OCCURRENCE AND FUNCTIONAL IMPORTANCE OF A RIBOFLAVIN-CARRIER PROTEIN IN THE PREGNANT RAT

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1. Introduction

During pregnancy in higher animals and humans, riboflavin, is actively concentrated by the conceptus with resultant increase in foetal:maternal plasma ratio of the vitamin [1,2]. The underlying molecular processes, including the possible involvement of a specific vitamin-carrier protein in this phenomenon are hitherto unknown though the impermeability of the placental membranes to the free vitamin and its coenzyme forms has been recognized [3]. In the chicken, on the other hand a reproduction-specific, high affinity carrier-protein mediates adequate deposition of riboflavin in the eggs for proper embryonic development and survival [4–6]. Earlier, we had purified and developed a specific radioimmunoassay (RIA) for this avian riboflavin-carrier protein (RCP) to study the kinetics of its hormonal induction in estrogenised birds [6,7]. High specificity and potency of the antiserum (a/s) employed therein prompted us to investigate the probability of the pregnant rat elaborating a similar carrier-protein presumably for transplacental delivery of riboflavin to developing foetuses. We have shown that sera from pregnant rats, but not from adult male or immature animals, contain a protein species which crossreacts with the specific a/s to the chicken RCP [8,9]. Here we show that estrogen (E) induces this protein in the rat and that in vivo passive immunoneutralization of the protein terminates pregnancy in this mammal.

2. Materials and methods

Inbred Wister rats, were fed a pelleted diet (Hindustan Lever Products, Bombay) and water ad

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libitum. They were exposed to a 14 h:10 h light–dark schedule. Adult female rats with regular 4 day oestrous cycle were mated with adult males and the day on which sperms were detected in the vaginal smear was taken as day 1 of pregnancy. The number of implantation sites on uterine horns were counted by laprotomy on day 7. Sera prepared from blood withdrawn by cardiac puncture were used for RIA. The rabbit a/s to purified chicken RCP, ovalbumin and ovomucoid were prepared and characterized as detailed in [6,10]. RIA was carried out using ^{125}I-labelled chicken RCP and its specific a/s [6] and the concentrations of crossreacting protein in suitably diluted rat sera were quantified using this RIA and are expressed in terms of the chicken RCP used as standard. The sources of chemicals and other materials used were the same as in [6,7].

3. Results and discussion

The data of fig.1 substantiates the validity of the heterologous RIA method for quantification of the rat serum RCP since a good parallelism between standard curves for dose-dependent displacement of ^{125}I-labelled antigen from its homologous antibody by the unlabelled chick RCP and the pregnant rat serum is clearly evident.

It is clear from the results of fig.2A, that in the pregnant rat, significant amounts of the rat RCP are present as early as day 4 of gestation, i.e, even before implantation, suggesting that the protein is of materal origin. Following a measurable fall on day 7 the concentrations of the specific protein markedly increase by day 10 with minor fluctuation in its levels thereafter till day 18. It is significant that high concentrations of the protein in circulation are more or less
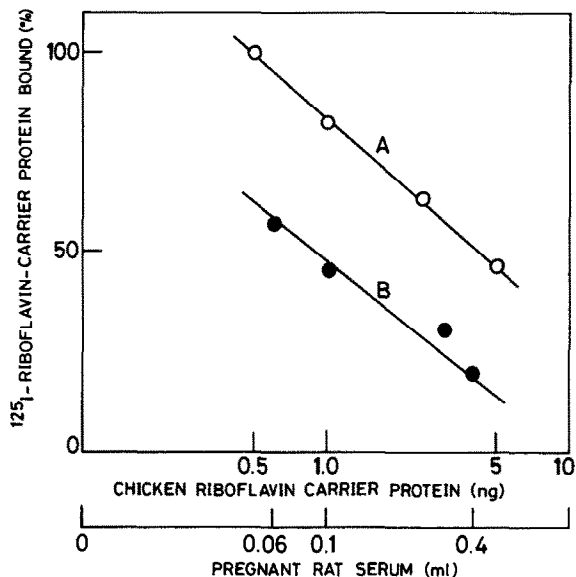
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Fig. 1. Displacement of ^{125}I-labelled purified chicken riboflavin-carrier protein to its homologous a/s in the presence of varying amounts of (A) chicken riboflavin-carrier protein and (B) pregnant rat serum. The RIA and the competition experiments were performed as detailed in [6]. The dilution of the specific a/s was 1:10 000 and the ^{125}I-labelled chicken protein had spec. act. 6×10^8 cpm/μg protein.

maintained during this critical period of rapid embryonic growth and organogenesis [11]. Assuming that, as in the chicken, circulatory E is the primary inducer of this reproduction-specific protein in the pregnant rat also, it would appear that the significantly elevated hormonal levels on day 4 and days 8–10 of pregnancy [12] are responsible for this pattern of protein levels. A strict temporal relationship between the serum E concentrations and the specific protein may not be apparent presumably due to multiple hormonal interaction during pregnancy. On the other hand, in cycling rats (fig.2B) a good correlation between serum concentrations of the steroid and the specific protein is apparent with pro-estrous surge of E [12] coinciding with the maximum concentration of the specific protein. The results obtained with ovarectomized females treated with estradiol-17β(E_2) (fig.2C) confirm this correlation in addition to demonstrating E involvement in induction phenomenon. Since the maximum levels are reached around day 4 following the hormone administration, it would appear that the effect of pro-estrous E surge manifests maximally during the next pro-estrous period in the cycling rat. Of relevance in

this context are the observations that in malnourished women taking combination contraceptive pills in some parts of the developing world riboflavin deficiency is aggrevated [13,14]. Since E appears to be the primary inducer of RCP in higher animals, it is conceivable that induction of this specific protein drains the vitamin from circulation consequent to binding to such carrier protein which has no normal physiological function except in pregnancy.

The importance of adequate circulatory concentrations of the specific protein for maintenance and progression of pregnancy was revealed in experiments wherein endogenous protein was neutralized in vivo by administering adequate quantities of the potent and specific a/s to the chicken RCP (table 1). In the a/s treated animals, the effect on pregnancy was dramatic; there was profuse vaginal bleeding within 24–48 h followed by a 100% foetal rejection as con-
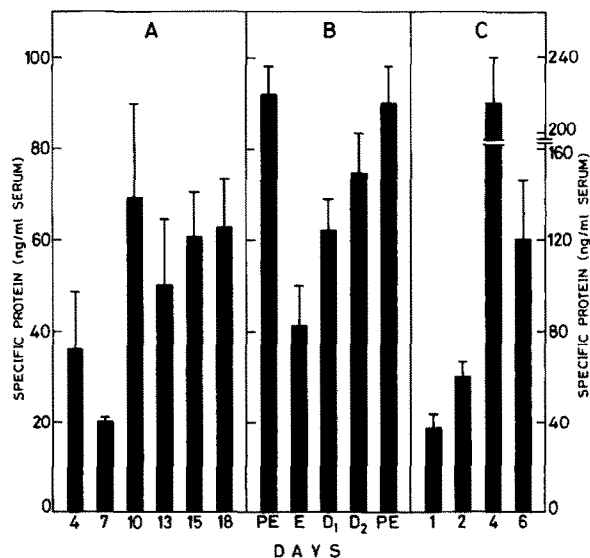

Fig. 2. The concentrations of the crossreacting protein in the rat sera: (A) during pregnancy; (B) during 4-day estrous cycle; (C) in ovarectomized female following a single injection of estradiol-17β. The pregnant rats were housed in individual cages, and the serum concentrations of the protein determined by reference to the standard curve in the heterologous RIA (fig.1). The cycling rats of the same age and body wt. are used in (B). PE, pro-estrous; E, estrous; D, diestrous days of the cycle. The animals of similar age and body wt. were ovarectomized and after 8 days were injected with estradiol-17β intramuscularly (0.5 mg/100 g body wt) and sera was analysed on subsequent days. Base line value prior to estrogen treatment, i.e., on day 0 was <6 ng/ml serum. Vertical bars represent SD (n = 4).

Table 1

The influence of the specific antiserum to the chicken riboflavin-carrier protein on pregnant rats

Group	No. of animals	At laprotomy on day 8 (nT/T)	Treatment	Autopsy on day 18	Remarks
1	4	28/9.3	Non-immune rabbit serum	28/9.3	Normal
2	3	27/9	Ovalbumin or ovomucoid a/s	27/9	No bleeding, viable pups
3	4	39/9.7	a/s to chicken ribo-flavin-carrier protein	0/0	Profuse bleeding during 24–48 h
4	3	28/9.3	Riboflavin-carrier protein a/s neutralised	28/9.3	No bleeding, viable pups

The pregnant rats (15 day, 100–150 g body wt) were treated with either the non-immune rabbit serum (0.5 ml), the a/s to ovalbumin or the a/s to the purified chicken riboflavin-carrier protein raised in rabbits (0.2 ml/rat). nT = total number of implantation sites. T = average implantation sites/animal determined at laprotomy. The a/s to either ovalbumin or ovomucoid could neutralize 400 μg respective protein/ml of serum while the a/s to chicken riboflavin-binding protein had a corresponding potency of 300 μg/ml. The sera was administered intraperitonially

firmed at autopsy. An identical pattern was observed, when the a/s was administered during the period from day 6–16 of pregnancy; however, after day 18, the a/s had no perceptible effect on foetal survival; the reasons for this relative refractoriness are not clear at present. Interestingly, equivalent amounts of either non-immune rabbit serum, the a/s to ovalbumin and

Fig.3. The relative sizes of uterine horns and inclusions dissected from pregnant rats which have been treated with (A) nonimmune rabbit serum (0.5 ml/rat) and (B) the rabbit a/s to the chicken riboflavin-carrier protein (0.2 ml/rat). The autopsy was performed at 24 h after intraperitonial serum administrations, i.e., at the beginning stages of vaginal bleeding. The a/s used neutralized 300 μg chicken riboflavin-carrier protein/ml at equivalence point.

ovomucoid or the pre-neutralized a/s to the chicken riboflavin-binding protein, uniformly failed to induce either vaginal bleeding or abortion demonstrating the specificity of immunoneutralization. Autopsy of the animals treated with the a/s to the chicken RCP during the early periods of vaginal bleeding showed significant foetal wastage as gauged by the relative sizes of the foetal inclusions in the uterine horns (fig.3). Further, it has been consistently observed that the animals which abort following the a/s treatment appear normal and resume their regular 4 day estrous cycle indicating that the maternal wellbeing including endocrine functions is unaffected by the passive immunoneutralization of the reproduction specific protein.

The above data clearly show that the pregnant rat sera contain a maternal protein, which is immunochemically similar to the chicken riboflavin-binding protein and functionally is of paramount importance for foetal wellbeing and progression of pregnancy. In agreement, this protein can be purified from the pregnant rat serum by affinity chromatography on lumiflavin–Sepharose [15] and binds [2-^{14}C] riboflavin with high affinity (in preparation). Employing a similar approach, we have been able to isolate such a protein from the sera of pregnant monkeys, *Macaca radiata* (unpublished). Furthermore with the recent discovery of the thiamin-binding protein in the chicken and development of a specific a/s to it [16,17], we have shown the presence in the pregnant rat, of yet another specific protein crossreacting with this a/s [8]; more
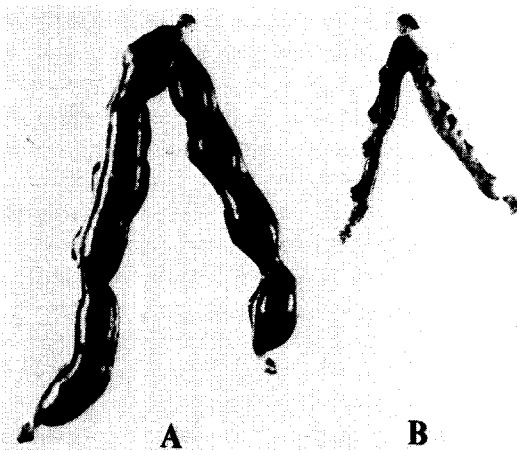
importantly administration of this a/s to the pregnant rat also leads to profuse vaginal bleeding and foetal rejection in a manner identical to that described with the a/s to RCP [8].

Apart from showing for the first time the presence and functional importance of reproduction-specific RCP in mammalian embryonic development these observations have other important implications:

(i) They emphasize that in addition to the satisfactory maternal vitamin supply, adequate and efficient pregnancy-specific delivery systems (carrier protein) ultimately determine the foetal vitamin nutrition;

(ii) A possible explanation for some of the ill-defined causes of premature foetal death and/or teratogenesis may lie in genetic or endocrine defects in the production and/or functionality of these carrier proteins.

Acknowledgements

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