

Studies on tissue distribution of M1 sperm antigen using sperm-specific monoclonal antibody

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Vol. 17, No. 2 (2006-05 - 2006-08)

Biomedical Research 2006; 17 (2): 133-138

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Key words: Tissue distribution, M1 antigen, Monoclonal antibody

Accepted May 17 2006

Abstract

It was reported that M1 monoclonal antibody (M1 mab) inhibited hamster sperm-egg fusion in a dose-dependent manner. The antigen was localized to the equatorial segment (ES) of the hamster sperm, and that after the acrosome reaction, it was exposed on the surface of the sperm plasma membrane overlying the ES. The M1 antigen therefore fulfilled the main requirements of a sperm component that might be involved in gamete fusion processes. Hence, it was necessary to fully characterize this sperm determinant in order to elucidate its role in fertilization and to assess its potential as contraceptive antigen. In this study, M1 mab was used to analyze the developmental expression of M1 antigen during spermatogenesis and during sperm maturation in the epididymal tract. Indirect immunofluorescent staining (IIF) assay revealed that the M1 antigen was detected only in the cross sections of seminiferous tubules containing spermatids and sperm. The specificity of M1 mab was examined also by IIF assay of cell suspensions prepared from various tissues of the hamster. The result showed that the M1 mab did not cross react with cells isolated from somatic tissues including lung, heart, kidney, spleen, brain and liver. Further detection by immunoblotting also showed that the M1 antigen appeared to be sperm-specific as it was not detected in a range of somatic cells. It is therefore proposed that the M1 antigen which is synthesized only by the germ cells of the testis, also found to be expressed at late spermatid stage and is a sperm-specific protein.

Introduction

Mammalian spermatogenesis and sperm maturation are highly programmed processes during which numerous components are synthesized and degraded to fulfill the structural, functional and developmental requirements of the sperm. To analyze these complicated developmental processes, it is helpful to have antibodies which react specifically with different antigenic components of spermatozoa [1]. Monoclonal antibodies have been particularly useful in this respect [2]. Information about the initial expression of sperm antigen can be useful in ascertaining sperm function. Generally, antigens expressed at or after meiosis are important for the development and function of the mature sperm rather than for maintaining the general survival of the germ cell [3,4]. Moreover, germ cell specific components are more likely to be expressed after meiosis [5].

Generally, the expression of membrane antigenic determinants of a cell can be derived either from the synthesis of novel components followed by their translocation to the membrane (possibly by vesicles) or from the modification of existing membrane components of the cell by post-translation processes such as glycosylation. This process usually occurs during spermatogenesis but additional changes may also be initiated during epididymal maturation as sperm are transported along the epididymis [4]. During this stage of sperm development many new components have been identified with monoclonal antibodies some of which originate from the epididymal epithelium. For this reason it is important to investigate sperm antigen expression in both the testis and epididymis.

As reported earlier M1 mab recognises the antigen specifically on the ES of hamster sperm but it is unknown whether this component is present elsewhere in the body. An essential requirement for a candidate antigen for a contraceptive vaccine is gamete (sperm) specificity [7]. The presence of antigen in other tissue could lead to undesirable side effects due to antibodies mounting an auto-immune response [8]. Thus, an important aspect of anti-gen characterisation is to establish its distribution in various tissues. When the antigen is unknown but a specific antibody has been generated then immunofluorescent and immunoblotting techniques are normally performed [9].

In the present study the nature of appearance of M1 sperm antigen was investigated by IIF assay of the testicular and epididymal cells and/or in the frozen sections as well as selected somatic tissues (lung, heart, kidney, spleen, brain and liver) using M1 mab. For further confirmation, sodium lauryl sulphate polyacrylamide gel electrophoresis ((SDS PAGE) in conjunction with immunoblotting technique was also performed.

Materials and Methods

Reagents

The M1 mab was produced at the Department of Molecular Biology and Biotechnology, University of Sheffield, UK [10]. All chemicals were purchased from Sigma Chemicals Ltd (Poole) unless otherwise stated.

Preparation of tissues for IIF and immunocytochemistry

Testes from mature hamster were decapsulated to isolate spermatogenic cells following a modified procedure of Lee and Wong [11]. Seminiferous tubules free from interstitial tissue were obtained by incubation in 0.1% collagenase and 0.2% hyaluronidase in phosphate buffered saline (PBS) containing 0.1% glucose (PBS-G) at 37°C for 30 minutes with constant shaking. Following incubation, the cell suspension was gently pipetted and filtered through nylon screen mesh. After three washes with PBS by centrifugation at 200 g for 10 minutes, the resulting cells were resuspended in PBS-G and subjected to IIF assay. Cell suspension from epididymis was also prepared according to the same method as above.

