Acta virologica **53**: 15 – 20, 2009
doi:10:4149/av_2009_01_15

BROADLY CROSS-REACTIVE MONOCLONAL ANTIBODIES AGAINST HA2 GLYCOPEPTIDE OF INFLUENZA A VIRUS HEMAGGLUTININ OF H3 SUBTYPE REDUCE REPLICATION OF INFLUENZA A VIRUSES OF HUMAN AND AVIAN ORIGIN

A. STROPKOVSKÁ, V. MUCHA, T. FISLOVÁ, M. GOCNÍK, F. KOSTOLANSKÝ, E. VAREČKOVÁ*

Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 05 Bratislava, Slovak Republic

Received November 14, 2008; accepted January 22, 2009

Summary. – The reactivity of monoclonal antibodies (MAbs) prepared to the HA2 glycopeptide (gp) of A/Dunedin/4/73 (H3N2) hemagglutinin was tested against influenza A viruses of H3, H4, and H7 subtypes. Only one (CF2) out of six MAbs reacted with influenza A viruses of all three subtypes (H3, H4 and H7). The inter-subtype reactivity of this MAb (CF2) is in accord with the highly conservative sequence in the previously defined MAb-binding site I, i.e. the aa 1-38 of N-terminus of HA2 gp. MAb CF2 as well as inter-subtype cross-reactive MAb IIF4, recognizing the binding site II of HA2 gp, were tested for their effect on replication of influenza A viruses. Both these MAbs reduced the number of plaques of viruses of homologous (H3) as well as heterologous (H4) virus subtypes, the latter less efficiently. The potential of these MAbs to influence *in vivo* replication of influenza A viruses of various subtypes is discussed.

Keywords: influenza A virus; avian influenza; human influenza; hemagglutinin; HA2; monoclonal antibody; cross-reactivity; plaque reduction

Introduction

Influenza A viruses cause acute respiratory infection in humans, which has an epidemic character with relatively high degree of morbidity and mortality. The high variability and broad host specificity of influenza A viruses are the reasons why until now no universal prevention against influenza exists (Arnon, 2006; Luke and Subbarao, 2006; Palese, 2006). The main source of influenza A viruses of various subtypes of hemagglutinin (H1-H16) are aquatic birds. Till now, only three (H1-H3) of 16 known HA subtypes have been found in viral isolates circulating in the human population. However, since 1997 several cases of human infections with avian influenza A viruses of H5, H7 and H9 subtypes have been described (Lipatov *et al*., 2004; Stephenson, 2004; Luke and Subbarao, 2006). Some of them, especially those of H5 subtype, had a severe course with fatal impact. Therefore, the presently circulating avian influenza viruses are carefully monitored and their potential to infect humans is the subject of many research projects.

To protect human population against influenza A viruses not only of human (H1-H3 subtypes) but also of avian origin (other HA subtypes), a new strategy for preparation of influenza vaccines has been recently developed (Palese

*Corresponding author. E-mail: viruevar@savba.sk; fax: +4212-54774284.

Abbreviations: gp = glycopeptide; DUN = A/Dunedin/4/73 (H3N2); MISS = A/Mississippi/1/85 (H3N2); DUCK = A/Duck/Czech/56 (H4N6); CHICK = A/Chicken/Germany/34 (H7N1); RCA = rapid culture assay; MAb(s) = monoclonal antibody(ies); GAM IgG-Px = goat anti-mouse IgG conjugated with horseradish peroxidase; HA = hemagglutinin; TPCK-trypsin = trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone.

and García-Sastre, 2002; Palese 2006). The new approach is based on selection of such an influenza antigens, which are conserved among various HA subtypes and stimulate hetero-subtype protective immune response (Neirynck *et al*., 1999; Mozdzanowska *et al*., 2003; Tamura *et al.*, 2005; Ben-Yedida and Arnon, 2007).

