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Neutralization activity of influenza A virus humanized antibodies against new subtypes of influenza viruses

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ABSTRACT

Antibodies are ideal for controlling the influenza A virus, but their effect on newly emerging strains is unclear. Here, we assessed the neutralization activity of the humanized monoclonal antibodies (mAbs) F10, H98 and H40 against circulating influenza viruses (H5N1, H1N1, H3N2 and H7N7 and new subtypes viruses H5N6 and H7N9). The results showed that all the three humanized mAbs (F10, H98 and H40) displayed different degrees of virus neutralization activities when encountered with different subtypes of influenza viruses. Remarkably, the humanized monoclonal antibody F10 produced higher and broader neutralization titers (range 25–1.56 μg/ml) than those of the other two humanized mAbs (H98 (range 50–3.12 μg/ml), H40 (range 50–5.56 μg/ml)) to against the viruses H5N1, H1N1, H3N2, H7N7, H5N6 and H7N9. This mAb may represent a new class of heterosubtypic neutralizing humanized mAb that could replace vaccines and chemical drugs.

In recent years, incidences of viruses H7N9, H5N1 and epidemic of viruses H3N2, H1N1 have increased, leading to dramatic impacts on human health. Currently, the main measures to combat with influenza viruses are vaccines and antiviral treatments. Vaccines can't provide a protective effect against the current new subtypes of influenza strains [1]. Antiviral drugs only exert therapeutic effects before drug-resistant strains emerge; but their effect when used as a late treatment is not clear [2,3]. As an effective complement to vaccines and chemical anti-viral drugs, high-affinity antibodies against conserved epitopes of viral proteins not only can provide immunotherapy to multiple influenza subtypes and but also can prevent future pandemic viruses [4,5]. The high titers of antibodies isolated from influenza-infected patients can be effectively used to treat patients with severe clinical symptoms resulting from influenza viruses; however, they provide limited rehabilitation, and it is not easy to obtain large amounts of plasma [6]. Furthermore, serum antibody titers are not uniform across different people. Therefore, to further industrializing the production of humanized antibodies is an inevitable trend.

Here, we performed experiments to analyze the microneutralization activity of the influenza A virus humanized mAbs F10, H98 and H40. These mAbs have been successfully prepared and preliminarily identified by our laboratory. Our investigation revealed that the humanized mAbs F10, H98 and H40 identified by our laboratory have active antiviral effects on four newly discovered (H1, H3, H5 and H7 viruses), four previously identified (H5N1, H1N1, H3N2 and H7N7) and two newly emerging (H5N6 and H7N9) strains.

The antibody variable-region amino acid sequences of the mAbs F10, H98 and H40 have been described elsewhere [7]. We use the computer analysis software Insight II2000 to analyses the binding properties to HA of mAbs. Humanized antibodies were produced in *Spodopterafrugiperda* cells (Sf9, Invitrogen, Carlsbad, CA, USA). Sf9 cells and Madin-Darby canine kidney (MDCK) cells were cultured as previously described [8]. The HPAI H5N1 virus A/meerkat/Shanghai/SH-1/2012 (SH-1; clade 2.3.2.1) (group 1) was isolated by our laboratory, and the mouse adapted H1N1 virus A/Changchun/01/2009 (group 1), mouse adapted H3N2 virus A/baikal teal/Shanghai/SH-89/2013 (group 2) and mouse adapted H7N7 virus A/Lesser White-fronted Goose/HuNan/412/2010 (group 2) were adapted by our laboratory. The H5N6 virus A/chicken/Vietnam/NCVD-15A59/2015 (clade 2.3.4.4) and the H7N9 virus A/shanghai/2/2013 were rescued by our laboratory using reverse genetic manipulation techniques. Humanized antibody concentrations were determined using an

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ASSAYS Micro BCA (tm) Protein Assay Kit (Thermo Fisher). The $TCID_{50}$ (median tissue culture infective dose) of each of these viruses was determined by infecting MDCK cells with serial viral dilutions and calculated by the Reed and Muench method [9].

