Ontogeny and tissue distribution of leukocyte-common antigen bearing cells during early development of *Xenopus laevis*

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Summary

To analyze the ontogenic emergence of leukocytes during early development, a mouse monoclonal antibody (IgG1), designated as XL-1, was produced against the peritoneal macrophages of adult *Xenopus laevis*. The XL-1 determinant was expressed on all types of leukocytes, including lymphocytes, granulocytes, thrombocytes and macrophages, but not on erythrocytes of either larvae or adults. Immunohistochemical observations of the hemopoietic organs revealed that the XL-1+ cells with granulocyte and/or macrophage morphology appeared at st.36-37 in the liver, at st.44-45 in the mesonephric and the thymus rudiments, and at st.47 in the spleen. The XL-1 determinant was expressed on the precursor cells of T lymphocytes in the thymus rudiments at st.46-47, on the pre-B cells in the liver rudiments at st.47, and on lymphocytes in the spleen at st.48-49. A few XL-1+ cells were present in the ventral blood island of the st.35/36 embryos, where differentiating erythrocytes had predominated since st.28. XL-1+ cells with a macrophage-like morphology were found in several locations of the mesenchyme in the st.32 embryos, before the establishment of vascularization at st.33/34 and far earlier than the emergence of lymphocytes.

Key words: leukocyte ontogeny, *Xenopus*, monoclonal antibody, leukocyte-common antigen, XL-1.

Introduction

It is generally accepted that various types of blood cells in vertebrates are derived from a common, pluripotent stem cell capable of differentiating into discrete lineages under the control of specific microenvironments. The initial population of hemopoietic stem cells is believed to arise in the yolk sac during embryogenesis (Metcalf and Moore, 1971), although more recent studies of birds emphasize the intraembryonic origin of lymphocytes, monocytes and definitive erythrocytes (Dieterlen-Lievre, 1975). Hemopoietic cell lineage studies both in *vitro* and *in vivo* on mammals and birds have been made with a variety of antibodies detecting the surface marker antigens specific to each type and/or subset of hemopoietic cells (Shaw, 1987; Holmes and Morse, 1988). No specific markers have been developed, however, which are both common to, and restricted to, leukocyte-series cells. Thus, the leukocyte-common antigen (L-CA) is expressed on T- and B-lymphocytes, thymocytes, granulocytes and macrophages, together with erythroblastic cells, but not on mature erythrocytes (reviewed by Thomas and Lefrancois, 1988).

Recent experiments employing grafts of cytogenetically labeled tissues in *Xenopus laevis* have established that stem cells of early larval erythrocytes and lymphocytes are localized in the ventral blood island (VBI) mesoderm, while those of their more advanced larval and adult counterparts gather in the dorsolateral plate mesoderm of tailbud embryos (Maeno et al. 1985a; 1985b; Kau and Turpen, 1983; Smith et al. 1989; Flajnik et al. 1984). Of these hemopoietic cells, erythrocytes undergo differentiation in the VBI (Mangia et al. 1970), whereas B- and T-lymphocytes start to express their differentiation markers IgM (Hadji-Azimi et al. 1982) and XT-1 antigen (Nagata, 1985, 1986) after migration to the rudiments of liver and thymus, respectively. Compared with what is known of lymphocytes, however, very few reports have been published about the embryonic origin, and the ontogenic emergence, of non-lymphoid leukocytes such as macrophages, granulocytes and thrombocytes; studies based on the classical criteria of cell identification are an exception (Manning and Horton, 1969, 1982).

The present study was intended to identify the differentiation markers for leukocytes in *X. laevis*, as clues for studying the ontogenic emergence of leukocytes in this animal. We report here a monoclonal antibody (mAb) named XL-1, which recognizes all *Xenopus* leukocyte-series cells but not those of the erythrocyte-series. Our immunohistochemical observations employing this mAb demonstrate that the cells that express the XL-1 determinant differentiate in the mesenchyme of embryos chronologically far earlier than has been previously supposed.
Materials and methods

Materials
The animals used in this study were outbred (HD-group) and MHC-homozygous J strain individuals (Tochinai and Katagiri, 1975) of Xenopus laevis. Embryos and larvae were reared at 23°C, and were staged according to the Normal Table of Nieuwkoop and Faber (1967).

