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A highly cytopathogenic influenza C virus variant induces apoptosis in cell culture

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An influenza C virus variant, C/AA-cyt, was identified as the agent responsible for highly effective induction of cytopathogenicity in MDCK cells. The cytopathogenic effect was manifested by cell rounding, cell shrinkage and foci of cell destruction leading finally to disruption of the monolayer in a virus dose-dependent manner. Virus-induced cytopathogenicity was suppressed by temperatures nonpermissive for virus replication. Maintenance of plasma membrane integrity post-infection, in connection with induction of a DNA fragmentation ladder, revealed the characteristic picture of apoptosis. In support of this, quantitative analysis demonstrated high levels of apoptosis-like oligonucleosomal DNA. The results indicate that influenza C viruses can induce programmed cell death, as formerly reported for influenza type A and B viruses.

Highly effective induction of cytopathogenic effect (CPE) in MDCK cells was observed after infection with a spontaneous variant of influenza C/Ann Arbor/1/50 virus, designated C/AA-cyt. This property is very unusual amongst influenza C viruses, which tend to follow a productive, non-lytic cycle. The C/AA-cyt virus variant was obtained in the laboratory by passaging the persistent virus C/AA-pi (Camilleri & Maassab, 1988; Marschall *et al*., 1993, 1996) 11 times in the allantoic cavity of embryonated hen's eggs. Virus adaptation and improvement of virus yield was achieved by variant selection in embryonated eggs, as shown for other influenza viruses (reviewed by Robertson, 1993; Hardy *et al*., 1995). Infectivity of the C/AA-cyt variant, as indicated by progeny virus production, was very high in comparison to other strains and to C/Ann Arbor/1/50 wild-type virus. Here, we present evidence for the mode of host cell death induced by this new virus variant.

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Host cell death has been reported to occur by either necrosis or apoptosis. Necrosis is typified by loss of membrane integrity which leads to an influx of extracellular fluid and consequently to swelling and cell disruption (Wyllie *et al*., 1980). In contrast, apoptosis is described as a physiological, programmed cell death typically correlating with preserved membrane integrity, specific endonucleosomal cleavage of the DNA (Jones *et al*., 1989), cell rounding and cell shrinkage (Wyllie *et al*., 1980). Apoptosis is induced by a wide variety of stimuli, as well as by viruses (reviewed by Shen & Shenk, 1995). Although, in the case of influenza viruses, the mechanisms are still unknown, experimental approaches have clearly shown that type A as well as type B viruses can induce apoptosis in cell culture (Hinshaw *et al*., 1994). In the case of influenza A virus, morphological CPE was demonstrated to result from apoptosis in cultured cells (Takizawa *et al*., 1993; Hinshaw *et al*., 1994) and also in experimental animal infections (Mori *et al*., 1995). CPE and apoptosis caused by influenza C virus have not been investigated in depth.

In our study, confluent MDCK cell monolayers were infected with decreasing concentrations [64 to 2×10^{-6} haemagglutinin (HA) U/ml] of C/AA-cyt virus inoculum. Cells were grown in Dulbecco's modified Eagle medium containing 10% (v/v) foetal calf serum and infected for 1 h, followed by rinsing and cultivation at 33 °C in fresh medium containing 2% foetal calf serum. The CPE of infected monolayers was determined by light microscopy and the haemagglutination titres of progeny virus were determined by the standard microtitre method using 1% chicken erythrocytes. Beginning at 1 day post-infection (p.i.), cells infected with high C/AA-cyt virus concentrations showed signs of severe cytopathogenicity. The cells rounded up, started shrinking and floating into the supernatant, and foci of cell destruction appeared. This kind of CPE was also found with high inoculum dilutions (up to 0·125 HAU/ml) at which wild-type virus C/AA-wt, in comparison, caused no CPE (data not shown). The dependence of CPE on infectious inoculum, that is active virus synthesis, was demonstrated by an *in situ* esterase neutralization assay (Fig. 1). C/AA-cyt virus was neutralized with various volumes of a specific antiserum by incubation for 1 h at room temperature with gentle agitation. After neutralization, volumes were made up to 500 µl by the addition of PBS and were inoculated into

