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Amino Acid Substitutions at Position 95 in GyrA Can Add Fluoroquinolone Resistance to *Mycobacterium leprae*

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Amino acid substitutions at position 89 or 91 in GyrA of fluoroquinolone-resistant *Mycobacterium leprae* clinical isolates have been reported. In contrast, those at position 94 in *M. tuberculosis*, equivalent to position 95 in *M. leprae*, have been identified most frequently. To verify the possible contribution of amino acid substitutions at position 95 in *M. leprae* to fluoroquinolone resistance, we conducted an *in vitro* assay using wild-type and mutant recombinant DNA gyrases. Fluoroquinolone-mediated supercoiling activity inhibition assay and DNA cleavage assay revealed the potent contribution of an amino acid substitution of Asp to Gly or Asn at position 95 to fluoroquinolone resistance. These results suggested the possible future emergence of quinolone-resistant *M. leprae* isolates with these amino acid substitutions and the usefulness of detecting these mutations for the rapid identification of fluoroquinolone resistance in leprosy.

Leprosy is a chronic human infectious disease caused by *Mycobacterium leprae* which may cause severe disabilities due to damage to the peripheral nerves (33). The World Health Organization (WHO) reported the global number of registered new cases in 2010 to be 228,474, while during 2009 it was 244,796 (37). Although the number of new cases detected globally fell by 16,322 (6.7%) during this period, new leprosy cases are still detected every year, mainly in Asia, Latin America, and Africa (21, 37). In the 1980s, the WHO introduced multidrug therapy (MDT), composed of dapsone (DDS), rifampin (RIF), and clofazimine (36). Recently, fluoroquinolones (FQs), especially ofloxacin (OFX), have been recommended for the treatment of leprosy with a single lesion. The emergence of multidrug-resistant (MDR) leprosy, resistant to both DDS and RIF owing to therapeutic failure or low compliance, has been reported (17, 29), and FQs are thought to be important. For appropriate treatment, early assessment of drug susceptibility is essential; however, *M. leprae* cannot be cultivated on artificial media and a drug susceptibility test depending on *in vitro* growth is not available. Consequently, antibiotic susceptibility tests have relied on the mouse footpad leprosy model, requiring 8 to 12 months because of the slow growth of *M. leprae* (18). Recently, genetic analysis of drug-resistant *M. leprae* substantiated the correlation of DDS, RIF, and OFX resistance with mutations in *folP1*, encoding dihydropteroate synthetase (5, 15, 19, 23–25, 35); *rpoB* (4, 6, 12, 19, 23–25, 33), encoding the beta subunit of RNA polymerase; and *gyrA*, encoding the A subunit of DNA gyrase (4, 19, 24, 26, 40), respectively. Among these, data for *folP1* in *M. tuberculosis* are not available as DDS is not used for the treatment of tuberculosis. Mutations in *rpoB* observed in *M. leprae* showed good agreement with those obtained from RIF-resistant *M. tuberculosis*. In contrast, the distribution of mutations in *gyrA* of FQ-resistant *M. tuberculosis* was distinct from that in *gyrA* of OFX-resistant *M. leprae* (Fig. 1). Namely, amino acid substitutions at position 94 in GyrA were found in approximately half of FQ-resistant *M. tuberculosis* isolates, whereas no amino acid substitutions at position 95, equivalent to position 94 in *M. tuberculosis*, have been reported in *M. leprae*, and 11 cases with amino acid substitutions at position 91, equivalent to position 94 in *M. tuberculosis*, were reported from a total of six countries (4, 19, 24, 26, 40). Thus, elucidation of the contribution of amino acid substitutions at position 95 of GyrA in *M. leprae* to FQ resistance is important for the gene-based detection of fluoroquinolone resistance.

FQs inhibit type II DNA topoisomerases, DNA gyrase, and topoisomerase IV, which play crucial roles in DNA replication during cell division (8). As *M. leprae* has only DNA gyrase, this is the sole target of FQs. DNA gyrase, consisting of two GyrA and two GyrB subunits, catalyzes the negative supercoiling of the circular bacterial chromosome by cleaving double strands and passing the enwrapped DNA, followed by resealing the double strands (8, 13). To reveal the significance of amino acid substitution at position 95 to FQ resistance, we conducted the FQ-mediated supercoiling activity inhibition assay and DNA cleavage assay using recombinant DNA gyrases having an amino acid substitution in GyrA at position 95, Asp to Gly (GyrA-Asp95Gly) or Asp to Asn (GyrA-Asp95Asn). These mutations are frequently found in FQ-resistant *M. tuberculosis* strains (1, 7, 9, 10, 32, 34, 39) but not in FQ-resistant *M. leprae* strains.

MATERIALS AND METHODS

Materials. The Thai-53 strain of *M. leprae* (22), maintained at the Leprosy Research Center, National Institute of Infectious Diseases (Tokyo, Japan), was used to prepare *M. leprae* DNA. *Escherichia coli* strains TOP-10 (Life Technologies Corp., Carlsbad, CA), Rosetta-gami 2, and BL21(DE3)(pLysS) (Merck KGaA, Darmstadt, Germany) were used for cloning and protein expression. GyrA and GyrB expression plasmids were constructed on the basis of pET-20b(+) (Merck KGaA). OFX and gatifloxacin (GAT) were purchased from LKT Laboratories, Inc. (St. Paul, MN); moxifloxacin (MXF) was from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Sitafloxacin (SIT) was a gift from Daiichisankyo Pharmaceutical, Co., Ltd. (Tokyo, Japan). Ampicillin was purchased from Meiji Seika Pharma, Ltd. (Tokyo, Ja-

Received 10 October 2011 Returned for modification 1 November 2011
Accepted 11 November 2011
Published ahead of print 21 November 2011
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Supplemental material for this article may be found at http://aac.asm.org/.

doi:10.1128/AAC.05890-11

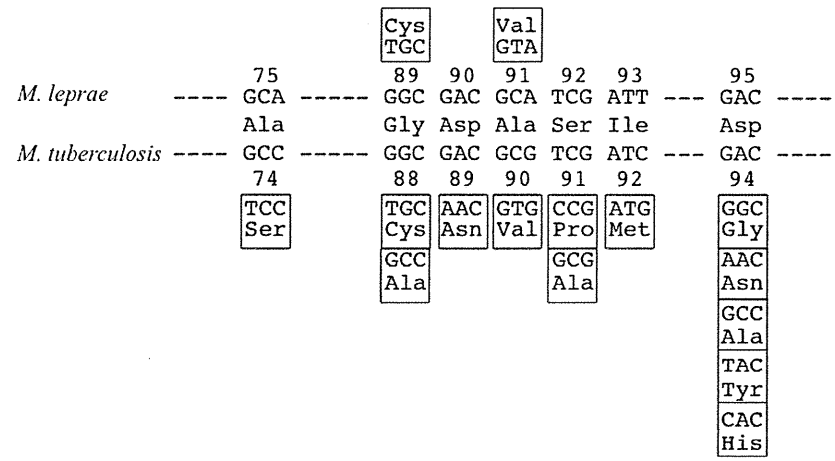

FIG 1 Nucleotide substitutions encoding the quinolone resistance-determining region in *gyrA* of WT and FQ-resistant *M. leprae* and *M. tuberculosis*. Nucleotide sequences encoding the quinolone resistance-determining region of WT *M. leprae* and *M. tuberculosis* GyrA were aligned with the amino acid sequence at the corresponding positions indicated by the numbers. Altered amino acids and the corresponding nucleotide substitutions of *M. leprae* and *M. tuberculosis* are placed above and below WT sequences, respectively.

pan). Oligonucleotide primers were synthesized by Life Technologies Corp. Restriction enzymes were obtained from New England BioLabs, Inc. (Ipswich, MA). The supercoiling assay kit and supercoiled and relaxed pBR322 DNA were purchased from John Innes Enterprises Ltd. (Norwich, United Kingdom).

Construction of recombinant wild-type (WT) and mutant DNA gyrase expression plasmids. DNA gyrase expression vectors were constructed basically as previously described (16), and Fig. 2 presents an overview of the procedure. The sequences of the primers used in the study

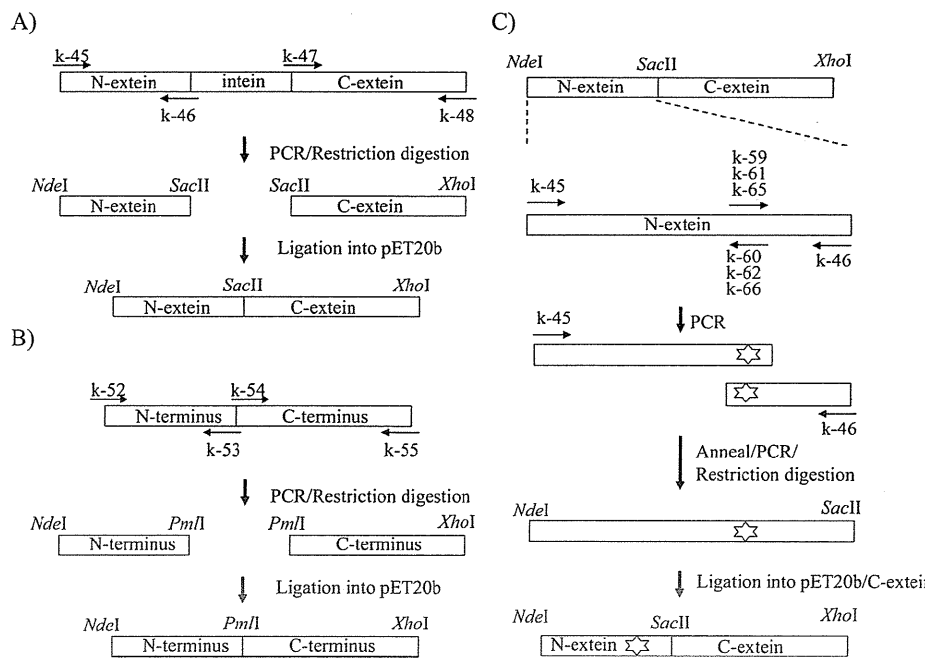

FIG 2 Construction of WT and mutant DNA gyrase expression plasmid. (A) DNA fragments encoding N-extein (amino acids 1 to 130) and C-extein of GyrA (amino acids 125 to 830) were amplified by PCR with primer pairs k-45/k-46 and k-47/k-48 (Table 1), respectively. Similarly, those encoding the N-terminal (amino acids 1 to 428) and C-terminal (amino acids 424 to 679) regions of GyrB were amplified with primer pairs k-52/k-53 and k-54/k-55 (Table 1), respectively. PCR products encoding N-extein and C-extein of GyrA were digested by NdeI-SacII and SacII-XhoI, respectively, and introduced simultaneously into NdeI-XhoI-digested plasmid pET-20b (+). (B) DNA fragments encoding the N-terminal and C-terminal regions of GyrB were digested by NdeI-PmaCI and PmaCI-XhoI, respectively, and introduced into pET20b as described above. (C) Primer pairs consisting of primer k-45 and primer k-60, k-62, or k-66 (Table 1) were used for amplifying the DNA fragment encoding the N-terminal portion (amino acids 1 to 94) of N-extein carrying Ala91Val, Asp95Gly, and Asp95Asn, respectively. Primer pairs consisting of primer k-46 and primer k-59, k-61, or k-65 (Table 1) were used for amplifying the DNA fragment encoding the C-terminal portion (amino acids 88 to 130) of N-extein carrying Ala91Val, Asp95Gly, and Asp95Asn, respectively. To complete the N-extein-encoding cassette, DNA fragments encoding the N-terminal and C-terminal regions of N-extein of GyrA were annealed and reamplified by PCR using the primer pair k-45/k-46. The mutated *gyrA*-N cassettes were digested with NdeI and SacII restriction endonucleases and ligated into the expression plasmid containing WT *gyrA* C-extein DNA fragment digested by the same enzymes.

TABLE 1 Nucleotide sequences of primers used in PCR

Primer name	Primer sequence (nucleotide positions)[a]
k-45	5′-GGCATATGACTGATATCACGCTGCCACCAG-3′ (1–25)
k-46	5′-ATAACGCATCGCCGCGGGTGGGTCATTACC-3′ (361–390)
k-47	5′-CACCCGCGGCGATGCGTTATACCGAGGCTCGGCTTACTC C-3′ (371–410)
k-48	5′-GGCTCGAGTTAATGATGATGATGATGATGACCGACACCG CCGTCGG-3′ (2471–2490)
k-52	5′-GGCATATGGCTGCCCAGAGGAAG-3′ (1–18)
k-53	5′-CTAACTCACGTGCTTTACGTGCAGCTATTC-3′ (1259–1288)
k-54	5′-CGTAAAGCACGTGAGTTAGTGCGTCGAAAAAGTGCC-3′ (1270–1305)
k-55	5′-GGCTCGAGCTAATGATGATGATGATGATGGACATCCAGG AAACGAACATCC-3′ (2013–2037)
k-59	5′-GCACGGCGAC**GTGT**CGATTTATG-3′ (261–283)
k-60	5′-CATAAATCGA**CACG**TCGCCGTGC-3′ (261–283)
k-61	5′-CATCGATTTAT**GGC**ACGTTAGTGC-3′ (272–295)
k-62	5′-GCACTAACGT**GCC**ATAAATCGATG-3′ (272–295)
k-65	5′-CATCGATTTAT**AAC**ACGTTAGTGC-3′ (272–295)
k-66	5′-GCACTAACGT**GTT**ATAAATCGATG-3′ (272–295)

[a] Six-histidine tag codons are underlined, and mutated codons are shown in bold type.

are shown in Table 1. All PCRs were carried out in a thermal cycler (Applied Biosystems) under the following conditions: predenaturation at 98°C for 2 min; 35 cycles of denaturation at 98°C for 10 s, annealing at 50 to 60°C for 15 s, and extension at 68°C for 1 to 2.5 min; and then a final extension at 68°C for 2 min. The nucleotide sequences of the DNA gyrase genes in the plasmids were confirmed using a BigDye Terminator (version 3.1) cycle sequencing kit (Life Technologies Corp.) and an ABI Prism 3130*xl* genetic analyzer (Life Technologies Corp.) according to the manufacturer's protocol.

