INTRODUCTION

Bat as a group of primitive mammals under the order Chiroptera are known to harbour different viruses; (Ito & Saito, 1952; Sulkin, 1962; Sulkin *et al.*, 1966*a,b* Oya *et al.*, 1982; Banerjee, Ilkal & Deshmukh, 1984; Bijlenga & Hernandez-Baumagarten, 1984;) and bacteria (Constantine, 1970) usually without manifesting any apparent sign of the disease (Sulkin & Allen, 1970). It is also established that there exists epidemiological association between bat and mycotic agents such as *Histoplasma capsulatum* (Klite and Young, 1965) and are also implicated to human infections (Wimsatt, 1977). Therefore, a better understanding of the immune system and immune response in these animals to a variety of antigenic stimuli was felt necessary (Heck, 1973). This is important not only for understanding the mechanism of how animals resist disease but for the possible use of the knowledge by man. Bats are of special interest in studies of this kind because as mammals they are more likely to be susceptible to diseases of man (Wimsatt, 1977).

Nevertheless, little is known about the immune response in bats. Reports are available on antibody development in response to some virus infection in bats (Leonard, 1968; Gadkari *et al.*, 1976; Seymour *et al.*, 1978) and the

influence of body temperature on antibody production (Allen et al., 1964; Sulkin et al., 1966) and there have been few systematic studies of the immune capability of Chiropterans (Heck, 1965). Recently, the immune response of bats to Histoplasma capsulatum infection was reported by McMurray et al., (1978) and McMurray & Greer (1979) and more recently the immunobiology of a frugivorous bat, Pteropus giganteus have been studied by Chakraborty (1982). It has been observed that the degree of humoral (Chakraborty, 1982; Chakraborty & Chakravarty, 1983a; Chakraborty and Chakravarty, 1984) and cell mediated (Chakraborty, 1982; Chakraborty & Chakravarty, 1983b) immune response in bat, P. giganteus, is lesser and the peak of responses delayed in comparison to other species of laboratory mammals inspite of their possession of a well defined immune system with bone marrow and thymus as primary and spleen and lymph nodes as secondary lymphoid organs. Similar low degree of immune responses was reported in big brown bats (Eptesicus fuscus fuscus) by Hatten et al. (1970), Leonard et al. (1968) and Heck (1965). McMurray and Thomas (1979) reported that in vitro PHA mediated transformation of peripheral blood lymphocytes shows maximum response on 5th day in two species of bats, Desmodus rotundus, a vampire bat and Carollia perspicillata, a frugivore. These and other work by Fletcher (1961)

and Sulkin (1963) on immune response of bat amply indicates that there exist a quantitative deficiency in immune responses and the peak of responses are delayed compared to other common laboratory mammals. Of the various causative mechanisms involved for the lesser and delayed immune responses, the process of activation can be one of the crucial step as activation is a prerequisite for cell differentiation and clonal expansion (Chakravarty, 1980). Thus, study of the kinetics of different events like blastogenesis and DNA synthesis and their control during the activation of B and T lymphocytes was in the main focus of the present investigation.

For activating bat lymphocytes different T and B cell mitogens were used instead of naturally occuring infecting antigen like JEV or rabies virus which are known to be carried by bats because i) changes inducible by an antigen in a small responsive population which is difficult to measure against a large background of non-responsive cells that are generally not detectable within the limits of accuracy of standard biochemical techniques; ii) mitogen will obviate variation due to antigen processing and antigen concentration on activating cell which are not known in bat system.

Several studies have shown that antigenic stimulus can be substituted by the mitogenic stimulus of lectins which can activate both B and T cells polyclonally for antibody secretion and cell mediated response respectively (Coutintio et al., 1975; Waterfield et al., 1975; Charmot et al., 1975; Heininger et al., 1976). Pokeweed mitogen (PWM) and lioppolysaccharide (LPS) have been used for activating murine B cells in vitro (Andersson et al., 1972; Parkhouse et al., 1972; Piguet & Vassalli, 1972; Shortman et al., 1973; Weber, 1973). For activating T cell in vitro, a number of workers have used mitogenic agents like Con A (Stobo & Paul,1973; Waterfield et al., 1975; Heininger et al., 1976; Chakravarty & Clark, 1977) and Phytohaemagglutinin (PHA) (Stobo & Paul, 1973; Haskovee & Agelisova, 1975). Initial observation with PHA was reported by Rigas & Osgood(1975), Beckman (1962), Barkham & Ballas (1963), Halland & Halland (1965), Barker (1969), Blomgren & Svedmyr (1971) and Owen et al., (1971).

