Human spermatozoa antigens in unexplained infertility

L. Karakoc Sokmensuer[1,2], B. Demir[3], D. Zeybek[2], E. Asan[2], S. Gunalp[1]

[1]Department of Obstetrics and Gynecology, [2]Department of Histology & Embryology, Hacettepe University, Faculty of Medicine, Ankara
[3]Department of Gynecology and Obstetrics, Haseki Training and Research Hospital, Istanbul (Turkey)

Summary

Objective: To determine and compare the immunolocalization of functionally important antigens in human spermatozoa in an unexplained infertility (UI) group. *Materials and Methods:* In this study, the sperm samples of 20 patients undergoing evaluation belonging to normozoospermic group, whose primary reason of infertility was under investigation for this purpose, were screened. CD46, CD55 and CD52, CD69, CD98, fMLP, HI307, and 80280 were stained on the spermatozoa through indirect immunofluorescence technique. *Results:* In addition to CD46, CD55, and CD52 antigens, which are known to be localized on human spermatozoa, significant immunolocalization of several novel antigens including: CD52, CD69, CD98, fMLP, HI307, and 80280 were determined on the spermatozoa of the unexplained infertility group, possibly reflecting important roles in the pathophysiology of such unresolved clinical situations. *Conclusion:* Identification and characterization of antigens present on sperm cells is crucial for understanding of the diagnosis and treatment of unexplained infertility. Further studies were conducted to evaluate a possible correlation between the expression of these antigens and clinical outcomes in different well-defined infertility groups.

Key words: Spermatozoa; Surface antigens; Unexplained infertility; Immunofluorescence.

Introduction

Fertilization is a complex process involving numerous molecules, cell-cell, and cell-matrix interactions. For successful fertilization, the spermatozoa must undergo a cascade of events including capacitation, hyperactivation, acrosome reaction, binding to the zona pellucida, penetration through the zona pellucida, and fusion with the plasma membrane of oocytes [1]. Several families of molecules such as complement regulatory proteins, tetraspans, ADAM proteins, integrins, and others have been shown to be involved in this process [2]. Most of these molecules are not restricted to the reproductive system, but also play essential roles in a variety of immune reactions. Thus the function of these molecules is still unclear and the mechanisms controlling this complex event is not yet completely understood. Chemotaxis and activation of reactive oxygen intermediates (ROI) are also important components of the fertilization process; consequently, chemotactic factors and their receptors on spermatozoa are under intensive investigation [3-5].

Unexplained infertility (UI) refers to a diagnosis made in couples where standard investigations including semen analysis, tests of ovulation, and tubal patency are normal. UI still accounts for some 10% to 25 % of all cases of infertility. The pathophysiology of unexplained male infertility is still poorly understood, and various diagnostic tests are unable to determine the underlying cause of sperm dysfunction. Most possible causes of UI seems to be any disorder in the molecular interactions between sperm and oocyte in the reproductive environment [6, 7]. Thus any information on these molecules and/or their functions is of critical importance. The authors attempted to determine the antigenic profile of spermatozoa of normal individuals and UI patients at the light-microscopy level using several monoclonal antibodies (mAbs), some of which are reactive with previously reported antigens, while some others are introduced in the present study. The aim of this study was to determine and compare the immunolocalization of these antigens in normal and UI groups. In the future, the authors intend to extend these studies to the ultrastructural level for more precise localization.

Materials and Methods

The human semen samples were collected in sterile plastic containers through masturbation by unexplained infertile patients (n = 20) each with three consecutive conception failures on intrauterine insemination (IUI) attending the ART clinic in Hacettepe University Medical School, as well as from healthy proven-fertile donors (n = 6) after an abstinence of three to five days. The ejaculates were allowed to liquefy for 30 minutes, and semen parameters were analyzed according to World Health Organization (WHO) guidelines [8].

Sperm counts of UI subjects were similar to those of men of the proven-fertile group. It was ensured that each subject in both groups was married and lived with his spouse for two or more years without any recorded conception. All spouses were found normal after strict gynecological assessment. The controls had at least one child and had routine semen analysis within the normal range, according to WHO 1999 guidelines. Necessary approval was given by the institutional review board to perform the study.

