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Chemotaxis and Chemokinesis of Human Spermatozoa to Follicular Factors[1]

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ABSTRACT

Human spermatozoa accumulate in vitro in diluted follicular fluids obtained from follicles from which the eggs have been fertilized. Using capillary assays under a variety of experimental conditions (ascending or descending gradients of follicular fluid, or no gradient at all) and microscopic assays in which individual spermatozoa could be followed, we found that the sperm accumulation in follicular fluid was the result of both sperm chemotaxis and chemokinesis and eventually hyperactivation-like motility. We determined the optimal conditions for sperm accumulation, which involved sperm preincubation (possibly to induce sperm capacitation) and proper dilution of follicular fluid. In all the assays, the net accumulation was low, probably reflecting the chemotactic responsiveness of only a small fraction of the sperm population at any given time. We partially fractionated follicular fluid in a Centricon microconcentrator (Amicon, Danvers, MA) and by acetone precipitation, and found that at least one of the chemotactic factors is a small (< 10-kDa) molecule that is probably nonhydrophobic. This is the first time that sperm chemotaxis and chemokinesis in response to a follicular factor(s) in mammals has been established and has been distinguished from other processes that might cause sperm accumulation. The physiological significance of these findings is discussed.

INTRODUCTION

The occurrence of sperm chemotaxis (i.e., a response of motile cells to the gradient of a chemical stimulus, resulting in modulation of the direction of travel so as to approach an attractant or to move away from a repellent) and chemokinesis (i.e., a change in swimming speed in response to a chemical stimulus) in species with external fertilization is well known, primarily in metazoa whose females spawn their eggs into sea water before fertilization [1–3]. The occurrence of sperm chemotaxis and chemokinesis in species with internal fertilization is still an open issue (see [4] for review). Thus, in vivo studies reporting sperm chemotaxis in mammals [5–7] could well reflect other processes, such as chemokinesis or trapping [4]. Similarly, none of the published in vitro studies proved that chemotaxis occurred. Two types of in vitro studies have been carried out in mammals: chemotaxis to synthetic peptides of the *N*-formyl-Met-Leu-Phe class and chemotaxis to follicular fluid. The synthetic peptides are derived from bacteria and are known to be attractants for leukocytes [8]. Attraction to these peptides was studied with bull [9] and human [10] spermatozoa. However, the attraction of bull spermatozoa was later shown to be the result of adhesion to glass [11], and the attraction of human spermatozoa was not controlled for the possibilities of trapping and/or chemokinesis (in this context trapping may involve adsorption of spermatozoa or a negative effect of the "attractant" on the sperm motility). Follicular fluids include the secretions of the egg and its surrounding cells, have a large number of bioactive substances [12–14], and have been shown to affect sperm physiology and motility (though with apparently conflicting observations) [4, 15–18]. Attraction of human spermatozoa to follicular fluid, correlated with egg fertilization, was indeed demonstrated, but the issue of whether or not the attraction was due to chemotaxis was not determined [19]. Other studies that claimed observation of sperm chemotaxis to follicular fluid (e.g., [20–22]), did not distinguish between true chemotaxis and other processes that might cause sperm accumulation. In addition to reports that unjustifiably claimed to have demonstrated chemotaxis, a recent study reported a random distribution of spermatozoa in a gradient of follicular fluid and was unable to demonstrate chemotaxis [23]. This ambiguity in both the in vivo and in vitro observations reflects the difficulties involved in studying sperm chemotaxis and chemokinesis in mammals [4].

Here we make a clear distinction between the processes that might cause sperm accumulation in follicular fluid. We

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demonstrate, for the first time, that chemotaxis of human spermatozoa to follicular fluid does indeed occur and that it is accompanied by chemokinesis and, eventually, hyperactivation-like motility (a motility pattern associated with sperm capacitation [24, 25]). We demonstrate that stringent experimental requirements must be fulfilled in order to observe these processes, and that unawareness of these requirements have contributed to the ambiguity of the previously published results. We also report on partial fractionation of follicular fluid as a first step towards isolation of the chemotactic factor(s).

MATERIALS AND METHODS

Spermatozoa

Human ejaculates were collected by masturbation from normal healthy donors. Each ejaculate was allowed to liquefy at room temperature and then was washed twice (by centrifugation at 120 × *g* for 15 min) with Biggers, Whitten, and Whittingham medium (20 mM sodium lactate, 5 mM glucose, 0.25 mM sodium pyruvate, BSA [fraction 96–99%] at 3 g/L, penicillin G at 0.08 g/L, streptomycin sulfate at 0.05 g/L, 95 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 1.2 mM $MgSO_4$ in 25 mM $NaHCO_3$ buffer, pH 7.4) [26] supplemented with HEPES (10 mM, pH 7.4) and 0.1% polyvinylpyrrolidone 40 (M_r 40 000; Sigma Chemical Co., St. Louis MO) to reduce sperm adsorption. (This solution is referred to hereafter as BWW.) The spermatozoa were resuspended in BWW to a concentration of 1–2 × 10^7 cells/ml (for capillary assays) or 1–5 × 10^8 cells/ml (for microscopic assays) and, unless indicated otherwise, were incubated for 2 h at 37°C under 5% CO_2.

Follicular Fluid

Human follicular fluids were obtained from women undergoing transvaginal aspiration for in vitro fertilization, who had been pretreated with human menopausal gonadotropins for ovarian stimulation [27]. Follicular fluids were filtered through a 0.45-μm Acrodisc filter (Gelman Sciences, Nes Ziona, Israel) to remove cells and cell debris. The filtrates were divided into 200-μl aliquots and stored at −20°C. Follicular fluids were tested fresh or within 2 wk of storage. After this period of time, their attractive activity for sperm gradually decreased.

Acetone Precipitation of Follicular Fluid

For 90% acetone precipitation, one portion of follicular fluid was mixed with nine portions of cold acetone. The mixture was incubated on ice for 60 min and centrifuged (640 × *g*, 10 min). The supernatant was dried in a SpeedVac concentrator (Savant Instruments, Farmington, NY), and the pellet was dried under nitrogen. The dried supernatant and pellet were stored at −20°C for no longer than 1 mo. Prior to the experiment, they were diluted with BWW to the original volume of the follicular fluid. For 100% acetone precipitation, a portion of the supernatant fraction from the 90% acetone precipitation was dried, and 100% acetone was added. The newly formed pellet was separated from the supernatant by centrifugation and then dried and resuspended in 100% acetone by sonication. Five such cycles of precipitation in 100% acetone were carried out. Each of the supernatants was filtered through a pipette plugged with cotton wool; then, each was dried and resuspended in water to the original volume. The five supernatants were pooled and the final pellet was examined for activity.

