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Immunohistochemical Study of Dendritic Cells and Kupffer Cells in Griseofulvin-Induced Protoporphyric Mice

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To determine whether protoporphyric liver injury affects hepatic dendritic cells (DC) and Kupffer cells, we examined liver tissues of griseofulvin-induced protoporphyric mice using histological and immunohistochemical methods. After 1 week of griseofulvin feeding, the protoporphyric liver showed prominent hepatomegaly and a gradual increase in histopathological changes such as hepatocellular hypertrophy, focal necrosis and brown pigment deposits. After 4 weeks of treatment, marked ductular reaction was observed in the liver tissue. Immunohistochemical analyses indicated that the density of NLDC-145-positive hepatic DC gradually decreased during griseofulvin feeding. However, the index of the number of DC in the whole liver appeared to fall sharply after 6 weeks. In contrast, the density of F4/80-positive Kupffer cells gradually increased during griseofulvin feeding. In the spleen and lung, no significant differences were noted in the distribution of NLDC-145-positive DC between experimental and control mice. These results suggested that griseofulvin-induced protoporphyria leads to a specific decrease in the density of hepatic DC due to hepatomegaly until 4 weeks of treatment and is substantial after 6 weeks. This substantial decrease of hepatic DC might have been induced by some alterations in protoporphyric liver injury including ductular reaction at a later stage in this experiment. Since hepatic DC were reduced in number, they seemed to have no significant relation to the progression of griseofulvin-induced protoporphyric liver injury. However, the decrease of hepatic DC might affect the cellular immune response in protoporphyria.

Key words: dendritic cell; griseofulvin; immunohistochemistry; Kupffer cell; protoporphyria

Although human porphyrias are often associated with the development of liver injury, they are relatively uncommon, preventing adequate investigation in patients. Griseofulvin, an antifungal drug, is known to inhibit hepatic ferrochelatase activity (Lochhead et al., 1967) and disturb porphyrin metabolism in the mouse (De Matteis and Rimington, 1963). This experimental mouse model shows liver injury with accumulation of brown protoporphyrin crystals, resembling human erythropoietic protoporphyria. This mouse model has therefore been used to study the pathogenesis of protoporphyric liver injury (Matilla and Molland, 1974; Gschnait et al., 1975; Denk et al., 1979; Kawahara et al., 1989; Woltsche et al., 1991; Cadrin et al., 1995). However, these studies have focused on changes in hepatocytes or Kupffer cells, so little is known about changes in dendritic cells (DC) in the liver.

DC are professional antigen-presenting cells, which contribute to the initiation of T cell-mediated immune responses. DC are intimately related to the liver in both normal and pathological conditions. Recently, DC have been shown to migrate from the blood to the lymph via the hepatic sinusoids (Matsuno et al., 1996; Kudo et al., 1997). It appears that this is the major migration pathway for DC from the blood, and Kupffer cells play an important role

Abbreviations: BSA, bovine serum albumin; DC, dendritic cells; PBS, phosphate-buffered saline

in this migration (Matsuno et al., 1996; Suda et al., 1996; Kudo et al., 1997). In addition, DC have been suggested to infiltrate into the liver tissues and then play a pivotal role in the development of chronic inflammatory liver diseases such as primary biliary cirrhosis, chronic hepatitis type B and type C, and autoimmune hepatitis (van den Oord et al., 1990; Kaji et al., 1997; Shinomiya et al., 1998; Tanimoto et al., 1999). However, there have been no attempts to quantify changes in hepatic DC or Kupffer cells in protoporphyric liver injury. Therefore, we examined changes in the numbers of these cells in griseofulvin-induced protoporphyric mice using immunohistochemistry to determine whether protoporphyric liver injury affects these cells and to speculate on the pathological role of these cells, particularly hepatic DC, in protoporphyric liver injury.

