were plated into a 48-well plate. HIV-1 CHNHLJ03009/Luc was added into the cells (2.5ng, 5ng, 10ng, 20ng, 40ng, 80ng/well). After incubation at 37°C for 1 hour, the non-entered virus was replaced with 1 ml of fresh D-MEM containing 2% FCS and the cells were cultured for another 48 h. The cells were lysed with Luciferase Cell Culture Lysis (Promega, USA). The luciferase activity was measured by using the Luciferase Assay System (Promega, USA) and a Luminometer TD-20/20 (Turner Designs, USA). Luciferase activity represents the capability of virus entry into the target cells [10-11].

Analysis of the secondary structure of the Env and the phylogenesis

The secondary structure, antigenicity and hydrophilicity of HIV-1 envelope proteins was analyzed using software DNAMAN (version 5.1, Lynnon Biosoft). Phylogenetic analysis was taken place with CLUSTAL X 1.8. Sequence homology was analyzed by using Blast program in GenBank.

RESULTS

The variability of the Env open reading frame

Two functional env clones named CHNHLJ03009c34 (GenBank Accession No: AY905493 and CHNHLJ03009c33 with open reading frame of 894 amino acids were obtained. Sequence comparison indicated that the amino acid sequences of the two clones are different at seven sites: two are located in V1/V2 region, one is located in V4 region (figure 1). Hence, CHNHLJ03009c34 was extensively analyzed as the representative.

Homology analysis using full-length Env amino acid sequences has shown that the highest homologies between CHNHLJ03009c34 and HIV-1 isolated in either China or the world (from GenBank) were subtype B′ strains, RL42 [12], LTG0218 [13] from Yunnan province and CNHN24 [14] from Henan province, which were 91.52%, 85.03% and 84.57%, respectively. Comparing the variable and the parts of the conserved regions of the Env of CHNHLJ03009 and RL42 strains, we found that the variation degree among the regions of V1/V2, C2, C2-V3, V3, C4 and V4 is much different. The homologies were 75.36%, 90.91%, 89.55%, 85.71%, 97.50%, 80.00%, respectively.

```
c33    MRVTGIRKNCQHLWRWGTMLLGMLMICSAAENLWVTVYYGVPVWKDAITTLFCASDAKAYDTEV
c34    ----------------------------------------------------------------
RL42   ---------Y-----------------N----------------E-T-----------------

c33    HNVWATHACVPTDPNPQEVVLGNVTENFNMWKNNMVDQMHEDIISLWDQSLKPCAKLTPLCVTL
c34    ----------------------------------------------------------------
RL42   -------------------------------------E-----------------V--------
                                    V1/V2
c33    NCTNLKNTTNTMSNTSGTTEGGEMKNCSFNITTSTSTKVTDYALFYKLDIIPIENDNTSYRLIN
c34    ---------S-----N------------------------------------------------
RL42   -----------S-TME-....--I----------IK---K-------V-VV--G--S-------

c33    CNTSVITQACPKVSFEPIPIHYCTPAGFAILKCNNKKFNGTGPCTNVSTVQCTHGIRPVVSTQL
c34    ----------------------------------------------------------------
RL42   ------------------------------I---------------------------------
                                                                  V3
c33    LLNGSLAEEEVVIRSVNFSNNAKVIIVQLKESVEINCIRPNNNTRKSIHMGPGQAWFATGDIIG
c34    ----------------------------------------------------------------
RL42   -------------KFS-TD---R------N-----K-------------L---K--YT--Q---
                                                gp120   ▼▼  gp41
c33    DIRQAHCNISRTKWNDTLRQITKKLREQFGNKTIIFNQSAGGDPEIVMHSFNCGGEFFYCNTSQ
c34    ----------------------------------------------------------------
RL42   --------L-S----N--K----------------V----S-------------------------
           V4
c33    LFNSTWNGNATWNNTAGNSNITLPCRIKQIVNMWQEVGKAMYAPPIRGQIRCSSNITGLLLTRD
c34    ---------T------------------------------------------------------
RL42   -------DTG---D-T---T--------------------------E-----------------

c33    GGTNKSENTETFRPGGGDMEDNWRSELYKYKVVKIEPLGIAPTKAKRRVVQREKRAVGTIGAMF
c34    -------------------K--------------------------------------------
RL42   --N-E-KP-----------R--------------------V-----R-----------------

c33    LGFLGAAGSTMGAASITLTVQARQLLSGIVQQQRNLLRAIEAQQHMLQLTVWGIKQLQARVLAV
c34    ----------------------------------------------------------------
RL42   ----------------------------------------------L-----------------

c33    ERYLKDQQLLGIWGCSGKLICTTAVPWNVSWSNKSLNEIWNNMTWMEWEREIDDYTREIYTLIE
c34    -----------------------------------------------------N----------
RL42   ----------------------------A-------H----------------N----------

c33    ESQNQQEKNELELLELDKWASLWSWFDISNWLWYIRIFIMIVGGLVGLRIVFAVLSIVNRVRQG
c34    ----------------------------R-----------------------------------
RL42   ----------------------N----TK--W--K------------------------------

c33    YSPLSLRTRFPTQRGPGRPEGIEEEGGERDRDRSERLVHGFLTLIWEDLRSLCLFNYHRLRDLL
c34    ------Q---------------------------------------------------------
RL42   ------Q----A-----------------------T---S------------S-----------

c33    LIVARIVELLGRRGWEALRYWWNLLQYWIQELKNSAISLLNATAIAVAEGTDRVIEVVQRAYRA
c34    ----------------------------------------------------------------
RL42   ----------------V--------------------G--------------------------

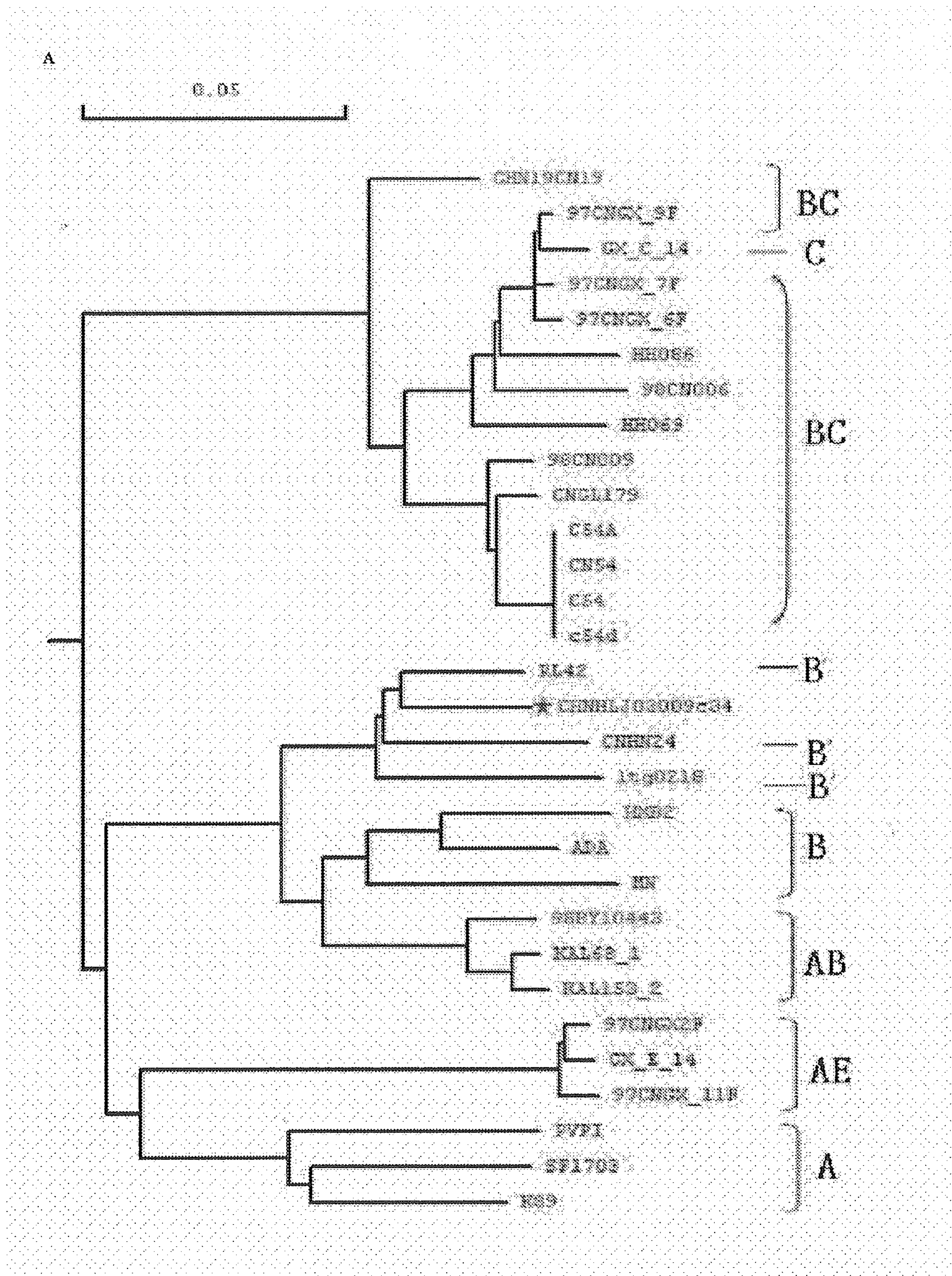
c33    ILNIPTRIRQGLERALL  849
c34    -----------------  849
RL42   --H--------------  845
```