The treated light (L) and heavy chain (H) variable region genes of mAbF10, mAbH98 and mAbH40 genes were optimized for Sf9 cells and then embedded into the vector PAC-k-CH3, which contains a humanized antibody Fc fragment, to obtain recombinant expression plasmids (Figure S1). The resulting recombinant expression plasmids were transfected into Sf9 cells to obtain recombinant baculoviruses. The third-generation recombinant baculovirus supernatants containing mAbF10, mAbH98 and mAbH40 were purified with GE HiTrap® Protein G High Performance liquid chromatography and then used to determine their neutralization activity according to a conventional method (Supernatants samples obtained from Sf9 cells infected with wild baculovirus after the same treatment were used as negative controls). Purified mAbF10, mAbH98 and mAbH40 were used at an initial concentration of 50 μg/ml to determine their neutralization activity according to a conventional method. The antibodies were serially diluted 2-fold and then incubated with 100 $TCID_{50}$ H5N1, H1N1, H3N2, H7N7, H5N6 or H7N9 viruses for 1 h at 37 °C temperature. The mixture was then added to MDCK cells grown in a 96-well plate to evaluate neutralization titers. The cells were cultured in a 37 °C, 5% CO_2 incubator for 3 days. After the cell monolayer was fully washed and fixed with acetone, the mouse anti-influenza virus NP IgG antibody was added. Supernatant was discarded and the unbound antibody was washed away. Then HRP-labeled goat anti-mouse IgG secondary antibody was used for color reaction. The highest dilution of the antibodies was used to represent the IC50.

All experiments with highly pathogenic viruses were carried out in biosafety tertiary (BSL-3) facilities that have biosafety-approved by the Changchun Veterinary Research Institute, in line with WHO recommendations recommendation standards.

This experiment analyzed only the mAbF10 spatial constellation (Figure 1). It is indicated that the antibody is recognized at the HA1 site. Using the www.bioinf.org.uk/abysis to perform a step-by-step analysis of the obtained mAbF10 heavy chain variable region sequence (data not show), two cysteines in the CDR3 of the heavy chain were identified. The other two antibodies were analyzed in the same way. Figure 2 showed

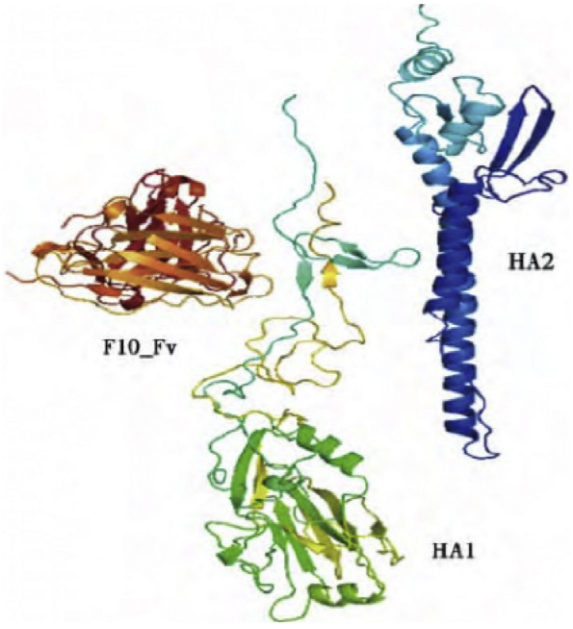

Figure 1. Schematic mode for interaction between mAbF10 and HA. The mode constructed by molecular docking method, the antibody F10 was recognized at the HA1 site.

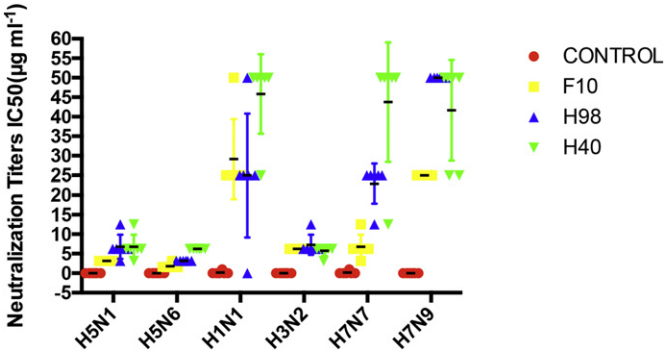

Figure 2. Microneutralization activity. Evaluation of mAbF10, mAbH98 and mAbH40 in microneutralization assays against H1, H3, H5 and H7 influenza viruses. The initial concentration of all antibodies was 50 μg/ml.

