ISOLATION AND IMMUNOCYTOCHEMICAL CHARACTERIZATION OF A LIBRARY OF MONOCLONAL ANTIBODIES DIRECTED AGAINST RAT TESTICULAR ANTIGENS

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Objective. To characterize immunocytochemically the antigens recognized by monoclonal antibodies (Mabs) in the library we have accumulated and to reveal their spatiotemporal distribution in testicular tissue in the course of testis development.

Methods. Female BALB/c 2-month-old mice were immunized intraperitoneally with isolated immature Sertoli and germ cells obtained from 20 day old male Wistar rats. The obtained Mabs were characterized by its cell type-specific binding reaction using light immunocytochemistry (avidin-biotin-peroxidase complex technique, immunogold-silver staining, indirect immunofluorescence).

Results. On the basis of immunocystochemical results the selected Mabs were divided into four classes: 1. Mabs of class 1 recognized the differentiation specific antigens appearing during germ cell development, two of them revealing a stage-specific expression of nuclear antigens from preleptotene to early pachytene stage. Other Mabs of this class 1 detected the antigens in pachytene spermatocytes and acrosomes of round spermatids until their elongation; 2. the labeling of class 2 Mab was restricted only to Sertoli cell cytoplasm; 3. the binding of class 3 Mabs was observed in the cytoplasm of germ and Sertoli cells; 4. Mabs of class 4 reacted with antigens distributed in the cytoplasm of primary spermatocytes, Sertoli and Leydig cells.

Conclusions. The Mabs from the library we have accumulated recognized the antigens in different cell types at various stages of testicular development and could be an useful tool for the characterization of cell- and development- specific molecules which may participate in germ cell differentiation and/or cell to cell interactions during testis development.

Key words: Immunocytochemistry – Testicular antigens – Monoclonal antibodies

The mammalian spermatogenesis is a complex process that involves a sequence of events such as mitosis (spermatogonia), meiosis (spermatocytes) and maturation of spermatids into spermatozoa (spermiogenesis). Most investigators agree that the Sertoli cell is a key cell supporting the spermatogenesis and plays a major role in establishment and maintenance of spermatogenic process (Skinner 1991). The functions of Sertoli cell are dependent on the stage of the cycle of the seminiferous epithelium and on specific stage in the course of testis development, but the germ cells in turn modulate Sertoli cell responses (Sharpe 1993). There is increasing evidence that during spermatogenic process the cell- and development-specific proteins are synthesized which may participate in germ cell differentiation and/or cell-cell testicular interactions (Jegou 1993). A major problem in understanding the processes that control male reproduction is the lack of suitable markers to be used as analytical tool for studying both the mechanisms by which the complex processes of spermatogenesis are triggered and the molecules participating in the cellular communication events.

Characterization of antigens using monoclonal antibodies (Mabs) is one of the approaches to isolate and identify the specific molecules of spermatogenesis and interaction mechanisms. Several cell-surface differentiation antigens have been detected on spermatogenic cells using polyclonal (Millette and Bellve 1977; O'Rand and Romrell 1981) or monoclonal antibodies (Bechtol 1984; O'Brien and Millette 1986; Watanabe at al. 1992; Koshimizu et al. 1993). Numerous reports were published on the characterization of the antigenic structures in mature spermatozoa 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 a number of antigens were identified by means of immunological probes, their molecular nature and function still remain unclear.

Cell- and development- specific molecules are of particular importance in the process of spermatogenesis and the availability of suitable antibodies could contribute to the characterization of proteins with essential properties. In this regard the aim of the present study was to characterize immunocytochemically the antigens recognized by Mabs in the library we have accumulated and to reveal their spatiotemporal distribution in testicular tissue in the course of testis development.

