long period. During the past few years, many investigators have investigated the forecasting factors of drug resistance and mutations, but without reaching a conclusion. Some factors concerned with YMDD mutations, could raise the curative effect of lamivudine. Recently many investigations have concentrated on the time of the YMDD mutation appearance, the mutation type, the YMDD mutation and ALT level, the DNA level, and the rate of HBeAg seroconversion, but no relationship between the mutations of HBV and the HBV genotypes has been reported. The viral genotype that represents the features of natural infection is the result of evolutionary variation of the virus. The HBV genotype has a certain association with the route of virus infection, genetic mutations, and the progression of chronic hepatitis B and the efficacy of antiviral therapies. The genotypes of HBV also have distinct geographical distributions. Genotypes B and C are were only found in those who originated from eastern Asia and Far East including Taiwan (14). In Japan, the great majority of HBV isolates belong to genotype B or C (15). Genotypes B, C are spread dominantly in China while genotypes A, D relatively rare. Genotype B is prevalent in South China and genotype C in North China (16). The clinical prognosis after infection with HBV varies according to the genotype. In the previous study, clearance of HBeAg occurred earlier and more frequently, and development of cirrhosis was less common in patients with genotype B compared with genotype C (15). Genotype C is associated with chronic liver disease, genotype B mostly associated with acute hepatitis in China (17). The present study analyzed 142 sera of chronic hepatitis B in North China. The results showed that genotype C occupied 88%, and genotype B 9.2% and genotype D 2.8%. Genotype C is predominant in patients with chronic liver disease in North China. A study in Japan reported that among 234 patients with chronic hepatitis B infected with genotype A, B, or C and treated with

lamivudine for more than 1 year, the emergence of mutations was not different among genotypes A, B and C by the Kaplan-Meier method. (18). In Japan, it was reported that the emergence rate of lamivudine resistance was independent of the genotype (A, B, and C), after treatment of patients with chronic hepatitis B with various genotypes with lamivudine,. In contrast, the emergence rate was significantly higher in the Ba ("a" stands for Asia) subgroup of HBV than in Bj ("j" for Japan) subgroup ($P<0.05$) (19). In the Hong Kong study, there was no difference in the antiviral response and the rate of development of YMDD mutations in 82 patients with chronic hepatitis B with genotype B and C after 1 year of lamivudine treatment (20). A report on 87 patients receiving lamivudine in Guiyang, results showed that, though genotype B was dominant in that region, there was a higher mutation rate of drug resistance among genotype C cases (21). In another report, 135 chronic hepatitis B patients received lamivudine for 1 year, and the YMDD mutation rate in genotype C cases was higher than that in genotype B cases in Guangdong (22). But Guiyang and Guangdong province located in Southern parts of China. In the present study, 9 YMDD mutations were found in genotype B, and 68 YMDD mutations were found in genotype C. The YMDD mutation rates were 69.2% and 54.4% in genotype B and in genotype C, respectively in North China（$P>0.05$）. It has been reported that the YIDD mutation occurs more frequently in genotype D, while YVDD is more common in genotype A (23). It is suggested that the YMDD mutation type is related to the genotype. But the present study showed that the YVDD mutation occurred more frequently in genotypes B, the YIDD mutation in genotypes C, the YMDD mutation showed significant differences between the HBV genotypes B and C.（$\chi 2=4.6$, $P<0.05$）. It has been reported that the replication ability of the YVDD mutant strain might be stronger than that of the YIDD mutant strain (24). The mutation types among different genotypes aren't identical, because

the genotypes are associated with virus replication and virus variation. Because of the small number of cases of genotype D, only HBV genotype B and C were examined in this study. Large-scale prospective studies of each genotype should be conducted in the future to confirm these findings.

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REFERENCES

1. Suzuki, Y., Kumada, H., Ikeda, K., Chayama, K., Arase, Y., Saitoh, S., Tsubota, A., Kobayashi, M., Koike, M., Ogawa, N. and Tanikawa, K. (1999): Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J. Hepatol., 30, 743-748.
2. Kidd-Ljunggren, K., Miyakawa, Y. and Kidd, A.H. (2002): Genetic variability in hepatitis B viruses. J. Gen. Virol., 83, 1267-1280.
3. Arauz-Ruiz, P., Norder, H., Robertson, B.H. and Magnius, L.O. (2002): Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J. Gen. Virol., 83, 2059-2073.
4. Moriyama, M., Taira, M., Matsumura, H., Aoki, H., Mikuni, M., Kaneko, M., Shioda, A., Iwaguchi, K., Arai, S., Ichijima, S., Iwasaki, H., Tanaka, N., Abe, K. and Arakawa, Y. (2003): Genotype analysis, using PCR with type-specific primers, of hepatitis B virus isolates from

patients coinfected with hepatitis delta virus genotype II from Miyako Island, Japan. Intervirology, 46, 114-120.

