Virchows Archiv A Pathol Anat (1989) 415:203–209

Virchows Archiv A
Pathological Anatomy and Histopathology

Mechanism of replacement of non-parenchymal liver cells (NPLC) in murine radiation chimeras

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Summary. The mechanism of cell replacement of non-parenchymal liver cells (NPLC) was investigated in an attempt to answer the question: to what extend are NPLC replaced by proliferation of local resident cells and to what extend by cells originating from the bone-marrow. The bone-marrow of mice was used, and $H2^k$ positive cells from F1 (B10.BR X B10.D2) hybrid mice were transplanted into irradiated $H2^k$ negative parent animals. Their NPLC were isolated and tested immunocytochemically with a monoclonal anti-$H2^k$ antibody for the presence of $H2^k$ positive cells. During the whole of the experiment (from the 5^{th} to the 20^{th} week following transplantation) $H2^k$ positive cells (macrophages and non-macrophages) were present, and made up an average of nearly one third of the NPLC. The $H2^k$ positive (immigrant) non-macrophages showed essentially more active DNA synthesis than these $H2^k$ negative cells. To increase cell turnover, we injected one group of animals with endotoxin. At the time of maximum replacement, more than 50% of the NPLC (macrophages and non-macrophages) were recruited from cells of bone-marrow origin. Noteworthy here was the proportionate level of the $H2^k$ positive macrophages (more than 70% of all liver macrophages). The DNA synthesis of both the $H2^k$ positive macrophages and non-macrophages was more than twice that of the $H2^k$ negative NPLC during the regeneration process following administration of endotoxin. Our observations suggest that the bone-marrow contributes significantly to the replacement of macrophages and non-macrophages of NPLC in both health and disease.

Key words: Non-parenchymal liver cells – Replacement – Bone-marrow – Endotoxin

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Introduction

The non-parenchymal liver cells (NPLC) consist of morphologically and functionally different types of cells, the identification of which is not complete. Today it is usual to distinguish the sinusoidal endothelium, macrophages and pit cells from the perisinusoidal fat-storing cells, and also, in the portal fields, the bile canalicular epithelium from connective-tissue cells.

The sinusoidal and perisinusoidal NPLC are of particular interest because of their close contact to the flowing blood on one side and to the hepatocytes on the other. The most numerous of the sinusoidal cells are the endothelial cells, which, in the mouse make up 50% of all NPLC (Freudenberg et al. 1989). The sinusoidal endothelial cells can be recognised under the electron microscope by the sieve-like structure of their cytoplasm. Their principal function is to serve as a selective barrier between the liver parenchyma and the blood. The macrophages are the second most numerous of the NPLC (23% in the mouse: Freudenberg et al. 1989). Morphologically they are seen as large mononuclear cells which are easiest to recognise when performing their principal function: phagocytosis. The endocytosis of a number of different substances and the liberation of mediators are today considered to be the most important functions of liver macrophages. The pit cells are morphologically lymphoid cells containing several typical organelles (Kaneda and Wake 1983). They are distributed among the cell population of the sinusoid and are regarded as "natural killer cells" (Bouwens and Wisse 1989). The perisinusoidal fat-storing cells can be recognised ultrastructurally by the lipid droplets in their cytoplasm. These fatty vacuoles contain vitamin A. It can also be shown that these

cells are capable of forming collagen under pathological conditions (Enzan et al. 1986). In addition to those mentioned above, we have recently been able to identify a further group of immunocompetent cells among the NPLC (Freudenberg et al. 1989). These include T cells, B cells and also asialo GM1 positive cells, which sometimes resemble typical pit cells, and sometimes appear as large vacuolated cells.

