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Scrapie and Transmissible Mink Encephalopathy: Search for Infectious Nucleic Acid

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Brain preparations from animals with scrapie or transmissible mink encephalopathy were phenol extracted and examined for the presence of pathogenic nucleic acid. Animals inoculated with various extracts remained healthy, and analysis on 2.6 to 5% polyacrylamide gels failed to detect a difference in extractable RNA species between infected and normal mink brain.

One of the greatest enigmas in microbiological research is the physicochemical nature of the scrapie agent. Scrapie is a natural slow virus disease of sheep and goats which has been experimentally transmitted to nine other species (9). The transmissible agent is nonpathogenic for a variety of tissue culture systems, produces no detectable antibody response, and has never been positively visualized.

Among the agent's properties is an unusual resistance to UV irradiation (1), nucleases (8), and β-propiolactone (11). Such characteristics have stimulated speculation that scrapie may be the first self-replicating agent devoid of nucleic acid (1). While most will be hesitant to accept such a hypothesis, it is evident that any nucleic acid contained within the scrapie agent is present in only a small amount or, possibly, in some unusual association, composition, or conformation, thus endowing it with structural and biological stability. The discrepancy between an operational size of 30 nm, as determined by filtration studies (14), and a radiation target size of 2×10^5 daltons (2) would indicate that the essential molecular structure or substance necessary for infectivity is but a small fraction of the total complex. Is this substance nucleic acid? If so, can it be separated from its intimate association with cell membrane and retain infectivity?

T. O. Diener has postulated that scrapie may be caused by a viroid (6), a low-molecular-weight RNA similar to that producing potato spindle tuber disease. To examine this possibility, we took two approaches. The first was to look for infectious nucleic acid after conventional phenol extraction of scrapie mouse brain. The second method was applied to mink brain from animals affected with transmissible mink encephalopathy (TME), a disease caused by an agent with properties indistinguishable from those of scrapie (16), and employed procedures used for the extraction and concentration of low-molecular-weight pathogenic RNA from citrus exocortis disease (18).

(A preliminary report on the extraction of mouse brain was presented at the Annual Meeting of the American Society for Microbiology, 6-11 May 1973, Miami Beach, Fla.)

MATERIALS AND METHODS

Brain tissue. Mouse brain containing ME7 scrapie agent was homogenized to 10% in saline by using disposable syringes and petri dishes. The homogenate was sonically treated five times for 3 min in an ice bath with a model LS-75 sonifier (Branson Instruments, Inc., Stamford, Conn.) at a setting of 4 at 4 A. this sonicated suspension was centrifuged for 8.4×10^5 *g*-min, the pellet was discarded, and the supernatant was retained for assay and extraction. Normal mouse brain was also prepared by using identical methods.

Mink brain was pooled from 24 animals killed in the terminal stages of TME and represented the 6th to 8th passages of the agent.

Extraction. (i) Mouse brain. A 2.6-ml amount of the scrapie brain preparation was placed in each of two tubes, and an equal amount of normal mouse brain was placed in a third tube. A suspension (0.1 ml) of purified encephalomyocarditis (EMC) virus containing 6.5×10^{10} PFU was added to one of the tubes containing scrapie brain. Each tube was then treated with 0.4 ml of TES (10 mM Tris, 5 mM EDTA, 0.14 M NaCl) at pH 7.5, 1.0 ml of purified benotonite suspension (5 mg), and 4 ml of a solution of liquid phenol containing sodium dodecyl sulfate (0.5%). This mixture was extracted at 45 C for 5 min and then centrifuged for 10^4 *g*-min, and the aqueous phase was removed. The interface was re-extracted, and the aqueous phases were combined. Dissolved

phenol was removed with ether, and the ether was removed by nitrogen, leaving a final volume of 3.8 to 4.0 ml in each of the three tubes.

(ii) Mink brain. Eighty grams of frozen brain was placed in a Waring blender together with 8.0 ml of 5% sodium dodecyl sulfate, 8.0 ml of purified bentonite solution (40 mg/ml), 1.0 ml of EDTA (0.1 M), 150 ml of cold (4 C) phenol, and 150 ml of 0.1 M Tris, pH 8.9. This mixture was blended thoroughly by three 30-s bursts and then centrifuged for 1.3×10^5 *g*-min. The aqueous phase was removed, and the interface and organic phases were re-extracted with 100 ml of water and 30 ml of phenol. The combined aqueous phases were then extracted with an equal volume of cold phenol.