5. Miyakawa, Y. and Mizokami, M. (2003): Classifying hepatitis B virus genotypes. Intervirology, 46, 329-338.

6. Chu, C.J., Keeffe, E.B., Han, S.H., Perrillo, R.P., Min, A.D., Soldevila-Pico, C., Carey, W., Brown, R.S. Jr., Luketic, V.A., Terrault, N. and Lok, A.S. (2003): Hepatitis B virus genotypes in the United States: results of a nationwide study. Gastroenterology, 125, 444-451.

7. Yuen, M.F., Sablon, E., Yuan, H.J., Wong, D.K., Hui, C.K., Wong, B.C., Chan, A.O. and Lai, C.L. (2003): Significance of hepatitis B genotype in acute exacerbation, HBeAg seroconversion, cirrhosis-related complications, and hepatocellular carcinoma. Hepatology, 37, 562-567.

8. Naito, H., Hayashi, S. and Abe, K. (2001): Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using type-specific primers. J. Clin. Microbiol., 39, 362-364.

9. Moskoviz, D.N., Osiowy, C., Giles, E., Tomlinson, G. and Heathcote, E.J.(2005): Response to long-term lamivudine treatment (up to 5 years) in patients with severe chronic hepatitis B, role of genotype and drug resistance. J Viral Hepat., 12, 398-404.

10. Atkins, M., Hunt, C.M., Brown, N., Gray, F., Sanathanan, L., Woessner, M., Lai, C.l., Dusheiko, G., Dienstag, J., Wright, T., Bamard, J., Bourne, E. and Condreay, L. (1998): Clinical significance of YMDD mutant hepatitis B virus in a large cohort of lamivudine-treated hepatitis B patients (Abstract). Hepatology, 8, 319.

11. Leung, N.W., Lai, C.L., Chang, T.T., Guan, R., Lee, C.M., Ng, K.Y., Lim, S.G., Wu, P.C.,

Dent, J.C., Edmundson, S., Condreay, L.D. and Chien, R.N; On behalf of the Asia. (2001): Hepatitis Lamivudine Study Group. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology, 33, 1527-1532.

12. Niesters, H.G, Honkoop, P., Haagsma, E.B., de Man, R.A., Schalm, S.W. and Osterhaus, A.D. (1998): Identification of more than one mutation in the hepatitis B virus polymerase gene arising during prolonged lamivudine treatment.J Ifect Dis.,177,1382-1385
13. Akuta, N., Suzuki, F., Kobayashi, M., Matsuda, M., Sato, J., Takagi, K., Tsubota, A., Suzuki, Y., Hosaka, T., Someya, T., Kobayashi, M., Saitoh, S., Arase, Y., Ikeda, K. and Kumada, H. (2003):Virological and biochemical relapse according to YMDD motif mutant type during long-term lamivudine monotherapy. J Med Virol., 71,504-510.
14. Kao, J.H., Liu, C.J. and Chen, D.S. (2002): Hepatitis Bviral Genotypes and lamivudine resistance. J Hepatol., 36, 303-304
15. Tomokuni, N., Tatsuji M., Tomofumi, N., Hiroki, N., Hiroshi, S., Fukunori, K., Etsuro, O. and Masashi, M. (2003): Difference in Prognosis Between Patients Infected With Hepatitis B Virus With Genotype B and Those With Genotype C in the Okinawa Islands: A Prospective Study. J. of Med Virol, 70, 350–354.
16. Gu, H.X., Xu, Z.L., Liu, J.Y., Zhong, Z.H., Wang, H.Q., Zhang, S.Y., Li, D., Zhang, H.H. and Abe, K. (2004): Epidemiology of HBV genotypes by nested PCR with multi-paired primers. World Chin J Digestol., 12, 1073-1076 (in Chinese).
17. Dong, M. and Zhong, Y.X. (2005):Distridution of hepatitis B virus Genotypes and its chinical significance.Chin J Hepatol., 13, 56-57 (in Chinese).
18. Suzuki, F., Tsubota, A., Arase, Y., Suzuki, Y., Akuta, N., Hosaka, T., Someya, T., Kobayashi, M., Saitoh, S., Ikeda, K., Kobayashi, M., Matsuda, M., Satoh, J., Takagi, K. and Kumada, H. (2003): Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology, 46, 182-189.
19. Akuta, N., Suzuki, F., Kobayashi, M., Tsubota, A., Suzuki, Y., Hosaka, T., Someya, T.,

Kobayashi, M., Saitoh, S., Arase, Y., Ikeda, K. and Kumada, H. (2003):The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J. Hepatol, 38,315-321.

