CHAPTER II

MATERIALS AND METHODS

1. Subjects

78 patients were recruited for the study who met the following criteria:

1.1 Age from 15-50 years

1.2 Nerver been immunized against rabies

1.3 Never taken any immunosuppressive drugs with 2 weeks befor rtabies vaccination (such as chloroquine, corticosteroid, cytotoxic durgs etc.)

1.4 Bitten by dogs or animals suspected of being rabid no later than 3 days before attending rabies clinic.

1.5 No severe wounds at the high risk areas such as face or arm.

1.6 Definite proof of rabies in the biting animals was not available, such as no post-mortem examination of animal brain or run-away animals.

The study was approved by the ethical committee of Faculty of Medicine, Chulalongkorn University.

2. Vaccines and Rabies Immune Globulins

2.1 Vaccines

Purified vero cell rabies vaccine (PVRV, Imovaxc Rabies Vero®, Institute Merieux, Lyon, France) lot A 1067 with a potency of ≥ 2.5 IU/0.5 ml dose was used in the study.

2.2 Rabies immune globulins

2.2.1 Human rabies immune glubulin (HRIG, Imogam® Istitute Merieux, Lyon, France), batch A 0038 at 150 Iu/ml was used in the study.

2.2.2 Equine rabies immune globulin (ERIG, Pasteur Antirabies serum®, Pasteur Vaccine, France) lot-L 5298 at 200 Iu/ml was used in the study.

3. Immunization Regimens

The patients were radomly allocated into 4 study groups as shown in Table 1.

Group A : 24 subjects (16 males and 8 females) ranging in age from 19-50 ($X \pm SD = 32 \pm 10.04$) received 5 intramuscular (I.M.) injection of one0.5 ml dose each of PVRV on day 0,3,7,14 and 28 at deltoid areas.

Group B : 22 subjects (7 males and 15 females) ranging in age from 15-50 ($X \pm SD = 28.54 \pm 10.6$) received I.M. 2-1-1 PVRV, i.e., 2 doses of PVRV injected I.M. into

Table 1 Immunization Regimens

Group	No.	Route	Immunization Day							RIG
			0	3	7	14	21	28	90	
A	24	IM	0.5	0.5	0.5	0.5	–	0.5	0.5	None
B	22	IM	2×0.5	–	0.5	–	0.5	–	–	None
C	14	IM	2×0.5	–	0.5	–	0.5	–	–	HRIG 20 IU/Kg.
D	17	IM	2×0.5	–	0.5	–	0.5	–	–	ERIG 40 IU/Kg.

both deltoids on day 0, followed by one dose on day 7 and 21

Group C : 14 subjects (8 males and 6 females) ranging in age from 15-45 ($X \pm SD = 27.21 \pm 11.32$) received I.M. 2-1-1 PVRV and HRIG at 20 IU/kg by injecting the entire amount of HRIG at gluteal ares on day 0.

Group D : 17 subjects (9 males and 8 females) ranging in age from 16-48 ($X \pm SD = 26.64 \pm 8.99$) received I.M. 2-1-1 PVRV and ERIG at 40 IU/kg in kthe ksame manner as HRIG.

4. Blood Collection

4.1 Ten ml of heparinized blood (52 units of heparin, Leo Pharmaceutical Products, Ballerup, Denmark, per 1 ml of blood) was obtained aseptically from each patient in group A and B on day 0,7,14,21,28 and 90 and used for lymphocyte transformation test.

4.2 Ten ml of clotted blood was obtained aseptically from all subjects of all groups on day 0,7,14,21,28,90 and 180. Sera were aliquoted in sterile tubes and stored at -70°C until tested for antirabies antibody.

