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Cell-Mediated Immunosuppression in Mice by Street Rabies Virus not Restored by Calcium Ionophore or PMA

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SUMMARY **It is known that rabies virus can suppress the host immune system. In this study we demonstrate a depression of cell-mediated immunity in mice, peripherally infected with Thai street rabies virus. The cell-mediated cytolysis of spleen cells from mice increased transiently on day 5 after infection and declined rapidly thereafter until death. The proliferation of spleen cells stimulated with a T-cell mitogen such as phytohemagglutinin or concanavalin A, was significantly suppressed during the course of infection. There was also a marked suppression of IL-2 secretion in parallel with a decrease of the T-cell proliferative response to mitogen. The suppression of T-cell proliferation was not restored by treatment with a calcium ionophore (A 23187) or phorbol 12-myristate-13 acetate (PMA).**

Rabies is an invariably fatal disease in humans yet the factors that determine rabies virulence in man have been poorly understood. Host defenses involve both humoral and cellular immune responses.[1,2] Infection of experimental animals with an attenuated strains can induce strong specific cell-mediated immunity (CMI) and antibody production which results in non-lethal infections.[3] However, mice lethally infected with street rabies virus show severe suppression of CMI.[4] The relationship between induction of protective immunity and immunosuppression is not clear. Suppression of CMI, seen in the course of rabies infection, may contribute to the severity of the disease. Studies in humans with rabies found defects in T-cell activation and in rabies-neutralizing antibody.[5-9] However, it is unknown which factors are responsible for these defective responses. The detailed mechanisms involved in immunosuppression are not fully understood. Elucidation of underlying mechanisms may help in the designs of strategies to reverse disease progression.

Proliferation of T-cells in response to antigens or mitogens involves production and response to IL-2. Following activation of T-cells, IL-2 is secreted and surface receptors for IL-2 are expressed. These two events are essential for subsequent lymphoproliferation.[10] An increase of cytosolic free-calcium (Ca^{2+}) is required for the production of IL-2 but not for the expression of IL-2 receptors.[11] This increase of Ca^{2+} mobilization is associated with several potential intracellular signaling events including increased activity of phospholipase C (PLC) and a resulting increases in the production of inositol phosphates and diacylglycerols. Diacylglycerol activates the enzyme protein kinase C (PKC)[12] and this subsequently induces IL-2 production from T-lymphocytes.[13]

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Activation of T-lymphocytes by ligands is accompanied by a rapid increase in cytosolic free Ca^{2+} and by activation of PKC.[14,15] The proliferation of T-cells induced by cation ionophore, A 23187, suggests a direct role of changes in cytosolic Ca^{2+} concentration in mitogenesis.[16,17] A23187 has the ability to increase cytoplasmic free Ca^{2+} by binding and moving Ca^{2+} across the cell membrane.[18] Also, it is known that PKC is directly activated by phorbol 12-myristate-13 acetate (PMA) treatment.[19] However, the effects of these drugs on T-cell suppression in rabies virus infection have not yet been tested. We, therefore, attempted to determine whether calcium ionophore A23187 or PMA involved in the signal transduction pathway of T-cell activation is able to restore the suppression of CMI.

MATERIALS AND METHODS

Infection of mice

The street rabies virus used was a salivary gland suspension from a naturally-infected dog. The infectivity of this virus stock was $10^{5.2}$ median mouse intracerebral lethal doses ($MICLD_{50}$)/ml. BALB/c mice (6 to 8 weeks of age) were infected peripherally in the left hind footpad using a volume of 30 µl.

Tissue culture medium and reagents

The tissue culture medium consisted of Dulbecco's modified Eagle's medium supplemented with 1 mM glutamine, 10 mM HEPES buffer and antibiotics (all from Gibco, NY, U.S.A.). The mitogenic lectins used were phytohemagglutinin (PHA-L; Seromed, Germany) and concanavalin A (ConA; Seromed). The calcium ionophore A23187 and phorbol 12-myristate-13 acetate (PMA) were purchased from Sigma (MO, U.S.A).