After initial wash with human tubal fluid (HTF) medium, the spermatozoa were smeared onto a clean glass slide coated with gelatine and the smear was allowed to dry at room temperature. Slides were fixed in methanol for ten minutes and air-dried for at least 30 minutes. Slides were then incubated for 60 minutes

Revised manuscript accepted for publication October 22, 2012

Table 1. — *Monoclonal antibodies used.*

Primary antibody	Clone	Isotype
CD46	122-2	mG1
CD52	FL-61	mG1
CD55	BRIC216	mG1
CD69	UN6	mG-2a
CD98	MEM-108	mG1
5F1	5F1	n/a
80280	n/a	n/a
HI307	HI307	n/a

with primary mAbs (Table 1). After washing in 0.01M phosphate buffered saline (PBS) pH 7.4, the slides were covered with mouse immunoglobulins/FITC labelled secondary antibody, except for CD52 monoclonal antibody for 30 minutes, washed in PBS 3 for ten minutes and covered by one drop of propidium iodide/antifade solution. Anti-rabbit IgG-FITCH secondary antibody was used for CD52 monoclonal antibody. Immunofluorescent labelled sections were then examined and photographed using a microscope.

Results

Complement regulatory/related proteins

CD46

Membrane cofactor protein (MCP; CD46) represented one of the most strongly-expressed antigens in human sperm. Extensive expression of this antigen in both groups provided a positive control for this technique. The main site of localization of the antigen was the acrosomal compartment of the sperm head (Figures 1A, B). In the control (normal) group a similar reaction was present. In the spermatozoa exhibiting abnormal head morphology (swollen or irregularly enlarged), a crescent-shaped reaction was confined to the tip of sperm head possibly representing an abnormal acrosome (Figures 1C-E).

CD52

CAMPATH-1 antigen exhibited a unique expression on the post-acrosomal membrane region in the UI group (Figures 2A, B). A similar but weaker reaction was observed in the control group (Figure 2C). There was also a weak reaction on the midpiece and initial segment of sperm tail in some spermatozoa of the control group (Figures 2D, E).

CD55

Decay accelerating factor (DAF) was expressed in the acrosomal region, midpiece, and the tail of the spermatozoa in the UI group being most strong in the midpiece (Figures 3A, B). There was a restricted reaction in the midpiece in some samples of the control group (Figure 3C). A weaker reaction was present in the acrosomal region in the control group (Figures 3D, E).

Activation antigens

CD69

Activation inducer molecule (AIM) was expressed in the acrosomal region, equatorial segment, midpiece, and tail in the UI group, being stronger in the equatorial segment and midpiece (Figures 4A, B). There was no significant reaction in the control group (Figure 4C).

CD98

Activation antigen 4F2 was expressed in the acrosomal region, midpiece, and tail in the majority of the spermatozoa in the UI group (Figure 5A). Both diffuse and patchy reaction patterns were present in the acrosomal region (Figures 5B, C). However different staining patterns were also observed in this group. In some of the spermatozoa, the reaction was confined to the midpiece and tail regions and absent in the acrosome (Figure 5D). No significant reaction was observed in the control group (Figure 5E).

Novel mAbs from human leukocyte differentiation antigens (HLDA) 7th and 8th Workshop blind panels

5F1(fMLP)

This antigen was another example of a very unique expression in the UI group. The reaction was present on the equatorial segment, being stronger at both edges, and in the midpiece resembling the corners of a triangle (Figures 6A, B). In some spermatozoa, a patchy reaction was present also in the acrosomal region (Figures 6C, D). There was a moderate reaction in the tail as well. No significant reaction was observed in the control group (Figure 6E).

80280

In the UI group, the acrosomal region was diffusely stained. There was also a moderate reaction in the tail (Figure 7). No significant reaction was seen in the control group.

HI307

The main reactive site for this antigen in the UI group was the midpiece and the tail (Figure 8A). Reaction intensity in the midpiece was quite strong (Figure 8B). No significant reaction was seen in the control group.

