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Herpesvirus, cytomegalovirus, human sperm and assisted fertilization

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BACKGROUND: The effect of viral particles on the motility of human sperm and the relationship between sperm and virus are of importance particularly in assisted fertilizations. METHODS: We incubated ejaculated sperm with or without seminal fluid with either herpes simplex virus type 2 (HSV2) or human cytomegalovirus (HCMV). For each experiment, 5×10^5 sperm were incubated with a viral load of between 10^4 and 10^6 plaque-forming units. RESULTS: We detected no apparent variations in the percentage of motile forms when sperm were incubated with either HSV2 or HCMV. Using a computer-aided semen analysis system, a slight difference was reported in the percentage of motile forms when seminal fluid-free sperm were incubated with HSV2 (57.18 versus 64.43 in the control). Although the mean amplitude of lateral head displacement and the curvilinear velocity were significantly higher in infected sperm, the difference in straight line velocity was not statistically significantly different. Few viral particles (HSV2 or HCMV) adhered to the sperm membrane in the presence of seminal fluid. However, more particles stuck when in the absence of seminal fluid, particularly with HSV2 (8% of sperm sections for HSV2; 4% for HCMV). CONCLUSIONS: The relationship between sperm and viruses depends on the type of virus present as well as the presence or absence of seminal fluid. Motility is not a good enough criterion on which to prove the presence of viral elements, either in the medium or on the sperm.

Key words: cytomegalovirus/Herpesviridae/human sperm/motility/seminal fluid

Introduction

Several successful new assisted reproductive techniques have been developed over the last decade. It is now possible to take a selected spermatozoon from a given point in the genital tract and to transfer it into the ooplasm. This technique, known as ICSI, involves the use of micromanipulations to move sperm across the zona pellucida and the plasma membrane. The ICSI technique has enabled sperm that would otherwise be unable to fertilize the oocyte to do so, resulting in successful pregnancies. The sperm can be extracted from different regions of the genital tract, e.g. from the testes or from the epididymal duct (Lewin *et al*., 1999), or from semen samples. The extraction of testicular sperm followed by ICSI is a convenient way of overcoming non-obstructive azoospermia (Devroey *et al*., 1996). Several methods can be used to process the extracted sperm. The choice of method depends on the facilities available and the physiological condition of the sperm. These methods include isolating a simple swab in a synthetic medium (De Croo *et al*., 2000), washing by centrifugation (Devroey *et al*., 1996), selection on a Percoll gradient (Mercan *et al*., 2000) and swim-up migration (Marina *et al*., 1998). Although these techniques can solve many fertility problems, they also raise questions about the risks of transferring viral particles into the oocyte.

Several viruses are present in the genital tract. The most common are the herpes simplex virus type 2 (HSV2) and human cytomegalovirus (HCMV), which are sexually transmissible and can lead to fetal and neonatal anomalies (Huraux *et al*., 1999; Ranger-Roger *et al*., 1999). Sperm are formed in seminiferous tubules in the testes, where they are protected from the environmental tissue by the haematotesticular barrier. We previously showed that no viral particles are found within the seminiferous tubules in MCMV-infected murine testes. However, the peritubular cells, Leydig cells and endothelial cells contained high viral loads (Tebourbi *et al*., 2001). Several other papers have reported the presence of HSV2 (Deture *et al*., 1976) and of CMV (De Paepe *et al*., 1990) in the testes, but this does not automatically mean that the seminiferous tubules were contaminated (Baskar *et al*., 1986; Stephanopoulos *et al*., 1989). However, viruses may be present in the epididymis (Dalton and Harcourt-Webster,

