Erythropoiesis and Lymphopoiesis in the Chick Yolk-Sac-Embryo Chimeras: Contribution of Yolk Sac and Intraembryonic Stem Cells

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Lymphocyte development and ontogenetic changes in erythroïd cells have been studied in chick–chick yolk-sac–embryo chimeras differing at the B locus antigens. Erythroïd cells derived from the yolk sac or from the intraembryonic mesenchyme were demonstrated by indirect immunofluorescence in the peripheral blood of these allogeneic chimeras. At 7 days of incubation, yolk-sacerythroid cells have been studied in chick-chick yolk-sac-embryo chimeras. After hatching, no yolk-sac-derived erythroïd cells were detected on bursa cells using a triple layer immunofluorescence system. These results obtained from the allogeneic chimeras indicate that the early chicken yolk sac produces only transiently erythroid stem cells, while intraembryonic stem cells are involved in the production of definitive erythroïd cells as well as of lymphocytes, both of T and B cells.

**Vertebrate Embryos** of different species show developmental changes in their erythropoiesis.1,2 The early embryo elaborates a primitive red cell population, which is succeeded by definitive red cells maturing further into adult type erythroïd cells. The chicken embryo yolk sac produces primitive erythroïd cells containing early embryonic hemoglobins. Their place in the circulation is taken after day 13 of incubation by the second and permanent red cell series carrying the adult hemoglobin types.1,2 The cellular origin of the precursors of the definitive red cells has been unknown. Recently their origin and the timing of the appearance has been studied in quail–chick chimeras.3,4 Using quail embryos grafted in ovo on a chick yolk sac, it has been demonstrated that stem cells formed within the embryo replace yolk sac stem cells in their erythropoietic function.3,4 The hemoglobin switch has been investigated in the two populations of red cells, i.e. quail, intraembryonic-derived, and chick, yolk-sac-derived. Both types of stem cells were observed to have similar potentialities in erythropoiesis; the hemoglobin pattern was evolved rather by a time program than the anatomical origin of erythroïd stem cells or the site of red cell production.4 However, if not qualitatively, yolk sac and intraembryonic stem cell contribution to erythropoiesis is quantitatively different. The yolk sac stem cells give rise to all or most primitive erythroïd cells and to declining proportions of definitive erythroïd cells, while the intraembryonic stem cells give rise to definitive erythroïd cells in an increasing number and only to a minimal amount of primitive erythroïd cells.

However, the period during which yolk sac stem cells give off an erythroid progeny has not been determined precisely. In the xenogeneic chimeras, the replacement of yolk-sac-derived by intraembryonic-derived red cells is irregular, rapid in some chimeras, and slow in others. Furthermore, the chimeras usually do not develop beyond day 13 of incubation. Thus, it has not been possible to determine whether yolk sac stem cells participate to the seeding of the permanent stem cell reserve destined to ensure erythropoiesis. Although the thymus and bursa of quail–chick chimeras were always entirely populated by intraembryonic hemopoietic cells, the spleen on the other hand harbored small and seemingly transient yolk-sac-derived cell populations.5

These problems can be approached using chick–chick yolk-sac–embryo chimeras capable of hatching and developing into adulthood.6,7 Studies on these chimeras, using sex chromosome analysis of specifically stimulated T and B lymphocytes and serum IgG allotype determination, have confirmed the early intraembryonic origin of stem cells for the lymphoid system.6,8 In the present study, chick–chick yolk-sac–embryo chimeras have been used to analyze the developmental shift in erythropoiesis from yolk sac stem cells to intraembryonic stem cells. For this purpose, alloantigens of the chicken major histocompatibility locus (B complex) have been used as markers to trace the origin of cells in allogeneically constructed chimeras. In the chicken, the B complex is considered to be analogous to the MHC of mammals.

In the three-locus model of chicken B complex, B-F

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determines the histocompatibility antigens on leukocytes and erythrocytes, B-L determines an antigen present on B lymphocytes, and B-G determines an antigen unique to erythrocytes.\textsuperscript{9,10} Chicken Ia-like alloantigens (products of the B-L locus) are by cellular expression and molecular weight analogous to Ia antigens of mammalian species.\textsuperscript{11,12} In the present study, anti-Ia alloantisera were used to detect chicken B lymphocytes, and anti-B antisera containing antibodies against alloantigens coded by B-F and B-G loci were used to detect histocompatibility antigens on erythrocytes and embryonic thymocytes in allogeneic chick chimeras.

**MATERIALS AND METHODS**

**Chick Embryos**

White Leghorn line V and P chick embryos from the colonies at the Department of Medical Microbiology, Turku University, were used. These lines are homozygous at the major histocompatibility locus; line V is of genotype $B^V B^V (Ia^V Ia^V)$ and line P of genotype $B^P B^P (Ia^P Ia^P)$. The fertile eggs were incubated in a Funki type 1 automatic egg incubator at 37°C and 65% relative humidity.

