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Chemotaxis of Capacitated Rabbit Spermatozoa to Follicular Fluid Revealed by a Novel Directionality-Based Assay[1]

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ABSTRACT

Precontact communication between gametes is established by chemotaxis. Sperm chemotaxis toward factor(s) in follicular fluid (FF) has been demonstrated in humans and mice. In humans, the chemotactic responsiveness is restricted to capacitated spermatozoa. Here, we investigated whether sperm chemotaxis to factor(s) present in FF also occurs in rabbits and, if so, whether only capacitated spermatozoa are chemotactically responsive. Chemotaxis assays were performed by videomicroscopy in a Zigmond chamber. We measured chemotactic responsiveness as a function of FF dilution by means of a novel directionality-based method that considers the ratio between the distances traveled by the spermatozoa both parallel to the chemoattractant gradient and perpendicular to it. A peak of maximal response was observed at 10^{-4} dilution of FF, resulting in a typical chemotactic concentration-dependent curve in which 23% of the spermatozoa were chemotactically responsive. In contrast, the percentage of cells exhibiting FF-dependent enhanced speed of swimming increased with the FF concentration, whereas the percentage of cells maintaining linear motility decreased with the FF concentration. The percentages of chemotactically responsive cells were very similar to those of capacitated spermatozoa. Depletion of the latter by stimulation of the acrosome reaction resulted in a total loss of the chemotactic response, whereas the reappearance of capacitated cells resulted in a recovery of chemotactic responsiveness. We conclude that rabbit spermatozoa, like human spermatozoa, are chemotactically responsive to FF factor(s) and acquire this responsiveness as part of the capacitation process.

acrosome reaction, fertilization, gamete biology, sperm capacitation, sperm motility and transport

INTRODUCTION

Chemotaxis is the modulation of the direction of movement of motile cells up a concentration of extracellular diffusible molecules (chemoattractant gradient) or down a chemorepellent gradient. Sperm chemotaxis up the gradient of follicular fluid (FF) has thus far been reported and distinguished from other processes that may cause sperm accumulation in only two mammalian species: humans [1] and mice [2, 3] (for a review of studies of mammalian sperm chemotaxis to FF, see [4]). In both species, only a small subpopulation (~10%) is chemotactically responsive [2–5], and at least in humans, this subpopulation consists of capacitated spermatozoa [6], such as spermatozoa that have the potential to undergo the acrosome reaction on stimulation [7]. Individual human spermatozoa can be capacitated/chemotactic only once in their lifetime, and even then only for a relatively short period of time (50–240 min) [5, 6]. Because spermatozoa get to this stage asynchronously, the outcome is a continuous replacement of capacitated/chemotactic cells within the sperm population. The consequence of this situation is a steady state of capacitated spermatozoa that can be maintained for at least 30 h [6]. It has been postulated that the role of sperm chemotaxis in mammals (at least in humans) is a selective recruitment of capacitated spermatozoa for fertilizing the egg and that the role of the continuous replacement of capacitated/chemotactic spermatozoa is to prolong the time during which capacitated spermatozoa are available in the female reproductive tract [6].

Several approaches have been used to study chemotaxis: the microporous filter assay, migration under agarose assay, accumulation in chemoattractant-containing capillaries, and choice assays (see [8] for a review). However, most of these techniques could not distinguish chemotaxis from cell accumulation caused by trapping or chemokinesis [8]. For example, sperm accumulation was demonstrated in FF of horses [9] and pigs [10], but this alone cannot be taken as evidence for sperm chemotaxis. Our aim in the present study was to establish a directionality-based, objective method for the evaluation of chemotaxis, independent of the speed and pattern of movement, and to employ it for determining whether rabbit spermatozoa respond chemotactically to FF as do human and mouse spermatozoa and, if so, whether only capacitated cells are responsive.

MATERIALS AND METHODS

Animals

New Zealand white strain rabbits (6–12 mo old) were used in this study and treated in accordance with the Guide for Care and Use of Laboratory Animals (NRC, National Academy of Science, 1996).