Materials and Methods

Twenty-nine male ICR mice, 5 weeks old and weighing about 30 g, were fed a powdered diet containing 3.5% griseofulvin (Sigma Chemical Co., St. Louis, MO) and water ad libitum. They were analyzed at 1 ($n = 5$), 2 ($n = 5$), 3 ($n = 5$), 4 ($n = 5$), 6 ($n = 5$) and 8 ($n = 4$) weeks after the commencement of griseofulvin feeding. Eight male ICR mice, 5 ($n = 5$) and 13 ($n = 3$) weeks old on a powdered diet without griseofulvin, served as controls. The 13-week-old control mice were the same age as the mice fed griseofulvin for 8 weeks. Animals were weighed and killed under diethyl ether anesthesia. The liver, spleen and lung were removed and the liver was weighed. Small pieces of these specimens were processed for histological and immunohistochemical studies.

Liver tissues were fixed in 10% formalin and processed using standard histological methods to yield paraffin sections 4 μm thick stained with hematoxylin and eosin.

Specimens of the liver, spleen and lung were snap-frozen in OCT medium with liquid nitrogen. Cryostat sections 4 μm thick were fixed in acetone for 10 min and single or double immunostaining was carried out. The single immunostaining method was as follows. After blocking nonspecific binding with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7.4) for 10 min and then with avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA), liver sections were incubated with rat anti-mouse macrophage monoclonal antibody F4/80 (diluted 1:50; Serotec, Oxford, United Kingdom) and those of the spleen and lung were incubated with rat anti-mouse dendritic cell monoclonal antibody NLDC-145 (diluted 1:25; Serotec) for 60 min (Austyn and Gordon, 1981; Witmer-Pack et al., 1995). Bound monoclonal antibody was detected with biotinylated rabbit anti-rat IgG (diluted 1:200 for F4/80, 1:1000 for NLDC-145; Vector) for 30 min. The sections were then treated with avidin-biotin-alkaline phosphatase complex (Vectastain ABC-AP Kit; Vector) for 30 min and labeled cells were colored red with alkaline phosphatase substrate kit I (Vector Red; Vector). Sections were counterstained with hematoxylin and mounted in Aquatex (Merck, Darmstadt, Germany). Since NLDC-145 recognizes not only DC but also proliferating bile ductules, we performed double immunostaining of the liver using anti-keratin antibody as follows to distinguish these structures. Endogenous peroxidase activity was eliminated by treatment with 1% H_2O_2 and 0.1% NaN_3 for 10 min. After blocking nonspecific binding with 1% BSA in PBS for 10 min, the sections were incubated with rabbit polyclonal anti-keratin, Wide Spectrum Screening (diluted 1:600; Dako, Carpinteria, CA) for 30 min to label the bile ductules (Shiojiri, 1994). The reaction of the antibody was detected using goat anti-rabbit immunoglobulin conjugated to peroxidase labeled-dextran polymer (Dako EnVision+; Dako) for 30 min and labeled cells were colored brown with 3,3'-diaminobenzidine tetrachloride (Dojin Chemical, Kumamoto, Japan). The sections were then treated with 5% normal rabbit serum in PBS for 20 min and with avidin-biotin blocking kit. Then, they were reacted with NLDC-145 and processed by the indirect immunoalkaline phosphatase method as described above. On double immunostaining, hepatic DC (red) could be distinguished from bile ductules (brown).

The number of NLDC-145-positive DC was quantified by counting cells per unit area on the sections in a blinded fashion, and the density of these cells was determined. Since the protoporphyric liver showed prominent hepatomegaly, we considered that hypertrophy of hepatocytes decreased the density of NLDC-145-positive DC. Therefore, we calculated the index of the number of DC in the whole liver as follows. We multiplied cell density (/mm^2) by liver weight (g) in each mouse and calculated the mean value of the products in each stage. Then the ratio of the value to that of the 5-week-old controls was evaluated. On the other hand, to quantify the density of Kupffer cells, we counted the number of F4/80-positive cells only in the sinusoidal area and excluded macrophages in the portal area in randomly selected fields. Since Kupffer cells were counted only in the sinusoidal area, we could not estimate the changes in Kupffer cell number in the whole liver. In these analyses, only the labeled cells where the nucleus could be identified were counted.

The density of hepatic DC and Kupffer cells was analyzed using the Mann-Whitney *U* test. A P value < 0.05 was taken to indicate statistical significance.

