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CHRONIC NON-CYTOPATHIC INFECTION OF HUMAN CONTINUOUS CELL LINES WITH MUMPS VIRUS

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Summary. — Chronic infection with a vaccine strain of mumps virus (MV) was produced and studied in human cell cultures L-41 and HEp-2. The establishment and course of the infection was not accompanied by cytopathic changes. Among probable protective factors (mechanisms) interferon (IFN) was detected in L-41 culture and defective interfering particles in either cell culture. Their role in the establishment and maintenance of chronic infection was not confirmed, however.

Key words: *mumps virus*; *chronically infected cultures*; *factors of persistence*

Introduction

Investigation of chronically infected systems and their components is of great interest because it provides an insight into biological and molecular bases of virus-cell interaction in their long-term coexistence. It also offers possibilities to study the evolution of persistent system components, to determine the regularities of appearance of protective factors and to elucidate the means of survival of both viral and cell populations.

The system of human continuous cells — mumps virus (MV) is a convenient model for such investigations because of high sensitivity of cells and poor cytopathic activity of the virus.

This paper presents data on the properties of L-41 and HEp-2 cultures chronically infected with MV as well as on protective factors detectable in them and the properties of persisting virus.

Materials and Methods

Cells. Continuous HEp-2 and L-41 cells, a cloned variant of J-96 cells (Gulevich, 1968), and a primary culture of Japanese quail embryo (JQE) cells were cultivated in medium 199 supplemented with 10% bovine serum and antibiotics. Chronically infected L-41-MV and HEp-2-MV cultures were grown in bottles of different capacity in the same medium. Subpassages were performed every 4—5 days at a ratio of 1 : 2 or 1 : 3 using 0.02% versene solution for dispersion of the cells.

Virus. The vaccine L-3 strain of MV adapted to JQE cell culture was used for cell inoculation

The infectivity of the virus was determined in 2-day-old JQE cell cultures grown in medium 199 with 2% bovine serum in tubes inoculated with 10-fold dilutions of MV. The infected cultures were incubated at 36 °C for 6—7 days. Then the virus-containing culture fluid was removed and a 0.5% suspension of guinea pig or chicken red blood cells was added. The presence of haemadsorption (HAD) was determined by examinations of the cultures in light microscope. Virus titres were calculated by the method of Reed and Muench and expressed in $\log_{10}$ HAD units/ml.

MV titrations by the plaque method were carried out in JQE cell cultures according to the procedure described for measles virus titrations (Shteinberg and Yakovleva, 1974).

Heterologous interference. In order to determine resistance of chronically infected culture (CIC) to superinfection with different viruses, control and infected cells were grown in tubes, 1—2 days later the culture fluid was removed and cells of each type were inoculated with 10-fold virus dilutions. Virus titres were read by the cytopathic effect (CPE). The presence or absence of interference was judged by the difference in virus titres in CIC and control cultures.

Determination of the interfering activity in culture fluid from chronically infected cultures. Samples of the culture fluid in a volume 5—7 ml were added to 100-ml bottles with JQE cell monolayers. MV in a dose of 10^4 HAD units per bottle was added 30 min later. At 72—96 hr samples of the culture fluid from mixed infection were titrated by HAD in JQE cell culture. The presence of the interfering activity was judged by differences in virus titres of culture fluids from the bottles infected with MV alone and of those infected with the mixture of virus and tested material.

Determination of IFN. The culture fluid from CIC was clarified by a low-speed centrifugation. MV was inactivated by the adjustment of pH of the medium to 2.0 and incubation at 4 °C for 48 hr. Samples of the culture fluid adjusted to pH 7.6 were titrated in L-41 cell culture against 100 TCD_{50} of encephalomyocarditis (EMC) virus.

