Virchows Arch (1996) 428:187–194

ORIGINAL ARTICLE

N. Freudenberg · P. Rahner · C. Darda · A. Kiss
G. Veres · T. Nees · R. Lamers · U.N. Riede
C. Kortsik · M. Schubert · K. Frenzer-Welle

Early detection of metastasis by alterations in the cellular immune system in the murine liver and blood

Received: 4 August 1995 / Accepted: 23 November 1995

Abstract We investigated the reaction of the cellular immune system of liver and blood in the C57BL/6 mouse to a metastasizing Lewis lung carcinoma. The cellular immune system of the liver consists of mature and immature macrophages, B-cells, T-cells including their subpopulations, and natural killer cells, and their percentage frequencies differ significantly from those in the corresponding mononuclear blood cell (MBC) compartment. This suggests that the hepatic immune cells represent a system with autonomous function showing a typical homing of its members. Imminent metastasis to the liver is signalled by impressive alterations in the percentage frequencies of nonparenchymal liver cells (NPLC). There are a dramatic loss of mature macrophages, an increase in immature macrophages, a reduction of T-helper cells leading to a low CD4/CD8 ratio, and an increase in natural killer cells. In the blood, the corresponding precursor cells show comparable changes with a delay of at least 2 days. Early metastasis is accompanied by a significant increase in mononuclear NPLC producing tumour necrosis factor α. The alterations in percentage frequencies of the NPLC during tumour metastasis differ markedly from the changes in these cells in the liver during endotoxinaemia.

Key words Cellular immune system · Liver · Blood · Lewis lung carcinoma · Liver metastases · Tumour necrosis factor

N. Freudenberg (✉) · P. Rahner · C. Darda
U.N. Riede · M. Schubert · K. Frenzer-Welle
Pathologisches Institut der Universität, Albertstrasse 19,
D-79104 Freiburg, Germany
Fax: (49) 761-203-67 69

A. Kiss
Ist Department of Pathology,
Semmelweis University of Medicine, Üllöi ut 26,
H-1085 Budapest, Hungary

G. Veres
IInd Department of Pathology,
Semmelweis University of Medicine, Üllöi ut 93,
H-1091 Budapest, Hungary

T. Nees
Universitäts-Augenklinik, Killianstrasse 5,
D-79106 Freiburg, Germany

R. Lamers
Max-Planck-Institut für Immunbiologie, Stübeweg 51,
D-79108 Freiburg, Germany

C. Kortsik
Abteilung Pulmologie, Medizinische Universitätsklinik,
Hugstetterstrasse 59, D-79106 Freiburg, Germany

Introduction

The liver is frequently affected by metastases. The cellular defence system in the liver that is directed against malignancies is composed of nonparenchymal liver cells (NPLC); pit cells and Kupffer cells have been thought to be the most important cells in antitumour defence in vivo [1, 14]. In vitro, cytotoxic effects of pit cells , Kupffer cells and hepatic T-cells have been observed in a series of tumour cell lines [3] but the mechanisms by which these cells operate in tumour defence are largely unknown. Possibly cytokines secreted from NPLC, such as interferon γ (IFN-γ) and tumour necrosis factor α (TNFα), play a key part in the regulation of tumour cell spread. Matthys et al. [20] have shown that IFN-γ is essential for the growth of Lewis lung carcinoma in mice; however, a tumour-necrotizing effect of TNFα has been shown in the same tumour [16]. Whether B-cells or some recently identified subpopulations of the NPLC (mature, immature macrophages, T-helper and suppressor cells) are involved in tumour defence has not been investigated. It was the aim of the present study to discover what reactions of the cellular hepatic immune system could be seen during metastasis of a Lewis lung carcinoma to the liver. The percentage frequencies of NPLC and of their TNFα-producing cells were compared in tumour-bearing mice and sham-inoculated animals. Since the results of a recent investigation dealing with the origin of NPLC indicated that a major part of this cell population originated from the bone marrow [6], possible changes in the percentages

of NPLC precursor cells (the mononuclear cells from the blood) were studied simultaneously during metastasis.

Materials and methods

C57BL/6 mice 6–12 weeks old and of both sexes were obtained from Charles River.

The tumour cell line of the rapidly growing Lewis lung carcinoma (3LL-HH) was obtained from Dr. L. Paku of the Ist Department of Pathology, Semmelweis Medical University, Budapest.

To identify NPLC the following antibodies were used: the rat-to-mouse monoclonal antibody 88a (macrophages and monocytes precursor cells; E. Michels and C. Sorg, Münster, personal communication), and the rat-to-mouse monoclonal antibody BM8 (mature macrophages [17]) were obtained from Prof. Dr. C. Sorg, University of Münster, Germany. The numbers of immature macrophages were calculated as $88a^+$ minus $BM8^+$ cells. T-cells were identified by means of monoclonal rat-to-mouse antibodies (a) against the Thy 1.2 antigen (pan T-cells), (b) against the YTS 191.1 antigen (corresponding CD4 T-cell helper subpopulation) and (c) against YTS 169.1 (corresponding CD8 T suppressor cells). B-cells were identified with a mixture of monoclonal rat-to-mouse biotinylated antibodies against lamda and kappa light chains and also against mu and delta heavy chains. The rabbit-to-mouse anti-asialo GM1 polyclonal antibody (WAKO) marks natural killer cells, including a definite percentage of T-cells and the precursors of cytotoxic T-cells [23]. Rat-to mouse monoclonal TNFα MP6-XT3 (GIBCO, no. 3300SA) antibody was used for the detection of TNFα.

To establish tumours, following premedication with atropine (0.1 mg/kg bodyweight) anaesthesia was carried out using a combination of ketamine (100 mg/kg body weight i.p.) and xylazine (5 mg/kg body weight i.p.). Once the peritoneal cavity had been opened, 6×10^3 tumour cells suspended in 60 µl sterile RPMI medium were injected into the convexity of the spleen. Following blood staunching using sterile, lyophilized collagen, the wound was closed with two Michel clips (ÄSKULAP 11×3 mm, art. no. 511). Control animals were sham-inoculated using 60 µl RPMI tumour-free medium with the same conditions of anaesthesia and preparation. In order to isolate NPLC, mice were killed with an i.p. of 60 mg Nembutal/kg body weight on days 4 and 6 (NPLC and MBC), on day 7 (only NBC) and on day 8 (only MBC) following inoculation of tumour cells or "sham inoculation".

