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Ontogeny of rat thymic macrophages. Phenotypic characterization and possible relationships between different cell subsets

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SUMMARY

In the present study we combined electron microscopy, immunohistology and primary stromal cell cultures to analyse the ontogeny of rat thymic macrophages (Mϕ) in an attempt to clarify the relationships between the different macrophage cell subsets described in adult rat thymus. Although phagocytic cells were observed in 15-day-old fetal thymus, monoclonal antibodies (mAb) which recognize different adult macrophage types were unable to identify positive cells until the end of embryonic life. However, our *in vitro* results from primary thymic stromal cell cultures of 16-day-old fetal rats, and the phenotyping of enriched thymic $CD2^-$ cell suspensions, demonstrated that monocyte-like cells which strongly expressed major histocompatibility complex (MHC) class II molecules colonized the embryonic thymus early, giving rise later to distinct macrophage subsets. During the process of maturation, macrophage precursors gradually lost their MHC class II expression, acquired other surface markers (CD45, Thy-1, CD25, CD4, etc.) and increased the acid phosphatase activity. In this respect, $ED1^+$ macrophages, which appeared for the first time in the last stages of embryonic life, consisted of a MHC class II molecule-expressing phagocytic cell population, presumably involved in the elimination of non-selected cortical thymocytes, and of non-phagocytic cells which, in the thymic cortex, might differentiate to $ED2^+$ macrophages throughout $ED1^+ED2^{lo/med}$ and $ED1^+ED2^{high}$ intermediate cell stages, observed *in vitro* in 16-day-old fetal thymic stromal cell cultures. At the end of embryonic life and during the postnatal period the numbers of thymic macrophages increased, particularly in the medulla and corticomedullary border (CMZ), and more slowly in the thymic cortex. This increase was presumably due to the arrival, through perivascular spaces, of new macrophage progenitors, rather than *in situ* proliferation of pre-existent mature macrophages. The possible function of different thymic macrophage subsets, as well as the relationships between themselves and with their presumptive monocyte-like precursors, are discussed.

INTRODUCTION

According to their morphology, phenotype and functional capabilities, thymic macrophages, which belong to the phagocyte mononuclear system, constitute a heterogeneous cell population. *In situ* immunohistological studies, using monoclonal antibodies (mAb) raised against specific cell markers, have identified different macrophage subpopulations in the thymus of adult rats.[1,2] These cell markers appear late during rat thymus ontogeny,[3,4] although indirect evidence supports early colonization of the thymus gland by macrophage cell progenitors.[5–8] In fact, the phenotypic characteristics of these cell precursors and their progeny, mainly their capacity for expressing major histocompatibility complex (MHC) class II molecules, is still not clear. Some authors emphasize that only the most primitive members of macrophage cell lineage can express class II antigens.[5] In contrast, both *in* situ[3,9] and *in vitro* studies[10,11] have demonstrated their expression on mature macrophages. Besides, Murawska *et al.*[12] point out that this MHC class II molecule expression on mature macrophages is not constitutively but passively acquired. Accordingly, we know very little about the origin, time of appearance and relationships between the different thymic macrophage cell subpopulations. In the present study we therefore combine electron microscopy, immunohistology and *in vitro* thymic stromal cell cultures to analyse the development of thymic macrophages during rat ontogeny, with special emphasis on the relationships between the different cell subpopulations.

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MATERIALS AND METHODS

Animals

Wistar rat thymic glands were sampled from day 13 of fetal life to 2 weeks after birth. Adult animals were also included in the study.

