Jikeikai Med J 2002 ; 49 : 3-11

In Vitro Neutralization of Human Immunodeficiency Virus Type-1 Subtype B and E with Humanized Monoclonal Antibody NM-01

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ABSTRACT

Humanization of certain antibody by transplanting the complimentarity determining region (CDR) to a human immunoglobulin framework attempts to reduce the response against a foreign molecule in human body during passive immunization.

We transferred the CDR from the murine monoclonal antibody (MoAb) NM-01 to a human IgG2b frame. The humanized NM-01 (hNM-01) neutralizes Human Immunodeficiency Virus Type-1 (HIV-1) as its murine progenitor. Moreover, hNM-01 shows enhanced neutralization of HIV-1. We have shown that this increase in reactivity may be attributed to residue 4 of the humanized κ chain, where the presence of a methionine residue rather than the murine leucine appears to promote a more advantageous conformation of the antigen binding site, perhaps via packing interactions with the Vκ CDR1.

The capacity of hNM-01 to neutralize both lab strains and clinical isolates was also examined with the expectation that hNM-01 will prove suitable for development as a therapeutic agent. This reshaped antibody reacted with several clinical isolates of HIV-1 tested, including subtype B and subtype E. Moreover, we proved the inhibitory activity for syncytium formation, which indicates the ability for the inhibition of cell to cell HIV transmission.

These findings may provide new opportunities for the development of a therapeutic immunoglobulin against AIDS. (Jikeikai Med J 2002 ; 49 : 3-11)

Key words : HIV, monoclonal antibody, neutralization, CDR grafting, clinical isolate

INTRODUCTION

Passive and active antibody based immunotherapy is important for protection against most virus diseases of humans, and the development of an effective HIV-1 vaccine is a major scientific priority[1,2]. It has been reported that the third variable region (V3) of HIV-1 envelope glycoprotein gp120 is the most critical determinant for host immunological responses[3,4] and cellular tropism[5,6]. It contains the epitopes for both humoral and cellular immune responses and thereby represents a potential target for HIV-1 vaccine development. A number of studies have demonstrated the protective role of antibodies against HIV challenge[7-9]. Passive immunization with anti HIV monoclonal antibody in chimpanzees[10] has also resulted in protection against HIV challenge. The studies of passive immunization showed decreased opportunistic infections[11] and prolonged survival[12].

The murine monoclonal antibody NM-01 is reactive to the core epitope of gp120 V3, which is

Received for publication, December 20, 2001
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relatively conserved Gly-Pro-Gly-Arg (GPGR) motif, correlating with the broad specificity of the antibody. Therefore, NM-01 has the ability to neutralize several strains of HIV-1[13] and may, therefore,be useful in the passive immunization of HIV-1 infected individuals.

Here we investigate the characterization of a human reshaped monoclonal antibody to confer upon it the gp120 binding and HIV-1 neutralizing activities of NM-01. It is expected that this humanization of NM-01 will reduce the immunogenicity of the reagent in human. Therefore it suggests the possibility of this antibody for passive immunization for HIV-1 positive individuals. Moreover, we describe the neutralization activity of humanized antibody against both HIV-1 subtype B and subtype E. This allows the development of a broad range of vaccine therapy.

MATERIALS AND METHODS

Vectors and primers

Vectors M13VHPCR1, M13VKPCR1, pSVgpt and pSVhyg have been described in detail previously[14] and were obtained from Dr. G. Winter, MRC Laboratory of Molecular Biology, Cambridge, UK.

For cDNA synthesis, the primers were

VHIFOR 5′ TGAGGAGACGGTGACCCTGGTCCCTTGGCCCCAG 3′

VKIFOR 5′ GTTAGATCTCCAGCTTGHGTCCC 3′

For PCR, the additional primers were

VHIBACK 5′ AGGTSMARCTGCAGSAGTCWGG 3′

VKIBACK 5′ GACATTCAGCTGACCCAGTCTCCA 3′.