Materials and Methods

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 consecutively with 0.5 % bovine serum albumin (BSA) and PBS. 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 10^6 isolated testicular cells (immature Sertoli and germ cells) five 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-peroxidase procedure (ABC). Those cultures positive by ABC were transferred to large 24 well-plates, allowed to grow in additional 6 days and then reassayed. Selected hybridoma cultures were cloned directly from one ml culture wells by limited dilution into 96-well dishes. Wells containing single hybridoma colonies were reassayed, and positives were subcloned. The selected hybridoma lines were cryopreserved in 95 % fetal calf serum (FCS) and 5 % DMSO in liquid nitrogen.

A secondary screening of the Mabs (positive ABC reaction with Sertoli cells and other cell types) was carried out by ELISA using Sertoli cell-conditioned medium (SCCM) as coating antigen. Additional screening of the obtained Mabs was performed using immunofluorescence and immunogold silver staining (IGS). On the basis of all immunocytochemical results ten Mabs were selected for our library. Immunoglobulin subclasses were determined using subclass-specific antibodies (Amersham, Buckinghamshire, UK). In addition, the obtained Mabs did not react with other tissues, e.g. kidney, lung, heart and liver.

Sertoli cell culture and medium collection. Sertoli cells were isolated according procedure by Kancheva et al. (1990). They were plated in tissue culture dishes at density 1-1.5x10^6 cells/ml in DMEM/Ham's F12 (1:1; vol/vol) supplemented with 5 % FCS (Flow Labs., UK). At 24 hour after seeding the cells were washed twice and given DMEM/F12 without serum. The SCCM was collected on 4th and 7th day after seeding. SCCM was concentrated 10-fold using a SpeedVac concentrator (Savant, USA). The amount of protein present in concentrated SCCM was generally 150 μg/ml as estimated according to Bradford (1976).

Avidin-biotin-peroxidase complex (ABC) technique. Paraffin sections from testes of 20- and 25-day-old and adult 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 peroxide 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 immunoglobulins). The sections were incubated with hybridoma supernatants (primary antibody) for 18 h at 4 °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) 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).

Immunogold-silver staining (IGS). Paraffin sections were incubated with hybridoma supernatant for 18 h at 4 °C. In the second step of immunostaining goat anti-mouse IgG – 5 nm gold conjugate (Amersham, UK) was applied for 3 h and in the third step IntenSe M silver enhancement kit (Amersham, UK) was used.

Indirect immunofluorescence. Hybridoma supernatant was overlaid on the testicular cryosections and cultured Sertoli cells grown on glass coverslips and then incubated for 1 h before a 30-min rinse in TBS and TBS-0.1 % Triton X-100. The sections were covered with a drop of FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co., USA) dilluted 1:50 with PBS and the reaction was allowed to continue for 30 min. After 45-min rinse in PBS, the sections were mounted in 90 % glycerol in PBS and observed in a Zeiss epifluorescence microscope equipped with interference filters for FITC. Photomicrographs were recordered using an Zeiss camera system and Kodak T-MAX 400 film.

Controls for light microscope observation. The immunohistochemical specificity was examined by substitution of the primary antibody either with PBS or normal mouse serum, or by omitting the secondary antibody. Further controls were performed with subclass-matched control Mabs (3D8 – IgM; 4E6- IgG) obtained against rat ovarian antigens, produced and characterized in our laboratory after immunization with granulosa cells and oocytes (Russinova et al. 1993, 1994). Additional controls were performed with Mabs preabsorbed with protein extract obtained from the cells utilized for immunization before being processed for immunohistochemistry. For preparation of protein extract the cell pellet was homogenized on ice in PBS containing 1 % Triton X-100 and a cocktail of protease inhibitors: 1mM PMSF and 5 μg/ml each of benzamidine-HCL, aprotinin and leupeptin. After the extraction the mixture was centrifuged at 20,000 x g for 30 min at 4 °C to yield supernatant.

Results

Each of the obtained ten Mabs was examined for its cell type- specific binding reaction using ABC peroxidase method. Additional screening of the selected Mabs was performed by IGS technique and immunofluorescence. All methods applied gave identical pattern of staining.

