microscopy after negative staining, as described previously.[11] To estimate the amounts of encapsulated plasmid DNA, refolded and purified VLPs were treated with 10 IU benzonase (SIGMA-ALDRICH, Irvin, UK) for 1 h at 20°C to remove DNA on the surfaces of VLPs and disrupted with EGTA (1 mM). Absorbance of the supernatant was measured for detection of plasmid DNA contents.

Density analysis of refolded VLPs

Refolded VLPs were separated on a CsCl equilibrium density gradient and fractioned into 0.2 ml aliquots. HEV-VLPs in each fraction were detected by ELISA as previously described,[10] as well as DNA contents.

Gene transfer in mammalian cells

Four cell lines (NIH/3T3 (mouse), RK13 (rabbit), COS-7 (monkey), HepG2 (human)) were used in transfection experiments. Sterilized coverslips were placed in six-well plates, and 5×10^5 cells per well were seeded in the plates. After overnight culture, cells were washed twice with a medium, and about 1 μg of VLP-encapsulated EGFP expression vector (BD Bioscience Clontech, CA, USA) diluted with 0.5 ml medium was added. After 2 h of incubation at 37°C, VLPs were removed. Cells were then incubated for 48 h at 37°C. At the end of the culture period, cells were removed from the culture medium and washed three times with PBS. Coverslips were then mounted onto microscope slide glasses. Fluorescence of the GFP-expressing cells was observed under a fluorescence microscope.

Immunization

Mice were orally immunized four times with 50 μg protein of HEV-VLP/DNA (pJWNL432) complex or 20 μg naked pJWNL432 DNA in 100 μl of potassium-MES buffer at 1 week intervals.

Immunohistochemical analysis

At 2 days after oral immunization, the mice were killed and tissues were collected. Cryostat sections were air-dried and incubated in 0.5% HIO_4 for 10 min to quench endogenous peroxidase activity. The sections were further pretreated with chicken anti-mouse IgG antibody (Chemicon International, Inc., CA, USA) to prevent nonspecific reactions of a secondary antibody. The sections were then incubated with an HIV env-specific mAb (HIV-1 IIIB gp120 mAb (902)), which was obtained through the AIDS Research and Reference Reagent Program,[31] for 30 min at 37°C. The bound antibodies were visualized with a biotinated secondary antibody, HRP-labeled avidin–biotin complex (ABC-peroxidase staining kit, Elite Vector Lab. Inc., CA, USA) and 3.3′-diaminobenzidine tetrachloride with 0.01% H_2O_2. Sections were slightly counterstained with hematoxylin. An mAb (A1/3D1, ANOGEN, Canada) against hepatitis C virus core, which is same isotype to 902, was used as a control.

ELISA

Serum and fecal samples were collected at 0 (preimmunization), 2, 4, 6 and 12 weeks after the first immunization. Feces were suspended in ice-cold PBS at 200 mg/ml, and the centrifuge supernatant was used as fecal extract. Culture plates (96-well) were coated with purified HEV-VLPs or synthesized oligopeptides (P18) at a concentration of 10 or 100 μg/well, respectively, overnight at 4°C followed by 30 min of blocking with PBS containing 0.1% FBS and 0.05% Tween 20. To determine the anti-HIV env gp120 antibody responses, CV-1 cells were seeded in 96-well plates and infected with recombinant Sendai virus expressing HIV env gp120 of NL432 strain (SeV gp120),[32] and then the plates were incubated at 37°C. At 3 days after infection, plates were washed and fixed with PBS containing 10% formalin for 10 min. Test samples were added to each well and incubated at room temperature for 1 h. For detection of anti-HIV env gp120 antibody, test samples were reacted with wild-type Sendai virus-infected CV-1 cells before addition to the wells to eliminate the nonspecific antibody. Biotin-labeled anti-mouse IgG (Vector, CA, USA) or IgA (CALTAG, CA, USA) was used as the detection antibody. Following 1 h incubation, the plates were washed and further incubated with avidin-HRP (Vector, CA, USA). The reaction was developed using an ABTS substrate (Roch Diagnostic, Mannheim, Germany).

Generation of CTL effector cells

Effector cells were derived from spleen, MLN and PP cells as precursor CTLs. Aliquots of 5×10^6 spleen cells were co-cultured with 2.5×10^6 mitomycin C-treated autologous spleen cells labeled with a peptide at 37°C in a CO_2 incubator. The effector cells generated were harvested after 5 days of culture.

Cytotoxicity assay

Target cells, A20.2J cells (2×10^6), were incubated at 37°C in a 5% CO_2 atmosphere with 10 μg/ml of P18 or control peptide for 16 h. The target cells were then washed and labeled with ^{51}Cr. The ^{51}Cr-labeled target cells were incubated for 5 h with effector cells. Spontaneous release varied from 5 to 10%. Percent lysis was calculated as ((experimental release−spontaneous release)/(100% release−spontaneous release)) × 100. All the experiments were performed at least four times, and each experimental group consisted of five mice.

Blocking of cytolysis

^{51}Cr-labeled target cells (10^6 cells) were preincubated at 4°C for 1 h with anti-H-2 K^d, D^d or L^d mAb (Meiji Institute of Health Science Ltd., Tokyo, Japan) (1 μg/ml), and effector cells were then added. In a separate experiment, effector cells (10^7 cells) were preincubated with anti-CD4 mAb (GK1.5) or anti-CD8 mAb (Lyt2.2) (10 μg/ml) at 4°C for 1 h, and then the labeled target cells were added. Blocking of cytolytic activities by these mAbs was assessed by a 5-h ^{51}Cr release assay.

Statistical analysis

Statistical analysis was performed using Mann–Whitney's U test and Kruskal–Wallis test. Values are expressed as means ± s.d.s. A 95% confidence limit was taken as significant ($P < 0.05$).

Acknowledgements

This work was supported by Health Science Research Grants from the Ministry of Health, Labor and Welfare of Japan and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Pediatrics International (2004) **46**, 236–244

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Feature Article

Molecular epidemiology of HIV: Tracking AIDS pandemic

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Abstract

Background: Human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) epidemic is a global threat to maternal and child health, especially in developing countries. It is estimated that 800 000 children are infected and 580 000 children die of AIDS-related illnesses every year. Molecular epidemiology has been a useful tool in analyzing the origin of HIV and tracking the course of global HIV spread. This article provides an overview of recent advances in the field of molecular epidemiology of HIV across the world, and discuss the biological implications.

Methods: Based on the near full-length or partial nucleotide sequence information, the phylogeny and recombinant structure of HIV strains are analyzed. Using genotype classification of HIV as a molecular marker, the origin and the genesis of HIV epidemic are investigated.