Sperm Collection

Semen samples were obtained with an artificial vagina [11]. The spermatozoa were separated from the seminal plasma by the migration-sedimentation technique, which avoids the centrifugation stress [12]. The separation was carried out in Biggers, Whitten, and Whittingham (BWW) medium (20 mM sodium lactate, 5 mM glucose, 0.25 mM sodium pyru-

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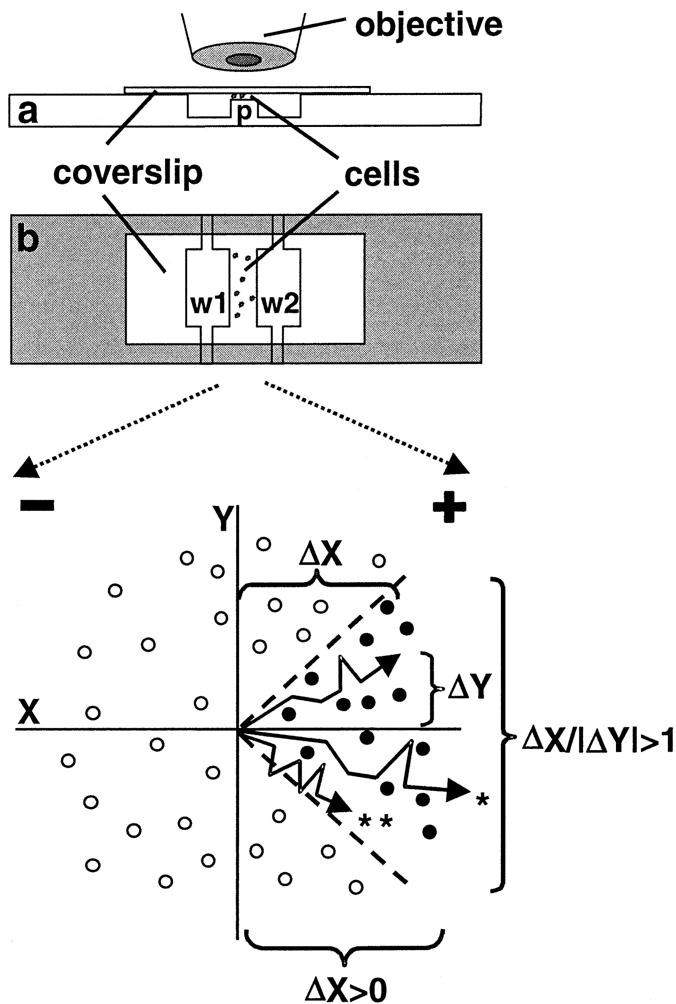

FIG. 1. Chemotaxis detection system. Zigmond chamber under a video microscopy equipment is shown in transverse section (**a**) and in a plane view (**b**). A concentration gradient is formed between the coverslip and the partition wall (p) separating two wells (w1 and w2). Bottom: a projection of the partition wall area is represented as an x,y-coordinate system, with the right side (+) facing the source of the chemoattractant (or culture medium = control). Cell tracks are shown with the starting point at the origin (0,0) of the x,y-system and the final point in an angular area of 90°, representing the area with higher probability of location of chemotactic responsive cells (●). ΔX represents the net distance parallel to the gradient, ΔY the net distance perpendicular to the gradient, $\Delta X > 0$ the number of cells with a net distance parallel to gradient higher than zero, and $\Delta X/|\Delta Y| > 1$ the number of cells with the quotient between the net distance in X over the absolute value of the net distance in Y higher than the unit. The percentage of cells with $\Delta X > 0$ is calculated using all the dots appearing on the right side (+). The percentage of cells with $\Delta X/|\Delta Y| > 1$ is determined by using the dots appearing in the 90° area on the right side (●). The net distance (ΔX) dependence is indicated: greater ΔX at higher cell speed (*) and lower ΔX at less linear pattern (**) (see *Materials and Methods* for details).

vate, 95 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM $NaHCO_3$, pH 7.4 [13], supplemented with 40 mg/ml of BSA and 50 mM Hepes) for 15–20 min at 37°C under an atmosphere of 5% CO_2 in air. Then, the sperm suspension was adjusted to 4×10^6 cells/ml (for chemotaxis assays) or 1×10^6 cells/ml (for capacitation assays) and incubated under the above conditions for 16 h, the time that results in the maximal proportion of capacitated spermatozoa both in vitro [14] and in vivo [15].

Collection of FF

Considering that the mating process in the rabbit induces ovulation (usually 10 h postcoitus), on the estrous day the female was joined with the male until copulation took place. The time of mating was considered as time zero. Then, females were killed by i.v. injection of 150–200 mg of sodium penthotate (Abbott, Buenos Aires, Argentina) 8–9 h postcoitus (e.g., before ovulation). The ovaries were placed in cold PBS, and the FF was aspirated with glass capillaries under a stereomicroscope. Fluids from several females were pooled and centrifuged at $3000 \times g$ for 10 min, keeping the supernatant at −20°C until utilization. The total protein concentration, determined by the Bradford method [16], was used as a measure of the FF concentration. Besides, the progesterone concentration of the FF was measured by radioimmunoassay with the Coat-a-Count kit (Diagnostic Products Corp., Los Angeles, CA). Before running the chemotaxis assays, dilutions of 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} of the pooled FF were prepared.