In our previous studies we focused on HA2 gp, the conserved part of influenza virus HA, which is characterized as a strong immunogen and inductor of specific antibody response (Styk *et al*., 1979; Kostolanský *et al*., 2002). We have shown that antibodies induced by HA2 gp protected mice against the lethal influenza A infection of the same HA subtype as that of HA2 gp used for immunization (Gocník *et al*., 2007, 2008). Three (IIF4, FE1, and CF2) of four tested MAbs specific to different antigenic sites on HA2 gp, inhibited the fusion activity of influenza HA and reduced *in vitro* and *in vivo* replication of the virus of homologous HA subtype (Varečková *et al*., 2003a,b; Gocník *et al*., 2007). This biological activity of HA2-specific MAbs is of interest with regard to relative antigenic stability of HA2 gp.

Based on the screening of selected HA2-specific MAbs with influenza A viruses of H1-H13 subtypes (Varečková *et al*., 2002, 2008) we showed that three of them, recognizing two different antigenic sites localized within aa 125–175 of HA2 gp, namely the sites II (MAb IIF4) and IV (MAbs FE1, FC12), bound equally to influenza A viruses of H3 and H4 subtypes. One of them, MAb IIF4, reacted even with the viruses of other subtypes (H5, H8, and some strains of H2, H6 and H13) including a highly pathogenic avian H5N1 virus isolated from humans in Hong Kong in 1997 (Varečková *et al*., 2002, 2008).

The aim of this work was to study the cross-reactivity of MAb CF2, prepared to HA2 gp of H3 subtype and recognizing its antigenic site I (aa 1–38), with influenza A viruses of different HA subtypes, namely H3 of human and H4 and H7 of avian influenza A viruses. Moreover, the potential of this MAb to influence the *in vitro* replication of avian influenza A virus of heterologous (H4) subtype was explored.

Materials and Methods

Viruses. A/Dunedin/4/73 (H3N2), A/Mississippi/1/85 (H3N2), A/Duck/Czech/56 (H4N6), and A/Chicken/Germany/34 (H7N1) (further abbreviated as DUN, MISS, DUCK, and CHICK, respectively), originated from the collection of viruses of Institute of Virology, Bratislava. The viruses were propagated in chick embryos and purified from allantoic fluid by differential gradient centrifugation (Russ *et al*., 1974).

Monoclonal antibodies (MAbs). IIF4, CF2, EB12, BB8, FC12, and FE1, specific to HA2 gp of DUN virus, were prepared by Russ *et al*. (1987). MAb specific to influenza A virus nucleoprotein (107L) and that specific to HA1 gp of H1 subtype (4L) were prepared by Varečková *et al*. (1995).

Rapid culture assay (RCA). Confluent monolayers of MDCK cells in 96-well cultivation microplates were washed with PBS and infected with appropriate virus dilution in PBS (100 µl/well). After 45 mins of adsorption at room temperature, the unadsorbed virus was removed and serum-free medium Ultra MDCK-DMEM containing 0.5 µg/ml TPCK-treated trypsin was added for overnight incubation at 37°C in 5% CO_2. Then the cell monolayers were washed with PBS, fixed with cold methanol at 4°C for 10 mins and specific MAbs (100 µl) at a concentration of 5 µg/ml (CF2, EB12, and BB8) or 1.5 µg/ml (107L and IIF4) were added. The bound MAbs were detected with goat anti-mouse IgG conjugated with horseradish peroxidase (GAM IgG-Px, Biorad). The reaction of each MAb was visualized after adding the substrate solution containing 3-amino-9-ethylcarbazole (Sigma) in 0.03% H_2O_2. The reaction was considered positive when differentiated red-stained cells were visible in light microsope (Tkáčová *et al*., 1997).

ELISA. A tested purified virus (300 ng/100 µl) was adsorbed to 96-well microplates at 4°C overnight. The adsorbed virus was then treated with McIlvaine's solution pH 5 at room temperature for 30 mins. After 1 hr of saturation with 0.5% ovalbumine in PBS, the microplates were washed with PBS and a tested MAb (100 ng/100 µl/well) was added. After 90 mins of incubation at 25°C, the microplates were washed with PBS containing 0.05% Tween 20 and GAM IgG-Px was added to detect the bound MAb. The reaction was visualised after adding o-phenylendiamine with 0.03% H_2O_2 and stopped with 3 M HCl (100 µl/well). A_{492} was read in Multiscan MCC/340 ELISA reader (Labsystem).

