STUDY OF IMMUNOCOMPETENT CELL TYPES IN THE BAT, *Pteropus giganteus*

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Thesis Submitted for the Degree of
Doctor of Philosophy (Science)
of the
UNIVERSITY OF NORTH BENGAL
1990

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November 17, 1990

This is to certify that Mr. Saurav K Sarkar, M.Sc. worked in my laboratory since October, 1982 for his dissertation on the topic, "Study of Immunocompetent Cell Types in the Bat, Pteropus giganteus"for fulfilment of the requirements of the Degree of Doctor of Philosophy (Science)of the University of North Bengal.

He is conversant with the techniques and literature cited in the dissertation and carried out the work thoroughly. It seems that the thesis is fit for submission for Ph.D. and he is worthy of the award of the degree.

Chakravarty

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A C K N O W L E D G E M E N T

I take the pleasure to express my heartfelt sense of gratitude and reverence to Prof. A.K. Chakravarty, without whose constant and ever watchful guidance this work would not have taken shape. I shall remain ever grateful to him for his constant encouragement and tireless interest despite his tight schedule as Director, Centre for Life Sciences, University of North Bengal.

I feel very much indebted to Dr. P.D. Gupta, Assistant Director, Centre for Cellular & Molecular Biology, Hyderabad, for the training in electron microscopy and later, for extending the full facilities of his laboratory for the TEM studies. I am also indebted to the Professors of our Centre for Life Sciences for providing different facilities and generous encouragement during my tenure as a research worker.

I am specially thankful to Drs. Amal K Chakraborty, T.K. Chaudhuri, U.K. Maitra and B.N. Paul for their invaluable suggestions and help from time to time. Special thanks are also

due to my labmates, Mrs. Asha Sinha, Miss Subhra Jha for thier kind co-operation during the course of work and Mr. A. Saha for his help in the final phases of the work.

I am also thankful to the staff of this Centre for their co-operation, to Mr. M. Chakraborty for his excellent typographical work and Mr. N.C. Ghosh for the superb drawing of figures.

Finally, I take the opportunity to express my deep sense of gratitude to my parents for their blessings and bearing with me throughout the course of work.

Centre for Life Sciences
University of North Bengal
Raja Rammohunpur
November, 1990.

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C O N T E N T S

INTRODUCTION

Bats are an evolutionarily old group of flying mammals under the order Chiroptera and interesting from several points of view. The ability of echolocation by ultrasonic sound waves, hibernation and aestivation in extreme weather conditions, ability of the females to store spermatozoa for long times in their genital tracts are only a few of the several peculiar characteristics of these animals (Young, 1962; Wimsatt, 1977). It was also known since the early fifties that bats harbour several dreadful pathogenic bacteria (Klite, 1965; Arata et al, 1968; Constantine et al, 1970), viruses like rabbies (Soave, 1966; Smith et al, 1967; Fischman and Ward, 1968; Correa-Giron et al, 1970), Japanese B encephalitis (Sulkin et al, 1963, 1966a, 1966b, 1970a, 1970b; Miura et al, 1970; Allen et al, 1970), St. Louis encephalitis and many other arthropod borne arboviruses (Ito and Saito, 1952; Sulkin, 1962; Banerjee et al, 1984); epidemiological association between bats and mycotic agents like Histoplasma capsulatum etc. was also shown (Klite and Young, 1965). However, most of these studies chiefly emphasized on the carrier role of bats. Although the bats carry such pathogens, they do not usually manifest any apparent symptoms of the diseases (Queiroz-Lima, 1934; Pawan, 1936; Burns et al, 1956; Smith et al, 1967; Sulkin

and Allen, 1970). This fact alone makes these animals an interesting subject for immunological analyses. However, a systematic approach towards the problem of immunity in bats was lacking.

Reports on the immune system of bats started to come when different groups of workers (Heck, 1965; Sulkin et al, 1966a,b; Leonard et al, 1968; Hatten et al, 1970) made investigations on the production of humoral immunity in bats against experimentally inoculated JBE or ϕX 174 viruses. Although no clear evidence of the presence of complement fixing antibodies in bats was obtained, naturally occurring antibodies detected by haemagglutination inhibition test to group B arboviruses in bats was reported (Whitney, 1963; Williams et al, 1964; Stanley and Choo, 1964; Pavri and Singh, 1965). One important finding by Leonard and his co-workers (1968) was that these animals have both 19S and 7S types of immunoglobulins, but the immune response was quantitatively deficient as compared to guinea pigs. McMurray and co-workers (1978, 1979, 1981, 1982) reported both humoral and cell mediated immune responses in bats in terms of blast transformations and other parameters (McMurray et al, 1978, 1979, 1982; Greer and McMurray, 1981).

A systematic approach to analyse the immune system and immune responses in bats was attempted by Chakraborty and

Chakravarty (1983) who demonstrated that bats, while being ranked as a group of evolutionarily old mammals, possess a well defined immune system almost similar to that of the primates. A study of the ontogency of the primary lymphoid organ thymus revealed a remarkable age dependent involution just like in human (Chakraborty and Chakravarty, 1984c). Analyses of the secondary lymphoid organs like spleen and lymph nodes of the Indian fruit bat Pteropus giganteus showed the presence of highly organized white pulps in spleen and lymph nodes of normal animals and of well differentiated germinal centres in immunized animals (Chakraborty and Chakravarty, 1984a). The organization of the lymphoid cells in these secondary lymphoid organs resemble very much the situation in the higher primates.

In spite of their possession of well evolved and well organized lymphoid organs, bats show a significant delay in the onset and reaching the peak of immune response (Chakraborty and Chakravarty, 1984b). Antibody mediated immune response as measured by plaque forming cell (PFC) assay was found to persist for a longer period and both the B-mercaptoethanol (BME) sensitive primary and BME resistant secondary responses were observed with a single antigenic challenge unlike the other animals which usually require a booster challenge for

the production of secondary response. Cell mediated immunity in these animals was also expressed at a lesser degree (Chakraborty, 1982), reaching a peak with a similar delay as in case of humoral response.

The understanding of the mechanism of the delayed onset and decay of immune responses of bats is yet at minimum. Cell types, their interactions among themselves and with ubiquitous factors in plasma may likely play crucial roles. Thus, a critical characterization of the different types of immunocompetent cells and their interactions in bats needs to be initiated.

The immunocompetent cells of higher vertebrates are the B and T lymphocytes and other accessory cells like macrophages and follicular dendritic cells. These cells can be categorized on the basis of their differential adhessibility to plastic substratum and nylon wool fibres. The macrophages have been shown to possess a high surface adhessibility to glass, plastic and several other substrata including Sephadex and Latex beads. (Sjoberg et al, 1972; Ly and Mishell, 1974; Lee et al, 1976; Steinman et al, 1979). The follicular dendritic cells are also known to possess similar adhessive property (Steinman et al, 1979). Thus in the present study attempts were made to isolate these cell types on the basis of adhession to plastic surface.

Julius and his co-workers (1973) showed that murine T and B cells differ in their surface adhessiveness to nylon wool fibres and that this differential surface adhessibility can be used successfully to separate the T lymphocytes from the more adhessive B lymphocytes. Since then, several workers have used columns of nylon wool fibres to separate the T and B lymphocytes of mouse, human and other mammals (Handwerger et al, 1974; Trizio and Cudkowicz, 1974; Tada, 1977, Tada et al, 1978). Similar procedures may also be applied in case of the immunocompetent cells of bat to see whether they can be categorized according to their differential cell surface adhessiveness.

The immunocompetent cell types not only differ in their adhessibility and function, but are also distinguishable by means of their characteristic cell surface morphology. This is why several workers in the past have used the scanning electron microscope (SEM) to study the different immunocompetent cell types (Lin et al, 1972; Polliack et al, 1973a,b; Lin and Wallach, 1974; Alexander and Wetzel, 1975; Alexander et al, 1976; Newell et al, 1976; Roath et al, 1978). Studies with murine and human lymphocytes from normal peripheral blood and tissues as well as from abnormal tissues including tumours and other cell lines (Wilson and Nossal, 1972; Holt et al, 1972;

Polliack and De Harven, 1975; Polliack et al, 1975, 1981; Kwock et al, 1976; Polliack et al, 1981) revealed detailed and characteristic surface morphology of the immunocompetent cells. Polliack and his associates reported the presence of cell surface microvilli on the B lymphocytes while the T lymphocytes were shown to possess a comparatively smooth cell surface (Lin et al, 1972; Polliack et al, 1973a, 1973b, 1975, 1976; Lin and Wallach, 1974; Polliack and DeHarven, 1975). Even some neoplastic cells of B lymphocyte origin revealed the presence of surface microvilli, while some leukemic cells of T lymphocyte origin demonstrated a smooth cell surface (Polliack and DeHarven, 1975; Polliack et al, 1981, 1983). This was also supported by immunofluorescence and other microscopic techniques (Sciorra and Eckert, 1974; Fagraeus et al, 1974; DeHarven et al, 1975; Reyes et al, 1975; Vila and Taub, 1975; Padnos, 1976; Mason et al, 1977; Renau-Piqueras, 1978; Renau-Piqueras and Knecht, 1979; Polliack and Gamliel, 1983). Wetzel and co-workers (Wetzel et al, 1974; Alexander and Wetzel, 1975). Barber and Burkholder (1975) Kwock and co-workers (1976) and Newell and associates (1976) stressed the importance of type of fixation, temperature and other parameters for the study of cell surface morphology. Kwock and associates (1976) also reported that some alterations in the surface topography of lymphocytes may be caused by the use of nylon wool columns.

The macrophages have been well characterized by their possession of irregular surface morphology and different types of pesudopodial projections (Albrecht et al, 1972 & 1978; Besis, 1973; Brynes et al, 1976). There is another type of accessory cells, the follicular dendritic cells, which are known to play major roles in trapping and modification of antigens and possibly retention of antigens for long time before presenting them to the lymphocytes (Nossal et al, 1968a, 1968b; Steinman et al, 1973, 1974a, 1974b, 1975, 1978; Klaus et al, 1980; Van Rooijen, 1980).

Thus the scanning electron microscopic study would help to characterize and distinguish the different immunocompetent cell types of bats.

Besides the cell surface characteristics, immunoreactive cells in higher vertebrates also differ by several cell surface antigenic markers. B lymphocytes have characteristic immunoglobulin (Ig) markers on their cell surface (Raff et al, 1970, 1971; Wilson et al, 1971; Rabellino et al, 1971; Padnos, 1976). T lymphocytes lack this marker, but possess other specific cell surface antigens such as θ or Thy 1, and Ly in mouse, CD in human etc (Reif and Allen, 1964; Shaw, 1987; Roitt, 1988). Similarly, macrophages of monocytic origin possess characteristic

Mac-1 or-2 surface markers in mouse (Klaus et al, 1983). Thus, characterization of different types of immunocompetent cells of bat on the basis of cell surface markers have been locked into in the present investigation. B lymphocytes in peripheral blood and secondary lymphoid organs of man and mouse bear predominantly IgM or IgG and also IgD molecules (Papamichail et al, 1971; Pernis et al, 1971; Cooper and Lawton, 1972; Fröland and Natvig, 1972; Teale et al, 1980; Lafrenz et al, 1986). As because both IgM and IgG mediated immune responses have been demonstrated in the bats (Leonard et al, 1968; Chakraborty, 1983a), the IgM and IgG bearing cells in bats equivalent to B lymphocytes have been investigated in the present study with fluorochrome conjugated antibodies to bat IgM and IgG.

Next, the immunocompetent cell types of bat bearing Thy-1 type of surface antigen were characterized with anti-Thy-1 type serum. Raising of anti bat thymocyte serum is difficult because of total involution of thymus in adult bats (Chakraborty and Chakravarty, 1984), but it is known that Thy-1 is often shared by brain cells in different vertebrates (Reif and Allen, 1964; Acton et al, 1974; Trowbridge et al, 1975; Mansour and Cooper, 1975; Williams et al, 1976) and it has also been shown that the Thy-1 molecule has been highly conserved during evolution (Cambell et al, 1981; Cotmore et al, 1981;

Mackenzie et al, 1981; Williams and Gagnon, 1982; Mansour et al, 1985, 1987; Shalev et al, 1985). Golub (1971, 1972) raised anti-mouse brain serum in rabbit and employed it successfully to identify the mouse thymocytes. Later, anti-brain Thy-1 serum have been used by several workers to detect T lymphocytes of many vertebrates (Claggart et al, 1973; Acton et al, 1974; Morris et al, 1975; Cotmore et al, 1981). Chakraborty and Chakravarty (1983a) took advantage of this fact by absorbing the rabbit anti-bat lymphocyte serum with bat brain homogenate and showed a differential susceptibility of the lymphocytic cell population of bat to this pre-absorbed anti serum, and suggested a possible dichotomy of lymphocytes in the line of B and T cells. In the present investigation, Thy-1 type antigen bearing lymphocytes of bat have been identified by their susceptibility to anti-bat brain serum, and then localization of these cell types in secondary lymphoid organs have been studied after repeated injections of the serum in the bat and making histological preparations of spleen and lymph nodes from the anti serum treated bats. Such studies for anatomic compartmentalization of B and T Cells and their differentiation in lymphoid organs were attempted in mouse, rat and lizard (Parrot and De Souza, 1966, 1969, 1971; Howard et al, 1972; Bhan et al, 1975; Gutman and Weisman, 1972; Pitchappan and Muthukkaruppan, 1977b; Barclay, 1981; Van Ewijk et al, 1981,

Rouse et al, 1982). In man, recent immunofluorescence and histological observations of primary and secondary lymphoid organs from normal subjects as well as patients with T or B cell deficiency have yielded important information regarding the T dependent and B dependent regions in these organs (Lamelin et al, 1978; Seymour et al, 1980; Bhan et al, 1980; Poppema et al, 1981).

For characterization of mouse and human immunocompetent cells, transmission electron microscopy (TEM) has been used since early 1960's (Zucker-Franklin, 1963, 1969; Inman and Cooper, 1963; Movat and Fernando, 1964, 1965; McFarland and Heilman, 1965; Harris et al, 1966; Hummeler et al, 1966; Heiniger, 1967; McFarland, 1969; Tanaka and Goodman, 1972; Besis, 1973). Analysis of cellular and nuclear volume from serial ultrasections revealed a significant difference between thymic and lymph node lymphocytes of mice (Heiniger, 1967). Later, the use of specific antibodies coupled to ferritin, horse raddish peroxidase and such other electron dense markers resolved two distinct populations B and T cells (De Petris et al, 1963; De Petris and Karlsbad, 1965; Storb et al, 1969; Reyes and Bach, 1971; Murphy et al, 1972). Using this technique, Matter and his associates (1972) distinguished different stages of differentiation of B and T cells depending on cell size and cellular content of organelles.

Cohn and his co-workers (Cohn and Benson, 1965; Cohn, Hirsch and Fedorko, 1966) and others (Sutton, 1967; Chapman et al, 1967) studied the structure and differentiation of monocytes and macrophages under the TEM. Further study of the structure and fate of several subcellular organelles in the macrophages were also made (Cohn, 1968; Nicholas et al, 1971; Allison et al, 1971). Allison and his co-workers (1971) studied in detail the formation of pseudopodia, the striking and characteristic feature of macrophages. To our knowledge there is as yet no report on the fine structure of the immunocompetent cells of bat and so, attempts were made in the present investigation to study the fine structure of these cells by transmission electron miscroscopy.

Summarily, the present study attempts first to isolate the different types of immunocompetent cells from the secondary lymphoid organs of the Indian fruit bat, Pteropus giganteus on the basis of their differential cell surface adhessibility to plastic and nylon wool. The subsets of immunoreactive cells thus isolated are further analyzed by scanning electron microscopy to reveal the cell surface topography. Then the different cell populations are characterized with reference to cell surface markers such as IgM, IgG and Thy-1. Anatomical compartmentalization of B and T cell type, is studied by rabbit anti Thy-1 type serum in vivo. Finally, transmission electron microscopy is used to reveal ultrastructural characteristics and organization of the immunocompetent cells in situ.

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.

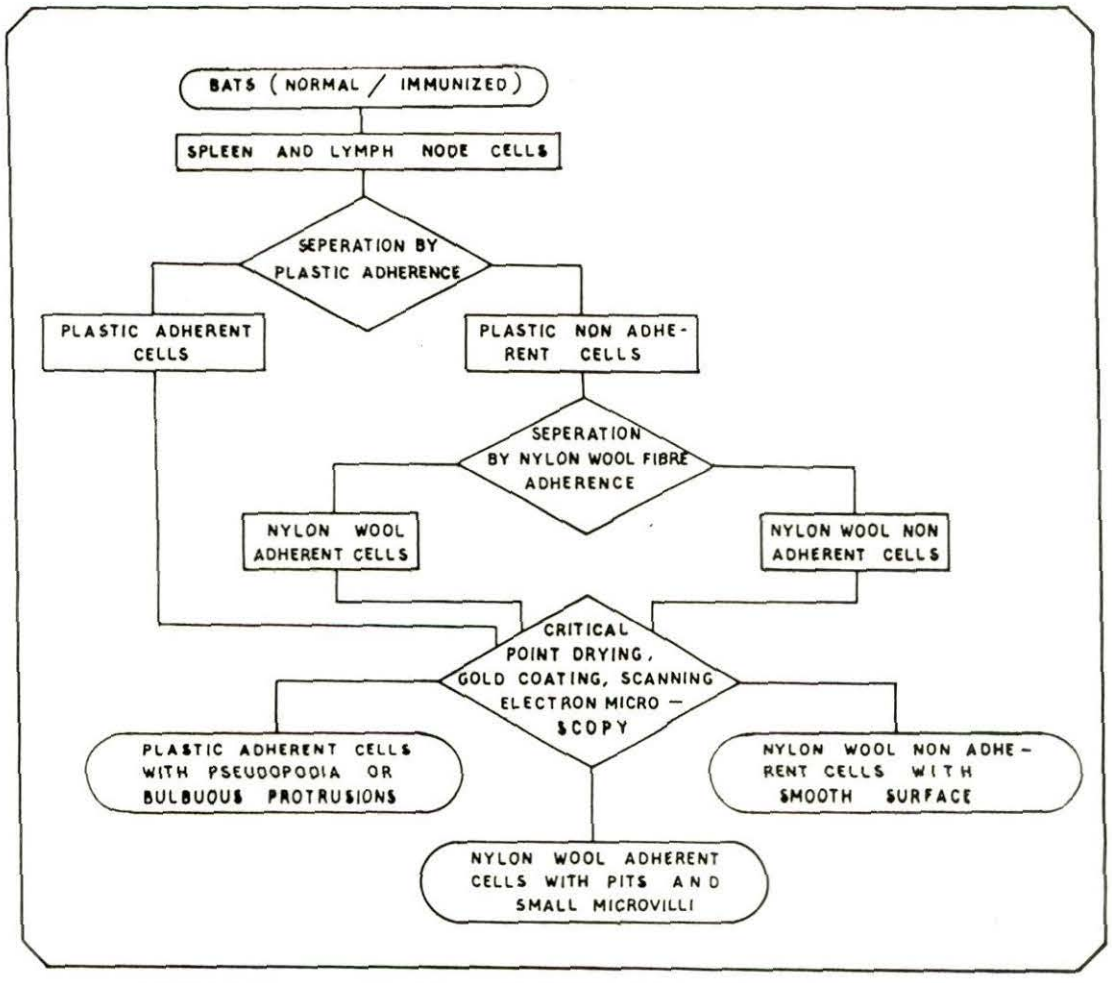
About 5 ml of the cell suspension containing upto 10^8 cells/ml in serum supplemented warm EBSS or MEM was carefully loaded in the presoaked and prewarmed nylon wool column and incubated for 1 hour at 37°C in a humidified atmosphere containing 5% CO_2. Then the nylon wool non adherent cells were eluted 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.

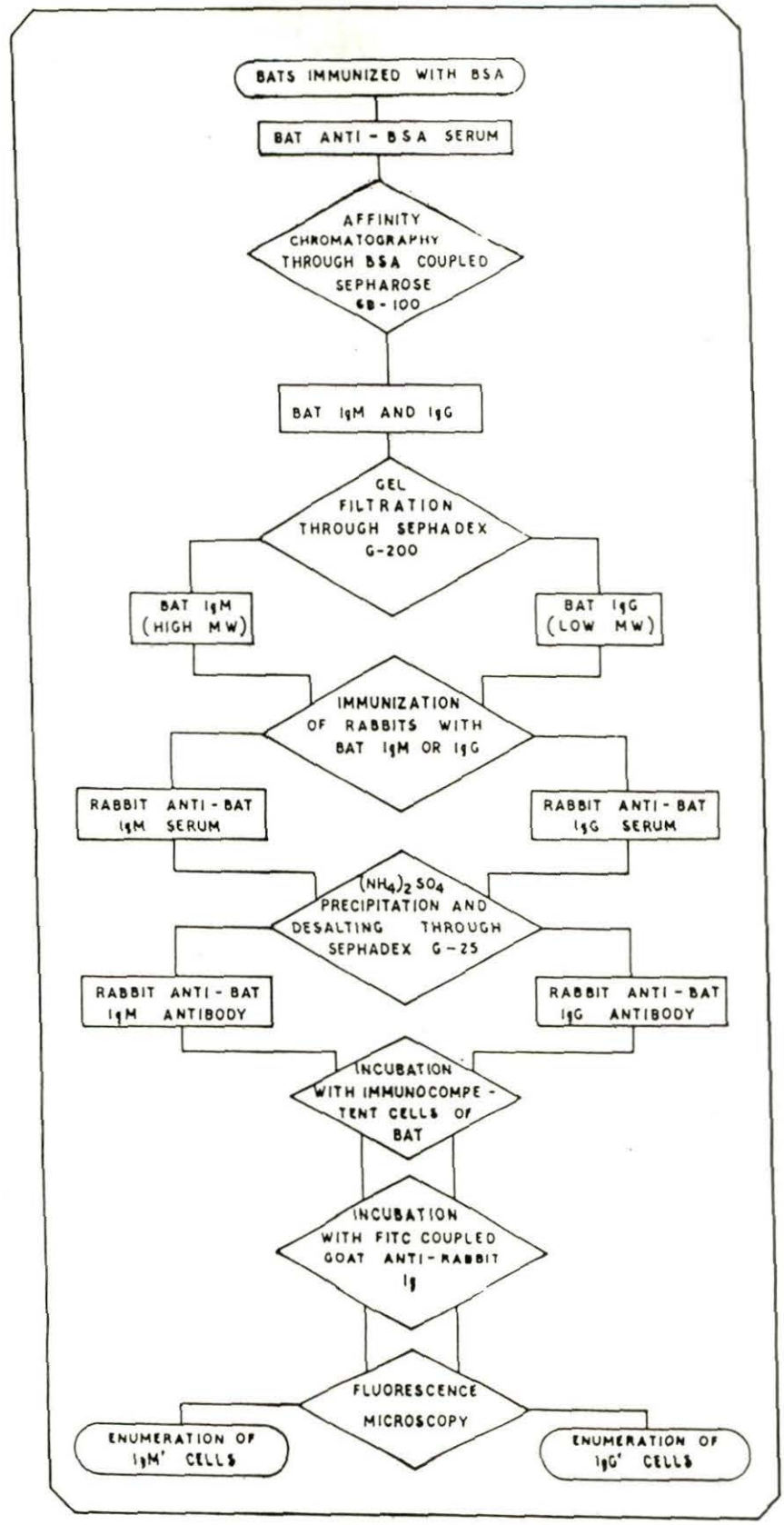
Ammonium sulphate precipitation of immunoglobulins raised in rabbit: 2 ml of the rabbit antiserum against bat IgM or bat IgG was mixed slowly with 2 ml of saturated ammonium sulphate under constant stirring in cold. The precipitate produced by such 50% ammonium sulphate saturation was collected by centrifugation 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.

RESULTS

PLASTIC ADHERENT CELLS FROM SPLEEN AND LYMPH NODES OF BAT

Spleen and lymph node cells from the bats were incubated in plastic petridishes under appropriate conditions to separate the group of cells adhering to the plastic substratum. It was found that some cells from spleen and lymph nodes adhered readily to the plastic and could be removed later by mechanical means. This plastic adherent cell population represented about 3.55% of the total spleen and lymph node cell population of lymphocytic and monocytic origin. This percentage was fairly constant from experiment to experiment even with varying numbers of cells from different bats (Figre 1).

NYLON WOOL ADHERENT AND NON ADHERENT CELLS

The plastic non adherent cells were further separated by nylon wool fibre column following two protocols. In the first porotocol, the nylon wool was not pretreated and in the second, the wool was pretreated with EDTA and $NaHCO_3$ following the suggestion by Henry (1980). In both the protocols, the results indicate two distinct populations of lymphoid cells — nylon wool adherent and non adherent populations. However, following the first protocol, the yield of adherent and non adherent cells was low, about 2.16% and 1.57% respectively of the initial cell population (Table 1). The pretreatment of nylon wool in

the second protocol led to higher yield of both the cell types, 8.77% adherent and 31.83% non adherent (Table 2), and in further experiments, this second protocol was used.

However, even after this increase in the efficacy of the pretreated nylon wool column, the cell recovery was about 41% in the present study, whereas 50-60% in case of mouse or human (Trizio and Cudkoicz, 1974).

The 3 cell types, plastic adherent, nylon wool adherent and nylon wool non adherent populations have been recovered in the ratio of 1:2:9 approximately (Table 3).

Neutral red positive cells were about 89.6% in the plastic adherent cell population, whereas the nylon wool adherent and non adherent cell populations showed about 12.3% and 6.8% contamination of the neutral red positive cells respectively (Table 4).