Indirect immunofluorescence (IF) procedure. The cells grown on mica plates were washed in phosphate buffer and fixed in cold acetone for 20 min. The cells were treated with rabbit hyperimmune serum to mumps virus in a dilution of 1 : 64 for 30 min at room temperature, washed in phosphate buffer and stained with fluorescein-labelled donkey antirabbit serum (N. F. Gamaleya Institute of Epidemiology and Microbiology of the U.S.S.R. AMS, Moscow) diluted 1 : 8 containing rodamine in a 1 : 16 dilution for 30 min. The preparations were examined in a luminescent microscope Luman R-3 (LOMO, Leningrad).

MV was concentrated as described elsewhere (Andzhaparidze *et al.*, 1982). It was purified in a step-wise sucrose gradient consisting of 1.5 ml 40%, 1 ml 30%, 1 ml 20% and 1 ml 10% sucrose in NTE buffer (0.1 mol/l NaCl, 0.01 mol/l Tris-HCl, pH 7.8, 0.001 mol/l EDTA) in MSE rotor 3×6 ml at 45,000 rev/min for 1 hr. In "double label" experiments, unlabelled haemagglutinating (HA) virus obtained after primary infection of JQE cells was mixed with ^{3}H-uridine-labelled virus from CIC and layered on the gradient in a volume of 0.2—0.3 ml. Viruses from CIC caused no HA even after concentration and purification.

Radioactive labelling of viruses with ^{3}H-uridine was done in Eagle's medium with 2% serum. At 24 hr post inoculation (p.i.) (primary infection) or 48 hr after cell transfer (chronic infection) 2 MBk of ^{3}H-uridine (specific activity 1 TBk/mM, "Isotope", Leningrad) were added into the medium and the same amount was added again 48 hr later. The culture fluid was harvested within 72—96 hr after initial addition of the isotope label.

Electrophoresis of proteins in 10% polyacrylamide gel was done as described elsewhere (Andzhaparidze *et al.*, 1982). For protein analysis the virus was concentrated from serum-free culture fluid. Coomassie R-250-stained gels were scanned in Joyce-Loebl microdensitometer. Analysis of RNA size was done by centrifugation in 15%—30% sucrose gradients as described previously (Andzhaparidze *et al.*, 1979*a*).

RNA-DNA hybridization. Viral RNA was isolated from purified virus (1024 HA units) and iodinated (Prensky, 1976) to the specific activity of 8.8×10^7 cpm/μg. Cellular DNA was isolated by treatment with 2% sodium dodecylsulphate (SDS) — proteinase K (250 μg/ml in a buffer of 0.01 mol/l NaCl, 0.1 mol/l Tris-HCl, pH 8.0, 0.1 mol/l EDTA) followed by extraction with phenol and chloroform. For DNA fragmentation and hydrolysis of the contaminating RNA, DNA preparations were treated with acid (2 mol/l sodium acetate, pH 4.2, 70 °C, 1 hr) and alkaline (0.25 N NaOH, 100 °C, 1 hr). The composition of the reaction mixture and hybridization conditions were as described earlier (Andzhaparidze *et al.*, 1979*a*). ^{125}I-RNA (12 pg, 880 cpm/min) was hybridized with equal amounts (160 μg) of DNA from CIC and thymus DNA (control) and treated with RNase A (50 μg/ml). Transfection of JQE or L-41 cells with preparations of DNA isolated from CIC was carried out as described (Andzhaparidze *et al.*, 1981).

Results

Establishment and course of chronic infection

Chronically infected cultures were obtained by inoculation of L-41 and HEp-2 cultures with MV at a multiplicity of infection of 0.1 and 3 HAD units/cell, respectively. No signs of destruction were observed after inoculation and upon subsequent cultivation of the cells. CIC were observed for approximately 3 years. Morphologically and by growth rate CIC did not differ from uninfected cultures. Throughout the observation period, virus-specific antigen was detectable in the cytoplasm of CIC in a form of large and small granules (Figs. 1, 2, Plate XXXVIII). The number of cells producing virus-specific antigen ranged from 20% to 98% in L-41-MV culture and from 50% to 100% in HEp-2-MV culture.

When erythrocytes were added to CIC, HAD was regularly observed in L-41-MV culture but could be specifically inhibited when the cells had been pre-treated with mumps antiserum. No HAD was demonstrable in HEp-2-MV culture despite the presence of virus antigen in the cytoplasm of cells of this chronically infected culture.