Results

Griseofulvin-induced protoporphyric mice showed retardation of growth. Mean body weight of these mice at 8 weeks was about 80% of that of the 13-week-old controls (Fig. 1). These mice showed prominent hepatomegaly, with the liver being dark brown in color. The livers of the mice fed griseofulvin for 8 weeks were about 3.6-fold as heavy as those of the 13-week-old controls (Fig. 1).

The intensity of histopathological changes in the liver gradually increased over the experimental period (Fig. 2). Hepatocytes showed nuclear and cytoplasmic hypertrophy, and vacuolated cytoplasm. Focal necrosis in the parenchyma occurred at all stages. Brown pigment deposits appeared 1 week after the commencement of griseofulvin feeding. They accumulated in hepatocytes, Kupffer cells, portal macrophages, bile ducts and bile ductules. Plugs of the brown pigment frequently obstructed the bile ducts and ductules. Proliferation of bile ductules was obvious at 3 weeks and became marked after 4 weeks. This was accompanied by a variable number of connective tissue elements and infiltration of neutrophils and mononuclear cells as reported previously (Weston-Hurst and Paget, 1963; Gschnait et al., 1975). These changes, the proliferation of bile ductules accompanied by inflammatory infiltration and connective tissue elements, are similar to so-called "ductular reaction". After 4 weeks of griseofulvin feeding, this ductular reaction became a marked pathological change in the injured liver. It extended from the portal tracts toward neighboring portal tracts and penetrated the parenchyma between the liver cell plates.

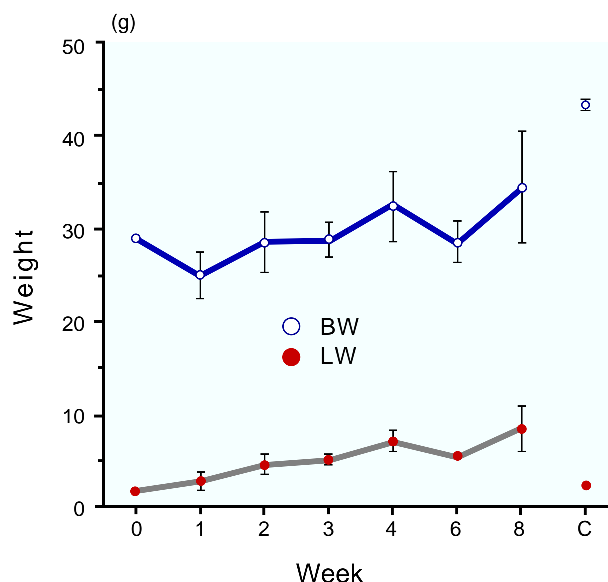

Fig. 1. Body weight (BW) and liver weight (LW) in control and griseofulvin-treated mice. Data are expressed as mean ± SD. Bars represent SD. C, 13-week-old control mice, identical in age to the mice treated with griseofulvin for 8 weeks.

