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Short communication

Humanized antibodies with broad-spectrum neutralization to avian influenza virus H5N1

Yingwei Chen[a], Wenxin Luo[a,*], Wai Lan Wu[b], Zhong Fang[a], Lin Xia[a], Xun Gui[a], Yixin Chen[a], Honglin Chen[b], J. Wai-Kuo Shih[a], Ningshao Xia[a,*]

[a] National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Life Sciences, Xiamen University, Siming South Road 422, Xiamen 361005, China
[b] State Key Laboratory for Emerging Infectious Diseases, Department of Microbiology, The University of Hong Kong, Pokfulam, Hong Kong, SAR, China

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ABSTRACT

Hemagglutinin (HA), the major antigen on the surface of influenza viruses, is the primary target for neutralizing antibodies and vaccine design. However, frequent mutations in this gene allow the virus to evade host immune responses and conventional prophylaxis and treatment. In this report, we humanized 4D1 and 10F7 mouse monoclonal antibodies (mAbs) that, we had previously shown to display broad-spectrum neutralization to avian H5N1 virus. The genes of variable (V) regions of 4D1 and 10F7 mAbs were combined with constant region of human antibody to construct the chimeric antibodies (cAbs). The results of hemagglutinin inhibition (HI) and neutralization assays showed that 4D1 and 10F7 cAbs were functional and retained broad-spectrum reactivity. Antibody competitive ELISA and affinity tests indicated that the cAbs recognized the same epitope as the parent mAbs with similar affinity. In animal experiments, the 10F7 cAb showed full protection against lethal challenge of highly virulent avian H5N1 virus, A/BH Goose/QH/15C/2005, in all infected mice. These humanized broad-spectrum antibodies may be potentially important for the control of both current and future antigenic variants of H5N1 virus.

Avian influenza A viruses may cause disease in poultry and occasionally transmitted to humans. The highly pathogenic avian influenza (HPAI) H5N1 virus has spread worldwide in recent years. The epizootic has been associated with sporadic transmissions of H5N1 virus infection from birds to humans. According to the recent report from World Health Organization (WHO), avian H5N1 virus has so far been responsible for 417 cases of human infection, among them 257 cases were, worldwide, fatal since 2003 (WHO, 2009). Hemagglutinin (HA) is the major antigen on the viral surface and responsible for virus binding to host receptors, enabling entry into the host cell through endocytosis and subsequent membrane fusion (Stevens et al., 2006). Therefore, HA is an important target for vaccine development because it is the primary target for neutralizing antibodies. However, frequent mutations in HA gene under the selection pressure of herd immunity in the host lead to the constantly antigenic drift in influenza virus. The segmented genome of influenza viruses also allows for genetic reassortmant to occur when two influenza viruses infect the same cell (Peiris et al., 2007). These mechanisms provide influenza viruses with powerful options for the generation of genetic diversity for interspecies transmission and immune escape mutants through a major antigenic change (Duan et al., 2008). The antigenic novelty and the highly pathogenic properties of H5N1 virus have the potential to result in infection of hundreds of millions of people worldwide with significant global health, social, and economic impacts if this virus gains the ability to transmit efficiently between humans.

The adamantanes (amantadine and rimantadine) and the neuraminidase (NA) inhibitors (oseltamivir and zanamivir) are the two currently available classes of drugs that are specifically active against influenza viruses. However, the efficacy of these drugs for the treatment of H5N1 virus infection is not clear and drug-resistant variants have been observed (Dharan et al., 2009). A wider range and more efficient way for the therapeutics is essential for the treatment of avian H5N1 influenza virus in humans.

Antibodies could mediate the cellular cytotoxic effect or directly neutralize the viruses. During the last ten years, more than 20 antibodies have been approved by the Food and Drug Administration (FDA) for therapeutic applications in humans. Furthermore, hundreds of clinical trials related to therapeutic antibodies are currently in progress (http://www.clinicaltrials.gov/ct). Treatment with antibodies against the virus responsible for the 1918 influenza pandemic has been considered for future H5N1 treatment (Luke et al., 2006). Recently, the humanized antibodies or fully human antibodies which were developed from B cells obtained from survivors of H5N1 infection or recipients of H5N1 vaccines showed definitive protection in mice when given before and after lethal H5N1 virus challenge (Hanson et al., 2006; Simmons et al., 2007; Throsby et al., 2008; Prabakaran et al., 2009).

