AN EPITOPE SHARED BY THE HEMAGGLUTININS OF H1, H2, H5, AND H6 SUBTYPES OF INFLUENZA A VIRUS

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Summary. – The membrane-inserted hemagglutinin (HA) is the most variable protein of influenza viruses. Here we describe the characterization of a shared epitope in the HA of influenza A virus H1, H2, and H5 subtypes which were completely neutralized by a monoclonal antibody (MAb), directed against this epitope. This MAb (C179) also efficiently precipitated the HAs of these viruses. In addition, MAb C179 did not neutralize H6 subtype strains despite complete amino acid homology of the epitope regions. Furthermore, only the non-glycosylated form of the HA of one of the H6 subtype strains could be precipitated by the MAb. The conformational epitope may be masked by glycosylation, although it could not be excluded that differences in the primary amino acid sequence may cause the decreased accessibility of the epitope in H6 subtype strains.

Introduction

Influenza viruses manage to cause yearly epidemics by their ability to change their antigenic makeup. The cumulative nature of these alterations results in a virus that will eventually evade the neutralizing antibodies in the human population, a mechanism referred to as antigenic drift. A more drastic change is complete replacement of surface glycoproteins by those of another subtype of influenza virus. Such an „antigenic shift" may result in the introduction of a new subtype of virus in a completely unprotected population (Murphy and Webster, 1990).

Protective immunity towards influenza virus infection is mainly based on the presence of neutralizing antibodies. The major virus neutralization-inducing moiety of an influenza virus is its HA. This viral membrane glycoprotein undergoes frequent antigenic changes under selective pressure of antibodies present in the population. Molecular studies suggest that the antibody binding sites are located predominantly in the HA1 globular domain (Wiley and Skehel, 1987). The amino acid sequences of these sites are highly variable not only among different subtypes of HA but also within a single subtype. On the other hand, some conserved amino acid sequences and cross-reactive antigenic determinants were observed in the HA2 subunit of the HA (Graves *et al.*, 1983; Laver *et al.*, 1980; Raymond *et al.*, 1986; Verhoeyen *et al.*, 1980). It may be speculated that highly conserved antigenic sites on different HA molecules induce neutralizing antibodies to different subtypes of influenza viruses. Furthermore, Okuno *et al.* (1993, 1994) generated and characterized a MAb directed against the HA of a virus strain belonging to H2 subtype that exhibited unique cross-neutralizing activity. This MAb, designated C179, neutralized all examined human influenza virus strains of the H1 and H2 subtypes. It was shown that a conformational epitope in the middle of the stem region of the HA that consists of two regions, aa 318-322 of the

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Abbreviations: CPE = cytopathic effect; DMEM = Dulbecco's Modified Eagle's Medium; HA = hemagglutinin; MAb = monoclonal antibody; PAGE = polyacrylamide gel electrophoresis

HA1 subunit (A region) and aa 47-58 of the HA2 subunit (B region). Both these regions were found to be conserved among the HAs of human influenza A viruses of the H1 and H2 subtypes (Okuno *et al.*, 1993; Sagawa *et al.*, 1996).

In the present study, we have further evaluated this cross-reactivity beyond the H1 and H2 subtypes. Determination of sequences and subsequent phylogenetic analyses of the different HAs revealed that influenza viruses of the H5 and H6 subtypes were most closely related to those of the H1 and H2 subtypes (Air, 1981; Nobusawa *et al.*, 1991). On the basis of biological studies using MAb C179 and sequence analyses we determined the conservation of the conformational epitope using avian influenza A viruses of the H5 and H6 subtypes.

Materials and Methods

Viruses. Human and avian influenza A virus strains A/Japan/305/57xA/Bel/42 (H2N1) (Jap/57), A/Black duck/NJ/1580/78 (H2N3) (Black dk/NJ/78), A/Tern/South Africa/61 (H5N3) (Tern/SA/61), A/Mallard duck/NY/189/82 (H5N2) (Mal. dk/NY/82), A/Mallard duck/PA/10218/84 (H5N2) (Mal. dk/PA/84), A/Mallard duck/Alberta/211/85 (H6N2) (Mal. dk/Alb/85), A/Shearwater/Australia/1/72 (H6N5) (Shearw/Austral./72), and A/FPV/Weybridge/27 (H7N7) (FPV/Weybr/27) were used in this study. The H2 subtype strains were included as positive controls as they were known to react with the MAb. The virus of the H7 subtype represented a negative control as this subtype was phylogenetically distant from the cluster of H1, H2, H5, and H6 subtype viruses (Air, 1981; Nobusawa *et al* , 1991). The viruses were propagated in the allantoic cavity of nine-day-old embryonated chicken eggs at 37°C for 48, 35 (Tern/SA/61) or 24 (FPV/Weybr./27) hrs. The allantoic fluids were harvested and used as virus stocks in the experiments.

