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INTERACTION BETWEEN JACKFRUIT LECTIN AND RAT EPIDIDYMAL SPERM

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ABSTRACT

Changes in the glycoproteins on the surface of rat epididymal sperm during maturation were investigated using Jackfruit lectin. Using the lectin-horse radish peroxidase conjugate, the lectin-binding glycoproteins were found to be localized mainly on the acrosomal region of the immature (caput) sperm. However, the lectin-binding glycoproteins were found to distribute over the whole surface of the mature (cauda) sperm. When the membrane fraction extracted by Triton X-100 was analyzed by SDS-PAGE and the lectin staining, 3 glycoprotein bands (M_r = 127,000, 118,000 and 62,000) were detected for the caput sperm but 4 glycoprotein bands (M_r = 118,000, 108,000, 99,000 and 62,000) were detected for the cauda sperm. Differences were also found in the association constants and the number of binding sites between these two groups of sperm. The data supported the contention that sperm surface underwent changes during epididymal transit (maturation).

INTRODUCTION

A N-acetylgalactosamine-specific lectin, called JFL, has been isolated from Jackfruit (*Artocarpus heterophyllus* or *Artocarpus integrifolia*) seeds[1]. It has been shown possessing numerous interesting immunological properties[2-6]. Many attempts were made to analyse structure[7-9], function[7,10] and carbohydrate specific binding[11,12] of the lectin. It was shown to induce IgE response[13] and bind serum and colostrum IgA[6,14-16]. And because JFL shows precipitating property with some serum proteins, it became an useful tool in isolation of various serum proteins[17,18]. Besides its interesting immunological properties, JFL could also induce sperm agglutination[1]. Our preliminary observation revealed that lectin bound to immature and mature epididymal sperm with different strength. Thus, the comparative studies were performed to establish the differences in the lectin binding. By using JFL and its conjugate with horse radish peroxidase (JFL-HRP), data on the location of JFL-receptor sites on sperm surface, JFL-binding glycoproteins and affinity in binding of JFL to rat caput and cauda epididymal sperm were collected. This paper will present the data and the interpretation.

MATERIALS AND METHODS

1. Preparation of JFL

Fresh Jackfruit seeds were chopped and homogenised in 50 mM phosphate buffer, pH 7.4 containing 0.1 M NaCl (PBS). The supernatant was precipitated with ammonium sulfate and, then affinity isolated through a N-acetylgalactosamine-agarose column as previously described[1].

2. Preparation of JFL-HRP conjugate

JFL and HRP were linked according to the procedure described by O'Sullivan *et al.*[19]. 3.0 mg of HRP was dissolved in 0.3 M sodium bicarbonate buffer and carbohydrate residues were removed by 1% dinitrofluorobenzene (FDNB). Any precipitate formed was removed before incubating HRP with 0.06 M sodium periodate for 30 min. Then 0.06 mM glycerol was added to stop the reaction.

An equal amount of purified JFL in powder form was added to the dialysed peroxidase-aldehyde solution and mixed for 2 hrs. The Schiff-base formation was reduced by the activity of 5 mg sodium borohydride. Separation of JFL-HRP conjugate from free HRP and free JFL was accomplished by chromatography on a column of Sephadex G-200 with PBS as the eluent.

3. Collection and washing of rat sperm

Mature male albino rats (Fisher strain) were used. Rats were anesthesized with ether. The epididymis was surgically removed and dissected free of fat. The epididymis was then divided into the proximal caput and distal cauda. Sperm were obtained by puncturing the epididymal tubule with a hypodermic needle and squeezing the sperm into the PBS. Sperm were then washed twice by centrifugation (2,000 rpm, 5 min) followed by resuspension in PBS. These sperm were immediately used in labelling studies or membrane isolation. Sperm concentration was determined by haemacytometer counts.

4. Isolation of epididymal sperm membrane proteins

Washed epididymal spermatozoa (10^8 cells) were pelleted by centrifugation and the pellet was resuspended in 1 ml of demembranating solution (25 mM Tris-HCl pH 9.0 containing 1% Triton X-100, 0.1 M NaCl and 2 mM dithiotheritol) and incubated at 37 °C for 30 min. The suspension was centrifuged at 2,000 rpm for 5 min and the supernatant was recentrifuged at 15,000 rpm for 10 min.

