and nine genetic subtypes (A to D, F to H, J, and K) within major group (M) (Fig. 1, Table 3).[6] The vast majority of HIV-1 strains belong to group M (for Major), which is the pathogen responsible for the current pandemic. Group O (for Outlier) consists of a pool of highly divergent, genetically related strains with no defined clades (Table 1).[7-10] Group O *infections* are limited to people living in Central Africa (mainly Cameroon and some neighbouring countries), but even in this area they represent a small minority of HIV-1 infections. Only a few cases of group N (for New, or non-M/non-O) infections have been identified, and these were in patients from Cameroon.[11]

Human immunodeficiency virus-1 group M viruses are further classified into nine different subtypes (A-D, F-H, J, K) (Fig. 1). Within some subtypes, further phylogenetic structure was identified, leading to a classification into sub-subtypes. The subtype F is subdivided into two subsubtypes, F1 and F2, and subsubtype A2 strains was identified within subtype A (Fig. 1). The subtypes B and D are better considered as subsubtypes within a single subtype, however they have been designated as subtypes for historical reasons. The amino acid distances among different subtypes of HIV-1 group M reach approximately 25–30% in the env gene sequence and 15% in the gag gene sequence.

It was determined that certain HIV-1 strains clustered with different subtypes in different regions of their genomes. Some of these mosaic HIV-1 genomes have been identified in several, apparently unlinked, individuals and play a major role in the global AIDS pandemic. These mosaic HIV-1 genomes are known as 'circulating recombinant forms' (CRFs).[12] A total of 15 CRFs are currently recognized (Fig. 1 and Table 4).[3] The recombinant structures of selected CRFs with their global distributions are shown in Figure 3. Under new nomenclature proposals, each CRF is designated by an identifying number, with letters indicating the subtypes involved. If the genome contains sequences originating from more than two subtypes, the letters are replaced by 'cpx', denoting 'complex'. To define a new subtype, subsubtype or CRF, the representative strains must be identified in at least three epidemiologically unlinked individuals. Three near full-length genomic sequences are preferred, but two complete genomes with partial sequences of a third strain are sufficient to designate a new subtype, subsubtype or CRF.

Besides CRFs, at least 30 other 'unique' recombinant forms (URFs) of HIV-1 have been identified, currently without evidence of epidemic spread.[13] Most URFs are detected in regions where multiple subtypes cocirculate, such as in Africa (A/D), India (A/C),[14] Thailand (CRF01_AE/B),[13] and Myanmar (various combination between subtypes B and C and CRF01_AE),[15-17] and China (B/C)[18,19] (Fig. 1).

Table 1 Global summary of human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) among women and children (aged <15 years)*

		Daily rates
Number of people living with HIV/AIDS		
Total	40 million	
Adults	37.2 million	
Women	17.6 million	
Children <15 years	2.7 million	
People newly infected with HIV in 2001		
Total	5 million	13700/day
Adults	4.3 million	11780/day
Women	1.8 million	4900/day
Children <15 years	800 000	2190/day
AIDS deaths in 2001		
Total	3 million	8220/day
Adults	2.4 million	6600/day
Women	1.1 million	3000/day
Children <15 years	580 000	1590/day

Daily rates represent the estimated incidence of new infection or AIDS deaths per day. *Source: UNAIDS/WHO. 2001. AIDS epidemic update December 2001.

Table 2 UNAIDS estimate of human immunodeficiency virus (HIV) infections among children (aged <15 years) in different geographic regions in 2001*

Region	No. HIV/AIDS cases as at end of 2001	No. new infections in 2001‡	No. AIDS deaths in 2001‡
Sub-Saharan Africa	2.4 million	700 000	500,000
South and South-east Asia	200 000	65 000	40,000
Latin America	40 000	10 000	8,000
Caribbean	20 000	6000	5,000
North Africa & Middle East	20 000	12 000	6,000
Eeastern Europe & Central Asia	15 000	1000	<100
North America	10 000	<500	<100
East Asia and Pacific	7000	3000	1,500
Western Europe	4000	<500	<100
Australia & New Zealand	<200	<100	<100
Total	2.7 million	800 000	580 000

*Source: UNAIDS/WHO. 2001. AIDS epidemic update December 2001. ‡Estimated annual incidence of new infection or AIDS death.

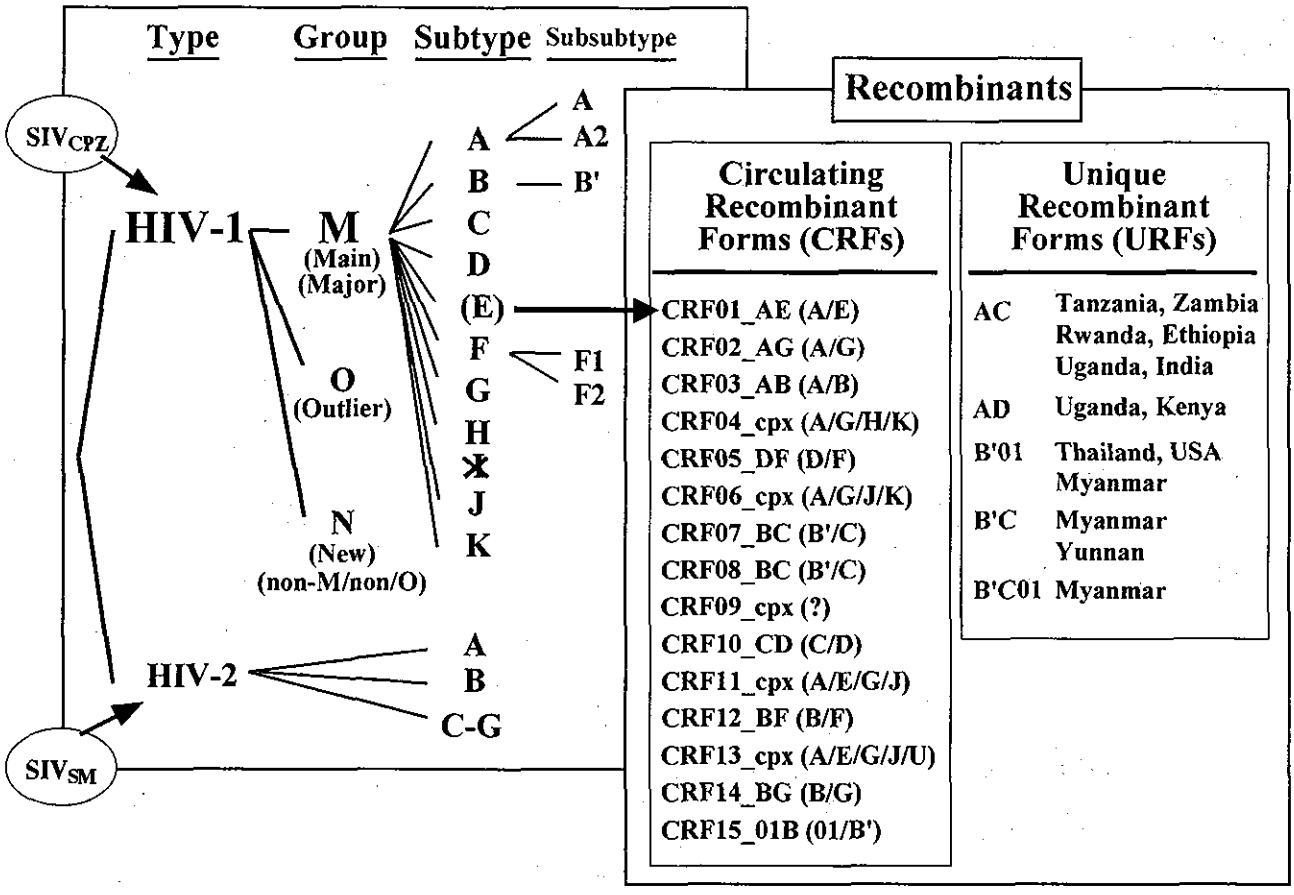

Fig. 1 Classification of human immunodeficiency viruses (HIV). HIV types, HIV-1 groups, subtypes, subsubtypes are shown. HIV-1 recombinants are categorized into two class: circulating recombinant forms (CRFs) and unique recombinant forms (URFs).