Neutralization of virus infectivity. MAb C179 was a generous gift from Biotechnology Research Laboratories, Takara Shuzo Co. Ltd, Otsu, Shiga, Japan. It was used for neutralization of infectivity of the influenza A virus strains of the H5 and H6 subtype according to a routine test based on inhibition of the cytopathic effect (CPE) in MDCK cells (Lipatov *et al.*, 1996). The virus stocks were diluted in Dulbecco's Modified Eagle's Medium (DMEM) to obtain a virus dose of 100 $TCID_{50}$ (approximately 70 PFU) in 1.0 ml. MAbs in concentrations of 100.0 μg/ml, 10.0 μg/ml, 1.0 μg/ml, and 0.5 μg/ml were mixed with 100 $TCID_{50}$ of virus and incubated at 37°C for 45 mins. Subsequently, the virus-MAb mixtures were serially 10-fold diluted in DMEM supplemented with 0.2% bovine serum albumin and 1.0 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_2 for 72 hrs, the cells were checked for CPE and the $TCID_{50}$ titers were determined.

Radio-immunoprecipitation assay (RIPA). MDCK cells were cultured in DMEM with 10% of fetal bovine serum, washed twice and infected with virus-containing allantoic fluid at multiplicity of infection of 15–20 PFU/cell. After adsorption of the virus for 45 mins at room temperature, the cells were washed twice with DMEM and incubated at 37°C. After 4.5 hrs, the medium was removed, the cells were washed twice with RPMI medium without methionine, and 50 μCi/ml [^{35}S]methionine (25 μCi per culture) in RPMI was added and incubated for 30 mins (pulse-labeling) and 1 hr (total labeling). The pulse-labeled cells were washed twice with DMEM containing an excess of unlabeled methionine and were further incubated for 30, 60, and 90 mins (chase), respectively. Next, the cells were washed with STE buffer pH 7.4, scraped off and resuspended in STE buffer. The cell suspension was centrifuged at 11,000 rpm for 1 min and disrupted in RIPA buffer (Dantas *et al.*, 1986). One third of the cell lysates were subjected to polyacrylamide gel electrophoresis (PAGE) and the remainder was used in RIPA.

RIPA was carried out essentially as described by Dantas *et al.* (1986) with a minor modification: cell lysates were incubated with 2.0 μl of MAb C179 for 30 mins at 0°C, *Staphylococcus aureus* A antigen was used instead of protein A-Sepharose, and the precipitation was carried out for 30 mins at 0°C. Cell lysates and precipitates were subjected to PAGE in the presence of sodium dodecyl sulfate (SDS, SDS-PAGE) in 15% gels at 15 mA for 6 hrs according to Laemmli (1970). The gels were placed in 11% sodium salycilate for 30 mins, dried and exposed to X-ray film for autoradiography.

Tunicamycin (Sigma) was used to inhibit the glycosylation of the HA (Nakamura and Compans, 1978). The viruses were grown in MDCK cells and labeled with [^{35}S]methionine for 30 mins at 4.5–5 hrs after infection (pulse) and further incubated for 1.5 hr with the medium containing an excess of unlabeled methionine (chase). Tunicamycin (1.0 μg/ml) was added at 2 hrs after infection (Orlich *et al.*, 1990). The labeled polypeptides were further examined in RIPA and PAGE as described above.

