DISCUSSION

Lymphoid Organs

So far there were fragmentary reports regarding the histological structure of lymphoid organs (Schiwatschewa, 1967; Forman, 1974; Bhide, 1979) and immune responses of bats to specific antigens (Heck, 1965; Leonard et al, 1968; Hatten et al, 1968, 1970; McMurray et al, 1978). The aim of the present study was to get a comprehensive picture of the lymphoid organs and immune responses in a megachiroptera, Pteropus giganteus. The study of secondary lymphoid organs like spleen and lymph nodes have been made in detail regarding their distribution, morphological and histological structures. Detail study of the thymus, an important primary lymphoid organ has also been included in this study.

Location and gross morphology of different lymphoid organs like thymus, spleen and different lymph nodes follow the general patterns as in other mammalian species (Bailey, 1975). Histological architecture of spleen reveals white pulps with compact mass of lymphoid cells, mostly concentric around splenic arteriole and surrounded by red parenchyma. Primary nodule or white pulp similar to these in spleen can

also be observed in the cortex of the lymph node.

The white pulps in spleen and lymph nodes gradually differentiated into germinal centre after immunization. It has been observed in serial sections that the germinal centres increase in number and size more significantly in spleen than in lymph node after immunization with sheep's erythrocytes (Table 1). It has been observed that the gradual differentiation and increment in size of the germinal centres in spleen and lymph nodes correlate well with the kinetics of antibody response in bats, reaching the maximum stage by 15 to 20 days when antibody response in bats reaches at the peak (Table 1).

Division of the lymphoid cells and their differentiation into plasma cells can also be visualized in the differentiating germinal centres. Differentiation of germinal centres in lymph nodes of the bats after 15 to 20 days of immunization is interesting as particulate antigen like SRBC was injected intravenously. It seems that by 15 days of immunization, antigen as such or in modified form might stimulate the lymphocytes <u>in situ</u> by reaching lymph nodes via lymphatic channels or sensitize the lymphoid cells in circula-

tion seeking lymph nodes.

Regarding thymus, details of the structure during development of this organ in bat from foetal to adult stage have been studied at macroscopic and microscopic level. Thymus is known as a source of T lymphocytes which become immunocompetent after seeding into peripheral lymphoid organ and are responsible for different cell mediated immune reactions (Good and Gabrielsen, 1964). We have observed that thymocytes remain in an unorganized fashion in the thymus of the foetal bat. The differentiation of cortex and medulla can be realized in the thymus of a neonatal bat (Plate 6, Fig. 1; Plate 7, Fig.1). With the advancement in development, the differentiation between cortex and medulla becomes more prominent. Distinct cortex and organized medulla with Hassall's bodies can be observed in the thymus of a neonatal bat. Hassall's bodies are squamous epithelium bound structure containing some degenerating cells and amorphous ground substance (Plate 8). The size of the Hassall's bodies are also maximum in the young neonates, when lymphopoiesis is at the highest level.

Another notable feature of the thymus of a bat is that alike in primates and other mammals it undergoes age

dependent involution. The size and weight of the thymus are at ~~a~~ the maximum level during neonatal and Young stage of the bat (Table 2). Rudiments of the thymus can only be seen in adult age. One of the factors responsible for this age dependent involution in other mammals are often considered as fall in the mitotic index of the thymic cells (Nakamura and Metcalf, 1961); in this process it has been observed that cortex of the thymus is more severely affected than the medulla (Metcalf, 1966).

Thus it can be said that the noteworthy features of the lymphoid organs of the bat are well organized white pulp in normal condition, germinal centres in immunized spleen and lymph nodes, Hassall's bodies in thymic medulla from neonatal stage and involution of thymus in adults. Interestingly most of the features are similar to these in the lymphoid organs of a recently evolved group like primate (Bailey, 1975; Bloom and Fawcett, 1976). On the basis of palaeontological evidences, the phylogenetic closeness of the order chiroptera and primates has been indicated by certain authors (Young, 1962). From the present investigation it seems that the striking similarities in the organization of the lymphoid organs of the bats and the primates possibly provide another

kind of evidence regarding phylogenetic closeness of the bats, an evolutionarily old group of animals and the recently evolved group like primate.

Although bone marrow is considered as another primary lymphoid organ for the source of B lymphocytes (Davis et al, 1973), detail study of bone marrow has not been considered in this study as it seems that will constitute a separate and elaborate investigation.

