Molecular cloning of the first human monoclonal antibodies neutralizing with high potency Swine-origin Influenza A pandemic virus (S-OIV)

Roberto Burioni, Filippo Canducci, Nicasio Mancini, Nicola Clementi, Monica Sassi, Donata De Marco, Diego Saita, Roberta Antonia Diotti, Giuseppe Sautto, Michela Sampaolo, Massimo Clementi

Laboratorio di Microbiologia e Virologia, Università Vita-Salute San Raffaele, Milano, Italy

―――――― SUMMARY ――――――

The pandemic caused by the new H1N1 swine-origin influenza virus (S-OIV) strain is a worldwide health emergency and alternative therapeutic and prophylactic options are greatly needed. Two human monoclonal antibody Fab fragments (HMab) neutralizing the novel H1N1 influenza strain at very low concentrations were cloned from a patient who had a broad-range anti-H1N1 serum neutralizing activity. The two HMabs neutralized S-OIV with an IC_{50} of 2.8 and 4 µg/mL. The genes coding for the neutralizing HMabs could be used for generating full human monoclonal IgGs that can be safely administered with the potentially of representing a novel drug to be used in the prophylaxis and the treatment of this human infection. This is the first report of molecular cloning of human monoclonal antibodies against the new pandemic swine-origin influenza virus.

KEY WORDS: Human monoclonal antibodies, Swine influenza, Influenza pandemic

INTRODUCTION

Recently, a new strain of human H1N1 influenza A virus (swine-origin influenza virus; S-OIV) was identified (Fraser *et al.*, 2009) and it is responsible of a worldwide pandemic, causing - as of November 9th 2009 - more than 482,300 cases and 6,071 deaths.

Human monoclonal antibodies with strong neutralizing activity against S-OIV can be of the greatest importance in this setting, as they are molecules potentially useful in prophylaxis and therapy (Trifonov *et al.*, 2009 and Simmons *et al.*, 2007). In a number of different viral infections antibodies are successfully used (Sawyer, 2000), and several reports demonstrated that anti-influenza mouse and human monoclonal antibod-

Corresponding author
Roberto Burioni
Laboratorio di Microbiologia e Virologia
Università Vita-Salute San Raffaele
Via Olgettina, 60 - 20132 Milano, Italy
E-mail: burioni.roberto@hsr.it

ies are effective in prophylaxis and therapy in a mouse model (Palladino *et al.*, 1995; Renegar *et al.*, 2004; Sminorv *et al.*, 2000), and vertical transmission of anti-influenza antibodies is causing a "natural" passive immunotherapy that resulted beneficial to the host both in animal models and in human subjects (Simmons *et al.*, 2007; Luke *et al.*, 2006; Martinez *et al.*, 2009; Sui *et al.*, 2009; Sun *et al.*, 2009; Throsby *et al.*, 2008; Yu *et al.*, 2008; Puck *et al.*, 1980; Reuman *et al.*, 1983; Sweet, Bird *et al.*, 1987; Sweet Jakeman *et al.*, 1987).This body of evidence indicates that passive antibody administration has the potential of representing an important treatment and preventive tool against the S-OIV infection. Furthermore, human monoclonal antibodies can constitute a useful alternative to current regimens in pregnancy, a clinical situation where the patient is more at risk for complication after S-OIV infection, and the utilization of vaccine and antiviral drugs is problematic (Burioni *et al.*, 2009).

In this paper, we describe for the first time the molecular cloning of two human monoclonal an-

tibody Fab fragments (HMab) directed against S-OIV endowed with a strong neutralizing activity. These antibodies can be crucial reagent to develop alternative therapeutic and prophylactic strategies in this worldwide health emergency.

