The Kupffer cells of the liver are the largest group of fixed tissue macrophages in the mammalian organism and are responsible for the phagocytosis of most circulating particles and micro-organisms (1, 2). They are an important constituent of the mononuclear phagocyte system (3); according to this concept, Kupffer cells, like other macrophages, originate from bone marrow pro-macrophages via the peripheral blood monocytes (4, 5). Previous studies from our group have established that Kupffer cells of rat liver exhibit an endogenous peroxidase activity in nuclear envelope (NE) and endoplasmic reticulum (ER) (6, 7). Rat monocytes, on the other hand, contain only peroxidase-positive granules but no activity in NE and ER (8). Attempts to find transition forms between monocytes and macrophages in various experimental models have been, for the most part, unsuccessful (9-13). This has led to the criticism of the view of the exclusive derivation of macrophages from monocytes.

Recently Bodel et al. have noted that monocytes of rat, rabbit, and man as well as exudate macrophages from rabbit peritoneal cavity cultured in vitro develop peroxidase activity in NE and ER after surface adherence (14, 15). These and similar in vivo observations of Beelen et al. (16) on rat peritoneal macrophages strongly support the concept of transformation of monocytes to tissue macrophages. The absence of such transition forms in the liver could have been due to the use of unsuitable experimental models (see also Discussion).

Glucan, a β-1,3-polyglucose, introduced by Riggi and Di Luzio, is a strong stimulant of the mononuclear phagocyte system (17, 18), which gives rise to massive accumulation of mononuclear phagocytes in the liver (19-21). In the present study we have used the glucan model for investigation of the relationship between peripheral blood monocytes and Kupffer cells. The results have revealed the existence of transition forms between the two cell types; in addition, evidence of self-replication of Kupffer cells has been found.

Materials and Methods

Animals. Male adult Sprague/Dawley rats (Ivanovas, Kissleg, West Germany) weighing 200-230 g and kept on a normal laboratory diet and water ad libitum were used. One group was given a single intravenous injection of 30 mg/kg body weight of glucan (Dr. Di Luzio, Germany).

Abbreviations used in this paper: DAB, 3,3'-diaminobenzidine; ER, endoplasmic reticulum; NE, nuclear envelope; SEM, scanning electron microscopy; TEM, transmission electron microscopy.
Tulane University, New Orleans, La.) and the livers were submitted to fixation 12, 24, 48, and 96 h thereafter. Another group received multiple, i.e. five consecutive daily injections, and was processed after 1, 5, 10, 30, 60, and 90 d. For each interval two to four rats were used. Controls were obtained by injection of 0.9% NaCl solution.

**Fixation.** After starving for 24 h before fixation livers were perfused through the portal vein for 1 min with 0.9% NaCl followed for 5 min by 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, containing 0.05% CaCl₂. Strips of liver tissue were rinsed three times in 0.1 M sodium cacodylate buffer and cut on a vibratome (Oxford Lab., Foster City, Calif.) into 40-μm sections.

**Incubation for Endogenous Peroxidase.** The medium contained 0.1% 3,3′-diaminobenzidine (DAB) in 0.1 M Tris-HCl buffer at pH 7.0, and 0.02% H₂O₂. The sections were first preincubated in the absence of H₂O₂ for 60 min followed by incubation in the complete medium for another 60 min at 22°C.

**Further Transmission Electron Microscopy (TEM)-Processing.** Specimens were postfixed for 90 min in 2% aqueous OsO₄ followed by dehydration in graded ethanol and embedding in Epon 812. Semithin sections were examined either unstained or were stained with methylene-toluidine blue. Thin sections were cut on an LKB Ultratome IV ultramicrotome, counterstained with lead citrate, and examined in a Philips 301 G transmission electron microscope.

