EXPERIMENTAL INFECTION WITH A PERSISTENT INFLUENZA C VIRUS VARIANT LEADS TO PROLONGED GENOME DETECTION IN THE CHICKEN LUNG

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Summary. – A persistent variant of influenza C virus was used to infect chickens by intraamniotic (i.a.) inoculation. The infected hatchings were analyzed for virus production in different tissues and for the continuous presence of viral RNA genomes. The permissiveness for infection was demonstrated primarily for the chicken lung, besides other organs. Viral antigens could be detected by indirect immunofluorescence staining for a period of 8 days and reisolates were obtained mainly at early time points post infection (p.i.). Nested reverse transcription-polymerase chain reaction (RT-PCR) directed to 3 genomic sequences was positive at least until day 53, whereby no distinct end point was determined. These experiments provide first evidence for the long-term stability of influenza C virus RNA segments *in vivo*.

Key words: influenza C virus; persistent variant; infection of chickens; nested reverse transcription-polymerase chain reaction; viral genome stability

Influenza C viruses are human pathogens, which are usually cleared from the respiratory tract 5 to 7 days p.i. (Kilbourne, 1987). Infection studies in ducks revealed the loss of genetic material of avian influenza A virus between days 7 and 14 p.i. (Wang and Webster, 1990). Similarly, the influenza C strain Ann Arbor/1/50 (wild-type) replicates in a transient cycle in cell culture for up to 17 days, as characterized by complete clearance of the viral RNA (Marschall *et al.*, 1996). In contrast to this, a long-term persistence in MDCK cells was obtained by selecting a variant of this virus strain by continued passaging of infected MDCK cultures (Camilleri and Maassab, 1988). We previously characterized the persistent state of infection by several aspects of viral replication, e. g. the viral genome level, protein function and life cycle (Lapatschek *et al.*, 1995; Marschall *et al.*, 1994). Of importance, the persistent C/Ann Arbor/1/50 (C/AA-pi) virus shows productive and non-productive phases in MDCK cells (Marschall *et al.*, 1993) and seems to be maintained in a minimal form of its genomic minus strand RNA (Marschall *et al.*, 1996). In this study, an *in vivo* transfer to chickens was performed with this selected variant. We specifically adressed the question, if the persistent variant's gene products might be stabilized for a longer period than that observed in the reports cited above.

Chicken embryos were infected i.a. on day 11 or 12 before hatching, which occurs on day 23 at 33°C. The ongoing virus replication was assayed during 3 days p.i. in samples of allantoic fluid for haemagglutination activity. In some hatchings leg and gait malformations were observed, as noted by others (Parker *et al.*, 1994). Downfeather abnormalities, described by Spence and O'Callaghan (1985), could not be detected. Embryos or chickens were sacrificed and examined at different times p.i. for virus RNA, virus protein synthesis and production of infectious progeny virus (Table 1). The materials (lung, trachea, kidney, spleen, muscle, heart, liver, pancreas, thyroid gland and blood cells) were analyzed in comparison to a mixture of total embry-