Discussion

Characterization of cell differentiation and maturation relies on structural observations and/or cell specific expression of specific transmembrane or cytoplasmic antigens. However data arising from recent studies revealed that different cell types share a number of antigens which have recently been classified into several familes of proteins according to their molecular structures and/or functions. Thus investigators work on anti-

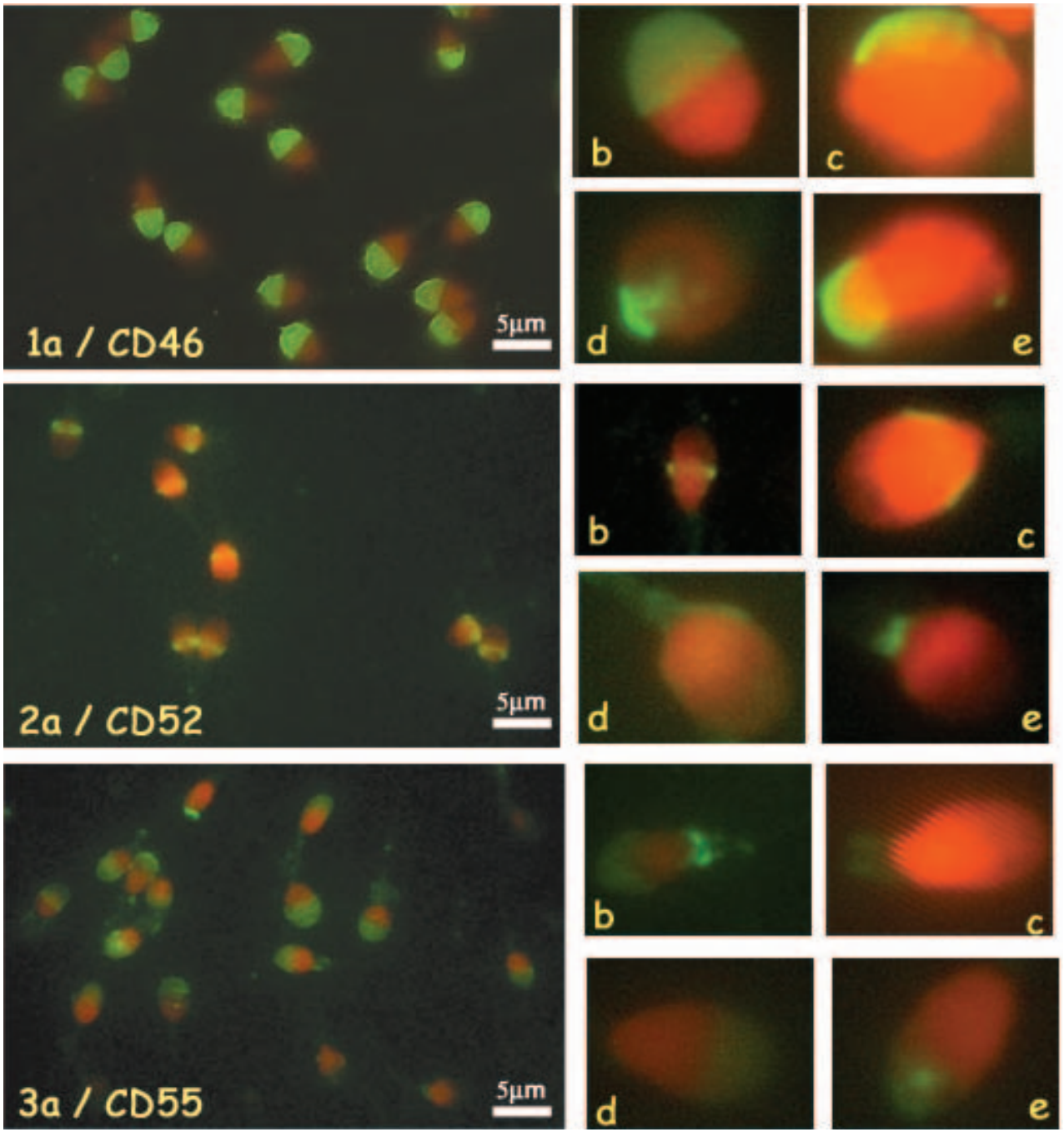

Figure 1. — Localization of CD46. **a, b:** CD46 localization on the acrosomal compartment of the spermatozoa head in the UI group; **c, d, e:** acrosomal localization of CD46 in the control group.
Figure 2. — Localization of CD52. **a, b:** CD52 antigen localization on the post-acrosomal membrane region in the UI group; **c:** post-acrosomal membrane CD52 expression in the control group; **d, e:** weak CD52 reaction on the midpiece and initial segment of tail in some spermatozoa of the control group.