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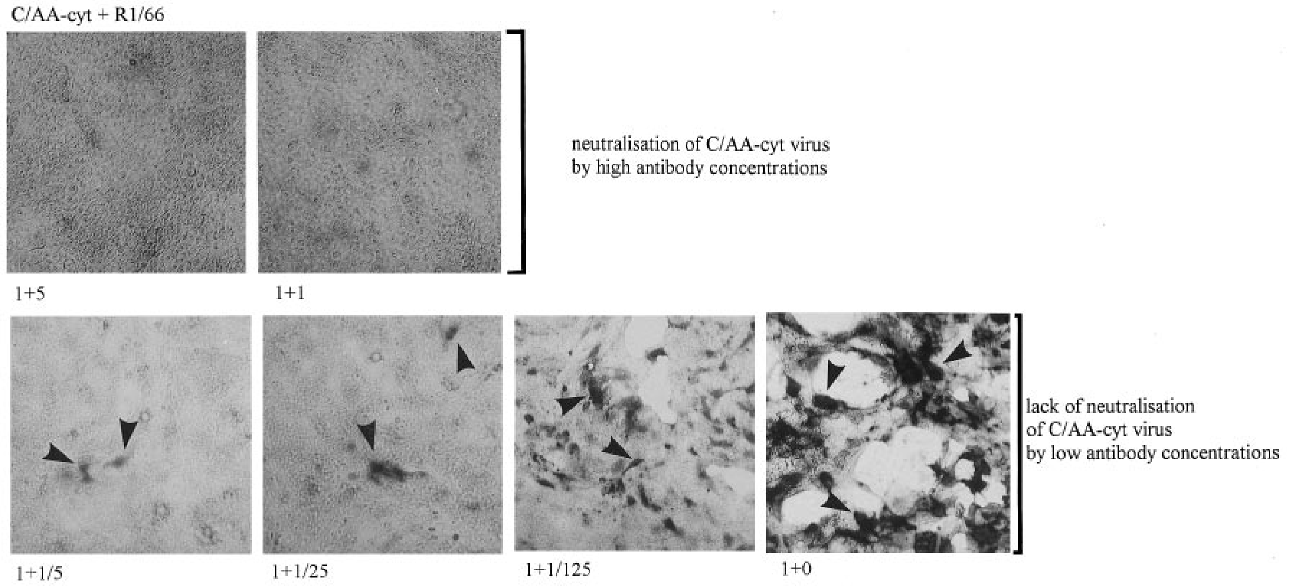

Fig. 1. Correlation between C/AA-cyt virus replication and CPE in MDCK cells, detected by an *in situ* esterase neutralization assay. C/AA-cyt virus (32 HAU/ml) was preincubated with different concentrations of specific antiserum R1/66 (1 + 5, 1 + 1, 1 + 1/5, 1 + 1/25, 1 + 1/125 and 1 + 0, volumes) before infection of MDCK cells in a total volume of 500 µl. *In situ* detection of infected cells was done 3 days p.i., visualizing virus esterase activity of the surface glycoprotein HEF (arrowed).

