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Ontogeny of plasma cells in the early rat yolk sac

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Summary. The present study investigated the development of plasma cells in the early rat yolk sac (days 10-16 of gestation) by light microscopy, transmission electron microscopy, immunoelectron microscopy, and indirect immunofluoresce techniques. Cells delineating the morphology of plasma cells in the yolk sac were observed as early as 12 days of embryonic life. As for positive immune staining for the intra-cytoplasmic immunoglobulin (Ig) production (IgA, IgM and IgG), the intensity of the immune staining was very weak on days 10 and 11 of gestation, while it turned very dense on day 12 of gestation. At 14 days of gestation, the number of positive cells was markedly reduced. Immunoelectron microscopy visualized products of the immune reaction in cisterns of the rough endoplasmic reticulum. Conventional electron microscopic examination of 12, 13, and 16-day yolk sacs confirmed the development and differentiation of plasma cells with their well-known ultrastructural features, making this the first study to demonstrate these in the early rat yolk sac. The development of plasma cells in the early yolk sac implies the ability of the yolk sac to effect a humoral immune response at this stage of fetal life. The probable role of plasma cells in the yolk sac is also discussed.

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Introduction

During mammalian ontogeny, the yolk sac is the first site of haematopoiesis (Moore and Metcalf, 1970). In the rat, yolk sac hematopoiesis commences around the day 9 of the embryonic life (Rugh, 1968). Macrophage development and differentiation have been studied precisely in the extra-embryonic yolk sac in different species (Enzan, 1986; Takahashi *et al.* 1989; Morris *et al.* 1991; Takahashi and Naito 1993; El-Nefiawy *et al.*, 2002), but not the ontogeny of the lymphocytes in this milien.

Lymphocytes are the effector cells of both humoral and cell-mediated immunity in adult systems. In the embryonic life, the liver is the dominant site of B lymphocyte hematopoiesis (Hardy *et al.*, 2000). The hepatic rudiment in the rat is colonized around days 11-12 of gestation, and hepatic hematopoiesis reaches its maximum around day 17 of embryonic life (Rugh, 1968). Since plasma cells are the late stages of differentiation of the B-lymphocytes, they are mature end-stage cells. They play a vital role in the humoral immune responses, being the sites of the synthesis and release of antibodies. Antigenic stimulation of B cells results in a complex series of interactions leading to their activation, proliferation, and differentiation into plasma cells (Zubler, 1997; Duchosal, 1997).

Subsequent elegant studies have revealed that hematopoietic stem cells in the early yolk sac constitute a unique cell population. They have a proliferation potential which exceeds that of later-appearing hematopoietic stem cells in the liver or bone marrow. When these cells are introduced into allogeneic immunocompromised hosts, they are able to give rise to all of the leukocyte lineages including T cells, B cells, and myeloid cells (Lu *et al.*,1996). Studies have also shown that the yolk sac can support the proliferation and differentiation of bone marrow-derived hematopoietic stem cells (Yoder *et al.*, 1994, 1995)

Numerous *in vitro* culture experiments have been conducted to generate mature functioning T and B-lympho-

cytes from yolk sac cells under appropriate culture conditions (Liu and Auerbach, 1991; Huang and Auerbach, 1993; Cumano *et al.*, 1993; Palacios and Imhof, 1993; Yoder *et al.*, 1997; Auerbach *et al.*, 1998). However, mature haematopoietic cells of the lymphoid lineage have not been previously demonstrated *in situ* in the yolk sac. In an earlier work, we reported on the first emergence of plasma cells in the rat yolk sac (El-Nefiawy *et al.*, 2003). In the current research, we extended our work to study the ontogeny of plasma cells during rat yolk sac hematopoiesis (days 10–16 of gestation). Light microscopy, transmission electron microscopy, indirect immunofluoresce, and immunoelectron microscopy techniques were employed in the study.

Materials and Methods

Animals

Dark Agouti (DA) adult rats about 200–220 g in body weight were purchased from Japan Sic Co. (Hamamatsu). The rats were maintained under specific pathogen-free conditions in the laboratory animal center of our university with free access to food and water. The temperature was maintained at 23 ± 1℃, humidity at 55 ± 5%, with a 12 h light and dark cycle. Adult female rats were mated overnight with fertile male rats, and the next morning was considered as gestational day 0. Pregnant rats were killed with a lethal dose of ether according to an animal protocol that was approved by the Animal Care and Use Committee of Hamamatsu University. Embryos with the yolk sacs were removed at daily intervals from days 10–16 of gestational life. Five animals were used at each gestational age.

Monoclonal antibodies

Because no plasma cell specific antibody is available in the rat, we used monoclonal antibodies (mAbs) to stain intracytoplasmic immunoglobulins for their identification. The primary monoclonal antibodies were heavy chain specific mouse anti-rat IgA, IgG, and IgM mAbs (Sigma Chemical, St. Louis, MO, USA) that were used at a dilution 1:500, 1:750, and 1:1000 in order (Bazin *et al.*, 1974; Voller *et al.*, 1976; Chayen and Parkhouse, 1982). Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig was used as the secondary monoclonal antibody at a dilution 1:40 (Sigma Chemical).

