Journal of Medical Virology 65:614–618 (2001)

Quantitative Study of the Infection in Brain Neurons in Human Rabies

Alan C. Jackson,[1,2]* Hongtao Ye,[1] Cecilia Ridaura-Sanz,[3] and Eduardo Lopez-Corella[3]

[1]*Department of Medicine, Queen's University, Kingston, Ontario, Canada*
[2]*Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada*
[3]*Department of Pathology, Instituto Nacional de Pediatria, Mexico City, Mexico*

Rabies virus is a highly neuronotropic virus that causes encephalomyelitis. Rabies virus infection was studied in neurons in the brain of an 8-year-old girl that died of rabies in Mexico. The extent of the neuronal infection was evaluated quantitatively in neuronal cell types of the brain using histologic staining for Negri bodies and immunoperoxidase staining for rabies virus antigen in the same neurons. Quantitative image analysis was used to compare the amount of infection in five different neuronal cell types, which was expressed as a percentage of neuronal area. Purkinje cells and periaqueductal gray neurons showed the largest percentage area for both Negri bodies and signal for rabies virus antigen. In general, there was a good linear relationship between the area of Negri bodies and the area of signal for rabies virus antigen. Many neurons with rabies virus antigen did not have Negri bodies, however, and some neurons with large antigen signals, especially Purkinje cells and periaqueductal gray neurons, lacked Negri bodies. Formation of Negri bodies is likely influenced by factors that vary in different neuronal cell types. ***J. Med. Virol. 65:614–618, 2001.***

KEY WORDS: immunohistochemistry; Negri bodies; pathogenesis; rabies

INTRODUCTION

Rabies virus causes an acute encephalomyelitis in humans and animals that generally has a fatal outcome [Jackson, 1997]. Rabies is usually associated with widespread infection of central nervous system (CNS) neurons. In 1903 Adelchi Negri [1903] described cytoplasmic inclusions in neurons infected with rabies virus. Since that time there have been many unanswered questions about the nature of these inclusions, which are now called Negri bodies [Kristensson et al., 1996]. It has been reported that Negri bodies are demonstrated most readily in Ammon's horn of the hippocampus, pyramidal cells of the cerebral cortex, and Purkinje cells of the cerebellum, and to a much more limited extent in neurons of the thalamus, pons, medulla, spinal cord, and sensory ganglia [Tierkel and Atanasiu, 1996]. Dupont and Earle [1965] assessed 49 cases of human rabies and found Negri bodies in 70.9% of the cases. They were found in the cerebellum in 59.5%, in the hippocampus in 42.9%, in the medulla in 14.3%, in pontine nuclei in 11.9%, in the spinal cord in 9.5%, and in the cerebral cortex in 7.1%. Other studies in animals, however, have demonstrated Negri bodies more frequently in the hippocampus [Horgan and McKinnon, 1937; Tustin and Smit, 1962]. Similarly, diagnostic assessment of animal tissues for the presence of rabies virus antigen, usually undertaken with the fluorescent antibody test, normally includes sampling of brain tissues from Ammon's horn, the cerebellum, and medulla [Dean et al., 1996]. Although it is clear that the histologic demonstration of Negri bodies is less sensitive than antigen-detection methods in the assessment of rabies virus-infected tissues, quantitative methods have not yet been used to compare infection in the same neurons using different techniques. In this study the infection of brain neurons in a fatal case of human rabies from Mexico was evaluated in situ using histologic staining for Negri bodies and immunoperoxidase staining for rabies virus antigen. Staining for Negri bodies and rabies virus antigen was carried out sequentially on the same tissue sections in different brain regions. The amount of neuronal infection was evaluated and compared using these techniques with quantitative image analysis.

Grant sponsor: Queen's University Violet E. Powell Research Fund.

*Correspondence to: Dr. Alan C. Jackson, Kingston General Hospital, Connell 725, 76 Stuart Street, Kingston, Ontario, Canada K7L 2V7. E-mail: jacksona@post.queensu.ca

Accepted 6 October 2000

MATERIALS AND METHODS

Case Report

An 8-year-old girl was bitten by a puppy in rural Mexico on May 30, 1986. The animal died 7 days after the bite. Twenty-six days after the bite the girl developed fever, dysphagia, and thoracic and abdominal pain, and she was transferred to Instituto Nacional de Pediatria in Mexico City. She exhibited periods of hyperexcitability, agitation, visual hallucinations, hydrophobia, aerophobia, generalized hyperreflexia, and upgoing toes. Rabies was diagnosed and she died on July 1, 1986. There was a post-mortem interval of 4 hr before the autopsy.

