

## INTRODUCTION

The vertebrate liver is an organ that has undergone very little changes in its function throughout the evolutionary progress of the group. This resistance to change reveals its important and complex role in the well being of the animals that possess it. The liver is called upon to perform several functions in the daily life of animals. So varied and complex are the functions, it is difficult to decide as to what is the specialized function of the liver. It could be reasonably said that 'non-specialization' is its specialization. Hence, it is not surprizing to see the liver performing excretory, secretory, metabolic, storage, phagocytic as well as many other physiological functions, routinely. This organ is also described as the guardian of the body as many toxic substances are filtered by the organ from the circulation. All these functions add to the welfare of the animals and are critical in adult life. Besides, in embryonic life, the liver is entrusted with some other activities too, especially the haematopoiesis. In many lower vertebrates, haematopoietic activities in the liver is protracted into the adult life too. But this is seen seldom in the liver of adult birds and mammals.

In many respects the mammalian and avian livers are believed to have many things in common, though structural variations are prominent. Recent studies, however, elicit doubts about such belief of equating avian liver to the mammalian counterpart. Even in the metabolic activities, differences are established by workers in the field. In birds, the liver is now considered to be the chief site of lipid synthesis (Goodrich and Ball, 1967). More interesting are the reports of haematopoietic activities in the liver of adults of some species of birds. Thus, George and Naik (1963) found haematopoietic nodules in the migratory starling, Sturnus roseus, while Pilo (1970) found such nodules in the pigeon, Columba livia. The nodules in the pigeon is reported to be producing lymphocytes while that of Sturnus roseus both erythrocytes and lymphocytes, predominantly the later<sup>t</sup>.

Lymphocytopoiesis merits a detailed study in birds, as they lack a well developed lymphatic system. Since, in birds, there are no specialized lymphoid organs like lymph nodes (Jordan, 1939), hepatic lymphocytopoiesis in them has some apparent significance.

The present study centres mostly on the lymphocytopoietic activity in the pigeon liver.

This was necessary as very little is known about the significance of such activity of the liver and also about the nature of function of the lymphocytes produced in the liver.

Tremendous progress has been achieved in the study of lymphocytes and their myriads of activities. The function of lymphocytes in immunological reactions is a knowledge too prosaic these days. Other equally important functions have also been ascribed to this versatile wandering cells in the body. The alluring nomadism coupled with the nondescript ability to change into various other cell types have rightly earned the lymphocytes the name "Cindrella cells" (Yoffey, 1966). Various experiments and observations have paved the foundation for the contention that this blood cell has an unusual ability to transform into macrophage, fibroblast, mast cell, fat cell and even into stem cell from which other blood cells could develop. Like their multitude functions the sites of production of lymphocytes are also numerous in the vertebrates. There are specialized lymphoid tissues like, thymus, bursa of Fabricius, lymph nodes, and spleen where these cells are produced in large numbers. Apart from these sites, other organs and tissues also take up secondary function of lymphocyte production. Thus, in various

vertebrates, intestine, kidney and caeca are known to become lymphocytopoietic in adulthood or at different stages of development of the animal. Some of these tissues and organs that show lymphocytopoiesis in the young or embryonic stages surrender this function to more potent and permanent lymphoid tissues on attainment of maturity. In higher vertebrates like birds and mammals, lymphocytopoiesis during embryonic period has been observed in yolk sacs, kidney and liver. These structures ~~may~~<sup>are</sup> also known to produce other blood cell types. However, none of these structures continue to show such activities in adult life.

Nevertheless, as mentioned earlier, the occurrence of lymphocytopoietic nodules in the liver of adults of two species of birds, Rosy Pastor, Sturnus roseus (George and Naik, 1963) and pigeon, Columba livia (Pilo, 1970) stand distinct. So far, such informations about similar feature in the liver of other species of birds are not met-with in the literature. In the starling, Sturnus vulgaris, haematopoietic activity in the liver was observed even after hatching (Dantschakoff, 1908). In pigeon, hepatic lymphocytopoiesis is found in the adults and is not confined to any stage in the development (Pilo, 1970).

He has also mentioned that this lymphocytopoiesis in the liver is not due to any pathological conditions.

