Interleukin 1 in Oviductal Tissues of Viviparous, Oviparous, and Ovuliparous Species of Amphibians

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ABSTRACT

In previous reports, we have shown that interleukin 1 (IL1), a cytokine associated with implantation in mice, is also expressed in reproductive tissues of viviparous squamate reptiles and cartilaginous fishes. In the present study, we investigated the expression of IL1B and its functional membrane receptor type I (IL1R1) in amphibians, a class of vertebrates that is characterized by different reproductive modes, including internal and external fertilization. In particular, we investigated the oviductal tissues of the aplacental viviparous Salamandra lanzai, the oviparous Triturus carnifex, and the ovuliparous Bufo bufo. Immunohistochemistry with anti-human IL1B and IL1R1 polyclonal antibodies found that in S. lanzai, most cells in the uterine mucosa were immunoreactive for IL1B and IL1R1. In T. carnifex, IL1B and IL1R1 were present in ciliated luminal cells, and there was evidence of IL1B in glandular cells. In B. bufo, the expression of IL1B and IL1R1 was limited to the apical cytoplasm of the ciliated oviductal cells. Western blot analysis showed that a putative mature form of IL1B, similar to that seen in mammals, was present in the oviductal tissues of S. lanzai, whereas different forms, which probably correspond to an inactive pro-IL1B protein, were found in T. carnifex and B. bufo. A band that corresponded to the predicted 80-kDa human IL1R1 was found in S. lanzai and T. carnifex. Although the present study shows that IL1B and IL1R1 expression occurs in all reproductive modes, the differential expression patterns noted between ovuliparity and oviparity and viviparity may reflect the different roles of IL1 in the various reproductive modes.

cytokines, female reproductive tract, fertilization, immunology, oviduct

INTRODUCTION

Viviparity is a reproductive mode that involves retention of the embryo within the female reproductive tract. Although viviparity is sometimes viewed as a typical mammalian phenomenon, many other groups of vertebrates (excluding agnathans and birds) include live-bearing species. In general, fishes, amphibians, and reptiles present various degrees of oviparity and viviparity [1–5]. Viviparity may involve the formation of a placenta, a structure formed by the apposition of extraembryonic membranes (chorion, allantois, yolk sac) and the maternal uterus [6, 7]. In anamniotes, yolk sac placentation is present in a few elasmobranch fishes of the family Triakidae [8], while there are no literature reports of placental structures in amphibians [9–11].

Although viviparity represents an evolutionary advantage for the developing embryo, it also represents a great risk for the embryo, which can be rejected by maternal tissues and thus not reach complete maturation [12]. Indeed, the semiallogeneic embryo bears antigens of paternal origin and thus, viviparity can be considered as a complex conflict between the selfish genes of the mother and those of her mate [13, 14]. Most studies on materno-fetal immunotolerance have been performed on murine and human placentas [13]. Among the immunological mechanisms proposed, the local secretion and action of cytokines at the materno-fetal interface appear to play a major role [15, 16].

Cytokines are peptides or glycopeptides with important activities in immune and inflammatory reactions [17]. Interleukin 1 (IL1) is a cytokine whose importance has been widely documented in human and murine reproduction [18]. Interestingly, data from mice show that blockage of the functional IL1 membrane receptor (IL1R1) prevents blastocyst implantation, which suggests a critical role for this cytokine in murine pregnancy [19].

Previously, we have shown that IL1 is present in the reproductive tissues of nonmammalian vertebrates, including squamate reptiles and cartilaginous fishes [20–22]. In particular, we have demonstrated that the IL1 system, including the IL1A and IL1B isofoms and the functional membrane receptor IL1R1, is expressed by the chorio-allantoic placenta of a squamate reptile, Chalcides chalcides, and by the yolk sac placenta of an elasmobranch fish, Mustelus canis [20, 21]. IL1 is also expressed in the uterine mucosa of Lacerta vivipara, which is a squamate reptile with both oviparous and viviparous populations [23].

In the present study, we examined the expression of IL1B and IL1R1 in the oviductal tissues of some amphibians, a vertebrate class that includes species with various reproductive modes [2]. Although many species exhibit external fertilization (oviparous, as defined by Blüm [24]), many other amphibians have internal fertilization and are oviparous or oviviparous. The coexistence within the same class (Amphibia) of species with such different evolutionary stages of reproduction means that these vertebrates are a good experimental model to investigate the role of immunoregulatory factors, such as cytokines, in materno-fetal immunotolerance.

To evaluate the expression of IL1 in amphibians with different reproductive modes, we used the following species: the aplacental viviparous urodèle Salamandra lanzai, in which embryos are retained until full metamorphosis; the oviparous urodèle Triturus carnifex, which lays eggs soon after
fertilization; and the oviparous anuran *Bufo bufo*, which lays eggs before fertilization.

**MATERIALS AND METHODS**

Animals and Sample Collection

Five females each of *B. bufo* and *T. carnifex* were collected in their natural habitat (environs of Florence, Tuscany, Italy) during the reproductive season from March 2005 through April 2005. The effective laying condition of the females was confirmed by the occurrence of at least some oocytes inside the oviducts. Four gravid females (with early embryos) of *S. lanzai* were collected in October 2005 (Park del Re, Monviso, Lombardy, Italy). Immediately after being transported to the laboratory, the animals were killed by deep anesthesia with 0.2% chlorobutanol. The body was then opened by a mid-ventral incision and the oviducts were isolated. Only the caudal portion of each oviduct was collected for the present study. In the oviparous species, this portion, which opens into the cloaca, is the region in which the unfertilized eggs are stored as clusters or strings before deposition. In the oviparous and viviparous species, the corresponding portion (known as the uterus) is where the eggs are fertilized and in the viviparous species, it is where embryonic development takes place. The caudal portion of the right oviduct was fixed in 10% buffered neutral formalin for 24 h, washed in running water for 12 h, and then dehydrated and embedded in paraffin wax for routine histological (hematoxylin-eosin; H&E), periodic acid, and Schiff reaction (PAS) followed by Mayer hemalum and orange G [25] or immunohistochemical staining. The caudal portion of the left oviduct was frozen and stored at −80°C until Western blot analysis.

The experiments and animal captures were performed with the approval of the institutional committees and the Italian Ministry of the Environment (DPI/2D/2006/16274).

Immunochemistry

Formalin-fixed, paraffin-embedded tissues were sectioned at 5-µm thickness. Only histologically normal tissues, as assessed by H&E staining, were processed for immunohistochemistry.

After deparaffinization and rehydration, the histological sections were washed in Tris-buffered saline (TBS) (50 mM Tris-Cl [pH 7.6], 150 mM NaCl) and preincubated for 20 min with normal rabbit serum (DAKO, Copenhagen, Denmark) diluted 1:10 in TBS, to prevent nonspecific binding. The slides were incubated overnight at 4°C with the following primary polyclonal antibodies: goat anti-human IL1B (50 ng/ml in TBS) or anti-human IL1R1 (50 ng/ml in TBS) (R&D Systems, Abingdon, UK). The slides were then washed three times with TBS for 5 min, and incubated for 30 min with rabbit anti-goat biotinylated secondary antibody at a dilution of 1:500 (DAKO). After three washes with 0.1% PBST for 10 min each, the secondary antibody, horseradish peroxidase (HRP)-conjugated rabbit anti-goat antibody, was applied at a dilution of 1:1000, and the signals on the membranes were detected by the West Pico chemiluminescent substrate (Pierce, Rockford, IL).

**RESULTS**

General Organization of the Caudal Portions of the Oviducts

In the aplacental viviparous *S. lanzai*, histological analysis of the uterus during early pregnancy revealed that the uterine wall contained numerous (often branching) folds separated by deep furrows (Fig. 1A). It consisted of three layers: an inner monolayered luminal epithelium, a thick layer of connective tissue, and an outer sheath of circular and longitudinal muscle fibers (Fig. 1A). The well-developed connective tissue was highly vascularized, and an extensive capillary network extended beneath the uterine lining. The latter comprised a monolayer of cuboidal or columnar cells of a single cell type. Their nuclei were basal and intensely heterochromatic, while the apical regions of most of the cells contained numerous ovoid PAS positive vesicles (Fig. 1B).

For the females of *T. carnifex*, which were collected during the reproductive season, cross-sections of the caudal portions of the oviducts revealed numerous radial plicae that projected towards the oviductal lumen (Fig. 1C). The luminal lining consisted of two cell types: columnar cells with basal nuclei filled with secretory material in their central and apical cytoplasm, and numerous ciliated cells inserted among the columnar cells (Fig. 1, C and D). The epithelial cells adhered to a thin sheet of connective tissue that formed the axis of each plica. A very thin layer of poorly vascularized connective and muscle tissue formed the outermost portion of the oviductal wall (Fig. 1C).