The selected Mabs were divided into four main classes according to the immunohistochemical results related to their specificity for the cell type staining reaction in rat testicular tissue. Thus, the reaction of class 1 Mabs was restricted only to germ cells, while that of class 2 only to Sertoli cells. The general target of binding class 3 Mabs were Sertoli and germ cells. The Mabs of class 4 recognized the antigens present in the germ, Sertoli and Leydig cells.

Class 1 – Immunocytochemical analysis with a group of two Mabs from this class (Mab 1F2 and 2F2) revealed identical results, e.g. strongly positive reaction in the nuclei of early meiotic cells from preleptotene to early pachytene spermatocytes in the testes of 20-day-old and adult rats (Figs.1A and 1B). These two Mabs identified a 44 kDa antigen using immunoblotting (Atanassova et al., 2000). The nuclear reactivity was associated with the meiotic chromosomes. The recognized antigen was not present either on spermatogonia or on more advanced spermatogenic cells after pachytene stage. In immature and adult rat testes a stage-specific nuclear expression of antigen recognized by these two Mabs can be immunocytochemically detected. No reaction was observed in Sertoli, Leydig and peritubular somatic cells. The subclass of these Mabs was determined to be IgM for 1F2 and IgG for 2F2.

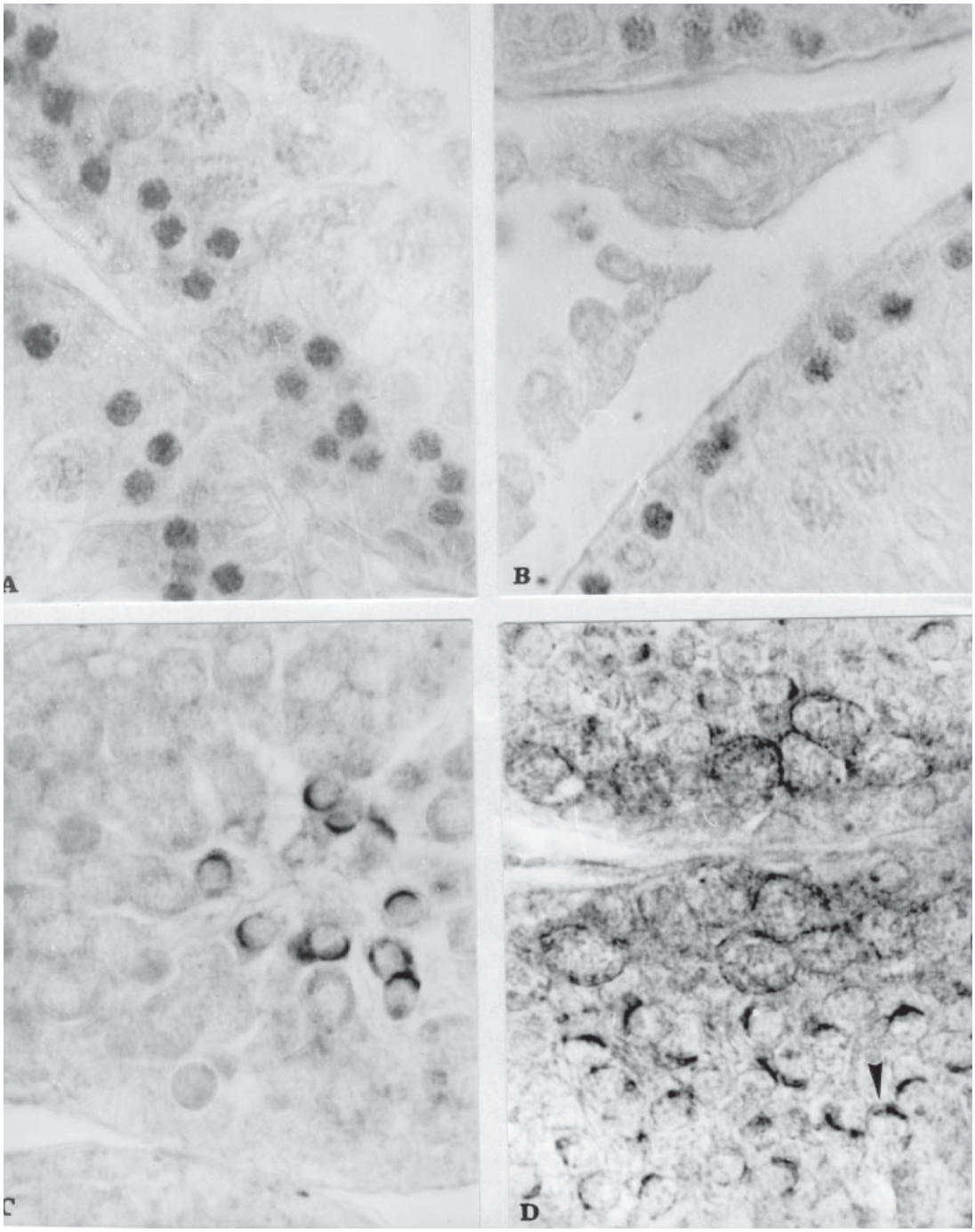

Fig.1 ABC staining of rat testis using Mab 2F2 from class 1. In cross section of seminiferous tubules from 20-day-old *(A)* and adult rat testes *(B)* a strongly positive reaction was observed in the nuclei of early meiotic spermatocytes. The antigen is present neither on spermatogonia nor on more advanced spermatogenic cells after early pachytene stage. *(C)*: Using Mab 3C2 from class 1 as immunocytochemical probe the ABC staining was confined to the pachytene spermatocytes and acrosomes of newly formed round spermatids in 25-day-old rat testis. *(D)*: In adult rat the immunostaining with the same Mab was observed in Golgi and cap phase of round spermatid acrosomes. x500