Distribution of the lymphoid cells in spleen, lymph nodes, peritoneal exudate cells and peripheral circulation of the bat was determined by complement dependent killing of leukocytes with bat anti-lymphocyte serum (ALS) raised in rabbit. Necessary control with normal rabbit serum was also maintained as sometimes this serum could be cytotoxic to the cells. Lytic indices with the cells from different sources have been indicated in figure 1 and was found maximum in lymph node cells and then followed serially by splenocytes, peritoneal exudate cells, bone marrow and peripheral blood lymphocytes; these were mostly obtained with the higher concentration of anti-lymphocyte serum. It seems that the lytic index can be correlated with the percentage of lymphocytes present in the

leukocyte population of different lymphoid organs and in circulation. Thus it may be proposed that in reference to the ALS mediated killing lymph nodes harbour highest number of lymphocytes than in other organs in the bats.

The specificity of bat ALS raised in rabbit was also tested by absorbing the ALS with lymphocytes before using it for cytolytic experiment (Table 3). It has been observed that the lymphocytes can specifically absorb out the antibodies in the serum raised against them, more specifically against their cell surface determinants.

It is well known from the works done in other species of mammals (Greaves et al, 1973; Roitt, 1975; Douglas, 1980) that the lymphocyte population basically consist of two types of lymphocytes, one derived from bone marrow and another from thymus. Thus it seemed that, it would be fruitful ~~in some way~~ to determine whether B and T cell types could be ascertained in different lymphoid cell populations of the bat. To our knowledge there is no systematic study in this direction in the bat. This could easily be done by subjecting the cells to the killing with anti-thymocyte serum. The difficulties to raise the anti-thymocyte serum in bat have been indicated

in the results section and these withhold us to perform the experiment with anti-thymocyte serum directly.

It was thought that brain cells of the bat might share some antigenic determinants with the thymus as in certain other mammalian species. Especially in mouse, thymus cells and brain cells share the Θ or thy-1 antigen (Reif and Allen, 1964, Roth, 1969). Thus in a set of experiment the lymphoid cells were subjected to the killing of ALS after prior absorption to brain cell homogenates. We observed this absorption caused decrease in the lytic index with ALS, indicating that certain antibody molecules normally effective against certain lymphocytes would have been possibly absorbed by the brain cell homogenate. The decrease in lytic index was again highest with the lymphocytes from lymph node. From this experiment, it seems that brain cells possibly share some antigen expressed on the thymocytes in the bat. Thus it seems that there is some kind of heterogenity in the lymphocyte population of the bat in the line of T and B cells which can be determined on the basis of cell surface determinants as in mice and other mammalian species.

The ALS absorbed with bone marrow cells was also tested for its cytotoxic ability against different population

of lymphoid cells. It has been noticed that prior absorption with bone marrow did not significantly affect the ALS mediated killing of the cells. On the basis of this data it can be suggested that the B cells, differentiated in the different lymphoid organs and in peripheral circulation, do not possibly bear the similar type of determinant as in the immature B cell in the bone marrow.

Antibody Mediated Immune Response

Antibody mediated immune response of the bats was measured in reference to the number of antibody secreting cells and hemagglutination titre of the serum from the immunized bats. For assaying the antibody forming or secreting cells, the technique of Cunningham & Szenberg has been modified in terms of time of incubation, concentration of SRBC and source of complement, etc. In case of bats longer period like 3 to 4 hr of incubation is needed for development of clear plaques, whereas 1 hr incubation is optimal in this regard in case of mice.

The antibody secreting cells of the immunized spleen of the bats have a notable degree of specificity in lysing the antigen in assay slides which becomes obvious from the Table 6. The lymphocytes immunized with SRBC could only respond effective-

ly against SRBC, not against pig erythrocytes during PFC assay.

Most effective lysis of SRBC and development of clear plaque by immunized spleen cells of bats have been observed in presence of homologous complement out of three different types of serum (Table 5).