SEM ANALYSIS OF SURFACE TOPOGRAPHY OF THE PLASTIC ADHERENT CELLS

Cells from normal bats: The plastic adherent cells from normal bats under the scanning electron microscope revealed an irregular shape, with a diameter usually centered around 5 μm. The conspicuous feature of these cells was the presence of pseudopodial projections. Sometimes they were in the form of a

uropodium at the back of the cell, or as flattened lamellipodia, or finger shaped filopodia all over the cell (Plate I, Figs. 1 & 2). Usually more than one type of pseudopodia were found on a cell. There were certain distinct plastic adherent cells which were comparatively bigger in size, 6-8 µm in diameter and with characteristic bulbuous protrusions of 2-4 µm diameter (Plate II, Figs. 1 and 2). These projections were different from the usual lamellipodia or filopodial pseudopodia.

Cells from immunized bats: Plastic adherent cells from bats immunized with 25% SRBC for 10 days showed an apparent increase of cell size to about 8 µm. The pseudopodia seemed not as prominent as in the cells from normal bats (Plate III, Figs. 1 and 2).

SURFACE TOPOGRAPHY OF NYLON WOOL NON ADHERENT CELLS

Cells from normal bats: About 80% of the cells in this group had a diameter ranging from 6µm to 7 µm. The cells were very regular and round in shape. The cell surface was relatively smooth except a few surface ridges (Plate IV, Fig. 1). Pseudopodia of any type were absent in these cells, but occasionally some localized membrane rufflings could be seen (Plate IV, Fig. 2).

Cells from immunized bats: Immunization caused slight increase in cell size which ranged from 7 to 8 μm and more, and there were some prominent cell surface rufflings (Plate V, Fig. 1). Occasionally some large cells, about 10 μm in diameter, showed some surface 'blebs' (Plate V, Fig. 2).

SURFACE TOPOGRAPHY OF NYLON WOOL ADHERENT CELLS

Cells from normal bats: The nylon wool adherent cells revealed a characteristic cell surface morphology that was distinctively different from that of plastic adherent or nylon wool adherent cells. The surface of these cells showed the presence of some small microvilli like projections along with some pit like formations with a diameter of 0.7 to 1.2 μm (Plate VI, Figs. 1 and 2). During screening, 70 to 80% of the cells were found to be large in size, having a diameter ranging from 7 to 9 μm.

Cells from immunized bats: After immunization, the nylon wool adherent cells showed a marked difference in cell surface topography from that of any other cell types examined. Average diameter of the cells was found normally to have increased to about 10 μm. The characteristic microvilli as seen in normal cells were absent, rather the cell membrane was highly ruffled (Plate VII, Figs. 1 and 2). Such degree of surface ruffling was not found in any other cell types. In addition, some long,

filamentous membrane projections or 'spikes' were present on these cells. The spikes had a length of 2.5 to 2.8 μm, and a width of about 0.3 μm.

Occasionally, very large cells, more than 10 μm in diameter, were found which had extremely ruffled membrane; the spikes were not prominent in these cells (Plate VII, Fig. 3).

IMMUNOFLUORESCENCE STUDY OF SURFACE IMMUNOGLOBULIN BEARING CELLS

Separation of bat Ig M and IgG: Bat anti-BSA immunoglobulins (Ig) were first isolated by affinity chromatography of whole bat anti-BSA serum in BSA conjugated Sepharose 6B column. When this bat Ig was fractionated by gel filtration in Sephadex G-200, two protein peaks were observed at 280 nm. One of the proteins was eluted in the void volume, while the other protein was eluted later (Fig. 1). When purified human Ig G (Sigma, U.S.A.) was chromatographed in the same column, a single protein peak was observed almost at the same position of the second protein peak of bat Ig.

Polyacrylamide gel electrophoresis of the bat Ig isolated by affinity chromatography also revealed two major protein bands, of which one band showed a relative mobility close to that of

purified human Ig G (Plate VIII, Fig. 1). For practical purpose, these two fractions of bat Ig may be called Ig G and Ig M respectively.

Enumeration of surface Ig M and Ig G bearing lymphocytes : Surface Ig M and Ig G bearing cells in the plastic adherent, nylon wool adhrent and nylon wool non adherent cell populations, as well as in spleen, lymph node, bone marrow and peripheral blood were detected by indirect immunofluorescence microscopy using rabbit anti-bat Ig M and anti-bat Ig G as first antibody, and fluoresceinated goat anti-rabbit Ig as second antibody.

Three different types of fluorescent staining were observed using either anti-bat-Ig M or -Ig G antibody — (a) ring like fluorescence around the periphery of the cell (Plate Ix, Figs. 1, 2 and 3) which was often discontinuous (Plate X, Fig. 1) (b) fluorescence spots or patches (Plate XI, Fig. 1) and (c) in case of dead cells, a diffuse and dull fluorescence all over the cell body (Plate IX, Fig. 1) When Ig from normal rabbit (obtained by ammonium sulfate precipitation of normal rabbit serum) was used as the first antibody, no fluorescence was observed, which indicated the specificity of the anti-bat Ig M and anti-bat Ig G antibodies.

Enumeration of positively labelled cells showing fluorescent rings and patches revealed 51-58% surface Ig M bearing cells and 30-39% surface Ig G bearing cell, together 81.89% positive cells in the nylon wool adherent population (Table 7).

In the plastic adherent population, only 16-24% of the cells were positively stained, of which 7.5% to 14.5% showed the presence of surface Ig M and the rest showed surface Ig G (Table 5). Similarly in the nylon wool non adherent population, only 10-20% cells showed fluorescence of which 6-10% cells showed the presence of surface Ig M and 2-9% cells showed surface Ig G (Table 6).

When cells from different tissues were examined it was observed that bone marrow contained 32-45% surface Ig bearing cells, of which 20-24% had surface Ig M and 10-20% had surface Ig G (Table 8). Spleen had a high number of surface Ig bearing cells, about 64-74%. Of this, 40-41% cells were Ig M positive and 24-31% Ig G positive. Mesenteric lymph node on the other hand, showed a lesser number of surface Ig bearing cells — only 29-35% of which 19-23% were positive for Ig M and the rest positive for Ig G (Table 8). In the peripheral blood however, many cells bearing surface Ig were observed; of the 69-90% cells bearing surface Ig, 44-54% were Ig M bearing cells and 25-37% were Ig G bearing cells.

In spite of variation in the number of Ig M and Ig G positive cells in a particular purified cell population or a lymphoid organ, the number indicated a characteristic range for the cell population or the organ.

DIFFERENTIAL SUSCEPTIBILITY OF THE IMMUNOCOMPETENT CELL TYPES TO RABBIT ANTI-BAT BRAIN SERUM

The different immunocompetent cell populations isolated on the basis of adhessiveness to plastic substratum or nylon wool column were tested for their sharing of the brain cell antigen which is usually common with the thymus cell antigen in most mammals, as Thy-1 in mouse (Raff, 1971; Golub, 1971).

Cytotoxic ability of the rabbit anti-bat brain serum was found to be the highest in case of nylon wool non adherent cell population of bat, about 63% at serum dilution of 1:10 as shown in Figure 3. Percent cytotoxicity in the plastic adherent and nylon wool adherent cell populations was always below 10%. When the anti serum was pre-absorbed with nylon wool non adherent cells, the cytotoxic efficacy of the anti serum towards this cell type decreased drastically to almost background level, thereby indicating the specificity of the anti serum for these cells.

EFFECTS OF IN VIVO ADMINISTRATION OF RABBIT ANTI-BAT BRAIN SERUM ON THE IMMUNOCOMPETENT CELL TYPES

Rabbit anti bat brain serum diluted 1:10 with PBS was injected intravenously into each bat every 24 hours for 5 consecutive days following the method of Pitchappan and Muthukkaruppan (1977) to deplete the cells bearing the Thy-1 type antigen shared by brain cells and thymocytes. Spleen and lymph nodes were taken out of bats sacrificed at 24, 72 and 120 hrs after the schedule of 5 injections and the proportions of plastic adherent, nylon wool adherent and nylon wool non adherent cells were determined. The nylon wool non adherent cells were most affected by the antiserum treatment as indicated by the reduction of its proportion from 24 hrs onward and significantly at 72 hrs (Table 9); this is revealed by comparing the data with that in Table 3.

IN SITU LOCALIZATION OF THE AREA OF ANTI-BRAIN SERUM SENSITIVE CELLS IN SECONDARY LYMPHOID ORGANS

In spleen : A primary white pulp follicle of a normal animal was a compact mass of cells with deeply stained nuclei, surrounding a splenic arteriole in the form of a periarteriolar lymphocytic sheath. The white pulps were distributed in the splenic red parenchyma as typical in the primates, which were

previously described in detail by Chakraborty and Chakravarty (1984). The secondary follicles or germinal centres have a prominent circular zone of lightly stained large dividing cells surrounded by a jacket like mantle layer of small lymphocytes (Plate XII, Fig. 1).

After the schedule of 5 injections of anti-brain serum, the average number of lymphocytes per unit area of 0.001 mm^2 in the periarteriolar sheath region decreased 24 hrs onwards and significantly at 72 hrs (Fig. 4). The cells were loosely organized in the white pulps and some pycnotic cells were observed (Plate XII, Figs. 2 & 3).

In lymph node: In normal lymph nodes of bat roughly three areas could be delineated - (a) the outer cortex just below the collagenous capsule and harbouring the white pulp follicles, (b) the deep or paracortex containing cords of lymphocytes and (c) the innermost medulla, mainly consisting of the medullary sinus. The primary lymphoid follicles appeared as concentric masses of lymphocytes and with antigenic stimulation, they converted to germinal centres having a central, less dense zone of large lymphocytes surrounded by small lymphocytes (Plate XIII, Fig. 1).

After the antiserum treatment, there was a significant reduction in the number of lymphocytes per unit area (0.001 mm^2) in the paracortical area, particularly at 72 hrs and 120 hrs (Fig. 5), leaving some empty spaces (Plate XIII, Fig. 3). The cell loss was more severe than that in the spleen.

TRANSMISSION ELECTRON MICROSCOPY OF THE IMMUNOCOMPETENT CELLS IN SPLEEN AND LYMPH NODES

Normal Spleen: Under the transmission electron microscope, normal spleen tissue of bat revealed a compact organization of the lymphoid cells (Plate XIV, Fig. 1). The ultrastructural organization of the cells was more or less similar to those found in mouse and human. The cells were usually spherical or cuboidal in shape. Under the TEM, the average diameter of the cells of different size was found to range from 5 to 7.0 µm. Cells differing in cytoplasmic content, nuclear-cytoplasmic ratio, nuclear heterochromatinization etc. probably represented different types of immunocompetent cells. Four distinct categories of cells could be identified — small lymphocytes, large lymphocytes, plasma cells and macrophages.

To begin with, a typical lymphoid cell may be described.

Typical lymphocyte - The plasma membrane was of usual thickness; microvillous projections as seen in the SEM were not as prominent in the sections. Cytoplasmic granulation varied, possibly depending on the abundance of organelles. Number of mitochondria varied, structurally they were spherical or elongated sac like, and resembled typical mitochondria of murine or human cells (Plate XVI, Fig. 2). Short flattened cisternae of endoplasmic reticulum were seen (Plate XV, Fig. 1). Free ribosomal particles could also be observed.

Although a full fledged Golgi apparatus was not seen in the micrographs, some small vesicular structures present in the sections possibly indicated part of the Golgi apparatus (Plate XVI, Fig. 1). Some membrane bound vesicles, about 0.15 μm in diameter and sometimes surrounded by an electron dense coat were seen with opening to the exterior (Plate XVI, Fig. 2), probably in course of endo or exocytosis. The average dimensions of the cell organelles observed in course of the TEM study were as follows:

Mitochondrial diameter	: 0.25 μm to 0.50 μm
Outer mitochondrial chamber (bound by outer and inner membrane)	: about 820 ^{o}A
Ribosome diameter	: about 140 ^{o}A
Endo or exocytotic vesicle diameter	: about 0.15 μm

Nuclear membrane thickness	: 120°A to 160°A
Perinuclear space	: about 300°A
Nuclear pores	: about 180°A
Nucleolus	: 1.80 to 2.10 µm
Nucleolar granules	: about 150°A.

The nuclear morphology was typical with a perinuclear space below the nuclear membrane and with nuclear pores (Plate XVIII, Fig. 1). Usually the nucleus was round, ovoid or polygonal in shape. The nuclear material could be easily distinguished into the lightly stained granular euchromatin and the darkly stained heterochromatin. The latter was usually present as broad uneven patches mainly along the nuclear margin and also as small patches inside the nuclear mass. Nucleolus of about 2 µm diameter was observed with a central lightly stained region containing some dark granular material which were possibly indicative of ribonucleoprotein synthesis (Plate XVIII, Fig. 2).

Small lymphocyte - These cells represented the majority of the lymphocytes in normal spleen and were around 5 µm in size, and showed a thin rim of cytoplasm containing very few organelles except a few free ribosome (Plate XIV, Fig. 1). The nucleus of these cells was not always round in outline and was slightly notched at some places and contained a fair

amount of heterochromatin. Some of these features resembled those of small T lymphocytes in mouse.

Large lymphocyte - These cells were larger in size, usually about 6.5 µm to about 8 µm in diameter (Plate XVI, Fig. 1), and had more cytoplasmic content. Number of mitochondria was variable. Occasional short profiles of endoplasmic reticulum, and some small vesicles, probably indicative of a Golgi complex, were observed. Free ribosomes were scattered in the cytoplasm. Nucleus was usually polygonal in shape and the marginal heterochromatic patches were less heavy than in the smaller lymphocytes.

Besides these cells, there were some cells that could be characterized as plasma cells and macrophages which were more in number after immunization. So they are described in detail later.

Spleen after immunization: The cells in the spleen from bats immunized with 25% SRBC for 10 days were not as compactly organized as in the normal spleen. The cells were usually 7 µm to 9 µm in diameter and were cuboidal or elongated in appearance (Plate XIX, Fig. 1). Most of these cells looked like the large lymphocytes seen in the normal spleen. Although some filamentous projections were observed by SEM on the nylon wool adherent

cells isolated from immunized bats, their presence was not revealed markedly in the tissue state of organization under the TEM.

Cytoplasm of the cells showed the presence of several mitochondria of usual shape and size, many free ribosomes and small vesicles as in the normal spleen cells (Plate XXI, Fig. 1).

Nucleus in these cells was large and often deeply invaginated (Plate XXI, Fig. 1). The average area wise ratio of nucleus to cytoplasm as calculated from planimetric measurements from the micrographs was about 0.7 which is slightly higher than the average of 0.6 obtained from normal small and large spleen cells. This condition was also reflected in the photograph of isolated nylon wool adherent cells from immunized bats (Plate XXII, Fig. 1). Nuclear heterochromatin in most of the cells was less in amount. Thin patches of heterochromatin were mostly distributed along nuclear margin and a few small heterochromatic patches were seen inside the nucleus. Occasionally a nucleolus was seen.

Plasma cells - Some of the nylon wool adherent large cells from immunized bats had a significantly higher cytoplasmic content almost equal to the nuclear amount, euchromatic nucleus and often with som

vesicles near to the plasma membrane. Although idealized ergastoplasmic reticulum was not prominent, but in all likelihood the cells represented the plasma cells (Plate XXII, Fig. 1). This type of cells were only occasionally seen in the normal spleen (Plate XVIII, Fig. 1).

Macrophage - Certain cells in the spleen from immunized bats showed an irregular outline (Plate XX, Fig. 1). Some of them were as big as 9 µm. A few vesicular structures of different sizes and containing granular or homogeneously osmophillic material were observed in these cells and resembled the lysosomal bodies seen in murine or human cells. Few microfilaments were also noticed. Possibly these cells represented the macrophages in bats.

Normal lymph node : The ultrastructural details of the lymphoid cells in lymph nodes of normal bats did not vary much from that of normal spleen cells. The cells, with a diameter usually ranging from 5 µm to 6 µm were roughly spherical or cuboidal in shape (Plate XXIII, Fig. 1 & 2).

Cytoplasm of the cells was light and granular, and contained some mitochondria. Sometimes, small membrane bound vesicles were seen, some of which were coated by electron dense

material. Some free ribosomes were observed. Full fledged Golgi apparatus or endoplasmic reticulum were not seen in the sections.

The centrally placed nucleus was varying in shape and in many cells, notched at some places. Nuclear heterochromatin was, as usual, chiefly distributed in thick patches along nuclear margin. Occasionally a nucleolus about 2 μm in diameter was seen.

Lymph nodes from immunized bats: Cells in the immunized lymph nodes were loosely organized with some intercellular space in between them. The cells ranged from 5 μm to 8 μm in diameter with a predominance of large lymphocytes and could be ranked as medium or large lymphocytes. Small lymphocytes and plasma cells were occasionally seen (Plate XXIV, Fig. 1). In a few regions, some surface irregularities were noted, otherwise the plasma membranes were simple in outline.

The cytoplasm of the cells was dark and granular in appearance. Number of mitochondria was comparatively more than in the normal lymph node cells (Plate XXIV, Fig. 1). Ribosomal particles and few vesicles were also observed (Plate XXV, Fig. 1).

The nucleus in these cells was usually large and often showed several deep invaginations (Plate XXIV, Fig. 1). Nuclear heterochromatin content was not changed much from the normal lymph node cells.

DISCUSSION

Understanding of the characteristics of immunocompetent cells is the basis for realization of any obtuse immune response in an organism. Besides having quite a few interesting and unique physiological characteristics already pointed out earlier (Wimsatt, 1977), bats show some deviations in the arena of immunity too; there is a notable delay in the onset and decay of immune responses (Chakraborty, 1982; Chakraborty and Chakravarty, 1983a, 1983b, 1984) and they act as carriers of dreaded pathogenic agents (Constantine, 1970; Banerjee et al 1984). In the present study, the immunocompetent cell types of the Indian fruit bat, Pteropus giganteus have been analyzed in terms of their differential cell surface adhessibility to certain substrata like plastic and nylon wool, their surface morphology and surface antigenic characteristics, relative proportions and organization in the secondary lymphoid organs, and finally their ultrastructural characteristics.

The results of cell separation studies indicate that all the major categories of cells responsible for the phagocytic, humoral and cell mediated arms of immune system are present in the bat. The immunocompetent cells categorized according to their differential cell surface adhessibility into three groups — plastic adherent, nylon wool adherent and nylon wool non

adherent populations appear in a ratio of about 1:2:9 (Table 3) which approaches the proportions of murine immunocompetent cells separated by similar techniques (Paul, 1986). Thus, it seems that the relative proportions of different cell types, equivalent to murine system, possibly cannot be responsible for the delayed onset of immune response in bat. It has also been shown that certain variations in the proportions of plastic adherent and nylon wool adherent cells with a fixed number of nylon wool non adherent cells did not influence much the kinetics of Con A mediated activation of the latter (Paul, 1986).

About 3.5% of the total mononuclear cells from spleen and lymph nodes adhere readily to plastic surface (Fig. 1) and take up the vital dye, neutral red (Table 4). Subsequently, these cells were shown to be negative for both cell surface Ig (Table 5) and Thy-1 type antigen (Fig. 3). These features are also true for the macrophages of mouse, man and many other mammals (Hardwenger, 1971; Steinman and Cohn, 1973; Biemesderfer et al, 1978, Klaus et al, 1980).

The plastic non adherent cells, can further be subgrouped into nylon wool adherent and nylon wool non adherent populations. The elution behaviour of the cells from nylon wool column follows the general pattern as observed in mouse, man and many other mammalian species, where the B lymphocytes

adhere to the nylon wool fibres in the column and T lymphocytes being less adherent, pass through (Julius et al, 1973; Trizio and Cudkowicz, 1974; Kwock et al, 1976; Danilovs et al, 1980; Lewin et al, 1985). As expected, both these cell populations are neutral red negative (Table 4).

For the technique of nylon wool column separation, two protocols were used; in the first protocol, the yield of cells was quite low, probably due to retention of some cells in the column. Some workers reported that some subsets of T cells may be retained in the nylon wool column under these conditions (Shortman et al, 1972; Tada et al 1977, 1978). In the second protocol using nylon wool pretreated with EDTA and $NaHCO_3$, there was a significant increase in the yield of nylon wool non adherent cells and the overall yield of cells was more than 40% as compared to 50-60% recovery in case of mouse (Trizio and Cudkowicz, 1974).

Subsequent SEM analyses of the cell surface morphology indicates that the cells differing in surface adhessiveness also differ in their surface topography. The plastic adherent cells possess pseudopodial projections (Plate I, Figs. 1 & 2) as observed on typical macrophages of man or mouse (Warfel and Elberg, 1970; Albrecht et al, 1972, 1978; Steinman and Cohn, 1973; Besis et al, 1973; Polliack and Gordon, 1975; Quan and Golde, 1977; Biemesderfer et al, 1978). Thus, from the cell surface properties, the plastic adherent cells of bat can

possibly be equated with the macrophages of other mammals. However, in general, the size of these cells appears to be comparatively smaller than typical macrophages of mouse or man.

Besides the macrophages, we observed another type of plastic adherent cells, the difference of which from typical macrophages can be recognized under SEM for their possession of bulbuous protrussions (Plate II, Figs. 1 & 2). They resemble the follicular dendritic cells of mouse described by Steinman and his colleagues (Steinman and Cohn, 1973, Steinman et al, 1974a, 1974b, 1975, 1978, 1979; Chen et al, 1978a, 1978b). Although their presence was hinted by Chakraborty (1983) from histological observations of bat's spleen, SEM observations identify these cells properly. The presence of these cells possibly has a strong bearing in the delayed decay of immune response and the appearance of secondary response with a single antigenic stimulation in bats. These cells, present in very small numbers in mouse spleen (Steinman and Cohn, 1973) are known to retain antigens for long time on their cell processes and to sensitize other immunocompetent cells for the generation of immune response (Nossal et al, 1968a, 1968b; Sjoberg et al, 1970; Steinman et al, 1974a, 1974b, 1975, 1978; Lee et al, 1976; Chen et al, 1978; Klaus et al, 1980). Recently these cells were shown to participate in allograft rejection by migrating from the graft to lymphoid organs of the host staying in the

vicinity of $CD4^+$ T cells (Larsen et al, 1990).

The difference in cell surface topography between the nylon wool adherent and non adherent cells too, becomes apparent from the SEM studies. The nylon wool adherent cells with their small surface microvilli and pits clearly differ from the smooth surfaced, nylon wool non adherent cells (Plates IV and VI). This is in agreement with the SEM findings of many other workers who observed surface microvilli on B lymphocytes but not on T lymphocytes (Polliack et al, 1973b, 1975, 1975a, 1975b, 1981; Lin et al, 1973a, 1973b; Brynes et al, 1976; Coleman et al, 1976; Dantchev and Belpomme, 1977). Experimental results from fluorescence (Fagraeus et al, 1974), interference phase and Hoffman modulation microscopy (Sciorra and Eckert, 1974; De Harven et al, 1975; Vila and Taub, 1975; Padnos, 1976), transmission immunoelectron microscopy (Reyes et al, 1975; Mason et al, 1977) and freeze etching techniques (Renau-Piqueras, 1978; Renau-Piquerras and Knecht, 1979) also support this view.

In course of the study of surface topography of the cells, it is natural that one should take interest in the changes in membrane organization after activation of the cells, as because activation or antigenic stimulation induces remarkable changes in the activity of the cell membrane and metabolic

functions of the cells. In the present investigation, nylon wool adherent cell population showed significant membrance rufflings and formation of filamentous spikes (Plate VII, Figs. 1 & 2). Similar changes as reflection of altered membrane activity after antigenic or mit ogenic stimulation of murine lymphocytes was observed by several workers (McFarland, 1969; Loor and Hagg, 1975; Schreiner et al, 1976; Bhalla et al, 1978, 1979; Ashman, 1980).

Increase in membrane fluidity associated with a reorientation of membrane lipids (Sackman et al, 1973; Inbar and Shinitzky, 1974; Bretscher, 1976; Harris, 1976; Collard et al, 1977; Plesser et al, 1979) is now an established fact. This has been implicated to influence several biosynthetic pathways involved in the early stages of lymphocyte transformation (Shechter et al, 1972; Toyoshima and Osawa, 1975; Collard et al, 1977; Cone, 1977; Farber and Resch, 1977; Szamel et al, 1985; Somers et al, 1987). Activity of respiratory enzymes especially LDH in activated bat lymphocytes have also been observed to be altered (Paul, 1986).

Significant changes in surface topography of macrophages and nylon wool non adherent cells have not been observed in this investigation, except an overall increase in cell size

(Plates III and V). However, other workers noted more pseudopodial projections on macrophages after antigenic or mitogenic stimulation in vitro (Chapman et al, 1967; McFarland, 1969; Cline et al, 1971, 1975; Albrecht et al, 1972; Polliack and Gordon, 1975; Biemesderfer et al, 1978).

Further analysis of the immunocompetent cells have been done on the basis of cell surface immunoglobulins (Ig). In course of this study, we first identified and characterized the major classes of serum Ig in the bat. Figure 2 shows that the affinity purified serum Ig can be separated into two major fractions according to their molecular weight during Sephadex G-200 gel filtration. The similarity of elution profile of a class of bat Ig with that of purified human Ig G indicates that the molecular weight of this fraction of bat Ig approaches that of human Ig G, i.e. about 150 KD, and so, this fraction probably represents the 7S Ig G in the bat. The other class of bat Ig was eluted in the void volume much like the other mammalian Ig M as reported by other workers (Pitchappan and Muthukkarupan, 1977). In all likelihood, this fraction possibly represents the Ig M class in bat. Polyacrylamide gel electrophoresis of affinity purified bat Ig also showed the presence of two major fractions of bat Ig; the Ig G fraction distinctly migrated with purified human Ig G (Plate VIII, Fig. 1). The presence of two

classes of Ig in bats was indicated by other workers (Leonard et al, 1968; Chakraborty, 1982). Thus, it seems that bats possess both Ig M and Ig G as typical of the recently evolved mammals, although the reptiles and birds do not possess the Ig G type immunoglobulins (Pernis et al, 1971; Natarajan and Muthukkaruppan, 1984).

