Properties of Intracytoplasmic A Particles Isolated from Oncornavirus-Producing Human Cells

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Received for publication 4 June 1974

A particles with the diameter of 70 to 80 nm were isolated from the cytoplasm of HEP-2, HeLa, and AO cells producing oncornavirus of Mason-Pfizer-like type. Most of the A particles banded at 1.23 to 1.24 g/ml, whereas 3 to 10% banded at 1.29 g/ml in equilibrium sucrose gradients. They banded at 1.30 g/ml in CsCl gradients suggesting that they contained 8% RNA. Individual A particles sedimented at 200 to 250S in velocity sucrose gradients, but their significant part was found aggregated and sedimented at more than 300S. They were resistant to RNase digestion. A particles possessed polymerase activity which was preferentially activated by Mn^{2+} rather than by Mg^{2+} , the RNA template being 60S RNA. Cross-hybridization with two DNA products and immunoassay showed that A particles and Mason-Pfizer-like oncornavirus produced by the same cells contained neither homologous RNA sequences nor common antigens, suggesting that A particles are not intracellular precursors of Mason-Pfizer-like oncornavirus but represent an independent oncornavirus. Hybridization of A particle RNA with excess of cellular DNA revealed about 20 proviral copies per HEP-2 cell genome and no proviral copies in human embryo and placenta cell genomes.

Intracellular A particles were found in a number of eukariotic cells producing oncornaviruses of B and C types (1, 3, 5, 10-12, 14-17). They were detected also in monkey cells producing Mason-Pfizer virus (6). However, the data on their relation to the reproduction of B and C particles are contradictory. A number of investigators (1, 6, 14, 15) regarded A particles as putative intracellular cores of B particles. Meanwhile, Smith and Wivel (12) reported that A particles in mouse carcinoma cells distinctly differed in polypeptide composition from MTV produced by the same cells, suggesting that they hardly represented its intracellular cores. However, since protein cleavage may occur during virus maturation, the question of intracellular precursors can be more properly solved by detection of the same RNA sequences in nucleic acid hybridization test.

We reported previously (2) the isolation of A particles and a fibrillar component from the cytoplasm of human continuous line cells producing oncornavirus of Mason-Pfizer-like type (9). The fibrillar component was shown to be a precursor of extra-cellular virus (2).

In this paper the properties of A particles are

described and a further evidence is presented that A particles are not the precursors of Mason-Pfizer-like oncornavirus.

MATERIALS AND METHODS

Cells. AO, HeLa, and HEP-2 cells were grown as described (2). Primary monolayers of human lung embryo cells were grown in Eagle medium supplemented with 20% calf serum.

Isolation of A particles. AO, HeLa, and HEP-2 cells were labeled with [3H]uridine (50 μ Ci/ml) or carrier-free $^{32}PO_4$ in phosphate-free medium (200 μ Ci/ml) for 24 h and disrupted by Dounce homogenization, nuclei were centrifuged at 2,000 rpm for 10 min and washed twice with 0.25 M sucrose prepared on RSB (2), and the supernatant fractions were pooled with cytoplasmic fraction and centrifuged at $15,000 \times g$. The supernatant ("cytoplasmic extract") was further used for A particle isolation. It was shown in special experiments where cytoplasmic fraction and nuclear washing fluids were analyzed separately that the washing supernatants contained an essential amount of A particles, up to 50% of that found in the cytoplasmic fraction.

The cytoplasmic extracts were centrifuged in 28-ml equilibrium sucrose gradients which consisted of a 22-ml linear 1.22 to 1.30 sucrose gradient and a 6-ml layer of 1.22 sucrose above. Such modification was

applied for better separation of virus-specific structures with a density higher than 1.22 g/ml from cellular material (mitochondria, microsomes, membraneous components, as well as both ribosomal subunits) which banded at 1.18 to 1.21 g/ml under the conditions used.

The gradients were centrifuged in a Spinco SW 27 rotor for 16 h at 25,000 rpm at +4 C. The radioactivity was determined in portions, the fractions corresponding to the peaks of radioactivity were pooled, and the structures were pelleted at 100,000 $\times$ g through a sucrose cushion with a density of 1.22 g/ml, resuspended in STE (Tris-hydrochloride, 0.01 M, pH 7.2; NaCl, 0.1 M; EDTA, 0.001 M) and used for further analysis.

Sedimentation and buoyant density analysis in CsCl was performed by using 15 to 35% linear sucrose gradients with 70% sucrose cushion prepared on STE and preformed CsCl gradients prepared on 0.01 M phosphate buffer containing 0.01 M KCl, 0.001 M EDTA, and 4% formaldehyde.

