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Enhanced tumour growth in the rat liver after selective elimination of Kupffer cells

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Abstract. The evidence that Kupffer cells are capable of controlling metastatic growth in the liver in vivo is largely circumstantial. The best approach when studying natural cytotoxicity activities of Kupffer cells is to investigate the effect of Kupffer cell elimination on tumour growth. Until now it has not been possible to eliminate Kupffer cells without affecting other cell populations. We have recently developed a new method to eliminate Kupffer cells selectively: intravenous injection of liposome-encapsulated (dichloromethylene)bisphosphonate (Cl_2MDP-liposomes) leads to effective elimination of all Kupffer cells, without affecting non-phagocytic cells. Wag/Rij rats were injected with Cl_2MDP-liposomes. After 48 h, rats were inoculated with syngeneic CC531 colon carcinoma cells by injection in the portal system. The results show a strongly enhanced tumour growth in the liver of the Cl_2MDP-liposome-treated rats. In these animals, livers were almost completely replaced by tumour and had increased in weight, whereas in the control groups only a few (four to eight) small (1-mm) tumour nodules were found. These data show that selective elimination of Kupffer cells results in enhanced tumour growth in the liver, implying that Kupffer cells play a crucial role in controlling tumour growth in the liver.

Key words: Liposomes – (Dichloromethylene)bisphosphonate – Kupffer cells – Metastatic growth – Liver

Introduction

It has been postulated that liver macrophages, the so-called Kupffer cells, act as effector cells in host defence against tumour growth and metastases. Since the liver is a major site of metastatic growth of primary colorectal cancer, Kupffer cell cytotoxicity against tumour cells is of interest. Experimental in vitro and in vivo studies have shown that Kupffer cells can be tumoricidal after activation with biological response modifiers [20, 8, 21, 6]. However, whether non-activated Kupffer cells are capable of controlling hepatic metastases (natural cytotoxicity) in vivo is not clear [21].

The best approach to the study of functional aspects of macrophage populations in vivo is to investigate the effect of selective macrophage elimination on defined processes in the body. Macrophages can be depleted by the administration of silica [12, 23], asbestos [12], carrageenan [26], and by a number of other treatments [22]. Earlier studies have shown that depletion or inhibition of macrophages affects tumour induction [14, 17, 19, 21]. However, none of these studies resulted in a complete and selective elimination of macrophages. The various methods for macrophage depletion appear to affect macrophages to different degrees and for different periods of time. Furthermore, most compounds used for elimination also have direct access to other immune effector cells, e.g. lymphocytes, which may be affected [37, 35].

Recently we have developed a method to eliminate macrophage populations selectively, using liposome-encapsulated (dichloromethylene)bisphosphonate (Cl_2MDP-liposomes) without affecting non-phagocytic cell populations [32, 33].

The aim of the present study was to investigate the effects of Kupffer cell elimination on tumour growth in order to determine the role of these cells during tumour colonisation in the rat liver. We also investigated the kinetics and repopulation of liver macrophages after Kupffer cell elimination in normal rats and after tumour cell inoculation. Furthermore, in order to establish whether the effect on tumour growth in the liver is a result of the Kupffer cell elimination or a direct effect on tumour cells, the in vitro effect of Cl_2MDP-liposomes on tumour cells was investigated.

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Materials and methods

Animals. Male Wag/Rij rats were obtained from Centraal Proefdieren bedrijf (T. N. O., Rijswijk, The Netherlands) and kept under routine laboratory conditions. The animals were 12 weeks old at the start of the experiments and weighed 241 ± 27 g.

Tumour cell line. The tumour cell line CC531 is a carcinoma originating in the colon of rats exposed to methylazoxymethanol. This well-defined cell line is syngeneic with Wag/Rij rats [39]. Tumour cells were cultured under standard incubator conditions in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (Flow Laboratories). Cell suspensions were prepared by enzymatic detaching of the CC531 cells with trypsin/EDTA solution (Life Technologies, Scotland, Gibco) at room temperature. After centrifugation, cell concentrations were resuspended in DMEM to the required concentration. The viability of the cells was assessed with trypan blue exclusion and was always more than 95%.

