Purification of ovine transferrin and study of the hormonal control of its secretion in enriched cultures of ovine Sertoli cells

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Summary. Ovine transferrin (o-transferrin) was purified from sheep serum by fractionated precipitation with ammonium sulphate, ion-exchange chromatography on DEAE trisacryl and finally by affinity chromatography on Affigel blue to remove albumin. Ovine transferrin was identified by its apparent molecular weight in sodium dodecyl sulphate polyacrylamide gel electrophoresis and by its *N*-terminal amino-acid sequence. The procedure presented in this report permits the preparation of highly purified o-transferrin with a good recovery (52% of initial total immunoactivity).

An antiserum against o-transferrin was then raised in rabbits, using this highly purified preparation. A specific radioimmunoassay was set up using ^{125}I -labelled o-transferrin. Its detection threshold (4 ng/ml) was low enough to measure o-transferrin in spent culture media of ovine Sertoli cells, which ranged between 15 and 600 ng/ml.

Sheep seminiferous tubule cells, containing ~80% Sertoli cells, were cultured at a high density (1.5×10^6 cells/cm²) on a thin layer of reconstituted basement membrane. Kinetic studies showed that basal daily secretion of o-transferrin was reduced by half (–49%) between Day 1 and Day 2 of culture, and progressively decreased thereafter. Under FIRT (500 ng ovine follicle-stimulating hormone (FSH)/ml + 10 µg insulin/ml + 500 ng retinol/ml + 5×10^{-7} mol/l testosterone) stimulation, the ratio of stimulated to basal secretions increased 11-fold between Day 1 (1.1) and Day 6 (12). When 10% fetal calf serum was added, mean o-transferrin secretion was a third of that in serum-free medium, suggesting that fetal calf serum contains factors that inhibit secretion of ovine Sertoli cell transferrin. In the presence of serum, the ratio of FIRT-stimulated to basal secretions doubled between Day 1 (1.0) and Day 4–6 (2.0).

Between Days 2 and 4 of culture, insulin had a slight stimulatory effect on o-transferrin secretion (128% of control at 10 µg insulin/ml), as well as epidermal growth factor (124% of control at 50 ng/ml). Testosterone at up to 5×10^{-7} mol/l had no effect; 500 ng retinol/ml doubled o-transferrin secretion (218% of control) as did 500 ng FSH/ml (220% of control). A combination of retinol and FSH increased the secretion 4-fold, indicating that maximal stimulation of o-transferrin secretion by ovine Sertoli cells requires the combined actions of mechanisms dependent and independent of cAMP.

Keywords: sheep; Sertoli cell; transferrin; retinol; FSH

Introduction

Sertoli cells are major secretory cells of the testis and constitute the only non-germinal cells of the seminiferous epithelium. They offer structural support to the tubule and are in intimate contact and communication with germinal cells. Tight junctional complexes formed between adjacent Sertoli

cells comprise a barrier that restricts access to serum components by leptotene spermatocytes and further-developed germinal cells inside the barrier. Sertoli cells are responsible for creating a unique environment in which germinal cells divide and differentiate into spermatozoa. Elucidation of the factors that regulate Sertoli function is required to understand the mechanisms involved in the maintenance and control of spermatogenesis (for review, see Bardin *et al.*, 1988).

In rats, a number of Sertoli cell secretory products have been identified and their functions determined, including the iron-transport protein transferrin (for review, see Griswold *et al.*, 1988). The protein was identified in cell culture media by its ability to bind iron and by its cross-reaction with antibodies to rat sero-transferrin. Further analysis of transferrins derived from Sertoli cells and serum revealed that their peptide backbones were identical, but they exhibited different glycosylation patterns. The proposed function of this protein in testicular cells is to transport iron. It has been postulated that Sertoli cells synthesize and secrete this protein into the seminiferous tubular lumen to provide a mechanism for transport of Fe^{3+} to developing germ cells. The demonstration of transferrin-specific binding to spermatocytes and of transferrin receptors on spermatids is consistent with this hypothesis.