**Allogeneic Yolk-Sac-Embryo Chimeras**

In the developing avian blastoderm, two defined zones are present (Fig. 1); the central area is destined to produce the embryo, amnion, and allantois, and the peripheral vascular area gives rise to the yolk sac. The central area of line P ($B^P B^P$) chick blastoderm was grafted onto the age-matched line V ($B^V B^V$) yolk sac, replacing the equivalent area removed, according to a technique as described previously.\textsuperscript{13} The embryos used were 36–42 hr of incubation (8–20 somites) at the time of grafting.

**Detection of Erythroid Cells With Indirect Immunofluorescence**

The erythroid cells from the chimeras were collected at appropriate intervals from the peripheral blood by venipuncture and washed two times with Dulbecco’s phosphate-buffered saline (PBS; Flow Laboratories, Irvine, Scotland). Each chimera was used only one time. To detect B blood group antigens on erythroid cells, 20 $\mu$l of a cell suspension (1–5 x $10^7$ cells/ml) was mixed with 20 $\mu$l of anti-B\textsuperscript{2} or anti-B\textsuperscript{15} (diluted 1:50 with Dulbecco’s PBS) and incubated 30 min at 4°C. Anti-B\textsuperscript{2} and anti-B\textsuperscript{15} antisera were produced by cross-immunizations of line P and V adult male chickens using weekly intravenous injections of adult peripheral blood erythrocytes for 5 wk. Anti sera were absorbed using pools of appropriate erythrocytes at 40°C for 2 hr and +4°C overnight. Normal chicken serum served as a control. After 2 washes with Dulbecco’s PBS, 20 $\mu$l of FITC-conjugated rabbit anti-chicken IgG (Miles-Yeda, Rehovot, Israel), diluted 1:20, was added and the cells were incubated for 30 min at 4°C. Thereafter the cells were washed twice and mounted in 1:5 glycerol-PBS and examined under a Leitz Ploemopak ultraviolet microscope. At least 400 cells were counted.

**Detection of Ia-Like Antigens by Triple Layer Immunofluorescence**

Peripheral blood lymphocytes were separated using slow spin centrifugation, 62 g, 30 min at room temperature and bursa cells were obtained as described previously.\textsuperscript{4} A triple layer immunofluorescence staining technique described by Galton and Ivanyi was used.\textsuperscript{14} In brief, 20 $\mu$l of cell suspension (1–5 x $10^7$ cells/ml) was incubated with Ia antisera diluted 1:20 for 30 min at 4°C; anti-Ia\textsuperscript{2} and anti-Ia\textsuperscript{15} used were prepared as described earlier.\textsuperscript{15} Details of immunization schedules and methods used to absorb these antisera with erythrocytes, specificity, and sensitivity of these anti-Ia antisera have previously been described.\textsuperscript{11,12} After 2 washes with Dulbecco’s PBS at 4°C, 20 $\mu$l of rabbit antiserum to chicken Fc-IgG, diluted 1:400, was added and the cells were incubated for 30 min at 4°C. After washing, the supernatant was removed and 30 $\mu$l of FITC-conjugated goat anti-rabbit IgG (Miles-Yeda, Rehovot, Israel), diluted 1:20, was added and the cells were incubated 30 min at 4°C. Thereafter, the cells were washed twice and mounted, and counted as described above. Normal chicken serum (NCS) diluted 1:20 was used as a control in each test.

**RESULTS**

Figure 1 describes the proportions of B\textsuperscript{2} and B\textsuperscript{15} positive erythroid cells in the peripheral blood of allogeneic chimeras at different stages of development. At 7 days of incubation, most of the red cells (80.5 ± 17.1; mean ± SD) are of type B\textsuperscript{15}, i.e. differentiated from yolk-sac-derived cells. Later, the proportion of embryo-derived B\textsuperscript{2}-positive erythrocytes increases rapidly so that they make up the majority of blood erythrocytes (93.2 ± 4.6) in 17–18-day-old chimeras. At the same time, erythrocytes taken from embryonic spleen and bone marrow are mostly embryo derived, expressing B\textsuperscript{2} surface antigens (Table 1). It should be noted, however, that a low percentage of yolk-sac-derived B\textsuperscript{15}-positive red cells is still found in these organs at 17–18 days of incubation.

B locus antigens were also studied on embryonic thymus cells and Ia antigens on bursa cells. At the stages investigated, about one-fourth of the thymus cells expressed embryo-type antigens (B\textsuperscript{2}), the rest were null cells; yolk-sac-type (B\textsuperscript{15}) cells did not exceed the values obtained with the normal chicken serum.

Fig. 1. Evolution of red cell populations in peripheral blood of yolk-sac-embryo chimeras constructed at 2 days of incubation of B\textsuperscript{2}B\textsuperscript{15} yolk sacs and B\textsuperscript{2}B\textsuperscript{15} embryos. Frequency of cells reacting with anti-B\textsuperscript{2} or anti-B\textsuperscript{15} is given.
used as control (Table 2). Bursa cells also expressed Ia antigens of embryonic origin (Ia$^{i}$), whereas values for the yolk-sac-type (Ia$^{5}$) cells did not exceed the control values (Table 3). The values obtained for bursa and thymus cells of these chimeras using anti-Ia and anti-B antisera, respectively, are within the range obtained for normal, unoperated, age-matched embryos of both chicken lines. The results about the specificity of different antisera used is this study are demonstrated in Table 4.

