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ORIGINAL ARTICLE

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Milky spots in the greater omentum are predominant sites of local tumour cell proliferation and accumulation in the peritoneal cavity

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Abstract The role that milky spots in the greater omentum play in tumour cell spread in the peritoneal cavity is presently not fully understood. To study whether intraperitoneally injected tumour cells appear preferentially in milky spots of the greater omentum and to study the changes in the greater omentum, and especially in the cell population of milky spots after tumour cell infiltration, the following study was performed. A detailed temporal sequences of changes in morphology and cellular composition in milky spots of the greater omentum of Wag/Rij rats 5, 15, 30, 60 min, 2, 4, 8, 16, 24 h, 2, 4, 8 days and 2 and 4 weeks after intraperitoneal administration of 2.0×10^6 CC 531 tumour cells was investigated by light microscopy and electron microscopy (pre-embedding labelling). Our data showed that the milky spots in the greater omentum were the sites to which tumour cells migrated preferentially from the peritoneal cavity. The tumour cells infiltrated the milky spots and formed clusters within. The cellular population in milky spots reacted by a very rapid influx of young macrophages during the first hour and an increase of the total number of cells ($P < 0.01$). After 4 h tumour cells were also located on the greater omentum outside the area of the milky spots. Around these tumour cell deposits, new milky spots are formed, which increased the total number of milky spots. The cells present in milky spots are not capable of reversing the growth of tumours and finally a solid omental cake of tumour cells is formed.

Key words Greater omentum · Milky spots · Tumour cells · Macrophages · Solid metastasis

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Introduction

Milky spots are performed accumulations of leucocytes, primarily macrophages, in the greater omentum of humans and of animals [3, 10, 18, 25]

Milky spots are involved in the clearance of particles, bacteria and tumour cells from the peritoneal cavity [9, 11, 15, 22]. Moreover, several authors have established that tumour cells are present in milky spots 24 h after intraperitoneal (i.p.) injection [7, 8, 13]. These studies, suggested that, within the peritoneal cavity, milky spots in the greater omentum are important sites of tumour cell metastases. However, these studies did not describe the importance of milky spots in the process of tumour cell arrest and proliferation into solid tumours within the milky spots. This is of clinical importance, since in the peritoneal cavity the greater omentum is an important site of metastases from tumours of the colon, ovaries and stomach.

It has been well established that macrophages exhibit tumoricidal activity [12, 20, 27]. Since, on one hand, omental milky spots are accumulations of macrophages and, on the other hand, tumour cells locate in milky spots, the macrophage population in the milky spots could have a specific function in the control of metastatic spread in the peritoneal cavity. Dullens et al. [7] reported that tumour cells located in milky spots could develop into solid tumours; however, we established recently that macrophages isolated from milky spots in the greater omentum are cytotoxic in vitro [19], as was earlier also noted by Dullens [8]. Moreover this could be enhanced by immunomodulation with granulocyte/macrophage-colony-stimulating factor (GM-CSF) [17].

From these studies the balance of the milky spots in facilitating or preventing metastatic spread of tumour cells is not clear. Therefore the possible role of the cell population of milky spots in the greater omentum, especially that of macrophages, in clearance and control of tumour cell spread in the peritoneal cavity in vivo, was investigated. We therefore determined whether tumour cells injected i.p. appear preferentially in milky spots and studied the changes in the greater omentum and especially in the cell population of the milky spots.

Materials and methods

Animals

Male WAG/Rij rats were obtained from Harlan CPB (Zeist, the Netherlands) and were kept under routine laboratory conditions in accordance with institutional guidelines. The animals were 10 weeks old at the start of the experiments and weighed 210 ± 30 g. Experimental procedures performed on the rats were reviewed and approved by the Institutional Committee for Animal Experiments.

Tumour cells

The tumour cell line 531 is a carcinoma originating from the colons of rats exposed to methylazoxymethanol. This well-defined cell line is syngeneic with Wag/Rij rats [29]. Tumour cells were cultured under standard incubator conditions in Dulbecco's modified Eagle's medium (GibcoBRL, Life Technologies Ltd., Paisley, Scotland), supplemented with 10% fetal calf serum (Integro b.v., Zaandam, the Netherlands), 50 IU/ml penicillin and 50 μg/ml streptomycin (both from GibcoBRL), 0.04 mg/ml gentamycin (Schering Plough, Heist op den Berg, Belgium) and 20 mM glutamine (GibcoBRL). Cell suspensions were prepared by enzymatic detaching of the CC 531 cells with trypsin/EDTA solution (0.25 mg EDTA and 1.88 mg trypsin both from GibcoBRL in 1 ml phosphate-buffered saline, PBS) at room temperature. After centrifugation at 300 *g* for 10 min at 4 °C, cells were resuspended in sterile PBS. Occasionally clumping of up to four cells was seen. The viability of the cells was assessed by trypan blue (0.1%) exclusion and was always at least 96%.