Expression and purification of recombinant DNA gyrase. DNA gyrase subunits were purified as previously described (2, 3, 16, 20, 21, 31). Expression plasmids carrying the *gyrA* (WT and mutants) and WT *gyrB* genes of *M. leprae* were transformed into *E. coli* Rosetta-gami 2 and BL21(DE3)(pLysS), respectively. Expression of GyrA and GyrB was induced with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries Ltd., Tokyo, Japan), followed by further incubation at 14°C for 16 h. The recombinant DNA gyrase subunit in the supernatant of the sonication lysate (by Sonifier 250; Branson, Danbury, CT) was purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Life Technologies Corp.) column chromatography. The protein fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

DNA supercoiling activities and inhibition by FQs. ATP-dependent and quinolone-inhibited DNA supercoiling assays were carried out as previously described (2, 3, 16, 20, 21, 31) with the following modifications. DNA supercoiling activity was examined with a reaction mixture (total volume, 30 μl) consisting of DNA gyrase reaction buffer, relaxed pBR322 DNA (300 ng), and purified GyrA and GyrB (50 ng each) subunits. Reactions were performed at 30°C for 1.5 h and stopped by adding an equal volume of chloroform-isoamyl alcohol (24:1 mixture) and 3 μl of 10× DNA loading dye. The total reaction mixtures were subjected to electrophoresis in a 1% agarose gel in 1× Tris-borate-EDTA (TBE) buffer, followed by ethidium bromide (0.7 μg/ml) staining. Supercoiling activity was evaluated by tracing the brightness of the bands with the software ImageJ (http://rsbweb.nih.gov/ij). Gyrase bearing an Ala91Val amino acid substitution in GyrA was used as a positive control for all assays (20). The inhibitory effect of FQs on DNA gyrases was assessed by determining the drug concentrations required to inhibit the supercoiling activity of the enzyme by 50% (IC_{50}s). All enzyme assays were performed at least three times to confirm reproducibility.

Quinolone-mediated DNA cleavage assay. DNA cleavage assays were carried out as previously described (16, 20, 21, 31). The reaction mixture (total volume, 30 μl) contained DNA gyrase reaction buffer, recombinant DNA gyrase subunits (50 ng), supercoiled pBR322 DNA (300 ng), and 2-fold serially increasing concentrations of FQs. After incubation for 2 h at 30°C, 3 μl of 2% SDS and 3 μl proteinase K (1 mg/ml) were added to the reaction mixture. After subsequent incubation for 30 min at 30°C, reactions were stopped by the addition of 3 μl of 0.5 mM EDTA, 30 μl chloroform-isoamyl alcohol (24:1 mixture), and 3 μl of 10× DNA loading dye. The total reaction mixtures were subjected to electrophoresis in 0.8% agarose gels in 1× TBE buffer, followed by ethidium bromide staining. The extent of DNA cleavage was quantified with ImageJ, and the quinolone concentrations required to induce 25% of the maximum DNA cleavage (CC_{25}s) were determined.

Temperature sensitivity of *M. leprae* DNA gyrase. The reactions with mixtures (total volume, 30 μl) consisting of DNA gyrase reaction buffer, relaxed pBR322 DNA (300 ng), and recombinant DNA gyrase subunits

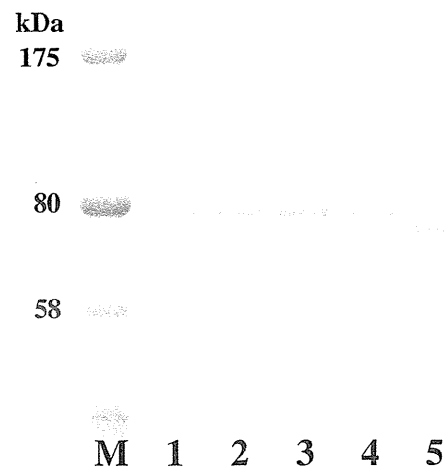

FIG 3 SDS-PAGE analysis of purified *M. leprae* DNA gyrases. The His-tagged recombinant DNA gyrases were overexpressed in *E. coli* and purified by Ni-NTA affinity resin chromatography. Lanes: M, protein marker (NEB); 1, WT GyrA; 2, GyrA-Ala91Val; 3, GyrA-Asp95Gly; 4, GyrA-Asp95Asn; 5, WT GyrB. Three hundred nanograms of each protein was loaded onto a 5 to 20% gradient polyacrylamide gel.

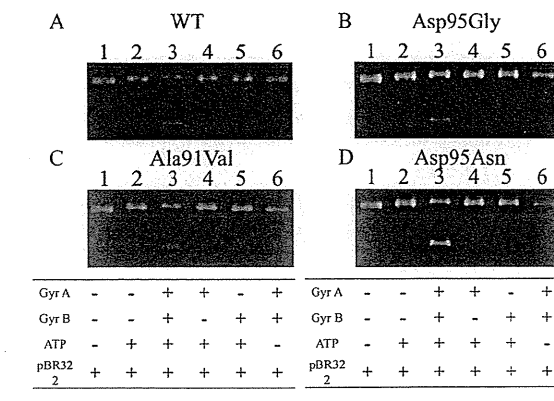

FIG 4 DNA supercoiling assay. Supercoiling activities of WT DNA gyrase (A) and DNA gyrases bearing GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D) were analyzed. Relaxed pBR322 (0.3 μg) was incubated with GyrA (50 ng) or GyrB (50 ng), or both. Lanes: 1, relaxed pBR322 alone; 2, relaxed pBR322 and ATP; 3, relaxed pBR322, ATP, GyrA, and GyrB; 4, relaxed pBR322, ATP, and GyrA; 5, relaxed pBR322, ATP, and GyrB; 6, relaxed pBR322, GyrA, and GyrB.

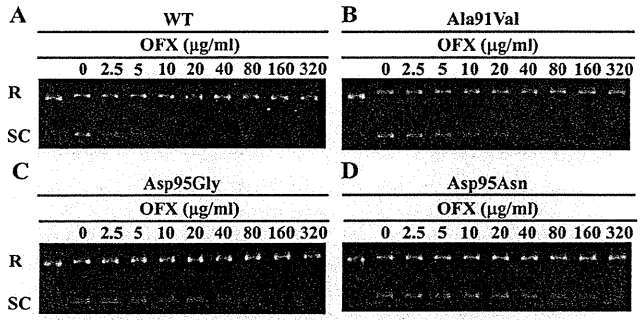

FIG 5 OFX-inhibited DNA supercoiling assay. Relaxed pBR322 (0.3 μg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. Quinolone-inhibited supercoiling activity assay was performed with combinations consisting of WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D). R and SC, relaxed and supercoiled pBR322 DNA, respectively.

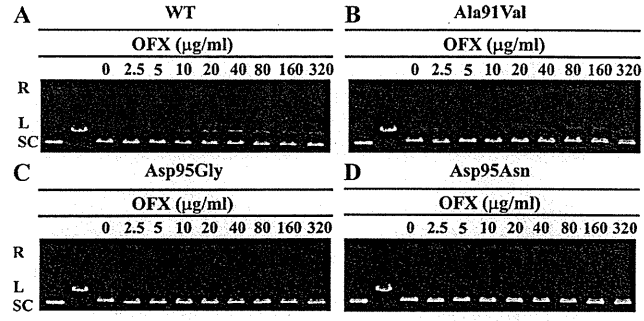

FIG 6 OFX-mediated DNA cleavage assay. Supercoiled pBR322 (0.3 μg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. DNA cleavage assay was performed with combinations consisting of WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D). R, L, and SC, relaxed, linear, and supercoiled pBR322 DNA, respectively.

(50 ng) were run at 25, 30, 33, 37, and 42°C for 1.5 h. Supercoiling activities of recombinant DNA gyrases were evaluated at each reaction temperature as described above.

RESULTS

Construction and purification of recombinant His-tagged GyrA and GyrB proteins. DNA fragments, including the *gyrA* and *gyrB* genes, were successfully amplified from *M. leprae* Thai-53 strain DNA and inserted in frame downstream of a T7 promoter in pET-20b (+). GyrA and GyrB were expressed as C-terminal hexahistidine-tagged proteins for ease of purification, as the His tag has been shown not to interfere with the catalytic functions of GyrA and GyrB (2, 3, 16, 20, 21, 31). Expressed recombinant WT and mutant DNA gyrase subunits were purified as 0.3 to 1.5 mg soluble His-tagged 80-kDa protein of GyrA and 75-kDa protein of GyrB from 500-ml cultures. The purity of the recombinant proteins was confirmed by SDS-PAGE (Fig. 3). All of the recombinant proteins were obtained with high purity (>95%).

DNA supercoiling activities. Combinations of GyrA WT, Ala91Val, Asp95Gly, or Asp95Asn and WT GyrB subunits were examined for DNA supercoiling activities using relaxed pBR322 DNA as a substrate in the presence or absence of ATP (Fig. 4). DNA supercoiling activities were observed in the presence of ATP and recombinant DNA gyrase subunits (Fig. 4A to D, lane 3), while neither subunit alone exhibited DNA supercoiling activity (Fig. 4A to D, lanes 4 and 5). In addition, no supercoiling activity was observed when ATP was omitted from the reaction mixture (Fig. 4A to D, lane 6).

Inhibition of DNA gyrase activities by FQs. The IC_{50}s of FQs were determined using the quinolone-inhibited DNA supercoiling assay (Fig. 5). Representative data showing the inhibitory effects of OFX against DNA gyrase are shown in Fig. 5, and data for other FQs are presented in Fig. S1 in the supplemental material. IC_{50}s of each FQ against WT and mutant DNA gyrases are summarized in Table 2. Each FQ showed dose-dependent inhibition, with IC_{50}s ranging from 0.4 to 262.3 μg/ml. DNA gyrases bearing GyrA-Asp95Gly and -Asp95Asn showed significantly higher IC_{50}s to quinolones (Table 2; Fig. 5; see Fig. S1 in the supplemental material) than WT gyrase (Table 2). These DNA gyrases also showed higher resistance than DNA gyrase bearing GyrA-Ala91Val, which was simultaneously analyzed as a positive control for resistance to FQs. Inhibitory effects of FQs were ranked SIT > GAT > MXF > OFX in all DNA gyrases.

FQ-mediated DNA-cleavable complex formation. The CC_{25}s of FQs were determined. Figure 6 shows the result of a DNA cleavage assay using OFX, and Fig. S2 in the supplemental material presents the results using GAT, MXF, and SIT. Table 2 summarizes the CC_{25}s of each DNA gyrase. DNA gyrases bearing GyrA-Asp95Gly and -Asp95Asn showed significantly higher CC_{25}s to quinolones than WT gyrase (Table 2). These DNA gyrases also showed higher CC_{25}s than gyrase bearing GyrA-Ala91Val (Table 2). Effects on cleavable complex formation were ranked SIT > GAT > MXF > OFX in all DNA gyrases.

Temperature sensitivity of *M. leprae* DNA gyrase. Figure 7 shows the effects of temperature on DNA gyrase activities. The highest DNA supercoiling activities were observed at 33°C in all DNA gyrases. WT and GyrA-A91V DNA gyrases showed reduced DNA supercoiling activities at 37°C, whereas Gyr-Asp95Gly and Asp95Asn DNA gyrases maintained activities comparable to those at 33°C. No supercoiling activities were observed in any of the DNA gyrases at 42°C.

TABLE 2 IC_{50}s and CC_{25}s of FQs against WT and mutant DNA gyrases[a]

Drug	IC_{50}				CC_{25}			
	WT	Ala91Val	Asp95Gly	Asp95Asn	WT	Ala91Val	Asp95Gly	Asp95Asn
OFX	6.8 ± 0.8	39.4 ± 15.5 (5.8)	161.2 ± 44.2 (23.7)	262.3 ± 105.8 (38.6)	7.3 ± 0.5	75.5 ± 16.8 (10.1)	240.5 ± 30.7 (32.1)	269.5 ± 76.5 (35.9)
GAT	1.0 ± 0.1	3.1 ± 0.7 (3.1)	7.5 ± 1.6 (7.5)	13.8 ± 1.6 (13.8)	1.1 ± 0.2	4.3 ± 0.2 (3.9)	15.6 ± 3.6 (14.2)	13.5 ± 3.1 (12.3)
MXF	1.5 ± 0.3	5.2 ± 1.0 (3.5)	21.5 ± 4.7 (14.3)	34.7 ± 3.1 (23.1)	1.0 ± 0.1	4.5 ± 1.0 (4.5)	25.5 ± 3.7 (25.5)	20.8 ± 5.0 (20.8)
SIT	0.4 ± 0.0	1.0 ± 0.2 (2.5)	2.2 ± 0.5 (5.5)	3.9 ± 0.6 (9.8)	0.3 ± 0.0	0.9 ± 0.0 (3.0)	2.2 ± 0.6 (7.3)	2.3 ± 0.4 (7.7)

[a] IC_{50}s and CC_{25}s are in μg/ml, and data in parentheses represent the fold increase compared to WT.

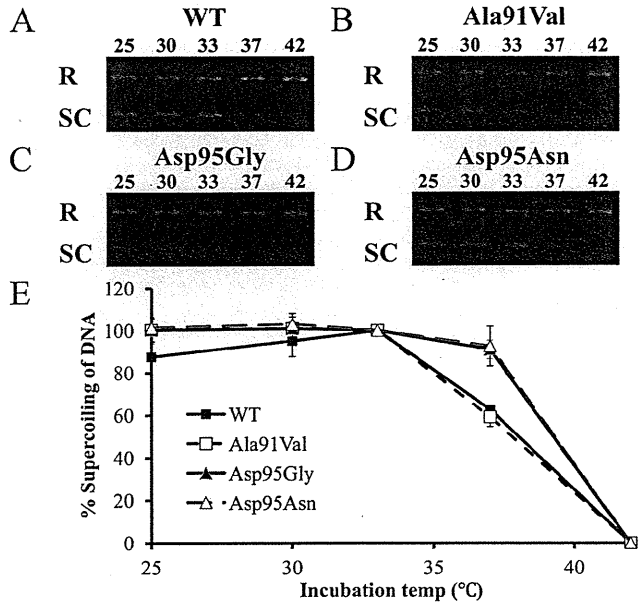

FIG 7 Temperature-dependent DNA supercoiling activity of DNA gyrases. Relaxed pBR322 (0.3 μg) was incubated with WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D) at the temperatures (in °C) indicated above the lanes. The proportion of supercoiled DNA compared to that of WT DNA gyrase at 33°C is plotted for each incubation temperature.

