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STABILITY OF RUBELLA VIRUS AFTER LONG-TERM PERSISTENCE IN HUMAN CELL LINE

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SUMMARY

Primary infection of HEp-2 cells with rubella virus resulted in non-cytophatic long-term persistent infection. During four years of persistence the virus was produced in sufficient quantities (up to 6 logs PFU/ml) and did not differ from the parental variant in its pathogenicity for BHK-21 or RK-13 cells, or hemagglutinating activity, but formed smaller plaques. Persistent virus preserved the original antigenicity as judged from reciprocal hemagglutination-inhibition or plaque reduction-neutralization tests with polyclonal antisera. Both original and persistent rubella viruses were thermoresistant (T 56° C) and sligthly temperature-sensitive. Clonal analysis revealed presence of ts-mutants among both original and persistent virus clones with different degrees of plating efficiency at 40°/34° C. RNA fingerprinting showed only minor changes in persistent rubella virus.

KEY WORDS *Rubella virus, persistent infection, cell culture*

Many animals viruses are often prone to change their original properties during long-term persistence. These changes may involve reduced virulence for animals and/or tissue cultures (Holland *et al.*, 1980; Weiss *et al.*, 1980), poor replicative capability (Holland *et al.*, 1982), acquired ts - or small plaque phenotype (Youngner and Preble, 1980), and, rarely, altered antigenicity (Holland *et al.*, 1982). Alterations in persistent virus properties are currently attributed to multiple viral genome mutations which continuously evolve in the course of viral persistence (for review see Holland *et al.*, 1982).

Rubella virus which is strongly implicated in human degenerative disease - subacute sclerosing panencephalitis (SSPE) (Weil *et al.*, 1975; Cremer *et al.*, 1975), can easily establish persistent infection in numerous cell lines

(Stanwick and Hallum, 1974; Norval, 1979). However, rubella virus changes during persistence have been confined only to emergence of ts-mutants, or rarely occurring phenotypic mixing with endogenous retrovirus (Sato *et al.*, 1976). We obtained long-term rubella virus infection of HEp-2 cells which consistently yielded infectious small-plaque virus for more than 4 years of observation. This investigation was undertaken in order to detect possible changes in biological properties of persistent rubella virus.

A persistently infected (p.i.) culture was obtained by infection of HEp-2 monolayer cell culture with C-66 rubella virus strain at a multiplicity of 2 PFU/cell (C-66 is a clinical isolate obtained in 1973, which had undergone 2 and 11 passages in RK-13 and BHK-21 cell cultures, respectively). No signs of cytopathology were observed either at initial infection or at further propagation of p.i. cells. 5.5 months after establishment the p.i. culture was split into three, and each one was propagated at different temperatures: 34°C, 36°C, or 40°C. The only difference between p.i. and uninfected HEp-2 cells was a slightly retarded (1,5-fold) growth of p.i. cells.

During the whole period of observation (more than 4 years) the p.i. culture produced infectious rubella virus within the titre range of 4 - 6.3 logs PFU/ml. Persistent virus preserved cytopathic activity for RK-13 and BHK-21 cells, and formed plaques twice as small in size (I mm or less) compared to original virus. Hemagglutinating (HA) activity could be determined in concentrated persistent virus preparation from p.i. culture; otherwise persistent virus had to be amplified on BHK-21 cells (2-3 passages) to be detected in unconcentrated cultural fluid.

Antigenic properties of original and persistent viruses were studied in reciprocal neutralization and hemagglutination-inhibition (HI) tests with hyperimmune rabbit antisera to each variant virus. Both tests (table 1) showed original and persistent viruses to be very closely related, the respective antisera for these viruses giving the same homologous and heterologous titres. To check the possibility of phenotypic mixing between persistent rubella virus and endogenous retrovirus, as reported by Sato *et al.*, (1976), we performed plaque reduction-neutralization test with antiserum to HEp-2 retrovirus (kindly supplied by Dr. K.V. Ilyin). At a dilution of 1:5 effective for radioimmunoprecipitation of HEp-2 virus proteins (Morozov and Ilyin, 1983), the serum did not prevent plaque formation by persistent of parental rubella viruses up to 10^{-4} end point dilution of either virus, compared to untreated control virus preparations (data not shown).

Both original and persistent rubella viruses revealed similar capability for antibody induction in rabbits. Antibodies were detectable two weeks after virus inoculation (1:8) and peaked five weeks post inoculation (1:128) in a similar fashion for both viruses.