Table 3 Classification and global distribution of human immunodeficiency virus (HIV)

HIV type	Group	Subtype	Global Distribution
HIV-1	Group M	A	East and Central Africa
		B	North and South America, Europe, Asia, Oceania
		C	South and East Africa, India, Brazil
		D	Central Africa
		F	Central Africa, Romania, Latin America
		G	Central Africa, Taiwan, Russia
		H	Central Africa, Belgium
		J	Congo, Gambia, Sweden
		K	Cameroon
	Group O		Cameroon, Gabon, France
	Group N		Cameroon
HIV-2	A–G		West Africa, Portugal, Spain, Germany, France, Sweden, UK, USA, India, Korea

Table 4 Distribution of circulating recombinant forms (CRFs) of HIV-1 Group M

CRFs	Region
CRF01_AE	Asia, Central Africa
CRF02_AG	West and Central Africa
CRF03_AB	IDUs in Kaliningrad, Russia and Ukraine
CRF04_cpx	Cyprus/Greece
CRF05_DF	Belgium, Congo
CRF06_cpx	Burkina Faso, Mali
CRF07_BC	Northwest China (Xinjiang)
CRF08_BC	Southeast China (Guangxi)
CRF09_BC	Senegal, USA
CRF10_CD	Tanzania
CRF11_cpx	Greece, Congo
CRF12_BF	Latin America
CRF13_cpx	Cameroon
CRF14_BG	Spain
CRF15_BF	Thailand

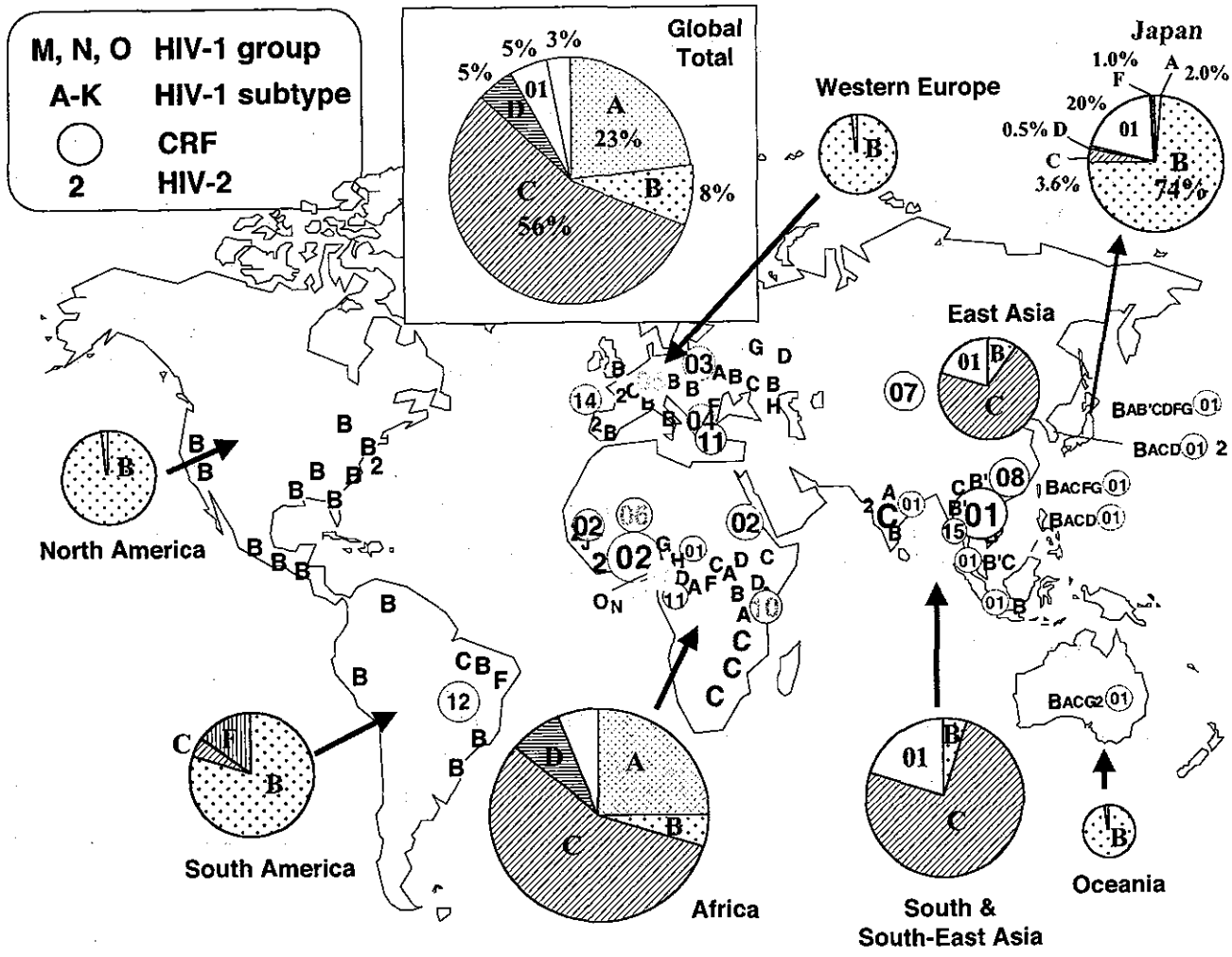

Fig. 2 Global distribution of human immunodeficiency virus (HIV) genotypes. Designations for types, HIV-1 groups, subtypes and circulating recombinant forms are shown in the inset. Genotype distribution illustrated in pie graphs is based on the data from Osmanov *et al.*[20]

Worldwide distribution of human immunodeficiency virus variants

On a global scale, the most prevalent HIV-1 genotypes are subtypes C (47%), A (27.2%), B (12.3%), D (5.3%) and CRF01_AE (3.2%).[20] The greatest genetic diversity of HIV-1 was found in Central sub-Saharan Africa. Subtype A and C are the most common in these areas, but all groups and subtypes have been identified (Fig. 2). This is consistent with the hypothesis that Africa is the source of the current pandemic. Subtype C is the predominant subtype in south and east Africa, which has the worst epidemic with more than 30% of the adult population infected with HIV.[21] In West and West-central Africa, the majority of circulating strains is CRF02_AG.[12] In North America and Europe, subtype B is predominant, showing a strong founder effect. In South America, subtype B is prevalent, while subtypes F and C, and CRF12_BF and the related B/F recombinants have been reported.[22] In Asia, subtype C predominates in India and CRF01_AE is predominant in South-east Asia.[14,23–25] Subtype B′ (Thailand variant of subtype B) is a unique subtype B regional variant that spread primarily through injecting drug user (IDU) networks in South-east Asia.[16,23,24,26,27] Two closely related CRFs, CRF07_BC and CRF08_BC are disseminating rapidly among IDU networks in North-western (Xinjiang Province) and South-eastern (Guangxi Province) China, respectively.[28,29] Injecting drug use triggered a new HIV-1 epidemic in Eastern Europe: CRF03_AB was identified among IDUs in Kaliningrad, and in cities in Ukraine and Belarus.[30]

Although the exact prevalence of recombinant strains is not known, preliminary data show that the proportions of discordant gag/env samples varied from less than 10% to up to 40% in Africa and 10–30% in some areas in Asia, including Central Myanmar and Western part of Yunnan Province of China.[15–18,31–33]

