

## Short Communication

# Immunosuppression in Rabies Virus Infection Mediated by Lymphocyte Apoptosis

Songsri Kasempimolporn\*, Thaweesak Tirawatnpong<sup>1</sup>, Wachiraporn Saengseesom, Somboon Nookhai<sup>2</sup> and Visith Sitprijia

*Queen Saovabha Memorial Institute, World Health Organization  
Collaborating Center for Research on Rabies Pathogenesis and*

*<sup>2</sup>AIDS Research Center, Thai Red Cross Society,  
1871 Rama IV Road, Bangkok 10330 and*

*<sup>1</sup>King Chulalongkorn Memorial University,  
Bangkok 10330, Thailand*

(Received April 26, 2001. Accepted August 22, 2001)

**SUMMARY:** Previously, we demonstrated a depression of cell-mediated immunity in mice by street rabies virus infection. In the present study, we investigated several events during the course of infection and looked for alterations in the host lymphoid cells for evidence of apoptosis. Total cellular RNA was extracted from muscle tissues at the inoculation site of peripherally infected mice at different intervals after infection. Rabies virus mRNA was monitored by reverse transcription-PCR. The length of virus localization at the site of exposure in the muscle was as long as 5 days post-inoculation before the virus entered the central nervous system. At this inoculation site, the virus disappeared transiently between days 7 and 9 after infection but then was restored thereafter until death. Annexin V-fluorescein isothiocyanate staining of splenocytes and thymocytes from mice revealed apoptotic changes in these cells with a marked increase after day 6 of infection. Rabies virus antigen in the brain became detectable 6 days after infection; this occurred parallel to the appearance of apoptosis in the lymphoid cells. There was atrophy of the spleen and thymus, with no evidence of infection. Our results suggest that the interaction between the rabies virus and infected neurons triggers the process of lymphoid cell apoptosis, which reflects the defective operation of the immune system.

One difficulty posed to a better understanding of the pathogenesis of rabies is the identification of the factors that affect lymphocytes, and how such changes lead to the cascade of events that ends in immunosuppression. Host defenses involve humoral and cellular immune responses (1,2). Infection of experimental animals with an attenuated strain can induce a strong specific immune response which results in a non-lethal infection, whereas mice lethally infected with street rabies virus show severe suppression of immunity (3). Currently it remains unclear which factors are responsible for this chain of events. Previous studies of human rabies have shown that the process of activation, proliferation, and sequestration of natural killer cells may not occur in rabies patients and may reflect a naive condition (4). Only a minority (25%) of nonvaccinated rabies patients had neutralizing antibody in their serum at the onset of symptoms (5). A defect in immune recognition to rabies virus nucleoprotein (N), in addition to a depressed cell-mediated immunity, may be involved in the inefficient production of rabies virus-neutralizing antibody (6). This defect also influences the development of specific cytotoxic T-lymphocytes, mitogen-induced proliferation of T-cells, as well as IL-2 secretion. This has been demonstrated in experimental mice (7). Moreover, treatment with a calcium ionophore (A23187) or phorbol 12-myristate-13 acetate was shown to have no influence on the unresponsiveness of T-cells to mitogens. This latter finding suggests that T-cell suppression appears to be dependent upon a change in T-cell function, rather than upon changes in the cytosolic free

Ca<sup>2+</sup> concentration or upon alterations in protein kinase C activation involved in the signal transduction pathway of T-cell activation (7).

The overall features of immunosuppression in rabies are still not clear. We therefore investigated the changes in host lymphoid cells for evidence of apoptosis, as well as in the normal sequence of events following infection. We attempted to thereby define the mechanisms involved in the immune suppression which occurs due to street rabies virus in mice during the course of infection.

