MATERIALS AND METHODS

Animals : Pteropus giganteus, a frugivorous bat supplied by an animal supplier at Calcutta was maintained in our laboratory with banana and water ad libitum and utilised for this study. The adult animals were fairly large; length from snout to the tip of the tail in average was 24 cm and across the patagium or wing was 97 cm. The average weight of animals were from 440 to 520 gms. Foetuses and neonatals were collected from gravid mothers in the season of parturition. As the mothers were wild , there was no way to ascertain the age of the foetuses or younge; thus the size of these immature animals was noted to indicate their chronological stages in course of our study instead of their real age.

Macro study of lymphoid organs : After dissecting the animals, location of different lymphoid organs was noted and the organs were removed in a petridish containing phosphate buffered saline (pH 7.2) for measurements and drawing. Fat from the periphery of the lymphoid organs was removed at first.

Histological preparation : The lymphoid organs were fixed in Bouin's fixative for 4 hrs, dehydrated in ascending grades of alcohol and cleared in xylol. Paraffin infiltration was done

initially in 1:1 (v/v) xylol paraffin mixture and then in paraffin. Finally blocks were made in paraffin (melting point 52-54°C). Sections were made with rotary microtome at 6 µm thickness.

During staining, paraffin of sections was removed by dissolving in xylol and then the slides were brought to distilled water through descending grades of alcohol. Sections were stained in Delafield's haematoxylin and differentiated by acid and alkali treatment. During dehydration the sections were counter stained in alcoholic eosin and finally cleared in xylol and mounted in D.P.X. They were viewed through a binocular microscope and photographed with OROW films (80ASA).

To study the secondary lymphoid organs like spleen and lymph nodes at immunized state, 0.5 and 1 ml of 25% sheep red blood cells (SRBC) as a standard antigen, was injected in the animals by intravenous route. The organs were fixed for histological preparations at 10, 15 and 20 days after immunization.

Raising of Anti-Lymphocyte Serum (ALS): The cell suspension from spleen and mesenteric lymph node were made following the procedure as indicated later. The suspension of the cell was centrifuged at 4000 rpm for 5 min and for lysing the erythrocytes 8-10 ml of

0.85% NH_4Cl was added to the cell pellet for 5 min. Then the cells in NH_4Cl solution were centrifuged, washed twice with Phosphate Buffer Saline (PBS). 1 ml of cell suspension containing 10^8 living cells was mixed with 1 ml complete Freund Adjuvant by sucking in and out through 22 gauge needle attached to a syringe. The mixture was injected subcutaneously in the thigh region of a rabbit. Reimmunization of the rabbit was done at an interval of 10 days for 7-8 times. Each time same number of cells were injected. After 7 days of last immunization, blood was collected from ear vein puncture and kept at room temperature for 1-2 hr. Clot was separated off from glass wall and centrifugation was made at 2000 rpm. Serum aliquots were collected and kept in deep freeze (-70°C) until use.

Determination of the percentage of lymphocytes in Different Lymphoid Organs and Cell Population by ALS :

Collection of Different Cell Types :

Spleen and Lymph Node Cells - Spleen and lymph node cells were obtained following the usual procedure of dissociation with a stainless steel grid of wire mesh (Chaudhuri and Chakravarty, 1981) and by centrifugation at 3000 rpm, layered on Ficoll-Hypaque (SIGMA, U.S.A. product # F.8628) gradient for 15 min. RBCs and debris precipitated at the bottom and lympho-

cytes were at the interface of Ficoll-hypaque and water.

Bone marrow (BM) cells - Long bones were collected after removing all the adherent muscles. Bone marrow was flushed out of the bones with a jet of PBS from a syringe fitted with a needle. An uniform suspension of the cells was made by passaging through a 27 gauge needle attached to a syringe. Cells were separated from the debris by flotation on Hypaque-Ficoll and washed two times with PBS.