Sperm Capacitation

The proportion of capacitated spermatozoa was determined from the difference between the levels of acrosome-reacted spermatozoa before and after an acrosome reaction induced with the calcium ionophore A23187 (Sigma Chemical Co., St. Louis, MO) [17, 18]. The acrosome-reacted spermatozoa were visualized by the acrosome marker *Pisum sativum* agglutinin labeled with fluorescein isothiocyanate (PSA-FITC; Sigma). At each experimental time point, two aliquots were removed from the sperm suspension. One aliquot was treated with 10 μM A23187 for 30 min at 37°C, and the other was incubated under the same conditions in the absence of the inducer. Then, the samples were labeled with PSA-FITC as previously described [19] and observed under an epifluorescence microscope at 800×. Two fluorescence patterns were clearly identified: one with the acrosome completely fluorescent (acrosome intact) and the other showing only an equatorial fluorescent band (acrosome reacted). Spermatozoa that were completely fluorescent or transparent (dead cells, verified with Hoeschst 33258; Sigma) were not included in the counting. Therefore, the total number of counted cells (considered as 100%) included live spermatozoa only.

Directionality-Based Assay for Sperm Chemotaxis

Chemotaxis assays were performed at 37°C in a home-made Zigmond chemotaxis chamber [20] consisting of two wells separated by a wall and closed with a coverslip sealed with paraffin (Fig. 1). This construction allowed the formation of a stable, one-dimensional concentration gradient of the chemoattractant between both compartments, which was verified fluorescently as described earlier [20]. The spermatozoa were put in the left well (Fig. 1, w1), whereas the "target well" (Fig. 1, w2) was filled with FF (or BWW as a control). Alternatively, the coverslip with attached leukocytes (which were assayed as a positive control) was mounted to close the chamber, then the left well was filled with Dulbecco modified Eagle medium (Sigma) as a culture medium and the right well with 10^{-8} M *N*-formyl-methionyl-leucyl-phenylalanine peptide (fMLP; Sigma) as the attractant [20]. The movement of cells in the capillary space (~10 μm) between the coverslip and the partition wall separating the wells (Fig. 1, p) was recorded at 10× in the middle of the field between both compartments. Our videomicroscopy system consisted of a CCD video camera, a time-lapse video recorder, and a monitor (Panasonic, Matsushita Ltd., Osaka, Japan) connected to a phase-contrast microscope as previously described [21, 22]. In the case of spermatozoa, the recording started 5 min after the sealing of the chamber, at which time the distribution of the cells was stable. Recordings were carried out at videotape speed of one image every 0.016 sec (continuous running) for spermatozoa or one image every 0.4 sec for leukocytes. During the video frame-to-frame playback, cell tracks (previously selected at random in the pause mode) were drawn by hand on an acetate sheet attached to the monitor screen during 3 sec (spermatozoa) or 10 min (leukocytes) of recording. No less than five microscopical fields were used for the analysis in each experiment. Taking into account that the chemotaxis parameters are meaningful when the cells are tracked for sufficiently long distances and that following cells traveling long distances is difficult by videomicroscopy, we monitored a large number of relatively short tracks. Then, the tracks were scanned, transferred into a computer, and analyzed with the SigmaScanPro image analysis software (SPSS, Inc., Chicago, IL). Considering that the chemoattractant material was always placed in the right well of the Zigmond chamber (Fig. 1, w2 and bottom: +), the starting point of each track was arbitrarily located at the origin (0,0) of the x,y-coordinates, and the net distances traveled both parallel to the chemoattractant gradient (Fig. 1, ΔX) and perpendicular to it (Fig. 1, ΔY) were measured.

