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Ontogeny and Phenotype of Macrophage and T-lymphocytes in Rat Yolk Sac and Embryonic Liver

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ABSTRACT

Background : Ontogeny of fetal macrophage and T-lymphocyte differentiation is important for understanding self-recognition and biophylaxis. Phenotypes of the cells were compared.

Materials and Methods : The yolk sac and embryonic liver were extirpated from DA/Slc rats and subjected to immunological studies.

Results : Macrophages expressing Mar1, Mar3, ED1, or ED2 antigens were detected in both the yolk sac and the liver. Whereas approximately 60% of cells expressed the antigens in the yolk sac, fewer than 5% of cells expressed antigens other than Mar3 in the liver. T-lymphocytes expressing CD3, CD4, CD8, TCR$\alpha\beta$, or TCR$\gamma\delta$ was observed in approximately 60% of the cells on day 16 in the yolk sac. However, expression was modest in the liver.

Conclusions : The patterns of surface marker expression during macrophage and T-lymphocyte development differ between the yolk sac and the embryonic liver. Pluripotent hematopoietic cells appear in the yolk sac and migrate to the embryonic liver. Because expression differed, factors that alter the surface marker expression may exist.

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Key words : macrophage, T-lymphocyte, ontogeny, yolk sac, and embryonic liver

INTRODUCTION

Macrophages and lymphocytes are multifunctional cells that participate in numerous biological processes[1]. Macrophages respond to local signals in inflammation and scavenge foreign pathogens. They also play a role in tissue remodeling by phagocytizing foreign particles or cellular debris. Lymphocytes are responsive for immunity by eliciting an immunological reaction to attack pathogens or transformed cells directly or in combination with macrophages. Both cells are present from early in embryonic development and are essential for self-recognition and the prevention of foreign invasion.

Serious discussion of the origin and development of macrophages has continued to the present day since the first designation of macrophages by Mechanikoff in 1892[2]. Kiyono (1914) proposed a histiocytic cell theory based on the results of his studies of vital staining[3]. Aschloff (1924) established the concept of the reticuloendotherial system and included macrophages (histiocytes) as part of this system together with reticular cells and reticuloendothelial cells[3]. However, this concept contained several errors aris-

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ing from the definition of reticuloendothelial system. In contrast, van Furth *et al*. proposed the theory of the mononuclear phagocyte system and insisted that all macrophages, both those appearing in inflammatory foci and those residing in tissues under normal conditions, are derived from monocytes that differentiated via promonocytes from monoblasts originating in the bone marrow[4]. Naito *et al*. have identified macrophages in oseteopetroic mice, which have a genetic defect in the production of macrophage colony-stimulating factor, an essential factor for monocyte development, causing these mice to have half the number of macrophages found in healthy mice[5]. Furthermore, from the ontogenetic and phylogenic viewpoints, macrophages are present in the yolk sac before the development of bone marrow, fetal liver, or other hematopoietic systems, and, on the basis of studies with original anti-rat macrophage Mar1 and Mar3 monoclonal antibodies, Yamashita has proposed that macrophages originate in the extraembryonic yolk sac membrane[6]. The migration of early macrophages from the yolk sac to the fetal viscera has been demonstrated with a tracer experiments, and possible migration routes have been proposed. In fact, the emergence of immature yolk sac macrophages has been confirmed to occur when the earliest hematopoietic cells formed in mammalian development[7].

In contrast to macrophages, little is known about lymphocytic populations. As with macrophages, the possibility of an endodermal origin of yolk sac lymphocytes has been investigated. A recent study has demonstrated the emergence of plasma cells in the early rat yolk sac[8]. Cells delineating the morphology of plasma cells in the yolk sac were observed as early as 12 days of embryonic life. Immunologic staining for intracytoplasmic immunoglobulin production weak on days 10 and 11 of gestation but was dense on day 12 of gestation ; however, by day 14 of gestation, the number of positive cells had markedly reduced[9]. Plasma cells play a pivotal role in the immune system and are responsible for the synthesis and release of immunoglobulins. Numerous *in vitro* culture experiments of the yolk sac have demonstrated the generation of mature cells of the myeloid and lymphoid lineages under appropriate conditions. A series of ontogenetic studies in birds and in mice have revealed that hematopoietic and lymphoid development involves the migration streams of primitive cells that colonize developing primary lymphoid organs as well as the spleen, marrow, and liver[10]. The yolk sac has been proposed as the ultimate origin of these lymphohematopoietic precursors. In this context, we investigated macrophage and T-lymphocyte populations in the rat yolk sac and embryonic liver. Surface markers of macrophages and T-lymphocytes in the yolk sac and embryonic liver on days 12 to 16 were compared.