20. Yuen, M.F., Wong, D.K., Sablon, E., Yuan, H.J., Sum, S.M., Hui, C.K., Chan, A.O., Wang, B.C. and Lai, C.L. (2003): Hepatitis B virus genotypes B and C do not affect the antiviral response to lamivudine. Antivir. Ther., 8, 531-534.
21. Wang, G.Y., Zhu, W.J. and Yang, X.L. (2005): YMDD mutation of hepatitis B virus and genotyping in Guiyang region. Chin J Hepatol., 13, 193-195 (in Chinese).
22. Zhou, J.L. and Wu, S.P. (2004): Effects of comparison of lamivudine therapy B virus genotype B and C. Chin J Hepatol., 12, 489-490 (in Chinese).
23. Zollner, B., Petersen, J., Puchhammer-Stockl, E., Kletzmayr, J., Sterneck, M., Fischer, L., Schroter, M., Laufs, R. and Feucht, H.H. (2004): Viral features of lamivudine resistant hepatitis B genotypes A and D. Hepatology, 39, 42-50.
24. Zhang, X., Liu, C., Gong, Q., Zhang, S., Zhang, D., Lu, Z. and Wang, Y. (2003): Evolution of wild type and mutants of the YMDD motif of hepatitis B virus polymerase during lamivudine therapy. J Gastroenterol Hepatol.,18, 1353-1357.

Competitive Study of Monoclonal Antibodies Against the HIV-1 Gp41 Core Structure

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Running title: UNIDIRECTIONAL COMPETITION OF MABS AGAINST GP41

Subject sections and specified fields: Virology, Viral immunology

Abstract: Monoclonal antibodies (MAbs) 50.69, 98.6 and T26 bind specifically to the core structure of the human immunodeficiency virus type 1 (HIV-1) envelope transmembrane glycoprotein (gp41). To clarify the specificity of the anti- core structure MAbs, we performed competitive assays using the MAbs to the H9 human T cell line infected with the IIIB strain of HIV-1 (H9/IIIB). Bound MAb 50.69 inhibited MAb 98.6 binding unidirectionally. The reason for the unidirectional cross competition between MAbs 50.69 and 98.6 is not clear, but these results help to define the antigenic structure of gp41 on the surface of infected cells.

Key Words: HIV-1, Gp41, 50.69 antibody, 98.6 antibody

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FACS, flow cytometry; Gp41, envelope transmembrane glycoprotein 41; Gp120, envelope glycoprotein 120; Gp160, envelope glycoprotein 160; HIV-1, human immunodeficiency virus type 1; H9/IIIB, the H9 human T cell line infected with the IIIB strain of HIV-1; MAbs, monoclonal antibodies; MFI, mean fluorescence intensity; PE-avidin, phycoerthrin-labeled avidin

The binding of envelope glycoprotein 120 (gp120) of human immunodeficiency virus type 1 (HIV-1) to cellular receptors on target cells leads to conformational changes of envelope transmembrane glycoprotein (gp41) that permit viral and cellular membrane fusion (12). Recent crystallographic studies have shown that fusion-active gp41 folds into a six-helix alpha-helical bundle, in which three N-terminal helices (N peptides) form an interior, parallel-coiled-coil trimer, while three C-terminal helices (C peptides) pack in the reverse direction into three hydrophobic grooves on the surface of this coiled-coil (2, 9, 16). We have previously reported that the decline of anti-DP107 (aa 553-590) (alpha-helical N-peptide) antibody is associated with the clinical progression in HIV-1 infected individuals, suggesting that antibodies against the structure may have a protective role (7). To define the epitopes within these regions to which infected humans respond during the course of infection, the specificity of human MAbs to these regions was studied. Using 10 human MAbs identified initially by their reactivity to whole gp41 in HIV-1 virion lysates, Xu *et al.* previously reported two immunodominant regions of gp41 that define the epitopes within these regions to which infected humans respond during the course of infection (5, 17). The first region of gp4l is in the vicinity of the cysteines between 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 approximately 100-fold more efficient antibody binding to cluster I than to cluster II in patients' sera, confirming the immunodominance of cluster I. Subsequent studies have disclosed that human MAbs against gp41 could recognize the gp41 core structure (14). We called these MAbs anti-core structure MAbs, because the exact antigenic structure of gp41 clarified so far is only the core structure and the native and fusion-active structure of all gp41 molecules have not been clarified yet. MAb 50.69, which is a cluster I MAb, reacts to a N51(aa 540-590) and C43(aa 624-666) peptide mixture (N51/C43) (6), but neither to N36(aa 546-581)/ C34(aa 628-661) nor a single C43 peptide (17). MAb 98.6, which is a cluster II MAb, reacts to N51/C43, N36/C34 and a C43 peptide (14). Both N51/C43 and N36/C34 are known to form a six-helical bundle (2). MAb T26 was reported to bind to the six-helix bundle N36/C34 (1, 3, 4). These studies suggest