Studies of seminal fluid in man suggest that >80% of seminal transferrin comes from the testes, and show that seminal transferrin is a reliable index of seminiferous tubular function because concentrations of this protein are lower in patients with oligo- and azoospermia (Holmes *et al.*, 1982; Orlando *et al.*, 1985). One study of seminal fluid in bulls indicated that 60% of seminal transferrin comes from the testes and that there is a relationship between total seminal transferrin and total sperm output (Gilmont *et al.*, 1990). These studies led to the suggestion that the protein might be a good marker of seminiferous tubule function.

In contrast to the abundant information on Sertoli cell function in rat testis, only a few studies have been published concerning domestic species such as sheep. Speight *et al.* (1984) showed that the proportion of [^3H]leucine-labelled proteins that were secreted by Sertoli cells of lambs 2–12 weeks old increased with age. Monet-Kuntz *et al.* (1991) demonstrated the secretion of plasminogen activator, which was enhanced by FSH in Sertoli cells in lambs 3–10 weeks old. In this species, however, nothing was known about the existence of secretion of transferrin by Sertoli cells nor its regulation by hormones.

In this study, we therefore investigated the regulation of transferrin secretion in Sertoli cells from 3-month-old sheep. For this purpose, we needed to set up a radioimmunoassay (RIA) for ovine transferrin (o-transferrin). As neither anti-rat transferrin antibodies (F. Guillou, unpublished data) nor anti-bovine transferrin antibodies (J. M. Guillaumin, personal communication) could recognize o-transferrin, transferrin had to be purified from sheep serum in order to prepare a specific antiserum. The aims of this work were to (i) purify transferrin from ovine serum; (ii) set up a specific RIA for use in spent culture media of ovine Sertoli cells; and (iii) study the regulation of its secretion by factors known to be regulators of its secretion in rats, i.e. follicle-stimulating hormone (FSH), insulin, retinol, epidermal growth factor (EGF and testosterone).

Materials and Methods

Animals

Ile-de-France or Préalpes lambs were born throughout the year and were subjected to natural variations in light and temperature. Testes were removed at ages of 83–130 days.

Chemicals

Iodogen was obtained from Pierce (Rockford, IL, USA). Nonidet P-40, bovine insulin, retinol, sodium selenate, sodium pyruvate, cycloheximide and sodium lactate were purchased from Sigma Chemical Company (St Louis, MO, USA). DEAE trisacryl and Ultrosor-G were obtained from IBF (Villeneuve la Garenne, France) and Affigel blue matrix from Amicon (Danvers, MA, USA). Dulbecco's minimum essential medium was purchased from Gibco

(Grand Island, NY, USA) and Matrigel from Sochibo (Velizy-Villacoublay, France). Fetal calf serum was purchased from Biomérieux (Charbonnières les Bains, France). Testosterone was obtained from Roussel-Uclaf (Romainville, France) and EGF from Serva (Heidelberg, Germany).

The sheep anti-rabbit γ -globulin serum was prepared by N. Martinat (INRA, Nouzilly, France), and ovine FSH (CY 1746; 38 $\times$ NIH-FSH-S1) was purified in our laboratory.

The 24-well culture plates were obtained from Nunc (Roskilde, Denmark).

Purification of o-transferrin

Ovine transferrin was first precipitated from sheep serum with 50–60% ammonium sulphate as described by Guerin *et al.* (1976) and was a gift from these authors. The precipitate was extensively dialysed against water and lyophilized; 2 g of protein was applied to a (7.5 $\times$ 3 cm) column of DEAE trisacryl previously equilibrated with Tris-HCl (25 mmol/l) (pH 8.8) containing 35 mmol NaCl/l. Bound proteins were recovered by stepwise elution, first with 25 mmol Tris-HCl/l (pH 8.6) containing 75 mmol NaCl/l and second with 25 mmol Tris-HCl/l (pH 7.6) containing 150 mmol NaCl/l. The first peak containing transferrin was detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and was desalted on a G25-Sephadex column (Pharmacia, Uppsala, Sweden); 12 mg of this fraction was passed through an Affigel blue (1 $\times$ 10 cm) column previously equilibrated with 50 mmol Tris HCl (pH 7) containing 100 mmol KCl/l, to remove the albumin contaminant. Albumin was eluted with 50 mmol Tris-HCl/l (pH 7) containing 1.5 mol KCl/l.

