Viruses of *Entamoeba histolytica*

IV. Studies on the Nucleic Acids of the Filamentous and Polyhedral Viruses

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Received for publication 27 July 1973

The nucleic acids of two amoebal viruses were studied by several independent methods. The filamentous virus, V_{ABRM} , was shown to be inhibited by bromodeoxyuridine, iododeoxyuridine, and cytosine arabinoside. With acridine orange staining, V_{ABRM} inclusions appeared greenish-yellow, indicating that these contained double-stranded nucleic acid. The polyhedral virus, V_{301} , was also inhibited by bromodeoxyuridine, iododeoxyuridine, and cytosine arabinoside. In addition, nucleic acid hybridization showed that a new DNA species was synthesized in infected amoebal cultures. The intracellular localization of this new DNA was consistent with previous electron microscope studies of the cytoplasmic maturation of V_{301} .

Since the identification of two morphologically different viruses, one filamentous and the other polyhedral, in axenically cultivated strains of *Entamoeba histolytica* (4, 11), and the investigation of a number of biological and chemical properties of the polyhedral virus (9), we have concentrated our attention on determining whether these viruses contained DNA or RNA. We have made only preliminary and unsuccessful attempts to purify the filamentous virus, V_{ABRM} , because it has not yet been separated from a contaminating polyhedral virus previously described (4, 11). Purified polyhedral virus was impossible to prepare, despite an intensive effort, because the virus became irreversibly bound to the cell debris of lysed amoebae and because of the extraordinary lability of the virus in concentrations of salts and nonionic substances ordinarily employed in gradient purification procedures (9). Therefore, several independent methods were employed to determine the nucleic acid composition of these morphologically distinct viruses. These included acridine orange staining of the characteristic intranuclear inclusions of the filamentous virus, inhibition of replication of both viruses by nucleic acid analogues, and incorporation of radioactive thymidine and nucleic acid hybridization in the case of the polyhedral virus. The results of these studies support the thesis that these viruses contain DNA.

MATERIALS AND METHODS

Virus strains. Two representative amoebal viruses were selected for study. One was the filamentous

agent which to date has only been obtained from the ABRM strain of amoeba. This virus isolate is known to be contaminated with a polyhedral virus and was originally designated (ABRM)₂-HK-9 (4, 11). Hereafter, it is simply referred to as V_{ABRM} . The second virus, a polyhedral agent with icosahedral symmetry (12) isolated from the HB-301 amoebal strain, has been described in some detail (9, 12) and is hereafter referred to as V_{301} . Both viruses were cultivated in HK-9 amoebae during these studies.

Assay system. A tube assay system employing susceptible amoebae and serial virus dilutions has been previously described (9) and was employed throughout these investigations.

Acridine orange staining. Control and virus-infected amoebae were grown on cover slips placed in glass scintillation vials filled with 10 ml of TPS-1 medium (9). This permitted monitoring cytopathology in the amoebae by means of an inverted microscope. At various time intervals, cover slips were removed and fixed for 15 min in Carnoy fluid (glacial acetic acid-chloroform-ethanol, 1:3:6). Subsequently, the cover slips were washed twice in absolute alcohol and rehydrated for 10 min in McIlvaine buffer (6 parts of 0.1 M citric acid mixed with 4 parts 0.15 M NaH_2PO_4 , pH 3.8). Staining (1) was performed for 30 min with 0.01% (wt/vol) acridine orange dissolved in McIlvaine buffer. The cover slips were washed with four changes of the buffer, mounted on a glass slide with a drop of buffer, and sealed with Permount. The slides were then examined and compared in a fluorescence microscope.

Separation of amoebae into "cytoplasmic" and "nuclear" fractions. Amoebae were washed two times in phosphate buffered saline (PBS) pH 7.2, and suspended in hypotonic reticulocyte standard buffer (0.01 M NaCl, 0.01 M Tris, 0.0015 M MgCl_2 , pH 7.5) for 10 min at 4 C. The suspension was homogenized with 10 strokes of a loosely fitting Dounce homoge-

nizer. A sample was examined in the microscope to verify that essentially all amoebae were disrupted. The sample was centrifuged for 15 min at 2,500 rpm in a refrigerated International Centrifuge (1,200 $\times$ g). The pellet was designated the "nuclear" fraction because only amoebal nuclei were recognized by light microscopy. The supernatant, called the "cytoplasmic" fraction, probably consisted of various cytoplasmic elements and some nucleoplasm which may have been liberated in the homogenization.

