Production of Borna Virus in Tissue Culture (36492)

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Borna disease, an enzootic meningoencephalomyelitis in horses, has been known for over 200 years in middle and eastern Europe. The disease derived its name from the main outburst in 1894 in the region of Borna, a Saxonian town. Identified as a virus by Zwick and others in 1926 (1), until recently very little (Table I) was known about the morphological and biochemical features of the Borna virus or its classification (2, 3). The wide host range of Borna disease, its long incubation period, and a general lack of serological reaction in afflicted animals raise the suspicion that the Borna virus may be more widespread than previously assumed and may possibly also occur in man. Only recently has Borna disease been attributed to the group of slow virus infections (4).

Experimental work with Borna virus is very difficult and time consuming. Practically the only animal suitable for the demonstration of the virus is the rabbit, although several other laboratory animals are susceptible to Borna virus. The incubation time in rabbits ranges between 3 weeks and 8 months after intracerebral infection. Virus production in embryonated chicken eggs has been described (5) but is not suitable for routine work. Until now, no report has been made on the production of the virus in tissue culture.

Materials and Methods. Laboratory animals. For demonstration of the virus, 6-week-old rabbits weighing an average of 400 g and fed a regular diet, were used.

Virus strain. Virus was taken from the brain of an afflicted horse. Through cultivation in the rabbit, we isolated the strain M27. Following its characterization as Borna virus (refer to Table II), it was passaged three times in rabbits. The third passage was used as virus material for the experiments. For this purpose, we prepared, as has been previously described (6), a 10% suspension of the rabbit brains.

Tissue culture. Primary lamb kidneys: Kidneys from approximately 4-month-old butchered lambs were treated with 0.25% trypsin using the method of Dulbecco and Vogt (7) and grown to stationary monolayers.

Lamb kidney subcultures: Primary monolayers of lamb kidney cells were trypsinized with 0.125% trypsin, centrifuged, resuspended at a ratio of 1:2 and seeded in dilution bottles and culture tubes.

Medium. The medium for primary and subcultivated lamb kidney cells was Earle's salt solution supplemented with 10% calf serum, 0.5% lactalbumin hydrolysate, 100 IU penicillin/ml, and 100 μg streptomycin/ml of medium.

The control of cultures was performed microscopically on native cultures and at certain intervals on stained tissue culture preparations. For this purpose, monolayers from the culture tubes were prepared using the celloidin technique which has been applied for routine work at the Institute for Epizootiology of the Veterinary University of Budapest and was communicated personally by Bartha. They were then stained with hematoxylin and eosin. To demonstrate infectivity, culture cells and medium were used separately. The cells were frozen and thawed several times and then centrifuged at 3000 rpm for 10 min.

Specificity assay for Borna virus. In general, the virus was demonstrated by intracerebral inoculation into rabbits. For specificity demonstration, the following criteria

TABLE I. Characteristics of Borna Virus.

Particle size	80 nm
Type of nucleic acid	?
Particle structure	Enveloped
Sensitivity against	
chloroform treatment	Labile
pH change	Stable at 5–12
heat	Nonresistant
Virus replication in	?
Formation of inclusion bodies	Intranuclear (Joest-Degen)
Antigenical composition	Complement fixing s-antigen (15–30 nm)
Serotypes	None

were used.

1. Typical sickness of rabbits with symptoms of meningoencephalomyelitis after an incubation period of 4–8 weeks.

2. Demonstration of Borna antigen in the brain of sick rabbits according to a modified Kolmer technique for a complement fixation reaction (CF test). The hyperimmune sera used in this test were produced in rabbits through repeated subcutaneous injections of brain extracts from horses that had died of Borna disease. As control antigens, Borna brain extracts of horses and rabbits and brain extracts of healthy rabbits were used.

3. Demonstration of characteristic pathohistological alterations in the brain of the sick rabbits,[1] resulting in perivascular lymphocytes, ganglion cell degeneration, and gliosis.

Experiments and Results. Preliminary experiments. In preliminary trials with primary lamb kidney cultures which had been infected with Borna virus after sheeting to a dense monolayer and maintained over a 2–3 week period, neither cell alterations nor infectivity in rabbits could be found. Also, subcultures of the Borna-infected primary cells failed to produce infective Borna virus. Only in one experiment, the fourth subpassage which was maintained over 43 days, did we find the intranuclear inclusion bodies (Fig. 1), that had not been observed in the control cultures. Inoculation into rabbits was positive and the Borna specificity of the disease could be demonstrated (CF test, histology).

