Chapter IV

Results

1. Preparation and Isolation of Rabbit Antirabies IgG

Serum was obtained 1 week after the last immunization from rabbit immunized with 3 weekly injection of PVRV in CFA and assayed for neutralizing antibody by RFFIT. It was found to be 2.1 IU/ml

The IgG fraction was isolated by affinity chromatography using Protein-A Sepharose CL-4B. Two peaks of protein were obtained when measured at 280 nm.(Figure 4). The majority of serum protein other than IgG passed through the column first (peak 1) since protein A has the capacity to bind specifically and with high affinity to the IgG of most species (103,104). The bound IgG were subsequently eluted with acid and recovered mostly in peak 2 (fraction No. 18-24). The purity of the IgG was determined by Immunoeletrophoresis (IEP) against swine anti-rabbit serum as well as goat anti-rabbit IgG (Figure 5). The protein concentration of the purified antirabies IgG was 3.5 mg/ml according to Lowry method and its neutralizing activity as determined by RFFIT was 3.8 units/ml . IgG from peak 2 was used in the subsequent tests and the optimal dilution of the dot-immunoblot was determined by checkerboard titration.

2. Study of Factors Affecting the Dot-Immunoblot System

2.1 Checkerboard titration to determined the optimal concentration of primary and secondary antibodies

Different dilutions of rabbit anti-rabies IgG (RRIG),i.e., 1:50, 1:100, 1:150, 1:200 was titrated against different dilutions of biotin-conjugated goat anti-rabbit IgG (1:100,1:200,1:300,1:400 dilution). Rabies antigen in the form of PVRV and PBS served as positive and negative controls respectively. As shown in Table 1A , the most satisfactory result was obtained by using a dilution of 1:100 of RRIG and 1:300 of biotin conjugated goat antirabbit IgG and these dilutions were therefore choosen for subsequent studies.

ERIG-BBL was also titrated in the same manner. A dilution of 1:200 of ERIG-BBL and 1:300 of biotin- conjugated rabbit antihorse IgG produced the best result (Table 1B).

Other parameters explored were diluent for primary (1^{ry}) and secondary (2nd)antibody as well as the incubation time. It was founded that diluting the 1^{ry} Ab (RRIG) in cocktail solution containing 2%goat serum (GS),5% chicken serum (CS) and 0.002% normal human AB serum was most satisfactory. In ERIG-BBL ,the satisfactory diluent for 1^{ry} Ab was 2%GS and 0.002 % NHS ,as well as 2% GS , 5 % CS in both 2nd Ab with an incubation time of 1 hr. at 37 C. All of these reagents were diluted in 0.1% Tween 20-PBS (0.1% PBS-T) pH 7.4. In such conditions, low background staining was obtained as compared to that diluted in 0.1% PBS-T alone without any serum protein.

The optimal incubation time for both the 1^{ry} and 2nd Ab reaction was 1 hr at 37 C. Long incubation period leaded to increase background staining.

2.1.1. The Specificity and sensitivity of Purified Rabbit Antirabies IgG (RRIG) and ERIG-BBL

The specificity of RRIG and ERIG-BL was determined by using rabies vaccine and other non-rabies antigens such as HBs Ag, HSV-2, HeLa cell and Vero cell. These protein was dotted onto the filter and then incubated with primary antibodies. Neither RRIG nor RIG-BBL reacted with non-rabies antigens at any concentrations. Optimal dilution (1:100) of RRIG was able to detect as little as 10^{-6} dilution of rabies antigens in PVRV which was equivalent to 40 ng of vaccine protein (Table 2A). Results with rabies antigen in PCEC and inactivated animal vaccine (Rabdomun) gave a much less sensitivity. Only 10^{-2} dilution of PCEC and Rabdomun could be detected. Sensitivity of RIG-BBL was carried out in similar fashion and 10^{-4} and 10^{-2} dilutions of rabies antigen in Rabdomun and PVRV as well as PCEC could be detected respectively (Table 2B).

2.3 Checkerboard titration to determine the optimal concentration of avidin biotin conjugated enzyme horse radish peroxidase (ABC)

Various dilutions of ABC (1:500, 1:1000, 1:2000, 1:3000) were titrated with appropriate dilutions of primary and secondary antibodies. PVRV (10^{-4}, 10^{-5}, 10^{-6} dilutions) served as positive control and HBsAg (10^{-1}, 10^{-2}, 10^{-3} dilutions) as well as 0.1 % PBS - T as negative controls. As shown in Table 3, it was found that ABC at 1:1000 concentration gave the most satisfactory result.

