RESULTS

STUDY OF THE LYMPHOID ORGANS

The cells responsible for immune responses are usually located in the secondary lymphoid organs like spleen and lymph nodes in higher vertebrates. So in this investigation the study of the spleen, lymph node of the bat has been made in normal condition and after immunization. Antigenic stimulation leads to proliferation, rapid division and increase in number of immunocompetent lymphoid cells mostly in spleen and lymph nodes. So change in histological structure of these organs after immunization have been studied.

Besides these, the study of the thymus, a primary lymphoid organ has also been made.

Spleen :

Spleen in bat is blackish red in colour and situated in the left abdominal cavity, beneath the diaphragm and attached by peritoneal folds to the wall of the stomach as usual as in other mammalian species. Size of the spleen varies from 4 cm x 0.5 cm to 5 cm x 1 cm in normal nonimmunized bats.

From histological sections, it reveals that the spleen is encapsulated by a thick collagenous layor which invaginates at some places to form distinct trabeculae. The trabeculae are often with blood vascular channels. It seems that the blood vascularization in spleen of bat is considerably rich in comparison to that of a mouse. The blood vessels enter in the spleen slightly away from the apex.

White pulps are the compact concentric organization of about 6 to 8 μm lymphoid cells and stained much deeply with haematoxylin. They surround splenic arteriole in the form of lobules and are distributed in the red pulp, throughout splenic parenchyma (Plate 1, Fig. 1,2). The reticular fibres and epithelial cells create zonation in the red pulp.

After immunization with SRBC, white pulps in spleen transform into distinct germinal centres. Germinal centres are comprised of jacket like compact zone of small lymphocytes surrounding the central lightly stained circular zone of dividing lymphocytes, macrophages and plasma cells (plate 1, Fig.3). The central zone and germinal centres as such increase with the days of immunization. The increments in the size of the germinal centres from white pulp stage after

immunization have been depicted in table 1. Vascularization and number of venous sinuses increase with immunization; increasing number of small lymphocytes and blood components give a spongy appearance to the red pulp.

Histological study of the spleen of the embryos of different ages and the neonatal bats were made to get an idea of the gradual organization of these organs during development. Lymphoid cells in the foetal spleen can be detected as dispersed throughout the red parenchyma. Lymphoid cells get organized into limited numbers of concentric small masses, the first stage of the developing white pulps, in the young neonates. Distinct trabeculae can also be observed from this stage (plate 2, Fig.1,2).

Lymph Node :

Cervical, maxillary, axillary, mesenteric, inguinal lymph nodes follow the general plan of distribution in other mammals. Here mesenteric and inguinal lymph nodes in particular have been studied in detail; the sizes of these nodes in normal animals range from 2.5 cm x 0.25 cm to 4 cm x 0.4 cm and 0.25 x 0.21 cm to 0.39 x 0.27 cm respectively. They are whitish in colour and most of the time covered with a

layer of fatty tissue. Lymph nodes of an adult animal are encapsulated by a thick layer of connective tissue fibres with subcapsular sinuses underneath. The capsule is thicker at hilus where ingoing and outgoing vascular channels are located.

Cortex and medulla are distinct. Primary lymphoid nodules are present in the cortex (Plate 3, Fig.1,2). Cortex is demarcated into outer and inner cortex. Demarcation of the two layers of cortex and medulla is more pronounced than in mice. Germinal centres with central lightly stained lymphocytes blasts and outer cover of deeply stained smaller lymphocytes as in the case of spleen, differentiate from white pulp in the outer cortex after immunization (Plate 3, Fig.3 & Plate 4, Fig. 1,2). Apparently difference in size of primary lymphoid nodule and germinal centre of mesenteric lymph nodes after immunization can be observed (Table 1), but their difference is not that significant as that in case of the spleen. Trabecular extensions from the capsule traverse through the cortex to some extent. Medulla is constituted by cells organized in medullary cords and medullary sinuses. It seems that the number of blood-borne cells increases in the medullary sinuses after 15 days of immunization.

Organisation of the lymphoid cells into white pulp cannot usually be seen in the histological sections of the foetal and the neonatal mesenteric lymph node. Deeply stained cells, similar to the small lymphocytes can only be seen throughout the mesenteric lymph node. Distinct demarcation of cortex and medulla cannot be found even in young stage. Rudiments of trabeculae are noticeable in the sections of the mesenteric lymph node of a neonatal bat.