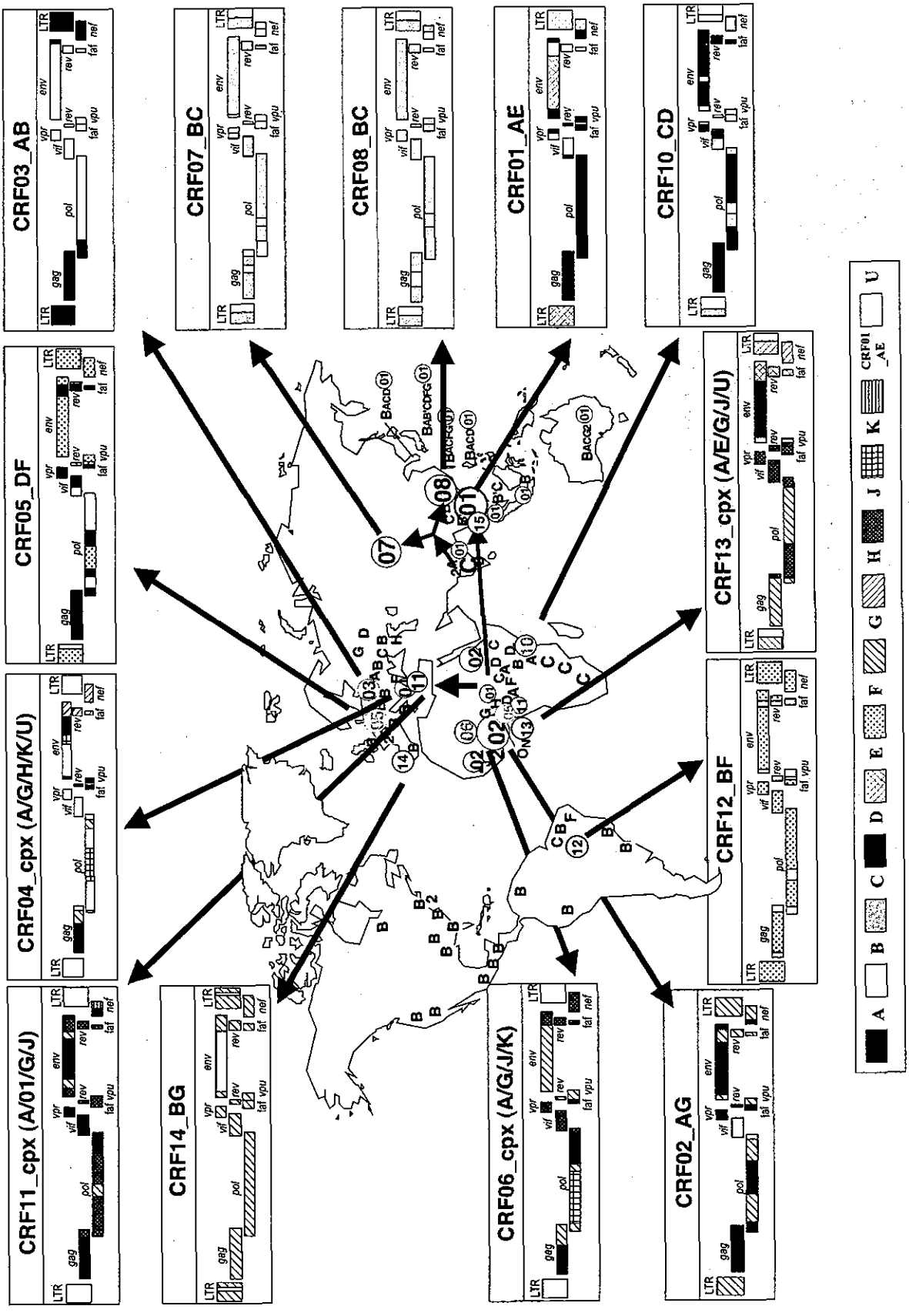

Fig. 3 Recombinant structure of selected circulating recombinant forms (CRF01 through 14) and their geographic distribution.

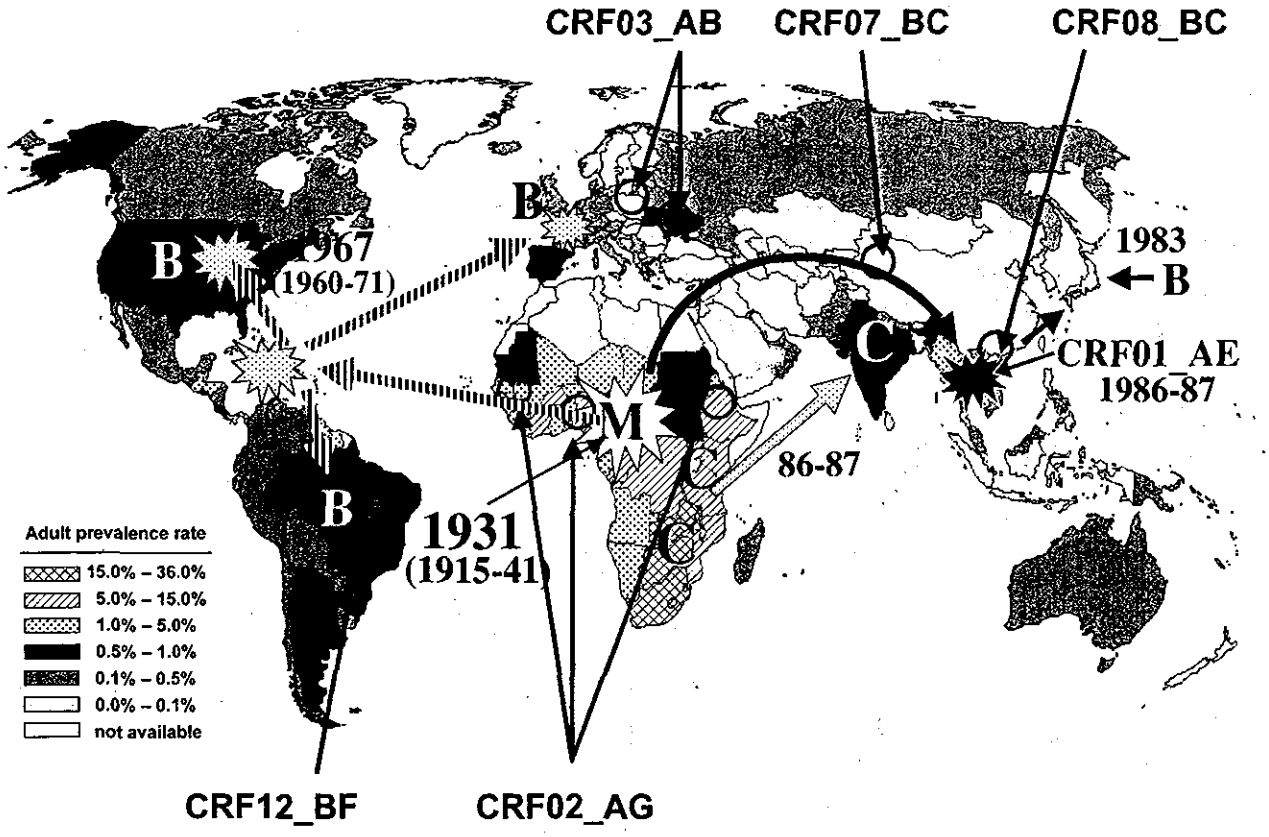

Fig. 4 Origin of HIV-1 group M and plausible route of spread of HIV-1 strains responsible for epidemic in Asia. The geographic focuses of newly emerging circulating recombinant forms are shown on the map.

The subtype distribution in Japan is as follows: subtype B (74%); CRF01_AE (20%); C (3.6%); A (2.0%); F (1.0%); D (0.5%) (Fig. 2). HIV-1 subtype B is distributed among infected hemophiliacs, who contracted HIV with contaminated blood products imported from the USA before 1985 when unheated coagulation factors products were banned. HIV-1 subtype B is prevalent among male homosexuals and in some individuals infected via heterosexual contact, while CRF01_AE is spread mainly through heterosexual contact.[27,34]

Origin of human immunodeficiency virus

Current evidence indicates that HIV-1 and HIV-2 entered into the human population through multiple zoonotic infections from non-human primates infected with simian immunodeficiency viruses (SIV).[35] HIV-1 is most closely related to SIVcpz isolated from the chimpanzee subspecies *Pantroglodytes troglodytes* (*P.t.t*).[35–38] The most diverse forms of HIV-1 are found in the geographic region corresponding to the range of *P.t.t.* in west equatorial Africa, and HIV-1 groups and SIVcpz sequences are interspersed in phylogenetic trees, suggesting that there are shared viral lineages in human and chimpanzees.[7,11,35–40] HIV-2 and SIV Sooty Mangabey (SIV sm) have a high degree of genetic and phenotypic homology.[41] This close relation between HIV-2 and SIVsm led to the hypothesis that HIV-2 infection is a zoonosis.

The study of Korber *et al.* estimated the date of the last common ancestor of HIV-1 group M to be 1931 (95% confidence interval (CI): 1915–1941), suggesting that HIV-1 group M began its expansion in the human population approximately 70 years ago.[42] The phylogenetic analyses assuming molecular clock suggested that the founder of subtype B in the USA originated in 1967 (95% CI: 1960–1971). Similarly, the last common ancestor of CRF01_AE in Thailand was dated to 1986 (95% CI: 1978–1989).[42] Plausible routes of dissemination of HIV-1 strains responsible for epidemic in Asia are shown in Figure 4.

