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Proliferation of Mononuclear Phagocytes (Kupffer Cells) and Endothelial Cells in Regenerating Rat Liver

A Light and Electron Microscopic Cytochemical Study

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The proliferation of littoral cells in regenerating rat liver after partial hepatectomy has been investigated using endogenous peroxidase and uptake of large (0.8-μ) latex particles as markers of Kupffer cells. Female rats were subjected to 2/3 partial hepatectomy and sacrificed at intervals up to 11 days. Prior to sacrifice, animals were injected with latex and their livers were fixed by perfusion and were processed for cytochemical localization of peroxidase. The sinusoidal cells exhibited a marked regenerative response after partial hepatectomy. Peroxidase activity persisted in endoplasmic reticulum of Kupffer cells during mitosis. Furthermore, latex particles were exclusively localized in such peroxidase-positive cells, thus confirming their identity as Kupffer cells. Quantitative counts revealed that the peak mitotic activity of Kupffer cells occurred at 48 hours, whereas that of endothelial cells was at 96 hours after partial hepatectomy. Our findings indicate that Kupffer cells are capable of dividing locally in the liver; no morphologic evidence of transformation of endothelial cells or monocytes to Kupffer cells was found. The significance of these observations concerning the origin of Kupffer cells is discussed, and it is concluded that in the model of liver regeneration after partial hepatectomy the Kupffer cells are formed predominantly by local cell division. (Am J Pathol 80:349–366, 1975)

RECENT FINE STRUCTURAL and cytochemical studies from this and other laboratories have established that rat hepatic sinusoids are lined by two distinct cell types: the wall-forming endothelial cells and the

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phagocytic Kupffer cells.[1-4] The Kupffer cells, which are an important part of the mononuclear phagocyte system,[5] are distinguished from endothelial cells on the basis of a) endogenous peroxidase activity in the endoplasmic reticulum [6] and b) their exclusive phagocytosis of large (0.8-μ) latex particles.

The origin of Kupffer cells remains controversial. Although the derivation of tissue macrophages from blood monocytes and precursor cells in bone marrow seems to be firmly established,[7-10] there is also some evidence suggesting that Kupffer cells are capable of dividing locally.[11-14] Thus, Kelly *et al.*[11] and Kelly and Dobson [13] have shown that the stimulation of the reticuloendothelial system with estradiol is associated with proliferation of phagocytic cells in liver sinusoids, and North [12] has demonstrated that mouse liver macrophages divide actively in the course of development of immunity to infection with *Listeria monocytogenes*. More recently, Warr and Sljivic [14] noted that some agents (such as stilbestrol, endotoxin, and zymosan) induce an influx of new cells into the liver sinusoids, whereas other agents (such as zymosan and *Corynebacterium parvum*) in addition induce the proliferation of the preexisting Kupffer cells. Finally, the possibility has also been raised that the endothelial cells may transform directly into phagocytic Kupffer cells.[15,16]

The present study was undertaken to investigate the mitotic potential and possible origin of Kupffer cells in the model of liver regeneration following partial hepatectomy using the combined peroxidase reaction and the phagocytosis of large (0.8-μ) latex particles as markers for Kupffer cells. The results indicate that Kupffer cells, which retain their peroxidase activity during the mitotic division, are capable of dividing locally. No morphologic evidence of transformation of endothelial cells or monocytes to Kupffer cells was found in this model.

Materials and Methods

Adult female albino rats (Charles River strain) weighing 300 to 350 g and kept on a regular laboratory diet and given water *ad libitum* were used. Animals were subjected to the standard 2/3 partial hepatectomy procedure of Higgins and Anderson [17] and were sacrificed between 1 and 11 days after surgery, using 4 rats for each time interval. Thirteen hours prior to sacrifice, each animal received an intravenous injection of .05 ml/100 g body weight of 0.81-μ latex particles [1] (Difco Laboratories, Detroit, Mich.), followed 1 hour later by 1 mg of vinblastine (Velban, Eli Lilly Company, Indianapolis, Ind.) to arrest the dividing cells in mitosis.[12] Animals were starved for 12 hours prior to sacrifice. Control animals included normal and sham-operated rats. These were injected with latex and vinblastine, and the sham-operated animals were examined on the second and the fourth day after surgery. The partial hepatectomies as well as the animal sacrifices were performed between 9 and 11 AM to prevent the influence of diurnal variations on the regenerative response.[18]

Livers were fixed by perfusion through the portal vein [19,20] with 2.5% purified glutaraldehyde [21] in 0.1 M cacodylate buffer, pH 7.4. Pieces of tissue from two different areas of each of the remaining lobes were sampled and processed for cytochemical localization of endogenous peroxidase activity in Kupffer cells using a modified Graham and Karnovsky technique,[22] as described previously.[1,6] This was followed by postfixation in 2% aqueous osmium tetroxide, dehydration in graded ethanol solutions, and embedding in Epon 812 (Luft).[23]

From each animal, ten to fifteen 1-μ-thick sections stained with 1% toluidine blue were examined by light microscopy, and dividing sinusoidal cells with and without peroxidase activity were localized. The corresponding tissue blocks were trimmed and sectioned for electron microscopy on an LKB Ultramicrotome (LKB Instruments, Rockville, Md.) with a diamond knife. Sections were examined in a Philips EM 200 electron microscope after staining with lead citrate.[24]

Cell Counts

Because of the zonal variation in the regenerative response in different parts of the liver lobule,[25-28] only periportal areas were considered for cell counts. For each time interval, 4 animals were used, and from each animal an average of 40 circular fields, bordering directly on portal tracts, were examined by light microscopy at 1000 times magnification using an oil immersion lens. For this purpose, ten to fifteen 1-μ-thick Epon sections originating from two different liver lobes of each animal were used, and 1500 to 2000 sinusoidal lining cells, all with a visible nucleus (or chromatin clumps for dividing cells), were counted. In nonoperated and sham-operated controls, the mitotic figures were counted in 120 to 140 high-power (1000×) fields obtained from two separate lobes of 2 animals for each time interval.