Nucleotide sequence analysis. Extraction of total RNA and subsequent amplification of viral RNA by reverse transcription-polymerase chain reaction (RT-PCR) were carried out as described previously (Claas *et al.*, 1993). In brief, total RNA was extracted from virus-containing allantoic fluid with a guanidine isothiocyanate solution and collected by precipitation with isopropanol. The viral RNA was then amplified with oligonucleotide primers that were selected from a consensus sequence of previously published sequences. The amplified products were subjected to nucleotide sequence analysis by cycle sequencing with a Thermo Sequenase™ Dye Terminator Cycle Sequencing Pre-Mix Kit (Amersham Life Sciences). Sequences of the HAs of the H5 (A/Mal.dk/PA/84) and H6 (A/Mal.dk/Alb/85) subtypes have been submitted to GenBank (accession numbers AF100180 and AF 100181, respectively). The nucleotide sequences were converted to amino acid sequences and aligned according to Kawaoka *et al.* (1984) and Feldmann *et al.* (1988) with previously published sequences and sequences kindly provided by Drs. E.A. Govorkova and N.V. Makarova, the D.I. Ivanovsky Institute of Virology, using the Wisconsin GCG Package.

Results

Neutralization of infectivity

Results of the infectivity neutralization assay are shown in Table 1. MAb C179 in different concentrations neutralized the CPE caused by both the H2 strains. In addition, this

Table 1. Infectivity neutralization test of strains of H2, H5, and H6 subtypes of influenza A virus with MAb C179

Strains	Concentration of MAb C179 (μg/ml)			
	100.0	10.0	1.0	0.5
Jap/57 (H2N1)	≥ 2 375*	2.25	0.375	ND
Black dk/NJ/78 (H2N3)	≥ 1.875	1.25	0.625	ND
Mal. dk/NY/82 (H5N2)	≥ 1 5	≥ 1.5	0.75	ND
Mal. dk/PA/84 (H5N2)	≥ 2.125	≥ 2.125	1.125	0.5
Tern/SA/61 (H5N3)	≥ 1.25	≥ 1.25	0.875	0.625
Mal. dk/Alb./85 (H6N2)	0	0	0	0
Shearw./Austral/72 (H6N5)	0.5	0.375	0.25	ND
FPV/Weybr./27 (H7N7)	0	0	0	ND

*Difference between log values of $TCID_{50}$ obtained with the concentration of MAb indicated and with the control.
ND = not done.

MAb efficiently neutralized all the three H5 strains. However, the neutralizing effect of the MAb on the infectivity of the H6 Shearw./Austral./72 strain was significantly lower, and no effect was observed with the H6 Mal.dk/Alb/85 strain. As expected, the MAb did not influence the infectivity of the H7 FPV/Weybr./27 strain, confirming the specificity of the virus neutralization.

RIPA

The results of the neutralization of subtype H2 and H5 strains were confirmed by RIPA. MAb C179 precipitated the H5 HA as efficiently as the H2 HA (Fig. 1A). The H6 HA of Mal. dk/Alb./85 strain, however, was precipitated less efficiently as shown in Fig. 1B. The Shearw./Austral./72 H6 HA was not precipitated at all. MAb C179 also failed to react with the H7 subtype strain, confirming the specificity of precipitation. The autoradiograph of precipitated virus-specific proteins revealed that, in general, the HA consisted of two somewhat diffuse bands. MAb C179 precipitated both HA bands of influenza virus strains of the H2 and H5 subtypes. In the case of the H6 HA of Mal. dk/Alb./85 strain, which was presented as one diffuse band, the MAb reacted only with the lower part of this band (Fig. 1B). Two HA bands after PAGE described previously (Sklyanskaya *et al.*, 1980) reflect the presence of an immature form of the HA in addition to its mature form. The HA of the H6 subtype was precipitated by MAb C179 when pulse-labeled for 30 mins and chased for 30 mins. However, after 60 mins of chase, the HA was precipitated less efficiently than the just pulse-labeled HA. In the course of further maturation (90 mins of chase) MAb C179 no longer precipitated the H6 HA (Fig. 2A). On the other hand, the precipitability of the H5 HA, labeled under the same conditions as the H6 HA, was not changed: Fig. 2B shows that the HA of Tern/SA/61 strain after a 90 mins chase was precipitated as efficiently corresponding as pulse-labeled HA. A part of the H5 HA of Tern/SA/61 strain, processed in MDCK cells, was cleaved. MAb C179 precipitated uncleaved HA as well as HA1 and HA2 (Fig. 2B).

Glycosylation is an important feature of the maturation of glycoproteins. The influence of glycosylation was investigated in a pulse-chase labeling experiment with Mal. dk/Alb./85 strain. In the presence of tunicamycin, an inhibitor of glycosylation, virus-specific H6 HA protein could be observed in a RIPA with MAb C179 (Fig. 3). However, without tunicamycin, the H6 HA was not precipitated (Fig. 2A). The H6 HA of the Shearw./Austral./72 strain that was not precipitated when labeled without tunicamycin (Fig. 1B) was also not precipitated by MAb C179 in the presence of tunicamycin (data not shown).

