BINDING ANTIBODY TO NEUTRALIZING EPITOPE GP41 IN HIV-1 SUBTYPE CRF 01_AE INFECTION RELATED TO STAGE OF DISEASE

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Abstract. The responsiveness of gp41 antibody against epitope ELDKWA in HIV-1 infected subjects is of importance in neutralizing viral infectivity and for being related to disease progression. In this study, antibody titers to this neutralizing epitope from HIV-1 infected subjects at asymptomatic and AIDS stages in Thailand were investigated by peptide ELISA. The results showed that the frequency of antibody production against this neutralizing epitope was low (15-35%). Moreover, antibody titers to this epitope in sera from AIDS patients were significantly lower than those in sera from asymptomatic subjects which were collected in the same year (p=0.001). Comparison between the past (1992-1994) and present (2002) sera from asymptomatic infected individuals revealed that the earlier panel contained lower antibody titers than the later panel did (p=0.05). In addition, random sera for HIV-1 infected subjects who were infected by diverse genetic subtypes, (A through G) including CRF 01_AE, had low titers of antibody to this region as well. It is assumed that antibody production to this epitope is low and related to the stage of HIV-1 infection.

INTRODUCTION

Human immunodeficiency virus-type 1 (HIV-1) infection elicits antibodies directed against several regions of gp120 and gp41 envelope glycoproteins. A highly conserved gp41 epitope (amino acid sequence ELDKWA) has been described to elicit antibodies neutralizing a broad variety of HIV-1 strains (Calarota *et al,* 1996). The natural occurrence of HIV-1-specific neutralizing antibodies in HIV-1 infected individuals was first described in 1985 (Robert-Guroff *et al*, 1985; Weiss *et al,* 1985). Most patients with primary HIV infection rapidly generated significant neutralizing antibody responses to early (0-39 months) autologous viruses (Richman *et al*, 2003). Consequently, neutralizing escape mutants were found rapidly in the majority of HIV-1 infected patients. The quality of the antibody response changed significantly and was not able to neutralize plasma virus (Albert *et al,* 1990). Only a few HIV- infected patients produced antibodies that neutralized a broad range of HIV-1 strains (Stiegler and Katinger, 2003). In this group, a correlation between the presence of neutralizing antibodies and the protective effect in HIV infection has been reported (Ariyoshi *et al,* 1992). In serological studies, evidence confirms that one of the broad and potent neutralizing antibodies reacted with an immunogenic epitope on gp41 defined by 2F5 human monoclonal antibody (Mab). This Mab also reacts with the short peptide ELDKWA, corresponding to the linear conserved region (ELDKWAS) of gp41 cluster II (Neurath *et al,* 1995; Purtscher *et al*, 1996). A previous study of Mab to this epitope reported that 2F5 and 2G12 passively administrated to macaques were able to confer protection from both intravenous and mucosal challenge with pathogenic HIV-simian immunodeficiency virus chimeric strains and has shown beneficial effects in phase 1 clinical trials (Wolbank *et al,* 2003). Other studies reported

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that the native structure of this epitope was unexposed and affected significantly by other regions (Nyambi *et al*, 1998; Biron *et al*, 2002). However, It has been observed that production of neutralizing antibody to this epitope (ELDKWA) has been related to slow disease progression (Stiegler *et al*, 2002). This may suggest that the antibody to this epitope is related to disease progression. In predicting disease progression, a number of biological markers, such as viral load, CD4+T lymphocyte counts, CD38 molecules and other activated molecules, have been followed. These confounding factors may be related to the level of neutralizing antibody against this epitope. However, the relationship between the levels of this antibody and the stage of disease is unclear. The dynamic response of antibody to this conserved region, and how this epitope is related to genetic subtypes, is poorly understood. Therefore, we investigated the frequency and titers of antibody to this region of gp41 in the sera of HIV-1 infected subjects at different stages of disease.