To isolate and fix NPLC, following preperfusion with RPMI 1640 medium (GIBCO) – which results in a total decolourization of the liver within a few seconds – perfusion with pronase E (Merck; 3 mg in 5 ml RPMI) and then with pronase/collagenase solution (7 mg collagenase CLS0, 400 U/mg, Seromed; Biochrom and 3 mg pronase (Merck), dissolved in 25 ml RPMI) was carried out, followed by incubation in collagenase (17 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 (GIBCO). For further investigation, $5–10\times10^4$ NPLC were individually anchored on reaction fields by the slide adhesion method introduced by Bross et al. [2]. For the detection of surface-bound antigens, the cells were treated with 0.05% glutaraldehyde (Merck) in 0.1 M Soerensen buffer, pH 7.8.

To isolate MBC, blood was obtained by puncturing the inferior cava. The blood was heparinized and diluted 1:2 with phosphate-buffered saline (PBS). The blood was carefully overlayed to Ficoll-separating solution (density 1.077 g/ml, BIOCHROM) and then separated by centrifugation (400 *g*, 30 min). The MBC that were enriched in the interphase were suspended in a solution of 10% newborn calf serum (virus and mycoplasma screened, GIBCO). Following a second washing with PBS and centrifugation (200 *g*), the pellet was resuspended in PBS. The percentage of viable isolated MBC was 98%, determined by the trypan blue test.

Using immunocytochemistry 88a and BM8 positive cells were detected by the ABC technique. After covering the nonspecific linkage sides with 20% normal sheep serum, the specimens were incubated for 1 h with diluted monoclonal rat-to-mouse antibodies (88a: 1:20; BM8: 1:50). After washing in NKH buffer, the cells were treated for 30 min with a (sheep-to-rat) biotinylated antibody (Amersham) diluted 1:200. Following washing in NKH buffer for 15 min, endogenous peroxidase was inhibited with 0.5% H_2O_2 in 70% methanol for 30 min. The cells were incubated for 30 min with diluted (1:500) Avidin-Biotin complex, washed, and stained for peroxidase activity with 0.03% H_2O_2 and 0.45% diaminobenzidine in NKH buffer. After further washing in the buffer, the specimens were postfixed with 2% OsO_4 in double-distilled water for 10 min. After the cells had been washed in double-distilled water they were placed in gelatin under a coverslip.

Detection of T-cells was performed using primary antibodies Thy 1.2 (1:50), CD4 (1:10) and CD8 (1:10), followed by a secondary biotinylated antibody (sheep-to-rat, 1:200). The immunocytochemical identification of B-cells was achieved with biotinylated primary antibodies using the following dilutions: anti-lambda (1:40), anti-kappa (1:80), anti-mu (1:20) and anti-delta (1:20). The remaining steps were identical for both T- and B-cell detection. Washing in the buffer and inhibition of endogenous peroxidase were followed immediately by the AB complex (1:500). All remaining steps have been described above.

Asialo-GM1-positive cells were identified by the indirect immunoperoxidase technique. The cells were first covered with 20% pig serum and then incubated in the anti-asialo GM1 antibody (a rabbit-to-mouse IgG: WAKO) for 1 h at a dilution of 1:1000. After thorough washing with NKH buffer, endogenous peroxidase was inhibited as described above. The cells were then incubated with a peroxidase-coupled pig-to-rabbit antibody (1:400, Dako), which was absorbed with 10% normal mouse serum, and the peroxidase activity was demonstrated. Treatment with osmic acid and covering was performed as described above.

TNFα was identified immunocytochemically in isolated NPLC on the 7th day after tumour cell inoculation using the ABC technique. Sham-inoculated mice served as controls. Briefly: following the inhibition of endogenous peroxidase the cells were washed in PBS, nonspecific binding sites were covered with 20% sheep serum for 40 min at room temperature, incubated overnight with diluted TNFα antibody (1:640) or with diluted (1:1280; corresponding value to the protein content of the TNFα antibody) nonimmune rat IgG (Sigma I-4131), washed with PBS, incubated with a diluted (1:200) sheep-to-rat biotinylated antibody (Amersham), absorbed with 10% normal mouse serum for 40 min at room temperature, then washed with PBS and stained with the ABC technique described above. After application of the immunocytochemical technique, the cells were washed in buffer and postfixed with 2% OsO_4 in double-distilled water at room temperature for 10 min. For light microscopy the cells were washed and then mounted in gelatin under coverslips.

For transmission electron microscopy the specimens were dehydrated in graded alcohols and embedded in Epon using a previously published technique [5]. Ultrathin sections were cut on an LKB ultramicrotome. All micrographs were primarily taken on unstained sections, and then a second time after counterstaining of the same object with lead citrate and uranyl acetate to characterize the cell types morphologically. For this procedure a Zeiss-EM-9A was used, operating at 60kV and with primary magnifications from 4,500 to 28,000.

For lectin histochemistry the metastatic tumour cells of Lewis lung carcinoma were detected selectively in the liver using soy bean agglutinin (SBA) lectin histochemistry according to the method of Kahn et al. [13].

The percentage frequencies of the identified cell populations were determined under the light microscope. The counting procedure was carried out on the reaction fields of the slides, each of which contained an average of 40,000 cells. Using a randomized procedure, a minimum of 1,000 cells from each compartment were counted. The mean values and standard deviations of at least three mice (in one experimental group) were calculated

Results

Using light microscopic and immunocytochemical methods, 37% of NPLC and 97% of MBC were identified. For comparison of the changes occurring in different tissues, each of these components (37% and 97%) was regarded as 100%.

Table 1 shows the percentages of the defence cells identified in the liver and the blood, and also the most significant ratios of these cell groups and of their subpopulations. Mononuclear cells of myeloid origin represent the largest cell population both in the liver and in the blood. The percentages of their subpopulations (mature and immature macrophages), however, display remarkable differences. This was best shown with the ratio mature/immature macrophages, which is 5 in the liver and 0.08 in the blood. T-cells are in second place in the liver, with 26%. Less than half the hepatic T-cells (11%) could be subclassified as $CD4^+$ and $CD8^+$ cells by immunocytochemistry. In contrast, the T-cells in the blood, third in percentage frequency, were readily subdivided into $CD4^+$ and $CD8^+$ cells, and the CD4/CD8 ratio of NPLC was nearly identical with that of MBC. The percentages of B-cells in the liver and blood differ widely. B-lymphocytes make up the smallest population amongst the NPLC, and the second largest in the blood. The T/B cell ratio is markedly higher in the liver (4) than in the blood (6). In both groups, the asialo $GM1^+$ cells make up the third largest population, being significantly smaller in the liver with a percentage of 10±1%, than that in the blood with 17±3%.

