VISUALISATION OF THE UPTAKE OF PROLACTIN (PRL) IN RAT EMBRYONIC TISSUES

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ABSTRACT

Objective: Evidences implicate roles for prolactin (PRL) in the regulation of embryonic growth. To clarify the roles of PRL in rat embryogenesis we examined the uptake and expression of the hormone in embryonic tissues.

Methods: Nine and a half day postimplantation rat embryos were cultured in vitro for 44h in rat serum and serum depleted of low molecular weight molecules (retenate). The embryos were transferred to M199 for the last 4h, and 12.8 ng/ml rat PRL was added to culture medium for different times (4h - 15 min) and/or different temperatures (37°C and 4°C). As a control tissue, pituitary glands from 11.5 and 18.5d pregnant rats were used. Embryos and tissues were then examined by an indirect immunofluorescence protocol.

Results: The pituitary glands showed positive immunoreactivity for anti-PRL antibody whilst there was no stain in the control brain tissue. Immunoreactivity was observed in embryos grown in rat serum, and intensity was much greater in the presence of additional rat PRL, whilst there was no immunoreactivity detected in those grown in retenate only. Shorter incubations and incubations at 37°C caused a greater immunoreactivity for PRL, suggesting that this is an active and temperature dependent metabolic process.

Conclusion: These results show the uptake and distribution of PRL by the yolk sac and embryonic tissues which might be interpreted for the presence of PRL receptors.

Key Words: Prolactin receptors; Organogenesis; Embryo culture; Development

INTRODUCTION

Evidence from several studies implicates roles for PRL in the regulation of embryonic and fetal metabolism. For instance it has been shown that PRL circulates in fetal as well as the maternal serum (1). Also the presence of lactogenic binding sites and PRL receptor mRNA in embryonic (2) and fetal rat tissues in late gestation (3-6), and the high levels of PRL in amniotic fluid (7) suggest that the PRL may exert direct effects on embryonic and fetal metabolism. In agreement with these, we observed that cultured rat embryos are able to utilise exogenous PRL when cultured in serum depleted of low molecular weight molecules, and the additional PRL increases all growth and developmental parameters (8, 9). This implies that functional PRL receptors might be expressed during organogenesis.

Binding sites for PRL, detected using labelled ligands, have been reported in a variety of tissues and animal species (10). Boutin et al. (11) demonstrated the expression of RPL receptor gene by Northern blotting in the adult rat liver, ovary, prostate, testis, kidney, adrenal and mammary gland. These findings have recently been extented by in situ hybridisation (12). In the fetal rat, similarly to adults the biological actions of PRL appear to be mediated through binding to PRL receptors (2). Recently Freemark et al. (5) and Royster et al. (4) demonstrated that PRL receptor mRNA is expressed widely in fetal tissues and is induced in late gestation (embryonic days 17.5-20.5). However, apart from the study carried out by Freemark et al. (2) there appears to be no published data which mentions the presence of PRL receptor mRNA in the early stages of development. They determined the presence of PRL, receptor mRNA in the whole rat conseptus as early as days 9 and 10 of gestation.

However, the mechanism of the growth promoting effects of PRL in embryonic tissues is still not clear. PRL may activate embryonic growth via its own receptors as mentioned above. Therefore, after observing the growth promoting effects of PRL from different species, especially rat PRL on developing rat embryos in culture (8, 9), we examined the uptake of rat PRL and the expression of the hormone in rat embryonic tissues to clarify the roles of PRL in rat embryogenesis.

MATERIALS AND METHODS

Male and female Wistar rats (Rattus norvegicus) used in this study were obtained from the breeding colony of the animal house at Queen's Medical Centre, Nottingham University. Rats were mated overnight and the following midday on which the vaginal plugs were detected was assigned as 0.5 day of gestation. In order to control the efficiency of the immunofluorescein staining system which was planned to be used in this study, as a test tissue, pituitary glands from 11.5 and 18.5 day pregnant rats were excised after decapitation of the animals under anaesthesia. Also brain tissue from those animals was excised to use as the control tissue. Tissues were then placed into small foil blocks containing sufficient OCT embedding compound (BHD Chemicals, Poole, UK) to cover the tissue. These blocks were then immediately frozen by immersion in liquid nitrogen and stored in a -20°C freezer until required.