Biological implications of human immunodeficiency virus-1 variability

It has been suggested that HIV-1 subtypes can influence viral transmissibility and pathogenecity. However, the existence of many other factors makes it difficult to establish the true

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Geographical Hotspots of Extensive Intersubtype Recombination in Asia: "Melting Pot" That Generates Diverse Forms of HIV-1 Unique Recombinants

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Summary

Molecular epidemiological investigation was conducted among high risk populations in two cities (Yangon and Mandalay) in Myanmar and three study sites (Wenshan, Honghe and Dehong) in Yunnan, China. The study identified the unique geographical hotspots in Central Myanmar and Western Yunnan, where diverse forms of recombinant strains appear to be arising continually. This may reflect the presence of highly exposed individuals and social networks in these regions. Precise mapping of recombination breakpoints revealed that significant proportion of unique recombinants found in Central Myanmar shared the breakpoints with CRF07/08_BC in China, suggesting that they may have been evolved from common ancestral recombinants. This represents the first evidence that may suggest the possible linkage between the epidemics in both regions.

Introduction

Genetic subtyping is a useful tool to track global spread of HIV. We investigate the molecular epidemiology of HIV in Myanmar and nearby

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Yunnan Province of China to study the genesis and the interrelationship of the epidemic in these two regions. HIV-1 epidemic broke out almost at the same time among injecting drug user (IDU) populations in Myanmar and western part of Yunnan Province in 1989, one year after Thai IDU epidemic in 1988. Both regions are becoming new epicenters of HIV epidemic in Asia.

Several lineages of HIV-1 strains are known to create the epidemics in Asia. HIV-1 subtype B' (Thailand variant of subtype B) and CRF01_AE are responsible for the epidemic in Thailand [1] and subtype C is the single major founder strain in India. Two closely related circulating recombinant forms (CRFs), CRF07_BC and CRF08_BC, are distributed among IDUs in northwestern and southeastern China, respectively [2, 3]. In addition to these strains that disseminate widely in population, various types of unique recombinant forms (URFs) have been reported in some areas in Asia, where different lineages of HIV-1 strains are cocirculating, including A/C recombinants in India [4], 01/B' in Thailand [5] and Myanmar, B'/C in Yunnan [6] and Myanmar [7-9].

In the present study, we describe the identification of unique geographical hotspots of extensive HIV-1 intersubtype recombination in Myanmar and western Yunnan and will discuss the biological implications of our findings.

Materials and Methods

Molecular epidemiological investigation was conducted among injecting drug users (IDUs) in two cities (Yangon in Central South, and Mandalay in Central) in Myanmar [8] and three study sites (Wenshan and Honghe in the east, Dehong in the west) in Yunnan, China [10]. The nucleotide sequences of 2.6-kb *gag*-reverse transcriptase (RT) region or near full-length HIV-1 genome were PCR-amplified and determined [6, 9]. The subtype structure and precise boundaries of HIV-1 recombination were determined by various recombination breakpoint analyses, including bootscanning, informative site and subregion tree analyses. The precise boundaries of the recombination breakpoints were compared extensively.

Results

Molecular epidemiological investigation revealed three distinct transmission clusters amongst high-risk populations in Myanmar [7, 8, 11] (Fig.1). CRF01_AE predominated among heterosexuals in eastern Myanmar near the border to Thailand. HIV-1 subtype B' showed unique predominance among IDUs in Yangon. In contrast, three HIV-1 strains, including subtype B' and C and CRF01_AE are cocirculating in Central Myanmar. Moreover, 10-30% of HIV-1 strains circulating in Central Myanmar

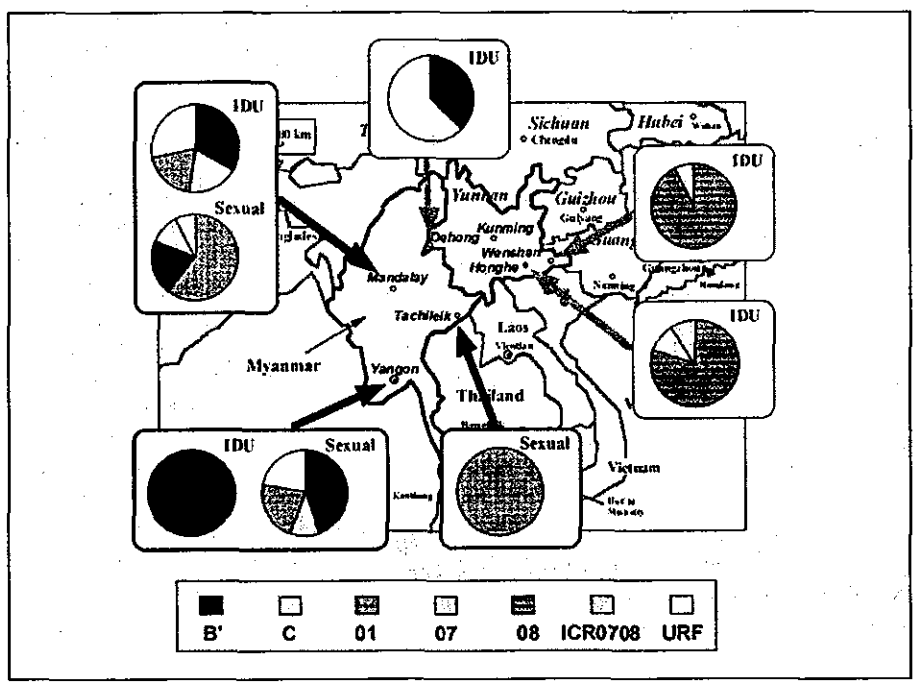

Fig. 1. Distribution of HIV-1 genotypes in Myanmar and Yunnan Province of China. HIV-1 subtype B' (B'); subtype C (C); CRF07_BC (07); CRF08_BC (08); Inter-CRF recombinant (ICR0708); unique recombinant form (URF).

were found to be unique recombinant forms (URFs) comprised of various combinations between three HIV-1 strains circulating in Myanmar, including subtypes B' and C and CRF01_AE [9].

The study in Yunnan showed the unique disparity of subtype distribution (Fig.1). CRF08_BC predominated among IDUs in eastern Yunnan. In contrast, approximately 2/3 of HIV-1 strains were diverse types of URFs between subtypes B' and C, and the remaining 1/3 was subtype B' [6] in western Yunnan (Dehong). In addition, a new class of HIV-1 recombinants (inter-CRF recombinants, ICR0708) in which further recombination occurred between the two previously established CRFs (CRF07_BC and CRF08_BC) were detected in southeastern Yunnan (Honghe), accounting for approximately 10% of HIV-1 strains distributed in this region [12].

Interestingly, fine mapping of recombination breakpoints revealed that significant proportion of Mandalay URFs shared the precise breakpoints with CRF07_BC and CRF08_BC in China, suggesting that they may have been evolved from common ancestral recombinants (Takebe *et al.* manuscript in preparation).

Conclusions

We identified unique geographical hotspots of extensive recombination in Central Myanmar and western part of Yunnan Province of China, where diverse forms of recombinant strains appear to be arising continually. This may reflect the presence of the highly exposed individuals and social networks in these regions. Our study also represents the first evidence that may suggest the possible linkage between the epidemic in Myanmar and China. The rapid emergence and evolution of diverse forms of HIV-1 recombinants could further complicate the development of effective vaccines to limit HIV-1 spread in these particular areas in Asia.

(This study is dedicated to the memory of late Dr. Hlat Htut Lwin)

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Isolation and Biological Characterization of An Infectious Molecular Clone of HIV-1 CRF08_BC from China

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Summary

We isolated the first HIV-1 infectious molecular clone (IMC) (designated as 00CN-HH040.NX22) for CRF08_BC that is responsible for IDU epidemic in southeastern China. 00CN-HH040.NX22 replicated to high titers in PBMCs and utilized only CCR5 as a coreceptor for entry. 00CN-HH040.NX22 showed the identical recombinant structure with CRF08_BC reference strain. A potential sequence signature unique to CRF08_BC was observed in the enhancer region of LTR. 00CN-HH040.NX22 will be a useful tool to delineate the biological and virological properties of CRF08_BC and may also be used in design for vaccine candidates to limit the epidemic in southeastern China.

Introduction

The infectious molecular clones (IMCs) of HIV-1 have been critical tools for systemic evaluation for delineating the mechanisms of viral replication and pathogenesis.

