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Electron microscopic study of the effects of endotoxin on the cells of the hepatic sinusoid in normal and BCG sensitized mice

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Summary. Electron microscopic studies were conducted to access ultrastructural alterations in Kupffer cells and other cells lining the hepatic sinusoids at the peak of mediator release two hours after challenge with low doses of endotoxin under various conditions including reticuloendothelial system (RES) expansion and activation with BCG. BCG is known to sensitize animals to endotoxin rendering normally innocuous, low doses of endotoxin lethal.

Low non-lethal doses (5 μg) of endotoxin activated Kupffer cells as well as caused isolated foci of cellular injury. However, animals which were treated with BCG had a highly activated and expanded RES system as evidenced by enlarged Kupffer cells with many extended cellular processes. Granulomas were prevalent and many reactive cells were present. After two hours marked cellular injury occurred to sinusoid lining and parenchymal cells when BCG treated animals were challenged with these same low doses of endotoxin. Cellular debris, fibrin, and platelets were observed in sinusoids often associated with Kupffer cells. These results suggest that the functional state of Kupffer cells is an important determinant in the host response to endotoxin. While there appears to be an effective clearance of endotoxin; the release of mediators by the highly activated Kupffer cells can be toxic causing hepatocellular injury.

Key words: Liver, Sinusoid, Endotoxin, BCG

Introduction

Kupffer cells, fixed macrophages adhering to the lining of hepatic sinusoids, are in a key position for monitoring and removing particulates and toxins which enter the portal circulation. These cells have been found to be responsible for the major uptake and detoxification of endotoxin (Morrison and Ulevitch, 1978; Mathison and Ulevitch, 1979; McCabe, 1980; Ruiter et al., 1981; Van Bossuyt and Wisse, 1988a; Wake et al., 1989). Endotoxin elicits the release of various substances (e.g., cytokines, eicosanoids and reactive free radicals) from Kupffer cells which affect a variety of host-defense mechanisms (Nolan, 1975, 1981, 1982; Bhatnager et al., 1981; Cohen et al., 1982; Decker et al., 1982; Leser et al., 1982; Kirn et al., 1982; Nolan and Cohen, 1988; Decker et. al., 1989). While many of these substances are beneficial, unfortunately some are inflammatory, cytotoxic and/or vasoactive causing disruption of the endothelium which leads to impaired microcirculation (Bhatnager et al., 1981; Leser et al., 1982; Keppler et al., 1985; McCuskey et al., 1982, 1987; Decker, 1989) and damage to hepatocytes (Nolan, 1975, 1981, 1982; Kirn et al., 1978; Ruiter et al., 1981; Nolan and Cohen, 1988; Van Bossuyt and Wisse, 1988b). The peak release of these mediators appears to be two hours after the administration of endotoxin (Berry, 1987).

Nolan (1975, 1981, 1982), Nolan and Cohen (1988), Liehr and Grün (1977, 1979) and Walter et al. (1981) have reviewed the relationship between the reticuloendothelial system (RES), gut-derived endotoxin, and liver injury. Studies by Kirn and co-workers (1978, 1988) and Ruiter et al. (1981) have explicitly related hepatocellular injury to altered Kupffer cell function due to endotoxins. Damaged Kupffer cells fail to clear endotoxin which results in systemic endotoxemia leading to hepatic dysfunction (Nolan, 1975, 1981, 1982; Nolan and Cohen, 1988). The mechanism of these events and their effects on total liver function has yet to be fully clarified.

McCuskey et al. (1982a,b, 1983) used *in vivo* microscopic methods to assess alterations in hepatic microcirculation and Kupffer cell function when challenged with low doses of endotoxin under a variety of conditions including RES expansion and activation with BCG. Under these conditions, dramatic alterations in microcirculation and Kupffer cell function were reported

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at the time of peak mediator release (two hours post-endotoxin) (Berry, 1977). To expand their report and our understanding of changes seen using *in vivo* microscopy, liver samples were fixed for electron microscopy at the same time periods as the *in vivo* microscopic observations. This paper describes the ultrastructural alterations that were observed and correlates them with the previously reported responses seen by light microscopy (McCuskey et al., 1982a,b, 1983).

Materials and methods

Male Swiss Webster mice which weighed 20-25 gm were fed food and water ad lib. These mice were divided into four groups with three animals/group. Endotoxin (*E. coli* 0111: B4, TCA extracted) BCG (*M. bovis,* 1029 Phipps, Lot A-17; Trudeau Institute, Saronac, N.Y.) and non-pyrogenic saline were administered intravenously via a tail vein as follows:

Group I. 5 μg endotoxin suspended in saline was given and animals were sacrificed two hours later.

Group II. 10^{-8} CFU BCG was administered 14 days prior to sacrifice.

Group III. 14 days after the administration of 10^{-8} CFU BCG, 5 μg of *E. coli* 0111:B4 (TCA extracted) was given and the animals were sacrificed two hours post-endotoxin.

Group IV. Control animals received an equivalent volume of saline and were sacrificed two hours later.

Two of the 3 animals in each group were injected with latex particles (0.81 μm) (Difco) via the tail vein just prior to sacrifice to label Kupffer cells.