Table 1. Monoclonal antibodies used in this study

mAb	Immunoglobulin subclass	Specificity	Source*
OX18	IgG1	MHC class I	1
OX17	IgG1	MHC class II	1
OX1	IgG1	CD45	1
OX55	IgG1	CD2	1
OX38	IgG2a	CD4	1
OX8	IgG1	CD8	1
OX7	IgG1	Thy-1	1
OX39	IgG1	CD25	1
OX44	IgG1	CD53	1
ED1	IgG1	Rat Mϕ and IDC	2
ED2	IgG2a	Rat cortical and CMZ Mϕ	2
R-MC41	IgG1	Rat cortical and CMZ Mϕ	3
R-MC42	IgG1	Rat cortical and CMZ Mϕ	3
R-MC44	IgM	Rat medullary and CMZ Mϕ	3

(1) Serotec, Oxford, UK; (2) Dr C. Dijkstra (Free University, Amsterdam, the Netherlands); (3) Dr M. Colic (Medical Faculty, Belgrado).

Electron microscopy

Thymi were removed aseptically from adults, neonates and 16–21-day-old fetuses. Fetuses from earlier embryonic stages were fixed *in toto*. Small pieces either from the thymic tissue or the anterior part of the fetal body were fixed by immersion in 2·5% glutaraldehyde, buffered to pH 7·3, with Millonig's fluid, post-fixed in 1% osmium tetroxide in the same buffer, and dehydrated in acetone for embedding in Araldite™. Ultrathin sections were obtained with a Reichert OM-U3 ultratome, double-stained with uranyl acetate and lead citrate and examined with JEOL 100B or ZEISS EM 902 electron microscopes.

Immunohistochemistry and enzymehistochemistry

The samples were frozen in liquid nitrogen to make cryostat sections. Slides were fixed for 10 min in acetone. Cell marker characterization was achieved by an indirect immunoperoxidase labelling assay using the mAb listed in Table 1.

Endogenous peroxidase was blocked with 1% H_2O_2 in phosphate-buffered saline (PBS) for 15 min. Sections were then incubated for 30 min with 1% normal rat serum in PBS and a 1/100 solution of peroxidase-conjugated rabbit anti-mouse immunoglobulins in PBS (Dako Co., Glostrup, Denmark). The peroxidase reaction was developed with 0·05%, 3,3′-diaminobenzidine (Sigma Co., St Louis, MO) in 0·05% Tris–HCl buffer (pH 7·6) with 0·1% H_2O_2 for 10 min. Sections were counterstained with methylene blue. Preparations incubated without primary antibodies were used as negative controls.

Acid phosphatase activity was determined using the method described by Barka & Anderson.[13] Sections incubated without

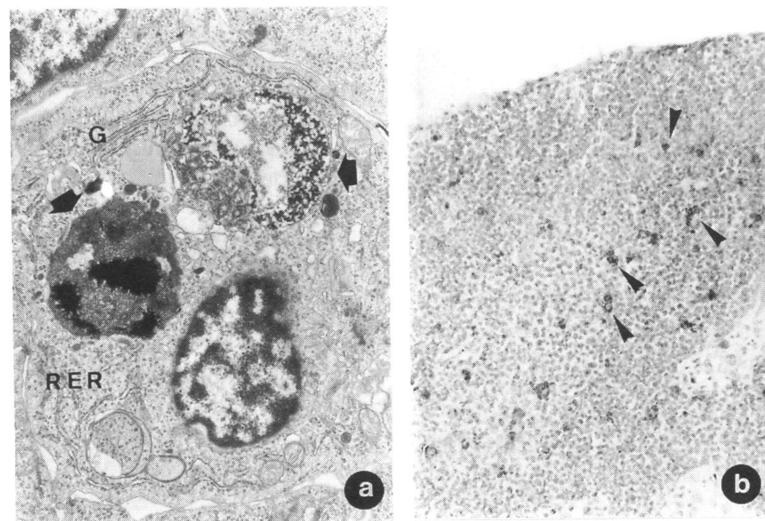

Figure 1. (a) Macrophage of a 15-day-old embryonic rat thymus. Note the presence of long profiles of rough endoplasmic reticulum (RER), a well-developed Golgi complex (G) and engulfed, degenerated cells (arrows) in its cytoplasm. Magnification ×12 938. (b) Acid phosphatase activity in a 19-day-old embryonic rat thymus (arrowheads). Magnification × 94.

