Archives of Virology 59, 285—298 (1979)

Archives of Virology

Molecular Basis of Infectivity and Pathogenicity of Myxovirus

Brief Review

By

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With 8 Figures

Accepted November 8, 1978

Introduction

Pathogenicity of a virus—that is its property to cause a disease in a given organism—comprises a multitude of factors determined by the biological, biochemical and genetic characteristics of the infecting virus on the one hand, and the reactivity of the host on the other hand. It is obvious that a molecular basis for viral pathogenicity is not easy to define. It is, however, reasonable to assume that clinical manifestation of a virus disease is expressed, if cells of vital functional significance are infected and are altered by the virus or are killed. Since the tropism of a virus for a host cell represents primarily an interaction between the surface components of the virus and receptors of the host cells, it is appealing to postulate that surface structures of a virus might determine infectivity and pathogenic properties of the virus. Recent results might be interpreted in this sense, because such an interrelationship could be demonstrated.

Structure of Myxoviruses

All myxoviruses follow the same structural principle (for ref. see 6, 21). There is a single-stranded RNA associated with a protein component to form the helical nucleocapsid. An RNA-dependent RNA polymerase activity is associated with this nucleocapsid. This structure is surrounded by a lipid-bilayer, which in turn is internally lined with a special protein, the so-called M-protein. This lipid envelope carries on its surface glycoproteins which have the morphological appearance of spikes (Fig. 1).

On the basis of specific structural and biological characteristics myxoviruses are divided into ortho- und paramyxoviruses. Thus, the genetic material of paramyxoviruses consists of an uninterrupted RNA while the RNA of orthomyxoviruses is composed of 8 segments each of which represents a gene that codes for a specific protein (for ref. see 27). This particular structure of the viral RNA explains

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several biological peculiarities of orthomyxoviruses, especially their high rate of recombination. After double infection with different influenza virus strains *in vitro* and *in vivo*, RNA segments of these viruses can be reassorted yielding virus progeny whose gene products have been exchanged accordingly (for ref. see 34).

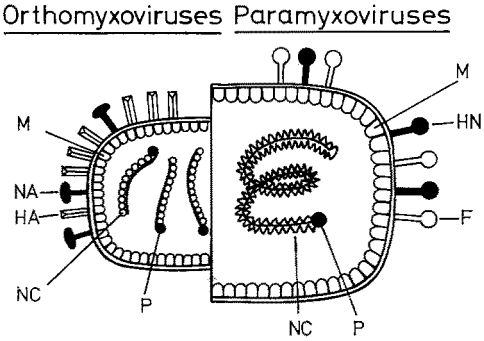

Fig. 1. Schematic diagram of the structure of myxoviruses. In the figure *P* stands for RNA polymerase, *NC* for nucleocapsid, *M* for membrane protein, *HA* for hemagglutinin, *NA* for neuraminidase, *F* for fusion, *HN* for hemagglutinin-neuraminidase complex

The glycoproteins located on the surface of myxovirus particles carry different biological properties. Of the two glycoproteins of orthomyxoviruses, the hemagglutinin (HA) adsorbs to sialic acid containing receptors of the host cell to initiate the replicative cycle of the virus. The second glycoprotein, the neuraminidase, attacks α-ketosidic linkages between sialic acid and other carbohydrates. The functional significance of this enzyme is not fully understood (for ref. see 20, 21). In paramyxoviruses both activities, the ability for adsorption and the neuraminidase activity, are located in the glycoprotein HN; the glycoprotein F is responsible for hemolysis, cell fusion, and virus penetration (for ref. see 21).

Posttranslational Modification of Myxovirus Glycoproteins

Viral envelopment and maturation is a multistep process involving sequential incorporation of viral proteins into the cellular membranes. Cell fractionation studies on myxovirus-infected cells have shown that the polypeptide chains of the glycoproteins are transported from the rough endoplasmic reticulum, where they are synthesized, to the cytoplasm. In the course of this transport the polypeptides undergo posttranslational modifications. These involve sequential glycosylation on the rough and smooth endoplasmic reticulum, and proteolytic cleavage which takes place in association with smooth internal membranes and the plasmalemma. Thus, the hemagglutinin of orthomyxoviruses consists of two separate subunits, HA1 and HA2, which are linked by disulfide bonds, and result from proteolytic cleavage of the precursor glycoprotein HA. The paramyxovirus glycoproteins F_1 and F_2 and HN are derived from the precursors F_0 and HN_0, respectively. Whether proteolytic cleavage occurs depends on the virus strain as well as on the host cells infected (for ref. see 21).

