Detection of Rabies Virus Genomic RNA and mRNA in Mouse and Human Brains by Using In Situ Hybridization

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Rabies virus RNA was detected in mouse and human brains by in situ hybridization. ³H-labeled single-stranded RNA probes were prepared which were specific for genomic RNA and mRNAs coding for the five rabies virus proteins (N, NS, M, G, and L). Paraffin-embedded brain tissues from human cases of rabies and mice experimentally infected with the challenge virus standard (CVS)-11 strain of rabies virus and street rabies virus were examined. In CVS-infected mice, genomic RNA had a multifocal distribution in the perikarya of infected neurons, perhaps reflecting concentration of genomic RNA in viral factories. The mRNAs were more abundant than genomic RNAs in CVS- and street virus-infected mouse brains and had a diffuse distribution in the perikarya. Similar amounts of signal were present in infected neurons for mRNAs coding for different rabies virus proteins. In brain tissues from human cases of rabies, genomic RNA was much more abundant than the mRNAs in infected neurons. This finding suggests either a relative block at the level of transcription or greater loss of mRNAs than of genomic RNA during the agonal period, postmortem interval, or prior to penetration of fixative during immersion fixation.

Rabies virus is highly neurotropic in humans and animals and causes an acute infection of the central nervous system (CNS). The detection of rabies virus antigen in CNS tissues, performed with either the fluorescent-antibody technique (11) or immunohistochemical methods (5, 6, 10), is very useful as a confirmation of rabies. The detection of mRNA that encodes rabies virus nucleocapsid protein in the CNS of experimentally infected mice by in situ hybridization was recently reported (10).

In this study, brain tissues from two fatal human cases of rabies and mice experimentally infected with either the challenge virus standard (CVS)-11 strain of fixed rabies virus or a fox salivary gland isolate of street rabies virus were examined for the presence of rabies virus genomic RNA and mRNAs coding for each of the five rabies virus proteins (N, NS, M, G, and L) by in situ hybridization. A difference in the subcellular distribution of genomic RNA and mRNA was found, and there was a major difference in the relative quantities of genomic RNA and mRNAs in mouse and human brains.

MATERIALS AND METHODS

Viruses. The CVS-11 strain of fixed rabies virus (Wistar Institute, Philadelphia, Pa.) was used. Stock CVS was grown in BHK-21 cells to a titer of 4.2×10^7 PFU/ml.

A salivary gland homogenate (10% suspension) of an Ontario fox isolate of street rabies virus was obtained from K. M. Charlton (Animal Diseases Research Institute, Nepean, Ontario). The titer was $10^{5.0}$ mouse intracerebral 50% lethal doses (LD₅₀) per 0.03 ml, calculated by the method of Reed and Muench (15).

Animals and inoculations. Six-week-old female ICR mice (Charles River Canada, Inc., St-Constant, Quebec) were used. Mice were inoculated either intracerebrally with 9.3×10^5 PFU of CVS (6.5×10^4 intracerebral LD₅₀) in 0.03 ml of phosphate-buffered saline with 2% fetal bovine serum or in

the left hind limb footpad with 0.03 ml of the 10% suspension of street rabies virus. Control mice were inoculated with 0.03 ml of phosphate-buffered saline with 2% fetal bovine serum by the same routes.

Preparation of tissue sections. Mice were killed 3 to 6 days after intracerebral inoculation with CVS or 5 to 13 days after footpad inoculation with street virus. Mice were anesthetized with methoxyflurane and perfused with buffered 4% paraformaldehyde. Brains were removed, immersion-fixed in the same fixative for 18 h at 4°C, dehydrated, and embedded in paraffin. Coronal sections of brain and transverse sections of brain stem 6 µm thick were cut at multiple levels on a microtome.

Human brain tissues. Formalin-fixed paraffin-embedded blocks of brain tissues from two human cases of rabies (one classical and one paralytic [18] rabies) that occurred in 1983 and 1984 were obtained from S. Roy (All-India Institute of Medical Sciences, New Delhi, India). In both cases the infections were transmitted by dog bites. The brains were fixed in 10% buffered Formalin for 10 to 14 days before the tissues were processed and embedded in paraffin for histopathologic examination. Brain tissue blocks from a patient who died without any neurologic or neuropathologic disease were also examined as a control.