TABLE 1

Comparison of antigenicity of persistent and original rubella viruses in reciprocal plaque reduction-neutralization and HI tests

VIRUS	plaque reduction-neutralization test						HI-test				
	anti-C-66 persistent		anti-C-66 original		non-immune serum		anti-C-66 persistent	anti-C-66 original	anti**-C-44	ref.*** antiser.	non-serum serum
	1:8*	1:16	1:8	1:16	1:8	1:16					
C-66, persistent	11****	33	12	33	123	143	512*****	2048	2048	1024	8
C-66, original	3	7	1	5	123	120	512	2048	2048	1024	8
C-44	9	51	13	17	232	243	256	2048	1024	512	8

Cross plaque reduction-neutralization test employed heated (30 min, 56°C) hyperimmune rabbit antisera against original and persistent C-66 strain, and equal doses of infectious virus. After 1.5 h incubation under double agar overaly, as described by Desyatskova (1977). Hemagglutination-inhibition (HI) test was performed in 0.6 ml volumes of glucose-gelatose-veronal buffer, pH 7.2, supplemented with 0.25% of pigeon red blood cells and 0.1% of bovine serum albumin (fraction Y), an 4 units of rubella virus HA antigen.

*antibody dilution

**C-44 is another clinical isolate of rubella virus with passage history similar to that of C-66

***sheep reference antiserum against Sp-2 rubella virus strain was characterized previously (Desyatskova, 1977)

****mean number of plaques obtained from three parallel flasks

*****titre of antiserum (reciprocal value).

TABLE 2

Temperature sensitivity of parental and persistent ruballa virus clones

VIRUS	Titer (lg PFU/ml)		EOP (40°C/34°C)
	40°C	34°C	
Parental, uncloned	1.0	2.0	-1.0
Clone No. 5	6.87	6.95	-0.08
Clone No. 6	1.0	6.4	-6.4
Clone No. 12	4.95	6.0	-1.05
Clone No. 13	3.3	6.1	-2.8
Clone No. 16	6.94	6.98	-0.04
Clone No. 25	6.98	6.86	0.12
Persistent, uncloned	1.2	3.7	-2.5
+Clone No. 1	1.0	3.47	-3.47
+Clone No. 3	2.4	6.0	-3.6
+Clone No. 5	4.36	4.75	-0.39
+Clone No. 9	1.0	5.43	-5.43
+Clone No. 11	5.36	5.19	0.17
+Clone No. 12	6.87	6.94	-0.07
+Clone No. 14	6.71	6.56	0.15
+Clone No. 17	3.62	3.4	0.22
+Clone No. 19	1.0	4.23	-4.23
+Clone No. 21	4.2	6.58	-2.38

+ persistent virus was cloned at the level of 112 passages of p.i. culture maintained at 36°C.

Single plaque were picked out and transferred to tubes with monolayer RK-13 cell cultures. After the development of cytophatic effect cultural fluids were inoculated into BHK cell monolayers which were kept in parallel at 34°C, 40°C and 36°C for every clone. Virus harvests were titered 72 h later by plaquing. Efficiency of plating (EOP) was determined as a ratio of virus titre at 40°C to that at 34°C.

Temperature sensitivity studies of viral replication showed moderate ts-pattern of both original and persistent virus populations (table 2). Clonal analysis revealed presence of ts-mutants among viral clones with different degrees of EOP (Table 2). Three out of six original virus clones and 5 out of 10 persistent virus clones were ts-mutants with various degrees of EOP ranging from less than 1 lg to more than 6 logs PFU/ml. Despite long-term maintenance of one of the p.i. cultures at 40°C viral production continued at the level comparable to that of 34°C - or 36°C - maintained p.i.

cultures (4-6 logs PFU/ml). Moderate temperature-sensitivity of rubella viruses was not associated with increased thermolability of virus particle since heating at 56°C for 10 min brought less than 1 lg reduction in infectivity. In contrast, thermosensitive rubella strain Cendehill, included in the experiment as a positive control, lost 5 logs of infectivity titre after heating. Rubella virus as well as many other viruses, is known to be able to generate defective interfering (DI) particles, both in primary (Bohn and Van Alstyne, 1981) and in persistent (Norval, 1979) infection. Typical DI particle-enriched viral preparations are characterized by increased HA/infectivity ratio, as compared to standard virus preparations (Huang and Baltimore, 1979). To consider the possibility of DI particle production in our p.i. cells, persistent virus taken directly from p.i. culture, and BHK-21-grown, low multiplicity passaged original C-66 virus were pelleted from cultural fluid at 110 000 g for 2 h. Pellets were suspended in STE buffer (0.1M NaCl; 0.01M tris, pH 7.8; O,001M EDTA), clarified at 5 000 g, 20 min and layered on 5-50% linear sucrose gradient made in STE buffer. Centrifugation was at 25 000 rpm for 18 h in 3 x 25 ml rotor of MSE SS 65 centrifuge. The virus was positioned by HA, and buoyant density of every fraction was determined by refractometry. Both viruses formed a single band which peaked at a density of 1.19 - 1.20 g/ml (not shown), characteristic cor rubella virus (Norval, 1979). Comparison of HA/infectivity ratio of peak fractions revealed similarity between the two viruses (2.4×10^{-7} and 3.9×10^{-7} for original and persistent virus, respectively). Titration of less dense fractions of gradients (1.17,1.15 g/ml) that reportedly may contain DI rubella virus particles (Bohn and Van Alstyne, 1981) did not reveal HA/infectivity ratio differences either. Coupled with our observations on the absence of autointerference phenomenon in persistent virus preparation, when titered by plaquing, these findings suggest the lack of detectable DI particle production in HEp-2 p.i. cells.