DISCUSSION

Mutations in the *gyrA* gene of quinolone-resistant *M. leprae* clinical isolates have predominantly been reported at codon 91, and a smaller number have been reported at codon 89 (4, 19, 24, 26, 40). Amino acid substitutions at other positions have not been reported, in strong contrast to the substitutions reported in *M. tuberculosis*, with predominant mutations in codon 94 (1, 7, 9, 10, 32, 34, 39), equivalent to codon 95 in *M. leprae* (Fig. 1). This study aimed to obtain basic data for the rapid detection of FQ-resistant leprosy by elucidating the correlation between mutations at codon 95 and quinolone resistance.

To explain the discrepancy described above, we first hypothesized that amino acid substitution at position 95 in GyrA of *M. leprae* has less of an influence on FQ resistance. Hence, we carried out a quinolone-mediated supercoiling activity inhibition assay and DNA cleavage assay at 30°C, the optimal temperature of *M. leprae* growth, using recombinant DNA gyrases and calculated IC_{50}s and CC_{25}s of four FQs, OFX, MXF, GAT, and SIT. The DNA gyrase bearing GyrA-Ala91Val, used as a control, exhibited resistance, having approximately 2- to 10-fold higher IC_{50}s and CC_{25}s of FQs than WT DNA gyrase, as has been reported previously (20, 21). Interestingly, DNA gyrases bearing GyrA-Asp95Gly or -Asp95Asn showed resistance, having approximately 5- to 40-fold higher IC_{50}s and CC_{25}s of FQs than WT DNA gyrase (Table 2). Namely, amino acid substitution from Asp to Gly or Asn at position 95 added higher resistance to DNA gyrase than that from Ala to Val at position 91. This was similar to the observation in *M. tuberculosis* (2, 3). These results suggested that a possible property of Asp95Gly and Asp95Asn amino acid substitutions in GyrA is to give higher FQ resistance to DNA gyrase in *M. leprae*.

We then hypothesized that amino acid substitutions at position 95 place a disadvantage on the enzymatic property of DNA gyrases, especially lower or abolished activity at higher temperatures, and thus, we conducted a DNA supercoiling assay at various temperatures: 25, 30, 33, 37, and 42°C. DNA supercoiling activities of WT and GyrA-Ala91Val DNA gyrase showed a similar temperature dependence, with the highest activity being at 25 to 33°C, reduced activity occurring at 37°C, and activity being completely abolished at 42°C. In contrast, DNA gyrases bearing GyrA-Asp95Gly or -Asp95Asn maintained their activities even at 37°C. Our hypothesis was rejected by these data.

The influence of the clear usage of FQs for the treatment of leprosy and tuberculosis might solve this question. For leprosy patients with a single lesion, a single application of 400 to 600 mg of OFX is used. For the treatment of MDR leprosy, two or three doses of 400 to 600 mg in combination with first-line drugs DDS and RIF (11) are applied. In contrast, for tuberculosis, OFX is taken twice daily at 400 mg each time with first-line drugs such as isoniazid and rifampin for several months (11, 36). The maximum serum concentration (C_{max}) of OFX has been reported to show a dose-dependent increase. The C_{max}s achieved with administration of 100 mg, 300 mg, and 600 mg of OFX in humans were 1.00, 2.81, and 6.81 μg/ml, respectively (14). The blood concentration of OFX is low in leprosy patients and is maintained at a high level in tuberculosis patients because of the treatment regimen. Thus, *M. leprae* carrying DNA gyrase with lower resistance, such as GyrA-Ala91Val, might be predominantly selected for various reasons in leprosy patients, whereas GyrA-Asp94Gly or -Asp94Asn is predominantly found in *M. tuberculosis*-infected patients (1, 7, 9, 10, 32, 34, 39); however, the possible emergence in the future of highly FQ-resistant *M. leprae* having an amino acid substitution at position 95 cannot be rejected, especially when MDR leprosy is treated by repeated administration of FQs.

We investigated the inhibitory effects of OFX, GAT, MXF, and SIT against WT and mutant DNA gyrases. IC_{50}s of OFX for WT and GyrA-Ala91Val, -Asp95Gly, and -Asp95Asn DNA gyrases were 6.8, 39.4, 161.2, and 262.3 μg/ml, respectively (Table 2). The order of FQ inhibitory activity was SIT > GAT > MXF > OFX. OFX does not have the ability to inhibit *M. leprae* with DNA gyrase carrying GyrA-Asp95Gly or -Asp95Asn. The IC_{50} of SIT was the lowest of the four quinolones, with IC_{50}s of 0.4, 1.0, 2.2, and 3.9 μg/ml for WT, A91V, D95G, and D95N gyrases, respectively. As the C_{max}s of OFX, GAT, MXF, and SIT at the 100-mg dosage were determined in clinical trials to be 1.00, 0.87 to 5.41, 4, and 0.3 to 1.9 μg/ml, respectively (14, 27, 28, 30), SIT might strongly inhibit *M. leprae* carrying GyrA-Ala91Val DNA gyrase and be a promising candidate for the treatment of the majority of cases of FQ-resistant leprosy.

In conclusion, we revealed the contribution of the GyrA-Asp95Gly and -Asp95Asn amino acid substitutions to FQ resistance in *M. leprae* by an *in vitro* assay. This suggested the possible emergence in the future of FQ-resistant *M. leprae* carrying GyrA with these amino acid substitutions, although further analysis is needed to clarify a direct relationship to *in vivo* resistance. Hence, we would like to propose analysis for these amino acid substitutions to detect FQ-resistant leprosy.

ACKNOWLEDGMENTS

We thank Haruka Suzuki, Yukari Fukushima, and Aiko Ohnuma for their technical support.

This work was supported by grants from the U.S.-Japan Cooperative

Medical Science Program, the Global Center of Excellence Program, the Establishment of International Collaboration Centers for Zoonosis Control, and the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and in part by grants from the Japan Initiative for Global Research Network on Infectious Diseases MEXT to Y.S. and by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science to Y.S. and C.N.

REFERENCES

1. An DD, et al. 2009. Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with high-level fluoroquinolone resistance in Vietnam. Antimicrob. Agents Chemother. 53:4835–4839.
2. Aubry A, et al. 2006. Novel gyrase mutations in quinolone-resistant and -hypersusceptible clinical isolates of *Mycobacterium tuberculosis*: functional analysis of mutant enzymes. Antimicrob. Agents Chemother. 50: 104–112.
3. Aubry A, Pan XS, Fisher LM, Jarlier V, Cambau E. 2004. *Mycobacterium tuberculosis* DNA gyrase: interaction with quinolones and correlation with antimycobacterial drug activity. Antimicrob. Agents Chemother. 48: 1281–1288.
4. Cambau E, Perani E, Guillemin I, Jamet P, Ji B. 1997. Multidrug-resistance to dapsone, rifampicin, and ofloxacin in *Mycobacterium leprae*. Lancet 349:103–104.
5. Cambau E, Carthagena L, Chauffour A, Ji B, Jarlierbl V. 2006. Dihydropteroate synthase mutations in the folP1 gene predict dapsone resistance in replaced cases of leprosy. Clin. Infect. Dis. 42:238–241.
6. Cambau E, et al. 2002. Molecular detection of rifampin and ofloxacin resistance for patients who experience relapse of multibacillary leprosy. Clin. Infect. Dis. 34:39–45.
7. Campbell PJ, et al. 2011. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 55:2032–2041.
8. Champoux JJ. 2001. DNA topoisomerases: structure, function, and mechanism. Annu. Rev. Biochem. 70:369–413.
9. Cui Z, Wang J, Lu J, Huang X, Hu Z. 2011. Association of mutation patterns in gyrA/B genes and ofloxacin resistance levels in *Mycobacterium tuberculosis* isolates from east China in 2009. BMC Infect. Dis. 11:78–82.
10. Feuerriegel S, et al. 2009. Sequence analyses of just four genes to detect extensively drug-resistant *Mycobacterium tuberculosis* strains in multidrug-resistant tuberculosis patients undergoing treatment. Antimicrob. Agents Chemother. 53:3353–3356.
11. Goto M, et al. 2006. Guideline for the treatment of Hansen's disease in Japan (second edition), Japanese leprosy. Nihon Hansenbyo Gakkai Zasshi 75:191–226. (In Japanese.)
12. Honore N, Cole ST. 1993. Molecular basis of rifampin resistance in *Mycobacterium leprae*. Antimicrob. Agents Chemother. 37:414–418.
13. Hooper DC. 2000. Mechanisms of action and resistance of older and newer fluoroquinolones. Clin. Infect. Dis. 31:S24–S28.
14. Ichihara N. 1984. Phase I study on DL-8280 (OFX). Chemotherapy 32: 118–149.
15. Kai M, et al. 1999. Diaminodiphenylsulfone resistance of *Mycobacterium leprae* due to mutations in the dihydropteroate synthase gene. FEMS Microbiol. Lett. 177:231–235.
16. Kim H, et al. 2011. Impact of the E540V amino acid substitution in GyrB of *Mycobacterium tuberculosis* on quinolone resistance. Antimicrob. Agents Chemother. 55:3661–3667.
17. Kyaw UK, Aye DKS. 2006. A case of multi-drug resistant leprogy-relapse or re-infection, Myanmar J. Curr. Med. Pract. 10:41–43.
18. Levy L, Ji B. 2006. The mouse foot-pad technique for cultivation of *Mycobacterium leprae*. Lepr. Rev. 77:5–24.
19. Maeda S, et al. 2001. Multidrug resistant *Mycobacterium leprae* from patients with leprosy. Antimicrob. Agents Chemother. 45:3635–3639.
20. Matrat S, Cambau E, Jarlier V, Aubry A. 2008. Are all the DNA gyrase mutations found in *Mycobacterium leprae* clinical strains involved in resistance to fluoroquinolones? Antimicrob. Agents Chemother. 52: 745–747.
21. Matrat S, et al. 2007. Expression and purification of an active form of the *Mycobacterium leprae* DNA gyrase and its inhibition by quinolones. Antimicrob. Agents Chemother. 51:1643–1648.
22. Matsuoka M. 2010. The history of *Mycobacterium leprae* Thai-53 strain. Lepr. Rev. 81:137.
23. Matsuoka M, et al. 2007. The frequency of drug resistance mutations in Mycobacterium leprae isolates in untreated and replaced leprosy patients from Myanmar, Indonesia and the Philippines. Lepr. Rev. 78:343–352.
24. Matsuoka M, Kashiwabara Y, Namisato M. 2000. A Mycobacterium leprae isolate resistant to dapsone, rifampin, ofloxacin and sparfloxacin. Int. J. Lepr. Other Mycobact. Dis. 68:452–455.
25. Matsuoka M, Kashiwabara Y, Liangfen Z, Goto M, Kitajima S. 2003. A second case of multidrug resistant Mycobacterium leprae isolated from a Japanese patient with relapsed lepromatous leprosy. Int. J. Lepr. Other Mycobact. Dis. 71:240–243.
26. Matsuoka M, et al. 2010. Possible mode of emerging drug resistant leprosy cases revealed in Mexican samples' analysis. Jpn. J. Infect. Dis. 63: 412–416.
27. Nakashima M, et al. 1995. Pharmacokinetics and tolerance of DU-6859a, a new fluoroquinolone, after single and multiple oral doses in healthy volunteers. Antimicrob. Agents Chemother. 39:170–174.
28. Nakashima M, et al. 1995. Single- and multiple-dose pharmacokinetics of AM-1155, a new 6-fluoro-8-methoxy quinolone, in humans. Antimicrob. Agents Chemother. 39:2635–2640.
29. Norman G, Joseph G, Ebenezer G, Rao PSSS, Job CK. 2003. Secondary rifampin resistance following multi-drug therapy–a case report. Int. J. Lepr. Other Mycobact. Dis. 71:18–21.
30. Ohnishi N, et al. 2005. Safety, pharmacokinetics and influence on the intestinal flora of BAY 12-8039 (moxifloxacin hydrochloride) after oral administration in healthy male subjects. Jpn. Pharmacol. Ther. 33: 1029–1045.
31. Pan XS, Yague G, Fisher LM. 2001. Quinolone resistance mutations in *Streptococcus pneumoniae* GyrA and ParC proteins: mechanistic insights into quinolone action from enzymatic analysis, intracellular levels, and phenotypes of wild-type and mutant proteins. Antimicrob. Agents Chemother. 45:3140–3147.
32. Ramaswamy S, Musser JM. 1998. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis 1998 update. Tuber. Lung Dis. 79:3–29.
33. Scollard DM, et al. 2006. The continuing challenges of leprosy. Clin. Microbiol. Rev. 19:338–381.
34. Sun Z, et al. 2008. Comparison of *gyrA* gene mutations between laboratory-selected ofloxacin-resistant *Mycobacterium tuberculosis* strains and clinical isolates. Int. J. Antimicrob. Agents 31:115–121.
35. Williams DL, Spring L, Harris E, Roche P, Gillis TP. 2000. Dihydropteroate synthase of *Mycobacterium leprae* and dapsone resistance. Antimicrob. Agents Chemother. 44:1530–1537.
36. World Health Organization. 2004. Multidrug therapy against leprosy. Report WHO/CDS/CPE/CEE/2004.46. World Health Organization, Geneva, Switzerland.
37. World Health Organization. 2011. Leprosy update, 2011. Wkly. Epidemiol. Rec. 86:389–399.
38. Reference deleted.
39. Yin X, Yu Z. 2010. Mutation characterization of gyrA and gyrB genes in levofloxacin-resistant *Mycobacterium tuberculosis* clinical isolates from Guangdong province in China. J. Infect. 61:150–154.
40. You E-Y, Kang TJ, Kim S-K, Lee S-B, Chae G-T. 2010. Mutations in genes related to drug resistance in *Mycobacterium leprae* isolates from leprosy patients in Korea. J. Infect. 50:6–11.