Peritoneal exudate cells (PEC) - We adopted the technique of Landahl (1974) for PEC preparation with some modifications. One day before sacrificing the bat, 2 ml of incomplete Freund's Adjuvant was injected intraperitonially. After sacrificing the animal, skin of the abdomen was removed. A volume of 15-20 ml PBS was injected in the peritoneal cavity. After gentle massage of the abdomen, exude cell suspension from the peritoneal cavity was drawn out with a syringe. After centrifugation of the suspension of peritoneal exudate cells, two washes with PBS were performed.

Peripheral blood lymphocytes (PBL) - About 10 ml of blood were collected by heart puncture and kept in tubes containing heparin (40 IU/ml of blood) and placed in room temperature

for about 2-3 hrs for settling RBC at the bottom. Upper buffy layer containing leukocytes was collected into another tube. Lymphocytes were purified by centrifugation on Hypaque-Ficoll and were washed 2 times with PBS.

Lytic Reaction with ALS :

After heat inactivation at 56°C for 30 min, ALS was diluted in 2 fold order from 1:20 to 1:80. To 0.4 ml of diluted serum in a small glass tube (Corning), 0.1 ml of suspension of a particular cell type adjusted to 10^7 cells/ml was added. An amount of 20 µl of fresh pre-absorbed guinea pig serum was added to each tube for the source of complement. For pre-absorption, 1 ml packed spleen and erythrocyte cells of bat was used for 10 ml of guinea pig serum. The mixture was allowed to stand for 30 min at 4°C and then centrifuged to take of the aliquot of the serum. Contents of the tubes were mixed well. Tubes containing bat's leukocytes, ALS and pre-absorbed guinea pig serum were incubated at 37°C for 1 hr. Control tubes with heat inactivated normal rabbit serum (NRS) diluted in the same order of experimental series and equal number of cells and guinea pig complement were also incubated for the same hr. The reaction was stopped by taking out the tubes from the incubators and keeping them on icebath. Living cells were ~~for the same hr~~ counted by dye

exclusion principle.

$$\text{Lytic Index \%} = \frac{\text{Total no. of living cells with NRS} - \text{Total no. of living cells with ALS}}{\text{Total no. of living cell added in each tube}} \times 100$$

Lytic Reaction after absorption of ALS with Bone marrow, Brain, Spleen & Lymph node cells : Bone marrow, spleen, lymph node cells were collected as previously described. Brain was collected after removing the skull bones and was homogenized in cold PBS with the help of tissue homogenizer. Cell suspension of each kind and brain homogenate were washed 2 times with PBS.

Absorption of different aliquots of ALS were done at the ratio of 0.1 ml packed cells or homogenate and 1 ml ALS at $4^{o}C$ for 30 min. Tubes were shaked intermittently, then centrifuged and absorbed serum separated and diluted in two fold order from 1:20 to 1:80 as indicated earlier. Then cytolytic experiments with these absorbed serum were carried as a usual to determine the specificity of ALS and sharing of cell surface determinants in different populations of lymphocytes and with brain.

Antigen and immunization : Blood from jugular vein of a sheep was drawn by a syringe in sodium citrate solution and then two

washes were made with phosphate buffered saline (PBS). The final pellet was diluted on volume to volume basis with PBS to make 25% solution of SRBC. Different doses of 25% SRBC were injected in bats by intravenous route in patagium.

The blood utilized throughout the study was collected mostly from a single healthy male sheep.

Collection of complement : Blood was collected from ear vein of rabbits and by heart puncture from anaesthetised bats and guinea pigs. The blood samples were kept in room temperature for 1 hour and then the clot was separated from the wall of the glass tube and the tubes were centrifuged. The serum aliquots in small volumeswere collected separately in small glass tubes and preserved at $-70^{\circ}C$ until use.

Preparation of spleen cell suspension : Bats were sacrificed at different days of immunization and the spleen was dissected out aseptically and kept in PBS. The spleen was dissociated by scraping against a fine stainless steel wire grid in 10 ml PBS. The cell suspension was passed through 27 gauge needle for further dissociation of the cells. Cell suspensions were utilized for primary and secondary plaque forming assays.