1991; MacCarthy *et al*., 1991), deferent duct (Kimura *et al*., 1993), prostate (Boldogh *et al*., 1983; Benson and Smith, 1992; Mastroianni *et al*., 1996) or seminal vesicles (Deture *et al*., 1976; Kimura *et al*., 1993). If the virus is present in the annexial glands, it may be discharged in the seminal fluid before coming into contact with sperm. The presence of the virus in the semen indicates a genital tract infection. One study (Moore *et al*., 1989) found infective HSV2 virus in the semen of a healthy donor with an asymptomatic primary infection, and showed that insemination led to the horizontal transmission of the virus. However, cell culture methods have rarely detected HSV2 in sperm (Deture *et al*., 1978; MacGowan *et al*., 1983), because semen contains inhibitors that may interfere with these methods (Deture *et al*., 1978; Sherman and Morgan, 1989). More sensitive methods, such as PCR and in-situ hybridization (ISH), have made it possible to detect viral DNA in the semen of men with genital HSV2 infection (Kotronias and Kapranos, 1998; Wald *et al*., 1999) and in the semen of a high-risk population (Tabrizi *et al*., 2000). HCMV has been identified in sperm (Lang and Kummer, 1972). The prevalence of HCMV elements was found to be variable in men who did not have human immunodeficiency virus (HIV) or other sexually transmitted diseases (Lang and Kummer, 1972; Bantel-Schaal *et al*., 1993; Shen *et al*., 1994; Mansat *et al*., 1997), but it may be high in high-risk populations (Biggar *et al*., 1983; Leach *et al*., 1993). Detection rates are generally higher for viral DNA than for infective particles. In a recent study involving men taking part in an IVF programme (Witz *et al*., 1999), 25% of ejaculates, including those from HCMV-negative men (who may or may not have gone on to become positive), were found to contain viral DNA.

Thus, sperm are protected from viral contact in the upper regions of the genital tract, i.e. the testes, whereas sperm in the semen can easily come into contact with viral particles (in the epididymis, deferent duct and annexial glands). This is important as sperm can be harvested from testicular biopsies for ICSI in cases of azoospermia. This then disrupts the haematotesticular barrier, thus removing the protective effect and placing the sperm in close contact with endothelial cells, Leydig cells and peritubular cells. Sperm can also be harvested from the other genital organs or from the semen. In this case, the fertilizing sperm for ICSI are choosen according to their motility. The relationship between the virus and sperm motility is not very clear and the interaction between sperm and virus are of importance.

Some authors have reported an inverse correlation between the presence of HSV2 in semen and sperm motility (Kotronias and Kapranos, 1998). Similar observations have been reported concerning CMV (Torino *et al*., 1987). A high concentration of HCMV was detected in the semen of a patient with CMV mononucleosis, and this was associated with a transient decrease in sperm motility (Lang and Kummer, 1972; Lang *et al*., 1974). We were interested to know whether this decrease in sperm motility was due to a direct effect of high concentrations of the virus on sperm or if these results were due to an indirect effect. Few papers have addressed the viral load of semen (Lang and Kummer, 1972; Biggar *et al*., 1983). Therefore it is not clear how the virus and sperm interact. No relationship has been found between sperm and HSV2 (Sherman and Morgan, 1989), whereas ISH has been used to demonstrate that there is a close relationship between sperm and HSV2 (Kotronias and Kapranos, 1998). Only one group has found CMV within human sperm (Huang *et al*., 1986); another group (Baskar *et al*., 1986) observed virus-like particles in mouse sperm.

We developed experimental models to analyse the effects of viruses on sperm motility and the interaction between viruses and sperm in assisted reproduction indications. On the one hand, we used semen, i.e. sperm plus seminal fluid, which is the physiological environment from which motile sperm are selected for intrauterine insemination, IVF and ICSI. On the other hand, we used sperm without seminal fluid (selected by use of a PureSperm gradient), which corresponds to sperm collected in the epididymis before the formation of seminal fluid or to sperm collected from testicular biopsies in azoospermic men for ICSI. The viruses used were HSV2 and HCMV. This study enabled us to study the relationship between viruses and sperm in different regions of the genital tract and to determine whether the new assisted reproduction techniques (especially ICSI in the case of azoospermia) affect the risks of virus adherence to sperm, leading to the introduction of viral particles into oocytes.

