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Development of monoclonal antibodies to highly pathogenic avian influenza H5N1 virus and their application to diagnostics, prophylaxis, and therapy

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Summary. – A panel of 17 monoclonal antibodies (MAbs) against highly pathogenic avian influenza virus (HPAIV) A/Duck/Novosibirsk/56/05 A/H5N1 (subclade 2.2) isolated in Russian Federation was developed. Immunoblot analysis showed that 12 MAbs were specific for the hemagglutinin (HA) and 5 MAbs for nucleoprotein (NP). All anti-HA MAbs were reactive in ELISA and immunofluorescence (IF) test and 10 of them were reactive in hemagglutination-inhibition (HI) and neutralization tests. Quantitative competitive ELISA revealed that anti-HA MAbs recognized at least 4 non-overlapping antigenic determinants and anti-NP MAbs recognized at least 3 non-overlapping antigenic determinants. Four sandwich ELISA procedures were developed using the obtained MAbs. These procedures are useful for 1) identification of avian, human, and swine influenza A viruses, 2) differentiation of avian influenza virus (AIV) from human and swine influenza viruses, 3) differentiation of AIV H5 from other AIV subtypes, and 4) differentiation between 2.2 and 2.3.2 subclades of H5N1 influenza viruses. Prophylactic and therapeutic efficacy of anti-HA MAbs with high neutralization activity was tested in BALB/c mice. A complete protection was achieved by single injection of MAbs (20 mg/kg) 24 hrs before challenge with 10 LD_{50} of HPAIV H5N1. Therapeutic efficacy was 90% that was similar to those of Rimantadine and Tamiflu.

Keywords: highly pathogenic avian influenza virus H5N1; monoclonal antibodies; hemagglutinin; nucleoprotein; rapid diagnostic tests; prevention; treatment

Introduction

Avian influenza A viruses (H5N1) have caused epizooties in Southeast Asia and other regions. Some variants of H5N1 virus induced serious infections in humans with high lethality. WHO reported 295 deaths out of 499 cases (WHO, 2010). The majority of H5N2 and H5N3 viruses isolated in Russian Federation from wild birds and poultries showed low pathogenicity. For the first time HPAIVs H5N1 were isolated in Western Siberia in 2005. In the years 2006–2007 these viruses circulated in European and Asian part of Russian Federation. Phylogenetic analysis showed that they belonged to the Qinghai type, subclade 2.2 (Lvov and Kaverin, 2008). New strains of HPAIV H5N1 isolated in 2008–2009 in Western Siberia from wild birds and poultries were attributed to the subclade 2.3.2 (Lvov *et al*., 2008).

At present AIVs circulating in Southeast Asia are thoroughly studied (Chen *et al*., 2006; Peiris *et al*., 2007). Analysis of RNA and investigation of antigenic and three-dimensional structure of H5 hemagglutinin revealed considerable difference between low pathogenic AIV (LPAIV) strain A/Duck/Singapore/3/97 and HPAIV strain A/Vietnam/1203/04 isolated from humans (Webster and Govorkova, 2006). Rapid

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Abbreviations: AIV = avian influenza virus; HA = hemagglutinin; HI = hemagglutination-inhibition; HPAIV = highly pathogenic AIV; IF = immunofluorescence; LPAIV = low pathogenic AIV; MAb(s) = monoclonal antibody(ies); NP = nucleoprotein

and sensitive laboratory and field tests for the diagnosis of HPAIV H5N1 are essential for the disease control. MAbs against the currently circulating H5N1 viruses are necessary for analysis of antigenic structure and diagnosis. Anti-HA MAbs to the different clades of H5N1 influenza viruses were successfully used for the HA mapping of viruses isolated in Southeast Asia (Smith *et al*., 2006; He *et al*., 2007; Kaverin *et al*., 2007; Wu *et al*., 2008; Du *et al*., 2009; Ho *et al*., 2009). MAbs to Qinghai-type H5N1 have not been prepared up till now.

This study reports epitope specificity of 17 MAbs to HPAIV H5N1 A/Duck/Novosibirsk/56/05 (subclade 2.2), their virus-neutralizing activity *in vitro* and protective efficacy *in vivo*.

Materials and Methods

Viruses. Influenza viruses used in this study are listed in Table 2 and 4. All viruses were obtained from the Virus collection of the D.I. Ivanovsky Institute of Virology, Russian Academy of Medical Sciences, Moscow, Russian Federation. The viruses were propagated for 48 hrs in the allantoic cavities of 10-day-old embryonated chicken eggs at 37°C. Strain A/Duck/Novosibirsk/56/05 was propagated in the pig embryo kidney (PEK) cells, concentrated, purified using a sucrose density gradient, and inactivated by treating with β-propiolacton (Kushch *et al*., 2008). This virus was used as an antigen for immunization of mice and production of MAbs. In addition, it was used also in some immunological assays.

Production of MAbs. Preparation of MAbs against HPAIV A/Duck/Novosibirsk/56/05 (H5N1) was described previously (Kushch *et al*., 2008). Large quantities of MAbs were prepared as ascitic fluid. Immunoglobulins were precipitated with ammonium sulphate and purified using Protein A-Sepharose CL-4B column (Sigma). Purified MAbs were conjugated with horseradish peroxidase (HRP, Sigma) by the periodate method (Nakane and Kawaoi, 1974).

Isotyping of MAbs. Isotyping was performed by using a Mouse-Hybridoma-Subtyping Kit (Boehringer Mannheim).

HI test was performed with human red blood cells (group 0) by standard method (WHO, 2004). Influenza virus doses presented 8 U.

Neutralization assay. MAb-containing ascitic fluids were inactivated, serially diluted, and incubated with 100 $TCID_{50}$ of HPAIV A/Duck/Novosibirsk/56/05 for 2 hrs at 37°C and added to the PEK cell monolayers. CPE was observed after 72–96 hrs, when the maximum CPE was visible in the infected cell culture without MAbs. The highest dilution of MAb showing 50% CPE was scored as the neutralization titer.

Indirect ELISA. The procedure for indirect ELISA was described previously (Masalova *et al*., 2002). Briefly, 96-well plates (NUNC) were sensitized with 2 μg/ml of A/Duck/Novosibirsk/56/05 antigen (overnight, room temperature) and incubated with serially diluted MAbs, washed, and incubated with HRP-conjugated rabbit anti-mouse Ig (DAKO). The enzyme activity was determined using the substrate 3,3′,5,5′-tetramethylbenzidin (Sigma) with H_2O_2 at 450 nm. Reciprocal dilution with A_{450} 2-fold as high as that of A_{450} of negative control (anti-hepatitis C MAbs with the same Ig subtype) was taken as MAb titer.

Competitive ELISA. Epitope specificities of the MAbs were analyzed in competitive ELISA as described elsewhere (Masalova *et al*., 2002). The A/Duck/Novosibirsk/56/05 antigen was immobilized on 96-well ELISA plates (2 μg/ml) and incubated with 100 μg/ml MAbs (or ascitic fluids in dilution 1:100) overnight at 4°C. Serial dilutions of competing MAbs were used in some experiments. HRP-labeled MAbs were added in dilutions providing A_{450} = 1.0–1.5 upon interaction with influenza virus in the absence of competing MAbs and incubated for 1 hr at 37°C. A_{450} determination was performed and the results were expressed as inhibition degree in % of the binding of the conjugated MAbs with virus in the presence of competing unlabeled MAbs.

Sandwich ELISA. MAbs (5 μg/ml in PBS) were immobilized on plastic plates (overnight, room temperature) and after washing were incubated for 2 hrs at 37°C with A/Duck/Novosibirsk/56/05 antigen or virus-containing fluids, then with HRP-labeled MAbs (1 hr at 37°C). Results were regarded positive if A_{450} was greater than cut-off value that was calculated as the mean of negative controls (blocking buffer without virus, native allantoic fluid or culture medium) plus two standard deviations.