A sample of the final aqueous phase was removed for animal inoculation before treatment with 3 volumes of ethanol to which was added 10 ml of 3 M sodium acetate (pH 5.5). This was allowed to sit at −20 C for 1 h, and the precipitate was removed by centrifugation for 7.2×10^4 *g-min*. The pellet was resuspended in 15 ml of TKM (0.01 M Tris, 0.01 M KCl, 0.0001 M $MgCl_2$, pH 7.4) and then dialyzed against 2 liters of TKM at 4 C for 2 h. After dialysis, a sample was removed for animal inoculation before treatment with 50 mg of DNase and 0.4 ml of 0.1 M $MgCl_2$. This mixture was incubated at 37 C for 2 h, reprecipitated in ethanol, resuspended in TKM, and dialyzed.

RNA was partitioned by the addition of an equal volume of 4 M LiCl (4 C, overnight). The precipitate, containing mainly high-molecular-weight, single-stranded rRNA, was removed by centrifugation for 1.3×10^5 *g*-min, resuspended in 2 ml of TKM, and dialyzed. The LiCl supernatant, containing mainly tRNA and low-molecular-weight or double-stranded species, was precipitated with ethanol (no acetate), resuspended in 2 ml of TKM, and dialyzed. The purity of each fraction was tested by UV absorbance ($A_{260/280}$). An equivalent amount (80 g) of normal mink brain was simultaneously carried through the same manipulations as the TME brain.

Gel electrophoresis. Fractions were tested by electrophoresis in polyacrylamide gels in 0.04 M Tris, 0.02 M sodium acetate, 0.001 M sodium EDTA buffer, pH 7.2, with 0.2% sodium lauryl sulfate (3). LiCl precipitate preparations were migrated on 2.6% gels with 0.5% agarose while the LiCl supernatant preparations were analyzed on 5% polyacrylamide gels. Gels were prerun for 60 min at 7 mA/gel. Samples (0.1 to 0.3 ml) were applied with 1 to 3 drops of saturated sucrose to 10-cm gels and electrophoresed for 4 h at 6 mA/tube at room temperature. Gels were scanned at 260 nm in a Gilford model 2410 linear transport coupled with a Beckman DU spectrophotometer.

Animal inoculation. (i) Mice. The aqueous phase from the scrapie mouse brain extraction was frozen at −70 C for 5 days before inoculating (0.03 ml) suckling, random-bred, Swiss white mice by an intracerebral route. The extract was divided into five fractions: (i) treated with 10 μg of DNase per ml for 10 min; (ii) treated with 10 μg of RNase per ml for 10 min; (iii) treated with 500 μg of DEAE dextran (DD) per ml, (iv) treated with 1,000 μg of DD per ml; and (v) untreated. Each fraction was diluted in TES, and samples from undiluted and from 10^{-1}, 10^{-2}, and 10^{-3} diluted preparations were each inoculated into 9 to 12 mice.

Extracted brain containing EMC RNA and a sample of the unextracted scrapie brain preparation were also inoculated intracerebrally into suckling mice at dilutions of 10^{-1} to 10^{-7}. The extracted EMC RNA was inoculated untreated and after treatment with RNase (10 μg/ml, 10 min) and DD (30, 300, 1,000, and 3,000 μg/ml). End points were calculated by the Spearman-Karber method (7).

(ii) Minks. The following samples were inoculated into minks: (i) aqueous phase before ethanol precipitation; (ii) aqueous phase after ethanol precipitation and resuspension of pellet in 15 ml of TKM; (iii) precipitate after LiCl treatment and resuspension in 2 ml of TKM; (iv) LiCl supernatant after precipitation with ethanol and resuspension of the pellet in 2 ml of TKM; and (v) identical samples after treatment with RNase (10 μg/ml, 10 min). Each sample was inoculated undiluted into six minks for a total of 48 animals. Inoculation was intracerebral (0.1 ml); minks were 6 months of age and of various color phases.

RESULTS

A summary of results is presented in Table 1.

Mouse brain. (i) EMC virus. The nucleic acid preparation from the EMC virus-seeded

TABLE 1. *Summary of results from animal inoculations with various preparations of scrapie mouse brain, encephalomyocarditis virus, and brain from mink affected with transmissible mink encephalopathy*

Material and treatment	Infectivity
Scrapie mouse brain, 10% in saline, sonified supernatant (8.4×10^5 *g*-min)	$10^{7.2}$ LD_{50}/ml
Scrapie mouse brain supernatant and, 6.5×10^{10} PFU of EMC virus, phenol at 45 C	$10^{5.9}$ LD_{50} EMC RNA
6.5×10^{10} PFU of EMC virus, phenol at 45 C	$10^{7.3}$ LD_{50} EMC RNA
Scrapie mouse brain supernatant, phenol at 45 C	0[a]
TME mink brain, 10% in saline	$\geq 10^{7.5}$ LD_{50}/ml
TME mink brain, phenol at 4 C	0[a]
TME mink brain, phenol, ethanol, and sodium acetate (7.2×10^4 *g*-min)	0[a]
TME mink brain, phenol, 4 M LiCl, 1.3×10^5 *g*-min (supernatant)	0[a]
TME mink brain, phenol, 4 M LiCl, 1.3×10^5 *g*-min (precipitate)	0[a]