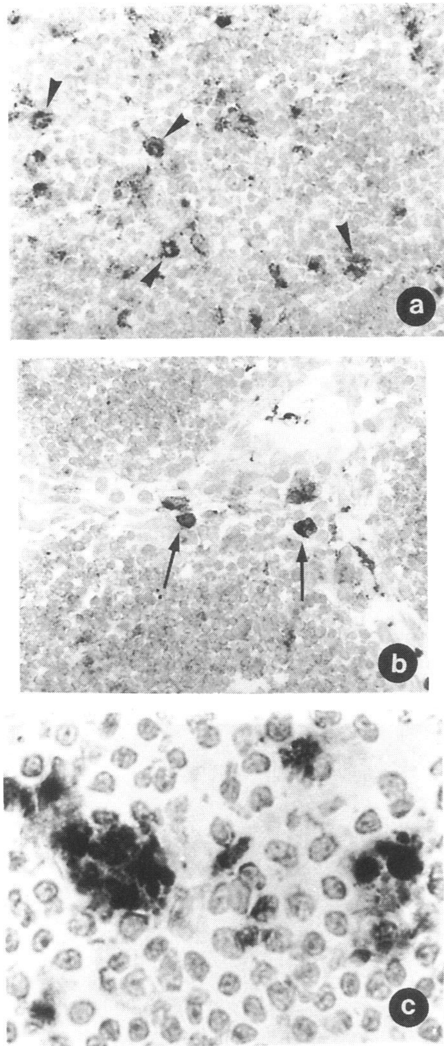

Figure 2. (a) $ED1^+$ macrophages (arrowheads) in the medulla and corticomedullary border of a 19-day-old embryonic rat thymus. Magnification ×188. (b) $ED1^+$ macrophages (arrows) in the connective tissue of a thymic trabeculae of a neonate rat. Magnification ×188. (c) $ED1^+$ macrophages containing engulfed material in their cytoplasm. Neonatal rat thymus. Magnification × 375.

the specific substrate—α naftil AS-biphosphate—were used as negative control.

Mϕ enrichment

Thymi were removed, minced with scissors and filtered through a stainless steel screen. The cell suspension obtained was washed twice in RPMI-1640/5% fetal calf serum (FSC; Sigma, Madrid, Spain) and resuspended in the same medium. 10^7 cells were incubated with anti-CD2 mAb for 1 hr at 4°. After two washes, cells were resuspended in 0·2 ml RPMI-1640/5% FCS containing rabbit anti-mouse IgG-coated magnetic beads (6 : 1 bead to cell ratio; Dynabeads, Dynal, Oslo, Norway). After 30 min in ice with intermittent shaking, 4 ml of ice-cold RPMI-1640/10% FCS was added and the rosettes formed ($CD2^+$ cells–Dynabeads) were separated from the non-rosetting cells by placing the tube on a magnetic particle concentrator (Dynal) for 3 min.

The cell composition of the enriched $CD2^-$ cell population (30–50% of enrichment in Mϕ) was analysed immunocytochemically on cytospin preparations by the above mentioned immunoperoxidase method, using the mAb listed in Table 1.

Primary stromal cell cultures

Sixteen-day-old fetal rat thymi were minced into small fragments and seeded in 25-cm^2 plastic flasks in RPMI-1640 medium plus 1% sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS. Cultures were maintained at 37° and 5% CO_2 and the medium was changed twice a week. Cell marker characterization was achieved on cytospin preparations of cell suspensions obtained from free cells of the culture medium by the above mentioned immunoperoxidase method using the following mAb: ED1, ED2, OX44 and OX17.