Lymphocyte proliferation assay

Spleens were removed at intervals from infected mice after infection. Spleen cells were cultured in 96-well flat-bottomed plates at 4×10^5 cells/well in 0.2 ml of tissue culture medium supplemented with 10% fetal bovine serum (FBS) in the presence and absence of 2 µg /ml PHA-L or 5 µg/ml Con A at 37°C for 48 hours. The cells were pulsed with 0.5 µCi of [^{3}H]-thymidine (Amersham, Buckinghamshire, England) for 18 hours. They were harvested and the amount of incorporated radioactivity was determined by liquid scintillation counter. The lymphocyte proliferative response was assessed by the uptake of [^{3}H]-thymidine by the mitogen-stimulated lymphocytes. The lymphocyte reactivity was expressed as a stimulation index (SI = mean counts per minute with mitogen / mean counts per minute without mitogen). SI higher than 2 were considered as evidence of proliferation. In some experiments, PMA (5×10^{-5} mM) or A23187 (0.5 µM) was added to the above culture 1 hour after mitogen addition.[20]

Interleukin (IL)-2 assay

IL-2 secretion was tested in 24-well flat-bottomed plates by culturing 4×10^6 splenocytes in 1.6 ml of tissue culture medium supplemented with 2% FBS and 10^{-6} M 2-mercaptoethanol (2-ME) in the presence of mitogen. Cell-free supernatants were harvested 24 hours later and measured by an enzyme-linked immunosorbent assay (ELISA) kit for quantification of murine IL-2 (Duoset; Genzyme, Cambridge, MA).

Cell-mediated cytolysis

L-929 mouse fibroblast cell line (Gift of Professor HCJ Ertl, Wistar Institute, PA), maintained in culture medium supplemented with 10% FBS was used as target cells. Rabies-infected target cells were plated into 96-well round-bottomed plates at a cell density of 1×10^4 cells/well and mixed with spleen cells at an effector/target (E/T) ratio of 50 in a total volume of 200 µl of culture medium supplemented with 2% FBS and 2-ME. The cultures were performed in triplicates. Killing of target cells was measured in a lactate dehydrogenase (LDH)-release assay (CytoTox96™; Promega, USA.). The colorimetric LDH-release assay was performed according to the manufacturer's protocol. Lactate dehydrogenase, a stable cytosolic enzyme, is released upon cell lysis. Released-LDH in culture supernatants was measured in an enzymatic assay which results in the conversion of a tetrazolium salt into a red formazan product. The amount of color formed was proportional to the number of lysed cells and determined by the absorbance at 490 nm. Specific lysis (% cytoxicity) was calculated as in a standard ^{51}Cr-release assay.

Statistics analysis

Statistical significance was determined by the Student's *t*-

test. A p-value of less than 0.05 was considered to be significant.

RESULTS

Induction of a cell-mediated cytolytic response upon infection of mice with street rabies virus

Splenocytes, derived from mice infected with street rabies virus during the course of infection, were tested for cytolytic activity. Kinetics of the induction of cell-mediated cytolysis was shown in Fig. 1. The cytolytic activity increased slightly on day 5 but declined rapidly during the onset of disease. All mice died on days 12-15 after infection.

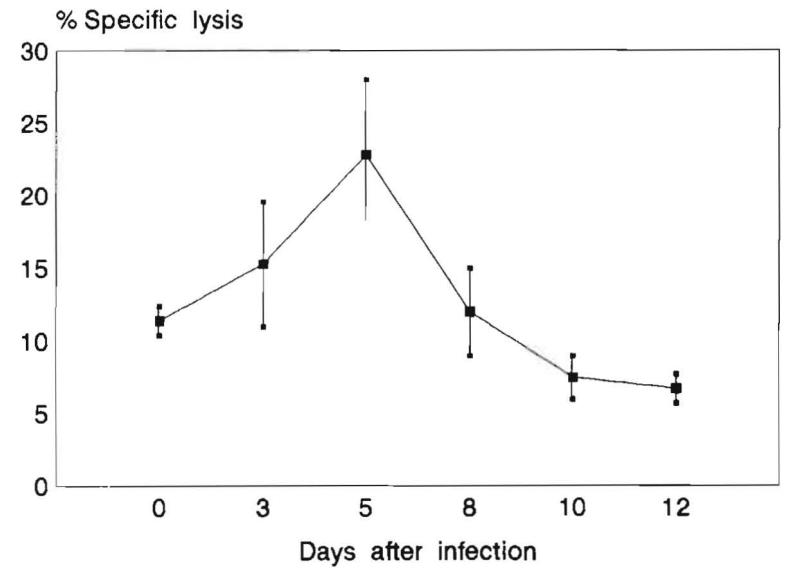

Fig. 1. Kinetics of the induction of cell-mediated cytolysis of spleen cells from infected mice. Cell-mediated cytolysis was determined by the colorimetric LDH-release assay. Results are means of six mice ± s.e.m.

