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Effect of intraperitoneal administration of granulocyte/macrophage-colony-stimulating factor in rats on omental milky-spot composition and tumoricidal activity in vivo and in vitro

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Abstract Milky spots in the greater omentum are small accumulations of leucocytes that consist mainly of macrophages and have recently shown to be a selective dissemination site of intraperitoneal (i.p.) inoculated tumour cells. However, milky-spot macrophages show tumoricidal activity and may, therefore, be an excellent source of effector cells suited for local immunotherapy. In the present study we first examined whether granulocyte/macrophage-colony-stimulating factor (GM-CSF) treatment of isolated milky-spot macrophages affects the cytotoxicity against syngeneic colon carcinoma cells (CC531) in vitro. Secondly, we studied the influence of intraperitoneal GM-CSF administration on the number and antitumour activity of milky-spot and peritoneal macrophages. All studies were performed in Wag/Rij rats in which a syngeneic colon carcinoma cell line (CC531) is available. The results of the in vitro study showed that GM-CSF treatment of the omental macrophages led to an increased cytotoxicity against the tumour cell line. Intraperitoneal administration of 1000 U GM-CSF daily for 7 consecutive days demonstrated both an enhanced antitumour activity of the milky-spot macrophages and an increase in the milky-spot macrophage population. An increase in the proliferative capacity, according to bromodeoxyuridine incorporation, was shown in the milky-spot macrophages. Taking into account both the enhanced macrophage number and their enhanced activity upon i.p. GM-CSF treatment, the milky-spot macrophages may provide a rationale for local intraperitoneal immunotherapy in the prevention of intra-abdominal tumour growth.

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Introduction

Milky spots in the greater omentum, now referred to as omentum-associated lymphoid tissue [1], are accumulations of leucocytes in which macrophages are the most numerous cell type [2, 4, 18, 30]. Milky spots are generally recognized as the site of origin of part of the free peritoneal macrophages [2, 6, 33], and are considered to have a microenvironment in which precursor cells of the mononuclear phagocyte system can home and proliferate [34]. Recently we have shown that isolated omental milky-spot macrophages from Wag/Rij rats are cytotoxic against the syngeneic CC531 colon carcinoma cell line in vitro [18]. However, there is increasing evidence that milkys spots in the omentum majus may be important in the early spread of intraperitoneally inoculated tumour cells and, moreover, may play a role in the defence against invading tumour cells [12, 19, 32].

In the last few years considerable attention has been paid to the use of biological response modifiers as an adjuvant immunotherapy to the conventional treatment of cancers and the prevention of metastatic disease, also with regard to neoplastic diseases of the abdomen [9]. Granulocyte/macrophage-colony-stimulating factor (GM-CSF) may be considered as a biological response modifier. Besides the established function of GM-CSF as a regulator of a haemopoiesis, it is capable of regulating the proliferation, differentiation and function of mature myeloid cells [23, 24]. GM-CSF may be important in antitumour defence as it induces macrophage and/or monocyte tumoricidal activity in vitro and in vivo [5, 11, 16]. Since macrophages are recognized as important non-specific effector cells in the resistance to and eradication of neoplasms, we have addressed the question whether GM-CSF may activate milky-spot-macrophage-mediated cytotoxicity.

In the clinical context, i.p. introduction of GM-CSF may be expected to result in a useful immunotherapy against disseminating gastrointestinal and gynaecological cancers by stimulation of peritoneal macrophages and, probably of greater importance, the milky-spot macrophages. Toner et al. [31] showed that GM-CSF can be safely administered into the human peritoneal cavity, having similar effects to i.v. administration in doses that have been used in other therapeutic settings.

The present study describes the influence of in vitro and in vivo GM-CSF administration with regard to omental and peritoneal macrophages. In vitro GM-CSF pretreatment of omental macrophages increased the antitumour activity against the CC531 tumour cell line. This prompted us to study and compare the effect of intraperitoneal and subcutaneous GM-CSF administration on cell number, cellular composition of the milky spots and cytotoxicity against the CC531 tumour cell line.

