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Ontogeny of Milky Spots in the Human Greater Omentum: An Immunochemical Study

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ABSTRACT ***Background:*** **Milky spots in the human greater omentum are preformed specific accumulations of primarily macrophages within the stroma of the greater omentum. To obtain a better understanding of milky spots in the human greater omentum, the development and the earliest forms of milky spots in the human greater omentum were studied, with special attention to the macrophage population.**

Methods: **Specimens of human greater omentum were obtained from fetuses of 20 to 40 weeks gestation and one newborn three days old (n = 6). Using mature macrophages (RFD 7), activated macrophages (RFD 1), B-lymphocytes (CD 22), and T-lymphocytes (CD 2), and immunoperoxydase labeling, the percentage of these cells in developing milky spots and the development of milky spots were studied by light microscopy. A time-dependent increase in the percentage of positive staining cells and the size of clusters was analyzed using the non-parametric Spearman rank correlation test.**

Results: **Small accumulations of cells with about 50% monocytes/macrophages were present at 20 weeks of gestation. With increasing gestational age the number of clusters of cells increased significantly ($P < 0.01$) as well as their size ($P < 0.01$). Starting at 29 weeks, vascularized clusters of cells were seen; true milky spots were present at 35 weeks. A significant ($P < 0.05$) increase in the percentage of mature macrophages was found in developing milky spots, whereas no activated macrophages were seen. The percentage of B-lymphocytes and T-lymphocytes found in the clusters of cells and milky spots increased significantly ($P < 0.05$) but did not exceed 10% of the total number of cells.**

Conclusions: **From our data it can be concluded that milky spots are specific structures in the greater omentum formed between the 20th and 35th week of gestation. Further, we concluded that immature cells (promonocytes) mature locally in developing milky spots. Anat. Rec. 249:399–404, 1997.**

Key words: human greater omentum; milky spots; ontogeny; macrophages; lymphocytes; light microscopy

Milky spots are opaque patches in the greater omentum. They were first described by von Recklinghausen (1863) as white spots in the omentum of rabbits and were named "taches laîteuses" by Ranvier (1874). Milky spots have been described in a variety of mammals, including man (Shimotsuma et al., 1989; Krist et al., 1995). In man, milky spots are relatively uniform, highly vascularized accumulations of mononuclear cells comprising macrophages (68%), B-cells (10%), T-cells (10%), and mast cells within the stroma of the greater omentum. Further, the macrophages are present in different stages of maturation (Krist et al., 1995).

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TABLE 1. Applied monoclonal antibodies

MoAb*	CD**	Cells	Dilutions	Source	Reference
EBM 11	68	monocytes/macrophages	1:20	Dako, Glostrup, Denmark	Kelly et al., 1988
RFD7	—	mature macrophages	1:5	Royal Free, London, UK	Poulter et al., 1986
RFD 1	—	activated macrophages	1:5	Royal Free, London, UK	Poulter et al., 1986
4KB128	22	B-cells	1:40	Dako, Glostrup, Denmark	Dörken et al., 1989
T11/1 6G4	2	T-cells (and NK-cells)	1:100	CLB, Amsterdam, The Netherlands	Beyers et al., 1989

*MoAb = monoclonal antibody.
**CD = cluster of differentiation.

Milky spots have a function in the clearance of particles, bacteria, and tumor cells from the peritoneal cavity (Hodel, 1970; Mandache et al., 1985; Dullens et al., 1991; Hagiwara et al., 1993; Krist et al., 1996). They are highly reactive structures originating from perivascular effusions upon induction of intraperitoneal inflammation and immunization or are newly formed around tumor cells located on the mesothelium of the omentum (Kaboth et al., 1966; Holub et al., 1970; Beelen et al., 1980; Krist et al., 1996). Moreover, special B-cell and T-cell areas are formed in milky spots after intraperitoneal immunization (Dux et al., 1986; Beelen et al., 1988, 1991; Mandache et al., 1987). In addition, milky spots are regarded as a source of peritoneal macrophages (Wijffels et al., 1992), whereas the omentum can be regarded as a primary site of B-cell development (Solvason and Kearney, 1992).

