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EFFECT OF DEPRIVATION OF VITAMIN A ON THE BASIC PROTEINS OF THE NUCLEI OF RAT TESTES

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SUMMARY: The relative concentrations of the major histones, namely, H1, TH1, H2A, H2B, H3 and H4 are not significantly changed in the testes of the vitamin A-deficient rats, as compared to those in the normal controls. But the testis-specific protein, TP, which is synthesized at the mid-spermatid stage, is markedly reduced in the testes of the deficient rats. On supplementation of the deficient rats with retinyl acetate for 28 days, there was a 50% recovery in the relative concentration of the TP with respect to the total amounts of H1 and TH1.

INTRODUCTION

It is being increasingly recognised in recent years that vitamin A is required for normal division and differentiation of epithelial cells (1,2,3). The process of spermatogenesis is a typical classical example of division and differentiation of epithelial cells. Here the original stem cells of the seminiferous tubules undergo several mitotic divisions ultimately yielding spermatogonia, which in turn divide and differentiate eventually giving rise to primary spermatocytes. The spermatocytes in turn undergo meiosis and produce spermatids, which contain haploid DNA and differentiate into mature spermatozoa.

Long ago Mason (4) had observed that the germinal epithelium of the testes of vitamin A-deficient rats undergoes degenerative changes, while the sperms do not develop beyond the spermatid stage. Subsequent histological examination of the testes of

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vitamin A-deprived rats has revealed that even at the early stages of the deficiency the germinal epithelium contains mostly spermatogonia with few spermatocytes, but no spermatids (5). It has also been reported that the testes of rats maintained on a vitamin A-free diet supplemented with retinoic acid show poor development with marked atrophy and contain mostly spermatogonia, with some spermatocytes, but no spermatids (6).

One of the important biochemical characteristics of spermatogenesis in mammals is attenuation of the cytoplasm and condensation of the chromatin giving transcriptionally inactive spermatozoa. During this process of condensation the somatic histones, namely, H1, H2A, H2B, H3 and H4 and the testis-specific histone H1 (TH1) are replaced by protamine(s) (7). Kistler et al. (8) have identified in rat testes a rather unique protein TP, which transiently appears at the mid-spermatid stage during spermatogenesis. These investigators have purified this particular protein and have shown it to be rich in lysine (19.1%) and arginine (20%), while later work of Shires et al. (9), Balhorn et al. (10) and of Mayer and Zirkin (11) has suggested that it may initiate condensation of chromatin.

Since the transition of the nuclear basic proteins is an important and characteristic event during the process of spermatogenesis, and since vitamin A deprivation exerts pronounced effects on spermatogenesis, particularly at the step of meiosis, we have compared the basic proteins of the nuclei isolated from the testes of normal and vitamin A-deprived rats and our findings reported here indicate a marked reduction in the TP fraction in the deficient testes.

MATERIALS AND METHODS

Male albino rats of this Institute strain were raised on a vitamin A-deficient diet after weaning, as described by Malathi

et al. (12) and were used when they ceased to grow. At the same time corresponding control male rats were maintained on the same diet, but they received daily supplements of 40 µg. of retinyl acetate per rat and were used at comparable age. Another group of male rats was also kept on the same diet till they ceased to grow, at which stage they started to receive daily supplements of 40 µg, of retinyl acetate per rat and were killed thereafter at weekly intervals. In those experiments where TP was isolated in pure form for use as reference, adult albino male rats raised on the normal stock diet were used.

Isolation of nuclei from the testes of normal and vitamin A-deficient rats and extraction of the acid-soluble proteins of the nuclei: All rats were killed by decapitation and the testes were quickly removed and decapsulated, following which the tissues were homogenized in a Potter-Elvehjem homogenizer in 10 volumes of 0.01M Tris HCl buffer of pH 7.4, which also contained 0.31M sucrose, 0.005M $MgCl_2$, 0.0001M phenyl methyl sulphonyl fluoride and 0.5% Triton X-100. Purified nuclei were isolated from this homogenate, as described earlier (13).

The acid-soluble proteins were isolated from the testes-nuclear pellet essentially according to the method of Platz et al. (14). At the same time the acid-soluble proteins were isolated from the liver nuclei of normal adult male rats by employing the same procedure.

Isolation of the reference TP from the testes of normal adult rats: While the procedure described above was used for the isolation of the total acid-soluble nuclear proteins, the TP was specifically isolated in pure form from the testes of 60 normal adult rats by the method described by Kistler et al. (8). This procedure involves homogenization of the testes in 0.4N H_2SO_4, differential precipitation of the proteins from the extracts with trichloroacetic acid, solubilization of the precipitated proteins in 0.1M sodium acetate buffer of pH 4.5 and chromatography of the solubilized proteins on CM-Sephadex followed by filtration through Sephadex G-50. This procedure gave pure TP which was used as reference during electrophoresis of the nuclear proteins isolated from the testes of the vitamin A-deficient and corresponding control rats.