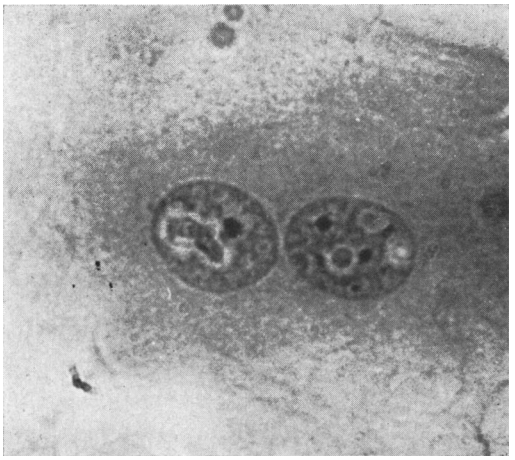

FIG. 1. Intranuclear inclusion bodies in Borna-infected lamb kidney subcultures, 43 days after seeding. Hematoxylin and eosin, ×600.

Main experiment. Based on the preliminary experiments, we assumed that Borna virus could be propagated and passaged in lamb kidney subcultures, whereas it occurs in an infectious form only after a longer incubation time. To confirm this, we cultivated lamb kidney subcultures with regular medium changes (about every 10 days). This allowed us to maintain the cultures up to 10–12 weeks and to subcultivate them further. This model was used for all further experiments. The cells were always infected at the time of seeding by adding 5% of the virus material

TABLE II. Identification of Borna Virus.

Clinical symptoms in horse
↓
Histological examination of horse brain
(nonpurulent meningoencephalomyelitis,
intranuclear inclusion bodies)
↓
Immunofluorescent test with horse brain
↓
Complement fixation test
(s-antigen)
↓
Intracerebral infection of rabbits
a. clinical symptoms
b. histological changes in brain
c. complement-fixation test with brain

[1] For the performance of the histological examinations, we express our gratitude to the Institute for General Pathology and Neuropathology for Animals, Munich (Chairman: Professor Dr. E. Dahme).

TABLE III. The Course of Borna Virus Cultivation in Lamb Kidney Subcultures.

Time post-infection	Assayed material	Results of intracerebral rabbit infection			Cell alterations compared to controls
		Clinical	CFT	Histology	
10 min	Medium	+	+	+	
after seeding	Cells	+	+	+	—
24 hr	Medium	—	+	+	Marked cell repro-
after seeding	Cells	—	+	+	duction
48 hr	Medium	+	+	+	Marked cell repro-
after seeding	Cells	+	+	+	duction
72 hr	Medium	—	—	—	Cultures sheeted
after seeding	Cells	—	—	—	earlier than controls
15 days	Medium	—	—	—	Cell and nuclear
after seeding	Cells	—	—	—	enlargement
25 days	Medium	—	—	—	Cell and nuclear
after seeding	Cells	—	—	—	enlargement
37 days	Medium	+	+	+	Rare nuclear
after seeding	Cells	+	+	+	inclusions
64 days	Medium	+	+	+	Rare nuclear
after seeding	Cells	+	+	+	inclusions
98 days	Medium	+	+	+	Cell degeneration
after seeding[a]	Cells	+	+	+	

[a] Day 34 after subcultivation.

to the medium.

In these experiments, we investigated the infectivity of the Borna virus from the time of seeding by regularly inoculating samples of the cells and the culture medium into rabbits. The results are compiled in Table III.

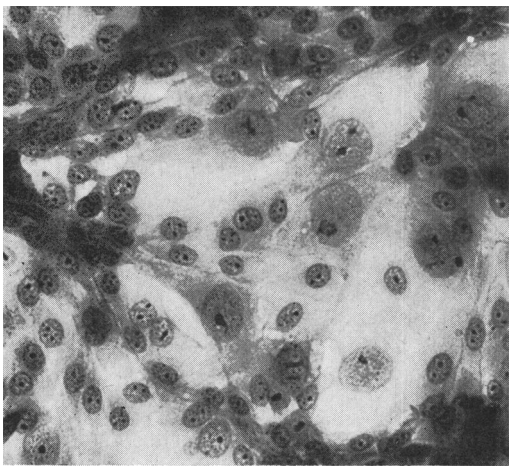

FIG. 2. Plaque-like area of enlarged cells and nuclei in Borna-infected lamb kidney subcultures, 16 days after seeding. Hematoxylin and eosin, ×250.

