Preliminary Characterization of an Organism Isolated from a Case of Viluy Encephalomyelitis Indicates a Protozoal, Rather Than Viral, Aetiology

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A microbial agent was isolated previously from a case of Viluy encephalomyelitis and named the 'KPN agent' after the initials of the patient. Here a detailed characterization of nucleic acids extracted from the purified KPN agent is presented. The agent contains both DNA and RNA, and has its own tRNAs and some other low- M_r RNAs, including 5S RNA. These findings, and the isolation of eukaryotic-type ribosomes, suggest that the KPN agent is not a virus, as believed before, but a more complex micro-organism, with protein-synthesizing capacity. The nucleotide sequence of the 5S RNA in the ribosomes of the KPN agent is identical with the sequence of 5S RNA of *Acanthamoeba castellanii*. The novel protozoan nature of the KPN agent is discussed in relation to other unusual properties of this micro-organism. Some implications of these results for the aetiology of Viluy encephalomyelitis are also discussed.

INTRODUCTION

Viluy encephalomyelitis (VE) is a severe neurological disease of man endemic for some regions of Yakutia (central Siberia) (Petrov, 1980). Epidemiological data suggest that the disease is transmissible, but its causative agent is unknown. Its incubation period may be several years. The disease is characterized by a slow degenerative process in the central nervous system, with limited inflammation. This suggested that VE may be caused by a slow virus. In the course of virological studies, several strains were isolated from VE patients, but most of them were found to be contaminants, bearing no relation to VE. One of the strains, KPN, named after the patient's initials, could not be identified with any known virus. Moreover, some of its properties were quite unusual (Sarmanova *et al.*, 1975; Karavanov *et al.*, 1975); for example, it was not neutralized by hyperimmune antisera raised against it. Infection of tissue cultures with the KPN agent induced an antigen that reacted in an indirect immunofluorescence assay with sera of VE patients (A. S. Karavanov, unpublished observations). This is an indirect indication of a possible involvement of KPN in VE. Another indication was obtained from the results of experimental infections of animals. The agent caused encephalitis in mice and monkeys. In the latter case the incubation period was 6–11 months; an agent very similar to KPN was reisolated (Zaklinskaya *et al.*, 1975). Nevertheless, there is no direct proof of the role of KPN in the aetiology of VE.

Irrespective of whether KPN is involved in VE, the biological nature of this specific agent is of interest. A number of its properties, including insensitivity to antibiotics and other drugs, suggested that it might be an RNA-containing virus (Sarmanova *et al.*, 1975). Nevertheless, an extensive electron microscopic investigation failed to reveal any virus-like particles. We showed

Abbreviations: VE, Viluy encephalomyelitis; PEK, porcine embryo kidney; 2-MET, 2-mercapthoethanol.

previously that several discrete high- M_r RNA species are formed in KPN-infected tissue cultures in the presence of actinomycin D (up to $10 \mu\text{g ml}^{-1}$) (Karavanov & Chumakov, 1983). Low- M_r RNAs (4–6S) are also present. In this communication we present further data on nucleic acids that can be specifically labelled in KPN-infected tissue cultures and extracted from the purified agent.

METHODS

Isolation of the KPN agent. The KPN agent was initially isolated in tissue culture from the brain of a victim of VE (Sarmanova *et al.*, 1975). It was grown in monolayers of porcine embryo kidney (PEK) cells cultured at 28°C as described by Karavanov & Chumakov (1983). To purify the agent, the cells were detached from the glass, washed in buffer A (150 mM-NaCl, 30 mM-Tris/HCl, pH 7.5) and disrupted by addition of non-ionic detergent (Nonidet P-40) to a final concentration of 0.5% at 0°C . The infectious agent was sedimented by centrifugation for 5 min at 3000 g, washed once with buffer A and applied to a 10–50% (v/v) Percoll (Pharmacia) density gradient. After centrifugation for 15 min at 3000 g, the KPN agent formed several closely located bands in the centre of the gradient, whereas the nuclei of the PEK cells remained on top of the gradient. In the tube containing material from the control, non-infected culture, no bands were visible in the middle of the gradient. Opalescent bands containing the infectious KPN agent were collected and diluted twice with buffer A. The agent was sedimented, washed with buffer A and extracted with water-saturated phenol/10% SDS (1:20, v/v). In some experiments, the material was digested with micrococcal nuclease (Sigma, $50 \mu\text{g ml}^{-1}$, 30 min at 25°C) in the presence of 1 mM- CaCl_2 , or with Pronase P (Serva, 2 mg ml^{-1} , 30 min at 25°C) before phenol extraction.

Agarose gel electrophoresis. This was done in a horizontal apparatus in 5 mm thick slabs. Agarose (1.5%, w/v) was prepared in 25 mM-sodium citrate, pH 3.5, containing $1 \mu\text{g}$ ethidium bromide ml^{-1} . Polyacrylamide gel electrophoresis was done in a Tris/borate/EDTA buffer system with 7 M-urea as described by Maniatis *et al.* (1982).