Immunoblot analysis. A/Duck/Novosibirsk/56/05 antigen was subjected to 10% SDS-PAGE and separated under both reducing and non-reducing conditions. Separated proteins were electroblotted onto nitrocellulose membrane (Schleicher&Schuell). After blocking, the blots were incubated with ascitic fluid or hybridoma supernatant in PBST with 5% nonfat milk for 2 hrs at and then incubated with HRP-conjugated rabbit anti-mouse Ig for 1 hr at room temperature. The blots were developed by incubation with 3, 3′-diaminobenzidine (DAB, Sigma) and H_2O_2 as described (Masalova *et al*., 2002).

Dot blot analysis. Influenza viruses were adsorbed onto nitrocellulose membrane (Schleicher&Shuell) using a Bio-Dot apparatus (Bio-Rad). The membranes were incubated with MAbs, HRP-conjugated rabbit anti-mouse Ig and DAB as described for immunoblot analysis. The reaction sensitivity and specificity were determined using serial dilutions of A/Duck/Novosibirsk/56/05 antigen and allantoic fluids containing various influenza viruses, respectively.

IF test. Acetone fixed PEK cells grown on slides and infected with HPAIV A/Duck/Novosibirsk/56/05 were incubated with MAbs for 1 hr at 37°C. Slides were washed with tap water. The secondary FITC-conjugated rabbit anti-mouse immunoglobulin (DAKO) diluted 1:50 in PBS with 0.3% Evans blue was incubated for 1 hr at 37°C. The results were observed under UV microscope (Olympus).

Determination of prophylactic and therapeutic efficacies of MAbs was done in BALB/c mice aged 4 to 6 weeks. Twenty mice were anesthetized with ketamine-xylazine and intranasally infected with 10 LD_{50} in 50 μl of PBS of HPAIV A/Chicken/Kurgan/Russia/2/05 (H5N1). LD_{50} was determined by the method of Reed and Muench (1938).

To determine the prophylactic efficacy of MAb 4F11, groups of mice (n = 10) were pretreated intraperitoneally with 2, 10, or

20 mg/kg of MAb in 100 μl of PBS 24 hrs prior to the viral challenge. The control group (n = 20) received 100 μl of PBS. After 24 hrs, the mice were challenged with 10 LD_{50} of HPAIV H5N1. To determine therapeutic efficacy of MAb 4F11, each group of mice (n = 10) was treated intraperitoneally with 2, 10, and 20 mg/kg of MAb in 100 μl of PBS 24 hrs after viral challenge. The control group (n = 20) received 100 μl of PBS. Rimantadine (Rozfarm, Russian Federation) and Oseltamivir (Tamiflu®, Roche, Switzerland) were used as controls. They were administered daily for 5 days before infection perorally at concentrations of 16 and 13 mg/kg in 50 μl of PBS (prophylactic study, n = 20). In addition, the mice were administered 30 and 25 mg/kg daily 5 days after infection (therapeutic study, n = 20). The mice were observed daily for 21 days after the viral challenge to monitor mortality.

Statistical analysis. Means and standard deviations of A_{450} determinations were calculated using Statistica 6.0 software. The significance of differences was evaluated by Fisher's test. The differences were regarded as statistically significant at $P < 0.05$.

Results

General characteristics of MAbs

A panel of hybridoma clones secreting MAbs to the HPAIV A/Duck/Novosibirsk/56/05 (H5N1) was generated by screening supernatants in ELISA and HI test. The highest antibody titers in these assays were obtained with 17 MAbs that were selected for subsequent experiments (Kushch *et al.*, 2008). The characteristics of previously received 14 MAbs and 3 new MAbs are summarized in Table 1. The majority of MAbs were IgG type, e.g. 7 MAbs were IgG1, 9 MAbs were IgG2a, and only one MAb was IgA type. MAbs reacted with A/Duck/Novosibirsk/56/05 antigen in ELISA in titers 10^{-5}–10^{-7}(except MAb 2C6). Twelve anti-HA MAbs and anti-NP MAb 4H4 stained PEK cells infected with the homologous virus in IF test. Specific staining of varying intensity was observed only in the cytoplasm. Ten of 12 anti-HA MAbs were reactive in HI test at the dilutions 1:320–1:20,480. These MAbs neutralized infectivity of the virus *in vitro.*

Reactivity of MAbs in immunoblot analysis

The specific recognition of both native and denatured NP of HPAIV A/Duck/Novosibirsk/56/05 by MAbs 4H4, 2E6, 1G7, 1G8, and 2A5 was demonstrated in the immunoblot analysis (Fig. 1, Table 1). However, interaction of the majority of MAbs except 2A5 with NP was weak. Twelve MAbs were reactive with the non-cleaved HA0 molecule. Only 2 MAbs 3G5 and 5E5 recognized HA1. Remaining MAbs were non-reactive under the reducing conditions.

Table 1. Reactivity of MAbs with HPAIV A/Duck/Novosibirsk/56/05 (H5N1) in various tests

MAb	IgG type, subtype	Reactivity of MAbs with HPAIV A/Duck/Novosibirsk/56/05					
		ELISA titer[a]	IF[b]	HI titer[a]	Neutralization titer[a]	Immunoblot analysis	
						Non-reducing conditions	Reducing conditions
4F11	IgG2a	10^7	++	20,480	>2,0420	HA0	–
7E11	IgG2a	10^6	++	5,120	2,560	HA0	–
4G10	IgG2a	10^6	++	5,120	40	HA0	–
3G9	IgG2a	10^6	+++	1,280	2,560	HA0	–
6E2	IgG2a	5 x 10^6	+++	1,280	10,240	HA0	–
5F12	IgG2a	10^7	+++	640	>10,240	HA0	–
5G9	IgG2a	10^6	++	2,560	2,560	HA0	–
7B3	IgG2a	10^5	++	320	320	HA0	–
6F3	IgA	10^7	++	10,240	5,120	HA0	–
3G5	IgG1	10^5	++	–	–	HA0	HA1
5E5	IgG1	5 x 10^5	++	–	–	HA0	HA1
2C6	IgG2a	10^2	+++	20,480	10,240	HA0	–
4H4	IgG1	10^6	+	–	–	NP (weak)	NP (weak)
2E6	IgG1	10^5	–	–	–	NP (weak)	NP (weak)
1G7	IgG1	10^6	–	–	–	NP (weak)	NP (weak)
1G8	IgG1	10^6	–	–	–	NP (weak)	NP (weak)
2A5	IgG1	10^6	–	–	–	NP	NP

Most of the MAbs (14) were prepared by Kushch *et al.* (2008). Newly prepared MAbs (3) are printed in bold. [a]Reciprocal dilution of ascitic fluids; (–) = negative result (< 1:20); [b]Intensity of fluorescence (+++) = bright; (++) = less bright; (+) = weak; (–) = negative.

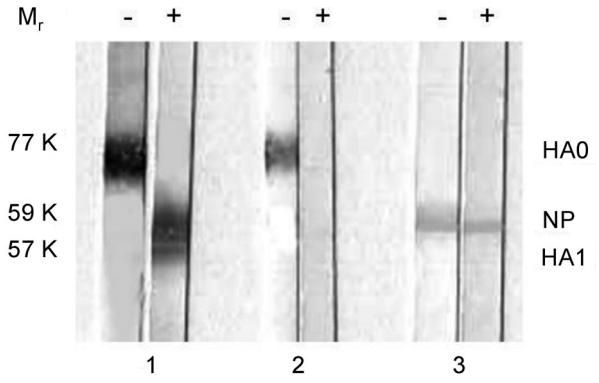

Fig. 1

Specificity of MAbs detected by immunoblot analysis

A/Duck/Novosibirsk/56/05 antigen was subjected to SDS-PAGE under both reducing (+) and non-reducing (–) conditions and electroblotted. Tested MAbs: 3G5 (lanes 1), 4F11 (lanes 2), 1G8 (lanes 3). Detected viral proteins and their Mr are indicated.