Radioactive isotope labeling. Isotopic labeling of both amoebal and viral DNA was accomplished by adding 20 μ Ci of thymidine-6- 3 H per ml to the growth medium and pulsing for appropriate periods of time. For the production of labeled viral DNA, [3 H]thymidine and virus were added to amoebae simultaneously, and the cultures were harvested when maximum lysis appeared. After harvesting, either normal amoebae or the amoebal lysates were pelleted at 4 C at 2,000 rpm and washed twice with PBS. Purified labeled DNA was prepared from some of these samples as subsequently described. Other samples were employed to determine nucleic acid precursor uptake and were treated as follows: all radioisotopically labeled samples were counted in a liquid scintillation counter (Packard Instruments Co., Inc.) after precipitation with 10% trichloroacetic acid or perchloric acid (PCA) and collection on membrane nitrocellulose filters (Millipore Corp.). The scintillation fluid was made by using "rpi scintillator" in toluene. The final concentration of phosphores was 4 g/liter for 2,5-diphenyloxazole and 50 mg/liter for 1,4-bis-(5-phenyloxazolyl)benzene.

Purification of nucleic acid. DNA was extracted from amoebal and viral pellets by the method of Gelderman et al. (6) with minor modifications. Initially, cells or amoebal lysates were washed three times in PBS and pelleted at 2,500 rpm for 15 min. The pellet was dissolved in 10 $\times$ (vol/vol) standard buffer (0.1 M NaCl, 0.05 M Tris, pH 8.0, and 0.025 M EDTA). Sodium dodecyl sulfate was added to a final concentration of 2%, and the tube was inverted gently 60 times at room temperature. After precipitation of protein with 1 M sodium perchlorate overnight at 4 C, the resulting supernatant was incubated with Pronase (1 mg/ml) at 60 C for 4 h. Subsequently, the solution was extracted with an equal volume of chloroform-isoamyl alcohol (24:1) for 10 min and centrifuged. After another Pronase and chloroform-isoamyl alcohol treatment, the supernatant was digested for 1 h at 37 C with 20 μ g of RNAase per ml (Worthington Biochemical Corp.). Any contaminating DNase was inactivated by preincubation of the RNAase at 80 C for 10 min.

After a final chloroform-phenol-isoamyl alcohol extraction (50:50:1), the DNA was reprecipitated three times with absolute ethanol. Typically such preparations revealed optical density ratios (260/280) of 1.8.

Nucleic acid hybridization. Nitrocellulose membrane filter hybridization according to the method of Green et al. (7) was used with some modifications to increase efficiency (3, 15). These changes included a decrease of the reaction mixture to 0.7 ml and an increase in the ionic strength of the hybridization

supernatant to 3 $\times$ 0.15 M NaCl plus 0.015 M sodium citrate.

RESULTS

Acridine orange staining of viral nucleic acid. Because V_{ABRM} formed large, characteristic intranuclear inclusions, seen in the electron microscope to consist of filamentous particles (4, 11), it was felt that acridine orange staining might identify the type of nucleic acid in these inclusions.

With acridine orange, the cytoplasm of normal HK-9 amoebae appeared reddish-brown, which is characteristic of single-stranded nucleic acid. The nuclear DNA of normal amoebae is known to be condensed within a small structure called the endosome, which in normal amoebae was observed to stain yellowish-green, this color being characteristic of double-stranded nucleic acids.

The acridine orange fluorescence pattern of HK-9 amoebae infected with V_{ABRM} differed from that of normal amoebae. The nucleus was larger and more irregular than normal and was completely filled with a yellowish-green fluorescence, which prevented the identification of the endosome. Other infected cells whose nuclei had lysed contained large yellowish-green fluorescent inclusions in the cytoplasm. This appearance is compatible with previous investigations in which cytoplasmic inclusions of filaments were identified by electron microscopy subsequent to rupture of the nuclear membranes (11).

Amoebal cultures were stained at various periods of time after infection. The number of V_{ABRM} -infected amoebae with abnormal fluorescent nuclear inclusions increased with time beginning with the 11th h of infection. Eventually all of the amoebae in the infected cultures exhibited the fluorescent nuclear inclusions, nuclear lysis with fluorescent cytoplasmic inclusions, or total lysis. The yellow-green fluorescence of the nuclear inclusions suggests the presence of an unusual quantity of double-stranded nucleic acid, either DNA or an unusual form of RNA, in nuclei infected with V_{ABRM} .

Observations by electron microscopy of polyhedral viruses replicating in amoebae failed to reveal highly specific inclusion bodies (11), although developing particles were seen in the perinuclear cytoplasm. HK-9 amoebae were also infected for similar periods of time with the polyhedral V_{301} and subsequently were stained with acridine orange. We have been unable to distinguish between V_{301} -infected and uninfected amoebae by their acridine orange staining.