5. Labelling of rat epididymal sperm with JFL-HRP conjugate

Air-dried preparations were made by spreading 10 μl of sperm suspension over the clean glass slides about 1 cm^2 using a pipette tip leaving at ambient temperature. Non-specific sites were blocked by preincubating the preparations in 0.5% periodate-treated BSA[20] for 30 min. Labelling was accomplished using 20 μl of 50 μg JFL-HRP /ml for 30 min in a humid chamber at 4 °C. After labelling, the preparations were rinsed three times in PBS and stained with an incubating medium containing 3-amino-9-ethylcarbazol as a substrate[21]. Incubation for peroxidase activity lasted for 30 min at 37 °C, the staining was then stopped by washing the slides in distilled water. All slides were mounted with 22x22

mm coverslips using a PBS:glycerol mixture (1:1 v/v). Microscopic examination was performed under a bright-field.

6. Detection of JFL-binding glycoproteins

The electrophoretically separated proteins were transferred from SDS-PAGE to a nitrocellulose sheet as described by Towbin *et al.*[22]. The transfer was accomplished by applying a current of 500 mA for 1 hr. at room temperature. The JFL-binding glycoproteins were then detected followed the published method [20]. The nitrocellulose sheet was saturated with periodate-treated BSA prior to overlayering with JFL-HRP solution (5 μg/ml). The binding was allowed to proceed for 1 hr. at room temperature. Then the JFL-binding glycoproteins were detected by staining for peroxidase activity in 50 mM Tris-HCl pH 7.5 containing 0.5 mg/ml of 3,3'-diaminobenzidine and 0.005% hydrogen peroxide.

7. Quantitative analysis of JFL-binding glycoproteins on sperm membrane

The experiment was performed in 3 sets and each set was done in duplicate. An aliquot of washed sperm suspension was pipetted to a microcentrifuge tube. The sperm pellet obtained after centrifugation was incubated with 1 ml of 0.5% periodate-treated BSA for 1 hr. at room temperature. The BSA solution was removed prior to adding JFL-HRP solution (200 μl) containing various amount of JFL-HRP (0-8 μg). The suspension was further incubated for 1 hr. To separate pellet from the supernatant, the suspension was layered on 10% Ficoll in PBS and centrifuged at 4,000 rpm. for 10 min at 4 °C. The pellet was directly assayed for peroxidase activity and average activity from 3 experiments with S.D. less than ±0.01 was calculated for concentration of bound conjugate. The supernatant could not be assayed directly because of the interference of Ficoll. So, the concentration of free conjugate was the concentration of total conjugate minus with bound conjugate.

8. Peroxidase activity assay

The activity of HRP was determined using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as substrate. An aliquot of supernatant was mixed with 2.0 ml of DAB solution in 50 mM potassium phosphate buffer pH 4.3. The reaction was started by adding 50 μl of 1 mM H_2O_2. The change in absorbance at 465 nm was recorded at various time.

9. Hemagglutination assay

Two-fold serial dilution of the lectin solution (50 μl) was incubated with an equal volume of a 2% rat red blood cell suspension in a 96-wells U-shaped plate at room temperature for 1 hr. The titer was calculated from the highest dilution of lectin showing 100% hemagglutination.

10. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS-PAGE)

Electrophoresis of proteins was performed in the presence of 0.1% SDS under reducing condition on 4-15% gels according to Laemmli's method[23].

11. Protein determination

Protein concentration was determined by the method of Lowry[24] using bovine serum albumin as standard.

RESULTS

1. Labelling patterns of the caput and the cauda epididymal sperm

To determine the lectin labelling patterns of the cell, sperm treated with JFL-HRP were examined under a microscope after being fixed by air-drying. Different patterns of color complex deposition were seen between the caput the and cauda sperm. A very intense color staining of JFL-HRP was found only in the acrosomal region of the caput sperm (Fig. 1A). However, the color staining was seen on all regions of the cauda sperm surface: head, midpiece and tail (Fig. 1B). A less intense color staining was observed on the acrosomal region of the cauda sperm in comparison with that on the caput sperm (Fig. 1). Binding of the JFL-HRP was prevented in the presence of 0.2 M D-galactose, a competitive sugar[1] (data not shown). Using HRP (20 μg/ml) instead of JFL-HRP, no color staining was seen on either sperm (data not shown).