Defining the x-axis as the direction of the gradient, the following directionality-based parameters were measured. First was the mean of the

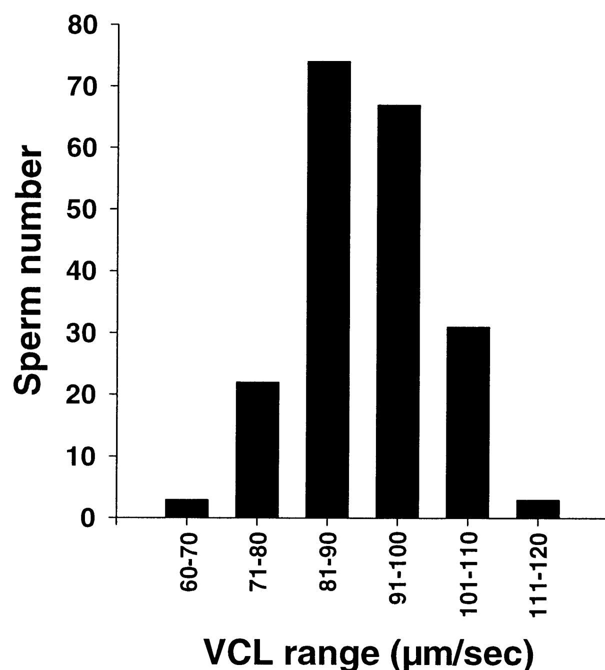

FIG. 2. Frequency distribution of VCL values in the control group.

net distances traveled by the cells parallel to the gradient ($\overline{\Delta X}$). The average net distance traveled by randomly moving cells in the direction of the gradient (as in any other direction) is expected to be approximately zero. However, the average net distance along the x-axis ($\overline{\Delta X}$) traveled by chemotactically responsive cells should be positive and substantial. The second parameter was the percentage of cells with a net distance greater than zero ($\Delta X > 0$), that is to say, cells swimming a larger distance toward the attractant/culture medium well than in the opposite direction. This parameter is expected to be approximately 50% for a population of randomly moving cells and significantly greater than 50% for a population of chemotactically responsive cells. The third parameter was the percentage of cells with the quotient between the net distance in X over the absolute value of the net distance in Y higher than the unit (Fig. 1, $\Delta X/|\Delta Y| > 1$), that is to say, cells swimming a larger distance toward the attractant/culture medium well than in a nongradient direction, perpendicular to the gradient. This parameter is expected to be approximately 25% for a population of randomly moving cells and significantly greater than 25% for a population of chemotactically responsive cells.

Other kinematic parameters (determined in the same system) were the curvilinear velocity (VCL) and the pattern of movement. For determining the percentage of chemokinetic spermatozoa (those having FF-dependent enhancement of swimming speed), a frequency distribution of VCL at 10-µm/sec intervals was performed. On the basis of the control values, cell speed greater than 110 µm/sec was considered to be chemokinetically responsive (Fig. 2). The pattern of movement was evaluated by means of the fractal dimension (FD), defined as the degree to which a line fills a plane [23]. On the basis of the FD values analyzed at 60 Hz, the motility pattern of spermatozoa was classified as linear (FD < 1.3), transitional ($1.3 \leq$ FD ≤ 1.8), or hyperactivated (FD > 1.8) (Fig. 3). These criteria were established by correlating the FD values with the corresponding cell trajectories for cases in which the linear tracks showed almost no fluctuations from the straight line, the hyperactivated tracks showed a "star-spin" trajectory, and the transitional tracks were considered to be between these two extreme patterns.

In all the experiments, the percentage of motile cells, estimated by videomicroscopy in three fields chosen at random, served as a measure of sperm viability, which in all cases was greater than 90%.

