CHAPTER V

DISCUSSION

The aim of this work was to determine the distribution of rabies virus in the various regions of the brain and spinal cord in both human and dog rabies, using an immunohistochemical technique.

We used immunoperoxidase staining (IP) for the detection of the rabies antigen because its a higher sensitivity compared to other conventional methods has been demonstrated (150,151). Avidin-biotin-peroxidase complex was used in this study to amplify the sensitivity of the IP system.

The sensitivity of the ABC method in our study was only 86.7% compared to 100% of FAT or MIT. The relative insensitivity of the IP staining may be due to different techniques in tissue preparations. FAT was done on brain impression smear whereas IP in this study was done on thin histological sections of 6 µm thickness. The impression smear may contain more rabies virus than the 6 µm section.

Previous studied by Kotwal and Narayan (150), using impression smears of mouse brain, showed the direct IP test to have a sensitivity of 93.3% compared to 99% for FAT.

Using paraffin-embedded sections, Anjaria and Jhala (151), showed a very high sensitivity for IP direct staining (97.92%) compared to Seller's stain (96.71%) and FAT (98%).

The use of ABC was found to dramatically increase the intensity of staining and provided a greater sensitivity, when compared to the direct IP method (153).

It does not only provide a suitable sensitive method for demonstrating rabies viral antigen in CNS specimens, but it also has other significant advantages. It is easy to perform, does not require expensive equipment, such as a fluorescent microscope, nonspecific staining is minimal, the structural integrity of the nervous tissue is maintained enabling a detailed study of cell morphology and hence sites of infection, and one has the additional benefit of a permanent record in the form of the stained slide.

Our attempts to produce rabbit anti-rabies IgG, as a primary antibody for staining, were somewhat dissappointing. Although the purity of our product, following chromatography of the hyperimmune serum using Protein A-Sephadex, was high, by IEP criteria, it was rather unsatisfactory in the staining procedure. We had problems with rather low staining intensities in the neurons and neuroglias and a relatively high background of nonspecific staining.

Equine anti-rabies globulin, labeled with fluorescein isothiocyanate (BBL) is prepared for use in the routine

fluorescent testing. However, the fluorescein component did not interfere with our staining procedures, and the reagent gave a high sensitivity, depicting very intensive brown inclusion bodies and excellent staining of the cytoplasmic neurons and neuropil, all against a very clear background.

This commercial reagent is of course rather expensive, but this is mitigated by the high dilution (1:500), at which it may be satisfactorily used.

We did attempt to look at any differences between our rabbit antibody and the BBL product using the Western blot technique, but our preliminary results were rather ambiguous and we decided to shelve this examination and return to the main study using the BBL antibody in our staining procedures.

The results of our studies showed that the rabies viral antigen is distributed in neuroglias as well as in neurons, in both rabid dogs and infected humans. Thus rabies is not an exclusive infection of neurons in either species. The data presented in Table 6 (dogs) and Table 9 (humans) provide good evidence for this proposition.

Further, our studies in both furious and dumb dogs and in human encephalitic and paralytic forms of the disease, showed that the distribution of the viral antigen is anatomically similar in each of these forms of infection.

The distribution of rabies virus has been previously studied in the dog, by Ito et al.(126). However, their examination

was restricted to the brain stem and spinal cord of the naturally infected dogs. Their results, so far as they go, are in agreement with our findings.

We found a large amount of antigen in the spinal cord of both furious and dumb dogs, and most particularly in the brain of Dog 4, which had the longest survival period (5 days).

Data obtained from the other dogs, with shorter survival periods, showed only moderate or little dissemination of the virus through the brain, the extent of which, roughly correlated with the time elapsed between the onset of the disease and ultimate sacrifice.

Studies in human rabies using light microscopy, have demonstrated inclusion bodies in astrocytes although this has been regarded as an uncommon finding (21,62). Seventeen percent of human rabies cases reported by Tangchai et al. (21) were found to have inclusion bodies positive astrocytes. These were mostly in the floor of the third ventricle, paraventricular area and brain stem. However, the amount of inclusion bodies positive astrocytes was usually minimal. Small aggregates of matrix and viral particles within astrocytes and oligodendrocytes have also been observed by electron microscopy (62,69). Identification of antigen in a significant number of glial cells in our study, especially in Patient 5, differs somewhat from previous reports (21). This discrepancy is most likely due to difference in technique.

There was no correlation between the distribution of rabies viral antigen in neurons or glial cells and clinical manifestations. Patients who had survival time of 7 days or less (Patients 1,3,4 and 6) had greater amount of antigen-positive neurons in brain stem and spinal cord regardless of the clinical type. Patients 1,3 and 4 had a classical presentation of encephalitic rabies with no demonstrable weakness of limbs during the pre-terminal phase. Deep tendon reflexes were preserved during the entire clinical course. Widespread neuronal involvement of similar degree in different neuroanatomical regions (supratentorial, infratentorial structures and spinal cord) was evident when the survival time exceeded 7 days as demonstrated in Patients 2,5 and 7. Further, the presence of viral antigen in cerebral cortex did not parallel with the degree of disturbance of consciousness. Rabies viral antigen was easily demonstrable by the immunofluorescent test in the frontal area of one paralytic patient (Patient 10 in reference 109) who had quadriplegia and weakness of respiratory muscle requiring ventilatory support and exhibited only sign of euphoria and was still conscious and rational at the time of brain biopsy. Interestingly, Patient 5 initially presented with episodic attacks of nonconvulsive absence-like seizures 5 days prior to the onset of limb weakness. It is not known whether seizure activity can be induced by the presence of virus in glial cells. This patient had no electrolyte abnormalities and intractable convulsions appeared when she lapsed into coma.