Table 1 Percentage frequencies of defence cells in the liver and the blood, and the ratios of their subpopulations and those of T/B cells in the untreated B57BL/6 mouse

	Liver	Blood
Macrophages/monocytes	58%	42%
Ratio mature:immature macrophages	5	0.08
T-cells	26%	19%
Ratio CD4:CD8	0.9	1
B-cells	6%	22%
Ratio T:B	4	0.9
Asialo GM1 cells	10%	17%

The first hepatic metastases were detected under the light microscope on the 7th day after intrasplenic tumour cell inoculation of 6×10^3 cells of the 3LL-HH. They were located in the periportal region of the liver lobule (Fig. 1). At the same time single tumour cells were found in the liver sinusoids.

The percentage frequencies of NPLC and of mononuclear blood cells were investigated as described. Animals that had received injections of RPMI medium instead of tumour cells served as controls ("sham inoculation").

Figure 2a shows that in the experimental animals the percentage frequencies of mature macrophages amongst NPLC were markedly reduced, showing a significant difference from the sham-inoculated mice. The difference persisted on day 6 (22±10.6%), though it had decreased

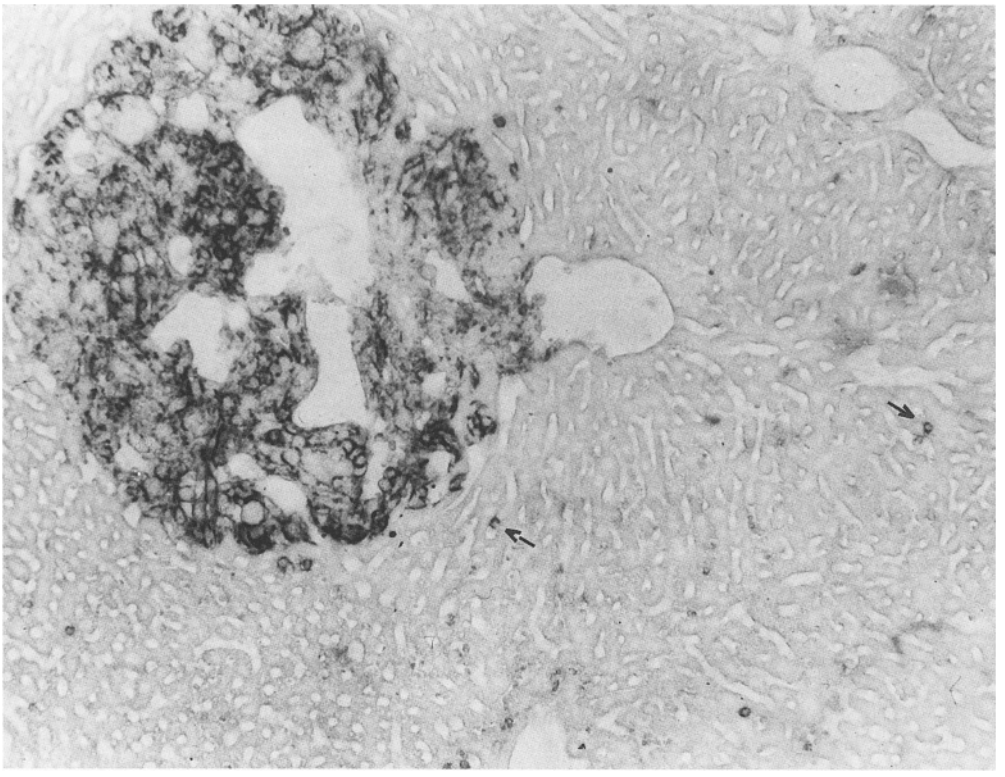

Fig. 1 Liver metastasation of the 3LL-HH tumour, 8 days after intrasplenic inoculation of tumour cells. Beneath a periportal metastatic nodule, several tumour cells can be seen within the liver sinusoids (*arrows*). SBA-lectin histochemistry, no counterstaining; primary magnification ×100

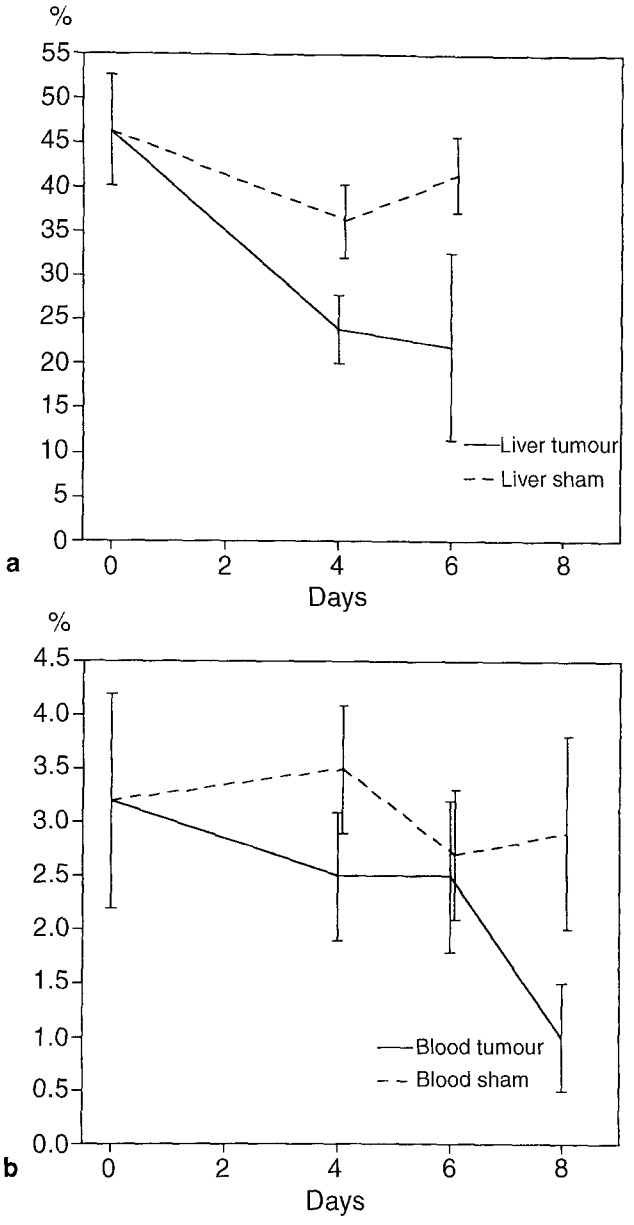

Fig. 2a, b Percentage frequencies of mature macrophages in the liver and the blood at different times after tumour and sham inoculation. **a** Immunocytochemically identified NPLC; **b** immunocytochemically identified MBC