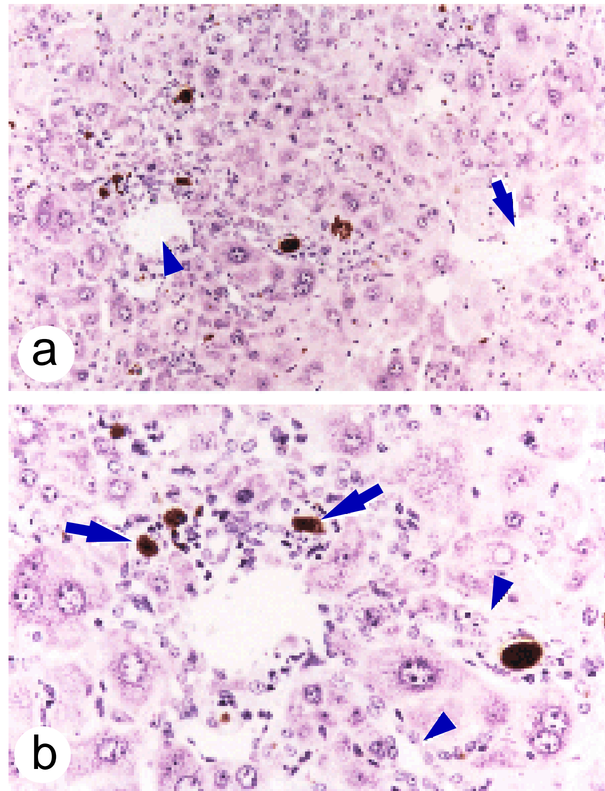

Fig. 2. Light micrographs of the liver of a mouse fed griseofulvin for 8 weeks. Hematoxylin and eosin. **a**: Brown pigment deposits are accumulated in the liver tissue. Central (arrow) and interlobular (arrowhead) veins are visible. × 120. **b**: Hepatocytes show hypertrophy. The proliferation of bile ductules is accompanied by inflammatory infiltrate and connective tissue elements, similarly to the so-called "ductular reaction". It penetrates the parenchyma between the liver cell plates (arrowheads). Note brown pigment deposits in the bile ductules (arrows). × 250.

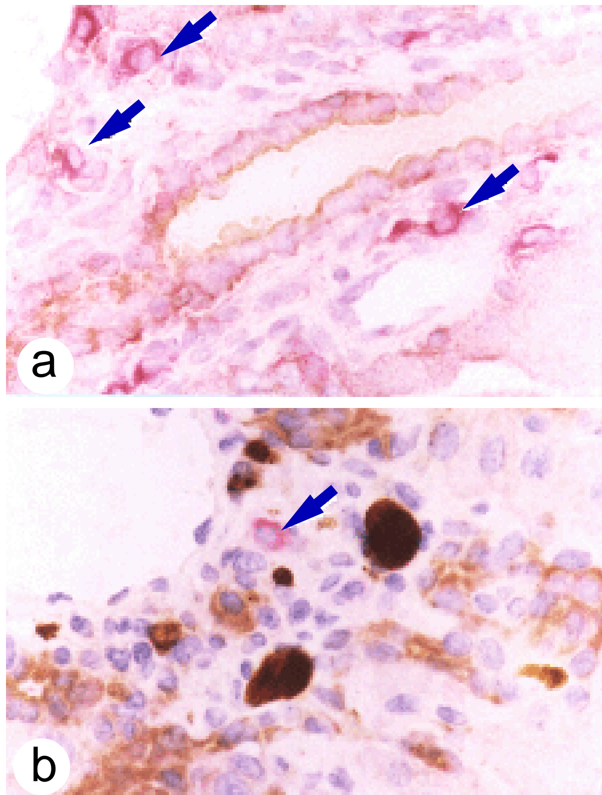

Fig. 3. Double immunostaining of the liver with monoclonal antibody NLDC-145 (red) and polyclonal anti-keratin, Wide Spectrum Screening (brown). **a**: A 5-week-old control mouse. Several NLDC-145-positive dendritic cells (DC) are visible in the portal area (arrows). × 500. **b**: A mouse treated with griseofulvin for 8 weeks. The number of NLDC-145-positive DC (arrow) is obviously decreased. Proliferating bile ductules are colored brown. × 500.

In the control liver, NLDC-145-positive hepatic DC were located mainly in the portal area and were rarely seen in the parenchyma (Fig. 3a). The density of these hepatic DC gradually decreased with the duration of griseofulvin feeding (Fig. 4). After 6 weeks, the DC disappeared in most of the portal area (Fig. 3b), and the density of the hepatic DC was less than 10% of that in the controls (Fig. 4). However, the index of the number of DC in the whole liver showed a different course of changes, increasing slightly until 4 weeks, but falling sharply at 6 weeks (Fig. 5).

On the other hand, F4/80-positive cells in the control liver were scattered in the hepatic parenchyma and the portal area (Fig. 6a). Accumulation of macrophages in the portal area was obvious at 3 weeks of griseofulvin feeding, but the increase in the density of Kupffer cells in the sinusoidal area was relatively gradual (Fig. 7). At 8 weeks on the griseofulvin diet, Kupffer cells were sometimes hypertrophic, and clusters of Kupffer cells often contained brown pigment deposits (Fig. 6b). In this stage, the density of Kupffer cells was significantly increased (Fig. 7).