Neither the viral distribution nor the amount of viral antigen paralleled inflammation. Inflammation was not confined to medulla and spinal cord in patients with paralysis or to cerebrum and brain stem in patients with encephalitis as had been previously described (17,21,40,62,118,161). Length of survival or incubation period did not affect the intensity of inflammation. Patient 1, who had a survival period of 5 days, had moderate inflammatory reactions comparable to that of Patient 5 who lived 16 days after onset of symptoms.

The pathogenesis of paralytic rabies is not yet fully understood. Transmitting animal host such as vampire bat and strain of the virus (39) are probably not the only important determinants. Hemachudha, et al. (109) reported 16 cases of human rabies; 7 of them presented with paralysis, and all of them had been bitten by cats or dogs. This is also true in the cases reported here as well as in those described by Chopra (17). The same dog that transmitted paralytic rabies to one patient caused classical encephalitic rabies in another, thus did not support the importance of viral strain (personal communication: Dr.T.Hemachudha).

Unsuccessful immunization attempts, a widely quoted impression as predisposing factor to paralytic rabies, were not found in all of these patients. Only 4 of 18 patients with paralytic rabies in two series reported were given postexposure prophylaxis (17,109). One of 3 paralytic cases presenting here had postexposure vaccination with Semple vaccine.

It has been found that medulla and spinal cord of human paralytic rabies are mainly involved with extensive neuronal damage and inflammation whereas in encephalitic rabies, it is the brain stem and cerebrum, particularly the limbic system (17,21,40,62,118,161). Inclusion bodies were found in the brain cortex in fewer patients with paralysis than with encephalitis (17,21). Our findings, on a limited number of patients, showed relatively little neuronal damage. We feel that viral localization and inflammation may not account for the differences in clinical presentations.

Our results did not support the hypothesis that differential distribution of rabies virus of the extent of inflammation is responsible for the different clinical manifestations of human and canine rabies. Its pathogenesis may be multifactorial, involving immunological as well as physiological disturbances. In fact, it should be noted that regional imbalance of neurotransmitters or neuromodulators leading to neurophysiologic impairment or lesion development remote from the actual site of viral replication has also been postulated (76,77,162).

Conclusions :

1. The immunoperoxidase staining using avidin-biotin- peroxidase complex (ABC) were developed for the detection and quantitation of rabies viral antigen in central nervous system with high sensitivity and specificity.

2. No correlation between distribution of rabies viral antigen in neurons or glial cells and clinical manifestations either in the rabid dogs at the early stage or in human patients.

3. Viral localization is correlated with survival period (interval between onset of disease). Human patients as well as rabid dogs, whose survival periods less than 7 and 5 days respectively had greater amount of antigen-positive neurons in brain stem and spinal cord, whereas those who had longer survival period had widespread dissemination of rabies viral antigen in the entire CNS.

4. Other mechanisms than viral localization may be responsible for this direct clinical manifestations.

Table 1. Checkerboard titration to determine the optimal dilution of antibodies

A. Between primary Ab - rabbit anti-rabies IgG and secondary Ab - biotinylated goat anti-rabbit IgG lot I (0.9 mg/ml) and lot II (0.68 mg/ml)
Trypsinization at 37°C 5 minutes

Sections	Secondary Ab dilutions	Primary Ab (dilution) 1:10	1:20	1:40	1:80	1:100	1:120
Patient 5: Cerebellum	lot I 1:100	-*	± (MOD)•	+ 1 (MOD)	+ 2 (SL)	+ 3 (NB)	+ 1 (NB)
	1:120	-	+ 1 (MOD)	+ 2 (SL)	+ 3 (NB)	+ 3 (NB)	+ 2 (NB)
Dog 6: Medulla	lot II 1:100	-	-	+ 1 (MOD)	+ 2 (SL)	+ 3 (NB)	+ 2 (NB)
	1:200	-	-	-	-	+ 1 (NB)	+ 1 (NB)

* Intensity of stain : - negligible, + 1, + 2, + 3, + 4 maximum obtained

• Background : MOD = moderate, SL = slightly, NB = no background

Table 1. B. Between primary Ab - equine anti-rabies globulin-BBL
and secondary Ab - biotinylated goat anti-horse IgG (1.5 mg/ml)
Trypsinization at 37°C 5 minutes

Sections	Secondary Ab	Primary Ab (dilution)					
		1:40	1:80	1:100	1:200	1:300	1:500
Patient 5: Cerebellum and Dog 6: Medulla	1:200	+ 3 (MOD)	+ 3 (SL)	+ 3 (SL)	+ 4 (SL)	+ 4 (NB)	+ 4 (NB)
	1:300	+ 3 (MOD)	+ 3 (SL)	+ 3 (SL)	+ 4 (SL)	+ 4 (NB)	+ 4 (NB)

* Intensity of stain : - negligible, + 1, + 2, + 3, + 4 maximum obtained

* Background : MOD = moderate, SL = slightly, NB = no background

Table 2. Determination of optimal concentration of trypsin
primary Ab - rabbit anti-rabies IgG dil. 1:80
secondary Ab- biotinylated goat antirabbit IgG, dil. 1:200
Trypsinization at 37°C 5 minutes

	Sections	Trypsin concentration (%)				
		0	0.02	0.05	0.01	0.15
Patient 1 :	Parietal	- *	-	-	+ 1	W
	Cerebellum	-	-	+ 1	+ 2	W
Patient 5 :	Parietal	-	-	+ 2	+ 2	W
	Cerebellum	-	-	-	+ 1	W

* Intensity of stain : - negligible, + 1, + 2 ,+ 3, + 4 maximum obtained
W : section washed off slide

Table 3. Duration and temperature for trypsin treatment
primary Ab - rabbit anti-rabies IgG dil 1:80
secondary Ab- biotinylated goat antirabbit IgG dil. 1:200
Trypsin concentration 0.1%