Fig. 1 The comparison of amino acid sequences of HIV-1 CHNHLJ03009 and RL42 envelope.

The dishes represent the same residues with that of c33.

The nonuniformity of the env variation

When we did phylogenetic analysis through comparing the amino acid sequences of full -length Env proteins of all the international reference strains of different HIV-1 subtypes, indexed in Los Alamos and GenBank sequence database, we found that CHNHLJ03009c34 has the most closest molecular relation with HIV-1 RL42, isolated from Yunnan province.



A
0.05
97CNGX_9F
GX_C_14
97CNGX_7F
97CNGX_6F
98CN006
98CN009
CNGL179
C54A
CN54
C54
c54d
RL42
CNHN24
ADA
MN
KAL68_1
KAL153_2
97CNGX2F
GX_E_14
97CNGX_11F
SF1703
BC
C
BC
B
B
B
B
AB
AE
A

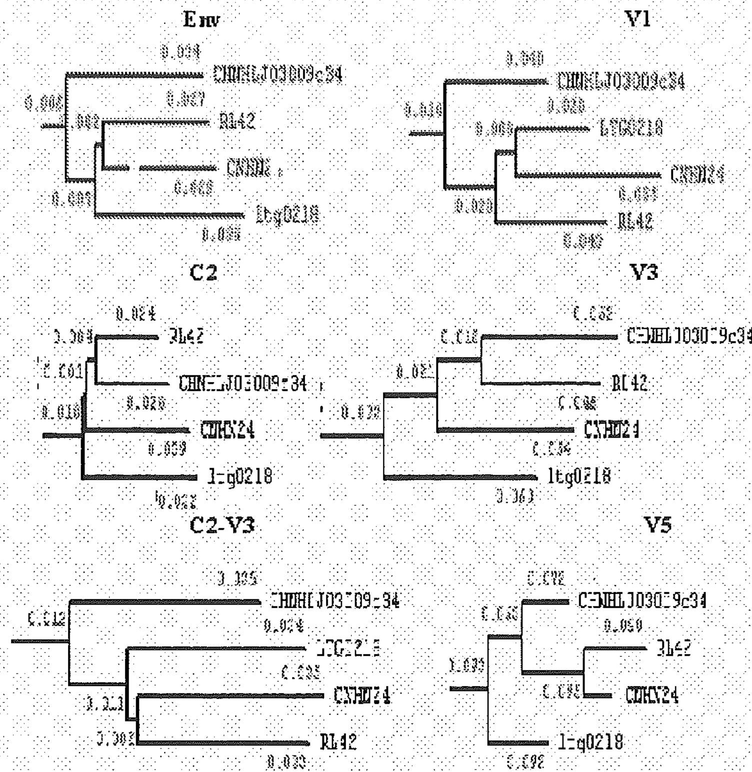


Fig. 2 The results of phylogenetic analysis
A. The phylogenetic tree from the nucleotides of full length env; B. The phylogenetic tree from the nucleotides of parts of env.

The phylogenetic tree is shown in the figure 2A. Meanwhile, we also analyzed the phylogenesis of different regions of the Env. It was found that there was apparent distinction in the phylogenesis results obtained from analyzing full-length Env and the variety of the Env regions (figure 2B). The genetic distance of CHNHLJ03009c34 and above-mentioned three strains of subtype B' has been changed when the phylogenetic tree was made by analyzing C2-V3 region. CHNHLJ03009c34 strain became an independent arm to the other strains.

The secondary structure analysis of the envelope protein

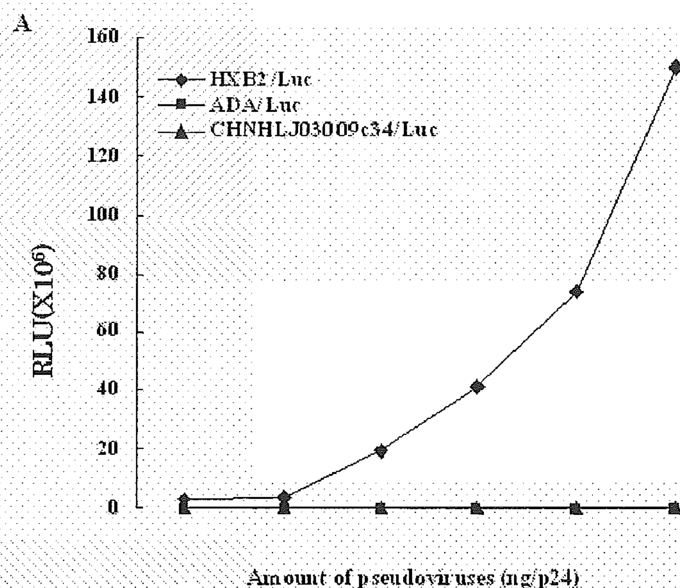
The antigenicity and hydrophilicity of CHNHLJ03009 did not have significant difference

from RL42 (Data not shown).

CHNHLJ03009c34 is a R5-tropic HIV

Infection assay showed that the pseudovirus can infect only U87.CD4.CCR5 cells indicating that it is a R5-tropic HIV-1 (figure 3).