Radiolabelling. RNA was labelled *in vitro* with cytidine 3',5'-[5'- ^{32}P]bisphosphate (^{32}P]pCp) (Amersham) by using RNA ligase of bacteriophage T4 (Ferment, USSR) as described by England & Uhlenbeck (1978).

Isolation of ribosomes. The KPN agent purified in a Percoll gradient was washed in buffer A, sedimented and suspended in buffer B (50 mM-KCl, 30 mM-Tris/HCl, pH 7.5, 5 mM- MgCl_2 , 5 mM-2-MET). Sodium deoxycholate was added to a concentration of 0.2% and after 5 min at 0°C the homogenate was centrifuged for 5 min at 30000 g. The supernatant was collected and applied to 5–20% (w/v) sucrose gradients prepared in buffer B. Centrifugation conditions are indicated in the figure legends. In some experiments, the ribosomes were additionally purified before gradient centrifugation by sedimentation through a 22% sucrose cushion in buffer B. To prepare ribosomal subunits, ribosomes were treated with 25 mM-EDTA and centrifuged in a sucrose gradient prepared in buffer C (50 mM-KCl, 30 mM-Tris/HCl, pH 7.5, 1 mM-EDTA, 5 mM-2-MET).

To determine their buoyant density, the ribosomes were treated with 6% (v/v) glutaraldehyde for 12 h at 4°C . Saturated CsCl was added to 30% of saturation ($\rho 1.186 \text{ g ml}^{-1}$) and the resulting solution was layered on an equal volume of 60% saturated CsCl ($\rho 1.785 \text{ g ml}^{-1}$). The centrifuge tube was stoppered with Parafilm and slowly inverted once; this resulted in the formation of a gradient. Tubes were centrifuged for 8.5 h at 70000 g in an SW-39 rotor (Beckman) at 4°C . The density of the fractions was determined by weighing 100 μl samples.

Nucleotide sequence of 5S rRNA. The low- M_r RNA fraction was labelled *in vitro* with ^{32}P]pCp as described, and subjected to preparative separation by electrophoresis in 8% (w/v) polyacrylamide gel (Maniatis *et al.*, 1982). The band of 5S RNA was visualized by autoradiography, and RNA was eluted from the excised zone with a solution containing 0.5 M-ammonium acetate, 0.5% SDS and 10 mM- MgCl_2 . The eluted RNA was divided into samples, and each was treated with base-specific chemical reagents (Peattie, 1979). After cleavage of the RNA by treatment with aniline, fragments were separated by electrophoresis in polyacrylamide gels 0.2 mm thick and 50 cm (for 12% gels) or 100 cm (for 6% gels) long.

RESULTS

Agarose gel electrophoresis of the nucleic acids of the KPN agent

Fig. 1 shows the results of 1.5% agarose gel electrophoresis of nucleic acids extracted from the purified KPN agent. rRNAs of *Escherichia coli* and Krebs-II ascites tumour cells were used as references. In addition to the three major RNA bands, and several minor ones, described in our previous paper (Karavanov & Chumakov, 1983), a further slow-migrating band was present. This band stained green with acridine orange (McMaster & Carmichael, 1977), indicating double-stranded material (not shown). Fig. 2 presents the results of agarose gel electrophoresis of nucleic acids of the KPN agent treated with various nucleases. These results showed that the

Fig. 1

Fig. 2

Fig. 1. Electrophoresis in a 1.5% agarose gel of nucleic acids extracted from the KPN agent (lane 1). Lanes 2 and 3 contain rRNAs of *E. coli* and Krebs-II cells, respectively.

Fig. 2. Electrophoresis in a 1.5% agarose gel of nucleic acids extracted from the KPN agent and treated with RNAase A (lane 1), RNAase T1 (lane 2) or DNAase I (lane 3). Lane 4, control, no enzymes added; lane 5, a mixture of rRNAs of *E. coli* and Krebs-II cells. All treatments with enzymes were for 15 min at 25 °C, 10 µg ml⁻¹.

slow-migrating band was composed of DNA. The sharpness of this DNA band is not an indication of its homogeneity since for a very high- M_r DNA the relative mobility in agarose gel is independent of the M_r ; all DNA larger than some critical value migrates anomalously as a single sharp band. To evaluate the M_r of this DNA and to determine whether it was a monodisperse molecule, we did electrophoresis in 1% and 0.6% agarose gels, using a low voltage gradient. The DNA band still migrated anomalously in these conditions (results not shown), whereas *EcoRI* restriction endonuclease fragments of bacteriophage λ DNA were clearly resolved. This showed that the DNA present in preparations extracted from the KPN agent was larger than 56 kb.

