CHAPTER II

Materials and Methods

2.1) Patient Selection

The study was carried out from July 1, 1988 to March 31, 1990, at the Queen Saovabha Memorial Institute (QSMI) and the Immunology Laboratory of the Department of Microbiology, Chulalongkorn University Hospital, Bangkok.

Inclusion Criteria of the patients in this study were as follows :-

1) Male or female patients between 15 - 60 years old who had the indication to receive equine rabies immunoglobulin (ERIG) at QSMI because of being biten by potentially rabid anmials. Informed consent was obtained from each patient.

2) Patients would come back for further treatments or follow - up.

3) Patients had no past history of severe anaphylaxis to horse serum products in the past.

4) Patients were not on corticosteroids or antihista mines.

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Cases ellgible by these criteria were then enrolled in the study. A history of any present illness and of the

animal bite, site of bite, past history of drug allergy, corticosteroid or antihistamine therapy and previous antiserum treatment was taken.

2.2) Blood collection and skin test.

Ten millilitres of blood was drawn from each patient as baseline serum. The skin test was then performed at the forearm by intradermal injection of approximately 0.02 ml of a 1:10 dilution of Pasteur ERIG to raise a 3 mm skin bleb. Any reaction was measured after 15 minutes using the ballpoint pen technique. (40) The wheal and flare diameters were averaged from 2 perpendicular readings. Physiologic saline and 0.01 % histamine solution were used as negative and positive controls respectively. After direct measurement,an imprint of the wheal and flare reaction site was made with transparent adhesive tape for permanent record.

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2.3) Criteria for positive skin test.

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

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

3) If ERIG produces only a positive wheal according to the criteria stated above without a flare, the skin test is read as borderline.

2.4) Patient Management.

Skin test-negative patients were injected with Pasteur ERIG at 40 IU/kg body weight. Half of the total volume of ERIG was injected around the bite site and the remaining half was given intramuscularly at the buttock. The patients were observed for 30 minutes for any adverse reactions before discharge.

Skin test-positive patients were offered human rabies immunoglobulin (HRIG) as alternative for ERIG. Those patients who could not afford HRIG were placed on a bed and an IV line was inserted. Vital signs i.e., blood pressure, pulse rate and respiratory rate were recorded as well as spirometry result. ERIG was given under close observation by the attending physician. The patient was observed for 1 hour before discharge for adverse effects. In case of adverse effects, the patient was given appropriate treatment and adverse effects were recorded. All patients were instructed to return immediately to Queen Saovabha Memorial Institute or to the Chulalongkorn Hospital emergency room if any side effects occured later.

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Patients were also asked to return for serologic and clinical follow - up on days 7 and 14 whether or not they had side effects.

If the patient later had adverse effects such as skin rash, fever, arthralgia, etc, a complete set of skin test was performed on the right and left forearms as diagramed

	Right forearm		Left forearm	
	ERIG (undiluted)	NSS	ERIG (1:10)	PVRV
ID 0.02 ml	/	/	/	/
ID 0.1 ml	/	/	/	/
SC 0.1 ml	/	/	/	/

The results of the skin test were read at15 minutes, 6 hours, 24 hours and 48 hours. The sizes of wheal and flare (at 15 minutes), erythematous swelling (at 6 and 24 hours) and induration (at 48 hours) were recorded as they appeared accordingly. Any inflammatory swellings of the injection site at 6 hours which lasted up to 24 hours, but not to 48 hours were considered as positive Arthus reaction.

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2.5) laboratory investigations

2.5.1) Heterophile antibody test

This test identifie the presence of serum antibody which will agglutinate sheep red blood cells and was carried out according to the method of Paul- Bunnell - Davidsohn sheep cell test. Diagram was shown in table I

a) Prepare 10 test tubes with normal saline 0.4 ml in the first tube and 0.25 ml from the second to the tenth tube.

b) Add 0.1 ml of heat (56° C x30 minutes) inactivated serum to the first tube mix well and transfer 0.25 ml of this mixture to the second tube, mix and transfer 0.25 ml of this mixture to the third tube and so on up to the ninth tube. The tenth tube serves as negative control. Discard 0.25 ml of the mixture from the ninth tube.

c) Add 0.1 ml Of 2 % sheep red blood cells to every tube, mix and keep the tubes at room temperature for 2 hours.

d) Observe the agglutination of sheep red blood cells by visual reading. This may also be confirmed by microscopic examination. Presence of agglutination is interpreted as positive test.

e) The antibody titer is the reciprocal of the highest dilution which results in agglutination.

f) If the difference of antibody titer on day 0 and

day 7 is greater than 2 fold dilution, a differential test of the heterophile antibody is performed to differentiate heterophile antibody from serum sickness from that of infectious mononucleosis.

2.5.2) Differential test for heterophile antibody.

a) Prepare 2 rows of test tubes, 10 tubes per row. The first row of tubes is for guinea pig - absorbed serum. The second row of the tubes is for ox red blood cell - absorbed serum. Diagram was shown in Table II.

b) The first tube of each row is blank, 0.25 ml normal saline is added from the second to the tenth tube of each row.

c) Prepare another two tubes.