In order to observe the uptake of rat PRL and the expression of binding sites of the hormone in rat embryonic and extra embryonic tissues postimplantation rat conceptuses were explanted at day 9.5 and cultured in vitro for 44 hours using the technique of New (13) in whole rat serum and serum which has low growth supporting capacity (8, 9) (retenate prepared by ultrafiltration of serum for a period of 8h using Macrosep centrifugal concentrators with a molecular weight exclusion of 30kDa, therefore it does not contain low molecular weight (< 30kDa) growth supporting molecules such as EGF, FGF, IGFI-II, prolactin or placental lactogen). 1 ml retenate used for each embryo was supplemented with 2 mg/ml glucose and 10 µl/ml MEM vitamin solution (Sigma, Poole, UK). The conceptuses were then transferred to phenol red free Medium 199 (Sigma, Poole, UK) for the last 4h, and 12.8 ng/ml rat PRL (rPRL; Biogenesis, Poole, UK) was added to the culture medium for different time intervals (4h, 2h, 1h, 30min and 15 min) at 37°C. After the culture period embryos with their yolk sacs were removed from Medium 199 and washed in 1 % saline. Conceptuses were then arranged in OCT embedding compound, frozen down and stored at -20°C until required.

In order to establish if the uptake was an active process, low temperature incubations were performed. The experiment above was repeated, but this time after transferring the embryos to phenol red-free Medium 199 for last 4h, and 12.8 ng/ml rPRL was added to the culture medium for different times (4h, 2h, 1h, 30min and 15 min) at 4°C. These conditions had previously been shown to inhibit both fluid phase and absorptive pinocytosis (14). In all cases conseptuses were equilibrated at 4°C for 30 minutes before the addition of rPRL. Embryos were blocked and stored at -20°C.

Six to eight µm sections from the blocked tissues were cut using a Microm HM 505 E cryostat and mounted on glass multiwell slides (10 well). The sections were dried for 15 minutes at room temperature, and then placed in petri dishes and washed in PBS twice (2 minutes each wash), and then fixed in 3% paraformaldehyde (in PBS) for 10 minutes at room temperature. The fixed tissues were washed with PBS (containing 3% bovine serum albumin; BSA) twice (2min each) and divided into two groups; first four well used as a control, and the last 6 well used as an experimental. An indirect immunofluorescence protocol was performed as follows:

Sections were permeabilised in 0.05% triton X-100 (BDH Chemicals, Poole, UK) in PBS for 10min at room temperature, then washed in PBS using several changes of PBS and left in PBS for 1 hour at 4°C. PBS was removed from slides using a vacuum driven suction pump and first stage antibody (anti rat prolactin at a dilution of 1:100, Biogenesis, Poole, UK) added, 30µl to each experimental well, and 30µl of PBS to each control well. Slides were then incubated overnight at 4°C. Excess antibody was removed from the wells and they were washed in PBS twice (2min each). Following the removal of PBS, the second stage antibody (FITC-conjugated Anti rabbit IgG at a dilution of 1:20, Biogenesis, Poole, UK) was added to all wells and the tissue incubated for 1 hour at 37°C. Antibody was removed from the wells and they were washed in PBS for 30 minutes, and PBS was then removed from the wells.

For better observation of the tissues on the slides the nuclei of the cells were stained with 1:3 dilution of propidium iodide for five minutes at room temperature, then washed twice (10 min each) with PBS. After drying, the slides were mounted with antiphotobleaching mountant (Citofluor, Agar Scientific Ltd, Stansted, Essex, UK). The slides were then examined using an inverted Nicon AFX fluorescence and Leica TCS 40 confocal microscopes, photographed on Fuji Provia 100 and 1600 colour transperancy film respectively. For each experimental condition triplicate conceptuses were incubated on

each occasions. Ten sections, which were selected at intervals through the tissue block were viewed from each conceptus. The results from different embryos of the series were very consistent for any experimental condition and the pictures demonstrated are representative samples. In each case an independent worker also viewed the slides to at least partially validate the conclusions.

RESULTS

The sections from the pituitary glands obtained from 11.5 and 18.5day pregnant rats showed positive immunoreactivity for anti-PRL antibody (Figs. 1b 2b) whilst there was no stain in the sections from control brain tissue and the tissue from pituitary gland which were not preincubated with the primary antibody (Figs. 3a-b, 4a-b, 1a, 2a).