Materials and methods

Animals

Male WAG/Rij rats, aged 8–10 weeks (weighing approximately 240 g), were obtained from the Netherlands Cancer Institute (Amsterdam, The Netherlands) and kept under standard conditions. The investigation protocol was approved by the ethical committee.

Agents

Purified recombinant murine GM-CSF with a specific activity of 2.4×10^7 units/mg was a gift from Sandoz. The lipopolysaccharide content, as measured in the LAL assay was below 26 pg/mg. Proliferative activity induced by GM-CSF was assessed on both rat and mouse peritoneal macrophages and led to a similar level of tritiated thymidine incorporation in vitro.

Administration regime

Rats were intraperitoneally injected with 1000 U GM-CSF (0.5 ml 2000 U/ml in phosphate-buffered saline, PBS) daily for 7 subsequent days. This dose was based on pilot studies (data not shown) and findings reported by other groups studying GM-CSF administration in vivo [16]. Control animals were injected with PBS. On day 8 the animals were sacrificed and the desired cell populations were collected and further processed.

Tumour cell line

The syngeneic CC531 carcinoma cell line was induced in the colon of WAG/Rij rats exposed to methylazoxymethanol [21]. CC531 tumor cells were cultured in DMEM+ (Dulbecco's modified Eagle's medium; GibcoBRL, Life Technologies Ltd., Paisley, Scotland) supplemented with 10% fetal calf serum (Integro b.v., Zaandam, The Netherlands), 5 ml penicillin (5000 IU/ml)/streptomycin 40 (5000 μg/ml; Schering Plough, Heist op den Berg, Belgium) and 5 ml glutamine (200 mM; GibcoBRL) at 37 °C with 5% CO_2. To obtain a tumour cell suspension, the adherent cells were enzymatically detached by trypsin/EDTA (0.25 mg EDTA and 1.88 mg trypsin in 1 ml PBS) incubation and collected in DMEM+. After centrifugation at 300 *g* for 10 min at 4 °C the desired cell concentration was prepared in supplemented DMEM+. Viability, as determined by trypan blue exclusion, was always more than 95%.

Collection of peritoneal macrophages

Total peritoneal cells were harvested by injecting 10 ml cold medium 199 (GibcoBRL, Life Technologies Ltd., Paisley, Scotland) into the peritoneal cavity followed by a gentle massage of the abdomen for 1 min and collection of the medium. Cells were kept on ice and washed in supplemented DMEM+ at 300 *g* for 10 min at 4 °C. Cell number and viability (always above 95%) were determined by trypan blue exclusion. Cellular subsets were analysed on cytocentrifuge preparations according to May-Grünwald-Giemsa and immunohistochemical stainings using specific monoclonal antibodies for the rat (c.f. immunohistochemical characterization).

Isolation of omental macrophages

Greater omenta were excised under sterile conditions from sacrificed rats ($n = 4-6$). To obtain a cell suspension the omenta were enzymatically and mechanically dissociated according to a slightly modified method of Krist et al. [18]. Briefly, the omentum was minced into small tissue fragments and incubated in DMEM+ supplemented with 1 mg/ml collagenase CLS3 (235 U/mg; Worthington Biochemical Corporation, Freehold, N.J., USA) and 0.1 mg/ml DNase 1 type IV (1680 U/mg; Sigma Chemical Co. St. Louis, Mo., USA) for 30 min at 37 °C on a rock/roller bank. Supernatant was removed, filtered through a 100 μm polyamide wire gauze, washed in DMEM+ and kept on ice. The remaining tissue was resuspended in the enzyme solution and again incubated and processed as mentioned above. The remaining tissue fragments were forced through an open-chamber filter (pore 200 μm), which was continuously rinsed with DMEM+. The suspension was filtered and collected. The cell suspensions were pooled and centrifuged at 300 *g* for 10 min at 4 °C. Viability and cellular subsets were examined as mentioned earlier.