Figure 3. — Localization of CD55. **a:** CD55 localization on the acrosomal region, midpiece, and tail of the spermatozoa in the UI group; **b:** strong reactivity with CD55 in the midpiece of the spermatozoa in the UI group; **c:** restricted CD55 reaction on the midpiece in some samples of the control group; **d, e:** weaker CD 55 reaction on the acrosomal region in the control group.

gens on the cell groups of their interest for two main goals: (i) determination of antigens which are specific to a cell reflecting their differentiation/maturation state for their characterization; (ii) determination of antigens with known functions in other systems of the organism to obtain evidence of a similar function in the cells of inter-

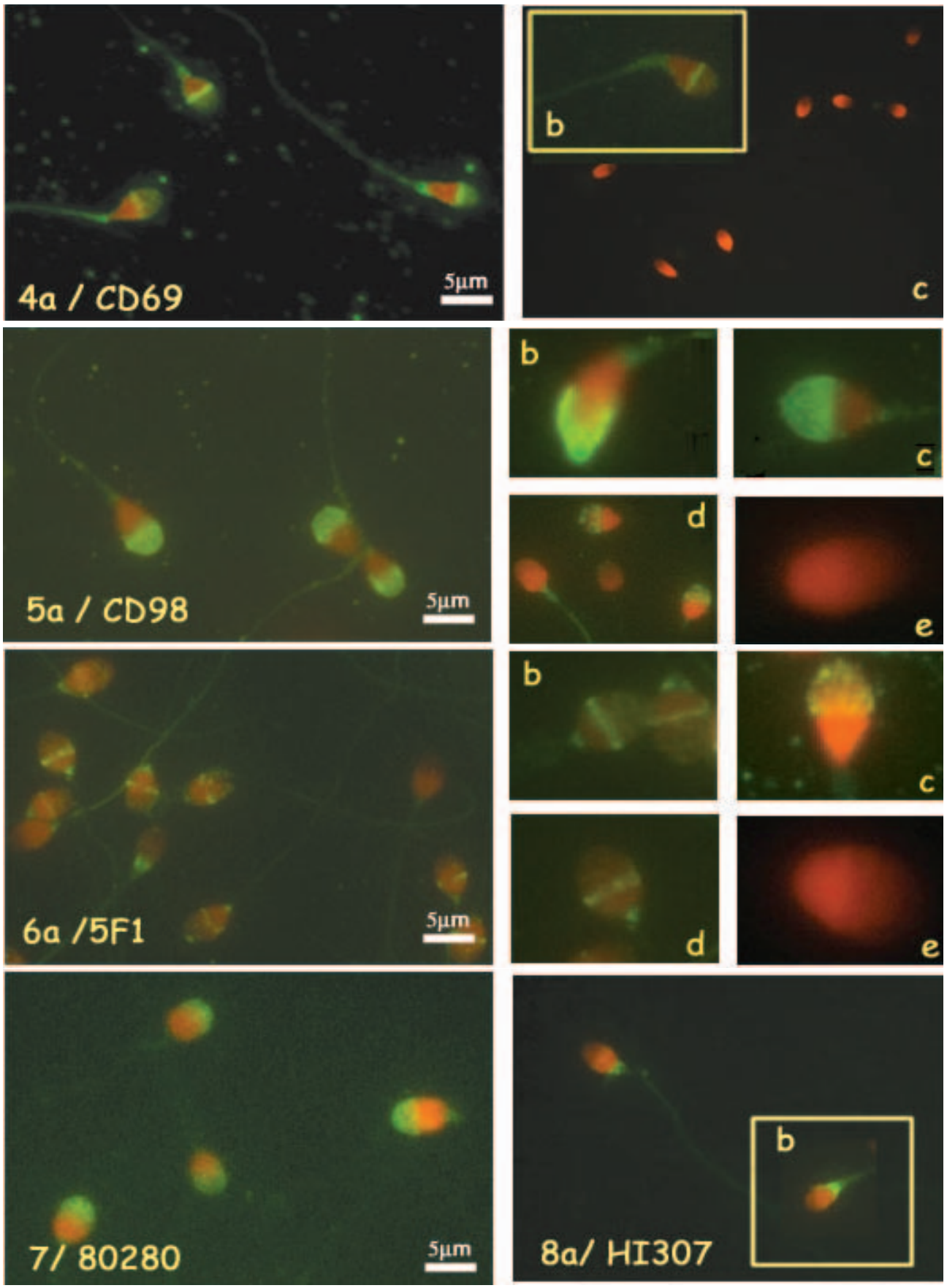
4a / CD69
5µm
b
c
5a / CD98
5µm
b
c
d
e
6a /5F1
5µm
b
c
d
e
7/ 80280
5µm
8a/ HI307
b