Indirect immunofluorescence

Yolk sac sections were deparaffinized in xylene baths, rehydrated through descending grades of alcohol, and rinsed in phosphate buffered saline (PBS, pH 7.2). Sections were then fixed in cold acetone for 10 min at room temperature. After washing in the PBS (three washes 5 min for each), primary antibodies were then applied at the appropriate dilutions and incubated in 2% bovine serum albumin (BSA, Sigma) in the PBS for 1 h at room temperature. After a rinse, slides were incubated with FITC conjugated secondary antibody for 30 min at room temperature. After a final wash, glass cover slips were mounted on glass slides with Bacto FA Mounting Fluid (Difco, Kansas, USA), and sealed with clear nail varnish. Slides were examined with a Leitz Ortholpan fluorescence microscope equipped with phase contrast optics and filters for the discrimination of fluorescein (Gathings *et al.*, 1977).

Light microscopy

The extracted rat fetuses with yolk sacs were fixed in 10% formaline in water, embedded in paraffin, and sectioned longitudinally in 4-μm thick slices. Paraffin sections were stained with the following standard histological stains: periodic acid-Schiff (PAS), May-Grünwald-Giemsa, and methyl-green-pyronin. Thin sections (1-μm thick) cut from Epon-embedded blocks for electron microscopy were stained with 0.05% toluidine blue in water.

Electron microscopy

Under a dissecting microscope, the yolk sacs were separated from the rat fetuses, and cut into small blocks. These blocks were fixed in 2.5% glutaraldhyde at 4℃ for 2 h. After washing with a 0.1 M cacodylate buffer (pH 7.4), they were post-fixed in 1% osmium tetroxide OsO_4 at 4℃ for 2 h, dehydrated in a graded series of ethanols, and embedded flat in Epon 812. Ultrathin sections were cut with a diamond knife on a Reichert OM-U3 ultratome and observed in a JEM 2000 EX electron microscope (JEOL, Tokyo) after staining with uranyl acetate and lead citrate. The cellular measurements of the plasma cells were done on electron micrographs at a final magnification of ×7,500 with a computer-coordinating area-curve meter (x-plan 360; Ushikata, Japan). The longest axis of the cytoplasm, rather than the diameter, was measured since many cells had oblong configurations. Ultrastructural characterization of cells was based on previously described cytological criteria (Bloom and Fawcett, 1968; Grossi *et al.*, 1988; Tanaka and Goodman, 1972; Beutler *et al.*, 2001)