Although the cellular structure of milky spots in humans has been described extensively (Shimotsuma et al., 1989, 1991; Krist et al., 1995) as of yet little is known about the function and development of milky spots in humans.

To obtain a better understanding of the milky spots in the human greater omentum, it is essential to know how these structures develop. To the best of our knowledge, no such study on the earliest forms of milky spots has been published. Therefore, the present study was performed. The objective of this study was to describe the development of milky spots in the human greater omentum and their earliest forms, with special attention to the macrophage population. To address this issue, we used omenta from fetuses which died of causes other than infection or malignancy and applied immunolabeling techniques to gain more information on the cellular composition of developing human milky spots in an unstimulated, steady state.

MATERIALS AND METHODS

Specimens of greater omentum were obtained from fetuses of 20 to 40 weeks gestation, of which one died three days after birth (n = 6). The fetuses and the newborn died of various causes but none of them had a history of infection or malignancy. The study was approved by the Hospital Ethical Committee.

Light Microscopy and Staining Techniques

The entire omenta were excised. To demonstrate the presence of macrophages, B-cells, and T-cells and their distribution, the selected omenta were stretched on glass slides, dried, and stained with an indirect immunoperoxidase technique, as described previously (Krist et al., 1995). Briefly, for this immunolabeling the specimens were fixed in acetone for 10 min and dried. Endogenous peroxidase activity was inhibited with incubation of the preparations in 0.03% HIO_4 in distilled water for 10 sec and rinsing in PBS (phosphate buffered saline 0.1 M; pH 7.4). The specimens were then incubated for 1 hr with monoclonal antibodies (MoAb; Table 1) against monocytes/macrophages (CD 68), mature macrophages (RFD 7), activated macrophages (RFD 1; RFD 7 and RFD 1 both kindly provided by Dr. L.W. Poulter, Royal Free Hospital School of Medicine, London, England), B-lymphocytes (CD 22) and T-lymphocytes (CD 2), diluted in PBS+ (PBS with 0.5% bovine serum albumin obtained from Sigma Chemical, St. Louis, MO). Specimens were then rinsed in PBS+ and incubated with a conjugate of rabbit anti-mouse antibody labeled with peroxidase (1:250 in PBS+) and 1% normal human serum in PBS+ for 1 hr. After rinsing in PBS, peroxidase activity was demonstrated with 3,3'diaminobenzidine (DAB, Sigma) at a concentration of 0.5 mg/ml in TrisHCl buffer (pH 7.6) containing 0.03% H_2O_2 and 0.05 M imidazole for 10 min, the latter to enhance staining quality. Subsequently, specimens were rinsed in NaCl 0.9% and put in 0.5% $CuSO_4$ in 0.9% NaCl for 15 min to enhance staining quality, washed in distilled water, counterstained with hematoxylin, dehydrated, cleared, and mounted in Entallan (Merck, Darmstadt, Germany). For control slides, the first step was omitted. The entire procedure was carried out at room temperature.

The number clusters was counted for each specimen. Of up to 25 randomly selected clusters, the total number of cells were counted.

The positive-staining cells in the clusters were quantified and expressed as the percentage of total number of cells. In each determination, up to 100 cells were evaluated.

Statistical Analysis

A time-dependent increase in the number and size of cell clusters, the number of cells, and the percentage of positive-staining cells were analyzed using the nonparametric Spearman rank correlation test (Siegel, 1956). A P-value < 0.05 was considered to be statistically significant.