that MAb T26 is specific for the core structure because MAb T26 was reported to bind only to the oligomeric form in immunoprecipitation assays. Therefore, these MAbs are expected to bind to different epitopes. These MAbs must bind to the infected cell surface gp41 in the human body, although it is not known whether these MAbs interfere with the bindings of other MAbs. To clarify this issue we did competitive assays among human MAbs 50.69, 98.6 and T26.

One half million H9/IIIB cells, a human T cell line infected with the IIIB strain of HIV-1, were pre-incubated with saturated concentrations of MAbs 50.69, 98.6 or human IgG (Calbiochem, La Jolla, CA) for one hour at 4 ℃, followed by washing. The effects of these MAbs on the binding of biotinylated MAbs (B-50.69, B-98.6 and B-T26) to H9/IIIB cells were studied. Biotinylated MAbs 50.69 and 98.6 were provided by Dr. Miroslaw K. Gorny. MAb T26 IgG was purified from culture supernatants of hybridoma cells obtained from Dr. P. L. Earl and biotinylated. Because MAb T26 bound little at 4 ℃, we did not use MAb T26 as a competing MAb. B-50.69 (2 μg/ml), B-98.6 (2 μg/ml) or B-T26 (32 μg/ml) at saturated concentrations were added to the cells and incubated for 30 min at 4 ℃ for MAb B-50.69 or MAb B-98.6, and for 15 min at 37 ℃ for B-T26. After washing the cells twice, 4 μl phycoerthrin-labeled avidin (PE-avidin) (Serotec Ltd, Kidlington, Oxford, UK) was added and the cells were incubated for 30 min at 4 ℃ followed by fixation. PE-avidin labeled biotinylated MAb was detected by flow cytometry (FACS).

Since we could not detect efficient MAb T26 binding to H9/IIIB at 4 ℃, we tried to detect the temperature dependency of the MAb T26 binding. MAb T26 bound H9/IIIB and yielded a low mean fluorescence intensity (MFI) at 4 ℃, and a high MFI at 37 ℃ (Fig. 1). The saturating concentrations were 64 μg/ml at 4 ℃ and 32 μg/ml at 37 ℃ and the saturated MFI at 4 ℃ was 11.5 and significantly lower than the saturated MFI 18.8 at 37 ℃ ($P<0.0167$).

Fig. 1

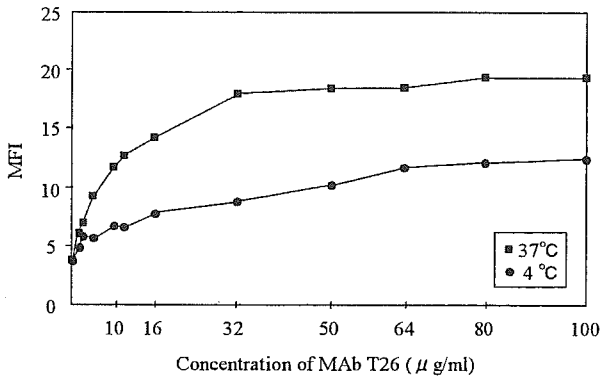

Fig. 1 The temperature dependency of MAb T26 binding. MAb T26 binding to H9/IIIB at 37 ℃ was higher than at 4 ℃.

We evaluated the MAb T26 binding at 37 ℃, and those of MAbs 50.69 and 98.6 at 4 ℃, and calculated the % inhibition (Fig. 2). MAb 50.69 pre-incubation blocked its own binding as a positive control. MAb 98.6 did not block MAb B-50.69 binding ($P \geqq 0.05$). MAb B-98.6 was also examined in a similar condition. MAb 98.6 pre-incubation blocked MAb B-98.6 binding. The MAb 50.69 pre-incubation blocked MAb B-98.6 binding ($P<0.0167$), though the former MAb inhibited the binding more efficiently than the latter did. We were able to observe that MAbs 50.69 and 98.6 bound at 4 ℃ and avoided non-specific binding, although the condition was not physiological (13). We also confirmed that MAb B-T26 binding at 37 ℃ was blocked by bound MAb T26. The MAb 50.69 pre-incubation did not show a significant blocking effect for MAb B-T26 ($P \geqq 0.05$). However, pre-incubation of MAb 98.6 blocked it's binding very efficiently ($P<0.0167$).