The macrophages in the omental suspension were subsequently enriched by counterflow centrifugal elutriation using a modified method as described by Heuff et al. [15]. The cell suspension was washed in elutriation buffer (Ca^{2+}/Mg^{2+}-free 1:10 diluted PBS containing 0.1% bovine serum albumin and 3 mM NaEDTA pH = 7.4). The elutriation procedure was performed at room temperature and the collected samples were kept on ice. The flow rate was calibrated at 23 ml/min. The fraction of interest, between flow rates 36 ml/min and 47 ml/min, was collected in 150 ml buffer. Cell number and viability (always more than 95%) were determined after the cells had been collected by centrifugation at 300 *g* at 4 °C for 10 min and pooled.

Characterization of cellular subsets

Immunohistochemical analysis of cytocentrifuge preparations was performed with the monoclonal antibodies mentioned in Table 1. Granulocytes, mast cells and mesothelial cells were differentiated by May-Grünwald-Giemsa staining.

MTT cytotoxicity assay

To determine macrophage cytotoxicity against the CC531 tumour cell line a modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used [20]. The assay is based on

Table 1 Monoclonal antibodies used to identify the different cellular subsets present in the cell suspensions. (*MPS* mononuclear phagocyte system)

mAb	Specificity	Source
ED1	MPS	Dr. Dijkstra [8, 4]
ED2	Mature macrophages	Dr. Dijkstra [8, 4]
OX6	MHCII Ia	Dr. Williams [21]
OX19/52	T cells	Serotec, Oxford, UK [7, 27]
OX33	B cells	Serotec, Oxford, UK [35]

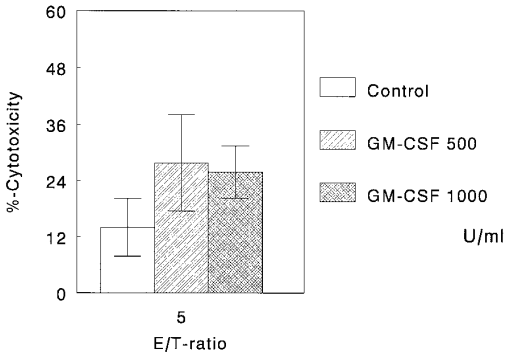

Fig. 1 Effect of granulocyte/macrophage-colony-stimulating factor (*GM-CSF*) pretreatment for 20 h of isolated omental macrophages on the in vitro cytotoxicity against the CC531 colon carcinoma cell line at an effector-to-target cell ratio (*E/T ratio*) of 5 ($n = 3$)

Table 2 Influence of i.p. administration of 1000 U granulocyte/macrophage-colony-stimulating factor (*GM-CSF*) daily for 7 subsequent days on the total cell number of the omentum suspension and peritoneal lavage. Results represent means ± SD, $n = 18-21$

Treatment	Cell number	
	Omental cells	Peritoneal cells
Control	$3.0 \pm 1.2 \times 10^6$	$3.8 \pm 1.1 \times 10^7$
GM-CSF	$5.8 \pm 2.9 \times 10^6$	$4.5 \pm 1.5 \times 10^7$

the fact that only viable cells can reduce the yellow-coloured formazan salt, MTT, into a purple formazan precipitate [26]. Briefly, in a 96-well flat-bottom microtitre plate (Nunc, Roskilde, Denmark) 5.0×10^4 macrophages/well were incubated together with different numbers of CC531 tumour cells to establish effector-to-target cell ratios (E/T) ranging from 1 to 5 in a total volume of 100 µl/well. Macrophages and the applied tumour cell numbers were incubated separately. All experiments were performed in triplicate in supplemented DMEM+. After the microtitre plates had been incubated for 20 h at 37 °C, 5% CO_2 and 90% relative humidity, 10 µl MTT (5 mg/ml; Sigma MTT M21 28) was added to of the wells, which were again incubated for 4 h under the previously mentioned conditions. Then the supernatant was aspirated from the wells and the remaining formazan crystals were dissolved in 175 µl (6/1) dimethylsulphoxide (Baker A 7033; Sigma D 8779 ACS)/glycine (0.1 M glycine, pH 10.5) buffer. The absorbance (*A*) was recorded on a microplate spectrophotometer (EL312e, Biotek Instruments) at 540 nm. Percentage cytotoxicity was calculated as follows:

$$\text{Cytotoxicity (\%)} = 1 - \frac{[\text{A CC531} + \text{macrophages}] - [\text{A macrophages}]}{[\text{A CC531}]} \times 100$$