Materials and methods

Preparation of the viral strains

The viral strains, HSV2 (ATCC VR-2019) and HCMV (AD-169), were prepared from human embryo lung cells (MRC5, primary cells; Biomerieux, Lyon, France) in modified Eagle's medium (MEM; Gibco BRL, Life Technologies, Paisley, UK). The viral suspensions were titrated by an enzymatic assay method (visualization of early viral antigens) at a concentration of 2×10^7 plaque-forming units (PFU)/ml.

Human sperm

Sperm samples were taken from male patients undergoing IVF or who attended the American Hospital in Neuilly (France) with fertility problems. The selected patients all had $\geqslant$50% motile sperm, as tested in seminal fluid (six samples) or after selection on PureSperm (JOD, Lyon, France) gradient (five samples). For PureSperm gradient selection, 0.2–2 ml of semen was passed over two successive layers of PureSperm. The culture medium was used to prepare a 45% dilution of the upper layer and a 90% dilution of the 1 ml lower layer. After centrifugation (600 *g* for 20 min), the sperm pellet was washed in 10 ml PBS (600 *g* for 10 min) and then diluted in MEM.

Sperm incubated with virus

Sperm (5×10^5 sperm in 125 µl of MEM) were incubated with the virus ($<10^4$ to 10^6 PFU for 10^5 sperm) in a Petri dish (Corning 35 mm, tissue culture) for 1 h at 37°C in a moist atmosphere containing 5% CO_2. In the controls, the viral suspension was replaced by the culture medium used to prepare the cells.

Interaction between sperm and virus

Sperm motility

Analysis at the optical level. After 1 h incubation, 50 µl of each sample was placed on a slide and the percentage of motile sperm was assessed under the microscope by three independent observers.

Table I. Comparison of the percentage of motile sperm between seminal fluid-free sperm incubated with HSV2 and with culture medium by optical analysis

HSV2	PFU		
	10^4	10^5	10^6
Samples			
1	50 (50)	50 (50)	50 (50)
2		70 (70)	
3		50 (50)	
4		60 (60)	

Values are percentage of motile sperm after incubation with HSV2. The values in parentheses correspond to percentage of motile sperm after incubation with culture medium.

Table II. Comparison of the sperm motility parameters with computer-assisted semen analysis between seminal fluid-free sperm incubated with HSV2 and with culture medium

	Culture medium	HSV2
% motile sperm	64.43 (9.84)	57.18 (2.26)
VSL (μm/s)	25.66 (11.6)	22.97 (14.0)
VCL (μm/s)	67.23 (20.0)	82.90[a] (34.4)
LIN (%)	0.38 (0. 2)	0.27[a] (0.0)
ALH (μm)	2.46 (1.3)	3.48[a] (1.9)
LA (m/s^2)	474.84 (126.94)	608.62[a] (249)

Values in parentheses are SD.
[a]Correspond to values significantly different from control values ($P = 0.05$, Student's *t*-test).
VSL = straight line velocity; VCL = curvilinear velocity; LIN = linearity; ALH = mean amplitude of lateral head displacement; LA = linear acceleration.

Analysis with a CASA system. We used a CASA system to analyse sperm motility. The system used was composed of a microscope (Olympus BH×200), a high speed video camera (Lhesa-Electronic) with a video recorder (Sony time lapse 168) associated with a sytem consisting of a pentium PC, a digitalized Matrox PIP card and an original Siam-Trac software. The following parameters were studied: percentage of motile sperm, straight line velocity (VSL), curvilinear velocity (VCL), linearity (LIN) mean amplitude of lateral head displacement (ALH) and linear acceleration (LA).

For this analysis, sperm were selected on PureSperm gradient and incubated with HSV2 for 1 h (10^6 sperm/10^6 PFU in 125 μl DMEM). The control was treated in the same way but replacing the viral suspension with culture medium.

Ultrastructural analysis

The sperm were then incubated with the viral suspensions for 1 h in a glutaraldehyde solution (2.5% in Sörensen buffer). After washing several times with the same buffer to which 1 mol/l sucrose solution had been added, the sperm were incubated in osmic acid (1% in Sörensen buffer) for 1 h before dehydrating by successive passages in 70°, 90°, 95° (3×10 min) and 100° (3×20 min) ethanol. This was followed by a final incubation in araldite for 3 days at 60°C. Ultrathin sections were recovered on 600 mesh grids, before being stained with uranyl acetate in ethanol and then with lead citrate. The sections were examined under a Hitachi electron microscope at a magnification of ×12 000. The sections analysed were 10 μm away from each other. The proportion of sperm sections (95 nm thickness) in which viral particles were attached to the membrane or contained in the cell was determined for each experimental condition.