As it stands, the lymphocytopoietic nodules in the liver of the above mentioned species of birds are normally occurring structures and have some specific functions to perform or have some peculiar reasons to be present there. These nodules having only lymphocytes in them could be naturally considered as additional centres where lymphocytes are being continuously produced. However, like other lymphocytopoietic centres such as spleen, these nodules may also have other functions to perform. Since no available informations could be extrapolated to explain the questions like why and what for these nodules are there in the pigeon liver, it became pertinent to perform certain experiments so as to evaluate the significant functions of these lymphoid structures in the liver.

The functions of these nodules could only be investigated when the development and structural characteristics are fully known. More or less the functions may become almost a corollary fact when the morphology of these nodules is determined. The main question that required circumvention is whether these nodules are permanent structures in the liver of pigeon

or not. No direct approach could be made to solve this. However, one could easily investigate the fact that whether the lymphocytopoietic nodules in the liver of a large number of adult pigeons possess similar structural characteristics or not. If these nodules are permanent, then they should have uniform structural features.

Thus in the present study the first attempt was to make a survey of structural aspects of nodules in the liver of various individuals of the same species of pigeon (Columba livia) reared under laboratory conditions (Chapter 1). These studies revealed that nodules in the liver vary greatly in structure, not only in different individuals but in the same individual. These facts rendered rather puzzlement at first, but when the various types of nodules were studied and arranged in a sequential manner with the most complex ones at one end and simplest at the other it could be discerned that the difference in structure are due to peculiarities of the different stages of developing nodules. An account of various stages in the development of nodules in the pigeon liver is given in Chapter 1. From these studies it could be perceived that the nodules first develop

as an aggregation of lymphocytes in the sinusoidal spaces. Proliferation of lymphocytes then increases the mass of these aggregates, pushing aside the parenchymal hepatic cords or plates. Later, they acquire connective tissue covering as well as a 'germinal centre'. Liberation of lymphocytes from a nodule may eventually result in an altogether disappearance of the nodule from the liver. Thus it is suggested that a lymphocytopoietic nodule in the pigeon liver is good for production of lymphocytes once only and does not remain active for further production ~~when~~ once it has liberated the lymphocytes produced therein. <sup>6</sup>One the "spent" nodules disappear new ones may start developing either in the old space itself or somewhere else in the liver, thus maintaining almost a constant number of nodules per area in a given time and season.

Considering that the liver, in this bird, functions as a lymphoid tissue to a certain extent, it would be worthwhile to investigate whether the nodules in it have any similarity, either in development or in ~~the~~ function, with those of other usual lymphoid organ like spleen. In birds, spleen is considered as <sup>the</sup> only ~~a~~ lymphoid organ that produces lymphocytes all throughout ~~the~~ life. Thymus and bursa

of Fabricius do produce lymphocytes but they become involuted in adult life. Some structures like intestine may obtain lymphoid follicles (Payers patches) in adult stage. In pigeon, apart from the liver (Chapter 1) the intestinal caecae are also known to have lymphoid follicles (Marshall, 1960). There is no information about when the lymphocyte production appears in the liver or from where the lymphocytes or the blast cells arrive there. Probably the cells liberated from the primary lymphoid organs like thymus and bursa populate these organs where they may divide and form follicles or nodules. However, this contention needs proof and hence a preliminary study was carried out on the liver, caeca, spleen, thymus and bursa <sup>of</sup> Fabricius of developing pigeons to elucidate possible relationships of the time when lymphocytopoietic nodules appear in the liver, spleen and caeca with the time of involution of thymus and bursa (Chapter 2). Various parameters such as weights of thymus, bursa and spleen, nodular count in the liver and histological picture of caeca of growing pigeons were determined for such studies. In fowl, the lymphoid nodules develop in the caeca by 14th day after hatching (Calhoun, 1933), but in the caeca of pigeon the lymphocytes appear by 6th day after hatching. However, in the liver, nodules were

seen in 1 day old young ones of pigeon. Thereafter, throughout growth period of the young ones, increase in the number of nodules in the liver was directly proportional to the increase in the weight of spleen. The thymus continued to increase in weight up to one to two months and then its involution took place. However, thymus involution in pigeon is not irreversible. During, atleast, moulting period the thymus weight was found to increase. Bursa on the other hand involuted during the period of one to two months after hatching and thereafter did not show any weight increase during either moulting or breeding seasons. From these facts it is concluded<sup>d</sup> that the development of nodules in the liver has no apparent dependency on the thymus or bursa since lymphocytes found in the liver were from prehatching period itself. It is interesting, however, to note that weight of thymus and spleen and number of nodules all increased during moulting. Perhaps the lymphocytes are required to be produced in large number during this period. Thus, for this, basically the liver in the pigeon could function as a lymphocytopoietic centre.

