Virchows Arch A [Pathol Anat] (1986) 410:1–7

Virchows Archiv A

Investigations into the origin of mouse liver sinusoidal cells

N. Freudenberg [1], M.A. Freudenberg [2], C.D. Hoess [1], H. Schrecker [1], and C. Galanos [2]

[1] Pathologisches Institut der Universität Freiburg, Albertstrasse 19, D-7800 Freiburg
[2] Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-7800 Freiburg, Federal Republic of Germany

Summary. The possibility that liver sinusoidal cells are derived from the bone-marrow was investigated in chimeric mice. $H2^k$-positive bone-marrow cells from F_1 (B10.BR × B10.D2) hybrid mice were transplanted into irradiated $H2^k$-negative parental mice (B10.D2), and the liver examined immunohistochemically for the presence of $H2^k$-positive cells, with the help of an anti-$H2^k$ monoclonal antibody. With the passage of time (from the fifth week onwards), increasing numbers of transplanted bone-marrow cells enter the liver sinusoids, undergo alteration in their shape, and remain there, probably replacing sinusoidal lining cells. DNA-synthesising cells in the sinusoids were observed, suggesting, in addition, local cell proliferation. The replacement of sinusoidal cells from bone-marrow was greatly accelerated after liver damage had been induced by sublethal doses of endotoxin (LPS), and proliferation was also enhanced after treatment with LPS. These results strongly suggest that the bone-marrow participates in the replacement of liver sinusoidal cells.

Key words: Liver sinusoidal cells – Origin – Bone-marrow cells

Introduction

Liver sinusoids are lined by at least four kinds of cells: Kupffer cells, endothelial cells, fat-storing (Ito) cells and pit cells. Kupffer cells and endothelial cells actively participate in clearing foreign material by different endocytotic mechanisms (Praaning-van Dalen et al. 1982; Dan and Wake 1986). Fat-storing cells contain lipid droplets which are rich in vitamin A, and play a role in collagen formation during specific pathological processes (Enzan and Hara 1986). The function of pit cells is hitherto unknown, but they are probably involved in liver-associated "natural killer" activity (Dan et al. 1986).

The origin and replacement of sinusoidal cells, particularly of Kupffer cells, have been in dispute for many years, and it is not clear whether these cells are formed locally in the liver or not. In accordance with the concept of a mononuclear phagocyte system (Van Furth et al. 1972; Van Furth et al. 1977), monocytes derived from the bone-marrow are the precursors of all macrophages, including Kupffer cells. The opponents of this theory maintain, however, that true Kupffer cells, i.e. "resident macrophages" (Naito and Wisse 1977) can be identified in the fetal liver before the initiation of bone-marrow haematopoesis (Fukuda 1974; Naito et al. 1986). The observation that Kupffer cells and other sinusoidal cells such as endothelial cells and fat-storing cells, show mitotic activity (Naito and Wisse 1977) supports the view of these investigators that these sinusoidal cells are independent and self-replicating.

The reasons for these contradictory theories lie in the difficulty of discriminating between the different kinds of sinusoidal cells and of identifying their extra-hepatic antecedents. The present study represents an attempt to answer two questions: (1) are there any indications that the hepatic sinusoidal cells are replaced by cells from the bone-marrow, and (2) can they (or their immediate precursors) multiply locally?

Materials and methods

Inbred mice of B10.BR ($H2^k$-positive) and B10.D2 ($H2^d$-positive) strains and their F_1 hybrids (B10.BR × B10.D2, $H2^{k/d}$-positive) were obtained from the breeding stock of the Max-

Offprints requests to: N. Freudenberg at the above address

Dedicated to Prof. Dr. W. Gerok on the occasion of his 60th birthday, March 27, 1986

Planck-Institut für Immunbiologie Freiburg, FRG. Mice of both sexes and between 6 and 10 weeks old were used.

Hybridoma culture supernatant containing the monoclonal rat to $H2^k$ *antibody* R1-21.2 was kindly provided by Prof. Dr. G. Hämmerling, Deutsches Krebsforschungszentrum Heidelberg, FRG. This antibody, which belongs to the IgG_2 class of the rat strongly binds to $H2^k$ cells of the B10.BR mouse strain (Koch et al. 1983). The streptavidin-biotin kit was purchased from Amersham International. It consisted of biotin-conjugated sheep antibody to rat immunoglobulin and of peroxidase conjugated biotin-streptavidin complex. The biotin-conjugated sheep to rat immunoglobulin was absorbed with mouse serum proteins (10%) before use.