Polyacrylamide gel electrophoresis: The patterns of the major histones and of TP of the testes nuclei of the various groups of rats were compared by subjecting the total acid-soluble proteins to electrophoresis on 15% polyacrylamide acid-urea gels by following the method of Panyim and Chalkley (15). At the end of the electrophoresis the gels were stained with amido black for 1 hr. and destained with the same solvent, after which densitometric scans of the stained gels were taken at 600 nm in a Beckman 26 spectrophotometer.

The protein contents of the nuclear acid-soluble fraction as well as of the purified TP were determined by measuring the turbidity at 400 nm after precipitation with 25% trichloroacetic acid, as described by Platz et al. (14).

RESULTS AND DISCUSSION

The electrophoretic patterns of the acid-soluble nuclear proteins isolated from the testes of the normal and vitamin A-

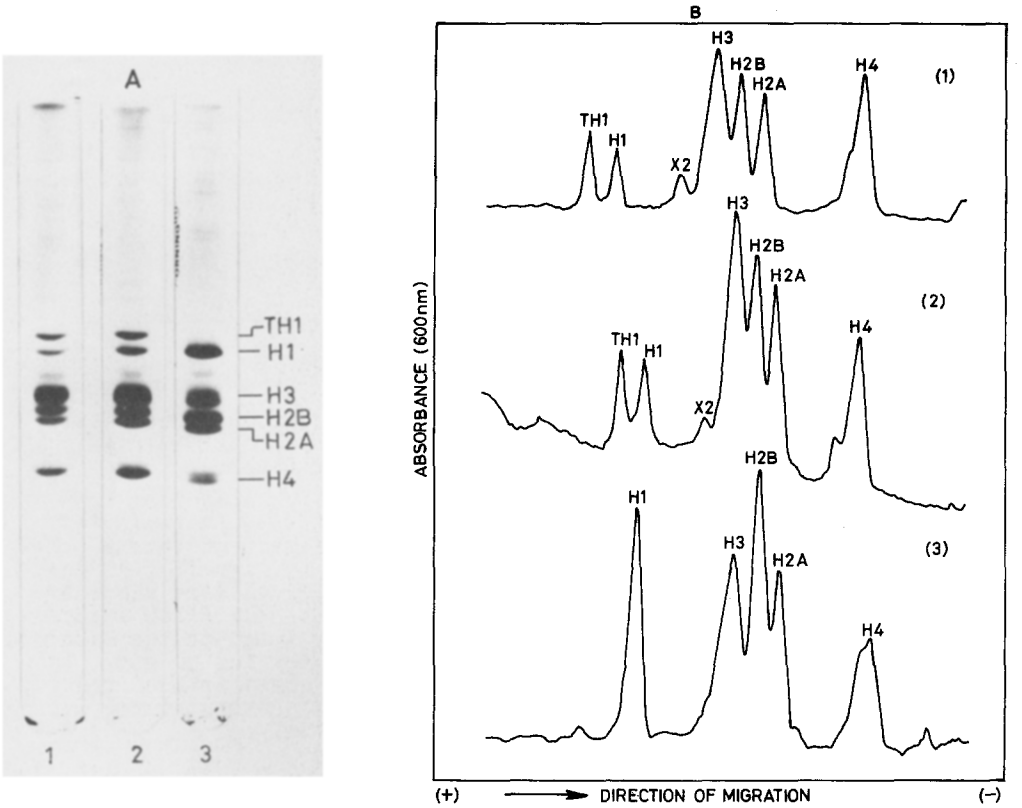

Fig. 1: Polyacrylamide Gel Electrophoresis of the nuclear Acid-soluble Proteins isolated from the Testes of normal and vitamin A-deficient Rats and from the Liver of normal Rats:

A. The acid-soluble nuclear proteins (60 µg each) isolated from the testes of normal (1) and vitamin A-deficient (2) rats, and from normal rat liver (3) were subjected to electrophoresis on 15% polyacrylamide acid-urea cylindrical gels (10cm x 0.4cm) according to the method of Panyim and Chalkley (1969), using pyronine Y as the tracking dye. The duration of the electrophoresis was 4 hr. during which time the pyronine Y had moved two-thirds of the gel. The gels were stained with 0.2% amido black in 40% methanol and 5% acetic acid and destained with the same solvent.