Immunoelectron microscopy for the anti-IgA mAb

The technique was applied as previously described (Takada

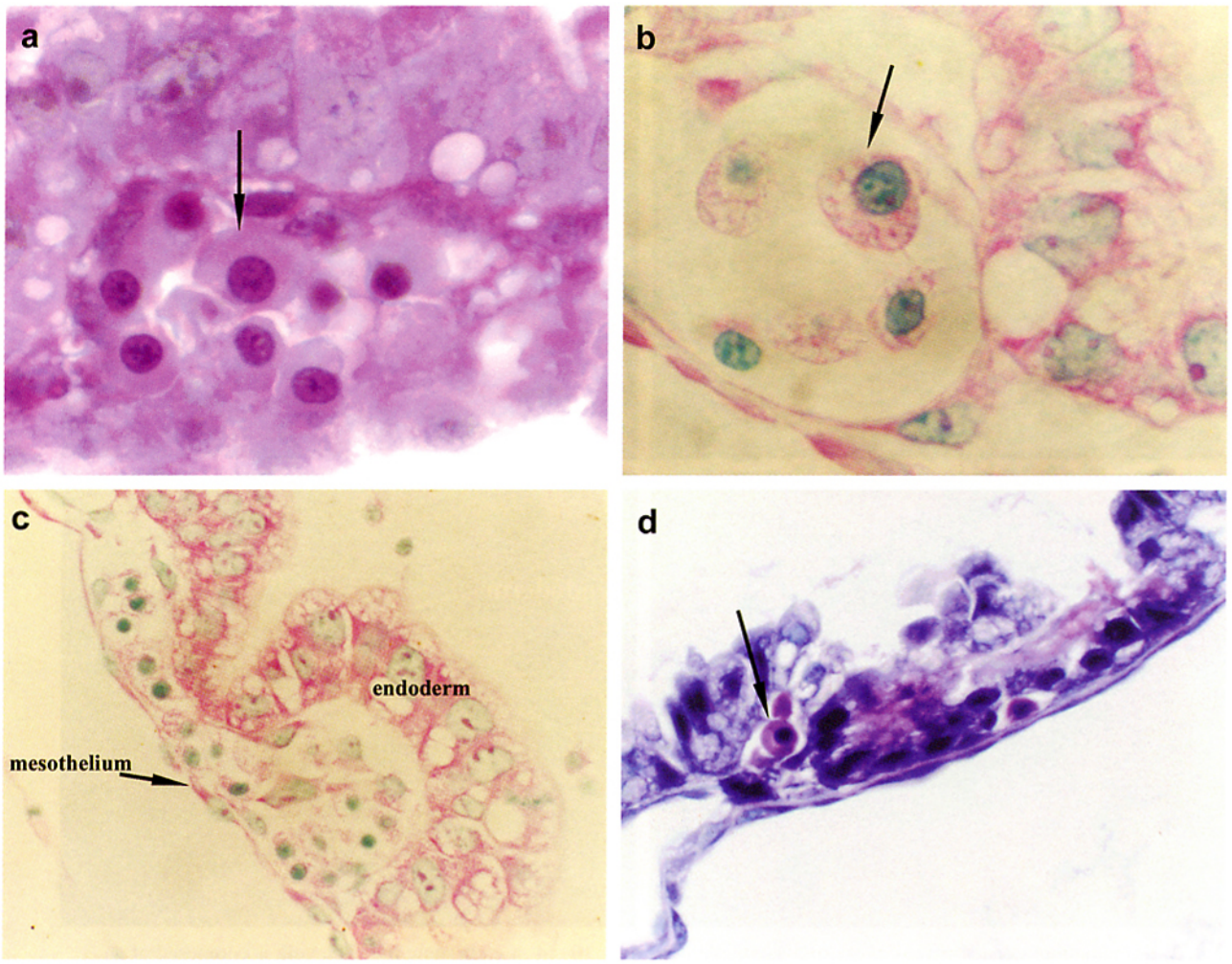

Fig. 1. Light micrographs of 5-μm thick paraffin sections of 12–16 day rat yolk sacs. The sections are stained with different histological stains to demonstrate plasma cells (↑). **a:** Periodic acid-Schiff reaction (14 days); **b, c:** Methyl green-pyronin stain (12 days); **d:** May-Grünwald-Giemsa stain (16 days). Three layers of the yolk sac can be seen: endoderm, mesothelium, and mesoderm containing blood cells inside. c, d: × 32, a, b: × 80

et al., 2001). In brief, after visualization of the immunoreactive products for anti-rat IgA mAb with the streptavidin-biotin-peroxidase complex method (SAB-method), sections were post fixed in 1% osmium tetroxide in a 0.1 M phosphate buffer (pH 7.4) for 1 h at 4°C. Then sections were dehydrated in a graded series of ethanol and embedded in Polybed 812 epoxy resin. Ultrathin sections were mounted on 150-mesh fine bar copper grids and observed in a JEOL 100 CX electron microscope operated at 100 kV. No counter staining with lead citrate or uranyl acetate was performed.

Results

Light microscopic study

At 10 and 11 embryonic days, no cells with the morphology of plasma cells were found in the yolk sac wall stained with different standard histological stains.

At 12–16 embryonic days, cells with the typical morphology of plasma cells were observed in the yolk sac wall at this stage (Fig. 1a–d). These cells were round or oval and had eccentrically placed nuclei with a coarse nuclear chromatin pattern like a characteristic cartwheel (Fig. 1a). With