A



B

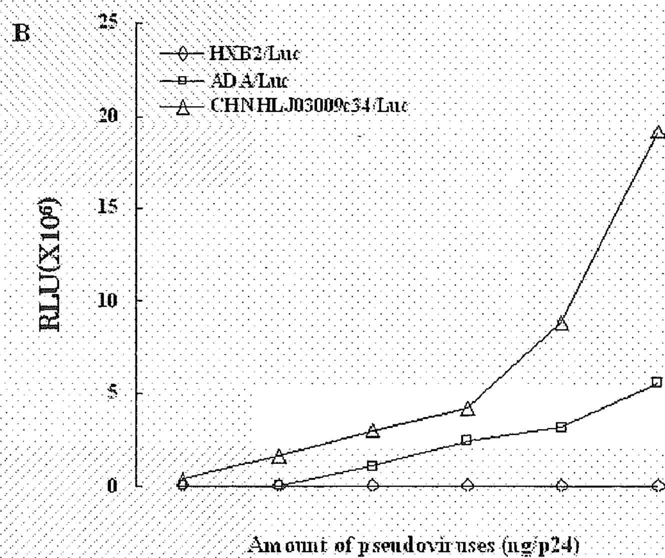


Fig. 3 The infectivity of the pseudoviruses.
A. The infection of the pseudoviruses into U87.CD4.CXCR4 cells; B. The infection of the pseudoviruses into U87.CD4.CCR5 cells.

DISUSSION

In this study, the env gene from the genome DNA of PBMCs of a HIV-1 seropositive individual in Heilongjiang province in China was isolated. Two functional env clones named CHNHLJ03009c34 (GenBank Accession NO: AY905493) and CHNHLJ03009c33 were obtained and the variability of the env gene as well as the open reading frame were analyzed. It was found that the homology between CHNHLJ03009c34 and HIV-1 B′ subtype RL42 strain, isolated from Yunnan province, belonging subtype Thailand B, is 91.5%. Phenotype analysis showed the pseudovirus bearing CHNHLJ03009c34 Env can infect only U87.CD4.CCR5 cells, indicating that it is a R5-tropic HIV-1. This is the first report about analyzing HIV-1 primary isolates in Heilongjiang province.

Sequence comparison indicated that the two env clones isolated have seven mutation sites at the amino-acid level. Three of them were located in the hypervariable regions of gp120, and three of them were located in gp41 region. The amino acid sequences of their V3 loop, which is a major determinant for viral tropism, are identical. This result suggested that the two env clones would have the same phenotype in the cell tropism though the structure of their surface protein may be different.

As the amino acid variation may cause the structure change and subsequently affect the functions of the protein, therefore, analyzing the Env structure is necessary to verify whether the sequence variation alters viral envelope functions. We found that though the Env amino acid sequence of CHNHLJ03009 was variable comparing to that of RL42 strain, their antigenicity and hydrophilicity do not have obviously distinction. Therefore, further analysis on epitopes and neutralization characteristics would be taken place to learn whether their neutralization characteristics have significant differences. Of note, the variation of the amino acid sequence of partial C2 region proximal to V3 region was higher than that in V3 loop itself.

It has been thought that a gene, in which the sequence evolution is not disproportional, should not be taken as a whole doing phylogenetic analysis as it may cause misunderstanding in the genetic evolution [15]. For instance, the conserved and the variable regions of HIV-1 env gene are located alternatively, and the diversify velocity of the gene

regions are considerably variable. Therefore, it would cause a deviation if the genetic distance is calculated upon the full-length sequence [15-17]. The results of this study showed that CHNHLJ03009c34 has the closest molecular relation with strain RL42 through analyzing the full-length env gene; while, it became independent branch in the phylogenetic tree made upon analyzing C2-V3 region. Homology analysis also showed that V1/V2 region has the highest variability (homology was 75.36%), following by V4 region, in which the homology is strikingly lower than full-length env (homology was 91.5%). Because of the high recombination of HIV-1 in population, comparison of the parts within the gene may actually reflect variation and recombination degree.

In the present study, a pseudovirus infection system was used to examine the coreceptor utilization. Because of highly variation of HIV-1, there is usually a mixture of polyclonal viruses in an individual of long-term infection. Utilizing molecular cloning approaches with combination of pseudovirus infection system is benefit in observing the biology phenotype of a single clone of the viruses. The HIV-1 pseudovirus CHNHLJ03009c34/Luc can infect only U87.CD4.CCR5 cells indicating it is a R5-tropic HIV-1. The other functional clone from the same infected individual has identical amino acid sequence in V3 loop (figure 4). The net charge of V3 loop of both two clones was 2.18.

```
HXB2              CTRPNNNTRKSIRIQRGPGRAFVTIGK.IGNMRQAHC
ADA               -------------H-..GPGR--Y-T-EI--DI-----
RL42              -I-----------HL..GPGK-WY-T-QI--DI-----
CHNHLJ03009c34    -I-----------HM..GPGQ-WFAT-DI--DI-----
```

Fig. 4 The comparison of amino acid sequences of HIV-1 envelope V3 region.
The dishes represent the same residues with that of HXB2.

It suggested the two clones have identical phenotype in the cell tropism and that R5-tropic HIV-1 strains possibly is the dominant virus population in the individual. Considering the fact that the infected individual had no clinical symptoms yet when the blood sample was

collected (CD4 count was 578 cell/ml) and did not enter progression stage, it may be close to the original virus prevalent at infection time, 1980s. Monitoring the capability of the HIV-1 strains in coreceptor utilization would be important for the prediction of the disease progress.

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SHORT COMMUNICATION

Low antibody response against tuberculous glycolipid (TBGL) in elderly gastrectomised tuberculosis patients

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SUMMARY

To evaluate differences in anti-tuberculous glycolipid (TBGL) antibody titers in patients who developed tuberculosis (TB) with and without gastrectomy, 11 gastrectomised patients who developed TB after surgery (GS-TB), 19 TB patients without any other complications (TB), 12 gastrectomised patients who did not develop TB after surgery (GS) and 27 healthy subjects (H) with normal findings on chest X-ray were evaluated, although there were no differences in the clinical findings at admission between the TB and GS-TB groups. The assay used here allowed us to find low anti-TBGL antibody titers in GS-TB patients.

KEY WORDS: TBGL; gastrectomy; tuberculosis

GASTRECTOMY is known as an associated co-factor in the development of tuberculosis (TB). Although the reasons for the association have not been clarified,[1,2] previous studies with a significant number of gastrectomised patients have reported prevalences of 1.7% to 2.5%. In addition, immunodeficiency and/or malnutrition contribute to the development of TB.[3] We therefore evaluated the clinical and laboratory findings, including purified protein derivative (PPD) reaction, in TB and/or gastrectomised patients and healthy controls. In the gastrectomised patients, group 2 lymph nodes were dissected, which could have affected the host immune responses. The glycolipid antigen trehalose 6, 6′-dimycolate (TDM) purified from *Mycobacterium tuberculosis* H37Rv has recently been reported as a useful diagnostic antigen.[4–6] We thus investigated specific immune responses against TDM by measuring anti-tuberculous glycolipid (TBGL) antibody titers.

Between 1999 and 2001, a total of 60 patients from Tohoku University Hospital enrolled in the study were divided into four groups: 1) gastrectomised patients who developed TB after surgery (GS-TB, n = 11); 2) TB patients without any other complications (TB, n = 19); 3) gastrectomised patients who did not develop TB after surgery (GS, n = 12); and 4) healthy subjects (n = 27) with normal findings on chest X-ray. For both TB and GS-TB patients, blood samples were taken before anti-tuberculosis drugs were given.

