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Identification of a Sperm Cell Attribute Responsible for Subfertility of Roosters Homozygous for the Rose Comb Allele[1]

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ABSTRACT

Fertility, sperm metabolism, sperm filling of oviductal sperm storage tubules (SST), and sperm motility were compared in subfertile roosters homozygous for the rose comb allele (*R/R*) and fertile controls (*r/r* or *R/r* males). As expected, fertility of *R/R* males was less than that of controls ($p < 0.0001$). The metabolic rate of spermatozoa from *R/R* males was also lower than that of controls ($p < 0.05$). Likewise, filling of SST in vivo was lower ($p < 0.0001$) for spermatozoa from *R/R* males than for control spermatozoa. However, in vitro filling of SST was not different between genotypes ($p > 0.05$). Motility of spermatozoa from *R/R* males was less than that of controls ($p < 0.001$) as determined by an objective spectrophotometric assay. Previous researchers have concluded that subfertility associated with homozygosity for the rose comb allele is attributable to a sperm-specific phenomenon. However, the cellular mechanism(s) responsible for the subfertile status were not defined. In contrast to previous researchers, we have demonstrated that subfertility of *R/R* males is explicable in terms of reduced sperm transport through the hen's vagina; reduced sperm motility appears to be the major contributing factor.

INTRODUCTION

In 1951, Cochez [1] reported that roosters homozygous for the rose comb allele (*R/R*) were subfertile. Subfertility was reported by several groups and determined to be a sperm-specific phenomenon [2–4]. Thereafter, sperm motility was implicated as a basis for subfertility. Sperm motility is essential for passage through the hen's vagina [5]. Poor sperm motility for *R/R* roosters was reported by Petitjean and Cochez [6]; however, Crawford and Smyth [7], Petitjean [8], and Petitjean and Servouse [9] reported normal motility for sperm from homozygous rose comb roosters. These conflicting observations may have been due to subjective estimates of sperm motility.

In contrast to subfertility following intravaginal insemination, intramagnal insemination of sperm from *R/R* males resulted in fertility equivalent to that of fertile males [10, 11]; consequently, the authors inferred that subfertility following intravaginal insemination was due to insufficient filling of the sperm storage tubules (SST). Maximal filling of SST occurs during the first 24–48 h after insemination and is essential for the series of fertilized eggs that typically follows a single insemination [12]. In previous research, neither an objective determination of motility nor an estimation of SST filling was made for sperm from *R/R* males.

Sperm metabolism also has been suspect. Buckland et al. [13] associated homozygosity for the rose comb allele with decreased spermatozoal fumarase, aconitase, and isocitric dehydrogenase activities. Fumarase activity was significantly correlated with fertility. However, Petitjean and Servouse [9] did not observe a difference in spermatozoal fumarase activity from subfertile *R/R* and fertile males. Likewise, estimates of sperm metabolism also have been contradictory. For example, neither Crawford and Smyth [7] nor Petitjean [8] observed a difference in sperm metabolism between subfertile *R/R* and fertile males. However, studies in our laboratory have indicated otherwise [11, 14].

The objective of the present work was to provide a definitive explanation for the subfertility associated with roosters homozygous for the rose comb allele. The present research was designed to do the following: 1) compare sperm filling of SST following intravaginal insemination of sperm from *R/R* and fertile males, 2) compare sperm filling of SST following incubation of oviduct explants with sperm from *R/R* and fertile males, and 3) compare spermatozoal motility from *R/R* and fertile males by an objective method. Provided that reduced sperm metabolism would be observed as reported previously [11, 14], we believed these objectives would enable us to definitively identify a sperm cell attribute that would account for subfertility in *R/R* males.

MATERIALS AND METHODS

Evaluation of Fertility

In order to establish homozygosity for the rose comb allele (*R/R*), Silver Laced Wyandotte roosters were bred to Single Comb White Leghorn (SCWL) hens (*r/r*), and the comb phenotype of progeny was determined. Roosters were designated homozygous if they sired only rose comb chicks (n = 25 to 40 chicks per male). New Hampshire roosters (*r/r*) of equivalent age were used as controls. Groups of 10 roosters per genotype were maintained under identical conditions.

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Fertility of individual roosters was determined in order to verify a difference between genotypes. Sperm concentration was determined according to Bilgili and Renden [15] for each male. Semen was diluted to 2×10^9 spermatozoa per milliliter with Beltsville Poultry Semen Extender (BPSE) prepared at our facility according to Sexton and Fewlass [16]. Twelve hens per male were inseminated intravaginally with 1×10^8 sperm per hen. Eggs were collected throughout a 21-day period and set for incubation twice weekly. Fertility was determined by breaking eggs open after 4 days of incubation and then examining the contents for embryonic development. Three replicate trials were performed per male. Fertility was analyzed as described by Kirby and Froman [17]. On the basis of fertility, representative roosters (n = 3 per genotype) were selected for subsequent evaluation of sperm metabolism, SST filling, and motility.

Evaluation of Sperm Metabolism

Metabolism was determined by a modification of the technique of Chaudhuri and Wishart [18]. Semen was pooled according to genotype. Each semen sample was diluted to 5×10^8 spermatozoa per milliliter in (*N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) buffer containing 111 mM NaCl, 25 mM glucose, and 4 mM $CaCl_2$, pH 7.4. A 200-µl volume of sperm suspension was added to 1.0 ml of reaction medium prewarmed to 41°C in a culture tube. The reaction medium contained 200 µM 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride (INT), 9.2 µM phenazine methosulphate, and 2.4 mM KCN in the buffer described above. Reaction mixtures, in triplicate, were incubated at 41°C for 1, 3, 5, or 7 min. Reduction of INT to formazan was terminated by the addition of 200 µl of 0.1 M HCl containing 5% (v/v) Triton X-100 (Sigma Chemical Co., St. Louis, MO). Culture tubes were placed in a boiling water bath for 30 sec and then centrifuged at $700 \times g$ for 10 min. Absorbance of the supernatant was measured at 520 nm. Data were evaluated by the method of least squares.

Evaluation of SST Filling In Vivo

Semen was pooled according to genotype. Semen was diluted with BPSE to 2×10^9 spermatozoa per milliliter. Each hen was inseminated intravaginally with 1×10^8 in a volume of 50 µl. Hens were killed 16–18 h after insemination, and oviducts were removed. Oviducts were split longitudinally, and tissue containing the SST was located by the technique of Bakst [19]. Tissue containing SST was excised and weighed. Tissue explants were mixed with 10 ml homogenization buffer per gram of tissue. The buffer contained 0.05% (v/v) Triton X-100 in 0.9% (w/v) NaCl. The tissue was homogenized for 3 min in a 25-ml stainless steel microcontainer (Eberbach Corp., Ann Arbor, MI). Formaldehyde, 37% (v/v), was added to bring the final formaldehyde concentration to 0.5% (v/v). This solution was homogenized for 1 min and then stored overnight at 4°C in a 10-ml Erlenmeyer flask. Sperm nuclei were counted with a hemacytometer. Six replicate counts were made per sample. The data were analyzed by the Kruskal-Wallis nonparametric test [20].

Evaluation of SST Filling In Vitro

Tissue explants were procured and weighed as above. Each explant was halved and incubated either with sperm from *R/R* or *r/r* males in a 25-ml Nalgene Erlenmeyer flask. Incubation conditions were based on Nash et al. [21], Bakst [22], and Steele and Wishart [23]. Incubation was performed at 41°C for 2 h in a humidified chamber filled with air containing 5% CO_2. Additionally, flasks were rotated at 55 rpm. The incubation medium was Eagle's Minimum Essential Medium (MEM; Sigma) prepared as described by Howarth [24]. Pooled semen from each genotype was diluted beforehand with BPSE to 2×10^9 sperm per milliliter. A volume of sperm suspension was added to the MEM so that the final concentration was 2.5×10^7 sperm per milliliter. MEM volume was based upon tissue weight: 4 ml MEM per gram explant. After incubation, explants were washed in excess isotonic saline for 3 min in order to remove sperm associated with the mucosal surface. Washed explants were homogenized, and spermatozoal nuclei were counted as described above. Data were analyzed by paired comparison [20].

Evaluation of Sperm Motility

Sperm motility was measured by using a modification of a swim-up technique [25]. Semen was pooled according to genotype. Spermatozoal concentration and viability were determined according to Bilgili and Renden [15]. Semen was diluted to 5×10^8 spermatozoa/ml with 50 mM TES buffer, pH 7.4, containing 111 mM NaCl, 25 mM glucose, and 4 mM $CaCl_2$. A 150-µl volume of sperm suspension was layered upon 1.5 ml 6% (w/v) Accudenz (Accurate Chemical & Scientific Corporation, Westbury, NY) solution prewarmed to 41°C in a disposable cuvette. The cuvette was incubated for 5 min in a 41°C water bath. Absorbance was measured at 550 nm 1 min after the incubated cuvette was loaded into a spectrophotometer (DU-640, Beckman Instruments, Fullerton, CA).

Each representative rooster used in the experiments described above was bred to 10 heterozygous rose comb hens (*R/r*). At sexual maturity, male progeny were bred to SCWL hens in order to establish homo- or heterozygosity for the rose comb allele (5 SCWL hens per male). Sperm motility was compared in homozygous (n = 29) and heterozygous (n = 29) rose comb males. Data were analyzed by single classification analysis of variance with the general linear model of SAS [26].

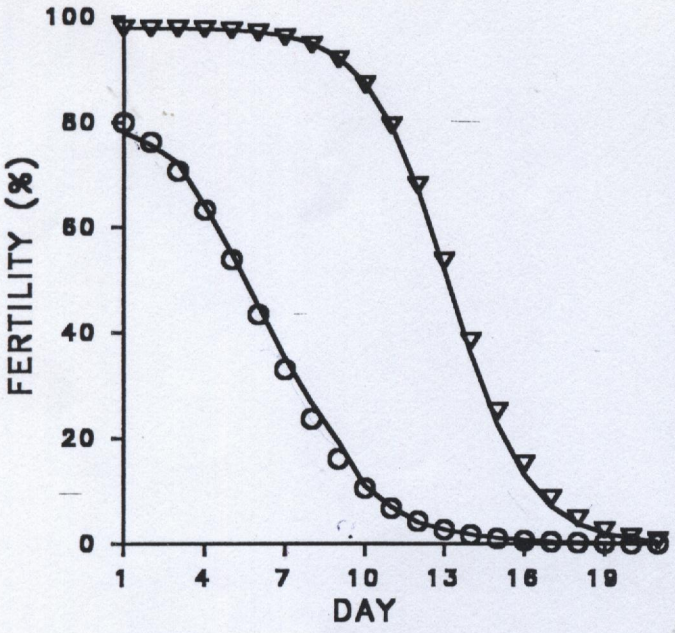

FIG. 1. Duration of fertility after a single insemination of SCWL hens with sperm from *R/R* male (circles) or *r/r* male (triangles). Solid lines represent functions $y(x) = [82.9]/[1 + e^{-.443(7.3-x)}]$, and $y(x) = [97.8]/[1 + e^{-.664(13.2-x)}]$, in which 7.3 and 13.2 are estimates of parameter τ, or time of half-maximal fertility. Each hen (n = 36 per treatment group) was inseminated with 1×10^8 sperm. There was a significant difference ($p < 0.0001$) in τ between *R/R* and *r/r* males.

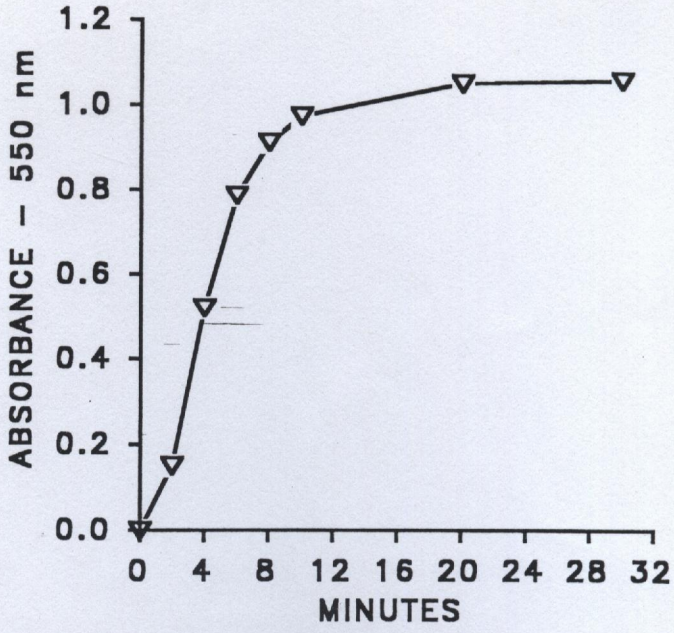

FIG. 3. Absorbance of 6% (w/v) Accudenz solution through time following an overlay of sperm suspension. The cuvette contained 1.5 ml of Accudenz prewarmed to 41°C. After a 0.150-ml volume sperm suspension (5×10^8 sperm/ml) was layered on Accudenz and an initial reading was taken, the cuvette was maintained at 41°C in a water bath. Motile sperm penetrate Accudenz layer, immotile sperm do not.

RESULTS

As shown in Figure 1, Silver Lace Wyandotte roosters homozygous for the rose comb allele were subfertile relative to controls. Both initial fertility and duration of fertility were reduced in each replicate trial. These observations were consonant with previous research [4]. Likewise, sperm from the homozygous rose comb roosters were characterized by a metabolic rate that was less than that of the controls ($p < 0.05$). As shown in Figure 2, the difference in formazan production observed for subfertile and fertile roosters was comparable to that observed previously [11, 14].

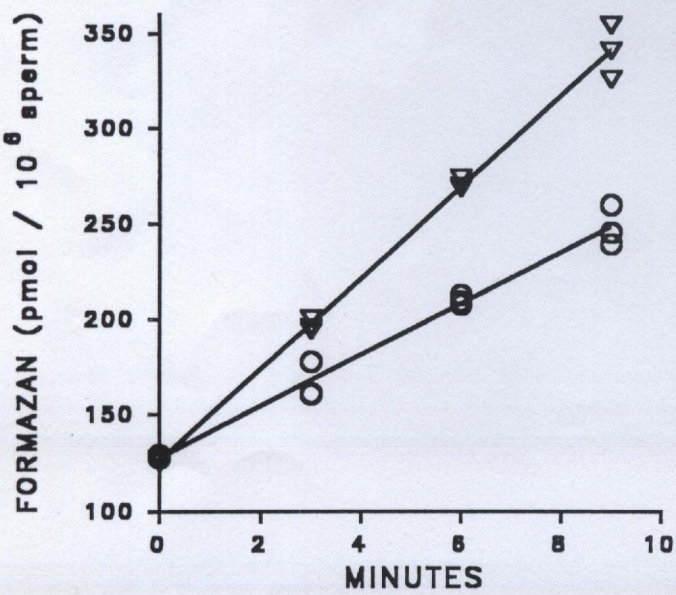

FIG. 2. Formazan production by sperm from *R/R* males (circles) and *r/r* males (triangles) incubated at 41°C. Solid lines represent 1) functions of *R/R* males: $y(x) = 127.68 + 13.41(x)$, $R^2 = 0.99$; and 2) functions of *r/r* males: $y(x) = 126.13 + 23.83(x)$, $R^2 = 0.99$.

At 16–18 h after intravaginal insemination, SST contained an average 2.9×10^6 sperm in the case of fertile males (Table 1). In contrast, SST from hens inseminated with sperm from *R/R* males contained only 41% ($p < 0.0001$) as many sperm (Table 1). However, when oviduct explants were incubated with sperm, there was no difference in SST filling between genotypes (Table 2).

Preliminary research demonstrated that sperm motility was essential for sperm penetration into 6% (w/v) Accudenz. Sperm immobilized by heat denaturation at 56°C did not penetrate. When a sperm suspension was overlaid on the Accudenz and the absorbance of the Accudenz layer

TABLE 1. Spermatozoa from *R/R* and *r/r* roosters present in SST after artificial insemination.

Genotype	Hens (n)	Spermatozoa in SST* ($\times 10^6$)
R/R	17	1.207 ± 0.539^a
r/r	17	2.934 ± 0.1811^b

*Each value is a mean ± SEM.
[a,b]Means differed at $p < 0.0001$.

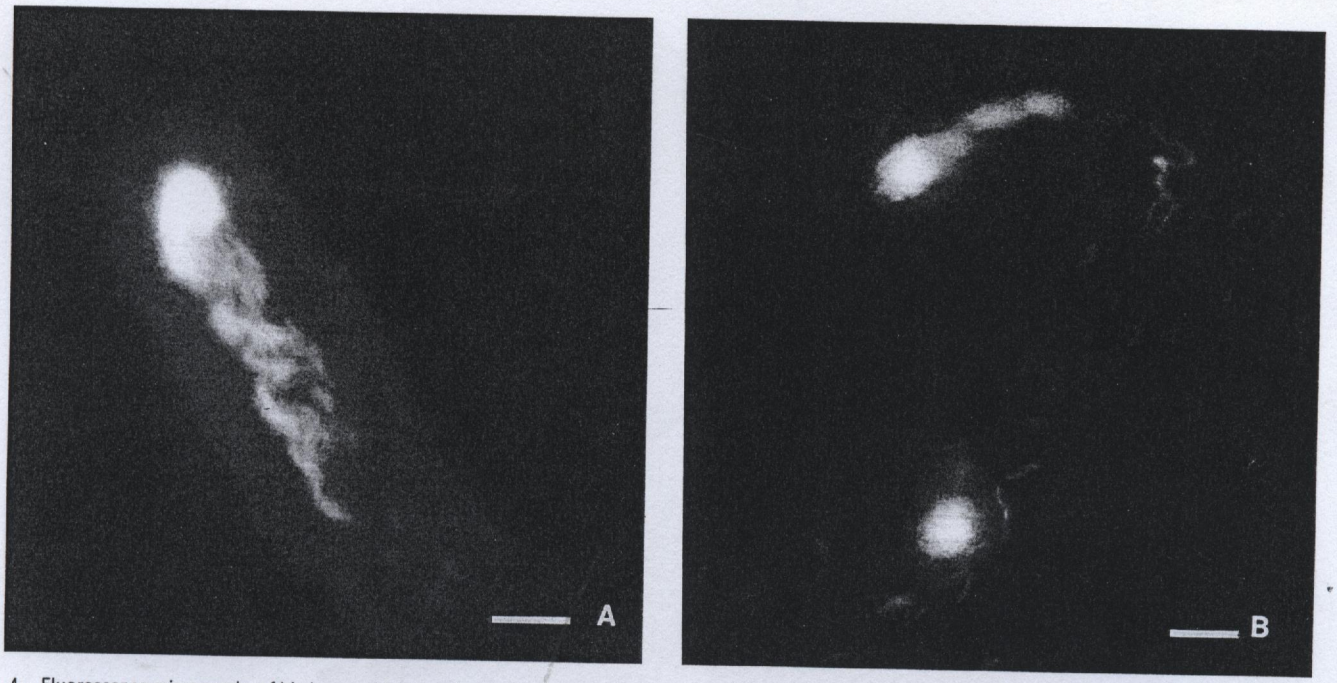

Fig. 4. Fluorescence micrographs of bis-benzamide-labeled sperm associated with SST. **A)** SST from a hen inseminated intravaginally with 1×10^8 sperm and killed 18 h after artificial insemination. Note fluorescence of sperm nuclei clustered at the distal end and scattered throughout the lumen of SST. Scale bar = 25 μm. **B)** SST from oviduct explant incubated for 2 h at 41°C with labeled sperm. Sperm nuclei are located at the distal end of both SST similar to in vivo results in **A**. Single sperm can be localized in the lumen of SST with addition of white light. Scale bar = 25 μm.

was measured as a function of time, data points approximated a logistic function (Fig. 3). The greatest change in absorbance occurred between 2 and 8 min of incubation. Therefore, an incubation interval of 5 min was chosen for subsequent motility measurements.

As shown in Table 3, the mean absorbance value of samples from *R/R* males was 67% of controls. Thus, the motility of sperm from *R/R* males was less than that of the fertile controls.

DISCUSSION

The cellular basis for the subfertility of roosters homozygous for the rose comb allele has perplexed researchers since the 1950s (see [11] for review). Previous research, when viewed collectively, has generated contradictory results. Nonetheless, sperm motility has been implicated by several groups [6, 11] but never objectively measured. Chaudhuri and Wishart [18] correlated sperm metabolic activity with sperm motility in normal roosters. Kirby et al. [11] reported that sperm from *R/R* roosters were characterized by reduced metabolic rate and inferred poor sperm motility. Suboptimal filling of SST by sperm from *R/R* males also was inferred from fertility data [11], but, as with sperm motility, objective measurements were not made.