* Corresponding authors. Tel.: +86 592 2184113; fax: +86 592 2181258.
E-mail addresses: wxluo@xmu.edu.cn (W. Luo), nsxia@xmu.edu.cn (N. Xia).

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We previously reported a panel of 52 broadly cross-reactive H5 specific mAbs which are potentially important for therapeutic purpose (Chen et al., 2009). Among these, mAb 4D1 and 10F7 were further evaluated by HI assay against a panel of forty H5N1 virus strains which were collected between 1997 and 2002. The reactive spectrum of these H5 antibodies, as determined by HI, was found to correlate with their reactivity in cell-based neutralization assays (Wu et al., 2008), suggesting that they were broadly cross-reactive neutralizing antibodies.

In this paper, the V region genes of 4D1 and 10F7 mAb were isolated by RT-PCR method using Ig-Prime kit from Novagen Company and inserted into the pcDNA3.1 vectors (Invitrogen) containing the constant region of human IgG gamma heavy or kappa light chain (Luo et al., 2007). The expression plasmids pcDNA3.1-H and pcDNA3.1-K were co-transfected into 293FT cells using standard method of calcium phosphate transfection for expressing 4D1 or 10F7cAb. One day after transfection, the transfected cells were cultured with serum-free medium. The supernatant was collected after 72 h and the expressed antibodies were purified by protein A chromatography affinity column. The chimeric antibodies were eluted by Mcilvaine buffer (pH 4.0) and dialyzed against 20 mM phosphate buffer (pH 6.7). The solution was concentrated by centrifugation in Millipore column (10 kD) to a final concentration of 7 mg/ml with the purity over 90%.

Antibody competition ELISA was performed to test whether the cAbs and the parental mAbs targeted the same epitope. H5N1 virus Ck/HK/YU22/2002 was captured by antibody coated on microplate and serial dilutions of competing antibodies with initial concentration of 50 μg/ml and HRP-conjugated parental antibody were added simultaneously to compete for available viruses. An unrelated cAb 4D11 against HBV preS1 was used as negative control and the parental mouse antibody used as positive control. As shown in Fig. 1, the binding of cAb was concentration-dependent and it could compete with the corresponding parental mouse mAb for binding to the virus of similar activity, while the negative control 4D11 cAb showed no competition. The result indicated that the chimeric antibody recognized the same epitope as its parental one.

The affinity of chimeric antibody to bind to HA was estimated by procedure as previously described (Golemis and Adams, 2005). In brief, the recombinant HA expressed by yeast was used as antigen in the ELISA test. Serial dilution of antibody with initial concentration of 50 μg/ml was reacted with HA protein coated on the plate. The overall affinity (K_D) was calculated by the reciprocal of concentration of antibody required for 50% binding to immobilized antigen. Analysis of binding data obtained using this method resulted in an overall affinity (K_D) of 2.98×10^{-8} M for 4D1 cAb and 7.47×10^{-8} M for 10F7 cAb, which were similar to those of 4D1 mAb (1.19×10^{-8} M) and 10F7 mAb (5.97×10^{-8} M).

The H5N1 virus strain, Ck/HK/Yu22/02, that was used in initial immunization for the developing of 4D1 and 10F7 mAbs was chosen to test the activity of the cAbs in HI assay. Virus titration and HI test were performed in accordance with the World Health Organization *Manual on Animal Influenza Diagnosis and Surveillance* and modified as previously described (Wu et al., 2008). For 4D1 and 10F7 cAbs, the minimum concentrations (100% heamagglutinin inhibition) against Ck/HK/Yu22/02 virus were 1400 ng/ml and 778 ng/ml respectively (Table 1), comparable to those of the parental mAbs. These results indicated that the chimeric antibodies assembled and migrated to the outside of the cells after co-transfection, and retained the same antigen binding activity and specificity as the parental mouse antibodies.

