MATERIALS AND METHODS

Animals: Big brown Indian frugivorous bats from natural populations were supplied by an animal supplier at Calcutta and maintained in our laboratory with adequate food and water ad libitum. Healthy adult bats of both sexes with body weight ranging from 440 to 520 grms were chosen randomly for the experiments.

Media: Isotonic Dulbecco's phosphate buffered saline (PBS), Hank's balanced salt solution (HBSS), Earl's balanced salt solution (EBSS) and minimal essential medium (MEM) were obtained from HI MEDIA, Bombay, India. All media and balanced salt solutions were supplemented with 10% heat inactivated goat serum as this serum was established in our laboratory to be a good and cheaper substitute for the more costly foetal calf serum (Chaudhuri and Chakravarty, 1983).

Collection of lymphocytes : The method of Chaudhuri and Chakravarty (1983) was followed with certain modifications. Briefly, secondary lymphoid organs like spleen, mesenteric, axillary and maxillary lymph nodes were dissected aseptically from anasthetized bats and rinsed in PBS. The organs were cut into small pieces and pressed against a stainless steel wire

mesh to dissociate the cells. Further dissociation of the small clumps of cells was brought about by passing the cell suspension through a 27 gauge hypodermic needle. Contaminant red blood cells were lysed by subjecting the cells to hypotonic shock in 0.84% NH_4Cl dissolved in 0.1M Tris-HCl (pH 7.2) for 10 minutes, followed by two immediate washes in excess PBS. The lymphoid cells were then resuspended in serum supplemented EBSS or HBSS containing 50 U/ml of penicillin-streptomycin and 50 U/ml of nystatin.

Separation of plastic adherent cells : About 5 ml of the cell suspension containing upto 2×10^8 cells was spread on a sterilised plastic petridish of 5" diameter and incubated at $37^{\circ}C$ for 1 hour. Then the supernatant containing the plastic non adherent cells was gently pipetted off. The plastic adherent cells were then removed from petridish by scrapping with a rubber policeman. Alternatively, treatment with 0.2% EDTA for a few minutes also released the cells from the plastic. The adherent and non adherent cells were then separately resuspended in serum supplemented EBSS for further experimentation.

Nylon wool column separation of lymphoid cells : Out of several techniques for separating the T and B lymphocytes of higher vertebrates, we opted for the nylon wool fibre column separation technique as outlined by Julius and coworkers (1973) because of

its simplicity and rapidity and also because the cells are not exposed to any harsh treatments during this procedure.

About 400 mg of nylon wool was cut into small pieces and teased into loose fibres devoid of knots. The wool was then boiled in 1N HCl for 10 minutes to remove any toxicity. Then washing in boiling tripple distilled water was done for 3-4 times to remove the acidity. The fibres were then air dried and loosely folded and packed in a 10 ml glass syringe upto the 6 ml mark. In another method, suggested by Henry (1980), the washed wool was soaked overnight in a mixture of 0.2% EDTA and 0.2% $NaHCO_3$ washed in distilled water, dried and packed in the syringe. In all cases, the nylon wool column was sterilised by autoclaving and then incubated in serum free EBSS for 45 minutes at 37°C.