Production of monoclonal and polyclonal antibodies
Peritoneal macrophages were collected from the J strain X. laevis by the method described previously (Sekizawa et al., 1984), and were suspended in phosphate-buffered saline (PBS). The viable macrophages (each 0.2-1.0×10⁶ cells) were injected intraperitoneally into BALB/c mice, five times at two-weekly intervals. Four days after the final injection, the spleen cells were removed and fused with P3-X63-Ag8.653 myeloma cells, according to the principle devised by Kohler and Milstein (1975), with minor modifications. Undiluted hybridoma supernatants were screened for the identification of antibodies by immunofluorescence on cells attached to the Terasaki plate or tissue sections as described below. The hybridoma line used in this study was cloned three times by the limiting dilution technique. The anti-larval hemoglobin monoclonal antibody (mAb) was provided by T. Enami of our laboratory. The polyclonal antibodies to Xenopus IgM were raised in a rabbit by injecting the periodate-treated purified IgM, according to the procedure devised by Mattes and Steiner (1978) and Hadji-Azimi et al. (1982).

Immunohistochemical staining
For screening of antibodies produced by hybridomas, various cells obtained from adult spleen, thymus, peritoneum and peripheral blood were attached to the poly-L-lysine-coated Terasaki plates by centrifugation. The cells were fixed either with 0.25% glutaraldehyde in PBS (10 min), absolute methanol (5 min) or 4% paraformaldehyde in PBS (30 min) at 4°C. Adult white blood cells were collected from the uppermost layer (buffy coat) of the centrifuged peripheral blood, smeared on glass slides and fixed with absolute methanol. Larval peripheral blood was smeared directly onto glass slides and fixed with absolute methanol. For subsequent study of the distribution of leukocytes, 10 individuals at various developmental stages were fixed with an ethanol and acetic acid mixture (1:3) (60 min), embedded in Tissue Prep, and serially sectioned at 7 µm. Five individuals each of embryos or larvae for whole-mount preparation were fixed with 4% paraformaldehyde in PBS overnight at 4°C.

For indirect immunofluorescent study, fixed cells, sectioned tissues or fixed embryos and larvae were incubated with PBS supplemented with 10% fetal calf serum (10% FCS–PBS) for blocking of nonspecific protein binding, and were incubated overnight at 4°C with undiluted hybridoma culture supernatants or a 1:1000 diluted rabbit antiserum to Xenopus IgM, followed by incubation with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse Ig antibodies or fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ig antibodies. Control samples were incubated either with 10% FCS–PBS, myeloma culture media or a diluted normal rabbit serum instead of mAbs or antisera, followed by incubation with the TRITC- or FITC-conjugated reagent. All controls were negative. Observations were performed with an epifluorescence microscope.

Electron microscopy
St.46–47 larvae were fixed with 4% paraformaldehyde in 0.1 M-phosphate buffer (pH 7.2), dehydrated in ethanol and acetone, and were embedded in LR-Gold (TAAB Lab., Berkshire) according to the procedure devised by Newman et al. (1982). Ultrathin sections were made with glass knives on a Porter-Blum MT-1 ultramicrotome, and were made to react for 2 h each at room temperature with mAb and the goat anti-mouse IgG antibodies coupled to colloidal gold particles (20 nm, E-Y Labs Inc.). Control samples incubated with only the colloidal gold-conjugated reagent showed distribution of an extremely few gold particles in nonspecific fashion. Along with these series, some specimens were processed by the ordinary method of glutaraldehyde–osmium tetroxide fixation and Epon embedding. Ultrathin sections were stained with uranyl acetate and lead citrate, and were observed with a JEOL JEM-100S electron microscope.

