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We have demonstrated in populations of HIV-2 and HIV-1 infected individuals that these related viruses have distinct biologic properties. We have shown over a 12 year period the differences in HIV-2 and HIV-1 incidence and prevalence; where HIV-2 appears to be less transmissible by heterosexual sex. In natural history studies we have shown significant differences in the progression to AIDS, where HIV-2 behaved as a less pathogenic virus. This led to the hypothesis that it might protect individuals from subsequent HIV-1 infection, which was shown in our recent studies. In preparation for future vaccine cohorts, we have characterized the HIV-1 subtypes in our seroconverting women and demonstrated differences in their progression to AIDS, with A subtypes showing a longer disease free incubation period compared to subtypes C,D, and G. Immunologic studies of these women have demonstrated that they are capable of recognizing subtype B targets, suggesting that vaccines based on subtype B could be broadly protective.

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Alyssa J. Kerb 4/15/97

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Introduction

As the HIV epidemic spreads globally, the need for HIV intervention becomes more acute. In the developing countries of Africa, it is clear that this epidemic will exact a significant toll in human life and resources. There is an immediate need for research on HIV pathogenesis and intervention strategies, that may be necessary for even modest control of the ever-growing epidemic. As the research and development of HIV-1 vaccine candidate progresses, the preparation of appropriate study populations for efficacy trials is also mandated.

Over the past twelve years, the University Cheikh Anta Diop in Dakar, Senegal and the Harvard School of Public Health in Boston, MA, USA have successfully collaborated on HIV research. A 12-year study of HIV-1 and HIV-2 incidence demonstrates that populations in Senegal are at risk for both HIV types (1). The HIV-1 epidemic is in its early phases, as evidenced by the rapid and significant increase of HIV-1 incidence since 1987. We have previously described a number of biologic and behavioral determinants for both HIV-1 and HIV-2 sexual transmission in the cohort studies of registered female prostitutes as well as risk factors for perinatal transmission in pregnant women (1-4). Our data gathered from this collaboration suggests that high risk populations in Senegal might be highly appropriate for future HIV vaccine trials.

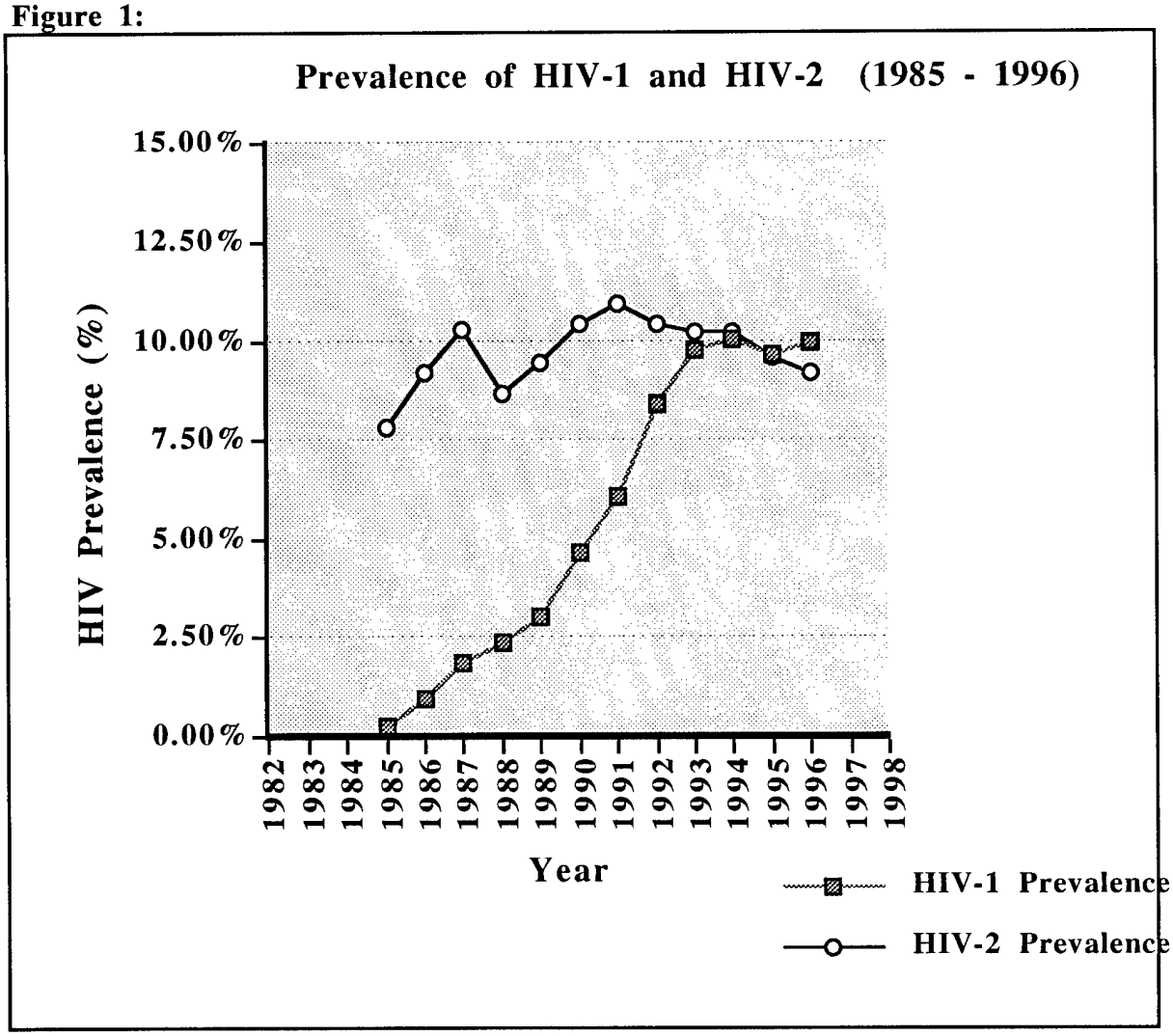
Since the first serologic description of HIV-2 in Senegal, one of the most important scientific questions asked how this related virus might differ from HIV-1 in its biological and clinical consequences (5-7). Critical to answering these questions has been the comparative examination of the epidemiology and pathogenicity of HIV-2 compared to HIV-1 (8-11). Our studies have described and compared the risk determinants for HIV-1 and HIV-2 in prevalent (2) and incident infection (1), distinct temporal trends in incidence (1), and different incubation periods to the development of AIDS (12, 13). We have also shown that HIV-2 infection may provide some protection from subsequent HIV-1 infection (14, 15).

