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ANALYSIS OF INTERNAL PROTEINS OF INFLUENZA A (H2N2) VIRUSES ISOLATED FROM BIRDS IN EAST GERMANY IN 1983

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Summary. - Proteins and RNAs of influenza A (H2N2) viruses isolated from birds in 1983 in East Germany were compared antigenically with those of H2N2 human strains. The electrophoretic mobility of the viral proteins and of the S1-treated double-stranded RNAs from two human and six avian strains, as well as the results of EIA-tests using monoclonal antibodies to their matrix protein and nucleoproteins indicate an antigenic relationship between the avian isolates and human strains of H2N2 subtype. One of the avian strains had a reduced amount of matrix protein.

Key words: influenza A/H2N2; internal proteins; antigenic specificity; ELISA; RNA-RNA hybridization

Introduction

The avian population is an enormous reservoir of influenza antigens. This i exceptionally significant because of the direct transmission from species t species and the formation of new antigenic variants by mutation and recomb nation. In the framework of our influenza surveillance programme in animal we isolated a strain group of H2N2 influenza A viruses from mallards whic show characteristics similar to those of human influenza viruses. During thi period many other strains were isolated containing a HA and/or NA usually nc present in human influenza A viruses. Considering that the influenza viruse isolated from mallards seem to have a similar antigenic structure to huma influenza viruses (H2N2), we compared the morphology, composition of poly peptides and antigenic properties of the internal proteins isolated from huma and mallard strains of influenza A viruses.

Previously, some authors using monoclonal antibodies in combination wit the ELISA have shown differences among internal proteins of differer influenza A virus strains (Air *et al.*, 1981; van Wyke *et al.*, 1980 *a,b*; Sonnenber

et al., 1984; Joassin *et al.*, 1987; Bucher *et al.*, 1989). Therefore, we focused our interest to the antigenic specificity of the matrix protein (M1-protein) using monoclonal antibodies in ELISA.

Materials and Methods

Viruses. Influenza A virus strains A/mallard/Potsdam/176/83, A/mallard/Potsdam/177-4/83, A/mallard/Potsdam/177-6/83, A/mallard/Potsdam/178-4/83, A/mallard/Potsdam/178-6/83, and A/mallard/Potsdam/179/83 were isolated from birds in 1983 and subtyped as H2N2. They were compared with human influenza A(H2N2) strains A/Singapore/1/57 and A/Leningrad/549/80.

The haemagglutination inhibition (HI) test was carried out as described by Palmer *et al.* (1975). The neuraminidase inhibition (NI) test was modified according to Aminoff (1961).

Viruses were grown in the allantoic cavity of 11-day -old embryonated hen eggs, concentrated by centrifugation with polyethyleneglycol, and purified by banding in sucrose gradients. The isolates used for our investigation were the subject of no more than 3 passages in embryonated chicken eggs. In contrast to the human strains the strains isolated from birds were propagated in chicken embryos at 42 °C.

The virus concentration was defined according to Peterson (1983). Bovine serum albumin (Sigma) was used as a standard.

Polyclonal hyperimmune rabbit antisera to the matrix protein and to the nucleoprotein were raised in rabbits. Chromatographic purification of the polyclonal antibodies was performed as described by Zagidullin *et al.* (1987).

Monoclonal antibodies to the nucleoprotein were produced and characterized by Busse *et al.* (1988). Monoclonal antibodies to different sites of the influenza A virus matrix protein were kindly provided by Dr. D. Bucher (Dept. of Microbiology and Immunology, Medical College, New York, U.S.A.). Monoclonal antibodies were prepared against the recombinant virus strain (H1N1) X-53a (Bucher *et al.*, 1989).

Polyacrylamide-SDS-gel-electrophoresis was used for comparing the electrophoretic mobility of the proteins from different influenza A virus strains. For our investigation, we used a modification of the polyacrylamide gel electrophoresis technique described by Laemmli (1970).

Analysis of homologous and heterologous RNA-RNA hybrids. Chick embryo fibroblasts were infected with influenza (H2N2) viruses isolated from birds and incubated in the presence of cycloheximide and/^{3}H/-uridine for 3.5 hr at 36 °C. The RNA from these cells was then isolated and hybridized with an excess of unlabelled virion RNA from the virus strain A/mallard/Potsdam/176/83 (H2N2). Resulting RNA-RNA hybrids were treated with S1 nuclease at 36 °C or at 42 °C and examined on a 7.5 % or 4 % polyacrylamide gel (Hay *et al.*, 1977*a*, *b*; 1979).