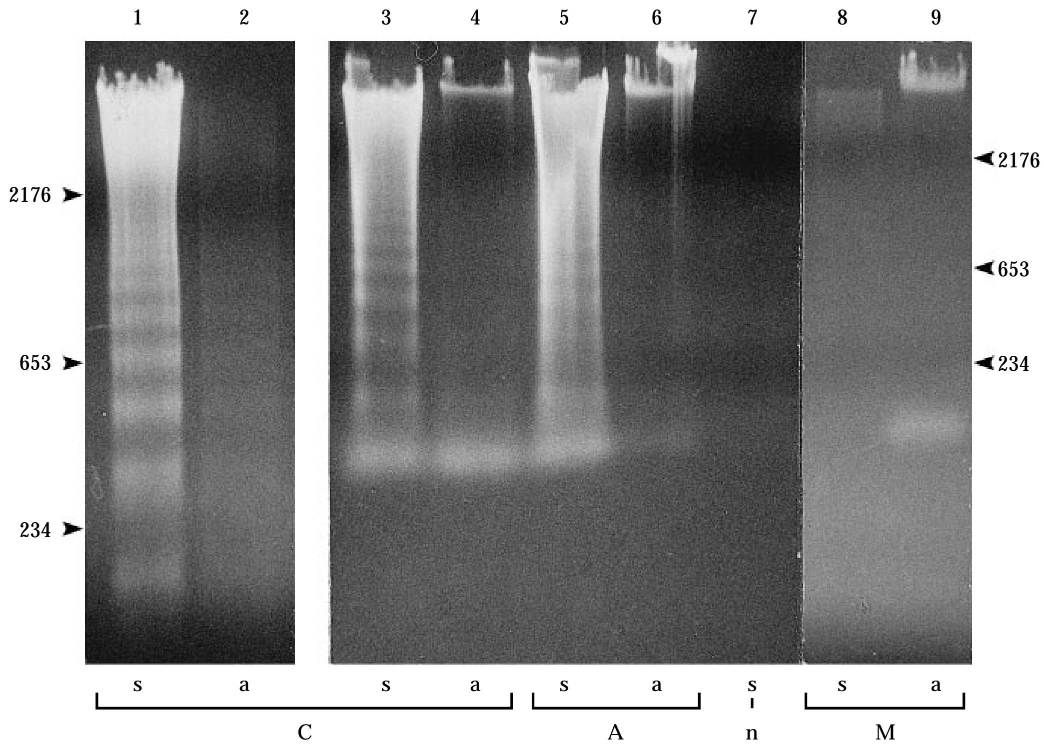

Fig. 2. DNA ladder assay showing virus-induced DNA fragmentation. MDCK cells were infected at 33 °C with C/AA-cyt virus (C) or A/PR/8/34 virus (A). Equal numbers of total cells were harvested in two fractions 2 days p.i. The two fractions [adherent cells (a) and cells floating into the supernatant (s)] were examined for apoptotic DNA. Uninfected cells (M) and necrotic cells (n, heat-treated) were used as controls. DNA fragments were visualized by ethidium bromide staining on 1·2% agarose gels. Molecular masses are in bp.

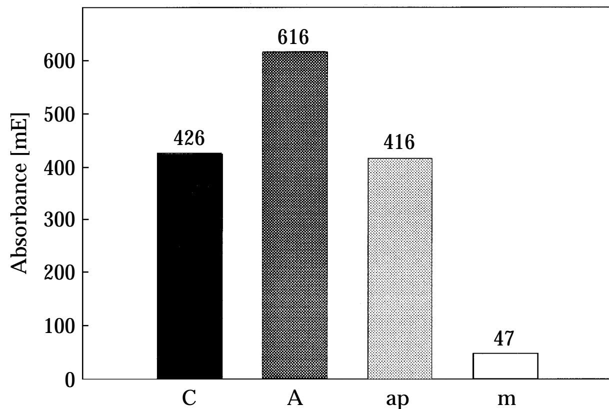

Fig. 3. Quantitative evaluation of apoptosis in infected MDCK cells by an ELISA that determines cell death. MDCK cells were infected at 33 °C with C/AA-cyt virus (C) and A/PR/8/34 virus (A) and the total supernatant and adherent cells were harvested 1 day p.i. Cells treated with hypertonic buffer (ap) and mock-infected cells (m) were assayed as controls. Cytoplasmic mono- and oligonucleosome release was detected by the use of antibodies in a quantitative ELISA procedure. The relative values of $A_{405}-A_{630}$ are given after subtraction of a blank control (*A*, 35 mE). The diagram represents experimental mean values.

confluent MDCK cells. Intracellular virus production was detected 3 days p.i. together with CPE formation. Staining of infected cells based on the esterase activity of the viral glycoprotein HEF was performed as described previously (Wagaman *et al.*, 1989; Marschall *et al.*, 1996). In this experiment, preincubation of virus with high antibody concentrations of R1/66 (hyperimmune rabbit antiserum raised against influenza C/Johannesburg/1/66 virus; 1:5 and 1:1, v/v) totally prevented infection of cells, as illustrated by the lack of esterase staining and of CPE (Fig. 1, upper part). Virus preincubation with decreasing volumes of R1/66 antiserum (1:0·2 to 1:0), however, led to increasing esterase activity and cell destruction (Fig. 1, lower part). Thus, CPE is linked to active virus synthesis in the host cell, as measured by the activity of expressed HEF protein. In addition, a nonpermissive incubation temperature of 37 °C instead of 33 °C suppressed virus production and CPE, although virus uptake did occur in this case as demonstrated by virus-specific RT–PCR (data not shown).

In order to characterize cell death induced by C/AA-cyt virus, the plasma membranes of infected cells were examined by staining with trypan blue. Dye exclusion was noted for C/AA-cyt virus- and influenza A/PR/8/34 virus-infected cells (a positive apoptosis-inducing control) from the culture supernatant whereas uninfected cells heat-treated at 90 °C for 15 min (a negative necrosis-inducing control), stained bright blue (data not shown). Therefore, virus infection did not interfere with the plasma membrane integrity, indicating that the mode of host cell death was not by necrosis.