Animals to be sacrificed were anesthetized using 25% urethane (0.03 mg/kg body weight). The livers were exteriorized and a small sample was taken from the edge of the left lobe to be processed for electron microscopy. This was the same area studied using *in vivo* microscopy (McCuskey et al., 1983). All tissue were fixed by immersion using standard Karnovsky's fixative (Karnovsky, 1965), post fixed in 1% osmium tetroxide, buffered with 0.1 M sodium cacodylate (pH 7.4). The blocks were dehydrated through a graded series of alcohols, and embedded in Epon. Thin sections were cut on a MT-5000 microtome (DuPont-Sorvall), stained with lead citrate and uranyl acetate and subsequently studied using a JEOL 100CX electron microscope.

Results

Group I

Low, non-lethal doses of endotoxin (5 μg) produced the following changes 2 hrs after administration. Kupffer cells were activated as evidenced by phagocytosis of numerous latex particles and frequently appeared injured as evidenced by the dilatation and ballooning of the nuclear envelope (Fig. 1). The cytoplasm of these cells contained many phagosomes filled with cellular debris; platelets often were entrapped by filopodia (Fig. 2). In scattered sinusoids, the endothelium was pulled away from hepatocytes widening the space of Disse while in other sinusoids it was missing altogether giving blood cells access to the hepatic parenchyma (Figs. 3, 4). Hepatocytes lacked microvilli along their vascular margins where cells had infiltrated the Space of Disse (Fig. 3). In addition to cellular debris, cytoplasmic blebs of hepatocytes, devoid of organelles, protruded into the lumen of sinusoids (Fig. 4). Lymphocytes frequently were found in sinusoids and occasionally were seen in the space of Disse (Fig. 3) having foot processes embedded in hepatocytes which were devoid of microvilli.

Group II

In BCG infected animals, the following changes were seen. Kupffer cells appeared greatly increased in number as well as more extensive in size possessing many processes which filled sinusoids (Fig. 5). There were many lysosomes in their cytoplasm as well as vacuoles which contained cellular debris and platelets were entrapped by filopodia (Fig. 6). A number of Kupffer cells were binucleate (Fig. 6) and a few were observed in mitosis (Fig. 7). Endothelial cells also appeared to have undergone change. Nuclear chromatin (Fig. 8) was condensed and an electron-dense cytoplasm (Fig. 6) contained large lysosomal like structures (Fig. 8). Large mononuclear cells (Figs. 6, 8) and lymphocytes (Fig. 6), some having an electron-dense cytoplasm, frequently were seen in sinusoids. Reactive cells, with many processes and lysosomes, had invaded the hepatic parenchyma wrapping their processes around hepatocytes resulting in extensive hepatocellular injury. A smooth border was formed on hepatocytes (a loss of microvilli) as the granuloma expanded in the parenchyma, collapsing the normal framework, and at the same time impinged upon sinusoids and incorporated them into the granuloma (Fig. 5). A rare megakaryocyte was observed in sinusoids.

Group III

Animals vaccinated with BCG 14 days prior to being given a 5 μg dose of endotoxin exhibited extensive hepatocellular damage 2 hours post endotoxin. Considerable cellular debris, fibrin and platelets were observed in sinusoids (Fig. 9). There was extensive damage to Kupffer cells. The nuclei of some were fragmented with condensed and irregularly arranged chromatin. In others, there was ballooning of the nuclear membrane (Fig. 10). The cytoplasm of Kupffer cells contained many lysosomes, digestive vacuoles, and dilated ER containing flocculent material (Fig. 10). Various cells (e.g., macrophages, lymphocytes, erythrocytes) had gained access to the space of Disse forming nests among hepatocytes

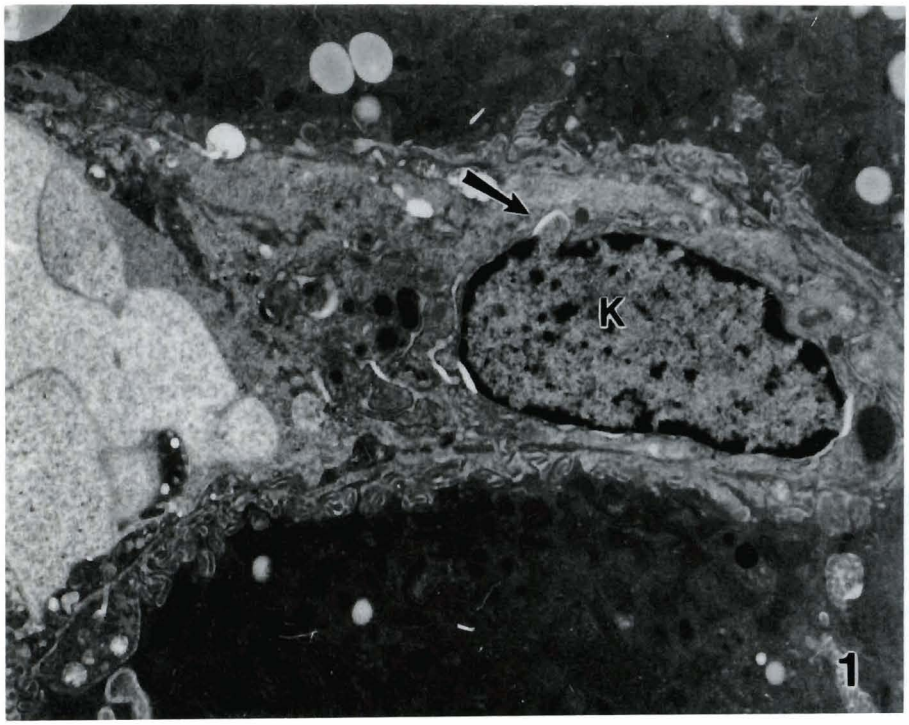

Fig. 1. Injured Kupffer Cell (K) following a 5 μg dose of endotoxin. Note: ballooning of nuclear envelope (arrow) × 3,300