MATERIALS AND METHODS

Study patients and serum bank

In 2002, after obtaining consent forms, 242 blood samples from HIV-1 infected subjects, consisting of 171 subjects in the AIDS stage and 71 subjects in asymptomatic stage, based on 1994 CDC staging system, were obtained. These samples were collected from HIV-1 infected individuals at Bamrasnaradura Institute in Bangkok, Thailand. The mode of transmission in these subjects was sexual transmission. All, except 9, asymptomatic persons were treated with antiretroviral therapy (using one protease inhibitor and two nucleoside reverse transcriptase inhibitors). Subtypes of these samples were determined by peptide ELISA. In addition, two other panels with known subtypes of HIV-1 sera, which were part of a serum repository collected in 1992-1994, were included. The first panel (n=58) belonged to asymptomatic HIV-1 subtype CRF 01_AE infected patients in Thailand. The second panel (n=184) collected from HIV-1 diverse genetic subtypes (A to G) was random. All sera were stored at -20ºC until tested.

Although this was a cross-sectional study, longitudinal information regarding plasma RNA levels, CD3+, CD4+, CD8+T-cell counts, antiretroviral therapy, opportunistic infections and modes of transmission, were abstracted from the medical records. Complete blood counts, CD3+, CD4+, CD8+ T-cell counts, HIV-RNA and levels of antibody to gp41 were performed simultaneously (Table 1). Lymphocyte

Table 1
Clinical and immunological characteristics of HIV-1 subtype CRF 01_AE infected patients.

Disease stage	Asymptomatic patients		AIDS
HIV-1 CRF 01_AE	1992-1994 (n=58)	2002 (n=71)	(n=171)
Antiretroviral treatment			
Yes	-	62	171
No	58	9	-
CD4 (x10^6 cells/l)			
Min-max	ND	212-514	2-198
Mean ± SD		356.4±127.3	63.4±65.9
CD8 (x10^6 cellsl)			
Min-max	ND	369-2936	8-2497
Mean ± SD		1183.5±564.2	677.6±445.2
Viral load (copies/ml)[a]			
Min-max	ND	50-185,000	50-650,000
Mean ± SD		862±838	80,435±50,034

[a]Viral load was not measured in all cases; ND; not done.

subpopulations, CD3+, CD4+ and CD8+T-cell counts were determined using manufacture protocol. Briefly, whole blood was stained by monoclonal antibodies (DAKO Denmark). After staining and washing, red blood cells were lysed by FACS-lysing reagent and analyzed by the FACScan cytometer using Simulset (BD biosciences, San Jose, CA).

Subtype identification

For sera collected in 2002, HIV-1 subtypes of these samples were identified by V3 peptides ELISA using the CRF 01_AE peptide-TSI TIGPGQVFYRTG and the Thai B peptide-KSI HLGPGQAWYTTG, as previously described (Louisirirotchanakul *et al,* 1998). Since subtypes CRF 01-AE and Thai B were in the majority in Thailand, two V3 peptides, corresponding to the consensus sequences of subtype CRF 01_AE and subtype B, were used. Subtypes of samples were determined when the OD value of antibody to the V3 of a particular subtype was two times higher than the other. Only subtype CRF 01_AE samples were furthered investigating for the frequency and level of antibody against the gp41 epitope.

Gp41 antibody titer

The levels of anti-gp41 titer were measured by using the peptide-DQELLELDKWALWNS corresponding to the gp41 conserved region. The procedure for peptide ELISA has been described elsewhere (Louisirirotchanakul *et al,* 1999). Briefly, peptide was diluted and coated on an ELISA plate at 1 μg/ml overnight at room temperature. Serial two-fold dilutions of HIV-1 subtype CRF 01_AE (diluted from 1:100 to 1:28,000) were added and incubated for 1 hour, washed, and added to enzyme-conjugated goat anti-IgG. After incubating and final washing, the substrate was added, and the reaction was stopped by sulfuric acid (1M). The optical density (OD) of the samples was measured. The OD of samples (at dilution 1:100) above the cut-off were considered to be positive. The antibody titers were expressed as the reciprocal of the serum endpoint dilution. The endpoint binding antibody titers were determined as the final dilution of serum which gave an OD greater than the cut-off value [(mean of 8-panel HIV-1 – negative control + 3 standard deviations) x 2]. Sera with indeterminate results were omitted from the analysis.