Immunoperoxidase staining. Tissue sections were stained for rabies virus antigen by the avidin-biotin-peroxidase method as previously described by Jackson et al. (10) with minor modifications. Deparaffinized slides were successively reacted with 0.001% pepsin (Boehringer, Mannheim, Germany) in 0.01 N HCl at 37°C for 30 min, with 5% normal goat serum, with rabbit anti-rabies virus serum diluted 1:2,000 (obtained from K. M. Charlton, Animal Diseases Research Institute, Nepean, Ontario), with biotinylated goat anti-rabbit immunoglobulin G diluted 1:200 (Vector Laboratories, Burlingame, Calif.), with 1% hydrogen peroxide in methanol, with Elite avidin-biotinylated horseradish peroxidase complex (Vector Laboratories), with 3,3'-diaminobenzidine tetrachloride (Polysciences, Inc., Warrington, Pa.) with 0.01% hydrogen peroxide, and finally with 0.5% cupric

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sulfate in 0.15 M sodium chloride, and the slides were counterstained with hematoxylin. Tissues from uninfected mice were used as a control. Normal rabbit serum diluted 1:2,000 was used as a primary antibody for tissues from infected mice as another control.

Preparation of RNA probes. ^3H -labeled RNA probes were used for localization of rabies virus RNA in tissues. cDNA clones containing the coding sequences for the five rabies virus proteins were used to prepare radiolabeled RNA probes. Fragments were excised from cDNA clones and subcloned into dual-promoter-containing Gemini vectors (Promega, Madison, Wis.). Radiolabeled probes were synthesized in the presence of [5,6- ^3H]UTP (ICN Radiochemicals, Irvine, Calif.) by using either SP6 or T7 RNA polymerase (Promega). Depending on the orientation of the inserted cDNA sequence, one polymerase was used to prepare probes specific for positive-sense mRNA (and positive-strand replicative intermediate), and the other polymerase was used to prepare probes specific for negative-sense genomic RNA. The cDNA for the nucleocapsid protein (N) subcloned into the pGEM-2 vector has been described (10). A 0.99-kb fragment of the sequence coding for the phosphoprotein (NS or M1) of the ERA (Evelyn-Rokinicki-Abelseth) strain (12) was excised with *Pst*I and *Eco*RI and subcloned into the pGEM-2 vector. A 0.65-kb fragment of the sequence coding for the matrix protein (M or M2) of the ERA strain (14) was excised with *Bst*XI and *Xba*I and subcloned into the pGEM-7 vector. A 1.5-kb fragment of the sequence coding for the glycoprotein (G) of the ERA strain (obtained from Connaught Research Institute, Willowdale, Ontario) (13) was excised with *Eco*RI and *Bam*HI and subcloned into the pGEM-2 vector. A 2.0-kb fragment of the sequence coding for the polymerase or large transcriptase molecule (L) of the PV (Pasteur virus) strain from pRb42 (positions 7500 to 9496) (obtained from Noel Tordo, Pasteur Institute, Paris, France) (17) was excised with *Pst*I and *Eco*RV and subcloned into the pGEM-5 vector. The ^3H -labeled RNA probes were reduced in size by alkaline hydrolysis (3). The probes had specific activities of 3.1×10^7 to 5.3×10^7 dpm/ μg . An irrelevant control template (Riboprobe Gemini positive control template; Promega) was used to prepare ^3H -labeled RNA transcripts as a control for the specificity of the hybridization.

In situ hybridization. In situ hybridization was performed as previously described by Jackson et al. (10) with minor modifications. Deparaffinized slides were treated by sequential immersion in 0.2 N HCl for 20 min, in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.4) for 30 min, in 10 μg (mouse brain) or 25 μg (human brain) of proteinase K per ml of 10 mM Tris-HCl–2 mM CaCl_2 (pH 7.4) at 37°C for 15 min, and finally in 0.25% (vol/vol) acetic anhydride in 0.1 M triethanolamine-HCl buffer (pH 8.0) for 10 min, and then were rehydrated in graded alcohols and air dried.