For site directed mutagenesis to transplant CDRs, the primers used were :

VHCDR1

5′ CTGTCTCACCCAGTGCATATAGTAGTCGCTGAAGGTGAAGCCAGACACGGT 3′,

VHCDR2

5′ CATTGTCACTCTCATTGTCACTCTGCCCTGGAACTTCGGGGCATATGGAACATCATCATTCTCAGGATCAATCCA 3′

VHCDR3

5′ CCCTTGGCCCCAGTGGTCAAAGTCACTCCCCCATCTTGCACAATA 3′

VKCDR1

5′ CTGCTGGTACCATTCTAAATAGGTGTTTCCATCAGTATGTACAAGGGTCTGACTAGATCTACAGGTGATGGTCA 3′

VKCDR2

5′ GCTTGGCACACCAGAAAATCGGTTGGAAACTCTGTAGATCAGCAG 3′

VKCDR3

5′ CCCTTGGCCGAACGTCCGAGGAAGATGTGAACCTTGAAAGCAGTAGTAGGT 3′

For site directed mutagenesis of the human Vκ framework, the oligonucleotide used was 5′ CTCCCCCATGAATTACAGAAATAGACCG 3′.

Sequences of the NM01 variable regions

Cytoplasmic RNA from the NM01 producing hybridoma was purified using Fast Track kit (Invitrogen, San Diego, CA) and the cDNA synthesis was initiated from primers VH1FOR and VK1FOR. The V_H and Vκ cDNAs were then amplified using the PCR as described[14]. Amplified V_H and Vκ DNA were purified, cloned into M13 for sequence analysis, and identified as variable regions by comparison with known sequences[15].

Construction of chimeric antibody genes

Restriction sites were placed to the termini of NM-01 variable region gene to clone murine V region gene into expression vectors as described previously[14]. These variable region genes were cut and cloned into M13VHPCR1 and M13VKPCR1, which contain an Ig promoter, signal sequence, and splice sites. The DNA was excised from M13 as HindIII-BamHI fragments and cloned into the expression vectors pSVgpt and pSVhyg containing human IgG1[16] and human κ constant region[17] genomic DNA as for the construction of the chimeric genes.

Transplantation of CDRs into human frameworks

The human variable domains chosen for reshaping were NEWM V_H[18] and REI Vκ[19]. The CDRs of NM-01, as defined on the basis of sequence hyper variability[15]. Two versions of the humanized Vκ

were made. The HuVK was derived only its CDRs from the murine domain. The HuVKF variant included, additionally, the murine residue Phe71[20]. Oligonucleotides containing the desired substitutions were used as primers for mutagenesis and the mutations were introduced by the method of Nakayama and Eckstein[21]. After site-directed mutagenesis, the DNA was transformed into competent TG1 cells, and the mutations were verified by DNA sequencing from single-stranded DNA prepared from individual plaques.

The CDR replaced V_H and Vκ genes were cloned into pSVgpt, which contains human IgG2b and human κ constant regions.

Antibody expression

VH and VK DNA were linealized by digestion with PvuI, and cotransfected into YB2/0 myeloma cells[22]. After 5 min on ice, the cells were given a single pulse of 170 V at 960 μF (Gene-Pulser, Bio-Rad) and left on ice for a further 20 min. The cells were then put into growth media. The transfectants were then selected for the presence of the selectable marker gpt, the Escherichia coli xanthine-guanine phosphoribosyltransferase gene[23] found on the heavy chain expression vector.

Preliminary analysis of transfectoma

For identifying the presence of human antibody in the supernatant, individual clones were examined by ELISA. Goat anti human IgG (gamma chain specific) antibody was coated on a 96 well plate at 4°C overnight. After washing and blocking with 0.1% Tween20/0.1% bovine serum albumin (BSA)/PBS, 100 μl of culture supernatant was added to each well for 1 hr at 37°C. The wells were then washed with 0.05% Tween20/PBS and peroxidase-conjugated sheep anti human IgG (Amersham) was added and incubated at 37°C for 1 hr. 20 mM citrate-phosphate buffer containing o-phenylenediamine was added, and the reactivity was determined by monitering absorbance at 492 nm and 630 nm.

Virus stock and cell lines

The supernatants containing the peak production of viruses were harvested and the infected cells were vortexed for 1 min. The supernatant was collected, added 50% Fetal Bovine Serum (FBS), aliquated, and stored at −80°C.