Capillary Assay

The assays were carried out in a system consisting of a series of teflon wells and "capillaries" (polyethylene tubes [Intramedic, Parsippany, NJ] PE-50, i.d. 0.058 mm) as shown in Figure 1. (The use of polyethylene tubes, instead of glass capillaries, prevented adsorption of spermatozoa to the tubes.) The wells were filled with 100 μl of spermatozoa at a concentration of 1–2 × 10^7 cells/ml suspended in BWW or in the test solution, as indicated. The capillaries were filled either with BWW or with the test solution and sealed at one end with a clamp. The open side of each capillary was inserted into the well, and the whole tray was incubated, unless mentioned otherwise, for a period of 30 min

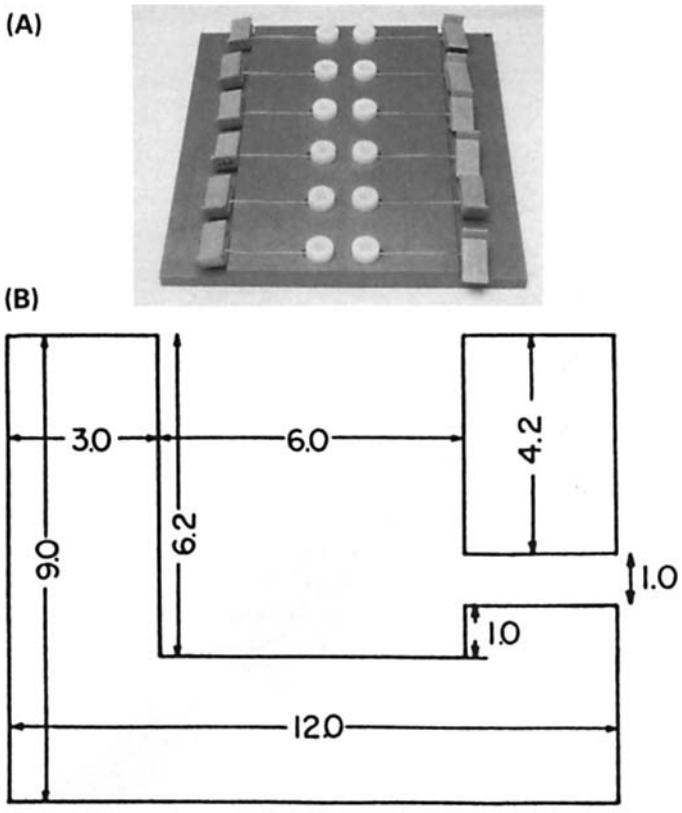

FIG. 1. Multicapillary system for measuring chemotaxis: A) top view of system; B) vertical cross section of a teflon well. Dimensions of well are given in mm. Length of polyethylene tubes ("capillaries") is 55 mm.

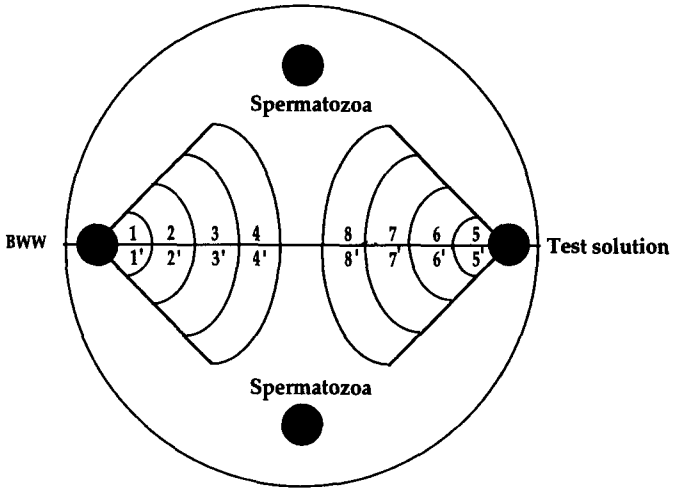

FIG. 2. Definition of zones in the sealed chamber.

either in a 37°C incubator under 5% CO_2 or at room temperature (22–25°C). (In a separate experiment we determined the diffusion time of a dye, Coomassie Brilliant Blue G [Sigma], from the capillary into the well. A gradient of the dye was established in the well within minutes and persisted for at least 90 min.) The distribution of spermatozoa in the capillary at the end of the incubation period was not homogeneous: the concentration was highest near the open end of the capillary and lowest near its sealed end. Therefore, the total content of each capillary was counted, and the average concentration of the spermatozoa was determined by direct counting or enzymatic assay. In direct counting, the contents of the capillary were transferred into an Eppendorf tube containing 5 μl of 8% glutaraldehyde in water, and the spermatozoa were counted under the microscope by a hemocytometer. In the enzymatic assay, the contents of each capillary (about 10 μl) were transferred to a microtiter plate, followed by addition of 200 μl endopeptidase substrate (3-carboxypropanoyl-alanyl-alanyl-leucine-4-nitroanilide, 0.4 mM, supplemented with *Streptomyces griseus* aminopeptidase [28]) in a solution of Tris-HCl (50 mM), NaCl (100 mM), and $CaCl_2$ (1 mM), final pH 7.5. The plate was incubated overnight at 35°C, and the absorbance was measured at 405 nm (due to release of 4-nitroaniline) by a Bio-Rad (Brussels, Belgium) microplate reader (model 450). Since the seminal fluid has a high level of endopeptidase activity, it is essential that the concentration of seminal fluid retained in the sperm suspension after washings should be less than 10^{-5} of the original concentration.

Microscopic Assay

Microscopic assays were carried out in a sealed chamber with a depth of 10 μm, so that the spermatozoa could only swim in two dimensions within the focal plane of a microscope throughout the observation period [23]. The chamber contained four wells (black circles in Fig. 2), of which two opposing wells (top and bottom) were filled with washed spermatozoa, and the other two (left and right) were filled with the control and the test media, respectively. A gradient of the test solution was established by diffusion [23]. The behavior of the spermatozoa in the chamber was recorded on video and then analyzed. The video recording commenced within 1 min of the sealing of the chamber and continued for 15 min. Although the observation field under the microscope was smaller than the field defined by the area between the four wells, we covered most of this area by recording two smaller areas (2.11 mm^2 each) in sequence. Each of these areas was divided into four serial zones. One area covered zones 1–4 (or 1′–4′), the areas of which were 0.22, 0.42, 0.62, and 0.85 mm^2, respectively; and the other area covered zones 5–8 (or 5′–8′; Fig. 2). The two areas were recorded alternatively for 10 sec each time. The number of spermatozoa in each zone was counted in 30-sec intervals, and the sperm density (number of spermatozoa per mm^2) was calculated.

Track Analysis

The experiments for track analysis were carried out in two steps. First, to ascertain the time period in which the sperm accumulation in zone 5 was higher than in zone 1, a regular microscopic assay was carried out as described above. Then, two experiments were conducted, one for the analysis of spermatozoa near the follicular fluid-containing well (zone 5), and one for the analysis of spermatozoa near the BWW-containing well (zone 1). The experiments of this step were performed with only (0.8–1) × 10^8 spermatozoa/ml (to avoid confusion between crossing tracks). At this sperm concentration, the numbers of spermatozoa that reached zones 1 and 5 were 6–14 and 13–32, respectively (equivalent to 11–25 and 23–56 spermatozoa/mm^2). The video recording was conducted for 10 min without shifting the microscope from one well to another. The trajectories made by the swimming spermatozoa were obtained by backward and forward tracing of the video-recorded tracks of spermatozoa found in zones 1 and 5. The tracings were made manually by drawing on transparencies. The starting point of each trajectory was its first appearance on the video screen. The trajectory ended either when the spermatozoon entered the well or when it left the video screen. Accordingly, the duration of the recorded tracks varied. The swimming speed of each spermatozoon was calculated for the period of time from its first appearance on the video screen until its entrance into zone 1 or 5.