**Scanning Electron Microscopy (SEM)-Processing.** After the perfusion small cubes of liver tissue were immersed in the same fixative for another 60 min and subsequently rinsed in 0.1 M sodium cacodylate buffer, pH 7.3. Specimens were postfixed for 4 h in 2% aqueous OsO₄ and dehydrated in graded ethanol solutions. Extracellular fractures were obtained by breaking the tissue in the glutaraldehyde fixative or after critical point drying. Intracellular fractures were prepared according to Haggis (22). Tissue was critical point dried after changing 100% ethanol with CO₂, sputtered with gold, and examined in a Philips PSEM 500 scanning electron microscope at 12 and 25 kV.

**Results**

**Light Microscopy.** The livers of glucan-injected rats showed prominent periportal mononuclear infiltrates and large numbers of mononuclear cells in periportal vessels and in central veins, adhering to the endothelial lining (Figs. 2, 4). Such adherent cells were observed in spite of the perfusion fixation, contrasting the almost empty blood vessels of control animals (Figs. 1, 3). This phenomenon was most prominent in early intervals (12-48 h) after a single injection and 1-5 d after multiple injections of glucan, decreasing subsequently, and becoming almost negligible after 30 d.

**SEM.** By SEM many elongated and rounded cells were noted in portal vessels and central veins adhering to the endothelium (Figs. 5, 6). The majority of these cells exhibited prominent lamellodopia (ruffles) and raised, ridge-like profiles on their surfaces. Whereas the cells in portal blood vessels showed occasional microvilli-like profiles and a few lamellodokia (Fig. 5), most cells in central veins were covered with ruffles and blebs (Fig. 6). Some of the adherent cells appeared elongated, being attached with foot-like processes (pseudopods) to the endothelial surface and stretching with a major portion of their cytoplasm along the vascular wall. In addition, many lymphocytes with a smooth surface or with numerous microvilli were found in large blood vessels.

**Peroxidase Cytochemistry.** By TEM most of the cells in portal blood vessels were identified as monocytes, exhibiting reniform nuclei, a well developed Golgi apparatus, and both peroxidase-positive and negative cytoplasmic granules (Fig. 7). Whereas the DAB reaction product filled the matrix of peroxidase-positive granules, thus obscuring the granule membranes, the membrane of most peroxidase-negative granules was accentuated by an electron lucent halo.
Figs. 1-4. Light micrographs of semithin sections of rat liver fixed by glutaraldehyde perfusion and stained with methylene-toluidine-blue. × 140

Figs. 1 and 2: These sections show portal triads, including the portal vessels and the surrounding connective tissue. Whereas in the control animal the portal vein is almost empty (Fig. 1), in the glucan-treated rat numerous mononuclear cells are attached to the endothelial lining (Fig. 2; 96 h after a single injection of glucan). Note also the prominent mononuclear infiltrate in the periportal region of the glucan treated animal.

Figs. 3 and 4: These illustrate the central veins (CV) in a control (Fig. 3) and in a glucan treated rat (Fig. 4). Whereas in the control the vessel is empty, numerous mononuclear cells adhering to the lining of the central vein are present in the glucan treated animal.
Fig. 5. Scanning electron micrograph of a branch of portal vein 12 h after a single injection of glucan. Numerous adherent cells with a rounded or an elongated cell body are present. Some have pseudopods which form spatial contacts with the endothelial wall. The luminal surface of these cells exhibits occasional short microvilli and a few lamellipodia and blebs, which are the typical features of monocytes and macrophages. × 4,700

Fig. 6. This scanning electron micrograph shows a central vein with several rounded and elongated cells adhering to the endothelium. Although these cells resemble in size and shape those in portal vessels, their surface is completely covered with blebs, lamellipodia, and ridge-like profiles. × 6,500
The central veins, on the other hand, contained many macrophages with positive peroxidase reaction in NE and ER (Fig. 8). These cells contained a prominent peroxidase-negative Golgi apparatus and numerous large lysosomes, but they lacked peroxidase-positive granules. Extensive microvilli-like projections were noted on the luminal surface of these cells, corresponding to the elaborate surface features seen by SEM. Macrophages were most prominent at intervals from 1 to 10 d in animals injected with five consecutive doses of glucan. In addition to macrophages, occasional lymphocytes and monocytes were present in central veins. These latter cell types were also noted in hepatic sinusoids, forming close contacts with Kupffer cells, which frequently contained phagocytized glucan particles (Fig. 9).