Cell suspensions of other hamster tissues (non-reproductive organ) were prepared by passing the tissue homogenates through a muslin filter. No specific enzymatic treatment was performed. After three washes with PBS by centrifugation at 200 g for 10 minutes the cells were resuspended in PBS at pH 7.2, 4°C and subjected to IIF assay.

To prepare frozen testis and epididymal sections, both the organs were removed from the sacrificed hamster and frozen immediately in liquid nitrogen. Subsequently, they were placed in an embedding solution (Tissue Tek II O.C.T Compound No. 4583; Miles Scientific, Naperville, IL) at 4°C and then plunged into liquid nitrogen for freezing. Sections of 10 µm thickness were cut with a cryo-tome. Tissue sections were placed on gelatin-coated glass slides, air-dried and fixed in 100% methanol prior to use for IIF, or they were stored at -20°C for later use.

M1 localization in hamster tissues by indirect immunofluorescence staining (IIF)

Cell suspensions from each tissue sample was subjected to two different IIF assays. Cells were either methanol-fixed on slides or were maintained as a cell suspension in microcentrifuge tube (unfixed cells). Briefly for the methanol-fixed procedure, cell suspensions were smeared and left to air-dry overnight before fixation in 100% methanol. Dried slides were subjected to IIF staining as describe by Ellis et al [6].

IIF staining of various cell suspensions was the same as described for live hamster sperm. Briefly, 20 µl of cell suspension in a microcentrifugation tube was incubated with 200 µl of M1 hybridoma supernatant (undiluted) or myeloma culture supernatant (negative control) overnight at 4°C. Following this incubation, each cell suspension was centrifuged at 200 g for 10 minutes and washed three times with PBS-BSA (0.1% BSA). Secondary antibody (FITC-labeled anti-mouse IgG) at 1:100 dilution in PBS was then added for a further 2 hours incubation at 37°C. Following three centrifugation washes with PBS, the cells were transferred to glass slides for observation under the epifluorescent microscope. The percentage of stained cells was recorded for each study.

The IIF staining of the frozen testicular and epididymal sections on slide was similar to that described for methanol-fixed spermatozoa. Culture media from myeloma cells and PBS were served as negative controls.

Tissue distribution of M1 antigen by immunoblotting.

Immunoblotting was carried out using eight different tissues of the hamster; testis, epididymis, lung, heart, kidney, spleen, brain and liver. The tissues were removed immediately after the animals were sacrificed and frozen in liquid nitrogen. Subsequently they were homogenized in PBS containing 2 mM phenylmethanesulfonylfluoride (PMSF), a protease inhibitor. Cellular pellets were solubilised in 10 volumes of 0.5% NP 40, precipitated in acetone and solubilised in 50 mM Tris-HCl buffer containing 2% SDS and 5% β mercaptoethanol. Ten microgram of each of the protein samples was submitted to SDS-PAGE and transferred to a nitrocellulose membrane. Blotted membranes were then incubated with undiluted M1 hybridoma supernatant and processed as described previously [10].

Protein determination

The amount of protein in the sperm and in the tissue extractions were determined using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL) according to the supplier instructions.

Results

Indirect immunofluorescence and immunohistochemical staining

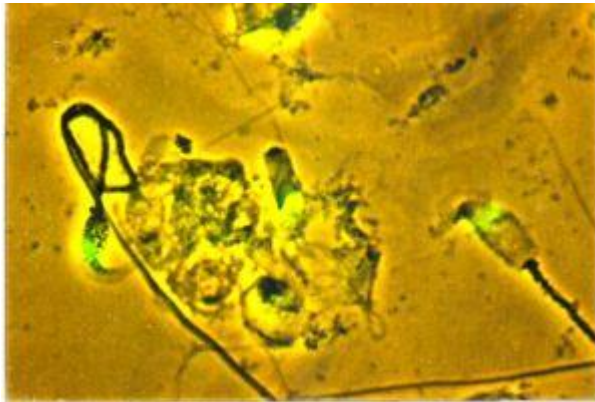
The specificity of M1 antibody was examined by IIF of fixed and unfixed cells that were prepared from various tissues of the hamster. As summarised in table 1.0, M1 antibody did not cross-react with cells isolated from somatic tissues including lung, heart, kidney, spleen, brain and liver but was spermspecific (see Table 1 & Figure 1). Additionally, immunohistochemical staining analysis of frozen sections of hamster testis and epididymis confirmed that the M1 antigen was only expressed on sperm cells at the very late spermatid stage. As shown in Figure 2 (a) M1 antigen was detected only on cross sections of testes seminiferous tubules containing spermatids and immature sperm.