MAb T26 was identified as an anti-gp41 antibody that binds only to oligomers, particularly to trimers, but not to monomers (4). Both MAbs 98.6 and T26 bind to a mixture of N36/C34, but their precise epitopes must be different from each other

because MAb 98.6 also binds to monomeric gp41. MAb 98.6 pre-incubation inhibited MAb B-T26 binding to H9/IIIB, indicating the epitopes of MAbs 98.6 and T26 may partially overlap.

Fig. 2

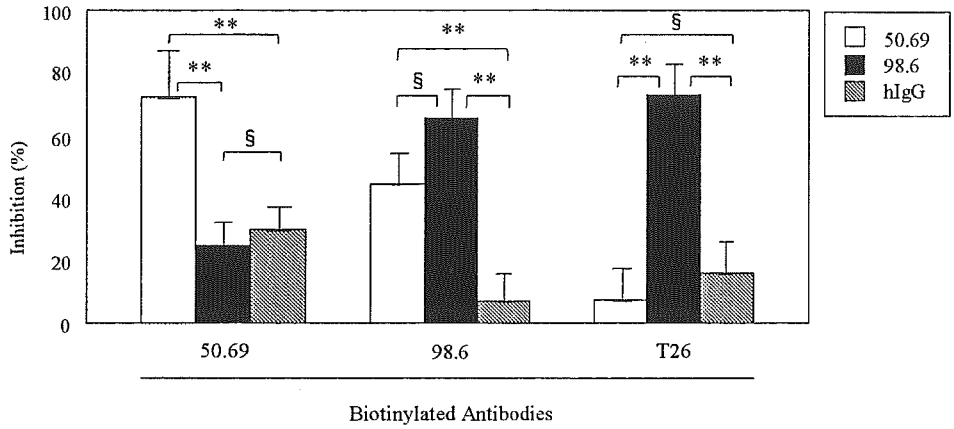

Fig. 2. The decreased binding of biotinylated MAb 50.69, 98.6 or T26 to H9/IIIB cells after preincubation with MAb 50.69 (open bars), 98.6 (filled bars) or hIgG (gray bars). The negative control was calculated as the MFI without preincubation MAb and with biotinylated MAb. The positive control was calculated as the MFI with biotinylated MAb blocked by the same preincubation MAb, the concentration of which was saturated. % inhibition was calculated as [1-(biotinylated MAb MFI - positive control MFI) / (negative control MFI - positive control MFI)] x 100 (%). Data are expressed as mean □S.D. Each bar (±SD) represents the mean of triplicate determinations. (**P<0.0167) (§ P≧0.05) To determine statistically significant differences among three groups, differences were considered to be statistically significant when P<0.0167 by Bonferroni/Dunn test. Data were analyzed using the CellQuest software (Becton Dickinson Biosciences).

On the other hand, MAb 50.69 did not compete with MAb B-T26 at all, indicating that the epitopes of the two MAbs are distant. These data accord with the findings that MAb 50.69 does not bind to a mixture of N36/C34 but MAb T26 does.

MAb 98.6 binds to a unique epitope shared with a mixture of N51/C43, N36/C34

and C43. But MAb 50.69 binds specifically to a mixture of N51/C43 but not C43. It is worthy to note that MAb 50.69 pre-incubation inhibited MAb 98.6 binding but the interference was not reciprocal. As shown previously, MAbs 50.69 and 98.6 did not compete in an enzyme-linked immunosorbent assay (ELISA) using viral lysate or recombinant proteins derived from gp41 (17). P. L. Earl *et al.* also performed a competitive assay for anti gp41 MAbs in ELISA. They also showed that the majority of the anti- gp41 MAbs are conformation dependent and most of determinant I as cluster I MAbs do not compete with determinant II as cluster II MAbs (3). MAb 50.69 was classified as a cluster I antibody, and MAb 98.6 was classified as a cluster II antibody. However, in the present study we performed competitive assays using an infected live cell line and FACS. The difference in the method used may explain the conflicting results. The epitopes of MAbs 50.69 and 98.6, which are expressed on the infected cell surface, possibly have a different conformation from the gp41 peptides previously used in ELISA, while it is known that HIV-1 envelope proteins form oligomers dominantly in viral lysate (6). The envelope proteins on the infected cell surface are heterogeneous with native gp120-gp41 complex, residual gp41 after gp120 shedding, and uncleaved envelope glycoprotein 160 (gp160) precursor, although gp160 derived from viral lysate forms a trimer (10). Atomic force microscopy investigation revealed that monomeric gp120 is dominant on H9/IIIB (8). The unidirectional competition of MAbs 50.69 and 98.6 may be explained by the positional relationship among epitopes on monomeric gp41. The size of the epitopes, the induced conformational changes upon binding MAb 50.69 and differences in the affinities of the MAbs also might be responsible for our results.

