A sex-limited serum protein of Syrian hamsters: Definition of female protein and regulation by testosterone

(serum $\alpha$-globulin/testosterone inhibition/diethylstilbestrol stimulation)

J. E. COE

National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana 59840

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ABSTRACT Normal Syrian hamster females contain a serum protein not found by simple gel diffusion assay in normal adult males. This sex-limited protein was called female protein (FP). Low levels of FP were found in sera from normal weanling male hamsters. Adult male hamsters castrated or treated with diethylstilbestrol also developed serum FP, which could be suppressed by administration of testosterone. Exogenous testosterone also suppressed serum FP in adult females, and ovarian function did not appear to be critical for maintenance of serum FP. Although FP is present in significant amounts (1–2 mg/ml) in adult females, its function is presently unknown.

Serum proteins specific for one sex have been described in many species. Numerous examples exist in insects (1) where hormonally controlled female specific proteins have been detected in sera during egg production and are necessary for egg maturation (2). Similarly, a phosphoprotein, called "serum vitellin" by Laskowski (3) was found in the plasma of laying hens. This protein was also found in cockerels after diethylstilbestrol (DES) treatment (4) and along with a lipoprotein component (5) apparently acts as a transport system for the developing yolk. Another sex-linked serum protein called "hormone-influenced protein" has been detected in some normal laying hens and in other chickens (male and female) after progesterone treatment (6).

In mammals, sex differences in serum levels of IgM (7, 8) have been described and two mouse complement components, C5 and C9, have been shown to be under sex hormone control (9, 10). Of particular interest are the sex-limited proteins of mice controlled by the Ss locus, an important marker for the S region of the H-2 complex (reviewed in ref. 11). The Ss locus is regulated by male hormone (12) and Ss protein has recently been shown to be the fourth component of complement (13).

The present report defines a new sex-linked serum protein in Syrian hamsters. This protein is called female protein (FP) as it is found in normal adult female hamsters but is not detectable by gel diffusion assay in adult male hamsters. However, FP does appear in adult male serum after castration or estrogen treatment. FP is a prominent constituent of female hamster serum although its function at present is unknown.

MATERIALS AND METHODS

Animals. Randomly bred Syrian hamsters were obtained from the Rocky Mountain Laboratory colony and New Zealand white rabbits were purchased locally. Chinese hamsters from Chick Line, Newfield, N.J. were used. Animals were fed Purina Laboratory Chow and given water ad lib.

Antisera and Affinity Chromatography. Rabbit antisera were produced by footpad injection of antigen emulsified in complete Freund's adjuvant followed 3 weeks later by two weekly subcutaneous injections of antigen in incomplete Freund's adjuvant. Specific antisera to FP were obtained either by direct addition of absorbing antigen (normal male hamster serum, NMHS) to the antisera and removal of precipitins by centrifugation or usually by passage of the antisera through a column of Sepharose 2B (Pharmacia, Uppsala, Sweden) to which NMHS had been coupled by cyanogen bromide (14). FP was isolated by passing normal female hamster sera (NFHS) through a column containing Sepharose 2B conjugated by cyanogen bromide to the one-half saturated ammonium sulfate precipitate of specific rabbit antisera against FP. The column was thoroughly washed with phosphate buffered saline at pH 7.2 and the FP was eluted with 0.5 M acetic acid, promptly neutralized with Tris base, and then concentrated by negative pressure and dialyzed against phosphate-buffered saline.

Immunodiffusion Studies. Agar electrophoresis and immunoelectrophoresis were performed on 2% agar covered microscope slides using 7 V/cm with barbital buffer at pH 8.2 (ionic strength 0.04). Agar electrophoresis patterns of FP were stained with various dyes according to Uriel (15). Simple gel diffusion was done on 1% agarose covered slides. FP was quantified by radial diffusion on glass slides covered with 1% agar containing specific rabbit antisera against FP. Five microliter samples were placed into 14 gauge needle holes and after 24 hr at room temperature the precipitin diameters were measured. The squared diameters were then compared with those produced by 2-fold dilutions of a standard serum containing a known amount of FP. Examination of replicate samples showed an experimental error of less than 5%.

Serum Fractionation and Other Procedures. Normal hamster serum was separated by Pevikon block electrophoresis (barbital buffer pH 8.6; ionic strength = 0.05) at 00 V for 18 hr and 1 cm fractions were eluted. Carbohydrate of FP was quantified by reaction with tryptophane (16). Ultraviolet absorption spectrum of FP was performed with a Beckman spectrophotometer Acta M6.

