Mumps Virus-persistently Infected Cell Cultures Release Defective Interfering Virus Particles

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SUMMARY

Two human cell cultures, HEp-2 and L-41, persistently infected with mumps virus, produced small amounts of slowly replicating small-plaque infectious virus and detectable amounts of defective interfering virus particles which were similar to standard mumps virus in polypeptide composition, contained subgenomic size RNAs and interfered with standard mumps virus replication in susceptible cells.

Defective interfering virus particles (DIP) have been recognized as inevitable products of virus replication, implicated in establishment and/or maintenance of virus persistence (Huang & Baltimore, 1970). This is particularly true for persistently infected cell types which are genetically deficient in some aspects of the interferon system (Kawai et al., 1975; Rima et al., 1977; Roux & Holland, 1979). However, the ultimate role of DIP in ‘cell-virus persistence’ (Sekellick & Marcus, 1979) is not so clear when a non-cytopathic type of virus persistence takes place (Andzhaparidze et al., 1981a). Mumps virus, a typical paramyxovirus, can easily establish a carrier state in several cell types (Walker & Hinze, 1962; Truant & Hallum, 1977; McCarthy et al., 1981), but DIP involvement in mumps virus persistence has only recently been documented (McCarthy et al., 1981). These data need confirmation and further extension in order to characterize extracellular mumps DIP. We report here on DIP of mumps virus produced by two long-term persistently infected (PI) human cell cultures.

Persistent infections were established in HEp-2 and L-41 [a clone of the J-96 cell line of Osgood & Brooke (1955)] human cells by the Leningrad-3 (L-3) vaccine strain of mumps virus (Andzhaparidze et al., 1981b). The original L-3 virus was maintained as a low-multiplicity passage virus in primary quail embryo (QE) cell cultures, and shown to be essentially free of detectable amounts of DIP (O. G. Andzhaparidze et al., unpublished results). No signs of cytopathology were observed during the establishment and further maintenance of these PI cultures, whereas about 80 to 100% of cells contained intracellular virus antigen, as revealed by immunofluorescence. Starting from early passages of PI cultures, infectivity titres gradually declined, until at the 100th passage they were $10^2$ to $10^3$ p.f.u./ml. On QE monolayers, persisting viruses induced the formation of slowly developing pin-point plaques (20 to 30 days post-inoculation), whereas the original QE cell-propagated virus produced clearly discernible plaques 1 to 1.5 mm in diameter 7 to 10 days after inoculation.

Culture fluids from PI cultures of various passages were tested for the presence of interfering activity. To this end, QE cell monolayers were infected with samples of undiluted culture fluid from PI cultures supplemented with $10^4$ p.f.u. of original L-3 mumps virus. The harvest was titrated 72 h later on QE cells by haemadsorption. A marked reduction of infectivity has been reproducibly observed in the mixed infections, compared to single infections by standard mumps virus alone. The degree of reduction ranged from 2-5 to 5 logs TCID$_{50}$/ml for unconcentrated culture fluid from PI cultures. For example, the final titres of the mixed infections with culture fluids from PI L-41 of passage 110, PI L-41 of passage 14, PI HEp-2 of passage 87, and a mock-infected L-41 culture were 4-5, 1-0, 2-0 and 7-0 logs TCID$_{50}$/ml respectively. After a 100-fold concentration of culture fluid by ultracentrifugation, the interfering ability increased from 5 to 7 logs TCID$_{50}$/ml. This observation suggested that the interfering activity was sedimentable. No haemadsorbing virus could be detected in QE cells infected solely with culture fluid from PI cultures. Culture fluids from uninfected control L-41 or HEp-2 cells displayed no interfering activity.
When culture fluids from PI cultures were exposed to u.v. light at 0.46 J/m²/s for 1 min, the interfering activity was reduced by a factor of 10².

The L-41, but not HEp-2, PI culture was found to produce interferon in low amounts detectable, at 1:8 dilution of culture fluid, by the conventional technique. However, its contribution to the interference phenomenon on QE cells seemed unlikely due to the species specificity of interferon. To investigate the possibility that the interfering activity observed was due to interferon induction in QE cells by persisting viruses (which presumably increased their interferon-inducing capacity after long-term persistence), we infected QE cells with 1:2 dilutions of culture fluids from PI cells. Vesicular stomatitis virus (VSV) was titrated on both infected and uninfected cells 72 h later. No differences in VSV titres were observed, suggesting that the decrease of standard virus titres in mixed infections was not due to interferon induction in QE cells by persisting viruses.

