Borna Disease Virus Replicates in Astrocytes, Schwann Cells and Ependymal Cells in Persistently Infected Rats: Location of Viral Genomic and Messenger RNAs by In Situ Hybridization

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Abstract. Borna disease (BD) is an immune-mediated neurological disease caused by infection of the nervous system with a negative strand RNA virus, Borna disease virus (BDV). The host range for BDV is broad and extends from birds to primates. A BDV-like agent may cause disease in humans. Until recently, BDV-infected neural cells could only be identified immunocytochemically using serum from BDV-infected animals. The advent of BDV cDNA clones allowed definition of the relationship between viral nucleic acids and viral proteins in vivo. In situ hybridization with strand-specific RNA probes from a BDV cDNA clone, pAP4, identified BDV genomic RNA and BDV mRNAs in neurons, astrocytes, Schwann cells and ependymal cells in an anatomic distribution consistent with that of BDV proteins. Genomic RNA was contained primarily within the nucleus, whereas mRNAs were found in both the nuclear and cytoplasmic compartments. Viral RNAs were demonstrated in neurons expressing BDV proteins and in glial cells by combined techniques of immunocytochemistry and in situ hybridization.

Key Words: Astrocytes; Borna disease virus; Ependymal cells; In situ hybridization; Schwann cells; Viral persistence; Virus.

INTRODUCTION

Borna disease (BD) is an immune-mediated neurobehavioral disease caused by infection of the nervous system with a negative strand RNA virus, Borna disease virus (BDV) (1–3). Although BD was originally described in horses, the experimental host range for BD is broad and extends from birds to primates, and possibly to humans (4–6). Antibodies that recognize BDV proteins have been reported in patients with neuropsychiatric illness and acquired immune deficiency syndrome (AIDS) encephalopathy (5, 6).

Rats infected with BDV exhibit a characteristic biphasic neurologic disease. The first signs of Borna disease are hyperactivity, ataxia and aggression (acute phase). Histopathological examination of these animals reveals an intense, mononuclear inflammation of the central (CNS) and peripheral nervous system (PNS) and the presence of BDV proteins in limbic system neurons. As the disease progresses, the rats become listless and may become blind or obese (chronic phase). There is neuronal loss in the brain resulting in ex vacuo hydrocephalus, presumably due to immune-mediated neuronolysis (1).

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205
The amount of infectious virus in brain is comparable in rats during the acute and chronic phases of disease in spite of the neuronal loss (1). Persistent virus replication in non-neuronal cells is one possible explanation for this observation. Support for this hypothesis came from studies demonstrating BDV proteins in astrocytes and Schwann cells in chronically-infected rats (7). However, the relationship of these proteins to the infectious agent was not clear because viral nucleic acids had not been isolated. It was possible, for example, that these proteins were encoded by the host and that their expression was induced by infection with the virus. In addition, one could not exclude the possibility that the proteins were produced by infected neurons and taken up by astrocytes or Schwann cells following neuronolysis.

We recently cloned cDNAs for BDV which allowed us to specifically detect either the 8.5 kb viral genome or its complementary 2.1 kb and 0.8 kb mRNA transcripts (2). RNA probes transcribed from one of these clones, pAF4, were used to determine whether the anatomic distribution of BDV nucleic acids was correlated with that of BDV proteins. In addition, by using immunocytochemical and in situ hybridization techniques in concert on individual tissue sections, we examined the association between BDV nucleic acids and BDV proteins at the level of single cells. In situ hybridization was done with strand-specific RNA probes to study the status of viral replication in the chronic phase of BD. Subcellular sites of viral replication were also discriminated using probes complementary to either the viral genome or viral mRNA.

MATERIALS AND METHODS

Virus

The virus stocks consisted of a 20% w/v BDV-infected rat brain homogenate prepared as described (1). The virus stock titer was $1 \times 10^8$ TCID$_{50}$/ml as determined by the infectivity of serial dilutions of virus for Fetal Rabbit Glia (FRG). Infection of FRG was determined by indirect immunofluorescence assay using serum from rabbits infected with BDV (1).

BDV Infection of Rats

Seven week old male and one day old neonatal Lewis rats (Charles River Laboratories, Wilmington, MA) were used. Seven week old rats were either inoculated subcutaneously in the footpad (FP) or intracranially (IC) with $5 \times 10^4$ TCID$_{50}$ BDV. Three uninfected rats served as controls. Two to three rats in the FP or IC group were deeply anesthetized and killed by exsanguination at each timepoint. The rats were then perfused with 4% paraformaldehyde and the PNS and CNS dissected. The tissues were post-fixed in 4% paraformaldehyde overnight and embedded in paraffin blocks. Four to six micrometer (μm) tissue sections were placed on chrom alum coated slides. After deparaffinization with xylene and alcohols, the sections were processed as below.

