

**Prevention and treatment of bronchopneumonia in mice caused by mouse-adapted variant of avian H5N2 influenza A virus using monoclonal antibody against conserved epitope in the HA stem region**

Brief Report

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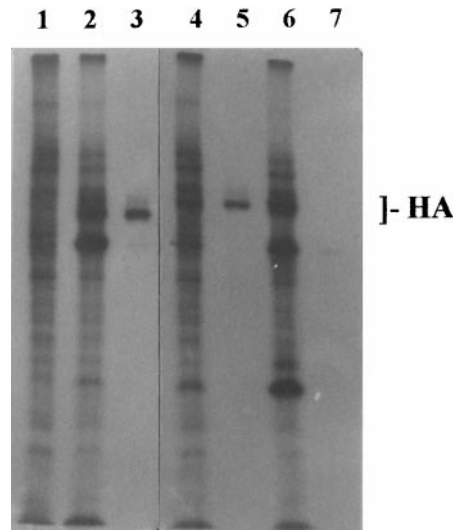
**Summary.** The effects of monoclonal antibody (MAb) C179 recognizing a conformational epitope in the middle of the hemagglutinine (HA) stem region were examined in a mouse model in the experiments of prevention and treatment of lethal bronchopneumonia caused by influenza A virus of H5 subtype. To model the lethal infection, avian nonpathogenic strain A/mallard duck/Pennsylvania/10218/84 (H5N2) was adapted to mice. This resulted in highly pathogenic pneumovirulent mouse-adapted (MA) variant, which was characterized. Three amino acid changes were found in the HA1 subunit of HA of MA virus. One of these was located inside the region of the conformational epitope recognized by MAb C179. However, this substitution was not significant for the recognition of HA and virus neutralization by MAb C179 in vitro and in vivo. Intraperitoneal administration of two different concentrations of MAb C179 one day before or two days after the virus challenge significantly decreased mortality rate. These results suggest that MAb C179 is efficient not only in the prevention and treatment of H1 and H2 influenza virus bronchopneumonia, as was reported previously, but also of H5-induced bronchopneumonia as well, and demonstrate in vivo the existence of a common neutralizing epitope in the HAs of these three subtypes.

Protective immunity toward influenza virus infection is mainly based on the presence of neutralizing antibodies. The viral envelope glycoprotein hemagglutinin (HA) is the major virus neutralization-inducing moiety of influenza virus [9]. The antibody binding sites of influenza A virus HA are located predominantly in the HA1 globular domain [20]. The amino acid sequences of these regions are extremely variable not only among different subtypes of HA, but also within a single subtype. On the other hand, some conserved and cross-reactive antigenic determinants were observed in the HA2 subunit of the HA [4, 6, 12, 19]. It may be supposed that highly conserved antigenic sites on different HA molecules induce neutralizing antibodies to different HA subtypes. Furthermore, Okuno et al. [10, 11] find and described a conformational antigenic epitope in the middle of the stem region of HA of H2 subtype consisting of two regions (aa 318–322 of the HA1 – A region, and aa 47–58 of the HA2 – B region), which was conserved among HAs of all studied viruses belonging of H1 and H2 subtypes. Monoclonal antibody C179 recognizing this epitope possessed unique broad cross-neutralizing activity among H1 and H2 influenza viruses [10], and was effective for prevention and treatment of experimental bronchopneumonia caused by viruses of these subtypes in mice [8, 11]. Recently, we described the existence of this conformational epitope in HA of H5 and H6 subtypes too [17]. After the outbreak caused by H5 influenza virus in Hong Kong in 1997, which clearly demonstrate the possibility of insertion of new influenza A virus subtypes into human population [1, 3, 18], such conserved cross-neutralizing determinants in HA may acquire significance as potential target for urgent prevention and treatment measures.

In the present study we have examined the interaction of MAb C179 with H5 influenza virus in animal model. The main aims of this study were to demonstrate in the experiments *in vivo* the existence of conformational neutralizing epitope in HA of H5 subtype, and to determine the efficacy of MAb C179 for prevention and treatment of experimental H5 influenza virus-induced bronchopneumonia in mice.

