A MONOCLONAL ANTIBODY SPECIFIC TO THE HA2 GLYCOPROTEIN OF INFLUENZA A VIRUS HEMAGGLUTININ THAT INHIBITS ITS FUSION ACTIVITY REDUCES REPLICATION OF THE VIRUS

E. VAREČKOVÁ[1*], S.A. WHARTON[2], V. MUCHA[1], M. GOCNÍK[1], F. KOSTOLANSKÝ[1]

[1]Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 05 Bratislava 4, Slovak Republic;
[2]National Institute for Medical Research (NIMR), London, UK

Received October 6, 2003; accepted November 14, 2003

Summary. – Monoclonal antibody (MAb) CF2, which binds to the fusion peptide of influenza A virus hemagglutinin (HA) (amino acids (aa) 1–35 of the N-terminus of the light chain of HA), inhibited the fusion activity of HA. This MAb preferentially bound to pH 5-treated virus (with conformationally altered HA) and bound only weakly to the native wild type (wt) virus. However, a significant binding of MAb CF2 to the amantadine resistant virus mutant Ab4 (with a mutation at aa 17 of HA1 leading to a destabilization of HA trimer) was obtained without pH 5 treatment. Exploiting the fusion-inhibition activity of MAb CF2 the effect of this antibody on the virus replication *in vitro* was followed using both the wt virus and the amantadine resistant mutant Ab4. No reduction of replication of wt virus and a low reduction of replication of Ab4 mutant (by about 20%) was detected by radioimmunoassay after preincubation of the virus with a high concentration of MAb CF2 at room temperature. An increased reduction of replication of Ab4 mutant (by about 40%) was observed in cell radioimmunoassay (RIA) and in plaque assay when the virus was preincubated with MAb at 37°C. Under these conditions a reduction of the wt virus replication also occurred by about 40%. This is the first report on the capacity of a MAb specific to HA2 gp, the light chain of influenza A virus HA, to reduce replication of the virus. This capacity in relation to (i) the affinity of the antibody to the virus, and (ii) the accessibility of corresponding epitopes on the virus surface as well as the proposed mechanism of inhibition of replication of the virus are discussed.

Key words: HA2 glycoprotein; influenza A virus; fusion; hemagglutinin; monoclonal antibody; virus neutralization

Introduction

The light chain of influenza A virus HA (HA2 gp) is responsible for the fusion of virus to cell endosomal membranes. Due to the low pH in endosomes, a HA trimer is destabilized and HA2 gp is refolded into the fusion-active form. The N-terminus of HA2 gp is exposed from the trimer and inserted into the target membrane. As a consequence, the fusion process of viral and target membranes starts (for review see Skehel and Wiley, 2000).

It was shown that both HA1 gp and HA2 gp are strong inducers of antibody response during the natural infection (Styk *et al*., 1979; Kostolanský *et al*., 2002). In contrast to HA1-specific antibodies, which are mostly virus-neutralizing, the biological significance of antibodies specific to HA2 gp is unclear (for review see Gerhard *et al*., 2001). The HA2 part of HA, in contrast to HA1 gp, is relatively conserved (Nobusawa *et al*., 1991), which results in the high

*E-mail: viruevar@savba.sk; fax: +4212-5477 4284.

Abbreviations: BHA = bromelain-cleaved HA; DMEM = Dulbecco's Modified Eagle's Minimal Essential Medium; HA = hemagglutinin; HA1 gp = heavy chain of influenza virus HA; HA2 gp = light chain of influenza virus HA; HAU = hemagglutinin unit; HI = hemagglutination-inhibiting; MAb = monoclonal antibody; PBS = phosphate-buffered saline pH 7.4; RIA = radioimmunoassay; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; wt = wild type

crossreactivity of antibodies elicited by HA2 gp (Graves *et al*., 1982; Becht *et al*., 1984; Russ *et al*., 1987; Sánchez-Fauquier *et al*., 1987). Therefore the possible effect of HA2-specific antibodies on the virus replication would be interesting from the view of anti-influenza immunity.

In our previous studies it was shown that three out of seven MAbs specific to HA2 gp were able to inhibit the fusion activity of influenza HA *in vitro*. Three experimental systems have been used to follow the inhibition of fusion: (i) hemolysis mediated by virus, (ii) virus-liposome fusion assay, and (iii) prevention of the cell-cell fusion of CHO cells expressing HA on their surface. These three MAbs recognized the N-terminal region of HA2 (Varečková *et al*., 2003). Only one MAb, CF2, which bound directly to the fusion peptide (aa 1–35 of the N-terminus of HA2), inhibited the fusion in all three experimental systems (Varečková *et al*., 2003).

To know whether the fusion-inhibition activity of this MAb has any impact on virus replication, the virus growth in the presence of MAb CF2 was followed in cell RIA and in plaque assay.

Materials and Methods

Virus strains. The following influenza virus strains from NIMR, London, UK and from the Institute of Virology, Bratislava, Slovak Republic were used: A/Dunedin/4/73 (H3N2) virus, recombinant X-31 containing the HA and NA glycoproteins from A/Aichi/2/68 (H3N2) virus, and the amantadine resistant mutant Ab4 with H to R mutation at position 17 in HA1 derived from influenza A virus X-31 (Daniels *et al*., 1985).