less than half of its original value (46.4±6.2%). In the blood (Fig. 2b), a reduction of the percentage frequency of mature macrophages was not seen until day 8 (1±0.5%), when there was a significant difference from the sham-inoculated animals (2.9±0.9%).

Immature macrophages (monocytes) were the first to show major alterations in the blood (Fig. 3a). Here, the percentage frequency of monocytes was still reduced by more than one-third of the basic value (38.3±2.2%) to 22.9±2.5% (4th day), remaining constant with a significant difference from that in the sham-inoculated mice until the end of the observation period (8th day). In the liver, however, a significant difference between tumour-bearing and sham-inoculated animals was only found on day 6, with an increase of the percentage frequency of immature macrophages (Fig. 3b).

The ratio of mature to immature macrophages shows a significant reduction to 1.1±0.5% in the liver only on day 4 compared with controls (1.9±0.2%). In the blood of tumour-bearing animals, this ratio increases on the 4th (0.12±0.02%) and 6th day(0.11±0.01%) showing a significant difference from sham-inoculated mice on day 6: 0.08±0.01%. Thereafter, the value decreases to little more than half the basic ratio (0.08±0.01%) showing a significant difference from controls.

T-cells of tumour mice showed no significant differences from those of control animals in their percentage

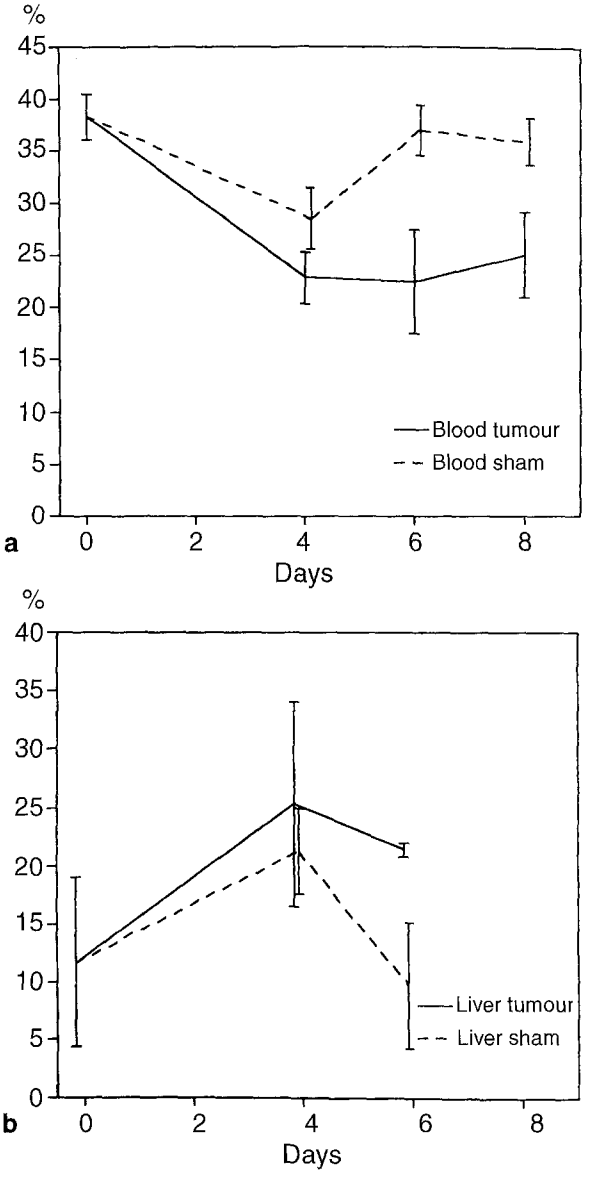

Fig. 3a, b Percentage frequencies of immature macrophages in the blood and the liver at different times after tumour and sham inoculation. **a** Immunocytochemically identified MBC; **b** immunocytochemically identified NPLC

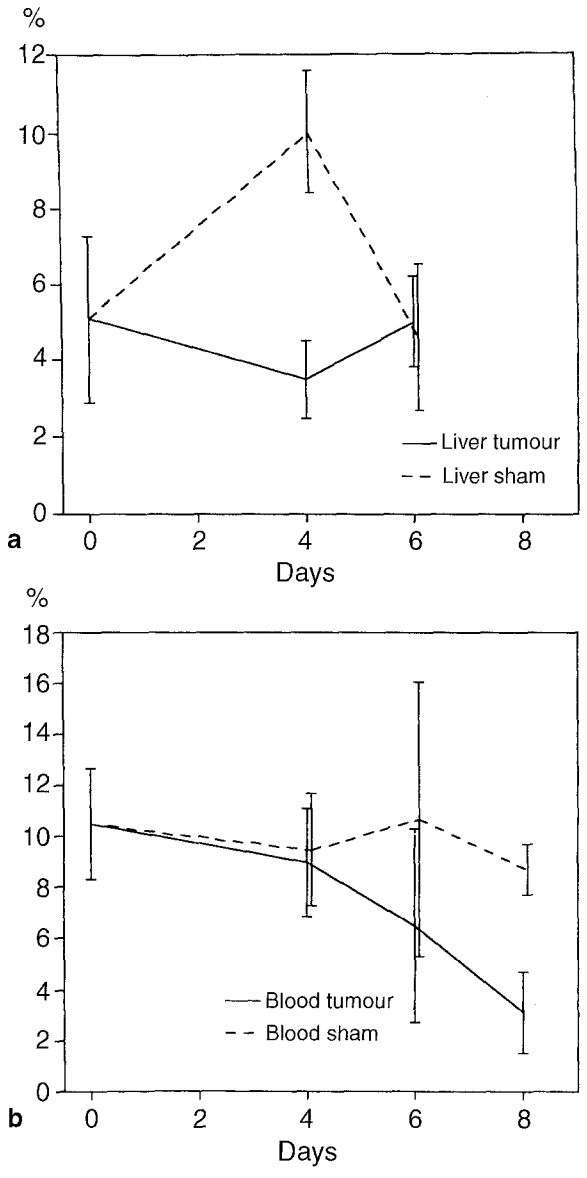

Fig. 4a, b Percentage frequencies of CD4+ cells in the liver and the blood at different times after tumour and sham inoculation. **a** Immunocytochemically identified NPLC; **b** immunocytochemically identified MBC

