CHAPTER III

MATERIALS AND METHODS

To search for explanations on the mechanisms of rabies virus effect upon human brain proposed at the end of introduction section. A conceptual framework of the experimental designs was presented in Figure 2.

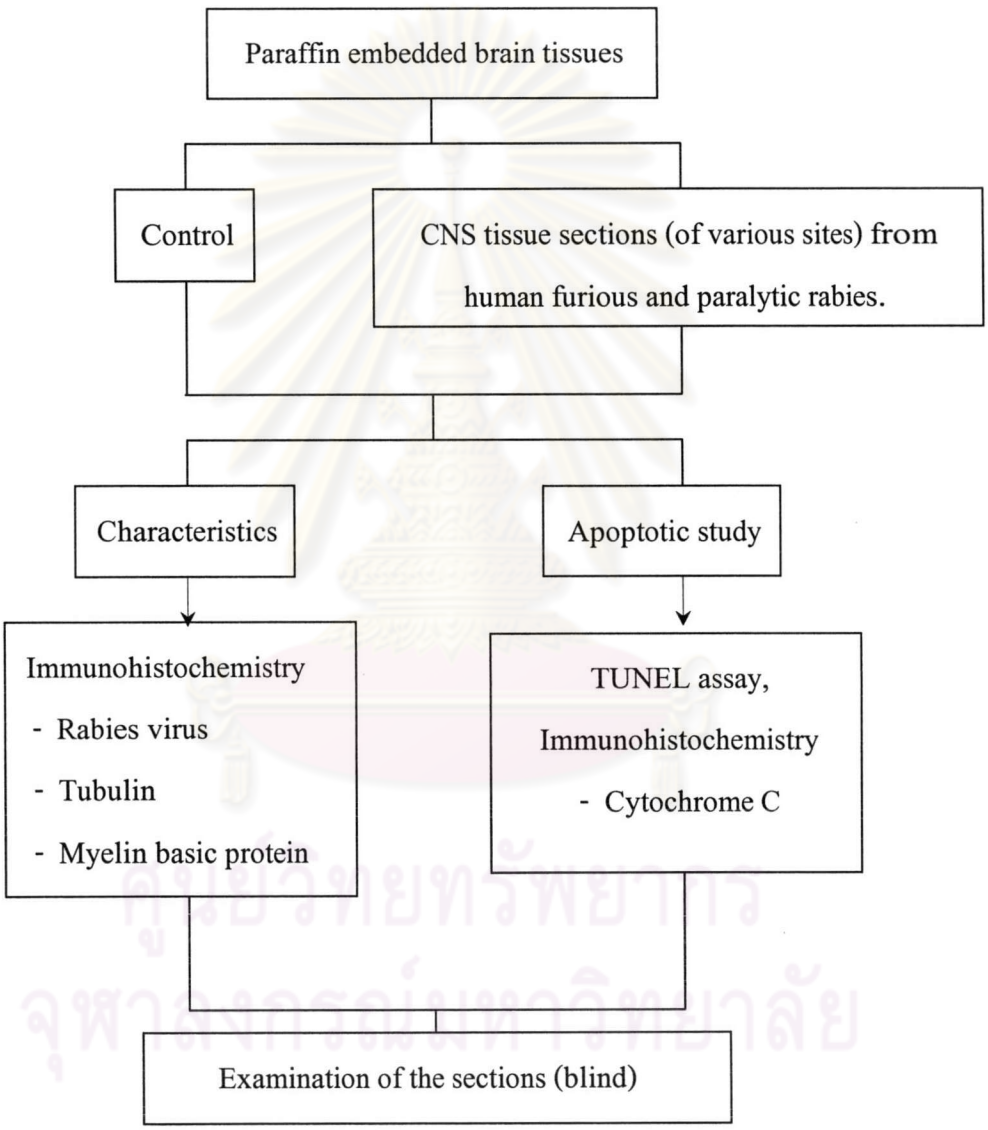

Figure 2 : Conceptual framework of the experimental designs.

1. Samples

Paraffin blocks of formalin-fixed tissue were obtained at autopsy from 7 patients studied who had been admitted to Bumrasanaradura and Chulalongkorn University Hospitals in Bangkok between May 1987 and April 1988. Four presented as encephalitis and the remaining had paralysis. The clinical diagnosis was based on a reliable history of a recent dog bite, and by typical clinical manifestations including aerophobia, hydrophobia and/or inspiratory spasms. The diagnosis was confirmed by DFA brain impression smear and mouse inoculation test.

Post-mortem examinations were performed within 24 h of death. Brain and spinal cord were fixed in formalin for 7 days. Sections of frontal, parietal, occipital, temporal, hippocampus, thalamus, basal ganglia, cerebellum, midbrain, pons, medulla, cervical, thoracic and lumbosacral enlargement were subsequently embedded in paraffin, sectioned and immunostained.(83)

Patient No.	Age (Yrs.), Sex	Time and location of bite	Survival period*	History of rabies immunization
Encephalitis				
H1	9,M	1 month before ; dog bite on right buttock	5	-
H3	22,F	3 months before ; dog bite on right ankle	8	-
H5	55,F	2 months before ; dog bite on right hand, left leg, left breast	4	-
H6	14,M	1 month before ; dog bite on buttock	5	-
Paralysis				
H2	15,F	3 months before ; dog bite on finger	16	-
H4	81,M	2 months before ; dog bite on left calf	7	+
H7	61,M	4 months before ; dog bite on right leg	13	-