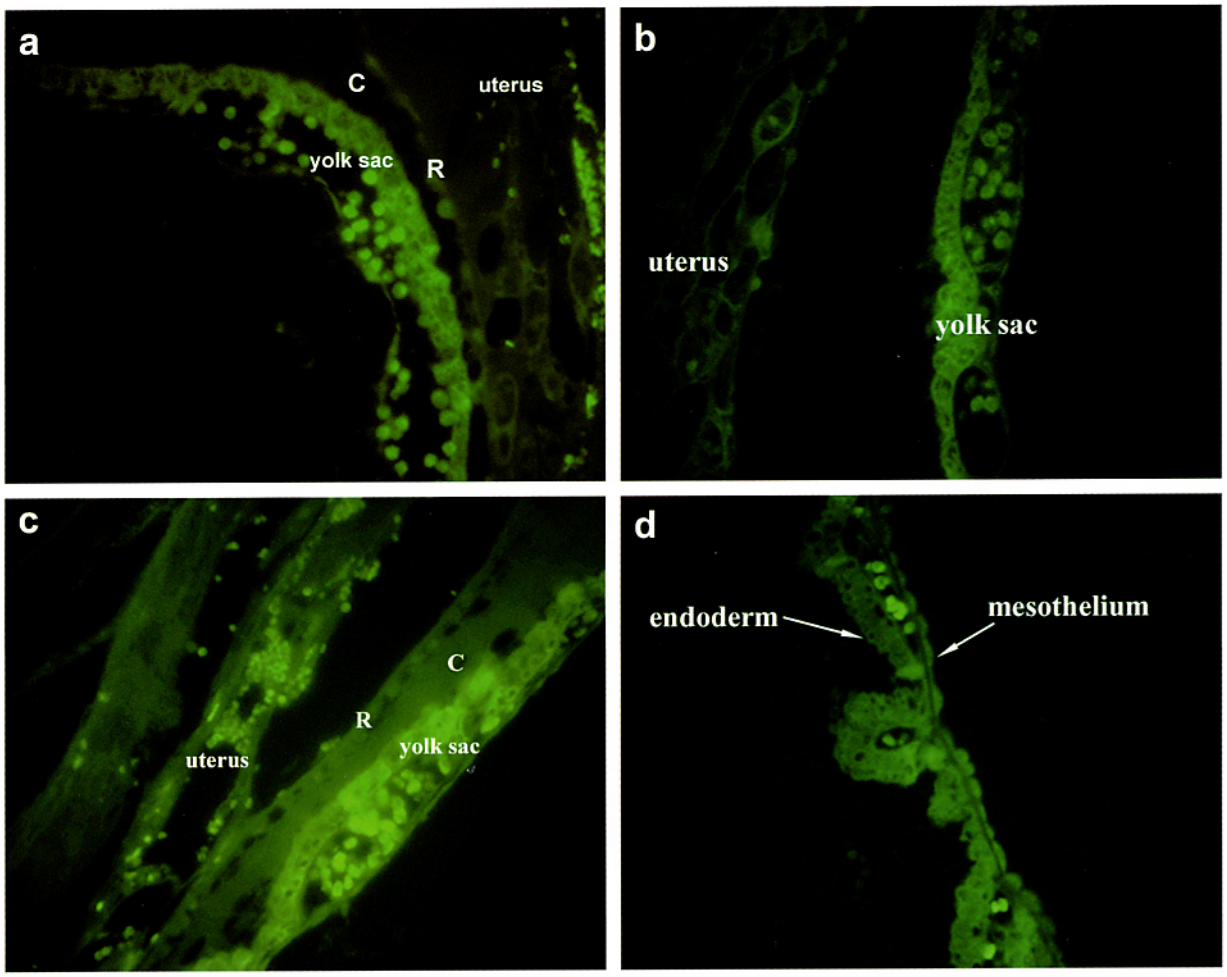

Fig. 2. Immunofluorescenct images of rat yolk sac sections on days 10–14 of embryonic life. The yolk is composed of an endodermal cell layer, viteline vessels, and a mesothelial layer. C: yolk sac cavity, R: Richert's membrane. **a, b:** 10 and 11-day yolk sacs revealing very faint immune staining. **c:** A 12-day yolk sac showing dense immune staining at the endo-dermal cell layer and hematopoietic cells inside vitelline vessels. The yolk sac cavity is also densely stained. Positive staining of IgG in the uterine vasculture can also be seen. **d:** A 14-day yolk sac showing few positive cells inside vitelline vessels and the endoderm. All sections were stained with an anti-rat IgG antibody (primary antibody) and. FITC conjugated rabbit anti-mouse antibody (secondary antibody). a–d: × 32

methyl green-pyronin stain, the nuclei of plasma cells stained blue-green, while their cytoplasm stained selectively red due to its high content of ribonucleoprotein (Fig. 1b). In contrast, the cytoplasm of other hematopoietic cells in yolk sac vitelline vessels was not stained (Fig. 1c). Using periodic acid-Schiff reaction, plasma cells a deep red color while other cells were light pink one (Fig. 1a). A paranuclear halo due to large well-developed Golgi zone was also observed (Fig. 1d).

Indirect immunofluorescence study

At 10 and 11 days of gestation, a very weak immune reactivity for immunoglobulin isotypes IgA, IgG, and IgM was observed in the yolk sac. The densest immune staining was then obtained at 12 days of gestation. Positive cells were localized to the endodermal cell layer of the yolk sac as well as to hematopoietic cells inside the yolk sac vitelline vessels. Positive staining was also observed in the yolk sac cavity. At 14 days of gestation, the degree of reactivity and

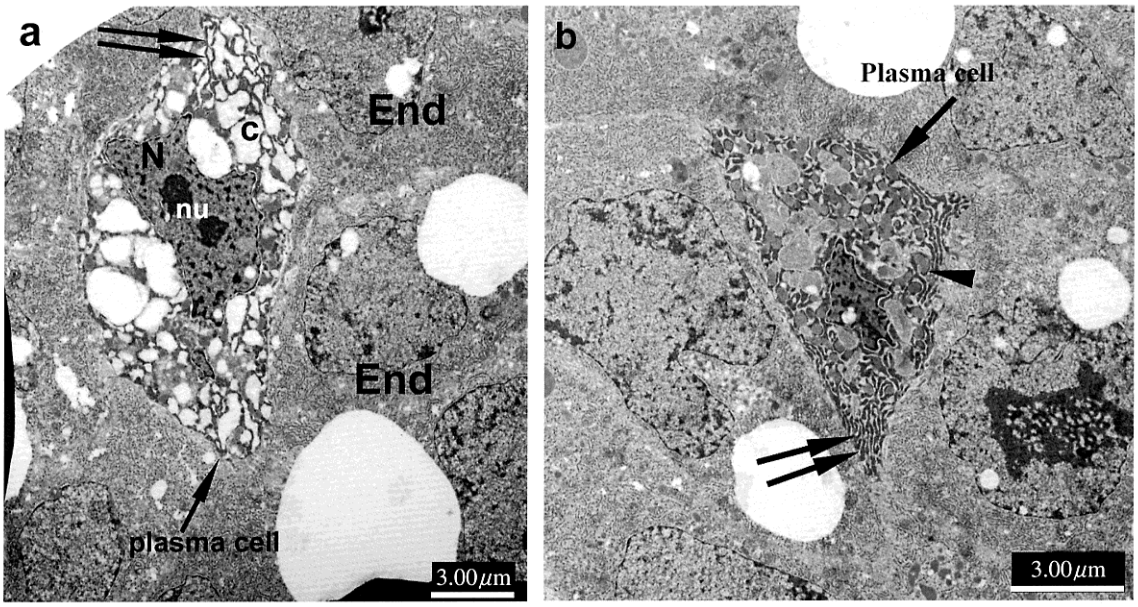

Fig. 3. Electron micrographs of a 12-day rat yolk sac endoderm. **a:** Plasma cell located inside the endoderm (↑). Note the well-packed sacks of endoplasmic reticulum (↑↑), and the dilated cisterns that show some flocculent materials (c). The nucleus (N) contains condensed heterochromatin and two nucleoli (nu). **b:** Other plasma cells of the same age inside the endoderm. Note the numerous mitochondria (▼) between cisterns of rough endoplasmic reticulum.