Results: The HIV-1 group M, a major HIV group responsible for current AIDS pandemic, began its expansion in human population approximately 70 years ago and diversified rapidly over time, now comprising a number of different subtypes and circulating recombinant forms (CRF). Of note, recent studies revealed that new recombinant strains are arising continually, becoming a powerful force in the spread of HIV-1 across the globe.

Conclusion: Global dissemination of HIV is a dramatic and deadly example of recent genome emergence and expansion. Molecular epidemiological investigation is expected to provide information critical for prevention and future vaccine strategies.

Key words acquired immunodeficiency syndrome, circulating recombinant form, genetic variability, human immunodeficiency virus, molecular epidemiology, recombination, subtype, vertical transmission.

The human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) pandemic continues to expand globally at a rate of 14 000 new infections every day. The Joint United Nations Program on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimated that more than 60 million individuals were infected with HIV worldwide by the end of the year 2002. Of these, approximately 25 million patients have already died, and 42 million people were living with HIV/AIDS.[1] It is known that HIV transmits through blood and sexual contacts and via mother-to-infant. Vertical infection occurs during pregnancy and delivery as well as through breast milk at an estimated rate of 25–30%. UNAIDS estimates that close to 3 million children under 15 years of age are now living with HIV/AIDS worldwide: 800 000 children are become infected and 580 000 children die of AIDS every year (Table 1).[2] Of approximately 14 000 new HIV infections which occur each day, 2000 are in children under 15 years of age and approximately 5000 are women in their reproductive age (Table 1), mostly in developing countries in Africa, Asia and Latin America (Table 2).[2] The HIV/AIDS epidemic has a serious impact in the health of mothers and children.

Molecular epidemiological investigations have been a powerful tool in analyzing the origin of HIV and in tracking the global spread of this pathogen. Since HIV-1 group M began its expansion in the human population early in the 20th century (1931, 95% confidence interval: 1915–1941),[4] it has diversified rapidly, now comprising a number of different subtypes and circulating recombinant forms (CRFs).[3–5]

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Received 7 October 2003.

Classification of human immunodeficiency virus

Phylogenetic analyses of globally circulating viral strains have identified three distinct groups of HIV-1 (M, N, and O),

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.

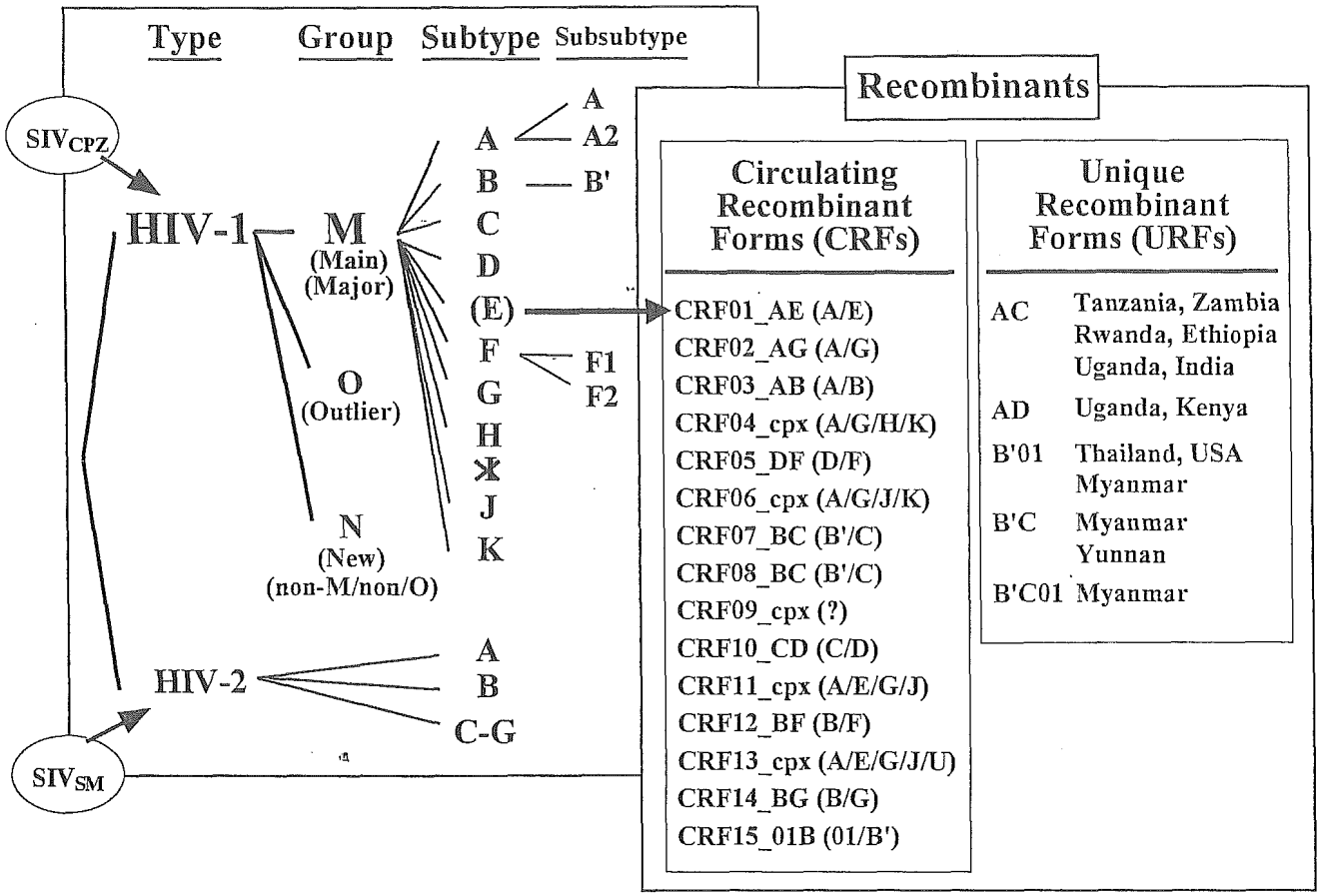

Fig. 1 Classification of human immunodeficiency viruses (HIV). HIV types, HIV-1 groups, subtypes, subsubtypes are shown. HIV-1 recombinants are categorized into two class: circulating recombinant forms (CRFs) and unique recombinant forms (URFs).