2. JFL-binding glycoproteins on the caput and the cauda epididymal sperm membrane

The membrane fragments prepared by extraction with a non-ionic detergent, Triton X-100, were found to contain a number of polypeptide bands on SDS-PAGE stained for protein with Coomassie Brilliant Blue R (data not shown). These proteins exhibited the molecular weights ranging from 12,000 to 70,000 which corresponded to those in other report[28]. Using JFL-HRP, different JFL-binding glycoproteins were found in the extracts of the cauda and the caput sperm. While the extract of the cauda epididymal sperm revealed 4 bands with Mr of 118,000, 108,000, 99,000 and 62,000, only 3 bands with Mr of 127,000, 118,000 and 62,000 were detected in detergent extract of the caput epididymal sperm (Fig. 2). No JFL-HRP binding band could be detected in the presence of 0.2 M D-galactose or HRP solution (2 μg/ml) (data not shown).

3. Quantitative analysis of the JFL binding on rat sperm

Our preliminary experiments suggested that JFL agglutinated rat epididymal sperm from the caput and the cauda regions with different strength. Using micro-agglutination technique, we found that the minimum concentration of JFL required for aggregation of the caput sperm was 1 μg/ml while the aggregation of cauda sperm required 4 μg/ml of JFL as the minimum concentration.

To examine JFL-binding affinity to the rat epididymal sperm, a suitable incubation time required for the maximum binding was first established. JFL-HRP was used instead of JFL so that quantitation of the bound lectin can be followed by measuring HRP activity bound. The binding of JFL-HRP to the cauda sperm increased with increasing incubation time and reached a saturation within 20 min whereas the binding to caput sperm was almost constant within 5 min (data not shown). Thus, the incubation time of 30 min was used to study the binding of JFL-HRP to the sperms.

From the binding assay performed as described in the materials and methods, the association constant (K), and the number of receptor sites per cell (n) were calculated according to Steck and Wallach[25]. If r = [LS]/[C] is the ratio of concentration of bound conjugate [LS] divided by the concentration of sperm [C], then at any free conjugate concentration, [L],