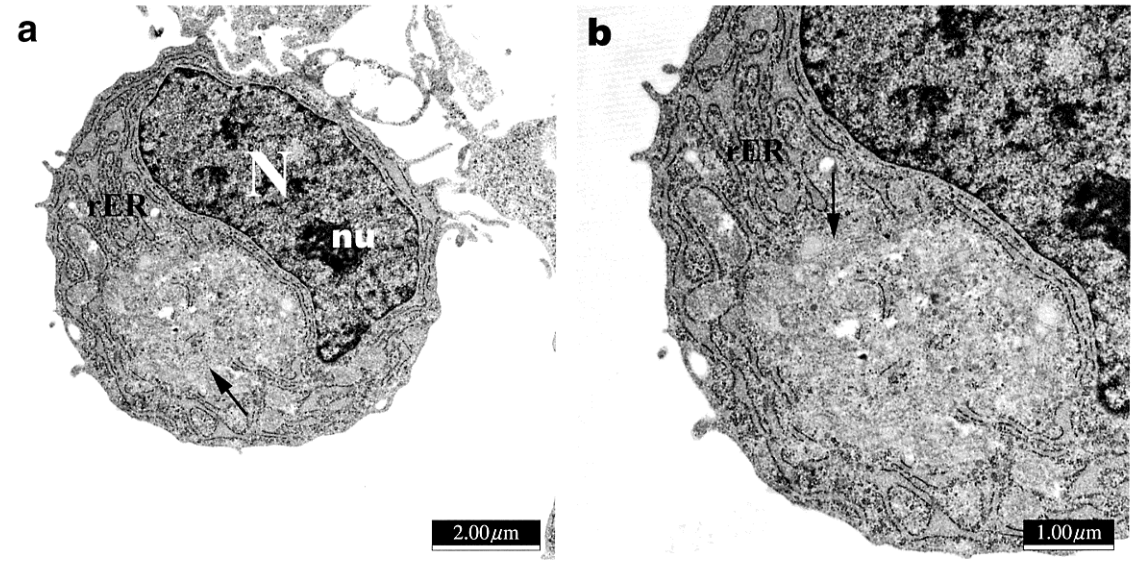

Fig. 4. Electron micrographs of in a mature plasma cell in a 12-day rat yolk sac. **a:** The cell reveals an eccentric nucleus (N), one nucleolus (nu), well-developed rough endoplasmic reticulum (rER), and a large Golgi zone at the perinuclear area showing lamellae (↑), granules, and flocculent material. All these are characteristic features of the mature plasma cell. **b:** A closer view of the Golgi zone of the cell in **a**.