Five newborn (NB) rats were inoculated IC with $2.5 \times 10^4$ TCID$_{50}$ BDV at birth. At seven weeks of age the sciatic nerve was sectioned as described (1). Two to four weeks after surgery, the rats were killed, perfused and dissected as above.

Avidin Biotin Immunocytochemistry (ABC)

The sections were hydrated in phosphate buffered saline (PBS, pH 7.4) and incubated in 2% normal goat serum in PBS. The primary antibodies consisted of either rabbit anti-S100 (Signet Laboratories, Cambridge, MA) or serum from BDV-infected rabbits. After incubation with primary antibodies the slides were washed with PBS and incubated with biotinylated goat anti-rabbit Ig (Vector Laboratories, Burlingame, CA). The sections were rinsed and endogenous peroxidase was quenched with methanol-hydrogen peroxide. After rinsing, the avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) was

applied. The slides were again rinsed in PBS and allowed to react with a diaminobenzidine-
hydrogen peroxide mixture. The sections were counterstained in Gill's hematoxylin, dehy-
drated and coverslipped. The slides were examined with light microscopy for a brown pre-
cipitate indicative of antigen expression. Uninfected tissues and normal rabbit serum were
used in control slides.

In Situ Hybridization with pAF4

The BDV cDNA clone, pAF4, was derived from BDV-infected rat brain (2, 3). RNA probes
labelled with ³²P-CTP, pAF4-SP6 and pAF4-T7, were synthesized as described (2). Previous
studies demonstrated that pAF4-SP6 hybridized to the 8.5 kb BDV genomic RNA whereas
pAF4-T7 hybridized to two smaller polyadenylated BDV mRNA transcripts with mw 2.1 kb
and 0.8 kb (2, 3).

In double-labeling experiments, tissue sections were stained using the ABC technique as
above with the modification that antibody reagents were treated with 0.01% DEPC. Duplicate
sections were hybridized with either pAF4-SP6 or pAF4-T7 as described (2). Slides were
counterstained with Gill's hematoxylin.

RESULTS

The rats inoculated in the footpad (FP) were killed on days 14, 21, 42, 49, 56 and
70. Borna disease developed in the FP rats at approximately day 50. BDV nucleic
acids were detected in neurons in the spinal cord and dorsal root ganglia by day 42,
prior to the onset of clinical disease (data not shown). No ependymal cells showed
BDV nucleic acids at this timepoint. By day 49, BDV nucleic acids and proteins
were detected in neurons in frontal cortex, brainstem, spinal cord and dorsal root
ganglia and in ependymal cells. By day 56, BDV nucleic acids were also evident in
neurons in the hippocampus, cortex and cerebellum (Figs. 1–3).

The rats inoculated intracranially (IC) with virus developed acute BD (ataxia,
agression, and hyperactivity) beginning day 17. Tissues were examined on days 23,
37, 56, 70 and 120. Rats killed from day 56 forward showed signs of chronic BD
including listlessness and wasting. Neonatally infected rats (NB) showed no signs
of BD.

At all timepoints in FP, IC and NB rats, pAF4-SP6 and pAF4-T7 detected BDV
RNAs in the same anatomical and temporal distribution as antibodies detected BDV
proteins (neurons in hippocampus, cortex, brainstem, spinal cord, dorsal root ganglia,
cerebellum, hypothalamus and olfactory bulb (Figs. 1–3)). BDV probes did not
hybridize to matched tissue sections from uninfected controls (Fig. 1A, 1B inset, 1C).

In both IC and FP rats, the anatomic distribution of hybridization signal within
the CNS and PNS was similar with both BDV probes, pAF4-SP6 and pAF4-T7.
Concordant with earlier reports (2), in instances where serial sections were obtained
through individual neurons, cells hybridizing to one BDV probe invariably hybrid-
ized to the other BDV probe (data not shown).