* mean days from onset to death

Table 1 : Characteristics of patients with rabies

2. Slides preparation

Three μm-thick paraffin sections of formalin-fixed tissue were mounted on silane–coated slides. (2% 3-aminopropyltriethoxysilane (Sigma, German))

3. Immunoperoxidase staining

Sections were stained by DAKO EnVision™ System kit, HRP (DAKO Corporation, CA, USA) after deparaffinized by xylene and ethanol and then antigen was retrieved by pressure cooker with citrate buffer for 1 min. Briefly, sections were incubated for 10 min with 3%H_2O_2 to eliminate endogenous peroxidase, washed in phosphate-buffered saline (PBS), incubated for 20 min with 3% horse serum to block nonspecific staining, and incubated for 60 min with primary antibody. After two 3-min rinses in PBS, sections were incubated in DAKO EnVision™ System kit, HRP reagent (DAKO Corporation, CA, USA) as secondary antibody for 30 min. Slides were washed with PBS again and incubated for 10 min with peroxidase substrate (diaminobenzidine (DAB) 50 mg/ml and 30%H_2O_2 in Tris-HCl buffer with 1 M Imidazole). After rinsed by tap water, the stain was couterstained with hematoxylin.

Primary antibody	Working dilution	Products from
anti-rabies nucleocapsid polyclonal antibody	1:80	Bio-rad, France
anti-myelin basic protein polyclonal antibody	1:800	Chemicon, USA
anti-beta tubulin monoclonal antibody	1:2000	Sigma, USA
anti-cytochrome c monoclonal antibody	1:4000	Santa Cruz Biotechnology,USA

Table 2 : Primary antibody.

4. Detection of apoptosis

To evaluate whether cell death was due to apoptosis, we used the ApopTag® Plus Peroxidase *In Situ* Apoptosis Kit (Intergen Company, USA) as well as TUNEL assay (Terminal deoxynucleotidyl transferse(TdT) –mediated dUTP-digoxigenin nick end labeling assay) for detection. The kit detects apoptotic cells by peroxidase staining detection of the digoxigenin-labeled 3'-OH DNA ends generated by DNA fragmentation, and typically localized in morphologically identifiable nuclei and apoptotic bodies.

Sections were stained by ApopTag® Peroxidase kit after deparaffinized by xylene and ethanol and then permeabilized cell by proteinase K. Briefly, sections were incubated for 5 min with 3%H_2O_2 to quench endogenous peroxidase and apply equilibration buffer. Sections were then incubated in a humidified chamber at 37°C for 1 hour with working strength TdT enzyme (TdT enzyme mediated digoxigenin-dUTP), put in a couplin jar containing working strength stop/wash buffer, and incubated for 10 min at room temp. Slides were washed with PBS, incubated with anti-digoxigenin peroxidase conjugate in humidified chamber for 30 min at room temp and washed again. Developing color was done with peroxidase substrate (diaminobenzidine (DAB) 50 mg/ml and 30%H_2O_2 in Tris-HCl buffer with 1 M Imidazole). Finally, slides were washed in water and couterstained with hematoxylin.

5. Controls

5.1 Negative control system in rabies infected tissues

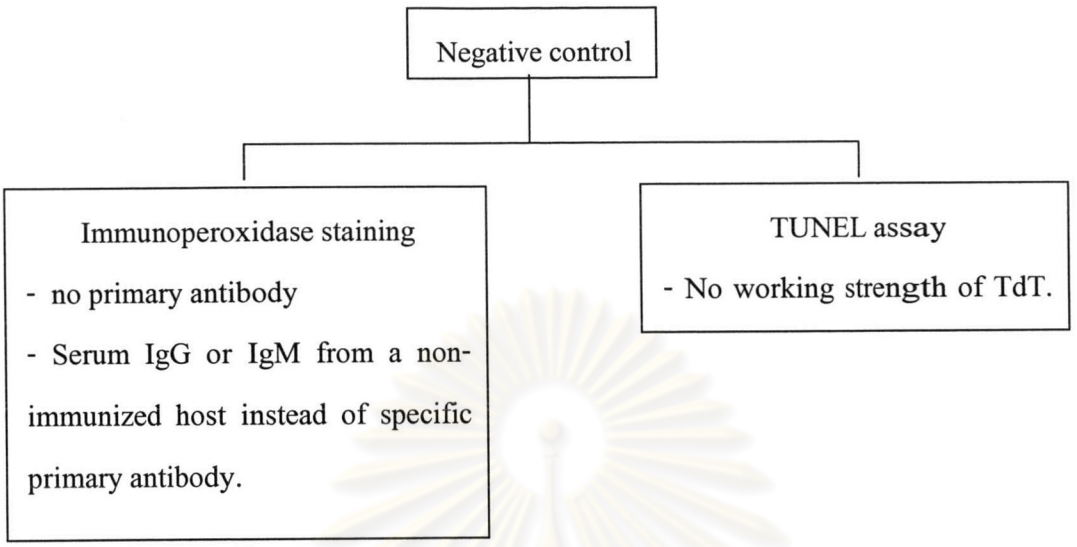

Figure 3: Negative control in experiment.

5.2 Negative control system in non-rabies infected tissues

5.2.1 Immunoperoxidase staining

Rabies virus detection : paraffin embedded brain tissue from a patient who died of lung cancer.

Myelin basic protein detection : same as above.

Beta tubulin detection : same as above

Cytochrome c detection : same as above.

5.2.2 TUNEL assay

Same as above.

6. Quantitation

The number of antigen-positive neurons from IHC and neuronal apoptosis from TUNEL assay in various areas was graded on a 0-4 scale from none to most abundant.(84)

7. Data analysis

Blind assay of all slides from furious and paralytic rabies.