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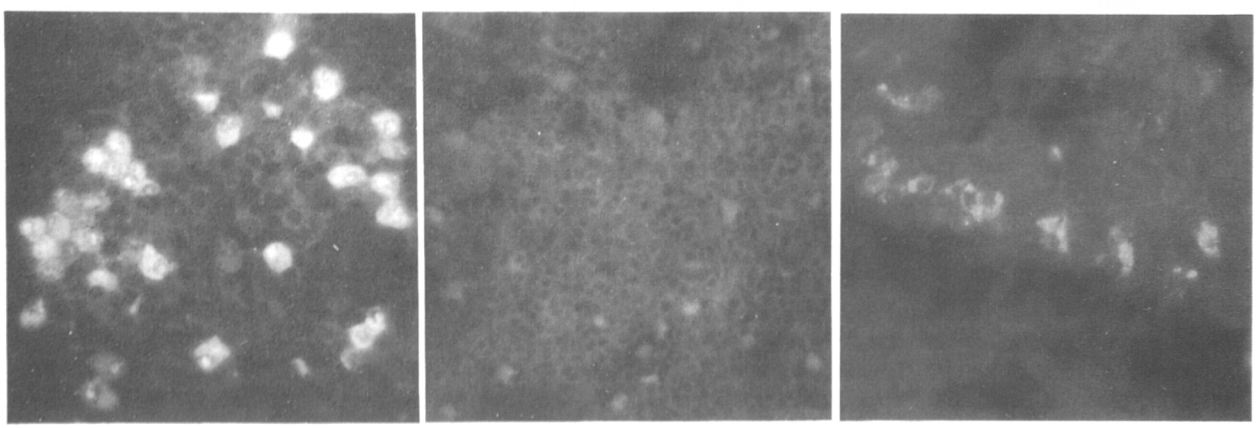
Abbreviations: HEF = haemagglutinin, esterase, fusion protein; HI = haemagglutination-inhibition; i.a. = intraamnionic(ally); i.n. = intranasal(ly); M = matrix; NP = nucleoprotein; NS = non-structural (proteins); p.i. = post infection; RT-PCR = reverse transcription-polymerase chain reaction

Table 1. Experimental set-up and summarized results

Chicken	Inoculum virus	Mode of inf.	Day of inf./ tissue prep[1].	Inf. period[2] (days)	Analyzed tissues	Serum antibody[3]	IF	RT-PCR	Nested PCR	Reiso-lates[4]
CH 2.3	C/AA-wt	i.n.	10,19, 38/43	5	lung, trachea, kidney	1:30	+	–	+	ND
CH 3.1	C/AA-pi	i.a.	-12/-7	5	total embryonic cells	ND	+	+	ND	+
CH 4.1	C/AA-pi	i.a.	-12/-4	8	lung, kidney, muscle, heart, liver	ND (lung)	+	+	ND (lung)	+
CH 6.3	C/AA-pi	i.a.	-11/10	21	lung	ND	–	+	ND	–
CH 6.5	C/AA-pi	i.a.	-11/38	49	lung, kidney	0	–	–	+	–
CH 6.6	C/AA-pi	i.a.	-11/42	53	lung, trachea, kidney, spleen, muscle, heart, liver, pancreas, thyroid gland, blood cells	1:80	–	–	+	+ (lung)[5]

[1](-) = days before hatching; [2]Period after the last infection; [3]Titers determined by HI test; [4]Reisolated virus was identified by the use of monoclonal antibody FC 1.16.3.3 against HEF in HI test; [5]Isolate was lost due to instability; ND = not done; IF = immunofluorescence test.



(a) (b) (c)

Fig. 1
Indirect immunofluorescent staining of influenza C virus-infected chicken lung cells

Monoclonal antibody F17 against NP (Sugawara *et al.*, 1991) was assayed on different tissue samples: (a) CH 4.1, infected with C/AA-pi virus, 8 days p.i. (homogenized and trypsinized embryonic lung cell preparation), (b) CH 6.6, infected with C/AA-pi virus, 53 days p.i. (sections) and (c) CH 2.3, 5 days after the last infection with C/AA-wt virus (sections). Cells were fixed in 3% formaldehyde for 20 mins and treated with 0.1% Triton-X 100 for 10 mins at room temperature. Primary and secondary antibodies (FITC-conjugated rabbit anti-mouse IgG), both in a 1:100 dilution, were incubated at 37°C for 90 and 45 mins, respectively, before microscopic analysis (magnification 630 x).

onic cells (CH 3.1), which served as a detection standard, and uninfected embryonic cells, which served as negative control. As a wild-type control of infection, a chicken was infected three times intranasally (i.n.) with allantoic fluid of the parental influenza C/AA/1/50 virus strain (C/AA-wt) at the age of 10, 19 and 38 days (Table 1, CH 2.3). It was bled at day 43 and prepared for tissue analysis.

