CHAPTER 6

ROLE OF LYMPHOCYTES IN THE WOUND HEALING AND INITIATION OF HEPATIC CELL PROLIFERATION AT THE WOUND SITE IN THE PIGEON LIVER

Wound healing and repair of epithelial tissues, especially skin, are extensively studied phenomena. However, knowledge about such activities in visceral organs is scanty. Epithelial tissue like skin has inherent mechanisms by which wound healing and repair processes are immediately initiated when injured. The ease with which such response takes place is due to the fact that there are many cells which are actively dividing all throughout the life. The presence of fibroblasts and connective tissue also speed up the wound healing processes. Such pre-existing conditions could not be expected in most of the visceral organs which usually do not show cell division in adult stages. Inspite of the fact that liver cells are endowed with the power to divide and regenerate, when a part of it is removed or damaged, the proliferation of cells is seen either all over the remnant part or is not seen at all, depending upon the amount of liver that is removed (see Butcher, 1963; Leduc, 1964). However, there are reports of wound healing and repair that take place at the site of wound in the mammalian liver

(Cameron, et al., 1957). But, even this local proliferative activity is found to occur only under certain conditions. Hence an attempt is made in this study to elucidate the mechanisms of wound healing and repair that take place in the pigeon liver in response to mechanical injury and/or surgical woumds.

MATERIAL AND METHODS

Healthy adult pigeons (domestic variety of Blue Rock pigeons, Columba livia) reared under laboratory conditions with balanced diet, were chosen for the experiments. Birds of the same age group, weighing about 250 to 300 g, were selected.

Surgical injury was inflicted by removing a small part of the right lobe of the liver. Then, the region subjacent to the wound surface was subjected to a high pressure by the aid of two metal plates arranged like a pinch cock, thereby causing a mechanical injury. Only 5 to 10% of the liver was removed, which is much below the threshold value, so as to prevent extensive regeneration response all over the remaining mass of the liver. The blood clot formed at the wound surface was removed keeping only a thin layer. The abdominal body wall and skin were sutured separately with fine nylon

thread. Operations were carried out under ether anaesthesia and adequate post-operative care was taken to prevent any infections.

At regular intervals viz., 6, 8, 12, 24, 36, 48, 72, 96 hrs, and 6, 8, 10, 15, 20, 25, 30, 40 days, each time, three of the experimental (operated) birds were sacrificed by decapitation under mild anaesthesia. The liver was removed and weighed immediately and a portion of it with a part of the injured region was fixed in formol saline, while another similar part in Bouin's fluid, for the preparation of paraffin sections. The sections of the formol saline fixed part were stained with Jenner-Giemsa, while those of the Bouin's fixed part with haematoxylin-eosin.

The spleen was also removed and weighed. The nodular count per unit area in the liver was determined from the fresh liver frozen sections selecting regions at random.

RESULTS

When only 5 to 10% of the entire liver was removed, the wound healed by mere extension of the connective tissue that covers the organ (Glissons capsule) and no proliferation of the hepatic cells at the wound site

was detected. When only high pressure was applied on the edge of the liver, that part became necrotic and the injury was irreversible. Such an effect due to high pressure is common to all tissues in the body (Tsanev, 1963). When a piece of the liver was removed and high pressure on the area subjacent to the cut surface was simultaneously applied, the proliferation of the cells next to the (mechanically) injured area was found to take place.

In the liver, following injury, certain changes like acidophilia and the beginning of necrosis in the region between the scab and normal liver cords, where the pressure was applied, could be detected as early as 6 to 8 hrs (Fig. 1). By about 12 hrs, the lymphocytes made their appearence in the region between normal and irreversibly injured tissue (Fig. 2). These lymphocytes were similar in size and shape as that are seen in the lymphocytopoietic nodules present in the liver itself. It is reported that such nodules regularly occur in the liver of healthy adult pigeons (Pilo, 1967; Chapter 1). From these facts in view it could be presumed that the lymphocytes that appeared near the injured part of the liver might have migrated from the nodules.