If the nodules found in the pigeon liver are lymphocytopoietic in function, then, such activity should be shown by them in experimental conditions where

an increased demand of lymphocytes is artificially created. In Chapter 3 various experiments described were performed and from the facts a reasonable conclusion was derived regarding the functions of these nodules. Haemorrhage naturally produces a shortage of circulating lymphocytes. The bone marrow is not found to produce lymphocytes in pigeon as reported by Jordan (1939) and hence could not be expected to play any part in bringing the lymphocytes number in the blood back to normal. In experimental birds following sublethal hamorrhage the number of nodules in the liver as well as the weight of spleen increased between 6th and 10th day, pointing to the fact that lymphocyte production was mainly carried out by spleen and liver. Perhaps the liver must be having only a supplementary function to that of spleen in this respect. To prove this, another experiment was performed in which from a few pigeons, spleen was completely removed by operation while in a few others only partial splenectomy was done. When the splenectomy was complete the nodular number in the liver increased about four fold by 30 days while no such great increase was recorded in the nodular count in the liver of those pigeons subjected to only partial splenectomy. The reason for the later<sup>t</sup> observation was that the remaining part of the spleen quickly got regenerated and hence

liver was not put into stress for the purpose. These data support the contention that the liver nodules supplement the splenic activity. The splenic nodules also have phagocytic function apart from lymphocytopoiesis. To find an answer to the question: "Do the liver nodules also perform phagocytic activity?" a third experiment was conducted wherein the liver of some pigeons was subjected to considerable damage by injecting carbon tetrachloride ( $\text{CCl}_4$ ). It was found that following administration of  $\text{CCl}_4$  the number of nodules in the liver increased greatly; while spleen registered no such increase in its weight. This finding in its own way pointed to the fact that the nodules in the liver also have some role in phagocytosis.

Since it was established that during increased requirement of phagocytic activity in pigeons lymphocytopoietic nodules increased in large number in their liver, it became necessary to elucidate the possible manner in which these nodules or the cells produced in them participate in the phagocytic activity. A phagocytic cell should take up both foreign as well as cellular fragments or proteins originated within the body of the animal. Hence in one set of pigeons, haemolyzed blood previously drawn from healthy pigeons

was injected intravenously. So injected erythrocytes were detected in the nodules of the liver as well as in those of spleen as brown particles that showed the presence of iron when stained by Perl's Prussian Blue method. Liver as well as spleen nodules in control birds in which only saline was injected did not react with the stain nor did they show any debris in the lymphocytes of the nodules. The ability of ingesting 'self' cellular fragments (autochthonous particles) was shown by nodules when a part of the liver was mechanically injured (by applying high mechanical pressure). Besides, the presence of 'germinal centres' in many of the nodules loaded with cellular debris was suggestive of an antibody producing function for these nodules. In Chapter 4 these functions of lymphocytopoietic nodules in pigeon liver are discussed.

Thus all the basic functions of a lymphoid tissue viz., lymphocytopoiesis, phagocytosis and antibody production could be ascribed to the nodules found in the pigeon liver. However, like in spleen or lymph nodes of mammals, the nodules in pigeon liver are not of permanent existence in the liver. Once they liberate their contents (lymphocytes) they disappear altogether (Chapter 1). Perhaps from the same site new ones may develop. Such cyclic nature is not new for lymphoid

nodules in birds. Jordan (1939) has described similar cyclic nature of the nodules in the spleen of birds. Thus it means that the lymphocytes produced by the liver invariably find their way into the circulating blood through which they could reach various parts of the body for varied functions according to requirements.

