Preliminary Studies on the Biology of Borna Disease Virus

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(Accepted 29 July 1989)

SUMMARY

Borna disease virus (BDV) is an unclassified agent that causes neurological disease in a wide range of animal species and possibly in humans. The infectious nature of BDV has been long established but, despite extensive progress on the pathogenesis of the infection, the aetiological agent is still uncharacterized. Recent studies have shown that BDV replicates productively in cultures of foetal rabbit glial cells (FRG) which produce a virus-specific protein that is easily detected immunocytochemically. This provides a marker for BDV infectivity. This cell culture system was used to investigate the replication cycle of BDV. The agent required at least 1 h to bind to and penetrate the cells and the antigen was detected 24 h later. Cycloheximide and actinomycin D inhibited production of the antigen in inoculated cells, indicating that both protein synthesis and a DNA-dependent function were required for the production of viral antigen. Cocultivation of BDV-infected FRG cells with Vero cells resulted in a persistent productive infection in the latter. Use of these cells showed that the infectious agent matured exclusively in the cytoplasm and within the plasma membrane of the cell. Antigen-laden nuclei did not have infectivity. These studies showed that BDV has the physical and replicative properties typical of conventional viruses but its mechanism of replication and site of morphogenesis may be unique.
these antigen-positive cells can be selected so that 100% of the cells show infection. This mechanism has been used to derive persistently infected cell lines such as BDV-infected MDCK cells described by Herzog & Rott (1980).

Despite its unique biology, BDV is uncharacterized structurally. It has never been visualized by electron microscopy (Anzil & Blinzinger, 1972) and its site of morphogenesis and its mechanism of replication are not yet understood. Moreover, it is not clear whether the BDV antigen, identified previously as a 38K non-glycosylated protein (Haas et al., 1986), is a structural component of the agent. In this report, using Vero cells persistently infected with BDV, we show that the agent is located almost exclusively within the plasma membrane of the infected cell, but only negligible infectivity was present in the antigen-laden nuclei. Foetal rabbit glial (FRG) cultures inoculated with lysates of infected Vero cells developed BDV antigens 24 h after inoculation. At least 60 min was required to accomplish entry of the agent into the cell, after which both DNA-dependent functions and new protein synthesis were essential for synthesis of the antigen.

Vero cells (ATCC) were propagated in minimal essential medium and 10% foetal bovine serum (FBS) and, while still at a subconfluent stage, inoculated with 1 × 10⁶ TCD₅₀ of BDV. The inoculum was an infectious brain homogenate derived from rats inoculated with a Giessen strain of BDV, kindly provided by Professor R. Rott, Institute for Virology, Giessen, F.R.G. The inoculum was maintained in the culture for 1 week, after which the cells were subcultured and examined for infection. BDV antigen was not identified in any of the cells. A suspension of 1 × 10⁵ Vero cells was then added to a culture of infected FRG cells and separated by gentle trypsinization 1 week later. Examination of these Vero cells showed that approximately 20% had now acquired BDV antigens. This ratio was maintained through several subcultivations. Cells from this mixed culture were then cloned in a 96-well plate and six clones expanded blindly into new stocks. Three of these new stocks had no antigen and three had BDV antigens in 100% of the cells. Although the sample size was small, the all-or-none segregation between infected and non-infected cells suggested that this cell-associated agent does not spread only from cell to cell, like other known cell-associated viruses, but that the agent spreads from parent to progeny.

The experiment suggested also that the cocultivation of the Vero cells with the infected glial cells resulted in infection by fusion, overcoming an apparent lack of receptors on Vero plasma membranes.

Using lysates of infected Vero cells, we next determined sensitivities of the agent to physicochemical denaturing procedures to obtain preliminary information on essential structural components of the particles. Sonicated lysates of 1 × 10⁶ infected Vero cells had an infectivity titre of approximately 1 × 10⁶ TCD₅₀ in FRG cultures. Treatment of this material with 250 μg trypsin for 15 min at 37 °C, u.v. light (22 J/m²/s) for 30 min or 0.5% NP40 for 60 min all resulted in loss of infectivity to < 1 × 10³ TCD₅₀ per ml (> 99.9% loss of infectivity). This suggested that the agent has essential proteins, nucleic acid and lipid. This sensitivity of BDV to lipids solvents had been observed more than 70 years ago (Nicolau & Galloway, 1928); we confirmed this. We next sought to determine whether infectious particles were present on the external portion of the plasma membrane of the infected Vero cells. Treatment of a suspension of infected, intact Vero cells with 250 μg/ml trypsin for 15 min at 37 °C followed sequentially by quenching of the enzyme with serum, sonication of the preparation and assay in FRG cultures, showed that there was no reduction in infectivity of the lysate compared to the titre in a lysate of untreated infected cells; identical titres of infectivity of 1 × 10⁶ TCD₅₀ were obtained. These data suggested that only negligible infectivity was present on the external portion of the plasma membrane. Further, intact plasma membranes of the infected cells protected the agent in the cell against proteolysis.