RESULTS

In situ appearance of Mϕ in the embryonic rat thymus

The electron microscopy study first identified phagocytic cells in the thymic parenchyma of 15-day-old embryonic rats (Fig. 1a). At that stage, the rat thymus consisted of an epithelial parenchyma, containing a heterogenous population of epithelial cells housing the first lymphoid cells. Ultrastructurally, macrophages were electron-lucent elements containing some cisternae of rough endoplasmic reticulum, numerous free ribosomes, patent mitochondria and a well-developed Golgi complex (Fig. 1a). In addition, distinct engulfed materials, some of them resembling pyknotic cells, occurred in their cytoplasm (Fig. 1a).

In contrast, we could not demonstrate the presence of phagocytic cells either immunohistologically or histoenzymatically in the rat thymus before days 16–17 of fetal life. Cells showing acid phosphatase activity appeared on following days (Fig. 1b), reaching the adult condition around birth in both the medulla and corticomedullary border, and 2 weeks later in the thymic cortex.

By using mAb raised specifically to macrophage cell markers, the first positive cells were found at the end of fetal life. $ED1^+$ macrophages, which in adult rat thymus occupy both cortex and medulla, appeared on day 19 principally in the corticomedullary border and in a lesser number in the thymic medulla (Fig. 2a). Occasionally, a few positive cells occurred scattered throughout the cortex. Gradually, the number of $ED1^+$ cells increased during the late part of fetal life but, around birth, there was an important increase in the medulla, corticomedullary border and in the connective tissue of both the capsule and trabeculae (Fig. 2b). In contrast, the amount of $ED1^+$ cells of thymic cortex increased slowly, reaching the adult condition only 2 weeks after birth. Some $ED1^+$ cells, scattered throughout the thymic parenchyma, contained engulfed materials, thus showing their phagocytic capacity (Fig. 2c). So, although during embryonic life the number of $ED1^+$ macrophages was always lower than that of cells showing acid phosphatase activity in the adult condition (i.e. 2 weeks after birth), their numbers had equalized.

The mAb ED2, R-MC41 and R-MC42, which in adult rats mainly recognized thymic macrophages of both cortex and corticomedullary border, showed a similar pattern of expression during rat thymus ontogeny. Positive cells occurred on