Experimental design I

To investigate whether i.p. administered tumour cells located initially in milky spots of the greater omentum and did not locate at other sites in the peritoneal cavity or at sites outside the peritoneal cavity, Wag/Rij rats (3 per assay time) received an i.p. inoculation of 2×10^6 tumour cells/rat in 0.5 ml sterile PBS. The animals were sacrificed 5, 15, 30 and 60 min after inoculation.

The number of tumour cells present in the peritoneal cavity in an undetached state was assessed by rinsing the abdominal cavity with Hanks buffered saline (4 °C). This cell suspension was centrifuged at 300 *g* for 10 min at 4 °C. Cells were resuspended and counted. Subsequently, cytospin preparations were used to characterize the cell population present and, furthermore, to assess the percentage of tumour cells present by immunohistochemical staining (described below). The entire greater omentum was excised and spread on glass slides and dried. Biopsies were taken of the diaphragm, parietal peritoneum and of the mesenterium of the small intestine, spread on glass slides and dried.

Spleen, liver, lung, thymus, parathymic, paratracheal, mesenteric and axillary lymph nodes were sampled and snap-frozen in liquid nitrogen, after which cryostat sections (8 μm) were prepared. Subsequently specimens were processed for immunohistochemical staining, as described below, and positively staining cells in the tissue samples were counted semi-quantitatively.

Control rats were injected with sterile PBS.

Experiment design II

To investigate the reaction of the milky spot cell population, especially that of the macrophages, to i.p. inoculated tumour cells over time, Wag/Rij rats (3 per assay time) received an i.p. inoculation of 2.0×10^6 CC 531 tumour cells/rat. The rats were sacrificed 5, 30, 60 min, 2, 4, 8, 16, 24 h, 2, 4, 8 days and 2 and 4 weeks after inoculation. The abdominal cavity was opened and the greater omentum dissected, washed in normal saline, spread on glass slides, dried and further processed for immunohistochemical labelling as described below. The total number of milky spots per omentum, the total number of cells per milky spot and the percentage of positively staining cells per milky spot were counted to assess the changes in the cell population of the milky spots.

As controls, rats ($n = 3$) were sacrificed without inoculation of tumour cells ($t = 0$) and at each assay time 1 was injected with sterile PBS.

Immunoperoxidase labelling

Characterization of cells present in the peritoneal lavage fluid and in the tissue samples was performed by immunophenotyping using the monoclonal antibodies (mAb) listed in Table 1. Slides with cytocentrifuge preparations were fixed in buffered formalin/acetone (1.67 g/l $Na_2HPO_4 \cdot 12H_2O$ and 3.33 g/l KH_2PO_4 in 30 ml distilled water with 45 ml acetone and 25 ml formalin, pH 6.6) for 30 s at 4 °C, while the tissue samples were fixed in acetone for 10 min and air-dried for 1 h. Specimens were subsequently washed in PBS (pH 7.4) and incubated with the mAb (dilutions see Table 1) for 60 m at room temperature. After washing in PBS, specimens were incubated with peroxidase-labelled rabbit anti-mouse IgG in PBS with 0.5% bovine serum albumin (Sigma, St Louis, USA) and 1% normal rat serum for 60 min. Peroxidase activity was demonstrated with 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M TRIS/HCl buffer (pH 7.6) containing 0.01% H_2O_2 for 10 min. After washing in PBS, the specimens were counterstained with Harris haematoxylin, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany). For controls, the first step (incubation with mAb) was omitted.

Counting of cells

The number of cells per milky spot was determined microscopically by measuring the surface area of 20 randomly selected milky spots per omentum. Subsequently the number of cells per surface area was determined. The number of cells per milky spot was obtained by the following formula:

$$\text{Number of cells per surface area} \times \text{surface area per milky spot} = \text{number of cells per milky spot}$$

Table 1 Monoclonal antibodies (mAb) used

mAb	Against	Dilution	Reference
ED1	Monocytes/all macrophages	1:400	[6]
ED2	Resident tissue macrophages	1:400	[6]
OX33	(Pan) B cells	1:500	[28]
OX19/OX52	(Pan) T cells	1:1	[5]
CC 52	CC 531 tumour cells	1:1	[14]

The percentage of cells of different type per milky spot was determined by counting 3 × 100 cells of 6 randomly selected milky spots per glass slide.

Statistical analysis

Statistical analysis, to compare the changes in the number of milky spots per omentum and in the number of cells per milky spot within the cell population of the milky spots in the two groups during the time periods studied, was carried out using the Wilcoxon matched-pairs signed-rank test.

Experimental design III

To determine the maturation stage of the expanding macrophage population in the milky spots during the first 2 h after i.p. inoculation of tumour cells, the endogenous peroxidase activity pattern of macrophages was demonstrated as described before [1, 2]. Cells were fixed for 10 min in 1.5% glutaraldehyde at 4 °C, washed three times in 0.1 M sodium cacodylate (pH 7.4), put into an Eppendorf tube and tested for peroxidase activity in 0.1% 3,3-diaminobenzidine tetrahydrochloride. Preincubation and incubation with 0.01% H_2O_2 were each done for 1 h in the absence of light at 20 °C. In addition, the cells were washed, postfixed with OsO_4 for 30 min at 4 °C, processed for electron microscopy and embedded in Araldite.

