CHAPTER 3

MATERIALS AND METHODS

1. PATIENTS

1.1 One hundred and thirty-one patients with severe rabies exposure who came to the rabies clinic at the Queen Saovabha Memorial Institute (QSMI) of the Thai Red Cross Society (TRCS) were the subjects of this study. They all required rabies immune globulin (RIG) according to the Institute's therapeutic guideline. However, because of their financial restraints, only equine RIG (ERIG) was affordable. All patients gave informed consent to take part in the study which had been passed by Chulalongkorn University ethical committee. Concomitently with ERIG, the patients also received a full course of purified Vero cell rabies vaccine (PVRV) according to the TRCS regimen. 77 were males and 54 were females. Their ages range from 15 to 50 ($\bar{X} \pm SD = 51 \pm 9.25$). Of the 131 patients, only 104 returned on day 7 and 14 after ERIG administration for follow-up sera.

1.2 Twenty seven patients with documented serum sickness after receiving horse immune globulin were used as positive controls. Serum sickness was diagnosed by the

history of receiving heterologous serum, followed in 5-10 days by fever, skin rashes, arthralgia, lymphadenopathy and proteinuria. The diagnosis was further confirmed by heterophile antibody test and CH_{50} levels. 14 were males and 13 were females. Their ages ranged from 15 to 53 with a mean of 33 (SD = 10.85). Their clinical data are summarized in Table 4.

2. Normal control

Sera obtained from 50 blood donors at the National Blood Center of the Thai Red Cross Society were used as normal controls.

3. Equine rabies immune globulin (ERIG)

ERIG manufactured by Pasteur Institute was used in this study at 40 IU/Kg body weight. Approximately half of the amount of ERIG was infiltrated around the bite sites and the remaining was injected intramuscular at the gluteal areas. The immunoglobulin was obtained from the serum of healthy horses immunized with the fixed Pasteur strain (PV 11) of rabies virus. The serum was highly purified by enzymatic treatment and successive selective precipitations, thus allowing the elimination of inactive proteins, and then concentrated and sterilized.

In ELISA test for the detection of horse gamma globulin, ERIG was used as the antigen representing horse gamma globulin.

4. Collection and storage of specimen

10 ml blood samples were collected before the injection of ERIG as well as 7 and 14 days after the injection of ERIG. Sera were divided into small aliquots and stored at -70°C until use.

5. Skin test

Before the administration of ERIG, intradermal skin test was performed in all patients. ERIG was diluted 1:10 in normal saline and 0.02 ml was injected intradermally into the forearm. 1% histamine and normal saline were used as positive and negative controls respectively. 15 minutes after the intradermal injection, diameters of the wheal and erythema (flare) on the test and control sites were measured.

Criteria of positive skin test

1. If saline has a wheal of 3 mm. or less, wheal of ERIG has to be at least 6 mm in diameter, surrounded by flare of any size.

2. If wheal of saline is larger than 3 mm., wheal of ERIG has to be at least twice the size of the wheal from saline, surrounded by flare of any size.

3. If only wheal from ERIG fulfils the criteria of a positive skin test as stated above, but no flare with ERIG, the skin test is read as boderlined.

Patients with negative skin test were injected with ERIG and observed for 30 minutes before discharge. For patients with positive skin test who could not afford HRIG (Human rabies immune globulin), ERIG would be given under close observation of a physician. The patients were observed for possible side effects for 4 hours before discharge. During the period of this study, of the 19 patients with positive skin tests were in this category.

Table 4 The clinical data of 27 serum sickness patients

Patient No.	Onset after serum administration (day)	Fever	Skin rash	lymphadenopathy	joint symptom
1	12	+	+	-	-
2	8	-	+	-	+
3	6	-	+	-	-
4	4	-	+	-	-
5	7	-	+	-	-
6	13	-	-	+	+
7	7	+	+	-	-
8	8	+	+	-	-
9	7	-	+	-	+
10	7	-	+	-	+
11	5	-	+	-	-
12	9	-	+	+	-
13	6	-	+	-	-
14	7	+	+	-	-
15	5	+	-	+	-
16	6	-	+	-	+
17	8	-	+	-	-
18	8	-	+	-	-
19	9	-	+	-	-
20	6	+	+	+	-
21	8	+	+	-	-
22	7	-	+	-	-
23	7	-	+	-	-
24	8	+	+	-	-
25	6	-	+	-	-
26	6	+	+	-	+
27	7	-	+	-	-