Salmonella abortus equi S (smooth) form *lipopolysaccharide* (LPS) was isolated from parent bacteria by the phenol-water method (Westphal et al. 1952), and purified by the phenol-chloroform-petroleum ether procedure (Galanos et al. 1969). The LPS was abministered intraperitoneally (1 μ/g bodyweight).

Bone marrow chimeras were prepared by injecting lethally irradiated (750 rad) recipient mice (B10.D2) with donor (F_1 hybrids) bone-marrow cells ($F_1 \rightarrow$B10.D2). 6 to 10 weeks old lethally irradiated B10.D2 mice were injected intravenously with 2×10^7 viable F_1 ($H2^{k/d}$) bone-marrow cells within 60 min of irradiation, the recipient and the donor being in each case of the same sex (Hood et al. 1984). For control experiments irradiated B10.D2 recipients received homologous B10.D2 bone-marrow cells.

During the first four weeks following irradiation and transplantation, the animals were maintained (one mouse per cage) under specific pathogen-free conditions and were given sterile food and sterile drinking water provided with antibiotics (Neomycin-sulfate, 10 mg/l, Serva, Polymyxin B-sulfate, 1 mg/l, Serva) and a special multivitamin preparation (Multivitamin-Kombination, Albrecht, Aulendorf, Württemberg, FRG). Survivors (about 80%) could be maintained under standard conditions of care without additional deaths. Animals were used for experiments after 4 weeks following chimera production.

Immunohistochemical investigations

Pairs of mice were used on each occasion. Between four and 11 weeks following irradiation and transplantation, the livers were investigated for the presence of $H2^k$-positive cells. The animals were perfused under ether anaesthesia through the left ventricle with periodate-lysine-paraformaldehyde (PLP) solution, pH 7.4 (McLean and Nakane 1974). The liver was removed, postfixed for three hours in PLP, washed three times for two hours in lysine phosphate buffer (pH 7.4 containing 1% saccharose), placed on aluminium discs on OCT-compound embedding medium (Tissue Tek, Miles), and snap frozen in 2-methylbutane in liquid nitrogen. Frozen specimens were stored at $-70°$ C until use. 6 μm cryostat sections were made, dried in air and fixed in acetone for 10 s and subjected to the immunohistochemical procedure. The avidin-biotin technique was employed to localize $H2^k$-positive cells in the liver. Briefly: after covering non-specific binding sites with 5% normal sheep serum, liver sections were incubated with diluted (1:50) anti-$H2^k$ monoclonal antibody (R1-21.2) and, after extensive washing in phosphate-buffered saline, treated with diluted (1:200) biotin-conjugated sheep-to-rat antibody. Endogenous peroxidase was inhibited by treating the sections with phenylhydrazine (0.05 g in 100 ml of 0.05 M Na_2HPO_4 solution) for 30 min at 37° C. Then the sections were incubated with diluted (1:300) peroxidase-conjugated biotin-streptavidin complex, washed and stained for peroxidase activity (Freudenberg et al. 1982). After peroxidase-staining the specimens were either counter-stained with haemalum of left unstained. $H2^k$-positive B10.BR mice made satisfactory positive controls. $H2^k$-negative B10.D2 animals served as negative controls, and were subdivided into four groups as follows: (1) untreated animals, (2) animals treated with endotoxin alone, (3) irradiated animals subjected to homologous bone-marrow transplantation and (4) irradiated animals subjected to both homologous bone-marrow transplantation and endotoxin treatment.

^3H thymidine labeling and autoradiography

Groups of three radiation chimeras were treated as described below 2, 4, 8 and 14 days after LPS injection. A group of three radiation chimeras and of untreated (non-irradiated) B10.D2 mice, both without LPS treatment served as a control.

Each experimental animal received three intraperitoneal injections of 0.5 μCi/g bodyweight of ^3H thymidine (New England Nuclear, 17 Ci/mmol) dissolved in 0.5 ml PBS. The labeling schedule was applied 17, 9 and 1 h prior to the death of each animal (Schwartz and Benditt 1973). The mice were sacrificed under ether anaesthesia, and the liver was removed, fixed in formaldehyde and embedded in paraffin in the usual way. 5 μm sections were made and mounted on cover slips.