Results

Light Microscopy

The sinusoids of liver after the perfusion fixation appeared patent and were free of circulating blood cells. Two distinct cell types lined the open sinusoids: a) the elongated, wall-forming endothelial cells, which were peroxidase negative, and b) the more rounded phagocytic Kupffer cells, which exhibited a positive-peroxidase reaction (Figure 1). The cytochemical reaction product appeared as a diffuse, homogenous, brown precipitate over the entire cytoplasm of Kupffer cells, sparing only the nucleus. In addition, most Kupffer cells contained numerous peroxidase-positive vacuoles in their cytoplasm (Figure 1).

Mitotic figures were extremely rare in nonoperated rats, but in partially hepatectomized animals, especially at 48 to 96 hours after surgery, both peroxidase-positive and peroxidase-negative cells were seen in mitosis (Figure 2). Such dividing cells appeared rounded, and some of them protruded into the sinusoidal lumen (Figures 2–4). The chromatin clumps were easily identified after the staining of sections with toluidine blue. Whereas the dividing endothelial cells had a distinctly clear and almost transparent cytoplasm, devoid of any inclusions (Figures 2 and 3), the dividing Kupffer cells appeared uniformly brown and contained numerous

inclusions (Figure 4). At higher magnification, ingested latex particles could be identified in the cytoplasm of some Kupffer cells (Figure 4).

Electron Microscopy

The nondividing sinus lining cells maintained their fine structural and cytochemical characteristics as described previously.[1] Thus, Kupffer cells, which exhibited peroxidase reaction product in the endoplasmic reticulum and the nuclear envelope, had an irregular, somewhat ruffled, luminal plasma membrane with numerous cytoplasmic vacuoles containing ingested latex particles (Figure 5). The endothelial cells, in contrast, were peroxidase negative, and each had an elongated shape with a fairly straight luminal plasma membrane with focal fenestrations; the cells contained many micropinocytosis vesicles and a few larger cytoplasmic vacuoles (Figure 5). The large (0.8-μ) latex particles were seen exclusively in the phagolysosomes of the peroxidase-positive Kupffer cells—never in the peroxidase-negative endothelial cells.

In dividing cells, the nuclear envelope was absent, and aggregates of chromatin were dispersed in the cytoplasm (Figures 6–9). The endoplasmic reticulum appeared as short dilated segments embedded in cytoplasm which contained many free ribosomes. Figure 6 shows two Kupffer cells next to each other, one in interphase with a distinct peroxidase-positive nuclear envelope and the other, in mitosis, containing chromatin clumps and short dilated segments of endoplasmic reticulum surrounded by a ribosome-rich cytoplasm. In addition, ingested latex particles are noted in a phagolysosome of this dividing Kupffer cell. Figure 7 shows, at higher magnification, the contrast between the normal endoplasmic reticulum of a nondividing and the slightly dilated endoplasmic reticulum of a dividing Kupffer cell. Because of this dilatation of the endoplasmic reticulum, the peroxidase reaction product in dividing cells appeared somewhat granular, whereas in nondividing cells it was more homogenous (Figures 6 and 7).

The dividing endothelial cells were recognized by their location and their ultrastructural resemblance to nondividing endothelial cells. These cells were consistently peroxidase-negative and never contained any ingested latex particles. In Figure 8, a peroxidase-positive Kupffer cell containing numerous phagolysosomes is seen next to a dividing endothelial cell with a chromatin aggregate in the cytoplasm. Such endothelial cells in mitosis contained many small mitochondria and several small cytoplasmic vacuoles surrounded by a ribosome-rich cytoplasm (Figure 9). In all sections examined, no cells with a partially positive peroxidase reaction in the endoplasmic reticulum were found. A conspicuous absence of

microtubules and filaments was noted in dividing and nondividing Kupffer cells. Even though autophagic vacuoles were frequently observed within parenchymal cells, they were seen only rarely in sinusoidal cells.

Counts of Mitotic Figures of Various Cell Types After Partial Hepatectomy

Mitotic figures were extremely rare in nonoperated control animals which received injections of latex and vinblastine (Table 1). However, a dramatic rise in mitotic activity of sinus lining cells was observed at 2 to 4 days after hepatectomy. Text-figure 1 shows the results of cell counts of peroxidase-positive and peroxidase-negative sinusoidal cells, expressed as percentage of cells of each category in mitosis. After an early silent period during the first day, there was a sharp rise in mitotic activity of Kupffer cells, which reached its maximum of 19% at 48 hours and returned to normal levels at about 8 to 11 days after surgery. In contrast, the endothelial cells exhibited a somewhat delayed and slower regenerative response, reaching their peak mitotic activity of 11% at about the fourth day after partial hepatectomy and returning to normal at the same time as the Kupffer cells. In sham-operated animals, there was also a short burst of marked mitotic activity of both endothelial and Kupffer cells at 72 hours which returned to normal levels at 96 hours after surgery (Table 1).