6. ELISA tests for IgG and IgE anti-horse gamma globulin antibodies

Indirect ELISA test was used to measure IgG and IgE antibodies to horse gamma globulins. The principle of the test is to immobilize antigen on the solid phase and allow the antigen to react with the antibodies in the test serum which can be detected by enzyme-labelled-anti-human immune globulin antibodies.

6.1 Indirect ELISA for the detection of IgG anti-horse gamma globulin.

One hundred microlitres of ERIG (Pasteur Institute, France, Lot 5298) representing horse gamma globulin, diluted in coating buffer (Appendix I) to 5 ug of protein per ml. were added to each well of 96-well microtiter plates (Costar. MA, USA). The plates were incubated at 37°C for 1 hour and then washed with PBS-Tween (Appendix I) 5 times, 3 minutes each time and tapped dry. 100 ul of serum sample diluted 1:800 in PBS-Tween albumin (Appendix I) were added to each well and the plates were incubated at 37°C for 1 hour. Each sample was assayed in duplicate. After 5 washes with PBS-Tween, 100 ul of rabbit anti-human IgG peroxidase conjugate (Dakopatts, Denmark) diluted 1:10,000 in PBS-Tween albumin were added and the plates were

incubated at 37°C for 1 hour and then washed with PBS-Tween 5 times. One hundred microlitres of O-phenylenediamine dihydrochloride (OPD) substrate solution (Appendix I) were added to each well. After 30 minute incubation at room temperature in the dark, the reaction was stopped by the addition of 50 ul of 1 N H_2SO_4. The absorbance was read by an ELISA reader at 492 nm.

In addition to positive and negative control sera, conjugate control and substrate control were also included in each plate. One hundred microlitres of PBS-Tween albumin were substituted for sample in the conjugate control and for both sample and conjugate in the substrate control.

Sera diluted 1:800 were used for initial screening of IgG anti-horse gamma globulin antibody. Any positive sera would be retested at two-fold dilutions to derive at the final highest positive dilution.

Interpretation of results

A dilution was considered positive for IgG anti-horse gamma globulin antibody if it yielded the absorbance value which was 3 standard deviations (SD) greater than the mean of the negative control specimens.

6.2 Indirect ELISA for the detection of IgE anti-horse gamma globulin

One hundred microlitres of horse gamma globulin diluted in coating buffer to a protein concentration of 40 ug/ml were used for coating the microtitre plates at 37°C for 2 hours, followed by washing 5 times, 3 minutes each time with PBS-Tween and tapped dry. One hundred microlitres of serially 5 fold diluted samples in PBS-Tween albumin were added to each well. Each sample was assayed in duplicate. The plates were incubated at 37°C for 2 hours and then washed 5 times. Rabbit anti-human IgE peroxidase conjugate (Dakopatts, Denmark) was diluted 1:500 in PBS-Tween albumin and 100 ul of this solution were added to each well. The plates were incubated at 37°C for 2 hours. After 5 washes with PBS-Tween, 100 ul of OPD substrate solution were added to each well and the plates were incubated at room temperature in the dark for 45 minutes. The enzyme-substrate reaction was stopped by addition of 50 ul of 1 N H_2SO_4. The absorbance was read with a spectrophotometer at 492 nm.

In addition to positive and negative control sera, conjugate control and substrate control were also included in each plate as described above.

Interpretation of results

A dilution was considered positive for IgE anti-horse gamma globulin antibody if it yielded the absorbance value which was 3 standard deviations (SD) greater than the mean of the negative control specimens.