Avian non-pathogenic strain A/mallard duck/Pennsylvania/10218/84 (H5N2) (mld/PA/84) was kindly provided by Dr. R. G. Webster. Highly pathogenic pneumovirulent mouse-adapted (MA) variant of this strain (mld/PA/84-MA) was obtained by 23 serial lung-to-lung passages and isolated by one passage in embryonated chicken eggs [7]. MA variant caused bronchopneumonia in mice with high mortality rate:  $\log_{10}$  of LD<sub>50</sub> for mld/PA/84-MA, determined by the method described previously [14], was equal to 6.0/0.1 ml. Original strain mld/PA/84 did not cause illness and mortality in infected mice. The HA gene of mld/PA/84-MA were sequenced and translated as described previously [17] (GenBank accession number AF 100179). Comparison of amino acid sequences of HA between original strain mld/PA/84 and MA variant revealed three amino acids substitutions: S<sub>203</sub>→F, E<sub>273</sub>→G and L<sub>273</sub>→P (H3 numbering system [21]). One of them at position 320 is located in the A region (aa 318–322) of the conformational epitope recognized by MAb C179.

MAb C179 was generously supplied by Takara Shuzo Co. Ltd., Biotechnology Research Laboratories, Japan as standard preparation of purified ascitic fluid



**Fig. 1.** Radioimmunoprecipitation assay of mld/PA/84-MA with Mab C179. Virus-infected MDCK cells were labeled with [ $^{35}$ S] methionine (50  $\mu$ Ci/ml) at 4.5 h post infection for 1 h. After that the cells were scraped off and suspended in STE buffer (pH 7.4). The cell suspension was centrifuged and disrupted in RIPA buffer. One third of cell lysates were subjected directly to polyacrylamide gel electrophoresis (PAGE) and the remainder was used in RIPA. PAGE and RIPA were carried out as described previously [17]. 1 Mock-infected cells control; 2, 4, 6 lysates of cells infected with black dk/NJ/78-MA (H2N3) (positive control), mld/PA/84-MA (H2N2) and FPV/Weybr./27 (H7N7) (negative control); 3, 5, 7 HAs of black d/NJ/78-MA, mld/PA/84-MA, and FPV/Weybr./27 respectively precipitated with MAb C179

with MAb concentration 10 mg/ml. Before the studies in animals mld/PA/84-MA virus was tested with MAb C179 in radioimmunoprecipitation assay (RIPA) and cytopathic effect (CPE) neutralization test in MDCK cells, which were carried out according to the methods described previously [17]. MA variant of avian H2 strain A/black duck/New Jersey/1580/78-MA (H2N3) (black dk/NJ/78-MA) reacting with MAb C179 [7, 8] was chosen as positive control in these experiments. A/FPV/Weybrige/27 (H7N7) (FPV/Weybr./27) phylogenetically distant from H2 and H5 viruses and not reacting with MAb C179 [17] was used as negative control. The results of RIPA are shown in Fig. 1. MAb C179 precipitated the H5 HA as efficiently as the H2 HA and failed to react with H7 subtype strain, confirming the specificity of the precipitation. The results of RIPA correlate with CPE neutralization test (Table 1). MAb C179 in different concentrations neutralized the CPE caused by H5 and H2 strains. As expected, the MAb did not influence the infectivity of H7 virus, confirming the specificity of virus neutralization. These results show that MAb C179 recognizes HA and neutralizes the infectivity of mld/PA/84-MA in experiments in vitro. These data allowed us to perform further studies of MAb C179 in mice.

Four-week-old unbred albino mice were inoculated intraperitoneally with 100  $\mu$ l of undiluted ascitic fluid (1000  $\mu$ g of C179 per mouse) or with 100  $\mu$ l of appropriate dilution of MAb in sterile 0.9% NaCl solution 24 h before