MATERIALS AND METHODS

Human monoclonal antibodies

A patient, of the age of 55 and with a serum neutralizing titer ($IC_{50}>1:20$) against a reference H1N1 1934 strain (A/PR/8/34) was used as a source of lymphocytes 3 weeks after a routine influenza seasonal vaccination, and after written informed consent was given. Fifteen B-cell lines producing antibodies reacting in immunofluorescence with influenza A-infected cells were obtained by Epstein-Barr Virus (EBV) transformation (Cole *et al.*, 1984), and subsequently cDNA coding for Fab fragments were PCR amplified and cloned in appropriate bacterial expression vector (Burioni *et al.*, 1998; Burioni *et al.*, 1997) for avoiding instability of antibody production of EBV-transformed cell lines and for DNA sequencing. Transformed bacteria were used for production of the recombinant Fabs (Perotti *et al.*, 2008; Burioni *et al.*, 1994), that were purified as previously described (Burioni *et al.*, 1998) . Only two of the Fabs were demonstrated to be endowed with neutralizing activity. An anti-hepatitis C virus Fab, named e509 (Burioni *et al.*, 1998), purified with an identical procedure was used as a negative control in all experiments.

Isolation and Identification of influenza A/Milan/UHSR1/2009 strain

All experiments were conducted in the BLS3 laboratory of the Università Vita-Salute San Raffaele. The swine origin influenza virus (S-OIV) used in this study was isolated from the pharyngeal swab of a 26-year-old Italian patient of our hospital referring fever and malaise after a recent travel to the United States. The swab was seeded on 80% confluent MDCK (Madin-Darby canine kidney) cells (ATCC n° CCL-34TM). The cells were infected in Modified Eagle Medium (MEM, GIBCO) with the addition of 2 µg/ml of trypsin. After one hour, 10% fetal bovine serum (GIBCO), 50 µg/ml of penicillin (GIBCO), 100 µg/ml of strep-

tomycin (GIBCO) and of L-glutamine (2 mM) (EuroClone) were added and cells were incubated at 35°C, in 5% CO_2 atmosphere for 5 days. Identification was performed directly on patient's swab sample and on culture supernatant by whole length amplification and sequencing of the S-HA gene by using a previously described RT-PCR protocol (Puthavathana *et al.*, 2005) with minor modifications. Primer forward was Bm-HA-1-Fw 5'-TAT TCG TCT CAG GGA GCA AAA GCA GGG G-3', primer reverse was Bm-NS-890-Rev 5'-ATA TCG TCT CGT ATT AGT AGA AAC AAG GGT GTT T-3'. The reaction was performed using the SuperScript III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen) and the following thermal profile: 30 min 50°C;10 min 94°C; 30 sec 94°C, 1min 53°C, 1 min 72°C (45 cycles). Sequencing was performed by using BigDye Terminators 3.1 with the automatic sequencer AbiPrism3130 (Applied Biosystems); the primers used in the amplification are: F1 5'-TAG GAA ACC CAG AAT GCG-3', F2 5'-TAC TGG ACC TTG CTA GAA-3', F3 5'-TCT ATT TGG AGC CAT TGC-3'.

Immunofluorescence assays

Seven hours after infection, the MDCK cells were trypsinized, washed twice in PBS and spotted on a slide by cytocentrifugation ($2x10^5$ cells in each spot). The cells were then fixed and permeabilized by cold methanol/acetone (-20°C) for 10 minutes at room temperature. After 3 washes in PBS, the cells were incubated for 30 minutes at 37°C with each one of the HMabs (10 µg/mL). The cells were then washed again in PBS, and incubated for 30 minutes at 37°C with a fluoresceinated anti-human Fab monoclonal diluted in Evans Blue. Commercially available anti-NP protein mouse monoclonal (Anti-influenza A group, Argene, cod 11-030) was used as an infection control, and as negative control e509Fab was used on infected cells.

