ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2001) 19: 259-265

Virological, Immunological and Host Factors in HIV-1 Discordant Couples in Thailand

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SUMMARY **The potential factors of resistance to HIV-1 infection were investigated in 23 HIV discordantly infected couples, of each, one partner had HIV infection and the matched spouse was not infected. Both partners of the HIV discordant couples possessed comparable number of $CD4^+$ cells expressing CCR5. Our study demonstrated that resistance to HIV-1 infection was not due to low level of HIV viral load in their infected-matched spouses. In addition, selective biological phenotype of HIV clinical isolates, which is indicative for risk of transmission, could not be determined in this study. However, we have demonstrated that the unknown genetic factor(s), and neutralizing antibody of broad and high activity could be taken into an account for resistance to HIV infection in the HIV discordant couples.**

According to the Bangkok Collaborative Perinatal HIV Transmission Study, it was reported that 26% of sexual partners of HIV infected women were not infected.[1] Multiple factors including host genetic polymorphism in the chemokine receptors,[2,3] MHC class I restricted cytotoxic T lymphocytes,[4,5] HIV-1 specific cellular immunity,[6] serum neutralizing antibody[7,8] and mucosal immunity[9,10] might be involved in the protection against HIV-1 infection. A homozygous mutation in the gene encoded for CCR5 (*Δ32 ccr5/Δ32 ccr5*) coreceptor has been related with protection from HIV-1 infection in the multiple exposed but not infected persons.[2] Single point mutation at m303 allele in CCR5 gene has been found to confer resistance to HIV-1 infection.[3] Other studies attempted to find a relationship between cytotoxic T lymphocyte response to HIV-1 specific epitopes and the lack of HIV-1 transmission in the HIV-1 exposed-seronegative persons.[5,6] Moreover, a high frequency of $CD8^+$ MHC class I restricted cytotoxic T lymphocytes to specific peptides and possession of HLA-A11 have been associated with HIV seronegative female prostitutes in Kenya.[4] Autologous neutralizing antibodies to primary HIV-1 isolates and broadly neutralizing antibodies to heterologous HIV-1 isolates have been found to reduce the risk of vertical transmission.[7,8] In addition, the presence of HIV-1 gp160 specific IgA in cervicovaginal lavage may provide protection against HIV-1 infection in the HIV-1 seronegative partners of some discordant couples.[9-11] According to the HIV-1 phenotype, the macrophage-tropic strains were likely to be selected for transmission.[12,13] One explanation may be the presence of massive number of the target cells bearing CCR5 over the number of cells expressing CXCR4 in the infected persons.[14]

This study aimed to investigate virological factors, host factors and the host specific antibody response in HIV discordantly in-

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fected couples as follows: 1) expression of CCR5 on CD4$^+$ lymphocytes of the couples; 2) biological characterization of HIV-1 isolates from the infected partners in term of the ability of the virus to replicate in T-cell line, autologous and heterologous peripheral blood mononuclear cells (PBMC) and primary macrophage/monocyte culture; 3) susceptibility of PBMC from the uninfected partners as the target cells for HIV infection; 4) presence of neutralizing antibodies in sera of the infected partners to the HIV-1 autologous isolates as tested in PBMCs derived from the uninfected–matched partners and to the T-cell line adapted (TCLA) subtype B and E strains; and (5) presence of the binding antibodies to HIV-1 *env* regions (gp120, gp41, CD4/gp120 binding site) in sera from the infected partners.

MATERIALS AND METHODS

Study population

HIV-1 discordantly infected couples (HIV discordant couples) were assigned as one partner had HIV infection, whereas the matched spouse was not infected in this study. Twenty-three HIV discordant couples, who attended the Antenatal Care Unit, Department of Obstetrics and Gynaecology, Siriraj Hospital during 1999 to 2000, were enrolled with informed consent. The infected spouses (19 pregnant women and 4 men) were infected with HIV subtype E based on V3 peptide ELISA.[15] The asymptomatic females had no history of antiretroviral drug treatment whereas 3 out of 4 males were on drug therapy. Only 4 (2 males and 2 females) infected cases had a history of other sexually transmitted disease. Questionnaires were administered for the information of sexual behavior and condom usage. All had regular unprotected sex during the last 12 months and had at least one offspring.