1st tube :- 0.2 ml inactivated serum + 1 ml 20 % guinea pig kidney suspension.

2nd tube :- 0.2 ml inactivated serum + 1 ml 20 % ox red blood cell suspension.

mix each tube well and kept at room temperature for 3 minutes, shaking it every minute.

d) Centrifuge these 2 tubes at 1,500 rpm for 10 minutes, the supernatant is used as the absorbed serum.

e) 0.25 ml of each absorbed serum is pipetted into the first and the second tubes, the mixture in the second

tube is mixed and transfer 0.25 ml to the third tube and so on up to the minth tube. The tenth tube serves as negative control. Discard 0.25 ml of the mixture from the ninth tube.

f) The next method is as 2.5.1 c-d.

Heterophile antibody may be found in healthy persons with titers $\leq$ 1:56. If antibody titer on day 7 or day 14 shows four fold rise from the titer on day 0, it is interpreted that serum sickness is in the active state. However the antibody titer does not correlate with the severity of the disease.(36)

g) Antibody titer after absorption are interpreted according to the criteria listed in Table III which differentiate the heterophile antibodies found in normal individuals, infectious mononucleosis and serum sickness.(36,38)

2.5.3) Radial immunodiffusion for C_3 level

a) Mix 2.5 ml each of 2 % agarose and 2 x barbitone buffer, boil and incubate at 56°C, then add 100 Ul of anti C_3 and mix well.

b) Pour 1 ml of the mixture into Radial Immunodiffusion plate with plastic cover. Size of plate is 9.5 cm x 3.8 cm, Hyland company.

c) Keep the plate at room temperature until the agarose is hard.

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d) Use the gel cutter to make 2 -mm holes on the plate, each 12 mm apart.

e) Fill 8 Ul of the serum and the standards into the holes in each plate.

f) Incubate these plates in a moist chamber for 16 - 18 hours at 37°C.

g) Read the diameter of precipitin ring using a magnifying lens.

h) Plot graph between log concentration and diameter.

i) Read the unknown serum from the graph.

2.5.4) Radiolabelled C_1q binding assay for the detection of soluble immune complex.

2.5.4.1 Preparation of serum for C_1q purification Collect 100 ml of whole blood from a single donor, leave it to clott at room temperature. Centrifuge at 4°C, 2000 rpm for 10 minutes and collect serum. Fasting Blood is used to avoid lipid interference in the serum. If there is lipid in the serum, the serum should be centrifuged at 10,000 rpm, and 4°C for 30 minutes. Lipid will be in the upper layer and discarded by suction. The serum should be used immediately or kept at-70°C until use.

2.5.4.2 Purification of human C_1q

a) Mix 40 ml serum with 10 ml of solution A. (0.1 M EDTA, pH 7.5) stir slowly and gently to dissociate C_1qrs complex in the serum at 37°C for 10 minutes.

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b) The mixtures in (a) is put in the icebath and adjust pH to 7.5.

c) Add 200 ml of solution B (0.005 M EDTA) into the mixture (b), stir slowly and keep in the icebath for one hour. Stir mixture every 20 minutes.

d) Centrifuge mixture from (C) at 10,000 rpm, and 4° C for 30 minutes. Discard the supernatant and keep only the precipitate.

e) Wash precipitate from (d) with solution C (0.02 M EDTA), stir and centrifuge at 10,000 rpm, and 4° C for 30 minutes. Repeat washing of the precipitate 2 times and keep only the precipitate.

f) Add 10 ml solution D (0.01 M EDTA, pH 5) into the precipitate from(e),mix well. Precipitate will dissolve in solution D and leave this mixture overnight. In the morning, centrifuge this mixture at 16,000 rpm, and 4° C for 30 minutes. Collect the supernatant into the dialysis tube and dialyse against solution E (0.1 M EDTA, pH 5.0)at 4° C, for 24 hours. Change solution E 2 times during dialysis with magnetic stirrer.

g) Centrifuge the mixture from dialysis tube at 12,000 rpm, and 4° C for 30 minutes. Wash the precipitate with solution E and centrifuge. Repeat washing the precipitate 2 times.

h) Dissolve precipitate from (g) in 3 ml of solution F (0.01 M EDTA, pH 7.5). Keep this mixture at 4° C for 2

hours, then centrifuge at 16,000 rpm, and 4° C for 30minutes. C_1q will dissolve in supernatant. The impurity will be in the precipitate.

i) Aliquot purified C_1q into small tubes and kept at -70° C untill use.

j) Measure the O.D. of the supernatant at λ 280 nm, cuvette 1 cm.