BrdU incorporation

Bromodeoxyuridine (BrdU; 40 µg/g body weight, Sigma), dissolved in 0.5 ml PBS was injected intraperitoneally. The BrdU incorporation of free peritoneal and milky-spot macrophages was detected by immunohistochemistry on cytocentrifuge and omentum preparations respectively, 1 h after the administration of BrdU.

Statistical analysis

Data are presented as means and standard deviations. Statistical analysis was performed by the two-sample Mann-Whitney *U*-test where values of $P < 0.05$ (two-tailed) were considered significant.

Results

Effect of GM-CSF pretreatment of isolated omental macrophages on the cytotoxicity of CC531 cells

The effect of GM-CSF pretreatment on the tumorigenicity of isolated omental macrophages against the CC531 tumour cell line in vitro was studied in an MTT assay. Figure 1 shows the percentage cytotoxicity of untreated and GM-CSF-treated omental macrophages at an E/T ratio of 5. GM-CSF pretreatment leads to an up-regulation of the cytotoxicity compared to untreated cells; the most pronounced effect was found at 500 U/ml GM-CSF.

Influence of i.p. GM-CSF administration on cell number and cellular subsets of the milky spots in the omentum and peritoneal cell suspension

To determine whether GM-CSF has an effect on the milky-spot or peritoneal cellular composition in vivo, rats were injected i.p. with 1000 U GM-CSF daily for 7 consecutive days. Both cell number and the presence of different cell populations in the omentum were investigated as well as the peritoneal cavity 24 h after the last injection. GM-CSF treatment resulted in an increase in both peritoneal and omental cell number (Table 2) of 18.3% and 91.7% respectively.

The cellular subsets were immunohistochemically examined in cytocentrifuge preparations according to the immunoperoxidase method and/or May-Grünwald-Giemsa staining. Lymphocyte and mast cell numbers, if present at

Table 3 Influence of i.p. administration of 1000 U GM-CSF daily for 7 consecutive days on the percentage of cellular subsets in the omentum suspension and peritoneal cells. Results represent means ± SD percentage

Cell type	Omental cells ($n = 13-16$)		Peritoneal cells ($n = 10-17$)	
	Control (%)	GM-CSF (%)	Control (%)	GM-CSF (%)
$ED1^+$	31.8 ± 6.3	49.6 ± 6.0*	64.6 ± 16.2	57.0 ± 10.9
$ED2^+$	22.7 ± 7.3	33.1 ± 7.3*	8.9 ± 4.5	8.2 ± 6.5
$OX6(MHCII)^+$	24.5 ± 6.9	35.0 ± 9.9**	8.1 ± 3.6	8.6 ± 4.6
Eosinophils, granular	5.6 ± 1.7	7.7 ± 4.8	22.5 ± 4.8	29.0 ± 5.2***
Neutrophils, granular	0.2 ± 0.2	1.4 ± 1.0	2.4 ± 3.6	5.5 ± 7.7

*–*** Significantly different from control at *$P < 0.001$, **$P < 0.005$, ***$P < 0.05$