The broad cross-reactivity of the cAbs against H5N1 virus isolates belonging to different clades/subclades were examined by HI and microneutralization assays. Twenty H5N1virus strains, isolated from chicken, domestic and wild ducks during 2002–2006, were used in the HI test. These viruses were selected not according

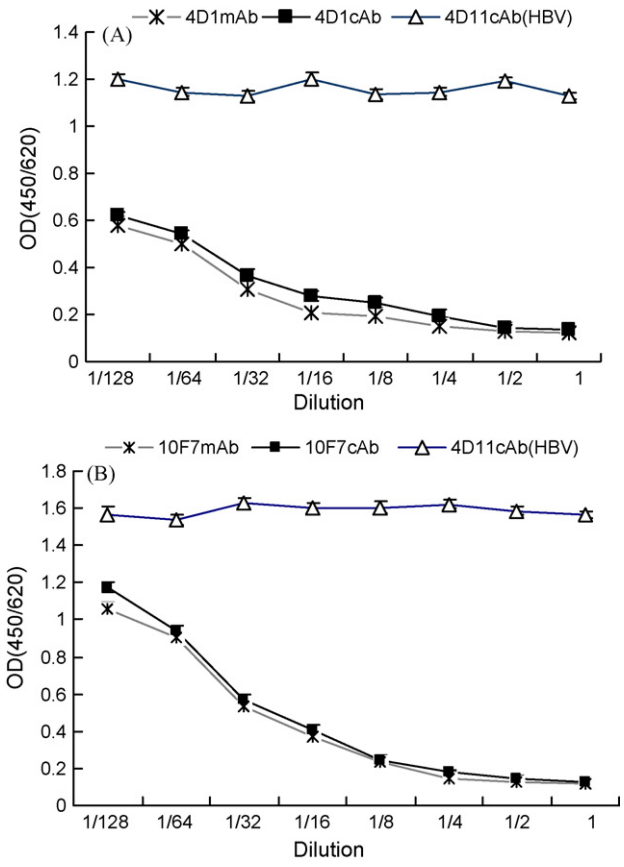

Fig. 1. Competitive ELISA test for chimeric Abs binding to H5N1 virus. The serial dilution of 4D1 (A) and 10F7 cAbs (B) competed with HRP-conjugated parent mAb for specifically binding to Ck/HK/Yu22/02 captured by another monoclonal antibody coated on the well. The cAb 4D11 unrelated to HBV preS1 and parental antibody were used as negative positive controls respectively. The binding affinity was calculated by the reciprocal of concentration of antibody required for 50% binding to immobilized recombinant HA expressed by yeast.

Table 1
Hemagglutinin inhibition assay of 4D1 and 10F7 cAbs.

H5N1 virus[a]	Clade	Minimum HI amount (ng/ml)			
		4D1cAb	10F7cAb	4D1mAb	10F7mAb
Rb pochard/HK/821/02	1	389	273	547	547
HK/213/03	1	1094	547	2188	547
VNM/1194/04	1	4375	2188	2188	2188
DK/VNM/283/05	1	1400	1094	1094	1094
CK/IDN/2A/04	2.1	2188	778	1094	547
IDN/5/05	2.1	1400	1400	1094	2188
CK/Malang/BBVet4/04	2.1	1400	547	1094	547
CK/Bantul/BBVet1/05	2.1	2800	2188	1094	1094
CK/Salatiga/BBVet1/05	2.1	273	273	273	273
Ck/ST/4231/03	2.2	778	1094	547	1094
BH Gs/QH/15C/05	2.2	273	273	273	273
DK/HN/101/04	2.3	547	547	547	547
Dk/GX/951/05	2.3	389	273	547	547
Ck/HN/999/05	2.3	1400	1094	1094	2188
Dk/HN/1265/05	2.3	1094	1094	547	1094
Common Magpie/HK/2256/06	2.3	4375	4375	4375	4375
Shenzhen/406H/06	2.3	8750	8750	8750	4375
CK/YN/115/04	2.4	4375	1400	1094	2188
Gs/GX/2112/04	5	2188	11,667	1094	2188
A/Ck/HK/Yu22/02	8	1400	778	1094	1094

The chimeric antibodies had HI activity to all of the 20 virus strains in different clades isolated from chicken, duck or wild avian during 2002–2006.

[a] *Abbreviations*: Gs, Goose; GX, Guangxi; HN, Hainan; Rb pochard, rosybilled pochard; ST, Shantou; SZ, Shenzhen; YN, Yunnan.

Table 2
Neutralization assay of 4D1 and 10F7 cAbs.