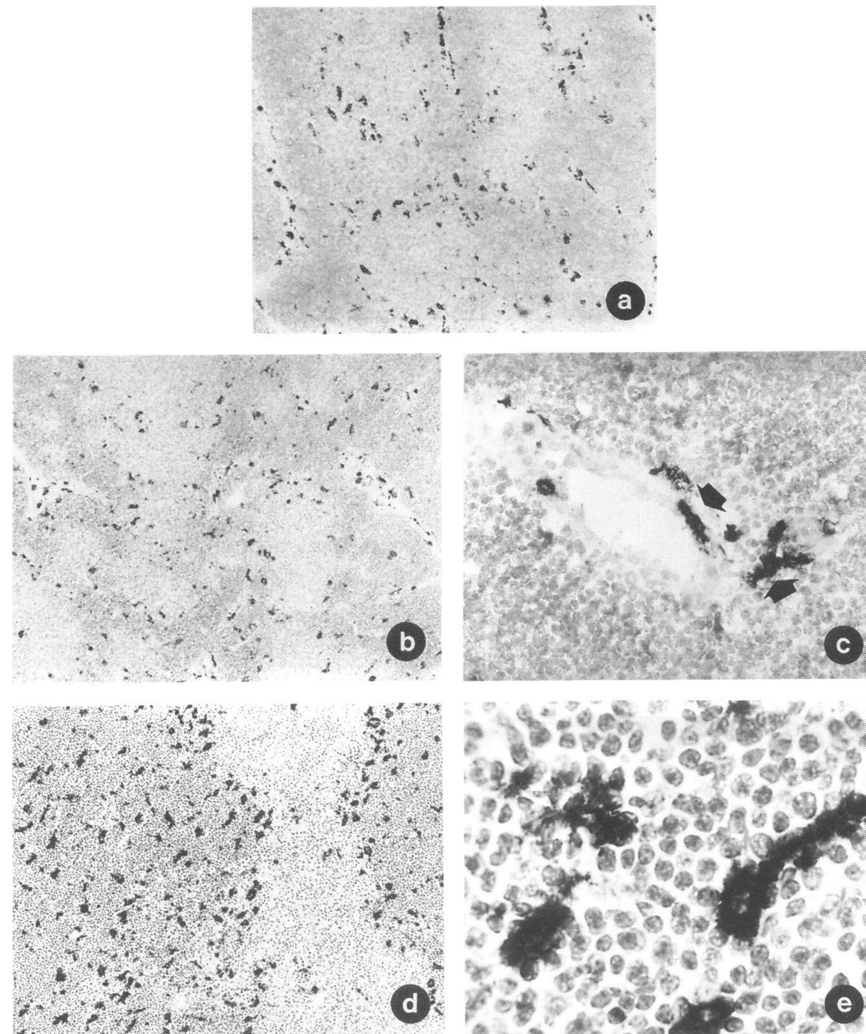

Figure 3. (a) R-MC42$^+$ macrophages in a 20-day-old embryonic rat thymus. Note their predominant distribution throughout the connective tissue capsule and trabeculae. Magnification × 49. (b) ED2$^+$ cells in both medulla and corticomedullary border of a neonatal rat thymus. Magnification × 49. (c) ED2$^+$ macrophages (arrows) in the perivascular space of a blood vessel of the thymic cortex. Neonatal rat. Magnification ×188. (d) R-MC42$^+$ macrophages largely restricted to the cortex and corticomedullary border of a 15-day-old rat thymus. Magnification × 49. (e) Cell clusters formed by R-MC42$^+$ macrophages and thymocytes in the thymic cortex of a 15-day-old rat. Magnification × 338.

fetal days 19–20, in the connective tissue of the thymic capsule, trabeculae and surrounding the blood vessels (Fig. 3a). A few positive cells also appeared in the cortical parenchyma. As shown for ED1$^+$ cells, the macrophages recognized by either mAb ED2, R-MC41 and R-MC42 increased around the time of birth, becoming especially evident in the corticomedullary border (Fig. 3b) and around blood vessels (Fig. 3c). Development of these macrophages in the thymic cortex was also slow, reaching an important number only in the second week of postnatal life (Fig. 3d). Morphologically, they were very irregular elements, the cell processes of which closely embraced the neighbouring thymocytes, forming true macrophage–thymocyte cell clusters (Fig. 3e).

Macrophages recognized by mAb R-MC44 also appeared on fetal day 19 in both the medulla and corticomedullary border. Apart from the numerical increase occurring around the time of birth, no important modifications were observed in this cell subpopulation.

Phenotypic characterization of Mϕ throughout rat thymic ontogeny

In order to improve the phenotypic characterization of these thymic macrophage subpopulations, we carried out cytospin preparations of enriched thymic CD2$^-$ cell suspensions from rats of different ages. All cells belonging to the monocyte–macrophage lineage observed in enriched thymic CD2$^-$ cell suspensions obtained from 17-day-old fetal rats expressed MHC class I molecules, CD45 and weak Thy-1, also exhibiting acid phosphatase activity. The expression of these molecules remained unchanged in cell preparations from older embryonic and neonatal rats. Likewise, a variable number of thymic