that the three humanized mAbs F10, H98 and H40 displayed varying degrees of neutralizing activities against H5N1, H1N1, H3N2, H7N7, H5N6 viruses and the H7N9 virus. Importantly, the humanized mAb F10 had higher and broader neutralization titers (range 25–1.56 μg/ml) (Figure 2) than the other two humanized mAbs. Moreover, all of the investigated humanized mAbs exhibited the highest activity against H5 influenza viruses (range 5.56–1.56 μg/ml) (Figure 2).

Influenza A viruses pose a pandemic threat because their antigenicity is complex and nonpreservative. If neutralizing humanized mAbs that can effective against all subtypes of pandemic potential could be produced, they would be useful against influenza strains. For this purpose, we performed a model analysis for the interaction between mAbF10 and HA. We observed that mAbF10 could recognized at the HA1 site, and this finding is consistent with previous reports [10]. There are two cysteines in the CDR3 of the mAbF10 heavy chain [7]. It is well known that introduction of cysteine residues will increase complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC) [11]. So, there need ADCC tests or NAI tests that effects mediated by BnAbs. The neutralization efficacy of the humanized mAbs F10, H98 and H40 indicated that mAbF10 produced higher and broader neutralization titers than were observed from mAbs H98 and H40. This result indicates that the HA protein not only contains a site for recognizing the mAbF10 antibody at HA1, but also has a conserved neutralizing epitope on the HA2 subunit. Furthermore, we found that all three humanized mAbs exhibited high efficacy against H5 influenza viruses. However, the efficacies of the three humanized mAbs against different subtypes of influenza viruses varied. This phenomenon is likely explained by the fact that their variable region nucleic acid sequences are diverse and the activity of the site is different, even though all of them were designed for H5 highly conserved epitopes and to inhibit viral and cell membrane fusion. Therefore, it is meaningful to do the binding competitive ELISA between the mAbs with mAbF10 to define their epitopes. It is unfortunately that we have not carried out the competitive binding ELISA for some objective reasons yet, however we will conduct in-depth research in this direction. The all above findings are consistent with those described in previous studies [12]. It is curious that mAbF10 can against the Group2 influenza viruses H7N7 and H3N2, but very weak effect on H1N1. Which is different from the previous study that mAbF10 is a well-known BnAb against Group1 HA2 [10]. This is a specific case, so it is necessary to clarify the mechanism of this phenomenon in the further experiment. Previous *in vitro* studies suggested that humanized mAbs exhibit reduced activity against influenza viruses, indicating that there is a need for *in vivo* studies [13]. In the identification of antibody versatility, the screened antibodies only inhibited several influenza viruses and could not completely interact with different subtypes of influenza virus. These findings suggested that different kinds of monoclonal antibodies could be applied simultaneously when treating disease.

In conclusion, the activities of the neutralizing humanized mAbs F10, H98 and H40 were evaluated in this study provide a foundation for future

in vivo studies. To our knowledge, none of these mAbs have been clinically licensured for human use. While there is no anti-influenza viral products that can be used in humans, the information presented here provides valuable support for the use of these mAbs as laboratory diagnostic reagents. Furthermore, these data will be important reference for the early treatment of influenza virus infection and should accelerate implementation of mAbs as excellent treatment candidates.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bsheal.2019.12.005.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

J. Liu, T. Wang, Y. Gao and X. Xia conceived and designed the experiments. J. Liu, T. Wang and Y. Xie performed the experiments. J. Liu and T. Wang analyzed the data. Y. Li, J. He, X. Zhang, W. Sun, N. Feng, C. Qin contributed reagents/materials/analysis tools. J. Liu and Y. Gao wrote the paper. X. Xia and Y. Gao requested financial support. All authors read and approved the manuscript.

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