Both CIC produced an infectious virus detectable by HAD in JQE cells. A titre of the infectious virus in early passages of CIC was 5.5—4.5 log HAD_{50} units/ml with a trend to decline in subsequent passages. In L-41-MV cell the infectious virus capable of producing HAD ceased to be detectable 7 months after infection and in HE-p-2-MV culture approximately within 11—12 months. The disappearance of HAD-producing virus was regular and reproducible upon initiation of new chronic infections in the same cell lines.

The virus from CIC which had lost its capacity for HAD in sensitive cells was nevertheless detectable by immunofluorescence or by the plaque method. In contrast to the original virus which produced plaques of 0.2—0.3 cm in diameter at 8—10 days p.i., the persisting viruses produced small plaques, 0.05—0.1 cm in diameter at 14—25 days post inoculation. A titre of the persisting virus in the culture fluid of CIC did not exceed 2.5—3.5 $\log_{10}$ PFU/ml. The persisting virus retained the small-plaque phenotype after 3 successive passages in JQE cell culture.

The virus produced by CIC of both early and late passages could be completely neutralized by antiserum to the original MV. It is of interest that the vi-

Table 1. Sensitivity of CIC to superinfection

Cell culture	Superinfecting virus			
	VSV	HS	EMC	MV
L-41-MV	2.7*)	1.7*)	4.0*)	0**)
L-41	5.5	3.5	6.5	4.7
HEp-2-MV	n. d.	2.0	2.5	2.7
HEp-2	n. d.	2.0	2.5	7.5

*) in log TCD_{50}/ml; **) in log HAD_{50} units/ml; n. d. — not done

Table 2. Interfering activity in nutrient medium of cultures chronically infected with mumps virus

Material used for interference	MV titre*
Culture fluid from L-41-MV at 110th passage	
unconcentrated	4.5
concentrated	1.7
Virus (control)	7.5
Culture fluid from HEp-2-MV at 87th passage	2.0
Virus (control)	5.0
Culture fluid from L-41-MV at 23rd passage	
UV-irradiated	5.0
non-irradiated	3.0
Virus (control)	6.5
Purified extracellular virus particles	
from L-41-MV (fractions 6 and 7 from sucrose gradient)	5.0
Virus (control)	6.3

* log HAD_{50} units/ml

rus from CIC acquired the capacity to destroy JQE cells, however, the cell destruction was incomplete, its intensity varying from passage to passage. In order to determine temperature sensitivity of the persisting virus it was propagated in JQE culture at 34 °C, 36 °C, and 40 °C. It was found that viruses from L-41-MV of the 27th and 40th passage and HEp-2-MV of the 16th and 87th passage replicated at all three temperatures similarly, whereas the virus from L-41-MV of the 114th passage did not multiply at 40 °C.

For the study of the effect of higher (40 °C) and lower (34 °C) temperatures on the virus carrier state, CIC were cultivated in parallel at 34 °C, 36 °C and 40 °C for 60 passages.

Every week the number of antigen-containing cells in CICs at different temperatures was counted (Fig. 3), and at various intervals a titre of infectious virus was determined by the plaque method. Cultivation at 40 °C was found not to cure CIC from the virus and cultivation at 34 °C not to favour the development of cell destruction, increase in infectivity titres, or in the number of antigen-containing cells as could have been expected in case of activation of infection at lower temperature. All the above data indicate that the virus carrier state is thermostable and the virus population is insensitive to temperature.