Sections of Patient5	Temperature	Duration of trypsin treatment (min)					
		0	5	6	7	8	10
Parietal	37°C	- *	+ 2	+ 3	+ 4	+ 4	W
	RT	-	+ 1	+ 2	+ 3	+ 3	W
Cerebellum	37°C	-	+ 1	+ 1	+ 1	+ 1	W
	RT	-	-	-	+ 1	+ 1	W

* Intensity of stain : - negligible, + 1, + 2, + 3, + 4 maximum obtained
W : section washed off slide

Table 4. Comparison of IP (ABC method), Seller's stain, FAT and MIT

Patient No.	Sections	IP (ABC) 1°Ab (rabbit)	IP (ABC) 1°Ab (equine-BBL)	Seller's Stain	FAT	MIT
Human						
2	Hippocampus	+ 2[a]	+ 3[a]	+[b]	+ 3[c]	12[d]
3	Cerebellum	-	+ 1	-	+ 3	17
4	Hippocampus	nd	+ 1	-	+ 3	19
6	Hippocampus	+ 1	+ 2	+	+ 3	15
7	Hippocampus	nd	+ 3	+	+ 3	12
Dog						
1	Hippocampus	+ 1	+ 3	+	+ 3	10
2	Hippocampus	-	+ 1	+	+ 3	9
3	Hippocampus	nd	+ 3	+	+ 3	18
	Cervical	nd	+ 3	-	+ 3	18
	Lumbar	nd	+ 3	-	+ 2	nd
4	Hippocampus	-	+ 3	+	+ 3	nd
5	Hippocampus	+ 1	+ 2	-	+ 3	11
	Cerebellum	+ 1	+ 4	-	+ 3	11
6	Hippocampus	-	-	-	+ 3	20
	Cerebrum	-	-	-	+ 3	20
	Cerebellum	+ 1	+ 2	-	+ 3	20
	Cervical	+ 1	+ 4	-	+ 3	20

[a] positive or negative staining and intensity
[b] positive or negative staining for Negri bodies
[c] positive staining and intensity
[d] days after inoculation into mice to die
nd not done

Table 5. Characteristics of dogs with rabies

Dog Number	Age(months), Sex	Days from onset to death	History of rabies immunization
Furious			
1	12, F	3	-
2	5, M	3	-
3	36, F	2	-
Dumb			
4	4, M	5	-
5	24, F	4	+ (vaccinated)
6	4, F	1	-

Table 6. Distribution of rabies antigen and inflammation in CNS of dog rabies

Dog	Antigen-positive cells and inflammation	Frontal	Temporal	Hippocampus	Parietal	Occipital	Thalamus	Basal ganglia	Cerebellum	Midbrain	Pons	Medulla	Cervical	Thoracic	Lumbar	Sacrum
Furious																
	Neuron	+1	+1	+3	+1	+1	+1	+1	+1	+3	+4	+4	+2	nd*	+3	+3
1	Neuroglia	0	0	0	0	0	0	0	0	+2	+2	+1	0	0	0	0
	Inflammation	0	0	+1	0	0	0	0	0	0	+1	0	0	0	0	0
	Neuron	0	+1	+1	+1	0	+1	nd	+1	+2	nd	nd	+3	+2	+4	+3
2	Neuroglia	0	+1	+1	0	0	0	0	0	+1	0	0	0	0	0	0
	Inflammation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Neuron	+2	+1	+3	0	0	+1	+1	+2	+3	+4	+4	+3	+3	+3	+3
3	Neuroglia	0	0	0	0	0	0	+1	+3	+2	+3	+1	+1	+2	+1	+1
	Inflammation	0	0	0	0	0	0	0	0	+1	0	+1	0	+1	0	0
Dumb																
	Neuron	+3	+3	+3	+3	+2	+3	+3	+3	+4	+4	+4	+4	+3	+3	+3
4	Neuroglia	0	+1	+1	+1	0	+2	+1	+2	+4	+4	+1	+1	+1	+3	+3
	Inflammation	0	0	0	0	0	0	0	0	+2	0	+1	0	0	0	0
	Neuron	+1	+2	+2	+2	+1	0	+1	+4	+3	+2	+4	+3	+4	+2	+3
5	Neuroglia	0	+1	0	0	0	0	+1	0	+1	+1	+1	0	+1	0	0
	Inflammation	0	0	0	0	0	0	0	0	0	0	0	0	+2	0	0
	Neuron	0	+1	0	0	0	0	+2	+2	+4	+3	+3	+4	+4	+3	+4
6	Neuroglia	0	0	0	0	0	0	0	0	+1	0	+1	0	+1	+1	+1
	Inflammation	0	+1	0	0	0	0	+1	+1	+3	+3	+4	0	+1	+1	+1

Antigen-positive microglia was rarely observed in all cases
* nd = not done

Table 7. Mean of amount of viral antigen in neurons and neuroglias in various CNS regions of dog rabies

Dog Number	Brain* (neuron/neuroglia)	Brain stem and cerebellum (neuron/neuroglia)	Spinal cord (neuron/neuroglia)
Furious			
1	1.3/0	3/1.25	2.7/0
2	0.7/0.3	1.5/1	3/0
3	1.1/0.1	3.25/2.25	3/1.25
Dumb			
4	2.85/0.85	3.75/2.75	3.25/2
5	1.3/0.3	3.25/0.75	3/0.25
6	0.4/0.1	2.75/0.5	3.75/1.3