Discussion

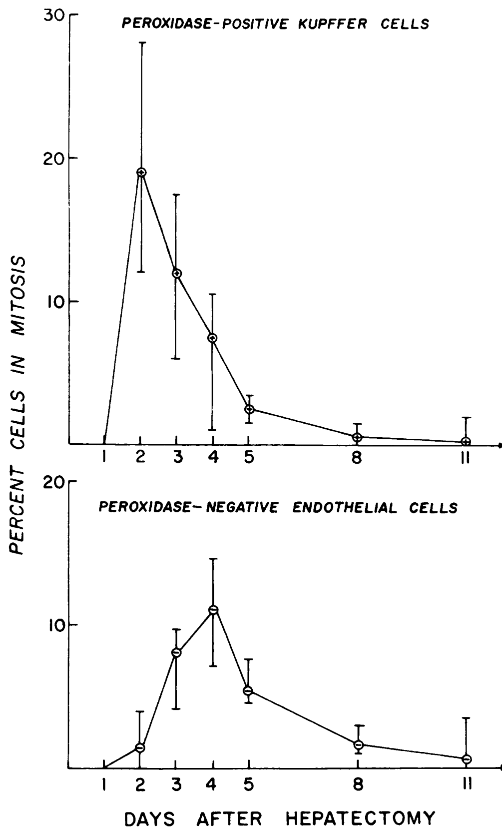
The observations reported herein indicate that the cytochemical and functional characteristics of endothelial cells and Kupffer cells during interphase are also maintained in dividing cells. The exclusive localization of large (0.8-μ) latex particles in peroxidase-positive dividing cells, which also share some morphologic features with the normal Kupffer cells, indicates that these are indeed dividing Kupffer cells. Since the peroxidase

Table 1—Proliferation of Sinus Lining Cells in Nonoperated and Sham-Operated Control Animals

	Mitotic index ± SD*			
		Sham-operated controls†		
	Nonoperated controls†	Day 2	Day 3	Day 4
Kupffer cells	0.5 ± 0.3	2 ± 1.5	51 ± 7	3 ± 1.5
Endothelial cells	1.3 ± 0.3	3 ± 2	13 ± 5	2.2 ± 1.5

* Mitotic index is the number of mitoses per 100 fields. The number of mitoses were counted in 120 to 140 high-power fields (1000 times magnification) for each interval, and 2 animals were examined for each time interval.

† All animals received 1 mg of vinblastine 12 hours prior to sacrifie to arrest the dividing cells in mitosis.



TEXT-FIGURE 1—Results of mitotic counts of peroxidase-positive Kupffer cells *(upper curve)* and peroxidase-negative endothelial cells *(lower curve)*. For each time interval, 4 animals were used, and 1500 to 2000 littoral cells were counted. The results are expressed as percentage of cells of each category in mitosis. The circles on each curve are average values and the bars demonstrate the range of individual variations. Note the sharp rise in mitotic activity of Kupffer cells after the first day with a peak at 48 hours and the slow increase in mitotic activity of endothelial cells with a peak at 4 days after surgery. Both cell types return to normal at about 11 days.

reaction product is also visible by light microscopy (Figures 1–4), it was used as a marker of Kupffer cells in quantitating the mitotic activity of the various sinus lining cells. Thus, it was revealed that Kupffer cells reach their peak mitotic activity at 48 hours and endothelial cells at 96 hours after partial hepatectomy (Text-figure 1).

Littoral Cells in Regenerating Liver

In all previous studies on liver regeneration, the littoral cells have been lumped together without any distinction between the endothelial cells and Kupffer cells.[18,25-28] These studies have shown that the peak mitotic activity of littoral cells is reached at about the end of the second day. Our results compare favorably with these observations, since the combined mitotic activity of endothelial cells and Kupffer cells in our material would also give a curve with a flat peak at about 48 to 72 hours after surgery. The slight delay in the regenerative response of our animals is probably related to the withdrawal of food for 12 hours prior to sacrifice and the older age

of our rats (300 to 350 g); the effects of nutrition and age upon the liver regeneration have been well established.[18] Our animals were fasted to reduce the content of hepatic glycogen, which interferes with the proper tissue processing in peroxidase cytochemistry.[6,20] The larger sized rats were preferred because their portal veins could be more easily cannulated for perfusion fixation. Furthermore, the delayed regeneration in our animals could be due to methodologic differences—in most previous studies, tritiated thymidine labeling has been used [25-28] and it is known that the peak incidence of the mitotic rate lags about 6 to 8 hours behind the corresponding point in the curve of labeled nuclei.[26]

Grisham mentioned the low incidence of mitosis in littoral cells of regenerating rat liver.[26] This problem was also noted by us in the early stages of this study, and we therefore used a low concentration of vinblastine to increase the mitotic yield.[12] This treatment was probably responsible for the conspicuous absence of microtubules in our material, especially in dividing cells. Vinblastine is known to bind to tubulin [29,30] and cause the disappearance of microtubules in a variety of cell types.[30,31] Recently Arstila *et al.*[32] described the formation of autophagic vacuoles in liver parenchymal cells of rats treated with higher doses of vinblastine. We also noted numerous autophagic vacuoles in hepatocytes but no significant increase in the autophagic activity of sinus lining cells. Similar findings were reported recently by Wisse in partially hepatectomized rats treated with colchicine.[33] In addition, Wisse mentioned that, following colchicine treatment, the dividing sinus lining cells appeared rounded and contained swollen rough endoplasmic reticulum cisternae. He admitted that these ultrastructural changes seriously hampered the identification of the various cell types by simple ultrastructual criteria.[33] His observations reemphasize the importance of the cytochemical peroxidase reaction for the identification of the various cell types in rat liver sinusoids and point out the shortcomings of ultrastructural criteria alone. The rounded appearance of the dividing cells and their projection into the lumen of the sinusoids, however, are not necessarily related to the treatment with antimitotic drugs, since similar changes have also been described in untreated animals.[34]

Partial hepatectomy is associated with a marked increase in the phagocytic function of the liver.[35,36] According to Leong *et al.*,[36] the uptake of radiolabeled chromic phosphate is increased to values 200% above controls within 48 hours after hepatectomy and remains elevated for at least 90 to 100 days postoperatively. Since the regeneration of sinusoidal cells is completed at about 11 days after surgery, it must be concluded that the increased phagocytic function is due not only to a numerical increase

of Kupffer cells but also to "activation" of existing cells. The only feature which could be attributed to such an "activation" in our study was the presence of large phagolysosomes containing damaged blood cells and debris. It should be emphasized, however, that in spite of enhanced phagocytic activity, the peroxidase-negative endothelial cells never showed evidence of uptake of large (0.8-μ) latex particles. This, plus the fact that no intermediate cell types were found and that endothelial cells and Kupffer cells had two distinct and separate peaks of mitotic activity (Text-figure 1), all argue against the transformation of endothelial cells to Kupffer cells in normal as well as in stimulated animals.

