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Spontaneous excretion of virus from MDCK cells persistently infected with influenza virus A/PR/8/34

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When MDCK cells in a semiconfluent monolayer were infected with 5 p.f.u. per cell of influenza virus A/PR/8/34 (H1N1), a majority of the cells continued to grow stably upon subsequent cultivation with a growth medium containing 50% foetal calf serum. While growing, the cells spontaneously excreted virus, the amount of which declined gradually as the passage number of the cells increased. The extent of virus shedding was significantly increased when the cells were subsequently maintained in a medium containing 0·2% bovine serum albumin. Within the cells, viral messenger RNAs for all eight genes of A/PR/8 were demonstrated by PCR indicating that endogenous viral genes were constitutively transcribed. However, viral proteins as well as viral genes were not demonstrable by radioimmunoprecipitation or ribonuclease protection assays, respectively.

Several investigators have described persistent infection with influenza virus (De & Nayak, 1980; Frielle *et al.*, 1984; Goshima & Maeno, 1989; Marschall *et al.*, 1993) or long-term persistence of viral genomes in susceptible host cells (Cane *et al.*, 1987; Cane & Dimmock, 1990; Urabe *et al.*, 1992, 1993). In addition, Urabe *et al.* (1994) presented evidence that persisting viral genes were self-amplified during their persistence within cells. These results clearly indicate that two quite heterogeneous genetic systems, namely that of influenza virus and that of host cells, are not mutually exclusive, and that the genome of even a highly cytolytic virus such as influenza virus can remain in the host cell for a considerable time under certain conditions. Defective interfering (DI) virus is reportedly involved in the establishment of persistent infection with influenza virus (De & Nayak, 1980; Frielle *et al.*, 1984).

We infected a semiconfluent monolayer of 5×10^5 MDCK cells with 5 p.f.u. per cell of plaque-purified wild-type influenza virus A/PR/8/34, free from a significant amount of DI virus. After 30 min at room temperature, Eagle's minimum essential medium (MEM) supplemented with 0·2% bovine serum albumin (BSA) (MEM + BSA) was added, and the cells were incubated for 1 h at 34 °C. The cells were then washed five times with PBS, and incubated with MEM supplemented with 50% foetal calf serum (FCS) (MEM + 50% FCS) at 34 °C. A majority of the cells continued to grow stably without any appreciable crisis. For stable growth of the infected cells, 50% FCS was required. Less concentrated FCS in the growth medium resulted in eventual destruction of the culture.

At appropriate times post-infection (p.i.), the culture medium was removed for infectivity assay. The cells were then washed five times with PBS and re-fed with fresh MEM + 50% FCS, or subcultured. Infectivity of the culture was more than 10 p.f.u. per cell during the first 4 days, and gradually decreased with time of incubation to less than 1 p.f.u. per cell on day 7 p.i. and later. The doubling time of the cells was 2 days. Morphologically, the cells were diffuse, thin, and less compact than MDCK cells, and were designated P/CK cells. After day 10 p.i., an aliquot of the cells was grown in the presence of an antiserum to A/PR/8 (anti-PR/8) to prevent secondary infection of the cells by virus consistently present in the culture medium (P/CK, S-line).

Semiconfluent monolayers of P/CK cells (S-line) at various passage levels were washed five times with PBS, and further incubated with MEM + 50% FCS or MEM + BSA without anti-PR/8 at 34 °C. Prominent cells lysis was observed in the latter (Fig. 1 *a*). At 20, 40 or 60 h p.i., the cultures were harvested and their infectivity was titrated by plaque assay in MDCK cells. The result with P/CK cells on day 20 p.i. is shown in Fig. 1(*b*). The amount of virus shed from the cells incubated with MEM + BSA was greater than that from the cells maintained in MEM + 50% FCS. Spontaneous excretion of virus from P/CK cells grown in the absence of anti-PR/8 was observed until 25 days p.i. However, the virus was inducible from the S-line of P/CK cells until 40 days p.i. but not later.