Thymus :

Thymus, one of the major primary lymphoid organs, is known to provide the lymphoid cells which potentiate the immunological capacities of other lymphoid organs. In foetuses and in neonatal milk suckling youngs, the thymus can be visualized easily, lying as usual at anterior mediastinum of the heart. Primarily the thymus is composed of two separate leaf like lobes joined by connective tissue and arising from a common stalk near the root of the conus arteriosus (Plate 5). It is white in colour. Two white lobes of the thymus are firmly bound together in the midline at anterior end. Each lobe is covered by connective tissue sheath having a lobulated margin showing the external expression of the investment of the sheath as septa to form several lobules. These lobules are

more prominent at the posterior margin of each lobe. In histological preparations each lobule follows the general plan of tissue distribution. The reticular fibers and epithelial cells provide the basic supportive network of the organ and the interstices of which are occupied by enormous number of lymphoid cells. The thymic parenchyma is ill differentiated into cortex and medulla in foetuses (Plate 6, Fig. 1,2). However, this distinction is easily recognizable in neonatal bats (Plate 7, Fig.1). The cortex is made of small lymphocytes and occupies the major area of the thymus in young ones.

Moreover, the prominence of medulla in neonatal thymus is accentuated with the presence of well defined Hassall's corpuscles. Hassall's bodies are aggregates of the epithelial cells in whorled pattern (Plate 7, Fig.2 & Plate 8) and very similar to the structure in primates (Arnesen, 1958). They take bright red stain of eosin; an amorphous substance and degenerating cells in the centre of the Hassall's bodies have been observed. These bodies were not observed in the foetal thymus studied by us. The diameter of Hassall's bodies in neonatal thymus varies from 47 to 125 µm. The size and number of these bodies decrease in adult stage where diameter varies from 17 to 48 µm (Plate 9, Fig. 1,2).

Another interesting feature has been observed in course of our study of the bat's thymus in different ages. There is a gradual increment in the size of the thymic lobules upto neonatal or young stage. After attending the maximum size in young stage, pronounced involution of thymus occurs in adult animal as in some other mammalian species including human (Plate 10). The size of the right and left lobe of the thymus in the foetal, neonatal and adult bats have been noted in Table 2. Thus in adult bats, the size of the thymus is minimum, and the islands of lymphoid cells are embededded in a mass of fatty tissue which infiltrated the original space occupied by thymic tissue in earlier life and it is not possible to visualize the small thymus in adult bats easily, unless the enormous mass of fatty tissue is removed.

SUSCEPTIBILITY OF THE LEUKOCYTES FROM DIFFERENT SOURCES TO ANTI-LYMPHOCYTE SERUM (ALS)

The percentages of cells from different lymphoid organs, bone marrow and peripheral blood susceptible to the anti-lymphocyte serum (ALS) mediated killing were determined in this series of experiment. ALS was raised in rabbit against bat's lymphocytes from spleen and lymph nodes. At first, specificity of raised ALS was tested against bat's lymphocytes. Experiments to test the heterogeneity in the lymphoid cell population of

the bats depending on the different cell surface determinants were also performed.

Specificity Test for Raised ALS :

Specificity of raised ALS to the lymphocytes was ascertained from the cytotoxicity experiment with splenocytes and lymph node cells after absorption of ALS with spleen and lymph node cells and the result of the experiment has been presented in Table 3. Absorption of serum caused sharp decrease in the efficacy of ALS to the target cells used for raising the anti-serum. After absorption, different dilutions of serum did not show gross variation in the percentage of lysis. Similar trends were observed in 3 repeated experiments.

Study of cell types of secondary lymphoid organs, peripheral circulation and bone marrow by ALS mediated cytotoxicity test :

ALS collected after last booster injection was diluted in two fold order from 1:20 to 1:80 with the addition of adequate amount of PBS. Lymphocytes from different organs were collected after usual Ficoll-hypaque purification and 10^6 cells added in each experimental tube. Lytic indices (%) for the lymphocytes collected from different organs have been presented in figure 1.

With three different dilutions of ALS, maximum lytic indices were obtained in case of lymphocytes from the lymph nodes. Susceptibility of the splenocytes to ALS was less than that of the lymph node lymphocytes. Lytic index was lower with (PEC) than lymphocytes from spleen and lymph nodes and there was not much variation in the lytic indices with three different dilutions of ALS. Lytic indices with bone marrow cells and lymphocytes collected from peripheral circulation did not differ appreciably in 3 dilutions of ALS.

Susceptibility of the lymphocytes from spleen and lymph node after absorption with bone marrow cells and brain homogenate :

On the basis of cell surface determinants the possible heterogeneity in the lymphoid cell population of the bats was tested. This has been done by absorbing ALS with bone marrow cells and brain homogenates prior to the cytotoxicity test. Anti-thymocyte serum (ATS) could not be used in this set of experiments as it was difficult to raise ATS due to non-availability of several neonatal bats at a specific time and adult thymus was very small due to age dependent involution. The brain homogenate was used for absorption of ALS in view of existence of similar antigenic determinants like θ or Thy-1 both in brain and thymus cells of mice (Golub, 1971) and usage

of anti-mouse brain serum for killing of mouse thymocytes.