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

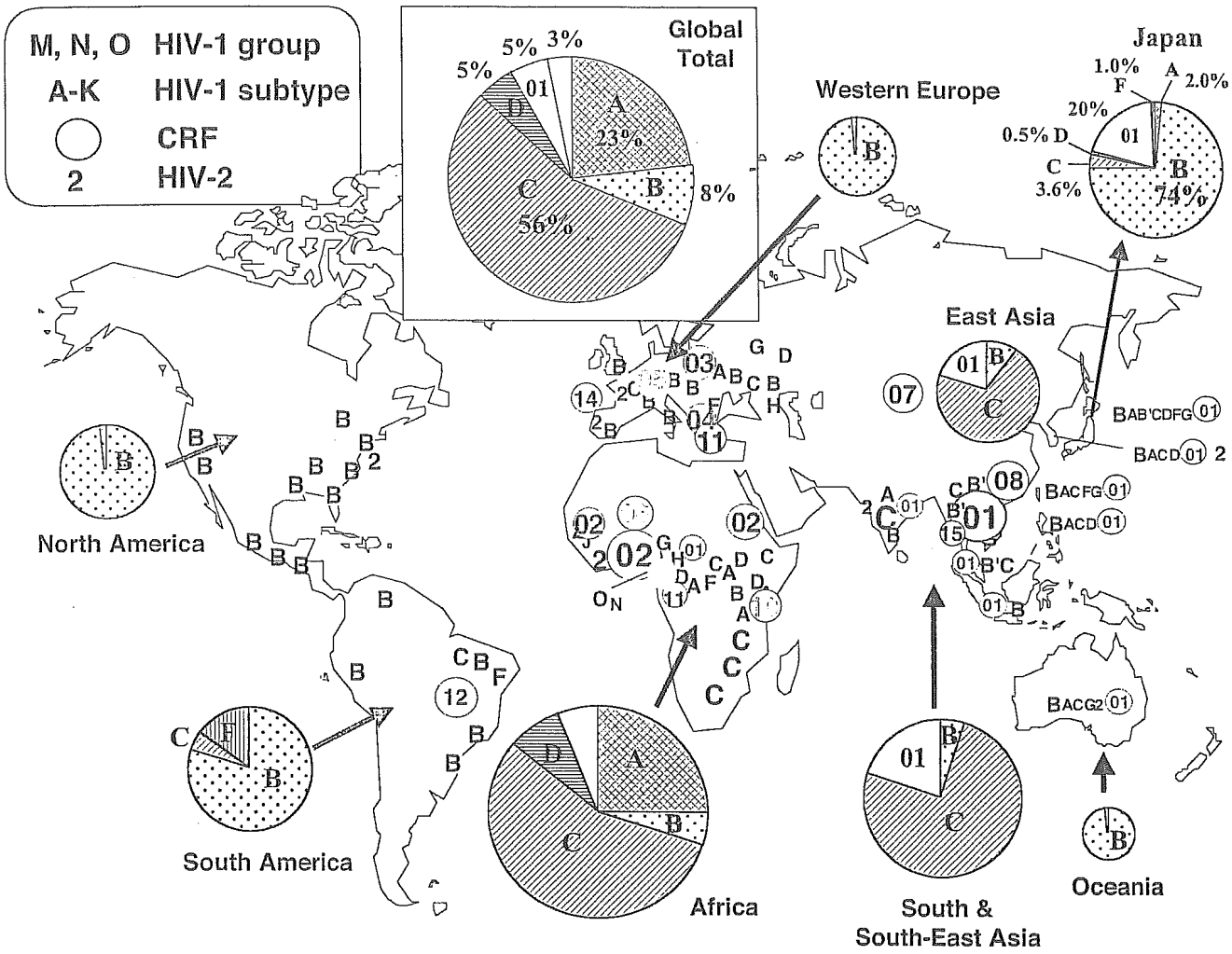

Fig. 2 Global distribution of human immunodeficiency virus (HIV) genotypes. Designations for types, HIV-1 groups, subtypes and circulating recombinant forms are shown in the inset. Genotype distribution illustrated in pie graphs is based on the data from Osmanov *et al.*[20]

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]

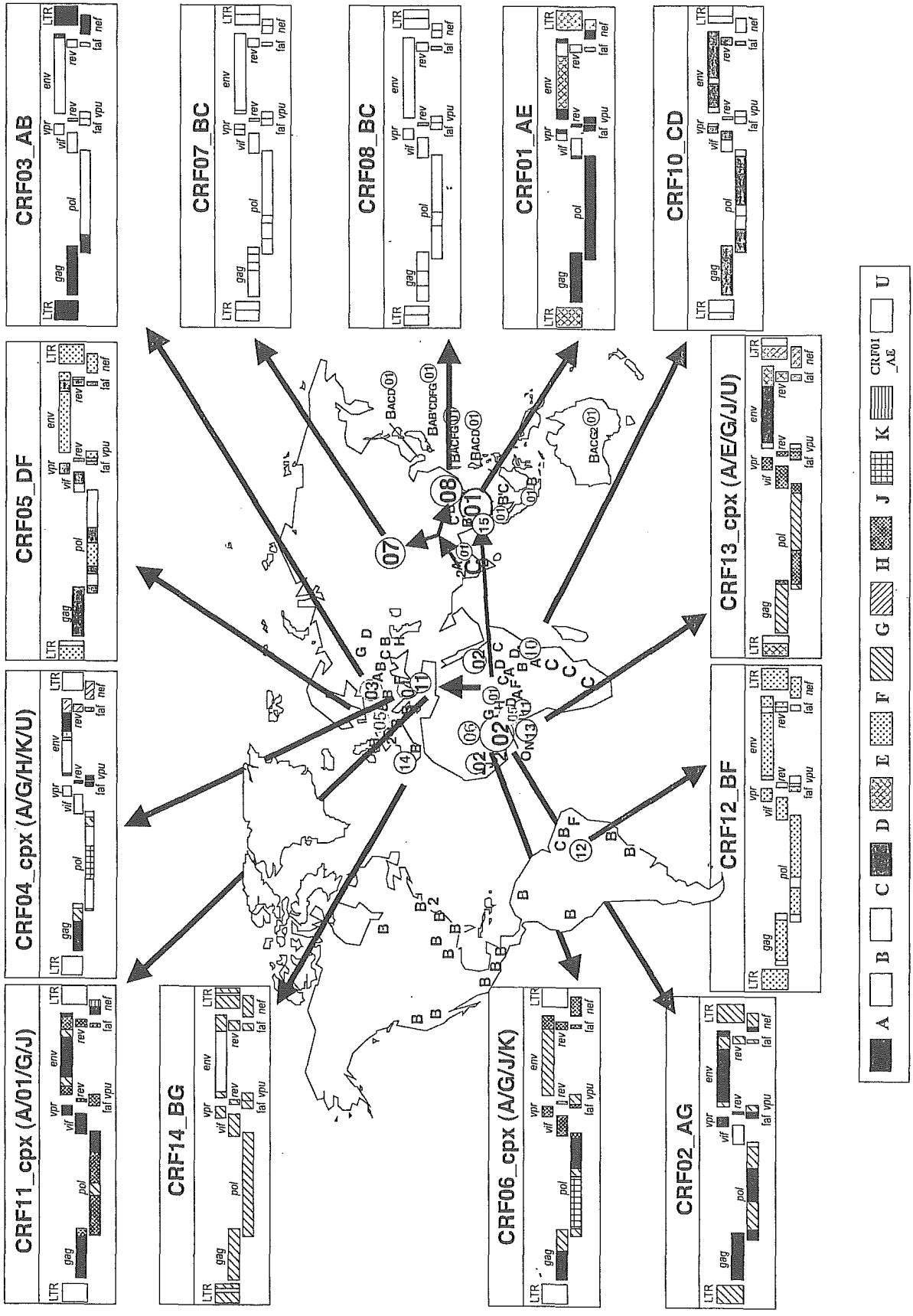

Fig. 3 Recombinant structure of selected circulating recombinant forms (CRF01 through 14) and their geographic distribution.

