Chapter III

Materials and Methods

1. Materials

1.-1. Dogs

Brains,saliva and parotid glands were collected from 40 dogs submitted to the Animal Rabies Diagnostic Unit of the Queen Saovabha Memorial Institute, Bangkok according to the following criteria :-

a. 20 with positive fluorescent antibody test (FAT) for rabies virus and positive mouse inculation test (MIT) using brain as the testing material (see the following Materials and Methods)

b. 20 with negative FAT and MIT

These dogs were submitted for rabies diagnosis because of the history of biting man. They might either die shortly before arrival or were well preserved with ice during their transportation and no signs of putrifaction were observed.

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Another 10 dogs quarantined for rabies at the same institute were sequentially examined for rabies virus excretion in the saliva. They were examined on the other day (three times a week) beginning from the first day of quarantine until death or until the animal were released from quarantine.

1-2. Collection of Saliva ,Parotid Glands and Brains from the Deceased Dogs

1-2-1. <u>Saliva</u>

Cotton swabs to be used for saliva collection were first immersed in 2 ml phosphate-buffer saline (PBS) pH 7.4 and the excess PBS was expressed against the wall of the tube. The swab was taken from the anterior and posterior aspects of the tongue as well as from the cheek mucosa. The swab was then placed back into the PBS tube and the excess fluid was expressed before discarding the cotton swab. The saliva was centrifuged at 3,000 rpm for 30 min at 10 C to get rid of the particulate materials and the supernatant was aliquoted and kept at -20 C.

1-2-2. <u>Parotid Glands</u>

Both parotid glands were collected by a skin flap incision over the parotid areas from each animal. A portion of the gland was submitted for FAT and the rest of the gland was homogenized in 5 ml PBS with a tissue grinder (Chatas Glass Co., Vineland, NJ). The homogenate was centrifuged at 3,000 rpm for 30 min at 10 C to eliminate cell debris and the supernate was aliquoted and kept at -20 C.

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1-2-3. <u>Brains</u>

Brain was exposed with a bone saw. Portions of cerebellum and hippocampus from both sides were examined by FAT whereas only a portion of hippocampus was examined by Sellers' stain (see the following Materials and Methods). The remaining portion of hippocampus was homogenized as described for parotid glands.

1-2-4. Saliva Collection from live dogs

Live animals were injected intramuscularly with 3 mg/kg of Xylazine hydrochloride (Rompun, 20 mg/ml, Bayer Vetchem, Korea) as an anesthetic agent. Ten to fifteen minutes later, saliva was collected by the method described above. Saliva samples were centrifuged at 3,000 rpm for 30 min at 10 C and a portion of the fresh supernatant fluid was used for MIT. The remaining saliva was frozen at -20 C for dot-immunoblot assay.

2. Sellers' stain for Negri body (86).

Part of hippocampus was smeared on slide and stained with Sellers' stain for 1 to 5 seconds. After rinsing with running tap water and drying at room temperature, the slide was examined under oil immersion lens. Negri bodies ground substance takes the fuchsin stain and appear magenta red or cherry red. The diameter is ordinary more than 10 um and the shape is oval, spherical or elongated. One neuron may contain several inclusion bodies of variable sizes. To be specific for rabies,Negri bodies must contain blue staining granules or inner bodies with a diameter of 0.2-0.5 um. (Figure 2)

3. FAT (Direct immunofluorescent antibody test) (92).

Brains and partially excised parotid glands from the dead animals were compressed onto slides.All slides were fixed in cold (4-10 C) acetone for 30 min. at 4 C. The slide was flushed with fluorescein-labelled equine rabies immunoglobulin (ERIG,BBL,Becton Dickinson,MD) and incubated in a humid chamber at 37 C for 30 min. The slides were then washed twice in PBS and rinsed finally

with distilled water before mounting with phosphate buffer glycerol (pH 8.5). The finished slides were examined under a fluorescent microscope. The presence of rabies antigen could be visualized as bright greenish intracytoplasmic inclusion body. They might be fine granular (<1 um) or masses of variable sizes (2-10 um) and shapes (oval,round or thread) (Figure 3)

rabies antigen in salivary gland tissue usually appeared as smaller and more irregular masses than those observed in brain tissue. The so called "dust-like appearance" was commonly found.