Determination of N-terminal amino-acid sequence

The purified molecule was reduced with β -mercaptoethanol and alkylated with 4-vinylpyridine according to Henschen (1986). N-terminal sequencing of the reduced and alkylated molecule was performed by automated Edman degradation of the whole protein following the original method of Hewick *et al.* (1981) on an Applied Biosystems 470A sequenator with an on-line PTH-amino acid analyser model 120A with the reagents and methods proposed by the manufacturer. This sequence determination was achieved by J. C. Huet and J. C. Pernollet (Protein Study Laboratory, INRA, Versailles, France).

Labelling of o-transferrin and preparation of antiserum

Anti-ovine transferrin serum was prepared by eight intradermic injections of transferrin (100 μ g/week in Freund's adjuvant) into two rabbits at Days 0, 7, 14, 21, 42, 49, 56 and 64. Four weeks after the last injection, 30 ml of blood was collected from each rabbit and the serum was stored at -20°C .

Ovine transferrin was labelled with ^{125}I as follows: 2 μ g of transferrin in 50 μ l of phosphate buffer (50 mmol/l, pH 7.4) was put into a tube coated with 25 μ g iodogen. Then 200 μCi ^{125}I was added and the tube left for 15 min at 20°C . The solution was transferred onto a Sephadex G-25 column, which was eluted with 25 mmol veronal buffer/l containing 2.5 (w/v) serum albumin. The mean incorporation of ^{125}I into the protein was 54%; so, the specific activity of iodinated transferrin was $\sim 54 \mu\text{Ci}/\mu\text{g}$.

Radioimmunoassay of o-transferrin

Standard curves were constructed from 12 doses of o-transferrin ranging between 0.1 and 500 ng in 100 μ l culture medium, and assayed in triplicate. When samples of culture medium containing fetal calf serum or fetal calf serum substitute (Ultrosor G) were assayed, fetal calf serum or Ultrosor G was added to the o-transferrin standards at the same final concentration. For unknown culture medium samples, duplicate 100 μ l-aliquants were put into 3-ml polycarbonate tubes. To each tube was added 50 μ l of iodinated transferrin (15 000 c.p.m.) in phosphate buffer (0.1 mol/l, pH 7.4) containing 0.1% bovine serum albumin and 0.5% Nonidet P-40. Then 50 μ l of rabbit anti-transferrin serum (dilution 1:10 000) plus normal rabbit serum (0.5%) were added and the tubes were left overnight at room temperature. Separation of bound from free transferrin was achieved by immunoprecipitation: 50 μ l of sheep anti-rabbit γ -globulin serum diluted 1:2, plus 1 ml polyethylene glycol₄₀₀₀ (6% in water) were added and the tubes were immediately centrifuged at 2000 *g* for 30 min at 4°C . The supernatants were discarded and the radioactivity in the pellet was determined using an LKB gamma counter. For calculations, a four-parameter single-binding-site model, which includes antibody concentration, affinity coefficient, non-specific-binding and tracer concentration (Edwards & Ekins, 1982), was used.

Isolation and culture of an enriched population of ovine Sertoli cells

The same method as used for the preparation of cells from pig seminiferous tubules was used (Monet-Kuntz & Fontaine, 1989). Cells were diluted in an appropriate volume of culture medium to yield $\sim 6 \times 10^6$ seminiferous tubule cells/ml. The culture medium was nearly identical to that described by Hadley *et al.* (1985) and consisted of

Dulbecco's minimum essential medium (DMEM) supplemented with glutamine (2 mmol/l), insulin (2 µg/ml), human transferrin (5 µg/ml), vitamin A (50 ng retinol/ml), vitamin E (200 ng/ml), sodium selenate (5 ng/ml), sodium pyruvate (1 mmol/l) and sodium lactate (22 mmol/l). Fungizone (2.5 µg/ml), penicillin (100 units/ml) and streptomycin (100 µg/ml) were added. In some culture experiments, 10% fetal calf serum or a calf serum substitute ('Ultrosor G') was added at a final concentration of 0.02, 0.2 or 2%, as data from the manufacturer indicated that the cell-growth-promoting activity of 2% Ultrosor G was the same as that of 10% fetal calf serum.