Preparation of viruses. The viruses were propagated in fertilized chicken eggs and purified from the allantoic fluid by sucrose density gradient centrifugation (Russ *et al*., 1974). The amantadine resistant mutant Ab4 was isolated, grown in eggs and purified as described by Daniels *et al*. (1985).

Cells. MDCK cells, provided by NIMR, London, UK, were cultivated in Dulbecco's modified Eagle's Minimal Essential Medium (DMEM) containing 5% fetal calf serum in a humid 5% CO_2 atmosphere.

MAbs. The hybridoma clone producing MAb CF2/2 (for simplicity designated in this paper as MAb CF2), specific to the light chain of influenza A virus hemagglutinin (HA2), was prepared by the fusion of mouse myeloma cell line Sp2/0 with spleen cells from BALB/c mice immunized with a purified HA2 isolated from the virus A/Dunedin/4/73 (H3N2) as described by Russ *et al*. (1987) and MAbs 107L (specific to influenza A virus nucleoprotein), and 4L (specific to HA) from BALB/c mice immunized with the virus A/Singapore/1/86 (H1N1), as described by Varečková *et al*. (1995). The hybridoma cells were cultivated in DMEM containing 10% calf serum, gentamicin (20 μg/ml), glutamine (4 mmol/l), and sodium pyruvate (50 μg/ml) at 37°C in a 6% CO_2 humid atmosphere. The cells were inoculated into BALB/c mice pretreated with incomplete Freund's adjuvant for the production of ascitic fluid. MAbs were purified from the ascitic fluid by affinity chromatography on protein A-Sepharose CL4B (Ey *et al*., 1978). MAb 4L, specific to the HA of influenza virus A/Singapore/1/86 (H1N1), was used as an irrelevant control MAb and was of the same IgG1 isotype as MAb CF2 (Russ *et al*., 1987; Ivanova *et al*., 1991).

Preparation of bromelain-cleaved HA (BHA). The purified X-31 virus was digested with bromelain and purified as described by Brand and Skehel (1972) and Wharton *et al*. (1986).

Radiolabeling of MAb. The purified MAb 107L was iodinated with $Na^{125}I$ (Amersham) using the chloramine T procedure (Hunter, 1967).

ELISA-binding assay was performed on 96-well microplates. The purified virus (400 ng/well in 100 μl), incubated at an appropriate pH for 5 mins and neutralized, was adsorbed to wells overnight at 4°C. The plates were blocked with 1% BSA in phosphate-buffered saline (PBS) and washed. Specific MAbs at a concentration of 100 ng/well were added and incubated with the virus at 37°C for 1 hr. After several washings with 0.05% Tween 20 in PBS, goat anti-mouse IgG conjugated with peroxidase was added and the plates were incubated at 37°C for 1 hr and washed. The peroxidase substrate TMB was added and the color reaction was stopped by adding 100 μl of 0.1 mol/l H_2SO_4. The absorbance was read in an ELISA reader at 450 nm (A_{450}).

Virus-neutralization in cell RIA. MAb CF2 (maximum amount 10,000 ng/100 μl) was preincubated with Ab4 mutant (0.3 HAU) or A/Dunedin/4/73 (0.25 HAU) virus at room temperature or at 37°C for 1 hr. The virus mixed with MAb (100 μl) or the same amount of virus without MAb was then adsorbed to MDCK cell monolayers in 96-well plates at room temperature for 45 mins. The cells were then washed with PBS and a serum-free DMEM containing 2 μg/ml TPCK-trypsin was added. The infected cells were incubated at 37°C for 18 hrs and the virus was detected with ^{125}I-labeled MAb 107L (100,000 cpm/well) specific to the nucleoprotein in the cell monolayer after the cell fixation with cold methanol. After washing the cells, the radioactivity bound to the cells was measured in a gamma counter.

Virus neutralization in plaque assay. Confluent MDCK cell monolayers on 6-well plates were infected with Ab4 mutant or with a mixture of virus and MAb (1 ml/well). Virus (0.01 HAU; i.e. 25 PFU) and MAb (100 μg/ml) were preincubated at room temperature or at 37°C for 20 mins. Then the virus (or the virus from the mixture with MAb) was adsorbed to the cells at room temperature for 45 mins. The cells were washed and an overlay medium (2 ml/well) containing serum-free DMEM, 1% agar and 0.4 μg/ml TPCK-trypsin was added. The cells were incubated at 37°C in a humid atmosphere containing 5% CO_2. On the third day the cells were fixed with 20% trichloroacetic acid, stained with 2% crystal violet and plaques were counted.