Interference and IFN production

L-41-MV culture was resistant to superinfection with MV, herpes simplex (HS) type 1, EMC and vesicular stomatitis (VS) viruses, while HEp-2-MV culture was resistant to superinfection with MV alone (Table 1). In the culture fluid of L-41-MV IFN was detected in a titre of 1 : 8 whereas none was found in HEp-2-MV culture. To elucidate the role of IFN in the maintenance of chronic infection experiments were carried out in which persistently

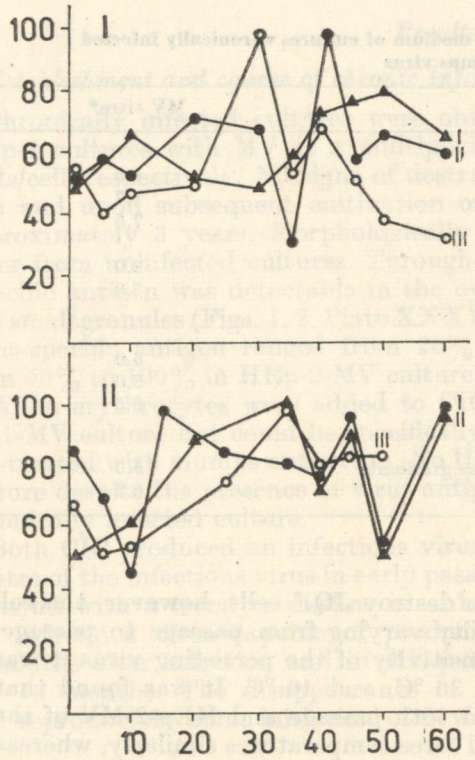

Fig. 3.
Viral antigen production by chronically infected L-41-MV (I, above) and HEp-2-MV (II, below) cultures at different temperatures 34 °C (○), 36 °C (●), and 40 °C (▲)
Abscissa: number of passages; ordinate: percentage of antigen-containing cells

infected cells were cultivated in the presence of anti-IFN serum [calf antiserum to HuIFN-β(F), 750 units per ml, Wellcome Research Laboratories, Beckenham, England, kindly supplied by Dr. Clemence]. After 1—2 passages of the infected cells in the presence of the anti-IFN serum no signs of cell destruction, increase of infectious virus titres, or the number of the antigen-containing cells were noted. Thus, in L-41-MV culture after 2 passages in the presence of anti-IFN serum the number of antigen-containing cells virtually did not differ from that in the control (82% and 85% respectively) and in HEp-2-MV culture it was even slightly lower (47% against 60% in control). The addition of antiserum to HuIFN-α-(Le) (NIH) to the cultivation medium of CICs in doses of 50 to 5,000 units did not affect the carrier state either.

Cultivation of both CIC in the presence of 140 units/ml of exogenous human commercial IFN [HuIFN-α(Le)] during 4 passages exerted no inhibiting effect on virus-carrier state. The number of antigen-containing cells in IFN-treated and untreated cultures was similar (HEp-2-MV 56% and 54%, L-41-MV 72% and 76%, respectively). These data permit a conclusion that IFN does not appear to be a critical factor responsible for the maintenance of MV persistence in these systems.

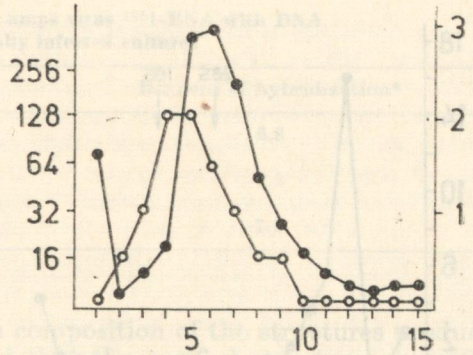

Fig. 4.
Comparative sedimentation analysis of MV propagated in JQE cells (○) and that produced in L-41-MV (●).
Abscissa: fraction number; left ordinate: haemagglutination titre; right ordinate: cpm $\times 10^{-3}$

Cloning of CIC cells

Experiments with cloning were carried out for the evaluation of cell populations in CIC. From L-41-MV culture 110 clones were recovered, of them 42 clones produced viral antigen and adsorbed erythrocytes, 68 clones were not infected and did not differ from the control culture in their sensitivity to the original MV. From HEp-2-MV culture 26 clones were isolated of which 14 were infected and 12 were free of virus. The latter were found to be sensitive to infection with MV while the infected clones were resistant to superinfection with this virus. These data suggest that no selection of more MV-resistant variants of cells ocurred in the course of persistence.