Reactivity of MAbs in HI test

Anti-HA MAbs were analyzed in HI test with a wide spectrum of AIV A/H5 (Table 2). The highest reactivity was recorded with 4 strains of HPAIV H5N1 (subclade 2.2) isolated in Russian Federation in the years 2005–2007. The MAbs displayed lower activity with H5N3 viruses isolated previously and with the WHO A/H5 antigen. Only one of the MAbs (2C6) inhibited hemagglutination of HPAIV H5N1, subclade 2.3.2. It is noteworthy that antiserum against AIV A/Chicken/Hong Kong/97 (H5N1) practically did not inhibit HA of subclade 2.3.2 viruses and less intensively reacted with Russian Federation strains of 2005–2007 than with AIV A/Tern/South Africa/61 and diagnostic A/H5 antigen from WHO 2003–2004. The anti-HA MAbs did not bind to other tested HA subtypes of influenza virus H1-H4, H6, H7, H9, H10, H11, and H13 (data not shown).

Reactivity of MAbs in dot blot analysis

The MAbs reacted with A/Duck/Novosibirsk/56/05 antigen in dot blot analysis with detection limit 30–90 ng of viral protein. Anti-NP MAbs 1G7, 1G8, and 2A5 were reactive with all tested human, avian, and swine influenza A viruses, but MAbs 4H4 and 2E6 reacted only with AIV (Table 3). All anti-HA MAbs recognized only HPAIV H5N1 (subclade 2.2) and LPAIV H5N3 in the allantoic fluids. The differences in the reactivity of anti-HA MAbs with HPAIV H5N1 (subclade 2.3.2) were demonstrated. Only MAbs 2C6, 3G5, and 5E5 were able to recognize 2–3 of these viruses in dot blot analysis. None of the MAbs reacted with influenza B virus. Some patterns of reactivity of two MAbs 4G10 and 1G7 are shown in Fig. 2.

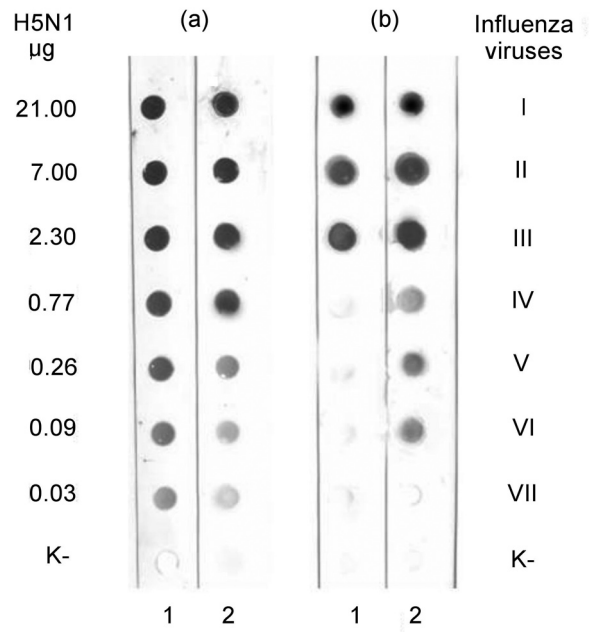

Fig. 2

Sensitivity and specificity of detection of various influenza viruses by MAbs 4G10 and 1G7 in dot blot analysis

a) Sensitivity of detection. HPAIV A/Duck/Novosibirsk/56/05 antigen was tested in dilutions 21–0.03 μg/dot and detected by MAbs 4G10 (lane 1) and 1G7 (lane 2). Non-infected allantoic fluid (K-). b) Specificity of detection. Allantoic fluids containing influenza viruses were adsorbed onto nitrocellulose membrane and detected by MAbs 4G10 (lane 1) and 1G7 (lane 2): I – A/Grebe/Tyva/Tyv06-1/06 (H5N1, clade 2.2), II – A/Chicken/Moscow/2/07 (H5N1, clade 2.2), III – A/Tern/South Africa/61 (H5N3), IV – A/Chicken/Primorje/1/08 (H5N1, clade 2.3.2), V – A/New Caledonia/20/99 (H1N1), VI – A/Aichi/1/68 (H3N2), and VII – B/Shanghai/361/02. Non-infected allantoic fluid (K-).

Reactivity of MAbs in competitive and sandwich ELISA

Epitope specificities of anti-HA MAbs were studied in competitive ELISA. The inhibition of interaction between HRP-conjugated Mabs and A/Duck/Novosibirsk/56/05 antigen by unlabeled MAbs was analyzed (Fig. 3a). A topology of antigenic sites for these MAbs is schematically illustrated in Fig. 4. Ten MAbs recognized at least 4 non-overlapping antigenic determinants I – IV (Fig. 4). Six epitopes recognized by MAbs of group II are similar but not identical, as evidenced by non-symmetric competitive relations between these MAbs (Fig. 3a). The same is true for the MAbs of group III (3G5 and 5E5). MAbs 4G10 and 7E11 actively inhibited binding of conjugated MAbs of group I (4F11) and group II, although in both groups blocking was not symmetrical (Fig. 3a).

In order to determine the epitope specificity of MAbs 4G10 and 7E11 more accurately we analyzed concentration dependence of competition (Fig. 5). When MAb 7E11 was

Table 2. Reactivity of MAbs with various AIV of H5 subtype in HI test

MAb	HPAIV H5N1 (Russian Federation)						LPAIV H5		
	Clade 2.2[a]			Clade 2.3.2[a]			H5N1	H5N3	
	A/Duck/Novosibirsk/56/05	A/Grebe/Tyva/Tyv06-1/06	A/Chicken/Moscow/2/07	A/Cygnus Cygnus/ Krasnodar/329/07	A/ Chicken/Primorie/1/08	A/Grebe/Tyva/3/09	Diagnostic antigen H5 [b]	A/Tern/South Africa/61	A/Duck/Primorie/2633/01
4F11	20,480[c]	10,240	10,240	20,480	–	–	640	640	1,280
7E11	5,120	10,240	5,120	5,120	–	–	640	640	1,280
4G10	5,120	10,240	5,120	5,120	–	–	2,560	2,560	1,280
3G9	1,280	20,480	2,560	2,560	–	–	1,280	1,280	320
6E2	1,280	320	320	320	–	–	640	640	320
5F12	640	320	320	320	–	–	320	320	80
5G9	2,560	5,120	2,560	2,560	–	–	640	640	320
7B3	320	20,480	2,560	1,280	–	–	640	1,280	320
6F3	10,240	40,960	10,240	2,0,480	–	–	5,120	5,120	5,120
2C6	20,480	40,960	20,240	20480	10,280	10,280	1,280	640	320
Anti-A/Chicken/Hong Kong/97[d]	1,280	5,120	2,560	320	80	80	40,960	20,480	2,560

[a]Clades are done according WHO (2008); [b] Antigen from WHO diagnostic kit 2003–2004 (H5 influenza kit, Atlanta); [c]Reciprocal dilution of ascitic fluids; (–) = negative result (< 1:20); [d]Polyclonal serum against AIV A/Chicken/Hong Kong/97 (H5N1) from WHO diagnostic kit.