The mechanism by which sinusoidal cells are replaced in the liver has long remained an unsolved problem. Because of their position as littoral cells and their well known mobility, it is considered likely that at least the macrophages are renewed from the bloodstream. According to the concept of a mononuclear phagocyte system (Van Furth et al. 1977), the macrophages are regarded as being derived from the monocytes of the bone-marrow. The opponents of this theory believe they can prove that socalled resident macrophages are replaced only by local proliferation (Bouwens et al. 1986). We were, however, able to demonstrate in an earlier investigation that in chimeras the sinusoidal liver cells can be replaced by cells from the bone-marrow (Freudenberg et al. 1986). In that study bone-marrow cells of F1 hybrids (B10.BR X B10.D2, $H2^{k/d}$ positive) were used to reconstitute lethally irradiated B10.D2 mice ($H2^{d}$ positive, $H2^{k}$ negative). The bone-marrow cells in such mice could then be identified by the presence of the $H2^{k}$ antigenic marker. Using a monoclonal $H2^{k}$ antibody, $H2^{k}$ positive resident cells were detected in the sinusoidal lining of the liver several weeks after transplantation.

In the present study using the above chimeric mice, the identity of the NPLC as macrophages and non-macrophages, their proliferative activity as well as the presence of the $H2^{k}$ antigen in these cells was investigated. It will be shown that the cells replacing NPLC to a larger extend are recruited from the transplanted bone-marrow and to a lesser extend derive from proliferating $H2^{k}$ negative cells.

Material and methods

Inbred mice B10.BR ($H2^{k}$ positive) and B10.D2 ($H2^{k}$ negative) strains and their F1 hybrids (B10.BR X B10.D2, $H2^{k}$ positive) were obtained from the breeding stock of the Max-Planck-Institut für Immunbiologie, Freiburg, FRG. Male and female mice between 6 and 10 weeks old were used.

Hybridoma culture supernatant containing the *monoclonal rat to $H2^{k}$ antibody* R1-21.2 was prepared in the Deutsches Krebsforschungszentrum Heidelberg, FRG. This antibody, which belongs to the IgG_2 class of the rat, binds strongly 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 peroxidase conjugated biotin-streptavidin complex. The biotin-conjugated sheep to rat immunoglobulin was absorbed with mouse serum protein (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 administered intraperitoneally (1 µg/g bodyweight) four days prior to the investigation of the liver cells.

Bone-marrow chimeras were prepared by injecting lethally irradiated (850 rad) recipient mice (B10.D2) with donor (F1 hybrids) bone-marrow cells (F1→B10.D2). The lethally irradiated B10.D2 mice were injected intravenously with 2×10^7 viable F1 ($H2^{k}$ positive) 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 were given 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 containing antibiotics (Neomycin-sulfate, 10 mg/l, Serva, Polymyxin-bisulfate, 1 mg/l Serva) and a special multivitamin-preparation (Multivitamin-Kombination, Albrecht, Aulendorf, Württemberg, FRG). Survivors (about 90%) could be maintained under standard conditions without additional deaths. Animals were used for experiments from 5 weeks or more after chimera production.

All animals used for isolation of NPLC were injected with latex beads intravenously and immediately thereafter with ^3H thymidine intraperitoneally. Five, 12, 15–16 and 20 weeks after irradiation and transplantation groups of two to four chimeras were treated as described below. A further group of three radiation chimeras (between the 15th and 16th week) were investigated on the fourth day after LPS injection.

20 µl latex beads, 1.1 µm mean diameter (LB-Polystyrene, Sigma) in 80 µl 0.9% NaCl solution, were injected into the tail vein, 1 h prior to perfusion of the liver. This period of time for maximum cellular phagocytosis had been decided in a preliminary investigation.

To isolate and fix NPLC, following preperfusion with Instamed RPMI 1640 medium (Biochrom KG) – which results in a total decolorisation of the liver within a few seconds – perfusion with 0.8% pronase (Merck), and then with a pronase/collagenase solution (9 mg collagenase Typ I CLS, 350 u/mg, Seramed, Biochrom KG, and 4 mg pronase, E. Merck, 9.500 u/g dissolved in 25 ml Instamed) was carried out, followed by incubation in collagenase (21 mg collagenase in 15 ml RPMI) at 37° C for 30 min.