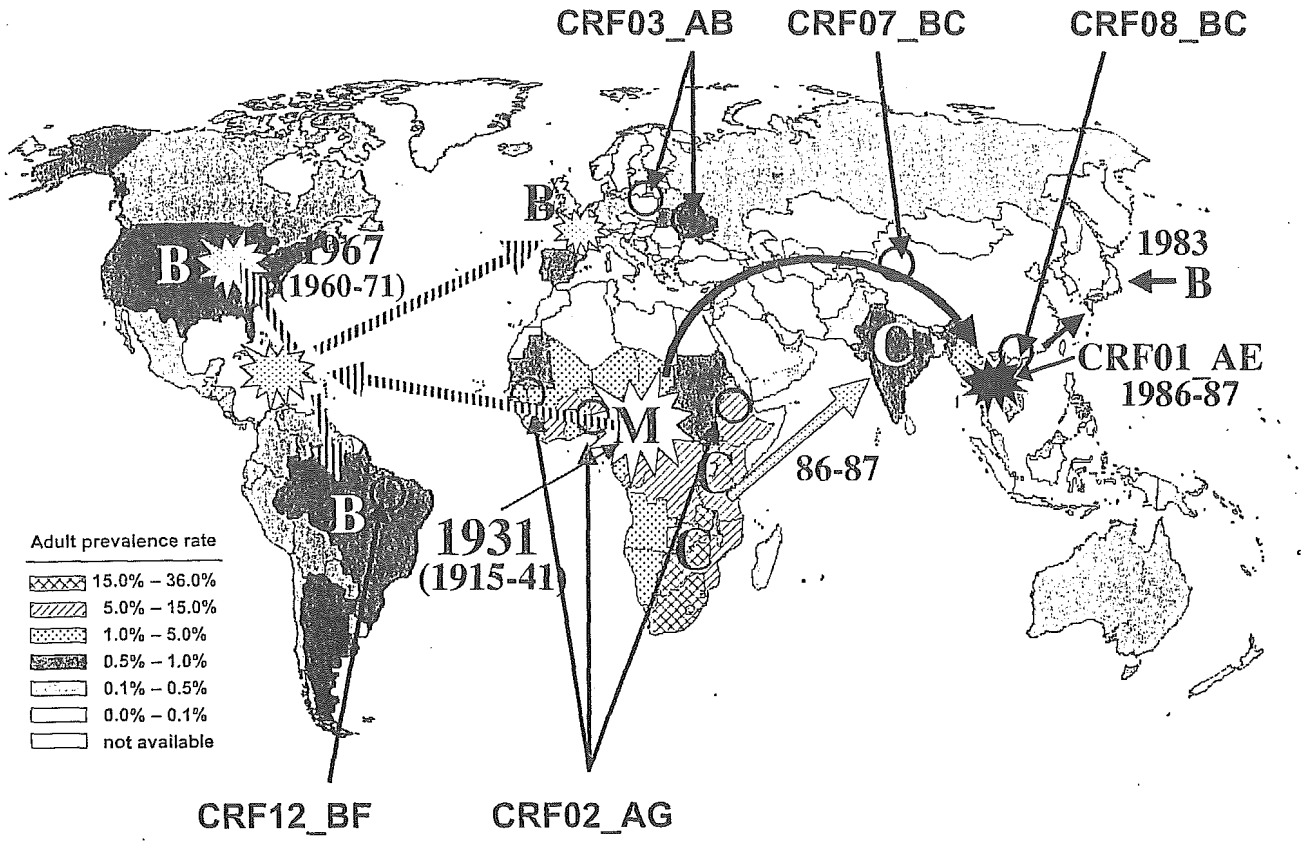

Fig. 4 Origin of HIV-1 group M and plausible route of spread of HIV-1 strains responsible for epidemic in Asia. The geographic focuses of newly emerging circulating recombinant forms are shown on the map.

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

effect of viral subtypes. A study in Thailand showed that the disease progression in patients infected with CRF01_AE is similar to those observed in subtype B-infected populations in the West.[43,44] In contrast, some studies showed that HIV-1 subtypes differ in rates of progression to AIDS. A prospective study of female prostitutes in Senegal showed women infected with subtypes C, D or G were eightfold more likely develop AIDS than women infected with subtype A.[45] In a cohort study in Kenya, where subtypes A, C, and D co-circulate, plasma RNA levels were found to be highest in subtype C.[46] A study conducted in Tanzania suggests that subtypes A and C and recombinant viruses are more likely to be transmitted perinatally than subtype D, suggesting that maternal subtype may play a role in the rate of vertical transmission.[47] The response to the proinflammatory cytokine TNFα is increased in subtype C LTR with triple NFkB configuration, suggesting that subtype C may have a replication advantage in individuals with chronic immune activation.[48] A matched case-control study showed that viruses containing subtype C LTRs were sixfold more likely to be transmitted than those with subtype D.[49] A study in Uganda showed subtype D was associated with faster progression to death and with a lower CD4 cell count than subtype A.[50] In contrast, a study from Sweden showed no differences in disease progression in subtypes A, B, C, or D.[51] However, it is not clear whether such differences are due to environmental factors such as the prevalence of other infectious diseases, including sexually transmitted diseases and parasitic diseases. Long-term prospective studies in recent seroconverters will be needed to elucidate the relationship between HIV-1 genotypes and clinical disease progression.

Subtype-specific difference in antiretroviral drug resistance

Human immunodeficiency virus type 2 (HIV-2) and HIV-1 group O viruses are known to be naturally resistant to non-nucleoside RT inhibitors (NNRTs). Current combination therapies for HIV-1 infection that include NNRTIs (nevirapine, delavirdine or efavirenz) would be less effective for treating HIV-2 infection. A recent X-ray crystal structure of HIV-2 RT shows the structural difference in NNRTI pockets.[52] It is known that substitution of isoleucine for tyrosine at position 181 is the factor that might account for the observed HIV-2 resistance to NNRTIs.[52] Because the single-drug regimens using azidothimidine (AZT, Zidovudine) or nevirapine have been widely used to prevent maternal transmission, it is important to access the natural resistance of those drugs in the target population.