5. Lymphocyte Transformation Test

5.1 Separation of Lymphocytes

Mononuclear cells were seprated from the heparinized blood by Ficoll-Hypaque density gradient. (see, Appendix II) (125). Four ml of heparinized blood were overlayered on 3 ml of Fivoll-Hypaque solution by aseptic technique and cintrifuged at 220 G for 30 minutes at 20°C. The mononuclear cells at Ficoll-Hypaque-Plasm interface were collected by the use of pasteur pipettes. The cells were washed twice with 5 ml of Hank's Balanced Salt Solution (HPSS) (Gibco, Grand Island, N.Y.,U.S.A) by centrifugation at 1800 rpm. (200 G) 4°C for 5 minutes. After the second wash, the mononuclear cell pellet was resuspended in 5 ml RPMI 1640 sepplemented with 5% heat inactivated pooled human AB serum, 100 ug/ml streptomycin (Dumex, Bangkok, Thailand), 100 units/ml penicillin (Dumex, Bangkok, Thailand) and 10 mM HEPES (Sigma, Mo., U.SA) (See, Appendix II). The cells were counted in a hemocytometer and adjusted to $2x10^6$ cells/ml with TCM.

5.2 In vitro Lymphocyte Transformation Test. (126)

The antigen-induced lymphocyte transformation test was used as the measurement of a specific cell-mediated immune response following rabies vaccination. The peripheral blood mononuclear cells separated as described above were cultured for 6 days in the microtiter plate (Falcon Beckton Dickinson Company, Oxnard, CA 93030, U.S.A)

both in the presence and absence of rabiesantigen. 100 ml of mononuclear cell suspension ($2x10^6$ cells/ml) in TCM were cultured in flat-bottomedmicrotiter plates with 100 ul of 1:5 PVRV diluted in TCM (124) as antigen. The cultures were performed in triplicate and were incubated at 37°C. in humidified atmosphere of 5% CO_2 and 95% air for 6 days. Eighteen hours before harvest, 20 ul of 0.5 uCi ^{3}H-thymidine (specific activity 8.3 mCi/mg Amersham, Buckinghamshire, U.K.) were added. ^{3}H-thymidine incorporation was determined by harvesting cells with a multichannel automatic cell harvester (CH-103, Dynatech Lab, Inc., Sussex, U.K.) and the radioactivity was measured in a liquid scintillation counter.

The degree of lymphocyte proliferative response was expressed as CPM and stimilation index (SI). The CPM is the difference between the stimulated and unstimulated CPM and the stmulation index is the ratio of the stimulted CPM and the unstimulated CPM (S.I. = Stimulated CPM/ Unstimulated CPM).

5. Titration of Rabies Neutralizing Antibody

Serum neutralizing antibodies to rabies virus were assayed by the rapid immunofluorescent focus inhibition test (RIFFIT) according to the modified method of Smith et al. (127). All serum specimens were heat-inactivated at 56°C 30 minutes before assay. The international standard antiserum to rabies was a gift from Dr. Urich Bijok, Behring

Institute, West Germany and included in each assay. The antibody levels were expressed as international units per millitre.

Serum samples were serially diluted 2 folds to eight dilutions in microtiter plate, Costar, 205 Broadway Cambridge. MA 02139 U.S.A) using BHK (Baby Hamster Kidney) cell growth medium (see, Appindix II). Stock virus suspension at 4 PFU/ml (Plaque Forming Unit) of CVS (Challage Rabies Virus Standard Strain) was diluted 1:10 and 50 ul of this suspension was adfded to equal volume of each serum dilution inj the microtiter plate. After gentle mixing, the plate was incubated at 36°C in a humudified atmosphere of 5% CO_2 and 95% air for 90 minutes. Trypsinize (0.05% Trypsin, see Appendix II) BHK cells in tissue culture flask (Nunclon, Nunc, Denmark) and adjust to a concentration of 1 x 10^5 cells/ml. Fill 50 ul of cell suspension to each well containing the virus-serum mixture and gently shake. Incubate at 36°C in a humidified atmosphere of 5% CO_2 and 95% air overnight (20 hrs). Remove medium and wash cell monolayer with PBS. Fix with 90% acetone (cold acetone), air dry. Stain the monolayer cells in each well with 20 ul FITC-labelled anti-rabies conjugate (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD 21030) for an hour at 36°C. Wash 2 times with PBS. Use an invertedmicroscope epi-fluorescence attachment (High pressur ;amp HBO 100 W/2, Nikon, Tokyo 100, JAPAN) to examine 8 fields of each well at a magnification of x 200. Each