The second group of two class 1 Mabs (3A3 and 3C2) detected the antigens that appear at specific stages of germ cell development and acrosomal biogenesis (Russinova et al. 1998). These Mabs reveal a similar pattern of staining. In 25 day old rat testis the immunoreactivity was confined to pachytene spermatocytes and to acrosomes of newly formed round spermatids (Fig.1C). In adult rat the immunostaining was observed in Golgi phase (steps 1-3 of acrosomal development) and cap phase (steps 4-7) of round spermatids (Fig.1D). The reaction was pronounced in round spermatid population until stage VIII of the cycle of the seminiferous epithelium. Immunostaining was absent in the acrosomes of elongating and mature spermatids. These Mabs were of IgG type.

Class 2 – Mab 1H9. Using Mab 1H9 as immunocytochemical probe the immunoreactivity was observed in Sertoli cell cytoplasm. The intensity of immune reaction in adult testis was more significant in the seminiferous epithelium region situated above spermatogonia which coincides with the location of Sertoli cell tight junctions (Figs. 2A and 2B). The immunostaining was also pronounced around condensing spermatids oriented in bundles deeply in Sertoli cell cytoplasm facing the basal lamina. This Mab was shown to be of IgG subclass.

Class 3 – Mabs 3A11, 4A9 and 3D6 (IgG). Under light microscopical observation this group of Mabs detected antigens in two cell types – spermatocytes and Sertoli cells. The reaction was restricted to the cytoplasm of the labelled cells. In 20 day old and adult rat testis the germ cells reacted with Mab 3A11 were identified as leptotene, zygotene and early pachytene spermatocytes (Figs. 3A and 3B). The immunostaing was not detected in late pachytene spermatocytes and more advanced stages of spermatogenic process. The recognized antigens were present in cytoplasm of Sertoli cells but Leydig cells remain unlabeled. Similar localizations were observed using Mab 4A9 and Mab 3D6 as immunocytichemical probe (not shown).

