

# Basement membrane heterogeneity during chick development as shown by tomato (*Lycopersicon esculentum*) lectin binding

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**Summary.** Basement membranes (BMs) constitute a distinct compartment of the extracellular matrix (ECM). All BMs show a similar structural appearance but differ in molecular composition. These variations have critical functional implications. The aim of this study is to establish the pattern of the tomato lectin (*Lycopersicon esculentum* agglutinin - LEA) binding sites in the BMs of the developing chick embryo (stages 4-21, Hamburger and Hamilton, 1951) in order to achieve a better understanding of the molecular heterogeneity of BMs. The study was performed with transmission electron microscopy (TEM) histochemistry, and confocal laser microscopy.

TEM showed that LEA bound to the lamina densa and to the lamina fibroreticularis of the BMs. Through the period studied, most of the LEA binding appeared in the ectodermal BM and its derivatives. In the limb bud, LEA binding to the ectoderm BM was more intense in the ventral half than in the dorsal half. Furthermore, LEA allowed the early (HH16) detection of the transverse fibrillar tracts. In the lens and in the inner ear primordium, the BMs were LEA positive through the placode and cup stages. The binding was progressively reduced through the vesicle stage. The BMs of the olfactory primordium, and of the Rathke's pouch were positive. In contrast, the BMs of the developing central nervous system were negative. The BMs of both the paraxial and the lateral plates of the mesoderm were negative, whereas the notochord and the BM of the Wolffian duct were positive. The endodermal BM and its derivatives were negative. The ECM located between the fusing endocardial tubes, and the BM of the fusion zone of the paired aortae, were positive. This suggested an active role of the LEA-positive glycoproteins in the fusion of endothelia. Our results show the heterogeneity

of the chick embryo BMs during development. In addition, LEA constitutes an excellent marker for the primordial germ cells.

**Key words:** Chick development, Basement membrane, *Lycopersicon esculentum* agglutinin, Wolff duct, Primordial cells

## Introduction

The extracellular matrix (ECM) is a complex assemblage of molecules. The different molecular components interact both with each other and with cells by means of integrin receptors (Hynes, 1992), to modulate a wide range of cellular functions. The repertoire of integrin expression and ECM composition appear to dictate whether a cell will survive, proliferate, or exit the cell cycle and differentiate in response to soluble factors (Beauvais-Jouneau and Thiery, 1997). Furthermore, some of the sulphated glycosaminoglycans (GAGs) present in the ECM interact with diverse growth factors to become part of their signalling pathway. Changes in GAG composition may function as critical temporal regulators of the activity of growth factors, and as morphogen signalling during embryogenesis (Allen and Rapraeger, 2003).

Basement membranes (BMs) are layers of specialized ECM associated with different cell types such as epithelial and endothelial cells. They have a cell-supporting role, are involved in the maintenance and regulation of the cellular phenotypes (Streuli, 1996), and appear to control macromolecular passage through epithelia and endothelia (Robinson and Walton, 1989). Electron microscope studies have shown few significant structural differences between the BMs from different anatomic sites (Inoue, 1989). However, the molecular composition of BMs is extremely heterogeneous (Damjanov, 1990; Erickson and Cauchman, 2000).

Several isoforms of laminin, collagen IV and entactin/nidogen (Ekblom et al., 1988; Sanes et al., 1990; Kohfeldt et al., 1998; Pierce et al., 2000; Virtanen et al., 2003), as well as different proteoglycans (Erickson and Couchman, 2000), are normal components of the BMs. Variations in sugar composition, size, and sulfatation pattern of the constituents of the proteoglycan chains, can generate a wide array of molecular species (Gallagher, 1986; Sanes et al., 1990). A large body of evidence indicates that the histochemical characteristics of BMs vary from one organ to another; that different anatomic structures within the same organ may be invested with different BMs; and, finally, that BM composition changes considerably during development and in relation to adult stages (Wan et al., 1984; Leu and Damjanov, 1988; Ojeda et al., 1993). The knowledge of the temporal sequence in which the developmental heterogeneity of the BMs is established is necessary to understand the possible regulatory roles played by BMs.

The carbohydrate moieties of the BM glycoconjugates vary during development. These changes have been implicated in cell-to-cell and in cell-matrix interactions (Shur, 1989). Also, carbohydrate domains may serve as markers of several physiological and pathological conditions (Rademacher et al., 1988; Salamat et al., 1993). Lectins are ubiquitous proteins of non-immune origin which show a high specificity for carbohydrate moieties. This quality of lectins has proved very useful in detecting both cellular and extracellular carbohydrate changes in BMs (Lis and Sharon, 1986). Distinct changes in the ability of BMs to bind to different lectins have been detected in the course of the morphogenesis of several organs (Gallagher, 1986; Laitinen et al., 1987; Ojeda et al., 1993). However, systematic studies of the BM glycosylation pattern during chick development are scarce.

The *Lycopersicon esculentum* (tomato) agglutinin (LEA) is a lectin which binds specifically to the poly-N-acetyl D-glucosamine sugar residues (Nachbar and Oppenheim, 1982) (nominal carbohydrate specificity: GlcNAc $\beta$ 1, 4GlcNAc $\beta$ 1, 4GlcNAc $\beta$ 1, 4GlcNAc>GlcNAc $\beta$ 1, 4GlcNAc $\beta$ 1, 4GlcNAc>GlcNAc $\beta$ 1, 4GlcNAc, - Goldstein and Poretz, 1986). This lectin has frequently been used as a marker for microglial cells (Acarin et al., 1994; Andjelkovic et al., 1998) and for non-professional phagocytes (Andjelkovic et al., 1998; Navarro et al., 2003). In the early chick embryo, LEA has shown affinity for the primordial germ cells (stage 4-5, Hamburger and Hamilton, 1951) and for the most lateral regions of the epiblast (6HH-7HH) (Griffith and Sanders, 1991). The developmental distribution of several lectins has been the subject of previous studies (Griffith and Sanders, 1991; Ojeda et al., 1993; Wilson and Wyatt, 1995; Yao et al., 1996; Götz and Quondamatteo, 2001). However, very little is known of the changes which may occur in the distribution of LEA during development (see: Griffith and Sanders, 1991; Hentschel and Walther, 1993). The aim of the present study is to establish the pattern of LEA-binding sites

during the development of the chick embryo (4HH-21HH). Special emphasis has been placed on BMs. A better understanding of BM heterogeneity will provide insights into the regulatory roles of BMs during development.