Induction of hepatic metastases. The animal model to study the development of tumour colonisation in the liver has been described previously [3]. Briefly, a laparotomy was performed under general ether anaesthesia and a loop of the small intestine was exposed. A suspension of 1×10^6 CC531 cells in 0.5 ml DMEM was slowly injected into a mesenteric vein under microscopic vision with a 0.4 × 12-mm needle and the vein was ligated.

Preparation of liposomes with entrapped Cl_2MDP. Multilamellar liposomes were prepared as described earlier [31]. Briefly, 86 mg phosphatidylcholine and 8 mg cholesterol, molar ratio 6:1 (Sigma, USA) were dissolved in 20 ml methanol/chloroform (1:1) in a round-bottom flask. The thin film that formed on the interior of the flask after low-vacuum rotary evaporation at 37° C was dispersed in 10 ml phosphate-buffered saline (PBS; 10 mM, pH 7.4), containing 2.5 g Cl_2MDP (a kind gift of Boehringer, Mannheim, Germany), by gentle rotation for 10 min. Free Cl_2MDP was removed by rinsing the liposomes with PBS and centrifuging them for 30 min at 100000 *g* at 16° C. The liposomes were then resuspended in 4 ml PBS.

Experimental design. Four rats were injected with 1 ml Cl_2MDP-liposomes i. v. and were killed 48 h later to determine whether Kupffer cells were eliminated. Eight rats were injected i. v. with 1 ml Cl_2MDP-liposomes on days 1 and 7. Another eight rats were injected with 1 ml liposome-encapsulated PBS as control, and finally eight rats were injected i. v. with 1 ml saline solution on days 1 and 7. On day 3, the three groups of rats received a single injection of tumour cells in a mesenteric vein. As another control, four rats were injected only with 1 ml Cl_2MDP-liposomes on days 1 and 7. All rats were sacrificed 2 weeks later and their livers were weighed. To determine the lowest dose of Cl_2MDP-liposomes necessary for total elimination of Kupffer cells, Cl_2MDP-liposomes were diluted in PBS: 100 µl (1/10), 50 µl (1/20), 20 µl (1/50) and 10 µl (1/100) in a total volume of 1 ml.

Four groups of two rats were injected with the various dilutions of Cl_2MDP-liposomes and killed 48 h later. We found that 50 µl (1/20) Cl_2MDP-liposomes was sufficient to eliminate all Kupffer cells with a minimal loss of spleen macrophages. With this Cl_2MDP-liposome dose, we performed the same experiment as described above.

Immunohistochemistry. The following mAb were used: ED1, a highly specific marker for cells of the mononuclear phagocyte lineage (monocytes, macrophages); ED2, a marker for resident macrophages (Kupffer cells in liver tissue); ED3, staining a macrophage subpopulation present in lymphoid organs only [7]; His14, a marker for B lymphocytes; Ox19, staining all T lymphocytes; Ox6, recognizing a monomorphic determinant of class II antigen of the major histocompatibility complex present on cells of all strains of rats. All these mAb are commercially available (Serotec, Oxford, UK). To detect the mAb a rabbit anti-(mouse IgG)/peroxidase conjugate (DAKOpatts, Copenhagen, Denmark) was used. Cryostat sections of liver and spleen of 8 µm were picked up on slides and air-dried. Sections were fixed in acetone for 10 min and air-dried. mAb were diluted in PBS with 0.5% bovine serum albumin (BSA) and used in optimal concentrations (ranging from 1:200 to 1:400). After incubation with the antibodies for 1 h at room temperature, the slides were rinsed thoroughly in PBS and incubated in conjugate for 30 min. Conjugate was diluted in PBS/BSA containing 1% normal rat serum. Slides were washed again in PBS and stained for peroxidase activity for 10 min with 0.5 mg/ml 3,3′-diaminobenzidinetetrahydrochloride (Sigma, St Louis, Mo.) in 0.05 M TRIS/HCl buffer, pH 7.6, containing 0.01% H_2O_2. After being rinsed in PBS, the samples were slightly counterstained with haematoxylin, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany). Control sections were incubated in PBS/BSA instead of mAb in the first step, with conjugate in the second step and examined for non-specific staining.