In the females of most anurans, such as *B. bufon*, the major component of the caudal portion of the oviductal wall is a high palisade of tubular jelly glands that open along the oviductal lumen (Fig. 1E). The glands consisted of cells with basal nuclei and abundant secretory products, which were almost transparent and weakly eosinophilic. At the luminal surface, cuboidal or low columnar ciliated epithelial cells were grouped to form shallow cup-shaped profiles between the glands (Fig. 1F). A blood vessel was present at the base of each group of ciliated cells. The outermost portion of the oviductal wall was formed by an extremely thin layer of poorly vascularized connective and muscle tissues.
Immunohistochemistry

Marked differences were noted between the viviparous, oviparous, and ovuliparous species with regard to immunostaining for IL1B and IL1R1 (Table 1).

*S. lanzai.* Immunohistochemistry for IL1B in the uterine wall showed strong and widespread immunoreactivity in the luminal epithelial cells, mostly in their basal portions and in the nuclei (Fig. 2A). The apical regions of the cells, which contained large amounts of secretory products, were unstained. Intense IL1B immunoreactivity was also detected in many cells of the connective tissue, in the endothelial cells of the uterine blood vessels, and in the muscle layer (Fig. 2A). IL1R1 was expressed in the luminal epithelial cells, mostly in the basolateral cytoplasm and on the luminal surface (Fig. 2B). The nucleus and the apical (secretory) compartment were mostly unstained. Connective cells, vascular endothelium, and numerous muscle cells were positively stained.

*T. carnifex.* Immunoreactivity for IL1B was detected in the ciliated luminal cells (Fig. 2C). Some immunoreactivity was also present in the cytoplasm of some glandular cells, as well as in some muscle cells. Low-level immunoreactivity was observed in the connective tissue, whereas the secretory granules were unstained (Fig. 2C). IL1R1 was present mainly in the luminal portions of numerous ciliated epithelial cells (Fig. 2D). Evidence of immunostaining was also observed in the basolateral areas of some secretory cells, in the endothelia of the blood vessels, and in the muscle layer. The nuclei of secretory and ciliated cells were unstained.

*B. bufo.* IL1B was detected in a very thin portion of the apical cytoplasm of the ciliated epithelial cells (Fig. 2E). Some immunoreactivity was detected in the endothelia of the vessels at the base of the groups of ciliated cells. No staining was observed in the large glandular portion of the oviductal wall or in the muscle layer.

Similarly, IL1R1 expression was detected in the luminal membrane of the ciliated epithelial cells (Fig. 2F). Slight immunoreactivity was observed in the blood vessels at the base of the ciliated cells and in the muscle sheet beneath the peritoneal layer. Control sections for all three species did not reveal any specific positive staining for IL1B or IL1R1 (Fig. 2, G and H).

Western Blot Analyses

We performed Western blot analyses on oviductal tissue lysates of *S. lanzai, T. carnifex,* and *B. bufo* to identify the
molecular masses of the immunoreactive IL1B and IL1R1 proteins (Fig. 3, A and B).

**IL1B.** Total proteins (50 μg) from each species were run in parallel with human rIL1B (Fig. 3A). Two bands were detected for IL1B, which correspond to the predicted 17-kDa mature form and the 34-kDa dimeric form. In oviductal tissues of *S. lanzai*, the polyclonal anti-human antibody recognized a putative IL1B protein of about 20 kDa, which most likely corresponds to the mature form of IL1B in this species. In *B. bufo* and *T. carnifex*, one band was observed at approximately 31 kDa, which probably corresponds to a pro-IL1B protein [27, 28]. An additional band at approximately 12 kDa was detected in all three amphibian species.

**IL1R1.** Total membrane proteins (50 μg) from each species were run in parallel with 30 μg of human placenta lysates at term (positive control) (Fig. 3B). A band that corresponds to the predicted 80-kDa human IL1R1 was revealed in human placental tissue, as well as in *S. lanzai* and *T. carnifex*. No band was detected in *B. bufo*.

**DISCUSSION**

IL1 is a key regulator of the inflammatory response and plays important roles in reproductive processes [29, 30]. Two

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**TABLE 1. Immunoreactivity for IL1B and IL1R1 in oviductal tissues of amphibians during the reproductive season.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Luminal epithelium</th>
<th>Blood vessels</th>
<th>Connective and muscle tissue</th>
<th>Secretory elements</th>
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<td><em>S. lanzai</em></td>
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<td><em>T. carnifex</em></td>
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<td><em>B. bufo</em></td>
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* Immunoreactivity is estimated on a scale of − to ++: − (unstained), +/− (weak), + (moderate), ++ (intense) staining.

