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Qualitative and Quantitative Analysis of Lectin Binding on the Epididymal Spermatozoa of Cat (*Felis catus)*

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ABSTRACT

The variations in the carbohydrate residues present on the surfaces of maturing epididymal spermatozoa of cat (***Felis Catus***) have been studied. Spermatozoa recovered from the caput, corpus and cauda epididymides were labeled with fluorescein isothiocyanate (FITC) linked lectins and examined with a fluorescence microscope and flow cytometer. Seven FITC-linked lectins viz. ***Canavalia ensiformis*** or ***Concanavalin A*** (Con A), ***Dolichos biflorus*** (DBA), ***Maclura pomifera*** (MPA), ***Arachis hypogaea*** or ***peanut agglutinin*** (PNA), ***Glycine max*** or ***Soybean agglutinin*** (SBA), ***Ulex europaeus*** (UEA), ***Triticum vulgaris*** or ***Wheatgerm agglutinin*** (WGA) were used. We find that as the spermatozoa move through the epididymis there is an increase in the lectin binding affinity for Con A and MPA and a decrease in SBA and DBA binding α-D glucose / α-D mannose residues populate the major portion of the cat sperm head and are present prominently on the plasma membrane covering the acrosome as revealed by Con A labeling. The N-acetyl-galactose specific lectin, MPA exclusively labels the equatorial segment. Flow cytometry indicates no change in the binding intensities of UEA, but microscopic examination reveals a distinct alteration in the labeling sites.

KEYWORDS

Lectins, Fluorescence microscopy, flow cytometry, spermatozoa, cat.

INTRODUCTION

The plasma membrane of mammalian spermatozoa undergoes structural transformations during the transport of the spermatozoa in the epididymis including modification of its surface glycocomponents. Glycocomponents on the sperm surface play an important role in fertilization, such as sperm-zona recognition and fusion [2, 22]. Studies on a number of mammals have shown that carbohydrate determinants involved in sperm-zona interactions are species specific.

Sperm membrane glyco-constituents have been localized with a wide variety of lectins [18, 19, 23]. Lectins conjugated with fluorescein, ferritin, peroxidase and colloidal gold have been fruitfully used to detect the exposed saccharides of the sperm surface [14, 15, 20, 34]. The agglutinability of the spermatozoa with lectins [17, 25, 35] and characterization of the sperm plasma membrane glycoproteins with lectin affinity columns [12, 30] and lectin blotting technique [31] have proved useful to analyse various aspects of sperm-lectin interactions. Flow cytometry has been employed to determine the intensity of lectin reactivity on the surfaces of spermatozoa [4, 5, 6, 21 and 31]

We have reported the lectin binding features on the sperm surfaces of a carnivore i.e. dog [4, 5]. In the present study, we report the distribution and relative densities of lectin binding on the spermatozoa isolated from the caput, corpus and çauda epididymis of another carnivore, cat (***Felis catus***). ***Fluorescein isothiocyanate*** (FITC) *linked* ***Concanavalin*** A (Con A), ***Dolichos***

biflorus (DBA), ***Maclura pomifera*** (MPA), ***Arachis hypogaea*** or ***peanunt agglutinin*** (PNA), ***Glycine max*** or ***soybean agglutinin*** (SBA), ***Ulex europaeus*** (UEA) and ***Triticum vulgaris*** or ***wheat germ agglutinin*** (WGA) have been used. Morphological observations of lectin binding sites have been supplemented with flow cytometric analysis to quantitate sperm-lectin interaction.

MATERIALS AND METHODS

Four adult male cats (***Felis catus***) were obtained from an animal collector in the months of March and April. The animals were caged and fed ad libitum for seven days prior to sacrifice. The epididymides were removed and rinsed in phosphate buffered saline (PBS) pH 7.4. Spermatozoa were obtained from the caput, corpus and cauda segments of the epididymis by gently mincing the tissue pieces in PBS. These cells were washed twice in the same buffer via centrifugation prior to use for further experimentation.

Lectin labeling procedure

FITC-conjugated lectins listed in Table (1) were obtained from EY Laboratories, San Mateo, Ca. USA and their specific sugars from Poly Sciences Inc. Pa. USA. For fluorescence microscopy, spermatozoa obtained from the three regions of the epididymis were smeared onto poly-L-lysine coated coverslips. Cells were labeled with FITC-linked lectin diluted in PBS at 10 µg/ml concentration in humidified chambers at 25°C in dark according to the procedure of Sinowatz et al. [34] and reported in our previous studies [4, 5]. Controls for the specificity of the lectin labeling were coincubated with 0.2M of the corresponding inhibitory sugar. The coverslips with the labeled spermatozoa were mounted with CITIFLUOR mountant on glass slides and examined with olympus Vanox-s with epifluorescence optics and photographed using Ilford HP5, ASA 400 black and white film.

Con A- a C-type lectin was diluted in TBS (Tris buffered saline) containing 1 mM each of Ca2+ and Mn2+[9]. Quantitative analysis of lectin labeling was performed via flow cytometry by the modified procedure of Magargee et al. [21]. Sperm aliquots (40 x 10^6 cells/ml) in PBS pH 7.4 were labeled with FITC conjugated lectins (4 mg/ml) in 500 µl reaction mixture at 25°C for 30 minutes. Parallel controls were run by adding 0.2M of the inhibitory sugars to the sperm-lectin reaction mixture. After incubation, the material was centrifugen at 1000 rpm for 5 minutes and resuspended in PBS and examined with Beckton Dickinson flow Cytometer in the FACSAN mode. The binding intensity was plotted on the x-axis, marked by channel numbers in the range of 0 to 1000 while the number of cells were plotted on the Y-axis.

Electron Microscopy

For scanning electron microscopy (SEM) the spermatozoa were smeared onto poly-L-lysine coated glass coverslips and fixed in 2% glutaraldehyde. The material was osmicated in cacodylate buffered 1% osmium tetroxide. After dehydration in graded alcohol, the samples were sputter coated with gold-platinum and examined with a Zeiss DSM-950 electron microscope.