Although we may expect differences in susceptibilities to antiretrovirals between other HIV-1 genotypes, *in vitro* studies have mostly shown similar susceptibilities of HIV-1 group M to currently used antiretrovirals with the exception of some strains of subtype G, which show decreased susceptibility to protease inhibitors, and some subtype F strains that show decreased susceptibility to the TIBO derivative.[53,54] Naturally occurring secondary resistance mutations related to RT inhibitors, including L10I/V/R, K20R, and M36I, are more prevalent in naive individuals with non-B subtype infections.[55] However, it is not known whether the presence of these polymorphisms is associated with increased likelihood of developing drug resistance. In addition, the study showed that D30N is a frequently found primary resistance mutation associated with Nelfinavir in subtype B strain, while it is rarely found in patients infected with CRF01_AE.[56] This may suggest that data derived from subtype B drug resistant genotypes may not always be applicable to non-B subtypes.

Biological implications of recombination

Recombinant viruses may have certain advantages over the parental strain, including modifications in tropism and replication efficiency ('viral fitness'). Under selection pressure imposed by antiretroviral drugs, recombination between strains with different drug sensitivity, resulting in new HIV-1 variants with dual or multiple drug resistance.[57] The discovery of large numbers of recombinant strains clearly suggests that co-infection with different HIV-1 strains is not as rare as once thought. Dual infections with different subtypes have been reported in regions where multiple variants cocirculate. Furthermore, a recent study showed that HIV-1 superinfection can occur in the setting of a strong and broadly directed virus-specific CD8+ T cell response, suggesting that the host immunological responses are not efficient against divergent strains.[58,59] These findings will provide important implications for public health and vaccine development.

Conclusion

The geographic distribution of subtypes is a dynamic and unpredictable process. The intermixing of HIV-1 variants is inevitable. Recombinant viruses have already contributed substantially to the global pandemic, and the likelihood of generating recombinant viruses will continue to increase as the different HIV-1 subtypes spread globally.[60] Even recombinant viruses will recombine, leading to the evolution of second-generation recombinants.[19] Continued monitoring is necessary to determine the emergence of new predominant subtypes and CRFs around the world. It is important to study in more detail the impact of viral genetic variability and recombination on viral properties. In order to develop an efficacious vaccine, it remains to be determined to what

extent humoral and cellular immune response are effective against divergent strains. Molecular epidemiological information about HIV-1 strains prevalent in different geographic regions is critically important in order to elucidate the dynamics of HIV spread and to formulate future vaccine strategies.

Acknowledgments

This study was supported by grants from the Ministry of Health, Labour and Welfare, the Ministry of Education, Science and Technology, and the Japanese Foundation for AIDS Prevention. We thank Drs Naoki Yamamoto, Yoshiyuki Nagai, Kouji Matsushima, Tsuyoshi Nagatake and Kazunori Oishi for their support and advice.

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Pediatrics International (2004) **46**, 236–244

Feature Article

9.

Molecular epidemiology of HIV: Tracking AIDS pandemic

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Abstract

Background: Human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) epidemic is a global threat to maternal and child health, especially in developing countries. It is estimated that 800 000 children are infected and 580 000 children die of AIDS-related illnesses every year. Molecular epidemiology has been a useful tool in analyzing the origin of HIV and tracking the course of global HIV spread. This article provides an overview of recent advances in the field of molecular epidemiology of HIV across the world, and discuss the biological implications.

Methods: Based on the near full-length or partial nucleotide sequence information, the phylogeny and recombinant structure of HIV strains are analyzed. Using genotype classification of HIV as a molecular marker, the origin and the genesis of HIV epidemic are investigated.

Results: The HIV-1 group M, a major HIV group responsible for current AIDS pandemic, began its expansion in human population approximately 70 years ago and diversified rapidly over time, now comprising a number of different subtypes and circulating recombinant forms (CRF). Of note, recent studies revealed that new recombinant strains are arising continually, becoming a powerful force in the spread of HIV-1 across the globe.

Conclusion: Global dissemination of HIV is a dramatic and deadly example of recent genome emergence and expansion. Molecular epidemiological investigation is expected to provide information critical for prevention and future vaccine strategies.

Key words acquired immunodeficiency syndrome, circulating recombinant form, genetic variability, human immunodeficiency virus, molecular epidemiology, recombination, subtype, vertical transmission.

The human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) pandemic continues to expand globally at a rate of 14 000 new infections every day. The Joint United Nations Program on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimated that more than 60 million individuals were infected with HIV worldwide by the end of the year 2002. Of these, approximately 25 million patients have already died, and 42 million people were living with HIV/AIDS.[1] It is known that HIV transmits through blood and sexual contacts and via mother-to-infant. Vertical infection occurs during pregnancy and delivery as well as through breast milk at an estimated rate of 25–30%. UNAIDS estimates that close to 3 million children under 15 years of age are now living with HIV/AIDS worldwide: 800 000 children are become infected and 580 000 children die of AIDS every year (Table 1).[2] Of approximately 14 000 new HIV infections which occur each day, 2000 are in children under 15 years of age and approximately 5000 are women in their reproductive age (Table 1), mostly in developing countries in Africa, Asia and Latin America (Table 2).[2] The HIV/AIDS epidemic has a serious impact in the health of mothers and children.

Molecular epidemiological investigations have been a powerful tool in analyzing the origin of HIV and in tracking the global spread of this pathogen. Since HIV-1 group M began its expansion in the human population early in the 20th century (1931, 95% confidence interval: 1915–1941),[4] it has diversified rapidly, now comprising a number of different subtypes and circulating recombinant forms (CRFs).[3–5]

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Received 7 October 2003.

Classification of human immunodeficiency virus

Phylogenetic analyses of globally circulating viral strains have identified three distinct groups of HIV-1 (M, N, and O),

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.