RESULTS

Physiological Conditions for Detecting Sperm Accumulation in Follicular Fluid

One characteristic of our earlier measurements of sperm attraction or accumulation, which employed a 48-well

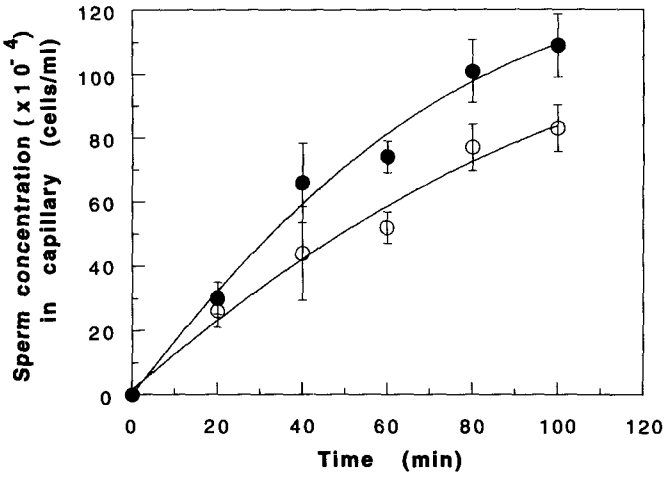

FIG. 3. Time-dependent accumulation of spermatozoa in the polyethylene capillaries. Results of a typical experiment (total of 13 experiments) conducted as described in *Materials and Methods*. Each well contained preincubated spermatozoa, suspended in BWW (2×10^7 cells/ml). Capillaries contained either follicular fluid diluted 3000-fold in BWW (solid circles), or BWW alone (open circles). The number of spermatozoa (determined by direct counting) that migrated into the capillaries within the indicated period of time is shown. Each point represents the average of six determinations ± SD.

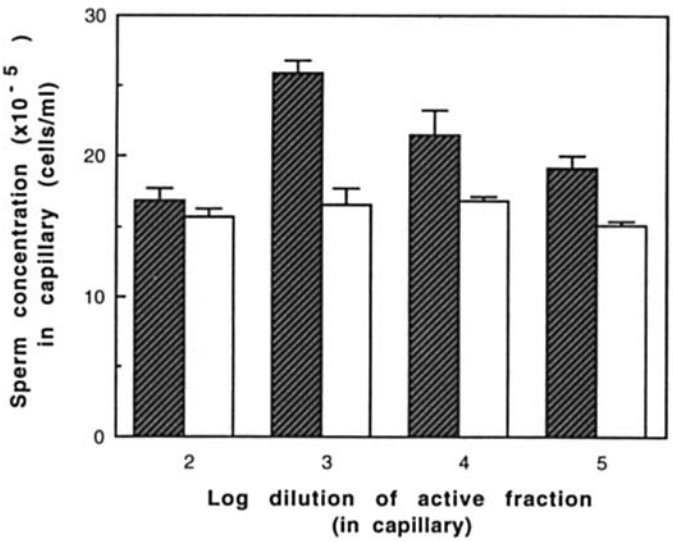

FIG. 4. Concentration dependence of sperm accumulation in capillaries containing an active fraction of follicular fluid. Assays were carried out at room temperature (25°C). The active fraction of follicular fluid was prepared by 90% acetone precipitation as described in *Materials and Methods* and diluted in BWW to the extent indicated in the figure. Results shown are of a single experiment (total of 11). Each point represents the mean of six capillaries ± SEM (SEM, rather than SD, is shown here for ease of comparison with Fig. 1 of ref. [19]). The number of spermatozoa in the capillaries was determined by direct counting. Hatched columns, active fraction in the capillary; empty columns, BWW in the capillary.

chemotaxis chamber [10], was a small signal and a large standard deviation, for which reason a large number of experiments was required to obtain statistically significant results [19]. In the present study, we initially wished to determine whether the low signal-to-noise ratio in our assays was a reflection of a nonoptimal assay, nonoptimal experimental conditions, or an intrinsic physiological property of the spermatozoa.

Capillary assays with an ascending gradient of follicular fluid. To address the first possibility, and to try to increase the signal-to-noise ratio, we tested a number of different assays, configurations, and techniques. The best results were obtained using the system (shown in Fig. 1) consisting of spermatozoa in teflon wells and various dilutions of follicular fluid in capillaries. This assay is essentially a modification of the capillary assay developed by Adler [29] for the study of bacterial chemotaxis. Figure 3 shows the time-dependent accumulation of spermatozoa in an active follicular fluid (diluted 3000-fold, the activity of a follicular fluid defined herein as its ability to attract spermatozoa [19]). When compared with the standard deviation in previous studies, in which a 48-well chemotaxis chamber was used [19], the standard deviation in this assay was indeed lower. However, the signal (i.e., the ratio or the difference between the follicular fluid-containing capillaries and BWW-containing capillaries) was as low as before (cf. Fig. 1 in ref. [19]). With all the techniques we tested, including this assay, there was a large variability in the degree of activity among the tested follicular fluids (all derived from follicles containing fertilizable eggs); a few follicular fluids had no detectable activity at all. Hereafter, we pretested follicular fluids by the capillary assay and used only the active fluids. The dependence of the sperm accumulation on the concentration in the capillaries of the follicular fluid's active fraction (described in the subsequent section), is shown in Figure 4. The dependence was bell-shaped, typical of chemotaxis [29].

Optimizing the sperm parameters. Since the assay was not the cause of the low signal-to-noise ratio, we examined whether or not the experimental conditions were the cause. In our earlier studies we used only "swim-up" spermatozoa (i.e., highly motile spermatozoa collected from the upper layer of a suspension that had not been stirred for 2 h) [19], as for current procedures of in vitro fertilization [30]. In the present study, we used the total sperm population rather than the swim-up subpopulation, since we observed that the responses of the two populations were similar (Fig. 5). (This observation is in apparent disagreement with our previous report [19], where we stated that only the swim-up fraction was responsive; the difference is due to the 2-h incubation period in the current study.) The similarity in responses is in accordance with recent findings that both populations are similarly effective in hamster egg penetration tests and in vitro fertilization ([31] and Y. Barak and L. Yogev, personal communication). Since spermatozoa are known to undergo a time-dependent process of capacitation [32, 33], we incubated the total sperm population for different periods of time and measured their responsive-