Cleavage protection assay. The purified virus (1 mg/ml) or BHA (0.6 mg/ml) with or without MAb CF2 was preincubated at room temperature for 30 mins and then at 37°C for 20 mins. The samples were exposed to low pH by dialysis against 0.1 mol/l citrate pH 5 or pH 7 at 4°C for 2 hrs. pH was adjusted to neutral by dialysis against PBS at 4°C overnight. The protein ratio of virus or BHA to MAb was 1.0 (w/w). Simultaneously MAb CF2 at the same concentration (1 mg/ml) without virus or BHA was incubated at pH 5 and adjusted to pH 7. All samples were then digested with TPCK-trypsin in a final concentration of 0.1 mg/ml at room temperature for 20 mins. The proteolytic cleavage was stopped by

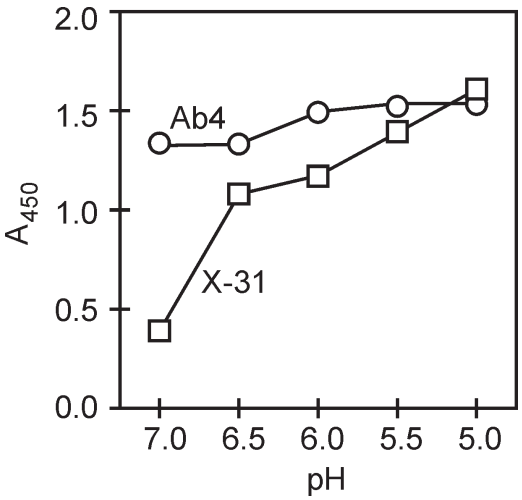

Fig. 1
pH dependence of MAb CF2 binding to X-31 virus and Ab4 mutant in ELISA

Concentration of MAb CF2 was 100 ng/100 µl. SD from two parallel experiments was calculated for each experimental point (average value ± 0.024).

adding a twofold molar excess of a trypsin inhibitor. The products of the proteolytic cleavage were analyzed by electrophoresis in 12% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) under the non-reducing conditions. The starting conditions for the electrophoresis were 80 V and 40 mA. The gels were stained with Coomassie Brilliant Blue R 250 and destained in a standard way.

Results

Binding of MAb CF2 to the wild type virus and its amantadine resistant mutant Ab4

MAb CF2 similarly to other HA2-specific MAbs previously tested (Kostolanský *et al*., 1988; Varečková *et al*., 1993, 2003), revealed increased binding to the pH 5-treated virus as compared to the native virus. The binding of MAb CF2 to the native X-31 virus was negligible and markedly increased after the acid treatment. However, the binding of MAb CF2 to the native amantadine resistant mutant Ab4, derived from X-31 virus, was comparable to that of the pH 5-treated parental virus X-31 (Fig. 1), i.e. the accessibility of the epitopes recognized by MAb CF2 on Ab4 mutant at neutral pH was about 80% of those of the epitopes accessible at pH 5. Mutation H to R at the aa position 17 of HA1 of Ab4 mutant destabilized the HA trimer (Daniels *et al*., 1985), which enabled a better accessibility of HA2 epitopes on Ab4 virus to the antibodies in the native

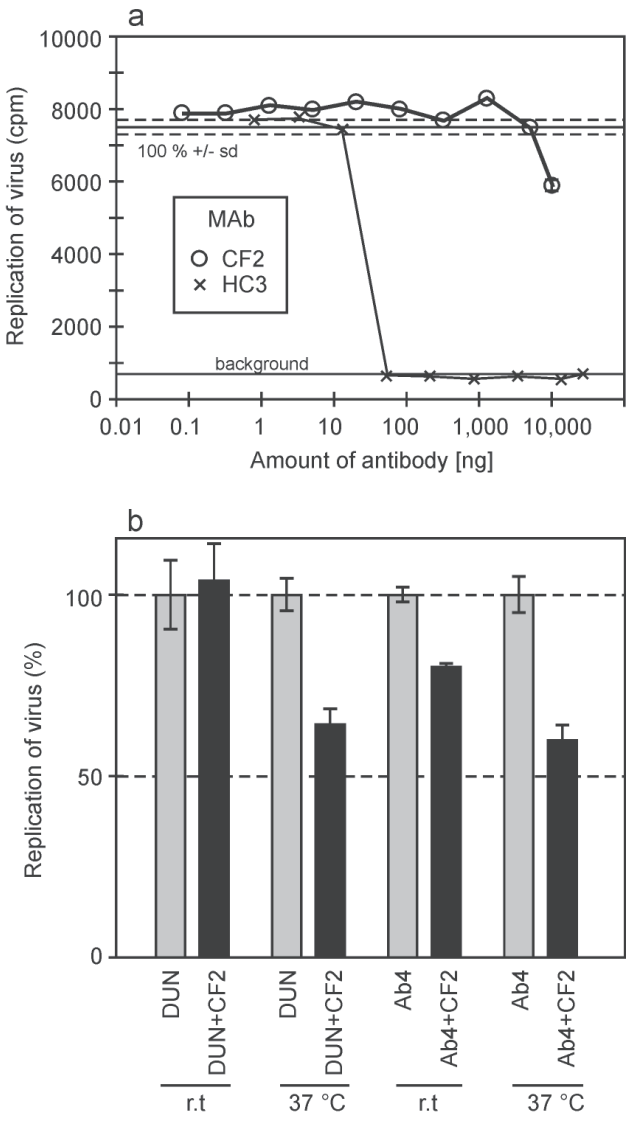

Fig. 2
Influence of MAb CF2 on virus replication as detected by cell RIA method

MAb CF2 at a concentration of 10,000 ng/100 µl/well in a serial dilution (a) or in a maximum concentration only (b) was preincubated with Ab4 mutant (a, b) or with A/Dunedin/4/73(H3N2) virus (DUN, b) at room temperature (r.t.) (a, b) or at 37°C (b). MAb HC3 (Daniels *et al*., 1983) was used as a positive control. The replicated virus was detected by MAb ^{125}I-107L specific to the nucleoprotein of influenza A virus (Varečková *et al*., 1995) (100,000 cpm/100 µl/well). Cell-bound radioactivity of replicated virus without MAb was taken as 100%. SD was calculated from three independent experimental values.

form, not treated with pH 5 (Varečková *et al*., 2003). This mutation did not cause a conformational change of the HA trimer to the pH 5-like structure as it was shown later in cleavage-protection experiments (Fig. 4a).