Paired *t*-tests were used to compare sperm motility (CASA) parameters of seminal fluid-free sperm incubated with either HSV2 or with culture medium. $P \leqslant 0.05$ was considered significant.

Results

Sperm motility

Analysis at the optical level

The percentage of motile sperm was evaluated after incubation with HSV2 (seven samples) or with HCMV (nine samples). Both viruses were tested on samples consisting of sperm plus seminal fluid (HSV2: three samples; HCMV: four samples), and samples of sperm separated from the seminal fluid on PureSperm gradient selection (HSV2: four samples; HCMV: five samples). Several viral concentrations were used ($<10^4$, 10^5 and 10^6 PFU). Table I summarizes the results obtained with seminal fluid-free sperm incubated with HSV2 culture medium. The percentage of motile sperm in the experimental and control samples did not differ significantly, regardless of the procedure, viral strain or viral concentration used.

Analysis with a CASA system

Table II summarizes the analysis of sperm motility parameters (CASA analysis) of seminal fluid-free sperm incubated with *HSV2* or with culture medium alone. A slight but insignificant decrease in the percentage motile sperm was reported when sperm were incubated with HSV2 (57.18 versus 64.43% for control). Although VSL was not different in the two groups (22.97 versus 25.66 μm/s for control), VCL differed (82.90 versus 67.23 μm/s for control; $P = 0.05$), lowering LIN in the experimental group (0.27 versus 0.38% for control; $P = 0.05$). ALH was significantly higher when sperm were in the presence of HSV2 (3.48 versus 2.46 μm for control; $P = 0.05$) as was the linear acceleration (LA; 608.62 versus 474.84 m/s^2; $P = 0.05$).

Ultrastructural analysis

Sperm with seminal fluid incubated with HSV2 or HCMV

HSV2. Two samples were subjected to ultrastructural analysis and viral particles were detected in both. The particles observed were in the form of complete virions and isolated capsids, but they were seldom found in contact with the sperm membrane (two particles in 310 sperm (0.6%) for sample 1, none in 154 sperm for sample 2).

HCMV. Two samples underwent ultrastructural analysis. Complete and incomplete viral particles, and dense bodies were found to co-exist. Only a few of the sperm were in close contact with viral particles [two out of 238 sperm for sample 1 (0.8 %), and two out of 319 sperm for sample 2 (0.6 %)].

Sperm without seminal fluid incubated with HSV2 or HCMV

A comparative study was carried out using sperm obtained from the same ejaculate and selected by PureSperm gradient. They were incubated with either HSV2 or HCMV.

HSV2. We found 28 viral particles in close contact with the 350 sperm sections observed (8%). The viral particles were either very near the membrane or directly stuck to the membrane of the sperm head (Figure 1E could correspond to