To check the possibility that the DNA was the result of contamination with host cell material, we compared the nucleic acids of two samples of the KPN agent, one treated with micrococcal nuclease and the other not. The two preparations showed similar sets of bands upon agarose gel electrophoresis, including the band of high- M_r DNA. Treatment of PEK cell nuclei with micrococcal nuclease under the same conditions resulted in complete destruction of DNA

(results not shown). These results suggest that the DNA in the KPN agent preparation was not the result of contamination, since it was protected from nuclease digestion. However, there still remains the unlikely possibility that the host cell DNA is protected in some specific way by the KPN agent. Hybridization experiments are needed for the final proof of the KPN-specific character of this DNA.

Characterization of low- M_r RNA

We have shown that along with a high- M_r RNA (sedimentation coefficient 20S), RNA preparations extracted from the KPN agent contain low- M_r RNA species (sedimentation coefficients 4–6S) (Karavanov & Chumakov, 1983). This RNA can be seen in Fig. 2, in which electrophoresis was done for a shorter period than usual. This method gave poor resolution of the high- M_r RNA, but made it possible to detect low- M_r species which otherwise would have migrated off the gel.

For a more detailed characterization of these small RNAs, we used polyacrylamide gel electrophoresis in the presence of 7 M-urea. RNA was labelled *in vitro* with [32 P]pCp by T4 RNA ligase. Two independent preparations of RNA from the KPN agent contained a group of bands with a mobility corresponding to tRNA, and two other bands, one of which had a mobility very close to that of 5S RNA, and the other, to that of 5.8S RNA (Fig. 3). These RNAs differed in their mobility from the 5S and the 5.8S RNAs of control, non-infected PEK cells. It can be seen from Fig. 3, lane 6, that the low- M_r RNA preparation from a whole KPN-infected PEK cell culture contained both the host cell and the KPN agent 5S RNA species. These differences in electrophoretic mobility argue against the possibility that the 5S and 5.8S RNA in the KPN agent nucleic acids were the result of contamination with host cell material.

To exclude this possibility for tRNAs we did two-dimensional gel electrophoresis of tRNAs extracted from the purified agent, and from control, non-infected and KPN-infected PEK cell cultures. This analysis showed that the two sets of RNAs were different. The tRNA preparation from the KPN-infected PEK cell culture contained RNA spots from both sets (results not shown). Thus we concluded that the small RNAs present in nucleic acid preparations extracted from purified KPN agent were not due to contamination, but represented the RNA species of the agent itself.

Isolation of ribosomes of the KPN agent

The presence of 5S and 5.8S RNA species in preparations of nucleic acids extracted from the purified KPN agent suggested that these RNAs might be components of the agent's putative ribosomes. To isolate these ribosomes we used a KPN agent preparation labelled with [3 H]uridine in the presence of actinomycin D and purified on a Percoll gradient as described in Methods. The agent was treated with sodium deoxycholate and the homogenate, clarified by centrifugation for 15 min at 30000 *g*, was fractionated on a sucrose gradient; unlabelled HeLa cell ribosomes with a sedimentation coefficient of 80S were run in the same tube as the standard. A sharp peak of radioactive material was observed, coincident with the peak of ultraviolet-absorbing material at a position corresponding to a sedimentation coefficient of 87S (results not shown). This value is consistent with a ribosomal nature for this material. To prove this assumption we determined the buoyant density after fixation with glutaraldehyde, and obtained a value of 1.57 g ml $^{-1}$. After treatment with EDTA this material gave two peaks in a sucrose gradient (Fig. 4), with sedimentation coefficients of 40S and 56S. We concluded that this material was KPN agent ribosomes.

Determination of the nucleotide sequence of 5S rRNA of the KPN agent

Due to its high degree of evolutionary conservation, the chemical structure of 5S rRNA of various organisms is suitable for use as a probe to determine the phylogenetic position at a high taxonomic level (Hori, 1975). In an attempt to establish the biological nature of the KPN agent, we determined the nucleotide sequence of the 5S RNA in a preparation from the purified agent. Fig. 5 presents the primary and the proposed secondary structure of this RNA. A comparison with the data listed in the collection of published 5S RNA sequences (Erdman *et al.*, 1983)

Fig. 3

Fig. 4

Fig. 3. Electrophoresis in an 8% polyacrylamide gel of low- M_r RNAs of two independently isolated preparations of KPN (lanes 1 and 2), 5S RNA of *E. coli* (lane 3), tRNA of Krebs-II cells (lane 4), total PEK-cell RNA (lane 5), and total KPN-infected PEK cell culture RNA (lane 6). The outlined area marked XC indicates the position of the dye xylene cyanol.