Plaque reduction assay. An appropriate dilution of virus in PBS (20–40 PFU/ml/well) was preincubated with purified antibody (100 µg/ml) at 37°C for 15 mins and applied to MDCK cell monolayer in 6-well cultivation plates (1 ml/well) for 1hr at 25°C. The unadsorbed virus was removed, the cells were washed with PBS and a serum-free overlay medium (MEM with 1% agar, non-essential amino acids, 0.01% dextran and 0.5 µg/ml TPCK-trypsin) was added. The cultures were incubated for 3 (DUN virus) or 4 days (DUCK virus) at 37°C in 5% CO_2. Plaques were counted after fixation of cells with 20 % trichloroacetic acid and staining with 2% crystal violet (Varečková *et al*., 2003b). Reduction of plaque number by a MAb was derived from average of duplicates and was expressed in % of control represented by plaque number in the presence of indifferent MAb (4L).

Alignment of amino acid sequences concerned the antigenic sites I and II of HA2 of DUN, MISS, DUCK, and CHICK viruses. Since the sequence of DUN virus accessible in GenBank was limited to aa 1–18 (Acc. No. AAF18089), for the rest a consensus sequence of 27 influenza A viruses of H3 subtype isolated from humans in 1972–1974 was employed. Since complete sequences of MISS, DUCK, and CHICK viruses were not available either, a consensus sequence of 20 influenza A viruses of H3 subtype isolated from humans in 1984–1986, a consensus sequence of 4 influenza A viruses of H4 subtype isolated from ducks (Acc. Nos. BAF48478, AAF99711, BAA14332, and AAA43216), and the sequence of A/Chicken/Rostock/8/1934 (H7N1) identical with CHICK virus (Acc. No. AAA43150), respectively, were used.

Results and Discussion

Cross-reactivity of HA2-specific MAbs

Six MAbs prepared to the HA2 gp of H3 subtype were tested by RCA for their cross-reactivity with influenza A viruses of H3, H4 and H7 subtypes. As expected, all of them positively reacted with the homologous DUN virus and with MISS virus of the same (H3) subtype. Four MAbs specific to three different antigenic sites on HA2 gp (CF2 to site I, IIF4 to site II, FC12 and FE1 to site IV) also reacted with avian influenza virus of H4 subtype (DUCK) and two MAbs (CF2 and EB12 to site I) also reacted with avian influenza virus of H7 subtype (CHICK). Only one MAb, namely CF2, was positive with all tested viruses, i.e. of H3, H4 and H7 subtypes (Table 1, Fig. 1).

Table 1. Reactivity of HA2-specific MAbs with influenza A viruses of various HA subtypes in RCA

MAb	Virus (HA subtype)			
	DUN (H3)	MISS (H3)	DUCK (H4)	CHICK (H7)
CF2	+++	+++	+	++
EB12	++	+	–	+
BB8	++	+	–	–
IIF4	+++	+++	+++	–
FC12	++	+	+	–
FE1	+	+	+	–
107L*	+++	+++	+++	+++

(+++), (++), (+), (–) = high, middle, low, no reactivity, respectively.
*MAb specific to the nucleoprotein of influenza A virus used as a positive control.