Reconstituted basement membrane (RBM) extracellular matrix prepared by high salt and urea extraction of the EHS mouse tumour (Kleinman *et al.*, 1986) is marketed under the name Matrigel; ~250 µl of RBM matrix diluted 1:1 v/v with DMEM was applied to the bottom of the wells, the excess of matrix was immediately aspirated and polymerization was achieved by incubation at 34°C for 30 min.

Samples of 500 µl of cell suspension were distributed into 24-well plates which had been coated with a thin layer of RBM matrix, yielding a density of 1.5×10^6 seminiferous tubule cells/cm².

Cells in coated culture plates were incubated at 34°C in a humidified atmosphere of 95% air:5% CO₂ (Day 0 of culture). Hormones and factors were added on either Day 0 or Day 2, depending on the experiment (see Results).

Identification of the different types of seminiferous tubule cells

Peritubular cells in the seminiferous tubule cell suspension were identified by examination of alkaline phosphatase activity. Cells were fixed and stained as described by Chapin *et al.* (1987), except that the reaction mixture was incubated for 5 min instead of 10 min.

Germ cells were identified according to morphological criteria using light microscopy (Ortavant, 1959). The cell suspension was fixed with 2% glutaraldehyde and 0.5% potassium hexacyanoferrate (III) in sodium cacodylate buffer (0.175 mol/pH 7.25). Cells were fixed in 2% osmium tetroxide, dehydrated in ethanol and embedded in Epon. Semithin (1 µm) sections were stained with toluidine blue dye.

A typical preparation of seminiferous tubule cells contained 3% peritubular cells and 5% germ cells, which indicated that it contained ~80% Sertoli cells.

Statistical analyses

To analyse the effects of different factors on transferrin secretion in different culture experiments, a two-way analysis of variance was performed.

Results

Purification of o-transferrin

During purification, o-transferrin was identified by its apparent molecular weight in SDS-PAGE. Transferrin was eluted in the first peak of ion-exchange chromatography on DEAE trisacryl (Fig. 1). As transferrin was still contaminated with albumin at this stage, we performed an affinity chromatography on Affigel blue matrix. Two peaks were separated (Fig. 2), and SDS-PAGE showed that only a single band with an apparent M_r of 75 000 (corresponding to transferrin) was present in the first peak. Albumin was present only in the second peak.

The N-terminal amino-sequencing of the purified molecule up to the 11th residue (Table 1) strongly suggested that it was o-transferrin.

The purification procedure yielded 64 mg of o-transferrin from 2 g of ammonium sulphate precipitate produced from 64 ml of sheep serum (Table 2). A recovery of 52% of the starting total immunoactivity was obtained.

Characteristics of the o-transferrin assay in culture media

Specificity of the antisera. Since human transferrin (h-transferrin) is a component of the culture medium, it is important to distinguish secreted o-transferrin from added h-transferrin. With the antiserum 188, h-transferrin exhibits a cross-reactivity of <0.2% (Fig. 3). This allows specific assay of o-transferrin in culture tubes to which 5 µg/ml (500 ng/assay tube) h-transferrin was added. Rat transferrin had a cross-reactivity of <0.5%. The second peak from Affigel blue elution was used as a source of o-albumin and exhibited a cross-reactivity of 1.4%. With antiserum 189 from the other

Fig. 1. DEAE trisacryl chromatography of proteins precipitated from sheep serum by ammonium sulphate. A sample of 1.2 ml (2 g) was applied to a 7.5×3 cm column equilibrated with Tris-HCl (25 mmol/l, pH 8.8) containing 35 mmol NaCl/l. The arrows show buffer changes at fraction No. 30 (25 mmol Tris-HCl/l, pH 8.6, containing 75 mmol NaCl/l) and at fraction No. 90 (25 mmol Tris-HCl/l, pH 7.6, containing 150 mmol NaCl/l). The flow was 1 ml/min, and 3-ml fractions were collected and monitored at 280 nm.