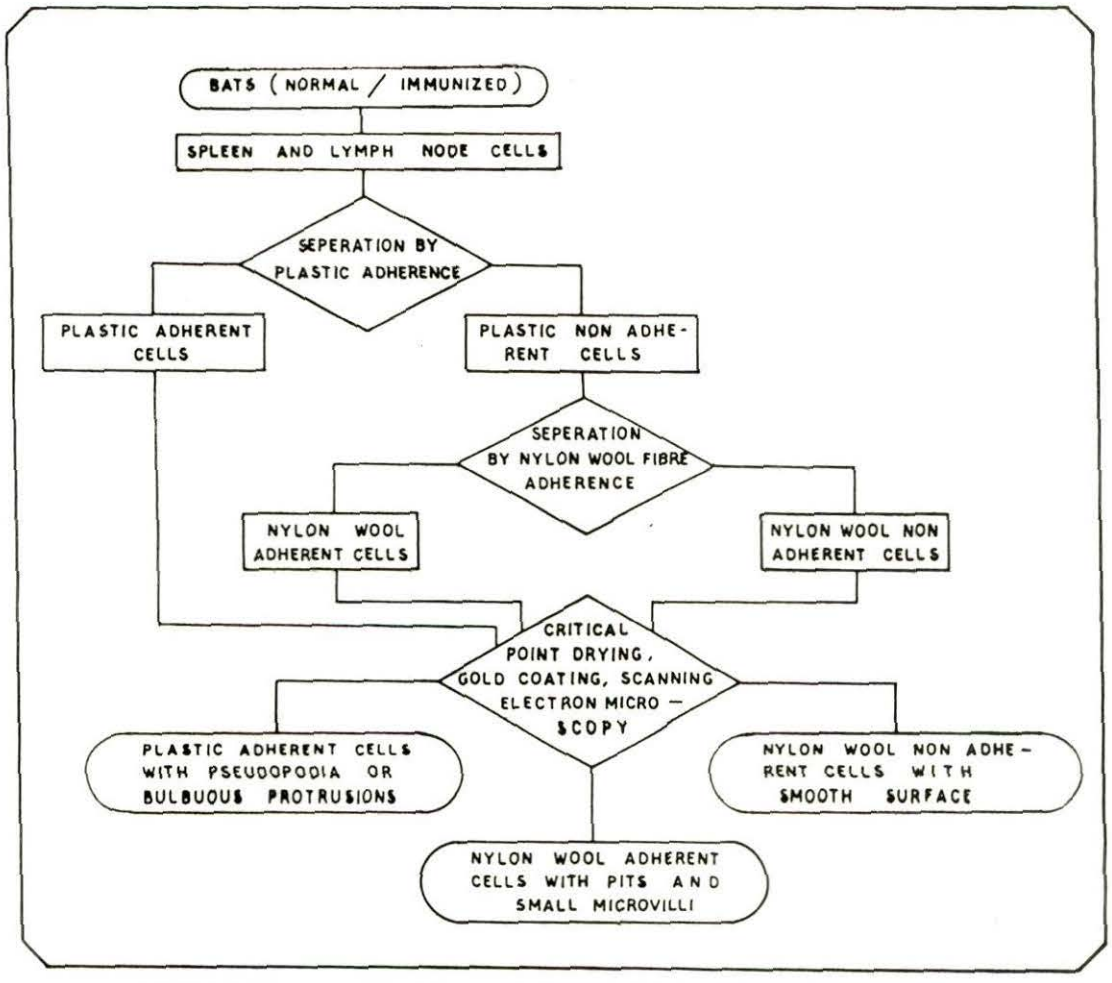
About 5 ml of the cell suspension containing upto 10^8 cells/ml in serum supplemented warm EBSS or MEM was carefully loaded in the presoaked and prewarmed nylon wool column and incubated for 1 hour at 37°C in a humidified atmosphere containing 5% CO_2. Then the nylon wool non adherent cells were eluted out with an excess amount of warm EBSS, and resuspended in fresh medium. The column was then filled with chilled HBSS and further incubated in ice for 10 minutes. Now the nylon wool adherent cells were eluted out with an excess

amount of cold HBSS by vigorous agitation of the wool and then resuspended in fresh medium.

Preparation for scanning electron microscopy :

A. Fixation and Dehydration The isolated cell populations were fixed and dehydrated following standard techniques (Pease, 1964). Briefly, the cells were resuspended in 0.2M Sodium cacodylate buffer (pH 7.2) and smeared on grease free cover-slips appropriately marked at the side with a diamond tipped pencil. After 10 minutes, when the cells settled on the glass surface, the overlying buffer was drained off and 2% Gluteraldehyde in the same buffer was overlaid. The cells were fixed for 3 hours after which the fixative was removed by several gentle rinses in cacodylate buffer. The cells were then post fixed with 1% Osmium tetroxide in cacodylate buffer for 10 minutes. Excess fixative was removed by several gentle rinses in distilled water after which the specimens were dehydrated in 30, 50, 70, 90 and 100% ethyl alcohol with two changes in each.