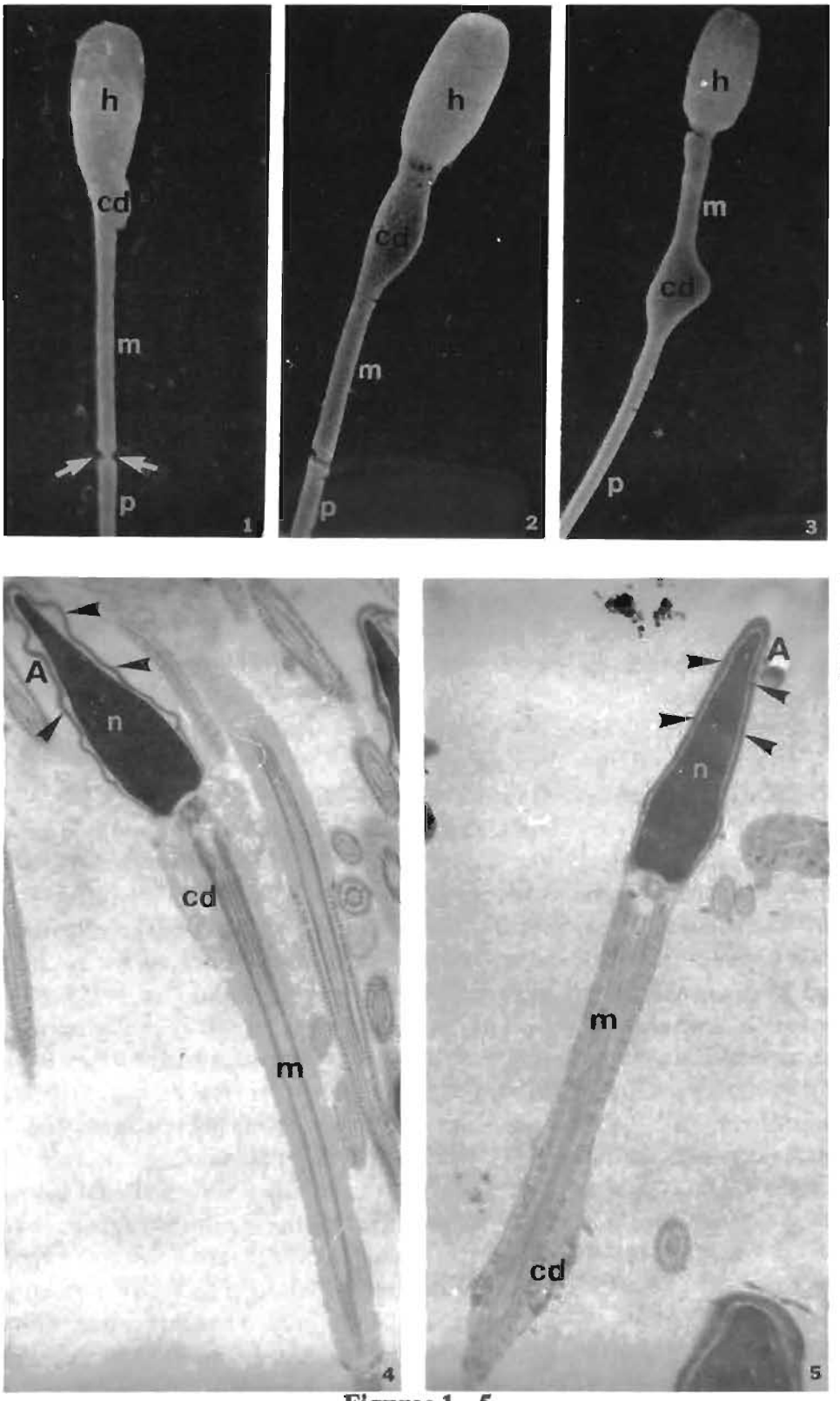
Specimens for transmission electron microscopy were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 containing 0.5% tannic acid and post fixed in 1% osmium tetroxide in cacodylate buffer. Subsequently the material was dehydrated in ascending series of acetone and embedded in araldite resin. Ultrathin section were cut with glass knives using Reichert-Jung Ultracut ultramicrotome, stained with lead citrate - uranyl acetate stain and examined under a Jeol 1200 EX electron microscope.

RESULTS

The nomenclature of Koehler [19] and Friend [13] has been followed to dileneate the various segments of the cat spermatozoa. The spermatozoa of the domestic cat have a paddle shaped head which measures approximately 3.5 µm.

Table 1: Lectins and their Sugar Specifities

Lectin	Sugar Specifities
Con A ***Canavalia ensiformis*** **Concanavalin A**	**α-D-glucose** **α-D-mannose**
DBA ***Dolichos biflorus***	**Methyl-2-acetamido-2 deoxy-D- galactose**
MPA ***Maclura pomifera***	**α-D-galactopyranoside**
PNA ***Arachis Hypogaea or*** **Peanut agglutinin**	**Methyl α-D-galactopyra-noside**
SBA ***Glycine max* or** **Soybean agglutini**	**α-or β-D-gal-N-Acetyl, α-D-galactose**
UEA ***Ulex europeaus***	**α-L-fucose**
WGA ***Triticum vulgaris* or** **Wheat germ agglutinin**	**N-Acetyl-β (1-4)-D-glucosaminide**



Figures 1 - 5

Figures 1-3: Scanning electron micrographs of epididymal spermatozoa of cat. Head (h), cytoplasmic droplet (cd), Middle piece (m) and principal piece (p) are marked.

Figure 1: Caput epididymal spermatozoon. Junction (arrows) between middle piece and principal piece is clear. SEM x 1100.

Figure 2: Corpus epididymal spermatozoon. Cytoplasmic droplet draws out along the tail. SEM x 1300.

Figure 3: Cauda epididymal spermatozoon, Cytoplasmic droplet drifts further along the tail. SEM X 10000.

Figures 4&5: Transmission electron micrographs of the epididymal spermatozoa of cat. Acrosome (A), nucleus (n), middle piece (m) and cytoplasmic droplet (cd) are marked.