Sequence analyses

The conformational epitope recognized by MAb C179 was shown to contain two regions (Okuno *et al.*, 1993): aa 318–322 in the HA1 subunit (A region) and aa 47-58 in the HA2 subunit (B region) (H3 numbering system, Wilson *et al.* (1981). Comparison of the amino acid sequences of the A and B regions in 13 subtypes of HA revealed that in H5 and H6 subtypes they were very similar to those in human H2 and H1 subtypes (Nobusawa *et al.*, 1991; Rohm *et al.*, 1995). Nucleotide sequencing of the HAs of the strains used in this study revealed identical sequences in the A region of the H1, H2, H5, and H6 subtypes, while two amino acid differences were observed in the H7 subtype (Table 2). The B region of the avian H2 subtype Black dk/NJ/78 strain differed from the human H2 subtype Jap./57 strain by a Ser (human) to Tyr (avian) mutation at aa 54. In the B region of the H5 and H6 subtypes, two differences at aa 55 (Val→Ile) and 57 (Glu→Asp) were observed in comparison to human H1 and H2 subtypes (Table 2). The B region of the H7 subtype contained five differences as compared to the H1, H2, H5, and H6 subtypes.

The number and location of potential glycosylation sites (Asn-X-Ser/Thr) in the HA proteins of the H1 subtype strains used by Okuno *et al.* (1993) and the H2, H5, and H6 subtype strains from the present study were determined. Eight N-linked potential glycosylation sites were present in the H1 HA, nine in the H2 HA, and seven in the H5 HA. The H6 HA of Shearw./Austral./72 strain contained eight potential glycosylation sites, and that of the Mal. dk/Alb./72 strain seven. Differences were only found in the HA1 subunit of the HA (Fig. 4). Asn_{298} in the HA of H1, H2, and H5 subtypes was not found in the H6 subtype, while Asn_{291} and Asn_{296} were unique for the H6 subtype. Furthermore, Asn_{169} was absent in the H1 and H6 subtypes.