* Brain represents all regions of supratentorial structures including thalamus

Table 8. Characteristics of patients with rabies

Patient Number	Age (years), Sex	Time and location of bite	Survival period (Days from onset to death)	History of rabies immunization
Encephalitis				
1	9, M	1 month before; dog bite on right buttock	5	-
2	22, F	3 months before; dog bite on right ankle	8	-
3	55, F	2 months before; dog bite on right hand, left leg, left breast	4	-
4	14, M	1 month before; dog bite on buttock	5	-
Paralysis				
5	15, F	3 months before; dog bite on finger	16	-
6	81, M	2 months before; dog bite on left calf	7	+ (Semple Vaccine-14 injections)
7	61, M	4 months before; dog bite on right leg	13	-

Table 9. Distribution of rabies antigen and inflammation in CNS of human rabies

Patient	Antigen-positive cells and inflammation	Frontal	Temporal	Hippocampus	Parietal	Occipital	Thalamus	Basal ganglia	Cerebellum	Midbrain	Pons	Medulla	Cervical	Thoracic	Lumbar	Sacrum
Encephalitis																
	Neuron	0	0	0	0	0	+3	+2	+1	+3	+3	+3	+2	0	+3	nd*
	Astrocyte	0	0	0	0	0	0	0	0	0	0	+1	0	0	0	nd
1	Oligodendrocyte	0	0	0	0	0	0	0	0	0	0	0	0	0	0	nd
	Inflammation	+1	0	0	0	0	0	0	0	0	+1	+2	+2	+2	+3	nd
	Neuron	+2	+3	+3	+4	+3	+3	+4	+2	+4	+4	+4	+4	+4	+4	+4
	Astrocyte	+1	+1	+1	+1	+1	+1	+2	0	+1	0	+1	+1	+2	+1	+1
2	Oligodendrocyte	0	+1	0	0	0	0	0	0	0	0	0	0	0	0	0
	Inflammation	0	0	+1	0	0	0	0	0	+1	0	0	0	0	0	+1
	Neuron	+2	+2	+2	+2	+2	+3	+3	+1	+4	+4	+4	+3	+3	+4	+4
	Astrocyte	+1	+1	+1	+1	0	+2	+1	0	+1	0	0	+1	0	0	+1
3	Oligodendrocyte	0	0	+1	+1	0	0	+1	0	+1	0	0	0	0	0	0
	Inflammation	0	0	0	0	0	0	0	0	0	0	0	0	0	+1	+2
	Neuron	+2	0	+1	+3	+1	+2	+2	+2	+2	+2	+3	+2	+3	+3	+3
	Astrocyte	0	0	0	+1	0	+1	+1	0	+1	+1	+1	+1	0	0	+1
4	Oligodendrocyte	0	0	0	0	0	0	+1	0	0	0	0	+1	0	0	0
	Inflammation	0	0	0	0	0	0	0	0	0	0	+4	0	0	+2	+2
Paralysis																
	Neuron	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	nd
	Astrocyte	+3	+3	+3	+4	+4	+3	+4	+4	+4	+2	+3	+4	+3	+2	nd
5	Oligodendrocyte	+2	+1	+1	+3	+4	+1	+3	+3	+1	+2	+1	+1	0	+1	nd
	Inflammation	+1	+1	+1	+1	0	+1	+1	0	+1	+2	+1	0	0	+1	nd
	Neuron	+1	+1	+2	+2	+2	+3	+2	+2	+4	+4	+3	+3	+3	+3	nd
	Astrocyte	0	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+2	+1	nd
6	Oligodendrocyte	0	0	+1	+1	0	+1	0	0	0	0	0	0	0	0	nd
	Inflammation	0	0	0	0	0	0	0	0	0	0	+2	0	0	0	nd
	Neuron	+2	+3	+3	+3	+3	+4	nd	+3	+4	+4	+4	+2	+3	+4	+4
	Astrocyte	+1	+1	+1	+1	+2	0	nd	0	+1	+1	+1	+1	+2	+2	0
7	Oligodendrocyte	+1	+1	+1	+1	+2	+1	nd	+1	0	0	0	+1	0	0	0
	Inflammation	0	0	0	0	0	0	0	0	+1	0	+1	0	0	0	0

Antigen-positive microglia was rarely observed in all cases
*nd = not done

Table 10. Mean of amount of viral antigen in neurons and neuroglias in various CNS regions of human rabies

Patient Number	Brain* (neuron/astrocyte/ oligodendrocyte)	Brain stem and cerebellum (neuron/astrocyte/ oligodendrocyte)	Spinal cord (neuron/astrocyte/ oligodendrocyte)
Encephalitis			
1	0.7/0/0	2.5/0.25/0	1.7/0/0
2	3.1/1.1/0.1	3.5/0.5/0	4/1.25/0
3	2.3/1/0.4	3.25/0.25/0.25	3.5/0.5/0
4	1.6/0.4/0.1	2.25/0.75/0	2.75/0.5/0.25
Paralysis			
5	4/3.4/2.1	4/3.25/1.75	4/3/0.7
6	1.9/0.9/0.4	3.25/1/0	3/1.3/0
7	3/1/1.2	3.75/0.75/0.25	3.25/1.25/0.25

* Brain represents all regions of supratentorial structures including thalamus

ENCEPHALITIC RABIES

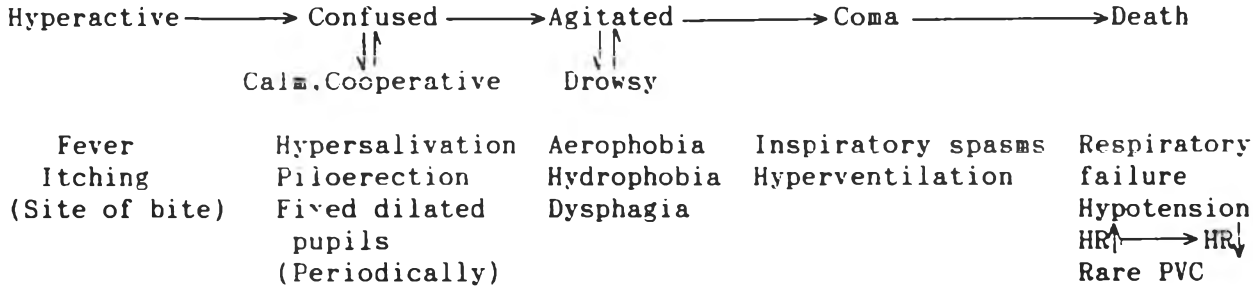

Figure 1. Clinical pattern of human encephalitic rabies.