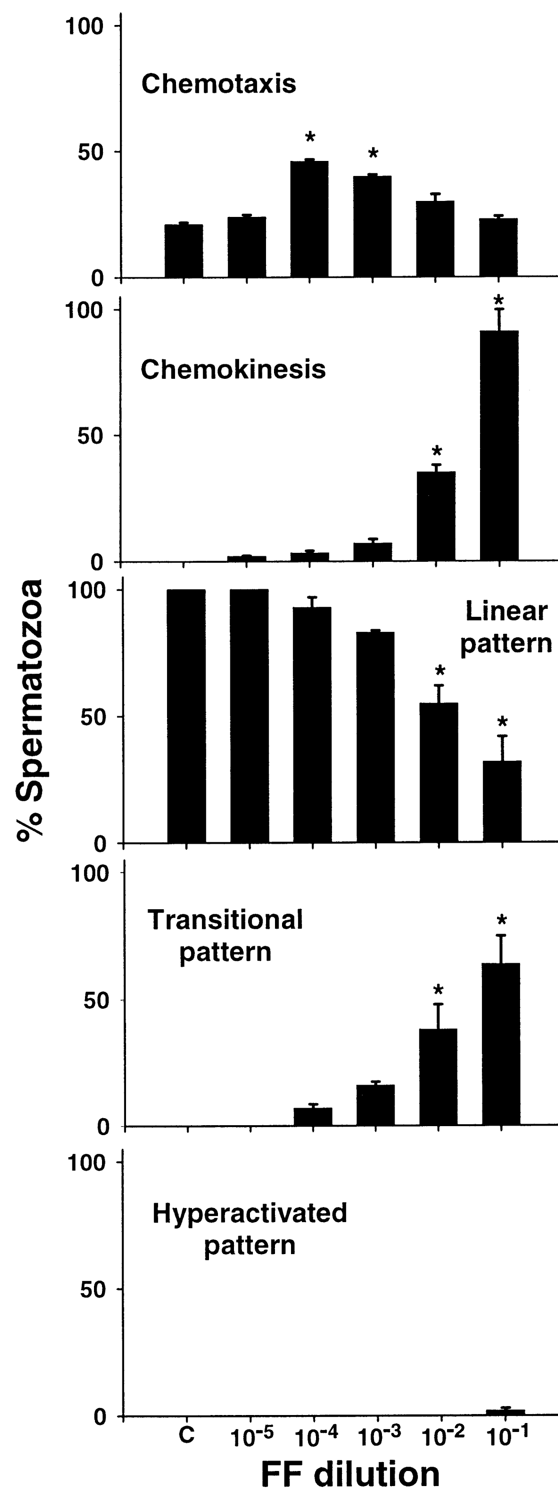

FIG. 3. Sperm dynamic response to FF dilutions. Chemotaxis (% $\Delta X/|\Delta Y| > 1$), chemokinesis (% cells with VCL > 110 µm/sec), linear pattern of movement (% cells with FD < 1.3), transitional pattern (% cells with $1.3 \leq$ FD ≤ 1.8), and hyperactivated pattern (% cells with FD > 1.8) are shown. The total number of cells analyzed was defined as 100%. All data are presented as the mean ± SEM. *Significantly different from the control group ($P < 0.05$).

Statistical Analysis

The experiments were performed with ejaculates from three different rabbits, each carried out in triplicate. The minimal number of analyzed cells per experiment was determined by incrementally increasing the number of cells until the average remained unchanged. Therefore, the minimal number of blindly analyzed spermatozoa per treatment, at each time point, was 50 cells for kinematic parameters and 200 cells for the capacitation test. The differences between the mean values were determined by the Student *t*-test, for which in the case of percentages the arcsine square-root transformation of the data was previously applied [24] using the SigmaStat software (SPSS, Inc.)

TABLE 1. Directionality-based chemotactic criteria in active leukocytes and in noncapacitated rabbit spermatozoa.[a]

Chemotactic parameter[b]	Human leukocytes		Noncapacitated rabbit spermatozoa	
	Control	fMLP (10^{-8} M)	Control	FF (10^{-4} dilution)
$\overline{\Delta X}$ (μm)	2.3 ± 11.5	26.1 ± 12.6[c]	7.3 ± 10.1	8.8 ± 11.7
% $\Delta X > 0$	52 ± 0.08	94 ± 0.12[c]	51 ± 0.02	51 ± 0.02
% $\Delta X \lvert\Delta Y\rvert > 1$	24 ± 0.01	69 ± 0.03[c]	23 ± 0.06	25 ± 0.43

[a] All data are presented as mean ± SEM.
[b] $\overline{\Delta X}$, Mean of the net distance traveled parallel to the gradient; % $\Delta X > 0$, percentage of cells with a net distance greater than zero; % $\Delta X/\lvert\Delta Y\rvert > 1$, percentage of cells with the quotient $\Delta X/\lvert\Delta Y\rvert$ higher than the unit.
[c] Significant difference vs. the control group ($P < 0.001$).

RESULTS

Validation of the Directionality-Based Method to Determine Chemotaxis

To determine experimentally whether rabbit spermatozoa are chemotactically responsive to FF, we employed a directionality-based assay in a Zigmond chamber, thus avoiding interference from processes other than chemotaxis that might cause sperm accumulation [8]. We assessed the occurrence of chemotaxis according to three criteria, as explained in *Materials and Methods*: 1) the average net distance traveled by the spermatozoa in the direction of the gradient ($\overline{\Delta X}$), 2) the percentage of cells with $\Delta X > 0$, and 3) the percentage of cells traveling a longer distance on the x-axis than on the y-axis ($\Delta X/\lvert\Delta Y\rvert > 1$).