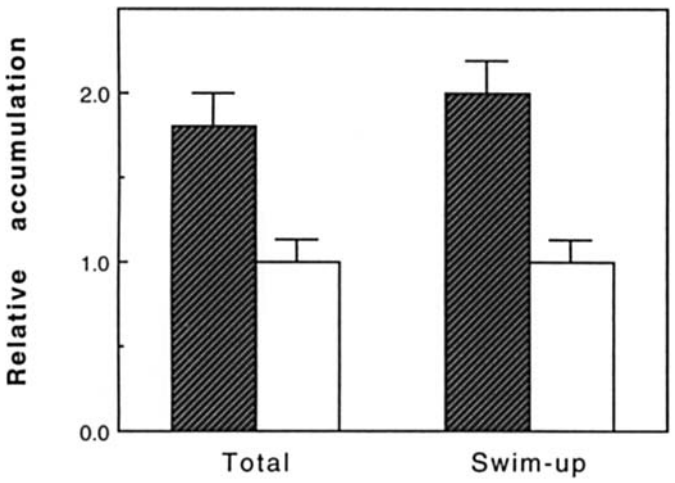

FIG. 5. Accumulation assay of the swim-up fraction and total population of spermatozoa. The swim-up fraction was isolated as previously described [19]. The experiment was carried out as described in the legend to Figure 3, except that the wells contained 1.5×10^7 cells/ml and the duration of the assay was 40 min. The total population was prewashed and incubated for 2 h at 37°C in a 5% CO_2 incubator as was the swim-up fraction. Both sperm populations were normalized according to the accumulation of spermatozoa in BWW. Relative accumulation is defined as the ratio between the number of spermatozoa accumulated in the capillary and the average accumulation in the control of the same experiment (BWW in both the well and the capillary). The number of spermatozoa in each capillary was estimated by the endopeptidase assay. Data shown are of a representative experiment (total of 5). Each point represents six determinations ± SEM. The results with the two populations were similar, as revealed from Student's *t*-test ($p = 0.51$). Empty columns, BWW-containing capillaries; hatched columns, follicular fluid-containing capillaries.

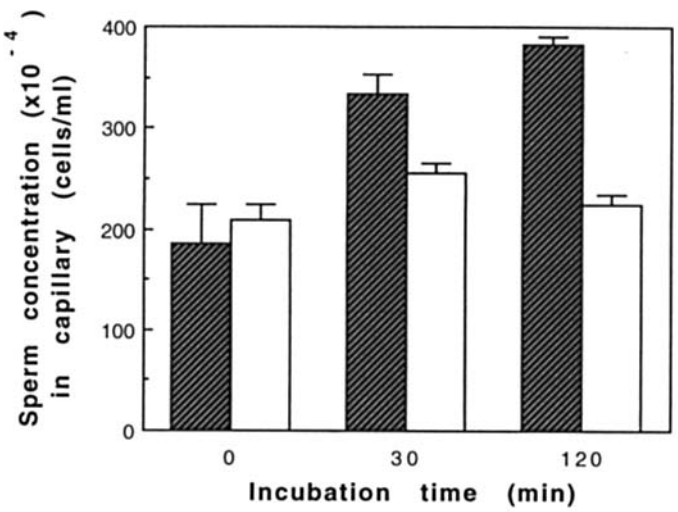

FIG. 6. Effect of sperm preincubation on sperm accumulation. The experiment was conducted as in Figure 3, except that spermatozoa were preincubated for the indicated periods of time and duration of each assay was 40 min (in Fig. 3, length of preincubation was constant and duration of the assay varied). The number of spermatozoa in each capillary was estimated by the endopeptidase assay. Results shown are of a typical experiment (total of 9). Empty columns, BWW-containing capillaries; hatched columns, follicular fluid-containing capillaries.

ness to follicular fluid. As shown in Figure 6, sperm incubation was a prerequisite for observing sperm accumulation in follicular fluid. These results suggest that spermatozoa undergo some physiological change, possibly capacitation, before they can respond to follicular fluid. We also examined the temperature effect on sperm accumulation in follicular fluid and found that the accumulation was similar at room temperature and at 37°C.

The observation of low signals and varied follicular fluid activities in all of our assays, even under optimal experimental conditions, indicated that the cause of the low signal and the varying activity was physiological rather than technical.

Determination of the Mechanism of Sperm Accumulation in Follicular Fluid

Having optimized the experimental conditions (with respect to the assay, temperature, and sperm incubation time), we examined the processes involved in sperm accumulation. In principle, apparent sperm accumulation in capillary assays could result from chemotaxis, chemokinesis, or trapping [4, 19]. (Trapping may result from a negative effect of follicular fluid on motility, from a change in swimming behavior at a particular concentration of follicular fluid [distinct from chemotaxis, where the change in swimming behavior is in response to a gradient of a chemical], from mechanical effects such as adsorption to glass or capillary, or from any combination thereof.) To distinguish among chemotaxis, chemokinesis, and trapping, we carried out a variety of assays, both macroscopic and microscopic.

Capillary assays with a descending gradient of follicular fluid. Distinction between these processes is difficult because of the low signal. Therefore, we carried out assays in which the direction of the chemotactic effect was opposite to that of the chemokinetic effect, and in which trapping (if it occurs) would affect both processes similarly. In other

TABLE 1. Sperm accumulation in capillary assays with a descending gradient of follicular fluid or no gradient at all.

Setting no.	Follicular fluid[a] in Well	Follicular fluid[a] in Capillary	Relative sperm accumulation in capillary[b]
1	+	−	1.05 ± 0.11
2	+	+	1.69 ± 0.16
3	−	−	1.00 ± 0.05

[a]The follicular fluid was 1000-fold diluted.

[b]Relative accumulation is defined as the ratio between the number of spermatozoa accumulated in the capillary and the average accumulation in the control of the same experiment (BWW in both the well and the capillary). The experiment was carried out as described in *Materials and Methods* using the system shown in Figure 1. Each well contained 1.5×10^7 spermatozoa/ml. The assays were carried out at 37°C for 40 min. Each result represents 36 determinations (3 donors of spermatozoa; 2 experiments with each donor; 6 capillaries in each experiment) ± SEM. The number of spermatozoa in the capillaries was determined by direct counting. The two-way factorial ANOVA test showed no significant interaction between the donors and the settings. The *p* value for the settings was 0.0001. According to the Fisher test, the differences between setting no. 1 vs. no. 2 and between no. 2 vs. no. 3 were significant at the 99%-level.

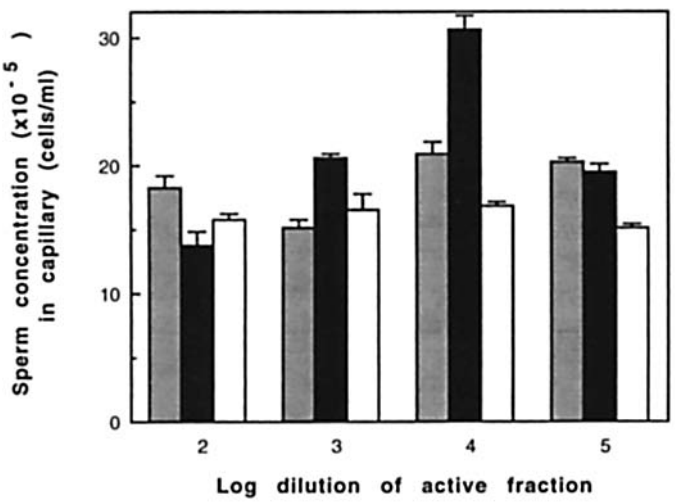

FIG. 7. Concentration dependence of sperm accumulation in capillary assays with a descending gradient of follicular fluid or no gradient at all. Capillary assays were carried out as in Table 1, except that they were performed at room temperature (25°C) and the active fraction of follicular fluid (described in Fig. 4 legend) was used instead of the whole fluid. Results shown are of a typical experiment (total of 13). Each point represents the mean of six determinations ± SEM. The active fraction was diluted in BWW to the extent indicated. Gray columns, active fraction in the well (like setting no. 1 in Table 1); black columns, active fraction in both the well and capillary (like setting no. 2); empty columns, BWW in both the well and capillary (like setting no. 3).