The study was approved by the ethics committee of the Tohoku University School of Medicine. Informed consent was obtained from patients and volunteers to participate in the study. For the diagnosis of TB, in addition to clinical features, acid-fast smear, culture and polymerase chain reaction tests (Roche Amplicor Mycobacterium Kit, Branchburg, NJ, USA) were performed using sputum or gastric fluid. Gastrectomised patients with recurring cancer, those treated with anticancer drugs and those with other diseases were excluded. Immunoglobulin G (IgG) antibodies against TBGL antigen was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Kyowa Medex Co, Tokyo, Japan). A cut-off value ⩾2 U/ml was considered positive.[4,5] Statistical analyses were performed using conventional methods.

Mean age and postoperative duration were 74.5 ± 9.2 years and 24.3 ± 6.3 months in the GS-TB group and 74.8 ± 3.0 years and 20.6 ± 9.2 months in the GS group, respectively. There were no significant differences between the two groups ($P > 0.05$). The mean ages in the TB patients and healthy controls were respectively 73.0 ± 9.4 and 73.2 ± 8.2 years. There were no significant differences in positive tuberculin test (6/11 vs. 10/19), lung infiltration shadow (⩾2 lobes) (5/11 vs. 14/19) or acid-fast smear (⩾1/field) (6/11 vs. 10/19) between the GS-TB and TB groups.

In this study, 55% of the GS-TB patients were positive for the anti-TBGL antibody titers by ELISA; this result was comparable to that obtained for the TB patients (68%). Maekura et al. found a significant number (17%) of TBGL-positive serum samples in healthy individuals.[5] In our healthy subjects, 22% of serum samples showed positive responses, but curiously none of the GS group was positive. Furthermore, the anti-TBGL antibody titers in the GS-TB patients were

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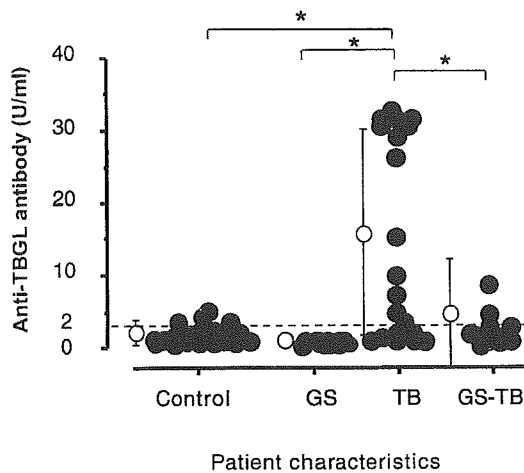

Figure 1 Individual anti-TBGL values in healthy controls and GS, TB and GS-TB patients. Circles = individual anti-TBGL values; bars = standard deviation; dotted line = cut-off; anti-TBGL = anti-tuberculous glycolipid; GS = gastrectomised; TB = tuberculosis. * $P < 0.05$.

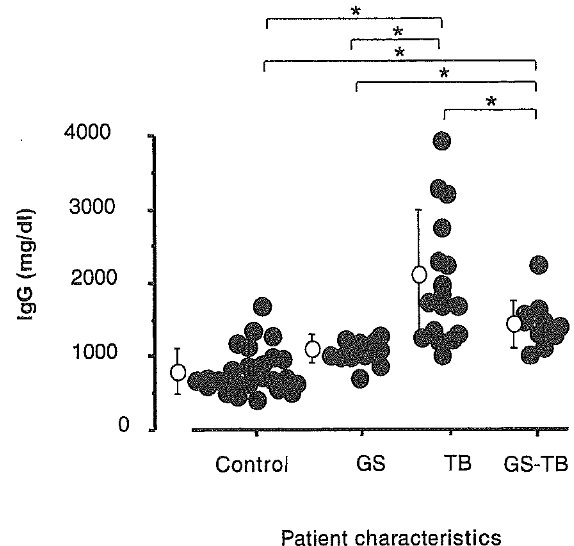

Figure 2 Individual IgG values in healthy controls and GS, TB and GS-TB patients. Circles = individual IgG values; bars = standard deviation; IgG = immunoglobulin G; GS = gastrectomised; TB = tuberculosis. * $P < 0.05$.

significantly lower than those of the TB patients ($P < 0.05$) (Figure 1). The serum IgG in both TB and GS-TB patients was significantly higher than in the GS patients and healthy controls (Figure 2), confirming that the decrease of anti-TBGL antibody in GS-TB patients could not be explained by the low IgG in their serum. It should also be noted that both anti-TBGL antibody and total IgG values were lower among GS-TB patients than in the TB group.

The reason for the lack of anti-TBGL antibody in GS patients and the low anti-TBGL antibody titers in GS-TB patients is not clear, but both suggest that the stomach may play a role in the production of anti-TBGL antibodies.

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RÉSUMÉ

Afin d'évaluer les différences entre les titres d'anticorps glycolipides anti-tuberculeux (TBGL) chez des patients ayant développé TB avec ou sans gastrectomie, ont été évalués 11 patients gastrectomisés ayant développé une TB après chirurgie (GS-TB), 19 patients TB sans autre complication (TB), 12 patients gastrectomisés n'ayant pas développé de TB après chirurgie (GS), et 27 sujets sains (H) présentant des radiographies thoraciques normales, bien qu'il n'y a eu aucune différence dans les investigations cliniques à l'admission entre les groupes TB et GS-TB. L'essai utilisé nous a permis de trouver de faibles titres d'anticorps d'anti-TBGL chez les patients GS-TB.

RESUMEN

Para evaluar la diferencia de títulos de anticuerpos para un glicolípido antituberculoso (TBGL), en pacientes infectados con TB con y sin antecedente de gastrectomía, se evaluaron 11 pacientes gastrectomisados (GS-TB), 19 pacientes infectados con TB sin otra complicación (TB), 12 pacientes gastrectomisados que no desarrollaron TB (GS) y 27 sanos (H) con exámenes radiológicos normales. No hubo diferencias significativas entre los grupos TB y GS-TB al ingreso. Los resultados mostraron bajos títulos anti-TBGL en pacientes GS-TB.

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Original article

Properties of anti-gp41 core structure antibodies, which compete with sera of HIV-1-infected patients

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Abstract

To determine the correlation between the immunoreaction against the core structure of human immunodeficiency virus type (HIV-1) transmembrane protein gp41 epitopes and the disease progression, it is essential to evaluate the anti-core structure antibody epitopes and the humoral immunity against the epitopes. For this purpose we evaluated monoclonal antibodies (mAbs) against the gp41 core structure such as mAbs 50.69, 98.6 and T26, by Western blotting (WB) and flow cytometry. WB showed mAbs 50.69 and 98.6 bound to both monomeric and oligomeric gp41, and mAb T26 exclusively bound to oligomeric gp41. We evaluated the sera from *Pneumocystis* pneumonia patients (PCP; $n = 7$) and long-term survivors (LTS; $n = 7$). Competition assay with sera and mAbs for binding to H9 cells infected with HIV-1 IIIB virus was done using flow cytometry. The results revealed that PCP sera as well as LTS sera inhibited the binding of all the three mAbs, and the PCP sera inhibited mAb T26 binding more efficiently than LTS. Therefore, PCP patients retain competing immunity to antibodies against not only the shared epitopes of the core structure (binding sites of mAbs 50.69 and 98.6) but also against oligomeric gp41 specific epitope (binding site of mAb T26).