It is customary to use guineapig's complement for best result in developing plaques with murine lymphocytes since the introduction of PFC assay by Jerne & Nordin (1963). Guinea pig serum complement was found most effective in PFC assay when erythrocytes from species like sheep, horse and ox rather than some other species were used (Kawaguchi, 1981). Rabbit serum complement was better in PFC assay with rat erythrocytes or bromelain treated mouse erythrocytes (Bretscher, 1978). Usually in lower vertebrates, the complement components of same species are required for lysing heterologous erythrocytes. For example, lizard (Kanakambika and Muthukkaruppan, 1972) and chicken (Soto, 1980) complement are most effective for PFC assay with respective lymphocytes. This can be attributed to compatibility between the antibody molecules and the first component of the complement (Jensen et al, 1968) Kawaguchi et al, 1978). Similar factors could be responsible for

better lysis of SRBC by the antibody secreting cells of bat in presence of homologous serum.

Kinetics of primary antibody mediated response was measured in terms of number of antibody secreting cells and level of HA titre in the serum. Different doses of the antigen was also used for this purpose. With the increment in the dose of antigen, PFC response in bat, was at higher level and the peak was reached earlier (Fig.4). These are similar to the events in other mammalian species (Ivanyi and Corny, 1969; Friedman, 1969; Mackaness et al, 1974; Kerckhaert, 1974). But the humoral antibody mediated response in bats has two notable features in comparison to this in other conventional laboratory mammals. The peak of the primary response and the decay of the response with one antigenic challenge are notably delayed.

The profile of HA titre follows the pattern of PFC response but remains at a significant level even on 50 days after immunization (Fig.6) when PFC response was almost at background level. Similarly the dose of 0.2 ml SRBC could not elicit a significant PFC response but augmented a reasonable HA titre level. This is possibly because the circulating antibody remains effective when the activity of antibody secreting cells is in wane.

We calculated and found that the number of PFC per one million leukocytes is much less in the bat than in mice (Friedman, 1965). As they are poor responder in PFC assay than other laboratory animals, our aim was to see whether two injections could enhance PFC response than single challenge. The response of the animals injected twice with 0.5 ml SRBC with 48 hr interval was similar to that of the animals injected with 1 ml SRBC at a time (Fig.5). Thus it seems, the lymphoid cells were not activated and proliferated to such an extent by the time the animals received their second immunization, that the second dose of antigen would be more effective. Rather both the doses of antigen became additive at 48 hr and acted similarly to 1 ml dose of antigen injected on day 0. Shalaby and Auerbach (1973) showed that in newborn BDF_1 mice, two injections of SRBC with 48 hr interval caused a good PFC response and they hypothesized that this could be due to earlier activation of T cells and or mobilization of immunocompetent cells. They did the experiment to analyse the maturation of immune responsiveness in the early life of the mouse when single injection of antigen did not elicit any response.

As the immune response in terms of PFC and HA titre was prolonged in the bats, the shift from primary to secondary

response was quite possible. That is why the assays for secondary PFC and HA-titre in presence of mercaptoethanol (ME) were performed. It has already been indicated with reasons that the use of the agar plating technique instead of Cunningham and Szenberg's method was necessary for assaying secondary PFC in the bats.

We observed the appearance of ME-resistant PFC and HA-titre during the primary PFC and HA-titre response (Fig.7). This phenomenon is interesting and could not be attributed to the prior experience of the antigen by the hosts as SRBC is not a naturally occuring antigen as bacteria and viruses. Thus it seems that there is an effective mechanism in bat by which a single challenge of antigen is sufficient to cause both ~~xx~~ primary and secondary immune responses upto a reasonable level. This could be due to several reasons including the retention of antigen within the system for a long time and some kind of involvement of dendric cells in germinal centres in this respect (Mandel et al. 1981)

The antigen binding follicular dendritic cells (FDC) were first described by Nossal et al.(1968a) in the rat popliteal lymph node. Ultrastructural studies by that group and others

defined that after immunization some of the injected antigen in the form of antigen-antibody complex eventually became localized on the surface of FDC situated in primary follicle of peripheral lymphoid tissue (Nossal et al, 1968b; Hanna and Szukal, 1968; Chen et al, 1978a, 1978b; Nossal and Ada, 1971). Veerman and Van Rooijen (1976) suggested that immune complex were carried to the spleen on B cells and they were shed from B cells following the process of capping and deposited on the FDC. Non specific nature of FDC for binding antigen has also been reported by van Rooijen (1972). Thus possible mechanisms of processing and storing of antigen in the lymphoid system of bats and involvement of FDC will be an interesting topic for studying in future.