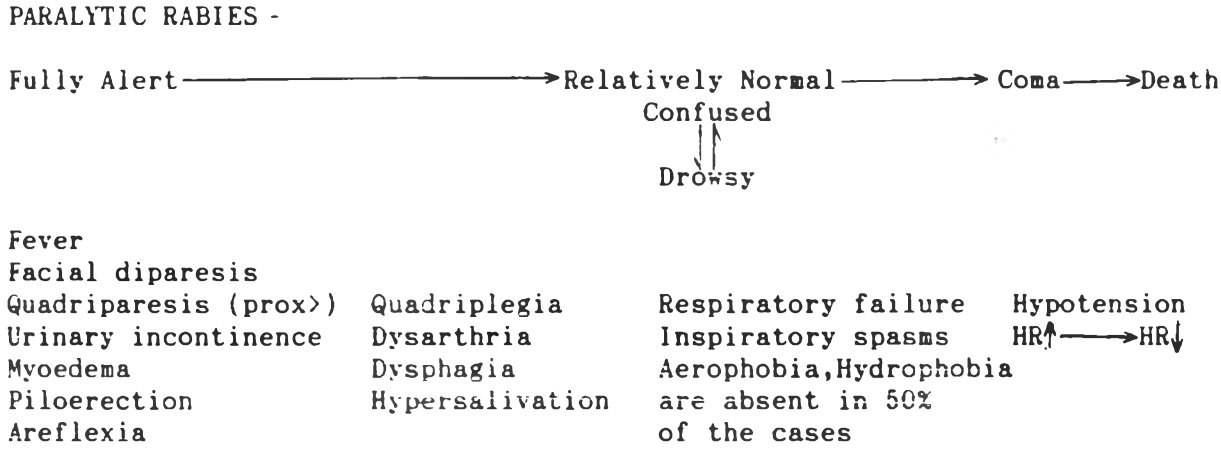

Figure 2. Clinical pattern of human paralytic rabies.

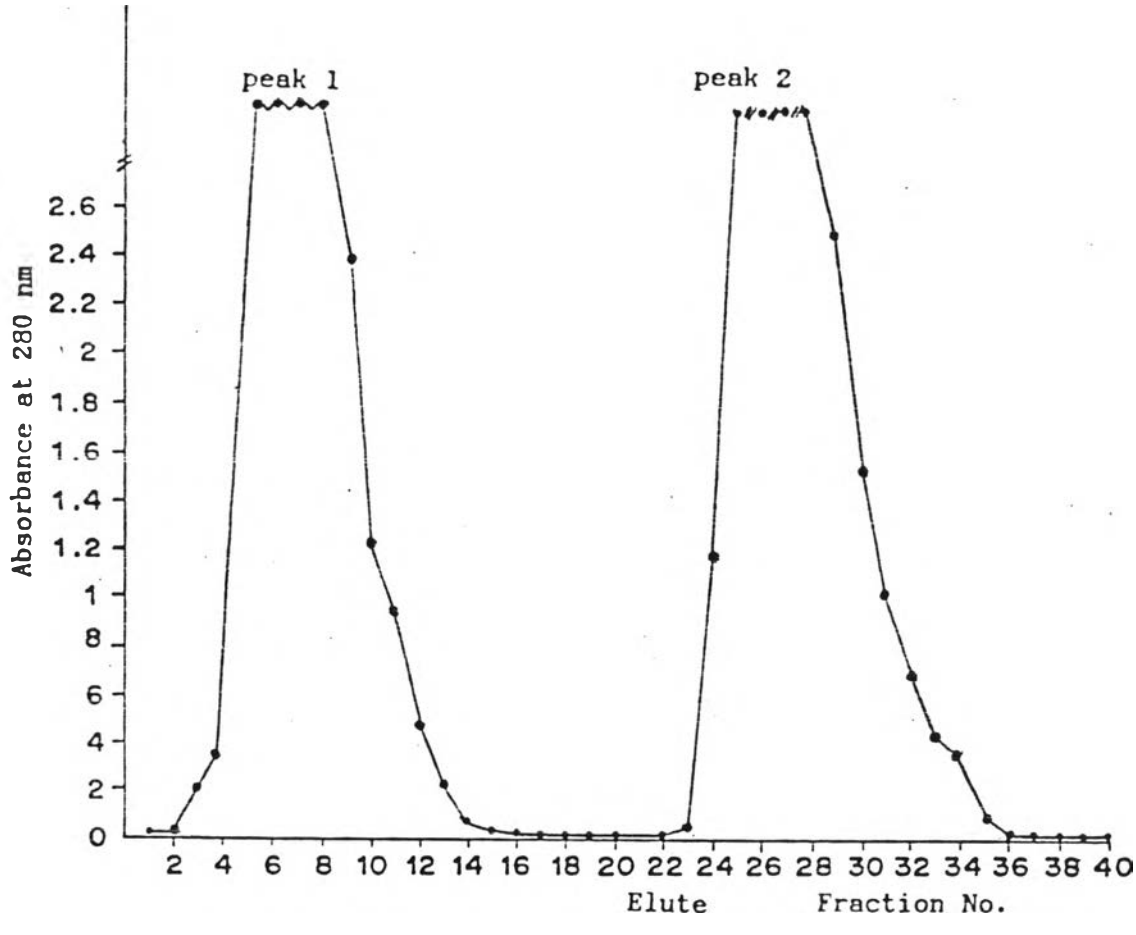

Figure 3. Affinity chromatography of rabbit anti-rabies on Protein A-Sepharose CL-4B column. The column was eluted with 0.05 M acetate in 0.15 M Nacl, pH 4.3, flow rate 50 ml/hr; volumn of fractions 3 ml.