In the present study, 6- to 8-week-old BALB/c mice were infected in the hind leg with 0.03 ml of a 10% suspension of salivary glands from a naturally-infected dog. The salivary glands were prepared by homogenization with diluent (phosphate buffered saline containing 10% fetal bovine serum, 500 units of penicillin/ml and 1 mg of streptomycin/ml) and centrifugation at 600 g for 15 min to discard cell debris. The infectivity titer of the suspension was 10<sup>5.2</sup> mouse intracerebral 50% lethal doses (LD<sub>50</sub>)/ml. Hindlimb paralysis is a major neurological sign of rabies infection. Signs of paralysis were observed 10-12 days after infection and all mice died by days 12-15. Five mice were sacrificed daily. At necropsy, the muscle tissues at the inoculation site, the brains, thymus glands, and spleens were collected.

Brains from mice were examined daily for evidence of infection. Impression brain smears were fixed in 95% acetone and stained with fluorescein isothiocyanate (FITC)-conjugated antirabies globulin (Thai Red Cross production, Thailand). All preparations were examined under a fluorescence microscope. The virus antigen in the brain became detectable after day 6 post-infection (data not shown).

\*Corresponding author: Tel: +662-2520161~3, Fax: +662-2540212, E-mail: qsmiskp@redcross.or.th

The time course of rabies virus RNA persistence in the muscle tissues at the inoculation site was monitored by reverse transcription (RT)-PCR. Total cellular RNA was extracted from the muscle tissues by homogenization in a monophasic solution of phenol and guanidine isothiocyanate, as specified by the manufacturer (TRIzol®, GibcoBRL, Gaithersburg, Md., USA). One step RT-PCR was carried out using a commercial kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's specifications. The RNA (2  $\mu$ g) was used in a 50  $\mu$ l reaction mixture. The mixture contained 50 pmol each of primers for rabies virus N gene (5'-GACATGTCCGGAAGACTGG-3', at position 319-337, and 5'-GTATTGCCTCTCTAGCGGTG-3', at position 823-842) or for  $\beta$ -actin gene (5'-ACCGTGAAAAGATGACCCAG-3', at position 266-285, and 5'-TCTCAGCTGTGGTGGTGAAG-3', at position 519-538). The nucleotide sequences of N and  $\beta$ -actin genes were obtained from the GenBank database, accession numbers NC\_001542 and X03765, respectively. Amplifications were performed in a thermocycler (Perkin Elmer Cetus, Norwalk, Conn., USA) for 35 cycles (denaturation: 60 s at 94°C; annealing: 60 s at 55°C for  $\beta$ -actin, and 50°C for rabies virus; extension: 60 s at 72°C). The  $\beta$ -actin and rabies virus primers led to the amplification of 272 bp and 524 bp fragments, respectively. DNA products were visualized on agarose gels by ethidium bromide staining. The  $\beta$ -actin mRNA was assayed as a control of mRNA purification, RT-PCR amplification, and of equal loading.

The duration of virus localization to the muscles at the site of exposure was as long as 5 days post-inoculation. Persistence of virus in muscle cells prior to invasion of the nervous system occurred, as determined by the intensity of the amplified rabies virus N gene on days 1-5 post-inoculation. Although virus-specific RNA almost disappeared from the site of infection at 6 days post-infection, it was detected again at this site in later stages of infection, starting approximately on day 10 (Fig. 1). It is difficult to determine whether virus persistence in muscle tissues is the result of primary replication in muscle cells or the result of a second cycle of replication initiated by virions transported centrifugally from the infected neurons back to the muscles. Most previous studies in laboratory animal-fixed rabies virus models have revealed the

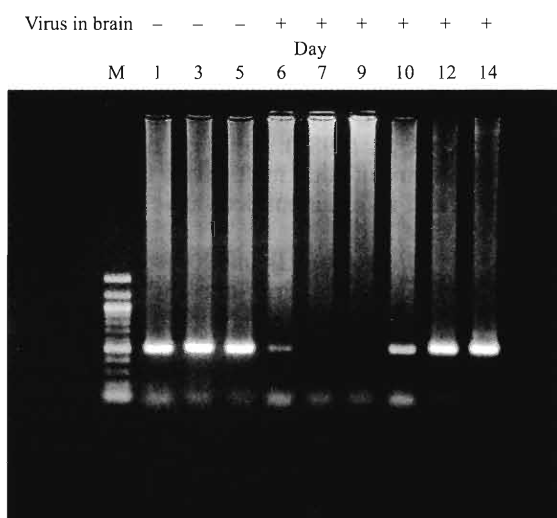


Fig. 1. The time course of rabies virus RNA persistence in the muscle at the site of exposure, compared to that of rabies virus antigen detection in the brain. A 524 bp fragment of rabies virus N gene was expressed. M: molecular size markers.