Virus neutralization assays

Fluorescence inhibition assay

S-OIV was titrated by the limiting dilution method, and the viral titer calculated by the Reed-Muench formula. 100 $TCID_{50}$ of SOI-V were preincubated 1h at 37°C with scalar concentrations of each human Fab (from 0.125 to 20 µg/mL). 250 µL of each pre-incubated solutions

were then added on MDCK cells and incubated 1 h at 37°C (5% CO_2). The cells were then washed twice in PBS; 1.5 mL of serum-free medium with trypsin (1 µg/mL) was added, and the cells incubated 6h at 37°C. The cells were then washed again with PBS, fixed and permeabilized with cold methanol/acetone. The number of infected cells on each slide was then revealed using the anti-NP monoclonal antibodies, as above. An infection control without antibody was added, as well as a negative control with e509 anti-HCV/E2 monoclonal Fab. Each neutralization assay was performed in triplicate, and repeated in three different sessions. The neutralization activity for each Fab concentration was expressed as the percentage reduction of fluorescent nuclei, compared with the nuclei counted in the infection control. The neutralization curves were then fitted by non-linear regression with the GraphPad Prism software, allowing IC_{50} calculation (HMab concentration giving 50% neutralization).

RESULTS

Two distinct HMabs, named PN-SIA28 and PN-SIA49, were cloned from a patient with a strong serum neutralizing activity against a 1934 influenza A isolate.

When tested in immunofluorescence assay, both MAbs recognized cells infected with the S-OIV strain (A/Milan/UHSR1/2009). No reactivity was demonstrated against the B/Lee/40 influenza B strain. In immunofluorescence staining on infected cells, both HMabs featured a clear cytoplasmic pattern with plasma membrane reinforcement (Figure 1) against S-OIV-infected cells. PN-SIA28 and PN-SIA49 showed a strong neu-
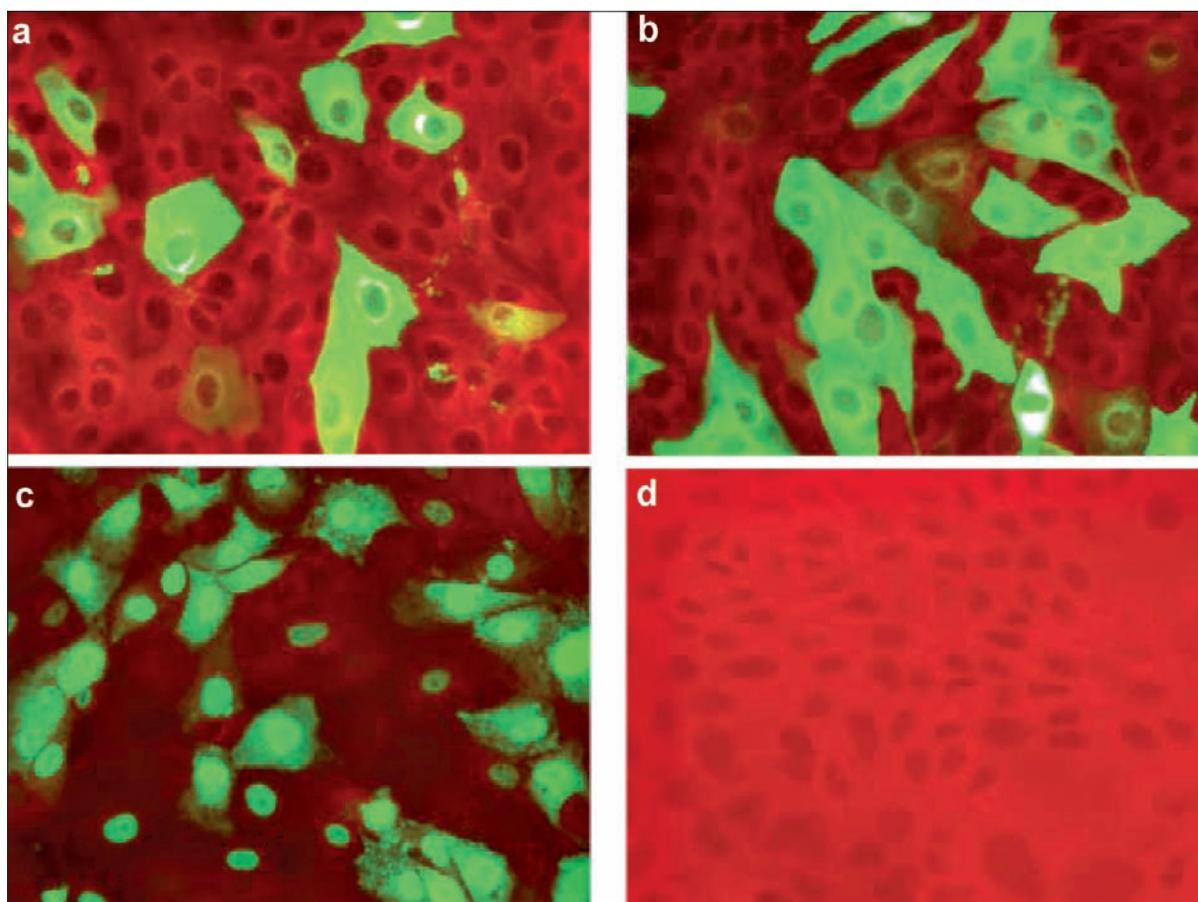