Western blot. Purified and concentrated viruses separated on 10 % polyacrylamide-SDS gels were transferred to nitrocellulose (0.22 μm pore size) sheets (Towbin *et al.*, 1979) in 25 mmol/l Tris-HCl (pH 8.3) - 192 mmol/l glycine - 20 % ethanol buffer; the electrotransfer was performed for 4 hr at 0.3 A (at 4 °C). The gel was stained with amido black 10B to locate residual peptides. The nitrocellulose sheets were soaked in 10 mmol/l Tris-HCl (pH 7.4) - 150 mmol/l NaCl - 0.01 % bovine serum albumin (TBS-BSA) at 37 °C for 2 hr. The sheets were cut into strips and soaked separatedly in diluted monoclonal antibodies against matrix protein or the nucleoprotein. After the washing procedures and incubating with anti-mouse-conjugate the strips were soaked in the substrate solution [4-chloro-1-naphthol (0.06 % - 20 % ethanol - 0.05 mol/l Tris-buffer, pH 7.4 - 0.012 % H_2O_2]. The reaction was stopped by washing the nitrocellulose strips with aqua dest.

Double antibody sandwich solid phase EIA was performed by using a modification of the procedure described by Voller *et al.* (1977). This test was used for standardizing the concentration of M1-protein or nucleoprotein (NP) (standardization EIA). Chromatographically purified polyclonal antibodies to the M1-protein or to NP were diluted in bicarbonate buffer (pH 9.5) and

adsorbed at 37 °C for 3 hr to polysterene microplates (Linbro, England; Nunc-Immuno Plate I, Denmark). Before the virus suspensions were adsorbed on primary polyclonal antibodies, the virus particles (1 mg/ml) were disrupted with 0.5 % Lauroyl sarcosine (LS, Sigma) at 37 °C for 1 hr. After incubation with LS the disrupted viruses were diluted and added into the wells in two-fold dilutions beginning with the concentration of viruses of 10 ng/well. The two-fold dilutions were performed directly in the wells. After washing procedures 100 μl of chromatographically purified polyclonal antibodies to M1-protein (or NP) conjugated with peroxidase (dilution 1:1000) were added. After incubation at 37 °C and washing, 100 μl of 0.05 % ortho-phenylenediamine solution (Sigma, U.S.A.) in 0.03 mmol/l Na-phosphate buffer (pH 5.3) containing 0.003 % H_2O_2 was given in each well. The reaction was stopped by adding 50 μl of 4 mol/l H_2SO_4. The extinction was measured at 490 nm.

EIA-test for investigation of antigenic properties of influenza viruses. The monoclonal antibodies to the M1-protein or the NP were used as secondary antibodies. In this case we used an anti-mouse-conjugate (Sigma). The test was performed like the EIA-test for standardizing the M1-protein or NP concentration. The concentrations for all viruses were chosen by the standardization EIA-test and the secondary antibodies were titrated. Titres were determined for all monoclonal antibodies versus a single virus strain on a given day. Titres like the end-point titrations were determined as the dilution which produced an absorbance value 3 fold above background. All assays were performed in duplicate.

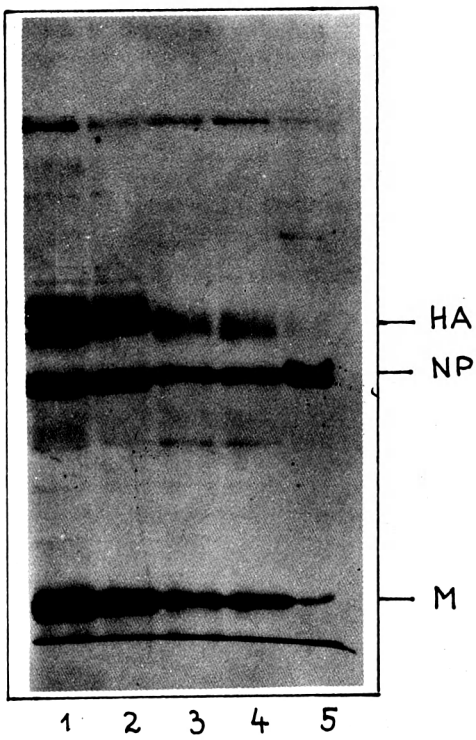

Fig. 1
Polyacrylamide-SDS-gel electrophoresis of proteins from different influenza A virus strains:
1 - A/Singapore/1/57
2 - A/Leningrad/549/80
3 - A/mallard/Potsdam/179/83
4 - A/mallard/Potsdam/178-4/83
5 - A/mallard/Potsdam/177-6/83