Figure 4: Caput epididymal spermatozoon showing electron dense acrosomal matrix irregular in outline (arrowheads) TEM x 9400.

Figure 5: Cauda epididymal spermatozoon with an increase in the acrosomal matrix (arrowheads) TEM x 10000.

The sperm tail is appoximately 57 µm long with the middle piece constituting 7 µm of its length. In most of the spermatozoa recovered from the caput epididymis (**Fig.1**), the cytoplasmic droplet lies immediately below the paddle shaped head. In the corpus epididymis spermatozoa the cytoplasmic droplet lies slightly away from the neck (**Fig. 2**) and in the cauda epididymal spermatozoa the cytoplasmic droplet further moves along the middle piece. Here the cytoplasmic droplet appears to be excentrically located on the middle piece (**Fig. 3**). The middle piece and the principal piece junction is marked by a notch.

Our transmission electron micrographs reveal that the plasma membrane is closely bound to the acrosomal membrane of the epididymal spermatozoa recovered from the caput, corpus and cauda epididymis. the acrosome covers approximately 75-80% of the nucleus of the epididymal spermatozoa of cat, a feature which is also seen in the ejaculated spermatozoa of cat [10, 29]

In the caput epididymal sperm there is an appreciable perinuclear space which is bound on the outside by an irregularly outlined acrosomal complex (**Fig.4**). In the later stages of maturation, that is, in the spermatozoa recovered from the cauda epididymis, the perinuclear space decreases and the acrosome appears thickened with an adherent plasma membrane (**Fig.5**).

Lectin Labeling

Labeling with FITC-ConA shows an increase in the intensity of fluorescence as the spermatozoa move through the caput to corpus and then finally to the cauda epididymis (**Figs. 6 a-c**). There is a corresponding increase in the intensity of fluorescence over the acrosome as the spermatozoa move from the caput to corpus (**Fig.13**) to cauda epididymis. The acrosome is moderately labeled in the caput epididymal spermatozoa. The head and neck are brightly labeled in the spermatozoa recovered from the cauda region (**Fig. 14**).

With FITC-DBA, there is a progressive decrease in the intensity of fluorescence on the transit of the spermatozoa from the caput to the terminal segment of the epididymis(**Figs.7 a-c**). The receptors for DBA show faint reaction over the entire surface of the caput and corpus epididymal spermatozoa. However, the label becomes prominent over the head and the middle piece of the cauda epididymal spermatozoa (**Fig. 15**).

Flow cytometry reveals that with FITC-MPA the cauda epididymal spermatozoa exhibit maxium intensity of fluorescence followed by the corpus epididymal spermatozoa and the caput epididymal spermatozoa (**Figs. 8 a-c**). The cytoplasmic droplet and the equatorial segment appear brightly stained in the caput and corpus epididymal spermatozoa (**Fig. 16**). Approximately 50% of the corpus epididymal spermatozoa loose reactivity of the cytoplasmic droplet. In the cauda epididymal spermatozoa the cytoplasmic droplet is non stainable, here only the equatorial segment is prominently labeled (**Fig. 17**).

With FITC-PNA the corpus spermatozoa show maximum intensity of fluorescence followed by the caput and cauda epididymal spermatozoa as examined by flow cytometry (**Figs 9 a-c**). PNA receptors exhibit a weak reaction over the entire surface of the caput epididymal spermatozoa, the head however is slightly more stainable. In the corpus epididymal spermatozoa PNA receptors are of moderate intensity over the entire surface of the spermatozoa. In the cauda epididymal spermatozoa the receptors over the acrosome and the middle piece are prominent (**Fig. 18**).

Flow cytometrically with FITC-SBA the caput epididymal spermatozoa reveal maxium intensity of fluorescence. Thereafter the intensity decreases, and the spermatozoa recovered from the corpus epididymis and cauda epididymis reveal similar intensities of fluorescence (**Figs. 10 a-c**). The caput epididiymal spermatozoa show faint staining with FITC-SBA. In the corpus and cauda epididymal spermatozoa the head and middle piece are prominent. The middle

