ lymphocytes expressed mRNA for IL-10. Recent studies have shown that $CD4^+CD25^+$

lymphocytes represent one of the various types of T regulatory cells (45), and bacterial flora play significant roles in the development and maintenance of T regulatory cells (46, 47). We also detected induction of mRNA for the transcription factor Foxp3 in the BV-LPS stimulated lymphocytes which is a unique marker for T regulatory cells (25, 26). In light of these previous findings, our results suggest that continuous stimulation with LPS of indigenous flora induces and maintains T regulatory type cells to suppress excess immune responses in the colon. It is also of great interest to ask whether or not $CD4^+CD25^+$ cells develop in an antigen (LPS)-specific manner. LPMCs consist of T cells, B cells, which include plasma cells, a small percentage of NK cells, and macrophages and dendritic cells. One group reported that LPS activated human T cells from PBMCs *in vitro* in the presence of monocytes or $CD34^+$ cells as antigen-presenting cells (APC) in a MHC-independent manner (48–50). Our preliminary study depleting $CD33^+$ cells from an LPMC population showed that LPS still induced CD25 on lamina propria lymphocytes (data not shown), suggesting that macrophages were not required for induction of $CD25^+$ lymphocytes in response to BV-LPS. Further investigations are necessary to know if $CD4^+$ lymphocytes directly respond to LPS, or if they require other types of rare cells such as dendritic cells, which may be included in the $CD33^-$ cell fraction.

In summary, we found that the hyporesponsiveness of colonic macrophages to LPS in the production of proinflammatory cytokines was possibly due to low expression of MD-2 and that LPS induced IL-10-producing $CD4^+CD25^+$ T cells in the colon. Our results provide important clues as to possible mechanisms to maintain colonic immunological homeostasis and also for the pathogenesis of chronic inflammation in the colon, such as IBDs.

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