RESULTS

Cell Clusters

At 20 weeks gestation no milky spots were seen in the omentum. However, a small number of non-vascularized clusters of cells of up to 100 cells were present (Fig. 1A; Table 2). At 26 weeks, larger clusters of cells were found. They were not vascularized but small vessels were seen in their proximity (Fig. 1B). Starting at 29

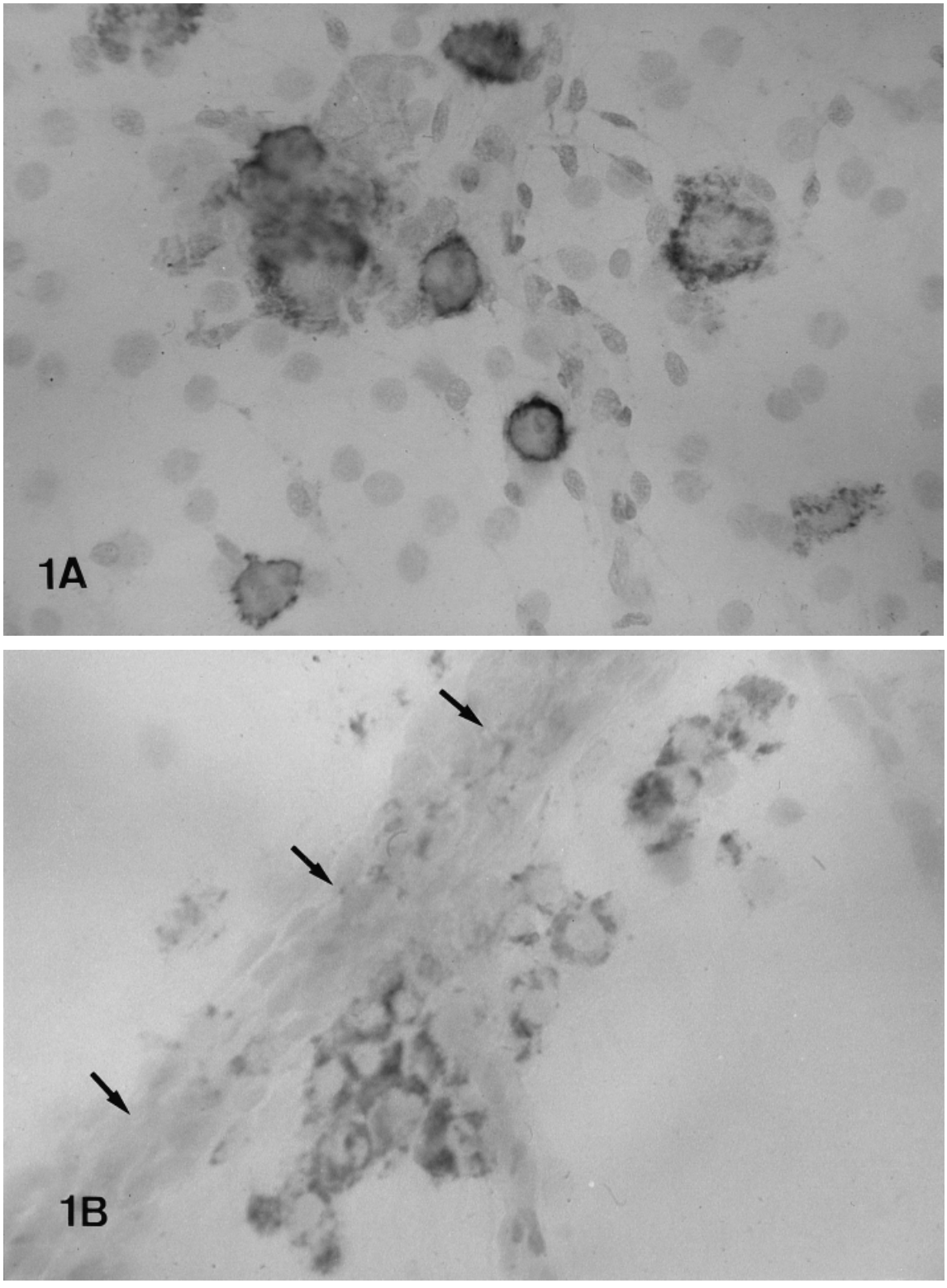

Fig. 1. Detail of piece of omentum of a foetus of 20 weeks gestation (**1A**) and 26 weeks of gestation (**1B**) stretched on a glass-slide and stained with a monoclonal antibody against macrophages (CD 68). At 20 weeks of gestation a small cluster of cells is seen. At 26 weeks, the size of the developing milky spot has increased and a small accumulation of cells is seen next to a blood vessel (arrow) (both × 1000).

TABLE 2. Number of clusters of cells and number of cells per cluster in developing milky spots in human fetuses

Weeks of gestation	Number of clusters per omentum	Number of cells per cluster	Mean of cells	SD of cells
20	±15	10–100	40	32
26	±25	20–200	86	59
29	±80	20–400	127	120
35	±90	20–400	132	132
40	±110	20–800	254	293
40*	±120	20–800	282	290

*Biopsy of an omentum of a child that died three days after birth (40 weeks gestation).
Abbreviation: SD = standard deviation.

weeks, incomplete vascularized clusters of cells were seen, whereas at 35 weeks vascularized clusters resembling true milky spots were found. With increasing gestational age the number of clusters of cells increased significantly ($P < 0.01$) as well as their size ($P < 0.01$).

Macrophages

In the non-vascularized clusters of cells present at 20 weeks gestation, 45% of the cells were positive for CD 68 (monocytes/macrophages). Furthermore, macrophages were found randomly dispersed over the entire greater omentum. At 26 weeks, the percentage of macrophages had increased to 55%. The percentage of macrophages in the clusters increased significantly ($P < 0.05$) to 70% at 29 weeks (Fig. 2A). At that moment, dispersed macrophages were still present in the omentum. However, with increasing gestational age a decreasing number of macrophages were seen dispersed over the omentum.