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Keywords: HIV-1; PCP; LTS; gp41; Core structure; Sera

1. Introduction

The outcome of exposure to human immunodeficiency virus type 1 (HIV-1) is affected by humoral and cellular immune responses against HIV-1. Blocking of virus entry by antibodies is thought to be critical for vaccine development. Even though HIV-1 transmembrane protein gp41 plays a key role in both virus-mediated cell–cell infection and infection by cell-free virus, the exact conformation of the native gp41 molecule is not well known. A structural model was proposed in which fusion-active gp41 folds into a six-helix alpha-helical bundle, with three N-terminal helices forming an interior, parallel-coiled-coil trimer, while three C-terminal helices pack in the reverse direction into three hydrophobic grooves on the surface of this coiled-coil [1–3]. Two immunodominant regions of gp41 core structure have been reported to define the epitopes within these regions to which infected humans respond during the course of infection [4]. The first is the region of gp41 in the vicinity of the cysteines at amino acids 598 and 604 (cluster I). The second immunogenic region position is between 644 and 663 (cluster II). Titration of sera from HIV-1-infected patients showed that there was an antibody that binds about 100-fold more efficiently to cluster I than to cluster II in the patients' sera, confirming the immunodominance of cluster I. Recently, we reported that there was an association between the clinical progression in HIV-1-infected individuals and the decline of anti-DP107 (alpha helical N-peptide) (aa 553–590) antibody, suggesting that the antibodies against the structure may have a protective role [5]. Because the DP107 peptide shows an alpha helical structure, we assumed that the humoral responses against the core structure might be associated with the clinical progression. Furuta et al. [6] showed that the DP178 (aa 638–673) peptide

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bound to gp41 and inhibits envelope protein mediated membrane fusion. Subsequent studies by Gorny et al. have disclosed that human monoclonal antibodies (mAbs) against gp41 could recognize the gp41 core structure. Among them, mAb 50.69 reacts only when N51 (aa 540–590) is mixed with C43 (aa 624–666), and mAb 98.6 reacts only when N36 (aa 546–581) is mixed with C34 (aa 628–661), and both combinations are known to form a six-helix bundle [7,8]. The previous studies proved that mAb T26 binds to the epitope of a six-helix bundle composed of N36 and C34 peptides [9–12] (Table 1). MAbs 50.69, 98.6, T26 have an epitope that composes the core structure so we call these mAbs anti-core structure mAbs because the exact antigenic structures of the native and fusogenic gp41 molecule have not been clarified yet. Among them, only mAb T26 has specificity to oligomeric gp41 so we called mAb T26 anti-oligomeric gp41 antibody because a previous study showed that mAb T26 binds specifically to oligomeric gp140 by immunoprecipitation [10]. These mAbs have been used to analyze the conformational changes induced by recombinant soluble CD4 (sCD4) (Immuno. Diagnostics, Woburn, MA) because sCD4 could mimic the interaction of gp120 with the cell membrane. This treatment is known to enhance mAb 50.69 expression [13,14]. Such anti-core structure mAbs as 50.69, 98.6 and T26 have not been studied extensively because they have less neutralizing activity [15,16], and because the gp41 core structure was believed to appear only after membrane fusion occurred [17]. However, to clarify the conformational properties and changes of the gp41 molecule in detail, we need to evaluate the binding properties of the anti-core structure mAbs, for which these mAbs are useful. In the present study we were able to clarify the properties of anti-gp41 mAbs.

Loomis-Price et al. [18] have reported that anti-gp41 immunoresponses have a more significant correlation with the disease progression than those of anti-gp120. It has also been reported that antibodies against gp41 epitopes on HIV-1 are lost or escape mutants arise and consequently control of HIV-1 is lost and the virus load increases, as in the case of CDC stage C [19]. Much evidence has accumulated supporting the correlation with anti-gp41 immunity and disease progression. However, the correlation between the immnoreactions against each core structure epitope and the disease progression remains unclear. Here, we studied the antigenicity of the core structure, which has two immunodominant regions of anti-gp41 antibodies, and the immunity against these epitopes in sera of HIV-1-infected patients.

Table 1
Presence of different immunoreactive mAbs that bind to gp41 derived peptide.

	C34	N36	N36/C34	C43	N51	N51/C43
50.69	– [24]	– [24]	– [24]	– [24]	– [24]	+ [24]
98.6	– [7]	– [7]	+ [7]	+ [8]	– [8]	+ [8]
T26	– [11]	– [11]	+ [11]	unknown	unknown	unknown

Each number indicates reference
–: No binding detected between mAb and peptide. +: Significant positive binding detected between mAb and peptide. unknown: no consensus on whether mAb binds to peptide or not.

2. Materials and methods

2.1. Subjects and sera tested

We enrolled seven healthy volunteers (mean age = 33: all of them were men) who were HIV-1 negative and whose peripheral CD4 counts were above 500 cells per ml. And we enrolled seven HIV-1-infected individuals (mean age = 26: all of them were men) as LTS. LTS were defined as patients who had not been treated with antiretroviral therapy and did not develop AIDS-related disease for more than 10 years and whose peripheral CD4 counts were above 500 cells per ml (LTS) [20–22]. Sera were drawn in 1997 from six male LTS who were hemophiliacs and estimated to have been infected at least before 1986. Six LTS were registered in the Collaborative HIV study of Japanese Natural History Committee. One sample was drawn in 2003 for LTS #7 who been infected for more than 10 years. And sera were drawn from seven AIDS patients (mean age = 45) who had developed *Pneumocystis* pneumonia (PCP) (mean CD4 count = 34) [23]. One PCP #1 patient has been infected with HIV-1 fore more than 10 years and the others were found to be infected when they developed PCP. Six PCP patients did not have antiretroviral therapy except PCP #2 who already had started AZT + ddC + IDV as ART (antiretroviral therapy) when his blood was drawn in 2003. PCP #1, #2, #3 and #6 were infected by heterosexual intercourse and the others PCP were by homosexual intercourse. In LTS #3, 5 and 6 the HIV strain was identified as subtype B but the others were unknown (Table 2). One male LTS #7 and one female PCP patient #1 from Tohoku University Hospital, Division of Infectious and Respiratory Diseases were enrolled. Sera from six male PCP patients were kindly provided by Dr. Sato of the Sendai Medical Center. LTS #1 and #2 sera were kindly provided by National Institute of Infectious Diseases, Tokyo, Japan. LTS #3, 4, 5 and 6 sera were kindly provided by Dr. Shin Nishihata at Kagoshima Seikyo Hospitaland. LTS #1–6 were registered in the Collaborative HIV study of Japanese Natural History Committee and those clinical information of LTS #1–6 was provided from HIV Infectious Diseases Integrated Database (https://www.aids.nih.go.jp/).

The plasma from the six PCP patients showed viral RNA loads above 50,000 copies per ml, except case patient #2 with 3300 copies per ml. All PCP patients were hospitalized and treated properly. Informed consent was obtained from all patients. Plasma was separated from heparinized blood by centrifugation and stored at –80 °C until use and thawed just before use. Approval by the Tohoku University Ethics Committee for Clinical Investigations and informed consent were obtained.