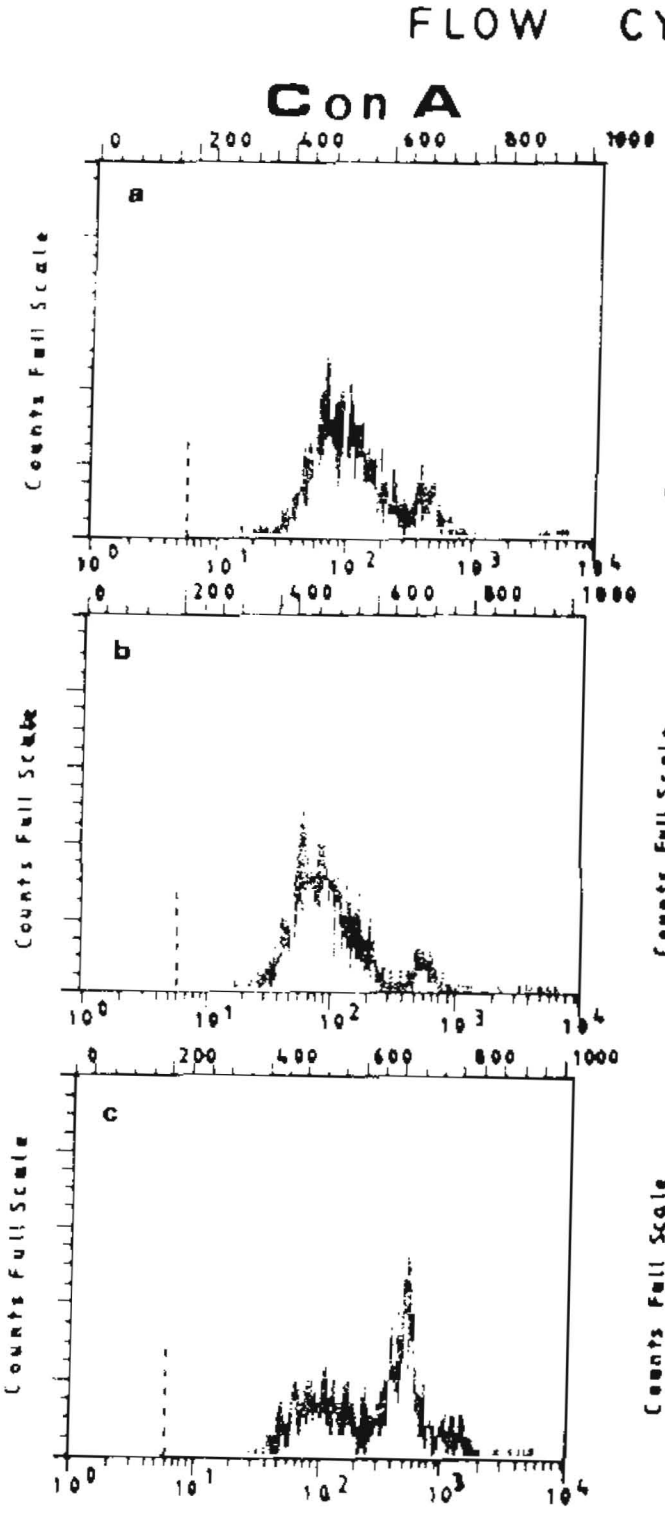


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Figure 6

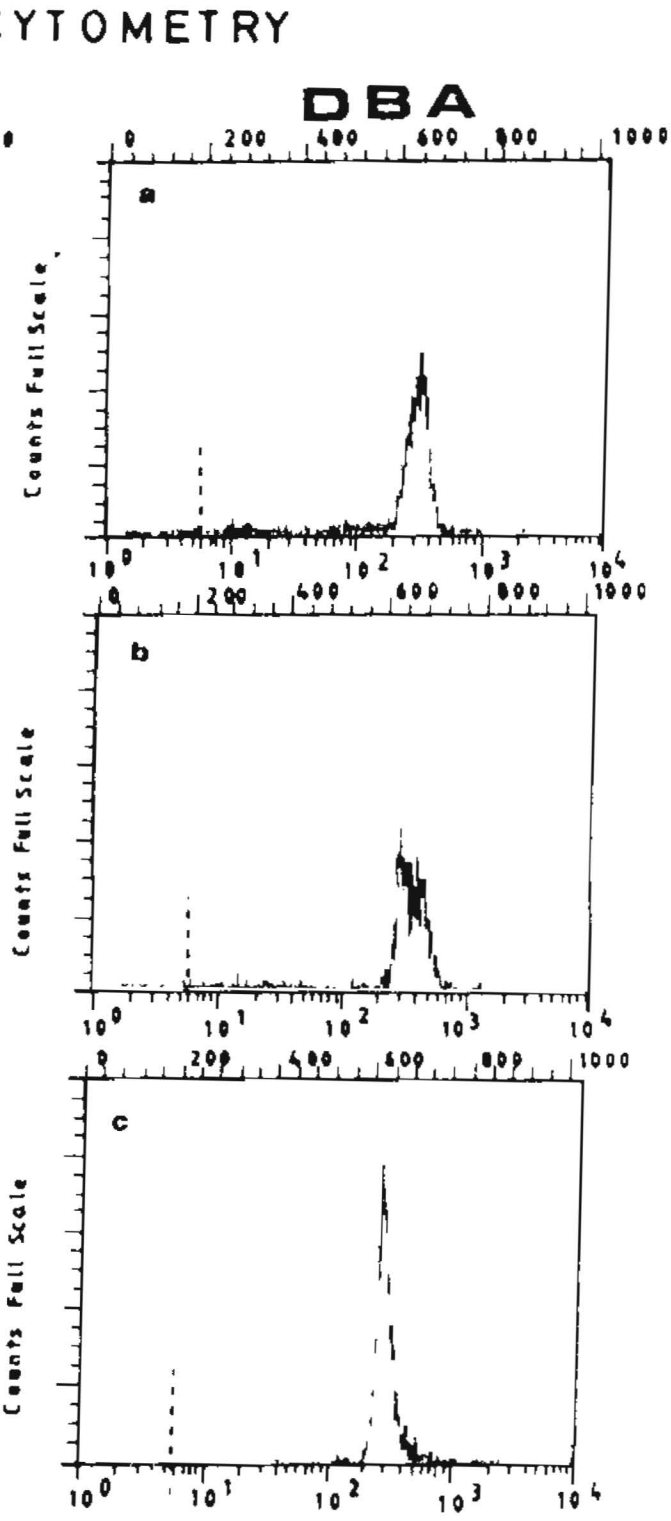

