Autoradiographic Evidence of Human Male Germ-cell Differentiation *in vitro*¹

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In vivo spermatogenesis of man has been thoroughly investigated recently by Heller and Clermont (1964). On the other hand, *in vitro* cultures of mammalian germ-cells have remained rather insufficient up to now, and the techniques currently available can hardly be used by those who are interested in genetic and endocrinologic problems of the testis. Under such a situation, efforts for successful culture of mammalian germ-cells have been made by some investigators (Katsh and Stackelburg 1961, Kodani 1962, Yao and Pace 1964), but clear evidence of cell growth and differentiation has not been obtained in most cases, since germ-cells present even after a prolonged period of culture may be only static survivors from the initial inoculum. With the application of autoradiographic technique, Lima-de-Faria *et al.* (1968) reported definite evidence for cell differentiation till the late pachytene stage. Because of his major interest being in the study of DNA replication, he did not try to maintain his cultures for more than 16 days.

In a previous paper we have described a method which afforded healthy human male germ-cells in culture for a period of 35 days (Matte and Sasaki 1968). In this paper is presented autoradiographic evidence of the germ cell differentiation *in vitro* till and including early spermatid stages.

Material and methods

A 62-year-old man who had an orchidectomy due to prostate cancer provided testicular tissue as material for this study. Half of the testis was minced with scissors and suspended in a total volume of 80 ml of culture medium (80% NCTC-109 and 20% calf serum). Labeled thymidine was then added at a final concentration of 50 μ C/ml and incubated for 15 hours at 30°C. Then the cell suspension was centrifuged at 1000 r.p.m. for 10 minutes and the supernatant was discarded. Fresh culture medium with excess cold thymidine was added, the cell suspension was centrifuged again and the precipitate was divided into 11 tubes each containing 7-8 ml of fresh culture

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medium. After incubation the gross of the testicular tissue was allowed to settle down for 1 minute to the bottom of the flask and the supernatant cell suspension was used for observation. The slides were prepared at different intervals starting from immediately after labeling (which represents 15 hours in Table 1) to 35 days. The slides were prepared according to the routine air-drying method after hypotonic treatment of 20 minutes and stained with carbol fuchsin. The autoradiogram was made by means of the dipping

Table 1. Labeling of germ cells in man after exposure to H^3 -thymidine for 15 hours

Stage	Time of culture										
	15 hrs.	3 days	5 days	14 days	20 days	22 days	25 days	27 days	28 days	32 days	35 days
Leptotene	###	###	###	+	+	+	+	+	+	+	+
Zygotene	###	###	###	+	+	+	+	+	+	+	+
Early pachytene	+	+	+	###	+	+	+	+	+	+	+
Mid pachytene	—	—	—	###	+	+	+	+	+	+	+
Late pachytene	—	—	—	—	###	###	+	+	+	+	+
Spermatocyte II	—	—	—	—	—	+	###	+	+	+	+
Early spermatid	—	—	—	—	—	—	—	+	+	###	###

method in Sakura photoemulsion (NR-M2), exposed for 25 days, and processed for analysis. For each sample 2-4 slides were prepared and a minimum of two hundred labeled cells were observed. The cells with less than 10 grains were considered as slightly labeled, cells with 10-15 grains as intermediately labeled and with more than 15 grains as heavily labeled. In Table 1 the above features are represented by + (slight), ++ (intermediate) and ### (heavy), and only the maximum labeling for each stage is expressed.

Results

Table 1 gave the summary of results obtained so far. Following the path of the heavily labeled cells through increasing duration of cultures we observed that leptotene and zygotene cells appeared heavily labeled after 15 hours, early and mid pachytene after 14 days, late pachytene after 20 days, secondary spermatocytes after 25 days and early spermatids after 32 days of culture (Figs. 2, 3, 6-9). In all cultures the proportion of such labeled cells was between 3 to 5 percent which implies the number of germ cells which have undergone their DNA synthesis in 15 hours where they were in contact with the isotope. Worth mentioning was the almost complete absence of non-germinal heavy labeled elements in the early culture and consecutively in all the cultures. Such non-germinal elements were found to be non or only slightly labeled and to be located either on the glass surface or in the precipitate, whereas the germ-cells occurred mostly in the supernatant. This is accepted as a confirmative feature for the results of Steinberger and Steinberger (1966).

Spermatogonia have not been included in Table 1, because they were difficult to identify in such meiotic preparations. However, in all preparations we have observed a population of large, slightly stained and sometimes heavily labeled cells; they are assumed to be spermatogonia on their characteristic configuration.