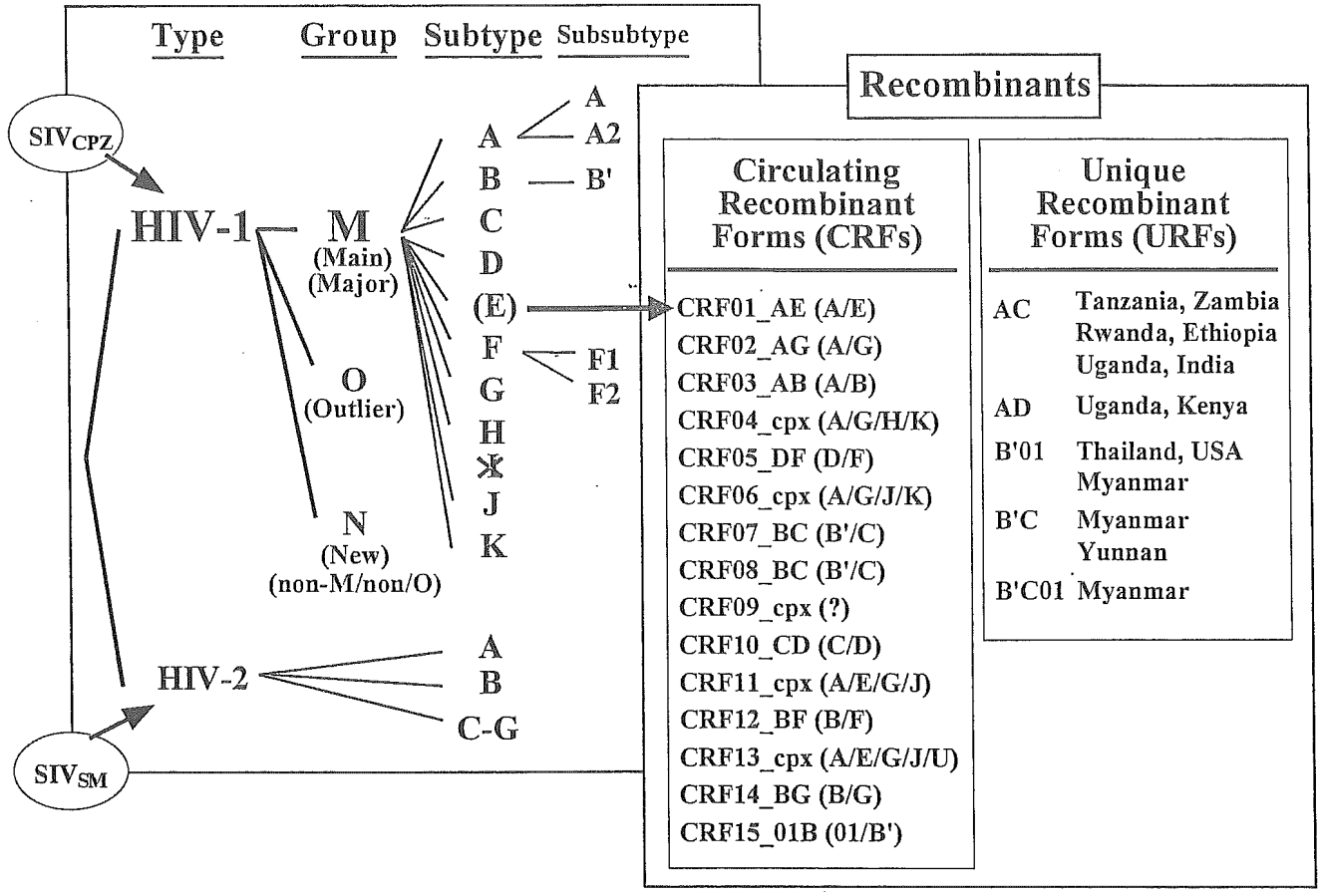

Fig. 1 Classification of human immunodeficiency viruses (HIV). HIV types, HIV-1 groups, subtypes, subsubtypes are shown. HIV-1 recombinants are categorized into two class: circulating recombinant forms (CRFs) and unique recombinant forms (URFs).

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

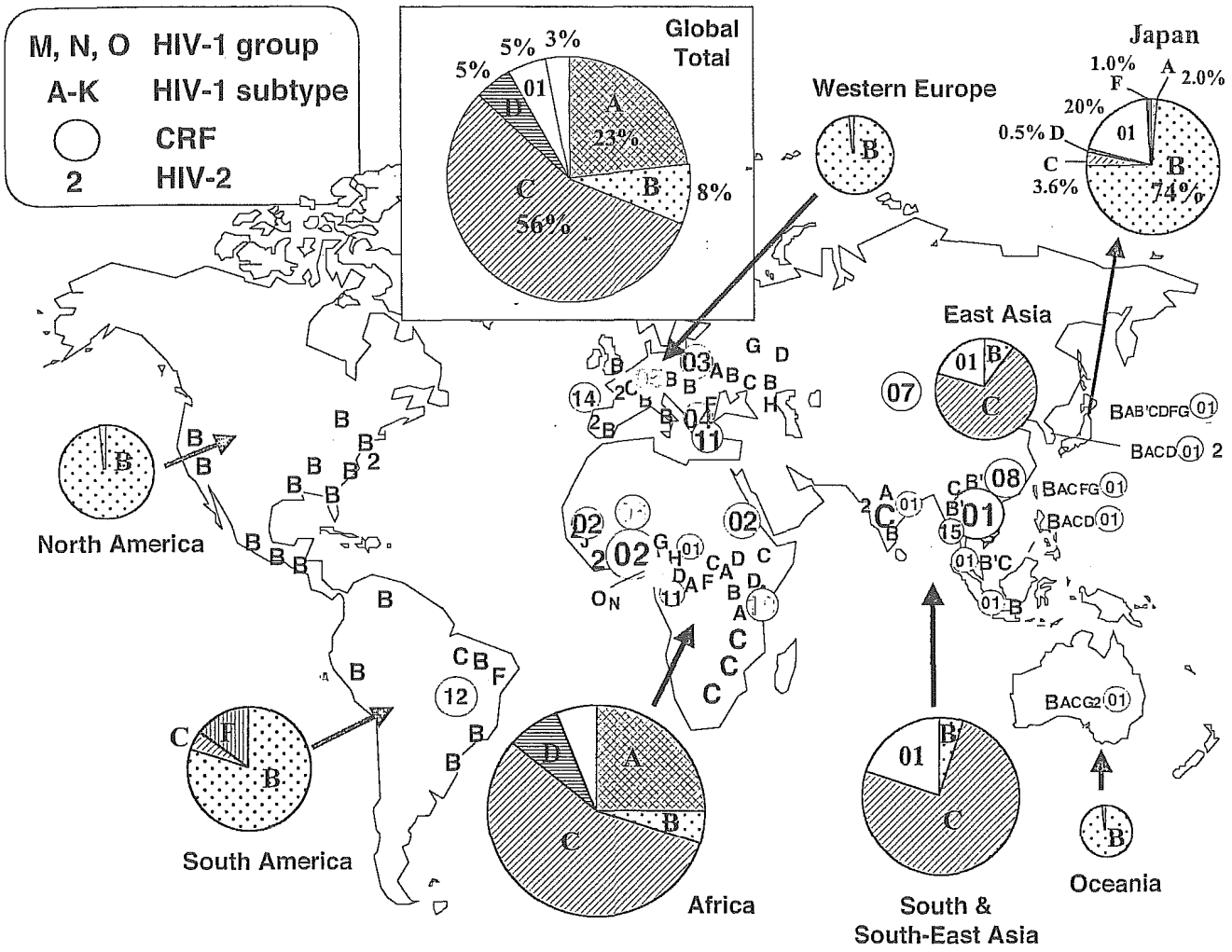

Fig. 2 Global distribution of human immunodeficiency virus (HIV) genotypes. Designations for types, HIV-1 groups, subtypes and circulating recombinant forms are shown in the inset. Genotype distribution illustrated in pie graphs is based on the data from Osmanov *et al.*[20]