Materials and Methods

Animals

Specific pathogen-free female DA/Slc rats, 8 to 10 weeks old and weighing 100-120 g, were purchased from Japan Slc Co. (Hamamatsu, Japan) and maintained in a specific pathogen-free animal facility at the Hamamatsu University School of Medicine. All the animal procedures were performed under the strict guidance of the animal care facility. The age of the embryos was determined by scoring for the appearance of a vaginal plug, with the morning on which the mating plug was observed regarded as day 0. Under ether anesthesia, embryos with a yolk sac were removed from the pregnant female rats at 12 to 16 days' gestation. Under a dissecting microscope, the yolk sac and embryo were separated for use in the investigation.

Antibodies

Monoclonal antibodies, Mar1 and Mar3, recognizing rat macrophages, were produced at Hamamatsu University School of Medicine[11]. The mouse monoclonal antibodies recognizing CD3 (W3/13), TCR$\alpha\beta$, TCR$\gamma\delta$, CD4 (W3/25), CD8 (OX8), ED1, ED2 (anti-macrophage), fluorescein isothiocyanate (FITC)-conjugated rabbit F(ab')2 fragment to anti-mouse IgG, and Phycoerythrin (PE)-conjugated monoclonal antibodies were purchased from Seikagaku Co. (Tokyo, Japan). Monoclonal antibodies specific for the adhesion molecules LFA-1α (WT-1), LFA-1β

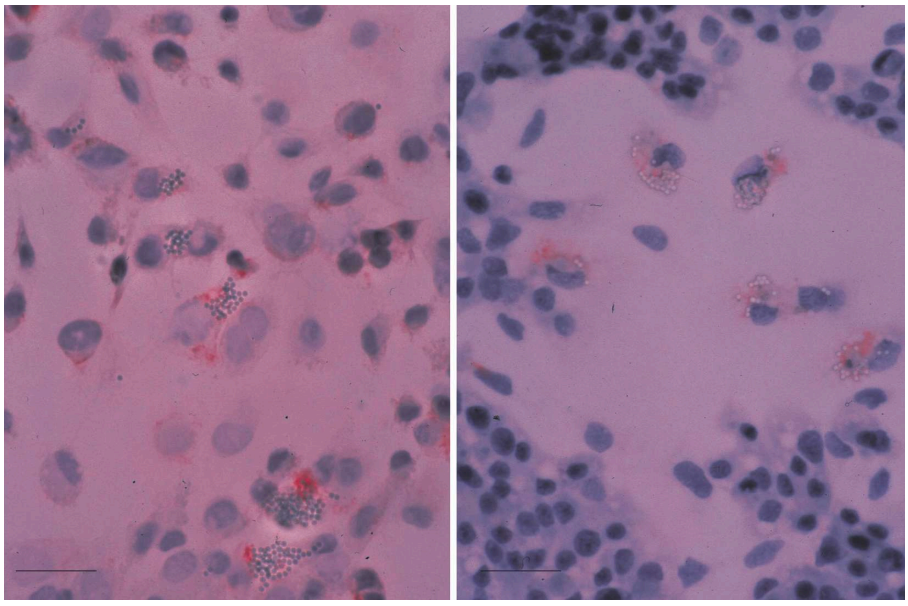

Fig. 1. Yolk sac and liver cells on day 16 of gestation. Mar3 staining.
Latex particles were injected into the yolk sac, and 10 hours later the yolk sac (left) and embryonic liver (right) were examined with Mar3 immunostaining. Although not all Mar3-positive cells took up the particles, the cells demonstrating phagocytosis were Mar3-positive. Bars, 100 μm.

(WT-3), and ICAM-1 (1A29) were provided by Dr. M. Miyasaka (Osaka University School of Medicine, Osaka, Japan). A second antibody, alkaline phosphatase-conjugated anti-mouse IgG, was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Immunohisotological staining

Cell smears were prepared by modified Hogan's method[12]. In short, yolk sac and embryo were separated in Dulbecco's MEM (Invitrogen Japan, Tokyo, Japan) under a dissecting microscope. Yolk sac or embryonic liver tissue were digested by 0.25% trypsin/EDTA and separated cell smears were prepared with a cytospin centrifuge (Shandon Ltd., Cheshire, UK). The unfixed specimens were embedded in an OCT compound (Miles Inc., IN, USA), frozen in −70°C, and sectioned at 5 μm with a cryostat. Tissues were incubated with antibodies for 60 min at room temperature. As a control, mouse IgG was used as negative staining. After being washed with 0.01 M cold phosphate-buffered saline (pH 7.4), the slides were incubated with second antibodies for 45 minutes at room temperature. The slides were developed with ABC alkaline phosphatase substrate kit 1 (Vector red) (Funakoshi Co., Tokyo, Japan). Specimens were counterstained with hematoxylin and observed under a light microscope.

Phagocytosis experiment

On day 16 of gestation, latex particles were injected into the yolk sac under anesthesia. Ten hours later, sections from the yolk sac and embryonic liver tissues were prepared and examined immunohistochemically.

Flow cytometric analysis

Yolk sac cells and embryo liver cells were dispersed and treated with monoclonal antibodies for 30 minutes at 4°C. After the cells were washed with phosphate-buffered saline, the fluorescein isothiocyanate-conjugated rabbit anti mouse IgG antibody was added and incubated for a further 30 minutes. After being washed, the cells were analyzed on an EPIC Profile (Colt Co. FL, USA). Controls consisted of unstained yolk sac cells or embryo liver cells as autofluorescence controls.