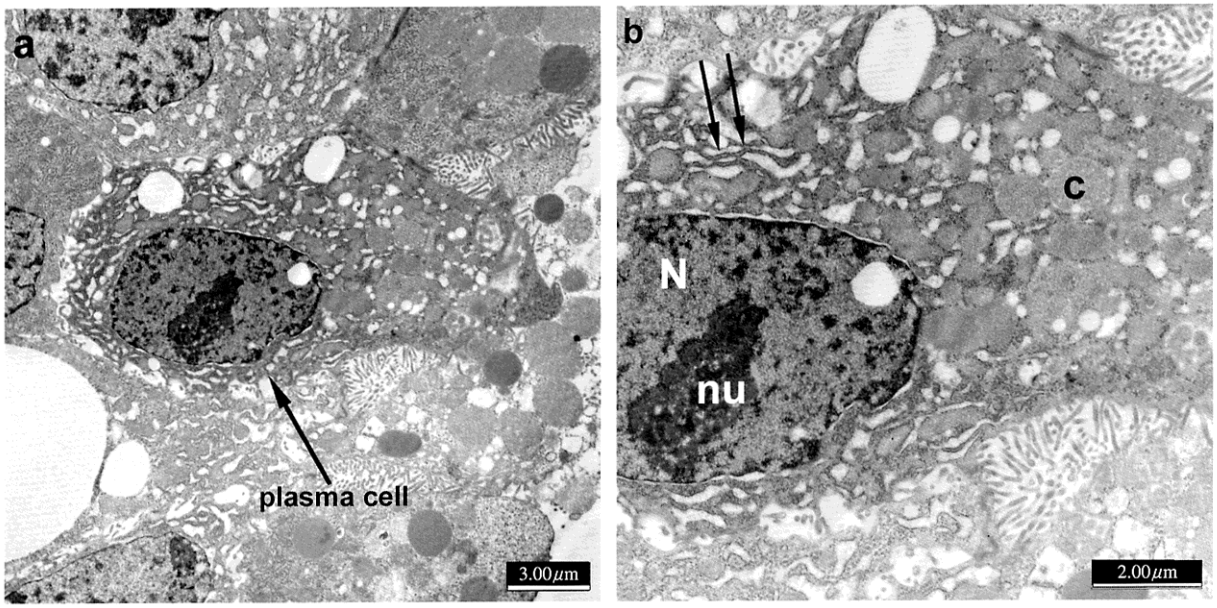

Fig. 5. Electron micrographs of a 13 day rat yolk sac. **a:** A plasma cell is situated within the yolk sac endoderm. **b:** High-power micrograph of the cell in **a**. The plasma cell retains a nucleus (N) with dense heterochromatin and one nucleolus (nu). Cisterns of rough endoplasmic reticulum (↑↑) are dilated and filled with flocculent material (c).

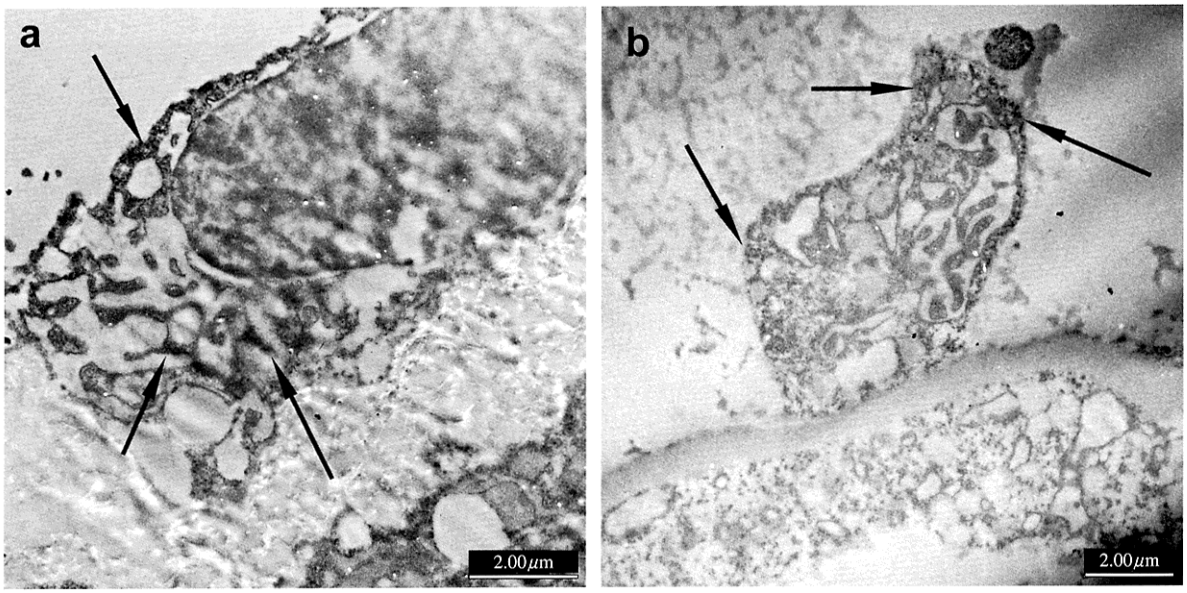

Fig. 6. Electron micrographs demonstrating IgA-positive reaction products in 12-day (**a**) and 13-day (**b**) yolk sac plasma cells. Note that the immune reaction products are localized at rough endoplasmic reticulum (↑).

the number of positive cells were much reduced as compared with these at 12 days. Figure 2a–d illustrates the results for IgG immune staining at 10–14 days of gestation.

Ultrastructural study

At 12 days of gestation, cells delineating the typical characteristics of plasma cells could be observed (Fig. 3a, b). The developing plasma cells revealed oval, round, or elongated configurations. Cell diameters were between 5.6–17.7 μm, and the mean nuclear diameter was around 5 μm. Plasma cells possessed nuclei with multiple dense areas of heterochromatin. The most conspicuous and characteristic feature of these cells was the well-developed saccules of rough endoplasmic reticulum. Parallel arrangement of the saccules of rough endoplasmic reticulum was seen to occupy most of the cytoplasm. Some of these saccules were dilated and revealed flocculent materials inside. The cytoplasm also contained some oval or round mitochondria found between the saccules of rough endoplasmic reticulum.

Mature plasma cells were characterized by the presence of a large well-developed paranuclear Golgi apparatus containing lamellae, vesicles, and granules (Fig. 4a, b). These cells were located in yolk sac vitelline vessels, while the vast majority of the developing plasma cells were localized inside the endodermal cell layer of the yolk sac.

At 13 days of gestation, numerous differentiating plasma cells were still observed (Fig. 5a, b). Secretion products could be seen inside cisterns of rough endoplasmic reticulum. However, plasma cells were only occasionally noticed in the yolk sac endoderm at 16 days of gestation.

Results of the immunoelectron cytochemistry for IgA antibody demonstrated immune products localized to the sacks of rough endoplasmic reticulum (the site of synthesis of the immunoglobulins) (Fig. 6a, b).