These findings confirmed the earlier observations that influenza viral genes remain (and retain their functions) within growing cells for periods of time. In addition, they suggest the presence of a host factor expressed only in growing cells that

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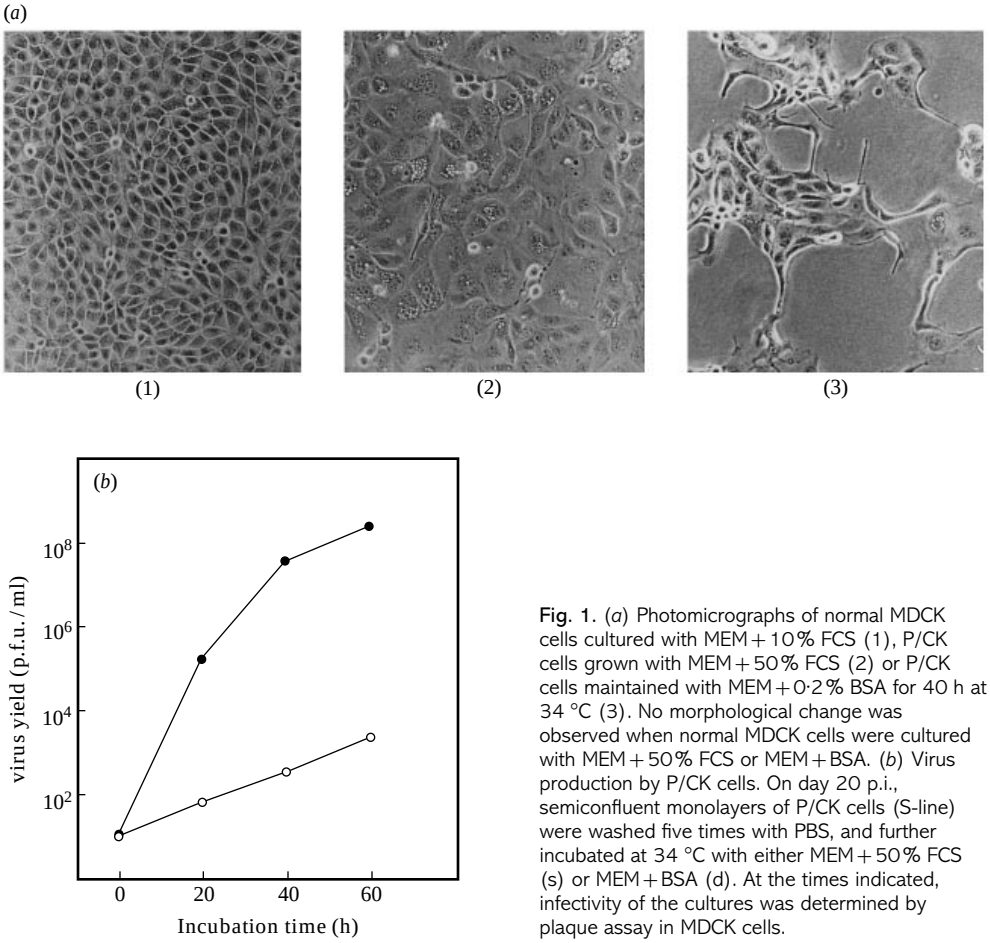

Fig. 1. (*a*) Photomicrographs of normal MDCK cells cultured with MEM + 10% FCS (1), P/CK cells grown with MEM + 50% FCS (2) or P/CK cells maintained with MEM + 0·2% BSA for 40 h at 34 °C (3). No morphological change was observed when normal MDCK cells were cultured with MEM + 50% FCS or MEM + BSA. (*b*) Virus production by P/CK cells. On day 20 p.i., semiconfluent monolayers of P/CK cells (S-line) were washed five times with PBS, and further incubated at 34 °C with either MEM + 50% FCS (s) or MEM + BSA (d). At the times indicated, infectivity of the cultures was determined by plaque assay in MDCK cells.

suppresses the function of endogenous virus genes (Fig. 1*b*). On the other hand, P/CK cells require 50% FCS for optimal growth and their doubling time was 2 days, indicating that persisting viral genes also in some way regulate division of host cells.