A secondary screening of the class 3 Mabs was carried out by ELISA using SCCM as coating antigen. Only Mab 3D6 was found to react intensely with SCCM. Additional screening of the Mab 3D6 was performed on cultured Sertoli cells grown on glass coverslips using immunofluorescence which revealed a labelling of Sertoli cell cytoplasm (Fig.3C).

Class 4 – Mabs 1A1 and 3B6 (IgG). These antibodies reacted with antigens of three testicular cell types – germ, Sertoli and Leydig cells. In immature and adult rat testis the antigens were distributed in cytoplasm of primary spermatocytes, Sertoli and Leydig cells. Using Mab 1A1, differences in staining intensity among the labelled cell types were registrated. The reaction product was more abundant in cytoplasm of Leydig cell clusters (Fig. 3D).

Control experiments by omitting the primary antibody or using the control Mabs (IgM, IgG) and preabsorbed Mabs were in all cases negative (not shown).

Discussion

To investigate the molecular basis of the complex interactions in mammalian testis we accumulated a library of monoclonal antibodies against rat testicular antigens using a suspension of Sertoli and germ cells as immunogen. The monoclonal antibodies from class 1, 3 and 4 recognized antigens that are expressed in germ cells at various stages of their development.

A group of two Mabs (1F2 and 2F2 from class 1) recognized nuclear antigen which exhibites a stage-specific expression in meiotic spermatogenic cells from preleptotene to early pachytene spermatocytes in immature and adult rat testes. The immune reaction is clearly associated with meiotic chromosomes. Several studies have reported a limited numbers of immunological markers which recognize germ cells in early meiotic stages of spermatogenesis. Watanabe et al. (1992) and Koshimizu et al. (1993) reported development specific expression of cell surface proteins in early meiotic germ cells using monoclonal antibodies against neonatal mouse testicular cells. Applying germ cell- specific antiserum Tsuchida et al. (1995) detected 20 antigenic molecules in adult mouse testis. According to the authors at least 12 of these are differentiation-specific antigens appearing during germ cell development from type A spermatogonia to spermatozoa. In our previous study (Hadjiolova et al. 1989) with a monoclonal antibody was shown that the nuclear matrix antigen p125/6.5 is expressed not only during mitosis but also during meiosis. Wrobel et al. (1996) revealed the dis-