Immunofluorescence study revealed the presence of the Ig determinants on the surface of only one category of immunocompetent cells of bat which are adherent to nylon wool column. This indicates the distinctiveness of this group of cells and their equity with the B lymphocytes of other mammals (Sell and Gel, 1965; Nigzell and Anderson, 1969; Byrt and Ada, 1969; Raff et al, 1970; Wilson and Nossal, 1972; Reaves and Renshaw, 1978; Lewin et al, 1985), birds (Pernis et al, 1971) and reptiles (Natarajan and Muthukkaruppan, 1985).

As usual with other mammals, the majority of the B lymphocytes in bat bear Ig M determinants (Table 7). Simultaneously, the presence of Ig G on some of the B lymphocytes of bat indicates their similarity with that of other mammals and distinctiveness from reptilian and avian B lymphocytes bearing Ig M and Ig Y. The possibility of coexpression of Ig M and Ig G on the same B lymphocytes of bat however, remains as the case is in mouse and man (Perlmutter and Gilbert, 1984; Lafrenz et al, 1986).

The immunofluorescence analysis shows the different proportions of surface Ig bearing cells in different lymphoid organs and peripheral blood (Table 8). Among the lymphoid organs, spleen shows the maximum number of B lymphocytes, about 64% to 74%, while lymph nodes harbour about 29% to 35% B lymphocytes. Such a reciprocal ratio of B cells is also observed in mouse (Raff et al, 1971; Rabellino et al, 1971; Chaudhuri, 1983). Bone marrow in bats contains 32% to 45% B cells. Earlier workers (Raff et al, 1971; Rabellino et al, 1971) observed nearly 15% B cells in mouse bone marrow. Osmond and Nossal (1973), using more sensitive radioactive tracers, observed 30% B cells in mouse bone marrow. Recent work (Hamaoka and Ono, 1986; Roitt, 1988) however, revealed up to 80% Ig containing cells in mouse bone marrow of which about 50% express surface Ig. It may be mentioned that the B lymphocyte proportion in bone marrow of bat is higher than that in reptiles (about 21%), possibly indicating that the major function of bone marrow as primary lymphoid organ evolved early in the mammalian evolution.

A strikingly high percentage of B lymphocytes, beyond 70%, has been observed in the peripheral blood of bats, while the normal values in mouse and man are respectively 15% and 30% (Cooper and Lawton, 1972; Grey et al, 1971; Rabellino et al, 1971; Papamichael et al, 1971; Siegal et al, 1971;

Wilson and Nossal, 1972; Cooper and Lawton, 1972; Preud'homme and Seligman, 1972). Such high percentage of B cells have been reported in women during 7th to 15th week of pregnancy (Strelkaukas et al, 1975) and this has been implicated with the requirement of maintaining the maternal immunocompetence at a reasonable state since the immune system in the mother at this stage is depressed temporarily. How far the situation in bats is similar or divergent from this situation in man is however, a matter of conjecture at this stage. Interestingly, patients suffering from certain immunodeficiency disorders have also been reported to possess 80-90% circulating B lymphocytes (Gatti et al, 1971; South et al, 1972; Cooper and Lawton, 1972).

The high rate of cytotoxicity of rabbit anti-bat brain serum for the nylon wool non adherent cells only (Fig. 3) indicates the separate category of these cells. Details of the equivalence of anti-bat brain serum with the anti-thymocyte serum has been indicated in the introduction; the brain and thymus cells in different species have been found to share a common, evolutionarily highly conserved cell surface marker known as Thy-1 (Raff, 1971; Douglas, 1971; Acton et al, 1974; Morris et al, 1975; Letarte-Muirhead and Williams, 1975; Zwerner et al, 1977; Ropke, 1977; Williams and Gagnon, 1982)

which is thought to represent the ancestral genetic unit from which all Ig and MHC genes evolved (Shalev et al, 1985; Mansour et al, 1987).

The specificity of the antiserum towards the nylon wool non adherent cells of bat is indicated by drastic decrease in cytotoxicity of the antiserum preabsorbed with these cells. Furthermore, the anti-bat brain serum does not cause much cytotoxicity to the plastic adherent macrophages or the nylon wool adherent B lymphocytes.

The reasons for non susceptibility of a fraction of nylon wool non adherent cells to the Thy-1 antiserum (Fig. 3) could be several. The expression of the antigen on these cells could be very low to nil. The degree of Thy-1 antigen expression is not same on T lymphocytes in different species. Only in case of mouse nearly all T cells express Thy-1, while about 46% of the peripheral T cells in rats and virtually no thymocytes in human are Thy-1 positive (Acton et al, 1974; Mckenzie et al, 1981; Mansour et al, 1987).

Repeated injections of anti-bat brain serum effectively removed the T cells in vivo (Table 9). Similar depletion of T cells from secondary lymphoid organs of mice have been shown by using anti-thymocyte serum and monoclonal anti-Thy-1

antibody (Martin and Miller, 1968; Zimmerman and Tsui, 1978, 1979, 1980; LeGross et al, 1983). Removal of the T lymphocytes in vivo with anti-brain serum helped in the localization of T cell populated areas in spleen and lymph nodes of bat by studying histological preparations of these organs from bats treated with the antiserum. The T lymphocytes populate around the central arteriole of splenic white pulps and in the paracortical regions of lymph nodes (Plate XIII, Figs. 2 & 3). By studying T cell deficient nude mice and mice treated with anti-thymocyte serum (Parrott et al, 1966; Nagaya et al, 1969; Donati et al, 1969), the murine T cells were also located in the periarteriolar lymphocytic sheath of splenic white pulps and the mid- or paracortical region of lymph nodes. Thus the disposition of the T cell organization in spleen and lymph nodes of bats is in conformity with other recently evolved mammals like mouse (Gutman and Weissman, 1972; Sprent, 1973; Hoffmann-Fezer et al, 1976; Van Ewijk et al, 1981), rat (Howard et al, 1972; Goldschneider and McGregor, 1973; Barclay, 1981), human (Lamelin et al, 1978; Seymour et al, 1980; Poppema et al, 1981) and reptiles (Pitchappan and Muthukkaruppan, 1977).

Furthermore, the cell depletion study after in vivo treatment with anti-brain serum indicates the higher content of T lymphocytes in lymph nodes than in spleen of bat (Figs. 4 & 5; Plates XII & XIII), which is again in conformity with other mammals.

Immunocompetent cells have also been characterized on the basis of detailed study of organization of the cell and subcellular organelles by transmission electron microscopy. The lymphoid cells of bat can be differentiated as small lymphocytes, large lymphocytes and plasma cells on the basis of existing criteria like size, nucleocytoplasmic ratio, nuclear heterochromatinization, cytoplasmic granulation etc. as in other mammalian species (De Petris et al, 1963; De Petris and Karlsbad, 1965; Movat and Fernando, 1965a, 1965b; La Via et al, 1968; Aoki et al, 1969; Mandel, 1977). The large lymphocytes differ from the small lymphocytes by their higher content of cytoplasm, mitochondria, ribosomes and lesser nuclear heterochromatin (Plate XVI, Fig. 1). A category of large lymphocytes with average diameter of more than 7 μm and larger euchromatic nucleus can be identified as plasma cells different from other large lymphocytes (Plate XXII, Fig. 1) as in other mammals (Zucker-Franklin, 1963, 1965; Inman and Cooper, 1965; Matter et al, 1972). Structure and distribution wise, the cellular organelles are typical as in the other mammals (Parker et al, 1965; Cohn et al, 1966; Hirsch and Fedorko, 1968; Van Furth et al, 1970, 1972, 1976; Matter et al, 1972; Steinman et al, 1974, 1975; Chen et al, 1978a, 1978b) and no obvious deficiency could be observed.

The scanning and immunofluorescence microscopy could detect the difference of B and T cells for sure which is not possible with precision in TEM study. In future this could be achieved by employing immunoelectron microscopic techniques by suitable markers. The characteristic microvilli on the B lymphocytes seen under SEM are not discernible in the tissue state of organization revealed by TEM. Besides subcellular organization, the TEM study elucidates cell-cell organization in the lymphoid organs of bat. The increased metabolic activity of cells as reflected from the increased cell size, nucleus to cytoplasm ratio, ribosomal content and less heterochromatinization of the nucleus in the lymphocytes from immunized bats is also discernible by TEM.

To our knowledge, the present investigation is possibly the first attempt of studying the immunocompetent cells of bat at ultrastructural level.

These qualitative studies along with quantitation of different cell types in bat show no reasonable deficiency in immune system of this animal. Thus the causative factor for delayed onset and decay of the immune responses of bats needs to be looked for in other aspects of the immune mechanisms in bats. This could be lower density of membrane receptors on bat lymphocytes as recently shown by Paul and Chakravarty (1988)

in connection of the sensitivity of the cells to Con A stimulation. Slower rate of energy turnover during activation of lymphocytes could also contribute to delayed response in bats as indicated by Paul (1986). These revelations point to the fact that genomic deficiency for receptor density or certain other molecules might explain peculiarities of immune responses in bats. Simultaneously, a low keyed response without any genomic deficiency as an adaptive feature in this animal can not also be ruled out.

Follicular dendritic cells, having capacity of retaining antigen for long time on their membrane have been identified in bats by our study; these cells may very well contribute towards the slower decay of immune response. The low titer of antibody could help in making the pathogens ineffective in low concentration and allow the bat to be a carrier.

In other words, the present study indicates the mechanism of delayed response in bats lies somewhere else than the deficiency on the part of the immunocompetent cells.

The major points transpiring from this investigation may be summed up here. For the first time, the immunocompetent cell types macrophages, B and T lymphocytes of bats have been characterized in great detail. They differ in adhessibility

to substratum, like macrophages to plastic, B lymphocytes to nylon wool fibres and T lymphocytes without any of these properties. Simultaneously, they can also be differentiated on the basis of specific markers on the cell membrane, B cells being positive for Ig M and Ig G, and T cells with Thy-1 type antigen. At the ultrastructural level, specific membrane characteristics like microvilli to the B lymphocytes, comparatively smooth surface to the T lymphocytes and pseudopodial projections to the macrophages can be ascribed. Differential characteristics of these cell types including plasma cells were also revealed by TEM.

Furthermore, the ultrastructural studies also indicated the state of differentiation of the lymphocytes after antigenic stimulation on the basis of changes in nucleus, cytoplasm and plasma membrane. The SEM studies quite distinctly established the formation of spikes on the surface of activated B cells which might indicate the change in physical state of the cell membrane from gel to sol as often encountered in course of hectic activity of the cell membrane following antigenic or mitogenic stimulus.

Besides having specific characteristics, the cell types have specific spatial distribution as revealed from the histological studies.

This study also shows the immunocompetent cell types in bats are in almost all aspects similar to those in highly evolved mammalian species like mouse and man. Chakraborty and Chakravarty earlier observed that organization of thymus in P. giganteus is pretty much similar to that in primates. These facts become revealing on the background that the chiropterans originated very early, almost at the point of origin of Class Mammalia. So, it may be projected that the lymphocyte had all its endowments including specific cell surface markers in different mammals since their origin, or they evolved in different orders of this class as a result of parallel evolution

S U M M A R Y

Bats, classified under the order Chiroptera which originated almost at the beginning of mammalian evolution, are known for their role as carriers of a number of dreaded pathogens without being infected. Previous studies in our laboratory revealed a noticeably delayed onset and decay of immune responses in these animals, but little was known about the cells mediating immunity in bats. In the present work, the major categories of immunocompetent cells in the Indian fruit bat Pteropus giganteus have been characterized in detail by several criteria, viz. their adhessibility to different substrata, cell surface topography, surface antigenic markers, organization in lymphoid organs, and ultrastructural details.

Cell separation techniques based on physical adherence to plastic and nylon wool distinguished three major groups of immunocompetent cells with differential surface adhessibility — the plastic adherent cells, the nylon wool adherent cells and the nylon wool non adherent cells. Intake of the vital dye neutral red by the plastic adherent cells but not by other cell types indicated the phagocytic nature of these

cells. Subsequent immunofluorescence and cytotoxicity experiments revealed that the plastic adherent cells were negative for both surface Ig and Thy-1 type antigen; the nylon wool adherent cells were surface Ig positive but Thy-1 negative, while the reverse was true for the nylon wool non adherent cells.

The plastic adherent, nylon wool adherent and nylon wool non adherent cells appeared in a ratio of about 1:2:9, which is pretty close to the ratio of murine macrophages, B and T lymphocytes obtained by similar techniques; this indicates that delayed responses in bats are probably not caused by a deficiency in the type or quanta of the immunocompetent cells.

Next, scanning electron microscopic analysis delineated significant differences in surface topography among these three categories of cells. The plastic adherent cells were characterized by their possession of different types of pseudopodia, the nylon wool adherent cells showed small microvilli, while the nylon wool non adherent cells characteristically had a smooth cell surface. These features are in compliance with the macrophages, B and T lymphocytes respectively of many other mammals.

A small group of plastic adherent cells possessing bulbuous protrusions were distinguished by SEM from the usual pseudopodia bearing plastic adherent cells; these cells resembled the murine follicular dendritic cells. Such cells, because of their proven role in antigen retention and presentation to other lymphoid cells in mouse, rat, man etc. led us to propose that they might have important implications for the delay in decay of immune responses in bats.

Significant changes in surface topography of especially the nylon wool adherent cells were noted after in vivo stimulation with a model antigen like sheep erythrocytes. A notable increase in surface ruffling and the production of long filamentous spikes on the surface of nylon wool adherent cells possibly reflected an alteration in membrane fluidity which is usually associated with transitional changes in the lipid bilayer of the membrane during lymphocyte transformation.

Further characterization of the cell types was brought about by analyses of cell surface antigenic moieties. In course of this study, serum immunoglobulins of bat isolated by affinity chromatography, were fractionated into two major classes by gel filtration. The molecular weights of these two fractions as judged by their chromatographic elution pattern and their

relative electrophoretic mobility resembled those of human Ig M and Ig G. These two classes of Ig thus represent the Ig M and Ig G in this animal. Subsequent immunofluorescence microscopy using rabbit antisera to bat Ig M and Ig G fractions revealed the presence of surface Ig M or Ig G specifically on the nylon wool adherent cells, thereby confirming further their equivalence to the B lymphocytes of other mammals. Majority of the B cells possessed cell surface Ig M as in other higher vertebrates while presence of Ig G on some B cells distinguished them from reptilian or avian Ig Y bearing cells.

Analysis of the tissue distribution of the B lymphocytes revealed that bone marrow of bats contained 32-45% B cells, probably indicating that bone marrow is the primary lymphoid organ for generation of B lymphocytes. Spleen and lymph nodes contained 64-71% and 29-35% B cells respectively; such a reciprocal proportion is also true for mouse and man. Number of B cells in peripheral blood is above 70% which is strikingly higher than in normal mouse or man; such a higher proportion is encountered in human patients with certain immunodeficiency disorders. The exact significance of it for the immune responses in bat is yet to be resolved.

The existence of a T cell compartment in the bat immune system was confirmed by the demonstration of susceptibility of

specifically the nylon wool non adherent cells to rabbit anti-bat brain serum, thereby indicating sharing of a Thy-1 type cell surface antigen between these cells and brain cells like the T lymphocytes of other mammals. Anti-thymocyte serum could not be used in this experiment because of non availability of thymus in adult bats due to thymic involution as in man. Thus a clear dichotomy of the lymphocytic population along T and B lines in the bat was established.

In vivo administration of anti-bat brain serum in bats effectively depleted the T lymphocyte population from secondary lymphoid organs such that the ratio of plastic adherent : nylon wool adherent : nylon wool non adherent cells came down to about 1:2:4 after 72 hours of treatment, as compared to the normal ratio of 1:2:9. This experiment then led us to investigate the localization of the T lymphocytes in the secondary lymphoid organs of bat. Histological study of spleen and lymph nodes of anti-brain serum treated bats revealed T cell depletion chiefly in the periarteriolar lymphocytic sheath of splenic lymphoid follicles and in lymph node paracortex; these regions may therefore be marked as T dependent regions in bats as in mouse or man.

Besides the surface topographic and antigenic characterization, the internal architecture of the cells was studied by

transmission electron microscopy of the cells in secondary lymphoid organs. On the basis of size and shape of the cells, nucleocytoplasmic ratio, nuclear heterochromatinization, cytoplasmic organelles etc., four types of immunocompetent cells were identified; small lymphocytes had a heavily heterochromatinized nucleus surrounded by a thin rim of cytoplasm, while the larger lymphocytes possessed a less heterochromatinized nucleus and a higher content of mitochondria, Golgi vesicles, endoplasmic reticulum, ribosomes etc. Plasma cells had characteristically large, mainly euchromatic nucleus and a large amount of cytoplasm while macrophages showed an irregular shape, less heterochromatimized nucleus and cytoplasm containing vesicles resembling lysosomes and phagosomes.

After immunization, an increase in cell size, nuclear cytoplasmic ratio and ribosomal content and a decrease in nuclear heterochromatimization indicated the differentiated state of these cells under TEM.

The present investigation tried to characterize the immunocompetent cells of Pteropus giganteus, an evolutionarily old mammal, from several points of view, such as structural endowments, specific cell surface antigenic markers and their differential distribution. All these features are comparable

to those of the highly evolved recent mammals like primates. So it appears that the characteristic features of mammalian lymphocytes evolved almost at the time of origin of mammals and since then they remained as permanent fixtures in course of evolution, or one needs to envisage parallel evolution of immunocompetent cells in different orders of mammals to accommodate the idea of constant changes during evolution.

Furthermore, the similarities in types and ratio of the immunocompetent cells of bat with those of other mammals suggest that the reason for delayed immune responses in bats lies somewhere else than in the possibility of deficiency in types or quanta of immunocompetent cells. Recent revelations in our laboratory about lower density of antigen/mitogen receptors on lymphocyte surface (Paul and Chakravarty, 1989) and slower energy turnover in lymphocytes during activation (Paul, 1986) in bats as genomic deficiency or adaptation might hold the key for explaining peculiarities of delayed immune response in this interesting animal.

Fig. 1 Proportions of plastic adherent cells (PA) in spleen and lymph node cell population of bat. Total mononuclear cells from spleen and lymph node. Plastic adherent cells.

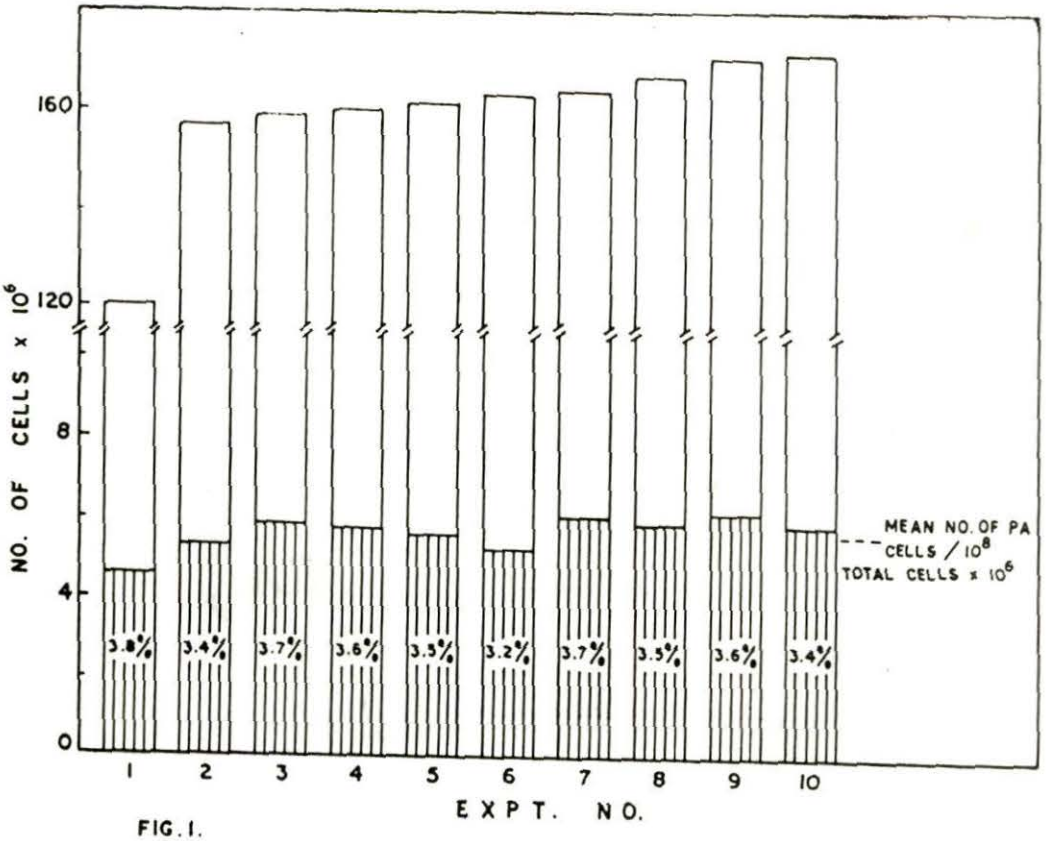

NO. OF CELLS x 10^6
160
120
8
4
0
3.8%
3.4%
3.7%
3.6%
3.5%
3.2%
3.7%
3.5%
3.6%
3.4%
1
2
3
4
5
6
7
8
9
10
EXPT. NO.
MEAN NO. OF PA CELLS / 10^8
TOTAL CELLS x 10^6

FIG. 1.

Fig. 2 Sephadex G-200 elution profile of bat immunoglobulins showing separation of two classes of Ig, one eluting in the void volume and another eluting later, almost in the same position as purified human Ig G run later under same conditions. O—O bat Ig, ●--● purified human Ig G.

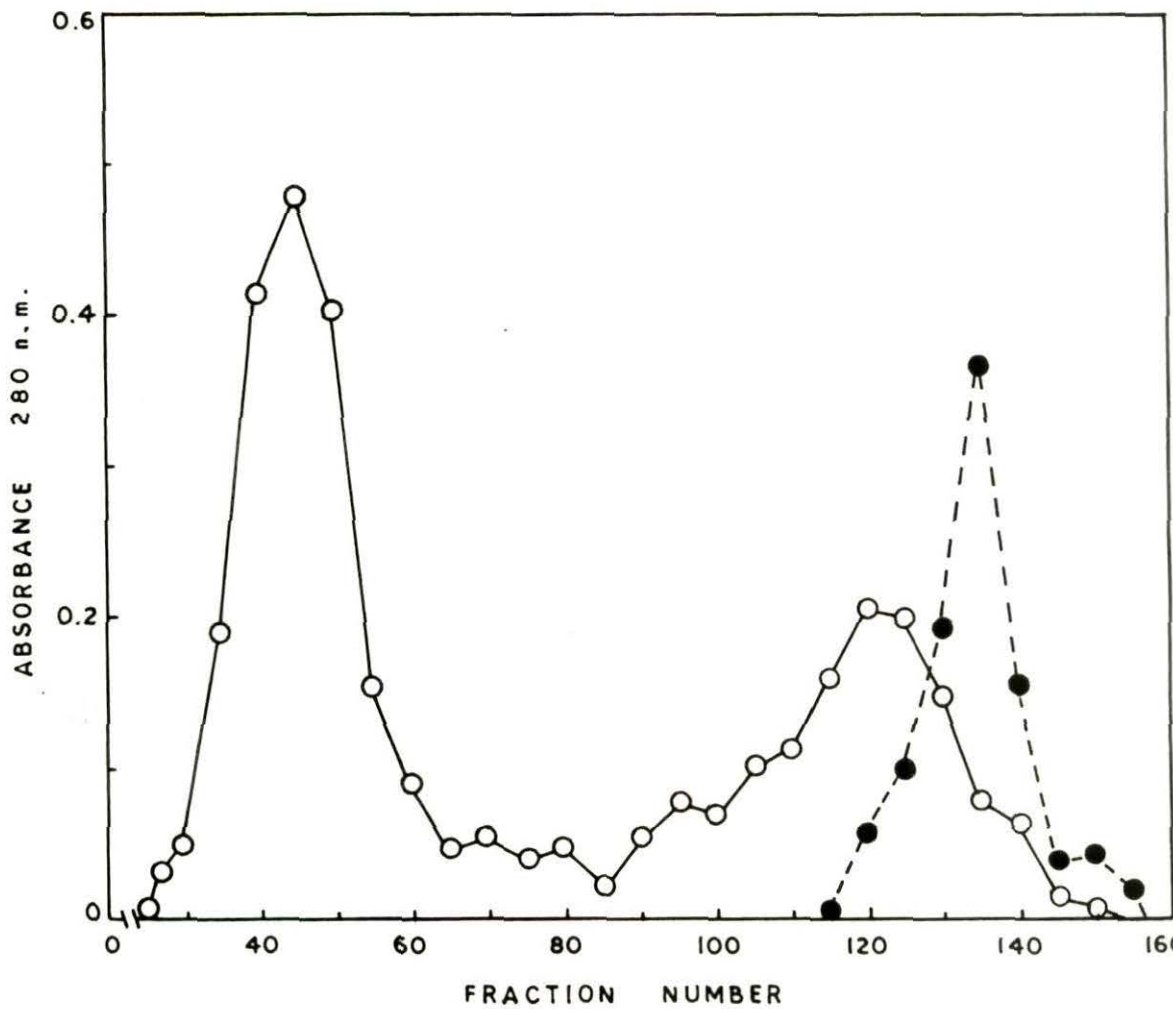

0.6
0.4
0.2
0
ABSORBANCE 280 n.m.
0
40
60
80
100
120
140
160
FRACTION NUMBER

FIG. 2.

