Journal of Steroid Biochemistry, Vol. 11, pp. 671 to 674
Pergamon Press Ltd 1979. Printed in Great Britain

QUANTITATIVE DETERMINATION OF SPECIFIC PROTEINS IN RAT EPIDIDYMIS

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SUMMARY

This paper describes the development of a radioimmunoassay for the measurement of specific proteins (SEP) in rat epididymis. Using [^{125}I]-SEP the assay is useful within the range 0.6–30 ng SEP. With this method we confirmed the organ specificity of SEP which could neither be detected in the cytosol of several other organs in the rat nor in rat blood plasma. Furthermore, SEP appear to be species specific since the epididymal cytosol from man, stallion, bull, dog and rabbit did not react with serum anti rat SEP. Castration decreased the SEP content of rat epididymis. Preincubation of anti-SEP with cauda epididymis spermatozoa sharply decreased the binding capacity of the antiserum. The same treatment did not alter the binding capacity of control anti-LH serum. Immunofluorescence experiments show that SEP are attached to spermatozoa obtained from the cauda epididymis.

INTRODUCTION

Sperm maturation is a physiological process whereby spermatozoa acquire fertilizing capacity during their transit through the epididymis [1]. Recent investigations have shown that this phenomenon is androgen-dependent and that it is mediated by factors produced by the epididymis [2, 3].

These findings prompted studies on the composition of epididymal fluid, leading to the identification of compounds, such as carnitine, glycerophosphoryl choline and sialic acid, which attain high concentrations in the luminal fluid [4]. As yet, however, a definite relationship between these compounds and sperm maturation has not been established.

The rat epididymis is also known to produce and secrete specific proteins (SEP) [5, 6]. Lea *et al.*[7] and ourselves [8] observed that the production of SEP is androgen dependent and that these proteins are attached to spermatozoa. That SEP might play a role in sperm maturation was recently suggested by the findings of Orgebin-Crist and Jahad[9] showing that the 5α-DHT-induced sperm maturation in cultured rabbit epididymal tubules was blocked by the simultaneous addition of RNA or protein synthesis inhibitors.

The present study deals with the quantitative determination of rat SEP using a radioimmuno-assay method.

EXPERIMENTAL

Carrier-free ^{125}INa was purchased from the New England Nuclear Company. Sepharose 4B and Sephadex G-75 were obtained from Pharmacia Fine Chemicals. Anti rabbit IgG serum labelled with fluoresceine isothiocyanate was purchased from the Institut Pasteur (Paris). All other chemicals used were of analytical grade.

Antisera

An antiserum against rat SEP was obtained from rabbits injected with portions of polyacrylamide gels containing SEP, emulsified in Freund complete adjuvant, as previously described [10]. The specificity of the antiserum was increased by passage through an affinity column of Sepharose 4B coupled to the cytosol proteins obtained from epididymides of adult rats castrated for 60 days. Coupling was obtained by the cyanogen bromide method [10]. The second antibody used was goat anti-rabbit IgG. Anti LH serum was kindly supplied and tested by Dr M. A. Rivarola, Children's Hospital, Buenos Aires.

Tissues

Tissues were minced with scissors and homogenized in 50 mM Tris buffer, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA and 0.25 mM dithiothreitol using a Polytron homogenizer.

The homogenates were centrifuged at 105,000 *g* for 1 h at 4°C to obtain the cytosol.

Labelling of proteins

SEP were isolated by precipitation of rat epididymal cytosol with $(NH_4)_2$ SO_4 followed by ion exchange chromatography on DEAE cellulose, as previously described [10]. SEP (3, 2 μg) were mixed in a siliconized test tube with 800 μCi^{125}INa, 7.5 nM Na ClO and 0.5 M phosphate buffered saline (PBS) to a final volume of 50 μl. After 1 min at room temperature the reaction was stopped by dilution with PBS. Unreacted ^{125}INa was removed by passage through a Sephadex G-75 column. The labelling efficiency was 14.75% and the specific activity 44 μCi/μg.

Radioimmunoassay

This was performed in triplicate using the antiserum at a final dilution 1/12,000 and adding 10,000

cpm ^{125}I SEP to each tube. Non radioactive SEP was added within the range of 0.6 30 ng. Incubation was carried out in a final volume of 0.3 ml for 72 h at 4 C and was followed by the addition of 1 vol. of a second antiserum at a dilution of 1/5. After further incubation for 24 h at 4 C the precipitate was removed by centrifugation and the radioactivity determined in both phases.