The NPLC suspension was freed of red blood cells by density centrifugation with Nycodenz (Nyceguard & Co., Oslo). For further investigation, $10–15 \times 10^4$ NPLC were each anchored on reaction fields by the slide adhesion method introduced by Bross et al. (1978). For the detection of surface-bound $H2^{k}$ antigen, the cells were treated with 0.05% glutaraldehyde (Merck) in 0.1 M Soerensen buffer pH 7.8.

$H2^{k}$ positive cells were identified by using the streptavidin-biotin technique. After the non-specific binding sites had been covered with 5% normal sheep serum, the NPLC were incubated with diluted (1:50) anti $H2^{k}$ monoclonal antibody (R1–21.2) and, after extensive washing in NKH buffer (4 g NaCl, 0.2 g KCl, 1 ml Hepes pH 7.4 ad 500 ml aqua bidest.), treated with diluted (1:200) biotin-conjugated sheep-to-rat antibody, being absorbed by 10% normal mouse serum. After rinsing, the NPLC were incubated with diluted (1:300) peroxidase-conjugated biotin-streptavidin complex, washed and stained for peroxidase activity with 0.03% H_2O_2 and 0.045% diaminoben-

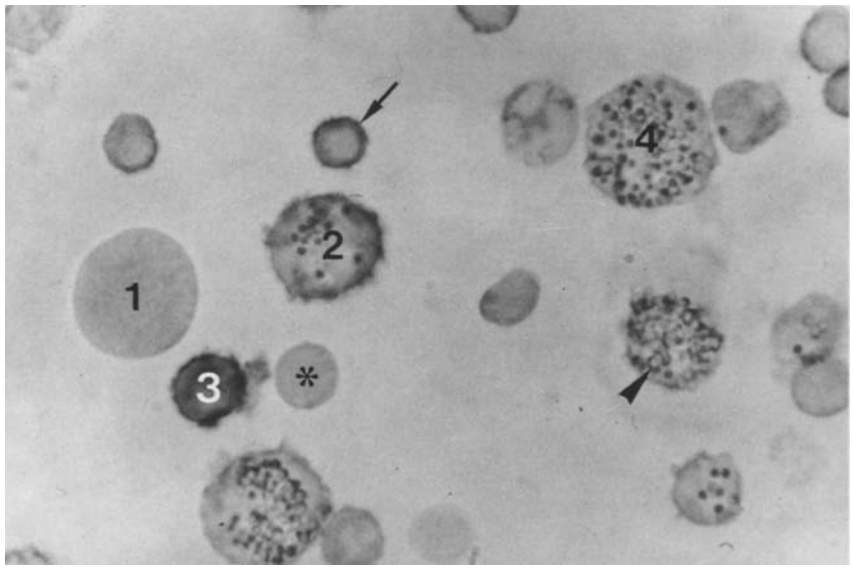

Fig. 1. Light microscopic demonstration of both macrophages (with phagocytosed latex beads ▶), non-macrophages (without latex beads ✱), and of $H2^k$ positive (black reaction product on the cell surface →) and $H2^k$ negative cells (without immuno-reaction). 1 = $H2^k$ negative non-macrophage, 2 = $H2^k$ positive macrophage, 3 = $H2^k$ positive non-macrophage, 4 = $H2^k$ negative macrophage

zidine in NKH buffer. After washing in buffer, the cells were postfixed with 2% OsO_4 in 0.1 M phosphate buffer (pH 7.4) on ice for 10 min. Cell preparations lacking the first (anti $H2^k$) antibody served as controls for the detection of endogenous peroxidase.