frequencies at any point of the observation, neither among the NPLC nor amongst the MBC. A look at T-cell subpopulations, however, reveals obvious alterations of CD4+ cells and of the CD4/CD8 ratio between tumour-bearing and sham-inoculated animals (Figs. 4, 5). Figure 4a shows that the CD4 values of tumour-bearing mice in the liver remain at the same level as in untreated animals (time 0), differing significantly (3.5±1%) from the markedly raised value in sham-inoculated mice (10±1.6) on day 4. The percentage of CD4+ cells amongst the MBC of tumour-bearing mice decreased significantly, with 3.1±1.6% on the 8th day compared with the 8.7±1% in sham-inoculated animals (Fig. 4b). The CD4/CD8 ratios in the liver and the blood show comparable changes, although in the case of the MBC a time shift is apparent.

In the liver of tumour-bearing mice (Fig. 5a), a marked reduction to one-third (0.34±0.04) of the basic value (0.9±0.1), in sham-inoculated animals (1.06±0.06) was found. In the blood (Fig. 5b), the fall in that ratio can be seen 2 days later, and it declines further on day 8, having its minimum at 0.2±0.1, giving a highly significant difference from controls (0.9±0.2).

B-cells of the tumour-bearing mice showed no significant differences from those of sham-inoculated controls in their percentage frequencies of NPLC or MBC.

The T/B ratio (basic value of MBC: 0.9±0.1) only increased in the blood of tumour-bearing animals on day 6 (1.4±0.2) and day 8 (maximum value with 1.6±0.2), displaying significant differences to the ratios in sham-inoculated mice (6th day: 0.8±0.1; 8th day: 1±0.3).

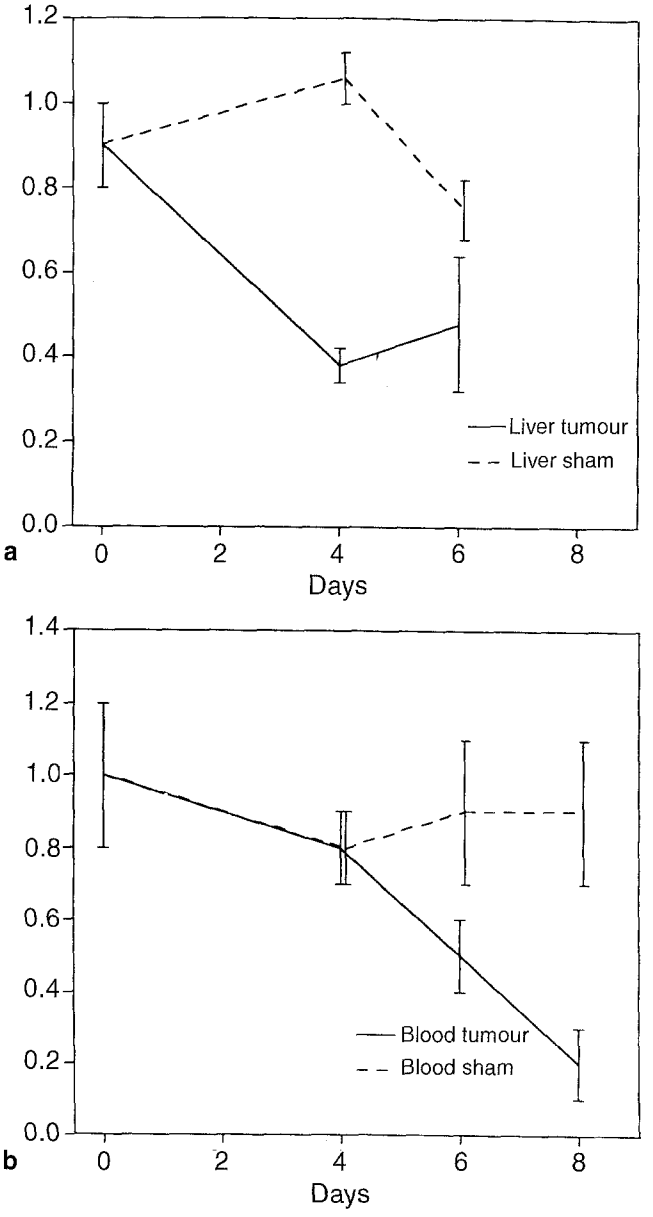

Fig. 5a, b CD4/CD8 ratio in the liver and the blood at different times after tumour and sham inoculation. **a** NPLC; **b** MBC

Marked alterations were observed in the percentage frequencies of asialo-GM1-positive cells of both groups (Fig. 6). Figure 6a shows a markedly increased percentage of this cell type amongst the NPLC of tumour-bearing animals (17.2±4.4%) by day 4 after inoculation, a significant difference from sham-inoculated mice (8.8±1%). In Fig. 6b a comparable increase in the percentage frequencies of this cell population can also be recognized in blood, but with a 2-day delay. In this case, with 26.2±1.5%, the percentage frequency in tumour-bearing animals on day 8 was almost twice the value in sham-inoculated mice (14.7±2.9%).