Table 2
Clinical characteristics of LTS (A) and PCP (B) patients

A

Case	Age	Gender	CD4 (cells/μl)	Viral load (copies/ml)	ART	Subtype	Infection route
1	20	M	648	280	Nothing	unknown	Blood Product
2	15	M	950	950	Nothing	unknown	Blood Product
3	34	M	665	80000	Nothing	B	Blood Product
4	38	M	552	<400	Nothing	unknown	Blood Product
5	32	M	501	2000	Nothing	B	Blood Product
6	19	M	898	<400	Nothing	B	Blood Product
7	32	M	547	<400	Nothing	unknown	unknown
Mean	28		648	400			

B

Case	Age	Gender	CD4 (cells/μl)	Viral load (copies/ml)	ART	Subtype	Infection route
1	30	F	9	50400	Nothing	unknown	Hetero
2	56	M	828	3300	AZT ddC IDV	unknown	Hetero
3	60	M	212	80000	Nothing	unknown	MSM
4	35	M	1	>100000	Nothing	unknown	Hetero
5	32	M	34	>100000	Nothing	unknown	MSM
6	59	M	3	>100000	Nothing	unknown	Hetero
7	40	M	544	79000	Nothing	unknown	MSM
Mean	45		648	400	Nothing		

2.2. Monoclonal antibodies

The anti-gp41 human mAbs 50.69, 98.6 and 246D which recognize the gp41, and the biotinylated mAbs 50.69 and 98.6 were also kindly provided by Dr. S. Zolla-Pazner and Dr. M.K. Gorny [4,24,25]. The mAb T26, which recognizes the oligomeric gp41, was kindly provided by Kilgore et al. [11]. The anti-gp120 mouse mAb 902, which recognizes the V3 loop of gp120 [26], was provided by Dr. B. Chesebro through the NIH AIDS Research References Reagent Program. MAb Chessie 8 was purified using HiTrap Protein G (Amersham Biosciences, Piscataway, NJ) from Chessie 8 hybridoma supernatant provided by Dr. George K. Lewis through the NIH AIDS Research References Reagent Program.

2.3. Western blotting (WB)

Immunoassays of each mAb confirmed their binding to envelope protein by LAV-BLOT1 (LOT No. 3C0175) (Bio-Rad, Hercules, CA). The WB procedure was done as described by the manufacturer. In brief, HIV-1 stain IIIB lysate blotted membranes were washed with diluted wash solution and incubated with diluted mAbs at 25 °C for 2 h The membranes were developed with alkaline phoshatase conjugated anti-human or -mouse IgG antibody (Sigma, St. Louis, MO), followed by 5-bromo-4-chlor-3-indoly-phosphate/nitroblue-terazulium reaction. The membrane images were acquired by flat scanner ES-2200 (Espon, Nagano, Japan) and analyzed using PhotoShop software (Adobe, San Jose, CA).

2.4. Biotinylation of antibodies

MAbs T26 and 902 were biotinylated as follows. Ten milligrams biotinamidohexanoic acid *N*-hydroxysuccinimide ester (Sigma) were resolved in 500 ml *N*,*N*-dimethyformamide (Sigma) and stocked at –20 °C. Sodium bicarbonate was added to the stock solution to adjust the pH to 8.3. One mg antibody was added to 10 ml pH adjusted solution and incubated at 4 °C for overnight. Then, biotinylated mAb was dialyzed with PBS 3 l at 4 °C for 24 h and detected with phycoerthrin-labeled avidin (PE-avidin) (serotec Ltd., Kidlington, Oxford, UK) using flow cytometry (FACS) (Becton Dickinson Biosciences, San Jose, CA). Data were analyzed using the CellQuest software (Becton Dickinson Biosciences).

2.5. Cell culture

A human T cell line, H9 cells and H9 cells infected with the IIIB strain of HIV-1 (H9/IIIB) and with the MN strain of HIV-1 (H9/MN) were obtained from Dr. R.C. Gallo. All cells were cultured in the presence of antibiotics (penicillin and streptomycin) at 37 °C in 100% humidified air containing 5% CO_2. The H9/IIIB and H9/MN cell lines were kept in RPMI 1640 medium containing 10% fetal calf serum. The viabilities of the cells were estimated using the trypan blue dye exclusion method and the cells that were more than 95% viable were used for the experiments. H9/IIIB and H9/MN cells were proved to be infected with IIIB and MN and the express the envelope protein more than 80%.

2.6. Sequence of HIV-1 strain analyzed

HTLV-IIIB (GenBank accession no., X01762) and HTLV-IIIMN (GenBank accession no., M17449) strain sequences were obtained from NIH AIDS Research and Reference Reagent program HIV-1 sequence database. Sequences were analyzed with GenetyxMAC software (Software Development, Shibuya, Japan).

2.7. Effect of sCD4 on the conformational changes of gp120 or gp41 by FACS

Conformational changes of the HIV-1 envelope glycoprotein induced by sCD4 were detected using FACS [27]. The H9/IIIB or H9/MN (5×10^5) cells were washed twice with PBS followed by incubation at 37 °C for 1 h in the absence or presence of 10 µg/ml sCD4 in RPMI 1640 medium containing 0.1% BSA and 25 mM HEPES. Thereafter, the cells were washed with PBS containing 0.1% bovine serum albumin and 0.01% sodium azide and incubated with anti-gp41 human or mouse mAb. The human IgG_1 (Calbiochem, La Jolla, CA) and mouse IgG myeloma proteins (R&D Systems, Minneapolis, MN) were used as controls for the mAbs. Subsequently, the cells were washed and detected by FITC-conjugated goat F $(ab')_2$ anti-mouse Ig's (FITC-aM) (Biosource, Camarillo, CA) and FITC-conjugated goat F $(ab')_2$ anti-human Ig's (FITC-aH) (Sigma), followed by fixation with a 4% solution of formaldehyde in PBS. The fixed cells were analyzed by FACS as previously described [27]. The results were obtained after subtracting the background staining of the control human or mouse IgG.

2.8. mAb competition assay

The effects of patients' sera on the binding of biotinylated 50.69, 98.6, T26 and 902 mAbs to H9/IIIB (5×10^5) cells were studied. Biotinylated mAbs at saturated concentrations, 50.69 (4 µg/ml), 98.6 (4 µg/ml), T26 (32 µg/ml) and 902 (4 µg/ml), were added to the cells, which were pre- incubated with 20 µl sera for 1 h at 4 °C and washed twice. Cells were incubated for 30 min at 4 °C for mAb 50.69 or 98.6 and 15 min at 37 °C for mAb T26. After being washed twice, 4 µl PE-avidin was added and the cells were incubated for 30 min at 4 °C with gentle agitation and then fixed. Binding of biotinylated mAbs to cells was detected by FACS as above. % Inhibition by sera competition was calculated as follows. The MFI of cells incubated with healthy volunteer sera and biotinylated detector mAb was defined as 100% positive control. The MFI of cells without sera and detector mAb was defined as background. Background MFI was subtracted from the patient sera MFI and positive control MFI, then calculated. [1-(patient sera MFI – background MFI)/(positive control MFI – background MFI)] × 100 (%). PCP/LTS % inhibition ratio was calculated as (% inhibition of PCP sera/% inhibition of LTS sera).

2.9. Statistics

Data are expressed as mean ± S.E.M. One-sided nonparametric analysis was performed with the Mann–Whitney U-test to determine statistically significant differences between two groups. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. The binding of anti-gp41 antibodies to monomeric or oligomeric gp41 molecules

The immunoassays of anti-gp41 antibodies binding to HIV-1 virus lysate were examined to assess whether the anti-gp41 antibodies can bind to monomeric or oligomeric gp41 molecules (Fig. 1). The mAbs 50.69, 98.6, T26, 246D, 902 and Chessie 8 were added. These antibodies were detected by alkaline phoshatase conjugated anti-human or mouse antibody. MAbs 50.69, 98.6, 246D and Chessie 8 detected monomeric gp41 as 41 kDa and oligomeric gp41 molecules as 120 and 160 kDa bands, suggesting that the epitopes of these mAbs are expressed in both monomeric and oligomeric gp41. MAb T26 preferentially detected oligomeric gp41 as 120 and 160 kDa bands but not monomeric gp41 as previously reported [11]. Since mAb 902 detected the band of 120 kDa gp120 but not that at 160 kDa, 160 kDa indicated the gp41 tetramer rather than gp160 as previously reported [28]. Binding of mAb Chessie 8, which binds to the gp41 cytoplasmic linear epitope [29], to gp41 was not influenced by the gp41 conformation.