**Table 1.** Infectivity neutralization test of mld/PA/84-MA with MAb C179

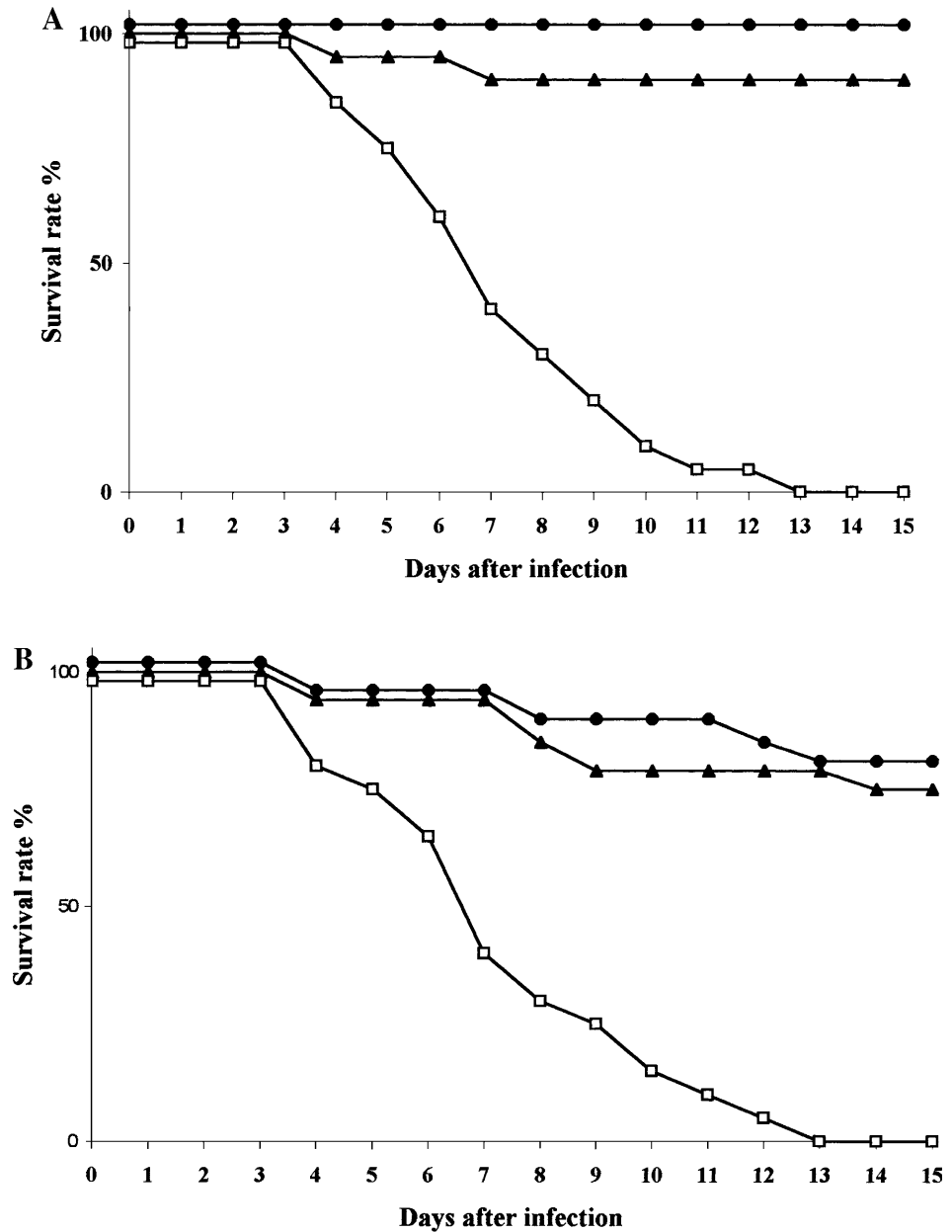
	Concentration of MAb C179 (µg/ml)			
	100.0	10.0	1.0	0.5
Viruses	100.0	10.0	1.0	0.5
black dk/NJ/78-MA (H2N3)	≥1.875	1.5	0.5	ND
mld/PA/84-MA (H5N2)	≥2.125	≥2.125	1.125	0.5
FPV/Weybr./27 (H7N7)	0	0	0	ND

Different concentrations of MAb C179 were mixed with 100 TCID<sub>50</sub> of viruses and incubated at 37 °C for 45 min. Subsequently, the virus-MAb mixtures were serially 10-fold diluted in DMEM supplemented with 0.2% bovine serum albumin and 1 µg/ml trypsin, and added to a confluent monolayer of MDCK cells in 96-well flat bottom microtiter plates. After incubation at 37 °C in 5% CO<sub>2</sub> for 72 h, the cells were checked for CPE and the TCID<sub>50</sub> titers were determined. The results presented as differences between log<sub>10</sub> values of TCID<sub>50</sub> obtained with the concentration of MAb indicated and with the control. *ND* Not done