Table 1. Plaque count reduction of Ab4 mutant by MAb CF2

Virus	MAb	No. of plaques/well[a]
Ab4 (RT)	None	21 ± 1
Ab4 (37°C)	None	25 ± 2
Ab4 (37°C)	CF2 (preincubation)	15 ± 2
Ab4 (37°C)	CF2 (preincubation) + CF2 (overlay)	14 ± 1
Ab4 (37°C)	4L (preincubation) + 4L (overlay)	26 ± 0
Negative control	None	0

[a]The values represent averages ± SD from two independent experiments. RT = room temperature. Ab4 mutant was preincubated with MAb CF2, MAb 4L or no MAb at given temperature for 20 mins. MAb CF2 was present/absent during preincubation with Ab4 mutant and in overlay.

Influence of MAb CF2 on virus replication detected by cell RIA

As the inhibition effect of the MAb on virus replication requires its binding to the native virus HA, in further experiments we included the amantadine resistant mutant Ab4 (derived from parental X-31 virus) which significantly bound MAb CF2 in its native form.

The effect of MAb CF2 on the replication of Ab4 mutant in MDCK cells was followed by cell RIA and the replicated virus was detected by radioactively labeled MAb (^{125}I-107L) specific to influenza A virus nucleoprotein (Varečková *et al*., 1995). As a positive control of inhibition, anti-HA1 gp MAb HC3 (Daniels *et al*., 1983, 1985) was used. This MAb inhibited the virus replication by preventing the virus binding to the cell (Daniels *et al*., 1985). As shown in Fig. 2a, MAb HC3 inhibited the replication of Ab4 virus (750 PFU) by 100% even at 60 ng/100 µl. Anti-HA2 MAb CF2 caused an inhibition of 20% when preincubated with virus at room temperature. However, this effect was achieved at a very high concentration of MAb (10,000 ng/100 µl) only. No inhibition of a homologous virus A/Dunedin/4/73 by MAb CF2 was observed under the same conditions (Fig. 2b). The inhibition of replication of Ab4 mutant increased to 40% when the virus was preincubated with MAb CF2 at 37°C. After the preincubation of homologous virus A/Dunedin/4/73 with CF2 at 37 °C, the virus replication was inhibited by about 40 % (Fig. 2b), too.

Plaque count reduction of Ab4 mutant by MAb CF2

In an attempt to confirm the previous observation of the inhibition effect of MAb CF2 on virus replication a plaque assay, more sensitive than cell RIA, was used. In this assay Ab4 mutant was used as it was found to be more susceptible to the influence of MAb CF2 than wt virus.

A reduction of plaque count of Ab4 mutant by more than 40% was observed in the presence of MAb CF2 and the preincubation at 37°C. (Table 1). The same plaque count reduction was achieved by MAb CF2 regardless the antibody presence in the overlay (Fig. 3). No change in plaque count occurred with an irrelevant MAb 4L of the same isotype IgG1 as compared to the virus control without antibody.

MAb CF2 did not prevent the conformational change of HA from the native to the fusion-active form

Since MAb CF2 did not inhibit hemagglutination (Kostolanský *et al*., 1989), it can be supposed that it did not prevent the virus binding but it acted in a later stage of infection. There are two possible mechanisms by which MAb CF2 can influence replication of the virus: (i) after binding to the fusion peptide it prevents the change of HA from native to fusion-active conformation during the exposition of virus to low pH, or (ii) it can block the insertion of fusion peptide into the target membrane.