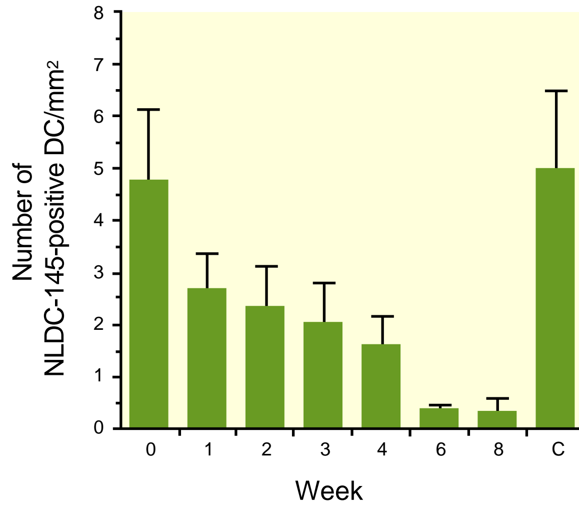

Fig. 4. Changes in the density of NLDC-145-positive DC in the liver after griseofulvin feeding. The density is significantly decreased after 1 week. Data are expressed as mean ± SD. Bars represent SD. C, 13-week-old control mice.

In the spleen and lung, no significant differences were noted in the distribution of NLDC-145-positive DC between experimental and control mice (Figs. 8 and 9). These DC were located mainly in the periarterial lymphoid sheath in the spleen, and studded the lung parenchyma.

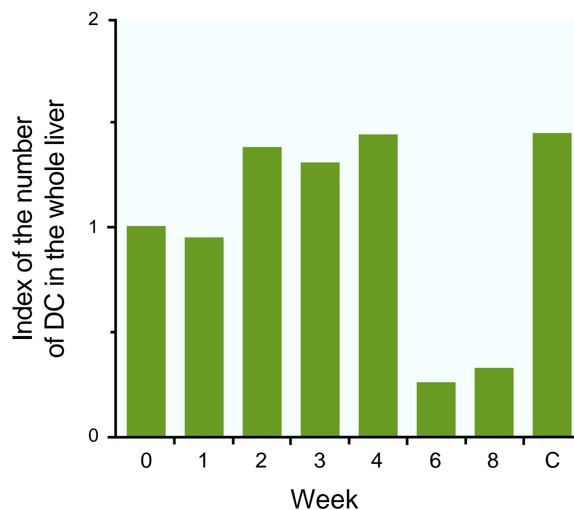

Fig. 5. Changes in the index of the number of DC in the whole liver after griseofulvin feeding. The mean value of the products [cell density (/mm^2) by liver weight (g)] was calculated in each stage, and the ratio of the value to that of the 5-week-old controls was evaluated. After 6 weeks, the index falls sharply. C, 13-week-old control mice.

Discussion

This study showed a decrease in density of NLDC-145-positive hepatic DC and an increase in that of F4/80-positive Kupffer cells in griseofulvin-induced protoporphyric liver injury.

The decrease in density of hepatic DC indicated a reduction of hepatic DC per unit volume of the liver. However, this change does not imply a decrease in number in the whole liver. Hepatomegaly, an increase in the volume of the liver, might be responsible for the decrease in density of hepatic DC. Therefore, we calculated the index of the number of DC in the whole liver. Hepatic DC number in the whole liver appeared to increase slightly until 4 weeks of griseofulvin treatment and fell sharply after 6 weeks. These results suggested that the decrease in density of hepatic DC was due to hepatomegaly until 4 weeks of treatment, and was substantial after 6 weeks.