## Materials and methods

Fertilised White Leghorn chicken eggs were incubated at 37.5°C in a humidified commercial egg incubator. Embryos ranging from stages HH4 to HH21 (Hamburger and Hamilton, 1951) were used according to the following procedures.

### Fluorescence microscopy

The embryos were fixed in cold ethanol-glacial acetic acid (99:1), dehydrated in cold ethanol, transferred to cold xylene and embedded in diethylene glycol diestearate (DGD) (Polysciences, Inc. Warrington, PA), as previously described (Ojeda et al., 1989). Briefly, the embryos were transferred from the xylene to a mixture of xylene/DGD of 2:1 (10 min.), and then 1:2 (10 min.), followed by two changes of 100% DGD (15 min. each). During the embedding procedure, the DGD was maintained at 60°C to keep it molten. After infiltration, the embryos were transferred to flat silicone embedding moulds filled with freshly melted DGD. The blocks were allowed to solidify at room temperature, and were stored at 4°C until sectioning.

Semi-thin sections with a nominal thickness of 1-2  $\mu$ m were obtained on a Jeol Jum-7 ultramicrotome using glass knives with a water bath. The sections were removed from the water with a glass rod and transferred to a water drop on a clean slide. The slides were placed on a warm plate (40°C) until dry. They were then stored at 4°C overnight. Dewaxed sections were stained with LEA fluorescein isothiocyanate (FITC)-conjugated (Sigma) (50 $\mu$ g/ml in phosphate buffered saline [PBS]) for 30 min, followed by three washes with PBS (10 min. each). In addition, selected sections were stained with a propidium iodide solution (1 $\mu$ g/ml) for 3 min. at room temperature to visualize the cell nucleus. All the staining procedures were carried out in the dark at room temperature. The slides were mounted with the antifading medium Vectashield (Vector, Burlingame, CA). All sections were examined with a confocal laser microscope (MRC 1024; BioRad) using argon (488nm) and HeNe (543nm) lasers. The specificity of the lectin binding was tested by incubating the FITC-conjugated lectin with 0.1M solution of N-acetyl D-glucosamine (Sigma) for 30 min. Then, selected sections were incubated with the mix lectin-sugar (Acarin et al., 1994). All the controls were routinely negative.

### Enzymatic digestion

Prior to LEA staining, a duplicate series of sections was treated with neuraminidase in order to expose the

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penultimate carbohydrate residues blocked by sialic acid (Uehara et al., 1985). These sections were washed in 0.05M acetate buffer pH 5.3. Neuraminidase from *Clostridium perfringens* (Type V, Sigma) was used at the concentration of 1U/ml, diluted in acetate buffer for 30 min., at 37°C.

### Electron microscopy histochemistry

To identify the LEA-positive sites of the BMs, several embryos in different stages were processed for lectin electron microscopy histochemistry. The embryos were fixed by immersion (2-3 h) in cold cacodylate-buffered 2% paraformaldehyde and 0.5% glutaraldehyde, pH 7.3, and washed with PBS for 2 h. The embryos were then dehydrated in graded methanol at -20°C, and embedded in Lowicryl K4M at -20°C. Ultra-thin sections were cut with a Leica Ultracut UCT ultratome, mounted on nickel grids, treated with 1% BSA-PBS for 10 min, and incubated with biotinylated LEA (20µg/ml) (Sigma) in 0.1BSA-PBS for 1h. After rinsing in PBS, the sections were incubated with streptavidin conjugated with 10-nm gold particles (Sigma) diluted 1:15 in PBS. The sections were then washed, air-dried, contrasted with uranyl acetate and lead citrate, and examined with a Philips SEM 501 microscope.

### Results

The precise location of the LEA-binding was defined at the ultrastructural level. On TEM, the gold particles were restricted to the lamina densa and to the collagen fibres and the amorphous material which constitute the lamina fibroreticularis of the BM (Fig. 1). A correlation was always found between the ultrastructural and the histological findings. On light microscopy, binding to LEA was detected in the BMs of the ectoderm (EBM), of most of the ectoderm derivatives, and in some mesoderm derivatives. The BMs of the endoderm and its derivatives were LEA-negative. As an exception, the endodermal BM appeared faintly LEA-positive at gastrulation stages (HH4-HH7). Neuraminidase digestion, to remove sialic acid residues, did not modify either the pattern or the intensity of the LEA binding. Significant temporal and spatial changes in the LEA-binding pattern occurred between developmental stages HH4 and HH21.

#### Ectoderm and its derivatives

During gastrulation (HH4-HH7), the BM of the more lateral zones of the epiblast was faintly LEA positive at all cranio-caudal levels (Fig. 2a). The zone of the epiblast near the primitive streak was negative. At neurulation stages (HH8-HH13), the entire EBM was LEA-positive (Fig. 2b,c). The intensity of the fluorescence was higher than that shown by the epiblast in the previous stages. Curiously, EBM positivity to

LEA was more intense in the ventral than in the dorsal half of the embryonic body (Fig. 2d).

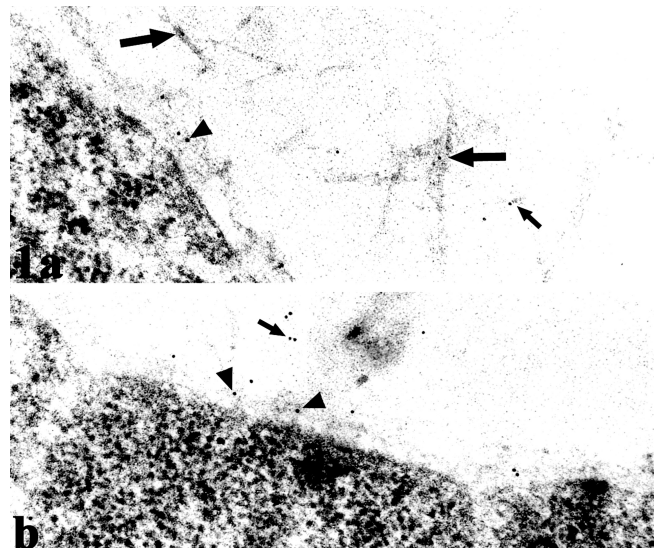
In the limb bud, the EBM was positive for LEA throughout the period studied. During the first stages of limb development (HH16-HH18), the EBM exhibited a rather uniform LEA-binding pattern (Fig. 2e). However, after stage HH18, the EBM binding was more intense in the ventral than in the dorsal half of the limb (Fig. 3). In addition, LEA revealed the presence of a system of transverse fibrillar tracts connecting the EBM to the mesodermal core of the limb. These tracts were first visible at HH16 (Fig. 2e). After stage HH18, the tracts became thicker and the fluorescence appeared more intense. Again, the intensity of the fluorescence of these tracts was higher in the ventral than in the dorsal half of the limb (Fig. 3b-c).