H9[24] and C8166[25] culture was maintained at densities between 10^5 and 10^6 cells/ml in RPMI1640 supplemented with 10% FBS.

Syncytium inhibition assay

The binding inhibition assay was performed with the modification of that described previously[26]. Briefly, H9 cells chronically infected with IIIB were incubated with serially diluted monoclonal antibodies for 1 hr at 37°C. C8166 cells were then added to each well and incubated for 2 hr at 37°C. Syncytium greater than three lymphocyte cell diameters were counted and compared to that obtained for control (absence of antibody).

HIV-1 lab strain neutralization

Purified NM-01 and HuVH/HuVKF were serially diluted in RPMI 1640 medium with 10% FBS, and incubated with 100 tissue culture 50% infective doses (TCID50) of HIV-1 SF-2, MN, LAI, NY5-LAV-1, and GUN strains in 96 well plates for 2 h at 4°C. H9 cells were then added to each well and the plate was incubated for another 1 hr at 37°C. The H9 cell suspension was then diluted in 10% FBS/RPMI 1640 (1.5×10^5/ml) and incubated in a 96 well flat bottom plate at 37°C. Virus production was determined by reverse transcriptase (RT) assay[27] on day 6.

Neutralization on several clinical isolates

Peripheral blood mononuclear cells (PBMC) were obtained from HIV sero negative donors with Ficoll-Paque centrifugation and stimulated by PHA (5 μg/ml) for 3 days. The Thai isolates (kindly provided by Dr. Takebe, National Institute of Infectious Diseases) and Massachusetts (MA) isolates (kindly provided by

Dr. Groopman, New England Deaconess Hospital) from PBMC cocultures were titrated described previously[28,29]. 100 μl of monoclonal antibody to be tested was added to a separate well in a 96 well plate and incubated with 20 μl of 10 TCID50 clinical isolates at 4°C for 2 hrs. PHA stimulated PBMCs (7×10^5 cells/well) were then added to the plate in 20% FBS/50 u/ml human IL-2/RPMI 1640. The amount of the progeny viruses was determined by RT assay on day 14. Neutralization was defined as a % of the control culture (no antibody).

Cloning and sequencing of Thai isolates

DNA was extracted from NH-1, 2, 3 infected PBMCs with QIAamp DNA purification kit (QIAGEN) and the V3 region of gp120 were amplified by PCR. The sequence of env primers were 5′-TAGTACTGCAGTCTCGACGCAGGACTCGGC-3′ and 5′-GAATCTAGATCCCAAGGAGCATGGTG

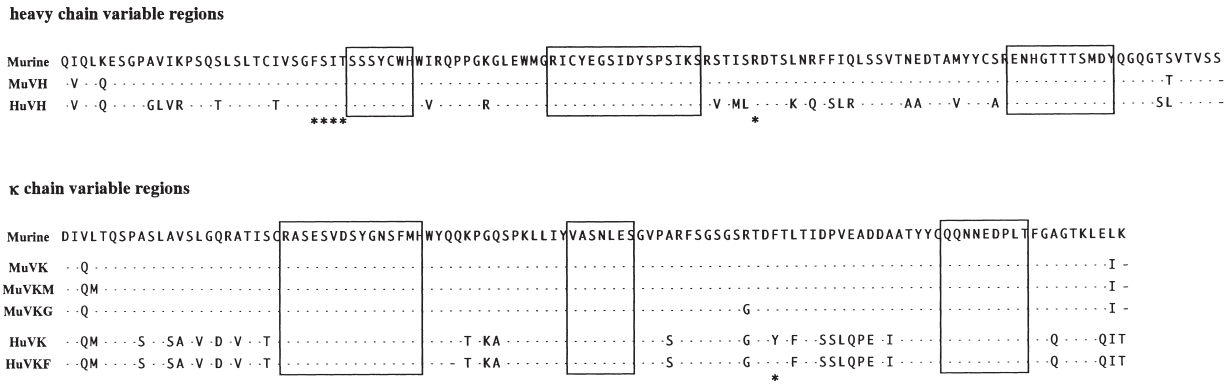

Fig. 1. Alignment of the amino acid sequences of the variable regions of murine NM-01.
A dash represents identity with the murine residue. The CDRs are boxed and murine residues included in humanized chains are indicated (*).