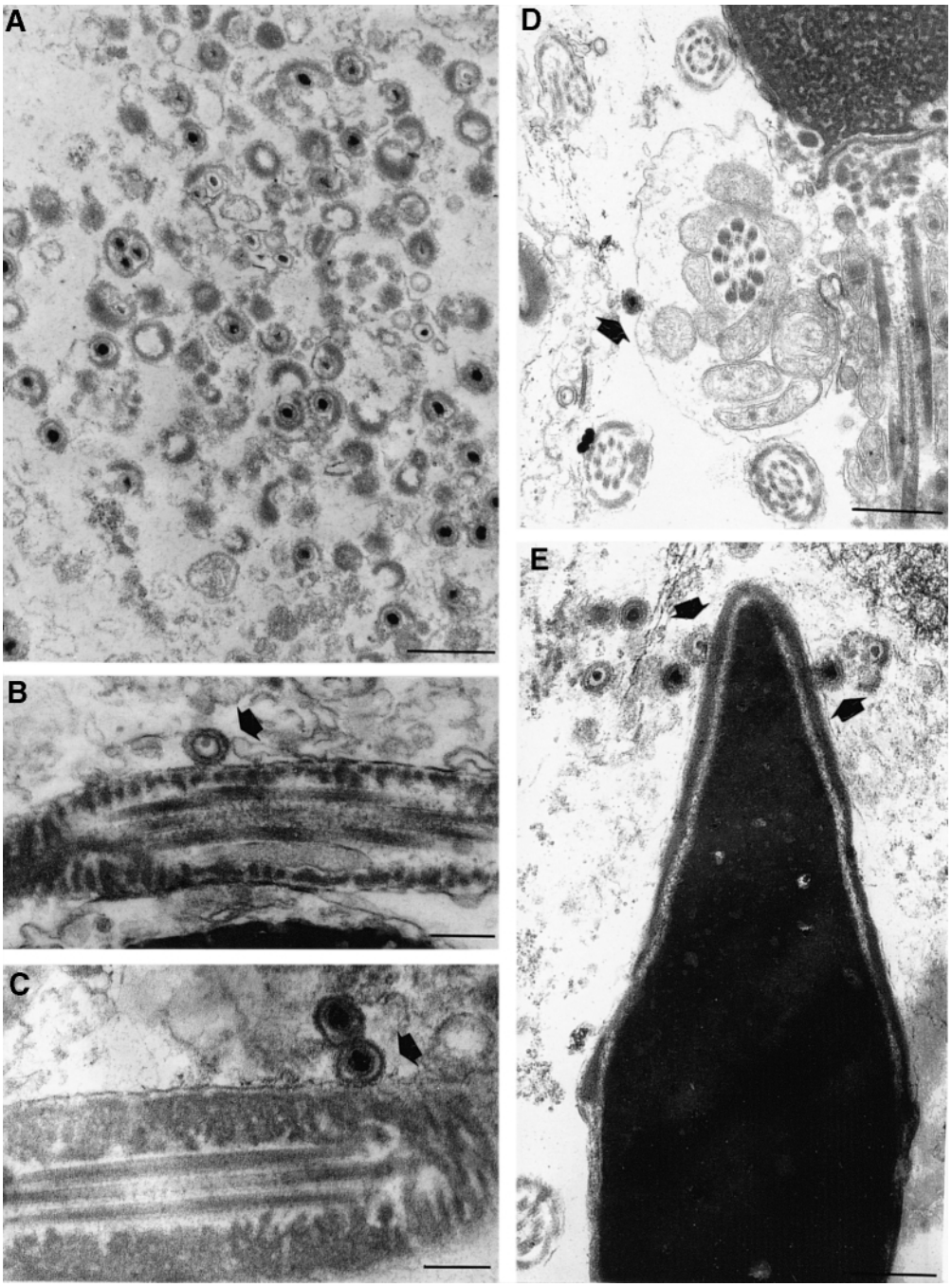

Figure 1. (**A**) Herpes simplex virus 2 (HSV2) viral particles in the sample. The viruses were present in the form of the complete virion with one or several capsids, or in incomplete forms with a full or empty capsid and thick electron-dense coat. Bar = 500 nm. (**B**, **C**) Two sperm with viral particles (arrows) attached to the membrane of the principal piece. Bar = 200 nm. (**D**) HSV2 viral particle (arrow) attached to the membrane of the intermediate piece. Bar = 500 nm. (**E**) Viral particles (arrows) attached to the sperm membrane of the acrosome. Bar = 500 nm.

fusion of HSV2 virion with the sperm membrane) or of the flagella (Figure 1B,C,D). We did not detect any viral particles inside the sperm. Viral particles were also observed either in isolation or in clusters in the preparation (Figure 1A), showing that they had not been removed from the sperm sample exposed to HSV2 despite the washes carried out before inclusion in araldite.