Fig. 2. Affinity chromatography on Affigel blue of fraction I from DEAE trisacryl containing ovine transferrin. A 2-ml sample (12 mg) was applied onto a $1 \text{ cm} \times 10 \text{ cm}$ column equilibrated with Tris-HCl (50 mmol/l, pH 7) containing 100 mmol KCl/l. The arrow shows a buffer change at fraction No. 16 (50 mmol Tris-HCl/l, pH 7, containing 1.5 mol KCl/l). The flow was 0.5 ml/min, and 1-ml fractions were monitored at 280 nm.

Table 1. Comparison of the *N*-terminal sequence of the ovine transferrin preparation described in this work with previously published sequences of transferrins (or ovotransferrins) from different species

This work	Reference	I	5	10
		Asp-Pro-Glu- Lys- Thr- Val- Ile-	Trp- Cys- Thr- Ile- ...	
Sheep	Guerin <i>et al.</i> , 1976	Ser- Pro-Glu- Lys- Thr- Val- Ile-	Trp- ...	
Cow	Brock <i>et al.</i> , 1980	Asp-Pro-Glu- Arg- Thr- Val- ...		
Pig	Graham & Williams, 1975	Val- Pro-Glu- Lys- Thr- ...		
Rat	Aldred <i>et al.</i> , 1984	Val- Pro-Asp- Lys- Thr- Val- ?	-Trp- Cys- Ala- Val- ...	
Man	MacGillivray <i>et al.</i> , 1983	Val- Pro-Asp- Lys- Thr- Val- Arg-	Trp- Cys- Ala- Val- ...	
Chicken	Graham & Williams, 1975	Ala- Pro-Pro- Lys- Ser- Val- Arg-	Trp- Cys- Thr- Ile- ...	

Table 2. Yield and recovery of ovine transferrin

	Yield (mg)	Activity*		Recovery (%)
		Specific (mg / mg)	Total (mg)	
Ammonium sulphate precipitate	2000	0.06	122	100
DEAE trisacryl elution—peak I	386	0.26	100	82
Affigel blue matrix elution—peak I	64	1†	64	52

*As determined by radioimmunoassay.

†Served as the standard for o-transferrin radioimmunoassay.

rabbit, cross-reactions were the same (data not shown), but all the o-transferrin assays reported in this study were performed using the antiserum 188.

Sensitivity of the assay. The standard curve sensitivity was 0.4 ng of o-transferrin, determined as the first dose of the standard curve which yielded a bound (B) value statistically different from unbound (B_0). As 100- μ l aliquots of culture medium samples were assayed, the sensitivity of the assay was 4 ng/ml cultured medium.

Specificity of the assay. A parallelism test was applied to a pool of culture media, obtained from enriched cultures of ovine Sertoli cells, which was assayed against o-transferrin standards diluted in culture medium (Fig. 4). The slope of the line obtained with the culture medium pool was not different from the slope of the standard curve ($P > 0.05$).

Precision of the assay. The intra- and interassay precisions were 15 ($n = 20$) and 22% ($n = 9$), respectively, for a culture medium pool yielding a B: B_0 ratio of 30%.

Influence of the day of culture on o-transferrin secretion: effect of serum supplementation

The influence of the day of culture on transferrin secretion by sheep Sertoli cells was investigated over 6 days (Fig. 5a). Cells were plated on Day 0, and an inhibitor of protein synthesis, cycloheximide (10 μ g/ml) or FIRT (500 ng o-FSH/ml + 10 μ g insulin/ml + 500 ng retinol/ml + 5×10^{-7} mol testosterone/l) was immediately added. The medium was changed and factors added every 24 h for 6 days. Basal daily transferrin secretion was halved (-49%) between Days 1 and 2 of culture, and decreased slightly thereafter. On Day 1, preformed transferrin represented the greater part (65%) of basal secretion, whereas it represented $<20\%$ on Days 2 to 4. Under FIRT stimulation, transferrin secretion increased sharply, the ratio of stimulated secretion to basal secretion increasing 11-fold between Day 1 (1.1) and Day 6 (12).