In contrast to the decrease in number of hepatic DC, there were no definite changes in the distribution of NLDC-145-positive DC in the spleen or lung. This implied that the decrease in number of NLDC-145-positive DC was specific in the liver. To evaluate these observations, we considered the results of previous reports showing the behavior of circulating DC and turnover time of tissue DC. Intravenously transferred DC migrate mainly to the liver and spleen, and partly to the lungs and other organs (Fossum, 1988; Kupiec-Weglinski et al., 1988; Suda et al., 1996). A physiological study further showed that the turnover time of mouse splenic DC is 8–11 days (Steinman et al., 1974). If the decrease of hepatic DC was due to a reduction in number of circulating DC, splenic DC should have decreased in number in the later stage. However, splenic DC showed no definite changes after 8 weeks of griseofulvin feeding, suggesting that the number of circulating DC does not decrease in protoporphyric mice at least until about 2 months. Accordingly, it is reasonable to speculate that some changes that occurred in the injured liver caused the specific decrease in hepatic DC number in protoporphyric mice.

The liver is an important organ for blood DC to migrate to the lymph. Blood DC attach to the vessel wall in the sinusoidal area, pass through the space of Disse to the connective tissue stroma of either the portal or hepatic vein area, and then enter the initial lymphatic ducts accumulating in the regional hepatic lymph nodes via the afferent lymph (Kudo et al., 1997). It

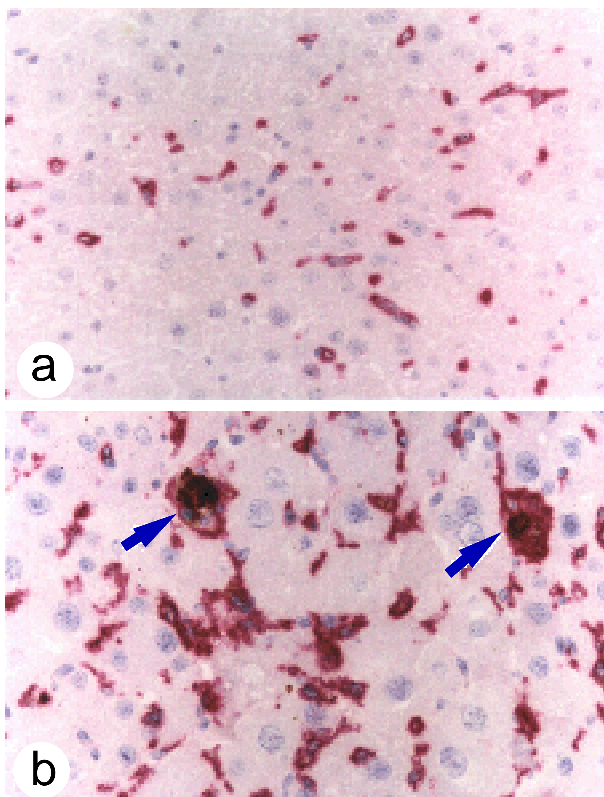

Fig. 6. Immunostaining of the liver with monoclonal antibody F4/80 for Kupffer cells (red). **a**: A 5-week-old control mouse. F4/80-positive Kupffer cells are distributed in the sinusoid. ×250. **b**: A mouse treated with griseofulvin for 8 weeks. F4/80-positive Kupffer cells are hypertrophic and markedly increased in number. Note clusters of Kupffer cells containing brown pigment deposits (arrows). ×250.

is probably important for migration that the normal hepatic structure and function are maintained. Alteration of liver structure after 4 weeks of griseofulvin feeding might interrupt the migration pathway and subsequently cause the decrease of DC in the whole liver. After 4 weeks of griseofulvin feeding, marked ductular reaction occurred in the injured liver (Shapiro and Wessely, 1984). This reaction is a characteristic change observed in the cholestasis of bile duct obstruction (Slott et al., 1990; Desmet, 1995; Roskams and Desmet, 1998). Recently, Tanimoto et al. (1999) reported that DC numbers were extremely low in the livers of patients with large bile duct obstruction. This implies that ductular reaction might cause a decrease in number of DC in the whole liver. However, ductular reaction has also been shown to occur in other liver diseases in which hepatic DC increase in number (Popper, 1986; Roskams and Desmet, 1998). Therefore, we could not conclude that the ductular reaction we found led to a decrease of hepatic DC in protoporphyric mice.