The autoradiographic procedure was carried out in the standard way, using Ilford^R K_5 photographic emulsion. After 9 days of exposure in the dark, the photographic film was developed and fixed. The specimens were stained with haematoxylin and mounted in resin.

In each liver section 2,000 sinusoidal liver cells were counted in subcapsular and intraparenchymal areas. For each time of observation the labelled cells (i.e. showing more than five silver granules overlying the nucleus) were exposed to a randomized schedule. In this way estimations were obtained for frequencies of occurrence of labelled cells for each time of observation.

Results

Cryostat sections of mouse livers were examined for the presence of $H2^k$ antigen, using a monoclonal anti-$H2^k$ antibody as described in Materials and methods. A very strong $H2^k$ antigenic activity was detected in livers of B10.BR parental mice (Fig. 1a). The activity was associated with cell membranes mainly in the region of the sinusoids, and was especially intense in the vicinity of the portal tracts. It was not possible to identify the type of the sinusoidal lining cells morphologically. Liver sections of the B10.D2 parental mice were, as expected, completely negative when tested for the $H2^k$ antigen (Fig. 1b). A positive $H2^k$ reaction with a pattern of the antigenic distribution comparable to that of the B10.BR strain was found in liver sections of F_1 hybrids. Here, the intensity of the antigen-specific staining – although strong – was evidently weaker than in the B10.BR parental strain.

The above results show that the monoclonal antibody was able to distinguish between sinusoidal cells of B10.D2 ($H2^k$-negative) and F_1 ($H2^k$-