In 1970, the government of the Republic of Senegal instituted a program for the registration of self-identified female prostitutes, legalizing their practice of providing sex for payment. This program required quarterly evaluation for sexually transmitted diseases at clinic centers and treatment if necessary. These centers, originally managed by social workers and nurse practitioners, were joined by our study physicians in Dakar (1985), Kaolack (1987) and Ziguinchor (1987). Three cohorts of registered female prostitutes from three different Senegalese cities were established and have been evaluated for seroprevalence, seroincidence and identification of risk factors for HIV-2 and HIV-1 infection (2). Sub-cohorts from each site have been monitored for clinical and immunologic abnormalities to characterize the natural history of HIV-2 infection (13, 16, 17). This is a unique study population with both HIV virus types and the longest and largest prospective study of HIV infected individuals conducted in Africa.

Prevalence and Incidence Trends of HIV-1 and HIV-2 Infection (1)

Our prospective studies conducted in the Dakar cohort has provided the unique opportunity of measuring the infection rates of both HIV-1 and HIV-2 in registered female prostitutes. From the beginning of our studies in 1985, there was already significant HIV-2 infection at about 8% which has remained stable over the 12 years. HIV-1 prevalence was quite low in 1985 and has increased to over 10% in 1996. Prevalence trends for both viruses are shown in Figure 1. Our original study published in Lancet, 1994 described incidence trends for HIV-1 and HIV-2 from 1985 to 1993 (1). We have continued our study of HIV-1 and HIV-2 incidence, most recently as a HIVNET international site (1994-1997). Current incidence data is for both HIV-1 and HIV-2 is shown in Figure 2. Over the twelve year period, we have evaluated over 1800 women that were initially enrolled in the study as HIV seronegative and followed with over 7500 person years of observation have been recorded.

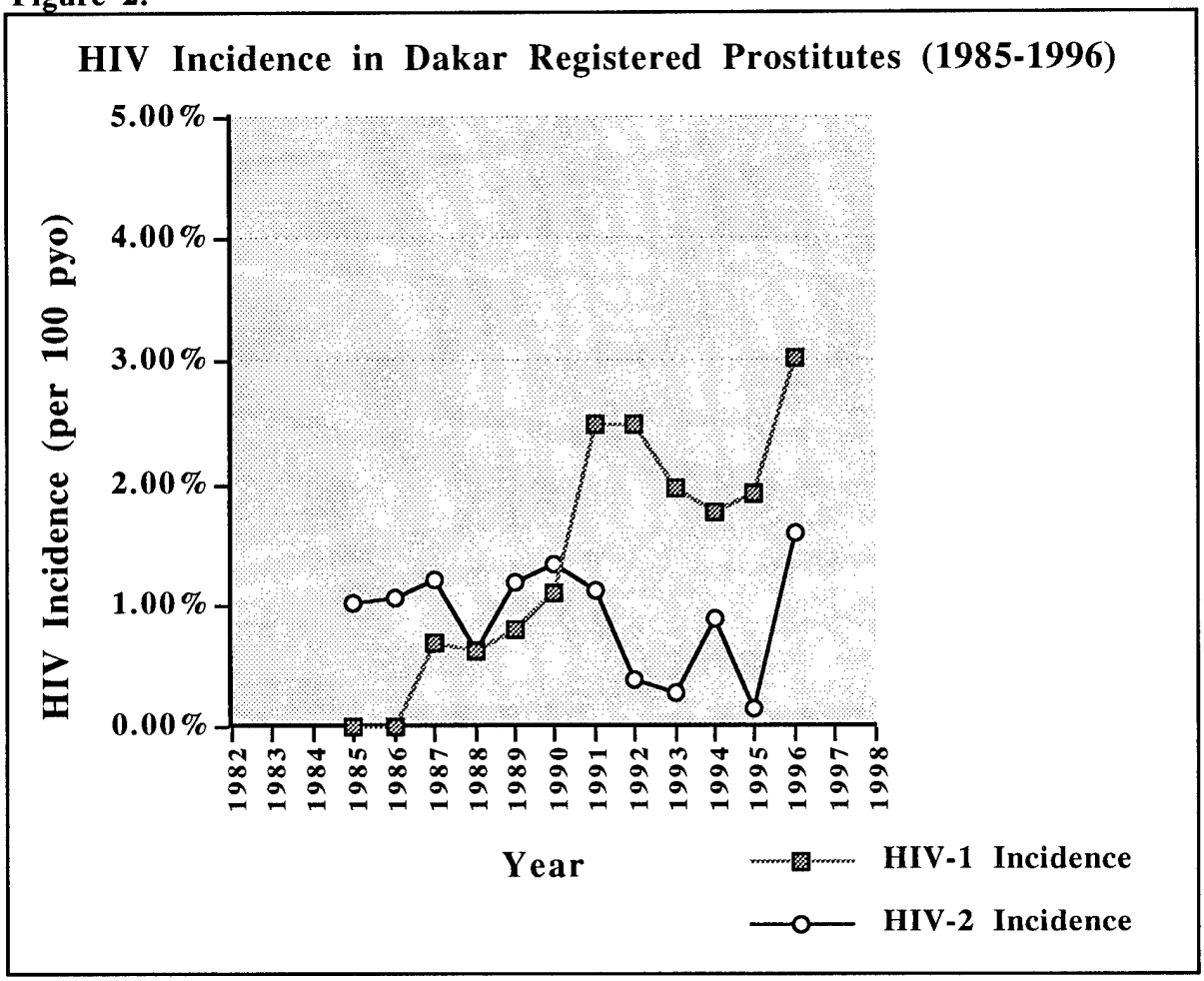
Figure 1:



HIV-1 annual incidence has risen dramatically from 0 per 100 pyo in 1985-86 to over 3.00 per 100 pyo in 1996. Since 1992, the HIV-1 incidence has oscillated between 1.9 and 3.10 per 100 pyo (Figure 1). The HIV-2 annual incidence has also shown annual fluctuations and had appeared to be decreasing until 1996, this was confirmed by a relatively constant prevalence of HIV-2 throughout the study period (Figure 2). Although this population is clearly experiencing new infections by both HIVs, the data indicates that HIV-1 is the more recent and increasing source of HIV infection. Similar to the analysis performed in the original Lancet paper, we have updated our analysis on risk determinants of HIV incident infection (1). A poisson regression model was constructed with incident HIV infection as the outcome and nationality, years of registration, age at registration and study year as variables. Ghanians

were at higher risk for HIV-1 infection compared to Senegalese and other West Africans (IRR=1.77; 95%CI= 1.01 - 3.08). The risk of HIV-1 seroconversion increased by 1.18 (95%CI = 1.10 - 1.26) for each additional study year. Based on this analysis we would predict that the HIV-1 incidence in our cohort will be 3.56 per 100 pyo in calendar year 1997.

Figure 2:



In order to evaluate changes in the risk of HIV-2 or HIV-1 infection during the study period, we obtained estimates for the increased risk associated with each additional calendar year of exposure. The estimates were obtained while simultaneously adjusting for age, nationality, years of registered prostitution, calendar year, and time in the study. The relative risk for HIV-2 infection associated with a subject entering a new calendar year from the previous one was not significant (relative risk, 1.04, 95% confidence interval, 0.89-1.21). There was, however, a significant trend for increasing risk of HIV-1 infection during the study period with

a relative risk of 1.43 (p value<0.002, 95% confidence interval, 1.15-1.78) associated with movement from one year to the next. This suggested that the risk of HIV-1 infection increased 12-fold over the first 8 years of the study period (1).

In areas of the world where the major mode of virus spread is via heterosexual transmission, incidence studies have been rarely conducted. A study of female prostitutes in Nairobi, Kenya has previously reported an HIV-1 seroincidence rate of 47% based on a total person-years of 176 (18), this rate being significantly higher than our study. There are a number of possible explanations for these differences. The prevalence of HIV-1 in our cohort was significantly lower than that of the Nairobi cohort, which is currently at close to 99% infection. Our incidence estimates are based on a significantly larger denominator of 7500 person-years, with narrow confidence intervals indicating relative lack of imprecision on the incidence estimates. In addition, the women in our study were regularly evaluated for sexually transmitted diseases and counseled on HIV prevention as part of the study design, which may have resulted in lower incidence rates. Nonetheless, these data are important in emphasizing the trends of HIV incidence outside of an actual intervention trial setting. Not only is this the only study of HIV-2 incidence but is one of the longest prospective studies of HIV-1 incidence worldwide. The fluctuations of HIV incidence in a high risk cohort provided with regular counseling and condom distribution, may serve as an important baseline indicator of what we may anticipate in clinical trial settings. The availability of such long-term incidence data will allow more precise predictions of future incidence rates and this data is critical to the ultimate success of HIV intervention trials.

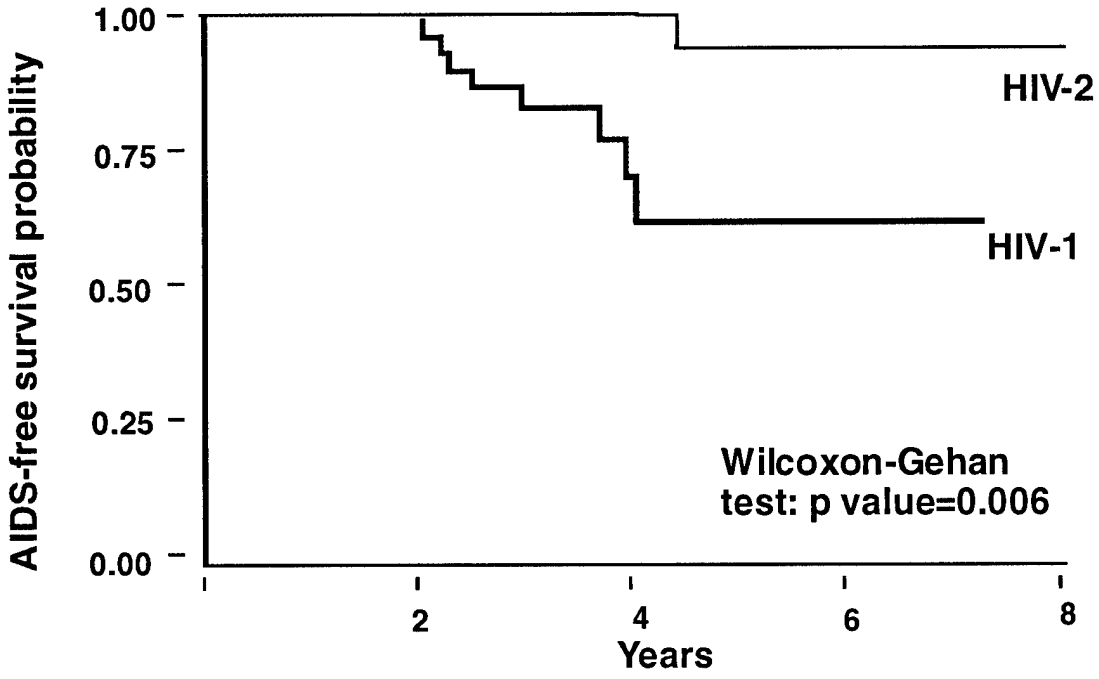
Natural History of HIV-2 (19)

This prospective study was undertaken to define and compare the natural history of HIV-2 and HIV-1 infection. This was the first study to evaluate disease progression rates of both HIV viruses in a cohort of asymptomatic individuals followed prospectively. We have analyzed disease progression in HIV-1 and HIV-2 infected women with both prevalent and incident

infection. Furthermore, we have utilized clinical outcomes of AIDS, CDCIV disease and drops in CD4+ lymphocytes below 400 cells/mm3, in these comparisons (19). Results from the original eight year study and an update now including 12 years of study, indicate that the rate of AIDS development is dramatically different between the two HIV viruses.