High Frequency of HIV-1 Dual Infections in Cameroon, West Central Africa

To the Editors:

The molecular epidemiology of HIV-1 is characterized by a complex and evolving genetic diversity. This results from a high rate of mutations and recombination and a high turnover of virions in HIV-1–infected individuals. Based on phylogenetic analysis, HIV-1 is classified into 3 known groups of HIV-1 (M, N, and O); a new group named as P has recently been identified in a Cameroonian patient.[1] The great majority of HIV-1 sequences belong to the M group, composed of 9 genetic subtypes (A–D, F–H, J–K), 6 subsubtypes (A1, A2, A3, A4, F1, and F2), and at least 48 circulating recombinant forms (CRFs). A myriad of unique recombinant forms have also been identified (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html). Most genomes of CRFs are highly complex intersubtype recombinants.[2] In sub-Saharan Africa, most HIV-1 types and subtypes cocirculate, although their geographical distribution is not uniform.[3] In west Africa, CRF02_AG accounts for the majority of HIV-1 infections. In Cameroon, the majority (>80%) of HIV-1 infections among blood donors are caused by CRFs and unique recombinant forms with 80% of the recombinant strains containing segments of subtype G.[4]

To date, a number of studies have been performed to determine the prevalence of dual infection in Cameroonian populations.[5–7] We set out to characterize HIV-1 subtypes and to determine the frequency of HIV-1 intersubtype and intrasubtype dual infections in antenatal attendees and blood donors from northwestern Cameroon. The study protocol was approved by the Ethical Review Board of the Ministry of Public Health of Cameroon and the National Science and Ethics Committee.

This is a cross-sectional clinical cohort study in a population with low-risk sexual behavior. A total of 120 participants in the northwestern region of Cameroon were enrolled in this study following their informed written consent. Sera were screened by a particle agglutination test (Serodia-HIV; Fujirebio, Tokyo, Japan). Proviral DNA extraction, amplification, and sequence analysis were performed using a modification of previously reported methods.[7] Of the 51 seropositive specimens subjected to nested polymerase chain reaction (PCR) using universal primers (Unipol-5 and Unipol-6 for first round, and Unipol-1 and Unipol-2 for second round) and a Super Mix premixed reagent (Invitrogen, Carlsbad, CA). The partial *pol* was amplified using first round PCR primers Unipol-5 (5'-TGGGTACCAG CACACAAAGGA ATAGGAGGAAA-3', at position 3434–3765 of HIV-1LAI) and Unipol-6 (5'-CCACAGCTGATCTC TGGCCT TCTCTGTAATAGACC-3', at position 4483–4516 of HIV-1LAI) and second round primers Unipol-1 (5'-AGTGGATTCATAGAA AGCA-GAAGT-3', at position 4052–4074 of HIV-1LAI) and Unipol-2 (5'-CCCCTAT TCCTTCCCCTTCTTTTAAAA-3', at position 4363–4388 of HIV-1LAI) were used to generate sequence fragment.[7] The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and inserted into Invitrogen TA-cloning vector (Invitrogen, Carlsbad, CA) for cloning and sequencing. Between 10 and 20 colonies per sample were analyzed for evidence of intrasubtype and intersubtype dual infections. Only proviral DNA amplification led to the identification of dual infections in most patient samples. Attempts to identify dual infections from reverse-transcribed and amplified HIV RNA were unsuccessful in most of our specimens. It is therefore possible that some of the sequences amplified are archived viruses that do not significantly contribute to a productive infection.

The nucleotide sequences were manually refined in Se-Al (http://tree.bio.ed.ac.uk/software/), and electropherogram sequence assemblages were obtained with Genetyx Mac 10.0 and Genetyx MacATSQ v3.0 (Software Development, Tokyo, Japan).[8] Subtype reference sequences of HIV-1 group M available from the Los Alamos Sequence database were aligned to the generated sequences using ClustalX. The MEGA version 4.0 software package was used to perform phylogenetic analysis, and the pairwise evolutionary distances were estimated using the HKY85 model.[9,10] The phylogenetic trees were constructed using neighbor joining method, and the reliability of tree topologies was estimated by bootstrap analysis (1000 replicates).[11,12] Epidemiologically linked transmissions were defined as those with bootstrap values >80%. For clarity of the figure, only 2 sequences were included in the tree for this letter, but an analysis was also performed including all clones per patient. An analysis including a good sampling of subtypes and including all the clones per patient may also reveal intrasubtype recombination, and only consensus sequences derived from intersubtype dual infections were included and deposited to GenBank with the accession numbers HQ714903 to HQ714956 (Fig. 1).

The results show that 11 HIV-1 strains are cocirculating in the northwestern region of Cameroon. HIV-1 group M CRF02_AG infections (35.0%), subtype A (4.0%), C (6.0%), D (4.0%), G (27.0%), H (4.0%), U (2.0%), CRF11.cpx (4.0%), CRF22.cpx (2.0%), CRF25.cpx (2.0%), and CRF37.cpx (10.0%). Four intersubtype (8.0%) dual infections were identified among the 51 samples analyzed (1 H/G, 1 C/CRF25.cpx, 1 H/CRF37.cpx, and 1 C/CRF37.cpx). We identified 3 potential transmission pairs (Fig. 1, unfilled circles), which were supported by epidemiological data. Moreover, there are 2 subclusters of 10 new Cameroonian and 1 outlier sequence.

In a study of HIV-infected blood donors in several regions of Cameroon from 1996 to 2004, subtype G accounted for 4.5% of HIV-1 infections.[13] Our findings confirm an earlier report that showed a relatively high proportion of subtype G and CRF02_AG in western Cameroon.[14] Identification of HIV-1 subtype H strains in our cohort is of substantial interest. Although subtype H has been formally recognized for many

The authors have no conflicts of interest and competing financial interest.

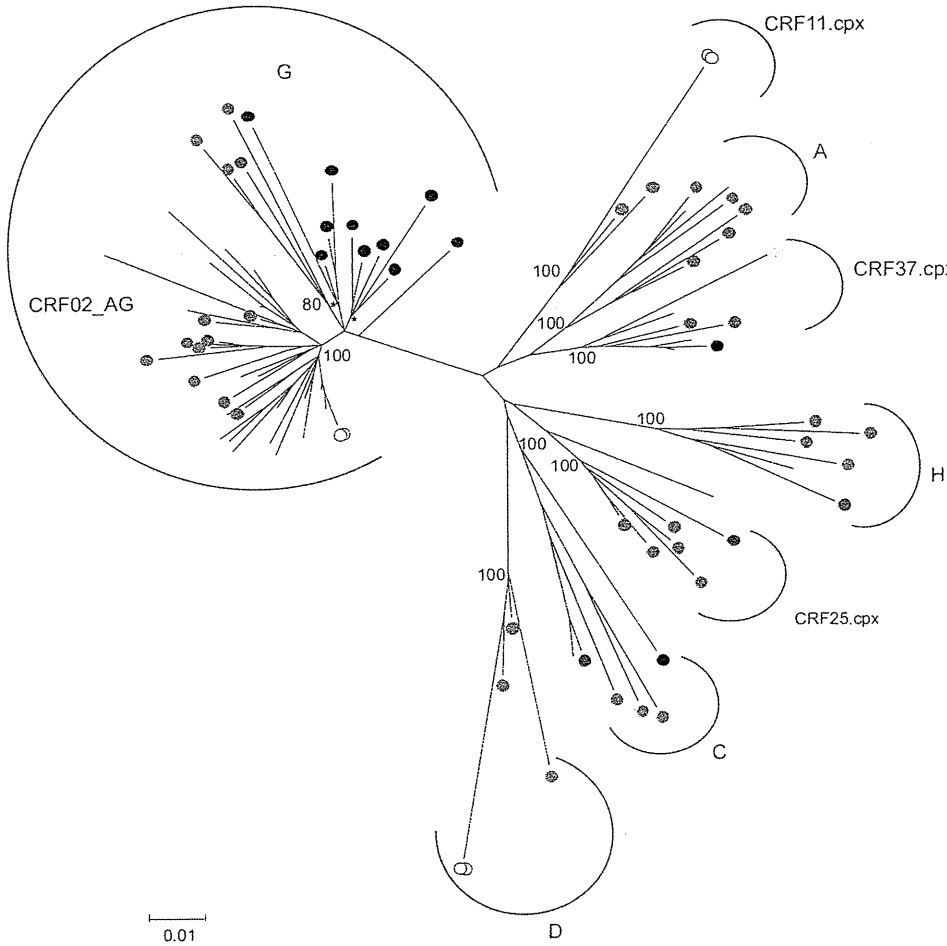

FIGURE 1. Unrooted neighbor joining tree derived from nucleotide alignment of clonal sequences of participants from northwestern Cameroon. HIV-1 group M is represented by at least 3 sequences for each subtype A, C, D, G, H, CRF02_AG, CRF11.cpx, CRF22.cpx, and CRF36.cpx (filled circles, gray); "subsubtype G" distinct clusters and outlier (filled circles; blue with bootstrap> 70% represented with asterisks); transmission pairs (unfilled circles); dual infection (1): subtype G/H (filled circles, red); dual infection (2): subtype H/CRF37.cpx (filled circles, yellow); dual infection (3): subtype C/CRF25.cpx (filled circles, pink); dual infection (4): subtype C/CRF37.cpx (filled circles, black); and nodes that do not have significant bootstrap support are not shown.

years, only 4 near full-length genomes and 2 recombinants with subtype H segment have been reported to date.[15] The most recent data available on the distribution and prevalence of subtype H suggest that it represents <0.2% of global HIV infections and is most prevalent in central Africa (3.14%).[4] This could indicate that dual infections observed are a major event among this heterosexual population.The relatively high frequency of intersubtype dual infections with subtype G seems to correlate with the high prevalence of subtype G (CRF02_AG) viruses in northwestern Cameroon.[16]

In conclusion, dual infections with divergent HIV-1 strains are more common than previously thought and are important because this information provides a more rational basis for developing AIDS vaccines and for predicting the global evolution of HIV in the future.

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ACKNOWLEDGMENTS

The authors are grateful to the participants and Laboratory staff at St Elizabeth General Hospital. They also thank Prof Frances Gotch, Dr Jean Carr, and Dr Catherine Brennan for reviewing the manuscript. This work was partly supported by Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. Vallari A, Holzmayer V, Harris B, et al. Confirmation of putative HIV-1 gGroup P in Cameroon. *J Virol.* 2011;85:1403–1407.
2. The circulating recombinant forms (CRFs). 2010. Available at: http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs/html. Accessed February 3, 2011.
3. Peeters M, Toure-Kane C, Nkengasong JN. Genetic diversity of HIV in Africa: impact on diagnosis, treatment, vaccine development and trials. *AIDS.* 2003;17:2547–2560.
4. Hemelaar J, Gouws E, Ghys PD, et al. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. *AIDS.* 2006;20:W13–W23.
5. Powell RL, Urbanski MM, Burda S, et al. High frequency of HIV-1 dual infections among HIV-positive individuals in Cameroon, West Central Africa. *J Acquir Immune Defic Syndr.* 2009;50:84–92.
6. Yamaguchi J, Bodelle P, Vallari AS, et al. HIV infections in northwestern Cameroon: identification of HIV type 1 group O and dual HIV type 1 group M and group O infections. *AIDS Res Hum Retroviruses.* 2004;20: 944–957.
7. Takehisa J, Zekeng L, Ido E, et al. Various types of HIV mixed infections in Cameroon. *Virology.* 1998;245:1–10.
8. Galtier N, Gouy M, Gaultier C. SEAVIEW and PHYLO_WIN: two graphic tools for sequences alignment and molecular phylogeny. *Comput Appl Biosci.* 119; 12:543–548.
9. Tamura K, Dudley J, Nei M, et al. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol.* 2007;24:1596–1599.
10. Hasegawa M, Kishino H, Yano T. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol.* 1985;22: 160–174.
11. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol.* 1987;4: 406–425.
12. Felsentein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution.* 1985;39:783–791.
13. Brennan CA, Bodelle P, Coffey R, et al. The prevalence of diverse HIV-1 strains was stable in Cameroonian blood donors from 1996 to 2004. *J Acquir Immune Defic Syndr.* 2008;49: 432–439.
14. Tebit DM, Zekeng L, Kaptué L, et al. Genotypic and phenotypic analysis of HIV type 1 primary isolates from western Cameroon. *AIDS Res Hum Retroviruses.* 2002;18: 39–48.
15. Holzmayer V, Aitken C, Skinner C, et al. Characterization of genetically diverse HIV type 1 from a London cohort: near full-length genomic analysis of a subtype H strain. *AIDS Res Hum Retroviruses.* 2009;25: 721–726.
16. Ndembi N, Mbanya D, Kamdem D, et al. Molecular epidemiology of HIV-1 in Cameroon: decrease in the proportion of mosaic CRF02_AG from west to east Cameroon. AIDS 2006-XVI International AIDS Conference: Abstract no. MOPE0534.

Original article

Activity of superior interferon α against HIV-1 in severe combined immunodeficient mice reconstituted with human peripheral blood leukocytes

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Keywords: *human immunodeficiency virus type 1; severe combined immunodeficient mouse; interferon-α*

Background Interferon (IFN) can inhibit human immunodeficiency virus type 1 (HIV-1) replication *in vitro* and in clinic. However, IFN therapy for HIV infection was limited by its moderate antiviral efficacy and its frequent adverse effects. In the present study we evaluated the anti-HIV efficacy of a novel synthesized superior interferon α (sIFNα).

Methods We performed *in vitro* experiments with HIV-1 IIIB infected MT4 cells, and evaluated *in vivo* anti-HIV efficacy of sIFNα in severe combined immunodeficient (SCID) mice reconstituted with human peripheral blood leukocytes (hu-PBL-SCID mice).

Results We found that the 50% effective concentrations (EC_{50}) of sIFNα against the replication of HIV-1 in MT4 cells was 0.06 ng/ml, representing stronger antiviral activity than interferon-α *in vitro*. In the hu-PBL-SCID mice, a dose-dependent protection pattern was observed: with 0.45 μg and 1.35 μg sIFNα daily treatments, parts of SCID mice were protected from HIV infection, whereas 2.25 μg sIFNα daily treatments resulted in a terminally complete protection.

Conclusions sIFNα shows good anti-HIV activity both *in vitro* and in SCID mice, may be a promising anti-HIV agent deserving clinical investigation, especially considering the potential of IFN-α to inhibit HIV replication in patients infected with drug-resistant variants or co-infected with hepatitis C virus (HCV).