Experimental Procedures. Plasma or serum was obtained from hamsters by bleeding from the retro-orbital area. Male and female hamsters anesthetized with sodium pentobarbital were castrated at 21 or 90 days of age by aseptic ligation and extirpation of the gonads. Sham control animals underwent a similar surgical procedure except the organ was left intact. Pellets of diethylstilbestrol (DES) (12 mg/Pfizer) were surgically implanted subcutaneously. Testosterone (Calbiochem, San Diego, Calif.) was injected intraperitoneally. Hamster milk was obtained (17) and hamster estrus was determined according to Orsini (18).

RESULTS

Definition of female protein

Comparison of IEP patterns of normal Syrian hamster sera developed with a sheep antisera against whole hamster serum (male plus female) revealed the presence of a fast alpha globulin
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female 

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(bottom) 

FIG. 1. Immunoelectrophoresis of a normal male (top) and female (bottom) hamster sera after development with sheep antibodies against whole hamster sera (outside troughs) and specific rabbit antibodies against female protein (center trough). The female protein precipitin line is detected in the fast alpha globulin region and only in the female serum. NHS, normal hamster sera.

precipitin line discernible in female but not in male sera (Fig. 1). A preliminary antiserum specific for this protein, female protein (FP), was made by simply adding NMHS to sheep antibodies against whole hamster serum and this antiserum was used to locate FP in the fast alpha fractions after Pevikon block separation of normal female hamster sera. This Pevikon isolated FP was injected into rabbits and the resulting antiserum was specific for FP after absorption with NMHS (Fig. 1). Male hamsters theoretically would be a convenient source of FP antiserum; however, antibody to FP was not detected in serum from male hamsters after inoculation with purified FP in complete and incomplete Freund's adjuvant.

FP purified by affinity chromatography produced only one precipitin line when tested by gel diffusion with various multivalent antisera, sedimented as a single homogeneous Δ 7S peak by sucrose density gradient analysis, and after determination of protein content by Kjeldahl was used as a standard for ring diffusion calibration. FP produced a UV absorption spectrum of a typical protein with maximal absorption at 278 nm and an absorptivity at 280 nm of 2.0. FP was stained with protein stains (amido-black and ponceau red) but not with oil red 0 on agar and cellulose acetate electrophoresis. Carbohydrate content measured 6.6% although some of this carbohydrate may have originated from the Sepharose affinity column.

Ontogeny of serum FP

Although FP was not detected by simple gel diffusion in normal serum from adult males, it was detected for a transient interval in young male hamsters. Sera obtained from male and female hamsters at various intervals after birth was quantified for FP by ring diffusion assay (Fig. 2). In both sexes small amounts of FP (≥0.1 mg/ml) were first detected 10 days after birth and rapidly increased to about 0.5 mg/ml by day 15. However, FP concentrations in females continued to increase to a mean value of 2.2 mg/ml by day 28 whereas in males it plateaued after day 15, started decreasing after day 21, and became undetectable after day 43. Examination of sera from older females revealed a decrease in the mean serum concentration and 0.1 mg/ml levels were not uncommon in 1-year-old animals (Fig. 2). On the other hand, serum FP sometimes appeared in old male hamsters, i.e., one of 14 males 365 days old had 0.3 mg of FP per ml of serum and 2 of 11 males between 548 and 660 days of age had approximately 0.1 mg of FP per ml of serum (not shown in Fig. 2).

The decrease of serum FP in male hamsters after weaning (day 21) suggested possible transfer via maternal milk. However, no FP in hamster milk was detected by gel diffusion analysis. To further determine whether hamster milk had a direct (i.e., subdetectable amounts of FP in the milk) or indirect (hormonal influence) effect on male FP serum concentration, a litter of Syrian hamsters were obtained immediately after parturition and grafted onto a post-partum Chinese hamster mother. Chinese hamsters were used as foster mothers because FP has not been detected in male or female Chinese hamster serum (data not shown). Table 1 shows that shortly after weaning, foster reared Syrian hamsters had significant amounts of serum FP (males 0.3 and 0.17 mg/ml, females 1.4 mg/ml) although the levels (especially the females) were less than two litter mates who remained with the Syrian mother (male no. 5 = 0.44 and female no. 6 = 2.2 mg/ml). However, foster reared hamsters were obviously malnourished with the small Chinese hamster mother, as they were significantly smaller in size and this may have contributed to the lower serum FP levels. Furthermore, three Chinese hamster babies suckled on a Syrian