Results
Expression of the XL-1 determinant in leukocytes
The monoclonal antibody (mAb) named XL-1 that was used in the present study reacted with all the leukocyte-series cells including lymphocytes, granulocytes, thrombocytes and macrophages (cf. Hadji-Azimi et al. 1987) from both adult and larval Xenopus laevis but not with erythrocytes (Figs 1, 2). The antibody subclass was determined to be IgG1. The reactivity of leukocytes with XL-1 on the indirect immunofluorescence technique did not differ significantly according to the fixatives used, either by smear or sectioning of the materials. The cytoplasm of leukocytes showed granular staining with various degrees of brightness, although large refractile cytoplasmic granules of eosinophils did not stain. Exactly the same staining pattern was obtained with viable leukocytes. Upon incubation of viable lymphocytes under conditions without NaN₃, a capping of the fluorescence was evident, suggesting that the XL-1 determinant is also localized on the surface of leukocytes. The leukocytes from outbred animals were also stained with XL-1 as strongly as those from inbred J frogs. Adult and larval leukocytes from Xenopus borealis were stained in a similar pattern, although more weakly than their X. laevis counterpart.
Western blot analyses revealed that XL-1 determinant comprises several molecules ranging from 100 to $300 \times 10^3 \text{Mr}$, showing both unique and sharing components with each other in splenocytes and thymocytes (Fig. 3). No reactivity was seen at all in erythrocyte lysates. Although no other tissue components showed reactivity against XL-1, the XL-1 determinant was found also in the serum expressing some unique electrophoretic mobilities (Fig. 3). Presumably reflecting this aberrant reactivity, XL-1 showed occasional, but not consistent, immunohistochemical localization in and along the epithelial lining of nephric ducts and along the inner surface of blood vessels in advanced larvae. Except these particular organs, the XL-1 determinant was expressed only on the leukocyte-series cells in *Xenopus*.

**Ontogenic emergence of XL-1$^+$ cells in early hemopoietic and lymphoid organs**

The ontogenic emergence and distribution of XL-1$^+$ cells were examined on sections of various hemopoietic tissues or whole-mount preparations of embryos and larvae. The XL-1$^+$ cells first appeared in the liver rudiments at st.36–37, following the establishment of a blood stream, scattered both in the parenchyma and sinusoids. The positive cells increased in number thereafter and, from st.47 on, some of them could be seen in the perihepatic layer as if lining the organ (Fig. 4).
During the succeeding stages, the positive cells were preferentially localized as clusters in the subcapsular area as well as in the blood vessels. The pre-B cells that possessed the cytoplasmic IgM but not any surface Ig (clg⁺ sIg⁻) were first detectable at st.47, but their localization was not identical with that found for the XL-1⁺ cells described above. Double immunofluorescence staining using antisera to IgM and XL-1 during these stages showed that some of these pre-B cells were also XL-1⁺ although eventually all B cells (clg⁺ sIg⁺) were XL-1⁺ in the later stages. Most of the positive cells at st.47 were identified as non-lymphoid leukocytes such as granulocytes and macrophages. At this stage, larval hemoglobin (LHb)-positive cells (larval erythrocytes) were few in number in the perihepatic layer.

In the mesonephric rudiment, XL-1⁺ cells appeared first at st.44-45 in the area surrounded by the dorsal aorta, the vena cava and the Wolffian ducts (Fig. 5). By st.47 numerous XL-1⁺ cells had aggregated in this area, where they underwent extensive mitosis. Electron microscopy of the mesonephric primordia revealed that these XL-1⁺ cells were granulocytes and macrophages, as defined by their lobulated or bean-shaped nuclei, unique chromatin condensation pattern, numerous cytoplasmic granules, as well as by their distinct pseudopodia (Figs 6, 7). None of the XL-1⁺ cell aggregates in the mesonephros possessed IgM prior to st.49.

In the thymus rudiments, a small number of XL-1⁺ cells appeared for the first time at st.44-45. The positive cells were regarded as macrophage/monocyte-series cells as defined by their dendritic shape and a stronger reactivity than the lymphocyte-series cells described below. Two types of XL-1⁺ cells appeared in the thymus at st.46-47: the large, macrophage-like cells with brightly stained dendritic cytoplasm and the lymphoblasts with weakly stained dots in the thin cytoplasm (Fig. 8). At this stage, the latter cells tended to locate preferentially in the outer region of the thymus (future cortex). The reactivity of these cells increased gradually as they differentiated into lymphocytes, so that by st.49 virtually all the thymic lymphocytes were stained uniformly with XL-1. In the thymus of the more advanced stages, as Fig. 9 shows, all thymic lymphocytes and macrophage-like cells were positive, but epithelial cells and myoid cells were entirely negative.