In ELISA, a low reactivity of these MAbs with native virus has been reported (Varečková *et al.*, 2003a). However, their reactivity is known to increase following a pH 5-treatment of virus due to irreversible conformational change of HA molecule leading to the exposition of HA2 out of the trimer and resulting accessibility of HA2 epitopes to antibodies (Skehel and Wiley, 2000; Chen *et al.*, 1995). Therefore, in this study in ELISA binding test, pH 5-treated purified viruses were used as antigens (Fig. 2). Similarly to results of RCA, all the MAbs strongly bound to the viruses of homologous H3 subtype. MAbs CF2, IIF4, FC12, and FE1 but not EB12 and BB8 also reacted with the virus of

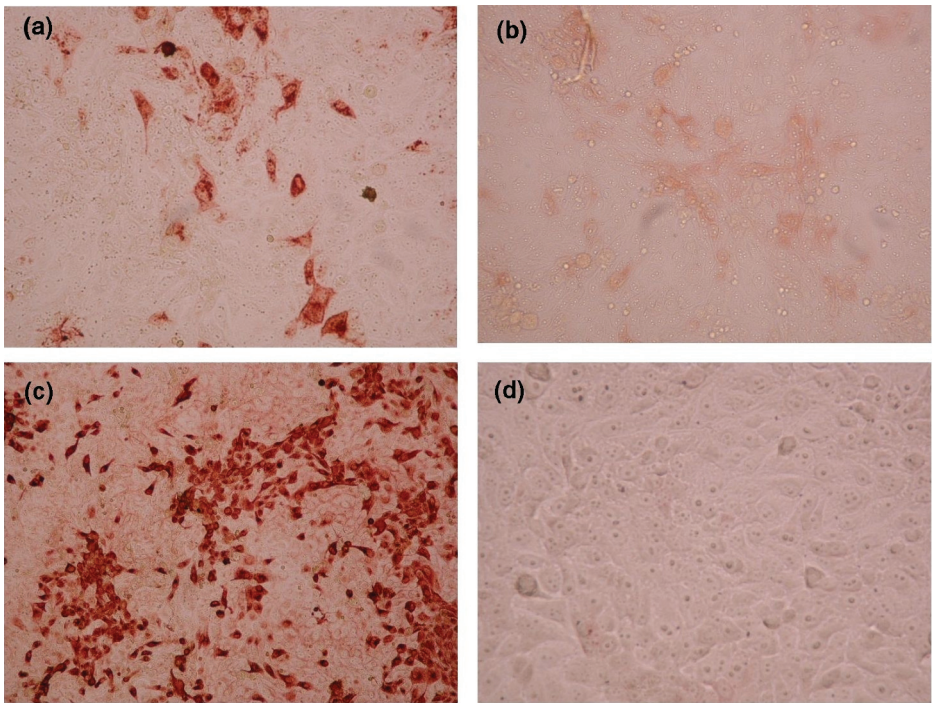

Fig. 1

Reactivity of HA2-specific MAbs with influenza A virus of H4 subtype in RCA

MAbs IIF4 (a) and CF2 (b) were tested in RCA with DUCK virus. MAb 107L (c) specific to the influenza A virus nucleoprotein was used as a positive control and no specific antibody was used in a negative control (d).