At 20 weeks just a few cells staining for RFD 7 (mature macrophages) were detected in the small clusters of cells and dispersed over the omentum. The percentage of RFD 7-positive cells increased ($P < 0.05$) from 10% at 26 weeks through 25% at 29 weeks to 70% at 35 and 40 weeks gestation and three days after birth (Fig. 2B). No cells staining for RFD 1 (activated macrophages) were seen.

Lymphocytes

B-cells and T-cells were seen dispersed over the entire greater omentum and in the cluster of cells. However, in the smallest clusters of up to 15 cells no B- and T-cells were seen. At 20 to 29 weeks, the percentage of B-lymphocytes and T-lymphocytes in the clusters of cells were both less than 5%. After 29 weeks, the percentage of B-lymphocytes and T-lymphocytes increased to 7% and 5%, respectively, at 35 weeks and increased ($P < 0.05$) up to 7% for both cell types at three days after birth (Fig. 2C,D).

Other Cells

The remainder of the cells were mesothelial cells, stromal cells, an occasional mast cell, and immature cells. The relative number of these cells decreased ($P < 0.05$) in time.

DISCUSSION

The main conclusion to be drawn from this study is that milky spots in the human greater omentum are specific structures formed between the 20th and 35th weeks of gestation. Milky spots, as described before (Krist et al., 1995), are present in the omentum at 35 weeks. Monocytes/macrophages represent the largest group of cells in the developing milky spots. The percentage of monocytes/macrophages increases significantly during the development of milky spots up to 70%, as does the percentage of mature macrophages. The percentage of B-cells and T-cells also increases significantly, but for both cell types remains below 10%. The remainder of cells in developing milky spots are stromal cells and immature cells.

The greater omentum develops from the eighth week of gestation onward from the dorsal mesogastrium (Liebermann-Meffert and White, 1983). Our material consisted of omenta of fetuses of 20 weeks of gestation and older. It was practically impossible to obtain omenta of human fetuses of a younger age. Therefore, no information could be obtained from this earlier period.