Fig. 4. Sedimentation of subunits of ^3H -labelled ribosomes of KPN agent (●) and unlabelled HeLa cell ribosomes (○) in a 5–20% sucrose gradient. Centrifugation was for 75 min at 60000 r.p.m. in an SW-65 rotor (Beckman) at 2 °C. The experiment was repeated twice; a representative profile is shown.

Fig. 5. Nucleotide sequence of 5S RNA of the KPN agent.

showed that the nucleotide sequence was identical with that of 5S rRNA of *Acanthamoeba castellanii*, so we concluded that the KPN agent contained an amoeboid protist. It should be mentioned that amoebae as well as some other protists contain three high- M_r RNA species instead of the two that are most common for the majority of organisms (see Stevens & Pachler, 1972; Cammarano *et al.*, 1982). The presence of three high- M_r RNA species in KPN agent ribosomes was therefore compatible with its suggested protozoan nature.

DISCUSSION

The main result of this investigation is that all the properties of the KPN agent described above are incompatible with its being a virus. The nucleotide sequence of 5S rRNA indicated the presence of an amoeboid protist in KPN agent preparations. The protozoan nature of the KPN agent can therefore be regarded as established. When this work had been completed, a morphological study revealed the presence of limax amoebae in infected PEK cell cultures (Yu. S. Chentsov, unpublished observations).

The coincidence of the nucleotide sequences of the 5S RNAs of the KPN agent and of *Acanthamoeba castellanii* implies that these are closely related, but not necessarily identical species, since 5S RNA structure is highly conserved in the course of evolution. Unfortunately, the *A. castellanii* 5S RNA sequence is the only one so far published for amoebae. More sequence data are needed to evaluate the taxonomic significance of the coincidence and differences in 5S RNA sequences. According to our preliminary data, the nucleotide sequence of KPN agent 5·8S RNA, another small rRNA species, differs somewhat from that of 5·8S RNA of *A. castellanii* and other small amoebae that we have studied (K. M. Chumakov & L. M. Gordeeva, unpublished). Further investigation is required to define whether the KPN agent represents a novel species or belongs to one already described.

The investigation described here has drastically changed our views on the nature of the KPN agent and prompted some questions. How could this large eukaryotic micro-organism mimic many viral properties and thus escape identification for many years? There are two precedents for amoebae being erroneously taken for viruses: in one case the cytopathic agent, called 'lipovirus' (Chang & Humes, 1962), was shown to be *Hartmannella* (Dunnebacke & Williams, 1967), and in the other, the so-called 'Ryan' virus (Pereira, 1966) was identified as *Acanthamoeba* in the course of a microscopic study (Armstrong & Pereira, 1967). In the case of the KPN agent, the morphological study was inconclusive, probably because the main aim was a search for virus-like particles. There were two major arguments in favour of the viral hypothesis: (i) multiplication of the agent was insensitive to antibiotics, including actinomycin D, and other drugs, and (ii) cell cultures showed cytopathic effects when inoculated with an agent that passed through filters with 100–200 nm pores (Sarmanova *et al.*, 1975; Karavanov *et al.*, 1975). The insensitivity of multiplication of the KPN agent to a broad range of drugs may be explained by a perfect selectivity of the outer membrane of this agent, protecting the cell from toxic substances. It is much more difficult to explain the results of the filtration experiments. Apart from the unlikely possibility that the KPN agent can pass through filters by means of a thin pseudopodium, all other explanations require additional assumptions. It is known that protists may produce toxins (Chang *et al.*, 1962) or contain factors pathogenic for mammalian tissue cultures (Dunnebacke & Schuster, 1977). If this is true for KPN, then the cytopathic effect caused by the filtered material may be not due to the agent itself, but rather to an associated toxic substance. Whichever possibility is true, it is clear that further investigations of the properties and morphology of the KPN agent are required. Such investigations are in progress.

The way in which the biological nature of the KPN agent was established deserves comment. It is quite unusual to establish the identity of an organism from the sequence of its rRNA. Nevertheless, this may not be just a curiosity, but rather an indication that this approach is a realistic one. This may be one of the first results of practical molecular determinative systematics. This approach may prove useful in complicated cases such as that described in the present study.

Elucidation of the protozoan nature of the KPN agent has important implications for the aetiology of VE. Small free-living amoebae of the genus *Acanthamoeba* and of the related species *Naegleria fowleri* are the causative agents of sporadic primary amoebic meningoencephalitis (Griffin, 1978; John, 1982). The ability of small amoebae to cause pathology of the human central nervous system supports the assumption about the causative role of KPN in VE. On the other hand, *Acanthamoeba* usually infects debilitated, immunocompromised persons. So there is a possibility that the KPN strain is not an aetiological agent, but one causing secondary complications. This question may be answered by further investigations. In any case, a more specific search for KPN in pathological materials is now possible. Another prospect is the search for KPN in the environment in order to understand its ecology and, probably, its role in the epidemiology of VE.

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