Street rabies virus infection suppresses the mitogen-induced proliferation of T-lymphocytes

Spleen cells from street rabies virus-infected mice were examined at various intervals after infection for their proliferative responses to T-cell mitogenic lectins such as PHA and Con A. Figs. 2A and B represent T-cell proliferative responses to PHA and Con A, respectively. Rabies virus significantly inhibited mitogen-induced murine T-cell proliferation ($p<0.05$). This was observed after day 5 of infection.

Reduced ability to produce IL-2 of mice infected with street rabies virus

Proliferation of T-cells in response to PHA or Con A involves production and response to IL-2. Therefore, IL-2 concentrations in the culture supernatants of spleen cells from street rabies virus-infected mice, which were stimulated with PHA or Con A, were measured (Fig. 3). There was a marked suppression of IL-2 secretion from splenic T-cells during the course of infection. This was in parallel with a decrease of their proliferative response to PHA or Con A.

The immunosuppression mechanism in street rabies-infected mice does not involve inhibition of protein kinase C (PKC) or cytosolic free-calcium ions

The effects of calcium ionophore A23187 and PMA on T-cell suppression were studied as shown in Figs. 2A and B. The suppression induced by street rabies virus infection was not restored by treatment with A23187 or PMA. Combination with A23187 or PMA induced no significant reversion when compared with single addition of mitogen. In addition, treatment by these drugs did not augment the IL-2 production (data not shown). These data suggest that the immune suppression by street rabies virus infection is not associated with PKC activation or changes in cytosolic free Ca^{2+} concentration.

DISCUSSION

Virus-induced immunosuppression can result from a variety of mechanisms. Although Rouse and Horokov[21] delineated four hypotheses, the actual mechanism by which rabies virus causes immunosuppression is still unclear. A previous study demonstrated that the suppressive effects, induced by street rabies virus infection in mice, did not involve a prosta-