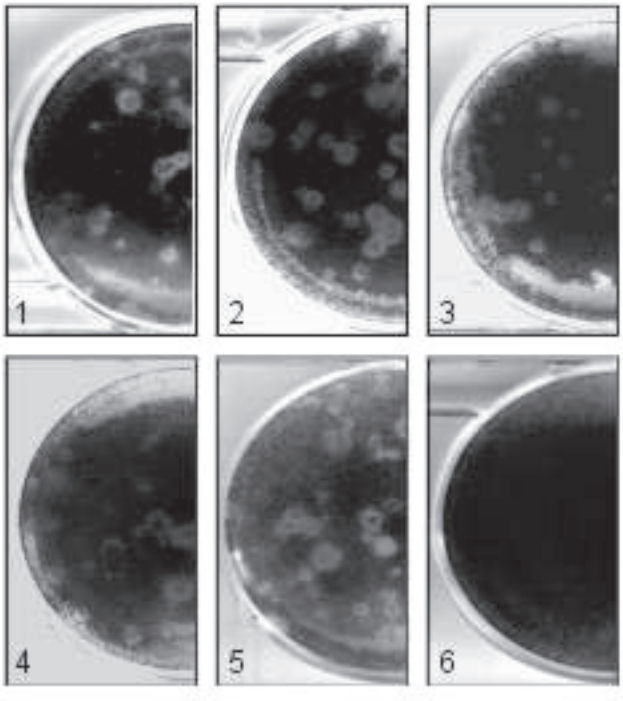

Fig. 3
Plaque count reduction of Ab4 mutant by MAb CF2

Ab4 mutant (0.01 HAU) was preincubated with MAb CF2 or with irrelevant MAb 4L, specific to the influenza A virus HA of H1 subtype of the same isotype (IgG1) (Ivanova *et al*., 1991; Varečková *et al*., 1995) at a concentration of 100 µg/ml at 37 °C (see also Table 1). Ab4 mutant (well 1). Ab4 mutant preincubated at 37°C (well 2). Ab4 mutant preincubated with MAb CF2 at 37°C (well 3). Ab4 mutant preincubated with MAb CF2 at 37°C, MAb CF2 present also in the overlay at a concentration of 100 µg/ml (well 4). Ab4 mutant preincubated with MAb 4L at 37°C, MAb present also in the overlay at a concentration of 100 µg/ml (well 5). MDCK cells without both virus and MAb (well 6).

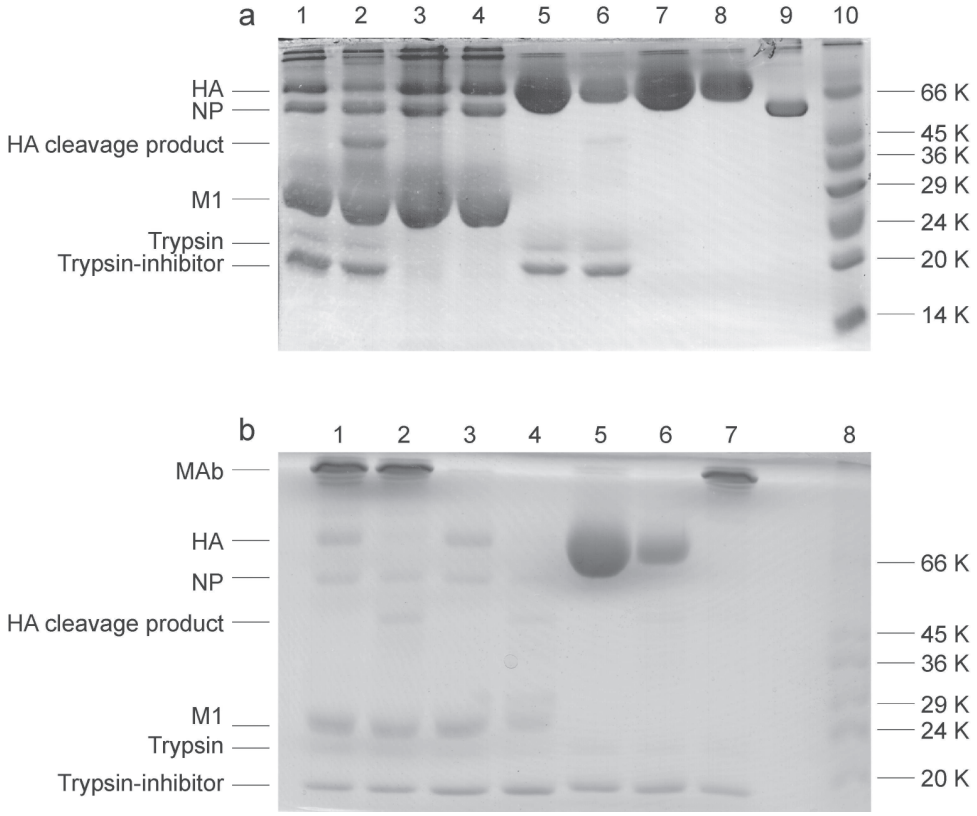

Fig. 4

SDS-PAGE of trypsin-cleaved products of Ab4 mutant treated with pH 5 or pH 7 in the presence (b) or absence (a) of MAb CF2

a. Ab 4 mutant (pH 7), trypsin (lane 1); Ab 4 mutant (pH 5), trypsin (lane 2); Ab4 mutant (pH 7) (lane 3); Ab4 mutant (pH 5) (lane 4); BHA (pH7), trypsin (lane 5); BHA (pH 5) trypsin (lane 6); BHA (pH 7) (lane 7); BHA (pH5) (lane 8); ovalbumin (lane 9); molecular mass standards (lane 10).
b. Ab 4 mutant, MAb CF2 (pH 7), trypsin (lane 1); Ab 4 mutant, MAb CF2 (pH 5), trypsin (lane 2), Ab4 mutant (pH 7), trypsin (lane 3); Ab 4 mutant (pH 5), trypsin (lane 4); BHA (pH 7), trypsin (lane 5); BHA (pH 5), trypsin (lane 6); MAb CF2 (pH 5), trypsin (lane 7); molecular mass standards (lane 8).

To decide by which of these two mechanisms MAb CF2 influenced the virus replication, the cleavage protection assay was done. It is known that the HA trimer in the native conformation is resistant to the trypsin cleavage; however, after its exposure to low pH (pH 5) it becomes susceptible to trypsin cleavage which results in the occurring new fragments originating from HA (Vanlandschoot *et al*., 1998). These products could be analyzed by SDS-PAGE.