Immunoreactivity was observed in the yolk sacs and the embryos grown in retenate in the presence of additional rat PRL, whilst there was no immunoreactivity detected in those grown in retenate only. The yolk sacs and embryos grown in whole rat serum expressed less or no immunoreactivity compared to those cultured in the presence of exogenous rat PRL. Shorter incubations showed a greater uptake of PRL by the yolk sacs and distribution of PRL to the rat embryonic tissues (Figs. 5a-f, 6a-f).

Immunoreactivity was observed in the yolk sacs and the embryos grown in retenate in the presence of additional rat PRL incubated at 4°C. However, the intensity of immunoreactivity was much lower compared to those incubated at 37°C (Figs. 7a-c) the results indicate that uptake is greatly decreased by low temperature.

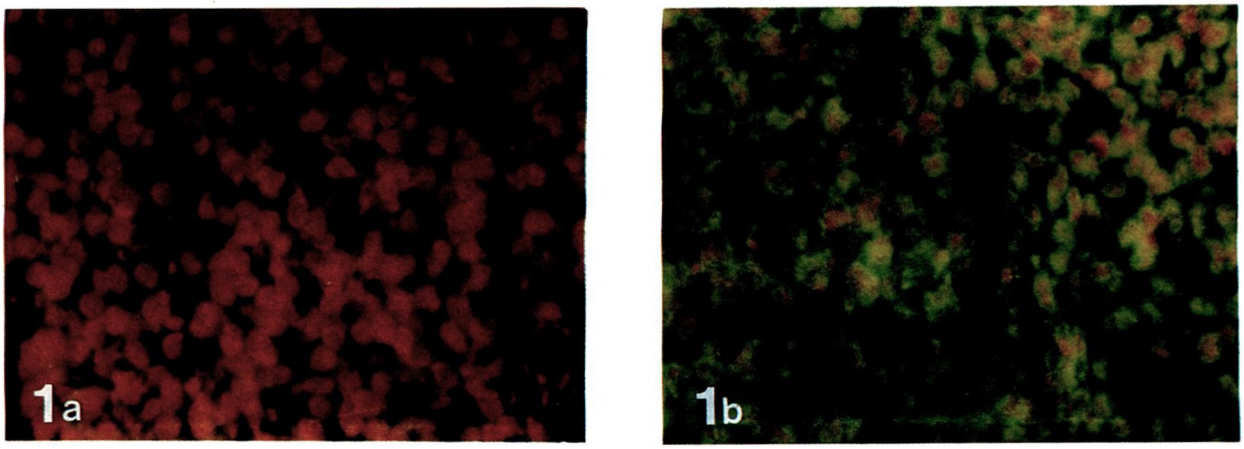


Fig. 1.: Immunofluorescence micrographs from 11.5 day pregnant rat pituitary tissue. Indirect immunofluorescent staining of the tissue with a) FITC alone, b) antibody recognising rat prolactin (magnification X 400).

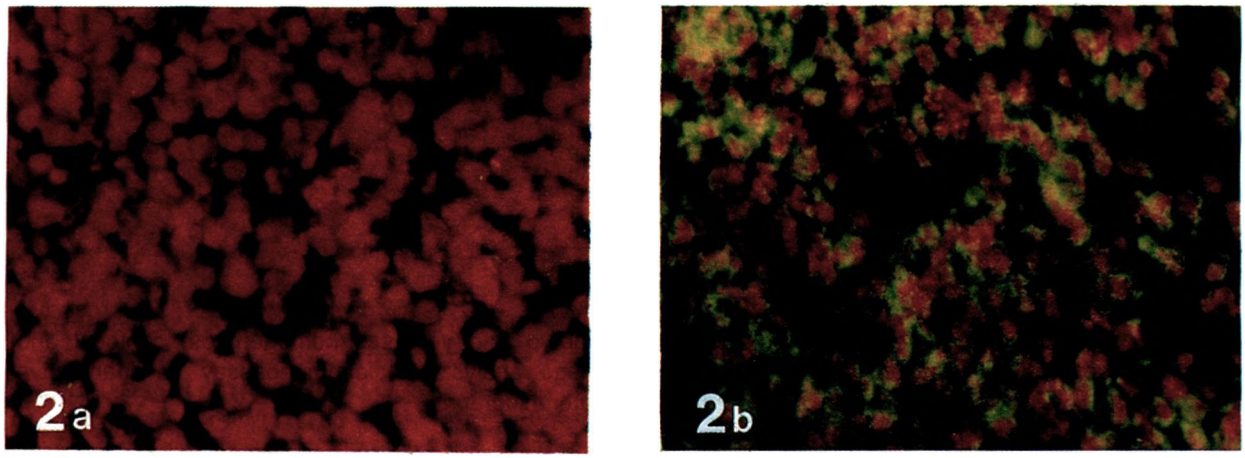
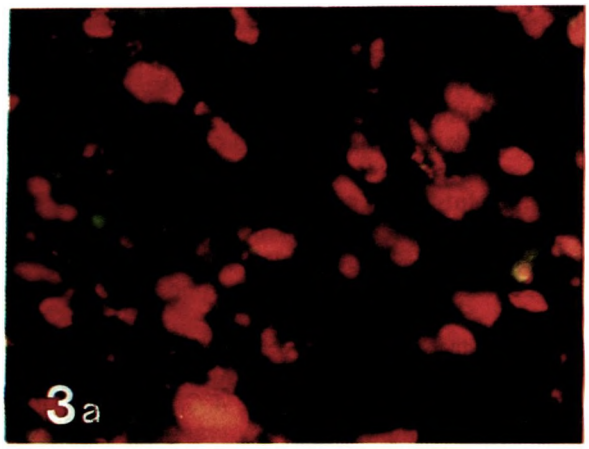