Discussion

The present study demonstrated for the first time the ontogeny of antibody producing plasma cells in rat yolk sac hematopoiesis. Substantial evidence for the presence of yolk sac plasma cells at sequential gestational days was obtained in this research as follows: 1) light microscopic examination of sections stained with standard histological stains showed cells having the morphology of plasma cells as early as 12 days of gestation; 2) the typical morphology of plasma cells at the ultrastructural level was found; 3) detection of the intracytoplasmic immunoglobulin production was done through conducting indirect immunofluorescence staining; 4) immune products for the anti-IgA antibody using the immunoelectron microscopic technique were demonstrated.

The characteristic morphological features of plasma cells at the light and electron microscopic levels have been described in details in the literature (Bloom and Fawcett, 1968; Tanaka and Goodman, 1972; Grossi *et al.*, 1988; Beutler *et al.*, 2001). In the present study, yolk sac plasma cells delineated all of these features even at 12 days of embryonic life.

It is well recognized that the yolk sac is the first site of haematopoiesis during mammalian ontogeny. However, the presence of mature haematopoietic cells of the lymphoid lineage was not previously demonstrated *in situ* in the yolk sac. A review of the literature shows only one study reporting on the development of plasma cells from the yolk sac (Weinberg and Ohlman, 1976). However, this study used the culture of 10.5 days mouse yolk sac cells into diffusion chambers.

The present study revealed, that the number of positive cells and the density of the immune staining reached a maximum at 12 days of gestation for the three immunoglobulin isotypes investigated. In addition, by electron microscopy, numerous differentiating plasma cells were observed in the yolk sacs at 12 and 13 days of gestation. However, from 14 days onwards, the number of plasma cells decreased which may be attributed to the decline of yolk sac hematopoiesis and the start of the hepatic hematopoiesis. These findings indicate that the peak of yolk sac plasma cell hematopoiesis occurs at 12–13 days of gestation. Similar observations have been reported on the peak of yolk sac macrophage hematopoiesis in the mouse and rat (Takahashi *et al.*, 1989; Takahashi and Naito, 1993). Interestingly, previous investigators (Tyan and Herzenberg, 1968; Moore and Metcalf, 1970) reported that spleen colony forming units are first detectable in the yolk sac of the 8-days mouse embryo, reaching their peak number at day 10 and falling to zero by day 13. These authors also found that 11-day-old yolk sac cells repopulated the marrow, spleen, thymus and peripheral lymphoid tissue of irradiated mice. These findings are in agreement with our present data.

Our data also coincide with previous investigations carried out on the yolk sac as a source of hematopoietic cells. Several *in vitro* studies (Liu and Auerbach, 1991; Huang and Auerbach, 1993; Cumano *et al.*, 1993; Palacios and Imhof, 1993; Yoder *et al.*, 1997; Auerbach *et al.*, 1998) have been conducted on yolk sac cell cultures to generate mature hematopoietic cells of the myeloid and lymphoid lineages. Previous electron microscopic studies of human (Hesseldahl and Larsen, 1971; Fukuda, 1973) and mouse (Haar and Ackerman, 1971) yolk sacs also demonstrated the existence of blast cells resembling large to medium-sized lymphocytes.

Plasma cells represent the late stages of differentiation of

B-lymphocytes. They are mature end-stage cells that have a relatively short life span. Antigenic stimulation of B cells results in a complex series of interactions leading to their activation, proliferation, and differentiation into plasma cells. Each B cell is programmed in its DNA to make an antibody molecule of a single specificity (Zubler, 1997; Duchosal, 1997). Plasma cells developed in the rat yolk sac may play a role in protecting the developing embryo against possible viral or bacterial in-utero infections (clinical or subclinical) by releasing their antibodies. Yolk sac plasma cells may also have a role in protecting the developing embryo from antigenic attacks by the maternal immune system when considering the embryo a successful allograft in maternal tissues, although the exact mechanisms involved in maternal tolerance are not fully understood.

Recognition of such immune competent cells in the early yolk sac signifies an additional important role of the yolk sac as a site that can effect a humoral immune response. Numerous reports have documented the development of mature macrophages in murine yolk sacs (Enzan, 1986; Takahashi *et al.*, 1989; Morris *et al.*, 1991; Takahashi and Naito, 1993; El-Nefiawy *et al.*, 2002). It is known that macrophages are one of the main antigen presenting cells (Grossi *et al.*, 1988). Beyond the scavenging function of macrophages, their exact roles in the yolk sac have not yet been delineated. Results of the present investigation also imply that a series of immunological stimulations and cellular differentiations occur in the yolk sac. However, the nature and the site of origin of the antigenic stimulus involved in this process were not confirmed in this study. Future research shall have to explore these questions.

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References

Auerbach R, Wang SJ, Yu D, Gilligan B, Lu LS: Role of endothelium in the control of mouse yolk sac stem cell differentiation. *Dev Comp Immunol* 22: 333-338 (1998).

Bazin H, Beckers A, Querinjean P: Three classes and four (sub)classes of rat immunoglobulins: IgM, IgA, IgE and IgG1, IgG2a, IgG2b, IgG2c. *Eur J Immunol* 4: 44-48 (1974).

Beutler E, Lichtman MA, Coller BS, Kipps TJ: Morphology of lymphocytes and plasma cells. In: *Wiliams Hematology,* 6th ed. McGraw- Hill, Medical Publishing Division, 2001.