Since Ab4 mutant with the destabilized HA trimer was used, first we confirmed the differences in electrophoretic profiles of native and pH 5-treated Ab4 mutant after trypsin cleavage. According to Fig. 4a, the trypsin cleavage product of HA appeared after Ab4 mutant treatment with pH 5 (lane 2) but not with pH 7 (lane 1). This allowed us to test whether the MAb CF2 binding prevented the conformational change of Ab4 mutant HA, triggered by pH 5.

The electrophoretic profile of Ab4 mutant treated with pH 5 in the presence (Fig. 4b, lane 2) or absence of MAb CF2 (lane 4) was not changed. MAb CF2 did not prevent the appearance of the cleavage product, i.e. CF2 being bound to HA very probably did not prevent the conformational change from the native to the fusion-active from of HA triggered by low pH. The possibility that the binding of MAb CF2 to HA was disrupted after lowering the pH was excluded by doing ELISA binding experiments at pH 5 (data not shown).

Discussion

The induction of HA2-specific antibodies has been demonstrated after the intramuscular immunization of rabbits with intact influenza virus (Russ *et al*., 1978) as well as experimental intranasal infection of mice (Kostolanský *et al*., 2002). HA2-specific antibodies have been detected in human convalescent sera after natural infection (Styk *et*

al., 1979) or in postvaccination sera (Cox and Brokstadt, 1999). However, the possible contribution of HA2-specific antibodies to the course of infection is unclear.

Our previous results (Varečková *et al*., 2003) have shown some HA2-specific MAbs to inhibit the fusion activity of HA in three various fusion-inhibition assays: polykaryon formation of CHO cells expressing HA, virus-liposome fusion, and hemolysis. Therefore the question whether such antibodies could influence the virus replication arose. MAb CF2 recognizing the N-terminal aa 1–35 domain of HA2 gp revealed the highest fusion inhibition activity in all three assays. Consequently, this MAb was chosen to follow its potential to inhibit replication of the virus *in vitro*. For this purpose the most sensitive conditions were selected, i.e. highly sensitive cell RIA and plaque assay using a mutant influenza virus with elevated fusion pH, and an increased preincubation temperature (37°C) of the virus-antibody mixture. The reduction of virus replication was repeatedly observed by both cell RIA and plaque assay. Though the plaque count reduction was achieved at high MAb concentration (100 μg/ml) only and did not reach 100%, it was clearly specific, because no reduction was obtained in the presence of irrelevant MAb 4L of the same isotype (IgG1) and concentration (100 μg/ml).

The reduction of virus replication by MAb CF2 appeared to be temperature-dependent; increasing the preincubation temperature from room temperature to 37°C led to the more efficient inhibition. Surprisingly, the elevated preincubation temperature resulted in the inhibition of wt virus replication comparable to that of Ab4 mutant. This result suggested that at 37°C MAb CF2 bound to wt virus similarly as to Ab4 mutant. This finding is consistent with the observation of Yewdell *et al*. (1993) that a prolonged incubation of an anti-HA1 MAb (capable of binding to HA monomer but not to native trimer) with virus at an elevated temperature resulted in the neutralization of viral infectivity. According to Yewdell *et al*. (1993) the corresponding epitope located at the interface of the adjacent subunits is transiently exposed in trimers for antibody binding at elevated temperatures. This temperature-dependent conformational flexibility of HA could explain the question of the accessibility of the CF2 epitope at 37°C on the wt virus at neutral pH. In contrast the released trimer structure of HA on the Ab4 mutant caused by the mutation at the position 17 of HA1 gp enables the accessibility of the CF2 epitope already at room temperature.

As MAb CF2 was shown not to exhibit hemagglutination-inhibition activity (Russ *et al*., 1987; Kostolanský *et al*., 1989), it could not prevent adsorption of the virus to the cell receptor in our experiments. Due to the epitope specificity of MAb CF2 it could be supposed that this antibody inhibits virus replication in a later, postattachment stage of the infectious cycle. Taking into account the fact that MAb CF2 has a significant fusion-inhibition activity (Varečková *et al*., 2003), we suppose that this MAb might inhibit virus-endosome membrane fusion, an essential step in initiation of virus infection. The HA-mediated fusion is a transient short-term process and therefore its inhibition by antibody requires its sufficiently high concentration to reach at least partial inhibition of replication. Moreover, the affinity value of MAb CF2 (4×10^8 l/mol) (Varečková *et al*., 2003) is near the border value (10^7 l/mol) needed for biological activity of an antibody (Kostolanský *et al*., 2000). This can explain the low reduction of virus replication by MAb CF2.

In our previous experiments, the virus-liposome fusion assay was the only experimental system, which has enabled quantitative analysis of the antigen-antibody interaction leading to the inhibition of fusion (Varečková *et al*., 2003). From those experiments it followed that at the used concentrations of Ab4 mutant (100 μg/ml) and MAb CF2 (100 μg/ml) and at the given MAb CF2 affinity all available epitopes were occupied by the MAb (Kostolanský *et al*., 2000) and that only under these conditions the fusion-inhibition could be observed. It could be expected that an increase in the affinity of MAb would lower the amount of MAb necessary for the fusion-inhibition. In the virus neutralization assay the MAb could not act directly as in virus-liposome fusion assay, but it would have to be endocytosed together with virus to prevent subsequently the fusion in the endosome. Therefore not only the affinity of MAb but also the accessibility of corresponding epitopes on virus before the pH 5 exposure are limiting for effective inhibition of virus replication. Taking into account the limitations mentioned above, it is probable that the concentration of MAb CF2 in endosomes was not sufficiently high for 100% inhibition.