words, rather than measuring the tendency of the spermatozoa to accumulate in follicular fluid, as in the experiments shown in Figures 3 and 4, we measured their tendency to leave the follicular fluid. The spermatozoa in the wells were suspended in follicular fluid, and the capillaries contained either BWW (Table 1, setting no. 1) or follicular fluid (setting no. 2). In the former setting, the spermatozoa in the well sensed a descending gradient of follicular fluid as they moved to the capillary; in the latter setting, they sensed no gradient at all. A control for lack of gradient in the absence of follicular fluid was also carried out (setting no. 3). The concentration dependence of the sperm accumulation in these settings is shown in Figure 7. The results suggest that both chemotaxis and chemokinesis occur. Chemotaxis is suggested by the lower accumulation in the capillary under conditions of a descending gradient of follicular fluid vs. no gradient at all (setting no. 1 vs. no. 2). Chemokinesis is manifested by the higher number of spermatozoa that reached the capillary when both the well and the capillary contained follicular fluid vs. when both of them contained just BWW (setting no. 2 vs. 3). Trapping as a sole cause may be ruled out because the spermatozoa in settings 1 and 2 were already suspended in the "trap" (i.e., in follicular fluid) prior to the assay.

Capillary assays with spermatozoa having exceptionally high motility. To determine unequivocally whether or not chemotaxis occurred on top of the chemokinetic effect seen in Table 1, we repeated the experiments with a follicular fluid and with an active fraction thereof (described in the legend to Fig. 4), but this time used spermatozoa from sperm donations with exceptionally high motility. (The motility of washed spermatozoa was measured by capillary assays in which both the well and capillary contained BWW [i.e., like setting no. 3 in Table 1]). The criterion for exceptionally high motility was that more than 10% (e.g., 15–25%) of the spermatozoa in the well accumulated within 1 h in the capillary. (The accumulation level of spermatozoa with regular motility varies between 1 and 10%.) We observed that the motility of such spermatozoa cannot be further enhanced by either follicular fluid or drugs that normally stimulate sperm motility (e.g., caffeine [34]). Table 2 contains the results.

TABLE 2. Capillary assays with spermatozoa having exceptionally high motility.

Setting no.	Follicular fluid or active fraction in[a]		Relative sperm accumulation in capillary[b]	
	Well	Capillary	Follicular fluid	Active fraction
1	+	–	0.57 ± 0.04	0.71 ± 0.03
2	+	+	1.11 ± 0.04	0.97 ± 0.07
3	–	–	1.00 ± 0.04	1.00 ± 0.04

[a]The follicular fluid and active fraction were diluted 10^4 and 10^3, respectively.

[b]The experiments were carried out as in Table 1. The numbers are the mean ± SEM of 6 determinations in each experiment. The difference between settings 1 and 2 was statistically significant ($p < 0.003$ according to Student's *t*-test); it was not significant between settings 2 and 3.

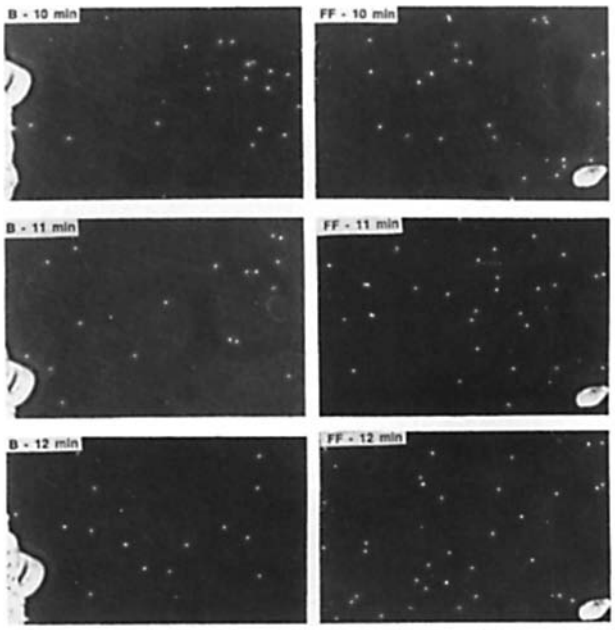

FIG. 8. Distribution of spermatozoa near wells containing follicular fluid and BWW in a microscopic assay for chemotaxis. Spermatozoa appear as small dots. The photographs were taken near the BWW-containing (B) and follicular fluid-containing (FF) wells at 1-min intervals, beginning 10 min after the chamber had been sealed (time at which each photograph was taken shown at upper left). The follicular fluid was 1:10 diluted in BWW. The rim of the B well is shown at left on each left-side photograph; the FF well was to the right of each right-side photograph (not seen in photographs). The figure represents a typical experiment from a total of 19. ×75.