The hybridization mixture contained 0.2 μg of ^3H -labeled RNA transcripts per ml, 50 mM dithiothreitol, 0.3 M NaCl, 50% (vol/vol) deionized formamide, 10% (wt/vol) dextran sulfate, 0.2 mg of sheared salmon sperm DNA per ml, 0.125 mg of tRNA per ml, 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinylpyrrolidone, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.1% Triton X-100. The mixture was applied to the tissue sections on slides for a 4-h incubation at 45°C. After hybridization, the slides were washed three times in $4\times$ SSC at room temperature for 5 min, once in $2\times$ SSC at 4°C for 5 min, once in 50% formamide–0.3 M NaCl–20 mM Tris-HCl (pH 8.0)–2 mM EDTA at 55°C for 15 min, once in

$2\times$ SSC at room temperature for 30 min, and once in $0.1\times$ SSC at 55°C for 15 min, dehydrated in graded alcohols (each containing 0.3 M ammonium acetate), and air dried.

The slides were dipped in NTB2 nuclear track emulsion (Eastman Kodak Company, Rochester, N.Y.) diluted 1:1 with 0.6 M ammonium acetate, exposed for 6 to 7 days at 4°C, developed with D19 developer (Eastman Kodak Company) for 5 min, fixed with 30% sodium thiosulfate for 5 min, and counterstained with hematoxylin. Controls included tissue sections pretreated with RNase A (Boehringer), in situ hybridization on uninfected tissues with the rabies virus RNA probes, and hybridization of rabies virus-infected tissues with the RNA probe prepared from the Riboprobe Gemini control template.

RESULTS

CVS-infected mouse brain. All probes gave strong signals with low backgrounds in sections of mouse brain infected with CVS. No definite signal was found in the controls (Fig. 1 to 3). Target rabies virus RNA was only detected by in situ hybridization in cells with the morphological appearance of neurons. Grains were present in perikarya and in dendritic processes of infected neurons. The distribution of rabies virus RNA detected by in situ hybridization was similar to the distribution of viral antigen demonstrated with immunoperoxidase staining, although the amount of signal was generally lower with in situ hybridization. In particular, antigen was demonstrated with greater sensitivity than viral RNA by in situ hybridization in dendritic processes (Fig. 2 and 3). More signal was detected with the mRNA probes than with the genomic RNA probes. Although there were differences in the sizes of the cDNA templates (0.65 to 2.0 kb) used for synthesizing the probes and in the specific activities of the probes, large differences in the amounts of signal in infected neurons were not noted with probes for individual mRNAs coding for the five rabies virus proteins (Fig. 2 and 3). However, the relative amounts of signal were not assessed quantitatively. The genomic RNA probe that contained the *G* gene sequence was as good as or better than the genomic RNA probe that contained the *N* gene sequence.

A fundamental difference was identified in the subcellular distribution of genomic RNA and the mRNAs in infected neurons of the mouse brains (Fig. 3). All of the mRNAs had a diffuse distribution of grains in the perikarya and proximal dendrites. In contrast, genomic RNA had a multifocal distribution in the perikarya and dendrites of many of the infected neurons. In areas where there were many infected cells, there were often many grains in the neuropil, suggesting the presence of significant amounts of rabies virus RNA in the processes of infected neurons.

Street rabies virus-infected mouse brain. Much less rabies virus RNA and antigen were detected in street virus-infected mouse brains than in the CVS-infected mouse brains. In particular, there were relatively few infected neurons. The infection was most prominent in the brain stem. As in CVS-infected mice, signals for the mRNAs were much greater than for genomic RNA (Fig. 4). Signals for genomic RNA were relatively weak for street virus in comparison with CVS. There was a fairly diffuse distribution of signal for genomic RNA in the neurons of street virus-infected brains, which contrasted with the multifocal distribution of signal in CVS-infected mice. Signals for mRNAs were also less than in CVS-infected mice. The relative amounts of genomic

FIG. 1. In situ hybridization (dark-field optics) and immunoperoxidase staining (antigen) of the cortex of a mouse 4 days after intracerebral inoculation with CVS virus. Probes were used for nucleocapsid protein (*N*) and glycoprotein (*G*) gene genomic RNA and mRNA, and a probe was derived from a control template (control). There was widespread rabies virus antigen in the cortex (antigen). Signals were similar for the *N* and *G* genes and greater for mRNA than for genomic RNA, indicating a high level of transcription in the infection. Antigen, Immunoperoxidase-hematoxylin. Magnification, $\times 55$.