Origin of Kupffer Cells

Because of basic differences in phagocytic and other functional properties of tissue macrophages and vascular endothelial cells, it has been proposed to abandon the term *reticuloendothelial system* and to replace it by the new term *mononuclear phagocyte system*.[5] The various members of this system share certain functional properties, such as sticking to glass and avid phagocytosis. Kinetic studies indicate that tissue macrophages are derived from precursor cells in bone marrow called the promonocytes via the peripheral blood monocytes.[8] Whereas the promonocyte is a potent, rapidly dividing cell, the monocyte and the tissue macrophage are believed to be mature end cells that are unable to divide.[37-39]

Mitotic figures are extremely rare in hepatic sinusoids of normal animals,[15] but estrogen,[11,13] endotoxin,[40] infection with *Listeria monocytogenes*,[12] injection of particles,[14,41] and partial hepatectomy [25-28] all have been associated with a marked rise in the mitotic activity of sinusoidal cells. In most previous studies, however, the dividing Kupffer cells were either lumped together with endothelial cells or were distinguished from the latter by means of labeling with small particles such as carbon.[12] As we have shown before, however, small particles are also taken up by endothelial cells and are, therefore, unreliable.[1] The present study is the first in which two separate methods of identification of Kupffer cells, peroxidase activity and phagocytosis of large latex particles, have been combined for identification of Kupffer cells, and it shows that indeed these cells are capable of division *in situ*. To reconcile our observations with those of van Furth and others [8,10,37-39] on the monocytic derivation and the end cell nature of Kupffer cells, three possibilities have to be considered.

The first hypothesis, which has been shared by most investigators who have observed evidence of mitosis in various types of tissue macrophages, is the following: Although Kupffer cells under normal steady-state condi-

tions do not divide, they may resume their mitotic activity after proper stimulation.[9,10,12] The validity of this theory, however, has been questioned by van Furth *et al.*,[39] who commented recently that the resumption of mitotic activity of macrophages already present in the tissues remains to be confirmed.

A second possibility, suggested by van Furth,[39] is that the dividing cells in liver sinusoids are promonocytes which, under the influence of specific stimuli, move into the tissues and multiply. Indeed, promonocytes in some mammalian species exhibit a positive peroxidase reaction in endoplasmic reticulum similar to rat Kupffer cells.[42] However, in these cells, peroxidase activity is also strongly positive in all the Golgi saccules and granules.[42] In contrast, the reaction in the Golgi apparatus of Kupffer cells is very scanty, being confined to a single saccule,[6] and the nature of the peroxidase staining in granules is controversial.[3]

In addition, the phagocytic function is less pronounced in promonocytes than in mature macrophages, and large lysosomes and dense bodies are rare in promonocytes.[43,44] The dividing peroxidase-positive cells in our preparations, on the other hand, contained large phagolysosomes and exhibited latex phagocytosis. Although species differences exist in the localization of peroxidase in mononuclear phagocytes,[45] the cited cytochemical and ultrastructural differences argue against the promonocytic nature of peroxidase-positive cells in rat liver.

A third possibility is that Kupffer cells belong to a separate line of macrophages which can multiply *in situ* independent from peripheral blood monocytes and their bone marrow precursors. Observations supporting this hypothesis are: a) the marked difference in the localization of peroxidase in Kupffer cells and mature blood monocytes; the monocytes contain only peroxidase-positive granules, but no enzyme, in the endoplasmic reticulum.[42,45] b) the recent observations of Cotran and coworkers from this laboratory[46,47] and those of Daems and collaborators,[48,49] favor the existence of at least two different lines of macrophages. Based on the peroxidase reactivity of cells harvested from the peritoneal cavity, these authors[49] have identified two separate populations of macrophages: the "resident macrophages" obtained from unstimulated animals exhibiting peroxidase activity in endoplasmic reticulum, similar to Kupffer cells; and the "exudate macrophages," obtained after stimulation, which show peroxidase-negative endoplasmic reticulum but contain peroxidase-positive granules, similar to monocytes. In all of our animals the Kupffer cells exhibited the same pattern of reaction as the resident macrophages, but occasionally cells, with peroxidase positive-granules, that are similar to blood monocytes have been observed

by us and others in hepatic sinusoids.[3,4] Although our findings do not rule out the possibility of an ancestral derivation of Kupffer cells from monocytes, we did not find any intermediate cell types exhibiting transitions from monocytes to Kupffer cells. In fact, our observations would indicate that in the model of liver regeneration after partial hepatectomy the Kupffer cells are formed predominantly by local cell division. Further studies on the kinetics of Kupffer cells and their relationship to blood monocytes and the possible existence of different lines of tissue macrophages are needed. Our observations have established the usefulness of endogenous peroxidase and latex-labeling as markers of Kupffer cells for such future studies.

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Legends for Figures

Figures 1 to 4 are light micrographs of rat liver 72 hours after partial hepatectomy. The tissue was fixed by perfusion with glutaraldehyde and reacted for cytochemical localization of peroxidase.