Structure of Myxovirus Glycoproteins and Infectivity

Proteolytic cleavage of the viral glycoproteins is not essential for maturation and liberation of myxovirus particles from the host cell. It is, however, of vital significance for infectivity of newly synthesized virions. After infection of chicken embryos or cultured cells of the chorioallantoic membrane, all myxovirus strains tested so far are synthesized in an infectious form. If these viruses, however, are produced in other cells, e.g. chicken fibroblasts or BHK cells, only a small number of virus strains of ortho- and paramyxoviruses are infectious, while the progeny of the other strains have little infectivity (Tables 1 and 2).

Analysis in polyacrylamide gel electrophoresis (PAGE) (Figs. 2 and 3) revealed a direct correlation between infectivity and the structure of the glycoproteins:

Table 1. *Activation of different influenza A virus strains by trypsin treatment*

Influenza virus strains	Rate of activation by trypsin[a]			
	Chicken embryo		Chicken fibroblasts	
	Hem-agglu-tinin	In-fectiv-ity	Hem-agglu-tinin	In-fectiv-ity
A/PR/8 (H0N1)	1.0	1.3	1.0	27
A/FM/1/47 (H1N1)	1.0	2.4	1.0	4.3
A/Singapore/1/57 (H2N2)	1.0	0.8	1.0	193
A/equine/Miami/1/63 (Heq2Neq2)	1.0	1.1	1.0	111
A/swine/1976/31 (Hsw1N1)	1.0	1.0	1.0	200
A/chick/Germany (Hav2Neq1)	1.0	1.0	1.0	150
A/fowl plague/Rostock (Hav1N1)	1.0	1.0	1.0	1.0

Purified virus particles grown either in chicken embryo or in chicken fibroblasts were tested for HA activity or infectivity before and after trypsin treatment (11)

[a] treated/non-treated

Table 2. *Effect of trypsin treatment on the biological activities of NDV grown in BHK 21-F cells (14)*

NDV strains	Pathogenicity	Mean embryo death time (hours)	Rate of activation by trypsin[a]			
			Hem-agglu-tinin	Neur-amin-idase	Hemo-lysis	In-fectiv-ity
Italien	pathogenic	50	1.0	1.01	0.62	1.13
Herts	pathogenic	49	1.0	0.96	0.76	0.79
Field pheasant	pathogenic	50	1.0	1.10	0.87	1.35
Texas	pathogenic	50	1.0	1.01	0.68	1.30
Warwick	pathogenic	50	1.0	0.99	0.79	1.80
La Sota	non-pathogenic	103	1.0	1.02	5.00	5.55
B1	non-pathogenic	120	1.0	0.97	5.34	7.10
F	non-pathogenic	168	1.0	1.00	5.58	—
Queensland	non-pathogenic	∞	2.0	2.03	5.34	18.1
Ulster	non-pathogenic	∞	2.0	2.72	9.20	144.0

[a] treated/non-treated

Only virions with cleaved glycoproteins are infectious. The non-infectious virions possess uncleaved glycoproteins. Cleavage of both glycoproteins is necessary for infectivity of paramyxoviruses. Virions containing F_0 have reduced infectivity which is even lower if both glycoproteins are present in uncleaved form (Table 2).