Table 1: Summary of the distribution of M1 antigen in hamster reproductive and non-reproductive tissues. Tissues containing sperm were positive after immunoblotting and IIF staining assay.

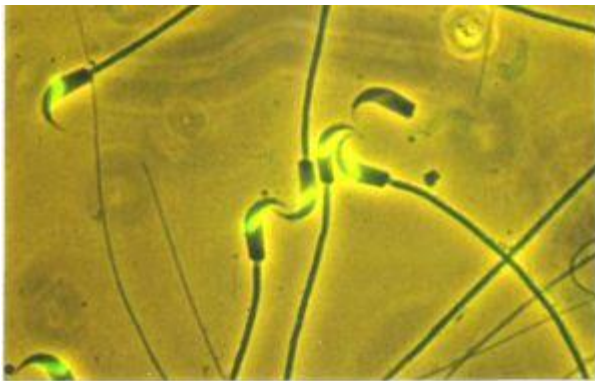
Tissues	Distribution on fixed and unfixed cells (IIF assay)
Testis	+ve
Epididymis	+ve
Liver	-ve
Kidney	-ve
Spleen	-ve
Heart	-ve

Brain	-ve
Lung	-ve

+ve : Cells with fluorescent staining
-ve : Cells without fluorescent staining



(a) (For larger image click [here](#))



(b) (For larger image click [here](#))

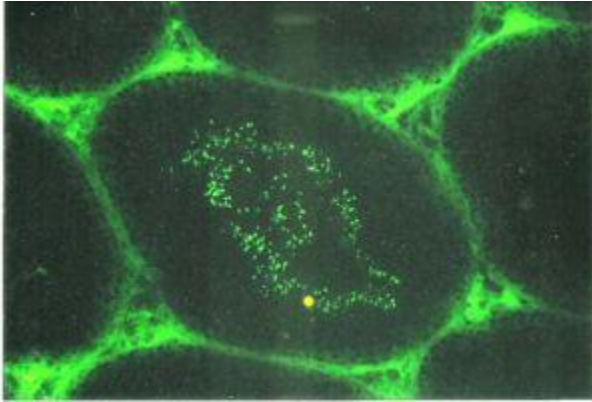
Fig. 1: Immunofluorescent localisation of M1 antigen on a methanol-fixed smear of the hamster

(a) testicular cells (late spermatids).

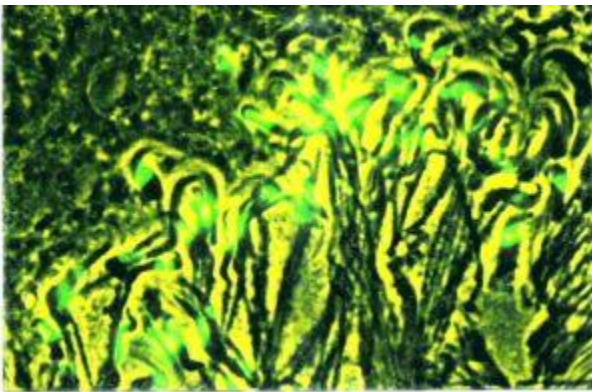
(b) epididymal sperm cells. M1 mab recognised antigen on the ES of the hamster sperm (X1000).

At a higher magnification, the fluorescent stain was localised to the ES of developing spermatids [Figure 2 (b)]. Control sections displayed some staining in the interstitial tissue and it was considered to be due to non-specific binding (Moore, personal communication). The formation of the ES could be followed by examining germ cells at various stages of development.

Intensed staining was exhibited by the late spermatid stage. On sections of epididymis, intensed staining was seen in the epididymal lumen in both the caput (data not shown) and cauda (Figure 3). There was no apparent change in the intensity of staining on the sperm in the epididymis.