4. Mouse Inoculation Test (MIT)

Supernatant fluids of brains ,parotid glands and saliva after centrifugation were examined for the presence of live virus by MIT, according to the method of Webster (101). Swiss Albino mice of 21-31 days of age (9-15 gm body weight) of either sex were used. Antibiotics (2 mg Streptomycin and 500 IU Penicillin/ml) were added to the specimens prior to inoculation. A group of 3-5 mice were inoculated intracerebrally with 0.03 ml material using tuberculin syringe and 26 or 27 gauge needles, 1/4 to 3/8 inch long. The mice were observed for signs of rabies and any deaths before the fifth day was regard as nonspecific ,i.e, either from microbial contamination or trauma during inoculation. Signs of rabies and death normally occured from 7 to 20 days after inoculation but might be as long as 28 days. Beyond 28 days, the MIT was considered negative. Rabies antigen in the brain of the dead mouse had to be confirmed by Sellers' stain and FAT.

5. Dot-Immunoblot Assay

Principle of the test is the detection rabies antigen in the test specimens bound on nitrocellulose membrane by anti-rabies immunoglobulin and with the use of avidin-biotin peroxidase conjugated secondary antibody to amplify the reaction.

5-1. <u>Test specimens</u>

The supernatant fluids of brain, parotid gland and saliva were examined in a blind fashion, i.e., without the knowledge of the results of other rabies tests. Three types of rabies vaccine were used as positive controls., namely, purified Vero cell rabies vaccine (PVRV, IMOVAX Rabies Vero, Institute Merieux, Lyon, France), purified chick embryo cell rabies vaccine (PCEC, Behring, Germany) and BHK-21 cell grown animal rabies vaccine (Rabdomun, Coopers, West Germany). Negative controls consisted of purified hepatitis B surface antigens (a gift of the Central Laboratory of Blood Transfusion Services of the Netherland), herpes simplex type 2 virus (HSV) grown in Hela cell (prepared in the Virology laboratory, Chulalongkorn University School of Medicine) and supernatant fluids from harvested uninfected Vero cell.

5-2. <u>Nitrocellulose paper</u>

The nitrocellulose paper (BA 85/21 SD pore size 0.45 um, Schleicher & Schull, West Germany) was cut into 0.6 cm disc with a paper hole puncher and placed in a 96 well microtiter plate (one disc per well). To avoid the unnecessary staining of the nitrocellulose paper with exogenous proteins and grease handling with gloves and forceps was required.

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5-3. Blocking agent

Before proceeding with the immune reaction, it was necessary to block nonspecific binding of the agents to the unoccupied binding site of the nitrocellulose filters. There was 2 aspect to the blocking step : it was essential to reduce nonspecific binding and it may promote renaturation of antigenic sites. To choosen the appropriate blocking agent, several types of the blocking agent were tested.

5-3-1. 5% BLOTTO (Bovine Lacto Transfer Technique Optimizer) composed of 5% suspension of skim milk (slim, Thailand) in PBS-0.1% Tween 20 (0.1% PBS-T). The suspension was filtered through a filter paper (Wattman No.2) and had to be prepared freshly before each use.

5-3-2. BSA (Bovine Serum Albumin, Faction V, SIGMA Chemical Co.,St.Louis,MO) Various concentration of BSA (1%, 3%, 5%, 7%, 10%) were tested for blocking activity.

5-3-3. 0.1% tween 20 in PBS pH 7.4

5-4. Anti-rabies antibody (The primany antibody)

Two types of antirabies antibody were studied, namely, purified rabbit antirabies IgG (self-prepared, see following methods and materials) and the commercial equine antirabies immunoglobulin (ERIG) fluorescein - labelled (BBL, MD).

5-4-1. Preparation of rabbit anti-rabies immunoglobulin (RRIG)(102)

One rabbit, 2-3 kgs body weight, was immunized

with purified Vero cell rabies vaccine (PVRV) in complete Freund's adjuvant (CFA). The rabbit was injected with 1 human dose of PVRV (0.5 ml) mixed with equal volume of CFA weekly x3. Serum was collected one week after the last injection and the IgG was purified by affinity chromatography on Protein A-Sepharose CL-4B column

Protein A - sepharose CL-4B beads (Pharmacia Fine Chemicals, Uppsala, Sweden) were resuspended in 8-10 ml PBS and rehydrated for 2 h at 4 C. After centrifuge at 2,000 rpm for 5 min, the beads were resuspended in PBS containing 0.02% sodium azide. All of following steps were to be 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 at the flow rate of 50 ml/hr. The hyperimmune serum was gently applied as soon as the fluid run almost to the top of the column. The sepharose was washed with four volume column size of 0.05 M Tris in 0.15 M NaCl, pH 8.6. The collected fluid (2 ml in each tube) was measured at 280 nm by a spectophotometer (Beckman ACTA III lv-visible spectophotometer, USA). Albumin was the first coming off and followed by IgG which was eluted with 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 purity of rabbit-antirabies IgG was tested by immunoelectrophoresis (IEP) and the protein content was determined by Lowry method. Neutralizing antibody activity to rabies was measured by RFFIT. The purified IgG were aliquoted and stored at -20 C.