appearance of rabies virus replication in muscle fibers prior to its invasion of the peripheral and central nervous systems (8,9). In contrast, other studies demonstrated that the virus can also gain entrance to the brain without prior local replication in the muscle cells, resulting in a short incubation period (10,11). The relevance of primary replication in muscle tissue remains unknown. Nevertheless, our study supports current recommendations for local infiltration of virus inoculation sites with human or equine rabies immune globulin (12).

Apoptosis is a form of programmed cell death and is a fundamental event in many biological processes (13). After initiating apoptosis, most cell types translocate the membrane phospholipid phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. PS can be detected by staining with an FITC conjugate of annexin V. Externalization of PS occurs earlier than the nuclear changes associated with apoptosis (14). To investigate apoptosis of lymphoid cells in infected mice, apoptotic cells were identified using the ApoAlert apoptosis kit (Clontech, Palo Alto, Calif., USA) according to the manufacturer's protocol. Thymus glands or spleens from infected mice were gently pressed through 50- $\mu$ m mesh stainless steel screens into Dulbecco modified Eagle medium (DMEM). The single-cell suspension was centrifuged at 1,000 g for 5 min. Erythrocytes were lysed by briefly suspending the cell pellet in a 0.84%  $\text{NH}_4\text{Cl}$  solution. After treatment, thymocytes or splenocytes were resuspended in DMEM and subjected to apoptosis analysis. Lymphoid cells prepared in this way were routinely 99% viable, as assessed by trypan blue exclusion assay. Splenocytes or thymocytes were incubated for 5 min with 1  $\mu$ g/ml annexin V-FITC in binding buffer (10 mM HEPES-NaOH; pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM  $\text{MgCl}_2$ , 1.8 mM  $\text{CaCl}_2$ ), and 30,000 cells were analyzed by FACScan (Becton-Dickinson, Paramus, N.J., USA) using a single laser emitting excitation light at 488 nm. To distinguish cells that had lost membrane integrity, propidium iodide dye was added to a final concentration of 2  $\mu$ g/ml before the analysis. Annexin V-FITC staining of splenocytes or thymocytes revealed apoptotic biological changes in these cells, with a marked increase after day 6 following infection (Fig. 2). The data obtained represent two separate experiments. There was atrophy of the spleen and thymus that was parallel to development of the evidence of apoptosis (data not shown). To further examine whether lymphoid cells are peripheral targets of the rabies virus, splenocytes and thymocytes were assayed by RT-PCR for rabies virus mRNA. Although murine lymphocytes have been shown to be permissive to fixed rabies virus infection *in vitro* (15), no evidence of virus infection in these cells was observed during the course of infection (data not shown).

In our mouse model using street rabies virus infection, the appearance of virus antigen within the brain ran parallel to the occurrence of lymphoid cell apoptosis; this sequence of events began 6 days after infection, when the early signs of disease appeared. A portion of lymphoid cells underwent apoptosis, reaching a rate of 50% of all such cells in the late stage of disease (Fig. 2). In other words, the distribution of rabies virus in the brain, as well as apoptosis of lymphoid cells, became more prominent as the disease advanced. There was also a marked decrease in the size of the spleen and thymus during the course of infection (data not shown). Hirai et al. (16) demonstrated a marked decrease in the number of mononuclear cells in the spleen of street rabies virus-infected mice, whereas that of mice infected with an attenuated rabies virus strain increased with the infection. Moreover, the