At about 24 hrs after inflicting the injury, the region between normal and injured areas of the liver

mass became clearly distinguishable (Fig. 3). To this region lymphocytes were seen migrating (Fig. 3). This region (hereafter it is referred as "zone"), comprising of both injured and non-injured hepatic cells, later becomes the site of certain activities such as phagocytosis of injured cells and laying down of collagen fibres. By 36 hours the "zone" area became heavily filled with lymphocytes, macrophages and fibroblasts (Figs. 4 & 5). At this time many mature erythrocytes were also found in the "zone" which indicated that a good supply of blood was directed to this region. Certain cells with metachromatic granular inclusions also made their appearance in the zone by this period (36 hours) (Fig. 6).

The fibroblasts increased in number in the "zone" by the 2nd day. At this time most of the lymphocytes, metachromatic cells and other cells in the "zone" were found to be enmeshed in the ground substance (Fig. 7) which indicated the beginning of the laying down of collagen fibres in the area that would separate the injured region from the non-injured one. By this time a good number of metachromatic cells were seen in ruptured condition spilling out the granular inclusions in the area (Fig. 7). On the 3rd day, the number of macrophages and fibroblasts greatly increased and were found

interspersed in fibrous matter (Fig. 8). The formation of the fibrous collagen materials which had started as early as 2nd day continued even by the 4th day. The wound healing was nearly completed by about 6 days after the infliction of injury when the formation of a continuous and compact band of connective tissue was completed, separating the injured area from the intact one (Fig. 9). At this stage the metachromatic cells disappeared completely from the "zone".

While process of wound healing was being completed, the repair and regeneration set in, in the region adjacent to the newly formed wound covering. Proliferation of the hepatic parenchymal cells had begun at this stage i.e., by 6th day (Fig. 10). This proliferation of hepatic cells continued further for three to four days and by the 10th day, this activity reached the maximum level (Fig. 11). The new hepatocytes were round in shape with a thin layer of cytoplasm around their nuclei and were seen adhering to each other (Fig. 12). Newly forming bile ducts (Fig. 13) and a few lymphocytopoietic nodules in different stages of their development (Figs. 14 & 15) were also seen in the mass of the new hepatocytes. This gave full explanation for an apparant increase in the number of lymphocytopoietic nodules in the liver at this stage (Table I).

By about 15 days the band of collagenous fibres

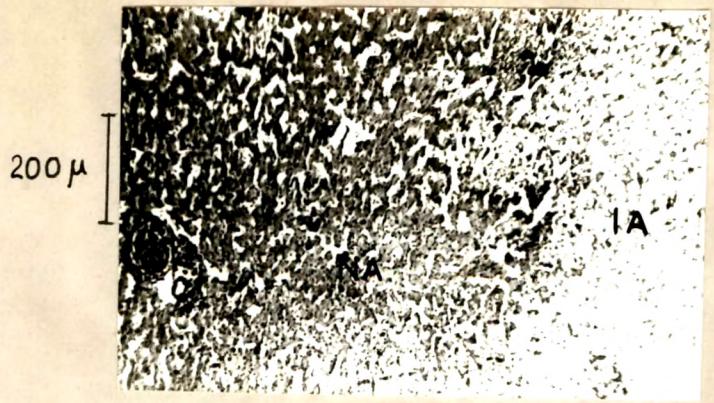
(Chapter 6: Figs. 1 to 4. Photomicrographs of the pigeon liver showing histological changes during wound healing and repair)

Fig. 1. 8 hrs after the infliction of the injury. Note the injured area (IA) on the right and normal intact area (NA) on the left.

Fig. 2. 12 hrs after the infliction of the injury. Note the appearance of lymphocytes in the hepatic sinusoids (arrow).

Fig. 3. 24 hrs after the infliction of the injury. Note the 'zone' (Z) that is distinguishable between the normal (N) and injured area (IA). Small groups of lymphocytes are seen migrating towards the 'zone' (arrows).

Fig. 4. 36 hrs after the infliction of the injury. The 'zone' (Z) is heavily filled with lymphocytes.