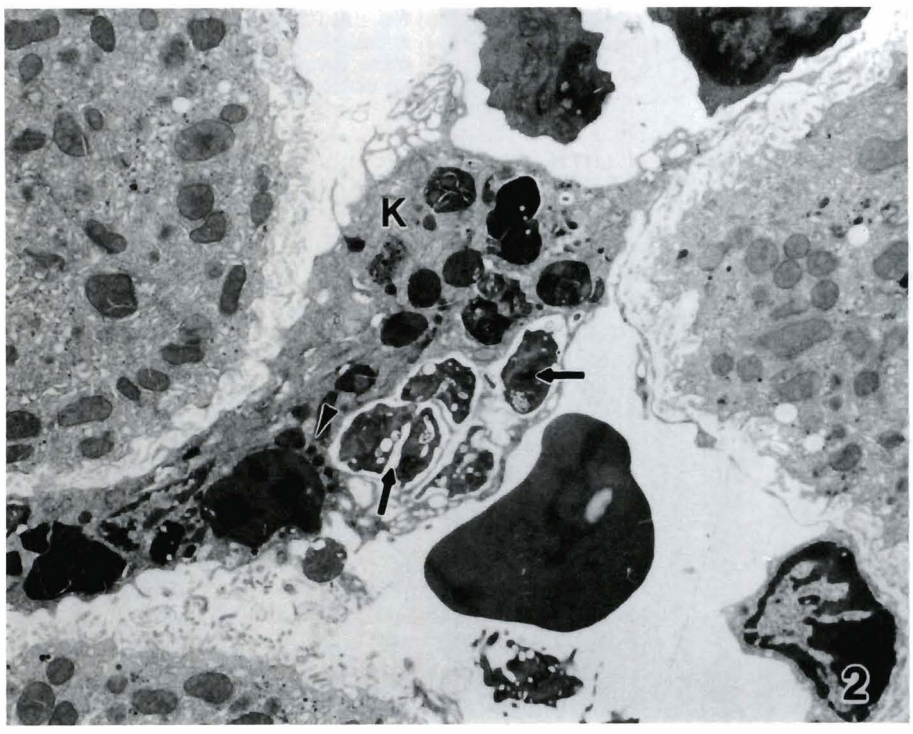

Fig. 2. Kupffer cell (K) with entrapped platelets (arrows) and heterogeneous phagosomes (arrowheads). 5 μg endotoxin. × 3,000

(Fig. 11). Sometimes foot processes of lymphocytes impinged upon hepatocytes. The hepatocytic degenerative changes were evidenced by vacuolization of vascular margins, loss of microvilli, nuclear enfolding and disorganized cristae in mitochondria; cytoplasmic blebs of hepatocytes protruded into sinusoids (Figs. 9 - 11) as well as many necrotic hepatocytes were seen in sinusoids.

Group IV

The livers of saline injected animals exhibited none of the changes seen above and were consistent with

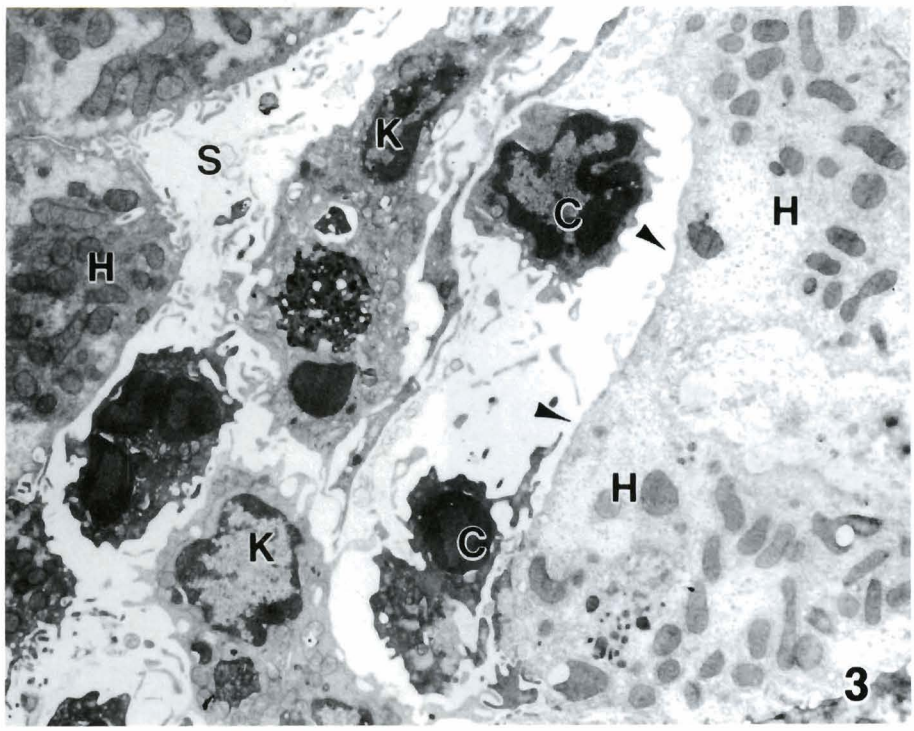

Fig. 3. Note hepatocytes (H) with loss of microvilli (arrowheads). Reactive cells (C) in space of Disse and activated KC (K) in sinusoid (S). 5 μg endotoxin. × 2,600