Detective interfering particles (DIP)

The inhibiting properties of the culture fluid from CIC with respect to the original MV were studied for the purpose of detecting DIP as described in

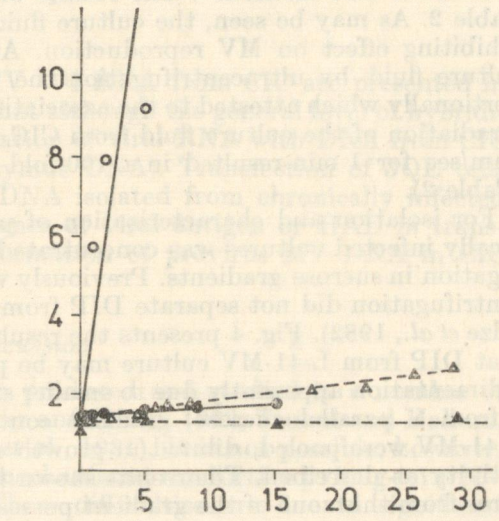

Fig. 5.
Polypeptides of MV propagated in JQE cells (I), produced in L-41-MV (II) and in HEp-2-MV (III)

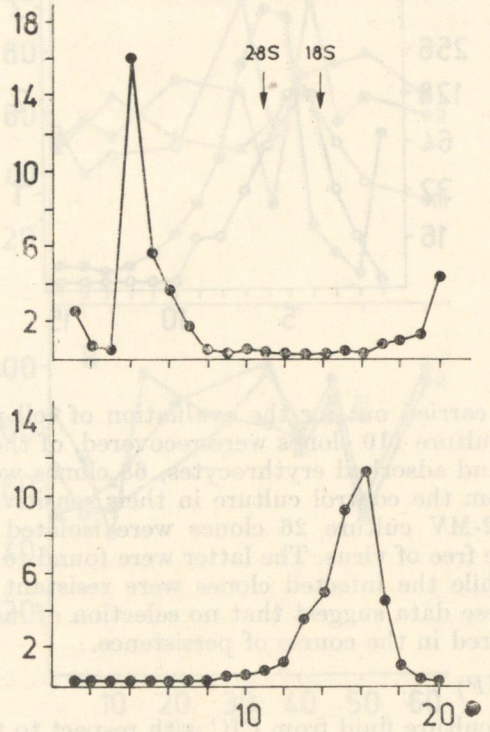

Fig. 6.
Sedimentation of RNA from MV propagated in L-41-MV cell culture (above) and from the virus produced in L-41-MV (below).
Abscissa: fraction number; ordinate: cpm $\times 10^{-2}$

"Materials and Methods". The results of the experiments are presented in Table 2. As may be seen, the culture fluid from both CIC exerted a marked inhibiting effect on MV reproduction. After 100-fold concentration of the culture fluid by ultracentrifugation the interfering activity increased proportionally which attested to the association of the latter with virus particles. Irradiation of the culture fluid from CIC with UV light in a dose of 4.6 erg/ /mm/sec for 1 min resulted in a 100-fold decrease of the interfering activity (Table 2).

For isolation and characterization of probable DIP the virus from chronically infected cultures was concentrated and purified by rate zonal centrifugation in sucrose gradients. Previously we had found that density gradient centrifugation did not separate DIP from infectious MV virions (Andzhaparidze *et al.*, 1982). Fig. 4 presents the results of one of experiments indicating that DIP from L-41-MV culture may be partially separated from virions by sedimentation apparently due to smaller sizes of the former. Fractions 6 and 7 from a parallel sucrose, gradient containing unlabelled structures from L-41-MV were pooled, diluted in growth medium and tested for interfering activity as described. The results shown in Table 2 indicate that the structures from this zone of the gradient possess the interfering activity.