To prevent the marked decrease in basal transferrin secretion during the 6 days of culture, 10% fetal calf serum was added to the culture medium (Fig. 5b). Transferrin secretion in the presence of

Fig. 3. Competition curves for the binding of purified ovine transferrin (●), human transferrin (◆), rat transferrin (▲) and ovine albumin (■) with ^{125}I -labelled ovine transferrin to rabbit anti-ovine transferrin antibody. Data are means $\pm$ s.d. ($n = 3$).

Fig. 4. Relationship between volume of a pool of culture medium (▲) and amount of ovine (o) transferrin diluted in culture medium (■). Data are means ($n = 3$). Logit (bound (B):unbound (B_0)) = $\text{Log} (B:B_0)/1 - (B:B_0)$.

serum was about a third of that in the absence of serum, from the first day of culture. Basal transferrin secretion was halved (-45%) between Day 1 and Day 2 of culture, and increased 5-fold between Days 2 and 6. On Day 1, preformed transferrin represented the greater part (77%) of the basal secretion, whereas it became undetectable afterwards. Under FIRT stimulation, transferrin secretion increased 5-fold from Day 1 to Day 6. However, the ratio of stimulated to basal secretion doubled between Day 1 (1.0) and Days 4–6 (2.0). Therefore, at the end of the culture period, this ratio was about six times lower in the presence of serum than in its absence.

Similar experiments were performed using the fetal calf serum substitute Ultrosor G for 4 days in culture. With 2% Ultrosor G, results were identical to those obtained with 10% fetal calf serum;

Fig. 5. Daily secretion of ovine (o) transferrin by enriched cultures of sheep Sertoli cells. Cells were cultured in the absence (a) or presence (b) of 10% fetal calf serum; (■) 10 µg cycloheximide/ml, (▨) control, (□) FIRT 500 ng follicle-stimulating hormone/ml + 10 µg insulin/ml + 500 ng retinol/ml + 5×10^{-7} mol testosterone/l). Data are means $\pm$ s.d. ($n = 4$ culture wells).

0.02% and 0.2% Ultraser gave intermediate results between 0 and 2% Ultraser, showing a dose-dependent inhibition of o-transferrin secretion (data not shown).

Since the aim of the study was to test the stimulating effect of different factors upon transferrin secretion, the following culture experiments were performed in serum-free medium. Cells were cultured for 2 days without any factor, and then 2 days in the presence of various factors.

Regulation of o-transferrin secretion

To investigate the effect of various factors, cells were cultured in medium lacking in insulin and retinol, and the factors were individually added on Day 2, at three concentrations. Secretion of transferrin was slightly stimulated by the higher concentrations of insulin and EGF and doubled by retinol and FSH (Table 3), but was not affected by testosterone.

To determine whether the two more efficient stimulating factors, retinol and FSH, have additive or synergistic effects on transferrin secretion, two culture experiments were conducted. Retinol (500 ng/ml) or FSH (500 ng/ml) increased transferrin secretion 2-fold, and a combination of the two factors at these concentrations increased the secretion 4-fold (Fig. 6).

Table 3. Effects of various factors on secretion of ovine transferrin

Number of experiments	Supplement	Concentration	o-transferrin (% control)
	None		100 ^a
4	Insulin	0.4 µg/ml	117 ^b
		2	119 ^b
		10	128 ^c
4	Epidermal growth factor	2 ng/ml	108 ^{ab}
		10	110 ^{ab}
		50	124 ^b
3	Retinol	20 ng/ml	118 ^b
		100	138 ^c
		500	218 ^d
4	Testosterone	0.2×10^{-7} mol/l	101 ^a
		1	97.5 ^a
		5	104 ^a
5	Follicle-stimulating hormone	20 ng/ml	102 ^a
		100	144 ^b
		500	220 ^c

Each factor was tested in three to five culture experiments. Cells were cultured for 2 days in culture medium lacking insulin and retinol; on Day 2, the medium was changed and factors were added at the indicated concentration to four culture wells. Spent medium was collected on Day 4. In each experiment, the concentration of transferrin secreted in the presence of cycloheximide (10 µg/ml) has been subtracted. For each factor, numbers with different letter superscripts are significantly different ($P < 0.05$) when tested by analysis of variance.