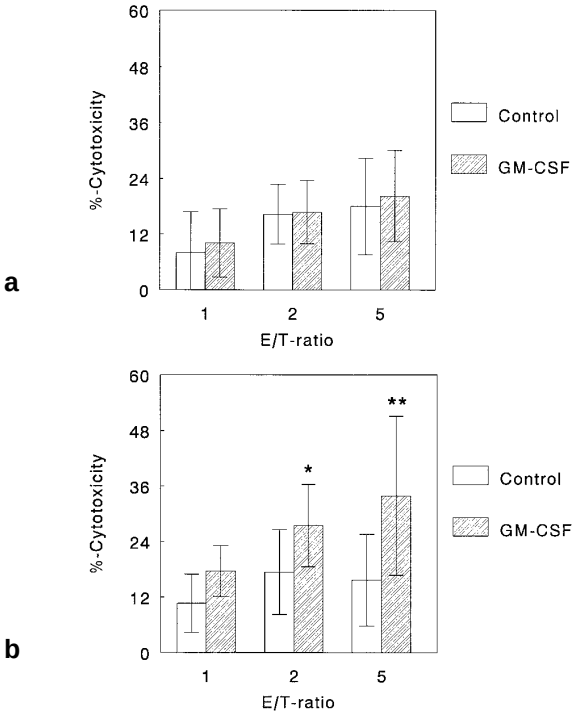

Fig. 2 a, b Influence of intraperitoneal administration of 1000 U GM-CSF daily for 7 consecutive days on the tumorigenicity of peritoneal macrophages (**a**) or omental cells (**b**) ex vivo. Data are expressed as mean ± SD percentage cytotoxicity. **P* < 0.05, ***P* < 0.005

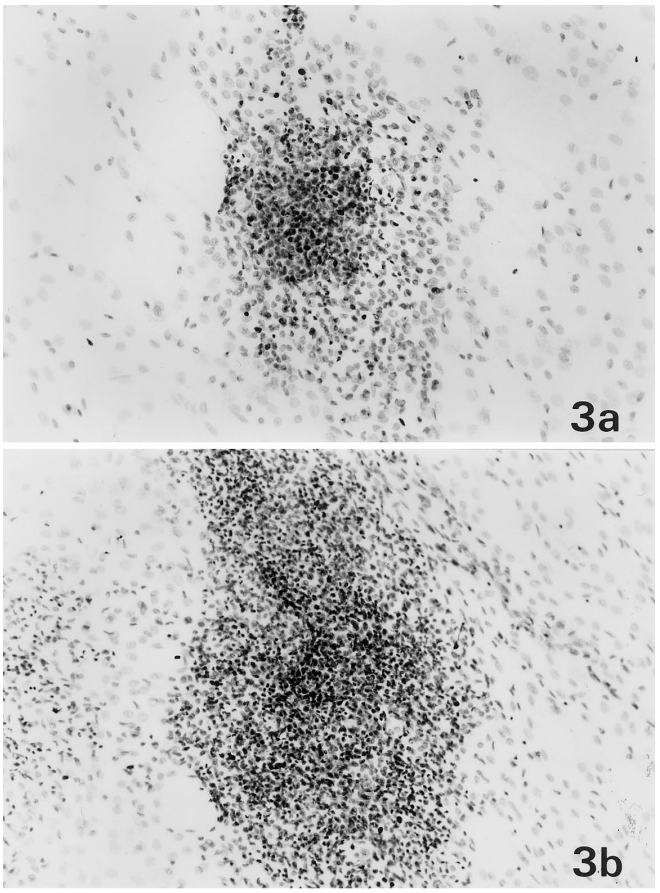

Fig. 3 a, b Bromodeoxyuridine (BrdU) incorporation 1 h after i.p. BrdU injection in the milky spot (magnification 200×) from omenta of animals previously i.p. injected with phosphate-buffered saline (**a**) or 1000 U GM-CSF (**b**) daily for 7 consecutive days. One representative spot for each group is shown; the dark-coloured cells have incorporated BrdU

all, in both the omentum and peritoneal cavity were hardly affected by GM-CSF treatment (data not shown). Table 3 compares changes in macrophage and granulocyte numbers of rats i.p. injected with GM-CSF or PBS. A significant increase in the percentage of ED1-, ED2- and OX6-positive cells was found in the omental cell suspension (Table 3). Granulocyte numbers in the omentum suspension were hardly affected.

With the exception of eosinophilic granulocytes, no significant changes in the cellular composition of the peritoneal lavage were found after GM-CSF treatment (Table 3).