FIG. 2. Ontogeny of female protein in normal male and female hamsters. Sera or plasmas from various aged hamsters were assayed by ring diffusion assay and the range of values (brackets), mean of values (dot on brackets), and number of sera examined (number on top of brackets) are presented. Note similar initial serum levels in 10- to 15-day-old male and female hamsters, although in males the serum female protein subsequently disappeared.
Table 1. Female protein levels in a litter of Syrian hamsters suckled on Chinese or Syrian hamster mothers

<table>
<thead>
<tr>
<th>Suckling animal*</th>
<th>Serum FP (mg/ml)</th>
<th>Weight (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Sex</td>
<td>Source of milk</td>
</tr>
<tr>
<td>1</td>
<td>♂</td>
<td>Chinese hamster</td>
</tr>
<tr>
<td>2</td>
<td>♂</td>
<td>Chinese hamster</td>
</tr>
<tr>
<td>3</td>
<td>♀</td>
<td>Chinese hamster</td>
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<tr>
<td>4</td>
<td>♀</td>
<td>Chinese hamster</td>
</tr>
<tr>
<td>5</td>
<td>♂</td>
<td>Syrian hamster</td>
</tr>
<tr>
<td>6</td>
<td>♀</td>
<td>Syrian hamster</td>
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</table>

* Individual Syrian hamster babies from one litter placed with Chinese hamster mother (or remaining with Syrian hamster mother) immediately postpartum (day 0).

Hamster mother did not contain serum FP at weaning. Therefore, Syrian hamster milk was not a likely source or stimulus for serum FP in suckling offspring.

**Effect of castration and testosterone on serum FP in adult male hamsters**

Male hamsters (groups of six each) were either castrated or sham castrated at 21 or at 90 days of age and plasma samples were obtained at 2 week intervals thereafter and quantified for FP. Hamsters castrated at 21 days of age showed increasing serum levels of FP 15 and 29 days postcastration (mean values of 1.0 and 1.6 mg/ml, respectively) in contrast to sham controls, whose levels of FP decreased to undetectable levels in normal fashion. FP was also detected 15 days after adult (90-day-old) orchietomy (mean 0.8 mg/ml serum) but not in the sham controls. FP levels were maintained in both castrated groups for 56 days although more serum FP was usually found in males castrated at weaning; on day 56, mean serum FP was 1.7 and 0.5 mg/ml in 21 and 90 day castrate groups, respectively. Therefore, FP was detectable in serum from castrated males and the higher levels found after castration at an early age were comparable to those found in normal females of a similar age (Fig. 2).

To determine the time required for appearance of serum FP, 90-day-old males were castrated and subsequently bled every other day. Fig. 3 shows that FP reached detectable levels 4 days post-castration and peak levels were achieved by 10 days. Commencing on day 35, these hamsters were given 50 μg of testosterone daily with a resultant decrease in mean serum FP level to 0.05 mg/ml 21 days later (day 56) (Fig. 3). When testosterone was discontinued on day 56, serum FP again increased to pretreatment levels (Fig. 3). Similarly, male hamsters given 18 daily injections of 50 μg of testosterone from the time of castration did not develop detectable serum FP although serum FP was found 23 days after testosterone was stopped (day 41) (not shown). These data indicate a suppressor function of testicles via testosterone on serum FP in adult male hamsters.

**Effect of diethylstilbestrol on FP in male hamsters**

Administration of DES to adult male hamsters consistently resulted in the prolonged appearance (at least 90 days) of 0.3–0.9 mg of FP per ml of sera. To determine if higher levels could be achieved with DES in castrated hamsters, 90-day-old hamsters were either castrated or sham castrated and both groups of six hamsters each were given a 12 mg pellet of DES subcutaneously. Higher levels of serum FP were seen on day 14 when DES was combined with castration (mean 0.7/ml versus 0.15 mg/ml in DES group), although on days 28, 42, 56, and 90 the FP concentration in both groups was similar (0.3–1.0 mg/ml). The FP response of normal male hamsters given DES was similar to that detected in sham castrated hamsters. Thus, DES did not augment the effect of castration and the serum concentration of FP achieved in male hamsters was similar regardless of the treatment, i.e., DES or orchietomy, or DES plus orchietomy.