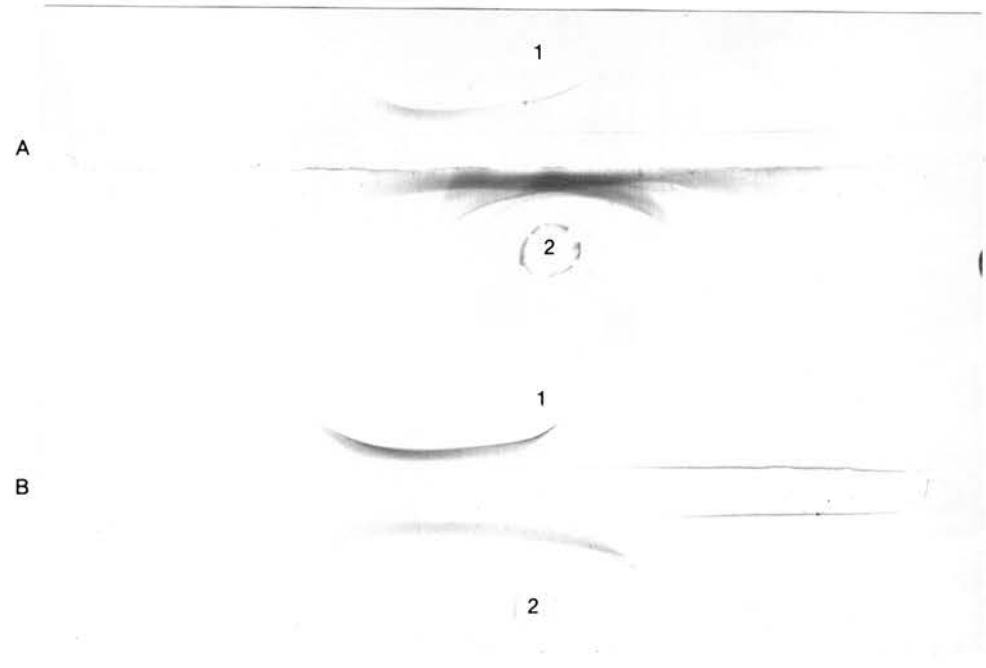

Figure 4. Immunoelectrophoresis of rabbit anti-rabies IgG (well 1) against
A. Swine anti-rabbit serum (trough)
B. Goat anti-rabbit IgG (trough)

Control - normal rabbit serum (well 2)

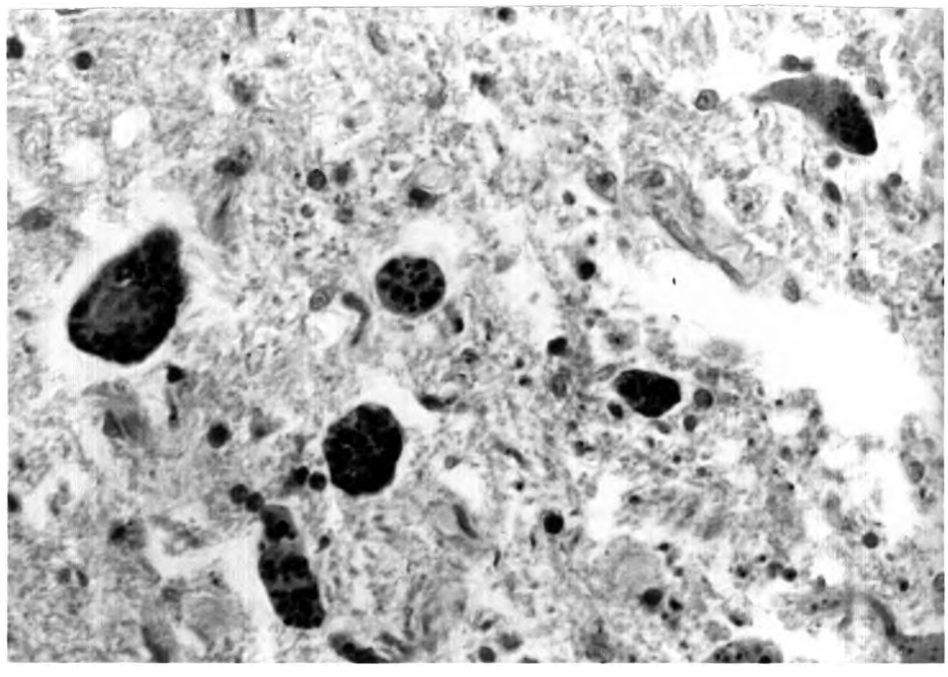

Figure 5. Immunoperoxidase staining of rabies viral antigen showing intensive brown inclusion bodies in neurons in the anterior horn of cervical segment of spinal cord from Patient 6, who presented as paralytic rabies (x 400).