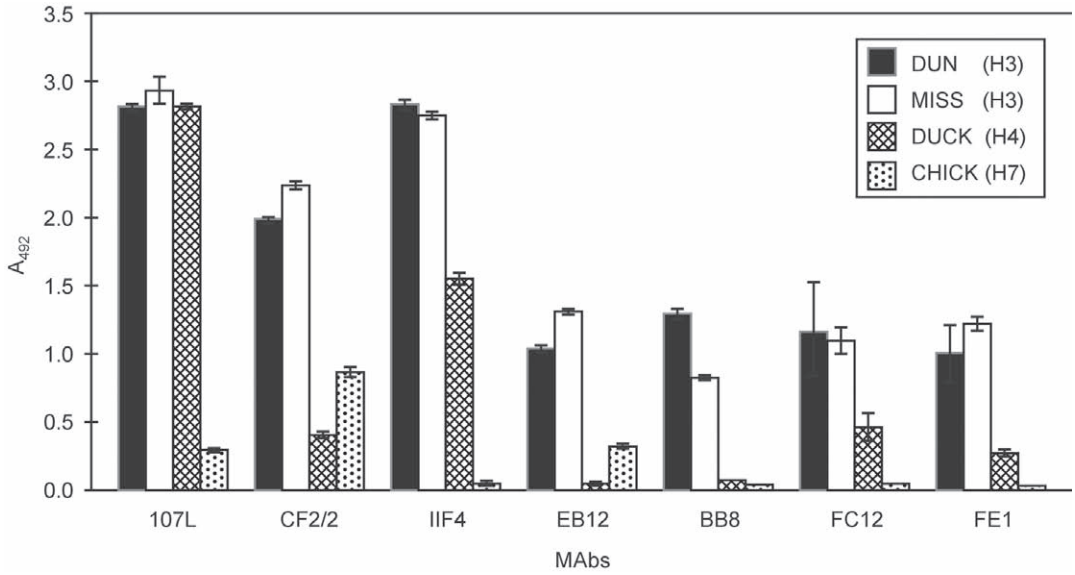

Fig. 2

Reactivity of HA2-specific MAbs with influenza A viruses of various HA subtypes in ELISA

Purified antibodies reacted with pH 5-treated viruses. MAb 107L specific to the influenza A virus nucleoprotein was used as a positive control.

H4 subtype. Two MAbs, CF2 and EB12, reacted even with the virus of H7 subtype. However, only one MAb, CF2, gave positive reaction with both avian viruses of H4 (DUCK) and H7 (CHICK) subtypes in ELISA and RCA as well.

In this study, the strongest binding to viruses of H3 and H4 subtypes exhibited MAb IIF4 (antigenic site II), while the broadest inter-subtype cross-reactivity, i.e. with viruses of H3, H4, and H7 subtypes, had MAb CF2 (antigenic site I). Therefore these two MAbs were selected for use in further experiments.

Sequence analysis of antigenic sites I and II of HA2 gp

Above we confirmed our previous observation (Varečková *et al.*, 2008) that MAb IIF4 recognizing the antigenic site located at aa 125–175 of HA2 gp (antigenic site II), reacted with human influenza A viruses of homologous H3 subtype

Ag site I

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>HA2          1.......10........20........30......38
DUN(H3)       GIFGAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADL
MISS(H3)      ......................................
DUCK(H4)      .L...........Q.LI.........A....T....
CHICK(H7)     .L............L..........AQ.E.T...Y
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Ag site II

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>HA2     125..130.......140.......150.......160.......170..175
DUN(H3)       QLRENAEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKG
MISS(H3)      ....................................................
DUCK(H4)      .......K.....E.F.....N..E..........I.....I......Q.
CHICK(H7)     .......ED.K...E.F....DD.MA....N....SK..E..MQ..I..DP
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Fig. 3

Alignment of amino acid sequences of antigenic sites I and II of HA2 gp of DUN, MISS, DUCK, CHICK viruses

DUN virus was used as a reference. For Acc. Nos see Materials and Methods.

as well as with avian virus of heterologous H4 but not H7 subtype. Sequence analysis of this region of HA2 gp revealed substitutions in H7 subtype at amino acid positions, which are conserved in H3 and H4 subtypes, particularly at aa 132, 135, 146, 149, 155, 160 164, 168, 171, and 175. These substitutions might cause the non-reactivity of MAb IIF4 with the virus of H7 subtype (Fig. 3).

On the other hand, we showed here that MAb CF2 recognizing the N-terminal region of aa 1–38 of HA2 gp (antigenic site I) reacted with human viruses of homologous H3 subtype, as well as with avian viruses of heterologous H4 or H7 subtypes in both assays used in this study. Sequence analysis of this part of HA2 gp showed that the regions of aa 3–14 and 19–28 were fully conserved in the tested viruses of H3, H4, and H7 subtypes. The inter-subtype reactivity of MAb CF2 with all these viruses was thus in accord with the sequence identity of the previously defined MAb-binding site I, i.e. the aa 1–38 terminus of HA2 gp.