In the cultures, infectious virus was present until 48 hr after seeding. The cultures lost their infectivity after 72 hr and remained negative until day 37 when inoculation into rabbits yielded positive results. Infectivity could also be demonstrated at day 64 after seeding. At this time, the cultures were subpassaged. When assayed for infectivity at day 34 after subpassaging, these cells proved to be positive (*i.e.*, day 98 after original seeding).

Morphologically, there was not much difference between the infected cells and the control cultures, although an accelerated growth of the infected cultures was observed (see Table III). After 10–20 days, there were distinct foci in the cultures with enlargement of the cells and nuclei (Figs. 2 and 3) and vacuolization of the nuclei. In some areas, the cytoplasm showed granulation and less distinct structures. After week 5, we occa-

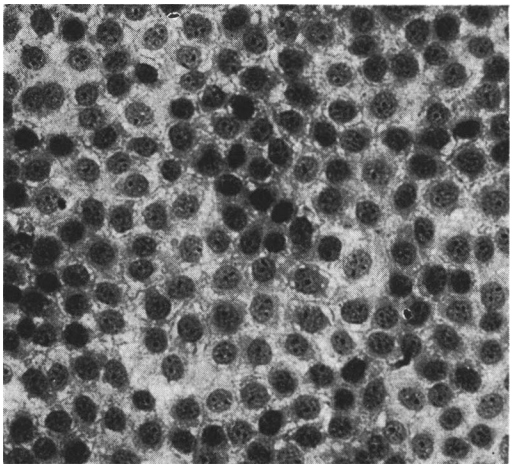

FIG. 3. Noninfected lamb kidney subculture, 16 days after seeding. Hematoxylin and eosin, ×250.

sionally detected nuclear inclusion bodies of the Borna type (small, compact, eosinophilic inclusions with a large halo). Their occurrence was not correlated with the infectivity of the cells for rabbits, as proved in other experimental series. Cytopathogenic effects such as degenerative or lytic processes were not observed in the cultures, nor was there any evidence of a cell transformation.

We confirmed the results described above in further experiments. At 48–72 hr postinfection, the infectious virus in rabbits regularly disappeared from the cultures. In one series, the cultures became Borna-positive 56 days after cultivation and remained infectious until subcultivation on day 76. In another series, infectivity occurred only after day 76. In all experimental assays, the alterations of the cell in comparison to the controls were similar to that described above—enhanced growth of the infected cells, enlargement of cells and nuclei in distinct areas of the tissue culture with no direct correlation between the nuclear inclusion bodies and the infectivity for rabbits.

Discussion. Borna virus can be multiplied in several laboratory animals, most easily in the rabbit and, under certain conditions, in the embryonated chicken egg. Cultivation in tissue cultures has not yet been achieved, although our experiments demonstrate that cultivation in tissue culture is possible. A suitable cell system is lamb kidney subcultures, if infected at the time of seeding and actively maintained for at least 5 weeks. At the beginning of infection, the infected cultures show an enhanced growth tendency as compared with controls, and subcultivation can be achieved without difficulty. Lytic or degenerative processes cannot be demonstrated during the virus reproduction period. After several weeks, distinct foci with enlargement of cells and nuclei appear in some areas of the tissue cultures. Inclusion bodies typical for Borna rarely occur. There is no correlation between the infectivity of the cells for rabbits and the inclusion bodies.

In general, the initial infectivity of the cultures disappears after approximately 72 hr. It reoccurs after the latent period of 5–10 weeks, remains demonstrable during the time of cell culture survival, and even persists during the subcultivation of originally infected cells.

The behavior of the Borna virus in cell cultures strongly resembles a slow virus infection. The study of this new model has some advantages for the investigation of slow viruses:

1. The cause of a persistent, slow virus infection can be observed *in vitro*.

2. Borna virus can easily be transmitted to rabbits.

3. The animals develop a typical meningoencephalomyelitis with characteristic histological brain alterations.

4. Complement-fixing antigen occurs at the site of the virus reproduction (rabbit brain).

Summary. This is the first report on the cultivation of Borna virus in tissue culture. In subcultures of lamb kidneys, the virus reproduces with a latent period of several weeks. At 48–72 hr postinfection, the infectivity of the subcultures for rabbits disappears but recurs after 5–10 weeks. The infectivity is not lost by subcultivation of the cells, and the infected cultures show an enhanced growth rate. After several weeks, plaque-like areas with enlarged cells and nuclei can be observed in the infected cultures. Nuclear inclusion bodies are found, but very rarely, and show no correlation to the infectivity of the cells for rabbits. The behavior

of Borna virus in cell cultures is typical of a slow virus infection, and thus, the Borna infection of tissue cultures offers itself as a new model for the study of slow viruses.

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