In this study, we analyzed the binding properties of anti- core structure gp41 human MAbs using infected cells. These analyses will contribute to understanding the structure of gp120-gp41 on the infected cell surface and the complex interactions of humoral antibodies against HIV-1. The unidirectional competition, which has not been able to be observed using gp41-derived peptides so far, suggests that the immune-response against the gp41 core structure varies much among patients and some of them are possibly dominant for certain exclusive epitopes, because patients derived

MAbs are considered to recognize the functional gp41 in the human body. We previously did competition assays between patients' sera and anti-core structure MAbs, because patients' sera may have a diverse competition with anti-core structure MAbs (15), and found that the patients' sera competed with MAb 50.69 more than MAb 98.6 (unpublished data), suggesting that the antibodies that compete with MAb 50.69 are greater in number than those that compete with MAb 98.6 in vivo.

According to the above results the antibodies that bind to the epitope of MAb 50.69 are expected to compete with MAb 98.6. The lower competition of MAb 98.6 with sera suggests that some antibodies that recognize the overlapping epitope of MAb 50.69 do not compete with MAb 98.6.

Our results also indicate that there are few antibodies that have the epitope of MAb 98.6 in vivo, and suggest that the antibodies with this epitope may hardly be induced, especially among cluster II antibodies. This is assumed to be partly due to the fact that cluster II is a more variable domain than cluster I (11).

The anti-gp41 antibodies in one patient's body are assumed to compose a population including such exclusivity and heterogeneity. Our data not only contribute to analyses of the functional epitopes, which are able to induce anti-gp41 antibodies in vivo, but also to establishing a method to evaluate the complex anti-gp41 immunity.

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References

1) Broder, C. C., Earl, P. L., Long, D., Abedon, S. T., Moss, B., and Doms, R. W. 1994. Antigenic implications of human immunodeficiency virus type 1 envelope quaternary structure: oligomer-specific and -sensitive monoclonal antibodies. Proc. Natl. Acad. Sci. U. S. A. **91:** 11699-11703.

2) Chan, D. C., Fass, D., Berger, J. M., and Kim, P. S. 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell **89:** 263-273.

3) Earl, P. L., Broder, C. C., Doms, R. W., and Moss, B. 1997. Epitope map of human immunodeficiency virus type 1 gp41 derived from 47 monoclonal antibodies produced by immunization with oligomeric envelope protein. J. Virol. **71:** 2674-2684.

4) Earl, P. L., Broder, C. C., Long, D., Lee, S. A., Peterson, J., Chakrabarti, S., Doms, R. W., and Moss, B. 1994. Native oligomeric human immunodeficiency virus type 1 envelope glycoprotein elicits diverse monoclonal antibody reactivities. J. Virol. **68:** 3015-3026.

5) Gorny, M. K., Gianakakos, V., Sharpe, S., and Zolla-Pazner, S. 1989. Generation of human monoclonal antibodies to human immunodeficiency virus. Proc. Natl. Acad. Sci. U. S. A. **86:** 1624-1628.

6) Gorny, M. K., VanCott, T. C., Williams, C., Revesz, K., and Zolla-Pazner, S. 2000. Effects of oligomerization on the epitopes of the human immunodeficiency virus type 1 envelope glycoproteins. Virology **267:** 220-228.

7) Hattori, T., Komoda, H., Pahwa, S., Tateyama, M., Zhang, X., Xu, Y., Oguma, S., Tamamura, H., Fujii, N., Fukutake, K., and Uchiyama, T. 1998. Decline of anti-DP107 antibody associated with clinical progression. Aids **12:** 1557-1559.

8) Kuznetsov, Y. G., Victoria, J. G., Robinson, W. E., Jr., and McPherson, A. 2003. Atomic force microscopy investigation of human immunodeficiency virus (HIV) and HIV-infected lymphocytes. J. Virol. **77:** 11896-11909.

9) Lu, M., Blacklow, S. C., and Kim, P. S. 1995. A trimeric structural domain of the HIV-1 transmembrane glycoprotein. Nat. Struct. Biol. **2:** 1075-1082.