Fig. 3 Cytotoxicity of rabbit anti-bat brain serum at different dilutions against different immunocompetent cell populations of bat. O—O nylon wool non adherent cells, ●—● nylon wool adherent cells, ◐—◐ plastic adherent cells, ●—● nylon wool non adherent cells but with antiserum preabsorbed with the same type of cells.

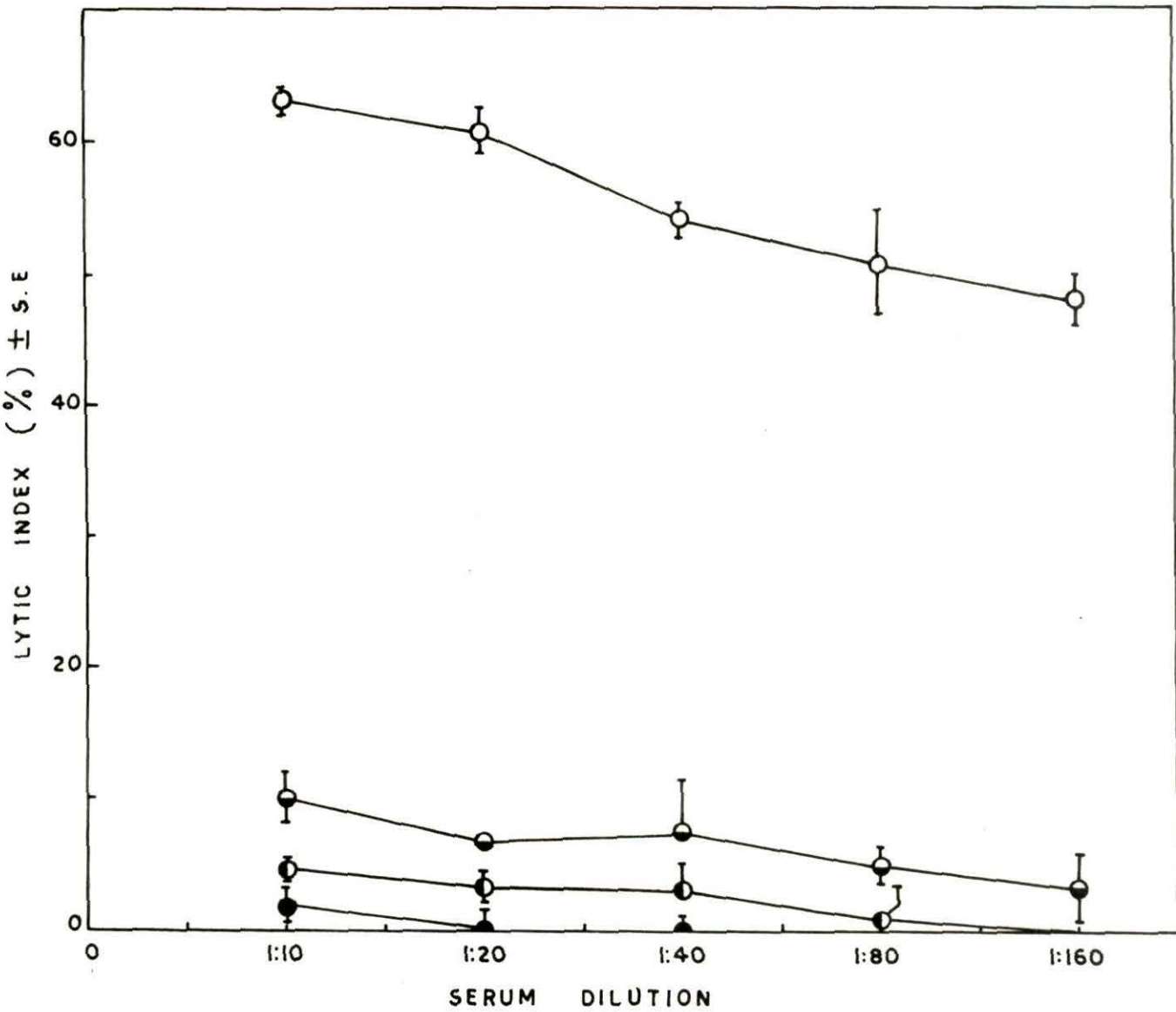


FIG. 3.

Fig. 4 Histogram showing density of lymphocytes per 0.001 mm^2 area in different regions of spleenic white pulp in normal bats and bats after the course of anti-brain serum treatrent. ▨ region adjacent to central arteriole, □ region distant to central arteriole.

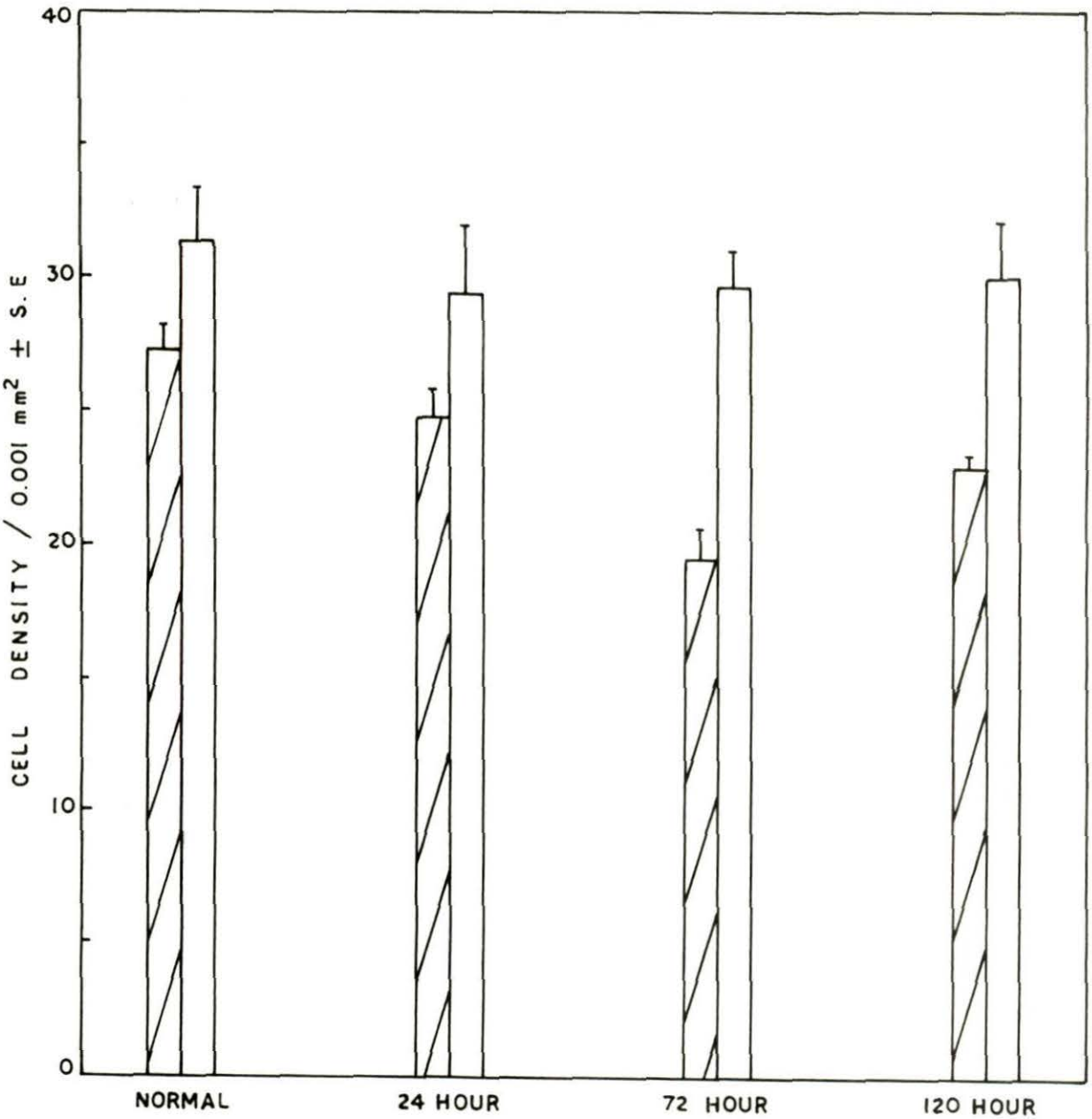

40
30
20
10
0
CELL DENSITY / 0.001 mm² ± S.E
NORMAL
24 HOUR
72 HOUR
120 HOUR

FIG. 4

Fig. 5 Histogram showing density of lymphocytes per 0.001 mm^2 area in different regions of lymph node from normal bats and bats after the course of anti-brain serum treatment ▨ paracortical area, □ follicular area.

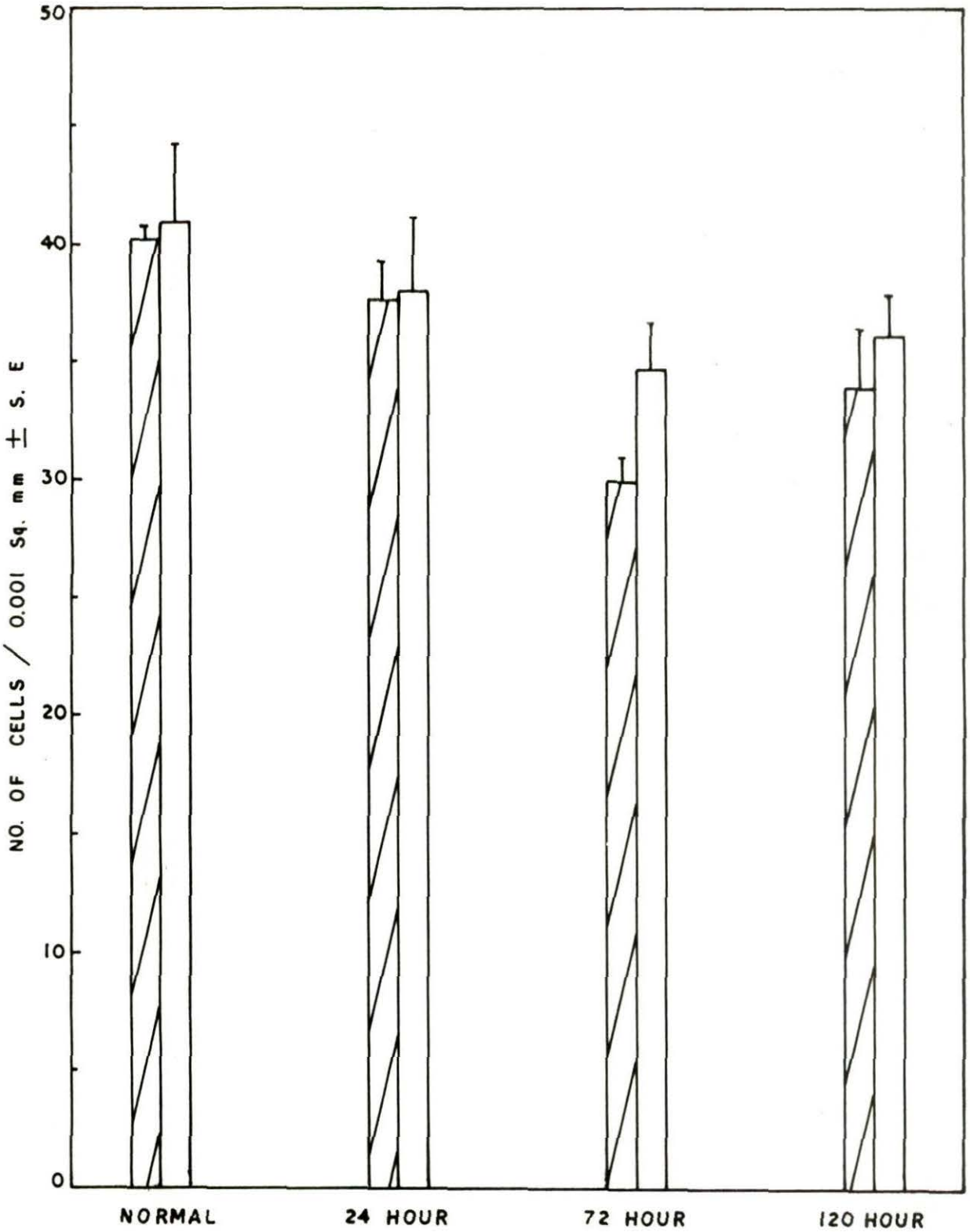

50
40
30
20
10
0
NO. OF CELLS / 0.001 Sq. mm ± S. E
NORMAL
24 HOUR
72 HOUR
120 HOUR

FIG. 5.

Table 1 . Lymphocytic sub populations of bat separated on untreated nylon wool column

Expt. No.	Total No. of cells incubated x 10^6	No. of cells (NA + NNA)[a] recovered x10^6 (% recovery)	Mean of % recovery (± SE)	No. of NA cells recovered x10^6	Mean (± SE)	No. of NNA cells recovered x 10^6	Mean (± SE)	Ratio of NA : NNA
1	150	4.81 (3.21)		2.31		2.50		
2	150	5.26 (3.51)		2.42		2.84		
3	150	6.35 (4.23)	3.840 (0.224)	2.43	2.51 (0.143)	3.92	3.248 (0.255)	1:1.4
4	150	6.37 (4.25)		2.42		3.95		
5	150	4.85 (3.23)		2.28		2.57		
6	150	6.93 (4.62)		3.22		3.71		

[a] NA and NNA stand for nylon wool adherent and nylon wool non adherent cells respectively in all tables.

Table 2. Lymphocytic sub populations of bat separated on nylon wool colum pretreated with EDTA and $NaHCO_3$

Expt. No.	Total cells incubated x 10^6	No. of cells (NA + NNA)[a] recovered x10^6 (% recovery)	Mean of % recovery (± SE)	No. of NA cells recovered x 10^6	Mean (± SE)	No. of NNA cells recovered x 10^6	Mean (± SE)	Ratio of NA : NNA
1	150	67.92 (45.28)		15.04		52.88		
2	150	72.45 (48.29)	40.60 (3.760)	15.44	13.15 (1.208)	57.01	47.75 (4.484)	1:3.63
3	150	55.29 (36.86)		11.15		44.14		
4	150	47.95 (31.97)		10.98		36.97		

[a] Abbreviations as in Table 1

Table 3 . Proportion of three different immunocompetent cell types of bat separated adhessibility to plastic and nylon wool[a]

Expt. No.	No. of PA cells[b] recovered x 10^6	No. of NA cells recovered x 10^6	No. of NNA cells recovered x 10^6	Ratio of three cell types PA:NA:NNA	Mean ratio PA:NA:NNA
1	4.60	11.58	40.82	1.00:2.52:8.87	
2	5.84	16.42	61.44	1.00:2.81:10.52	1.00:2.35:8.83 (1:2:9 approx.)
3	6.20	12.30	48.62	1.00:1.98:7.84	
4	5.80	12.18	46.94	1.00:2.10:8.09	

[a] Each experiment was done with the total cells obtained from secondary lymphoid organs of an individual bat.

[b] PA stands for plastic adherent cells. Other abbreviations as in Table 1.

Table 4. Neutral red positive cells in three different immunocompetent cell populations of bat separated by adhessibility.

Cell type	Expt. No.	No. of cells x 10^6	Neutral red positive cells	
			No. of cells x 10^6	% of cells
Plastic adherent	1	4.60	4.05	88.04
	2	5.80	5.30	91.38
	3	5.84	5.22	89.38
Nylon wool adherent	1	11.60	1.25	10.78
	2	12.16	1.50	12.34
	3	16.64	2.31	13.88
Nylon wool non adherent	1	40.80	2.61	6.40
	2	46.96	3.66	7.79
	3	61.44	3.81	6.20

Table 5 . Enumeration of surface Ig M and Ig G bearing cells in the plastic adherent cell population of bats.

Expt. No.	Percentage of surface Ig bearing cells			
	Ig M^+ cells	Mean (± SE)	Ig G^+ cells	Mean (± SE)
1(a)*	4.00	8.25 (2.63)	4.17	7.43
	13.04		3.85	(3.43)
	7.69		14.29	
1(b)*	13.64	7.45 (3.12)	8.00	7.08 (1.82)
	3.70		3.00	
	5.00		9.68	
2(a)	11.76	14.51 (3.28)	10.53	7.82 (2.03)
	21.05		3.85	
	10.71		9.09	
2(b)	20.00	12.33 (4.12)	4.17	12.13 (4.34)
	5.88		22.22	
	11.11		10.00	

*(a) and (b) represents two separate experiments with cells from an animal. In each set three tubes containing 10^6 cells were used.

Table 6 . Enumeration of surface Ig M and Ig G bearing cells in the nylon wool non adherent cells of bats.

Expt. No.	Percentage of surface Ig bearing cells			
	Ig M positive cells	Mean (± SE)	Ig G positive cells	Mean (± SE)
1(a)*	4.17	6.08 (0.98)	8.69	8.19 (1.85)
	7.41		11.11	
	6.67		4.76	
1(b)*	8.00	7.17 (1.73)	4.17	7.44 (3.43)
	9.68		3.85	
	3.85		14.29	
2(a)	19.05	10.32 (4.64)	5.56	9.43 (2.34)
	3.23		13.64	
	8.69		9.09	
2(b)	8.00	9.57 (0.81)	7.69	6.42 (1.13)
	10.71		4.17	
	10.00		7.41	
3	3.13	7.91 (4.72)	3.57	2.17 (0.89)
	3.45		0.00	
	26.32		3.45	
	6.67		0.00	
			3.85	

* As in Table 5

Table 7. Enumeration of surface Ig M and Ig G bearing cells in the nylon wool adherent cells or bats.

Expt. NO.*	Percentage of surface Ig bearing cells			
	Ig M^+ cells	Mean (± SE)	Ig G^+ cells	Mean (± SE)
1(a)	58.33	57.48 (1.35)	33.33	36.51 (3.17)
	54.84		42.86	
	59.26		33.33	
(b)	61.54	58.46 (6.58)	40.74	39.35 (5.59)
	45.83		48.28	
	68.00		29.03	
2(a)	50.00	50.86 (4.37)	28.57	30.26 (2.19)
	58.82		34.62	
	43.75		27.59	
(b)	57.89	53.04 (2.78)	36.84	33.18 (4.72)
	48.28		23.81	
	52.94		38.89	
3	58.33	52.91 (5.93)	30.43	34.61 (1.98)
	33.33		36.84	
	64.29		33.33	
	63.16		31.25	
	45.45		41.18	

* As in Table 5

Table 8. Surface Ig M and Ig G bearing lymphocytes from different lymphoid organs & peripheral blood of bats.

Source of cell	Expt. No.	Percentage of surface Ig bearing cells			
		Ig M^+ cells	Mean (± SE)	Ig G^+ cells	Mean (± Se)
Bone marrow	1a)	24.00	20.16 (2.38)	23.53	13.34 (5.12)
		20.69		7.41	
		15.79		9.09	
	b)	25.00	22.36 (1.64)	10.71	9.64 (0.98)
		22.73		10.53	
		19.35		7.69	
	2a)	20.69	24.47 (1.92)	28.57	20.04 (4.55)
		25.81		18.52	
		26.92		13.04	
	b)	24.24	23.40 (2.31)	22.73	18.93 (1.91)
		19.05		16.67	
		26.92		17.39	
Spleen	1a)	44.44	42.99 (0.80)	27.27	26.95 (1.04)
		41.67		28.57	
		42.86		25.00	
	b)	45.45	41.19 (5.24)	27.78	24.05 (4.13)
		30.77		28.57	
		47.37		15.79	
	2a)	45.83	41.99 (4.96)	36.00	30.80 (3.94)
		48.00		23.08	
		32.14		33.33	

Contd..

Table 8 (Contd..)

Source of cell	Expt. No.	Percentage of surface Ig bearing cells			
		Ig M^+ cells	Mean (± SE)	Ig G^+ cells	Mean (±SE)
Spleen	2b)	40.91	40.41 (3.29)	30.77	30.32 (6.67)
		34.48		18.52	
		45.83		41.67	
Mesenteric lymph node	1a)	18.18	19.38 (1.03)	9.52	9.66 (1.18)
		21.43		11.76	
		18.52		7.69	
	1b)	30.77	21.27 (4.77)	4.17	13.24 (5.21)
		15.79		22.22	
		17.24		13.33	
	2a)	17.39	22.77 (2.70)	7.41	11.20 (1.89)
		25.93		13.16	
		25.00		13.04	
	2b)	19.23	20.81 (1.67)	12.50	10.28 (1.29)
		24.14		8.00	
		19.05		10.34	
Peripheral blood	1a)	46.67	44.34 (4.10)	10.00	24.92 (8.02)
		36.36		37.50	
		50.00		27.27	
	1b)	33.33	51.85 (9.79)	44.44	34.74 (5.61)
		66.67		25.00	
		55.56		34.78	

Contd..

Table 8 (Contd..)

Source of cell	Expt. No.	Percentage of surface Ig bearing cells			
		Ig M^+ cells	Mean (± SE)	Ig G^+ cells	Mean (± SE)
Peripheral blood	2a)	43.75	49.58 (4.68)	38.89	36.57 (1.67)
		46.15		33.33	
		58.82		37.50	
	2b)	57.14	53.59 (7.05)	35.29	35.93 (0.79)
		40.00		35.00	
		63.64		37.50	

Table 9. Proportion of three different immunocompetent cell types of bat after *in vivo* administration of rabbit anti-bat brain serum

Time after antiserum treatment	No. of PA[a] cells $x10^6$	No. of NA cells $\times 10^6$	No. of NNA cells $\times 10^6$	Ratio of three cell types PA:NA:NNA
24 hrs	6.8	10.4	48.2	1:1.53:7.09
72 hrs	5.6	10.7	24.18	1:1.91:4.32
120 hrs	8.6	8.8	38.4	1:1.02:4.47

Plate - I

Fig. 1 & 2 Scanning electron micrographs of plastic adherent cells, i.e. macrophages of normal bat.

1) One with flattened pseudopodial projections (arrow) and (2) another with finger like filopodial projections.
X 11,000, X 10,500

[Bar equals to 2 μm in these and subsequent SEM photographs.]


