CHAPTER III

MATERIALS AND METHODS

1. Places of Study

1.1 Queen Saovabha Memorial Institute (QSMI), Rabies Diagnostic Unit, Thai Red Cross Society : for observation of clinical symptoms of rabid dogs and for collection of central nervous system (CNS) specimens.

1.2 Bumrasanaradura Communicable Disease Control Hospital : for collection of clinical data from human rabies cases and CNS specimens.

1.3 Immunology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University : for laboratory work-immuno-peroxidase staining.

2. Materials

2.1 Rabid Dogs

Dogs that had history of attacking people with no known provoked stimuli or those exhibited abnormal behavior were kept at quarantine unit at QSMI. Clinical signs were observed twice daily by two experienced veterinarians. They were sacrificed by curare as soon as the first definite rabies signs developed. Brain and spinal cord were removed promptly and then

kept in 10% formalin for 7 days, except for some portions of hippocampus, cerebellum and cervical enlargement which were saved for Seller's stain, FAT staining and mouse inoculation test (MIT). Sections of the following regions: frontal, parietal, occipital, temporal, hippocampus, thalamus, basal ganglion, cerebellum, midbrain, pons, medulla, cervical, thoracic, and lumbosacral enlargements were subsequently embedded in paraffin, sectioned and immunostained.

A total of 6 dogs, three in each clinical form namely encephalitic (furious) and paralytic (dumb), were studied.

2.2 Rabies Patients

The 7 patients in this report were admitted to Bumrasanaradura and Chulalongkorn University Hospital between May 1987 to April 1988. Four presented as encephalitis and the remaining had paralysis or a Guillain-Barre´ like syndrome. The typical clinical manifestations including aerophobia, hydrophobia and/or inspiratory spasms.

Postmortem examinations were performed within 24 hours of death. Brain and spinal cord, except some portions of hippocampus, cerebellum and cervical enlargement which were saved for Seller's stain, FAT staining and MIT, were fixed in 10% formalin for 7 days. Sections of the following regions: frontal, parietal, occipital, temporal, hippocampus, thalamus, basal ganglion, cerebellum, midbrain, pons, medulla, cervical, thoracic and lumbosacral enlargements were subsequently embedded in

paraffin, sectioned and immunostained.

3. Preparation of Paraffin - Sectioned Slides

3.1 Tissue Processing and Embedding

After fixation, tissue specimens were dehydrated, cleared and embedded according to routine processing procedured. Briefly, the cassettes containing tissues were immersed in Sorenson's buffer and processed on the Autotechnicon machine by the following schedule :

- 70% ethyl alcohol 1 hour x 2 times
- 95% ethyl alcohol 1 hour x 2 times
- 100% ethyl alcohol 1 hour x 3 times
- Xylene 1 hour x 2 times
- Paraffin at 59°C 1 hour x 3 times

The tissues were subsequently removed from paraffin and proceeded to embedding in melting paraffin about 30 minutes at 59°C, and refrigerated to cool.

3.2 Preparation of the Tissue-Sectioned Slides

The paraffin - embedded blocks were sectioned, by 6-8 microns thick, and placed on glass slides precoated with 0.3% gelatin (type I, approx.300 Bloom, Sigma Chemicals Co., St. Louis, MO,USA.) These slides were then incubated at 60°C for 1 hour and kept at room temperature till the time of immunostaining.

4. Preparation of Rabbit Anti-Rabies Serum (154)

Two rabbits, weighing 2-3 kgs., were immunized with rabies viral antigen in Vero cell vaccine (IMOVAX RABIES VERO "Merieux" : inactivated rabies vaccine prepared by culture Wistar rabies PM/W1 38-1503-3M strain on Vero continuous cell lines and inactivated with beta-propiolactone). Briefly described, rabies antigen was mixed with complete Freund's adjuvant (CFA) in the ratio of 1:1 and injected to the rabbits subcutaneously at 4 sites, 0.2 ml each. After 7 days, the rabbits were boosted with the same protocol. Rabbits were bled one week after the last injection and sera were assayed for neutralizing activity by Rapid Fluorescent Focus Inhibition test (RFFIT) (15). The booster was repeated once since the antibody level was still undesirable.