DC have been suggested to infiltrate the liver and then play a pivotal role in the development of chronic inflammatory liver diseases such as primary biliary cirrhosis, chronic hepatitis type B and type C, and autoimmune hepatitis (van den Oord et al., 1990; Kaji et al., 1997; Shinomiya et al., 1998; Tanimoto et al., 1999). In contrast, hepatic DC seem to have no significant relation to the progression of griseofulvin-induced protoporphyric liver injury as these cells decreased in number in this type of liver injury.

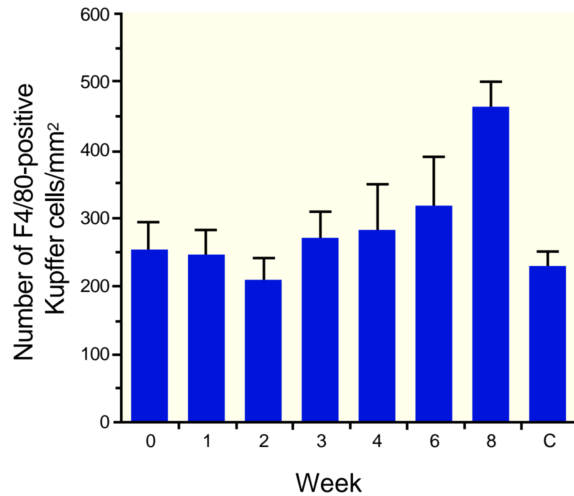

Fig. 7. Changes in the density of F4/80-positive Kupffer cells after griseofulvin feeding. The density of these cells shows a significant increase at 8 weeks. Data are expressed as mean ± SD. Bars represent SD. C, 13-week-old control mice.

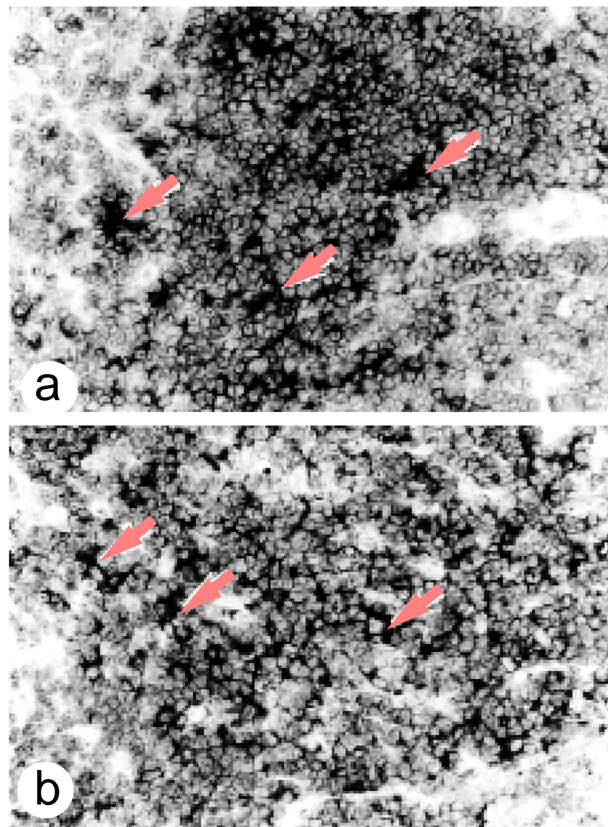

Fig. 8. NLDC-145-positive DC in the spleen of a 5-week-old control mouse (**a**) and a mouse treated with griseofulvin for 8 weeks (**b**). There are no significant differences in the distribution of splenic DC between the control and experimental animals. The splenic DC are distributed in the periarterial lymphoid sheath (arrows). × 280.