B. Critical point drying : Although a few samples were dried in air, most of the cell samples were dried by the critical point drying technique since this technique has been shown to preserve the cell surface details better (Polliack et al, 1973a;



EXPERIMENTAL PROTOCOL FOR STUDY OF CELL SURFACE MORPHOLOGY OF IMMUNOCOMPETENT CELLS OF BAT.

Bartlett and Burstyn,1975). The cell samples on the coverslips were transferred from absolute ethyl alcohol to anhydrous amyl acetate and critical point dried using liquid CO_2 as the transitional fluid in a Polaron Critical Point Drier at the Regional Sophisticated Instrumentation Centre, Bose Institute, Calcutta, following standard methodologies (Albrecht, Jordan and Hong, 1978).

C. Gold coating : The critical point dried cell samples on the coverslips were coated with 150-200 Å gold layer in a sputter coater at the R.S.I.C., Calcutta.

D. Scanning electron microscopy : The gold coated cell samples were scanned in a Phillips Scanning Electron Microscope with an accelerating voltage of 20 KV at the R.S.I.C., Calcutta. Photographs were taken on ILFORD FP4 panchromatic film and appropriately developed.

Preparation of BSA-Sepharose immunoadsorbent for isolation of bat Ig: Bovine serum albumin (BSA, obtained from Sigma, USA) was immobilised on Sepharose 6B-100 dextran following standard methodology (Porath et al, 1967; March et al, 1974). Briefly, 20 gm of Sepharose 6B-100 (wet weight) was washed with distilled water on a sintered glass funnel and then suspended in 40 ml of 2M K_2CO_3. 2 gm of Cyanogen Bromide

(CNBr) was dissolved in 1 ml acetonitrile and mixed with the Sepharose slurry. The mixture was stirred slowly in cold for 2 minutes, then washed immediately with cold distilled water in a coarse sintered glass funnel. Finally, the mixture was washed 2 times with 0.1M $NaHCO_3$. BSA (300 mg in 60 ml of 0.1M $NaHCO_3$) was then mixed with the activated Sepharose; the reaction mixture was stirred gently for 18 hours at 4°C. Ethanolamine (100 µl) was added to the conjugate to neutralise any unbound active groups generated by CNBr on the Sepharose molecules. The mixture was again stirred for 30 minutes in cold, then washed with 0.1M $NaHCO_3$ until the filtrate showed near zero absorbance at 280 nm. Finally, the BSA-Sepharose immunoadsorbent was suspended in 0.1M PBS for further use.

Raising of bat anti-BSA serum : BSA from the same lot as used for the preparation of immunoadsorbent was injected in bats at a dose of 10 mg in 1 ml PBS by intravenous route. The bat anti-BSA serum was collected after 12 days and pooled.

Isolation of bat anti-BSA immunoglobulins: The BSA conjugated sepharose was washed 3 times with 0.01M PBS pH 7.2 and packed in a 2.3 x 14 cm chromatography column avoiding air bubbles. 4.0 ml of bat anti-BSA serum previously dialysed for 48 hours against 0.01M PBS, was carefully layered on the column and allowed

to enter the gel. Then elution was started with 0.1M PBS pH 7.2 until the absorbance of the eluate at 280 nm went below 0.01. The eluate was passed through the column two more times to ensure complete binding of the antibodies with the BSA in the column. The column was thoroughly washed with PBS, and elution of the anti-BSA antibodies was started with 3M ammonium thiocyanate (NH_4SCN). The eluate was taken in 1 ml quantities in small glass tubes containing 4 ml distilled water. Elution was continued until the eluate showed an absorbance less than 0.02 at 280 nm. The eluted fractions were immediately pooled and dialysed in cold against triple distilled water with several changes a day for two days, and then concentrated by vacuum dialysis at low temperature.