To investigate the possibility of apoptotic cell death induced by C/AA-cyt virus, the fragmentation of nuclear DNA in infected cells was examined (Fig. 2). A DNA ladder assay was performed, as basically described by Bissonnette *et al.* (1992). For this, $1{\cdot}2\times10^6$ MDCK cells were infected with C/AA-cyt virus (8 HAU/ml), and with A/PR/8/34 virus (optimal dose generating 90% cell destruction, as a positive apoptosis-inducing control). Heat-treated cells and uninfected cells served as negative controls. Cells were harvested 2 days p.i., when infected monolayers showed strong cell destruction. Detached cells, harvested from the culture supernatant (s), and adherent cells (a) were analysed separately. Typical 200 bp DNA ladders were visualized in the cell fractions from the supernatants of C/AA-cyt virus-infected cells (lanes 1, 3) and of A/PR/8/34 virus-infected cells (lane 5). DNA fragmentation was also detected for adherent cells (lanes 2, 4 and 6), but to a much weaker extent. Necrotic cells (lane 7) and mock-infected cells (lanes 8 and 9) were devoid of any ladder formation. These findings indicate that the mode of host cell death for C/AA-cyt-infected cells was by apoptosis.

Quantitative evaluation of apoptosis in infected MDCK cells by an ELISA that determines cell death was done as per the manufacturer's protocol (Boehringer Mannheim), with 2×10^5 cells per reaction (Fig. 3). This assay is based on detection of mono- and oligonucleosomes by coated anti-histone antibody– and anti-DNA antibody–peroxidase conjugate. As described above, MDCK cells were infected with C/AA-cyt virus (C) and, for comparison, with an apoptosis-inducing agent, influenza A/PR/8/34 virus (A). Cells were harvested 1 day p.i. To provide a positive apoptosis control, cells were treated with hypertonic buffer (ap). Mock-infected cells (m) served as a negative apoptosis control. All absorbance values were corrected using a blank control without cell lysate. As shown in Fig. 3, significant absorbance values were obtained in C/AA-cyt virus-infected cells, A/PR/8/34-infected cells and the apoptosis control. Mock-infected cells and a necrosis-inducing control (heat-treated cells; data not shown) did not produce increased apoptosis values. The data demonstrate that mono- and oligonucleosome formation occurs during C/AA-cyt virus-induced cell death.

The role of apoptosis within the life cycle of viruses has been assessed in several ways. The fact that many viral infections can trigger programmed cell death is considered to represent a host defence mechanism (reviewed by Williams & Smith, 1993). It is interesting, however, that in the case of influenza A virus apoptosis may be important for optimal virus production (Olsen *et al.*, 1996). Moreover, there is mounting evidence for some viruses that the induction of apoptosis contributes directly to their pathogenesis, a model that is also discussed in the context of influenza A virus infections (Hinshaw *et al.*, 1994). In the case of influenza C viruses, there are conflicting ideas as to whether virus infection leads to clinical symptoms in humans. In some cases, influenza C viruses have been isolated from patients suffering from severe influenza symptoms (Manuguerra *et al.*, 1993; J. C. Manuguerra, Genetique Moleculaire Virus Respiratoire, Institut Pasteur, Paris, personal communication). Two of these

isolates, which were included preliminarily in our studies, were categorized as *in vitro* cytopathogenic viruses. The morphological signs of CPE following infection of MDCK cells, under the conditions described above, were comparable to those in C/AA-cyt-infected cells (unpublished observation). Whether the described CPE-positive phenotype of influenza C viruses is generally connected with increased pathology in humans remains to be investigated.

As a working concept for the C/AA-cyt virus variant, the course of cell death *in vitro* develops as follows: firstly, cell rounding, shrinkage and floating into the supernatant occurs; secondly, the main process of nuclear DNA fragmentation becomes evident in detached cells. This concept is based on the finding that adherent cells expressed only rare and limited apoptotic signals. However, the presence of weak apoptotic signals in adherent cells indicates that DNA fragmentation may begin in the first phase.

In summary, a cytopathogenic influenza C virus was characterized by the following: (i) highly effective induction of CPE, (ii) correlation of CPE with active virus synthesis, (iii) maintenance of plasma membrane integrity and (iv) nuclear DNA fragmentation. These findings indicate that, despite the profound differences with respect to influenza virus types A and B, influenza C viruses are also capable of inducing apoptosis.

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