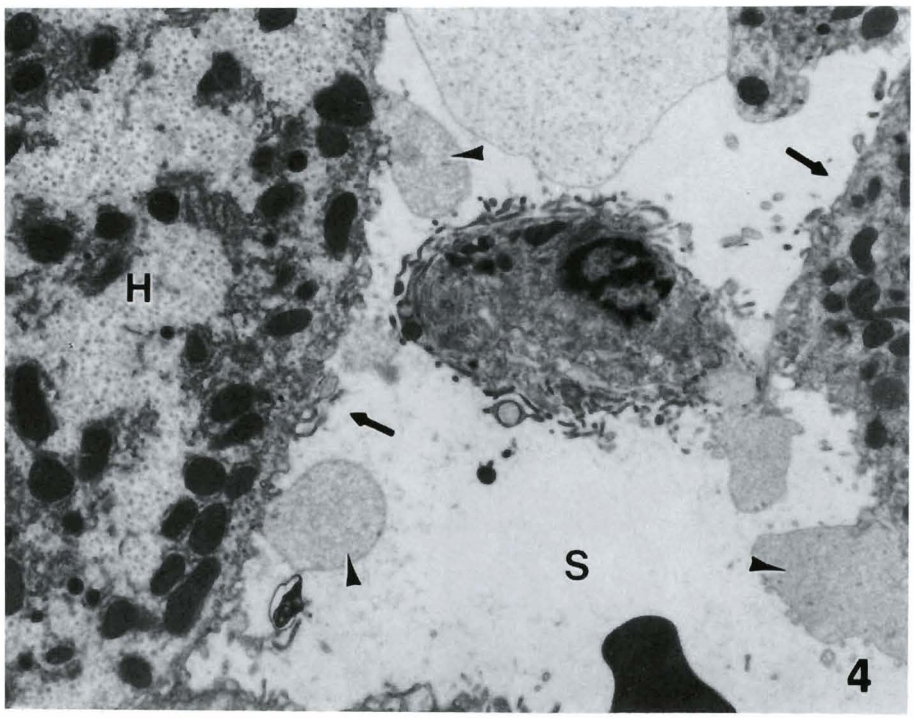

Fig. 4. Sinusoid (S). Note lack of endothelium (arrows) and cytoplasmic blebs (arrow heads) of hepatocytes (H). 5 μg endotoxin. × 3,300

previous reports of normal hepatic ultrastructure (Fig. 12).

Discussion

The results of this study document ultrastructural changes which occurred in livers of mice at the same time interval as previous reported *in vivo* microscopic studies (McCuskey et al., 1983). The livers in both studies were examined at the peak of mediator release which resulted from low doses of endotoxin given to normal and BCG infected mice.

In vivo light microscopic studies of McCuskey et al.

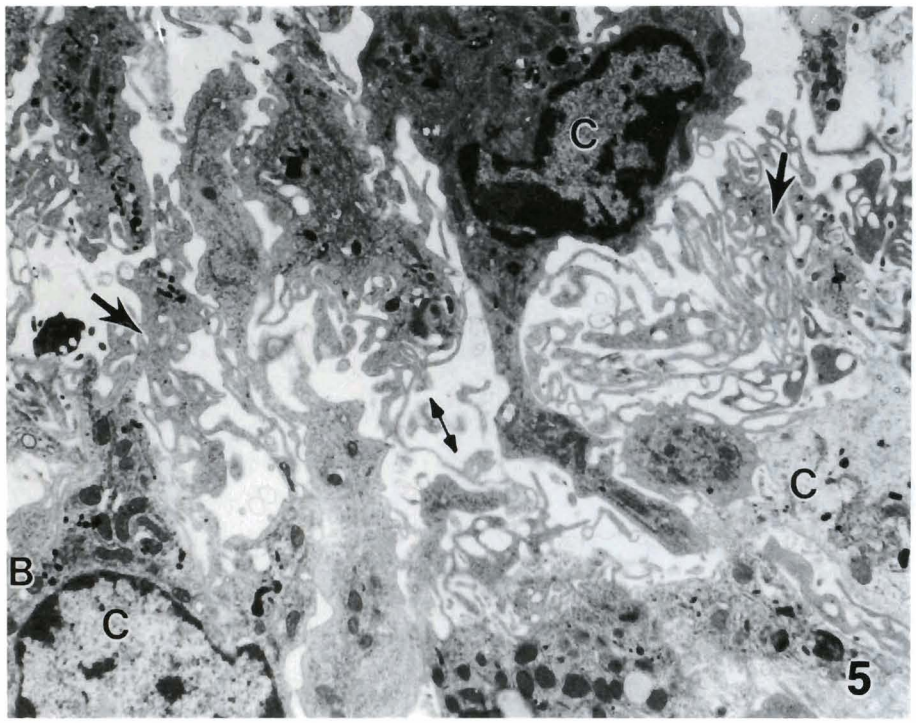

Fig. 5. Widened sinusoid filled with highly activated cells (C) possessing many processes (arrows) and lysosomes (arrowheads). BCG + 14 days. × 2,600