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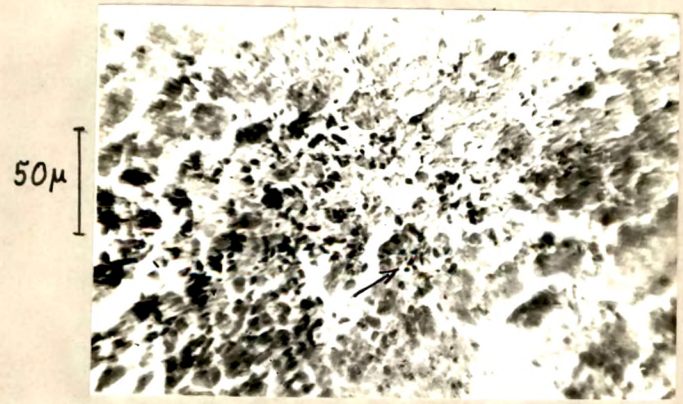

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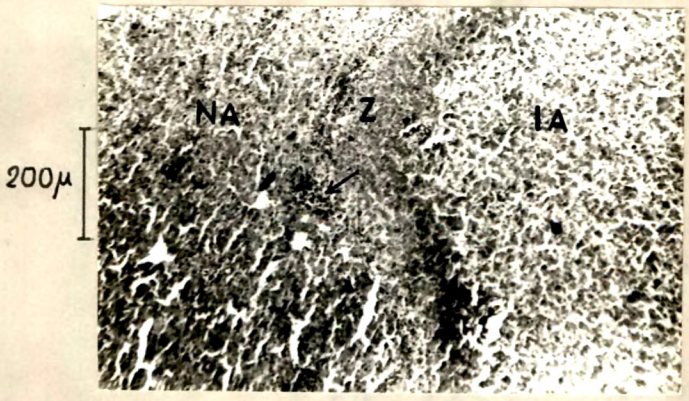

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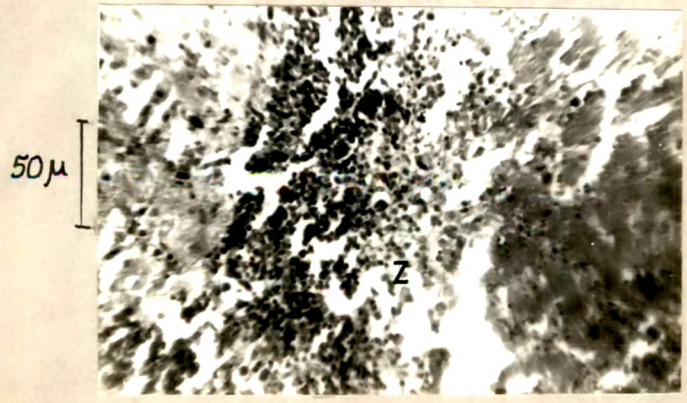

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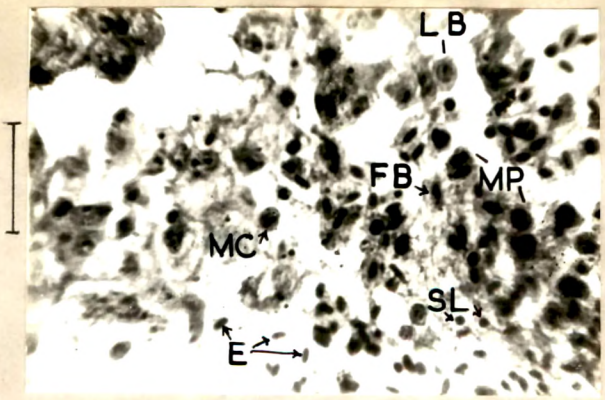
(Chapter 6: Figs. 5 to 8. Photomicrographs of the pigeon liver showing histological changes during wound healing and repair)

Fig. 5. 36 hrs after the infliction of the injury. Higher magnification of 'zone' where small lymphocytes (SL), lymphoblasts (LB), macrophages (MP), erythrocytes (E), metachromatic cells (MC) and fibroblasts (FB) are seen.

Fig. 6. 36 hrs after the infliction of the injury. Cells with metachromatic granules (arrow) are found in good number in the 'zone' (Z).

Fig. 7. 48 hrs after the infliction of the injury. The cells with metachromatic granules (MC) are found in large numbers. Some of them are seen in disrupted condition (arrows).

Fig. 8. 72 hrs after inflicting the injury. Most of the lymphocytes, macrophages and fibroblasts are seen interspersed with collagen fibres.