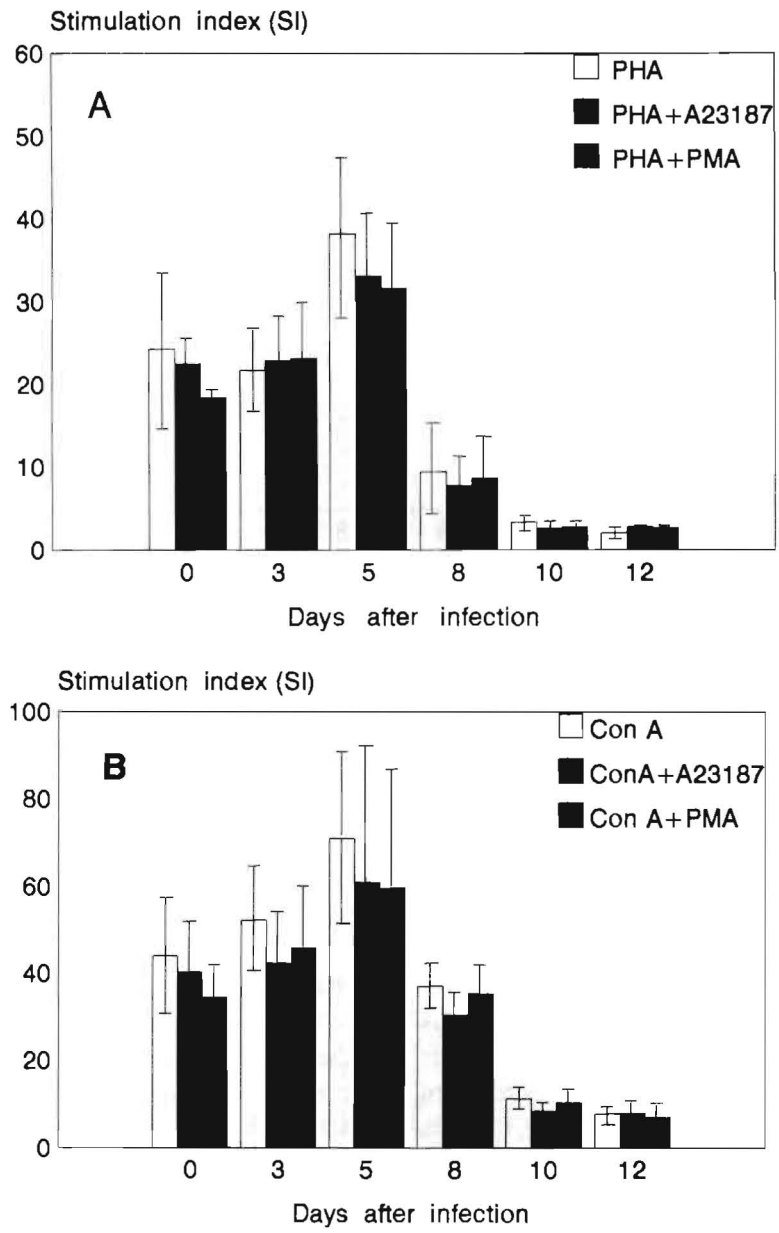

Fig. 2. Effects of A23187 and PMA on the proliferative response to PHA (A) or con A (B) of spleen cells from rabies-infected mice. Results are expressed as SI of tritiated thymidine incorporation ± s.e.m. (n = 6).

glandin-mediated mechanism.[22] There was no abnormality in the synthesis of prostaglandin E which has been shown to inhibit Con A-induced proliferation of murine T-cells. Prostaglandin E was released from adherent cells such as macrophages and lymphocytes and this was suppressed by treatment with indomethacin.[23]

Mice were lethally infected with street rabies virus. In this study, hindlimb paralysis is a major neurological sign of rabies infection. Signs of paralysis were observed 7-9 days after infection and all mice finally died around on day 12. Street-rabies virus infected mice failed to develop cytotoxic T-cells specific for rabies virus-infected target cells (Fig. 1). Also, there was a decrease of the T-cell proliferative response to mitogens during the course of infection. The proliferative response decreased significantly from day 8 comparing with that of T-cells from normal mice ($p<0.05$). This suppression could not be reversed by the addition of A23187 or PMA with a mitogen (Figs. 2A and B). In parallel with the depression of T-cell response, IL-2 production of spleen cell became suppressed (Fig. 3). In addition, the reversion effects of A23187 and PMA on IL-2 secretion were not observed (data not shown).

Although street rabies virus infection suppressed PHA-or Con A-induced proliferation of murine T-lymphocytes, the infected-mice exhibited a CMI response as early as 5 days after infection. This suggests that the process of immune recognition and development of cellular immunity occurs earlier than the onset of disease. Rabies virus antigens may be 'seen' at the inoculation sites or at ganglion cells where initial viral replication occurs. The unresponsiveness of splenocyte to Con A reported by Hirai *et al.*[22] is consistent with our finding. However, this differs from that described by Perrin *et al.*[24] The last authors found that spleen cells of symptomatic mice infected by pathogenic lyssaviruses lost the capacity to produce cytokines *in vitro* in response to rabies antigen. In contrast, Con A response was still preserved. The discrepancy might