For immunocytochemistry (immunoperoxidase staining at the ultrastructural level) we used the method described before [4]. Briefly, the cells were fixed in 0.05% glutaraldehyde in PBS for 5 min at 4 °C, washed, incubated with the mAb ED1 or ED2 for 45 min, washed, incubated in peroxidase-labelled rabbit anti mouse IgG for 30 min, washed, postfixed for 5 min in 1% glutaraldehyde at 4 °C, and thereafter prepared for electron microscopy as described above.

Results

Experiment I

To determine whether i.p. administered tumour cells initially locate in milky spots of the greater omentum and not at other sites in the peritoneal cavity nor at sites outside the peritoneal cavity, the peritoneal lavage fluid, the greater omentum and the peritoneal cavity, as well as lung, thymus and lymph nodes were studied.

A gradual decline of the tumour cells present in the peritoneal lavage fluid was seen during the first hour. Of the 2.0×10^6 administered tumour cells 60%–80% were still present in the lavage fluid after 5 min whereas, after 60 min, this had decreased to 20%–40%.

On examination of the entire greater omentum, tumour cells were only found in the milky spots. Within 5 min after inoculation of tumour cells these cells were present in the milky spots (Fig. 1). The greater omentum outside the area of the milky spots showed sporadically the presence of a tumour cell. Nearly all the tumour cells were located in milky spots.

The biopsies taken of the diaphragm, parietal peritoneum and mesenterium of the small intestine as well as the spleen, liver, lung, thymus and the parathymic, paratracheal, mesenteric and axillary lymph nodes were all negative for tumour cells (Table 2).

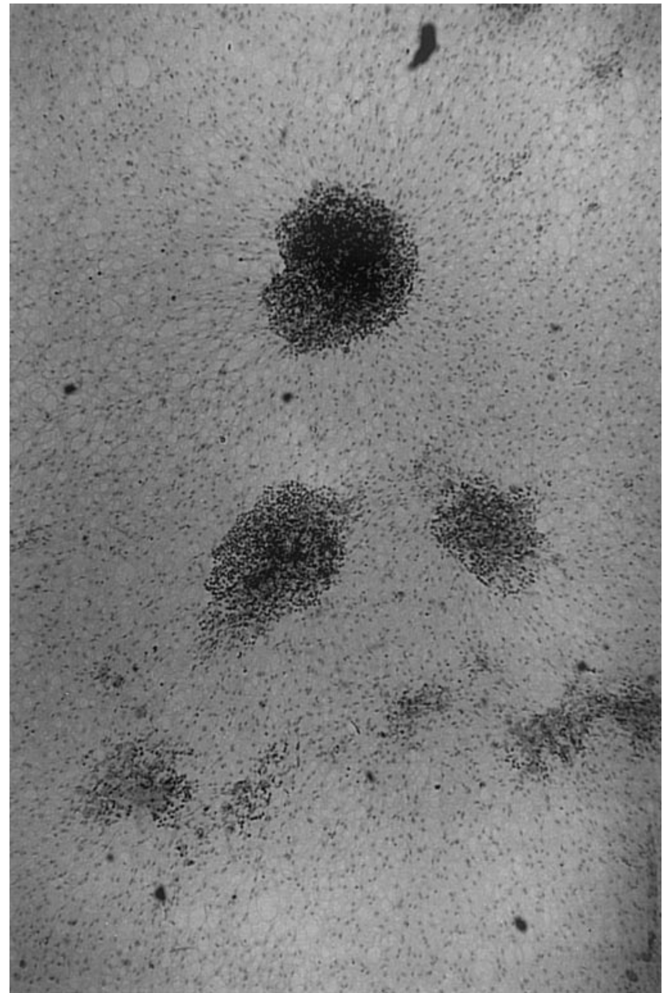


Fig. 1 This detail of a piece of omentum stretched on a glass-slide and stained with a monoclonal antibody (CC 52) against the tumour cells shows that, 5 min after intraperitoneal injection of tumour cells, these cells are found only in milky spots of the greater omentum. During the first 4 h, tumour cells are rarely found outside the area of milky sports (× 50)

Experiment II

Experiment II was performed to determine the reaction of the cells present in milky spots with the administered tumour cells during a period of up to 4 weeks. Macroscopic observations were made of the lavage fluid, the greater omentum and the peritoneal cavity. Microscopic observations were made of the tumour cells in the greater omentum, the changes in milky spots and in the cell population of the milky spots in particular.

Macroscopic observations

In the lavage fluid, no changes were seen macroscopically for up to 4 days. After 4 days the lavage fluid was blood-stained in 3 out of 3 rats (3/3), which changed into gross haemorrhagic ascites in 2/3 rats after 4 weeks.