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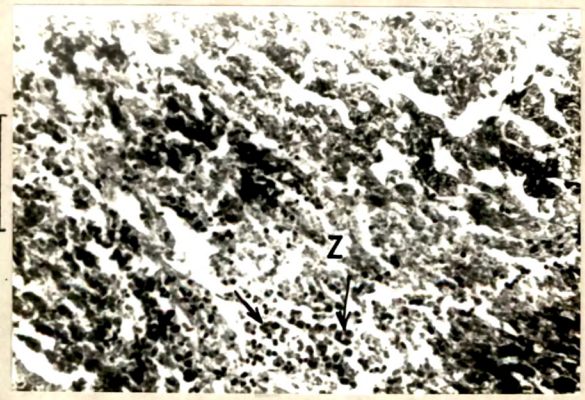

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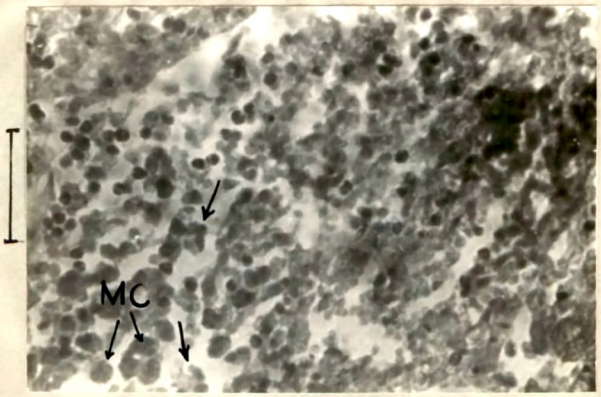

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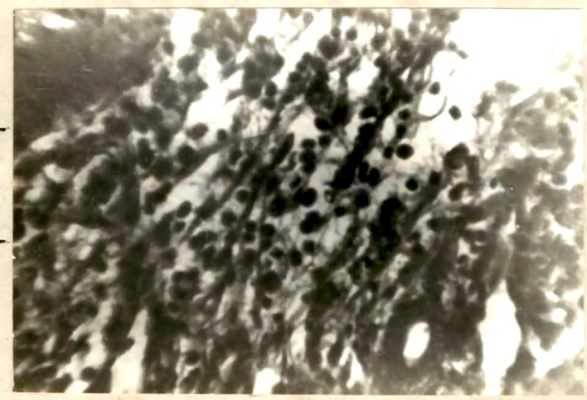

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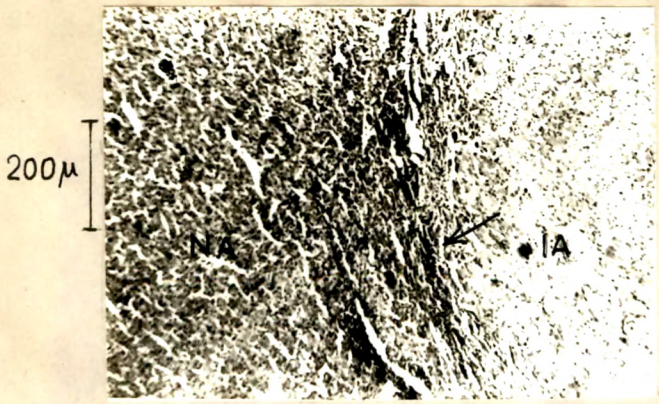
(Chapter 6: Figs. 9 to 12. Photomicrographs of the pigeon liver showing histological changes during wound healing and repair)

Fig. 9. 6 days after the infliction of the injury. The collagen fibres have formed into a band (arrow) separating normal (NA) and injured areas (IA).

Fig. 10. 6 days after the infliction of the injury. Note the proliferating hepatic cells (HC) adjoining the connective tissue band (CB) that separates the injured area (IA) from the non-injured area (NA).

Fig. 11. 10 days after the infliction of the injury. The connective tissue band (CB) has increased in thickness while the formation of new hepatic cells (HC) by the division nearby hepatic cells continues.

Fig. 12. 10 days after the infliction of the injury. The higher magnification of a part of Fig. 11 showing closely packed newly forming hepatocytes with nuclei and thin layer of cytoplasm.



