ACROSOMAL COMPONENT OF RAT ROUND SPERMATIDS RECOGNIZED BY A NOVEL MONOCLONAL ANTIBODY

A.I. Russinova, N.N. Atanassova, M.L. Paskaleva, L.S. Kancheva

Institute of Experimental Morphology and Anthropology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

Objective. To characterize immunocytochemically the antigen recognized which appears at specific stages of germ cell development and acrosomal biogenesis by the novel monoclonal antibody (Mab 3C2).

Methods. The novel monoclonal antibody (Mab 3C2) raised against testicular Sertoli and germ cells.

Results. The immunoreactivity of this Mab in testicular sections from immature 20-day-old rats was confined to the pachytene spermatocytes. In adult testis the Mab 3C2, besides meiotic cells, recognized also acrosomal component of round spermatids. The immune reaction was observed in Golgi and cap phases of acrosomal development until the stage VIII of the cycle of the seminiferous epithelium. Immunostaining was absent in acrosome of elongating and mature spermatids and indicated that some modifications in acrosomal protein may exist in subsequent stages of acrosomal development.

Conclusions. Novel Mab 3C2 shares a common antigen in pachytene spermatocytes and round spermatids. Therefore, it may be a marker of meiotic and postmeiotic germ cells.

Key words: Immunocytochemistry - Novel monoclonal antibody - Spermatogenesis - Acrosome - Spermatocytes - Round Spermatids

The mammalian spermatogenesis provides an unique system for the study of cell proliferation and differentiation and involves a sequence of events such as mitosis (spermatogonia), meiosis (spermatocytes) and maturation of spermatids into spermatozoa (spermiogenesis). The process of spermiogenesis is characterized by progressive spermatid elongation and nuclear condensation, and development of the acrosome. The acrosome is an important organelle of spermatids and is essential for a normal fertilization. Cytochemical and autoradiographic studies showed that, during the Golgi and cap phases of acrosomal development, the Golgi apparatus contributes to the acrosome by some constituents (Clermont and Tang 1985). The investigation by Kierszenbaum et al. (1988) demonstrated that rat Sertoli cell secretory proteins and components of the acrosome share antigenic homology. The previous studies have been directed mainly on the characterization of the antigenic structures in mature sperm and their membranes or acrosomal components have been used for immunization (Jassim and Festenstein 1987; O'Brien et al. 1988; Gallo et al. 1991; Bermudez et al. 1994). Although the main role of the sperm acrosome in fertilization is well understood, little is known about molecular aspects of acrosomal development during spermiogenesis. A major problem in understanding of the processes that control male reproduction is the lack of convenient markers as an analytical tool for studying the mechanisms by which the complex processes of spermatogenesis are triggered as well as the molecules participating in the cellular communication events. Stage- and/or cell-specific molecules are of particular importance in the processes of differentiation and interaction mechanisms. Characterization of antigens using monoclonal antibodies (Mabs) is one of the approaches to isolate and identify the specific molecules of spermatogenesis

and interaction mechanisms. In addition, such Mabs are useful tools for defining the developmental steps of spermatogenic cells (YOSHIKI et al. 1993).

Our interest was focused to the differential expression of antigens in relation to specific stages of spermatogenesis during the testis development and to specific stages of the cycle of the seminiferous epithelium. In order to understand the intratesticular mechanisms of spermatogenesis we accumulated a library of monoclonal antibodies against rat testicular antigens using Sertoli and germ cells as immunogen. The aim of the present paper was to characterize immunocytochemically the antigen recognized by Mab 3C2 which appears at specific stages of germ cell development and acrosomal biogenesis.

Materials and Methods

Animals: Male 20-day-old and adult Wistar rats and female 2-month-old BALB/c mice were purchased from the animal breeding farm of the Bulgarian Academy of Sciences, Sofia.

Cell preparation: Cell suspension was prepared from the seminiferous epithelium of 20-day-old Wistar rats by enzymatic digestion as previously described (KANCHEVA et al. 1990). Briefly, decapsulated testis fragments were consecutively digested with collagenase (0.5 mg/ml) and trypsin (0.5 mg/ml). The dispersed cells were washed with 0.5 % bovine serum albumin (BSA). Cell preparations from 20-day-old rats contained predominantly Sertoli cells (75 %) and germ cells (20 %) - spermatogonia and meiotic cells (leptotene, zygotene and pachytene spermatocytes).

Production of monoclonal antibodies: Female BALB/c 2-month-old mice were immunized intraperitoneally with 5 x 106 isolated testicular cells (immature Sertoli cells and germ cells) ten times at intervals of 2 weeks. Three days after the booster injection the mouse spleen was processed and the fusion was performed with SP2/0 mouse myeloma cell line. Fusion cultures in RPMI 1640/hypoxanthine/ aminopterin/thymidine medium supplemented with 10 % fetal calf serum (FCS) were seeded in 96-well microtiter plates. On day 13-16 after seeding hybridoma supernatants were screened for antibody production using Bouin's fixed and paraffin-embedded testicular sections by means of avidin-biotin complex (ABC) peroxidase procedure. Those cultures positive by ABC were transferred to large 1-ml culture wells (24 well-plates), allowed to grow in additional 6 days and then reassayed. Selected hybridoma cultures then cloned directly from 1-ml culture wells by limited dilution into 96-well dishes. Wells containing single hybridoma colonies were reassayed, and positives were subcloned. By immunocytochemical screening of the hybridoma supernatants a line was found to react intensely only with germ cells. This selected Mab 3C2 was of IgG type.

Immunocytochemistry: Paraffin sections from testes of 20-day-old and sexually-mature Wistar rats were processed for immunocytochemistry using ABC technique of HSU et al.(1981) as described previously (RUSSINOVA et al. 1995). In this procedure methanol hydrogen peroxidase solution was used to block endogenous peroxidase activity and normal rabbit serum was used to block nonspecific binding of the secondary antibody (biotinilated rabbit anti-mouse immunoglobulines). Sections were incubated with hybridoma supernatants (primary antibody) for 18h at 4 ^{o}C then rinsed with phosphate-buffered saline (PBS) and incubated for 60 min with biotinilated anti-mouse immunoglobulins (Vector, Burlingame, CA) diluted 1:250 in PBS. After rinsing in PBS avidin-biotin-peroxidase conjugate (Vector, Burlingame, CA) diluted 1:250 in PBS was applied for 60 min. Visualization of binding sites was accomplished with 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-HCl-buffered saline (pH 7.6) with 0.01 % hydrogen peroxide, dehydrated and coverslipped. Parallel sections were stained with haematoxylin. Morphological identification of germ cells was based on the criteria of RUSSELL et al. (1990).

Controls for light-microscope observation were examined by the substitution of primary antibody with PBS.

Results

By the light microscope, the immunoreactivity of Mab 3C2 in the sections of immature testis from 20-day-old rats was confined to pachytene primary spermatocytes in seminiferous tubules. A positive reaction was detected mainly in the spermatocyte nuclei and occasionally in perinuclear region of pachytene cells (Fig. 1). According to CLERMONT and PERREY (1957), in this stage of rat testis development three types of seminiferous tubules can be observed depending on the ar-