2.4 Blocking agent for dot-immunoblot

To block the nonspecific staining reaction, BLOTTO, 0.1 %

Tween 20 in PBS and various concentrations of BSA (1%, 3%, 5%, 7%, 10%) were compared. BLOTTO appeared to be the best among these three blockings materials. 5% BLOTTO (w/v) in 0.1% PBS-T was the optimal concentration in decreasing the background without disturbing the specific signal.

3. <u>Dot-immunoblot results</u>

3.1 <u>Freshly prepared parotid gland suspensions and fresh saliva</u>

After having developed the dot immunoblot assay using rabies vaccine as the antigen, the system was then tested with freshly prepared parotid gland suspension from dogs suspected to have rabies. The test was performed in a blind fashion ,i.e.,without the knowledge of the FAT and MIT results on the brain tissue. Then the immunoblot results were compared with the FAT and MIT results. As shown in Table 4. Of the 19 rabid dogs, all were positive on parotid gland immunoblot and all but one were positive on saliva immunoblot, accounting for a sensitivity of 100% and 94.7% ,respectively. Of the 15 nonrabid dogs (as proved by negative FAT and MIT), all were negative on parotid gland immunoblot and only 1 out of 12 was positive on saliva immunoblot, giving the specificity of parotid gland and saliva immunoblot 100% and 91.6% respectively.

As compared to Sellers' stain, dot-immunoblot was much more sensitive than Sellers' stain. That was, of the 19 FAT and MIT positive brains, 11 were Sellers' stain positive, a sensitivity of only 57.9%. However, the specificity of Sellers'stain was 100%,

namely none of 15 FAT and MIT negative brains was Sellers' stain positive.

3.2 Stored parotid gland suspensions and saliva on the dot-immunoblot assay

In order to study the effect of storage parotid gland suspensions and saliva kept at 4 C preservative or cold room without bacteriostatic agent 3 months were retested on dot-immunblot. These specimens were the same as used in the initial testing (freshly prepared) but with some additions. Table 5 shows the results with RRIG as primary antibody. Of the 29 rabid dogs, all but one were positive on parotid gland suspension immunoblot and 26 were positive on saliva immunoblot, giving the sensitivity of 96.5% and 89.6% respectively. Of the 25 nonrabid dogs, 21 and 19 were negative on parotid suspension and saliva respectively, accounting for a specificity of 84% and 76% respectively with ERIG-BBL as primary antibody, the sensitivity was even less. Of the 22 rabid dogs, 18 were positive on parotid gland suspension immnoblot and 16 on saliva immunoblot, giving the sensitivity of 81.8% and 72.7% respectively (Table 6). Of the 21 nonrabid dogs, 18 were negative on parotid gland suspension immonoblot as well as on saliva immunoblot, accounting for the specificity of 85.7% for both stored parotid gland suspension and stored saliva.

3.3 Stored whole brain

Dot immunoblot was performed on 21 FAT and MIT proved rabid brains and 21 non-rabid brains. Brain suspensions were prepared from the whole dog brains and stored at 4 c for 3 months

before dot immunoblot assay. Using RRIG as the primary antibody, a sensitivity of 61.9% (13/21 rabid brains were positive) and a specificity of 95.2% (20/21 non rabid brains were negative) were found. With ERIG-BBL as primary antibody, the sensitivity and specificity of dot immunoblots were 75% (18/24 true positive) and 100% (23 /23 true negative) respectively.

3.4 Fresh brainstem suspension

One of the reasons for the sensitivity of dot immunoblot assay on stored whole brain may be the " diluting out effect " due to the uneven distibution of the rabies virus in the brain. Therefore, brainstem , the area which is most abundant with rabies virus were tested with the immunoblot technique. Brainstem suspensions were prepared from 19 and 9 rabid and non-rabid dogs respectively and tested unstored (fresh) on dot - immunoblot with both RRIG and ERIG- BBL as primary antibodies. This way, a sensitivity of 100% (19/19 true positive) and specificity of 88.8% (8/9 true negative) were found by using ERIG- BBL and a sensitivity of 94.7% (18/19 true positive) , and specificity of 88.8% (8/9 true negative) by using RRIG. (Table 7)

3.5 The study of viral excretion in the saliva of quarantined dogs

Ten quarantined dogs were the subjects in this study. Six dogs had developed signs of rabies before entry. Saliva was collected only once in 5 of these 6 dogs and twice in the remaining dog,i.e.,again before its death. These 6 dogs were all confirmed to be rabid by FAT and MIT of the brain. All of the 7 specimens from

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

these dogs were positive on saliva immunoblot, concordant with the results of FAT and MIT of brain as well as with MIT of same saliva specimen. The other 4 dogs were found none rabid and saliva was sequentially collected , 4 times in 2 dogs and twice in the other, before their releases from quarantine. All the 12 saliva specimens from these dogs were negative on saliva immunoblot, confirming the results of MIT of saliva (Table 8)

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย