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Sex steroid sensitivity of developing bursa of Fabricius

TIMO YLIKOMI and PENTTI TUOHIMAA

Department of Biomedical Sciences, University of Tampere
Box 607, 33101 Tampere, Finland

ABSTRACT. Sex steroid sensitivity of the bursa of Fabricius (BF) was studied from the early embryonic time until its regression. Expression of progesterone receptor (PR) served as a dual marker: first, as a marker for progesterone sensivity and second, as a marker for estrogen action, since it is an estrogen-induced protein. The progesterone binding molecule in the bursa was characterized by different chromatography methods and by steroid binding studies. We showed that it fulfils the criteria of a progesterone receptor by binding, structural and immunological properties. With immunohistochemistry and with the combined techniques of immunohistochemistry and autoradiography we demonstrated two cell types which express the PR: smooth muscle cells surrounding the BF and stromal cells located under the bursal epithelium and between the lymphoid follicles. The epithelium and the cells inside the lymphoid follicles were negative. Using immunoelectron microscopy the PR-expressing stromal cells were shown to be fibroblasts. The cloacal mesenchyme, from which the BF develops, was shown to be sensitive to exogenous estrogen very early during the embryonic time. The mesenchyme around and inside the developing BF reached estrogen sensitivity a few days later. The estrogen-sensitive mesenchymal cells were first seen surrounding the bursal primordium and later in the center of the plicae. During a natural sexual maturation without exogenous estradiol an expression of the PR was detected much later, at the age of 10-12 weeks after hatching. This expression correlates with the onset of the bursal regression and with the increase of the sex steroid levels in the blood. In the oviduct stroma PR was undetectable before the onset of sexual maturation. In the oviduct stroma PR becomes detectable a few weeks earlier than in the bursa.

KEY WORDS: *progesterone receptor, chick, ontogeny, bursa of Fabricius*

Introduction

The bursa of Fabricius (BF) is an immunological organ in birds, required for B lymphocyte maturation. The BF, as well as the other central immunological organs, undergoes regression during sexual maturation (Wolfe *et al.*, 1962). When the sex steroids are exogenously administered, bursal involution is accelerated (Kirkpatrick and Andrews, 1944; Erickson and Pincus, 1966). In the oviduct, as well as in the other genital organs, the phenomenon is the opposite. These organs start to grow and differentiate during puberty and by exogenous sex steroid administration. The mechanism whereby these opposite effects are mediated has not been established.

The first anlage of the bursa appears in the chick embryo on the 4th day of incubation as a thickening of the cloacal epithelium. The bursa then starts to form a sac-like structure dorsal to the cloaca and later longitudinal folds of the surface epithelium project into the lumen (Metcalf and Moore, 1971). When the hemopoietic cells reach the epithelium, it starts to proliferate and the epithelial buds are formed. These develop into follicular medullas and the surrounding mesenchyme forms the cortex and the interfollicular space (Le Douarin *et al.*, 1982; Ratcliffe, 1985; Toivanen and Toivanen, 1987). The medulla and the cortex are separated by a basement membrane, but in the cortex the network of the mesenchymal cells is continuous with the surrounding connective tissue. The outermost layer of the bursal wall is a thin serosa under which there are two layers of obliquely-running smooth muscle (Frazier, 1974; Hodges, 1974). The size of BF increases up to the age of 10-12 weeks. Thereafter it starts to involute. The subepithelial and interfollicular connective tissue increases (fibrosis) and the follicular cortex narrows. The involution process is almost complete by the age of 24 weeks when the degenerated lymphoid tissue is replaced by fibrotic tissue (Naukkarinen and Sorvari, 1984).

Hemopoietic stem cells enter the BF at the 8-14th days of embryonic development. The cells which enter the epithelial buds differentiate into lymphocytes and those which remain in the surrounding mesenchyme differentiate into granulocytes. After reaching a certain degree of maturity they seed out to other lymphoid organs. B-lymphocyte maturation takes place before the age of 3-5 weeks after hatching and thereafter it functions as a peripheral immunological organ (Le Douarin *et al.*, 1982; Naukkarinen and Sorvari, 1984; Ratcliffe, 1985; Toivanen and Toivanen, 1987).

Exogenous sex steroids have been shown to affect embryonic development of the BF. Effects of androgens have been most extensively studied. When high doses of testosterone are injected into eggs at 5 days of incubation, there is a complete absence of the bursa after hatching. This has been used as a hormonal bursectomy. Hormone treatment at the time of follicle formation (11-12 days of incubation) stops bursal maturation and lymphopoiesis never occurs. When the hormone is administered after follicle development, these disappear and bursal epithelium returns to an undifferentiated state (Erickson and Pincus, 1966; Glick, 1977; Le Douarin *et al.*, 1980; Verheul *et al.*, 1986). These works suggest that sex steroids may affect bursal development *in vivo* during embryonic life. The results, however, only suggest steroid sensitivity, but tell nothing of the *in vivo* effects of the endogenous steroids or the mechanism through which the steroids mediate their effects.

We have studied the expression of the progesterone receptor (PR) from embryonic time until bursal regression. The expres-

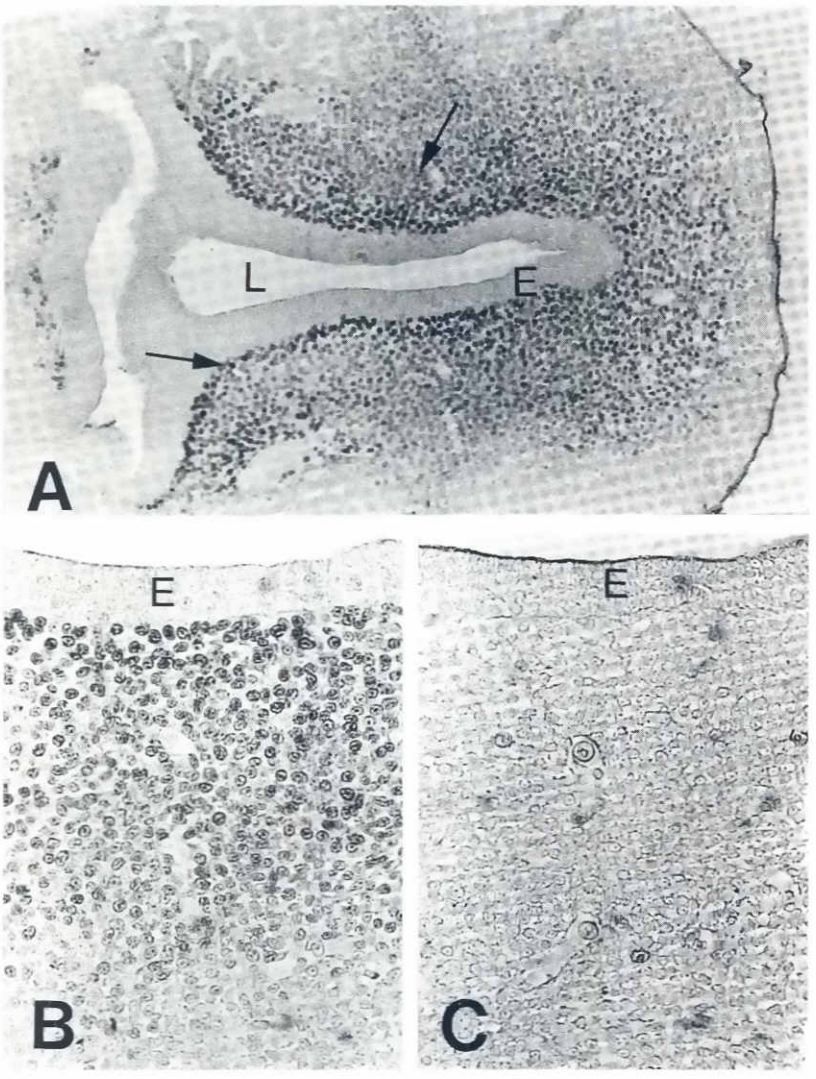

Fig. 1. *Progesterone receptor immunohistochemistry of cloacal region of estrogen-treated chick embryo. The embryo received estradiol benzoate (0.1 mg;embryo) in the allantoic sac on day 3 of incubation and was killed on day 6 Figs. A and B are stained with the progesterone receptor antibodies. Fig. C is a control stained with ovalbumin, which is a secretory protein in the oviduct and is not expressed in the cloaca. The PR staining is seen in the nuclei of mesenchymal cells, but not in the epithelium. The cloacal area, from which the bursa is derived, seems to be estrogen-sensitive very early. Arrows, PR-expressing cells; E, cloacal epithelium; L, cloacal lumen.*

sion of PR has been used as a marker for two phenomena. First, since it mediated the progesterone effects, it serves as a marker for progesterone sensitivity. Second, since it is induced by estradiol it serves as marker for estradiol action. We have shown that the bursa of Fabricius is sensitive for exogenous estradiol early during the embryonic time, but effects of endogenous progesterone and estrogen on the BF are minimal during embryonic life. The appearance of PR in the bursal and oviductal stromal fibroblasts during the course of sexual maturation correlates with regression of the bursa of Fabricius and with the growth and differentiation of the oviduct. This suggests that the BF is also affected by sexual maturation-associated factors and that the steroid effects in these organs are mediated through sex steroid sensitive stromal cells.

Results and discussion

Characterization of PR in the bursa of Fabricius

After demonstrating progesterone binding sites in the BF, we studied whether the properties of the binding fulfil the criteria of a steroid receptor. This was approached by comparing the properties of the binding site with the properties of the progesterone receptor of chick oviduct, which is a known progesterone target organ. Basic criteria for a steroid receptor are specificity for the appropriate steroid and limited binding capacity. The bursal PR binds progesterone with high affinity, the dissociation constant being approximately the same as with the PR in the oviduct. The binding is specific for progestins. The binding capacity in the bursa is also heat-labile (Ylikomi *et al.*, 1985; Ylikomi, 1986).

A typical feature of steroid receptors is the formation of an oligometric structure at a low ionic strength. This structure is transformed into a monomeric form at a high ionic strength (Grody *et al.*, 1982). In the presence of Ca++ ions, probably by calcium-activated proteases, steroid receptors are cleaved into a mero form (Sherman *et al.*, 1978). When the bursal receptor was analyzed with size-exclusion HPLC or by sucrose gradient centrifugation, the oligomeric form, the monomeric form and the mero were identical with corresponding forms of the chicken oviduct progesterone receptor (Ylikomi, 1986). The surface charges of steroid receptors have been studied by DEAE

chromatography. The DEAE-chromatography of the bursal PR gave a similar two-peak elution pattern as the chromatography of the oviduct PR (Ylikomi, 1986). By using immuno-blotting and sucrose density gradient centrifugation we showed that the bursal PR was detected by the antibodies to chicken progesterone receptor (Ylikomi *et al.*, 1987c).

Thus, the progesterone-binding molecules in the bursa of Fabricius share the general properties of steroid receptors. In addition, the binding properties, structural properties and immunological properties are similar to those of the chick oviduct progesterone receptor.

Localization and characterization of the cells expressing PR in the Bursa of Fabricius

Gasc and Stumpf (1981 a, b) have demonstrated mesenchymal cells which concentrate estrogen and androgen in the embryonic bursa. We have shown that these mesenchymal cells express the PR after estrogen treatment, indicating the presence of functional estrogen receptor. The cells expressing PR were first seen surrounding the bursal primordium and later inside the bursal plicae (Fig. 1; Fig. 2; Ylikomi *et al.*, 1987a). After hatching, two cell types expressing PR were detected: subepithelial-interfollicular cells and smooth muscle cells surrounding the BF. Neither the bursal epithelium nor the cells inside the lymphoid follicles expressed the PR (Fig. 3; Ylikomi *et al.*, 1987b). With a combined technique of immunohistochemistry and autoradiography we showed that the cells which were stained with the antibody are also able to concentrate tritiated progestin in their nuclei, indicating that they are real target cells for progesterone (Ylikomi *et al.*, 1987b).

Immunoelectron microscopy and non-specific esterase histochemistry were used to characterize the cells which express PR. By electron microscopy these resemble fibroblasts described by Frazier (1974). Their cytoplasm was rich in rough endoplasmic reticulum indicating active protein synthesis. Since fibroblasts cannot be distinguished from macrophages by histological criteria, we used non-specific esterase histochemistry to stain the macrophages. This showed that the macrophages and the PR-expressing cells were located in different compartments of the BF (Ylikomi *et al.*, 1987c).

It is concluded that in the BF there are two estradiol-sensitive cell types expressing progesterone receptor: interfollicular-subepithelial fibroblasts and smooth muscle cells surrounding the bursa.

Estrogen and progesterone sensitivity of the BF

We have shown that the PR is expressed in the cloacal mesenchyme after estradiol treatment at the age of 6 days of incubation (Fig. 1). This indicates the presence of functional estrogen receptors very early. A few days later some mesenchymal cells in the cloacal area were shown to express low levels of the PR without exogenous estradiol (Ylikomi, 1987a). Thus the cloacal area, from which the BF is derived, is sex steroid-sensitive very early. It is conceivable that these mesenchymal cells mediate sex steroid-related morphogenetic changes in the cloaca during embryonic development. In the bursal mesenchyme PR is also estradiol-inducible from the age of 10-11 days of incubation, but is not expressed spontaneously during embryonic development without exogenous estradiol (Fig. 3; Ylikomi *et al.*, 1987a). Since the endogenous estrogens are not able to induce PR in the BF, they probably do not have a pronounced effect on the embryonic bursa. The lack of detectable amounts of the PR in the BF also implies that the embryonic bursa is probably not directly progesterone sensitive.

The progesterone receptor is spontaneously expressed in the BF during sexual maturation at the age of 10-12 weeks after hatching. The PR is similarly expressed in the oviduct stroma at the age of 8-10 weeks after hatching. The bursal stroma thus expresses the PR a few weeks later than the oviduct stroma, probably indicating lower sensitivity to estrogens or other factors associated with sexual maturation. The onset of bursal regression takes place at the age of 9-12 weeks, thus correlating with the expression of PR in the bursal stroma (Naukkarinen and Sorvari, 1984). The oviduct epithelium starts to proliferate

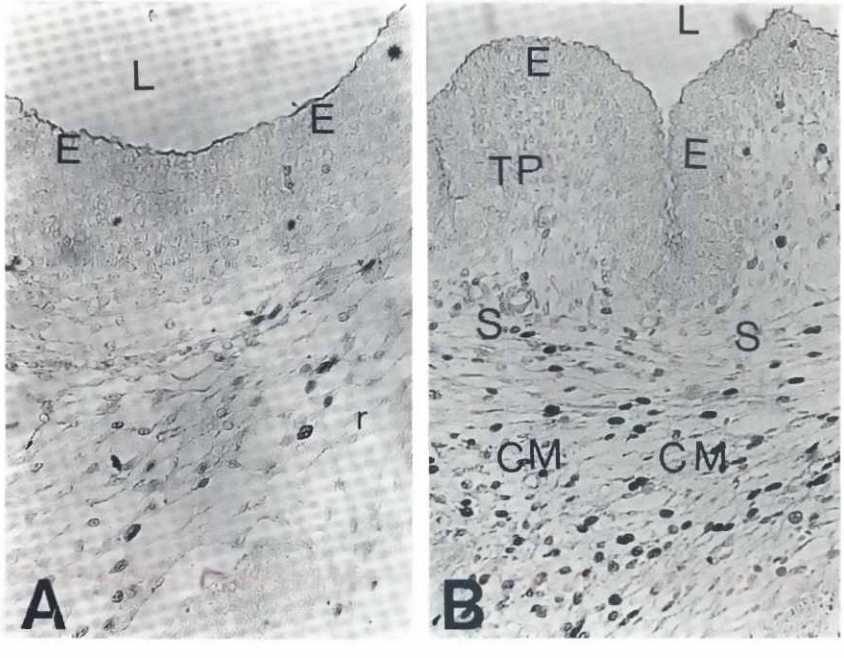

Fig. 2. *Immunohistochemistry of the progesterone receptor in the mesenchyme surrounding the bursal primordium in 9-day old embryos (A) and in 11-day old embryo (B). The embryos received 0.1 mg of estradiol 3 days before sacrifice. At the age of 9 days there is a weak positive staining and at the age of 11 days there are numerous strongly positive cells. This indicates that the mesenchyme around bursal primordium becomes estrogen sensitive later than the mesenchyme surrounding the cloaca (see Fig. 2). Arrows, PR-expressing cells; E, bursal epithelium; L, bursal lumen; TP, tunica propria of bursal primordium; S, serosal and muscular layers of bursal primordium; CM, cloacal mesenchyme.*

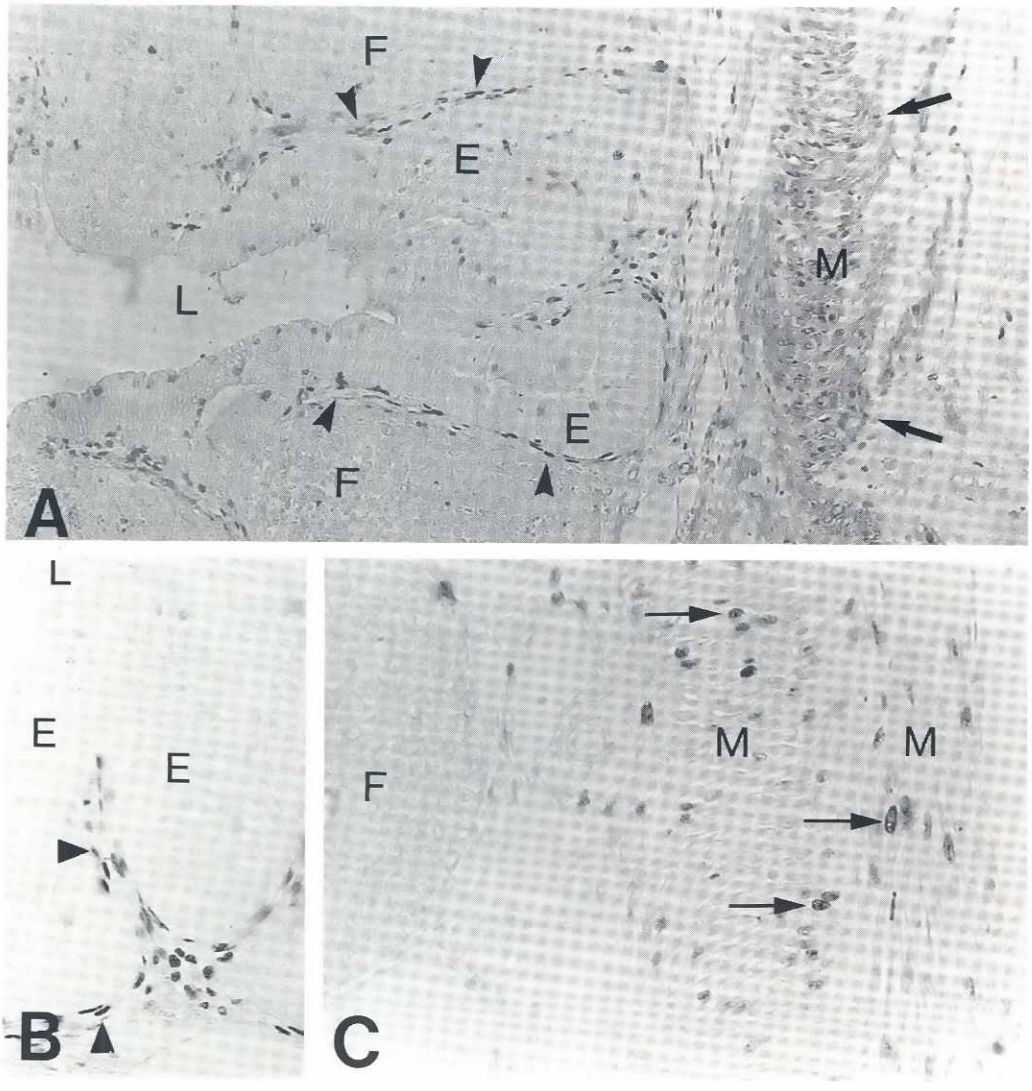

Fig. 3. *Progesterone receptor immunohistochemistry of the bursa of estrogen-treated chicks after hatching. There are two cell types which express the progesterone receptor: smooth muscle cells surrounding the BF (arrows) and stromal cells under the epithelium and between the lymphoid follicles (arrow heads). The receptor is not expressed in the bursal epithelium or inside the lymphoid follicles. M; smooth muscle cells at the bursal wall; F, lymphoid follicle; E, bursal epithelium; L, bursal lumen.*

A, B, C, different magnifications (50x, 80x, 100x).

at the age of 8-10 weeks correlating with the expression of the PR in the stroma. Later the PR-expressing stromal cells are seen in the center of oviduct plicae. When the epithelium starts to differentiate the stromal cells expressing PR are seen in the near vicinity of these epithelial cells (Ylikomi and Tuohimaa, 1988). These phenomena correlate with the increase in the sex steroid level in the blood. It is thus conceivable that the effects of sex steroids on oviduct differentiation and bursal growth are mediated through these stromal cells. PR was also inducible by exogenous estrogen in the male BF, although it is not spontaneously expressed to detectable levels during maturation (Ylikomi *et al.*, 1987b). Estrogen treatment has been shown to inhibit cell profileration in male and female ducklings (Gupta *et al.*, 1981; Bhat and Maiti, 1982).

Thus the bursa of Fabricius in both sexes is sensitive to exogenous estrogen from a very early embryonic stage. Notable effects of the endogenous estrogens on the BF, however, start only with the onset of sexual maturation. Direct progesterone sensitivity is likewise probably not achieved until the onset of sexual maturation. This difference between inducibility and natural expression of genes has also been detected in other systems. For instance, vitellogenin (a precursor for different egg yolk proteins) is estrogen-inducible in *Xenopus Laevis* larva during metamorphosis. The natural expression of the protein takes place only in sexually mature females, probably induced by endogenous estrogens (May and Knowland, 1981).

Possible effects of estrogen in the BF

There are studies demonstrating that exogenous estrogens and progesterone affect the development of the BF when given *in ovo*. We have shown that estrogen administration actually exerts a specific effect on the BF by inducing the PR in mesenchymal cells. However, since the effect of endogenous estrogens is minimal and PR is not present in the embryonic bursa, it is likely that direct effects of endogenous estrogen and progesterone on embryonic development of the bursa are minimal.

There are no studies on the effects of sex steroids on lymphocyte maturation. BF is required for B-lymphocyte maturation up to the age of 3-6 weeks after hatching (Toivanen and Toivanen, 1987). Since the proper action of estrogen and progesterone is expected at the age of 10-15 weeks it is probable

that these steroids do not greatly affect functions of the BF as a primary immunological organ. The lack of PR expression in bursal lymphocytes and macrophages indicates that sex steroids probably do not directly affect these cells

After the age of 6 weeks the BF starts to function as a peripheral immunological organ serving as a site for antigen contact and antibody production until the age of about 16 weeks (Naukkarinen and Sorvari, 1984). Antigens are transported into the BF from the cloaca by the action of cloacal and bursal smooth muscle (Sorvari *et. al*, 1975) Thus it would be conceivable that the estrogen and progesterone affect antigen contact by the sex steroid-sensitive smooth muscle. The lumen becomes plugged during involution. It may be that estrogen and progesterone affect peristaltic movements of the bursal and cloacal musculature to favor the accumulation of tissue debris and mucus in the bursal lumen.

It is probable that the most pronounced effects of progesterone and estrogen are associated with bursal involution during sexual maturation. As in the oviduct, the bursal mesenchymal cells start to express PR after the onset of sexual maturation. The PR expression in the BF takes place a few weeks later than in the oviduct and coincides with the onset of bursal involution. The timing of the PR expression in the BF falls together with the onset of oviduct growth. This is probably a consequence of proliferation of the sex steroid-sensitive stromal cells. The BF undergoes mucoid degeneration during involution. The degenerated lymphoid tissue is then replaced by fibrotic tissue, the amount of which begins to increase subepithelially (Naukkarinen and Sorvari, 1984). We showed that the cells expressing the PR are fibroblasts, and that they are in the active state characterized by pronounced proteinsynthesis machinery. In analogy with the oviduct it is possible that sex steroids (estrogen and progesterone in females and androgens in males) stimulate the bursal fibroblasts. The whole tissue is then replaced by fibroblasts and extracellular matrix components synthesized by these. It is not known whether the stromal cells produce some factors, induced by sex steroids to depress lymphoid functions in the BF, or whether bursal involution is due solely to replacement of lymphoid tissue by fibrotic tissue. Thus in the oviduct the sex steroids provoke growth and differentiation through the stromal cells, whereas in the BF they provoke involution of the organ.