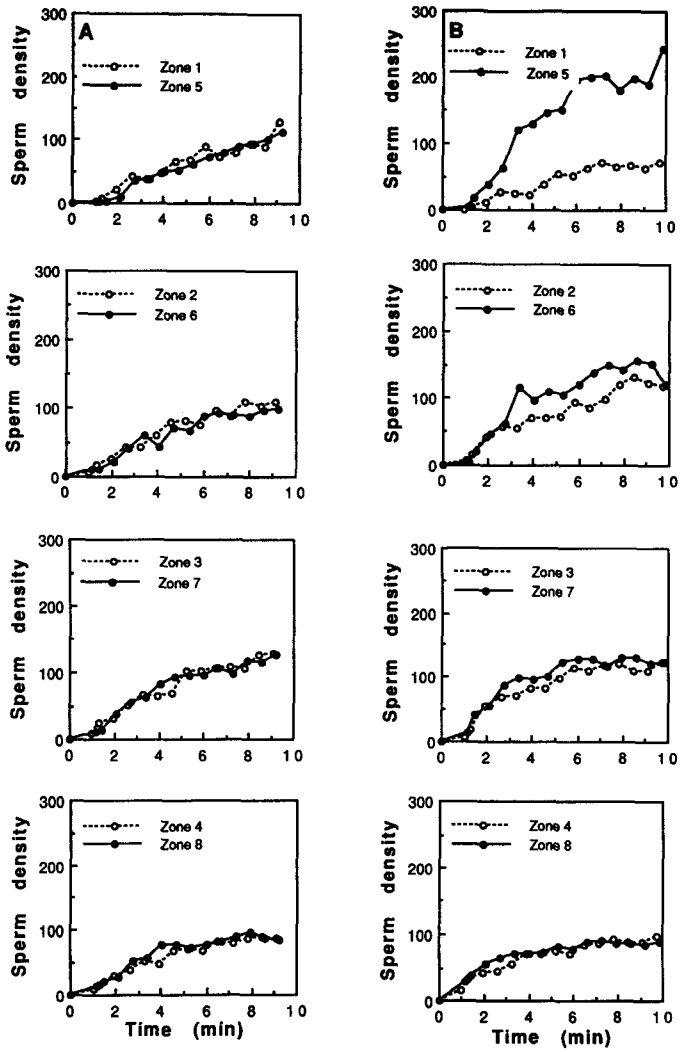

FIG. 9. Time-dependent distribution of spermatozoa in a microscopic assay for chemotaxis. Sperm density is expressed in spermatozoa per mm^2. Sperm concentration in the wells was 5×10^8 cells/ml. Open circles, zones 1–4; solid circles, zones 5–8. A) both wells containing BWW; B) left well (near zones 1–4) containing BWW, right well (near zones 5–8) containing the active fraction (diluted 10-fold) described in the legend to Figure 4. The figure represents a typical experiment (total of 6), in which both parts A and B were carried out with the same sperm sample. Additional experiments were carried out with different sperm samples (from 10 donors) that included part A only (14 experiments) or part B only (129 experiments).

sults of two typical experiments of this kind. Even though the chemokinetic effect was very small or even nonexistent with such spermatozoa (setting no. 2 vs. no. 3), their accumulation in the capillaries under conditions of a descending gradient of follicular fluid vs. no gradient at all was significantly lower (setting no. 1 vs. no. 2). This observation, made in the absence of chemokinesis, provides strong evidence in favor of the occurrence of chemotaxis.

Sperm accumulation in a sealed chamber. We followed microscopically the response of spermatozoa to a spatial gradient of follicular fluid in a sealed chamber that had been developed for this purpose ([23]; Fig. 2). As shown

TABLE 3. Pattern types of trajectories made by spermatozoa entering zone 1 or 5.

Behavioral category[a]	Spermatozoa entering zone #1 (BWW)		Spermatozoa entering zone #5 (active fraction)	
	%[b]	Swimming speed[c]	%	Swimming speed
1	12	44 ± 7	21	51 ± 14
2	7	46 ± 7	15	48 ± 13
3	36	nonapplicable	38	nonapplicable
4	45	47 ± 12	26	47 ± 12

[a]The categories (cf. Fig. 10) are:
#1—Distinct directional changes towards the well.
#2—As category #1, but the spermatozoa did not stay near the well (i.e., they left zone 1 or 5).
#3—Trajectories of spermatozoa which, at the moment of the initiation of video recording, were close to or already in zone 1 or 5.
#4—The rest of the trajectories.
[b]The total numbers of trajectories analyzed (100%) were 42 and 89 for zones 1 and 5, respectively. The sample size was chosen as such that the average would not change upon further increase in the number of tracks analyzed.
[c]The swimming speed (in μm/s) was calculated for the section of the trajectory out of zone 1 or 5 and is the mean ± SD of all the trajectories of the category.

in Figure 8, the number of spermatozoa near the follicular fluid-containing well was 1.3-fold to-2.2-fold higher than near the BWW-containing well, which was free of follicular fluid. All the spermatozoa seen in the chamber were motile. These results are in line with sperm chemotaxis; if the only effect of follicular fluid on spermatozoa had been enhancement of motility, the accumulation near the follicular fluid-containing well would be expected to be lower than near the control well. As before, the difference between the sperm numbers near the wells was not dramatic. This is not surprising in the light of our finding that, at a given time, only a small fraction of the sperm population responds to follicular fluid (see below). In this assay, as in the capillary assay, the correct dilution of follicular fluid was essential.

The time-dependence distribution of the spermatozoa in the sealed chamber is shown in Figure 9 for the active fraction of follicular fluid described in the legend to Figure 4. When the content of both wells was identical (either BWW [Fig. 9A] or follicular fluid [not shown]), the numbers of spermatozoa near each of the wells were similar. When the content of the wells was different, the sperm density near the well with the active fraction was higher from the very beginning. In accordance with the diffusion of the active fraction from the well, the difference was the largest and first apparent between zones 5 and 1 (closest to the wells); in the more remote zones, the difference was smaller and apparent at later stages (Fig. 9B).

Sperm trajectories in a gradient of follicular fluid. Chemotaxis, unlike chemokinesis and trapping, is expected to involve typical directional changes of the spermatozoa towards the source of the attractant. We therefore compared the trajectories of the spermatozoa found near the well containing the active fraction (i.e., the test well containing the presumed attractant), with those found near the BWW-containing well (the control well). Various patterns