Determination of tumour load. The livers of the rats that had been pretreated with Cl_2MDP-liposomes and control livers were weighed to determine tumour load. The tumour load in the liver of rats of the PBS-liposome group and saline group was assessed by counting the number of tumour nodules.

Serum liver function tests. Blood samples for serum liver function tests were obtained from all rats at sacrifice. The following markers for liver function were assayed: alkaline phosphatase, γ-glutamyltransferase, aspartate aminotransferase, alanine aminotransferase and bilirubin.

In vitro effect of the liposomes on tumour cell growth. The effect of Cl_2MDP- and PBS-liposomes on tumour cell growth was studied in vitro by the use of the sulphorhodamine B protein stain [27, 13]. Briefly, CC531 tumour cells were harvested from exponential-phase cultures by trypsinisation, counted and plated in 96-well flat-bottomed microtitre plates (100 µl/well). Optimal seeding densities for the tumour cells (2×10^3/well) were determined to assure exponential growth during a 4-day assay. Experiments were performed in triplicate. Following plating and a 24-h recovery to allow cells to resume exponential growth, 100 µl culture medium or culture medium with Cl_2MDP- or PBS-liposomes (diluted 250× and 500×) was added to the wells. The cells were fixed by means of protein precipitation with 50% trichloroacetic acid at 4° C (50 µl/well) for 1 h 1, 2 or 3 days after Cl_2MDP- and PBS-liposome administration. After five washing steps with tap water, the cells were stained for 15 min with 0.4% sulphorhodamine B dissolved in 1% acetic acid (50 µl/well) and subsequently washed four times with 1% acetic acid to remove unbound stain. The plates were air-dried and the bound protein stain was solubilised with 150 µl 10 mmol/l unbuffered TRIS base [tris(hydroxymethyl)aminomethane]. Microplate absorbance was read at 540 nm on a Multiskan MCC/340.

Statistical analysis. The statistical significance of the data was analysed with an unpaired Student's *t*-test.

Results

Effect of Cl_2MDP-liposome treatment on Kupffer cells and spleen macrophages

In an earlier study it was found that all ED1- and ED2-positive cells from the rat liver and almost all ED1-, ED2- and ED3-positive cells from the spleen, had disappeared 48 h after i. v. administration of 2 ml Cl_2MDP-liposome suspension. It was shown that the first ED1-positive macrophages reappeared in the liver on day 4 [34].

In the present experiments, rats that had been injected with 1 ml Cl_2MDP-liposomes, showed a complete elimination of ED2-positive macrophages (Kupffer cells), whereas a limited number of small round ED1-positive macrophages were observed intravascularly (monocytes) and perivascularly in the liver tissue (newly recruited macrophages). The majority of splenic macrophages had disap-

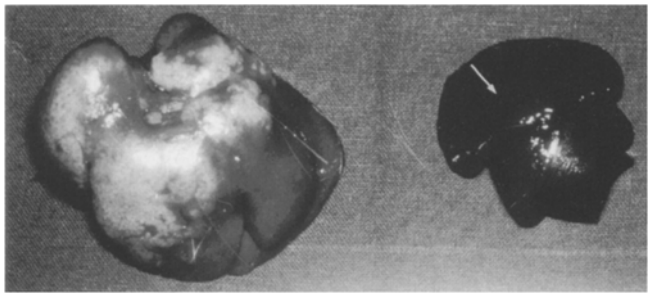

Fig. 1. *Left:* enhanced tumour growth in the liver of a rat where the Kupffer cells had been selectively eliminated by liposome-encapsulated (dichloromethylene)bisphosphonate (Cl_2MDP-liposomes) before tumour cell inoculation. *Right:* a control liver of a rat that had been treated with a saline solution before tumour cell injection. Small tumour nodules of 1 mm are present on the liver capsule (*arrow*)

peared. Rats that had been injected with a low dose (1/20) Cl_2MDP-liposomes still showed a complete elimination of Kupffer cells. However, an increased number of ED1-positive macrophages was observed intra- and perivascularly in the liver compared with the livers of rats injected with 1 ml Cl_2MDP-liposomes. The spleen slides showed that only a few spleen macrophages had disappeared.