Fig. 6. Effects of retinol and follicle-stimulating hormone (FSH) on secretion of ovine (o) transferrin. Cells from two experiments were cultured for 2 days in medium lacking insulin and retinol. On Day 2, the medium was changed and retinol or FSH was added to four replicate culture wells. Spent medium was collected on Day 4. In each experiment, the concentration of pre-formed transferrin secretion has been subtracted from all the values of transferrin secretion. Data are means $\pm$ s.d. ($n = 8$ culture wells).

Discussion

This paper describes an efficient purification scheme permitting the preparation of large quantities of o-transferrin. During its purification, the presence of the protein in the different fractions was detected only by its behaviour in SDS-PAGE. Indeed, there is generally no cross-reaction in the immunoassays of transferrins from different species and, accordingly, we found that the ovine molecule was not detectable in the assays for bovine and rat transferrins. The identification of o-transferrin was finally confirmed by *N*-terminal sequencing of its polypeptide chain. Eleven residues were determined, while only eight *N*-terminal amino-acid residues had been previously determined (Guerin *et al.*, 1976). The comparison with the sequence found in the present work shows complete identity except for the *N*-terminal residue (Asp instead of Ser). Bovine transferrin possesses an *N*-terminal sequence similar to the one we found here for the ovine molecule (including the terminal Asp), but with Arg instead of Lys in the fourth position in all the other sequences. Taken together, the comparison of the sequences (Table 1) strongly indicates that the molecule we purified is o-transferrin.

This o-transferrin preparation was used to set up an RIA which appeared specific. The parallelism test did not reveal any interference of compounds secreted by the cells into the culture medium. The fact that o-albumin exhibited some cross-reactivity in the assay might be explained in two ways: (a) peak II from Affigel blue elution (o-albumin) might still contain 1–2% transferrin; or (b) peak I from Affigel blue chromatography might be slightly contaminated with albumin, which would have led to the production of some anti-o-albumin antibodies in addition to anti-o-transferrin antibodies. The two hypotheses, which are not mutually exclusive, cannot be tested as neither pure o-albumin nor anti-o-albumin are available.

The RIA developed in our laboratory is sensitive enough to detect o-transferrin in spent culture medium of Sertoli cells. Only occasionally were the concentrations of o-transferrin in spent media from cells cultured in the presence of cycloheximide under the limit of detection of the assay (4 ng/ml).

In the absence of serum, basal secretion of o-transferrin decreased 2-fold between Days 1 and 2 of culture and decreased little thereafter, though our culture medium contained insulin (2 µg/ml) and retinol (50 ng/ml). In 20-day-old rats, Skinner & Griswold (1982) reported a dramatic fall in r-transferrin secretion during 8 days in culture in F12 medium, which occurred even after the addition of retinol (100 ng/ml), but was prevented by the addition of retinol plus insulin (5 µg/ml). In the same species, Perez-Infante *et al.* (1986) observed that r-transferrin secretion by cells derived from young animals (≤ 19 days old) markedly decreased during 10 days in culture in basal culture medium. The addition of retinoic acid (50 ng/ml) maintained r-transferrin secretion throughout the culture period. The discrepancy between these two groups could be due either to the form of vitamin A used, or to the composition of the basal medium. Janecki & Steinberger (1987) also observed that in basal medium r-transferrin secretion gradually decreased from Days 1–3 to Days 11–13. In the present study, after FIRT addition, the ratio of stimulated o-transferrin upon basal secretion increased sharply between Days 1 and 6 of culture. In rat Sertoli cells cultured on plastic or on a Millipore filter, the magnitude of r-transferrin secretion stimulation by FIRT was maximal between Days 4 and 6 (Skinner & Griswold, 1982; Janecki & Steinberger, 1987; Skinner *et al.*, 1989a). When cells were plated onto RBM matrix, stimulated r-transferrin production rose from Day 2 to Day 4 and decreased gradually from Day 4 to Day 8 of culture (Tananis Anthony *et al.*, 1989). In human Sertoli cell cultures, Holmes *et al.* (1984) observed maximal FIT-stimulated secretion (i.e. no retinol) of h-transferrin on Day 5 of culture. Thus it appears that in cultures of sheep Sertoli cells as well as in other species, transferrin secretion can be more efficiently stimulated by the combination of FSH, insulin, retinol and testosterone and that the ratio of stimulated to basal secretions increases up to Day 6 of culture.