Tissues

Tissue sections (6 μm) were cut on a microtome for examination of neurons in the third layer of the cerebral cortex, pyramidal neurons of the hippocampus (CA1 region), Purkinje cells of the cerebellum, periaqueductal gray neurons, and neurons in the trochlear nucleus. Formalin-fixed paraffin-embedded blocks from the brain of an individual who died of a non-neurologic cause in Mexico City were used as negative controls. Sections were stained with hematoxylin and eosin. After image analysis of the Negri bodies, the eosin stain was removed with 70% ethanol and the immunoperoxidase staining method (see below) was carried out on the same tissue sections for rabies virus antigen.

Immunoperoxidase Staining For Rabies Virus Antigen

Tissue sections were stained for rabies virus antigen by the avidin-biotin-peroxidase method as previously described by Jackson and Wunner [1991] with minor modifications. Deparaffinized slides were reacted successively with 5% normal rabbit serum in PBS, mouse monoclonal anti-rabies virus nucleocapsid protein IgG (5DF12) (obtained from Dr. Alexander I. Wandeler, Animal Diseases Research Institute, Nepean, ON, Canada) diluted 1:160 in 2% normal rabbit serum in PBS, biotinylated rabbit anti-mouse IgG diluted 1:100 (Zymed Laboratories, South San Francisco, CA), 1% hydrogen peroxide in methanol, Elite avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA), 3,3′-diaminobenzidine tetrachloride (Polysciences, Inc., Warrington, PA) with 0.01% hydrogen peroxide, 0.5% cupric sulfate in 0.15 M sodium chloride, and the slides were counterstained lightly with hematoxylin and examined using light microscopy.

Quantitative Image Analysis

A Sony 3CCD color video camera (model DXC-930) was attached to an Olympus BX60 microscope and Northern Exposure image analysis software release 2.9 (Empix Imaging, Mississauga, ON) was used for the computerized analyses. Tissues were analyzed under a 100 × oil immersion objective using bright-field optics. The operator (HY) visually identified positively labeled cells and captured the images digitally as bitmaps. Outlines of the cells, Negri bodies, and signals for rabies virus antigen were drawn manually. The areas of the cells in pixels were determined and the proportions of the areas occupied by Negri bodies and signals for rabies virus antigen were calculated.

RESULTS

Negri Bodies

Negri bodies were found in a subset of neurons in all five of the brain regions studied. There was a large variation in the quantity of Negri bodies in different neuronal cell types. For example, the area of Negri bodies in Purkinje cells ranged between 0–13.1% with a mean of 6.9±0.9 (SEM) of the neuronal area (Fig. 1A). Negri bodies occupied a mean of 6.1% of periaqueductal gray neurons and smaller areas in hippocampal neurons (CA1 region) (mean 4.6%), cortical neurons (mean 4.1%), and neurons in the trochlear nucleus (mean 1.1%) (Table I).

Immunostaining For Rabies Virus Antigen

Immunostaining for rabies virus antigen was found to be a much more sensitive technique for the detection of viral infection in neurons than the detection of Negri bodies, and rabies virus antigen was detected in many neurons that lacked Negri bodies. There was substantial variation in the amount of antigen detected in different neuronal cell types. For example, Purkinje cells showed signal over a wide range of 2.4–40.0% of the neuronal area (Fig. 1B). Antigen was most abundant in periaqueductal gray neurons and Purkinje cells and was less abundant in neurons in other brain regions (Table I), including the caudate nucleus and globus pallidus (data not shown).

Negri Bodies and Rabies Virus Antigen in the Same Neurons

Neurons containing Negri bodies demonstrated larger mean amounts of rabies virus antigen than neurons lacking Negri bodies (Table I). Linear regression analyses indicated that there was a good linear relationship between the area of Negri bodies and antigen signal in infected cortical ($R = 0.89$) and hippocampal ($R = 0.84$) neurons, including neurons without Negri bodies (Fig. 2A,B). In the other areas there was a linear relationship in neurons with Negri bodies: Purkinje cells ($R = 0.84$) (Fig. 3A), periaqueductal gray neurons ($R = 0.83$) (Fig. 3B), and trochlear nucleus neurons ($R = 0.72$). The goodness-of-fit became worse when neurons without Negri bodies from these regions were included in the analyses because of the presence of highly variable amounts of antigen signal (Fig. 3A,B). For example, among periaqueductal gray neurons and Purkinje cells there were neurons without Negri bodies with as high as 41.8% and 26.8% of the neuronal area containing signal for antigen, respectively. Linear regression analyses of the percentage