Table 3. Hybridization of mumps virus ^{125}I-RNA with DNA from chronically infected cultures

Source of DNA	Per cent of hybridization*
HEp-2-MV	6.8
L-41-MV	7.2
Calf thymus DNA (control)	7.2

* after treatment with RNase

A comparative analysis of protein composition of the structures produced by CIC and the original MV showed that the purified structures from CIC have a polypeptide composition similar to that of MV (Fig. 5) with the exception of reduced HN polypeptide in virus particles from CIC. Viral polypeptides were designated on the basis of their molecular weight corresponding to that described for MV (McCarthy and Johnson, 1980) and being 74,000 (HN), 68,000 (NP), 59,000 (F), 45,000 (P_{45}), and 39,000 (M).

Analysis of RNA size in virus particles produced by CIC was done for L-41-MV culture which had undergone over 50 passages, because at these passage levels incorporation of radioactive precursor into extracellular virus particles in the culture was sufficiently high. Fig. 6 presents profiles of viral RNAs in sucrose gradients. RNA isolated from purified mumps virus in primary infection of L-41 cells had a size of 50 S typical of MV (East and Kingsbury, 1971), whereas RNA in virus particles from chronically infected L-41-MV culture was represented by low molecular weight RNAs of subgenomic sizes from 22S to 9S. The virus specificity of RNAs isolated from the corresponding zones of the gradients had been previously confirmed by hybridization with MV cDNA (Andzhaparidze *et al.*, 1982).

Search for provirus DNA in CIC

The results of hybridization of MV ^{125}I-RNA from CIC are presented in Table 3 from which it may be seen, that although the general level of hybridization is low, the degree of hybridization of viral RNA with DNA from CIC does not exceed that in control (thymus DNA). Transfection of JQE cells or L-41 cells with preparations of DNA isolated from chronically infected cultures did not induce the appearance of viral antigen or HAD in transfected cells. Thus no evidence of formation of provirus MV DNA in CIC under study was obtained.

Discussion

Mumps virus persistence has been produced and studied in a number of cell cultures (Henle *et al.*, 1958; Walker and Hinze, 1962; Truant and Hallum, 1977; Ogino *et al.*, 1980; Mc Carthy *et al.*, 1981). It is not possible, however, to define clearly the critical factors (mechanisms) conductive to long-term MV coexistence with cell population. These difficulties are due to significant

differences in the results depending on cell origin, virus strains, cultivation conditions, etc.

We studied MV persistence in human L-41 and HEp-2 cells. The establishment of the virus carrier state did not require the addition of specific antibodies, IFN, DIP, or any other treatments. Chronic infection in the cultures was observed for 3 years in which time no signs of degeneration of the infected cultures were noted. Both CIC produced infectious virus and had a higher per cent of cells containing intracellular virus antigen. HAD was observed in L-41-MV culture but not in HEp-2-MV culture.

IFN is one of the protective factors the role of which in the establishment and perpetuation of virus persistence has been clearly defined (Friedman and Ramseur, 1979). Ogino *et al.* (1980) demonstrated IFN production in a mouse tumours cell culture chronically infected with MV. At the same time, no IFN was found in other cultures carrying this virus (Walker and Hinze, 1962; Truant and Hallum, 1977; McCarthy *et al.*, 1981). In our studies L-41-MV cells were resistant to superinfection with heterologous VS, EMC and HS type 1 viruses as well as with the homologous MV. IFN was demonstrated in the culture fluid of these cells. The HEp-2-MV system was resistant to superinfection with MV alone. No IFN was found in the culture fluid.

Cultivation of L-41-MV and HEp-2-MV with anti-IFN serum did not result in activation of the infection and passages of the CICs in medium to which exogenous IFN had been added did not cure the cultures from the virus. These results showed IFN not to be the mandatory factor for perpetuation of the virus-carrier in these systems. In the process of persistence, the virus and the cells are under conditions of complicated dynamic equilibrium exerting selective pressure on each other which in number of CIC results in changes of the properties of both components. Evolution of the systems is directed towards selection of a less cytopathic virus variant and a more resistant cell population. Cloning was performed for the evaluation of the cell populations of L-41-MV and HEp-2-MV. The former yielded 110 clones of which 38% were infected. HEp-2-MV yielded 26 clones 54% of which contained virus antigen. The uninfected clones did not differ in their sensitivity to the original virus from the control cultures which permits to rule out selection of virus-resistant cell population in the process of persistence.