Since lymphocytes produced in nodules in pigeon liver show phagocytic activity (Chapter 4) it is then reasonable to believe that these lymphocytes may aggregate in regions where demolition work is necessary, especially where extensive damages have occurred. In liver such focal damages are of frequent occurrence due to helminth parasitic infections. It was then decided to study such infected liver which could be ideal for determining the role of lymphocytes at the damaged areas. A large number of infected pigeon liver were collected and investigated with suitable histological techniques (Chapter 5). The results obtained were quite interesting. Not only a possible role of lymphocytes, that were found to aggregate around the necrotic or damaged part, in phagocytic reactions, but also their participation in the formation of a connective tissue covering around the injured or infected regions protecting the healthy areas was

discernible. Moreover, many nodules were found in close proximity with the connective tissue formed around the damaged part (Fig. 2, Chapter 5). Participation of lymphocytes in connective tissue formation is a known fact (Petrakis, 1961). Earlier it was also stated that each nodule in the liver, on maturing, forms connective tissue covering around it (Chapter 1). At this juncture a natural question that comes up is whether such connective tissue covering around the damaged part in the liver is restricted to pigeon or is of wide spread occurrence in other vertebrate groups. It is then imperative that a survey of damaged livers of other groups has to be made. Thus, frog's liver, infected with parasites were taken for examination. In frog's liver, though lymphocytes were seen around the damaged or infected parts but were not in such large number as noticed <sup>in</sup> the similar cases in pigeons. Massive formation of connective tissue around the damaged part in frog liver was not observed as was in pigeon liver also (Chapter 5). This was surprising enough, since frog (Rana tigrina) is one amongst a few other vertebrates which shows lymphocytopoietic nodules in its liver (Fig. 5, Chapter 5). Since these nodules, unlike those in pigeon, do not possess a connective tissue covering

around themselves, it was surmised that the lymphocytes produced in the liver of frog have no apparent ability to transform into fibroblasts or do not participate in the formation of connective tissue, while those produced in the nodules of the pigeon liver have greater capacity for such activities.

Being evident that lymphocytes from the nodules in the pigeon liver, retain the mesenchymal property to transform into fibrous collagen producing fibroblasts, these cells are in a position to play greater role in the process of wound healing in the visceral organs where (indigenous) fibroblasts or fibrocytes are usually absent. In the skin of vertebrates fibroblasts are plenty and hence the wound covering or granulation tissue is quickly formed from the existing fibroblasts. In the visceral organs, perhaps, there is a need for summoning fibroblasts to the wound site or damaged region from the other sites. However, there is no reason to believe that fibroblasts could be sent to the various parts of the body through circulating blood. In all probability the lymphocytes which possess the ability to get transformed into macrophages and/or fibroblasts when required are sent to such places where damage or injury have occurred.

In any attempts to obtain more information about the above mentioned function of lymphocytes,

it is necessary to cause damages in the internal organs. The liver itself is then a suitable material since the effect would be local and more intense. By inflicting injury to the liver two purposes could be served; firstly, to gather more data about the role of lymphocytes in the wound healing process and secondly, to have complete details about the mechanisms of wound healing and repair in the visceral organ.

The wound healing and repair in the pigeon liver was studied in detail following the infliction of a combined surgical and mechanical injury to a small part of the liver. A small part of the liver was removed surgically and the region subadjacent to the wound surface was subjected to high pressure. Histological features of wound healing and repair processes in the liver of pigeon ~~are~~ described in Chapter 6. It was interesting to note that wound healing in this organ was more or less depended on lymphocytes that aggregate at the wound site. These cells in course of time transformed into macrophages and also fibroblasts. As mentioned earlier, these cells ultimately form the connective tissue demarcating the irreversibly injured part (due to high pressure applied on this part) from the adjacent intact or uninjured one. Another interesting phenomenon noticed, was the local proliferation of hepatic cells

situated near the injured area which could be described as the repair process. Such proliferation occurred in spite of the fact that only a subthreshold portion of the liver was removed. Teir et al. (1967) could initiate hepatic cell proliferation by injecting disintegrated hepatic cells. Thus, the reason for the initiation of local proliferative activity was considered to be due to the presence of a large number of damaged cells in the irreversibly injured area of the organ.

The migration of lymphocytes to the injured area of the liver could be due to the release of some leucotactic substances at the site. Tsanev (1963) reported that it was the nucleic acid breakdown products that act as the leucotactic substance. A study of nucleic acids employing histochemical methods (Chapter 7), revealed that there was an apparent increase in the RNA content in the intact region adjacent the injured area soon after the infliction of injury. According to Tsanev (1963) this increase of RNA is due to increased synthesis of RNA to replace the broken down nucleic acids. An increase in DNA content was seen only later and that too just as the formation of the connective tissue band separating the injured and the intact areas was nearing completion. Thus, though there

are reports (Woessner and Roberts, 1961) that collagen synthesis is preceded by higher levels of DNA, the increased concentration of DNA seen here could not be correlated to the collagen synthesis. Since increased DNA content was noticed during the period when hepatic proliferation was observed, the increased DNA content could be due to newly formed hepatic cells. Since fibroblasts were involved in collagen synthesis, the hepatic cells near the wound site could not have any direct function in connective tissue formation apart from supplying certain essential metabolites or vitamins required for the collagen synthesis.

