DISCUSSION

In the analysis of the causative mechanism for lesser and delayed immune response in vivo (Hatten et al. 1970; Leonard et al., 1968; Heck, 1965; Chakraborty, 1982; Chakraborty & Chakravarty, 1983; Chakraborty & Chakravarty, 1984a, 1984b) the process of lymphocyte activation in terms of blastogenesis and DNA synthesis in vitro and its controlling factors have been studied in the present investigation. T cell mitogens like Con A and PHA and B cell mitogens, the LPS have been adopted for studying the process of activation and we demonstrate that bat lymphocytes when stimulated in vitro by these mitogens can undergo both blastogenesis and DNA synthesis. Furthermore, upon stimulation, these cells can undergo differentiation into functional cells as evident from the PFC response (Fig. 10) and cytotoxicity results (Figs. 7 & 9) Hence, as in mice, bats also display Con A - PHA- and LPS-responsive lymphocytes which in otherwords reflect the functional heterogeneity of bats lymphocyte population. This was further evident from: 1) diminished kinetics of ^{3}H-TdR incorporation in antithymocyte serum treated and untreated cells following Con A or PHA stimulation and the similar kinetics in both antithymocyte serum treated and untreated cells following LPS stimulation (Table 4); ii) the differential adherence property of bat lymphocytes to nylon wool column revealed an adherent type known as B cell and a non-adherent type as T cells in man and mouse (Trizio & Cudkowicz,1974; Stinson,Corkle & Click,1978; Kowalczyk et al., 1981).

Thus the results of our study strengthen the earlier suggestion of the existence of atleast two distinct subpopulation of lymphocytes in terms of T and B cell lineages (Chakraborty & Chakravarty, 1983). Furthermore, besides T and B cells, there also exists another cell type adherent to plastic surface and stained deeply with neutral red dye which in all likeliness is macrophase population. Thus, the lymphoid organs of bat possesses functional equivalents to the interacting T, B and MØ system of man and mouse and are capable of mounting both humoral and CMI responses and thereby contradicts the view expressed earlier by several authors (Hasenclever, 1972; Hasenclever et al., 1967; Emmons et al., 1966) where they reported that bats were not capable of mounting an immune response to the invading organisms.

Study of the activation kinetics following stimulation by Con A show that 10 µg dose appears to be optimal for blastogenesis and the peak of responses were attained at 120 h (Fig. 1). Similar pattern of ^{3}H-TdR incorporation with this dose of Con A was observed (Fig.3). PHA, the other T cell mitogen, used in the study, also reflected a similar prolonged activation phase and 10 µg appears to be the optimal dose for both blastogenesis and DNA synthesis. In mice, however, these peaks are attained at 48 h. Hence, bat lymphocytes which exhibits delayed immune response also display a delayed T cell activation process.

At the other end, it has been found that LPS could induce B cells for maximum blastogenesis between 72 to 96 h while maximum DNA synthesis was found to occur at 96 h (Figs. 5 & 6). In mice, the peak of these responses were found at 48 h and thus like T cells, B cells of bat lymphoid organs also reflect a prolonged activation phase.

The prolonged activation phase thus characterised in T and B cells of bats suggest us to investigate further whether there exist a lag period in between activation phase and functional phase. To resolve this, T cell function was measured by ^{51}Cr-release cytotoxicity assay and the B cell function by PFC assay. It was found that bat lymphocytes assumed function only after mitogennic stimulus and the peak of cytotoxic - and PFC - response corroborated well with the peak of activation, as measured by blastogenesis and DNA synthesis, in T and B cells respectively. Hence, in bat lymphocytes there exists no 'functional lag' between activation phase and functional differentiation in T cells or B cells.

It is revealed from our work that bat lymphocytes when stimulated with varying doses of different T cell mitogen, graded response with the increment in doses was not observed. In case of Con A, 20 µg dose

could induce DNA synthesis but could not transform significant number of cells into blast while in case of 20 μg of PHA stimulation, blastogenesis was observed reasonably but no DNA synthesis. The disparity thus observed in blastogenesis and DNA synthesis can be due to the fact that these processes are not always a correlative phenomena. It was shown earlier (Chakravarty & Chaudhuri, 1983) that 50 μg of Con A injected per mouse caused a good degree of blastogenesis but a low level of DNA synthesis. Earlier, Chakravarty and Clark (1977) also reported that blocking DNA synthesis in mouse memory T cells with hydroxyurea had essentially no effect on Con A mediated blastogenesis in vitro; similar observation was made earlier by Nedrud, Touton and Clark (1975) incourse of MLC reaction and blocking DNA synthesis. Contrary to the findings with PHA, higher dose of Con A was ineffective in inducing blastogenesis but could induce DNA synthesis in bat lymphocytes in vitro (Fig. 3). This asynchrony may be the reflection of an usual phenomenon in cell cycle. It is known that DNA synthesis occurs mostly in the S-phase of the cell cycle when size-wise the cell may not be a large one. The commonest pattern for cell growth in volume is due to increase in total protein (Mitchison, 1971) as observed during blastogenesis.