#### The central nervous system

The BMs of the developing central nervous system, except for the floor of the diencephalon (HH14-HH21), were LEA-negative (Fig. 2b-d). The fluorescence associated with the EBM ended abruptly at the boundary between the ectoderm and the lateral borders of the invaginating neural plate (Fig. 2b).

#### The eye

The lens first appeared (HH13-HH14) as an ectodermal thickening, the lens placode, at the area of contact between the ectoderm and the optic vesicle. In

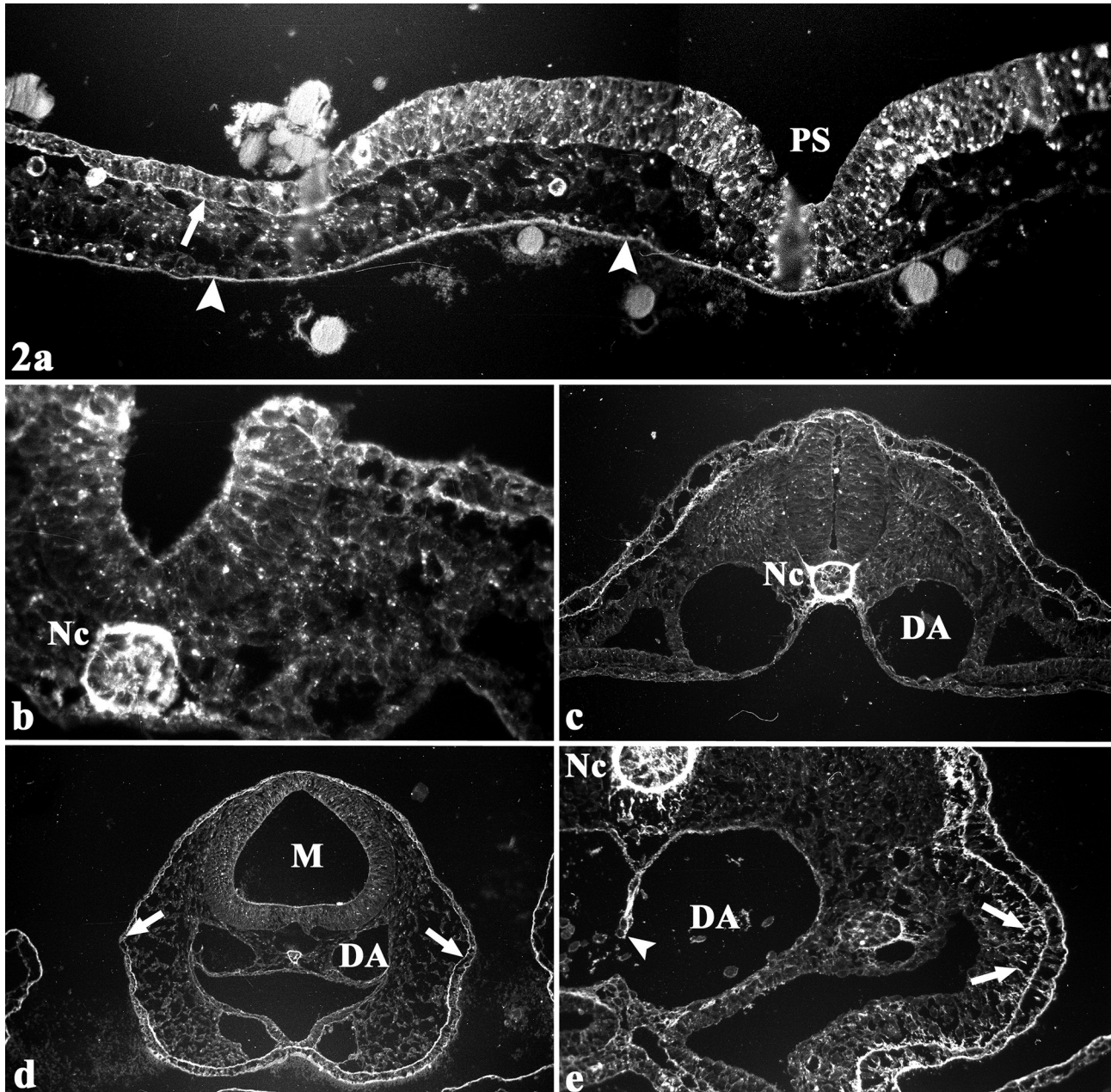


**Fig. 1.** Electron micrographs illustrating localization of LEA binding to BMs in the developing chick. **a** and **b** show LEA localization under the ectoderm and under the lens, at HH13 and HH17, respectively. The gold particles localize to the lamina densa (arrowheads), and to the amorphous material (small arrows) and collagen (large arrows) of the lamina fibroreticularis. x 31,500

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this area, the EBM was very thick and was intensely LEA-positive (Fig. 4a). During the invagination of the lens placode (HH15-16), the placode BM remained intensely positive (Fig. 4a,b). This positivity was lost only at the boundary between the lens primordium and

the ectoderm (Fig. 4c). At the time of formation of the lens vesicle, the BM covering the posterior surface of the lens was LEA-positive (Fig. 4d). The BM associated with the anterior surface of the lens vesicle was LEA-negative throughout the period studied. The intensity of



**Fig. 2.** LEA-binding pattern in the BM of the epiblast and of the ectoderm. Transverse sections. **a.** Stage HH5. In the primitive streak (PS) stages, the lateral zone of the epiblast (arrow), and the endoderm (arrowheads), are faintly positive. **b-c:** During the formation (**b**, HH8) and closure (**c**, HH10) of the neural tube, the ectoderm BM is positive. The BMs of the central nervous system, somites and endoderm are negative. **d.** Section through the mesencephalon (M) (HH12). The LEA binding to the ectoderm BM is more intense in the dorsal than in the ventral half (arrows indicate the boundary between the two halves). **e.** Stage HH16. The early wing bud contains LEA-positive transverse fibrillar tracts (arrows). The paired dorsal aorta (DA) is in the process of fusion (arrowhead). Nc, notochord. a, e: x 250; b: x 400; c: x 200; d: x 100.