In the presence of fetal calf serum, the average concentration of secreted o-transferrin was about a third of that in serum-free medium. This result is different from those reported in rats,

where fetal bovine serum is the most effective stimulant of r-transferrin secretion (Perez-Infante *et al.*, 1986; Janecki & Steinberger, 1987). Thus it seems that fetal calf serum contains one or more factors that inhibit transferrin secretion in sheep Sertoli cells. The partial purification of fetal calf serum to yield the substitute Ultrosor G does not eliminate this effect. The complex nature of serum makes it impossible to ascertain which factor is responsible for the inhibition of o-transferrin secretion. It would probably be easier to look for this factor in Ultrosor-G if the manufacturer disclosed its composition. However, we do not know whether the negative effect of serum is specific for o-transferrin secretion or whether it extends to other secretions or functions. After FIRT addition, the ratio of FIRT-stimulated to basal secretions was lower than in serum-free cultures. Similarly, in cultures of rat Sertoli cells, Mather *et al.* (1984) reported that serum supplementation markedly reduced the adenylate cyclase response to FSH. Likewise, Monaco *et al.* (1989) reported that both FSH-dependent stimulation of c-AMP accumulation and androgen aromatization were inhibited in a dose-dependent fashion by fetal bovine serum. They hypothesized that serum contains heat-stable, non-dialysable factors that affect the response of Sertoli cells to FSH, possibly by activation of polyphosphoinositide turnover, or by stimulating cAMP degradation by activating a calcium-calmodulin-dependent phosphodiesterase. However, neither the identity of the serum factor(s) nor its mechanism of action have been determined.

O-transferrin secretion by sheep Sertoli cells was slightly increased by insulin and EGF and was doubled by retinol and FSH. Testosterone had no effect. In rats, factors that regulate transferrin secretion have been studied extensively (Skinner & Griswold, 1982; Perez-Infante *et al.*, 1986) as well as factors regulating transferrin mRNA synthesis *in vitro* and *in vivo* (Huggenvik *et al.*, 1987; Hugly & Griswold, 1987; Skinner *et al.*, 1989a). FSH, insulin, retinol and, to a lesser extent, EGF have been shown to induce significant increases in r-transferrin secretion. The case of testosterone is somewhat controversial, since some authors reported a small stimulatory effect (Perez-Infante *et al.*, 1986; Huggenvik *et al.*, 1987), whereas others found no significant effect (Skinner *et al.*, 1989a). In cows, transferrin secretion by cultures of immature Sertoli cells is stimulated by FSH, retinol, insulin, EGF and, to a lesser extent, testosterone (Jenkins & Ellison, 1989). In man, only FSH and insulin have been tested in Sertoli cell cultures and they both exhibited stimulatory effects (Holmes *et al.*, 1984). So, it appears that the hormonal control of testicular transferrin secretion is similar in these mammalian species.

The action of insulin on Sertoli cells requires high concentrations, and has been shown to act through the insulin-like growth factor I receptors. Insulin-like growth factor I is probably the primary mediator of most of the effects of insulin on Sertoli cells, such as the increased production of lactate and synthesis of cell proteins (for a review, see Bellve & Zheng, 1989). The mechanism of action of EGF on Sertoli cell function is unclear. Receptors for EGF and growth factor- α exist in rat peritubular cells, but not in Sertoli cells or germ cells; it is therefore, hypothesized that the effects of EGF on Sertoli cell functions might be indirect via contaminating peritubular cells (Skinner *et al.*, 1989b).

In sheep, two of the principal factors regulating transferrin secretion are FSH and retinol, and their effects are additive. FSH action is mediated via the adenylate cyclase-protein kinase A system. Retinoids exert their actions through binding to an intracellular DNA-binding protein belonging to the thyroid/steroid hormone receptor family. This indicates that maximal stimulation of transferrin secretion requires the combined actions of mechanisms dependent and independent of cAMP.

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