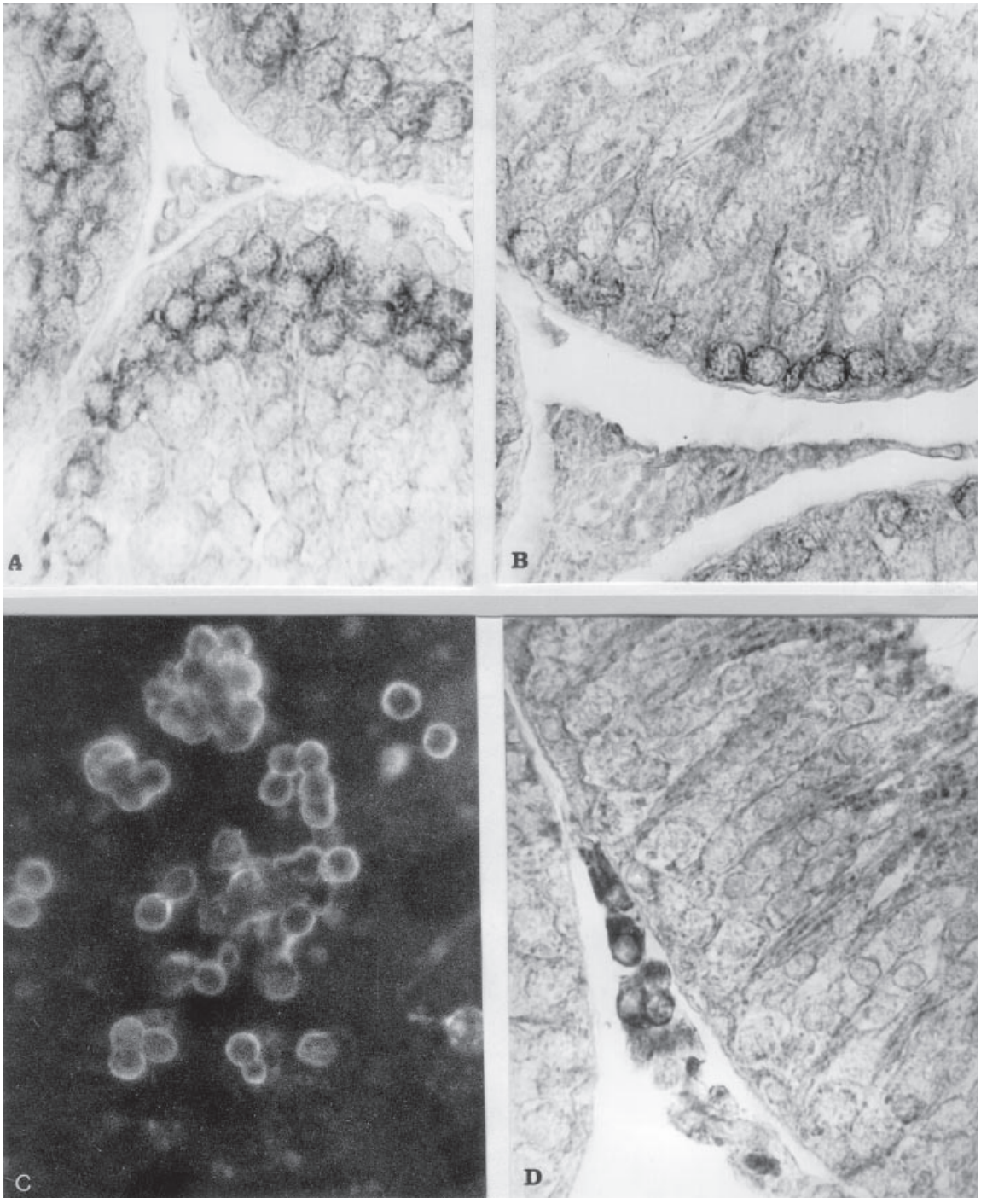

Fig.2 Using Mab 3A11 (class 3) the immunostaining (IGS) was observed in cytoplasm of early spermatocytes and Sertoli cells in immature *(A)* and adult *(B)* testes. *(C)*: Immunofluorescence with Mab 3D6 of cultured Sertoli cells grown two days on coverslips. Note that the reaction is associated with Sertoli cell cytoplasm. *(D)*: ABC staining of adult rat testis applying Mab 1A1 from class 4 revealed immunoreactivity in the cytoplasm of germ, Sertoli and Leydig cells. The reaction was more abundant in Leydig cell clusters. x500 (A,B); x400 (C); x630 (D)

tribution pattern of proliferating cell nuclear antigen (PCNA) and Ki-67 protein in adult bovine seminiferous epithelium by means of immunocytochemistry. Both antibodies reacted with spermatogonial nuclei. PCNA reactivity was present during leptotene through pachytene but Ki-67 expression was absent in leptotene and zygotene and appeared again during pachytene stage.

Immunocytochemical assessment of the stage-specific expression of antigen recognized by our Mabs 1F2 and 2F2 from class 1 suggests that this 44 kDa antigen may take part in regulating the events occuring in the male germ cells from the early stages of meiotic prophase. The absence of the antigen from mitotically dividing spermatogonia and more advanced spermatogenic cells and its association with meiotic chromosomes support the conclusion that this nuclear protein may be involved in the structural reorganization of germ cells entering meiotic division. Further characterization of these nuclear antigens during spermatogenesis will be published separately.