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the fluorescence of the lens vesicle BM was progressively reduced, and, at stage HH21, it was no longer identified (Fig. 4e). Intense LEA binding was also observed on the apical surface of the lens cells during the formation and invagination of the lens placode (Fig. 4b-c). This binding had almost disappeared at the lens vesicle stage (Fig. 4d).

The ectodermal cells containing phagosomes were always LEA-negative. It was only when the lens vesicle was completely detached from the ectoderm surface (HH20) that some intensely LEA-positive cells with characteristics typical of macrophages appeared in the extracellular space located between the ectoderm and the anterior lens epithelium (Fig. 4e). The rest of the eye components were LEA-negative throughout the period studied.

### *The ear*

The primordium of the inner ear was first observed at stages HH9-HH10 as a slight thickening of the ectoderm, the otic placode, on either side of the rhombencephalic region (Fig. 5a). The BM of the otic placode (OBM) was LEA-positive. The intensity of the fluorescence was similar to that observed in the rest of the EBM (Fig. 5a). As the otic placode invaginates and forms the otic vesicle (HH14-HH15), the OBM binding intensity increased in the area close to the rhombencephalon (Fig. 5a,b). The non-uniform distribution of the LEA binding along the OBM was more clearly detected during the early development of the otic vesicle (Fig. 5c). At stages HH17-HH20, concomitant with the appearance of the endolymphatic duct, the shape of the otic vesicle changed from rounded to elliptical (Fig. 5d-e). Then, the OBM underwent a progressive loss of the LEA-binding that followed a precise spatial and temporal pattern. The binding loss was first detected in the BM of the developing endolymphatic duct (Fig. 5d) and, then, in the BM of the medial wall of the otic vesicle (Fig. 5e). In contrast, the BM of the lateral wall of the otic vesicle remained intensely LEA-positive. In addition, the ECM associated with the periotic mesenchyme was also LEA-positive (Fig. 5d-e). During the invagination of the otic vesicle, the apical cell surface appeared LEA-positive (Fig. 5b). At stages HH17-HH20, the otic cells lost the LEA binding following a spatial and temporal sequence similar to that observed in the OBM (Fig. 5d-e). The cells of the otic primordium containing phagosomes were LEA-negative.

### *The olfactory primordia*

The olfactory placodes appeared as ectoderm thickenings on the lateral wall of the head, anterior to the lens primordia (HH14-HH15) (Fig. 6a). The thick BM of the olfactory placode was intensely LEA-positive (Fig. 6a). In addition, LEA-positive material was observed at the apical cell surface and in the intercellular spaces.

Subsequently, the olfactory placodes invaginated and formed the olfactory pits. At stages HH18-HH19, the olfactory pits became deeper and contacted the forebrain. During this process, the LEA binding in the more lateral zone of the olfactory pit BM was seen to decrease (Fig. 6b). In addition, the BM in the zone close to the forebrain showed a small area which was LEA-negative (Fig. 6b).

### *The hypophysis*

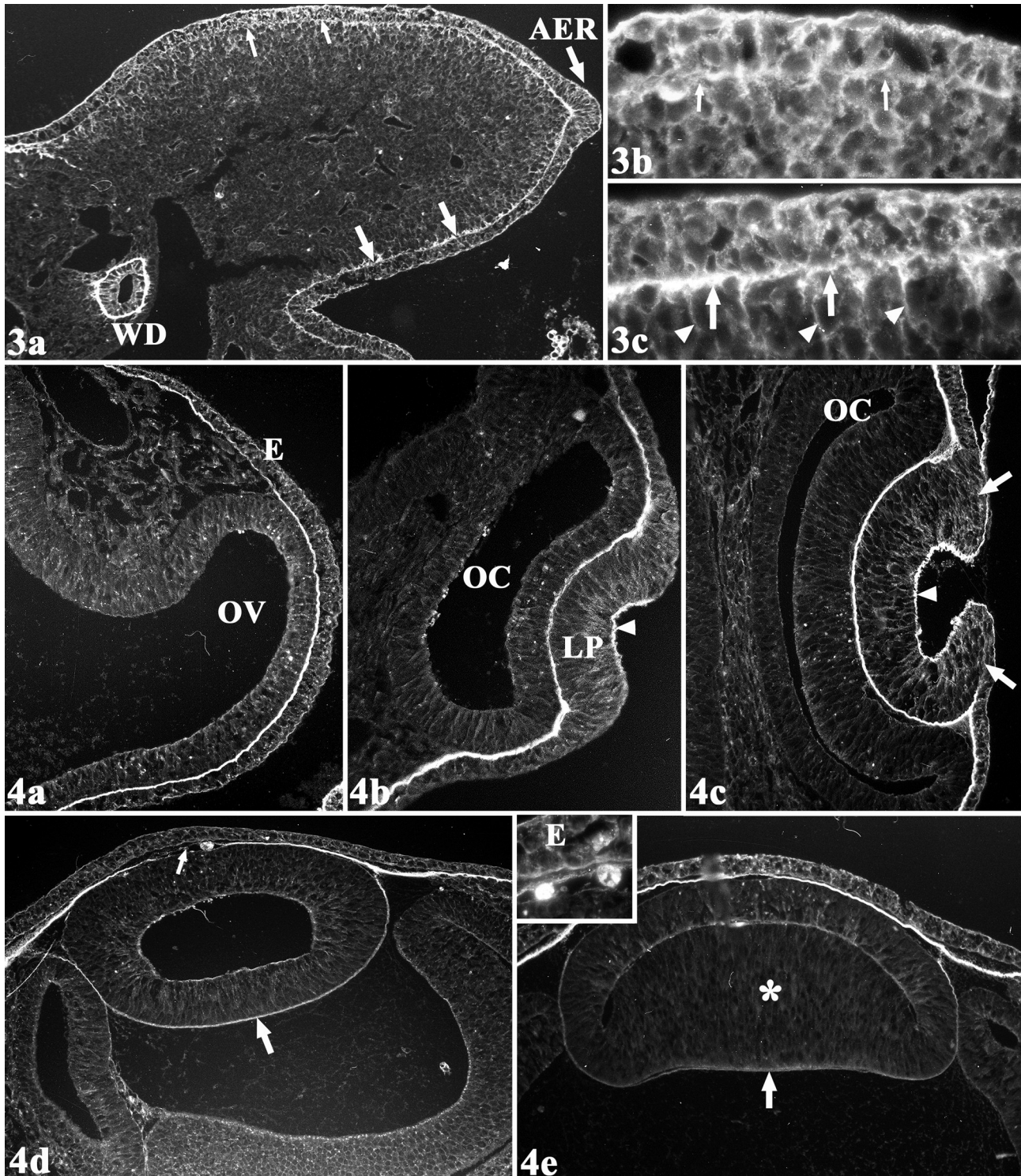
The epithelial component (adenohypophysis) of the hypophysis appeared before the neural component (neurohypophysis). The adenohypophyseal primordium was first seen at stages HH14-HH15 as an evagination of the ectoderm of Rathke's pouch from the roof of the oral cavity (Fig. 7). Subsequently, the pouch approached the anterior tip of the notochord and contacted the floor of the diencephalon. The pouch then expanded laterally (Fig. 7). During the initial stages of Rathke's pouch development, the pouch BM showed a faint, patchy binding pattern at the apical cell surface (Fig. 7a). This pattern was similar to that observed in other areas of the oral ectoderm. At stages HH17-HH19, the LEA binding intensity increased greatly in both the BM and in the apical cell surface (Fig. 7b). Furthermore, the area of the diencephalic floor in contact with Rathke's pouch showed a moderately positive BM. Faint LEA binding was also observed at the apical cell surface and in the intercellular spaces of this diencephalic area (Fig. 7b-c). When Rathke's pouch expanded laterally (HH20-HH21), the BM of the oral ectoderm lost most of the LEA binding (Fig. 7c). In addition, the binding of LEA to both the BM and the apical cell surface of the diencephalon became attenuated (Fig. 7c).

