CHAPTER IV

RESULTS

1. Immunoperoxidase Staining for Detection of Rabies Viral Antigen in Tissues

The immunoperoxidase staining for detection of viral antigen by using avidin-biotin-peroxidase complex was modified from methods previously described by Hsu and co-workers (153) and Johnson et al.(157).

1.1 Preparation and isolation of rabbit anti-rabies IgG

Sera obtained from 2 rabbits immunized with rabies antigen and CFA were assayed for neutralizing antibody on day 7 after first booster. Levels of neutralizing antibodies as determined by RFFIT in both rabbits were 5.4 and 6.0 IU/ml. After the second booster, antibody levels rose to 7.95 and 8.5 IU/ml respectively.

The IgG fraction was isolated from the pooled hyperimmune sera from both rabbits by salt precipitation and affinity chromatography using Protein A-Sepharose CL-4B. Two peaks of protein were obtained when measured at 280 nm (Figure3). The majority of serum proteins other than IgG passed through the column (peak 1), since protein A has the capacity to bind specifically and with high affinity to the IgG of

most species (159,160). The bound IgG was subsequently eluted with acid and recovered mostly in peak 2 (fraction No.25-28). Purity check was made by immunoelectrophoresis with swine anti-rabbit serum as well as goat anti-rabbit IgG (Figure 4A,B). The protein concentration of purified anti-rabies IgG was 5.4 mg/ml according to Lowry method and neutralizing activity was 5.99 IU/ml. IgG from Peak 2 was used in the subsequent tests and the optimal dilution for staining was determined by checkerboard titration.

1.2 Determination of factors affecting the immunoperoxidase staining

Various concentrations of rabbit anti-rabies IgG (1:10, 1:20, 1:40, 1:80, 1:100 and 1:120 dilution) were titrated against 2 different concentrations of 2 lots of biotinylated goat anti-rabbit IgG (1:100 and 1:200 dilutions) as shown in Table 1A, dilutions of 1:80 and 1:100 of primary antibody (1°Ab) and 1:200 of secondary antibody (2°Ab) of lot I resulted in a greater intensity of cytoplasmic staining of Purkinje cells and gave a clearer background when used to stain the cerebellum section of Patient 5. 1:100, 1:120 dilutions of 1°Ab also gave good results with 1:100 dilution of 2°Ab of lot II on medulla of Dog 6. A greater dilution of the 1°Ab resulted in weaker staining (Table 1 A). As compared to 1°Ab of rabbit anti-rabies IgG the equine anti-rabies globulin-BBL at 1:300 or 1:500 dilution gave a more sensitive result on both sections. Secondary antibody used was 1:300 dilution (Table 1 B). Equine anti-rabies

globulin-BBL was thus chosen for further study.

Other parameters explored were diluent for 1°Ab and period of incubation. It was found that diluting of 1°Ab in 2% normal goat serum in fresh PBS, pH 7.4 and an incubation time of 45 minutes at room temperature were most satisfactory. A very weak or false negative staining was shown when PBS, pH 7.4 alone was used as diluent. When the incubation time was increased,e.g. up to 2 hours, the intensity of staining was not improved.

The optimal concentration of trypsin that was used for digesting the aldehyde linkages after formalin fixation was 0.1% (Table 2). A 0.05% concentration was inadequate to improve staining intensity, and concentrations of trypsin of 0.15% and over caused sections to drop off the glass slides.

The duration of trypsin treatment that resulted in highest signal and low background staining was 7-8 minutes, at 37°C, when stained on the parietal and cerebellum sections of Patient 5 (Table 3).

Inhibition of nonspecific background staining and endogenous peroxidase activity by incubating the sections either in chilled acetone or 0.3% H_2O_2 before trypsinization was also tried but results were not superior than those using methanol and H_2O_2 after trypsin treatment.

2. Specificity of Reaction in Immunoperoxidase staining, ABC Technique

Control studies included using anti-Herpes simplex virus type II as 1°Ab or with normal rabbit serum instead of 1°Ab. Sections of normal brain and brain of AIDS patient also served as controls. There was no staining reactions on these control sections, thus confirming the specificity of the staining system.

3. Sensitivity of Immunoperoxidase Staining, ABC Technique

Seller's stain, FAT and MIT were also performed on various regions of the brains (were kindly done by Dr.W. Tepsumethanon and Dr. C. Polsuwan, QSMI). Table 4 demonstrated the comparative results between conventional methods and IP staining. Seller's stain for Negri bodies was positive in 6 of the 15; a 40% sensitivity. FAT and MIT had a sensitivity of 100% (15 out of 15). The IP staining showed a sensitivity of 63.6% (7 of 11). using rabbit anti-rabies IgG, and 86.7% (13 of 15) when equine anti-rabies Ig-BBL was used.