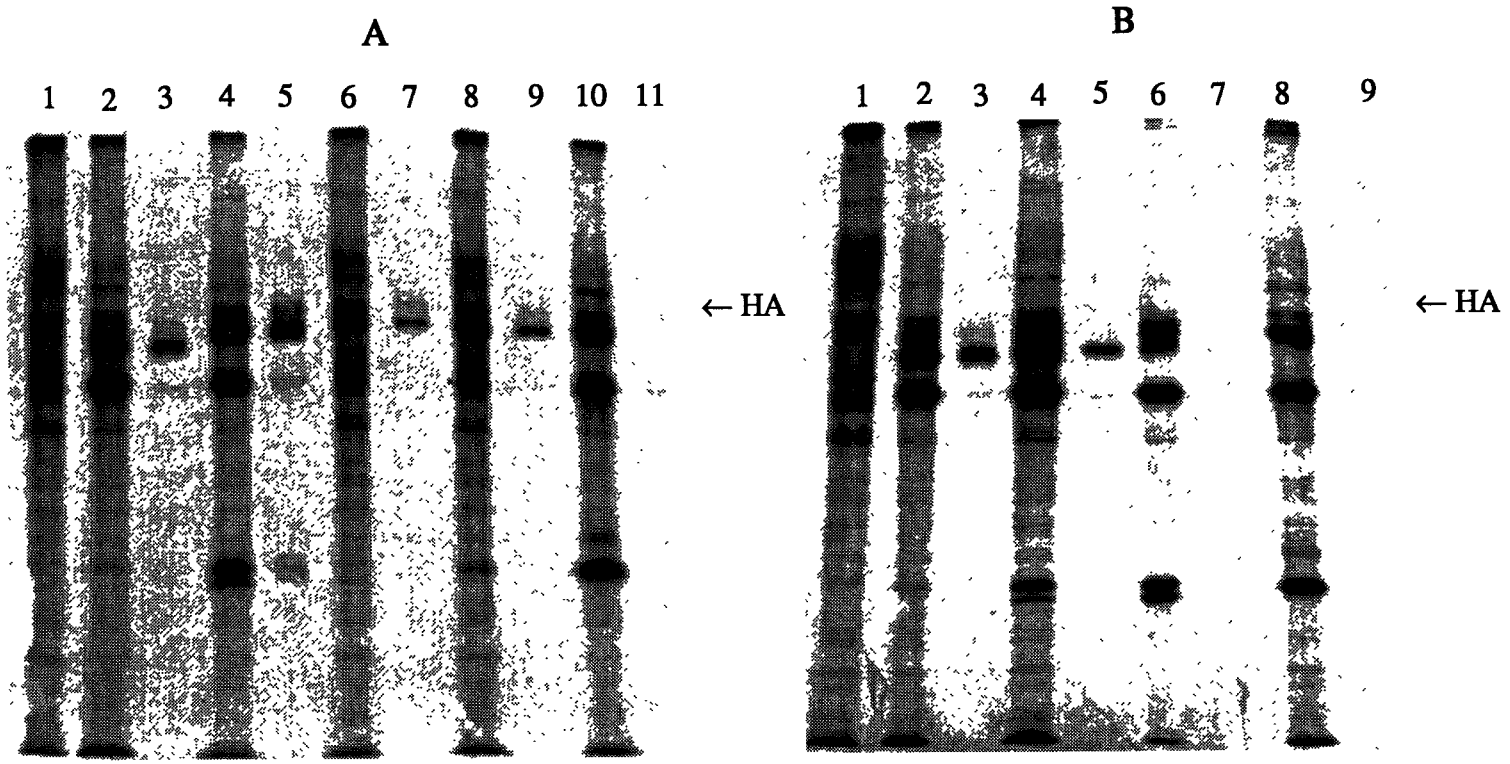

Fig. 1
Autoradiograph of RIPA after PAGE

MAb C179 used in precipitation. **A.** Lane 1, lysate of mock-infected cells (negative control); lanes 2, 4, 6, 8, and 10, lysates of cells infected with strains Black dk/NJ/78 (H2N3) (positive control), Tern/SA/61 (H5N3), Mal. dk/NY/82 (H5N2), Mal. dk/PA/84 (H5N2), and FPV/Weybr./27 (H7N7) (negative control); lanes 3, 5, 7, 9, and 11, HAs of the lysates from lanes 2, 4, 6, 8, and 10. **B.** Lane 1, lysate of mock-infected cells; lanes 2, 4, 6, and 8, lysates of cells infected with strains Black dk/NJ/78 (H2N3) (positive control), Mal. dk/Alb/85 (H6N2), Shearw./Austral./72 (H6N5), and FPV/Weybr./27 (H7N7) (negative control); lanes 3, 5, 7, and 9, HAs of the lysates from lanes 2, 4, 6, and 8.