Effects of metabolic inhibitors on viral replication. Metabolic inhibitors such as bromodeoxyuridine (BUdR) and cytosine arabinoside have been previously employed to study nucleic acid synthesis of DNA viruses of the herpes (10, 14), pox (5), and papova virus (2) groups. Similar studies were undertaken with the two types of amoebal virus. Amoebae were infected for different periods of time in the presence and absence of inhibitor and were homogenized to assay for virus replication. The replication of V_{ABRM} is substantially inhibited by BUdR (Fig. 1). That this effect is probably not due to amoebal death is suggested by the viable amoebal count, determined by trypan blue staining, being decreased only 30% after 60 h of BUdR treatment. To further eliminate this possibility, the same experiment was repeated except that infected amoebae were periodically removed from the BUdR, washed, and titrated as whole amoebal infectious centers. The BUdR effect was found to be partially reversible (Fig. 2), when infectious centers were assayed. Hence, in the presence of BUdR, virus did not replicate but remained "eclipsed" within the amoebal host cell; when the BUdR was removed, virus replication proceeded, and an appreciable quantity of virus was synthesized.

BUdR is not unique in its ability to arrest

amoebal virus replication. Cytosine arabinoside and iododeoxyuridine (IUdR) equally inhibited both V_{ABRM} and V_{301} (cf. Fig. 3). Thus, studies with metabolic inhibitors support the hypothesis that both of the amoebal viruses contain DNA.

[^3H]thymidine incorporation studies in V_{301} infected amoeba. Because the two previous experimental methods suggested that the amoeb-

FIG. 2. Effect of BUdR on V_{ABRM} replication as measured by infectious centers. Conditions were as in Fig. 1 except that the infected cells were not homogenized before titration. This allowed any eclipsed virus to continue replication after the BUdR was removed.

FIG. 1. Effect of BUdR on V_{ABRM} replication. Culture tubes of 10^6 HK-9 amoebae were infected with 10^5 mean tissue infective doses (TCID_{50}) per tube. A $66\text{-}\mu\text{g}$ portion of BUdR per ml was added to each experimental sample immediately before infection. Periodically the amoebae were harvested, washed once, and homogenized before titration. Unpublished studies showed that 100 to 200 μg of BUdR per ml were needed to completely inhibit DNA synthesis of normal HK-9 amoebae.

FIG. 3. Effect of cytosine arabinoside on V_{301} replication. Conditions were as in Fig. 1 except 300 μg of cytosine arabinoside per ml was added to each experimental sample. Unpublished studies showed that greater than 100 μg of cytosine arabinoside per ml were needed to completely inhibit DNA synthesis of normal HK-9 amoebae.

bal viruses contained DNA, its synthesis was measured in infected cells by the utilization of [^3H]thymidine. Amoebae infected with V_{301} incorporate three to four times as much [^3H]thymidine into acid-insoluble material as an uninfected control amoebal culture (Fig. 4). To rule out the possibility that the increased amount of DNA synthesis was due to nonspecific stimulation by the cell debris normally found in the viral inoculum, controls were run with and without additional debris from homogenized normal amoebae. All tubes had equal numbers of amoebae, as determined by counting with a hemocytometer, and appropriate tubes contained equal amounts of cell debris, as determined by optical density at 280 nm.

The distribution of newly synthesized DNA was different in controls than in V_{301} -infected cells (Fig. 5). In the control cells, most of the labeled DNA was intranuclear. In infected cells, however, the labeled DNA appears to be largely associated with the cytoplasmic fraction. This result was consistent with the observation by electron microscope that this polyhedral virus assembled in the perinuclear cytoplasm (11). It could be argued, however, that the labeled DNA in the cytoplasmic fraction results from an unusual fragility of nuclei of infected cells.

An additional noteworthy observation resulted from the experiments. Assay of viral infectivity showed that 50% of the infectivity was associated with the cytoplasmic (supernatant) fraction. This was unexpected because previous studies had shown that after complete lysis, 99% of the infectivity was associated with

FIG. 4. Thymidine incorporation in V_{301} -infected HK-9 amoebae. A 20- μCi portion of [^3H]thymidine per ml was added to culture tubes of infected and normal HK-9 amoebae and the cumulative amount of PCA-precipitable counts was periodically measured. Typically, $10^{6.5}$ amoebae were infected with 10^6 TCID $_{50}$ of V_{301} . All counts were normalized to the initial amount of amoebae by means of optical density readings of the PCA precipitate at 280 nm.

FIG. 5. Ratio of "cytoplasmic" to "nuclear" [^3H]thymidine incorporation in normal and V_{301} -infected HK-9 amoebae. Conditions were as in Fig. 4 except that each sample was homogenized and separated into the above two fractions. See Materials and Methods for a description of how "cytoplasmic" and "nuclear" fractions were obtained.

membrane debris and sedimented in the low-speed pellet (9). Thus, it appeared that in an early stage of replication prior to lysis, most of the virus was not attached to membranes, but was free in the cell cytoplasm.