Results and Discussion

In polyacrylamide gel electrophoresis, adequate proteins of different human and avian strains showed the same electrophoretic mobility (Fig. 1). The electrophoresis was performed with the same virus concentration for all virus strains (80 μg virus/lane). The results showed a weak band corresponding to the matrix protein of the virus strain A/mallard/Potsdam/177-6/83 (H2N2) and on the other side a strong band corresponding to the NP for this virus strain. The same results were observed for concentrated and purified virus of A/mallard/Potsdam/177-6/83 (H2N2) from different dates of concentration and purification. Electron microscopy showed a large number of incomplete virus particles for this strain (results are not shown), while other mallard strains had mainly complete particles. The existence of many incomplete virus particles indicates the instability of the virus particle. It can further be related to the presence of less matrix protein for the A/mallard/Potsdam/177-6/83.

We hawe shown previously that, to investigate antigenic properties of influenza virus internal proteins, it is necessary to standardize the concentration of adsorbed antigen (Khristova *et al.*, 1989; Schäfer *et al.*, 1990). Standardization of matrix and nucleoprotein concentration was performed by using the double sandwich EIA-test with polyclonal antibodies. Figs. 2 and 3 show the relationship of chromophoric responses in the EIA-test for the M1-protein and the NP for the virus concentrations of different strains. Obviously, in the M1-protein EIA-test, all of the mallard strains except the virus strain A/mallard/Potsdam/177-6/83, have the same extinction, whereas A/mallard/Potsdam/177-6/83 has only half of the extinction of the other strains (Fig. 2).

A corresponding but reversed ratio could be found for the above mentioned strain in the EIA-test for the NP (Fig. 3). When investigating the antigenic

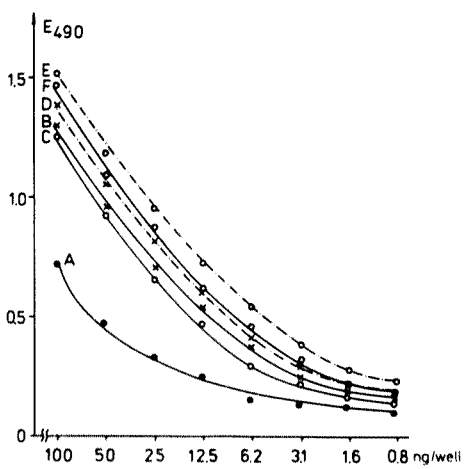

Fig. 2
Relationship of chromophoric responses in EIA-test for M1-protein:
A: A/mallard/Potsdam/177-6/83
B: A/mallard Potsdam/179/83
C: A/mallard/Potsdam/178-4/83
D: A/mallard/Potsdam/176/83
E: A/Singapore/1/57
F: A/Leningrad/549/80

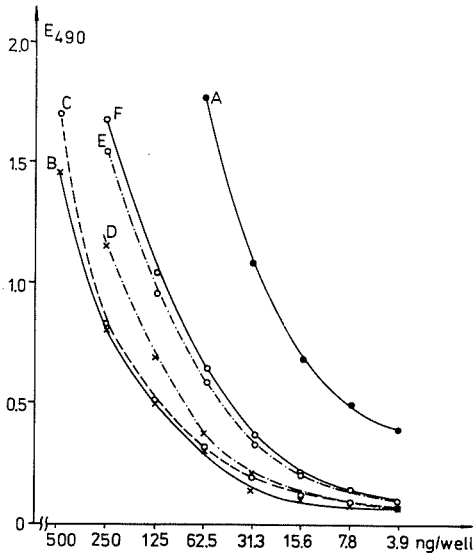

Fig. 3
Relationship of chromophoric responses in EIA-test for nucleoprotein
For legends see Fig. 2

properties of the M1-protein and the NP with monoclonal antibodies, virus concentrations were chosen to yield the same values in the EIA with polyclonal antibodies. The chosen concentration of the A/mallard/Potsdam/177-6/83 was 200 ng/well for the M1-protein-EIA and 50 ng/well for NP-EIA. The concentrations for the other mallard virus strains were 80 ng/well in both EIA-test. We could not find any antigenic difference between the reaction of 16 monoclonal antibodies (against M1-protein) with the M1-protein of all avian and human influenza virus strains under the investigation (Table 1). The same results were obtained for nucleoprotein (data are not shown). The EIA results were confirmed by Western-blot analysis with five monoclonal antibodies (2BB10-G9; 9E8-B2; 821-B8-A8; 823-B8-B11, 611-G10-D3).