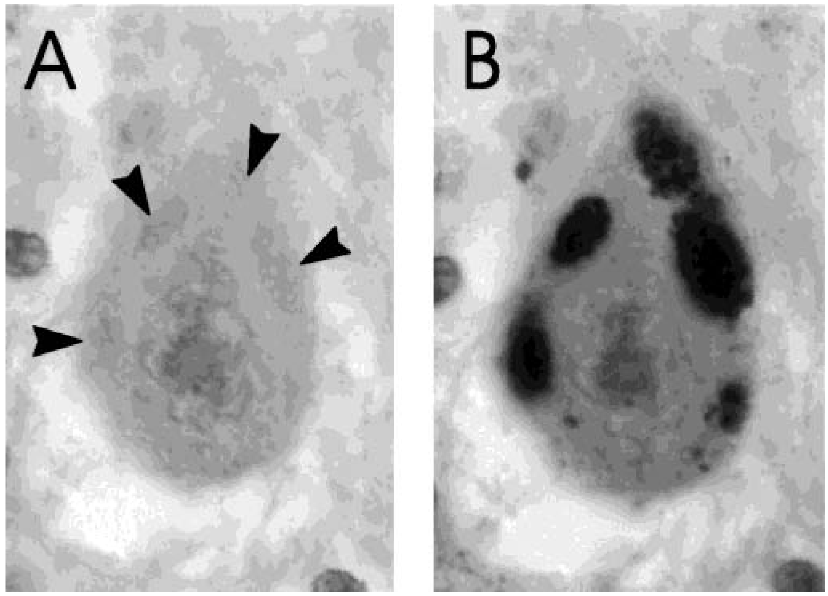

Fig. 1. Hematoxylin and eosin staining of the cerebellum (**A**) showing a Purkinje cell with four Negri bodies (arrowheads) in the cytoplasm, which occupy 11.5% of the cellular area. Immunoperoxidase staining of the same Purkinje cell (**B**) showing accumulations of rabies virus antigen in the cytoplasm, which occupy 25.4% of the cellular area (counterstained with hematoxylin). Magnification × 1,450.

area occupied by Negri bodies vs. percentage area occupied by signal for viral antigen also indicated substantial variability in the slopes of the lines of best fit (e.g., 1.55±0.24 for Purkinje cells, 1.97±0.15 for cortical neurons, and 2.23±0.35 for periaqueductal gray neurons).

DISCUSSION

Negri bodies are eosinophilic staining inclusions in the cytoplasm of rabies virus-infected neurons. Although Negri bodies often have a widespread distribution in neurons in human rabies, they are most commonly observed in large neurons in a number of specific brain regions [Perl and Good, 1991]. In this study Negri bodies were found to have the largest proportional size in Purkinje cells, where they occupied a mean of 6.9% of the two-dimensional area of the neurons in which they were observed. Negri bodies were also large in the periaqueductal gray matter, intermediate in size in pyramidal neurons of the hippocampus (CA1 region) and cortical neurons (third layer), and relatively small in the trochlear nucleus.

By undertaking sequential histologic and immunoperoxidase staining on the same tissue sections, it was demonstrated that all neurons containing Negri bodies also expressed detectable rabies virus antigen. Many infected neurons that contained rabies virus antigen, however, did not contain Negri bodies. In general, the proportional area of Negri bodies paralleled the amounts of viral antigen in the brain regions examined. Neurons without Negri bodies in some regions, however, showed large signals for antigen, and occupied a considerably higher percentage of the neuronal area than the mean for neurons containing Negri bodies.