EDTA blood and clotted blood were collected from all spouses. Within 4 hours of collection, PBMC's were separated and isolated immediately; whereas the remainder was stored in a liquid nitrogen tank (for biological analysis). Plasma and sera were kept at –70°C (for viral load) and –20°C (for antibody response) until tested. HIV-1 infection was diagnosed by positive anti-HIV testing as determined by 3 HIV-1/2 screening assays (Axsym HIV1/2 MEIA, Abbott, USA; Uniform I HIV1/2 EIA, Organon Teknika, Belgium and Serodia, Fujirebio, Japan) according to a WHO recommendation (WHO, 1997).[16] In this study, the uninfected spouses were ruled out of HIV infection by: 1) HIV seronegative results as determined by 2 screening assays; 2) negative result for the detection of *gag/pol* by polymerase chain reaction (PCR); and 3) absence of p24 antigen in serum by ELISA (Organon).

CD4$^+$ T-lymphocytes were determined by a FACScan flow cytometry (Becton Dickinson, San Jose, CA, USA) using CD3$^+$/CD4$^+$ monoclonal antibodies (Becton-Dickinson).

HIV-1 plasma viral load was assayed by reversed transcriptase PCR using an Amplicor HIV-1 Monitor (version 1.5) (Roche Diagnostic Systems, Branchburg, NJ, USA) test kits.

The second group of subjects consisted of 50 infected females whose matched spouses also had HIV infection. All were asymptomatic pregnant women who attended the Antenatal Care Unit, Siriraj Hospital. This group was used as control of the study on the antibody response in comparison with the infected females from discordant group.

Enumeration of activated CD4$^+$ lymphocytes expressing CCR5

CD4$^+$ lymphocytes and activated CD4$^+$ cells (HLA-DR$^+$ marker) bearing CCR5 (as indicated by 2D7 monoclonal antibody) were measured by a three-color flow cytometry including fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin, phycoerythrin (PE)-conjugated anti-CD4 and peridinin chlorophylly protein-conjugated anti–HLA-DR+) as previously described.[14]

Viral isolation and biological characterization

HIV-1 was isolated from PBMC's of the infected spouses by using standard co-culture method as previously described.[17] Positive isolation was shown by the presence of at least 1 ng/ml of p24 antigen in the culture supernatants collected on 2 consecutive time points as tested by ELISA (Vironostika, Belgium). The supernatants containing HIV-1 were stored in liquid nitrogen until tested. The biological phenotype of HIV-1 isolates was characterized as syncytium inducing (SI) or non-SI (NSI) in MT-2 cell line.[18]

The ability of HIV-1 to replicate on primary monocyte-derived macrophage (MDM) culture was performed as previously described.[19] Replication of HIV-1 in the

adherent macrophage cells was demonstrated by the presence of p24 antigen in the culture supernatant.

Kinetic replication of the autologous HIV-1 isolates in PBMCs from the uninfected-matched spouses

Kinetic replication of HIV-1 isolates obtained from the infected spouses was performed in PBMCs cultures either derived from blood of the matched-uninfected spouse or from blood donors using standard co-culture technique. Briefly, viral stock (75 µl) was added into a 96-well tissue culture plate containing 10^5 PHA-activated PBMC (150 µl) for overnight. After the washing step, supernatant was taken for p24 antigen determination on days 0, 7, 14, 21 and 27. The reference subtype E (SL17) was used as the virus control in order to check the quality of PBMC cultures in supporting HIV replication.

Neutralizing (NT) antibodies to HIV-1 primary isolates and T-cell line adapted (TCLA) strains

NT antibodies were performed on PBMCs target cells derived from the uninfected–matched spouses or healthy donors by using end point dilution assay as previously described.[18] The final serum dilution giving 50% reduction of p24 antigen concentration in the supernatant collected after 7 days of infection were considered positive for NT antibodies.