To verify that these criteria truly reflect chemotaxis, we studied the response of leukocytes to the chemoattractant fMLP. In leukocytes, a large fraction of the population is chemotactically responsive to *N*-formylated peptides [25–27]. As shown in Table 1, all three criteria clearly revealed a strong chemotactic response to fMLP. Then, capacitated rabbit spermatozoa were exposed to different concentration gradients of FF (10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1}). In the absence of an FF gradient, all three directionality parameters showed the expected values for a random movement, whereas they increased with the FF concentration and then gradually decreased, yielding a chemotactic-typical concentration dependence [28, 29] (Table 2). In contrast, the speed of swimming (VCL) and the pattern of movement (FD) did not exhibit such variations but increased with the FF concentration. As a negative control for the above results, we used noncapacitated spermatozoa, which are expected to be unable to respond chemotactically to FF [14]. For each of the studied parameters, no significant difference was observed between FF and the control condition (Table 1), which further substantiates the notion that the evaluated parameters are true criteria for chemotaxis. Although all three directionality-based parameters studied herein yielded a concentration dependence typical of chemotaxis [28, 29], independent from the cell speed and the pattern of movement, the percentage of cells with $\Delta X/\lvert\Delta Y\rvert > 1$ results in a higher signal-to-noise ratio. Therefore, in the following experiments, we evaluated the chemotactic spermatozoa by means of the percentage of cells having $\Delta X/\lvert\Delta Y\rvert > 1$.

Sperm Response to Follicular Fluid Dilutions

We investigated the dynamic response of capacitated rabbit spermatozoa with several dilutions of FF. The proportions of chemotactic and chemokinetic cells as well as the proportion of cells having a transitional pattern of movement were dependent on the FF dilution. Maximal chemotactic responsiveness was observed at the 10^{-4} dilution of FF (Fig. 3), with a difference of 23% versus the control group ($P < 0.01$), suggesting the existence of a subpopulation of chemotactically responsive spermatozoa. The percentage of chemokinetic cells or of cells that had a transitional pattern of motility increased with the FF concentration, whereas the percentage of cells exhibiting a linear swimming pattern decreased (Fig. 3). In contrast, the level of hyperactivated spermatozoa (cells showing a nonprogressive, vigorous movement with marked lateral displacement of the head [7]) was close to zero and apparently independent of the FF dilution. This was consistent with the low level of progesterone (the factor in FF that causes hyperactivation of human spermatozoa [30, 31]), which in our FF sample was 0.125 μg/ml. As a whole, these observations suggest that the sperm displacement developed at the 10^{-4} and 10^{-3} FF dilutions (Fig. 1) resulted from chemotaxis rather than from chemokinesis or hyperactivation.

Correlation Between Capacitation and Chemotaxis

In human spermatozoa, only capacitated cells are chemotactically responsive [6]. To examine whether this is the case for rabbit spermatozoa, we depleted a sperm population of capacitated cells by 30-min incubation with the calcium ionophore A23187, which induces the acrosome reaction only in capacitated spermatozoa [17, 18]. After removing the ionophore by centrifugation, the sperm population was totally depleted of both capacitated and chemotactically responsive cells (Table 3). Two hours later, capacitated and chemotactically responsive spermatozoa were again found, and as before, the levels of both were very similar (Table 3). In a control sperm population that was similarly incubated for an additional 2 h but in the absence of ionophore, the percentages of capacitated and chemotactically responsive spermatozoa equally augmented further (Table 3). This incremental increase (the difference between the fourth and first lines of Table 3) was very similar to the percentages of recovered cells 2 h after de-

TABLE 2. Criteria for sperm chemotaxis in capacitated rabbit spermatozoa.[a]

Parameter[b]	Control	Follicular fluid dilutions 10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
$\overline{\Delta X}$ (μm)	5.5 ± 11.5	23.4 ± 11.4	63.6 ± 10.6[c]	38.9 ± 10.2[c]	2.4 ± 10.5	−13.3 ± 12.2
% $\Delta X > 0$	50 ± 0.01	55 ± 0.32	67 ± 0.02[c]	60 ± 0.25[c]	54 ± 0.01	48 ± 0.02
% $\Delta X/\lvert\Delta Y\rvert > 1$	21 ± 0.01	24 ± 0.02	46 ± 0.02[c]	40 ± 0.03[c]	30 ± 0.29	23 ± 0.31
VCL (μm/sec)	90.1 ± 0.7	91.6 ± 0.7	92.9 ± 0.8	96.2 ± 0.7[c]	110.2 ± 0.9[c]	155.4 ± 1.2[c]
FD	1.08 ± 0.01	1.07 ± 0.01	1.12 ± 0.01	1.21 ± 0.7[c]	1.27 ± 0.01[c]	1.40 ± 0.01[c]

[a] All data are presented as mean ± SEM.
[b] See Table 1 for definitions.
[c] Significant difference vs. the control group ($P < 0.05$).