In addition, some cells exhibited features of both monocytes and resident macrophages, i.e. peroxidase-positive granules as well as a weak to moderately positive peroxidase reaction in NE and ER (Figs. 10-12). These intermediate cell types were attached either to the endothelium of the portal vessels (Figs. 10, 11) or to the lining of sinusoids (Fig. 12). The attachment sites in large vessels showed two patterns: (a) the two cells formed numerous small contacts over a long stretch of their surfaces (Fig. 10), or (b) fingerlike projections from intermediate cells extended towards the endothelium, touching the luminal surface of the latter (Figs. 11, 11a). Similar contacts were also noted between adherent monocytes and the endothelium.

In sinusoids some of these intermediate-type cells formed elaborate interdigitations with Kupffer cells (Fig. 12). The intercellular clefs between the plasma membranes of these cells measured 200-300 Å with a finely granular or fibrillar deposit exhibiting no distinctive features of specialized cell junctions. A prominent accumulation of peroxidase negative granules was noted consistently in that portion of the cytoplasm, which bordered the cell contacts (Figs. 11a, 12a).

In addition to the above-mentioned intermediate cells which were in the lumen of blood vessels or sinusoids, a few typical Kupffer cells bordering the space of Disse and with strong peroxidase activity in NE and ER also contained peroxidase-positive granules (Figs. 13, 14). Because of the small size and rarity of such granules, their distribution varied in different sections of the same cell. This is shown in Figs. 13a-13c, which are selected serial sections from the same Kupffer cell, containing two peroxidase-positive granules (Fig. 13a), which disappear in a deeper section (Fig. 13b). At a still deeper level (Fig. 13c) we note the appearance of two new peroxidase-positive granules. This cell also contains peroxidase-negative granules with varying distributions in different sections of the same cell (Figs. 13a-13c). The peroxidase positive granules were also found in Kupffer cells with erythrophagocytosis and phagocytosis of glucan particles (Fig. 14). It should be added that the typical monocytes did not exhibit any erythrophagocytosis, although they occasionally contained phagocytized glucan particles.

Another interesting finding in the livers of glucan-treated animals was that of dividing Kupffer cells (Fig. 15). The nuclear envelope was absent in such dividing cells, but peroxidase activity persisted in the ER during the mitotic division.

Discussion

The massive migration of mononuclear phagocytes into the rat liver as caused by glucan treatment (19–21) has served in this study as a model for investigation of the relationship of peripheral blood monocytes and Kupffer cells. The extent of this
migration was best revealed by SEM-examination of blood vessels (Figs. 5, 6), which showed cells with ruffles, lamellipodia, and blebs, the typical surface features of mononuclear phagocytes (23-27). It is of interest that cells in portal vessels exhibited fewer lamellipodia and blebs (Fig. 5) compared to those in central veins (Fig. 6). By TEM and cytochemistry the majority of these cells in portal vessels were monocytes (Fig. 7), whereas most of those in central veins were macrophages (Fig. 8). These findings demonstrate the basic similarity of the surface features of these two cells types, with macrophages appearing more differentiated than monocytes.