The antisera were aliquoted and stored at -20°C until use.

5. Rabbit Anti-Rabies Immunoglobulin G Preparation

Immunoglobulin G (IgG) was extracted from hyperimmune sera according to method previously described (155). Crude protein mixtures were obtained by precipitation of the hyperimmune sera with 33% saturated ammonium sulphate $\{(NH_4)_2SO_4\}$,pH 7.3, in cold temperature by stirring overnight. The precipitate was dissolved in 5 ml of 0.9% sodium chloride (NaCl) solution, and dialysed against several changes of 0.01 M and 0.02 M phosphate-buffered saline (PBS), pH 7.4 at 4°C. The

dialysated sample was then purified for IgG by affinity chromatography on Protein A-Sepharose CL-4B Column (Pharmacia Fine Chemicals, Uppsala, Sweden).

The Protein A-Sepharose CL-4B beads was soaked in excess volume (8-10 ml) of PBS, pH 7.4 and rehydrated for 2 hours at 4°C. The beads was centrifuged at 2000 rpm, 5 minutes and resuspended in PBS, pH 7.4, containing 0.02 % sodium azide. All further steps were carried out at 4°C. Five millilitres of swollen Sepharose beads were packed into a column of 13 mm diameter, and equilibrated with 20 ml of 0.05 M Tris in 0.15 M NaCl, pH 8.6. The flow rate of which was adjusted to 50 ml/hour. The dialysed hyperimmune rabbit serum 5 ml was then slowly applied as soon as fluid ran almost to the top of the column. As soon as added serum front reached the bottom of column, the Sepharose was washed with about four bed volumes of 0.05 M Tris in 0.15 M NaCl, pH 8.6. The collected fluid (1.5 ml in each tube) was measured at 280 nm by a spectrophotometer (Beckman ACTA III UV-visible spectrophotometer, USA). First peak will be albumin followed by IgG which will be eluted by different buffer i.e. 0.05 M acetate in 0.15 M NaCl,pH 4.3. The second peak eluents were pooled and dialysed against 0.05 M PBS, pH 7.4, containing 0.02% sodium azide at 4°C overnight with three changes of buffer. The protein solution was concentrated by polyethyleneglycol to the original volume (5 ml).

The purity of rabbit anti-rabies IgG was tested by immunoelectrophoresis. Neutralizing antibody activity to rabies

was measured by RFFIT and the protein content was determined by Lowry method (156). The purified antisera were aliquoted and stored at -20°C.

6. Reconstitution of Commercial Equine Anti-Rabies Globulin (BBL)

The commercial anti-rabies globulin was determined for the optimal dilution and sensitivity of the immunoperoxidase staining, simultaneously with the self-prepared rabbit anti-rabies IgG.

The reagent was an immune globulin of equine origin labeled with fluorescein isothiocyanate which was routinely used for FA test (purchased from BBL, Becton Dickinson and Co., Cockeysville, MD, USA). The lyophilized contents of the vial was reconstituted with 1 ml sterile distilled water, aliquoted and stored at -20°C.

7. Methods of Immunoperoxidase Staining Using Avidin-Biotin-Peroxidase Complex (ABC) Technique

Rabies viral antigen in brain and spinal cord sections was detected by immunoperoxidase staining, avidin-biotin -peroxidase complex technique as described by Hsu and co-workers (153) and Johnson et al. (157) with some minor modifications.