HCMV. Isolated and clustered viral particles were also observed in the sperm sample incubated with HCMV (Figure 2A). We observed viral particles close to the membrane (Figure 2B) in 15 of the 381 sperm sections analysed (4%), implying that the relationship between the sperm and viral particles may be weaker for HCMV than for HSV2.

Discussion

We have clearly shown that: (i) high concentrations of HSV2 or HCMV do not have a strong effect on the percentage of motile sperm regardless of the viral concentration used or the method used to prepare the sperm; however, motility para-

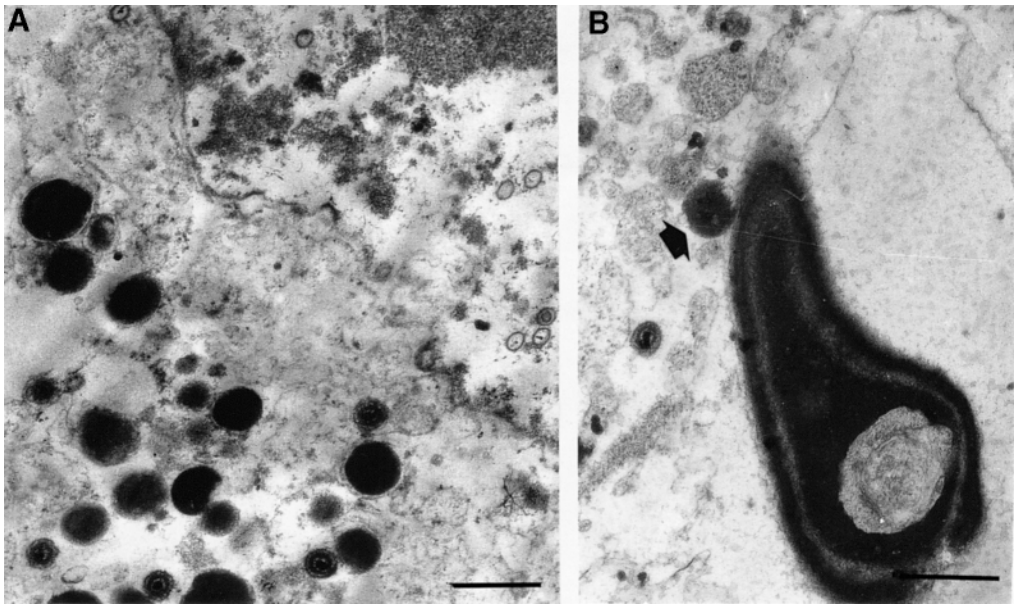

Figure 2. (**A**) Human cytomegalovirus particles. The viruses were present in the form of complete virions with one capsid, or in incomplete forms with empty isolated capsids, or dense bodies. Bar = 500 nm. (**B**) Dense body (arrow) attached to the sperm membrane. Bar = 500 nm.

meters detected by CASA differed when HSV2 was added to seminal fluid-free sperm; (ii) there was little interaction between HSV2 or HCMV and human sperm when the viruses were incubated with the semen (sperm plus seminal fluid); HSV2 and HCMV behaved differently when incubated with sperm in the absence of seminal fluid; more HSV2 adhered to sperm than HCMV.

Some studies have shown that the presence of viral particles in the semen is correlated with a lower percentage of motile sperm (Lai *et al*., 1997; Kotronias and Kapranos, 1998). Our study shows that the presence of HSV2 or HCMV in the sperm suspension does not inhibit sperm motility, regardless of the viral concentrations used and the method used to treat the sperm: sperm with seminal fluid, sperm selected by PureSperm gradient (no seminal fluid), or sperm selected by centrifugation and migration (data not shown). These observations were the same for all the samples studied, and CASA system applied to HSV2 strengthened our results. It would therefore appear that the in-vivo correlation between the reported reduction of percentage of motile sperm and the presence of viral particles (Lai *et al*., 1997; Kotronias and Kapranos, 1998) is due to indirect interactions between the virus and the sperm rather than to a direct effect on motility. However, we observed modifications of motility parameters such as VCL, LIN, ALH and LA, showing that in the presence of HSV2, sperm moved more rapidly with a greater ALH.