1

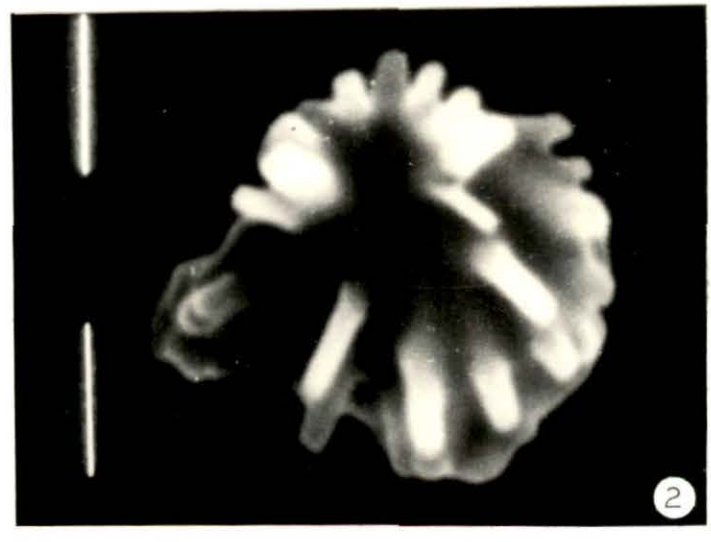

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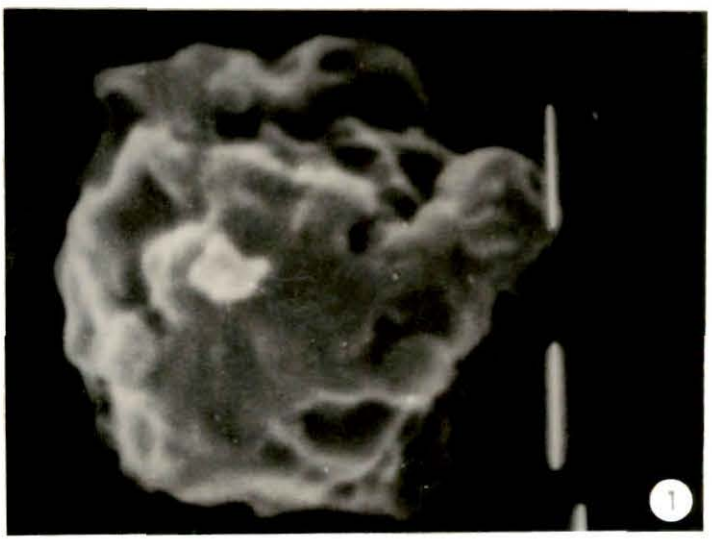
Plate - II

Fig. 1 & 2 SEM photographs of two plastic adherent cells from a normal bat with bulbuous protrusions, similar to follicular dendritic cells; size of the cells is bigger than most other plastic adherent cells. X 8,500 X 5,000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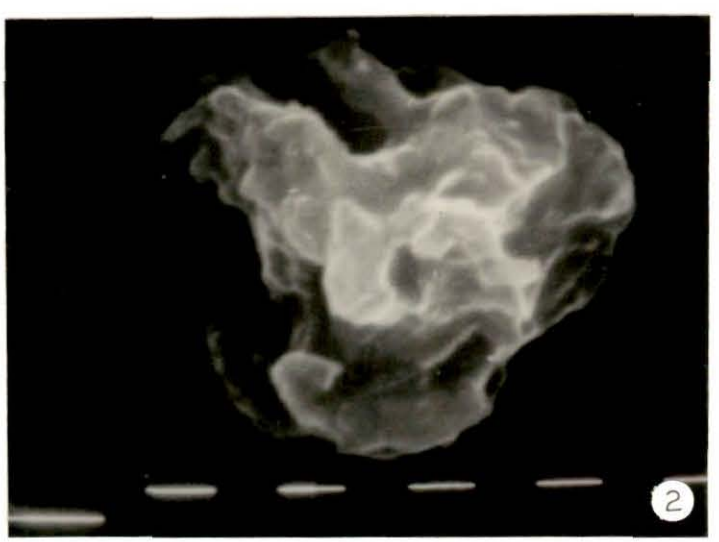
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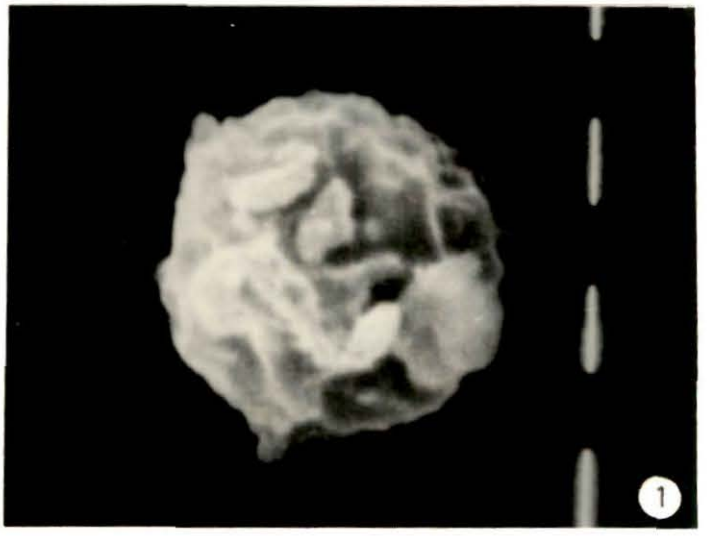
Plate - III

Fig. 1 & 2 SEM photographs of plastic adherent cells from bat immunized with SRBC showing that pseudopodial projections are not as prominent as in normal cells. X 6,000, X 6000.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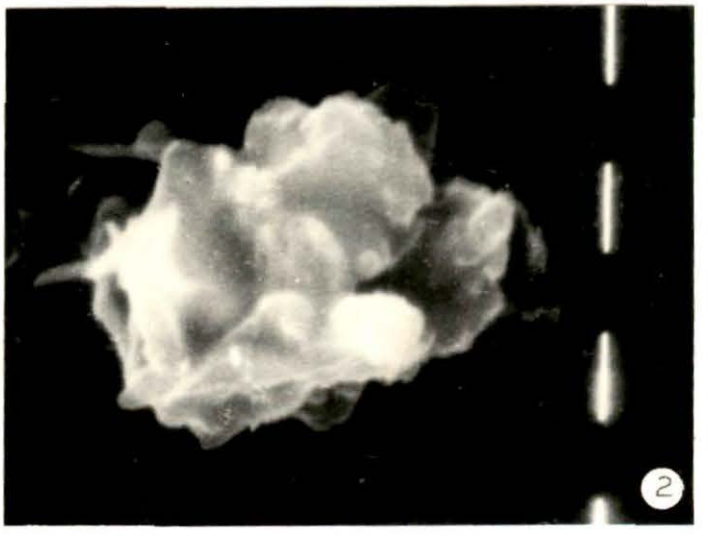
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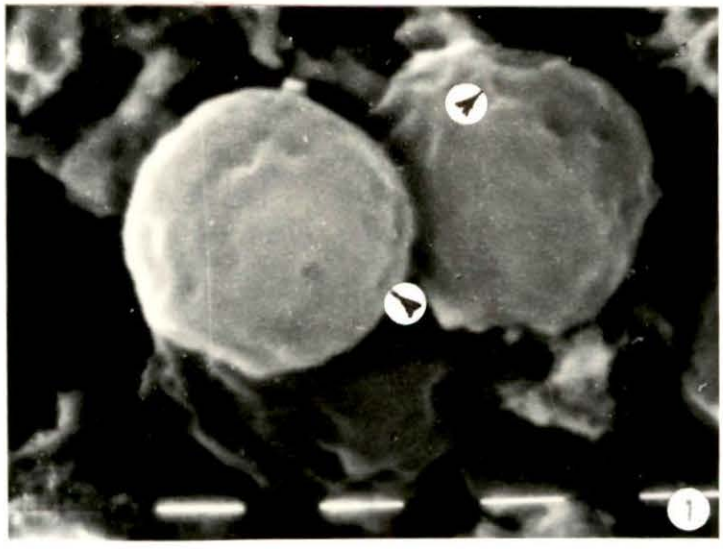
Plate - IV

Fig. 1 & 2 Typical nylon wool non adherent cells (equivalent to T cells) from a normal bat showing smooth cell surface devoid of any projections but occasional surface ridges (arrow). Diameter of the cells is about 7 μm in Fig. 1 and about 8 μm in Fig. 2. X 5,500, X 4,250.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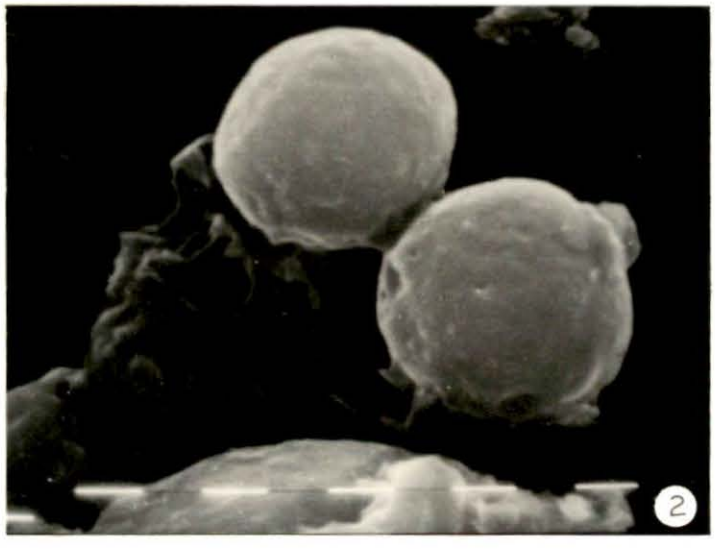
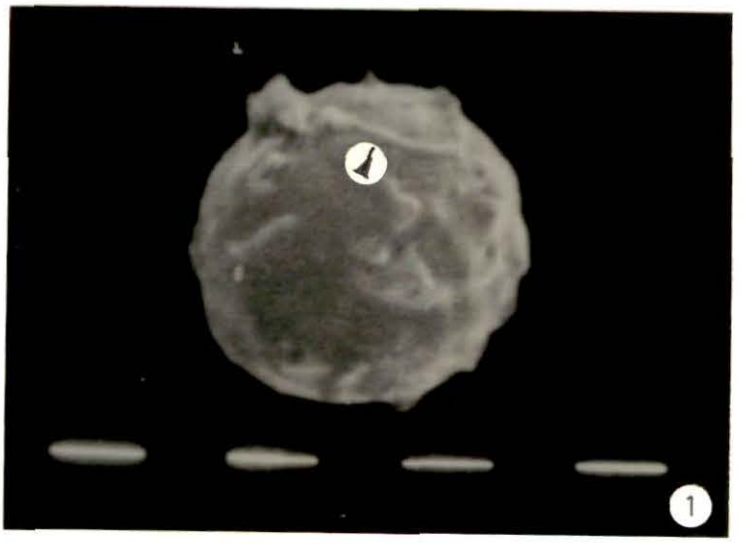

2

Plate - V

Fig. 1 & 2 SEM photographs of nylon wool non adherent cells from immunized bat showing some surface rufflings (arrow). Size of cell is comparatively bigger and about 7 μm in Fig. 1 and about 10 μm in Fig. 2.
X 6000, X 5,250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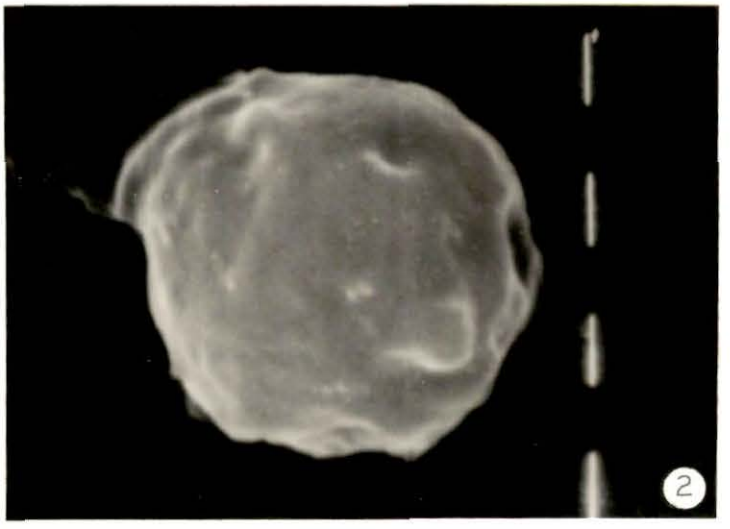
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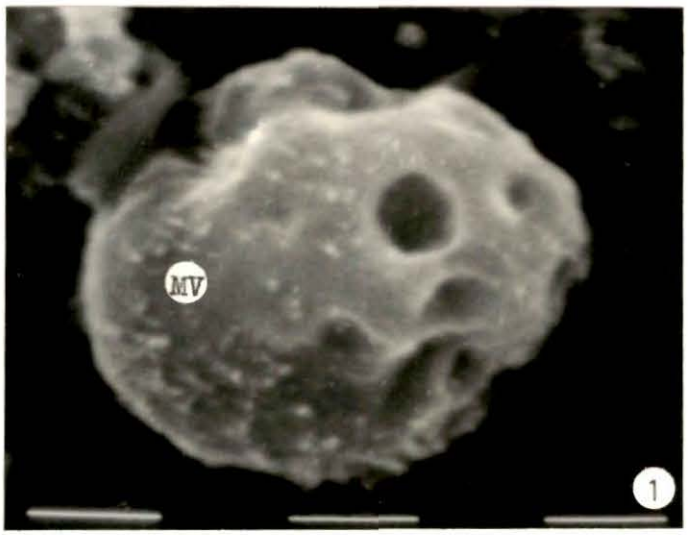
Plate - VI

Fig. 1 & 2 SEM photographs of nylon wool adherent cells from normal bat. Some small microvilli (MV) and pits (P) are seen on the surface. Size of cell is about 9 µm. X 8000, X 5500


MV
1

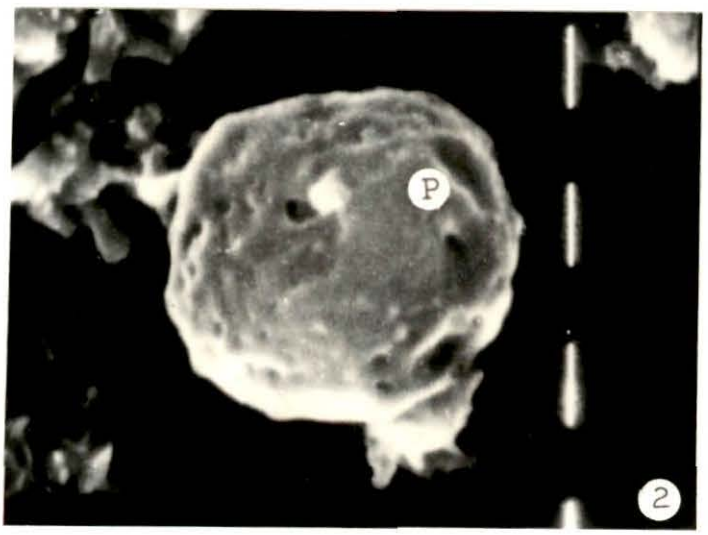
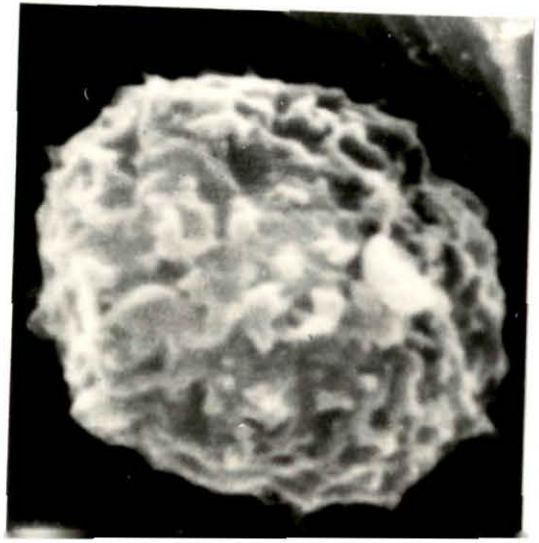
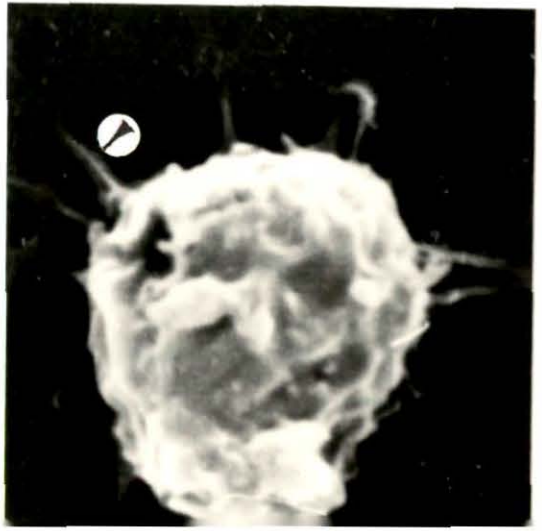

P
2

Plate - VII

Fig. 1,2 & 3 SEM photographs of nylon wool adherent cells from an immunized bat. The cell surface is highly ruffled. Long filamentous surface projections can be seen in Fig. 1 and 2 (arrow). Size of the cells about 9 µm in Fig. 1 and 2, and about 12 µm in Fig. 3. X 6000, X5250, X 5000.





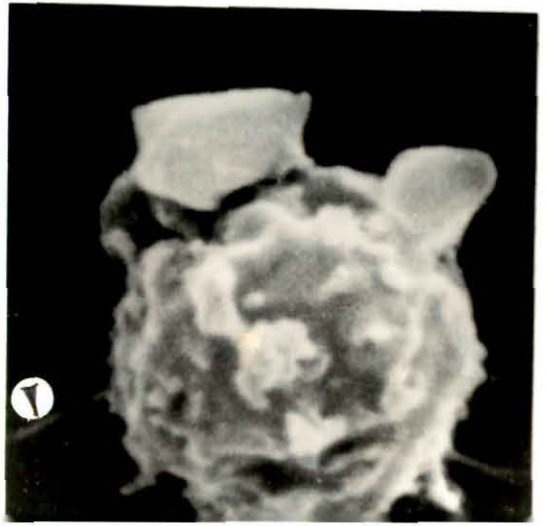
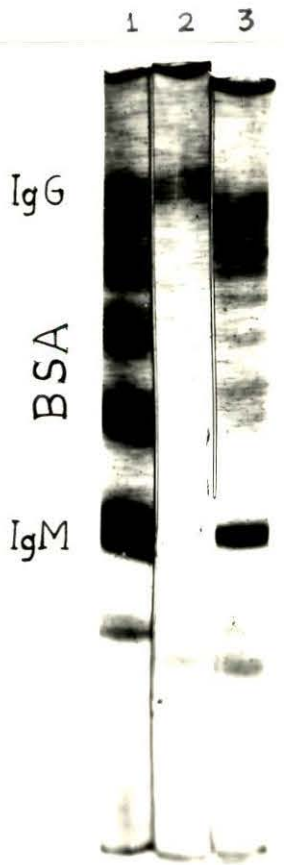


Plate - VIII

Fig. 1 Photograph showing the separation of major immunoglobulin classes of bat by polyacrylamide gel electrophoresis. Lane 3 contains bat Ig obtained by affinity chromatography, lane 2 contains purified human Ig G and lane 1 contains a mixture of bat Ig, human Ig G and BSA.

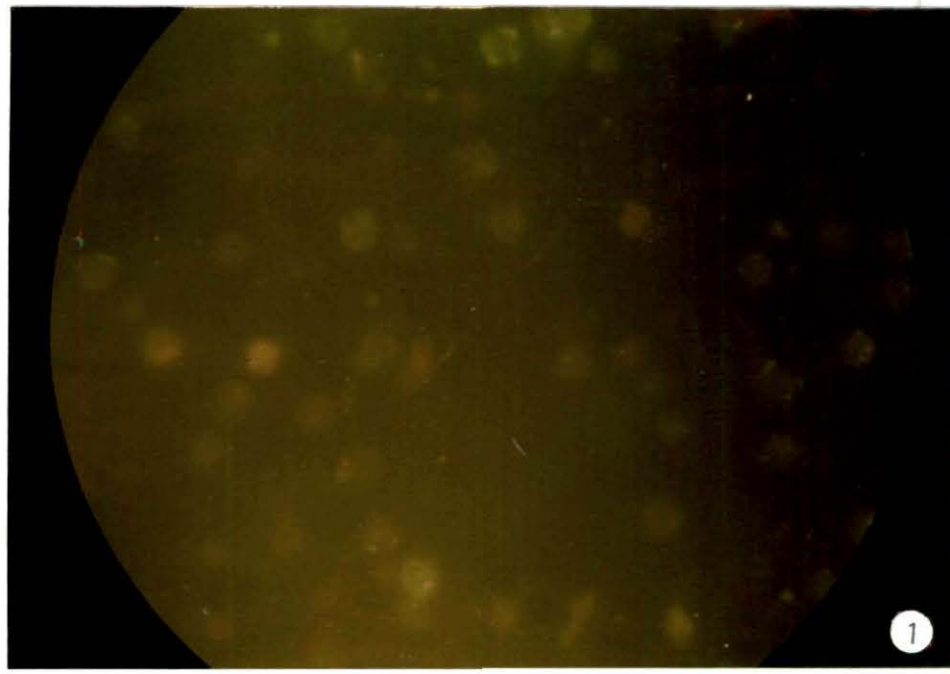


1

Plate - IX

Fig. 1 Photomicrograph showing indirect immunofluorescence of spleen cells of bat, treated with rabbit anti-bat Ig M (anti-B Ig M) and then fluoresceinated goat anti-rabbit Ig (Fl-anti-R Ig). Fluorescence on the cells is in the form of ring and patches. X

Fig. 2 & 3 Photomicrographs of spleen cells treated with rabbit anti-bat Ig G (anti-B Ig G) and then Fl-anti-R Ig showing a distinct ring type of immunofluorescene on the central cell, which is shown in magnified view in Fig. 3. X 700, X 750, X 900.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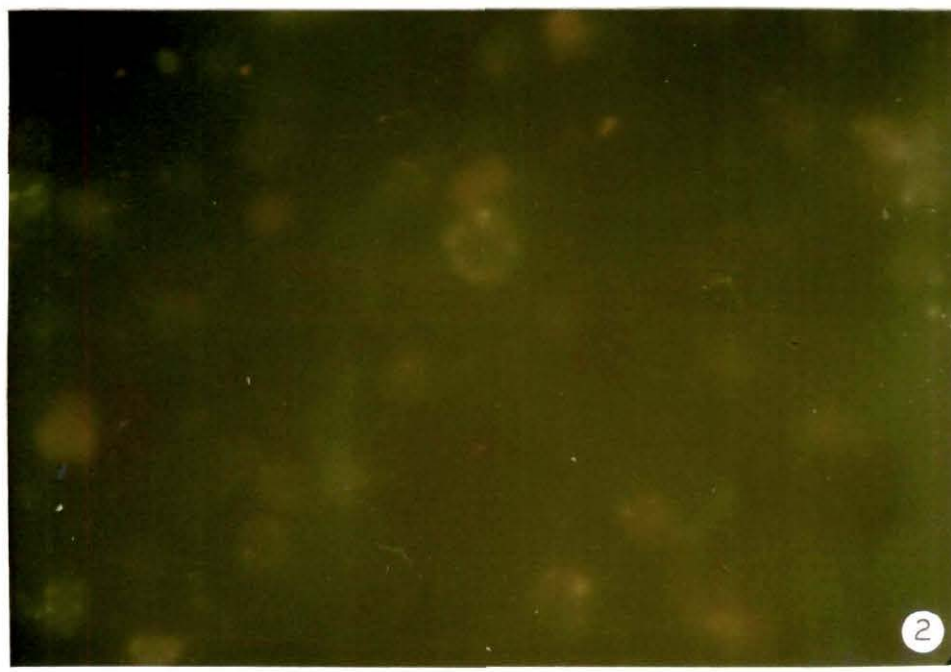
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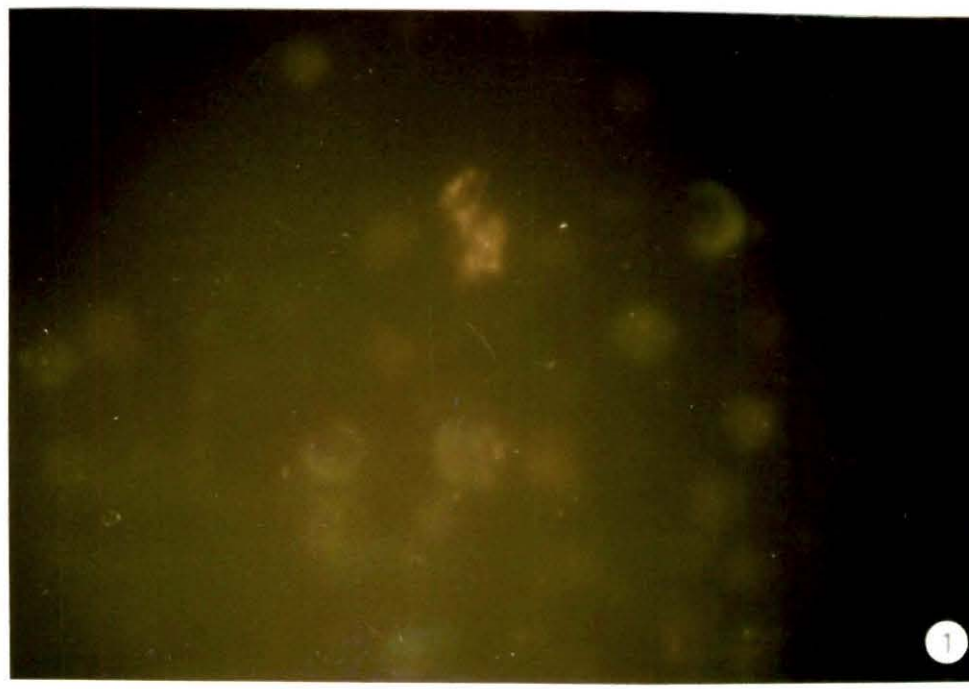
3

Plate - X

Fig. 1 Photomicrograph of peripheral blood lymphocytes of bat, treated with anti-B Ig M and then Fl-anti-R Ig showing incomplete ring and patches of fluorescence . X 700.

Fig. 2 Photomicrograph of peripheral blood lymphocytes treated with anti B Ig G and then Fl-anti-R Ig showing ring like fluorescence. X 700.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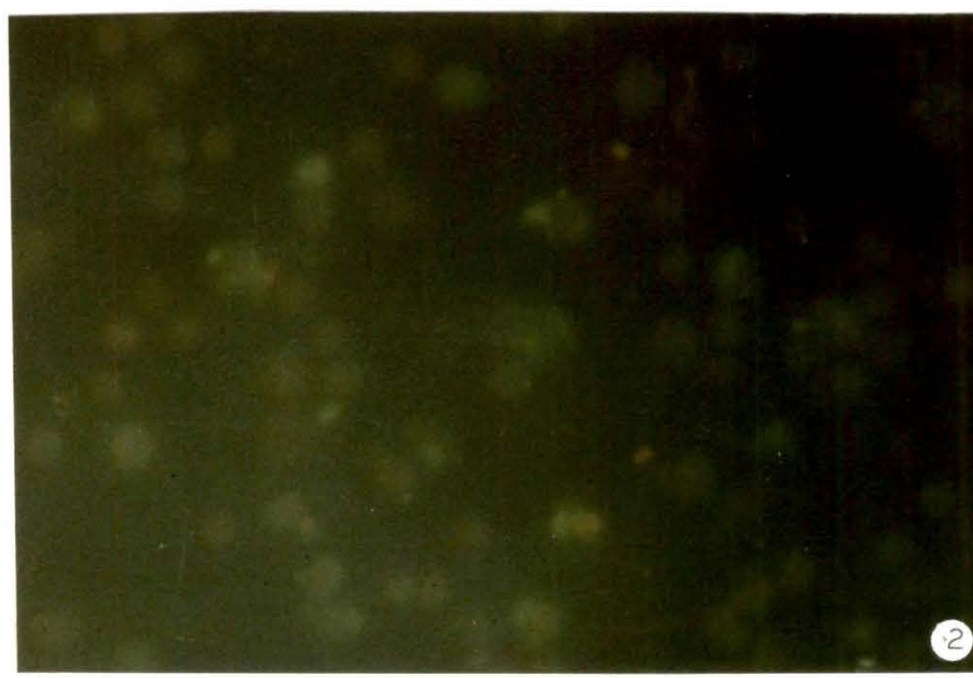
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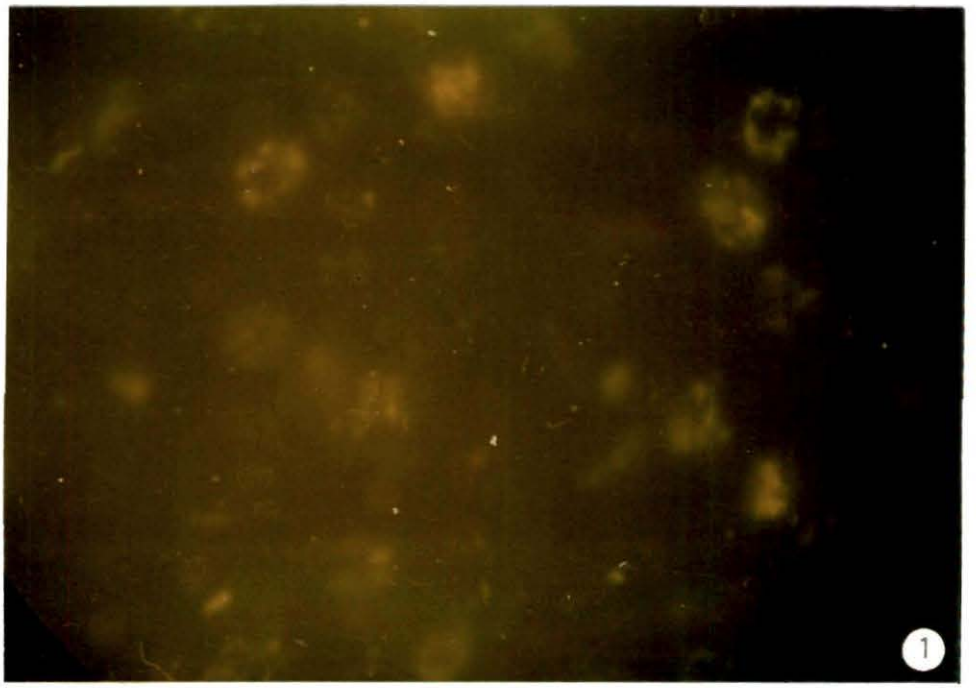
Plate - XI

Fig. 1 Photomicrograph of nylon wool adherent cells of bat treated with anti-B Ig M and then Fl-anti-R Ig showing indirect immuno-fluorescent staining of membrane Ig in the form of ring and patches. X 700.