Since the discovery that persistent infection generates multiple viral genome mutations (Holland *et al.*, 1979) the latter have been reported to occur in other viral p.i. systems including togaviral ones (Meinkoth and Kennedy, 1980; Eaton, 1982). We compared two representative non-ts viral clones of original and persistent rubella virus (namely, No. 16, original, and No. 14, persistent, see table 2) by T_1 oligonucleotide mapping. RNA was isolated from cloned, concentrated and gradient-purified virus by proteinsas K-sodium dodecylsulfate-phenol treatment, as detailed elsewhere (Andzhaparidze *et al.*, 1982). Labelling the RNAse T_1 digest with 5'-hydroxyl polynucleotide phosphokinase and γ-^{32}P ATP, and two-dimensional gel electrophoresis were performed essentially as described by Pederson and Haseltine (1980). T_1 oligonucleotide maps revealed the complex nature and almost complete identity of original and persistent rubella virus RNAs, but only one spot unequivocally missing from the former (data not

shown). Since no evidence of DI particle presence in persistent virus preparation was found the contribution of defective genomes into these negligible ologonucleotide maps changes seems unlikely. Therefore, one ologonucleotide loss from parental virus RNA may attest to only slight genome mutations of persistent virus, thus providing another indication of rubella virus stability during persistence.

The general conclusion that can be drawn from the data pesented is that rubella virus remained biologically stable despite long-term persistence in cell culture. One clear difference from the parental variant - diminished plaque size of persistent virus, is a characteristic feature of almost every persistent virus type, and might be due to numerous factores, e.g., reduced ability to spread within infected cells, slow-down of viral RNA synthesis, or altered capacity of virion glycoproteins to bind to sulfated polyanions in the overlay during plaque formation (Eaton, 1982).

Other viral properties, namely, HA ability, antigenicity, immunogenicity and thermostability, remained unchanged. In fact, there are few reports on viral antigenic changes during persistence, as revealed by polyclonal (Holland *et al.,* 1982) or monoclonal (Prabhakar *et al.,* 1983; Sheshberadaran *et al.,* 1985) antibodies. Much more often persistent viruses remain antigenically indistinguishable from their parental variants (Igarashi, 1979; Weiss *et al.,* 1980; Peeples and Levine, 1981; Rozhon *et al.,* 1983).

Acquisition of temperature-sensitivity by persistent viruses and selction of ts-mutants in the course of persistence is a characteristic feature of numerous p.i. cultures (for review see Youngner and Preble, 1982), and is considered one of the general mechanisms of viral persistence. Williams *et al.,* (1981) presented the evidence of ts-mutant selection in persistent rubella virus infection of human cells culture. In our case, however, both original and persistent rubella viruses contained ts- and non-ts viral clones. This finding suggest that selection of ts-variants, if any, did not lead to substitution of the entire persistent virus population for ts-mutants. Moreover, maintenance of p.i cultures at 40°C, a temperature non-permissive for ts-clone replication, allowed the rest of persistent virus to replicate at a rate comparable tot hat of the virus from 36°C - or 34°C - maintained p.i. cultures. These results suggest the lack of interferring ability of these ts-mutants against non ts-virus variants, and argue against a critical role of ts-mutants in maintenance of rubella virus persistence in this p.i. culture.

Stability of biological properties of rubella virus after year of persistence could explain the relative ease with which the virus could be isolated and further propagated in rubella SSPE (Weil *et al.,* 1976; Cremer *et al.,* 1976), compared to measles SSPE (Katz and Koprowski, 1973), where many isolation attempts have failed, or isolated measles virus remained strongly cell-associated.

Our results also support the contention that no antigenic variants of rubella virus exist (and have been observed, as yet) in nature and among vaccine strains.

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