For autoradiography each experimental animal received one intraperitoneal injection of 0.5 μ Ci/g bodyweight of ^3H thymidine (New England Nuclear, 17 Ci/mmol) dissolved in 0.5 ml PBS, one h before death. It was known from earlier investigation (Freudenberg et al. 1986) that maximum of DNA synthesis is found four days after the administration of endotoxin. A group of three untreated B10.D2 mice served as controls, in which the percentages of macrophages and non-macrophages, as well the DNA synthesis were determined. The autoradiographic procedure was carried out in the standard way, using Ilford^R K5 photographic emulsion. After 8 days of exposure in the dark, the photographic film was developed and fixed. The specimens were mounted unstained in resin.

The percentage frequency of macrophages and non-macrophages as well as of the $H2^k$ positive and of the ^3H labelled cells was determined among the NPLC. The counting procedure was carried out under the light microscope on the reaction fields of the slides, each of which contained an average number of 40.000 cells. Using a randomizing procedure, 2.000 NPLC from each animal were counted. The mean values and standard deviations of these values were calculated.

Results

Chimeras were produced by transplanting $H2^k$ positive bone-marrow cells of F1 hybrid mice (B10.BR X B10.D2) into irradiated $H2^k$ negative animals (B10.D2). 84% of the transplanted bone-marrow cells from F1 hybrids showed surface expression of the $H2^k$ antigen. By using both latex phagocytosis carried out in vivo, and the immunocytochemical demonstration of the $H2^k$ antigen, it was possible to distinguish in isolated NPLC between $H2^k$ positive and $H2^k$ negative macrophages and non-macrophages (Fig. 1).

The untreated B10.D2 animals had a percentage distribution of 77% non-macrophages and 23% macrophages among the NPLC. In the 5th week after transplantation, the NPLC of radiation chimeras were composed of 68% non-macrophages and 32% macrophages. From the 12th to the 16th week the percentage distribution had nearly reached the values of untreated B10.D2 mice, with 75% non-macrophages and 25% macrophages. In the 20th week after transplantation, however, the non-macrophages showed an increase in number to 79%, corresponding to 21% macrophages.

During the whole observation period of the experiment (from the 5th to the 20th week following transplantation), $H2^k$ positive cells were present in the liver. In the 5th week, 40 ± 6% of all NPLC were $H2^k$ positive. In the later weeks of the experiment, the number of $H2^k$ positive cells that had wandered into the liver had been reduced to 28 ± 5% (12th week), 30 ± 2% (15th/16th week) and finally to 27 ± 3% (20th week).

If one calculates the percentages of $H2^k$ positive macrophages and non-macrophages among the total populations of macrophages and non-macrophages (Fig. 2), it can be seen that, in the case of macrophages, the number of $H2^k$ positive cells has fallen by almost half between week 5 (67 ± 6%) and week 20 (36 ± 6%). However, the $H2^k$ positive non-macrophages remained unchanged (average percentage 22%).

DNA synthesis of NPLC was measured by au-

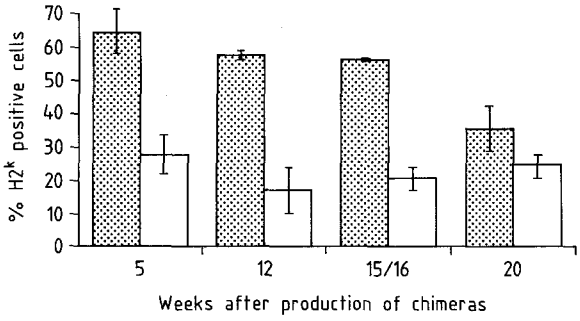

Fig. 2. Percentages of $H2^k$ positive macrophages and non-macrophages among the total populations of macrophages and non-macrophages in the NPLC from radiation chimeras. ▨ Macrophages; □ Non-macrophages