Filling of SST by spermatozoa is paramount for fertility in poultry [12]. However, only about 1% of inseminated sperm from fertile roosters reach the SST [27]. In this study, sperm from fertile control roosters were recovered from the SST at a rate of 2.9% after artificial insemination, comparable to previously published values [27]. We attribute our slightly higher value to differences among breeds of roosters and hens [28]. In contrast, the recovery rate for sperm from *R/R* males was only 41% ($p < 0.0001$) of that observed for fertile control sperm. We inferred that sperm from *R/R* males had a reduced ability to traverse the vagina to reach the SST. We hypothesized that poor sperm motility accounted for suboptimal SST filling.

TABLE 2. Spermatozoa from *R/R* and *r/r* roosters present in SST after incubation in vitro.

Genotype	Hens (n)	Spermatozoa in SST* ($\times 10^6$)
R/R	20	2.32 ± 0.305
r/r	20	2.27 ± 0.285

*Each value is a mean ± SEM.

TABLE 3. Results of the evaluation of sperm motility for homozygous (*R/R*) and heterozygous (*R/r*) rose comb roosters.

Genotype	Roosters (n)	Absorbance*
R/R	29	0.4962 ± 0.048[a]
R/r	29	0.7417 ± 0.047[b]

*Absorbance at 550 nm was directly proportional to the extent to which spermatozoa entered a solution of 6% Accudenz. A sperm suspension was overlaid upon the Accudenz within a cuvette. The cuvette was incubated at 41°C for 5 min, then the absorbance of the Accudenz layer was measured.
[a,b]Means different at $p < 0.001$.

An alternative hypothesis was that sperm from *R/R* males traversed the vagina but failed to enter the SST. Consequently, sperm from subfertile and fertile males were incubated with oviduct explants containing SST. In this case, however, there was no difference in the extent of SST filling between genotypes. Preliminary work (Fig. 4) using spermatozoa stained with the fluorochrome bis-benzamide [19] and incubated with explants indicated that the majority of spermatozoa associated with the explant after washing were within the SST. Therefore, we concluded that incubation of sperm with explants followed by explant homogenization and hemacytometer counts of homogenization-resistant sperm nuclei provided an estimate of sperm that had entered the SST. In summary, the alternative hypothesis that sperm from *R/R* males traversed the vagina but failed to enter the SST was rejected.

Results from the in vivo and in vitro SST experiments along with the observations of Kirby and Froman [14] as well as Kirby et al. [11] with respect to sperm metabolism lead us to believe that decreased sperm motility was the most likely explanation that could account for the subfertility associated with *R/R* roosters. Our initial attempts to test this hypothesis were informative, in that sperm from *R/R* roosters did not penetrate a 6% Accudenz solution to the extent that sperm from fertile controls did. Penetration of the Accudenz layer was dependent on sperm motility (Fig. 3). We inferred a difference in sperm motility based upon observable differences in sperm cell mobility. However, testing the motility hypothesis required a larger number of roosters. Therefore, each of three homozygous rose comb roosters was bred with approximately 10 heterozygous hens. Male progeny (n = 80) were tested for homozygosity for the rose comb allele. Therefore, males heterozygous for the rose comb allele were used as fertile controls in the motility experiment. As shown in Table 3, homozygotes were characterized by reduced ($p < 0.001$) motility. While the sperm motility assay did not provide any assessment of variables such as beat frequency or velocity, it did provide an evaluation of the mobility of a sperm cell population. The results from this experiment provided definitive evidence that the motility of sperm from *R/R* males is reduced compared to that of fertile controls (Table 3). However, the basis for this difference in motility is unknown.

Sperm are highly compartmentalized cells. In the case of rooster sperm under physiological conditions, motility appears to depend upon an influx of extracellular calcium [29, 30] in addition to the synthesis of ATP in the midpiece and the hydrolysis of ATP by dynein ATPase associated with the axoneme. Therefore, several cellular compartments may warrant investigation in the case of sperm from males homozygous for the rose comb allele. For example, it is not known whether plasma membrane calcium permeability, ATP production, ATP consumption, or axonemal structure differ according to genotype.

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Sulfated Polysaccharides Inhibit Lymphocyte-to-Epithelial Transmission of Human Immunodeficiency Virus-1[1]

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ABSTRACT

We have previously suggested that sulfated polysaccharides could be used in a vaginal formulation to inhibit infection by human immunodeficiency virus (HIV-1). This supposition was based on studies in which we developed and employed an in vitro model to simulate the mechanism of HIV-1 transmission during coitus. We found that adhesion of mononuclear cells to epithelia was the initial step in infection and speculated that blocking adhesion would prevent HIV-1 transmission. We observed that certain sulfated polysaccharides prevented adhesion of lymphoma cell lines to epithelial cell lines, which were derived from the genital tract, in concentrations of a few milligrams per milliliter; and we theorized that sulfated polysaccharides could thus be used as active ingredients in a topical "microbicide."