(prevention) or 48 h after (treatment) the virus challenge. Control groups received intraperitoneally 100 µl of sterile 0.9% NaCl solution alone. To confirm specific action of MAb C179 additional control groups of mice were inoculated intraperitoneally with 100 µl of mouse ascitic fluid without MAbs 24 h before or 48 h after the virus challenge. Ether anaesthetized albino mice were infected by intranasal inoculation with 50 µl of PBS-diluted allantoic fluid contained 10LD<sub>50</sub> of mld/PA/84-MA. Effects of MAb C179 for prevention and treatment of lethal bronchopneumonia were estimated on the basis of differences in survival rates calculated for groups of animals having received different concentrations of MAb and control groups after an observation period of 15 days.

The survival rates in mice, which received MAb C179 a day before the challenge with mld/PA/84-MA, are shown in Fig. 2A. A total of 70 mice were randomly divided into four groups: three of 20 mice and one of 10. Two experimental groups received 1000 and 100 µg of C179 per mouse, respectively. The control groups received 0.9% NaCl solution (20 animals) or mouse ascitic fluid (10 animals). The survival rate in the group of mice that received 1000 µg of MAb was 100%. In the group that received 100 µg of MAb C179 survival rate was 90%. Mortality in the group treated with 0.9% NaCl solution as well as with mouse ascitic fluid (data not shown) was equal 100% at the thirteenth day after infection. The statistical evaluation of the results presented in Fig. 2A by sign test, an unparametrical statistical test [5], revealed that the differences in the survival rates between the groups of mice injected with MAb (1000 or 100 µg) and the control groups was significant at 0.01 level. These results indicate that MAb C179 efficiently protected mice from lethal infection with mld/PA/84-MA.

The therapeutical effect of MAb on the infection of mice caused by H5N2-MA virus is shown in Fig. 2B. Three groups of 20 mice were injected with 1000 or 100 µg of MAb C179 or with 0.9% NaCl solution (control) two days after the virus infection. Additional control groups of 10 animals received mouse



**Fig. 2.** Survival rates in mice received MAb C179 24h before (A) or 48h after (B) the challenge with mld/PA84-MA (H5N2). Four-week-old albino mice were inoculated intraperitoneally with 1000.0 (-●-) or 100.0 (-▲-) µg of MAb per mouse. Control groups (-□-) received sterile 0.9% NaCl solution

ascitic fluid without MABs at the same time (data not shown). Survival rates in mice received 1000 and 100 µg of C179 were equal 80 and 75% respectively. In both control groups 100% mortality rate was observed. The differences in the survival rates among the groups of mice that received 1000 and 100 µg of MAB and the control groups were significant at 0.01 level. These data show that C179

is effective for treatment of H5-induced pneumonia. The administration of MAb C179 significantly decreases mortality in animals even when infection is initiated.

The mechanism underlying the action of MAb C179 is known in general [10, 11]. The binding of this MAb with the conformational epitope in the middle of the HA stem region, most likely, prevents the low pH-induced conformational changes in HA molecule and inhibits the fusion activity of HA. In vivo experiments for prevention of lethal pneumonia caused by H1 influenza virus in mice revealed that MAb C179 does not prevent the initiation of viral infection, however it efficiently inhibits the spread of infection in mouse lungs [11]. These results correlate with the location of haemorrhagic lesions in the lungs of infected mice observed in our previous [8] and present studies. The lesions in the lungs of mice that received 1000 or 100  $\mu\text{g}$  of MAb C179 a day before the virus challenge were localized in small areas only at the sites adjacent to the bronchi, while in the infected control group those were spread all over the lungs (data not shown).