Table 2 Presence of tumuor cells after intraperitoneal inoculation. *MS* milky spots, *PC* biopsies taken from peritoneal cavity, *LN* lymph nodes sampled, *PLF* lavage fluid from peritoneal cavity. – no tumor cells detectable, + 0–20% tumor cells present, + + 20%–40% tumor cells present, + + + 40%–60% tumor cells present, + + + + 60%–80% tumor cells present

Time (min)	*MS*	Omentum	*PC*	Liver	Spleen	*LN*	PLF
5	+	–	–	–	–	–	+ + + +
15	+	–	–	–	–	–	+ + +
30	+	–	–	–	–	–	+ +
60	+ +	–	–	–	–	–	+ +

A hyperaemic greater omentum with large accumulations of cells was present in 1/3 rats after 4 days. After 1 week 3/3 rats had large cellular accumulations, which remained constant for up to 2 weeks. At 4 weeks large areas of tumour cell accumulation were present in the omentum of 2/3 rats, whereas in 1/3 rats small areas of tumour cell accumulation were present. In this omental cake of tumour cell accumulation it was not possible to recognize milky spots.

In the peritoneal cavity, only a few small areas of tumour cell accumulation were present on the liver capsula and on the mesenterium measuring 0.5–1.0 mm after 2 weeks. At four weeks 2/3 rats had large deposits of tumour cells in the peritoneal cavity. Tumour cell accumulations were seen on each of the liver lobes and on the spleen. Large deposits were found around the intra-abdominal part of the oesophagus and on the diaphragm. The same pattern, but to a lesser extent, was seen in 1/3 rats.

Microscopic observations

Tumour cells. The localization of tumour cells on the greater omentum as well as in the milky spots was studied by light microscopy.

Within 5 min after administration the tumour cells were present in milky spots. During the first hour there was a gradual increase in the percentage of tumour cells present in the milky spots to 20%, which further increased to a maximum of 30% after 4 days (Fig. 2a). The greater omentum outside the milky spots showed sporadically the presence of a tumour cell during the first 4 h. However, after 4 h solitary, tumour cells were seen on the greater omentum, which increased in number. Within the milky spots, tumour cells had formed large clusters after 24 h.

From 4 h to 4 days, clusters of tumour cells surrounded by leucocytes were formed in the greater omentum outside the milky spots. The leucocytes were mainly macrophages.

After 4 days, tumour cells were located within milky spots as large aggregates, whereas on the greater omentum a few loose-lying tumour cells were present.

After 1 and 2 weeks the situation within the milky spots as well as in the rest of the greater omentum had not changed from that existing after 4 days.

At 4 weeks many tumour cells were present throughout the greater omentum and deformed the omentum into an omental cake. Large, bulky deposits of tumour cells were seen. Because of the number of tumour cells present, milky spots had lost their characteristic appearance and the number of cells and the percentage of cells could not be assessed any more.

Milky spots. The number of milky spots, expressed per omentum, was almost equal to that of the PBS group for up to 8 hours. The number of milky spots in the tumour cell group increased almost twofold after 24 h compared to the number in the PBS group. This difference disappeared at 1 week (Fig. 2b), while at 4 weeks the greater omentum had changed remarkably into an omental cake.

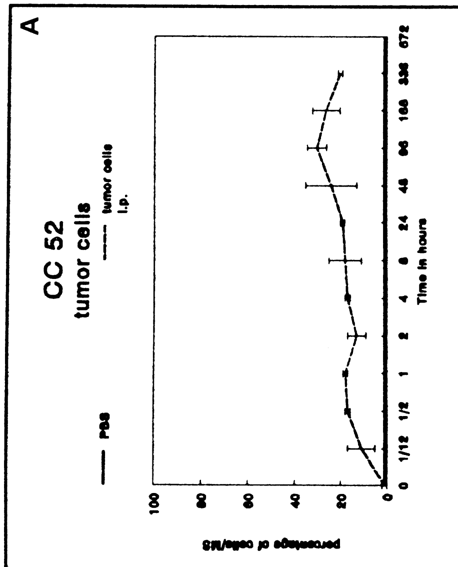
The number of cells per milky spot increased twofold within 30 min in comparison with PSB group, A maximum fivefold increase could been seen in the cells present in the milky spots after 1 week (Fig. 2c). There was a significant increase in the number of cells per milky spot in the tumour cell group ($P < 0.01$).

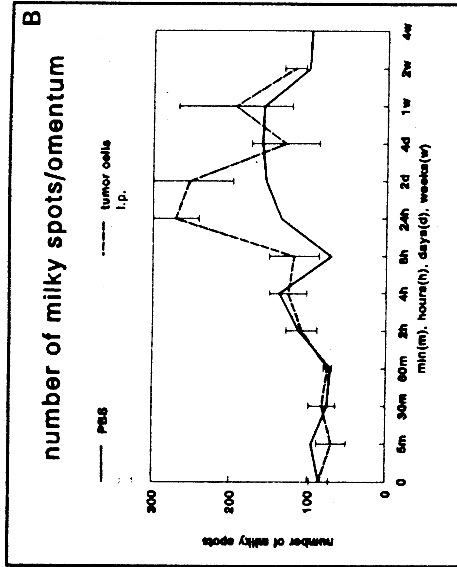
Cell population of milky spots. On examination of the cellular composition of milky spots after intraperitoneal inoculation of tumour cells, the following results were found.