Primary spermatocytes were very easily recognized on account of their characteristic chromatid configurations at different stages. No secondary spermatocytes at metaphase II was observed in our preparations. However, the interphase nucleus of secondary spermatocytes and early spermatids could be readily recognized because of their smaller size and of the appearance of

Figs. 1-6. Autoradiographs of human male germ-cells in culture. All figures were reproduced with the same magnification. 1, two pachytene nuclei slightly labeled at 15 hours. 2, heavily labeled leptotene nucleus at 3 days. 3, pachytene with more than 15 grains at 14 days. 4, pycnotic labeled pachytene cell at 20 days. 5, lysis of a slightly labeled pachytene at 22 days. 6a, two secondary spermatocytes at 25 days, one being heavily labeled. On the left leptotene slightly labeled. Note difference of nuclear size. 6b, same after removing the grains.

heavy labeling at a period which corresponds almost exactly to what was expected from *in vivo* studies (Figs. 6-9).

In many spermatids a clear hemispherical zone was sometimes apparent along the periphery of the nuclear surface which corresponds eventually to the acrosomal structure (Fig. 9). All cultures showed apparently normal unlabeled late spermatids (Figs. 7 and 10). At 15 hours, in addition to the presence of heavily labeled leptotene and zygotene, many early pachytene cells were seen to be slightly labeled (Fig. 1).

Degenerative cells somewhat increased in number as the length of culture increased. However, this was not a major problem since most of the degenerated elements were in the precipitate and that even in the last culture

many hundreds of healthy germ-cells could be observed. In general, pachytene cells appeared to be the most fragile and after 14 days of cultures and afterwards many of them were seen degenerated. The degenerative picture seems to be represented by two forms: in some cases we noticed a pycnotic degeneration of the nuclear material, while in others a lysis of the cells occurred in association with a low staining affinity (Figs. 4 and 5).

Figs. 7-10. Autoradiographs of human male germ-cells in culture. All figures were reproduced with the same magnification, except for Fig. 10 which was reduced to 2/3 of the others. 7a, heavily labeled secondary spermatocyte beside two unlabeled early spermatids and one late spermatid at 25 days. 7b, same after removing the grains. 8a, heavily labeled young spermatid at 28 days. 8b, same after removing the grains. 9a, heavily labeled young spermatid at 35 days. Note the eventual acrosomal structure. 9b, same after removing the grains. 10, many late spermatids at 35 days.

Discussion

We assume on the basis of the above findings that the present results give certain evidence of germ-cell differentiation till and including early spermatid stages under specific conditions of culture. The presence of labeled pachytene cells after 15 hours of culture are in agreement with the data of Lima-de-Faria *et al.* (1968) suggesting a slight DNA synthesis at the leptozygotene stage which could represent traces of the crossing-over phenomena. The occurrence of many heavily labeled young spermatids at 32 and 35 days corresponds to the results given by Heller and Clermont (1964) and suggest that the sequences of *in vitro* spermatogenesis seem to be quite similar to

what has been observed *in vivo*. What happens after the early spermatid stages is still a matter of speculation. Steinberger and Steinberger (1966) using a different condition of culture noted a rapid degeneration of early spermatids in contrast to late spermatids which could survive for many weeks. While many labeled pachytene cells underwent degeneration without going through more advanced stages, we have not observed such a degeneration at the early spermatid stage. We could not conclude, however, whether many late spermatids observed after 35 days of culture are just surviving cells, or those came from *in vitro* differentiation and maturation. Further study by increasing the length of culture is requested to solve this question. The impossibility to observe labeled metaphases I and II is in our opinion related to the facts that these stages are of very short length, and that even *in vivo* the relative number of cells at these stages is all the time very low. We have empirically tried to use colchicine on the 20th days' preparation in a hope to observe these stages, but with no success.

Slight labeling of the early stages seen in the late cultures could represent cells which have undergone more than one DNA synthesis since their contact with the isotope, such division evidently occurring at the spermatogonial level.

As stated before the most critical stage is in our opinion the pachytene which is a long and complex one. While germ-cells did seem to progress normally till early or mid pachytene, many degenerated afterwards. In an earlier paper (Matte and Sasaki 1968) we have described this incomplete block and stressed that in a study of azoospermic males (Kjessler and Matte 1969) we have observed a similar phenomenon occurring *in vivo*. In the present case, however, it is difficult to conclude whether this relative block was due to culture conditions, or was a result of an inherent abnormality of the germ-cells cultivated. Such previous *in vivo* deficiency of the germ-cell elements would not be surprising when we consider that the testis used for this experiment derived from a 62-year-old male suffering from a prostatic cancer.

In the culture conditions described we assume that the important modifications were the use of a large amount of testicular tissue and the relatively infrequent change of culture medium. Under these circumstances any hormonal production by other cellular elements (for instance Leydig cells) could be optimal and the hormonal dependence of the germinal epithelium is a well known fact *in vivo*. In some previous pilot experiments, we have tried to stimulate the germ-cells with exogenous hormone (testosterone) but with no success. This may be due to many factors, such as the solubility, dose and accessibility to the cells among others being theoretically possible.

Despite the partial success of germ-cell culture the present method has the obvious disadvantage of needing a large amount of testicular tissue which cannot be obtained by standard testis biopsy. Further improvement of the

technique is undoubtedly requested.

Summary

A method of culture of human male germ-cells is described. Autoradiographic evidence of germ-cell differentiation *in vitro* till the early spermatid stage is presented.

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