Chin Med J 2011;124(3):396-400

Interferon-α (IFN-α) was one of the first drugs to be tested against HIV in the 1980s.[1] It is a well-known antiviral protein and mediator of innate antiviral response, induced by virus infection.[2,3] Reportedly, IFN can interfere with mRNA transcription, protein synthesis, assembly and release of human immunodeficiency virus type 1 (HIV-1) in human cells cultivated *in vitro*,[4-9] and delay the appearance of AIDS and prolonging survival in asymptomatic patients.[10-13] However, IFN-α is seldom used for HIV treatment now due to its modest antiviral efficacy and dose-related side-effects.[1,14,15] In this study, we investigated the anti-HIV activity of a new synthesized superior interferon α (sIFNα) evolved through DNA shuffling.

Animal models are important preclinical systems to better predict clinical outcomes of antiretroviral treatments. In AIDS research, the HIV infected humanized severe combined immunodeficient (SCID) mouse model is widely used. The human peripheral blood lymphocyte-SCID (hu-PBL-SCID) mouse model is reconstituted by engrafting with hu-PBLs into SCID mice.[16] In these mice, the human T and B lymphocytes survive and persist in the peritoneal cavity and organs for several months, human immunoglobulin is produced, and secondary antibody response can be elicited.[17] The xenochimeric SCID mice reconstituted with hu-PBL[18-20] or other human organs such as fetal liver and thymus, lymph nodes (LN)[21-24] are highly susceptible to HIV-1 infection, and is the only small animal model allowing the study of the *in vivo* interaction between HIV-1 and human cells now. Here, we also further investigated the anti-viral effectiveness of sIFNα in hu-PBL-SCID mice model.

METHODS

Cell lines and agents

The T-cell lines MT-4 were cultured in RPMI 1640 medium (GIBCO/BRL, USA) supplemented with 10%

DOI: 10.3760/cma.j.issn.0366-6999.2011.03.014
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This work was supported by the grants from the National Grand Program on Key Infectious Disease (No. 2008ZX10001-002), the China National Program on Key Basic Research Projects (973 Program) (No. 2005CB22903 and No. 2006CB504202), and the China Ministry of Science and Technology.
Conflicts of interest: None.

fetal bovine serum (GIBCO/BRL), glutamine 290 mg/ml, penicillin 100 U/ml, and streptomycin 50 mg/ml. sIFNα was supplied by Sichuan Huiyang Life-Engineering Co., Ltd. (China) and AZT was purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd (Shanghai, China).

Virus stock

HIV-1 IIIB was used for *in vitro* antiviral assays and SCID mice experiments. Virus was prepared by limited passage through MT4 cells. Briefly, cell-free virus supernatant was used to infect MT4 cells and medium was changed in half every 3 days. Virus growth was monitored with HIV P24 enzyme-linked immunosorbent assay (ELISA). Virus stock was stored at −80°C.

In vitro antiviral assays

In vitro antiviral assays were performed with MT4 infected with Lab-adapted strains HIV-1 IIIB. MT4 cells (5×10^4) were infected with 100 $TCID_{50}$ cell-free virus and then culturing them in 96-well cell culture plates in the presence or absence of different concentrations of test compounds. After 6 days, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well and incubated at 37°C for 4 hours, and then acidified isopropyl alcohol was added to dissolve formazan crystals. The optical density was determined with a microplate reader (model 680; Bio-Rad Laboratories, USA).

HIV infected hu-PBL-SCID model

SCID mice

SCID/beige female mice, aged 4–6 weeks, were purchased from Vital River Lab Animal Co. Ltd. (Beijing, China) and housed in microisolator cages in an animal biosafety level 3 (ABSL-3) containment facility and kept under sterile conditions. The weight of the mice was 16–17 g. All food, water and bedding were autoclaved prior to use.

Immune reconstitution of SCID/beige mice

Peripheral blood mononuclear cells (PBMC) were isolated from HIV-1 and hepatitis B virus-seronegative donors, using a Ficoll gradient (Tianjin Haoyang Biological Manufacture Co., Ltd, China). Mice were immunologically reconstituted by intraperitoneal injection of 2.0×10^7 human PBMC (re-suspended in 0.5 ml of RPMI 1640 medium). To confirm successful engraftment, mouse were examined for human immunoglobulin production in serum by a sandwich ELISA with rabbit antihuman Ig capture antibody (I-8010; Sigma) and rabbit anti-human IgG-detecting antibody. Human IgG was used for positive control. Mice having high levels of serum human IgG were counted in the drug evaluation assay.

Drugs administration and HIV-1 infection

Solutions of sIFNα and azidothymidine (AZT) for injection were prepared in PBS, filtered through a 0.22-mm-pore-size filter, and stored at 4°C until use. PBS was as placebo compound. Two weeks after animal reconstitution, 200 μl of each compound was injected intraperitoneally (i.p.) at 2 hours before HIV infection and then either daily or every 3 days one time for two weeks after infection to each groups. The strain HIV-1 IIIB (10^3 50% tissue culture infective dosage into 200 μl), previously expanded in MT4 cells, was injected i.p.. Animal toxicity was evaluated by means of hair ruffling, weight loss and mortality at the time of mice sacrifice.

Determination of virus infection

The hu-PBL-SCID mice were sacrificed 14 days after infection; HIV was detected by cocultivation of human T cells obtained from peritoneal cavities and spleens with MT4 cells in RPMI medium with 20% fetal bovine serum (FBS) in the presence of recombinant interleukin (IL)-2 (50 U/ml).[16] Lymphoid cells from the peritoneal cavities and spleens were collected by the following methods: a two-step peritoneal lavage was performed with RPMI 1640 medium, first with 1 ml, followed by a second 4-ml wash. The recovered volume was centrifuged, and the cells were re-suspended in 1 ml of RPMI 1640 medium. Spleen was disrupted with the blunt end of a 5-ml plastic syringe plunger, the connective tissue and debris were allowed to settle and the single-cell suspensions were collected and washed twice in PBS, diluted in RPMI 1640 medium, centrifuged twice at 1200 r/min for 10 minutes and re-suspended in 1 ml of RPMI 1640 medium. Peritoneal lavage cells and spleen cells were co-cultivated with MT4 cells for 4 weeks. Every 3 days, half of co-cultivation medium was refreshed, and virus replication in culture supernatants was determined by detection of HIV P24 antigen by ELISA kit.

Statistical analysis

The effects of compounds on inhibition of HIV-1 replication were expressed mean ± standard deviation (SD) and compared using the Student's *t* test in Excel.[25] *P* value less than 0.05 was considered statistically significant.

RESULTS

sIFNα efficiently inhibits replication of HIV-1 IIIB *in vitro*

sIFNα efficiently inhibited replication of laboratory strain HIV-1 IIIB cultured in MT4 cells by MTT assay, with a 50% effective concentration (EC_{50}) of 0.06 ng/ml, while EC_{50} of the IFN-α and AZT were respectively no less than 100 ng/ml and 8 ng/ml, respectively (Figure). Thus, sIFNα displayed an obviously improved anti-HIV ability compared to common IFN-α. Moreover, sIFNα showed low toxicity *in vitro*; it did not affect the viability and proliferation of MT4 cells, even at concentrations up to 10 μg/ml (data not shown).

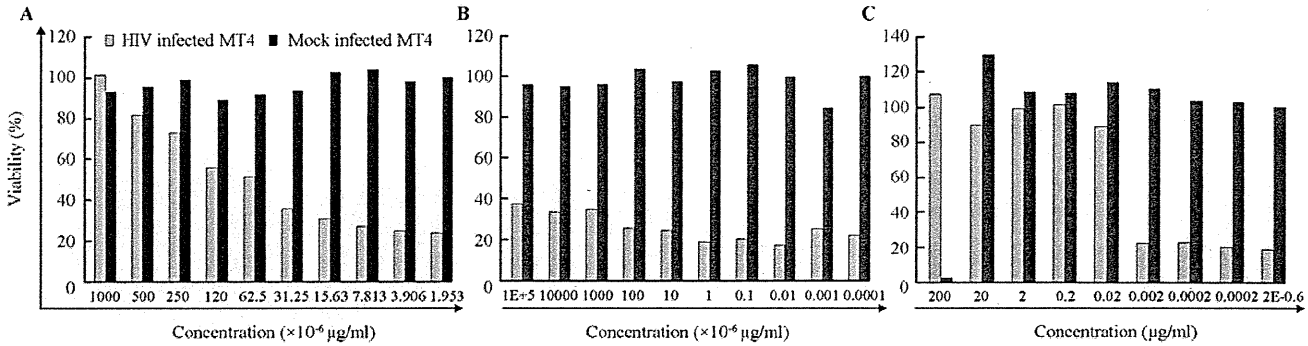

Figure. Anti-HIV effects of sIFNα (**A**), IFN-α (**B**) and AZT (**C**). MT4 cells were exposed to 100 $TCID_{50}$ of HIV-1 IIIB in the presence of various concentrations of drugs in 96-well microculture plates and incubated at 37°C for 5 days. After 5 days, the anti-HIV activity and cytotoxicities of the drugs were determined based on the viability and proliferation of MT4 cells, as determined with MTT.

sIFNα inhibits replication of HIV-1 IIIB in hu-PBL-SCID mice

For detailed evaluation of the anti-HIV efficacy of sIFNα in a small animal model, 23 hu-PBL-SCID beige mice were constructed and inoculated i.p. with the laboratory strain HIV-1 IIIB two weeks following PBMC reconstitution. All HIV-challenged humanized mice were subsequently divided into 6 groups to be administrated with various concentrations of drugs for two weeks (Table). During the two weeks of drug treatment, no clinical signs of drug toxicity in mice were observed in all drug-delivered groups. Mice were then sacrificed to determine whether the immune reconstruction of hu-PBL-SCID mice was successful, and whether the hu-PBL-SCID mice were HIV-positive.

Table. Results of HIV co-culture assay *in vitro*

Drug treatments	*n*	HIV^+ mice/mice tested (*n/N*)
PBS placebo	6	6/6
AZT dose (μg/d)	5	
100	3	3/3
2500	2	0/2
sIFNα dose (μg/d)	12	
0.45	4	3/4
1.35	4	1/4
2.25	4	0/4

Human T cells obtained from SCID mice peritoneal cavity and spleen were co-cultivated with MT4 cells in 24-wells plates in RPMI 1640 medium contained 50 U/ml recombinant IL-2 for 4 weeks. Every 3 days, medium was changed in half. Four weeks later, cultural supernatants were detected by HIV p24 ELISA assay to determine the infection of HIV.

To determine whether the mice were immunologically reconstituted successfully by human PBMC, serum human IgG were detected. All 23 mice were serum human IgG^+ by ELISA assay, proving that they were immune-reconstructed successfully. The presence of infectious HIV in the humanized mice was determined by cocultivation human cells recovered from the mice peritoneal cavity and spleen with MT4 cells *in vitro*.[16] The results indicated that, compared with the PBS-control group in which all six mice were HIV^+ (Table), the proportion of HIV-positive mice in both AZT and sIFNα groups were directly related to the dosage of drugs delivered. In the AZT treated group, all three mice treated at the dose of 100 μg once daily were HIV-positive, while the two mice treated at the dose of 2500 μg once daily were identified as HIV-negative indicating complete suppression of HIV replication. For the sIFNα treated groups, the proportions of HIV-positive mice were respectively 3/4, 1/4, 0/4 for 0.45 μg, 1.35 μg, and 2.25 μg sIFNα daily treatment, suggesting an uptrend in the protective rate. With low dosages of sIFNα, partial protection was noticed; whereas as the dosage of sIFNα increased to 2.25 μg once daily, none of four mice was HIV-positive, all the hu-PBL-SCID mice were protected completely. In conclusion, our results demonstrated that high dosage of sIFNα could protect the hu-PBL-SCID from HIV infection very well.

DISCUSSION

In the present study, we evaluated the anti-HIV activity of a new IFN-α, created by DNA shuffling. Results of MTT assay demonstrated that sIFNα had an improved anti-HIV effect compared to common IFN-α. Results of hu-PBL-SCID mice experiments further showed that appropriate doses of sIFNα could inhibit the replication of HIV in mice completely, and that sIFNα could exert its anti-viral effect effectively *in vivo*, with a potential for clinic use, alone or combined with Highly active antiretroviral therapy (HAART).

Treatment against HIV with IFN-α is reportedly most efficacious at the initial stages of HIV infection.[10,11,14] However, as the disease develops, the antiviral efficacy of IFN-α therapy diminishes dramatically. In patients progressing into AIDS, IFN-α treatment showed no effect on viral load. These observations underscore the importance of drug timing. It is not known whether the high effectiveness of sIFNα against HIV transmission we obtained in hu-PBL-SCID mice is tightly related to the sIFNα pretreatment before challenging mice with HIV IIIB. Early treatment might help block transmission of HIV in mice to some degree. Additional experiments to evaluate and define the effective timing of sIFNα treatment relative to virus exposure will be important.

It is still disputed whether treating the patients infected

with HIV with IFN-α is beneficial so far. Studies manifested high titers of IFN-α present in AIDS patients.[26-28] And, at the time of inhibition HIV-1 replication, IFN-α sometimes induces decrease in $CD4^+$ cell counts.[29-32] These phenomena make evaluate the anti-HIV effect of IFN-α more difficult. It is not known whether the widespread immune activation induced by IFN-α would favor viral replication by providing large numbers of activated target cells and by dampening the immune response, and ultimately attenuate the immunologic and clinical benefits of antiretroviral therapy and drive HIV disease progression.[27] However, the existence of drug-resistant HIV strains and co-infection of HIV and hepatitis C virus (HCV) highlight the importance of developing new IFN.