It has been shown that thymocytes develop maximal responsiveness to different mitogens such as PHA, Con A and PWM at different stages of ontogeny starting from the embryonic state (Howe & Manziello, 1972; Stobo &

Paul, 1972; Mosier, 1974; Chakravarty, 1980; Chakravarty et al., 1975; Chakravarty, 1977). Most of the experiments for activating T cells with polyclonal agents like PHA and Con A have been done in vitro (Winkelstein & Craddlock, 1967; Stobo, 1972; Greaves & Janossy, 1972; Shortman et al., 1973; [redacted]; Oliver et al., 1977).

However, extensive works have been done with murine T cells treated with either Con A (Sjorberg et al., 1973; Waterfield et al., 1975; Heininger et al., 1976; Bevan et al., 1976; Tartof & Fitch, 1977; Bonavida,1977; Chakravarty & Clark, 1977; Pilarski et al., 1977; Chakravarty, 1978; Lomnitzer & Rabson, 1979; Hunig, 1984) or PHA (Kay, 1969; Kay, 1971a & 1971b; Lindahl & Mattson, 1971; Stobo et al., 1972) or with B cells stimulated by LPS (Greaves & Janossy, 1972; Andersson et al., 1972; Janossy et al., 1972). This mitogen mediated activation of murine lymphocytes has been considered as a model in our analysis of the basic mechanisms involved in delaying the process of activation of lymphocytes of P. giganteus in humoral and cell mediated immune responses following binding with mitogen. In course of activation of lymphocytes, several events starting from binding of the mitogen have been observed (Peters & Hausen, 1971a and 1971b; Mendelsohn et al., 1971; Cross & Ord, 1971; Quastel &

Kaplan, 1971; Dent, 1971; Allwood et al., 1971). It is considered that most of these events are in common with the process of activation of lymphocytes initiated with specific antigen. It has been shown by many workers (Clark, 1975; Waterfield et al., 1975; Bevan et al.,1976) that Con A can drive mouse T cells to the expression of antigen receptor mediated cytotoxic function which is indistinguishable from that developed by reaction to alloantigen, with the exception that former is polyclonal while the latter is clonally restricted.

In addition, Chakraborty and Clark (1977) have shown that Con A stimulus can not only cause the differentiation of cytotoxic T cell in mouse but can bring about the expression of specific cytotoxicity in memory T cell which are qualitatively similar to that generated by alloantigen.

As the process of activation of lymphocytes leading to functional differentiation induced with mitogenic substances is similar to that induced with alloantigen and devoid of the step of antigen processing; substances like Con A and PHA for T cells and LPS for B cells were used in this study. Although Con A is a good T cell mitogen, PHA has been also chosen as human T lymphocytes were better stimulated with PHA than Con A

(Pienkowski et al., 1973) and this is important in view of the anatomical resemblance of bat thymus to that of priamates than of mouse (Chakraborty & Chakravarty, 1984). PHA mediated activation and generation of cytotoxic T lymphocytes has also been studied and compared to that of Con A to understand whether delay in the activation phase of immune response is a general phenomenon or differ with the substances used for T cell stimulation. Similarly, the process of activation of B cell was studied by using LPS or SRBC as the stimulator.

In order to understand the delayed immune responses in bat, the process of activation and the associated controlling factors were studied. The controlling factors are: a) different quantum of immunocompetent and accessory cells; b) serum suppressor factor(s) c) Suppressor T cells; d) mitogen receptors on cell surface and e) the involvment of energy requirements by measuring the kinetics of three respiratory enzymes. Their effects mainly on blastogenesis and DNA synthesis, the major two events during activation of lymphocytes were investigated.

Blastogenesis is one of the indicator of activation of lymphocytes. The intial interaction between lymphocytes and mitogen is the binding of the mitogen to the receptor on the lymphocyte surface. Normally the lymphocyte after getting stimulated become larger in size or form blast cells and undergo differentiation to become effector cells. There are many changes in the metabolic activity of lymphocytes between the initial stimulation by the mitogens and the start of DNA synthesis. DNA synthesis is needed for cell division. As DNA synthesizing cells are rare in many resting population of lymphocytes, the initiation of DNA synthesis has provided sensitive low background assay that has become the standard method for determining whether or not lymphocyte transformation has occured (Bender & Presscott, 1962; Cooper et al., 1963; Marshall & Roberts, 1965; Ribas-Mundo, 1966; Jasinska et al., 1970; Loeb et al., 1970; Zank et al., 1971; Soubil & Panijel, 1970; Hardy & Ling, 1973). Thus it seems that measure of blastogenesis and DNA synthesis at different hours following mitogen stimulation will help in analysing the process of activation of lymphocytes of bat, either T or B cells, in vitro by using Con A and PHA or LPS.