Statistical analysis

The pattern of distribution of each panel of infected sera was tested by the Kolmogorov-Smirnov test. Data from all 3 panels of HIV-1 infected sera had non-Gaussian distributions. Base on the non-parametric analysis, the differences in the antibody titers for the 3 independent panels were compared by the median test (SPSS version11.5). For the frequency of antibody in the diverse genetic subtypes, chi-square (Kruskal-Wallis test) was tested because of the limited number of sera in this panel. All p-values ≤0.05 were considered statistically significant.

RESULTS

The range of antibody titers against gp41 in AIDS patients and asymptomatic HIV-subtype CRF 01_AE infected subjects

In this report, anti gp41 was screened at a dilution of 1:100. The frequencies of anti-gp41 positive antibody in all the infected sera were quite low (15-35%). The range of antibody titers from all 3 panels of infected sera from HIV-1 subtype CRF 01_AE was from less than 100 to 6,400. The median titers of these 3 panels were at 100. In the AIDS group, a total of 171 infected sera (14.6%) had a positive titer greater than 100. Among the higher titers of anti-gp41, a titer of 200 was found in 10.5%, at titer of 400 was found in 3.5%, and a titer of 800 was found in only 1 sample (0.3%). The highest titer, of 1,600 was found in only 1 sample (0.3%). The mean titer and standard deviations for the sera are 133±138 (Fig 1).

There were two other panels of sera collected separately from the HIV-1 asymptomatic patients. The first panel was for 58 subjects collected in 1992-1994. An antibody titer of greater than 100 was found in 17.2%. A titer of 200 was found in 6.9%, of 400 in 5.2%, and of 800 in 3.4%. A titer of 1,600 was found in 1 sample (1.7%). The mean titer and standard deviations were 172±238 (Fig 1). For the second panel, 71 sera were collected from asymptomatic patients in 2002. The data showed that 35.2% of samples had an anti-gp41 titer greater than 100. A titer

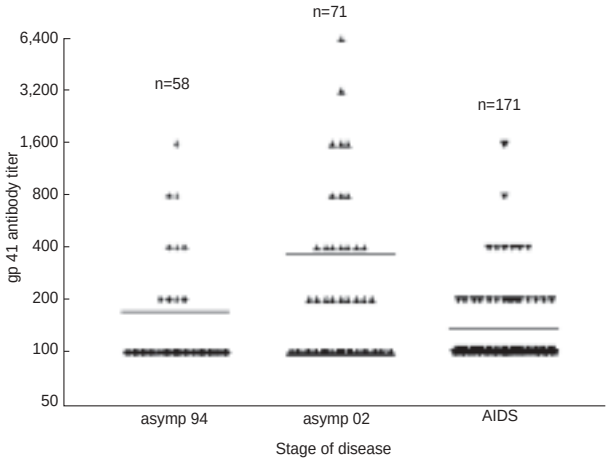

Fig 1–The frequencies of anti-gp41 titers among the three panels of sera of HIV-1 subytpe CRF 01_AE infected patients compared.

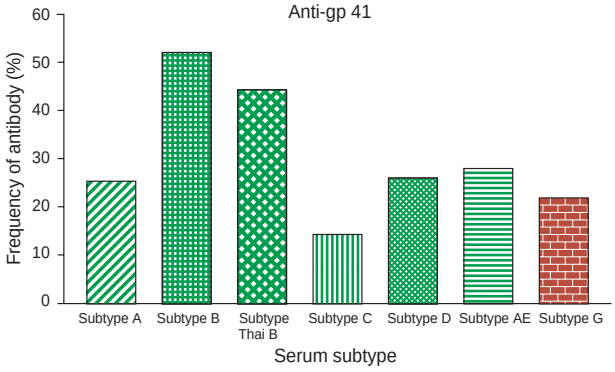

Fig 2–The frequencies of anti-gp41 in various genetic subtypes of HIV-1 infection. All sera in each subtype were collected at random.

of 200 was seen in 12.7%, of 400 in 8.5%, of 800 in 4.2%, of 1,600 in 4.2%, of 3,200 in 2.8%, and the highest titer at 6,400 was seen in 2.8%. The mean and standard deviations are 495±1,173 (Fig 1). An increase in the mean antibody titers was seen between 1994 and 2002 (172±238 in 1992-1994, 495±1,173 in 2002).