Seller's stain was the least sensitive method with IP staining being the intermediate as compared to FAT and MIT. Equine anti-rabies globulin-BBL gave a higher sensitivity than rabbit anti-rabies IgG (86.7% compared to 63.6%).

Based on the above mentioned results, equine anti-rabies globulin-BBL was chosen for this study due to its higher sensitivity, specificity and satisfactory pattern of antigen staining.

4. Regional Distribution of CNS Infection and Inflammation in Infected Dogs

4.1 Characteristics of dogs

Details of clinical features were shown in Table 5. The dogs were those brought to the QSMI as suspected rabid. Once the symptoms compatible with rabies developed, the dogs were sacrificed and CNS removed for examination.

4.2 Regional distribution of CNS infection and inflammation

Antigen-containing neurons were present in almost all regions of the brain and spinal cord of the rabid dogs (Table 6). Generally, the greatest number of antigen-positive neurons was found in the brain stem and spinal cord rather than neurons of the cerebral cortex. Moderate number of antigen- containing neurons in supratentorial structures was found only in dog number 4 that came with dumb presentation. Inflammatory reaction consisted of perivascular and, occasionally, parenchymal infiltrations with mononuclear cells.

For simplificity, this data were condensed in Table 7. This clearly showed the preponderance of rabies viral antigen in the brain stem and spinal cord compared to the cerebral cortex, in the early stage of rabies infection in both clinical forms of the diseass. However in dog number 4 that survived to 5 days after the first appearance of clinical signs, viral antigen was also demonstrated in supratentorial structure.

5. Regional Distribution of CNS Infection and Inflammation in Human Patients

5.1 Characteristices of human patients

As summarized in Table 8, of 7 patients with rabies, 4 had classical manifestations with alternating intervals of confusion and lucid calm, aerophobia and hydrophobia, and signs of autonomic disturbances such as generalized piloerection. Rapidly progressive course to coma and death was observed in all these patients. The remaining 3 cases had pure motor weakness of all limbs, as an early presentation, with predominant involvement of proximal muscles, which subsequently progressed to flaccid quadriplegia. Aerophobia and hydrophobia were absent but inspiratory spasms were observed during the late stage. Only one patient from the paralytic group received a complete course of brain tissue derived (Semple) vaccine. MIT confirmed the presence of rabies virus in the brain of all patients.

5.2 Regional distribution of CNS infection and inflammation

Rabies viral antigen was detected in almost all regions assessed from patients with rabies (Table 9 and 10). Although the overall regional distribution of antigen was roughly similar, the number, location and morphologic characteristics of antigen-positive cells varied. Antigen-containing neurons were found throughout the whole neuraxis. The number of antigen-positive neurons located in supratentorial, infratentorial

structures as well as in spinal cord were comparable in 2 of 3 patients with paralysis (Patients 5 and 7) and in only one patient from the encephalitic group (Patient 2). The remaining (Patients 1,3,4,6) had greater involvement of neurons in brain stem and spinal cord. Antigen-positive neurons found in cerebral cortex were usually located in all layers of the gray matter. Involvement of pyramidal neurons of the hippocampus was not greater than that of the other regions irrespective of clinical manifestations. This was also true in the case of Purkinje neurons and dentate nuclei in the cerebellum. Antigen was detected in dorsal and ventral horn neurons of the spinal cord in both clinical forms of the disease. The site of the inflicting bites in these patients did not have any influence on the distribution of antigen (Table 8 and 9). In neither of the cases were evidenced the extensive destructive anatomical changes.

Antigen-containing cells were not limited exclusively to neurons. Neuroglias were also found to contain antigen, particularly those of supratentorial structures of Patient 5 of which the number of antigen-positive glial cells was almost comparable to that of neurons. Antigen-positive cells consistent in appearance with astrocytes were found to be predominantly involved among glial cells. Amount of antigen observed in neurons and glial cells in CNS seemed to be dependent on the length of survival time noted after the first appearance of prodromal symptoms (Table 8 and 10). Survival times after onset of symptoms to death were longer in paralytic group (average of 12 days compared with 5.5 days in the encephalitic group).

Inflammation was scant in all cases in relation to the amount of viral antigen and it was not limited to the spinal cord in paralytic cases (Table 9). Inflammation, when present, was usually found in the brain stem and/or spinal cord. Inflammatory reactions consisted of perivascular and, occasionally, parenchymal infiltrations with mononuclear cells. Satellitosis, neuronal degeneration and glial nodules were rarely observed. The IP staining of antigen-containing neurons, neuroglias and inflammatory reaction are shown in Figure 5-10.