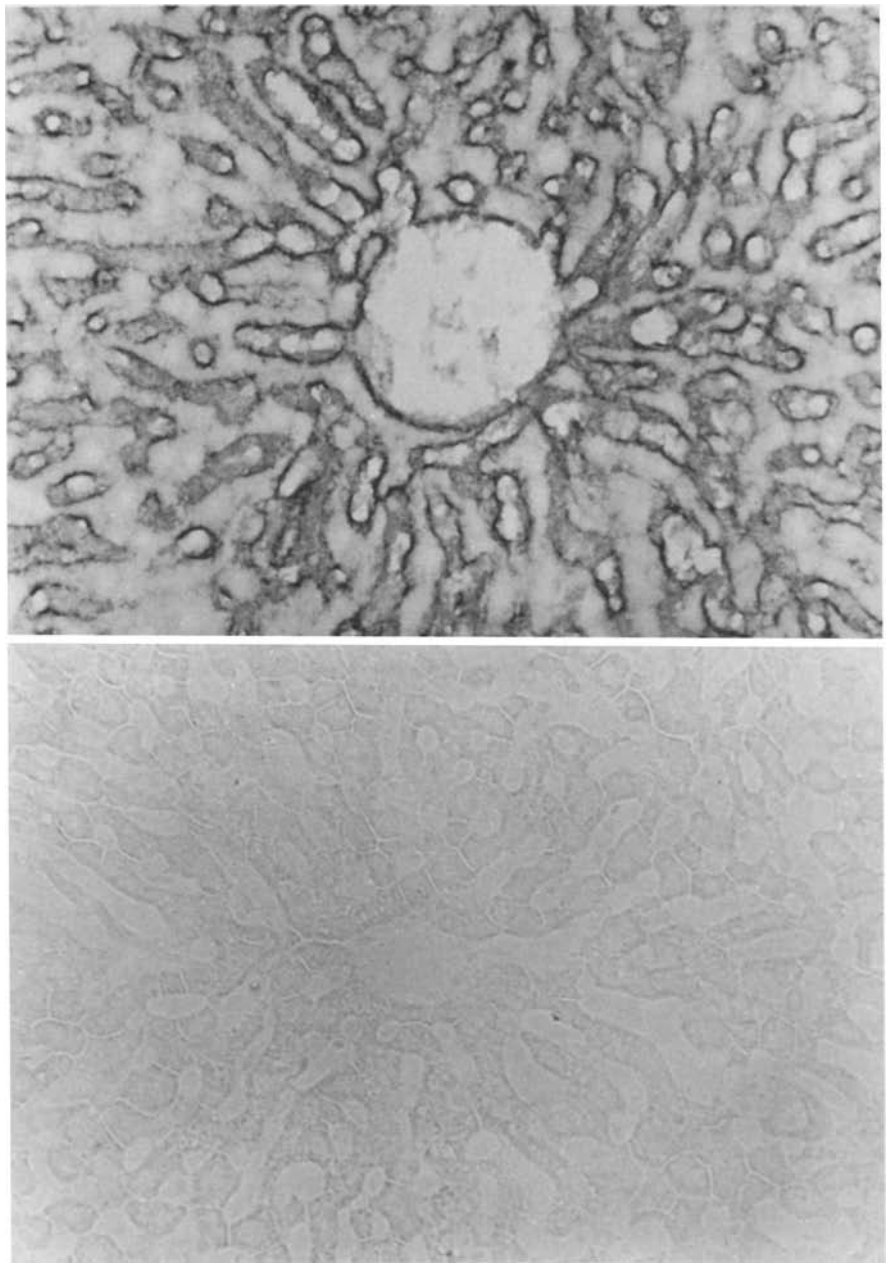

Fig. 1 a, b. Immunohistochemical staining of the $H2^k$ antigen in mouse liver with a rat-to-mouse monoclonal antibody (R1-21.2) using the avidin-biotin technique. No counterstaining. Original magnification ×250. **a** B10.BR ($H2^k$-positive: black staining of cell membranes). **b** B10.D2 ($H2^k$-negative)

positive) origin. In the following experiments, this antibody was used for the detection of the possible appearance of $H2^k$-positive cells in livers of B10.D2 mice injected with bone-marrow cells of F_1 hybrids.

When $F_1 \rightarrow$B10.D2 chimeras were maintained under special pathogen-free condition for four weeks, $H2^k$ antigen activity could already be detected in the liver during the following (i.e. the fifth) week. Thereafter an increasing number of $H2^k$-positive cells could be observed in the sinusoids. Initially, these cells were round in appearance, but changed into rod-like structures during the later stages of the experiment (Fig. 2). They persisted in the liver attached to the sinusoids for the whole time of observation (up to the 11^{th} week after chimera production).

These results suggest a gradual replacement of sinusoidal lining cells by bone-marrow cells. In order to increase the process of replacement of sinusoidal cells, liver damage was produced by sublethal amounts of endotoxin, four weeks after irradiation and transplantation. Animals received LPS (1 μg/g bodyweight) intraperitoneally, and the liver was examined histologically and immunohistochemically at different times thereafter.

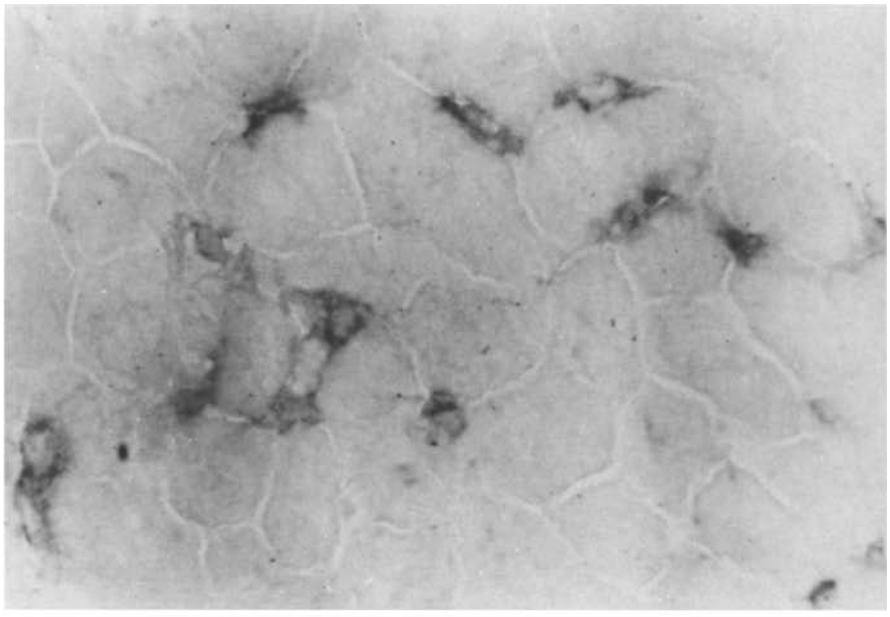

Fig. 2. Liver section of a mouse radiation chimera ($F_1 \rightarrow$B10.D2), 11 weeks after irradiation and transplantation alone. Note the $H2^k$-positive cells (black, mostly rod-like in shape) lining the liver sinusoids. Immunohistochemical staining with the avidin-biotin technique. No counterstaining. Original magnification ×400