### *Mesoderm derivatives*

The BM of the paraxial mesoderm, and that of the mesoderm lateral plates, were negative to LEA throughout the period studied.

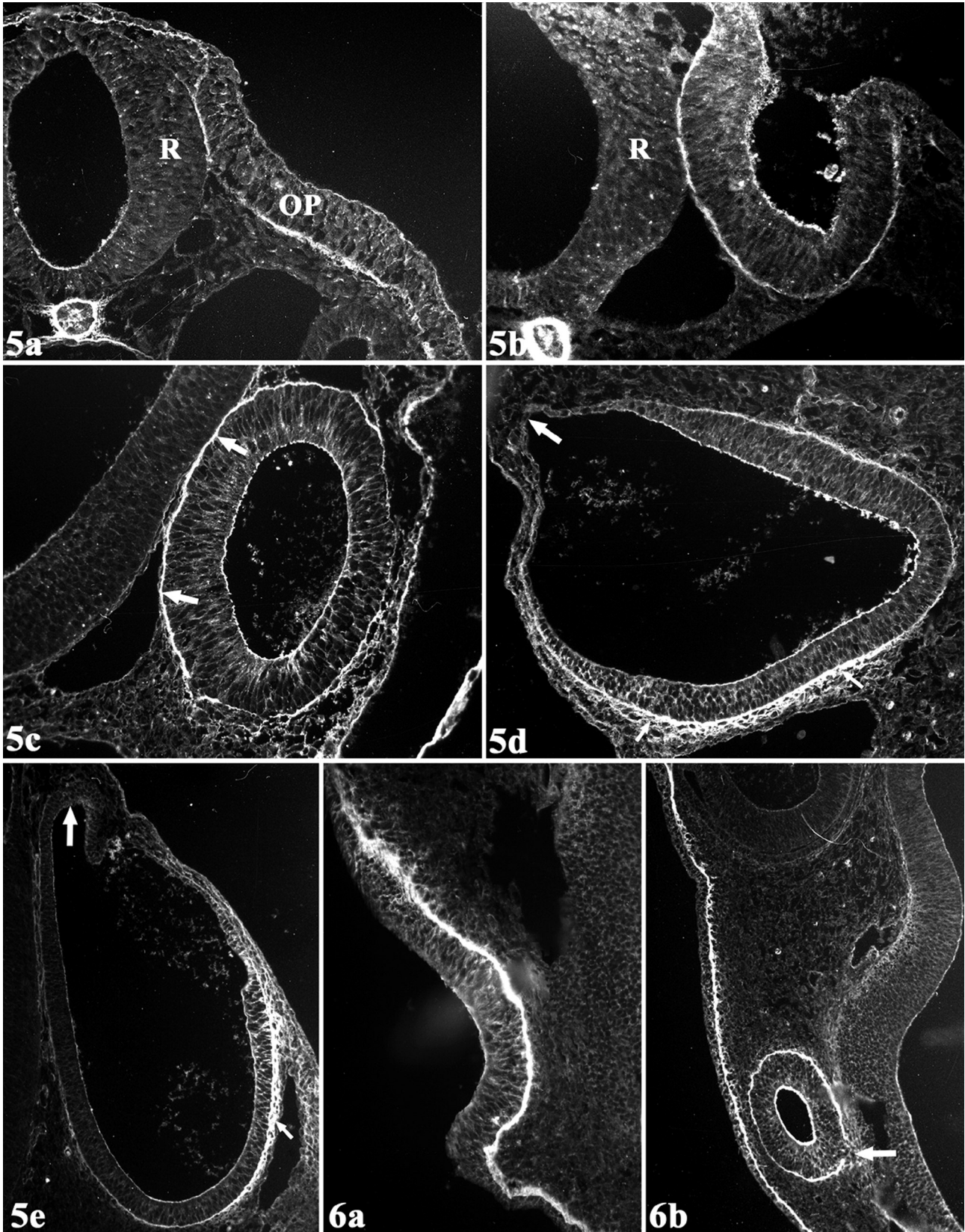
### *The notochord*

During the developmental period studied here, the anterior part of the notochord underwent three successive morphogenetic steps (Fig. 8). Initially, the notochord was formed by loosely arranged cells. The notochord cells, and the corresponding intercellular spaces, appeared positive to LEA (Fig. 8a). Gradually, the cells compacted, the size of the intercellular spaces decreased, and the notochord was encircled by the notochordal sheath. At these stages, the notochord cell coat and the notochordal sheath were moderately positive for LEA. LEA-positivity in the developing notochordal sheath was distributed in a patchy pattern (Fig. 8b). In the fully compacted notochord, the notochordal sheath (Fig. 8c) appeared very thick and was intensely and uniformly LEA-positive.