To characterize the putative DIP released from PI cultures, serum-free culture fluids were clarified by centrifugation at 5000 g for 20 min. Infectious virus was pelleted at 23 500 rev/min for 2 h in the 10 × 100 ml rotor of an MSE SS-65 centrifuge. The pellet was resuspended in 0-5 to 0-8 ml of NTE buffer (0.1 M-NaCl, 0.01 M-Tris–HCl pH 7.8, 0.001 M-EDTA) and clarified again at 5000 g for 20 min. The virus suspension was layered on a 10 to 55 % linear sucrose gradient prepared in NTE and centrifuged in a 3 × 25 ml MSE rotor at 23 000 rev/min for 18 h. L-3 mumps virus from QE primary-infected cells was similarly processed and used as a control standard virus. The position of the virus in the gradients was determined by haemagglutination with chick red blood cells or by haemadsorption on QE cells, and was found to be in the region 1.18 to 1.20 g/ml, corresponding to the buoyant density of mumps virus (McCarthy & Johnson, 1980b). It should be pointed out that only QE cell-propagated mumps virus caused haemagglutination. The L-41 or HEp-2 cells upon primary infection produced up to 10⁸ to 10⁹ p.f.u./ml of infectious virus which did not haemagglutinate, even after concentration and purification. This absence of haemagglutinating ability correlated well with a reduced amount of haemagglutinin (HN) polypeptide in L-41- or HEp-2-propagated mumps virus (Fig. 1) and the appearance of spikeless virions upon electron microscopic examination (not shown).

For protein analysis, the virus was pelleted from NTE-diluted fractions by centrifugation and lysed in sample electrophoresis buffer (5 M-urea, 2 % SDS, 0-1 M-dithiothreitol). Electrophoresis was performed in a 10 % polyacrylamide slab gel, with a 5 % stacking gel, at 100 V for 5 h. Mol. wt. standards were bovine serum albumin (68K), ovalbumin (45K), pepsin (35K) and trypsinogen (24K). Gels were stained with Coomassie Brilliant Blue R-250, destained and scanned in a Joyce–Loebl microdensitometer.

Purified extracellular material from PI cultures had a polypeptide composition very similar to that of purified infectious virus (Fig. 1). The protein profile of the material from PI cultures could not be attributed solely to concentrated infectious complete virions since there was no polypeptide profile when the equivalent of 10⁵ to 10⁶ p.f.u. of dissociated infectious mumps virus (the calculated amount of infectivity after 10³-fold concentration of persisting viruses) was run on a gel (not shown).

The reduction of HN protein content in L-41- or HEp-2-propagated mumps virus may be explained in terms of host cell-induced modification of the virus envelope and deserves separate investigation.

Next, we performed an analysis of the RNA present in extracellular material from PI cells. Cultures were labelled in phosphate-free medium with [³²P]orthophosphate (Amersham International) for 72 h, starting 24 h post-infection (L-41 cells, primary infection, 50 μCi/ml), or 48 h after cell transfer (L-41 PI cells, 100 μCi/ml). Labelled virus was concentrated, purified and pelleted from gradient fractions of density 1.18 to 1.20 g/ml. RNA was isolated by proteinase K treatment as described by Wunner & Clark (1980) and two subsequent deproteinization steps with phenol–chloroform (1 : 1). Ethanol-precipitated RNA was washed in 70 % ethanol containing 50 mM-NaCl, dried in vacuo and denatured in glyoxal–dimethyl sulphoxide (DMSO) buffer, as described by Meinkoth & Kennedy (1980). Electrophoresis was performed in 1-2 % agarose vertical slab gels at 35 V for 14 h. Gels were soaked in 95 % ethanol, dried on to Whatman paper and scanned in a Dünnschicht-Scanner II (Berthold LB 2723 Model, Camlab, Cambridge, U.K.).
Fig. 1. Microdensitometer tracings of Coomassie Brilliant Blue-stained polyacrylamide gel of purified mumps virus proteins. The traces show (a) QE cell-propagated original L-3 mumps virus, (b) L-41 cell-propagated mumps virus (primary infection), extracellular structures from (c) p.i. L-41 and (d) p.i. HEp-2, and (e) similarly processed extracellular material from L-41 mock-infected cells. Designations of polypeptides were made on the basis of reported mol. wt. estimates of mumps virus proteins (McCarthy & Johnson, 1980a).

Fig. 2. Autoradiogram of agarose gel RNA analysis. RNA was isolated from 32P-labelled purified mumps virus from (a) L-41 cells, primary infection, and (b) persistently infected L-41 cells of passage 145.

Figure 2 shows that instead of a 50S (mol. wt. $4.7 \times 10^6$) full-size genomic RNA (Fig. 2a), low mol. wt. RNA species of subgenomic size, ranging from 14S to 5S ($0.4 \times 10^6$ and less) (Fig. 2b), are present in extracellular virus preparations from L-41 PI cells. These RNAs were unlikely to be derived from the host since they could not be found in extracellular material from mock-infected cells (not shown).