Isolation of bat IgM and IgG by Sephadex G-200 gel filtration:

Sephadex G-200 dextran beads (Pharmacia Fine Chemicals, Sweden) were washed successively in 1N HCl, distilled water, 1N NaOH and again distilled water until the pH of the wash was neutral. The beads were swollen in 0.1M Tris-HCl, pH 7.3 containing 0.15M NaCl for 3 days. The swollen gel was packed in a 2.5 x 60 cm chromatography column avoiding air bubbles, and washed with the same buffer using a hydrostatic pressure of about 12 cm. The bat immunoglobulin solution obtained by affinity chromatography was carefully layered on the gel bed and elution started. A flow rate of about 5 ml/hour was maintained and the eluate

was collected in 3 ml fractions. Absorbance of the eluate at 280 nm was recorded and plotted against the fraction number. Two absorbance 280 peaks were obtained (fig. 4), the first peak eluting in the void volume and the second peak just after. The fractions corresponding to the two absorbance peaks in the elution profile were pooled, dialysed against several changes of cold tripple distilled water, lyophilised by vacuum dialysis and stored desiccated at -20°C in 2 mg aliquots for further use.

Raising of rabbit anti sera against bat IgM and bat IgG : 2 mg of either bat IgM or IgG was dissolved in 0.5 ml PBS and mixed well with Freund's Complete Adjuvant (FCA) in 1:1 ratio. The emulsion was injected through subcutaneous route in the left thigh of a rabbit from which normal serum had already been collected. The rabbits were given 6 such weekly injections and bled 72 hours after the last booster dose and respective rabbit antisera against bat IgM or IgG were collected.