In the present investigation antibody mediated secondary immune response was defined on the basis of sensitivity of the antibody and plaque forming cells to the mercaptoethanol. Usually mercaptoethanol sensitive antibody are classified as 19S type of immunoglobulin and resistant ones are classified in 7S category (Stanworth and Turner, 1978). Leonard and his associate (Leonard et al, 1968) have already characterized these two types of immunoglobulins on the basis of molecular weight in case of big brown bat immunized with live Japanese B encephalitis virus. It seems that the bats

are capable of producing these two types of antibodies as in most other mammals.

Besides the PFC assay and HA titre methods of estimating antibody mediated immune response, individual protein fractions of serum of bats immunized with 0.5 ml 25% SRBC were separated by electrophoretic method and the amount of protein in each class was estimated. It has been observed in most of the cases slight increase of the total protein and no significant quantitative difference in γ globulin level in the immunized sera.

Electrophoretic analysis of serum from the bats injected with 1 ml of 25% SRBC was also performed twice; change in the amount of total protein and serum protein components with immunization follows the similar trend (unpublished observation).

Heck (1965) also reported the electrophoretic analysis of the sera of bats immunized with KLH and he could not find any gross variation in total protein content or quantitative difference in globulin. Thus it seems that difference in the

amount of γ globulin with immunization might not be revealed by the electrophoretic analysis.

Cell mediated immune response

The involvement of T cells in different immune reactions such as allograft rejection, graft versus host reaction, mixed lymphocyte reaction, delayed type hypersensitivity reaction and for the immune response to fungi, certain microorganisms and certain viruses are well known fact (Elves, 1972; Marchalonis, 1977b).

Delayed hypersensitivity in mammals is characterised by T cell involvement resulting in erythematous, maximum indurating lessions normally appearing some 24 to 48 hr after secondary exposure to a sensitizing agent (Erard et al, 1979). Like other mammals bats showed maximum induration in 48 hr (Fig.9). We studied skin sensitivity of 12 bats to DNFB. Except 3 bats, rest of all showed slight response which are accompanied by small induration.

Mixed lymphocyte culture to assay the cell mediated immunity in the bats indicates that the response is better with longer period of incubation of the effector and stimulator cells. Thus the MLC response is also comparatively delayed

in the bats like their humoral response. This kind of delay of 7 days in MLC reaction can be observed in the lower vertebrates like marine toad, B. marinus (Goldshein and Cohen, 1972). In mice the peak of the MLC response can be obtained by 4 to 5 days (Bach et al, 1971).

In conclusion, it can be said that although the order Chiroptera originated in way back in the evolutionary time scale, the bat, P. giganteus possesses an organised lymphoid system very much equivalent to the primates by serval counts. However, it has been observed in the present study, the degree of immune responses in bat is lesser and delayed in comparison to these in the other species of mammals. It seems that the onset of response was slower, and peak of the responses was delayed. This could be for several reasons, such as inefficiency of handling of antigen, delay in activation of lymphocytes, absence of certain cell components, delay in the biosynthesis of antibody molecules.

Again the amount of antigen required to induce low or high zone of tolerance in bat is not presently known. We have only observed that higher dose of antigen like 1 ml of SRBC per animal caused the immune response to reach the peak

earlier. It seems that delayed immune response and prolonged state of immunization in bats might be correlated with the role of the bats as the reservoir of dreaded virus and bacteria. It seems that no single mechanism is sufficient to account for the phenomena, but rather a combination of the events is possibly required to insure the continued existence of these agents. Thus it becomes necessary to study the causative mechanisms of the delayed response and prolonged state of immunisation in bats to unravael the factors responsible for their role as reservoir hosts. Some of the possible factors for delayed onset of response have been indicated above and the possible role of Follicular Dendritic Cells in the prolongation of the immune response have also been discussed earlier. The present investigation in conjunction with the findings of Sulkin, Leonard and others (Leonard et al, 1968; Hatten et al, 1970; McMurray et al, 1978) have just possibly laid down the base line for future studies of immunobiology in Chiroptera. Moreover bats are already known as physiologically unique mammals being active flier, hibernator and estivator. Thus the further study of the immune system and the immune response of the bats will contribute to understand hitherto least known aspect of the bats and in future enable the researcher to understand the immunological phenomena with different physiological conditions.