The observation of a postattachment inhibition of fusion of endocytosed virus with intracellular membrane by a non-hemagglutination-inhibiting (HI) antibody resulting in neutralization of viral infectivity or reduction of virus-replication, has been also described by other authors. In contrast to our results they have observed neutralization of virus by non-HI anti-HA1 antibodies (Kida *et al*., 1983; Imai *et al*., 1998; Edwards and Dimmock, 2000; Edwards and Dimmock, 2001) or by a MAb against common epitope comprising HA1 and HA2 gp (Okuno *et al*., 1993) but not by a HA2-specific MAb as it was obtained in this study. The proposed mechanism of fusion-inhibition was the blocking the conformational change of HA. Our experiments showed that occurrence of the trypsin cleavage product of the pH 5-treated HA trimer in the presence of HA2-specific MAb CF2 was not inhibited. Therefore the most probable mechanism of reduction of virus replication by MAb CF2 seems to be the direct blocking of the insertion of the fusion peptide into the target membrane. This statement is supported also by fine specificity of MAb CF2.

From our results it follows that antibodies specific to HA2 gp can reduce the virus replication, however, their effect on

the course of natural infection might depend on their fine specificity as well as on their affinity of binding. It should be stressed that it is for the first time that the reduction of virus replication by a HA2-specific antibody was demonstrated. Though the inhibition effect on the virus replication was observed only at the high concentration of the antibody it cannot be excluded that under the physiological conditions such antibodies (anti-HA2 gp) might play role in the recovery of the organism from the infection. As it is known that HA2-specific antibodies are broadly cross-reactive inside a subtype (Graves *et al*., 1982; Becht *et al*., 1984; Russ *et al*., 1987; Sánchez-Fauquier *et al*., 1987) and even among subtypes (Kostolanský *et al*., 1994; Varečková *et al*., 2002), this can be an important factor especially when the infection is caused by the new antigenic variant of influenza virus.

Acknowledgements. The excellent technical assistance of Mrs. E. Uličná is greatly appreciated. The authors are indebted to Dr. G. Russ, Institute of Virology, Bratislava, for providing hybridoma producing monoclonal antibody CF2 and for valuable comments to the manuscript. We thank Dr. J.J. Skehel, NIMR, London, UK, for providing amantadine resistant mutant Ab4 of influenza A virus and for kind support of this project. This research was supported by the grants Nos. 2/3044/23 and 2/1103/21 from the Scientific Grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences, the Science and Technology Assistance Agency under the contract No. APVT-51-007802, Slovak Republic and by grants from The Royal Society, and The National Institute for Medical Research, UK.

References

Becht H, Huang RTC, Fleischer B, Boschek CB, Rott R (1984): Immunogenic properties of the small chain HA2 of the haemagglutinin of influenza viruses. *J. Gen. Virol*. **65**, 173–183.

Brand CM, Skehel JJ (1972): Crystalline antigen from the influenza virus envelope. *Nat. New. Biol*. **238**, 145.

Cox RJ, Brokstadt KA (1999): The postvaccination antibody response to influenza virus proteins. *Acta Pathol. Microbiol. Immunol. Scand*. **107**, 289–196.

Daniels RS, Douglas AR, Skehel JJ, Wiley DC (1983): Analysis of the antigenicity of influenza haemagglutinin at the pH optimum for virus-mediated membrane fusion. *J. Gen. Virol*. **64**, 1657–1662.

Daniels RS, Downie JC, Hay AJ, Knossow M, Skehel JJ, Wang ML, Wiley DC (1985): Fusion mutants of the influenza virus haemagglutinin glycoprotein. *Cell* **40**, 431–439.

Ey PL, Prowse SJ, Jenkin CR (1978): Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using Protein A-Sepharose. *Biochemistry* **15**, 429–436.

Edwards MJ, Dimmock NJ (2000): Two influenza A virus-specific FAbs neutralize by inhibiting virus attachments to target cells, while neutralization by their IgGs is complex and occurs simultaneously through fusion inhibition and attachment inhibition. *Virology* **278**, 423–435.

Edwards MJ, Dimmock NJ (2001): A haemagglutinin (HA1)-specific Fab neutralizes influenza A virus by inhibiting fusion activity. *J. Gen. Virol*. **82**, 1387–1395.

Gerhard W (2001): The role of the antibody response in influenza virus infection. *Curr. Top. Microbiol. Immunol*. **260**,171–190.

Graves PN, Schulman JL, Young JF, Palese P (1983): Preparation of influenza virus subviral particles lacking the HA1 subunit of haemagglutinin: unmasking of cross-reactive HA2 determinants. *Virology* **126**, 106–116.

Hunter WM (1967): The preparation of radioiodinated proteins of high avidity, their reaction with antibody in vitro. In Weir DM (Ed.): *Handbook of Experimental Immunology*. Blackwell Scientific Publication, Oxford, pp. 608–612.