**Fig. 3.** Longitudinal sections through the wing bud (HH20). **a.** Panoramic view. AER, apical ectodermal ridge. Wd, Wolffian duct. **b-c.** Detail of the dorsal (**b**) and ventral (**c**) halves. The fluorescence is more intense in the ectoderm BM of the ventral half (large arrows) than in the dorsal half (small arrows). The BM of the APR is also positive. Arrowheads, fibrillar tracts. a: x150 ; b-c: x 650

**Fig. 4.** LEA-binding pattern in the developing eye. Transverse sections. **a.** Stage HH10. The area of contact between the optic vesicle (OV) and the ectoderm (E) is positive before the formation of the lens placode. **b.** Developing lens placode (LP) (HH13). **c.** Invaginating lens placode (HH15). Note the loss of fluorescence in the EBM at the boundary between the LP and the ectoderm (arrows). Arrowhead in b and c indicate LEA binding to the apical cell surface. **d.** Lens vesicle stage (HH18). Large arrow, BM of the posterior lens surface. Small arrow, lens stalk. **e.** Developing lens (HH20). Note the loss of LEA binding in the posterior lens BM (arrow). Asterisk, differentiating lens fibers. Inset: Subectodermal (E) macrophages are intensely LEA-positive. E, ectoderm. a, d, e: x 200; b, c: x 250. Inset: x 650



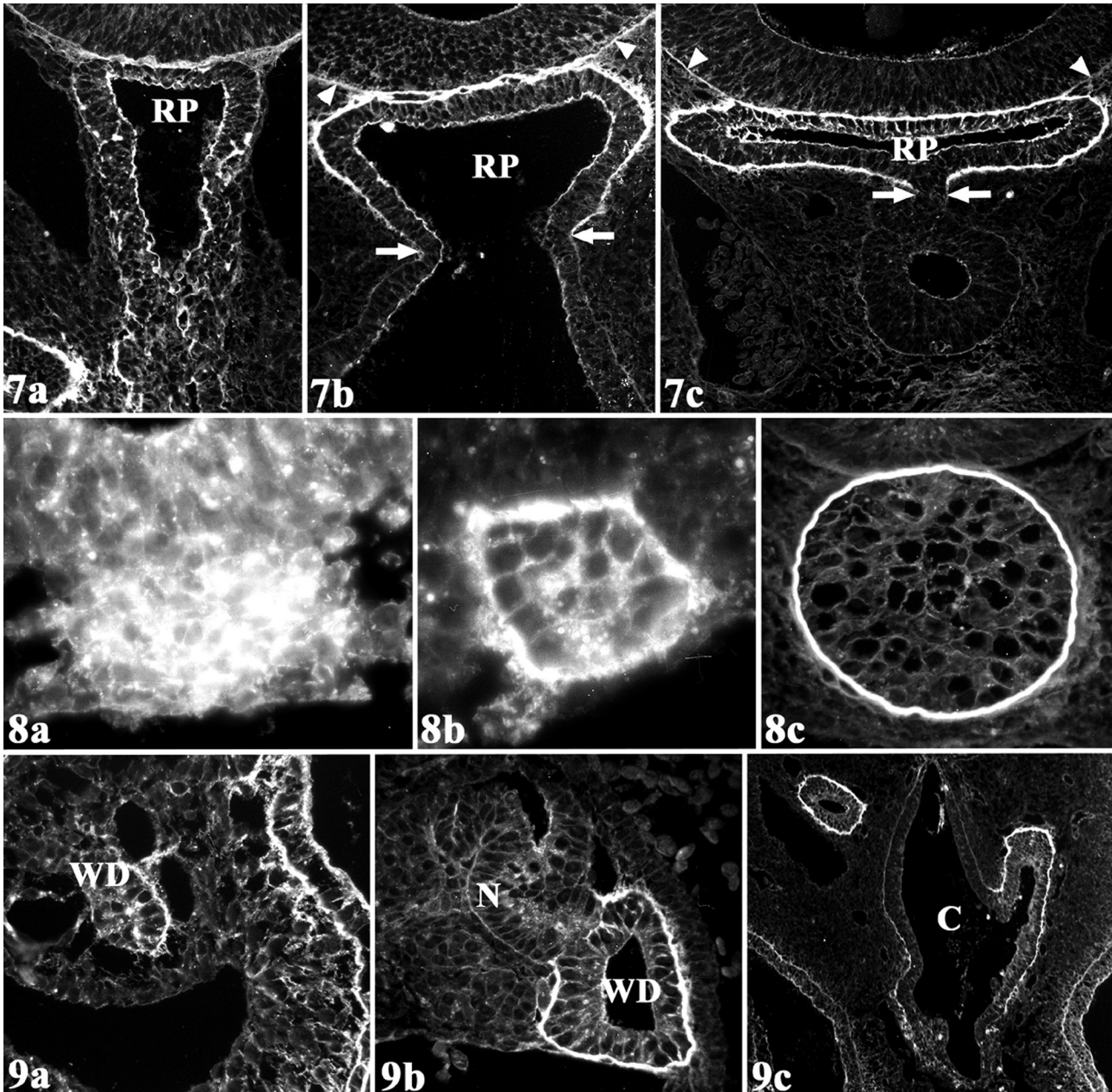
**Fig. 5.** LEA binding pattern in the developing ear. Transverse sections. **a.** Stage HH10. The otic placode (OP). **b.** Invaginating otic placode (HH14). **c.** Otic vesicle stage (HH16). Note that the fluorescence of the otic BM is more intense (arrows) near the rombencephalon; **d and e.** The developing endolymphatic duct (large arrow) at stages HH18 and HH20, respectively. The periotic mesenchyme (small arrows) is LEA-positive. **a, d:** x 200; **b, c:** x 250; **e:** x 150

**Fig. 6.** Transverse sections through the olfactory placode (**a**, HH14) and the olfactory pit (**b**, HH18). Arrow indicates the zone of the BM LEA-negative. **a:** x 200; **b:** x 170

Subsequently, the notochordal cells underwent vacuolization and the cell coat became faintly LEA-positive (Fig. 8c).