Table 3. Reactivity of MAbs with various influenza viruses in dot blot analysis

Influenza viruses[a]	Clade	Reactivity of MAbs with influenza viruses in dot blot analysis																
		4F11	7E11	4G10	3G9	6E2	5F12	5G9	7B3	6F3	3G5	5E5	2C6	4H4	2E6	1G7	1G8	2A5
HPAIV H5N1, 4 strains	2.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HPAIV H5N1 A/Chicken/ Primorje/1/08	2.3.2	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
HPAIV H5N1 A/Grebe/Tyva/3/09	2.3.2	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
HPAIV H5N1 A/Bean Goose/ Tyva/10/09	2.3.2	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
LPAIV H5N3, 2 strains		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AIV, 8 strains: H3N8, H4N6, H6N2, H7N1, H9N2, H10N7, H11N6, and H13N6		–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
Human influenza viruses, 5 strains: H3N2, H1N1 and H2N2		–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
Pandemic influenza viruses H1N1swl, 2 strains		–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
A/Swine/Wisconsin/67 H1N1		–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
B/Shanghai/361/02		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a]Influenza viruses are listed in Tables 2 and 4.

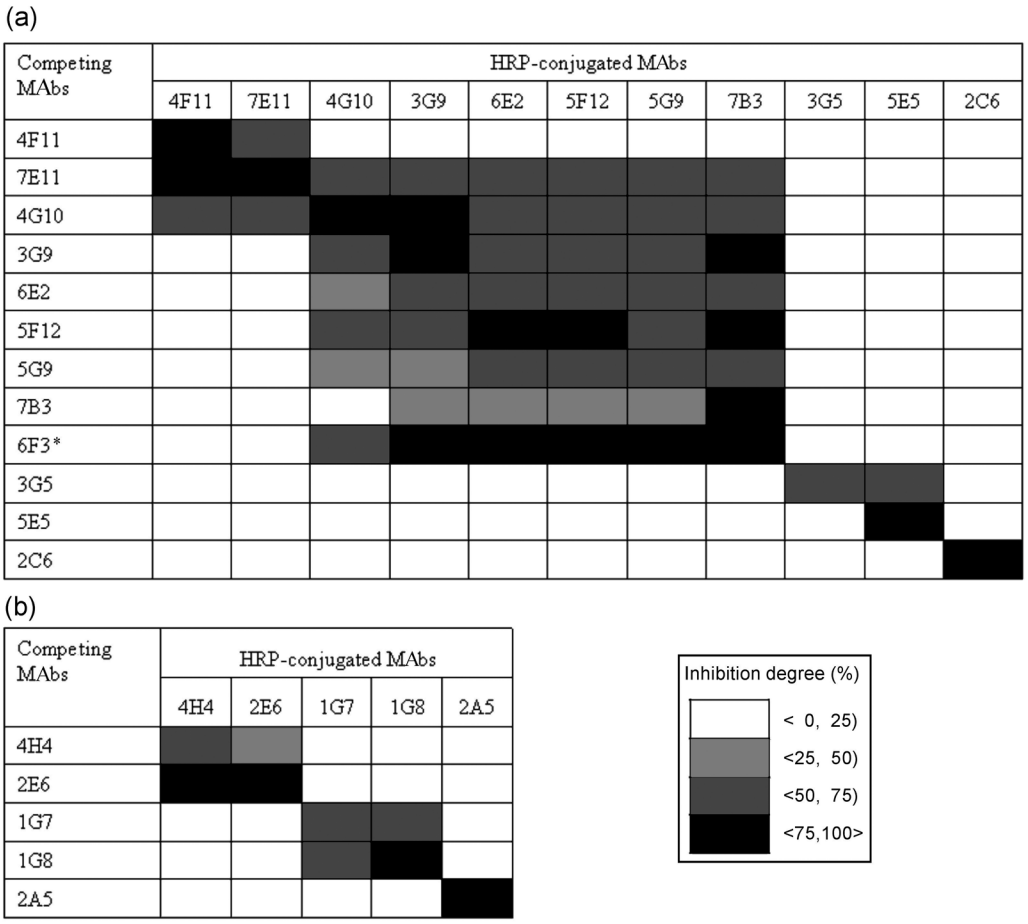

Fig. 3

Reactivity of anti-HA (a) and anti-NP (b) MAbs with HPAIV A/Duck/Novosibirsk/56/05 (H5N1) in competitive ELISA

The binding of HRP-conjugated MAbs with HPAIV A/Duck/Novosibirsk/56/05 antigen was inhibited**.** Competiting MAbs are purified Ig in concentration 100 μg/ml. *Ascitic fluid dilution 1:100.

used as a competitor, 50% inhibition of binding between the virus and conjugated MAbs 4F11 and 7E11 occurred at the MAb 7E11 concentration of 2–3 μg/ml, and group II conjugated MAbs at 7E11 concentrations of 10–20 μg/ml (Fig. 5a). By contrast, MAb 4G10 employed as a competitor inhibited the binding of conjugated group II MAbs at the concentration of 0.04 μg/ml and that of MAbs 4F11 and 7E11 at 7–20 μg/ml (Fig. 5b). Thus, MAbs 7E11 and 4G10 recognized partially overlapping antigenic sites on HA molecule. MAb 7E11 demonstrated greater affinity for group I and MAb 4G10 for group II (Fig. 3, 4, and 5).

Competitive analysis of anti-NP MAbs has shown that they recognize at least 3 non-overlapping antigenic determinants of the protein. One of them was recognized by MAbs 4H4 and 2E6, second one by MAbs 1G7 and 1G8, and the third one by MAb 2A5 (Fig. 3b). It should be noted that MAbs 4H4 and 2E6 displayed non-symmetric inhibition of binding that pointed to the partial overlapping, but not to the same epitopes.

Various combinations of MAbs were tested in sandwich ELISA in order to identify MAb pairs with the highest sensitivity of virus detection. More than 150 combinations of the MAbs as capture and detecting (HRP-conjugated) antibodies were analyzed. First, we evaluated detection limit for the homologous HPAIV A/Duck/Novosibirsk/56/05 antigen. The highest sensitivity (about 1 ng/ml viral protein) was observed with anti-NP MAbs: capture MAb 4H4 – detecting MAb 2A5. The combination of MAbs capture 1G7 – detecting 2A5 had a similar sensitivity. Sandwiches composed of anti-HA MAb combinations detected purified homologous virus with a lower sensitivity compared with the anti-NP MAb combinations. The highest sensitivity (about 5 ng/ml viral protein) was observed using two compositions: capture MAb 5G9 – detecting MAb 4F11 and capture MAb 5E5 – detecting MAbs 4F11 and 5F12 combined.

The sensitivity and specificity of influenza viruses detection were analyzed in sandwich ELISA using allantoic fluids

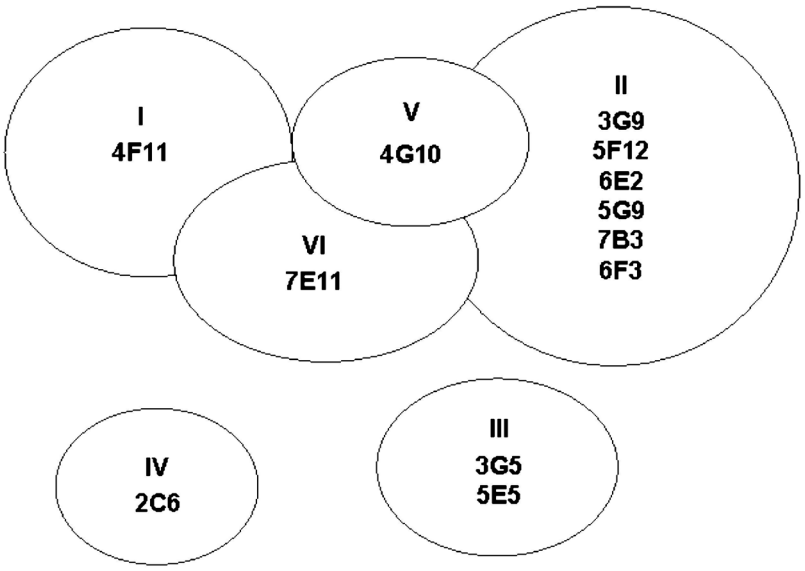

Fig. 4

Topology of antigenic sites on the HA of HPAIV A/Duck/Novosibirsk/56/05 (H5N1)

The antigenic sites (I – VI) are depicted by ellipses with corresponding MAbs inside. The degree of ellipse overlapping provisionally represents the degree of epitope overlapping.