RNA and mRNAs were similar in both street virus-infected fox and skunk brains and in mouse brains (data not shown).

Rabies virus-infected human brain. Rabies virus RNA was detected in the brain tissues of both human cases of rabies examined. Backgrounds were low, and no definite signal was found in the normal human brain sections. Histopathologic examination did not reveal changes suggesting significant postmortem autolysis. Rabies virus antigen was abundant in the brains of the human rabies cases. As in the mouse brains, antigen was more abundant than the in situ hybridization signals. Contrary to what was found in rabies virus-infected mouse brains, in situ hybridization signals in human brains were much stronger with probes for genomic RNA than with those for the mRNAs coding for the rabies virus proteins (Fig. 5). A multifocal distribution of signal was also observed

in perikarya of the human brains with the genomic RNA probes. The signals for the mRNAs were much weaker than for genomic RNA, and the distribution of mRNA signal was more focal in the perikarya. The findings were similar in human cases of rabies acquired in Thailand and the Dominican Republic and in these cases from India, although the in situ hybridization signals were less intense (data not shown).

DISCUSSION

In situ hybridization with single-stranded RNA probes allows the specific detection of rabies virus genomic RNA (negative strand) and the monocistronic mRNAs (positive strand) coding for the five rabies virus proteins in infected tissues. The detection of *N* mRNA in the central nervous

FIG. 2. In situ hybridization (dark-field optics) and immunoperoxidase staining (antigen) of cerebellar Purkinje cells of a mouse 4 days after intracerebral inoculation with CVS virus. Antigen and in situ hybridization signals were present in perikarya and dendritic processes in the molecular layer for genomic RNA and each of the mRNAs (*N*, *NS*, *M*, *G*, and *L*). There was less genomic RNA than mRNA, and signals were similar for each of the mRNAs. Antigen, Immunoperoxidase-hematoxylin. Magnification, $\times 130$.

FIG. 3. In situ hybridization (bright-field optics) and immunoperoxidase staining (antigen) of Purkinje cells of a mouse 4 days after intracerebral inoculation with CVS virus. Signals were strong with probes for genomic RNA, each of the mRNAs, and antigen. Antigen was demonstrated better than in situ hybridization signals in dendritic processes. Probes for genomic RNA showed a multifocal distribution of grains in the perikaryon and dendritic processes, and probes for the mRNAs showed a diffuse distribution. The amount of signal was greater for the mRNAs. Although signals could not be compared in the same Purkinje cells, many grains were found over the perikarya of Purkinje cells with probes for each of the mRNAs. Hematoxylin and immunoperoxidase-hematoxylin (antigen). Magnification, $\times 400$.

system of rabies virus-infected mice was reported previously (10). In this study, detection of genomic RNA and all five mRNAs individually was demonstrated by in situ hybridization with sequence-specific radiolabeled RNA probes in mice experimentally infected with fixed and street rabies virus and in human cases of rabies.

From the signal strengths, it is apparent that the mRNAs were more abundant than genomic RNA in mouse brains infected with CVS and street rabies viruses. A high level of viral transcription is attained in an active infection 3 to 4 days after intracerebral inoculation. The quantities of signal for the individual mRNAs were similar. Although the relative quantities of the mRNAs coding for the individual proteins in either cell culture or animals have not been reported previously for rabies virus, these findings are in contrast to the report that gene transcripts are produced in a

gradient of decreasing mRNA abundance that reflects the gene order (N, NS, M, G, and L) in infected cell cultures of the closely related vesicular stomatitis virus (19). This may be due to attenuation of transcription at or near the intergenic regions (9, 19). However, it has not been definitely established whether rabies virus mRNA synthesis is initiated only at the 3' end of the genomic RNA by the RNA polymerase to sequentially produce transcripts of the downstream genes, as described by Flamand and Delagneau (7). Alternatively, multiple initiations might occur at the different gene start sites, which has been proposed as a mechanism for vesicular stomatitis virus transcription (2). This study indicates that the relative abundance of gene transcripts may be different for rabies virus infection in animals. Quantitative data are needed about the relative amounts of the individual mRNAs in rabies virus infection in both cell culture and

FIG. 4. In situ hybridization (bright-field optics) and immunoperoxidase staining (antigen) of brain stem neurons of a mouse 7 days after hind limb footpad inoculation with street rabies virus. Antigen was abundant, and signals were less for genomic RNA than for N and G gene mRNAs. Hematoxylin and immunoperoxidase-hematoxylin (antigen). Magnification, $\times 400$.