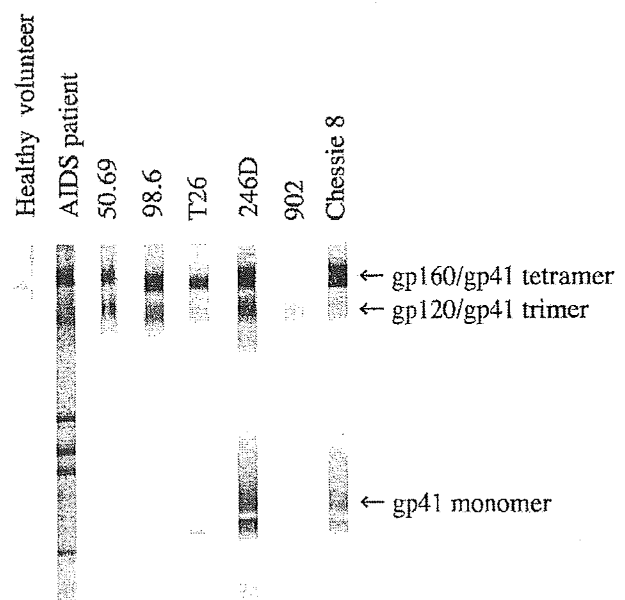

Fig. 1. WB analysis of mAbs. Each WB strip was incubated with 20 µl sera, 32 µg T26 mAb or 4 µg other mAbs diluted with 2 ml wash solution. The healthy volunteer sera as negative control and AIDS patient sera as positive control reference were performed.

3.2. Soluble CD4 induces the conformational change of gp120 and gp41 on H9/IIIB or MN cells

In this study, we used sCD4 which mimicked the interaction of cell-anchored CD4 with the HIV-1 envelope glycoprotein expressed on the surface of H9/IIIB cells in a dose-dependent manner and attained a maximum mAb binding at 10 μg/ml of sCD4 as previously described [13,14,27]. We measured the change in the gp120 and gp41 epitope exposure after the binding of sCD4 to H9/IIIB or MN cells using FACS. Fig. 2a shows the binding of mAbs to H9/IIIB cells. The binding of mAbs 50.69, 98.6 and 246D, gave a strong signal and pretreatment of sCD4 increased the signal as follows: control vs. mAb 50.69 ($P = 0.046$), mAb 98.6 ($P = 0.032$) and mAb 246D ($P = 0.016$). The binding of mAb T26 gave a weak signal and sCD4 pretreatment slightly increased the signal ($P = 0.121$). The binding of mAb 902 gave a weak signal and sCD4 pretreatment slightly decreased it ($P = 0.21$). Fig. 2b also shows the binding to H9/MN cells. The binding of mAbs 50.69 and 246D gave a low signal and pretreatment of sCD4 dramatically increased the signal for mAb 50.69 ($P = 0.03$) and mAbs 246D ($P = 0.001$). The binding of mAbs 98.6, T26 and 902 gave no signal regardless of sCD4 pretreatment because there was no significant difference between their MFIs and negative control MFI. This result suggests that mAb 50.69 cannot bind to MN strain gp41 before sCD4 pretreatment, although mAb 50.69 binds to activated IIIB strain gp41 on the cell surface. MAb 98.6 binds to N36/C34 peptide mix or C43 peptide derived from IIIB strain, but does not bind to H9/MN cells. Therefore, the seven amino acids of C34, which are different between the IIIB and MN strains, are possibly responsible for determining whether mAb 98.6 binds to the gp41 molecule (Table 3).

3.3. PCP sera compete with anti-oligomeric gp41 antibody T26 more efficiently than LTS

We performed a competition assay to evaluate humoral immno-responses to gp41 epitopes in LTS and PCP. Fig. 3a, b show the results of the competition assay between pre-bound sera and anti-core structure mAbs. Biotinylated mAbs were detected with PE-avidin. Biotinylated mAbs 50.69, 98.6 and T26 binding were significantly decreased after PCP and LTS sera pretreatment for mAb 50.69 ($P = 0.00006$), mAb 98.6 ($P = 0.028$), mAb 902 ($P = 0.013$) and mAb T26 ($P = 0.0006$) after PCP sera, and for mAb 50.69 ($P = 0.0003$), mAb 98.6 ($P = 0.051$), mAb T26 ($P = 0.11$) and mAb 902 ($P = 0.45$) after LTS sera.

In Fig. 3a, the average of LTS % inhibition was 52.5% for mAb 50.69, 34.9% for mAb 98.6, 31.6% for mAb T26 and –12.0% for mAb 902. The average of PCP % inhibition was 66.9% for mAb 50.69, 48.8% for mAb 98.6, 59.4% for mAb T26 and –31.8% for mAb 902. PCP/LTS % inhibition ratios were 1.27 for mAb 50.69, 1.39 for mAb 98.6, 1.87 for mAb T26 and 2.64 for mAb 902. PCP sera blocked these mAbs bindings more efficiently than LTS for mAb 50.69 ($P = 0.24$), mAb 98.6 ($P = 0.54$) and mAb T26 ($P = 0.17$). This suggests PCP sera contain more competitive antibody against mAbs 50.69, 98.6 and T26 than LTS sera. PCP sera competed with mAb T26 at the same level as with mAbs 50.69 and 98.6. On the other hand, anti-gp120 mAb 902 binding was enhanced after PCP and LTS sera pretreatment. PCP sera pretreatment more significantly enhanced mAb 902 binding to H9/IIIB cell compared to LTS sera pretreatment ($P = 0.27$).