Polymerase assay. Endogeneous polymerase activity of A particles with a density of 1.23 to 1.24 g/ml was assayed by using about 50 to 100 μ g of protein per sample as determined by the method of Lowry et al. (7). The standard assay was done in 1 ml containing 50 mM Tris-hydrochloride, pH 7.8, 60 mM NaCl, 6 mM MgCl₂, or 0.8 mM MnCl₂, 2 mM DTT, 60 μ g each of dATP, dGTP, and dCTP, 10 μ Ci of [³H]TTP, (specific activity 26 Ci/mM), 0.015% Triton X100, and 20 μ g of actinomycin D. All assays were at 37 C for 18 h. Determination of reaction products was described (2). The product represented single-stranded DNA which banded in caesium sulfate at 1.45 to 1.46 g/ml. Yeast RNA (50 μ g/ml) was added as carrier. Some assays were done for 20 min; nucleic acids were extracted from the mixture and analyzed in 15 to 30% sucrose gradients as described (2).

Nucleic acid hybridization. Two DNA products of endogeneous polymerase reaction were used for hybridizing the DNA product obtained on extracellular virions (DNA-V) and on intracytoplasmic A particles (DNA-A). The hybridization buffer (HB) contained 0.4 M NaCl, 0.05% sodium dodecyl sulfate (SDS), 0.001 M EDTA, and 0.05 M Tris-hydrochloride, pH 7.2. Annealing was carried out at 68 C in 20 μ liters of HB. Two thousand to 5,000 counts/min of [³²P]RNA (specific activity 2,000 counts per min per ng) and dilutions of DNA products were used. The extent of hybridization was analyzed by digestion with 30 μ g of RNase per ml (Calbiochem) at 37 C for 30 min after a 10-fold dilution of the samples with 0.1 M NaCl. The amount of trichloroacetic acid-precipitable ³²P counts was determined.

Preparation of cell DNA and hybridization with RNA from A particles. HEP-2, human embryo cells, and human placenta cells were suspended in STE buffer, treated with 0.2% SDS and Pronase (200 μ g/ml, predigested) at 37 C for 3 h, then nucleic acids were extracted with phenol, fragmented in an MSE ultrasonic desintegrator (10 μ m, 2 cycles 20 s each), and RNA was destroyed by alkali digestion (0.5 M NaOH at 37 C for 16 h). The mixture was neutralized

and DNA was precipitated by ethanol. DNA preparations had a sedimentation coefficient of 4 to 8S and an A₂₆₀:A₂₈₀ ratio >1.8.

A 2- to 4-mg amount of such DNA was hybridized with ³H-labeled RNA of A particles (1,000 to 2,000 counts/min) in 1 ml of HB. The mixture was incubated at 68 C. Portions were taken at various intervals corresponding to C₀t values from 10 to 4,000 mol per s per liter. The extent of hybridization was evaluated as described above.

Electron microscopy. A particles were pelleted from equilibrium and velocity sucrose gradient fractions, dissolved in STE buffer, negatively stained with uranyl acetate, and investigated in a JEM 100B electron microscope. Ultrathin sections of the cells were prepared as described (2).

Immunodiffusion test. Immunodiffusion tests were performed with antisera against extracellular virions produced by HEP-2 cells. The preparation of antisera and conditions of immunoassay were previously described (4).

RESULTS

Distribution of A particles in equilibrium sucrose gradients and their electron microscope investigation. Figure 1 shows the distribution of radioactivity in equilibrium linear 1.18 to 1.30 sucrose gradients after centrifugation of cytoplasmic extracts obtained from the strains of HeLa cells which produce Mason-Pfizer-like oncornavirus (b) and do not produce oncornavirus as judged by electron microscopy (a). It is seen that the main peak of radioactivity in both cases is observed in the 1.19 to 1.21 region, a shoulder of radioactivity is revealed in (b) at 1.23 g/ml, and minor peaks of radioactivity appeared in the regions of higher density.

When extracts were analyzed in modified equilibrium sucrose gradients as described in Materials and Methods, the minor components were better separated. Figure 1c and d shows that small peaks of radioactivity are found at 1.29, 1.25 to 1.26, 1.24, and 1.23 g/ml (in c) when HeLa and HEP-2 cytoplasmic extracts were used and the same distribution of radioactivity was observed with extracts from AO cells.