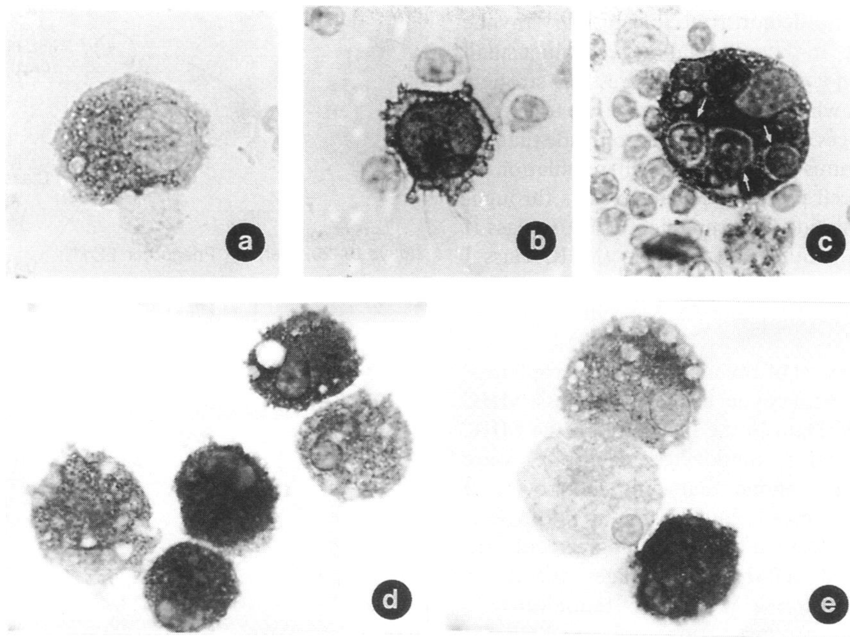

Figure 4. (a) Interleukin-2 receptor-α (IL-2Rα) (CD25)-positive macrophage isolated from an enriched thymic CD2$^-$ cell suspension of 19-day-old embryonic rats. Magnification × 750. (b) Monocyte-like cell expressing MHC class II molecules from an enriched thymic CD2$^-$ cell suspension of 17-day-old embryonic rats. Magnification × 825. (c) Strongly MHC class II-positive macrophage-containing engulfed thymocytes (arrows) in the cytoplasm from an enriched thymic CD2$^-$ cell suspension of 18-day-old embryonic rats. Magnification × 750. (d) ED1 expression in macrophages obtained from primary thymic stromal cell cultures of 16-day-old embryonic rats. Magnification × 750. (e) Differential ED2 expression in macrophages obtained from primary thymic stromal cell cultures of 16-day-old embryonic rats. Magnification × 750.

macrophages in all embryonic and postnatal stages studied showed weak and homogeneous expression of both CD4 and CD25 (Fig. 4a).

Monocyte-like cells, appearing mainly in the cell suspensions obtained from the younger fetal rats (17-day-old rats), expressed MHC class II molecules (Fig. 4b). Also, macrophages which exhibited a strong phagocytic activity with degenerated thymocytes in their cytoplasm showed a strong class II expression (Fig. 4c). Although phagocytic macrophages occurred in more or less numbers in all cell suspensions analysed, they increased considerably in those from 18- and 22-day-old rats, when the numbers of OX17- and OX44-positive macrophages also increased. Double staining for a direct demonstration of the phagocytic activity and MHC class II molecule expression was not carried out. On the other hand, we found that ED1$^+$ cells included both phagocytic and non-phagocytic elements, while ED2$^+$ macrophages did not exhibit phagocytic capacity.

In vitro evolution of Mϕ in stromal cell cultures from embryonic thymus

In order to determine whether cell precursors for ED1 and ED2 macrophages were present in thymic developmental stages in which the immunohistological studies had failed to find ED1$^+$ and ED2$^+$ cells, but the ultrastructural analysis had demonstrated the existence of thymic phagocytic cells, we carried out primary thymic stromal cell cultures from 16-day-old fetal rats. Fifteen days after culture establishment, phenotypical analysis of non-adherent cell populations demonstrated a minor (2–3%) cell population of interdigitating/dendritic cells (IDC/DC); the most non-adherent cells were macrophages containing a variable number of cytoplasmic vehicles. Most of these expressed ED1 and OX44 (Fig. 4d), but 15% of the total population corresponded to ED2^{high} cells whereas 35% expressed the cell marker weakly (Fig. 4e). On the other hand, 90% of the population was OX17^{low} but 10%, which showed monocyte-like features, expressed MHC class II antigens strongly in a similar way to that found in enriched CD2$^-$ thymic cell suspensions from 17-day-old fetal rats (Fig. 4b).