Fig. 2 & 3 Photomicrographs of nylon wool adherent cells of bat treated with anti-B Ig G and Fl-anti-R Ig showing typical fluorescent ring for membrane Ig G on a cell, magnified view of which is shown in Fig. 3. X 700, X 900.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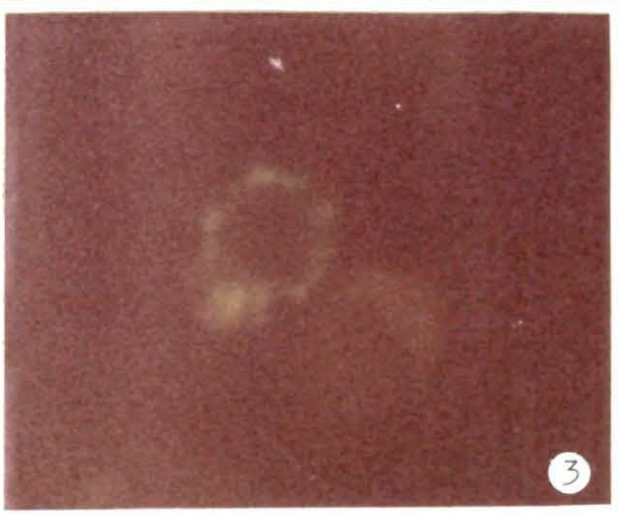
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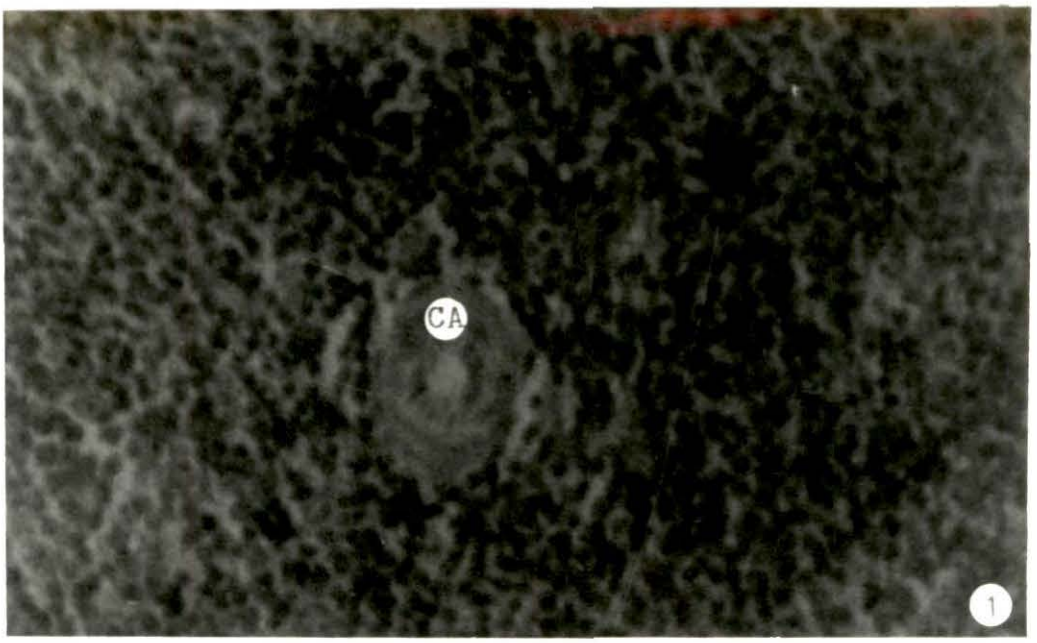
Plate - XII

Fig. 1 Photomicrograph of a histological section of spleen from normal bat showing a white pulp follicle. The periarteriolar lymphocytic sheath (PS) contains a good number of cells. CA-central arteriole. X 600

Fig. 2 Photomicrograph of a section of bat spleen removed 24 hours after in vivo anti-brain serum treatment. Density of lymphocytes in the PS region is slightly less than in the normal spleen. X 600.

Fig. 3 Photomicrograph of a section of bat spleen removed 72 hours after anti-brain serum treatment. PS region is loosely organized, with less number of lymphocytes than in normal, indicating depletion of cells in this region. X 600.

[All sections stained with Haematoxylin and Eosin]


CA
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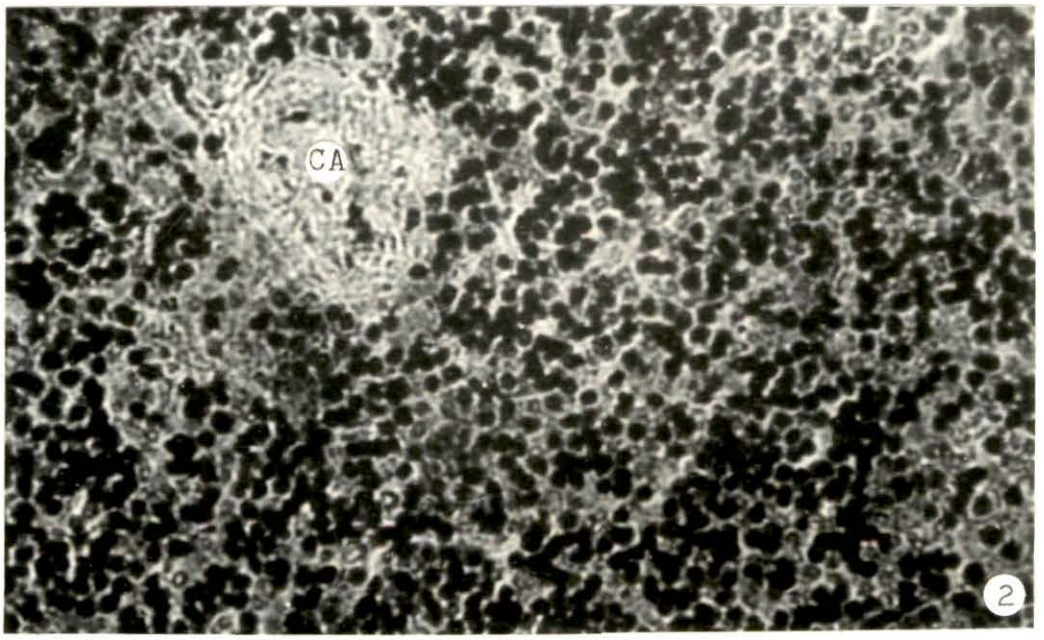
CA
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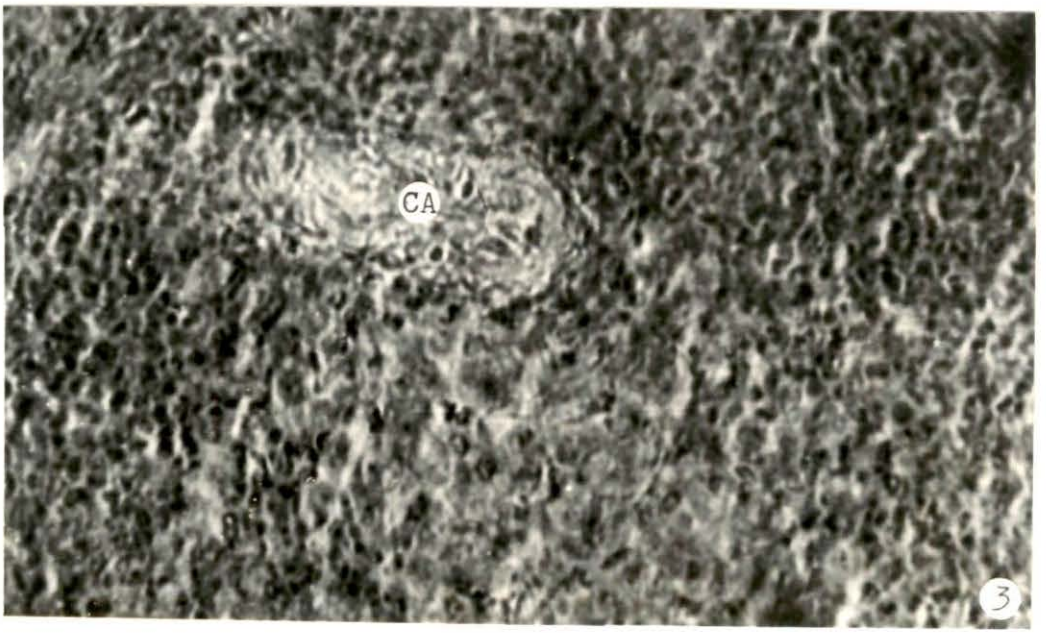
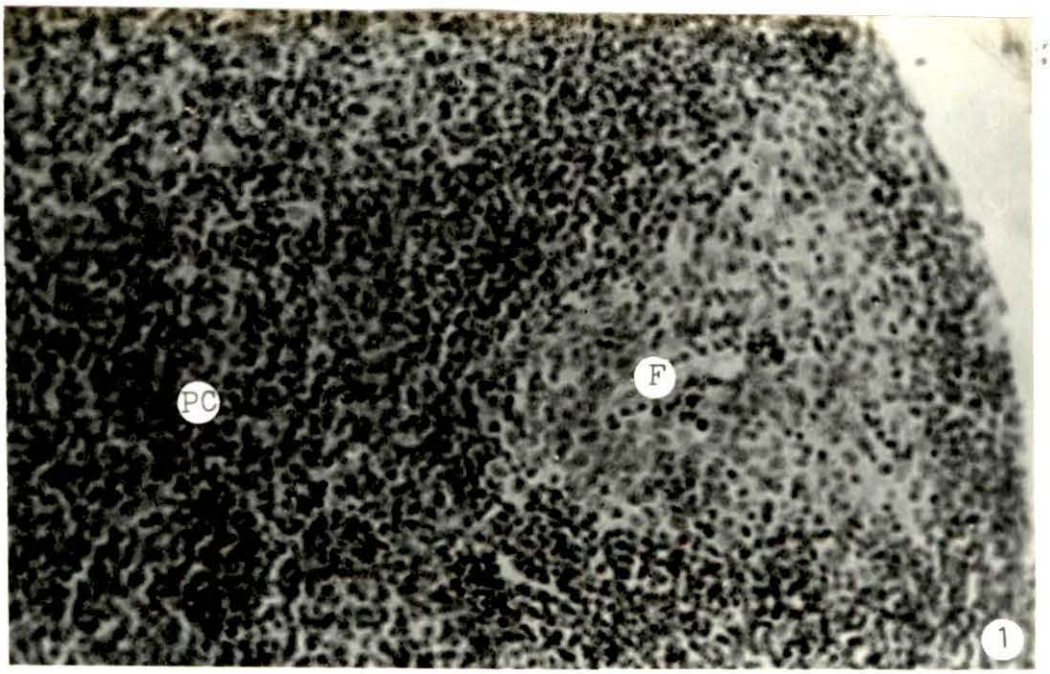

CA
3

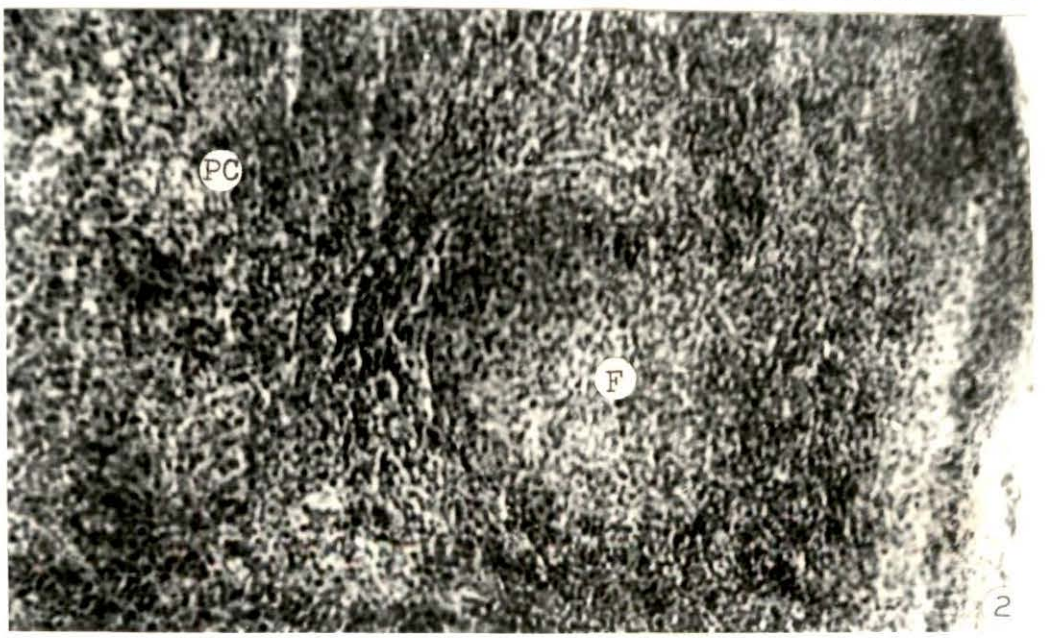
Plate - XIII

Photomicrograph of a section of mesenteric lymph node from normal bat, showing distribution of lymphocytes in lymphoid follicle (F) and paracortex region (PC). Haematoxylin-Eosin stain. X 450.

Fig. 2 Photomicrograph of a section of mesenteric lymph node of bat removed 24 hours after anti-brain serum treatment. Density of lymphocytes in follicular and paracortical regions is slightly less than in normal lymph node. Masson's Trichrome stain. X 300.

Photomicrograph of a section of mesenteric lymph node of bat removed 120 hours after anti-brain serum treatment. The paracortical region (PC) shows empty spaces indicating significant depletion of cells in this region. Masson's Trichrome stain. X 300.


PC
F
1


PC
F
2

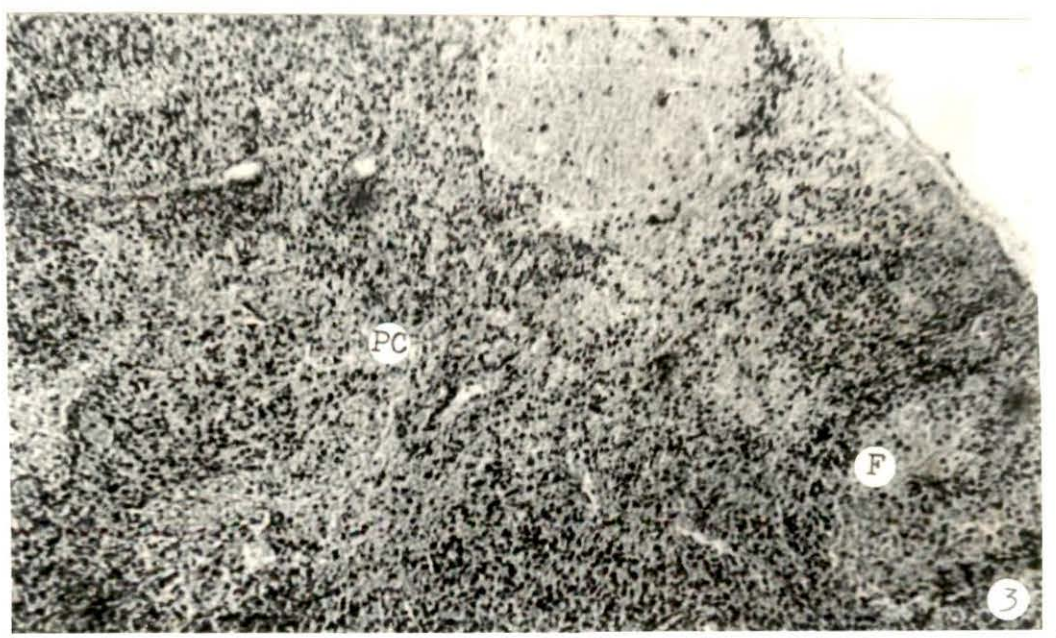

PC
F
3

Plate - XIV

Fig. 1 Transmission electron micrograph of a section of spleen from normal bat. Lymphoid cells differ in size and cytoplasmic content. The smaller cells (SL) show a thin rim of cytoplasm with very few organelles. Nucleus shows thick patches of darkly stained heterochromatin mainly along nuclear margin, and nuclear pores. The larger lymphocytes (LL) show more amount of cytoplasm containing mitochondria (M), vesicles (V), some ribosomes and few lamellae of endoplasmic reticulum. Cytoplasmic extensions from other cells can be seen (arrow). X 12,300.

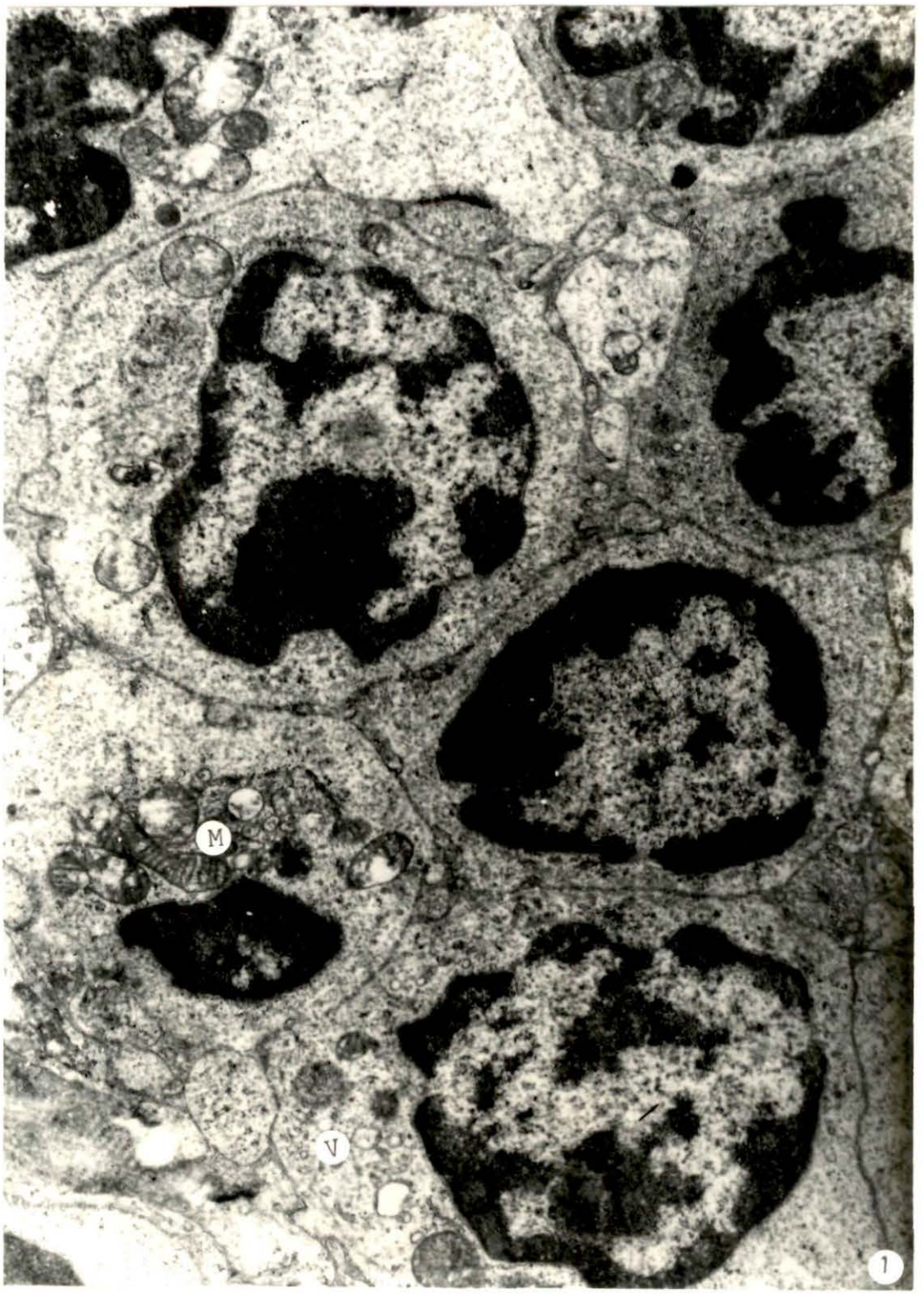

M
V
1

Plate - XV

Fig. 1 TEM photograph of lymphoid cells in normal bat spleen. Two large sized lymphocytes are seen with noticeable amounts of cytoplasm containing mitochondria (M) and vesicles (V). Occasional short profiles of endoplasmic reticulum (ER) can be seen. The cell nucleus is polygonal in shape, having fair amount of heterochromatin and a nucleolus (Nu). At the lower side, part of a capillary lined by endothelial cel (E) with typically elongated nucleus and microfilaments can be seen. X 12,300.