Another group of two Mabs from class 1 recognized antigens in pachytene spermatocytes and acrosomal region of round spermatids until to their elongation. Using monoclonal antibody 1D4 raised against mouse spermatogenic cell membranes, O'Brien et al. (1988) detected acrosomal constituents of round and condensing spermatids in the mouse, rabbit, and guinea pig but not in rat. 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. A 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). The immunoreactivity of antigens recognized by our Mabs (3A3 and 3C2 from this class) found in pachytene spermatocytes of immature and adult rat testis as well as in acrosomes of round spermatids suggested that some synthesis of the antigens 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

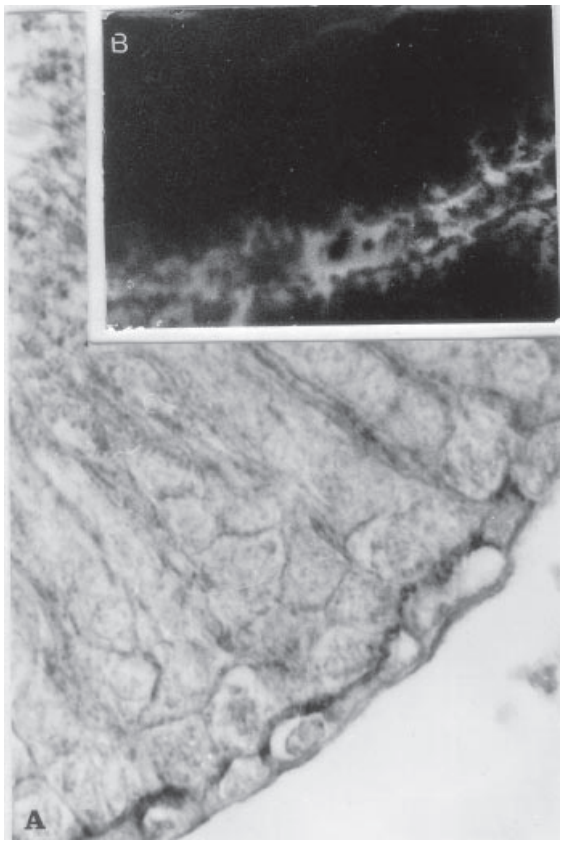

Fig.3 IGS staining *(A)* and immunofluorescence *(B)* of adult rat testis using Mab 1H9 from class 2. The intensity of immune reaction is more pronounced at the periphery of the seminiferous tubules, especially in the region situated above spermatogonia. x630

acrosomal proteins exist. Therefore, these novel antibodies probably recognize a common antigens in meiotic and postmeiotic germ cells and could be useful markers for germ cell differentiation.

Our immunocytochemical findings revealed that Mab 1H9 from class 2 recognized a Sertoli cell cytoplasmic antigen. In adult rat testis the reaction product was seen in apical Sertoli cell cytoplasm surrounding bundles of elongating spermatids. The close apposition of Sertoli cell surfaces to the plasma membrane of spermatogenic cells raises the possibility that Sertoli cell secretory proteins may be transported to developing germ cells (Kierszenbaum et al. 1988). In the present investigation using Mab 1H9, the reaction revealed a more significant labeling in seminiferous epithelium region situated above spermatogonia that coincides with the location of Sertoli cell barrier. It is

known that the basally located tight junctions between Sertoli cells in the postpubertal testis are the major structural component of this barrier (Dym and Fawcett 1970). Despite the importance of Sertoli cell tight junctions in spermatogenesis very little is known about its molecular formation and dissolution. Tight junction-associated protein ZO-1 (zonula occludens 1) has been characterized as a peripheral membrane protein associated with the cytoplasmic surface of tight junctions (Byers et al. 1991). Several other molecules associated with junctional complexes have been identified in the testis (Pelletier and Byers 1992). In this regard Mab 1H9 could be a helpful tool for further characterization of the recognized antigen and its role in formation and function of inter-Sertoli cell tight junction barrier.