The results show that small accumulations of macrophages, but no milky spots, are present at 20 weeks of gestation. These accumulations grow into milky spots. Starting at 29 weeks, vascularized clusters of cells are seen resembling milky spots; true milky spots, as described before (Krist et al., 1995), are present in the omentum at 35 weeks. Moreover, no activated macrophages were present at any stage. These RFD 1-positive cells would have been found if either infection or tumor growth were present in the investigated omenta. The development of milky spots in fetuses in the absence of infection or tumor growth, therefore, made us conclude that milky spots are primarily preformed structures.

Macrophages in fully grown milky spots form about 70% of the cell population (Krist et al., 1995). However, in the unvascularized clusters of cells up to 55% of the cells were CD 68-positive, whereas no large percentages of B-cells and T-cells were found. This leaves a population of cells of up to 50% which cannot be classified as either monocytes/macrophages or lymphocytes. A relatively small part of this population is stromal cells forming the structure of the early forms of milky spots. The remainder of cells are most likely immature cells. In the developing milky spots, monocytes/macrophages form the largest population of cells. Within the population of unlabeled cells the different cell types will be proportional to the labeled cells. Therefore, a substantial population of pro-monocytes must be present in developing milky spots, which might form a population of proliferating cells. The presence of proliferating cells could be the explanation for the observation that the monocyte/macrophage population is a rapidly expanding population of cells in relative and absolute numbers, although the early forms of milky spots are not yet vascularized and therefore these monocytes cannot be recruited directly from the circulation. Further, an increasing percentage of mature macrophages (RFD 7-positive cells) are seen in the developing milky spots and at 35 weeks are equalling the percentage of CD 68-positive cells. This increase of the percentage of mature macrophages made us conclude that within developing milky spots monocytes mature into macrophages.

The percentage of B-cells and T-cells found in developing milky spots was small and did not exceed 10%.