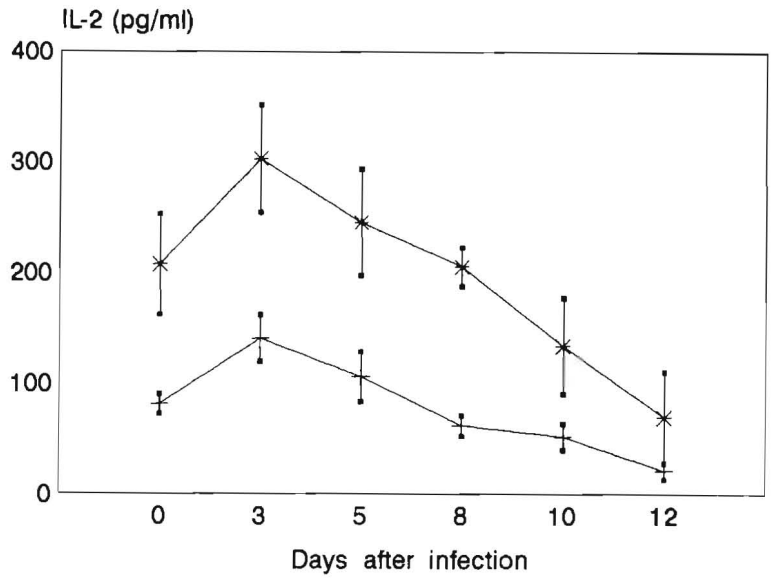

Fig. 3. IL-2 concentration in the spleen cell culture supernatants of PHA (+) or con A (*)-induced proliferation. Results are means of six mice ± s.e.m.

be due to the virulence of lyssaviruses used. They used rabies viruses (including both wild and fixed viruses) adapted to BHK-21 cells. On the other hand, we used the street rabies virus isolated from a naturally-infected dog without passage on cell cultures. The unresponsiveness of T-cells to IL-2, as demonstrated by the failure in recovery from immunosuppression after addition of exogenous IL-2,[22] might be one mechanism in T-cell suppression. Recent studies have demonstrated that tyrosine phosphorylation is an additional activation pathway for T-cell proliferation, which is not a consequence of PLC activation.[25] The responses of T-lymphocytes to IL-2 are mediated by tyrosine kinase activity and triggered by stimulation of the β-chain of IL-2 receptor.[26] The inhibition of tyrosine phosphorylation in T-cells involves a down-regulation of immune responses. Also, it may be associated with down-regulation of IL-2 receptor.[20] Interaction of IL-2 with its high-affinity membrane receptor complex (IL-2 receptor) is sufficient to induce proliferation of T-lymphocytes. This might be one of the possible explanations for their non-responsiveness to IL-2. The suppression of mitogen-induced T-cell proliferation did not appear to depend upon inhibition of Ca^{2+} movement as it was not reversed by adding A23187. Moreover, treatment with PMA also did not influence the unresponsiveness of T-cells to mitogens. These findings suggest that the T-cell suppression appears to be dependent upon a change of T-cell function, rather than changes in cytosolic free Ca^{2+} concentration or PKC activation.

Recently, Hemachudha[27] has summarized the hypothetical mechanisms for rabies neuropathogenesis. This thoughtful review emphasizes the interactions between the nervous and immune systems, which may affect different sets of neurotransmitters that in turn modulate variable neurobehavioural patterns and neuroendocrine-immune cascades. The immune system dysfunctions may be affected by manipulation of the neuroendocrine control at the level of the hypothalamo-pituitary-adrenal (HPA) axis during the course of infection. The pro-inflammatory cytokines (IL-1, tumor necrosis factor-α) can modify several hippocampal functions including electrical and HPA axis activities, and serotonin metabolism; which in turn affects the acetylcholine system and desensitizes muscarinic acetylcholine receptor. Futhermore, several neuropeptides and hormones may also be released from the neural axis during infection. They can exert either inhibitory or stimulatory effects on activation, proliferation, migration and secretions of various cell types including T-, B- and NK-cells and macrophages. Also, there is evidence that the cytokine-nitric oxide damaging process may trigger apoptosis and consequently alterations in host immune response.

It is still obscure which factors obviously affect the alteration of T-cells that lead to the cascade causing in immunosuppression. Conclusion awaits further investigations.