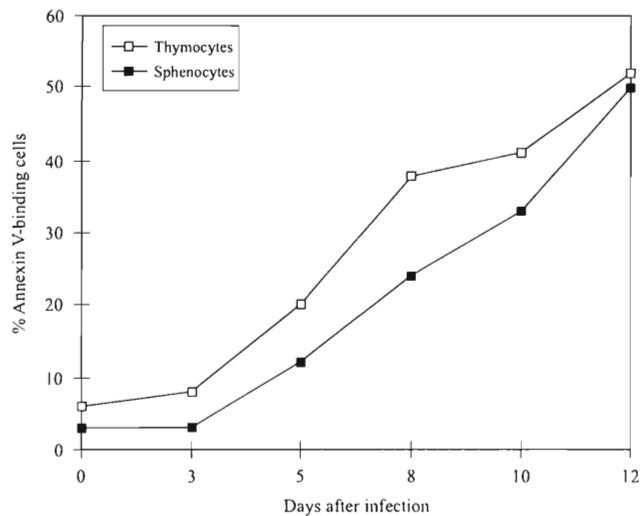


Fig. 2. Detection of early-stage apoptosis in thymocytes and splenocytes from street rabies-infected mice at specific intervals after infection. Annexin V-FITC binding to apoptotic lymphocytes was analyzed by flow cytometry. Propidium iodide (PI) was included in the assay mixture as a control for membrane integrity; less than 20% of the cells were positive for PI staining in this experiment (data not shown). Each data point was derived from an analysis of 30,000 cells from two separate experiments.

proportions of CD4<sup>+</sup> and CD8<sup>+</sup> cells in the spleen of street rabies-infected mice increased with time, although the absolute number of both types of cell decreased significantly as animals approached death. They thus proposed that the suppression of T cell functions observed during infection was not due to a selective mechanism of lymphocyte depletion. However, the study did not clarify the mechanism(s) involved.

Many viruses have been found to trigger apoptosis in the cells that they infect. Infections by lymphotropic viruses are known to trigger lymphocyte apoptosis (17). Both acute and persistent infections can establish a state of immune deficiency in infected individuals. Transient immune deficiency has been reported during many viral infections, e.g., Epstein-Barr virus, cytomegalovirus, human immune deficiency virus, and lymphocytic choriomeningitis virus. Infection with these viruses leads to the failure of host T cell response to mitogens or the failure to mount memory recall responses to other antigens. This transient immunodeficiency has been correlated with the induction of apoptosis in the highly activated T cells. However, after resolution of infection, or after the acute phase of the antiviral immune response has passed, responsiveness to recall antigens returns (17,18). In contrast, the triggering of lymphocyte apoptosis by neurotropic rabies virus may not depend upon direct infection by the virus. Although there are many hypothetical mechanisms for neurophathogenesis and consequent alteration in host immune response (19,20), our results have provided convincing evidence that immunosuppression in neurotropic virus infection results from a cascade of events. A certain unknown triggering event in the infected neurons initiates the process of neuronal dysfunction that in turn modulates variable sets of neuroendocrine-immune cascades. Therefore, apoptotic biological changes in lymphoid cells might be, at least in part, a major outcome of such interactions, resulting in an irreversible immunosuppression. The failure to recover from immunosuppression during rabies virus infection may in part contribute to the severity of this fatal disease.

## ACKNOWLEDGMENTS

The authors are grateful to Prof. Henry Wilde for reviewing the manuscript, and to Drs. Tamotsu Satoh and Motohide Takahashi for their kind encouragement.