When the structures were pelleted from these gradient regions and investigated in an electron microscope, typical A particles with a diameter of 70 to 80 nm were seen in the material with a density of 1.23, 1.24, and 1.29 g/ml, whereas fibrillar structures with a diameter of about 3 nm were revealed in the material with a density of 1.25 to 1.26 g/ml (these structures were identified as cytoplasmic precursors of Mason-Pfizer-like virions and described in details elsewhere).

We found that approximately 90 to 97% of the total A particle population banded at 1.23 to

FIG. 1. Distribution of radioactivity after equilibrium density centrifugation of cytoplasmic extracts of HeLa cells which do not produce oncornavirus (a), and HeLa cells (b and c) and Hep-2 cells (d) which produced oncornavirus of Mason-Pfizer-like type. Cytoplasmic extracts from ^3H -labeled cells were centrifuged in equilibrium linear sucrose gradients with a density of 1.17 to 1.29 g/ml (a and b) and with a density of 1.22 to 1.30 g/ml and a layer of 1.22 sucrose above (c and d) in an SW 27 bucket rotor at 25,000 rpm for 16 h at 4°C. After fractionation, acid-insoluble radioactivity was counted in 1/40-volume portions.

1.24 g/ml and the rest at 1.29 g/ml. Both materials were seen in an electron microscope as highly purified A particles, intact or at various stages of destruction.

In some experiments A particles with a density of 1.24 g/ml were isolated separately from cytoplasmic fraction and nuclear washing fluid. In both cases they looked similar being found

either in small aggregates or as free particles. Their presence in a nuclear fraction is apparently explained by cosedimenting of large A particle aggregates with nuclei as was the case with murine intracytoplasmic A particles (12). However, these aggregates seem to be readily destroyed by washing, giving rise to free particles and small aggregates. In fact, neither washing fluid pellets obtained at $15,000 \times g$ nor the fraction of washed nuclei contained A particles when examined in an electron microscope.

When A particles were investigated immediately after their isolation, most of them looked like hollow spheres formed by helical threads with a diameter of 2.5 nm regularly packed (Fig. 2a and b). Uncoiled threads were also revealed in individual partly destructed particles. A particles kept overnight at 0 to 2°C underwent the various stages of destruction (Fig. 2c and d) indicating that these structures were extremely fragile.

Sedimentation patterns. A particles were pelleted from equilibrium sucrose gradient fractions with a density of 1.24 g/ml and recentrifuged in a velocity sucrose gradient with a 70% sucrose cushion. Radioactivity was found at 200 to 250S as well as on the sucrose cushion (Fig. 3a), and typical A particles were revealed in both zones of the gradient when the pellet was investigated in an electron microscope. Figure 3b shows that radioactivity in both regions is conserved after preliminary treatment of A particles with RNase indicating that A particles are resistant to the enzyme.

Buoyant density in a CsCl gradient. When A particles with a density of 1.24 g/ml were recentrifuged in a preformed CsCl gradient, radioactivity was distributed heterogeneously in a broad zone of the gradient. Meanwhile, after preliminary treatment of the material with RNase, a distinct peak of radioactivity was usually revealed at 1.29 to 1.31 g/ml (Fig. 3c). It is likely that RNase eliminates disrupted particles which acquire sensitivity to the enzyme, as well as possible cellular contaminants.

Polymerase activity. Polymerase assay was done with the preparations of A particles with a density of 1.24 g/ml which did not contain or contained in trace amounts a cellular contaminated material when examined in an electron microscope. The same samples which did not contain the three nonlabeled deoxynucleoside triphosphates (dATP, dCTP, and dGTP) were used as controls. To compare polymerase activity of A particles to that of extracellular virions, their polymerase activity was also assayed under the same conditions.

Since endogenous RNA-dependent DNA pol-

ymerase activity of murine intracisternal A particles was shown to be preferentially activated by Mn^{2+} rather than by Mg^{2+} (17), we also compared the endogenous polymerase activity in the presence of Mg^{2+} and Mn^{2+} . Figure 4 shows that Mn^{2+} more than Mg^{2+} was also required for activation of intracytoplasmic A particle polymerase. By contrast, the endogenous polymerase activity of extracellular virions was preferentially activated by Mg^{2+} . Thus, in this respect A particles significantly differ from extracellular Mason-Pfizer-like virions.

It has been reported previously that A particles contain 60, 45, and 35S RNA species (2). To identify RNA template in polymerase reaction, the complete polymerase mixture was incubated for 20 min, and nucleic acids were extracted by SDS and analyzed in 15 to 30% linear sucrose gradients. Figure 5 shows that newly synthesized DNA is associated with high-molecular-weight RNA but not with 45 and 35S RNA. When the fractions containing high molecular weight RNA were diluted with 0.1 M NaCl and treated with RNase, 3H -radioactivity was revealed only at 60S indicating that 60S RNA is a real template for the synthesis of acid-insoluble DNA product.