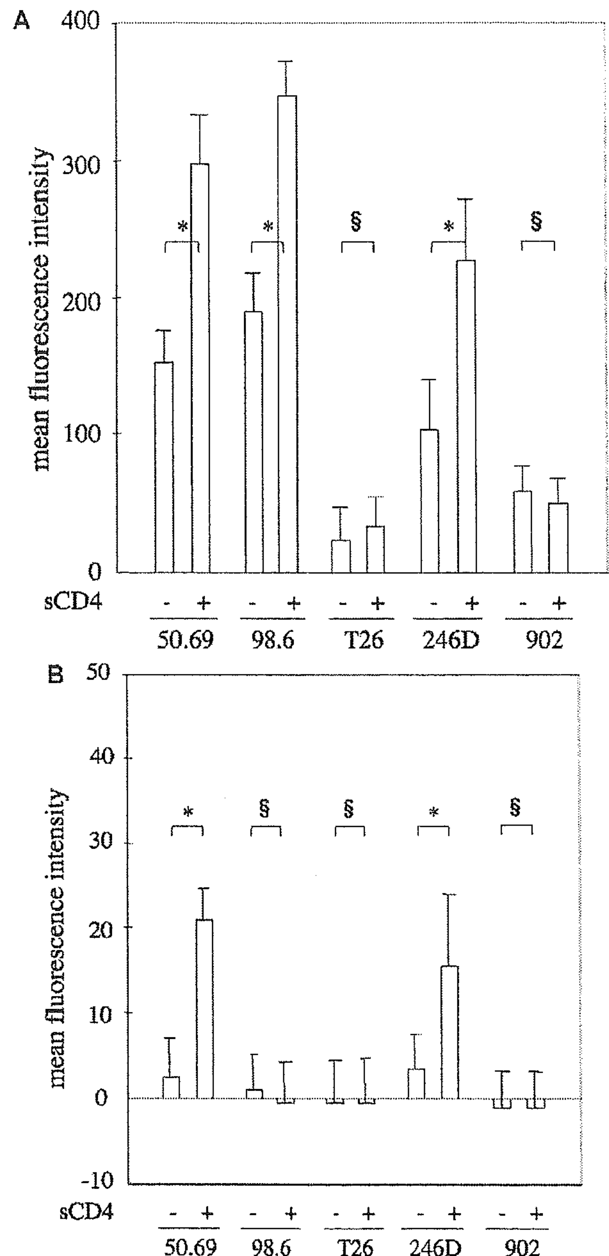

Fig. 2. Properties of mAbs binding to H9/IIIB or MN. Effect of sCD4 to env protein on (A) H9/IIIB and (B) H9/MN cells. Cells were incubated with 32 μg/ml mAb T26 and 4 μg/ml other mAbs, followed by FITC-aH or -aM incubation. Signals were detected by FL1. Each bar (± S.E.M.) presents the mean of triplicate determinations. * $P < 0.05$ and § $P > 0.1$ as determined by the Mann–Whitney U-test.

Table 3
Sequence of IIIB and MN strain gp41 N and C hapted domains

```
        N51   N36     DP107
IIIB : QARQLLSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ
  MN : ...L.....................M.............V..........

       C43 C34        DP178
IIIB : NNMTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF
  MN : ......Q.E...D......Y..L.K..T.....................
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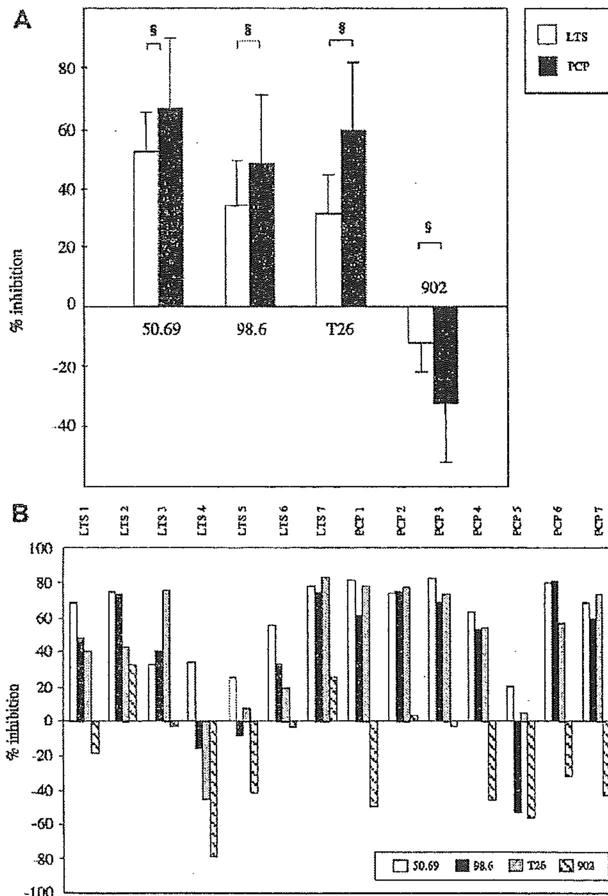

Fig. 3. % Inhibition of sera against binding of mAbs 50.69, 98.6, T26 and 902. (A) shows the average of competition between pre-bound sera and mAbs. Each bar (± S.E.M.) presents the mean of seven sera determinations. § $P > 0.1$ as determined by the Mann–Whitney U-test. (B) each patient's results between pre-bound sera and mAbs.

4. Discussion

The gp41 core structure to which mAbs 50.69 and 98.6 are assumed to bind has been considered to form its unique structure after receptor molecule binding. We showed that mAbs 50.69 and 98.6 bind to both monomeric and oligomeric gp41, and mAb T26 binds only to oligomeric gp41 in WB. Specific recognition of the oligomeric form gp41 by mAb T26 but not by other mAbs was already described previously [10]. The difference suggests that mAbs 50.69 and 98.6 bind also to the monomeric structure on the cell surface but mAb T26 react to only the oligomeric form. In FACS analysis of H9/IIIB cells, MFI or % of positive cells, given by mAb T26, was lower than by mAbs 50.69 and 98.6. This may be due to the fact that mAb T26 binding affinity is lower than those of mAbs 50.69 or 98.6. But this possibility is less likely because we used a saturating concentration of mAb T26 in FACS. The low MFI of H9/IIIB cells by oligomeric specific mAb T26 indicated that there were only a few oligomeric structures on H9/IIIB. It was already shown that most of the envelope structures on H9/IIIB are monomeric [30]. Furthermore, we could not find the slightest increment of mAb T26 positive cells by sCD4 treatment, though mAbs 50.69 and 98.6 reacted generally after the treatment. Therefore, not all the populations of gp41 on the cell surface are ready for immediate fusion even after sCD4 treatment. In fact, we found only 1 of 10^3–10^4 H9/IIIB cells caused fusion in the MAGI cell assay (Yi Lui, unpublished observation).

We attempted to detect anti-core structure antibodies in HIV-1-infected sera using a competition assay of mAb T26 and other mAbs because mAb T26 binds specifically to oligomeric gp41 epitope. We used mAb 902 (anti-gp120 V3 IIIB virus) as a control and found that pretreatment by sera enhanced its binding though the cause(s) of the finding is not clear. We showed that both LTS and PCP sera contain competitive antibodies with anti-core structure mAbs. We could not find any distinguishable relationship between each patient's clinical characteristics and each competition. And in all LTS and PCP patients' analyses, since the % inhibition of mAb T26 in LTS and PCP was at the same level as that of mAb 50.69 and 98.6, LTS and PCP appear to retain anti-oligomeric gp41 immunity. PCP pre-bound sera competed with the anti-core structure antibodies mAbs 50.69, 98.6, T26 more efficiently than LTS. And anti-gp41 immunity was induced more efficiently in PCP than LTS because PCP/LTS % inhibition ratio of mAb T26 was higher than that of mAbs 50.69 and 98.6 by sera competition.

It was clarified that there is the inverse correlation between antibody titers and disease progression when the peptide corresponding to gp41 immunodominant region was used [18]. We also described similar results using DP107 peptide [7]. The reason why the results here were different from those of previous reports is not known. One reason is the specific clinical features used in this study. Excessive immunoreactive factor in PCP lung was reported to induce developing pneumonia [31] and enhances local anti-PCP IgG production [32]. In addition, HIV-1 production by alveolar lymphocytes is increased during PCP [33]. Enhanced immunoreactions and higher HIV-1 copies in PCP lung possibly explain the enhanced production of anti HIV-1 antibodies. On the other hand, the reason why the increase of anti-core structure mAbs failed to prevent the disease progression is also unknown. Only a few reports demonstrated neutralizing activities of these antibodies, probably because they recognize the final structure and did not prevent the conformational change of gp41 upon virus entry [24]. Recently, we have noted one-

way interaction between mAbs 98.6 and 50.69 (O. Usami, unpublished observation, April 04). More careful analysis of the interactions of these antibodies would give us more information on fusion core structures of gp41.

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