The difference between anti-gp41 titers in sera from AIDS and asymptomatic persons

By using the median test, it was found that the differences between the titers of anti-gp41 antibodies in the sera of these three panels were statistically significant. For the comparison between each pair of infected sera, the Mann-Whitney *U* test was used. A significant difference between the frequency of the anti-gp41 titers in the sera from the AIDS and asymptomatic persons collected in 2002 was observed (p=0.001). In contrast, the difference in the frequency of the antibody titers to this epitope in the sera from the AIDS and asymptomatic subjects collected in 1992-1994 was not statistically significant (p=0.6).

Comparison between the two panels of sera from the 2 separate groups of asymptomatic patients infected with HIV-1 subtype CRF 01_AE in 1992-1994 and 2002

A higher concentration of anti-gp41 was observed in the panel of sera collected in 2002. The patients in these two asymptomatic groups were age and sex matched. The difference in frequency of the anti-gp41 antibody titers between the two asymptomatic groups was significant (p=0.05).

Qualitative study of the level of antibody to envelope gp41 in HIV-1 subtypes A-G

By evaluating sera from HIV-1 diverse genetic subtypes, the low seroreactivity of these epitopes has also been demonstrated. The gp41antibody in each subtype was screened at dilution 1:100. The binding reactivity of these samples with the gp41 peptide was 52.4% for subtype B (11 of 21), 44% for Thai B (8 of 18), 28% for subtype CRF 01_AE (24 of 87), 26% for subtype D (5 of 19), 25% for subtype A (4 of 16), 22% for subtype G (2 of 9), and 14% for subtype C (2 of 14). Our study indicates that among the various genetic subtypes of HIV-1, subtype B infected patients had the highest frequency of samples with titers were more than 100. Sera from HIV-1 subtype C had the lowest frequency of titers more than 100 (Fig 2). By statistical analysis (chi-square test), there was no significant difference between antibody titers in each pair of these subtypes (p=0.088).

DISCUSSION

In general, serum from HIV-1 infected persons contains antibodies that bind to an array of epitopes found on the surface envelope glycoproteins of whole viruses (Krause *et al*, 1997). Examining sera of HIV-1 infected patients in our study showed that binding antibody to this

epitope gp41-ELDKWA was low to non-existent. These low responses were observed even in a subset of well characterized individuals with high levels of CD4+ and CD8+ T-cell responses. The relative rarity of such antibody specificity in human serum suggests that this region is poorly immunogenic and may be masked, or not exposed, on the surface of intact virions (Neurath *et al,* 1995; Sattentau, 1996; Nyambi *et al,* 1998; Sreepian *et al,* 2004). Despite the low antigenicity, the critical role of antibody against this epitope has been confirmed for its broad and potent protective activity (Burton *et al,* 1994). Previous studies indicated that antibody to this epitope showed neutralizing activity against primary isolates of several genetic subtypes of HIV-1 (Zhang *et al*, 1997; Nyambi *et al*, 2000; Kitabwalla *et al*, 2003). In investigation the binding activity of antibody to this epitope, our results indicated that asymptomatic patients, whose sera were collected in 2002, showed higher levels of this specific antibody than sera from AIDS patients did. These observations suggest that antibody to this epitope is related to the stage of disease. To support this idea, another study has shown a sustained HIV-1 specific antibody response to HIV-1 primary isolates in HIV-1 positive non-progressors (Cao *et al,* 1995). In general, a correlation is seen between the decline in HIV-1 specific antibody and a poor prognosis. As shown in Table1, most of the patients with CD4+ T cell count less than 200 cells/μl had lower levels of antibody to this epitope than asymptomatic persons. Lowering the CD4 count will likely contribute to a poor immune response in controlling viral load. In contrast, it had been reported that high levels of this neutralizing antibody can actively neutralize virus, lower the viral load, and maintain CD4+ T cells (Nokta *et al,* 2000). One possible interpretation for these results is that the presence of high viral loads in the serum has bound the available anti-gp41 antibodies, leaving a low concentration to react with the gp41 peptide. Reduction in antibody titers due to excess viral antigen has been described for various antibodies, particularly late in the natural history of HIV infection or in symptomatic HIV disease (Levy *et al*, 1985; Garland *et al*, 1996). An increasing viral load may affect the quantity and quality of antibody producing cells in recognizing specific antigens. In AIDS patients, viral loads may present B cells from responding to some epitopes and turn down antibody production by an unclear mechanism. Antibody production varies enormously in rate and efficiency, depending on several host and viral factors.