The subcellular pattern of hybridization was different for the two BDV probes
(Figs. 1C, 2). Probe pAF4-T7 hybridization signal was distributed diffusely over the
cytoplasm of infected cells. In contrast, pAF4-SP6 probe hybridization signal was
focused over the nuclei of infected cells. This difference in hybridization pattern was
most apparent in large dorsal root ganglion cells. Probe pAF4-SP6, which is com-
plementary to the BDV genome, hybridized over the nucleus (Fig. 2A); pAF4-T7,
which is complementary to BDV mRNAs, hybridized in a diffuse pattern to the
nucleus and cytoplasm (Fig. 2B). In the spinal cord, pAF4-SP6 hybridized discretely
Fig. 1. Distribution of viral nucleic acids in BD rat brain and spinal cord. A, pAF4-SP6 hybridization to BDV-infected rat hippocampal neurons (large arrows) and cells surrounding parenchyma (small arrow), (day 56, IC inoculation). U. pAF4-SP6 hybridization to uninfected rat hippocampus. Probe does not hybridize to normal tissues. In situ hybridization, Gill’s hematoxylin. ×80. B. pAF4-SP6 hybridization to BDV-infected rat spinal cord (day 56, IC inoculation). Probe hybridizes to ependymal cells as well as to parenchymal cells. ×260. Inset: pAF4-SP6 does not hybridize to ependymal cells from normal rat. In situ hybridization, Gill’s hematoxylin. ×200. C. Hybridization of pAF4-SP6 and pAF4-T7 to Lewis rat brain. a. pAF4-T7 hybridization to a sagittal section from BDV-infected rat brain (day 56, FP inoculation). b. pAF4-SP6 hybridization to duplicate section of rat brain from (a). Probe hybridizes to olfactory, cortex, cerebellum, hippocampus and brainstem. c. pAF4-SP6 hybridization to normal Lewis rat brain. Probe does not hybridize to uninfected rat brain. In situ hybridization, Gill’s hematoxylin. ×5.

to cell nuclei (Fig. 2C), whereas pAF4-T7 hybridization signal was distributed over both nuclear and cytoplasmic grey matter (Fig. 2D).

To analyze the relationship between the presence of BDV nucleic acids and the expression of BDV proteins, individual sections were stained for proteins, then
Fig. 2. Subcellular localization of BDV genome and mRNAs. A. pAF4-SP6 hybridization to dorsal root ganglion from NB-infected rat. Probe hybridizes primarily to neuronal nuclei (arrowheads), indicating the nuclear location of genomic BDV RNA. *In situ* hybridization, Gill's hematoxylin. × 380. B. pAF4-T7 hybridization to dorsal root ganglion from NB-infected rat. Probe hybridizes to cell cytoplasm (small arrowheads) as well as to nuclei (large arrowheads), indicating the presence of viral mRNAs in both locations. *In situ* hybridization, Gill's hematoxylin. × 380. C. pAF4-SP6 hybridization to BDV-infected rat spinal cord (day 56, IC inoculation). Hybridization of probe to cell nuclei indicating cells that contain BDV genomic RNA. *In situ* hybridization, Gill's hematoxylin. × 35. D. pAF4-T7 hybridization to duplicate section from (D). Diffuse cytoplasmic signal between nuclear signals in the grey matter (outlined by arrowheads) indicating cells that contain BDV mRNAs. *In situ* hybridization, Gill's hematoxylin. × 35.

All cells expressing BDV proteins also hybridized to BDV probes (Fig. 3A, B).

In all rats with BD, both probes hybridized to cell nuclei in sections of optic nerve, an area devoid of neuron cell bodies (Fig. 4). To confirm that cells other than neurons, such as astrocytes and Schwann cells, contained BDV nucleic acids, sections of brain, sciatic nerve and distal stump of transected sciatic nerve were immunocytochemically stained for the glial antigen, S100, and then hybridized to either pAF4-SP6 or pAF4-T7. Both probes hybridized to S100-immunoreactive astrocytes (brain) and Schwann cells (distal stump of sciatic nerve) (Fig. 4 insets).

In rats with acute BD and in NB rats, the majority of cells showing BDV nucleic acids were neurons. By day 56, however, in rats with chronic BD, astrocytes containing BDV nucleic acids were found throughout the brain. In the sciatic nerve of
IC rats, the number of Schwann cells containing BDV nucleic acids gradually increased over time, coincident with lysis of dorsal root ganglion neurons and Wallerian degeneration. The sciatic nerve in NB animals remained intact and Schwann cells containing BDV nucleic acids were infrequent. When Wallerian degeneration was induced in NB rats by transection of the sciatic nerve, the majority of Schwann cells in the distal stump contained BDV nucleic acids and proteins.