Furthermore, to test the functional differentiation of bat lymphocytes after mitogenic stimulus *in vitro* and to understand whether or not there is a 'lag period' in between activation phase and functional phase, antibody mediated response was measured by Jerne's plaque technique PFC, modified by Cunningham and cell mediated immune (CMI) response by ^{51}Cr-release in cytotoxicity assay.

The following sets of experiment were performed to understand the role of different factors during activation of bat lymphocytes:

a) Cell-cell cooperation in different quantum has been studied. Three types of cells are implicated in the induction of antibody forming responses *in vivo* and *in vitro* (Feldman & Globerson, 1971; Playfair, 1971; Auerbach, 1972; Amos, 1971). Bone marrow derived lymphocytes (B cells), the direct precursors of antibody producing cells require for stimulation by some antigens both thymus derived lymphocytes, T cells, *in vivo* (Claman *et al.*, 1966; Mitchell & Miller, 1968) or *in vitro* (Globerson & Feldman, 1969; Hartman, 1970; Hartman, 1971; Dutton *et al.*, 1971) and an adherent cell type both *in vivo* (Gorczynski *et al.*, 1971) and *in vitro* (Mosier, 1967; Mosier and Coppleson, 1968; Hoffman, 1970; Mosier *et al.*, 1970; Munro & Hunter, 1970; Shortman *et al.*, 1970; Cosenza *et al.*, 1971).

Therefore, it was of interest to see the existing ratio of T, B, and macrophage (MØ) in bat lymphoid organs and to find out whether addition of different quantum of T, B and MØ in different combination can alter the activation kinetics of bat lymphocytes.

Chakraborty and Chakravarty (1984) reported the possible dichotomy of lymphocytes in P. giganteus in terms of T and B cells as bats are capable of mounting both humoral (Chakraborty & Chakravarty, 1983a; Chakraborty & Chakravarty, 1984) and CMI response (Chakraborty & Chakravarty, 1983b). These authors also reported that a part of the lymphoid cell population from secondary lymphoid organs were susceptible to anti-brain or anti-thymocyte serum.

Niederhuber (1980) while characterization of bone marrow derived macrophages as accessory cells in antigen induced T cell proliferation in mouse reported that 4 X 10^5 primed lymph node purified T cells would give optimal ^{3}H-TdR incorporation CPM with 5 x 10^4 bone marrow derived macrophages. Similar report was forwarded by Levis and Robbins (1970) that highly purified cultures showed marked dependence on the presence of macrophages for their response to even optimal concentration of PHA.

The number of macrophages required for activation are very small, often no more than 1% of the number of lymphocytes in the cultures. Therefore, in the analysis of factors associated with activation phase, cell-cell cooperation in various ratio of different cell type following mitogen mediated stimulation in vitro has been studied in medium containing autologous serum.

b) To find out whether autologous serum of bat has suppressor factor(s) for the process of activation, the kinetics of blastogenesis and DNA synthesis in vitro in medium supplemented with autologous or heterologous serum were studied. Heterologous serum source was from goat. Goat serum was preferred instead of fetal calf serum as it is cheap, readily available and has been shown earlier (Chaudhuri and Chakravarty, 1983) that viability, percentage blastogenesis and DNA synthesis in the in vitro cultures induced by Con A are quite similar in both cases.

Several specific and non specific suppression of delayed hypersensitivity reaction and mitogen responsiveness in human and experimental animals with malignant tumours have been observed to be caused by serum factors

(Waldman et al., 1976). In bat what appears more significant is the presence of interferon type II a soluble f factor produced during viral infections (Salvin et al., 1975), and this substance is known to inhibit response to T cell mitogen and the mixed lymphocyte reaction. Although no one has characterised the presence of Interferon type II in bat, this is important for consideration as bats have been known to carry several pathogens like bacteria (Constantine, 1970) and viruses (Sulkin, 1962; Ito & Saito, 1952; Sulkin et al.,1966; Gadkari et al.,1976 Seymour et al.,1978).

On the other hand, the precise role of suppressor cells in bats immune responses has not been worked out so far and hence their modulatory role in the activation of bat lymphocytes was also not known.

It is known that suppressor cell systems have been involved in virtually all of the immunoregulatory mechanisms that are recognised since the finding of it in mouse (Gershon, 1974). Suppressor cells have been shown to play an active role in the maintenance of immunological tolerence, in the phenomenon of antigenic competition, in the genetic control of immune responses, in the phenomenon of chronic allotype suppression and in the regulation

of antibody response to antigens (Waldman et al. 1976). More recently, it has been reported that many immunological deficiencies and autoimmune diseases are associated with disorders of the suppressor cell systems (Engel et al.,1984; Gupta et al., 1980; Ishizaka & Ishizaka, 1983; Kus et al.,1984). Suppressor cells have also been implicated in the immunological enhancement of tumour growth (Fujimoto et al., 1976). Hence the role of suppressor cells has been looked into by comparing the normal kinetics of activation with the activation kinetics following removal of suppressor cells by cyclophosphamide administration. It has been reported that administration of cyclophosphamide can cause either immuno-potentiation or immunosuppression depending on its dose, timings and the route of administration (Hengst et al., 1985). Immunopotentiation was achieved by the direct action of the drug on Suppressor T cells and several workers (Ray et al., 1980 ; Askenase, Hayden & Gershon, 1975; Moitra, 1986) have shown that cyclophosphamide at low dose can eliminate/ inactivate T suppressor Cell.