Figure 4. — Localization of CD69. **a, b:** CD69 localization on the acrosomal region, equatorial segment, midpiece and tail in the UI group; **c:** no significant CD69 reaction in the control group.

Figure 5. — Localization of 4F2. **a:** 4F2 localization in the acrosomal region, midpiece and tail in the majority of the spermatozoa in the UI group; **b:** 4F2 expression in the midpiece and tail regions being absent on the acrosome in UI group; **c, d:** both diffuse and patchy staining patterns with 4F2 in the acrosomal region in UI group; **e:** no significant reaction with 4F2 in the control group.

Figure 6. — Localization of 5F1. **a, b:** 5F1 localization on the equatorial segment being stronger at both edges and in the midpiece resembling the corners of a triangle in the UI group; **c:** patchy staining pattern with 5F1 in the acrosomal region in the UI group; **d:** moderate 5F1 reaction in the tail of the sperm in the UI group; **e:** no significant reaction in the control group

Figure 7. — Localization of 80280. Diffuse acrosomal and moderate tail staining with 80280 in UI group

Figure 8. — **a, b:** localization of HI307. HI307 reactivity on the midpiece and tail in the UI group.

All scale bars represent five μm.

est. Regarding the yet unsettled mechanisms of the complex reproduction process, the authors studied the antigenic profile of spermatozoa belonging to fertile and unexplained infertility groups to obtain some evidence to direct further studies. For this purpose, they used both monoclonal antibodies to known antigens and some others which have not been studied on spermatozoa previously and obtained valuable data. Following a screening study using large numbers of monoclonal antibodies, only those of interest which provide initial findings to explain some of the mechanisms leading to IU are presented in this paper.

Expression of complement regulatory proteins CD46, CD55, and CD59 on inner acrosomal membrane of spermatozoa has been previously reported [9-15]. The authors studied CD46 and CD55 expression together with CD52, a GPI-anchored surface glycoprotein, which is also known to be expressed on spermatozoa for comparison of spermatozoa from fertile and UI groups, also serving as a positive control. Both CD46 and CD55 were expressed on the acrosome in control and IU groups, however the intensity of CD55 expression in the fertile group is relatively weaker. CD46 is strongly expressed also on the spermatozoa with structural abnormalities, reflecting the structural deformities of the acrosomal vesicle. Thus CD46 antigen can be considered as a constitutive antigen being present in the spermatozoa, also providing a positive control for the technique used. CD55 expression shared variations, especially in the control group as a sign of maturational change reflecting the heterogeneity of the spermatozoa population in the smears. In the control group, reaction intensity was weaker in the acrosomal region, being the strongest in the midpiece in both groups. This observation leads to a conclusion that strong expression of CD55 on acrosome may be involved in a mechanism leading to IU, which should be confirmed.