TABLE 3. Depletion and recovery of chemotactic and capacitated spermatozoa.[a]

Time post-ejaculation (h)	Experimental timing	Capacitated cells[b] (%)	Chemotactically responsive cells[b] (%)
14	Before depletion by A23187	9 ± 0.01	9 ± 0.02
14	Immediately after depletion[c]	0 ± 0.0	0 ± 0.01
16	2 h after depletion	5 ± 0.06[d]	5 ± 0.02[d]
16	2-h incubation without depletion	14 ± 0.03	14 ± 0.02

[a] All data are presented as mean ± SEM.
[b] The total number of cells analyzed in each time point (defined as 100%) were at least 2600 for capacitation and 300 for chemotaxis assays. Chemotaxis was determined as the percentage of cells showing $\Delta X/|\Delta Y| > 1$ after subtracting the corresponding control value.
[c] Before assaying for chemotaxis and capacitation, the ionophore was removed by centrifugation and the cells resuspended in fresh BWW medium.
[d] Significant difference with respect to "Immediately after depletion" and "2-h incubation without depletion" ($P < 0.005$).

pletion (Table 3, third line). This observation suggests that the postdepletion increase of capacitated and chemotactically responsive spermatozoa resulted from newly formed capacitated cells. When capacitated cells were eliminated by the calcium ionophore 20 h after ejaculation, only 1–2% of the spermatozoa became capacitated (data not shown), suggesting that by this time, very few spermatozoa were competent to become capacitated. Moreover, when the cells are not fully capacitated (see Table 1), they are incapable of the chemotactic response. Taken together, these results suggest that in rabbit spermatozoa, as in human spermatozoa [6], only capacitated cells are chemotactically responsive.

DISCUSSION

In this study, by employing a novel directionality-based assay, we provided evidence that a subpopulation of rabbit spermatozoa, consisting of capacitated cells, responds chemotactically to defined dilutions of FF.

Directionality-Based Criteria for Chemotaxis

Chemotaxis, an extracellular gradient-guided cell locomotion, is essential for many biological processes [8, 14, 25, 32, 33]. However, to demonstrate chemotaxis, it is not sufficient to show cell accumulation, because cell accumulation could be biased by effects on the cell speed, pattern of movement, or trapping of any kind [8].

Several approaches have been used to study sperm chemotaxis (for a review, see [8]). Most of these approaches relied on sperm accumulation in an ascending gradient of FF and, therefore, could not distinguish chemotaxis from cell accumulation caused by trapping or chemokinesis [8]. This difficulty has been especially severe in the measurement of sperm chemotaxis in humans and mice, where only a small fraction of the cells are chemotactically responsive at any given moment [3, 5]. An optimal chemotaxis assay for mammalian spermatozoa should be based on directional changes of the cell's locomotion toward the source of the chemoattractant [1–3, 8, 25, 27, 29]. Such a criterion was previously used [1, 31]; however, those measurements were carried out in two-dimensional gradients, rendering the analysis both cumbersome and subjective. In contrast, the one-dimensional chemotactic gradient established in a Zigmond chamber [20] allows a simpler and more accurate measurement of cell directionality through a real-time recording of swimming cells, thus providing detailed information on a variety of dynamic parameters [2, 3, 26]. Recently, this chamber enabled us to demonstrate chemotaxis of mouse spermatozoa by means of the net distance traveled by the cell toward the source of the attractant [2, 3]. However, the net distance on the x-axis can be perturbed by changes in the speed or pattern of movement. For example, the increase of the speed results in a larger ΔX (Fig. 1, *), and a less linear cell movement causes a smaller ΔX (Fig. 1, **). In the present study, the proposed chemotactic criteria involve determination of the net distance on the *x*-axis, but because their computation is based on directionality, they appear to reflect chemotaxis without perturbation from other processes. For example, a significant increase in the curvilinear velocity at the 10^{-1} dilution of FF (~70% over the control) did not induce an augmentation in the chemotaxis values (Table 2).