Phylogenetic analyses of globally circulating viral strains have identified three distinct groups of HIV-1 (M, N, and O), and 11 genetic subtypes and subsubtypes (A, A2, B, C, D, F1, F2, G, H, J, and K), and 15 circulating recombinant forms (CRFs) within the major group (M). Two closely-related CRFs, CRF07_BC and CRF08_BC, are emerging strains that play a critical role in the epidemic among injecting drug users (IDUs) in China [1, 2], where the cumulative number of HIV cases would be expected to reach 10 million by 2010 with the current

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rate of increase (30%) [3]. CRF07_BC was distributed among IDUs in Xinjiang Province in northwestern China [2], while CRF08_BC was circulating widely among IDUs in Guangxi Province [1] and eastern part of Yunnan Province in southeastern China [4].

In the present study, we report the construction of the first replication-competent molecular clone of CRF08_BC and will discuss on its structural and biological properties.

Materials and Methods

A CRF08_BC strain (00CN-HH040) was isolated from an IDU in Yunnan Province. The infectious molecular clone was reconstituted by PCR-based amplification-cloning method [5, 6], involving the direct ligation of 8.3-kb proviral amplicons into a recovery vector carrying two functional LTRs (Fig. 1). The infectivities to peripheral blood mononuclear cells (PBMCs) and primary macrophages and coreceptor usages were examined. The complete HIV-1 nucleotide sequence was determined and subjected to the recombination breakpoint analyses (bootscanning and informative site analyses) to verify the subtype structure.

Results

Reconstitution and biological characterization of an infectious molecular clone of CRF08_BC

An infectious molecular clone for CRF08_BC was isolated by two-

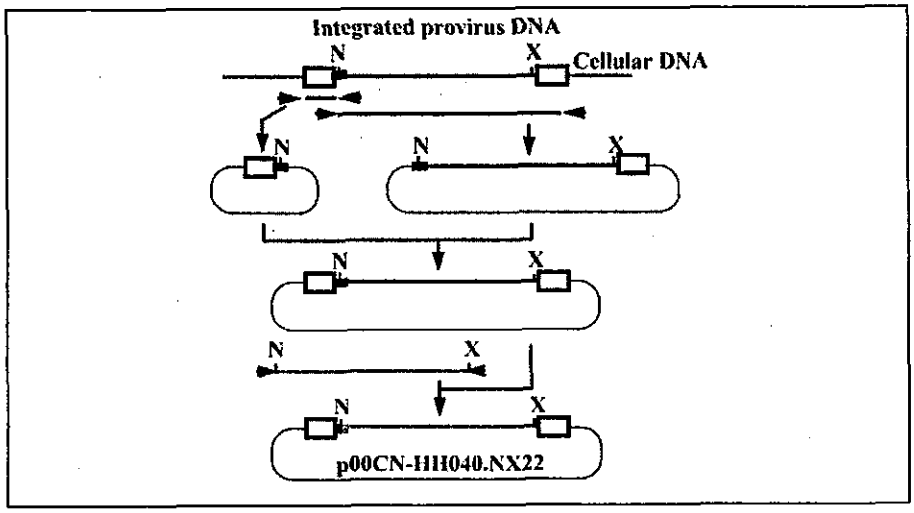

Fig. 1. Schematic representation of the genetic reconstitution of infectious molecular clone of CRF08_BC (00CN-HH040.NX22). Nar*I (N);* Xho*I (X).*

step reconstitution strategy, as illustrated in Fig. 1. We identified one clone, designated 00CN-HH040.NX22, that was capable of replicating in PHA/IL-2-stimulated PBMCs to high titers, comparable to parental primary isolate. This clone utilized CCR5 as a coreceptor for entry, but did not replicate in primary macrophages.

Structural characterization of infectious molecular clone 00CN-HH040.NX22

The complete nucleotide sequence of 00CN-HH040.NX22 was determined to ensure that this clone indeed belongs to CRF08_BC. The phylogenetic tree analysis of the complete genome of 00CN-HH040.NX22 showed that it was clustered tightly with CRF08_BC reference strains with high bootstrap support (100%). The recombination breakpoint analyses (bootscanning and informative site analyses) corroborated that 00CN-HH040.NX22 shared identical structural profile with CRF08_BC reference strain (98CN006) (Fig.2).

Sequence signatures specific to CRF08_BC

The nucleotide sequence signatures unique to CRF08_BC were identified in enhancer-promoter region in LTR. All known CRF08_BC (5 of 5) harbored three complete sets of NFκB sites in LTRs, which are common among most of subtype C family. 00CN-HH040.NX22 dis-

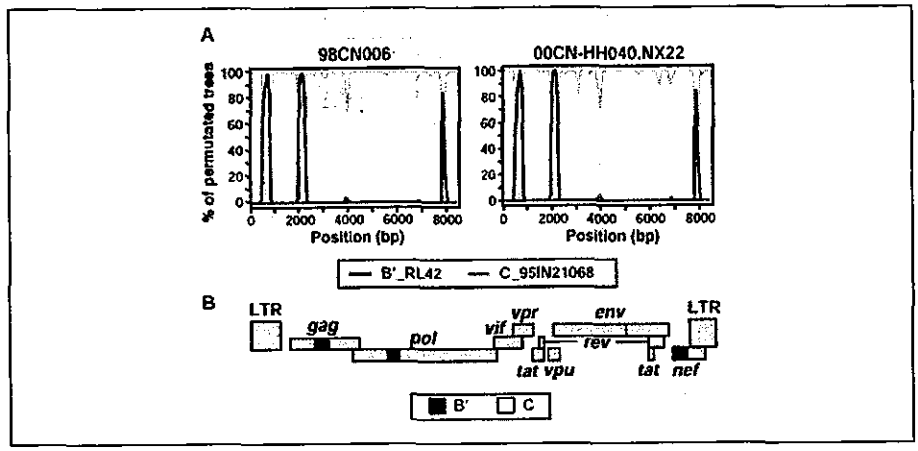

Fig. 2. Recombinant structure of 00CN-HH040.NX22. (A) Bootscanning analyses of CRF08_BC reference strain (98CN006) (left) and 00CN-HH040.NX22 (right) with subtypes B' (RL42) and C (95IN21068) reference strains. subtype D (NDK) and CRF01_AE (93TH253) are used as distantly related references. The bootstrap values are plotted for a window of 500-bp moving in increments of 50-bp along the alignment. (B) Deduced subtype structure of 00CN-HH040.NX22.

plays three-NF-κB configuration similar to other subtype C strains, but it harbors a C to T substitution in proximal NFκB binding motif in both 5' and 3' LTR regions. 00CN-HH040.NX22 retains the other sequence signature that appears to be specific to CRF08_BC [7]: *i.e.*, the spacer sequence between two distal NFkB sites (NFκB II and NFkB III) of 00CN-HH040.NX22 was comprised of two nucleotides (5'-GC-3'), similar to all known CRF08_BC strains (4 of 4) [7].

Conclusions

In the present study, we described the structural and biological characterization of the first infectious molecular clone of CRF08_BC (00CN-HH040.NX22). This clone was reconstituted from primary HIV-1 CRF08_BC strain isolated from eastern part of Yunnan Province of China, where CRF08_BC is a principal circulating strain among IDUs [4]. This represents the first report of the isolation of a replication-competent HIV-1 molecular clone of CRF08_BC. It may facilitate the study to investigate the differences in the virological and immunological properties and to develop clade-specific molecular and immunological reagents. This clone may also be used for designing novel immunogens to limit the epidemic in southeastern China.

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Dermal dendritic cells sensitized with plasmid DNA encoding immunostimulatory sequence by gene gun efficiently prime murine HIV-1-specific CD8$^+$ cytotoxic T lymphocytes

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ABSTRACT

When gold beads coated with plasmid DNA encoding the β-galactosidase (β-gal) gene having a strong immunostimulating sequence (ISS) with CpG-motif were inoculated intradermally by gene gun, blue colored cells producing β-gal could be seen within regional lymph nodes and spleen. Electron microscopic analysis suggested that cells expressing the β-gal gene in the regional lymph nodes look like activated interdigitating dendritic cells (DC). In addition, multiple gold beads were observed in blue colored cells in the regional lymph nodes but not in the spleen where β-gal was actively produced. The DC expressing the β-gal gene may prime directly β-gal epitope peptide (TPHPARIGL)-specific, CD8$^+$ cytotoxic T lymphocytes (CTL) without requiring Th1 type of help in this priming step. Moreover, HIV-1 gp120-specific CD8$^+$ CTL could be primed when mice were immunized with gold beads coated with gp120 plasmid DNA together with the β-gal plasmid, though gold beads coated with HIV-1 gp120 plasmid alone or a mixture of two distinct gold beads coated with either β-gal or gp120 plasmid could not prime HIV-1-specific CTL. These results suggest that intradermal immature DC, like Langerhans cells, activated by ISS-containing plasmids encoding strong CpG-motif such as β-gal DNA, can efficiently prime CTL specific for not only original β-gal epitope but also the products of another plasmid without encoding ISS when both plasmids are captured simultaneously by the same dermal DC.