CD52 (CAMPATH-1) antigen is known as an antigen exclusively expressed by immune system cells and epididymal cells transferred on spermatozoa [16-21]. Recently, this antigen was also shown on the mature cumulus cell mass [22]. In the present study, CD52 was shown on the post-acrosomal region of the spermatozoa in both groups. A significant expression on the midpiece in some spermatozoa of the control group was also evident. The localization of CD52 antigen strongly suggests a specific role for this molecule in sperm-oocyte contact, especially through their glycan moieties. Further ultrastructural studies should provide added evidence for this suggestion.

CD69 (activation inducer molecule) is a type II transmembrane glycoprotein with a lectin domain being mainly expressed on activated immune system cells, similar to CD52 [23, 24]. Expression of this antigen on spermatozoa, functioning as a signal transmitter on spermatozoa, has not previously been reported. This antigen was expressed on the acrosome, equatorial segment, midpiece, and tail of the spermatozoa in the IU group while no significant expression was determined in the control group. Thus, CD69 is another candidate molecule leading to signals initiating some mechanisms that result in IU.

CD98 (4F2), another activation antigen, was also broadly expressed in the spermatozoa of the IU group, however its expression was extensively variable when compared to the other antigens examined. It is reported to be expressed by a number of activated cells including neoplastic ones [25-28]. It is also expressed by trophoblastic lineage (the authors' unpublished observations). The function of this molecule is not entirely known, however it is believed to serve as an amino acid transporter in some cells. Expression of this antigen in the IU group, but not in the fertile group, apparently reflects a deviation in sperm activation leading to IU.

Another antigen with a unique expression on the spermatozoa of the IU group, which has not previously been reported was fMLP. The fMLP receptor family represents a group of molecules that receive recently chemotactic signals from bacteria and mitochondria [29, 30]. Although it is postulated that members of this receptor family direct leukocyte traffic, their physiological role is poorly understood.

Presence of such receptors on sperm is not previously reported. The authors determined a unique expression of this antigen on the spermatozoa of the IU group, however no significant reaction was determined in spermatozoa of the fertile-normal group. This finding apparently reflects

a targeting mechanism for the spermatozoa of IU patients leading to a decreased number of normal spermatozoa incapable of fertilization.

Another novel mAbs from HLDA (human leukocyte differentiation antigens) 7th and 8th Workshop blind panels was 80280. In the UI group the acrosomal region was diffusely stained. There was also a moderate reaction in the tail. No significant reaction was seen in the control group. Further studies on the characterization of this antigen recognized by this antibody need to be evaluated.

Human leukocyte antigens (HLA) coded by human major histocompatibility complex on chromosome 6 represents a group of transmembrane glycoproteins carrying out immunological recognition function [31]. Previous reports on studies in different species including humans, display controversial findings regarding their expression [32-35]. The authors detected a significant reaction with an anti-MHC Class II monoclonal antibody on the post-acrosomal zone, midpiece, and tail of the spermatozoa reflecting a possible non-immunological function for these molecules.

In conclusion, as discussed briefly above, most of the antigens the authors studied were related to the immune system, but were also present on spermatozoa. Though the function of reproductive and immune systems are separate, some overlapping molecular mechanisms for similar functions in the organism are not really surprising and has been demonstrated for the neuro-endocrine system. Information on such molecules will help to better understand their functions, assisting in revealing the physiological mechanisms in the complex process of both systems. The findings of the present study for CD52, CD69, CD98, 80280, and fMLP will lead to further studies including immuno-electron microscopy for the precise localisation of the antigens, comparison of patient groups of unexplained infertility, and some functional studies.

Acknowledgements

This project is supported by the Hacettepe University Research Foundation, Ankara, Turkey, with Grant number: 05 01 101 010.

Preliminary data of this project was partly presented in American Society of Reproductive Medicine 62nd Annual Meeting in USA and 16th International Microscopy Congress in Japan in 2006.