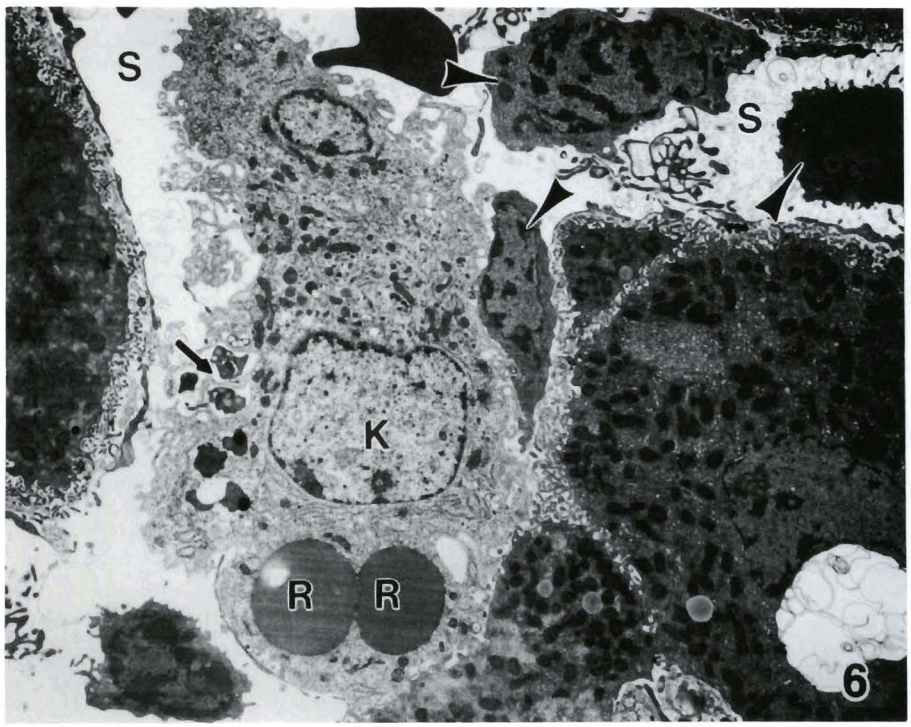

Fig. 6. Sinusoid (S) containing enlarged Kupffer cell (K) with entrapped platelets (arrows) and red cells (R). Note reactive cells (arrowheads). BCG + 14 days. × 2,000

(1983) reported reduced flow in scattered hepatic sinusoids, lengthening of the time required for Kupffer cells to phagocytose of latex particles, and cells (leukocytes and platelets) transiently adhering to the sinusoidal lining two hours following the intravenous injection of low, non-lethal doses of ET.

Whereas 5 μg endotoxin is non-lethal in the non-BCG treated mouse (LD 125 μg), 5 μg was found to be a LD100 in the BCG treated mice. The increased sensitivity to endotoxin in the BCG treated mice has been attributed in a large part to their expanded and activated RES system (Howard et al.,

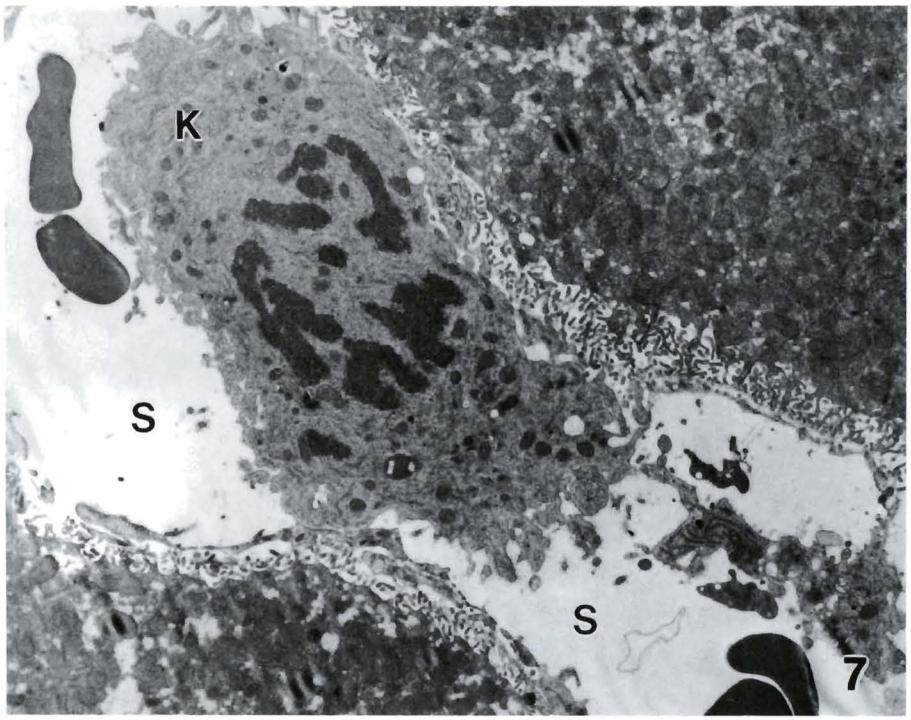

Fig. 7. Kupffer cell (K) in mitosis within hepatic sinusoid (S) BCG + 14 days. × 3,000