Results

Cells recognized by Mar1 and Mar3 appear in

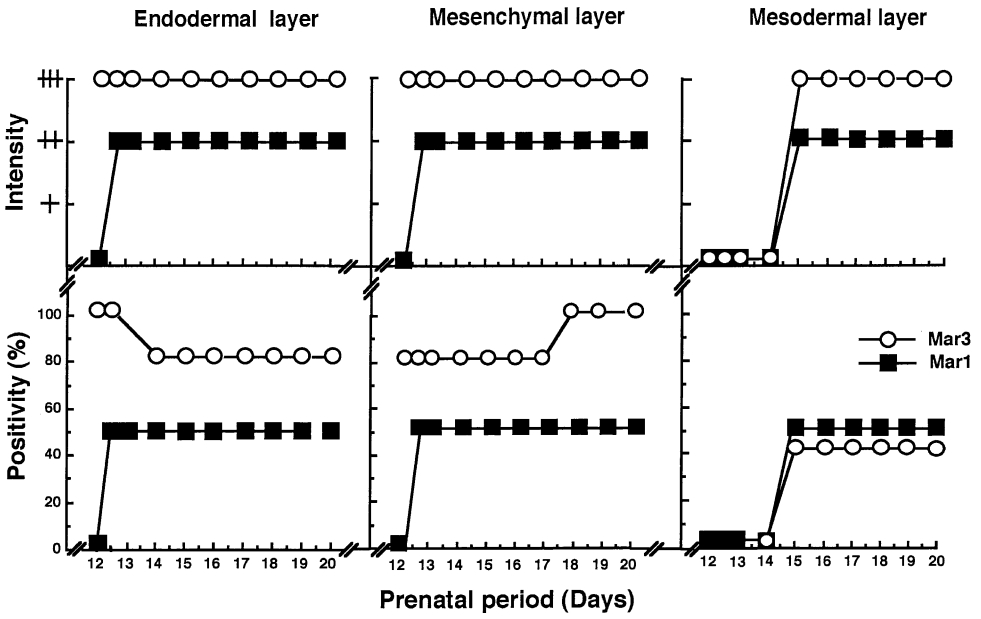

Fig. 2A. Distribution of Mar1-positive and Mar3-positive cells in the yolk sac in 12- to 20-day-old rat embryos. The intensity of the expression of Mar3 and Mar 1 antigens and the percentage of positive cells were examined with immunohisotochemistry. The distribution and intensity of Mar3 and Mar1 were different. In the mesodermal layer, Mar1- and Mar3-positive cells appeared later than in the endodermal and mesenchymal layers. Although the numbers of Mar3-positive cells were higher in the endodermal and mesenchymal layers, Mar1-positive cells were also abundant in the mesodermal layer.

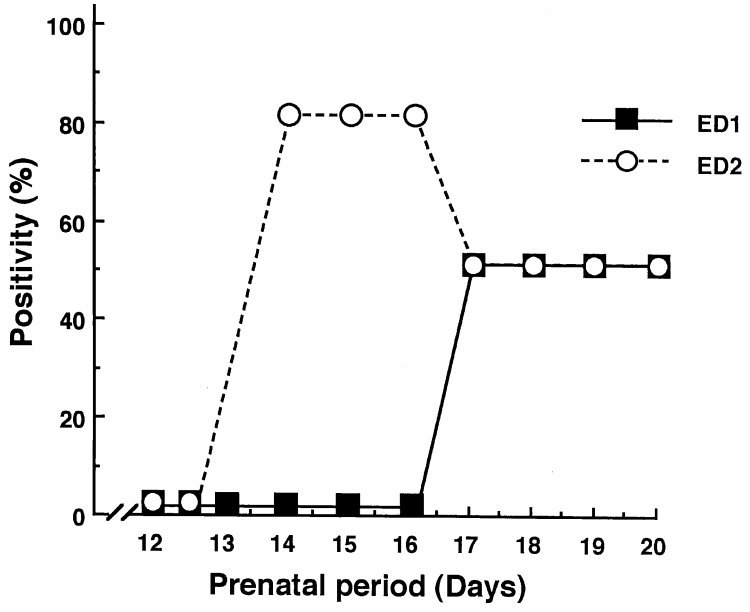

Fig. 2B. Percentages of ED1-positive and ED2-positive cells in the endodermal layer of the yolk sac in 12- to 20-day-old rat embryos.
Cells positive for the monocytic markers ED1 and ED2 also appeared at different stages of embryonic development. Whereas ED2-positive cells appeared on day 14 of gestation, ED1-positive cells appeared on day 17.

both the yolk sac and the embryonic liver. Uptake of the latex particles, indicating macrophage properties, by macrophage-like cells was observed both in the yolk and the liver. The Mar3 antibody indicated the expression of macrophage antigens by the cells (Fig. 1). Similar results were obtained with Mar1 (data not shown).