The incidence rates of AIDS development in 36 seroincident HIV-2 infected women was 0.63 (95%CI = 0.1-4.48). Among the 53 seroincident HIV-1 infected women AIDS incidence was 5.11 per 100 pyo (95%CI=2.55-10.22). Kaplan-Meier analysis comparing HIV-2 and HIV-1 seroincident women, showed a 60% AIDS-free survival for HIV-1 incident women, at 5 years post infection, compared with 97% AIDS-free survival for HIV-2 (Wilcoxon-Gehan test; p value = 0.006)(Figure 3). These differences in survival probabilities between HIV-2 and HIV-1, were also seen for CDC IV disease and CD4+ lymphocyte counts below 400 cells/mm^3 as outcomes. (19, 20).

Figure 3: AIDS-free Survival Probability comparing HIV-1 and HIV-2



The rate of HIV progression in African populations has been studied less frequently and, until this study, the average observation period was not longer than 2 years and none had described

AIDS progression in incident cohorts (21). Studies concerning the natural history of chronic infections such as HIV are difficult to achieve and our study has been successful in minimizing loss to follow-up, despite the developing country setting. We feel that our study will continue to document the distinct natural histories of HIV-2 versus HIV-1 infection. Importantly, these studies may provide data on the rates of AIDS development in heterosexually acquired HIV infection where antiviral therapy is not administered. Our experience and data from this natural history study may also be useful to consider in evaluation of future HIV vaccine candidates. Although, protection from infection is the ultimate goal, the use of other outcomes including disease development have been suggested (22). The data we have collected may be useful in planning trials that evaluate such alternative outcome measures.

Evaluation of HIV recombinant-env peptides [39]

We have evaluated recombinant-HIV env peptides in a dot-blot format for use as a type-specific HIV screening assay. Semi-purified recombinant-expressed HIV-1 (566) and HIV-2 (996) env proteins, homologous with the N-terminal region of gp41 (HIV-1) and gp35 (HIV-2), have been described (23, 24). These antigens were first evaluated in an immunoblot assay and then adapted to a dot-blot miniblotter technique. The 566 (HIV-1) peptide showed 100% sensitivity and specificity, and the 996 (HIV-2) peptide performed similarly, but showed the presence of HIV-1 cross-reactive epitopes. When the two env peptides were used together, there was high specificity and sensitivity for detecting HIV positive sera both in immunoblot and dot blot formats. The relative cost ($0.70/sample) of this assay was 10-fold lower than conventional commercial assays and could be easily performed in less than 2 hours. This assay has been extensively field tested, and we have continued to perform quality control of the method with back to back immunoblot testing performed in Boston over the past 7 years.

HIV-2 Protection from HIV-1 in Senegal (14, 15)

Demonstrated differences in the infectivity and disease potential of HIV-2 compared to HIV-1 support the notion that the mechanism for such protection might be analogous to the attenuated

virus vaccine model. We have shown that HIV-2 positive women were at lower risk of HIV-1 infection than were HIV negative women, controlling for changes in sexual behavior using incident sexually transmitted disease. The original report of 70% protection was based on 9 years of observation (14).

The re-analysis of our data with two additional years of observation and alternative methods of analyses have shown that HIV-2 positives were at lower risk of HIV-1 infection compared to HIV negatives with an adjusted IRR ranging from 0.48 to 0.26 (52 to 74% protection), completely consistent with our published results (15). In all analyses, a statistically significant protective effect of HIV-2 was demonstrated. Further studies on the mechanism of how HIV-2 infection appears to protect over half of the population at risk for HIV-1, should aid in the future design of vaccine candidates that are broadly protective across HIV subtypes.

Table 1: Adjusted HIV-1 rate ratios comparing HIV-2 positives to HIV negatives derived from multivariate Poisson regression models simultaneously adjusting for HIV-2 status, years of registered prostitution, age at registration, nationality, study year, CD4+ lymphocyte count and incident sexually transmitted disease (15)

Study Design/Analysis	HIV-2 Serostatus	# of Subjects	Adjusted Rate Ratios	95% Confidence Interval	Fraction Protected (%)
All registered female sex workers	Negative	1264	1.00		
	Positive	199	0.36*	(0.13 to 0.99)	64%
All registered female sex workers - Sensitivity analysis	Negative	1264	1.00		
	Positive	199	0.48*	(0.24 to 1.00)	52%
Suggested cohort analysis matched 2:1	Negative	374	1.00		
	Positive	187	0.27*	(0.10 to 0.76)	73%
Updated cohort analysis matched 2:1	Negative	398	1.00		
	Positive	199	0.26*	(0.09 to 0.72)	74%

* $p < 0.05$.

HIV Genital Shedding and genetic variation (25)

We evaluated the virus from blood and cervico-vaginal samples of 25 HIV-2 infected and 11 HIV-1 infected women in our cohort study. The prevalence of viral shedding in genital secretions was 16% and 36.4% for HIV-2 and HIV-1 infected women, respectively. We amplified and sequenced multiple clones of the C2-V3-C3 region of the surface envelope glycoprotein from cervical secretions and PBMC samples from three HIV-2 infected individuals, and the C2-V3 region from four HIV-1 infected individuals (25). A phylogenetic analysis showed that in most cases the sequences from the blood and the genital secretions were intermingled and subclusters were not discerned according to the sample site. In one HIV-1 individual, tissue specific sequences were observed. The average nucleotide distance was similar in both genital secretions and blood, as was the distance between sequences from the two sites in the majority of the women. Viral shedding appeared to be more common in HIV-1 than in HIV-2 infected individuals. Viral compartmentalization between sites was not evident in most individuals.