In addition, NT antibodies to TCLA strains (subtypes B: MN/H9 and Thai E: 42368/H9) were determined using a syncytium based assay as described above. The final serum dilution giving 90% reduction in syncytium formation on C8166 cell line after 3-5 days of infection as compared to the viral control culture was considered positive for the presence of NT antibodies.

HIV-1 binding antibodies to gp120, gp41 and CD4/gp120 binding site (BS)

Antibodies binding to recombinant gp120 HIV-1 IIIB (rHIV-1_{IIIB}, Medical Research Council, ADP, UK) and gp41 (DQELLE-LDKWASLWN) consensus peptides (Protein & Peptide Research, Devon, UK) were determined by indirect ELISA as previously described.[15] Positive antibodies in the test serum were determined by the optical density (OD) of greater than the cut-off value ([mean OD of a panel of HIV-1 negative normal control +3 S.D.] x 2) as assayed in 15 negative sera.

Antibodies to CD4 binding site on gp120 (CD4/gp120 BS) were determined by the ability of the antibodies to inhibit the binding of gp120 to soluble recombinant CD4 (srCD4) as previously described.[15] The antibody titer was determined as the final serum dilution at which 50% blocking of sCD4 binding occurred when compared to a panel of HIV-1 negative control sera (n = 7, 100% sCD4 binding activity). Internal positive (HIV-1 positive sera) and blank (without the addition of sCD4 and sera) controls were included.

Statistical analysis

Level of HIV-1 antibody in the infected females from the discordant couples and the concordance couples were compared and analyzed for the statistically significant value ($p < 0.05$) by non-parametric Mann-Whitney U test.

RESULTS

Demographic data of the subjects

Median of $CD4^+$ lymphocyte counts in the HIV-1 infected female (n = 19) and male (n = 4) subjects of the discordant couples were 232 (13%) and 74 (5%) cells/ml, respectively, while those of the uninfected-matched male (n = 19) and female partners (n = 4) were 616 (29%) and 624 (33%) cells/ml, respectively. Median of CCR5 expression on $CD4^+$ cells in the infected females and males was 21% and 22%, respectively, whereas that of uninfected males and females was 19% and 20%, respectively. The number of activated $CD4^+$ bearing CCR5 in the infected females and males with median of 5% and 14% was compared to those of the uninfected partners in males and females 2% and 4%, respectively. Median of viral load among the infected females and males were 60,800 and 171,300 copies/ml, respectively.

Biological characterization and kinetic replication

Ten out of 23 HIV-1 clinical isolates were selected for further investigation on biological characterization due to the availability of high infectivity stocks. Of the 10 isolates, 6 were NSI in MT-2 cells, and 5 could replicate in primary MDM culture.

In this study, all 10 isolates could replicate in their matched–uninfected target cells and donor PBMCs *in vitro*. Kinetic replication of 9 HIV-1 in PBMC culture

derived from the matched uninfected spouse or from blood donors was comparable to that of the SL17 virus control growing in the same types of cell system. Kinetic replication of the virus designated DA23 the representation of the 9 isolates as shown in Fig. 1A. Interestingly, DB13 an HIV isolate replicated poorly in PBMC culture derived from her matched uninfected spouse, but replicated well in culture derived from blood donor as shown in Fig. 1B.