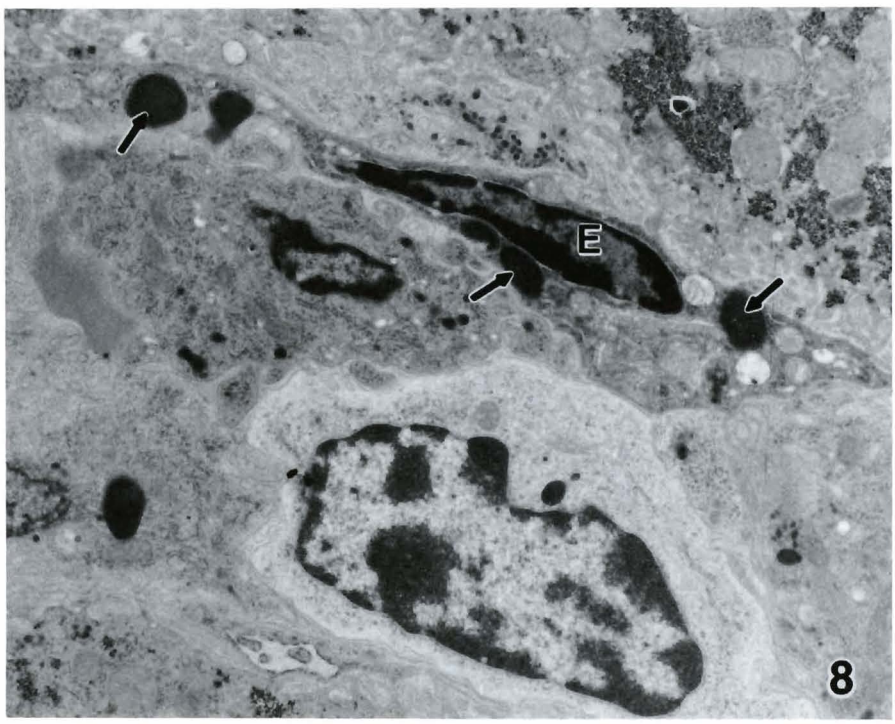

Fig. 8. Endothelial cell (E) having condensed nuclear chromatin and large lysosomes (arrows). BCG + 14 days + 5 μg endotoxin. × 5,000

1959; Rosentreich and Vogel, 1980).

In the current study, leukocytes and platelets were seen in scattered sinusoids; of particular interest was the close association of platelets and lymphocytes to Kupffer cells. This may be due in part to the induced release of mediators (thromboxane, tumor necrosis factor, interleukin 1, etc) (Reviewed by Decker, 1982; McCuskey, 1986; McCuskey et al., 1987; Decker, 1989) from Kupffer cells following stimulation or damage to them by endotoxin. In contrast, granular leukocytes usually were associated with endothelium further suggesting endothelial damage perhaps due to mediators released

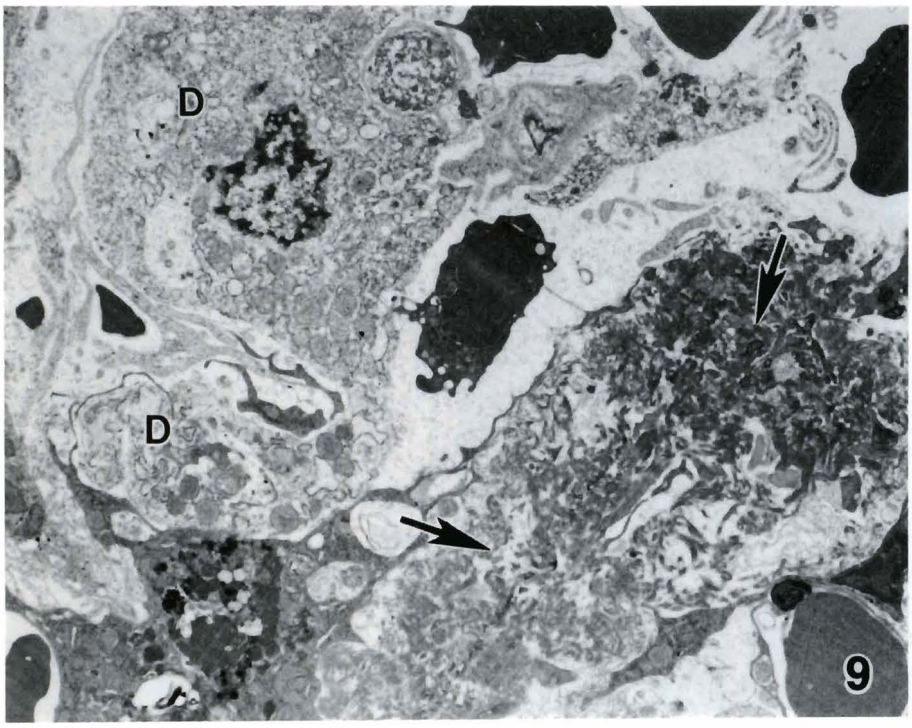

Fig. 9. Following BCG and endotoxin cellular debris (D) and fibrin (arrows) was observed within sinusoids (S). BCG + 14 days + 5 μg endotoxin. × 2,600