The spleen rudiments possessed XL-1⁺ cells for the first time at st.47. Similar to the thymus rudiments at st.47, the positive cells in the spleen at st.48-49 consisted of large, irregularly shaped cells with a macrophage-like morphology and faintly stained lymphocytes (Fig. 10). At st.56 all the lymphocytes and large den-
dritic macrophage-like cells were stained well with XL-1 both in white and red pulps (Fig. 11), whereas the cytoplasm of LHb+ cells was always XL-1 negative. The occasional staining with XL-1 of intercellular spaces among erythrocytes in red pulp (Fig. 11) may be ascribable to the exudates from adjacent leukocytes and/or deposition of serum components which were positive to XL-1 (cf. also Fig. 3).

Occurrence of XL-1+ cells in the mesenchyme of embryos and early larvae

Previous studies have indicated that the major population of lymphocytes and erythrocytes in early larvae are derived from the mesodermal cells localized in the ventral blood island (VBI) of st.22 embryos (Maeno et al. 1985a; Smith et al. 1989). Immunofluorescence staining that employs anti-LHb mAb revealed that the LHb+ cells did occur exclusively in the VBI mesoderm as early as st.28. These LHb+ cells were the major constituents of the VBI region during st.31–36/37 (vascularization started in embryos at st.33/34). On the other hand, XL-1 did not react with these hemopoietic series cells in the VBI. Only a few XL-1+ cells could be found scattered along the inner surface of the epidermal cells in VBI at st.33/34. The flattened shape and pseudopodia of these XL-1+ cells differed from those found on the erythroid cells.

XL-1+ cells were found in early embryos outside the hemopoietic regions. From st.32 on the XL-1+ cells were detectable as isolated cells in the mesenchyme in such locations as beneath the epidermis, around the notochord or somites, and above the spinal cord. At the initial stages of their appearance during st.32–35/36, most XL-1+ cells still possessed yolk platelets, and were of either round or highly flattened shape with occasional prominent pseudopodia (Fig. 12C,D). The shape of their nuclei was also variable, either round, indented or lobulated. The number of these cells rose sharply during st.32–36/37, so that they were easily detectable in the whole-mount preparations at later stages as shown in Fig. 12. It is thus clear that, chronologically, these mesenchymal XL-1+ cells appear before the differentiation of any hemopoietic or lymphoid organs.

Discussion

The determinant detected by our monoclonal antibody (mAb) XL-1 is similar to the leukocyte-common antigen (L-CA) reported in mammals (reviewed by Thomas and Lefrancois, 1988) and birds (Houssaint et al. 1987) in that it is expressed on all leukocytes but not on mature erythrocytes. However, the XL-1 determinant differs from the L-CA that is also expressed on both immature erythroid cells and leukocyte-series cells. In fact, our present study shows that the XL-1 determinant is not expressed on any erythroid precursor cells in the embryonic ventral blood island (VBI), which is the amphibian counterpart of the avian and mammalian extraembryonic yolk sac. We can therefore regard XL-1 as a unique probe for studying the differentiation of leukocyte-series cells during embryonic and larval development. The L-CA reportedly comprises a family of surface glycoproteins with relative molecular masses between 180 and 240×10^3, whose expression is variable according to the cell types and stages of hemopoiesis. Similarly, our XL-1 determinant was detected as several different molecular mass entities in splenocytes and thymocytes. It should be mentioned that the XL-1 determinant is not exclusively specific to leukocytes, as observed in the adult serum showing electrophoretic mobilities different from those in leukocytes. Appar-
ently this reactivity is responsible for the occasional immunohistochemical localization along the inner wall of the blood vessels and intercellular spaces in splenic red pulps. In this sense, XL-1 also resembles the quail anti-MB1 mAb (Peault et al. 1983; Labastie et al. 1986), which reacts with endothelial cells and plasma components as well as with all mature leukocytes.

Another important issue is the determination of the stages when embryonic or larval lymphoid cells express the antigen(s) detected with XL-1. Previous studies by Nagata (1977) and Tochinai (1980) have demonstrated that lymphoid precursor cells immigrate into the thymus rudiment during the restricted period of st.42–45. The T-cell-specific determinant XT-1 is first expressed on thymocytes at st.48 (Nagata, 1986) when morphologically mature thymus lymphocytes appear for the first time. The present study shows that the earliest XL-1* cells in the thymus rudiments at st.44–45 are macrophage-like dendritic cells, and that the lymphoid-series cells with XL-1* can first be observed at st.46–47, followed by an increasing fluorescence intensity until st.48–49. This means that the XL-1 determinant is expressed on the lymphoid precursor (pre-T) cells at st.46–47. Similarly, the XL-1* cells first occurred in the liver at st.47 in the pre-B cells as defined by their coexpression of cytoplasmic IgM (cf. Hadji-Azimi et al. 1982). It should be noticed that these T- and B-lymphocytes in the primary lymphoid organs have emigrated from the VBI of tailbud embryos (Mafno et al. 1985a,b). In view of the present observations with XL-1, it is clear that lymphoid precursor cells, although ‘committed’ to some extent during their migration, start to express the XL-1 determinant as a result of residence in the microenvironments provided by the thymus or the liver rudiments.