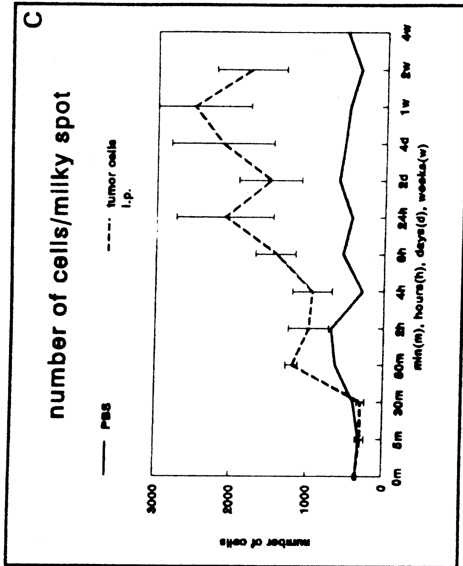
Macrophages: the percentage of macrophages showed a steady decline in both the tumour cell group and the PBS group (Fig. 2d). However, there was a remarkable difference between the two groups in the subpopulation of mature ED-2-positive macrophages during the first 2 h. In the tumour cell group the percentage of ED-2-positive cells in milky spots declined rapidly to 20% at 60 min, whereas in the PBS group about 50% ED-2-positive cells were present during the

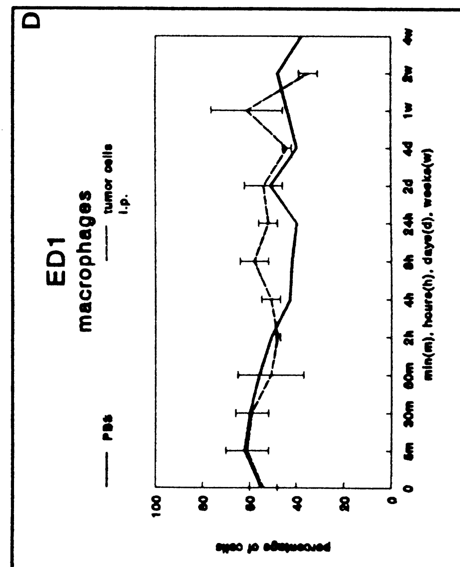
▶

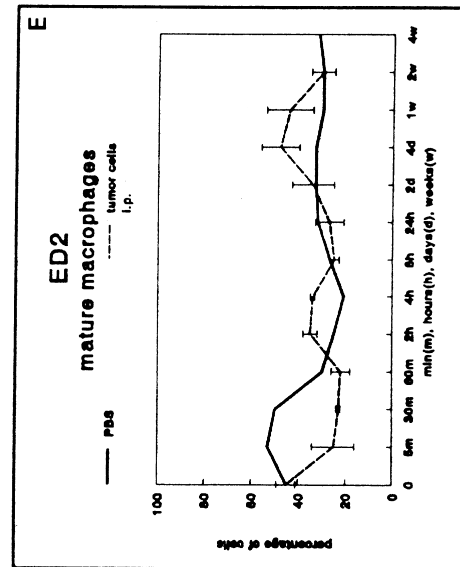
Fig. 2 A–F The groups of rats were formed. One group ($n = 3$) in which tumour cells were injected into the peritoneal cavity (tumour cells i.p.) and, as a control, one group in which phosphate-buffered saline was injected, the substance in which the tumour cells were dispersed. **A** During the period studied there is a gradual increase in the percentage of tumour cells in milky spots. **B** The total number of milky spots doubled and then returned to the same number as in the control group. However, after 4 weeks an omental cake of tumor was formed, in which milky spots were indistinguishable. **C** The number of cells per milky spot increased significantly ($P < 0.01$). **D–F** Interestingly, the percentage of macrophages and lymphocytes did not change significantly, although an early decline in the percentage of mature macrophages (ED2) can be seen (**E**), which indicates an influx of young macrophages (T lymphocyte data not shown; same pattern as **B** lymphocytes)

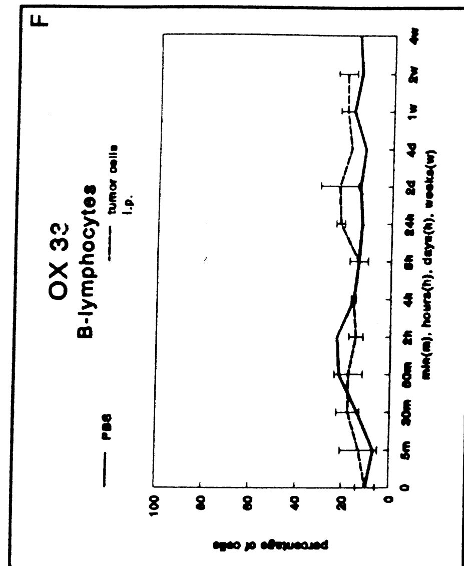

A
CC 52
tumor cells
PBS
tumor cells i.p.
percentage of cells/MS
Time in hours


B
number of milky spots/omentum
PBS
tumor cells i.p.
number of milky spots
min(m), hours(h), days(d), weeks(w)