Effect of Kupffer cell elimination of metastatic growth in the liver

The livers of all rats that had been injected with either dose of Cl_2MDP-liposomes were almost completely replaced by tumour, and had increased in volume (Fig. 1). The liver weights had markedly increased as compared to the control livers (Fig. 2). The mean liver weight of rats that had been injected with the Cl_2MDP-liposomes, was 32 g (27–52 g). The livers of rats that had been injected with PBS-liposomes or a saline solution before tumour cell inoculation and the livers of rats that had only been injected with Cl_2MDP-liposomes, weighed 10.1 g, 9.8 g and 10.3 g (mean). Rats, that had been injected with the saline solution before tumour cell inoculation showed 4–8 macroscopical tumour nodules 1 mm in diameter at the surface of the livers. The group of rats that had been injected with 1 ml PBS-liposomes showed a higher number of tumour nodules (30–50) in the liver, as compared to the rats injected with the saline solution. However, this increase was much smaller than the increase of tumour load of the rats that had been injected with the Cl_2MDP-liposomes. When the rats had been pretreated with the diluted PBS-liposomes (1/20), no increase in the number of tumour nodules was found as compared to the rats injected with a saline solution. Immunohistochemical examination of liver slides of rats injected with Cl_2MDP-liposomes, showed an infiltrating adenocarcinoma with a well-differentiated glandular structure. A marked increase of ED1-, ED2-, and ED3-positive macrophages was found in the remaining liver tissue (Fig. 3) as compared to slides from both control groups of rats. The ED1-positive cells were found intra- and perivascularly and in the portal and central areas of the liver. The ED2-, and ED3-positive macrophages were

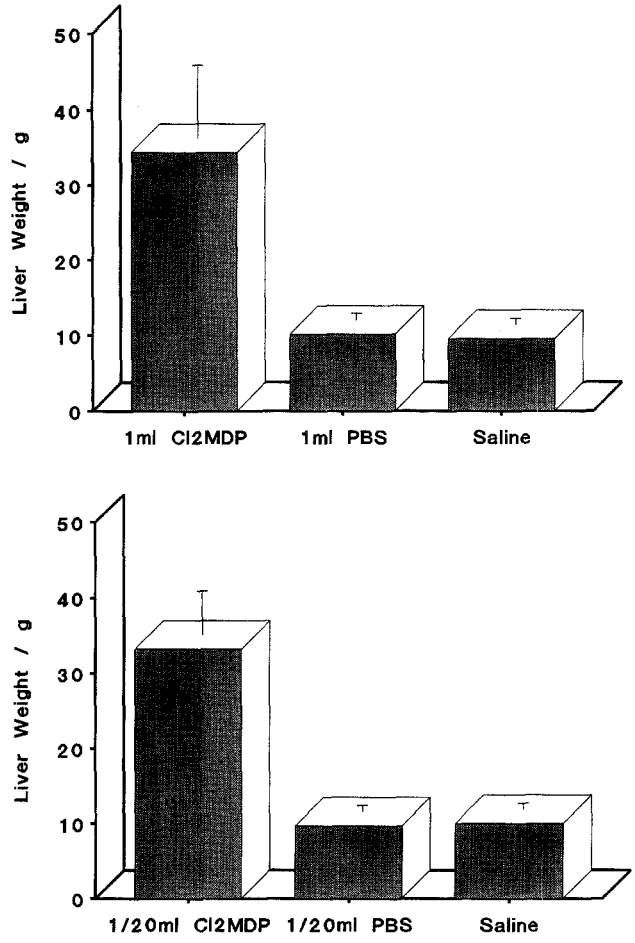

Fig. 2 A, B. Influence of Kupffer cell elimination on tumour growth in the rat liver after colonization. To determine tumour load the livers were weighed. The livers of all rats that had been i.v. injected with 1 ml Cl_2MDP-liposomes (**A**) and with the lower dose (1/20) of Cl_2MDP-liposomes (**B**) 48 h before tumour inoculation via the portal system had increased in weight (P <0.0001) in comparison to the livers of both control groups. **A** Rats were sacrificed on day 17. **B** Rats were sacrificed on day 21

present in the portal and central hepatic areas. The ED2-positive macrophages showed large cell extensions (like Kupffer cells).