References

[1] Primakoff P., Myles D.G.: "Penetration, adhesion, and fusion in mammalian sperm-egg interaction". *Science,* 2002, *296*, 2183.

[2] Takahashi Y., Bigler D., Ito Y., White J.M.: "Sequence-specific interaction between the disintegrin domain of mouse ADAM 3 and murine eggs: role of b1 Integrin-associated Proteins CD9, CD81, and CD98". *Mol. Biol. Cell.*, 2001, *12,* 809.

[3] Isobe T., Minoura H., Tanaka K., Shibahara T., Hayashi N., Toyoda N.: "The effect of RANTES on human sperm chemotaxis". *Hum. Reprod.,* 2002, *17*, 1441.

[4] Babcock D.F.: "Smelling the roses?". *Science,* 2003, *299,* 28.

[5] Eisenbach M., Giojalas L.C.: "Sperm guidance in mammals - an unpaved road to the egg". *Nat. Rev. Mol. Cell. Biol.,* 2006, *7,* 281.

[6] Davajan V., Nakamura R.M., Saga M.: "Role of immunology in the infertile human". *Biol. Reprod.*, 1972, *6,* 443.

[7] Tiitinen A., Isaksson R.: "Present concept of unexplained infertility". *Gynecol. Endocrinol.*, 2004, *18*, 278.

[8] WHO Laboratory manual for the examination of human semen and sperm-cervical mucus interaction, 4th ed. Cambridge: Cambridge University Press, World Health Organization, 1999.

[9] Simpson K.L., Holmes C.H.: "Differential expression of complement regulatory proteins decay-accelerating factor (CD55), membrane cofactor protein (CD46) and CD59 during human spermatogenesis". *Immunology,* 1994, *81*, 452.

[10] Taylor C.T., Johnson P.M.: "Complement-binding proteins are strongly expressed by human preimplantation blastocysts and cumulus cells as well as gametes". *Mol. Hum. Reprod.,* 1996, *2,* 52.

[11] Riley R.C., Kemper C., Leung M., Atkinson J.P.: "Characterization of human membrane cofactor protein (MCP, CD46) on spermatozoa". *Mol. Reprod. Dev.*, 2002, *62,* 534.

[12] Liszewski M.K., Kemper C., Price J.D., Atkinson J.P.: "Emerging roles and new functions of CD46". *Springer Sem. Immun.,* 2005, *27,* 345.

[13] Mizuno M., Harris C.L., Suzuki N., Matsuo S., Morgan B.P.: "Expression of CD46 in developing rat spermatozoa: ultrastructural localization and utility as a marker of the various stages of the seminiferous tubuli". *Biol. Reprod.,* 2005, *72*, 908.

[14] Riley-Vargas R., Lanzendorf S., Atkinson J.P.: "Targeted and restricted complement activation on acrosome-reacted spermatozoa". *J. Clin. Invest.,* 2005, *115,* 1241.

[15] Cummerson J.A., Flanagan B.F., Spiller D.G., Johnson P.M.: "The complement regulatory proteins CD55 (decay accelerating factor) and CD59 are expressed on the inner acrosomal membrane of human spermatozoa as well as CD46 (membrane cofactor protein)". *Immunology*, 2006, *118,* 333.

[16] Diekman A.B., Norton E.J., Klotz K.L., Westbrook V.A., Shibahara H., Naaby-Hansen S., Flickinger C.J., Herr J.C.: "N-linked glycan of a sperm CD52 glycoform associated with human infertility". *FASEB J.*, 1999, *13,* 1303.

[17] Ermini L., Secciani F., La Sala G.B., Sabatini L., Fineschi D., Hale G., Rosati F.: "Different glycoforms of the human GPI-anchored antigen CD52 associate differently with lipid microdomains in leukocytes and sperm membranes". *Biochem. Biophys. Res. Commun.*, 2005, *338,* 1275.

[18] Hasegawa A., Koyama K.: "Antigenic epitope for sperm-immobilizing antibody detected in infertile women". *J. Reprod. Immunol.*, 2005, *67*, 77.