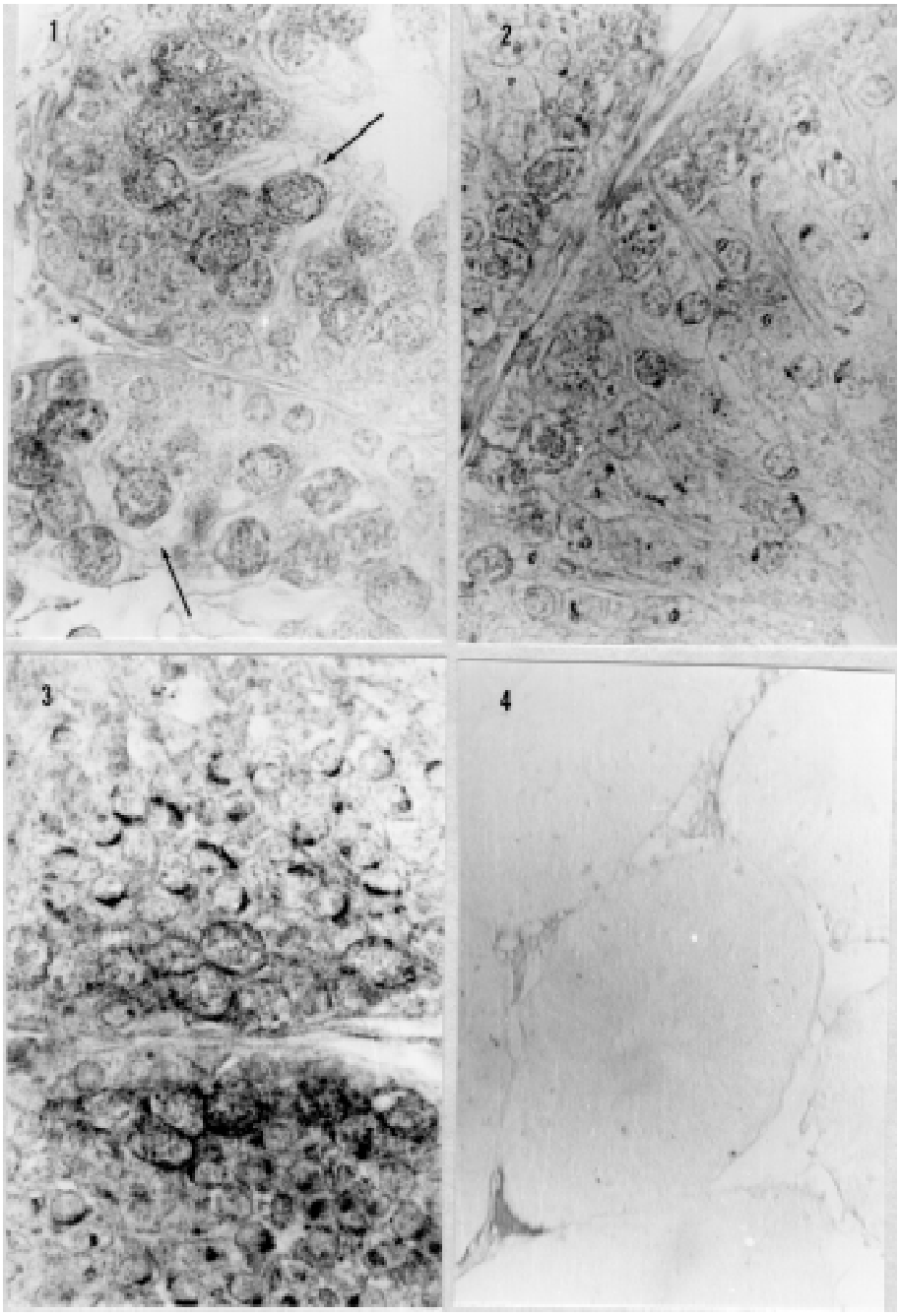

Fig.1
Immunoperoxidase staining (ABC method) of 20-day-old rat testis using Mab 3C2 as immunocytochemical probe. Staining was confined to the pachytene spermatocytes (arrows) x400.

Fig.2
Adult rat testis stained with Mab 3C2 showing immunoreactivity in the pachytene spermatocytes and acrosomes of round spermatids in two seminiferous tubules during V stage of the cycle x300.

Fig.3
Immunoreactivity with the same Mab in two seminiferous tubules during VI stage (bottom) and VII stage (top). The acrosomal reaction displayed more flattened appearance in the course of acrosomal development x400.

Fig.4
Immunoperoxidase staining of control section from the same testis after omitting of the primary antibody. The immune reaction was abolished x160.

rangement of Sertoli and spermatogenic cells. The reaction is characteristic for the pachytene spermatocytes in the three types of seminiferous tubules.