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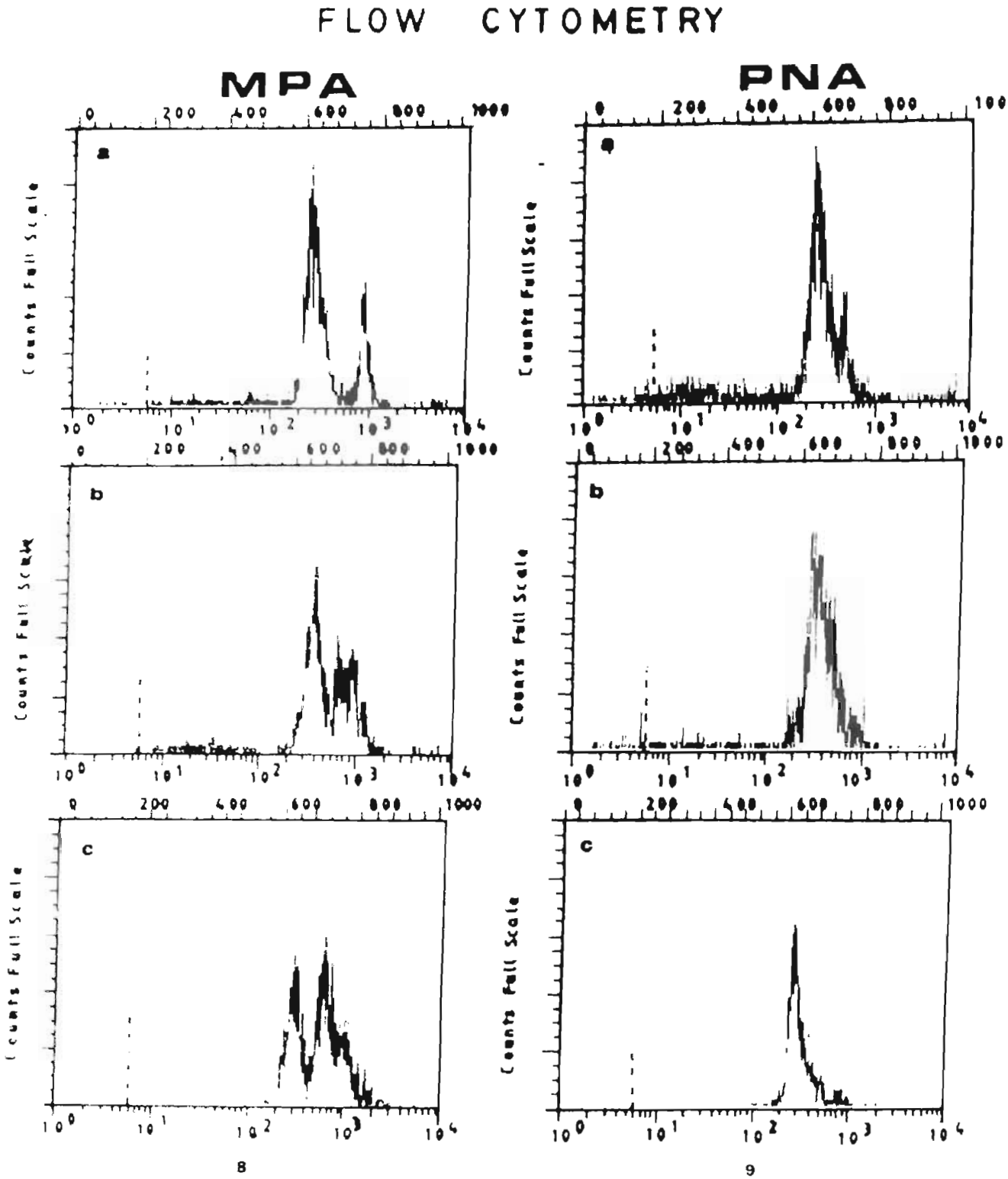
Figure 7



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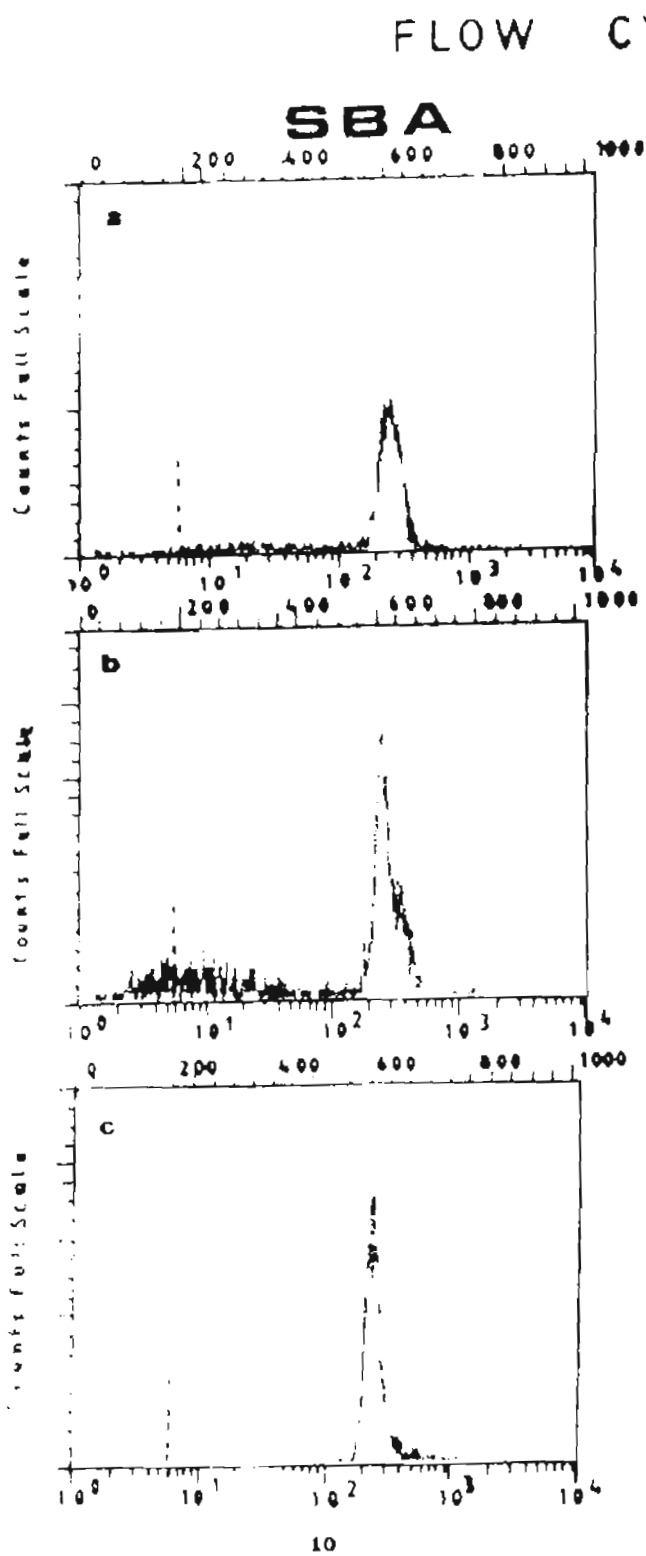

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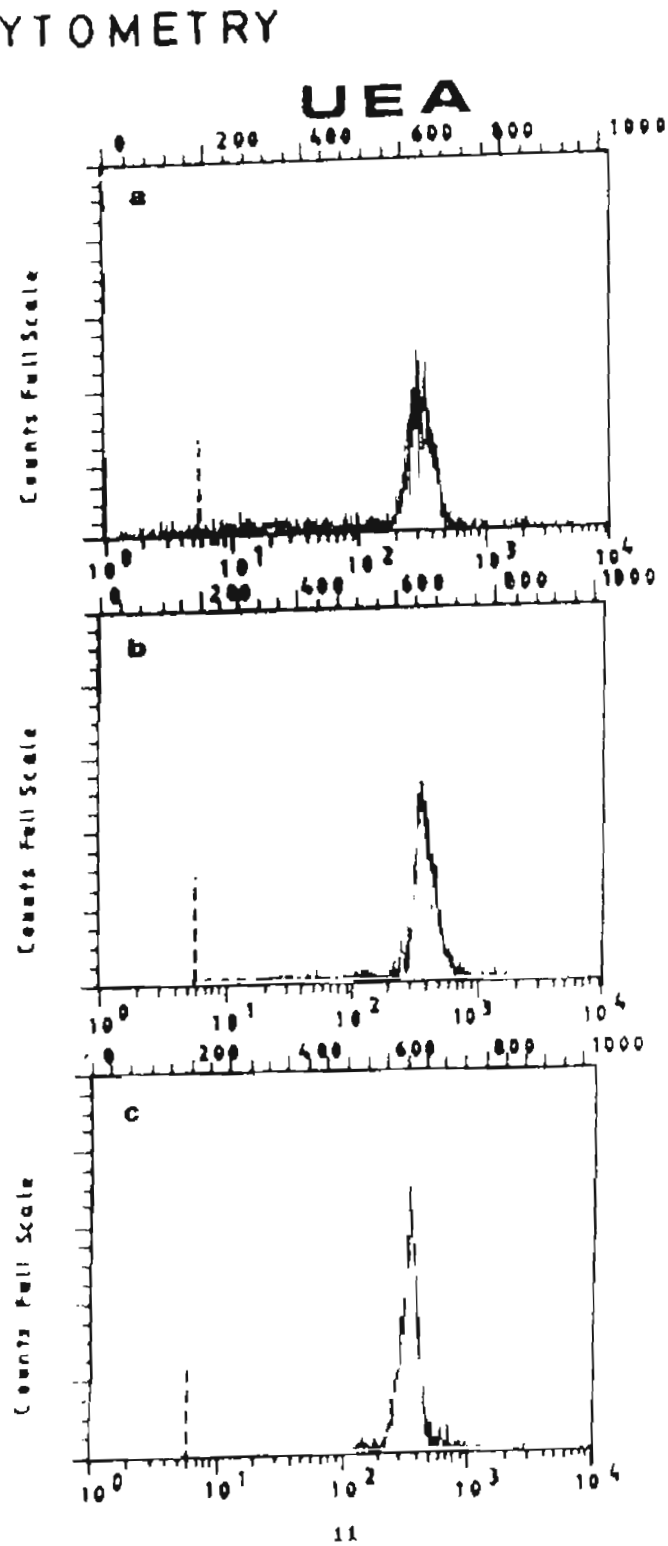

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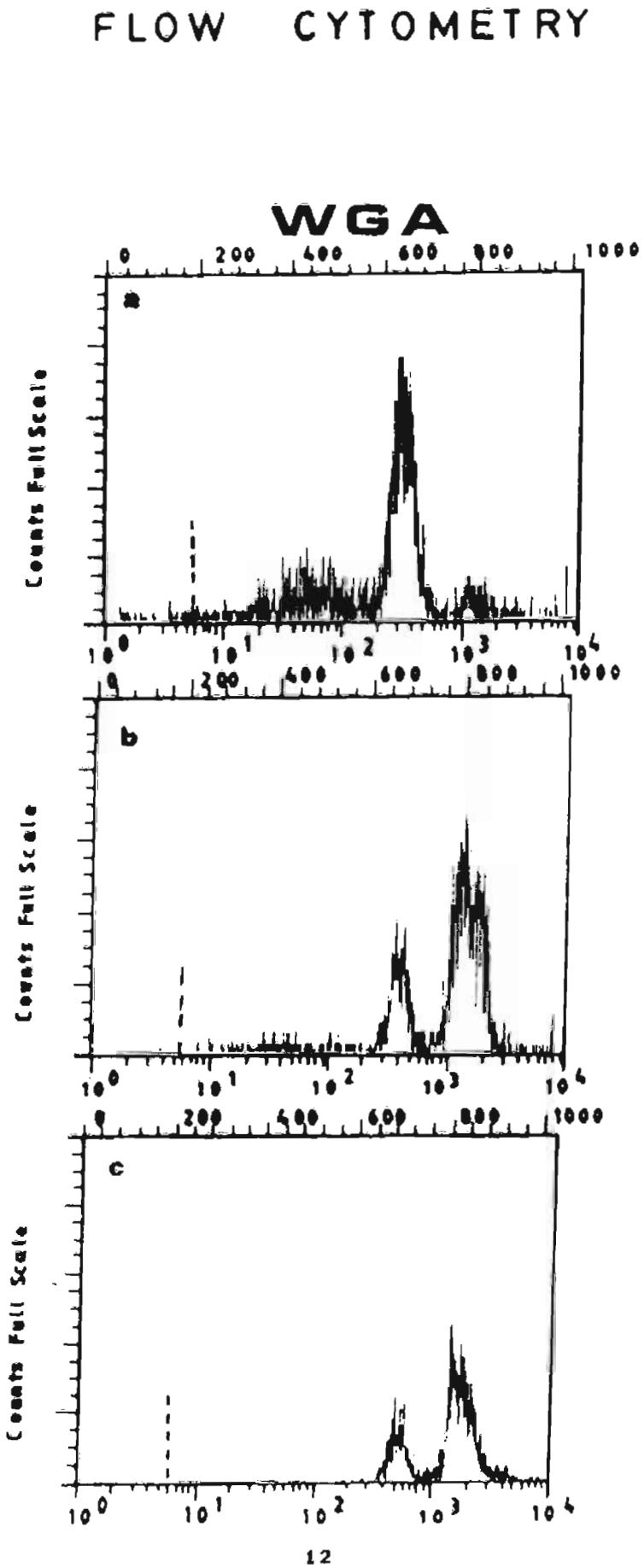