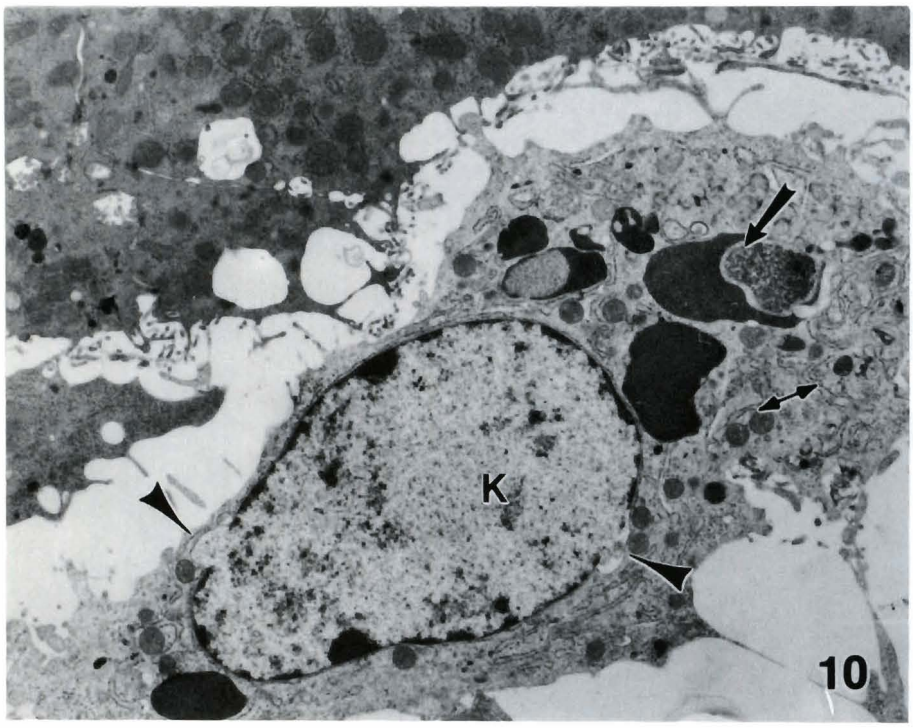

Fig. 10. Following BCG and endotoxin Kupffer cell (K) appeared injured as evidenced by ballooning of nuclear envelope (arrowheads). Also note lysosomes and ER containing flocculent material (arrows). BCG + 14 days + 5 μg endotoxin. × 3,000

from adjacent Kupffer cells. That endothelial damage occurred under these conditions is further supported by the lack of endothelium in focal areas demonstrated by electron microscopy. This also would provide an entry for blood borne substances and cellular elements to gain access to the hepatic parenchyma which may cause hepatocellular damage. Such damage was evidenced by nuclear and mitochondrial changes and cytoplasmic blebs that protruded into sinusoids. Vacuoles suggestive of hypoxic injury were observed in hepatocytes. These may be the result of transient plugging of sinusoids as observed by *in vivo* microscopy

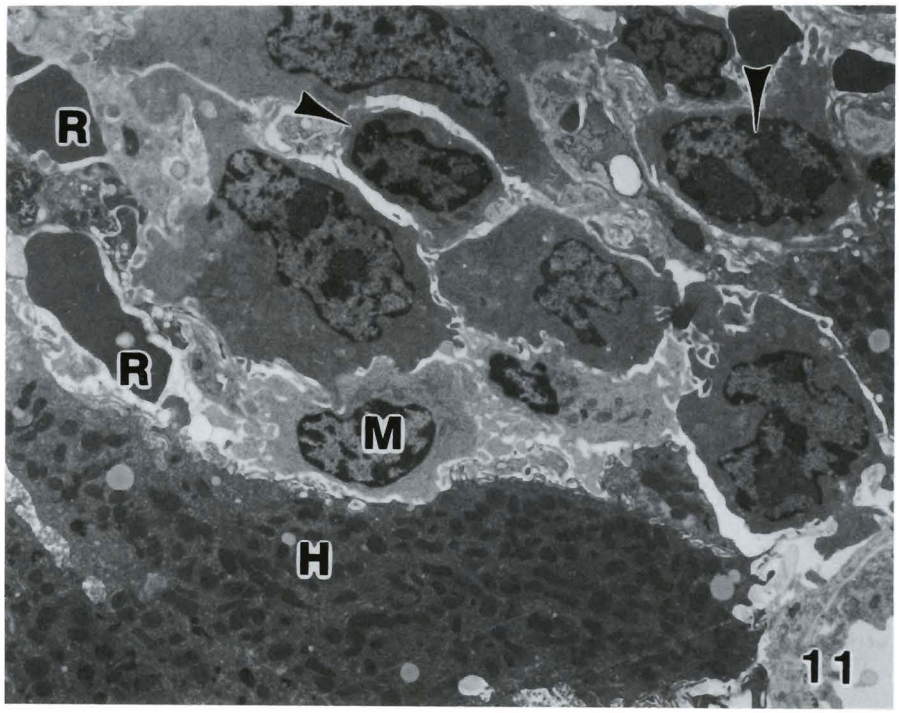

Fig. 11. Granulomatous area in hepatic parenchymal (H) containing many types of reactive cells. Lymphocyte (arrow heads), RBC (R), Macrophages (M). BCG + 14 days + 5 μg endotoxin. × 2,000