Usually, a typical chemotactic response does not result in a saturation curve, because when the chemoattractant concentration increases beyond saturation, the fully occupied receptors cannot sense the rising gradient and, consequently, the chemotactic response drops [27, 28, 32]. Although the sperm chemoattractants and their receptors have not yet been identified, spermatozoa are probably no exception in this regard. In the present study, all three directionality-based parameters yielded a concentration dependence typical of chemotaxis [28, 29], configuring a reliable method to determine a chemotactic behavior. We think that, among these parameters, the percentage of cells with $\Delta X/|\Delta Y| > 1$ is more accurate, because the expression $\Delta X/|\Delta Y|$ normalizes the distance run by a cell in the direction of the concentration gradient in relation to the distance traveled by that same cell in the absence of a gradient, resulting in a higher signal-to-noise ratio. This conclusion holds even when the measurement is carried out over a high background of nonchemotactic cells. Because this method for chemotaxis determination is effective in mammalian spermatozoa, in which the signal-to-noise ratio is low, the same approach likely will be adequate for any type of chemotactic cells showing a higher signal-to-noise ratio, as was indeed demonstrated in the present study with human leukocytes.

Sperm Response to Follicular Fluid

A subpopulation of rabbit spermatozoa showed the highest chemotactic response at FF dilutions of 10^{-4} and 10^{-3}, which is in agreement with the results of earlier studies in humans [1] and mice [3]. The independence between chemotactic responsiveness and other kinetic behaviors arose from the different responses obtained when the spermatozoa were exposed to FF concentrations. For example, the maximal percentage of chemotactically responsive cells was higher than that of chemokinetic cells, suggesting diverse responsible factors, or that the chemokinetic and chemotactic responsiveness of sperm cells were independent.

Rabbit spermatozoa, unlike the human sperm cell [1, 34], did not exhibit hyperactivated motility when exposed to FF; instead, they maintained a rather linear and progres-

sive locomotion, becoming increasingly transitional with the FF concentration (Fig. 3). The reason for not becoming hyperactivated is probably the low level of progesterone in the rabbit's FF (0.125 μg/ml as determined in the present study), because, at least for human spermatozoa, progesterone is the factor in FF (5–6 μg/ml [35]) that causes hyperactivation [30, 31].

Sperm chemotaxis, widely distributed and long known in marine species [36, 37], was neglected for many years in terrestrial mammals because of the general agreement about the competence of a large number of spermatozoa to fertilize the egg (see [4, 8] for reviews). However, in recent years, the occurrence of sperm chemotaxis has been reported in humans [8], mice [2, 3], and rabbits ([14], and present study) as being tightly associated to the capacitate state, which suggests that sperm chemotaxis may be widespread even in mammals.

Capacitated Spermatozoa Are Able to Respond by Chemotaxis

The present results indicate that, in the rabbit, only capacitated spermatozoa are chemotactically responsive. This conclusion is supported by the following: 1) the percentages of capacitated and chemotactically responsive spermatozoa were very similar, 2) the removal of capacitated cells resulted in a total loss of chemotactic responsiveness, 3) the reappearance of capacitated cells resulted in a recovery of chemotactic responsiveness (Table 3), and 4) noncapacitated spermatozoa were unable to respond by chemotaxis (Table 1). This idea agrees with our previous observation that the levels of capacitated and chemotactically responsive spermatozoa are essentially the same at any time point [14], and it strongly supports the notion that, in rabbits as in humans [6], spermatozoa acquire their chemotactic responsiveness as part of the capacitation process and lose it when the capacitated state ends.

As in all previous chemotaxis experiments, the FF in the present study was used as a source of substances secreted from the egg or its surrounding cells. Because small quantities of FF are transported to the fertilization site [38] and assuming that these biologically active molecules could also be secreted after ovulation, the present results support a hypothesis about what might happen under in vivo conditions. Whereas at a low concentration of biologically active molecules the spermatozoa swim at random, detection of a concentration gradient of chemotactic factor(s) is followed by a directional locomotion of only capacitated spermatozoa toward the source of the attractant, keeping a linear movement. Afterward, when the cells sense an oversaturated concentration of active molecules, they cannot sense the gradient, increasing their velocity and acquiring a less linear pattern of movement that could allow them to cross the egg coats. These physiological processes should happen during a short temporal window (lasting 12 h) of egg availability in the female genital tract [14].

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