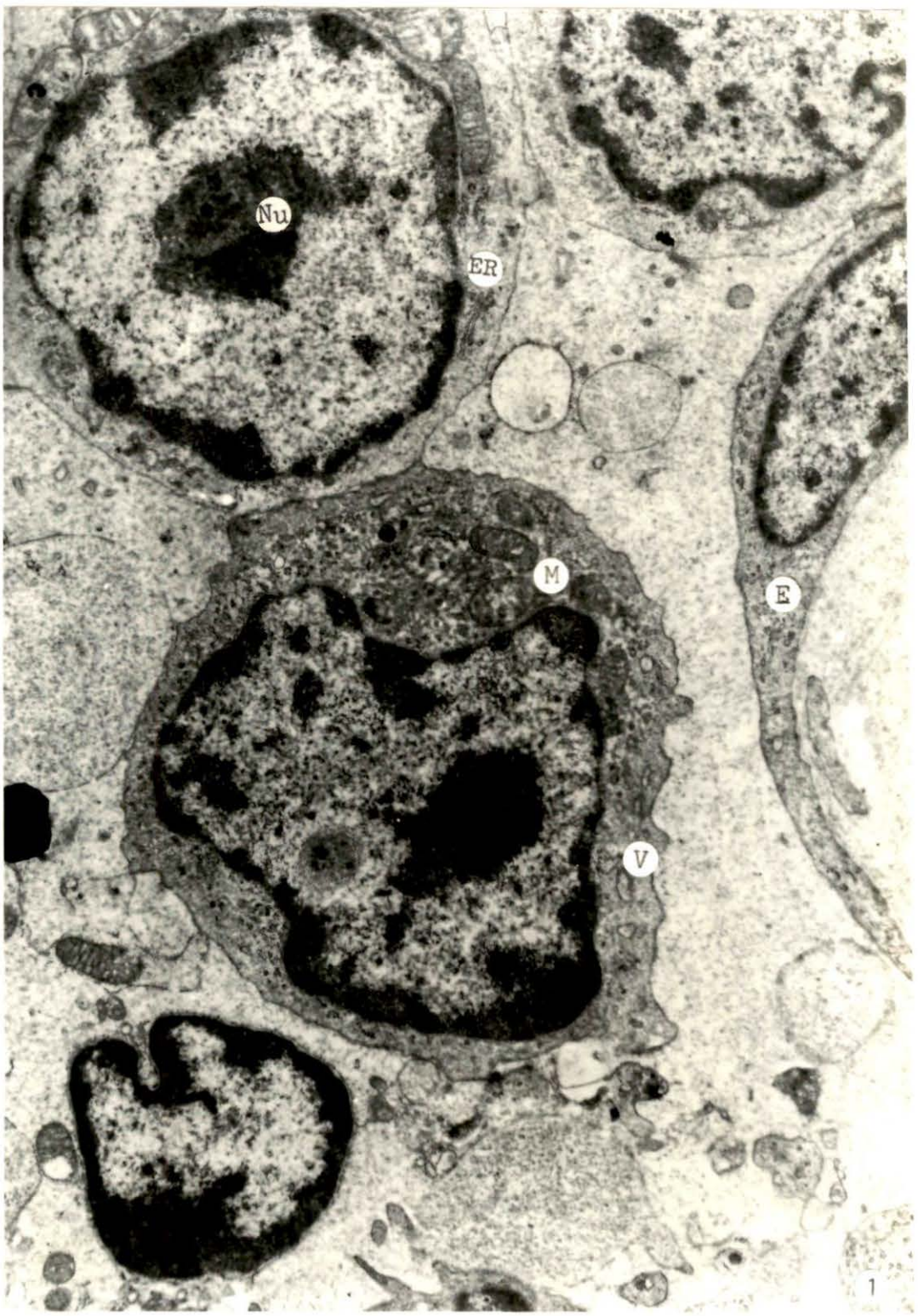
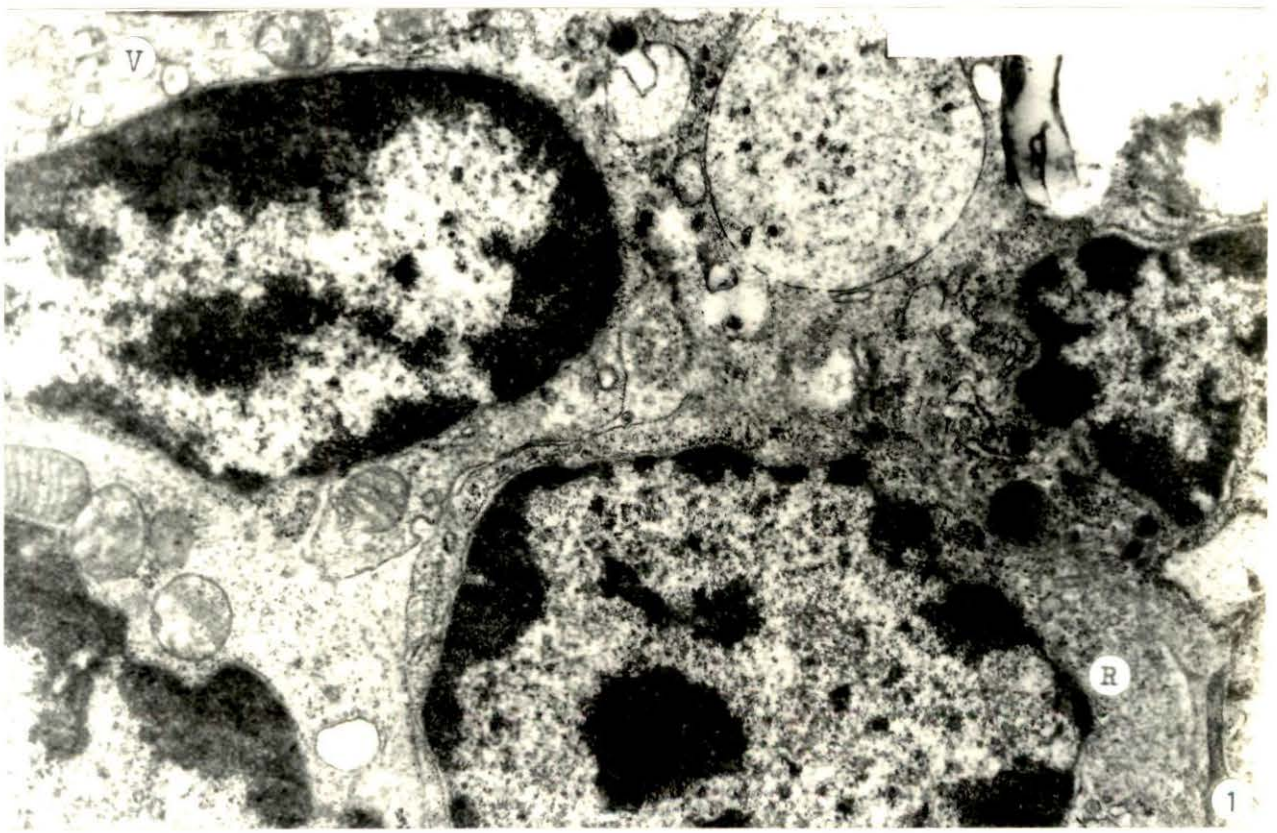
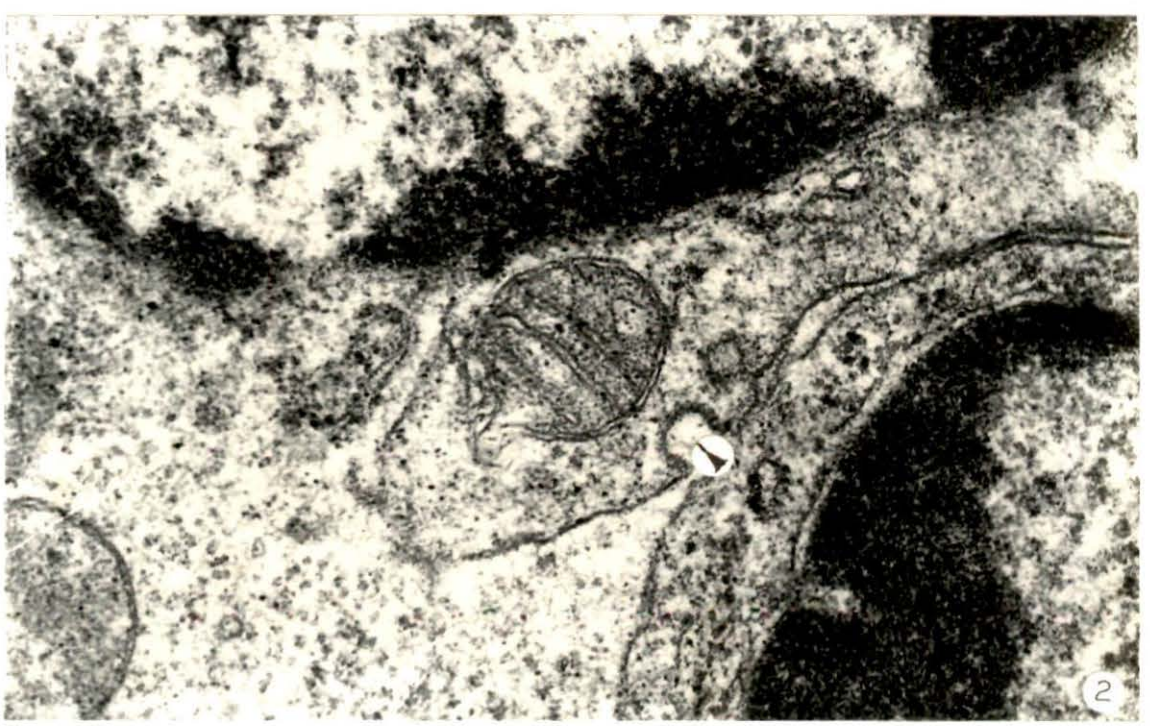

Nu
ER
M
E
V

Plate - XVI

Fig. 1 TEM photograph of normal bat spleen showing large sized lymphocytes. Many free ribosomes (R) and mitochondria indicate that the cells are metabolically active. The cell at the upper side of the photograph has some vesicular structures (V) which may be part of a Golgi apparatus. Some short profiles of ER are also seen. X 22,500.

Fig. 2 Magnified view of a part of Figure 1 showing the details of a mitochondria and endo- or exocytotic vesicles. The vesicles with openings to the exterior, are surrounded by electron dense material (arrow). X 60,000.


V
R
1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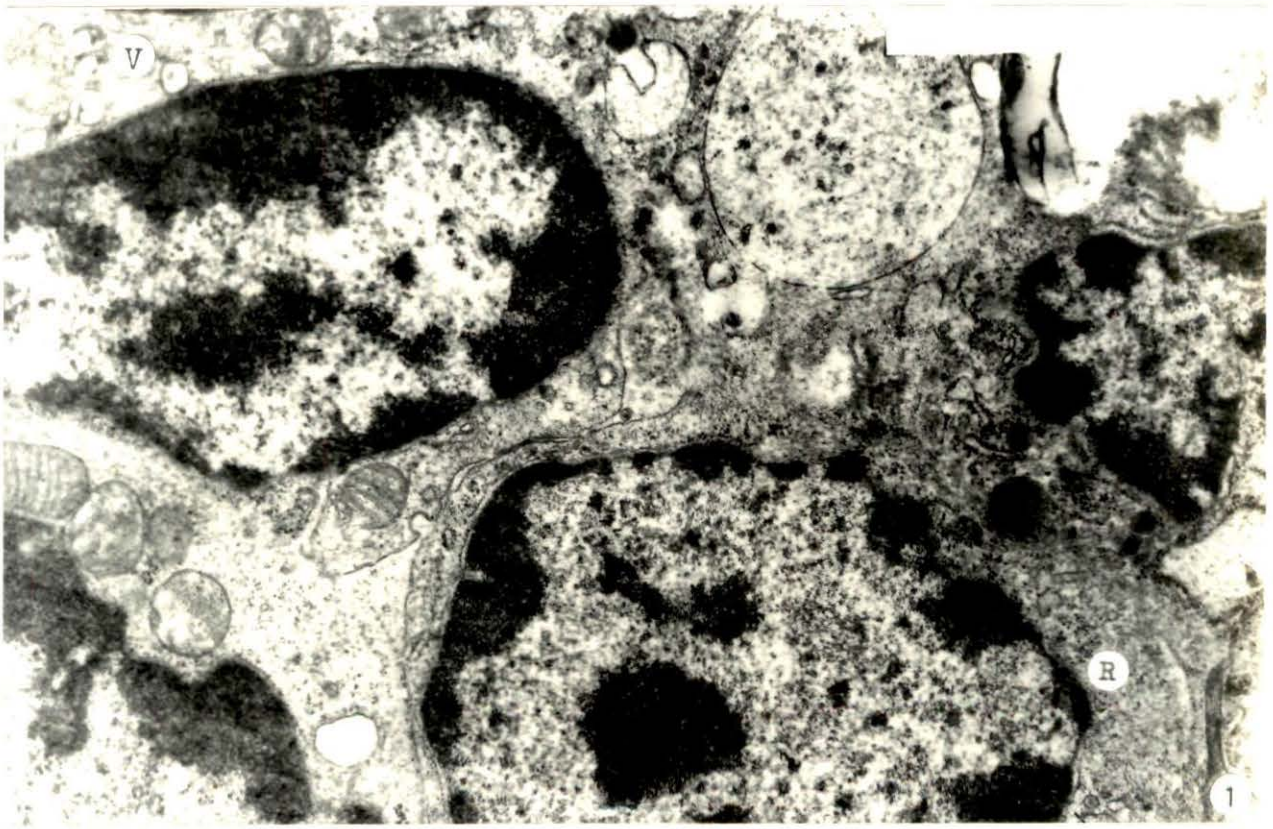
V
R
1

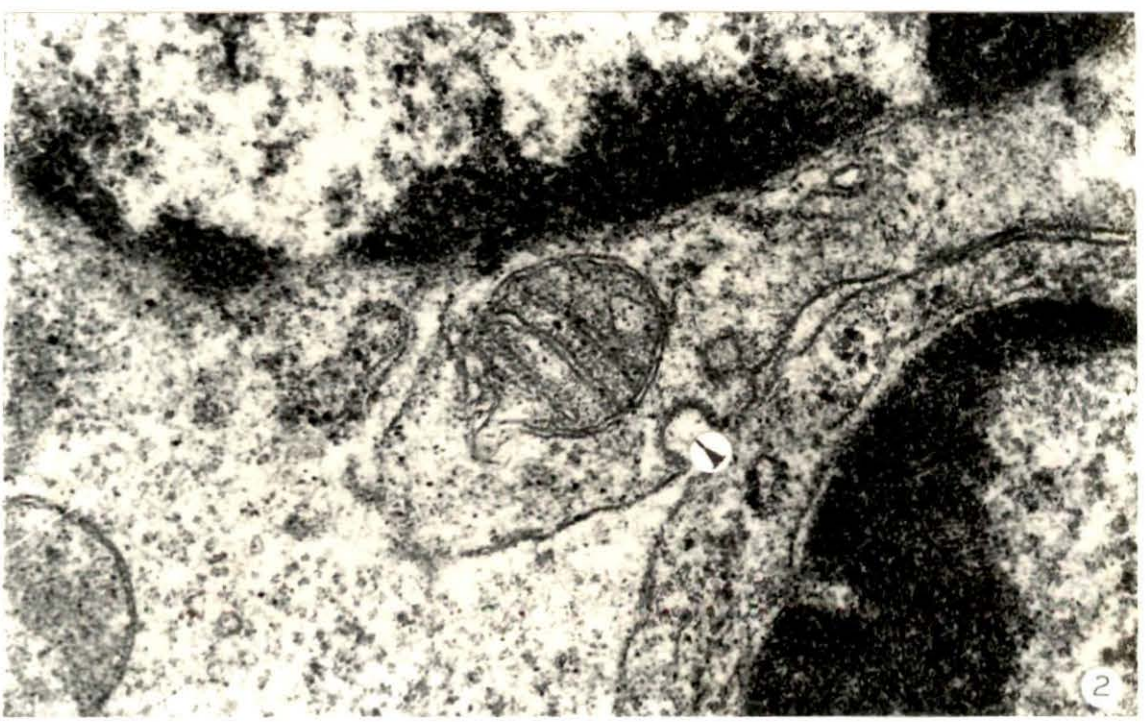
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Plate - XVII

Fig. 1 TEM photograph of normal bat spleen. Two large cells with paucity of cytoplasmic organelles are seen. Differences in nuclear morphology and heterochromatin content among the cells is noticeable. Cytoplasmic extensions from adjacent cells can be seen. X 14,000.

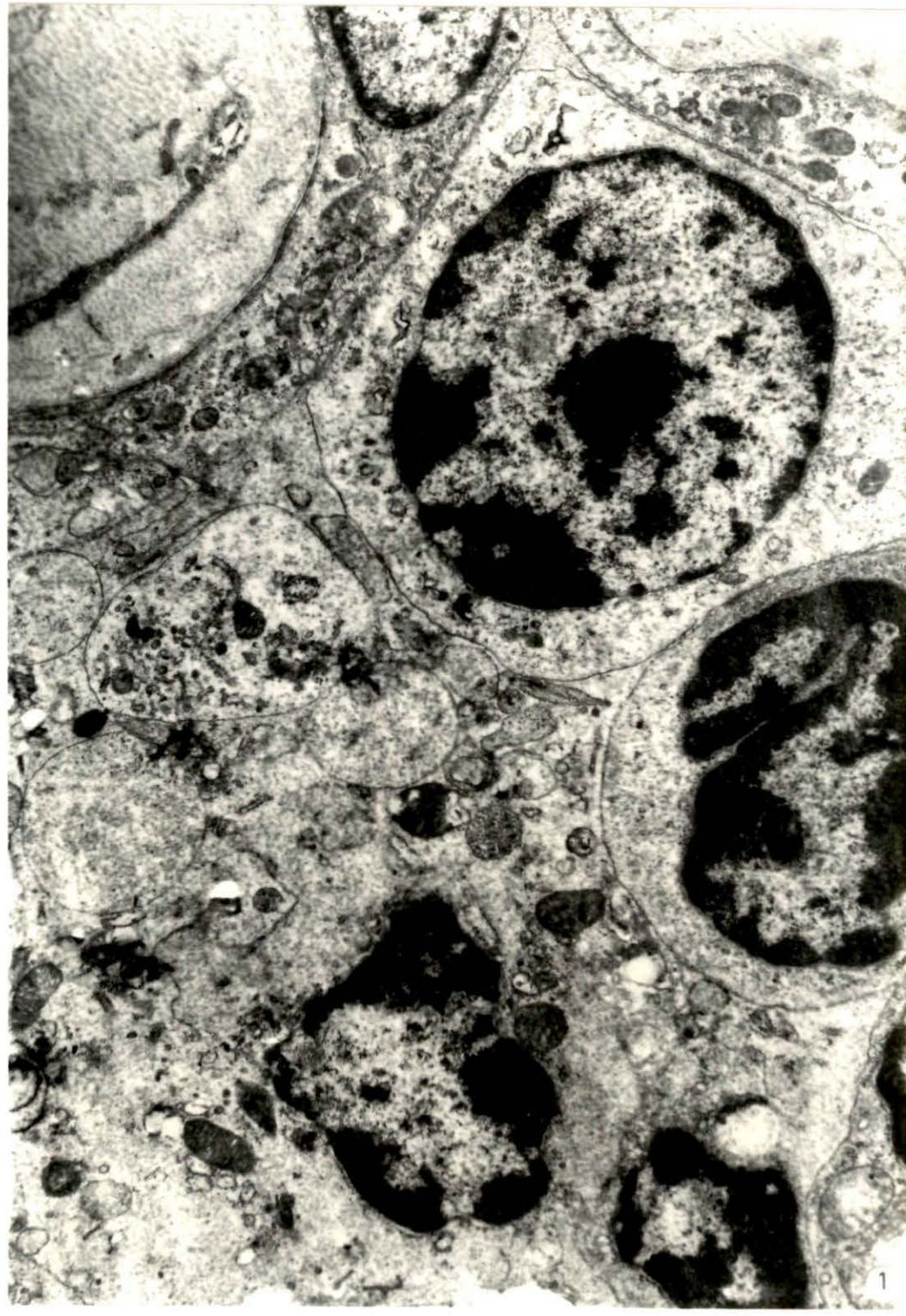
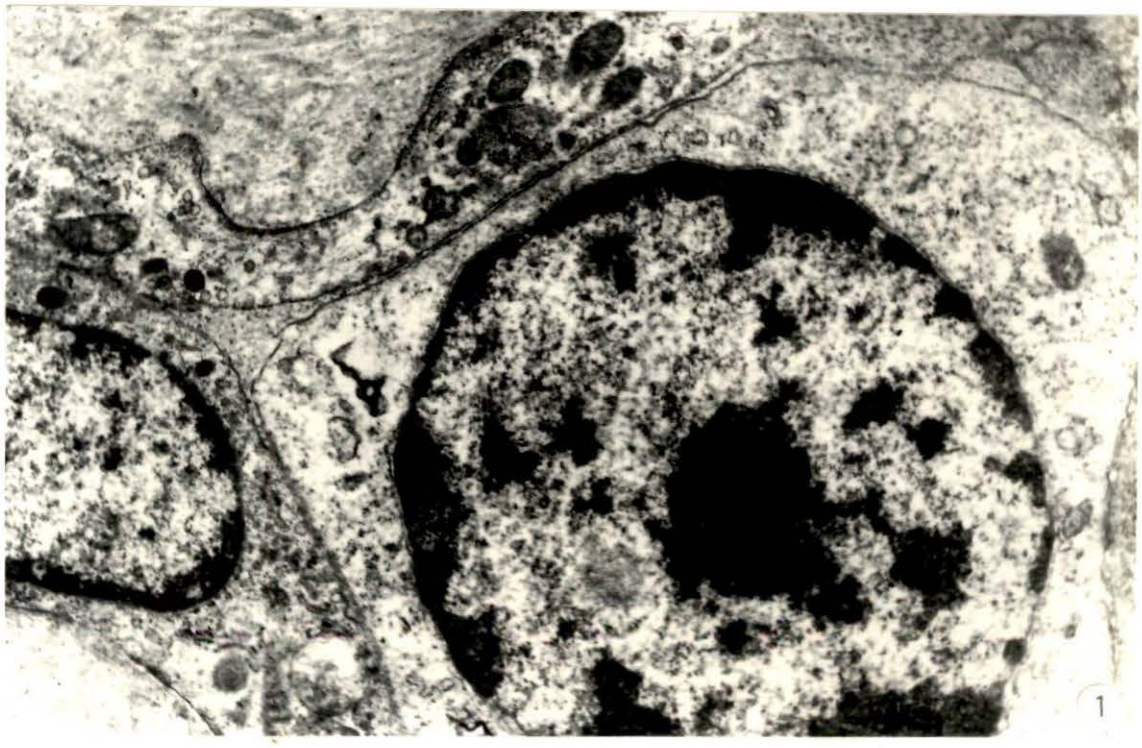


Plate - XVIII

Fig. 1 High power view of a part of the figure in Plate-XVII. The large cell has a round nucleus, many ribosomes and a very few other organelles. Few small vesicles are present. The cell looks like a plasma cell. X 18,200.

Fig. 2 Magnified view of a nucleolus in a lymphocyte of normal bat spleen. Dark granules (arrow) inside the nucleolus are visible, probably indicative of ribonucleoprotein particle synthesis. X 18,800.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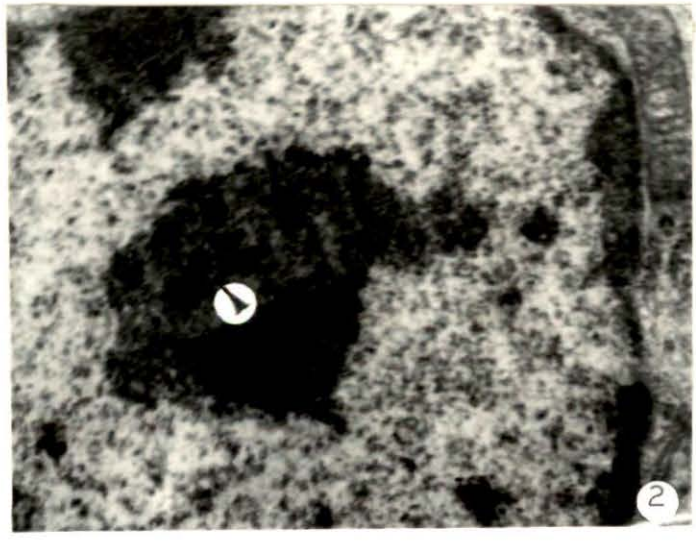

2

Plate - XIX

Fig. 1 TEM photograph of spleen from bats immunized with 25% SRBC for 10 days. Large cells, elongated in appearance are seen. The nuclei show less heterochromatin clumps. Cytoplasmic organelles are less prominent, except some ribosomes and mitochondria. X 19,900.

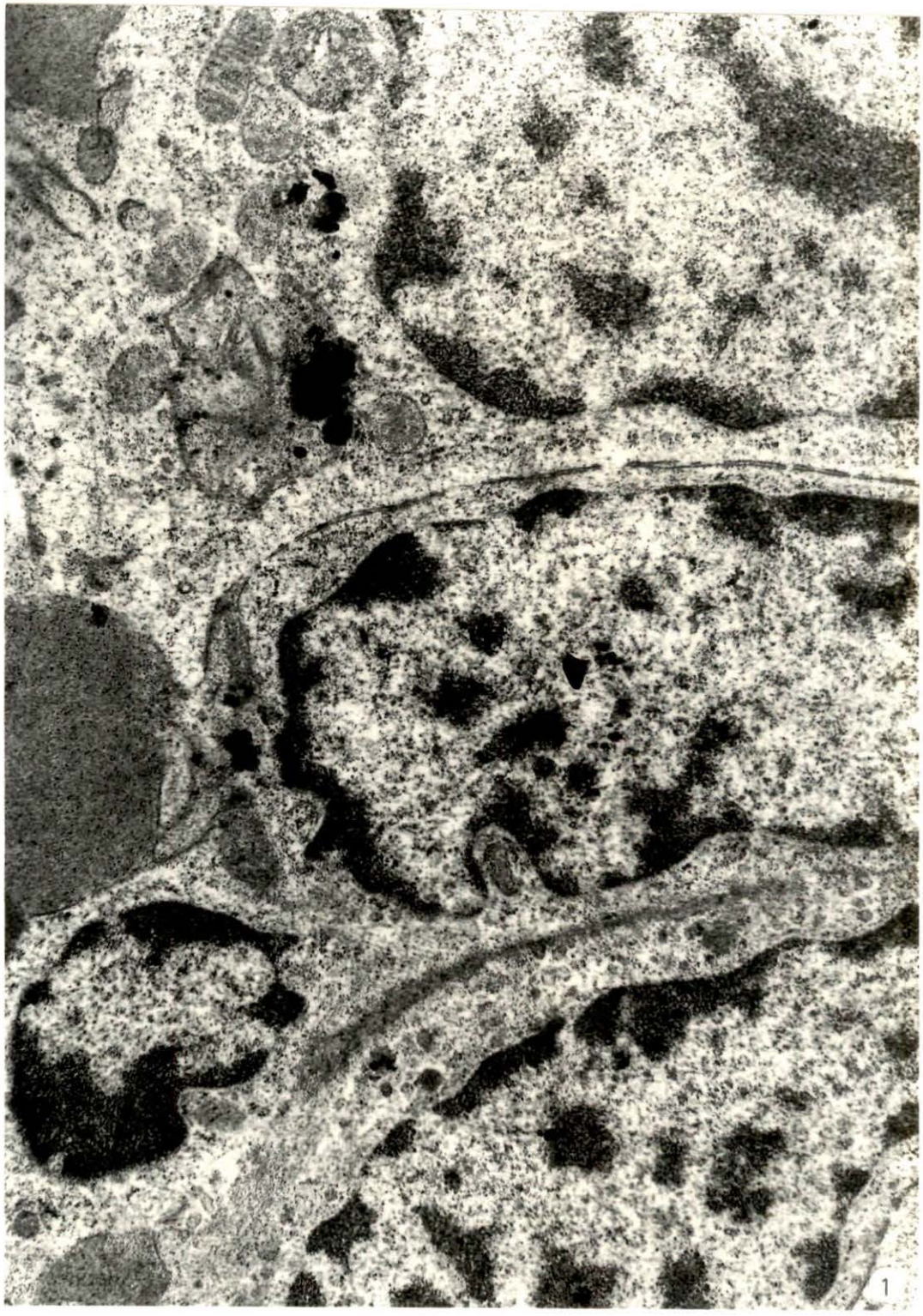


Plate - XX

Fig. 1 TEM photograph of spleen from immunized bat. A cell, resembling a macrophage is seen with irregular outline. Nucleus is with scanty heterochromatin. Membrane bound vesicles containing granular or homogeneously osmophillic material probably represent lysosomes (L). Faint outlines of microfilaments may be seen (arrow). X 16,700.