Figure 1—An elongated wall-forming endothelial cell appears next to a rounded phagocytic Kupffer cell, which contains numerous cytoplasmic vacuoles. The peroxidase reaction product in tissue sections appears as a diffuse brown precipitate localized over the cytoplasm of the Kupffer cell, sparing the nucleus. (In black and white reproductions, Kupffer cells are the same shade of gray as parenchymal cells). (Epon, toluidine blue, × 2000)

Figure 2—A dividing Kupffer cell *(K cell mitosis)* and endothelial cell *(E cell mitosis)* are shown here next to each other. Whereas the dividing endothelial cell has a distinctly clear cytoplasm, the cytoplasm of the dividing Kupffer cell is stained dark brown due to endogenous peroxidase activity. (Epon, toluidine blue, × 2000)

Figure 3—An endothelial cell in mitosis with the clear, almost transparent cytoplasm. Note the round shape and protrusion of this dividing cell into the lumen of the sinusoid, in contrast to the slender shape of normal endothelial cells seen in Figure 1. (Epon, toluidine blue, × 2000)

Figure 4—A Kupffer cell in mitosis exhibiting diffuse brown peroxidase reaction in the cytoplasm and containing numerous ingested latex particles *(LATEX)*. (Epon, toluidine blue, × 2000)

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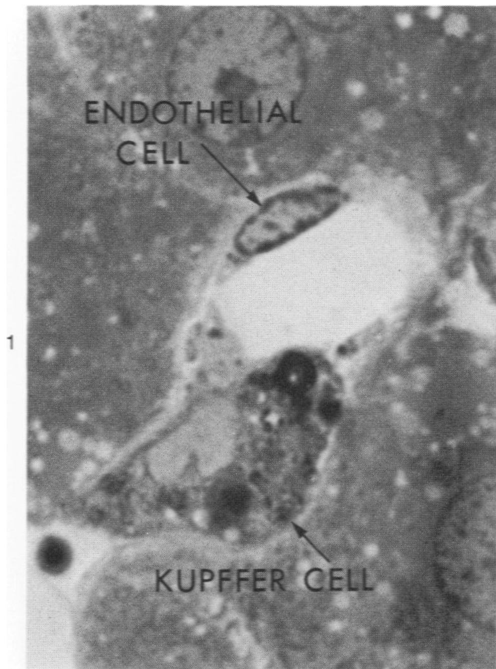

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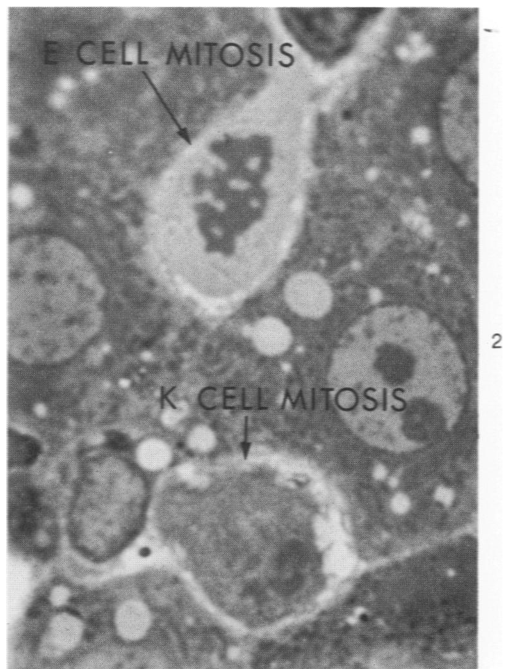

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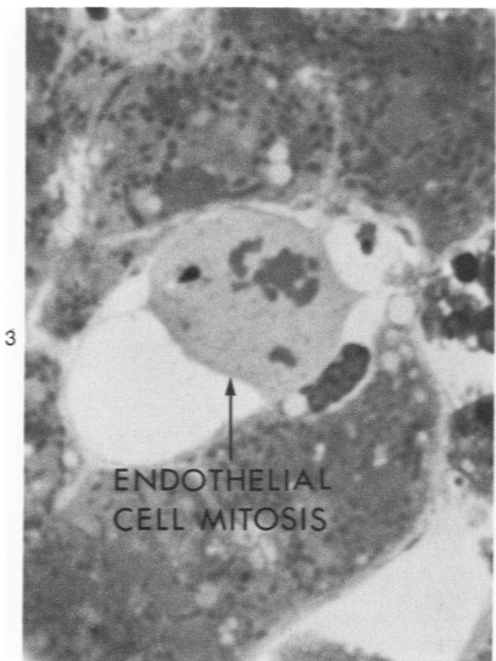

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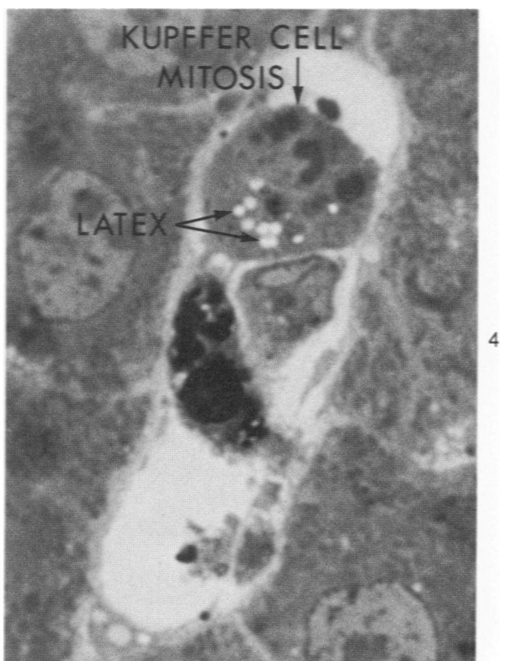


Figures 5 to 9 are electron micrographs of regenerating rat liver after partial hepatectomy, fixed by glutaraldehyde perfusion and processed for cytochemical localization of peroxidase activity. All sections are counterstained with lead citrate.[24]