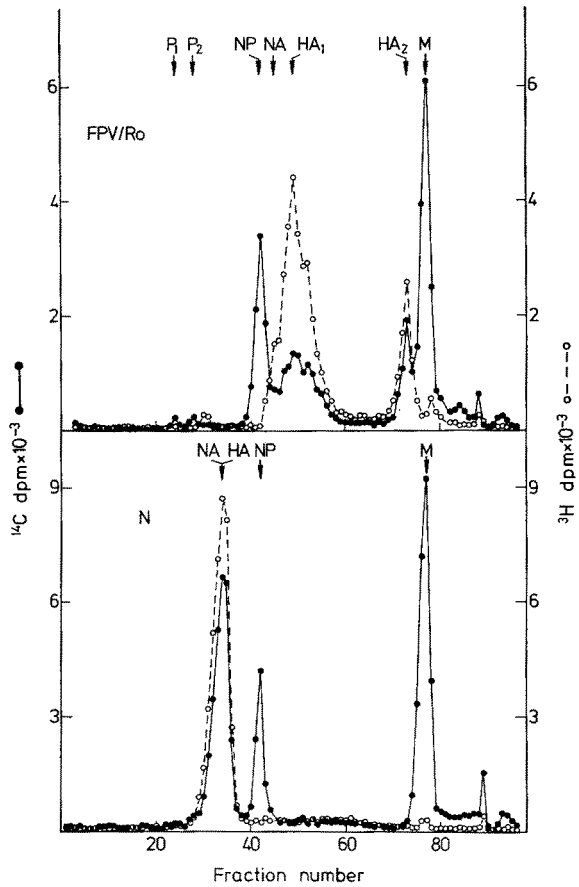

Fig. 2. Polypeptides of fowl plague virus (FPV/Ro) and virus N (N) after PAGE. After replication in chicken fibroblasts and labeling with [^{3}H]glucosamine (o– – – –o) and [^{14}C]amino acids (•————•) glycoprotein HA of FPV/Ro is cleaved into HA_1 and HA_2 while that of virus N remains uncleaved [For detail see (12)]

Viruses formed as non-infectious particles in a given host cell system can be converted into infectious particles by treatment *in vitro* with trypsin or trypsin-like enzymes which characteristically cleave the glycoproteins (Figs. 4 and 5) (9, 12, 14, 15, 25).

When cells were doubly infected with two myxovirus strains which are produced in these cells as infectious virus or as virus with reduced infectivity, the progeny virus behaved as if the cells had been infected separately: The glycoproteins of the highly infectious strain were cleaved while those of the other remained

uncleaved (Figs. 6 and 7). One strain, thus, cannot activate the cleavage process of the other (12, 15). This means that structural characteristics of the individual glycoproteins determine whether cleavage occurs rather than an activation of cellular enzymes by the infecting virus.

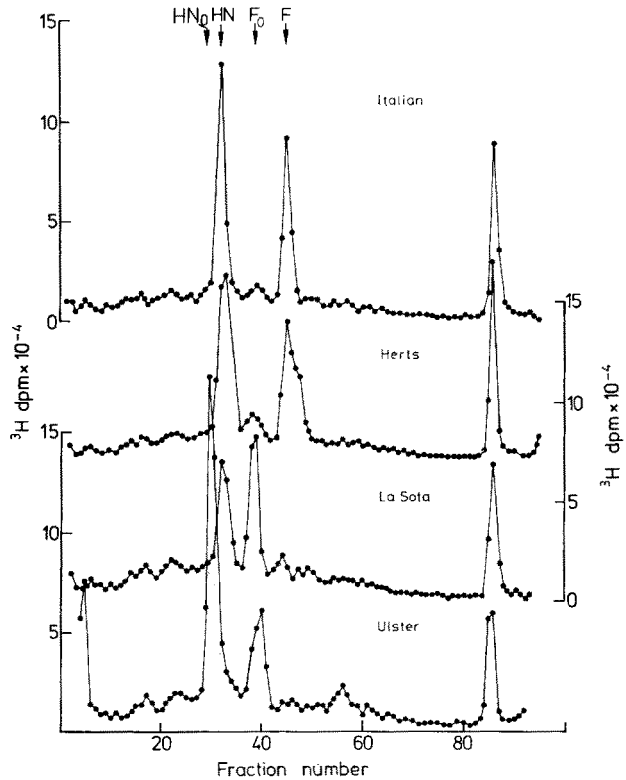

Fig. 3. PAGE of glycoproteins of different NDV strains. After replication in BHK 21-F-cells glycoproteins HN_0 and F_0 of strains Italien and Herts are cleaved into HN and F. In strain La Sota glycoprotein F_0 and in strain Ulster glycoproteins F_0 and HN_0 are uncleaved. Labeling with [^{3}H]glucosamine. [For detail see (15)]