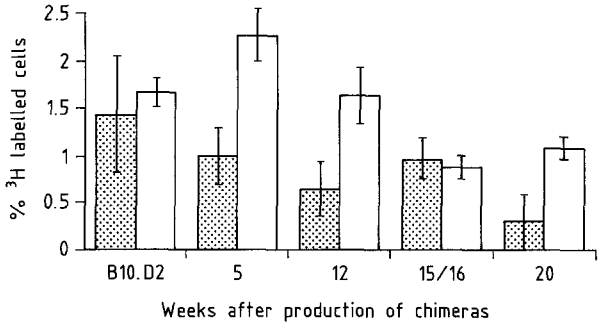

Fig. 3. ^3H thymidine indices of the macrophages and non-macrophages in NPLC from B10.D2 mice and from radiation chimeras (F1 B10.D2). ▨ Macrophages; □ Non-macrophages

toradiography following injection of ^3H thymidine, and calculated with the ^3H thymidine index, which is the percentage labelling frequency of each cell type investigated.

In non-irradiated B10.D2 mice macrophages showed $1.42 \pm 0.65\%$, non-macrophages $1.72 \pm 0.12\%$ ^3H thymidine labelled cells (Fig. 3). Figure 3 also shows that, apart from the duration of the $15^{th}/16^{th}$ week, the macrophages of the radiation chimeras have significantly lower ^3H thymidine indices than the non-macrophages. It is also apparent that DNA synthesis in both macrophages and non-macrophages of chimeric mice decreases in common from the 5^{th} until the 20^{th} week of the observation period.

Figure 4 shows the comparison of ^3H thymidine indices in $H2^k$ positive and $H2^k$ negative macrophages and non-macrophages of radiation chimeras during the 5^{th} and 20^{th} week after transplantation. It can be seen that the proliferating NPLC belong to both groups, to the transplanted $H2^k$ positive as well as to the $H2^k$ negative. The highest proliferative activity can be observed in the 5^{th} week and the lowest in the 20^{th} week after transplantation. In both groups the non-macrophages generally show a more active DNA synthesis than do the macrophages. In the case of $H2^k$ positive cells this difference is more pronounced, and true for the whole observation period, but for $H2^k$ negative cells it is less distinct, and not recognisable in week 15/16 after chimera production. If one compares the ^3H thymidine indices of the $H2^k$ positive and $H2^k$ negative macrophages, it can be seen that a more active DNA synthesis in the $H2^k$ negative cells occurs only in week 15/16. Comparison of the ^3H thymidine indices of the $H2^k$ positive and $H2^k$ negative non-macrophages shows that there is a significantly higher DNA synthesis in the positive than in the negative cells in week 5 and in week 15/16.

In order to increase the NPLC-turnover, liver damage was produced by an injection of shock-inducing sublethal amounts of endotoxin, in the $15^{th}/16^{th}$ week after transplantation. Four days following LPS treatment, the NPLC were isolated and investigated as described above.

The number of macrophages increased from $25 \pm 0.1\%$ (controls) to $40 \pm 10\%$ four days after the administration of endotoxin. This means that due to the LPS effect, the ratio of non-macrophages to macrophages had shifted from 3:1 (control chimeras) to 1.5:1 (after LPS).

The number of $H2^k$ positive cells had nearly

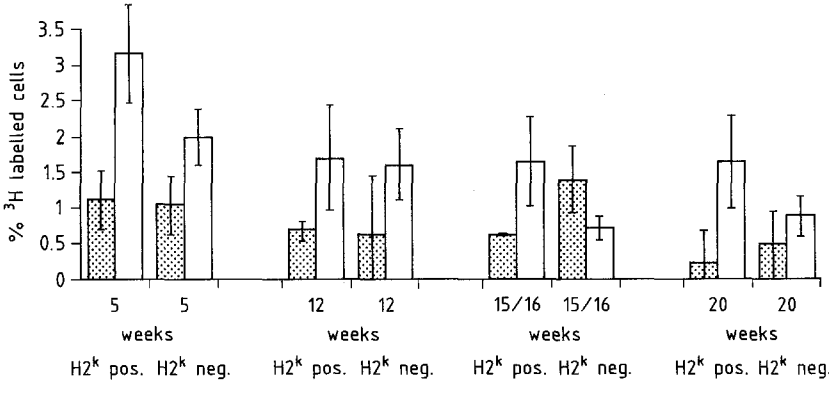

Fig. 4. ^3H thymidine indices of the $H2^k$ positive and $H2^k$ negative NPLC (macrophages and non-macrophages) from radiation chimeras. ▨ Macrophages; □ Non-macrophages