On day 7 of the experiment (Fig. 7), the tumour-bearing mice displayed a three-fold increase in the percent-

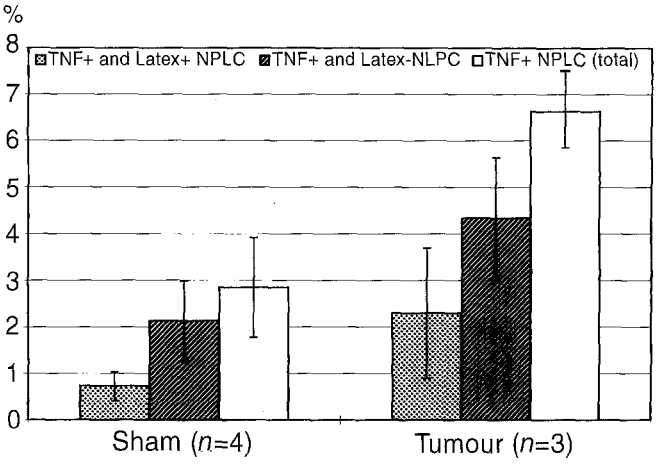

Fig. 7 Percentage frequencies of TNFα⁺ NPLC on day 7 after sham or tumour cell inoculation

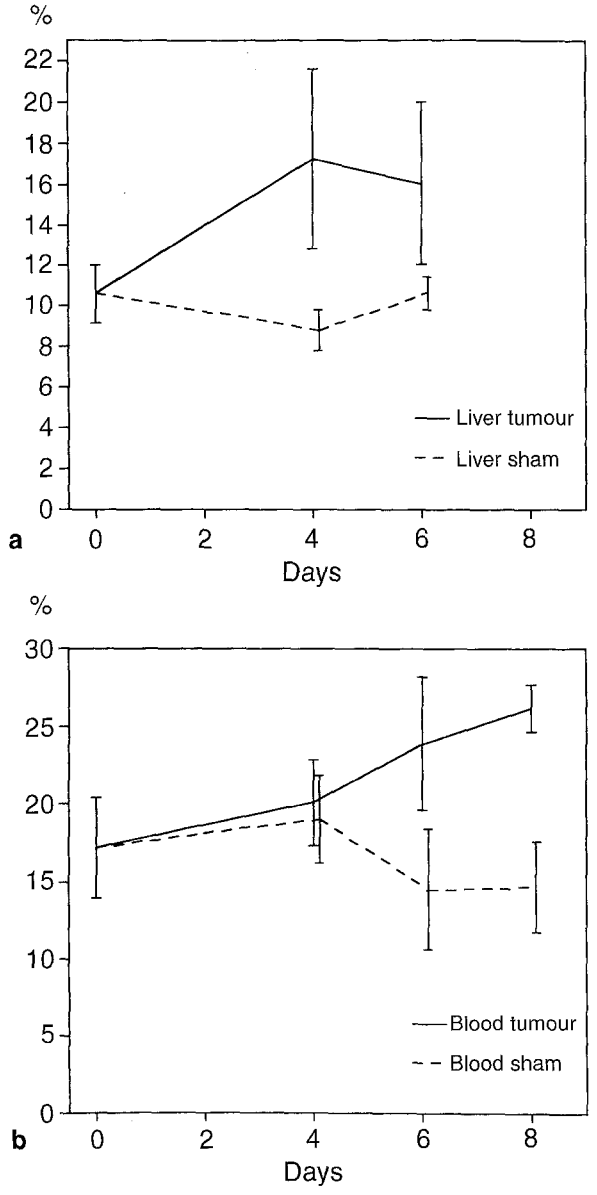

Fig. 6a, b Percentage frequencies of Asialo GM1⁺ cells in the liver and the blood at different times after tumour and sham inoculation. **a** Immunocytochemically identified NPLC; **b** immunocytochemically identified MBC

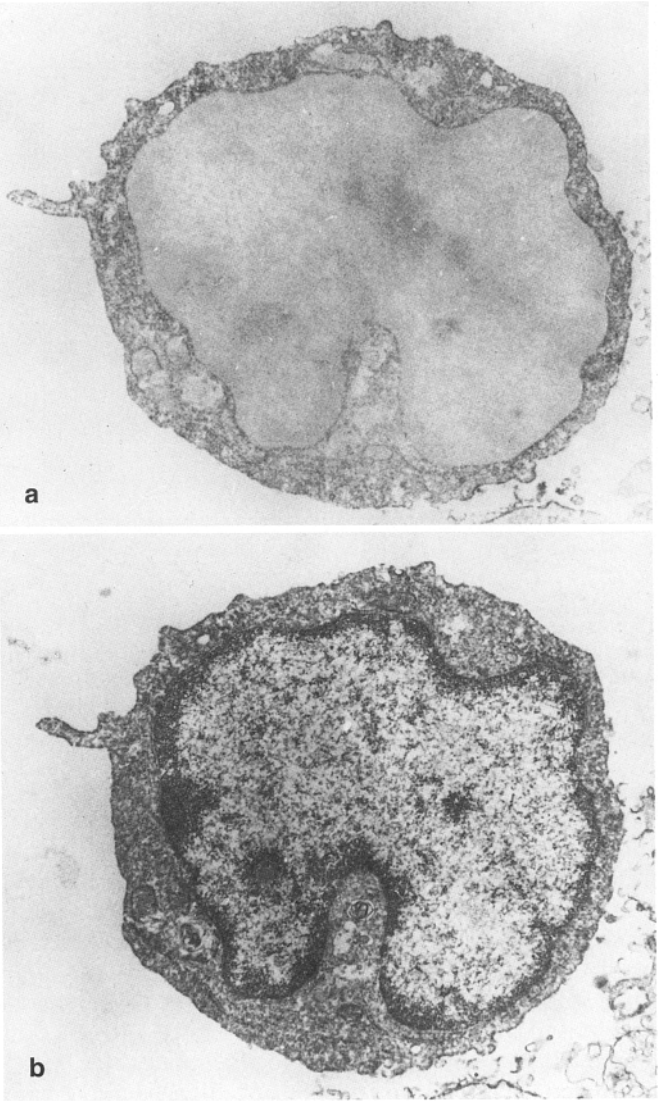

Fig. 8a, b Ultrastructural immunocytochemical demonstration of TNFα (*dark reaction product*) in the cytoplasm of a large mononuclear cell amongst the NPLC. Primary magnification ×4500. **a** No counterstaining; **b** counterstained

age of TNFα positive cells, with 6.6±0.8%, compared with the sham-inoculated animals (2.9±1.1%). TNFα-positive cells were categorized ultrastructurally into large (Fig. 8) and small mononuclear cells. The latex-storing component (Fig. 7) of the large cell group was classified as macrophages.