Imai M, Sugimoto K, Okazaki K, Kida H (1998): Fusion of influenza virus with the endosomal membrane is inhibited by monoclonal antibodies to defined epitopes on the hemagglutinin. *Virus Res*. **53**, 129–139.

Ivanova VT, Yakhno MA, Soláriková L, Stumpa A, Varečková E, Slepushkin AN (1991): Study of antigenic and biological properties of influenza A (H1N1) viruses in the USSR and CzFSR, 1988–1989. *Vopr. Virusol*. **36**, 378–380.

Kida H, Yoden S, Kuwabara M, Yanagawa R (1985): Interference with a conformational change in the HA molecule of influenza virus by antibodies as a possible neutralization mechanism. *Vaccine* **3**, 219–222.

Kostolanský F, Russ G, Mucha V, Styk B (1988): Changes in the influenza virus haemagglutinin at acid pH detected by monoclonal antibodies to glycopolypeptides HA1 and HA2. *Arch. Virol*. **101**, 13–24.

Kostolanský F, Styk B, Russ G (1989): Inhibition of influenza virus haemolytic and haemagglutination activities by monoclonal antibodies to haemagglutinin glycopolypeptides HA1 and HA2. *Acta Virol.* **33**, 504–512.

Kostolanský F, Mucha V, Betáková T, Varečková E (1994): Antibody response to hidden epitope of influenza A haemagglutinin elicited by anti-idiotypic antibodies. *Acta. Virol*. **38**, 215–222.

Kostolanský F, Varečková E, Betáková T, Mucha V, Russ G, Wharton SA (2000): The strong positive correlation between effective affinity and infectivity neutralization of highly cross-reactive monoclonal antibody IIB4, which recognizes antigenic site B on influenza A virus haemagglutinin. *J. Gen. Virol.* **81**, 1727–1735.

Kostolanský F, Mucha V, Slováková R, Varečková E (2002): Natural influenza A virus infection of mice elicits strong antibody response to HA2 glycopolypeptide. *Acta Virol*. **46**, 229–236.

Nobusawa E, Aoyama T, Kato H, Suzuki Y, Tateno Y, Nakajima K (1991): Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. *Virology* **182**, 475–485.

Okuno Y, Isegawa Y, Sasao F, Ueda S (1993): A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *J. Virol*. **67**, 2552–2558.

Russ G, Varečková E, Styk B (1974): Steric effects in the reaction of influenza virus neuraminidase with antibodies. *Acta Virol*. **18**, 299–306.

Russ G, Styk B, Poláková K (1978): Antigenic glycopolypeptides HA1 and HA2 of influenza virus haemagglutinin. II. Reactivity with rabbit sera against intact virus and purified undissociated haemagglutinin. *Acta Virol*. **31**, 374–386.

Russ G, Poláková K, Kostolanský F, Styk B,Vančíková M (1987): Monoclonal antibodies to glycopeptides HA1 and HA2 of influenza virus hemagglutinin. *Acta Virol*. **31**, 374–386.

Sánchez-Fauquier A, Villanueva N, Melero JA (1987): Isolation of cross-reactive, subtype-specific monoclonal antibodies against influenza virus HA1 and HA2 hemagglutinin subunits. *Arch. Virol.* **97**, 251– 65.

Skehel JJ, Wiley DC (2000): Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu. Rev. Biochem*. **69**, 531–569.

Styk B, Russ G, Poláková K (1979): Antigenic glycopolypeptides HA1 and HA2 of influenza virus haemagglutinin. III. Reactivity with human convalescent sera. *Acta Virol*. **23**, 1–8.

Vanlandschoot P, Beirnaert ER, Barrere B, Calder L, Millar B, Wharton S, Min Jou W, Fiers W (1998): An antibody which binds to the membrane-proximal end of influenza virus haemagglutinin (H3 subtype) inhibits the low-pH-induced conformational change and cell-cell fusion but does not neutralize virus. *J. Gen. Virol*. **79**, 1781–1791.

Varečková E, Mucha V, Čiampor F, Betáková T, Russ G (1993): Monoclonal antibodies demonstrate accessible HA2 epitopes in minor subpopulation of native influenza virus haemagglutinin molecules. *Arch. Virol.* **130**, 45–56.

Varečková E, Betáková T, Mucha V, Soláriková L, Kostolanský F, Waris M, Russ G (1995): Preparation of monoclonal antibodies for the diagnosis of influenza A infection using different immunisation protocols. *J. Immunol. Methods* **180**, 107–116.

Varečková E, Cox N, Klimov A (2002): Evaluation of the subtype specificity of monoclonal antibodies raised against H1 and H3 subtypes of human influenza A virus hemagglutinins. *J. Clin. Microbiol*. **40**, 2220–2223.

Varečková E, Mucha V, Wharton SA, Kostolanský F (2003): Inhibition of fusion activity of influenza A haemagglutinin mediated by HA2-specific monoclonal antibodies. *Arch. Virol*. **148**, 469–486.

Wharton SA, Skehel JJ, Wiley DC (1986):. Studies of influenza haemagglutinin-mediated membrane fusion. *Virology* **149**, 27–35.

Yewdell JW, Taylor A, Yellen A, Caton A, Gerhard W, Bächi T (1993): Mutations in or near the fusion peptide of the influenza virus hemagglutinin affect an antigenic site in the globular region. *J. Virol.* **67**, 933–942.