Samples to be measured were added to the assay at concentrations of up to 50 ng total protein. Protein was measured according to Lowry *et al.*[12], using bovine serum albumin as standard.

Immunofluorescence

Spermatozoa were expressed from the cauda epididymis and washed 3 times by suspension and centrifugation (1000 *g* for 10 min) in PBS. Washed sperm were smeared on microscope slides, dried, fixed in 5% formaline solution (10 min) and incubated with anti-SEP or normal rabbit serum for 30 min at 4°C. After 4 washings with cold PBS, fluorescein-labelled anti rabbit IgG was added and incubated for 30 min at 4 C. After washing 4 times the preparation was observed in a Carl Zeiss fluorescence microscope.

RESULTS

The optimal antiserum concentration to be used in the assay of SEP was determined by means of a titration curve at dilutions between 1/1000 and 1/50,000. The best sensitivity was attained at a dilution of 1/12,000 and it was routinely used at this concentration throughout this study.

Figure 1(a) shows the standard curve obtained after the addition of increasing concentrations of SEP in the presence of a fixed quantity of ^{125}I SEP. Curve b was obtained when each tube was supplemented with cytosol from the epididymides from rats castrated for 60 days, to bring the final protein concentration to 50 ng. The differences in binding between these two curves were not significantly different.

Using this technique, we tested rat plasma and the cytosol from several rat organs for the presence of SEP. The results in Table 1 confirm previous findings [8, 10] indicating that SEP are organ specific and not found in other tissues or fluids in the rat. Furthermore, SEP appear to be species-specific since the proteins in the cytosol of epididymides from man, stallion, bull, dog and rabbit did not cross-react with our antiserum. The androgen dependence of rat SEP, evidenced by the absence of displacing proteins in the cytosol of epididymides from 60 day castrated rats, was further investigated in rats castrated for 2, 7, 14, 21 and 28 days. The results in Table 2 show an initial rapid decrease of the SEP content, 42% after only 2 days of castration, followed by a slower decrease which reached 75% after 28 days of castration.

In attempting to detect the presence of SEP attached to spermatozoa we used an indirect determination since cells could not be introduced into the assay. For this purpose the binding of ^{125}I-SEP to anti-SEP was compared before and after exposure of the serum to graded numbers of spermatozoa. As a control, anti-LH serum was also incubated with sperm and subsequently tested for binding in its radioimmunoassay.

The results, shown in Table 3, demonstrate the decrease in binding activity of anti-SEP serum after exposure to spermatozoa while the binding capacity of anti-LH remained unaltered by this treatment.

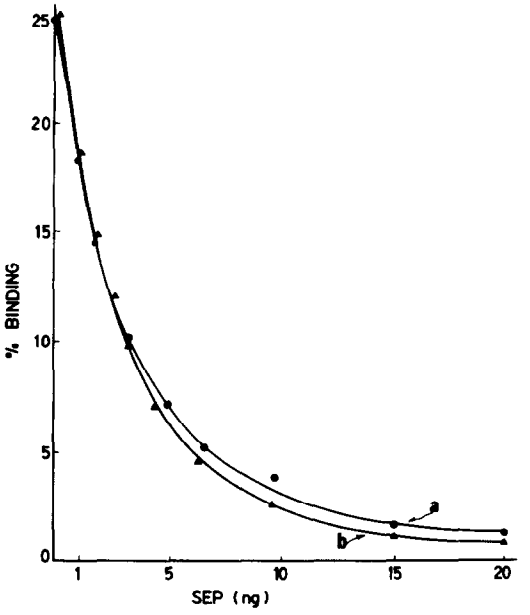

Fig. 1. Standard curve for the radioimmuno assay of SEP. Each tube (triplicates) contained 10,000 c.p.m. ^{125}I-SEP (100 µl, equivalent to 0.1 ng) and non radioactive SEP (100 µl) to bring the final concentration to the amounts shown, between 0.6 and 30 ng. Serum anti SEP (100 µl) was added at a dilution 1/12,000 and the mixture incubated for 72 h at 4°C. This was followed by addition of 300 µl of anti rabbit IgG serum at a dilution 1/5 and further incubation for 24 h at 4°C. The samples were then centrifuged and radioactivity measured in both the supernatant and the precipitate.