HAART helps long-term suppression of HIV-1 loads in many patients to low or undetectable levels, representing a significant advance in the treatment of AIDS.[33-35] Unfortunately, a major problem of this continual antiviral treatment is that the emergence of drug-resistant variants, which may lead to a rebound in virus load and treatment failure.[36,37] Every year, a significant proportion of patients die from AIDS with multi-resistant viruses. However, resistance selected by conventional antiretroviral drugs is unlikely to affect IFN activity on HIV. So far, in lots of conventional anti-HIV compounds, only IFN-α has not yet been found to induce drug resistance, which perhaps reflects the nonselective or pleuripotent mechanism of action of IFN-α.[33] This may be particularly useful in blocking production of multi-drug resistant viruses selected by other antiretrovirals in HIV patients.[13]

Moreover, due to shared common transmission routes, co-infection with HIV and HCV is common in China.[38-40] Reportedly, In China, HCV infection rates among HIV-1 positive injection drug users (IDUs), former blood donors (FBDs) and people who acquired HIV-1 through sexual contact are reportedly 97%, 93% and 20%, respectively.[41] HCV-related liver disease is a major cause of morbidity and mortality among HIV-infected persons. The high rate of HCV infection among HIV-1 positive patients highlights the importance of developing new anti-HIV treatment modalities. Currently, IFN-α, administered either alone or in combination with ribavirin is the only way to cure HCV infection in HIV/HCV co-infected patients.[42] However, almost all patients on treatment with interferon plus ribavirin will experience side effects such as flu-like symptoms, myalgia, sleep disturbances, asthenia, gastrointestinal disorders and depressive mood changes, which increase the risk of treatment discontinuations. Thus, developing of a novel IFN-α with higher efficacy and lower adverse effects is necessary. As a new synthesized IFN-α, sIFNα has been shown to suppress the HIV replication *in vitro* and in SCID mice; further exploring its anti-HIV mechanism and potential to inhibit HIV replication in patients infected with drug-resistant HIV strains or co-infected with HCV may offer useful new treatment modalities for HIV-1-infected people in China.

Acknowledgments: We thank Sichuan Huiyang Life-Engineering Co. for supplying sIFNα; we are grateful to all the staff in our laboratory for their help with the preparation of PBMCs.

REFERENCES

1. Lane HC. Interferons in HIV and related diseases. AIDS 1994; 8 Suppl 3: s19-s23.
2. Samuel CE. Antiviral actions of interferons. Clin Microbiol Rev 2001; 14: 778-809.
3. Brassard DL, Grace MJ, Bordens RW. Interferon-alpha as an immunotherapeutic protein. J Leukoc Biol 2002; 71: 565-581.
4. Hartshorn KL, Neumeyer D, Vogt MW, Schooley RT, Hirsch MS. Activity of interferons alpha, beta, and gamma against human immunodeficiency virus replication *in vitro*. AIDS Res Hum Retroviruses 1987; 3: 125-133.
5. Yamada O, Hattori N, Kurimura T, Kita M, Kishida T. Inhibition of growth of HIV by human natural interferon *in vitro*. AIDS Res Hum Retroviruses 1988; 4: 287-294.
6. Bednarik DP, Mosca JD, Raj NB, Pitha PM. Inhibition of human immunodeficiency virus (HIV) replication by HIV-trans-activated alpha 2-interferon. Proc Natl Acad Sci U S A 1989; 86: 4958-4962.
7. Hartshorn KL, Vogt MW, Chou TC, Blumberg RS, Byington R, Schooley RT, et al. Synergistic inhibition of human immunodeficiency virus *in vitro* by azidothymidine and recombinant alpha A interferon. Antimicrob Agents Chemother 1987; 31: 168-172.
8. Ho DD, Hartshorn KL, Rota TR, Andrews CA, Kaplan JC, Schooley RT, et al. Recombinant human interferon alfa-A suppresses HTLV-III replication *in vitro*. Lancet 1985; 1: 602-604.
9. Pitha PM. Multiple effects of interferon on HIV-1 replication. J Interferon Res 1991; 11: 313-318.
10. Rivero J, Fraga M, Cancio I, Cuervo J, Lopez-Saura P. Long-term treatment with recombinant interferon alpha-2b prolongs survival of asymptomatic HIV-infected individuals. Biotherapy 1997; 10: 107-113.
11. Rivero J, Limonta M, Aguilera A, Fraga M, Lopez Saura P. Use of recombinant interferon-alpha in human immunodeficiency virus (HIV)-infected individuals. Biotherapy 1994; 8: 23-31.
12. Hatzakis A, Gargalianos P, Kiosses V, Lazanas M, Sypsa V, Anastassopoulou C, et al. Low-dose IFN-alpha monotherapy in treatment-naive individuals with HIV-1 infection: evidence of potent suppression of viral replication. J Interferon Cytokine Res 2001; 21: 861-869.
13. Aguilar Marucco D, Veronese L, de Requena DG, Bonora S, Calcagno A, Cavecchia I, et al. Antiretroviral activity of pegylated interferon alfa-2a in patients co-infected with HIV/hepatitis C virus. J Antimicrob Chemother 2007; 59: 565-568.
14. Haas DW, Lavelle J, Nadler JP, Greenberg SB, Frame P, Mustafa N, et al. A randomized trial of interferon alpha therapy for HIV type 1 infection. AIDS Res Hum Retroviruses 2000; 16: 183-190.

15. Chevaliez S, Pawlotsky JM. Interferons and their use in persistent viral infections. Handb Exp Pharmacol 2009; (189): 203-241.
16. Mosier DE, Gulizia RJ, Baird SM, Wilson DB, Spector DH, Spector SA. Human immunodeficiency virus infection of human-PBL-SCID mice. Science 1991; 251: 791-794.
17. Boyle MJ, Connors M, Flanigan ME, Geiger SP, Ford H Jr, Baseler M, et al. The human HIV/peripheral blood lymphocyte (PBL)-SCID mouse. A modified human PBL-SCID model for the study of HIV pathogenesis and therapy. J Immunol 1995; 154: 6612-6623.
18. Ruxrungtham K, Boone E, Ford H Jr, Driscoll JS, Davey RT Jr, Lane HC. Potent activity of 2′-beta-fluoro-2′,3′-dideoxyadenosine against human immunodeficiency virus type 1 infection in hu-PBL-SCID mice. Antimicrob Agents Chemother 1996; 40: 2369-2374.
19. Lapenta C, Santini SM, Proietti E, Rizza P, Logozzi M, Spada M, et al. Type I interferon is a powerful inhibitor of *in vivo* HIV-1 infection and preserves human $CD4^+$ T cells from virus-induced depletion in SCID mice transplanted with human cells. Virology 1999; 263: 78-88.
20. Mosier DE, Gulizia RJ, MacIsaac PD, Corey L, Greenberg PD. Resistance to human immunodeficiency virus 1 infection of SCID mice reconstituted with peripheral blood leukocytes from donors vaccinated with vaccinia gp160 and recombinant gp160. Proc Natl Acad Sci U S A 1993; 90: 2443-2447.
21. McCune JM, Namikawa R, Shih CC, Rabin L, Kaneshima H. Suppression of HIV infection in AZT-treated SCID-hu mice. Science 1990; 247: 564-566.
22. McCune J, Kaneshima H, Krowka J, Namikawa R, Outzen H, Peault B, et al. The SCID-hu mouse: a small animal model for HIV infection and pathogenesis. Annu Rev Immunol 1991; 9: 399-429.
23. Naziruddin B, Shiroki R, Shishido S, Howard T, Mohanakumar T. Biochemical and functional characterization of xenoreactive natural antibodies in hu-PBL-SCID mice. J Clin Invest 1996; 97: 1267-1275.
24. Okamoto Y, Eda Y, Ogura A, Shibata S, Amagai T, Katsura Y, et al. In SCID-hu mice, passive transfer of a humanized antibody prevents infection and atrophic change of medulla in human thymic implant due to intravenous inoculation of primary HIV-1 isolate. J Immunol 1998; 160: 69-76.
25. Kodama E, Shigeta S, Suzuki T, De Clercq E. Application of a gastric cancer cell line (MKN-28) for anti-adenovirus screening using the MTT method. Antiviral Res 1996; 31: 159-164.
26. Gringeri A, Santagostino E, Cusini M, Muca-Perja M, Marinoni A, Mannucci PM, et al. Absence of clinical, virological, and immunological signs of progression in HIV-1-infected patients receiving active anti-interferon-alpha immunization: a 30-month follow-up report. J Acquir Immune Defic Syndr Hum Retrovirol 1996; 13: 55-67.
27. Tilton JC, Manion MM, Luskin MR, Johnson AJ, Patamawenu AA, Hallahan CW, et al. Human immunodeficiency virus viremia induces plasmacytoid dendritic cell activation *in vivo* and diminished alpha interferon production *in vitro*. J Virol 2008; 82: 3997-4006.
28. Soumelis V, Scott I, Gheyas F, Bouhour D, Cozon G, Cotte L, et al. Depletion of circulating natural type 1 interferon-producing cells in HIV-infected AIDS patients. Blood 2001; 98: 906-912.
29. Lane HC, Kovacs JA, Feinberg J, Herpin B, Davey V, Walker R, et al. Anti-retroviral effects of interferon-alpha in AIDS-associated Kaposi's sarcoma. Lancet 1988; 2: 1218-1222.
30. Krown SE, Gold JW, Niedzwiecki D, Bundow D, Flomenberg N, Gansbacher B, et al. Interferon-alpha with zidovudine: safety, tolerance, and clinical and virologic effects in patients with Kaposi sarcoma associated with the acquired immunodeficiency syndrome (AIDS). Ann Intern Med 1990; 112: 812-821.
31. Berglund O, Engman K, Ehrnst A, Andersson J, Lidman K, Akerlund B, et al. Combined treatment of symptomatic human immunodeficiency virus type 1 infection with native interferon-alpha and zidovudine. J Infect Dis 1991; 163: 710-715.
32. Edlin BR, Weinstein RA, Whaling SM, Ou CY, Connolly PJ, Moore JL, et al. Zidovudine-interferon-alpha combination therapy in patients with advanced human immunodeficiency virus type 1 infection: biphasic response of p24 antigen and quantitative polymerase chain reaction. J Infect Dis 1992; 165: 793-798.
33. Flexner C. HIV drug development: the next 25 years. Nat Rev Drug Discov 2007; 6: 959-966.
34. Bernstein A. AIDS and the next 25 years. Science 2008; 320: 717.
35. Fauci AS. 25 years of HIV. Nature 2008; 453: 289-290.
36. Zhang FJ, Maria A, Haberer J, Zhao Y. Overview of HIV drug resistance and its implications for China. Chin Med J 2006; 119: 1999-2004.
37. Li J, Li L, Li HP, Zhuang DM, Liu SY, Liu YJ, et al. Competitive capacity of HIV-1 strains carrying M184I or Y181I drug-resistant mutations. Chin Med J 2009; 122: 1081-1086.
38. Garten RJ, Zhang J, Lai S, Liu W, Chen J, Yu XF. Coinfection with HIV and hepatitis C virus among injection drug users in southern China. Clin Infect Dis 2005; 41 Suppl 1: s18-s24.
39. Zhang C, Yang R, Xia X, Qin S, Dai J, Zhang Z, et al. High prevalence of HIV-1 and hepatitis C virus coinfection among injection drug users in the southeastern region of Yunnan, China. J Acquir Immune Defic Syndr 2002; 29: 191-196.
40. Lu Y, Robinson M, Zhang FJ. Human immunodeficiency virus and hepatitis C virus co-infection: epidemiology, natural history and the situation in China. Chin Med J 2009; 122: 93-97.
41. Shang H, Zhong P, Liu J, Han X, Dai D, Zhang M, et al. High prevalence and genetic diversity of HCV among HIV-1 infected people from various high-risk groups in China. PLoS One 2010; 5: e10631.
42. Manns MP, Foster GR, Rockstroh JK, Zeuzem S, Zoulim F, Houghton M. The way forward in HCV treatment -- finding the right path. Nat Rev Drug Discov 2007; 6: 991-1000.

(Received September 28, 2010)
Edited by SUN Jing

Biochemical and Biophysical Research Communications 408 (2011) 615–619

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Dominant induction of vaccine antigen-specific cytotoxic T lymphocyte responses after simian immunodeficiency virus challenge

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ARTICLE INFO

Article history:
Received 12 April 2011
Available online 21 April 2011

Keywords:
AIDS vaccine
HIV
SIV
CTL
Immunodominance

ABSTRACT

Cytotoxic T lymphocyte (CTL) responses are crucial for the control of human and simian immunodeficiency virus (HIV and SIV) replication. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. We previously developed a CTL-inducing vaccine and showed SIV control in some vaccinated rhesus macaques. These vaccine-based SIV controllers elicited vaccine antigen-specific CTL responses dominantly in the acute phase post-challenge. Here, we examined CTL responses post-challenge in those vaccinated animals that failed to control SIV replication. Unvaccinated rhesus macaques possessing the major histocompatibility complex class I haplotype *90-088-Ij* dominantly elicited SIV non-Gag antigen-specific CTL responses after SIV challenge, while those induced with Gag-specific CTL memory by prophylactic vaccination failed to control SIV replication with dominant Gag-specific CTL responses in the acute phase, indicating dominant induction of vaccine antigen-specific CTL responses post-challenge even in non-controllers. Further analysis suggested that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses post-viral exposure but delays SIV non-vaccine antigen-specific CTL responses. These results imply a significant influence of prophylactic vaccination on CTL immunodominance post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

1. Introduction

In human and simian immunodeficiency virus (HIV and SIV) infections, cytotoxic T lymphocyte (CTL) responses exert strong suppressive pressure on viral replication but fail to control viremia leading to AIDS progression [1–5]. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. It is important to determine how prophylactic CTL memory induction affects CTL responses in the acute phase post-viral exposure.

We previously developed a prophylactic AIDS vaccine (referred to as DNA/SeV-Gag vaccine) consisting of DNA priming followed by boosting with a recombinant Sendai virus (SeV) vector expressing SIVmac239 Gag [6]. Evaluation of this vaccine's efficacy against a SIVmac239 challenge in Burmese rhesus macaques showed that some vaccinees contained SIV replication [7]. In particular, vaccination consistently resulted in SIV control in those animals possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [8]; $Gag_{206-216}$ (IINEEAADWDL) and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses were shown to be responsible for this vaccine-based SIV control [9]. Furthermore, in a SIVmac239 challenge experiment of *90-120-Ia*-positive macaques that received a prophylactic DNA/SeV vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), all the vaccinees controlled SIV replication [10]. This single epitope vaccination resulted in dominant $Gag_{241-249}$-specific CTL responses with delayed $Gag_{206-216}$-specific CTL induction after SIV challenge, whereas $Gag_{206-216}$-specific and

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doi:10.1016/j.bbrc.2011.04.071

$Gag_{241-249}$-specific CTL responses were detected equivalently in unvaccinated *90-120-Ia*-positive animals.