Besides the infectivity other biological functions which are also carried on the envelope glycoproteins are activated by proteolytic cleavage (Table 2). It could be shown that the Newcastle disease virus (NDV) glycoprotein HN possesses cell-adsorbing and neuraminidase activities only after cleavage of the biologically inactive precursor HN_0 (15). The glycoprotein F of Sendai virus and NDV has hemolytic and cell fusing activities only after proteolytic cleavage (9, 15, 25). Since penetration obviously depends on fusion between the virus envelope and the host cell membrane, in the infectious process of paramyxoviruses activation of infectivity is determined by the fact that only cleaved glycoprotein F initiates penetration (for ref. see 6). There is evidence that the hemagglutinin glycoprotein

of orthomyxoviruses has a decisive function in penetration for which a cleaved HA is required, in addition to its role in adsorption (13, 17, and unpublished results).

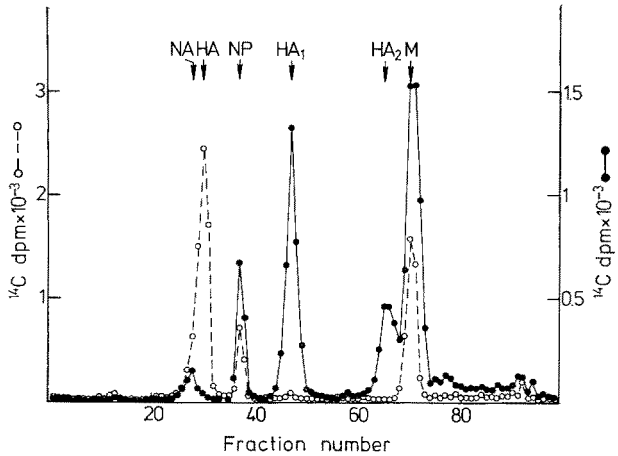

Fig. 4. Cleavage of glycoprotein HA of virus N, an orthomyxovirus, by treatment of isolated virus particles with trypsin. Purified virus N grown in chicken fibroblasts and labeled with [^{14}C]amino acids was treated with trypsin (•———•) and analysed in PAGE. (o — — — o) non-trypsin-treated control. [For detail see (12)]

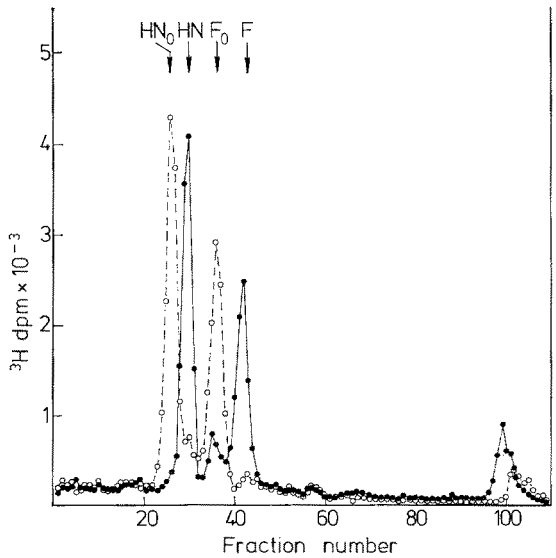

Fig. 5. Modification of glycoproteins HN_0 and F_0 of isolated NDV, strain Ulster by treatment with trypsin. Purified virus grown in MDBK cells and labeled with [^{3}H]-glucosamine was treated with (•———•) or without (o — — — o) trypsin and subjected to PAGE. [For detail see (15)]

Role of Proteolytic Cleavage of Paramyxovirus Glycoproteins in Pathogenicity

NDV comprises a wide range of strains which differ markedly in pathogenicity for their natural host, the chicken. It has long been recognized as an excellent model for investigations of virus pathogenicity (2, 33). A comparative analysis of pathogenic and non-pathogenic NDV strains revealed striking differences in the range of host cells that produce biologically active virus. These differences are based on the susceptibility of the NDV envelope glycoproteins to proteolytic cleavage in an infected cell (15).