Plaque forming cell (PFC) assay : Cunningham and Szenberg's (1968) technique was used with some minor modifications for assaying the primary antibody secreting plaque forming cells. Initially original spleen cell suspension in 10 ml PBS was further diluted with PBS at 1:9 ratio. 0.1 ml of this diluted suspension was mixed with 50 µl of 10% SRBC and 50 µl of complement by vortexing in a vortex mixer and with a Pasteur pipette. The mixture was transferred with a fine tip Pasteur pipette into the chamber made by two slides, fixed face to face with a bi-gummed tape (3M Co., Minnesota, U.S.A.). Two sides of the slide chamber were sealed by dipping into a molten mixture of paraffin and petroleum jelly at 70°C. After 3 to 4 hours of incubation of the slides at 37°C, typical plaques developed and they were counted under dissecting binocular microscope with transmitted light.

Number of leucocytes in spleen cell suspension was counted after lysis of RBCs with 2% glacial acetic acid.

Secondary plaque forming cell assay : The method of Plotz et al., 1968 (Cf. Williams and Chase, 1976) was adopted with certain modifications for assaying secondary PFC. 1.4% Agar (Difco, U.S.A.) in distilled water was autoclaved and after lowering the temperature to 45°C in water bath it was mixed with the same volume ~~mx~~ of pre - warmed

doubly concentrated MEM (HI-MEDIA, Bombay) to make 0.7% of final agar concentration in MEM. With preheated pipette, 0.5 ml of agar suspension was transferred into small glass tube containing 25 µl of 1% DEAE Dextran (Pharmacia Fine Chemicals, Sweden) in MEM, placed in the water bath at 45°C. 50 µl of 10% SRBC and 50 µl of 10% spleen cell suspension were added into the tube in rapid sequence, mixed well with cyclo-mixer and the mixture was poured on a clean, lipid free glass slide and spreaded uniformly. The slide was incubated 1 hr at 37°C in humidified atmosphere of 5% Co_2 and air. Then experimental slides were flooded with 0.15M 2-mercaptoethanol (2-ME) and control slide with PBS. After incubation for another 30 min at 37°C, ME was decanted off and the slides were washed with PBS. Four generous saline washings spaced over 30 to 60 min were sufficient for complete removal of ME. Then the slides were developed with 10% bat's complement for 3 hrs at 37°C. Complement was poured off and plaques were counted under dissecting binocular microscope.

Hemagglutination test : Blood from immunized bats was collected at different days from a vein of patagium and sometime from the heart of an animal sacrificed for PFC assay. Serum was collected following usual method as indicated earlier and kept at -20°C until use within a few days. Before use, complement of the

serum sample was inactivated at 56°C for 30 min. Then the twofold order gradual dilution of the serum from 1:10 to 1:1280 was made with saline in small glass tubes. Total volume of the diluted serum in each tube was 0.5 ml. An amount of 0.1 ml of 1% SRBC was added to each tube. Tubes were kept in 37°C water bath overnight. The degree of hemagglutination was marked according to the size of agglutinated particles of SRBC. The last dilution of serum giving positive hemagglutination reaction has been considered as agglutination titre value.

Hemagglutination reaction in presence of 2-mercaptoethanol (2-ME) : Usually ME-resistant antibody molecules belong to 7S immunoglobulin class and thus represent a product of secondary response. We used three different concentrations of 2-ME like 0.1, 0.2 and 0.3 M solution in the assay of secondary hemagglutination titre and found 0.1M2-ME was most effective for the purpose. Thus, 5 µl of 0.1 M 2-ME was routinely added in each of one set of tubes containing serially diluted serum at the beginning of incubation for assaying secondary hemagglutination titre. Incubation was at 37°C for overnight.

Electrophoretic analysis of the serum and quantitation of different classes of serum proteins : Normal sample of blood was collected from bats before immunization. After one day

the same bats were immunized with 0.5 ml of 25% SRBC via intravenous route. Immunized blood samples were collected on 5th, 10th and 20th days of immunization. After collection, the blood samples were allowed to clot at room temperature. Clots was separated from glass wall after 3 hrs and centrifuged for 10 min in 2000 rpm to obtain the serum. 0.2 ml of serum was mixed with 7.8 ml of 23% Na_2So_4 solution. From the mixture 2 ml was taken out for estimation of total protein and mixed with 4 c.c. of Biuret reagent. Colour intensity was measured in a colorimeter ("Systronics" model 103).