To verify the virus-specific nature of these small-sized RNAs isolated from defective particles we performed spot hybridization tests with a 32P-labelled cDNA probe of mumps virus 50S RNA and defective virus RNAs. 32P-labelled cDNA was obtained essentially as described by Taylor et al. (1977), using 2 µg of mumps virus RNA, 15 units of reverse transcriptase, and DNase-treated calf thymus DNA as a primer. After synthesis, cDNA was fractionated on Sephadex G-50 to separate the highly polymerized fraction (i.e. polynucleotides of more than 500 bases, as determined by agarose gel electrophoresis), which served as a probe.

To obtain RNAs for hybridization, the virus particles were concentrated and purified from large volumes of culture medium (about 3 l). The RNA was isolated in the presence of carrier tRNA (25 µg/ml), denatured and run on SDS–sucrose gradients as previously detailed (Andzhaparidze et al., 1979). Gradient fractions corresponding to RNA size classes of 52S to 48S and 16S to 5S were collected, dialysed free of sucrose against 0.1 x NTE buffer, lyophilized, dissolved in 100 µl of acetate buffer (0.3 M-NaCOOH, 0.1 M-MgCl2, 0.1 mM-EDTA) and precipitated with ethanol.

The conditions for hybridization were as follows. Unlabelled RNA samples were denatured with glyoxal–DMSO, brought to the same concentrations, and 1 µg amounts were bound in spots
Fig. 3. Spot tests for hybridization of 32P-labelled cDNA of mumps virus RNA to (a) 48S to 52S RNA of standard QE-propagated virus, (b) 16S to 5S RNA from defective particles released by L-41 and (c) HEp-2 PI cultures. The position of the negative hybridization between cDNA and rabies virus RNA is indicated.

to a strip of nitrocellulose filter by the method of Thomas (1980). The filter strip was then incubated overnight at 65 °C in 5 × SSC-0.1% SDS solution containing 0.02% each of Ficoll, bovine serum albumin and polyvinylpyrrolidone. It was then transferred to 4 ml of this same solution, but containing 2.6 × 10⁷ ct/min of previously-prepared 32P-labelled cDNA. The mixture was annealed at 65 °C for 24 h, and the filter strip washed twice in 2 × SSC-0.1% SDS, twice in 1 × SSC-0.05% SDS and once in 0.5 × SSC-0.025% SDS for 1 h at 65 °C, washed exhaustively in a large volume of 2 × SSC at room temperature, dried in an oven and autoradiographed. Figure 3 shows equally strong hybridization of cDNA to either virion 50S RNA, or to 18S to 5S defective RNAs isolated from PI L-41- and PI HEp-2-produced virus particles. There was no trace of hybridization to rabies virus RNA obtained as previously described (Andzhaparidze et al., 1981a). No appreciable amounts of RNA could be recovered either from the 50S fractions of a defective-virion RNA gradient, or from the 18S to 5S fractions of a standard virus RNA gradient.

The small size of these DIP RNAs is in agreement with previously made observations on Newcastle disease virus (13S defective RNA; Maeda et al., 1978) and mumps virus (10S to 18S defective RNA; McCarthy et al., 1981). Nevertheless, although the usual criteria for the authenticity of small-sized RNAs are that it was isolated at the same time as, and by the same method as, standard virus RNA, we cannot exclude the possibility that the defective RNA broke during isolation, due to its increased fragility.

Thus, we conclude that human cell cultures persistently infected with mumps virus released defective interfering mumps virus particles which (i) were similar to standard mumps virus in polypeptide composition, (ii) contained several sub-genome-sized RNA species, and (iii) strongly interfered with standard mumps virus in susceptible cells. Though we were not able to obtain DIP preparations free from infectious virions, the contribution of the latter to the DIP population seems to be negligible for two reasons. First, there was a lack of detectable amounts of virus proteins in preparations of 10⁶ p.f.u. of mumps virus (the calculated amount of contaminating infectious virus in concentrates from PI cells) and, second, there was an absence of full-size virion RNA in preparations of purified extracellular structures from PI cultures. However, the results presented here do not rule out the possibility of non-infectious, non-interfering virus particles being present in preparations of the interfering ones.

The defectiveness of mumps virus laboratory strains has been shown earlier in biological experiments (St Geme et al., 1978). McCarthy et al. (1981) recently presented biochemical evidence for defective nucleocapsid accumulation in mumps virus-PI Vero cells. The interfering activity of DIP against the original virus might account for the gradual decline in virus titre observed in the course of mumps virus persistence (Andzhaparidze et al., 1981b; McCarthy et al., 1981). However, the ultimate role of DIP in the non-cytopathic pattern of infection of cell cultures remains obscure. Perhaps additional factors, e.g. neuraminidase activity (Merz & Wolinsky, 1981) might play a role in establishment of non-cytopathic, persistent mumps virus infection.
Short communications

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