For the non-pathogenic strains only a few cell systems are permissive and thus yield infectious virus progeny. Most cell systems investigated are non-permissive for these strains, i.e. they produce non-infectious virus containing

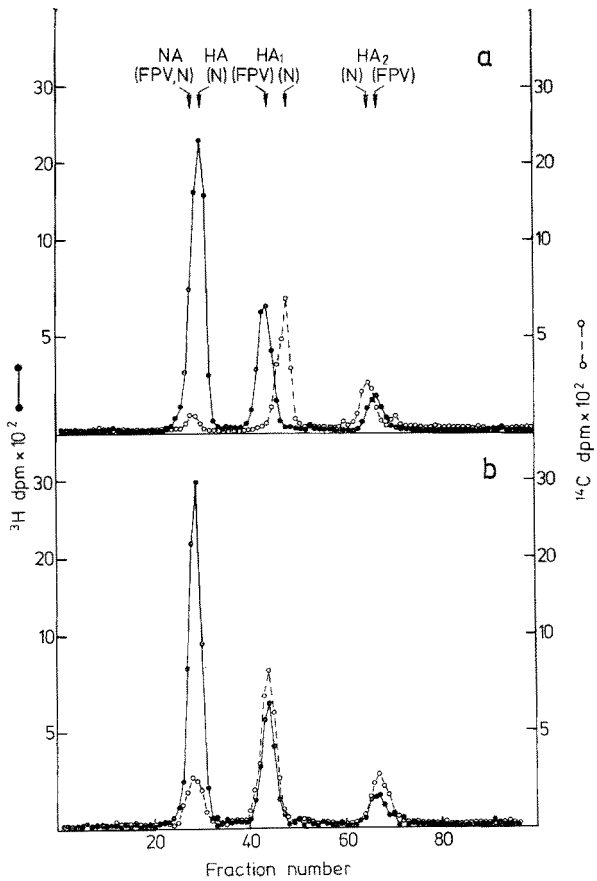

Fig. 6. Glycoproteins of progeny virus obtained after double infection of chicken fibroblasts with virus N and FPV. The virus was labeled with [^{3}H]glucosamine and purified (•———•). a) Co-electrophoresis with a purified preparation of [^{14}C]glucosamine labeled virus N treated *in vitro* with trypsin to cleave the glycoprotein HA. b) Co-electrophoresis with purified FPV labeled with [^{14}C]glucosamine. [For detail see (10)]

uncleaved glycoproteins. In contrast, all systems are permissive for pathogenic strains. These results explain earlier observations made by several groups (8, 19, 26) that, in contrast to avirulent NDV strains, only virulent ones form plaques and induce fusion of cells, such as chicken fibroblasts.

Formation of highly infectious virus is a prerequisite for spread of infection in a given host system. This idea is supported by the finding that all strains had identical rates of multiplication in the chicken embryo, but they differed strikingly in their capacity to penetrate the different embryonic tissues (3, 16). After infection of the ectodermal layer of the chorioallantoic membrane only pathogenic strains spread through the whole membrane, gaining entrance to the blood vessels to disseminate through viremia. In contrast viral material of the non-pathogenic strains was only found in this area of the ectodermal cell layer which was inoculated. Furthermore, PAGE analysis revealed that under these conditions of infection the glycoproteins of the pathogenic strains were cleaved, while the non-pathogenic viruses contained uncleaved glycoproteins (16). Evidently, there is a strict correlation between the structure of the NDV glycoproteins and the pathogenetically so important capacity to spread in the infected host.