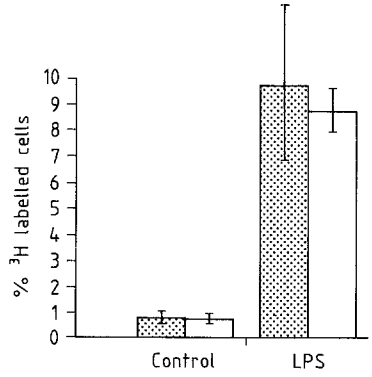

Fig. 5. ^{3}H thymidine indices of the macrophages and non-macrophages among NPLC from radiation chimeras in the 15th/16th week following bone-marrow transplantation: without treatment (Control) and 4 days after a shock-inducing injection of endotoxin (LPS). ▨ Macrophages; □ Non-macrophages

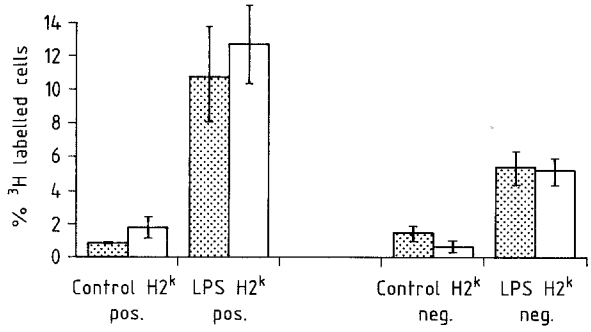

Fig. 6. ^{3}H thymidine indices of H2^k positive and H2^k negative NPLC (macrophages and non-macrophages) from radiation chimeras in the 15th/16th week following bone-marrow transplantation: without treatment (Control) and 4 days after a shock-inducing injection of endotoxin (LPS). ▨ Macrophages; □ Non-macrophages

doubled from $30 \pm 2\%$ (control chimeras) to $52 \pm 7\%$ (after LPS).

An increase in H2^k positive cells was observed in both populations of the NPLC, in macrophages (from $58 \pm 0.1\%$ in controls to $73 \pm 7\%$ after LPS) and in non-macrophages (from $20 \pm 3\%$ in controls to $42 \pm 7\%$ after LPS).

Figure 5 shows that, after the administration of endotoxin, macrophages and non-macrophages of the NPLC population revealed a 10-fold increase of their ^{3}H thymidine indices (macrophages from $1 \pm 0.2\%$ in controls to $9.8 \pm 2.7\%$ after LPS; non-macrophages from $0.9 \pm 0.1\%$ in controls to $8.6 \pm 0.8\%$ after LPS), which shows the strong proliferative activity of NPLC in response to endotoxin-induced damage. If one observes DNA synthesis separately in the H2^k positive and H2^k negative cells following endotoxin administration (Fig. 6), it can be seen that the ^{3}H thymidine indices of both the H2^k positive macrophages and non-macrophages are more than twice those of the H2^k negative NPLC.

Discussion

The radiation chimeras used for this investigation provide a model that is particularly suitable for examining the replacement of NPLC both in health and disease. Between the 12th and 16th week following bone-marrow transplantation, a percentage frequency of macrophages and non-macrophages is found in the chimeric NPLC which, accompanied by the DNA synthesis in these cell populations, is altogether comparable with the situation in untreated B10.D2 mice.

The immunocytochemical demonstration of the H2^k antigen on 84% of the F1 hybrid bone-marrow cells, which have been transplanted into irradiated B10.D2 mice, provides a cytogenetic marker by which the extrahepatic origin of the major part of these cells can be demonstrated in the H2^k negative livers of the latter.