7.1 Incubated the paraffin-sectioned slides at 60°C for 10 minutes.

7.2 Deparaffinized by immersion in xylene 2 times, two and one minute respectively.

7.3 Rehydrated briefly by immersion in 100%, 95% and 70% ethyl alcohol, twice in graded alcohol, each for one minute.

7.4 Washed twice with PBS, pH 7.4, at room temperature and once at 37°C, for 5 minutes each time.

7.5 Trypsinized by immersion of tissue slides in warm trypsin solution of appropriate concentration in PBS,pH 7.4, adjust pH to 7.8 with 0.1 N sodium hydroxide. Incubated at 37°C for appropriate interval.

The following steps (from 7.6-7.18) were carried out at room temperature.

7.6 Rinsed sections very gently by using PBS, pH 7.4, for 5 minutes, two times.

7.7 Blocked non-specific reaction by applying on to the sections with 2% normal goat serum in PBS, pH 7.4, for 20 minutes in a humidity chamber, rinse once in PBS, pH 7.4 for 1 minute.

7.8 Incubated with 100 µl of primary antibody (rabbit anti-rabies IgG and equine anti-rabies globulin-BBL at the optimal dilution in 2% normal goat serum in PBS,pH 7.4, left in a humidity chamber for 45-60 minutes, and gently rinsed with PBS, pH 7.4 for 1 minute and placed sections in two buffer baths, each for 5 minutes.

7.9 Incubated with 100 µl of secondary antibody [biotinylated goat anti-rabbit IgG (Sigma Chemicals Co., St.Louis, MO) for rabbit anti-rabies IgG, and biotinylated goat anti-horse IgG (Vector Co., Burlingame, CA) for equine anti-rabies globulin -BBL] at the optimal dilution in 2% normal goat serum in PBS, pH 7.4, in a humidity chamber for 30 minutes, then washing as described above (7.8).

7.10 Blocked the endogenous peroxidase activity by 1% hydrogen peroxide in methanol for 30 minutes, rinse in PBS, pH 7.4, two-5 minute times.

7.11 Applied the preformed avidin-biotinylated horseradish peroxidase complex (ABC) (Dakopatt, France) on the sections, incubated for 30 minutes, washed as step 7.8. (The ABC was made up more than 30 minutes before use by mixing 1% "A" and 1% "B" in PBS, pH 7.4.)

7.12 Substrate reaction:incubated slides with freshly prepared substrate, 3-3′-diaminobenzidine (DAB) 0.5 mg/ml and 0.1% hydrogen peroxide in PBS, pH 7.4, for 1-3 minutes or until the color become light brown. Drained off DAB into diaper and rinsed immediately in distilled water two times, 2 minutes each.

7.13 Darkened the sections by soaking in 0.5% copper sulphate in 0.15 M NaCl for 5 minutes and rinsed in distilled water for 5 minutes.

7.14 Counterstained with Gill's hematoxylin for 1-2 minutes, rinsed in distilled water.

7.15 Placed in tap water substitute (constituent of sodium bicarbonate and magnesium sulphate) for 2-3 minutes then rinsed in distilled water.

7.16 Dehydrated the sections twice in graded ethyl alcohol (70%, 95% and 100%) 30 seconds to 1 minute each.

7.17 Cleared in xylene for 1 minute.

7.18 Mounted the sections in permount and covered with cover glasses.

8. Determination of Factors Affecting the Immunoperoxidase Staining, ABC Technique

To achieve meaningful results in obtaining the greatest intensity of specific staining with the least amount of back ground interference, avoiding false negative and false positive, the staining technique should be tested in different conditions as followings :

8.1 Affecting on Antibodies

8.1.1 Checkerboard titration to determine the optimal dilution of primary antibodies prepared from rabbit by using rabies vaccine and commercial product of equine anti-rabies antibodies (BBL) and secondary antibody

The primary antibodies : rabbit anti-rabies IgG was diluted 1:10, 1:20, 1:40, 1:80, 1:100 and 1:120, and equine anti-rabies globulin (BBL) was diluted 1:40, 1:80, 1:100, 1:200,

1:300, 1:500 in diluent and 100 µl of each dilution was applied onto the brain sections previously trypsinized and proved to contain rabies viral antigen by FAT and MIT. Further steps were the same as previously mentioned in protocol. Tissue slides with different concentrations of primary antibody of each type (home-made vs commercial) were incubated with varying concentration of each secondary antibodies at 1:100, 1:200 and 1:200, 1:300 in 2% normal goat serum.