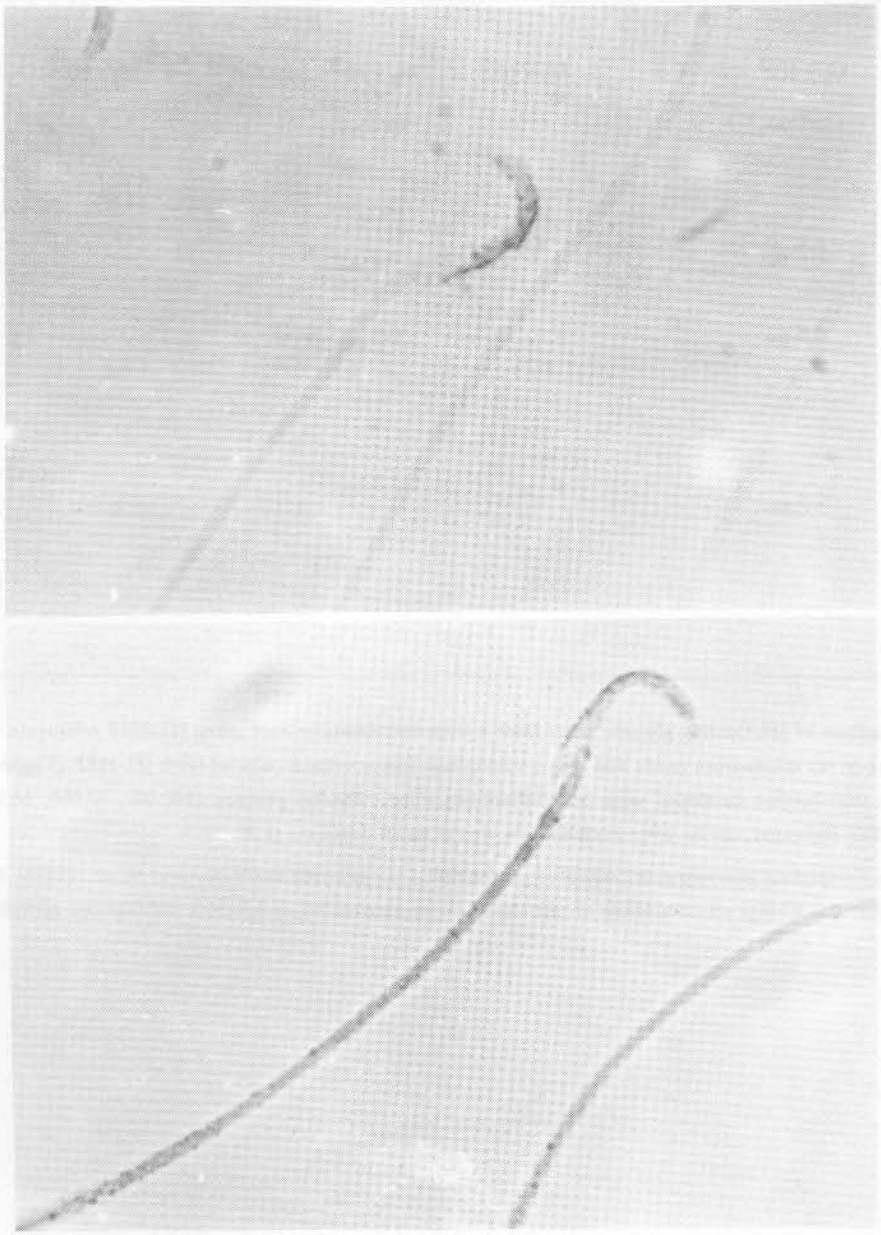

Fig. 1. Binding of JFL to sperm isolated from caput (A) and cauda (B) regions.

Rat sperm were isolated from the epididymis and processed for labelling with JFL-HRP. The distribution of JFL-HRP was examined under bright-field microscopy (x100). The labelling is very intense in the acrosomal region.