CsCl equilibrium density centrifugation. All of the DNA-labeling evidence up to this point was circumstantial and did not indicate whether the newly synthesized DNA was viral or host. To attempt to resolve this point, purified samples of normal HK-9 amoebal DNA and DNA from V_{301} -infected HK-9 cultures were centrifuged to equilibrium in CsCl density gradients in attempts to differentiate the two DNAs by their buoyant densities. However, the DNA from infected cultures was of the same density (1.68 g/ml) as that of control HK-9 amoeba. On the other hand, sucrose gradient velocity sedimentation showed that the DNA from infected cells was smaller in size than DNA from normal amoebae. Therefore, either the DNA from infected cells represented degraded host cell DNA, or the newly synthesized DNA was a small viral DNA molecule with a guanine plus cytosine content similar to the amoebal DNA, as indicated by their similar buoyant densities.

DNA-DNA hybridization studies of V_{301} and HK-9 DNA. Hybridization studies were undertaken to establish whether or not the newly synthesized DNA in V_{301} -infected cultures was viral. Table 1 illustrates these data. Normal amoebal DNA self-hybridizes to 27%. DNA from infected amoebae self-hybridizes to 45%. However, labeled DNA from infected amoebae hybridizes to unlabeled normal amoe-

bal DNA to only 4%, which is 10-fold less than the self-hybridization. This 4% hybridization means that the labeled DNA from infected amoebae is approximately 70 to 90% viral and that the host DNA contamination is only approximately 10 to 30%. The high percentage of hybridization of sonically treated and labeled amoebal DNA to unlabeled DNA from V_{301} infected amoebae (26%) can be explained by this 10 to 30% contamination with host DNA. Because the filter contained about a 25-fold excess of cold DNA from infected amoebae over the labeled normal DNA in the supernatant, a 20% contamination represents about a fivefold excess of contaminating amoebal DNA on the filter over that added as labeled, fragmented, normal amoebal DNA. The latter DNA therefore "sees" the former (contaminating) DNA as self-hybridizable, both qualitatively and quantitatively.

In addition, it is possible that the virus may turn off the synthesis of amoebal DNA. Consequently, although 70 to 90% of the newly synthesized ^3H -labeled DNA in a viral pellet may be viral, the DNA preparation may still contain additional high concentrations of unlabeled amoebal DNA. Naturally when this DNA is hybridized to amoebal DNA on a filter, only the newly synthesized DNA or viral DNA will be followed because it is the labeled one. However, if unlabeled DNA from a viral pellet is placed on the filter, both the DNA from virus and from amoebae will be present in high concentrations and will give saturating levels of hybridization similar to that found when amoebal DNA is hybridized to amoebal DNA.

DISCUSSION

The affinity of the polyhedral amoebal viruses for the membranous debris of lysed amoebae has hampered our attempts to purify these agents; nor have we been able to purify the filamentous virus. Consequently, it was not possible to characterize them by such direct tests as radioactive labeling or chemical analysis of their nucleic acids. However, indirect tests, including the green-yellow staining of the characteristic filamentous virus inclusions with acridine orange and the inhibition of replication by inhibitors of DNA synthesis led us to conclude that the V_{ABRM} is a double-stranded DNA virus. In the case of the V_{301} , similar inhibition of replication and nucleic acid hybridization studies suggests that this virus also contains DNA. Furthermore, the observation of self-hybridization of its DNA suggests that it is probably double stranded. The only way that self-hybridization could occur with a single-

TABLE 1. Hybridization studies of HK-9 amoebal DNA and V_{301} infected HK-9 DNA^a

Unlabeled alkali-treated DNA on filter (3 μg)	^3H -labeled DNA in sonically treated solution (0.2 μg)			
	HK-9 amoeba		V_{301} -infected HK-9	
	Hybridized (%)	Counts/min	Hybridized (%)	Counts/min
HK-9 amoeba	27	370	4	107
V_{301} -infected HK-9	26	365	45	1,140
Salmon sperm	1.8	25	0.4	10
Blank filters	0.4	5	0.3	8

^a Reactions were performed with an excess of unlabeled DNA on the filters. Further reductions in the amount of ^3H -labeled DNA in solution yielded no better percentage of hybridization. Reaction mixtures were incubated at 60 C for 18 to 20 h, at which time maximal hybridization was observed.

stranded nucleic acid would be for the crude viral preparation to contain nearly equal amounts of "minus" and "plus" strands, a situation so far found only in certain parvoviruses (8). By these criteria, it seems highly probable that both the filamentous V_{ABRM} and the polyhedral V_{301} are DNA viruses and that their DNA is double stranded.

ACKNOWLEDGMENT

We wish to thank Hilton Levy for his helpful discussions and comments, and I. Louise Bartgis and Wendell Daniel for their technical assistance.

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