Effect of MAbs specific to the antigenic sites I and II of HA2 gp on in vitro replication of viruses of H3 and H4 subtypes

The effect of cross-reactive MAbs CF2 and IIF4 on replication of homologous (DUN virus, H3) and heterologous (DUCK virus, H4) influenza A viruses was tested by plaque reduction assay. Both MAbs reduced the number of plaques of both viruses, however, the effect on homologous virus was slightly stronger than that on heterologous one (Fig. 4). Based on their cross-reactivity, we assume that MAbs CF2 and IIF4 might be able also to reduce replication of viruses of other HA subtypes, particularly those viruses with which they reacted in RCA or ELISA.

These two MAbs, which reduced the replication of the two influenza viruses, also inhibited the fusion activity of HA (Varečková *et al.*, 2003a). Therefore, a supposed mechanism of inhibition of replication can involve blocking of the second stage of infection, particularly the fusion of viral and endosomal membranes. The accessibility of HA2 epitopes to antibodies in the native virus is low (Kostolanský *et al.*, 1988; Varečková *et al.*, 1993). We suppose that at a higher temperature (37°C), some epitopes, which are hidden in the native HA trimer, are disclosed due to the flexibility of HA molecule (Yewdell *et al.*, 1993; Vaccaro *et al.*, 2005). The exposition of these epitopes enables their reaction with antibodies. This reaction is dependent on the temperature, affinity of antibody binding to the epitope, and duration of the contact between epitope and antibody. The virus-antibody complex is then internalized into the cells and the bound antibody prevents the fusion of viral and endosomal membranes most likely sterically (Varečková *et al.*, 2003b).

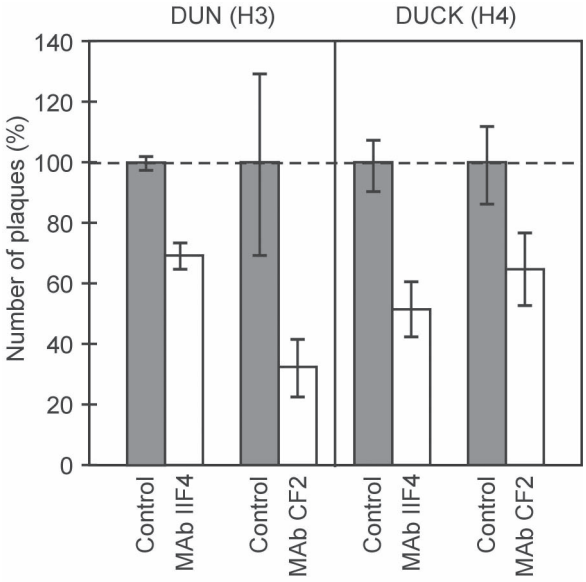

Fig. 4

Effect of HA2-specific MAbs on *in vitro* replication of influenza A viruses of H3 and H4 subtypes

The number of plaques (%) of DUN or DUCK viruses in MDCK cell monolayer in the presence of HA2-specific MAbs IIF4 or CF2 was compared with the number of plaques in the control (100 %), i.e. without specific MAb. Each value means an average from two parallel experiments. The reference value of 100% represents approx. 20–40 plaques.

This mechanism is in accord with the observation that the plaque reduction is relatively low and that HA2-specific antibodies do not block the infection (Gerhard, 2001). However, their protective effect on the *in vivo* influenza infection with different strains of homologous subtype has already been described (Gocník *et al.*, 2007, 2008). Regarding the broad inter-subtype cross-reactivity of HA2-specific MAbs described above and their protective potential *in vivo,* we suppose that they might accelerate the healing process and mediate a sooner elimination of infectious virus from lungs of mice infected not only with influenza A virus of homologous HA subtype (Gocník *et al.*, 2007, 2008), but also of heterologous HA subtype. This remains to be proven in future experiments.

Acknowledgement. The authors thank Dr. E. Závodská for preparing the photo documentation. Authors are indebted to Dr. G. Russ for valuable comments to the manuscript and for providing hybridomas producing HA2-specific MAbs. This work was supported by the grants Nos. 2/6077/6, 2/7065/7, and 2/0154/09 from Scientific Grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences, and by Slovak Research and Development Agency under the contract No. APVV/51–021605.

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