[a] No infectivity detected in 0.03-ml (scrapie) or 0.1-ml (TME) samples from an undiluted sample.

mouse brain contained $10^{5.9}$ mean lethal doses (LD_{50}) EMC RNA. An equivalent amount of EMC virus extracted in the absence of brain yielded $10^{7.3}$ LD_{50} RNA. Thus, the yield of RNA infectivity from the EMC-seeded brain extract was about 7% of theoretical. No infectivity survived treatment with RNase, and inoculation in the presence of various concentrations of DEAE dextran did not enhance infectivity.

(ii) Scrapie. The unextracted scrapie brain preparation contained a titer of $10^{7.2}$ LD_{50}/ml. None of the mice inoculated with any of the samples of the phenol-extracted scrapie brain developed signs of disease. After 1 year, the experiment was terminated and brain material was collected from mice inoculated with undiluted samples. None of these animals showed histopathological evidence of infection, and mice subinoculated with their pooled brains were unaffected after 8 months of observation.

Mink brain. (i) Gel electrophoresis. No anomalous RNA species were observed after polyacrylamide electrophoresis of samples from TME-infected brain tissue. The quality of the preparations was sufficient to permit detection of native rRNA species as well as 4 and 5*S* RNA in the LiCl supernatant.

(ii) Animal inoculation. Unextracted TME brain from the same pool as that treated with phenol was found to contain $\geq 10^{7.5}$ LD_{50}/ml in a separate experiment conducted 1 year earlier. The titer of the TME agent has not been found to decrease in pieces of whole tissue stored at −70 C for periods up to 20 months (16). Mink inoculated with the various samples of extracted brain have shown no signs of disease after 12 months of observation. One animal in each group was killed at 10 months. Histopathological examination revealed no lesions suggestive of infection.

DISCUSSION

Previous attempts at phenol extraction of scrapie (13) and TME (16) brain have also been unsuccessful in demonstrating an infectious nucleic acid. The present experiments were stimulated by new information on the existence of low-molecular-weight RNA viruses capable of producing disease in plants (5, 19). This would not be the first time that new phenomena operable in animal virus-host relationships were first observed or suggested by the study of plant and bacterial viruses.

We monitored our ability to extract and measure infectious nucleic acid by using EMC virus. Infectivity would appear to be the most sensitive indicator of an intact nucleic acid chain since there is evidence that single-chain RNA molecules may suffer loss of infectivity through one or more breaks with no change in particle size as determined by sedimentation coefficient (4, 10, 12). Our results indicated that the procedures used were relatively efficient in extracting pathogenic EMC RNA. Since a viroid, or other small nucleic acid, would have a molecular weight roughly 1/25th ($\sim 10^5/2.6 \times 10^6$) that of EMC RNA, it would sustain even less damage by random scissions due to exposure to nucleases in mouse brain during extraction procedures, assuming equal susceptibility. We can therefore conclude that the failure to detect an infectious scrapie nucleic acid was not likely due to destruction by nucleases during extraction.

One can only speculate as to the reasons why we were not successful in demonstrating an infectious scrapie or TME nucleic acid. The scrapie agent has been found to be intimately associated with cell membrane (17); therefore, it may be more difficult to dissociate from host tissue than any of the plant viroids or animal viruses which are present in the nucleus or cell sap. Association with cell membrane also limits the concentration of scrapie agent. To compensate for low titer, we extracted a quantity of mink brain which was approximately 300 times greater than in the mouse experiment, but this is still considerably less than concentrations available with more conventional viruses.

Unfortunately, these agents can only be detected by using in vivo procedures. Although we have shown that nucleic acids from viruses which produce acute encephalitides, such as EMC, can be assayed by intracerebral inoculation, we do not know whether or not slow infections, such scrapie and TME, can be established in the same manner. Membrane components may be essential in protecting these agents during an initially long cell-free period in the host. It is also possible that the membrane serves as a recognition site for absorption and penetration into susceptible cells. Without the membrane, the nucleic acids are diluted in "nonpermissive" cells.

We cannot conclude from these experiments that the agents of scrapie and TME contain no pathogenic nucleic acid. We must explore new methods of detection to overcome the formidable problems presented by an in vivo assay. Experiments to reconstruct an infectious membrane complex are indicated. This can perhaps be most easily accomplished by attempting to infect tissue culture cells with nucleic acid extracts in the presence of DEAE dextran. These cells can then be tested in susceptible animals for their ability to produce disease. It

may also be possible to improve upon the animal assay by detecting an earlier, more sensitive effect of the scrapie agent (15) which is not dependent on a long incubation period.

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