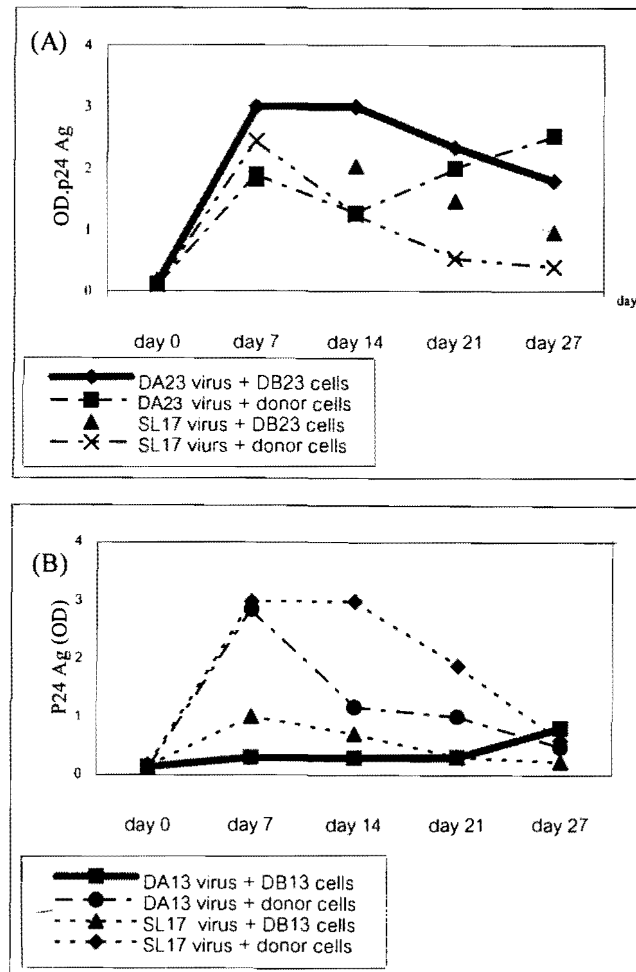

Fig. 1 Kinetic replication of HIV-1 clinical isolates (DA) in target cells (DB) from the uninfected-matched partners and blood donors. SL17 (subtype E) clinical isolate was used as the control virus.

NT antibodies to HIV-1 clinical isolates and TCLA strains; and binding antibodies to envelope region

Among the 23 infected spouses of the discordant couples, NT antibodies to their autologous HIV-1 from clinical isolate on PBMC's-based cultures (either derived from matched-uninfected spouse or blood donor) were low or absent (≤ 10) as tested. In order to compare the immune response in matched control, all 4 males in infected discordant couples would be excluded at this point. Significantly, higher NT antibodies to TCLA strains (subtypes B and E) were found in the infected females from discordant couples, compared to those of the control infected females from concordant couples ($p < 0.05$) (Table 1). Among the infected females from the discordant and concordant groups, the median of binding antibody levels to gp120 (12,800 *vs* 12,800), gp41 (<100 *vs* <100) and CD4/gp120 BS (80 *vs* 40) were not of significant difference ($p > 0.05$) (Table 2).

Table 1 Level of HIV-1 neutralizing antibodies among the infected females from HIV-1 discordantly infected couples as compared to those females from HIV-1 concordantly infected couples

	Neutralizing antibodies in HIV-infected females*			
	Discordant group (n = 19)		**Concordant group** (n = 50)	
	HIV-1$_B$	**HIV-1$_E$**	**HIV-1$_B$**	**HIV-1$_E$**
Median	20	10	< 10	< 10
Mean	32	32	15	< 10
Range	< 10-80	< 10-160	< 10-80	< 10-40

*Antibody titers are expressed as the reciprocal of endpoint serum dilution and p value was < 0.05 as analyzed by non-parametric Mann-Whitney U test.

DISCUSSION

The potential mechanism

Table 2 Level of HIV-1 binding antibodies among the infected females from HIV-1 discordantly infected couples as compared to those females from HIV-1 concordantly infected couples

	Binding antibodies in HIV infected females*					
	Discordant group (n = 19)			Concordant group (n = 50)		
	gp120	gp41	CD4/gp120 BS	gp120	gp41	CD4/gp120 BS
Median	12,800	< 100	80	12,800	<100	40
Mean	21,389	824	222	18,164	550	83
Range	1,600-51,200	< 100-12,800	20-1,280	< 100- 51,200	< 100-6,400	< 10-320

*Antibody titers are expressed as the reciprocal of endpoint serum dilution and p value was < 0.05 as analyzed by non-parametric Mann-Whitney U test.

of resistance to HIV-1 infection in the discordant couples including host factors, virus factors and host specific antibody response was investigated. Owing to a report of persistent HIV-1 seronegative results in a few HIV-infected subjects, the uninfected partners were screened not only by anti-HIV testing but also by p24 antigen assay and PCR for HIV *gag/pol* DNA.