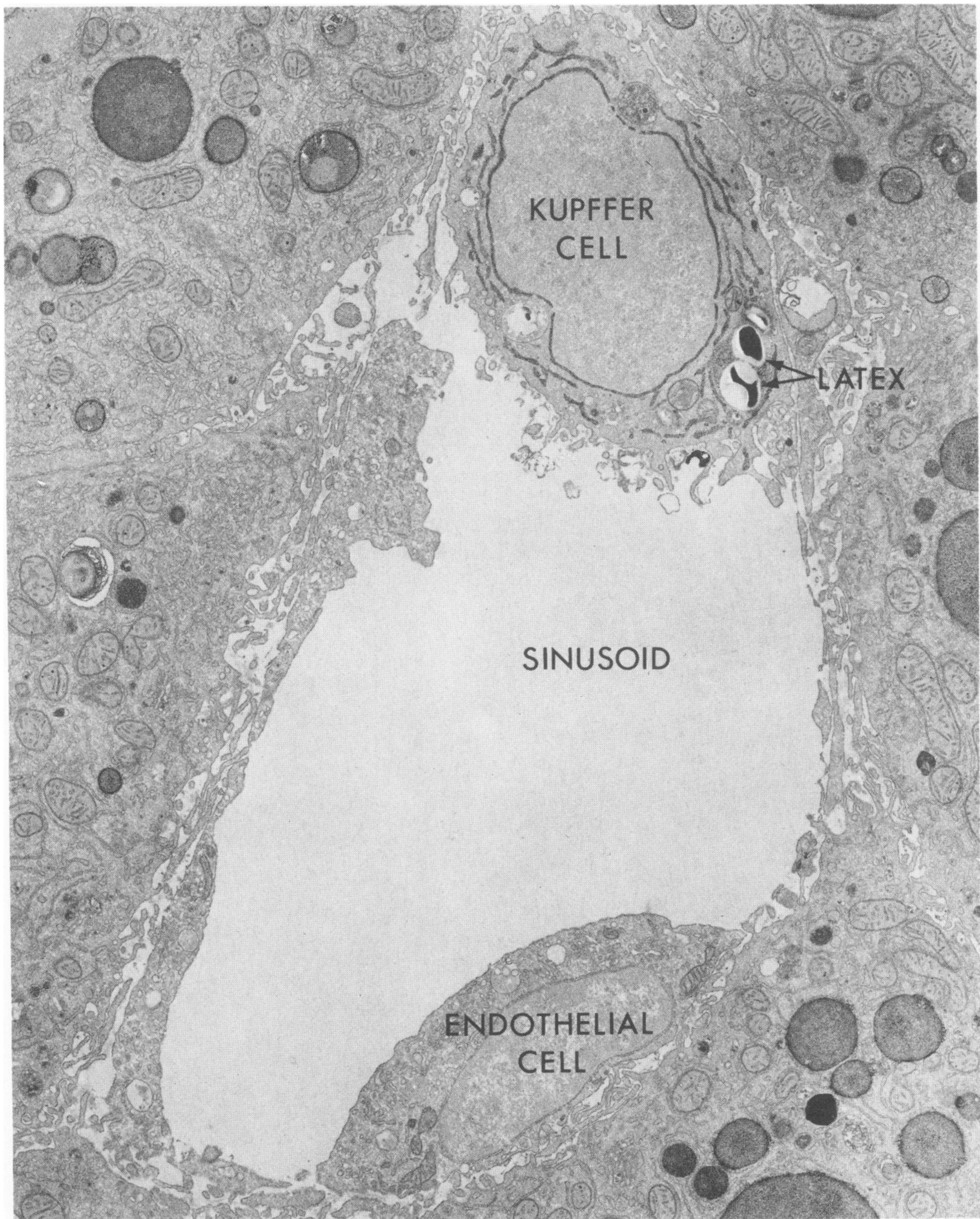


Figure 5—Low-power view of a liver sinusoid with a Kupffer cell, exhibiting peroxidase reaction product in endoplasmic reticulum and the nuclear envelope and containing phagocytized latex particles *(LATEX)*, and a peroxidase-negative endothelial cell. Note the ruffled luminal plasma membrane of the Kupffer cell in contrast to the smooth membrane of the endothelial cell. (× 8300)