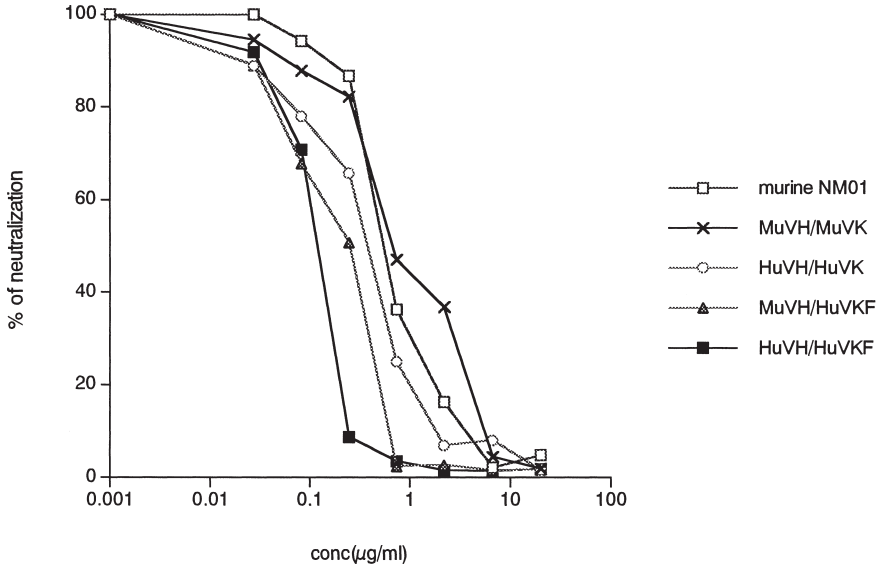

Fig. 2. Neutralization of MN virus infectivity with hNM-01.
The indicated concentrations of murine or human NM-01 were incubated with 100TCID50 of MN strain. H9 cells were added and then maintained for 6 days. Progeny viruses produced were measured by reverse transcriptase assay. The results are expressed as percent of inhibition compared to control activity(in the absence of antibody).
□murine, ×MuVH/MuVK, ○HuVH/HuVK, ▲MuVH/HuVKF, ■HuVH/HuVKF

CC-3′, as previously reported[30]. The PCR products were cloned into PCRscript and sequenced by dideoxy method.

Results

Complementary DNAs encoding the variable regions of murine monoclonal antibody NM-01 were cloned, sequenced, and identified by comparison with known sequences[15]. Fig. 1 shows the deduced amino acid sequences of the V segment of NM-01.

The resulting monoclonal antibodies were tested for their neutralizing activity against HIV-1 MN strain by monitering RT activity of culture supernatant. Fig. 2 indicates that the yield of progeny viruses was inhibited antibody concentration dependently. The construction of a chimeric Ab (consisting of murine variable regions, human IgG1 and κ constant regions) designated MuVH/MuVK, and demonstration of its approximate equivalence to the murine MoAb showed that these were the correct variable regions. Surprisingly, the HuVH/HuVKF MoAb was approximately ten fold more effective than its murine parent. This increase in neutralizing ability was also seen for the mixed antibody MuVH/HuVKF.

In order to confirm the superiority of the HuVH/HuVKF antibody, we next examined murine and HuVH/HuVKF NM-01s of their ability for neutrali-

Table 1. Neutralization of NM-01 against HIV-1 lab strains

Strain	Neutralization Titre (μg/ml)	
	murine NM-01	HuVH/HuVKF
SF-2	103	20
LAI	19	4
NY5-LAV-1	155	0.7
GUN	58	55

The results indicate the concentration of monoclonal antibody required to neutralize 1000TCID50 of virus of the strains listed, which all contain GPGR epitope in their V3 loops.