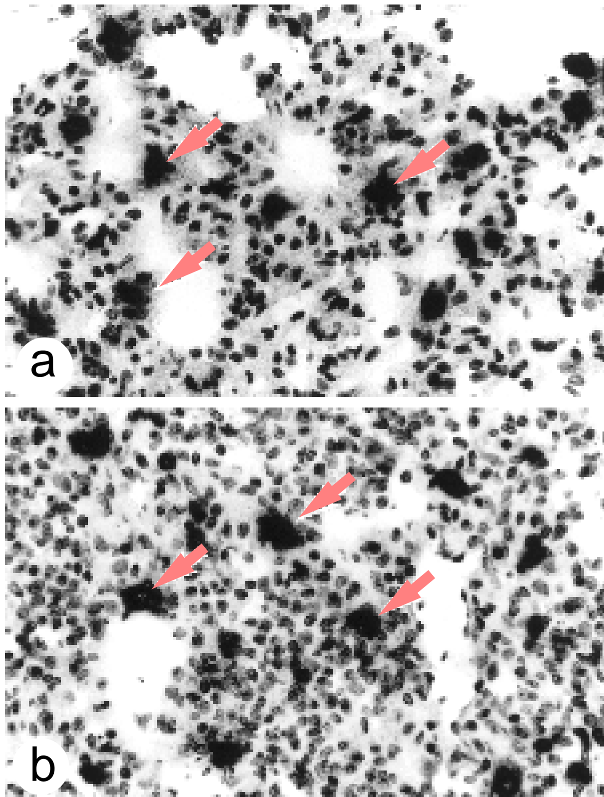

Fig. 9. NLDC-145-positive DC in the lung of a 5-week-old control mouse (**a**) and a mouse treated with griseofulvin for 8 weeks (**b**). In these sections, DC show a similar distribution in the parenchyma (arrows). × 280.

These observations raise the question of whether the decrease in hepatic DC number affects the immune system in protoporphyric mice. Previous studies showed that griseofulvin-treated mice were susceptible to bacterial infection (Weston-Hurst and Paget, 1963; Mitchell et al., 1973), and suggested a selective depressing effect of griseofulvin on the cellular immune response in mice (Mitchell et al., 1973). Recently, Matsuno et al. (1996) suggested that DC phagocytosed intravenously injected particles in the blood marginating pool and translocated from the sinusoidal area to the hepatic lymph, and these cells might initiate immune responses against pathogenic organisms such as bacteria and fungi. The susceptibility to bacterial infection and the depression of the cellular immune response in griseofulvin-treated mice might be due to the decrease in number of hepatic DC.

In protoporphyric mice, Kupffer cells are activated to phagocytose protoporphyrin crystals (Gschnait et al., 1975). However, quantitative analysis of Kupffer cells has not been performed. There are no precise markers for mouse Kupffer cells. In this study, we used a monoclonal antibody, F4/80, which recognizes macrophages including Kupffer cells in mice. To estimate the density of Kupffer cells in the liver, we counted the F4/80-positive cells only in the sinusoidal area and excluded macrophages in the portal area. Our results showed that the density of Kupffer cells gradually increased in the injured liver. Considering the effect of hepatomegaly on the density of Kupffer cells, the number of Kupffer cells in the whole liver probably increased more significantly than their density revealed. However, we could not calculate the index of the number of Kupffer cells in the whole liver from the product of their density by liver weight because they were located only in the sinusoidal area.

Recently, it was reported that Kupffer cells are involved in the migration of hepatic DC; i.e. Kupffer cells were shown to be capable of selectively trapping DC from the blood (Suda et al., 1996; Kudo et al., 1997). In the present study, since densities of DC and Kupffer cells showed opposite changes in the injured liver, the decrease of hepatic DC seemed to have no relation to Kupffer cells. However, we could not eliminate the possibility that Kupffer cells failed to trap circulating DC in the protoporphyric liver. Further studies are needed to determine whether Kupffer cells in protoporphyric mice can trap DC from the blood.

In conclusion, we demonstrated a decrease in number of hepatic DC and an increase in number of Kupffer cells in protoporphyric liver injury using an experimental mouse model and immunohistochemical methods. It seems that

the decrease in density of hepatic DC is due to hepatomegaly until 4 weeks of griseofulvin treatment, and is substantial after 6 weeks. Alteration of the liver structure might cause this substantial decrease in number of hepatic DC. Although hepatic DC seemed to have no relation to the progression of the griseofulvin-induced protoporphyric liver injury, their decrease might affect the cellular immune response in protoporphyria.

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