Effect of Cl_2MDP- and PBS-liposomes on tumour cell growth in vitro

The absorbance readings of the experiments ranged from a value of 0.2 to 1.2, which are optimal values to determine the exponential growth of the CC531 tumour cells. Growth of tumour cells did not significantly increase or decrease in comparison to control tumour cells, when cells were incubated 1, 2 or 3 days with Cl_2MDP- or PBS-liposomes (Table 1).

Serum liver function tests

No increase of serum liver enzymes was found 48 h after i.v. injection of Cl_2MDP-liposomes. The rats pretreated

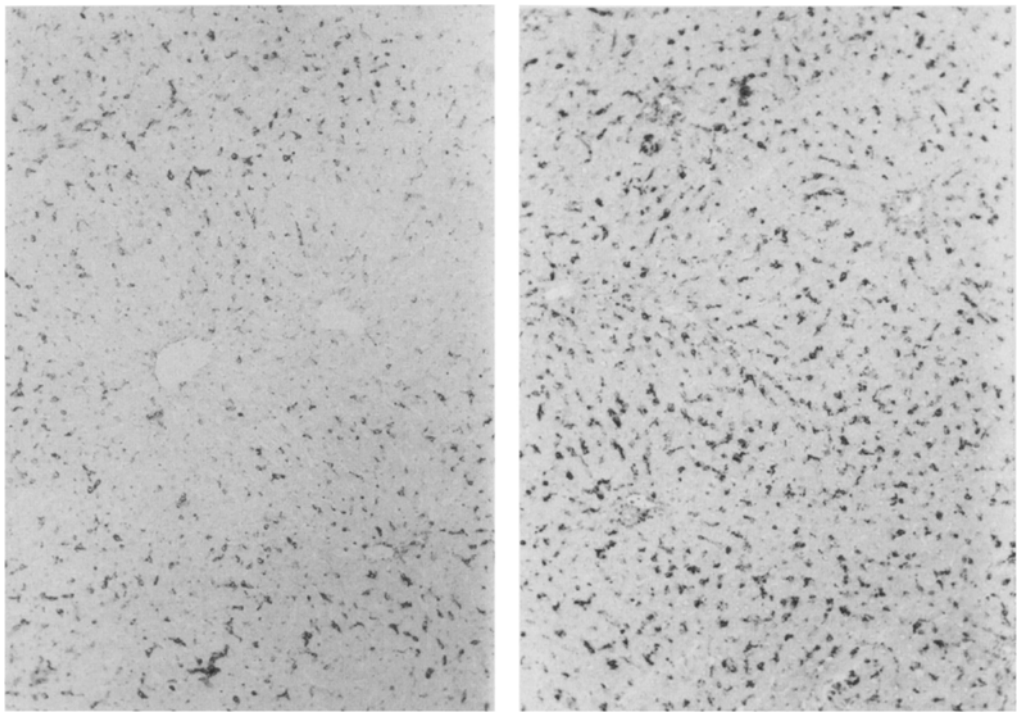

Fig. 3. *Left:* distribution pattern and number of Kupffer cells in the liver sinusoids of a control rat. *Right:* marked increase in number of newly recruited macrophages and Kupffer cells; stained with the mAb ED1 (monocytes and all macrophage populations), 100×

Table 1. Influence of liposome-encapsulated (dichloromethylene)bisphosphonate (Cl_2MDP-liposomes) and phosphate-buffered-saline(PBS)-liposomes on tumour cell growth in vitro[a]