The amino acid sequences of A and B regions forming conformational epitope in HA of H1 and H2 viruses studied previously in mice model with MAb C179 [8, 11], and H5 strains are presented in Table 2. These parts of HA molecule are highly conserved among the presented subtypes. MAb C179 was obtained against HA of human H2N2 strain, and it was shown that amino acid sequences of A and B regions are identical among human H1 and H2 viruses [10]. The MA-variant of avian strain black dk/NJ/78-MA (H2N3) possesses one amino acid replacement

**Table 2.** Comparison of amino acid sequences of A and B regions forming the conformational epitope in the HA stem region among H1, H2 and H5 strains

Viruses	Amino acid positions			
	HA1 A region		HA2 B region	
	<b>318</b>	<b>322</b>	<b>47</b>	<b>58</b>
FM/1/47 (H1N1)	TGLRN		GITNKVNSVIEK	
USSR/90/77-MA (H1N1)	TGLRN		GITNKVNSVIEK	
black dk/NJ/78-MA (H2N3)	IGLRN		GITNKVNSVIEK	
mald/PA/84-MA (H5N2)	TGPRN		GITNKVNSIIDK	
mald/PA/84 (H5N2)	TGLRN		GITNKVNSIIDK	
tern/SA/61 (H5N3)	TGLRN		GITNKVNSIIDK	
Hong Kong/97 (H5N1)	TGLRN		GVTNKVNSIINK	

The HA gene of mld/PA/84-MA was sequenced and submitted to GenBank under accession number AF100179. Published sequences: A/FM/1/47 (FM/1/47) [16], A/USSR/90/77 [2] (sequences of these regions in HA of USSR/90/77-MA is identical [22]), A/mallard duck/Pennsylvania/10218/84 (parental, avirulent for mice strain) [17], A/tern/South Africa/61 [13], and A/Hong Kong/156/97 [1]. Sequence of HA of A/black duck/ NJ/1580/78-MA is a personal communication of E. A. Govorkova and N. V. Makarova

in A region (T<sub>318</sub>→I) deffering the MA strain from the parental virus and other H2 viruses. This amino acid change in HA of avian H2-MA virus is a result of adaptation to mice. It had no effect upon the reaction of MAb C179 with black dk/NJ/78-MA in vitro (Fig. 1; Table 1) and in vivo [8]. In the case of H5 virus mld/PA/84-MA there are three amino acid differences (one in A region and two in B region) which differ the epitope from that in H1 and H2 viruses (Table 2). Two amino acid replacements in B region (V<sub>55</sub>→I and E<sub>57</sub>→D) are typical for most of avian H5 strains (Table 2) and do not influence the recognition of H5 HA by MAb C179 [17]. The HA of avian-like H5 viruses isolated from humans in 1997 in Hong Kong possess two substitutions in B region (I<sub>48</sub>→V and D<sub>57</sub>→N) which differ this part of molecule from that in other avian H5 strains (Table 2). The amino acid substitution in A region at residue 320 (L→P) is characteristic of H5 HA of MA strain as a result of adaptation to mice. The A region in the HA of the original virus mld/PA/84 as well as in other H5 viruses is identical to that in H1 and H2 strains (Table 2). Two other substitutions (S<sub>203</sub>→F, E<sub>273</sub>→G) in HA of H5 MA virus also do not influence the interaction of MAb with the epitope. These results indicate that conserved conformational epitope in the middle of HA stem region of H5 and avian H2 strains may be changed in the course of virus adaptation to new host. It is possible to assume that these changes in HA during adaptation of these avian strains to mice are result of selection by the organism of new host and lead to the acquisition of virulence. These changes have no effect on the recognition of HA and viral neutralization by MAb C179.

The results of the present study confirm our data that HAs of H5, H1 and H2 influenza A viruses possess common neutralizing epitope [17]. MAb C179 directed to this epitope is efficient in the prevention and treatment of lethal influenza virus bronchopneumonia caused by H5 virus in mice as well as the one caused by H1 and H2 viruses [8, 11]. Present results together with previously reported data about cross-neutralizing epitope and MAb C179 [8, 10, 11, 15, 17] make it possible to suppose that this highly conserved determinant in HA might be unique potential target for prevention and treatment of influenza A virus infection caused at least by H1, H2 and H5 strains. A search of such highly conserved antigenic and cross-neutralizing epitops among phylogenetically closed subtypes of influenza A viruses might be useful approach for development of vaccines and antiviral drugs of broad activity.

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