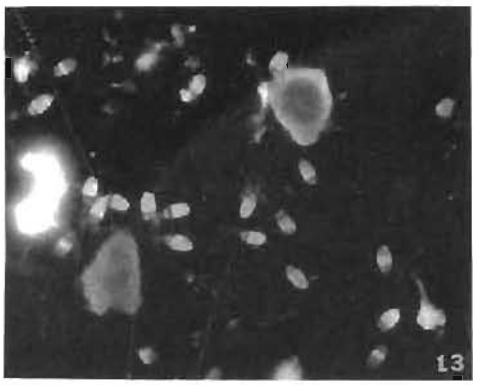
Figure 12

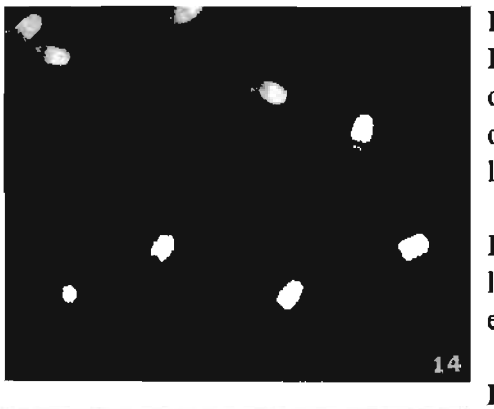
Figures 6-12:

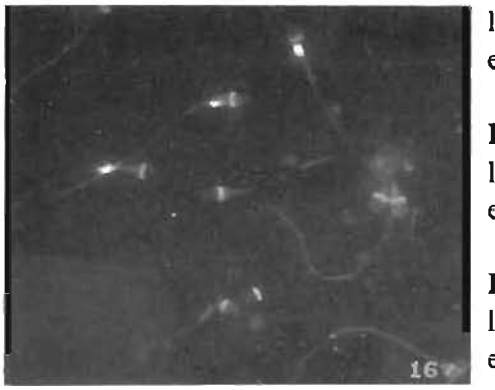
Flow cytometry of lectin binding intensity of the epididymal spermatozoa of cat. The intensity of fluorescence (X axis) has been plotted against the cell count (Y axis). The intensity of fluorescence (F1) is graduated on the upper line as channel numbers in the range of 0 to 1000 and lower line is in logarrithmic scale. Channel numbers between 0 to 200 indicate weak fluorescence.

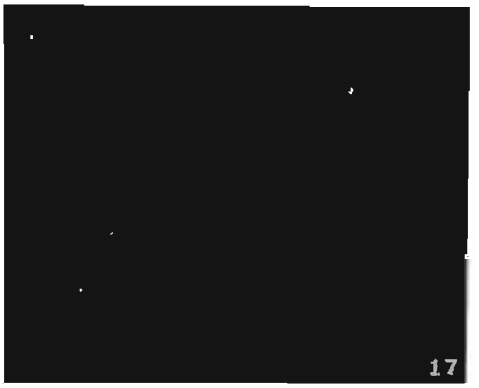
Figure 6: Con A
Figure 7: DBA
Figure 8: MPA
Figure 9: PNA
Figure 10: SBA
Figure 11: UEA
Figure 12: WGA

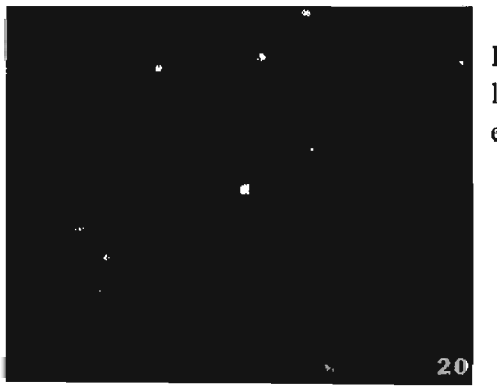
(a) Caput Epididymal spermatozoa.
(b) Corpus epididymal spermatozoa.
(c) Cauda epididymal spermatozoa.











Figures 13-20: Fluorescence micrograph of epididymal spermatozoa of cat labeled with FITC-lectins.

Figure 13: FITC- Con A labeling on the corpus epididymal spermatozoa.

Figure 14: FITC- Con A labeling on the cauda epididymal spermatozoa.

Figure 15: FITC-DBA labeling on the cauda epididymal spermatozoa.

Figure 16: FITC-MPA labeling on the corpus epididymal spermatozoa.

Figure 17: FITC-MPA labeling on the cauda epididymal spermatozoa.

Figure 18: FITC-PNA labeling on the cauda epididymal spermatozoa.

Figure 19: FITC-UEA labeling on the cauda epididymal spermatozoa.

Figure 20: FITC-WGA labeling on the cauda epididymal spermatozoa.

Figures 13- 20

piece of the spermatozoa recovered from the cauda epididymis exhibit maximum fluorescence.

With FITC-UEA, there is no change in the intensity of fluorescence in the caput, corpus and cauda epididymal spermatozoa as analyzed flow cytometrically (**Figs. 11 a-c**). The caput epididymal spermatozoa display moderate staining over the head and weak staining over the tail region. The reactivity over the tail increases as the spermatozoa move to the corpus epididymis. In the cauda epididymal spermatozoa (**Fig. 19**), labeling over the post acrosome is more as compared to the caput and corpus epididymal spermatozoa.

Flow cytometry reveals a gradual decrease in the intensity of WGA fluorescence as the spermatozoa move from the caput to corpus to cauda epididymis (**Fig. 12 a-c**). In the caput epididymal spermatozoa the acrosome is moderately labeled with FITC-WGA (**Fig. 20**). The head and tail of the spermatozoa appears distinct when the spermatozoa reach the corpus epididymis. In the cauda epididymis there is loss of reactivity of the sperm head and only the cytoplasmic droplet remains positively stained in approximately 50% of the spermatozoa.