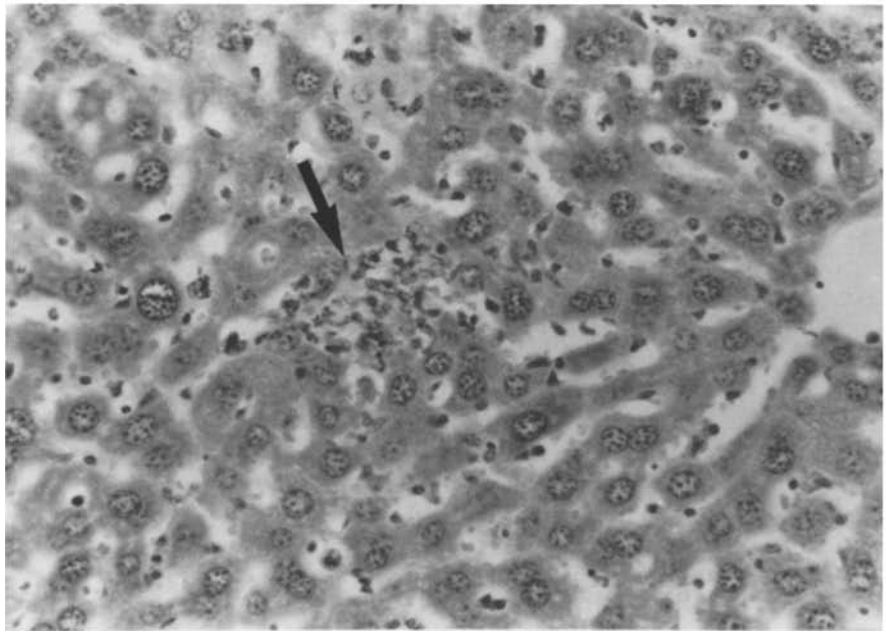

Fig. 3. Infiltration of damaged liver tissue with mononuclear cells and granulocytes (*arrow*), 12 h following LPS injection H&E staining. Original magnification ×250

Between 40 min and 6 h a pronounced increase in the number of leukocytes was seen in the sinusoids. From the 6th h onward liver cell necroses were detected, the damaged hepatic areas showing infiltration with mononuclear cells and granulocytes (Fig. 3). The intensity of the inflammatory tissue reaction began to decrease one week after endotoxin injection, and on day 15 histological alterations were no longer detectable.

Treatment of the animals with LPS caused a rapid influx of a large number of $H2^k$-positive cells (i.e. cells originating from transplanted F_1-hybrid bone-marrow) into the liver. 40 min after LPS injection such cells were first detectable in the periportal regions of the liver; 60 min after LPS numerous cells were seen in the sinusoids, some of them also near central veins. 90 min after endotoxin the liver showed a dense infiltration with $H2^k$ positive cells, mainly within the sinusoids and close to the intima of the central veins. Morphologically these cells could be identified as mononuclear cells and granulocytes. Infiltration of $H2^k$-positive granulocytes was seen mostly in the necrotic areas of the liver tissue. These cells persisted for about one week after LPS injection and their disappearance paralleled the recovery of necrotic lesions. In