Table 2(a). Amino acid sequences of MA isolates

9435	H I G P G R A F V T T R Q T I G D I R Q
JR/CSF	H I G P G R A F Y T T G E I I G D I R Q
9487	S I G P G R S F Y A T R Q I I G D I R Q
9532	Y I G P G R A F Y A T D R I T G D I R R
9617	T M G P G R V Y Y T T T G G I V G D I R
9664	S I G P G R A F R T T G N I G N I R Q
10001	S I G P G R A F Y T T G R I I G D I R Q
9938	R V G P G R T L Y A T R R I I G D I R Q
9874	G I G P G R T V Y A T D R I I G D I R Q
9446	H I G P G R A L Y A T R K I I G D I R Q
9440	H I G P G R A L Y A T G K I I G D I R Q
9434	H I E P G R A L Y A T G K I I G D I R Q

Table 2(b). Amino acid sequances of Thai isolates

SubtypeE:	C T R P S N N T R T S I T I G P G Q V W E Y R T G D I T G N I R K A Y C	Number of sequences
NH-1:	−−−−−−−K−−−−P−−−−R−−−−−−−−−−D−−−−−−	3/16
	−−−−−−−K−−RV−M−−−R−−−−−−−−−V−D−−−−−−	3/16
	−−−−−−−I−−−−P−−−R−−−−−−−−−−D−−−−−−	2/16
	−−−−−−−R−−−−P−−−R−−−−−−−−−−−−−Q−−−	1/16
	−−−−F−−R−−R−−M−−−R−−−−−−−E−V−D−−−−H−	2/16
	−−−−F−−R−−R−HM−−−R−−−−−−−E−I−D−−E−H−	1/16
	−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−	2/16
	−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−Q−−−	2/16
NH-2:	−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−	15/17
	−−−−−−−−−−−A−−−−−−−−−−−−−−−−−−−−−−−	1/17
	−−−−−−−−−−−−−−−−−−−−−*−−−−−−−−−−−−−−	1/17
NH-3	−−−−−−−−−−−−−−−−−−−−−−−−−I−−−−−−−−	11/15
	−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−	3/15
	−−−−−−−−−−−−−−−−−−−−I−−−−−−−I−−−−−−−−	1/15

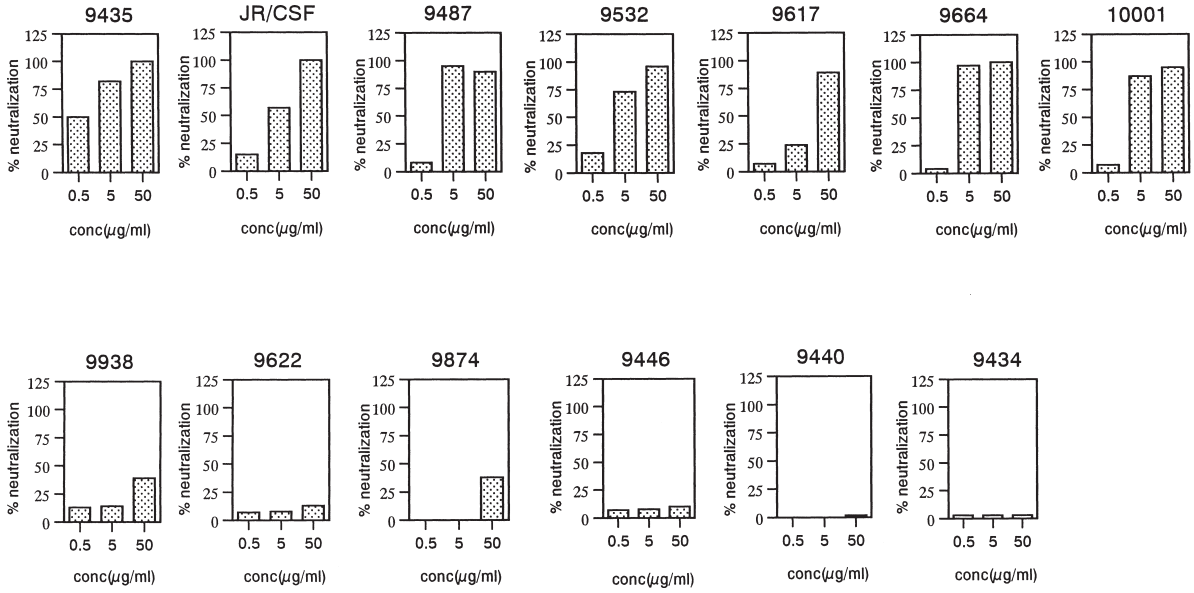

Fig. 3(a)