7. ELISA test for IgM anti-horse gamma globulin antibody

The conventional indirect ELISA test is not always effective for IgM antibody assay. This is partly due to the false positive reaction caused by the rheumatoid factor in the test serum which will react with the enzyme-labelled anti IgM conjugate. False negative reaction can also occur if there is a vast excess of high affinity IgG antibody with will compete for the limited amount of immobilized antigen, thus reducing the availibility of antigen to react with the IgM antibody.

The anti IgM method (Capture ELISA) can be useful for detection of specific IgM antibody. The procedure is as follow :-

1. The solid phase is coated with antibody to IgM.
2. Test sera are incubated with the anti-IgM solid plase.

3. Unlabeled antigen followed by enzyme-labeled specific antibody are then added.

4. Enzyme-substrate is added at the final step.

Capture ELISA for the detection of IgM anti-horse gamma globulin

The polystyrene microtitre plates coated with 100 ul of rabbit anti-human IgM (Dakopatts, Denmark) diluted in coating buffer to 10 ug of protein per ml. were incubated at 37°C for 2 hours. After washing 5 times, 3 mintues each time, 100 ul of sample diluted 1:25 in PBS-Tween albumin were added to each well in duplicate. The plates were incubated at 37°C for 1 hour and then washed 5 times. One hundred microlitres of horse gamma globulin diluted in PBS-Tween albumin to 20 ug of protein per ml. were added to each well. The plates were incubated at 37°C for 2 hours. After 5 washes with PBS-Tween 100 ul of rabbit anti-horse IgG peroxidase conjugate (Sigma chemical Co.Ltd., USA) diluted 1:500 in PBS-Tween albumin were added to each well and the plates were incubated at 37°C for 2 hours and then washed 5 times. One hundred microlitres of OPD substrate solution were added to each well. After 30 minutes at room temperature in the dark, the reaction was stopped by adding 50 ul of 1 N H_2SO_4. The absorbance was read at 492 nm in an ELISA reader.

In addition to positive and negative control sera, conjugate control and substrate control were also included in each plate. One hundred microlitres of PBS-Tween were substituted for sample in the conjugate control and for both sample and conjugate in the substrate blank.

Sera diluted 1:25 were used for initial screening of IgM anti-horse gamma globulin antibody. The sera that gave positive results would be tested at two-fold dilutions and titrated for final positive dilution.

Interpretation of results

A dilution was considered positive for IgM anti-horse gamma globulin antibody if it yielded the absorbance value which was 3 standard deviations (SD) greater than the mean of the negative control specimens.

Standardization of the ELISA test

1. Precision analysis of the assay

1.1 within-plate precision was determined by using positive and negative sera. The precesion of each serum was determined by running 20 replicates in one microtiter plate.

1.2 A between-plate precision was determined by testing positive and negative sera on 20 different occasions.

2. Specificity of the ELISA test

Sera from 10 persons without any history of allergic symptoms to horse gamma globulin and from 10 persons with positive antibodies to horse gamma globulin by ELISA test were used to assure the specificity of the antibody. An inhibition test was used by incubating 0.5 ml of sera with an equal volume of 100 ug/ml horse gamma globulin at 37°C for 1 hour and 4°C overnight. Sera were diluted 1:2.5, 1:12.5 and 1:400 for IgE, IgM and IgG anti-horse gamma globulin antibodies respectively and were undiluted for IgE anti-horse gamma globulin. The mixtures were centrifuged at 11,500 rpm (Eppendof, Eppendork Geratebau, West Germany) for 30 minutes and the supernates were used as in the routine assays.

Statistical analysis

1. Sensitivity, specificity, positive predictive value, negative predictive value and efficiency.

$$\text{Sensitivity} = \frac{\text{True Positive}}{\text{True Positive + False Negative}}$$

$$\text{Specificity} = \frac{\text{True Negative}}{\text{True Negative + False Positive}}$$

$$\text{Positive predictive value} = \frac{\text{True Positive}}{\text{True Positive + False Positive}}$$

$$\text{Negative predictive value} = \frac{\text{True Negative}}{\text{True Negative + False Negative}}$$

$$\text{Efficiency} = \frac{\text{True Positive + True Negative}}{\text{True Positive + False Positive + False Negative + True Negative}}$$

2. Fisher's exact test

3. Chi-squar test