**FIG. 2.** Immunohistochemical localization of IL1B (A, C, E) and IL1R1 (B, D, F) in oviductal tissues of *S. lanzai* (A, B), *T. carnifex* (C, D), and *B. bufo* (E, F). Positive staining is shown in red. **A** The expression of IL1B is intense and widespread in the luminal epithelial cells, mainly in the basal portion (large arrows) and in the nucleus (asterisk in the insert). Cells of connective tissues (bifurcated arrows) and endothelial cells of the blood vessels (thin arrows) are also highly immunoreactive. The apical portions of the epithelial cells are completely unstained (arrowhead). **B** Strong immunoreactivity for IL1R1 in the basolateral and perinuclear portions of most luminal epithelial cells (large arrows) and in the cells of the connective tissue (bifurcated arrow). The apical portions of the luminal cells are unstained (arrowhead). **C** IL1B immunoreactivity is present in the ciliated epithelial cells (big arrows) and, to some extent, in the cytoplasm of the secretory cells (thin arrow). Secretory granules are unstained. Insert shows intranuclear immunoreactivity in ciliated epithelial cells (arrowhead). **D** IL1R1 immunoreactivity is mainly present in the luminal portions of ciliated cells (large arrows). No staining is detected in the nuclei. **E** and **F** Immunoreactivities are confined to the upper portions of the ciliated epithelial cells (large arrows), whereas the glands are completely unstained. Note the immunoreactivities for IL1B and IL1R1 in the blood vessel at the base of the ciliated epithelial cells (arrowheads). **G, H** Control sections of *S. lanzai* (G) and *T. carnifex* (H). No staining is observed when the anti-IL1B or anti-IL1R1 primary antibodies are substituted with Tris-buffered saline.
forms of IL1 agonists (IL1A and IL1B) bind to the same membrane receptor (IL1R1) and have similar, if not identical, biological activities [31]. The third member of the IL1 ligand family is the natural IL1 receptor antagonist (IL1RN), which can block the binding of IL1 agonists to the specific membrane receptor, thereby inhibiting signal transduction [32]. Numerous studies have shown the contribution of the IL1 system to uterine receptivity in mammals [33, 34]. In humans, IL1B and IL1R1 have been detected in the endometrium throughout the menstrual cycle, with maximal expression of protein and mRNA during the luteal phase, which is the period of embryonic implantation [35]. More recently [36], expression of the IL1 system, which includes IL1B, IL1RN, and IL1R1, has been demonstrated in the fallopian tubes, which are the sites of oocyte fertilization and early embryonic development. Similarly, studies in mice have shown that the IL1 system is expressed by the uterus, with maximal expression during the peri-implantation period [37]. Interestingly, animal experiments have shown that blockage of the IL1 receptor by intraperitoneal injection of IL1RN from Day 3 to Day 6 of pregnancy (the time of blastocyst implantation) inhibits embryonic implantation in mice [19]. It has been reported that IL1RN interferes with embryonic attachment via a direct effect on the endometrial epithelium as a result of downregulation of integrins $\alpha_4$, $\alpha_5$, and $\beta_3$ [38]. These data suggest that the IL1 system plays a critical role in maternal receptivity to the semi-allogeneic embryo.

In the last ten years, we have conducted an evolutionary study of the reproductive tissues of nonmammalian vertebrates, including squamate reptiles and elasmobranch fishes, in which different forms of placentation have evolved [20, 21]. We have also studied $L$. vivipara, a species that is characterized by reproductive bimodality with oviparous and viviparous populations, depending on the habitat [23]. We have shown that the IL1 system, including the two IL1 isoforms and the specific membrane receptor IL1R1, is expressed by the uterine mucosa irrespective of the reproductive mode [39].

In the present study, we detected expression of the IL1 system in the oviductal tissues of amphibians, a class of vertebrates in which the role of cytokines in reproduction has never been investigated. Amphibians include species with internal fertilization, in which the fertilized eggs merely transit through the uterus (oviparity) or are retained for some time or until full development of the embryo (viviparity) [2, 3, 10, 24]. In all these species, paternal-derived antigens are in contact with maternal reproductive tissues, albeit for different periods of time. Amphibians also include species in which the eggs are fertilized outside the female body (oviparity) and thus, paternal antigens do not come into contact with the maternal tissues. Given their various reproductive modes, amphibians are of particular interest for studies on the roles of cytokines, e.g., IL1, in the evolutionary transition towards viviparity.