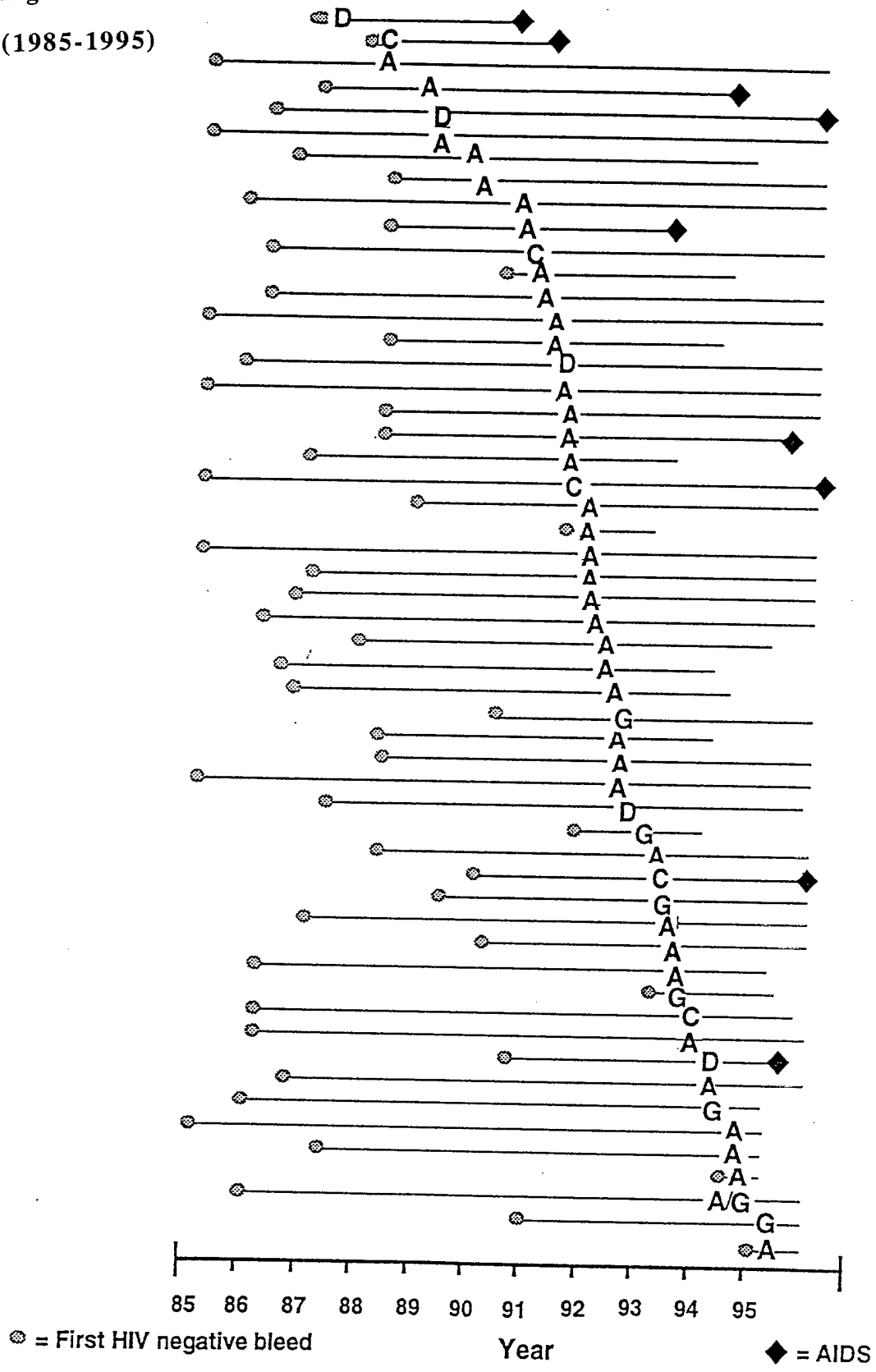
HIV-1 Subtypes in Senegal (26)

Many studies have now documented that multiple HIV-1 subtypes may be present in populations sampled at the same time, and this is particularly true in sub-Saharan Africa (27). However, to date, our appreciation of different subtypes has been limited to cross-sectional studies. Over the period of February 1985 to December 1995, we have evaluated the HIV-1 subtype in 54 of 81 HIV-1 seroconverters (Figure 4). HIV-1 subtype was assigned based on the C2-V3 sequence in 53 of 54 samples (26). Subtype designation was confirmed with phylogenetic tree alignment, and relatively high bootstrap values demonstrate the branching order of sequences with known HIV-1 subtypes A, C, D and G. Subtype A was found in 37 of 54 seroconverters (68.52%), 5 of 54 were subtype C (9.26%), 5 of 54 were subtype D (9.26%), and 6 of 54 were subtype G (11.11%). One (1.85%) of the V3 envelope sequences demonstrated significant variation from other subtype A sequences and was examined by boot-

strap scanning (28) which revealed a mosaic virus composed of subtypes A and G. This was further verified with sequencing of the complete gp120 portion of the envelope. The majority of the gp120 aligned with subtype A but approximately 300bp in the V2 to C3 region aligned with subtype G. Further studies are underway to investigate the role of the A/G recombinant in HIV-1 transmission in this cohort.