#### The kidney

Throughout the period studied, only the BM of the



**Fig. 7.** Frontal sections through the developing Rathke's pouch (RP) at stages HH14 (a), HH18 (b) and HH20 (c), respectively. Note in b and c that the LEA positivity of the BM abruptly ends at the boundary between the RP epithelium and the oral epithelium (arrows). The BM of the diencephalic floor (arrowheads) is moderately LEA-positive. a: x 250; b, c: x 200

**Fig. 8.** LEA binding-pattern in the developing notochord. a. Early development. b. Compaction stage. c. Fully compacted notochord. a-c: x 650

**Fig. 9.** LEA binding to the developing Wolffian duct (WD). a. The lateral wall of the duct shows a higher LEA-positivity than the medial wall. b. WD with a well formed lumen and an attached nephron (N). The fluorescence associated with the BM duct is very regular. c. The caudal end of the WD opens into the cloaca (C) (HH20). a-b: x 400; c: x 150



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Wolffian duct was LEA-positive (Fig. 9). The Wolffian duct first appeared (HH10-HH12) as a solid cell cord originating from the intermediate mesoderm. At these stages, the Wolffian duct BM exhibited an asymmetric LEA-binding pattern (Fig. 9a). While the lateral duct zone was moderately positive, the ventromedial zone was only faintly positive. Faint LEA-binding was also observed in the intercellular spaces. After stage HH15, concomitant with the appearance of the lumen in the Wolffian duct, the intensity of the LEA binding to the duct BM increased (Fig. 9b). The caudal end of the Wolffian duct opened into the dorsolateral wall of the cloaca at stages HH20-HH21. Then, the LEA binding to the duct BM ended abruptly at the boundary between the Wolffian epithelium and the cloacal epithelium (an endoderm derivative) (Fig. 9c).

### Heart and blood vessels

During cardiac development, only the cardiac jelly located in the heart midline, between the fusing endocardial tubes (HH9-HH10), was moderately LEA-positive (Fig. 10). Moderate LEA binding was also observed during the fusion of the paired dorsal aortae (Fig. 11). LEA binding to the paired aortae was first detected at stage 12. The binding was limited to the area

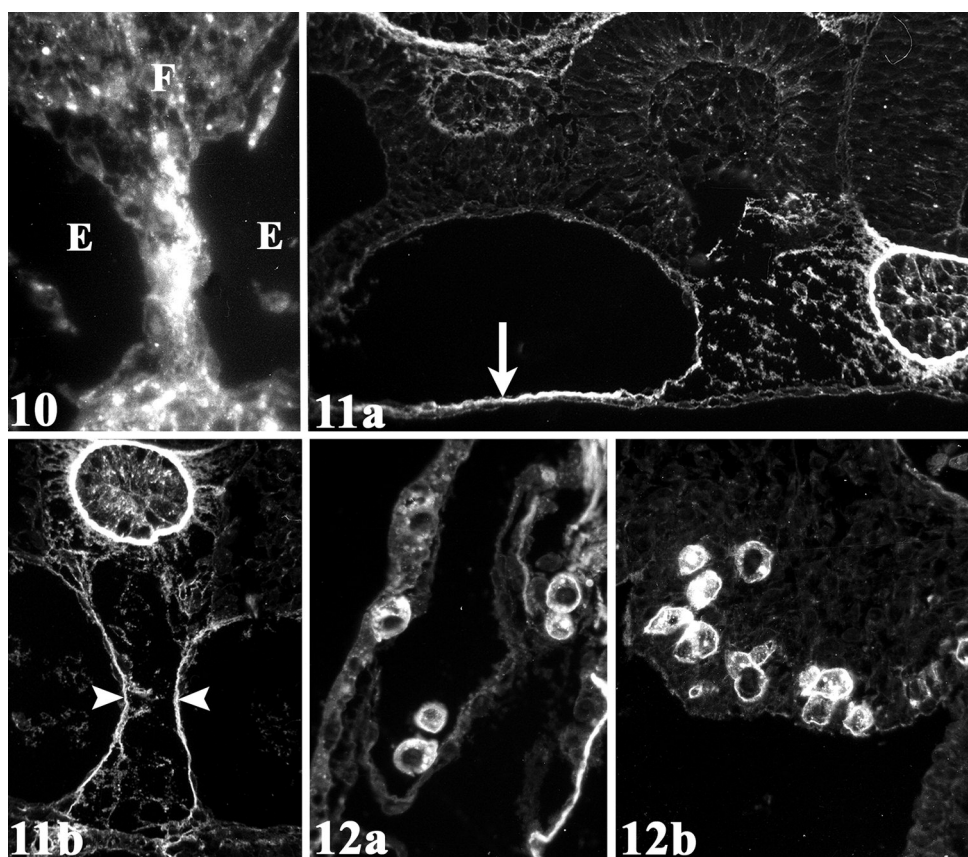
of contact between the vessels and the endoderm, i.e., the presumptive zone of fusion (Fig. 11a). Concomitant with the folding of the endoderm, the LEA-positive area was displaced to the embryonic midline (Fig. 11b). When the two aortae contacted and fused, binding to LEA disappeared from the aortic wall (Fig. 1e). On the whole, the embryonic blood vessels were LEA-negative throughout the period studied. Only after stage HH20, moderate LEA affinity could be observed in the primitive cerebral veins associated with the forebrain (not shown).

### Primordial germ cells (PGCs)

Regardless of their location or migratory state, PGCs were intensely LEA-positive throughout the period studied (Fig. 13). The LEA binding was located in both the cytoplasm (with a granular distribution) and in the cell coat (Fig. 13).

### Discussion

Our results clearly show the heterogeneity of the extracellular matrix of the developing chick with regard to its content in glycoconjugates rich in N-acetyl D-glucosamine residues. The LEA binds to the BMs of the



**Fig. 10.-** The extracellular matrix located between the fusing endocardial (E) tubes is intensely LEA positive. F, endodermal foregut. x 600

**Fig. 11.** Modifications of the LEA-binding pattern during the formation of the dorsal aorta. **a.** In the paired aorta, only the presumptive zone of fusion (arrow) is positive for LEA (Compare to figure 2c). **b.** During the approaching of the two vessels, LEA-positivity remains restricted to the fusion area (arrowheads). a: x 300; b: x 250

**Fig. 12.** The primordial germ cells located in the germinal crescent area (**a**, HH8) and in the gonadal primordium (**b**, HH20) are intensely LEA positive. a-b: x 400

ectoderm and its derivatives (excluding the BM of the developing nervous system), and to the BMs of several mesodermal derivatives. However, the BMs of the endoderm and its derivatives were LEA-negative throughout the period studied.