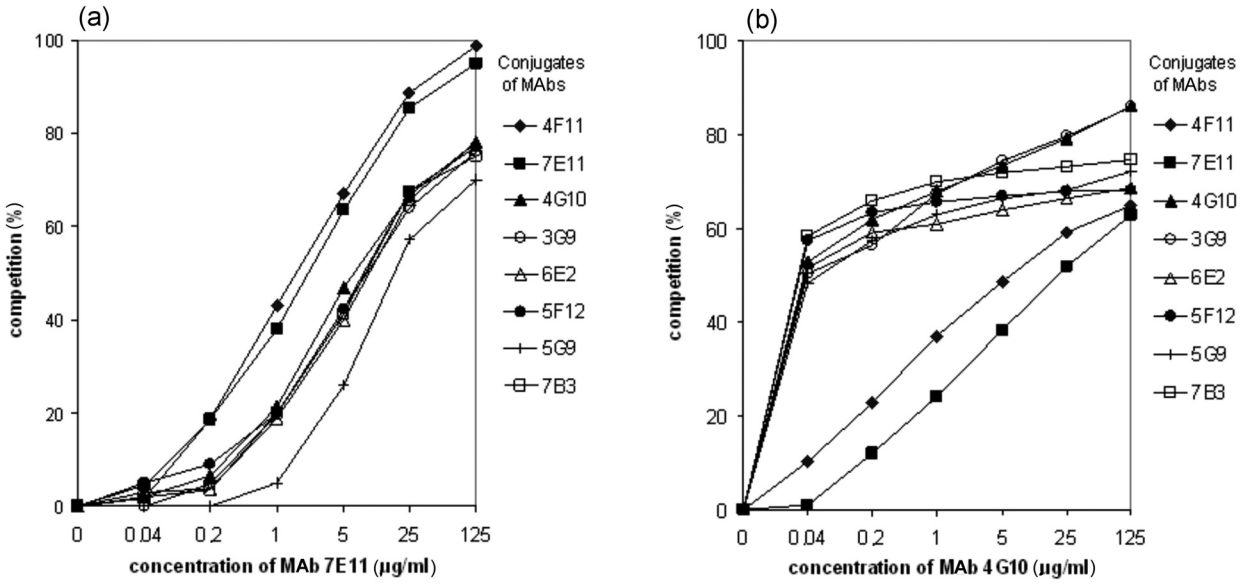

Fig. 5

Effect of concentration of MAbs 7E11 (a) and 4G10 (b) on their reactivity with HPAIV A/Duck/Novosibirsk/56/05 (H5N1) in competitive ELISA

x-axis: concentration of MAbs in µg/ml; y-axis: competition in %.

(Table 4). The pair capture MAb 1G7 – detecting MAb 2A5 recognized all tested subtypes of human and animal influenza A viruses including new pandemic H1N1 viruses. Several pairs of anti-NP antibodies differentiated AIV of various subtypes from human and swine influenza viruses. Pairs of anti-HA MAbs selectively identified only H5 AIV. The adding of MAb 2C6 in sandwich allowed revealing subclade 2.3.2 of HPAIV H5N1.

To determine the sensitivity of sandwich ELISA, allantoic fluids containing various influenza viruses with known HA

Table 4. Reactivity of anti-NP and anti-HA MAbs with various influenza A viruses in sandwich ELISA

Subtype	Clade	Influenza virus strain	Composition of sandwich (capture MAb – detecting MAb[a])			
			MAbs to NP		MAbs to HA	
			1G7-2A5[a]	4H4-2A5[a]	5G9-4F11[a]	5E5+2C6-4F11[a]+5F12[a]+2C6[a]
H5N1	2.2	A/Duck/Novosibirsk/56/05	2.5 [b]	2.7	1.7	1.9
H5N1	2.2	A/Grebe/Tyva/Tyv06-1/06	2.5	2.1	1.8	1.6
H5N1	2.2	À/Chicken/Moscow/2/07	2.3	2.5	2.0	1.8
H5N1	2.2	A/Cygnus Cygnus/ Krasnodar/329/07	2.7	2.3	2.1	2.1
H5N1	2.3.2	A/Chicken/Primorje/1/08	2.6	2.2	–[c]	3.2
H5N1	2.3.2	A/Grebe/Tyva/3/09	1.5	1.7	–	1.9
H5N1	2.3.2	A/Bean Goose/Tyva/10/09	2.2	2.0	–	2.2
H5N3		A/Duck/Primorie/2633/01	2.7	1.9	1.5	1.5
H5N3		A/Tern/South Africa/61	1.2	1.8	1.3	3.4
H3N8		A/Duck/Ukraine/63	2.1	1.7	–	–
H4N6		A/Duck/Czechoslovakia/56	1.5	1.9	–	–
H6N2		A/Turkey/Massachusetts/65	1.2	1.5	–	–
H7N1		A/FPV/Rostock/34	0.8	0.7	–	–
H9N2		A/Turkey/Wisconsin/66	1.0	0.9	–	–
H10N7		A/Chicken/Germany/49	1.6	1.2	–	–
H11N6		A/Duck/England/56	0.9	1.3	–	–
H13N6		A/Gull/Maryland/707/77	0.8	1.5	–	–
H3N2		A/Aichi/1/68	0.8	–	–	–
H3N2		A/Hong Kong/1/68	0.5	–	–	–
H2N2		A/Singapore/1/57	0.6	–	–	–
H1N1		A/USSR/90/77	1.2	–	–	–
H1N1		A/New Caledonia/20/99	0.9	–	–	–
H1N1swl		A/California/07/09	0.8	–	–	–
H1N1swl		A/Moscow/01/09	0.7	–	–	–
H1N1		A/Swine/Wisconsin/67	0.5	–	–	–

[a]HRP-conjugated MAbs; [b]A_{450} in ELISA with influenza viruses as allantoic fluids diluted 1:50 or A/Duck/Novosibirsk/56/05 antigen in concentration 1 μg/ml. Values represent the mean A_{450} from triplicate wells; [c] negative result (< cut off = 0.05).

titer were serially diluted and tested in the sandwich ELISA. The comparison of viral titers in ELISA and HA test showed that the sensitivity of virus detection in the allantoic fluids by ELISA was 4 to 160-fold (mean 41-fold) higher than in the HA test (Fig. 6).

Prophylactic and therapeutic efficacies of MAb 4F11

High neutralizing MAb 4F11 was tested for prophylactic and therapeutic efficacy (Fig. 7). To evaluate its prophylactic efficacy, the groups of experimental mice (n = 10) were treated with the indicated dosages of MAb 24 hrs prior to lethal virus challenge with 10 LD_{50} of HPAIV H5N1 strain. The mice given low doses of MAb 4F11 (2 mg/kg bodyweight) demonstrated 50% protection effect (Fig. 7a). Increased amounts of this MAb (10 mg/kg) protected 90% of mice from infection. Twenty mg/kg of MAb 4F11 completely protected mice from death and any clinical signs after challenge with the H5N1 strain. Tamiflu® protected 65% (13/20) and Rimantadine 60% (12/20) of the treated mice.