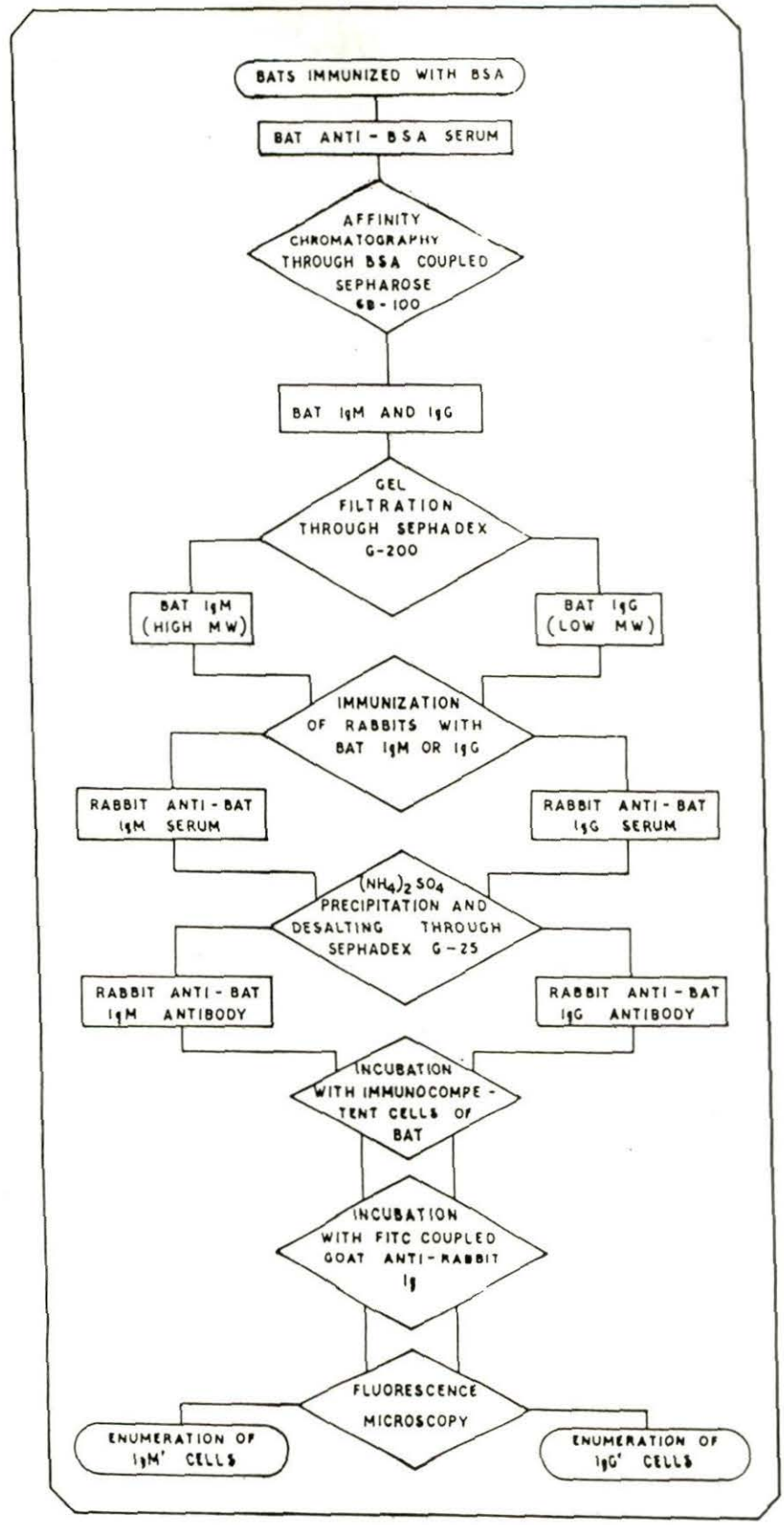
Ammonium sulphate precipitation of immunoglobulins raised in rabbit: 2 ml of the rabbit antiserum against bat IgM or bat IgG was mixed slowly with 2 ml of saturated ammonium sulphate under constant stirring in cold. The precipitate produced by such 50% ammonium sulphate saturation was collected by centrifugation at 10,000 g and reconstituted to original volume (2 ml) in tripple distilled water. To this, 1 ml of saturated ammonium

sulphate was slowly added under constant stirring in cold. The precipitate formed by such 33% ammonium sulphate saturation was collected by centrifugation, reconstituted to original volume in tripple distilled water and passaged through a 15 cm x 2.5 cm column of sephadex G-25 to remove the ammonium sulphate molecules. The protein solutions were concentrated by vacuum dialysis and stored in small aliquots at -20°C.

Polyacrylamide gel electrophoresis of bat Ig: The method of Davis (1965) disc gel electrophoresis was used with little modifications. About 9 cm long separating gel column was formed of 10% acrylamide; the overlying 1 cm long stack gel column contained 2.5% acrylamide. Polymerization was brought about by TEMED and freshly prepared ammonium persulfate. For separation of the Ig classes of bat, 20 µl of affinity purified bat Ig was mixed with 20 µl of 2M sucrose and 5 µl of 0.05% Bromophenol blue indicator dye. The mixture was layered over the stack gel and the tubes were gently filled up with Tris glycine electrode buffer. BSA and purified human IgG (obtained from Sigma, USA) were used as standard markers. Gels were run for 3-4 hours in cold at 3-4 mA/tube current at 300V. After the run, gels were briefly treated with 10% TCA, stained with Coomanic Brilliant Blue and destained in methanol-acetic acid mixture. Photographs were taken on 24 ASA black and white film.