With the recent progress in the development of DNA vaccines, it has become clear that the direct injection of plasmid DNA expressing a gene encoding the protein of a pathogen elicits specific responses in both cellular and humoral immunity (9). Unlike with live vectors, with bacterial DNA plasmid does not seem to induce any pathogenic risks concerning potential integration into the genome of the host cells (11). A number of studies have shown that DNA vaccines can elicit antigen-specific cytotoxic T lymphocytes (CTL), helper T cells (Th) and antibodies in mice and primates. Yet, most of these studies are only focused on the positivity of the results without clarifying possible mechanisms for the DNA vaccination or the optimal amount of the DNA, times and best route for immunization. Consequently, a large number of bacterial DNA plasmids were usually injected into the animal model to elicit various immunities randomly. Therefore, methods

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for immunization that reduce the amount of plasmid needed as well as the mechanism of priming with bacterial DNA should be investigated to establish this promising strategy.

Accumulating evidence indicates the importance of cellular immunity, in particular $CD8^+$ CTL including their precursors (CTL-p) to prevent various viral infections and associated diseases progressions. For example, the initial viremia in HIV-1 infected individuals usually ceases with the emergence of a virus-specific CTL response (13) and the appearance of CTL is correlated with the maintenance of a low virus load (12, 18, 20). Thus, we have been focusing on the effect of plasmid DNA on the priming of CTL (2, 3) in which dendritic cells (DC) seem to have a critical role (6, 16, 23).

To reduce the amount and pursue the localization of inoculated DNA for the priming of CTL, we immunized mice intradermally at sites where many DC such as Langerhans cells should be present with gold beads coated with less than 1 μg of plasmid DNA using a gene gun which will preferentially prime antibody responses by inducing Th2 dominance (7). To overcome this dominance, which is unfavorable for $CD8^+$ CTL induction, and to mark the position of the inoculated plasmid, we utilized a β-galactosidase (β-gal) plasmid containing a strong immunostimulating sequence (ISS) having the CpG motif as adjuvant DNA for enhancing cellular immunity (17, 25, 26) as well as a tool for identifying the localization. After confirming the difficulty in achieving a stable CTL induction by multiple (more than 3 times) injections of gold beads coated with HIV-1 gp120 plasmid DNA having a strong CMV promoter, we found that HIV-1-specific CTL could be primed when mice were immunized with gold beads coated with both gp120 plasmid DNA and the β-gal plasmid.

In the present study, we would like to demonstrate using a murine system that gold beads coated with less than 1 μg of plasmid DNA having the ISS with strong CpG motif will efficiently activate dermal DC which may directly prime $CD8^+$ CTL without evoking a Th1 type of response.

MATERIALS AND METHODS

Mice and Antibodies. Female BALB/c ($H\text{-}2^d$) mice were obtained from Charles-River Japan Inc. (Tokyo, Japan). Mice were used at 6 to 8 weeks of age. All experiments were performed according to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals. For DC staining, FITC-conjugated CD11c was purchased from PharMingen (San Diego, CA, USA).

Vaccine DNA plasmids. The pCMV-lac plasmid, a derivative of pRC/CMV (Invitrogen, SanDiego, CA, USA), encoding the β-gal gene was generously provided by Dr. Thomas Kipps. The pCMV-lac contains the *E. coli* lacZ gene under the control of the CMV immediate early promotor, the ampicillin resistance gene, and the simian virus 40 polyadenylation signal. As for the HIV-1 gp120 plasmid, a mammalian expression vector, pJW4303 (27), containing the CMV immediate early promotor, and the ampicillin resistance gene, kindly provided by Dr. James I. Mullins (Stanford Univ., USA), was used for expression of the HIV-1 envelope gp120 gene of NL432 strain (1). To produce a secreted gp120, the native signal sequence for the gp120 gene was replaced with the human t-PA signal in plasmid pJW4303 and a termination codon was created at the processing site for gp120-gp41 cleavage (4). The resultant plasmid was designated pJWSUNL2.

Immunization. Plasmid DNA was affixed to 1.0-μm-diameter gold particles (Bio-Rad Laboratories, CA, USA) using calcium chloride and spermidine. Gene gun immunizations were performed with a Helios gene gun (Bio-Rad Laboratories) at a helium discharge pressure of 400 psi. One shot contained 0.2 μg of each plasmid coupled with 0.75 mg of gold particles. Sodium pentobarbital-anesthetized BALB/c mice received three nonoverlapping shots per immunization to depilated abdominal skin.

CTL generation. For the generation of β-gal-specific CTL or HIV-1 envelope-specific CTL, immunized BALB/c spleen cells (5×10^6/ml) were restimulated *in vitro* with either 10^6/ml mitomycin C (MMC: Kyowa Hakko, Tokyo, Japan)-treated epitope peptide (TPHPARIGL) (8)-pulsed BALB/c 3T3 fibroblasts or gp160 gene-transfected BALB/c.3T3 fibroblasts (15–12) (21), respectively. The restimulated cells were further cultured at 37°C for 5–6 days in 24-well culture plates in complete T cell medium (CTM) (RPMI1640 medium containing 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 10 mM Hepes, 100 μM non-essential amino acids, 10 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol (ME)) in the presence of 10% rat concanavalin A (ConA)-stimulated spleen cell culture supernatant (Rat T- STIM without ConA, Collaborative Research Inc., Bedford, MA, USA) as described

previously (22). The β-gal epitope peptide was synthesized using an Applied Biosystems Model 430A (Foster City, CA, USA) peptide synthesizer to a purity > 95% as determined by high pressure liquid chromatography and amino acid analysis.

Assay for CTL. The cytolytic activity of the cells was assayed as described (24) using the ^{51}Cr-labeled targets indicated in the figure legends. The percent specific ^{51}Cr release was calculated as 100 × (experimental release − spontaneous release) / (maximum release − spontaneous release). Maximum release was determined from the supernatants of cells lysed with 5% Triton-X 100. Spontaneous release was determined from target cells incubated without effector cells.

Enzyme-linked immunosorbent assay (ELISA). Anti-β-gal specific antibodies of the IgG subclasses IgG1 and IgG2a, were measured by ELISA. Microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were coated with 5 μg/ml of β-gal in carbonate buffer (pH 9.6) overnight. The plates were washed with phosphate buffered saline (PBS) and blocked with 25% Block-Ace (Dainihon Seiyaku, Osaka, Japan) for 2 h. After washing twice with 10% Block-Ace containing 0.5% Tween 20, a 50 μl aliquot of appropriately diluted sample serum was added to the well for 1 h at 37°C. Then the plates were washed three times and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG1 or IgG2a (Zymed, San Francisco, CA, USA) for another 1 h at 37°C and the amount of alkaline-phosphatase bound to the well was determined by measuring the hydrolysis of *p*-nitrophenyl phosphate (Sigma Chemical Co., St Louis, MO, USA) to the yellow product, *p*-nitrophenolate, which was quantitated by absorbance at 405 nm with a microplate reader (Model 3550) (Bio-Rad Laboratories). Each plate included a previously screened standard mouse serum that contained a high titer of anti-β-gal-specific antibodies. The results are expressed in units per ml, calculated based on the units/ml of the standard serum, and represent the mean ± SD of four animals in each group.