5-5. Secondary antibody

The types of secondary antibody varied according to the sources of antirabies antibody. Biotin conjugated rabbit anti-horse IgG (SIGMA) and biotin conjugated goat anti-rabbit IgG (SIGMA) were used for ERIG-BBL and purified rabbit anti-rabies IgG, respectively.

5-6. Avidin and biotin conjugated horseradish peroxidase reagents (ABC, DAKOPATTS, DENMARK)

The system of ABC for amplification the reaction was widely used to increase the sensitivity by attribute to a large complex formation containing multiple peroxidase molecules. High concentration of ABC may causing troblesome nonspecific background which was reduced by the use of appropriate dilution.

Equal volumes of avidin and biotin conjugated horseradish peroxidase were prepared in PBS for 30 min before use in appropriate dilutions.

5-7 Substrate solution

4-chloro-1-naphthol (SIGMA) was dissolved in anhydrous methanol as stock solution (see Appendix). Immediately before use, 5 ml of PBS and 1 ul of 30% H_2O_2 were added to 1 ml of stock solution.

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6. Test Procedure

6.1) 5 ul of test specimens was dotted on nitrocellulose paper discs and allowed to air dried.

6.2) The dried filters were incubated with 100 ul BLOTTO for 30 min at 37 C to saturate the protein-binding sites.

6.3) The solution was removed by aspiration with a needle connected to a suction line and replaced with 100 ul of primary antibody.

6.4) After 1 hr incubation at 37 C, the solution was removed by suction. The test filters were washed three times with 0.1 % PBS-T under agitation on a shaker for 5 min each at room temperature, and then allowed to react for 1 hr at 37 C with 100 ul of secondary antibody.

6.5) After another cycle of aspiration and washing, 100 ul of ABC was added and the filters were further incubated for 45 min at 37 C.

6.6) After three more washings, the filters were incubated for 30-45 min with substrate solution (see Appendix) before stopped reaction by washing the discs with distilled water and allowed them to dry.

6.7) The positive test appeared as blue dot on the disc by visual inspection

7. Determination of factor affecting the dot - immmunoblot assay

7.1 Affecting on antibodies

7-1-1 Checkerboard titration to determined the optimal concentration of primary antibodies prepared rabbit and commercial product of equine antirabies antibody (BBL) and secondary antibody.

The primary antibody :rabbit antirabies IgG was diluted 1:50, 1:100, 1:150, 1:200 and equine antirabies immunoglubulin (BBL) was diluted 1:100, 1:200, 1:300, 1:400. The

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diluted antibody was applied on the nitrocellulose filter previously dotted with PVRV. Further steps was follow in protocol as mention above. The filter with different dilution of primary antibody of each type were incubated with varying dilution of each secondary antibody at 1:100, 1:200, 1:300 and 1:400.

7-1-2 Choice of diluent for antibodies

All antibodies were diluted in different diluent: 0.1%PBS-T alone, 0.1%PBS-T plus 2% goat serum (GS) and 5% chicken serum, 0.1% PBS-T plus 2% GS 5%CS and 0.002% normal human serum(NHS)

7-1-3 Incubation time of the antibodies

The incubation time of the primary and secondary varied from 30 min., 45 min, 1 hour and 2 hour in 37 C.

8. Specificity and sensitivity of RRIG and ERIG by dot-immunoblot

Both primary antibody was determined for specificity and sensitivity by using several types of rabies vaccine ,i.e., PVRV, PCEC, Rabdomun, and non-rabies vaccine (HBs Ag, HSV-2, Vero cell, Hela cell) 0.1% PBS - T also served as negative control. Further steps were the same as previously mentioned in protocol.

9. Methods of quantitation

The test filters were examined by visual inspection. The positive reaction demonstrated by a blue dot,absence the color defined as negative. The intensity of the color was graded as 0 to 4 scale from non to abandance.

10. Analysis of Data

The sensitivity and specificity were evaluated according to the method described by Mausner & Bahn (105),using the following formular :-

$$\text{percentage sensitivity} = \frac{\text{no of true positive}}{\text{total no of true positive and false negative}} \times 100$$

$$\text{percentage specificity} = \frac{\text{no of true negative}}{\text{total no of true negative and false positive}} \times 100$$

The following definitions were used in these calculation:

true-positive = specimen that was positive for rabies antigen by FAT and MIT and positive immunoblot.

true-negative = specimen that were negative for rabies antigen by FAT and MIT and negative immunoblot.

false-positive = specimen that were negative for rabies antigen by FAT and MIT but positive immunoblot.

false-negative = specimen that was positive for rabies antigen by FAT and MIT but nagative immunoblot.

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