In order to obtain information about initial virus production and immune responses, three chickens (CH 2.3, CH 6.5 and CH 6.6) were examined for serum antibodies by con-

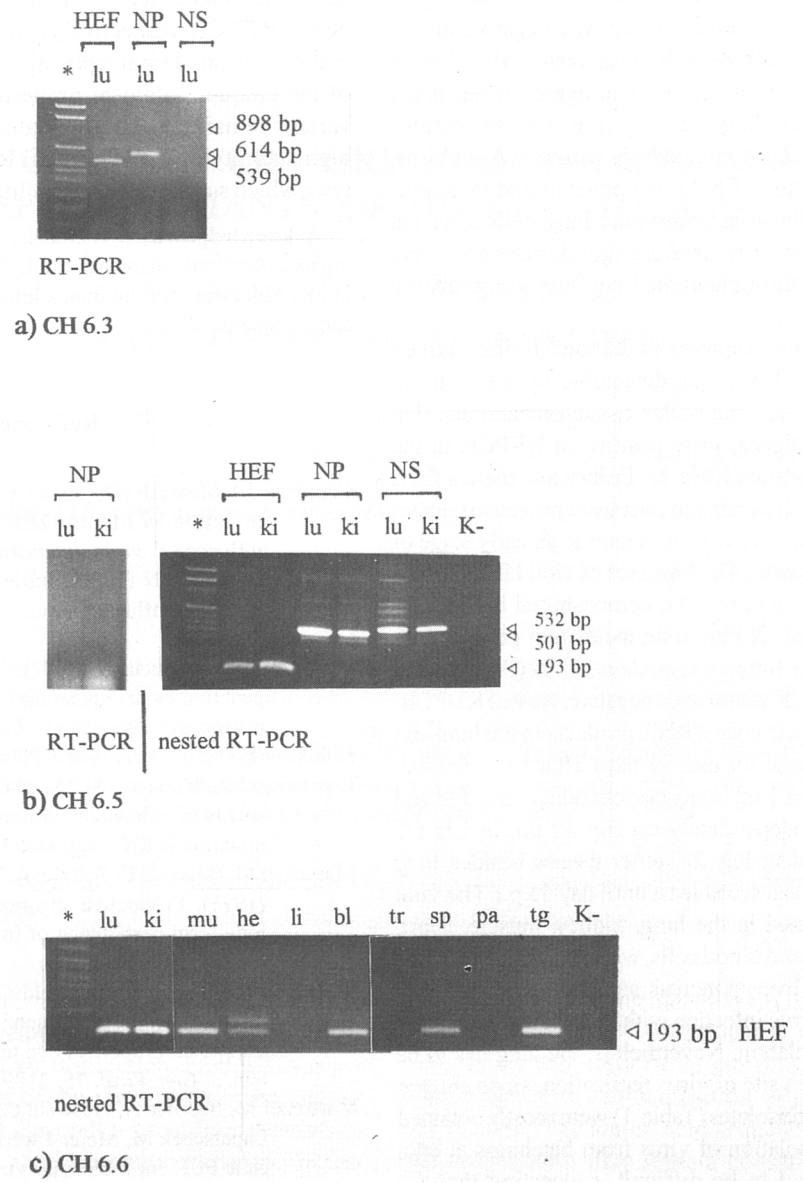


Fig. 2
Viral RNA detection by RT-PCR techniques

Distinct tissue samples from chickens infected with C/AA-pi virus were tested: lung (lu), kidney (ki), muscle (mu), heart (he), liver (li), blood cells (bl), trachea (tr), spleen (sp), pancreas (pa), thyroid gland (tg); (a) day 21 p.i. by primary RT-PCR, (b) day 49 p.i. by primary and nested RT-PCR, and (c) day 53 p.i. by nested RT-PCR. RNA extraction from homogenized tissue samples was done according to the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). RNA was reverse transcibed and amplified by PCR as described before (Marschall *et al.*, 1995), using oligonucleotide primers for three different influenza C genome segments. HEF amplification: nt 510 to nt 1048 and nt 820 to nt 1012; NP amplification: nt 18 to nt 606 and nt 119 to nt 606; NS amplifiction: nt 27 to nt 887 and nt 105 to nt 636. Control from uninfected chickens (K-). Boehringer Mannheim DNA molecular weight marker VI (*).

ventional haemagglutination-inhibition (HI) test. The i.n. infected control chicken showed low antibody titers of 5 HIU/ml after the first infection. A reinfection increased the titer to 30 HIU/ml, while sera from uninfected chickens were negative. In chickens infected with persistent virus, titers from 0 to 80 HIU/ml were observed (Table 1).