Incubation	Tumour cell growth (A_{540})			
	Day 1	Day 2	Day 3	Day 4
2×10^3 CC531 tumour cells	0.2±0.01	0.39±0.02	0.74±0.03	1.10±0.02
PBS-liposomes 1:500		0.36±0.01	0.69±0.03	1.10±0.04
PBS-liposomes 1:250		0.35±0.02	0.65±0.03	1.10±0.03
Cl_2MDP-liposomes 1:500		0.35±0.03	0.68±0.03	1.09±0.04
Cl_2MDP-liposomes 1:250		0.33±0.02	0.65±0.02	1.00±0.02

[a] Samples of 2×10^3 CC531 tumour cells were added to 96-well plates for adherence. After 24 h Cl_2MDP- and PBS-liposomes were incubated with the tumour cells for 1, 2 and 3 days. Tumour growth was measured after the various intervals with a sulphorhodamine B assay (mean ±SD); the absorbance at 540 nm was measured. In the wells with the Cl_2MDP- or PBS-liposomes and the tumour cells, no significant increase or decrease of tumour cell growth was found in comparison to the control tumour cell growth. $n = 8$

with Cl_2MDP-liposomes before tumour cell inoculation showed disturbance in liver function at the end of the experiments. All liver function tests had significantly increased in value as compared to those of rats that had received PBS-liposomes or a saline solution. Obstructive jaundice caused by tumour was obvious in these rats (Table 2).

Discussion

Our data show clearly that selective elimination of Kupffer cells with Cl_2MDP-liposomes, before inoculation of tumour cells via the portal system, results in enhanced tumour growth in all rat livers. Almost the whole liver was replaced by tumour as compared to control livers. The enormous enhancement of tumour growth in the liver indicates a crucial role of non-activated Kupffer cells in controlling the growth of tumour in the liver after colonisation.

Various in vitro and in vivo studies have shown that Kupffer cells can be cytotoxic against tumour cells after activation. However, it is still controversial whether non-activated Kupffer cells play a role in the host defence mechanisms against tumour cells. It is suggested that, in general, macrophages require activation signals to express tumoricidal functions [8]. On the other hand it would appear that macrophages have a limited capacity to kill tumour cells without prior stimulation (e. g. via phagocytosis) [20]. Recently, Cl_2MDP-liposome pretreatment before tumour cell administration has been used in a murine liver metastasis model and has been shown to be without effect on tumour cell growth. The same study has shown that activation of Kupffer cells with liposomes containing muramyl peptide derivative before tumour cell injection

Table 2. Serum liver function tests[a]

Rats	Bil (μmol/l)	GT (U/l)	OT (U/l)	PT (U/l)	AF (U/l)
Normal rats	1.3±0.5	1.7±0.8	42±3	45±7	90±12
Rats treated before tumour inoculation with					
Saline solution	1.6±0.5	2.6±1.3	74±14	56±16	138±6
PBS-liposomes	1.2±0.4	1.7±0.8	65±25	48±5	135±5
Cl_2MDP-liposomes	7.8±1.5	7.4±1.3	560±145	178±49	218±25

[a] Blood samples to determine serum liver enzymes and bilirubin were obtained from rats when they were sacrificed. The following liver functions were determined: alkaline phosphatase (AF), γ-glutamyltransferase (GT), aspartate aminotransferase (OT), alanine aminotransferase (PT) and bilirubin (Bil). All liver enzymes of the rats that had been pretreated with Cl_2MDP-liposomes before tumour cell inoculation, were elevated. Obstructive jaundice was obviously present. $n = 14$

was effective in inhibiting tumour growth in the liver [21]. However, in this murine model a reticulum cell sarcoma was used, which was monocytic/macrophage in origin; this may explain the discrepancy between their and our observations. It is reasonable to suspect that tumour cell lines of monocytic/macrophage origin may have been affected by Cl_2MDP-liposomes via phagocytosis, since it is not known what time it takes before liposomes are cleared from circulation after i.v. injection. We may add that, in our approach, the Cl_2MDP-liposomes had no effect on the CC531 tumour cell line when tested in vitro.