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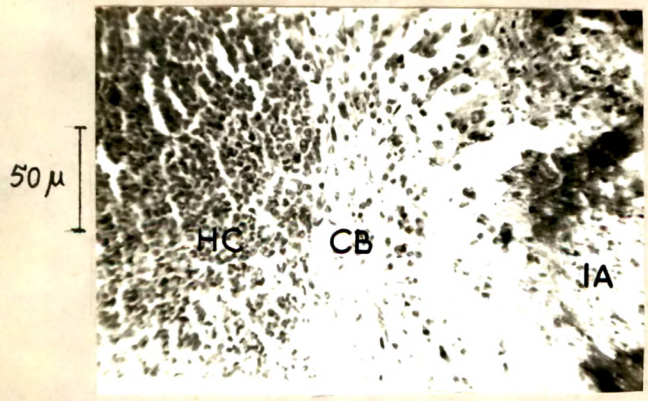

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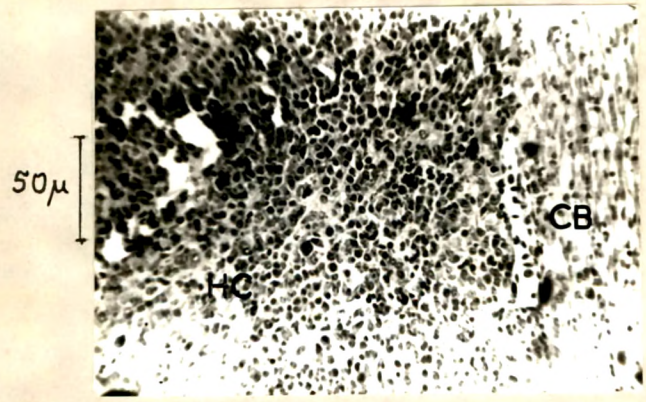

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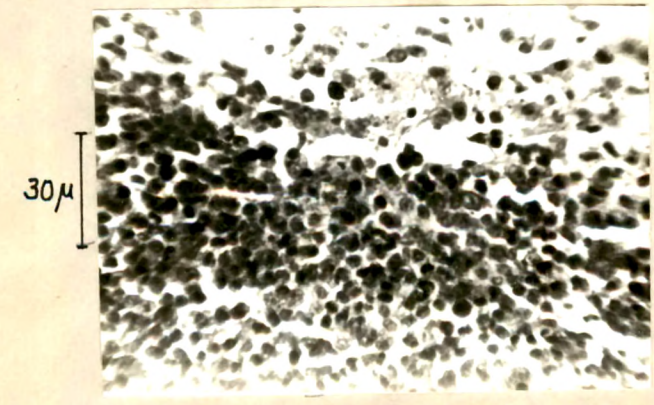

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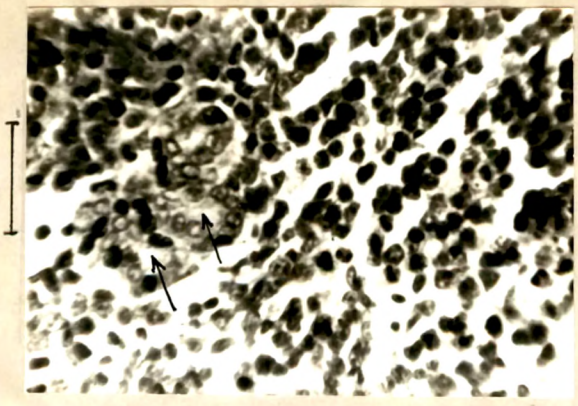
(Chapter 6: Figs. 13 to 16. Photomicrographs of the pigeon liver showing histological changes during wound healing and repair)

Fig. 13. 10 days after the infliction of the injury. Amongst the proliferating hepatic cells newly forming bile ducts (arrows) are clearly seen.

Fig. 14. 10 days after the infliction of the injury. A developing nodule among the dividing hepatic cells.

Fig. 15. 10 days after the infliction of the injury. The intact liver mass far away from the wound site also shows the presence of many developing lymphocytopoietic nodules (arrows).