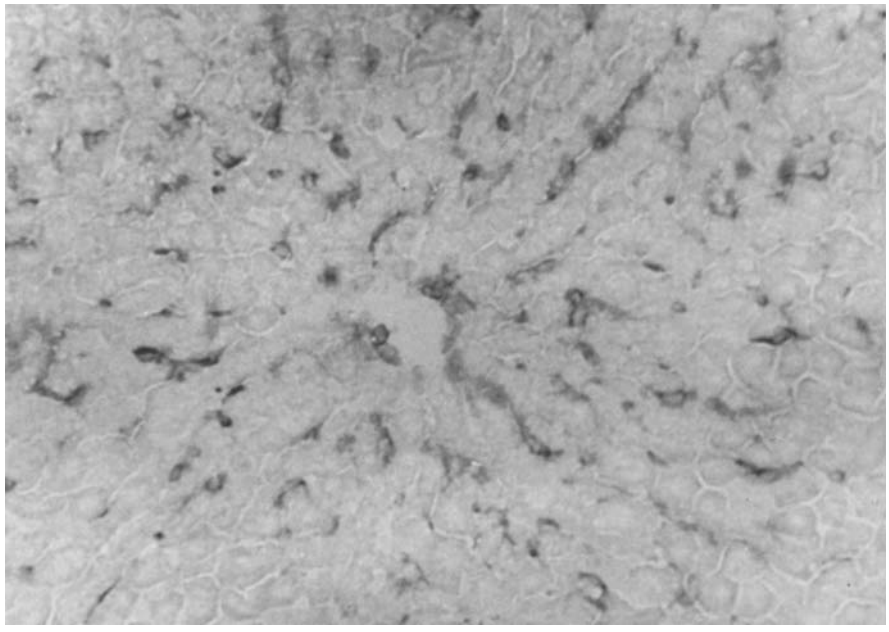

Fig. 4. Liver section of a mouse radiation chimera ($F_1 \rightarrow$B10.D2), 4 weeks after chimera production, and 5 days following i.p. injection of endotoxin. Note the dense infiltration with $H2^k$-positive bone-marrow derived cells in the sinusoids and in the intima of the central vein. Immunohistochemical staining with the avidin-biotin technique. No counterstaining. Original magnification $\times 160$

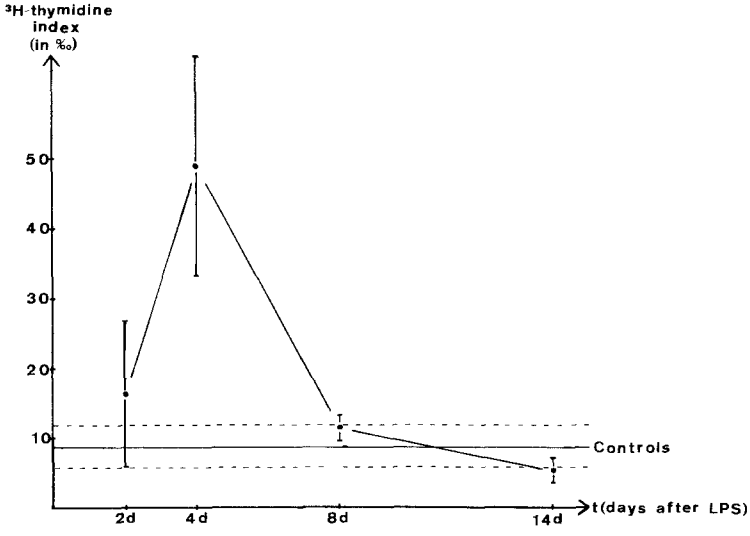

Fig. 5. Incorporation of ^3H thymidine in sinusoidal liver cells (in ‰) following an intraperitoneal injection of a sublethal amount of endotoxin into mouse radiation chimeras. Controls: chimeras without endotoxin

contrast, $H2^k$-positive mononuclear cells persisted in the sinusoids during the 7 week observation period after LPS treatment. From the fifth day after endotoxin injection, they progressively changed in shape from round to rod-like (Fig. 4), and appeared to be attached to the sinusoids.

DNA synthesis in sinusoidal cells was measured by autoradiography following injection of ^3H thymidine. Non-irradiated B10.D2 mice (untreated with LPS) showed $1 \pm 0.7‰$ ^3H thymidine-labelled sinusoidal cells.

Incorporation of ^3H thymidine in sinusoidal liver cells of LPS treated chimeras is illustrated graphically in Fig. 5. In the control animals (chimeras untreated with endotoxin) an average of 8 per 1,000 sinusoidal cells showed ^3H-labelled nuclei. After LPS treatment the number of radiolabelled cells increased with time, showing a maximum on day 4 with 49 ± 15.6 labelled cells. Thereafter the number of DNA synthesising cells in the sinusoids decreased, reaching nearly the control level on day 8 with $11.8 \pm 1.8‰$.

Discussion

The results of the present study suggest strongly that sinusoidal lining cells in the liver may be, at least in part, derived from bone-marrow precursor cells.