Using a novel approach for selective elimination of Kupffer cells by Cl_2MDP-liposomes [32, 33], we were able to study functional aspects of the Kupffer cells, without affecting non-phagocytic cell populations. All other known methods to eliminate Kupffer cells have major disadvantages as discussed earlier [12, 23, 26, 22]. The liposomes used in this study, containing (dichloromethylene)bisphosphonate (Cl_2MDP-liposomes), when injected i.v., are ingested by the macrophages in the liver and the spleen. The drug is released into the interior of the cell as soon as the liposomal phospholipid bilayers are disrupted under the influence of phospholipases, present in the lysosomal compartments of the cells, which results in their destruction [31]. This technique eliminates all Kupffer cells and almost all spleen macrophages selectively and efficiently within 24 h [32]. Free Cl_2MDP does not enter macrophages in the concentration required to eliminate or even affect such cells [9]. The elimination of the Kupffer cells and spleen macrophages has been determined by immunohistochemical and immunocytochemical analysis with the macrophage-specific mAb ED1 and ED2 [7], and has been confirmed at an ultrastructural level, showing that the effect of the Cl_2MDP-liposomes is not based on a loss of antigens of these cells [33]. No effects of intravenously administered Cl_2MDP-liposomes are seen on the macrophage populations of other organs including the bone-marrow macrophage precursors [34, 10]. Non-phagocytic cells, such as T and B lymphocytes, are not affected by Cl_2MDP-liposomes [4]. To our knowledge, no alternative methods have been described for the effective and complete elimination of the Kupffer cells in the liver.

In our study the effects of the Cl_2MDP-liposome treatment on Kupffer cells and spleen macrophages were determined 48 h after i.v. administration. Both Cl_2MDP-liposome doses (1 ml and 1/20 ml) used in the experiments cause a complete elimination of Kupffer cells. On reducing the dose of Cl_2MDP-liposomes, we observed fewer eliminated macrophages in the spleen. Thus, by diminishing the dose of Cl_2MDP-liposomes, the method of macrophage elimination becomes more selective in eliminating Kupffer cells only.

Immunohistochemical analysis of liver tissue of rats at the end of the experiments in which Kupffer cells were eliminated before tumour cell inoculation showed a marked increase in number of newly recruited macrophages and Kupffer cells in the surrounding liver parenchyma and within the tumours as compared to control livers. This effect can not be explained by the administration of Cl_2MDP-liposomes, since macrophage populations reappeared in the liver and returned to normal values after selective elimination, as shown previously [34]. The regulation of macrophage infiltration and function in tumour tissue is a complex, multifactorial system [16]. This effect could be due to obstructive jaundice, since it is known from an earlier immunohistochemical study that the numbers of liver macrophages were increased in bile-duct-ligated animals [5]. The rapid repopulation of monocytes/macrophages (tumour-associated macrophages) could be a result of (a) an extensive influx [29, 17] from the blood, since it is known that several murine and human tumour lines produce chemotactic factors for mononuclear phagocytes [16, 2], and (b) in situ proliferation. Within the last few years the ability of tissue macrophages to proliferate has gained general acceptance [38, 36]. Various cytokines have been shown to stimulate the proliferation of macrophages, like granulocyte-macrophage-colony-stimulating factor (GM-CSF) and macrophage-colony-stimulating factor (M-CSF) [28, 25]. The knowledge that many tumours produce factors with (M-)CSF activity [16, 1, 15] suggests the possibility that the tumour may induce macrophage proliferation.

The administration of 1 ml PBS-liposomes resulted in an increased metastatic growth as compared to results in the control rats that received a saline solution. When the dose of PBS-liposomes was reduced (1/20) no difference in metastatic growth was noted between the two control groups. This may well be explained by the fact that macrophage functions are temporarily suppressed after phagocytosis of the PBS-liposomes. Blocking of macrophage functions by overload with liposomes has been described in an earlier study [11, 24].

In conclusion, this study clearly shows that selective elimination of Kupffer cells with Cl_2MDP-liposomes, re-

sults in strongly enhanced tumour growth in the rat liver. These results suggest that Kupffer cells play a crucial role in controlling the growth of tumour after liver colonization and form strong evidence for such a role of Kupffer cells in vivo. The exact role and the mechanism of the various liver macrophage subpopulations, involved in the growth control of tumour in the liver after colonization, is under investigation.

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