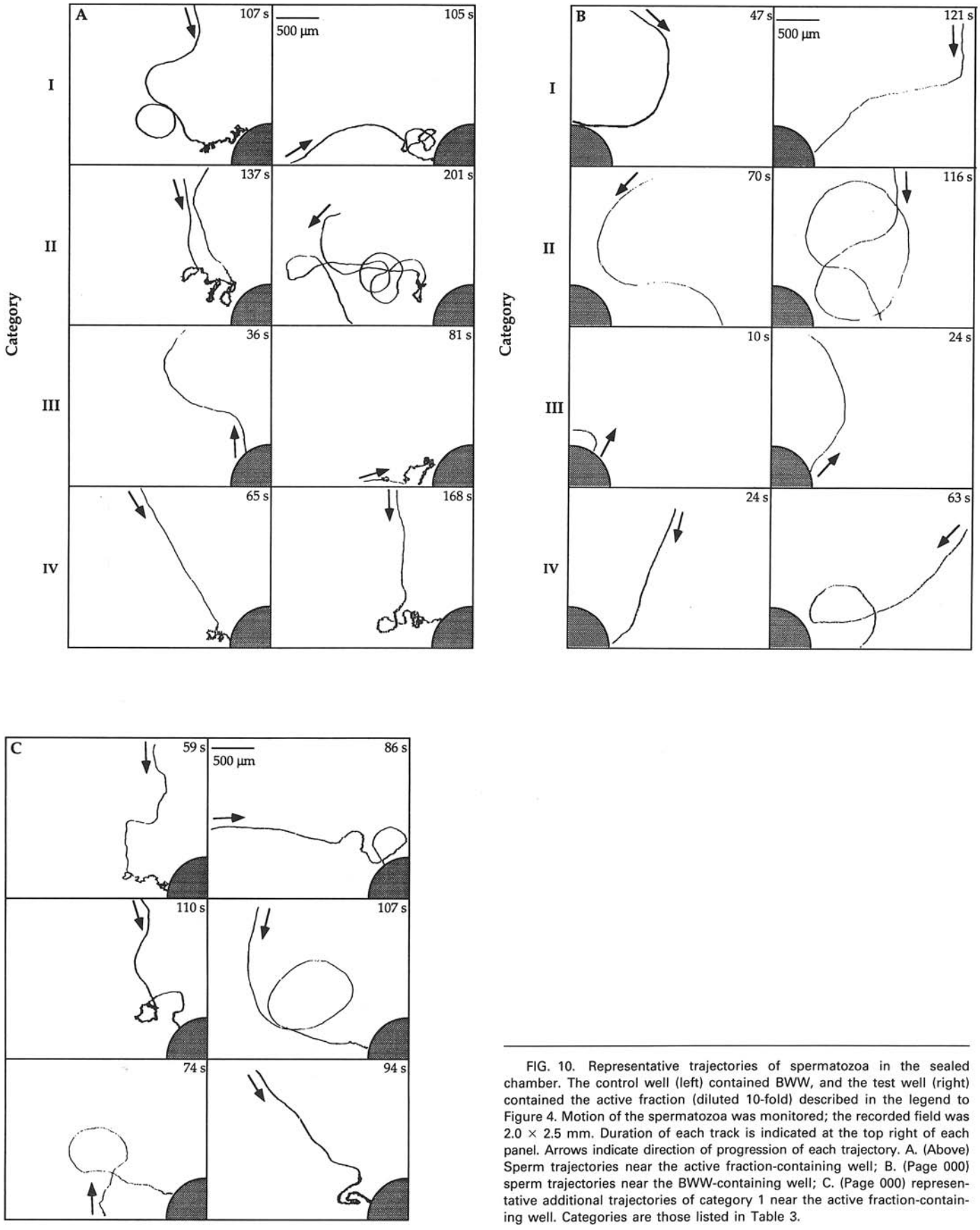

FIG. 10. Representative trajectories of spermatozoa in the sealed chamber. The control well (left) contained BWW, and the test well (right) contained the active fraction (diluted 10-fold) described in the legend to Figure 4. Motion of the spermatozoa was monitored; the recorded field was 2.0 × 2.5 mm. Duration of each track is indicated at the top right of each panel. Arrows indicate direction of progression of each trajectory. A. (Above) Sperm trajectories near the active fraction-containing well; B. (Page 000) sperm trajectories near the BWW-containing well; C. (Page 000) representative additional trajectories of category 1 near the active fraction-containing well. Categories are those listed in Table 3.

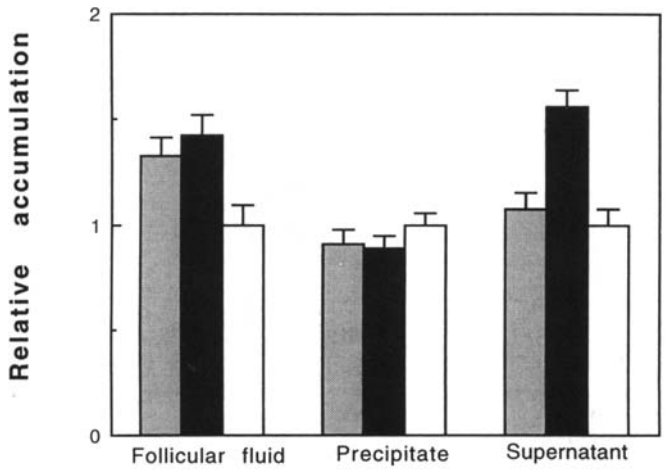

FIG. 11. Chemotactic and chemokinetic activities per volume of follicular fluid before and after precipitation by 90% acetone. Chemotactic and chemokinetic activities of supernatant and pellet fractions were determined (diluted 10^3-fold) by assays carried out as in Table 1. Protein concentrations of the original fluid, precipitate, and supernatant in the assay (after dilution) were 15, 8.5, and 0.24 μg/ml, respectively. Each column represents 36 determinations (3 donors of spermatozoa; 2 experiments with each donor; 6 capillaries in each experiment), normalized according to the accumulation of spermatozoa in BWW ± SEM. Gray columns, test solution in the well (like setting no. 1 in Table 1); black columns, test solution in both well and capillary (like setting no. 2); empty columns, BWW in both well and capillary (like setting no. 3).

of trajectories were observed, which were categorized by us into four groups. Representative examples of these groups observed near the test and control wells are shown in Figure 10A and 10B, respectively. The prevalence of each group, as well as its average swimming speed, is shown in Table 3. The following conclusions could be drawn from the figure and table. 1) A significantly larger number of spermatozoa exhibited distinct directional changes towards the test well than towards the control well (Table 3, category 1). 2) Within this category, there were marked differences between the trajectories near the test and control wells: the trajectories approaching the test well had more frequent and sharper directional changes (Fig. 10, A vs. B). This is further demonstrated in Figure 10C, which includes additional typical trajectories of category-1 spermatozoa near the test well. Of the spermatozoa approaching the test well, 84% acquired, near the well, motility patterns resembling hyperactivation (e.g., wide amplitude and marked lateral displacement of the head [25, 35]; Fig. 10A); none acquired hyperactivation-like motility near the test well (Fig. 10B). 3) Some spermatozoa exhibited distinct directional changes towards the wells but left the zone after a while (Table 3, category 2); most of the spermatozoa belonging to this category spent more time near the test well (Fig. 10A) than near the control well (Fig. 10B). Again, 69% of the spermatozoa of this category acquired hyperactivation-like motility near the test well, but none did near the control well. 5) Hyperactivation-like motility was also observed in the other categories (71–80%), but only near the test well (Fig. 10). These results are in line with sperm chemotaxis and with the notion that, at any given time, only a small fraction of the sperm population is responsive.

Partial Fractionation of Follicular Fluid

From the above studies it appeared that, under well-controlled conditions, sperm attraction can be observed and that this attraction is the result of chemotaxis and chemokinesis and, ultimately, hyperactivation-like motility. At this stage we initiated studies towards the identification of the attractant(s) (denoted below as chemotactic factor[s]) in the follicular fluid. Using a Centricon-10 microconcentrator, we determined that the molecular size of at least one of the chemotactic factor(s) was smaller than 10 kDa. We precipitated the proteins of the follicular fluid with 90% acetone and examined chemotactic and chemokinetic activities in the pellet and supernatant fractions after evaporation of the acetone. Figure 11 shows the results of capillary assays, like those described in Table 1, which indicate that both the chemotactic and chemokinetic activities were found only in the supernatant. This was confirmed by microscopic assays (Fig. 9). The specific chemotactic and chemokinetic activities (per protein content; in most other cases of sperm chemotaxis the attractant is a peptide [1–3]) of the supernatant were >60-fold higher than those of the original follicular fluid, and both were concentration-dependent (Fig. 7).

Acetone at 90% concentration does, indeed, precipitate proteins, but it is impossible to make a distinction between hydrophobic and hydrophilic substances at this concentration. After 100% acetone precipitation, the chemotactic and chemokinetic activities (determined by both the capillary and microscopic assays) were mainly in the pellet; in some experiments, a little activity was also observed in the supernatant (not shown). This appears to indicate that the potential chemotactic factor(s) is nonhydrophobic, possibly a short peptide or a similar molecule.

DISCUSSION

In this study, we have demonstrated that follicular fluid stimulates both chemotactic and chemokinetic activities of the spermatozoa and, ultimately, hyperactivation-like motility. We have found that these activities are due to a factor(s) within the follicular fluid that is probably nonhydrophobic, smaller than 10 kDa (at least one of the factors, if there are more than one), and apparently not a protein (but possibly a short peptide). We have shown that, because of specific physiological requirements fulfilled by only a fraction of the sperm population, the chemotaxis and chemokinesis phenomena are not easily detectable. Below we discuss these findings in detail.