While Mar1 and Mar3 both reacted to the macrophage lineage, the antigens recognized by these antibodies were different. To confirm that the embryonic tissue contains different types of phagocytic cells, endodermal, mesenchymal, or the mesodermal layer of the yolk sac wall were stained with Mar1 and Mar3. Yolk sac wall consisted of endodermal, mesenchymal, and mesodermal layers. The features of these layers in the stage were described previously[13]. The intensity of expression and percent of positive cells in each location varied with these antibodies. Cells recognized by Mar3 appeared at an earlier stage of development in the endodermal and mesenchymal layer (Fig. 2A, left and middle). In addition, the intensity of staining and the percent of positive cells were greater with Mar3 than with Mar1. However, expression was different in the mesodermal layer. The intensity of Mar3 was stronger, but unlike the endodermal and mesenchymal layers, there were fewer positive cells (Fig. 2A, right).

Because temporal and spatial expression differed between the antigens recognized by Mar1 and Mar3, other antigens associated with macrophage differentiation were investigated. When yolk sac endodermal cells were stained with ED1 or ED2, temporal expression was also different (Fig. 2B). Abundant ED2-positive cells were observed from day 14 to 16, although extremely few ED1-positive cells were detected in the area. These results were consistent with the emergence of yolk sac macrophages as the earliest event of hematopoietic cell development[7].

Lymphocytes, especially T-lymphocytes, are responsible for cellular immunity and they can attack pathogens or transformed cells directly when activated by macrophages. Since macrophages are present from an early stage of hematopoietic cell development, cells expressing T-lymphocyte markers were investigated. In the yolk sac, both CD3-expressing cells (Fig. 3A upper) and Mar3-expressing cells (Fig. 3A lower) increased from day 12 to 16. In contrast, in the liver, Mar3-positive cells also increased, but there were few CD3-positive cells (Fig. 3B). Because obtaining a sufficient number of cells for flow cytometric analysis was difficult and adequate cell dispersion was sometimes troublesome, percentage of cells positive for a marker was determined by immunohistochemistry in view of the fact that the populations determined by flow cytometry and immunohistochemistry were comparable. For example, the percentage of positive cells were 45.3% on day 12, 61.1% on day 14, and 66.7% on day 16 as determined with flow cytometry (Fig. 3 upper) and were 40.2% on day 12, 59.8% on day 14, and 63.3% on day 16 as determined with immunohistochemistry (other data not shown).

Immunohistochemical staining of the yolk sac and embryonic liver with macrophage markers showed that Mar1-, Mar3-, ED1-, and ED2-positive cells were increased in the yolk sac. More than 40% of the cells expressed some kind of macrophage markers until day 14, and the percentage of positive cells were approximately 60% until day 16 (Fig. 4A left). In general, the percentages of cells expressing Mar1, Mar3, ED1, or ED2 were similar. In contrast, fewer cells expressed macrophage markers in the embryonic liver. On day 12, fewer than 5% were positive for a marker (Fig. 4A right). Cells positive for Mar3 appeared approximately 2 days later than in the yolk sac and rapidly increased, reaching 95.3% on day 16. However, neither Mar1-, ED1-, nor ED2-positive cells increased in liver tissue, and the numbers of positive cells decreased from day 14 to day 16. The marker expression pattern differed between the yolk sac and the embryonic liver.

In the yolk sac, the expression of cell adhesion molecules ICAM-1, LFA-1α, and LFA-1β increased over time (Fig. 4B left). Percentages of positive cells gradually increased and reached 98.1%, 60.1%, and 95.8%, respectively (Fig. 4B left). In the embryonic liver, a similar increase in ICAM-1 and LFA-1β expression occurred (Fig. 4A right). However, conversely, LFA-1α positive cells decreased.