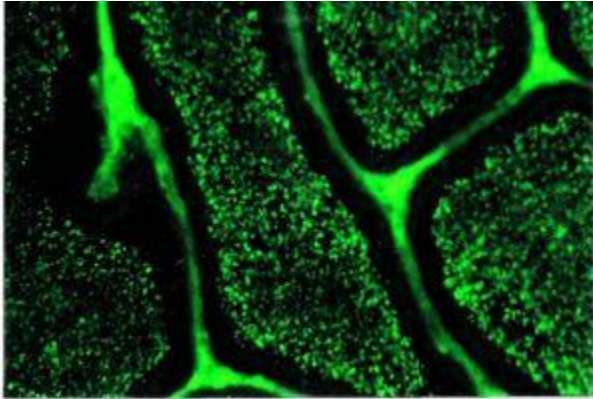


2a (For larger image click [here](#))



2b (For larger image click [here](#))

Fig. 2: Immunocytochemical localisation of M1 antigen within the seminiferous tubules of the mature hamster testis; (a) immunofluorescent localisation of M1 mab in a frozen section of the hamster testicular seminiferous tubules, showing fluorescent staining in the adluminal compartment containing sperm (X100), (b) same as (a) under UV and ordinary light at high magnification (X1000). Immunofluorescent localisation analysis apparently shows the M1 antigen is post-meiotically expressed.



(For larger image click [here](#))

Fig. 3: Immunofluorescent localisation of the M1 mab in a frozen section of cauda epididymis, showing fluorescent staining on caudal sperm in the adluminal compartment (X400).

Immunoblotting for M1 antigen in the testes, epididymis and other tissues

Since SDS-PAGE analysis only represents the pattern of total protein present in the sample, immunoblotting was undertaken to detect whether a protein was recognized by M1 antibody. The antibody was used to probe immunoblots of SDS-PAGE gels of protein samples obtained from the hamster; testis and epididymis, and other tissue, such as lung, heart, kidney, spleen, brain and liver. The M1 mab only reacted against the testis (79kDa) and epididymis (37.5 and 34 kDa) (see Table 2 and Figure 4).

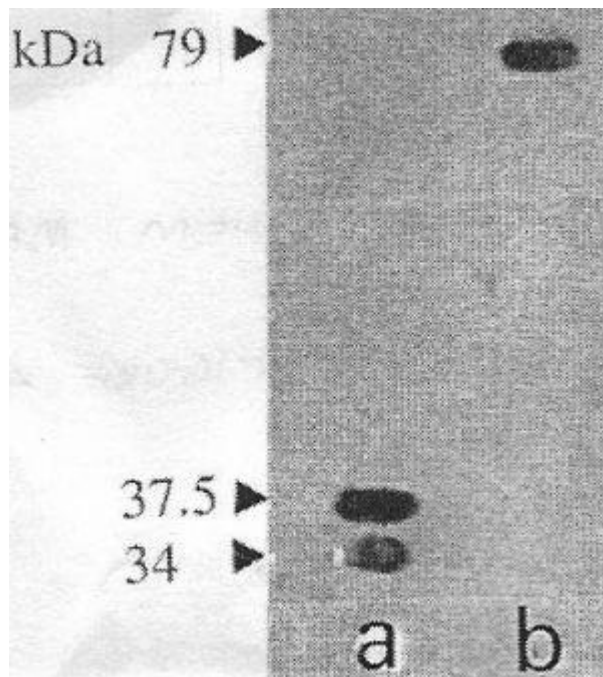


Fig. 4: Detection of M1 antigen on the hamster spermato-zoa after immunoblotting procedure utilizing M1 mab; Lane a M1 protein from the epididymis appears as 37.5 and 34kDa protein bands. Lane b, M1 protein from the testis appears as 79kDa band protein

Table 2: Summary of the distribution of M1 antigen in the hamster reproductive and non-reproductive tissues. Tissues containing sperm were positive after the immunoblotting procedure.

Tissues	Biochemical characterization (Immunoblotting)
Testis	+ve (79kDa)
Epididymis	+ve (37.5 & 34 kDa)
Liver	-ve
Kidney	-ve
Spleen	-ve
Heart	-ve
Brain	-ve
Lung	-ve

+ve: Protein band present
-ve: Protein band absent

For all other samples no immunoreactivity was detected at any level of the molecular weight. Control immunoblots incubated with myeloma medium showed no immunoreactivity at all (data not shown).

Discussion

IIF of cells released from the testis demonstrated that M1 antigen was only present in the developing (post-meiotic) or mature testicular sperm. The absence of M1 antigen in other testicular cells indicated that it was not secreted by Sertoli cells for example and taken up by the germ cells. Since the preparations were smears of the cell suspension, the developmental progress of M1 antigen during spermatogenesis could not be seen. Therefore, a more detailed immunohistochemical study with frozen sections of the hamster testis and epididymis was undertaken.