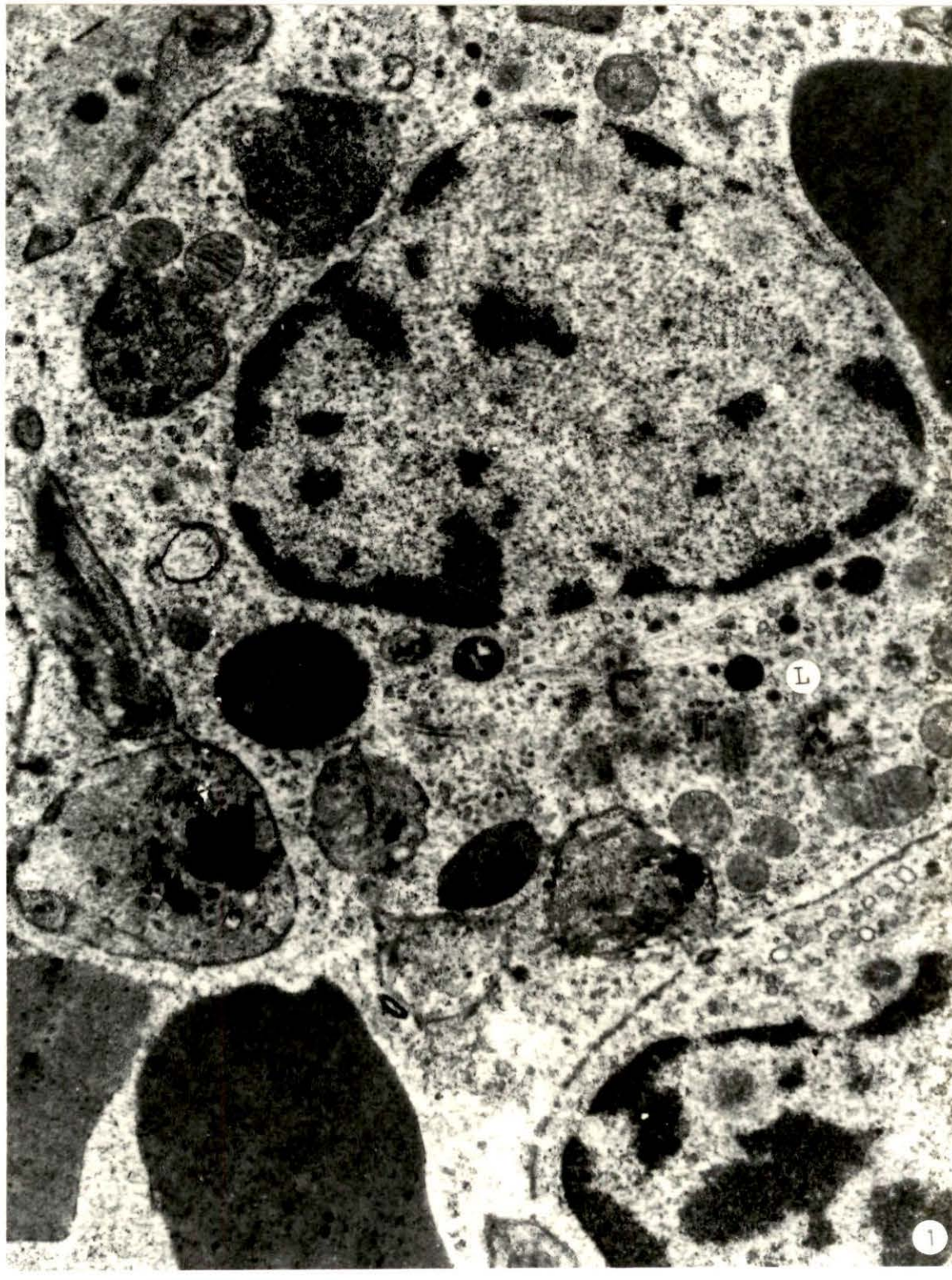

L
1

Plate - XXI

Fig. 1 TEM micrograph of spleen from immunized bat. A small lymphocyte is easily recognized from its thin cytoplasmic rim lacking organelles. In the adjacent cell, the nucleus is large and indented deeply (arrow). Euchromatic nucleus in the large lymphocyte is in contrast to the heterochromatic nucleus of the smaller cell. X 12,500.

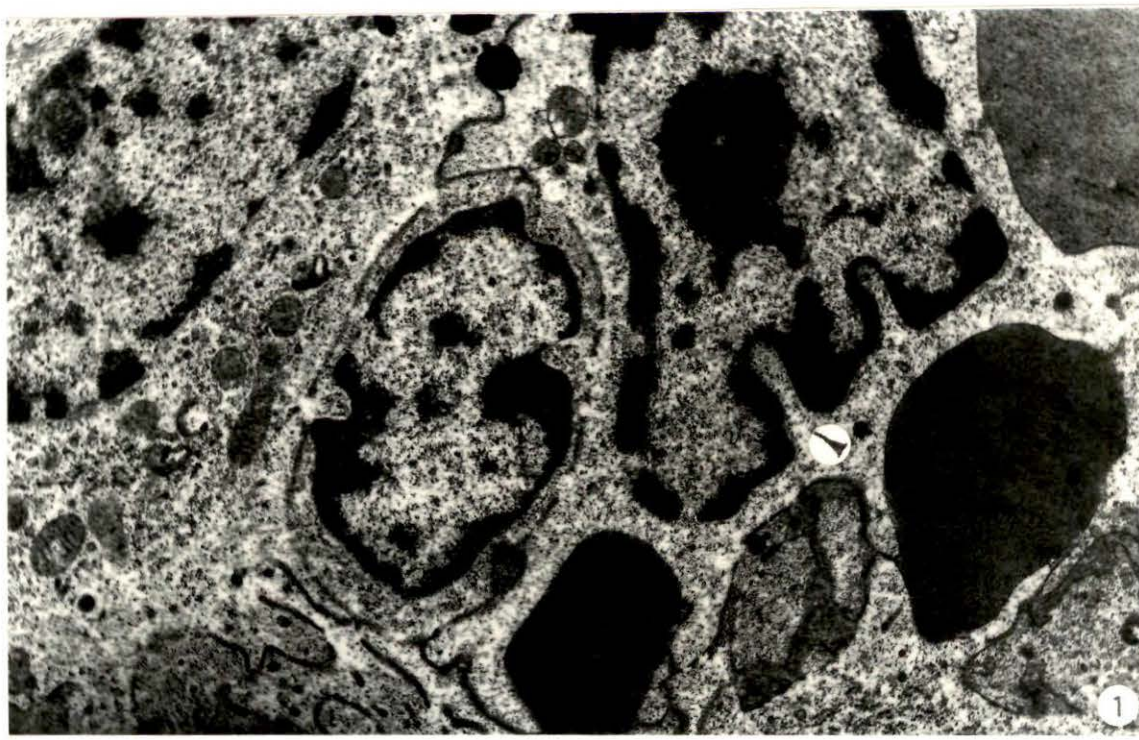
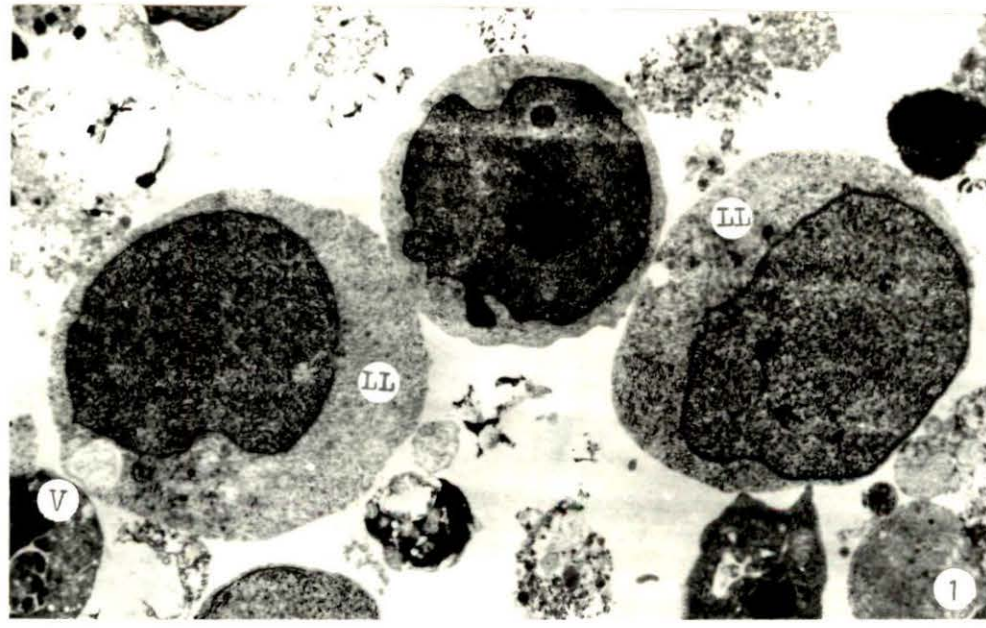

1

Plate - XXII

Fig. 1 TEM photograph of isolated nylon wool adherent lymphocytes from immunized bat. Two large lymphocytes (LL) with abundant cytoplasm are seen along with some small lymphocytes having less cytoplasm. Surface projections as observed in SEM photographs are not prominent, certain vesicles (V) adjacent to the plasma membrane are vissible. X 9000.

Fig. 2 TEM photograph of spleen from immunized bat. The cells are loosely organized, show large nuclei with scattered smaller clumps of heterochromatin. Cytoplasmic organelles are not very much prominent. X 14,000.


LL
LL
V
1

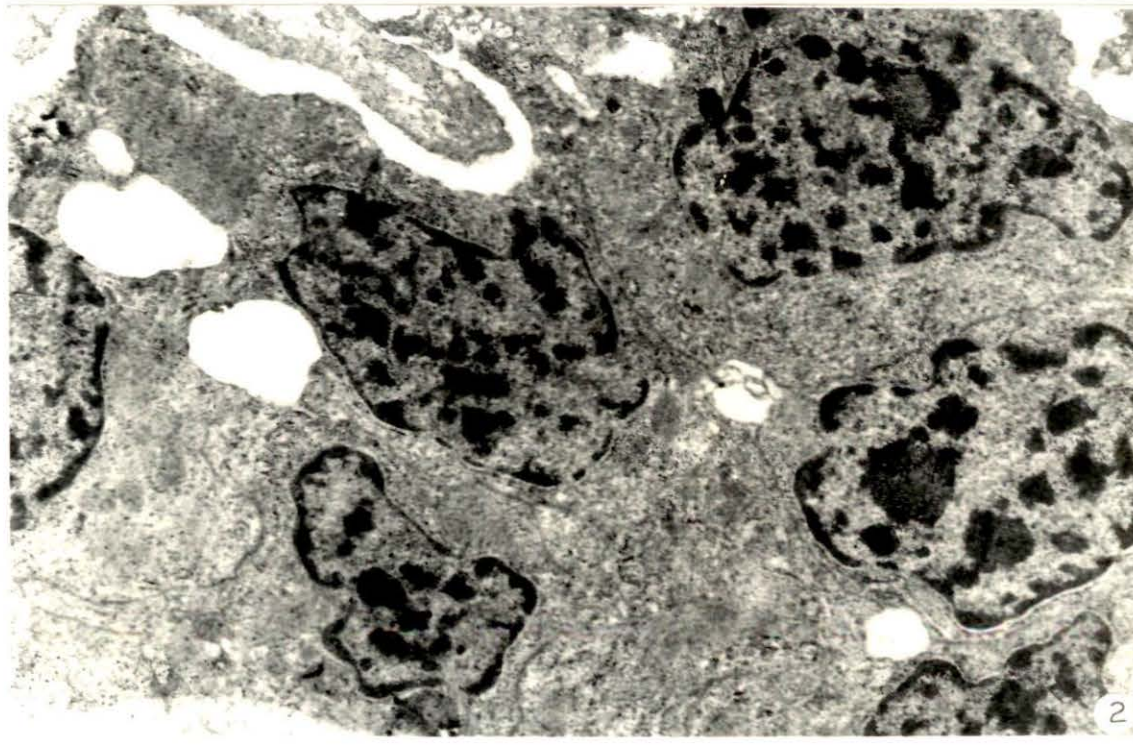
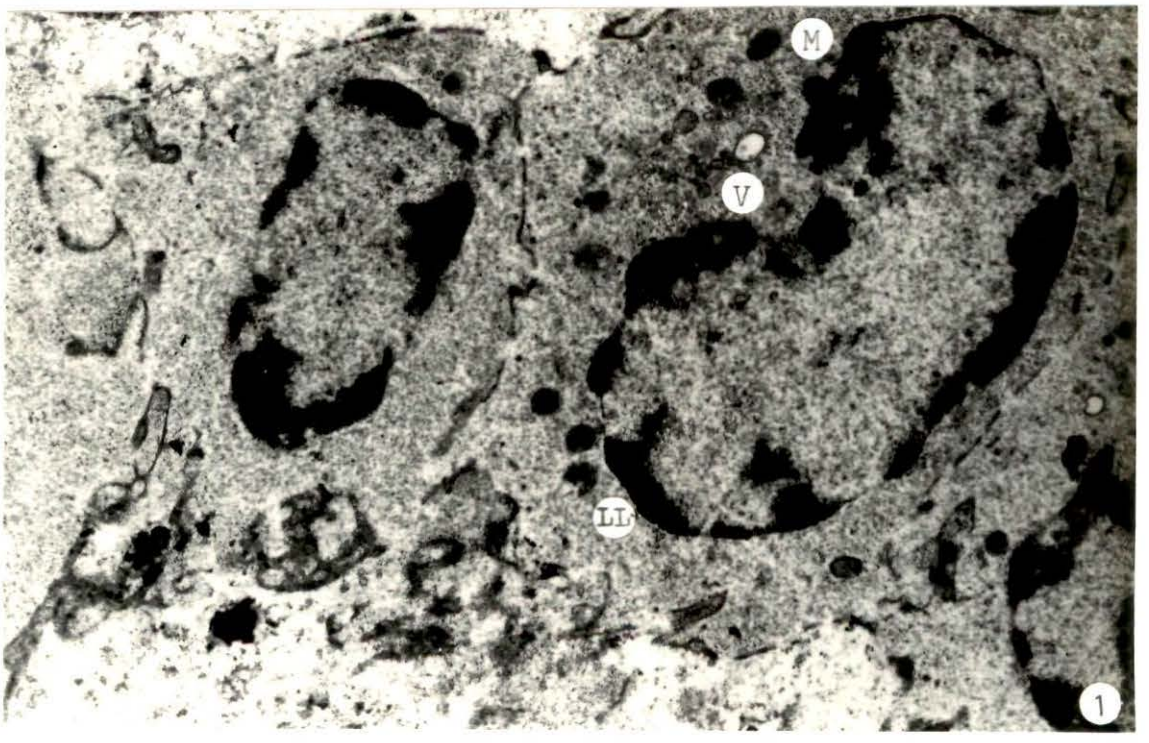

2

Plate - XXIII

Figs. 1 & 2 TEM micrographs of mesenteric lymph node from immunized bat. The small lymphocytes (SL) show less cytoplasm and notched nuclei (arrow) containing thick heterochromatin patches, while the larger lymphocytes (LL) have more cytoplasm containing several mitochondria (M) and vesicles (V). Nuclei in these cells have less heterochromatin, and are less indented. X 13,500, X 9,250.


M
V
LL
1

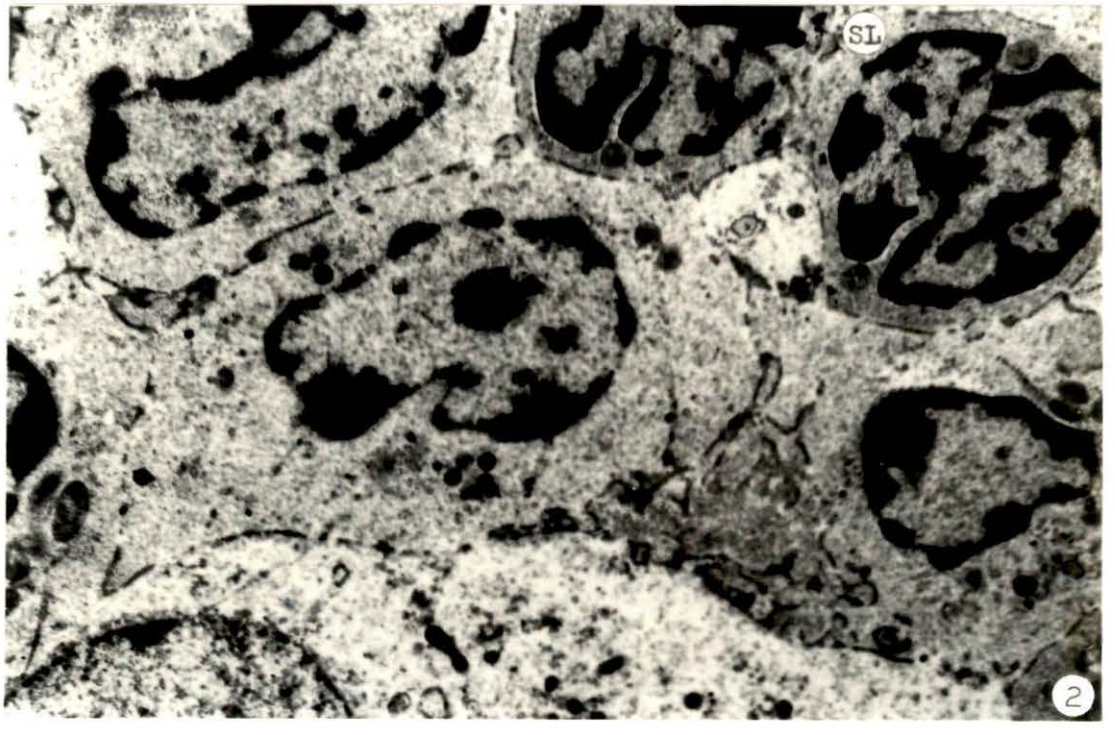

SL
2

Plate - XXIV

Fig. 1 TEM photograph of mesenteric lymph node from immunized bat showing cells differing in size, cytoplasmic content, nuclear morphology and heterochromatinization. The small lymphocytes (SL) typically has less cytoplasm while large lymphocytes (LL) have more cytoplasm and deeply indented nucleus. Several mitochondria and vesicles are seen in these cells. In one cell, faint outlines of ER can be seen. X 13,600.

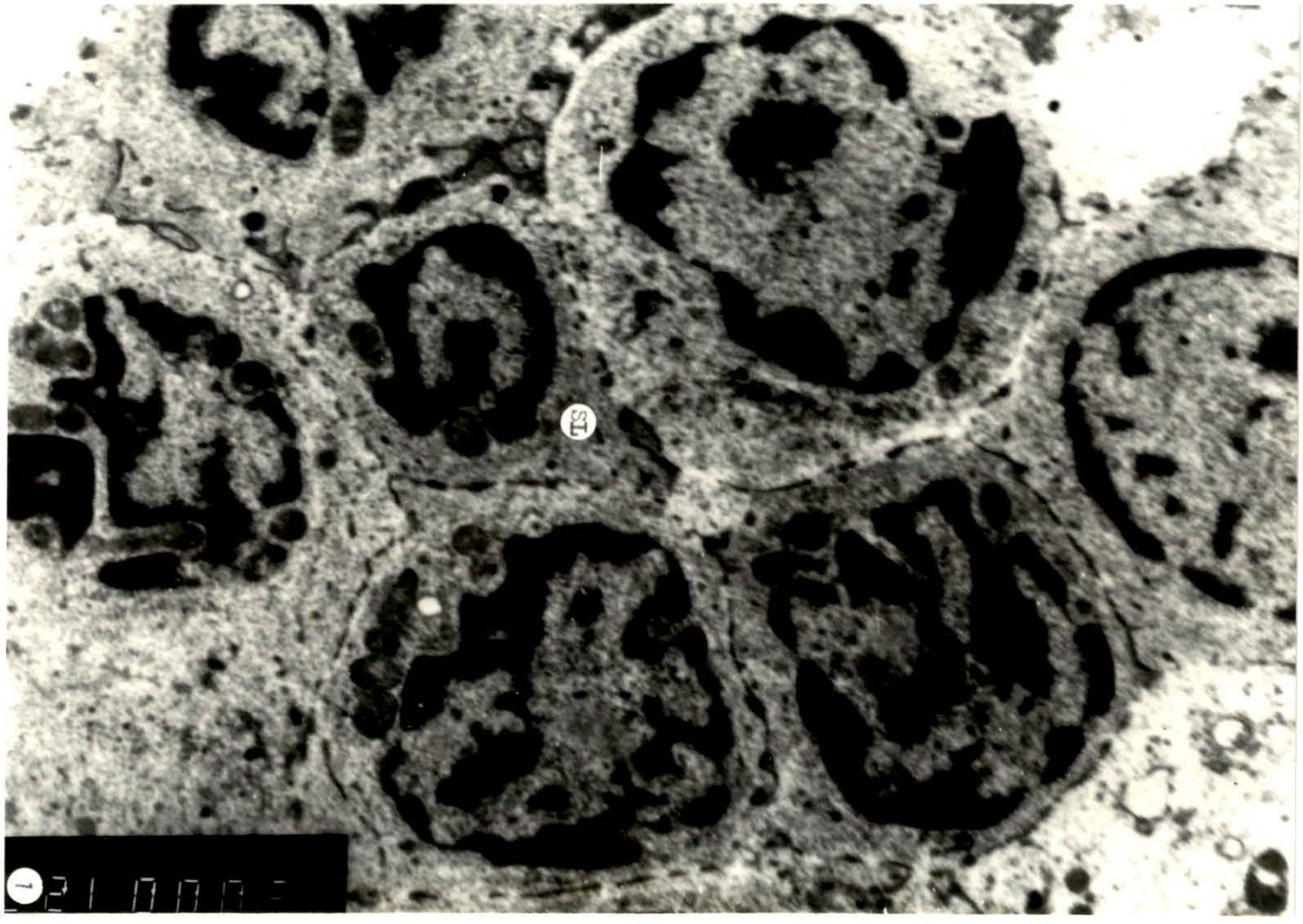
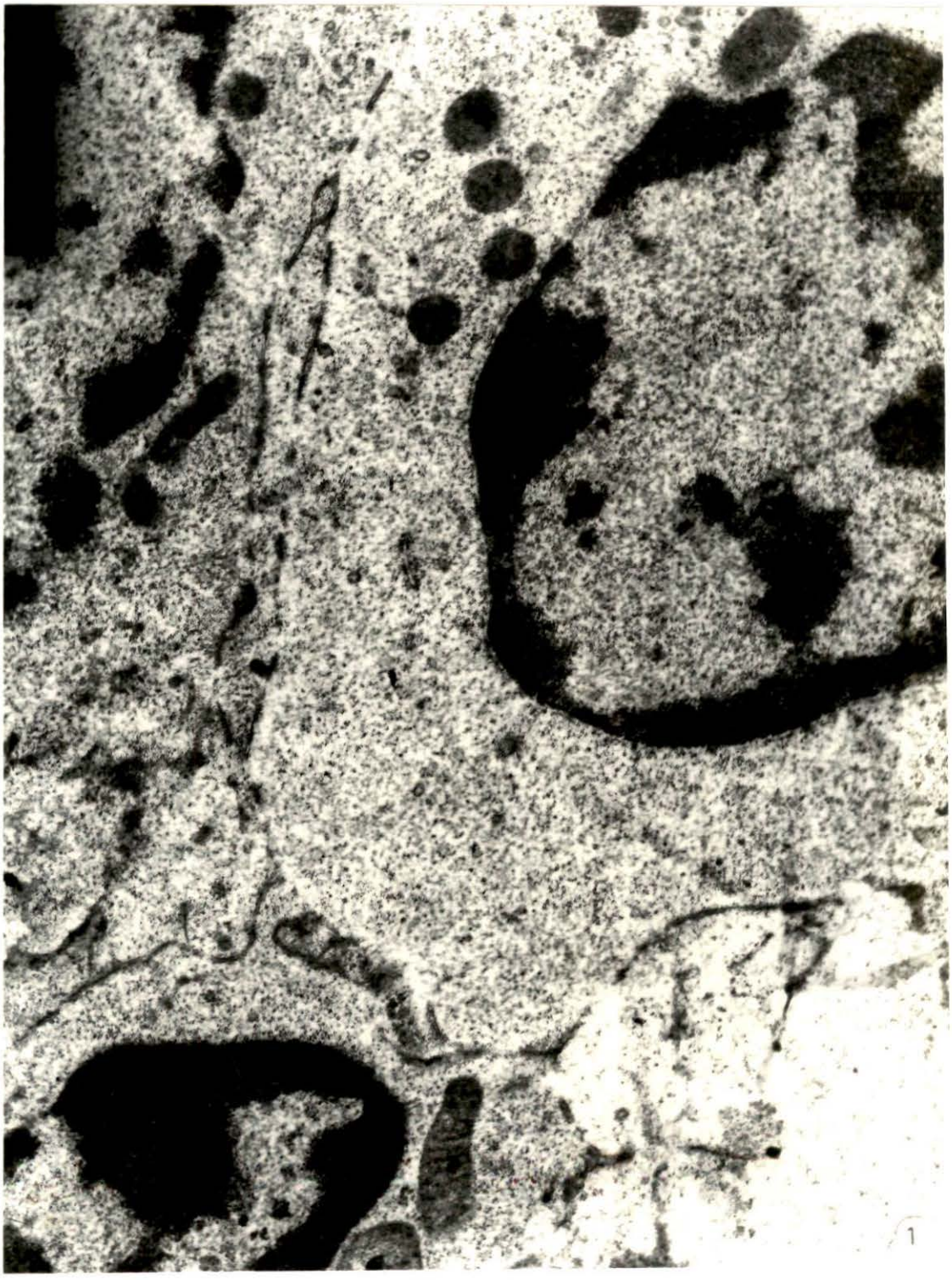


Plate - XXV

Fig. 1 TEM micrograph of mesenteric lymph node from an immunized bat, showing part of a large lymphocyte. Some mitochondria, vesicles and scattered ribosomal particles are seen. Nucleus shows moderate amounts of hetero-chromatin. X 23,000.



R E F E R E N C E S

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