sample was performed in duplicate. Count the number of uninfected fields. (uninfected field + negative field). Calculate the dilution which gives a 50% reduction in the number of negative fields using the Karber formala :

$$\text{Log } TCD_{50} = L - d\,(s - 0.5)$$

L = Log of strongest dilution

d = Difference between log dilutions

s = Sum of proportion of negative fields

TCD = Tissue culture dose

For example,

if the negative field count is :	7	3	2	0
then,the proportion of negative field=	0.87	0.37	0.25	0.00

S = 1.47

The starting dilution is 1:32, log = -1.5

The dilution are two-flod + 1:2, log = +0.3

Then, Log TCD 50 = -1.5-0.3 (1.47-0.5)

= -1.797

Antilog = dilution giving 50% reduction in negative fields

= 1/62

Convert to International Units using the standard serum. The negative and positeive field were illustrated in Figure 3 and 4 respectively.

6. <u>The Indirect Enzyme Linked Immunosorbent Assay (ELISA)</u>

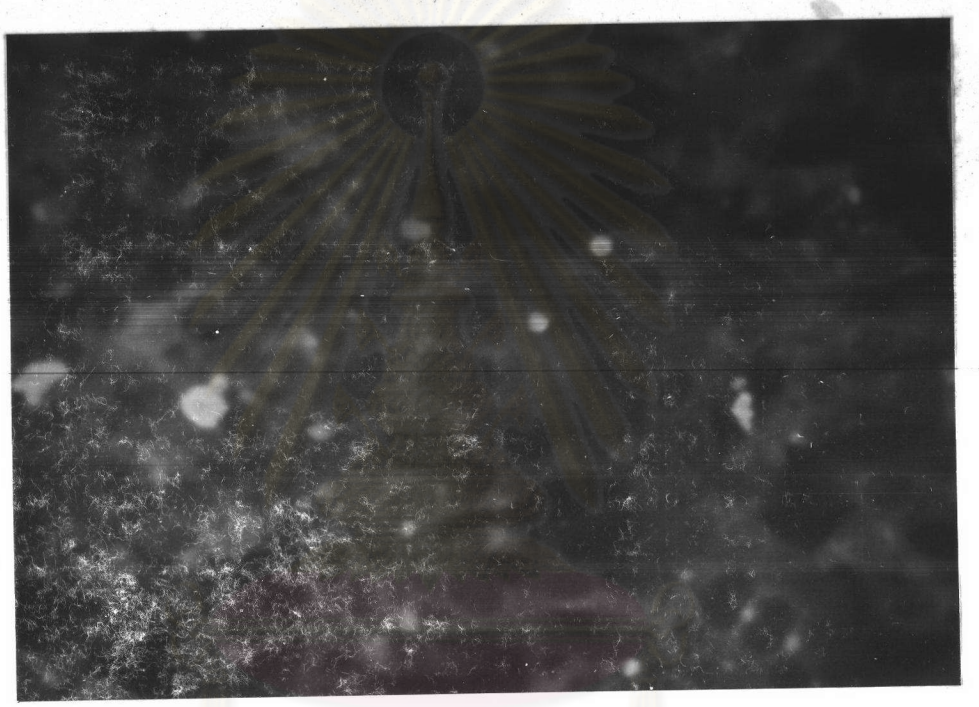

Figure 3. The immunofluorescent staining of rabies non-infected BHK-21 cells (negative fields) by FITC-antirabies immunoglobulin at the magnification 200X.