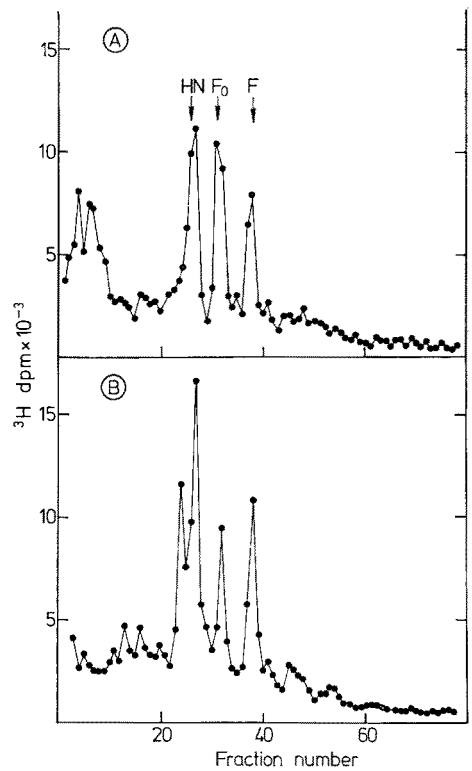

Fig. 7. NDV glycoproteins synthesized in BHK 21-F cells after double infection with the pathogenic strain Italien (A) and the non-pathogenic strain Ulster (B). Labeling with [^{3}H]glucosamine. [For detail see (15)]

Factors Required for Pathogenicity of Orthomyxovirus

Evidence was presented for a polygenic nature of orthomyxovirus pathogenicity (for ref. see 22). Genetic analysis of a large number of recombinants with single gene exchange revealed that an exchange of any RNA segment of a pathogenic strain can modify pathogenicity. The pathogenic properties of recombinants are determined by the virus strain from which the corresponding gene was derived as well as by the gene which was replaced (4, 29). As a rule, recombinants with defined multiple gene exchanges produce less severe signs of clinical illness as the gene exchanges increase (7, 18, 24). These data indicate that an optimal constellation of all RNA segments is required for a genome of a highly pathogenic virus strain.

If this were true, one should expect that some of the recombinants of non-pathogenic viruses should have pathogenic properties, or, in contrast, that reassortment between highly pathogenic parent strains may lead to pathogenic as well as non-pathogenic viruses. This is indeed the case as the following findings prove: 1. It could be demonstrated by recombination of orthomyxovirus strains [A/FPV/Rostock (FPV, Hav1N1) × A/England/1/61 (H2N2) or FPV × A/PR/8/34 (H0N1)] which by themselves are non-neurovirulent for mice that neurovirulent recombinants can be isolated (32). Analysis of the gene composition of the neurovirulent recombinants revealed that all these recombinants carry the HA gene of FPV, which was shown to be cleaved in a broad spectrum of host cells. The composition of the other RNA segments involved in neurovirulence depended on the parent virus strains used as donor for reassortment. It seems, however, that only genes of the RNA polymerase complex are important in this context (31). 2. Using two highly pathogenic avian influenza viruses [FPV × A/turkey/England/63 (Hav1Nav3)] for reassortment, non-pathogenic recombinants were isolated which carried the HA of either parent strain. Here again the loss of pathogenicity seemed to be dependent from the RNA segments associated with the RNA polymerase complex (23).

The implications of these observations with recombinant influenza viruses are: 1. There is not one single gene responsible for pathogenicity. 2. It is impossible to establish a rule for the combinations of the different genes indicative of pathogenicity for all influenza viruses. 3. In each reassortment an optimal composition might be achieved which depends on the parent virus strains used.

As mentioned above, there is little doubt that pathogenicity depends on the host range of the respective virus. From host range mutants and host range recombinants of FPV which were isolated and characterized recently (1, 28, 30), it emerged that the host range properties were dependent not only on the hemagglutinin but also on influenza virus genes involved in viral RNA synthesis. One might conclude, therefore, that a host factor is necessary for the function of the RNA polymerase complex in orthomyxovirus RNA synthesis. This idea is compatible with the observation that a given recombinant is only pathogenic for a specific host species. Thus, recombinants that are neurovirulent for mice, are not pathogenic for chickens or *vice versa* (31).

Recombinants obtained *in vitro*, however, can be looked at as artificially constructed viruses. The conclusion drawn from these experiments might therefore

not hold for naturally occurring influenza viruses, particularly since not every of the 254 possible combinations of RNA segments from a pair of strains will result in a functional genome (22).