DISCUSSION

Lectin binding studies in cat epididymal spermatozoa reveal that there is a preponderance of glucose/mannose residues on the surface of the maturing spermatozoa as revealed by Con A binding. This observation is similar to that observed in epididymal spermatozoa of another carnivore, i.e. dog, where ConA receptors are confined to the plasma membrane overlying acrosome [5]. In the epididymal spermatozoa of bull and ejaculated spematozoa of boar and bull most of the lectins viz., ConA, BPA (***Bauhinia purpurea***), MPA, PNA, SBA, DBA, GSI (***Griffonia simplicifolia I***) and WGA appear prominently on the plasma membrane overlying the acrosome excluding the region over the equatorial segment [1, 6, 34]. It appears that conA labels the entire head of the epididymal spermatozoa of cat but it is actually present only on the acrosome. Con A receptors occupy a major portion of the cat sperm head since we find that they are present on the acrosome which constitutes a very large segment of the sperm head. The transmission electron micrographs clearly show the acrosome covering approximately 80% of the nuclear region of the cat spermatazoa [10, 29, 32]. During the passage of the spermatozoa from the caput to the cauda epididymis there is an increase in Con A binding. We find that epididymal pasage of the cat spermatozoa is accompanied by progressive increase of glucose residues on the surface and progressive decrease of its N-acetylated form. An increase in Con A and concomitant decrease of WGA binding has also been reported in the maturing spermatozoa of monkey [11].

A similar increase in the galactose residues labeled with MPA and a decrease in its acetylated and methylated residues labeled by SBA and DBA respectively has been observed in the epididymal spermatozoa of cat as determined flow cytometrically. However, microscopic observations reveal that these residues are located on different regions of the sperm surface. While the galactose residues are exclusively present on the equatorial segment, the acetylated and methylated residues are localized on the sperm tail.The importance of MPA residues in fertilization events has been highlighted by Ahuja (1982). In cat the MPA label is confined to the equatorial segment while in other mammalian species studied, viz., dog, goat and bull, this lectin labels the acrosomal region of the epididymal spermatozoa [4, 5, 6, 34].

UEA an L-fucose specific lectin - displays alterations in its distributions on the surface of the maturing spermatozoa as they move down the epididymis. Although the UEA binding remains unaltered during epididymal transit of the spermatozoa when observed via a flow cytometer, microscopic observations reveal new labeling sites on the post acrosome region and a decrease in the UEA binding over the acrosome of the cauda epididymal spermatozoa.

PNA labeling sites are restricted to the acromosome region of the cauda epididymal spermatozoa in cat. The epididymal spermatozoa of bull and goat [1, 4, 5, 6, 34] also reveal preferential staining of the acrosome with PNA. Unlike other lectins such as ConA and MPA which show progressive increase and DBA, SBA and WGA which show progressive decrease in lectin binding, PNA does not follow an ordered pattern and the corpus epididymal spermatozoa reveal maximal intensity. This feature of lectin binding was earlier reported in goat (Bains et al. 1993a) in which the epididymal spermatozoa do not show an increase or decrease in the lectin binding as they travel from the caput to cauda epididymis.

Rearrangement of lectin binding sites on the surfaces of maturing spermatozoa in other species is not uncommon [4, 5, 11, 21, 24]. There may be many factors influencing the distribution of the glycoproteins on the passage of the spermatozoa in the epididymis. These surface modifications may be due to the interaction of the epididymal milieu with the sperm surface components which may lead to removal, masking or unmasking of preexisting surface components, adsorption and incorporation of macromolecular components by the excurrent duct [8, 26, 27, 38] These factors contribute to allocate various macromolecules to well defined domains of the sperm plasma membrane [14, 28]. It is well established that fucose residues form part of the recognition signal for sperm-zona pellucida attachment in mammals [16]. Ahuja et al. (1982) reported the involvement of other carbohydrates besides fucose to be involved in hamster fertilization. The plasma membrane overlying the equatorial segment is an important locus with regard to the membrane changes induced during capacitation [13, 36]. Since the equatorial segment provides a membrane capable of fusing with egg plasma membrane and it may also be the initial site of sperm-egg interactions, modifications of the molecular make up or distribution of components in this region of the sperm head may be of prime importance in the process of sperm-egg fusion [7].

We find that in cat sugar residues other than fucose were also expressed at the equatorial segment as demonstrated by Con A and MPA labeling of this region. Preponderance of Con A and MPA labeling sites suggest a possible role of these sugar in the gamete binding events in cat. This view is in consonance with the report by (Shalgi et. al 1986) where in sperm-egg interactions are brought about by fucose in concert with other carbohydrates.

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RESUMEN

Se estudió la variación en los residuos de carbohidratos presentes en la superficie de espermatozoides epididimales maduros de gato (***Felis Catus***). Los espermatozoides recuperados del ***caput***, el ***corpus*** y la ***cauda*** epididimales fueron marcados con isotiocianato de fluoresceina (**FITC**) unido a lectinas y examinados con un microscopio de fluorescencia y un citómetro de flujo. Se utilizaron siete lectinas unidas a **FITC:** ***Canavalia ensiformes*** o ***Concanavalina*** (Con A), ***Dolichos biflorus*** (DBA), ***Maclura pomifera*** (MPA), ***Arachis hypogaea*** o ***aglutinina de cacahuete*** (PNA), ***Glyscina max*** o ***aglutinina de soya*** (SBA), ***Ulex europaeus*** (UEA) y ***Triticum vulgaris*** o ***aglutinina de germen de trigo*** (WGA). Encontramos que a medida que los espermatozoides se mueven a través del epididimo hay un incremento en la afinidad de enlace de Con A y MPA y un decremento en el enlace con DBA y SBA. Los residuos α-D-glucosa/α-D-manosa constituyen la mayor por-

ción de la población de estosresiduos en el esperma de gato y se encuentran presente predominantemente la membrana citoplasmástica, cubriendo el acrosoma, tal como se demuestra por el marcaje con Con A. La lectina especifica para N-acetil-galactosa, MPA, marca exclusivamente el segmento ecuatorial. La citometria de flujo indica que no hay cambios en la intensidad del marcaje para UEA. Sin embargo, la observación microscópica revela diferentes alteraciones en los sitios de marcaje.

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