DISCUSSION

Until recently, the only available markers for BDV infection at the cellular level were proteins presumed to be of BDV origin. The isolation of BDV cDNA clones made it possible to ask whether cells expressing BDV proteins contained BDV nucleic acids and, further, whether these nucleic acids represented the BDV genome or BDV.
mRNAs. In the studies reported here, the anatomic distribution of BDV nucleic acids in the nervous system after IC and FP inoculation of virus was consistent with that of BDV proteins. In addition, after FP inoculation, both BDV nucleic acids and proteins followed centripetal neuronal pathways from site of inoculation (foot) to dissemination in the brain. The appearance of BDV nucleic acids and BDV proteins in the hippocampus coincided with the onset of clinical disease. This concordance between BDV nucleic acids and BDV proteins has also been observed in the early stages of infection (Lipkin and Carbone, unpublished observations).

In situ hybridization studies revealed that ependymal cells contained BDV nucleic acids and prompted a confirmatory immunocytochemical examination of these cells for BDV proteins. The significance of BDV infection in ependymal cells is not clear. Following FP inoculation, BDV nucleic acids and proteins were seen in neurons before they were seen in ependymal cells. Although BDV has been isolated from cerebrospinal fluid (CSF) (8), one would expect early infection of ependymal cells if the CSF were a major route for dissemination of BDV infection. Rather, BDV appears to spread along neuronal pathways after FP inoculation (1).

Both viral transcripts and proteins were detected in astrocytes and Schwann cells in rats with chronic BD. Thus, BDV protein expression in astrocytes and Schwann cells indicated infection with virus rather than uptake of viral proteins from lysed, infected neurons. Infected glial cells were infrequent in the absence of inflammation and necrosis (e.g., early BD, NB rats). These findings suggest that astrocytes and Schwann cells may become secondarily infected after BDV is released from degenerating neurons. Earlier studies indicated that infectious virus can be recovered from

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Fig. 4. BDV nucleic acids in astrocytes and Schwann cells. Large arrows indicate pAF4-SP6 hybridization to cell nuclei in the optic nerve from a BDV-infected rat (day 23, IC inoculation). In situ hybridization, Gill's hematoxylin. ×420. Inset, top left. Small arrows indicate pAF4-SP6 hybridization to astrocytes in the brain from BDV-infected rat (day 56, IC inoculation). Immunocytochemical stains demonstrate that these cells also contain S100 proteins, a marker for astrocytes. Note, four cells in a row at top which express S100 but do not hybridize to BDV probes (uninfected astrocytes). Arrowhead indicates hematoxylin-stained nucleus from a cell which contains neither S100 proteins nor BDV RNA (uninfected cell, not an astrocyte). In situ hybridization, ABC using rabbit anti-BDV, and Gill's hematoxylin. ×575. Inset, bottom right. Small arrow indicates pAF4-SP6 hybridization to Schwann cell in distal stump of sciatic nerve from BDV-infected NB rat. This cell also expresses S100 proteins. Arrowhead indicates hematoxylin-stained nucleus from a cell which contains neither S100 proteins nor BDV RNA (uninfected cell, not a Schwann cell). In situ hybridization, ABC using rabbit anti-BDV, and Gill's hematoxylin. ×575.

Chronically infected rats (9). Our results suggest that astrocytes and Schwann cells may be major sites for viral replication in chronic BD.

It was not possible to assess the relative abundance of individual BDV RNAs over the course of infection using the technique of in situ hybridization. However, Northern hybridization experiments using RNA extracted from brains of NB, acutely infected and chronically infected rats have shown no difference in the relative abundance of the 8.5 kb, 2.1 kb and 0.8 kb BDV RNA (Lipkin, unpublished observations).

Cell fractionation experiments have shown that the 8.5 kb genomic RNA is nucleus-associated (3), whereas the 2.1 kb and 0.8 kb mRNAs are primarily cytoplasmic. In situ hybridization studies presented here supported those findings. Probe pAF4-SP6, which is complementary to the 8.5 kb genomic RNA, hybridized primarily to the cell nucleus. In contrast, probe pAF4-T7, which is complementary to the 2.1 kb and 0.8 kb mRNAs, hybridized diffusely to the cytoplasm as well as to the nucleus.

The presence of BDV mRNAs in both nucleus and cytoplasm was consistent with transcription from genomic RNA in the nucleus and transport to the cytoplasm for
translation. Previous studies identified little infectious virus in the nucleus (9). These data suggest that the genomic nuclear RNA detected by in situ hybridization may not be associated with the full complement of components required for infectivity.

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