Fig. 16. 15 days after the infliction of the injury. The completed connective tissue band that covers the wound surface.



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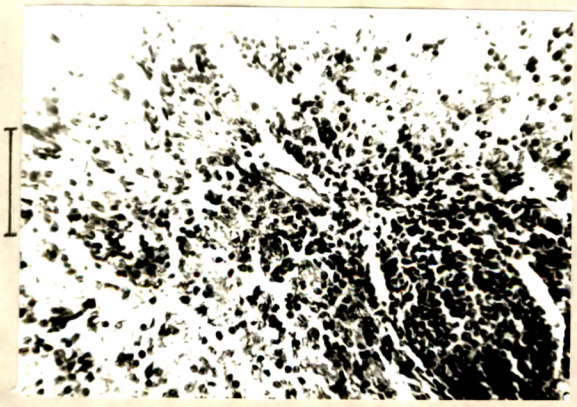

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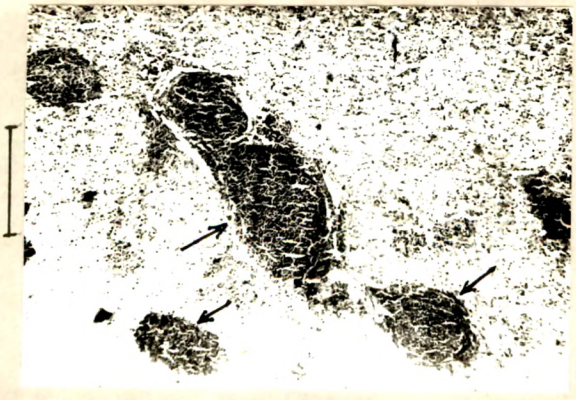

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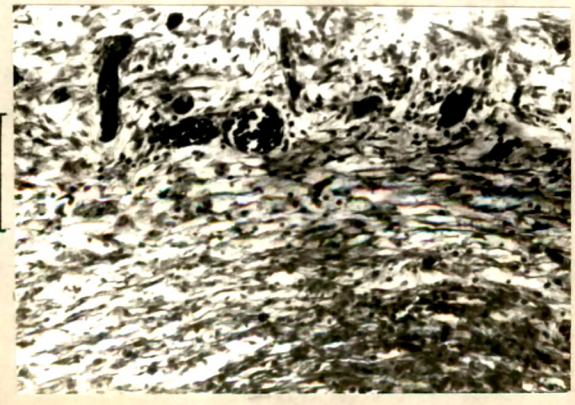

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TABLE I

The weight of the spleen and the nodular count in the liver of pigeon during the wound healing and repair after the infliction of the experimental injury

Time after the injury	Average spleen weight per 100 g body weight ± S.D.	Average nodular count in the liver ± S.D.
6 hrs	0.15 ± 0.02	6 ± 1
8 hrs	0.19 ± 0.02	6 ± 1
12 hrs	0.13 ± 0.05	8 ± 2
24 hrs	0.22 ± 0.03	8 ± 2
36 hrs	0.38 ± 0.06	8 ± 2
48 hrs	0.31 ± 0.04	8 ± 2
72 hrs	0.21 ± 0.06	8 ± 1
96 hrs	0.26 ± 0.08	8 ± 2
6 days	0.25 ± 0.06	9 ± 2
8 days	0.38 ± 0.05	9 ± 3
10 days	0.37 ± 0.06	20 ± 5
15 days	0.36 ± 0.08	21 ± 5
20 days	0.25 ± 0.04	8 ± 6
25 days	0.19 ± 0.03	9 ± 2
30 days	0.16 + 0.06	8 + 3

(connective tissue) increased in thickness (Fig. 16). The proliferative activity of the hepatic parenchyma, started earlier had ceased by this time and the necrotic area was off or absorbed up by this period. The newly formed hepatocytes, by 20th day, grouped themselves to form the hepatic cords, thus attaining the characteristic pattern of the normal liver. At this stage the number of lymphocytopoietic nodules decreased considerably (Table I) and compared to 20 to 21 per unit area noticed during ten to fifteen days after inflicting injury only about six to eight were present, a number which is comparable to the nodular count in the normal pigeon liver. The newly formed connective tissue band over the wound surface reached the characteristic nature comparable to that of capsule of Glisson that surrounds the entire liver mass. Thus 25th day after the infliction of the injury, the process of repair (proliferation of the hepatic cells) at the wound site reached culmination.