[19] Flori F., Ermini L., La Sala G.B., Nicoli A., Capone A., Focarelli R., Rosati F., Giovampaola C.D.: "The GPI-anchored CD52 antigen of the sperm surface interacts with semenogelin and participates in clot formation and liquefaction of human semen". *Mol. Reprod. Dev.*, 2008, *75,* 326.

[20] Parry S., Wong N.K., Easton R.L., Panico M., Haslam S.M., Morris H.R. *et al.*: "The sperm agglutination antigen-1 (SAGA-1) glycoforms of CD52 are O-glycosylated". *Mol. Reprod. Dev.,* 2008, *75,* 326.

[21] Koyama K., Hasegawa A., Komori S.: "Functional aspects of CD52 in reproduction". *J. Reprod. Immunol.,* 2009, *83*, 56.

[22] Hasegawa A., Takenobu T., Kasumi H., Komori S., Koyama K.: "CD52 is synthesized in cumulus cells and secreted into the cumulus matrix during ovulation". *Am. J. Reprod. Immunol.,* 2008, *60*, 187.

[23] Ziegler S.F., Ramsdell F., Alderson M.R.: "The activation antigen CD69". *Stem Cells*, 1994, *12*, 456.

[24] Marzio R., Mauël J., Betz-Corradin S.: "CD69 and regulation of the immune function". *Immunoph. Immunotoxicol*, 1999, *21,* 565.

[25] Deve's R., Boyd C.A.R.: "Surface antigen CD98(4F2): not a single membrane protein, but a family of proteins with multiple functions". *J. Membrane Biol.*, 2000, *173,* 165.

[26] Papetti M., Herman I.M.: "Controlling tumor-derived and vascular endothelial cell growth: role of the 4F2 cell surface antigen". *Am. J. Pathol.*, 2001, *159*, 165.

[27] Nawashiro H., Otani N., Shinomiya N., Fukui S., Nomura N., Yano A. *et al.*: "The role of CD98 in astrocytic neoplasms". *Hum. Cell.*, 2002, *15*, 25.

[28] Miyamoto Y.J., Mitchell J.S., McIntyre B.W.: "Physical association and functional interaction between beta1 integrin and CD98 on human T lymphocytes". *Mol. Immunol.*, 2003, *39,* 739.

[29] Migeotte I., Communi D., Parmentier M.: "Formyl peptide receptors: a promiscuous subfamily of G protein-coupled receptors controlling immune responses". *Cytokine Growth Factor Rev*., 2006, *17,* 501.
[30] Panaro M.A., Acquafredda A., Sisto M., Lisi S., Maffione A.B., Mitolo V.: "Biological role of the N-formyl peptide receptors". *Immunoph. Immunotoxicol*, 2006, *28*, 103.
[31] Hutter H., Dohr G.: "HLA expression on immature and mature human germ cells". *J. Reprod. Immunol.*, 1998, *38,* 101.
[32] Schaller J., Glander H.J., Ladusch M., Westhoff U., Grosse-Wilde H.: "Lack of HLA-molecules on human spermatozoa and in seminal plasma". *Andrologia*, 1993, *25*, 77.
[33] Fiszer D., Kurpisz M.: "Major histocompatibility complex expression on human, male germ cells: a review". *Am. J. Reprod. Immunol*., 1998, *40*, 172.
[34] Omu A.E., al-Qattan F., Ismail A.A., al-Taher S., al-Busiri N.: "Relationship between unexplained infertility and human leukocyte antigens and expression of circulating autogeneic and allogeneic antisperm antibodies". *Clin. Exp. Obstet. Gynecol.,* 1999, *26*, 199.
[35] Paradisi R., Neri S., Pession A., Magrini E., Bellavia E., Ceccardi S., Flamigni C.: "Human leukocyte antigen II expression in sperm cells: comparison between fertile and infertile men". *Arch. Androl.*, 2000, *45*, 203.

Address reprint requests to:
L. KARAKOC SOKMENSUER, M.D.
Ziya Gokalp Cad. Cemre Sok
9/4 06420 Kolej, Ankara (Turkey)
e-mail: lalekarakoc@hacettepe.edu.tr