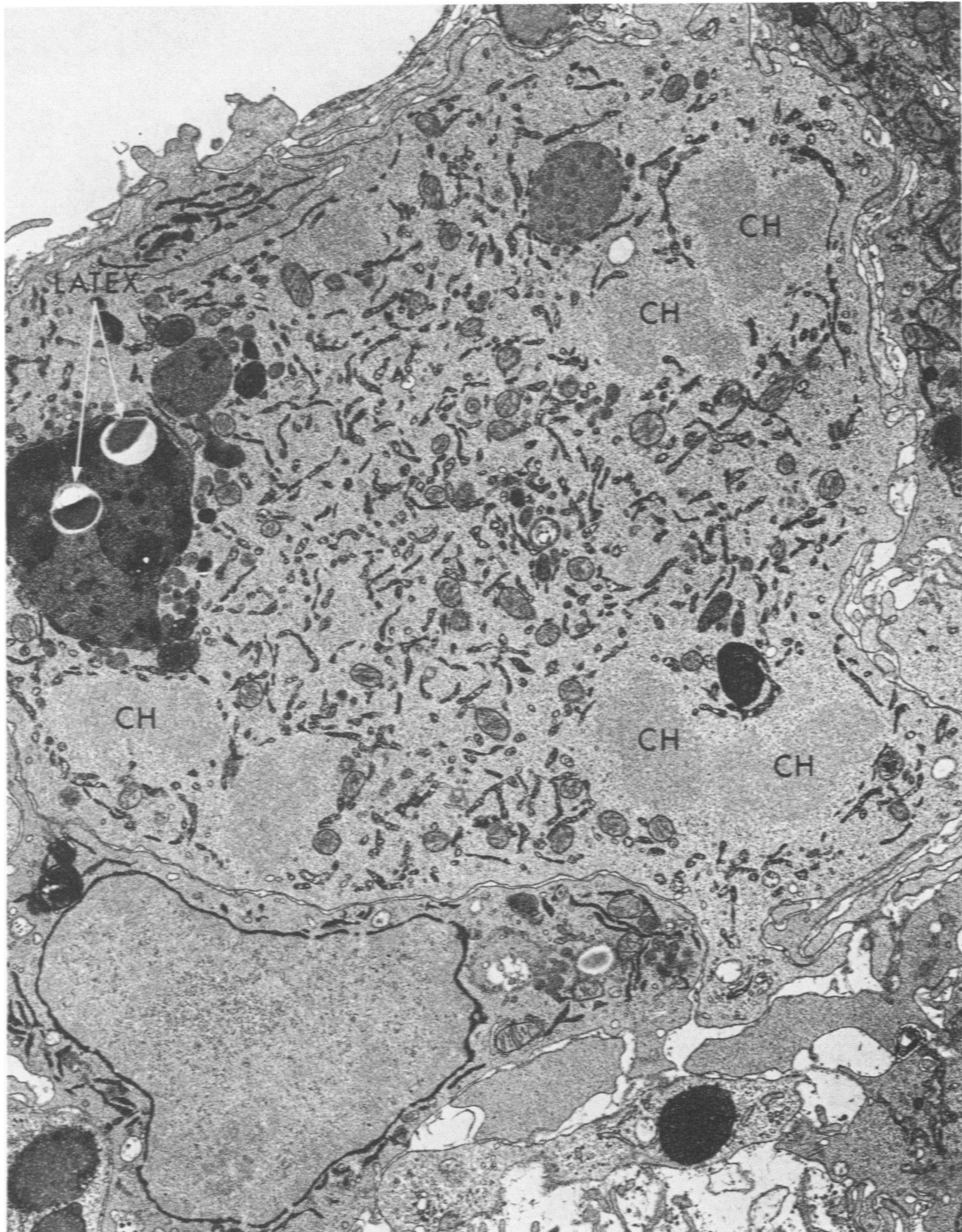
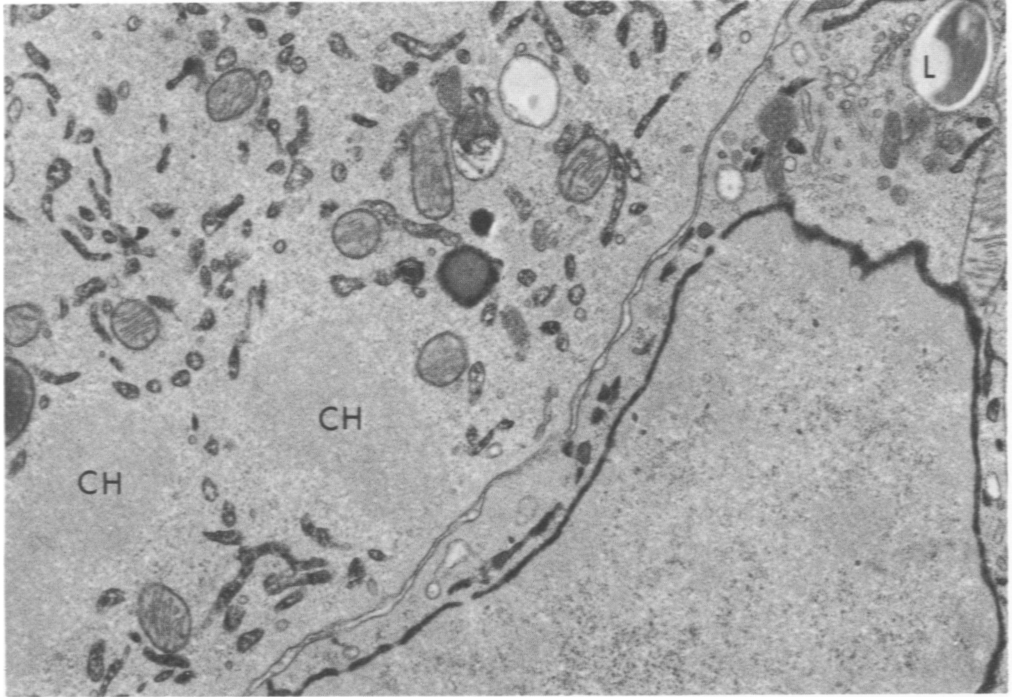


Figure 6—This figure, from an animal at 72 hours after hepatectomy, shows two Kupffer cells, one in the interphase with a distinct peroxidase-positive nuclear envelope and the second in mitosis containing chromatin clumps *(CH)* and short dilated segments of endoplasmic reticulum surrounded by a ribosome-rich cytoplasm. In addition, ingested latex *(LATEX)* particles are noted in one of the several large phagolysosomes of this dividing Kupffer cell. The presence of these lysosomes indicates that this is an avidly phagocytizing mature phagocyte in mitosis. (× 11,000)

Figure 7—This higher power view illustrates the contrast between the normal endoplasmic reticulum *(ER)* of a nondividing and the slightly dilated ER of a dividing Kupffer cell. Because of this dilation of ER, the peroxidase reaction product appears somewhat granular, whereas in the nondividing cell it is more homogeneous. *CH* = chromatin. (× 19,200)

Figure 8—This figure illustrates a dividing endothelial cell *(EC)* next to a normal Kupffer cell *(KC)*. Note the small mitochondria and the chromatin clumps *(CH)* in the endothelial cell. Whereas the Kupffer cell is peroxidase positive and contains numerous large phagolysosomes with latex particles *(LATEX)*, the endothelial cell is peroxidase negative and devoid of any evidence of avid phagocytic activity. (× 10,200)