As a model for such a natural virus-host system avian influenza viruses were investigated, because these viruses occurred in many different HA subtypes and in many different hemagglutinin-neuraminidase constellations (35). Besides the genes coding for hemagglutinin and neuraminidase there are considerable differences in base sequence homologies of the other genes as well (SCHOLTISSEK, personal communication). In Table 3 are summarized the results of investigations on the cleavability of the HA glycoprotein in MDCK cells, as well as chicken, duck, turkey, and quail fibroblasts, plaque formation in these cells as indicators for viral infectivity, and pathogenicity for chickens of 9 different avian influenza hemagglutinin subtypes (5). Indeed, a strict correlation between structure of the HA of these viruses and their pathogenicity for chickens exist. As was the case with paramyxoviruses those orthomyxoviruses which are produced in an infectious form in a broad spectrum of host cells are pathogenic.

Table 3. *Correlation between hemagglutinin structure, infectivity, and pathogenicity of avian influenza viruses for the chicken. [For detail see (5)]*

Virus strains	Surface antigens	HA present in cleaved form	Plaque forming capability without trypsin	Pathogenic for chicken
A/FPV/Rostock	Hav 1 N 1	+	+	+
A/Parrot/Ulster/73	Hav 1 N 1	−	−	−
A/FPV/Dutch/27	Hav 1 Neq 1	+	+	+
A/Fowl/Victoria/75	Hav 1 Neq 1	+	+	+
A/Turkey/Oregon/71	Hav 1 Nav 2	−	−	−
A/Turkey/England/63	Hav 1 Nav 3	+	+	+
A/Chick/Germany/49	Hav 2 Neq 1	−	−	−
A/Duck/Memphis/546/74	Hav 3 Nav 6	−	−	−
A/Duck/Czecho-Slovakia/56	Hav 4 Nav 1	−	−	−
A/Turkey/Ontario/7732/66	Hav 5 Nav 6	+	+	+
A/Duck/Germany/1862/68	Hav 6 N 1	−	−	−
A/Duck/Ukraine/1/63	Hav 7 Neq 2	−	−	−
A/Turkey/Ontario/6118/68	Hav 8 Nav 4	−	−	−
A/Turkey/Wisconsin/66	Hav 9 Neq 1	−	−	−

The narrow host range of avian influenza viruses nonpathogenic for chickens is clearly demonstrated after infection of the chorioallantoic membrane of chicken embryos. After inoculation of the ectodermal layer with non-pathogenic viruses only these cells produce viral material, while with pathogenic strains under the same conditions all cell layers become infected. The infection remains localized in the endodermal layer after inoculation of non-pathogenic virus into the allantoic cavity, but dissemination occurred with pathogenic strains (Fig. 8).

The decisive role of the hemagglutinin in the pathogenicity of avian influenza viruses does not contradict the results obtained with recombinants of ortho-

myxoviruses produced *in vitro*. It rather means that in the avian organism only viruses with an optimal gene constellation might be selected and survive. If, in addition to this optimal gene constellation, the viruses possess HA which is cleaved in many different host cells and thereby becomes activated, they always are pathogenic.

Non-pathogenic avian influenza viruses with a limited host cell range multiply in infectious form in cells of at least the upper respiratory and in the intestinal tract without inducing signs of disease (5, 36). This observation is of epidemiological importance, since infected birds shed virus which can be spread to other hosts for perpetuation of the infcction in the population. The probability that some