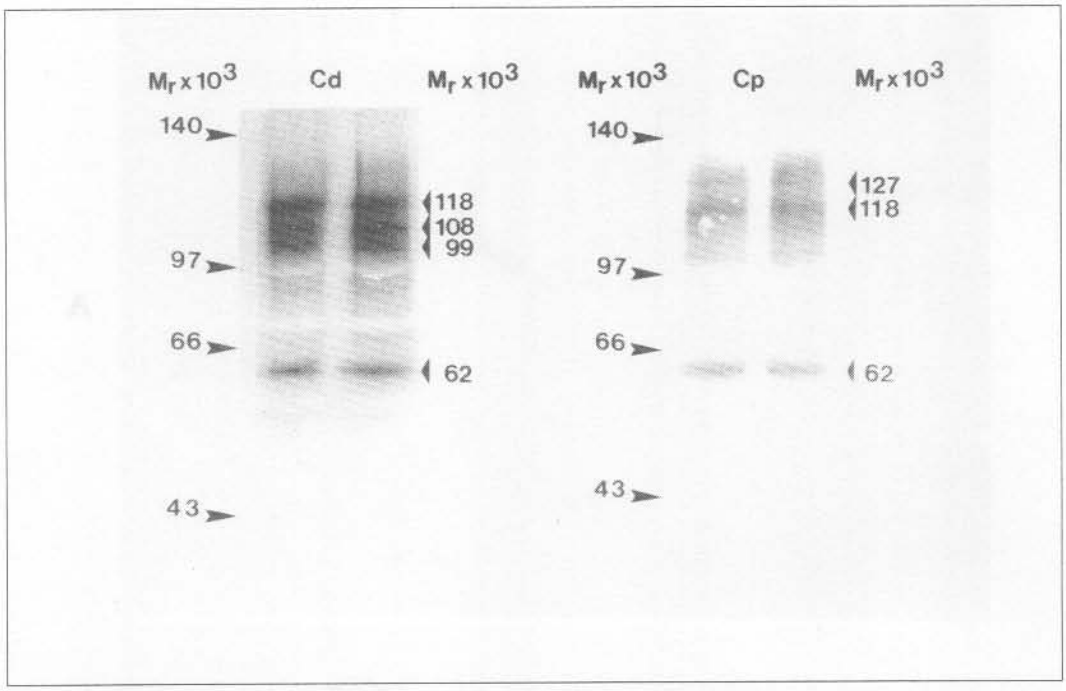

Fig. 2. Detection of JFL-binding glycoproteins from sperm membrane extract using JFL-HRP conjugate. Protein blots of rat sperm from cauda and caput epididymal region were incubated with JFL-HRP (5 μg/ml). The blot was further developed with 3,3'-diaminobenzidine. Marker proteins (140.000, 97,000, 66,000 and 43,000) indicated on the left. Each sample was done in duplicate (1,2).

The JFL-binding glycoproteins (indicated on the right) of the cauda sperm showed M_r of 118,000, 108,000, 99,000 and 62,000 whereas those of the caput sperm showed M_r of 127,000, 118,000 and 62,000.

$$1/r = (1/Kn \cdot 1/[L]) + 1/n \quad \text{.....(1)}$$

Using regression analysis, the straight line was obtained when plotting 1/r against 1/[L] (Fig. 3). By extrapolating to 1/r = 0, the intercept on the horizontal axis of the plot = -K. The K values for JFL-HRP binding were found to be 0.04 μg^{-1} and 0.14 μg^{-1} for the caput sperm and the cauda sperm respectively. The n values were determined from the intercept on the vertical axis where 1/[L] = 0 and 1/r = 1/n. From the extrapolation, the n values were found to be 1.8×10^{-8} μg/cell for the caput sperm and 4.3×10^{-8} μg/cell for the cauda sperm.

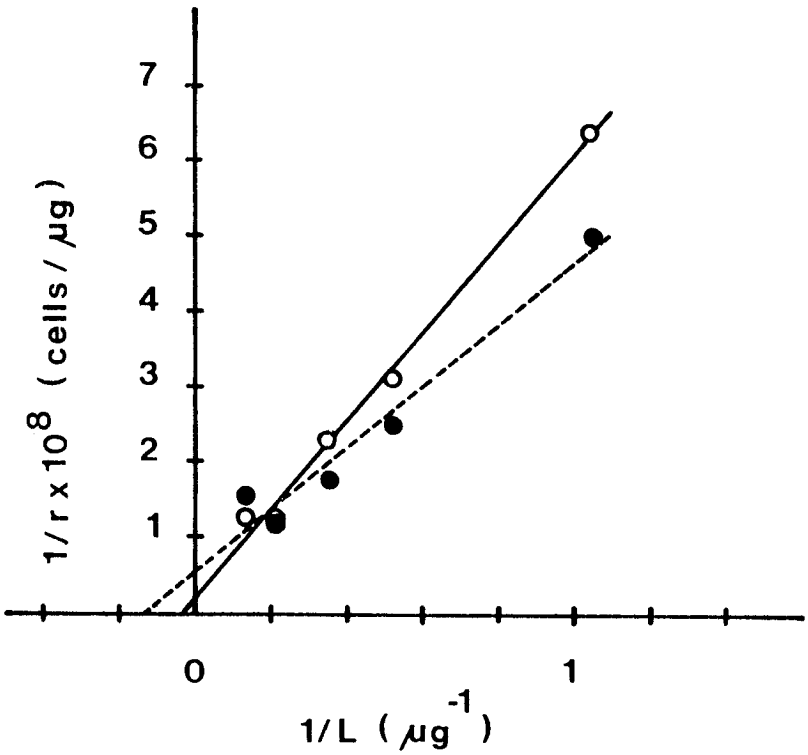

Fig. 3. The determination of K and n for the binding between JFL-HRP and the rat epididymal caput (o—o) and the cauda (●—●) sperm reaction. Rat sperm (2.5×10^6 cells) were incubated with various amount of JFL-HRP in fixed volume (200 µl) of solution for 30 min at room temperature. Pellet obtained by centrifugation through 10% Ficoll were assayed for peroxidase activity to determine [LS]. Data came from 3 sets experiment as described in the Materials and Methods. The straight lines were plotted by using regression analysis.