Hybridization of A particle RNA with DNA products. Cross-hybridization was performed with two ^{32}P -labeled RNA, RNA of A particles and extracellular virions, and two 3H -labeled DNA products obtained on these structures. Figure 6 shows that RNA of A particles with a density of 1.24 g/ml is fully protected from RNase by DNA-A and only 20% is protected by DNA-V. About 80% of RNA extracted from the 1.29 A particles is protected by NA-A indicating that A particles of both densities possess the same RNA sequences.

Meanwhile, RNA of extracellular virions is protected up to 80% by DNA-V and only up to 20% by DNA-A. Thus, A particles and extracellular virions possess different RNA sequences, and A particles can hardly be regarded as intracellular precursors of Mason-Pfizer-like virus.

Hybridization of A particle RNA with cellular DNA. Figure 7 depicts the kinetics of hybridization of 3H -labeled RNA of A particles with a density of 1.24 g/ml with DNA obtained from HEP-2 and human placenta cells. It is seen that RNA readily associates with HEP-2 DNA reaching a $C_0t_{0.5}$ value at about 180 mol s per liter. The data nearly follow a theoretical hybridization kinetic curve estimated for about 20 complete proviral copies per haploid genome (8). Meanwhile, RNA was not annealed to DNA obtained from human placenta cells (Fig. 7) as

FIG. 2a and b

FIG. 2c and d

FIG. 2. Negatively stained preparations of A particles with a density of 1.23 to 1.24 g/ml. Free (a) and aggregated (b) A particles. (a) Magnification $\times 170,000$; (b) $\times 380,000$. (c and d) Partially disrupted particle; (c) $\times 380,000$; (d) $\times 760,000$. Arrows in d show a helix with a diameter of 25 nm which is despiralized in strand with a diameter of 7 to 8 nm.

FIG. 3. Sedimentation (a and b) and buoyant density analysis in a CsCl gradient (c) of A particles. Material containing A particles with a density of 1.23 to 1.24 g/ml was divided in two parts, and one part was treated with RNase (20 $\mu\text{g}/\text{ml}$, 20 min at 18 C) (b and c). (a and b) The samples were centrifuged in SW 27.1 bucket rotor in 15 to 35% (wt/vol) velocity sucrose density gradients prepared on STE buffer at 24,000 rpm for 1.5 h at 4 C. (c) The sample was diluted with phosphate buffer containing 0.01 M KCl, fixed with 4% formaldehyde and centrifuged in a preformed CsCl gradient in SW 40 rotor at 35,000 rpm for 12 h at 4 C.

well as to NA from human embryo cells (not shown).

Immunodiffusion test. As shows Fig. 8, antisera against Mason-Pfizer-like virus produced

by HEp-2 cells do not form any band with 0.1% SDS disrupted A particles, whereas they form a single precipitin band with Mason-Pfizer-like virus.

FIG. 4. Kinetics of the endogenous RNA-dependent DNA polymerase activity of A particles (A) and extracellular virions (B) in the presence of Mn^{2+} and Mg^{2+} . All assay conditions were described in Materials and Methods. Assay mixture contained either Mn^{2+} , 0.8 mM (●) or Mg^{2+} , 6 mM (○). Control sample without three unlabeled triphosphates (Δ).

FIG. 5. Analysis of DNA product of endogenous polymerase reaction on A particles. The polymerase reaction was performed for 20 min and the nucleic acids were extracted with SDS-phenol and analyzed by centrifugation in 15 to 30% (wt/vol) velocity sucrose gradient prepared on STE buffer containing 0.5% SDS in an SW 40 bucket rotor at 36,000 rpm for 2 h 20 min. Acid-insoluble radioactivity was determined in samples; the rest of the material of the first 15 fractions was treated with RNase (15 µg/ml, 30 min at 37°C). Filled circles, untreated material, empty circles, RNase-treated material.

FIG. 6. Cross-hybridization of [³²P]RNA of A particles (RNA-A) and extracellular virions (RNA-V) with two ³H-labeled DNA-products. The specific activity of [³²P]RNA was estimated as 2,000 counts per min per ng. Hybridization was performed in 0.2 ml volume in hybridization buffer at 68°C for 48 h. Then samples were digested with RNase and acid-insoluble radioactivity was determined. Symbols: ●, DNA-V with RNA-V; ○, DNA-A with RNA-V; ▲, DNA-A with RNA-A; △, DNA-V with RNA-A; ■, DNA-A with RNA, extracted from A particles with a density of 1.29 g/ml.