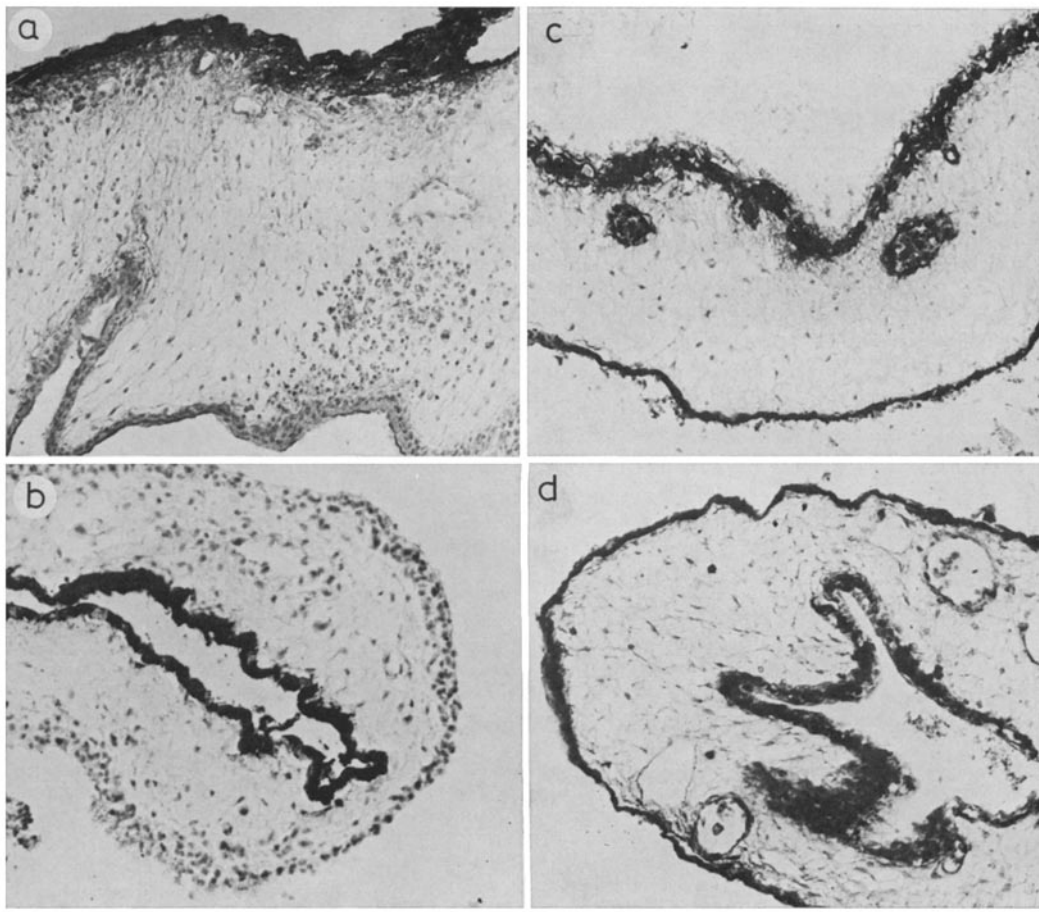

Fig. 8. Spread of avian influenza viruses in the chorioallantoic membrane of chicken embryos

Chicken embryos were inoculated with the non-pathogenic virus N (a, b) or the pathogenic FPV (c, d) onto the ectodermal layer (a, c) or into the allantoic cavity (b, d). Following incubation for 24—48 hours after infection virus-specific antigens were demonstrated in the membranes by the peroxydase-antiperoxidase method (Micrographs taken by M. REINACHER)

respiratory viruses multiply in cells of the intestine suggests a relationship which may be more common between respiratory and enteric viruses than had been recognized.

Conclusion

The objective of this study was to define a molecular basis for infectivity and pathogenicity of myxoviruses. It was demonstrated in both ortho- and paramyxoviruses that activation of the glycoproteins by posttranslational proteolytic cleavage is indispensable for the formation of infectious virus. Cleavage of the precursors to the active glycoproteins is a host-specified phenomenon, which depends on the presence of an appropriate protease. Whether proteolytic cleavage occurs in a given host cell is determined primarily by the individual structural characteristics of the viral glycoproteins rather than by the activation of cellular proteases.

Formation of highly infectious virus is a precondition for the spread of infection in the host. It is, therefore, a fair assumption that infection with a pathogenic strain which produces virus of full biological activity in a wide spectrum of different host cells, spreads more rapidly than infection with a virus strain of a narrow host cell range. In other words, the more cells in an organism are producing virus in infectious form, the greater is the chance for the virus reaching the target organ to exert pathogenic action.

The available evidence indicates that the acute course of the disease in naturally occurring infection with myxoviruses derives from the rapid multiplication and spread of the virus in the host. This seems the most critical factor in the pathogenesis of NDV and avian influenza viruses which have been studied in sufficient detail. The host defense mechanism is outmanuvert in this scheme of events. In contrast, virus strains which are produced in infectious form by a limited range of different host cells only, remain prime candidates for induction of persistent infections.

Acknowledgement

I thank Drs. H.-D. Klenk, C. Scholtissek, and J. Storz for discussion and criticism. Own work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 47).

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Received November 1, 1978