Immunohistochemical studies and electron microscopic observations. The localization of DNA-coated gold particles in cells or tissues was determined by regular hematoxylin-eosin staining. β-gal-expressing cells or CD11c-positive DC were identified as follows. In brief, tissue samples were embedded in OCT Compound (TissueTek) (Miles, Elkhart, IN, USA) and stored at −80°C. Cryostat sections (8 μm) were cut from the fresh frozen specimens, air dried, mounted on silanized glass slides, and stored at −80°C. For β-gal staining, frozen sections were incubated with X-Gal substrate (1 mg/ml X-Gal in PBS/5 mM potassium ferricyanide/5 mM pottasium ferrocyanide/2 mM magnesium chroride) at 37°C until positive cells (blue) appeared. For staining CD11c$^+$ DC, cryosections were incubated with FITC-conjugated CD11c monoclonal antibody for 1.5 h at room temperature and observed with a Nikon MICROPHOT-TXA (Nikon, Tokyo, Japan). For electron microscopic analysis, tissue specimens were fixed in 2.5% glutaldehyde overnight. Following fixation for 2 h in 1% osmium tetroxide, they were dehydrated using graded concentrations of alcohol and embedded in Epon (TAAB, Berks, England). Sliced sections were counterstained with 2% uranyl acetate and 1% lead citrate and observed using a HITACHI H7100 microscope (HITACHI, Tokyo, Japan).

Purification of DC. As described previously (23), the DC were isolated from non-adherent spleen cells after overnight culture of fresh adherent cells in tissue culture plates. In brief, spleens were digested with collagenase (Wako Pure Chemical Industry, Tokyo, Japan) at 400 U/ml for 30 min at 37°C and then spleen cells were fractionated on a discontinuous gradient of dense BSA (ρ = 1.080) and the low-density fraction was allowed to adhere to 60 mm diameter plastic dishes for 1.5 h. After the removal of non-adherent cells with cold PBS, the plastic dishes were further incubated with fresh medium for 18 h. After the incubation, non-adherent cells were collected and contaminating macrophages and B cells were removed by rosetting with antibody-coated sheep red blood cells. About ninety percent of the remaining cells were confirmed as CD11c-positive DC by FACScan® (Becton Dickinson, Bedford, MA, USA) analysis.

RT-PCR and PCR. Total RNA was prepared from mouse tissue using Isogen (Nippon Gene Co., Toyama, Japan). Each sample was incubated for 1 h at 42°C after the addition of 14 U of RNase inhibitor (Takara), 11 U of Rous-associated virus-2 reverse transcriptase (Takara), 50 mM DTT (Gibco BRL, Santa Clara, CA, USA), 0.2 mM dNTP, and reverse transcriptase buffer to a final volume of 20 μl. cDNA was then amplified in the presence of 0.5 U of Taq polymerase (Takara), 0.2 mM dNTP, and 1 μM of each primer listed below for 35 cycles. Then, nested PCR was performed for β-gal mRNA

detection. One thermal cycle consisted of 30s at 94°C, 45s at 58°C, and 45s at 72°C. PCR products were resolved on agarose gel containing ethidium bromide and visualized with UV light. Primers used in the present study are listed as follows.

β-gal sense: 5'-CAGATGTGGATTGGCGATAAA-3'

β-gal antisense: 5'-TCAAAACAGGCGGCAGTAAG-3'

β-gal sense for nested PCR: 5'-GCAGATACACTTG CTGATGC-3'

β-gal antisense for nested PCR: 5'-GCTCGCCACTT CAACATCAA-3'

RESULTS

Movements of gold beads after the gene gun inoculation

As shown in Fig. 1, the plasmid coated gold beads remained mainly in the epidermis and upper part of the dermis 4–8 h after three shots of the gene gun inoculation. Then the gold beads disseminated deep in the dermis (1 d), and further invaded the dermis and subcutaneous tissue where they seemed to form small clusters (3 d). After 7 d, no gold beads could be observed within the epidermis, whereas many large bead clusters were still seen in the dermis and subcutaneous tissue.

Expression of β-gal in various tissues

To study the effect of the inoculated DNA encoding the β-gal gene, we searched for cells expressing β-gal protein in frozen sections from various tissues by incubation with X-Gal substrate as indicated in the Materials and Methods section. Seven days after immunization, blue colored cells were seen within regional lymph nodes and spleens, but not in the skin (Fig. 2). In addition, we could not detect any blue colored cells in the liver, kidney, or thymus of the immunized mice on day 1, 3, 5, and 7 after the immunization (data not shown). It is of note that multiple gold beads were frequently seen in blue colored cells of the regional lymph nodes but not at all in the spleens (Fig. 2).

Type of cells that captured gold beads in the regional lymph nodes

Based on the observation (see Fig. 2) that gold beads were frequently present in β-gal-expressing blue-colored cells in the regional lymph nodes, we tried to identify the cells in the immunized mice by microscopic and electron microscopic analysis using gold beads as indicators. As indicated with arrows in Fig. 3A, relatively lightly stained large cells appeared to capture the gold beads. The electron microscopic analysis strongly suggests that the cells are

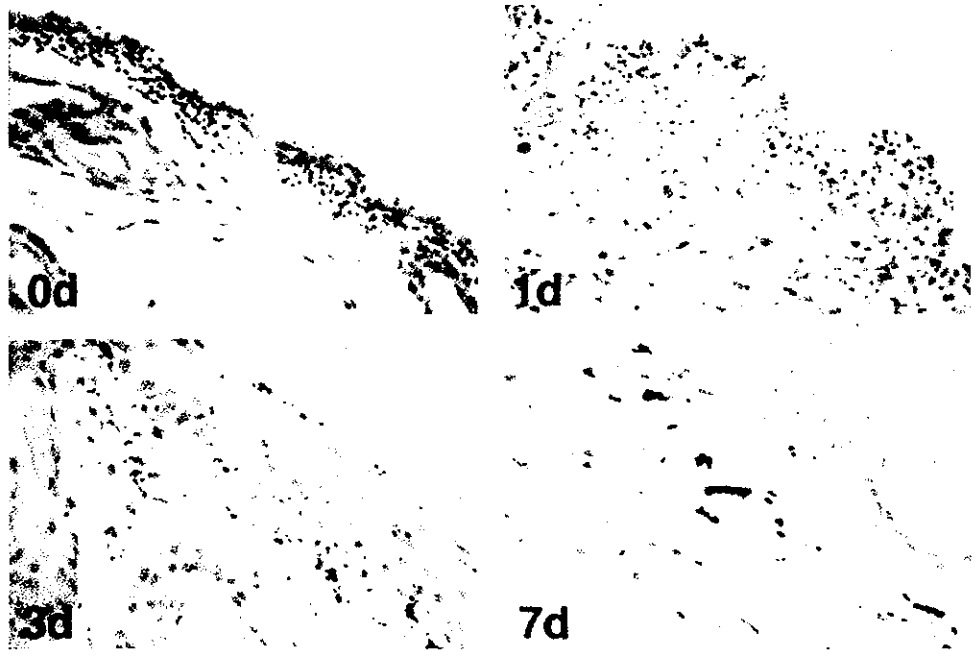

Fig. 1 Movements of gold beads after the gene gun inoculation.
Plasmid DNA affixed to 1.0-μm-diameter gold particles was inoculated subcutaneously three times to anesthetized BALB/c mice through depilated abdominal skin with a Helios gene gun at a helium discharge pressure of 400 psi and the localization of DNA-coated gold particles in cells or tissues was determined by regular hematoxylin-eosin staining.

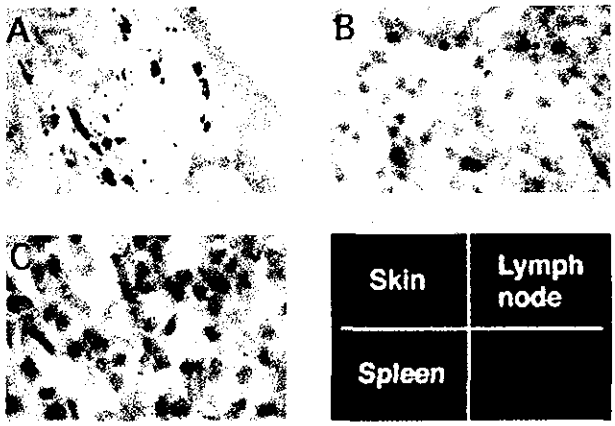

Fig. 2 Expression of β-gal in various tissues.
Seven days after the immunization, cells expressing the β-gal protein from various tissues such as the the skin (A), regional lymph nodes (B) and spleen (C) were examined. For β-gal staining, frozen sections were incubated with X-Gal substrate (1 mg/ml X-Gal in PBS/5 mM potassium ferricyanide/5 mM pottasium ferrocyanide/2 mM magnesium chroride) at 37°C until blue colored cells appeared.

interdigitating dendritic cells (IDC) (Fig. 3B).