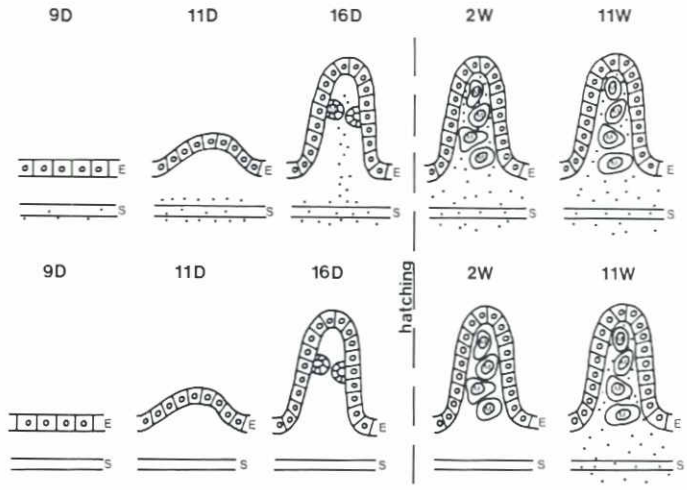

Fig. 4. *A schematic presentation of the ontogeny of estrogen-sensitive cells in the bursa of Fabricius. The progesterone receptor (PR) expressing cells are labeled with black dots. The upper panel shows cells which express PR after exogenous estrogen treatment. The lower panel shows cells which express PR during normal maturation (without exogenous estrogen). At 9 days of incubation (9 D) there are few PR-positive mesenchymal cells surrounding the bursal primordium after estradiol treatment. On day 11 (11 D) the reaction is stronger and the PR-expressing cells are more numerous. PR-expressing cells are mostly located in the muscle and serosal layer. On day 16 of incubation (16 D) estradiol induces the PR also in the center of the plicae. After hatching (2 W, two weeks after hatching or 11 W, 11 weeks after hatching) the estrogen-induced PR is seen in the subepithelial-interfollicular stromal cells and in the smooth muscle cells surrounding the bursa. During normal development (lower panel) PR is not seen in the bursa without exogenous estrogen before the onset of sexual maturation. First PR-expressing stromal cells are seen at the age of 10-11 days after hatching. The full expression of PR begins at the age of 15-17 weeks at the same age as the expression of PR in the oviduct stroma and the onset of growth and differentiation of the oviduct and the onset of the regression of the bursa of Fabricius. E, epithelium; S, serosal and muscular layers; D, days of incubation; W, weeks after hatching.*

Conclusions

The bursa of Fabricius seems to be a direct target organ for estrogen and progesterone. It is estrogen-sensitive from early embryonic time, indicated by the expression of the progesterone receptor by exogenous estrogen administration. There was, however, no sign of the effects of endogenous estradiol until the onset of sexual maturation long after hatching. Similar dissociation between inducibility and natural expression of the genes has also been detected in some systems.

Stromal cells, both in the bursa and oviduct, start to express progesterone receptor at the onset of sexual maturation. The expression correlates with the onset of bursal regression and with the growth and differentiation of the oviduct. Thus, it is probable that the sex steroid-induced regression of the bursa and differentiation of the oviduct is mediated through the sex steroid-sensitive stromal cells.

Materials and methods

1-23 week old chicks or chick embryos (at the age of 6 to 16 days of incubation) were used either untreated or treated with estradiol benzoate. The progesterone receptor was quantified by studying the binding of radioactive-labeled progestin in the cytosolic fraction. The receptor was characterized by three criteria. First, the binding properties were revealed by studying the ability of different steroids to bind the receptor. Second, the structural properties were studied by different chromatographic methods (sucrose gradient centrifugation, gel filtration, DEAE-cellulose, high performance liquid chromatography). Third, the immunological properties were studied by testing the ability of different progesterone receptor antibodies to recognize the receptor (For details, see Ylikomi *et al.*, 1985; Ylikomi, 1986). The receptor was localized by immunohistochemistry and by the combined technique of immunohistochemistry and autoradiography (Ylikomi *et al.*, 1978 a, b, c). The cells expressing the progesterone receptor were characterized by immunoelectron microscopy and by non-specific esterase histochemistry (Ylikomi *et al.*, 1978 c).

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