The most important vitamin necessary for the formation of connective tissue at the wound site is the ascorbic acid. According to recent evidences, ascorbic acid is very essential for the differentiation of lymphocytes into fibroblasts, as well as for the synthesis of collagen by the fibroblasts (Gould, 1963). A detailed study of ascorbic acid (Chapter 8) at the wound site in the liver showed that this vitamin was provided by the liver itself. Not only an accumulation of ascorbic acid was noticed in the hepatic cells near the wound site but a depletion of it from other parts was also evident. During the time of active collagen synthesis most of the lymphocytes and fibroblasts were loaded with ascorbic acid.

Vitamin C was also seen in the newly formed connective tissue preferably for maintaining the tensile strength of the connective tissue as suggested by Chen and Rostlethwait (1961).

Only after the formation of connective tissue or in other words only when the wound healing was complete the proliferative activity of hepatic cells adjoining the injured area began. Could there be any triggering mechanism ?. Nervous participation could be visualized since there are reports about nerves having direct influence on wound healing and repair (Thornton, 1970 & Hey, 1960). Histochemical demonstration of cholinesterases (an indirect method of showing nervous activity in tissues) was carried out in the present study (Chapter 9) which revealed that nerves are very much stimulated during the repair process in the liver. Perhaps, it was this nervous activity that triggered the hepatic cell proliferation at the injured area, in addition to the stimulation caused by the damaged cells present at the wound site.

Thus, it could be said that during wound healing and repair there are three major activities taking place at the injured region of the liver viz., (1) phagocytosis, of injured cells and other cellular components, (2) connective tissue formation and

(3) the hepatic cell proliferation. All these may simultaneously or separately cause many biochemical changes at the injured region in the liver. Amongst such biochemical processes, changes in the activities of various enzymes are of utmost importance.

The most important enzymes that were active during the wound healing and repair processes were acid and alkaline phosphatases. Histochemical studies of these two enzymes (Chapter 10) revealed that both acid and alkaline phosphatases became very active following the infliction of injury but not simultaneously. The first one that reached maximum intensity was acid phosphatase at a period when active phagocytic reaction was taking place at the wound site. Increased acid phosphatase activity was not only found in the intact hepatic cells that border the injured area but also in the lymphocytes and macrophages amassed near the injured area. Such increase in the enzyme activity was also reflected in the lymphocytopoietic nodules situated in the normal region of the liver of experimental birds. The alkaline phosphatase became active during the earlier phase of wound healing in the region where the connective tissue was actively being formed and hence could be easily correlated to the collagen synthesis as there are reports that this

enzyme plays an active rôle in the synthesis of the fibrous protein (Verzar and McDougall, 1936; Moog, 1946; Bradfield, 1950 and Danielli, 1954). Later the same enzyme became active in the region where proliferative activity was seen. Since there are much evidences that point to the part played by the alkaline phosphatase in the transport of metabolites, especially ~~the~~ glucose, it could be concluded that this enzyme might be aiding the metabolic activities of the cells that were under active division. A dividing cell falls on anaerobic metabolism rather than on aerobic one.

With a view to gather an insight into the metabolic activities taking place at the wound site in the liver, histological studies on fat, glycogen, lipase, esterase,  $\beta$ -hydroxy butyrate dehydrogenase, succinate dehydrogenase and lactate dehydrogenase were carried out (Chapter 11). It is quite evident that dividing cells did not utilize fat for energy purposes. On the contrary, fat, that would hinder the mitotic activity, was effectively removed by lymphocytes and macrophages. Thus there occurred a general reduction in the fat content in the parenchymal cells prior to cell division during the repair phase. During hepatic cell proliferation at the wound site (repair phase) glycogen got progressively depleted from the nearby liver lobules. It is reasoned

that the glycogen was utilized for energy purpose as evident from the observations that lactate dehydrogenase was highly active during same period when the proliferative activity was on and glycogen was being depleted.