FIGURE 1 - *Immunofluorescence on A/Milan/UHSR1/2009-infected MDCK cells stained after 8 hours of infection with HMabs PN-SIA28 and PN-SIA49 (a, b), mouse anti-NP1 (c), the negative control Fab e509 (d).*

tralization activity against S-OIV. When neutralization activity was evaluated with immunofluorescence reduction assay PN-SIA49 neutralized S-OIV strain (A/Milan/UHSR1/2009) with an IC50 of 2.8 µg/mL. PN-SIA28 neutralized S-OIV strain with an IC_{50} of 4.0 µg/mL. No neutralizing activity was demonstrated for both MAbs against the B/Lee/40 influenza B strain (Figure 2). Similar results were obtained with plaque reduction assay (Data not shown).

DISCUSSION

The current influenza pandemic caused by the newly emerged S-OIV strain is a worldwide emergency with potentially catastrophic consequences. Even if the pathogenic potential of the novel strains appears low, the possibility of a further evolution of the pathogen cannot be excluded (Lipstch *et al.*, 2009). The availability of adjunctive novel tools for preventing and treating this disease may be crucial in contrasting the current situation.

The HMabs described here for the first time could be of greatest importance in treatment and prophylaxis given the current S-OIV pandemic scenario. In a mouse model of H5N1 influenza infection, neutralizing human monoclonal antibodies were proven to be efficient in protecting mice, causing a milder disease and reducing virus dissemination (Simmons *et al.*, 2009). Several reports related to the 1918 "Spanish" pandemic indicated as administration of blood products from recovered patients was associated with a strong reduction in mortality of infected individuals, par-
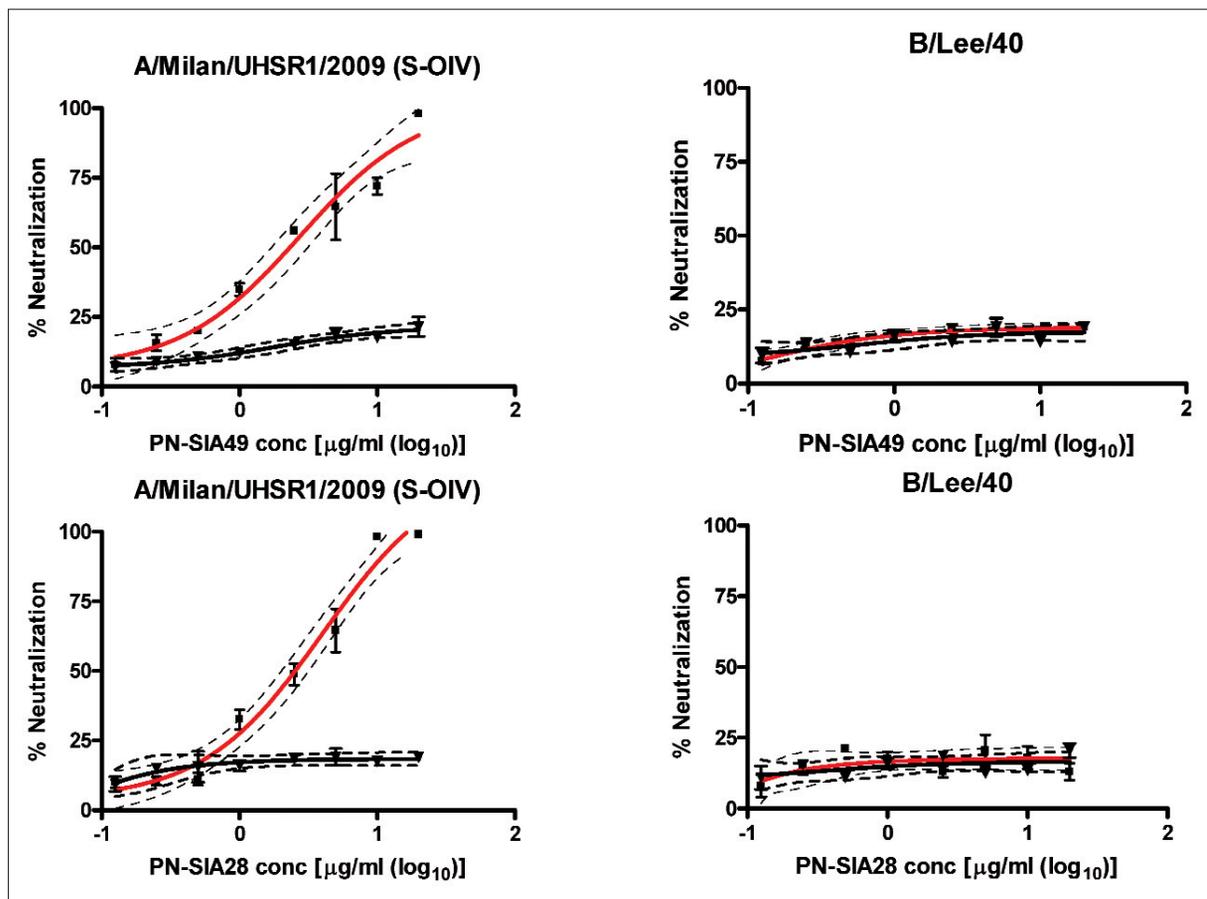