Fig. 2.: Immunofluorescence micrographs from 18.5 day pregnant rat pituitary tissue. Indirect immunofluorescent staining of the tissue with a) FITC alone, b) antibody recognising rat prolactin (magnification X 400).



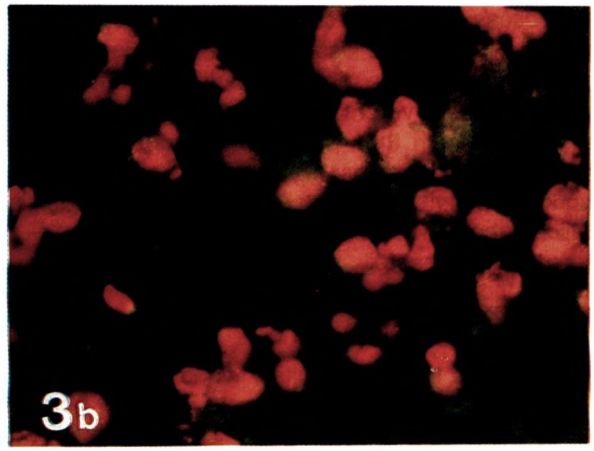
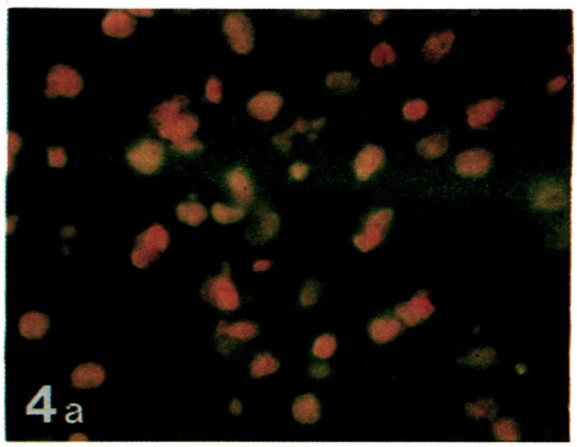


Fig.3.: Immunofluorescence micrographs from 11.5 day pregnant rat brain tissue. Indirect immunofluorescent staining of the tissue with a) FITC alone, b) antibody recognising rat prolactin (magnification X 400).



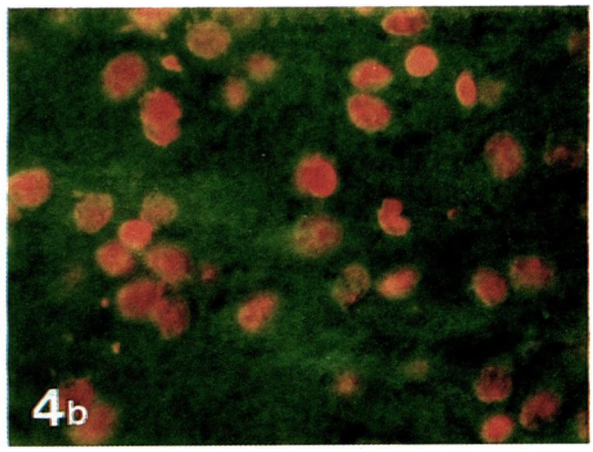


Fig.4.: Immunofluorescence micrographs from 18.5 day pregnant rat brain tissue. Indirect immunofluorescent staining of the tissue with a) FITC alone, b) antibody recognising rat prolactin (magnification X 400).