Bone marrow cells were also used for absorption prior to the cytotoxicity test with lymphocytes from the secondary lymphoid organs to determine whether or not both the cell types share the same type of cell surface determinants.

It is apparent that prior absorption of ALS with bone marrow cells does not reduce the cytolytic ability of ALS as it is revealed from the lytic indices in Table 4. Whereas absorption of ALS with brain cell homogenate causes significant reduction in lytic activity of ALS in most of the cases. The percent of reduction of lytic activity of ALS after absorption was approximately upto 40% in case of spleen cells and more than 50% in case of lymph node cells. Absorption of ALS with bone marrow and brain homogenate at a time also shows the reduction in the efficacy of ALS; the percent of reduction in this case is comparable or may be slightly higher than the indices obtained with ALS absorbed with brain homogenate only.

STUDY OF ANTIBODY MEDIATED PRIMARY IMMUNE RESPONSE

Antibody mediated immune response has primarily been measured in two ways, enumerating the antibody secreting cells by Cunningham and Szenberg's PFC method (1968) in which antibody

secreting cells form plaques by lysing the antigens in presence of complement and by agglutination reaction pattern of the secreted out antibodies in the serum.

Effect of complement from different sources on the formation of plaques :

Complement is necessary for antibody mediated lysis of the target cells or antigen. Three different types of serum including the homologous serum had been used in PFC assay to select the most effective source of complement. From Figure 2, it reveals that though guineapig complement is of wide use in PFC assay of lymphoid cells from different species, homologous serum shows better response in case of bats. From Table 5, it reveals that the number of PFC obtained with the use of bat's complement is always higher than that with two other types of complements; but the number is not significantly different from that obtained with guinea pig's complement. Rabbit complement was least effective. Thus we decided to use bat complement in all other PFC assays in this study.

Development of a typical plaque :

Very small clear spaces were visible after 2 hrs of incubation of the plaque slides and by 3 to 4 hrs typical

plaques developed. The plaques were quite similar to those in mouse and other species.

Antibody secreted out of the splenic lymphoid cells lysed the surrounding particulate antigen (SRBC) in the monolayer suspension of an assay slide. The lysis occur in a radial fashion and a circular clear space develops as plaque in the opaque monolayer of the suspension of lymphoid cells and SRBC; most of the time the antibody secreting lymphoid cell was present at the centre of the clear space of plaque and visible under microscope (Fig.3). Certain other lymphoid cells are sometimes present in the clear space of the plaque; but most likely they are not antibody secreting.

Specificity of the antibody secreting cells of the bats immunized with SRBC was tested. On different days of immunization with SRBC, PFC assays were performed with SRBC and pig's erythrocytes. There was no significant PFC response against pig's erythrocytes although a significant response was observed against SRBC antigen (Table 6).

PFC response with different doses of SRBC :

Antibody mediated response in bats was measured in terms of number of antibody secreting plaque forming cells

at different days of immunization after a single challenge of different antigenic doses. Spleen cells were used for the purpose. The kinetics of PFC response with three different doses of SRBC, 0.2, 0.5 and 1 ml per animal have been presented in figure 4.

The PFC response was dose dependent. The dose of 0.2 ml SRBC per animal did not elicit a marked response uptil 30 days after immunization. There was a peak of the PFC response on 20th day with the dose of 0.5 ml SRBC; whereas challenging with 1 ml dose a notable peak was observed around 10 days. With all three doses, the decay of the PFC response was slow and at a reasonable level upto 30 days, then the response fell almost to the background level by 50 days. The average number of PFC for one million leucocytes in a non-immunized bat was 6; this was considered as background count.

PFC response to double antigenic challenge with a short interval :

In this experiment the animals were injected twice with an interval of 48 hrs with 0.5 ml of 25% SRBC. Assays were made on 5, 10 and 15 days of immunization and the degree

of PFC response on these days were compared with that of the bats immunized with only 0.5 ml and 1 ml of 25% SRBC. PFC response was minimal on day 5 in all the cases. The PFC response of the animals injected only once with 0.5 ml SRBC, was lower than that in other two groups and the response was on way to increase even on 15th day of immunization. Whereas the response in other two groups injected twice with 0.5 ml SRBC and 1 ml SRBC were comparable and the peaks in both cases were observed on day 10 (Fig.5).