Influence of i.p. GM-CSF administration on the ex vivo cytotoxicity of omental milky-spot cells and peritoneal cells against the CC531 tumor cell line

The observed in vitro increase (Fig. 1) in cytotoxicity prompted us to study the influence of i.p. GM-CSF administration on the tumoricidal capacity of both omental as well as peritoneal cells against the syngeneic CC531 tumour cell line. As shown in Fig. 2a, b a significant increase in cytotoxicity as a result of GM-CSF treatment was found for the omental cell population at effector target cell ratios of 2 and 5 (17.4±9.2% compared to 27.5±8.9% and 15.7±9.9% compared to 35.7±17.2% respectively). No significant increase could be demonstrated in antitumour activity of the peritoneal cells against the CC531 cell line.

Changes in proliferative capacity of the milky spots after GM-CSF treatment

One hour after intraperitoneal BrdU injection a comparison was made between both the BrdU incorporation of peritoneal macrophages and milky-spot macrophages of animals that had previously been treated with PBS and 1000 U GM-CSF daily for 7 subsequent days. Intraperitoneal GM-CSF administration did not affect the incorporation of BrdU of the free peritoneal macrophages (data not shown). In contrast to the peritoneal macrophages a significant increase (576±116 compared to 962±373; $P < 0.001$, $n = 3$) in the number of positively labelled macrophages per square millimetre of milky spot was found as a consequence of GM-CSF treatment. In Fig. 3a, b the proliferative activity of the milky spots after GM-CSF administration is illustrated.

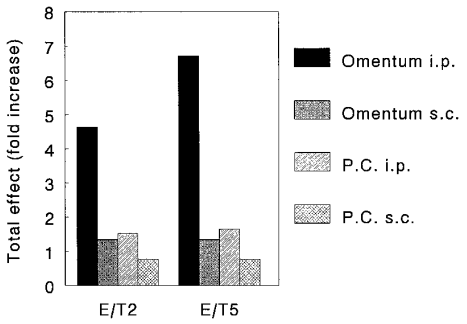

Fig. 4 Comparison of the combined effect of the increase in cell number, relative increase in macrophages ($ED1^+$) and cytotoxicity with values for the CC531 tumour cell line at E/T ratios of 2 and 5 after intraperitoneal (i.p.) and subcutaneous (s.c.) GM-CSF administration. No increase in cytotoxicity was found as a consequence of s.c. GM-CSF injection

Discussion

In this study we showed that GM-CSF administration both in vitro and in vivo leads to enhanced activity of milky-spot macrophages. Pretreatment of isolated milky-spot macrophages with GM-CSF increases the cytotoxic activity against the CC531 tumour cell line in vitro. In vivo, the administration of GM-CSF has many effects; we have shown an increase in omental macrophage number, ex vivo cytotoxicity and proliferative activity. In Fig. 4 the combined effect of in vivo GM-CSF administration is compared for intraperitoneal (i.p.) and subcutaneous (s.c.) injection; depending on the E/T ratio, i.p. administration leads, to a 4.5- to 6.8-fold increase in net effect of the omental cells.

GM-CSF is well known for its established myelopoietic activity and differentiation, proliferation and stimulation of the function of myeloid cells [13, 23]. The latter especially may provide an important way in which the antitumour activity of macrophages can be enhanced. Here we have shown that the cytotoxicity of omental rat macrophages against the syngeneic colon carcinoma cell line was up-regulated by GM-CSF both in vitro and in vivo. While the percentage of macrophages in the peritoneal cavity was hardly influenced by i.p. GM-CSF treatment, the milky-spot macrophage number significantly increased. In various murine studies the influence of i.p. GM-CSF administration on changes in peritoneal cell population revealed controversial results [13, 24, 25], which may be partly explained by differences in administration regime and/or the GM-CSF dose used.