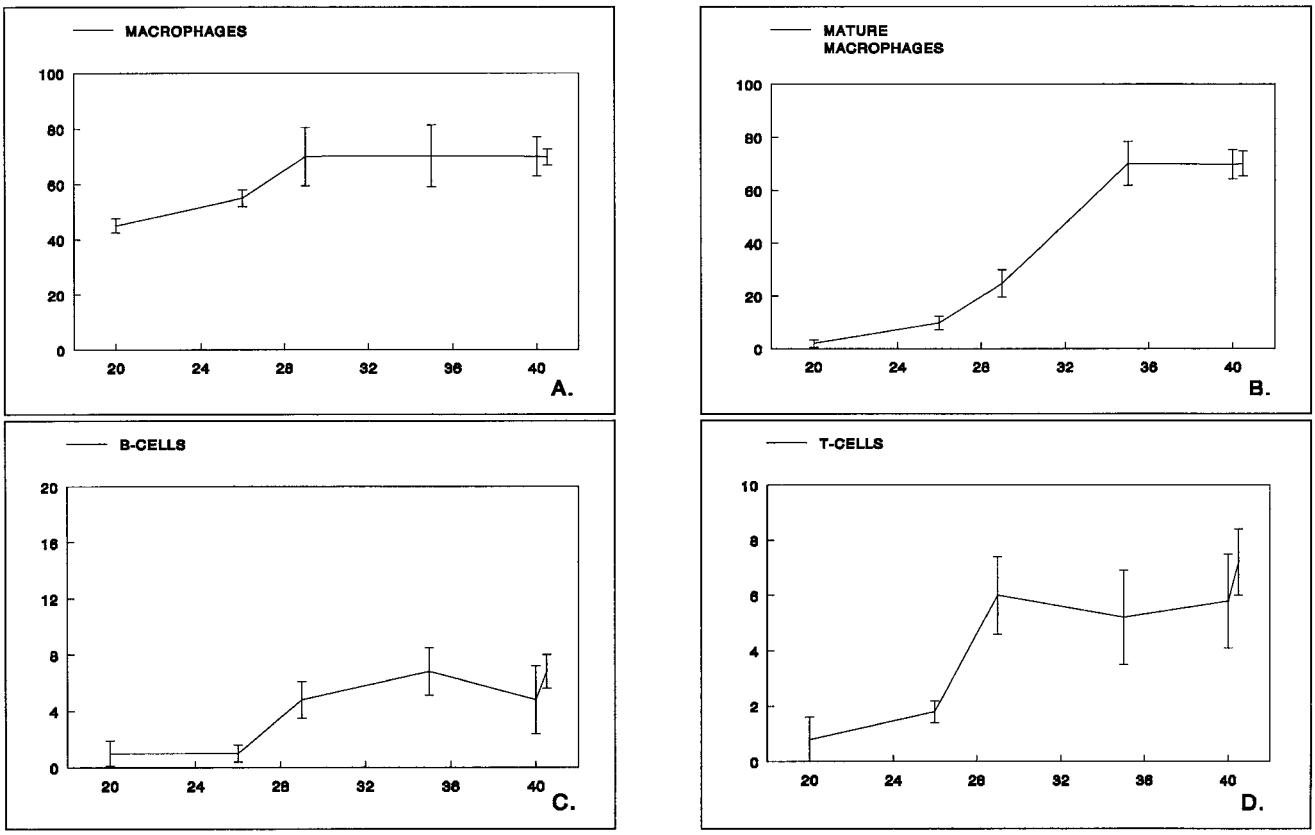

Fig. 2. The positive staining cells in the clusters were quantified and expressed as the percentage of total number of cells. In each determination up to 100 cells were evaluated. A time-dependent increase in the percentage of positive-staining cells were analyzed. In each graph the x-axis represents weeks of gestation and the y-axis the mean percentage of positive staining cells (±1 SD). Macrophage (CD 68) (2A) and B- and T-lymphocyte populations (CD 22 and CD 2 respectively) (2C & 2D) increase significantly in developing fetal milky spots ($p < 0.05$). Furthermore, a significant increase in mature macrophages (RFD 7) is seen (2B).

Solvason and Kearney (1992) demonstrated that the human greater omentum is a primary site of B-cell development. They found a decrease of pre-B-cells in the investigated period up to 23 weeks and suggested that development of B-cells in the fetal omentum is a transitory process. Although our study was not intended to investigate B-cells specifically, our results do not contradict this.

Little is known about T-lymphocytes and their subpopulations in fetal omentum and peritoneal cavity. This study shows that T-cells form a distinct population in developing milky spots. However, no lymphocytes were seen in the smallest clusters of cells, indicating that the development of milky spots starts with a cluster of macrophages and immature cells, most likely pro-monocytes.

In animal studies, too, it has been shown that milky spots develop in the fetus (Trebichavsky et al., 1981; Shimotsuma et al., 1994). Moreover, in pigs (Trebichavsky et al., 1981) and sheep (Shimotsuma et al., 1994) the same pattern of development is seen as in humans concerning the monocyte/macrophage population in milky spots. However, the presence of B-cells and T-cells varies considerably. In developing milky spots, B-cells appeared late in pigs and were absent in sheep, whereas T-cells were present in sheep and rarely present in pigs. In animals and humans, milky spots are primarily an accumulation of macrophages; B-cells and T-cells form but a small portion of the cells are present. This indicates that the development of milky spots in humans and animals as a macrophage organ are comparable. However, in different species the milky spots might have a different function in respect to the lymphocyte populations.

Future studies will be directed at investigating the population of immature cells present in developing milky spots as well as the role of macrophages in human milky spots in intra-abdominal pathology, especially in tumor cell metastasis.

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