C
number of cells/milky spot
PBS
tumor cells i.p.
number of cells
min(m), hours(h), days(d), weeks(w)


D
ED1
macrophages
PBS
tumor cells i.p.
percentage of cells
min(m), hours(h), days(d), weeks(w)


E
ED2
mature macrophages
PBS
tumor cells i.p.
percentage of cells
min(m), hours(h), days(d), weeks(w)


F
OX 33
B-lymphocytes
PBS
tumor cells i.p.
percentage of cells
min(m), hours(h), days(d), weeks(w)

first 30 min, declining to 30% at 60 min. After 2 h the percentage difference between the two groups had diminished (Fig. 2e).

Lymphocytes: there were no differences seen in the percentage of B cells (Fig. 2f) and T cells (data not shown) between the tumour cell group and the PBS group.

Experiment III

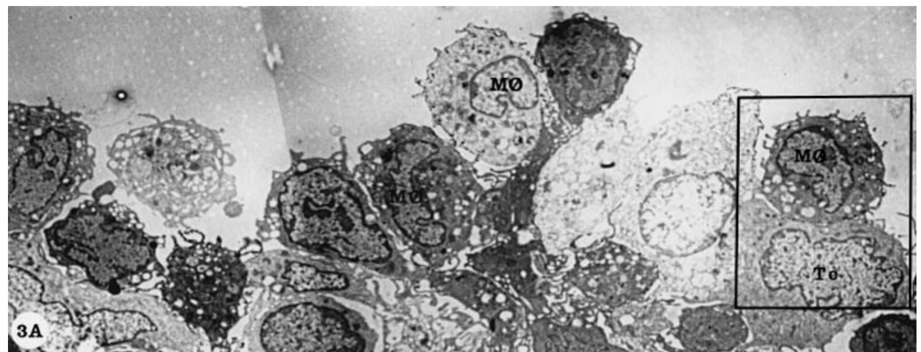
Ultrastructural analysis was performed to determine the maturation stage of the expanding macrophage population in the milky spots during first 2 h as determined by peroxidase activity staining characteristics [4].

In the tumour cell group exudate macrophages (monocyte-derived), exudate resident macrophages and resident macrophages were present. The peroxidase staining characteristics and the material used meant that quantification was not possible on the tissue level using these characteristics [16]. However, clearly more exudate macrophages were seen in the tumour cell group, confirming the influx of young macrophages on the ultrastructural level.

Since mAb ED2 is located on the plasma membrane, a qualitative analysis was possible by immunocytochemistry. In the tumour cell group about 30%–35% of the total macrophage population were mature ED2-positive macrophages whereas 60%–70% ED2-positive cells were seen in the PBS group. Moreover, from these results it is clear that the tumour cells were in good morphological conditions (Fig. 3).

Discussion

The main conclusion to be drawn from this study are that milky spots in the greater omentum are primarily the site where tumour cells locate in the peritoneal cavity



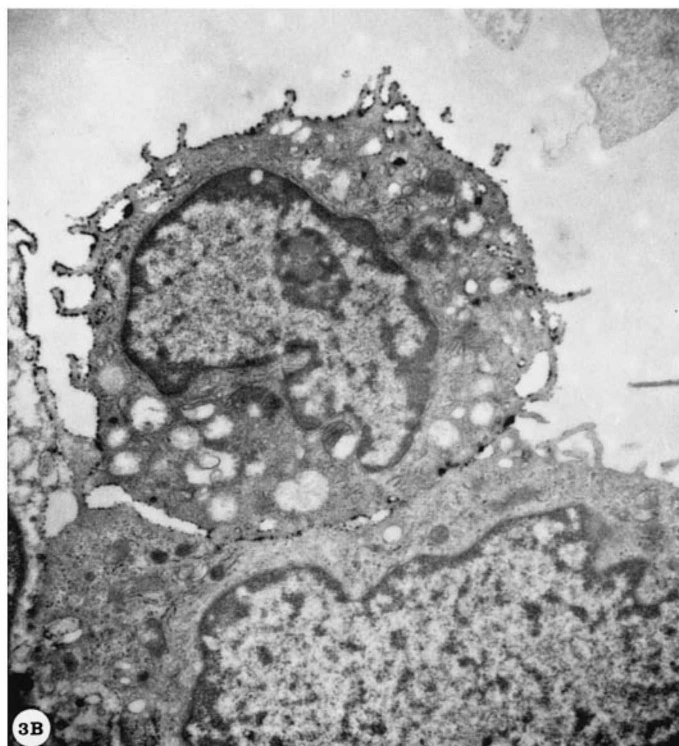


Fig. 3 A Overview of a milky spot stained on the ultrastructural level for ED2. Macrophages ($M\phi$) are present and tumour cells (*Tc*) are seen (× 2000). **B** In this detail a macrophage staining for ED2 is seen (membrane staining) closely associated with a tumour cell. Mature ED2-positive macrophages from 30%–35% of the total macrophages in the tumour cell group about 70% in the control group. Tumour cells in the milky spot are always located next to a mature macrophage (× 7500)

and that the tumour cells infiltrate the milky spots and form clusters within. Equally important, the cellular population in milky spots reacts with a very rapid inflow of young macrophages and an increase of the total number of cells. Furthermore, new milky spots are formed in the greater omentum around deposits of tumour cells. The cells present in milky spots are not capable of reversing the growth of the tumour cells into solid tumours and finally an omental cake of tumour cells is formed.