10) Pinter, A., Honnen, W. J., Tilley, S. A., Bona, C., Zaghouani, H., Gorny, M. K., and Zolla-Pazner, S. 1989. Oligomeric structure of gp41, the transmembrane protein of human immunodeficiency virus type 1. J. Virol. **63:** 2674-2679.

11) Robinson, W. E., Jr., Gorny, M. K., Xu, J. Y., Mitchell, W. M., and Zolla-Pazner, S. 1991. Two immunodominant domains of gp41 bind antibodies which enhance human immunodeficiency virus type 1 infection in vitro. J. Virol. **65:** 4169-4176.

12) Sattentau, Q. J., and Moore, J. P. 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J. Exp. Med. **174:** 407-15.

13) Sattentau, Q. J., Zolla-Pazner, S., and Poignard, P. 1995. Epitope exposure on functional, oligomeric HIV-1 gp41 molecules. Virology **206:** 713-717.

14) Taniguchi, Y., Zolla-Pazner, S., Xu, Y., Zhang, X., Takeda, S., and Hattori, T. 2000. Human monoclonal antibody 98-6 reacts with the fusogenic form of gp41. Virology **273:** 333-340.

15) Usami, O., Xiao, P., Ling, H., Liu, Y., Nakasone, T., and Hattori, T. 2005. Properties of anti-gp41 core structure antibodies, which compete with sera of HIV-1-infected patients. Microbes. Infect. **7:** 650-657.

16) Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J., and Wiley, D. C. 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature **387:** 426-430.

17) Xu, J. Y., Gorny, M. K., Palker, T., Karwowska, S., and Zolla-Pazner, S. 1991. Epitope mapping of two immunodominant domains of gp41, the transmembrane protein of human immunodeficiency virus type 1, using ten human monoclonal antibodies. J. Virol. **65:** 4832-4838.

Cloning and analysis of the envelope of HIV-1 CHNHLJ03009 from an infected individual in Heilongjiang province

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Abstract To analyze the variability and phenotype of envelope glycoprotein (env) of human immunodeficiency virus type 1 (HIV-1) prevalent in Heilongjiang province, cloning of full-length env gene from PBMCs (peripheral blood mononuclear cell) of a HIV-1 positive individual in Heilongjiang province in China was performed using conserved region primers. The amplified PCR products were cloned into a plasmid vector and sequenced. Phylogenetic analysis was done upon full-length env amino acid sequence. Subsequently, a HIV-1 pseudotyped virus bearing the envelope protein was constructed and the infectivity was examined using U87 cell lines expressing CD4 with either CCR5 or CXCR4. As the result, two functional env clones named CHNHLJ03009c34 (GenBank Accession No: AY905493) and CHNHLJ03009c33 were obtained. It was found that the homology between CHNHLJ03009c34 and a HIV-1 subtype B′ strain, RL42, which was isolated from Yunnan province, is 91.52% through comparing and analyzing full-length

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Env amino acid sequence of HIV-1 isolated from either China or abroad. Phylogenetic analysis indicated that CHNHLJ03009c34 has the closest molecular relation with strain RL42 based on analyzing full-length of the Env, while it became an independent branch upon analyzing the sequences of C2-V3 region of the Env. The secondary structure analysis of the envelope protein showed that the antigenicity and hydrophobicity of the strain do not have definite difference from that of RL42. Infectivity examination showed that pseudovirus CHNHLJ03009c34 can infect only U87.CD4.CCR5 cells indicating that it is a R5-tropic HIV-1. In the conclusion, two HIV-1 env clones from an infected individual CHNHLJ03009 in Heilongjiang province have been identified as subtype B' and R5-tropic HIV-1. This is the first report on the analysis of primary isolates in Heilongjiang province.
Key words: Human immunodeficiency virus type 1 (HIV-1) Envelope Variability Subtype Infection

Envelope glycoprotein (Env) of HIV-1 is a complex of two noncovalently associated subunits, Gp120 and Gp41. Gp120 is an external subunit that binds the cellular receptor CD4 and a chemokine receptor such as CXCR4 or CCR5. Gp41 is a transmembrane subunit, which is responsible for the receptor-mediated membrane fusion [1-2]. Because of the high variability of HIV-1, the amino acid sequence as well as the structure of viral envelope can vary and result in the change of viral tropism with time extension and disease progression after infection. During the early phase of HIV infection, macrophage (M)-tropic HIV-1 strains, which infect macrophages, mainly utilize CCR5 as a coreceptor, while from later stages of disease, T-tropic HIV-1 strains, which infect lymphocytes and T-cell lines, utilize CXCR4 as a coreceptor. Accordingly, the accumulation of the viruses of CXCR4 utilization may associate with the rapid progress of the disease, or AIDS stage [3]. The mechanism of HIV-1 tropism has not been clearly clarified. Many studies have previously demonstrated that the third hypervariable (V3) loop of gp120 is the major determinant for HIV-1 tropism and the coreceptor usage [4]. These studies have identified that V3 loop consists of a surface structure with other elements on the surface of *env*, which is responsible for its binding to the coreceptors. The infectivity of the viruses with V3 loop