In adult rat testis the immunoreaction was localized in pachytene spermatocytes and in the acrosomal region of round spermatids. According to morphological changes in the acrosome of rat testis LEBLOND and CLERMONT (1952) distinguished 19 steps of acrosomal development. The immunostaining of Mab 3C2 was observed in Golgi phase (steps 1-3) and cap phase (steps 4-7) in the areas representing the acrosomal region of round spermatids. In Golgi phase the immune reaction was localized in acrosomal granules (Fig. 2). In the next stage of spermatid development the immunostaining of this antigen showed a more flattened appearance. At the beginning of the acrosomal cap phase the spermatids maintain their spherical nuclei. Immunocytochemically positive reaction was detected at the extensions from either side of acrosomal granule around the nuclei (Fig. 3). The immunoreactivity was most pronounced in round spermatid population until the stage VIII of the seminiferous epithelium cycle. Immunostaining was absent in the acrosomal region of elongating and mature spermatids. No immunoreaction was observed in the control sections of rat testis (Fig. 4).

Discussion

The results of this study revealed that Mab 3C2 labeled pachytene spermatocytes and acrosomal region of round spermatids from the Golgi phase until late cap phase share the same antigen. The labeling in perinuclear region of pachytene spermatocytes may correspond to the localization of Golgi complex in this cell type (RUSSELL et al. 1990) and probably confirms the contribution of Golgi complex in addition to that of acrosomal components. Mab 3C2 recognized acrosomal antigen that are detected in round spermatids until to their elongation. No immunoreactivity was observed in condensing and condensed spermatids.

The monoclonal antibody 1D4 (O'BRIEN et al. 1988) revealed some similarities to the antibody characterized in this study. The Mab 1D4 raised against mouse spermatogenic cell membranes recognized acrosomal constituents of round and condensing spermatids in the mouse, rabbit and guinea pig. The recognized antigens are no longer detectable in late spermiogenesis. Little or no immunoreactivity was seen in prepubertal testis and immature Sertoli cells. The antigens recognized by Mab 1D4 are not detected in rat acrosomes and their properties distinguish Mab 1D4 from Mab 3C2 antibody obtained in the present investigation.

The monoclonal antibody (MHS-10) raised against sperm protein SP-10 localized this antigen in spermatids during the six stages of the cycle of the seminiferous epithelium in man (KURTH et al. 1991). Until the condensation of spermatid nuclei the labeling was associated with the dense acrosomal granules and later SP-10 appeared to change its distribution in association with acrosomal membranes by the end of spermiogenesis. Another monoclonal antibody HS-19 (FLORMAN et al. 1984) prepared against human spermatozoa binds only to the acrosome of mouse spermatozoa. KIERSZENBAUM et al. (1988) identified antigenic sites in rat testis using polyclonal antibodies raised against Sertoli cell secretory proteins. The antibodies recognized immunoreactive sites in acrosome of developing spermatids and in apical Sertoli cell cytoplasm in contact with spermatids. According to the latter authors the close apposition of Sertoli cell surfaces to the plasma membrane of spermatogenic cells raises the possibility that Sertoli cell secretory products may be transported to developing spermatogenic cells.

The monoclonal antibody 3C2 is raised against cell suspension containing Sertoli cells (75 %) and germ cells - spermatogonia through pachytene spermatocytes (20 %) without spermatids. The immunoreactivity in pachytene spermatocytes of immature and adult rat testis as well as in acrosomes of round spermatids suggested that some synthesis of the antigen recognized by Mab 3C2 occurs in meiotic germ cells. The lack of immunoreaction in condensing and mature spermatids during the subsequent stages of spermatid development indicates that some modifications of acrosomal protein exist.

Recently it was shown that some acrosomal proteins are synthesized in pachytene spermatocytes like guinea pig acrogranin (ANAKWE and GERTON 1990), proenkephalins in rat, hamster, sheep and man (KEW et al. 1990) and human proacrosin (ESCALIER et al. 1991; BERMUDEZ et al. 1994). However, the opposite suggestion by MARTINEZ-MENARGUES et al. (1996) that pachytene spermatocytes are mainly involved in the synthesis of lysosomal constituents, whereas sperma-

tids synthesize acrosomal proteins is not consistent with above mentioned data. Many questions concerning the acrosome formation like a characteristic organelle of mammalian spermatids remain to be resolved. Immunocytochemical localization of a monoclonal antibody received against rat testicular cells revealed that this novel antibody recognizes a common antigen in meiotic and postmeiotic germ cells and could be an useful marker for germ cell differentiation.