FIGURE 2 - *Fluorescence inhibition assay. Sigmoid dose-response curve fit non-linear regression are reported for PN-SIA28 and PN-SIA49 against S-OIV isolate A/Milan/UHSR1/2009 studied in this paper (red curves) and B/Lee/40 influenza B issolate. Fab e509 (black curves) was also used as negative control.*

ticularly when administered early (Luke *et al.*, 2006), with the speculation that the neutralizing antibodies present in the blood-derived products modified favorably the virus-host balance with a milder disease and decreasing the frequency of respiratory complications (Luke *et al.*, 2006). Administration of human monoclonal antibodies neutralizing S-OIV has the potential of having the same effect. Moreover, even if most of the currently circulating S-OIV strains are yet today sensitive to antiviral drugs, their use will probably give rise to resistant strains, as already happened with seasonal H1N1 strains that have now started to become resistant (Shetty, 2009). In the current situation, not knowing in advance the efficacy and of an anti-S-OIV vaccine and ignoring how long the usefulness of antiviral drugs will last, the availability of an alternative therapeutic and prophylactic option could be of paramount importance, also considering that mass production of human monoclonal antibodies is feasible, scalable, the final product is free of adventitious agents and it is utilizing well-known procedures that are used for many drugs with a minimal risk of unwanted effects, being the compound of human origin and thus well tolerated.

In summary, this is the first report of the molecular cloning of human monoclonal antibodies neutralizing the pandemic H1N1 S-OIV strain. These molecules have a paramount importance and can constitute the basis, alone or in a combination with other monoclonal antibodies, for a new class of drugs to be used in the treatment and in the prophylaxis of influenza infection.

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