In order to investigate further the intracellular location of the agent, we subjected suspensions of persistently infected Vero cells to three cycles of freezing and thawing, followed by Dounce homogenization and sedimentation of nuclei by centrifugation at 1000 g for 10 min. The supernatant fluid containing the enucleated cells and cell fragments was sonicated and assayed for infectivity. Fig. 1 shows that all of the nuclei had BDV antigen but sonicated lysates of these structures had < 10³ TCD₅₀ per ml. Nearly all of the infectivity (1 × 10⁶ TCD₅₀) was located in
the enucleated cell fraction (Fig. 2). This experiment suggested that maturation of the BDV particle occurs mainly in the cytoplasm. These data also support previous findings that the pathognomonic BDV-induced nuclear inclusion bodies recognized in histological sections and by immunocytochemical staining procedures do not represent aggregated virus particles (Joest & Degen, 1909; Anzil & Blinzinger, 1972; Blinzinger & Anzil, 1973). Whether the antigen is a precursor structural protein or a non-structural protein of the agent or even an aberrant virus-specified host protein is not known.

Assuming that the synthesis of the antigen is an integral part of the virus life cycle, we next performed experiments to determine the kinetics of entry of the agent into glial cells and some of the biochemical events essential for production of the antigen. Using trypsin protection
experiments, we asked whether there was a delay in entry of the agent into the cell where it becomes refractory to the effect of trypsin. Replicates of FRG cultures were inoculated with infectious cell lysates and cultures were treated with 250 μg/ml trypsin for 15 min starting immediately after inoculation and at 15 min intervals thereafter. In this experiment FRG cultures treated with trypsin between 0 and 60 min after inoculation did not develop antigen. This experiment showed that the agent required 1 h to bind and penetrate cells and that this process was complete by 4 h. These findings established that the agent does have a time requirement for binding and penetration into the cell, a property shared by all viruses. We next asked whether new protein synthesis and DNA-dependent cell functions were essential for synthesis of the antigen. We chose subtoxic doses of cycloheximide (100 μg/ml) and actinomycin D (6 μg/ml) and treated replicates of inoculated FRG cultures for 4 h periods spanning the 24 h period required for appearance of the antigen. This experiment showed that no BDV antigen was produced in those cultures treated with cycloheximide during the 4 to 8, 8 to 12 and 12 to 16 h time intervals. Treatment from 0 to 4, 16 to 20 and 20 to 24 h did not prevent appearance of the antigen. Cultures treated with actinomycin D between 8 and 12 h did not develop BDV antigen at 24 h but treatment at other 4 h intervals did not prevent appearance of the antigen. Thus, there was a requirement for synthesis of new proteins before and after the requirement for DNA function, suggesting a possible requirement for two different proteins. However, since so little is known about the agent, it is difficult to interpret the precise meaning of these data. Nevertheless, at this stage it is apparent that DNA-dependent functions and new protein synthesis are required in the cell for the production of BDV antigen.

In summary, the preliminary experiments described in this report show that BDV has many properties in common with conventional viruses. These include the physical components of the agent, a suggestion for the requirement of cellular receptors and dependence on the biosynthetic machinery of the cell for replication. The strict cytoplasmic site of assembly of the infectious
particle with minimal budding from plasma membranes, the lack of infectivity in antigen-laden nuclei and the suggestion that the agent spreads only from parent to progeny cell are properties that have been observed individually among other viruses. However, their combined expression in the life cycle of BDV makes this a unique agent. These properties probably contribute to the remarkable neurotropic nature of the virus and its propensity for spread from neuron to neuron via interconnecting cytoplasmic (dendritic) processes.

We thank Carol Thacker for assistance in preparation of the manuscript. These studies were supported by grants NS21916, NS07000 and NS23100 from the National Institutes of Health.

REFERENCES


(Received 1 February 1989)