Physiological Parameters that Affect Detectability of Chemotaxis and Chemokinesis

The main difficulty in the detection of chemotaxis and chemokinesis in human spermatozoa is that, at any given

time, only a small fraction of the sperm population is responsive. Although this fractional response is very important physiologically (see next section), it constitutes a technical nuisance in that it leads to a low signal in the assays, and sometimes even to false-negative results. This difficulty makes the assays very vulnerable to any deviation from the optimal experimental and physiological conditions. These involve preincubation of the spermatozoa (presumably to ensure fractional capacitation) and proper dilution of the follicular fluid. Insufficiently diluted follicular fluid may lead to both false-negative and false-positive results. False-negative results, in all types of assays, may be obtained when the concentration of the chemotactic factor(s) (i.e., of follicular fluid) is too high and may saturate the putative receptors on the sperm surface and prevent them from sensing the gradient. (In accumulation assays [e.g., Fig. 4], the receptors are saturated outside of the capillary, and the spermatozoa are not able to detect the concentration gradient of the chemotactic factor[s] and follow it into the capillary; in capillary assays with a descending gradient of follicular fluid [setting no. 1 in Table 1 or Fig. 7], the spermatozoa cannot sense the descending gradient and freely leave the well; in the microscopic assay [e.g., Fig. 9], the optimal concentration of the chemotactic factor[s] is established at too great a distance from the well, beyond the observation zone.) False-positive results may be obtained because non-diluted, or diluted less than 10-fold, follicular fluids (or even human or bovine serum) may cause nonspecific sperm accumulation by virtue of trapping [4]. In view of this information, earlier conclusions about the occurrence of sperm chemotaxis to follicular fluid [20–22] and about the inability to observe chemotaxis [23] should be evaluated with great caution. All these published studies used nondiluted or only slightly diluted (1:4) follicular fluids.

Distinction between Processes that Can Cause Sperm Accumulation

Two general criteria distinguish chemotaxis from chemokinesis and trapping. 1) A gradient of attractant is a prerequisite for the occurrence of chemotaxis but not of chemokinesis and trapping. (Note, however, that the latter two may be concentration-dependent.) 2) Chemotaxis involves directional changes of swimming within the gradient so as to approach the attractant. The first criterion (i.e., dependence on the presence of a gradient) was tested by capillary assays and confirmed by microscopic assays. Both chemotaxis and chemokinesis were found to be involved. (Due to the variability between the swimming speeds of individual spermatozoa in the microscopic assays, the 16% increase in the average of swimming speeds near the well containing the active fraction vs. the well containing BWW [Table 3, category 1] was not statistically significant; the difference was statistically significant in the macroscopic assays [capillary assays], where the total sperm population was measured [Table 1].) This is reminiscent of sea urchin spermatozoa, where chemotaxis is accompanied by chemokinesis [36]. The second criterion (i.e., the occurrence of gradient-dependent turnings of motile cells towards the source of the attractant) was tested by individual track analysis of all the spermatozoa found near the wells (zones 1 and 5) at time periods during which the sperm accumulation in zone 5 was higher than in zone 1. The analysis indeed revealed frequent directional changes of some of the spermatozoa towards the test well (containing the presumed attractant), ending near the well with motility patterns resembling hyperactivation. Fewer spermatozoa swam towards the control well (containing BWW); they made less frequent and more moderate directional changes (apparently coincidental), and they did not at any stage acquire hyperactivation-like motility. Interestingly, some of the spermatozoa that exhibited distinct directional changes towards the wells left zone no. 5 after a while. This may indicate desensitization of some spermatozoa by the active fraction or loss of responsiveness to it [37].

A likely sequence of events may be that the spermatozoa sense a gradient of a chemotactic factor and follow the factor up the gradient while enhancing their speed of swimming; when they reach an optimal concentration of the factor (in vivo it is presumably a concentration that prevails near the egg), they become hyperactivated and consequently remain essentially in place in spite of their vigorous motility. It is not yet known whether the attractant itself causes hyperactivation or whether a different substance in the follicular fluid (e.g., progesterone [38]) is responsible for it. It is also not known whether the hyperactivation-like motility is part of the chemotaxis mechanism (as tumbling with no net translational movement is part of the chemotaxis mechanism in bacteria such as *Escherichia coli* [39, 40]) or a separate mechanism.

Physiological Significance

The significance of sperm chemotaxis and chemokinesis in humans, as in any other organisms in which the fertilization is internal, is not straightforward. Recently we were able to establish that, at any given moment, only 2–12% of the sperm population is responsive to the active fraction of follicular fluid [37]. This was done by letting spermatozoa be attracted to the active fraction and then examining the attracted and remaining sperm subpopulations. This result is well in line with the track analysis made in this study, which showed that only a fraction of the sperm population changed the direction of swimming towards the active fraction (Table 3). The responsive spermatozoa do not belong to an exclusive subpopulation; we found that, with time, the identity of the responsive spermatozoa changes [37]. Assuming that the responsive spermatozoa are the fertilizing spermatozoa (i.e., capacitated spermatozoa [32, 33] or a subfraction thereof), these results suggest physiological roles for the processes of chemotaxis and chemokinesis in humans. Accordingly, the role of chemotaxis is to select fer-

tilizing spermatozoa and to ensure their continuous availability for an extended period of time rather than the availability of all at once for a short period of time. The role of chemokinesis is to enhance the movement of the selected spermatozoa and thus improve their competitiveness with respect to the unselected cells. We have already put forward this possibility as a working hypothesis [4]. This proposed function of human sperm chemotaxis is different from the function of sperm chemotaxis in sea urchins (or in any other organism with external fertilization), where it is probably to direct as many spermatozoa in sea water to the egg as possible, or to attract rare spermatozoa or highly diluted spermatozoa to the egg. Although other interpretations cannot be ruled out, it seems that, in species in which the role of sperm chemotaxis is sperm selection, only the selected spermatozoa respond to the chemotactic factor; but in species in which the role is a directed movement, most (if not all) of the spermatozoa respond.

So far, most chemotactic factors or attractants for spermatozoa that have been identified are peptides or proteins of various sizes (1–20 kDa) that are heat stable and sensitive to proteases [1, 2]. An exception are the sperm attractants of corals, which are lipids of 140–250 Da [41]. The partial fractionation of follicular fluid in this study identified at least one of the chemotactic factor(s) as a < 10-kDa molecule(s). The observation that the chemotactic activity was only in the supernatant after 90% acetone precipitation and mostly (but not only) in the pellet after 100% acetone precipitation suggests either that the chemotactic factor(s) is not a large protein, lipid, or steroid-like molecule but rather a nonhydrophobic substance such as a short peptide, or that there is an additional chemotactic factor that is hydrophobic (therefore retained in 100% acetone supernatant). Although in this study chemotaxis was accompanied by chemokinesis, at this stage it is not yet known whether both activities can be attributed to one factor or whether separate chemotactic and chemokinetic factors are involved. In the case of sea urchin spermatozoa, the attractant (resact, a 14-amino acid peptide, isolated from the jelly layer of the sea urchin egg) has a dual function [3, 36].

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