The emergence of temperature-sensitive mutants of viruses interfering with reproduction of the original virus is considered to be of great importance for the maintenance of persistent infection in cell cultures (Youngner *et al.*, 1976). Truant and Hallum (1977) observed the emergence of temperature-sensitive mutants in BHK-21 cells chronically infected with MV.

Chronically infected L-41-MV and HEp-2-MV cultures produced infectious virus, its titre decreasing with passages. At 7 months (L-41-MV) and 11 months (HEp-2-MV) the infectious virus was undetectable by HAD in JQE cell cultures and could be demonstrated by the plaque method alone. The virus from CIC was not sensitive to temperature and replicated to approximately similar titres at 34 °C, 36 °C, and 40 °C in JQE cultures.

Persistence was not sensitive to temperature either as indicated by the results of long-term (60 passages) cultivation of both CIC at 40 °C and 34 °C. In the course of passages, however, there was a tendency of increasing sensitivity of persisting virus reproduction from L-41-MV cells to higher temperature. The manifestation of temperature sensitivity in the population of persisting virus at later passages only as well as the stability of the virus carrier state at 40 °C and 34 °C suggest that this feature is not a mandatory condition for the existence of CIC.

Production of provirus DNA has been demonstrated in some chronic infections of cell cultures with RNA viruses (Zhdanov *et al.*, 1974; Andzhaparidze *et al.*, 1979*b*). We failed to detect provirus DNA in chronically infected L-41-MV and HEp-2-MV cultures using hybridization of highly labelled mumps virus RNA with DNA from CIC and by transfection of MV-sensitive cells with DNA prepared from CIC.

Both L-41-MV and HEp-2-MV cultures were found to produce DIP which inhibited reproduction of the original MV in JQE cells, had a polypeptide composition similar to that of the virus but contained RNA of subgenomic size. McCarthy *et al.* (1981) also observed DIP production in Vero cells chronically infected with MV. DIPs are an important factor for maintenance of stable carrier state in a number of persistently infected systems (Holland *et al.*, 1976).

What is then the role of DIP in MV persistence in chronically infected L-41-MV and HEp-2-MV cultures? The chronic infection was initiated with MV containing no detectable DIP. Nevertheless, persistent infection was established without cytopathogenicity and DIP in detectable amounts emerged after several passages of CIC. Rapid generation of DIP in primary infections of the continuous cells or amplification of the insignificant amount of DIP, present in the inoculum, to amounts capable of protecting against viral cytopathic effect seem to be little likely, because in primary infection of L-41 cells virus preparations, apart from virion 50S RNA, contained no RNA of subgenome size. Apparently the noncytopathic pattern of infection in the systems under study is due to the features of MV interaction with these cell cultures.

The formation of protective factors in the process of existence of a carrier culture depends on the properties of the host cell. Vero and BHK cells are genetically incapable of interferon production. Therefore, when these cells are components of chronically infected culture, DIP or temperature sensitive virus mutants are generated. L cells are good producers of IFN but poor producers of DIP. Therefore, in chronic infection of L cells with different viruses the factors participating in persistence include IFN or the mutants. The importance of the host cell in generation of DIP or ts mutants has been convincingly demonstrated by Youngner *et al.* (1981).

The role of DIP, IFN, or ts mutants in the estabishment and maintenace of persistent infection can be well demonstrated in lytic infection. In the case of noncytocidal infection, similar protective factors may be demonstrated, however, their role in the maintenance of virus persistence is not patent-

Apparently the generation of DIP, IFN, and ts mutants may be considered to be a host-cell regulated mechanism of antiviral protection manifested irrespective of the outcome of their primary virus infection.

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Explanation of Micrographs (Plate XXXVIII):

Figs. 1—2. Cytoplasmic fluorescence of MV antigen in cells of chronically infected L-41-MV (1) and HEp-2-MV (2) cultures.