For electrophoretic separation of different classes of protein in the serum, 20 µl of each serum were applied near middle portion of a strip of cellulose acetate paper (4.0 x 35 cm) in vertical electrophoretic unit. Two end of the paper were dipped into barbiturate buffer of pH 8.6 and ionic strength of 0.075. The unit was run for over night with a constant supply of 18 volts per strip. After removing the strips from the unit, they were dried in an oven at 100-110°C for 1 hr. The strips were then immersed in bromophenol blue overnight. They were washed in 5% acetic acid 2 to 3 times, then dipped into fixative solution containing 300 mg Na-acetate in 5% acetic acid and dried in air. Each band separated and eluted in 6 ml of 0.01N NaoH for 30 min with constant shaking and

colour was measured in colorimeter at 540 mμ for determining the amount of protein in each band.

Delayed type hypersensitivity test with 2,4-Dinitrofluoro-benzene (DNFB) : Bats were first immunized by application of 0.1 ml of 2% DNFB in acetone on the shaved surface just below the armpit region of left side. On 7th day after first application the bats were re-sensitised with 0.1 ml of 0.02% DNFB on the same region of the right side. The size of second spot was measured for 7 days from 7th day to 13th day at 24 hr interval for enumerating degree of delayed type hypersensitivity reaction.

One way mixed lymphocyte culture (MLC) : Minimum essential medium (MEM) (HI-MEDIA, Bombay) supplemented with 50 μ/ml of penicilin and streptomycin (Sarabhai, Bombay) and 50 U/ml of Nystatin (Sigma Chemical Co.) was used for washing the cells and 5% autologous serum was added to the supplemented MEM for culturing the cells.

A normal bat was dissected and spleen, lymph node were collected aseptically. They were suspended in 10 ml media with the help of fine stainless steel grid. The cells were further dissociated by passaging through 27 gauge needle fitted on a syringe. Lymphocytes were separated by Ficoll

hypaque (Sigma Ch. Co.) gradient and centrifugation. The cells at the interface of Ficoll hypaque gradient and water were collected with a pasteur pipette and washed two times with media. Number of cells was adjusted to 10^6 cell/ml, 5×10^5 cell/ml and 10^5 cell/ml for conducting experiment with three different concentrations of responder cells.

Stimulator cells were collected from another normal bat following the same procedure described above and treated with mitomycin C (Biochem Pharmaceutical Industries, Bombay, 25 µg/ml) for 25 min at 37°C. Then the cells were washed twice with media and adjusted to 10^6 cell/ml. 1 ml of stimulator cell suspension was added to 1 ml of responder cell suspension in a loosely capped culture tube (Corning, sized 12.2 x 1.3 cm). 5% autologous serum of responder cell type was added in each culture tube. Triplicate cultures for each concentration of the responder cells were set up. Tubes were incubated at 37°C in humidified atmosphere containing 5% Co_2 and air. After 3 days of incubation, the medium in the culture tubes were taken off after centrifugation and 2 ml of fresh media supplemented with antibiotics and 5% autologous serum was added to each tube. Further incubation was made for 4 days at 37°C in humidified atmosphere containing 5% Co_2 and air. 1 µCi/ml of ^3H Thymidine (specific activity

15 '8 Ci/mM; Bhaba Atomic Research Centre, Bombay) was added to each tube 16 hr prior to the termination of the culture. The cells were harvested on the marked disc of Whatman filter paper No.3 under the suction pressure. In another set of experiment the culture was labelled 16 hr prior to the termination of culture at the end of 4th day. Rest of the procedure were same as indicated before. The filter papers with the cell samples were dried in an incubator and placed separately in the Scintillation counting vials. 5 ml of omnifluor, made according to the standard technique (Hartzman et al, 1971) was added to each tube and the extent of incorporation of ^{3}H-TdR in each sample was measured by Scintillation Counter (Packard).

Statistical Calculations : Statistical calculations throughout this study was made according to the standard procedures (Steel and Torrie, 1960). The significance of a particular result in comparison to the control group (p value) was calculated by Student's t test.