DISCUSSION

As shown for other thymic stromal cell components, including epithelial and dendritic cells,[14] macrophages can be ultrastructurally identified early during rat thymus ontogeny, although they do not express surface molecules recognized by specific mAb in adult thymus. Furthermore, our current results support the existence of monocyte–macrophage cell precursors from the early developmental stages of rat thymus. Enriched CD2$^-$ thymic cell suspensions from 17-day-old embryonic rats contain MHC class II molecules-expressing monocyte-like cells that also express MHC class I antigens, CD45 and Thy-1 and gradually exhibit increasing acid phosphatase activity, an enzymatic marker of phagocytic cells which we and other authors[15] have identified *in situ* in 17-day-old fetal thymus sections.

Other authors had previously described the occurrence of monocyte–macrophage precursors early in thymus ontogeny[5–8]

and our *in vitro* results also demonstrate that primary thymic stromal cell cultures from 16-day-old fetal rats contain a small percentage of these monocyte-like cells, expressing strongly MHC class II molecules, which *in vitro* can give rise to mature ED1 and ED2 macrophages. Accordingly, we conclude that the early embryonic rat thymus contains a small population of monocyte–macrophage cell precursors that matures throughout ontogeny, down-regulating the expression of MHC class II molecules while acquiring other cell markers (MHC class I molecules, CD45, Thy-1, CD4, CD25), including those recognized by mAb such as ED1 and ED2 specific to mature macrophage subsets.

Nevertheless, a percentage of thymic mature macrophages, mainly those exhibiting phagocytic capacity, express MHC class II molecules as well. Data in the literature on the MHC class II molecule expression on monocytes–macrophages are conflicting. Some authors assume that only monocytes *in situ* identified in both embryonic and postnatal rat thymus can express Ia molecules.[5] Others, however, in agreement with our results, have detected mature macrophages, mainly in the thymic cortex, which express MHC class II molecules[3,9] and, *in vitro*, a certain percentage of macrophages obtained from enriched rat thymic cell suspensions[10] or from primary thymic stromal cell cultures of adult rats[11] are Ia positive. It is important to remark, however, that up- and down-regulation[16,17] of these molecules on cultured macrophages has been reported, and Murawska *et al.*[12] consider that macrophages do not express MHC class II molecules constitutively but acquire them passively.

The ED1 molecule [equivalent presumably to the determinant(s) recognized by mAb R-MC43] is the first specific cell marker appearing on thymic macrophages during rat ontogeny. Apparently this cell marker is expressed by the majority of cells belonging to the monocyte–macrophage cell lineage, including most thymic macrophages of adult rats,[1,3,11,18] and might be related with their phagocytic capacity.[18] Nevertheless, our results demonstrated that thymic ED1$^+$ macrophages represent a heterogeneous cell population containing phagocytic and non-phagocytic elements. The first ones gradually increase their acid phosphatase activity to constitute finally the phagocytic macrophage cell population which occupies the thymic cortex in adult rats. This cell population, previously described in both adult[19] and fetal rat thymus,[3] shows certain homology with the tingible body macrophages of germinal centres involved in the engulfment of non-selected B cells, and might be related to the first phagocytic cells identified ultrastructurally in our study in 15-day-old rat thymus.

Non-phagocytic ED1$^+$ macrophages occur throughout the distinct thymic compartments. In both the medulla and corticomedullary border they might be the equivalent of the R-MC44$^+$ macrophages, as previously described by Colic *et al.*,[2] whereas in the thymic cortex they could differentiate to ED2$^+$ cells through an intermediate ED2^{low} cell stage. To support this view, the primary stromal cell cultures established from 16-day-old rat thymus contain, according to our results, a variable proportion of both ED1$^+$ ED2$^{low/med}$ cells and ED1$^+$ ED2^{high} cells.