The weight of spleen showed variations during wound healing phase (Table I). The first peak of increase in weight was noted between 24 and 48 hrs after injury and the second peak between 8th and 15th day. The second peak of increased splenic weight corresponded with increased nodular count in the liver (Table I). Later during the repair phase the weight of spleen was similar to that in the normal bird.

DISCUSSION

Wound healing:

The migration of lymphocytes to the irreversibly injured or inflammated areas is a well known fact (Tsanev, 1963). The cordening off of the area that is damaged through the application of high pressure from that of the intact one by the aggregation of lymphocytes may be to ward off any bacterial infection that may gain entry through the injured cells. Since lymphocytes could develop into macrophages, the possibility of phagocytosis of injured cells could not be excluded. However, it appears that another important function of such aggregated lymphocytes during the repair and wound healing could be their part taking in the formation of fibroblasts.

There are number of experimental data from which a lymphocytic origin of fibroblasts could be inferred. From the study of lymphocyte culture, Maximow (1928) stated that after twelve to forty days, the cultures became closely resembling a connective tissue, with fibroblasts and macrophages dispersed in the matrix. Tuttle and Cannon (1935) also observed that in the areas of inflammation, macrophages developed from lymphocytes transformed into fibroblasts. Rebuck and Crowley (1955) and Riis (1959) also suggested such possibilities. Several workers using tissue

culture techniques of plasma clot type, had observed the transformation of lymphocytes through macrophages into fibroblasts and regarded that it is the hypertrophied lymphocytes that became macrophages (Awrorowa and Timofjewski, 1914; Bloom, 1927; Maximow, 1927; Maximow, 1928, Berman, 1942; Algower, 1956).

During wound healing process in the pigeon liver too, such a massive aggregation of lymphocytes was observed. In the early stages (10 to 12 hr), only the lymphocytes could be detected in the "zone" that separated the intact region from the injured one. But later macrophages and shortly afterwards fibroblasts too appeared. The presence of lymphocytes and the delayed appearance of macrophages and fibroblasts could be suggestive of the origin of the latter ones from the former. Such transformations are possible as the lymphocytes are endowed with certain embryonal characteristics. Petrakis et al. (1961) suggested that the "mesenchymal" capacity of mononuclear leucocytes (lymphocytes and/or monocytes) enable them to differentiate into macrophages and collagen forming fibroblasts.

Just prior to the formation of collagen fibres (36 hours) in the "zone" demarcating the injured area from the intact one in the pigeon liver, large number of cells with metachromatic granules made their appearance.

The metachromasia of these cells could be due to the presence of mucopolysaccharides. This is in agreement with the observation that mucopolysaccharide content increases during healing of wounds (Bunting and White, 1949; Bradfield and Kodicek, 1951; Persson, 1953; Kimoto et al., 1959). It is well known that metachromatic materials (presumably sulphated mucopolysaccharide) accumulate during wound healing processes shortly after fibroblastic proliferation but prior to collagen fibre formation. Such accumulation continues for a few days and rapidly disappears with the onset of the collagen fibre formation (Gould, 1963). In the pigeom liver too, the metachromatic cells that made their appearance by 36 hours, disappeared by about 6th day, a period characterised by the active laying down of collagen fibres. Though, the significance of cells containing mucopolysaccharides could be easily discernible, information regarding the nature and origin of these cells remains obscure. Since, many of them were found in disrupted conditions, a sort of trephocytic action could be ascribed to them which is definitely a characteristic activity of lymphocytes. Agreeing with the observation that lymphocytes taking part in the inflammatory reactions develop UDPG-glycogen transglucosylase, an enzyme concerned with the production of polysaccharides,

which is usually absent in the normal circulating lymphocytes (Wulff, 1963), it could be suggested that the metachromatic cells might have been formed from the lymphocytes.