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]

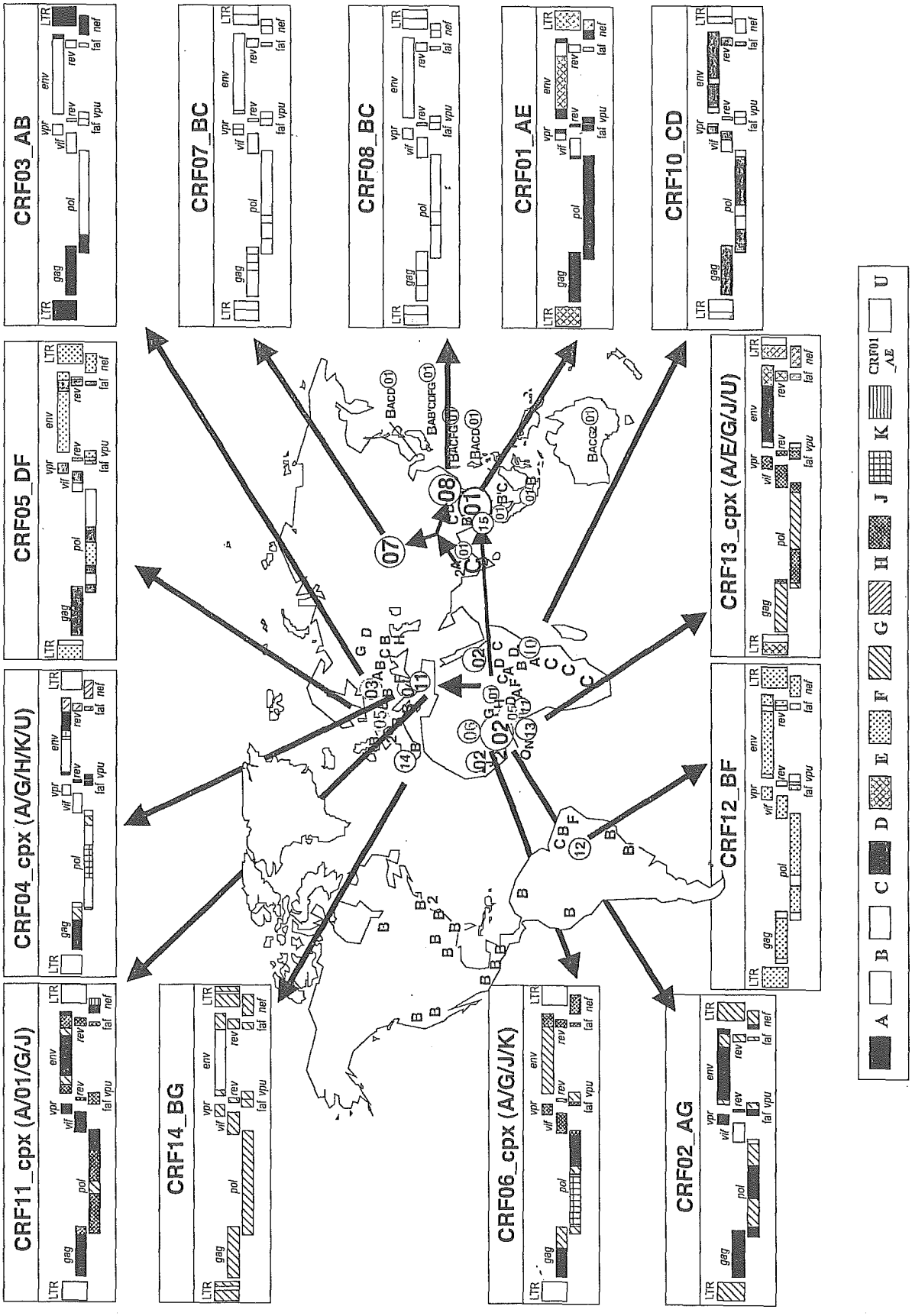

Fig. 3 Recombinant structure of selected circulating recombinant forms (CRF01 through 14) and their geographic distribution.