Discussion

The investigations of Malter and Süss [18] and of our working group [6] have shown homing of B- and T-cells in the liver sinusoids. Thus there are other sessile cells apart from the macrophages and pit cells previously described by Wisse et al. [28], and our present results show that the liver has a complete and autonomous immune system. When earlier ultrastructural findings in NPLC [5] and different phagocytotic activities of macrophage subpopulations [7] are taken into consideration, the cell types in mice of this system can be characterized more distinctly.

Among the macrophages, mature ($BM8^+$) and immature ($88a^+$ minus $BM8^+$) cells can be distinguished, the mature cells showing a much stronger phagocytosing activity than the immature ones. Compared with the immature types, mature hepatic macrophages are underrepresented in the pericentral lobular area [7]. Liver macrophages show a marked numerical predominance amongst the hepatic immune cells at 58% compared with the corresponding compartment of mononuclear cells in the blood (42%).

The ratios of mature/immature macrophages differ widely the value in the liver being 60-fold that in blood. This finding is evidence of the high phagocytic power of the hepatic mononuclear cell system compared with their precursors in the blood. T-cells are represented significantly more strongly among the NPLC, making up 26% of this group compared with 19% of the MBC. We focused on the T-cell subpopulations in the liver. It is noteworthy that 58% of the T-cells could not be subclassified into $CD4^+$ and $CD8^+$ lymphocytes by immunocytochemistry. This suggests the existence of an additional group of NK cells amongst the $CD4^-/CD8^-$ T-cells [21]. In contrast, no difference between liver and blood was found in the CD4/CD8 ratio, which was nearly identical in NPLC and MBC. The CD4/CD8 ratio of cells detected earlier in bronchoalveolar lavage from B10.D2 mice, which showed a comparable value of 1.3 [8], suggests that this ratio could be an immunogical constant of the T-cell system; it seems to be constant in all tissues investigated.

NK cells are cells that mediate cytolytic reactions without the need for class I major histocompability complex (MHC) molecules expressed on target cells and in certain circumstances the activity of NK cells is of prognostic significance in metastasizing tumours [24]. To identify NK cells in mice, asialo-GM1, as well as NK.1.1/NK-2–1 antibodies are used [11]. Although the asialo-GM1 molecule can be detected in more than 90% on the NK cells [27], it is found in certain percentages on T-cells, particularly on precursors of cytotoxic T-cells [23]. In the liver, an additional group of cells with NK activities may exist, which could increase the value of 10% asialo-$GM1^+$ cells. The $CD4^-/CD8^-$ cells, which are possibly T-cells showing NK cell-like activity should be considered [21]. The percentage frequency of 14.5% Asialo-GM1 positive cells amongst the cells of bronchoalveolar lavage in the B10.D2 mouse [8] is comparable to the values in the blood and possibly in the NPLC that show NK activity. A globally constant ratio for the cell compartment which shows cytolytic activity independent of MHC may also be suggested.

The first microscopically detectable metastases were observed on the 7th day after tumour cell inoculation in the periportal area. Previous immunohistochemical findings showed a marked decrease of mature macrophages in that region of the hepatic lobule at this time of observation [9].

Opinions concerning the role of macrophages in tumour processes are still controversial. On the one hand, cytotoxic and cytostatic effects from macrophages are well known [12]. On the other, factors have been described that are essential for tumour growth [4], and are able to suppress the reactions directed against the malignancy of tumour-infiltrating lymphocytes [26]. The heterogeneity of macrophages is one possible explanation for these contradictory findings. The different reactions of mature and immature macrophages in the liver of the C57BL/6 mouse during metastasis strongly suggest functional heterogeneity of at least two populations of macrophages in one organ. The decrease in the percentage of mature macrophages that had occurred by day 4 after tumour cell inoculation may be interpreted as an early effect on this macrophage population produced by signals from metastasizing tumour cells. At this point, hepatic metastases were not detectable, although single tumour cells must have been present in the liver. In contrast to the effects of metastasis from a malignant tumour, hepatic macrophages show an increase in their percentage frequencies following the application of bacterial endotoxins, which leads to a disease characterized by an exponential increase of mature cell types [7].

The observed increase in the percentages of immature macrophages during the metastatic process should be interpreted as a reaction to the loss of mature cells. The reduction of the number of blood monocytes at the same time demonstrates the function of replacement for NPLC from the MBC compartment.

The marked arrest of the percentages of T-helper cells amongst the NPLC on the 4th day after tumour cell inoculation, which leads to a decrease in the CD4/CD8 ratio, should be regarded as a further early signal of impending metastasis. The increase in the percentages of asialo-GM1-positive cells, which represent mainly NK cells, cannot be looked upon as a marker of a more effective tumour defence. However, a cytokine-induced increase of NK cell activity is suggested to have a tumoricidal effect [15]. The increase in the proportion of asialo-GM1-positive cells in the blood only after a time lapse sug-

gests dependence of this population amongst the NPLC on the bone marrow.

The 7th day of the experiment was the earliest time at which metastases were detectable in the liver, and, our investigation has been focused on the frequency of TNFα-producing cells in the organ. This cytokine has long been regarded as a potent substance with tumoricidal effects [16, 19]. Surprisingly, TNFα-positive cells in the liver's defence system increased numerically early in metastasis. Under the electron microscope, the large, cytokine-producing, mononuclear cells were shown to be macrophages, especially when latex phagocytosis was observed. Their ultrastructural features, however, suggested that the small mononuclear cells were mainly lymphocytes. This unexpected result of an increase in TNFα-positive cells suggests that the recently described property of TNFα as a growth factor for metastasizing tumours in man [11, 25] and in mice [22] should also be taken into consideration during the early stage of metastasis of Lewis lung carcinoma.

Acknowledgements This investigation was supported by a grant of the Deutsche Forschungsgemeinschaft (SFB 154, Teilprojekt E2) and of the Forschungskommission des Universitätsklinikums Freiburg. We are very grateful to Mrs. X. Ludwigs-Krayer for expert technical assistance. We express our thank to Mrs. J. Burk for discussions on the preparation of the English manuscript.