H5N1 virus	Clade	Minimum neutralizing concentrations (ng/ml)	
		4D1cAb	10F7cAb
CK/IDN/2A/04	2.1	875	438
Indonesia/5/05	2.1	1750	3500
A/BH Goose/QH/15C/2005	2.2	nd	1750
CK/YN/115/04	2.4	1750	875
CK/GX/2439/04	5	875	3500
A/Ck/HK/Yu22/02	8	nd	875

The chimeric antibodies were tested for neutralization to six representative virus strains in clade 2.1, 2.2, 2.4, 5, and 8 isolated from wild avian in several places during 2002–2005. 10F7 cAb had neutralizing activity to all of the six virus strains and 4D1 cAb neutralized four tested viruses.

to their phylogenetic position rather on the antigenic properties to cover all antigenic groups of H5N1 virus, as determined by HI and cell-based neutralization assays (Wu et al., 2008). The minimum concentrations of cAbs for HI are shown in Table 1. We found that both cAbs had reactivity to all of the 20 virus strains at different levels. This indicated that the cAbs had broad HI reactivity to avian H5N1 virus isolates from different genetic clades/subclades which have been prevalent in recent years. Six representative H5N1 virus strains in clades 2.1, 2.2, 2.4, 5, and 8, which were isolated from different geographical regions during 2002–2005, were used to test the neutralizing activity of 4D1 and 10F7 cAbs in cell-based microneutralization assays as previously described (Kaverin et al., 2002). The results showed that 10F7 cAb had neutralizing activity to all of the six virus strains and the minimum concentration to a strain of H5N1 virus isolated from chicken in Indonesia, CK/IDN/2A/04, was 438 ng/ml. Another cAb, 4D1, exhibited neutralizing activity for four out of six tested virus strains and the minimum concentration to CK/IDN/2A/04 and CK/GX/2439/04 was 875 ng/ml (Table 2).

To demonstrate its potential application for virus inhibition in vivo, 10F7 cAb was further tested for protection in animal experiment as previously described (Chen et al., 2009). Groups of 6 BALB/c mice were challenged intranasally with 10 MLD_{50} of H5N1 virus strain A/BH Goose/QH/15C/2005 (QH). Without intervention, all animals exhibited weight loss after virus challenge and other disease symptoms (Fig. 2A); death of mice were observed on day 6th or 7th post-infection (Fig. 2B). In the antibody treated group, 10F7cAb at a dose of 20 mg per kg of body weight in 100 μl of PBS was intravenously injected through the tail vein of animals 24 h after viral inoculation as previously described (Chen et al., 2009). Mice rapidly recovered from infection, gained weight (Fig. 2A), and full protection was observed in the group receiving 10F7 cAb on day 1 after infection (Fig. 2B). While a direct comparison of treatment efficacy between cAbs and the parental mouse antibodies was not conducted, the body weight change and survival rate from with and without 10F7 cAb treated mice after challenge with QH H5N1 virus showed that the efficacy of this cAb may be comparable to another broadly cross-protective H5 mAb, 13D4 (Chen et al., 2009).

Avian H5N1 viruses exhibit significant genetic and antigenic diversities (Chen et al., 2006). Vaccine strains for H5N1 virus have been continuously updated by WHO for matching the variable HA antigen of the circulating H5N1 influenza virus strains. However, the vaccine may fail to match the circulating H5N1 influenza virus strains if significant antigenic changes occur not to mention the slow and inadequate supply. Neuraminidase inhibitors are the main composition of current anti-influenza regimens. However, the efficacy of these drugs for the highly pathogenic H5N1 virus infection is not confirmed. There are also concerns about side effect and the emergence of resistant strains in using these drugs (Dharan et al 2009).