FIG. 7. Kinetics of hybridization of ^3H -labeled RNA from A particles with DNA from HEp-2 and human placenta cells. RNA (2,000 counts/min) was hybridized with DNA (2 mg) in volumes of 1 ml at 68 C. Portions were taken at intervals and the percentage of RNA-DNA hybrids was calculated.

Attempts to cultivate A particles in human cells. Primary monolayers of human lung embryo cells were infected with highly purified fresh preparations of A particles and incubated at 35 C. Three passages of the cells were made in a week interval and then ultrathin sections of the cells were prepared and investigated in an electron microscope. No A particles were observed in three analogous experiments.

DISCUSSION

Intracytoplasmic A particles, though repeatedly described in a number of systems, were isolated only from the cells of mouse tumors and investigated in details by Tanaka et al. (14), Kerckaert et al. (5), and Smith and Wivel (11, 12). These authors showed that A particles are found in large aggregates and band in equilibrium sucrose gradients at 1.27 or 1.29 g/ml.

Our results suggest that intracytoplasmic A particles isolated from the cells of human continuous lines distinctly differ from A particles of mouse origin. Only a small amount of them (3 to

10%) bands at 1.29, whereas their essential part bands at 1.23 to 1.24 g/ml. Most A particles are found in postmitochondrial fractions partly as free individual particles sedimenting at 200 to 250S in velocity sucrose gradients, partly in small aggregates, and least of all involved in large aggregates which cosediment with nuclei. These aggregates appear to dissociate readily during washing of nuclei with 0.25 M sucrose; twofold washing was shown to be enough to separate A particles from nuclei. The washing fluids were successfully used throughout the work essentially increasing the yield of the particles.

A particles were shown to be extremely fragile, and this peculiarity produced difficulties for their further analysis. However, it was partially avoided by their preliminary treatment with RNase which eliminated all disrupted particles. In this way their buoyant density in a CsCl gradient was determined as 1.30 g/ml in accordance with the data published for A particles of mouse origin (14). This density allowed to conclude that A particles contain about 8% of RNA (13).

The idea that intracytoplasmic A particles in mouse cells represent immature forms of the extracellular B particles is widely discussed in the literature though a number of facts does not confirm this hypothesis (12, 14).

FIG. 8. Immunodiffusion test with antiserum against extracellular virus produced by HEp-2 cells. SB, Solution buffer; AGS HEp-2V, extracellular viruses treated with ether; p1.24, A particles with a density of 1.24 g/ml; p1.29, A particles with a density of 1.29 g/ml. Completely disrupted A particles treated with 0.1% SDS were used.

As to our system, the number of data evidences that A particles and Mason-Pfizer-like virus are not genetically related. They require different optimal conditions for polymerase reaction. Hybridization of A particle RNA with HEp-2 DNA reveals about 20 proviral copies per cell genome, whereas the number of copies of Mason-Pfizer-like genome in the same system is estimated as 40 (Zhdanov, personal communication). Finally, the results of immunodiffusion and cross-hybridization clearly show that a bulk of A particles is not intracellular precursors of Mason-Pfizer-like virus. However, it can not be excluded that some part of them does represent putative nucleoprotein cores of some other virions.

It seems reasonable to suggest that A particles represent an independent intracellular oncornavirus (or virus cores) which is possibly defective in some biological activities. In fact, the numerous attempts to transmit A particles to human lung embryo cells were unsuccessful, where as Mason-Pfizer-like virus from the same cell system was accumulated in essential amounts under the same conditions, A particles being not revealed in the cytoplasm (M. Alstein, *Acta Virol.*, in press). However, the failure to cultivate A particles could be due to the fact that they do not penetrate cells. The question of the relationship between A particles and Mason-Pfizer-like virus remains unclear, and future experiments will show whether A particles serve as a helper of this virus or are not related to its reproduction. In this respect the data on the similarity of A particles isolated from different human cells are important. This work is now in progress. The suggestion that A particles represent some putative human endogenous virus was not confirmed by hybridization experiments with human embryo and placenta DNA.

ACKNOWLEDGMENTS

We are indebted to V. M. Zhdanov, O. N. Berezina, and A. D. Alstein for their help and fruitful discussion, and to Natalie Vorkunova and Tatiana Morosova for excellent technical assistance.

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