Repair (regenerative process) at the wound site:

In pigeon, the onset of proliferation of hepatic parenchymal cells /subadjacent to "zone" took place by about 6th day and continued further for several days. Kellner and Sugar (1963) observed hyperplasia on the 10th day near the wound that was experimentally inflicated on the epithelial surface of the rat skin. Thus, the lag period, before the proliferative activity starts at any wound site during which time wound healing processes nears completion, could be suggested to last about 8 $\pm$ 2 days. Since, in the pigeon liver the mitotic activity began just after the laying down of the collagen fibres, to be more precise, after the formation of the connective tissue covering over the wound surface, it could be inferred that the triggering off of the proliferative mechanisms in the adjoining healthy hepatic cells would only take place when the wound covering is formed.

It is generally accepted that in an injured tissue or organ the presence of disintegrating cells also

initiate proliferation of adjacent healthy cells (Kellner and Sugar, 1963). Lehtiharju (1961) and Teir et al. (1967) noted mitotic activities in the post-hepatectomy liver when homogenized and autolysed part of the liver was injected. Hence, the presence of disintegrating cells at the wound site in the pigeon liver due to injury caused by high pressure, might have also influenced the division of the healthy hepatic cells adjacent to the injured ones. Such a contention could be further supported by the fact that when the removal of a piece of liver was not coupled with the application of high mechanical pressure to produce irreversible injury, no proliferation of the parenchymal cells occured as excision alone may not be providing sufficient disintegrating cells at the wound surface.

The absence of mitotic activity when only a part of liver was removed (below threshold value) could be explained by taking into consideration the chalone theory of Bullough (1966), which assumes the production of some substances (Chalones) by the cells, that act in a negative feed back mechanism to suppress selectively the mitotic processes. Thus, removal of a small part of the liver may not be reducing the concentration of chalones below the critical level. The present observation is that the disintegrating cells at the site of wound

stimulate the proliferation of adjacent normal cells, inspite of the fact that only a very small part of the liver was removed. Here the possible mechanism may be that the damaged cells liberate certain substances which either bind up the chalone produced by the adjacent normal cells or inhibit the continuous production of it. In this case, the tissue breakdown products or autolysates (Teir et al., 1967) or the nucleic acid breakdown products (Tsanev, 1963) could play a vital role in the initiation of the cell division by preventing the chalones from exerting their inhibitory effect on mitosis.

Lymphocytopoiesis during wound healing:

It is interesting to note that the lymphocytes that play an important role in the wound healing in the liver could be provided by the organ itself. The pigeon liver is found to have large number of lymphocytopoietic nodules (Pilo, 1970; Chapter 1). As suggested earlier (in Chapter 1) ~~that~~ these nodules assist in the phagocytic reactions. However, it is puzzling to note that the increased nodular count in the liver was observed only by about 10 to 15 days after the infliction of the injury and not at a time when there was a massive accumulation of lymphocytes at the wound site (36 hrs. after injury). The spleen, however, showed two peaks of increased weight

differences, one between 24 and 48 hrs. and the other between 8 to 15 days (Table I). The second peak (between 8 to 15 days after inflicting the liver injury) undoubtedly coincides with the increase of nodular count in the liver during this period. The increase of splenic weight between 24 and 48 hrs could be due to something other than lymphocyte production, probably erythrocyte production. It is reasonable to suggest that the migration of lymphocytes to the wound site in the liver might be taking place from the existing nodules and their replenishment may be taking place after 8 to 10 days.

In conclusion it could be said that for wound healing, the liver may have to depend on lymphocytes and other cells especially when the wound site is having a large amount of damaged cells sandwitched between the cut surface and the intact cells. Presence of the irreversibly injured cells prevents the connective tissue covering (Glissons capsule) from extending over the wound. The wound healing process starts with the cordening off of the damaged area by the aggregated lymphocytes some of which later may give rise to fibroblasts. The first wound healing reaction is the laying down of collagen fibres. Such reaction would also takes place when the localized necrosis occurs due to toxic substances or parasites. Laying down of collagen fibres is accompanied

by phagocytosis of damaged cells and is followed by proliferation of intact parenchymal cells adjacently present. The hepatic cell proliferation undoubtedly is triggered off by the presence of damaged cells either directly influencing the division of intact cells or by the inhibition of chalone production or binding of the chalones already present in them.