The cardinal finding of the present study is the discovery of cells with peroxidase patterns intermediate between monocytes and Kupffer cells, i.e. peroxidase activity in granules as well as in NE and ER. This concurs with the observations on monocytes and peritoneal macrophages of Bodel et al. (14, 15) and Beelen et al. (16), supporting the concept of derivation of tissue macrophages from blood monocytes (4, 5). It should be emphasized, however, that the finding of peroxidase-positive granules alone in Kupffer cells is not sufficient evidence of transformation of monocytes to tissue macrophages. Indeed, sequestered erythrocytes, phagocytized neutrophilic granules, or even peroxisomes of Kupffer cells (28) could all exhibit a positive peroxidase reaction (Fig. 14) and thus be confused with peroxidase-positive monocyte granules. The intermediate cells described in the present study (Figs. 10-12) contained both peroxidase-positive granules as well as peroxidase-negative granules with halo and were apparently circulating blood monocytes, becoming focally attached to the lining of sinusoids or portal blood vessels. In addition, the weak to moderately positive peroxidase reaction in NE and ER indicates that these cells differed from regular tissue macrophages and were monocytes probably in process of transformation. This notion is further supported by the observation of peroxidase-positive and negative granules, similar to those in monocytes, in typical Kupffer cells after serial sectioning (Figs. 13a-13c).

Based on peroxidase cytochemistry Daems and associates (12) have suggested the existence of two separate lines of macrophages: the exudate macrophage with features similar to monocytes; and the resident macrophage resembling the Kupffer cells. Our observations, which are in agreement with those of Bodel et al. (14, 15) demonstrate that peroxidase cytochemistry alone does not provide sufficient basis for advocating the existence of two separate cell lines. The usefulness of the peroxidase cytochemistry in identification of monocytes and macrophages, however, remains indisputable. The reasons why transition forms between monocytes and Kupffer cells were not found in
Fig. 10. This cell, which is adherent to the wall of a portal vein branch, shows a peroxidase distribution intermediate between a monocyte and a macrophage: distinct peroxidase-positive granules (PPG), and slight to moderate peroxidase activity in the NE and ER. Typical monocytic peroxidase-negative granules (PNG) are also present. The plasma membrane of the cell forms numerous focal contacts (arrows) with the endothelial lining over a long stretch of the cell surface. 96 h after a single glucan injection. × 20,500

Fig. 11. Another cell with peroxidase features intermediate between a monocyte and a macrophage in a branch of the portal vein. The contact with the endothelium consists of a single fingerlike cytoplasmic extension, being attached to a small fold over the endothelial surface. 96 h after a single glucan injection. × 16,000

Fig. 11a. A high power view of the cytoplasmic extension in Fig. 11 showing an accumulation of peroxidase-negative granules (PNG) near the attachment site. × 44,000
previous studies (11, 29) could be due to: (a) the long life span of Kupffer cells (5),
(b) the rapidity of the transitional process, and (c) the inadequacy of previous
experimental models. These problems are further compounded by the use of the EM-
technique, which is not ideal for screening large fields in search of ca. 1,000 Å
monocytic granules in liver sections. The present finding of such transitional forms is
most probably related to the high influx of monocytes into the liver and possibly to
an increased turnover of Kupffer cells after glucan treatment. This latter assumption
is based on the finding of many mature macrophages in the lumen of central veins
(Fig. 8) but still requires further confirmation. The release of mature Kupffer cells
into the blood has been proposed in a few instances (30).

Another interesting finding of the present study was that most cells with peroxidase
features intermediate between monocytes and Kupffer cells were adherent to the
lining of portal blood vessels or the sinusoids (Figs. 10–12). At some of these adhesion
sites extensive membrane interdigitations were noted between intermediate and
Kupffer cells (Figs. 12, 12b). Somewhat similar close junctions have been described
in leukemic monocytes (31) and phagocytizing hairy cells (32). A modified membrane
contact resembling septate junctions was described recently by Bainton and Golde
(33) in human bone marrow macrophages in culture. The junctions in the present
study did not exhibit any distinctive features of specialized cell junctions. Functionally
they appeared to be involved in anchoring the monocytes to the vascular walls (Fig.
12).

A marked accumulation of peroxidase negative granules was noted in cytoplasmic
extensions of intermediate cells bordering directly the attachment sites (Figs. 11a,
12a). This could suggest that the content of these granules is involved in the process
of attachment of monocytes to vascular linings under in vivo circulatory conditions.