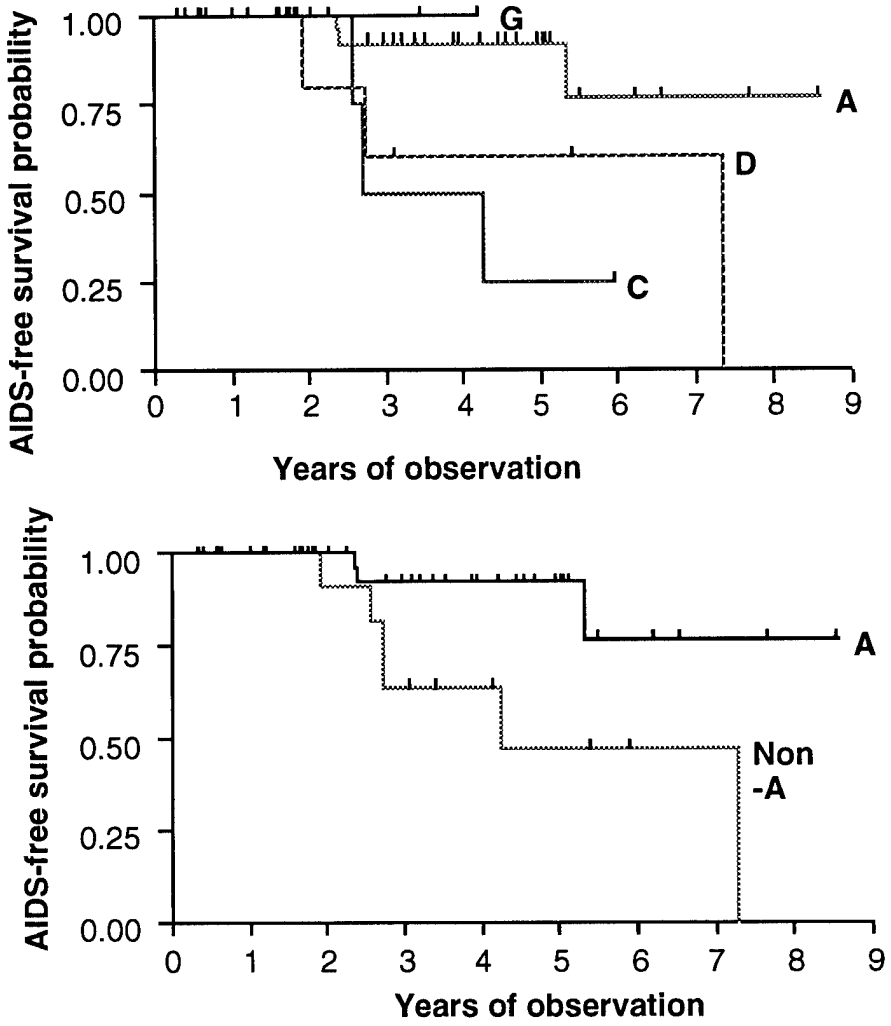
The distribution of seroconverters over the 10 year period is illustrated with the HIV subtype designated at the estimated seroconversion date (Figure 4). In this high risk group of women, the first seroconversions to HIV-1 occurred in late 1987. Throughout the study period, subtype A was the most common HIV-1 subtype. The first seroconversion to subtype C was in late-1987 and the last seroconversion was observed in late 1993. Similarly, subtype D was observed early in 1987 and then again sporadically until early 1994. Subtype G infections first appeared in mid-1992, and since that time have represented 20% of the HIV-1 subtypes.

Figure 4: HIV-1 Subtypes in Seroconverters, Dakar registered prostitutes (1985-1995)



This is also the first study that has evaluated disease progression in non-B subtype infections with known time of infection (26). In evaluation of the AIDS-free survival curves of women with incident A, C, D, and G infection, we have found significant differences in the AIDS-free survival (Figure 5a). Due to the small sample size per subtype, with few AIDS cases despite a lengthy study period, our estimate of AIDS incidence for each subtype may be considered imprecise, and further study is clearly warranted. The grouped comparison of non-A versus A subtypes demonstrated a significantly longer AIDS-free survival for women infected with subtype A. The 5 year AIDS-free survival probability was 92% in subtype A infected women compared to 50% for non-subtype A infected women (Figure 5b). Although, subtype A was the major subtype contributing to HIV-1 incidence in this high risk cohort, the AIDS-free survival was significantly longer for women with this subtype infection.

Figure 5a & b: AIDS-free Survival in HIV-1 seroconverters by Subtype



Differences in HIV subtype transmission rates may require that HIV subtype diagnosis be part of intervention trials, whether they be vaccine or non-vaccine based. Gilbert et al. have described the "sieve analysis" which may be important in analysis of candidate HIV vaccines in populations with multiple subtypes (29). The prior knowledge of circulating subtypes and their dynamics will lend confidence to analyses aimed at evaluating differential susceptibility of HIV subtypes to various intervention strategies. We are currently completing the sequence analysis of all HIV-1 infected women in our cohort, including all prevalent infections. Therefore, in

response to this SFP, we anticipate having complete subtype data on this cohort, which we feel will be invaluable.

Cross-reactive CTLS across diverse HIV-1 subtypes - (30)

The development of an effective HIV/AIDS vaccine will require the induction of immune responses that target specific viral subtypes in a given geographic region, or the induction of broadly cross-reactive immune responses. Most vaccines currently in development are based on clade B immunogens. Although this is the predominant clade in the US and Western Europe, clades A, C, D, E and others predominate in the areas of the world with the greatest burden of infection (31) To evaluate the CTL cross-recognition in individuals infected with non-clade B HIV-1, we obtained cryopreserved PBMC from HIV-1-infected individuals from Senegal, West Africa. The PBMC were selectively expanded into CD8+ cells and tested for HIV-specific CTL activity using autologous B-LCL infected with subtype B vaccinia constructs. Fourteen individuals were evaluated of whom ten were infected with clade A virus, three with clade G and one with clade C. All 14 subjects tested demonstrated recognition of clade B vaccinia constructs, using vectors expressing Gag, Env, RT and Nef proteins. Seven seronegative Senegalese individuals were evaluated concomitantly and no HIV-1-specific CTL activity was detected using similar protocols.

The relative contributions of CTL to protective immunity are not clear but most vaccine strategies include the induction of CTL responses as a primary endpoint. A number of vaccines capable of inducing CTL responses are presently in field trials or soon to begin, including both canarypox vectors and polynucleotide vaccines. Our data demonstrate significant cross-reactivity for CTL responses and provide support for initiating trials with clade B immunogens in Africa.

Laboratory Methods:

HIV serodiagnosis

All serum samples will be evaluated by immunoblot to HIV-2 (MS-U937) and HIV-1 (IIIb-Molt); no screening test will be employed (2, 5). Specimens will be classified as positive if antibodies directed at *env*, ± *gag*, ± *pol* antigens are recognized. Samples with antibodies to envelope antigens only, will be classified as positive if two or more *env* antigens are recognized (32). All nitrocellulose sheets impregnated with viral antigens are pre-tested with control sera to assure uniformity in serodiagnosis. Radioimmunoprecipitation analysis (RIPA) of S35-cysteine labeled whole cell lysates of HIV-1 and/or HIV-2 will be performed on sera with indeterminate immunoblot results, as previously described (33).