If O.D. of standard = 6.82

The concentration of standard is equal to 1 gm % (10 mg/ml)

Therefore OD unknown 2.82 (at λ 280 nm) x Factor 1.466

= concentration C_1q mg/ml

= 4.134 mg/ml

k) Test for purity of C_1q

The degree of purity is determined by immunoelectrophoresis

2.5.4.3 Radiolabelling of C_1q

a) 50 Ul C_1q mixed with solution F 500 ml in the flask, put this flask into the icebath. Add the following agents

a.1 5 μl I^{125} solution (= 500 μCi)

a.2 5 μl 0.006 mg/ml NaI in VBS.

a.3 5 μl 1 mg/ml lactoperoxsidase

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a.4 5 µl 0.003 % H_2O_2

b) Shake the tube well and incubate in icebath for 25 minutes.

c) Add 10 µl 6 mg/ml NaI

10 µl 0.03 mg/ml NaN_3

2 ml VBS.

d) Aliquot the mixture from (c) into 2 tubes, 5 µl each tube. The less is dialysed.

d.1 The first tube; add 20 % trichloracetic acid 500 Ul mix well, centrifuge in Ependof microcentrifuge for 5 minutes. Discard the supernatant. Measure the γ -ray from precipitate. This precipitate is I^{125} bound C_1q

d.2 The second tube, this tube will be I^{125} bound C_1q and free I^{125} in mixture. Measure γ -ray from the second tube.

$$\text{Percent Yield} = \frac{\text{CPM from precipitate}}{\text{CPM from the mixture}} \times 100$$

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$$= \frac{351212}{962039} \times 100$$

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Percent of radioactivity uptake = 36.5 %

e) Dialyse labelled C_1q in VBS at 4° C overnight with magnetic stirrer.

f) Add 5 ml VBS into the dialysis tube after dialysing.

g) Aliquot the solution from (f) into a small tube, 50 µl/tube.

These are radiolabelled C_1q and are kept at -70°C until use.

2.5.4.4 I^{125} bound C_1q binding assay for circulating immune complex.

The principle of this test is to measure the amount of C_1q which can be bound by the immune complexes in the test serum. Radiolabelled C_1q (I^{125} C_1q) is mixed with the test serum and free c_1q is separated from C_1q bound complexes by precipitation with polyethylene glycol (PEG).

EDTA is used to prevent the integration of I^{125} C_1q into the intrinsic C_1qrs complexes.

PEG will suspend free I^{125} C_1q in the mixture where as I^{125} C_1q bound to macromolecular substances (immune complex) will be precipitate.

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Stock I^{125} C_1q 100 µl is thawed and added with 3900 µl 0.1 % Bovine Serum Albumin in VBS. Mix well, then centrifuged at 18,000 rpm, 4°C, for 40 minutes. The supernatant is used in the test.

a) 50 µl test serum is mixed with 100 Ul of 0.2 M EDTA

incubated at 37°C for 30 minutes.

b) Add 50 μl I^{125} C_1q (labelled C_1q) and 1000 μl of 3 % W/V PEG in borate buffer.

c) Incubate the tube at 4°C for one hour without shaking or mixing the tube.

d) All tubes are centrifuged at 12,000 rpm for 5 minutes.

e) Discard supernatant and place the tube up-side down for overnight.

f) In the morning, these tubes along with the precipitate are put into the counter to count the γ- ray which is emitted from the labelled complexes.

g) Serum from healthy blood donors and standard aggregated human immunoglobulin are used instead of test serum as negative and positive controls respectively.

h) The TCA control tube is prepared by mixing 50 μl of I^{125} C_1q, 50 μl of normal healthy serum and 1 ml of 20 % TCA, % C_1q binding with CIC

$$= \frac{\text{CPM from } I^{125}\ C_1q - \text{CIC precipitate}}{\text{CPM from TCA control}} \times 100$$

Table I Diagram for Presumptive Heterophile Ab test.

Tube	1	2	3	4	5	6	7	8	9	10
0.85% Saline	0.4	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Serum	0.1	0.25	0.25							->discard 0.25
2% Sheep cells	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final serum dilutiom	1:7	1:14	1:28	1:56	1:112	1:224	1:448	1:869	1:1792	

Table II Diagram for Davidsohn Differential test by using absorbed serum.

Tube	1	2	3	4	5	6	7	8	9	10
0.85% Saline	–	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Absorbed serum	0.25	0.25	0.25	0.25						->discard 0.25
2% Sheep cells	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final serum dilutiom	1:7	1:14	1:28	1:56	1:112	1:224	1:448	1:869	1:1792	

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Table III Interpretation of differential heterophile antibody test.

	Presumptive test	Absorbed with G.P. kidney	Absorbed with OX Rbc
Normal or other conditions	0-1 : 56	titer decreased more than 2 dilutions	no change of titer or slighthy decreased
Infectious mononucleosis	1:28-1 : 7168	no change of titer or slightly decreased	titer decreased more than 2 dilutions
Serum sickness Syndrome	1 : 56	titer decreased more than 2 dilutions	titer decreased more than 2 dilutions

GP. = Guinea Pig

Rbc = red blood cell

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