FIG. 5. In situ hybridization (bright-field optics) and immunoperoxidase staining (antigen) of neurons from a human who died of rabies and a neuron from a human brain without rabies (control). There was abundant signal for genomic RNA in a multifocal distribution in the perikaryon. Occasional neurons had signals for mRNAs, and grains were present at a focal site in the perikaryon. Hematoxylin and immunoperoxidase-hematoxylin (antigen). Magnification: *N* and *G* genomic RNA, *N* mRNA, control, and antigen, $\times 400$; *NS*, *M*, *G*, and *L* mRNA, $\times 950$.

animals. Differences in the stability of individual mRNAs could compensate for differences in the amounts of mRNA synthesized.

There is a high level of viral replication in the brains of mice infected with CVS. The signals obtained with probes for mRNAs were distributed diffusely in the perikarya and proximal dendrites of infected neurons in CVS-infected mouse brains. In contrast, the signals obtained with probes for genomic RNA had a multifocal distribution in the perikarya and dendrites of infected neurons. This indicates that there are specific sites in the cytoplasm with relatively large amounts of genomic RNA, perhaps reflecting concentration of genomic RNA in viral factories.

The main difference in the brains of mice infected with CVS and street rabies virus was a reduction in the number of infected neurons and a reduction in the amount of signal for both genomic RNA and mRNAs in the street virus-infected mice. In street virus infection there was also a greater amount of signal for the mRNAs than for genomic RNA, which likely reflects efficient transcription. The lack of a multifocal distribution of genomic RNA in the perikarya in street virus infection may be due to the low intensity of signal rather than to a fundamental difference in local virus replication.

This is the first report describing the detection of rabies virus RNA in the brains from fatal cases of human rabies. Routinely prepared Formalin-fixed paraffin-embedded blocks were used for this study. Therefore, the in situ hybridization technique can be performed on routine autopsy specimens and may be done retrospectively.

A greater abundance of genomic RNA than the mRNAs was found in the human brains. This was an unexpected finding, since it contrasted with the observations for street virus-infected animal brains. There are at least three possible explanations for this observation. (i) A relative block might occur at the transcriptional level in street virus infection of humans but not in all mammalian species. (ii) Changes in levels of specific RNAs may have occurred in the human cases during the agonal state (8), although the effects of the agonal state would be very difficult to evaluate. (iii) Loss of RNA in tissues might have occurred, preferentially affecting mRNAs, in autolyzed infected neurons prior to fixation.

The duration of the postmortem period and the temperature of the body during this interval may influence the integrity or recovery of RNA. Even after removal of the brain and immersion in Formalin, there is a further delay while the fixative penetrates the relatively large human brain. Postmortem autolysis occurs during this period and could result in degradation of RNA by RNases or diffusion of RNA out of autolyzed infected cells prior to fixation. Degradation of RNA could occur in the absence of morphological changes observed by light microscopy. Degradation and diffusion may occur more efficiently for mRNA than genomic RNA because of its smaller size and reduced tendency for aggregation and because less protection is provided by bound protein in mRNA-protein complexes (16, 20). The close packing of genomic RNA by the nucleocapsid protein in the ribonucleoprotein complex protects the genomic RNA against RNase digestion (20, 21). Ermine et al. (4) performed Northern (RNA blot) analyses with cDNA

probes and found that significant degradation of rabies virus RNA extracted from infected mouse brains did not occur until at least 120 h postmortem. In a study with *in situ* hybridization, Arai et al. (1) found that signals for vasopressin mRNA were reduced in experimental rats subjected to a postmortem delay of only 24 h at room temperature. Hence, diffusion of RNA may be more important than degradation of RNA in explaining reduced *in situ* hybridization signals. Studies are now in progress examining the effects of postmortem autolysis on *in situ* hybridization signals for genomic RNA and mRNA in mice experimentally infected with rabies virus.

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