References

1. Bouwens L, Charels K, Van Dale P, Wisse E (1989) Sinusoidal cell reactions associated with colon cancer metastases in rat liver. In: Wisse E, Knook DL, Decker K (eds) Cells of the hepatic sinusoid, vol 2. Kupffer Cell Foundation, Rijswijk, pp 237–238
2. Bross KJ, Pangalis GA, Staatz CG, Blume KG (1978) Demonstration of cell surface antigens and their antibodies by the peroxidase-antiperoxidase method. Transplantation 25: 331–334
3. Cohen SA, Salager D, Nolan JP (1982) Natural cytotoxicity of isolated rat liver cells. J Immunol 129: 495–501
4. DeLarco JE, Todaro GJ (1980) Sarcoma growth factor (SGF): specific binding to epidermal growth factor (EGF) membrane receptors. J Cell Physiol 102: 267–277
5. Freudenberg N, Schalk J, Galanos C, Katschinski T, Datz O, Pein U, Freudenberg MA (1989) Identification and percentage frequency of isolated non-parenchymal liver cells (NPLC) in the mouse. Virchows Arch [B] 57: 109–115
6. Freudenberg N, Galanos C, Datz O, Hämmerling G, Katschinski TH, Schalk J, Pein U, Stübig H, Freudenberg MA (1989) Mechanism of replacement of non-parenchymal liver cells (NPLC) in murine radiation chimeras. Virchows Arch [A] 415: 203–209
7. Freudenberg N, Piotraschke J, Galanos C, Sorg C, Askaryar FA, Klosa B, Usener HU, Freudenberg MA (1992) The role of macrophages in the uptake of endotoxin by the mouse liver. Virchows Arch [B] 61: 343–349
8. Freudenberg N, Konarske K, Riede UN, Lamers R, Zeller S, Hämmerling G, Sorg C, Usener HU, Katschinski T, Galanos C, Freudenberg MA (1993) Function and "homing" of the lung macrophages. I. Evidence of functional heterogeneity of mobile cells of the murine lung parenchyma in the bronchoalveolar lavage (BAL). Virchows Arch [B] 64: 191–197
9. Freudenberg N, Rahner P, Kiss A, Sorg C, Lamers R, Veres G, Herrmann O, Pein U (1993) Die Reaktion der Nicht-Parenchymzellen der Leber (NPLC) der C57BL/6-Maus auf Tumormetastasierung. Verh Dtsch Ges Pathol 77: 423
10. Gambel P, Croy BA, Moore WD, Hunziker RD, Wegmann TG, Rossant J (1985) Characterization of immune effector cells present in early murine decidua. Cell Immunol 93: 303–314
11. Goillot E, Combaret V, Ladenstein R (1992) Tumor necrosis factor as an autocrine growth factor for neuroblastoma. Cancer Res 52: 3194–3200
12. Higushi M, Higashi N, Taki H, Osawa T (1990) Cytolytic mechanisms of activated macrophages. Tumour necrosis factor and L-arginine-dependent mechanism act synergistically as the major cytolytic mechanisms of activated macrophages. J Immunol 144: 1425–1431
13. Kahn HJ, Brodt P, Bammal R (1988) Lectin binding by liver and by metastasizing variants of the murine Lewis lung carcinoma. Am J Pathol 132: 180–185
14. Kaneda K, Natsuhara J, Oka S, Uehara K, Yano I, Wahe K (1990) In vivo defensive role of pit cells in the liver. In: Wisse E, Knook DL, Decker K (eds) Cells of the hepatic sinusoid, vol 2. Kupffer Cell Foundation, Rijswijk, pp 221–223
15. Lewis CE, McGee JD (1992) Natural killer cells in tumour biology. In: Lewis CE, McGee JOD (eds) The natural immune system: the natural killer cell. IRL Press at Oxford University Press, Oxford New York Tokyo, pp 175–203
16. Männel DN, Moore RN, Mergenhagen SE (1980) Macrophages as a source of tumoricidal activity (tumor-necrotizing factor). Infect Immun 30: 523–530
17. Malorny U, Michels E, Sorg C (1986) A monoclonal antibody against an antigen present on mouse macrophages and absent from monocytes. Cell Tissue Res 243: 421–428
18. Malter M, Süss R (1986) Immunocompetent cells in the liver: macrophages, NK-,T-, and B-cells. In: Kirn A, Knook DL, Wisse E (eds) Cells of the hepatic sinusoid, vol 1. Kupffer Cell Foundation, Rijswijk, pp 439–444
19. Martinet N, Charles T, Vaillant P, Vignaud JM, Lambert J, Martinet Y (1992) Characterization of a tumor necrosis factor-alpha inhibitor activity in cancer patients. Am J Respir Cell Mol Biol 6: 510–515
20. Matthys P, Heremans H, Opdenakker G, Billiau A (1991) Anti-interferon gamma antibody treatment, growth of Lewis lung tumours in mice and tumour-associated cachexia. Eur J Cancer 27: 182–187
21. Ohteki T, MacDonald HR (1994) Major histocompatability complex class I related molecules control the development of $CD4^+8^-$ and $CD4^-$ 8^- subsets of natural killer 1.1^+ T cell receptor -α/β^+ cells in the liver of mice. J Exp Med 180: 699–704
22. Orosz P, Krüger A, Hubbe M, Rüschoff J, Vonhoegen P, Männel DN (1995) Promotion of experimental liver metastasis by tumor necrosis factor. Int J Cancer 60: 867–871
23. Robles CP, Pollack SB (1986) Regulation of the secondary in vitro antibody response by endogenous natural killer cells: kinetics, isotype preference, and non-identity with T-suppressor cells. Immunology 137: 2418–2424
24. Schantz SP, Brown BW, Lira E, Taylor DL, Beddingfield N (1987) Evidence for the role of natural immunity in the control of metastatic spread of head and neck cancer. Cancer Immunol Immunother 25: 141–148
25. Schiller JH, Bittner G, Spriggs DR (1992) Tumor necrosis factor, but not other hematopoetic growth factors, prolongs the survival of hairy cell leukemia cells. Leuk Res 16: 337–346
26. Ting C, Hargrove ME (1982) Tumour cell-triggered macrophage-mediated suppression of the T-cell cytolytic response to tumour-associated antigens. I. Characterization of the cell components for induction of suppression. J Natl Cancer Inst 69: 867–872
27. Trinchieri G (1989) Biology of natural killer cells. In: Dixon J (ed) Advances in immunology. Academic Press, San Diego, pp 187–303
28. Wisse E, Van t' Noordende JM, Van der Meulen J, Daems WT (1976) The pit cell: description of a new type of cell occurring in rat liver sinusoids and peripheral blood. Cell Tissue Res 173: 423–443