$$1/[L] = 1/[L_T] - [LS] \; ; \; 1/r = [C]/[LS]$$

K = -1/[L] when 1/r = 0 ; 1/n = 1/r when 1/[L] = 0
[L] = concentration of free conjugate
[LT] = concentration of total conjugate
[LS] = concentration of bound conjugate
[C] = concentration of sperm

DISCUSSION

JFL has been shown to have a sperm-agglutinating activity[1]. The experiments in this paper have further revealed the differences in the localization (Fig. 1), the glycoproteins responsible for the JFL binding (Fig. 2) and the binding affinity (Fig. 3) between the cauda and the caput epididymal sperm. The sperm acrosomal region has been previously reported to bind other lectins like PNA[26] and ConA[27]. So it is not surprising that JFL also binds to the acrosomal region (Fig. 1). Besides the acrosome, the receptor sites of JFL were also found on the body and tail surface of the cauda epididymal spermatozoa but not with the caput sperm. The JFL-binding glycoproteins on both the cauda and the caput epididymal sperm appear to be the intrinsic components of sperm membrane since they are extractable by Triton X-100 which is used to remove membrane proteins of various spermatozoa[28]. They are not extracted by a high ionic strength solution (data not shown) which is used to remove the extrinsic proteins from rat sperm membrane[29].

The change in the localization of the JFL-receptor sites from the acrosomal region in the caput epididymal sperm to a more spread-out distribution of the sites all over the whole surface of the cauda epididymal sperm could be due to 3 mechanisms. Firstly, the JFL-binding glycoproteins may move from the acrosomal region to other parts of the body and the tail surface. Secondly, new JFL-binding glycoproteins in the body and the tail region became exposed after loss of absorbed proteins. Thirdly, new JFL-binding glycoproteins on the body and the tail surface may be absorbed and ankered to the sperm membrane during epididymal transit. Our data do not allow us to eliminate any of the 3 mechanisms. By any mechanism, our data further support the contention that sperm surface will be modified during epididymal transit which is necessary to transfer the immature caput sperm to the mature cauda sperm possessing the fertilizing capacity.

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บทคัดย่อ

ผลการตรวจสอบการเปลี่ยนแปลงของไกลโคโปรตีนบนผิวตัวอสุจิของหนูด้วยเลคตินจากเมล็ดขนุน โดยใช้คอนจูเกตระหว่างเลคตินกับเอนไซม์เปอร์ออกซิเดส เป็นเครื่องมือในการตรวจสอบ พบไกลโคโปรตีนที่เลคตินจับได้อย่างหนาแน่นบริเวณผิวเยื่อหุ้มส่วนอะโครโซมของตัวอสุจิที่ไม่เจริญตัว และพบบนผิวเยื่อหุ้มโดยทั่วไปของตัวอสุจิหนูที่เจริญตัวแล้ว เมื่อทำการวิเคราะห์เยื่อหุ้มตัวอสุจิที่ได้จากการสกัดด้วย Triton X-100 โดยวิธี SDS-PAGE และ lectin staining พบความแตกต่างของแถบไกลโคโปรตีนที่สามารถจับกับเลคตินได้ โดยพบแถบไกลโคโปรตีนดังกล่าวจำนวน 3 แถบ ขนาดน้ำหนักโมเลกุล 127,000, 118,000 และ 62,000 ในเยื่อหุ้มที่สกัดจากตัวอสุจิที่ไม่เจริญตัว ในขณะที่พบแถบไกลโคโปรตีนดังกล่าวจำนวน 4 แถบ ขนาดน้ำหนักโมเลกุล 118,000, 108,000, 99,000 และ 62,000 ในเยื่อหุ้มที่สกัดจากตัวอสุจิที่เจริญตัวแล้ว นอกจากนี้ ยังพบความแตกต่างของค่าคงที่ของการจับกันระหว่างเลคตินกับบริเวณจับ (K) และจำนวนบริเวณจับของเลคติน (n) บนผิวของตัวอสุจิทั้งสองกลุ่ม ข้อมูลที่ได้สนับสนุนการเปลี่ยนแปลงของผิวเยื่อหุ้มตัวอสุจิที่เกิดขึ้นระหว่างเกิดกระบวนการเจริญของตัวอสุจิในท่อเอพิดิไดมิส