An increase in the number of $ED1^+$, $ED2^+$ cells in the omentum was shown following 7 days of GM-CSF administration (Table 3). The increase in $ED1^+$ cells was shown to be somewhat higher than the $ED2^+$ number. The monoclonal antibody ED2 is specific for mature macrophages, while ED1 recognizes all macrophages. This implies that, besides an influx of monocytes, new macrophages appear by local proliferation of existing macrophages. The GM-CSF-induced local proliferation of macrophages was confirmed by the significant increase of BrdU incorporation, especially in the milky spots. These findings underline the assumption of other studies that milky-spots have an adequate microenvironment for the maturation of precursor cells from the mononuclear phagocyte system and contribute to the generation of free peritoneal macrophages [2, 34]. The fact that an increase in milky-spot macrophage number is not followed by enhanced peritoneal cell number may be the result of a lack of inflammatory stimuli in the abdominal cavity [2]. The only cell type in the peritoneal cavity that increased significantly upon GM-CSF treatment were eosinophilic granulocytes. Such an increase upon i.p. GM-CSF injection was also found by others [23] but, in contrast to our results, they showed an accompanying rise in other cell types present in the peritoneal cavity. Again these discrepancies may be the result of differences in GM-CSF dose, administration regime and/or the animal model used.

Macrophage/monocyte-mediated cytotoxicity against neoplastic cells has been reported by several investigators in human and animal studies [10, 11, 14, 29]. Recently, Krist et al. [18] showed that macrophages isolated from the milky-spots in the omentum majus of Wag/Rij rats are cytotoxic against syngeneic colon carcinoma cells (CC531) in vitro. It has been postulated that monocyte/macrophage tumoricidal activity can be up-regulated by GM-CSF [5, 11, 16, 17, 28, 31] in vitro and in vivo. This study confirms the occurrence of an enhanced tumorigenic activity of isolated omental macrophages after in vitro GM-CSF treatment.

Only a few reports exist on the in vivo administration of GM-CSF with respect to the function of macrophages in the prevention of cancer. Hill et al. [16] demonstrated a reduced tumor growth after i.p. GM-CSF injection in a murine model, which was parallelled by the enhanced production of oxygen radicals and nitric oxide. In our study, intraperitoneal injection of GM-CSF for 7 days led to a significant increase in ex vivo cytotoxicity of the omental cell suspension at effector/target cell ratios of 2 and 5. No significant increases was found for the peritoneal cells. This difference in cytotoxicity after GM-CSF administration between peritoneal and omental cells may be explained by the parallel rise in the number of mature macrophages ($ED2^+$) and MHC class II^+ cells, presumably representing the same cellular subset in the omental suspension (Table 2). The mature $ED2^+$, MHC-II-positive macrophages may be better capable of combating the CC531 carcinoma cells. Moreover, the earlier mentioned microenvironment created by harbouring omental cells, also present in the culture wells, may be responsible for the increased cytotoxic activity. Subcutaneous administration of GM-CSF (separate data not shown, for total response see Fig. 4) did not alter the cytotoxicity when compared to control animals. Local administration (i.e. intraperitoneal) of GM-CSF appears to be a necessity for the induction of an increase in cytotoxicity.

Toner et al. [31] demonstrated that GM-CSF can be safely administered i.p. in humans, which has similar effects to i.v. administration in doses that have been used

in other therapeutic settings. Instead of an increase in macrophage/monocyte number they found an enormous increase in neutrophilic granulocytes [31]. Unfortunately, data were available from one patient only. Moreover, the immunological status of this patient's peritoneal cavity could be considered as stimulated because of the continuous presence of an i.p. catheter and intra-abdominal malignancies.

In conclusion, pretreatment of isolated milky-spot macrophages with GM-CSF leads to an enhanced tumoricidal activity in vitro. Intraperitoneal administration of GM-CSF leads to an increase in milky-spot macrophage number and an enhanced cytotoxicity of omental milky-spot macrophages. The combination of these effects leads to a 4.5- to 6.8-fold increase in the total response (Fig. 4). Given the fact that milky spots are an important site for tumour cell dissemination [12, 32], our results support the need for a continued investigation of GM-CSF as an intraperitoneally administered immunotherapeutic agent in the stimulation of milky-spot macrophages in the prevention of intra-abdominal tumour growth and metastatic disease. The objective of future studies should be directed to this issue.

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