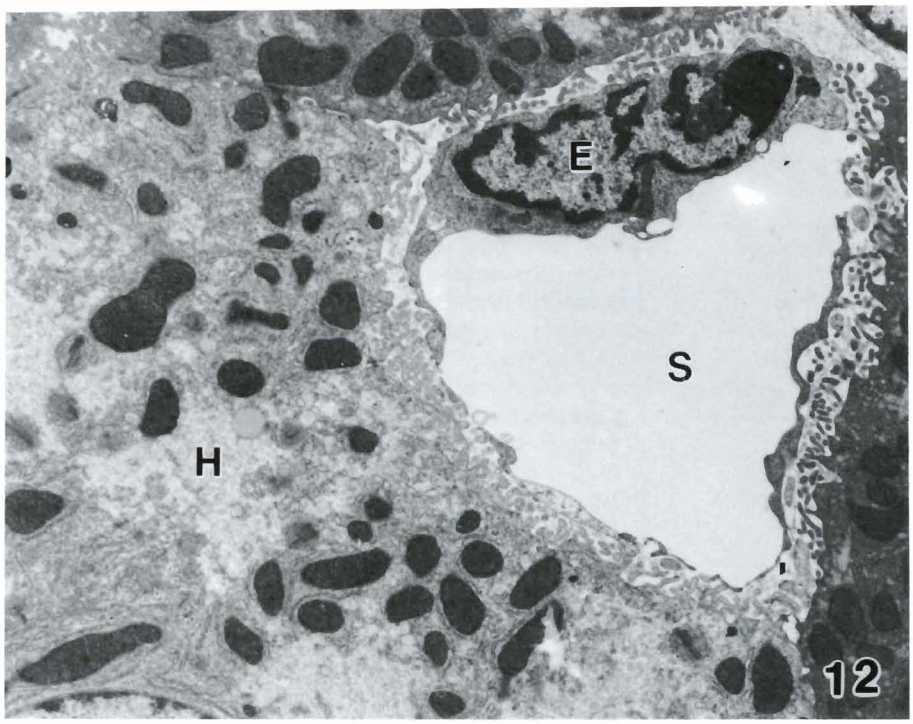

Fig. 12. Normal murine hepatic sinusoid (S). Endothelium (E) and hepatocyte (H). × 4,000

(McCuskey et al., 1982a, 1987; McCuskey, 1986).

In the livers of mice infected with BCG 14 days prior to sacrifice, activation and expansiton of the RES system was evidenced by marked increase in size, numbers of processes, and lysosomal structures in Kupffer cells as well as the influx of large mononuclear cells into the hepatic parenchyma forming granulomas. This is consistent with previous studies using zymosan, glucan, and BCG which are known activators of the RES (Howard et al., 1959; Mooton, 1959; Suter et al., 1961). The enlarged Kupffer cells as well as the increase and influx of mononuclear cells did not

markedly alter circulation in the sinusoids except in granulomatous areas, where there was little or no blood flow (McCuskey et al., 1982b, 1983). The association of platelets to Kupffer cells suggests that their activation was accompanied by the release of thromboxane or some similar substance. Previous *in vivo* microscopic studies confirmed the increase phagocytic activity in such animals; both in rate and amount of uptake of latex particles (McCuskey et al., 1982a,b, 1983).

Whereas 5 μg of endotoxin alone produced only minor focal changes; when given to animals with BCG activated RES systems marked responses occurred throughout the liver. Nuclear and cytoplasmic injury to Kupffer cells, endothelial cells, and hepatocytes was pronounced. In addition, the stasis noted by *in vivo* microscopy was evidenced at the ultrastructural level by fibrin, platelet aggregates, leukocytes, and enlarged KC contained with Kupffer cells. These responses are similar to responses seen in animals given lethal doses of endotoxin not previously vaccinated with BCG (McCuskey et al., 1982a, 1987).

In conclusion, the results of this study further support the central role of Kupffer cells in endotoxin induced injury to the liver. It is suggested that the state of activation of these cells determines the degree of injury. The degree of injury may be related to the release of toxic substances by these activated Kupffer cells including lysosomal enzymes (Saito and Suter, 1965; McCuskey et al., 1984). A relationship between lysosomal enzymes and hepatocellular injury has been proposed by others (Thorbecke, 1961; Saito and Suter, 1965). In the endotoxin resistant C_3H/HeJ mouse there is a paucity of activated Kupffer cells as well as deficiency of lysosomal enzymes (McCuskey et al., 1984). Normal Kupffer cell activity can be restored in these animals by infection with BCG (McCuskey et al., 1982b, 1984). The role of Kupffer cells was further evidenced by studies of mice infected with FV_3 virus which selectively destroyed Kupffer cells leading to hepatocellular injury of toxic origin, presumably by endotoxin (Kirn et al., 1978, 1988) which can no longer be cleared by Kupffer cells.

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