The delayed nature of T cell activation persist even when optimum proportion of MØ or both MØ and B cells were added to the constant number of T cells in vitro. The optimum level of different cell types were calculated in reference to mouse, considering it as a model animal. Further it was revealed that when T cells were alone stimulated by Con A, the peak of blastogenesis and DNA synthesis occured at 120 h as was seen in case of unfractionated lymphocyte population or in condition when cell types were adjusted to optimum ratio. Thus the variation in the number of cell types in secondary lymphoid organs of bat may not be the controlling factor for delayed activation of bat lymphocytes. In view of the 'recognition hypothesis' which advocates that mitogen mediated activation by binding to MHC encoded antigens on the surface of accessory cells (AC) in such a fashion that the resting T cell will recognise the modified AC via their antigen/MHC receptor (Kimura and Ersson, 1981; Beretta et al., 1982) our result suggest that polyclonal activation of Bat T lymphocytes is independent of MØ interaction and hence the requirement of for recognition of H-2 encloded antigens on MØ is possibly bypassed by bat lymphocytes also, as in mouse (Hunig,1984).

Next, we tested the hypothesis whether the serum suppressor factor(s) or a subset of T cells, namely the suppressor T cells, are involved in the delayed activation of T or B cells of the bat.

The possibility of serum suppressor factor(s) mediated prolongation of activation phase of lymphocytes in bat gets ruled out as the kinetics of both T and B cell activation in medium supplemented with serum from heterologous source were found same as when lymphocytes were cultured in medium with autologous serum (Figs. 11 to 16). Although there was no early attainment of blastogenesis and DNA synthesis peak by using heterologous serum, amplification of these parameters were observed. These amplifications may be due to foreign protein moieties of heterologous serum having lymphocyte stimulating property.

On the other hand, treatment of bats with CY prior to subject the lymphocytes for in vitro stimulation with T or B cell mitogen suggest the existence of CY sensitive cells or suppressor T cells in an evolutionary old group of mammals. The attainment of peak for both blastogenesis and DNA synthesis were significantly enhanced (Figs. 17 - 22) and shifted earlier in CY - treated lymphocytes stimulated either with Con A, PHA or LPS. The 10 µg dose of Con A or PHA which were

found to be optimal in normal lymphocytes stimulation, by and large, remained the most effective dose in CY pretreated lymphocytes also. In case of 5 µg dose of Con A or PHA, which could not induce any peak of blastogenesis or DNA synthesis even at 168 h in normal lymphocytes, were however effective in doing so in CY treated lymphocyte population.

Furthermore, normal lymphocyte when challenged *in vitro* with SRBC, the peak of PFC response was wanting even at 120 h where as with CY pretreated cells the peak was apparent at 96 h. Peak of SRBC induced PFC response *in vivo* usually take atleast 10 days to develop (Chakraborty & Chakravarty, 1983). Thus it seems that suppressor T cells of bat possibly play a dominant role in causing delay by suppressing the attainment of the peak of blastogenesis and DNA synthesis induced with Con A, PHA or LPS or PFC response induced by SRBC.

Besides the existence of suppressor T cells, we observed that Con A binding sites bat per T lymphocyte is low compared to 10^7 molecules of Con A on the surface of murine thymocyte or lymphocyte and also on rat lymph node cells (Edelman & Millette, 1971; Betel & Vandenberg, 1972). This decresed number of Con A receptor sites is possibly reflecting the altered cell surface which contribute to the prolonged activation phase of bat

lymphocytes. This is in consistent with the early finding that lymphocytes from patients with chronic lymphocytic leukemia showing both a delayed and an impaired blastogenic response to phytohaemagglutinin (Robbin, 1964) also exhibits decreased PHA receptor sites (Kornfeld, 1969).

Furthermore, Stobo et al., (1972) have calculated that the number of Con A molecules required for murine lymphocyte activation lies with in 0.8 to 3×10^7 range. Interestingly, our calculated value of Con A molecules that can bind on T cell surface of bat is even lesser than the lowest value. However, we do not know in bat lymphocytes the minimum number of Con A receptors in terms of Con A molecules which is optimal for blastogenesis and DNA synthesis. But it transpires that Con A receptors are less closely packed than murine lymphocytes and as the ability of an immunocompetent cell to respond to an external stimuli depend mostly on optimal concentration of the corresponding receptors on the cell surface, therefore, possibly lesser number of receptors donot allow sufficient binding of Con A molecules at the receptors which is a crucial factor for initiation of activation in normal situation (Heininger et al., 1976). Moreover, the less closely spaced receptors possibly answers why Con A mediated activation of bat lymphocytes requires a higher dose of the order of

10 $\mu g.ml^{-1}$ (Paul & Chakravarty, 1986) as against 2-5 $\mu g.ml^{-1}$ in mouse (Heinger et al., 1976; Williams and Benacerraf, 1972; Bevan & Cohn, 1976) for optimal response. This type of critical analysis of receptors for alloantigen on B lymphocytes of bat is necessary to understand the mechanism of delayed humoral response as reported earlier (Chakraborty & Chakravarty, 1984; Chakravarty & Paul, 1986).