Conjugation of goat-anti-rabbit IgG with fluorescent dye : The method of Goding (1976) was used with brief modifications. Briefly, fluorescence in Isothiocyanate (FITC), isomer II on celite (obtained from Sigma, USA) was dissolved in 0.15M $Na_2HPO_4 \cdot 2H_2O$ (pH 9.0) buffer to a final concentration of 1.0 mg/ml immediately before use. 0.2 ml of this solution was mixed under constant stirring with 2 ml of goat anti-rabbit-IgG immunoglobulin solution (concentration about 10 mg/ml in the same buffer) at room temperature. The pH of the reaction mixture was adjusted to 9.5 by addition of 0.1M $Na_3PO_4.10\ H_2O$. The reaction was continued for 2 hour in dark, after which the mixture was charged on a Sephadex G-25 gel filtration column (15 x 2.5 cm). The fluorochrome conjugated immunoglobulins were eluted out in the void volume using 0.1M PBS. This fraction was quickly concentrated by vacuum dialysis in dark and stored at -20°C. To evaluate the labelling ratio, a small part of the conjugate was diluted in distilled water and subjected to spectrophotometry at 280 nm and 495 nm. The ratio of the absorbance 495 to absorbance 280 was found to be 1.87 which was quite satisfactory for our purpose.

Immunofluorescence microscopy for detection of IgM and IgG bearing cells of bat: An indirect immunofluorescence technique using incident illumination was adopted. Immunocompetent cell types from secondary lymphoid organs, peripheral blood and bone



EXPERIMENTAL PROTOCOL FOR STUDY OF Ig+ IMMUNOCOMPETENT CELLS OF BAT.

marrow were taken in 0.1 ml cold PBS with cell concentration at 10^7 cells/ml and incubated at 4°C for 30 min. with 100 µl of 1:10 dilution of rabbit anti bat IgM or rabbit anti-bat IgG antibodies in presence of 0.1% NaN_3. The cells were then washed twice with cold PBS containing 0.1% NaN_3 and further incubated with 100 µl of 1:10 dilution of goat anti rabbit Ig coupled with FITC for 30 min. at 4°C in presence of 0.1% NaN_3. After incubation, cells were washed with PBS at 4°C two times and resuspend in 0.1 ml of 1:1 mixture of PBS and glycerol at 4°C. For control, cells were first incubated with normal rabbit serum, washed twice, and incubated further with fluorescinated goat anti-rabbit Ig followed by two washes, and the cells were finally resuspended in glycerol PBS. Cells were then taken on a haemocytometer slide, covered with a cover slip and examined in a Zeiss FLUOVAL fluorescence microscope equipped with epi-illumination from a HBO 200 UV lamp. D224 excitation filter for blue excitation and BG47 barrier filter was used. Fluorescent cells were photographed on Kodak 400 ASA film exposed for 4-6 minutes and boosted to 800 ASA during development.

Raising of rabbit anti-bat brain serum : Brain cells of mouse, rat etc. are known to share the θ or Thy-1 cell surface antigen with T lymphocytes and anti-brain serum has been shown to recognise the T cells in these animals (Reif and Allen, 1964; Golub, 1971, 1972). To raise antiserum against bat brain cells,

the technique of Chakraborty and Chakravarty (1983) was followed with little modifications. Briefly, pieces of cerebral cortex of bats were homogenized in cold PBS (pH 7.2) in a glass homogenizer. Then 1 ml of brain homogenate was emulsified with 1 ml of Freund's Complete Adjuvant (Difco Laboratories, Detroit, U.S.A.), and 1 ml of this water-in-oil emulsion was injected subcutaneously in the thigh region of the hind leg of a healthy rabbit from which normal serum had already been collected. The rabbit was given 6 such weekly injections. After 72 hours of the last booster injection, the rabbit was bled by ear vein puncture and antiserum was collected as outlined previously. Serum aliquotes were preserved frozen at -20°C until use. Before every use, the antiserum was decomplemented by heat inactivation at 56°C for 30 minutes.

Collection of guinea pig complement : Blood was collected from guinea pigs aseptically by heart puncture and allowed to stand at room temperature for 45 minutes following the method of Herbert (1978). After clotting of the blood, serum was collected by centrifugation and aliquotes of serum were preserved at -20°C until use as source of complement.