For influenza viral genes to persist within cells, endogenous virus genes must be self-amplified to some extent so that they are not diluted out by cell division. For viral genes to be amplified, viral polymerase in addition to NP protein are essential (Luytjes *et al.*, 1989; Huang *et al.*, 1990). Consequently, transcription and translation of endogenous viral genes must be constitutively taking place. To see if viral mRNAs could be readily detected in P/CK cells, we extracted RNA from the S-line of P/CK cells on day 25 p.i., according to the methods described by Chomczynski & Sacchi (1987); the poly(A)-containing fraction was selected with a plastic plate to which oligo(dT) had been fixed (GenePlate, Hitachi Chemicals, Japan) (Mitsuhashi *et al.*, 1992). DNA complementary to mRNA was synthesized on the same plate by adding Moloney murine leukaemia virus reverse transcriptase (Wako), and dATP, dCTP, dGTP and dTTP for 1 h at 37 °C. Gene-specific sequences were amplified from the products by PCR using AmpTaq polymerase (Takara, Japan), and primer pairs that were specific to the genes of influenza virus A/PR/8 (Table 1), by 25 cycles of denaturation at 95 °C for 1 min, followed by annealing at 55 °C for 30 s and elongation at 72 °C for 2 min on a DNA Thermal Cycler (Perkin-Elmer-Cetus). The same procedure was repeated twice. The PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide (Fig. 2). With every primer pair, PCR product with the expected molecular size was unambiguously demonstrated, indicating that endogenous viral genes were routinely transcribed. However, the same RNA preparations did not contain sufficient amounts of vRNA to be detected by a ribonuclease protection assay (RPA) (data not shown).

On day 30 p.i., S-line P/CK cells were metabolically labelled with 120 μCi/ml [^{35}S]methionine (1175·0 Ci/mmol, DuPont/NEN) for 4 h at 34 °C. Viral proteins were precipitated with a 1 : 200 dilution of anti-PR/8 and analysed by PAGE (RIPA). MDCK cells labelled for 30 min at 5 h after infection with 10 p.f.u. per cell of A/PR/8 and then similarly treated were included. Although PB1, PB2, PA, HA, NP, M1 and NS1 were evident in MDCK cells acutely infected with the virus, none was demonstrable within P/CK cells (data not shown), probably reflecting a low rate of viral protein synthesis within the cells.

Table 1. PCR primers used to detect viral mRNAs in P/CK cells

The primers were synthesized on an automated DNA synthesizer model 380B, Applied Biosystems. The numbers above oligonucleotides indicate their positions on the respective genes in the plus sense.

Gene	Plus sense	Minus sense	Expected molecular size (nt) of product
PB2	52 5'-AATCTAATGTCGCAGTCTC-3' 70	868 5'-ATAAAGATGCTAGTGGGTC-3' 850	817
PB1	81 5'-CACAACTTTCCCTTATACC-3' 99	815 5'-TCACATATACTCCTTGCCA-3' 797	735
PA	26 5'-TGGAAGATTTTGTGCGAC-3' 43	731 5'-AATCCATCCACATAGGCT-3' 714	706
HA	27 5'-ACCAAAATGAAGGCAAACC-3' 45	640 5'-TTACTGTTAGACGGGTGAT-3' 622	614
NP	72 5'-TTACGAACAGATGGAGACT-3' 90	622 5'-ATTCCATCACCATTGTTCC-3' 604	551
NA	53 5'-ATCAATCTGTCTGGTAGT-3' 79	425 5'-CTTAACAGTCCCATTTGA-3' 408	373
M	26 5'-ATGAGTCTTCTAACCG-3' 41	315 5'-ACTGCTTTGTCCATGT-3' 300	290
NS	3 5'-CAAAAGCAGGGTGACAAA-3' 20	260 5'-TTTAAGTGCCTCATCCGA-3' 243	258

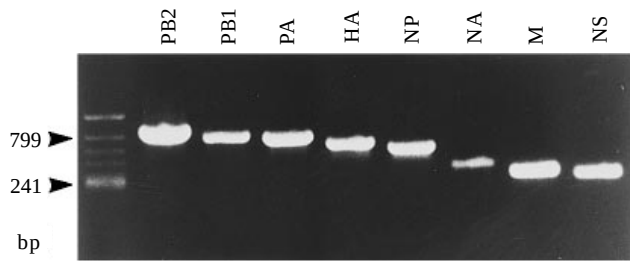

Fig. 2. Detection of viral mRNAs in P/CK cells by PCR. On day 25 p.i., poly(A)-containing RNA was selected from the S-line of P/CK cells, and reverse transcribed to complementary DNA. Virus-specific sequences were amplified by PCR, using gene-specific primer pairs and AmpTaq DNA polymerase. PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.

Possible, endogenous viral genes were regulated in growing P/CK cells so as to be expressed to an extent that is only sufficient to enable gene amplification, but not to cause cell destruction or allow either viral genes or viral proteins to be detectable by conventional RIPA or RPA.

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