Four weeks after transplantation of $H2^k$-posi-

tive bone-marrow cells in irradiated $H2^k$-negative recipients, sinusoidal lining cells showed signs of proliferation, the number of cells undergoing DNA synthesis being 8 times as high as in the non-irradiated control animals. During this time $H2^k$ antigen was present in a significant number of cells, indicating that "homing" of transplanted cells had already taken place in the liver. It could not be decided whether the DNA synthesising cells observed in the sinusoids represented local proliferating cells, cells derived from transplanted bone-marrow or both. The continuous increase in the number of $H2^k$-positive cells seen in the liver of chimeric mice suggests that transplanted bone-marrow cells were participating in the replacement of sinusoidal cells.

The results also demonstrate that the bone-marrow reservoir may play an important role in the replacement of sinusoidal cells during acute liver damage. Treatment of mice with sublethal amounts of endotoxin resulted in a very fast influx of a large number of $H2^k$ positive granulocytes and mononuclear cells into the liver. On account of the persistence of mononuclear cells attached to sinusoids and the change in their morphology it is concluded that they replaced damaged sinusoidal cells. The appearance of granulocytes in the liver and their disappearance after histological lesions had subsided, is interpreted as an inflammatory response to the damage caused by endotoxin.

Following endotoxin treatment, an increase in the number of ^3H thymidine-incorporating cells in the sinusoids with maximum on day 4 was also seen, which suggests increased local proliferation. However, the identity of these proliferating cells is not known. A comparable proliferation of liver sinusoidal cells after endotoxin treatment has already been observed in rats (Freudenberg et al. 1983). The present findings are in agreement with results of earlier investigations, which support the bone-marrow origin of sinusoidal macrophages. Shand and Bell (1972) showed in their immunohistochemical study that some hepatic macrophages in xenogeneic radiation chimeras are derived from the bone-marrow stem-cells. Using sex-chromatin as a marker Gale and his co-workers (1978) were also able to demonstrate the bone-marrow origin of liver macrophages. With the help of ^3H thymidine labelling, Van Furth and his co-workers (1977) demonstrated the continuing replacement of Kupffer cells by circulating monocytes.

The opponents of this concept of a mononuclear phagocyte system emphasize the necessity of distinguishing between true Kupffer cells (also called "resident macrophages") and "exudate-type macrophages" in the liver, which can be recognised by the distribution of their peroxidase activity (Naito and Wisse 1977). These authors claim that "resident macrophages" (i.e. Kupffer cells) are self-replicating cells, and not derived from the bone marrow. "Exudate-type macrophages" are believed to be monocytes originally, which differentiate in the liver and leave the organ after a short margination time (Bouwens et al. 1986).

Our results showing a continuous increase in the number of bone-marrow cells in the sinusoidal lining, a change in morphology and long persistence in the liver strongly suggests differentiation of bone-marrow cells into liver sinusoidal cells. These suggestions are in good agreement with the cytochemical and ultrastructural findings of Deimann and Fahimi (1979), who described the appearance of transitional forms between monocytes and Kupffer cells in the liver of rats treated with glucan. Whether bone-marrow cells are really differentiated into Kupffer cells in our case is a question which requires further investigation.

To summarize: these results emphasize the importance of the bone-marrow reservoir, not only for the "physiological" replacement of sinusoidal cells in chimeras, but also for the emergency replacement of littoral cells following liver damage.

Acknowledgments. We express our thank to Dr. J. Guzman for valuable advice in performing special tissue preparations for immunohistochemistry, and to our colleague Dr. F. Steel for discussions on the preparation of the English manuscript. We are very grateful to U. Pein, H. Stübig, S. Chaudhuri, and U. Wiehle for expert technical assistance. This study was supported by grants of the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg (SFB 154) to N.F. and C.G.

References

Bouwens L, Baekeland M, Wisse E (1986) A balanced view on the origin of Kupffer cells. In: Kirn, A., Knook, DL, Wisse E (eds) Cells of the hepatic sinusoid. Rijswijk: The Kupffer cell foundation, vol 1: Proceedings of the 3rd Int. Kupffer cell Symposium held in Strasbourg, pp 7–12

Dan CH, Wake K (1986) Endocytosis of latex particles in the endothelial cells of perfused liver. In: Kirn A, Knook DL, Wisse E (eds) Cells of the hepatic sinusoid. Rijswijk: The Kupffer cell foundation, vol 1: Proceedings of the 3rd Int. Kupffer cell Symposium held in Strasbourg, pp 503–504