Sperm cells have unusual membranes compared to those of the somatic cells. As discussed by Phelps et al [12], the initial organisation of the sperm surface into discrete membrane domains begins during spermiogenesis and continues as the sperm cells traverse through the epididymal duct. A delicate reorientation and modification of plasma membrane molecules then take place in the female tract before the fertilization

occurs [13]. Previous studies also suggest that antigens appear on the sperm at different times from the beginning of spermiogenesis. In guinea-pigs for example, PH20 first appears at step 6-7 of the spermiogenesis [14] while PH30 antigen first appears on the sperm in the cauda epididymis [15]. However, immunofluorescence localisation on the fixed cryosections of the hamster testis indicated that M1 antigen was first expressed during postmeiotic differentiation at elongating spermatid, concomitant with the development of the apical acrosomal and equatorial region (Figure 1a). The M1 antigen was well established when sperm entered the epididymis. There was no fluorescent staining on epididymal epithelial cells. Although the connective tissue surrounding the epididymal tubules exhibited staining, this was also seen in controls and thus represented nonspecific binding as also seen in the testis. Therefore, it can be concluded that the M1 antigen is synthesised only by germ cells in the male reproductive tissue.

Immunoblot analysis with protein extracts from the testis and epididymis sperm revealed different molecular weight was detected by M1 antibody. Two main bands were detected at 37.5 and 34 kDa for epididymal sperm samples and 79kDa for testicular sperm samples, suggesting that M1 antigen is initially present as a protein and then under certain proteolytic condition it splits into two subunits 37.5 and 34 kDa. Multiple forms of peptides that are identified by immunoblotting may be the consequence of (i) post-translational modification, (ii) proteolytic processing of the proteins within the acrosome, (iii) multiple gene product, or several of these possibilities acting in concert.

The likelihood of sperm antigen undergoing proteolytic cleavage during maturation is quite high. For example cleavage of PH-30 antigen occurs while the sperm are in the epididymis. This may be caused either by epididymal secretions [4] or due to some dissociated sperm acrosome proteases that affect intact cells. Because the population of acrosome-intact sperm, immediately after removal from the male guinea-pig normally contain about 10-15% cells without acrosomes [16].

The molecular profile of M1 antigen exhibited some similarity with the mouse sperm antigen (40 kDa) detected by M29 mab [17] and also the guinea-pig sperm antigen (34 kDa) [18]. Both these antigens are localised to the ES of the sperm. Although M1 antigen displays the same molecular weight as DE protein [19], it is reported to be involved in the rat sperm-egg fusion [20], and it is unlikely to be the same molecule. The reason for this is that M1 antigen is produced by the sperm during the late spermatid stage while DE is a sperm-coating protein secreted by the epididymis. Furthermore, M1 antibody does not cross-react with the rat sperm. It has been reported by Calvete et al [21], that horse sperm plasma 3 (HSP-3) is involved in sperm-egg fusion since it possesses a high amino-acid sequence similarity with the DE protein [19]. Calvete and his colleagues [21] moreover demonstrated a 16 kDa stallion spermadhesin which is present in another membrane fraction (HSP-7), localised on the ES of the ejaculated stallion sperm and it may possibly be a putative sperm-egg fusion protein. Again, based on the present result M1 antigen is found not to be similar to HSP-3 and HSP-7 proteins even though they are structurally conserved in several mammalian species.

Based on IIF and immunoblotting results, M1 antigen appeared to be sperm-specific as it was not detected in a range of somatic cells. The result is entirely in agreement with the proposed function in relation to fertilization, since gamete interactions are thought to be tissue specific [22].

In conclusion, the results to date suggest that M1 antigen is sperm specific, synthesized only by the sperm cells and is located in the crypt of the ES. The M1 mab against this protein was proven to be involved in sperm-egg fusion by reducing fertilization rate in vitro [10] and in vivo without any detectable side effects [23]. This protein is, therefore, likely to be important for the development of a potential and safe candidate for an immunocontraceptive vaccine. Investigations at the molecular level are essential and proteomic of M1 antigen is in progress.

Acknowledgements

The editorial assistance of Professor Dr. Mohd Hamim Rajikin is gratefully acknowledged. This work was supported by research grant from Ministry of Science and Technology Malaysia.

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