Another aspect emerging from our results is the increase in the number of macrophages, principally ED2$^+$ cells, which occurs in the rat thymus during the first 2 weeks of postnatal life. This process could be associated mainly with the arrival of new macrophage precursors, which gain access into the thymus through the perivascular space, as suggested by our immunohistochemical results and as proposed previously by Sminia *et al.*,[3] rather than with an *in situ* proliferation of pre-existent intrathymic cortical ED2$^+$ cells, which seem to exhibit a low proliferative capacity.[20] In accordance with this, indirect evidence demonstrates that ED2$^-$ macrophage cell precursors which colonize various lymphoid organs during both embryonic and postnatal life need about 1 week prior to ED2 expression[3,21,22] in a process requiring connective tissue–macrophage interaction.[23] In addition, cultured macrophages only express ED2 under adherent conditions[23] and mAb recognizing this molecule, including ED2, R-MC41 and R-MC42, immunoprecipitate a molecule presumably involved in cell–cell and/or cell–matrix interactions.[23] Proposed relationships between monocyte-like precursors, the ED1$^+$ cell subset and ED2$^+$ macrophages during rat thymus ontogeny are summarized in Fig. 5.

Finally, the function of thymic ED2$^+$ macrophages is largely unknown. Their close association with cortical lymphocytes demonstrated in this study, and previously observed in the bone marrow and splenic periarteriolar lymphoid tissue, suggests that ED2$^+$ macrophages could promote T-cell maturation and/or differentiation. In accordance with this,

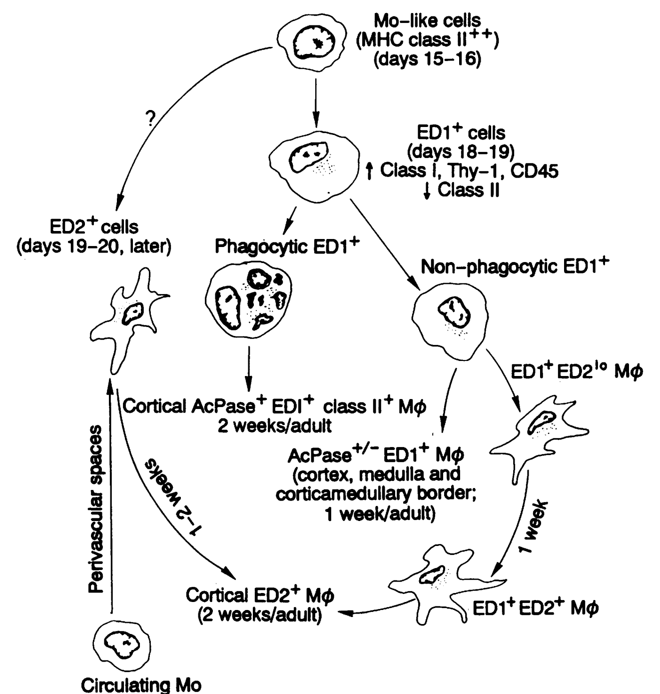

Figure 5. Relationships between macrophage precursors, ED1$^+$ cell subsets and ED2$^+$ macrophages during rat thymus ontogeny. Monocyte (Mo)-like cells, which colonize the embryonic thymus early and express MHC class II molecules strongly, differentiate at the end of fetal life to ED1$^+$ and ED2$^+$ cells. ED1$^+$ cells constitute a heterogeneous cell population including phagocytic (MHC class II$^+$ cells) and non-phagocytic cells. These latter ones could differentiate through intermediate stages to ED2$^+$ macrophages. Another important source of ED2$^+$ cells in postnatal rat thymus is circulating monocytes that, around birth, reach the organ through the perivascular spaces, becoming mature ED2$^+$ macrophages in 1–2 weeks.

other authors have demonstrated the capacity of ED2$^+$ macrophages from rat thymic cortex to form cell complexes with immature double-positive cortical thymocytes,[12] and van Rees & Dijkstra[24] describe a reduced number of thymic cortical ED2$^+$ macrophages associated with T lymphopenia in autoimmune diabetes-prone BB rats.

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