In the ontogeny of macrophage appearance in the

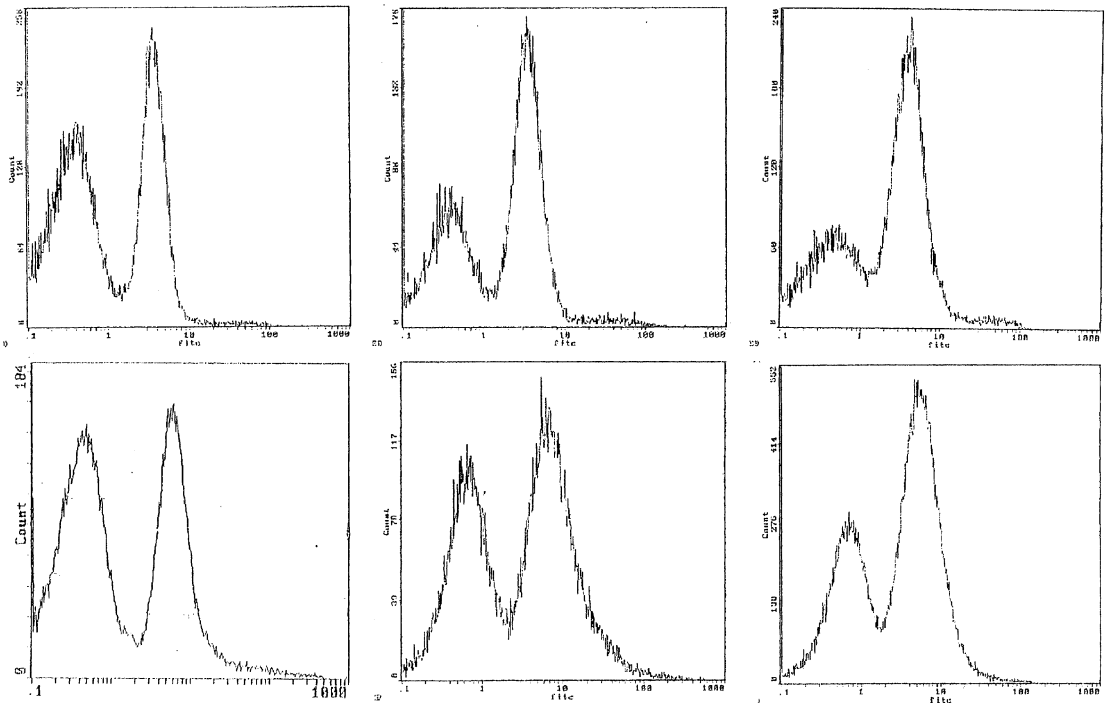

Fig. 3A

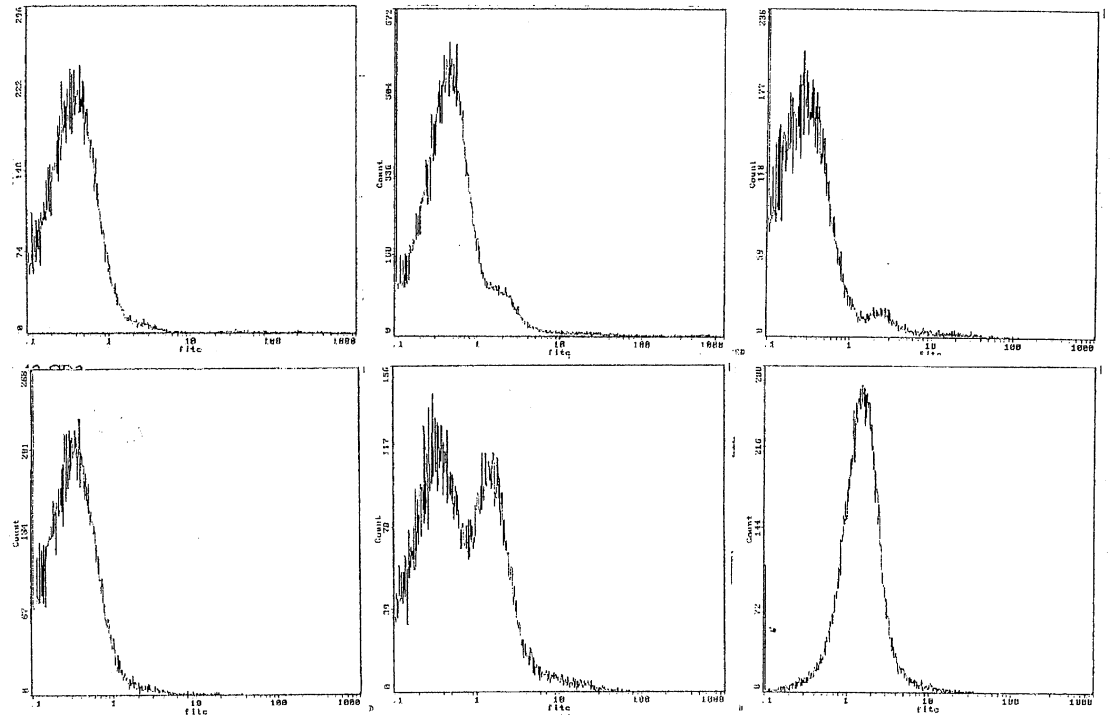

Fig. 3B

Fig. 3. Flow cytometery of yolk sac cells (A) or embryonic liver cells (B).
Cells were dispersed, stained with fluorescent-conjugated antibody, and then analyzed with flow cytometry. Upper column, reacted with anti-CD3 antibody and, lower, with anti-Mar3. Left, day 12 ; middle, day 14 ; and right, day 16.
A : In the yolk sac, the number of CD3-positive cells and the number of Mar3-positive cells increased over time. B : In contrast, although Mar3-positive cells also increased in the liver, CD3-positive cells were not prominent.