8.1.2 Choice of diluent for primary antibodies

Both primary antibodies were diluted as 8.1.1 in two different diluents : fresh PBS, pH 7.4 and 2% normal goat serum in fresh PBS, pH 7.4.

8.1.3 Time course for incubating with primary antibody

Incubation time for primary antibody varied from 45 minutes, 1 hour and 2 hours in a humidity chamber, at room temperature.

8.2 Trypsinization for Digesting the Aldehyde Linkages

8.2.1 Determination of optimal concentration and pH of trypsin

After deparaffinized the tissue sections, immerse the slides in the trypsin containing PBS bath. The concentrations of trypsin used were 0.02%, 0.05%, 0.1% and 0.15 % (0.2, 0.5, 1.0 and 1.5 g/litre) in fresh PBS, pH 7.4.

Trypsin concentrations were adjusted to pH 7.8 with 0.1 N sodium hydroxide.

8.2.2 Temperature and time course for trypsinization

To determine the optimal temperature and time, the digesting procedures were carried out in room temperature and 37°C for a period varying between 5 and 10 minutes (5,6,7,8 and 10 minutes).

8.3 Inhibition of Nonspecific Background Staining and Endogenous Peroxidase Activity

To better acheive the least background activity, each of the following steps was tried in addition to that already used routinely.

8.3.1 Incubated the sections in chilled acetone bath for 10 minutes before trypsinization.

8.3.2 Placed the sections in 0.3% hydrogen peroxide in PBS, pH 7.4, at 37°C for 5 minutes before trypsinization.

9. Specificity of Reaction in Immunoperoxidase staining, ABC Technique

To characterize the specificity of immunoperoxidase staining to rabies antigen.

The sections of brain and spinal cord of the rabies patient at some regions proved to contain rabies antigen by FAT

and MIT : frontal, occipital, cerebellum, medulla and cervical enlargement were incubated with 100 µl of anti-Herpes simplex virus type II (Dakopatt, France) at dilution of 1:20, 1:40 and 1:80 in 2% normal goat serum and normal rabbit serum diluted at 1:100, 1:200 and 1:500 in PBS, pH 7.4 for 45 minutes. A normal human brain section also served as a control.

Further, sections of brain of a patient died from other disease than rabies i.e. Acquired Immunodeficiency Syndrome (AIDS), that caused by HIV-I, also served as negative control by using rabbit and equine anti-rabies IgG of 1:80 and 1:300 dilution in 2% normal goat serum, respectively.

10. Sensitivity of Immunoperoxidase Staining, ABC Technique

The use of both of primary antibody (homemade vs commercial) in staining for rabies antigen in some regions of human and dog brain were compared with routine analysis i.e. Seller's stain, FAT and MIT by using the same specimens. The results were analysed for sensitivity of the tests and both primary antibodies.

11. Methods of Quantitation

Sections were examined under high power objective (40x) by light microscope. The antigen-positive cells contained either dark brown granules or diffuse staining reaction in the cytoplasm.

Number of antigen-positive cells in various areas was graded as 0 to 4 scale from none to most abundance. Inflammatory

cellular reactions at perivascular cuffs and parenchymal tissue were also quantitated in the same manner.

12. Analysis of Data

The sensitivity were calculated according to the method described by Mausner & Bahn (158), using the following formula :

$$\text{Percentage sensitivity} = \frac{\text{No. of true positives}}{\text{total No.of true positives and false negatives.}} \times 100$$

The following definitions were used in this calculation :

true positive = specimen that was positive for rabies antigen by immunoperoxidase-ABC staining and MIT

false negative = specimen that was positive for rabies antigen by MIT, but gave a negative immunoperoxidase-ABC staining