Although the greater omentum is a well-known site of metastases of carcinomas of the ovaries, stomach and colon, few studies have been performed that elucidate the cellular reactions and mechanism involved. Lawrence et al. [20] demonstrated that a reduction in tumour take after peritoneal seeding was achieved from omentectomy. Furthermore, Hagiwara et al. [13] demonstrated that tumour cells infiltrate milky spots while Dullens et al. [7] demonstrated that tumour growth occurs in milky spots and that the parathymic lymph nodes are more effective at eradicating the tumour cells than are milky spots. Moreover Tsujimoto et al. [26] showed growth of tumour cells and distinct metastatic lesions within the milky spots after 1 week. The latter three studies used morphological characteristics and non-specific tumour cell labelling to identify the tumour cells. With the newly developed mAb against the CC 531 tumour cell line [14] we were able to identify the tumour cells specifically. Moreover, we studied the earlier period after inoculation, which showed that tumour cells initially adhere to milky spots (Fig. 1), as also has been shown by Dullens [8], and subsequently to the rest of the omentum where they induce the formation of milky spots.

Milky spots largely consist of an accumulation of macrophages. Macrophages exhibit tumoricidal activity in general [21, 27]; moreover, macrophages from milky spots in the greater omentum also demonstrate tumoricidal activity in vitro [8, 19]. However, the present study demonstrated that the macrophages in vivo could not prevent the growth of tumours. Remarkably, over time, the percentage of the different subpopulations of macrophages did not change and no specific organization occurred within the macrophage population. Only in the early stage after tumour inoculation was an influx of immature macrophages seen (Figs. 2e, 3) which, however, could not reverse the tumour growth in vivo. Macrophages not only have cytotoxic properties but also produce growth-stimulatory factors [23, 24]. Therefore, it might be argued that tumour cells find a microenvironment within milky spots in which they are capable of survival and growth into solid metastases.

An accidental observation, but no less significant for clinical practice, is that tumour cells attach to milky spots very tightly. In the method applied in this study the omental specimens were rinsed twice: once by i.p. lavage and once in normal saline, yet the tumour cells remained attached to the milky spots. This has significant clinical importance, indicating that tumour cells spilled during a curative resection of an intra-abdominally located tumour are not removed by an adequately performed lavage of the peritoneal cavity. The tumour cells are tightly attached to the milky spots in the greater omentum and find a microenvironment that enables them to form solid metastases. In this respect our data support the current practice of surgical resection of the greater omentum in patients with ovarium carcinoma. Moreover, it could be argued that this procedure should be done in all cases of malignancy known to be at risk of forming metastasis in the peritoneal cavity.

In conclusion, our findings indicate a major role for milky spots in the greater omentum in arresting tumour cells in the peritoneal cavity. The cell population in the milky spots increases partly through an early influx of young macrophages, which suggests a role for newly recruited macrophages in early metastatic development. However, the macrophages of the milky spots are only partly able to control the process of the expanding tumour cell population and tumour growth.

In this respect we have already shown that GM-CSF given intraperitoneally results in an increase both in the number of macrophages and in their cytotoxic capacity [17]. Further studies will be directed at enhancement of the cytotoxic abilities of the cell population of the milky spots and especially of the macrophage population.

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References

1. Beelen RHJ, Fluitsma DM, Hoefsmit ECM (1978) Identification of exudate-resident macrophages on the basis of peroxidatic activity. J. Reticuloendothel Soc 23:103
2. Beelen RHJ, Fluitsma DM, Van der Meer JWM, Hoefsmit ECM (1979) Development of different peroxidatic activity patterns in peritoneal macrophages in vivo and in vitro. J Reticuloendothel Soc 25:513
3. Beelen RHJ, Fluitsma DM, Hoefsmit ECM (1980) Milky spots in the omentum. I. Cellular composition of milky spots and fine structure of milky spot macrophages and reticulum cells. J Reticuloendothel Soc 28:585
4. Beelen RHJ, Eestermans IL, Döpp EA, Dijkstra CD (1987) Monoclonal antibodies ED1, ED2, and ED3 against rat macrophages: expression of recognized antigens in different stages of differentiation. Transplant Proc 19:3166
5. Dallman MJ, Mason DW, Webb A (1982) The roles of host and donor cells in the rejection of skin allografts by T cell-deprived rates with syngeneic T cells. Eur J Immunol 12:511
6. Dijkstra CD, Döpp EA, Joling P, Kraal G (1985) The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. Immunology 54:589
7. Dullens HFJ, Rademakers LHPM, Cluistra S, Os R van, Dux K, Besten PJ den, Otter W den (1991) Parathymical lymph nodes during growth and rejection of intraperitoneally inoculated tumour cells. Invasion Metastasis 11:216
8. Dullens HFJ, Rademakers LHPM, Doffemont M, Veen PT van, Bulder R, Otter W den (1993) Involvement of the