STD Diagnostic Assays

Serologic testing for syphilis will be done with a rapid plasma reagent assay (Becton Dickinson, Cockeysville, MD) and a microhemagglutination assay for antibodies to *T. pallidum* (Miles, Elkhart, IN). Chocolate medium supplemented with isovitalex, colistin, nystatin and with and without vancomycin will be inoculated and incubated in CO2 atmosphere for the diagnostic of *Neisseria gonorrhoeae.* Wet preps and Gram staining of genital secretions will also be examined for the diagnostic of *Trichomonas vaginalis*, *Candida albicans* and non-specific vaginitis (clue cells and *Mobiluncus sp.*). In the presence of genital ulceration, dark field microscopy will be used to detect *T. palladium*, and material collected from the base of the ulcer will be examined after Gram staining and inoculation onto GC agar (GIBCO Laboratories, Grand Island, NY) supplemented with isovitalex, hemoglobin, horse serum and vancomycin and incubated at 33°C in CO2 atmosphere to detect the presence of *Haemophilus ducreyi* infection. Chlamydia
trachomatis diagnosis is currently performed with an antigen ELISA assay (Syva, Palo Alto, CA). Over the past year, we have also developed the PCR assay in Dakar, which appears to be more sensitive (described below).

PCR diagnosis of Chlamydia trachomatis

Cervical secretions will be collected by wiping the cervix with 3 cotton swabs followed by dilution in 2 ml of PBS per swab (34). After vortexing and centrifugation the cell pellet is resuspended in 1% SDS, boiled for 15 min. and subjected to standard phenol-chloroform extraction. All DNA samples are first evaluated for integrity with a b-globin PCR. The primers used for generating a 201-bp fragment of the *Chlamydia trachomatis* endogenous plasmid were CTP1 (sense; 5'-TAGTAACTGCCACTTCATCA-3') and CTP2 (antisense; 5'-

TTCCCCTTGTAATTCGTTGC-3). DNA extracted from a *C. trachomatis* culture was used as positive control for PCR and subsequent hybridization.Chlamydia PCR products were visualized on a 1% agarose gel stained with ethidium bromide. The blots were confirmed by southern blotting and hybridization with a specific internal oligonucleotide probe (sense; 5'-ATCTCATTACCATGCATTAGCAGCTA TCCA-3') (35). In addition, samples can be evaluated by more standard direct immunofluorescence assay from endocervical secretions (Chlamydia DST, Syva, Palo Alto, CA).

Storage and processing of serum blood clots for DNA extraction

Blood specimens will be drawn at each clinic visit, in a 14- ml vacutainer clot tube, labeled with the subject number and date. After the clot has formed, the sera will be withdrawn from the tube using a disposable plastic pipette, leaving the clot which will be processed later for DNA extraction. The sera will be aliquoted into 2 appropriately labeled cryopreservation tubes, one of which will be shipped to the Boston lab for serology while the other will remain frozen at -20° C in Dakar. The clot remaining in the tube will be frozen at -70° C for processing at a later time according to the following protocol adapted from the method described by Everson, et al. (36).

After thawing the clot in a 37° C water bath, the clot will be vortexed until homogenized, and the red blood cells lysed by washing with saline solutions as follows: the clot will be mixed with 20 ml of cold (4° C) 0.2 M NaCl, and after sitting for 20 s, an additional 20 ml of a cold 1.6 M NaCl solution will be added. After mixing, the tube will be spun at 400 g for 10 minutes in a refrigerated centrifuge, and the supernatant will be aspirated. The saline wash step will be repeated at least once, or more, until the pellet shows no red appearance which would indicate the presence of intact red blood cells. After the final supernatant aspiration, the pellet will be resuspended in 3 ml PBS, pH 7.2, and mixed with 3 ml STE buffer. Then 50 U proteinase K will be added and the sample will be incubated at 37° C for at least 4 hrs. At this point, the samples will be frozen for shipment to the Boston lab, where the DNA will be extracted from the samples using standard phenol/chloroform extraction procedures.

DNA Extraction and PCR methods

Whole blood samples are collected and peripheral blood mononuclear cells (PBMCs) will be separated by centrifugation in Leukoprep tubes (Becton-Dickinson, Mountainview, CA) then lysed with Proteinase K (Boehringer Mannheim; Indianapolis, IN). DNA will be extracted twice with equal volumes of phenol-chloroform, and once with chloroform, and then precipitated with two volumes of ethanol. DNA pellet will be resuspended in TE buffer

(10mM Tris-HCl, 1 mM EDTA, pH 7.5), and concentrations measured by optical density (OD) at 260 nm on a spectrophotometer. These procedures will be conducted in the laboratory in Dakar. DNA samples can then be easily shipped or transported to Boston at regular intervals. All DNA samples are additionally evaluated for quality of PCR quality DNA by B-globin PCR on separate assays (37). This assures that a PCR negative result is not due to inadequate DNA in the sample, irrespective of OD readings.

HIV-1 and HIV-2 nested primer pairs will be used for both env and gag regions. For HIV-2, the primers used were described previously (38) and are summarized in the following table:

NESTED PRIMER PAIRS FOR AMPLIFICATION OF HIV-2 SEQUENCES

REGION	POSITION (ROD)	NAME	SEQUENCE (ROD) 5'-3'
GAG	539-561	OG53	GTGGGAGATGGGCGCGAGAAACT
GAG	637-660	OG63	TAAAACATATTGTGTGGGCAGCGA
GAG	810-833	OG81	CACGCAGAAGAGAAAGTGAAAGAT
GAG	1062-1085	OG106	GGATTTCAGGCACTCTCAGAAGGC
ENV	7782-7805	OG778	GGGATAGTGCAGCAACAGCAACAG
ENV	7837-7861	OG783	TGTTGCGACTGACCGTCTGGGGAAC
ENV	7950-7973	OG795	GTCTGCCACACTACTGTACCATGG

The HIV-1 primers used have been previously described (39) and are summarized in the following table:

NESTED PRIMER PAIRS FOR AMPLIFICATION OF HIV-1 SEQUENCES

REGION	POSITION (HXB2)	NAME	SEQUENCE (HXB2) 5'-3'
GAG	1214-1234	881	GTGACATCAGGCCATATCACC
GAG	1669-1686	882	ACCGGTCTACATAGTCTC
GAG	1407-1426	883	GAGGAAGCTGCAGAATGGG
GAG	1646-1672	990	GGTCCTTGTCTTATGTCCAGAATGCTG
ENV	6539-6559	401	GAGGATATAATCAGTTTATGG
ENV	6976-6999	404	AATTCCATGTGTACATTGTACTG
ENV	6560-6579	402	GATCAAAGCCTAAAGCCATG
ENV	6876-6895	403	CAATAATGTATGGGAATTGG

HIV-2 PCR

One microgram (μg) of DNA template or H6/H8 fragments of JSPH-27 plasmid (40) will be added to a 50 μl PCR reaction mixture of: 50 mM KCl, 10mM Tris-HCl, 6.7 mM MgCl2, 200 μM dNTPs, 5 μM outer primers, and 1.25 units Amplitaq DNA polymerase (Perkin-Elmer, Norwalk, CT). This mixture is then subjected to the following cycles: 95° x 1", (95° x20 sec, 55° x20 sec, 70° x20 sec) x 30 cycles, 70° x2". Hot start method will be used (41) and cycles performed on a MJ Research PTC-100 cycler (MJ Research, Watertown, MA). Five microliters will then be taken from the first round reaction and added to an identical reaction mixture with the 32P-labeled inner primer pair and undergo the same temperature cycling.

HIV-1 PCR

One microgram (μg) of DNA template or 8E5 cell line for copy number control (42) will be added to a 50 μl PCR reaction mixture of as described above. This mixture will then be subjected to the following cycles: (95° x 1", 50° x1", 68° x3") x 25 cycles. Again, 5 μl will be removed upon completion of the cycles and added to the above reaction mixture with the 32P-labeled inner primer pair, and given the same temperature cycling.

Upon completion of nested PCR, 15μl of amplified DNA will be run on a 2% agarose gel, stained with ethidium bromide, visualized by UV illumination, referenced to Gel Marker size standards (Research Genetics, Huntsville AL) and excised. The gel piece containing the band of 32P-labeled amplified DNA will be counted in a scintillation counter and compared to a standard curve generated by copy number controls (H6/H8 plasmid fragments or 8E5 cell DNA). Copy number for each input sample will be calculated using the equation of the line generated by the standards.

HIV-1 Genotyping by PCR

To amplify the HIV-1 C2-V3 region, a nested PCR is performed using two sets of primers: WT1:5'GCTGGTTTTGCGATTCTAAAGTGTA 6884-6908, positions relative to HXB2) and WT2:5'CAATAGAAAAATTCCCCTCCACAAT (7353-7377), for the first round; and published primers, KK30-KK40 (43) for the second round. The PCR reactions are performed in a 100μl volume reaction mixture containing the DNA, 10μl of 10X PCR Buffer II (Perkin-Elmer PCR Reagents, Roche Molecular Systems, Branchburg, NJ), 2.5 units of Taq Polymerase (Perkin-Elmer PCR Reagents, Roche Molecular Systems, Branchburg, NJ), 0.2 mM of each dideoxy-nucleotide and 2 mM of $MgCl_2$. Each reaction is subjected to 30 cycles of denaturation (45 seconds at 94°C), annealing (45 seconds at 57°C and 55°C for the first and second round respectively) and extension (45 seconds at 72°C) followed by a final

extension of 3 minutes at 72°C in an automatic thermal cycler. Negative controls include deionized water, and negative PBL DNA in all experiments.

The PCR products are purified by agarose gel electrophoresis and purification columns (Qiaquick gel extraction kit, Quiagen Inc., Chatsworth, CA) and directly sequenced using the second round primers (KK30 and KK40). When necessary the purified product is cloned in pCR2.1 vector (T/A cloning, Invitrogen, San-Diego, CA). Positive colonies are chosen based on X-gal metabolism or after PCR screening. Plasmid preparation for double-stranded DNA sequencing are performed by alkaline lysis, using DEAE columns (Qiagen plasmid minikit, Quiagen Inc., Chatsworth, CA). Sequence is determined by dye terminator cycle sequencing using Taq polymerase (Perkin-Elmer, Applied Biosystem Division, Foster City, CA). and an automatic sequencer ABI 373A (Perkin-Elmer, Applied Biosystem Division, Foster City, CA).

Conclusions:

Our studies of HIV-1 and HIV-2 infection in people have demonstrated important biological differences that may help to focus further virologic and immunologic investigations. As research results have accumulated, we have grown to appreciate the tremendous diversity these viruses possess, at the level of genetic variability, epidemiologic patterns, and host pathogenesis. In order to better understand the virologic properties of these viruses, we must now attempt to link our observations from in vitro and genetic analysis studies to studies in infected populations. New and innovative technologies will be required to more readily diagnosis subtypes, recombinants, and quantitate viral burden. Our understanding of HIV-1 subtype epidemiology is evolving and future studies are likely to provide better data on the subtype distribution and their epidemiology. These studies will be critical to future predictions of the epidemic, and they may also indicate differences in transmission and disease potential that will impact the design of future global HIV interventions.

The data that we have recently published on HIV-2 protection and HIV-1 clade differences are highly relevant to HIV vaccine development. In one case we have demonstrated natural protection from HIV-1 infection, which might indicate that vaccine candidates evaluated for similarity to HIV-2 and HIV-1 cross-reactive epitopes would be reasonable. Our studies with

HIV-1 clades suggest that different biologies of the clades will complicate the future HIV-1 epidemic in the future. It also suggests that evaluation of vaccine trials in cohorts with mixed clades will require consideration of these potential differences. Finally, our demonstration that HIV-1 non-B clade individuals are capable of mounting cellular immune responses to B clade targets would suggest that a single clade vaccine may provide protection from a broad number of genetically diverse clades. Further studies will be critical to the design and development of candidate HIV vaccines, which we hope will be broadly efficacious.

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