deletion is strikingly degraded [5-7], while their Gp120 can still bind to antibody 17b whose epitope is partially overlapped with coreceptor binding sites [8]. It is important to clarify the correlation between the sequence variation of viral env and the biological characteristics of HIV-1 prevalent in China. In the present study, we cloned the full-length env gene from PBMCs of a HIV-1 positive individual from Heilongjiang province, in China, analyzed the nucleotide sequence, examined the cell tropism using a pseudovirus infection system. This work will be helpful to understand the biological phenotype of the prevalent strains in China and viral infection mechanism.

MATERIALS AND METHODS

Cells and cell culture

Human embryonic kidney cell line 293T and human astroglia cell lines expressing CD4 antigen and either coreceptor CXCR4 (U87.CD4.CXCR4) or CCR5 (U87.CD4.CCR5) were maintained in D-MEM supplemented with 10% fetal calf serum (FCS). 0.3mg/ml G418 (Gibco, USA) and 1μg/ml puromycin (Sigma, USA) were added as the selection antibiotics for CD4 or coreceptors, respectively [9]. Viabilities of the cells were examined by the trypan blue dye exclusion method and always exceeded 95% when used for the experiments.

Isolation of PBMCs and extraction of the genome DNA

The peripheral blood from a female HIV-1 seropositive in Heilongjiang province, who had been a blood donor since 1980s, was collected and the PBMCs were isolated using Lymphocyte Separation Medium (TBD biotechnology development center, China) upon the manufacturer. The CD4+ and the CD8+ cell counts of the patient were 578 and 1376 cells /ml, respectively.

Amplification of HIV-1 env gene and construction of expression vector

The genome DNA was extracted from the PBMCs by using DNeasy Tissue Kit (Qiagen,

USA). The full-length env gene was amplified using nested PCR with pairs of conserved primers: forward outer primer, env-1, 5'-TAGAGCCCTGGAAGCATCCA-3'; reverse outer primer, env-2, 5'-TTGCTACTTGTGATTGCTCC-3'; inner forward primer, env-IFP, 5'-GAGCGAATTCACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAG - 3'; and inner reverse primer, env-IRP, 5'-ATCTGTCGACTCTCGAGATACTGCTCCCACCCATC TGCT–3' (Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd, China). The first PCR was performed in 20μl reaction mixture as follows: PCR buffer, 0.35mM of each dNTP, 0.3μM of env-1 and env-2, 1.0 unit of DNA polymerase (Roche, Switzerland), and 100ng of DNA templates in the condition of 5 min of initial denaturation at 94°C, followed by 1min of denaturation at 94°C, 1min for annealing at 55°C and 6 min of extension at 68°C for 25 cycles. The second PCR was performed in a 50μl reaction mixture as follows: PCR buffer, 0.35mM of each dNTP, 0.3μM of env-IFP and env-IRP, 2.5 units of DNA polymerase, and the first PCR products in the condition of 5 min of initial denaturation at 94°C, followed by 1 min of denaturation at 94°C, 1 min for anneal at 60°C and 6 min of extension at 68°C for 25 cycles. After being digested by EcoR I and Xho I, the second PCR products were inserted into the plasmid pSM and the recombinant plasmids were transformed into *E.coli* JM109. Bacterial colonies were identified by PCR. Five positive clones were selected and sequenced by Shanghai Sangon.

Pseudovirus generation and infectivity assay

Luciferase reporter pseudotype viruses CHNHLJ03009/Luc, HXB2/Luc and ADA/Luc were generated by co-transfection of 293T cells with luciferase-expressing plasmid pNL4-3-Luc-E-R- and an envelope-expressing plasmid, pSM-03009, pSM-WT (bearing HIV-1 HXB2 env gene) or pSM-ADA (pSM-WT and pSM-ADA were kindly provided by Dr. D. Littman), respectively, using Lipofectamine™ Reagent (Invitrogen, USA) as described [9]. The supernatants containing pseudoviruses were collected after 48 hours after transfection and frozen at -80°C. The viruses were quantified by using a p24 enzyme-linked immunosorbent assay kit (ZeptoMetrix, USA) and used for the infection.

One day before infection, U87.CD4.CXCR4 or U87.CD4.CCR5 cells (4×10^4/well)