Two of 23 histoincompatible yolk-sac–embryo chimeras hatched, and their peripheral blood erythrocytes were analyzed at 2 and 4 wk of age. No cellular chimerism was observed, either according to the B antigens expressed on erythrocytes (Fig. 1) or to Ia antigens expressed on peripheral blood lymphocytes (Table 3). All erythrocytes bore embryodervied antigens (B$^i$), so did all lymphocytes (Ia$^5$).

**DISCUSSION**

The erythropoietic system in the chick embryo provides a fascinating model for studies on erythroid cell populations during ontogenesis. In the present study, the replacement of yolk-sac-derived erythrocytes by embryo-derived erythrocytes was analyzed in homospecific chimeras composed of an embryo and yolk sac differing by the major histocompatibility locus (B complex) antigens. The replacement pattern appeared consistent and rapid. At 10 days of incubation the chimeras retained only about 30% of red cells derived from yolk sac stem cells. This finding should be compared to the development shift in erythropoiesis previously established in heterospecific chimeras, i.e., in quail embryos with chick yolk sac.35 In these chimeras the replacement of chick by quail red cells in individual embryos obeyed an unpredictable time course. This was probably due to species differences, such as the larger size of chick yolk sac or the quicker developmental rhythm of the quail. Thus, the analysis of homospecific chimeras not only confirms the primordial role of intraembryonic stem cells in definitive erythropoiesis but also discloses a calendar of...
replacement probably very similar to that in normal development (Fig. 1). Furthermore, it was demonstrated that yolk sac stem cells become entirely extinct; in other words none of them participate in seeding the permanent erythropoietic pool.

The study of histocompatibility marked cell populations in hemopoietic organs further confirms that yolk sac stem cells colonize transiently some organs only, as was shown by means of the quail–chick nuclear marker. In both types of chimeras, only a small contribution of yolk sac lineage cells is observed for a short period in the bone marrow and spleen, though it is possible that yolk-sac-derived erythroid cells observed in these organs are circulatory erythrocytes and thus do not represent any true colonization. Both chimeric models demonstrate that the thymus and bursa of Fabricius are not colonized by yolk sac stem cells, although in the present study it cannot be totally excluded due to the presence of null cells.

Interference by the rejection mechanism with the cellular evolution observed in this study can be excluded. The period when erythropoiesis shifts most rapidly from yolk sac to intraembryonic stem cells, i.e., 7–10 days of incubation, precedes the ontogeny of T-cell-mediated immune functions in the chick embryo.

Colonization of the thymus and bursa by intraembryonic cells and their increasing involvement in erythropoiesis indicate that they become active in the second wave of embryonic hemopoiesis. Hemopoiesis in normal chick embryo up to 5 days of incubation is present mostly in the yolk sac, producing mainly primitive erythrocytes. However, on the basis of hemoglobin switching in quail–chick chimeras and of in vitro cultures of “deembryonated” chick blastoderms, the yolk-sac-derived cells give also a small progeny of definitive erythrocytes. It has been demonstrated that yolk sac stem cells at head-fold stage of blastoderms are capable of colonizing the thymic rudiment. On these bases we can postulate existence of two categories of stem cells with equal qualitative potentiality. Yolk sac stem cells produce early, transient erythroid differentiation, while most intraembryonic stem cells remain unactivated until a later phase leading to definitive erythropoiesis and lymphopoiesis. Finally, it is interesting to compare the kinetics of replacement of yolk-sac-derived erythrocytes by embryo-derived erythrocytes with the quantitative evolution of primitive versus definitive erythrocytes as described by Bruns and Ingram. At all stages of development, this comparison reveals a close parallelism between our data and those of Bruns and Ingram. These findings indicate that since the first definitive erythrocytes are produced, the participation of yolk sac stem cells to erythropoiesis becomes extremely small.

Diffuse hemopoiesis occurs within the early chick embryo during days 6–10 of incubation. The extravascular hemocytoblasts form hemopoietic foci in the mesenchyme around the heart and aorta, in the mesonephros and in the mesentery of 6–8-day-old chick embryo. It is likely that some of the cells involved in these hemopoietic processes colonize the organ rudiments. In fact, we have demonstrated that cells obtained from 7-day intraembryonic chick mesenchyme are capable of developing to functional T and B lymphocytes after transfer into irradiated chick embryos or into cyclophosphamide-treated immunodeficient chick embryos.

Our results do not comprise the earlier events of hemopoietic differentiation and possible cell exchange between the embryo and the yolk sac prior to the grafting. At least no vascular route exists for the cell migration from the yolk sac to the embryo before or at the time of grafting.

In conclusion, this study on allogeneic chick–chick chimeras demonstrates that intraembryonic stem cells are the only progenitors of lymphocytes and of permanent erythropoiesis. Yolk sac stem cells give off primitive erythrocytes and may participate shortly in the production of the first definitive erythrocytes.

REFERENCES

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