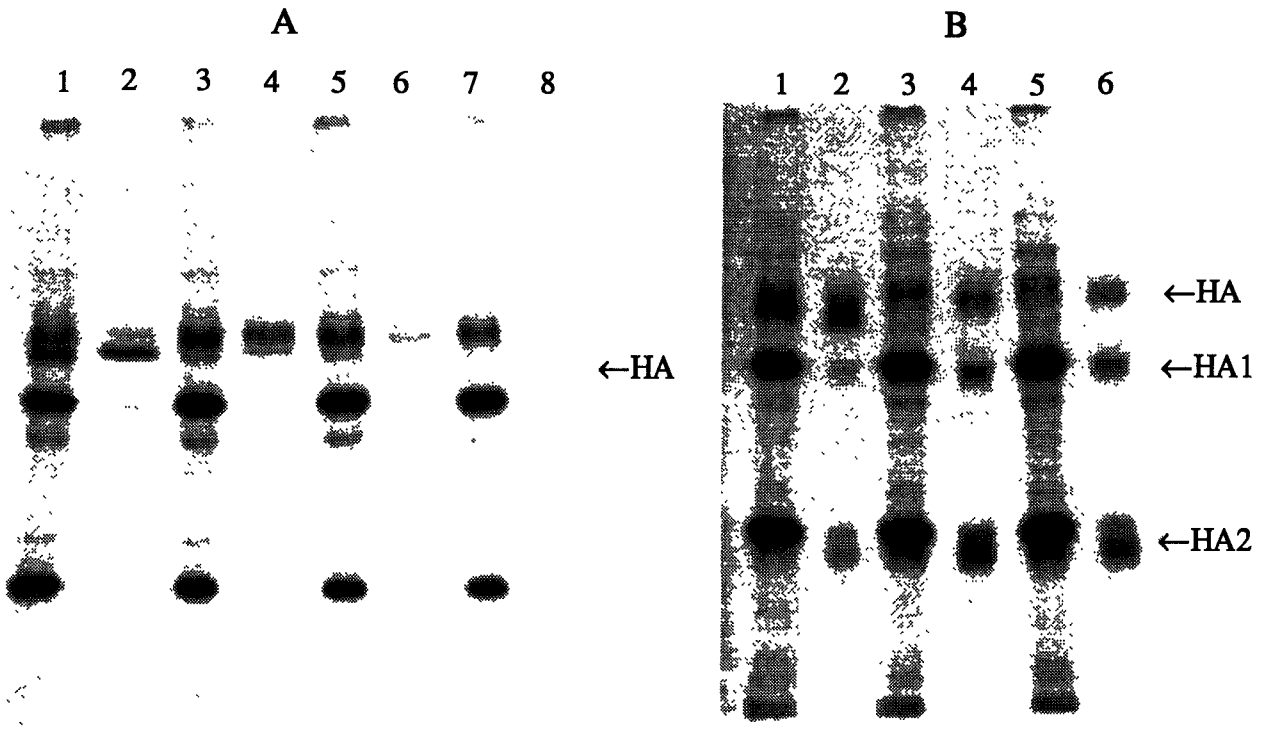

Fig. 2
Autoradiograph of RIPA after PAGE
Precipitation with Mab C179 of HAs of H6 subtype Mal. dk/Alb./85 strain (**A**) and H5 subtype Tern/SA/61 strain (**B**), labeled under condition of pulse-chase. **A.** Lysates of cells infected with Mal. dk/Alb /85 strain after a 30 mins pulse (lane 1) and a 30 mins chase (lane 3), a 60 mins chase (lane 5) or a 90 mins chase (lane 7) HAs from these lysates after a 30 mins pulse (lane 2) and a 30 mins chase (lane 4), a 60 mins chase (lane 6) or a 90 mins chase (lane 8). **B.** Lysates of cells infected with Tern/SA/61 strain after a 30 mins pulse (lane 1) and a 30 mins chase (lane 3) or a 90 mins chase (lane 5). HAs from these lysates after a 30 mins pulse (lane 2) and a 30 mins chase (lane 4) or a 90 mins chase (lane 6).

Discussion

Variability in the HA is the major factor that determines the success of influenza viruses in continuously spreading and causing epidemics. But even in this highly variable protein, MAb C179 recognized a cross-reactive epitope between viruses of the H1 and H2 subtypes. This site appears to be a conformational epitope in the middle of the stem region of the HA molecule (Okuno *et al.*, 1993, 1994).

The neutralization experiments in this paper suggest that a similar conformational epitope is present on influenza A virus of the H5 subtype. Analysing the amino acid sequence revealed that the A and B regions of the epitope are more or less conserved among H1, H2, H5, and H6 subtypes. One amino acid substitution in the B region of the avian H2 HA and two in the B region of the H5 HA do not decrease binding of MAb C179 to the epitope, which is in line with its conformational nature. However, despite complete amino acid homology between the regions of the epitope in the H5 and H6 subtypes, binding of MAb C179 to the latter proved to be less efficient.

The H5 HA was precipitated equally efficiently by MAb C179 as H2 HA in a RIPA. As shown previously by Sklyanskaya *et al.* (1980) and also in our experiments, immature and mature forms of the HA protein can be detected by PAGE and autoradiography. These forms can be observed as two bands or as one diffuse band, where the immature form of HA appears as the lower band or the lower part of the diffuse band. MAb C179 exclusively precipitated the immature form of HA of the H6 subtype Mal. dk/Alb./85 strain. This suggests that the conformational epitope is accessible to MAb C179 in the immature HA only. However, MAb C179 did not precipitate either form of HA of the other H6 subtype strain, Shearw./Austral./72.

It is known that the accessibility of antigenic epitopes can be changed by glycosylation of the HA (Skehel *et al.*, 1984; Munk *et al.*, 1992). When tunicamycin, an inhibitor of glycosylation, was added in the pulse-chase experiment, the HA of Mal. dk/Alb./85 strain was precipitated even after

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