In vitro cytoxcity of rabbit anti-bat brain serum : For the serum cytotoxicity test, the method outlined by Chakraborty and Chakravarty (1983) was adopted with few modifications. Cytotoxicity of the rabbit anti-bat brain serum was tested against the

plastic adherent, nylon wool non adherent and nylon wool adherent cell populations separately. Heat inactivated normal rabbit serum, rabbit anti-bat brain serum and non inactivated guinea pig complement were absorbed with packed (10% of the volume of serum) erythrocytes, liver and kidney cells of the experimental bats for 1 hour at 4°C. An aliquote of 0.1 ml of bat immunocompetent cell suspension at a concentration of 10^7 cells/ml was mixed with 0.1 ml of 1:10, 1:20, 1:40, 1:80 and 1:160 dilutions of rabbit anti-bat brain serum. In the control tubes, bat immunocompetent cells were mixed with similar dilutions of normal rabbit serum. In another set of control tubes, cells were mixed with 0.1 ml HBSS only. Triplicate tubes for each serum dilution were first incubated at 37°C for 30 minutes after which 20 µl fresh guinea pig serum was added to each tube as source of complement. The tubes were then incubated at 37°C for further 1 hour. The reaction was stopped by placing the tubes on ice for 10 minutes. Viable cells were counted by using Trypan Blue dye exclusion technique. The lytic index of the antiserum for each dilution was calculated from the following formula :

% lysis =

$$\frac{\text{No. of live cells with normal serum} - \text{No. of live cells with antiserum}}{\text{Total No. of cells added in each tube}} \times 100.$$

In vivo treatment with rabbit anti-bat brain serum: The method described by Pitchappan and Muthukkaruppan (1977) was employed. Briefly, bats were given daily intravenous injections of 1/10 dilution of rabbit anti-bat brain serum in 1 ml quantity. After 5 consecutive injections, bats were sacrificed at 24, 72 and 120 hours and their secondary lymphoid organs were removed for histological observations and cell separation studies.

Histological study: Small pieces of spleen and lymph nodes from normal and experimental bats were fixed with Bouin's fixative for overnight. Excess fixative was then removed by 4-5 changes in 70% ethanol. Tissues were then dehydrated by passing through increasing grades of ethanol, cleared in xylene and embedded in paraffin. Sections of 6 µm thckness were cut in rotary microtome and stained with haematoxylin and eosin. For better staining of the fibrilar materials, some of the sections were stained with Masson's Trichrome stain containing Biebrich scarlet, fast green and Weigert's haematoxylin.

Transmission electron microscopy : Small pieces (about 1 mm^3) of spleen and lymph node tissues were first fixed with 2.5% Gluteraldehyde in 0.2M Sodium cacodylate buffer (pH 7.2) for 3 hours in cold. Excess fixative was removed by several washings in chilled cacodylate buffer and the specimens were

post fixed with 1% Osmium tetroxide in the same cacodylate buffer for 2 hours at 4°C. After washing out the excess osmium tetroxide, with the same buffer, the samples were dehydrated by passing through increasing grades of cold acetone, and infiltrated with a 1:1 mixture of acetone and Epon embedding medium for 48 hours at room temperature. Finally, the samples were embedded in the Epon embedding medium containing 13 ml Epon 812, 7 ml Nadic Methyl Anhydride, 8 ml Dodecyl Succinic Anhydride and 16 drops of DMP 30 as per standard methodology (Pease, 1964). Polymerization of the resin was done for 48 hours at 60°C. Ultrathin sections obtained from a LKB NOVA IV Ultramicrotome were taken on naked copper grids and stained with 5% aquous Uranyl acetate for 1/2 hour and then with 2% Lead citrate for 5 minutes (Pease, 1964). The stained sections were examined in a JEOL 100 CX Transmission Electron Microscope at 60, 80 and 100 KV accelerating voltages at the Centre for Cellular and Molecular Biology, Hyderabad.