Despite the similarity of host factors and viral factors between the two groups of asymptomatic subjects, binding antibody to this region was significantly higher in asymptomatic subjects whose sera were collected in 2002. In fact, improved responsiveness in both humoral and cellular immunity in antiretroviral treated patients was observed in most cases. Viral load decreased to undetectable (less than 50 copies/μl) levels while the CD4 count has been increased, stable or slowly decreased (Lempicki *et al,* 2000; Riou *et al,* 2000; Flepp *et al,* 2001). In the era before antiretroviral drugs, many infected persons were not treated or received inadequate treatment. Thus, the immune system deteriorated rapidly. Our data suggests that significantly higher levels of anti-gp41 in asymptomatic sera collected in 2002 may be due to antiretroviral treatment. This study supports the results of the HAART (Highly-Active Anti-Retroviral Therapy) trial showing immune restoration in HIV-1 infected persons (Kaufmann and Rosenberg, 2003). Considering viral factors, it can be seen that later viral sequences can be distinguished from earlier sequences (Markham *et al,* 1998; Srisurapanon *et al,* 2001). Although this epitope is highly conserved in various subtypes, mutation in this region (ELDKWA) had been described. Changing of amino acid in this region affects both epitope expression and/or cell-cell fusion (Cao *et al,* 1993; Poumbourios *et al*, 1995). An improvement in immunoresponsiveness by antiretroviral therapy and/or changing in the amino acid sequence may affect antibody responses. For these reasons, the concentration of anti-gp41 in the present is different from the past.

By epitope mapping, this epitope (ELDKWA) in gp41 has been highly conserved in many genetic subtypes. Therefore, antibody to this epitope shows a stronger degree of cross subtype reactivity. In our study, the antibody response to this region in infected patients has not been effective in all the diverse subtypes of HIV-

1. The approximate frequency of antibody in subtypes A, D, E and G was about 25%. These results indicate that this region of gp41 was not accessible. However, slight differences in frequencies are seen in subtypes B and C. Several factors may account for these differences. First, some of these epitopes may be more immunogenic in one subtype than in others. Second, variance in ethnicity may account for the difference in immune response. Last, better treatment and more appropriate knowledge may have influenced immunity. HIV-1 subtype B infected patients are predominant in developed countries, while people infected with subtype C are usually found in developing countries. Considering viral factors, viral phenotype has also been associated with stages of disease (Koot *et al,* 1993). Unfortunately, viral phenotypes were not determined in this study. The frequency of antibody to gp41 (ELDKWA) does not significantly discriminate between genetic subtypes, since it reacts directly to a relatively conserved feature of the envelope glycoprotein.

In conclusion, our data indicate that immunogenicity of this region is poor and highly conserved in diverse subtypes; concentration of anti-gp41 is related to the clinical stage of HIV-1 infection; concentrations of anti-gp41 in asymptomatic infected person from the past and in the present are different; and the responsiveness of the antibody to this region is not different in diverse genetic subtypes.

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