In the present in vitro study, evidence is presented that a number of sulfated polysaccharides, including carrageenan, dextran sulfate, heparin, fucoidan, and pentosan polysulfate, are capable of blocking infection by mechanisms other than adhesion at concentrations of a thousand times lower than the dosages that are needed to block cell adhesion. One of these compounds, iota carrageenan, is capable not only of blocking infection of epithelia at concentrations of 1–2 μg, but of blocking adhesion to a far greater extent than the other sulfated polysaccharides tested. For this reason, as well as for considerations of safety, stability, and gelling properties, we suggest that iota carrageenan may be the best choice of the sulfated polysaccharides tested for use as a vaginal microbicide.

The same in vitro model was employed to decipher the cell surface molecules involved in lymphocyte-to-epithelial adhesion. To accomplish this, we screened for the presence of cell adhesion molecules (CAMs), carbohydrates, proteoglycans, and carbohydrate-binding sites. HIV-1-infected lymphocytic cells expressed a CAM profile typical of activated, infected cells (e.g., HLA-DR$^+$, CD4$^-$, LFA-1$^+$, ICAM-1$^+$, LFA-3$^+$, CD2$^+$) whereas epithelia expressed few CAMs (LFA-3, ICAM-1, VLA-5, CD44, CD26, s^{LEX}). Both cell types expressed heparan sulfate and chondroitin sulfate proteoglycans. A variety of sugars (mannose, fucose, galactose, Nac-galactosamine, Nac-glucosamine) were also present, but these cells expressed few carbohydrate-binding sites; lymphocytes bound β-galactose. We were unable to block the adhesion with anti-CAM antibodies or with exogenous sugars. When enzymes were used against sulfated cell surface molecules, chondroitinase was found to block the adhesion. Our evidence suggests that this CAM-independent adhesion may be a lectin-glycosaminoglycan interaction.

INTRODUCTION

Recently there has been increasing awareness among reproductive biologists of the importance of relating laboratory findings to reproductive health concerns. There has been a parallel recognition of the natural association between contraceptive technology and reproductive health issues and of the appropriateness of integrating family planning with prevention of sexually transmitted diseases. Perhaps the most pressing problem and challenge in the field of reproductive health is AIDS. Recently, new complexities in developing a vaccine against human immunodeficiency virus (HIV-1) have been brought to light [1]. Thus, there is an urgent need for strategies other than vaccines to prevent the sexual spread of HIV-1. One alternative approach is the development of a female-controlled, intravaginal formulation to prevent HIV-1 infection. Such formulations are now generally referred to as "microbicides" [2].

To study HIV-1 transmission and identify compounds that could block HIV-1 transmission, we developed an in vitro model to mimic what may happen during sexual transmission of HIV-1 in vivo [3–5]. The model consists of a confluent monolayer of CD4-negative epithelial cells to which chronically HIV-1-infected T lymphocytic cells are added. The epithelium becomes productively infected after the adhesion of lymphocytes to epithelial cells, since adherent lymphocytes secrete HIV-1 onto the surface of the epithelial cells at the region of contact between the T cell and epithelial cell.

Our in vitro model supports the hypothesis that sexual transmission of HIV-1 is a cell-mediated phenomenon whereby HIV-1-infected mononuclear cells, present in genital secretions, attach to and infect epithelial cells lining the genital tract, negating the requirement for lesions to breach an epithelium in vivo. (For a review of these studies, see [4].) Further evidence from a number of in vivo [6–9] and in vitro studies [3, 10–15] also suggests that epithelial cells are targets of HIV-1 infection, and that HIV-1-infected mononuclear cells are the primary source of infection rather than cell-free virus.

If sexual transmission of HIV-1 is primarily a cell-mediated process, there is a pressing need to develop appropriate assays that reflect the situation in vivo. To date, in vitro assays commonly assess the antiviral activity of a compound in terms of cell-free virus, CD4-positive target cells, and syncytium formation. These assays have less relevance when

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