Studies by Goldwasser and Kissling [1958] showed that Negri bodies contain rabies virus antigens in street virus-infected animal brains. Electron microscopic

TABLE I. Quantitative Assessment of Signals for Negri Bodies and Immunostaining for Rabies Virus Antigen in Infected Brain Neurons

Anatomical region	Number of neurons	Negri bodies (% signal±SEM)	Immunostaining (% signal±SEM)
Cerebral cortex (3rd layer neurons)	22	4.1±0.5	10.8±1.0
	26	None	1.6±0.4
Hippocampus (CAI region)	19	4.6±0.6	11.8±1.1
	21	None	2.6±0.7
Cerebellum (Purkinje cells)	20	6.9±0.9	18.5±1.6
	21	None	10.5±1.4
Brainstem			
Trochlear nucleus	9	1.1±0.3	10.2±1.0
	20	None	7.6±1.0
Periaqueductal gray	20	6.1±0.8	27.0±2.1
	13	None	19.9±3.3

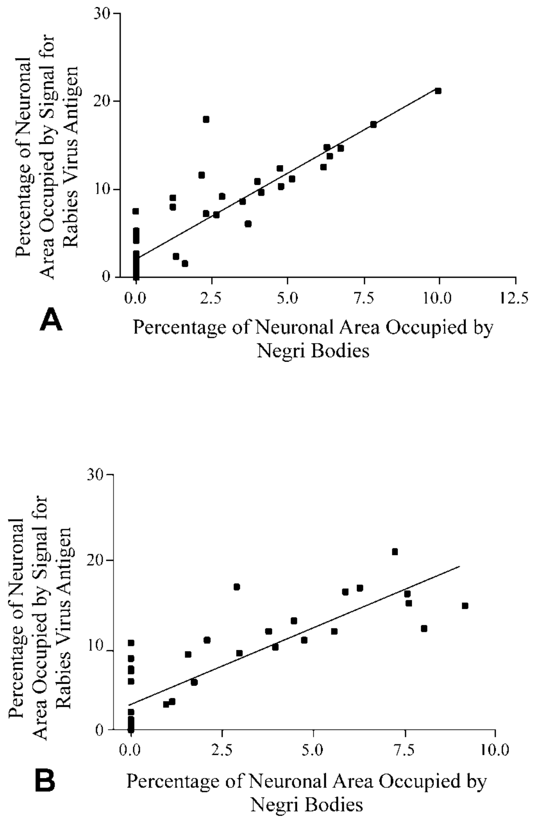

Fig. 2. Graphs of percentage area occupied by Negri bodies vs. percentage area occupied by signal for rabies virus antigen in neurons in the third layer of the cerebral cortex (**A**) and pyramidal neurons (CA1 region) of the hippocampus (**B**), including neurons without Negri bodies. Lines of best fit are shown based on linear regression analyses.

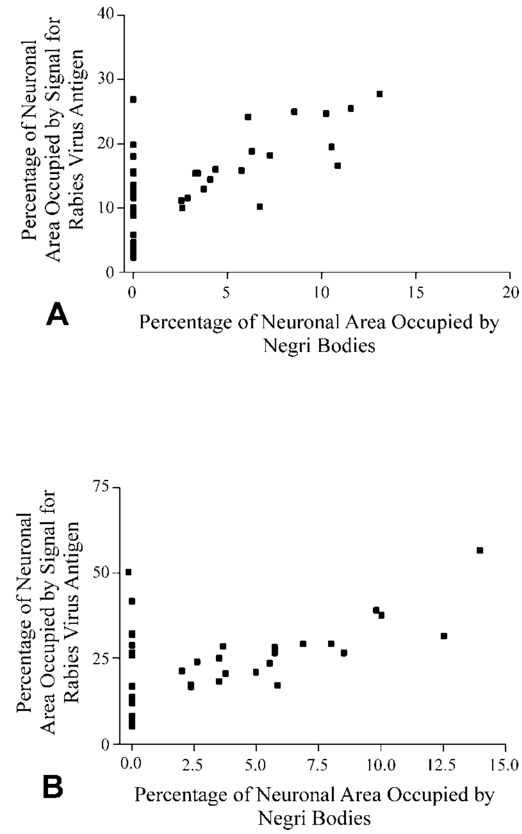

Fig. 3. Graphs of percentage area occupied by Negri bodies vs. percentage area occupied by signal for rabies virus antigen in neurons in Purkinje cells of the cerebellum (**A**) and periaqueductal gray neurons (**B**), including neurons without Negri bodies.