In the analysis of different factors affecting the process of activation in bat lymphocytes, mitogen receptor density has also been considered because mitogenic transformation of lymphocytes is initiated by a

primary interaction between the mitogen and receptors exposed on the cell surface (Greaves & Janossy, 1972; Stobo, 1972; Stobo, 1977). More than 25 electrophoretically distinct proteins occur within the lymphocyte membranes (Marchalonis et al.,1971) and of these only the glycosyl containing molecules are known to combine with lectins, (Lis and Sharon, 1973). Con A binds glucose, mannose, glucosamine and other structurally related sugar residues and complexes between Con A and the sugar residues of glycoproteins can be dissociated by the addition of the hapten sugar, α-methylmannoside (Lis & Sharoh, 1973).

A range of Con A binding glycoproteins have been identified (Iwata et al., 1977) and among which were components precipitated by specific antisera directed against the mouse major histocompatibility antigens ($H\text{-}2^k$ and $H\text{-}2^d$) and the Ia antigens (Henkert & Fisher, 1975; Nilsson & Waxdal, 1976). Further, Schmidt-Ullrich et al. (1975) and Yamashita et al. (1978) reported that Con A reactive cells express atleast two different I-region determinants by virtue of which mitogen interact with the cell surface to elicit their biological function.

The number of lectin molecules that can bind to the lymphocyte surface have been determined by several groups. Human lymphocytes have been found to bind upto 2.7×10^6 molecules of H-PHA per cell (Kornfeld,1969) and either 2.4×10^6 or 6.6×10^6 molecules of lentil mitogen per cell (Kornfeld et al., 1971; Stein et al., 1972). Mouse spleen cells, bone marrow cells, thymocytes and lymphocytes and rat lymph node cells have all been found to bind about 10^7 molecules of Con A per cell (Edelman & Millette, 1971; Stobo et al., 1972; Betel & Vanden Berg,1972). In contrast, lymphocytes from patients with chronic lymphocytes leukemia binds only 1.15×10^6 molecules of PHA per cell (Kornfeld, 1969).

We have examined the number of Con A receptor sites on the cell surface of bat T lymphocytes to see whether causative factor for delayed immune response could be reflected in terms of receptor density also.

Finally, the increase in membrane transport and protein and nucleic acid synthesis that accompany lymphocyte activation require increased energy production; therefore the molecular strategies for releasing energy during the process of activation in the lymphocyte of bats showing delayed immune response have been undertaken in our

94613
16 JAN 1987

study. Peters and Hausen (1971) reported the doubling of the rate of glucose uptake within 15 to 30 mins of PHA addition and the rate of lactate production also shows similar increase in the same period (Roos & Loos, 1970). After 24 h, lactate production rises to about 4 times the initial rate and majority of the glucose utilised is converted to pyruvate or lactic acid (Pachman, 1967; Hedeskov & Esmann, 1966) indicates that the activating lymphocytes undergo both anareobic and aerobic respiration. On the other hand, the revealation that 5% of the glucose taken up is oxidised by the pentose phosphate pathway after PHA stimulation (Hedeskov & Esmann, 1966; MacHaffie & Wang, 1967) also indicate that generation of pentoses for nucleic acid synthesis and reducing power in the extra-mitochondrial cytoplasm in the form of NADPH is necessary. The involvement of Hexose-mono-phosphate (HMP) shunt during respiratory burst following phagocytosis (Pachman et al., 1973) also predict that a similar involvement may be seen in case of lymphocyte stimulation. These and other references (Roos & Loos, 1973) amply indicate that membrane transport, protein synthesis and nucleic

acid synthesis which accompany lymphocyte stimulation are energy requiring process and therefore the state of delayed cellular function can arise if the energy requirement to support a given function exceeds that which is available. In view of this, the enzyme activity and substrate affinity of three respiratory enzymes - lactate dehydrogenase (LDH) of anaerobic pathway, mitochondrial citrate synthase (m-Cs) of aerobic pathway and glucose -6-phosphate dehydrogenase (G6PDH) of HMP-pathway at different phases of <u>in vitro</u> activation of bat lymphocytes have been studied.