The results of the present investigation show that, in the radiation chimeras, about one third of the NPLC are derived from precursor cells in the bone-marrow, and that these immigrant cells consist of macrophages and non-macrophages. The number of H2^k positive non-macrophages remains unchanged with an average percentage of 20%, against which the number of H2^k positive macrophages decreased continuously during the observation period from 67% (week 5) to 36% (week 20). Recent reports from other laboratories (Paradis et al. 1989; Steinhoff et al. 1989) have verified the origin of liver macrophages in the bone-marrow. Paradis and his coworkers (1988) found that more than 85% of mature macrophages were derived from the bone-marrow in a similar mouse/bone-marrow transplantation model. Bouwens et al. (1986), however, have suggested that the "resident" Kupffer cells are replaced exclusively by local proliferation, and that the exudative (immigrant) macrophages only appear during acute disease. Our observations suggested that cell replacement of the NPLC is brought about both by immigrant bone-marrows cells and by the proliferation of (at least partly) resident cells (see below). The finding that besides the H2^k negative also the H2^k positive NPLC divide, probably means that an immigrant bone-marrow cell can become resident and then reproduce by proliferation locally. Among the non-macrophages, the H2^k positive (immigrant) cells are basically more liable to proliferate than the H2^k negative NPLC. The ^{3}H thymi-

dine indices of the $H2^k$ positive and negative macrophages differed only during the 15th and 16th weeks of the experiment, when the $H2^k$ negative cells multiplied more rapidly than the immigrants. The reproduction of both immigrant and resident cells has also been described following the administration of liposomal encapsulated muramyl dipeptide (Daemen et al. 1989).

The reason for the disagreement concerning the mechanism of replacement of macrophages, is to be sought in the obviously variable definitions of a "resident" macrophage. The definition of a resident macrophage put forward by Bouwens et al. (1986) depends upon the demonstration of a special peroxidase pattern in these cells. In our own investigations, demonstration of what are probably resident macrophages in radiation chimeras depended upon their $H2^k$ negativity, together with the in-vivo incorporation of big latex particles. Furthermore, the heterogeneity of macrophages (Dougherty and McBride 1984; Martin et al. 1989) may also help to explain the frequent disparity between the various attempts to demonstrate the mechanism of replacement of the Kupffer cell. We have not been able to identify with any certainty the origin of $H2^k$ negative NPLC, which show themselves to be capable of DNA synthesis. One possibility is that some of the original $H2^k$ negative NPLC in the host animal maintained their ability to reproduce in spite of the lethal irradiation. It could also be that the 16% $H2^k$ negative cells from the F1 bone-marrow and/or bone-marrow cells left over from the host animal, having survived the irradiation, are again capable of synthesising DNA. Our observation of NPLC following endotoxin administration have established the time of maximal proliferation following shock-inducing injury (Freudenberg et al. 1986). On the 4th day after administration of LPS, the following principal changes occur: the ratio of non-macrophages to macrophages has shifted from 3:1 (controls) to 1.5:1 (after LPS) in favour of the macrophages. The proportion of the $H2^k$ positive cells, which by this time has risen to 50% (controls: 30%), is particularly impressive, and means that 73% of all the macrophages and 42% of the non-macrophages have originated in the bone-marrow. Furthermore, our results show that cells from the transplanted bone-marrow ($H2^k$ positive) were more effectively involved in the proliferative response seen after endotoxin-induced damage than were the $H2^k$ negative cells. The presented data are very much in favour of a bone-marrow origin of the liver macrophages and also of some other NPLC. The identity of bone-marrow derived non-macrophages is currently being studied in our laboratory.

Acknowledgements. We express our thanks to our colleague Dr. F. Steel for discussions on the preparation of the English manuscript. We are very grateful to X. Ludwigs for expert technical assistance. This study was supported by grants of the Deutsche Forschungsgemeinschaft (SFB 154) to N.F. and C.G.

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Received January 23, 1989 / Accepted March 28, 1989