To evaluate therapeutic potential of MAb 4F11, the mice were treated with indicated doses of MAb 4F11 24 hrs after challenge with the virus. Two mg/kg and 10 mg/kg of MAb 4F11 provided 50% and 60% protection, respectively (Fig. 7b). At the concentration 20 mg/kg, MAb 4F11 protected 90% of mice against 10 LD_{50} of H5N1 strain. In therapeutic study Tamiflu® protected 70% and Rimantadine 45% of mice. The differences between protective efficacy of MAb 4F11 at the highest concentration and Rimantadine were statistically significant both in prophylactic (P = 0.029) and therapeutic (P = 0.024) studies. Control mice (untreated with MAb) mice showed 100% mortality within 10 days after the viral challenge.

Discussion

HPAIVs H5N1 cause systemic disease of the wild birds and poultry with a high mortality (Spackman, 2008). Avian

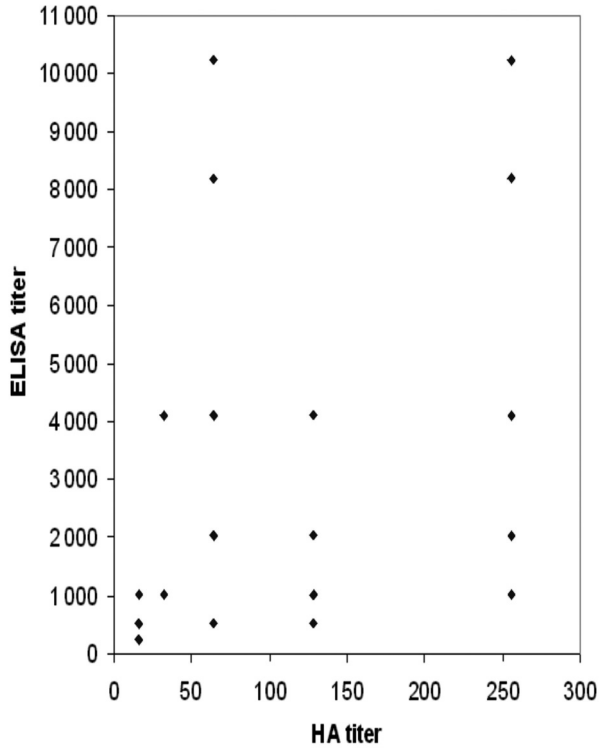

Fig. 6

Comparison of virus titers obtained by hemagglutination test and sandwich ELISA

x-axis: HA titer of influenza viruses as allantoic fluids in the hemagglutination test, reciprocal dilutions; y-axis: the titer of the same viruses in sandwich ELISA, reciprocal dilutions. Sandwich ELISA: mixture of MAbs 5E5 and 2C6 represents capture MAbs and mixture of HRP-conjugated MAbs 4F11, 5F12, and 2C6 detecting MAbs.

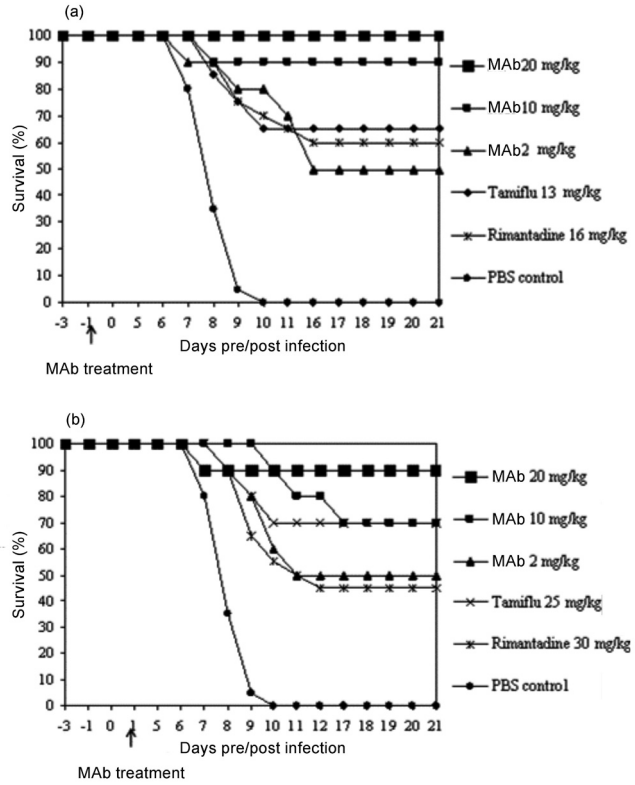

Fig. 7

Protection of mice from lethal H5N1 virus infection by MAb 4F11

Prophylactic efficacy (a) and therapeutic efficacy (b) of MAb 4F11. Mice were monitored for survival throughout 21 day observation period. The results are expressed as % of survival.

influenza became a serious problem after direct transmission of AIV to the humans. High lethality was documented among humans in Hong Kong in the years 1997–1998 (Claas *et al.*, 1998; Subbarao *et al.*, 1998). Studies of antigenic structure of AIV have shown that the viruses isolated during recent years differ considerably from those isolated previously (Chen *et al.*, 2006; Wu *et al.*, 2008). Therefore, the monitoring of H5N1 virus variations is necessary.

From the competitive analysis data we concluded that anti-HA MAbs characterized in this study were directed against at least 4 non-overlapping antigenic determinants on the HA molecule. Escape mutant mapping 7 out of 12 anti-HA MAbs has shown that the epitopes recognized by MAbs 5G9, 6E2, 5F12, 3G9, and 6F3 are located in an area adjacent to the antigenic site B in globular head of the HA molecule with antigenically relevant positions of amino acid residues 113, 115, 117, 118, 120, 121, 123, and 162. The epitopes recognized by MAbs 4F11 and 7E11 were located in the site A (aa 141 and 139) (Rudneva *et al.*, 2010). Preliminary data have shown that MAb 4G10 recognized also the site A (145 aa) (I. Rudneva, personal communication). Thus, a comparison of these data with those of competitive analysis indicated that the site II corresponded to the site B and the site I corresponded to the site A. It could be suggested that MAbs 7E11 and 4G10 partially overlapped both sites A and B on the HA molecule. We did not use Mabs 2C6, 7B3, 3G5, and 5E5 for the escape mutant selection, since these MAbs demonstrated low or no activity in HI test with wild type A/Mallard/10218/84 (H5N2) strain used to obtain the escape mutants. Two MAbs (3G5 and 5E5) did not react with H5 viruses in the HI and virus neutralization tests suggesting that they interacted with aa residues other than those forming the receptor-biding site of HA. Since the MAbs 2C6, 3G5, and 5E5 did not compete with the MAb of groups I and II (sites A and B) and differed from them in immunological and biological properties, we supposed that they were specific for other sites on the HA molecule. It was interesting that all MAbs reactive in HI and virus neutralization tests recognized the conformational epitopes and belonged to the IgG2a subtype. By contrast, MAbs 3G5

and 5E5 non-reactive in these biological tests recognized the linear epitope and belonged to the IgG1 subtype.

It was remarkable that the MAbs had different reactivity with H5 influenza viruses. Ten MAbs reacted in the HI test with LPAIV isolated in the years 1961 and 2001. Three of them (MAbs 2C6, 4F11, and 6F3) more effectively detected Russian Federation strains of the years 2005–2007 compared with the reference antiserum to the Southeast Asia H5N1 strain 1997. This antiserum displayed extremely low reactivity with Russian Federation strains of the years 2008-2009. The only anti-HA MAb 2C6 reacted with HPAIV of subclade 2.3.2 in HI test and MAbs 2C6, 3G5, and 5E5 reacted with these viruses in dot blot analysis. Thus, the epitopes recognized by these MAbs were the most conserved. Further investigation, however, is needed to determine the exact residues that constitute the 2C6, 3G5, and 5E5 epitopes. Taken together, these findings indicated that viruses H5N1 belonging to the subclade 2.3.2 had considerable antigenic change in sites A and B compared to the LPAIV H5N2 and A/H5N3 and HPAIV H5N1 of the subclade 2.2.