The RNA-segments of the strains isolated from mallards in 1983 show the same electrophoretic mobility after hybridization except A/mallard/Potsdam/176/83. This strain definitely deviates in its NA/NP-gene (segments 5 and 6) from the other mallard isolates (Fig. 4). Segment 7 coding for the matrix protein shows the same electrophoretical mobility in 7.5 % and 4 % polyacrylamide gels for all of the mallard strains. In this case we probably have complete homology of virus and complementary RNA between these viruses. This means that mutations in RNA segments 7 were not detected. The results suggest that no differences in the antigenic properties of the matrix protein of these avian influenza A viruses of subtype H2N2 could be detected by the methods in question. One can conclude that the smaller matrix protein can be considered a marker for the A/mallard/Potsdam/177-6/83. No differences have been found for the other strains in comparison to the human H2N2 strains.

Table 1. ELISA titres of influenza viruses

Antigenic site	Monoclonal antibody	A/mallard/Potsdam/strains						Human strains	
		176/83	177-4/83	177-6/83	178-4/83	178-6/83	179/83	A/Singapore/ 1/57	A/Leningrad/ 549/80
# 1A	2BB10-G9	64	64	64	64	64	64	64	256
	1G8-A11	64	64	64	64	64	64	64	128
	3G12-C12	64	64	64	64	64	64	64	256
	9E8-B2	128	128	128	128	128	128	128	128
	821-B8-A8	128	128	128	128	128	128	128	128
# 1B	2E5-C1	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
	961-G8-H3	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
	963-D3-G10	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
	6B9-B8	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
# 2	1G11-D11	4	4	4	4	4	4	4	4
	951-C4-G2	32	32	32	32	32	32	32	32
	823-B8-B11	64	64	64	64	64	64	64	64
# 3	611-G10-D3	8	8	8	8	8	8	8	8
	951-D10-B3	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
unclassified	611-B12-D10	2	2	2	2	2	2	2	2
	961/6-B10	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2

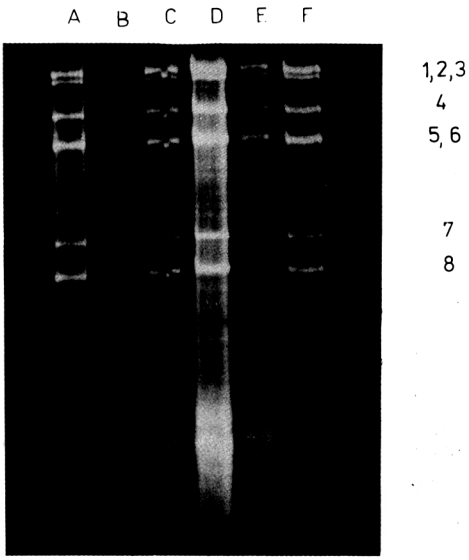

Fig. 4
Analysis of the electrophoretic mobility of S1-treated doublestranded hybrids after RNA-RNA hybridization in the 4 % polyacrylamide gel:
[^{3}H]- labelled complementary RNA of:
A: A/mallard/Potsdam/177-4/83
B: A/mallard/Potsdam/177-6/83
C: A/mallard/Potsdam/176/83
D: A/mallard/Potsdam/178-4/83
E: A/mallard/Potsdam/178-6/83
F: A/mallard/Potsdam/179/83
were hybridized with the virion RNA of A/mallard/Potsdam/176/83.
1-8 are the RNA-segments coding for antigens: 1-3: P1-3; 4:HA; 5-6: NP/NA; 7: M; 8: NS

Our investigations point at the absence of any relevant differeces between human and mallard influenza strains as far as the H2N2 subtype is concerned. If there are any differences in the internal and surface proteins, they must to be detected with more sensitive methods.

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