Acknowledgements

This study was supported by the grant B-616 from the National Foundation "Scientific Researches".

References

Anakwe OO, Gerron GL: Acrosome biogenesis begins during meiosis: evidence from the synthesis and distribution of an acrosomal glycoprotein, acrogranin, during guinea pig spermatogenesis. Biol Reprod **42**, 317-328, 1990

Bermudez D, Escalier D, Gallo JM, Viellefond A, Rius F, Perez de Vargas I, Schrevel J: Proacrosin as a marker of meiotic and post-meiotic germ cell differentiation: quantitative assessment of human spermatogenesis with a monoclonal antibody. J Reprod Fertil **100**, 567-675, 1994

Clermont I, Perey B: Quantitative study of cell population of the seminiferous tubules in immature rats. Am J Anat **100**, 241-267, 1977

Clermont I, Tang XM: Glycoprotein synthesis in the Golgi apparatus of spermatids during spermatogenesis of the rat. Anat Rec **213**, 33-43, 1985

Escalier D, Gallo JM, Albert M, Merduri G, Bermudez D, David G, Schrevel J: Human acrosome biogenesis immunodetection of proacrosin in primary spermatocytes and of its partitioning pattern during meiosis. Development **113**, 779, 1991

Florman HM, Bechtol KB, Wassarman PM: Enzymatic dissection of the mouse egg's receptor for sperm. Dev Biol **106**, 243-255, 1984

Gallo JM, Escalier D, Grellier P, Precigout E, Albert M, David G, Schrevel J: Characterization of a monoclonal antibody to proacrosin and its use in acrosomal status evaluation. J Histochem Cytochem **39**, 273-282, 1991

Hsu S, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase technique: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem **29**, 577-585, 1981

Jassim A, Festenstein H: Molecular dissection of human testicular germ cell differentiation with monoclonal antibodies. J Reprod Immunol **12**, 173-189, 1987

Kancheva LS, Martinova YS, Georgiev VD: Prepubertal rat Sertoli cells secrete a mitogenic factor(s) that stimulates germ and somatic cell proliferation. Mol Cell Endocrinol **69**, 121-127, 1990

Kew D, Muffly KE, Kilpatrick DL: Proenkephalin products are stored in the sperm acrosome and may function in sperm fertilization. Proc Natl Acad Sci USA **87**, 9143-9147 1990

Kierszenbaum AL, Ueda H, Ping L, Abdullah M, Tres L: Antibodies to rat Sertoli cell secretory proteins recognize antigenic sites in acrosome and tail of developing spermatids and sperm. J Cell Sci **91**, 145-153, 1988

Kurt B, Klotz K, Flickinger C, Herr J: Localization of sperm antigen SP-10 during the six stages of the cycle of the seminiferous epithelium in man. Biol Reprod **44**, 814-821, 1991

Leblond CP, Clermont I: Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann NY Acad Sci **55**, 548-573, 1952

Martinez-Menargues JA, Geuze HJ, Ballesta J: Evidence for a nonlysosomal origin of the acrosome. J Histochem Cytochem **44**, 313-320, 1996

O'Brien DA, Gerton GL, Eddy EM: Acrosomal constituents identified with a monoclonal antibody are modified during late spermatogenesis in the mouse. Biol Reprod **38**, 955-967, 1988

Russell LD, Ettlin RA, Sinha HA, Clegg ED: Histological and histopathological evaluation in the testis. Clearwater Fl, Cache River, 1-40, 1990

Russinova A, Atanassova N, Kancheva L: A monoclonal antibody raised against rat ovarian antigen recognizes Leydig cell surface: An immunocytochemical study. Exp Cell Res **218**, 485-489, 1995

Corresponding author: Dr. Angelina I. Russinova
Institute of Experimental Morphology and Anthropology
Bulgarian Academy of Sciences
Acad. G. Bonchev Str., bl.25
1113 Sofia, Bulgaria
Fax number: 00359 2 71 90 07

Accepted: June 15, 1998