Bloom W, Fawcett DW: *Textbook of histology.* 9th ed. Philadelphia, W.B. Saunders, 1968.

Chayen A, Parkhouse RM: Preparation and properties of a cytotoxic monoclonal rat anti-mouse Thy-1 antibody. *J Immunol Methods* 49: 17-23 (1982).

Cumano A, Furlonger C, Paige CJ: Differentiation and characterization of B-cell precursors detected in the yolk sac and embryo body of embryos beginning at the 10- to 12-somite stage. *Proc Natl Acad Sci USA* 90: 6429-6433 (1993).

Duchosal MA: B-cell development and differentiation. *Semin Hematol* 34 (Suppl 1): 2-12 (1997).

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* 80: 441-447 (2002).

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 in Press).

Enzan H: Electron microscopic studies of macrophages in early human yolk sacs. *Acta Pathol Jpn* 36: 49-64 (1986).

Fukuda T: Fetal hemopoiesis. I. Electron microscopic studies on human yolk sac hemopoiesis. *Virchows Arch B Cell Pathol* 14: 197-213 (1973).

Gathings WE, Lawton AR, Cooper MD: Immunofluorescent studies of the development of pre-B cells, B-lymphocytes and immunoglobulin isotype diversity in humans. *Eur J Immunol* 7: 804-810 (1977).

Grossi CE, Ferrarini M, Greaves MF: Normal lymphocytes In: *Atlas of blood cells.* (Zucker-Franklin D, Graves MF, Grossi CE, Marmont AM ed) Edi Ermes, Milano, 1988 (p.383-443).

Haar JL, Ackerman GA: A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse. *Anat Rec* 170: 199-223 (1971).

Hardy RR, Li YS, Allman D, Asano M, Gui M, Hayakawa K: B-cell commitment, development and selection. *Immunol Rev* 175: 23-32 (2000).

Hesseldahl H, Larsen JF: Hemopoiesis and blood vessels in human yolk. *Acta Anat* 78: 274-278 (1971).

Huang H, Auerbach R: Identification and characterization of hematopoietic stem cells from the yolk sac of the early mouse embryo. *Proc Natl Acad Sci USA* 90: 10110-10114 (1993).

Liu CP, Auerbach R: Ontogeny of murine T cells: thymus-regulated development of T cell receptor-bearing cells derived from embryonic yolk sac. *Eur J Immunol* 21: 1849-1855 (1991).

Lu LS, Wang SJ, Auerbach R: In vitro and in vivo differentiation into B cells, T cells, and myeloid cells of primitive yolk sac hematopoietic precursor cells expanded > 100-fold by coculture with a clonal yolk sac endothelial cell line. *Proc Natl Acad Sci USA* 93: 14782-14787 (1996).

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* 18: 279-296 (1970).

Morris L, Graham CF, Gordon S: Macrophages in haemopoietic and other tissues of the developing mouse detected by monoclonal antibody F4/80. *Development* 112: 517- 526 (1991).

Palacios R, Imhof BA: At day 8-8.5 of mouse development the yolk sac, not the embryo proper, has lymphoid precursor potential in vivo and in vitro. *Proc Natl Acad Sci USA* 90: 6581-6585 (1993).

Rugh R: *The mouse: Its reproduction and development.* Burgess Publ Co., Minneapolis, 1968.

Takada K, Aiyama S, Ikeda R: Morphological and histological changes in the secretory granules of mucous cells in the early postnatal mouse parotid gland. *Arch Histol Cytol* 64: 259-266 (2001).

Takahashi K, Naito M: Development, differentiation, and proliferation of macrophages in the rat yolk sac. *Tissue Cell* 25: 351-362 (1993).

Takahashi K, Yamamura F, Naito M: Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: A light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. *J Leukoc Biol* 45: 87-96 (1989).

Tanaka Y, Goodman JR: *Electron microscopy of human blood cells.* Harper and Row Publishers, New York, 1972.

Tyan ML, Herzenberg LA: Studies on the ontogeny of the mouse immune system. II. Immunoglobulin-producing cells. *J Immunol* 101: 446-450 (1968).

Voller A, Bidwell DE, Bartlett A: Enzyme immumoassays in diagnostic medicine. *Bull World Health Organ* 53: 55-56 (1976).

Weinberg SR, Ohlman F Jr: Growth of mouse yolk sac cells cultured in vivo. *Br J Haematol* 32: 543-555 (1976).

Yoder MC, Papaioannou VE, Breitfeld PP, Williams DA: Murine yolk sac endoderm-and mesoderm-derived cell lines support in vitro growth and differentiation of hematopoietic cells. *Blood* 83: 2436-2443 (1994).

Yoder MC, King B, Hiatt K, Williams DA: Murine embryonic yolk sac cells promote in vitro proliferation of bone marrow high proliferative potential colony-forming cells. *Blood* 86: 1322-1330 (1995).

Yoder MC, Hiatt K, Mukherjee P: In vivo repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 postcoitus. *Proc Natl Acad Sci USA* 94: 6776-6780 (1997).

Zubler RH: Key differentiation steps in normal B cells and in myeloma Cells. *Semin Hematol* 34 (Suppl 1): 13-22 (1997).