Hemagglutination (HA) titre :

With a single antigenic dose of 0.5 and 1 ml SRBC, HA titre levels follow the pattern of PFC response with the respective dose of antigen (Fig.6). The peak of the response was reached on 10th day after injecting 1 ml SRBC and with 0.5 ml dose it was on 15 to 20 days after immunization. Although there was no notable peak of PFC response with 0.2 ml antigenic dose, the peak of HA titre index for this dose was on the 20th day and the response level in general was at reasonable height. HA titre for the different antigenic doses were at a significant level even on 50th day after immunization.

ANTIBODY MEDIATED SECONDARY IMMUNE RESPONSE

As the duration of PFC response in bats was prolonged with a single dose of antigen the secondary response was also studied. This was tested by PFC assay and HA titre in presence of mercaptoethanol (ME). Though Cunningham and Szenberg's monolayer technique is of wide use for PFC assay but assay of ME - resistant plaque is not feasible with this method. Thus the agar plating method has been considered suitable for assaying secondary plaque forming cells.

Agglutination test in presence of three different concentrations of ME :

To select the most effective concentration of ME, agglutination assays were performed in presence of three different concentrations; 0.1M, 0.2M, 0.3M and results have been presented in Table 7. Degree of agglutination reaction was scored by a single or multiple of + sign depending on the size of the agglutinated particles. From our experimental results it is cleared that 0.1M ME showed best response in all cases out of three concentrations. So we used this concentration for further assay of HA titre in presence of ME.

ME-resistant PFC response and HA titre :

The profile of the ME-resistant PFC and HA titre response with 0.5 ml and 1 ml have been presented in figure 7. The number of ME-resistant PFC gradually increased to the maximum level by 15 days in case of 1 ml antigenic dose and by 20 to 30 days with the dose of 0.5 ml. ME-resistant HA titre values follow the similar pattern. Both the types of responses with two doses of antigen falls almost to the bottom by 50 days after immunization.

ANALYSIS OF THE DIFFERENT CLASSES OF SERUM PROTEIN DURING ANTIBODY RESPONSE

The electrophoretic pattern of normal and immunized sera is presented in the figure 8. It shows clear separation of 5 major serum protein components. Apparently the normal and experimental strips do not show any difference in intensity of bands. Quantitative analysis of the different protein components of serum of bats in normal condition and 5, 10, 20 days after immunization with 0.5 ml SRBC is presented in Table 8. Clear change in the amount of total protein after immunization was observed in all cases. Although no significant variation in the proportion of γ globulin was observed

in last two cases, but the first case shows slight increase in the amount of γ globulin at immunized state.

CELL MEDIATED IMMUNE RESPONSE

Skin sensitivity test to 2,4-Dinitrofluoro benzene (DNFB) :

The skin sensitivity to DNFB is a delayed hyper sensitivity type of reaction. The reaction depends on overall T-lymphocyte function. Resensitization of the animals with 2,4-DNFB was done on the 7th day of the first application of this chemical on the skin. Size of the experimental or the resensitization spot was measured upto 13 days at 24 hr intervals (Fig.9). The degree of erythema and induration was measured in terms of the diameter of the resensitized spot in mm. Size of the reaction spot was measured in two directions at right angles and the average was taken as index for the reaction. Maximum induration was observed at 48 hr of 2nd application accompanied by maximum erythema also. Then gradually the size of the reaction spot decreased to normal.

Mixed lymphocyte culture (MLC) reaction :

Immune response of T cells can be measured in vitro by MLC method. Differences in Major histo-compatibility

(MHC) locus between allogenic lymphocytes are mainly responsible for this reaction. Although nothing is known about MHC locus of bat, the bats used in this study were wild and they were outbred and thus it was expected that they would differ from each other at MHC locus.

For each set of experiment, the responder cells at three different concentrations, 10^5, 5×10^5, 10^6 cells per tube were cultured with Mitomycin-C (Mito-C) treated 10^6 stimulator cells. Triplicate cultures for each concentration of the responder cells were maintained. Cultures were incubated for 4 days and 7 days and radioactive thymidine was added in the culture 16 hr prior to the termination of the culture. As the culture period was longer, 5% serum autologous to responding cells was added in the culture medium. Increase in the uptake of ^{3}H-Thymidine by the responder cells leads to the increase in the stimulation index. The stimulation index at 1.2 or more was considered positive as the same index was taken into account as positive in graft-versus-host type of cell mediated reaction in mice (Simonsen, 1962; Chakravarty, 1977). Out of three experiments with three different concentration of effector cells, only in two cases of the cultures, incubated for 4 days showed

positive reaction and one of them was marginal (Table 9). Whereas assay on the 7th day of culture showed enhancement of the incorporation of ^{3}H-Thymidine at higher cell concentrations in both the experiments (Table 10).