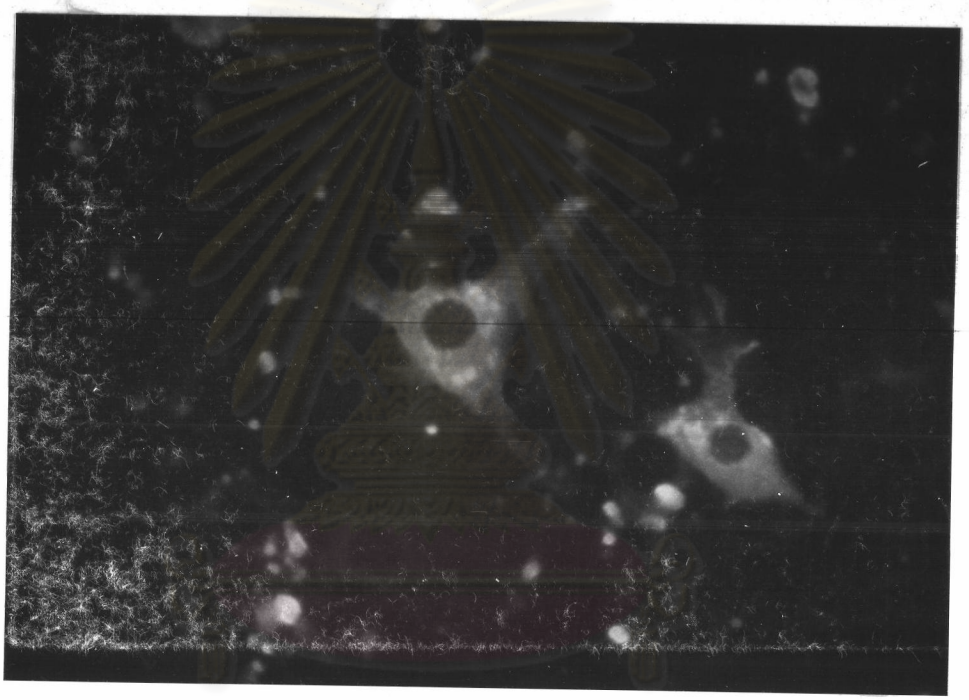

Figure 4. The immunofluorescent staining of rabies infected BHK-21 cells (positive fields) by FITC-antirabies immunoglobulin at the magnification 200X.

for Antirabies Antibody Titration (128,129)

Rabies antigen used for antibody estimation by ELISA was the same PVRV used for immunization. It was diluted in carbonate buffer (0.06 M, see Appendix II) and a dilution of 1:150 was found to be optimal in a preliminary study. Each well of microtiter plate (Costar, 9j6 well-flat buttom,USA) was coated with 200 ul of 1:150PVRV diluted in carbonate buffer, pH 9.6. Incubate overnight at 37°C and wash 4 times with PBS containing 0.05% Tween 20 (see Appendix II). Serum samplewere diluted in PBS-Tween 20 and 1% bovine serum albumin. Add 100 ul of 1:20, 1:40, 1:80 diluted sera and incubate 2 hrs at 37°C. After 4 washings with PBS-Tween 20. The rabbit antihuman immunoglobulin peroxidase conjugate (Lot 088, code P212, Dako, Denmark), diluted 1:4,000, 100 ul/well was added, incubated 90 minutes at 37°C. After washing, 100 ul of the subtrate (Orthophenylene diamine dihydrochloride, OPD, Sigma : in citric acid-phosphate buffer, see Appendix II) was added. Incubate at room temperature for 30 minutes in the dark. Stop the reaction with 50 ul of 0.5 N H_2SO_4 in each well. The absorbance values were obtained by ELISA Processor Photometer (Behring ELISA processor II, Behring Werke, W.Germany) wavelength 492 nm.

Conversion of antibody titers to the internationl untis was perfromed by creting a stardard curve from Imogam (HRIG) 150 IU/ml, which was used as a standard antirabies

antibody, diluted at various concentrations : 0.06, 0.12, 0.24, 0.47, 0.95, 1.9, 3.8 and 7.5 IU/ml. These concentrations corresponded to the dilutions of 1:20 respectively. Simultaneously assay the negative control sera (negative neutralizing antibody by RIFFIT) at the corresponding dilutions. Plot OD of Imogam VS the concentrations, where the OD is the difference between the OD to the test serum and the OD of negative control serum at the same dilutions.