Dan CH, Kaneda K, Wake K (1986) Pit cells and liver associated natural killer activity following administration of OK-432. In: Kirn A, Knook DL, Wisse E (eds) Cells of the hepatic sinusoid. Rijswijk: The Kupffer cell foundation, vol 1: Proceedings of the 3rd Int. Kupffer cell Symposium held in Strasbourg, France, pp 427–432

Deimann W, Fahimi HD (1979) The appearance of transition forms between monocytes and Kupffer cells in the liver of rats treated with glucan. J Exp Med 149:883–897

Enzan H, Hara H (1986) Ito cells (Fat-storing cells) and collagen formation in carbon tetrachloride induced liver fibrosis. In: Kirn A, Knook DL, Wisse E (eds) Cells of the hepatic sinusoid. Rijswijk: The Kupffer cell foundation, vol 1: Proceedings of the 3rd Int. Kupffer cell Symposium held in Strasbourg, pp 233–238

Freudenberg MA, Freudenberg N, Galanos C (1982) Time course of cellular distribution of endotoxin in liver, lungs and kidneys of rats. Br J Pathol 63: 56–65

Freudenberg N, Riese KH, Freudenberg MA (1983) The vascular endothelial system. Gustav Fischer Verlag, Stuttgart New York

Fukuda T (1974) Fetal haemopoesis. II Electron microscopic studies on human hepatic haemopoesis. Virchows Arch [Cell Pathol] 16:249–270

Galanos C, Lüderitz O, Westphal O (1969) A new method for the extraction of R-lipopolysaccharides. Eur J Biochem 9:245–249

Gale RP, Sparkes RS, Golde DW (1978) Bone marrow origin of hepatic macrophages (Kupffer cells) in humans. Science 201:937–938

Hood LE, Weissman IL, Wood WB, Wilson JH (1984) Tissue transplantation. In: Immunology 2nd edition, Chapter 11. The Benjamin/Cummings Publishing Company, INC. Menlo Park, California, Reading Massachusetts, London, Amsterdam, Don Mills, Ontario, Sidney, pp 402–435

Koch S, Koch N, Robinson P, Hämmerling G (1983) Comparison of allogenic and xenogeneic determinants on the H-2K^k molecule. Transplantation 36:177–180

McLean IW, Nakane PF (1974) Periodate-lysine-paraformaldehyde fixative; a new fixative for immunoelectron microscopy. J Histochem Cytochem 22:1077–1083

Naito M, Wisse E (1977) Observations on the fine structure and cytochemistry of sinusoidal cells in fetal and neonatal rat liver. In: Wisse E, Knook DL (eds) Kupffer cells and other liver sinusoidal cells. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 497–505

Naito M, Yamamura F, Takeya M, Takahashi K (1986) Ultrastructural analysis of Kupffer cell progenitors. In: Kirn A, Knook DL, Wisse E (eds) Cells of the hepatic sinusoid. Rijswijk: The Kupffer cell foundation, vol 1: Proceedings of the 3rd Int. Kupffer cell Symposium held in Strasbourg, pp 13–20

Praaning-van Dalen DP, De Leeuw MA, Brouwer A, De Ruiter CF, Knook DL (1982) Ultrastructural and biochemical characterisation of endocytic mechanisms in rat liver Kupffer and endothelial cells. In: Knook DL, Wisse E (eds) Sinusoidal liver cells. Elsevier Biomedical Press, Amsterdam, pp 271–278

Schwartz SM, Benditt EP (1973) Cell replication in the aortic endothelium: A new method for study of the problem. Lab Invest 28:699–707

Shand EL, Bell EB (1972) Studies on the distribution of macrophages derived from rat bone marrow cells in xenogeneic radiation chimeras. Immunology 22:549–556

Van Furth R, Cohn ZA, Hirsch JG, Humphrey, JH, Spector WG, Langevoort HL (1972) The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. Bull WHO 46:845–853

Van Furth R, Crofton RW, Diesselhoff-den-Dulk MMC (1977) The bone marrow origin of Kupffer cells. In: Wisse E, Knook DL (eds) Kupffer cells and other sinusoidal cells. Elsevier/North Holland Biomedical Press, Amsterdam, pp 471–480

Westphal O, Lüderitz O, Bister F (1952) Über die Extraktion von Bakterien mit Phenol/Wasser. Z Naturforsch 7b:148–155

Accepted June 25, 1986