In Xenopus larvae, the liver and the mesonephros are
known to be the major hematopoietic organs. Manning and Horton (1969, 1982) have shown that the granulo-
poiesis and lymphopoiesis in these organs initiate from
st.49–50 on. The present study reveals that prior to the
initiation of liver leukopoiesis at st.47, a cluster of XL-
1+ cells, defined electron microscopically as typical
granulocytes and macrophages, are first detectable at
st.44–45 in the mesonephric primordium. Although a
few XL-1+ pre-B cells occur in the liver at st.47, as we
note above, most, if not all, perihepatic XL-1+ cells
during st.47–49 represent macrophages and granulo-
cytes as well. In comparison with the positioning of
their lymphoid precursors, the exact localization of
precursor cells for these non-lymphoid leukocytes in
early embryos has not been elucidated.

An intriguing finding derived from the present study
is that a fairly large number of the XL-1+ cells with
macrophage-like morphology were found in the mesen-
chyme at st.32 and in the blood vessels at st.36–37, prior
to the appearance of lymphocytes. Because it is not
until st.48 that Xenopus lymphoid immune responses
are activated (Horton and Manning, 1972; Kidder et al.
1973), the presence of these leukocytes draws attention
to their possible relevance as nonspecific defense mech-
isms required for the free-living early tadpoles. The
following findings may support this view: Turner (1969)
reported that pericardial and peritoneal free macro-
phages are the first line of defense against intra-
peritoneally injected particles in the st.48 larvae. Lehman
(1953) also observed the ingesting activities of macro-
phages in the transparent tail fin of the larvae whose
stages correspond roughly to st.45–49. Besides their
early occurrence in Xenopus, macrophages are report-
edly present in the liver of Rana pipiens at Shumway
Stage 22, equivalent to st.41 of X. laevis before the
onset of hemopoiesis (erythropoiesis, granulopoiesis
and lymphopoiesis; Turpen et al. 1979). The classical
observations by Metchnikoff (1893) and Clark and
Clark (1930) offered evidence for migratory and phago-
cytic macrophages in the connective tissue of the tail fin
of the axolotl, Hyla and Rana embryos immediately
after hatching. Although their functional characteriza-
tion is still insufficient, the present mesenchymal
XL-1+ cells found in early tadpoles most likely function
in the defense against pathogens. Compared with the
study devoted to lymphocytes and erythrocytes, not
much attention has hitherto been paid to the embryonic
origin of these phagocytic leukocytes. An explanation
for the origin of the extremely early-emerging mes-
enchymal XL-1+ cells may be that prior to the onset of
heart beating, the precursor cells emigrate from the
VBI to undergo subsequent differentiation into leuko-
cytes. Our experiments including grafting of labeled
VBI-mesodermal cells, however, have excluded this
emigration which could account for the occurrence of
many mesenchymal XL-1+ cells at st.32–35/36 (Ohi-
nata et al. in preparation). It is thus reasonable to
postulate a population of XL-1+ cells that do not share

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Fig. 12. Immunofluorescence micrographs of the whole-mount preparations of st.35/36 embryos, showing distribution of
cells reactive to XL-1 (A,C,D) and anti-larval hemoglobin monoclonal antibody (B). Lateral views, anterior to the right.
Arrowheads in B indicate the ventral blood island region where at this stage the cells producing larval hemoglobin are
boundedly distributed. C and D, higher magnification views of XL-1+ cells in the areas shown in Fig. 12A (C, posterior box;
D, anterior box). ey, eye. Bars (A,B), 0.5 mm; Bars (C,D), 40 μm.
the embryonic origin with other hemopoietic cells. Experiments designed to give a definite answer to this question are currently under way.

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References