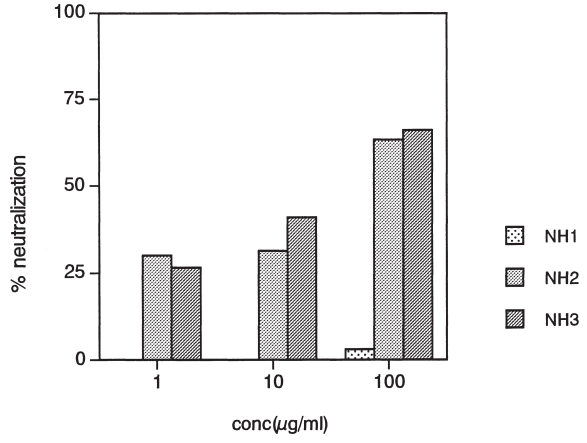

Fig. 3(b)

Fig. 3. Neutralization of hNM-01 against clinical isolates.
10TCID50 clinical isolates and hNM-01 was incubates at 4°C for 2 h, PHA stimulated PBMCs were then added, and incubated at 37°C for 14 days. Progeny viruses were measured by RT assay. The data represents as percent of inhibition compared to control activity (in the absence of antibody). The value is the average of triplicate experiments.
(a) Neutralization against MA (subtype B) isolates.
(b) Neutralization against Thai (subtype E) isolates.

zation against 4 lab strains of HIV-1 type B. As listed in Table 1, HuVH/HuVKF was able to neutralize type B lab strains, which contain GPGR motif and its efficacy relative to the murine NM-01 was much higher.

We next examined humanized NM-01 of its ability for neutralization against 16 clinical isolates, 13 from MA isolates and 3 from Thai isolates, respectively. The sequences of each isolate was listed in Table 2(a) and (b). As shown in Fig. 3(a), the IC50 of hNM-01 against #9435 was approximately 0.5 μg/ml, and IC50 against JR/CSF, #9487, 9532, 9617, 9664, and 10001 were less than 5 μg/ml. Therefore, hNM-01 was effective at its concentration of 5 μg/ml for 46% MA clinical isolates. As shown in Fig. 3(b), NM-01 could neutralize 2 out of 3 Thai clinical isolates (type

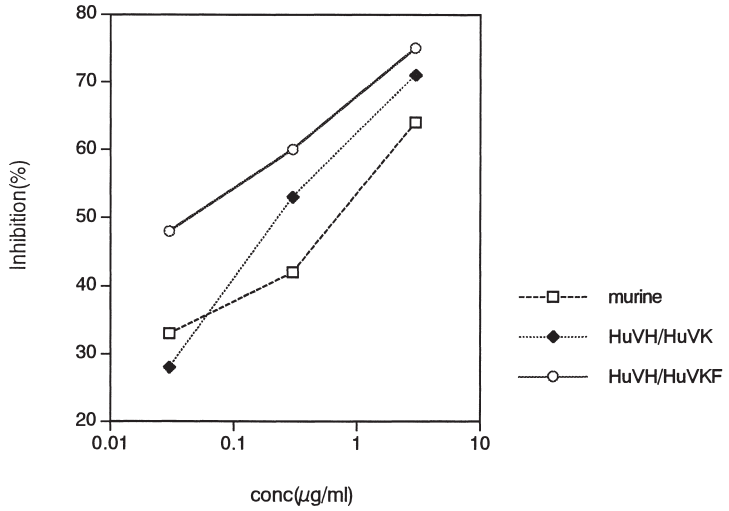

Fig. 4. Inhibitory effect of hNM-01 on syncytium formation.
H9 cells infected with MN virus were incubated with the serially diluted NM-01 for 1hr at 37°C. C8166 cells were then added to the culture for 2 hr. The number of syncytia was scored after 72 hrs. Results are expressed as percecnt inhibition compared to control.
□murine, ◆HuVH/HuVK, ○HuVH/HuVKF

E). Neutralization was less effective than for type B and IC50 was approximately 30 μg/ml.