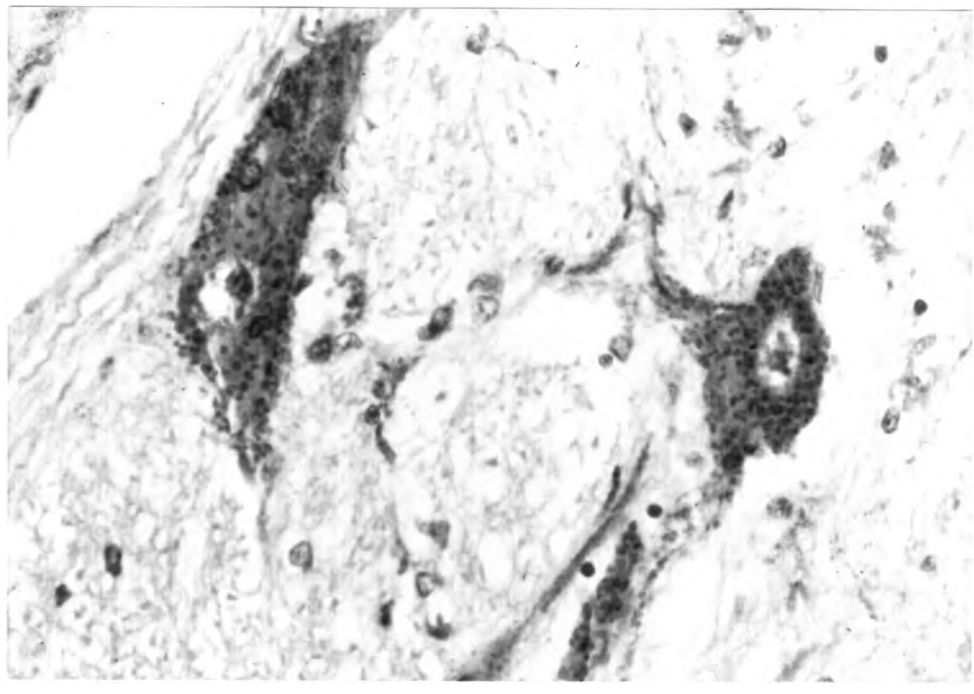

Figure 6. Rabies viral antigen in neurons in the midbrain of a dumb dog, Dog 3. Dense peroxidase reaction, in the form of inclusions, is present in the cytoplasm (x 400).

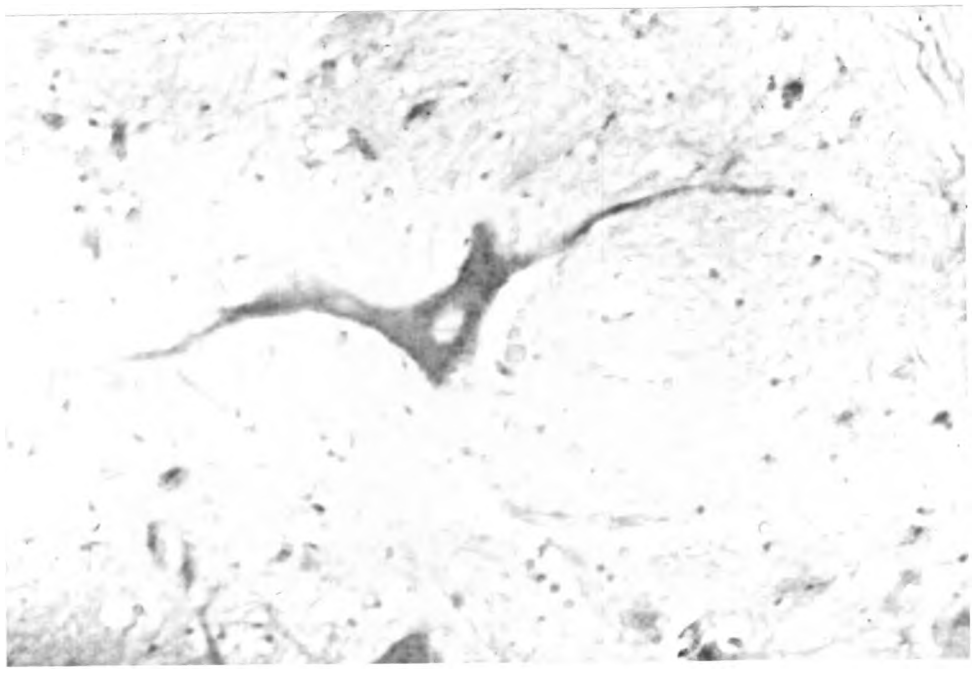

Figure 7. Diffuse staining of rabies viral antigen in neuron and neuropiis in the medulla of a furious dog, Dog 1 (x 200).

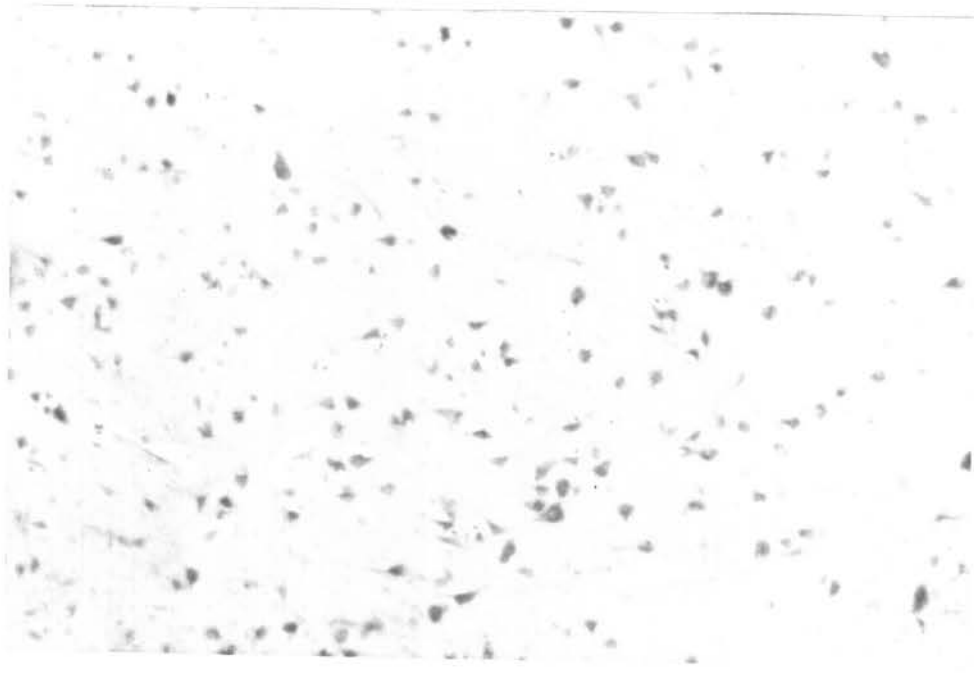

Figure 8. Rabies infection of neurons and astrocytes in the occipital region of Patient 5 (x 100).