**Effect of castration and testosterone on FP in female hamsters**

Female hamsters in groups of six were castrated or sham castrated at 90 days of age and plasma samples obtained at 14-day intervals (until day 56) were quantified for FP. No significant alteration of serum FP levels was detectable after this treatment although average FP levels in castrated females were slightly less than controls (1.8 mg/ml versus 2.3 mg/ml on day 56). Therefore, intact ovaries were not necessary for maintenance of normal FP serum levels.

Daily injection of 50 μg of testosterone to a group of six adult female hamsters resulted in a gradual decrease in serum FP levels from 0.9 to 0.4 mg/ml (mean values day 0 and 28, respectively). Increasing the testosterone to 200 μg daily abolished detectable serum FP within 14 days. These data indicate testosterone has a suppressor effect on serum FP in female as well as male hamsters.

**DISCUSSION**

FP of Syrian hamsters is a remarkable protein in a number of ways. First of all, significant serum levels (1–2 mg/ml) are found only in normal adult females, and sexes can easily be distinguished by serum immunoelectrofocusing even when developed with rabbit or sheep antisera raised against whole female sera because these antisera usually contain antibodies to FP. However, adult males are capable of FP synthesis, and suppression of this protein by testosterone was clearly shown by the prompt appearance of FP in male serum 4 days after castration; testosterone administration commencing at the time of castration blocked appearance of serum FP or decreased serum levels when initiated 35 days after castration. This indicated that FP synthesis was normally inhibited in the adult male by testicular testosterone. On the other hand, serum FP levels quantitatively similar to those found in castrated males also appeared in normal males given DES; the combination of castration plus DES did not result in over-all higher levels than castration alone. This suggested that DES acted more as a testosterone antagonist rather than a primary stimulus for FP synthesis. The concept that male and female hormones tend to neutralize each other has been utilized to explain the finding that testosterone will inhibit estrogen-induced renal tumors in hamsters (19, 20). This primary renal adenocarcinoma is of interest because it can be induced in male hamsters by chronic treatment with DES in doses similar to those used in the present report. Renal tumor can also be produced in female hamsters but only in special circumstances such as after castration (19). Studies with 14C added to tissue culture and fluorescent microscopy techniques have not been successful in defining the site of FP synthesis although the kidney is an intriguing possibility considering the unusual occurrence of an estrogen induced tumor in this nonendocrine organ. Androgen–estrogen imbalances in the hamster also have influenced the incidence of many other hamster tumors (19–22) and even splenic bone marrow hematopoiesis (23).
Although estrogen receptors and estrogen stimulated protein-RNA synthesis in target tissue are well known (24), the studies herein have not demonstrated a direct control of the serum FB by ovarian estrogen in the female; FP levels were maintained in castrated females and administration of testosterone to noncastrated females markedly decreased their serum FP levels, again suggesting a primary suppressor effect of androgens. These preliminary observations unfortunately shed little light on the actual means of androgen control of the FP genome which could be by an indirect feedback mechanism or by a direct multistage pathway.

Assay for FP by more sensitive inhibition techniques may show that small amounts of FP also are present in adult NMHS. NMHS occasionally contained small amounts of FP which were not detectable directly on simple gel diffusion assay but could show slight interference with an intersecting FP precipitin line. Also, the presence of quantifiable amounts of FP in very young and occasional old males, and in some tumor bearing or complete Freund's adjuvant injected males (unpublished data), suggest that small amounts may be continually synthesized in males. This would also explain immunological tolerance of adult males to FP although the neonatal exposure of males to autoantogenic FP should suffice for tolerance induction.

Hormonal control of FP in Syrian hamster females appears almost diametrically opposite to sex limited protein control in mice (Mus musculus) (12); sex-limited protein alloantigen is normally detected only in males although it appears in females after treatment with testosterone. Ss protein carries the sex limited protein marker and is of great interest because the Ss gene is in the H-2 gene complex and Ss protein is now known to be the fourth component of mouse complement (13). Although the function of FP is presently unknown, a role in the hamster complement system is an attractive speculation in light of the hormonal control of mouse complement components.

The expert technical assistance by Mary Jane Ross and Emery Beker is gratefully acknowledged.

Fig. 3. Development of serum FP in castrated male hamsters and its suppression by testosterone administration. Five 90-day-old hamsters were castrated on day 0 and FP levels at various intervals thereafter charted as in Fig. 2. Daily testosterone (50 µg) on days 35-56 depressed FP levels which returned to preinjection levels after cessation of testosterone. See legend to Fig. 2 for other details.