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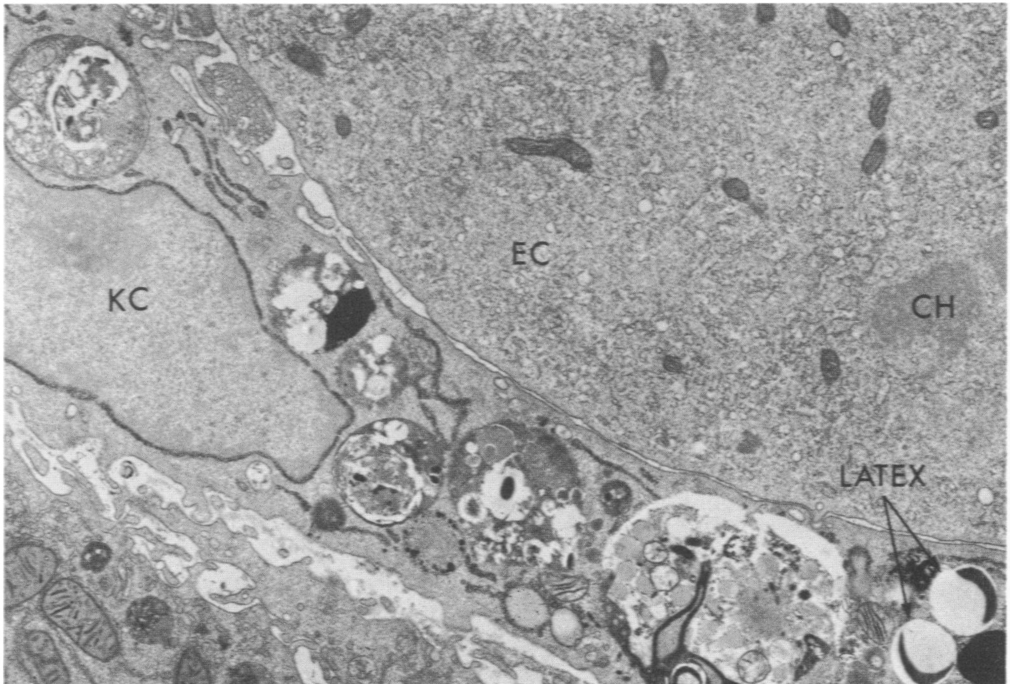

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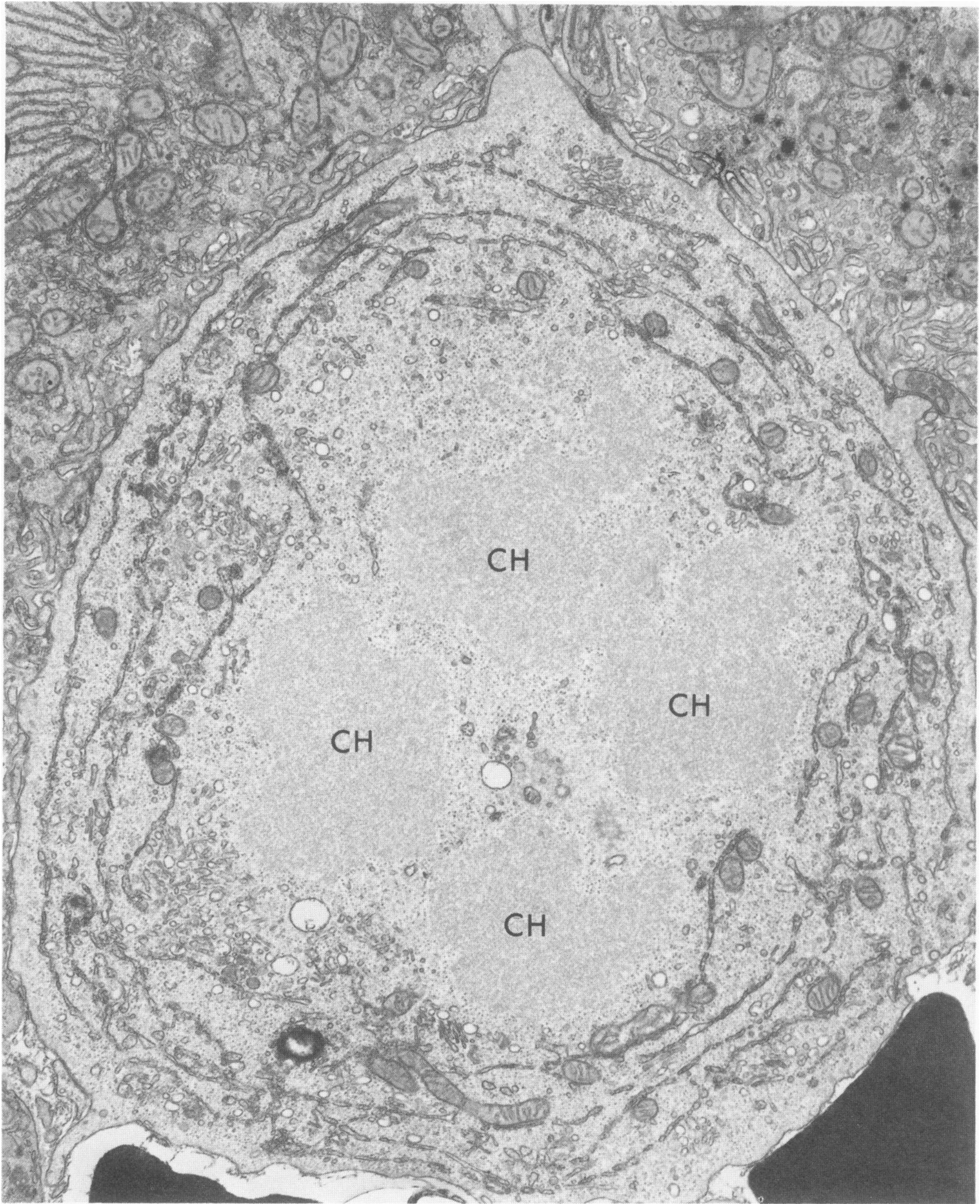

Figure 9—An endothelial cell in mitosis with several chromatin clumps *(CH)*, small mitochondria and focally dilated segments of ER embedded in a ribosome-rich cytoplasm. Note the rounded appearance of this cell. (× 21,000)