DISCUSSION

This study has demonstrated that there is an uptake and distribution of rat prolactin by the rat yolk sac and embryonic tissues at the stage of early organogenesis. Before trying to determine the uptake and distribution of PRL by rat yolk sac and embryo, it was necessary to test the reliability of the immunostaining protocol used in this study. Therefore, pituitary glands and control brain tissues were excised from 11.5 and 18.5 day pregnant rats, and an indirect immunofluorescence protocol was carried out. There was an intense staining for the rat anti-PRL antibody in the pituitary tissue whilst control brain tissue did not show any staining suggesting that the method used in this study is adequate to demonstrate the localisation of PRL. Previous studies have detected PRL receptor mRNA in the adult (12, 15) and fetal (4, 5) rat pituitary gland.

The study performed did not attempt to quantitate the extent of staining by any objective methods such as image analysis. However, although this study was qualitative it was performed in such a way as to give an indication of the extent of PRL binding under different conditions. Further studies would be performed to provide quantitative data, such as FACS (Fluorescence Activated Cell Sorter) assay of the embryo and yolk sac homogenates, confocal and image analysis, radioimmunoassay or quantitative autoradiography.

PRL uptake and distribution were detected in the yolk sacs and the embryos grown in rat serum in the

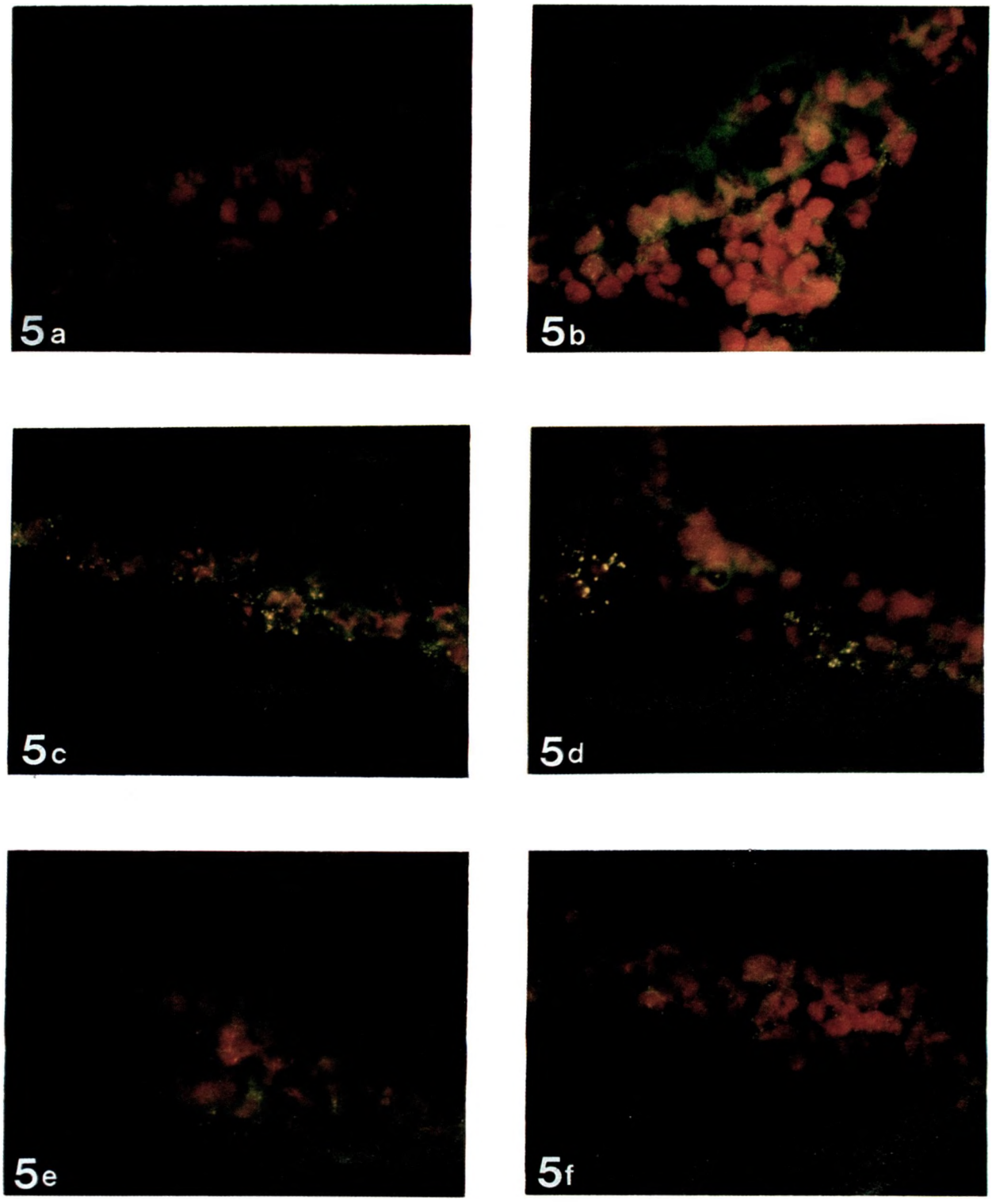


Fig.5.: Uptake of rat prolactin by 11.5 day rat visceral yolk sacs. Immunofluorescence micrographs from yolk sacs cultured in (a) retenate, (b) 4h, (c) 2h, (d) 1h, (e) 30 min and (f) 15 min incubations with rat prolactin at 37°C (magnification X 400). Showing increased uptake of rat prolactin in shorter incubations.

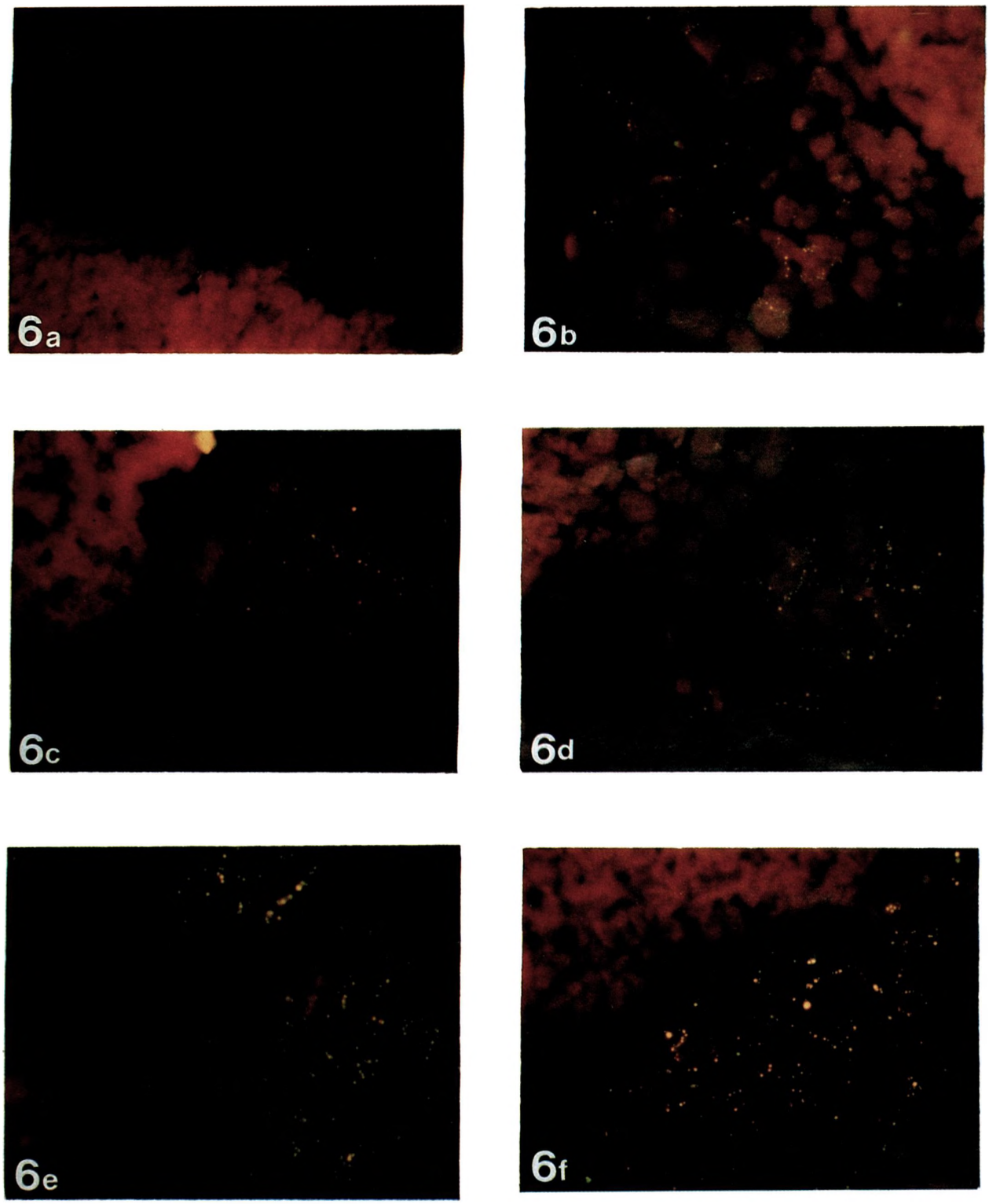
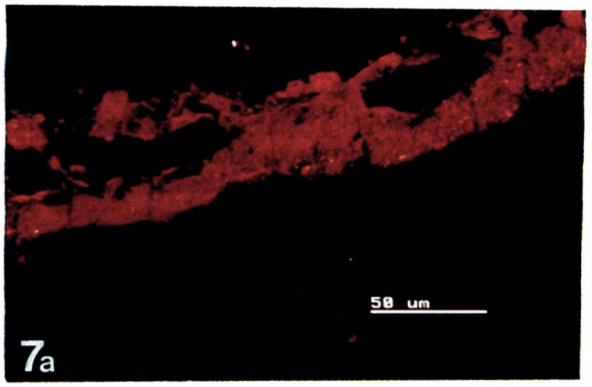
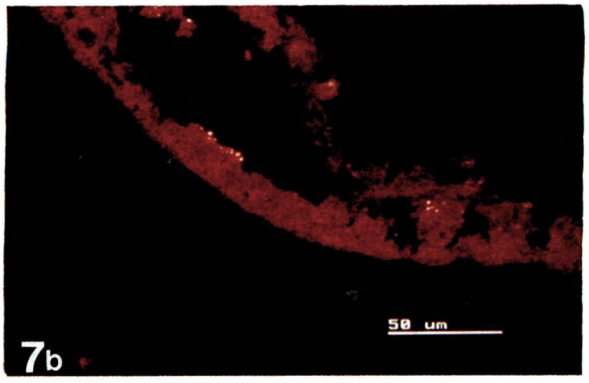