Rapid, early, and reliable detection of A/H5 viruses is the key issue in control of influenza disease. Since the majority of our MAbs displayed a high reactivity in immunochemical tests, they could be employed in the development of various immunoassays. All anti-HA and anti-NP MAbs differentiated between influenza A and B viruses. MAbs 2C6, 3G9, 6E2, and 5F12 that have shown high reactivity in IF test are prospective reagents for the direct virus detection or virus identification in specimens of infected birds, animals, and humans in rapid culture assay. These MAbs could be also suitable for the development of rapid test based on the dot blot assay and immunochromatography for the use in field.

Several sandwich ELISA procedures (antigen capture ELISA) based on anti-HA and anti-NP antibodies have been suggested for the detection of influenza viruses (Chomel *et al.*, 1989; Hornsleth and Jankowski, 1990; Scalia *et al.*, 1995; Tkáčová and Varečková, 1996; Varečková *et al.*, 2001; Zhang *et al.*, 2006; He *et al.*, 2007; Yang *et al.*, 2008; Du *et al.*, 2009; Yang *et al.*, 2009). Various immunochemical properties and broad epitope specificity of MAbs analyzed in this study allowed us to develop several sandwich ELISAs for the detection of influenza A viruses including new pandemic H1N1 influenza viruses, for typing of AIV, and for differentiating HPAIV H5N1 of subclades 2.2 and 2.3.2. It should be noted that the use of anti-HA MAbs 2C6 and 5E5 against conserved epitopes and detecting combination of MAbs (4F11 + 5F12 + 2C6) to the non-overlapping antigenic determinants enhanced the sensitivity and specificity of sandwich ELISA. Viruses in allantoic fluid samples with HA titer 1:16–1:256 were detected in the dilution up to 1:10,000 that was several times higher than the sensitivity of other sandwich ELISAs (He *et al.*, 2007; Ho *et al.*, 2009). However, further evaluation of the H5N1 sandwich ELISA using field samples is needed to determine sensitivity and specificity of the assay in a more quantitative way.

Special attention has been focused on the virus-neutralizing anti-HA antibodies. MAb 4F11 was tested in prophylactic and therapeutic experiments in the mice infected with 10 LD_{50} HPAIV A/Chicken/Kurgan/Russia/2/05 (H5N1, subclade 2.2) that displayed antigenic properties similar to the HPAIV A/Duck/Novosibirsk/56/05 (Lvov and Kaverin, 2008). This antibody was chosen since it neutralized most effectively a homologous virus *in vitro* (titer >1:20,000). A single intraperitoneal injection of 20 mg/kg MAb 4F11 produced 100% and 90% protective effects in prophylactic and therapeutic studies, respectively. However, this effect was dose-dependent. Similar results have been obtained also by other researchers. For example, passive immunization of mice with the anti-HA antibodies of different origins (murine, humanized, chimeric and human) produced various degree of protection against lethal doses of HPAIV H5N1 (Hanson *et al.*, 2006; Chen *et al.*, 2009; Koudstaal *et al.*, 2009; Prabhu *et al.*, 2009a, b; Sun *et al.*, 2009). The protective efficacy of MAb 4F11 was similar or higher than that of Tamiflu and Rimantadine. This finding opened prospects for the production of humanized antibodies based on this MAb for the prevention and therapy of disease caused by the H5N1 influenza virus in humans.

Thus, the panel of MAbs tested in this study could allow the detection of antigenically significant epitopes in HA of H5 viruses, to develop immunoassays for rapid differential diagnosis of avian, animal, and human influenza A viruses, and to demonstrate a high prophylactic activity and therapeutic effectiveness. In addition, these MAbs can be used for the influenza A virus detection, analysis of the antigenic drift of H5 viruses, and for the design of effective measures needed for the prevention and therapy of influenza.

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References

Chen H, Smith G, Li K, Wang J, Fan X, Rayner J, Vijaykrishna D, Zhang J, Zhang L, Guo C, Cheung C, Xu K, Duan L, Huang K, Qin K, Leung Y, Wu W, Lu H, Chen Y, Xia N, Naipospos T, Yuen K, Hassan S, Bahri S, Nguyen T, Webster R, Peiris J, Guan Y (2006): Establishment of multiple sublineages of H5N1 influenza virus in Asia: Implications for pandemic control. Proc. Natl. Acad. Sci. USA 103, 2845–2850. doi:10.1073/pnas.0511120103

Chen Y, Qin K, Wu WL, Li G, Zhang J, Du H, Ng MH, Shih JW, Peiris JS, Guan Y, Chen H, Xia N (2009): Broad cross-protection against H5N1 avian influenza virus infection by means

of monoclonal antibodies that map to conserved viral epitopes. J. Infect. Dis. 199, 49–58 doi:10.1086/594374

Chomel JJ, Thouvenot D, Onno M, Kaiser C, Gourreau JM, Aymard M (1989): Rapid diagnosis of influenza infection of NP antigen using an immunocapture ELISA test. J. Virol. Methods 25, 81–91. doi:10.1016/0166-0934(89)90102-X

Claas E, Osterhaus A, van Beek R, De Jong J, Rimmelzwaan G, Senne D, Krauss S, Shortridge K, Webster R (1998): Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 351, 472–477. doi:10.1016/S0140-6736(97)11212-0

Du A, Daidoji T, Koma T, Ibrahim M, Nakamura S, de Silva U, Ueda M, Yang C, Yasunaga T, Ikuta K, Nakaya T (2009): Detection of circulating Asian H5N1 viruses by a newly established monoclonal antibody. Biochem. Biophys. Res. Commun. 378, 197–202.

Hanson B, Boon A, Lim A, Webb A, Ooi E, Webby R (2006): Passive immunoprophylaxis and therapy with humanized monoclonal antibody specific for influenza A H5 hemagglutinin in mice. Respir. Res. 7, 0928–0936.

He Q, Velumani S, Du Q, Wee Lim C, Kheong Ng F, Donis R, Kwang J (2007): Detection of H5 avian influenza viruses by antigen-capture enzyme-linked immunosorbent assay using H5-specific monoclonal antibody. Clin. Vaccine Immunol. 14, 617–623. doi:10.1128/CVI.00444-06

Ho H, Qian H, He F, Meng T, Szyporta M, Prabhu N, Prabakaran M, Chan K, Kwang J (2009): Rapid detection of H5N1 subtype influenza viruses by antigen capture enzyme-linked immunosorbent assay using H5- and N1-specific monoclonal antibodies. Clin. Vaccine Immunol. 16, 726–732. doi:10.1128/CVI.00465-08

Hornsleth A, Jankowski M (1990): Sensitive enzyme immunoassay for the rapid diagnosis of influenza A virus infections in clinical specimens. Res. Virol. 141, 373–384. doi:10.1016/0923-2516(90)90009-8

Kaverin N, Rudneva I, Govorkova E, Timofeeva T, Shilov A, Kochergin-Nikitsky K, Krylov P, Webster R (2007): Epitope mapping of the hemagglutinin molecule of a highly pathogenic H5N1 influenza virus by using monoclonal antibodies. J. Virol. 81, 12911–12917. doi:10.1128/JVI.01522-07

Koudstaal W, Koldijk M, Brakenhoff J, Cornelissen L, Weverling G, Friesen R, Goudsmit J (2009): Pre- and postexposure use of human monoclonal antibody against H5N1 and H1N1 influenza virus in mice: Viable alternative to oseltamivir. J. Infect. Dis. 200, 1870–1873. doi:10.1086/648378

Kushch A, Klimova R, Masalova O, Fedorova N, Botikov A, Fediakina I, Burtseva E, Isaeva E, Deriabin P, Ľvov D (2008): Monoclonal antibodies to highly pathogenic avian influenza A(H5N1) strain isolated in the Russian Federation: development and properties. Vopr. Virusol. 53, 9–14 (in Russian).