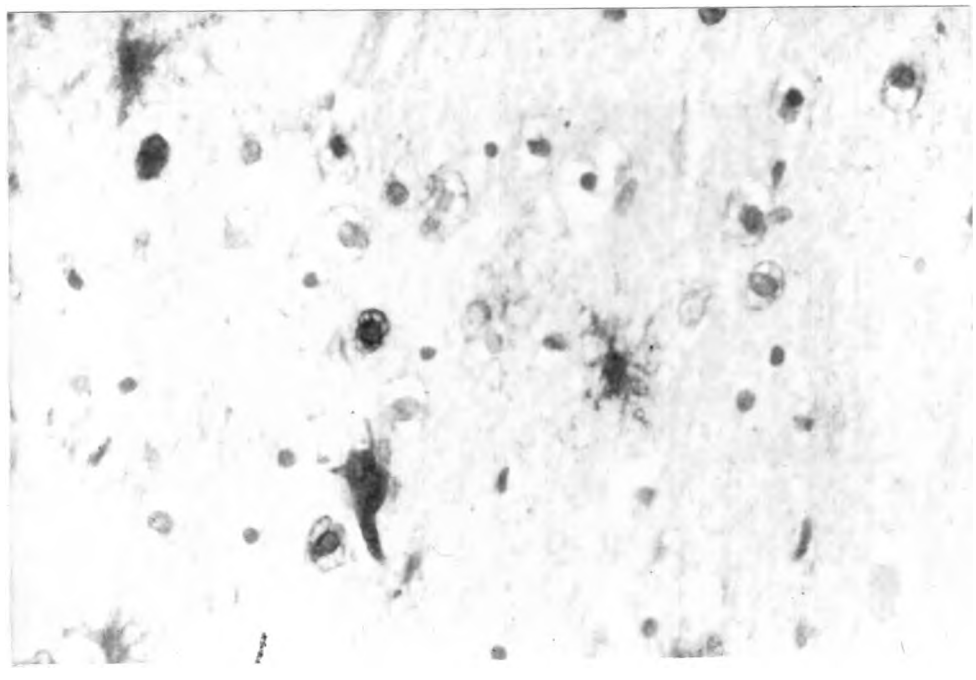

Figure 9. Diffuse staining of rabies viral antigen in astrocytes in the midbrain of Dog 4 (x 400).

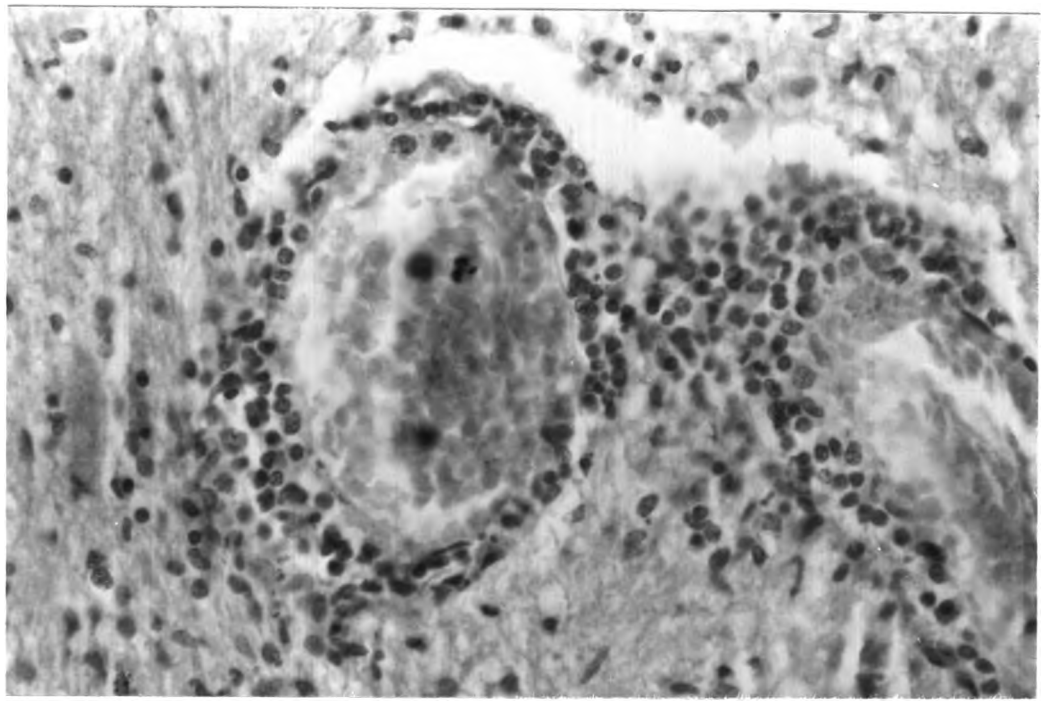

Figure 10. Dense perivascular infiltrations of mononuclear cells in the brain stem of Patient 4 (x 400).