Fig.6.: Indirect immunofluorescent staining of 11.5 day rat embryonic tissues. Immunofluorescence micrographs from embryos cultured in (a) retenate, (b) 4h, (c) 2h, (d) 1h, (e) 30 min and (f) 15 min incubations with rat prolactin at 37 C (magnification X 400). Showing increased distribution of rat prolactin in shorter incubations.





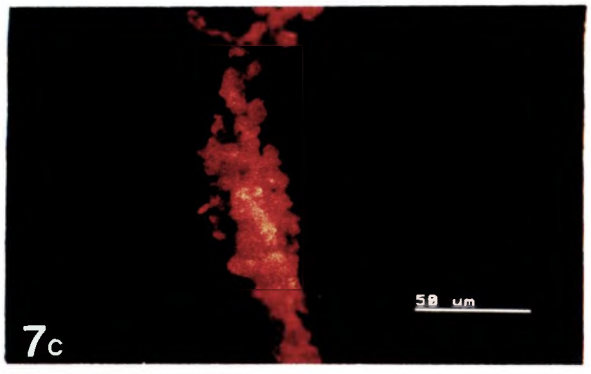


Fig.7.: Effect of incubation at low temperature on uptake of rat prolactin by 11.5 day rat visceral yolk sacs. Confocal micrographs from yolk sacs cultured in (a) retenate, (b) 30 min and (c) 15 min incubations with rat prolactin at 4°C. Incubations at lower temperature caused a delay on the uptake of prolactin.

presence and absence of additional rat PRL, suggesting the presence of receptors for PRL in the embryonic tissues. However, the intensity of immunoreactivity of PRL in embryonic tissues was much greater in the presence of exogenous PRL. This is not surprising because rat serum contains a certain amount of the circulating forms of PRL which may have been taken up by the yolk sac as well as the exogenous PRL. The immunoreactivity observed in the embryonic tissues was widespread and was not tissue or organ specific; only the neural tube did not show any staining. At this stage of development, it is difficult to distinguish the organs from each other on the sections, and also the preparation of the tissues in our study did not allow a standardisation on the position of the embryo. It was therefore impossible to ensure that the sections were obtained at similar levels from each sample and to compare them. Therefore from our observations it can only be said that there is an uptake of rat PRL by the yolk sac and binding sites for rat PRL are expressed by embryonic tissues at this stage. Several authors have demonstrated that the yolk sac endoderm possesses mechanisms for the specific uptake and transport of maternal macromolecules including IgG, EGF, albumin, rat transferrin, insulin and IGF I (16-21).

Increased immunoreactivity was observed in shorter incubations (especially 30min and 15min), which suggests that the uptake and distribution of PRL is time dependent and this may be considered as a preliminary data. However, this would need to be further investigated by a more sensitive and quantitative approach. Considering the short half-life of rPRL this was not suprising. Shorter incubations may cause accumulation of PRL in the yolk sac and the tissues before it is broken down. Incubations at 4°C caused less immunoreactivity in embryonic tissues and a delay in the uptake of PRL by the yolk sac, suggesting that the uptake of rPRL is an active metabolic process and is temperature dependent. This can be explained by the effects of low temperature which may reduce the speed of the uptake process. The data represented here fit in well

with the results of experiments with both yolk sacs and macrophages, using PVP and BSA, which show that below 20°C there is little or no internalisation of substrate (14, 22). Similarly to those, Cumberland and Pratten (19) showed the lower rate of uptake of the transferrin by the yolk sac at 4°C compared to uptake at 37°C. These effects were attributed to reduced mobility of membrane constituents as well as metabolic activity of the cells.

There was no detectable immunoreactivity for PRL in the embryos grown in retenate showing that ultrafiltration of serum for 8 hours is an effective method to remove rat PRL molecules from the serum. A further experiment would be to investigate the distribution of the PRL receptors which can be studied with anti-PRL receptor antibodies, which may be more informative for this kind of study. At the time of this experiments carried out we could not locate any commercially available anti-receptor antibody for rat prolactin from the chemical companies despite an intensive research. However, the method used in this study provides an opportunity to observe the uptake of PRL as well as the distribution of the hormone.

Previous studies demonstrated the presence of PRL receptors in fetal rat liver (3) and in uteroplacental tissues (23). The widespread distribution of the bovine PRL receptor transcripts in bovine fetal and uteroplacental tissues has also been reported (24). Freemark et al (2) demonstrated the distribution and expression of PRL receptor mRNA transcripts in fetal rat adrenal, kidney, small intestine, liver, lung, and brain on day 20 of gestation and in the whole conseptus on days 9 and 10 which is in agreement to the observations in the present study, suggesting that PRL receptors are present at the early stage of development. Similarly to this study, using the method of reverse transcription-PCR, Freemark et al (5) demonstrated the expression of PRL receptors in numerous tissues on day 19 of gestation. Recently, Royster et al. (4) examined the cellular distribution and developmental expression of the PRL receptor in the late gestational fetal rat (days 17.5-20.5) by in situ hybridisation, immunohistochemistry, and radioligand binding, and demonstrated the widespread expression of mRNA encoding the two isoforms of receptor in tissues derived from all three germ layers. They also observed an increase of the levels of expression of PRL receptor mRNA and protein between 17.5 and 20.5 days of gestation in a number of fetal tissues. More recently Freemark et al. (6) compared the expression of PRL receptor mRNA in the olfactory system of fetal and lactating rats, and demonstrated that the levels of PRL receptor mRNA of the lactating rats was lower than those in the fetal and neonatal rats. It has been well established that PRL-dependent tyrosine kinase activation is an early event in the signal transduction pathway for PRL in Nb2 cells (25-32). Recently, we have investigated the relationship between the PRL receptors and tyrosine kinases in the mitogenic action of PRL, and in agreement with the previous studies, we observed that, addition of tyrosine kinase inhibitors genistein and tyrphostin A47 to the PRL-supplemented retenate abolished the PRL-induced increase in growth and development of rat embryos (9). These results fit well with the observations in the present study indicating that functional PRL receptors are present in rat embryos at this stage, and the growth promoting effects of PRL observed in retenate cultures (8) is likely to be mediated by PRL-receptors which play essential role during organogenesis.

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