B. Densitometric scans of the stained gels of (A) at 600 nm.

deficient rats and also from the liver of normal rats, as given in Fig. 1A, reveal that the normal testes contain an additional histone H1 (TH1) which is not found in the liver and this is in agreement with the earlier observations (7,9,16). It should be noted from the densitometric scans presented in Fig. 1B that the amounts of the TH1 and H1 together of the testes are equivalent to

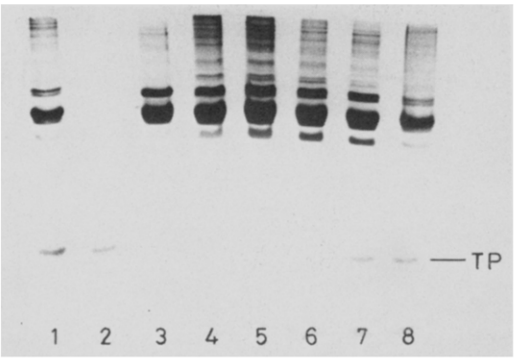

Fig. 2: Comparison of the Testis-specific Protein (TP) in the Acid-soluble Extracts of the Nuclei isolated from the Testes of normal, vitamin A-deficient and vitamin A-replenished Rats and from normal Rat Liver:

Acid-soluble nuclear proteins (160 µg each) isolated from the testes of normal rats (1); normal rat liver (3); vitamin A-deficient testes (4); testes of vitamin A-deficient rats supplemented with retinyl acetate for 7 days (5), 14 days (6), 21 days (7) and 28 days (8); 10 µg of the purified TP (2) were subjected to electrophoresis on 15% polyacrylamide slab gels (11cm x 13cm x 0.2cm) as described in the legend for Fig. 1 and in the Methods, except that the electrophoresis was carried out for 90 min., by which time the pyronine Y had moved to only one-fourth of the running gel. The protein bands were located after staining with amido black as described in the legend for Fig. 1.

the total amounts of H1 in the liver. The scans also reveal the presence of one additional peak of a minor protein X_2 in the testes which is not found in the liver. But X_3, which is a variant of H2B (16,17), has not separated from H3 in these gels, and therefore the peak given by H3 is relatively higher in the testes than in the liver.

It was interesting that the patterns of the histones isolated from the normal and deficient testes were rather similar (Figs. 1A and 1B). This is not surprising, because vitamin A deficiency affects spermatogenesis only at the later stages, particularly at the step of conversion of spermatocytes to spermatids, while according to Branson *et al.* (16) and Grimes *et al.* (17) the histones TH1, X_2 and X_3 are synthesized at an early stage of spermatogenesis.

Typical electrophoretic patterns obtained from a run for a shorter time of the acid-soluble proteins isolated from the normal and deficient testes and from normal rat liver given in Fig. 2

demonstrate that the normal testes contain a protein fraction which comigrates with the pure TP isolated from the testes of normal adult rats. Fig. 2 also shows that the TP is testis-specific, because it was not found in the acid-soluble nuclear proteins of the liver. But it was most significant that the band corresponding to that given by the TP from normal testes was absent in the nuclear extracts of the deficient testes. The same figure further demonstrates that following daily supplementation of the deficient rats with retinyl acetate the TP starts to reappear around day 21 of the supplementation.

The amount of the TP was estimated by scanning the gel strips of Fig. 2 followed by calculation of the areas under the peaks given by the TP as well as by H1 and TH1, after which the molar ratio of the TP with respect to that of the total of H1 and TH1 was ascertained in the samples isolated from the testes of normal, vitamin A-deficient and retinyl acetate-replenished rats. The results given in Table 1 show that, while the molar ratio was 1.1 in the normal testes, its value declined to 0.07 in the deficient

Table 1: Relative Concentrations of TP in the Testes of the normal, vitamin A-deficient and vitamin A-replenished Rats

Treatment	Molar ratio of TP/H1+TH1*
Control	1.10
Vitamin A deficient	0.07
Vitamin A replenished for 21 days	0.20
Vitamin A replenished for 28 days	0.58

* The polyacrylamide gel strips of Fig. 2 were scanned at 600 nm. The areas under the peaks given by TP, and H1 and TH1 were calculated and divided by their molecular weights, namely, 13,000, 21,000 and 21,000, respectively. The relative concentration of the TP is expressed on the basis of the molar ratio of TP/H1+TH1.

tissue. On the other hand, on continued supplementation with retinyl acetate the ratio started to improve in that, while it was 0.20 on day 21 of supplementation, it became 0.58 on day 28. Such slow process of TP regeneration would suggest that the spermatocytes already formed in the deficient testes are probably defective and that a fresh batch of the stem cells has to undergo division and differentiation to produce normal spermatocytes and spermatids.

It has already been discussed in the Introduction that the testis-specific protein TP might take part in the condensation of chromatin during the formation of the spermatozoa from spermatids. It has also been consistently observed that the germinal epithelium of the vitamin A-deprived rats contains mostly spermatogonia, few spermatocytes and no spermatids. On the other hand, recent electron microscopic investigations of Sobhon et al. (18) and Unni, Dass and Ganguly (unpublished observations) have revealed that the chromatin in the spermatocytes of the testes of vitamin A-deficient rats is disorganized. It is possible that such ultrastructural defects of the chromatin might be due to the absence of TP in the deficient testes. It would therefore be of considerable importance to investigate the role of TP in the replacement of histones by protamine(s) in the testes and the role of vitamin A in this process.

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