At the end we attempted to find out whether changes in molecular mechanism associated with energy liberation occurs in the T lymphocytes of bat secondary lymphoid organs could have any bearing with delayed activation of the cells. Persistence of activity of LDH, G6PDH and m-Cs were noted in course of activation and hence reflect the role of anaerobic, aerobic and hexose monophosphate (HMP) shunt pathway to the energy provision during lymphocyte activation. It is also indicative that delayed activation phase is no way associated with the absence of either of theenzymes under study. However, there has been marked decrease in LDH activity in bat lymphocytes at 120 h following Con A stimulation when the peak of blastogenesis, DNA synthesis and cytotoxicity was found. This implies that confer of energy from the anaerobic apparatus fail to support increased energy demand imposed on the cells by the activation of the biosynthetic pathways. The m-Cs and G6PDH activity, however shows no appreciable change at all phases of activa-

vation and is speculated that at 48 h and onwards the normal activity of these enzymes keeps providing energy, whatever it can, for the process of activation in order to transform and differentiate T cells in vitro. Probably due to this mode of energy supply, the process of activation of T cells leading to cytotoxic differentiation of the cells in bat lymphocyte population is prolonged.

Low activity of lymphocyte LDH at 120 h of Con A stimulation, however, suggest another possibility that serum LDH can play the compensatory role. The persistence of normal activity of G6PDH at all phases of activation possibly reflect, apart from confering energy, the conversion of hexose to pentose, particularly D-ribose-5-phosphate which will be utilised in due course for the synthesis of nucleic acid. This step is necessary for lymphocyte proliferation and polyclonal expansion by Con. A.

The binding affinity of enzymes to its corresponding substrate as estimated by K_m values show that with the progress of activation, the catalytic function at low substrate concentration is enhanced in cases of LDH, m-Cs and in case of G6PDH at 120 h. which is the peak time for blastogenesis, DNA synthesis and cytotoxicity by bat lymphocytes and not at 48 h which is the

peak time for all these parameters in mice. Therefore, it is conclusive that although specific activity did not increase significantly, the enzyme molecules prepare themselves to increase their catalytic efficiency to meet the energy demand. Presently, we donot have any information regarding the K_m values of these enzymes in the lymphocyte of normal mice or man which would otherwise enabled us to say whether or not, the degree of change of K_m at different phases of activation is at par or different in P. giganteus. Besides other implications the comparative K_m study with other systems would answer the question of why bat lymphocytes exhibits a low degree of immune response as reported earlier by Chakraborty and Chakravarty (1984).

Thus in course of the analysis of the controlling factors associated with delayed activation phase, we have found that in bat lymphocytes Con A receptor sites are less in number; role of suppressor T cell manifested and the mode of energy confer by the respiratory enzymes, representative of different pathways, differ significantly and the magnitude of activity was found comparatively less. In the process it was also revealed that the dose requirement for polyclonal expansion by mitogen is comparatively high and interestingly bat T lymphocytes can be equally induced by Con A and PHA.

But the most striking revealation that surfaces from this study is the significant role of suppressor cells, the removal of which enhances the attainment of the peak of both blastogenesis and DNA synthesis at an early hour. Question therefore arises why in bats the suppressor cells are operative right from the initial phase of activation by virtue of which the degree of immune responses were lesser and the peak of responses were delayed.

Apart from bat, similar diminished proliferative T cell responses were reported in many haemophiliacs receiving clotting factor concentrates (Lederman et al., 1983; Davis et al., 1983; Elliot et al., 1983; Poon et al., 1983; DeShazo et al., 1983) where depressed helper/suppressor T cell ratio was observed. Similarly, in Acquired immune deficiency syndrome (AIDS) depressed proliferative response to mitogens have been found to be associated with abnormal ratio of immunoregulatory T cells (Ammann et al., 1983; Gupta & Safai, 1983; Gupta, 1985; Laurence, 1985; Dobzin et al., 1986). Furthermore, in various clinical situations like EB virsus-induced infection mononucleosis, CMV-induced infection mononucleosis and acute phase toxoplasmosis with lymphadenopathy (Reinherz et al., 1980; deWaele, Thielemans & VanCamp, 1981a,

1981b; Carney, Iacoviello & Hirsch, 1983) all have reported decrease helper/suppressor cell ratio and this is important as bats are also associated with certain viral and parasitic infections (Sulkin,1962; Sulkin et al., 1966; Klite & Young, 1965; Constantine, 1970; Oya et al., 1982; Banerjee, Ilkal & Desmukh, 1984; Bizlenga & Hernandez-Baumgarted, 1984). Possibly the incidence of (re) infection by these organism or unrelated ones seems to generate suppressor T cells in bat thus causing alteration or suppression of the normal kinetics of activation leading to a delayed immune response.