## REFERENCES

1. Miller, A., Morse, H. C., Winkelstein, I. and Nathanson, N. V. (1978): The role of antibody in recovery from experimental rabies. *J. Immunol.*, 121, 321-326.
2. Mifune, K., Takeuchi, E., Napiorkowaki, P. A., Yamada, A. and Sakamoto, K. (1981): Essential role of T cells in the post-exposure prophylaxis of rabies in mice. *Microbiol. Immunol.*, 25, 895-904.
3. Wiktor, T. J., Doherty, P. C. and Koprowski, H. (1977): In vitro evidence of cell-mediated immunity after exposure of mice to both live and inactivated rabies virus. *Proc. Natl. Acad. Sci. USA*, 74, 334-338.
4. Panpanich, T., Hemachudha, T., Piyasirisilp, S. and Manatsathit, S. (1992): Cells with natural killer activity in human rabies. *Clin. Exp. Immunol.*, 89, 414-418.
5. Hemachudha, T., Phanuphak, P., Sriwanthana, B., Manatsathit, S., Phanthumchinda, K., Sriprasomsup, W., Ukachoke, C., Rasameechan, S. and Kaoroptham, S. (1988): Immunologic study of human encephalitic and paralytic rabies. A preliminary study of 16 patients. *Am. J. Med.*, 84, 673-677.
6. Kasempimolporn, S., Hemachudha, T., Khawplod, P. and Manatsathit, S. (1991): Human immune response to rabies nucleocapsid and glycoprotein antigens. *Clin. Exp. Immunol.*, 84, 195-199.
7. Kasempimolporn, S., Saengseesom, W., Mitmoonpitak, C., Akesowan, S. and Sitprija, V. (1997): Cell-mediated immunosuppression in mice by street rabies virus not restored by calcium ionophore or PMA. *Asian Pac. J. Allergy Immunol.*, 15, 127-132.
8. Charlton, K. M., Nadin-Davis, S., Casey, G. A. and Wandeler, A. I. (1997): The long incubation period in rabies: delayed progression of infection in muscle at the site of exposure. *Acta Neuropathol.*, 94, 73-77.
9. Murphy, F. A., Bauer, S. P., Harrison, A. K. and Winn, W. C. (1973): Comparative pathogenesis of rabies and rabies-like viruses: viral infection and transit from inoculation site to the central nervous system. *Lab. Invest.*, 28, 361-376.
10. Coulon, P., Derbin, C., Kucera, P., Lafay, F., Prehaud, C. and Flamand, A. (1989): Invasion of the peripheral nervous systems of adult mice by the CVS strain of rabies virus and its avirulent derivative AvO1. *J. Virol.*, 63, 3550-3554.
11. Shankar, V., Dietzschold, B. and Koprowski, H. (1991): Direct entry of rabies virus into the central nervous system without prior local replication. *J. Virol.*, 65, 2736-2738.
12. World Health Organization (1992): Expert committee on rabies. Technical report, 824. WHO, Geneva.
13. Nagata, S. (1997): Apoptosis by death factor. *Cell*, 88, 355-365.
14. Martin, S. J., Reutelingsperger, C. P. M., McGahon, A. J., Rader, J. A., Van Schie, R. C. A. A., LaFace, D. M. and Green, D. R. (1995): Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition

- by overexpression of Bcl-2 and Abl. *J. Exp. Med.*, 182, 1545-1556.
15. Thoulouze, M. I., Lafage, M., Montano-Hirose, J. A. and Lafon, M. (1997): Rabies virus infects mouse and human lymphocytes and induces apoptosis. *J. Virol.* 71, 7372-7380.
  16. Hirai, K., Kawano, H., Mifune, K., Fujii, H., Nishizono, A., Shichijo, A. and Mannen, K. (1992): Suppression of cell-mediated immunity by street rabies virus infection. *Microbiol. Immunol.*, 36, 1277-1290.
  17. Razvi, E. S. and Welsh, R. M. (1995): Apoptosis in viral infections. *Adv. Virus Res.*, 45, 1-60.
  18. Zarozinski, C. C., McNally, J. M., Lohman, B. L., Daniels, K. A. and Welsh, R. M. (2000): Bystander sensitization to activation-induced cell death as a mechanism of virus-induced immune suppression. *J. Virol.*, 74, 3650-3658.
  19. Ader, R., Cohen, N. and Felten, D. (1995): Psychoneuro-immunology: interactions between the nervous system and the immune system. *Lancet*, 345, 99-103.
  20. Reichlin, S. (1993): Mechanism of disease: neuroendocrine-immune interactions. *N. Engl. J. Med.*, 329, 1246-1253.