In curve b each tube was supplemented with enough cytosol obtained from the epididymides of rats castrated for 60 days to bring the final protein concentration to 50 ng.

Table 1. Determination of SEP in blood serum and the cytosol of different organs from the rat

Sample	SEP (ng/100 ng protein)
Epididymis	12.93 ± 2.07*
Serum	ND
Liver	ND
Prostate	ND
Testis	ND
Pituitary	ND

* Results are expressed as the mean ± SD of three determinations. All samples containing 50 ng total protein were assayed as described in Fig. 1.

ND: not detectable.

Table 2. Influence of castration for different periods on the SEP content of rat epididymis

Period of castration (days)	SEP (ng/100 ng protein)
0	12.93 ± 2.07*
2	7.50 ± 0.89
7	5.38 ± 0.53
14	4.90 ± 0.07
21	4.10 ± 0.19
28	3.30 ± 1.12
60	ND

* Results are expressed as the mean ± SD of three determinations. All samples contained 50 ng total protein and were assayed as described in Fig. 1.
ND: not detectable.

Table 3. Effect of preincubation of antisera with spermatozoa on their binding capacity

Sperm preincubated (10^6)	Binding of SEP (% ± SD)	Binding of LH (% ± SD)
0	24.55 ± 0.78	26.40 ± 2.43
1.0	6.30 ± 0.81	—
2.0	4.25 ± 0.35	—
4.0	2.23 ± 0.07	24.45 ± 0.1

Spermatozoa obtained from the cauda epididymis were washed twice with PBS and counted in an hemocytometer. Graded numbers of spermatozoa were incubated 16 h at 4°C with aliquots of sera anti-SEP and anti-LH and after removal of the sperm by centrifugation the sera were tested for binding as described in Experimental.

In order to confirm the presence of SEP on spermatozoa we applied the immunofluorescence technique. The results, shown in Fig. 2, demonstrate that SEP covers most of the acrosomal region of spermatozoa as well as portions of the tail.

DISCUSSION

A better understanding of the nature of the physiological processes involved in the development of fertilizing capacity by spermatozoa will facilitate the treatment of some types of male infertility and provide the knowledge necessary for the development of effective means of interference with this process.

The aim of the present study was the development of a quantitative technique for the measurement of rat specific epididymal proteins which might be part of the "epididymal factors" that intervene in sperm maturation.

In view of the androgen dependency of this process [3], it is likely that the epididymal factors involved are also directly or indirectly regulated by androgens. The results of the present study confirm that the concentration of SEP in the rat epididymis

Fig. 2. Immunofluorescence of rat spermatozoa exposed to serum anti-SEP. Spermatozoa obtained from the cauda epididymis were washed three times with phosphate buffered saline, smeared onto microscope slides, fixed with 5% formaline and incubated with anti-SEP or normal rabbit serum for 30 min at 4°C. After 4 washings the preparations were incubated 30 min at 4°C with anti rabbit IgG serum labelled with fluoresceine isothiocyanate. The arrows show zones of intense fluorescence.

depends on the adequate provision of androgens (Table 2) and that these proteins interact with spermatozoa (Table 3, Fig. 2).

Several independent groups have recently described glycoproteins of epididymal origin which attach to spermatozoa and, in some cases, the characteristics of the moieties described by each group are similar. For example, Lea *et al.*[7] found an acidic epididymal glycoprotein (AEG) (mol. wt 40,000) which resembles the characteristics of SEP (mol. wt. 37,000 for proteins D-E) [10] which are also glycoproteins [10]. Both AEG and SEP are eluted from DEAE-cellulose columns at the same molarity of salt [7, 10] and are androgen dependent. Olson and Hamilton[13] described the existence of a glycoprotein (mol. wt. 37,000) which can be found on the surface of rat spermatozoa obtained from the cauda epididymis but that is absent in caput epididymis sperm, suggesting that it is added during epididymal transit.

This evidence, when analyzed in the light of the fact that protein synthesis inhibitors can block the androgen-induced maturation of spermatozoa [9], suggests that epididymal proteins which attach to the maturing sperm might play an important role in the acquisition of fertilizing capacity.

Acknowledgements—This work was partially supported by Grant 780-0610 from The Ford Foundation. The authors wish to express their gratitude to Drs M. A. Rivarola and E. H. Charreau for their helpful suggestions and discussion.

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