These previous results in vaccine-based SIV controllers indicate dominant induction of vaccine antigen-specific CTL responses post-challenge, implying that prophylactic vaccination inducing vaccine antigen-specific CTL memory may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as non-vaccine antigens) post-viral exposure. In these SIV controllers, the reduction of viral loads could be involved in delay of SIV non-vaccine antigen-specific CTL responses. Then, in the present study, we examined the influence of prophylactic vaccination on immunodominance post-challenge in those vaccinees that failed to control SIV replication. Our results showed dominant induction of vaccine antigen-specific CTL responses post-challenge even in these SIV non-controllers.

2. Materials and methods

2.1. Animal experiments

The first set of experiment used samples in our previous experiments of six Burmese rhesus macaques (Macaca mulatta) possessing the MHC-I haplotype *90-088-Ij* (macaques R02-004, R02-001, and R03-015, previously reported [7,11]; R04-014, R06-022, and R04-011, unpublished). Three of them, R02-001, R04-011, and R03-015, received a prophylactic DNA/SeV-Gag vaccine [7]. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian–human immunodeficiency virus $SHIV_{MD14YE}$ [12] molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV chimeric Vpr, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 6×10^9 cell infectious units (CIUs) of F-deleted replication-defective SeV-Gag [13,14]. All six *90-088-Ij*-positive animals including three unvaccinated and three vaccinated were challenged intravenously with 1000 50% tissue culture infective doses (TCID50) of SIVmac239 [15] approximately 3 months after the boost. At week 1 after SIV challenge, macaque R03-015 was inoculated with nonspecific immunoglobulin G as previously described [11].

In the second set of experiment, unvaccinated (R06-001) and vaccinated (R05-028) rhesus macaques possessing the MHC-I haplotype *90-120-Ib* were challenged intravenously with 1000 TCID50 of SIVmac239. The latter R05-028 were immunized intranasally with F-deleted SeV-Gag approximately 3 months before the challenge.

In the third, three rhesus macaques received FMSIV plus mCAT1-expressing DNA vaccination three times with intervals of 4 weeks. The FMSIV DNA was constructed by replacing *nef*-deleted $SHIV_{MD14YE}$ with Friend murine leukemia virus (FMLV) *env*, carrying the same SIVmac239-derived antigen-coding regions with SIVGP1, as described before [16]. Vaccination of macaques with FMSIV and a DNA expressing the FMLV receptor (mCAT1) [17] three times with intervals of a week was previously shown to induce mCAT1-dependent confined FMSIV replication resulting in efficient CTL induction while vaccination three times with intervals of 4 weeks in the present study resulted in marginal levels of responses (data not shown). These three DNA-vaccinated animals were challenged intravenously with 1000 TCID50 of SIVmac239 approximately 2 months after the last vaccination.

Some animal experiments were conducted in the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates, in accordance with the guidelines for animal experiments at the National Institute of Infectious Diseases, and others were in Institute for Virus Research, Kyoto University in accordance with the institutional regulations.

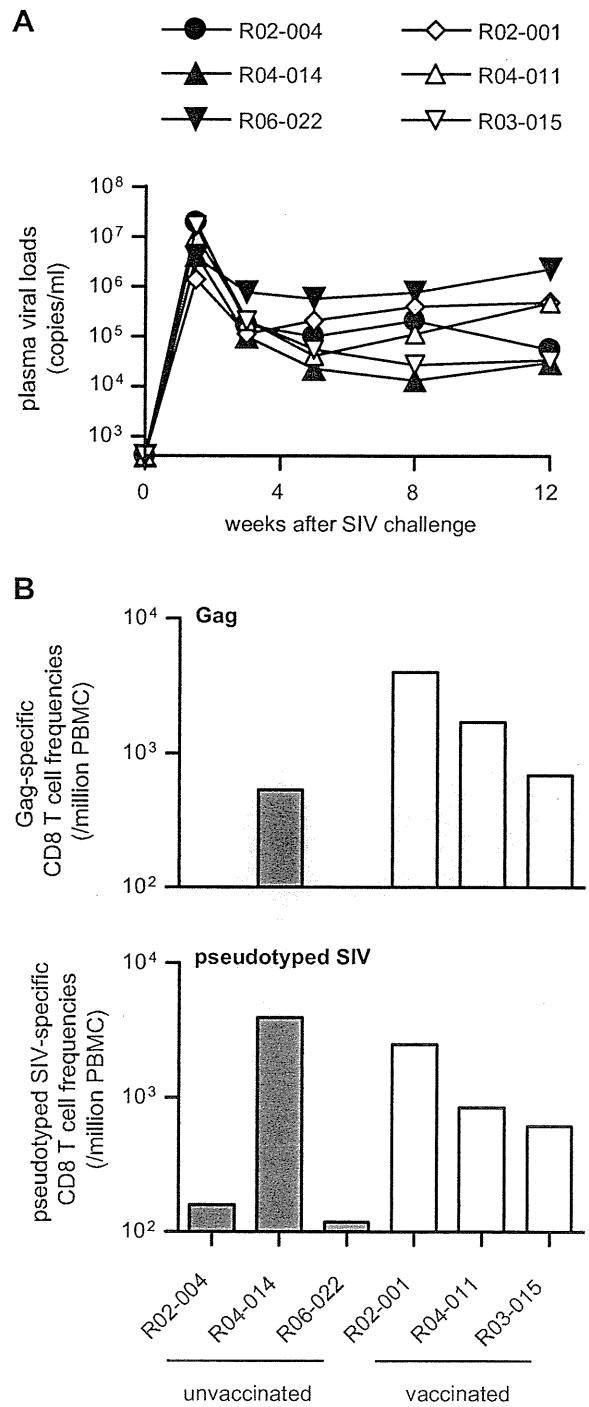

Fig. 1. CTL responses after SIVmac239 challenge in *90-088-Ij*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated (R02-004, R04-014, and R06-022) and DNA/SeV-Gag vaccinated animals (R02-001, R04-011, and R03-015). The viral loads (SIV gag RNA copies/ml) were determined as described previously [7]. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at week 2 after SIV challenge.

2.2. Analysis of virus-specific CTL responses

We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific

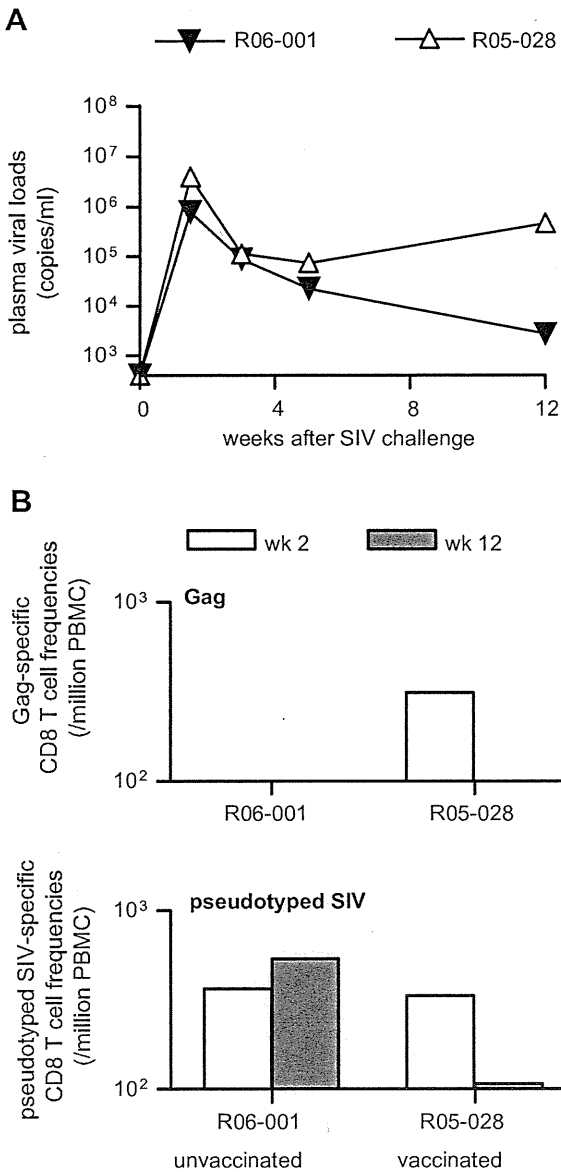

Fig. 2. CTL responses after SIVmac239 challenge in *90–120-Ib*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated R06–001 and SeV-Gag-vaccinated macaque R05-028. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge.

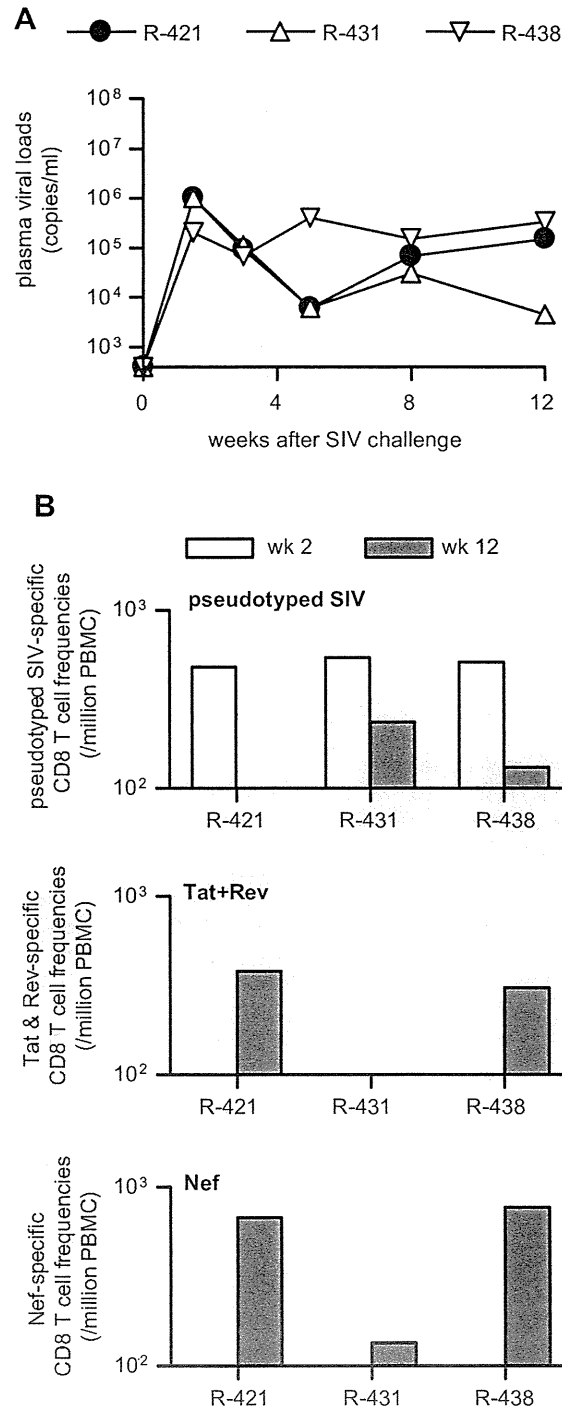

Fig. 3. CTL responses after SIVmac239 challenge in DNA-vaccinated macaques. The DNA used for the vaccination has the SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx and is expected to induce pseudotyped SIV-specific CTL responses. (A) Plasma viral loads after SIV challenge in DNA vaccinated macaques R-421, R-431, and R-438. (B) Vaccine antigen (pseudotyped SIV)-specific (top panel), Tat-plus-Rev-specific (middle panel), and Nef-specific $CD8^+$ T cell frequencies (bottom panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge. In macaque R-438, CTL responses at week 5 instead of week 12 are shown.

stimulation as described previously [18,19]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIV for pseudotyped SIV-specific stimulation. The pseudotyped SIV was obtained by cotransfection of COS-1 cells with a VSV-G-expression plasmid and SIVGP1 DNA. Alternatively, PBMCs were cocultured with B-LCLs pulsed with peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Tat, Rev, and Nef amino acid sequences. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ monoclonal antibodies (Becton Dickinson). Specific $CD8^+$ T-cell levels were calculated by subtracting nonspecific IFN-γ^+ $CD8^+$ T-cell frequencies from those after Gag-specific, pseudotyped

	vaccine antigen					non-vaccine antigen										
	Gag				Vif	Vpr	Tat					Rev		Nef		
	165	333	375	376	143	73	23	115	120	122	125	45	50	63	100	124
wk 5																
R- 421					++											
R- 431					+											
R- 438	++		+							++						
wk 12																
R- 421		++			++				+		+	+	+			++
R- 431					+		+			++						
R- 438	++			++		+		++						++	++	

Fig. 4. Viral mutations in DNA-vaccinated macaques. Plasma viral genome sequencing was performed as described previously [18] to determine mutations resulting in amino acid substitutions in SIV Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef antigens (except for Env) at weeks 5 and 12 in DNA-vaccinated macaques. The amino acid positions showing mutant sequences dominantly (++) or equivalently with wild type (+) are shown. While we found a mutation leading to a lysine-to-arginine alteration at the 40th amino acid in Rev in all animals, this mutation is not shown because the wild-type sequence at this position in the SIVmac239 molecular clone is considered to be a suboptimal nucleotide that frequently reverts to an alternative sequence in vivo [18,23].

SIV-specific, or peptide-specific stimulation. Specific $CD8^+$ T-cell levels lower than 100 per million PBMCs were considered negative.

3. Results and discussion

In our previous SIVmac239 challenge experiments, the prophylactic DNA/SeV-Gag vaccination did not result in viral control in rhesus macaques possessing the MHC-I haplotype *90-088-Ij*. These vaccinated animals showed similar levels of plasma viral loads as those in unvaccinated *90-088-Ij*-positive animals after SIV challenge (Fig. 1A). Analysis of virus-specific $CD8^+$ T-cell responses using PBMCs at week 2 after challenge showed equivalent Gag-specific and pseudotyped SIV-specific (Gag-, Pol-, Vif-, and Vpx-specific) CTL responses in all three vaccinees (Fig. 1B). Pseudotyped SIV-specific CTL responses were also detected in all three unvaccinated animals, but Gag-specific CTL responses were undetectable in two out of the three; even the Gag-specific CTL responses detected in macaque R04-014 were much lower than pseudotyped SIV-specific CTL responses, indicating dominant induction of CTL responses specific for SIV antigens other than Gag (Fig. 1B). Thus, in the acute phase of SIV infection, SIV non-Gag antigen-specific CTL responses were dominantly induced in unvaccinated *90-088-Ij*-positive macaques, whereas vaccine antigen (Gag)-specific CTL responses were dominant in *90-088-Ij*-positive vaccinees.