Analysis of β-gal gene expression in regional lymph nodes and spleens of the immunized mice

To confirm whether the β-gal was actively produced in the spleens of the immunized mice, we conducted RT-PCR analysis to detect β-gal mRNA expression. As shown in Fig. 4, although the window to catch the mRNA seems narrow, we detected β-gal-specific mRNA in the spleen and confirmed the sequences of mRNA as β-gal by sequence analysis (data not shown).

Detection of β-gal-specific CTL activity

These findings suggest that β-gal plasmid DNA inoculated in association with gold beads by the gene gun are captured by dermal Langerhans cells which change into activated DC while they are moving into regional lymph nodes and the encoding gene product, β-gal, might be transcribed and translated in the DC. Moreover, we observed β-gal production as well as β-gal-specific mRNA in the spleens of the immunized mice where the injected gold beads could not be detected. These results strongly indicate that the plasmid encoding β-gal has been transferred to the spleen from the regional lymph nodes by an unknown mechanism probably mediated by antigen presenting cells (APC), such as antigen-captured DC, which may prime antigen-specific CTL systemically *in vivo*.

Therefore, we measured β-gal-specific CTL activity to confirm whether the β-gal epitope was presented in conjunction with class I MHC molecules by the splenic APC of immunized mice. As demonstrated in Fig. 5, at least two weeks after the immunization when β-gal had already been produced in the spleens, we measured strong CTL activity against β-gal epitope peptide (TPHPARIGL)-sensitized syngeneic BALB/c.3T3 fibroblasts expressing only class I MHC molecules when the immunized spleen cells were restimulated *in vitro* with the epitope peptide-pulsed MMC-treated BALB/c 3T3 cells. The CTL were conventional $CD8^+$ CTL since their activities were totally abrogated by the treatment with anti-CD8 mAb plus rabbit complement but not with anti-CD4 mAb plus complement (data not shown).

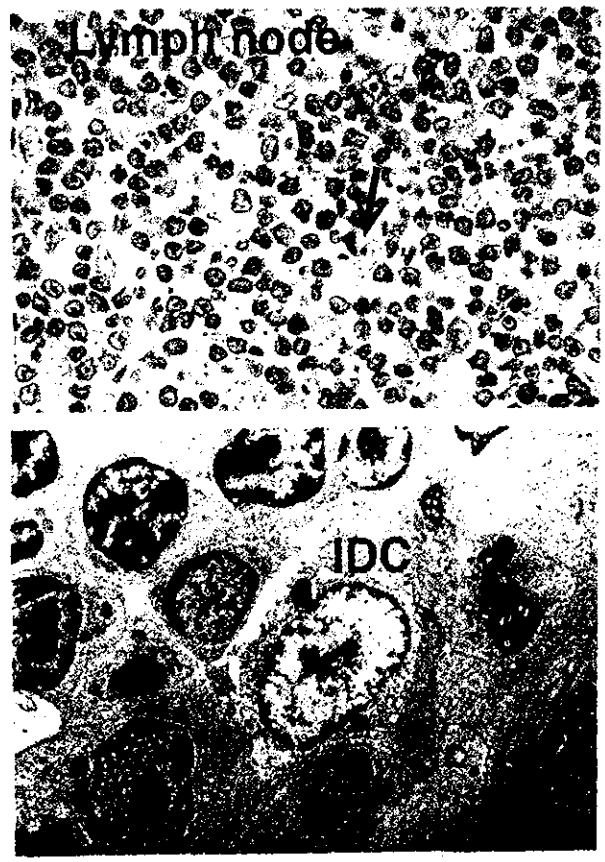

Fig. 3 Identification of cells that captured gold beads.
The regional lymph node of BALB/c mouse immunized with β-gal DNA plasmid-coated gold beads by gene gun were analyzed with microscopic (upper panel A, original magnification × 400) and electron microscopic (lower panel B, original magnification × 15,000) analysis using gold beads as indicators (arrow).

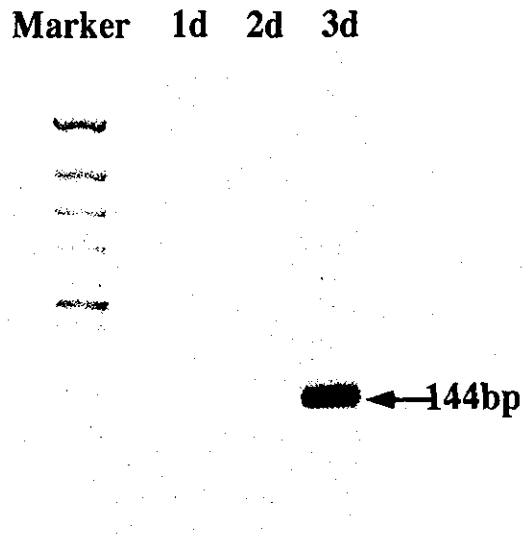

Fig. 4 Detection of β-gal mRNA expression in the spleen of mice immunized by gene gun using RT-PCR analysis. PCR products were resolved on agarose gel containing ethidium bromide and visualized with UV light. Three days after the immunization, β-gal-specific mRNA expression in the spleen was confirmed with the detection of a 144 bp product using nested PCR analysis.

Kinetic of β-gal-specific IgG1 and IgG2a antibody production

Because we could detect β-gal-specific CTL activity as early as two weeks after the gene gun immunization, we were interested to know whether the Th1 type of help is necessary for the priming. We compared the response to β-gal-specific IgG1 and IgG2a antibody, reflecting the Th2 type and Th1 type of immunity, respectively. As shown in Fig. 6, although β-gal-specific IgG1 responses were observed two weeks after the immunization, IgG2a responses could not be detected at that time. Similar results were obtained in three distinct sets of experiments. Thus, there seems to be no correlation between β-gal-specific CTL priming and the β-gal specific Th1 type of immunity. These results strongly indicate that some professional APC such as activated DC may directly prime β-gal-specific $CD8^+$ CTL in the spleen without Th1 type of help as has been reported (15, 19).

Induction of HIV-1 gp120-specific CTL by DNA plasmid of HIV-1 gp120 and β-gal expressing the CpG motif

Since we failed to elicit measurable HIV-1-specific CTL activity with HIV-1 gp120 plasmid DNA using the gene gun system, we immunized mice with three shots of gold beads coated with gp120 DNA plasmid together with an equal amount β-gal plasmid having the CpG motif using the gene gun. The results clearly show strong CTL activity three weeks after the immunization (Fig. 7) and that the priming effect remained at least 3 months (data not shown). However, when the mice were imuunized with a mixture of two distinct gold beads coated with either β-gal or gp120 plasmid did not prime HIV-1-specific CTL (Fig. 7). These results suggest that intradermal immature DC, like Langerhans cells, activated by the ISS-containing plasmids encoding strong CpG-motif such as β-gal DNA, can efficiently prime the CTL specific for not only original β-gal epitope but also the product of another plasmids without encoding ISS when both plasmids are captured simultaneously by those dermal DC.

DISCUSSION

In the present study, it appears that intradermal DC, such as Langerhans cells, preferentially capture the injected gold beads coated with β-gal plasmid having the strong ISS with CpG motif within a few days after the gene gun immunization. Then, the DNA-bearing DC seem to move into regional lymph nodes where they become activated IDC, which express various co-stimulatory molecules and may transcribe and translate the β-gal DNA. Thus, the activated IDC may present endogenously synthesized antigenic epitope(s) of β-gal protein in association with their class I MHC molecules which could prime β-gal-specific $CD8^+$ CTL locally without evoking a Th1 type of response. We observed β-gal-expressing $CD11c^+$ IDC capturing gold beads in the regional lymph nodes by both microscopically and electron-microscopically. Moreover, the fact that β-gal mRNA as well as β-gal protein but not the gold beads could be detected in the spleen of the immunized mice suggests that β-gal DNA is transferred from regional lymph nodes to spleen where the DNA was actively transcribed and translated by some APC which may prime $CD8^+$ CTL systemically. The cells having the capacity to transfer the genetic information of β-gal gene from regional lymph nodes to spleen remain to be elucidated.

Raz *et al.* have shown that immunization with plasmid DNA encoding β-gal in a CMV-based expression vector (pCMV-LacZ) mainly induced an IgG2a response, whereas that with β-gal in saline or alum elicited an IgG1 response, and thus concluded that plasmid DNA immunization preferentially generates a Th1 type of response, whereas protein immunization induces a Th2 type of response to the