The results of neutralization prompted us to perform further studies to determine whether hNM-01 can inhibit syncytium formation. In order to confirm the superiority of HuVH/HuVKF, H9 cells chronically infected MN strain were incubated with NM-01s (murine, HuVH/HuVK, HuVH/HuVKF) and then mixed with C8166 cells. Syncytium formation as compared to that for infected H9 cells not treated with NM-01 was inhibited. The antibody concentrations resulting in 50% inhibition was 0.05 μg/ml for HuVH/HuVKF, 0.2 μg/ml for HuVH/HuVK, and 0.8 μg/ml for murine, respectively. This observation corresponded to the results of neutralization (Fig. 4).

Discussion

We have previously demonstrated that murine MoAb NM-01 binds to and neutralize several strains of HIV-1[13], and a higher neutralization was observed in incubation with NM-01 in the presence of complement[31].

The important issue for passive immunization for HIV is that the antibody should be highly potent and be cross-reactive with a wide range of isolates. Moreover, human antibody should be administrated to patients since foreign immunogloblins can elicit an anti-immunoglobulin response which cause allergic or immunecomplex hyper sensitivity. We have attempted to reshape murine NM-01 to humanized NM-01 for serotherapy of AIDS, because it would be more worthy to have available human MoAbs of the desired specificity for administration. Demonstration of approximate equivalence of HuVH/HuVK to the murine MoAb in the neutralizing and syncytium inhibition assays with MN strain showed that cloned variable regions were equivalent to those of parental antibody.

Two versions of the humanized Vκ were made. The HuVK derived only its CDRs from the murine domain. The HuVKF variant included, additionally, the murine residue Phe71 since residues at this position interact with Vκ CDR1[20]. HuVH/HuVKF demonstrated greater efficiency than HuVH/HuVK.

The change in reactivity between the HuVH/HuVK and HuVH/HuVKF MoAbs shows that the identity of the residue at position 71 of the κ chain has a great influence on the antigen-binding of the humanized NM-01. However, the Tyr to Phe change cannot be responsible for the difference between the murine and HuVH/HuVKF MoAbs since both possess

Phe71. Furthermore, we examined the murine and humanized κ chain amino acid sequences for variance which might manifest in an alteration to the antigen binding site. Residue 4 was highlighted due to possible packing interactions with Vκ CDR1[32] and residue 68 because of its proximity to the antigen binding surface. The HuVKF residues Met4 and Gly68 were separately introduced into the chimeric κ chain to see whether either could bestow increased binding activity upon it. Moreover, the Arg68 to Gly change was found to offer no advantage over the original chimeric κ chain but the binding of the HuVH/MuVKF antibody was seen to be at least equivalent to that of the HuVH/HuVKF, suggesting that the inclusion of Met4 via the humanization process had modified the antigen binding site to one of higher affinity.

The increase in inhibition efficacy by both HuVH/HuVKF and MuVH/HuVKF confirms that the difference could be attributed to the κ chain. The antigen binding properties of the engineered antibodies, which incorporate amino acid changes within their CDRs, provide a direct measure of the success of the design strategy by which the mutations were chosen. The CDRs of NM-01 and five other murine residues were introduced into the human frameworks during the creation of the humanized V_H (HuVH). These additional amino acids included residues 27-30 which are part of the structural CDR1 loop[20]. The residues prior to CDR1 are part of the structural loop and are likely to be important either by directly interacting with antigen or by affecting the conformation of the loop. The side-chain of the fifth residue, 71, has contacts with both CDR1 and CDR2 of the VH and can affect their relative position[33].

A number of studies in animal models demonstrate a protective role of antibodies against HIV-1 challenge[34,35]. Adoptive immunotherpy with HIV specific immunoglobulins[36] and passive immunization with anti-HIV envelope MoAbs in chimpanzees[37] have also resulted in protection against HIV challenge.

It is notable that humanized NM-01 can neutralize several HIV-1 strains including clinical isolates subtype B and subtype E. The irregularity in their relative effectiveness may indicate that variations in the V3 loop, outside the core Gly-Pro-Gly-Arg epitope. Moreover, we proved the ability of modified hNM-01 of its higher efficiency against HIV. These results indicate that humanized NM-01 is suitable for development as a therapeutic agent against AIDS to prevent vertical transmission and in cases of accidental exposure.

Acknowledgment: This work was supported by the grant of "Bio-Venture Research Fund Project Aid" from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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