studies in human Purkinje cells indicate that Negri bodies consist of large numbers of virus particles with different morphologies embedded in an amorphous or slightly electron-dense matrix [Gonzalez-Angulo et al., 1970]. The matrices are thought to contain rabies virus antigen, which is based on correlative studies undertaken with fluorescent antibody staining [Hummeler et al., 1967]. In contrast, infected neurons without Negri bodies showed small groups of virus particles without the amorphous matrix. The appearance of viral particles by electron microscopy has been noted to correspond with significant increases in the quantity of intracellular infectious virus [Hummeler et al., 1967]. It is likely that both the concentration and distribution of viral proteins in the cytoplasm are important for the histochemical staining of Negri bodies. This study has demonstrated that there is a fairly close linear relationship between the proportional area of Negri bodies and the proportional area of staining for viral antigen in neurons. This linear relationship also applied to neurons without Negri bodies in the cerebral cortex and hippocampus, but it did not apply in other regions where neurons without Negri bodies contained highly variable amounts of rabies virus antigen. Some Purkinje cells and periaqueductal gray neurons without Negri bodies contained very high antigen signals. Clearly, the formation of Negri bodies varies in different neurons, and it is not closely related to the quantity of viral antigen in some neuronal cell types. Neurons in the trochlear nucleus were found to have a much smaller area of Negri bodies than neurons in all of the other regions examined, despite a similar amount of signal for viral antigen (expressed as a percentage of area). Also of interest, neurons in the trochlear nucleus from the same case showed relatively high signals for genomic RNA using in situ hybridization with quantitative image analysis compared with neurons in other regions (D. Browman and A.C. Jackson, unpublished observations). Clearly, the formation of Negri bodies varies in different neurons, and the presence or amount of Negri bodies is not always closely related to the quantity of viral antigen in the neurons. Quantitative methods have not been used previously to assess the formation of Negri bodies, but similar approaches in the future should give us greater insights into the nature of these distinctive viral inclusions in rabies.

ACKNOWLEDGMENTS

The authors are grateful for the gift of monoclonal antibody 5DF12 from Dr. Alexander I. Wandeler (Animal Diseases Research Unit, Nepean, ON). This work was supported by the Queen's University Violet E. Powell Research Fund.

REFERENCES

Dean DJ, Abelseth MK, Atanasiu P. 1996. The fluorescent antibody test. In: Meslin F-X, Kaplan MM, Koprowski H, editors. Laboratory techniques in rabies, 4th ed. Geneva: World Health Organization. p 88–95.

Dupont JR, Earle KM. 1965. Human rabies encephalitis. A study of forty-nine fatal cases with a review of the literature. Neurology 15:1023–1034.

Goldwasser RA, Kissling RE. 1958. Fluorescent antibody staining of street and fixed rabies virus antigens. Proc Soc Exp Biol Med 98:219–223.

Gonzalez-Angulo A, Marquez-Monter H, Feria-Velasco A, Zavala BJ. 1970. The ultrastructure of Negri bodies in Purkinje neurons in human rabies. Neurology 20:323–328.

Horgan ES, McKinnon RM. 1937. A comparison of the mesencephalon and hippocampus as sites of election for Negri bodies in rabies. J Hyg 37:340–344.

Hummeler K, Koprowski H, Wiktor TJ. 1967. Structure and development of rabies virus in tissue culture. J Virol 1:152–170.

Jackson AC. 1997. Rabies. In: Nathanson N, Ahmed R, Gonzalez-Scarano F, Griffin DE, Holmes K, Murphy FA, Robinson HL, editors. Viral pathogenesis. Philadelphia: Lippincott-Raven. p 575–591.

Jackson AC, Wunner WH. 1991. Detection of rabies virus genomic RNA and mRNA in mouse and human brains by using in situ hybridization. J Virol 65:2839–2844.

Kristensson K, Dastur DK, Manghani DK, Tsiang H, Bentivoglio M. 1996. Rabies: interactions between neurons and viruses. A review of the history of Negri inclusion bodies. Neuropathol Appl Neurobiol 22:179–187.

Negri A. 1903. Beitrag zum Studium der Aetiologie der Tollwuth. Z Hyg Infektionskr 43:507–528.

Perl DP, Good PF. 1991. The pathology of rabies in the central nervous system. In: Baer GM, editor. The natural history of rabies, 2nd ed. Boca Raton, Florida: CRC Press. p 163–190.

Tierkel ES, Atanasiu P. 1996. Rapid microscopic examination for Negri bodies and preparation of specimens for biological tests. In: Meslin F-X, Kaplan MM, Koprowski H, editors. Laboratory techniques in rabies, 4th ed. Geneva: World Health Organization. p 55–65.

Tustin RC, Smit JD. 1962. Rabies in South Africa. An analysis of histological examination. J S Afr Vet Med Assoc 33:295.