We showed that human sperm do not bind to HSV2 in the presence of seminal fluid, although free viral particles were found in the seminal fluid. These findings are similar to those of other investigators (Sherman and Morgan, 1989) who did not find a close relationship between sperm and HSV2, and those of another study (Deture *et al*., 1978), which observed free viral particles in the seminal fluid. The seminal fluid seems to impede the adherence of HSV2 with sperm. Sherman and Morgan (1989) hypothesized that spermine phosphate is involved. Seminal fluid contains high molecular weight proteins that could impede the interaction of HSV2 with sperm or behave as a receptor for HSV2 thus preventing further contact by sperm.

Conversely, in the absence of seminal fluid, sperm were able to adhere to HSV2. It was difficult to evaluate the exact percentage of sperm with HSV2 adhering to their surface because we observed sections of sperm (95 nm thick) and not whole sperm (4–5 μm thick). The percentage of sperm bearing viral particles was 8% but may have been as high as 45% if only one viral particle adhered to each sperm. This percentage is within the range described for ISH HSV2 DNA-positive sperm cells (Kotronias and Kapranos, 1998). These results also raised questions about the nature of the relationship between HSV2 and sperm. It may consist of a simple passive attachment that is able to resist the repeated washes. This is highly possible given our results. However, in one spermatozoon, the virus particle seemed to be closer to the sperm membrane, and this could even be a beginning of fusion. Thus, we cannot exclude the possibility that this is a true ligand/receptor relationship that only occurs when sperm have been separated from their seminal fluid. Numerous HSV2 receptors have been identified, including heparan sulphate-like glycosaminoglycans (Trybala *et al*., 2000), nectins and a receptor belonging to the tumour-necrosis factor family (Campadelli-Fiume *et al*., 2000). It is possible that the sperm have one of these membrane receptors, which is not accessible to the virus when seminal fluid is present.

In conclusion, sperm motility, which is the parameter used to select sperm for ICSI, is not a good enough criterion on which to prove the presence of viral elements in the medium or on the sperm.

From our experiments with virus and sperm with seminal fluid, we can conclude that when the virus is mixed with semen in the annexial glands *in vivo*, HSV2 does not interact

with the sperm and the virus remains in the seminal fluid. In these conditions, the techniques used in assisted reproduction to eliminate the seminal fluids also eliminate the viral particles and improve the sperm preparation. This means that either PureSperm gradient selection or swim-up migration can be used efficiently.

Sperm inside the seminiferous tubules are protected from viruses by the haematotesticular barrier. When this barrier is ruptured, sperm can come into contact with viral particles. Indeed, this barrier is always destroyed when sperm are collected from the testis. This increases the risk of sperm coming into contact with high local concentrations of HSV2 due to contamination from the blood or interstitial tissues. The washing procedure cannot eliminate viral particles, meaning that they are directly injected into the cytoplasm.

The HCMV virus particle did not interact with the sperm when HCMV was added to the semen. However, the relationship between sperm and virus in the absence of the seminal fluid was not so clear as for HSV2, perhaps due to the fact that this virus binds non-specifically. However, as for HSV2, the viral particle may be accidentally injected into the ooplasm during ICSI, either as a result of passive transport by the sperm or from the surrounding environment as washing did not eliminate the virus from the preparation.

Some studies used sperm as vectors (Perry *et al*., 1999). These experiments concerned purified DNA or plasmids, and we did not study this aspect of the problem as we focused only on infectious virions visible under the electron microscope.

We have attempted to investigate whether there may be a specific risk of viral infection associated with ICSI. Our main conclusion is that ICSI creates a new risk of contaminating sperm with viruses in cases of ICSI for azoospermia. Sperm inside the seminiferous tubules are protected from viruses by the haematotesticular barrier. When this barrier is ruptured, sperm can come into contact with viral particles. Indeed, this barrier is always destroyed when sperm are collected from the testes. This increases the risk of sperm coming into contact with high local concentrations of HSV2 due to contamination from the blood or interstitial tissues. The washing procedure cannot eliminate viral particles, meaning that they are directly injected into the ooplasm.

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