Viral antigens could be detected by indirect immunofluorescence in total embryonic cells 5 (CH 3.1) and 8 days p.i. in embryonic lung cells (CH 4.1). Monoclonal antibodies against the viral surface protein HEF (haemagglutinin, esterase, fusion protein), the matrix (M) protein (both data not shown) and the nucleoprotein (NP) (Fig. 1a) reacted

positively in the lung tissue. Immunofluorescent staining of muscle, heart, liver and kidney tissue was negative at this time point. Likewise, all animals investigated after longer infection periods were negative for antigens in the listed organs (Table 1), including the lung (Fig. 1b). In sections from the lung of chickens infected i.n. with C/AA-wt virus, however, the production of NP was demonstrated in quantities comparable to those in embryonic lung cells after i.a. infection with C/AA-pi virus. Interestingly, positive cells were arranged along lines throughout the lung (Fig. 1c), probably indicating the lung pipes.

In all persistent-type samples that showed viral antigen production, the viral RNA was detectable by RT-PCR. In contrast to this, in later time points tissue preparations that were negative for antigens, were positive in RT-PCR in the secondary nested reaction (Table 1). Embryonic tissues from the lung, kidney, muscle, heart and liver were proven to contain viral RNA, indicating virus dissemination at an early stage of infection (data not shown). The presence of viral HEF, NP and NS segments in the lung could be demonstrated by a single round of RT-PCR (Fig. 2a) up to an incubation period of 21 days (CH 6.3). In later times, i. e. on days 28, 49 (Fig. 2b) and 53 p.i., primary RT-PCR was always negative. Nested RT-PCR, however, revealed specific viral RNA products in the lung and the kidney of hatchings, 49 and 53 days after i.a. infection. The sequences of three viral segments, encoding HEF, NP and NS, were detected independently on day 49 p.i. in CH 6.5 (Fig 2b). As depicted in Fig. 2c, other tissues besides lung and kidney could be positive at least until day 53 p.i. The viral HEF gene was detected in the lung, kidney, muscle, heart, spleen, thyroid gland and blood cells, while no virus RNA was demonstrated in the liver, pancreas and trachea of CH 6.6. This suggests a systemic infection with virus spread to several organs after i.a. inoculation. Nevertheless, the lung has to be considered as the main site of virus replication, since antigen detection and the rare reisolates (Table 1) were mostly obtained from this organ. Reisolation of virus from hatchings at later time points turned out to be difficult, suggesting that the continued presence of viral RNA may take place without production of infectious progeny virus. This behaviour of C/AA-pi virus had already been described in non-productively infected MDCK-pi cells (Marschall *et al.*, 1993).

Taken together, the persistent C/AA-pi virus strain remained detectable in our studies on the RNA level for more than 7 weeks. A general intracellular stability of influenza RNA segments was reported for experimentally inactivated virus, too (Cane and Dimmock, 1990). Our findings, however, provide a novel aspect with respect to long-term viral persistence. They do not conform to an earlier publication, which states the opposite effect, namely the rapid loss of genetic information of a non-persistent influenza virus strain in ducks (Wang and Webster, 1990). In the latter and in our study the experimental procedures had similarly high detection sensitivity (RT-PCR Southern blot analysis or nested RT-PCR, respectively), proposing a basic difference in the duration of viral replicative events. Hereby, the impact of the unique biological properties of the persistent virus variant remains to be elucidated in detail. However, the high stability of C/AA-pi viral RNA was demonstrated *in vivo*, which suggests the possibility of persistent RNA forms.

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