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REFERENCES

1. Miller A, Morse HC, Winkelstein J, Nathanson N. The role of antibody in recovery from experimental rabies. J Immunol 1978; 121: 321-6.
2. Mifune K, Takeuchi E, Napiorkowaki PA, Yamada A, Sakamoto K. Essential role of T cell in mice. Microbiol Immunol 1981; 25: 895-904.
3. Smith KA. Interleukin-2: Inception, impact and implications. Science 1988; 240: 1169-76.
4. Wiktor TJ, Doherty PC, Koprowski H. Suppression of cell-mediated immunity by street rabies virus. J Exp Med 1977; 145: 1617-22.
5. Hemachudha T, Phanuphak P, Sriwanthana B. Immunologic study of human encephalitic and paralytic rabies. A preliminary study of 16 patients. Am J Med 1988; 84: 673-7.
6. Hemachudha T, Panpanich T, Phanuphak P, Manatsathit S, Wilde H. Immune activation in human rabies. Trans R Soc Trop Med Hyg 1993; 86: 106-8.
7. Sriwanthana B, Hemachudha T, Griffin DE, Manatsathit S, Tweardy D, Phanuphak P. Lymphocyte subsets in human encephalitic and paralytic rabies. Acta Neurol Scand 1989; 80: 287-9.
8. Kasempimolporn S, Hemachudha T, Khawplod P, Manatsathit S. Human immune response to rabies nucleocapsid and glycoprotein antigens. Clin Exp Immunol 1991; 84: 195-9.
9. Panpanich T, Hemachudha T, Piyasirisilp S, Manatsathit S, Wilde H. Phanuphak P. Cells with natural killer activity in human rabies. Clin Exp Immunol 1992; 89: 414-8.
10. Greene WC, Leonard JW. The human interleukin-2 receptor. Annu Rev Immunol 1986; 4: 69-95.
11. Mills GB, Cheung RK, Grinstein S, Gelfand EW. Increase in cytosolic free-calcium concentration is an intracellular messenger for the production of interleukin-2 but not for expression of interleukin-2 receptor. J Immunol 1985; 134: 1640-3.
12. Gelfand EW, Mills GB, Cheung RK, Lee JWW, Grinstein S. Trans-membrane ion fluxes during activation of human T lymphocyte: role of Ca^{2+}, Na^{+}/H^{+} exchange and phospholipid turnover. Immunol Rev 1987; 95: 59-87.
13. Gelfand EW, Cheung RK, Mills GB, Grinstein S. Mitogens trigger a calcium-independent signal for proliferation in phorbol ester-treated lymphocytes. Nature 1985; 315: 419-20.
14. Tsien RY, Pozzan T, Rink TJ. T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocyte. Nature 1982; 295: 68-71.
15. MacDonald HR, Nabholz M. T cell activation. Annu Rev Cell Biol 1986; 2: 231-53.
16. Luckasen JR, White JG, Kersey JG. Mitogenic properties of a calcium ionophore, A23187. Proc Natl Acad Sci USA. 1974; 71: 5088-90.
17. Akerman KEO, Andersson LC. Direct mitogenic effect of ionophore A23187 on isolated human T helper lymphocytes. Eur J Immunol 1984; 14:286-8.
18. Reed PW, Lardy HA. A23187: a divalent cation ionophore. J Biol Chem 1972; 247: 6970-7.
19. Castagna M,Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem 1982; 257:7847-51.
20. Matsui K, Arai T. Immunosuppression induced by salmonella involves inhibition of tyrosine phosphorylation in murine T lymphocytes. FEMS Immunol Med Microbiol 1993; 7: 344-5.
21. Rouse BT, Horohov DW. Immunosuppression in viral infection. Rev Infect Dis 1986; 8: 850-73.
22. Hirai K, Kawano H, Mifune K, Fujii H, Nishizono A, Shichijo A. Suppression of cell-mediated immunity by street rabies virus infection. Microbiol Immunol 1992; 36: 1277-90.
23. Webb DR, Nowowiejski I, Mitogen-induced changes in lymphocyte prostaglandin levels: a signal for the induction of suppressor cell activity. Cell Immunol 1978; 41: 72-85.
24. Perrin P, Franco MTD, Jallet C, Fouque F, Morgeaux S, Tordo F, Colle JH. The antigen-specific cell-mediated immune response in mice is suppressed by infection with pathogenic lyssaviruses. Res Virol 1996; 147: 289-99.
25. Stanley JB, Gorczynski R, Huang CK, Love J, Mills GB. Tyrosine phosphorylation is an obligatory event in IL-2 secretion. J Immunol 1990; 145:2189-98.
26. Fung MR, Scearce RM, Hoffman JA, Peffer NJ, Hammes SR, Hosking JB, Schmandt R, Kuziel WA, Haynes BF, Mills GB, Greene WC. A tyrosine kinase physically associates with the β-subunit of the human IL-2 receptor. J Immunol 1991; 147:1253-60.
27. Hemachudha T. Rabies. In: Roos KL, ed. Central nervous system infectious diseases and therapy. New York: Marcel Dekker Inc., 1997: 573-600.