In the present study the antigens identified by Mabs from class 3 and 4 are expressed in germ and Sertoli cells (class 3) and germ, Sertoli and Leydig cells (class 4). Using ELISA Mab 3D6 from class 3 was found to react with concentrated SCCM. Each protein excreted at the adlumenal compartment of the seminiferous epithelium is likely to interfere the spermatogenesis directly via the germ cells or by creating special contitions in the microenvironment. There is increasing evidence for the role of specific secretory proteins in the testicular cell-cell interactions which are essential for the maintenance of spermatogenesis (Skinner 1991; Sharpe 1993).

A precise interpretation of our results using Mabs from class 3 and 4 as immunohistochemical probes is difficult at the present time. However, our observations raise two possibilities: 1) the uptake and processing of Sertoli cell secreted proteins and 2) the antigenic homology between Sertoli cell secreted proteins and protein components of germ and Leydig cells. An experimental analysis of these two possibilities is being carried out in our laboratory.

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BOOK REVIEW

THYROID CANCER
A COMPREHENSIVE GUIDE TO CLINICAL MANAGEMENT

EDITED BY LEONARD WARTOFSKY (WASHINGTON, DC)

HUMANA PRESS (TOTOWA, NEW JERSEY) 1999
E-MAIL: HUMANA@HUMANAPR.COM, 515 PAGES, HARD COVER US $ 175.00,

"It sometimes seems that thyroid carcinoma is a neglected orphan among human cancers, which is at the root of some important issues. Thyroid carcinomas comprise a diverse group of malignancies ranging from indolent microscopic papillary carcinomas that pose no treat to survival to anaplastic carcinomas that are the most vicious carcinomas afflicting humans. Yet, because of its low incidence, there have been no prospective randomized clinical trials of the treatment of thyroid carcinoma." This is a fragment of the "Foreword" written by outstanding thyroid surgeon Ernest L. Mazaferri.

This comprehensive book brings an instructive review of present knowledge on the pathology, etiology, epidemiology, diagnostic methods and the methods of surgical, radioiodine and chemotherapeutic treatment of various forms of thyroid cancer. Written by outstanding experts and professionaly edited by Leonard Wartofsky, it may be considered of substantial utility to all physicians dealing with thyroid diseases.

A total of 52 comprehensive chapters is divided into 9 sections dealing with the diagnostics and management of thyroid nodule (Part I), general considerations on the thyroid cancer (Part II), clinical aspects, pathology, treatment, follow-up, prognosis and special aspects in children of papillary carcinoma (Part III), the same aspects of follicular (Part IV) and anaplastic carcinoma (Part V) and lymphoma (part VI). Next sections are devoted to medullary carcinoma (Part VII), unusual thyroid cancers (Part VIII) and future directions (Part IX). A number of up to date references and instructive tables, figures and photos are attached to each chapter. In addition to basal theoretical knowledge on each problem discussed in individual chapters, there is a number of practical and handy instructions such as a detailed description of fine needle aspiration technique, detailed descriptions of external irradiation techniques and doses, sonography, various imaging procedures and strategies of follow-up the patients after the surgical and radiation treatment. However, some actual questions on the extent of thyroidectomy such as lobectomy versus subtotal or ner-total thyroidectomy would perhaps deserve more detailed discussion.

Of special value may be considered the chapters on molecular pathology of thyroid cancer including significant recent achievements of molecular genetic analysis of inheritance pattern in families with medullary carcinoma.

Finaly, again few words of Ernest L. Mazaferri: *"I believe the knowledge contained in Thyroid Cancer will give the practicing clinicians the necessary information to provide patients the latest and best diagnostic and therapeutic techniques."*

Pavel Langer