Our results on the LEA binding affinity to the BMs of the ectoderm and of the developing nervous system are in agreement with previous studies (Griffith and Sanders, 1991). During the development of the lens, otic and nasal placodes, the LEA-binding pattern followed a similar trend, with LEA localized to the BM and the apical cell surface. A similar pattern was also observed during the formation of Rathke's pouch. The deposition of LEA-positive glycoconjugates at the apical side of the invaginating epithelia supports the notion (Sinning and Olson, 1988; Hilfer and Randolph, 1993; Yao et al., 1996) that glycoproteins constitute an important (physical and/or chemical) factor in the signalling pathway during the formation of the placodes. The progressive loss of LEA binding in these structures must be considered a sign of developmental maturation. The loss of LEA binding could be explained by sialylation masking. Masking of sugar residues has been reported during the differentiation of several tissues (Zieske and Bernstein, 1982; Ojeda et al., 1993). However, we did not detect any LEA binding after neuraminidase digestion. Another possibility is that the LEA-positive glycoproteins could have been replaced by newly synthesized glycoproteins during the differentiation process. However, the loss of LEA binding in the BM of the lens vesicle occurred both in the undifferentiated anterior epithelium and in the more differentiated posterior epithelium. Finally, pre-existing LEA-positive glycoproteins could have been degraded by glycosidases. While we favour the last hypothesis, experimental testing is necessary to prove the existence of glycosidase activity.

The non-uniform distribution of the LEA-binding sites along the EBM during limb development should be emphasized. The dorso-ventral asymmetric distribution of the LEA-binding pattern observed in the EBM has not previously been described for lectins with N-acetylglucosamine specificity (WGA and WGAs). Our results provide further support in favour of the existence of a dorso-ventral compartmentalization in the developing chick limb. According to Altabef et al. (1997, 2000), the ectoderm layer may be bisected by a plane passing from the forelimb to the hindlimb region, and thus dividing the limb into two cell lineage restriction compartments, dorsal and ventral. Indeed, the apical ectodermal ridge of the developing limb is formed at the boundary between these two compartments. On the other hand, the LEA detects the fibrillar tracts extended between the EBM and the mesodermal core of the developing limb earlier (HH18) than previously (HH26) reported (Hurle et al., 1988). Furthermore, we show that the tracts are thicker (and more intensely fluorescent) in the ventral than in the dorsal half of the limb. Again, this speaks in favour of the existence of a

dorso-ventral compartmentalization in the developing chick limb. The lectins of the LEA group appear to be a better tool to study the development of the limb fibrillar tracts. To gain a complete understanding of the developmental and compositional changes undergone by these fibrils is of crucial importance since they appear to modulate the activity of the intercellular signals during the development of the limb (Arteaga-Solis et al., 2001).

The notochord and the BM of the Wolffian duct were the mesodermal derivatives that showed the greatest LEA positivity. The increasing amount of LEA-positive glycoconjugates may represent a factor of stabilization during notochord compaction. Our results on the LEA binding to the chick notochord differ from previous (Griffith and Sanders, 1991) observations. However, these differences could be explained by the different lectins used. The human notochord has been shown to be positive for wheat germ agglutinin (WGA) (Götz and Quondamatteo, 2001). Both WGA and LEA belong to the N-acetylglucosamine group.

Between stages HH14 and HH21, the Wolffian duct BM was LEA positive. However, both the pronephric and mesonephric nephrons were negative. To our knowledge, this is the first lectin-binding study of the Wolffian duct. Throughout the period studied, the Wolffian duct underwent compaction of the loose mesenchyme into a solid epithelium, generation of a lumen (canalization), establishment of the cell polarity, and cranio-caudal migration of the Wolffian duct tip to join the cloaca. Several ECM glycoproteins, especially fibronectin and laminin (Jacob et al., 1991), appear to play an important role in all these processes (Hay, 1991). Our results indicate that glycoconjugates having N-acetyl D-glucosamine residues play a key role during the development of the Wolffian duct.

The presence of LEA-positive material associated with the fusion of endothelia is worth comment. During heart development, LEA-positive material accumulates between the fusing endocardial tubes (the rest of the extracellular matrix -cardiac jelly- of the early heart is negative for LEA). During the fusion of the paired aortae, LEA positivity is restricted to the BM of the fusion area. In the two cases, the presence of LEA-positive material may mediate the fusion process. At the time of endocardial fusion, apoptosis of the midline endocardium plays a decisive role in the fusion process (Ojeda and Hurle, 1975, 1981). Endothelial cell death also occurs during formation of the single aorta (Berry, 1973). The concurrent accumulation of LEA-positive material and the presence of endothelial apoptosis suggests a causal relationship. It is well established that the ECM has an essential function in the control of cell survival. The ECM signals for cell survival are specific and are mediated, at least in part, by the integrin family of cell surface receptors (Prince et al., 2002). The late appearance of moderate LEA affinity in the wall of the cerebral veins suggests that the presence of glycoproteins rich in N-acetyl D-glucosamine residues may constitute a sign of maturation of the venous

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endothelium (see: Navarro et al., 2003).

The PGCs are intensely LEA-positive from the time of their appearance in the central zone of the area pellucida (Griffith and Saunders, 1991). These cells express LEA-positive sites along their entire migratory path, and when they settle into the germinal crescents. This suggests that the presence of glycoproteins containing N-acetyl D-glucosamine residues may play an important role in the processes of cell surface recognition and cell migration during PGC development. LEA is revealed as an excellent marker for PGCs in the chick embryo.

Apoptotic cell areas appear in several developing structures such as the lens vesicle (García-Porrero et al., 1979), the otic vesicle (Lang et al., 2000), and the neural tube (Weil et al., 1997; Hirata and Hall, 2000). In these areas, healthy neighbouring cells function as non-professional phagocytes. While LEA has been defined as a macrophage marker in several animal species (Andjelkovic et al., 1998; Navarro et al., 2003), we did not observe any LEA binding associated with non-professional phagocytes. However, a few LEA positive cells with the characteristics of free macrophages were observed in the final stages of lens development. Our results suggest that the presence of N-acetyl D-glucosamine residues may constitute an important difference between the two types of phagocytes.

Finally, the nature of the glycoproteins recognized by the LEA binding remains mostly unknown. Previous studies have identified lymphocyte cell surface (Kilpatrick et al., 1986) and sperm adhesion (Zhang and Martin-DeLeon, 2003) molecules as LEA targets. We have shown here that LEA binds to the collagen associated with the lamina fibroreticularis of the BMs (presumably, some type IV-collagen isoform). We could also infer that, during the fusion process of the heart anlage, LEA may be binding to carbohydrate chains associated to the fibronectin molecule. Monoclonal antibodies recognize large fibronectin deposits in this area of the developing heart (Icardo and Manasek, 1983). In addition, the LEA could be recognizing the glycoprotein fibrillin in the developing limb (see: Handford et al., 2000), and laminin-1 receptors at the cell surface (Botti et al., 2003). However, full identification of the LEA-bound glycoproteins is out of the scope of the present paper.

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