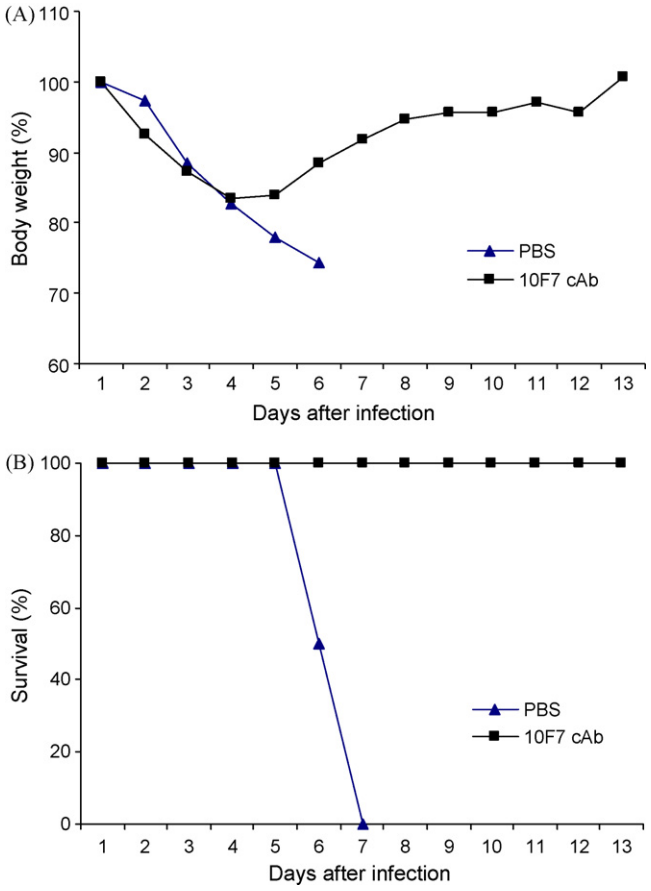

Fig. 2. Body weight change and survival rate in mice infected with H5N1 virus. Group of six mice were challenged intranasally with 10 MLD_{50} of A/BH Goose/QH/15C/2005 H5N1 virus. Mice then received 20 mg per kg of body weight of 10F7 cAb intravenously at 24 h after inoculation. Animals in the control groups were injected PBS at 24 h after infection. Mice were monitored daily for 13 days for body weight change [percent of the averaged body weight as compared with averaged body weight at day 1] (A) and survival (B).

Neutralizing antibodies are extremely effective in blocking virus attachment to the cell receptor and abrogating virus infection. Because of the circulation of antigenically distinct groups of H5N1 viruses in different regions, ideally, H5 MAbs generated for prophylactic or therapeutic purposes should be able to neutralize both currently circulating and future antigenic drift variants. In our study, two mAbs, 4D1 and 10F7, were identified and characterized by HI against 20 H5N1 virus strains from major clades/subclads representing virus that isolated in 1997 and those prevalent since 2002. It is postulated that the antibodies targeted at conserved epitopes on the HA protein of H5N1 virus would be a potential effective treatment for the antigenically diversified H5N1 viruses. Immunogenicity is a critical concern when developing an antibody-based drug. Humanization has played a crucial role in the progress of using antibodies as therapeutic reagents, and the chimeric method by the combination of the mouse variable region with the human constant region, represents one of the most efficient and effective ways in this application. Recently, using chimeric antibody for potential application in the treatment of H5N1 virus infection has been investigated in a mouse model (Prabakaran et al., 2009).

In the present study, we produced two chimeric antibodies of 4D1 and 10F7 mAbs. The cAbs proved to have similar activity as the parental antibodies in the HI and binding affinity tests. These cAbs recognized the same epitope as the parental antibodies in the competitive ELISA. On the other hand, it is very important that cAbs retain the broad-spectrum properties of 4D1 and 10F7

mAbs after humanization. Our results showed that both 4D1 and 10F7 cAbs retained the broad-spectrum cross-reactivity to 20 antigenically distinct H5N1 virus strains in HI test, and neutralized 6 representative H5N1 virus strains in the cell-based assay. In animal experiments, the 10F7 cAb showed protection against lethal challenge of H5N1 virus, A/BH Goose/QH/15C/2005, in all animals when it was given to mice 24 h after virus challenge. Chimeric antibody could successfully retain the parental antibody activity with diminished immunogenicity. It has been shown that the immunogenicity of chimeric antibody is low and rarely induces neutralizing anti-chimeric antibodies after treatment (Waldmann and Morris, 2006). The broadly cross-reactive properties of both humanized 4D1cAb and 10F7cAb and the procedures described here may be important for the control of infections caused by current and further evolving antigenic variants of H5N1 virus. The broad-spectrum neutralizing property of H5 specific antibodies described in this study may aid in understanding the antigenic properties of avian H5N1 virus and ultimately enhance treatment of emerging infections in humans.

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