In agreement with our previous studies (11, 29), we noted that Kupffer cells in
glucan-treated animals undergo mitotic division (Fig. 15). The occurrence of dividing
Kupffer cells has been noted in normal as well as in a variety of experimental
conditions (34–39) and has been interpreted by some as evidence of the existence of
Kupffer cells as a self-replicating cell line independent of blood monocytes. Recently

Fig. 12. A composite made up of three separate electron micrographs illustrating an intermediate

Fig. 12a. This higher magnification reveals in more detail the fine structure of the granules (PPG,

Fig. 12b. The contact between the intermediate cell and the Kupffer cell, enlarged in this view,

Figs. 13a–c. These are selected views from 10 serial sections (Si–S10) of a Kupffer cell with a typical

Fig. 13a. At this level (S1) two distinct peroxidase-positive granules are seen.

Fig. 13b. In this view (S5), which is 300–400 nm deeper, the peroxidase-positive granules have
disappeared, but typical peroxidase-negative granules (PNG) with halo are revealed.

Fig. 13c. In this still deeper section (S10) new peroxidase-positive and negative granules are
revealed side by side.
Fig. 14. A Kupffer cell containing peroxidase reaction product in NE and ER with two ingested glucan particles (G). Note in addition peroxidase activity in cytoplasmic granules (PPG), in a phagocytized erythrocyte (RBC), and in cytoplasmic granules of an adjacent neutrophilic granulocyte (PMN). \( \times 7,000 \)

Fig. 15. This micrograph shows two Kupffer cells, one in interphase (KC) and the other undergoing mitotic division. Whereas the NE has disappeared during mitosis, the ER still exhibits peroxidase activity. Note the clumps of chromatin in the cytoplasm (CH). \( \times 4,500 \)
Crofton et al. (5) also confirmed that some Kupffer cells are capable of mitosis and added that these dividing Kupffer cells were derived recently from bone marrow.

Our observations indicate that although Kupffer cells may be derived from blood monocytes, they still maintain the capability of self-replication in situ. Thus, depending upon the type of stimulus, new Kupffer cells could be provided either by local division or by recruitment from the blood and the bone marrow. Further studies combining peroxidase cytochemistry and autoradiography in rat chimeras are now in progress to assess the validity of this hypothesis.

Summary

A massive accumulation of mononuclear phagocytes in the rat liver was found after the injection of glucan, a β-1,3-polyglucose. Portal vessels and central veins contained large numbers of rounded and elongated cells which were adherent to the endothelium. By scanning electron microscopy most of these cells exhibited prominent lamellipodia, raised ridge-like profiles and blebs, the typical features of mononuclear phagocytes. Peroxidase cytochemistry revealed that whereas most cells in portal vessels were monocytes with peroxidase positive and negative granules, the majority of cells in central veins were macrophages exhibiting peroxidase activity in nuclear envelope (NE) and endoplasmic reticulum (ER). In sinusoids monocytes and macrophages were seen side by side. The major finding of the present study was that some cells, adherent to the endothelium of portal vessels or to the lining of sinusoids, exhibited a peroxidase pattern intermediate between monocytes and Kupffer cells, i.e. strong peroxidase activity in cytoplasmic granules, as well as a weak to moderately positive peroxidase reaction in NE and ER. These intermediate cells also contained peroxidase-negative granules with halo, which are usually seen in monocytes. Furthermore, examination of serial ultrathin sections of typical Kupffer cells revealed numerous peroxidase-positive granules and peroxidase-negative granules with halo in their cytoplasm. Finally, dividing Kupffer cells with positive peroxidase reaction in ER were found. These in vivo observations provide ultrastructural and cytochemical evidence in support of the concept of derivation of Kupffer cells from monocytes, demonstrating in addition that Kupffer cells are capable of self-replication in situ.

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