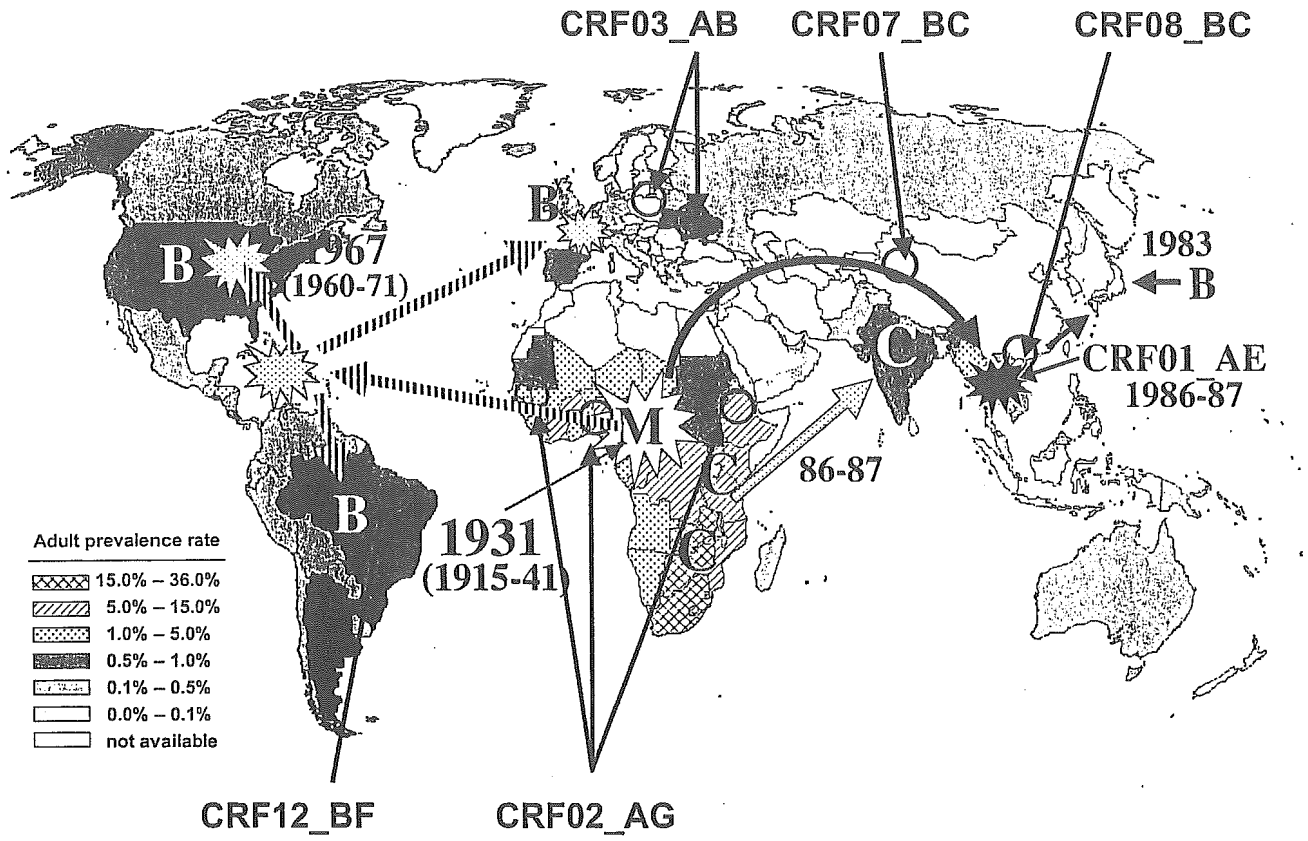

Fig. 4 Origin of HIV-1 group M and plausible route of spread of HIV-1 strains responsible for epidemic in Asia. The geographic focuses of newly emerging circulating recombinant forms are shown on the map.

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

effect of viral subtypes. A study in Thailand showed that the disease progression in patients infected with CRF01_AE is similar to those observed in subtype B-infected populations in the West.[43,44] In contrast, some studies showed that HIV-1 subtypes differ in rates of progression to AIDS. A prospective study of female prostitutes in Senegal showed women infected with subtypes C, D or G were eightfold more likely develop AIDS than women infected with subtype A.[45] In a cohort study in Kenya, where subtypes A, C, and D co-circulate, plasma RNA levels were found to be highest in subtype C.[46] A study conducted in Tanzania suggests that subtypes A and C and recombinant viruses are more likely to be transmitted perinatally than subtype D, suggesting that maternal subtype may play a role in the rate of vertical transmission.[47] The response to the proinflammatory cytokine TNFα is increased in subtype C LTR with triple NFkB configuration, suggesting that subtype C may have a replication advantage in individuals with chronic immune activation.[48] A matched case-control study showed that viruses containing subtype C LTRs were sixfold more likely to be transmitted than those with subtype D.[49] A study in Uganda showed subtype D was associated with faster progression to death and with a lower CD4 cell count than subtype A.[50] In contrast, a study from Sweden showed no differences in disease progression in subtypes A, B, C, or D.[51] However, it is not clear whether such differences are due to environmental factors such as the prevalence of other infectious diseases, including sexually transmitted diseases and parasitic diseases. Long-term prospective studies in recent seroconverters will be needed to elucidate the relationship between HIV-1 genotypes and clinical disease progression.

Subtype-specific difference in antiretroviral drug resistance

Human immunodeficiency virus type 2 (HIV-2) and HIV-1 group O viruses are known to be naturally resistant to non-nucleoside RT inhibitors (NNRTs). Current combination therapies for HIV-1 infection that include NNRTIs (nevirapine, delavirdine or efavirenz) would be less effective for treating HIV-2 infection. A recent X-ray crystal structure of HIV-2 RT shows the structural difference in NNRTI pockets.[52] It is known that substitution of isoleucine for tyrosine at position 181 is the factor that might account for the observed HIV-2 resistance to NNRTIs.[52] Because the single-drug regimens using azidothimidine (AZT, Zidovudine) or nevirapine have been widely used to prevent maternal transmission, it is important to access the natural resistance of those drugs in the target population.

Although we may expect differences in susceptibilities to antiretrovirals between other HIV-1 genotypes, *in vitro* studies have mostly shown similar susceptibilities of HIV-1 group M to currently used antiretrovirals with the exception of some strains of subtype G, which show decreased susceptibility to protease inhibitors, and some subtype F strains that show decreased susceptibility to the TIBO derivative.[53,54] Naturally occurring secondary resistance mutations related to RT inhibitors, including L10I/V/R, K20R, and M36I, are more prevalent in naive individuals with non-B subtype infections.[55] However, it is not known whether the presence of these polymorphisms is associated with increased likelihood of developing drug resistance. In addition, the study showed that D30N is a frequently found primary resistance mutation associated with Nelfinavir in subtype B strain, while it is rarely found in patients infected with CRF01_AE.[56] This may suggest that data derived from subtype B drug resistant genotypes may not always be applicable to non-B subtypes.

Biological implications of recombination

Recombinant viruses may have certain advantages over the parental strain, including modifications in tropism and replication efficiency ('viral fitness'). Under selection pressure imposed by antiretroviral drugs, recombination between strains with different drug sensitivity, resulting in new HIV-1 variants with dual or multiple drug resistance.[57] The discovery of large numbers of recombinant strains clearly suggests that co-infection with different HIV-1 strains is not as rare as once thought. Dual infections with different subtypes have been reported in regions where multiple variants cocirculate. Furthermore, a recent study showed that HIV-1 superinfection can occur in the setting of a strong and broadly directed virus-specific CD8+ T cell response, suggesting that the host immunological responses are not efficient against divergent strains.[58,59] These findings will provide important implications for public health and vaccine development.

Conclusion

The geographic distribution of subtypes is a dynamic and unpredictable process. The intermixing of HIV-1 variants is inevitable. Recombinant viruses have already contributed substantially to the global pandemic, and the likelihood of generating recombinant viruses will continue to increase as the different HIV-1 subtypes spread globally.[60] Even recombinant viruses will recombine, leading to the evolution of second-generation recombinants.[19] Continued monitoring is necessary to determine the emergence of new predominant subtypes and CRFs around the world. It is important to study in more detail the impact of viral genetic variability and recombination on viral properties. In order to develop an efficacious vaccine, it remains to be determined to what

extent humoral and cellular immune response are effective against divergent strains. Molecular epidemiological information about HIV-1 strains prevalent in different geographic regions is critically important in order to elucidate the dynamics of HIV spread and to formulate future vaccine strategies.

Acknowledgments

This study was supported by grants from the Ministry of Health, Labour and Welfare, the Ministry of Education, Science and Technology, and the Japanese Foundation for AIDS Prevention. We thank Drs Naoki Yamamoto, Yoshiyuki Nagai, Kouji Matsushima, Tsuyoshi Nagatake and Kazunori Oishi for their support and advice.

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