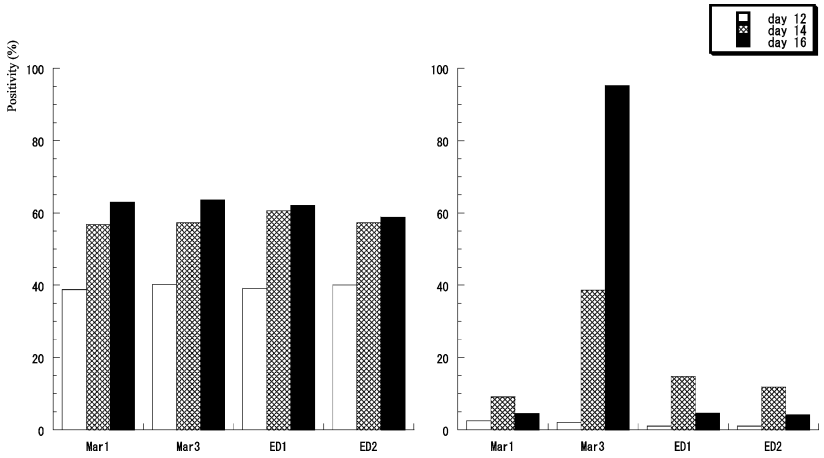

Fig. 4A

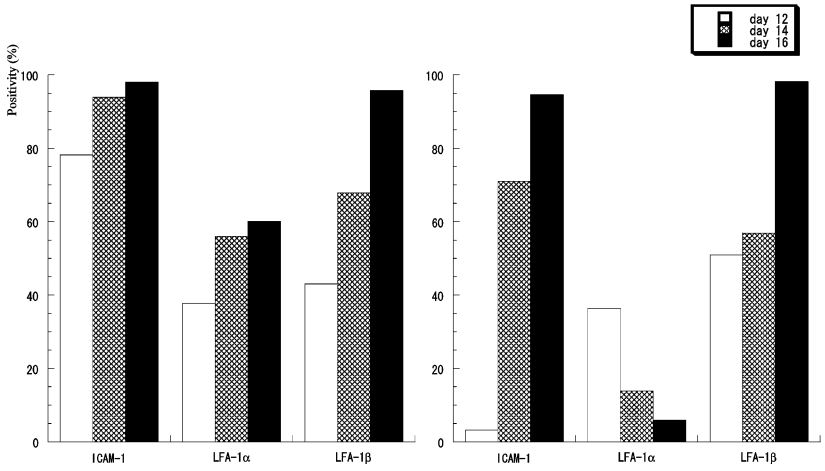

Fig. 4B

Fig. 4. Yolk sac (left) and embryonic liver cells (right) were stained immunohistochemically.
A : Expression of macrophage differential markers.
Mar1-, Mar3-, ED1-, and ED2-positive cells gradually increased in the yolk sac. The increase in Mar3-positive cells was more rapid in the embryonic liver. These cells appeared approximately 2 day later than in the yolk sac. Unlike Mar3-positive cells, cells positive for Mar1, ED1, or ED2 did not increase in the liver.
B : Expression of ICAM1 and LFA-1
Cells positive for ICAM1, LFA-1α, or LFA-1β increased in the yolk sac. In the embryonic liver, ICAM1- and LFA-1β-positive cells also increased, but ICAM1-positive cells appeared 2 days later than in the yolk sac. Unlike cells positive for ICAM1 or LFA-1β, LFA-1α-positive cells decreased from day 13 to day 16. The expression pattern in the embryonic liver differed from that in the yolk sac.

embryonic liver, it was considered that the macrophages had migrated from the yolk sac. However, surface marker distribution as well as expression of LFA molecule differed significantly quite between the yolk sac and the embryonic liver. To investigate the ontogeny of other types of hematopoietic cells, the markers of the T-lymphocytes were examined.

In the yolk sac, CD3, CD4, CD8, TCR$\alpha\beta$, and TCR$\gamma\delta$ were expressed in approximately 40% of cells on day 12 of gestation (Fig. 4C lower, left). The percentage of positive cells reached around 60% on day 14 or day 16. In the embryonic liver, the number of positive cells was smaller than in the yolk sac (Fig. 4C lower, right). Although the percentage of positive cells increased from day 12 to day 14, expression decreased on day 16. The maximum percentages of