We then analyzed another vaccinees that failed to control a SIVmac239 challenge; these macaques were vaccinated with SeV-Gag alone or DNA alone. First, we compared post-challenge CTL responses in unvaccinated and SeV-Gag-vaccinated macaques possessing the MHC-I haplotype *90-120-Ib*. Both macaques failed to control SIV replication after challenge (Fig. 2A). In the unvaccinated animal R06-001, Gag-specific CTL responses were undetectable but pseudotyped SIV-specific CTL responses were induced efficiently at weeks 2 and 12 (Fig. 2B). In contrast, Gag-specific CTL responses were induced efficiently at week 2 in the SeV-Gag-vaccinated animal R05-028 (Fig. 2B). At week 12, Gag-specific CTL responses became undetectable while pseudotyped SIV-specific CTL responses were still detectable in this animal. These results indicate that, in the acute phase after SIVmac239 challenge, the unvaccinated *90-120-Ib*-positive macaque dominantly elicited SIV non-Gag antigen-specific CTL responses whereas the SeV-Gag-vaccinated *90-120-Ib*-positive macaque dominantly induced vaccine antigen (Gag)-specific CTL responses.

Next, we analyzed post-challenge CTL responses in three DNA-vaccinated macaques. These animals failed to control SIVmac239 replication after challenge (Fig. 3A). The DNA used for the vaccination and the pseudotyped SIV genome both have the same SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx, thus expected to induce pseudotyped SIV-specific CTL responses. Pseudotyped SIV-specific CTL responses, namely vaccine antigen-specific CTL responses, were induced efficiently at week 2 but diminished after that in all three animals (Fig. 3B). In contrast, Tat/Rev- and Nef-specific CTL responses were undetectable at week 2 but induced later (Fig. 3B). Again, vaccine antigen-specific CTL responses were dominantly induced in the acute phase after SIV challenge and non-vaccine antigen-specific CTL responses were elicited later.

All three animals showed viral genome mutations leading to amino acid substitutions in Gag or Vif at week 5 (Fig. 4). Further analysis indicated that viral mutations in vaccine antigen-coding regions appeared earlier than those in other regions. These results may reflect selective pressure on SIV by vaccine antigen-specific CTL responses dominantly induced in the acute phase, although it remains undetermined whether these mutations are CTL escape ones. Disappearance of vaccine antigen-specific CTL responses at week 12 may be explained by rapid selection of CTL escape mutations in vaccine antigen-coding regions. However, analysis using peptides found Gag-specific CTL responses in macaques R-421 and R-431 that had no gag mutations at week 5 (data not shown), suggesting involvement of immunodominance [20] in the disappearance of vaccine antigen-specific CTL responses at week 12.

In summary, the present study indicates that vaccine antigen-specific CTL responses are induced dominantly in the acute phase after viral exposure, with delayed induction of CTL responses specific for SIV non-vaccine antigens (SIV antigens other than vaccine antigens). While this delay previously-observed in vaccine-based SIV controllers [10] can be explained not only by immunodominance but also by reduction in viral loads, the delay in vaccinated non-controllers in the present study might reflect the immunodominance in CTL responses. Thus, in development of a prophylactic, CTL-inducing AIDS vaccine, it is important to select vaccine antigens leading to effective CTL responses post-viral

exposure [21,22]. These results imply a significant influence of prophylactic vaccination on the immunodominance pattern of CTL responses post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

Acknowledgments

This work was supported by Grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, Grants-in-aid from the Ministry of Health, Labor, and Welfare, and a Grant from Takeda Science Foundation in Japan.

References

[1] R.A. Koup, J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, D.D. Ho, Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome, J. Virol. 68 (1994) 4650–4655.
[2] P. Borrow, H. Lewicki, B.H. Hahn, G.M. Shaw, M.B. Oldstone, Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection, J. Virol. 68 (1994) 6103–6110.
[3] T. Matano, R. Shibata, C. Siemon, M. Connors, H.C. Lane, M.A. Martin, Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques, J. Virol. 72 (1998) 164–169.
[4] X. Jin, D.E. Bauer, S.E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C.E. Irwin, J.T. Safrit, J. Mittler, L. Weinberger, L.G. Kostrikis, L. Zhang, A.S. Perelson, D.D. Ho, Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques, J. Exp. Med. 189 (1999) 991–998.
[5] P.J. Goulder, D.I. Watkins, HIV and SIV CTL escape: implications for vaccine design, Nat. Rev. Immunol. 4 (2004) 630–640.
[6] T. Matano, M. Kano, H. Nakamura, A. Takeda, Y. Nagai, Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen, J. Virol. 75 (2001) 11891–11896.
[7] T. Matano, M. Kobayashi, H. Igarashi, A. Takeda, H. Nakamura, M. Kano, C. Sugimoto, K. Mori, A. Iida, T. Hirata, M. Hasegawa, T. Yuasa, M. Miyazawa, Y. Takahashi, M. Yasunami, A. Kimura, D.H. O'Connor, D.I. Watkins, Y. Nagai, Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial, J. Exp. Med. 199 (2004) 1709–1718.
[8] Y. Takahashi-Tanaka, M. Yasunami, T. Naruse, K. Hinohara, T. Matano, K. Mori, M. Miysazawa, M. Honda, Y. Yasutomi, Y. Nagai, A. Kimura, Reference strand-mediated conformation analysis (RSCA)-based typing of multiple alleles in the rhesus macaque MHC class I Mamu-A and Mamu-B loci, Electrophoresis 28 (2007) 918–924.
[9] M. Kawada, T. Tsukamoto, H. Yamamoto, N. Iwamoto, K. Kurihara, A. Takeda, C. Moriya, H. Takeuchi, H. Akari, T. Matano, Gag-specific cytotoxic T lymphocyte-based control of primary simian immunodeficiency virus replication in a vaccine trial, J. Virol. 82 (2008) 10199–10206.
[10] T. Tsukamoto, A. Takeda, T. Yamamoto, H. Yamamoto, M. Kawada, T. Matano, Impact of cytotoxic-T-lymphocyte memory induction without virus-specific CD4+ T-Cell help on control of a simian immunodeficiency virus challenge in rhesus macaques, J. Virol. 83 (2009) 9339–9346.
[11] H. Yamamoto, M. Kawada, A. Takeda, H. Igarashi, T. Matano, Post-infection immunodeficiency virus control by neutralizing antibodies, PLoS ONE 2 (2007) e540.
[12] R. Shibata, F. Maldarelli, C. Siemon, T. Matano, M. Parta, G. Miller, T. Fredrickson, M.A. Martin, Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing, J. Infect. Dis. 176 (1997) 362–373.
[13] H.O. Li, Y.F. Zhu, M. Asakawa, H. Kuma, T. Hirata, Y. Ueda, Y.S. Lee, M. Fukumura, A. Iida, A. Kato, Y. Nagai, M. Hasegawa, A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression, J. Virol. 74 (2000) 6564–6569.
[14] A. Takeda, H. Igarashi, H. Nakamura, M. Kano, A. Iida, T. Hirata, M. Hasegawa, Y. Nagai, T. Matano, Protective efficacy of an AIDS vaccine, a single DNA priming followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model, J. Virol. 77 (2003) 9710–9715.
[15] H.W. Kestler 3rd, D.J. Ringler, K. Mori, D.L. Panicali, P.K. Sehgal, M.D. Daniel, R.C. Desrosiers, Importance of the nef gene for maintenance of high virus loads and for development of AIDS, Cell 65 (1991) 651–662.
[16] T. Matano, M. Kano, T. Odawara, H. Nakamura, A. Takeda, K. Mori, T. Sato, Y. Nagai, Induction of protective immunity against pathogenic simian immunodeficiency virus by a foreign receptor-dependent replication of an engineered avirulent virus, Vaccine 18 (2000) 3310–3318.
[17] L.M. Albritton, L. Tweng, D. Scadden, J.M. Cunningham, A putative murine retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection, Cell 57 (1989) 659–666.
[18] M. Kawada, T. Tsukamoto, H. Yamamoto, A. Takeda, H. Igarashi, D.I. Watkins, T. Matano, Long-term control of simian immunodeficiency virus replication with central memory CD4+ T-cell preservation after nonsterile protection by a cytotoxic T-lymphocyte-based vaccine, J. Virol. 81 (2007) 5202–5211.
[19] N. Iwamoto, T. Tsukamoto, M. Kawada, A. Takeda, H. Yamamoto, H. Takeuchi, T. Matano, Broadening of CD8+ cell responses in vaccine-based simian immunodeficiency virus controllers, AIDS 24 (2010) 2777–2787.
[20] S. Tenzer, E. Wee, A. Burgevin, G. Stewart-Jones, L. Friis, K. Lamberth, C.H. Chang, M. Harndahl, M. Weimershaus, J. Gerstoft, N. Akkad, P. Klenerman, L. Fugger, E.Y. Jones, A.J. McMichael, S. Buus, H. Schild, P. van Endert, A.K. Iversen, Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance, Nat. Immunol. 10 (2009) 636–646.
[21] P.J.R. Goulder, D.I. Watkins, Impact of MHC class I diversity on immune control of immunodeficiency virus replication, Nat. Rev. Immunol. 8 (2008) 619–630.
[22] H. Streeck, J.S. Jolin, Y. Qi, B. Yassine-Diab, R.C. Johnson, D.S. Kwon, M.M. Addo, C. Brumme, J.P. Routy, S. Little, H.K. Jessen, A.D. Kelleher, F.M. Hecht, R.P. Sekaly, E.S. Rosenberg, B.D. Walker, M. Carrington, M. Altfeld, Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells, J. Virol. 83 (2009) 7641–7648.
[23] L. Alexander, L. Denekamp, S. Czajak, R.C. Desrosiers, Suboptimal nucleotides in the infectious, pathogenic simian immunodeficiency virus clone SIVmac239, J. Virol. 75 (2001) 4019–4022.

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Measuring Enzymatic HIV-1 Susceptibility to Two Reverse Transcriptase Inhibitors as a Rapid and Simple Approach to HIV-1 Drug-Resistance Testing

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Abstract

Simple and cost-effective approaches for HIV drug-resistance testing are highly desirable for managing increasingly expanding HIV-1 infected populations who initiate antiretroviral therapy (ART), particularly in resource-limited settings. Non-nucleoside reverse trancriptase inhibitor (NNRTI)-based regimens with an NRTI backbone containing lamivudine (3TC) or emtricitabine (FTC) are preferred first ART regimens. Failure with these drug combinations typically involves the selection of NNRTI- and/or 3TC/FTC- resistant viruses. Therefore, the availability of simple assays to measure both types of drug resistance is critical. We have developed a high throughput screening test for assessing enzymatic resistance of the HIV-1 RT in plasma to 3TC/FTC and NNRTIs. The test uses the sensitive "Amp-RT" assay with a newly-developed real-time PCR format to screen biochemically for drug resistance in single reactions containing either 3TC-triphosphate (3TC-TP) or nevirapine (NVP). Assay cut-offs were defined based on testing a large panel of subtype B and non-subtype B clinical samples with known genotypic profiles. Enzymatic 3TC resistance correlated well with the presence of M184I/V, and reduced NVP susceptibility was strongly associated with the presence of K103N, Y181C/I, Y188L, and G190A/Q. The sensitivity and specificity for detecting resistance were 97.0% and 96.0% in samples with M184V, and 97.4% and 96.2% for samples with NNRTI mutations, respectively. We further demonstrate the utility of an HIV capture method in plasma by using magnetic beads coated with CD44 antibody that eliminates the need for ultracentifugation. Thus our results support the use of this simple approach for distinguishing WT from NNRTI- or 3TC/FTC-resistant viruses in clinical samples. This enzymatic testing is subtype-independent and can assist in the clinical management of diverse populations particularly in resource-limited settings.

Citation: Hoffmann D, Garcia AD, Harrigan PR, Johnston ICD, Nakasone T, et al. (2011) Measuring Enzymatic HIV-1 Susceptibility to Two Reverse Transcriptase Inhibitors as a Rapid and Simple Approach to HIV-1 Drug-Resistance Testing. PLoS ONE 6(7): e22019. doi:10.1371/journal.pone.0022019

Editor: Clive M. Gray, University of Cape Town, South Africa

Received March 17, 2011; **Accepted** June 12, 2011; **Published** July 20, 2011

Funding: This study was supported by intramural CDC funds. Dieter Hoffmann was partially supported by an International Emerging Infectious Diseases Fellowship from CDC. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding was received for this study.

Competing Interests: JGGL and WH are named on patents related to the Amp-RT assay (US patents 6787126, 7,691,572, 5849494, 6136534). Miltenyi Biotec played no role in the design and execution of the study.

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Introduction

Antiretroviral therapy has significantly improved life expectancy and quality of life in persons living with HIV [1]. Currently there are 24 antiretroviral drugs approved by the US Food and Drug Administration (FDA) for the treatment of HIV-1-infected persons, including 11 reverse transcriptase (RT) inhibitors, 10 protease inhibitors, 1 fusion inhibitor, 1 entry inhibitor, and 1 integrase inhibitor. The selection of a combination regimen that maximally suppresses virus replication is critical for treatment success, since persistent virus replication due to suboptimal therapy may result in the selection of viruses carrying drug-resistance mutations. The emergence of drug-resistant viruses can be one of the most important factors leading to therapy failure [2]. Accumulating data from various retrospective and prospective studies support the use of drug-resistance testing in many clinical situations, and several agencies and expert panels such as the IAS-USA Panel [3], the EuroGuidelines Group for HIV Resistance [4], and the U.S. Department of Health and Human Services (http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf) recommend drug-resistance testing for the management of antiretroviral therapy.

NNRTI-based ART regimens containing efavirenz (EFV) or nevirapine (NVP) are frequently used in first regimens worldwide. These regimens typically include a nucleoside RT inhibitor backbone containing either lamivudine (3TC) or the closely related emtricitabine (FTC). Resistance to 3TC/FTC is primarily associated with mutations at position 184 of the HIV-1 RT, in which the wild-type (WT) Methionine (M) is frequently replaced by Valine (V) and less commonly by Isoleucine (I). The presence of the M184V mutation results in >100-fold decreased susceptibility to both drugs [5,6]. EFV and NVP have overlapping resistance profiles conferred by a number of mutations. K103N and Y188L confer high-level resistance to NVP and EFV, while Y181C/I/V