omental lymphoid organ in the induction of peritoneal immunity against tumor cells. Invasion Metastasis 13:267
9. Dux K (1969) Role of the greater omentum in the immunological response of mice and rats to the intraperitoneal inoculation of Ehrlich ascites tumor. Arch Immunol Ther Exp 17:425
10. Dux K (1986) Proliferative activity of macrophages in the greater omentum of the mouse in relation to the early postnatal development of the vascular structures. J Leukoc Biol 40:445
11. Dux K, Janik P, Szaniawska B (1977) Kinetics of proliferation, cell differentiation, and IgM secretion in the omental lymphoid organ of B10/Sn mice following intraperitoneal immunization with sheep erythrocytes. Cell Immunol 32:94
12. Evans R, Alexander P (1970) Cooperation of immune lymphoid cells with macrophages in tumour immunity. Nature 228:620
13. Hagiwara A, Takahashi T, Sawai K, Taniguchi H, Shimotsuma M, Okano S, Sakakura C, Tsujimoto T, Osaki K, Sasaki S, Shirasu M (1993) Milky spots as the implantation size for malignant cell in peritoneal dissemination in mice. Cancer Res 53:687
14. Heuff G, Steenbergen JJE, Van de Loosdrecht AA, Sirovich I, Dijkstra CD, Meyer S, Beelen RHJ (1993) Isolation of cytotoxic Kupffer cells by a modified enzymatic assay: a methodological study. J Immunol Methods 159:115
15. Hodel Ch (1970) Ultrastructural studies on the absorption of protein markers by the greater omentum. Eur Surg Res 2:435
16. Hoefsmit ECM, Beelen RHJ (1987) Letter to the editor. Authors' reply to Dr. Ginsel. J Histochem Cytochem 35:1169
17. Koenen HJPM, Smit MJ, Simmelink MMJA, Schuurman B, Beelen RHJ, Meijer (1996) Effect of intraperitoneal administration of granulocyte/macrophage-colony-stimulating factor in rats on omental milky-spot number composition and tumoricidal activity in vivo and in vitro. Cancer Immunol Immunother 42:310
18. Krist LFG, Eestermans IL, Steenbergen JJE, Hoefsmit ECM, Cuesta MA, Meyer S, Beelen RHJ (1995a) Cellular composition of milky spots in the human greater omentum: an immunochemical and ultrastructural study. Anat Rec 241:163
19. Krist LFG, Kerremans M, Koenen HJPM, Blijleven N, Eestermans IL, Calame W, Meyer S, Beelen RHJ (1995b) Novel isolation and purification method to study functional cytotoxicity of macrophages from milky spots in the greater omentum. J Immunol Methods 184:253
20. Lawrence, R.J, Loizidou M, Cooper AJ, Alexander P, Taylor I (1991) Importance of the omentum in the development of intra-abdominal metastases. Br J Surg 78:117
21. Loosdrecht AA van de, Ossenkoppele GJ, Beelen RHJ, Langenhuysen MMAC (1992) Monocyte mediated cytooxicity against a human histiocytic lymphoma cell line. Role of interferon-δ and tumor necrosis factor-α. Cancer Immunol Immunother 34:393
22. Mandache E, Moldoveanu E, Savi G (1985) The involvement of omentum and its milky spots in the dynamics of peritoneal macrophages. Morphol Embryol 31:137
23. Mantovani A (1990) Tumour associated macrophages. Curr Opi Immunol 2:689
24. Mantovani A, Bottazzi B, Colotta F (1992) The origin and function of tumour-associated macrophages. Immunol Today 13:265
25. Shimotsuma M, Kawata M, Hagiwara A, Takahashi T (1990) Milky spots in the human greater omentum. Macroscopic and histological identification. Acta Anat 136:211
26. Tsjujimoto H, Hagiwara A, Shimutsuma M, Sakakura C, Osaki K, Dasaki S, Ohyama T, Oghaki M, Imanishi T, Yamazaki J, Takahashi T (1996) Role of milky spots as selective implantation sites for malignant cells in peritoneal dissemination in mice. J Cans Res Clin Oncol 122:590
27. Withworth PW, Pak CC, Esgro J, Kleinerman ES, Fidler IJ (1989) Macrophages and cancer. Cancer Metastasis Rev 8:319
28. Woollett GR, Barclay AN, Puklavec M, Williams AF (1985) Molecular and antigenic heterogeneity of the rat leukocyte-common antigen from thymocytes and T and B lymphocytes. Eur J Immunol 15:168
29. Zedeck MS, Sternberg SS (1974) A model system for studies of colon carcinogenesis: Tumour induction by a single injection of methylazoxymethanol acetate. J Natl Cancer Inst 53:1419