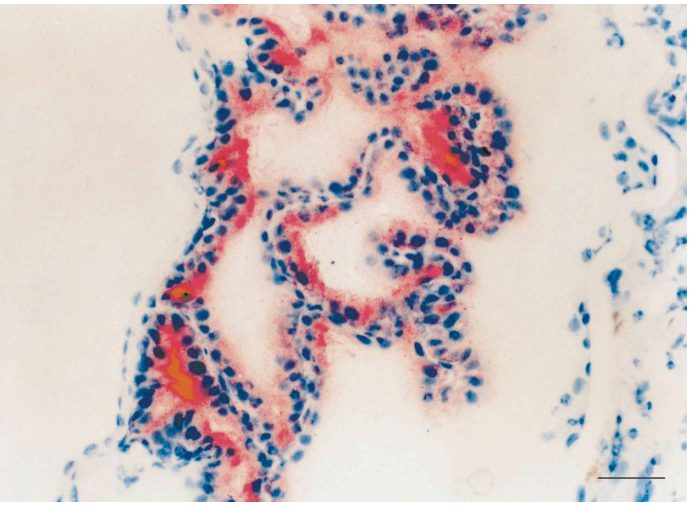

Fig. 4C Upper

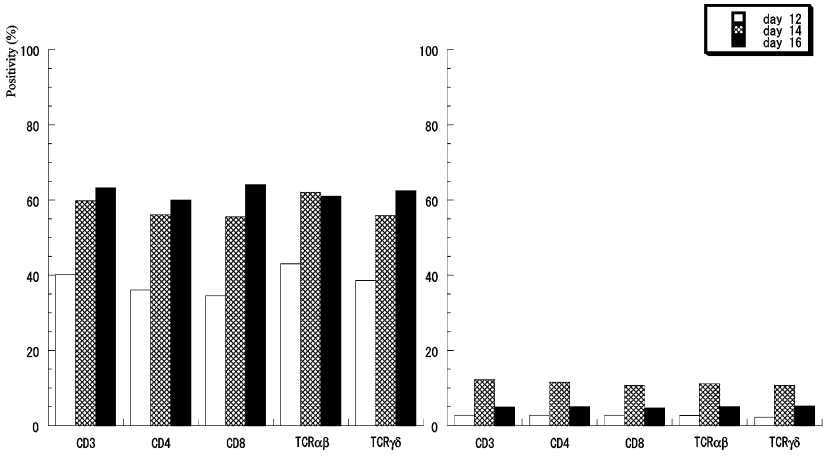

Fig. 4C Lower

Fig. 4. C: Expression of T-lymphocyte markers
Upper: Yolk sac cells on day 16 of gestation were stained with CD4. The picture is representative of immunostaining experiments. Note the preferential staining of CD4 on the surface of yolk sac endoderm. Indirect immunoalkaline phosphatase staining using anti-CD4 monoclonal antibody. ×450.
Lower: In the yolk sac, the percentages of CD3-, CD4-, CD8-, TCR$\alpha\beta$-, TCR$\gamma\delta$-positive cells increased to more than 60% (left). However, the percentages of positive cells were much lower in the embryonic liver (right). In the liver, the percentages of cells expressing T-lymphocyte markers peaked at 14 days of gestation.

cells positive for CD3, CD4, CD8, TCR$\alpha\beta$, and TCR$\gamma\delta$, obtained on day 14, were 12.2%, 11.6%, 10.7%, 11.2%, and 10.7%, respectively.

Discussion

According to Moore, in the mouse, pluripotential hematopoietic stem cells are first detected in the yolk sac[10]. Colony-forming assays have shown that these cells can migrate to the fetal liver and colonize the bone marrow[14]. If yolk sac stem cells comprise a truly pluripotent population, they should be able to differentiate into lymphocytes as well as macrophage. More recent observations have shown that the ontogeny of macrophages in early mouse and human embryos differs from that during adult development and that embryonic macrophages do not follow the monocyte pathway. Fetal macrophages are thought

to differentiate from primitive yolk sac-derived macrophages before the development of adult monocytes[15].

As for development of the yolk sac in embryonic rat, yolk sac epithelium of the rat is physiologically a placenta that function as an organ for maternal and embryo exchange. The formation of yolk sac placenta begins on day 7 with the proliferation of at the periphery of inner cell mass of hypoblast. The visceral portion of the yolk sac becomes trilaminar by an interposition of extraembryonic mesoderm while more peripheral portion or parietal wall of the yolk sac remains bilaminar. By day 10, visceral yolk sac mesoderm has split to form an extraembryonic coelom and undergoes angiogenesis. Thus the yolk sac is the first hematopoietic organ[16].

Our present immunohistochemical studies using the Mar series monoclonal antibodies showed that cells stained with Mar3 were observed first in the proximal endoderm on fetal day 6 ; this area is destined to differentiate into the yolk sac endodermal cell layer[6,17]. According to the results of the present study, the change in surface marker expression on yolk sac cells gradually increased from day-12 to day-16. Although both the Mar and ED series monoclonal antibodies are anti-macrophagic, ED1 monoclonal antibody recognizes a single chain glycoprotein of 110 kD that is expressed predominantly on the lysosomal membrane. The antigen recognized by ED1 is the rat homologue of human CD68. ED2 antibody reacts with a membrane antigen (175, 160 and 95 kDa) on resident rat macrophages and the antigen is identical with CD163. Mar1 binds specifically to the cells constituting the mononuclear phagocyte system and Mar3 antigen is a phagocytosis-associated molecule. Application of these antibodies in both *in vivo* and *in vitro* studies has allowed the functional capability and differentiation of fatal macrophage to be assessed[6]. Expression of the adhesion molecule phenotypes of yolk sac cells and embryo liver cells has demonstrated that these molecules are necessary for the homing of these cells[18,19].