Viral load has been reported as a risk of heterosexual transmission. Transmission rate is dose dependent such that no transmission occurred at viral load 1500 copies/ml.[21] In our study, most of the infected subjects had enough viral load for transmission except one infected female from HIV discordant group with a low viral load (698 copies/ml). Therefore, escape from infection as seen in the uninfected matched spouses should be explained by other mechanism.

Observation for CCR5 expression in $CD4^+$ cells showed that numbers of cells bearing CCR5 in the uninfected partners of the discordant couples were similar to those observed in the infected matched spouses in this study. These numbers were also within the normal range as compared to that previously reported in the healthy uninfected subjects.[14] Other investigators have demonstrated that resistance to HIV infection was associated with homozygous mutation deletion in gene encoded for CCR5 (*Δ32 ccr5/Δ32 ccr5*).[2] Our study showed normal level of cells expressing CCR5 in the uninfected partners of the HIV discordant couples and showed no deletion in gene encoded for CCR5 molecules (unpublished data).

In our limited number of HIV clinical isolates in primary macrophage culture, these MDM isolates could be identified regardless of differences in biological phenotypes. Replicate capacity in MDM has been suggested to be an important selective factor for transmission of HIV-1.[13] Escape from infection as seen in the uninfected partners of the discordant couples could not be explained by the absence of the appropriate viruses. Thus, the study on biological phenotypes including growth in macrophage culture also could not reveal a selective indication for risk of transmission.

PBMC culture from 9 of the 10 uninfected partners were susceptible to HIV-1 isolates obtained from their matched infected spouses with the kinetic replication similar to that observed in normal healthy donors *in vitro*. PBMC from one subject, DB13, poorly support growth of HIV-1 isolates from the matched spouse, and also resisted to the control SL17 virus. Thus, genetic factor might play role in the resistance mechanism. Our observation showed no Δ32 CCR5 deletion in this DB13. Other host genetic mutation on chemokine receptors such as CCR5 promoter and coding region as well as CCR2 mutation had been associated with the resistance of HIV-1 transmission.[3,22] And again, DB13 subjects had not shown any mutation in other gene encoding chemokine receptors i.e., CCR5m303 and CCR5 at promotor gene, CCR2-64I and SDF-1 3'UTR were not found as well (data not shown). Therefore, the unique feature of genetic mutation might not account for the resistant of HIV-1 infection in this uninfected case.

Regarding the humoral immune response to HIV infection, HIV infected females from discordant (n = 19) and concordant (n =

50) groups did not develop NT antibodies to their autologous HIV isolates. However, infected females in discordant group had significantly higher and broader NT activity than those infected females in concordant group as assayed against TCLA subtype B and E strains. Lower NT antibody titer in the infected females from the concordant couples should not be due to low capacity of these subjects to synthesize the specific immunoglobulin, since the level of binding antibodies to various envelope regions were not significant different from those of the infected females from the discordant couples.

In conclusion, our data demonstrated that unknown genetic factor(s) and neutralizing antibodies of broad and high activity may be attributable to resistance to HIV infection.

ACKNOWLEDGEMENTS

This study was granted supported by Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand and Japanese Foundation of AIDS Prevention. We are grateful to Dr. Yutake Takebe (National Institute of Infectious Disease, Tokyo for his suggestion on finance. The biological reagents were provided by the EUA/MRC Centralized Facility for AIDS Reagents, NIBSC, UK (Grant Number QLK2-CT-1999-00609 and G822102).

The authors would like to thank the nurses' team from the Department of Obstetrics and Gynaecology and all the volunteers for participation in this study. We also acknowledge Professor T. Shioda, Drs. H. Liu and E.E. Nakayama (Research Institute for Microbial Diseases, Osaka University) for the host genetic assessment and Professor K. Pathanapanyasak and Dr. P. Auewarakul for technical advice on cellular immunity; and Dr. R. Chuachoowong for some helpful suggestion. We are grateful to Prof. P. Puthavathana and Dr. P. Auewarakul for the useful comments and to Drs. S. Ratanavichitrasin and C. Komoltri for statistical analysis.

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