Ľvov DK, Kaverin NV (2008): Avian influenza in Northern Eurasia. In Klenk H-D, Matrosovich MN, Stech J (Eds): Avian Influenza. Monogr. Virol. Vol. 27. Karger, Switzerland, pp. 41–58.

Ľvov DK, Zhchelkanov MIu, Vlasov NA, Prilipov AG, Deriabin PG, Fediakina IT, Galkina IV, Zaberezhnyĭ AD, Liapina OV, Shliapnikova OV, Kireev DE, Fesenko EE, Kalmykov MV, Vitkova ON, Morozova TN, Proshina ES, Grebennikova TV, Akanina DS, Samokhvalov EI, Al'khovskiĭ SV, Volkov VA, Semenov VI, Gaponov VV, Shmakov NI, Kushnir AT, Kazarian AS, Starikov NS, Petrenko MS, Slavskiĭ AA, Litvin KE, Shcherbakova LO, Frolov AV, Manin TB, Umanets OA, Bandeev VV, Khvan AM, Dunaev VG, Cheledina TP, Abgarian SR, Mikhaĭlovich VM, Zasedatelev AS, Liubchenko EN, Fliagin VN, Tikhonova IF, Maslov DV, Anan'ev VIu, Baranov NI, Gorelikov VN, Iakovlev SS, Aliper TI, Nepoklonov EA, Suarez D (2008): The first break-through of the genotype 2.3.2 of high-virulence influenza A virus A/H5N1, which is new for Russia, in the Far East. Vopr. Virusol. 53, 4–8 (in Russian).

Masalova O, Lakina E, Abdulmedzhidova A, Atanadze S, Semiletov Yu, Shkurko T, Burkov A, Ulanova T, Pimenov V, Novikov V, Khudyakov Yu, Fields H, Kushch A (2002): Characterization of monoclonal antibodies and epitope mapping of the NS4 protein of hepatitis C virus. Immunol. Lett. 83, 187–196. doi:10.1016/S0165-2478(02)00081-0

Nakane P, Kawaoi A (1974): Peroxidase-labeled antibody. A new method of conjugation. J. Histochem. Cytochem. 22, 1084–1091.

Peiris J, de Jong M, Guan Y (2007): Avian influenza virus (H5N1): a threat to human health. Clin. Microbiol. Rev. 20, 243–267. doi:10.1128/CMR.00037-06

Prabhu N, Prabakaran M, Ho H, Velumani S, Qiang J, Goutama M, Kwang J (2009a): Monoclonal antibodies against the fusion peptide of hemagglutinin protect mice from lethal influenza A virus H5N1 infection. J. Virol. 83, 2553–2562. doi:10.1128/JVI.02165-08

Prabhu N, Prabakaran M, Hongliang Q, He F, Ho H, Qiang J, Goutama M, Lim A, Hanson B, Kwang J (2009b): Prophylactic and therapeutic efficacy of a chimeric monoclonal antibody specific for H5 haemagglutinin against lethal H5N1 influenza. Antivir. Ther. 14, 911–921. doi:10.3851/IMP1413

Reed L, Muench H (1938): A simple method for estimating fifty percent end-points. Am. J. Hyg. 27, 493–497.

Rudneva I, Kushch A, Masalova O, Timofeeva T, Klimova R, Shilov A, Ignatieva A, Krylov P, Kaverin N (2010): Antigenic epitopes in the hemagglutinin of Qinghai-type influenza H5N1 virus. Viral Immunol. 23, 181–187. doi:10.1089/vim.2009.0086

Scalia G, Halonen PE, Condorelli F, Mattila ML, Hierholzer JC (1995): Comparison of monoclonal biotin-avidin enzyme immunoassay and monoclonal time-resolved fluoroimmunoassay in detection of respiratory virus antigens. Clin. Diagn. Virol. 3, 351–359. doi:10.1016/0928-0197(94)00050-5

Smith G, Naipospos T, Nguyen T, de Jong M, Vijaykrishna D, Usman T, Hassan S, Nguyen T, Dao T, Bui N, Leung Y, Cheung C, Rayner J, Zhang J, Zhang L, Poon L, Li K, Nguyen V, Hien T, Farrar J, Webster R, Chen H, Peiris J, Guan Y (2006): Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia

and Vietnam. Virology 350, 258–268. doi:10.1016/j.virol.2006.03.048

Spackman E (2008): A brief introduction to the avian influenza virus. Methods Mol. Biol. 436, 1–6. doi:10.1007/978-1-59745-279-3_1

Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, Bender C, Huang J, Hemphill M, Rowe T, Shaw M, Xu X, Fukuda K, Cox N (1998): Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 279, 393–396. doi:10.1126/science.279.5349.393

Sun L, Lu X, Li C, Wang M, Liu Q, Li Z, Hu X, Li J, Liu F, Li Q, Belser JA, Hancock K, Shu Y, Katz J, Liang M, Li D (2009): Generation, characterization and epitope mapping of two neutralizing and protective human recombinant antibodies against influenza A H5N1 viruses. PLoS One 4, e5476. doi:10.1371/journal.pone.0005476

Tkáčová M, Varečková E (1996): A sensitive one-step immunocapture EIA for rapid diagnosis of influenza A. J. Virol. Methods 60, 65–71. doi:10.1016/0166-0934(96)02046-0

Varečková E, Tkáčová M, Mattila ML, Mucha V, Waris ME (2001): Comparison of 44/107L one-step immunocapture enzyme-immunoassay and time-resolved fluoroimmunoassay for influenza A diagnosis. J. Virol. Methods 91, 131–137. doi:10.1016/S0166-0934(00)00257-3

Webster R, Govorkova E (2006): H5N1 influenza-continuing evolution and spread. N. Engl. J. Med. 355, 2174–2177. doi:10.1056/NEJMp068205

World Health Organization (2004): Laboratory Biosafety Manual. 3rd ed. World Health Organization, Geneva, Switzerland.

World Health Organization (2008): Toward a unified nomenclature system for highly pathogenic avian influenza virus (H5N1). Emerg. Infect. Dis. 14, e1.

World Health Organization Epidemic and Pandemic Alert and Response (2010): Online report: Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO on 8 June 2010 Available from http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_06_08/en

Wu WL, Chen Y, Wang P, Song W, Lau SY, Rayner JM, Smith GJ, Webster RG, Peiris JS, Lin T, Xia N, Guan Y, Chen H (2008): Antigenic profile of avian H5N1 viruses in Asia from 2002 to 2007. J. Virol. 82, 1798–1807. doi:10.1128/JVI.02256-07

Yang M, Berhane Y, Salo T, Li M, Hole K, Clavijo A (2008): Development and application of monoclonal antibodies against avian influenza virus nucleoprotein. J. Virol. Methods 147, 265–274. doi:10.1016/j.jviromet.2007.09.016

Yang M, Clavijo A, Graham J, Salo T, Hole K, Berhane Y (2009): Production and diagnostic application of monoclonal antibodies against influenza virus H5. J. Virol. Methods 162, 194–202. doi:10.1016/j.jviromet.2009.08.006

Zhang A, Jin M, Liu F, Guo X, Hu Q, Han L, Tan Y, Chen H (2006): Development and evaluation of a DAS-ELISA for rapid detection of avian influenza viruses. Avian Dis. 50, 325–330. doi:10.1637/7473-111605R.1