B-lymphocytes positive for OX33 were observed in the yolk sac. OX33-expressing cells were almost undetectable until day 12 of gestation. Then expressing cell number suddenly and transiently increased. The results were consistent with those of previous studies that indicated the presence in the yolk sac of plasma cells producing intra-cytoplasmic immunoglobulin (IgA, IgG, and IgM) as early as 12 days of embryonic life[8,9].

T-lymphocytes positive for CD3 were also observed in the yolk sac (Fig. 4C). Other than CD3, CD4, CD8, TCR$\alpha\beta$, and TCR$\gamma\delta$ were also fully developed. The expression of T-lymphocyte surface markers, except for TCR$\alpha\beta$, gradually increased on yolk sac cells from day 12 to day 16. We propose on fetal days 12 to 16 that the expressions of T-lymphocyte, macrophage, and adhesion molecule antigenic phenotypes are related in yolk sac cells. The pattern of surface marker expression on embryonic liver cells differed greatly from that on yolk sac cells.

Although pluripotent hematopoietic stem cells first develop in the yolk sac and then migrate to the embryonic liver, other factors might also alter surface marker expression. In addition, the ontogeny of T lymphocytes in the early rat embryo may not be consistent with postnatal or adult T-cell development. The ontogeny and function of macrophages and lymphocytes are critical for self-recognition and biophylaxis. Further study is warranted.

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References

1. Geodon S. The macrophage. BioEssays 1995 ; 17 : 977-86.
2. Frolov VA. I.I. Mechnikoff's contribution to immunology. J Hyg Epidemiol Microbiol Immunol 1985 ; 29 : 185-91.
3. Wake K, Kawai Y, Smedsrod B. Re-evaluation of the reticulo-endothelial system. Ital J Anat Embryol 2001 ; 106 : 261-9.
4. Furth Rv, Cohn ZA, Hirsch JG, Humphery JH, Spector WG, Langevoort HL. The mononuclear phagocyte system : a new classification of macrophages, monocytes, and their precursor cells. Bull WHO 1972 ; 46 : 845-52.
5. Naito M. Macrophage heterogeneity in development and differentiation. Arch Histol Cytol 1993 ; 56 : 331-51.

6. Yamashita A. Role of yolk sac endodermal cells with special reference to the fetal macrophage differentiation. J Leukoc Biol 1996 ; 59 : 139–44.
7. El-Nefiawy N, Abdel-Hakim K, Yamashita A, Kanayama N. Embryonic macrophages of early rat yolk sac : Immunohistochemistry and ultrastructure with reference to endodermal cell layer. Immunol Cell Biol 2002 ; 80 : 441–7.
8. El-Nefiawy N, Abdel-Hakim K, Kanayama N, Suganuma N, Terao T. Identification of mature plasma cells in early rat yolk sac. A possible origin from the endodermal cell layer : immunohistochemistry and immunoelectron microscopic study. Immunol Cell Biol 2003 ; 81 : 335–42.
9. El-Nefiawy N, Abdel-Hakim K, Kanayama N, Suganuma N, Terao T. Ontogeny of plasma cells in the early rat yolk sac. Arch Histol Cytol 2003 ; 66 : 327–35.
10. Moore MA. Commentary : the role of cell migration in the ontogeny of the lymphoid system. Stem Cells Dev 2004 ; 13 : 1–21.
11. Yamashita A, Hattori Y, Kotani M, Miyasaka M, Fukumoto T. A novel monoclonal antibody, Mar1, directed specifically against mononuclear phagocyte system cells in rats. Immunology 1990 ; 70 : 262–71.
12. Hogan BLM, Tilly R. Cell interaction and endoderm differentiation in cultured mouse embryos. J Embryol Exp Morphol 1981 ; 62 : 379–94.
13. Dai J, Tsujimura K, Imada M, Yamashita A. Morphological and functional features of endodermal cells in rat yolk sac, with special reference to the fetal macrophage differentiation. Acta Anatomica Nipponica 1991 ; 66 : 114–29.
14. Moore MA, Metcalf D. Ontogeny of the haemopoietic system : yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. Br J Haematol 1970 ; 18 : 279–96.
15. Shepard JL, Zon LI. Developmental derivation of embryonic and adult macrophages. Curr Opin Hematol 2000 ; 7 : 3–8.
16. Suckow MA, Weisbroth SH, Franklin CL, editors. The laboratory rat. 2nd ed. Burlington : Elsevier Academic Press ; 2006. p. 818–35.
17. Dzierzak E, Medvinsky A, de Bruijn M. Qualitative and quantitative aspects of haematopoietic cell development in the mammalian embryo. Immunol Today 1998 ; 19 : 228–36.
18. Kusterer K, Ludwig R, Hochschulz P, Stegmuller M, Bojunga J, von Minckwitz G. Macrophage and lymphocyte homing in experimental diabetes. Exp Clin Endocrinol Diabetes 1999 ; 107 Suppl 3 : S102–7.
19. Yue L, Bao Z, Li J. Expression of MacMARCKS restores cell adhesion to ICAM-1-coated surface. Cell Adhes Commun 2000 ; 7 : 359–66.