To investigate the role of IL1 in amphibian reproduction, we evaluated the expression of IL1B and its functional membrane receptor IL1R1 in oviductal tissues of the viviparous $S$. lanzai, the oviparous $T$. carnifex, and the ovuliparous $B$. bufo. In $S$. lanzai, most of the cells in the uterine mucosa were immunoreactive for IL1B and IL1R1. These included the epithelial cells lining the uterine lumen, the cells of the underlying connective tissue, and numerous blood vessels. Expression of IL1R1 in the epithelial cells was mainly localized to the basolateral cytoplasm and the luminal surface. In $T$. carnifex, the ciliated cells intercalated with the glandular cells showed immunoreactivities for IL1B and IL1R1. Evidence of IL1B was also found in glandular cells, mainly in the nucleus. The connective tissue was very scarce and did not show significant immunoreactivity. In the ovuliparous $B$. bufo, the expression of IL1B and IL1R1 was limited to the apical cytoplasm of the ciliated epithelial cells.

Our findings are the first evidence of expression of the IL1 system in the oviductal tissues of amphibians and, more interestingly, of vertebrates with external fertilization. In species with external fertilization, there is no contact with paternal-derived antigens. However, in ooviparity, as in oviparity and viviparity, the reproductive tract undergoes hormonally mediated cyclical changes, particularly by means of steroid modulation [2, 40, 41], to ensure that ovulation and egg envelope formation occur. Therefore, the expression of the IL1 system in reproductive tissues of vertebrate species with external fertilization may be an integral part of this hormonal control. Moreover, it is noteworthy that the caudal portion of the oviduct readily comes in contact with antigens in the aqueous environment. Therefore, the expression of IL1 and of its functional membrane receptor in the oviductal epithelial lining may suggest a mucosal immune response to environmental antigens, possibly mediated by IL1.

In both oviparity and viviparity, paternal-derived antigens are present in the maternal reproductive tract when: 1) the spermatophore is stored inside the female reproductive apparatus, and 2) when the fertilized eggs transit through the cloacal lumen or are retained in the maternal uterus until full or partial embryonic development. In terms of immune challenge,
these two reproductive modes differ with regard to the duration of exposure of maternal tissues to paternal antigens. Our present study of amphibians shows that IL1B and IL1R1 expression is present in oviparity and in viviparity, which suggests a common role for the IL1 system in species with internal fertilization, which is potentially crucial for the maternal immune response to paternal-derived antigens.

In the Western blot analyses, we showed that a putative mature form of IL1B of approximately 20 kDa was present in the oviductal tissues of S. lanzai, whereas a different form of approximately 31 kDa, which likely corresponds to an inactive pro-IL1B protein, was detected in T. carnifex and B. bufo. In amphibians, the gene for IL1B has been cloned from Xenopus laevis and shown to have high (48%) homology with the corresponding human gene [42, 43]. Furthermore, IL1 bioactivity has been reported in the same species, in different cell types, including peritoneal cells and thymocytes [44]. Jelaso et al. have demonstrated in embryos of X. laevis the presence of a putative IL1B protein, with molecular masses of 17 kDa and 31 kDa for the mature and precursor forms, respectively [45]. Our current findings show a putative mature form of IL1B in the oviductal tissues of S. lanzai and a putative pro-IL1B in T. carnifex and B. bufo, which together may indicate an active biological role for IL1B in the reproductive processes of viviparous species. Further studies are required to define the bioactive form of IL1B in the reproductive tissues of vertebrates with different reproductive modalities.

An immunoreactive band of 80 kDa that corresponds to the putative IL1 type I receptor was detected in both S. lanzai and T. carnifex, which suggests a potential role for IL1B in the oviductal tissues of these amphibian species. The scarce IL1R1 immunoreactivity in B. bufo, being confined to the outermost portion of the ciliated epithelial cells, may explain the lack of a band that corresponds to a putative IL1R1 in the oviductal tissues of this oviparous species.

The present study provides the first evidence of an immunological response, mediated by cytokines, in the female reproductive tissues of amphibians during the reproductive phase. We hypothesize that the observed variations in cytokine expression reflect the different roles these molecules play in the various reproductive modes. In external fertilization, expression of the IL1 system can be a sign of uterine mucosal immune response to antigens present in the aqueous environment. This response is amplified in oviparity and viviparity, possibly because of the presence of paternal-derived antigens in the maternal reproductive tissues. On these bases, it can be speculated that the IL1 system is an important mediator of the evolution from oviparity to viviparity.

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REFERENCES